Influence of Histone Deacetylase Inhibitors on Polymer Mediated Transgene Delivery

by

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ABSTRACT

The effects of specific histone deacetylase inhibitors (HDACi) on transgene expression in combination with a novel polymer as a delivery vehicle are investigated in this research. Polymer vectors, although safer than viruses, are notorious for low levels of gene expression. In this investigation, the use of an emerging chemotherapeutic anti-cancer drug molecule, HDACi, was used to enhance the polymer-mediated gene expression. HDACi are capable of inhibiting deacetylation activities of histones and other non-histone proteins in the cytoplasm and nucleus, as well as increase transcriptional activities necessary for gene expression. In a prior study, a parallel synthesis and screening of polymers yielded a lead cationic polymer with high DNA-binding properties, and even more attractive, high transgene expressions. Previous studies showed the use of this polymer in conjunction with cytoplasmic HDACi significantly enhanced gene expression in PC3-PSMA prostate cancer cells. This led to the basis for the investigation presented in this thesis, but to use nuclear HDACi to potentially achieve similar results. The HDACi, HDACi_A, was a previously discovered lead drug that had potential to significantly enhance luciferase expression in PC3-PSMA cells. The results of this study found that the 20:1 polymer:plasmid DNA weight ratio was effective with 1 μM and 2 μM HDACI_A concentrations, showing up to a 9-fold enhancement. This enhancement suggested that HDACi_A was effectively aiding transfection. While not an astounding enhancement, it is still interesting enough to investigate further. Cell viabilities need to be determined to supplement the results.

DEDICATION

To my granddad,
my mom, dad, and brother

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Without the help and support of many people, this research could not have happened, and I would like to ensure that they are acknowledged for all their contributions. First of all, I would like to extensively thank Dr. Kaushal Rege for allowing me to conduct research in his laboratory at Arizona State University. I came to him as a sophomore in my undergraduate studies to let me volunteer in his lab in order to gain experience conducting research. This opportunity allowed me to enter the Fulton Undergraduate Research Initiative, which provided funding and additional experience working as an undergraduate researcher. That experience was invaluable and taught me more than I could ever have learned in a classroom and gave me the confidence to pursue an undergraduate thesis for the Barrett Honors College. Aside from the lab space, Dr. Rege's undying belief in me and my work kept me motivated to achieve the results I obtained for this thesis. I would also like to thank Dr. Sutapa Barua for being my mentor as soon as I stepped foot into the lab. Her guidance from basic proper laboratory protocol to superior techniques in conducting this research was more than I could ever have hoped for. Somehow, on top of all her many projects, she was able to find the time and patience to train me, without a hint of hesitation. She became my guru in transfection studies and was always able to provide answers to any problems I encountered. Her passion for this field of study is limitless and I hope that part of her dedication and expertise has rubbed off onto me in the process of working alongside her. I would also like to acknowledge the Rege lab group, especially for their emotional support and general day to day camaraderie. I

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Chapter 1

INTRODUCTION

Prostate cancer was estimated to present in 240,890 new cases and cause 33,720 deaths in 2011, making it a leading cancer-related cause of death (second to lung cancer) in males in the United States (Howlader *et al*., 2010). While it can be treated adequately if caught early enough, there still exist the not uncommon possibilities that the cancer may be inoperable, unresponsive to current treatments, or even recur after initial treatments. The problem, thus, lies in not only the effectiveness of drug and treatment options for prostate cancer, but also in the quality of life for the patient. While conventional techniques have proven successful in the eradication of this disease, the side effects are detrimental to the patient (Madalinska *et al*., 2001). Such major health risks arise from the high toxicity of the drugs and the higher doses needed to overcome their low efficacy nature (Fisher *et al*., 1999). In order to lessen these harmful health risks, a shift in alternative approaches to combat cancer has become exceedingly more prevalent in the last few decades. Gene therapy, in particular, has become the primary approach to do just that.

Chapter 2

BACKGROUND

2.1 Gene Therapy

Gene therapy aims at correcting a disease at a genetic level in order to alter the particular cells that cause disease, implant a new gene to kill the disease, or change other cells in the body in order to fight the disease more efficiently (Mayo Clinic, 2010). It is basically the process of delivering exogenous nucleic acids to diseased cells, which has emerged as a unique process to treat diseases that are both acquired and genetic. Recent studies have shown substantial progress in effective gene therapy that could prove its widespread use to treat diseases in the near future. Gene therapy in eye diseases has shown great success, for example. Recombinant adeno-associated virus vectors have a range of effects for retinal gene transfer, lentiviral vectors mediate efficient sustained expression in retinal pigment epithelium, corneal endothelium and trabecular meshwork, and even non-viral methods are used in improving gene transfer in the retina (Bainbridge, Tan, & Ali, 2006). More specifically, a recent study of canines with Leber congenital amaurosis (a severe inherited form of retinal degeneration that induces severe visual impairment that leads to blindness), found that 16 of 18 treated eyes showed rescue vision and improved retinal function, after treatment of a genetically altered recombinant adeno-associated viral vector (Annear *et al.*, 2011). Progress such as these show just how prospective gene therapy is for disease treatments.

Gene therapy most often uses a genetically altered DNA (in viral methods) and the negatively charged plasmid DNA (pDNA). A plasmid DNA is a large macromolecule (3-15 kbp) that is capable of encoding for a specific gene that could express its

complement protein within a target cell via transcription. The major obstacles preventing delivery, however, are the biological barriers presented by the target cell. An adequate vehicle is essential to propel the plasmid DNA through the cell membrane (without succumbing to attack by the immune system), transport it through the cytoplasm, and finally facilitate it through the nuclear membrane to achieve efficient transcription and translation. Therefore, in order ensure optimal transfection of a gene, the optimal delivery system should be able to the following: bind and transport DNA to target cells, evade the body's immune response, release DNA in the cytoplasm, transport DNA through the cytoplasm, and transfer DNA across the nucleus (Barua, 2011).

2.2 Cancer Gene Therapy

The application of gene therapy in cancer diseases has been a rapidly growing research field since the first clinical trial was performed by Steven A. Rosenburg and R. Micahel Blaese's group in 1990. Successful tumor regression in patients with metastatic melanoma was achieved in their study, which paved the way for future use of gene transfer in cancer therapies (Rosenberg *et al*., 1990). Over the past couple of decades, substantial progress has been made in this field, with about 65% of the approved 1644 gene therapy clinical trials used to treat cancer (Edelstein, 2012).

There are four phases of clinical trials, where phase I is screening for safety, phase II is establishing the testing protocol, phase III is final testing, and phase IV is post-approval studies. Currently, phase I, phase II and a combination of phase I/II trails are the most common clinical trials conducted with gene therapy. Most of these gene therapy applications are in the primary stages of clinical trial, but show great promise towards the latter stages.

In order to achieve efficient gene delivery, an appropriate vector is necessary, as stated previously. Currently, the most commonly used vectors in clinical trials are viral, followed far behind by non-viral (Edelstein, 2012). However, these do not possess all the desirable properties of effective and safe delivery of transgenes to their target cells in large quantities. Yet, both types of vehicles have their unique set of advantages and disadvantages that determine their use in gene therapy.

2.3 Viral Vectors

Viral vectors are viruses that have evolved over time to specifically function as a vehicle to deliver genetic data into their hosts in order to replicate. This specialized evolution has made viruses the most efficient delivery system of genes and it is used by many researchers who thought that domesticating viruses was a feasible approach. Many viral vectors in current use are (in order of most abundant) adenovirus, retrovirus, vaccinia (pox) virus, adeno-associated virus (AAV), herpes simplex virus (HSV), and lentivirus (Edelstein, 2012). While they may be the most efficient vector, viruses pose major safety risks, primarily due to their high immunogenicity effect. This became a well-known problem when a young patient died in 1999 after being treated with adenoviral vectors in an effort to treat an inherited enzyme deficiency (Marshall, 1999). Again in 2002 and 2003, young children suffering from an X-linked SCID-XI syndrome were treated with a retroviral gene therapy and later developed leukemia as a result of the viral mutagenesis (Thomas, Ehrhardt, & Kay, 2003). Clinical trials, such as these, have demanded a shift away from the use of viral vectors, since gene therapy is aimed to provide an alternative to current detrimental anti-cancer methods, not induce more harmful side effects. Aside from posing major health risks, viral vectors have additional

drawbacks such as: low DNA load capacity, relatively expensive production, and difficulty to produce in mass quantities (Atkinson & Chalmers, 1010). Despite these implications, viruses still remain the most widely used vectors in over two-thirds of clinical gene therapy (Edelstein, 2012).

