CUSTOMIZATION AND OPTIMIZATION OF A HISTONE H2A-BASED VECTOR FOR TARGETED GENE TRANSFER TO CANCER CELLS

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ABSTRACT OF THE DISSERTATION

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Developing an efficient and safe system for gene delivery is considered the bottleneck of gene therapy, where a successful delivery of the nucleic acid can reverse a defective cellular pathway to normal, eradicate cancer at molecular level or simply make it more susceptible to current chemotherapies. Not only have the intracellular events played a crucial role in a successful gene delivery, but the interaction of nano-particles with extracellular factors should be studied as well.

The goal of this study was to design, produce and optimize a non-viral gene delivery system for targeted delivery of nucleic acid such as reporter genes (e.g., green fluorescent protein) or therapeutic genes (e.g., suicide genes) to cancer cells. The

system was designed in a way to be easily customized for different cancer types, still presenting a high level of targeted delivery.

The first chapter of this thesis will focus on the concept of gene therapy and current systems used for this modality. Different types of vectors including viral and non-viral polymeric vectors will be discussed briefly and the advantages and disadvantages of each will be mentioned. We also discussed natured inspired biopolymers such as peptides and amino acid based vectors which are the fundamental premise of this study.

In chapter II, the concept of suicide gene therapy will be explained. Additionally, the current enzyme/prodrug systems, different methods for delivery of suicide genes will be elaborated.

In chapter III and IV, the new nanotechnology platform for targeted delivery of plasmid DNA to HER2-positive ovarian cancer cells and HER2-negative prostate cancer cells will be presented. In chapter III, the method for optimization of nanotechnology platform will be discussed and the features of optimized particles will be presented. Chapter IV explains the modification we introduced to vectors' primary structure to customize it for a different cell line. Also another method for optimization of amino-acid based vectors for in vivo delivery will be introduced. In these two chapters, the methods of designing and the efficiency of each vector to fulfill the expected goals as well as their safety will be discussed in details and supportive data will be presented.

*This is the **LORD**'s doing; it is marvelous in our eyes*

All this and Heaven too

To my grandmother, Leila

The bravest and kindest woman I have known in my life

To my parents, Hossein and Fatemeh,

The Angels of my Life, whose unconditioned Love keeps me alive

To my dearest precious sisters, Maral, Parisa and Sarah

My Soul Mates, my best Friends whose Love guides me through the darkest moments of my life

To Alexander Eden Guy

My beloved Root and Guide in this vast generous land

And to all my Teachers at School of Pharmacy, Esfahan University of Medical Sciences, Esfahan, Iran

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I have learned a lot, and it is not only about science. It was also about how to tumble but not fall, how to be pushed to my boundaries but not break. I passed the highest mountains and deepest seas and saw the darkness and light, both in human soul.

Sometimes I thought I would not make it but I did. And I owe this to my family and friends. Today, I am here at the end of this dark road, because of the people who have been there for me;

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I am ready to close this dark chapter of my life and take nothing but good memories. It is always darkest before the dawn….

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1. Gene therapy: a background

Gene therapy is defined as a method in which a group of products "mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome and that are administered as nucleic acids, viruses, or genetically engineered microorganisms" (FDA definition). The products may be used to modify cells in vivo or transferred to cells ex vivo prior to administration to the recipient[1]. The idea of altering the inherent features of a living organism by transferring the genetic material came from Griffith's study on bacteria[2]. Later, other groups tried the same concept to cure hyperargininemia and β-thalassemia by transferring the correct version of the affected gene. These studies, though important as the pioneers, brought no success[1, 3, 4].

September 14th 1990 is an important day to remember in the history of gene therapy. On that day, four-year-old Ashanti de Silva became the first patient in the first NIH-approved gene therapy trial. De Silva suffered from severe combined immunodeficiency (SCID), a severe immune disease caused by inheriting two defected copies of Adenosine deaminase (ADA) gene which leads to immature T cells. In this trial, performed by Dr William French Anderson and colleagues, the patient's T cells were infected ex vivo with a retroviral vector carrying a functional adenosine deaminase gene. The cells then were re-injected to the patient's body. Although the response was described as temporary, it raised hopes for gene therapy as an effective modality to

cure various diseases with genetic basis[4]. Later on, some setbacks happened which slowed down the progress: one clinical trial performed in 1999 to correct ornithine transcarbamylase (OT) deficiency by injecting an adeno viral vector carrying OT gene caused the death of the 19-year old patient, Jesse Gelsinge who received a dose of viral vectors directly injected to his hepatic artery. Four days after injection, he died of multiple organ failure which was the direct result of extensive immune reaction to adeno-viral vector[5].

One year later, Alain Fisher and Marina Cavazzana-Calvo started another clinical trial for the treatment of X-linked SCID (SCID-X1). A total of twenty children received the gene encoding Interleukin-2 receptor gamma chain encoded by a retroviral vector. The trial was reported successful with no side effect on that time[6]. However, five patients were reported later to develop a type of T cell lymphoma which is a direct consequence of activation of a proto oncogene promoter by viral vector[4, 7, 8].

Despite all the tragic side effects ranging from inflammation to death and limited rate of success in early clinical level, today more than 1800 clinical trials have been approved worldwide[9]. The international effort to improve viral vectors safety and maintain their efficiency is still going on. Also, the challenging nature of viral vectors raised the idea of finding a replacement for them with collective features of an ideal vector: i.e. low toxicity, low immunogenicity, high efficiency of DNA and RNA delivery and flexibility to be targeted to the target cells[10, 11].

2. Extra and intracellular barriers to gene delivery vectors

The objective of gene therapy is to design a vector with low immunogenicity, low toxicity and high efficiency. In order to gain this goal, many researchers have a glimpse on nature to find out the keys for a successful gene therapy system.

Viruses are masters of gene delivery[12]. To imitate their sophisticated abilities which have been gained during million years of evolution, a gene therapy vector should be equipped to pass a number of biological obstacles to deliver its cargo to cell nucleus, where it dissociates from DNA and lets it be transcribed and expressed as a separate episomal DNA or integrate into host's genome. These obstacles can be divided into two groups: Extracellular Barriers and Intracellular Barriers [13]. At the first place, the vector should facilitate the internalization of DNA and protect against destructive agents such as cellular nucleases. Such propertied must be accompanied by packing the large and extensive DNA molecules into packed stable particles. For this purpose, negative charge of DNA backbone is exploited for designing positively charged vectors which condense DNA through electrostatic interactions with DNA. Each complex comprises of several DNA molecules and a certain number of vector molecules. Complex formation reduces the particle size down to several hundred nanometers or less [13]. Condensation increases the cytosolic half-life of packed DNA significantly, which is only 50-90 minutes for naked DNA [14]. However, this protecting effect of vector should be reversible in a controlled fashion to allow release of DNA molecule after entry to the nucleus[15].

The attachment of particles carrying DNA to the cell surface is the first step of particle internalization. The attachment can be either through non specific electrostatic interaction between positively charged particles and negatively charged proteins on the cell surface or mediated by specific ligand/receptor interaction. Also the Particle size defines the nature of route of internalization[16]. The cell membrane can be quite selective in uptaking the foreign materials. Usually the small hydrophobic molecules easily cross the membrane. Larger or more hydrophilic molecules should actively be transported through the cell membrane. The negatively charged DNA/vector complexes cannot inter the cells directly as the cell membrane has lipophilic structure and is totally impermeable to charged and large molecules[16, 17]. Uptake of such particles occurs through clathrin-mediated endocytosis (CME), caveolae/lipid-raft, macropinocytosis or clathrin/caveolae-independent endocytosis [18]. Receptor-mediated endocytosis involves the Clathrin mechanism and is considered to be the main route of internalization of viruses, toxins, macromolecules such as growth factors (including VEGF, EGF, FGF and insulin) and hormones. It was shown that particles less than 200 nm in size are internalized via Clathrin-dependant endocytosis[19]. The internalization pathway determines the fate of particles, which is lysosomes in clathrin-mediated endocytosis, caveosomes or lysosomes from caveolae and macropinosomes from macropinocytosis[20]. Most of these pathways end in late endosomes which proceed to lysosomes, although there are slight difference among the nature of late endosme for each pathway[20]. The particles are internalized through early endosomes which is a membrane pinched-off vesicle coated with Clathrin. Early endosomes, fuse with other

pre existing sorting endosomes to form late endosome[13, 21]. A significant pH reduction from neutral to about 5.9-6 occurs due to the activity of ATPase-dependant proton pumps. Some lysosomal vesicles from trans-Golgi network join late endosome to form lysosomes. Lysosomic hydrolytic enzymes and other degrading enzymes besides continuous acidification to pH 4 terminates vector/DNA journey by degrading it[13, 22]. This phenomenon can explain the observation that in spite of internalizing of vector/DNA by more than 95% of cultured cells, only less that 50% express the transgene [13].

Regardless of the pathway, the vector for gene delivery should be equipped with efficient endosomal escape mechanisms. Endosomal escape happens through two mechanisms; “proton sponge” phenomenon and lipid depletion by fusogenic peptides. Some polymers such as Polyethylenimine (PEI) and polyamidoamine (PAMAM) denderimers contain amine groups which undergo protonization when endosomal environment falls to acidic pH. So, ATPase dependant proton pump has to transport more hydrogen ion to reach the desired pH. This excess of positive ions causes more counter ions to transfer from outside to inside. This phenomenon leads to osmotic swelling and endosomal disruption. In this way, complexes get released into cytsol [13]. In the other hand, animal viruses use endosomolytic or fusogenic proteins located in their envelops to disrupt endosomal membrane. The interaction between viral envelop and endosomal membrane of host cell facilitates viral genome transport through the cell. These destabilizing peptides naturally are observed in defense toxins, antibiotic peptides or vertebrate innate immune system as well. It has been proved that endosomes move

to the nucleus along the microtubule highway, safe from cytoplasmic nucleases. Microtubules are also involved in direct trafficking of macromolecules such as DNA/vector complexes. Therefore, it is important that DNA molecule remains attached to the vector before reaching to nucleus[20].

It has been known now that the dividing cells at the end of prophase and the onset of pro-metaphase are more indulgent to gene transfer because of disassembly of nuclear envelope (NE)[22-24].During the mitosis, gene transfer occurs in a passive manner. However, in normal cell population, large macromolecules encounter active transport through biological gates or nuclear pore complexes (NPCs). Small molecules and small oligonucleotide (ODNs) which are at most 200-310 pb in size and 40 KDa in weight may readily pass through aqueous channels [23]. However, large plasmids and other macromolecules such as proteins are excluded as they should be transported actively. In addition to size, nucleic acids lack nucleus signal to guide them through NPCs. The transportation through NPCs relies mostly on specific nuclear transporting proteins called importins (or karyopherins) to pass. To be recognized and attach to importins, macromolecules should be equipped with some sequences which act as signals for importins. These signaling peptides are called nuclear localizing signal (NLS). They are basically cationic peptides, rich in basic amino acids. Viruses contain some well-known peptides of this group such as SV40 T-ag (large tumor antigen) [16]. There are also naturally occurred NLSs with intrinsic ability of DNA condensation and translocation through the NPCs. Histones and HMG (high mobility group)-box proteins are examples of such group[16].

3. Vectors for gene delivery

As mentioned before, it is difficult, if not impossible for nucleic acid molecule to diffuse directly through the cell membrane because of its highly charged and hydrophilic structure. Therefore, designing a vector which facilitates the diffusion of such molecule is a crucial step in gene delivery. So far, three major categories of vectors have been introduced: viral, non viral and physical. In the following parts each category will be discussed in detail.

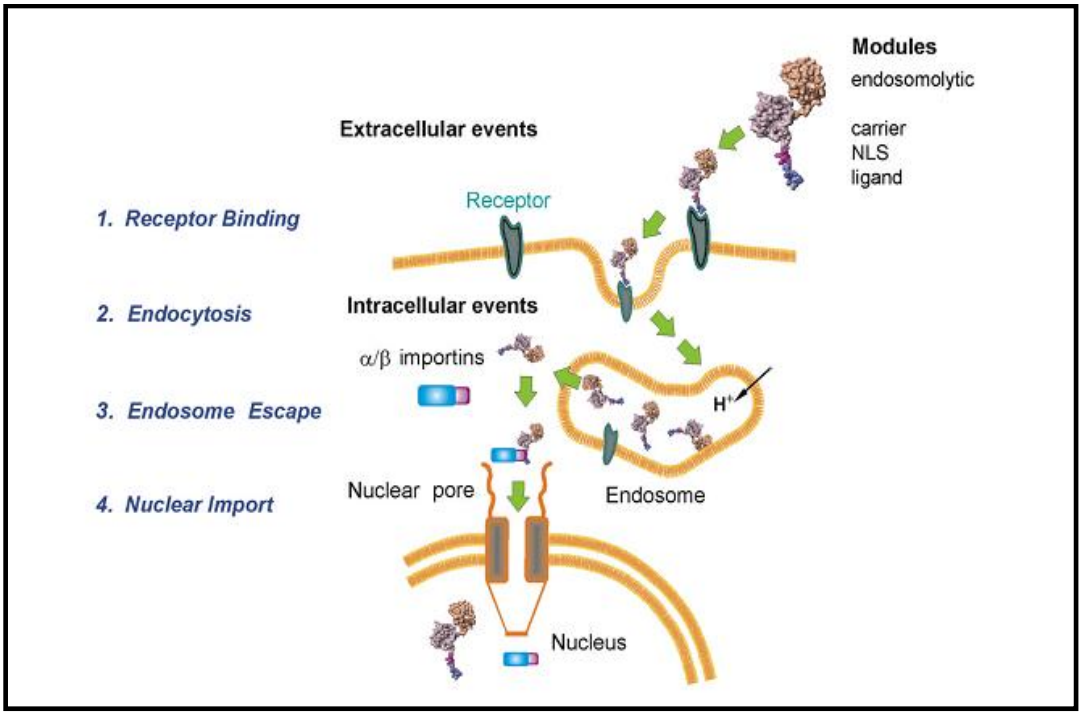

Figure 1: The main steps of gene delivery: (1) binding to cell surface, (2)endocytosis, (3)escaping from endocytic vesicles, (4)reaching the nucleus and entering through the nuclear pore complex. The picture was adapted from Sobolev et.al (2009) [25].

3.1. Physical methods for DNA delivery

Physical methods for DNA delivery have gained a lot of attention because of their simplicity and cost effectiveness. The common basis of all physical methods is using a physical force to transfer DNA to the cells. Since DNA molecule is the only material which is actually transferred during the process, the concerns regarding immunogenicity and toxicity will be alleviated, unless the immunogenicity comes from the DNA molecule itself. In addition, in some cases the efficiency of these methods is comparable with viral vectors[26, 27].

In The first experiment of this type, DNA was injected directly to muscle tissue[28]. The method is applicable for some other tissues such as skin, heart and liver[29-31]. However, the drawbacks of this method are low efficiency and restricted transduction limited to the point of injection. Also, due to rapid clearance, multiple doses are needed. Therefore, the use of this method is usually restricted to vaccination[26, 27]. Later on, another method called biolistic DNA injection, microprojectile gene transfer or gene gun was introduced for genetic manipulations of mammalian cells, both in vitro and in vivo[32]. This method was originally designed for gene transfetion to plant cells [26]. In this method, the cells are bombarded with gold particles which have been coated with plasmid DNA. The Driving force to accelerate the particles comes from pulses from helium gas or high-voltage electronic discharge pressure discharge[26]. The two major disadvantages of this method are cell damage at the center of discharge site and limited permeability to the deeper tissues. For these reason, the application of gene gun is

mainly for gene transfer to the skin for DNA vaccination purposes. However, few examples of using this method for the heart, tumor or even mouse embryo have been published[27, 33-35].

Another method which is designed based on physical forces is electroporation. By definition, electroporation is the formation of temporary hydrophilic pores in the cell membrane as a result of electric pulses to make it permeable to plasmid DNA [26]. this method has been studied on different types of tissues including skin, lung, liver, skeletal and cardiac muscle, brain, retina, cornea and the vasculature[27]. Although the method faces less effectiveness for the internal organs, it has been extensively used for direct DNA transfer to tumors[26, 27].

Another method in this category, Magnetofection, is the use of magnetic field to facilitate the transfection. In this method, the paramagnetic nano-particles which are made of Iron oxide are coated with a cationic polymer which can bind to DNA through electrostatic forces. After adding the particles to the target cells in vitro, a magnetic field is applied to concentrate the particles[36]. The internalization of particles is through the same pathway as non viral vectors, i.e. receptor –mediated endocytosis[37]. The method is also applicable for in vivo studies. Shortly after intravenous injection of nano-particles, the magnetic field is applied to capture the particles from the circulation and concentrate them close to the site of action. The particles are held in place by the magnetic field until they get absorbed by the target tissue[37]. The in vivo method have

been used successfully for transfection the cells in gastrointestinal track and the ear vasculature [36].

The last method which will be discussed here, namely sonophoresis/sonoporation, is based on the fact that ultrasound can perturb the cell membrane and increase the permeability by inducing temporary pores[38]. It has also been shown that ultrasound contrast agents or microbubbles can enhance the efficiency of ultrasound-mediated gene delivery, a technique which is called Ultrasound-targeted microbubble destruction (UTMD)[39]. The damage caused by microbubbles is temporary and does not lead to cell destruction. This method has been used successfully to deliver naked DNA or even adeno-associated viral vectors into hard-to-transfect cells[14, 39]. Figure 2 showed a diagram of physical methods for gene delivery.

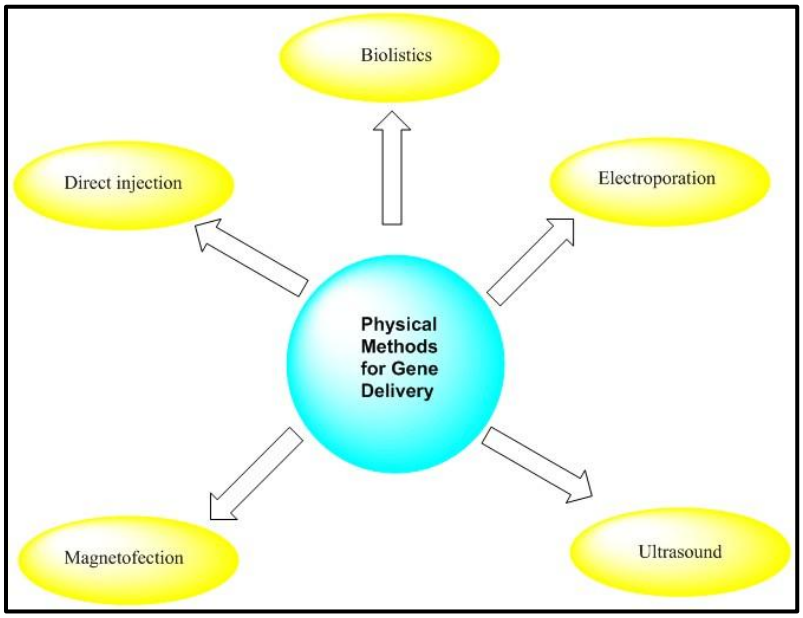

Figure 2: Physical method for gene delivery. Adapted from Jin et. al (2014) with modifications [40].

3.2. Viral-based gene delivery systems

More than 50% of all clinical trials approved in 2012 are using viral vectors as vectors for gene delivery[9]. The type of application determines the duration of transgene expression and as a consequence, the type of viral vectors that should be used. For examples, when the goal of gene therapy is to correct a genetic disorder, long term expression of transgene is required. In this case the viral vectors which can integrate the transgene in the genome of host cells are in priority. This type of vectors are called integrating vectors with retroviruses and lenti-viruses as two major groups in this family.

Retroviral vectors have been powerful and remunerative modalities for gene therapy. They are able to trigger a stable expression of transgene in different mammalian cells, independent of their origin[41]. Usually retroviruses, especially the ones derived from murine leukemia virus (MLV), require cell division for infection which provides a level of specificity to neoplastic tissue. Along with this property, the ease with which they can be manipulated, favors their extensive use in ex vivo gene therapy for hematopoietic stem cell gene therapy and clinical cancer gene therapy to correct genetic disorder [6, 42, 43]. Blease and Andesron's trial to correct severe combined immunodeficiency (SCID) was the first clinical trial used a retoviral gene therapy[44]. However, gene insertion to the host genome increases the chance of insertional mutagenesis. A known example is Cavazzana-Calvo's gene therapy clinical trials which led to the death of one patient due to vector-caused leukemia[6]. Another drawback of retroviral vectors is their limited capacity to accommodate big size gene. To overcome

this drawback, a hybrid of retrovirus with adenovirus has been designed which has a cloning capacity of up to 36 kb[45].

The other major family in integrating viral vectors is Lentiviruse family. Lentivirus (LV) belongs to a family of retroviridae which are RNA viruses. Human immunodeficiency virus (HIV) is the most well known member of this group. Lentiviruses offer many advantages: they provide a stable expression of transgene even in non dividing terminally differentiated cells such as neurons[46]. Compared to retroviruses, they have been reported to show less genotoxicity and a site selection profile while integrating[47, 48]. The immune reaction toward LV is also low compared to Adeno-associated viruses. Additionally, the LV therapeutic payload can be 10 kb [47]. Lentiviruses were successfully used to transfect retinal and cardiac cells in vitro and to restore the function of a genetic disorder in both cell types[49, 50]. The only drawback of lentiviruses is their low titer in production process which increases the cost of production.

Contrary to integrating viral vectors, in some cases such as cancer, a high but transient level of expression might be sufficient to render the desired effect. Therefore the choice for vectors is non-integrating vectors[12]. The viral vector in this category tend to sustain their genomic material as an independent episomal chromosome[11]. The major groups in this family are Adenoviruses, Adeno-associated virus (AAV) and Herpes Simplex virus (HSV).

Adenoviruses are the most abundantly used vectors in gene therapy. Based on Wiley data bank on gene therapy In 2012, about 23% of all clinical trials were based on adeno viral vectors [9]. Although the first death in the history of gene therapy is directly

associated with severe immune reaction to adeno viral vector, after many years of modifications and recognition of mechanism of adeno viral toxicity, the use of these vectors are an acceptable fact in the field of gene therapy[51]

Adenovirus offers certain advantages over retrovirus. It includes a far greater transfection ability, ability to infect dividing as well as non-dividing cells such neurons and capability to produce gene expression without integration into the host genome, providing safety advantage [52].Moreover, they can accommodate up to 37 kb of DNA gene. They are also stable and relatively easy to be manufactured compared to other common viral vectors like Adenovirus Associated Vector (AAV).

The internalization of Adeno viral vectors mainly happens through the interaction of its capsid with coxsackievirus-adenovirus receptor (CAR)[53]. Many attempts have been made to modify the capsid proteins to redirect the internalization though other cell receptor. The main rationale for such attempt is the low expression of CAR in some cells which are potentially the target for adenoviral vectors[51].

The second group in non-integrating viral vectors is Adeno-Associated Virus (AAV). AAV is a human non pathogenic virus from Parvoviredae with a single-stranded DNA genome of approximately 4.7 kb. It has also been placed in the family of Dependoviruses because It requires co-infection of a helper virus (e.g. adenovirus or herpes virus) for efficient DNA replication[54].

Because of two favored features of non pathogenic and defective replication, AAV vectors are among the most frequently used viral vectors for gene therapy[54, 55].

Recombinant AAV is able to infect cells both ex vivo and in vivo. In the absence of helper virus, AVV can integrate into host chromosome in a site-specific manner[54]. One of the subtype of AVV, namely AVV2, is used more frequently for developing of viral vectors for gene delivery[54].

Herpes Simplex virus (HSV) is a highly infective DNA virus with an inherent affinity to neural tissue [56]. They persist in a lifelong non-integrated latent state without causing disease in the immune-competent host[57]. The genome is a 152 Kb DNA, with many genes superfluous for replacement; therefore the genome can accommodate large transgene or multi transgenes.

Replication-defective HSV is non-pathogenic and able to maintain in episomal state in the cytoplasm of host cells, in this case both neural and non-neural tissues. Replication-defective HSV as gene delivery vector has been promising in pre-clinical studies performed on models of neurological disease, including glioma, peripheral neuropathy, chronic pain and neuro degeneration [56].

HSV-1 is the most commonly used vectors in this category. It has been genetically manipulated for many purposes such as for producing oncolytic effect. Other alterations include the insertion of reporter genes such as GFP or lacZ for fluorescent or histologic detection, or genes to improve the infectivity and cytotoxicity of oncolytic HSV-1, insertion of genes encoding interleukins, GM-CSF, cytosine deaminase, and fusogenic peptides.

3.3. Non-viral based gene delivery system

In spite of great achievements in viral based gene therapy, the major drawbacks of viral system, such as immunogenicity, high cost of mass production and mutagenicity slowed down the progress in this field. Although the non viral vectors for gene therapy were far less efficient than their viral counterparts, recent developments in nona-medicine, particularly nano-medicine for gene therapy draws a brighter picture on the potentiality of such systems for a safe, targeted and efficient delivery of nucleic acid material to cancer cells[58]. Non viral vectors are classified into two main categories; cationic lipids and cationic polymers.

Lipids are amphiphilic molecules consisting of a hydrophilic head and a hydrophobic tail. The hydrophilic head can be anionic, cationic or neutral. In case of gene delivery, the cationic lipids gain a lot of attentions because of their ability to bind to negatively charged DNA molecule. Each hydrophilic side of a lipid bilayer can electrostatically react with DNA molecule to form a multi-layer structure called lipoplex (Figure 1, panel A)[59]. The most frequently used lipoplex structure is liposome which consists of a bilayer sphere with the hydrophilic phase encapsulated inside (Figure 1, panel B). The nucleic acid can easily be entrapped inside the liposome and be protected against nucleases, sharing the same bio-distribution and half life with intact liposome in blood circulation[60] the integration of liposome/lipoplex with negatively charged cell membrane happens through the same electrostatic interaction between negative proteoglycans on cell surface and positively charged outer layer of liposome and lipoplex which finally cause the internalization through different mechanisms of

endocytosis (clathrin-mediated, clathrin independent or caveolae-mediated)or other mechanisms include phagocytosis and macropinocytosis [61, 62].

The two common lipids used frequently for DNA delivery are 1,2 dilexyloxy-3-trimethylammonium propane (DOTAP) and N-(1-(2,3-diolexyloxypropyl)-N,N,N trimethylammonium chloride (DOTMA). DOTAP is usually mixed with a neutral helper such as 1,2-dioleoyl phosphatidyl ethanolamine (DOPE) to enhance the transfection [63]. The efficiency of gene transfection not only depends on physico-chemistry of each structural component of lipid, i.e head group, hydrocarbon chain and linkage between head and chain but also on lipid to DNA charge ratio, the structure and proportion of helper lipid, the complex size and charge and the cell type[64, 65].

As mentioned before, the nature of lipoplex internalization depends on the electrostatic interaction between cell membrane and positive charges at the outer layer of liposome. This mechanism has a dual effect, as it can increase the efficiency of transfection and at the same time, renders off-target transfection and also systemic toxicity due to interaction with negatively charged blood components such as Albumin. The latter phenomenon can alter or even reverse the positive charge of liposome/lipoplex and trigger the fast and early clearance of liposome/lipoplex structure from the blood circulation before the particles reach site of action; hence reduce the effectiveness of system in gene delivery [62, 66].

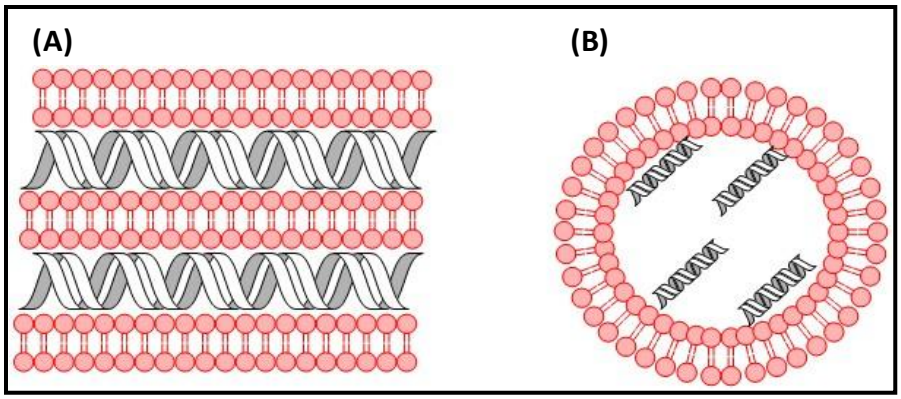

Figure 3: Schematic diagram of supramolecular lamellar structures of lipoplexes (panel A) and cationic liposome (Panel B).

Many attempts have been made to redirect the interaction of lipoplex/liposome with cell membrane to a more targeted fashion and avoid side effects and toxicity. One approach is to attach targeting ligands to lipoplex through covalent band or complexation with cationic lipids. In this context, adding targeting moiety also reduces the surface charge of the liposome and as a result the possibility of interaction with blood components. Transferrin, folate, asialofetuin, epidermal growth factor (EGF) and sugar residues are among the numerous ligands which have been used successfully so far[62, 67].

Poly ethylene glycol (PEG) is the other molecule which has been used vastly to add “stealthy” characteristics to polyplex/liposome systems, therefore enhance the retention time in blood circulation upon intravenous injection. However, some drawbacks such as the susceptibility to oxidation, an IgM-mediated immune response followed by accelerated blood clearance (ABC) halt the use of PEG in case of long-term repeated administration[68-70].

The other groups of non viral vectors which show promising results in gene delivery are cationic polymers. Despite cationic lipids, cationic polymers lack the hydrophobic moiety, therefore completely soluble in water. They can be synthesized in variety of molecular weights, lengths and substituted/added functional groups[71]. Cationic polymers are categorized into natural DNA binding proteins such as protamine and histone, synthetic peptides and polymers such as Poly L-lysine (PLL), Polyethyleneimines (PEI), polyamidoamine dendrimers (PAMAM) and carbohydrate-based polymers such as chitosan. Among them PEI and PLL with different molecular weights and geometry (branched vs. linear) have been more frequently used for non viral gene delivery, thank to their high charge density which facilitates the complexation and condensation of pDNA[72]. An interesting feature of PEI is its proton sponge effect which is related to the structural amine groups (primary, secondary or tertiary). As a result, PEI/pDNA nonoparticles are able to render endosomal escape mechanism and release their DNA cargo in cytoplasm, the ability that lipoplex structures lack per se or show only at a low level through disruption of endosomal membrane[72]. The other advantage of polyplexes is the condensation of DNA, although it is more limited to high molecular weight PEI such as 25KDa[71]. This ability leads to smaller nano-particles (approximately 20-40 nm) compared to lipoplexes which favors higher yield of transfection[61].

In spite of all advantages of PEI and PLL polymers in gene delivery, there are still several drawbacks that should be addressed. As it mentioned before, DNA condensing ability of PEI is more eminent in high molecular weight PEI such 25 KDa compared to low MW PEI (for example 10 KDa)[73]. Two types of cytotoxicity have been reported for PEI; the first

one is immediate cytotoxicity which is the result of free PEI and occurs through non specific interaction with blood albumin and red blood cells, causing the big clusters on the cell surface[74]. After internalization and release of DNA cargo, the free PEI inside the cell reacts with intracellular components leading to shrinking, reduced number of mitoses and vacuolization of the cytoplasm[74].

PLL polymers possess a biodegradable nature, however the transfection efficiency is much lower than PEI, mainly because this polymer only has primary amines which are not able to cause a strong endosomal escape mechanism[75]. Therefore, a helper component, such as a lysosomal disrupting agent Chlroquine or pH-responsive peptides such as INF7 or GALA should be added to the medium. The former causes toxicity when the latter add more instability and possible immunogenicity[72]. The half-life of PLL in blood circulation is very low, due to its rapid interaction with blood component and subsequent clearance from blood circulation[76].

In order to overcome the above mentioned disadvantages, many modifications have been introduced to non-viral systems. For example, in order to decrease nonspecific interaction, PEGylation has been used widely as a well-established technique to increase the bioavailability of polyplexes[72]. Other molecules such as poly-(2-methacryloyloxyethyl phosphorylcholine) (PMPC) and cyclodextrin have also been used to decrease toxicity and increase the efficiency[77, 78].

In conclusion, many developments have been achieved to make non-viral vectors as efficient as their viral counterparts where the beneficial features of them such as low

immunogenicity and safety are preserved. However, the progress toward clinical trials was slow, indicating that still hurdles should be removed to achieve the robust non-viral gene delivery system.

4. Biologically inspired motifs for gene delivery

Biopolymers are defined as polymers that are produced by a living organism. There are three groups of biopolymers; polysaccharides, proteins/peptides and nucleic acids[79]. Here we are more interested in the second group, i.e proteins and peptides that can overcome one or all obstacles in gene delivery. Here, we will discuss protein/peptide sequences able to render a targeted delivery, the ones which can condense and protect DNA and the finally the ones that are able to cause a membrane destabilizing effect and consequently, endosomal escape of nano-particles.

