Electrostatic Targeting of Feraheme Using Doxorubicin Conjugates for Prostate Cancer

Nancy Luo

Yorktown High School

Acknowledgments

I would like to thank Dr. Jan Grimm and Edwin C. Pratt for their guidance and feedback throughout the completion of this project. I would also like to acknowledge the support of my teachers, Mr. Blueglass and Mrs. McNelis, as well as the encouragement of my parents.

Table of Contents

List of Figures and Tables

Abstract

Prostate cancer is a deadly disease that lacks effective treatment. Many existing chemotherapeutics are non-specific, meaning they kill off both cancerous and healthy cells. Targeting agents have been utilized in order to increase specificity of these treatments. Studies performed by Kaittanis in 2017 demonstrated the conjugation of a prostate-specific membrane antigen (PSMA) targeting peptide onto iron oxide nanoparticles followed by loading of therapy. They found increased uptake for PSMA-expressing cells after treatment with the targeting nanoparticles. Effective peptide conjugation to the particle surface was shown, but they did not show if the targeting peptide could be directly conjugated to the chemotherapeutics. In this study, a PSMA targeting agent was created and attached to Doxorubicin, a cancer treatment drug, through a peptide linker. LNCaP, 22Rv1, Du145, and PC3 prostate cancer cells were treated in order to determine uptake by these cells. The cells were treated for 24 hours with Doxorubicin, Doxorubicin loaded onto Feraheme, Doxorubicin conjugate, and the conjugate loaded onto Feraheme. LNCaP cells showed the highest fluorescence under microscopy, which was expected because they expressed the highest level of PSMA. Fluorescence signals were lower in Du145 and PC3 cells, which did not express PSMA at all. Data from flow cytometry showed that the Doxorubicin conjugate was taken up by the prostate cancer cells but did not exceed the amount of free drug taken up by the same cells. These findings suggest that more experiments need to be performed in order to determine how the targeting agent affected cell uptake.

Introduction

Prostate cancer is one of the leading cause of death in cancer death amongst men, and approximately 1 in 41 men will die of prostate cancer (Ruggiero, 2011). Typically, death by prostate cancer is caused by late diagnosis, which can result in the cancer becoming resistant to treatment and metastasizing to other organs of the body.

In order to prevent delayed diagnosis of the disease, researchers have discovered prominent biomarkers for the disease. Prostate-specific membrane antigen (PSMA) is a common marker of prostate cancer in aggressive forms of the disease. Prostate cancer is traditionally treated by surgical removal of the prostate gland followed by continuous chemotherapy in order to prevent recurrence. A problem with the traditional method is that many of the cancer treatment drugs such as Doxorubicin and Enzalutamide are non-specific, meaning they can kill off healthy cells rather than just prostate cancer cells. In order to increase the uptake of these drugs by the cancer cells and prevent uptake by healthy cells, it is possible to conjugate targeting agents onto these drugs through coupling reactions (Kalia, 2010). It is difficult to find an optimal binding method that will increase drug efficiency while avoiding scrutiny from the immune system (Spicer, 2014). The addition of a targeting component to the drugs would reduce the dosage, which reduces the adverse effects that may be caused by the non-specific drugs.

Review of Literature

Nanotechnology is a rising field in the treatment of cancers where it has been utilized for the delivery of chemotherapeutics. Feraheme, a type of iron oxide nanoparticle, is able to encapsulate multiple drugs simultaneously for uptake by cancer cells through phagocytosis, and these particles will release the drugs intracellularly to deliver cell death. These iron oxide particles are between 10-300 nm in size and contain an iron core, which makes them easy to

track using magnetic resonance imaging (MRI; Daldrup-Link, 2017). Iron oxide nanoparticles will exit the body through the liver and spleen, meaning they will be expelled and will not remain in the body for an extended period of time (Weissleder, 2014). Past studies have shown the use of conjugation reactions in order to attach fluorescent agents to these particles in order to achieve detection with flow cytometry (Yuan, 2018). Utilizing these particles for delivering therapy can reduce the toxicity of drugs to healthy cells and increase the effectiveness of treatment (Heckert, 2017).

Delivery of Targeted Therapy with Iron Oxide Nanoparticles. The cancer treatment drug, Taxol, and a near infrared fluorescent dye were encapsulated within the nanoparticle. In addition, a folic acid ligand was conjugated to the surface of the nanoparticles in order to target the overexpressed folate receptors in A549 lung carcinoma cells (Santra, 2009). The targeting ligands were successfully attached through click chemistry conjugation reactions. (Figure 1) The lipophilic dyes were successfully encapsulated within the particle coating, and as a result, the particles were able to increase uptake by the lung cancer cells and deliver effective treatment. Since the addition of a targeting agent onto the surface of the iron oxide nanoparticles successfully increased uptake by lung cancer cells, it is likely that the addition of a PSMA targeting agent will increase the uptake by prostate cancer cells because of their high expression of prostate-specific membrane antigen (PSMA; Santra, 2009).