2.4 Non-Viral Vectors

Non-viral vectors, on the other hand, have no limitations with transgene load capacity and provide a safer alternative to viruses. However, non-viral vehicles are notorious for the low efficiency of target and delivery to specific tissues, and tend to invoke dose-dependent inflammatory responses (Zhao *et al*., 2003; Norman *et al*., 2000). Many non-viral vectors have been explored for use in gene delivery including cationic molecules of lipids (Wansungu & Hoekstra, 2006), polyamines (Osland & Kleppe, 1977), and polymers (Lungwitz *et al*., 2005) Such polymers are capable of condensing DNA into complexes on a nanoscale level by neutralizing negative charges on DNA in aqueous solutions (Smedt, Demeester, & Hennink, 1999). The formed complexes also possess a positive charge that can interact with the cellular membrane to effectively achieve cellular uptake.

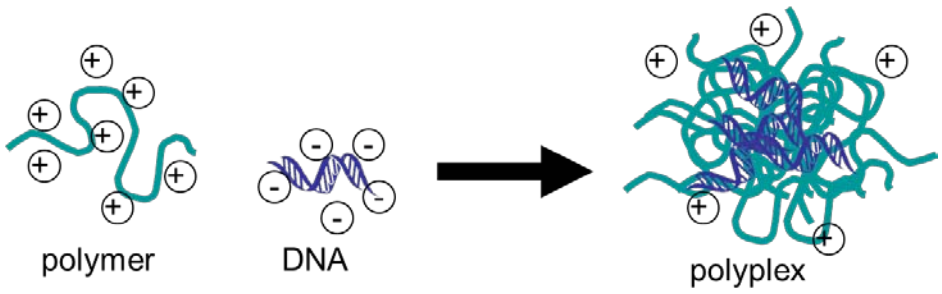

Figure 2.1. Formation of a polyplex through electrostatic interactions between the positively charged polymer and negatively charged DNA.

Cationic polymers, in particular, are of great interest due to their potential advantages of easy production, stability, low immunogenicity and toxicity, and large DNA loading capacity (Ledley, 1995). Most cationic polymers contain a positively charged, protonated amine group that has electrostatic interactions with the negatively charged phosphate groups of DNA which allow for spontaneous formation of complexes, termed polyplexes (see Figure 2.1). When these positively charged polyplexes come in contact with the negatively charged cell membrane, they allow for increased DNA uptake that can substantially enhance transfection. The most widely used cationic polymer traditionally used in transfection studies are poly(L-lysine) (PLL) (Wu & Wu, 1998), polyethyleneimine (PEI) (Abdallah *et al*., 1996), chitosan (Roy *et al*., 1999), poly(vinyl pyrrolidone) (Mendiratta *et al*., 1999), and polyamidoamide (PAMAM) (Qin *et al*., 1998). The main drawback to the use of these particular cationic polymers is their high cytotoxicity. This problem led to a parallel synthesis and screening of polymers by Barua *et al*. in 2009, to identify a cationic polymer that was biocompatible, degradable, and could effectively transport DNA to enhance transgene expression. The study pointed to a one such polymer, 1,4C-1,4Bis (Figure 2.2), which was based on the monomers 1,4-cyclohexanedimethanol diglycidyl ether (1,4C) and 1,4-bis(3-aminopropyl) piperazine (1,4Bis). The properties of this particular cationic polymer showed transfection enhancements much higher than the currently employed, pEI-25, in human prostate cancer cells and murine osteoblasts and thus was chosen as the polymer for all transfections in this study (Barua *et al*., 2009).

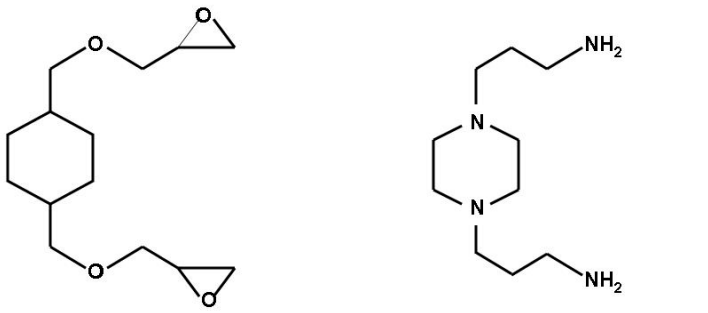

Figure 2.2. Monomers of the identified lead polymer 1,4C-1,4Bis.

2.5 Cellular Barriers

Efficacy of delivery is lost at each step of the gene transfection process, so it is important to understand the barriers within the cell and with cellular delivery in order to develop successful vectors for an efficient gene delivery method. While there are biological barriers prior to arriving at the target cell, including serum stability, cell-specific targeting and route of administration, the primary focus of this investigation is on intracellular uptake of polyplexes as depicted in Figure 2.3.

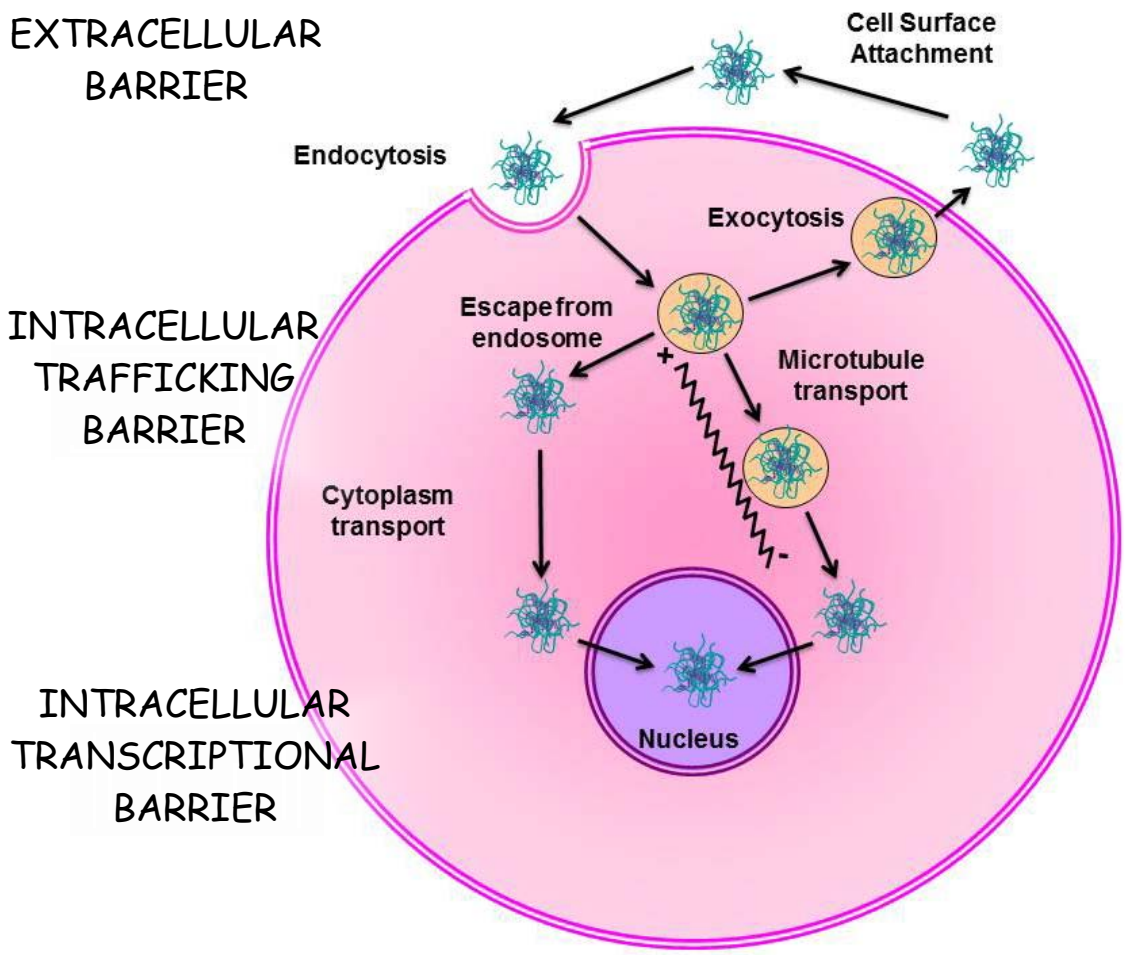

Figure 2.3. The major cellular barriers that non-viral delivery methods must overcome to achieve transgene transfection: extracellular, intracellular trafficking, and transcriptional (adapted from Pack *et al*., 2005).

2.5.1 Extracellular Barrier

The first biological roadblock, once the vector reaches the target cell, is the extracellular barrier. The cell's plasma membrane is comprised of a lipid bilayer, laden with integral proteins that selectively regulate molecules. Polyplexes tend to electrostatically interact with the cellular membrane, due to the positively charged cationic polymer and negatively charged glycoproteins, proteoglycans and glycerophosphates on the cell surface (Morille, 2008). The method of cellular uptake of

polymer complexes seems to point towards non-specific adsorptive endocytosis followed by the clathrin-coated pit mechanism (Morille, 2008).