4.1. Peptides for active targeting of tumors

Nanomedicine has been based on pathophysiological features of cancer as well as its molecular aspects. Some common features of tumor tissues include poorly differentiated vasculature, extensive but heterogeneous extravasations and poor, if any, lymphatic recovery[80]. A considerable numbers of nanomedicine systems have been designed to exploit these characteristics, known as enhanced permeability and retention effect (EPR) for accumulation of drug in tumor niche over time, generally called “passive targeting”.

This effect, first described by Maeda et al explained the reason underlying the longer retention time of a high molecular weight antitumor drug in tumor site[81]. Another

groups also showed that leaky vessels and distorted lymphatic drainage stabilized liposomes in tumor niche. It seems that not only these two physiological features, but also particle properties such as size and surface charge plays a role [82].

However, designing a strategy merely based on EPR might be risky because EPR is very variable from tumor to tumor, person to person and even between different parts of the same tumor[83]. Also, EPR effect does not alter the deposition of the drug, it only increases the retention time. As reported by Maeda et al, the major part of administrated dose is accumulated in spleen and liver with less than 5% in tumor[81, 83].

Contrary to passive targeting, active targeting is based on the interaction between gene/drug delivery system and target cells, usually through receptors expressed (or over-expressed) on the target cell surface. Although active targeting does not lead to a higher accumulation of nano-particles in tumor site, but their uptake by tumor cells increase significantly, probably because receptor/ligand interaction prevents their rapid clearance to blood stream[84]. For example it has been shown that HER2 targeted liposomes are taken more than 20 times than non-targeted ones by HER2 over-expressing breast cancer xenografts[85]. Based on these primary results many molecules were considered as candidates for active targeting therapies, among them only antibodies reach clinical trials and even approved for clinical use[83], [80]. The target on the cell can be either a tumor specific ligand or a tumor associated antigen. The famous examples of first group are folate receptor, integrins, vascular endothelial growth factor receptors (VEGFR), Endoglin (CD105) and CD13/aminopeptidase N as targeting ligands[86-88]. The most well-known tumor associated antigens are PSA (prostate associated

antigen), prostate stem cell antigen (PSCA), prostate cancer membrane antigen (PSMA) and the receptor of Urokinase plasminogen activator (uPA) which is involved in tissue degradation, cell migration, angiogenesis, cancer invasion, and metastasis.[89].

In spite of all advances in targeted delivery so far, it has been reported that only 5% of administrated drug accumulate in target organ whereas the other 95% is cleared from blood stream by liver, spleens and lungs. No matter how selective the interaction between ligand and cancer marker is, the targeted reaction between cell and nano-particles only happens when the nano-particles reach the site of action after enough retention time in the blood[83]. Although all currently available nano-particle possess a rational design but this rationale is normally based on biochemical principle whereas a deeper understanding of other factors such as regulation of blood distribution, dynamic aspects of tumor spatial, tumor heterogeneity, and complexities of diffusion barriers in solid tumors might be taken into account for designing a successful nano-particle system.

4.2. DNA condensing motifs

DNA molecule, as one of the longest molecules in nature, undergoes a packing process called condensation inside the cells. A packed DNA molecule occupies less space, as well as becomes more protected against nucleases. Different proteins and peptides are in charge of DNA condensation, such as protamine in sperm, histones in chromatin, and adenovirus core peptide Mu peptide in adenoviruses[90]. All the proteins mentioned above share a feature, i.e the interaction between condensing agent and DNA molecule

happens through positive charge in basic amino acids such as histidine, arginine and lysine and negative charge of DNA molecule. Through these interactions in Eukaryotic cell, DNA molecule is folded and coiled 10000 times in a highly ordered organization.

DNA condensation is a crucial step in gene delivery, since DNA molecule is vulnerable to environmental factors such as nucleases. Also, because of highly charged hydrophilic nature and enormous size, DNA is not able to penetrate through hydrophobic cell membrane. Therefore, the essential role of DNA condensing module is to pack and condense DNA to stable nano-particles which are able to enter the cell.

Although a group of sequence-specific DNA-binding proteins have been used for DNA condensation, the cationic protein/peptides are more abundant, simply because their use does not require the addition of any specific sequence to DNA cargo.

The combination of a positively charged peptide (or lipid) and negatively charged DNA can lead into two architectures; aggregation and self-assembled hierarchical structure. As it has been shown for other molecules, DNA aggregation tend to have an increasing particle size, as more cationic lipid is added to pass beyond critical micelle concentration (CMC). At this point the particle size is approximately a couple of hundreds nanometers and as a result, the turbidity of solution increases. DNA aggregates are not a desired structure in gene delivery as these unordered tangles significantly decrease DNA availability and expression.

In contrary, usually the formation of hierarchical structures is characterized by stable, soluble DNA-self assembling peptide structure above nanoparticle CMC value. The other characteristic of such architecture is the core-shell structure which is visible under

electron microscopy. As also indicated in elegant work of Tirell et. Al, the peptide/DNA interaction follow a saturation binding curve which is correlated with the number of positive charge in peptide vs. the number of negative charges in DNA molecule[91]. We refer to this stoichiometry of binding as NP ratio. For each certain DNA/peptide combination there is a certain NP ratio in which the degree of binging approaches to the saturation point. It has been speculated that at this point DNA is close to be fully packed and particle size shows a decrease. At higher NP ratios (e.i higher concentration of peptides), the extra peptide molecule might loosely bind to outer surface of the nanoparticles or simply dissolve in the medium[92].

Many natural peptides are able to render a self-assembled hierarchical structure when binded to DNA. The 19-amino acid Mu peptide (MRRAHHRRRRASHRRMRGG), 23-amino acid peptide derived from the adenovirus core protein V or PepV (RPRRRATTRRRTTTGTRRRRRRR), $Tat_{49–57}$ derivatives (RKKRRQRRR), POLYTAT $(CGRKKRRQRRRGC)_n$ and histone H1 (34 mer: ATPKKSTKKTPKKAKKPAAAAGAKKAKSPKKAKA, 16mer: ATPKKSTKKTPKKAKK) are among them and have been vastly used for DNA condensation in vitro[15, 93]. However, for the purpose of this work only a few of the most studied DNA condensing motifs will be discussed. A more complete list can be found in table 1.

Adenovirus core peptide, Mu (u) is an arginine rich sequence with multiple binding sites per peptide. Keller *et.al* (2002) compared the binding ability of Mu with another positively charged protein, protamine. They found out that the interactions between mu and DNA result in the formation of significantly more size-stable condensed particles of

the size of 80-100 nanometers than protamine. They also reported the ease of particle formation with pDNA over a broad range peptide:DNA ratios[94]. However, the same group indicated that in spite of strong NLS properties of mu peptide, it is not able to lead a DNA trafficking mechanism which is major pitfall for any gene delivery vector to be considered ideal[95].

The other natural protein family with inherent potential for gene delivery is nuclear proteins, histones. The building block of eukaryotic chromosomes, i.e. nucleosomes, consist of 146 base pair of DNA wrapped around a complex of core histones, H2A, H2B, H3 and H4. The stretch part of DNA between nucleosomes interacts with linker histone H1[96]. Histones have a rich content of basic amino acids such as arginine and lysine which interact with DNA through electrostatic forces between positively charged amino acids and negatively charged phosphate groups. Through this interaction, the volume of DNA package is much smaller than the DNA molecule itself. During S phase of cell cycle, all new synthesized histone proteins are imported to nucleus where they are getting involved in packing and reconstruction of newly replicated DNA. In spite of their small size, importing of histones is through an energy-dependant mechanism which indicates that their entrance to nucleus is by the aim of nuclear pore complexes[97]. Actually, the nuclear localization signal effect of histone H1 and all core histones have been proved in many studies, although different sequences and mechanism cause this effect in each histone subgroup [97, 98].

Because of their innate ability in DNA protection, condensation and packaging and their NLS effect, histones have always been attractive proteins for non-viral gene delivery[99]. In spite of their potentials, the use of whole protein sequence may trigger immune response against DNA/vector complex and also limit their application in large scale gene therapy trials due to problems related to purification of recombinant proteins[99].

A great percentage of histone-based gene delivery studies have been focused on histone H1. This histone is shown to be more efficient in delivering a variety of nucleic acids, including DNA, RNA and small interfering RNA (siRNA). Its efficiency is comparable or even better than liposome-based systems[100]. However, H1 (and not core histones) is greatly dependant on Ca^{2+} ion for better efficiency. Also, transfection efficiency is increased in the presence of Chloroquin, an evidence to prove that this type of Histone might not be able to induce a strong endosomal escape per se [16].

Many attempts have been made to identify the exact sequence of each histone which is actively involved in DNA condensation. Khadake et al. (1997) showed condensing ability is directly related to an octapeptise within C-terminal of Histone H1. This octapeptide contains S/TPKK motifs which have been showed to be a crucial factor in DNA binding[80]. They also compared K<u>SPKK</u>AKK which contains one repeat of mentioned motif with A<u>TPKK</u>STKK<u>TPKK</u>AKK (two units of S/TPKK motifs). The results demonstrated that only 16-mer peptide possesses the ability to condense DNA and lead chromatin to higher structure[80]. To shed more light on detailed structure of C-terminal and the significance of different combination of S/TPKK motifs in direct interaction with DNA, Bharath *et al*.

generated a series of mutants of a 34-mer stretch within the C-terminal of Histone H1d. In this stretch, the first two S/TPKK motifs are located sequentially (144-151 and 152-159) whereas the third one (170-177) is separated by a 10 amino acid spacer. Their results showed that deletion of a single motif reduces the DNA condensation by 25%, whereas double and triple deletion causes a reduction of 40% and 45% respectively[101]. These results agree with the outcomes of previous studies which show the role of S/TPKK motifs in DNA condensation.

Interestingly, in attempt to find the sequence involved in NLS mechanism, Schwamborn et al (1998) suggested that this sequence (PVKKAKKKLAATPKKAKK) contains S/TPKK motif as well[102].

Other groups have been focused on the potentials of core histones, especially histone H2A as module for gene delivery. Balicki et al. (1997) reported the significant increase in DNA delivery to COS-7 cells when Histone H2A was added to the liposome. This effect was not observed for other histones subclasses[103]. They used Histone H2A to successfully transfer the gene coding for IL-12 fused to a single chain IL-12 to a murine neuroblastoma model. They observed the superior ability of histone H2A over commercially available transfection agent. The immune reaction to the tumor is more efficient in groups received H2A based-DNA delivery[104]. Later on the same group generated a series of short peptides, each embedded a number of amino acids from Histone H2A (129 amino acids) to elucidate which part of this histone is involved in DNA delivery and NLS activity. A segment of 37 amino acid (GRGK

QGGKARAKAKTRSSRAGLQFPVGRVHRLLRKG) corresponding to amino acid 1-37 of the N-terminal of histone H2A sequence was recognized to mediate transfection successfully[105]. Four tandom repeats of this sequence were chosen as the DNA condensing module in our studies.

4.3. Membrane destabilizing peptides

Cell uptake of nano-particles can mediated either by endocytotic or non-endocytotic mechanisms; however receptor-mediated endocytosis is the preferred mechanism since it confers cell specificity. It is strongly believed that all endosome end up in late lysosome, where DNA cargo is exposed to degradation by lysosome enzymes[21]. This pathway, as destructive as it is for DNA, can be useful because microtubule transport which is a part of this pathway protects DNA against cytoplasm nucleases[22].

Regardless of the precise cell-entry mechanism, the expression of transgene enhance dramatically if the DNA/vector combination be able to promote an endsomal escape mechanism. Pathogenic viruses and bacteria are equipped with such natural peptides. Their ability to facilitate membrane passing occurs through four major mechanisms: membrane fusion, membrane disruption, translocation and pore formation (Figure 4). The first two mechanisms seem to be more dominant in viruses or viral-derived peptides[106].

The ability of cell penetrating can be mapped to certain domains or short peptide sequences in spite of remarkable differences among virus-driven peptides; they

show some similarities such as length of about 15-30 amino acid and an alternating pattern of hydrophobic amino acids interrupted by hydrophilic amino acids. Most of them can form amphipathic α-helices at acidic pH[107]. However, even at neutral pH, an equilibration exists between protonated (active) and non protonated (inactive) forms of the peptide, resulting in cell membrane disruption and toxicity[108].

Perhaps one of the most well-known of these peptides is hemaglutinin HA-2. It has been demonstrated that N-terminal fusion domain of hemaglutinin HA-2 of influenza virus improves transgene expression. This hydrophobic peptide deprotonates under acidic circumstance of endosome and change its conformation. As a result, some inner parts come to the surface and its destabilizing effects appear[24]. Many synthetic peptides derived from the N-terminus of influenza virus hemaglutinin have been made and tested. Among them, some sequences are shown to increase endosomal escape significantly with minimal toxicity, of which one of the best known is GALA sequence. GALA is a 30 amino acid synthetic peptide with a glutamic acid-alanine-leucine-alanine (EALA) repeat that also contains a histidine and tryptophan residues as spectroscopic probes (WEAALAEALAEALAEHLAEALAEALEALAA)[109]. Its conformation changes from random coil to α-helix when the pH drops from 7 to 5. GALA mediated membrane permeabilization occurs through pore formation mechanism. The trans-membrane cores consist of then GALA α-helix monomers and leads to membrane leakage[109]. GALA has been used in several studies to improving in vitro and in vivo transfection both for pDNA and siRNA [110, 111].

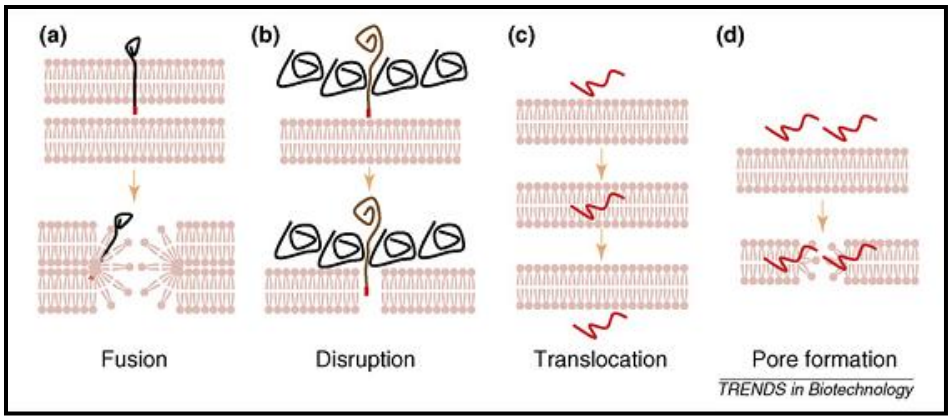

Figure 4: Schematic representation of known membrane-crossing mechanisms. (a) Membrane fusion is promoted by fusogenic peptides (red segments) (b) Membrane disruption is promoted by endosome-disrupting peptides (red segments). (c) Translocation allows the membrane crossing of peptides and short proteins (red segments) without altering the physicochemical integrity of the cell membrane (d) Pore formation is promoted by several types of antimicrobial peptides (red segments) that act cooperatively. The picture was adapted from Ferrer-Miralles et. al (2008) [106].

5. Recombinant multi-functional proteins as vectors for gene delivery

In all gene delivery systems discussed earlier in this chapter, a delivery system may not be able to perform DNA condensing, cell receptor binding, cell entry, endosomal escape and nuclear internalization all at the same time, therefore, different components accounts for each step should be added to the system separately. In contrast, proteins can be designed in a way to contain all the functional modalities mentioned above in one single molecule. Two basic architectural strategies have been suggested to design such proteins for gene delivery; in the first one the structure of a protein which is inherently cable of DNA delivery is manipulated to add more biological functions to it. One example is restricting the tropism of mouse polyomavirus (MPyV) virus like particles by inserting the 18 kDa dihydrofolate reductase (DHFR) into HI-loop of MPyV structural

coat protein[112]. If this approach is the choice, the ability of viral constructional protein to spontaneous assembly to virus like particle (VLPs) after insertion of foreign sequence must be preserved. Usually the recombinant virus-like particles possess exact size and symmetry of viruses of natural viruses.

Overall, some shortcomings such as the variation in size of encapsulated DNA each VLP can accommodate, and the restricted ability to fully assemble when another sequence fused to constructional protein prevent the vast use of this architectural approach to design new protein cage gene delivery vectors. Also it has not been proved yet whether all VLPs from different viruses are able to accommodate a foreign nucleic acid[106, 113].

An alternative method is to use conventional DNA recombinant technology to combine all the functional motifs require for cell entry and accurate gene delivery to the nucleus in a single macromolecule. The nucleoprotein particles resulted from incubation of nucleic acid with multifunctional protein are reported to be amorphous, lacking the symmetrical structure of virus/VLPs, however theirs sizes are still within the range of viruses[106, 114]. Prediction of non specific protein-protein interaction between different peptide motifs in one protein molecule might not be easy to be speculated, especially because occasionally the sequence of some peptides are totally based on in silico design with no counterparts in nature. These interactions should be avoided since it leads to aggregation of protein vector or sometimes, deactivation of one or two motifs because of masking effect of other motifs[115].

The flexibility to test infinite combinations of bio-inspired peptides together in one molecule, along with biocompatibility, low toxicity and homogeneity while produced in a living organism have been appealing for many groups[20, 116]. A variety of sequences have been used to perform basic tasks, i.e DNA condensation, cell targeting, endosomal escape and nuclear internalization. Table 1 illustrates the peptides used in the body of fusion protein for gene delivery.

In line with all attempts to combine all gene delivery modules in one single molecule, Wang et al reported the efficiency of another fusion protein, mainly consists of three major components; a membrane disrupting sequence GALA and a single chain affibody against HER2 receptor connected to each other through four repeats of residues 1-37 of histone H2A as DNA condensing and NLS module. The chimeric protein was reportedly able to render a targeted gene transfer to $HER2^{+}$ ovarian cancer cell line SKOV3 with high efficiency and minimum toxicity[117]. The same group reported different combination of different modules, for example Mu peptide as DNA condensing motif flanking by a NLS sequence and an endosomal disrupting motif called H5WYG. A cyclic targeting peptide to target ZR-75-1 breast cancer cell line was also added to the C-terminal of the protein molecule[116].

The same design has been used further to produce more proteins of this type for targeted gene delivery to $HER2^{+}$ SKOV3 cell line and to evaluate the effect of PEGylation of resulted nucleoprotein complexes on efficiency of gene transfer and decrease the chance of possible immune reaction (Chapter 3).

Another protein of this family has been suggested in this thesis which takes advantage of a specific ligand on Androgen independent prostate cancer cell line PC3. The ability of the suggested protein to be incorporated in suicide gene therapy for above mentioned cell line will be discussed in chapter 4.

Function	peptide
DNA Condensation	Polylysine
	Polylysine containing peptides
	Protamine
	Histones (H1 and H2A)
	TAT (TGRKKRRQRRR)
	PolyTAT
	Tyr-TAT (TTGRKKRRQRRR)
	Segment of *Antennapedia* homeodomain
	Mu (MRRAHHRRRRASHRRMRGG)
Endosomolytic/Fusogenic	Histidine-rich peptides Influenza HA-2 GALA (WEAALAEALAEALAEHLAEALAEALEALAA) KALA (WEAKLAKALAKALAKHLAKALAKALKACEA) Transportan Penetratin TAT (48-60) Melittin
Cellular targeting	RGD
	Integrin α,β
	Secretin
	Folate receptotr
	Transferrin receptor
	HER2 (ligand: anti-ErBb2 (Her2) mAb)
	EGF
	Neurotensin
	Lectin-like oxidized LDL receptor.
	CD13 (ligand:Cyclic Asn-Gly-Arg (cNGR) peptides)
	CD44
	CD105
	Prostate-specific antigen (PSA)

Table 1: known peptide motifs for multi-functional protein-based gene delivery vectors**.**

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Chapter II: Gene Directed Enzyme Prodrug Cancer Therapy*

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1. Introduction

Cancer chemotherapy has been through an evolutionary history since the beginning of its modern period in 20th century. Though the unsophisticated chemotherapy agents of 1940s and 1950s, like nitrogen mustard, elevated the hope for cancer treatment, their undesirable cytotoxic effects along with the limited remission of cancer and their uncontrollable "off-target" side effects intensified the search for more selective anti-cancer drugs.

Because of the cytotoxic nature of chemotherapeutic agents, finding a way to limit the lethal effect only to cancer cells and reduce the adverse effects on normal tissues has been of great interest. Heidelberger *et al* (1957) were the first to practically show the concept of "selective toxicity" [1]. They showed that targeting the uracil uptake pathway is an efficient way of targeting tumor cells since the uracil pathway is more active in cancer cells than normal cells [1].

In 1970s, the emergence of monoclonal antibodies and later their conjugations with cytotoxic agents started a new era of "targeted therapy" in cancer treatment[2, 3]. The main goal of targeted therapy is to exploit unique features of cancer cells, like altered pathways and overexpressed receptors, to narrow down the toxic effects only to the tumor cells and reduce toxicity in normal tissues [4, 5]. In order to achieve this goal, a

better knowledge of cancer biology and genetics becomes necessary. In the past decades, scientists have gained a better insight about the signaling molecules involved in cell cycle and apoptosis, altered transcription and metastasis mechanisms. Such discoveries have led to the identification of more target molecules for selective drug or gene delivery[4, 6].

Among new emerging strategies for cancer gene therapy, one of the prominent ideas is the triggering of a self-destructive mechanism by transferring an exogene (transgene) to cancer cells. The product of the transgene might be naturally toxic for cancer cells (toxin gene therapy) or be a harmless enzyme which catalyzes the conversion of a non-toxic prodrug to a toxic compound[6, 7]. The latter approach is also known as gene directed enzyme prodrug therapy (GDEPT) and is basically a two-step process. First, the enzyme-coding gene is selectively delivered to tumors followed by the systemic or intratumoral administration of a prodrug in a sub-therapeutic dose[8, 9]. The accumulation of the prodrug's toxic metabolites then causes target-specific death in the enzyme-bearing cells or the surrounding cells (Figure 1). This method of gene therapy - also known as gene/prodrug activation therapy (GPAT) or suicide gene therapy (SGD) - aims to improve the therapeutic ratio (benefit versus toxic side-effects) of cancer therapy [10].

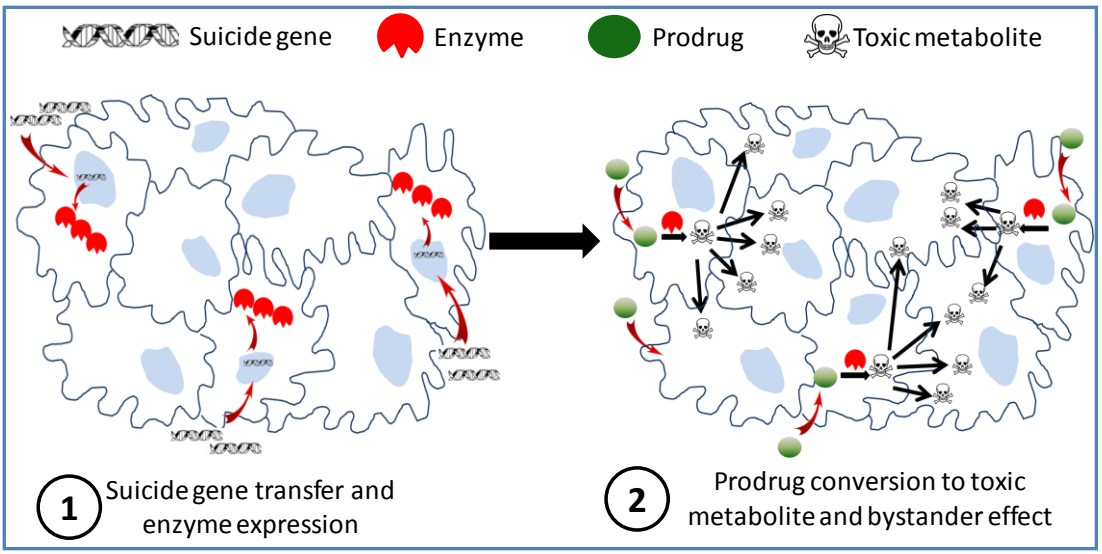

Figure 5: Schematic representation of the two step process in suicide gene therapy. In step 1, with the help of a vector the cancer cells are transduced by suicide genes resulting in expression of an enzyme. In step 2, prodrug is administered which can be converted into its cytotoxic form by the enzyme and kill not only the transduced cells but also the neighboring ones.

The two main factors that have a significant impact on the clinical success of GDEPT are the choice of enzyme and prodrug. Each year, a few new or modified enzyme/prodrug systems are introduced and among them some find their way into preclinical and clinical studies. Section III of this chapter will discuss in details some of the most widely used enzyme/prodrug systems namely: 1) Herpes simplex virus thymidine kinase/ganciclovir (HSVTK/GCV), 2) Cytosine deaminase /5-fluorocytosine (CD/5FC), 3) Nitroreductase/CB1954 (NTR/CB1954), 4) Carboxypeptidase G2 / Nitrogen Mustard (CPG2/NM), 5) Purine nucleoside phosphorylase (PNP)/ 6-Methylpurine deoxyriboside (MEP) and 6) Cytochrome-P 450 (CYP450)/Oxazaphosphorine.

One of the critical elements that govern the success of any enzyme/prodrug system is a potent bystander effect. Unlike other gene therapy systems where only the cells which receive the therapeutic genes undergo apoptosis or necrosis, in GDEPT the

therapeutic effect and cytotoxicity spreads from affected cells to neighboring cells as well. The following section discusses this phenomenon which includes both bystander and distant bystander effects.

2. The Bystander Effect

The success of GDEPT is heavily dependent on bystander effect. Bystander effect phenomenon can explain the observations that transduction of even less than 10% of cells are sufficient to eradicate the whole cancer cell population[11, 12]. The main suggested mechanism for bystander effect is through diffusion of toxic metabolites from transduced cells to non-transduced ones, either passive or active. For example, ganciclovir triphosphate (GCV-TP), the final toxic metabolite of HSVTK/GCV system is a charged molecule. The bystander effect induced by this metabolite is entirely dependent on active transport via gap junctional intercellular communication (GJIC)[13]. This can be a drawback for such systems because compared to normal tissue, tumor tissues usually lack highly ordered cell junctions. Beside diffusion of toxic metabolites, another interesting mechanism is endocytosis of apoptotic bodies released from dying cells by neighboring untransfected ones. It has been shown that by preventing the release of such apoptotic vesicles bystander effect could effectively be shut down[14].

The bystander effect which has been discussed so far is effective only in vicinity of transduced cells where cells are connected through cell to cell communication or located in a short distance to receive the apoptotic bodies or toxic metabolite of prodrug. Another type of bystander effect, namely distant bystander effect, involves the activation of immune system [15]. This phenomenon has been observed when CD^{4+} and

CD^{8+} T cells and NK cells are attracted into the primary tumor site followed by systemic immune response to the metastatic sites which are anatomically far from the primary suicide gene/prodrug treated tumors[16]. Distant bystander effect can be quite effective in preventing formation of secondary tumors[17]. It is believed that death of tumor cells causes the release of tumor-associated Antigens (TAA) which are stimulators for an anti tumor immune response[18]. Agard et .al (2001) showed the adoption of inhibition response to tumor growth when splenocytes from animals treated with a AdV-TK and GCV are transferred. The same response was not observed when splenocytes from AdV-TK (no prodrug) treated animals were transferred, indicating that the cell killing step is required to trigger an immune response against tumor[19]. Both necrosis and apoptosis can trigger immune response however the immune reaction after necrosis has shown to be stronger [20]

In the following section, we will discuss the important factors that play roles in the success of suicide gene therapy.

3. Pillars of an Effective Suicide Cancer Gene Therapy

As of June 2012, 149 out of 1843 clinical trials (8.1%) conducted worldwide focused on cancer suicide gene therapy, indicating that suicide gene therapy is considered one of the major approaches for cancer gene therapy[21]. The success of a cancer suicide gene therapy is highly dependent on each of its three components, i.e. enzyme, prodrug and the delivery system (vector). The choice of each component becomes especially critical at the clinical level because only a few numbers of vectors are deemed safe and efficient to elicit a significant therapeutic response.

Enzymes that are used in GDEPT can be categorized into two groups. The first group includes enzymes that can be found in normal human cells such as cytochrome P450. This group of enzymes is less probable to induce any immune response; however, the presence of enzymes in normal cells could result in off-target toxicity. The second group of enzymes usually originate from viruses or bacteria[22]. Thymidine kinase (TK, Viral), cytosine deaminase (CD, bacterial and yeast), carboxypeptidase G2 (CPG2, bacterial) and nitroreductase (NTR, bacterial) are examples of this group[23] . In contrast to the first group, enzymes in this group are more likely to be immunogenic but there is less probability for observing toxicity in non-target tissues [24]. In the past decades many attempts such as site directed mutagenesis and protein recombination techniques have been made to introduce new versions of enzymes with not only higher affinity to prodrug, but also more stability and less immunogenicity. Besides the enzymes, the prodrug should also possess several important characteristics in order to help maximize therapeutic response. The prodrug should be stable under physiological conditions, show low toxicity profile before activation and high toxicity to cancer cells after activation. In addition, the activated prodrug should posses a high bystander effect in order to overcome the deficiencies related to vectors' low transduction rates. Based on the activation mechanism, the prodrugs are categorized into two groups; direct-linked and self-immolative[22, 25]. Direct-linked prodrugs become activated in one straightforward reaction to produce the active component. GCV and CB1954 are examples of prodrugs in this group which are targets for TK and NTR enzymes, respectively. In Self-immolative mechanism, the prodrug is converted to an

intermediate form which spontaneously changes to active form through fragmentation process. The most well-known example of this category is doxorubicin prodrug which can break into doxorubicin by CPG2 enzyme[26]. Self-immolative mechanism makes it possible to use many different well-known anticancer drugs as prodrugs for GDEPT[27, 28].

As it was mentioned before, the third important component of a suicide gene therapy system is the vector. Until this date, most vectors used for suicide gene therapy are viral vectors; amongst them, adenovirus occupies the highest share of vectors used for gene delivery in clinical trials (23%)[21]. Adenovirus offers certain advantages over other viral vectors such as greater transduction level, ability to infect dividing as well as non-dividing cells, transduction of neuronal tissue without clinical toxicity and capability to produce gene expression without integration into the host genome[29]. Also, the transduction efficiency is quite high after local injection [30]. In spite of all advantages, the bio-distribution of adenovirus is mostly restricted to liver. The toxicity and the patient immune response to the viral protein remained the main concerns for wide use of this vector in gene therapy[30, 31].

Retrovirus vectors are the second preferred vectors for suicide gene therapy. Unlike adenovirus vectors, retro viruses trigger less abrupt immune response and are able to integrate their genome into host cell's genome. As useful as this feature is for a long term expression of transgene, the risk of oncogenicity remains to be addressed in retrovirus gene therapy. For genome integration, the nuclear membrane needs to be disrupted; therefore retroviral vectors transduce only the actively dividing cells[30]. In malignancies such as glioma, the percentage of non-dividing cells is significantly high

and therefore this group of cells will not be targeted by retroviral vectors. However, the pioneer clinical trials of Oldfield *et al.* Raffel *et al.* Kun *et al.* and later Klatzmann *et al.* treating brain cancer patients with HSVtk suicide gene therapy brought into attention the role of retroviral mediated suicide gene therapy as a complementary therapy[32-36]. The main focus of all these studies was using retrovirus producing packaging embryonic mouse fibroblast cells (Virus packing cells or VPCs) directly to the tumor or to the surgical cavity after tumor cyto-reduction followed by intra venous ganciclovir therapy[34]. The safety of such therapy was confirmed by all the above-mention studies. In Klatzman study, the therapy led to a significant increase of survival time[34].

Where viral vectors provide an entry level of targeted gene transduction, another group of vectors, cell-based vectors, are mostly vectors targeted toward the cell niche. Engineering T cells, fibroblast stem cells, outologous cancer cells and even bacterial cells are examples of cell-based vectors.

The concept of cell-based suicide gene therapy is mostly known for the use of stem cells as a vector to carry the suicide gene to tumor surrounding environment and not to tumor cell. The pioneer works of KS Aboody and others illustrated the proof of principle for stem cell tropism for tumors and metastasis lesions[37-41]. All types of stem cells, including neural stem cells, mesenchymal stem cell (MSCs) and embryonic stem cells are capable to render the tropism which is independent from tumor type, route of administration or the situation of the host's immune system[39 42]. The ability of "suicide gene armed" human embryonic stem cell to pass the blood brain barrier and reach hard-to-access tumors such as brain tumors raised many hopes for an effective therapy

for these types of cancer[40]. However, some obstacles stagnate the process; in spite of ease of stem cells isolation from patients, some inherent characteristic such as limited number of passages before differentiation and the short lifespan after injection restricts their extensive use in clinical trials[39, 43, 44]. Considering these facts, finding a suitable vector to transduce stem cells at lower passages becomes crucial. So far, retroviral vectors have been used to transduce stem cells *ex vivo*. In a recently published paper, Park *et al*. showed the feasibility of using retroviral vector to transduce mesenchymal stem cells. In this study, human mesenchymal stem cells were transduced with a retroviral vector coding for bacterial cytosine deaminase. Retroviral vectors had no effect on the growth pattern, differentiation or genetic stability in up to 11 passages [45].

