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Multivalent targeted theranostic liposomes for chemo-resistant aggressive CaP

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14. ABSTRACT

While diagnosis and treatment methods of prostate cancer (CaP) continue to improve, drug resistance pathways in advance stage disease still requires new methodologies to better identify, and treat CaP with fewer side effects for patients. The research objective was to develop new theranostic (Th_x) liposomes capable of independently targeting three protein receptors expressed at different stages of advanced PCa cells for delivery of imaging and therapeutic components in a single package. The multi-valent liposomes were loaded with a therapeutic (docetaxel) and PIM1 inhibitor (SGI-1776) to resensitize docetaxel resistant cells. This approach was examined to provide a targeted delivery of encapsulated therapeutics in comparison to systemic delivery methods in vitro.

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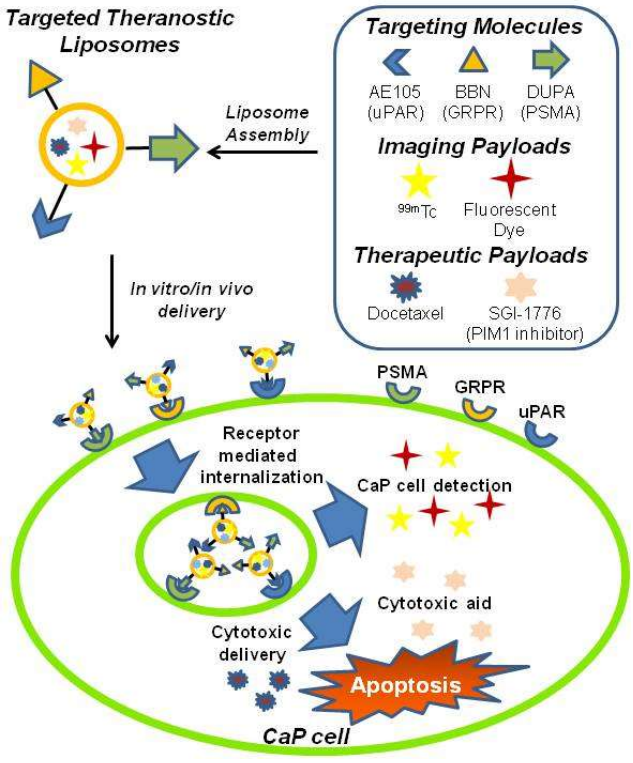
Abstract:

While diagnosis and treatment methods of prostate cancer (CaP) continue to improve, drug resistance pathways in advance stage disease still requires new methodologies to better identify, and treat CaP with fewer side effects for patients. The research objective was to develop new theranostic (Th_x) liposomes capable of independently targeting three protein receptors expressed at different stages of advanced PCa cells for delivery of imaging and therapeutic components in a single package. The multi-valent liposomes were loaded with a therapeutic (docetaxel) and PIM1 inhibitor (SGI-1776) to resensitize docetaxel resistant cells. This approach was examined to provide a targeted delivery of encapsulated therapeutics in comparison to systemic delivery methods.

Introduction:

The heterogeneous nature of metastatic and circulating tumor CaP cells presents significant challenges in the overall assessment and therapeutic effectiveness. Combined with drug resistance in aggressive CaP and dose limitations of systemically administrated therapeutics limited effective treatments are available to address each of these issues. This research seeks to increase the targeting spectrum of heterogeneous CaP while enhancing treatment efficacy through co-delivery of synergistic therapeutics using targeted liposomes. By simultaneously targeting three biomarkers (uPAR/PSMA/GRPR), *a greater number of CaP phenotypes can be selected to more effectively target aggressive CaP than a single biomarker alone*. Each of these biomarkers is overexpressed in aggressive CaP, including metastatic or circulating tumor cells.[1,2] Such a strategy is envisioned to more effectively target CaP phenotypes expressing single or multiple receptor types.

