PEPTIDES AS THERAPEUTICS AND ACTIVE GENE DELIVERY VEHICLES FOR CANCER TREATMENT

by

LAKSHMI DEEPTHI UPPALAPATI

M.S., Kansas State University, 2010

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy & Physiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2015

Abstract

Over the years proteins/peptides have evolved as promising therapeutic agents in the treatment of cancer. Considering the advantages of peptides such as their small size, ease of synthesis, tumor-penetrating ability and bio-compatibility, present report discusses proof of concept for 1. C1B5 peptide of protein kinase Cγ and a low dose of gemcitabine combination treatment for peritoneally disseminated pancreatic cancer and 2. dTAT peptide nanoparticles mediated gene (angiotensin II type 2 receptor gene) therapy for lung cancer. 1. A significant reduction in intraperitoneally (IP) transplanted pancreatic carcinoma growth was demonstrated with C1B5 peptide and gemcitabine co-treatment in an immunocompetent mouse model. Increased number of Granzyme B positive cells was observed in treated mice ascites, suggesting the involvement of immune response in tumor attenuation. The strong effect observed in combination treatment might be because of increase in lymphocyte recruitment by gemcitabine followed by C1B5 peptide mediated CD8+ T-cells or NK cells activation apart from direct cancer cell apoptosis. 2. To test dTAT peptide nanoparticles (dTAT NPs) mediated therapeutic gene delivery, luciferase reporter gene containing dTAT nanoparticles were synthesized (dTAT/pLUC/Ca^{2+}). Synthesis conditions for nanoparticles were optimized based on dTAT/pLUC/Ca^{2+} nanoparticles transfection efficiency. With the optimized conditions, dTAT NPs containing AT2R, TRAIL or miR-34a pDNA (dTAT/pAT2R, dTAT/TRAIL or dTAT/miR-34a) were synthesized. Therapeutic potential of these NPs was analyzed in lung adenocarcinoma containing mice by administering them intravenously (IV) or/and intratracheally (IV). Combination treatment with the IV injection of the new dTAT/pAT2R/Ca^{2+} formulation and the IT injection of the original dTAT/pAT2R/Ca^{2+} formulation is effective in attenuation of developed human bronchioloalveolar carcinoma in the SCID mouse lungs. Findings from the

above mentioned studies have vital clinical relevance as it implies that peptides alone or when used as gene delivery systems may prove to be beneficial in the treatment of various stages of cancer.

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Acknowledgements

I would like to acknowledge my major professor, Dr. Masaaki Tamura, for giving me the opportunity to work in his lab. I would like to thank Dr. Michael J. Kenney, Dr. Duane Davis and Dr. Deryl L. Troyer for devoting their time as my committee members. I would like to thank my present and former lab members Dr. Susumu Ishiguro and Dr. Atsushi Kawabata for their help in my research work. I would also like to thank the members of Dr. Cory Berkland lab at KU for their help and input in my projects. I would like to acknowledge Sivasai Balivada for his valuable suggestions.

Chapter 1 - Introduction & Literature Review

Introduction

Cancer continues to be of the most deadliest diseases and a major cause of death worldwide [1]. The early understanding about cancer was that the normal healthy cells lost control over their cell dividing capacity that lead to tumor formation and cells would later migrate and metastasize leading to failure of body organs and eventually causing death [2]. Since then, numerous research groups studying cancer biology to clarify the underlying pathways / biochemical reactions provided us with better understanding of the concept, but also with tough challenges to formulate an effective treatment [3]. Cancer cells, by deregulating cell growth-and-division cycle signals chronically proliferate in an abnormal fashion creating a niche within the supporting tumor-associated stroma, which supply the cancer cells with various growth factors [4, 5]. Sandra S. McAllister & Robert A. Weinberg [6] examined that the tumor-derived factors and underlying pathophysiological processes including immune cells and cytokines play an important role in nearly all aspects of cancer progression and metastasis supporting the hallmarks of cancer such as: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, deregulating cellular energetics and avoiding immune destruction [7]. The recruitment of many of the stromal cell types that are found within the tumor microenvironment of most primary adenocarcinomas involves the release of tumor-derived factors that may also mobilize host cells from distant tissues, notably the bone marrow and spleen [8].

Currently, available clinical therapies for cancer include surgical resection, radiation therapy and chemotherapy. Surgical resection is usually the most common and first line of

therapy for cancer. Radiation therapy uses high-energy ionizing radiation to kill the tumor cells and this procedure generally reduces the risk of cancer recurrence by approximately 70%. However, it often induces adverse side effects on normal tissues adjacent to the radiation site. For both of the above therapies the tumor should be within clinical detection range and accessible to therapeutic procedures. Chemotherapy is a major post-surgical treatment used to reduce the risk of cancer recurrence. Chemotherapy decreases the tumor size by affecting rapidly dividing and highly metabolizing cells, which are the main characteristics of malignant cells. A large number of chemotherapeutics are clinically available like Gemcitabine, doxorubicin, paclitaxel etc. In many cases, a combination of two or more drugs will be used as a treatment regimen. In general, chemotherapeutic drugs are found to a) lack specificity, b) exhibit resistance, c) low drug clearance, d) varied bio-distribution and e) biotransformation making them common issues compelling to use high concentrations of these chemotherapeutic drugs. Therefore, targeted treatments are emerging as powerful tools via maximizing therapeutic index and reducing toxicity [9]. However, chemotherapy also causes other severe side effects like hair loss (alopecia), fatigue, anemia etc., by acting on dividing cells and cells, which have high metabolic activities. Although strategies targeting primary malignancies have improved markedly, there are no cures for clinical relapse and metastatic disease, which remains the underlying cause of death for the majority of cancer patients. Several new therapies based on biological agents such as nanoparticle drug delivery, stem cell based anticancer therapy, targeted antibody therapy, and cancer vaccines, which are currently in development, have been very effective in preclinical research, but they have not yet been shown to be effective in human clinical trials. Accordingly, a novel powerful cancer therapy with fewer side effects is urgently needed.

Current cancer statistics

Cancer is a major public health problem in the United States and many other parts of the world. One in 4 deaths in the United States is due to cancer. A total of 1,658,370 new cancer cases and 589,430 cancer deaths are projected to occur in the United States in 2015. Among men, cancers of the prostate, lung and bronchus, and colorectum will account for about 50% of all newly diagnosed cancers. Lung and bronchus cancer alone will account for 28% (86,380) of estimated death cases in men. The three most commonly diagnosed types of cancer among women in 2015 will be breast, lung and bronchus, and colorectum, accounting for 50% of all cases in women with 26% (71,660) estimated deaths due to lung and bronchus cancer alone [1].

Figure 1.1 Leading cancer types for the estimated new cancer cases and deaths by sex, United States, 2015.

*Estimates are rounded to the nearest 10 and cases exclude basal cell and squamous cell skin cancers and in situ carcinoma except urinary bladder (Siegel, 2015).

Estimated New Cases

Males				Females		
Prostate	220,800	26%		Breast	231,840	29%
Lung & bronchus	115,610	14%		Lung & bronchus	105,590	13%
Colon & rectum	69,090	8%		Colon & rectum	63,610	8%
Urinary bladder	56,320	7%		Uterine corpus	54,870	7%
Melanoma of the skin	42,670	5%		Thyroid	47,230	6%
Non-Hodgkin lymphoma	39,850	5%		Non-Hodgkin lymphoma	32,000	4%
Kidney & renal pelvis	38,270	5%		Melanoma of the skin	31,200	4%
Oral cavity & pharynx	32,670	4%		Pancreas	24,120	3%
Leukemia	30,900	4%		Leukemia	23,370	3%
Liver & intrahepatic bile duct	25,510	3%		Kidney & renal pelvis	23,290	3%
All Sites	**848,200**	**100%**		**All Sites**	**810,170**	**100%**

Estimated Deaths

Males				Females		
Lung & bronchus	86,380	28%		Lung & bronchus	71,660	26%
Prostate	27,540	9%		Breast	40,290	15%
Colon & rectum	26,100	8%		Colon & rectum	23,600	9%
Pancreas	20,710	7%		Pancreas	19,850	7%
Liver & intrahepatic bile duct	17,030	5%		Ovary	14,180	5%
Leukemia	14,210	5%		Leukemia	10,240	4%
Esophagus	12,600	4%		Uterine corpus	10,170	4%
Urinary bladder	11,510	4%		Non-Hodgkin lymphoma	8,310	3%
Non-Hodgkin lymphoma	11,480	4%		Liver & intrahepatic bile duct	7,520	3%
Kidney & renal pelvis	9,070	3%		Brain & other nervous system	6,380	2%
All Sites	**312,150**	**100%**		**All Sites**	**277,280**	**100%**

Literature review

Emergence of biologics as cancer drugs

The biological treatment option involves the treatment of cancer using a) the living organisms like bacteria and vaccines to primarily stimulate the host body immune response, b) substances derived from living organisms like proteins, monoclonal antibodies (mAbs), and peptides or c) their synthetic versions produced in the laboratory to interfere and target the specific molecules or pathways involved in cancer growth and progression.

Biological understanding of cancer came to a point that design of cancer treatments need to consider myriad hallmarks of tumor and its environment [7]. In recent years, the use of large molecules like therapeutic mAbs for cancer therapy has achieved great success in human clinical trials[10-15]. The development of these mAbs require a good understanding of multiple hallmarks of cancer tissue and its interaction with the surrounding microenvironment along with the other physiochemical and protein-engineering aspects [10]. These antibody-drug conjugates are now included in the treatment regimens of lymphomas and solid tumors, mainly[16-19]. Even today, for most of the cancer treatments, small molecules or chemicals remain the most viable option[20].

Figure 1.2 Size comparison between small and large biomolecules ("crowsandcats.blogspot.com")

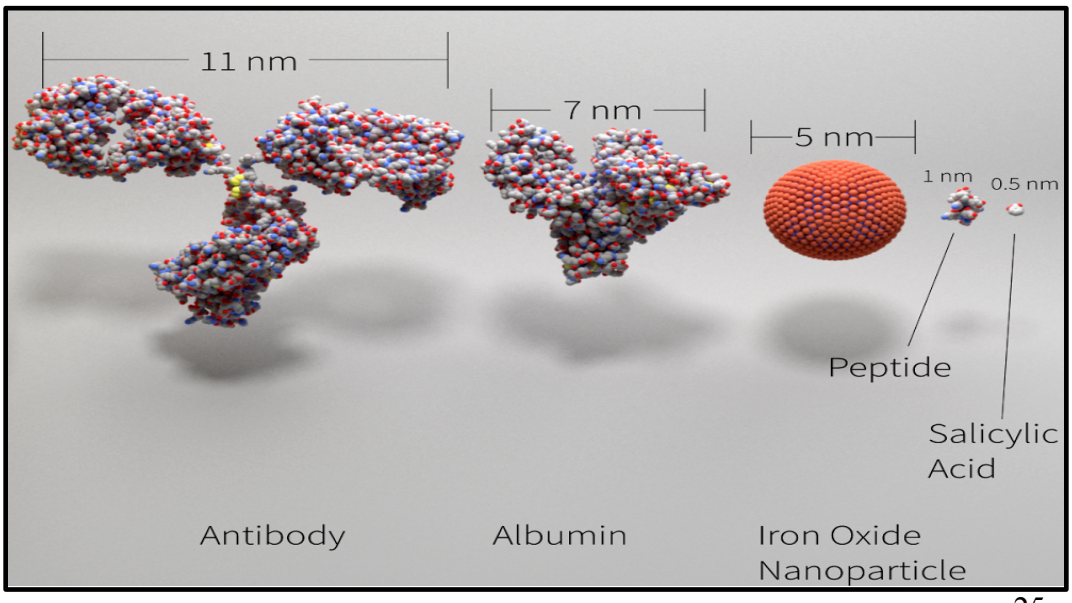

Fig 1.2 provides a clear understanding of size wise comparison between a small molecule like salicylic acid measuring as low as 0.5 nm or peptide measuring around 1 nm and a large

molecule like antibody measuring around 11 nm. Each module has its own pros and cons. The mAbs and large protein ligands can be tumor specific but has few major limitations compared to small molecules like peptides; a) poor diffusion and distribution to tumors due to their large size, b) activating host immune response and c) toxicity to the liver and bone marrow due to their uptake into the reticulo-endothelial system. On the other hand, very small molecules have increased tissue diffusion into and do not cause host immune response. However, it is hard to achieve multifunctionality and specificity. Peptides usually measuring around 1 nm possess many advantages, such as small size, ease of synthesis and modification, biocompatibility, tumor penetrating ability and low or no host immune response to successfully treat the site of primary tumor and the distant metastatic sites [21]. Peptide degradation by proteolysis or host immune reaction can be easily masked by structural and chemical modifications, such as incorporation of D-amino acids or cyclization [9]. Out of four blockbuster peptide drugs available in the market, three of them namely, leuprolide, octreotide and goserelin are anti-cancer peptides used directly or in the treatment of separate clinical stages associated with certain tumors[22].Peptides can be designed as: direct anti-cancer drugs, vaccines, hormones, cytotoxic drug or radionuclide carriers, and drug targets [23]

Peptides in cancer treatment

Peptide based anti-cancer agents depending on their biological function can be classified as peptide hormones, peptide as radionuclide carrier, peptide vaccines, peptide as cytotoxic drug carrier, and anti-cancer drug peptides[23]. On the other hand, depending on the properties of peptides they can be broadly divided into three groups; Anti-microbial peptides (AMPs) or "pore-forming" peptides, cell penetrating peptides (CPPs), and tumor targeting peptides (TTPs)[24].

Peptide hormones

The classic examples of peptide hormones are LHRH (luteinizing hormone-releasing hormone) agonists and antagonists. Schally et al. introduced LHRH agonists as prostate cancer therapeutics leading to the downregulation of LHRH receptors, inhibition of follicle-stimulating hormone (FSH) and LH release, and decrease in testosterone production efficaciously [25-29]. Later, the discovery of LHRH antagonists caused an immediate and dose-dependent inhibition of LH and FSH by antagonist blockade of the LHRH receptors. Cetrorelix was the first LHRH antagonist available clinically [30, 31]. Abarelix and degarelix are the new generation clinically available antagonists [32, 33].

Peptide as radionuclide carrier

Most neuroendocrine tumors (NETs) are found to overexpress somatostatin subtype 2 (sst2) receptors[34, 35]. Therefore, sst2 receptors are attractive targets for delivery of radioactivity via radiolabeled somatostatin analogs. [111In-DTPA]-octreotide (Octreoscan) and NeoTect (tc-99m depreotide) are the only radiopeptide tracers approved by the FDA[36, 37]. Octreotide is indium-111 radiolabeled and injected into the bloodstream intravenously. Octreotide attaches to cells that have sst2 receptors. Then using a radiation-measuring device octreotide binding tumor cells were detected. NeoTect is a radioactive imaging test used to identify specific cells that may be associated with lung cancer or with other conditions[37]. Peptide receptor radionuclide therapy (PRRT) is another highly specialized technique which combines somatostatin analogs like octreotide with a radionuclide to form powerful radiolabeled somatostatin analogues or radiopeptides specifically binding to carcinoid cells killing them by emitting radiation[38-43]..

Peptide Vaccines

In the recent years, active cancer immunotherapies like peptide-based cancer vaccines against tumor-associated antigens (TAAs) have been considered to be an effective strategy to eradicate cancer cells from the host body. This therapeutic strategy elicit TAA-specific cytotoxic T lymphocytes (CTLs) activation eventually leading to cancer cell clearance, is being developed further[44, 45] Tumor cells express antigens called as tumor-associated antigens (TAAs) that can be recognized by the host's cytotoxic T-lymphocytes. Any protein/peptide synthesized in a cancer cell that has an abnormal structure due to mutation can work as a tumor antigen. These TAAs can also be injected into host body to induce a systemic immune response that may result in the inhibition of tumor growth in different body tissues [46]. This procedure of inducing a systemic immune response is called active immunotherapy or active vaccination as the patients immune system is either activated or re-stimulated to develop an effective, tumor-specific immune response that may ultimately lead to tumor regression[47, 48]..

Some of the notable peptide vaccines that have been evaluated in human clinical trials include Mucin-1 (MUC-1, Stimuvax) peptide for breast or colon cancer treatment [49, 50].,HER-2/neu immunodominant peptide for lung, breast, or ovarian cancer treatment [51, 52]. Ras oncoprotein peptide for colorectal and pancreatic carcinoma treatment[53-55] and Melanoma antigens for Melanoma therapy [56-58].

Anti-cancer drug peptides

The usage of peptides as anti-cancer therapeutic molecules in targeted drug delivery and as a diagnostic tool in tumor biology is evolving. Cancer cell targeting exploits the differences between the normal and tumor cell characteristics and their relation to their surrounding microenvironment. With the developments in controlled delivery of cancer therapeutics, tumor

targeted peptides have emerged as the most valuable non-immunogenic approach to target and eradicate tumor cells. Peptides can also be added into different multicomponent gene delivery complexes for enhanced cell-specific targeting.

Cancer gene therapy

Altered bio-distribution and drug resistance in the host body is a critical reason to most of the adverse side effects and poor therapeutic efficacy in conventional cancer treatments like chemotherapy. Hence, targeted therapeutic strategies aimed at transformed tumor tissue have emerged as favorable alternatives over the non-targeted conventional approaches. Anti-cancer specific effects can be obtained by either blocking a tumor growth promoter gene expression, or by delivering drug molecules along with an over-expressed tumor growth suppressor gene into cancer cells. Various research studies have shown that gene delivery systems are made more efficient through improved gene transfection efficiency, intracellular stability with minimum toxicity [59-63]. Different viral vectors such as retrovirus, adenovirus, adeno-associated virus (AAV), herpes virus, pox virus, human foamy virus (HFV), and lentivirus have been used for this purpose[64]. For gene delivery purpose, viral vector genomes have been modified so that their replication is unhinged making them safer for gene transduction purposes. However, while viral vector systems have shown to have potent transgene expression in tumor cells [65-67], their effectivity is limited by host immunogenic response leading to inflammation the elimination of transducted tissue and toxin production limiting transgene expression *in vivo [68]*. In severe conditions the virus causes death, mutagenesis; and also limit transgenic product size [69]. Viral vectors with specific receptors have been constructed in the recent years to transfer the transgenes to retarget specific cells (Wickham TJ, 2003) but they have not been translated to *in*

vivo clinical trails. Therefore, non-viral vectors are widely researched and believed to have very promising roles in therapeutic gene delivery [70].