In spite of many successful preclinical trials, the use of bioengineered stem cell in clinical setting for gene therapy remains very limited due to their pro-angiogenesis and immunosuppressive properties which in some cases expedite tumor growth[46]. For some types of stem cells, such as MSCs, the bio-distribution of cells after intravenous injection is still under debate [44]. More studies should be done to rule out tumorogenesis of stem cells before they reach their full potential in clinical trials.

The other group of cell-based vectors is autologous tumor cells. In a reprehensive study, Okada *et al.* suggested the feasibility of using a vaccine consisting of autologous tumor glioma cells and normal fibroblast cells carrying both HSVtk and interleukin-4 (IL-4) on patients with recurrent supratentorial glioblastoma or anaplastic astrocytoma. IL-4 showed immunomodulatory effect in brain tumors such as increasing MHC II, increasing the growth rate of both B and T cells[47]. In this study, the tumor cells were

transduced with IL-4 and HSVtk genes. The fibroblast cells were isolated from skin and were transduced with the same gene cassette. After *in vitro* tests confirmed the sensitivity of cells to GCV and IL-4, the patients received two shots in two weeks interval, followed by systemic GCV administration[47]. No data has been released on the result of this study yet.

The other group of vectors which gains a lot of attention because of their tropism to tumor niche is bacteria. The idea of using bacteria as a vector of gene therapy was emerged based on observation occurred in 1813 in which the cancer patient who got contaminated with Clostridium perfringens showed tumor regression. Later on, it was proved that many anaerobic strains such as Bifdobacterium, Salmonella, Escherichia coli, Vibrio cholerae and Listeria monocytogenes only germinate under hypoxia condition which is a typical condition of many tumors, although the extent of hypoxia is different [48]. Most recently, the non-invasive strains have been genetically modified to carry various genes such as enzymes for GDEPT or toxins specifically to tumor site. For a comprehensive review, the reader is referred to Baban *et al.* recent article[48].

4. Enzyme/Prodrug Systems: From Bench to Bed

In the following sections, we take a close look at the most commonly used enzyme/prodrug systems and highlight their most significant accomplishments as well as shortcomings at both preclinical and clinical levels. To remain focused, we have refrained from elaboration on the history, use of less common enzyme/prodrug systems and also application of enzyme/prodrug systems in cancer imaging which can be found

elsewhere [24, 49]. An overview of the most widely used enzyme/prodrug systems is presented in Table 1.

Enzyme	Prodrug	Mechanism of Action	Bystander Effect	Distant Bystander Effect
Herpes Simplex Virus Thymidine Kinase (HSVTK)	Ganciclovir	Blocks DNA synthesis. S and G2 phase arrest. Mitochondrial damage. Active in dividing cells.	High, when GJIC exists Low, when GJIC doesn't exist	Yes
Cytosine Deaminase (CD)	5-fluorocytosine	Blocks DNA and RNA synthesis. Inhibits thymidylate synthetase. Decline in Bcl-2 level. Active mostly in dividing cells.	High, independent of GJIC	Yes
Nitroreductase (NTR)	CB1954 and analogues	DNA interstrand cross linker. Active in both dividing and non-dividing cells	Very High, independent of GJIC	Yes
Carboxypeptidase G2 (CPG2)	Nitrogen mustard CMDA ZD-2767P	DNA interstrand cross linker. Active in both dividing and non-dividing cells.	High, independent of GJIC	Yes
Purine Nucleoside Phosphorylase (PNP)	6-methylpurine deoxyriboside	Inhibits DNA, RNA and protein synthesis. Active in both dividing and non-dividing cells.	High, independent of GJIC	Yes
Cytochrome P450 (CYP450)	Oxazaphosphorines; Cyclophosphamide	DNA interstrand crosslinking agent. Active mostly in dividing cells.	Medium, independent of GJIC	unknown

Table 2: This table summarizes the most important features of six main enzyme/prodrug systems that are used in GDEPT.

4.1. Herpes Simplex Virus Thymidine Kinase/Ganciclovir (HSVTK/GCV) System

Thymidine kinase plays an important role in reactivation of herpes simplex virus-1 (HSV-1) from latent phase [50]. TK initiates the phosphorylation of deoxythymidine to deoxythymidine monophosphate which is then turned into deoxythymidine

thriphosphate by cell's endogenous kinases[51]. This mechanism, as necessary as it is for viral DNA replication in infected cells, has been used for activation of guanosine analogue, GCV, to its toxic metabolite GCV-TP by endogenous kinases[52]. The incorporation of GCV-TP in DNA structure leads to single-strand breaks and eventually inhibition of cellular DNA polymerase (Figure 2)[52]. In comparison to cytosolic TK, the affinity of HSVTK is 1000-fold higher to GCV. Therefore, the first step of GCV phosphorylation occurs predominantly by viral TK[53].

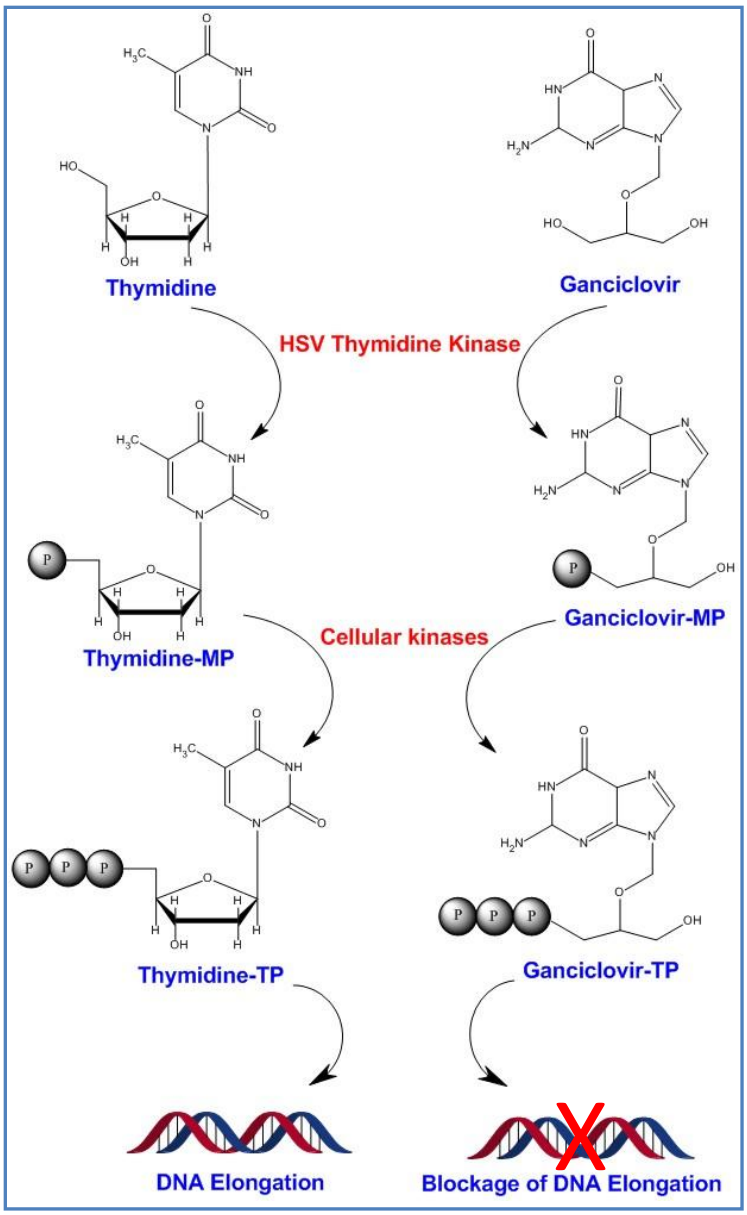

Figure 6: Mechanism of action for ganciclovir (GCV). HSVTK phosphorylates thymidine to thymidine monophosphate (thymidine-MP) which undergoes more phosphopylation steps by cell endogenous kinases. The final product, thymidine triphosphate (thymidine-TP) is one of the building blocks of DNA structure. GCV competes with thymidine and gets phosphorylated to ganciclovir-MP first and then ganciclovir-TP. Ganciclovir-TP blocks DNA elongation by inhibiting DNA polymerase.

HSVTK/GCV system is the most abundantly used system in preclinical cancer suicide gene therapy studies. The mechanism of GCV mediated cell death is involved necrosis

and apoptosis rather than direct chemical toxicity[54, 55]. The evidence of innate and adaptive immune responses and a synergistic effect with radiation has also been reported[18, 55, 56].

In spite of great literature on applications of HSVTK/GCV in cancer therapy, there are a number of shortcomings which are highlighted by several groups[57, 58]. Firstly, as mentioned earlier, the cytotoxic bystander effect of GCV is heavily dependent on the GJIC; thereby, its anticancer activity is significantly limited as GJIC is considerably compromised in many tumor tissues[57, 59]. The second concern is the relatively high affinity (indicated by K_m) of HSVTK for its natural substrate thymidine. K_m of HSVTK for GCV is 100 folds higher than it is for thymidine, therefore the dose of GCV required to win the competition for active site is still too high. This subsequently generates nonspecific toxicity such as severe bone marrow and immune system suppression, slow cancer cell killing kinetics and to some extent incomplete killing[12, 60]. In order to alleviate this problem, Black *et al.* (2001) modified HSVTK's active site at five different residues generating a mutant, namely SR39. This mutant showed 294-fold decrease in the dose of prodrug needed to render the same effect as wild type HSVTK (wt-HSVTK) (Table 2) [61]. Ardiani *et al*. (2010), later developed a construct made of HSVTK mutants fused to the second enzyme in GCV phosphorylation pathway, mouse guanylate kinase (MGMK). The combination of MGMK/SR39 showed stronger bystander effect where only 1% of transduced cells were enough o render 60% cell death. The same study indicated that in the presence of GCV (as low as 0.1 mg/Kg), the combination of SR39 with guanylate kinase showed slightly stronger tumor growth inhibition in comparison to SR39 alone[62].

Amino acid position	159	160	161	168	169
HSVtk	L	I	F	A	L
SR39	I	F	L	F	M
TK007	L	I	F	H	L

Table 3: Sequence differences among wild type HSVTK, SR39 and TK007 mutants. In comparison to wild-type, SR39 has five amino acid mutations at positions 159, 160, 161, 168 and 169, whereas TK007 bears only one mutation at position 168.

Other groups have also made attempts to enhance the affinity of HSVTK for GCV. Balzarini et al. (2006), reported a mutant TK (A168H mutation) which showed significantly non-detectable thymidine kinase activity but with an elevated activity towards GCV by 4 fold[63]. Preuss et al. (2011) splice corrected A168H mutation version of HSVTK to make another mutant, namely TK007 (Table 2). The in vitro results showed an improved cancer cell killing efficiency and significantly higher bystander effect over the wt-HSVTK. The in vivo studies also showed complete remission of glioblastoma xenograft tumors in the presence of GCV with doses as low as 10 mg/kg[58].

Considering the outstanding cancer cell killing efficiency of HSVTK/GCV system, it has been successfully used for therapy of leukemia[64], glioma[65], bladder cancer[66], oral cancer, as well as others in various animal models. The promising preclinical results encouraged a number of clinical trials in the past decade, in which HSVTK/GCV system been used as an adjunct therapy in patients with glioblastoma, prostate, hepatocarcinoma, head and neck carcinoma and others (Table 3) [67-69]. Most of the clinical trials so far have been in phase I/II with the focus on the safety and toxicity of suicide gene therapy treatment. This is mostly due to the use of adenoviral vectors in

these clinical trials. Review of the data shows that a number of these studies have addressed the safety of adenoviral vectors when used locally[68]. For example, Nasu *et al*. (2007) injected escalating doses from 10^9 to 10^{10} of viral particles directly to prostate of patients with prostate cancer. Their study showed neither viral DNA in systemic circulation nor any change in phenotype distribution in peripheral blood samples[68]. Xu et al. (2009), later tried intratumoral injection with dose escalation from 2.5×10^{11} to 1×10^{12} in patients with head and neck carcinoma. In spite of minor side effects, the authors demonstrated the safety of the study and also reported a partial response in the loci of injection[70] . In a more recent study by Sangro *et al.* (2010), patients with hepatocellular carcinoma received different intratumoral doses of first generation adenoviral vector bearing HSVTK followed by systemic administration of GCV. Even at the high dose of 2×10^{12} viral particles per patient no serious side effects was observed. In patients who received high doses, partial tumor stabilization and intratumoral necrosis was also reported. The authors confirmed the safety and feasibility of such local therapy in patients with hepatocellular carcinoma[71]. As the first in this category, Cerepro® (Sitimagene ceradenovec) an adenoviral vector-based HSVTK/GCV system with the potential for the treatment of high grade glioma has been introduced. In a very recent study, Chiocca et al. (2011) investigated the effect of AdV-TK/valacyclovir as an adjuvant therapy to surgery and chemo radiotherapy in newly diagnosed glioma patients. No toxicity related to viral dose or the treatment itself was reported, not any adverse interaction with radio chemotherapy. An improvement is survival time and quality of life has also been reported. One patient with poor prognosis survived 46.4

months. The promising results of this study have encouraged on ongoing Phase II trial[72]. So far, in none of the clinical trials based on localized HSVTK suicide gene therapy any serious vector related toxicity has been reported. Cerepro® (Ark Therapeutics; UK and Finland) has passed preclinical and phase I/II clinical trials (2010) in patients with operable high-grade glioma[73, 74]. Although significant increase in survival rate has been observed in patients who received intra-cavity injection of Cerepro® after tumor resection, but the efficacy results so far have not been satisfactory to receive approval by the European Committee for Medicinal Products for Human Use [74].

Overall, it seems that the efficiency of therapy and not the safety is the main problem that should be addressed. The bottleneck of the most studies is the low percentage of transduced cells by viral vectors. To address this problem, redirecting the tropism of adenoviral vectors has been suggested to be a potential solution for higher rate of transduction, especially in cancer tissues which barely express CAR[75]. Redirecting the tropism of adenoviral vector through changing the surface ligands has been reported in various studies[76-78]. In a recent phase I clinical by Kim et al. (2012), the application of this technique has been demonstrated by using a tropism modified adenovirus carrying RGD sequence in the fiber knob[79]. As a result, the transfection of adenovirus depended on integrin receptors rather than CAR. The safety profile of this adenoviral vector was tested before the clinical trial[75]. The results of this clinical trial have not been posted and still in progress.

In parallel to the strategies mentioned above for the enhancement of vectors' transduction efficiency and therapeutic efficacy, tissue- or cell-specific promoters with

elevated activity in tumor tissue have also been utilized to increase vectors' safety as they tend to accumulate in non-target tissues such as liver and lungs. The most common promoters which have been used to enhance the expression of suicide genes at the target tumor site are human telomerase reverse transcriptase (hTERT) promoter, carcinoembryonic antigen (CEA) promoter, osteocalcin promoter (OC), hypoxia and radiation-responsive elements[24, 80-83]. The latest clinical trial using this approach was conducted by Kubo *et al.* which goes back to year 2003. A DNA construct with HSVTK under the control of osteocalcin promoter was designed in an adenoviral vector backbone for local suicide gene therapy of prostate cancer[84]. Although the treatment was well-tolerated but due to the low expression of CAR in prostate tumors of the patients, this study did not produce significant results in terms of efficacy and did not move forward.

Enzyme/Prodrug System	Clinical Stage	Tumor Type	Vector	Reference
HSVTK/GCV	I/II	Glioblastoma multiform	Liposomal	67
HSVTK/GCV	I	prostate cancer	Adenoviral	68
HSVTK/GCV	I	Head and neck cancer and other malignant tumors	Adenoviral	70
HSVTK/GCV	I	Prostate cancer	Adenoviral	84
HSVTK/GCV	I	Hepatocellular carcinoma	Adenoviral	71
HSVTK/GCV	I	Recurrent Gynecologic Cancer	Adenoviral	79

HSVTK/VCV+Chemoradiotherapy	IB	Malignant Glioma	Adenoviral	[72]
CD/5-FC and HSVTK/GCV	I	Prostate cancer	Adenoviral	[85]
CD/5-FC and HSVTK/GCV	I	Prostate cancer	Adenoviral	[86]
CD/5-FC and HSVTK/GCV	I	Prostate cancer	Adenoviral	[87]
NTR/CB1954	I	Gastrointestinal	Adenoviral	[88]
NTR/CB1954	I	Liver cancer	Adenoviral	[89]
NTR/CB1954	I/II	Prostate cancer	Adenoviral	[90]
CPG2/Nitrogen Mustard	I	Colorectal carcinoma	ADEPT	[91]
P450/ Oxazaphosphorine	I/II	Pancreatic carcinoma	Encapsulated Allogeneic Cells	[92]
P450/ Oxazaphosphorine	I	Breast cancer or Melanoma	MetXia-P450 (Retroviral)	[93]

Table 4: Clinical trials for the major enzyme/prodrug systems in past decade. Clinical trials before year 2003 are not shown.

4.2. Cytosine Deaminase/5-Fluorocytosine (CD/5-FC) System

The anticancer drug, 5-flourouracil (5-FU) has been used over four decades for the treatment of colorectal, breast and head and neck solid tumors. Its side effects include myelosuppression, mucositis, dermatitis, diarrhea and cardiac toxicity among others[94]. A precursor of 5-FU, 5-FC is shown to be converted to 5-FU by bacterial or fungal CD which does not exist in mammalian cells[95]. Once converted to 5-FU, it can easily diffuse to neighboring cells thanks to its small size and neutral charge[96]. 5-FU is converted by intracellular enzymes to several metabolites which lead to formation of fraudulent 5FU-

RNA and 5FU-DNA, inhibition of thymidylate synthase and eventually apoptosis (Figure 3). The level of mRNA inhibition is reported to be higher than systemic administration of 5-FU, leading to to protein starvation in non dividing cells[97]. CD/5-FC system has a number of advantages over HSVTK/GCV including gap junction independent bystander effect and induction of significant distant bystander effect[98]. 5-FC, but not 5-FU, is able to diffuse through blood brain barrier; hence, many studies have focused on using this property for treating hard-to-reach tumors such as glioblastoma[98]. The other advantage of CD/5-FC system is the radiosensitizing ability of 5-FU which can enhance its tumor killing efficiency in combination with radiotherapy[99]. A distance bystander effect and anti tumor immune response was reported by Haak et .al (2000) mediated by massive filtration of $CD8^+$ T cells to solid tumor induced by low immunogenic tumorigenic rat adenocarcinoma cell line AS stably expressing CD. This effect is independent of 5-FC treatment which is in contrast with other studies showing that cell death as a result of conversion of 5-FC to 5-Fu is an essential step for immune response[100] . This feature might be beneficial in terms of vaccination but in the context of suicide gene therapy the reaction is against the bacterial protein (CD) expressed by tumor cells but not the suicide gene/prodrug system.

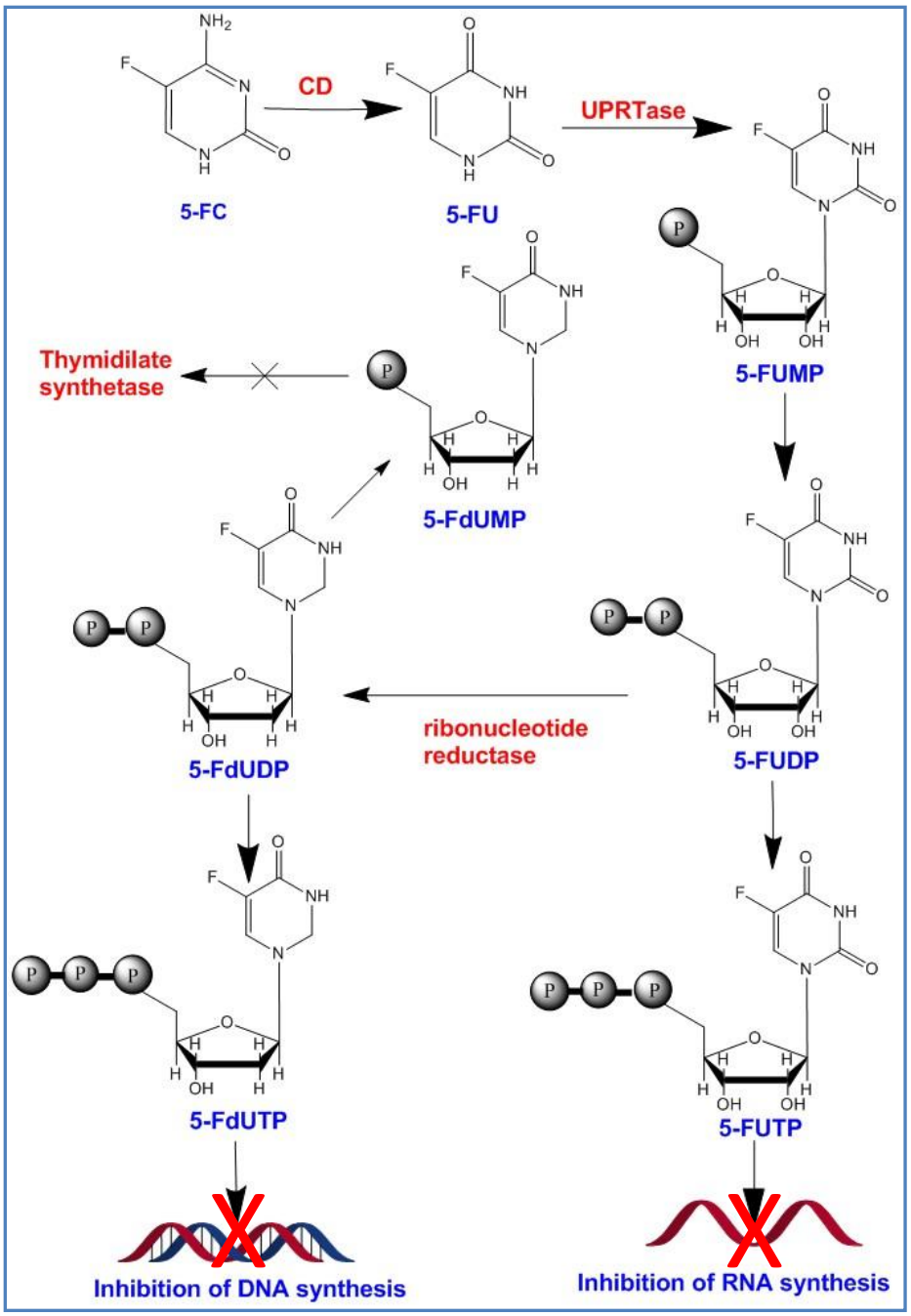

Figure 7: Deamination of 5-FC by cytosine deaminase (CD). The product, 5-FU is converted to 5-FUMP and eventually 5-FUTP which blocks RNA synthesis. Other byproducts such as 5-FdUMP and 5-FdUTP block DNA synthesis after incorporation into DNA structure.

In spite of the above mentioned advantages, there are few drawbacks which overshadow the clinical usefulness of CD/5-FC system. One is that 5-FC might be converted to 5-FU by normal flora in the gut resulting in side effects. The other is the

higher affinity of bacterial cytosine deaminase (bCD) for its natural substrate, cytosine, which necessitates the higher dose of 5-FC. Several attempts have been made to improve bCD kinetics characteristics such as higher affinity and lower IC_{50} for 5-FC. Kaliberova et al. (2008), reported the sequence of a mutant, bCD-D314A, which showed a significant increased specificity y towards 5-FC with a lower IC_{50} in comparison to wt-bCD. This mutant has been successfully used in combination with low dose radiation to reduce tumor sizes in various cancer models[101, 102]. Another mutant, reported by Fuchita et al. (2009), has showed the most desirable features for suicide gene therapy in terms of significant shift to 5-FC, decrease in IC_{50} and an outstanding bystander effect compared to wt-CD[103].

While many studies have been focused on improving bCD toxicity profile, cytosine deaminase from the yeast saccharomyces cerevisiae (yCD), possesses the natural characteristics which overtakes bCD in enzyme kinetics. Its K_m for 5-FC is significantly lower (by 22 folds) than bCD. However, the thermolability of yCD and its short half life in vivo limits its use at the clinical level[12]. New mutants of yCD, namely yCD double and yCD triple seem to overcome these problems, imparting more thermostability and in case of yCD triple, even greatest enhancement in sensitivity[104, 105]. In another approach, the combination of yCD with uracil phosphoribosyltransferase (UPRT) gene which converts 5-FU to 5-fluorouridine 5'-monophosphate produces a higher sensitivity to 5-FC in comparison to yCD alone[106,107]. While CD/5-FC alone has been tested successfully on a variety of tumors in preclinical settings, there are studies that show the significant advantages of using of CD along with HSVTK/GCV, or as a radiation sensitizer in

combination with radiotherapy[85, 108]. Although the exact mechanism of radiosensitization is not clear but it is hypothesized that induction of cell apoptosis in S phase where the cells are more resistant to radiotherapy may play a major role.

Despite the vast use of CD/5-FC system in preclinical level, examples of clinical studies are more limited than HSVTK/GCV system (Table 3). Freytag et. al (2003 and 2007), in two separate studies examined the combination of CD/HSVTK in phase I clinical trials against prostate cancer using first and second generation adenoviral vectors [86, 87]. The expression of transgene was detectable up to three weeks after injection and no serious side effects related to adenoviral vector or prodrug were reported. It was observed that PSA doubling time increased from 17 to 31 months in these patients, delaying androgen salvage therapy for 2 years[86, 109]. These findings established adenoviral mediated double suicide gene therapy as a potentially safe and effective treatment for prostate cancer. These studies also raised the possibility that it may have the potential to improve the outcome of conformal radiotherapy. Recently (2012), a commercialized viral vector (Toca 511) for heat stabilized yCD suicide gene therapy was introduced by Tocagen Inc. Toca 511 is a non-lytic retroviral vector armed with yCD which has been deemed suitable for clinical trials. Tocagen Inc. is currently conducting a phase I/II clinical trial in the United States on patients with recurrent or progressive Grade III or Grade IV gliomas after tumor resection (http://www.clinicaltrials.gov; see NCT01156584 and NCT01470794). Although no data has been posted yet but their protocol was based on in vivo data demonstrating the

safety and effectiveness of Toca 511 therapy followed by oral dose of extended-release 5-FC in animals [110 111].

To date, significant progress has been made in stabilizing and optimizing the cytosine deaminase enzyme. Given that the 5-FU is able to kill both dividing and non-dividing cancer cells and has a significant bystander effect, this enzyme/prodrug system emerges with great potential to succeed. The major limitation at this point appears to come from the low efficiency of the targeted vectors.

4.3. Nitroreductase/CB1954 System

CB1954 is a DNA alkylating agent which becomes activated after conversion to its toxic metabolite by E. coli NfsB nitroreductase (NTR)[112]. After a non-enzymatic reduction by cellular thioesterase, CB1954 becomes a potent DNA chelating agent which can freely diffuse to surrounding cells and trigger extensive DNA damage and a P53 and cell cycle-independent apoptosis in both replicating and non-replicating cells (Figure 4)[113]. The effect on cells is described very fast; the effect was manifested after 24 hours, in comparison to HSVTK/GCv system which usually takes 1 or 2 weeks for any detectable effect[114]. Because NTR enzyme needs NADPH or NADH as an electron donor, the activation of prodrug happens only intracellularly; therefore, side effects are limited to modified cells and the cells in vicinity. The only drawback of NTR/CB1954 system is the low activation rate of prodrug, as CB1954 is not the natural substrate of NTR,.

These observations provoked others to focus on the improvement of enzyme kinetics[115]. In a study by Grohmann et al. (2009), the bacterial NTR was codon optimized

to enhance and stabilize its expression in mammalian cells[116]. Other groups have also worked to assess other prokaryotic enzymes which are able to activate CB1954 or design nitroaromatic derivatives which are potentially better prodrugs for nitroreductase [113, 117, 118]. For example, Prosser et al. (2010), evaluated all E. coli nitroreductases with the ability to activate CB1954 and reported few new NTRs such as AzoR, NFsA and NemA. In comparison to NfsB nitroreductase, NfsA in the form of purified protein was 10 fold more efficient in CB1954 activation[115]. In addition, they observed wild type and mutated version of other nitroreductase enzymes such as flavin reductase FRase I from Vibrio fischeri are able to activate CB1954 and its analogues[113, 119]. In parallel, others have developed new generation of nitroaromatic prodrugs such as nitro-CBI-DEI and PR-104A which can be activated both by hypoxia and bacterial nitroreductases [120]. Under hypoxic conditions (e.g., tumor environment), these two compounds undergo a one-electron transfer reduction reaction by human endogenous oxidoreductases but in normal cells they rapidly get reoxidazed[118]. Bacterial nitroreductases catalyze the same reaction through a two step electron transfer in an oxygen-independent fashion which results in toxic metabolites[118]. Therefore, these two mechanisms induce synergistic cytotoxic effects.

So far, a limited number of clinical trials have been conducted with NTR/CB1954 system (Table 3). In a phase I/II clinical trial conducted in 2009, replication-defective adenoviruses encoding nitroreductase and systemic administration of prodrug CB1954 were used for the treatment of patients with localized prostate cancer[90]. The trial assessed the effect of dose, route of administration and volume of injection on bio-

distribution of viral vectors. Although immune response against adenoviral vectors was detected, but signs of decrease in tumor sizes were also observed in some patients (decrease in PSA >10% and in two patients >50%). This effect lasted for six months. Another piece of evidence which supported the effectiveness of therapy was a delayed PSA progression[90]. In a 2012 phase I clinical study which has conditionally been approved in UK, a tumor specific promoter (human telomerase) has been used in an adenoviral vector along with NTR/CB1954 system. The system was injected i.p to the patients with advanced intra-abdominal cancer, followed by the injection of prodrug CB1954 [121]. This clinical trial (UK-0125) is still in progress and no data has been released so far.

This enzyme/prodrug system overall has the advantage of being able to kill both dividing and non-dividing cancer cells with significant distant bystander effect; however, concerns related to CB1954 conversion rate and dose-dependent hepatotoxicity has slowed down its progress. Although, new generation of prodrugs such as PR-104A (Proacta Inc.) in recent years have revived hope and several new studies are underway to evaluate their therapeutic potential in clinical trials[122], but their limited availability through commercially available sources has limited the number of studies that are necessary for comprehensive preclinical evaluation.

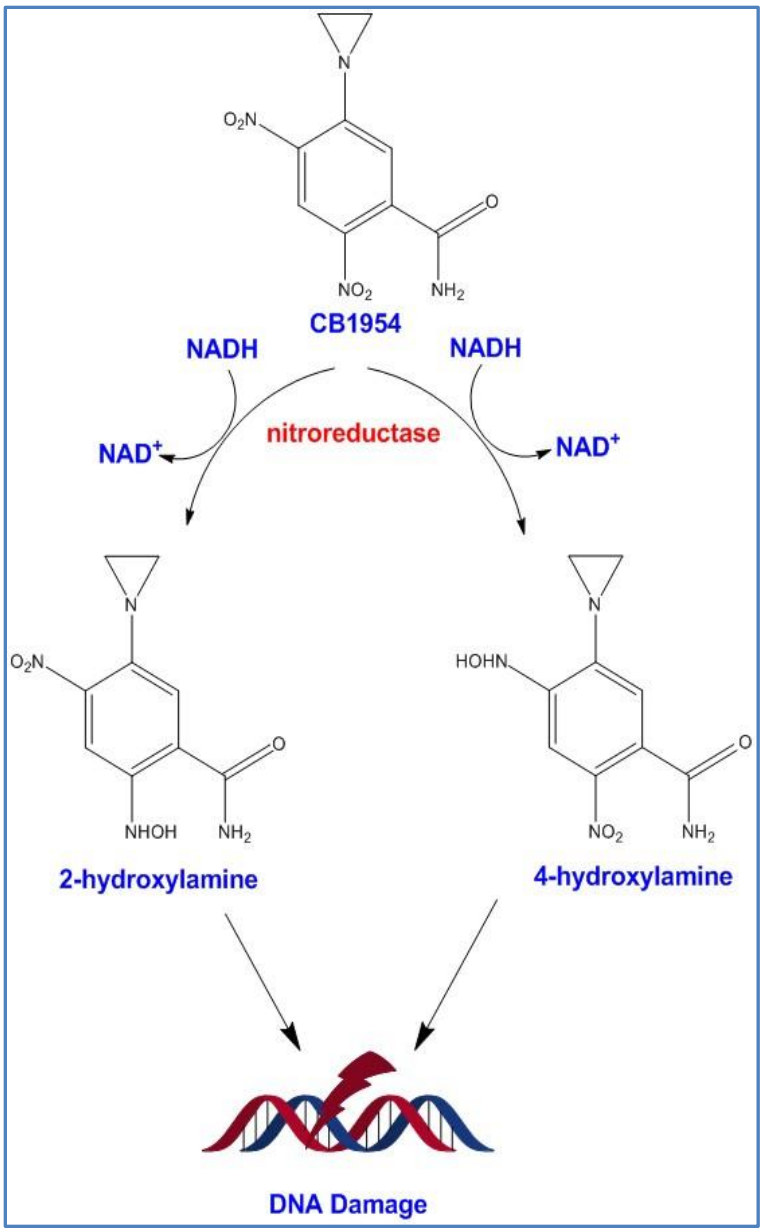

Figure 8: Activation of CB1954 by bacterial nitroreductase. After activation, both 4-hydroxylamine and 2-hydroxylamine metabolites cause DNA damage.