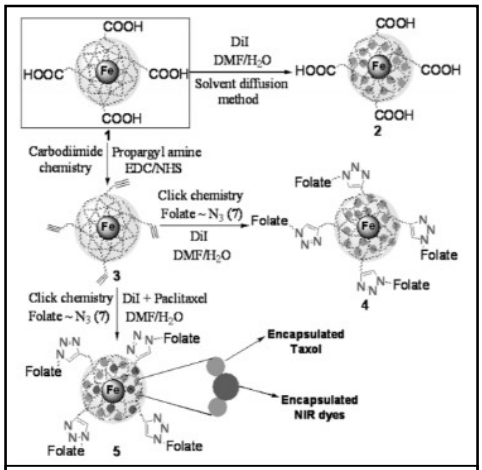

Figure 1: Taxol drug and a near infrared dye were encapsulated with in the iron oxide nanoparticle. Carbodiimide and click chemistry were used for the conjugation of the folic acid ligand (Santra, 2009).

Targeting the Intracellular Domain of PSMA. The 7E11 monoclonal antibody targets an internal epitope of PSMA, meaning the antibody will only be able to attach after membrane disruption of the prostate cancer cells. A past study aimed to radiolabel 7E11 with ^{89}Zr in order to monitor the effects of different chemotherapy treatments under positron emission tomography. The monoclonal antibody J591 recognizes all PSMA-positive cells and was used as a control for the detection of membrane disruption by the treatments (Ruggiero, 2011). It was found that a higher percentage of the LNCaP prostate cancer cells were labeled after treatment compared to the untreated cells (Figure 2). However, the study did not address targeting for the delivery of the chemotherapeutics in order to increase specificity of the drugs (Ruggiero, 2011).

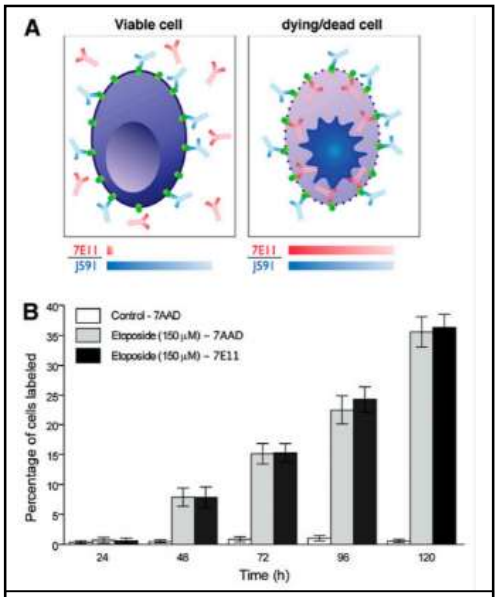

Figure 2: A) 7E11 targets the internal epitope while J591 targets all PSMA-expressing cells **B)** Etoposide treatment was monitored with the radiolabeled 7E11. 7AAD was used to monitor cell viability (Ruggiero, 2011)

Delivery of Chemotherapeutics with Feraheme through PSMA Targeting. A PSMA, prostate-specific membrane antigen, targeting peptide was successfully conjugated to the surface of Feraheme nanoparticles. These particles were then loaded with the drugs, Etoposide, Paclitaxel, and Doxorubicin, for the targeted delivery to PSMA-positive prostate cancer cells. PC3 control cells were used for comparison in treatment because they do not express PSMA (Kaittanis, 2017). It was found that a

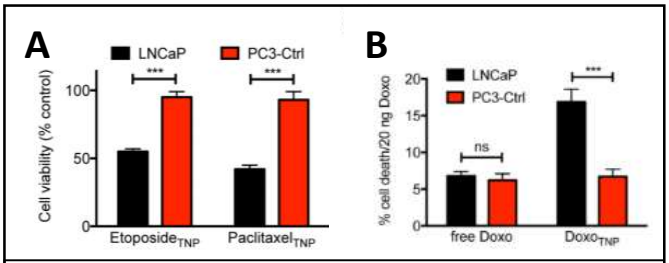

Figure 3: A) Cell viability decreased after treatment with the Etoposide-loaded targeting Feraheme and Paclitaxel-loaded targeting Feraheme for PSMA-positive LNCaP cells. Cell viability was not affected for PSMA-negative PC3 control cells **B)** Doxorubicin-loaded targeting Feraheme successfully increased the delivery of cell death to LNCaP cells compared to the free drug treatment (Kaittanis, 2017)

greater percentage of uptake and cell death was seen in PSMA-expressing cells compared to the controls after treatment with the targeting nanoparticles (Figure 3). This study showed the effective conjugation of a targeting peptide onto the surface of nanoparticles, but did not show if the targeting peptide could be directly attached to the drug for loading onto Feraheme (Kaittanis, 2017).

Problem Statements (2018)

P_1: There has been a limited number of drug combinations tested on prostate cancer cells.

P_2: Not all drugs load onto iron oxide nanoparticles the same, which can affect treatment efficacy.

P_3: Numerous combinations of chemotherapeutics exist, but not all are effective in inducing cell death. The effects of different drugs need to be tested in combined loading in order to test each combination's effectiveness.

Objectives (2018)

Obj_1: Utilize new combinations of drugs that are thought to induce Ferroptosis, a type of programmed cell death dependent on iron and lipid peroxide accumulation, such as Fluvastatin with Erastin

Obj_2: 22Rv1 cells are used to test if the combinational therapy of Erastin and Sildenafil citrate delivered by iron oxide nanoparticles will cause more cell death than free drug. An enhanced effect shown with Feraheme, an iron oxide nanoparticle, would mean these loaded particles can be tested *in vivo* to see if they will have similar effects in reducing the tumor volume.