Endocytosis is basically the invagination of the cellular membrane to transport extracellular molecules through the cytoplasm. Adsorptive endocytosis does not necessitate ligand-receptor interactions, but rather an unspecified physical adsorption of the complex at the cell surface (Jung *et al*, 2000). Complexes that are positively charged on the surface seem to undergo adsorptive endocytosis due to the electrostatic interactions (Merdan, Kopecek, & Kissel, 2002). Clathrin-dependent endocytosis occurs at a specific domain in the cellular membrane where a clathrin protein is present (Mukherjee, Ghosh, & Maxfield, 1997). This area of the membrane is referred to as a clathrin-coated pit (Soenen *et al*., 2009). Invagination of the plasma membrane at this particular point yields a vesicle that is clathrin-coated, but the vesicle tends to lose the sheath once it encounters other endosomes in the cytoplasm (Lungwitz *et al*. 2005).

Endocytotic pathways are largely dependent upon cell membrane composition, surface charge, and size of the complexes (Rejman *et al*., 2004). This can make it hard to pinpoint the exact mechanism of the endocytotic uptake of polyplexes into the target cell. This complication is most likely attributed to the highly complicated and iterative events that sort molecules for transportation across the membrane to compartments within the cell (Jung *et al*., 2000).

2.5.2 Intracellular Trafficking Barrier

The next major hurdle for gene delivery is the intracellular trafficking barrier. Cytoplasmic trafficking also plays a large role in determining whether or not the complexes will be efficiently delivered. Once the polyplexes have been endocytotically

uptaken into the cell, microtubules fibers essentially carry the encapsulated endosomal vesicle down its 'tracks' (Rejman *et al*., 2004; Musch, 2004). There are two motor proteins, dynein and kinesin, that facilitate the direction the vesicles are carried within the cytoplasm (Suomalainen *et al*., 1999). Dynein transports vesicles towards the nucleus (minus end of microtubules) while kinesin transports vesicles in the opposite direction, away from the nucleus and towards the cell membrane (plus end of microtubules). Bi-directional movement can also occur with utilization of both motor proteins (see Figure 2.4). In a particular study, when the microtubules were disrupted (using colchicine) or either of the motors was inhibited, transgene expression declined drastically (Drake & Pack, 2008). This finding suggests that microtubules and their motor proteins are essential to effective polymer-mediated gene delivery.

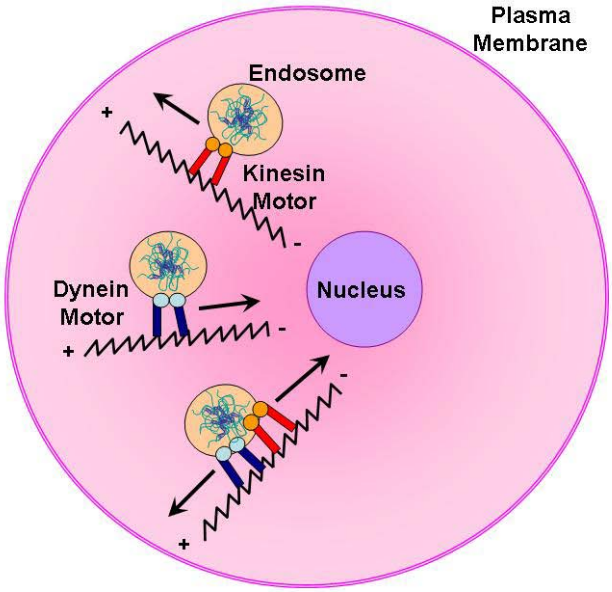

Figure 2.4. Intracellular trafficking of endosome vesicle, containing polyplexes, along microtubule 'tracks' via kinesin motor, dynein motor, or switching between both.

Transportation problems associated with intracellular trafficking also occur with the need for DNA to escape the endosomes that encompass the polyplexes. The complexes generally follow the endolysosomal pathway that begins after endocytotic uptake, with the early endosomes, to the late endosomes, and finally ending in the lysosomes where the complexes have no effect in the cell (Merdan *et al*., 2002). The problem is that polyplexes readily accumulate in the lysosomal compartment so the DNA is unable to reach the nucleus (Bieber *et al*., 2002). Early endosomal escape is one way to achieve this, so the DNA can safely evade degradation by the lysosomal environment of nucleases and acidic pH (Barua, 2011). One proposed theory of how this can occur is by the 'proton sponge hypothesis' (Boussif *et al*., 1995). The proton sponge effect (shown in Figure 2.5) is theorized to inhibit acidification of endosomal pH by neutralization of protons pumped by an active membrane transporter, which results in chloride counter ions flooding into the endosomes, thus increasing the osmotic pressure that causes swelling, and eventually leads to bursting of the membrane (Akinc *et al*., 2005;, Yamashiro, Fluss, & Maxfield, 1983; Al-Dosari & Gao, 2009). This theory has been supported indirectly by a noticeable decrease of transfection efficacy of polyplexes containing the polymer, PEI, in cells containing drugs that prevented endosome acidification (Rittner *et al*., 2002; Kichler *et al*, 2001). Further support of this theory has been through the research by Sonawane and colleagues which revealed polymers with protonable amines induced substantial increases in chloride ion concentration and the number of endosomes containing polyplexes, while a polymer without protonable amines had no remarkable effects (Sonawane, Szoka, & Verkman, 2003).

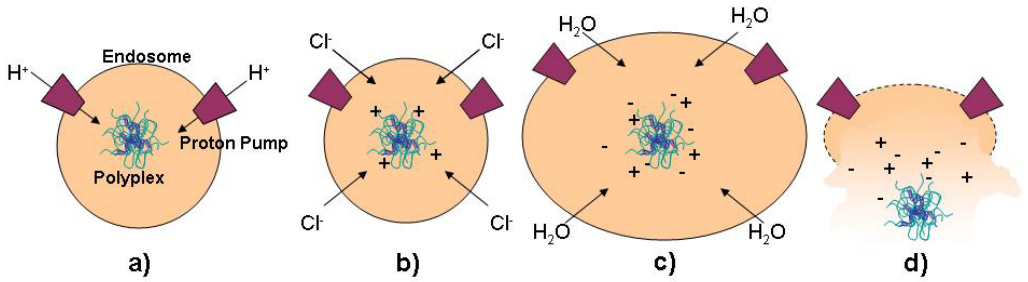

Figure 2.5. Proton sponge theory where the proton pump draws protons inside the endosome (a), which results in an influx of chloride ions (b), followed by an increased osmotic pressure (c) that ultimately causes the membrane to burst and expel the contained polyplex (d) (adapted from Pack *et al*., 2005).

2.5.3 Transcriptional Barrier

After passing all the way through cell, the final obstacle for the pDNA to overcome is the transcriptional barrier. A vector carrying the pDNA may pass through the cellular membrane, evade lysosomal degradation, and have proper microtubule transport through the cytoplasm. But without the pDNA actually penetrating through the nuclear membrane, absolutely no expression can take place. This physical characteristic of the nuclear membrane seems to be one of the limitations since it is composed of nuclear pores that are quite small (~25 nm diameter). Thus the translocation of pDNA from the cytoplasm to the inside of the nucleus is size-dependent as depicted by the nuclear diffusion of oligonucleotides through nuclear pore complexes (NPC) (Elouahabi & Ruysschaert, 2005).

The proposed mechanisms as to how polyplexes can cross the nuclear membrane are passive diffusion, active transport through DNA nuclear pores, or DNA entry during cell division (Elouahabi & Ruysschaert, 2005). The passive mechanism is effective for DNA fragments less than 250 bp via simple diffusion with no specific interactions (Mesika *et al*., 2005). The active transport method usually caters to larger DNAs (40 – 60 kDa) through nuclear pore complexes (NPCs). The two possible mechanisms that

facilitate the active transport are *cis*-acting, DNA sequence-dependent transport and *trans*-acting mechanism requiring addition of peptides (Elouhabi & Ruysschaert, 2005). However, this active transport has not been a very efficient process to transport large DNA fragments across the nuclear membrane. Finally, there is the possibility of DNA translocation via simple diffusion during cell cycle division. It has been shown that there is higher transfection efficiency when cells are at or near the M phase (mitosis) of the cell cycle at the time of polyplex transfection (Brunner *et al*,, 2000; Brunner *et al*., 2002; Wilke *et al*., 1996; Tseng, Haselton & Giorgio, 1999; Mortimer *et al*., 1999). However, this is a variable factor with different types of polymers, even between linear and branched PEI, that was seen in the work conducted by Brunner and his group (Brunner *et al*., 2000). Some cationic polymers, however, are independent of cell cycle stage in efficiently delivering transgenes to the nucleus. Since DNA cannot survive very long in the cytoplasm due to the presence of cytoplasmic nucleases, the uptake of pDNA into the nucleus is vital to effective gene expression, regardless of the method used (Lechardeur *et al*., 1999).