4.4. Carboxypeptidase G2/Nitrogen Mustard (CPG2/NM) System

In all other enzyme/prodrug systems discussed so far, the prodrug undergoes more than one step to get activated. Usually, one of these steps, especially when dependant

on intracellular enzymes, might be the rate limiting step, decelerating prodrug activation reaction. In spite of them, bacterial enzyme CPG2 from Pseudomonas RS-16 with no mammalian equivalent cleaves glutamic acid from nitrogen mustard-based drugs to release the cognate drug which unlike the product of other systems such as HSVTK/GCV or CD/5-FC is active by itself and does not require further modification (Figure 5). [123]. The final alkylating metabolite is lipophilic which freely diffuses from cell to cell, independent from gap junctions and eventually makes inter- and intra-strand DNA linkage. CPG2/NM system affects both dividing and quiescent cells and potent bystander effect has been observed both in vitro and in vivo[123 89]. Sribbling et al. (2000) reported that after treating xenograft breast tumors that expressed CPG2 with prodrug CMDA, most of the tumor cells were in apoptotic phase, even though the percentage of transduced cells was low[124].

CMDA (4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamicacid) is among the first developed NM-based prodrugs which can be hydrolyzed to glutamic acid and DNA alkylating agent 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoic[112]. Other nitrogen mustard derivates such as ZD2767P with at least 300 times more potency than CMDA have also been introduced (Figure 5)[91]. Friedlos et al. (2002), reported the design of three new prodrugs for CPG2 which showed much greater cytotoxicity in breast and colon carcinoma cell lines as compared to CMDA. Surprisingly, these prodrugs were more effective when the lower percentage of cells (10% to 50%) expressed CPG2 enzyme[125]. In another attempt to develop a new prodrug for CPG2, Mancini et. al (2009) designed a new non-toxic derivative suitable for evaluating gene delivery and expression

of enzyme. The conversion of compounds to their cleaved byproducts was detectable by ^{19}F magnetic resonance spectroscopy, thanks to the fluorine groups[126]. One of the drawbacks of CPG2/Nitrogen Mustard system is the toxicity caused by secretion of the enzyme from transduced cells into blood circulation. In addition, due to the bacterial origin of the enzyme, a potent immune reaction might be triggered. In order to prevent these problems, the secretory tag has been cut off from the enzyme to restrict its localization to cytosol while maintaining its activity[124].

The main application of CPG2/Nitrogen Mustard system in clinical studies has been in combination with antibodies in Antibody-Directed Enzyme Prodrug Therapy (Table 3). In this approach, the enzyme is attached to a tumor-specific antibody via chemical reaction or recombinant protein fusion technology[127]. Early clinical trials with this kind showed the feasibility of technique, with the evidence of localized CPG2 activation only in tumor tissues[128]. The main reported toxicity was myelosuppression due to long half life of the drug and its leakage to the blood. Later, in another dose-escalating phase I clinical trial, Francis et al. (2002) used A5CP, a conjugation of bacterial carboxypeptidase to a Fab_2 fragment of a mouse monoclonal antibody for carcinoembryonic antigen (CEA) in 27 patients with advanced colorectal carcinoma. The prodrug ZD2767P was used because it had higher potency than CMDA and shorter half life after conversion to its toxic metabolite[91]. Overall, no significant therapeutic effect was observed because of an inadequate localization of enzyme/antibody conjugate and a humoral immune reaction against CPG2 and mouse antibody. However, the prodrug had a fast clearance and no active form of drug was detectable in blood which resulted in less Myelosuppression[91].

From the review of literature, it appears that this enzyme/prodrug system has a significant potential for success in clinic, if a prodrug with suitable half-life (e.g., ZD2767P) in combination with non-secretory CPG2 and an efficient targeted vector are used. Unfortunately, there are no recent clinical trials performed with this system to discuss at this point.

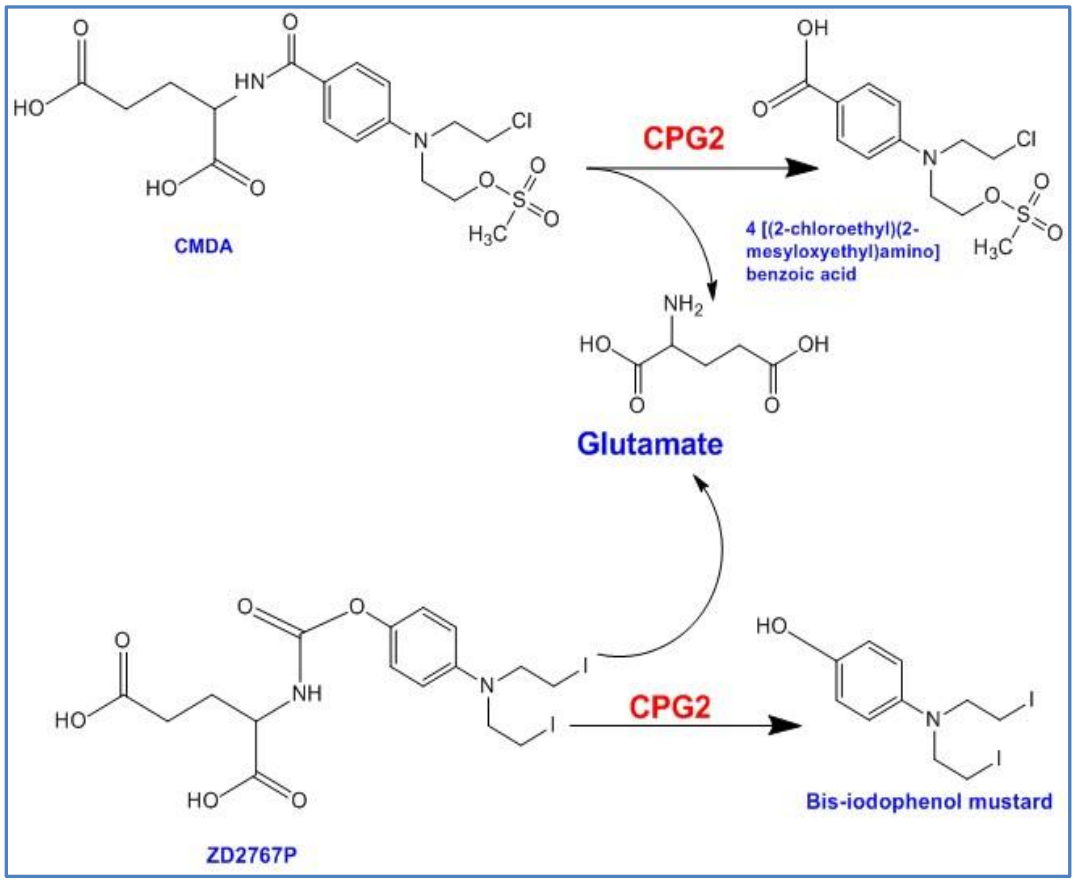

Figure 9: Activation of CMDA and ZD2767P to their toxic metabolites by bacterial carboxypeptidase G2 (CPG2).

4.5. Cytochrome P450 (CYP450)/Oxazaphosphorine System

P450 enzymes are the most important super family in metabolism of xenobiotics. They are located mostly in liver; however, their activity in other organs and to some extent in tumor has been detected. The role of P450 enzymes in metabolism and inactivation of chemotherapy agents is well-understood[129], however, the effect of P450-mediated metabolism on oxazaphosphorine drugs such as cyclophosphamide (CPA) and ifosphomade (IFO) is quite different. While most of the chemotherapeutics are deactivated by P450 enzymes, these enzymes convert CPA and IFO to their unstable but active metabolite, 4-hydroxy cyclophosphamide (4-OH-CPA) which is later decomposed to phosphoramide mustard and acrolein (Figure 6). Phosphoramide mustard is a potent DNA alkylating agent affecting both dividing and non dividing cells, but the manifestation of effect is more noticeable in dividing cells[129]. Although CPA and IFO are able to penetrate through blood brain barrier, this ability ceases after conversion to their active metabolites. The metabolites also show a strong bystander effect quite independent of gap junctions[129]. With a high level of P450 activity in liver, the goal of GDEPT therapy has been set on inhibition of hepatic P450 while localizing the activity of recombinant P450 enzymes in tumors. Therefore, the systemic side effects of oxazaphosphorine drugs such as neurotoxicity, nephrotoxicity, and urotoxicity could be avoided. Huang et al. (2001) used CYP2C inhibitors to inhibit the conversion of CPA in liver while gliosarcoma xenografts were able to express CYP2B1. Unfortunately, the results were not significant indicating that the inhibitors were not specific for hepatic P450[130]. Another approach to shut down hepatic P450 is co-administration of cytochrome P450 reductase (CPR) inhibitors and anti-thyroid drugs such as Methimazole

since thyroid hormone controls the expression of CPR[131]. Utilizing this strategy, a number of clinical trials have been carried out worldwide for P450/oxazaphosphorine system (Table 3). In one of the most successful phase I/II trials conducted in 2003 for this system, fourteen patients with pancreatic tumor were treated with genetically modified allogenic cells to express CYP2B1. The cells were delivered to the tumors via tumor vasculature followed by administration of low dose iphosphamide two days after cell injection. In four patients the tumor regression was complete whereas other 10 patients had a stabilized tumor size. The median survival time was doubled from 22 weeks to 44 weeks[92]. In another phase I/II clinical trial (2005), human CYP2B6 a commercial retroviral vector, MetXia®, was used to deliver CYP2B6 (human cytochrome P450 type 2B6 gene)[93]. A total of nine patients with breast cancer and three with melanoma received Metxia® followed by oral administration of cyclophosphamide. The results showed safety and efficiency of MetXia, supporting the idea of using P450 isoforms/CPA in future clinical trials. However, no other clinical trial with this enzyme/prodrug system has been reported in recent years.

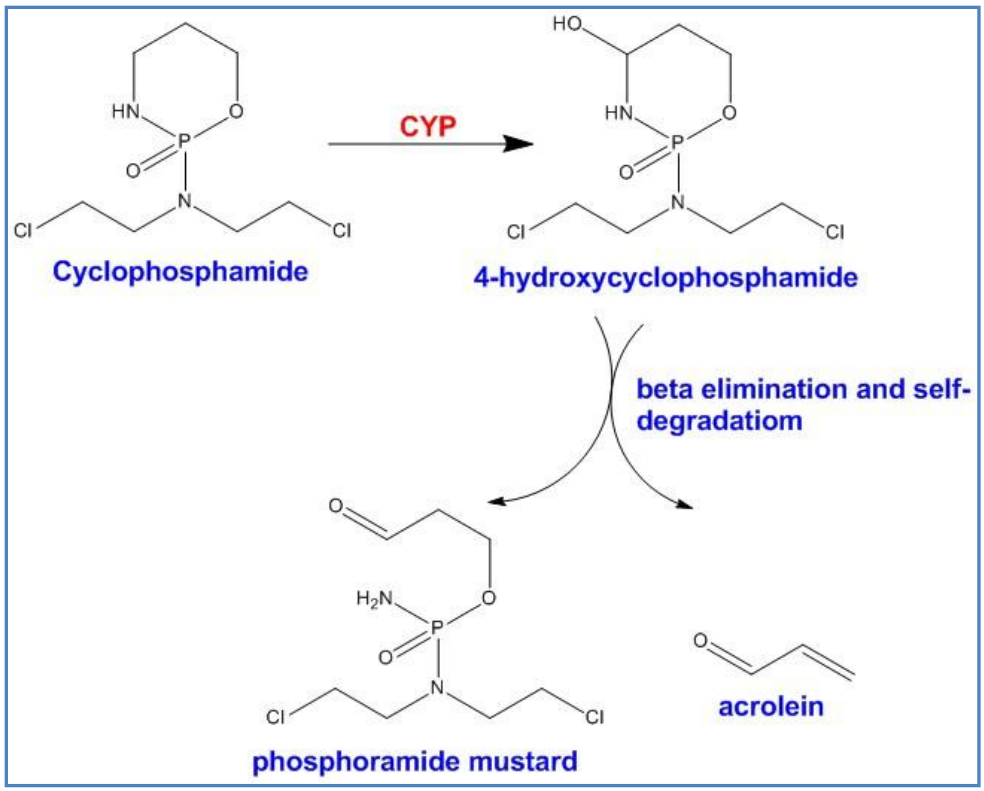

Figure 10: The two-step process which produces the toxic metabolites, phosphoramide mustard and acrolein from cyclophosphamide. CYP enzyme super family catalyzes the first step whereas the second step is a self-immolative reaction.

4.6. Purine Nucleoside Phosphorylase/6-Methylpurine Deoxyriboside (PNP/MEP) System

The E. coli purine nucleoside phosphorylase (PNP) is a hexameric enzyme, catalyzing the glycosidic cleavage of purine ribonucleoside prodrugs such as 6-methylpurine 2-deoxyriboside and fludarabine to 2-deoxyribose-1-phosphate (or arabinose-1-phosphate) and free base compounds such as 6-methylpurine (MEP) and 2-fluoroadenine (F-Ade), respectively (Figure 7). It has been reported that F-Ade is 100 fold more potent than MEP in terms of cell growth inhibition[132]. Both compounds are freely diffusible across cell membranes, allowing their spread from PNP transduced to

untransduced cells and are toxic to both proliferating and non-proliferating cells; thereby, achieving a potent bystander effect[133, 134]. The bystander activity is facilitated by the nucleotide and nucleobase transporters across membranes in both directions not requiring cell-cell contact or gap junctions. Metabolites from PNP are incorporated during RNA synthesis and hence eventually block protein synthesis. Because of the inhibitory effects of this system on DNA replication, some studies point at its usefulness in treating slow growing cancers such as prostate[132].

Limitations related to immunogenicity from the bacterial PNP led investigators to develop human PNP (hPNP) mutants that can cleave adenosine based prodrugs which are not recognized by wild-type hPNP[135]. In human cells, adenine is salvaged from the extracellular environment by adenine phosphoribosyl transferase (APRT) which is responsible for the first and rate-limiting step in the activation of both MEP and F-Ade. Preclinical studies with PNP have shown that the enzyme activity from PNP transfected tumor cells are more than that of endogenous APRT possibly causing MEP to diffuse away from the tumor. However, unlike CD/5-FC system where UPRT plays a role in increasing sensitivity of the prodrugs, APRT overexpression did not improve the efficiency of the PNP system[136]. In an effort to improve anti-tumor activity, designer nucleosides have been reported in combination with a structurally modified PNP enzyme[137, 138]. Unfortunately, this approach has not yet been successful in identifying prodrug/enzyme combinations that demonstrate better in vivo anti-tumor activity. To date, there has been only one phase I clinical trial which started in 2011 by PNP Therapeutics®. This trial investigates the safety of E.coli PNP/fludarabine phosphate in

patients with head and neck cancers or other solid tumors (http://clinicaltrials.gov, identifier NCT01310179). This is an ongoing clinical trial and no data has been released yet. The usefulness of this enzyme/prodrug system is unclear at this point and yet to be determined.

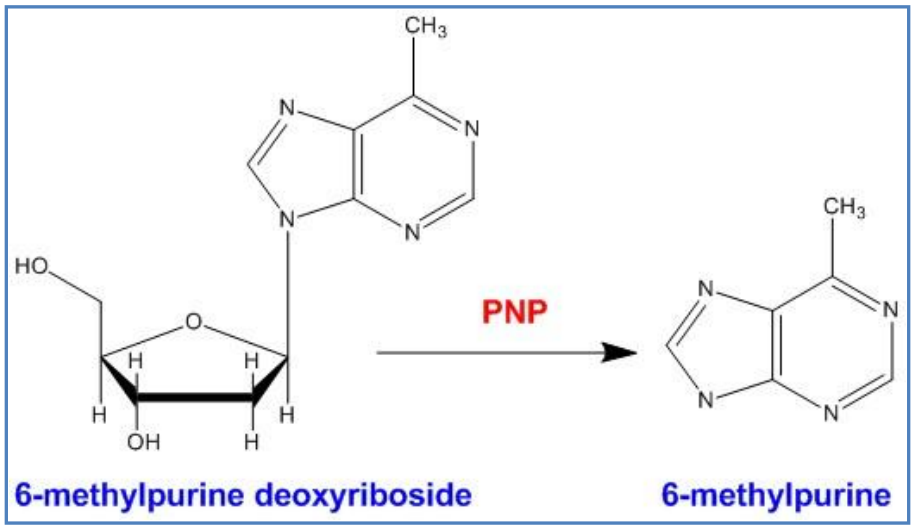

Figure 11: the conversion of 6-methylpurine deoxyriboside to 6 -methylpurine by bacterial purine nucleoside phosphorylase.

5. Conclusion

Significant progress has been made in the field of cancer suicide gene therapy since its introduction in 1984. Vector development technology have come a long way but designing an efficient targeted vector is still of great need, especially for solid and dense tumors where the cells at the inner layers of tumor are not easily accessible. Due to the significant limitations imposed by vectors' low transduction efficiencies, enzyme engineers and medicinal chemists are expected to devise new strategies to compensate for this shortcoming. Therefore, engineering of more stable and high affinity enzymes in

combination with safe prodrugs capable of inducing more potent bystander effect appears to be of great need and key to success. While promising, but the progress appears to be slow since only 45 clinical trials have reached phase III, one in phase IV and none approved for clinical use. This indicates that there is still a vast room for improvement and more investment in this field is needed.

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Chapter III

Vector Optimization: Development of Highly Efficient and Uniform Targeted and Shielded Vector for Gene Delivery to SKOV-3 Cancer Cells*

*A version of this chapter has been published. Please see: Karjoo Z, McCarthy HO, Patel P, Nouri FS, Hatefi A. **Systematic Engineering of Uniform, Highly Efficient, Targeted and Shielded Viral-Mimetic Nano-particles.** Small (Impact factor 8.5, Weinheim an der Bergstrasse, Germany). 2013.

1. Introduction

For nucleic acid based drugs such as siRNA and plasmid DNA (pDNA) to reach their target tumor site, they must overcome several extracellular as well as intracellular barriers. In attempts to overcome these obstacles, non-viral gene delivery systems (vectors) such as liposomes and polymers have been developed. These vectors are designed to protect nucleic acids from endonucleases by condensation into nanosize particles, facilitate their accumulation in the tumor environment via enhanced permeation and retention (EPR) effect, enhance their internalization into the cancer cells through use of targeting ligands and mediate efficient transgene expression. In order to extend the half-life in blood circulation, the surfaces of such particles are usually decorated with highly hydrophilic polymers such as polyethylene glycol (PEG). The role of PEG is to sterically stabilize the surface of the nano-particles, minimize interaction with plasma proteins (opsonization) and enhance the probability for accumulation in tumors via EPR effect. [1, 2]. PEG helps to achieve this goal by reducing the surface positive charge of the nano-particles resulting in minimum interaction with negatively charged blood compounds such as albumin and erythrocytes; hence, reduced

clearance by the mononuclear phagocyte system[3]. Because the target sites of the nucleic acid delivery systems (e.g., siRNA and pDNA) are inside the cells, accumulation in the tumor environment is not enough and it is essential for the nano-particles to be internalized by the cells. For this purpose, the nano-particles surfaces also need be equipped with targeting ligands (e.g., antibodies)[4]. Therefore, an optimum balance of PEG to targeting ligand is necessary to be expressed on the surface of nano-particles in order to achieve maximum shielding without compromising internalization activity.

Although a number of PEGylated but non-targeted drug delivery systems for small molecules (e.g., Doxil™, Oncaspar™) have reached the clinic, there has been no approved PEGylated and targeted delivery system for nucleic acids for clinical use. As a matter of fact, there is only one PEGylated and targeted formulation in clinical trials (Phase I), namely CALAA-01[5, 6]. It has been reported that the translation of nanotechnology-based cancer therapy into the clinic has been hampered by the lack of delivery systems (vector) that are not only clinically safe and efficient but from manufacturing standpoint cost-effective and compliant with criteria for batch-to-batch uniformity[7]. A critically important consideration with regard to formulation development for PEGylated-targeted nanomedicines relates to the fact that many promising nanomedicines reported in the literature are quite complex, and therefore difficult to synthesize and scale-up by the pharmaceutical industry. This is especially true for systems that are based on physicochemically different components, such as polymers, lipids, antibodies and peptides, because their production involves multiple synthetic and purification steps. This increases the costs, the complexity and the batch-

to-batch variance of such formulations, and as a result decreases their commercial attractiveness and their clinical relevance[8].

The characteristics of a desirable vector and importance of batch-to-batch uniformity without which the clinical translation of the final product is unlikely was also discussed by a panel of scientists and physicians from industry and academia during the Image-Guided Drug Delivery Summit organized by the National Institutes of Health, USA (Figure 1)[9].

Characteristics of an Ideal Nucleic Acid Delivery System

Clinical Level

Safety: Manageable vector related toxicity & immunogenicity

Efficacy: Maximum efficiency with minimum dose

Ability to Target: localizes the therapeutic cargo to the target site

Production Level

Cost-effectiveness: cheap raw materials, minimum number of steps involved to produce, conform to batch-to-batch uniformity

Figure 1: Desirable characteristics of a clinically translatable nanotechnology-based drug/gene delivery system.

The objective of this research is to develop a formulation of highly efficient targeted and shielded virus-mimetic nano-particles with an almost neutral surface charge that can be produced in a simple manner and meet the criteria for batch-to-batch uniformity. To achieve the objective, we first genetically engineered a single chain multifunctional biopolymer in one step that could provide cell targeting, DNA condensation, endosome membrane disruption, nuclear localization and efficient gene expression, namely THG.

To provide shielding, we then synthesized PEGylated histone H2A and adenovirus *Mu* peptides using solid phase peptide synthesis approach. The VMPs were constructed as a result of complexation of pDNA with the multifunctional biopolymer in combination with histone H2A or adenovirus *Mu* peptides. Various formulations of the virus mimetic particles (VMPs) were prepared and characterized using physicochemical and biological assays in order to find a few that are highly efficient, stable, bear almost neutral surface charge and can be manufactured in a reproducible fashion.

2. Materials and Methods

2.1. Production of THG Recombinant vector

THG single chain multifunctional fusion biopolymer consists of three main domains: HER2 targeting affibody (T), Histone H2A derived DNA condensing sequence (H) and pH sensitive fusogenic peptide (GALA). The gene encoding the THG vector was synthesized by IDT® integrated DNA technologies with N-terminal *NdeI* and C-terminal *XhoI* restriction sites. A C-terminal 6x histidine tag was also designed in the vector sequence to facilitate purification. The THG gene was then cloned into a pET21b expression vector (Novagen®) and transformed into *E.coli* BL21 (DE3) p*LysS.* One BL21(DE3) plysS colony bearing THG-hisx6:pET21b was inoculated in 5 ml Circlegrow® medium supplemented with Carbenicillin (50 ug/ml). The culture was incubated overnight at 30^{oC}. The day after, 5 ml overnight culture was added to 500 ml of Ciclegrow® medium and was shaken to reach OD_{600}>0.5. IPTG was added to final concentration of 0.4 mM and the incubation was continued for 6 hours at $30^{o}C$. Cells

were then collected at 5000xg for 20 minutes. For the purification, 20 ml lysis buffer (5 M Urea, 1 M NaCl, 100 mM NaH2PO4, 10 mM Tris, 1% Triton X-100 and 10 mM imidazole, pH 8) per gram of cell pellet was added and the solution was stirred for 1 hour followed by centrifugation at 20000 rpm for 1 hour at 4 ^{o}C. The supernatant was collected and added to 1 ml Ni-NTA agarose (Qiagen) pre-equilibrated with lysis buffer. The slurry was shaken for 1 hour on ice then added to the column. The column was washed with 100 ml of lysis buffer and 40 ml of wash buffer (5 M Urea, 1 M NaCl, 100 mM NaH2PO4, 10 mM Tris and 40 mM imidazole, pH 8). The protein was eluted by Elution buffer (3 M Urea, 500 mM NaCl, 100 mM NaH2PO4, 10 mM Tris and 200 mM imidazole, pH 8) and stored at -20 ^{oC}.

2.2. Desalting and preparation of THG stock solution

In order to prepare a working protein solution, extra salt, urea and Immidazole were removed by passing the purified protein through Sephadex G-25 prepacked column (GE Healthcare) followed by washing the column with Bis Tris propane/NaCl (10 mM, 5 mM, pH 7) to collect the protein. The concentration of protein was measured after desalting by Nanodrop 2000 spectrophotometer (Thermoscientific), using molecular weight and extinction coefficient of the protein.

2.3. Preparation of targeted and PEGylated-targeted VMPs

To prepare VMPs, total amounts of vector needed for a given N:P ratio was calculated. To prepare targeted VMPs, predetermined amounts of THG vector in HEPES buffer (100 mM, pH 7.4) were flash mixed with 1 ug of pDNA (pEGFP) to form complexes

at different N:P ratios (1 to 12) in a total volume of 100µl. Flash mixing (flash nanopercipitation) means addition of peptide solution to pDNA solution in an instant. For example, to prepare N:P ratio of 1, 1.3 ug of THG vector was used to complex with 1µg of pEGFP.

To prepare PEGylated-targeted VMPs, first truncated Histone H2A (H2A) with the amino acid sequence of RGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGG and adenovirus *Mu* peptide with amino acid sequence of MRRAHHRRRRASHRRMRGG with >98% purity were synthesized by American Peptide Company (Sunnyvale, CA). Then, the synthesized peptides were PEGylated by using 2000 and 5000 daltons PEGs to make: H2A-PEG2K, H2A-PEG5K, *Mu*-PEG2K and *Mu*-PEG5K. The covalent conjugation of PEG to C-terminus of the *Mu* and H2A peptides were conducted by the American Peptide Company. A mix of THG with H2A-PEG2K, H2A-PEG5K, *Mu*-PEG2K and *Mu*-PEG5K at different weight/weight (ug/ug) ratios was prepared and complexed with pEGFP to make PEGylated-targeted VMPs. For example, at N:P 12, THG and PEGylated peptides were mixed at weight/weight ratios of 16:0, 14:2, 12:4, 10:6, 8:8, 6:10, 4:12, 2:14 and 0:16, respectively. The schematics of the method are shown in Figure 4.

2.4. Particle size and charge analysis and evaluation of reproducibility

Targeted and PEGylated-targeted VMPs were prepared as described above and the mean hydrodynamic particle size and zeta potential of the particles were measured at room temperature using Nano-ZS Zetasizer (Malvern Instruments, U.K). The data are

presented as mean ± s.d (n=3). While for routine particle size and charge measurements number of independent batches prepared was set at 3, for reproducibility measurements the number of samples was set at 10 (n=10). Ambisome™ vials (n=4) were kindly provided as a gift by the pharmacy store at the Cancer Institute of New Jersey (New Brunswick, NJ). To measure reproducibility, the average and standard deviation of polydispersity index (PDI) of ten samples was determined by the zetasizer and from that we calculated the coefficient of variation (CV) using the following formula: CV= standard deviation/ mean × 100

2.5. Particle shape analysis by transmission electron microscopy

To study the shape of the VMPs, one drop of sample was put on a carbon type B coated copper grid (Ted Pella, USA) for 5 minutes. The sample was dried and the grid was stained for 1-3 minutes depending on the need with 1% Sodium phosphotungstate solution. The grids were imaged using transmission electron microscope (1200EX electron microscope, JEOL®, USA) at UMDNJ TEM core imaging facility. This method was adapted with slight modifications from a previously published method for imaging viruses[10].

2.6. Particle stability over time and in the presence of salt

To measure the stability of the VMPs over time, the particle size measurements were performed every 30 minutes on each sample for 180 minutes. For particle stability studies in the presence of salt (NaCl), VMPs were prepared in HEPES buffer (pH 7.4) and considered as zero molar salt. From a 2M NaCl stock solution, aliquots were taken and

added to the VMPs in HEPES buffer until the desired NaCl concentration is obtained. The particle sizes at each salt concentration was measured and reported as mean±s.d. (n=3).

2.7. Cell transfection studies

The above mentioned VMPs were used to transfect HER2 positive SKOV-3 ovarian cancer cells using the previously reported methods[11, 12]. In brief, SKOV-3 cells were seeded in 96-well plates. Cells were transfected with vector/pEGFP complexes at various N:P ratios (equivalent of 1 µg pDNA). The green fluorescent protein (GFP) was visualized using an epifluorescent microscope to evaluate GFP gene expression. To quantify transfection efficiency, percent transfected cells and total green fluorescence intensity was measured using F500 Flow Cytometer (Beckman Coulter, USA). Each time 10,000 cells were counted and the total fluorescence intensity of GFP positive cells was normalized against the total fluorescence intensity of untransfected cells (background control).The data are presented as mean ± s.d, (n=3). Percentage of GFP positive cells was determined by Kaluza flow analysis software (Beckman Coulter, USA) using 99% gating. Total green fluorescence intensity (TFI) which is a measure of green fluorescent protein expression was calculated using the following formula: TFI= mean fluorescence value of each GFP positive cell (measured by flowcytometer) × total number of transfected cells.

2.8. Cell viability study

SKOV-3 cells (4 x 10^4 per well) were seeded in a 96-well plate and incubated in McCoy's 5A full medium supplemented with 10% FBS. 24 hours later, 100 ul of freshly prepared vector/pEGFP was added to each well. The cells were incubated with the VMPs for 2 hours. Then the medium was replaced with fresh McCoy's 5A medium supplemented with 10% FBS. The cells were incubated at 37°C for another 48 hours, before WST-1 cell proliferation reagent (Roche Applied Science, Indianapolis, Indiana) was added to each well. The absorbance at 450 nm was measured 2 hours after adding the reagent. Considering the viability of untreated cells as 100%, the viability of other samples was reported accordingly. The data are reported as mean ± s.d. (n =3).

2.9. Immunogenicity study

All in vivo steps have been revised and approved by The Animal Care and Facilities Committee, Rutgers University. For immunogenicity study, Balb/cJ male mice, 5-6 weeks of age (Jackson labs, Maine, USA) were housed one week after arrival and grouped in 5 per cage. A blood sample was taken from tail vein after one week of acclamation (day 0). Each mouse receives two doses of THG NP 12 and THG/Mu5K NP12 4:12 weight/weight ratio on day 7 and 21 via retro-orbital injection. Each dose consisted of NPs prepared for 5 ug of pDNA in a total volume of 100 ul. The particles were prepared in HEPES buffer (100 mM). The buffer itself was previously prepared in injection grade water and passed through 0.2 um syringe filter. The pDNA used for this study (namely pBUD-FireFlyluc-SR39) was also purified using EndoFree plasmid Maxiprep Kit (Qiagen, USA). The particle size was measured before each injection.

On day 35, the animals were euthanized using CO_2 chamber and the blood was collected by cardio puncture. The blood samples were centrifuged at 2000 rpm for 15 minutes and the plasma was collected and stored at -80 ^{o}C for further analysis.

2.10. ELISA test for evaluation of IgG and IgM elevation

Enzyme linked immunosorbent assay (ELISA) to determine IgG and IgM levels was performed as per manufacturer's instructions using the IgG and IgM ELISA kits from Bethyl laboratories (Texas, USA). Plasma was used at a 1:1000 dilution for ELISA. Briefly Immulon2 plates (Dynatech) were coated with the capture antibody and incubated for 1h. Plates were washed and blocked overnight in blocking buffer containing 1% BSA. The following day, plates were washed 3 times, standard and samples were added in duplicates and plates incubated for 1h. Subsequently plates were washed and secondary antibody conjugated to HRP was added. OPD Easy tablets (2mg/ml; Acros) were used as detection substrate and the plates were read at 490 nm.

The data was analyzed in OriginPro 9.0 software. For each standard concentration point, the absorbance was calculated as A490 on that point minus A490 of blank. Each standard concentration was plotted against its A490 and the best fit was chosen for each set of data based on R Square. The concentration for each blood sample before and after injection was calculated using the plot equation and given A490 for each sample.

3. Results and Discussion

Numerous publications have previously explained the advantages of using recombinant techniques to synthesize biopolymers allowing the production of such bio macromolecules in a cost-effective manner[13-15]. In terms of production costs, the recombinant vectors can be produced far cheaper than the viral counterparts. In addition, given the fact that there is no need for the removal of toxic solvents or un-reacted monomers, such recombinant vectors could be just as, if not more cost-effective than synthetic counterparts[14].

