Advancing Treatments for Leukemia and Metastatic Pancreatic Cancer with Targeted, Synchronized Delivery of Drug Combination Stabilized in Nanoparticles

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Abstract

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Cancer continues to be a leading cause of death for all Americans due to an inability of the treatment drugs to properly target and accumulate at the cancer and tumor sites in the body. Leukemia (blood cancer) treatments that can temper the disease without truly curing it are now common first-line regimens, with some patients even experiencing treatment-free remission (TFR) for up to 10 years after their initial treatment. However, not all patients experience this TFR due to the inability of treatment drug to remain at sufficiently toxic levels in the body over time because of fast clearance, limited distribution of drug in the body, and poor patient compliance. Pancreatic cancer, on the other hand, has not benefited from treatment advances that

improve outcomes of patients with leukemia, primarily due to its often-late diagnosis, meaning most patients present metastatic symptoms at diagnosis. Gemcitabine (G) and *nab*-paclitaxel (T, a protein-based nanoparticle bound paclitaxel) is a leading combination therapy involving the sequential administration of hydrophobic, nanoparticle-bound T followed by hydrophilic, free G in solution. However, GT combination therapy generally only grants a median overall survival of about 9 months from treatment initiation. While somewhat successful relative to other treatments, this limited survival time extension is likely due to insufficiently drug concentrations at the tumor site that related to poorly vascularized tumors with limited drug access and overall high drug clearance from the body. If anticancer small molecule drugs could be assembled and co-localize in cancer laden tissues and cells for an extended duration, some of the current limitations (drug access and retention) for treating both leukemia and pancreatic cancer could be addressed in improving patient outcomes. Following this hypothesis, we first assembled a novel drug combination nanoparticle (DcNP) which carry two novel small molecule drugs, venetoclax and zanubrutinib (VZ-DcNP). The VZ-DcNP tested in mice were shown to prolong the exposure of each drug by 43-fold and 5-fold, respectively. Currently, there is no immune-competent pancreatic tumor mice model that reflects pancreatic cancer development in pancreas. In searching for an experimental pancreatic cancer model, we found that simple intraperitoneal, but not intravenous, inoculation of a pancreatic ductal adenocarcinoma cell Pan02 resulted in development of pancreatic tumors in their pancreases, mimicking pancreatic cancer in humans. The use of intensive survival surgeries, foreign matrices, or unreliable genetic induction events, which typically pose challenges in therapeutic evaluation, is no longer a limitation with this novel Pan02 orthotopic pancreatic tumor model. Pancreatic Pan02 cells were shown to home into the pancreas, resulting in a 100% take-rate of tumors in mice. Following an intraperitoneal

administration of drug combination nanoparticles with GT (GT-DcNP) at 20 mg/kg G and 2 mg/kg T, pancreatic tumor-bearing mice had greatly improved overall survival compared to mice receiving intravenous GT-DcNP, intravenous free drug, or no treatment. Further pharmacokinetic analysis of mouse tissue and plasma following intraperitoneal drug administration demonstrates the extended exposure of nanoparticle-bound drugs in blood and enhanced drug exposure in tumor-laden pancreas by GT-DcNP, compared to that dose with equivalent free GT combination. These results and accomplishments described in the thesis serve as the foundation for translating the novel pancreatic tumor models, enabling drug-combination technology for VZ and GT for safer and more effective treatments for leukemia and pancreatic cancer in the future.

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Introduction

Chapter 1. THE STATE OF TREATMENT FOR LEUKEMIA AND METASTATIC PANCREATIC CANCER: CURRENT AND NOVEL APPROACHES TO TREATMENT OF CHRONIC DISEASE

1.1 BACKGROUND AND SIGNIFICANCE

Cancer continues to be a leading cause of death for all Americans, regardless of genetics or lifetime exposures[1]. Among these cancers, leukemia and pancreatic cancer are especially prevalent in the United States and throughout the world. Utilizing state-of-the-art research, a multitude of drugs have been employed to treat these cancers in patients, including targeted small molecule drugs and biologic analogues, though limitations concerning fast drug clearance rates, synchronized drug delivery, and limited tissue penetration remain[2,3,4]. Overcoming these limitations through a "systems approach" aimed at fully understanding the dynamic distribution and elimination of a drug and a fit-for-purpose drug delivery system that modulates on-target and off-target drug level persistence over time is vital to progressing cancer treatment research[5].

Leukemias are cancers of the blood, affecting over 2 million people worldwide every year[2]. Leukemia has numerous forms, but advancements in some of the most common chronic forms have ameliorated the disease into a form similar to a chronic illness[6]. These tamed forms of leukemia, which include CML and CLL, cannot be reliably fully cured, but the best current treatments can keep the cancers suppressed for many years[7,8].

Pancreatic cancer remains one of the deadliest forms of cancer due to its often-late diagnosis and resistance to treatments that have improved outcomes for other forms of cancer[9,10]. For decades, pancreatic cancer incidence rates have been near-equal to mortality rates[11]. The most recent data from the Surveillance, Epidemiology, and End Results (SEER) Program at the National Cancer Institute in the United States reports that around 13 in 100,000 Americans were diagnosed with pancreatic cancer in 2018 while an additional 11 in 100,000 Americans succumbed to the disease[12]. Furthermore, though 11% of pancreatic cancer patients can be expected to survive 5 years from their diagnosis, this survival rate drops to 3% when only considering patients with advanced metastatic disease[13].

Historically, most medications were "small molecule drugs", meaning the drugs are discreet molecules typically under 1,000 Daltons in size, like aspirin or acetaminophen. Advancements in the understanding of living systems has allowed the development of biologics or molecular mimics that can treat disease, with the most common form being antibody mimics or monoclonal antibodies that can be constructed to selectively interact or bind to specific proteins and cell surface markers in modulating their functions. Advancements in biologic drugs have greatly improved patient treatments and outcomes in nearly all forms of cancer with some exceptions, such as pancreatic cancer with limited progress. Trastuzumab, for example, is a monoclonal antibody, which bind to truncated epidermal growth factor receptor (EGFR, referred to as HER) that is a primary driver behind the nearly 90% survival rate seen in patient with breast cancer cell expressing HER2-biomarker[12]. Pancreatic cancer, however, has not benefited from these therapeutic advancements for other cancer types due to its unique symptom presentation and tumor morphology that limits drug penetration and retentions in pancreatic tumor diagnosed in patients. Furthermore, despite its high mortality rates, pancreatic tumors grow slowly, presenting

few symptoms during early-stage development[14]. As a result, tumors are not usually detected until they are large enough to begin disrupting organs other than the pancreas[9]. Desmoplasia, a condition in which excessive fibroblast growth and extracellular matrix protein production results in an exceptionally dense coating around the tumor, is another hallmark of pancreatic cancer[15], and in some cases, the high internal pressures of some tumor may greatly limit vascularization and drug penetration, especially that of larger biologic molecules and vehicles[16]. As a result, the only approved drugs for use against pancreatic cancer remain small molecule-based monotherapies or combination regimens. Innumerable antibody and small molecule drugs have been examined for patients, but none have presented better outcomes than existing small molecule combination therapies[17,18,19].

Currently, there are two common regimens composed of small drug molecules for metastatic pancreatic cancer treatment: (1) FOLFIRINOX[20] (a four-drug combination of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin) and (2) gemcitabine in combination with *nab*-paclitaxel[21]. These regimens composed of multiple drugs used in sequence and combination are described in detail in Table 1.1. *Nab*-paclitaxel is composed of paclitaxel molecules bound to protein molecules to form nano-size particles. The water-insoluble or hydrophobic paclitaxel is co-formulated with human serum albumin (protein) to produce biocompatible, water-soluble nanoparticles in suspension. The formation of paclitaxel-protein nano-suspension has improved blood circulation time, delivery, and tumor-uptake of paclitaxel[22]. Compared to FOLFIRINOX, a 4-drug combination treatment, *nab*-paclitaxel with gemcitabine (2-drug combination) provides comparable median life expectancy. However, due to relatively milder untoward effects, *nab*-paclitaxel and gemcitabine is an attractive alternative for patients.

If small molecule chemotherapeutic drugs such as paclitaxel and gemcitabine could be efficiently delivered to their targets while minimizing their off-target burden to patients, treatment of metastatic pancreatic cancer could be greatly improved. This idea is not unique to pancreatic cancer, and multiple potential avenues have been explored in the literature. Two avenues of note are (1) drug combination nanoparticle (DcNP) platform technology developed by the Targeted and Long-acting Combinational Anti-Retroviral Therapeutic (TLC-ART) program at the University of Washington[23] and (2) Hypothermic Intraperitoneal Chemotherapy (HIPEC)[26], a technique explored through infusion of chemotherapeutic through intraperitoneal dosing in patients with advanced ovarian cancer. Drug combination nanoparticles (DcNP) were originally invented to allow the University of Washington team to transform short-acting antiretroviral drug combination to long-acting products for treating people living with human immunodeficiency virus (HIV). The DcNP platform technology is novel in its ability to stabilize multiple, physically disparate drugs of varying hydrophilicities/hydrophobicities to provide an injectable dosage form. Subcutaneous administration of drug combination in DcNP dosage form has resulted in extending from daily oral to many weeks before each dose is required to keep patients in care[24]. The platform has now been demonstrated to enable formulation of disparate cancer drug combinations for the treatment of metastatic breast cancer. Additional studies revealed that the nanoparticles' abilities to both associate with multiple varying drugs as well as traverse and retain in the lymphatic system may have contributed to controlling tumor growth and potentially lead to regression as breast cancer metastatic spread initially involves lymph nodes before spreading at high growth rate in blood and being trapped in highly perfused blood capillaries such as those in the lung[25]. HIPEC was also developed to improve drug delivery, namely that of small molecule chemotherapeutics to ovarian tumors. Like pancreatic tumors, ovarian tumors grow slowly and interact little with their

environment, resulting in an often-late diagnosis and an extensive, difficult-to-treat cancer. Due to their limited vascular perfusion, HIPEC takes advantage of the ovarian tumors' location in the peritoneal cavity by flooding the patient's peritoneum with warm, chemotherapy-laced fluid to directly reach the cancer. The treatment, though rudimentary, has been surprisingly successful in both extending patients' progression-free survival and overall survival at 1 year following diagnosis[27]. While it is more challenging for implementing IP infusion such as that for HIPEC, which required infusion clinics and specialized setting, IP single or multiple dosing of drugs and antibody therapeutics, including antibodies for rabies, are typically done in outpatient settings.

The primary goal of this thesis dissertation is to build on the lessons learned from the research relating to intricate details of how drug combination nanoparticles can be made, how the DcNP impact or modify the intrinsic properties of drugs with disparate properties to co-localize in cancer cells, and how the DcNP extend the presence of drug molecules beyond that of oral or infusion regimens to make impact on currently dismal outcome of existing therapeutic modalities for pancreatic cancer. This thesis research also addresses whether long-acting drug-combination composed of molecularly targeted molecules can be produced in DcNP dosage form for leukemia. The study on long-acting drug combination for clinically used anti-leukemia (molecularly) targeted drug is particularly important as having all-in-one two drug combination could improve durability of chronic drug effects through suppression of drug resistance potential.

To attain this goal, we first create anti-cancer nanoparticles composed of multiple drug substances in combination for potential use as a drug-combination injectable product. The anti-cancer drug-combination products made by DcNP technology are evaluated for the effects of DcNP modulating parent drug effects *in vitro* against various immortalized human leukemic cancer cell lines. Select anti-cancer DcNP products are further characterized in animal (*in vivo*)

experiments to determine the role of DcNP on pharmacokinetics of each drug substance in the anti-cancer drug combination in DcNP. While pharmacokinetic studies in healthy mice may provide intricate details on the time course of each drug in blood as well as target and off-target tissue distribution over time, a pancreatic cancer model suitable for therapeutic evaluation is lacking in the field. Recognizing this gap, we have searched and discovered a mouse pancreatic tumor cell called Pan02, derived from C57BL/6 mice, that can be inoculated within the peritoneal cavity and consistently populate in the pancreases of the host. This discovery and characterization of this pancreatic tumor model enables the evaluation of test and control anti-cancer DcNP products-of-interest. We employed this model for the first time to demonstrate the capabilities of anticancer drug combination nanoparticles containing gemcitabine and paclitaxel to suppress pancreatic cancer beyond that achievable by equivalent free drug combinations. In a series of experiments, we found that the DcNP formulation has enhanced both pharmacokinetics (drug exposure per dose) and efficacy against metastatic pancreatic cancer. These results provide a strong support to further develop this product concept through preclinical testing to support clinical development of anti-cancer DcNP products based on drug combination nanoparticle technology for the treatment of leukemia and metastatic pancreatic cancer.

In the following sections, I will provide additional details on (1) Pathophysiology of Pancreatic Cancer, (2) Pathophysiology of Leukemia, (3) Modern First-line Treatments for Cancer, and (4) Novel Research into Long-Acting Drug Delivery Systems before discussion of the research scope and aims of this thesis.

1.2. Pathophysiology of Pancreatic Cancer

Cancers of the pancreas occur due to unregulated and excessive growth of healthy host cells giving rise to malignant cells in the pancreas. As a result of uncontrolled growth, malignant

pancreatic cells overtake the pancreas function and eventually spread to other tissues and organs, referred to as metastatic spread of cancer. While disease etiology remained elusive, it is thought that a combination of factors including exposure to carcinogenic substances, like excessive alcohol and tobacco consumption, are risk factors for developing the disease; genetic sources are also suspected, but unconfirmed[14]. Regardless of how pancreatic cancer triggers in host, it is well-documented that most cases are late onset with most people diagnosed at older age, with frequencies rising around age 60[12].

The pancreas is a glandular organ with two primary functions: (1) endocrine regulation through the release of hormones in the blood and (2) exocrine digestion of ingested food through the secretion of digestive enzymes into the duodenum of the small intestine. Hormones released from the pancreatic are almost exclusively related to blood sugar regulation and include insulin, glucagon, and somatostatin. An additional hormone, pancreatic polypeptide, that is thought to be related to feelings of hunger is also released, but in extremely small quantities in comparison to the other three. These hormones are generated and released by pancreatic islets, which total less than 1% of the pancreas total mass[28]. Cancer of pancreatic islets are very rare, composing about 2% of total diagnoses pancreatic cancer cases; an even smaller percentage of these cancers are endocrine active, with most being inert. The remaining 99% of the total pancreas mass is devoted to the production and secretion of enzymes responsible for the digestion of proteins, fats, and carbohydrates. Acinar cells form smalls clusters that generate and secrete these enzymes, which are then shuttled out of the pancreas via endothelial tissue composed of ductal cells. The vast majority of pancreatic neoplasms occur in these endothelial cells, with the most common being pancreatic ductal adenocarcinoma[29]. Ductal cells overall play a major role in pancreatic function, but they are numerous and redundant, unlike endocrine cells that produce glucose-regulating

hormones, so a loss of function in a small section of the pancreas due to cancer is unlikely to cause symptoms in early disease stages.

1.2.1. *Development, Early Detection, and Typical Progression of Pancreatic Tumors*

Due to the central location of the pancreas within the body, tumors developing on or in the pancreatic tissue often go undetected until they become large enough to disrupt surrounding organs and tissue[9]. Despite tumor growth, most patients do not experience any significant disruption in blood glucose regulation or suffer digestive issues until late in cancer development as the cancer cells do not usually affect organ function until they are large enough to encapsulate and suffocate the tissue; at late stages, however, nearly 50% percent of patients experience diabetic-like symptoms[30]. As a result of this insidious development, most patients are diagnosed as Stage 3 or higher at diagnosis, indicating metastatic spread of the disease in addition to the primary tumor[9].

Pancreatic cancer has few available biomarkers for use in early detection[31]. The only FDA-approved biomarker for pancreatic cancer progression is CA-19-9, which is a polysaccharide cell surface antigen involved in human blood group systems. Despite its use in monitoring some disease progression, it has been deemed not useful for screening due to its presence in less than 0.9% of asymptomatic patients, minimizing its usefulness in the clinic. Instead, CA-19-9 is relegated to a marker for cancer progression during treatment for those few that express it[32]. Other potential markers, like those of commonly upregulated and/or mutated proteins in pancreatic cancer, have been examined, though none have shown sufficiently high concentrations in plasma for detection or relatively non-invasive monitoring[33].

Though the exact events that induce pancreatic cancer are widely varied, one of the most common precursors to ductal adenocarcinomas of the pancreas is pancreatic intra-epithelial

neoplasia (PanIN), a type of lesion typically discovered during unrelated surgery procedures. PanIN is rarely detected prior to diagnosis, so most research is retrospectively performed on pancreatic cancer patients or when lesions are serendipitously found during other surgical procedures. Evidence of PanIN lesions was found in over 82% of patients with pancreatic cancer compared to only 16% in control populations, and evidence strongly suggests that the PanIN lesions develop from the same acinar cells that later undergo metaplasia[34]. PanIN also shares many typical mutations in common with pancreatic ductal adenocarcinomas, including *KRAS*, TP53, *CDKN2A* , and Smad4. *KRAS* (Kirsten rat sarcoma virus) and *CDKN2A* (cyclin-dependent kinase inhibitor 2A) are proto-or-onco-genes that express proteins involved in signal transduction and cell growth, while TP53 and Smad4 are expressed proteins that regulate cell growth and division.

Tissues of PanIN typically converts to an adenocarcinoma over time; genetic profile and gene expression studies suggest that modulation of various proto-oncogenes affect cell growth and cancer progression. *KRAS*, a tumor suppressor gene, is commonly mutated in many forms of cancer. In nearly all examined human patient tissues, *KRAS* is found to be mutated, indicating its association with cancer progression[35]; however, *KRAS* mutations are seemingly also common in healthy patient showing no signs of cancer, limiting its use in the quantification or identification of cancers[36]. Other commonly mutated genes include *TP53* and *CDKN2A*, though these genes are typically found mutated late in the cancer development process and are not necessarily essential for carcinogenesis[35,37,38]. As the cancer progresses, the aforementioned mutations become more likely to occur, eventually culminating in metastatic spread throughout the patient's body.

1.2.2. *Treatment Limitations Due to Primary Pancreatic Tumor Morphology*

Pancreatic tumors are extremely dense due to the severe overgrowth of extracellular matrix (ECM) proteins and endothelial cells due to excessive growth signaling from malignant cells in a

phenomenon known as “desmoplasia”. In developed pancreatic tumors, cancer cells are thought to compose roughly 10% of the tumor mass with the remainder being the excessive ECM and overgrown, benign endothelial cells[15]. Due to the frequent late diagnosis of pancreatic cancer, tumors typically have developed, established, and encapsulated surrounding tissue with dense and impermeable extracellular matrix, preventing drug access to tissue and blood vessels supplying to pancreatic tumor cells. Resection, which can be considered the simplest way to remove a cancer, is difficult due to the overgrowth and frequent capturing of important vasculature, like the superior mesenteric artery; it is nearly impossible for surgical removal of pancreatic tumor even with therapy intended to shrink tumor and provide a margin for surgical incision[15].

An additional consequence of the desmoplasia seen in pancreatic adenocarcinomas is the constriction and prevention of tumor blood vessel development, limiting the internal perfusion of oxygen and nutrient supply to the tumor tissue. Poor perfusion is thought to also limit access, penetration, and exposure of chemotherapeutic molecules to suppress tumor growth as most therapeutics are given by intravenous routes and composed small molecule compounds that needed to be distributed through the blood vessels supplying the pancreatic tissues[16].

1.2.3. *Pre-clinical Safety and Therapeutic Assessments Enabled by Animal Models and Pancreatic Tumor Models*

Pre-clinical models are essential to assist in determination of efficacy and safety of product candidates in early development. For novel drug combination delivery systems, understanding of on-target and off-target drug distribution, as well as the need for the drug delivery system to modify on-and-off target drug distribution as it may relate to their safety and potential effects is vital for improving the outcome of a specific cancer type, i.e., pancreatic cancer in this case. This information and positive, quantifiable outcome of such studies are critical to arrive to decisions

relating to significant investments in time and resources essential for translating the novel therapeutic concepts into impactful therapeutic products. Even with molecular designs that generate molecules (compounds) that precisely target and inhibit specific enzymes critical for tumor growth, these novel or new foreign compounds in the patient body may result in unexpected events ranging from mild to severe toxicities due to unpredicted pharmacokinetics or biological interactions[39,40]. A new drug product has typically undergone numerous levels of preclinical and clinical testing prior to its release into the public market for use in patients, making the process very time-consuming and significantly expensive. Due to the massive influx of novel drug products enabled by novel research in the molecularly targeted small molecule drugs, monoclonal antibodies and their derivatives, and genetic modification-based therapies like (chimeric antigen receptor-modified T-cells) CAR-T cell therapy, there is not enough money and time to accommodate the appropriate trials for all new drugs and their potential combination regimens with other drugs[41]. This is especially costly when most new drug compounds fail, either due to toxicity or ineffective outcomes from clinical trials, resulting in significant financial and time losses. Pre-clinical testing seeks to limit these losses by attempting to reduce clinical failure and provide early indications that a product candidate is pharmacologically active *in vivo* and at a relatively safe dose range provide effectiveness against cancer growth or metastatic speared in appropriate tumor model mimicking human cancer type, i.e., pancreatic cancer growth in pancreas. In this context, pre-clinical animal models, which integrate biodistribution and biotransformation, may provide both safety and efficacy signals early in research and development of any therapeutic candidate. While various organoid or semi-functional organ systems may provide additional details, they may not provide information on the biotransformation and biodistribution impact on candidate drug products. Thus, animal cancer models mimicking pancreatic cancer will be critical

for finding effective and safe therapeutics and accelerating preclinical development process as well as success rate if such models can be developed and validated.

Animal models for cancer can be generally split into two categories: (1) cancer models externally implanted into the animal or (2) cancer models induced in animals through genetic stimulation or chemical induction. External implantation requires the recipient animal's immune system to accept the foreign cells or tumor mass to prevent host rejection of the foreign implant. A common solution for implanting human cancer cells is to use animal lines with deficient immune systems, allowing for relatively uninhibited growth of foreign (human cancer) cells; however, the lack of an immune system severely limits translation of any pre-clinical results into human patients[42,43]. Additionally, implantation of larger tumor pieces, as seen in many patient-derived xenograft models, can be physically traumatic for the animal, potentially leading to skewed or inaccurate results. Alternatively, animals that have been genetically modified into expressing specific proteins in order to induce cancerous-like growths have also been used to model human cancer as this type of model circumvents the aforementioned implantation issues involving immune system rejection or physical trauma unrelated to the damage caused by implantation. Genetic models are not without their flaws, namely the difficulties in synchronizing or controlling the growth rates of the cancer-like growths. The KPC genetically modified pancreatic tumor model, named for the mutated genes responsible for its behavior, is a murine pancreatic cancer that has modified *KRAS* and p53 expression; the mouse and all of its progeny (the KPC mouse line) develop pancreatic intraepithelial neoplasia (PanIN) lesions over their lives which eventually progresses to pancreatic adenocarcinoma-like growths over time[44]. Despite the near-guaranteed production of pancreatic neoplasms, KPC mice may not develop tumors at the same time, or the subsequent growths may grow at varying unpredictable rates. KPC mice typically begin showing

PanIN lesions around 8-10 weeks of age, whereas evidence of invasive pancreatic ductal adenocarcinoma materializes around 14-16 weeks of age. Additionally, though pancreatic tumors in KPC mice are reminiscent of human cancers, KPC tumors do not present desmoplasia (extracellular matrix protein and fibroblast overgrowth) to the same degree as that in humans. As a result the KPC pancreatic tumor model is not suitable for evaluation of therapeutic test candidates, a process that required synchronized, consistent, and predictable tumor growth to design experiments with rigorous control and test animal numbers to discern therapeutic and toxicologic impact of test articles.

Other novel pre-clinical research has focused on modeling subsets of systems, including organoids produced from adult stem cells and "Organs-on-a-Chip". Organoids, as their name may suggest, are pseudo-replicants of individual organs using single or multiple cell types. Given the appropriate growth medium and matrix, adult stem cells were found to divide and differentiate into 3-dimentional multicellular constructs; these constructs were found to resemble their parent tissue in terms of stable genetic and protein expression, though the overall tissue lacks the cellular organization and organ sub-structures required for a normal, functional organ[45]. Organoids can be developed from both normal and cancerous tissue, allowing for comparative investigations into cell expression data[46]; some pre-clinical work has been performed in organoids, though the field is still new and has frequent problems in translating results to real human patients[47]. Another novel avenue of pre-clinical research is modeling individual organs through use of a plastic "chip" to promote the growth, differentiation, and organization of administered cells into organ-like structures[48]. Unlike organoids, the chip platform allows for structural assembly of the cells to develop similarly to natural organs, making them immediately more applicable for potential research involving the use of anticancer compounds and how they can affect specific tissues or

mutations. Work into these "organs-on-a-chip" has been promising with current work focusing on linking together multiple "organs" to simulate more complex environments[49].

Though models are rarely a complete replication of the human patient, they have been employed for years in the successful develop of new drugs, including those in the PD-L1 ecosystem[50], an important new class of drug targets that have greatly advanced cancer treatment for a variety of sub-types, demonstrating the usefulness of the practice. . KPC mice and other genetic models do not fully model cancers due to their late and progressive onset around 4 months of age; though nearly guaranteed to produce pancreatic tumors, individual mice have different tumor growth rates, making them less favorable to use than more predictable models. We were fortunate to obtain the Pan02 murine pancreatic cancer cell line as it is one of the few available immortalized pancreatic cell lines; other lines, like the MIA PaCa-2 and PANC1, are derived from human tissues and are thus require less useful immunodeficient animals. Thus, a main gap in modeling human cancers through mice is the inability to properly synchronize and grow murine tumors in immunocompetent mice as only KPC mice and immunodeficient mice are currently employed at large.