Nanoparticle based gene-delivery system

Nanoparticles (NPs) are a class of versatile molecules with diameters ranging in between 1–100 nm which can act as carriers for chemotherapeutic drugs, imaging agents, and receptor targeting ligands. Various types of NPs coated with polyethylene glycol (PEG) to evade host immune system have been developed as carriers for therapeutic agents or drugs [71]. Nano-sized particles tend to accumulate in tumor tissues with leaky vasculature, without attaching to any tumor-specific moiety known as the enhanced permeability and retention (EPR) effect [72]. This therapeutic effect is achieved because of the hyper vasculature with abnormal leaky structure and impaired lymphatic drainage in tumor tissues, arising as a result of superfast malignant cell growth and insufficient nutrient supply [73, 74]. Beyond the passive EPR effect, nanoparticles provide a surface for the attachment of specific molecular motifs or receptors to enable facilitated drug internalization and thereby, active tumor targeting. Naked plasmid DNAs (pDNA) do not easily pass through the biological cell membranes, and hence, pDNAs combined with cationic polymers are now commonly employed as non-viral gene delivery vehicles [75]. Recent research has highlighted many advantages of a targeted nanomedicine approach in a combined therapy for treating HNSCC, such as enhanced preferential tumor-killing efficiency and reduced toxicity to healthy tissues [76].

Further, apart from pDNA, studies have shown that HIV-Trans-activating transcriptional activator (HIV-TAT) peptide, which is a protein transduction domain also shows promising translocational abilities as it effectively traverses biological membranes unaffected by either the

type or number of receptors and temperature [77]. HIV-Tat was among the first discovered cell penetrating peptides (CPPs) [78]. Tat sequence: GRKKRRQRRRPPQ could enable the internalization of drug nanocarriers by various cell types[79, 80]. Several studies have found that Tat fusions to membrane to enable the cell penetration of biological molecules[81]. However, TAT based Pre-clinical cancer studies are limited due to the lack of tumor specificity. In one such example mice implanted with human cancerous tissue was treated by TAT conjugated to a peptide that activate p53[82]. In a more recent study, either TAT or Antp (Antennapedia homeodomain) delivered Met peptide into Kaposi's sarcoma cells. At optimum concentrations of 10–25 μM TAT-Met or Antp-Met peptides inhibited tumor growth and further higher doses resulted in cytostatic activity [83]. Several research studies indicate the potential anti-cancer application of TAT and similar CPPs stressing the need for modifications for tumor targeting and concentration of toxic cargo. Here, we have developed a modified nanoparticle delivery system by combining polycationic dimerized TAT peptide and plasmid DNA (pDNA) deliver therapeutic genes AT2R, TRAIL and miR34a, which are known to inhibit cancer proliferation[84, 85].

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Chapter 2 - Co-treatment with a C1B5 peptide of protein kinase Cγ and a low dose of gemcitabine caused an effective inhibition of peritoneally disseminated pancreatic cancer growth in mice

Abstract

Although gemcitabine is an effective chemotherapeutic agent for pancreatic cancer, unacceptable side effects often accompany its use. Since we have discovered that locally administered C1B domain peptides effectively control tumor growth without any side effect [86], we sought to examine the efficacy of co-treatment with this peptide and a low dose of gemcitabine on the growth of pancreatic cancer. Cell culture studies clarified that both C1B5 peptide (1μM) and gemcitabine (20nM) effectively attenuated growth of PAN02 mouse pancreatic ductal adenocarcinoma cells, but not normal mouse mesothelial cells and fibroblasts. However, a combination treatment with 1μM C1B5 peptide and 20nM gemcitabine also effectively attenuated growth of PAN02 cells. The C1B5 peptide-induced cell growth inhibition was determined to be due to an induction of apoptosis. Furthermore, a combination treatment with C1B5 peptide at 20 mg/kg and a low dose of gemcitabine at 15 mg/kg markedly inhibited the growth of PAN02 allografts in the mouse peritoneal cavity (94% inhibition) despite the low dose of gemcitabine alone (76%) or C1B5 peptide alone (39%) inhibited tumor growth moderately. Peritoneal cavity infiltrated lymphocytes and granzyme B^+ (Granzyme B is a serine protease most commonly found in the granules of cytotoxic T cells (CTLs) and natural killer cells (NK cells)) lymphocyte numbers were significantly higher in combination treatment group than in control group. Remarkably, clinical symptoms associated with these treatments, such as loss of appetite and body weight, were not noticeable in the combination treatment group. Taken together, the current study suggests that C1B5 peptide offers a remarkably effective combination treatment strategy to reduce side effects associated with gemcitabine without losing its tumoricidal effect.

Introduction

Pancreatic cancer is the fourth leading cause of cancer mortality in the US with 48,960 estimated new cases and 40,560 estimated deaths in 2015 [2]. It is often termed as silent killer with an estimated 6% five-year survival rate remains a malignancy with increased incidence and mortality rates in recent years[1]. With its low diagnosis rate, invasive metastatic ability paired with poor efficacy of conventional therapies, pancreatic cancer prognosis is extremely poor and often inadequate[87]. Although genetic predisposition-dependent familial incidences have been recognized, the majority of human pancreatic cancer is shown to be associated with environmental factors such as smoking, heavy alcohol consumption and is sporadic. Novel targeted combination treatments that can elicit multiple anti-tumor responses are needed to treat pancreatic cancer[1]. Currently available treatments for pancreatic cancer have high degree of side effects apart from variability in their therapeutic effect [88, 89]. Gemcitabine (Gemzar), a nucleoside analogue of cytidine (Figure 2.1), a first-line standard chemotherapeutic agent for pancreatic cancer treatment induces cell apoptosis by DNA synthesis inhibition[90, 91] It is shown to regulate and modulate immune functions, such as depletion of B-lymphocytes[92], MDSC (myeloid-derived suppressor cells) [93], the decrease in regulatory T-cells[94], and the reinforcement of T-cell responses with an increase in the cross-presentation of tumor specific antigens[95]. However, the efficacy of gemcitabine as a single agent remains modest, with a median survival of approximately 6 months in randomized trials [96, 97]. In addition, unacceptable side effects often associate with gemcitabine treatment, which include myelosuppression, muscle pain, fever, *etc*. Due to these reasons, there has been an interest in developing strategies that reduce systemic toxicity while improving the therapeutic potential of gemcitabine, which includes the development of novel combination chemotherapies. However,

despite a number of clinical trials of the combination therapy with gemcitabine and other compounds like docetaxel, cisplatin, oxali-platin, fluorouracil, and irinotecan [88, 98-102] only a little benefit has been noticed as compared to gemcitabine monotherapy. Accordingly, it is clear that the development of less toxic and more efficient treatment strategies are urgently needed to improve the clinical management and prognosis of pancreatic cancer patients.

Considering their easy availability, convenient purification, storage techniques and possible positive outcomes in various in vitro and in vivo models, peptides have various applications in cancer diagnosis, prognosis and therapeutics [103]. Recently, synthetic C1B subdomain peptides of protein kinase C (PKC) γ isozymes, C1B1 and C1B5 [104], are demonstrated to be effective in the attenuation of the growth of human colon carcinoma cells in both cell culture and mouse study without any recognizable side effects [86]. The primary mechanism by which these peptides attenuate cancer growth is associated with an induction of apoptosis by stimulation of p53 phosphorylation and an attenuation of cell proliferation [86]. These peptides were also found to interact with PKC βI/βII isozymes and stimulate apoptosis in carcinoma cells. With evidence for the ability of C1B peptide alone to increase apoptosis of cancer cells [86], C1B peptides could enhance other chemotherapeutic agents, such as gemcitabine. Since the C1B1 or C1B5 peptide appears to act as decoys and interact with multiple C1B domain-associated signaling proteins, thus affecting cell proliferation and apoptosis through PKC-dependent manner, it is of interest to study the effect of a combination treatment with these mild anti-cancer peptides and gemcitabine in pancreatic cancer therapy[86].

In the present study, we sought to determine the effects of C1B5 peptide and gemcitabine, either alone or in combination on pancreatic cancer cell proliferation, tumor spheroid growth and *in vivo* tumor growth in PAN02 murine pancreatic carcinoma cell line.

PAN02 cells are derived from pancreatic ductal adenocarcinoma in C57BL/6 mice and widely used as a model cells for the human pancreatic ductal adenocarcinoma. Data shown here demonstrate remarkable anti-cancer effect of low dose of gemcitabine in the presence of C1B5 on the tumor growth of the PAN02 cells in the peritoneal cavity. Furthermore, data also indicate that the combination treatment with C1B5 and gemcitabine showed negligible side effects, such as weight loss and rough hairs in mice, which are commonly observed by the effective dose of gemcitabine alone treatment. Therefore, the present study suggests that the combination treatment with the C1B peptide and gemcitabine could be applied in therapy of patients with pancreatic cancer that may significantly improve the efficacy of treatment and reduce toxicity to normal tissues.

Materials and Methods

Materials

C1B5 (PKC residues 141–151, RCVRSVPSLCG) peptide was synthesized by the University of Ioannina Chemistry department (University of Ioannina, Ioannina, Greece). Gemcitabine was purchased from Sigma (St. Louis, MO). Antibody against Granzyme B was purchased from Spring Bioscience (Pleasanton, CA). Biotin-conjugated anti-rabbit IgG antibody was from Vector Laboratories (Burlingame, CA). RPMI 1640 and high glucose Dulbecco's Modified Eagle Medium (DMEM) medium was obtained from Mediatech, Inc. (Herndon, VA). Fetal bovine serum (FBS) was from EQUITECH-BIO Inc. (Kerrville, TX). Penicillin/streptomycin and trypsin-EDTA were from Invitrogen (Grand Island, NY). MTT reagent [tetrazolium compound; inner salt] was from Promega (Madison, WI).

Cell lines and cell culture

Murine pancreatic ductal adenocarcinoma cell line, PAN02, and human pancreatic ductal adenocarcinoma cell line, PANC-1 were purchased from American Type Culture Collection (Manassas, VA). PAN02 cells were cultured with RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. PANC-1 cells were cultured with DMEM medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. These cells were cultured in 5% CO_2 humidified air at 37°C.

Effect of C1B5 and gemcitabine on PANO2 and PANC-1 cell growth in vitro in a two dimensional (2D) cell culture model

To test the combination effect of C1B5 peptide and gemcitabine, PAN02 cells were seeded at 0.5 x 10^3 cells per well in 96-well plates in 10% FBS-containing RPMI 1640 culture medium. Cells were allowed to attach to well plate for 24 hours and all the wells were divided into four different treatment groups. Culture medium was aspirated, medium containing 1μM C1B5 peptide and 20nM gemcitabine was added to one of the group. Either C1B5 or gemcitabine containing medium was added to two of groups and fresh medium containing wells were used as controls. After 48hrs of medium change, MTT cell proliferation assay was performed to evaluate the effect of treatment as described previously [7]. Similar protocol was used to evaluate the combination effect of 1μM C1B5 peptide and 50nM gemcitabine on PANC-1 cells with a cell seeding density of 3 x 10^3 cells per well.

Three dimensional (3D) tumor spheroid assay

Fifty μl of 1.5% soft agar in 1% medium was gently dropped into each well of a 96-well plate and allowed to solidify at room temperature for 10 min. Either 2 x 10^3 PANC-1 or 0.5 x 10^3

PANO2 cells mixed in 200 µl of 1x growth medium are placed on top of the agar layer. The cells were incubated for 4 days in 5% CO2 humidified air at 37°C. On day 4, C1B5 (1µM) and/or gemcitabine (20nM for PANO2, 50nM for PANC-1) in 10 µl of 1x growth medium was added to the newly formed tumor spheroids. Spheroid images were acquired on day 6, 8, 10 and 12 by Olympus 1X51 inverted microscope equipped with FV11 camera and cellSens analysis software. Area of the tumor spheroids was analyzed using Olympus cellSens software and day 12 to 6 spheroid ratio (relative increase in spheroid area) was compared between groups.

Animals

Five to six week-old wild-type female C57BL/6 mice were purchased from Charles River Laboratories International, Inc. All mice were housed in a clean animal facility and maintained for 10 days to acclimatize. All *in vivo* animal experiments were conducted and carried out under strict adherence to the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC) protocols set by Kansas State University (Manhattan, KS).

C1B5 and Gemcitabine combination effect on intraperitoneal PANO2 tumor growth

PAN02, murine pancreatic adenocarcinoma cells ($2.5x10^5$ cells in 200µl PBS) were intraperitoneally injected into 18 C57BL/6 mice (Charles River Laboratories, Inc. Wilmington, MA). On day-5 after cancer cell inoculation, all the mice were divided into four groups (Figure 2.3); Group 1- PBS control (4 mice), Group 2- C1B5 peptide alone (5 mice), Group 3- gemcitabine alone (4 mice), Group 4- C1B5 and gemcitabine combination treatment (5 mice). C1B5 (20mg/kg body weight) was injected intraperitoneally to group 2 and 4 on day 5,7,11 and 13. Gemcitabine (15mg/kg body weight) was injected intraperitoneally to group 3 and 4 on day-5, 8, 11 and 14. At the same time PBS was injected to group 1 (control). On day-17, all the mice

were sacrificed by CO_2 inhalation followed by cervical dislocation. Tumor nodules (milky spot), mesentry were collected and weighed. All the tumor tissues were fixed in 10% buffered neutral formalin. Formalin fixed tissues were further embedded in paraffin for histological examination. Four µm thick sections were prepared and stained with hematoxylin and eosin (H&E) for histological analysis. Tumor nodules were counted from above prepared sections, five 4x magnified fields from each section. Total tumor area was estimated from the same H&E slides using Olympus cellSens software and compared between groups.

Effect of C1B5 peptide and gemcitabine combination treatment on leucocytes present in peritoneal ascites fluid

Peritoneal ascites fluid was collected from above treated mice on day-17 before euthanasia. Briefly, 10mL of sterile PBS was injected intraperitoneally through catheter to all of the mice. After injection, gently massaged on the peritoneum so that injected PBS distributes inside peritoneal cavity. Through the same catheter peritoneal fluid was collected and centrifuged at 1240rpm for 5min to pellet out cells. Red blood cells (RBCs) were lysed by using ACK lysing buffer (Lonza, Walkersville, MD) and the remaining cells were washed with PBS twice. Total cell number was estimated by using Hemacytometer. Differential counts were conducted on these same cells after spreading on slides followed by air-drying, ice cold methanol fixation and Giemsa (Thermo Scientific, Waltham, MD) staining. Total ten 40x magnified fields from each sample were counted and percentage of cells were estimated based on their histological features.

Granzyme B immunocytochemistry on peritoneal ascites fluid cells

Above collected peritoneal ascites fluid cells were further analyzed for the presence of Granzyme B positive cells by using immunocytochemistry. Briefly cells were air dried and fixed with ice-cold methanol. Fixed cells were blocked with 10% non-fat dry milk containing PBS for 20mins, followed by 1hr incubation with Rabbit anti-Granzyme B antibody. Slides were further incubated with Biotinylated anti rabbit secondary antibody after three PBS rinses. Granzyme B positive cells were visualized by using Vector labs elite ABC kit following manufacturer's protocol.

Statistical analysis

Data were analyzed using Stat View software, version 5.0.1. (JMP, Cary, NC). All the experiments were planned and performed with multiple sample (n) determinations. A p-value less than 0.05 was considered significant for all the experiments. All the *in vitro* and *in vivo* (tumor size) data values were expressed as the mean ± standard error (SE) on graphs. A statistical evaluation was carried out by ANOVA using Fisher's protected least significance difference (PLSD) student t-test as a *post-hoc* analysis to compare and determine the significance of overall differences in tumor size between two data sets.

Results

C1B5 peptide in combination with gemcitabine effect on PANO2 and PANC-1 cells in 2D cell culture

To evaluate the direct combination effect of C1B5 and gemcitabine on pancreatic cancer cells, mouse (PAN02) and human (PANC-1) pancreatic tumor cells were co-treated with C1B5

and a low-dose gemcitabine in 2D cell culture. Cell proliferation was estimated by using MTT assay 48hrs after the treatment. C1B5 alone and gemcitabine alone treatment slightly decreased cell growth in PANO2 cell line (Figure 2.2B). Gemcitabine alone slightly decreased cell growth in PANC-1 cell line (Figure 2.2A). Combination treatment with C1B5 and gemcitabine further decreased the growth of PANC-1 and PAN02 cells (Figure 2.2). In the 2D cell culture, the inhibitory effect of combination treatment by C1B5 and gemcitabine was small and not statistically significant, suggesting combination treatment may not have any direct effect on PANO2 and PANC1 cell proliferation.

C1B5 peptide enhanced inhibitory effect of gemcitabine on PANO2 and PANC-1 spheroid growth

To test combination treatment effect, PANO2 and PANC-1 cells were cultured as spheroids. C1B5 peptide and gemcitabine combined effect was evaluated by analyzing change in spheroid growth after treatment. Gemcitabine alone and C1B5 peptide alone decreased PANO2 and PANC-1 spheroids growth (Figure2.2). Decrease in spheroids growth was much higher in C1B5 gemcitabine combination treatment compared to C1B5 peptide alone or gemcitabine alone for PANO2 cells (Figure 2.2B). For the PANC1 cells combination treatment effect was not as prominent as PANO2 cells (Figure 2.2).

C1B5 peptide and low dose of gemcitabine combination treatment markedly attenuated PANO2 tumor growth

To test C1B5 and gemcitabine combination effect on pancreatic cancer growth, intraperitoneal PANO2 pancreatic tumor containing mice were treated with C1B5 peptide and low dose of gemcitabine. Tumor growth was analyzed by calculating the tumor weight

containing milky spot tumor nodules and the relative area of mesenteric tumor compared to control PBS group. C1B5 peptide treatment alone (Figure 2.5, not statistically significant) and gemcitabine treatment alone (Figure 2.5, statistically significant –$p<0.05$) showed decrease in tumor growth. But C1B5 in combination with gemcitabine treated mice showed much higher decrease in tumor growth when compared to C1B5 alone or gemcitabine alone (Figure 2.5, p-value <0.001). These results suggesting, that C1B5 peptide has shown to be effective on PANO2 pancreatic tumor growth, when co-treated with low dose of gemcitabine. Representative H&E stained mesentry images are presented in Figure 2.6.