We have previously reported the genetic engineering of a single chain multifunctional fusion biopolymer (vector) composed of a pH responsive fusogenic peptide, four repeating units of Histone H2A with an inherent nuclear localization signal and a human epidermal growth factor receptor 2 (HER2) targeting affibody (Figure 2)[14]. For simplicity, we will refer to this vector as THG. It has been demonstrated that this single chain recombinant fusion vector could perform a series of functions including: 1) condensation of pDNA into nanosize particles, 2) targeting cancer cells via HER2 receptors, 3) pH-dependent disruption of endosome membranes, 4) active translocation of pDNA towards cell nucleus, and 5) transfection of cancer cells in vitro [14]. We used THG vector as a base to formulate highly efficient targeted VMPs that are stable and can be produced by a simple mixing process and in a reproducible fashion. First, the gene coding for THG was designed and optimized to be synthesized in *E.coli* expression system. Western blot analysis and SDS-PAGE confirmed the expression and purity of THG protein (>95%) after Ni-NTA affinity chromatography (Figure 2).

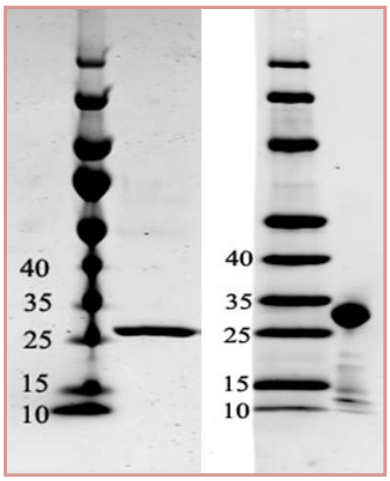


Figure 2: SDS-PAGE and western blot analysis of THG vector. SDS-PAGE (left panel) and western blot analysis by using anti His-tag antibody (right panel) shows the expression and purity of THG (MW: 27.76 KDa).

In the next step, the THG vector was desalted and used to complex with pDNA (pEGFP) to form targeted nano-particles (VMPs) and characterized in terms of hydrodynamic particle size and charge. The results of this study showed that the sizes of the particles formed at N:P ratio of 2 or higher are below 100 nm. It seems that at N:P 2, DNA molecule is not thoroughly compacted.

The desalting step is highly critical because it helps to remove the ions from the system and stabilize the particles' sizes by minimizing the potential for salt bridge formation and ensuring aggregation during formulation. The results of the zeta potential study revealed that the VMPs surface charge increased to ca. +20mV at N:P ratios 2 or higher (Figure 3). These VMPs are considered targeted due to the presence of HER2 affibody as demonstrated before [14].

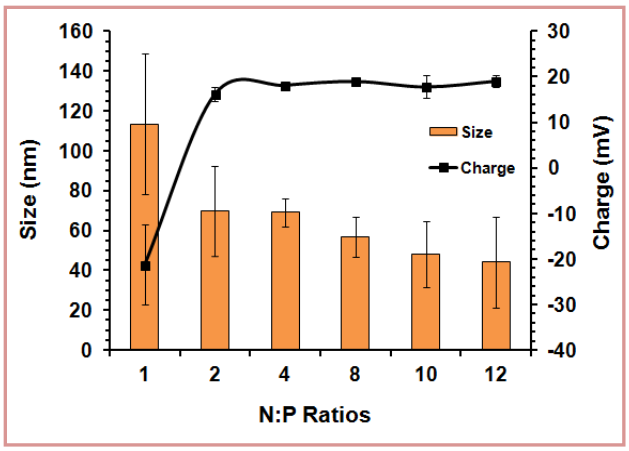


Figure 3: Particle size and charge analysis of THG in complex with pEGFP at different N:P ratios

To stabilize and reduce the surface charge of targeted VMPs and make them shielded, we mixed the THG vector with two different PEGylated peptides (i.e., H2A and *Mu*). These two peptides were purposely chosen because their efficient DNA condensation capabilities have previously been examined and reported[16, 17]. PEG2K and PEG5K were covalently attached to H2A and *Mu* peptides to make H2A-PEG2K, H2A-PEG5K, *Mu*-PEG2K and *Mu*-PEG5K and used in combination with THG at various ratios to complex with pEGFP and form PEGylated-targeted VMPs (Figure 4).

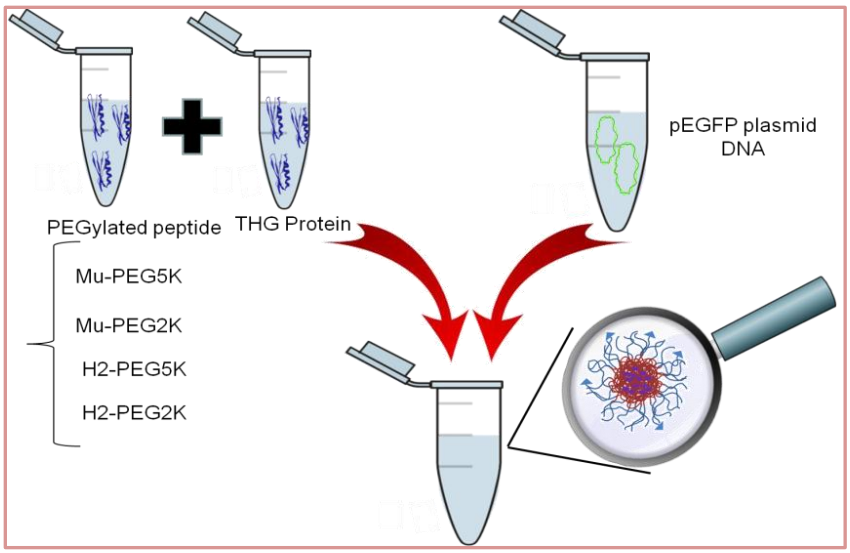


Figure 4: Schematics of PEGylated-targeted VMPs prepared through mixing of THG with H2A-PEG2K, H2A-PEG5K, *Mu*-PEG2K, or *Mu*-PEG5K followed by complexation with pDNA (pEGFP).

At first, we characterized particles formed as a result of complexation of PEGylated peptides (e.g., H2A-PEG2K, etc.) and pDNA at various N:P ratios to evaluate their DNA condensation ability and particle surface charge. The results of the complexation studies showed that H2A-PEG2K, H2A-PEG5K, *Mu*-PEG2K and *Mu*-PEG5K in complexation with pEGFP can form nano-particles with sizes of less than 100nm and with almost neutral surface charges (Figure 5). The surface charge neutrality of these PEGylated nano-particles could be attributed to the presence of PEG on the nano-particles surface. In addition, it could be observed that the presence of PEG did not interfere with nano-particle formation process.

So far, these results show that at one end targeted nano-particles (THG/pEGFP) stand with ca. +20mV surface charge (Figure 3) and at the other end PEGylated nano-particles (Figure 5) with surface charges of almost zero.

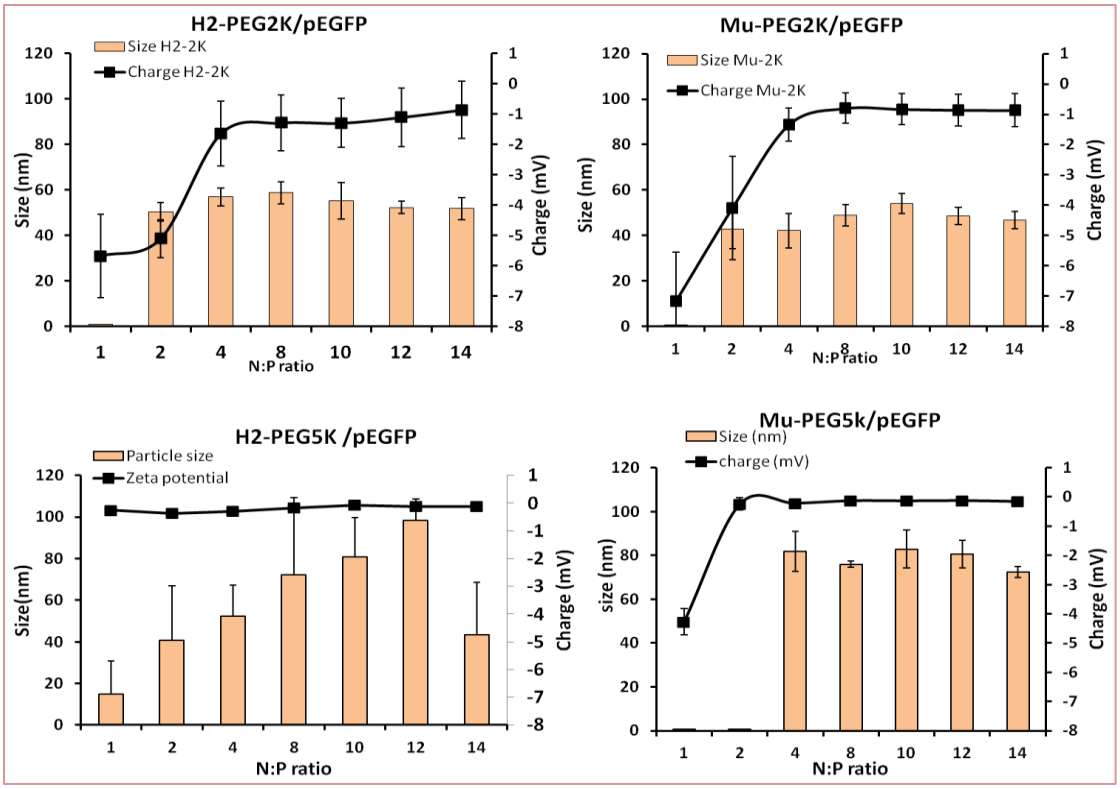


Figure 5: Particle size and charge analysis of PEGylated H2A and Mu peptides in complex with pEGFP at various N:P ratios.

To prepare targeted-shielded VMPs, Various amounts of THG was mixed with PEGylated peptides at N:P ratios of 8, 10 and 12. These ratios were selected because in both targeted nano-particles and PEGylated nano-particles maximum pDNA condensation was observed. The results of the particle size and charge characterization study for targeted-shielded VMPs revealed that as the PEG content increased and THG content decreased, the nano-particles surface charge gradually decreased (Figures 6 and 7). This indicates that the PEG molecules could present themselves on the nano-particles surface reducing the VMPs surface charge. Overall, all the prepared nano-particles had sizes between 40-80nm and we did not observe any significant difference among the fully condensed nano-particles. This suggests that the presence of PEG2K and

PEG5K in the nano-particles did not interfere with complexation process and all combinations were efficient in pDNA condensation.

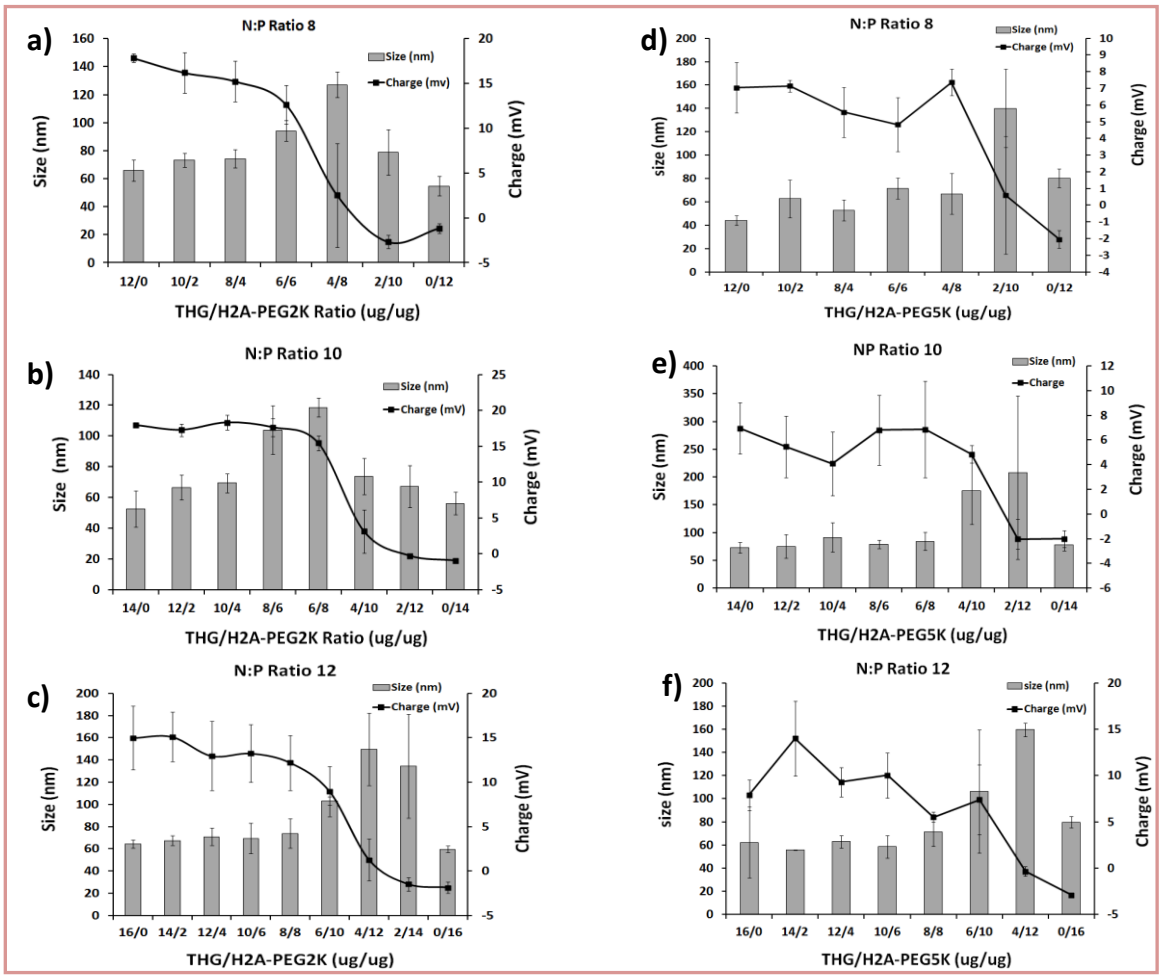


Figure 6: Particle size and charge analysis of THG mixed with H2A-PEG2K and H2A-PEG5K at different weight/weight (ug/ug) ratios and in complex with pEGFP.

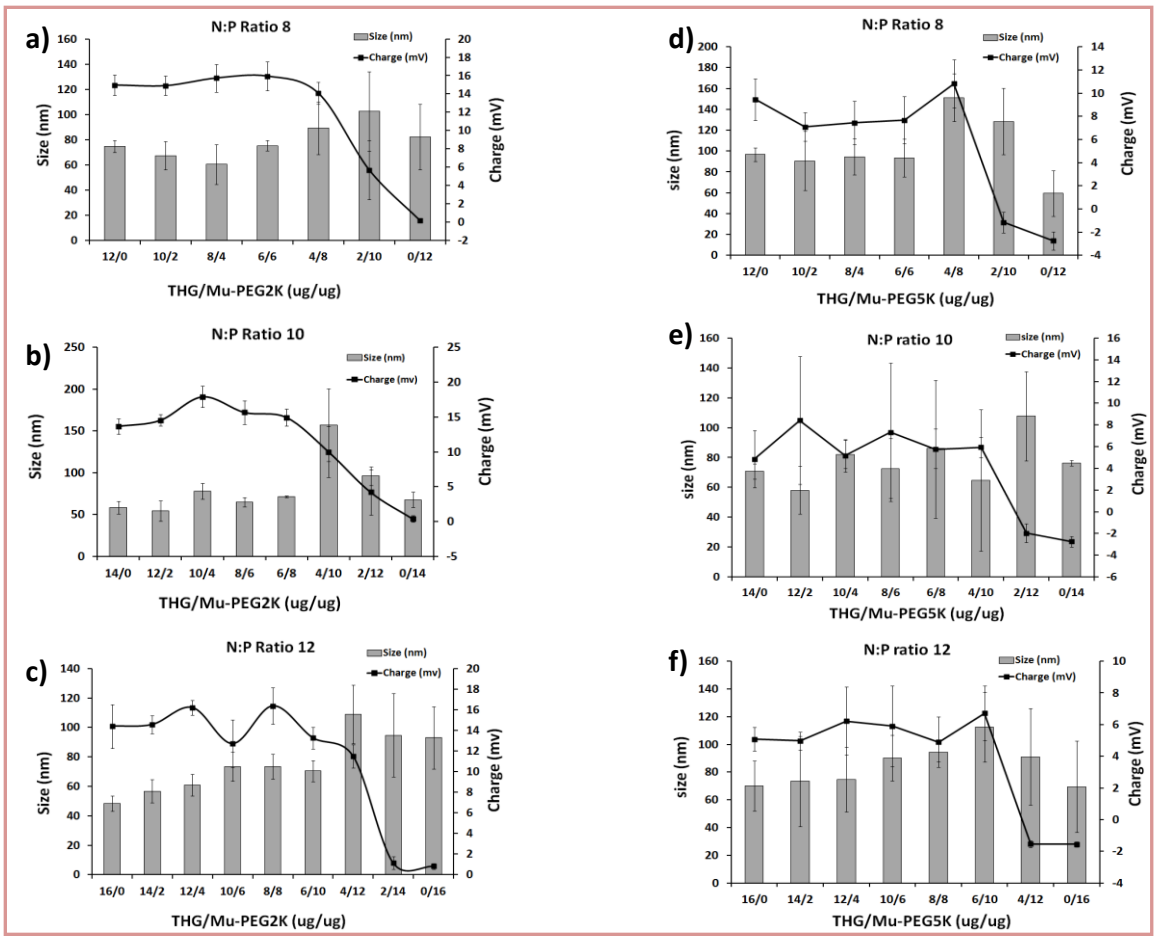


Figure 7: Particle size and charge analysis of THG mixed with *Mu*-PEG2K and *Mu*-PEG5K at different weight/weight (ug/ug) ratios and in complex with pEGFP.

Having a plethora of nano-particles with different surface properties at hand, we investigated the ability of these PEGylated VMPs to internalize and transfect SKOV-3 cancer cells. This cell line was used as a model HER2 (human epidermal growth factor receptor 2) positive ovarian cell line for this study because we have previously demonstrated that the affibody in the THG structure could recognize the HER2 on the surface of the cells and internalize[11]. In order to find the formulations of VMPs with lowest surface charge and highest transfection efficiency, first we used THG/pEGFP nano-particles at N:P ratios 1 to 12 to transfect cells. This was to determine the ratio at

which the nano-particles exhibit the maximum transfection efficiency. The results of the transfection studies showed that THG/pEGFP nano-particles had maximum efficiency at N:P ratios of 8, 10 and 12 (Figure 8).

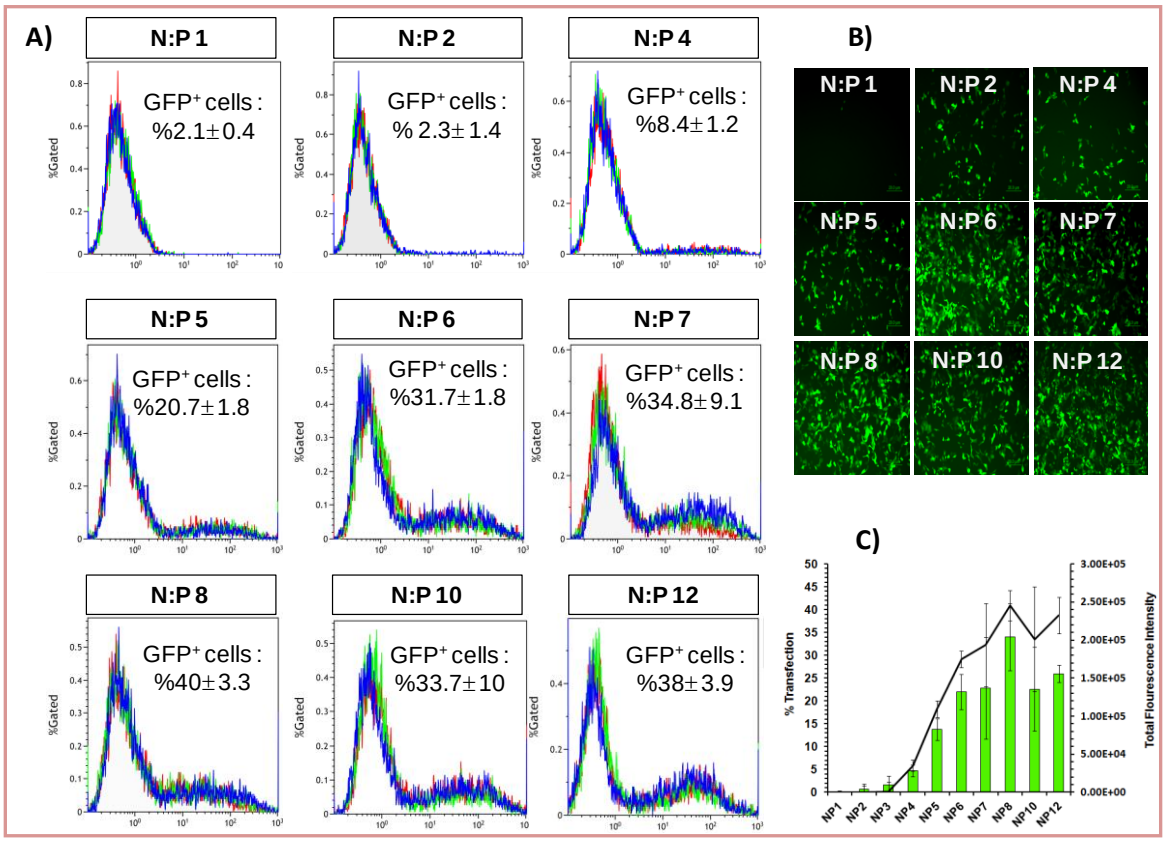


Figure 8: Evaluation of transfection efficiency of THG/pEGFP nano-particles at N:P ratios of 1 to 12. A) Flow cytometry graphs showing the percentage of GFP positive cells. Each graph is an overlay of three repeats. B) Fluorescent microscopy images of the transfected SKOV-3 cells illustrating the extent of gene expression. C) A bar chart that summarizes and quantitatively demonstrates the percentage of transfected cells and total green fluorescent protein expression.

To examine the effects of PEG content in targeted-PEGylated VMPs on transfection efficiency, all the targeted-PEGylated VMPs prepared at N:P ratios of 8, 10 and 12 were used to transfect SKOV-3 cells. For example, for N:P 8, all THG/PEGylated peptides combinations at weight/weight ratios of 12:0, 10:2, 8:4, 6:6, 4:8, 2:10 and 0:12 were

examined. The weight/weight ratio of 12:0 indicates 12ug of THG and 0ug of PEGylated peptide (e.g., H2A-PEG2K or *Mu*-PEG5K, etc.), whereas weight/weight ratio of 0:12 indicates 0ug of THG and 12ug of PEGylated peptide. The general pattern of transfection efficiency demonstrated that particles with higher or equal amounts of THG to PEGylated peptide had higher transfection efficiency (weight/weight combinations of 10:2, 8:4, 6:6) in comparison to the nano-particles with lower amount of THG (4:8, 2:10, 0:12), (Figure 9A-D)**.** This was to an extent that none of the PEGylated nano-particles (non-targeted) was able to transfect SKOV-3 cells. This is most likely due to the presence of PEG on nano-particles surface which inhibits interaction between cell membranes and nano-particles. In contrast, very high rate of transfection efficiency (>%95) was observed with THG/Mu-PEG5K (8:8) at N:P ratio of 12. We believe that at this particular ratio, the number of targeting peptides exposed on the nano-particle surface is at its optimum density providing this opportunity for the nano-particles to interact with cells very effectively and internalize. It has previously been shown there is an optimum number of ligands that should be present on the surface of each nano-complex to achieve maximum efficiency[18]. Increasing ligand number yields a corresponding increase in receptor binding associated with increased avidity. However, above a certain level, this increased avidity could result in increased receptor down regulation due to endosomal sorting to lysosomes for degradation, and therefore, decreases the number of receptors available for binding the vector. This concept has been elegantly shown in a study by Wagner et al. (1990) using transferrin conjugated onto polylysine[19].

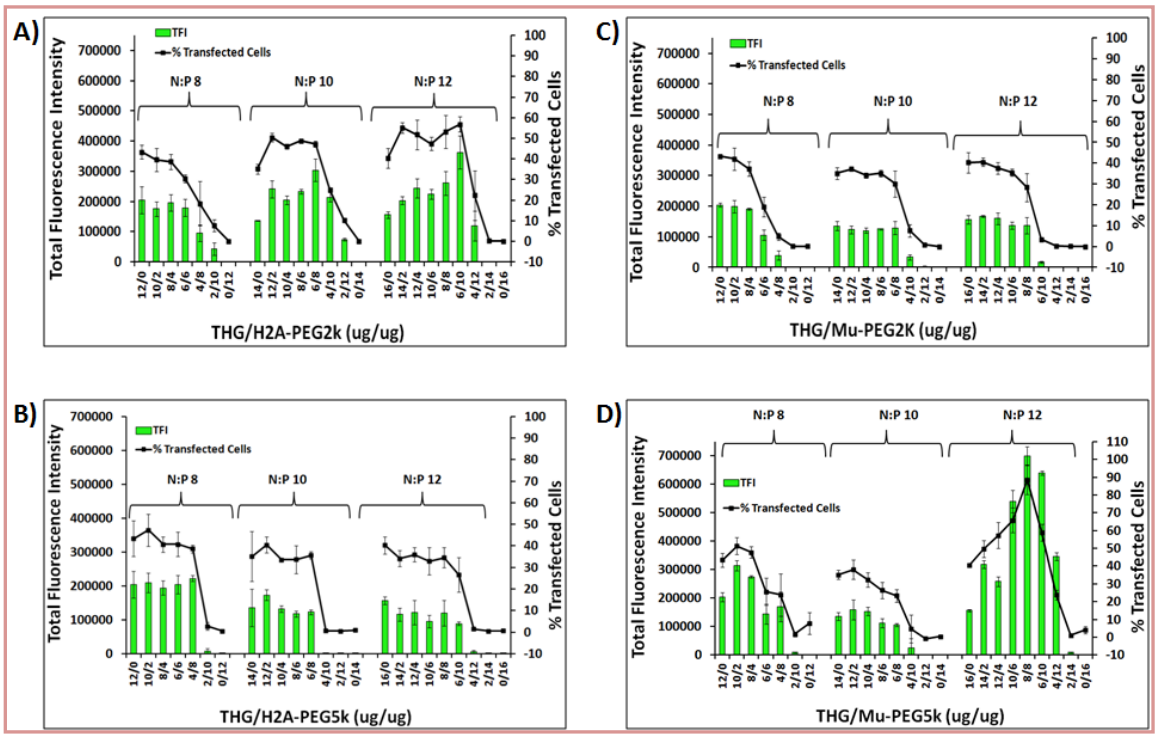


Figure 9: Percentage of transfected cells and total green fluorescent protein expression (TFI) for SKOV-3 cells transfected with vector/pEGFP complexes at various N:P ratios and in different combinations.

To examine whether formulation cytotoxicity was the cause of the low transfection of the cells, for example, with THG/Mu-PEG5K (4:12) in comparison to THG/Mu-PEG5K (8:8), a cytotoxicity assay was performed. This study was performed on some of the most efficient nano-particles prepared as a result of complexation of THG/Mu-PEG5K or THG/H2A-PEG2K with pEGFP. The results of our cytotoxicity study showed that none of the tested formulations were toxic; therefore, the observed transfection efficiency was not negatively affected by the PEGylated-targeted VMP formulations (Figure 10). Our

previous studies with THG also show that the vector does not have any significant impact on cell viability[11, 12].

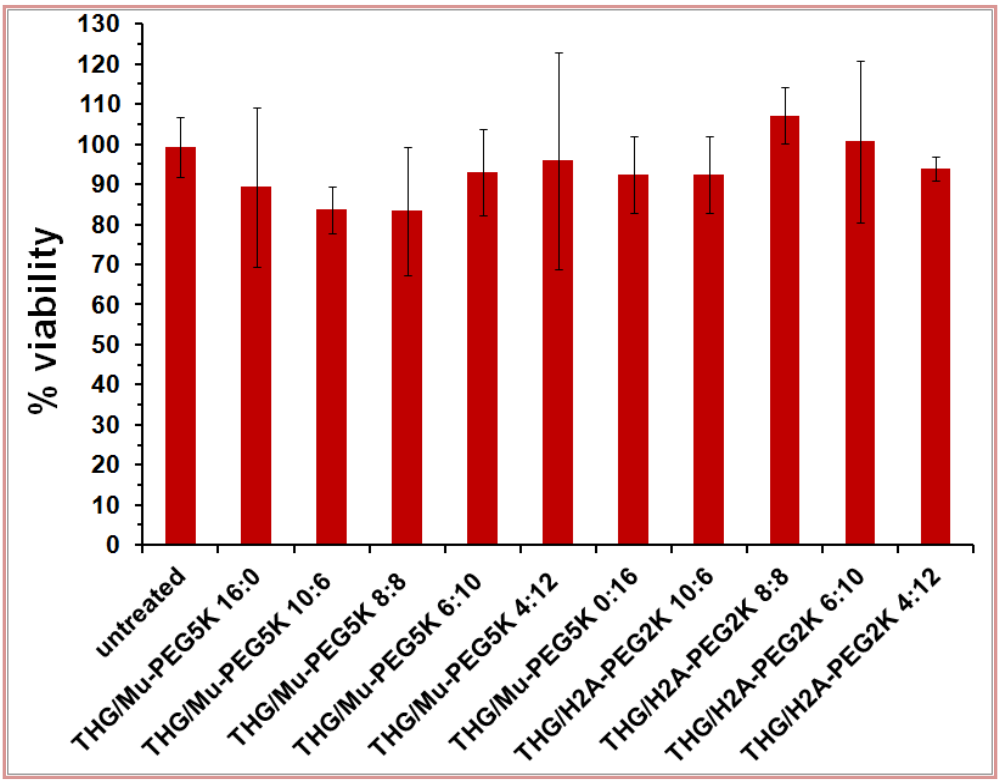
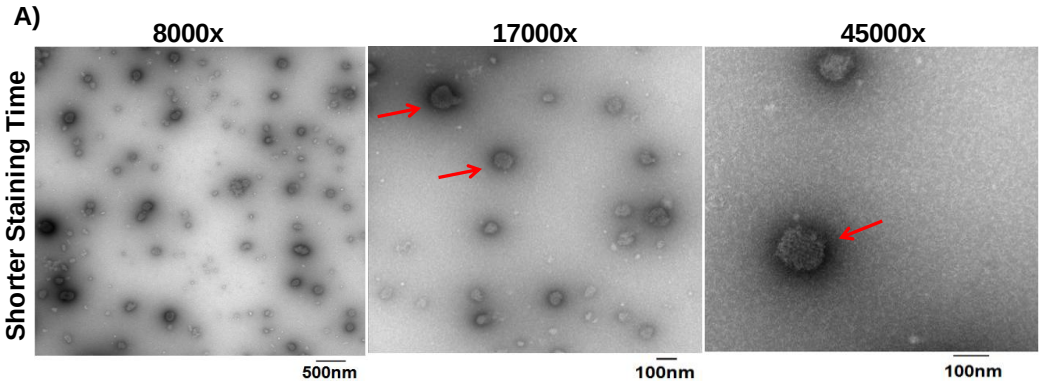
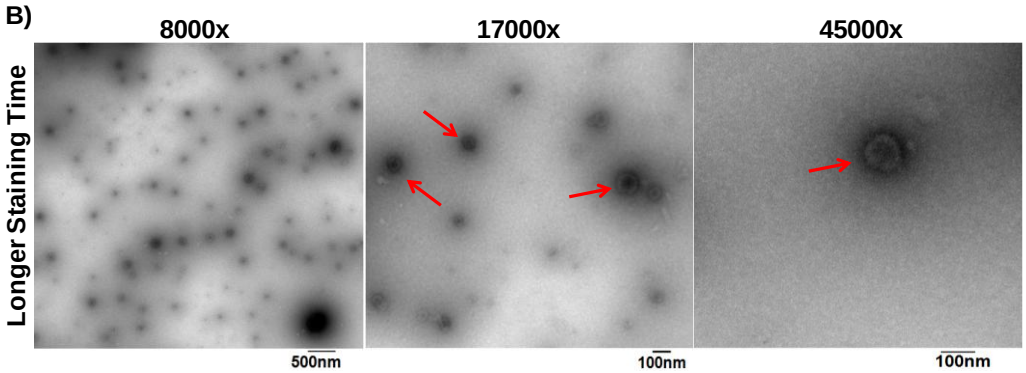


Figure 10: Evaluation of the viability of SKOV-3 cells after transfection with targeted and PEGylated- targeted VMPs.

The remarkably high efficiency of the THG/Mu-PEG5K (8:8) VMPs formed at N:P 12 prompted us to characterize them further in terms of shape and surface morphology and examine particle size uniformity. We adapted a previously published method for transmission electron microscopy (TEM) of viruses in order to study the surface morphology and internal structures of our formulated VMPs[10]. Brief staining was used to observe the surface morphology of the particles and the results showed that the prepared VMPs are somewhat floccus, uniform in size and spherical (Figure 11A). To investigate internal structures, the staining time was extended which helped to visualize

the packaged pDNA inside VMPs. In this case, the condensed pDNA could be clearly observed at the nano-particle's core (Figure 11B). For comparison's sake, we searched the literature to find clear TEM images of a model virus and compare with our VMPs. The results of the side-by-side shape comparison study illustrates that the surface morphology and internal structure of the formulated nano-particles (Figure 11A-B and 11C right panel) are very similar to poxvirus (Figure 11C, left and middle panels). Overall, these results show that the nano-particle formation process could produce uniform, compact and spherical nano-particles with similar morphology to viruses.