1.3. Pathophysiology of Leukemia - Blood Cancers

Leukemia is a very broad term for a large group of blood cancers typically originating from malignant bone marrow cells. Normally, stem cells in the bone marrow are constantly dividing and differentiating into new leukocytes to replenish those naturally lost over time in the blood at a controlled rate. In other words, each blood cell type derived from stem cells has its own set turnover rate. Due to some mutations, some blood cell-type become leukemic with the inability to differentiate into their mature terminal forms that cause overpopulation in blood or inability to

control leukocyte production rates, or a combination of both. Both mechanisms in dis-regulation of blood white cell balance can result in patient leukemic or blood cancer[51].

The severity and incidence of forms of leukemia may vary with the patient's age. Regardless, acute aggressive forms of leukemia like acute lymphoblastic leukemia (ALL) occur primarily in children whereas other less aggressive chronic forms like chronic lymphocytic leukemia (CLL) are more likely to occur as the patient ages[51,52]. Risk factors for leukemia are broad and include exposure to environmental carcinogens, like radiation, as well as genetic elements due to how common family history of the disease exists in diagnosed patients[53,54,55].

1.3.1. Pharmacological interventions for Leukemias- Current Strategies and Limitations

Early forms of leukemia treatment were centered around broadly toxic anticancer drugs that carried significant off-target patient side effects. Small molecule drugs, including chlorambucil, fludarabine, and cyclophosphamide, are proven to be effective for leukemias but exhibit dose-limiting and significant, severe toxicities to many tissues in the body, thus impacting patient quality-of-life[56,57]. Major turning points for leukemia treatment were the introduction of the tyrosine kinase inhibitor (TKI)[58] and B-cell receptor signaling pathway inhibitors[59], which were able to greatly improve patient outcomes as well as carried far fewer off-target toxicities. These novel compounds, which are targeted to specific tumor-associated proteins, function at molecular level and are thus referred to as being "molecularly targeted" or a "targeted therapy". Targeted therapy has made in-roads in replacing traditional chemotherapeutics in treating many forms of leukemia. These molecularly targeted agents (such as venetoclax and zanubrutinib) are favored by prescribers and patients as safer choices with less untoward effects when given chronically, as many forms of leukemia are treated as a chronic illnesses requiring more than 5-10 years therapeutic commitments to remain in remission and good health[7,8]. Exciting novel

advancements in leukemia treatment within the last 5 years have further improved treatment outcomes with the real possibility of treatment-free remission being available for many patients. Unfortunately, the long-term commitments required for long-term daily oral dosing are not a true cure; resistance to drugs may occur at any point in treatment, and daily oral dosing can be burdensome for patients, leading to pill fatigue. Strategies to circumvent these issues are expanded in the next section concerning modern treatment strategies for pancreatic cancer and leukemia.

1.4. Modern Treatment Strategies for Pancreatic Cancer and Leukemia

Modern chemotherapy treatments for virtually all forms of cancer have shifted towards utilizing drugs targeted to specific enzymes and molecular motifs and away from broadly toxic drugs and treatments that indiscriminately damage patient cells and tissue. Chemotherapy, itself, is often a limiting factor in determining optimal cancer treatments due to the frequently heavy drug burden that patients must endure in addition to their illness. Targeted agents used in modern chemotherapy regimens alleviate much of their off-target toxicities by ideally only killing their target cells and leaving patient tissue relatively healthy, which allows more patients to tolerate and chronically use these drugs longer than the traditional broadly toxic ones.

Most forms of leukemia have greatly benefitted from advances in small molecule drugs, with many being orally active, allowing for less invasive regimens that patients can self-administer outside of a hospital setting. Biologic advancements in antibody therapeutics have also allowed for more patient freedom due to the very long half-lives and resulting long term effects from a single subcutaneous injection or intravenous infusion, meaning patients only need monthly administrations.

Pancreatic cancers have not experienced the same positive developments as treatments for blood cancers. The lack of significant development in small molecule and biologic drugs is primarily due to the unique tumor morphology and late diagnosis of the cancers, resulting in an often-unresectable cancer with few treatment options at diagnosis. Though some headway has been made with certain small molecule drug regimens, these treatments remain untargeted and broadly toxic, severely limiting their application in most patients. Radiation therapy has also been mostly discarded due to a general lack of benefit in clinical trials. As a result, most current research into pancreatic cancer has focused on combinations of classically effective drugs with newer targeted agents.

1.4.1. *Current Therapies for Pancreatic Cancer*

Introduced into clinical use in 1995, gemcitabine is the first drug to show significantly improved efficacy against pancreatic cancer over the previously used drug, 5-fluorouracil. Gemcitabine monotherapy extends patient medium overall survival to 6 months from diagnosis, a 50% increase over 5-fluorouracil monotherapy[60]. The typical administration regimen is 1,000 mg/m^2 weekly for three out of four weeks per cycle, delivered each time as a 30-minute intravenous infusion; these frequent administrations are a consequence of the drug's fast clearance from plasma (roughly 1 hour)[61]. Gemcitabine is a small molecule drug that is toxic to human cells due to its nucleoside analogue structure. Due to this similar structure, gemcitabine molecules can enter cells by taking advantage of naturally occurring nucleoside transporter proteins on cellular surfaces, including hENT1 (human equilibrative nucleoside transporter 1) and hCNT3(human concentrative nucleoside transporter 3), which are located throughout the body[61].It is structured similarly to cytidine, save for two fluorine substitutions on the sugar ring that, when tri-phosphorylated, prevents chain elongation during DNA replication. A less significant contribution

to its toxicity is due to the diphosphate form of gemcitabine, which can somewhat inhibit Ribonucleotide Reductase, an enzyme responsible for replenishing cellular concentrations of deoxynucleotides, thus increasing the likelihood of incorporating gemcitabine into DNA replication machinery over regular nucleotides[62].

Despite its limited understanding in mechanisms of actions against various cancer types, and fast half-life, injectable gemcitabine monotherapy has remained largely unrivaled since its introduction. Though only a few advancements have been made in treating pancreatic cancer, most involve gemcitabine. One of the most effective combination therapies is the combination of gemcitabine with paclitaxel, a taxane that stabilizes microtubules and prevents cytoskeletal reorganization, a pivotal process required for cell division and replication. Though paclitaxel monotherapy is only weakly effective against pancreatic cancers[63], it has significant synergy with gemcitabine, increasing median overall survival to about 8.5 months[21]. In addition to its base toxicity, paclitaxel is also thought to inhibit Cytidine Deaminase, the enzyme primarily responsible for the metabolism of gemcitabine[64]. Paclitaxel, originating from Pacific Yew tree bark, is a very hydrophobic drug with a complex organic structure, resulting in a half-life ranging from 30 minutes to about 6 hours after a 30-minute infusion[65]. To circumvent the high clearance, paclitaxel is now typically administered in a nanoparticle form, known as Abraxane or *nab*-paclitaxel, that is composed of human serum albumen protein in order to extend clearance by avoiding natural elimination process within the particle[66]. Though combination regimens of drugs can be excessively taxing for patients, gemcitabine in combination with *nab*-paclitaxel is generally well-tolerated by most patients, making it a common first-line treatment for pancreatic cancer even if it is not considered the most pharmacologically effective regimen[21].

Other attempts at combination therapies involving gemcitabine have not been very successful. Due to a general lack of options, radiation is often employed in treatments against pancreatic cancer; as a result of the pancreas's central location in the body and the common occurrence of widespread metastases, radiation is not terribly effective against cancer growth and worsens patient well-being[67]. Attempts to combine radiation therapy with gemcitabine were not successful as the combination reduced life expectancy compared to gemcitabine monotherapy[68]. Erlotinib and capecitabine have been approved for combination use with gemcitabine, though erlotinib is only able to extend median overall survival by 1 week compared to gemcitabine monotherapy[18], and capecitabine is usually only used in conjunction with gemcitabine as a neoadjuvant prior to tumor resection attempts[69].

The most pharmacologically effective drug regimen available today is the FOLFIRINOX regimen as it can extend median patient survival to about 11 months, nearly doubling the expected overall survival compared to gemcitabine monotherapy[20]. Its name is a portmanteau of its multiple active constituent agents: folinic acid (leucovorin), 5-flourouracil, irinotecan, and oxaliplatin. Unfortunately, FOLFIRINOX is an extremely burdensome regimen, greatly limiting the number of patients that can tolerate both it and the cancer. Oxaliplatin, folinic acid, and irinotecan are each administered via intravenous infusion over 90-120 minutes followed by an additional bolus and 48-hour infusion of 5-fluorouracil. The regimen is delivered once every other week for an initially undetermined length of time with cessation occurring at either cancer elimination, progression, or deterioration in patient health. Despite its improved theoretical efficacy, FOLFIRINOX is used less frequently than gemcitabine in conjunction with *nab*-paclitaxel due to heavy off-target toxicities[70].

1.4.2. Current Therapies for Leukemia

In recent years, many forms of leukemia have received dramatically improved treatment outcomes. The best possible outcome of modern leukemia treatments is to extend treatment-free remission (TFR) as much as possible following the discoveries that many patients ceasing chemotherapy treatment did not show signs of remission for over 5-10 years after cessation. Originally observed in patients with chronic myeloid leukemia (CML) taking tyrosine kinase inhibitor (TKI) therapy in 2004[71], TFR was later confirmed to be possible through the STIM and TWISTER clinical studies around 2010[72,73,74]. TKI's are a broad class of targeted small molecule drugs that inhibit very specific enzymes or group of enzymes (from the tyrosine kinase family) present at higher levels in cancer cells, greatly reducing off-target toxicities that are seen in broadly toxic regimens while maintaining significant toxicity against cancer cells. In many forms of leukemia, neoplasia is caused by improperly differentiated cells due to the overexpression or absence of specific checkpoint proteins[51,52]. TKI's are able to target and inhibit specific developmental pathway proteins, eliminating the production of new cancer cells and stabilizing the disease. Largely due to these targeting abilities of TKI's, this mode of therapy has replaced a previously popular and effective treatment consisting of stem cell transplantation. Allogenic hematopoietic stem cell transplantation (alloHSCT) has been considered a cure for various forms of leukemia due to the complete elimination of a patient's cancerous cells through intense radiotherapy; many forms of leukemia result from stem cells producing poorly differentiated cells that can accumulate in a patient's body, resulting in leukemia. In alloHSCT, radiation is used to fully eliminate a patient's stem cell population in their bone marrow, effectively destroying their immune system as well as the cancerous stem cells, followed by the regrowth of the patient's bone marrow cells through the implantation of a donor's stem cells. Though this treatment is virtually a cure for leukemia, this treatment is highly burdensome for patients, and many patients may not

qualify due to the highly specific requirements needed to find a matching donor[104]. With the advent of TKI's, alloHSCT is primarily relegated to treating patients that have relapsed due to TKI resistance events. Recently, TFR has been induced in others forms of leukemia, including hairy cell leukemia[75], acute promyelocytic leukemia[76,77,78], acute myeloid leukemia[79], and chronic lymphocytic leukemia[80], indicating the potential to treat and potentially cure a variety of leukemias using the same or similar small molecule TKI's and similar targeting drugs.

Chronic lymphocytic leukemia (CLL) is caused by the overproduction and buildup of B cells in the patient's bone marrow. CLL is considered a milder form of leukemia due to its very gradual onset and lack of significant symptoms in early stages. As a person ages, their stem cells will naturally accrue mutations over time, resulting in an increased likelihood of cancer in the elderly compared to the young. CLL is a common product of these mutations, but its progression is slow with many patients not beginning any form of treatment until symptoms develop or worsen. Conventional chemotherapy has difficulty reaching distant tissue compartments where malignant cells reside while also avoiding significant off-target toxicities in the patient due to the extremely high doses required to reach these areas. As such, CLL was treated as more of a chronic illness than a true cancer for decades[81]. However, recent developments in Bcl-2 (B-cell lymphoma 2, protein regular of cell apoptosis) and BTK (Bruton's tyrosine kinase, protein regulator of B cell differentiation) inhibitor small molecule drugs have enabled new treatment regimens that can reliably reach and kill their target leukemia cells, resulting in a real opportunity for treatment-free remission. While Bcl-2 is a widely expressed family of proteins that promotes cell survival across numerous cell types[82], BTK is a protein crucial for B cell development and differentiation from stem cells[83]. Normal Bcl-2 function can vary between both inducing or inhibiting apoptosis, though Bcl-2 mutations in leukemia typically result in the prevention of apoptosis in blood cells, leading

to neoplastic growth. Numerous inhibitors have been developed for BTK, including the widely used ibrutinib[84], though zanubrutinib is the newest and likely safest BTK inhibitor developed so far[85]. While BTK inhibitor monotherapy has been examined as a second-line treatment for patients with treatment-resistant cancers[86], its novel use as a first-line combination therapy with a Bcl-2 inhibitor has become a likely first-line candidate for future CLL patients as seen in the ongoing SEQIOUA trial[87]. As previously mentioned, combination therapies are preferred over monotherapies due to the decreased likelihood of emerging drug resistance, and leukemia treatments are no different. Venetoclax is an orphan drug that was only recently developed for use against cancer[88]. It targets Bcl-2 by mimicking one of its binding proteins, BH3, that would normally promote cell survival through the activation of Bcl-2. By itself, venetoclax has been weakly effective against some cancers, but its real value comes from its combination regimens where it can greatly potentiate the regimen through a coordinated multi-target attack on the cancer. When administered with zanubrutinib, these two drugs together can greatly treat the disease while minimizing off-target toxicities[87]. Both drugs are orally active, allowing for patients to self-administer, though this naturally leads to a high clearance rate for both drugs as well as the risk of improper patient adherence. Though research is early, this combination regimen may result in treatment-free remission of CLL.

Acute myeloid leukemia (AML) is a family of cancers derived from the improper differentiation of precursor myeloid stem cells in bone marrow[89]. One notable subset of this family is acute promyelocytic leukemia (APL), a significantly more aggressive form of the disease characterized by specific stem cell mutations. When it was first characterized in the 1950's, patients with APL were estimated to have a median survival time of one week. However, with modern treatments consisting of all-trans retinoic acid (ATRA) combined with arsenic trioxide, a

potent chemotherapeutic agent, median patient survival has greatly improved, with over 80% of patients expecting to survive more than ten years after the initial diagnosis[90]. ATRA promotes the terminal differentiation of cancerous cells into their benign, fully-differentiated forms that can no longer divide and propagate without limit; however, while ATRA can deplete the pool of cancerous cells, an additional chemotherapeutic agent is needed to simultaneously kill the cancer cells at their source. Arsenic trioxide (ATO) was found to be the most effective agent, and so ATRA + ATO treatment has effectively produced treatment-free remission in most APL patients[76]. ATRA is an orally-active compound as opposed to the intravenous administration required for ATO. As such, even though the regimen is highly effective, it is dependent on the patient's willingness and ability to self-administer ATRA throughout treatment. Poor patient adherence is seen in virtually every form of self-administered treatments, often due to reasons beyond the patient's control, including fatigue, faulty memory, and the unwillingness to subject themselves to further treatments[91].

"Treatment-free remission" is synonymous with a true cure for leukemia, which has become possible in some leukemia patients due to the advent of tyrosine-kinase inhibitor (TKI) small molecule drugs and other novel therapies. Unlike their broadly toxic predecessor drugs, TKI's are designed for the inhibition of one specific enzyme or closely related family of enzymes to exert their anti-cancer effects. As previously mentioned, cancers are classified and treated based on their protein expression profiles, enabling various targeting regimens consisting of either single or combination therapies of small molecule and/or biologic drugs. Numerous TKI's and novel treatment methods have been developed in the last 20 years, allowing for an almost-personalized ability to treat the disease in the many patients who present it. The primary limitation for these ground-breaking drugs is that they are typically orally active and thus require patients to take the

drug daily at home for as many years as they are alive following their initial diagnosis. Pill fatigue is well-documented across myriad chronic diseases, which is understandable due to the physical burden that these drugs present. Altogether, this suggests that the next step in advancing leukemia treatment is to utilize these highly effective drugs and regimens to develop long-acting formulations. With a true cure for leukemia that does not require a full immune system restart through stem cell replacement therapy is within reach, the final frontier of treatment appears to be developing a form of drug or drug system capable of enabling long-term delivery that is not reliant on daily patient regimen adherence.

1.5. Novel Long-Acting Treatments and Strategies for Cancer and Chronic Disease

Many chemotherapeutic regimens utilizing small molecule drugs fail in patients due to insufficient delivery of the component drugs to the appropriate tissue and cellular locations at sustained and therapeutic meaningful levels. Even with very high intravenous dosages of drug, limited distribution into organs and distant compartments can restrict adequate accumulations of the drugs, limiting toxicity against cancer tissue; instead, the drugs are typically shuttled to clearance organs, like the liver and kidneys, where they exert their toxicities instead of the cancer locations. Intravenous infusion or subcutaneous dosing of a small molecule typically results in significant plasma concentration fluctuations, with peak C_{max} concentrations often reaching toxic or near-toxic levels and C_{min} levels being well below effective concentrations, both of which impede the patient's tolerability to treatment. These complications can mostly be attributed to the very fast clearance of small molecule drugs, which requires more frequent administrations; as a result, patients experience frequent peak and trough drug concentration-related issues. The therapeutic window or index is a range of plasma concentration at which the administered drug is both strong enough to be an effective anti-cancer agent while remaining weak enough to not

severely hurt the patient[92]. As opposed to multiple repeated administrations with severe peaks and troughs, a main goal of drug delivery has been to maintain the chosen drug concentrations over time within the therapeutic window for as long as possible without damaging the patient. In addition to the global drug levels measurable in the blood and plasma, it is important to consider the fraction of drug or drug-in-combination localized and exposed specifically to cancer cells, which is the target and goal for clearing cancer from the body. As a result, many formulations now exist to maximize the effects of constituent small molecule drugs via the extension of their half-lives and enabling deeper penetration into target tissue, which will be a major goal of this thesis dissertation.

1.5.1. *Goals and Strategies for the Sustained Delivery of Small Molecule Drugs*

Sustained release drug delivery systems are developed with the intent to reduce peak and trough plasma drug concentration fluctuations and to reduce frequency of dosing. Long-term, sustained delivery is achieved through the association of the component small molecule drugs to a form of scaffolding composed of lipids, carbohydrates, or other biodegradable or biocompatible molecular structures, including biologics. The effect of this scaffolding is to allow the small molecule drugs to evade clearance by preventing their full dissolution into patient plasma or subcutaneous space; depending on the desired method of association, small molecule drugs may be covalently bound to the scaffold structure or may simply attached through intermolecular electrostatic forces. Most base structures used for long-term delivery are synthetic polymers/nanoparticles, biologic nanoparticles, or antibody-drug conjugates (ADC's).

Synthetic polymer molecules have been used to modulate long-acting drug release for several decades. Some of the most commonly used types of synthetic polymers are polylactic acid (PLA), polylactic-co-glycolic acid (PLGA), and polyethylene glycol (PEG); recently, poloxamer

338 has also been implemented. Introduced in 1985, Lupron Depot was one of the earliest approved long-acting treatments for prostate cancer; it functioned by creating solid microspheres out of PLA, leuprorelin, and several excipient compounds[93]. These microspheres were solubilized and injected subcutaneously to create a depot of drug that would slowly release systemically over 1 month following administration. A 3-month formulation was also developed, but relied on using PLGA instead of PLA due to concerns with acid build-up at the injection sites when using too much PLA[94]. Acid formation at the depot site has long been a concern for PLA and PLGA products, limiting their applications for very long-term delivery despite their usefulness at shorter intervals[95]. CABENUVA, a novel treatment for HIV prevention and treatment consisting of rilpivirine and cabotegravir, uses the same principles as PLA and PLGA products, but instead uses the more patient-tolerant polymer poloxamer 338 to form its microspheres[96]. Liposomes and micelles developed from synthetic PEG molecules have also been extensively used in the clinic, but unlike the solid microspheres, these bubble-like nanoparticles can be administered intravenously rather than subcutaneously or intramuscularly. PEG molecules are very hydroscopic, allowing for the creation of a shell-like layer of water molecules around them. When bound to individual molecules or when present as entire micelle or liposomes, the water shell created by the PEG molecules shields its attached compound/inner cargo from immune recognition and enzyme clearance, thus greatly extending the half-lives of associated drugs[97]. Doxil, a liposomal formulation containing doxorubicin, was one of the earliest forms of liposomal drug delivery[98]; the liposomal shell containing the doxorubicin payload was able to deliver the drug over a long time (as opposed to a free formulation) while reducing buildup and clearance, enabling fewer drug administrations and significantly less off-target toxicities in patients.

Biologically derived compounds have also been used to enable long-term delivery of drugs, with the most common form being antibody-based systems. The underlying logic of using biological components is that they may be more tolerable to patients than exogenous alien compounds that the body is unfamiliar with. Antibody-drug conjugates (ADC's) mimic naturally occurring immunoglobulin proteins that can freely circulate in plasma without clearance due to immune response or significant enzymatic degradation as a base structure[99]. Due to their very long circulation times, immunoglobulin G (IgG) is the typical base choice when designed an ADC. In addition to their very long circulation times, antibodies can also be designed to target specific protein motifs or structures, enabling a highly targeted delivery system that can function over an extended period of time. The drug or small protein-of-interest can be covalently bound to the antibody utilizing an ester or another bond that is easily broken *in vivo*, allowing for the drug to release and exert its effects once the antibody base has found and bound its target motif[100]. The first ADC approved in the United States was gemtuzumab ozogamicin in 2000, a drug system called Mylotarg®[101]. Though Mylotarg® was later pulled from the market due to inferiority compared to other products, multiple ADC's have since been introduced, including drugs like trastuzumab[102] and cetuximab[103], that are still used today as first-line drugs. Another interesting biologic delivery mechanism is the formation of nanoparticles from biological compounds to create an immunologically inert nano-carrier. As previously discussed, paclitaxel is a widely used drug in chemotherapy for myriad cancers due to its ability to inhibit microtubule function in fast-growing cells. However, due to its highly hydrophobic structure, free paclitaxel is difficult to formulate without toxic excipients; additionally, paclitaxel has an extremely high clearance rate following intravenous administration, greatly limiting its applications[65]. To overcome this high clearance and poor solubility, nanoparticles developed from human serum albumen were

successfully developed. This albumen nanoparticle system is known as *nab*-paclitaxel or Abraxane® in a clinical setting[66]. In brief, paclitaxel is solubilized into an albumen solution, which is then freeze-dried, re-hydrated, and size reduced to create albumen nanoparticles containing paclitaxel. Due to the human origin of albumen, these nanoparticles are resistant to immune and enzymatic clearance, allowing for a prolonged delivery of paclitaxel.

1.5.2. *Intra-Peritoneal Infusion Chemotherapy*

A surprising innovation in ovarian cancer came from a treatment that delivers anticancer drugs directly to the peritoneal space in a method reminiscent of dialysis, called Hyperthermic Intra-Peritoneal (infusion) Chemotherapy (HIPEC)[26]. With HIPEC, cancer drugs are dissolved in a sterile, isotonic solution that is warmed to body temperature before it is infused into the patient's peritoneal cavity via a tube connected to a peristaltic pump. The solution fills the cavity, at which point a second tube can begin to drain the cavity fluid to be re-heated while it is simultaneously re-administered to the patient, creating a loop. This occurs over the course of about 30-60 minutes and was initially used as a form of neoadjuvant prior to attempted surgical resection of ovarian tumors.

Ovarian cancer is similar to pancreatic cancer in that tumor growth typically goes undetected until the cancer has reached late-stage progression at which point metastases and interrupted organ function become more apparent. As a result, patients with ovarian cancer face similar treatment difficulties as those with pancreatic cancer, including the need for treatments capable of treating both the primary tumor and metastases as well as treatments that are mild enough to be tolerated by already-weakened patients. Despite the somewhat archaic methodology of HIPEC, the treatment was both effective in delaying patient symptom-free progression and rated favorably by the patients who received it[27]. The median recurrence-free survival was over 3.5

months longer in the group receiving HIPEC therapy before surgery compared to surgery alone. More significantly was the difference in overall survival with patients receiving HIPEC plus surgery averaging 46 months versus 34 months in patients receiving surgery alone. Due to some similarities between ovarian and pancreatic cancers, HIPEC approach may be considered for the treatment for metastatic pancreatic cancer. With good adaptation for ovarian cancer care with HIPEC intraperitoneal infusion, the proposed intraperitoneal therapeutic injection (not infusion that may require visit to hospital and clinic for extended time) for pancreatic cancer treatment candidate described in this thesis, the proposed drug combination and targeted pancreatic therapy may be feasible and likely to improve patient acceptance.

1.5.3. *TLC-ART101 and Related Nanoparticles as a Long-Term Delivery Platform for Antiretroviral and Anticancer Drugs*

As mentioned earlier, the TLC-ART program and laboratory at the University of Washington has demonstrated the ability to synthesize lipid nanoparticles capable of associating with both hydrophilic and hydrophobic drugs through a novel method of controlled solvent removal and subsequent nanoparticle size reduction and homogenization in aqueous buffer. In brief, drugs are co-dissolved with lipid excipients into organic solution, followed by solvent removal via either rotary evaporation or spray drying. The resulting powder can be dissolved into aqueous buffer and homogenized, producing drug combination nanoparticles (DcNP's)[24]. Despite their lipid base structure, DcNP's bear a similar resemblance to grains of rice with a longene shape rather than liposomes or micelles. Figure 1.1 displays a graphical model of these nanoparticles utilizing the hydrophilic gemcitabine and hydrophobic paclitaxel as drugs representative of varying hydrophilicities. The core of the DcNP's is thought to be hydrophobic, attracting the less soluble drugs, while the outer layers containing PEGylated polar head groups attracts hydrophilic

drugs. These two layers allow the incorporation of drugs across a wide range of LogP values (i.e., aqueous solubility). In the TLC-ART101 formulation, this allows for the association of tenofovir (LogP = -1.6), lopinavir (LogP = 4.7), and ritonavir (LogP = 5.2).