Obj_3: Efficacy will be tested of free drug and loaded nanoparticles for different combinations of treatment such as Fluvastatin and Erastin, Sildenafil citrate and

Erastin, and Erastin alone (Hao, 2018).

Hypotheses (2018)

H_1: The loading of different combinations of drugs such as Sildenafil citrate and Erastin will enhance treatment delivered to prostate cancer cells because Erastin delivered with Feraheme will induce ferroptosis to cause cell death.

H_2: Feraheme loaded with therapeutics can provide a better therapeutic outcome than delivery of free drug because they will be taken up by the prostate cancer cells through phagocytosis and release the drugs intracellularly.

H_3: A combination of Erastin and Fluvastatin will have a synergistic effect in reducing cell viability *in vitro* by inducing ferroptosis, which is a programmed cell death dependent on iron accumulation in the cells.

Problem Statements (2019)

P_1: Free chemotherapeutic treatment can be non-specific and cause harm to healthy cells.

P_2: It is unknown if the targeting drug loaded onto Feraheme will increase uptake by the prostate cancer cells.

Objectives (2019)

Obj_1: Attach a PSMA, prostate-specific membrane antigen, targeting agent onto Doxorubicin and utilize Feraheme for delivery to various prostate cancer cells.

Obj_2: Assess the uptake of Doxorubicin, Doxorubicin conjugate, and Doxorubicin conjugate loaded into Ferhame by the cancer cells with fluorescence assays and microscopy.

Hypotheses (2019)

H_1: The Doxorubicin conjugated with a PSMA targeting agent will load well onto Feraheme because past studies have shown that free Doxorubicin loads well onto Feraheme (Kaittanis, 2014). The PSMA targeting agent will increase uptake of the drug by the cancer cells because of their high expression of PSMA.

H_2: There will be a difference in the method of uptake by prostate cancer cells for Feraheme with the targeting agent loaded onto the nanoparticles compared to the drug alone because the cells take in drugs and iron through different mechanisms.

Methodology

Role of Student vs. Mentor

Over the course of 2 summers, I spent 16 weeks in the laboratory in order to perform cell culture studies, cell assays such as protein assays, viability assays, and iron digestion, flow cytometry, and microscopy. Additionally, I was responsible for performing drug loading, microscopy, and all cell culture studies such as reviving cell lines and plating cell lines. The mentor provided guidance in the conjugation reaction for creating the compound of Doxorubicin with a PSMA targeting agent and performing flow cytometry. I performed statistical analysis on the flow cytometry data in order to examine percent positive cells with the guidance of my mentor.

Phase 1 (2018)

Loading Erastin and Sildenafil Citrate onto Feraheme. Stock Feraheme was diluted with buffer that had a pH of 8, and different concentrations of drug in dimethyl sulfoxide were added dropwise to the Feraheme dilution on a vortex (Kaittanis, 2014). The drugs, Sildenafil

Citrate and Erastin, were loaded at the highest concentration without visual precipitation. Loading was confirmed by comparing the cell viability of the free drug to the drug-loaded Feraheme treatment. The particles were washed twice in order to filter out buffer that was used to dilute the stock Feraheme and any unloaded Sildenafil Citrate and Erastin without disturbing the Feraheme nanoparticles.

Assays for 22Rv1 Prostate Cancer Cells. After treating the 22Rv1 prostate cancer cells with free drug and loaded drug, the MTS and Resazurin assays were performed in order to test cell-viability. After the cells were incubated for either 24 or 48 hours, the cells were washed twice with Roswell Park Memorial Institute (RPMI) media to remove any drug and Feraheme. Absorbance and fluorescence data were normalized to the control, untreated cells, in order to determine the normalized viabilities for the cells in each treatment condition tested.

Western Blot. Cell protein was obtained after treatment with Sildenafil Citrate and was mixed with the NuPAGE LDS sample buffer (Catalog number: NP0007). The 10μM and 100μM Sildenafil Citrate concentrations and their corresponding controls were tested in the western blot in order to detect the amount of specific proteins present after treatment. 20μL of each lysate mixture was loaded into 12 separate wells and ladder control was loaded into 3 different wells. A previous western blot performed by my mentor and I showed that loading 20μL of lysate mixture made it easier to read compared to loading 10μL which was suggested. Although the primary goal was to stain for ferroportin (FPN), the samples were also stained for glutathione peroxidase 4 (GPX4) and prostate-specific membrane antigen (PSMA) in order to see the expression of these proteins after treatment with Sildenafil Citrate. β-actin signal was also stained for as a standard for the protein levels after treatment. The membranes were read with the Li-Cor Odyssey CLx infrared imaging system (LI-COR, Inc., 2018).

Phase 2 (2019)

Extracting Doxorubicin Free Base and Reaction with Peptide Linker. The Doxorubicin free base had to be extracted from the stock, which contains Doxorubicin, sucrose, and hydrochloride. The stock was first dissolved in a small amount of methanol and then transferred to a filter. 10mL of sodium bicarbonate was added to the filter followed by the addition of chloroform until the filter was filled in order to separate the Doxorubicin from sucrose and hydrochloride. The component of the mixture that contains Doxorubicin free base and chloroform was collected in a 500mL Erlenmeyer flask. Multiple washes were performed with chloroform in order to collect as much of the Doxorubicin free base as possible. The chloroform and methanol were evaporated using a rotary evaporator. The Doxorubicin free base was transferred to a reaction vial for the reaction between Doxorubicin and Mc-Val-Cit-PABC-PNP (CAS Number: 159857-81-5), a peptide linker. 0.02g (1 eqv.) of Doxorubicin free base, 0.0271g (1 eqv.) of the peptide linker, and 0.01g (2 eqv.) of hydroxybenzotriazole reacted overnight with 5mL of dimethyl sulfoxide (DMSO) while stirring in order to conjugate the peptide linker to Doxorubicin (Figure 4). The peptide linker was used to conjugate the Doxorubicin drug and the PSMA targeting agent. High-performance liquid chromatography was performed in order to separate unreacted material and recover the product of the reaction.