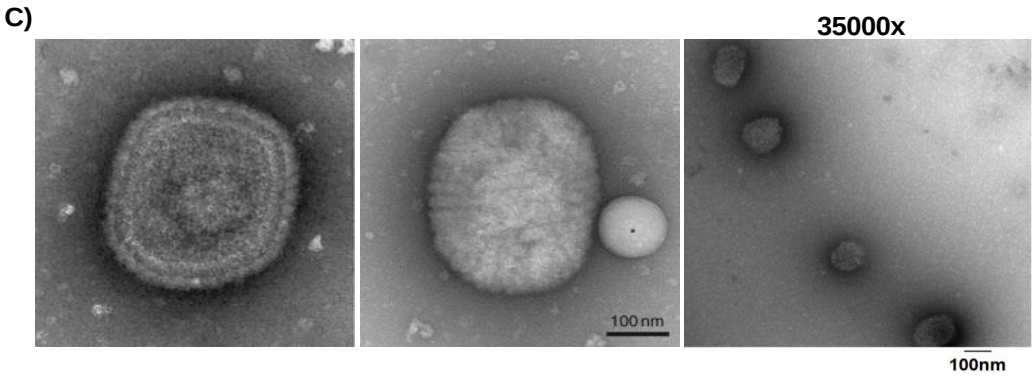


Figure 11: Transmission electron microscopy of the negatively stained targeted nano-particles, PEGylated-targeted nano-particles, and poxvirus. A) THG/pEGFP targeted nano-particles stained briefly to emphasize on the representation of the surface structures. B) THG/pEGFP targeted nano-particles with long staining times revealing internal structures. C) Right panel: PEGylated-targeted VMPs formed as a result of complexation of THG/Mu-PEG5K (8:8) with pEGFP. Middle and left panels: poxvirus surface and inner structures revealed as a result of negative staining. In the middle panel, a latex bead with a diameter of 100nm is localized close to poxvirus. The poxvirus images are adapted with permission from reference 19.

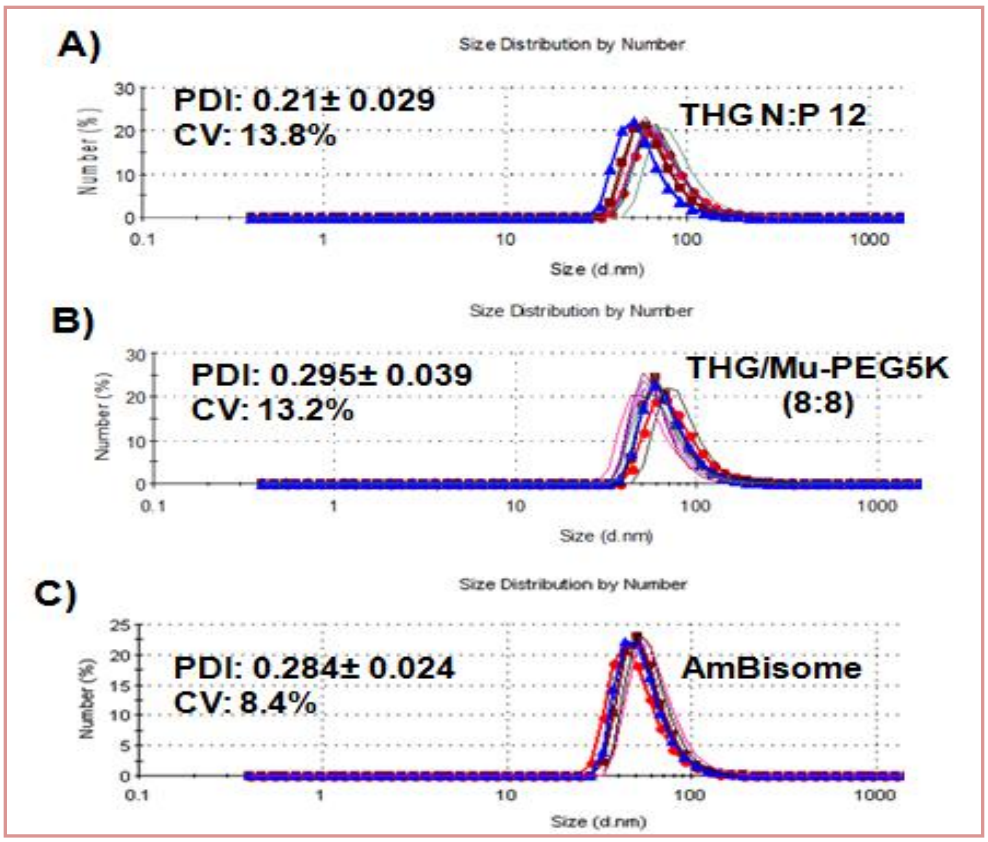


Figure 12: Polydispersity index analysis and measurement of coefficient of variation. A) Particle size distribution of THG/pEGFP at N:P 12 (n=10). B) Particle size distribution analysis of nano-particles formed through complexation of THG/Mu-PEG5K (8:8) at N:P 12 with pEGFP (n=10). C) Particle size distribution of Ambisome™ (n=4).

As mentioned in the introduction section, for a drug formulation to receive FDA approval, it needs to pass quality control tests and meet the criteria for batch-to-batch uniformity. Basically, the formulator needs to demonstrate that the product can be made in a reproducible fashion. To demonstrate reproducibility, we prepared ten independent batches of the VMPs which were formed as a result complexation of pEGFP with THG/Mu-PEG5K (8:8) or THG and evaluated the polydispersity index (PDI) and the corresponding coefficient of variation (CV). The results of the study demonstrated that the nano-particle formation process is reproducible because the coefficient of variance

remained below 15% as per guidelines for parenteral suspensions (Figure 12). As a control, we also purchased a few samples of Ambisome™ (n=4) which is an FDA approved nanotechnology-based product and evaluated the PDI and CV. As expected, Ambisome™ met the FDA requirements for batch-to-batch uniformity.

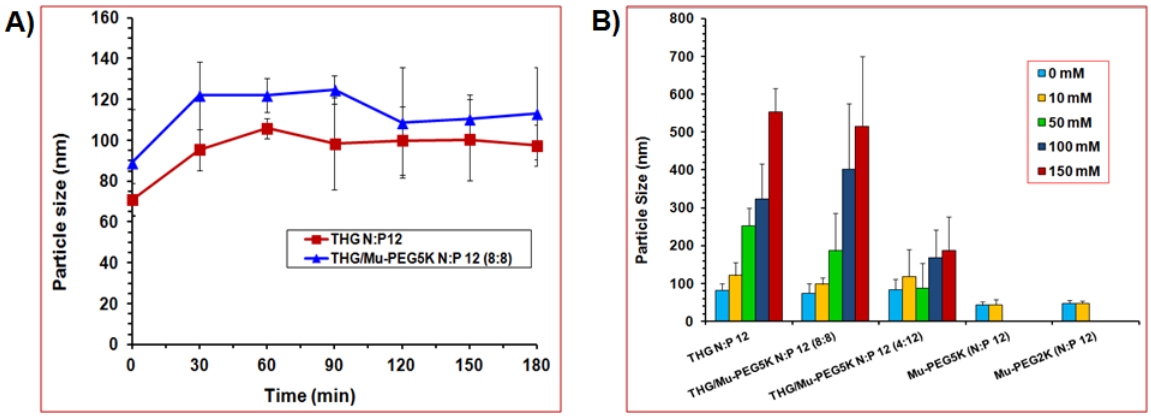


Figure 13: Particle size stability studies. **A)** Stability over time of the nano-particles prepared through complexation of THG with pEGFP and THG/*Mu*-PEG5K (8:8) with pEGFP. **B)** Particle size stability at various salt concentrations.

To avoid the complexities and stability problems associated with the long-term storage of nanosuspensions (e.g., aggregation), in the above mentioned formulation, the targeted THG vector and shielded Mu-PEG5K can be stored in one vial while the genetic material can

be stored in another. A pharmacist or physician can simply add sterile buffer to each vial and mix the components of the two vials by simple shaking to make ready for injection nanosuspensions (VMPs). This process is similar to what pharmacists and clinicians have to reconstitute AmBisome™ prior to use. In this approach, the need for long-term stabilization of the nanosuspensions is eliminated and the suspension needs to remain stable and unaggregated only for a short period of time until injected. The particle stability study data over time shows that the formulated VMPs are stable for at least three hours with no statistically significant increase in size (Figure 13A).

The approaches that are commonly used in preparation of physically stable suspensions fall into two categories. One is the use of a vehicle to maintain deflocculated particles in suspension and the other is to apply the principles of flocculation to produce flocs that, although they settle rapidly, are easily resuspended with a minimum of agitation. Parenteral suspensions are usually formulated as deflocculated system in order to avoid potential clogging of arteries. The results in Figure 13A indicate that the nano-suspension is a deflocculated system because no significant size increase was observed over three hour storage time.

In addition to storage time stability inside the vial, the stability of the nano-particles in the presence of salt at physiological concentrations (150mM) and resisting dissociation is also important. Salt stability study is especially important for nano-particles that are formed predominantly through electrostatic interactions because the presence of ions could easily interfere with the vector/pDNA attractive forces and result in particle dissociation[20]. For this purpose, the impact of salt concentration on the

stability of the nano-particles formed as a result complexation of pDNA with THG/*Mu*-PEG5K (N:P 12, 8:8) which had the highest rate of transfection efficiency was studied. Other vectors such as THG (N:P 12), THG/*Mu*-PEG5K (N:P 4:12), *Mu*-PEG5K and *Mu*-PEG2K were also studied as controls. After exposing the nano-particles to increasing concentrations of the NaCl (up to 150mM), it was observed that the nano-particles of *Mu*-PEG5K and *Mu*-PEG2K were not stable at salt concentrations beyond 10mM and rapidly dissociated at 50mM NaCl or higher concentrations (Figure 13B). Basically, we could not detect any particles at high salt concentrations with these two peptides. In contrast, the THG/pEGFP (N:P 12) and THG/*Mu*-PEG5K (8:8) nano-particles were stable in physiological salt concentrations (i.e., 150 mM), although we observed that as the salt concentrations increased the particle sizes started to increase. This size increase in the presence of salt was expected because it is known that salt ions could form salt bridges in between nano-particles and result in formation of loose floccules[21]. It is noteworthy that after injection into the body, the VMPs get diluted in the blood stream and as long as they don't dissociate, the potential for aggregation is very low. We also examined the stability of the nano-particles created through complexation of pEGFP with THG/*Mu*-PEG5K (4:12). At this ratio, the nano-particles contain significant amount of PEG with almost neutral surface charge and should resist aggregation. As it can be observed in Figure 13B, these nano-particles not only resisted aggregation but also dissociation. One factor that may have contributed to the salt stability of the THG-containing VMPs that could resist dissociation is the presence of hydrophobic residues in the THG sequence. These hydrophobic residues could contribute to the stabilization of hydrophobic pockets

in the nano-particle structure and block the penetration of water and ions into the nano-particle core. As a result, the salt ions may have difficulty interfering with the electrostatic interactions in between pDNA and vector's cationic residues.

Based on all the observed results, VMP formulations with different efficiencies and physicochemical properties are at hand which could potentially demonstrate different pharmacokinetic profiles in vivo. Although the in vitro results suggest that THG/*Mu*-PEG5K (8:8) at N:P ratio of 12 may be the most efficient formulation but only in vivo studies could determine which formulation is most effective one in reaching tumors and transfecting cancer cells. This is due to the fact that in tumor targeting, at first the targeting is achieved via the EPR effect which is a form of passive targeting[22]. Once accumulated in the tumor environment, the presence of targeting motif could facilitate internalization.

Aiming to use the current system for in vivo application, one should investigate the immunogenicity of an amino-acid based product before proceeding to in vivo efficacy studies. Immunogenicity is defined as the ability to trigger an immune response. like many biopharmaceuticals with protein structure, an immune reaction to the designed vector can lead to adverse reactions such as anaphylaxis, altered pharmacokinetics/pharmacodynamics, production of anti-drug-antibodies (ADA) and eventually partial or complete loss of efficacy[23].

Based on the type of immune reaction they can induce, protein based biopharmaceuticals can be divided into two major categories; Thymus dependent (TD) and thymus independent (TI). Thymus dependant immune reaction or "classical immune

response" is defined as the activation of one B cell clone in response to one epitope of the antigen which usually entails receiving necessary signals from an activated helper T cell, antibody class switching from IgM to high affinity IgG and production of antibody secreting B memory cells. Obviously a large bio-molecule can have more than one epitope which each of them may or may not active a B cell/T cell clone.

The other type of response to a protein based biopharmaceutical is Thymus independent (TI) which is defined as activation of polyclonal B cells in response to antigen's epitopes without activation of T cells. Such a reaction is described as an early and immediate response to antigen in order to buy more time for TD reaction to take over. TI reaction is characterized by rapid production of low affinity IgM without any or negligible production of IgG. Not only the epitope from protein product but also contamination with bacterial Lipopolysaccharides, protein aggregations and an array of repeated protein sequence (such as virus like particles) can produce such a reaction[24].

In order to determine any possible immune reaction, THG VMPs at NP of 12 and the PEGylated VMP (THG/Mu5k 4:12) were injected intravenously according to the method mentioned before in this chapter. pBUD plasmid DNA was used as a positive control, considering the fact that due to the presence of CpG islands on the plasmid sequence and different pattern of methylation in bacterial DNA, the plasmid is likely to trigger an immune response. As panel A in picture 14 suggests, there is no significant increase in the level of IgM in any of the groups. However, Panel B at the same figure shows a

significant increase in IgG level at pBUD group whereas, VMP groups did not trigger any significant increase in IgG level.

Considering the fact that both VMP groups contain the same dose of DNA (5 ug per injection), the results in figure 14 suggest a uniform condensing of plasmid DNA in a way to be inaccessible to immune system. This result may also suggest the lack of a T-dependant immune reaction against the VMPs, where the vectors not only conceal the immunogenic DNA out of the reach of immune system but the structure of vectors themselves did not trigger any immune reaction. However, to completely rule out the possibility of any immune reaction, one should also look at TI reaction by monitoring the level of IgM in time point shorter than 2 weeks, as well as measuring cytokines such as IFN γ, granulocyte macrophage colony-stimulating factor (GM-CSF) and B-cell activating factor which are the hallmarks of TI reaction might be helpful to draw a complete picture of immunogenicity of such VMPs.

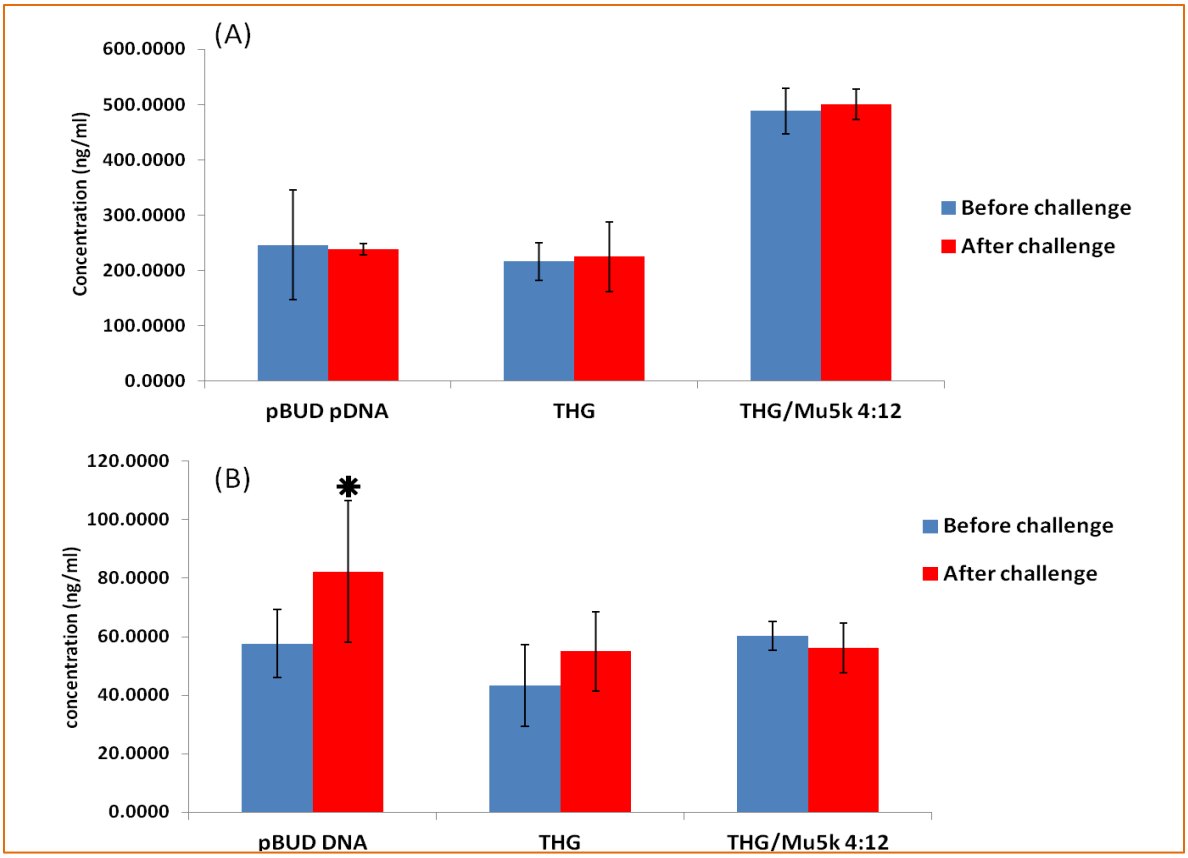


Figure 14: the effect of intravenous injection of pBUD plasmid DNA, THG NP 12 and THG/Mu5K 4:12 virus mimetic particles on IgM (Panel A) and IgG level (panel B).

4. Conclusion

Recent discussions among scientists in academia and pharmaceutical industry indicate that pharmaceutical and biotech companies face a significant challenge in making highly efficient targeted and shielded nanomedicines that are cost effective, stable and compliant with batch-to-batch uniformity[9]. This non-compliance appears to stem from the number of production steps and limited control over the chemical reactions involved in the attachment of targeting ligands (antibodies) and shielding

motifs (PEG) and subsequent formulation development for long-term stabilization of the nano-suspensions.

In this study, we demonstrated simple way of formulating highly efficient PEGylated and targeted VMPs that are stable and can be produced in a reproducible fashion. In an attempt to ensure homogeneity in VMP construct, we used only amino acid-based vectors. Because we utilized recombinant DNA technology to create a multifunctional biopolymer in a single step, the need for conjugation of various natural motifs to the vector backbone in multiple steps was eliminated. One draw-back that one may perceive in the developed formulation is the use of PEG which is a polymer and is conjugated to the peptides via chemical synthetic methods. At present, we have to accept this draw-back since there is no other viable alternative to PEG in the market. Nonetheless, the developed VMP formulation is unique and the first of its kind which can be produced through a simple mixing step without the need for long-term nanosuspension stabilization. Here, we have tried diligently to embrace the phrase coined by Prestwich which states “embrace complexity, engineer versatility, and deliver simplicity”[25].

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CHAPTER IV

Vector Customization: Development of a Highly Efficient Vector for Targeted Gene Delivery to PC-3 Prostate Cancer

1. Introduction

Prostate cancer is the second cause of death among men in the united state[1-3]. If diagnosed in early stages, the disease is still localized and can be cured with chemotherapy regimen and radiotherapy. However, almost one third of patients will progress to metastatic stage which is known to be incurable and treated only with palliative cares [3]. This fact emphasizes the lack of an effective systemic treatment modality for prostate cancer especially at late stages [4]. Although prostate cancer is extremely sensitive to hormone ablation therapy at the first stages, but eventually in most patients the cancer progresses to a hormone refractory stage after a relapse followed by anti androgen therapy[1]. This stage is believed to be fatal with high probability of metastasis[5].

The majority of human prostate cancer cell lines including the classical cell line PC3 are reported to be androgen independent, hence a good model to address the unique

progression of prostate cancer[6 7]. PC3 cell line is also characterized to be CAR^{-}/HER2^{-}. As a result, they are not a candidate for either adenoviral gene therapy or anti-HER2 immunotherapy. Therefore, development of a therapeutic protocol for this type of prostate cancer is highly beneficial to those patients who are suffering from hormone refractory prostate cancer.

Among new emerging strategies for cancer therapy, gene directed enzyme prodrug therapy (GDEPT) is an attractive method in which the enzyme-coding gene is selectively delivered to cancer cells followed by the systemic or intratumoral administration of a prodrug[8, 9]. The accumulation of the prodrug's toxic metabolites then causes target-specific death in the enzyme-bearing cancer cells and the surrounding ones. Unfortunately, one critical problem that currently restricts progress in cancer suicide gene therapy is poor target selectivity of the vectors, resulting in unwanted transfection of non-cancer cells. Consequently, several groups have investigated methods to confer tumor tropism on the vectors mainly employing targeting systems based on tumor-selective ligands[10, 11]. Recently, the amino acid sequence of a new cyclic peptide CPGDRGQRRLFSKIEGPC was reported with ability to bind to PC3 prostate cancer cells specifically but not normal human prostate cells[11]. This targeting peptide is reported to have no sequence similarity to bombesin, LHRH and prostate-specific antigen as confirmed by a search through Swiss Prot databases and European Molecular Biology Laboratory[11]. This indicates that this peptide could be binding to an antigen that is specifically expressed on PC3 cells and has not been used for targeted therapy before.

Our group has recently reported the structure of a genetically engineered histone H2A-based vector that could efficiently target and transfect cancer cells in vitro and in vivo[12, 13]. This vector consists of four repeating units of Histone H2A peptide (H) in fusion with a pH-responsive fusogenic peptide GALA (G). For simplicity, we refer to this vector as HG. Using HG vector as a base, the objective of this research was to engineer a targeted vector that can target and efficiently transfect PC3 prostate cancer cells with minimal impact on normal epithelial prostate cells. To achieve the objective, the multifunctional HG vector was genetically engineered in fusion with PC3 targeting peptide for cell targeting and efficient transfection. Because one of the major hurdles facing nano-medicines is their recognition by the immune system and production of IgG antibodies after repeated injections, an elastin-like peptide was also designed in the vector structure for reducing potential IgG/IgM response. The vector's transfection efficiency was evaluated by using a reporter gene whereas PC-3 targeted killing efficiency was evaluated through use of a suicide gene. The ability of the vector to transfect normal prostate epithelial cells was also assessed. Ultimately, the potential immunogenicity of the vector in immune-competent mice was studied.

2. Materials and Methods

2.1. Gene design and cloning

Three DNA constructs were designed and synthesized by Integrated DNA technology, IDT (Iowa, USA). The first and second constructs consist of targeting moiety (namely Tp), ELP sequence, four repeats of Histone H2 (namely H) and GALA fusogenic

sequence (G). The difference between two sequences lies in their ELP sequence, with the first one has amino acid Serine as the guest amino acid in ELP sequence (VPGSG) and the second one has Glutamic acid (VPGEG). These two vectors were named TpE_EHG and TpE_SHG respectively. As a control, the third sequence in this family consists only of targeting moiety, Histone repeats and fusogenic peptide (namely TpHG, figure 1). The DNA sequences were cloned into pET21b (+) expression host (Novagen®) using *NdeI* and *XhoI* restriction enzymes (Thermo scientific, USA). The sequence of recombinant plasmids were verified by sequencing (GENEWIZ, USA).

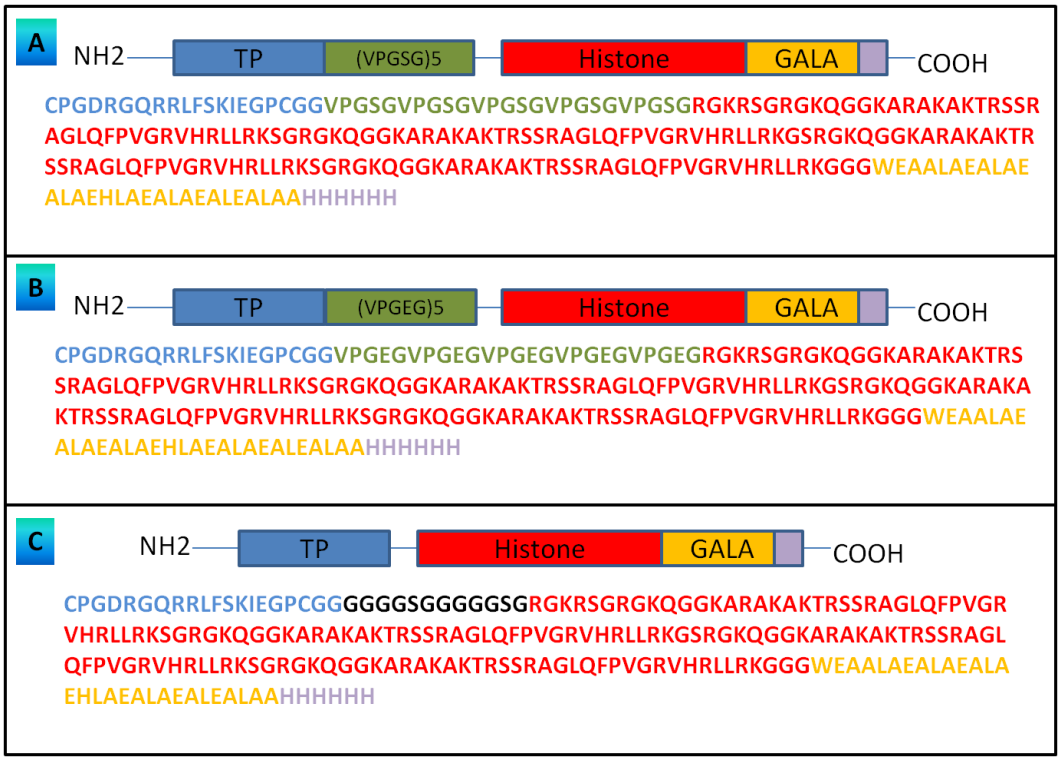

Figure 1: the schematics representing the structure of (A) T2EsHG, (B) $T2E_EHG$ and (C) T2HG and their protein sequences.

2.2. Expression and purification

One BL21(DE3) plysS colony bearing one of the constructs TpHG-hisx6:pET21b(+), TpE_EHG-hisx6:pET21b(+) and TpEsHG-hisx6:pET21b(+) was inoculated in 5 ml Circlegrow® medium supplemented with Carbenicillin (50 ug/ml). The culture was incubated overnight at 37°C. The day after, 5 ml overnight culture was added to 500 ml of Ciclegrow® medium and was shaken at 37°C to reach OD600>0.5. IPTG was added to final concentration of 0.4 mM and the incubation was continued for 6 hours at 30°C. Then cells were collected by centrifuging at 5000xg for 20 minutes. For protein purification, 20 ml lysis buffer (100 mM NaH_2PO_4 , 10 mM Tris , 5 M urea , 1.5 M NaCl, 1% Triton X and 10 mM Imidazole, pH 8) per gram of cell pellet was added and the solution was mixed for 1 hour. Then it was centrifuged at 20000 rpm for 1 hour at 4°C. The supernatant was collected and added to 1 ml Ni-NTA agarose (Qiagen), pre equilibrated with lysis buffer. The slurry was shaken for 1 hour on ice then added to the column. The column was washed with 100 ml of lysis buffer and 50 ml of wash buffer (100 mM NaH_2PO_4, 10 mM Tris, 5 M Urea, 1.5 M NaCl and 40 mM Imidazole, pH 8). The protein was eluted by Elution buffer (100 mM NaH_2PO_4, 10 mM Tris, 3 M Urea, 500 mM NaCl and 200 mM Imidazole, pH 8) and stored at -20 ^{o}C.

2.3. Desalting and preparation of Protein stock solution

The elution fractions were pooled together and concentrated before desalting using 10,000 NMWL Centrifugal Filter Units (Millipore). In order to prepare the working stock solution, the protein was desalted in a pre-packed Sephadex G-25 column

separately. Cold HEPES buffer (100 mM, pH 7.4) was used for preconditioning of column and eluting the protein. The final desalted fractions were concentrated and the final concentration was measured by Nanodrop 2000 spectrophotometer (Thermo scientific). The prerequisite factors, i.e molecular weight and extinction coefficient were calculated in Expasy Protparam online tool and used to calculate the final protein concentration.

2.4. Particle size and charge analysis

The combination of purified protein with pEGFP plasmid DNA were prepared at different N:P ratios of 1, 4, 8, 10 and 12 and in HEPES buffer (100 mM, pH 7.4). As for N:P ratio calculation, the number of negative charge in one molecule of pDNA was calculated. The number of positive charge needed to neutralize the number of negative charge for a given N:P ratio, such as N:P 4 is 4 fold the number of negative charge. Considering Lysine and Arginine residues as positively-charged amino acids, the number of protein molecules and eventually ug of protein needed to neutralize 1 ug of DNA was calculated for each N:P ratio. The particles were prepared in total volume of 100 ul HEPES buffer (100 mM, pH 7.4) and incubated at room temperature for 15 minutes before size and surface charge were measured by Nano-ZS Nonosizer (Malvern® instrument U.K). For the measurement of surface charge, the particles were diluted in HEPES buffer to reach the final volume of 700 ul. The data is presented as mean$\pm$ s.d (n=3).

2.5. Cell Transfection study

For each protein, the ability of transiently transfecting prostate cancer cell line, PC3 (ATCC, USA) with three NP ratios 8, 10 and 12 were tested. Transfection was done according to the following protocol; 4 x 10^4 PC3 were seeded in a 96-well plate and incubated at 37 ^oC in F12K complete medium supplemented with 10% serum. 2 hours before transfection, the medium was changed to serum free medium consisting of F12K medium supplemented with Dexamethasone, Fibronectin, Ovalbumine and ITS (insulin, transferrin, selenium). The particles were prepared as described before and added to each well. The cells were incubated with the particles for 4 hours; finally the medium was changed again to F12K full medium (supplemented with 10% serum). The plate was incubated for 48 hours before the cells were visualized by epifluorescent microscopy, harvested and resuspended in 500 ul of PBS+2% Formaldehyde for Flow cytometry analysis to quantify percentage of transfected cells and total fluorescence intensity.

2.6. Testing the targeting ability of Tp vector family

Biomarker positive cell line PC-3, biomarker negative cell line SKOV-3 and prostate epithelia cell line RWPE-1 were grown in the media according to vendor's recommendation. All of the cell lines were seeded in 96 well plates with a 10^4 seeding density. Cells were transfected with TpHG/pEGFP complexes formed at various N:P ratios (equivalent to 1ug of plasmid pEGFP) in media supplemented with insulin, transferrin, selenium, Ovalbumin, Dexamethasone, and fibronectin. After 4 hrs, the media was removed and replaced with fresh media supplemented with 10% serum. RWPE-1 cells were transfected in Keratinocyte serum free media (Lonza, Switzerland).

The green fluorescent protein (GFP) was visualized after 48 hours using an epifluorescent microscope to evaluate gene expression. Lipofectamine 2000 (Invitrogen) was used as a positive control to validate the transfection process. GFP expression level and percent of transfected cells was quantified with flow cytometry.

2.7. Preparation of pBUD recombinant constructs

The dual expression system pBUD 4.1 plasmid (Life technologies, USA) is equipped with two separate promoters, P_{CMV} and $P_{EF-1\alpha}$. The reporter gene dcsGFP-T2A- Firefly Luciferase was cloned under P_{CMV} using HindIII/XbaI restriction enzymes. The genes coding for bacterial Nitro reductase (NTR), viral SR39 thymidine kinase and yeast cytosine deaminase (yCD) were cloned separately under $P_{EF-1\alpha}$ using XhoI/NotI restriction enzymes to prepare pBUD-NTR-GFP-FireLuc, pBUD-SR39-GFP-FireLuc and pBUD-yCD-GFP-FireLuc respectively. The sequences of all cloned enzymes and reporter genes were confirmed by sequencing (GENEWIZ, USA).

2.8. Toxicity assay

NP ratios of 8, 10 and 12 for each protein were chosen for WST-1 toxicity assay. The test was done as followed; 4 x 10^4 PC3 cells were seeded per well in a 96-well plate and incubated in F12K full medium supplemented with 10% FBS. 24 hours later, 100 ul of freshly prepared Vector/ pDNA was added to each well. 2 hours after the transfection, the medium was replaced with fresh f12K medium supplemented with 10% FBS. The cells were incubated at 37°C for another 48 hours, before WST-1 cell proliferation

reagent (Roche Applied Science, USA) was added to each well (10 ul per 200 ul of medium). The absorbance at 450 nm was measured 1 hour after adding the reagent, with Absorbance at 600 nm as the reference wavelength. The average of absorbance at (A450-A600) for untreated cells was considered as 100% of viability, based on which the viability of other samples were calculated. The data was reported as mean ± SD (n =3).