C1B5 alone or in combination with gemcitabine caused increase in infiltrated neutrophils

To find immune cells involvement in observed tumor growth decrease, leucocytes in peritoneal ascites fluid (represents tumor infiltrated leukocytes in the present tumor model) were analyzed. Although, gemcitabine alone slightly increased total number of peritoneal leucocytes there is no significant difference in total number of peritoneal leucocytes between any of the treated groups (Figure 2.7A). But, C1B5 alone or in combination with gemcitabine substantially increased infiltrated neutrophils percentage suggesting that C1B5 might be potentiating tumor associated pro-inflammatory response (Figure 2.7B).

Granzyme B positive cells in the ascites fluid might be involved in the observed anti-tumor effect

Certain types of tumor-associated neutrophils were known to show antitumor activity through recruiting and/or activating CD8+ T-cells [105]. To find the involvement of activated CD8+ T-cells or natural killer cells, ascites fluid cells were stained for Granzyme B (Figure 2.8).

Percentage of Granzyme B positive cells in total peritoneal ascites cells were compared between groups and plotted in Figure 2.8B. The percentage of Granzyme B+ cells in peritoneal fluid was significantly increased in C1B5 treated and C1B5 and gemcitabine treated mice compared to remaining groups. Although, gemcitabine alone treated group showed increase in Granzyme B+ positive cells, it is not statistically significant. Further, combination treatment has substantially higher percentage of Granzyme B+ cells compared to C1B5 or gemcitabine treatment alone.

Discussion

Although gemcitabine is a commercially available strong chemotherapeutic agent for many types of cancer including pancreatic cancer [106], which inhibit DNA replication, it is also known for its severe side effects. Peptide-based anti-cancer vaccines have been considered to be a promising therapeutic option eliciting specific anti-tumor responses in pancreatic cancer models[48]. In recent years, some unique solutions have been proposed, such as modified Tumor-associated antigens (TAAs) are a novel class of peptide vaccines, which are recognized by the immune system resulting in the tumor growth inhibition in combination with chemotherapy [46]. One such specific combination phase I study of Wilms tumor gene (WT1) peptide-based vaccine with gemcitabine was found to be more effective than chemotherapy alone. The median survival rate of the combination therapy was 8.1 months[107]. WT1 peptide-based vaccine induced specific cytotoxic T lymphocytes (CTLs) against pancreatic cancer cells overexpressing WT1. Through the expression of perforins, granzymes the tumor cells were efficiently eliminated[108]. The side effects of combination therapy were similar to those of gemcitabine alone except for topical skin reactions[107]. In similar combination studies, a KIF20A-derived peptide in combination with gem increased the number of peptide-specific IFN-

γ producing cells[109]. The combination treatment of a telomerase (GV1001) vaccine and gem was found to be safe, however, with a weak transient immune response[110]. In another study, a bio-active peptide ACBP-L combined with a lower dose of Cisplatin, significantly improved host quality of life (QOL) and the efficacy of the treatment[111]**.**

Currently available treatments for pancreatic cancer have high degree of side effects apart from variability in their therapeutic effect [88, 89]. To overcome these problems, a novel targeted treatment strategy with low-dose gemcitabine that can elicit multiple anti-tumor responses is urgently needed to treat pancreatic cancer. Our previous study suggested that peptides derived from regulatory domain of PKCγ protein (C1B1, C1B5) attenuates human colon carcinoma growth in xenograft mouse model [36], which induced cell cycle arrest and apoptosis via P53 pathway [36]. Effect of these peptides on immune cells is currently unknown. But, it is highly probable that these peptides can also modulate immune responses by interacting with PKC associated proteins in immune cells. If combination treatment with low-dose gemcitabine and C1B peptide showed enhanced anti-tumor effect via different signaling pathways [86, 112, 113], it will be an effective cancer therapeutic. To confirm our hypothesis, combination effect of low-dose gemcitabine and C1B5 peptide on the growth of pancreatic cancer was evaluated *in vitro* and *in vivo*.

In the first experiment, combination effect of low-dose gemcitabine and C1B5 on the growth of human and mouse pancreatic cancer cell line was evaluated using 2D and 3D cell culture assay. *In vitro* evaluation of this combination with 3D tumor spheroid assay clearly showed that C1B5 peptide with gemcitabine could effectively decrease PANO2 spheroid growth (Figure 2.3B). It is previously known that cells in tumor spheroid assay have more features that are similar to *in vivo* tumor cells than the usual 2D culture system [114, 115]. Although, the

combination of C1B5 and gemcitabine didn't show the same effect on 2D *in vitro* culture model (Figure 2.3A), observations from spheroid assay might be more representative of the *in vivo* situation [115]. PKCγ is known to interact with proteins involved in cell migration, cell-cell and cell-ECM contacts [116, 117]. It might be possible that C1B5 peptide mediated increased effect observed in 3D models is by direct interaction of this peptide with above-mentioned proteins.

To assess the *in vivo* effect of proposed combination, we used intraperitoneal metastatic pancreatic cancer model. Gemcitabine alone and in combination with C1B5 peptide significantly attenuated PANO2 pancreatic tumor growth *in vivo* (Figure 2.5 & 2.6). The combination treatment has increased anti-tumor effect compared to gemcitabine alone (Figure 2.5 & 2.6). As shown in 3D cell culture, it is suggest that this enhanced effect may be occurred by C1B5 effect on cell-cell interaction. As another mechanisms of tumor attenuating effects by this combination therapy, it is expected to the involvement of immune cells attracted by the gemcitabine treatment. To find immune system effects of these treatments, we evaluated the immune cells composition in intraperitoneal ascites fluid. The combination and C1B5 alone treatment increased number of immune cells in ascites compared to control group, while it was lower than gemcitabine alone treatment. However, C1B5 peptide alone and in combination with gemcitabine significantly increased neutrophil percentage in total peritoneal cells in tumor containing mice (Figure 2.7B). Suggesting, C1B5 peptide might be causing pro-inflammatory response in these mice. It is possible that this response might be tumor associated pro-inflammatory response than direct peptide induced immune response; more studies are needed to confirm this. The ability of neutrophils to influence CD8+ T-cells has been studied in cancer [118, 119]. Neutrophils were known to have both pro-tumorigenic (N2 type) and anti-tumorigenic (N1 type) character[120]. N1 type of pro-inflammatory neutrophils can increase the recruitment and activation of CD8+ T-

cells [120, 121]. Conversely, pro-tumorigenic N2 type neutrophils can inhibit T-cell mediated functions [120].

As mentioned above, neutrophil can is found to induce the activation of CD8+ T cells. To evaluate involvement of this immune response in our combination treatment, ascitic cells collected from tumor bearing mice were analyzed by immunocytochemistry using anti-granzyme B antibody. Granzyme B is a serine protease found in the granules of cytotoxic CD8 T cells (CTLs) and natural killer cells (NK cells). It is secreted by cytotoxic lymphocytes along with the pore forming protein perforin to mediate apoptosis in target cells through caspase 3 cascade [122]. To further identify the immune system mediated mechanism in the observed treatment effect; ascitic fluid cells were analyzed for Granzyme B expression. Granzyme B positive cells were significantly increased in combination treatment mice (Figure 2.8). Gemcitabine is known to increase circulating lymphocytes in treated animals [123]. In conjunction, the strong effect observed in combination treatment might be because of increase in lymphocyte recruitment by gemcitabine followed by C1B5 peptide mediated CD8+ T-cells or NK cells activation apart from direct cancer cell apoptosis. This study is among the first to suggest that C1B5 might be involved in antitumor immune response. Further studies are required to confirm its role in immune modulation.

In conclusion, this study demonstrates that C1B5 in addition with low dose concentrations of gemcitabine significantly attenuate growth of human and murine pancreatic cancer cells in 3D cell culture compared to C1B5 or gemcitabine single treatment. C1B5 peptide modulates the host immune response by increased neutrophil population in the peritoneum. Tumor growth is significantly regressed in the combination group by activated cytotoxic lymphocytes expressing granzyme B. The absence of such effect in C1B5 or gemcitabine alone

1 treatment groups provides the underlying inter-relation between neutrophils and lymphocyte
2 activation. The correlation of these two mechanisms showed anti-tumor effect of combination
3 therapy with a low-dose gemcitabine and C1B5 peptide.
4
5
6

Figures

Figure 2.1 Chemical structure of gemcitabine

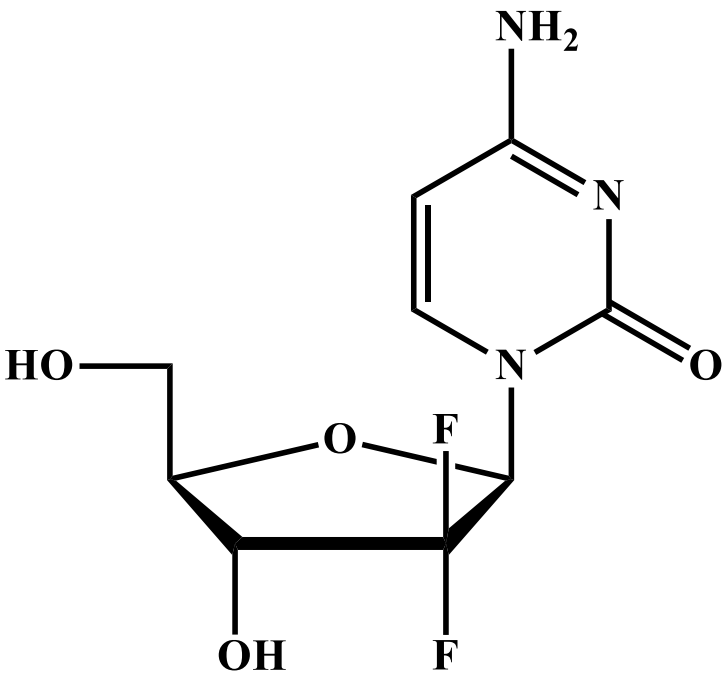


Figure 2.2 Combination treatment with C1B5 and gemcitabine didn't show any effect on PANC-1 human and PAN02 mouse pancreatic cancer cell growth in 2D cell culture system

C1B5 alone or Gemcitabine alone or combination of C1B5 and Gemcitabine didn't show any significant decrease in PANC-1 cell growth in 2D culture system (A). Similar results were observed with PANO2 cell line (B)

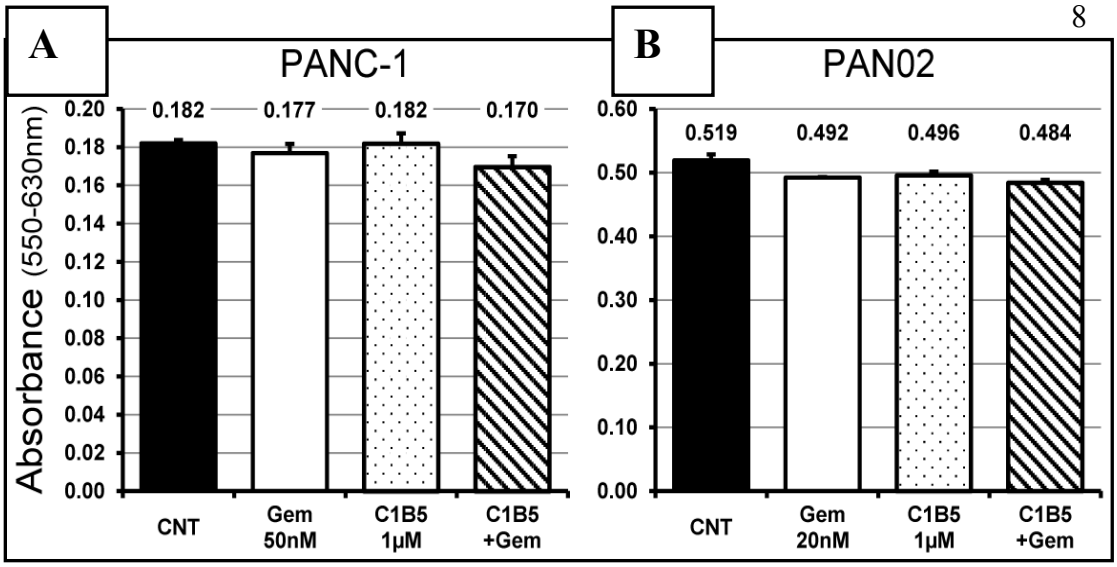


Figure 2.3 Combination treatment with C1B5 and gemcitabine decreased the PANC-1 human and PAN02 mouse pancreatic tumor spheroid growth in 3D cell spheroid assay

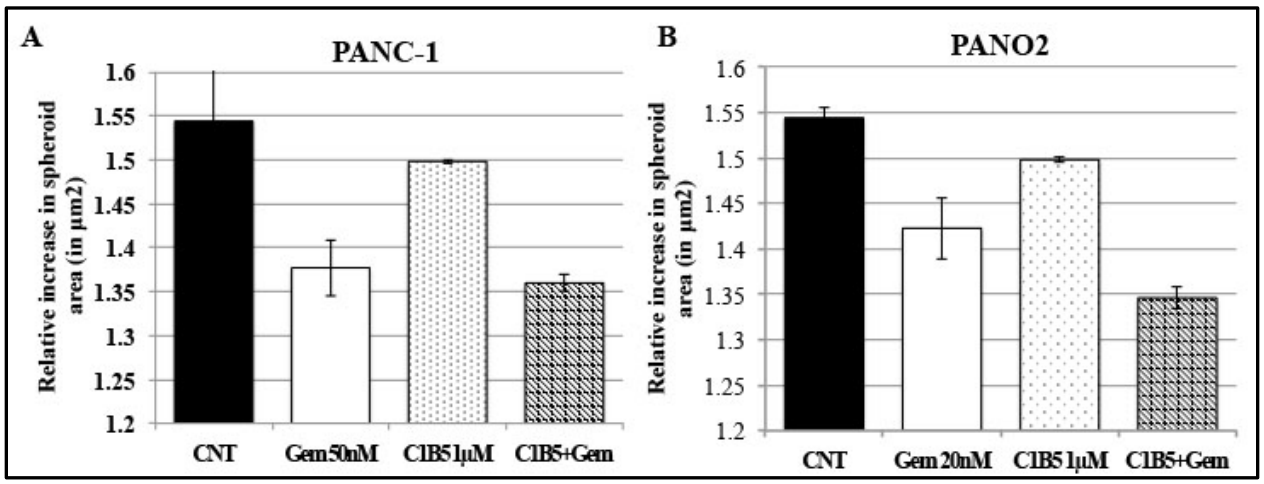


Figure 2.4 C1B5 and Gemcitabine combination effect on intraperitoneal PANO2 tumor growth experimental design schematic illustration.

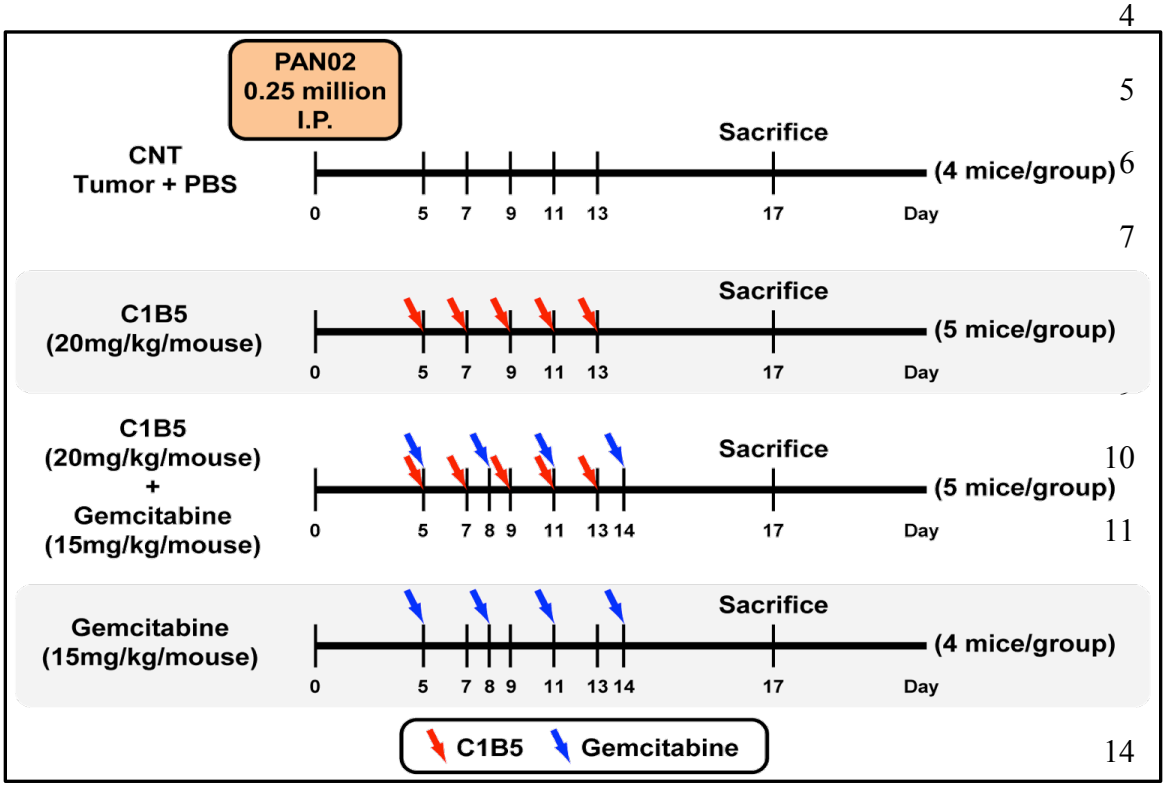


Figure 2.5 C1B5 and Gemcitabine combination treatment effect on intraperitoneal PANO2 tumor growth