An additional facet of the DcNP's is the ability to avoid extravasation into blood vessels following a subcutaneous injection due to their individual particle size, instead slowly entering the lymphatic system[5,23]. At the end of their production, DcNP's are sized to a length of 40 nm, which is too large for simple diffusion after a subcutaneous injection. In HIV infection, the virus is thought to evade drug treatment by hiding in sanctuary sites throughout the body, including the lymphatic system, preventing a full cure from traditional antiretrovirals. DcNP's are able to enter the lymph nodes and lymphoid tissue sites, where they can deposit their drug load over time and better suppress HIV infection. The outer PEG decorated DcNP serves as a protective hygroscopic shell for the drug-combination nanoparticles, limiting their systemic clearance and preventing any immune response from targeting the nanoparticles. These properties have led to the investigation of these nanoparticles as a potential long-term treatment for HIV in humans with early trials in non-human primates yielding positive results.

Building on this platform and understanding of lipid-drug and DcNP particles ability to stabilize drug combination of desperate properties, anti-cancer drug combinations were engineered and tested to provide stable formulation for evaluation in metastatic breast cancer treatment. Mu, *et al* from our research team have reported that anti-cancer drug combination nanoparticles composed of gemcitabine and paclitaxel, two commonly used chemotherapeutic agents with significant off-target side effects and high clearance rates[25], can be assembled to be stably associated with DcNP and formulation do not require complicated and costly removal of free-and-unbound drugs. The resulting anti-cancer DcNP's provide longer plasma drug concentration

profile compared to each parent drug when administered intravenously. In an aggressive metastatic 4T1 tumor model in mice, the anticancer drug combination nanoparticles significantly reduced the lung tumor nodules formed by 4T1 metastatic tumor advancing into the lung at levels that cannot be accomplished with equivalent dose of non-DcNP parent drugs. In an additional study to probe potential mechanisms, the team discovered that DcNP's were able to shield the drug molecules from metabolic transformation[25]. Also, with a combination of experimental and pharmacokinetic modeling approaches, additional mechanistic insights were reported about how drug combination nanoparticles are able to protect their drugs over time *in vivo,* leading to enhanced localization of synchronized exposure in the 4T1 tumor laden lungs. This work demonstrates the potential of the lipid-stabilized drug-combination nanoparticles, specifically DcNP platform for use in other forms of cancer, including cancers of the blood and pancreas.

1.6. Hypothesis and Aims of this Thesis Research

The overarching goal of this thesis research is to develop and characterize a drug combination dosage form that will be stable, effective, and safe to provide next-generation treatment for pancreatic and blood cancers. These factors will be assessed using a pre-clinical model of cancer to examine the formulation effect *in vivo*. To accomplish this goal, this thesis focuses on 3 aims to test the following hypothesis:

An anti-cancer drug-combination nanoparticle product can be made and demonstrated to exhibit long-acting pharmacokinetics that is also suitable for the treatment of currently uncurable pancreatic cancer.

Aims to test this hypothesis are as follows:

Aim 1: To develop and characterize a novel, reproducible syngeneic pancreatic tumor model suitable for preclinical testing that will enable evaluation of drug-combination efficacy and safety.

Aim 2: Develop, characterize, and examine the anticancer effects of drug combination nanoparticles containing venetoclax and zanubrutinib.

Aim 3: Evaluate the feasibility of gemcitabine and paclitaxel drug combination nanoparticles as a potential pancreatic cancer treatment.

As some of the methods for DcNP have been published[23,24,25], readers are referred to these manuscripts for details. As previously mentioned, there is no currently available animal model of pancreatic cancer in which tumors can be consistently (~100%) formed at the pancreas without requiring invasive surgeries or variable genetic induction; in this dissertation, we have searched and discovered a novel pancreatic tumor cell model and route of administration combination to consistently produce a cancer-like disease state in pancreas tissue and suitable for therapeutic evaluation, as described in Chapter 2. This novel orthotopic pancreatic cancer model has been developed and characterized utilizing the Pan02 cell line in C57/BL6 mice. Leveraging the DcNP technology, we have evaluated feasibility of transforming short-acting molecularly targeted drug combination into long-acting injectable drug combination product in DcNP dosage form. The study results for this aim 2 is described in Chapter 3. The development and characterization of this pancreatic cancer model was used to test the inhibitory effects of the nanoparticles on the model to demonstrate the anticancer effects of the DcNP's while also providing the first pharmacokinetic data for intraperitoneally administered DcNP's (Chapter 4). Finally, this thesis reviewed and summarized the overall accomplishments as well as potential future research direction concerning

the pancreatic cancer model and the two forms of drug combination nanoparticles for translating into synchronized delivery of drug combinations that are more effective and tolerable for cancer patients.

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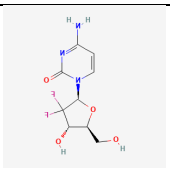
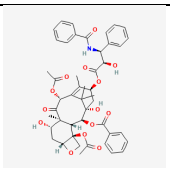
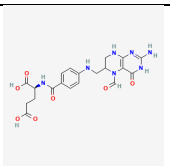
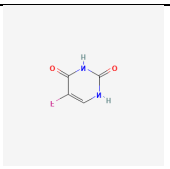
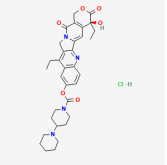
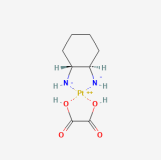
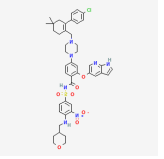
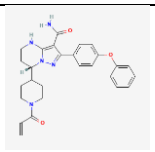
Table 1.1 Pharmacologic Properties of Nanoformulation Candidate Small Molecule Drugs

Regimen	Cancer Use	Drugs	Target or Mechanism	Molecular Weight (Da)	LogP	Structure
Gem and Ptx	Pancreatic	Gemcitabine	Nucleoside analogue	263.2	-1.5	
		Paclitaxel	Microtubule Inhibitor	853.9	2.5	
FOLFIRINOX	Pancreatic	Folinic Acid	Potentiates 5-FU	473.4	-1.2	
		5-Fluorouracil	Nucleoside Analogue	130.1	-0.9	
		Irinotecan	Topoisomerase Inhibitor	586.7	3.94	
		Oxaliplatin	General Cytotoxicity	397.3	-0.47	
VZ Combination	Leukemia	Venetoclax	Bcl-2	868.4	8.2	

		Zanubrutinib	BTK	471.5	3.5	

- Structures courtesy of PubChem public database

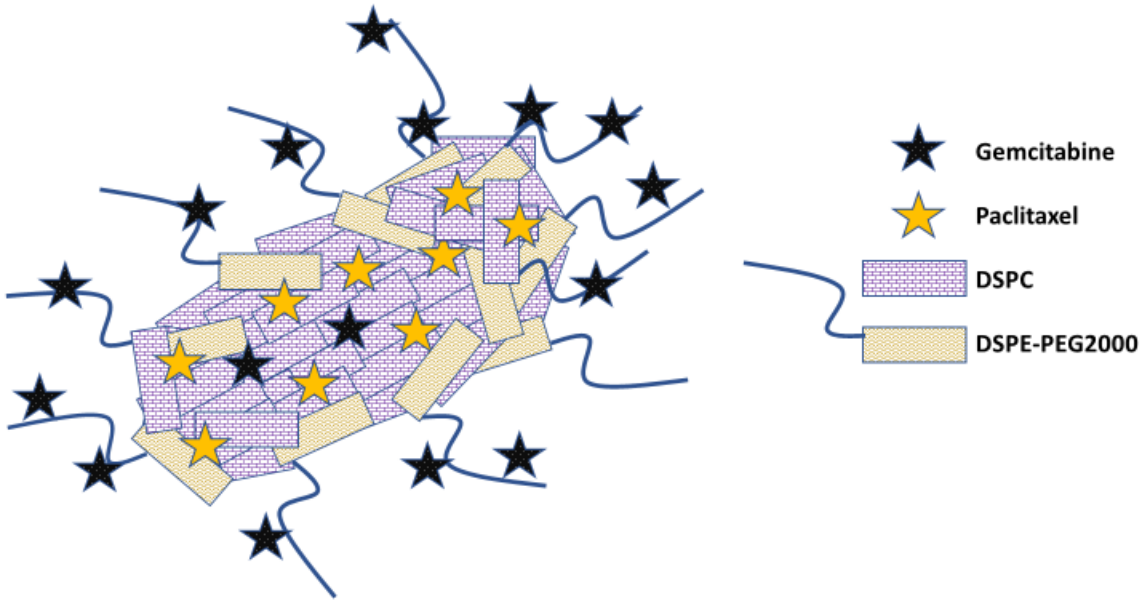


Figure 1.1 A graphical representation of the structure of a drug combination nanoparticle (DcNP) containing the anticancer drugs, gemcitabine (black star) and paclitaxel (orange star). Note the different distribution patterns of the constituent drugs: hydrophobic paclitaxel molecules remain within the similarly hydrophobic lipid core, while hydrophilic gemcitabine is primarily located within the outer water "shell" generated by the very hydrophilic polyethylene glycol (PEG) groups covalently attached to the lipid base structure. The DcNP is a solid, longene-shaped lipid nanoparticle of about 40 nm in length, which is dissimilar to the bubble-like structures observed in micelles and liposomes.

Chapter 2. Inoculation of Pan02 Cells Produces Pancreatic Tumor Nodules in Mice: Design and Characterization of a Novel Orthotopic Pancreatic Ductal Adenocarcinoma Model that May Enable Therapeutic Evaluation

2.1 Abstract

Preclinical models of cancer are vital for assessing and predicting efficacies and toxicities of novel treatments prior to testing in human subjects. While many pancreatic tumor models exist, they require long and unpredictable growth periods with resultingly varied tumor size, implantation in non-native sites, or requiring direct implantation with surgery that may illicit confounding inflammatory responses. A murine model of metastatic pancreatic cancer that is (1) reproducible, (2) time-efficient, and (3) can be grown in a wildtype animal with a fully functioning immune system is needed for improved future preclinical research into pancreatic cancer. In searching for such a model, we found that Pan02, a murine ductal pancreatic adenocarcinoma cell line derived from a cancerous pancreatic lesion in a C57BL/6 mouse, can be administered to C57BL/6 mice to produce tumors in the mouse pancreas. We found that only the introduction of Pan02 cells by intraperitoneal injection and not intravenous injection led to the attachment and growth of Pan02 in the mouse pancreas before subsequently spreading to other tissues. Time-course tissue analysis indicates that the Pan02 cells first home to, infiltrate, and grow within the pancreas, producing a pancreatic tumor model. This model appears to mimic late-stage metastatic pancreatic cancer in humans. This is the first reported use of Pan02 cells to produce orthotopic pancreatic and metastatic neoplasms in a mouse model without the need of tumor implantation within matrices or survival surgeries. This orthotopic pancreatic tumor model, with consistent tumor take, synchronized tumor development and survival, and predictable outcomes, may enable and accelerate preclinical evaluation of treatment candidates for pancreatic cancer.

2.2 Introduction

Despite 30 years of life-extension advancements with small molecule and biologic drugs for many types of cancers, there has been little change in life-expectancy for patients with pancreatic cancer. Starting from the day of pancreatic cancer diagnosis, less than 50% of patients survive for 1 year or more with less than 5% alive at 5 years[1]. Even with a relatively lower incidence rate than other cancer types (such as breast or lung), the higher mortality rate has led to over 500,000 annual pancreatic cancer deaths reported worldwide. The number of annual deaths attributed to pancreatic cancer is roughly equivalent to its incidence rate. In contrast, other more common cancers do not share these high mortality rates; for example, even though breast cancer is diagnosed in roughly 300,000 people per year in the United States alone, the mortality rate is a mere fraction of these diagnoses (only about 45,000 deaths per year). The cause of this discrepancy is partly due to (1) the anatomical location and morphology of tumors within the pancreatic tissue that limits the uptake and retention of drug molecules through limited vascularization and a high pressure internal environment due to overgrown extracellular matrix proteins (known as desmoplasia)[16], (2) long-circulating monoclonal antibody molecules that, while accessible to the tumor, are limited to cell surface-mediated functions, and (3) limited information on druggable targets for pancreatic cancer. Numerous antibody drugs targeted to specific cancer cell surface markers have been developed and employed in patient care, including the revolutionary trastuzumab for cancers of the breast and stomach that targets HER2 (human epidermal growth factor receptor 2); however, pancreatic cancer usually lacks the surface markers that have been so useful in the treatments of other cancers. Molecularly targeted small molecule drugs (typically intended to block specific enzymes or receptor functions) have also been introduced for the inhibition of specific tyrosine kinases or related proteins that control cell growth or differentiation

with the intent of restricting and preventing cancer cell propagation. While pancreatic tumor cells may express some of these druggable molecular targets, the low-to-moderate levels of target protein expression means that the drugs that work for other cancer type may be insufficient to significantly improve treatment outcomes of pancreatic cancer. A low response rate may be partly attributable to frequent late-stage diagnosis where pancreatic cancer has progressed to metastatic disease by the time of diagnosis. As such, current pancreatic cancer treatments are relegated to broadly cytotoxic yet highly potent small molecules. The small molecule treatments are often complicated and require multiple treatment cycles with different drugs combinations, resulting in significant, intolerable untoward side-effect for patients[2,3,4]. Drug intolerability and drug burdens are often cited by patients as to why they discontinue treatment, limiting potential therapeutic effects and treatment response. Intolerability and significant side-effects limit current chemotherapeutics' effectiveness in a real-world setting. Therefore, there is an urgent need for developing tolerable, safe, and highly potent treatment product for pancreatic cancer. Such a novel product candidate would need to undergo rigorous evaluation in cell culture work *in vitro* and appropriate animal models of pancreatic cancer in order to support pre-clinical testing for the determination of dose and potential treatment outcomes before progressing to human testing.

Pre-clinical testing of novel treatments is vital to the development of new drugs. Despite the advanced capabilities and predictive abilities of modern computers for designing novel drugs, difficulties with accurately predict how a living system will react to a foreign compound remain[5]. Additionally, predictive pharmacokinetic models typically require pre-clinical data from animals to inform and scale drug dosage appropriately for future work involving pharmacokinetic and tissue distribution profile in human[6]. Pre-clinical models are very effective even though there are

myriad differences between human and pre-clinical model gene expression and resulting physiology[7].

Unfortunately, there are limited preclinical animal model available for use in pancreatic cancer research, and those model that are available are typically not reflective of a true pancreatic cancer disease state that could enable therapeutic evaluation in a consistent and accelerated manner. Table 2.1 lists a number of pancreatic tumor models have been previously reported to enable efficacy assessment of drug candidates with some major limitations. The distinctions and limitations of the listed mouse pancreatic tumor model will be discussed below.

As listed in Table 2.1., the three most common types of pancreatic cancer models are patient-derived xenografts (PDX), genetically modified animals that are more predisposed to certain forms of cancer, and models utilizing immortalized human and murine cell lines to grow orthotopic (within the same tissue of origin, i.e., pancreatic cells growing in the pancreas) or heterotopic (an accessible area, for example beneath the skin or as a subcutaneous tumor mass) tumors in a live animal. These models and their limitations are explored below.

PDX models, in which human-derived tumors are implanted in immunodeficient mice, are unappealing due to their dependence on primary human cells taken from patients; these models can be unpredictable and are difficult to scale up for experiments involving larger animals and animal numbers needed for each test group[9]. One model by Principe, *et al* involved the creation of a primary cell line, G-68, from a human patient, which was then implanted into severely immunodeficient mice, the NSG strain, to create an *in vivo* environment suitable for drug testing. This was successful, though the long development of the primary cell line as invasive surgical implantation limits its use in larger studies. Additionally, while drugs are capable of attacking and killing cancer cells, the host immune system is also though to play a role in the clearance of both

healthy and dying cancer cells, meaning that the immune system and foreign drugs can work together against the cancer[7]; the lack of an intact immune system removes this element, which may lead to an underprediction of the drugs' cancer killing abilities within the context of a healthy human.

Genetically modified models of pancreatic cancer in mice that can mimic the conditions presented by human pancreatic cancer have also been described in the literature, with one of the most common being the KPC strain[8]. The animals used in genetic pancreatic mouse tumor models must be screened shortly after birth to verify they possess the required genetic defects; even with these defects present, it is common for some mice to still not develop tumors. These complicate breeding schemes and genetic screening add cost, time, and uncertainty of tumor development time. In addition, genetically modified mice that develop pancreatic tumors may not represent the complex array of mutations that exist in genetically heterogenous human tumor pancreatic tumors. Though these genetically modified animals are likely to develop tumors at some stage in life, tumors can begin growing at different times and at different rates, even for mice in the same litter[8].

Immortalized cell line-based models derived from pancreatic neoplasms are available for the development of an orthotopic model of pancreatic cancer, though not all are useful in the context of intraperitoneal tumor growth and subsequent treatment. Human lines, like Panc1 and MIA PaCa-2, are valuable for their similarity to natural human tumors; however, they can be difficult to grow, even in immunodeficient mice[10].

To date, all cell line-based models of pancreatic cancer in mice have required the use of a matrix to hold or stabilize the injected cells during implantation into pancreatic tissue, a requirement that does not represent a natural tumor environment and also requires invasive survival surgery, as seen in Jiang, *et al*'s model[11] and Markovic, *et al*'s model[12]. A murine

pancreatic cell line that can be grown in a "close-to-wildtype" strain of mouse without any foreign matrix would be ideal for generating tumors *in vivo* as these mice are often inexpensive and are immunocompetent, making them more similar to a human host than an immunodeficient mouse. Additionally, a cell line that could be monitored externally through luminescent imaging without disrupting or euthanizing the mouse would also greatly help with model development. Unfortunately, murine cell lines of pancreatic cancer are limited and a simple cell-based orthotopic model has not yet been reported in the literature. In summary, current pancreatic mouse tumor models of pancreatic cancer are limited to unreliable genetic models or invasive models that require surgical implantation of foreign cells or tissue into the tissue of interest. These models can be unpredictable in terms of both growth rates and time-tumor as well as cost-prohibitive, meaning that pancreatic cancer research would greatly benefit from a novel model that can circumvent these problems.

To develop a pancreatic tumor model in immunocompetent mice that mimics pancreatic cancer growth in pancreas tissue in a consistent, predictable manner, we have evaluated the ability of Pan02 cells, a pancreatic ductal adenocarcinoma cell line originally derived from cancerous pancreatic tissue in a C57BL/6 mouse[13], to form pancreatic tumors in an *in vivo* setting. Pan02 cells carry many common mutations also seen in human pancreatic cancers, including *Muc1* and *Muc4*[15], genes coding mucin proteins that can be overexpressed and lead to the commonly seen desmoplasia, or overgrown extracellular matrix proteins, in human patients. Unexpectedly, we found that intraperitoneal inoculation of Pan02 cells resulted in significant Pan02 tumor uptake and growth, starting in the pancreas before spreading to other tissues. Due to the consistent and nearly 100% tumor take-rate of Pan02 cells, these cells can be used to form an orthotopic mice model that may enable therapeutic and immunologic evaluation of pancreatic cancer treatments.

2.3 Materials and Methods

2.3.1 Materials and Cell Culture

Pan02 cells that were previously transfected to express luciferase as a luminescence marker were a generous gift from Dr. Mien-Chie Hung of MD Anderson in Houston, Texas. Cells were cultured in DMEM/F12 (1:1, high glucose) media (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 250 µg/mL G418 sulfate (Fisher Scientific, Pittsburgh, Pennsylvania, USA). A G418 or neomycin resistance gene was included in the luciferase transfection plasmid, resulting in the positive selection of Pan02 cells with plasmid when grown in the presence of G418; this ensures the continued expression of luciferase in culture. These Pan02 cells can be externally monitored and tracked via luminescence following an administration of luciferase substrate, D-luciferin (Sigma-Aldrich, Burlington, Massachusetts, USA), which can provide luminescence both *in vitro* in cell culture as well as in a living mouse and murine tissue with an IVIS imaging instrument (described below). Gemcitabine ("G"; CAS: 95098-81-4) and paclitaxel ("T"; CAS: 33069-62-4) were purchased from LC Laboratories of Woburn, Massachusetts.

2.3.2 Verification of Pan02 Cells' Luminescence Signal in Cell Culture

Pan02 cells, described above, were first grown overnight in a black 96-well plate (Corning, Corning, New York, USA) at 10 different concentrations: 80,000, 40,000, 20,000, 10,000, 5,000, 2,500, 1,250, 6,100, and 0 cells per well. These were assayed against 5 different concentrations of D-luciferin (300, 150, 30, 5, and 0 µg/mL) dissolved in cell culture media to confirm and quantify cell luminescence capabilities. 15 minutes following

the introduction of luciferin to the cells, the culture plate was placed into a Lumina II IVIS (*In Vivo* Imaging System) instrument (PerkinElmer, Waltham, Massachusetts, USA) to measure luminescence over a 30 second imaging window. Bioluminescence data, along with digitized images was collected and integrated using Living Image software, also from PerkinElmer.

2.3.3 Effects of Gemcitabine, Paclitaxel, and Fixed Dose-Combination in inhibit Pan02 Pancreatic Adenocarcinoma Cells

To determine if Pan02 cells were susceptible to the same chemotherapeutics used in a clinical setting, Pan02 cells were seeded into each well in a 96-well plate and allowed to grow overnight. Varying concentrations of gemcitabine and paclitaxel, alone or in combination, were used as representatives of clinically used chemotherapeutic agents to determine dose-response of Pan02 cells to these drugs. Drugs were examined either independently or in combination (ratio of gemcitabine to paclitaxel was 10:1, 1:1, or 1:10; range: 0-500ng/mL gemcitabine) in triplicate. Pan02 cells were incubated with the media and varying concentrations of chemotherapeutics for 5 days. Media was then removed and replaced with a media containing 10% AlamarBlue (Fisher Scientific, Pittsburgh, Pennsylvania, USA) to determine drug effects on cell viability. The conversion of AlamarBlue into its metabolite by active cellular mechanisms was quantified using a fluorescence spectrophotometer (excitation: 530-560; emission: 590nm), generating relative cell inhibitory response. Dose-cell viability response curves were fitted with an Emax model to estimate mid-point of drug concentration that reduced growth of cells by 50%, also known as IC_{50} values.

2.3.4 Route of Pan02 Inoculation on Tumor Establishment and Tissue Localization in Mice

To evaluate if and to what extent the route of tumor inoculation contributes to pancreatic tumor take-rate and distribution, we first purchased C57BL/6 (SPF free) mice of 4 weeks of age from Jackson Laboratory (Bar Harbor, Maine); Pan02 cells were derived from C57BL/6 mice, so the two can be considered "syngeneic", enabling cell growth in the mouse without immune system interference. All mice were the same sex (female) to remove variability due to sex differences; additionally, female mice were selected to remain consistent with our previous nanoparticle work in breast cancer[14]. Mice were divided into 2 groups of 5. In each group, 4 mice received a single Pan02 inoculation dose of 10 million Pan02 cells (depending on group, intravenous or intraperitoneal) in 200uL of sterile Hank's Buffered Salt Solution (HBSS; Thermo Fisher Scientific, Waltham, Massachusetts, USA), while the 5th mouse would receive an equivalent injection volume of sterile HBSS. Cells were administered in the first group as an intravenous dose through the tail vein, while the second group received an equivalent intraperitoneal dose. Mouse health was assessed through a combination of daily weight monitoring, body scoring, and IVIS imaging to characterize disease progression and to assess the extent of cancer growth *in vivo*. Mouse imaging with the IVIS took place every 3 days until either 28 days following initial inoculation or before the mice reached euthanasia criteria (ie, excessive tumor burden of more than 10% of body mass, more than a loss of 20% body weight, or other obvious indicator of suffering). To systematically evaluate Pan02 cell (expressing luciferase) in the mice body, we followed the bioluminescence marker, luminescent light produced by luciferase, after dosing with the substrate D-luciferin. Briefly, mice were first given intraperitoneal injection (0.15mg D-luciferin per 20g mouse), and IVIS image were collected 12 minutes after substrate dosing; bioluminescence IVIS images were based on

a 3-minute exposure. These data were expressed as fluorescent units, which are directly proportional to the amount of light emitted.

2.3.5 Effects of Cell Number on Pancreatic Tumor Development in Mice

To evaluate cell dose response on tumor take-rate and subsequent tissue distribution, 15 mice were divided into 3 groups of 5. 4 of 5 mice in each group received a single, intraperitoneal Pan02 inoculation dose (1, 5, or 10 million cells, IP) in 200µL sterile Hank's Buffered Salt Solution (HBSS) while the 5th mouse would receive an equivalent injection volume of sterile HBSS (these 3 mice served as no-tumor controls). Mouse health was assessed through a combination of weight monitoring, body scoring, and IVIS imaging to assess cancer progression over time as described above. Mouse imaging with the IVIS (as described above) took place every 3 days until either 28 days following initial inoculation or before the mice reached euthanasia criteria.

2.3.6 Histopathological Analysis and Serum Biochemistry in Mice Inoculated with Pan02 Pancreatic Ductal Adenocarcinoma Cells

To prospectively determine the time-course and extent of cancer cell distribution in murine tissues, we intraperitoneally inoculated 12 C57BL/6 mice with 5 million Pan02 cells in 200uL HBSS to follow tumor progression. One mouse remained noninjected as a negative control. Following confirmation of tumor growth using the aforementioned IVIS and luciferase method, mice were randomly divided into 3 groups of 4. Each group of tumor-burdened mice was sacrificed at a different time point after inoculation: 7 days, 14 days, and 21 days. Blood was drawn from mice prior to death, and mouse tissues were placed into 10% neutral-buffered formalin (Richard-Allan Scientific, Kalamazoo,

Michigan, USA) for 24 hours to fix the tissues. Tissues were then washed with 70% ethanol in water for 6 hours, and the ethanol solvent was replaced every 2 hours. These fixed tissues were then sent to Histology Consultation Services (Everson, Washington, USA) for sectioning and classical H&E staining to assess Pan02 cancer infiltration into tissues. The formalin-fixed tissues were analyzed with NIH Elements BR 5.11.01 software on a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan). Blood samples were spun down at 1,000 G for 15 minutes at 4°C to isolate serum. Serum was submitted to Moichor (San Francisco, California, USA) for biochemistry analysis. The data are presented with reference value based on previous work from Loeb, *et al*[17].