2.6 Histone Deacetylase Inhibitors

Histone deacetylases, (HDACs) are a type of enzyme that deacetylase lysine residues of histones, which remove charge-neutralizing acetyl groups from histone lysine tails, and subsequently cause the tight coiling of chromosomes around the histone proteins. Many HDACs have been discovered thus far and can be organized into four major classes (see Table 2.1). All classes, except class III, are zinc-dependent HDAC (Marks & Xu, 2009). The main difference between class I and class II HDACs appear to be that class I seem to primarily work with cell survival and proliferation, while class II

work with specific tissues (Marks & Xu, 2009). What makes the HDACs 1 and 2 seem to be found exclusively in the nucleus, is the characteristic nuclear localization signal (NLS), but no nuclear export signal (NES) (de Ruijter *et al*., 2003). Class II HDACs, however, possess both NLS and NES motifs, allowing for shuttling between cytoplasm and nucleus (Hess-Stumpp *et al*., 2007). Class III HDACs, also referred to as selective internal radiation therapies (SIRTs), are NAD+-dependent (Johnstone, 2002). Class IV HDACs have recently been discovered to show features of class I and II enzymes (Hess-Stumpp *et al*., 2007).

Table 2.1 Known HDAC classes, functions and relevant HDACi that inhibit their functions (adapted from Rasheed, Johsntone & Prince, 2007)

Class	Enzyme	Location	Function	Relevant HDACi
I	HDAC1 HDAC2	Nucleus	Participate in Sin3, NuRD and Co-REST complex	SK-7041, SK-7068, MS-275, VPA, romidepsin butyrate, trapoxin, SAHA, TSA, PXD-101, LBH-589, LAQ-824, and MGCD-0103
	HDAC3	Nucleus, rarely in cytoplasm	Participate in SMRT, N-CoR complex	MS-275, VPA, butyrate, trapoxin, SAHA, TSA, PXD-101, LBH-589, LAQ-824 and MGCD-0103
	HDAC8	Nucleus		VPA, butyrate, trapoxin, HDACI_B, TSA, PXD-101, LBH-589 and LAQ-824
IIa	HDAC4	Nucleus, Cytoplasm	Interaction with SMRT/N-CoR and co-repressors BcoR and CtBP	Romidepsin, VPA, butyrate, trapoxin, SAHA, TSA, PXD-101, LBH-589 and LAQ-824
	HDAC5			VPA, butyrate, trapoxin, SAHA, PXD-101, LBH-589, and LAQ-824
	HDAC7			VPA, butyrate, trapoxin, SAHA, PXD-101, LBH-589, and LAQ-824
	HDAC9		Muscle differentiation	VPA, butyrate, trapoxin, SAHA, PXD-101, LBH-589, and LAQ-824
IIb	HDAC6	Cytoplasm	Tubulin deacetylase	Romidepsin, tubacin, SAHA, TSA, PXD-101, LBH-589 and LAQ-824
	HDAC10	Nucleus, Cytoplasm		Tubacin, SAHA, TSA, PXD-101, LBH-589 and LAQ-824
III	SIRT1-7			
IV	HDAC11	Nucleus, Cytoplasm		SAHA, TSA, PXD-101, LBH-589, LAQ-824 and MGCD-0103

Histone deacetylase inhibitors (HDACi) are a new class of chemotherapeutic agents that inhibit HDAC action. Extensive research has shown that HDACs are related to repressed gene transcription and repressed expression of tumor suppressor genes (Marks *et al*., 2001; Carew, Giles, & Nawrocki, 2008). This is noted by the high HDAC levels that result in an increase of cancer cell proliferation, tight histone binding around DNA, and transcription inhibition (Marks *et al*., 2001). Since HDACs, HDAC1 in particular, are observed to be over-expressed in prostate cancer, they are an attractive target for anti-cancer gene therapy (Halkidou *et al*., 2004). While most HDACi in current clinical trials are being used for their primary enzymatic inhibition property, HDACi have also been found to have other mechanisms of action, based off of the structural diversity among the HDACi (Marks & Xu, 2009). HDAC can also bind to, deacetylate and regulate the activity of many other non-histone proteins , such as transcription factors (p53, E2F transcription factor (1E2F1), and nuclear factor-κB (NF-κB)) and other cellular proteins (α-tubulin, Ku70, and Hsp90) (Bolden, Peart, & Johnstone, 2006).

Zinc-dependent HDACi include most of the HDACi in classes I, IIa, IIb, and V. There are generally three common structural characteristics in these molecules: a zinc binding moiety, an opposite capping group, and a straight chain alkyl, vinyl, or aryl linker that connects the two. It has been found that these functional groups interact with three conserved regions of the active site for certain zinc-dependent HDACi (Finnin *et al*, 1999; Somoza *et al*., 2004; Vannini *et al*., 2004). The zinc ion facilitates amide hydrolysis and is found at the bottom of the catalytic pocket, the hydrophobic tunnel is penetrated by an acetyl-lysine substrate, and the channel opening contains the rim interaction with the hydrophobic capping group (Marks & Xu, 2009).

2.7 Combinational Therapy

Conventional cancer treatments that utilize only one therapy are rarely effective curative measures for the disease. However, when gene therapy is used in conjunction with traditional methods, outcomes of patients have shown clear benefits in clinical trials (O'Shaughnessy *et al*., 2002). While the use of cationic polymers to deliver exogenous DNA into cancer cells have proven to be somewhat effective by themselves, a combination with chemotherapeutic drugs is hypothesized to synergistically enhance transgene expressions (Kasman, Lu, & Voelkel-Johnson, 2007). In this research, it is proposed that a combinational therapeutic approach that uses the novel cationic polymer, 1,4C-1,4Bis and new class of chemotherapeutic agents (HDACi) can enhance transgene expression by altering intracellular trafficking and transcription regulation.

Chapter 3

PREVIOUS STUDIES

3.1 Cytoplasmic HDACi Investigations

3.1.1 Introduction

In a previous study conducted by Barua and Rege in 2010, the HDAC6 inhibitors, tubacin (cytoplasmic HDAC inhibitor) and Trichostatin A (TsA) (cytoplasmic and nuclear HDAC inhibitor) were evaluated for their combinational gene therapy effectiveness. Cytoplasmic HDACs, specifically HDAC6, deacetylates α-tubulin and regulates dynein motor transport on microtubules, thus mediating intracellular trafficking of cargo to the microtubular organizing center (MTOC), which had the potential to increase polyplex transport to deliver DNA for transcription (see Figure 3.1).

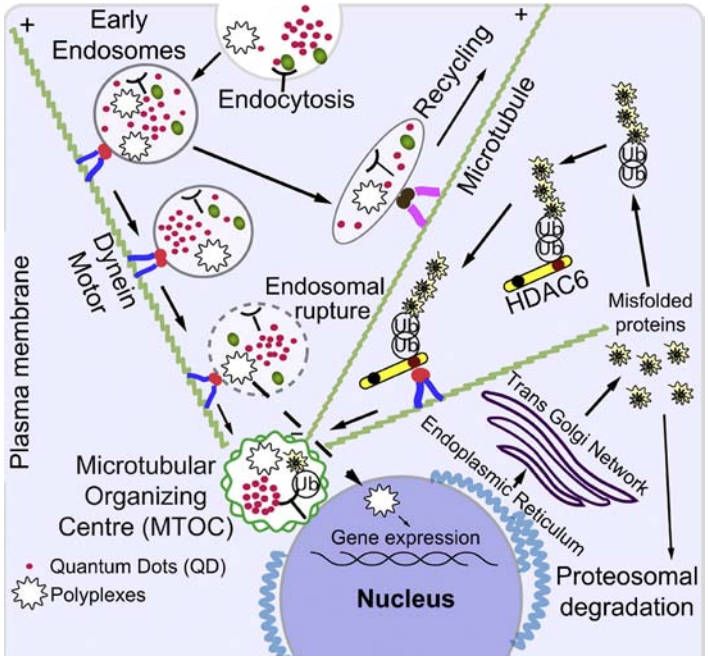

Figure 3.1. Schematic of the uptake, sorting and localization of polyplex and quantum dots inside a cell. Intracellular localization of polyplexes at or away from the perinuclear recycling compartment (PNRC)/microtubule organizing center (MTOC) was equated to transfection in PC3-PSMA cells. (Barua & Rege, 2010).

3.1.2 Research Findings

Tubacin acts as an inhibitor of this α-tubulin deacetylation, which results in more stable microtubules and better support within the cell, due to the increased dynein and kinesin motors for the microtubules. In this particular study, tubacin showed up to a 40-fold transgene expression enhancement at 4 μM concentration and a polyplex ratio of 25:1 (Figure 3.2) in PC3-PSMA cells. This is a significant enhancement, but the 10:1 polyplex ratio only gave up to a 5-fold enhancement. This suggested that while mediators of intracellular trafficking have been known to enhance transgene expression, the overall enhancement may also be due to polyplex size, intracellular localization profiles, and corresponding basal levels of transgene expression. Tubacin seems to have opposing motor activity when in a polyplex in cellular vesicles, resulting in more time for polyplexes or dissociated plasmid DNA to escape into the cytoplasm. This may have led to higher transgene expression as seen for the 25:1 polyplexes.