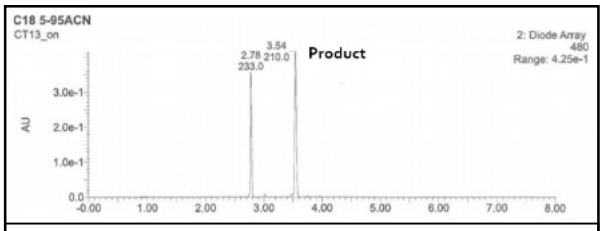

Figure 4: Liquid chromatography showing the product from the reaction on the right peak and unreacted Doxorubicin on the left peak (Figure by N. Luo).

Making the PSMA Targeting Agent and Conjugation to Doxorubicin Maleimide. In order to make the PSMA targeting agent, the following reaction was performed. 1.0g (1 eqv.) of H-Glu(OtBu)-OtBu·HCl (CAS Number: 32677-01-3), 0.87g (1 eqv.) of N,N'-disuccinimidyl carbonate (CAS Number: 74124-79-1), and 1.02g (3 eqv.) of triethylamine reacted in 20mL of

acetonitrile under argon for 1 hour in order to prevent the mixture from reacting with components of the air. Then, 1.26g (1 eqv.) of H-Lys(Z)-OtBu (CAS Number: 5978-22-3) and an additional 1.02g (3 eqv.) of triethylamine were added to the reacting mixture in order to complete the targeting agent. The reaction continued overnight, and the product PSMA targeting agent was collected. Next, 3-(Tritylthio)propionic acid (CAS Number: 27144-18-9) was added to the PSMA targeting agent in order to achieve conjugation with Doxorubicin maleimide. The reaction was performed with 1.299g (1 eqv.) of the product PSMA targeting agent, 1.02g (1.1 eqv.) of 3-(Tritylthio)propionic acid, 2.02g (2 eqv.) of HATU (CAS Number: 148893-10-1), and 0.688g (2 eqv.) of N,N-diisopropylethylamine. The reaction was performed in 10mL of dimethylformamide (DMF) overnight. DMF was chosen as the solvent for these reactions because organic compounds dissolve easily in DMF due to its polarity and hydrophilic properties. Trityl deprotection was performed by the mentor under hydrogen gas because the process could cause serious damage if not careful in dealing with the hydrogen gas. Finally, the Doxorubicin maleimide reacted with the PSMA targeting agent, and the new chemotherapeutic was successfully created for testing effects on cell uptake. 0.02g (1 eqv.) of the Doxorubicin maleimide, 0.0143g (2 eqv.) of the PSMA targeting agent after the addition of a thio group, and 0.0068g (3 eqv.) of N,N-diisopropylethylamine reacted in DMF under argon overnight (Figure 5). HPLC was then performed in order to retrieve the Doxorubicin conjugate product.

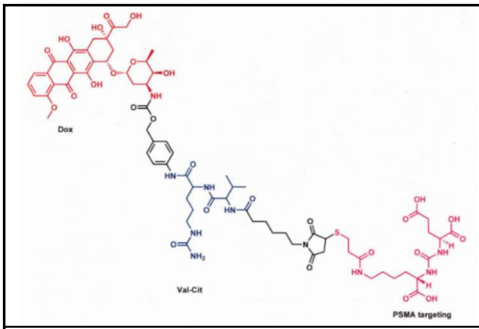

Figure 5: The Doxorubicin conjugate after reaction of Doxorubicin maleimide with the PSMA targeting agent (Figure by N. Luo).

Drug Loading onto Feraheme. Doxorubicin and the Doxorubicin conjugate were diluted in DMSO for loading onto Feraheme iron oxide nanoparticles. The Doxorubicin

conjugate was expected to load well onto Feraheme because of previous research showing the successful loading of Doxorubicin alone (Kaittanis, 2014). 30µL of 30mg/mL stock Feraheme was diluted in 370µL of 1x phosphate-buffered saline (PBS). The diluted Feraheme was then placed over a vortex mixer while 100µL of the Doxorubicin drug was loaded dropwise. A 100µM concentration of the drug could be loaded without obvious signs of precipitation of drug. Three washes were performed with PBS in order to filter out unloaded drug and PBS to obtain 100µL of the drug loaded onto Feraheme.

Plating and Treating Prostate Cancer Cells. The study utilized four different prostate lines: 22Rv1, LNCaP, Du145, and PC3. 22Rv1 and LNCaP cells (ATCC) were cultured in Roswell Park Memorial Institute (RPMI) media. Du145 cells were cultured in minimum essential medium (MEM), and PC3 cells were cultured in F-12K media. All cells were plated on 6-well plates for microscopy and flow cytometry. The treatment conditions tested were untreated, DMSO, Feraheme, Doxorubicin, Doxorubicin conjugate, Doxorubicin loaded onto Feraheme, and Doxorubicin conjugate loaded onto Feraheme. All cell lines were treated for 24 hours and were washed once before performing microscopy and flow. The 50µM and 100µM concentrations of the conjugate loaded onto Feraheme were tested, and 100µM treatments were repeated twice in order to obtain more data points and verify the results.