2.4 Results

2.4.1 Characterization of Pancreatic Ductal Adenocarcinoma Pan02 Cells with Luciferase Marker

To verify the function of the luciferase enzyme marker expressed in the Pan02 cells, we first evaluated these cells for their luciferase enzyme's ability to catalyze the transformation of D-luciferin substrate into luminescence product. The Pan02 cell study to confirm the presence and high luminescent activity of expressed luciferase enzymes was done in a cell dose-dependent manner as well as in a substrate D-luciferin concentration-dependent manner. As shown in Figure 2.1, we found that Pan02 cells express functional luciferase enzymes in a cell-concentration dependent manner. Within the context of increasing the substrate concentration from 6-300 µg/mL D-luciferin, very low numbers of Pan02 cells (~1.25-2.5 x 10^3) can be detected within 15 min. At 300 µg/mL luciferin, Pan02 cells were luminescent at numbers as low as 1,000 cells. Similarly, at larger cell numbers

(around 80,000), cells were detectable with at least 6 μg/mL luciferin. When administered intraperitoneally for tumor imaging in other models, D-luciferin is dosed intraperitoneally at a concentration of 30 mg/mL, over three orders of magnitude higher than the concentrations used in this experiment, after initial injection. Assuming the D-luciferin is fully taken up into blood over time, the concentrations of D-luciferin in the blood would still be very high at around 1 mg/mL. Thus, a tumor grown *in* vivo using Pan02 cells (themselves present in numbers exceeding several million) would likely produce significant luminescent from a standard dose of D-luciferin, allowing for the ability to detect these cells *in vivo*, making them a very promising cell line candidate. Collectively, these data indicates that Pan02 cells express functional luciferase that convert D-luciferin into luminescence suitable for real-time tracking of Pan02 tumor development and spread in mice. Considering the need to ensure sufficient bioluminescence detectable through the mouse body and tissues, we used 5 x 10^6 Pan02 cells for future *in vivo* mouse studies.

2.4.2 Sensitivity of Pan02 to Pancreatic Anticancer Drugs, Gemcitabine and Paclitaxel

We next determined the sensitivity of the Pan02 cells (with luciferase marker) to two commonly use pancreatic cancer drugs, gemcitabine and paclitaxel. As these two drugs are often used as a combination therapy given in sequence, we have thus explored drug sensitivity alone and in combination. For the combination test, we used different drug ratios to elucidate differences resulting from fixed-dose combinations of the two drugs involving two different pharmacologic mechanisms and sensitivity in Pan02 growth inhibition. In a series of dose-response studies, the degree of inhibition data was calculated through the IC_{50}, mid-point of drug concentrations that exhibit maximum inhibition. The IC_{50} data were summarized in Table 2.2. Pan02 cells strongly responded to gemcitabine

and paclitaxel, both independently and also more favorably in combination. Overall, Pan02 appeared to be very sensitive to gemcitabine (G) with IC_{50} recorded at 3.2 ng/ml (or 10.7 nM). In contrast, the cells are less sensitive to paclitaxel (T) with IC_{50} = 199 µg/ml (or 233 µM). As a result of the differential sensitivity, the overall drug-combination sensitivity appeared to be driven by gemcitabine, regardless of the varying ratios of the two drugs G:T from 10:1, 1:1 or 1:10 (Table 2.2). However, with increasing paclitaxel fraction in the G-T ratio, a trend toward lower (5-to-6-fold gemcitabine IC_{50} value was noted (G:T 10:1 vs 1:10) (Table 2.2). Taken together, Pan02 cells show a high sensitivity to gemcitabine, which is furthermore potentiated by the presence of paclitaxel. In tandem with their expression of luciferase marker that provides high luminescence values, Pan02's susceptibility to these currently use chemotherapeutic agents, gemcitabine and paclitaxel, further supports our intent to use these pancreatic tumor cells to determine whether Pan02 can produce a consistent pancreatic cancer in pancreas of syngeneic C57BL/6 mice.

2.4.3 Route of Pan02 Inoculation on Pancreatic Tumor Development in Mice

With Pan02 cells confirmed to consistently express luminescence marker and that these cells are sensitive to clinically used anticancer drugs, gemcitabine and paclitaxel, we next determined whether inoculating these cells would result in pancreatic tumors in a syngeneic C57BL/6 mouse. While other studies have used the subcutaneous route to establish subcutaneous Pan02 tumors or used surgical cell implantation to establish tumors in the pancreas, none have provided consistent and convenient inoculation methods to provide reproducible and synchronized pancreatic tumors. Therefore, we have evaluated two different Pan02 inoculation routes – intravenous (IV) or intraperitoneal (IP) - using (10 million cells in 200µL) in syngeneic mice. The IV route was intended to mimic

metastatic spread of pancreatic cancer cells that could form metastatic tumor nodules in the lung capillary (as in breast cancer models), while the IP route was intended to evaluate whether Pan02 could grow in the mouse peritoneum or home to its original pancreatic tissue. Mice were split into 2 groups (intravenous vs. intraperitoneal cell inoculation; n = 4; Figure 2.2), and the development of tumors in the mice was monitored by tracking Pan02 luminescence. Mouse body weight did not significantly change over the course of the Pan02 tumors' growth compared to control mice, making weight a poor biomarker for animal health.

The group of mice given intravenous Pan02 did not appear to develop tumors in the lung, pancreas, or in the lungs (Figure 2.2 C). The luminescence analysis of IVIS images revealed that only 1 of the 4 injected mice developed a solid tumor, but it was localized to a single kidney. None of the mice exhibited tumor growth in the pancreas based on tissue analysis and IVIS analysis. The remaining 3 mice showed growths in their tails around the sites of inoculation, but nowhere else in the body. No further tumors or cancer in other tissue was noted during necropsies of the animals in the IV group of mice.

To our surprise, mice inoculated with Pan02 cells through an intraperitoneal injection developed tumors in a consistent and predictable growth rate over 21 days (Figure 2.2 D). All mice developed metastatic cancer-like symptoms as the Pan02 cells colonized and invaded parent host pancreatic issue. Interestingly, all mice initially developed cancerous growths in their pancreas, the origin tissue of the Pan02 cells; these pancreatic growths appeared early in course, around 3-5 days post-inoculation. Despite early tumor growth seen in the pancreas, mouse body weight remained relatively normal for the first 14-17 days. At this point, the cancer rapidly spread through the peritoneum (requiring early

termination within 24 hours to limit animal suffering), indicating weigh loss (or change) may not be useful to estimate pancreatic cancer progression in mice as the effects only become noticeable less than 24 hours before euthanasia. Common metastatic locations included the liver, the entire gastrointestinal tract, and the peritoneum wall, while the kidneys were typically spared from metastases (as seen through IVIS imaging and necropsy). Taken together, the intraperitoneal route of Pan02 provides consistent tumor development in mice, while the intravenous route cannot. Pan02 cells given through the intraperitoneal route appeared to first populate in the pancreas before spreading into other tissues. Given that the simple IP inoculation of Pan02 in mice leads to tumor uptake and growth in pancreas, these data provide the basis for subsequent studies to define dose and pathogenic time-course characterization of this Pan02 pancreatic tumor model as a potential orthotopic model suitable for therapeutic evaluation.

2.4.4 Effects of Pan02 Cell Dose Number on Pancreatic Tumor Development in C57BL/6 Mice

We next determined an optimal Pan02 cell dose number needed to consistently produce pancreatic tumor in this intraperitoneal inoculation model. The intent is to define a dose that is sufficient to produce pancreatic tumors in a majority of mice while also trying to prevent excessive cell numbers that induce a far too-rapid disease progression. Defining an optimal dose would enable consistent and reproducible evaluation of therapeutic effects of a drug product candidate of interest. An additional goal is to determine the number of cells that would reliably induce tumor growth at a steady rate over a 2-3 week growth period, in most, if not all mice, to serve as an orthotopic pancreatic tumor model. Via intraperitoneal injection, mice were administered 1 million, 5 million, or 10 million Pan02

cells (Figure 2.3 panel B). As seen in the initial inoculation experiment with 10 million cells, all mice given Pan02 cells developed detectable cancer nodules within their intraperitoneal cavities within 3 days. The mice shown in Table 2.3 were imaged 7 days after inoculation. Additionally, all mice were shown to develop nodules specifically in the pancreas, typically before other nodules were established. All mice shared similar symptoms as the disease progressed; mice remained largely active and health in appearance until a very rapid decline in health necessitated euthanasia.

Mice given the maximum dosage of cells (10 million) were euthanized by day 18 due to rapid tumor growth, whereas mice given the smallest dose of 1 million cells did not reach euthanasia criteria within the 28-day study window. In contrast, mice given 5 million Pan02 cells developed tumors simultaneously and reached euthanasia criteria at the same time: roughly day 21 following inoculation. In both the 5 million and the 10 million cell groups, tumor distribution at euthanasia was very extensive, resulting in tumor nodules and masses on nearly all organ/tissues, including the abdominal wall and diaphragm. In the 1 million cell group, 2 mice exhibited small traces of the tumors on their pancreatic tissue, while 2 mice had no signs of tumor nodules at euthanasia. If this model is to be used going forward, the best measure for therapeutic efficacy would likely be the change in tumor cell luminescence over time as luminescence appears to remain stable throughout the animal model's lifespan. In other words, a decrease in tumor luminescence would likely correspond to an effective therapeutic's ability to inhibit or even reduce tumor growth. Taken together, a dose of 5 million Pan02 cells given IP provided nearly all mice exhibiting Pan02 tumors in pancreas, and this dose may be useful for generating an orthotopic pancreatic tumor in syngeneic mouse.

2.4.5 Histological Analysis of Tissues and Serum Chemistry of Pan02 Pancreatic Tumor Model

To further characterize the Pan02 pancreatic orthotopic tumor model, we next determined the time-course of tumor take and pathological consequences of intraperitonially inoculated Pan02 cells; another set of mice was inoculated with 5 million Pan02 cells, and tissues were collected at days 7, 14 or 21. Another objective is to also verify data reproducibility of the results stated above. Blood was collected via retro-orbital bleeding immediately prior to euthanasia to determine changes in serum chemistry due to tumor progression. Histopathological analyses were done on tissues collected at necropsy.

As seen in Figure 2.4 and 2.5, the only notable Pan02 colonization in these mice in non-pancreatic tissues was in the liver and possibly spleen more than 14 days after inoculation based on examination of tissues stained with H&E. A time-sequence analysis indicates that Pan02 cells appeared to minimally colonize and localize only in the periphery of the liver. On day 21, tumor ladened mice appeared to have significant loss of white pulp within spleen which is likely due to the murine body recognizing an active disease state caused by the inoculated cells. Pan02 tumor nodules are apparent in the pancreas as early as 7 days, though tissue invasion does not occur until around 14 days. Pancreatic tissue appears relatively healthy throughout the 21-day period, and infiltration of Pan02 deep into the pancreatic tissue by day 21 is noted (Figure 4D). Notably, pancreatic tissue does not appear necrotic, and tissue is likely functional up until the final stages of tumor progression.

The liver and spleen both show little colonization by Pan02 cells, even at 21 days post-inoculation. Figure 4E demonstrates the minimal colonization of the liver tissue, with only small portions of Pan02 cells visible on the tissue surface with no invasion. The spleen

shows no apparent colonization by Pan02 cells (Figure 4C and 4F) 21 days after inoculation, though an immune response has drastically changed the underlying spleen structure: virtually all red pulp has been overtaken and eclipsed by white pulp, indicating an immune response. This change does not occur until the final stages of cancer progression and is likely a generic immune response to massive cell death and resulting toxic epitopes circulating the body. Pan02 cells likely evade the immune system even at these late stages.

To evaluate the impact of pancreatic Pan02 tumor growth on serum chemistry, enzymatic, and biochemical changes, we have evaluated several biomarker parameters that may relate to respective tissue or organ. The data is presented in Table 2.3 with an n=1 for each value. Based on the day 21 data presented, we noted there are significant change (>50% change in value) in enzymes, such as aspartate transferase and creatine kinase, as well as basic markers like glucose that result from damage to a number of tissues and organs. These values are calculated based on the changes in the parameter between a control mouse given no cancer compared to cancer-burdened mice at day 21. Previously reported standard values from Loeb, *et al*[17] are also given. Serum protein and chemistry as well as liver alanine aminotransferase appeared to remain in the reference range in early days (not shown), before shifting between days 14 and 21. These data suggest that by day 21, there is a Pan02 tumor burden on multiple serum health markers, including blood urea nitrogen, alanine transferase, and total protein, reflecting the overall decline in mouse health. This dataset also supports the notion that most murine host tissues are left relatively functional throughout the cancer model's duration, which is reminiscent of human pancreatic cancer. However, this dataset is limited by the low number of mouse blood and plasma samples that were analyzed, thus requiring further research in order to confirm

these suspicions. Regardless, these changes are also alluded to in figure 2.4 in how cancer does not appear to invade most organs until the model's late stages, thus leaving most tissue function seemingly intact, as seen in Figure 2.5. The data suggests that Pan02 cells can consistently inoculate C57BL/6 mice through an initial pancreatic colonization before development in other nearby tissues. Clearly, a homing mechanic of some sort is at play based on the consistent initial colonization of the pancreas in all mice given Pan02 through intraperitoneal injection, but this requires further investigation as to what factors cause this effect. Figure 2.6 examines the invasion of the Pan02 cells over time in the pancreatic tissue; at day 7, Pan02 tissue is visibly evident (shown as dark purple due to the higher number of cell nuclei and disordered structure of the tumor cells) though has not yet invaded the lighter pink tissue representative of normal pancreatic tissue. By day 14, Pan02 cells have begun to enter the pancreatic tissue as they grow between the diffuse folds of the normal pancreatic tissue. By day 21, Pan02 cells have now fully encapsulated entire portions of the pancreas, effectively cutting them off from other normal tissue in the body and resulting in their disfunction.

Collectively, there time-course histopathological analysis indicates that at $5x10^6$ Pan02 IP inoculation in syngeneic mice produce orthotopic pancreatic tumor consistently by day 7 and would be suitable evaluation of interventional agent. These data also confirm the reproducibility of the model based on IP dosing of Pan02 cell homing to pancreas as a primary tissue target of colonization.

2.5 Discussion

Here we describe a novel murine model of metastatic pancreatic cancer utilizing the Pan02 cell line to reliably generate and produce tumor-bearing mice with a progression and symptom array similar to those observed in human pancreatic cancers for use in pre-clinical anticancer drug development. Importantly, the cells initially colonize the pancreas due to an unexplained homing phenomenon before taking root elsewhere in the murine body; in tandem with the slow progression of symptoms, the Pan02 model's symptoms are reminiscent of the progression seen in human patients, including uninterrupted organ function until very late stages of the model. Following an intraperitoneal of Pan02 cells, tumor nodules are detectable as early as 3 days post-inoculation, with mice typically reaching euthanasia criteria around day 21. This 3-week window provides ample time for future pre-clinical work due to its relatively fast rate of progression as well as harmless external tumor monitoring via luminescent imaging. These aggressive models that can quickly produce significant tumor masses from a single inoculation injection are needed to replicate the aggressive pancreatic cancers seen in human patients.

Pancreatic cancer is notorious for its seemingly rapid progression and its resistance to most modern chemotherapy regimens. Poor outcomes for patients are primarily due to late diagnosis, with most patients presenting metastatic symptoms at diagnosis, greatly restricting already-limited treatment options. Expanded access to pre-clinical models of metastatic pancreatic cancer can be essential to early research and development of novel drugs and treatment systems.

Pre-clinical drug development strategies often begin by exposing novel compounds or drug candidates to cells grown in culture, either to immortalized cells representing a specific tissue or cancer type or primary cells derived from human patients. In a living system, there are myriad factors at play affecting metabolism and distribution of a foreign compound, including different cell types organized in inter-connected tissues and organs in the body. While cancerous and

healthy cell lines are useful to assess the potential toxicity or effectiveness of a test compound, the experiments performed in cell-based in vitro systems lack key attributes of compounds, particularly those barriers relating to drug localization in the cancer-laden tissues and cells. The route of drug administration may also play a role. For example, after oral dosing, drug formulated in pills or tablets must first dissolve in the gastrointestinal tract, penetrate the gut epithelial cells to reach or extravasate into the blood vasculature, which then must distribute through blood to tissues and organs. Intravenous administration bypasses the gastrointestinal barrier, gaining direct access to the blood. In addition to differential compound distribution, uptake, and retention, each compound may be subjected to inactivation (also known as "elimination") by metabolic conversion and excretion. While the pharmacokinetic and disposition profile are considered intrinsic and unique for each compound, they may be influenced by specific drug delivery systems, route of administration or both.

Once a drug candidate is established as sufficiently potent in cell culture *in vitro*, researchers may consider scaling up experiments to more advanced living systems, including organoids and animal models such as mice. Though more similar to a human patient than an *in vitro* cell culture, these systems are still not accurate replicas of humans. Furthermore, these mice used in early pre-clinical work are usually required to be immunodeficient in order to accommodate foreign cancer cells to establish and develop in a living mouse; the lack of this immune system, while helpful to the cells, greatly limits how much results can be translated into human patients as the immune system is thought to play a role in many forms of cancer treatment. The final bridge to human studies from pre-clinical work usually involves "humanized" mice, meaning mice that have been genetically altered or surgically implanted with human cells or tissue; many different versions of murine and human cancers have been modeled in mice in the literature,

but pancreatic cancer has long had few models usable for research. This is an important deficiency as animal models provide unique insights into a foreign compound's pharmacokinetics in a living system that cannot be fully represented in cultured cell lines due to the presence of discrete organs, varying digestive enzymes, and cell surface transporters throughout a topologically diverse environment.

A murine model of metastatic pancreatic cancer that is (1) reproducible, (2) time-efficient, and (3) can be grown in an immune-competent syngeneic animal with a fully functioning immune system is needed for improved future preclinical research into pancreatic cancer. One major limitation of existing pre-clinical models is the reliance on many immunodeficient mouse strains in order to allow the growth of foreign cells from human or other animal tissue. As Pan02 cells were originally derived from the C57/BL6 strain (immune competent in-bread syngeneic strain), C57/BL6 mouse immune systems do not recognize the Pan02 cell as foreign, allowing for its unrestricted growth in the murine body. As previously stated, colonization of the pancreas prior to most other tissue sites is a hallmark of this model, further promoting its use a model for human pancreatic cancer research. Histological and serum biochemistry panels show that most murine tissue remains relatively unincumbered throughout the model progression, again demonstrating similarities with natural human cancers. However, for verification of therapeutic outcomes in animal models, human pancreatic cancer cells or tissues implanted in immune-deficient mouse may be used as a complementary information to ensure the treatment outcomes are relevant for human pancreatic tumor with higher heterogeneity and complexity in tumor development and progression.

Regardless, we were able to provide a consistent method for establishing Pan02 pancreatic tumor nodules in a C57/BL6 mouse to be 5 million cells administered via an intraperitoneal injection in

200μL of sterile Hank's Buffered Saline Solution. Unlike xenograft models that utilize immunodeficient mice or genetic models that are spontaneous and tricky to predict, the Pan02 model of metastatic pancreatic cancer can use immunocompetent mice that generate tumors at apparently equal rates and sizes. Additionally, an active plasmid within the cells additionally contains a gene for the enzyme, luciferase, allowing for noninvasive tracking of Pan02 growth *in vivo.* With these tools at our disposal, we propose that our Pan02 murine model of metastatic pancreatic cancer is a novel avenue of research for future pre-clinical work due to its (1) reproducibility, (2) time-efficient, and (3) can be grown in a near-wildtype animal. This orthotopic Pan02 pancreatic tumor model may serve as simplified and physiologically relevant pancreatic tumor model to evaluate promising therapeutic interventions in an accelerated and immune competent manner.

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Table 2.1 Analysis of Pancreatic Tumor Models Currently Available for Therapeutic Assessment

Model (Example)	**Advantages**	**Disadvantages**	**Gaps in Modeling Ability**
Genetic Modification (KPC strain[8])	Spontaneous	Inconsistent time-to-tumor and resulting difficulty in planning experiments. Often difficulties with breeding due to genetic deficiencies or modifications.	Variability in tumor phenotype
Orthotopic Xenograft (Jiang, *et al*[11])	Can recreate tumor within native environment (i.e., growing pancreatic tumor cells within the pancreas).	Direct implantation of cells (or tissue) requires difficult and laborious survival surgeries	Implant variability can lead to inconsistent tumor take-rates and growth, if any.
Patient-Derived Xenograft (PDX) (G-68 Primary Cell Line in NSG Mice[9])	Using human cells or patient tissue recreates actual tumor of origin for near-direct experimentation.	PDX typically requires subcutaneous inoculation to form/grow tumor as well as immunodeficient animals.	Subcutaneous tumors do not represent actual tumor environment in addition to the lack of normal immunological functions that may have an important role in treatment.
Heterotopic Cell Line Inoculation (Pan02 cells[15])	Inexpensive, predictable, fast, and potentially realistic growth environment with an active immune system. Cell lines may also be transfected with luminescent or fluorescent proteins for noninvasive external tracking.	Cell lines may eliminate transfection vehicles over time *in vivo* once selection agent is absent. Cell lines often have multiple mutations that may not best represent the cancer in question.	Models, while effective, may be purely murine in origin, limiting upscaling ability to other animal models or in human trials.

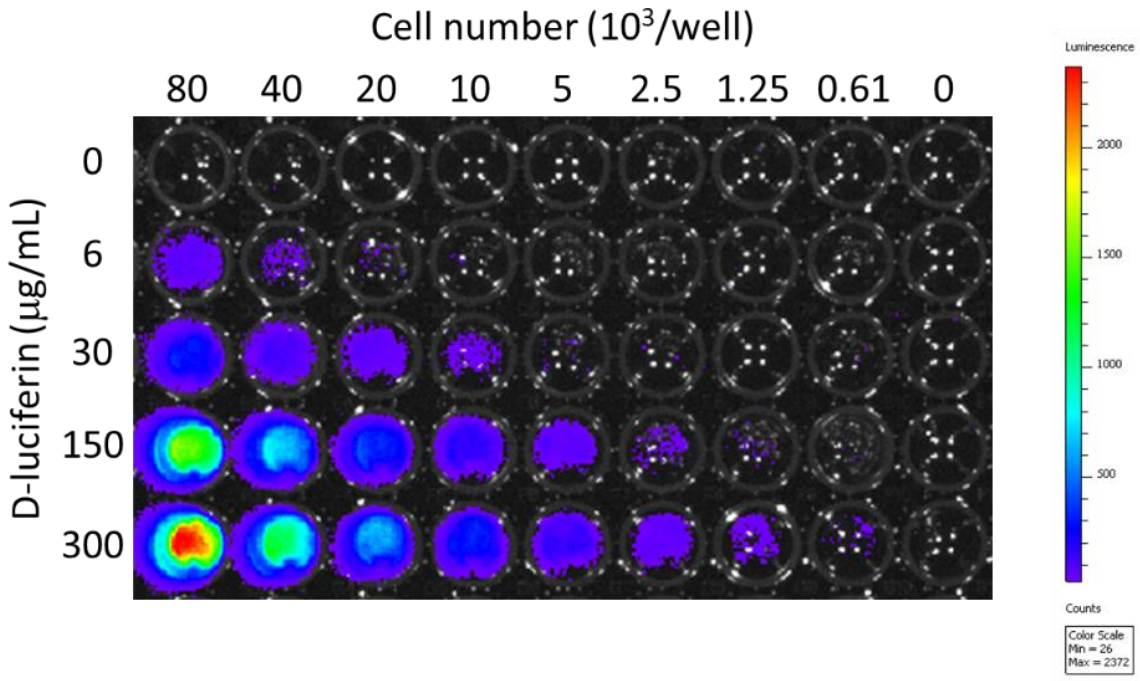


Figure 2.1 Effects of D-luciferin and Pan02 cell concentrations on Pan02 cell luciferase conversion to luminescence. The X-axis reflects 80-0 x 10^3 cells and the Y-axis reflects 300-0 µg/ml D-luciferin substrate in each well. The luminescence product is recorded and expressed in color scale (on the right panel 26-2372 blue-to-red) for reference. The luminescence was recorded and integrated over 30 seconds, 15 min after adding the substrate.