The other HDACi evaluated in this study was Trichostatin A, a class I and II HDACi, which allows for inhibition of cytoplasmic and nuclear activity. In the cytoplasm, it acts similarly to tubacin in inhibiting HDAC6. In the nucleus, it acetylates histones to enhance transcription and transgene expression. TsA has shown to act in repositioning plasmid DNA towards sites that are transcriptionally active in the nucleus. In PC3-PSMA cells, TsA showed up to a 35-fold transgene expression enhancement at a 250 nM concentration and a 25:1 polyplex ratio (Figure 3.2). Similar to tubacin, the 25:1 polyplexes showed a significantly higher expression enhancement than the 10:1 polyplex ratio. These results seem to support the mechanisms mentioned above. However, other

mechanisms, such as promoter activation, may also play a role in the enhancement seen in the study.

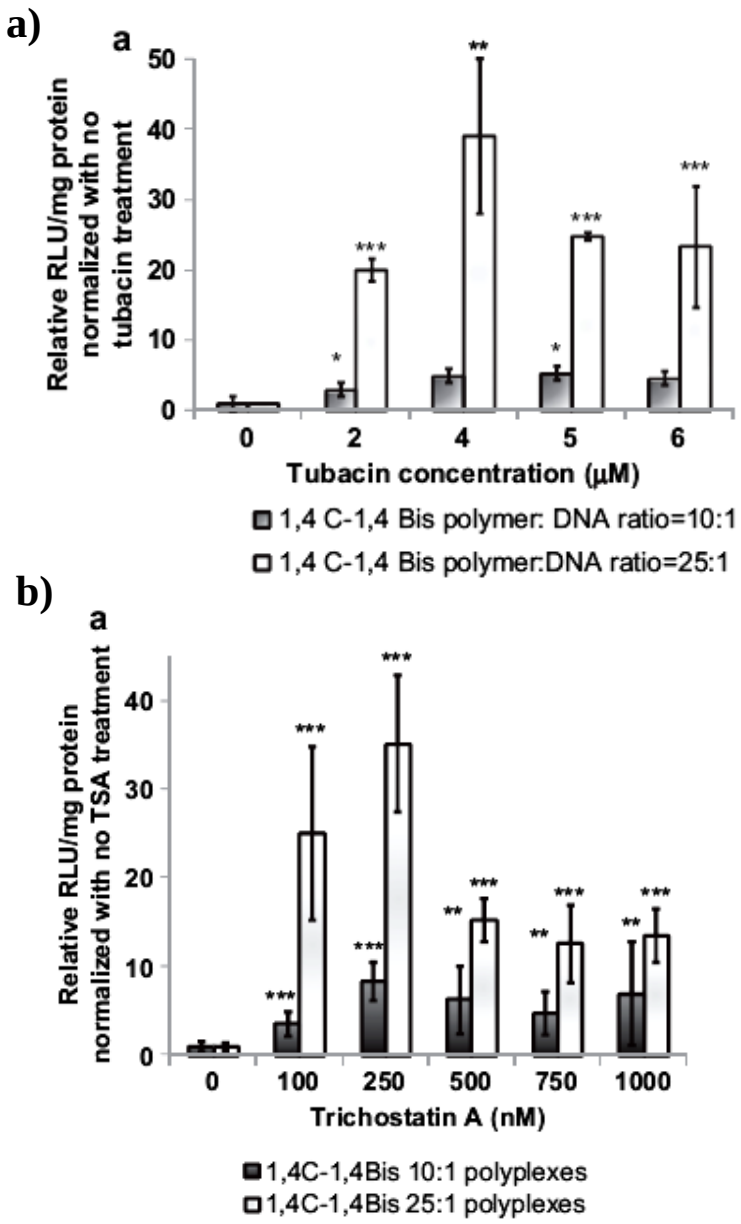

Figure 3.2. Transgene expression enhancement in PC3-PSMA cells transfected with pGL3 and treated with varied concentrations of the HDAC6 inhibitor a) Tubacin and b) Trichostatin A (Barua & Rege 2010).

3.2 Nuclear HDACi Investigation

3.2.1 Introduction

In a similar method to the previous studies for TsA and tubacin, this research involved the use of three nuclear HDACi's in combination treatments with polymer-mediated transcription to enhance transgene expression in PC3-PSMA prostate cancer cells. For purposes of this investigation only, the names of the drugs were referred to as HDACi_A (a HDAC1, 2 and 3 inhibitor), HDACi_B (a HDAC1, 2 and 3 inhibitor), and HDACi_C (a pan-HDAC inhibitor).

3.2.2. Research Findings

PC3-PSMA cells were treated with different concentrations of HDACi_A, HDACI_B, and HDACi_C (0-1000 nM) at a 25:1 weight ratio of 1,4C-1,4Bis polymer and pGL3 polyplexes. Up to a 14-fold transgene expression enhancement was seen at a concentration of 500 nM (Figure 3.3) for HDACi_A, but because only n=2 experiments were carried out at this concentration, this was a result that needed additional study to verify its validity. The next highest enhancement at 1000 nM for HDACi_A was approximately 8-fold, whereas HDACi_B and HDACi_C showed only up to a 4-fold enhancement. While there was some noticeable enhancement with the combination therapy, it was not as significant as previous studies showed with TsA (35-fold) and tubacin (40-fold). HDACi_C and HDACi_B both showed very little enhancement, making them less promising chemotherapeutic drugs to use in further research. However, despite results showing little synergistic enhancement in PC3-PSMA cells, compared to prior studies, the HDAC1, 2 and 3 inhibitor (HDACi_A) showed the most potential as the lead molecule to for additional studies with polymer-mediated transgene delivery.

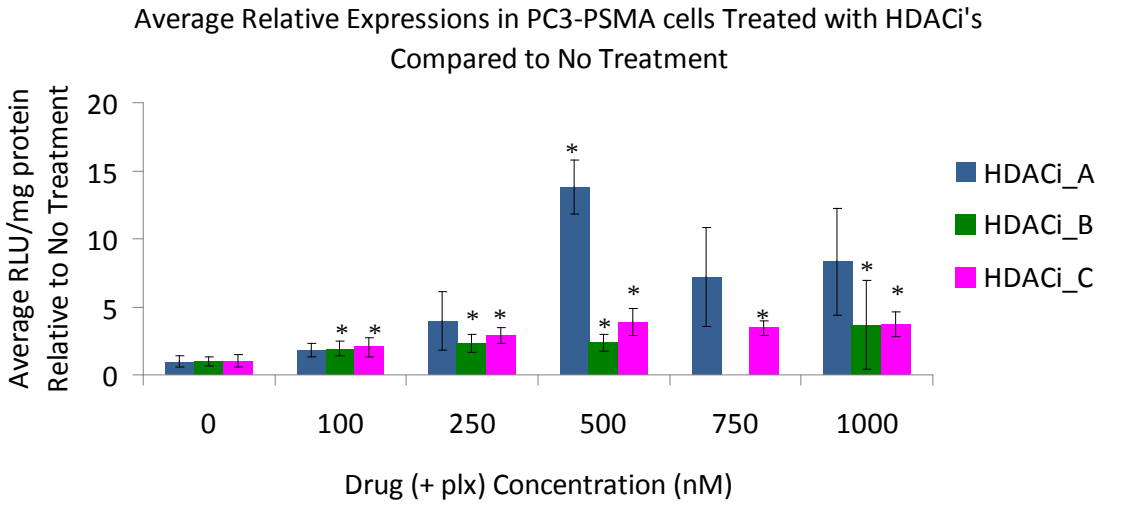

Figure 3.3. Transfection of PC3-PSMA cells using luciferase gene and 1,4C-1,4 Bis polymer in presence of HDACis: HDACi_A, HDACi_B, and HDACi_C. The luciferase gene containing pGL3 DNA of 200ng and the polymer:pGL3 DNA ratio of 25:1 (w/w) were used. Luciferase expression was measured as RLU/mg protein using luciferin and BCA, giving the values with respect to no treatment for the corresponding drug (0 nM and in presence of polyplexes for samples of same color). HDACi_A at 500 nM was carried out only as n = 2. Data was statistically significant for all of HDACi_B, HDACi_C, and HDACi_A at 500 nM concentrations ($p < 0.05$ with Bonferroni correction).

Cell cytotoxicities for all the HDACi molecules tested were within an acceptable range as depicted in Figure 3.4. HDACi_C displayed a slightly higher cell death (up to 40% for 1000 nM concentration) than the rest, which may have decreased the RLU expression seen at this concentration, but not significantly enough to reconsider the results. HDACi_B had up to a 25% cell death, which would very unlikely skew the RLU expressions either. With a less than 10% cell death across all concentrations, HDACi_A made itself a better candidate for future studies as it displayed the lowest cytotoxic levels, even at the higher concentrations.