2.9. Immunogenicity study

All in vivo steps have been revised and approved by The Animal Care and Facilities Committee, Rutgers University. For immunogenicity study, Balb/cJ male mice, 5-6 weeks of age (Jackson labs, Maine, USA) were housed one week after arrival and grouped in 5 per cage. A blood sample was taken from saphaneous vein after one week of acclamation (day 0). Each mouse receives two doses of nano-particles on day 7 and 21 via tail vein injection. Each dose consisted of nano-particles prepared for 1 ug of pDNA in a total volume of 100 ul. The particles were prepared in HEPES buffer (100 mM). The buffer itself had been prepared in injection grade water in advance and passed through 0.2 um syringe filter. pDNA was also purified using EndoFree plasmid Maxiprep Kit (Qiagen , USA). The particle size was measured before each injection to check for any possible aggregation.

On day 35, the animals were euthanized using CO_2 chamber and the blood was collected by cardio puncture. The blood samples were centrifuged at 2000 rpm for 15 minutes and the plasma was collected and stored at -80 ^{o}C for further analysis. A schematic of timeline is presented in figure 2.

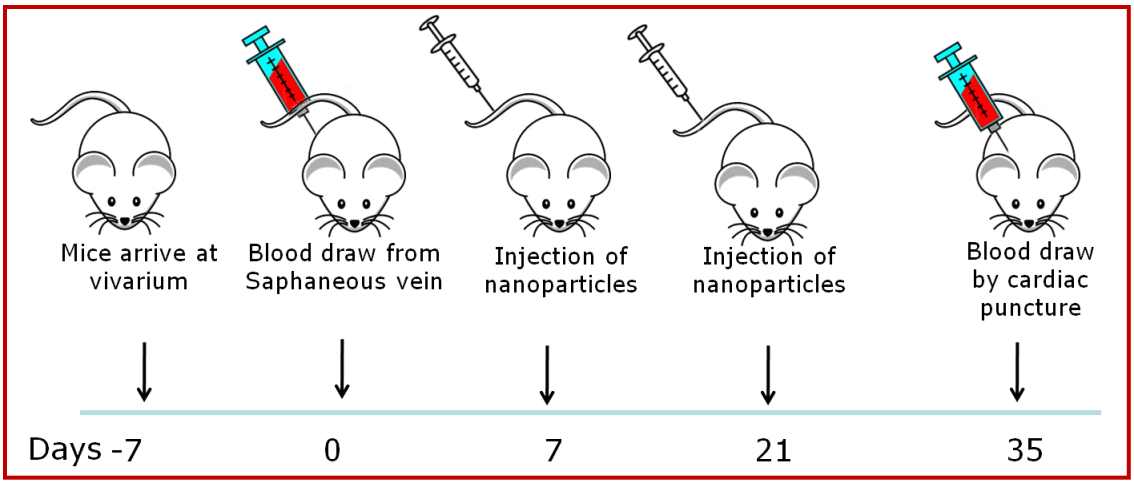

Figure 2: A schematic showing the steps of immunogenicity study.

Enzyme linked immunosorbent assay (ELISA) to determine IgG and IgM levels was performed as per manufacturer's instructions using the IgG and IgM ELISA kits from Bethyl laboratories (Texas, USA). Plasma was used at a 1:1000 dilution for ELISA. Briefly Immulon2 plates (Dynatech) were coated with the capture antibody and incubated for 1h. Plates were washed and blocked overnight in blocking buffer containing 1% BSA. The following day, plates were washed 3 times, standard and samples were added in duplicates and plates were incubated for 1h. Subsequently plates were washed and secondary antibody conjugated to HRP was added. OPD Easy tablets (2mg/ml; Acros) were used as detection substrate and the plates were read at 490 nm.

The data was analyzed in OriginPro 9.0 software. For each standard concentration point, the absorbance was calculated as Absorbance at A_{490} on that point minus Absorbance at A_{490} of blank. Each concentration was plotted against its A_{490} and the best fit was chosen for each set of data based on R Square. The concentration for each blood sample before

and after injection was calculated using the plot equation and given A_{490} Absorbance for each sample.

2.10. In vitro cell toxicity of suicide genes/prodrug system

To find the most effective suicide gene/enzyme system for targeted killing of PC3 cells, three suicide gene/Enzyme systems were chosen; SR39 (the codon optimized version of herpes Simplex Thymidine Kinase)/Gancyclovir (GCV), bacterial nitroreductase (NTR)/ CB1954 and yeast Cytosine Deaminase (yCD)/ 5-FlouroCytosine. It should be noted that each of the components alone, i.e prodrug and pDNA should not show any toxicity effect on the cell when administrated alone. Only the combination of both should render a killing effect. In order to find the maximum non-toxic dose, each prodrug was tested separately on different concentration range on 2x10^4 PC3 cells. GCV was tested at the range of 0-2000 uM, CB1954 at 0-400 uM and 5FC at the range of 0-100 uM. The cells were incubated with prodrugs, 24 hours after seeding and the incubation continued for 96 hours before WST-1 proliferation assay was carried out as mentioned before.

Considering the viability of untreated cells at 100%, the viability of PC3 cells incubated with different concentration of prodrug was calculated and the concentrations with >80% viability was considered non-toxic.

The same procedure was followed for plasmid DNA since transfection with high dose of pDNA can be toxic too. Each 2x10^4 cells were transfected with nanoparticles made of 0, 0.15, 0.25, 0.5 and 1 ug pBUD-SR39 plasmid DNA (As a sample Plasmid) and

subsequent amount of TpEsHG vector at N:P ration of 12. 48 hours after incubation, the viability of cells was measured using WST-1 cell proliferation test as described before.

In transient expression, the level of transfection is important since the transgene expression lasts only for a few cell cycles before it gets diluted to the point that the expression becomes undetectable. Therefore, not only the recognition of nontoxic DNA dose is crucial, but the non-toxic dose should be effective as well. Therefore the above mentioned doses of pDNA were also tested for their transfection efficiency. The transfection was done as described in section 2.5 of this chapter.

For the same reason, recognition of the highest transgene expression level is the key to get the highest effect. In order to do so, the Luciferase activity was measured in time intervals after transfection of PC3 cells with pBUD-SR39/TpEsHG nanoparticles, starting 4 hours after transfection.

After spotting the non toxic range of DNA dose and prodrug, the cells were transected with nano-particles consisting of pBUD 4.1 plasmid (doses of 0.15, 0.25 and 0.5 ug plasmid per well) coding for suicide gene and T2EsHG protein at NP 12. For each prodrug, the pBUD plasmid coding subsequent enzyme was used; i.e SR39 for GCV, yCD for 5FC and NTR for CB1954. 24 hours after transfection, the medium was changed to fresh F12K medium supplemented with 10% serum and prodrugs. Each prodrug concentration were testes in triplicate. The cells were incubated with prodrug for further 48 hours before WST-1 assay.

2. Results and Discussion

With the versatility offered by nano-particle modality, designing a tailor-made system with desired physico-chemistry and targeting properties is much simpler than before. However, a close observation of critical properties of particles such as size, charge and surface ligands make the dynamic interactions of nano-particles with the microenvironment components more predictable and guarantees a higher chance of success in delivering the nanoparticles' cargo (drug or nucleic acid) to the site of action[14]. In order to have a full control on the properties of nano-particles, the system should be kept simple with few straight forward steps for preparation.

It has been the focus of our lab to design and produce a nature-inspired vector for gene delivery with the ability to be easily tailor made to different cancer types and as the same time enjoys the possibility of a fast, large-scale production. The core of this system which consists of 4 repeats of Histone H2 and a fusogenic peptide namely GALA can overcome the intracellular barriers that the nano-particle encounters after internalization[15].

In this study, we added and modified two new components already reported HG to produce three new recombinant amino acid based vectors, namely TpEsHG, TpE_EHG and TpHG; these two components are a targeting moiety (T) and an Elastin Like Peptide (ELP). ELP sequences introduce unique characteristic to the protein such as a reversible thermal precipitation above a special temperature called transition temperature (T_t) [16]. The same phase transition has been observed when ELP containing polypeptides are in buffers with a high salt content[17]. In order to avoid the aggregation in desalting step, a

buffer with minimum amount of salt (HEPES 100 mM) was chosen for desalting because ELP containing proteins showed the tendency to aggregate even in buffers with low salt concentration such as Bis-Tris Propane 10 mM/NaCl 5 mM (pH 7). We observed that during the desalting step, when the protein starts to aggregate due to sudden decrease in salt concentration, cooling the column and the buffers on ice help to increase the yield of desalting and recovering more protein from size exclusion chromatography process (Figure 3).

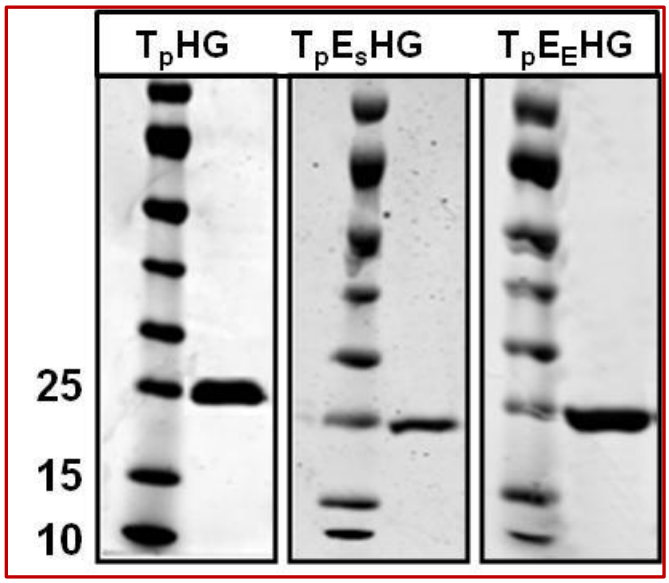

Figure 3: the purified TpHG (MW 23.25 KDa, TpEsHG (MW 24.48 KDa) and TpE_EHG (MW 24.69)

Once the stable protein solution was obtained, the ability of each member of Tp vector family in condensing plasmid DNA was tested for NP ratios of 1, 4, 8, 10 and 12. Also the surface charge for each group of nano-particles was measured. As expected, as the condensation of DNA become more complete by adding more protein at higher NP

ratios, the particle size decreases. The results also showed the close to zero charge of particles (less than 10 mv) which is a favored characteristic since the neutral charge limits the internalization of the nano-particles to endocytosis rather than the non specific interaction between cell surface's negative charges and nano-particles positive charge. Among Tp family nano-particles, T2EsHG has the lowest positive charge (3.27 mV at NP 12) which makes it more favorable for a receptor mediated gene transfer to PC3 cells since it renders less non-targeted interaction with blood components (figure 4).

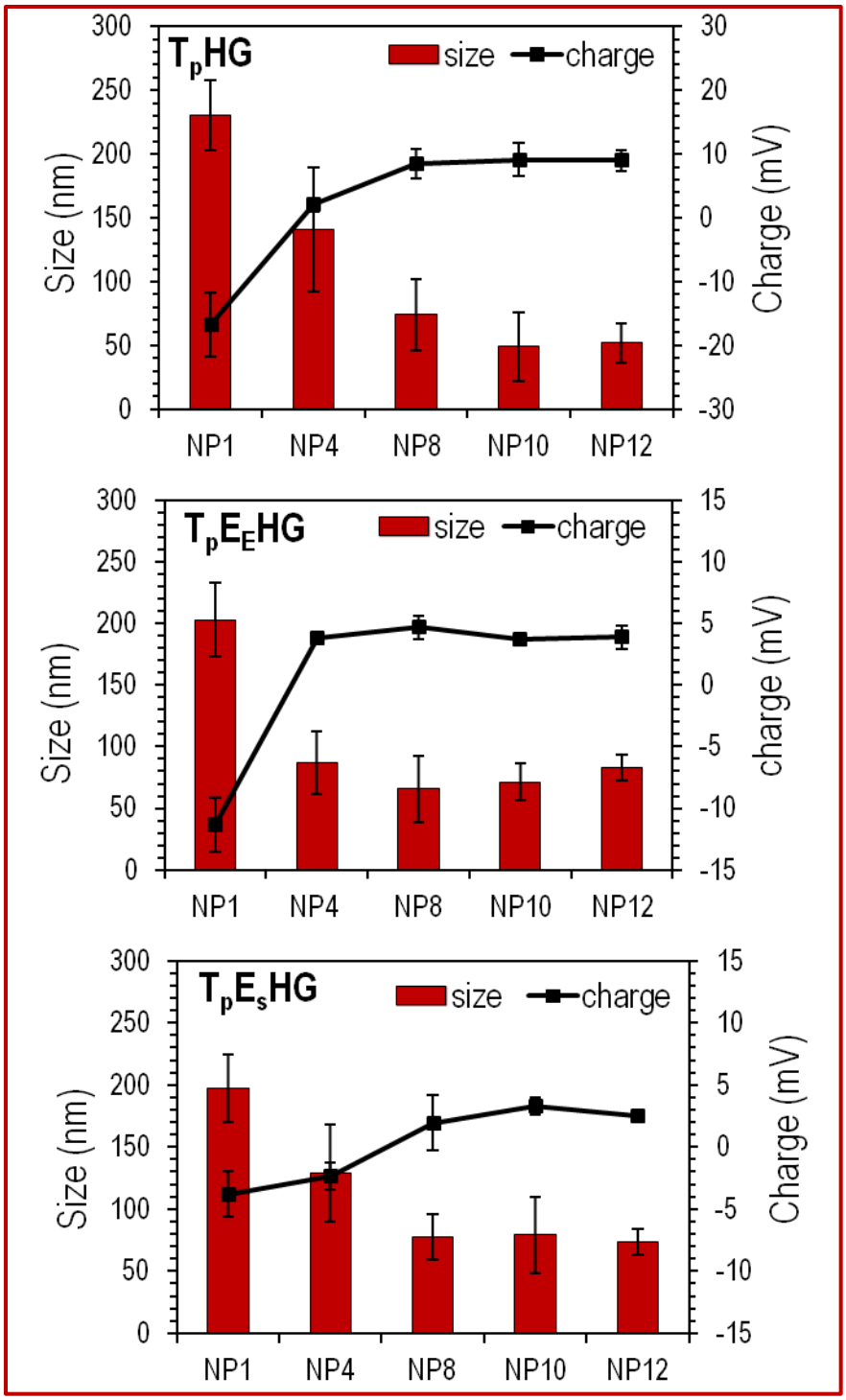

Figure 4: Particle size and charge for TpHG, TpE_EHG and TpEsHG

In the next step, all three amino acid based vectors were tested on their overall ability to transfect PC3 prostate cancer cells. PC3 cells express the biomarker which actively reacts with targeting moiety in the vectors' structures, however the nature of this biomarker is not known[11]. PC3 cells were transfected with nano-particles consisted of pEGFP plasmid DNA and each of the amino acid based vectors (TpHG, TpE_SHG and TpE_EHG) in three NP ratios of 8, 10 and 12. As Figure 5, panel C suggests, TpEsHG seems to render a significant higher transfection level compared to other two proteins ($P< 0.05$).

Knowing the efficiency of nano-particles for transfection, at the next step their ability in targeted delivery of therapeutic gene to PC3 cell line of human prostate cancer has been evaluated. Since the nature of the over-expressed biomarker on PC3 cells is not known yet, it has been hypothesized that such biomarker should not be present in normal prostate cells or should be expressed at a negligible level. The biomarker was also assumed to be specific to prostate cancer and not abundantly expressed on other cancerous cell lines. To check the hypothesis, TpHG nano-particles were tested on prostate normal epithelia cell line (RWPE1) and an ovarian cancer cell line (SKO3). When TpHG nano-particles were used to test the targetability, the transfection level is significantly higher in PC3 cells compared to SKOV3 and RWPE1 cell lines ($P<0.05$), (Figure 6). This data indicates the ability of designed system to effectively deliver its DNA cargo only to targeted prostate cancer cells but not the normal prostate tissue. It also demonstrates that TpHG vector is not interacting with HER2 receptors, which are

abundantly expressed by SKOV3 cells. The nature of interaction of targeting peptide with PC3 cells is left to be elucidated.

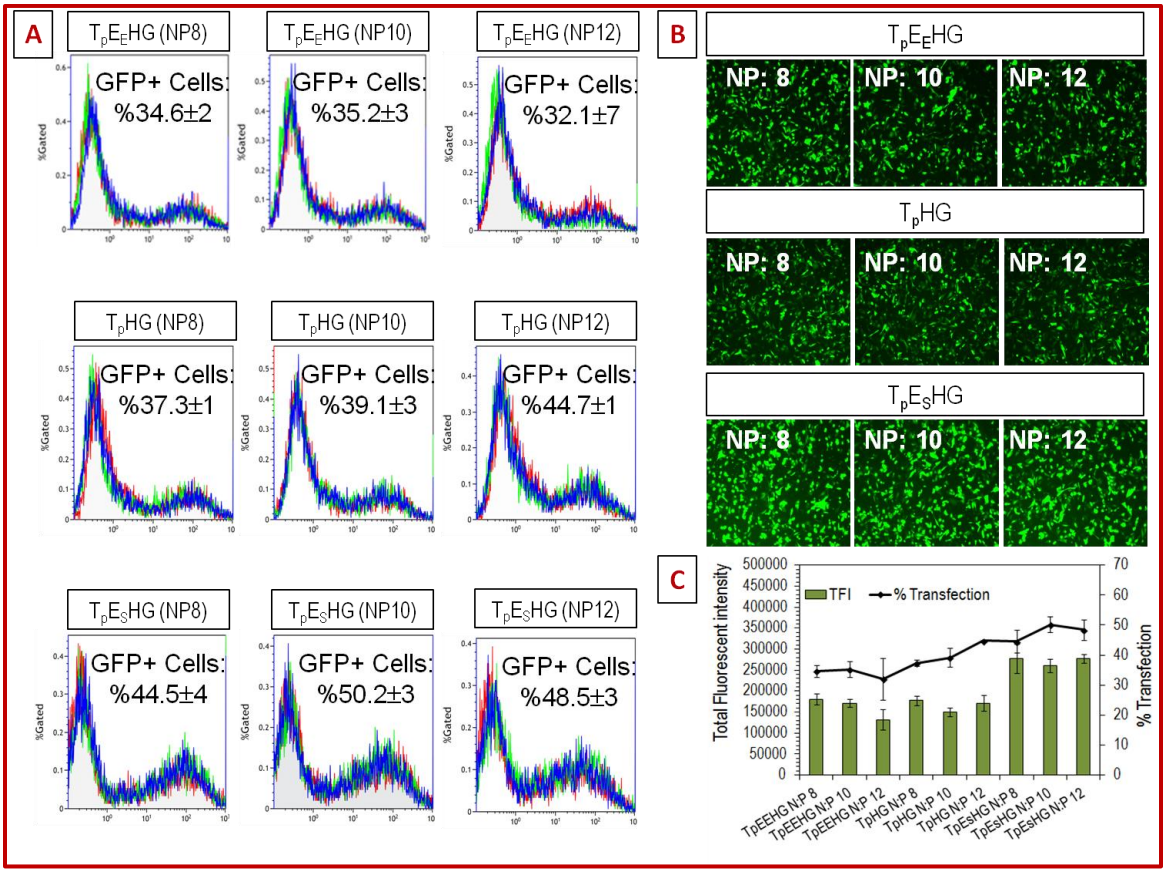

Figure 5: Percentage of transfection (Panel A), microscopic view of transfected cells (panel B) and total fluorescence intensity (TFI, panel C) for PC3 cells transfected with nano-particles consisting of pEGPF plasmid DNA and TpHG, TpEsHG and TpE_EHG at NPs 8, 10 and 12.

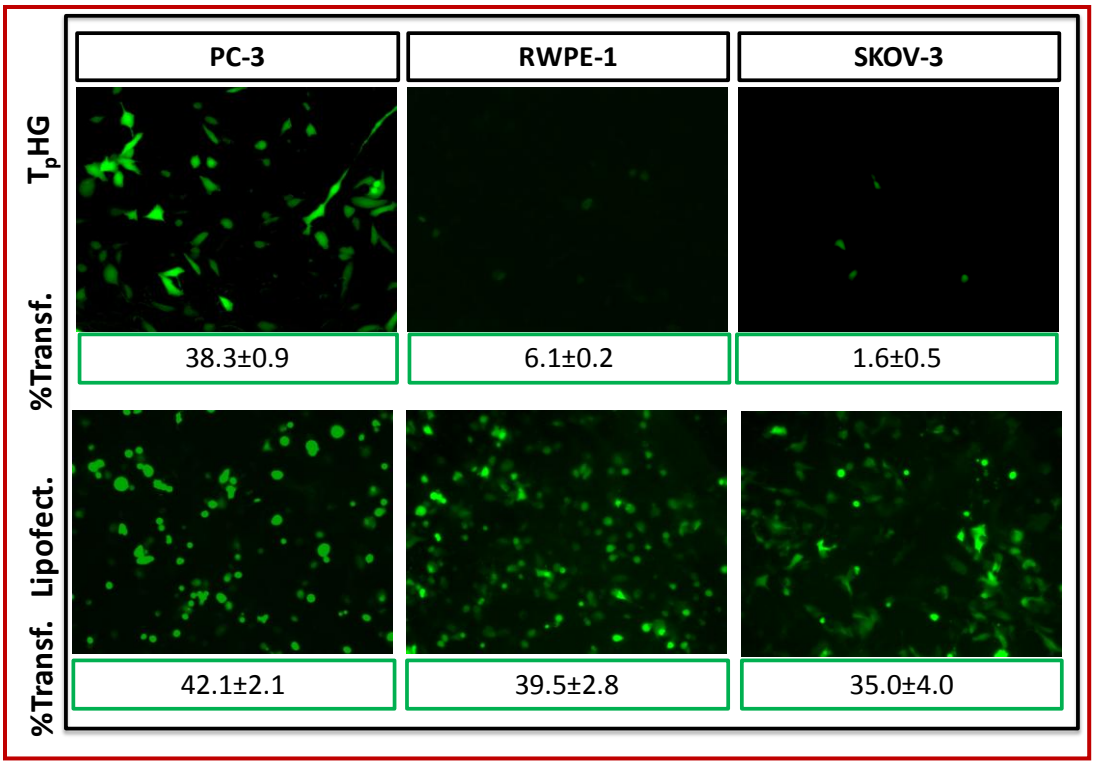

Figure 6: targeted transfection of TpHG nano-particles. The transfection level is significantly higher in PC3 cells transfected with TpHG/pEGFP nano-particles compared to RWPE-1 and SKOV-3 (upper panel), whereas there is no significant difference in transfection level among the same cell lines transfected with Lipofectamin (lower panel).

Each nano-particle system interacts with many extracellular and intracellular components during the process of gene delivery. These interactions define the efficacy of the system as well as its biocompatibility[18]. Therefore, evaluation of toxic interaction with intracellular component (cell toxicity) is the first crucial step that should be taken into consideration before proceeding to in vivo level.

We tested the toxicity of the proposed nano-particle system using WST-1 cell toxicity assay. The viability of PC3 cell transfected with Tp family vectors were compared to the viability of PC3 cells which were only treated with HEPES buffer (as a negative control).

The results showed no significant toxicity for Tp vectors compared to HEPES-treated cells ($p>0.05$) (Figure 7). The other type of toxicity, immunotoxicity is a result of nano-particles' interactions with extracellular components especially opsonins that could potentially start a cascade of immune reactions which eventually leads to the recognition of nano-particles as foreign objects and as a consequence, activation of one of the adaptive immune system through T-dependant or a T-independent immune reaction [19]. At worst-case scenario, the production of high affinity anti drug antibodies (ADA) leads to a decrease in drug efficacy and rapid clearance. To avoid such consequences, a better understanding of the nature of immune reaction after the systemic administration of nano-particles is necessary. We evaluated the Immuno-compatibility of nano-prticles after intravenous injection of nano-particles prepared from 1 ug of plasmid DNA. Each testing group consisting of five mice received two injections in a time interval of two weeks. IgG and IgM levels were measured by ELISA, two weeks after the last injection by euthanizing the animals and collecting the blood via cardiac puncture (figure 2). In this study, we chose pCpGfree plasmid DNA in HEPES buffer as an immunologically inert molecule. This plasmid is completely devoid from CpG dinucleotide (invivoGen). In contrast, pBUD plasmid DNA, originated from normal bacterial strain contains unmethylated CpG islands and like any DNA with unmethylated CpG islands is likely to induce both innate and adaptive immune systems[20]. It has been shown that removal of unmethylated CpG islands improve the duration of gene expression in gene therapy studies[21].

Here we are presenting IgG and IgM level before and after the injection of pCPGfree and pBUD plasmid DNA (1 ug), TpHG, TpEsHG and TpE_EHG nano-particles (NP12) containing pBUD plasmid. As expected and also shown in chapter III, the group received two i.v doses of pBUD plasmid have developed an immune reaction against it. Such a reaction was not detected in group received pCpGfree plasmid DNA, nor did the groups which received the Tp-based nano-particles and pBUD (N=5±S.D, Figure 8). It is worth to mention that when these results suggested the complete condensation of plasmid pBUD DNA and its inaccessibility to immune system, they do not give any clue if any IgM-mediated thymus independent reaction or any innate immune reaction had been occurred against the nano-particles. Perhaps, measurement of an array of cytokines which are indicators of other types of immune reactions could be helpful.

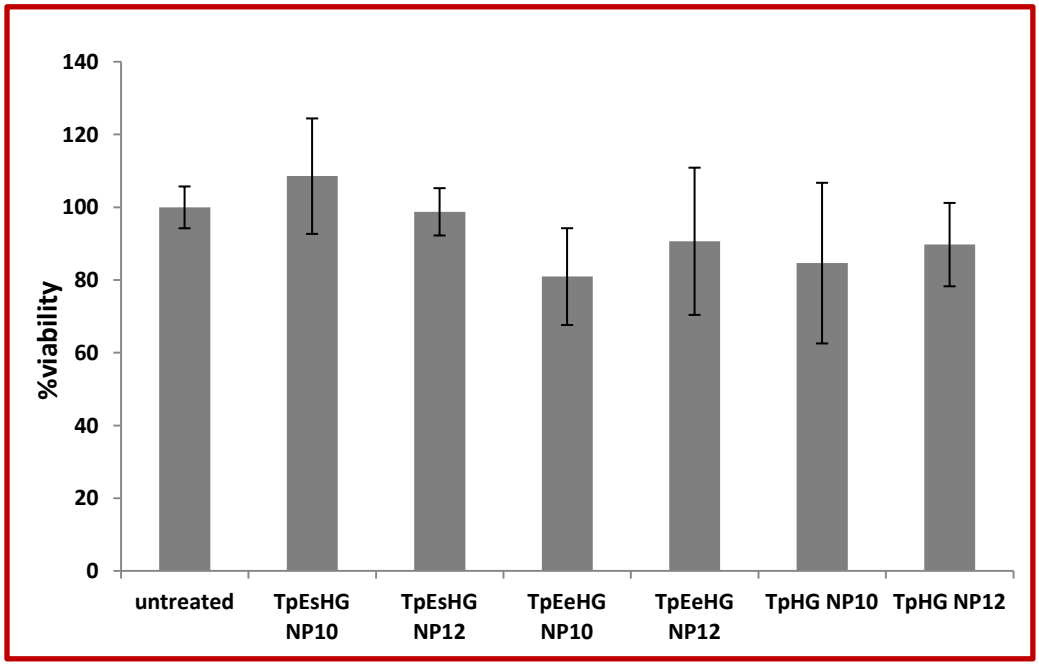

Figure 7: percentage of viability in PC3 cells transfected with Tp family vectors. The cells treated only with HEPES buffer were considered as control, with 100% viability.

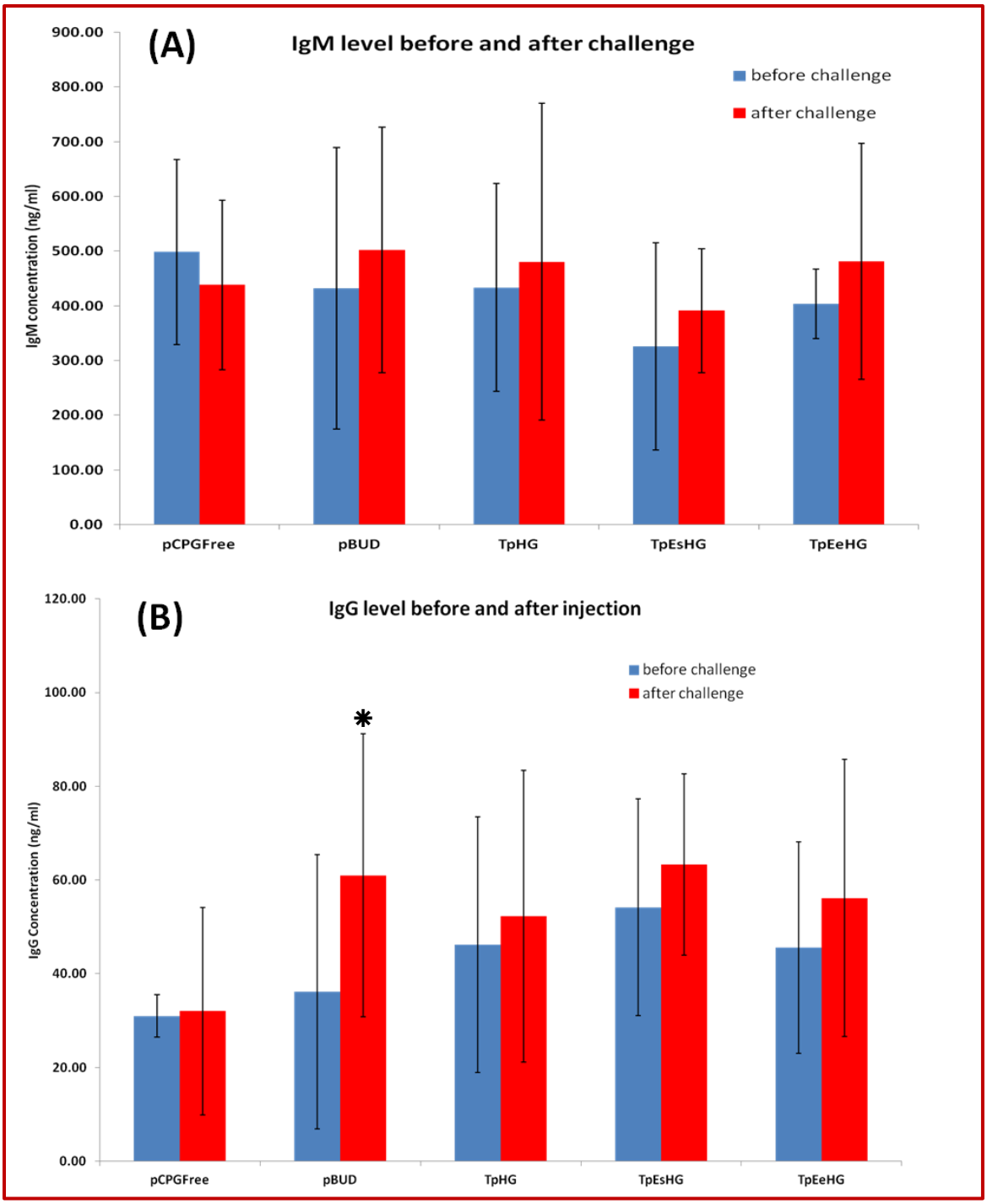

Figure 8: IgM (panel A) and IgG (panel B) level before and after injection of nano-particles. pCGPGFree DNA which is free of CPG islands was used as a plasmid DNA with no known immune-triggering effect.

Overall, considering the targeting ability and the absence of toxicity and lont term immune reaction, it seems that the designed system has the capability to be used in vivo.

The ultimate goal of designing a nano-particle system is to provide a manifold, highly stable, high capacity and compatibility platform for gene/drug delivery. To practice the features above, we decided to use Tp protein vectors for delivering a group of therapeutic gene to PC3 prostate cancer cells. Among different gene therapy modules, we chose suicide gene therapy. The concept of gene therapy and its premises have been explained in detail in chapter II.

Based on transfected data which has been presented in Figures 5 and 6, all three vectors, namely TpHG, TpEsHG and TpE_EHG, are able to render a considerable level of transfection in PC3 prostate cancer cells in vitro. Among them, TpEsHG seems to provide a significant higher transfection level compared to other two proteins ($P< 0.05$). Therefore, this vector was chosen for suicide gene-mediated cell toxicity.