C1B5 and Gemcitabine combination treatment effect was analyzed by comparing tumor weight (A), number of tumor nodules (B) and relative tumor area (C) between treated groups. Although gemcitabine alone showed significant antitumor activity, combination treatment with C1B5 effectively decreased the tumor growth (*-<0.05, **-0.01, ***-0.001 p-value)

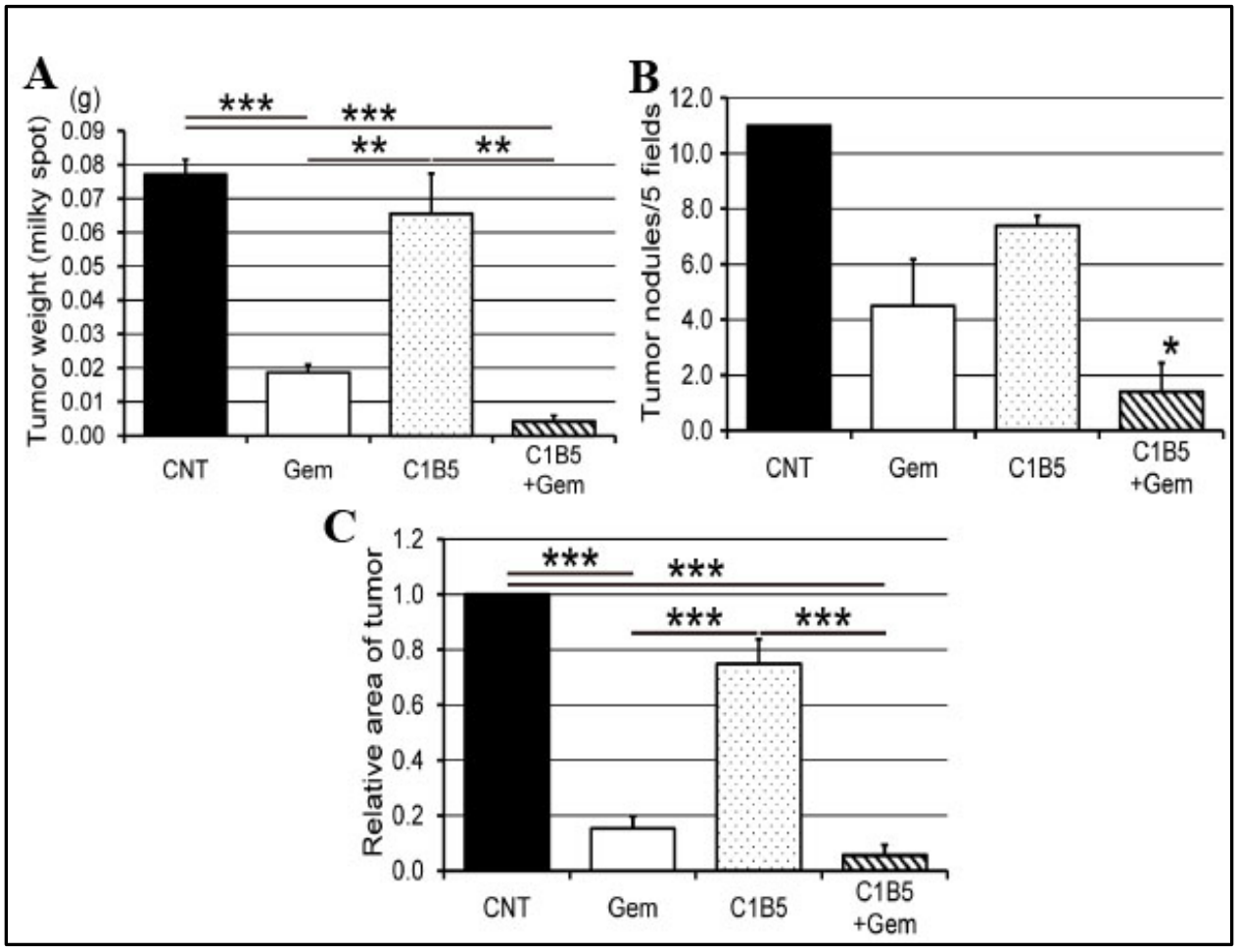


Figure 2.6 Histological analysis of PANO2 microtumors in C57BL/6 mouse peritoneal model

PANO2 pancreatic ductal adenocarcinoma allografts were treated with C1B5 and/or gemcitabine four-five times with two days interval as described in the Methods section. Four days after the last treatment, peritoneal tumors were dissected and fixed in 10% formalin. Paraffin embedded tissue sections were then stained by H&E staining. Morphologies of peritoneal pancreatic tumors in four different treatments are presented in the panels below.

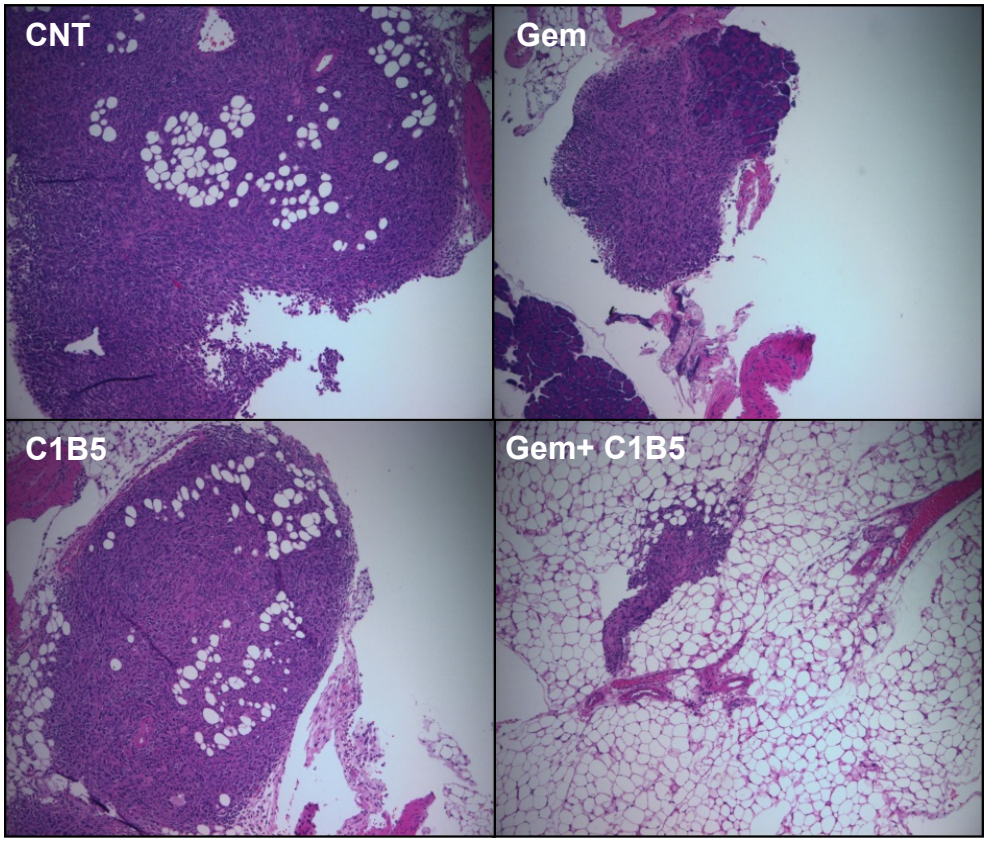


Figure 2.7 C1B5 alone or in combination with Gemcitabine treatment caused increase in neutrophil population in peritoneal ascites fluid

Total leukocytes infiltrated into peritoneal cavity (represents tumor associated leucocytes) were not different between treatment groups (A), But the percentage of neutrophils in total collected ascites cells were significantly increased in C1B5 alone and C1B5 & gemcitabine treated groups (*p-value <0.05). Suggesting, C1B5 peptide might be increasing the tumor associated inflammatory response.

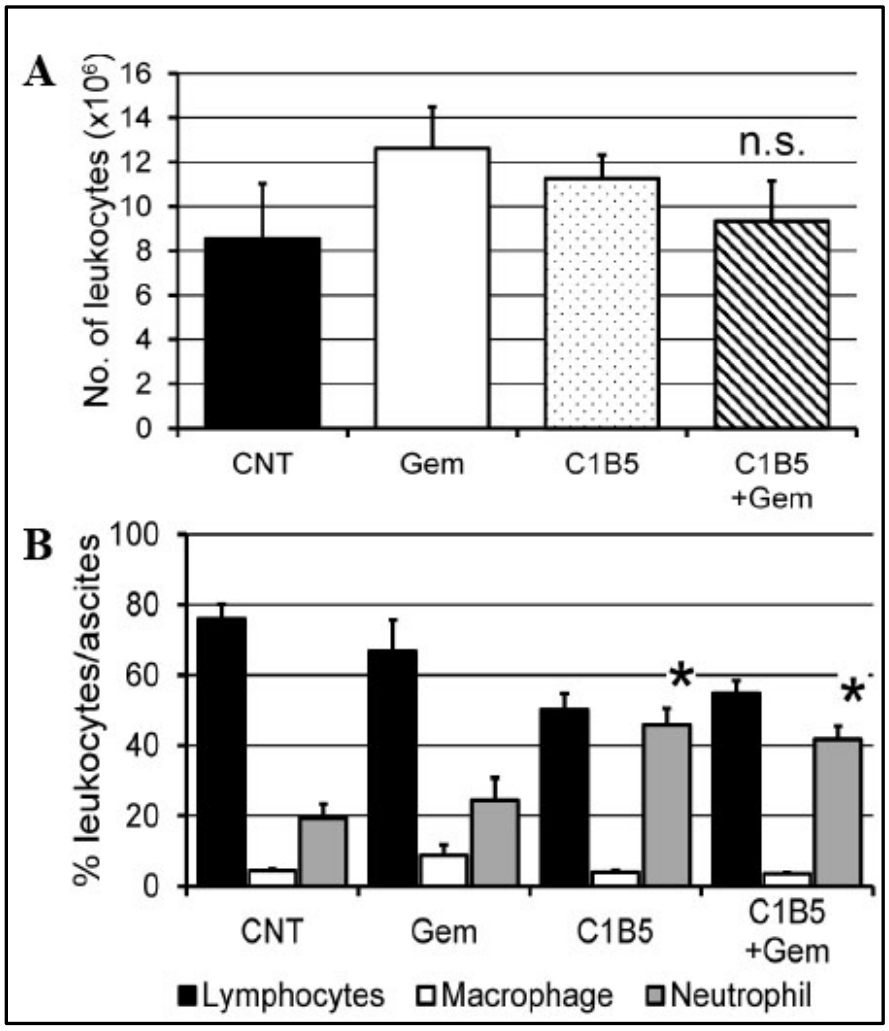


Figure 2.8 Granzyme B immunocytochemistry on peritoneal fluid cells.

Representative images of PBS (A) and C1B5 and Gemcitabine treated (B), Granzyme B antibody stained ascites fluid cells of PANO2 tumor bearing mice. Granzyme B positive cells were marked with arrow. C. Granzyme B positive cells percentage comparison between different treatment groups. C1B5 treatment alone or in combination with Gemcitabine substantially increased tumor infiltrating Granzyme B positive cells (*- <0.05, **-<0.01 p-value)

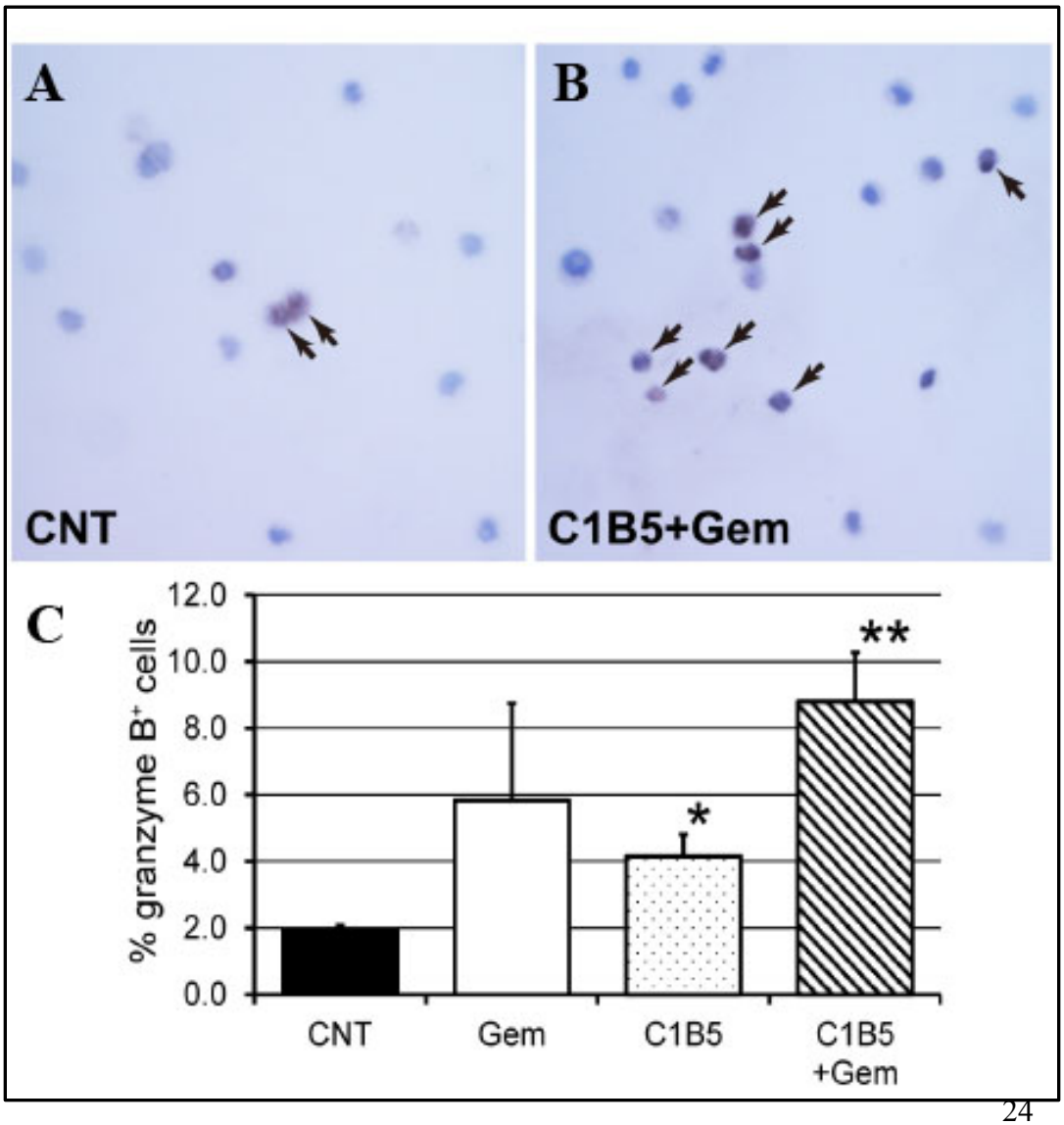
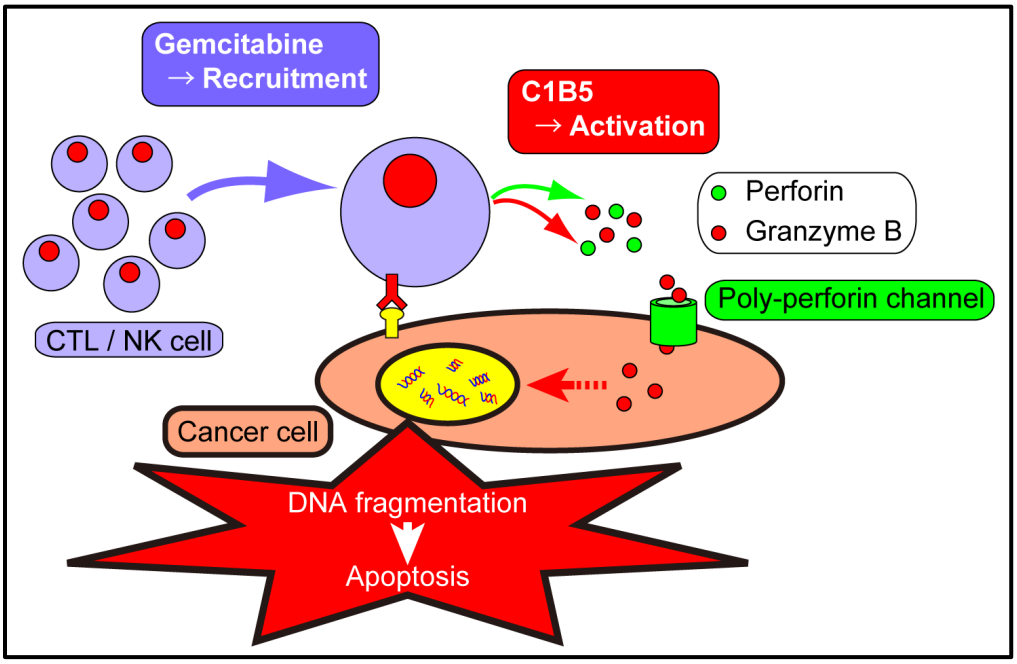


Figure 2.9 Schematic illustration of the proposed mechanism of anti-tumor action involved in C1B5 and gemcitabine combination treatment.



Chapter 3 - Intravenous and intratracheal administration of a nanoparticle-based therapy (dTAT/pDNA) with the angiotensin II type 2 receptor gene attenuates lung cancer growth.