Table 2.2 Sensitivity of Pan02 pancreatic ductal carcinoma to gemcitabine (G) and paclitaxel (P), alone or as combination at different G-to-P fixed ratios. The data are expressed as Effective Dose 50 (EC_{50}) based on an Emax model

	Gemcitabine		**Paclitaxel**	
	EC_{50} (ng/mL)	EC_{50} (nM)	EC_{50} (ng/mL)	EC_{50} (nM)
Gemcitabine Only	3.2	10.7	-	-
Paclitaxel Only	-	-	199,000	233,000
G : T :: 10 : 1	2.8	9.5	0.3	0.3
G : T :: 1 : 1	2.2	7.2	2.2	2.5
G : P :: 1 :10	0.5	1.7	5.2	6.0

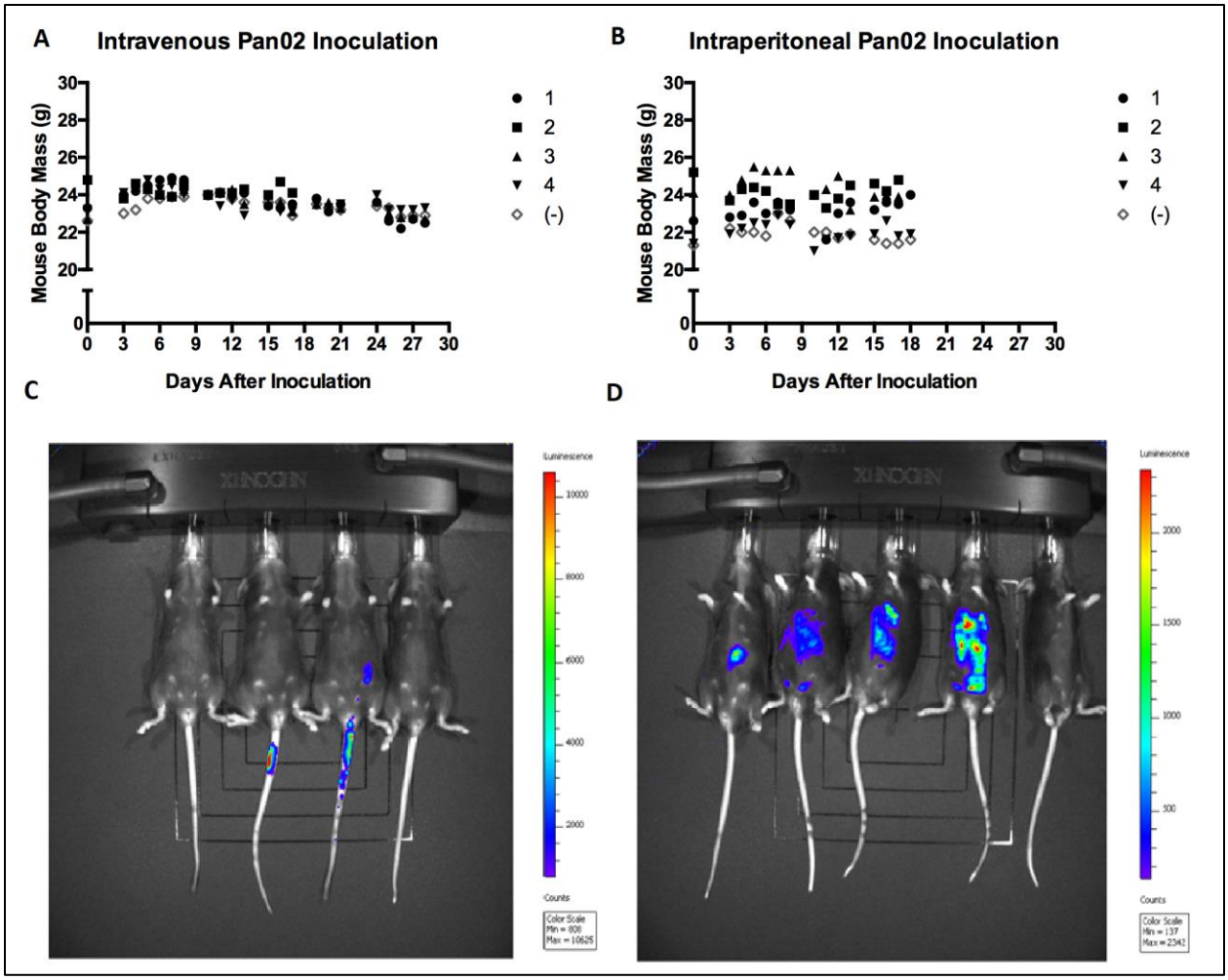


Figure 2.2 Effect of route of administration on Pan02 cells' ability to colonize murine tissue. A and B demonstrate mouse mass (Y-axis) over time from inoculation (X-axis). Intraperitoneal mice were euthanized before the end of the 28-day study period due to cancer burden. Mice given 5 million Pan02 cells intravenously in 200µL HBSS (C) and mice given 5 million Pan02 cells intraperitoneally in 200µL HBSS (D) were scanned at day 17 for luminescence. The luminescence product is recorded and expressed in color scale (right panel displays gradient of color versus the strength of signal, with "hotter" colors like red indicating higher concentrations compared to "colder" colors like blue). Luminescence was recorded 15 minutes after D-luciferin administration, collected over 3 minutes, and integrated into final reported values.

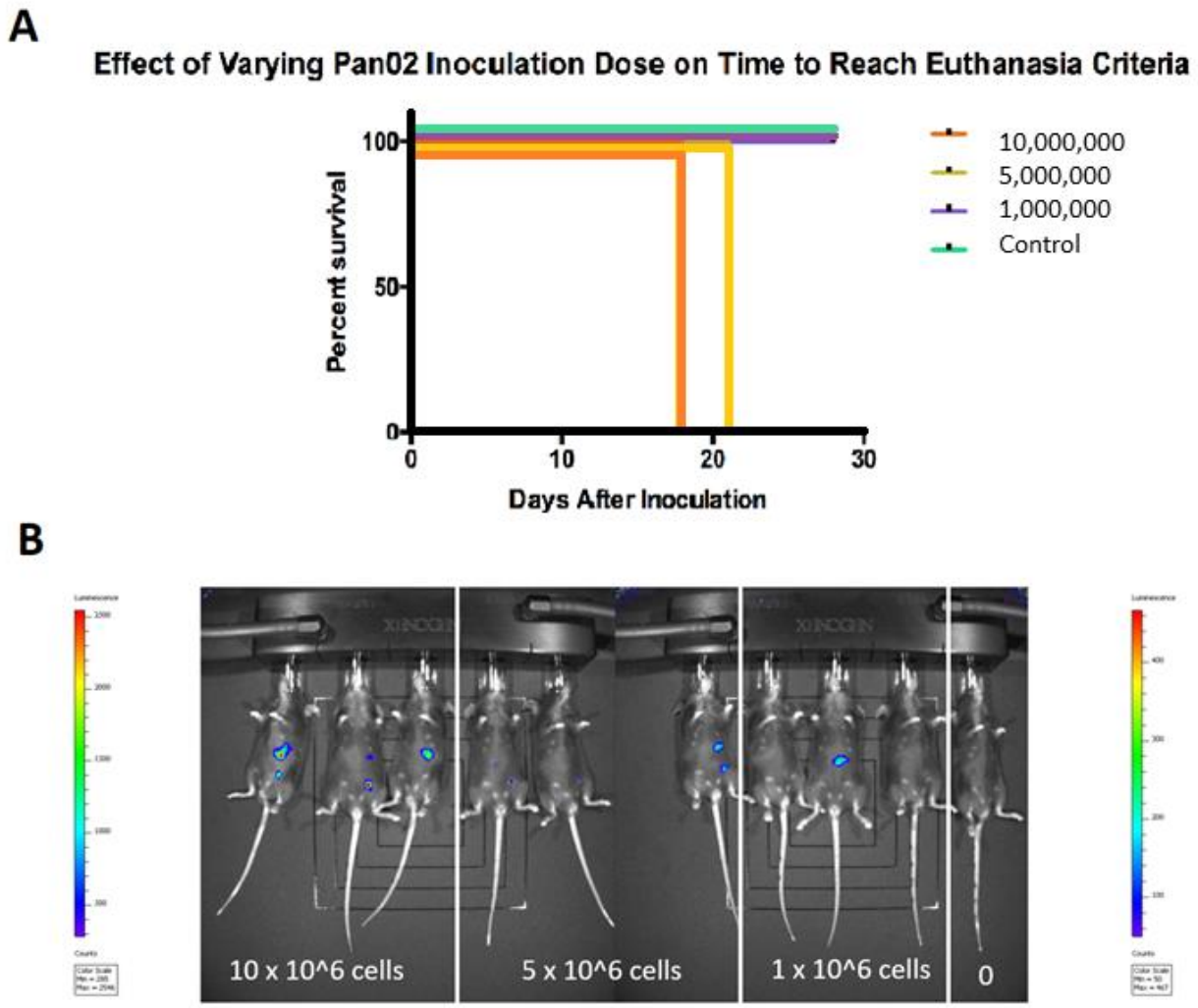


Figure 2.3 Effect of number of Pan02 cells used for Pan02 model inoculation on the progression and time-to-euthanasia of C57BL/6 mice. Mice were inoculated with 1, 5, or 10 million Pan02 cells, which were weighed daily (A). The luminescence product is recorded and expressed in color scale (right panel displays gradient of color versus the strength of signal, with "hotter" colors like red indicating higher concentrations compared to "colder" colors like blue). Luminescence was recorded 15 minutes after D-luciferin administration on day 7, collected over 3 minutes, and integrated into final reported values (B).

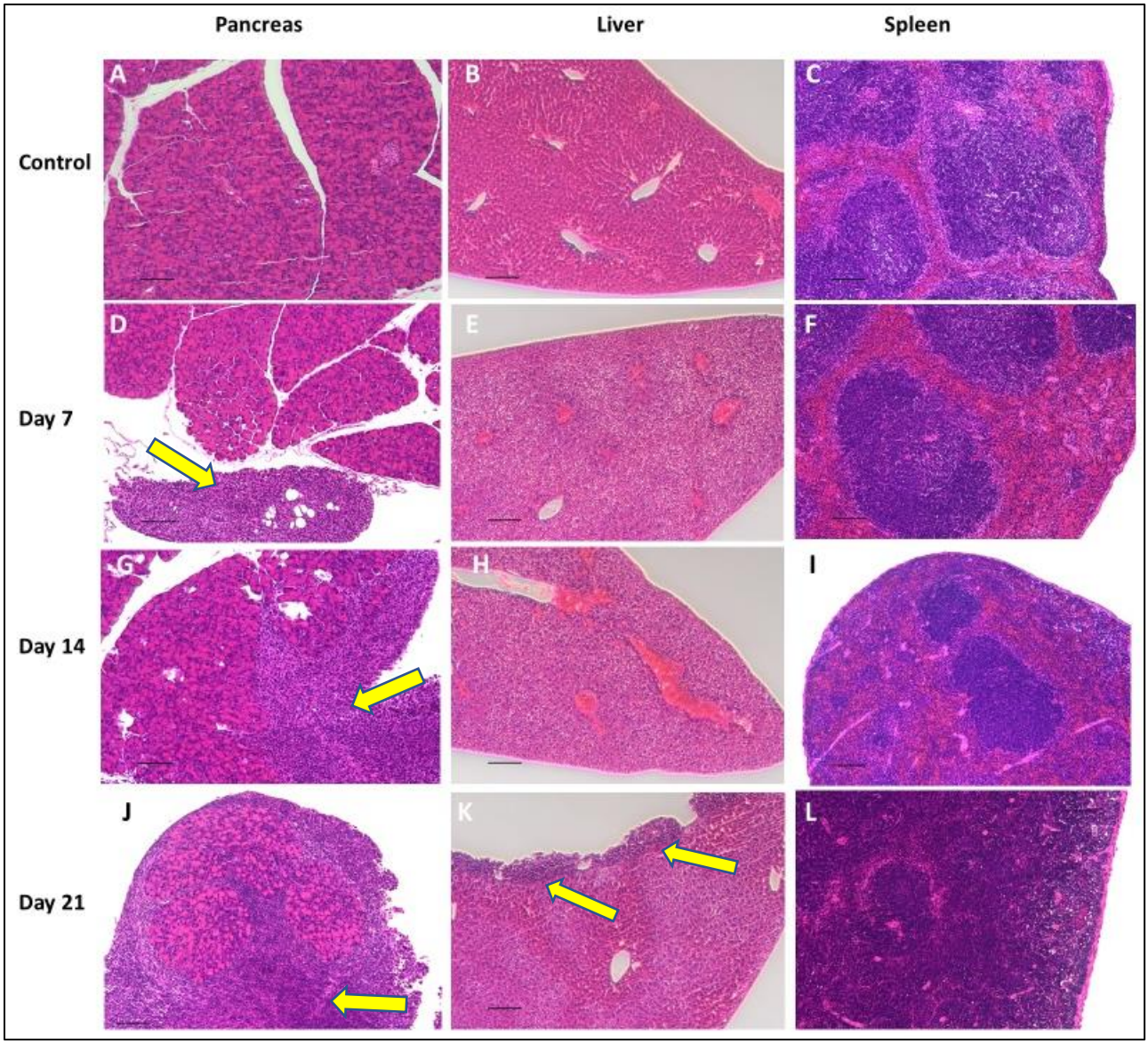


Figure 2.4 Histology of Control and Pan02-Burdened Mouse Tissues. The extent of Pan02 cell-based tumor growth and invasion of healthy tissue 7, 14, and 21 days after a peritoneal inoculation of the cells was compared to control tissue with no tumors using basic H&E staining for cellular structures (bar: 150nm). Arrows indicate the tumor mass (dark purple in the pancreas and liver) growing on healthy tissue (lighter pink in the pancreas and liver).

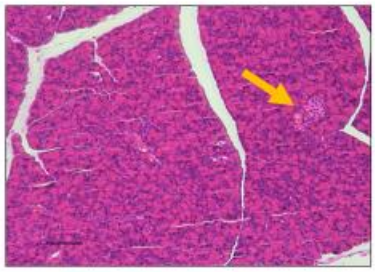
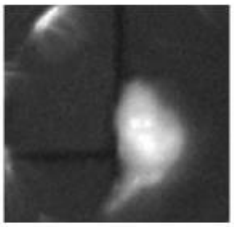
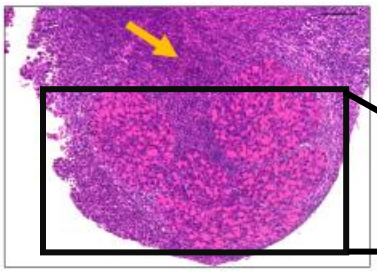
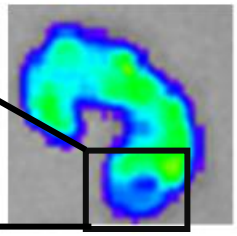
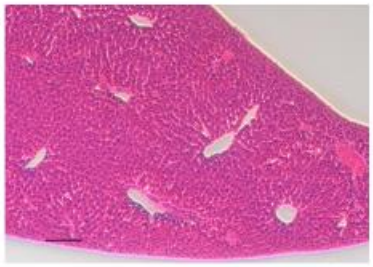
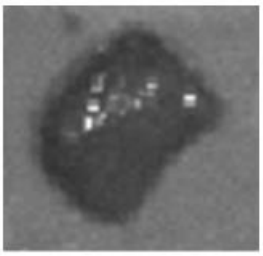
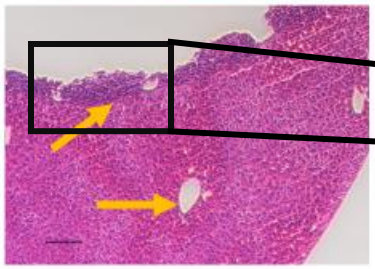
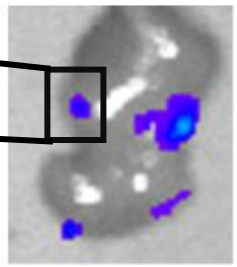
Tissue	Mouse	Histology	IVIS	Notes
Pancreas	Control			Diffuse structure; highly organized sub-structures (arrow: islet)
	Day 21			Solid structure due to cancer encapsulation (arrow: cancer); loss of tissue organization
Liver	Control			Well-ordered tissue
	Day 21			Only surface-level metastases; no disruption to overall tissue structure or function

Figure 2.5 Histology of control and Pan02-burdened murine liver and pancreatic tissue. Pan02 distribution is shown 21 days after inoculation as compared to the control. Note the complete loss of cellular organization in the pancreas by day 21 while the liver only has surface-level metastases, as seen in both IVIS and histological microscope images (bar: 150nm).

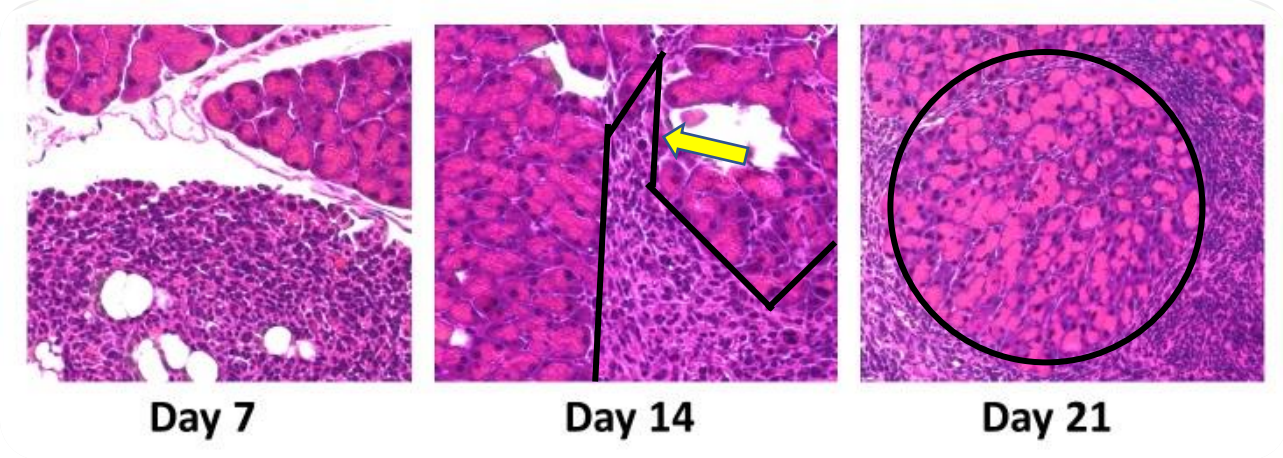


Figure 2.6 Progression of Pan02 tumor mass growth and invasion into pancreatic tissue following intraperitoneal inoculation. Using classic H&E staining, normal pancreatic tissue is shown as a bright pink color in contrast to the disordered, dark purple Pan02 cancerous tissue. Arrow in “Day 14” indicates the invasion of Pan02 cells into the pancreas tissue.

Table 2.3 Serum biochemistry of Pan02 Tumor-Burdened Mice 21 Days After Inoculation

Biomarker	**Related Tissue/Organ**	**% Change from Value at Day 0**	**True Value at Day 21**	**Reference Control Values (Loeb)**
Aspartate Transferase	Liver, muscle tissue	+ 61%	87 U/L	44-221 U/L
Glucose	General, pancreas, stressed tissue	+ 57%	320 mg/dl	60-133 mg/dl
Creatinine	Muscle, kidney	+ 470%	3.4 mg/dl	0.1-1.8 mg/dl
Creatine Kinase	Muscle, general	+ 290%	237 U/L	N/A
Blood Urea Nitrogen	Kidneys, liver	+ 29%	27 mg/dl	2-71 mg/dl
Total Protein	General stressed tissue	- 7.1%	3.9 g/dl	4.6-7.3 g/dl
Alanine Aminotransferase	Liver	0%	27 U/L	14-140 U/L
Sodium	General stressed tissue	- 3.2%	125 mmol/L	153-175 mmol/L
Cholesterol	Liver	+ 15%	69 mg/dl	34-173 mg/dl

Chapter 3. Design and Characterization of a Novel Venetoclax-Zanubrutinib Nano-Combination for Enhancing Leukemic Cell Uptake and Long-Acting Plasma Exposure

3.1 Abstract

Leukemia remains incurable partly due to difficulties in reaching and maintaining therapeutic drug concentrations in the target tissues and cells. Next-generation drugs targeted to multiple cell checkpoints, including the orally active venetoclax (Bcl-2 target) and zanubrutinib (BTK target), are effective and have improved safety and tolerability compared to conventional, nontargeted chemotherapies. However, dosing with a single agent frequently leads to drug resistance; asynchronous coverage due to the peak-and-trough time-course of two or more oral drugs has prevented drug combinations from simultaneously knocking out the respective drugs' targets for sustained leukemia suppression. Higher doses of the drugs may potentially overcome asynchronous drug exposure in leukemic cells by saturating target occupancy, but higher doses often cause dose-limiting toxicities. To synchronize multiple drug target knockout, we have developed and characterized a drug combination nanoparticle (DcNP), which enables the transformation of two short-acting, orally active leukemic drugs, venetoclax and zanubrutinib, into long-acting nanoformulations (VZ-DCNPs). VZ-DCNPs exhibit synchronized and enhanced cell uptake and plasma exposure of both venetoclax and zanubrutinib. Both drugs are stabilized by lipid excipients to produce the VZ-DcNP nanoparticulate (d ~ 40 nm) product in suspension. The VZ-DcNP formulation has enhanced uptake of the two drugs (VZ) in immortalized leukemic cells (HL-60), threefold over that of its free drug counterpart. Additionally, drug-target selectivity of VZ was noted with MOLT-4 and K562 cells that overexpress each target. When given subcutaneously to mice, the half-lives of venetoclax and zanubrutinib were extended by approximately 43- and 5-fold, respectively, compared to an equivalent free VZ. Collectively, these data suggest that VZ in VZ-DcNP warrant consideration for preclinical and clinical development as a synchronized and long-acting drug-combination for the treatment of leukemia.

3.2 Introduction

Roughly 500,000 cases of leukemia are diagnosed worldwide every year, with about 300,000 patients succumbing to the disease [1]. Leukemia is a general group of blood cancers derived from malignant bone marrow cells. Historically, treatments for leukemia have not been able to truly cure the disease due to the cancer's persistence in other systems within the body, including the lymphatic system and lymph nodes, to which cancer drugs may have limited access [2]. Molecularly targeted small molecule and antibody-based drugs have been developed to eliminate malignant cells. However, their cellular and cancer-laden tissue distribution and retention can be limited due to high clearance and tissue barriers [3]. Only a fraction of orally administered small molecule drugs are able to reach the leukemic cell target as significant percentages of such drugs are subject to metabolic or excretory elimination.

Chemotherapeutic agents, including chlorambucil (alkylating agent), fludarabine (purine analogue), and cyclophosphamide (alkylating agent), are effective treatments for leukemia. However, at therapeutic doses these chemotherapeutic agents may carry significant side effects that can limit their application, particularly in weaker or older patients [4]. With the introduction of molecularly targeted agents for specific druggable proteins that are overexpressed in leukemia, an additional safety margin is added. Newer clinical treatments for B-cell leukemia targeted to molecular checkpoints can be divided into three groups of compounds that inhibit the uncontrolled growth of B cells: (1) Bcl-2, a mitochondrial antiapoptotic protein, (2) Bruton's tyrosine kinase (BTK) inhibitors (TKI's), (3) monoclonal antibodies, several of which are targeted to CD20, a surface antigen on B cells [5]. As molecularly targeted agents, these three compounds reduce off-target toxicities compared to earlier drugs, making them a preferred treatment option for patients from a wide demographic range [6]. Biologic drugs have been largely successful, though their

inherent structure limits them to only binding a single target per drug molecule. Resistance events to molecularly targeted single agent treatments are well documented in chronic use. Therefore, combination regimens (with multiple drugs targeted to varying mechanisms of action) are often used to reduce the risk of single-drug resistance [7]. Combination regimens may provide synergy derived from two or more drug substances that both inhibit multiple pathways and improve potency [8]. While the newer molecularly targeted drugs are typically approved as monotherapies, combination therapies with these newer drugs are also being considered for treatment durability.

Zanubrutinib is a second-generation TKI of Bruton's Tyrosine Kinase (BTK) that has been recently introduced and approved by the FDA for several B cell-based blood cancers, including mantle cell lymphoma (MCL). The BTK inhibitor zanubrutinib is currently administered daily in an oral dosage form (considered an attractive treatment for patients). Due to a short plasma half-life of 2–4 h, oral zanubrutinib is administered twice daily to maintain adequate plasma concentrations of the drug [9]. As chronic twice-daily oral dosing may cause pill fatigue, patient compliance is often an issue due to the physically taxing nature of such chemotherapeutics [10]. Zanubrutinib is currently approved for patients with refracted MCL or similar diseases only as a monotherapy treatment, but combination therapy regimens consisting partly of zanubrutinib have been explored in a prominent phase 3 clinical trial, the SEQUOIA study [11]. This study, currently in progress, is focused on chronic lymphocytic leukemia (CLL) treatment; it has so far reported improved progression-free survival in patients receiving zanubrutinib monotherapy compared to bendamustine-rituximab, a commonly used treatment targeting CD20-positive cells. An additional arm of the study is exploring patient tolerance of zanubrutinib in conjunction with venetoclax, a small-molecule inhibitor of Bcl-2; results have been positive, with 50/51 patients responding to

treatment, but the study is ongoing [12]. Studies have also indicated that the zanubrutinib-venetoclax combination can be used on leukemias beyond CLL.

As previously noted, even if oral venetoclax and zanubrutinib can be administered together, the asynchronized peak and time course of the two drugs will not provide consistent, sustained intracellular levels to maximally suppress leukemic cell growth. The percentage of oral drugs absorbed into blood from the gut mucosa is typically lower than that of intravenous injections. Drug metabolic enzymes found in the gut and liver may also reduce the percentage of active drug in the blood, leading to limited drug bioavailability, and, in some cases, sub-therapeutic plasma and intracellular dug levels. As a result, these events may lead to an increased risk of inducing drug resistant cells in tumor sites [13]. In addition, daily (or more frequent) dosing, typically necessary for oral dosage form, can be cumbersome for patients, as high local concentration in the gut after oral dosing may lead to gastrointestinal injury. Additionally, zanubrutinib is typically administered twice daily, which over time can lead to pill fatigue in patients, further limiting the treatment due to missed doses. To address these limitations, we have evaluated the feasibility of a drug delivery system in which lower but sustained therapeutic levels persist in the blood for an extended period of time through the development of a combination of drugs that are targeted to multiple proteins in the leukemic cells. Drug combinations composed of molecularly targeted drug substances could greatly improve both the potency and patient tolerance of the combination drug product.