Microscopy and Flow Cytometry. Nikon Eclipse Ti-S microscope was used for the imaging of the four cell lines after 24 hours of treatment in order to examine the uptake of each treatment. 10x images were taken of the treated cells with the inverted microscope. Bright-field images were taken of all treatment conditions, and images under the 561nm channel were taken of the Doxorubicin treatments in order to detect Doxorubicin's natural fluorescence. The MACSQuant Analyzer 10 flow cytometer was used for performing flow cytometry in order to

quantify uptake of Doxorubicin by detecting fluorescence. The cells were trypsinized from the 6 wells and resuspended in 1mL of PBS because cells in PBS were easier to see under the microscope compared to cells in media. The B2 channel was used to detect the fluorescence of Doxorubicin. The flow cytometer was set to forward scatter 325V, side scatter 500V, and B2 channel (585/40nm filter) 700V for 22Rv1, LNCaP, and Du145 cells.The side scatter was decreased to 400V for PC3 cells in order to capture more of the cells in the data since the PC3 cells were smaller in size than the other cell lines.

Statistical Analysis

The p-values for cell viability assays from phase 1 of the study were found with unpaired t tests in order to determine if the changes in viability were significant. Each drug-loaded Feraheme condition was compared to cells treated with Feraheme alone. The t-test was performed with the analyze tool in the Prism software (GraphPad Software, 2018). In phase 2 of the study, analysis was performed on the flow cytometry in order to determine the percentage of cells showing fluorescence from Doxorubicin uptake.

Results (2018)

Cell Viability 24 Hour Treatment

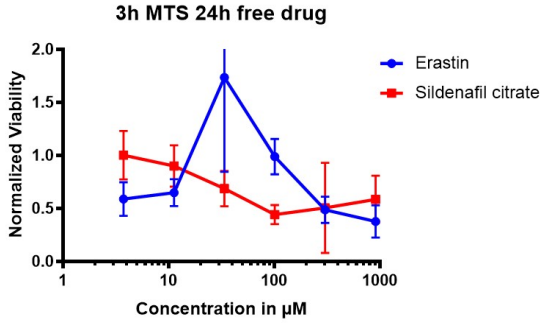

Figure 6: 22Rv1 cells treated with free Erastin experienced a drop in cell viability. A large error bar was observed for a concentration of 33.67 µM. Cells treated with free Sildenafil citrate also experienced a drop in viability. Error bars represent range of measured values. (Figure by N. Luo)

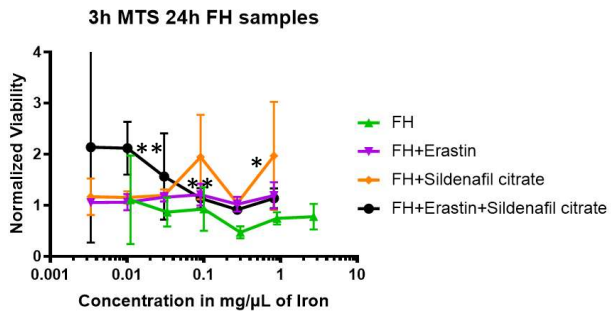

Figure 7: 22Rv1 cells treated with Feraheme showed no correlation between concentration and viability. A drop was seen in viability for cells treated with Feraheme loaded with both drugs. ($p<0.05$; $p<0.01$) Error bars represent range of measured values. (Figure by N. Luo)

22Rv1 cells treated with free Erastin showed a drop in viability because Erastin starved the cells of the necessary amino acid, Cystine (Figure 6). However, a huge error bar was seen for cells treated with Erastin at a concentration of 33.67 µM. As a result, the drop seen for cell viability with Erastin treatment is not reliable. A drop in the cell viabilities of cells treated with Sildenafil Citrate was seen from a concentration of 11.22 µM to 101.0 µM (Figure 6). The cancer cells show a significant drop in viability from treatment with Erastin-Sildenafil Citrate loaded Feraheme from concentrations of 0.010 mg/µL of iron to 0.27 mg/µL of iron ($p<0.01$; Figure 7). At similar iron concentrations, cell viability dropped for Feraheme loaded with both Erastin and Sildenafil Citrate but not Feraheme alone. Cells treated with Erastin-loaded Feraheme showed constant cell viabilities after treatment, indicating cell death after 24 hours ($p<0.01$; Figure 7). Cell viability was constant for most concentrations ($p<0.05$; Figure 7).