Abstract

Targeted delivery, high transfection efficiency and low toxicity are important challenges in conventional cancer treatments. Recent research by Kawabata *et al.* [85], has highlighted many advantages of intratracheally administered HIV-1 TAT peptide formulated nanoparticle vector (dTAT/pDNA/Ca^{2+}) as a local therapeutic approach with high gene transfection efficiency and low toxicity. In this study, we examined the new dTAT/pDNA/Ca^{2+} formulation to leverage the efficiency for tumor-targeted gene delivery in the setting of intravenous (IV) or/and intratracheal (IT) administration using mouse bronchioloalveolar carcinoma model. Addition of a small amount of albumin (2mg/ml) to the original dTAT/pDNA/Ca^{2+} formulation increased the nanoparticle stability in serum containing cell culture medium without losing gene transfection efficiency. In orthotropic tumor allograft models in immunocompetent mice, a single IV administration of new dTAT/pDNA/Ca^{2+} encapsulating plasmid DNA such as the pTRAIL, pAT2R or microRNA, pmiR34a, attenuated murine Lewis lung carcinoma (LLC) tumor growth without showing any significant adverse effects on mouse health conditions. Therefore the new dTAT/pDNA/Ca^{2+} formulation is suited for IV treatment. Further, a single or co-treatment of IV or/and IT administration of the dTAT/pDNA/Ca^{2+} containing pAT2R attenuated human H358 xenograft growth by two-fold in the lung confirming that dTAT/pAT2R/Ca^{2+} gene therapy is effective in inhibition of developed human lung bronchioloalveolar carcinomas in SCID mice. Taken together, these mouse studies indicate that our newly developed dTAT/pDNA/Ca^{2+} formulation may be applicable as a systemic formulation for the treatment of lung cancer.

Introduction

Lung cancer remains the third leading cause of cancer-related morbidity and mortality in the United States representing the largest annual financial burden of any cancer type (ACS, 2015). The American Cancer Society estimates 221,200 new lung cancer incidence cases in the United States with 158,040 expected deaths in 2015[1]. Cancers of the lung and bronchus, prostate, breast and colorectum account for almost half of the total cancer deaths among men and women, with more than one-quarter of all cancer deaths due to lung cancer [1]. Although its prognosis has improved due to latest advances in diagnostic and surgical techniques and early surveillance, however currently available therapies have had minimal impact in reducing lung cancer related mortality or survival rate. From 2004 to 2010, the relative 5-year survival rate of patients with lung and bronchus cancer was still quite low (18%), with minimal improvement since the 1970's (12%) [1]. Therefore, more robust treatment strategies for lung cancer are needed.

With the latest advancements in gene delivery strategies, gene therapy is found to be a promising approach for cancer treatment by expressing the desired genes in the defective cells[59, 124]. The vector carrying the desired gene has an important role in the gene therapy system. Several viral vectors were studied and found to be potentially pathogenic causing viral infections[69]. Therefore, non-viral vectors may be a safer alternative to viral vectors. One such non-viral system is the cationic-based (lipids:lipoplexes and polymers:polyplexes) nanoparticle gene delivery system[125]. Considering their ease of synthesis, cost-affectivity, specific cell targeting approach and low degree of immunogenicity they have gained increasing attention.. The naked plasmid DNA (pDNA) easily forms polyelectrolyte complexes with these cationic polymers through condensing pDNA into nanoparticles, protecting pDNA from enzymatic

degradation, and facilitating and improving the cellular uptake and mediate desired gene transfection via endocytosis[126]..

Polyethylenimine (PEI) and chitosan are widely used cationic, non-viral, polymer vectors in gene delivery systems. PEI is considered to be the gold standard, however; its high molecular weight related dose dependent cytotoxicity has discouraged its use in human trials[127-129]. On the other hand, chitosan is a well-known biodegradable non-toxic cationic polymer with less effective gene delivery ability[130]. Therefore, to investigate a complex that has high gene transfection efficiency, low cytotoxicity and maximum desired gene transfection stability would be promising[131]. The HIV-1 TAT peptide is one such cationic polymer widely used as a non-viral vector for therapeutic gene delivery. They TAT peptide representing a HIV-I protein transduction domain and a nuclear localization sequence[132, 133]. Owing to its unusual translocation properties it directly, freely crosses the biological cell membranes independent of receptors and temperature [77] and the TAT/pDNA complexes escape into the cytosol and are subsequently transported into the nucleus (endocytosis). The addition of calcium chloride ($CaCl_2$) to TAT/pDNA complexes produced more stable TAT-Ca/pDNA nanoparticle complexes even in the presence of 10% serum[84]. Kawabata et al., investigated and evaluated the effeciency of a dimerized TAT peptide (dTAT) complexed with pDNA and condensed with calcium chloride (dTAT/pDNA/Ca^{2+} complex) for a tumor-targeted gene delivery in the intratracheal administration setting [85]. A single intratracheal administration (treatment) of dTAT/pDNA/Ca^{2+} complex encapsulating a therapeutic gene caused strong gene expression preferentially in tumor cells significantly attenuated the growth of fast growing Lewis lung carcinoma tumors *in vitro* and *in vivo[85]*. These benefits have made therapeutic dTAT

nanoparticles a promising candidate to investigate in different drug administration routes like intravenous alone or together with intra tracheal administration.

Angiotensin II (Ang II), an octapeptide hormone, is an important component of the local renin-angiotensin system (RAS) [134]. Ang II type 1 (AT1R) and type 2 (AT2R) are the well-defined receptors of Ang II [135]. AT2R, a seven transmembrane G-protein-coupled receptor highly expressed in the fetal tissues and to a lesser extent in adult tissues like ovarian follicule atresia[136-138]. It is studied for its functional role related to cell growth inhibition, apoptosis and repair of cardiovascular and neuronal tissues *in vitro[139, 140]*.

In the context of its role in cancer malignancy, our previous research studies revealed that AT2R deficiency significantly altered chemical carcinogen-induced tumorigenesis in mouse colon [141] and lung [142]. Murine pancreatic carcinoma grafts implanted into *ATR2*-knockout mice demonstrate faster tumor growth related to high VEGF production in stromal cells[143]. In a prostate cancer model, recombinant adenoviral mediated AT_2R overexpression in cancer cells induced apoptosis[139]. This was found to be independent of AngII, mainly occurring through p38 MAPK, caspase 8 and caspase 3 pathways. It was found to be partially dependent on p53 but not p21 activation[139]. In a similar experiment, AT_2R overexpression in A549, a human lung adenocarcinoma cell line also inhibited the cell growth and increased apoptosis associated with a decrease in procaspase 3 levels[140]. In both these studies apoptosis was mediated by AT2R through an extrinsic cell death-signaling pathway. Further, administration of a selective AT2R agonist caused apoptosis of AT2R expressing PAN02 murine PDAC tumor cells in a syngeneic mice model[144]. Taken together, targeting AT2R might prove to be an effective therapeutic target for cancer treatment.

TNF-related apoptosis inducing ligand (TRAIL) is a tumor necrosis factor (TNF) family member that is known to induce apoptosis by binding as a homotrimer to Death Receptor (DR)-4 or -5 and recruiting Fas-Associated protein with Death Domain (FADD), an adaptor receptor or caspase-8, activating extrinsic apoptotic pathway[145]. The specificity of TRAIL to induce apoptosis only in transformed or tumorigenic cells but not normal cells and its expression in many tissue types, including spleen, thymus, colon, ovary, prostate, small intestine, placenta and peripheral blood lymphocytes has made TRAIL a strong tumor therapeutic gene[146]. In the last decade, preclinical and clinical evidence of recombinant human TRAIL (rhTRAIL) and DR4/DR5 agonistic MAbs as cancer therapeutics bought them to phase I-II trials analyzing their therapeutic efficacy, pharmacological properties and safety as single agents or in combination with cancer chemo therapeutics[147-149]. Several gene delivery vectors including nanoparticles have been well studied to deliver TRAIL to the tumor[150-153]. TRAIL gene transfer into tumor has effectively inhibited the tumor growth without any major side effects[152-155], even in a liver metastases model[156]. Retroviral-mediated TRAIL gene transfer in the bone marrow was shown to regress the tumor growth[157]. The efficiency of lentiviral-mediated TRAIL gene transfer *in vivo* has been found to be lower compared to *ex-vivo* studies[153]. The combination of TRAIL gene therapy with antitumor agents like HDAC inhibitors enhanced the anti-tumor effect of the treatment[158].

From the first study reported by Calin in 2002 microRNAs are extensively researched for their role in human cancer[159]. miR34 family consists of miR34a, miR34b, and miR34c members which act as significant downstream transcriptional targets of onco-suppressor p53 gene function in normal and cancer cells [160]. miR34 family, thus, contributes to p53 downstream effects like cell proliferation arrest and induction of apoptosis, by targeting c-MYC,

CDK6, and c-MET signaling pathways [161]. Low expression of miR34a in chronic lymphocytic leukemia (CLL) is found to be not only associated with p53 inactivation but also impaired DNA damage. In contrast, up-regulation of miR34a after irradiation is associated with induction of Bax and p21 but not p53 upregulated modulator of apoptosis (Puma) [162]. Phase I clinical study of MRX34 using liposome based miR34a delivery is ongoing, bringing the first miRNA to human clinical trials [163].

In the present study, we have tested the modified TAT peptide by connecting two TAT peptides in tandem (dTAT). Here we show that dTAT NP incorporating AT2R, TRAIL or miR-34a pDNA (dTAT/pAT2R, dTAT/TRAIL or dTAT/miR-34a) administered *via* intravenously and/or intratracheally attenuated the growth of lung carcinoma grafts in mice models. Therefore, dTAT NP is a realiable gene delivery system, and the dTAT and an apoptosis inducer therapeutic gene NP can be used as a powerful and less toxic therapeutic for lung cancer.

Materials & Methods

Materials

Plasmid DNA (pDNA) encoding firefly luciferase (pGL3, 4818 bp) was obtained from Promega (Madison, WI). Plasmid DNA (pDNA) encoding human AT2R (agtr2 pcDNA3.1+) was obtained from the UMR cDNA Resource Center (University of Missouri, Rolla, MO). The pDNA encoding human TRAIL (TRAIL pCMV-SPORT6) was purchased from Open Biosystems. dTAT (RKKRRQRRRHRRKKR; Mw = 2201.7 Da) peptide was purchased from Biomatik corp. Branched polyethylenimine (PEI, 25 kDa) was obtained from Sigma-Aldrich (Milwaukee, WI). RPMI-1640 were purchased through Mediatech, Inc (Manassas, VA).

Dulbecco's Modified Eagle's Medium (DMEM) was purchased through Invitrogen/Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Penicillin/Streptomycin was purchased from MB Biomedical, LLC (Solon, OH). Trypsin-EDTA, Tris-acetate-EDTA (TAE) buffer (10x) were purchased from Invitrogen (Carlsbad, CA). Luciferase Assay System Freezer Pack and CellTiter 96® AQ ueous one solution cell proliferation assay (MTS) were obtained from Promega (Madison, WI). BCA Protein Assay Reagent (bicinchoninic acid) was purchased from Thermo Fisher Scientific Inc. Sterile water (DNase, RNase free), Calcium chloride dihdrate ($CaCl_2$. $2H_2O$) were purchased from Fisher scientific. Mouse serum albumin (MSA) and glucose were obtained from Sigma-Aldrich (Milwaukee, WI).

Animals

All animal experiments were done under strict adherence with Kansas State University Institutional Animal Care and Use Committee protocols. Wild-type female C57BL/6 mice obtained from the Jackson Laboratory (Bar Harbor, Maine) were housed in a clean facility and held for 10 days to acclimatize. CB17/SCID mice were purchased from Charles River laboratories (Wilmington, MA) and housed in barrier rooms.

Cell lines and cell culture

LLC (Mouse lewis lung carcinoma; CRL-1642), H358 (human bronchioalveolar carcinoma; CRL-5807) cell lines were obtained from ATCC (Manassas, VA). LLC cells were cultured with high glucose DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. H358 cells were cultured in RPMI 1640 medium supplemented with 10%

FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. These cells were cultured in 5% CO_2 humidified air at 37°C.

dTAT/pDNA/Ca^{2+} nanoparticles - in vitro evaluation

dTAT/pGL3//Ca^{2+} nanoparticles preparation

dTAT/pGL3 nanoparticles (firefly luciferase plasmid nanoparticles) were prepared by adding 15 µL of dTAT solution (N/P-10, polymer nitrogen to pDNA phosphate ratio) to 10 µL (0.1 µg/µL) of pGL3 plasmid DNA (pDNA) in 1XTAE buffer followed by fast pipetting for 20 seconds. Different ratios of polymer nitrogen to pDNA phosphate were tested previously and N/P ratio 10 was used in current *in vitro* study. "Soft" calcium cross-links were known to enhance TAT peptide mediated transfection efficiency (Baoum, 2009). To find optimal Ca^{2+} concentration three different dTAT/pGL3/Ca^{2+} nanoparticles were prepared 15 µL of 0, 300, and 600 mM $CaCl_2$ was added to above prepared dTAT/pGL3 nanoparticles and mixed by fast pipetting. After preparing the nanoparticles, they were stored at 4°C for 20-25 minutes. Particle size (effective diameter in nm) and Zeta potential of dTAT/pGL3 nanoparticles with or without calcium chloride was determined by Zeta PALS dynamic light scattering apparatus (Brookhaven Instrument, Holtsville, NY). Samples intended for particle size measurements were prepared using nuclease Free Water or serum free culture medium. All samples intended for zeta potential measurements were prepared using KCL (1mM).

PEI/pGL3 nanoparticles preparation

PEI/pDNA nanoparticles were prepared by adding 15 µL of PEI solution (N/P ratio 10) to 10 µL (0.1 µg/µL) of pDNA followed by fast pipetting for 20 seconds. After preparing the

nanoparticles, they were stored at 4°C for 20-25 minutes. These nanoparticles were prepared immediately before each experiment.

Evaluation of dTAT/pGL3/Ca^{2+} nanoparticles transfection efficiency on LLC cancer cells

To determine the transfection efficiency of dTAT/pGL3/Ca^{2+} nanoparticles, 100,000 LLC cells/mL were seeded in gelatin coated 96-well plate, 24hrs prior to transfection. Wells were washed once with serum-free media (SFM) and 100µL of medium (20 µL of nanoparticle and 80 µL of SFM) was added to each well. After 5 hours incubation in humidified incubator at 37°C, nanoparticles solution was replaced with 100 µL of serum containing medium followed by further incubation for 48hrs. Luciferase expression level was measured by using Luciferase Reporter Assay (Promega) following manufacturer's protocol. Transfection efficiency was expressed as Relative Light Units (RLU) per mg of cellular protein. BCA Protein Assay Reagent (Bicinchoninic acid) was used to measure total cellular protein concentration in the cell extracts. pGL3 plasmid alone, PEI/pGL3 nanoparticle treated LLC cells were used as a controls. At the same time, LLC cells were treated with dTAT/pGL3/Ca^{2+} nanoparticles that were prepared with increasing concentrations of calcium and luciferase expression was estimated.

Assessment of dTAT, Calcium chloride, PEI mediated cytotoxicity on LLC cancer cells

Cytotoxicity of dTAT, PEI, and $CaCl_2$ was determined using a CellTiter 96® AQ ueous Non-Radioactive Cell Proliferation Assay (MTS) obtained from Promega (Madison, Wisconsin). LLC cells were seeded in a 96-well plate as described previously. After 24hrs, the medium was replaced with increasing concentrations of dTAT or Calcium chloride or PEI containing medium. Cells were further incubated for 24hrs and the media were replaced with a sample consisting of 100 µL of serum containing medium and 20 µL of MTS. Then, the plate was incubated for 3hrs in the incubator at 37°C. To determine cell viability, the absorbance of each well was measured

by a microplate reader (SpectraMax; Molecular Devices Crope, CA) at 490 nm and normalized to untreated control cells.

dTAT/pDNA/Ca^{2+} nanoparticles solvent optimization for in vivo intravenous administration

To find the ideal solvent for dTAT/pDNA/Ca^{2+} nanoparticles intravenous administration, N/P ratio 10 & 30 dTAT/pGL3/Ca^{2+} nanoparticles were prepared as mentioned above. These particles were suspended in increasing concentrations of dextrose, NaCl, FBS or human serum albumin. Effective diameter of the particles after re-suspension was used as metric to identify the solvent for *in vivo* use. Particle size was determined by Zeta PALS dynamic light scattering apparatus and compared between different solvents.

AT2R or TRAIL or miR34a nanoparticles synthesis and characterization

AT2R or TRAIL or miR34a plasmid containing dTAT/pDNA/ Ca^{2+} nanoparticles were prepared. N/P ratio 30 and 100mM calcium chloride preparative conditions were used. Mouse serum albumin (1%) containing nuclease free water was used as solvent. Particle sizes were measured with DLS apparatus at different time points.

dTAT/pDNA/Ca^{2+} nanoparticles – in vivo evaluation

dTAT/pDNA/ Ca^{2+} nanoparticles preparation for in vivo intratracheal use

Our lab previously has shown that intratracheally administered dTAT/AT2R nanoparticles attenuates lung cancer growth [85]. Nanoparticles that were prepared in the present study were similar to the ones we used previously [85], except small differences in the preparation to improve particles stability. From here on, nanoparticles that were tested by

Atsushi et al., will be called as old formulation and the particles that have increased stability will be described as new formulation.

dTAT/pDNA (old formulation) nanoparticles were prepared following the protocol of Atsushi et al. [85]. Briefly, 10 µL pDNA (AT2R or TRAIL or miR34a) (0.1 µg pDNA/µL) and 15 µL dTAT (N/P ratio 30) solutions mixed by fast pipetting. The resultant dTAT/pDNA solution was stabilized by adding 25 µL of 10% glucose and15 µL 0.3M $CaCl_2$. The final solution was mixed vigorously by pipette. Before use, dTAT/pDNA nanoparticles were allowed to equilibrate for 20 min at 4° C.

dTAT/pDNA (new formulation) nanoparticles were prepared following the protocol using 1%MSA to test and optimize new v/s old formulation for IT. *in vivo* experiment. Briefly, 10 µL pDNA (AT2R or TRAIL) (0.1 µg pDNA/µL) and 15 µL dTAT (N/P ratio 30) solutions mixed by fast pipetting. The resultant dTAT/pDNA solution was stabilized by adding 25 µL of 1% MSA and15 µL 0.3M $CaCl_2$. The final solution was mixed vigorously by pipette. Before use, dTAT/pDNA nanoparticles were allowed to equilibrate for 20 min at 4° C. Nanoparticles were freshly prepared before each experiment.

dTAT/pDNA/ Ca^{2+} nanoparticles preparation for in vivo intravenous use

For intravenous use, dTAT/pDNA/Ca^{2+} nanoparticles solution was prepared by adding 15 µL dTAT peptide solution (N/P ratio 30) to 10 µL pDNA (AT2R or TRAIL or miR34a) (0.1 µg /µL DNase and RNase free water), followed by fast pipetting for 20 seconds. Calcium chloride solution (15µL 0.1M) was added and mixed by fast pipetting. Resultant complex solution 160 µL was mixed with 40 µL 1% MSA (the final volume of the complex is 200 µl, 4 µg pDNA and 52.8 µg dTAT peptide). Nanoparticles were freshly prepared before each experiment.