The large error in most of the HDACi_A results and 1000 nM HDACi_B concentration results could have been attributed to the luciferin used in these experiments. Probably the most notable source of this error was due to the D(-)-Luciferin

powder to make 30 mg/mL aliquots re-suspended in 1X PBS, instead of dimethyl sulfoxide (DMSO). The luciferin powder was only soluble in DMSO and slightly soluble in water, and by re-suspending in 1X PBS, the powder could never fully dissolve. Thus, the luciferin was probably distributed unevenly in the 15 mL aliquot tubes used in preparing a luciferin mixture with medium prior to luciferase expression reading on the plate reader. Not only were the tubes probably unevenly distributed with the powder, the wells that were read may also have contained uneven amounts, giving variable readings from well to well. This error could have been prevented and subsequent experiments may have shown less variability in the results.

The other possible source of error could be due to the protocol used for the luciferin preparation. Previous studies in the lab had used a luciferase assay which had a standardized protocol to measure luciferase expression. This experiment, however, used new luciferin powder assay system, instead of the luciferase assay and buffer system previously employed. While the protocol used for measuring luciferase expression was thought to have been reliable, it was a new procedure that had not been standardized. This may have also contributed to the variability noted in the RLU expressions for the HDACi used.

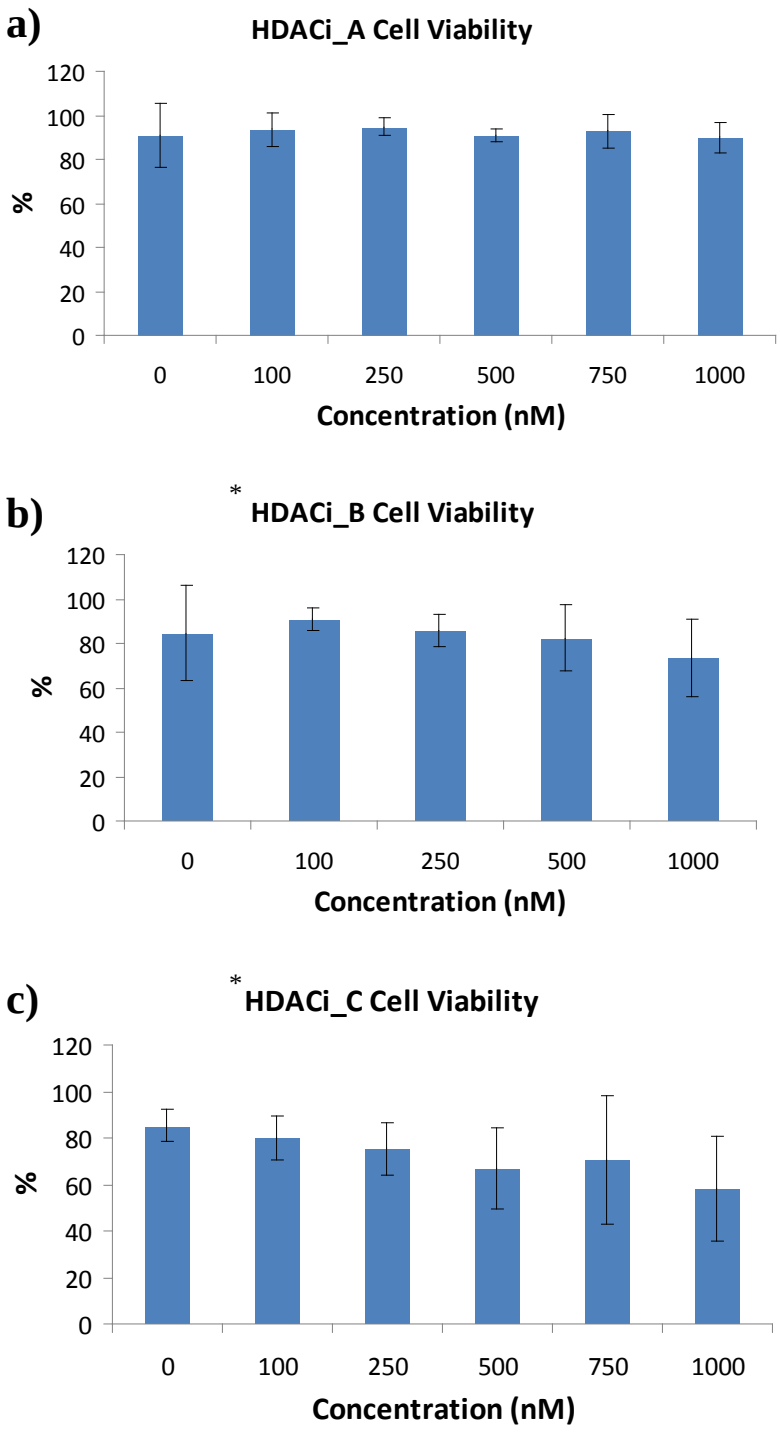

Figure 3.4. Cell viability of PC3-PSMA cells, measured in percentages, when treated with the labeled HDACi. The concentrations represent those of the drugs with a 25:1 polyplex ratio. a) HDACi_A displayed less than 10% cell death for all concentrations, b) HDACi_B showed up to 25% cell death for the highest concentration (1000 nM) and c) HDACi_C displayed up to 40% cell death for the highest concentration (1000 nM), which makes the cytotoxicities of each drug acceptable. An ANOVA analysis showed statistical significance in the data for HDACI_B and HDACi_C, but not HDACi_A (*$p < 0.05$).

3.3 Conclusions

In both studies, an attempt was made to achieve synergistic enhancement of transgene expression through polymer-mediated gene delivery with the use of HDACi. The cytoplasmic HDACi were novel findings that spurred the interest in studying the possibility of similar effects in nuclear HDACi. This nuclear HDACi study, unfortunately, yielded results that were less than extraordinary. Initial findings of the small molecule, HDACi_A, seemed to be the best candidate to pursue for initial studies, despite its relatively low synergistic enhancement, compared to the 40-fold enhancements seen in Tubacin and TsA. However, with its highest enhancement of 14-fold, it was still a promising candidate to evaluate further.

The data for HDACi_B and HDACi_C showed little enhancement and large sources of error which are likely attributed to a luciferin protocol that is not effective and the resuspension error made with the luciferin powder. This inconsistency of protocol made the use of luciferin powder not very attractive. Thus, a switch back to luciferase assay for expression readings was made for the following study.

With these findings, a nuclear HDACi lead drug was identified and an alteration in assay system to evaluate further findings was made. The following chapters discuss further the investigation that used HDACi_A as part of combinational therapy in polymer-mediated transgene delivery in PC3-PSMA cells.

Chapter 4

MATERIALS AND METHODS

4.1 Cell Culture

The PC3-PSMA (Prostate Specific Membrane Antigen) human prostate cancer cell line was a generous gift from Dr. Michel Sadelein (Memorial Sloan-Kettering Cancer Center, New York, NY). Cells were cultured at 37 °C and in a humidified 5% CO_2 incubator in RPMI-1640 medium (HyClone®, UT) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% peni-strep (100 units/mL penicillin and 100 μg/mL streptomycin).

4.2 Purification of Plasmid DNA

The pGL3 control vector (Promega Corp., Madison, WI) is a plasmid DNA that encodes for the modified firefly luciferase protein under control of an SV40 promoter. The pGL3 was added to a culture of DH5α *Escherichia coli* (XL1 Blue) and incubated overnight (16 h, 37 °C, 225 rpm) in 150 mL of Terrific Broth (MP Biomedicals, LLC) containing 150 μg/mL antiampicillin (Research Products International, Corp.). Cultures were centrifuged at 6000 rcf at 4ºC for 15 minutes and the QIAprep Maxiprep Kit (QIAGEN Inc., Valencia, CA) protocol was followed to purify the plasmid DNA. Concentration and purity of the plasmid DNA was based on absorbance at 260 and 280 nm, which was determined via a NanoDrop Spectrophotometer (ND-1000; NanoDrop Technologies). Plasmid DNA within the acceptance range of 1.8 – 2 of the 260/280 nM ratio were the only DNA used for experiments in this research. Plasmid DNA was stored at -20 ºC and thawed on ice upon use.

4.3. Transfection Protocol

In a 24-well plate, PC3-PSMA cells were seeded at a density of 50,000 cells/well in 500 μL serum-containing growth medium (RPMI-1640 medium with 10% FBS and 1% peni-strep) and allowed to attach overnight. Growth medium was aspirated from the 24-well plate and replaced with 500 μL serum-free medium (RPMI-1640 and 1% peni-strep). The HDACi was added in 1 μL amounts of specified concentrations to the cells. Polyplexes of 1,4C-1,4Bis (1,4-cyclohexanedimethanol diglycidyl ether-1,4-bis(3-aminopropyl) piperazine) polymer and pGL3 control vector in specified polymer:pDNA w/w ratio (200 ng pGL3 concentration) were prepared by adding the polymer to DNA in 1.5 mL centrifuge tubes, then incubated at room temperature for 20 min. The resulting polyplexes were added to the cells for 6 h. Serum-free medium was pipetted out of the wells and replaced with 500 μL serum-containing growth medium. Another 1 μL of the HDACi drug at the varied concentrations was added to the corresponding wells. Cells were incubated for 48 h at 37 °C and 5% CO_2.