This strategy also examines the delivery of a synergistic combination of taxane and PIM1 therapeutics. The PIM1 kinase inhibitor SGI-1776 has been shown to resensitize aggressive CaP (resistant) towards taxanes.[3] Using the targeted liposome as a vehicle, co-delivery of both therapeutics, taxane and PIM1 inhibitor, to a single cell would be accomplished, while mitigating toxicity issues observed with systemic administration. Incorporation of diagnostic agents (i.e., fluorescence, SPECT) would also provide a useful tool for external detection and localization assessment. Overall, the combination of targeted delivery and synergistic therapeutics addresses a key treatment issue of aggressive heterogeneous CaP.



This research addresses the PCRP overarching challenges of 1) developing better tools for detection of disease, and 2) developing effective treatments and addressing mechanisms of resistance in aggressive CaP. Specifically, targeted Th_x liposomes encompasses the focus areas of 1) *imaging* by incorporating a SPECT radionuclide for non-invasive whole body imaging and/or a fluorescent dye for high-resolution cellular imaging and surgical marking; 2) *mechanisms of resistance* by employing a PIM1 kinase inhibitor to re-sensitize chemoresistant cells; and 3) *therapy* by combining taxanes with a PIM1 kinase inhibitor to increase cytotoxic efficacy. Targeted liposomes are expected to enhance the therapeutic index by selectively depositing high localized concentrations of the synergistic components to CaP, while minimizing toxicity limitations and side effects in normal tissues.

The hypothesis/Rationale of the experiment focused on the

Multi-valent Th_x liposomes are hypothesized to provide enhanced assessment of heterogeneous aggressive CaP through imaging, while co-delivering docetaxel and PIM1 inhibitor (SGI-1776) at therapeutically relevant **concentrations is expected to overcome resistance and induce cell death in CaP.** The **rationale** for these targeted Th_x agents would provide a more potent system that is flexible to different receptor expression levels to better addresses the heterogeneity of CaP. The combination of docetaxel and SGI-1776 would treat taxane sensitive/resistant cells by resensitization through PIM1 inhibition to provide an improved treatment outcome. Inclusion of imaging components would provide a facile method for external detection of localization or as a marker for surgical removal.

Key words: Chemotherapeutic resistance, Liposome, Drug Delivery

Accomplishments:

During the course of the award period, the project encompassed the synthesis of the individual components for preparing the targeted liposomes for evaluation, assembly, therapeutic loading, and eventual in vitro testing. The targeted liposomes were constructed from individual components, particularly the small molecule and peptide targeting agents, were synthesized and conjugated to lipophilic groups to allow for insertion into the lipid bilayer. Synthesis of the biologically active molecules was done either through classic synthetic methods or solid phase peptide synthesis (SPPS). The first steps of the research involved the synthesis of the individual components of the three targeting agents for GRPR, PSMA and uPAR receptors expressed on the cells. A new lipophilic ligand and $^{99m}Tc(CO)_3$ complexes were also developed to incorporate the SPECT imaging agent into the liposome for external monitoring of in vivo localization and delivery.

Synthesis of Components

2-(3-(5-(6-azidohexanamido)-1-carboxypentyl)ureido)pentanedioic acid, (*PSMA targeting agent*, **1**).

1 was synthesized following previously reported procedures (WO 2013028664). Briefly, H_2N-Glu(t-Bu)-Ot-Bu hydrochloride salt was dissolved in dichloromethane (DCM) at -78 °C with triphosgene. Triethylamine (Et_3N) was added and the reaction was stirred for 2 h. H_2N-Lys(CBz)-Ot-Bu was added along with more Et_3N and the reaction was stirred overnight. The resulting product was isolated by extraction followed by column chromatography.The CBz (Benzyl carbamate) group was then removed by hydrogenation with 5% palladium on carbon in the presences on H_2 at atmospheric pressure. The resulting solution was filtered through celite, concentrated to dryness and used without further purification. The tert-butyl protected (t-Bu) **1** was then conjugated to a clickable linker, 5-hexynoic acid. This was achieved by under standard amide coupling conditions with O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIPEA) in DCM. The reaction was mixed under N_2 overnight and the resulting product was purified by silica gel flash chromatography, using potassium permanganate ($KMnO_4$) stain to identify the final product. The t-Bu groups were then removed using a 20% solution of triflouroacetic acid (TFA) in DCM for 3 hours, the resulting solution was concentrated to dryness, redissolved in methanol (MeOH) dried completely to give the clickable derivative of (**1**-hexyne).