Effect of intravenously administered dTAT/pDNA/Ca^{2+} nanoparticles on LLC lung tumor growth

dTAT/pDNA/Ca^{2+} nanoparticles were first tested on syngeneic LLC lung tumor model to evaluate safety and therapeutic effect, when administered intravenously. On day-0, ~$1.2X10^6$ LLC lung cancer cells were intravenously injected to 38 C57BL/6 mice. Day-5 all the mice were divided into five different groups; Group 1- PBS control (5 mice), Group 2- dTAT/Ca^{2+} (6 mice), Group 3- dTAT/AT2R/Ca^{2+} (6 mice), Group 4- dTAT/TRAIL/Ca^{2+} (6 mice) and Group 5- dTAT/miR34a/Ca^{2+} (5 mice). On day-7, 200 µl dTAT/pDNA/Ca^{2+} nanoparticles containing above mentioned plasmids were injected to groups 3, 4 and 5 through tail vein. At the same time, dTAT/Ca^{2+} complex (plasmid control) was injected to group 2 and PBS was injected to group-1 (nanoparticle control). Treatment was repeated again on day-11. All the mice were euthanized using CO_2 inhalation followed by cervical dislocation. On day 23 and 24. Lung, liver, spleen tissues were collected, weighed and fixed with buffered neutral formalin. Mouse weights were measured every other day of the experiment and compared between groups to find nanoparticles mediated acute side effects. Lung, liver weights were compared between groups to find nanoparticles therapeutic effect.

Comparison between old and new formulation dTAT/pDNA/ Ca^{2+} nanoparticles using a LLC lung tumor model

To find differences between old and new formulation dTAT/pDNA/Ca^{2+} particles, seven week old C57BL/6 mice were intravenously injected with $1.2x10^6$ LLC cells suspended in 200µl PBS *via* the tail vein. All the mice were divided into seven groups (n=5); Group 1- PBS control, Group 2- dTAT/Ca^{2+} (old), Group 3- dTAT/Ca^{2+} (new), Group 4- dTAT/AT2R/Ca^{2+} (old), Group 5- dTAT/AT2R/Ca^{2+} (new), Group 6- dTAT/TRAIL/Ca^{2+} (old) and Group 7- dTAT/TRAIL/Ca^{2+}

(new). After 7 days of LLC injection, 50μL nanoparticles were sprayed intratracheally to all the mice. PBS and dTAT/Ca^{2+} solution without pDNA were used as control. Mice were sacrificed by CO_2 inhalation followed by cervical dislocation 14 days after treatment. The lungs were dissected, and tumor burden was analyzed.

Effect of intravenously and/or intratracheally administered dTAT/pDNA/Ca2+ nanoparticles on mouse LLC lung tumor growth

On day-0, ~1.2X10^6 LLC lung cancer cells were intravenously injected to 41 C57BL/6 mice. Day-5 all the mice were divided into five different groups; Group 1- PBS control (5 mice), Group 2- dTAT/Ca^{2+} IT (5 mice), Group 3- dTAT/Ca^{2+} IV (6 mice), Group 4- dTAT/miR34a/Ca^{2+} IT (5 mice), Group 5- dTAT/miR34a/Ca^{2+} IV (5 mice), Group 6- dTAT/AT2R/Ca^{2+} IT (5 mice), Group 7- dTAT/AT2R/Ca^{2+} IV (5 mice) and. Group 8- dTAT/AT2R/Ca^{2+} IT+IV (5 mice). On day-7, 50 μl dTAT/pDNA/Ca^{2+} nanoparticles containing above mentioned plasmids were intratracheally and/or 200 μl dTAT/pDNA/Ca^{2+} intravenously administered to respective groups. Nanoparticles prepared through old formulation were used in intratracheal groups. At the same time, dTAT/Ca^{2+} complex (plasmid control) was sprayed intratracheally to group 2 , intravenously to group 3 and PBS was sprayed intratracheally to group-1 (nanoparticle control). All the mice were euthanized on day-14 after treatment and tissues were collected. Lung weights were measured and compared between groups.

Effect of dTAT/pAT2/Ca^{2+} nanoparticles on H358 human lung tumor growth

To find dTAT/pDNA/Ca^{2+} nanoparticles effect on established tumors, H358 human lung tumor model was used. One million H358 cells were injected twice with an interval of eight weeks through lateral tail vein to establish lung tumors in 25 mice as described previously [164]. One week after second injection all the mice were divided into five different groups; Group 1-

intratracheal (IT) PBS (5 mice), Group 2- IT dTAT/Ca^{2+}(5 mice), Group 3- IT dTAT/AT2R/Ca^{2+} (5 mice), Group 4- IV dTAT/AT2R/Ca^{2+} (5 mice) and Group 5- IT&IV dTAT/AT2R/Ca^{2+} (5 mice). On the same day, dTAT/pDNA/Ca^{2+} nanoparticles were administered to groups 3, 4 and 5 through designated routes. At the same time, dTAT/Ca^{2+} complex (plasmid control) was sprayed intratracheally to group 2 and PBS was sprayed intratracheally to group-1 (nanoparticle control). Intratracheal sprayer (Penn-Century Inc) was used to give particles into the trachea as mentioned previously in [85]. Lateral tail vein was used for intravenous injections. Treatment was repeated again on day-11 after H358 second injection. All the mice were euthanized on day-22 after H358 second injection and tissues were collected. Lung weights were measured and compared between groups.

Histological analysis

Fixed tissues were paraffin embedded, 4 μm sections were prepared and stained with H&E. Tumor nodules were counted from above prepared sections and compared between groups. Nodules in five 4x magnified fields from each section were used as measurement variable.

Statistical analysis

Data were analyzed by using GraphPad software. All values were expressed as the mean ± standard error of the mean. All experiments were conducted with multiple sample determinations. A statistical evaluation comparing the significance of the difference in gene expression (RLUs/mg protein) between the means of two data sets was performed using a t-test. One-way ANOVA, Tukey post-test was used to analyze the differences when more than two data sets were compared.

Results

dTAT/pDNA/Ca^{2+} Nanoparticles in vitro evaluation

Characterization of dTAT/pGL3/Ca^{2+} nanoparticles

The effect of calcium chloride concentration on the particles surface charge and particle size of the dTAT/pGL3 nanoparticle (N/P ratio-10) was investigated. Calcium chloride was added to decrease the nanoparticle size through "soft" cross-links of dTAT and pGL3. Calcium chloride addition to the dTAT/pGL3 nanoparticles induced an extensive decrease in the particle size. Figure 3.1A shows that the added calcium chloride concentration range of 0 to 300 mM produced small (around 300 nm) and stable nanoparticle, with relatively narrow polydispersity (~0.1) in serum free medium (SFM). Zeta potential of the dTAT/pGL3/Ca^{2+} nanoparticles increased significantly from ~14 to 20 mV with increasing concentration of calcium chloride, suggesting calcium "soft" cross-links increased the stability of dTAT/pGL3 particles (Figure 3.1B).

dTAT/pGL3/Ca^{2+} nanoparticles caused efficient gene transfection in vitro on LLC cancer cells with minimal toxicity

To confirm nanoparticles transfection efficiency LLC cells were treated with dTAT/pGL3/ Ca^{2+} nanoparticles. Plasmid expression was estimated by using luciferase assay. As shown in Figure 3.2A, LLC cells treated with dTAT/pGL3/ Ca^{2+} nanoparticles showed increased luciferase activity compared to dTAT/pGL3, PEI/pGL3 particles and pGL3 plasmid alone treated cells. This data clearly shows, proposed particles efficiency as plasmid transfection

agents. Figure 3.2B further confirms, effectiveness of dTAT peptide in transfecting LLC cells with pGL3 plasmid. To examine whether dTAT, PEI, and calcium chloride affected the viability, LLC cells were incubated with up to 5 mg/mL dTAT, PEI, calcium chloride for approximately 24 hours. Figure 3.3 displays the cytotoxicity of above mentioned chemical agents. dTAT and calcium chloride showed comparatively cytotoxicity, whereas PEI induced significant cytotoxicity on LLC cells.

Serum albumin increased the stability of dTAT/pDNA/Ca^{2+} nanoparticles

Blood mononuclear cells are known to engulf nanoparticles that are more than 100nm upon intravenous injection [165]. To find solvent that can increase circulation half-life of dTAT/pDNA/ Ca^{2+} nanoparticles, dTAT/pGL3/ Ca^{2+} nanoparticles were dissolved in different solvents that were known to increase the particle stability in body fluids and their sizes were compared. As shown Figure 3.4 A&B, particles that were dissolved in FBS and BSA containing solutions were more uniform and smaller in size. Increasing N/P ratio to 30 further increased size uniformity (Figure 3.4C) of dTAT/pGL3/ Ca^{2+} nanoparticles. Particles stability was confirmed by storing them in different conditions (Figure 3.5). dTAT/pDNA/ Ca^{2+} nanoparticles containing AT2R or TRAIL or miR34a plasmids were prepared and resuspended in 1%MSA. Size of these particles was compared in Figure 3.6 as a function of time after preparation.

dTAT/pDNA/ Ca^{2+} nanoparticles - in vivo evaluation

Inhibition of LLC tumor growth by intravenous dTAT/pDNA/ Ca^{2+} nanoparticles administration

dTAT/pDNA/ Ca^{2+} nanoparticles gene delivery efficiency and safety were tested by intravenously injecting them to LLC metastatic tumor containing mice. AT2R, TRAIL, miR34a

were used as therapeutic genes. As shown in Figure 3.7A, intravenous administration of nanoparticles didn't cause any change in mouse body weights suggesting nanoparticles may not have any acute side effects on treated animals. Although, body weight loss was observed in dTAT/AT2R/Ca^{2+} group after particles injection, the weight loss was transient and not statistically significant (Figure 3.7A). Intravenous injection of LLC cancer cells leads to tumors formation mainly in lung. Considering the metastatic ability of LLC, apart from lung they can form tumors in other tissues upon intravenous transplantation. In these metastatic tumor models, body weight of animals can be a reliable parameter to assess treatment effect[166]. Compared to the PBS group the remaining treatment groups didn't show any loss in body weight. Therefore, dTAT/ Ca^{2+} itself and in combination with therapeutic genes may have inhibitory effect on LLC tumor growth. A trend for decreasing lung weight was observed in all dTAT/pDNA/Ca^{2+} treated groups (Figure 3.7 c). Similar tumor growth attenuation effect was observed when the number of tumor nodules was compared (Figure 3.7d). Two of the mice in PBS group were died before the date of euthanasia, possibly because of tumor burden. Due to reduction of sample size in the PBS group and variation in lung weights treatment effects were not statistically significant. Replication of this experiment with increased sample size might confirm the observed trend.

Effect of dTAT/pDNA/ Ca^{2+} nanoparticles route of administration on mouse LLC lung tumor growth

First, old and new formulation dTAT/pDNA/Ca^{2+} nanoparticles treatment effect was compared. As shown in Figure 3.8 A&B, AT2R plasmid containing old formulation particles LLC tumor attenuation effect was higher than new formulation when given intratracheally. So, old formulation particles were used for IT and new formulation particles were used for IV. AT2R and miR34a plasmid containing particles administered through IT and IV showed similar

attenuation effect on LLC lung tumor growth (Figure 3.9 A&B). Combination of IV and IT didn't show any additional effect.

Effect of dTAT/pATR2/Ca2+ nanoparticles on established H358 human lung tumor growth

To find dTAT/AT2R/ Ca^{2+} nanoparticles treatment effect on larger size lung tumors, H358 human lung tumor model was used. To establish more than one cubic millimeter sized lung tumors, CB17/SCID mice were inoculated with two million H358 cells followed by 8 weeks of tumor growth. dTAT/AT2R/ Ca^{2+} nanoparticles were administered through either intratracheal or intravenous or combination of both the routes. Compared to PBS, dTAT alone and dTAT/AT2R/Ca^{2+} nanoparticles treated mice showed less body weight loss (Figure 3.8A). Mice treated with IT and combination routes showed decrease in lung weights compared to PBS treated mice (Figure 3.8C). But only combination route group lung weight decrease was statistically significant. Similar pattern was observed when number of tumor nodules was used as parameter (Figure 3.8 B&D). Suggesting, dTAT/AT2R/ Ca^{2+} nanoparticles can inhibit larger size lung tumor growth and they are therapeutically efficient when they are given intratracheally than intravenously.

Discussion

In order to develop an anti-cancer therapy for effective therapeutic gene delivery, several trials using viral- and non-viral vector-based trials have been conducted[124]. Owing to safety concerns with viral vectors, plasmid DNA (pDNA) is expected to be a non-viral, safety vector for therapeutic gene delivery. However, naked plasmid DNAs (pDNA) do not easily pass through cell membranes, and hence, pDNAs combined with lipid polymers are now commonly employed as gene delivery vehicles [75]. Previous studies have shown that TAT peptide can

traverse through cell membrane and several groups have used TAT peptide as DNA delivery agent [77]. Here, we have developed a modified nanoparticle delivery system by combining polycationic dTAT peptide and plasmid DNA (pDNA) to deliver AT2R gene, which is known to inhibit lung cancer proliferation[84, 85]. Previous studies from our lab showed evidence for dTAT mediated gene delivery [85]. A bolus intratracheal administration of dTAT/pAT2R/ Ca^{2+} or dTAT/pTRAIL/Ca^{2+}significantly attenuated the growth of fast growing Lewis lung carcinoma tumors, suggesting dTAT/pDNA/ Ca^{2+}-based gene therapy is effective and tumor specific [85]. The main goal of this work is to increase the stability of the nanoparticles, examining the safety and therapeutic efficacy of the dTAT/pDNA/Ca^{2+} complex injected intravenously and/or sprayed intratracheally and to determine whether pDNA (pAT2R, pTRAIL, pmiR34a) can inhibit the lung tumor growth in different mouse models.

The effect of calcium ions addition has been well studied in different nanoparticle vector systems like calcium phosphate nanoparticles, cationic liposomes etc.,[167-169]. The addition of calcium ions enhanced the transfection efficiency of nanoparticles encapsulating pDNA by mediating the delivery of a large amount of pDNA per cell while overcoming the serum mediated gene transfection inhibition[168, 170]. Baoum et al., found that the introduction of calcium “soft “crosslinks in TAT/pDNA complexes condensed and maintained the stability of TAT nanoparticle size, high transfection efficiency with no detectable cytotoxicity. Gene transfection levels were found to be as high as that of PEI particles, suggesting the possible translation of the TAT-Ca/pDNA complexes by the addition of calcium chloride[84]. The addition of calcium chloride to dimerized TAT (dTAT)/AT2R pDNA complexes have been found to enhance the gene expression in Lewis lung carcinoma tumors *in vitro* and *in vivo,* in turn exerting strong tumor inhibition. Hence, in the first step, we characterized dTAT

nanoparticle on their physical feature and efficacy for gene transfection. As we previously reported[84] , dTAT/pDNA complexes condensed and were more stable overtime with Ca^{2+} dose-dependently (Figure 3.1) and transfection efficiency was also increased in the presence of Ca^{2+} (Figure 3.2) compared to PEI control nanoparticle group. After the introduction of PEI by Behr *et al*. in 1995, it was considered the most important cationic polymer next to PLL (Poly-l-lysine)[127]. PEI is the positively charged dense polymer found to have high transfection efficiency *in vitro* and moderate efficiency *in vivo[171]*. Specific advantages of PEI include a) Formation of istoroidal polyplex particles, which are stable under physiological buffer conditions, b) Strong buffering capacity at almost any pH[172]. However, PEI is non-biodegradable[173] in nature and highly toxic *in vivo* compared to other nanoparticle delivery systems restricting its use in human trials. The cytotoxicity of dTAT was significantly lower than PEI, these results suggest that dTAT/Ca^{2+} complexes were safety and effective gene transfection vector. The, stability of dTAT/pDNA/ Ca^{2+} with serum albumin was evaluated for the purpose to optimize dTAT/pDNA/ Ca^{2+} complexes for *in vivo* IV and IT injection.

Finally, effect of dTAT/pDNA/ Ca^{2+} complex on the lung tumor was evaluated using mouse study. IV administration of new formulation dTAT/pDNA/Ca^{2+} did not show any significant adverse effect in mice indicating that this new formulation is well suited for IV administration (Figure 3.7A). All three therapeutic gene treatments (pAT2R, pTRAIL, pmiR34a) attenuated tumor growth in the lung to the normal lung weight level (Figure 3.7 C). This mouse study indicates that new dTAT/pDNA/Ca^{2+} formulation containing therapeutic pDNA with mouse albumin is an injectable and effective for lung cancer treatment. Further, we compared both old and new formulations in LLC lung cancer model to validate the new formulation for IT experiments. IT treatment using old formulation was more effective in decreasing tumor volume

with fewer tumor nodules (Figure 3.8). It is probable that albumin component of new formulation might have caused this difference. After evaluating these results we carefully planned to inject new formulation intravenously and old formulation intratracheally in the combination effect study. A single IV and IT combination treatment did not markedly attenuate lung tumor growth. IT and IV single treatments were more effective than the combination treatment group (Figure 3.9). In another mouse study using human bronchioloalveolar carcinoma (H358) xenografts in SCID mice, we have examined whether established lung tumors with approximately 1mm diameter is treatable with our dTAT/pDNA/Ca^{2+} nanoparticles. dTAT/pAT2R/Ca^{2+} NP treatment through IT and IV showed significant attenuation in lung tumor growth(Figure 3.10). This study suggests that dTAT/pAT2R/Ca^{2+} therapy is effective in inhibition of the developed lung carcinomas.