Western Blot of Sildenafil Citrate-Treated 22Rv1 Prostate Cancer Cells

A western blot was performed on 22Rv1 cells treated with free Sildenafil Citrate for 24 hours in order to determine how Sildenafil Citrate affected Ferroportin (FPN) levels. Three bands of FPN were seen in the western blot (Figure 8). In the first band, average normalized intensity decreased for both drug concentrations of 10 µM and 100µM Sildenafil Citrate compared to the control. It decreased from 7583.892 arbitrary units (A.U.) to 6974.17

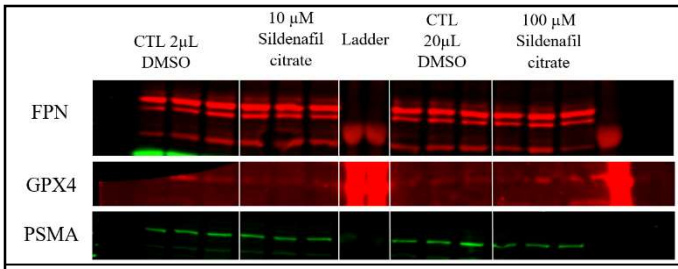

Figure 8: Western blot showed 3 bands of FPN signal. Band 1 of FPN refers to the top band, and band 3 refers to the bottom band of FPN. The reading revealed single bands of GPX4 and PSMA (Figure by N. Luo)

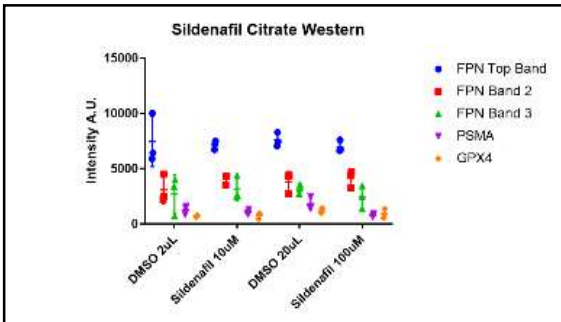

Figure 9: Changes in intensity of each band are shown. No changes were large enough to be significant. Intensity was measured in arbitrary units. (Figure by N.Luo)

A.U. in average FPN intensity for 100 μM drug (Figure 9). However, there was an increase in average Ferroportin (FPN) intensity in the second band for both concentrations. The third band showed an increase in intensity for a 10μM concentration of Sildenafil citrate while a decrease in intensity was shown for the 100 μM concentration. A decrease in average GPX4 and PSMA intensity was seen for both drug concentrations.

Discussion (2018)

Cell Viability 24 Hour Treatment

Both free Erastin and Sildenafil citrate induced cell death in 22Rv1 cells after 24 hours, and drug-loaded Feraheme was also shown to deliver cell death to 22Rv1 cells successfully. Cell death was induced in most cells treated with Erastin-loaded Feraheme because of the constant viabilities shown. The hypothesis that a combined loading of different drugs such as Sildenafil Citrate and Erastin would enhance treatment was refuted because Feraheme loaded with both Sildenafil citrate and Erastin did not show better performance compared to Erastin-loaded Feraheme and Sildenafil-citrate-loaded Feraheme. The hypothesis that drug-loaded Feraheme will perform better than free drug treatment was refuted due to the unknown amount of drug loaded onto the Feraheme particles, which meant it was not possible to compare their effects on cell viability.

Similar to Kaittanis in 2014, this study found that drug-loaded Feraheme induced cell death to prostate cancer cells. However, the previous study used LNCaP prostate cancer cells while 22Rv1 cells were used for this study, and this is important because different cell lines can have different sensitivities to similar treatments.

Western Blot

The western blot showed that FPN levels were inconsistent. It is possible that different concentrations of Sildenafil Citrate can affect FPN levels differently. Both PSMA and GPX4 expression decreased after treatment with Sildenafil citrate. FPN is a protein in charge of transporting iron out of a cell. A decrease in FPN levels would enhance the effects of ferroptosis, which is a type of programmed cell death associated with iron accumulation. Determining how Sildenafil citrate affects FPN levels in cells shows how it induces cell death.

Results (2019)

Microscopy after 24 Hour Treatment

LNCaP Cells. 10x images of these cells were taken in order to investigate the uptake of the different treatments such as Doxorubicin alone, Doxorubicin conjugate, and the drugs loaded onto Feraheme. Doxorubicin has a natural fluorescence which can be detected under the microscope. Most of the cells were alive after administering the treatments for 24 hours. This was expected because the cells were treated with low concentrations of Doxorubicin. The DMSO-treated and Feraheme-treated cells were similarly confluent as the untreated cells. The LNCaP cells showed a greater fluorescence with the Doxorubicin conjugate treatment under the microscope, indicating greater uptake of the drug by the cells (Figure 10). Signal was seen from various parts of the image for the Doxorubicin-treated cells and Doxorubicin conjugate FH-treated cells (Figure 10). The experiment was repeated with 10 μM treatments of the Doxorubicin drug. Lighter signal was

Figure 10: Microscopy images of the LNCaP cells after 24 hours of treatment with different controls and the 100 μM Doxorubicin treatments showing high fluorescence from Doxorubicin conjugate treatment (Figure by N. Luo).

seen from the LNCaP cells compared to the 100 μM treatments. However, bright spots of signal can still be seen from the cells which indicates uptake of the drug.

22Rv1 Cells. The cells were treated with the same conditions and similar concentrations of Doxorubicin as the LNCaP cells. 22Rv1 cells showed the greatest amount of fluorescence after treatment with Doxorubicin alone. The cells showed some signal after treatment with the Doxorubicin conjugate, Doxorubicin loaded onto Feraheme, and the conjugated loaded onto Feraheme. There was uptake of these treatments but not as much as the free Doxorubicin treatment.

PC3 Cells. These cells showed the most abundant signal after treatment with Doxorubicin alone. Fluorescence decreased when the drug was delivered with Feraheme which shows a decrease in uptake by the cells. No fluorescence was seen from the Doxorubicin conjugate and Doxorubicin conjugate loaded onto Feraheme treatment. The lack of fluorescence means that there was little to no uptake of the drug for these two conditions.