4.4 Luciferase Assay

Following ~48 h incubation of treated cells, medium was collected in 1.5 mL centrifuge tubes and washed with 150 μL of 1X phosphate buffered saline (PBS: 10mM Na_2HPO_4, 140mM NaCl, pH ~7.4). Each well was then treated with 150 μL of 1X cell lysis buffer reagent (Promega) for no longer than 2 min, but long enough to detach cells. Content from centrifuge tubes was then added back to lysate. For samples that did not need to be diluted, 25 μL of the cell suspension was put into a 96 well non-sterile half area white plate in triplicates. For samples needing dilution, 100 μL of the cell suspension was put back into the centrifuge tubes and had 900 μL of nanopure water added to dilute

10X. If further dilution was still needed, 50 μL of this 10X dilution was taken into new 1.5 mL centrifuge tubes in order to dilute 200X. With either dilution, 25 μL was taken and placed in the 96-well non-sterile half area white plate. Luciferase Glo Kit (Promega) was thawed at RT and mixed via vortexing, and then 50 μL was added to white plate as quickly as possible. The samples were read by Bio-Tek Synergy 2 plate reader immediately with excitation 360/40 filter plugged and emission 528/20 filter holed. Protein contents of each well were measured by Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). The relative luminescence values (RLU) were normalized from these measured protein contents for each sample.

4.5 Enhancement of Polymer-mediated Transgene Expression Using HDACi

PC3-PSMA cells were treated with 1 μL HDACi_A (0 – 2000 nM) at one of the six w/w ratios (1:1, 5:1, 10:1, 20:1, 25:1) of 1,4C-1,4Bis polymer and pGL3 plasmid DNA polyplexes. Following the ~48 h incubation period in the presence of the HDACi_A, PC3-PSMA cells were washed, lysed, and re-suspended in the medium, and prepared as the luciferase assay describes in section 4.4. Transgene expression was reported as fold increase in normalized RLU/mg protein relative to no treatment (cells in the presence of polyplexes but no HDACi).

4.6 Statistical Analysis

All values are expressed as the mean ± one standard deviation (S.D.). All experiments were carried in at least triplicates, unless mentioned otherwise. A two-tailed, paired Student's t-test was used to analyze the significance of the difference between the control and each experimental test condition ($\alpha = 0.05$ and 0.005), with a Bonferroni correction ($\alpha/29$ comparisons), giving the equivalent of $\alpha = 0.00172$ and 0.000172,

respectively. ANOVA was run for cell viability data to test statistical significance. All analyses were conducted using Excel 2003.

Chapter 5

RESULTS AND DISCUSSION

A combinational approach was taken to investigate the effects of a lead nuclear HDACi and lead polymer for a non-viral gene delivery system. It was hypothesized that an HDACi that works inside the nucleus could overcome transcriptional barriers to enhance transgene expression in prostate cancer. In this research, four different polymer:pDNA weight ratios were used to deliver the plasmid DNA, pGL3, into PC3-PSMA cells treated with varied concentrations of HDACi_A (a nuclear HDACi). The efficacy of the transfections were evaluated by reading luminescence (relative luminescence units or RLU) due to expression of the luciferase protein and normalized to the corresponding protein content of the sample.

The results from the transfections (see Figure 5.1) showed that the 20:1 polymer:pDNA ratio was the most effective compared to the other polyplex ratios tested. The trend seems to show little increase in transfection efficacy between 1:1 and 10:1, a significant spike at 20:1, and a decrease with 25:1. This could be indicative of a possible size correlation with the transgene efficacy, but a size-zeta potential analysis would need to be conducted to prove this. Especially interesting would be to see if the 20:1 polyplex ratio is well within the endocytotic limits compared to the other ratios.

At this 20:1 polyplex ratio, the two highest HDACi_A concentrations tested (1, 2 μM) gave up to a 9-fold enhancement, with 750 nM HDACi_A not too far behind with around an 8-fold enhancement. All other combinations of polyplex ratio and drugs were of little interest with less than 5-fold enhancements.

While the standard deviations for the 20:1 polyplexes were higher than most, the 1000 and 2000 nM concentrations were statistically significant as denoted by the Bonferonni correction, which takes into account the entire sample amount. The enhancement at the higher HDACi_A doses is consistent with the drugs effectiveness seen in clinical trials at the micromolar scale (Bolden, Peart, & Johnstone, 2006). Additional experiments are needed to further understand the reasoning behind this enhancement.

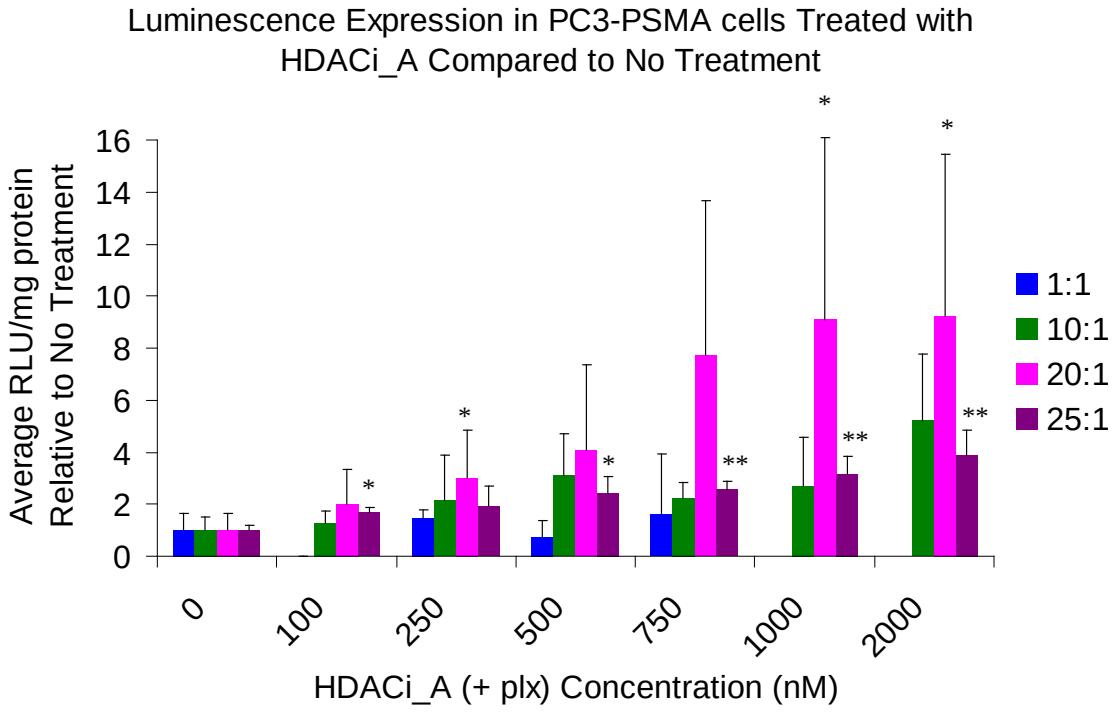

Figure 5.1. Luciferase expression in PC3-PSMA cells treated with HDACi_A using different polymer:pDNA weight ratios. Enhancement shown is related to no treatment (0 nM HDACi_A) but in the presence of polyplexes. Luciferase activity was measured as RLU/mg protein using means ± standard deviation ($n \geq 3$). Statistical significance of * and ** indicated $p < 0.05$ and $p < 0.005$ (with Bonferroni correction), respectively using two-tailed, paired Student's t-test.

Actual luminescence expression values (RLU/mg protein) showed an increasing trend with increasing polyplex ratios (Table 5.1). This finding may support that these larger delivery devices may be more effective at overcoming biological barriers that are characteristic on non-viral delivery. One possibility is that the larger molecule increases endosomal escape due to proton sponge effect, since there is more cationic polymer to buffer against the acidic endosomal environment. However, additional studies would need to be conducted to verify this hypothesis.

Table 5.1 Normalized RLU/mg protein with S.D. x 10^3 of polyplex ratios at the six drug concentrations

	RLU/mg protein ± S.D. x 10^3			
HDACi_A Concentration (nM)	1:1 plx	10:1 plx	20:1 plx	25:1 plx
0	0.04 ± 0.03	3.0 ± 2.0	51.0 ± 75.0	4000.0 ± 800.0
100	0 ± 0	3.8 ± 1.1	90.0 ± 72.0	6200.0 ± 870.0
250	0.06 ± 0.01	7.0 ± 7.0	120.0 ± 101.0	6000.0 ± 240.0
500	0.03 ± 0.03	9.6 ± 6.2	180.0 ± 145.0	9500.0 ± 3100.0
750	0.07 ± 0.1	7.3 ± 3.2	270.0 ± 145.0	11000.0 ± 530.0
1000		8.5 ± 8.0	250.0 ± 230.0	12000.0 ± 1000.0

Standard deviations were fairly high for nearly all polyplex and drug combinations, but more so at the 20:1 polyplex ratio. The reason behind this could be due to the larger sample size taken for 20:1 (n = 12) compared to the other polyplexes (n = 3 for 1:1 and n = 6 for 10:1 and 25:1). Additionally it has been witnessed in our laboratory that plasmid DNA, from different purifications has a variation in expression levels. Half the transfections at 20:1 used pGL3 were from one recently made stock and the other half from one from a year-old stock stored at -20ºC. Interestingly, though, it was noted that while the recent plasmid DNA gave higher luminescence values, it also gave lower

transfection enhancements. Further speculation is needed to understand the reason behind this, and may spur additional experiments to compare different plasmid DNA stocks.