Figure 1. PSMA targeting agent **1**-hexyne

Bombesin (GRPR targeting agent)

Solid phase peptide synthesis was used for the construction of the bombesin targeting molecules (NH_2-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2, **2**, Hexyne-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2, **2-yne**) using iterative

coupling of amino acids. A Rink amide resin was used to could the desired sequence stepwise. The Rink resin was initially deprotected with 20% piperidine in DMF. Coupling was performed using fluorenylmethoxy carbamate protected amino acids with acid labile side chain protection. O-(Benzotriazol-1-yl)-N,N,N',N-l-tetramethyluronium hexafluorophosphate (HATU) and DIPEA was mixed for 15 minutes with the protected amino acid in 1:1 DCM/DMF prior to addition to the resin and the Kaiser test (phenol/sodium cyanide, ninhydrin) was used to asses complete coupling of the amino acid to the solid phase. 20% piperidine in DMF was then used to remove the Fmoc protecting group for further coupling and the steps repeated until the entire peptide sequence was complete. After the full sequence was synthesized it was either directly cleaved off the resin (**1**) or a 5-hexynoic acid group was attached for click functionality (**1**-hexyne).

1 was conjugated to a N-hydroxysuccinimide ester (NHS) of dibenzoazacyclooctyne (DIBAC) either before or after cleavage to the resin using a TFA/anisole/ thioanisole/triisopropylsilane mixture. It was found that conjugation of the DIBAC to the resin prior to cleavage caused rearrangement of the clickable portion of the molecule resulting in no reaction with an azide. Addition of the NHS ester in DMF after cleavage from the resin yielded the desired DIBAC-bombesin conjugate (**2**-DIBAC).

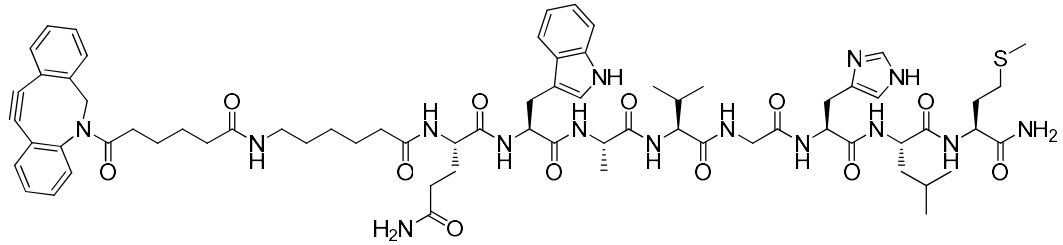


Figure 2. Bombesin peptide **2**-DIBAC

AE105(uPAR targeting molecule)

Solid phase peptide synthesis was used for the construction of the AE105 peptide (NH_2-Hex-Asp-Cha-Phe-D-ser-D-arg-Tyr-Leu-Trp-Ser-NH2, **3**) for targeting of uPar receptors. A Rink amide resin was used to could the desired sequence stepwise. The Rink resin was initially deprotected with 20% piperidine in DMF. Coupling was performed using fluorenylmethoxy carbamate protected amino acids with acid labile side chain protection. O-(Benzotriazol-1-yl)-N,N,N',N-l-tetramethyluronium hexafluorophosphate (HATU) and DIPEA was mixed for 15 minutes with the protected amino acid in 1:1 DCM/DMF prior to addition to the resin and the Kaiser test (phenol/sodium cyanide, ninhydrin) was used to asses complete coupling of the amino acid to the solid phase. 20% piperidine in DMF was then used to remove the Fmoc protecting group for further coupling and the steps repeated until the entire peptide sequence was complete. Fmoc-6-aminohexanoic acid was then appended to the peptide, the Fmoc was removed with piperidine in DMF and the peptide was cleaved from the resin using a TFA/anisole/thioanisole/TIPS mixture. Dibenzoazacyclooctyne (DIBAC) was added using the NHS ester in DMF after cleavage from the resin to give a clickable peptide (**3**-DIBAC).