In conclusion, our newly developed dTAT/pDNA/Ca^{2+} formulation is applicable as a systemic formulation to the treatment of lung cancer animal model. Combination treatment with the IV injection of the new dTAT/pAT2R/Ca^{2+} formulation and the IT injection of the original dTAT/pAT2R/Ca^{2+} formulation is effective in attenuation of developed human bronchioloalveolar carcinoma in the SCID mouse lungs. Such findings have vital clinical relevance as it implies that nanoparticle delivery systems may prove to be beneficial in the treatment of various stages of lung cancer.

Figures

Figure 3.1 Characterization of dTAT/pGL3/Ca^{2+} nanoparticles.

dTAT/pGL3/Ca^{2+} nanoparticles prepared at increasing concentrations of $CaCl_2$ were analyzed by using dynamic light scattering apparatus. Estimated particle size (hydrodynamic diameter) in nanometer was plotted as a function of $CaCl_2$ concentration in graph A, NFW-nuclease free water, SFM- serum free medium. dTAT/pGL3/Ca^{2+} nanoparticles Zeta potential was measured in 1mM KCl, and plotted in graph B, as a function of $CaCl_2$ concentration; dTAT complex- dTAT/pGL3/Ca^{2+} nanoparticles, PEI complex- PEI/pGL3 nanoparticles.

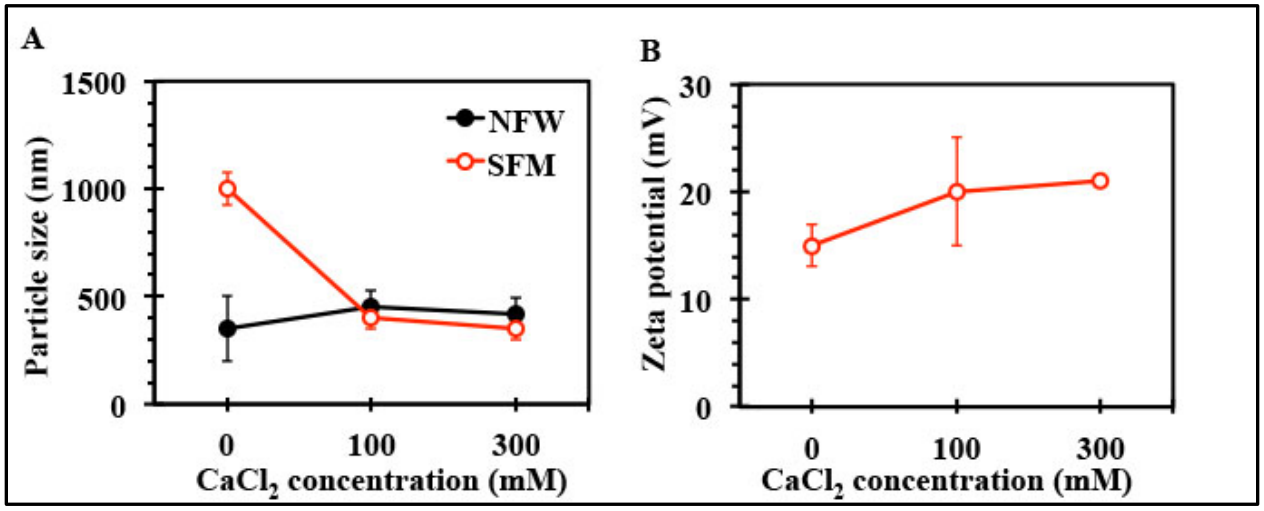


Figure 3.2 dTAT/pGL3/Ca^{2+} nanoparticles transfection efficiency on LLC lung cancer cells

LLC cancer cells were treated with dTAT/pGL3/Ca^{2+} nanoparticles and transfection efficiency was estimated by luciferase assay. Luciferase activity was expressed as relative light units (RLUs) per mg of treated LLC cells protein. dTAT/pGL3/Ca^{2+} nanoparticles incubated LLC cells showed higher luciferase activity compared to dTAT/pGL3, PEI/pGL3 particles treated LLC cells (A), confirming dTAT/pGL3/Ca^{2+} nanoparticles as effective plasmid transfection agents. pGL3/ Ca^{2+} complex and dTAT/pGL3/Ca^{2+} nanoparticles transfection efficiency was compared in plot B. (*-<0.05, **-0.01, ***-0.001 p-value)

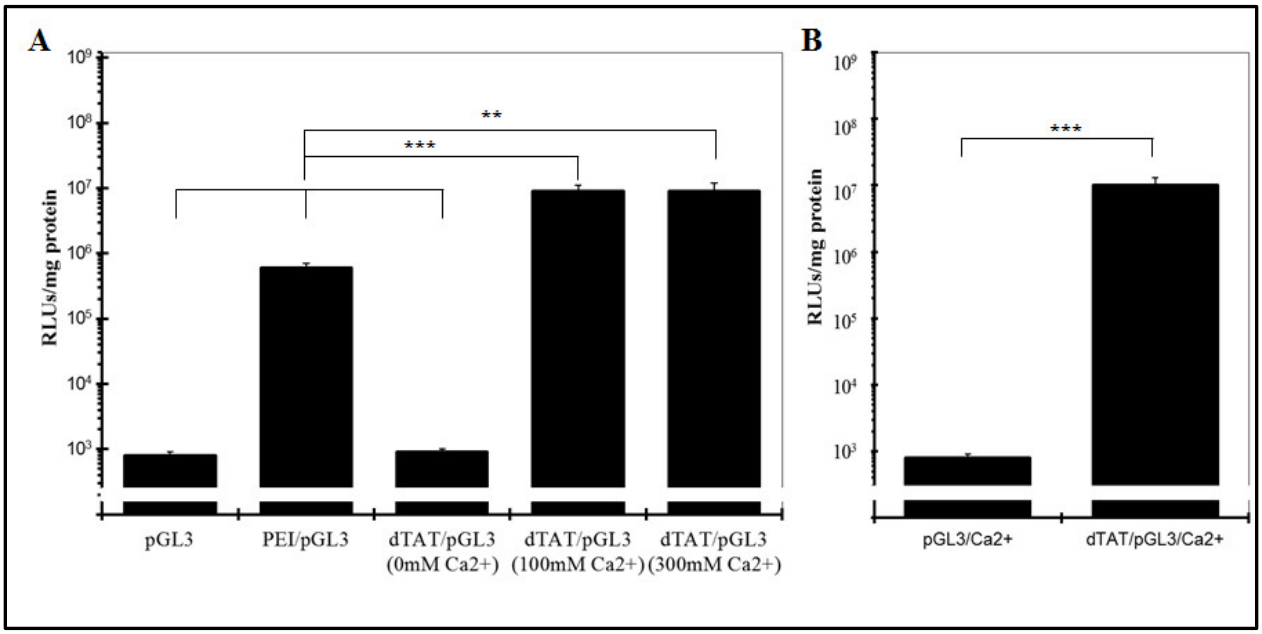


Figure 3.3 dTAT, $CaCl_2$, PEI toxicity profile on LLC cancer cells

LLC cells incubated with increasing concentrations of Calcium chloride or dTAT or PEI for 24hrs. MTS cell proliferation assay was conducted and optical density measurements were converted into viability percentages relative to control cells. Percentage of LLC cells viability was plotted as a function of tested chemical agent's concentration. $CaCl_2$ and dTAT toxicity was minimum compared to PEI cell toxicity as shown below.

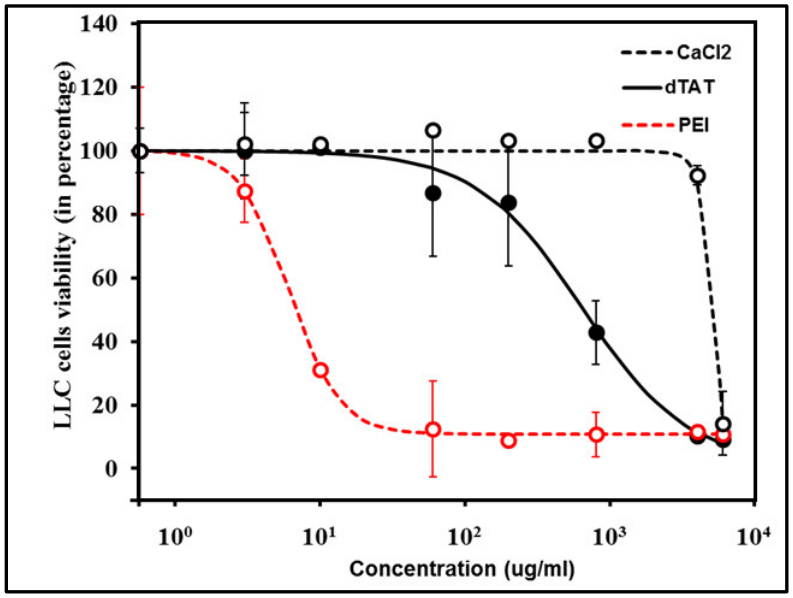


Figure 3.4 dTAT/pDNA/Ca^{2+} nanoparticles solvent optimization for *in vivo* intravenous injection use

dTAT/pGL3/Ca^{2+} nanoparticles (with 100mM calcium chloride) were prepared and resuspended in different solvents. Effective diameter measured by dynamic light scattered and compared between different preparations. Addition of FBS or HSA decrease particle size compared to other tested solvents (A &B). dTAT/pGL3/Ca^{2+} nanoparticles prepared with N/P ratio 30 displayed more uniform particles in HSA containing solvent than particles that were prepared with N/P ratio 10. NFW- Nuclease free water, SFM- serum free medium, FBS- Fetal bovine serum, HAS- Human serum albumin

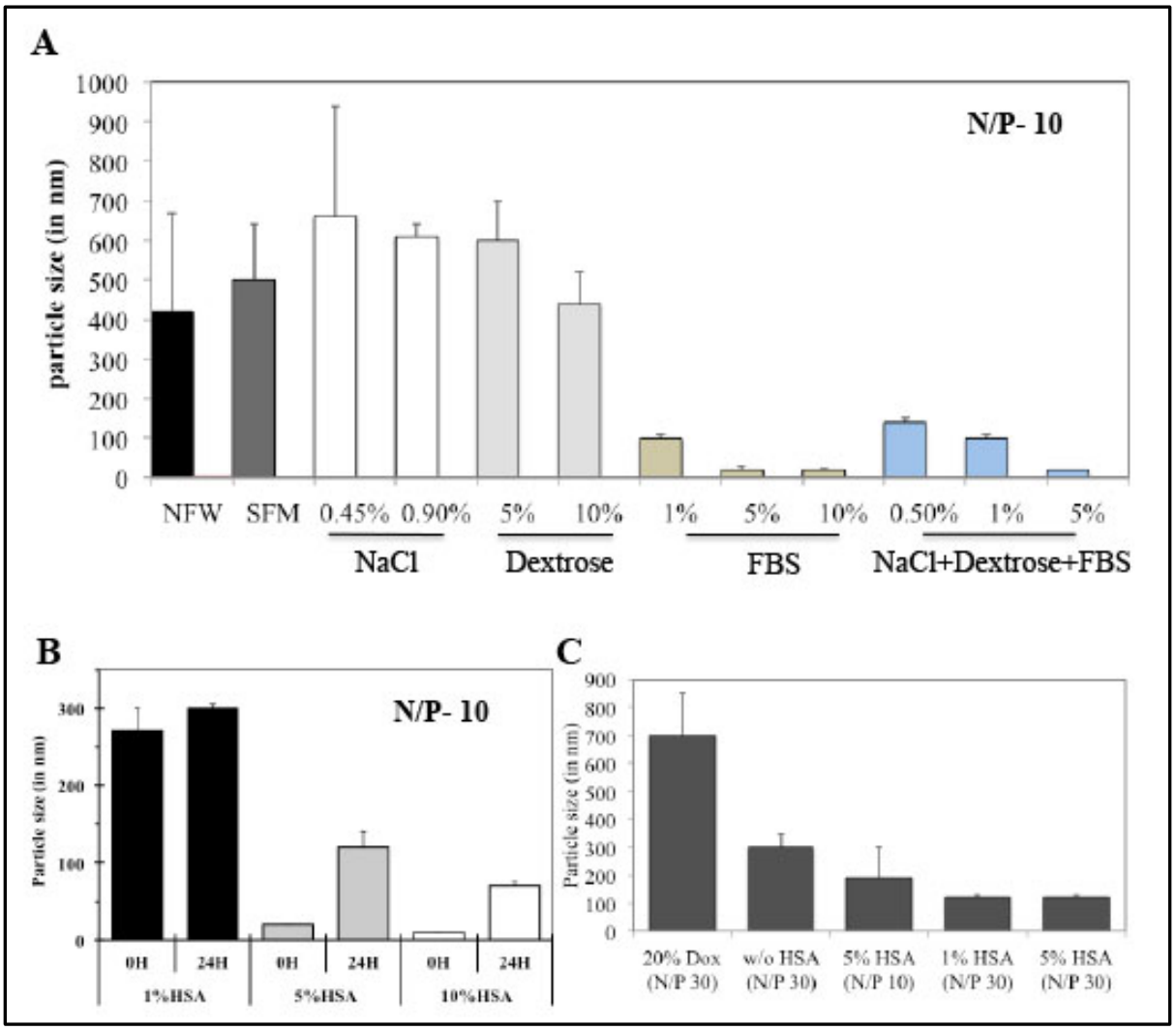


Figure 3.5 Effect of storage conditions on dTAT/pGL3/Ca^{2+} nanoparticles size and transfection efficiency

dTAT/pGL3/ Ca^{2+} nanoparticles with calcium chloride concentration 300 mM at N/P ratio 30 and HSA 1% were prepared and stored at different conductions. Their particle size (A) and transfection efficiency were compared (B).

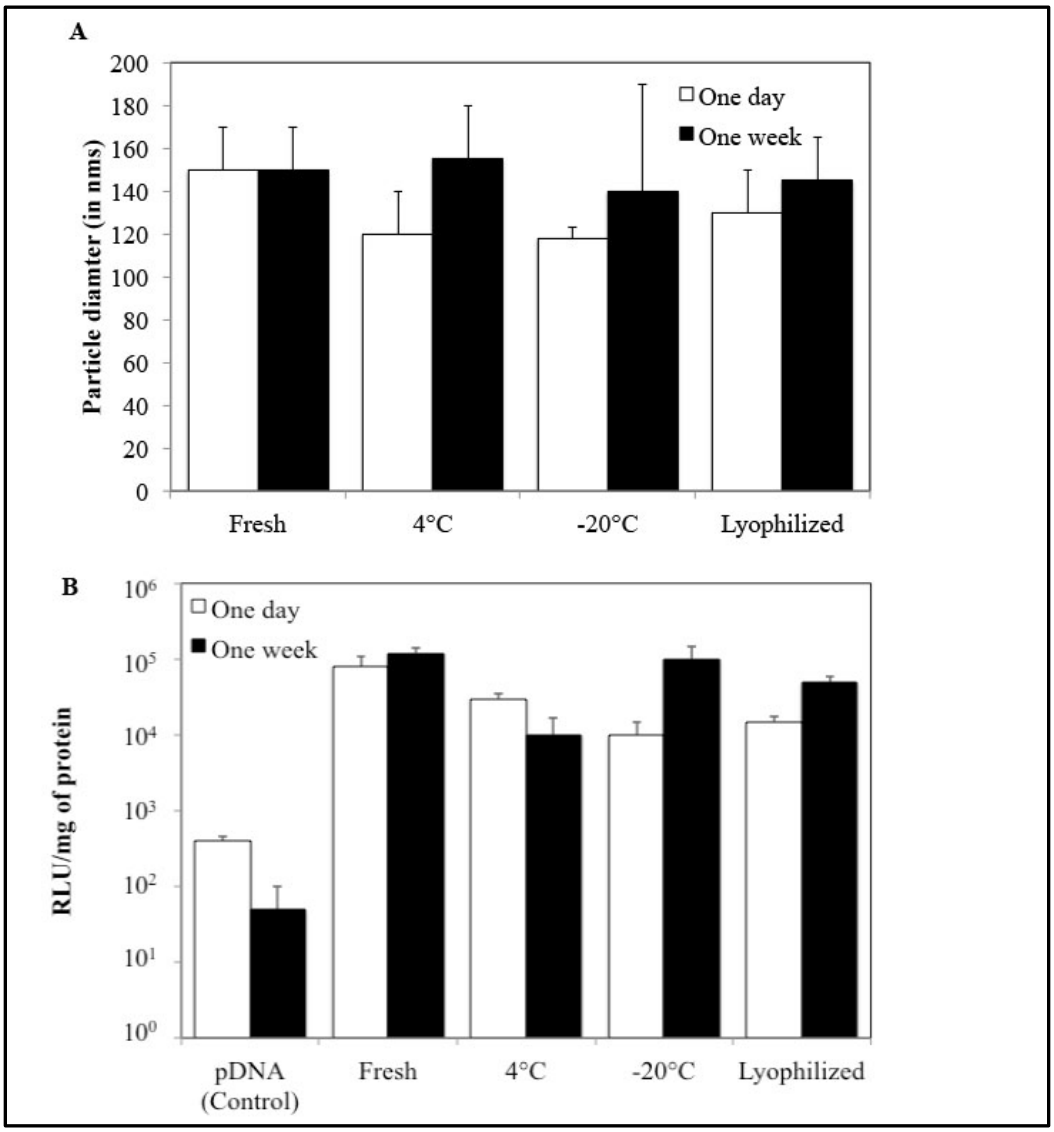


Figure 3.6 AT2R or TRAIL or miR-34a plasmid containing dTAT/pDNA/Ca^{2+} nanoparticles characterization.

Particle size of dTAT/pDNA (TRAIL, miR34a, and AT2R) complexes with calcium chloride concentration (100 mM) at **N/P ratio 30** with 1% MSA.