Chapter 6

CONCLUSION

The goal of this research was to evaluate the effectiveness of combinational therapy with a specific nuclear HDACi and polymer-mediated transgene delivery on transgene expression. Prior studies with HDACi that inhibit functions of HDAC in the cytoplasm (tubacin and TSA) have shown up to 40-fold enhancement over polymer delivery, alone. This same principle was thought to have similar results with HDACi that are found to work in the nuclear realm.

A preliminary screening of a few potential molecules yielded one HDACi that showed promise for further studies. This chemotherapeutic agent, HDACi_A, showed up to a 9-fold enhancement in PC3-PSMA cells at a 20:1 polymer:pDNA ratio for doses of 1 and 2 μM. This dose range was consistent with effective doses used in current clinical trials. It is hypothesized that the 20:1 polyplex ratio may have a size-correlation with transfection efficacy. Additional studies still need to be conducted to further understand the reason behind this enhancement.

Chapter 7

FUTURE WORK

7.1 Cell Cytoxicity

To further analyze the effect of HDACi_A on polymer-mediated transgene delivery, it is important to collect data on cell cytotoxicity, since this is an important factor in achieving an effective gene delivery system. Without acceptable cell viability, the combinational therapy proposed would not be useful in clinical applications. These experiments would be conducted for all combinations of polyplex ratios and drug concentrations in the same method used in previous studies, via MTT assay.

7.2 Size and Zeta Potential

Another important part of this study will be to collect data on size and zeta potential in order to ensure a correlation to the obtained luciferase results. This information will give further insight as to possible size barriers that could decrease the uptake and efficiency of transgene expression.

The methods for determining the polyplex size and zeta potential would be through the use of dynamic light scattering (DLS) via Malvern Zetasizer Nano Series (Malvem Instruments Inc., Westborough, MA). Polyplexes are incubated at R.T. for 20 min at 0.4 μg/ml pDNA at the w/w ratios used in the transfection study in 1X PBS.

7.3 Additional Cell Lines

Additional cell lines would need to be transfected with the same polyplexes and HDACi to evaluate the effectiveness of the combinational effects that can transcend only PC3-PSMA. This is essential because prostate cancer cell lines have shown to have various outcomes due to phenotypic differences (Barua & Rege, 2009). Also, an

investigation of breast cancer and pancreatic cell lines could open up additional research that could prove more effective. An initial dose response would be conducted for each cell line to establish the scale for HDACi_A concentrations (such as nano- or micro-molar). Then the transfections would be carried out as discussed in section 4.3 with same polymer and plasmid DNA at the same polyplex w/w ratios and the ascertained HDACi_A concentrations from the dose response.

7.4 Alternative Novel Polymer

In the laboratory, polymer synthesis has been conducted to find a new lead polymer that is highly efficient as a vector for gene therapy. One such polymer has been discovered and has shown significant enhancements in transgene expression. Use of this polymer with HDACi_A could have a possible synergistic effect. The same method as depicted in section 4.5 would be used to evaluate the best combination of polyplex ratio and drug concentration.

7.5 Use with TRAIL Gene

Finally, to test the effectiveness in an actual cancer therapy, the lead polyplex ratio and drug concentration obtained in this study, with the least cytotoxicity, would need to be tested with the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene. Past studies have shown effective use of HDACi that enhance the TRAIL gene in LNCap prostate cancer cell line using viral methods (Kasman, Lu, & Voelkel-Johnson, 2007). The combinational therapy used in this research could have similar outcomes in PC3-PSMA cell lines with use of TRAIL.

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APPENDIX A

1,4C-1,4BIS POLYMER

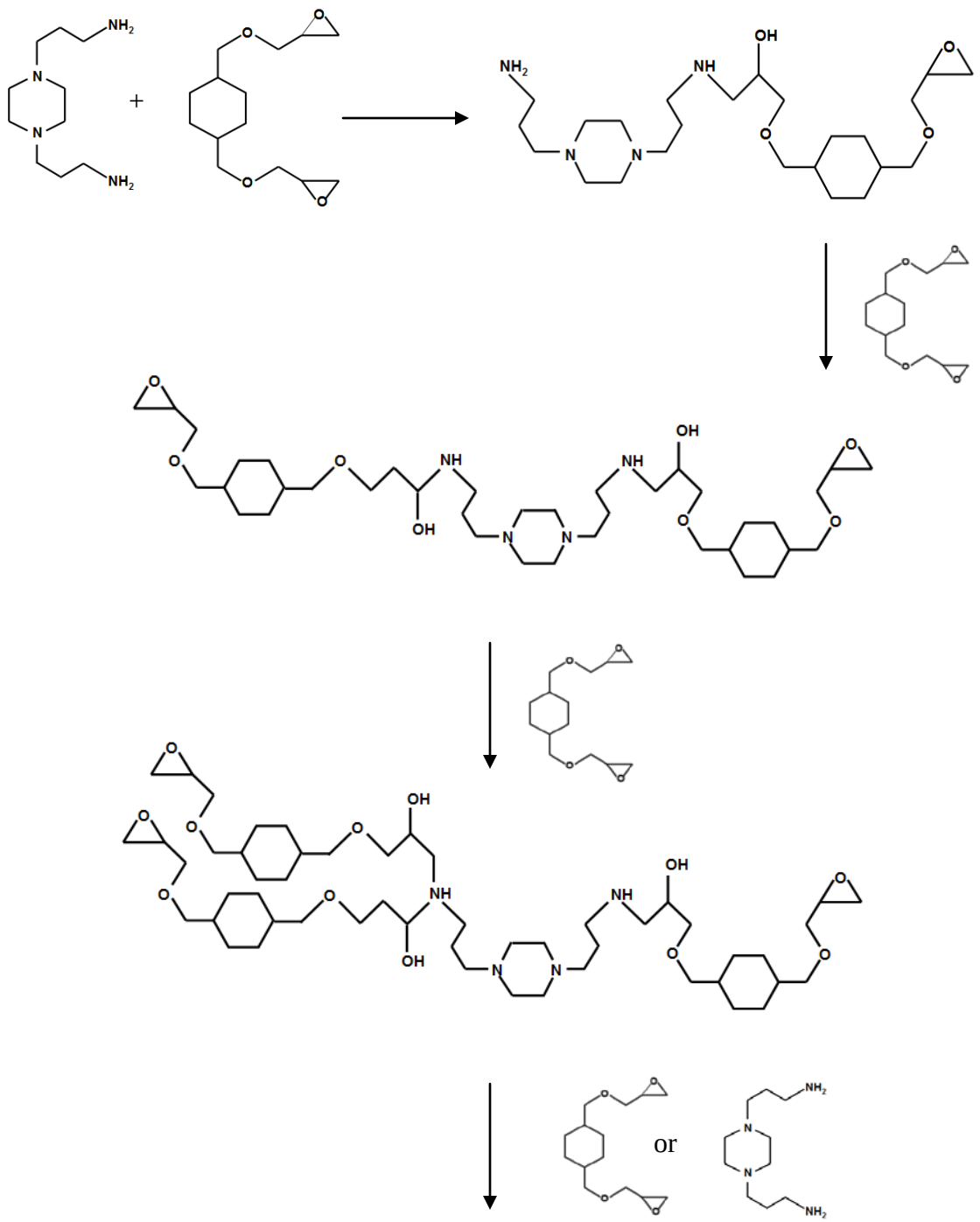

Figure A1. Proposed schematic of polymerization of 1,4C-1,4Bis polymer based on the ring opening of the diglycidyl ether by the amine (adapted from Barua *et al.*, 2009).

Table A1. Characteristics of 1,4C-1,4Bis compared to pEI-25 (Barua, 2011).

		1,4C-1,4Bis	**pEI-25**
M_n (kDa)		3.9	10
M_w (kDa)		23.5	25
Polydispersity		5.96	2.5
Size (nm)	10:1 plx ratio	154.5 ± 13	190.7 ± 6.3
	25:1 plx ratio	244.8 ± 17	261.7 ± 9.3
Zeta Potential (mV)	10:1 plx ratio	22.8 ± 2.7	20.7 ± 2.2
	25:1 plx ratio	27 ± 4.3	25.7 ±1.6