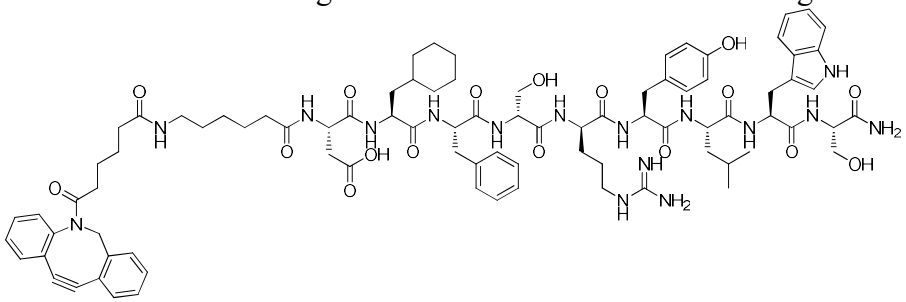


Figure 3. uPar targeting agent **3**-DIBAC

Conjugation to Lipid Framework

Liposomes consist of lipid bilayers with interlocking polar tails and a charged head groups facing into the water layers. For the prepared liposomes in our study we used distearoyl phosphotidylcholine (DSPC), a

molecule containing two C-18 unsaturated fats connected to glycerol, which was in turn connected to a phosphate and a 2 linker ending with a protonated tetra-substituted amine. In order to mimic DSPC and also place the targeting molecule far from the lipid surface, the tetra-substituted amine was substituted with an amide functionality. This was then attached to a 2000 molecular weight polyethylene glycol (PEG_{2000}) unit with an azide (N_3) terminal group (DSPE-PEG_{2000}-N_3). In order to integrate a targeting molecule onto a lipid platform, the azide group incorporated in the liposome was reacted with copperless click conditions to the sterically strained alkynes that were appended to the aforementioned targeting molecules.

PSMA targeting agent conjugate (DSPE-PEG_{2000}-**1**)

1-hexyne and DSPE-PEG_{2000}-N_3 were dissolved in methanol with stirring. Copper acetate and sodium ascorbate were then added in water and the reaction was allowed to stir overnight. A small amount of EDTA was added to coordinate the copper and the resulting solution was extracted with chloroform to remove any remaining DSPE-$PEG_{2000}N_3$ and the DSPE-PEG_{2000}-**1**. This compound was then concentrated, massed and dissolved in chloroform for use in liposome preparation. Due to extreme lipophilicity, low quantity and difficulty of NMR characterization, identification of the desired product was performed using high resolution mass spectroscopy (HRMS).