We have previously demonstrated that DcNP can stabilize combinations of hydrophobic (lopinavir and ritonavir, LogP = 5.9 and 6, respectively) and hydrophilic (tenofovir and emtricitabine, LogP = −1.6 and −0.6, respectively) HIV drugs with amphipathic lipid excipients [14]. When given subcutaneously to nonhuman primates, DcNP both extends the plasma time

course (long-acting behavior) of all three HIV drugs and leads to higher drug levels in lymphocytes than in plasma (demonstrating preferential cell targeting effects) [15].

In this study, we evaluated whether a venetoclax and zanubrutinib drug combination could be assembled into a similar DcNP lipid nanoparticle to provide both long-acting plasma exposure and adequate drug concentrations; in addition, we evaluated if enhanced, synchronized cell uptake could be achieved. We have found that a nanoparticle formulation can greatly extend the half-lives of certain drugs when administered subcutaneously in mice compared to equivalent free drugs or DcNPs administered intravenously. As their protein targets are expressed across multiple forms of leukemia, both venetoclax and zanubrutinib have shown efficacy against different types of leukemia in phase III clinical trials [11–13]. Thus, a DcNP approach for the venetoclax-zanubrutinib combination manifests broad treatment potential for several different types of leukemia beyond CLL.

3.3 Materials and Methods

3.3.1. Reagents

N-(carbonylmethoxypolyethyleneglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (DSPE-mPEG$_{2000}$), and 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) (GMP grade) were purchased from Corden Pharma (Liestal, Switzerland). The two drug substances, zanubrutinib (BGB 3111) and venetoclax (ABT 199) were supplied by MedChemExpress (Monmouth Junction, NJ, USA). All other chemicals and solutions were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

3.3.2. Preparation and Characterization of Drug Combination Nanoparticles

To prepare the drug combination containing venetoclax and zanubrutinib, 9.6 mg venetoclax and 9.6 mg zanubrutinib, plus 33.6 mg DSPE-mPEG$_{2000}$ and 85.4 mg DSPC, were dissolved together in 1 mL organic solvent in a glass tube. For the first production attempt, the chemical components were dissolved in an organic solution of ethanol with 5% ammonia, and were then subjected to rotary evaporation, followed by reconstitution in 0.9% NaCl and 20 mM $NaHCO_3$ buffer using a stir bar. An Avanti Polar Lipids sonicator (Avanti, Alabaster, AL, USA) was used to reduce particle size after reconstitution in aqueous solvent. Sonication was performed at 40–45 °C for 5 min, followed by 5 min rest, followed by a final 5 min of sonication. The suspended drug combination was stabilized by lipid excipients and referred to as the drug combination nanoparticle, or VZ-DcNP. This suspension was diluted to defined concentrations with buffer solution. The second production method used tert-butyl alcohol (TBA) as an organic solvent, which was then removed via rotary evaporation and an additional 4 h of lyophilization to remove residual TBA solvent. The diameter of particles in suspension was estimated with a NICOMP 380 ZLS (NICOMP, Chicago, IL, USA). The final production method, consisting of solvent rotary evaporation, lyophilization, and sonication, was selected for assessment both in vitro and in vivo.

To estimate the percentage of venetoclax and zanubrutinib association to DcNP, the VZ-DcNP in suspension was first dialyzed (6–8 kDa molecular weight cutoff) in buffer under sink conditions. The sink conditions were generated by dialyzing 200 μL of VZ-DcNP suspension in 200 mL buffer solution (1000-fold volume change) for 4 h. The VZ-DcNP drug association efficiency (AE%) was determined by comparing the pre- and post-dialysis drug concentration ratios of each drug (V and Z). To determine VZ either in the

dialysate or retentate, the samples were first extracted with organic solvent and analyzed with an LC-MS/MS assay as described below. Nanoparticles were also visually assessed for consistency using transmission electron microscopy with negative staining; sample suspensions containing the VZ-DcNPs were placed onto a TEM grid (copper, 300-mesh, coated with carbon and Formvar film), allowed to settle for 5 min, and then stained with 5% uranyl acetate as a negative stain. A Tecnai G2 F20 electron microscope (FEI, Hillsboro, OR, USA) was used at 200 kV.

3.3.3. Drug Extraction from VZ-DcNPs and LC-MS/MS Analysis

An extraction protocol was established to quantify concentrations of venetoclax and zanubrutinib in both nanoparticle-bound and free forms. Briefly, the drugs were solubilized by diluting the sample with ethyl acetate, which extracted them from either the DcNP complex, mouse plasma, or both. Following centrifugation, the supernatants were dried with nitrogen gas and then reconstituted in acetonitrile. Extracted drug solutions were then loaded onto a Shimadzu HPLC system coupled to a 3200 QTRAP mass spectrometer (Applied Biosystems, Grand Island, NY, USA). The HPLC system consisted of two Shimadzu LC-20A pumps, a DGU-20A5 degasser, and a Shimadzu SIL-20 AC HT autosampler. A Synergi Polar-RP column (100 × 2.0 mm) with a C_8 guard column (4.0 × 2.0 mm) (Phenomenex, Torrance, CA, USA) was used for separations. Mobile phase A used water with 20 mM ammonium acetate and B used acetonitrile. The separations were done at room temperature with a flow rate of 0.55 mL/min. The mass spectrometer was equipped with an electrospray ionization (ESI) TurboIonSpray source, and the system was operated using Analyst software, version 1.5.2 (ABSciex, Framingham, MA, USA). Drug

concentrations in various samples were calculated with standard curves prepared from normal mouse plasma containing known drug concentrations.

3.3.4. Drug Potency against Cancer Cells

K-562, a human chronic myelogenous leukemia cell line, was purchased from ATCC (Manassas, VA, USA). Human leukemic cell line MOLT-4 (of acute lymphoblastic leukemia origin), and acute promyelocytic leukemia HL-60 cells were obtained from Carrie Cummings at Fred Hutchinson Cancer Research Center (Seattle, WA, USA). They were grown in RPMI medium 1640, which contained 1% 100× Antibiotic-Antimycotic (Thermo Fisher Scientific, Waltham, MA, USA) and 10% fetal bovine serum. These were selected for evaluation due to their different degree of drug target expression, specifically Bruton's tyrosine kinase (BTK) and B-cell lymphoma 2 (Bcl-2). HL-60 cells express both BTK [16] and Bcl-2 [17], while K-562 [18,19] cells only express BTK and MOLT-4 [20,21] cells express only Bcl-2.

Each cell line was first allowed to grow in black 96-well assay plates (Corning, NY, USA). After 1 h, 200 µL of varying concentrations of each drug (venetoclax or zanubrutinib), a combination of both free drugs (w/w 1:1), the same combination in DCNPs (VZ-DCNPs), or no drug (medium control) were added in RPMI medium to the cells. On day 5, drug treatment effects were estimated using an AlamarBlue Cell Viability Assay (Thermo Fisher Scientific, Waltham, MA, USA). The viable cells that produced positive fluorescence signals were quantified with a PerkinElmer 1420 Multilabel Counter plate reader. AlamarBlue was diluted 10-fold with cell media; the existing cell culture media in the plates was replaced with the 10% AlamarBlue media and allowed to incubate for 4 h. The cell culture media was then assessed for fluorescence, (λ_{ex} = 570 nm, λ_{em} = 585 nm).

Employing GraphPad software (Version 7.05) and using an Emax model, the mid-point of the inhibitory concentration curve (or IC_{50}) was determined.

3.3.5. Effect of DcNP on Leukemic Cell Drug Uptake and Retention

One million HL-60 cells were aliquoted into several 1.5 mL Eppendorf tubes. To evaluate the effects of DcNP on VZ, we made a free-drug solution of venetoclax and zanubrutinib (1:1 w/w). A VZ-DcNP solution of identical drug concentrations was made and also added to each tube. Following drug exposure in a CO_2 incubator, cells were removed at preselected timepoints (15 min, 40 min, 1 h, 1.5 h, 2 h, 3 h, and 4 h), and were then washed twice with media to remove external drug and VZ-DCNPs. Cells were then lysed with acetonitrile. Drugs in the cells were quantified according to the aforementioned extraction protocol and LC-MS/MS methods.

3.3.6. Pharmacokinetic Analysis of VZ-DcNP Versus Free Drugs

All animal studies were performed under a protocol approved by the University of Washington Institutional Animal Care and Use Committee. Female BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA, USA). They were housed in a pathogen-free facility until use with a 12 h light/dark cycle. Three groups of three mice each were tested as follows: (group 1) an IV dose containing 30 mg/kg venetoclax and 30 mg/kg zanubrutinib in 0.9% NaCl, 20mM $NaHCO_3$ buffer with 5% DMSO and 5% Cremophor EL as solubilizing agents; (group 2) an intravenous dosing of venetoclax and zanubrutinib DCNPs equivalent in volume and drug molar concentration to that received by group 1; and (group 3) a subcutaneous injection of venetoclax and zanubrutinib DCNPs in the inner thigh of the right back leg. Plasma samples were collected at 5 min, 1 h, 3.5 h,

24 h, 48 h, 72 h, and 1 week through retro-orbital bleeding. The drugs in plasma samples were extracted and analyzed with an HPLC-MS/MS.

3.4 Results

3.4.1 Design and Characterization of Nanoformulation and Production

3.4.1.1. Effect of Solvent Removal, Size Reduction, and Drug/Lipid Ratio on Particle Size

To develop a venetoclax and zanubrutinib drug combination in a stable nanoparticle suitable for delivery to leukemia patients, biocompatible and biodegradable lipids were chosen for their nanoparticle structural ability as well as their ability to non-covalently associate with drugs across a wide range of hydrophilicity and hydrophobicity. The biocompatible lipids DSPC and DSPE-mPEG2000 were first mixed and dissolved together in organic solvent (either ethanol with 5% ammonia or tert-butyl alcohol); subsequently, the solvent was removed to form a lipid-drug complex. Using ethanol as the base solvent, the lipid-drug film was subjected to sonication as a particle size reduction process to form drug combination nanoparticles (diameter ~ 40 nm). This size range was chosen to enhance lymphocyte and leukemic cell uptake.

As outlined in Table 1, we first examined the process of solvent removal. Initially, we used rotary evaporation under reduced pressure for medium batches before later using lyophilization on a larger scale (>30 mL). We found that lyophilization did not seem to affect particle size or shape, but the process was retained to ensure complete removal of residual organic solvent.

We then determined whether varying ratios of drugs (venetoclax and zanubrutinib) to lipids (DSPC and DSPE-mPEG2000) influenced the particle size of DcNPs. Venetoclax and zanubrutinib were dissolved in a 1:1 molar ratio in small- and medium-sized batches as described above. The 1:1 ratio was selected due to its similarity to clinical treatments in human patients, while the drug amounts used were to approximate IC_{50}'s of the drugs in a living system. Lipid mass was kept consistent throughout the experiment to assess the effect of drug mass on particle size, with the drug/lipid ratio ranging from 0 to 0.3. No effect on particle size was observed, so the selected drug concentrations were 3.63 mM venetoclax and 6.67 mM zanubrutinib with a drug/lipid ratio of 0.26 for later characterization and experimental use.

3.4.1.2 Physical Characteristics of VZ-DcNPs

To further characterize VZ-DCNPs, we evaluated the degree of VZ association to DcNP under sink conditions and visualized VZ-DCNPs using transmission electron microscopy. Following a 4-h dialysis to remove any unbound drug, we found that venetoclax drug association was nearly 100% and that of zanubrutinib was 98.5%, demonstrating a stable association of the drugs to DcNP. This strong drug association to DcNP may allow the nanoparticles to fulfill their purpose of synchronized drug delivery to cells.

As seen in Figure 1, VZ-DCNPs were visually examined using a transmission electron microscope, which revealed their consistent lozenge shape, with an average length of 39 ± 4 nm and an average width of 20 ± 2 nm. The nanoparticles appeared discrete and homogenous. The TEM image both supported

our understanding of the nanoparticles and set a benchmark for future nanoparticle production.

3.4.2 Effect of DcNP on Venetoclax–Zanubrutinib Combination to Inhibit Leukemic Cell Growth

To test the ability of VZ-DCNPs to kill cancer cells, three immortalized cell lines representing different types of leukemia were incubated with each test drug, or drugs in combination, for five days before measuring their relative growth. Respective drug effects are presented as the half-maximal inhibitory concentration, or IC_{50}, and are summarized in Table 2. Free VZ combinations at equivalent concentrations and ratios were used as controls. HL-60 cells had the highest sensitivity to both free venetoclax alone (IC_{50}: 1.92 ng/mL) and to the free combination drug 1:1 mass ratio with zanubrutinib (IC_{50}: 0.181 ng/mL). HL-60 cells express both Bcl-2 and BTK, the respective targets of venetoclax and zanubrutinib, so their strong response to these drugs was expected. MOLT-4 and K-562 were less sensitive to venetoclax: 1.96 μg/mL and 15.9 μg/mL, respectively. This low response was also expected, as MOLT-4 cells express Bcl-2 at very low levels, and K-562 cells do not express the protein at all. Additionally, both MOLT-4 and K-562 were less sensitive to the free drug 1:1 mass ratio combination: 2.0 and 8.0 μg/mL, respectively. Zanubrutinib exhibited similar sensitivities for all cell lines tested; however, zanubrutinib is less potent with IC_{50}s recorded for HL-60: 10.3 ug/mL, K-562: 8.3 μg/mL, and MOLT-4: 4.0 μg/mL. In contrast, the same set of drugs in DCNPs exhibited a much lower IC_{50} recorded value for HL-60 cells: 2.2 pg/mL (each at 1:1 *w/w* fixed ratio). These results demonstrate that VZ in DcNP remained active for their respective pharmacological targets

and that they had enhanced potency to kill cancer cells when compared to free-drug equivalents.

3.4.3 Enhanced Uptake and Retention of Nanoparticle-Associated Drugs into Immortalized Leukemic Cell Lines

Cancer cell toxicity due to the nanoparticles (specifically, how the vehicle affects the associated drugs and their uptake) was then examined to assess the mechanism of toxicity in leukemia cell lines (HL-60, K-563, and MOLT-4). We found that both free drugs and those in DCNPs were rapidly taken up into all tested leukemic cells, reaching their peak intracellular drug concentrations within 1 h (Figure 2).

Intracellular venetoclax concentrations peaked at 1 h and were maintained for the 4-h study (terminal time point). Peak intracellular drug concentration was recorded at nearly 200 ng of drug per million cells (HL-60: 192 ng/million cells; K-562: 192 ng/million cells; MOLT-4: 176 ng/million cells) for cells exposed to the free-dosage form, compared to approximately 700 ng of drug per million cells for those incubated with an equivalent dose of DCNPs (HL-60: 674 ng/million cells; K-562: 647 ng/million cells; MOLT-4: 718 ng/million cells). These data suggested DcNP-associated venetoclax was taken up 3.5-fold compared to the uptake of the free-dosage form.

Intracellular zanubrutinib concentration also peaked at 1 h and persisted for the duration of the 4-h study. Cells incubated with the free drug reached an average maximal concentration of approximately 75 ng of drug per million cells (Hl-60: 69 ng/million cells; K-562: 109 ng/million cells; MOLT-4: 42 ng/million cells), compared to those treated with an equivalent dose of DcNP, which provided maximal values of 200–650 ng drug per

million cells (HL-60: 256 ng/million cells; K-562: 647 ng/million cells; MOLT-4: 208 ng/million cells). Thus, DcNP-associated zanubrutinib was taken up 3-fold to 9-fold compared to free zanubrutinib.

These in vitro cell-uptake kinetic data suggest that both the increased rate and extent of drug uptake due to nanoparticle association will positively affect the drugs' ability to kill cancer cells. This was further explored through how the nanoparticle vehicle affected the pharmacokinetics of the associated drugs in vivo.

3.4.4. Effect of DcNP on Venetoclax and Zanubrutinib Pharmacokinetics in Mice

To investigate the effects of the DcNP nanoparticle on the pharmacokinetics of venetoclax and zanubrutinib, three groups of mice were intravenously administered with equivalent dosages of intravenous free drug, intravenous VZ-DCNPs, or subcutaneous VZ-DCNPs, all in 180 µL. Venetoclax was detectable in plasma for up to seven days for mice treated with the DcNP-dosage form, while those treated with an equivalent dose of free zanubrutinib were detectable for less than one day (Figure 3). Among the mice tested, those treated with subcutaneous VZ-DcNP had the highest extended plasma levels over the seven-day study (Table 3). This group of mice also exhibited the highest AUC values for both drugs: venetoclax in DcNP = 232 $\mu g \cdot mL^{-1} \cdot h$ (as compared to free drug = 88.8 $\mu g \cdot mL^{-1} \cdot h$) and zanubrutinib in DcNP = 49 $\mu g \cdot mL^{-1} \cdot h$ (as compared to free drug = 8.3 $\mu g \cdot mL^{-1} \cdot h$). Intravenously administered VZ-DCNPs had consistently higher venetoclax AUC's than those treated with the free-drug counterpart. Venetoclax AUC in VZ-DcNP was 216 $\mu g \cdot mL^{-1} \cdot h$ (as compared to a free-drug AUC of 88.8 $\mu g \cdot mL^{-1} \cdot h$) and zanubrutinib AUC was 11.3 $\mu g \cdot mL^{-1} \cdot h$ (as compared to a free-drug AUC of 8.3 $\mu g \cdot mL^{-1} \cdot h$). It appeared that subcutaneously delivered VZ in DcNP provided the highest plasma drug exposure for

both drugs. These results suggest that a subcutaneous administration of the VZ-DCNPs may be a safe and effective way to treat leukemia with infrequent administrations due to the long-acting plasma drug time-course extension of both venetoclax and zanubrutinib in combination.

3.5 Discussion

Taking advantage of our ability to co-formulate venetoclax and zanubrutinib in a drug combination nanoparticle (DcNP), we have characterized the VZ-DcNP as stable with a high degree of drug association to DcNP, indicating that further purification is not necessary (Table 2). In addition, the resulting VZ combination in DcNP is biologically active and shown to enhance the overall potency of VZ in fixed-dose combination at a 1:1 mole ratio (Table 3). The overall enhanced potency against HL-60 leukemic cells appeared to enhance cell uptake by 42- and 5-fold for V and Z, respectively. We found that VZ-DCNPs are stable, scalable, and biocompatible, as the lipid excipients, DSPC and DPSE-mPEG$_{2000}$, provide a structural base to support the drug combination in nanoparticles appropriate for patient administration. These VZ-DcNPs are shown to extend plasma time-course and enhance the overall drug exposure of both venetoclax and zanubrutinib.

Unlike carriers that require drug encapsulation, it is noteworthy that both venetoclax and zanubrutinib were nearly completely associated to the DcNP lipid base structure in the VZ-DcNP. Thus, there is little or no drug loss during their synthesis, meaning that a final step to eliminate residual free drug is no longer necessary. In fact, our data indicate that the degree of drug association of both drugs to the VZ-DCNPs is 98% or more. Having a high degree of stable drug association may both reduce drug wastage in VZ-DcNP preparation and potentially minimize the

risk of contamination while making the injectable VZ-DcNP product. In addition, we found that a lyophilized dosage form of VZ-DcNP could be produced, and that upon resuspension the product exhibited a mean diameter of ~30–40 nm, suitable for both intravenous and subcutaneous dosing. Coupled together, the high degree of association and the option of storing the drug product in lyophilized form (for producing a suspension on site) make VZ-DcNPs realistic for human testing.

While the exact mechanisms responsible for the enhanced potency of VZ-DcNP compared to that of its free VZ counterpart are still unclear, it is likely that the small nanoparticles promote leukemic cell uptake and retention. The DcNP-associated drugs are taken up faster and are maintained at higher concentrations compared to equivalent soluble (free) VZ (Figure 2). The enhanced uptake of VZ in DcNP parallels the improved potency of VZ in HL-60 leukemic cells expressing both VZ targets (Bcl-2 and BTK). This potency was also seen in K562 cells (BTK-expressing cell line) and MOLT-4 cells (Bcl-2-expressing cell line), indicating that both drugs were present and active. We found a roughly 82-fold enhanced potency for VZ-DcNP compared to the free formulation, and a 42- and 5-fold enhancement in cellular uptake for V and Z, respectively. Once again, the mechanisms leading to the disparity in the changes in cellular uptake are not clear, and they will be a subject of our future investigations. Regardless of the exact mechanisms, however, it is clear that both V and Z in DcNP are localized in the cells and are biologically active, leading to enhanced leukemic cell-growth suppression.

When delivered subcutaneously to BALB/c mice, VZ-DcNP was able to extend the presence of both drugs in plasma over an extended period of time, resulting in a significant extension of drug half-lives compared to those of free drugs given intravenously; a 42-fold increase for venetoclax and a 5-fold increase for zanubrutinib were observed. Intravenous VZ-DCNPs did not significantly alter the two drugs' pharmacokinetics. VZ-DCNPs administered subcutaneously

can greatly extend the plasma half-lives of associated drugs, demonstrating their ability to safely administer drugs over a longer period from a single injection, compared to the oral dosage forms that require more frequent dosing to achieve the same effect.

Currently, oral zanubrutinib (taken twice daily) and venetoclax (taken once daily) are in a phase 2 clinical study as a potential combination therapy for treating MCL (mantle cell lymphoma) and CLL (chronic lymphocytic leukemia) (NCT 05168930). Producing a long-acting and effective fixed-dose combination therapy intended to improve leukemic cell uptake and extend the time between doses may improve both patient uptake and acceptance.

New administration strategies for long-acting delivery of drugs that can overcome these limitations have been introduced. Long-acting cabotegravir with long-acting rilpivirine, explored through the CUSTOMIZE Hybrid III implementation-effectiveness study, is a novel formulation strategy that has been successfully implemented in HIV patient treatment, as the formulation can overcome the common problems with long-term drug treatment, namely patient adherence to the drug and maintaining adequate plasma drug levels. The VZ-DCNPs reported here can overcome these limitations imposed by daily oral dosing via subcutaneous administration of the nanoparticles: association with the biocompatible lipids safely retains the drugs in the subcutaneous space, protecting them from gastrointestinal and plasma metabolism while also slowly releasing the drugs over time into the plasma either through direct extravasation or through lymphatic uptake. These routes are likely responsible for the observed extended half-lives of the drugs, though more research is needed.

In leukemic cells, a fixed-dose combination of venetoclax and zanubrutinib exhibited good potency, with IC_{50}s in the low nanogram per milliliter range for HL-60 cells, which have high expression levels of both drugs' targets. When formulated as a free drug with the same fixed-dose

combination as VZ-DCNPs, the IC_{50} value for HL-60 cells was enhanced by about 1000-fold. The improvement in potency of the DCNPs over the free-drug combination is likely due to enhanced uptake and retention of the DcNP-bound drug as compared to the free drug. The pharmacokinetic study results indicate that in mice, subcutaneously administered VZ-DcNP was more favorable than both intravenous VZ-DcNP and intravenous free drug. Both drugs were detectable for a longer period in the plasma of subcutaneous VZ-DcNP-treated mice those that received free drug or DcNP-associated drug through an intravenous injection.

Using lipid excipients to stabilize both venetoclax and zanubrutinib in drug combination nanoparticles, we successfully developed and characterized a VZ-DcNP injectable formulation that can be made in simple steps without the need for free-drug removal. VZ-DcNP is biologically active, is capable of enhancing leukemic cell uptake, and can extend both the drugs' plasma half-lives and overall exposure per dose in mice. Both subcutaneous and intravenous administration of the nanoparticles provided larger overall exposure and drug half-lives than an equivalent intravenous administration of free drug, though subcutaneous administration provided the largest effect; in contrast, the effect from intravenous administration was minor. The VZ-DcNP fixed-dose drug combination may provide a long-acting pharmacokinetic profile and enhance overall drug exposure per dose; most importantly, it may synchronize the uptake and retention of both drugs in leukemic cells for a durable leukemia suppression, though these results remain preliminary and require further validation. We hope to further examine VZ-DcNP pharmacokinetics and safety in both mice and larger model organisms to explore the feasibility of VZ-DcNP as a future long-acting treatment for leukemia.

3.6 Bibliography

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Table 3.1. Effect of variations in solvent removal, particle size reduction, and drug/lipid ratios on nanoparticle size.

Preparation Method	Production Scale	Batch	Drug Concentration (mM)		Drug/Lipid Molar Ratio	Size (nm)
			Venetoclax	Zanubrutinib		
Solvent Evaporation > Hydration > Sonication	Medium	1	10	10	0.2	28
	(5–30 mL)	2	15	15	0.3	25
		3	5	5	0.1	11
		4	10	10	0.2	38
Lyophilization > Hydration	Large	1	3.63	6.67	0.26	30
	(>30 mL)	2	3.63	6.67	0.26	14

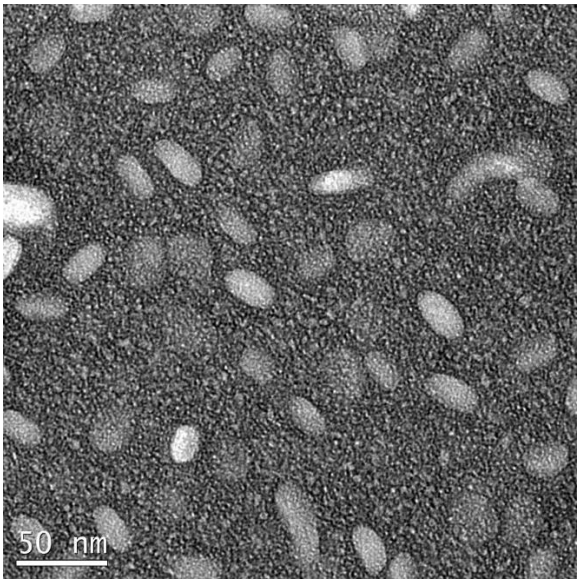

Figure 3.1. VZ-DCNPs examined with transmission electron microscopy with negative staining by 5% uranyl acetate. The drug combination nanoparticle, composed of the two drugs, venetoclax and zanubrutinib, appeared to assemble into solid, discrete, lozenge-shaped particles of approx. 20 nm × 39 nm with no apparent membrane structures.