Du145 Cells. Similar to the PC3 cells, Du145 cells did not show fluorescence with most of the conditions. The signal was faint even with the Doxorubicin alone treatment. It is possible that the Du145 cells have trouble taking up Doxorubicin itself which is why there is a lack of fluorescence for all treatment conditions. The Du145 cells did not show much fluorescence under the microscope after treatment with 10 μM Doxorubicin.

Flow Cytometry

Flow cytometry was performed on all four cell lines in order to quantify the uptake of Doxorubicin into the cells. Since the microscopy images only show a small portion of the entire plate, it cannot be used to evaluate uptake accurately. The data shows that Doxorubicin alone showed the most signal as a result of uptake in all cell lines. The geometric mean recorded for

Doxorubicin was approximately four times greater than the geometric mean for the other treatments such as Doxorubicin conjugate and the conjugate loaded onto Feraheme. The only other significant difference between geometric means was seen in Du145 cells for the Doxorubicin conjugate treated and the untreated group. The Doxorubicin conjugate and the conjugate loaded onto Feraheme had a geometric mean of 9.99, while the untreated group

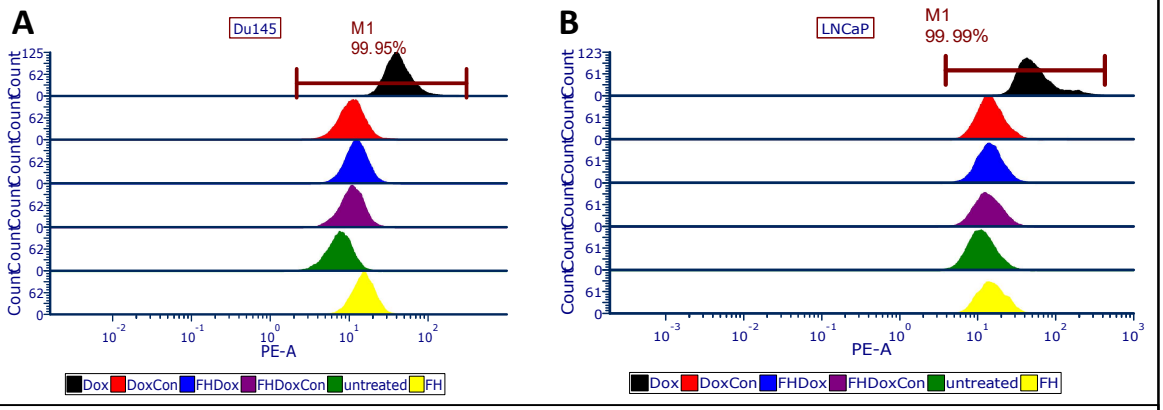

Figure 11: Flow cytometry data for 100 µM treatments of **A)** Du145 and **B)** LNCaP cells. The Y-axis represents cell count, and the PE-A signal of the X-axis represents signal from Doxorubicin (Figure by N. Luo).

showed a geometric mean of 6.95 (Figure 11A). The greater mean intensity indicates higher uptake of the treatment. The LNCaP cells did not show much variation between the geometric means for each treatment excluding Doxorubicin alone, and the values for geometric mean were only marginally different (Figure 11B).

Discussion (2019)

Microscopy Images after Treatment

The LNCaP cells showed the most fluorescence after treatment with the 100 µM Doxorubicin conjugate. This was expected because LNCaP cells express high levels of PSMA. 22Rv1 cells express low levels of PSMA while PC3 and Du145 cells do not express PSMA at all. The lack of PSMA in these two cell lines explain why there was no fluorescence after treatment with the Doxorubicin conjugate. However, the Du145 cells did not show much fluorescence even with the Doxorubicin alone treatment. This indicates that the Du145 cells have

a difficult time taking up Doxorubicin itself. The PC3 and Du145 cells showed little to no fluorescence with the Doxorubicin conjugate treatment at all the concentrations tested. The first hypothesis that the PSMA targeting agent could be attached to Doxorubicin was supported. The LNCaP cells treated with the Doxorubicin conjugate showed high fluorescence under microscopy, which represents high uptake of the treatment. Since LNCaP cells express high levels of PSMA, it supports the hypothesis that the targeting agent was successfully attached to Doxorubicin.

Flow Cytometry

The data from the flow cytometry shows that Doxorubicin alone had the most uptake in all four cell lines. The LNCaP cells showed a similar amount of signal for the 100 μM Doxorubicin conjugate and different concentrations of the conjugate Feraheme treatment. The data shows that the Doxorubicin conjugate was taken up by the prostate cancer cells, but the uptake did not exceed the amount of Doxorubicin alone taken up by the same cell lines. In addition, the Du145 cells showed a major difference between the 100 μM Doxorubicin conjugate and untreated cells, which is odd because Du145 cells do not express PSMA so, theoretically, there should not have been uptake. It is possible that the larger size of the Doxorubicin conjugate compared to free Doxorubicin hindered its uptake by the cells. The second hypothesis that the Doxorubicin conjugate loaded onto Feraheme can increase uptake by prostate cancer cells that express PSMA was refuted. The results show that the Doxorubicin conjugate and the loaded conjugate had similar uptake by both LNCaP and PC3 cells. Higher uptake was expected in LNCaP cells because these cells express PSMA while the PC3 cells do not.