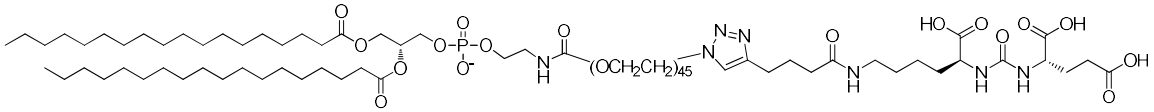


Figure 4. DSPE-PEG_{2000}-**1**

*Bombesin and AE105 conjugates (DSPE-PEG_{2000}-**2**, DSPE-PEG_{2000}-**3**)*

Initial attempts to use the hexyne derivative of bombesin (**2**-hexyne) were unsuccessful due to the lipophilicity of the peptide in relation to the copper catalyst needed for the reaction. It was unclear if the peptide was incorporated non-specifically in the liposome and an additional precipitate observed in the reaction vessel. In order to account for this, DIBAC was used, as no copper catalyst is required for the click reaction to function. Reaction of the DIBAC functionalized peptides with DSPE-PEG_{2000}-N_3 was performed by dissolving the species in an equimolar ratio in chloroform. Reaction for 18 hours gave complete reaction as evidenced by the disappearance of the starting peptide peak by HPLC analysis. The samples were then dried, a mass obtained, and redissolved in chloroform for incorporation into the liposomes. Final characterization was achieved using HRMS.

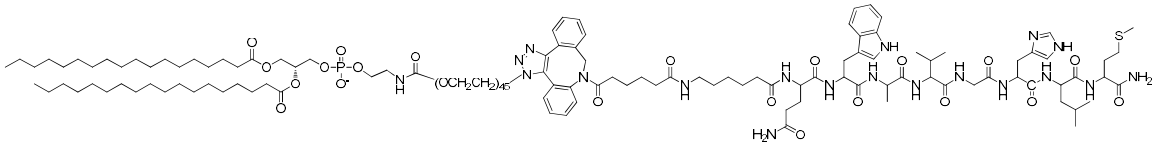


Figure 5. DSPE-PEG_{2000}-**2 product**

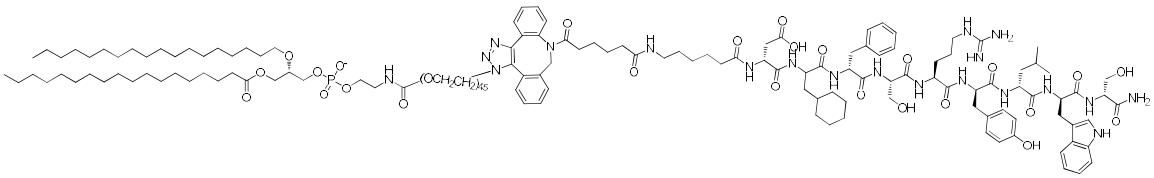


Figure 6. DSPE-PEG_{2000}-**3**

Liposome synthesis

Lipids for the liposomes were purchased from Avanti Polar Lipids. DSPC, cholesterol, DSPE-PEG_{2000}-OME, DSPE-PEG_{1000}-OMe and DSPE-PEG_{2000}-N_3 were acquired either as powders or dissolved in chloroform and used without further purification. Liposomes were formed by dissolving DSPC and cholesterol in a mixture of chloroform and methanol. At this time DSPE-PEG units or targeting agents were added and the solution was concentrated to dryness. The lipid film that forms is then hydrated with 10 mM HEPES buffer at 65 °C for 30 minutes with vortexing every 5 minutes. The resulting large unimallelar vesicles are then extruded through a 0.2 µm porous filter which creates liposomes of the desired particle size. Dynamic light scattering was used to determine that the liposomes formed using this method have a diameter of approximately 90 nm when using PEG_{2000} derivatives.

Labeling of Liposomes with $^{99m}Tc(CO)_3$

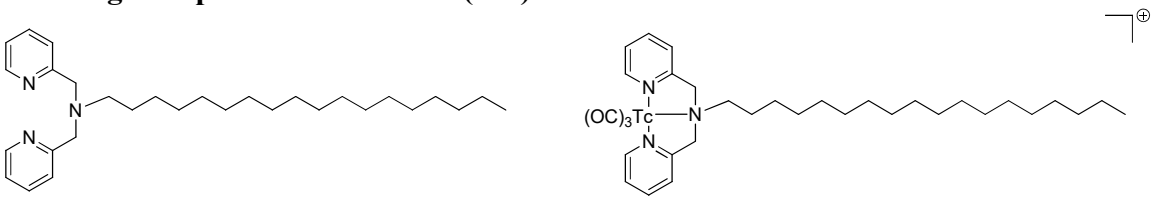


Figure 7. DPA-C18 (**4**) and its associated $Tc(CO)_3$ product (**5**)