Table 3.2. Effects of VZ-DCNPs and free drugs (V, Z, or both) on leukemic cell growth inhibition.

	Cell Line	HL-60	K-562	MOLT-4
	Bcl-2 Expression	+	-	+
	BTK Expression	+	+	-
Inhibitory Effects (IC_{50}, pg/mL)	Venetoclax	1.9×10^3	1.6×10^7	2.0×10^6
	Zanubrutinib	1.0×10^7	8.4×10^6	4.0×10^6
	V+Z Free Combo	180.6	8.0×10^6	1.9×10^6
	VZ-DcNP	2.2	-	-

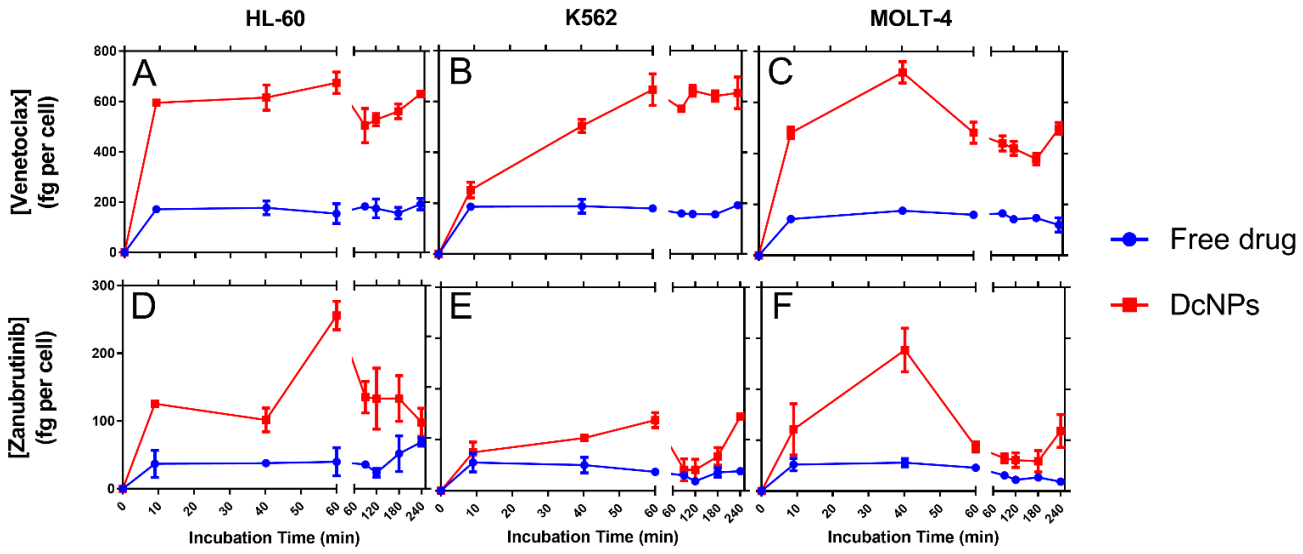

Figure 3.2. Effect of DcNP vehicle on rate and total uptake of venetoclax (A, B, and C) and zanubrutinib (D, E, and F) into leukemic cells. Drugs were incubated at a 1:1 mass ratio (3.63 mM venetoclax and 6.67 mM zanubrutinib) as either free drugs or nanoparticle-associated drugs.

Table 3.3. Effect of DcNP on plasma half-lives and exposures (AUCs) of venetoclax (V) and zanubrutinib (Z) in mice.

	Drug Route and Vehicle					
	Intravenous-Free		**Intravenous-DcNP**		**Subcutaneous-DcNP**	
	V	Z	V	Z	V	Z
$t_{1/2}$ (h)	0.39	0.33	0.45	0.95	16.71	1.62
AUC ($\mu g \cdot mL^{-1} \cdot h$)	400.6	15.1	338.7	14.2	98.9	7.2

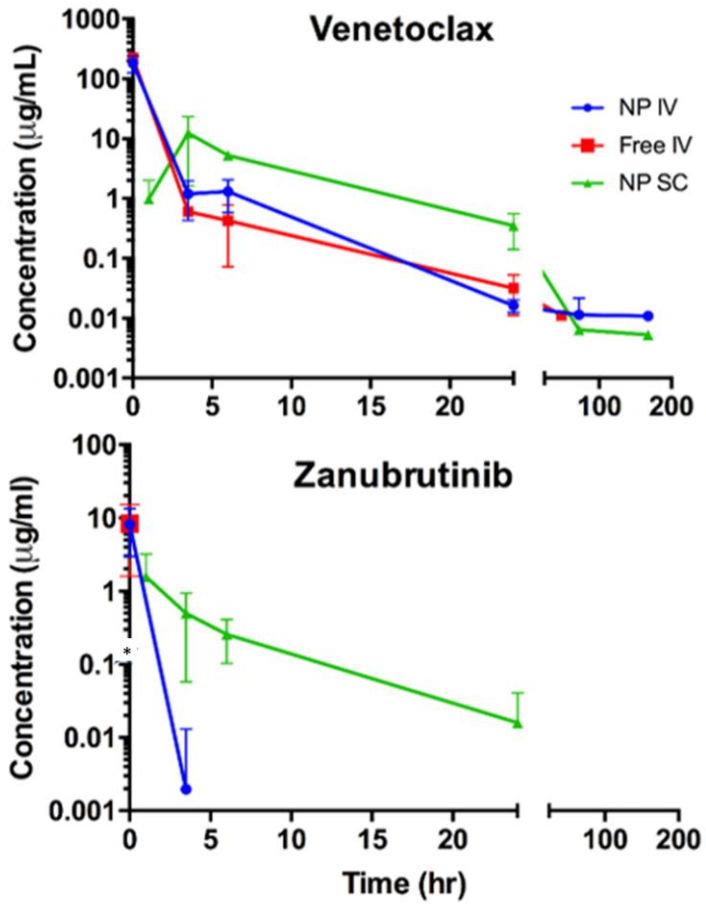

Figure 3.3. Effects of DcNP on pharmacokinetics of venetoclax and zanubrutinib. The venetoclax-zanubrutinib (1:1 *w/w*) nanoparticle (NP) combination was given by IV at 30 mg/kg each for route: NP-IV, NP-SC, or free form (Free-IV), respectively, to BALB/c mice. Plasma samples were collected at the indicated times; drug concentrations were determined over a 1-week study. Note: Drug concentrations below the limit of quantification were not plotted. * indicates an undetectable level of zanubrutinib following intravenous free administration.

Chapter 4. A Novel Drug Combination Nanoparticle Stabilizes Gemcitabine and Paclitaxel *in vivo* Leading to Enhanced Plasma Drug Exposure and Inhibition of Pancreatic Tumor Growth in an Orthotopic Mouse Model

4.1 Abstract

Due to detection at late stages and limited effects of current drugs for cancer, partly attributed to limited drug penetrations into the neoplastic tumor tissues, pancreatic cancer remains one of the deadliest cancers with less than 5% surviving after 5 years from diagnosis. This dismal statistic has been largely unchanged over 50 years[1]. The current standard of clinical care typically includes combinations of small molecule drugs to reduce cancer growth and reduce drug resistance risk. These highly potent regimens require very high intravenous dosages, given in sequence to kill the cancer through broad exposure to healthy cells and tissues in the body, resulting in off-target toxicities which can be intolerable; thus, dose-reductions are often needed to preserve patients' quality of life. Consequently, many patients may not gain full therapeutic benefit of these drug-combinations due to intolerable toxicity and premature treatment termination that may lead to disease progression. Therefore, less burdensome or more tolerable treatments that could enhance drug-combination efficacy in pancreatic tumors for an extended time are needed to prevent pancreatic cancer progression. In addition, a strategy that can synchronized multiple drugs and facilitate preferential delivery and retention in the pancreatic tumor tissues may extend both safety and effectiveness of current potent cancer drugs. We investigated the ability of a lipid nanoformulation to form stable drug-combination particles composed of two highly potent cancer drugs with desperate physical-chemical properties, gemcitabine (G) and paclitaxel (T). We initially found that formation of stable GT in drug-combination nanoparticles, or GT-DcNP, is possible, and in mice, pharmacokinetic data indicates that DcNP extends the presence of the two small molecule drugs. Following an intraperitoneal injection, mice dosed with GT-DcNP exhibit detectable plasma gemcitabine and paclitaxel levels for up to 1 week and 3 hours, respectively, post-injection, while those dosed with an equivalent free form reached undetectable levels of

gemcitabine and paclitaxel after only 30 minutes and 5 minutes. The DcNP formulation has enhanced gemcitabine plasma exposure by 45-fold and paclitaxel exposure by 50-fold. Additionally, despite low overall plasma concentrations, gemcitabine and paclitaxel were detectable in the pancreas and kidneys of mice dosed with GT-DcNP after 1 week, indicating the ability of the nanoparticles to deliver preferentially to pancreas tissue over an extended period. Employing a novel, orthotopic Pan02 pancreatic ductal carcinoma tumor model, we have evaluated the route and dosage form of GT combination on pancreatic tumor progression. We found that only when given by peritoneal injection (but not intravenous injection), GT in DcNP dosage form prevented pancreatic cancer progression within the study window. At an equivalent dose of GT (20:2 mg/kg), free and soluble dosage form did extend pancreatic cancer survival in Pan02 orthotopic model. Tissue distribution studies indicate that DcNP enhanced the uptake and retention of GT in pancreatic tissues, which may be related to enhanced pancreatic tissue and cell exposure and improved pancreatic cancer treatment outcomes. Therefore, GT in DcNP may present a potential treatment for metastatic pancreatic cancer that warrants further consideration for clinical translation.

4.2 Introduction

Pancreatic cancer is one of the deadliest forms of cancer, with the annual diagnostic rate of pancreatic cancer being roughly equivalent to its mortality rate, roughly 50,000 per year in the United States alone[1]. Definitive diagnosis of pancreatic cancer often occurs at late stages due to the combination of few noticeable symptoms in early stages of the disease and the lack of routinely testable biomarkers (those typically used for other cancer types such as breast and prostate), limiting the ability to deploy drug interventions for pancreatic cancer in early stages[3,10]. As a result, pancreatic tumors have typically grown unchecked for months before they are noticed, presenting an unusual situation where over 47% of patients' cancers have reached metastatic stages at diagnosis.

The pancreas is a glandular organ composed of two main cell types: epithelial ductal cells and hormone-releasing islet cells. Ductal cells are over 98% of the organ's mass, and the most common form of pancreatic cancer (over 95% of diagnoses) is pancreatic ductal adenocarcinoma (PDAC). Endocrine-inactive cancers derived from islet cells are uncommon, with endocrine-active tumors being even rarer[7]. Due to the pancreas's location in the inner, upper peritoneal cavity, pancreatic tumors often grow unnoticed by the patient until the tumor is large enough to disrupt pancreatic functions, such as blood glucose regulation or the production of digestive enzymes. By this stage, the tumor typically encapsulates the prominent nearby vasculature, making tumor resection too dangerous to perform; tumors in the pancreas head can grow around the superior mesenteric artery and inferior mesenteric vein, while tumors in the tail can grow around the celiac artery and the hepatic artery. Despite the cancer's proximity to these major blood vessels, surgical resection is not a good option and has poor interventional outcomes. In addition, due to often poorly vascularized environment of pancreatic tumors, many drugs predominantly delivery

through drug in plasma and blood vessels may not be sufficiently perfused with limited access to the tumor. Additionally, the tumor stroma is exceptionally dense due to overproduction of extracellular matrix proteins by fibroblast cells in the tumor in a phenomenon known as "desmoplasia"[8,9]. Both poor vascularization and desmoplasia limit the penetration of drug molecules (either biologic macromolecules or small-chemical molecules) administered to the patients. Poor drug penetration and/or retention for both larger biologic drugs and small molecules may limit the effectiveness against pancreatic tumor. Due to their smaller size, small molecule drugs may provide a higher degree of tissue access and thus are typically preferred for pancreatic cancer treatment. As a result, though many new chemotherapeutic and also biologic regimens have developed and effective for other forms of cancer, pancreatic cancer has few available treatment options due to limited drug distribution to and retention in the pancreas and its neoplasms.

The current standard of care is somewhat limited to small molecule chemotherapies given in sequential combination[3,6]. While a number of drug combination chemotherapeutic regimens have been developed and are generally available as pancreatic cancer treatment guidelines, these regimens are often inadequate to make a significant impact on survival outcomes. Most regimens available result in less than a 1-year median survival from the initial pancreatic cancer diagnosis. Even with the most effective four-drug regimen, FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan, and oxaliplatin), the median survival for patients from diagnosis is approximately 11 months[5]. Due to the off-target drug distributions of high dose FOLFIRINOX and its resultingly intolerable toxicities, many patients are unable to complete the treatment cycle and cannot benefit from it. To overcome intolerability, patients are often prescribed less-effective regimens with more acceptable side-effects.

A new drug combination that (1) can localize to and (2) be retained in cancer-laden tissues in a synchronized and sustained manner may reduce the overall off-target drug exposure and related untoward effects[4]. With the base idea of long-term drug exposure via intraperitoneal administration, we explored the effect of a novel nanoparticle carrier that provides stable association with associated drugs, both in a test tube and in the body for two currently used pancreatic cancer drugs, gemcitabine and paclitaxel. The pharmacokinetics and subsequent tissue distribution and impact on the growth of pancreatic tumors in mice was analyzed using mass spectroscopy and non-invasive luminescent imaging of tumors. We found that a single intraperitoneal dose of this novel drug-combination nanomedicine may potentially eliminate pancreatic tumors at a level that cannot otherwise be achieved with an equivalent free drug dose or the same nanomedicine given by IV injection.

4.3 Materials and Methods

4.3.1 Materials

DSPC (1,2-Distearoyl-sn-glycero-3-phosphocholine) and DSPE-PEG2000 (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000]) were purchased from Corden Pharm (Liestal, Switzerland). Paclitaxel (>99.5%; CAS 33069-62-4) and gemcitabine hydrochloride (>99%; CAS 95058-81-4) were purchased from LC Laboratories. All other chemicals were of analytical grade or higher.

4.3.2 Preparation and Characterization of Drug-combination Particles Composed of Gemcitabine (G) and Paclitaxel (Taxol or T)

Preparation of sterile injectable GT-DcNP has been reported previously by Mu, *et al*[13]. Briefly, in a sterile test tube, 710.7 mg DSPC, 280.5 mg DSPE, 213.2 mg gemcitabine

hydrochloride, and 21.3 mg paclitaxel were dissolved into 20 mL 100% ethanol at 60°C; gemcitabine and paclitaxel were present in a 10:1 ratio by mass. Ethanol in the drug-lipid mixture in the solution was removed by rotary evaporation under controlled conditions to produce lipid and drug complex. After subjecting vacuum desiccation for at least 24 hours to remove residual ethanol, the lipid-drug complex was hydrated at 60°C in 2 mL buffer containing 0.45% NaCl with 20mM $NaHCO_3$ for 3 hours. The DcNP drug-lipid complex particles containing G-T in suspension were subjected to sonication for size reduction to obtain approximately 55nm diameter. This is achieved with about 3 cycles of bath sonication at 40°C (1 cycle: 5 minutes on, 5 minutes off) using an Avanti Polar Lipids, Inc. sonication bath.

Nanoparticle diameter was determined by a NICOMP 380 ZLS (Particle Sizing Systems, Santa Barbara, CA). The concentration of drugs in the hydrated nanoparticles was quantified using an acetonitrile-based extraction and subsequent HPLC; the association efficiencies of drugs in the hydrated nanoparticles were quantified via dialysis of the nanoparticles using a 6-8kDa membrane against a 0.9% NaCl with 20mM $NaHCO_3$ for 4 hours and subsequent HPLC drug analysis.

4.3.3 Preparation of Free Drug Combination Formulation

Paclitaxel and gemcitabine were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL and 50 mg/mL, respectively. Injection solvent was prepared by diluting whole mouse plasma with 9 equivalent volumes of sterile Hanks Buffered Saline Solution (HBSS) to produce a 10% mouse plasma solvent suitable for injection. Paclitaxel and gemcitabine were then added to the solvent, ultimately producing several milliliters of

2mg/mL gemcitabine and 0.2 mg/mL paclitaxel. Free drug formulation was prepared the same day it was used due to inherent instability.

4.3.4 Pharmacokinetic Study of Intraperitoneal Administration of Free vs. Nanoparticle-bound Drug in vivo

Animal studies were conducted in accordance with the Institute of Animal Care and Use Committee (IACUC) at the University of Washington. 29 female 4-week-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine); mice were allowed 1 week to acclimatize to new research facility conditions before use, and female mice were selected for the aim of consistency with past research involving DcNP and mice[13].

Mice were divided into 3 groups: 12 mice to assess free drug, 15 mice to assess GT-DcNP, and 2 mice to remain as noninjected controls. In the free drug group, 12 mice were administered the free drug formulation via intraperitoneal administration to yield an injection dose of 20mg gemcitabine and 2 mg paclitaxel per kg mouse. Blood was then collected via retro-orbital bleeding using EDTA-coated collection tubes from 3 mice at 5 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 6 hours, and 24 hours post-injection; at most, blood was drawn once from the conscious mouse and a second time as a part of the terminal procedure while mice were under anesthesia. Additionally, 3 mice were sacrificed at 1 hour, 3 hours, 6 hours, and 24 hours post-injection via CO_2 asphyxiation and cervical dislocation; their blood and tissues were collected shortly after death and placed on ice for later analysis. Additionally, one of the control mice was euthanized at 24 hours post-injection for direct blood and tissue comparisons.

In the GT-DcNP drug group, 15 mice were administered GT-DcNP via intraperitoneal administration to yield an injection dose of 20mg gemcitabine and 2 mg paclitaxel per kg mouse. Blood was then collected via retro-orbital bleeding using EDTA-coated collection tubes from 3 mice at 5 minutes, 1 hour, 2 hours, 3hours, 6 hours, 24 hours, 2 days, 4 days, and 1-week post-injection; at most, blood was drawn once from the conscious mouse and a second time as a part of the terminal procedure while mice were under anesthesia. Additionally, 3 mice were sacked at 1 hour, 3 hours, 6 hours, 24 hours, and 1-week post-injection via CO_2 asphyxiation and cervical dislocation; their blood and tissues were collected shortly after death and placed on ice for later analysis. The second control mouse was also euthanized after 1 week for direct blood and tissue comparisons.

4.3.5 Drug Extraction from Murine Plasma and Tissues

Paclitaxel and gemcitabine were extracted from plasma and tissue via liquid-liquid extraction using acetonitrile. 50µL of plasma or homogenized tissue (tissue diluted with 9 volumes of HBSS) were placed into 1.5mL microcentrifuge tubes; plasma and tissue may also be diluted further using blank matrix to reach the detectable range on the mass spectrometer. Samples were then spiked with internal standard (deuterated gemcitabine and docetaxel) to ensure sample consistency during processing. Additionally, blank matrix was spiked with both internal standard and known quantities of gemcitabine and paclitaxel (dissolved in DMSO) to produce a standard curve by which to measure the unknown quantities in the samples. Blank DMSO was also added to samples to ensure solvent consistency between the samples and the standard curve.

Five hundred and forty µL acetonitrile was added to each prepared standard and sample to begin the extraction of the two drugs from the plasma and tissues. Samples were

vortexed for 15 minutes, following by centrifugation at 14,000 RPM for 15 minutes at 4°C. 400µL supernatant was removed from each sample and dried using a Biotage Nitrogen Evaporator. Each dry sample was then re-dissolved in 50µL of 20% methanol in water.

4.3.6 Quantification of Gemcitabine and Paclitaxel by LC-MS/MS

Drug separation and quantification was performed using a Shimadzu HPLC system coupled to a 4500 Triple Quad mass spectrometer (ABSciex, Redwood City, CA, USA). The HPLC system consisted of 2 Shimadzu LC-20AD pumps, a DGU-20A5 degasser, and a Shimadzu SIL-20AC HT autosampler (Shimadzu Corporation, Kyoto, Japan). The samples were cooled to about 4 degrees Celsius prior to analysis, and the mass spectrometer was equipped with an electrospray ionization (ESI) technology. The system used Analyst software, version 1.5.2 (ABSciex, Framingham, MA, USA). A Sunfire column (150 x 4.6mm; 4µm in particle size; Waters) was used. The flow rate was set to 0.6 mL/min with a 5µL sample injection volume. The mobile phase for separation consisted of pump A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The needle was washed with isopropanol after each injection. Analytes were monitored using multiple-reaction monitoring (MRM) for positive ions. The following ion transitions were monitored, i.e., G (m/z 264.066→112.000) and T (m/z 854.266→286.200); a stable labeled isotope (C8 13CH12ClF2N15N2O4; m/z 267.067→115.100) was used as an internal standard for G; docetaxel (m/z 830.312→549.3) was used as an internal standard for T.

4.3.7 Cells and Cell Culture

Pan02 cells were a generous gift from Dr. Mien-Chie Hung of MD Anderson in Houston, Texas. This cell line was previously stably transfected with a luciferase gene

using G418 drug resistance gene. These cells were grown under G418 to ensure retention of luciferase expression in Pan02 cells to enable monitoring luminescence following an administration of D-luciferin as a Pan02 marker. Cells were cultured and maintain in DMEM/F12 (1:1, high glucose) media with 10% fetal bovine serum and 250 ug/mL G418.

4.3.8 Attenuation of Murine Metastatic Pancreatic Tumor Growth

20 female 4-week-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine); mice were allowed 1 week to acclimatize to new conditions before use. Mice were divided into 4 groups of 5 mice each; 4 mice in each group were inoculated with 5 million Pan02 cells, delivered via intraperitoneal injection in 200µL of HBSS, while 1 remained as a noninjected control. Tumor growth was confirmed via luminescent imaging three days following the inoculation; in brief, each mouse was administered 150mg/kg D-luciferin via intraperitoneal injection 12 minutes prior to imaging. Image exposure was 180 seconds. Total Pan02-luc luminescence was quantified using Live Image software (PerkinElmer, Waltham, MA, USA).

Five days following inoculation, group 1 was administered 100L HBSS intravenously. Group 2 was administered 20mg/kg gemcitabine and 2mg/kg paclitaxel in 100µL as a free formulation intraperitoneally. Group 3 was administered 20mg/kg gemcitabine and 2mg/kg paclitaxel as drug combination nanoparticles, delivered intraperitoneally in 200µL. Group 4 was administered 20mg/kg gemcitabine and 2mg/kg paclitaxel, delivered intravenously in 100µL. Following drug administration to all 4 groups, Pan02 luminescence was assessed every 3 days until 28 days post-inoculation or upon euthanasia. If not euthanized on one of the typical imaging dates, mice were imaged one last time before sacrifice.

4.3.9 Statistical Analysis

Pharmacokinetic and tissue data concerning the concentrations of exogenous drugs is presented as the arithmetic mean ± SD as determined by Prism ver. 9.3.1 (Graphpad). The number of mice in all groups ranged from 3-4. Statistical analysis was done using GraphPad Prism version 9.3.1 (GraphPad Software Inc., San Diego, California, USA).

4.4 Results

4.4.1 Effects of DcNP Dosage Form in Extension of Gemcitabine and Paclitaxel Plasma Time Course and Pharmacokinetics after Intraperitoneal Injection

Previously, we have reported that IV dosing of gemcitabine (G) and paclitaxel at a 10:1 (w/w) fixed ratio in DcNP provides extended plasma time course and enhanced cancer laden tissue localization and exposure. It is not clear whether GT-DcNP could provide similar degree of enhancement in PK profile. Therefore, to evaluate plasma time course of GT-DcNP after intraperitoneal (IP) administration, we dosed C57BL/6 mice with free or DcNP form at 20mg/kg gemcitabine and 2mg/kg paclitaxel. The blood samples were collected over 200 hr to plot plasma drug concentration-time plot to determine the effects of DcNP carrier on the plasma uptake and exposure of both G and T (Figure 4.2), and select pharmacokinetic parameters are summarized in Table 4.1.

As noted in Figure 4.2 and Table 4. DcNP dosage form has extended GT plasma time course of both gemcitabine and paclitaxel to different degree. Thus, DcNP has converted short-acting GT free dosage form into long-acting after IP dosing. The non-compartmental PK parameters summarized in Table 4.2 indicate DcNP has enhanced gemcitabine exposure (AUC: 98,000 v 2,200 for free, ng/mL*h), C_{max} (14,300 v 6,300 for

free, ng/mL), and increased apparent $T_{1/2}$ (24h v 0.25h, for free). While less prominent effects on these parameters were observed for serum protein bound paclitaxel, the overall drug exposure per given dose of GT, as measured by AUC for the duration of study, appeared to be enhanced by about 45-50-fold, which is attributed to DcNP formulation of GT. As can be seen in Table 4.1, the GT nanoformulation increased gemcitabine exposure by 45-fold and extended the $T_{1/2app}$ 12-fold. For paclitaxel presented an increased exposure by 50-fold and the $T_{1/2app}$ was increased 12-fold in the nanoparticle formulation compared to the equivalent free drug administration. Collectively, these data indicates that IP dosing of GT-DcNP is viable, and no notable untoward effects were noted in treated mice. We used this 20:2 (G:T) mg/kg dose in DcNP for IP dosing in an orthotopic pancreatic tumor model as described in Chapter 2 of this thesis for evaluation of therapeutic outcomes.