Since the TpEsHG mediated transfection leads to a transient expression of transgene and not a stable one, a suitable setup to make the best use of highest level of enzyme becomes important. In addition, the concentration of prodrug and nano-particles chosen for the study should not be toxic for the cells per se if they are administrated separately. Figure 9 shows the toxicity of different doses of GCV, CB1954 and 5FC based on uM with the red line in each graph indicating the IC50. For each prodrug, doses with

≥80% of viability were chosen as the safe doses which are 100, 200, 400 and 1000 uM for GCV, 5, 10, 20 and 25 uM for 5FC and 10, 20, 40 and 100 uM for CB1954.

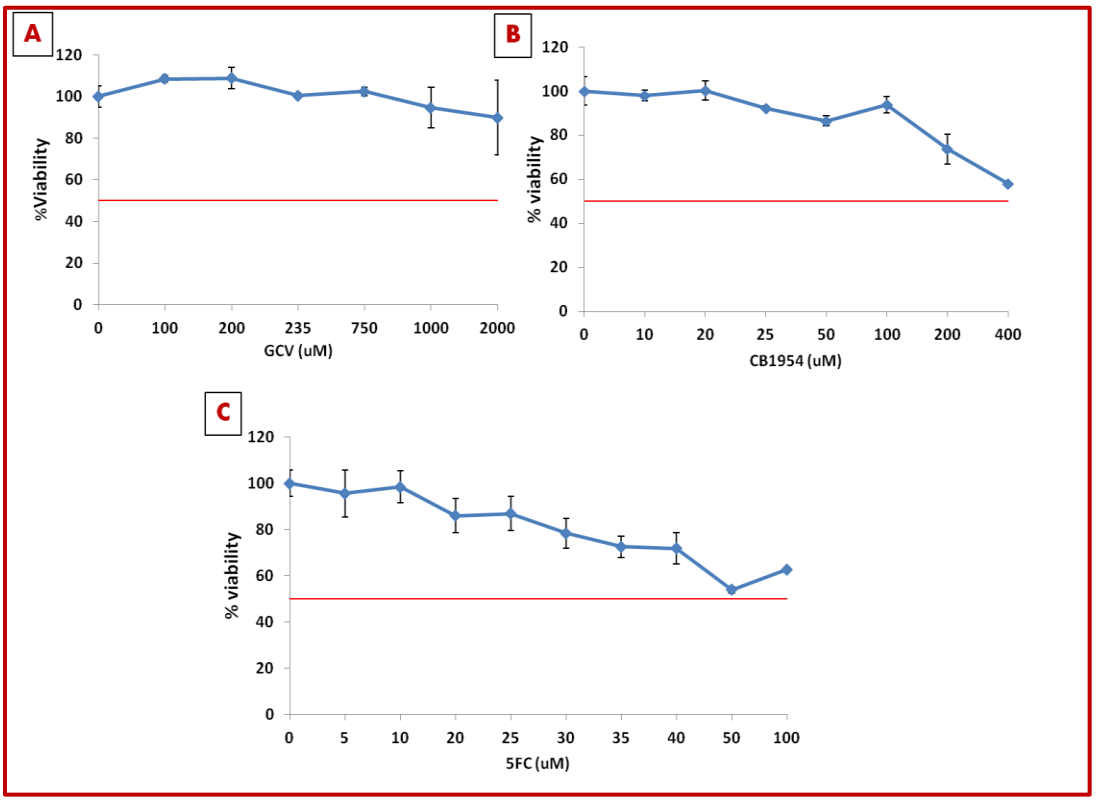

Figure 9: percentage of viability in PC3 cells incubated with different doses of GCV (panel A), CB1954 (panel B) and 5FC (panel C) prodrugs. The red line in each graph indicates IC50 for each prodrug.

The same experiment was carried out to identify a range of DNA/amino acid-based vectors with no significant toxicity on PC3 cells. For this purpose, nanoparticles with different doses of DNA (0.15, 0.25 and 0.5 ug DNA per $2X10^4$ cells) were tested. These nano-particles were prepared from TpEsHG and pBUD plasmid DNA. The aim was to find a DNA dose which is not toxic and at the same time renders a high level of transfection. As panel A figure 10 suggests, no significant differences between transfection efficiency

of DNA doses of 0.25, 0.5 and 1 ug/well (P>0.05) was observed. However the transfection efficiency of 0.15 ug DNA/well was significantly lower. This dose was removed for the next step which is toxicity of nanoparticles on PC3 cells. On the other hand, the viability of PC3 cells transfected with 1 ug DNA were significantly lower than the control group(P< 0.05), where the other two groups (transfected with 0.25 and 0.5 ug DNA) did not show any significant decrease in viability. These two doses (0.25 ug and 0.5 DNA/2X10^4 cells) along with 0.15 were chosen for the further experiments (figure 10). Although 0.15 showed very low transfection efficiency, it was still kept for suicide gene/prodrug toxicity since it was speculated that this dose might still be partially effective at some prodrug concentrations.

Another question that should be addressed is the duration of transgene expression, as the transgene remains episomal and its expression is lost during cell division or by environmental factors[22]. Transfected PC3 cells were tested for Luciferase activity as an indicator of transgene expression. The results illustrated in figure 11 shows the maximum expression of transgene after 24 hours, with a noticeable decline in day 2 post-transfection. Based on suggested data in figures 9-11, PC3 cells were transfected with TpEsHG nano-particles embedded doses of 0.15, 0.25 and 0.5 ug plasmid DNA coding for SR39, NTR and 5FC enzymes per 2X10^4 cells. This protocol is highly similar to another study performed by O'Keefe et.al. in which PC3 cells were transiently transfected with pDNA coding for Cytosine Deaminase[23].

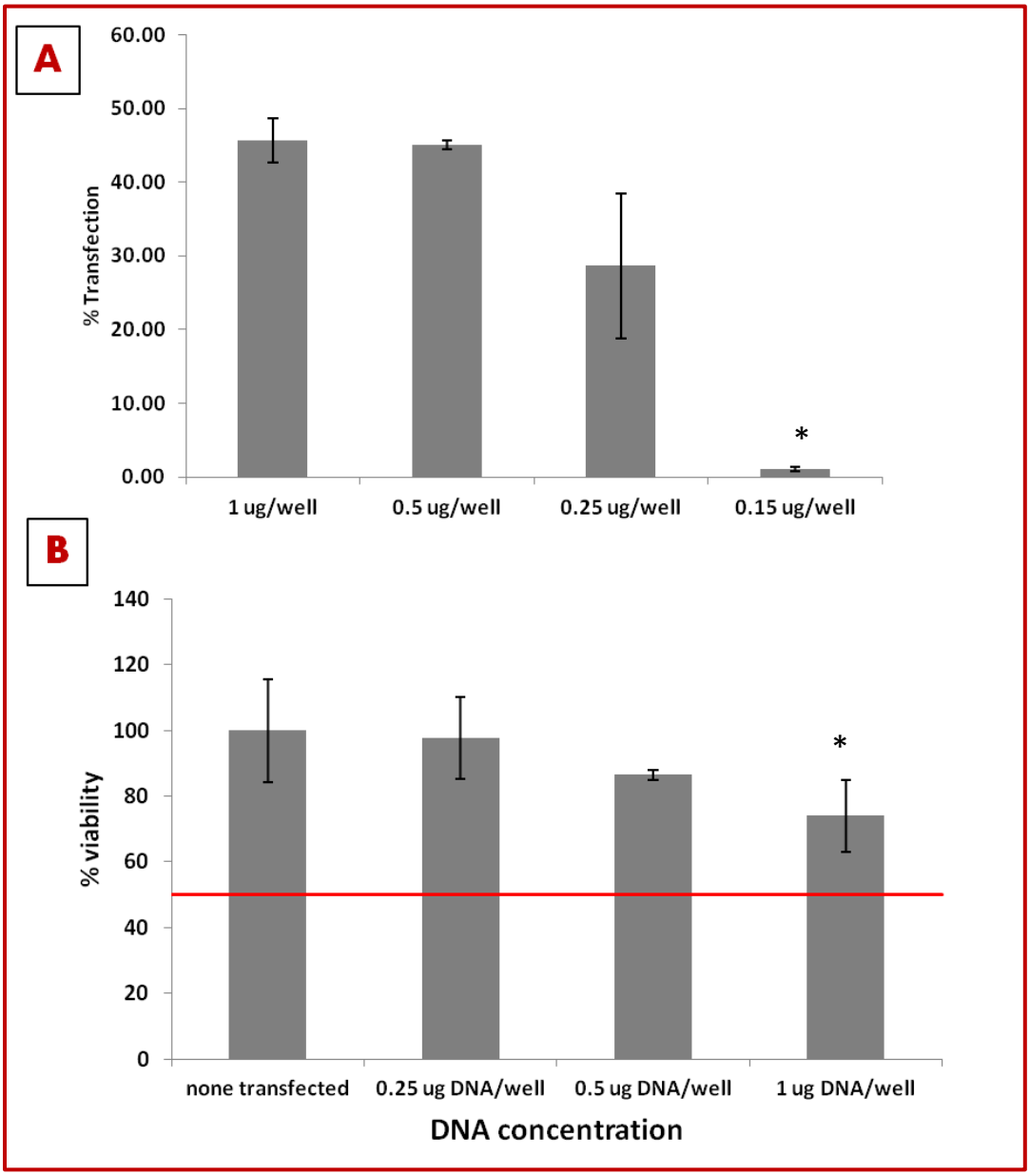

Figure 10: transfection percentage of PC3 cells and their viability after transfection with different doses of pBUD plasmid DNA and TpEsHG. The red line in panel B shows IC50. In Panel A, all samples were compared with 1 ugDNA/well. In panel B, samples were compared to non transfected group.

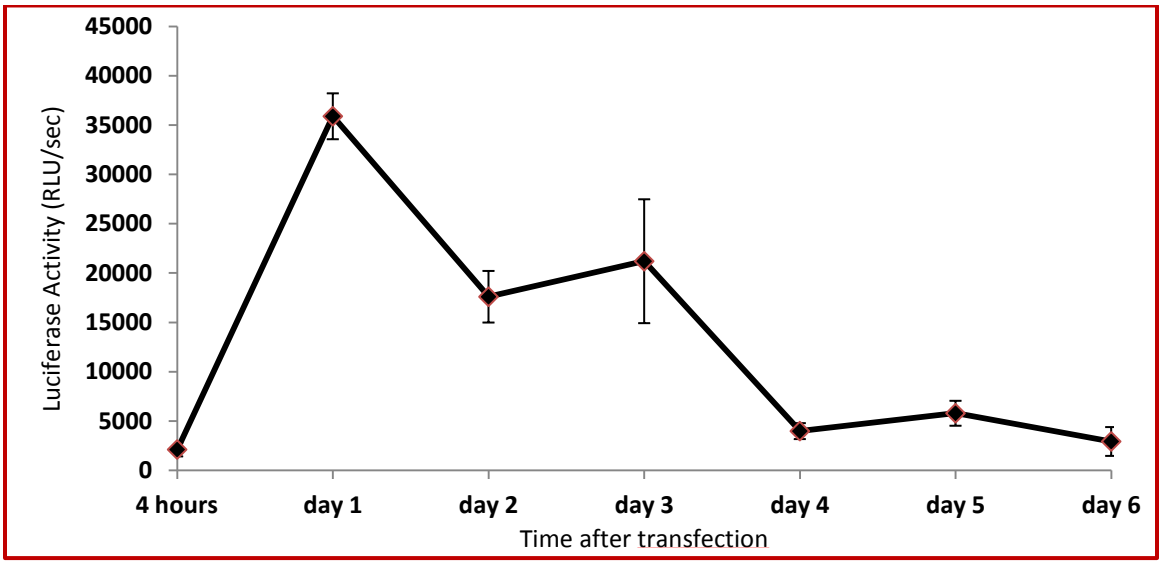

Figure 11: Luciferase activity in PC3 cells transiently transfected with nano-particles consisted of TpEsHG protein and pBUD DNA plasmid coding for luciferase enzyme.

The result of the viability of transfected PC3 cells after 24 hours incubation with different concentrations of prodrug are summarized in Figures 12-14.

As these figures suggest both yCD/5FC and NTR/CB1954 showed more effectiveness on PC3 cells than SR39/GCV system. The most effective dose for SR39/GCV leads to only (27%) cell death (73% cells survival) after 48 hours incubation with prodrug (Figure12). For CB1954 and 5FC systems, the percentage of survival was 59.26% and 59.38% (Figure 13 and 14). Overall, none of the treatments reached the IC50 level which is quite surprising since based on flow cytometry data almost 45% and 28.64% of cells showing the expression of transgene (green fluorescence protein) when transfected with 0.5 and 0.25 ug DNA /2 10^4 cells respectively. It seems that many factors should be optimized to increase the toxicity of transient suicide gene therapy. Our experiment was not the

first in its kind to use transient transfected cells instead of stable cell line for suicide gene therapy. In transient transfection, the level of transgene expression and its duration as well as genetic of cell line and as a result its susceptibility to toxic metabolites of prodrug plays an important role in success of suicide gene therapy. To demonstrate the effect of cell line characteristics, O'Keefe et al (2000) used Lipofectamine to transiently transfect prostate cancer cell line LNCaP, and its androgen-independent derivative C4-2. LNCaP cells showed less sensibility to 5FC treatment (IC50 of mM in LNCaP compared to IC50 of μM in C4-2) which can be related to the genetic differences between two cell lines and the longer time LNCaP needs to recover after transfection. They also studied the effect of adding a prostate specific promoter to the DNA construct, namely Prostate-specific membrane antigen (PSMA). Both LNCaP and C4-2 showed higher sensitivity to 5FU as a result of higher enzyme expression whereas PC3 cells showed no significant difference[23]. This study as well as another studies in this type emphasized the effect of cell line genetics and the regulatory elements of suicide gene to increase the effectiveness of suicide gene therapy[23-25].

While our results suggested partial effectiveness of current set up for suicide gene therapy in PC3 cells, the major modification which possibly leads to a higher level of cell death can be the modification of DNA construct. We suggested choosing a more cancer specific promoter for expression of suicide enzyme which is currently under the control of $P_{EF\text{-}1\alpha}$.

A good candidate can be Osteocalcin (OC) promoter which is upregulated in bone metastasis of prostate cancer. It has been showed that PC3 cells but not LNCaP cells are positively express OC gene[26]. The other suggestion might be to evaluate the expression level of enzyme directly through western blotting instead of measuring the expression level of reporter gene as the expression of the reporter gene (which is Luciferase in this study) is under the control of different promoter (CMV) and might not be necessarily reflective of expression level of the suicide enzyme.

4. Conclusion

The present study aimed to establish a novel and efficient non-viral strategy for transferring a therapeutic gene to a metastatic CAR^-/HER^- model of prostate cancer. The amino acid-based vectors introduced in this study are able to render a level of transfection which is quite comparable to commercial cationic lipids. Provided the known toxicity of such commercially available transfection agents, the nano-particles we are introducing here have been shown to be non-toxic and can be considered as an emerging group of vectors in the family of non viral vectors for gene delivery.

To use this nanoparticle system for a therapeutic application, one Tp protein, namely TpEsHG with significant higher transfection efficiency was chosen for suicide gene therapy. Because of the nature of the vector, the trasngene remains episomal; therefore as expected the expression of enzyme was transient with a peak of expression after 24 hours. All of the enzyme/prodrug combinations used in this study impose their partial or complete effect through interaction with DNA molecule, either by blocking the synthesis

of new strand (GCV and 5FC metabolites) or causing damage (CB1954 metabolites). The doubling time of PC3 cells is about 25 hours. Therefore it is very likely that the duration of expression was not enough to cause a robust killing effect. To overcome this problem, two approaches can be suggested; first, increasing the expression level of transgene, so even after one or two cell cycles, there is enough concentration of toxic metabolites to render toxic effect. The second method is to prolong the duration of transgene expression by integrating it into the cells chromosomes. Increasing the expression level of enzyme will be possible by adding prostate cancer promoter/enhancer before suicide gene. In order to prolong the expression, a transposone element can be included in the structure of plasmid DNA. One well-known transposone system, sleeping beauty, has been successfully used in combination with polymers for sustained expression of transgene[27]. Such a combination can be used to improve the efficiency of suicide gene therapy mediated by Tp vectors.

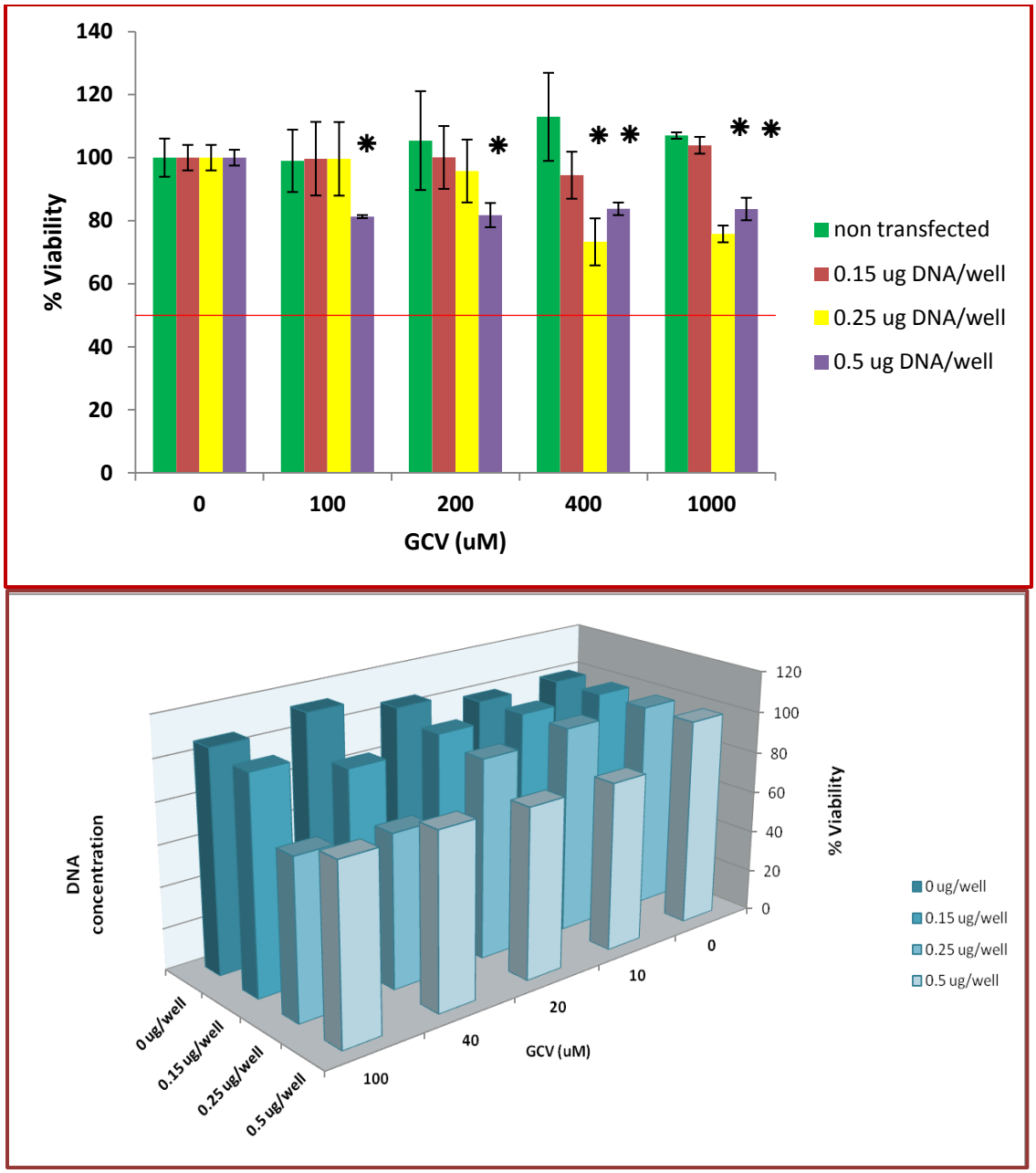

Figure 12: Bar chart (top panel) and 3D column chart (bottom panel) of percentage of viability in PC3 cells transiently transfected with three different doses of pBUD plasmid DNA (0.15 ug, 0.25 ug and 0.5 ug DNA/2 x 10^4 cells) coding for SR39 and 4 different prodrug concentrations. The lowest viability was recorded for cell transfected with 0.25 ug DNA and incubated at 400 and 1000 uM of GCV.

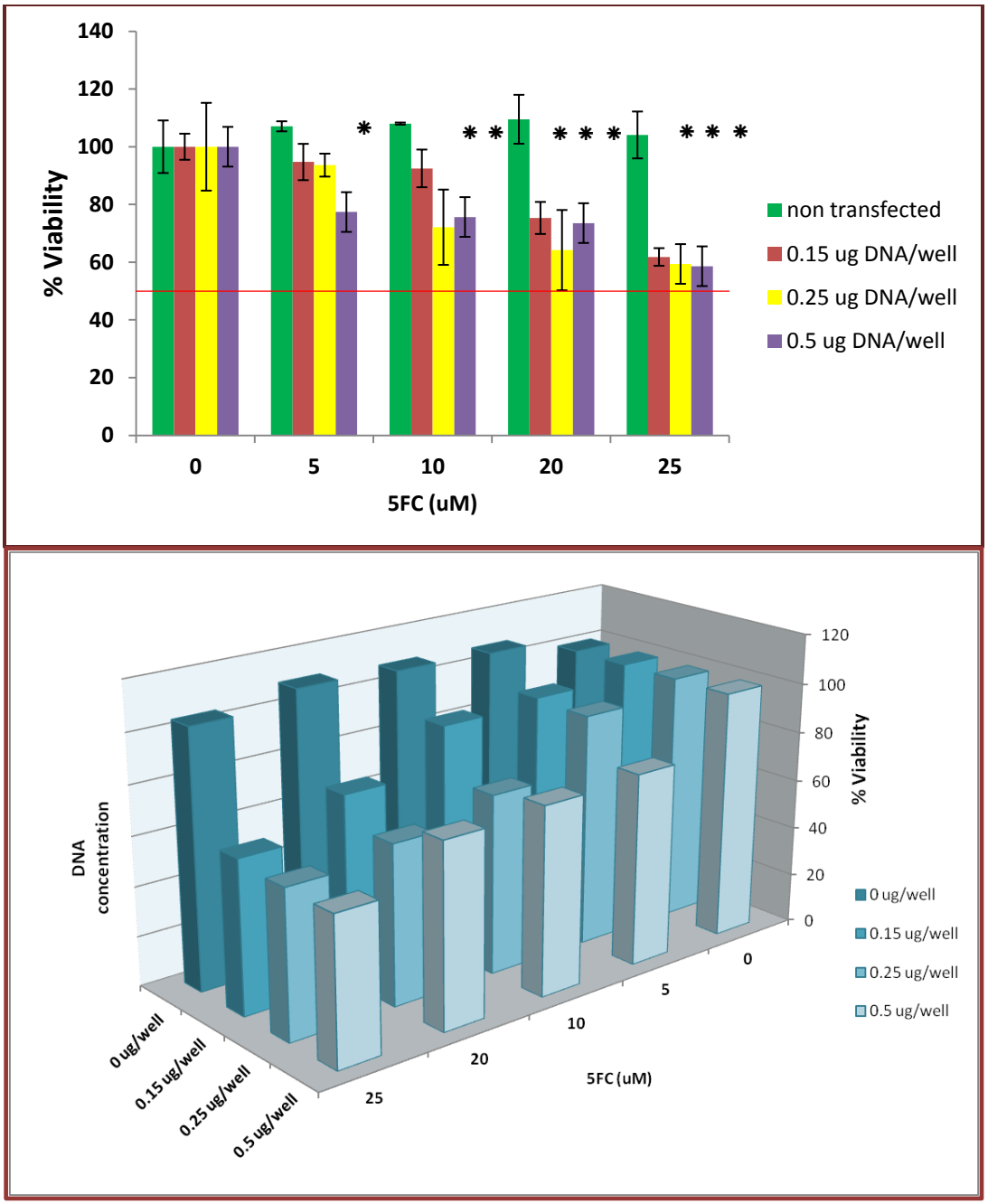

Figure 13: Bar chart (top panel) and 3D column chart (bottom panel) of percentage of viability in PC3 cells transiently transfected with three different doses of pBUD plasmid DNA (0.15 ug, 0.25 ug and 0.5 ug DNA/2 x 10^4 cells) coding for yCD and 4 different prodrug concentrations. The lowest viability was recorded for cell transfected with 0.5 ug DNA and incubated at 25 uM 5FC.

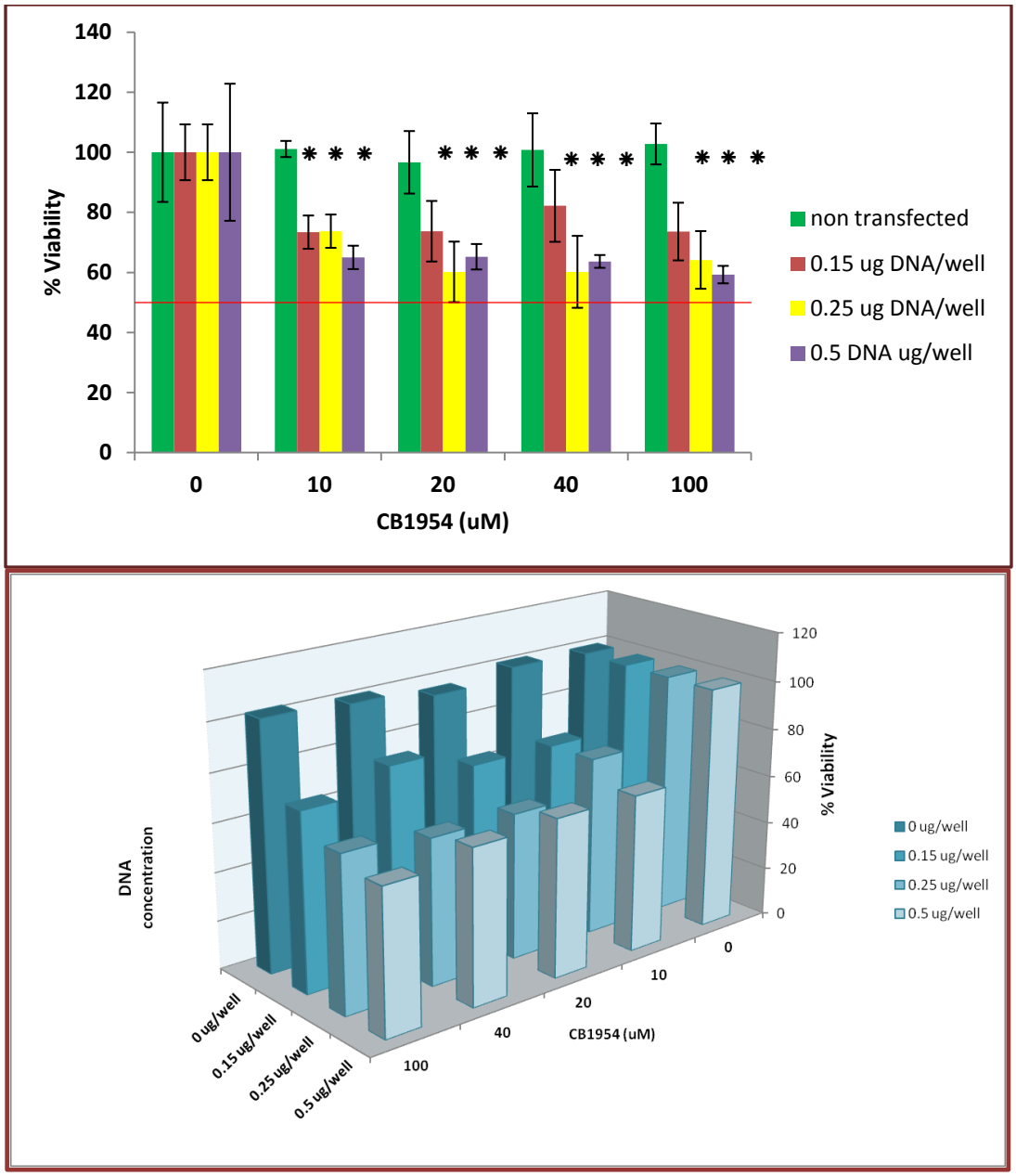

Figure 14: Bar chart (top panel) and 3D column chart (bottom panel) of percentage of viability in PC3 cells transiently transfected with three different doses of pBUD plasmid DNA (0.15 ug, 0.25 ug and 0.5 ug DNA/2 x 10^4 cells) coding for NTR and 4 different prodrug concentrations. The lowest viability was recorded for cell transfected with 0.5 ug DNA and incubated at 100 uM of CB1954.

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Chapter V: Conclusion

The advent of nanotechnology has not only revolutionized the diagnostic methods of cancer, but has also changed the design and formulation of chemotherapy agents drastically[1, 2]. With the versatility offered by nano-particle modality, designing a tailor-made system with desired physico-chemical and targeting properties is much simpler than before. Many scientists see the junction of nanotechnology and cancer molecular biology as a path to personalized medicine, where nanotechnology can refine and translate the science of cancer biomarkers and pathways to a more accurate diagnosis, safer medicine and better assessment of the therapy effects.

Since the introduction of first nanomedicine formulation in 1995, numerous systems have been introduced. However, only a few reach the clinical level. Many of them failed in late preclinical or early clinical levels. One of the reasons behind the failure of such sophisticated systems is the complexity exploited in design which is based on a very particular application and under defined (and sometimes unrealistic) condition. In this case, re-adjusting the nano system for different types of applications such as various cancer types and stages, different patient population and even a multi-drug chemotherapy regimen will be a labor intensive job and prone to failure, let alone a scale up process[3].

The more flexible and straightforward a nano system is, the more chance it has to reach preclinical and clinical levels, since the manufacturing process and design of preclinical and clinical trials are more facilitated. Also keeping the process simple with few straight

-forward steps for preparation guarantees a higher rate of success and a better control over the nano-particles' properties, if adjustment is required.

Having mentioned this fact, amino acid based vectors are promising modality for both gene and drug delivery because of their biodegradability, biocompatibility, low toxicity and ease of manipulation. The list of different polypeptides used for this purpose includes both natural and synthetic sequences[4].

It has been the focus of our lab to design and produce a nature-inspired amino acid vector for gene delivery with the ability to be easily customized and personalized based on tumor specific conditions and as the same time enjoys the possibility of a fast and easy to scale up production. Keeping these features in mind, we adapted a simple bio-production method which provides a high degree of reproducibility while keeps the production process in one step.

The core of this multi-domain amino acid based vector which consists of 4 repeats of Histone H2 and a fusogenic peptide has been shown previously to be able to overcome the intracellular barriers that a nano-particle encounters after internalization to cancer cells.

In this study, we aimed to optimize the nano particles to overcome extracellular barriers, increase the biocompatibility and eventually introduce the potential for a more efficient in vivo delivery.

The first step to achieve this goal was to optimize the physicochemical properties of particles such as surface charge. it is worth to mention that surface charge plays a crucial role in non specific interaction with the cell surface or elements of cellular microenvironment resulting in toxicity[5]. In chapter III, we presented the PEGylated particles with almost neutral surface charge. By adding PEGylated peptide to the structure of nano-particles, the surface charge dropped to neural level (Figures 6 and 7, chapter III), which potentially decreases, if not eliminates, the possibility of non-specific interaction. In same chapter, we showed that optimization of nano-particles by PEGlylation, not only increase the efficiency of transfection but also introduce the desirable features for an optimal in vivo delivery, such as stability in salt concentration close to physiological conditions.

To be loyal to the concept of simplicity in design, in chapter IV, we replaced the PEGylated peptide with an Elastin like peptide (ELP). In this case, the need for PEGylation, which to some extent introduce lower reproducibility and an extra step in production, will be eliminated. The nano-particles with a certain ELP sequence in their structures (TpHsG) have been shown to render a higher level of transfection but the same neutrality and safety as PEGylated peptides (Chapter IV). However, to answer the question of *in vivo* efficiency, a detailed animal study is required.

When optimizing the system for higher biocompatibility, we should keep in mind that the key concept for improving biocompatibility is not only reducing the adverse interaction with blood component but also with non-target cells[6]. While a neutral

surface charge prevents electrostatic interaction of nano-particles with the non-target cell surface, the active interaction of targeting moiety and specific biomarkers on the surface of target cell is the major driving force of internalization. In two different designs for HER2 positive ovarian and HER2 negative prostate cancer cells, we showed a straight-forward method to customize the amino acid-based vectors for each different cell line which is through DNA cloning techniques. This approach can be used to personalize each vector based on the type of biomarkers each cancer type dominantly expresses.

Many indications may be defined for such a flexible system, as we chose suicide gene therapy in this study. As it was mentioned before, in suicide gene therapy, the maintenance of enzyme expression plays a crucial role, as it helps the toxic metabolite concentration to reach minimum toxicity concentration. Also, the effectiveness of a complicated therapeutic modality such as suicide gene therapy depends not only on the vector's efficiency but also on the sensitivity of tumor cells to the treatment, the expression enhancing elements such as tumor specific promoters and transcription enhancers. In this study, we showed the plausibility of using the first amino-acid based vector for suicide gene therapy. Future studies will be required to introduce our amino-acid based vectors as a robust and reliable modality for suicide gene therapy.

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