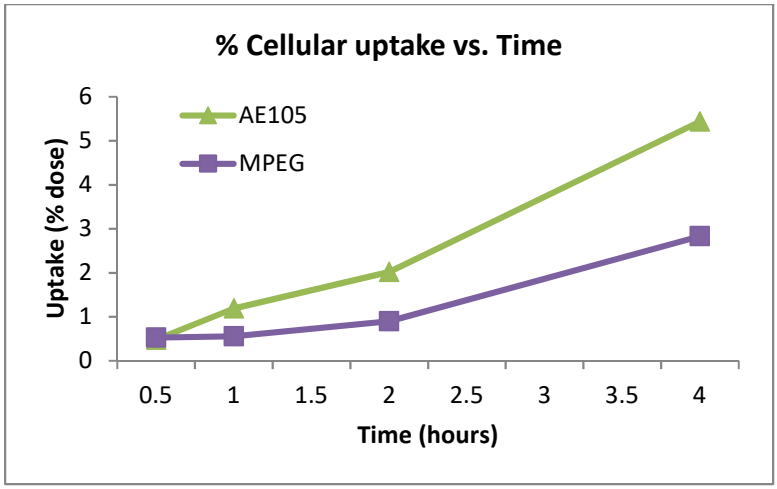
A dipicolylamine based chelator was used for incorporation of the $^{99m}Tc(CO)_3$ into the liposomes for nuclear imaging applications. A 18 carbon chain was appended to the central nitrogen to facilitate insertion into the lipid membrane (DPA-C18, **4**). Coordination to **4** was performed using $^{99m}Tc(CO)_3$ produced from an Isolink© kit at neutral pH and isolated by radio-HPLC to yield (**5**) in high specificity in excellent radiochemical yields >95%. Incorporation of the DPA-C18 into the liposome was achieved by addition to the lipids solution prior to the removal of the methanol chloroform mixture.

Cellular uptake studies

Studies were performed with targeted liposomes to determine receptor targeting effectiveness. Blocked liposomes were used to account for non-specific cell uptake of liposomes. Liposomes were radiolabelled with ^{99m}Tc and final aliquots were counted on a cobra counter. Activity of isolated cell aliquot was plotted as a percentage of the total radioactivity for each trial. Experiments were performed in triplicate and error bars were plotted as the standard deviation. Multiple time points were measured (0.5-4 hours) and increased cellular uptake with increased incubation times were observed.

Experimental procedure

Cells were harvested at 90% confluence and were plated at 300,000 cells/well in cell culture treated 12-well plates. Cells were allowed to adhere overnight and spent media was replaced with fresh media ~1hr before experiment. Wells were incubated with ~0.2µCi of targeted or blocked liposomes. Blocked liposomes involve a coating of MPEG and the absence of targeting molecules to mimic nonspecific cellular uptake. Plates were incubated at 37°C, 5% CO_2 in a humidified atmosphere for desired time point(0.5-4 hours) and were subsequently washed with PBS. Cells were lysed with 1M NaOH and all aliquots were counted on a cobra counter. Cellular uptake was plotted as a percentage of the total radioactivity and plotted by excel.