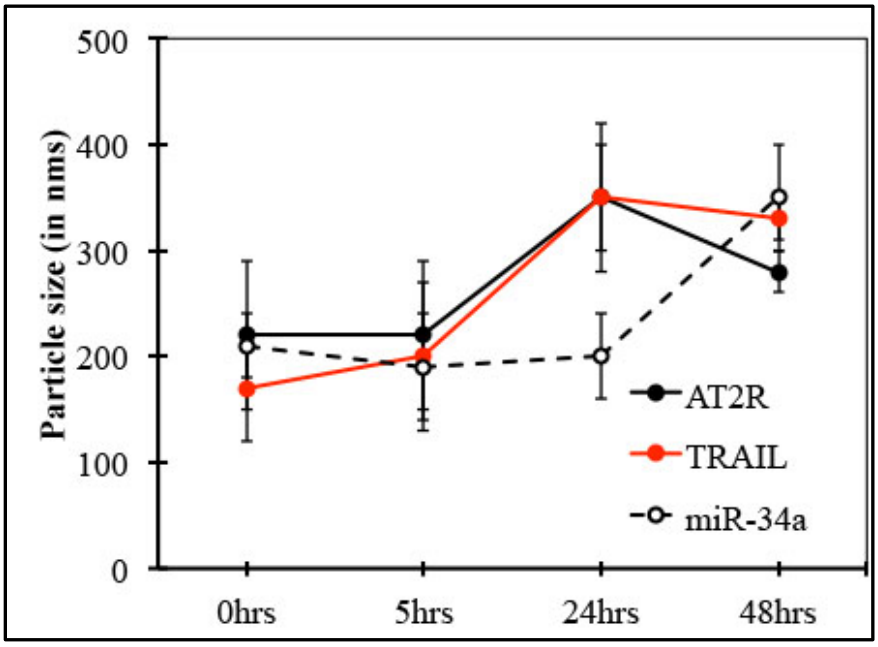


Figure 3.7 Intravenously administered dTAT/pDNA/Ca^{2+} nanoparticles effect on LLC lung tumor growth

To find the safety and therapeutic efficacy of dTAT/pDNA/Ca2+ nanoparticles, LLC syngeneic tumor model was used. A. Average body weights of each group were plotted as a function of experimental day and compared between groups. Treatment days were marked with red arrows. Except PBS group, remaining groups didn't show any change in their body weight (*-<0.05 p-value); Lung photographs were presented in B; C. Lung weights were compared between groups to find intravenously administered nanoparticles treatment effect; D. Tumor nodules from lung and liver sections were counted and compared between groups.

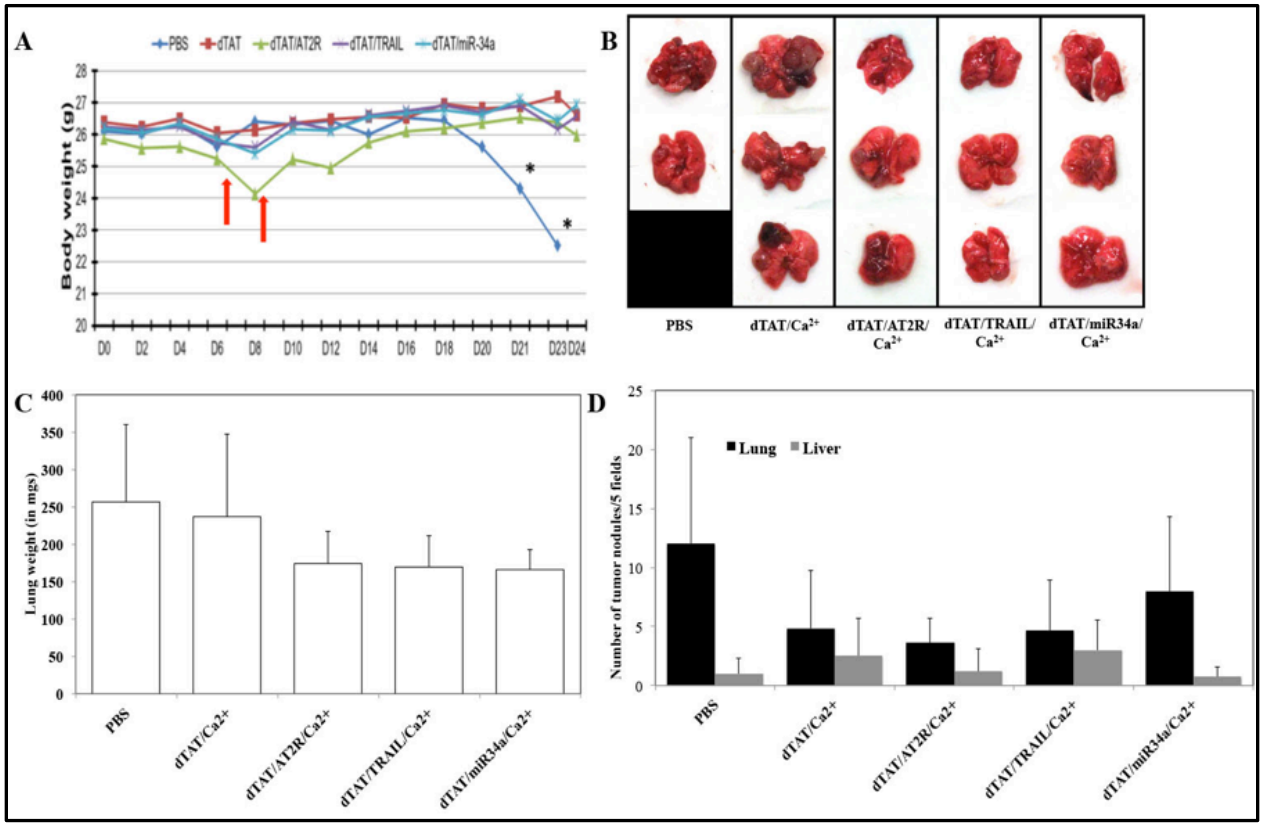


Figure 3.8 Comparison between old and new formulation dTAT/pDNA/Ca^{2+} nanoparticles by using LLC lung tumor model

Gene delivery efficiency difference between old and new formulation dTAT/pDNA/Ca^{2+} nanoparticles was tested by using AT2R and TRAIL as therapeutic genes. Particles were administered intratracheally. Lung weights (A) and number of tumor nodules (B) were compared between groups. AT2R plasmid delivered through old formulation nanoparticles showed higher tumor attenuation than new formulation nanoparticles.

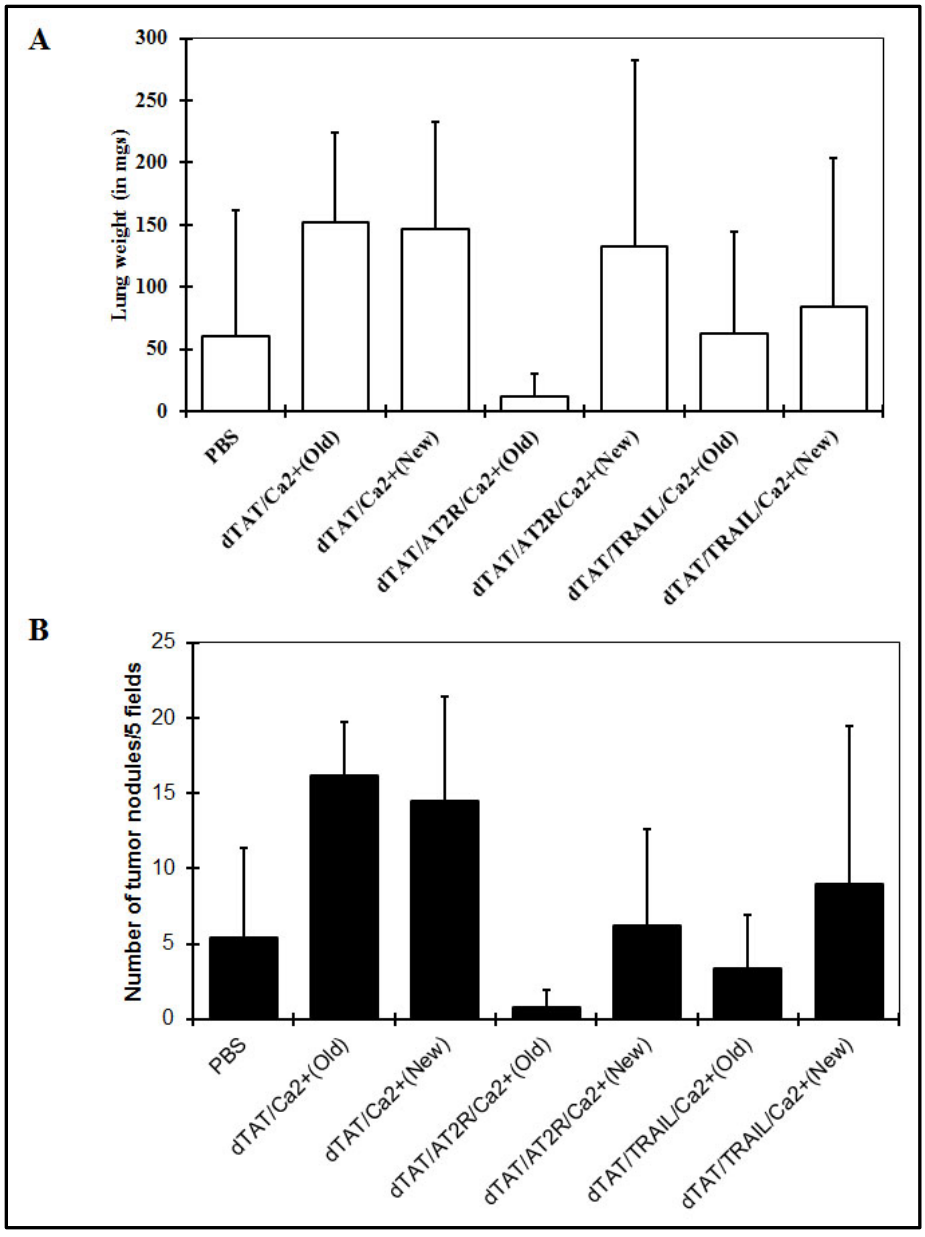


Figure 3.9 Effect of intravenously and/or intratracheally administered dTAT/pDNA/Ca2+ nanoparticles on mouse LLC lung tumor growth

To find administration route associated difference in nanoparticles treatment effect, LLC lung tumor containing mice were treated with dTAT/pDNA/Ca2+ nanoparticles intratracheally or intravenously or both the routes. Lung weights (A) and number of tumor nodules (B) were compared between groups. Nanoparticles administered through intratracheal and intravenous showed similar tumor attenuation effect for both AT2R and miR34a plasmids.

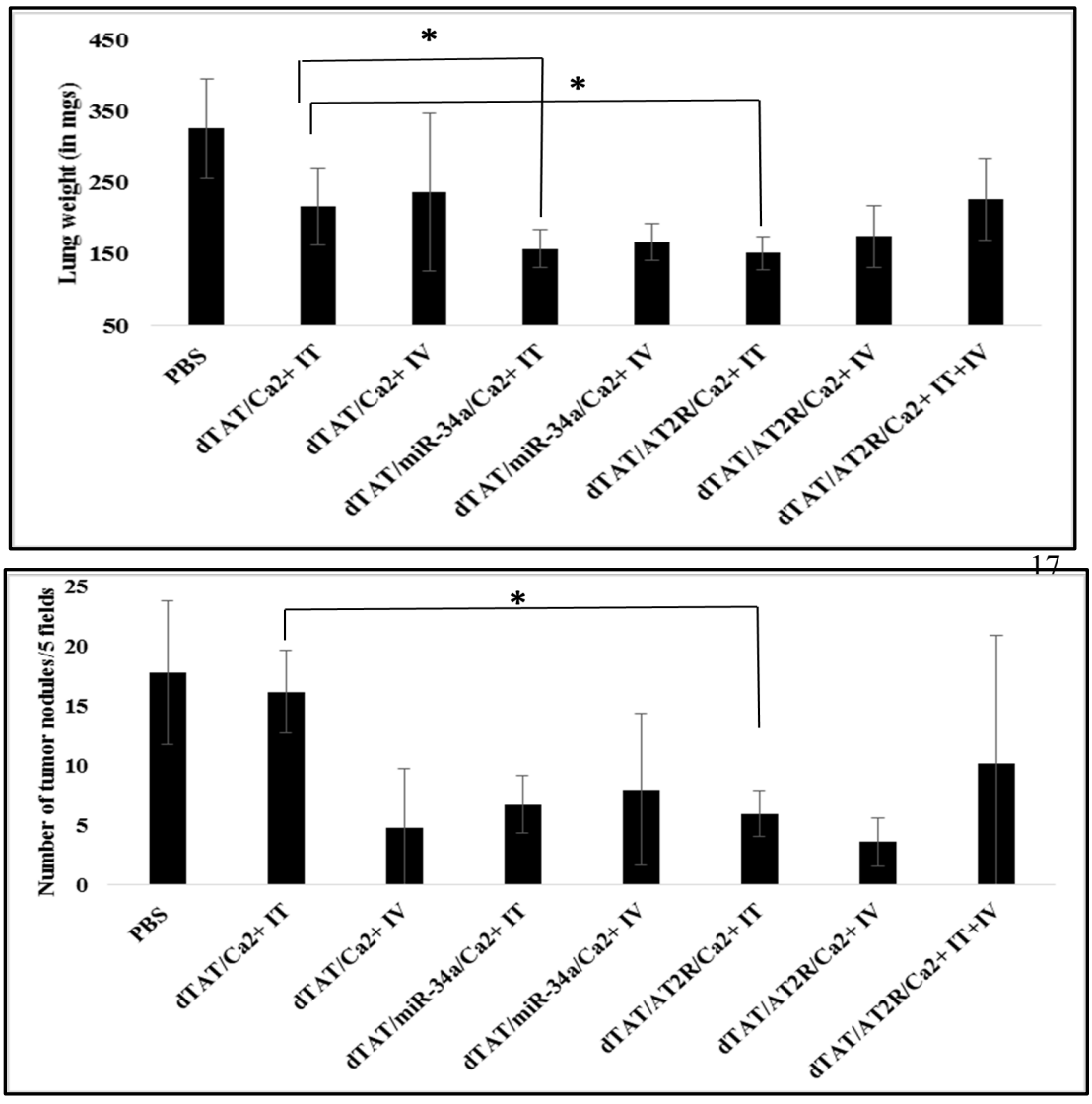
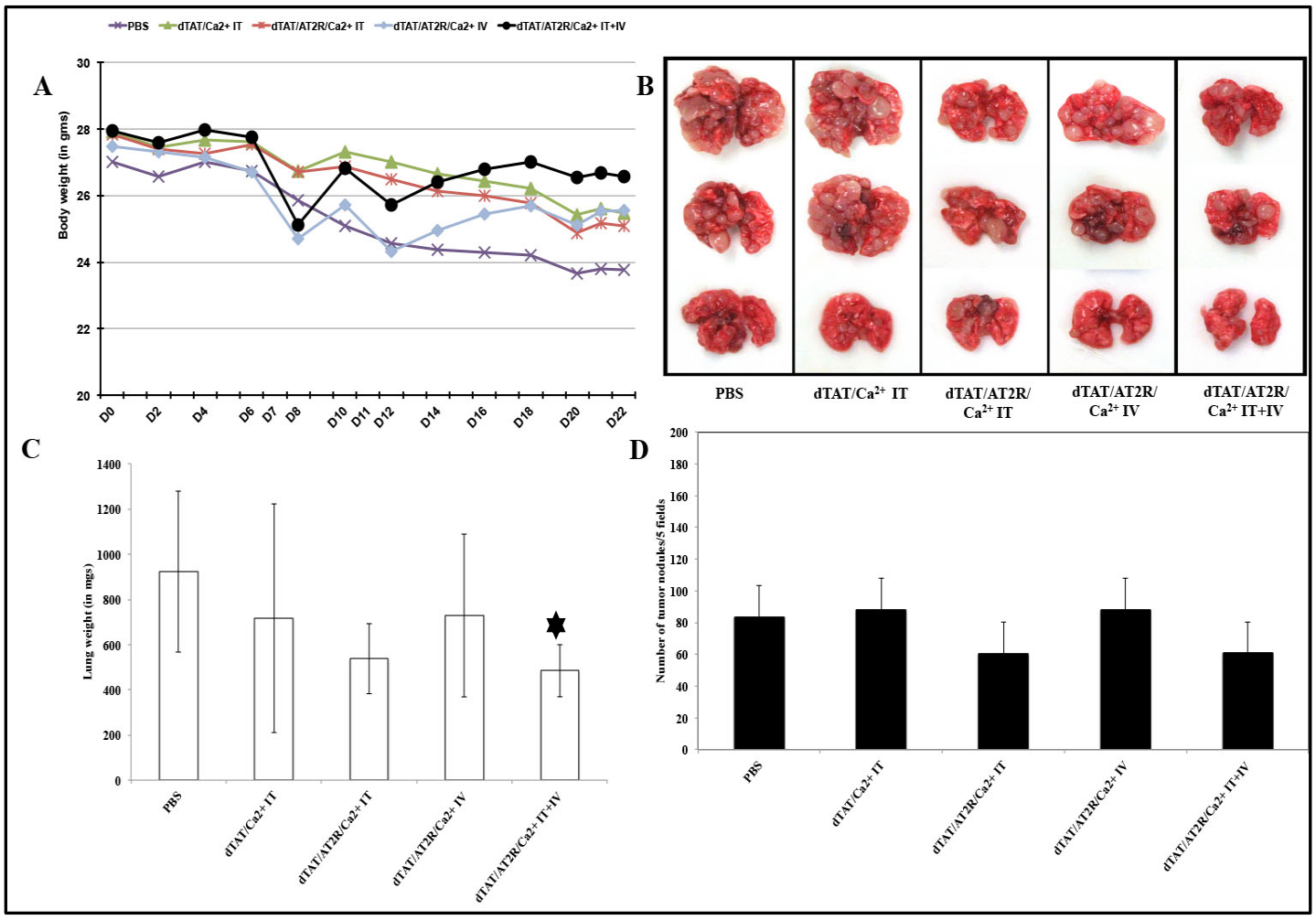


Figure 3.10 Effect of dTAT/pAT2/Ca^{2+} route of administration on H358 human lung tumor growth

A. Change in body weights of the mice compared between groups over time; B. Representive lung images from each group showing decrease in tumor nodules in AT2R treated groups; C. Comparison of average lung weights (C) and number of tumor nodules (D) between groups. (*-<0.05)



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