Applications

The PSMA targeting agent was successfully attached to the Doxorubicin drug and delivered treatment to various prostate cancer cell lines. The targeting property of this compound will increase the specificity of the Doxorubicin drug and uptake of the drug by PSMA expressing cancer cells. It was shown that the Doxorubicin conjugate compound successfully loaded onto Feraheme with the fluorescence shown in the cells after treatment with the loaded conjugate. The addition of the targeting property and the loading onto Feraheme greatly allows the drug to be delivered safely to the cancer cells. There will be a decrease in the risks involving the drug going off-target and killing healthy cells as opposed to cancer cells. The conjugation reactions used to attach the targeting agent onto Doxorubicin can also be tested on other cancer treatment drugs. Doxorubicin was chosen because it has a natural fluorescence that can be easily detected inside the cells to determine uptake of the drug. It is also a common type of chemotherapy for many types of cancer.

Future Research

The microscopy images and flow cytometry data were all collected after 24 hours of treatment in this study. Different treatment times need to be tested on the LNCaP, 22Rv1, PC3, and Du145 cells in order to see how uptake differs as time passes. Also, it should also be determined where exactly the Doxorubicin fluorescence originates from the cells. It is possible that the fluorescence could be emitted from the surface of the cells, floating in the media, or actually coming from within the cells. Furthermore, higher resolution images could help make more accurate interpretations of the data. It was hard to differentiate signal from Doxorubicin and background signal in this study. Lastly, additional trials need to be performed on the different cell lines in order to confirm the results that were collected, such as performing

experiments with different concentrations of control treatments and repeating the 10 μM Doxorubicin treatments. This data will determine if the high fluorescence signal seen from LNCaP cells after treatment with the Doxorubicin conjugate is representative of cell uptake.

Conclusion

Most existing cancer treatments are non-targeted, meaning they attack both cancer cells and healthy cells. The goal of the study was to create a compound of Doxorubicin with a PSMA targeting agent attached in order to increase uptake by prostate cancer cells and decrease adverse effects to healthy cells. Microscopy and flow cytometry was then performed on these cells in order to determine and compare uptake of the compound to different control treatments. The Doxorubicin conjugate was made with numerous overnight reactions to link the targeting agent with Doxorubicin free base through a peptide linker. Dilutions of the resulting compound were made and were loaded onto Feraheme for the testing of different treatments. The results show that the Doxorubicin conjugate successfully delivered to PSMA expressing prostate cancer cells such as the LNCaP cells which was seen from the microscopy images. Flow cytometry data shows that Doxorubicin showed the greatest fluorescence for all 4 cell lines. The LNCaP cells did not show much higher uptake of the Doxorubicin conjugate compared to the PC3 cells, which do not express PSMA. In conclusion, this study showed that a targeted drug was successfully created and was able to deliver therapy to prostate cancer cells in vitro. It is possible to use similar conjugation reactions for other therapeutics in order to decrease their harmful effects to healthy cells while still delivering treatment to cancer cells. This can increase the efficacy of all chemotherapeutic treatments and save countless lives.

Works Cited

Daldrup-Link, H. E. (2017, September). Ten Things You Might Not Know about Iron Oxide Nanoparticles. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/28825888

Heckert, B., Banerjee, T., Sulthana, S., Naz, S., Alnasser, R., Thompson, D., . . . Santra, S. (2017, March 21). Design and Synthesis of New Sulfur-Containing Hyperbranched Polymer and Theranostic Nanomaterials for Bimodal Imaging and Treatment of Cancer. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/29104818

Kaittanis, C., Bolaender, A., Yoo, B., Shah, N., Ouerfelli, O., & Grimm, J. (2017, November 08). Targetable Clinical Nanoparticles for Precision Cancer Therapy Based on Disease-Specific Molecular Inflection Points. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/29035540

Kaittanis, C., Shaffer, T. M., Ogirala, A., Santra, S., Perez, J. M., Chiosis, G., . . . Grimm, J. (2014, March 04). Environment-responsive nanophores for therapy and treatment monitoring via molecular MRI quenching. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/24594970

Kalia, J., & Raines, R. T. (2010, January). Advances in Bioconjugation. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2901115/

Ruggiero, A., Holland, J. P., Hudolin, T., Shenker, L., Koulova, A., Bander, N. H., . . . Grimm, J. (2011, October). Targeting the internal epitope of prostate-specific

membrane antigen with 89Zr-7E11 immuno-PET. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/21908391

Santra, S., Kaittanis, C., Grimm, J., & Perez, J. M. (2009, August 17). Drug/dye-loaded, multifunctional iron oxide nanoparticles for combined targeted cancer therapy and dual optical/magnetic resonance imaging. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/19384879

Spicer, C. D., & Davis, B. G. (2014, September 05). Selective chemical protein modification. Retrieved from https://www.nature.com/articles/ncomms5740

Weissleder, R., Nahrendorf, M., & Pittet, M. (2014, February). Imaging macrophages with nanoparticles. Retrieved July 19, 2018, from https://www.ncbi.nlm.nih.gov/pubmed/24452356

Yuan, H., Wilks, M. Q., Normandin, M. D., El Fakhri, G., Kaittanis, C., & Josephson, L. (2018, February). Heat-induced radiolabeling and fluorescence labeling of Feraheme nanoparticles for PET/SPECT imaging and flow cytometry. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/29370158