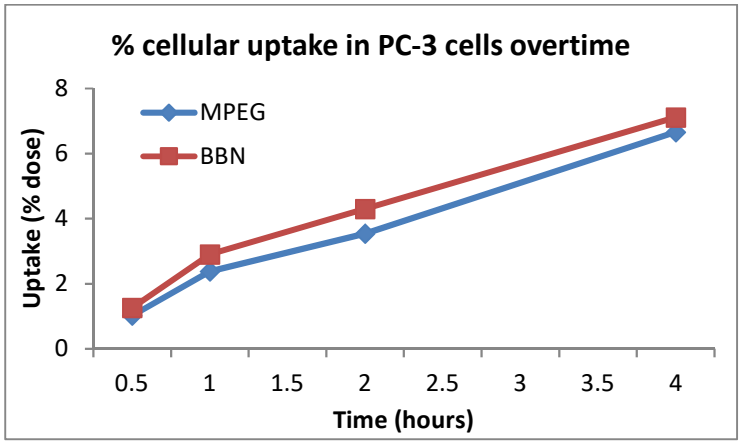


Figure 8. Percent cellular uptake of radioactively labeled liposomes over time. Experiments were performed in triplicate and error bars are the standard deviation. A) DU 145 cells incubated with AE 105 targeted liposomes and MPEG liposomes. B) PC-3 cells incubated with BBN targeted liposomes and MPEG liposomes.

Fluorescence Cell studies

To determine the effective imaging capabilities of these liposomes, samples were prepared with bodipy-stained phospholipids to allow for fluorescent imaging. Fluorescent microscopy is used to image the qualitative liposomal uptake into the cells. Cells were plated on culture treated coverslips and were allowed to grow to 90% confluence. Plates were incubated with liposomes for 1 h followed by paraformaldehyde fixation and 2 PBS washes. Coverslips were transferred to glass slides with DAPI mounting media to allow for easy identification of cell nuclei. Fluorescence was observed at three different excitation/emission wavelengths (488nm/510nm, 405nm/461nm, and 543nm/608nm) and pictures were taken at each including an overlay of all three. The 405nm excitation corresponds to the DAPI dyed nuclei and is shown in the upper left corner of the micrograph, 488nm and 543nm are each relevant to Bodipy and are shown in the upper right and lower left quadrants. The overlay of these three images in the lower right shows effective cellular membrane fluorescence due to the cell uptake of the liposomes.

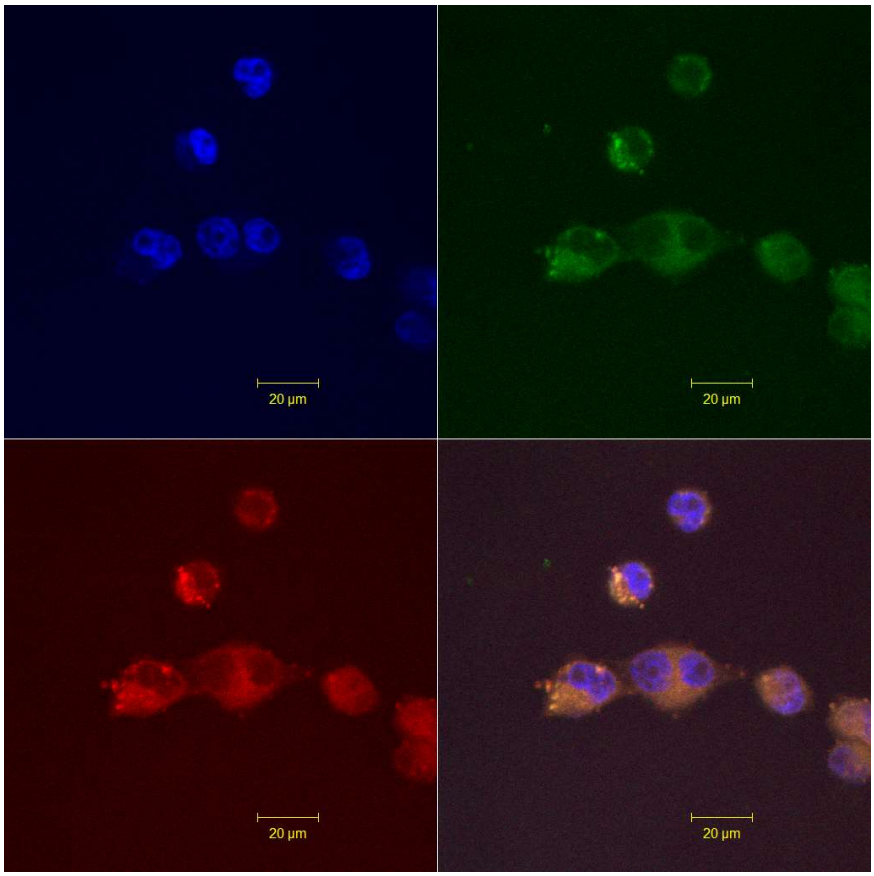


Figure 9. Micrograph image of PC-3 cells incubated with AE-105 targeted liposomes.

Cell Viability Assays

A secondary goal of the award was to examine the loading capacity of liposome for selective delivery of the targeted vehicles. The liposomes were to be loaded with docetaxel and/or SGI-1776. Both of the compounds have high effectiveness to treat prostate cell lines, but have limitation in systemic delivery due to side effects impacting other organs (heart, kidney, liver) function. The combination of the two compounds would provide a unique combination to enhance treatment of docetaxel resistant prostate cancer cells. To determine the optimal drug-loading concentration in the liposomes, control experiments will be performed with Docetaxel or SGI-1776 and a combination of the two. Two CaP lines (DU-145 and PC-3) were examined with the dose escalation studies with both compounds. MTT assays were performed to determine cell viability following drug incubation. Absorbance of the reduced MTT compound directly correlates to the amount of viable cells remaining. Results of these experiments performed on PC-3 cells and DU-145 cells show optimized docetaxel and SGI-1776 concentration to be 1nM and 1µM, respectively.

Impact:

The impact of the project thus far demonstrates the investigation of the development of a multi-targeting liposome for improved selectivity. Thee inherently complex nature of the synthesis While the inherent goal of the project was to examine a multi-valent system targeting each of the aforementioned receptors, individual testing of these liposome constructs illustrated only the AE-105 had a viable positive differentiation from untargeted liposomes. These studies demonstrate that imaging (radiochemical and fluorescence) agents can be incorporated in the AE-105 system.

Changes/problems:

Several challenges initially plagued the synthesis of the clickable analogs. An unexpected difficulty was the performance of the bombesin peptide in the synthesis of the clickable bombesin analogs for liposome insertion. Several approaches were utilized a standard click conditions of an alkyne with an azide in combination with a copper catalyst. While the click reaction was easily successful with other peptide analogs, each of the methods examined that varied concentrations, reaction conditions, order of operations and types Cu catalysts yielded little to no product. In each of these attempts, it was observed that a micro precipitate formed upon introduction of the Cu to the reaction vessel no matter how careful the addition.

Despite the goal of targeting each of the different receptors (GRPR, PSMA and uPAR) expressed on cell surfaces of prostate cancer cells with different targeting ligands in a single system, the independent assessment clear illustrated under the conditions of the experiment that no difference between the non-functionalized liposomes and the targeted analogs for bombesin (GRPR) and urea based small molecule inhibitor (PSMA). While this was an unfortunate outcome, it limited the ability to produce a multivalent targeting system as considerable effort was expended to prepare the clicked analogs, this highlights the critical importance of receptor affinity of modified peptide or small molecules. However, the uPAR functionalized liposomes did have a positive improvement for targeting in both PC-3 and DU-145 CaP cell lines.

Products:
Three different targeted liposomes were prepared and examined for targeting different receptors (GRPR, PSMA and uPAR) expressed on cell surfaces of prostate cancer cells. Methods were developed to incorporate in each of these liposomes with two different types of imaging agents (radiochemical and fluorescence) to monitor uptake in vitro and in vivo.

Participants and other collaborating institutions:

Zhen Cheng at Stanford University helped prepare the peptides for click reactions and assisted in the development of the cell assays for cell uptake.

Special reporting requirements: Final

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Appendices: NONE