4.4.2 Effects of DcNP on the Ability of Gemcitabine and Paclitaxel Combination Regimen to Attenuate Pan02 Tumor Growth and Pancreatic Tumor Progression

We next determined whether enhanced plasma exposure and stable association of water-soluble gemcitabine and water-insoluble paclitaxel were effective in inhibiting pancreatic tumor growth. As previously reported in Table 2.2, Pan02 cells are highly susceptible to gemcitabine alone and in combination with paclitaxel. To test this sensitivity in the *in vivo* setting, we employed an orthotopic tumor model through IP inoculation of Pan02 ductal pancreatic adenocarcinoma that led to development of pancreatic tumors within 3 days in a reproducible model. For additional details on this orthotopic tumor model, please referred to Chapter 2 of this Thesis. The experimental design and enrollment criteria are schematically presented in Figure 4.1. After confirmation of the inoculated Pan02 cells in the pancreas of mice following bioimaging, the mice with pancreatic tumor

signals verified on day 4 were divided into the following control or treatment groups: treatment of GT in DcNP given by IV or IP route, treatment of soluble GT given by IP route, and a saline placebo given IP. The treatments were given as a single dose on day 5 and disease progression was monitored by weight change, clinical observation and tumor imaging with IVIS to follow luciferase bioluminescence.

Mouse body mass and behavior was consistently normal in the Pan02 treatment groups in comparison to the mouse given no Pan02 nor treatment for roughly the first 2 weeks of treatment despite apparent tumor growth seen in IVIS images taken every 3 days. However, tumor burden became apparent in all groups around day 14, excluding the Pan02-burdened mice treated with intraperitoneal GT-DcNP, as body weight began to drop and mouse behavior became slower and less reactive to external stimuli. All 4 mice in the group receiving no treatment (i.e., the saline group) were euthanized between 18-22 days after their saline injection due to loss of body weight and excessive tumor burden. All 4 mice in the group receiving intravenous GT-DcNP's were euthanized between 18-24 days after their GT-DcNP administration. Similarly, all 4 mice given intraperitoneal free GT drugs were euthanized between 18-22 days after their DcNP administration. In contrast, no Pan02-burdened mouse administrated intraperitoneal DcNP's was euthanized during the 28-day treatment study. Following euthanasia, mice were dissected and imaged with the IVIS at two points during dissections: (1) following initial euthanasia after mouse skin, abdominal muscle walls, and thoracic cavity were opened to assess the gross distribution of Pan02 growth and (2) following organ excision to assess the spread of Pan02 to individual organs. Tumor luminescence signaling is examined in Figure 2.5 and 2.6, demonstrating the attenuating effects of DcNP through disparate cancer growth.

4.4.3 Effects of DcNP on Tissue Distribution of the Two Small Molecule Drugs, Gemcitabine and Paclitaxel Following Intraperitoneal dosing

To elucidate potential mechanisms relating to enhanced drug exposure in the plasma as well as tumor clearance, we next administered (intraperitoneally) free or DcNP formulated 20mg/kg gemcitabine and 2mg/kg paclitaxel fixed-dose combination to C57BL/6 mice and collected their tissues over time. The tissues are the pancreas (target of drug localization), liver, kidneys, and spleen of each mouse. These tissues were analyzed for tissue concentrations of gemcitabine and paclitaxel at pre-determined timepoints. They are presented in Figure 4.3 and Table 4.2.

For both the free and DcNP formulations, maximum tissue concentrations occurred at 1-hour post-injection, the earliest time-point assessed post-injection. Free drug was detectable in liver, kidney, and spleen up to 6 hours post-injection. DcNP-formulated drugs were detectable in kidney and spleen for up to 1-week post-injection. Notably, both drugs were detectable in plasma up to 50x higher when delivered as a DcNP formulation as opposed to the equivalent free drug dosage. This mirrored extension in half-life time may reflect the prolonged stability and resulting co-delivery of DcNP-associated drugs over several days following intraperitoneal administration.

4.5 Discussion

In this work we explored the effect of a synchronizing pancreatic cancer drug combination with clinically used drugs that are stabilized into drug-combination lipid nanoparticle on enhancing pharmacokinetics and tissue distribution for improving target pancreatic tissue distribution, exposure, and overall therapeutic outcomes. Employing the small molecule pancreatic cancer

chemotherapeutics, gemcitabine (G) and paclitaxel (T), and following an intraperitoneal administration of the GT combination stabilized in drug-combination nanoparticles or GT-DcNP, we found that a single dose of GT-DcNP, but not equivalent dose of soluble GT, can inhibit the progressive growth of a novel Pan02 pancreatic ductal adenocarcinoma model and improve pancreatic cancer mouse survival. Plasma time-course analysis revealed that mice dosed with the two chemotherapeutic drugs - gemcitabine and paclitaxel - presented in the drug combination nanoparticles (DcNP or GT-DcNP) formulation provided detectable and quantifiable concentrations of both drugs up to 1 week and 3 hours, respectively. This is a significantly longer duration of detectable drug levels compared to only 30 minutes and 5 minutes for an equivalent free dose of the free-soluble gemcitabine and paclitaxel dosage form. The DcNP formulation has enhanced gemcitabine's total exposure (Area Under the Curve; AUC) by 45-fold while for paclitaxel 50-fold over that of free-drug dosage form. Additionally, compared those treated with free drug combination, DcNP formulated drugs in mice presented much higher tissue concentrations in the pancreas, and also smaller increase in spleen, kidney. The overall drug exposure to liver in mice treated with GT-DcNP was slightly lower than that treated with free drug. The enhanced pancreatic drug localization and reduction in liver may be related to enhance pancreatic tumor reduction and survival in the Pan02 pancreatic tumor laden mice. The long-acting pharmacokinetic of gemcitabine and paclitaxel attributed to DcNP dosage form in C57/BL6 mice were similar to that reported by Mu, *et al*[13] in a breast cancer model in BALB/C mice given by intravenous instead of intraperitoneal dosing. Mu, *et al*'s use of GT-DcNP in mice achieved a similar half-life extension of the DcNP formulated GT drugs, though both gemcitabine and paclitaxel were detectable for up to 2 weeks after IV injection in contrast to the shorter periods observed for both drugs in C57/BL6 mice after given GT-DcNP by intraperitoneal route in this

work. Whether the different degree of plasma drug concentration extension differences in the two different mice is due to the route of administration or different strain of mice is not clear and it is a subject for future investigation.

Once we had characterized the long-acting pharmacokinetics, we then examined the effects of an intraperitoneal administration of the GT-DcNP's on limiting the growth of and potentially treating a murine model of metastatic pancreatic cancer derived from Pan02 cells. In brief, mice were inoculated with Pan02 cells through an intraperitoneal injection; after allowing the cells to colonize the host for 3 days, the mice were then given one of three treatments that were equivalent, single-dose administrations: (1) an intravenous dose of GT-DcNP, (2) an intraperitoneal dose of a free formulation of GT, and (3) an intraperitoneal dose of GT-DcNP. Mice given an intraperitoneal dose of GT-DcNP fared far better than their counterparts given intraperitoneal free drug or intravenous GT-DcNP; all mice given intraperitoneal DcNP did not reach euthanasia criteria over the course of the 4-week study, whereas all other mice had cancers progress at similar rates to mice given no treatment at all. Interestingly, 2 of the 4 mice given intraperitoneal GT-DcNP displayed no trace of Pan02 cancer cells or tumors upon post-mortem exploration at day 28, the conclusion of the study. The effects of multiple drug administrations rather a single dose are not known but warrant further investigation.

Due to late-stage diagnosis in humans, pancreatic cancer has often progressed to an advanced, metastatic stage at the time of diagnosis or soon after. As such, the developed tumors are typically spread throughout the patient's body as well as lacking proper vasculature, greatly restricting the ability to treat the disease. To overcome this problem, we have developed a commonly used drug combination regimen, gemcitabine and paclitaxel, that is effective against pancreatic cancer into a drug-combination lipid nanoparticle formulation, resulting in

synchronized delivery to tissues and cells of interests in order to sustained levels of drug over time from a single injection. Due to complex tumor physiology, intravenous administration of drugs formulated in nanoparticles may limit how much drug/nanoparticles can reach and enter the tumor. This limitation likely depends on the route of administration; GT-DcNP in particulate form may reach and penetrate to different tissues where cancer cells may colonize into metastatic nodules depending on their initial injection route. The intraperitoneal route may have circumvented these limitations of GT-DcNP by directing the GT-DcNP to gain tumor access through blood vessels supplying the pancreas. This hypothesis is consistent with the finding that a single dose of GT-DcNP given IP (but not IV) can significantly reduce pancreatic tumor size and provide 100% survival in orthotopic pancreatic tumor model within the study window. Given that the primary and metastatic pancreatic tumors can be inoculated with good success and home to the pancreas through IP injection, IP dosing of GT-DcNP is an appropriate and suitable route of administration. As the small molecule drugs are co-formulated into these nanoparticles, the drugs are also co-delivered together to the tumors simultaneously, thus synchronizing their delivery and increasing their toxicity against cancer cells. This may lead to lower off-target drug exposure and related untoward effects that may improve patient tolerability.

Drug combination lipid nanoparticles composed of similar lipid excipients but carrying different drugs have been used successfully as a carrier for antiretroviral drugs for HIV as well as other small molecule chemotherapeutics for breast and blood cancers. However, this is the first reported success in the DcNP dosage form to modulate pharmacokinetics of gemcitabine and paclitaxel resulting from a single dose of intraperitoneal administration. As seen with alternate dosage administration routes, we demonstrate the ability of the nanoparticles to retain and sustain drug exposure in plasma and target tissues over a much longer time than free drug. As a result of

these properties, the lipid nanoparticles may be useful as a treatment for metastatic pancreatic cancer in humans. Additional studies are needed to further characterize the tumor-inhibiting effects of GT-DcNP, including examining the exact mechanisms of toxicity of the nanoparticles as well as the pathology of mice given GT-DcNP in comparison to other treatments.

With respect to intraperitoneal dosing, it is a route that is used in acute dosing or continuous infusion for a number of drugs, saline for rehydration and more recently for continuous infusion of chemotherapeutic for ovarian cancer with good patient acceptance. Thus, this route of administration in intermittent dosing with the possibility of outpatient clinic or even home care can be envisioned to further improve patient acceptance and tolerance of drugs.

In summary, taking advantage of the ability of DcNP to co-formulate water-soluble gemcitabine and water-insoluble paclitaxel together in drug-combination nanoparticles, and intraperitoneal route to enhance uptake and retention in the pancreas, we found for the first time that a single dose of GT-DcNP (G:T, 20:2 mg/kg) suppressed a Pan02 ductal pancreatic adenocarcinoma tumor model, and all treated mice survived for the 28 day study while placebo and equivalent dose of free drug combinations were unable to do so. With additional validation and safety studies, GT-DcNP may be considered for preclinical and clinical development to find effective and safe treatments for pancreatic cancer.

4.6 Bibliography

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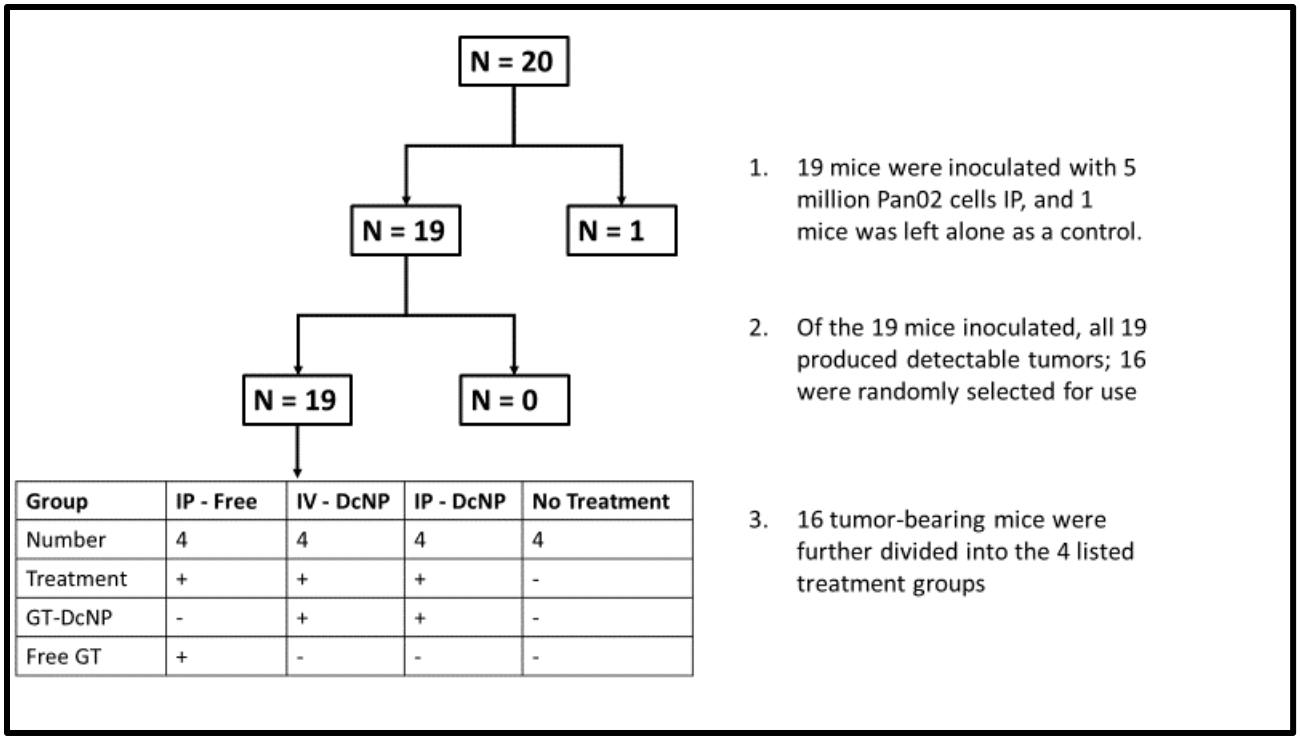


Group	IP - Free	IV - DcNP	IP - DcNP	No Treatment
Number	4	4	4	4
Treatment	+	+	+	-
GT-DcNP	-	+	+	-
Free GT	+	-	-	-

Figure 4.1 Experimental design scheme to assess the treatment effect of gemcitabine and paclitaxel on the Pan02 pancreatic tumor in mouse. A total of 19 C57/BL6 mice were inoculated with Pan02 cells by IP; followed by verification of 19 mice experessing Pan02-luciferase marker by whole-body luminescence in mice, 16 of 19 mice were enrolled in 3 gemcitabine and paclitaxel treatment groups and 1 placebo group. Remaining mice were kept as controls, but not used. The gemcitabine and paclitaxel drug combination was given on day 5 at 20mg G and 2mg T for each kg mouse of mix as a single dose in IP-free (soluble form by intraperitoneal injection), IV-DcNP (GT-DcNP dosage form by intravenous injection), IP-DcNP (GT-DcNP dosage form by intraperatoneal injection). The placebo or no-treatment group was used as a control. The mouse with no tumor inoculation was also used as a reference.

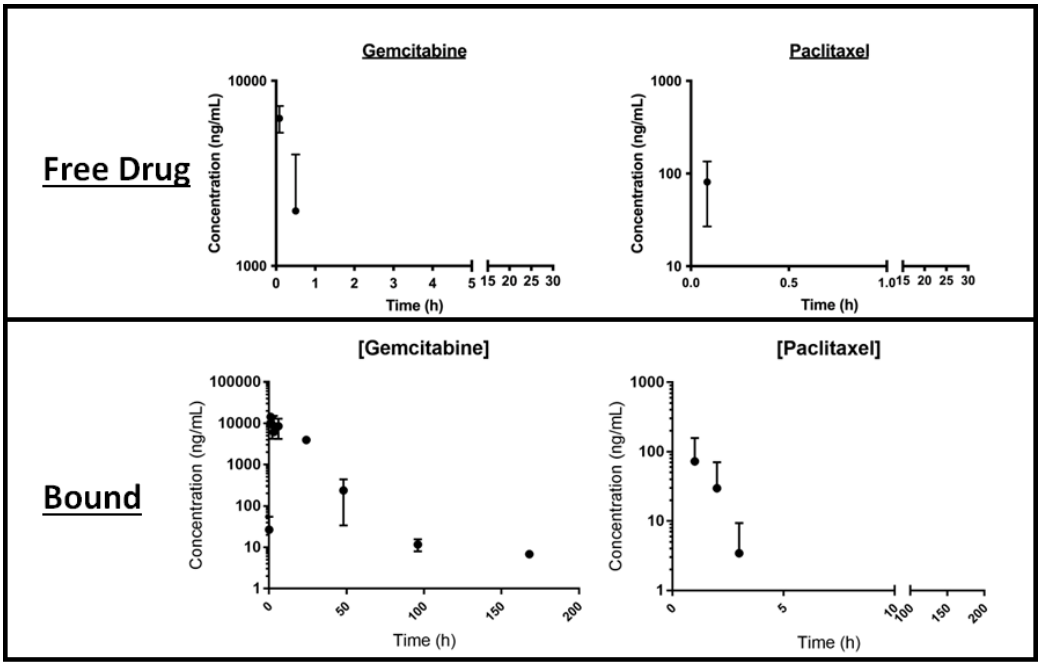


Figure 4.2 The effect of the DcNP formulation on the plasma pharmacokinetics of gemcitabine and paclitaxel following an intraperitoneal administration compared to an equivalent free drug dosage following either an intraperitoneal injection of either in DcNP or free gemcitabine (20 mg/kg) and paclitaxel (2 mg/kg); n = 3. Plasma was analyzed for up to 1 week after administration; both drugs quickly became unquantifiable or undetectable after the free dosage administration.

Table 4.1 Select pharmacokinetic parameters of gemcitabine and paclitaxel following either an intraperitoneal injection of either nanoparticle-bound or free gemcitabine (20 mg/kg) and paclitaxel (2 mg/kg); n = 3. Plasma was collected over 200 hours from the initial injection, and parameters are expressed as the median values ± standard deviations. AUC was calculated through non-compartmental modeling.

Drug	Injection State	C_{max} (ng/mL)	$t_{1/2}$ (h)	AUC (ng/mL*h)
Gemcitabine	Free GT	6,300 ± 8,900	0.25	2,200 ± 690
	GT-DcNP	14,000 ± 3200	24	98,000 ± 15,000
	DcNP/Free	2.3	96	45
Paclitaxel	Free GT	81 ± 54	N/A	17 ± 11
	GT-DcNP	73 ± 85	3.7	850 ± 75
	DcNP/Free	0.90	N/A	50

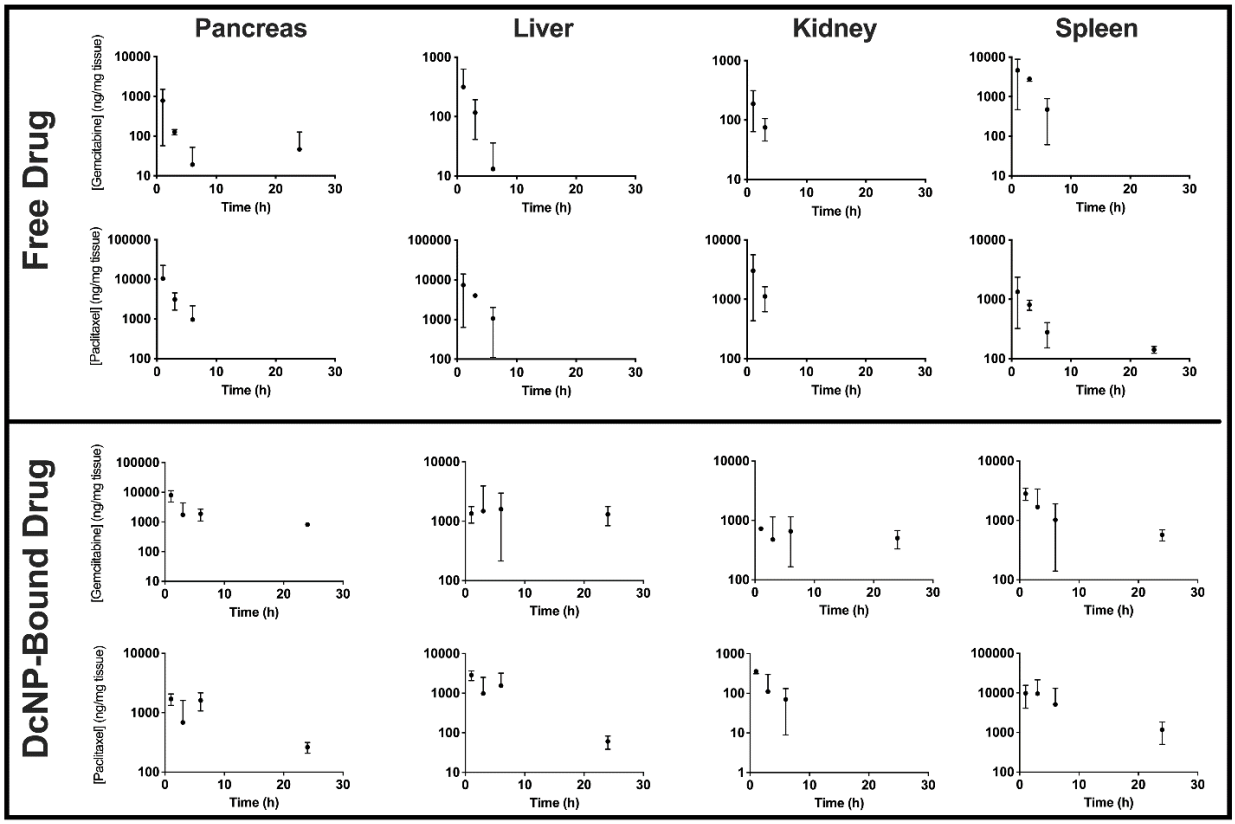
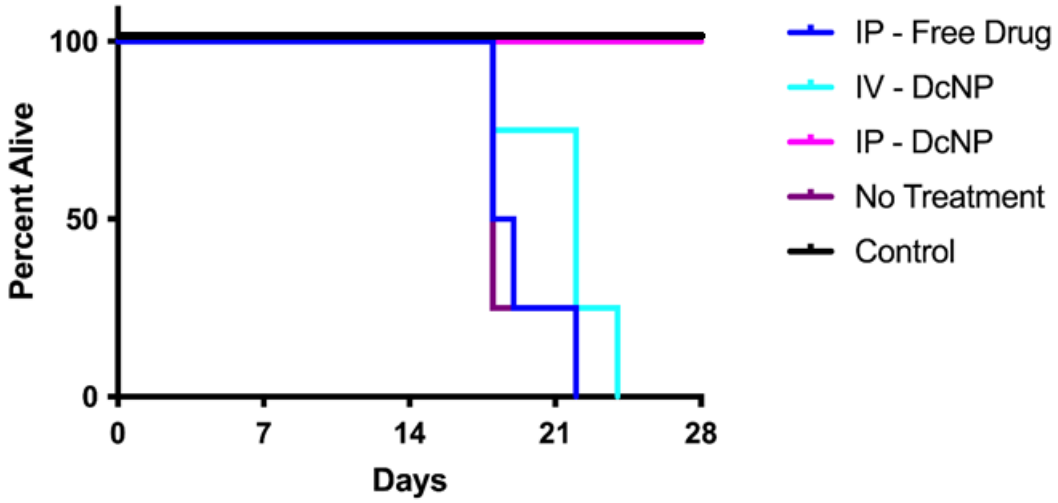


Figure 4.3 Distribution of gemcitabine and paclitaxel in select murine tissues following an intraperitoneal injection of free or DcNP dosage form. Mice were administered 20 mg/kg gemcitabine and 2 mg/kg paclitaxel, either as a free formulation or as a DcNP formulation. 3 mice were sacrificed at each pre-selected time point in order to harvest and quantify drug in tissue. Tissues were assessed up to 24 hours following administration.

Table 4.2 Tissue distribution of drug following an intraperitoneal injection of free or DcNP-formulated gemcitabine (20 mg/kg) and paclitaxel (2 mg/kg) (n=3).

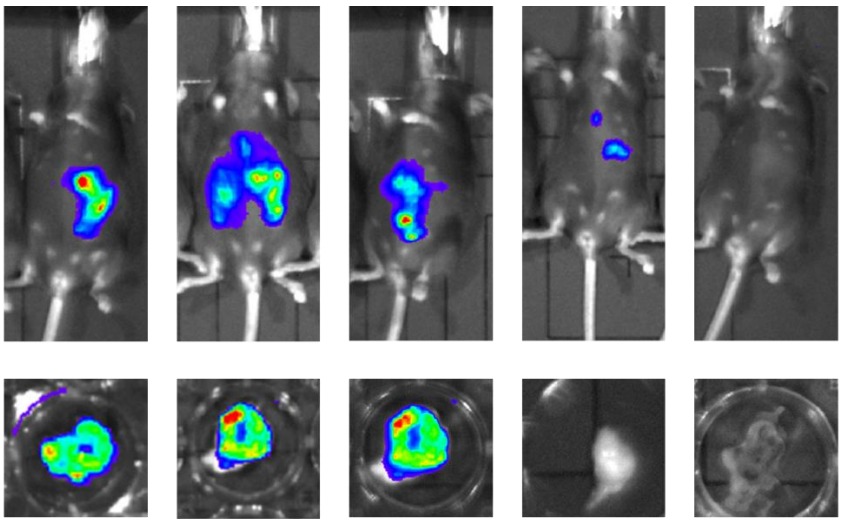
Concentration at 3 Hours (ng/mL)	Injection State	Plasma	Pancreas	Liver	Kidney	Spleen
Gemcitabine	Free	0 ± 0	130 ± 19	120 ± 76	75 ± 31	2,800 ± 350
	Bound	6,300 ± 8,900	1,800 ± 2,600	1,500 ± 2,400	780 ± 670	1,100 ± 1,500
Paclitaxel	Free	0 ± 0	3,100 ± 1,400	4,000 ± 110	1,100 ± 510	810 ± 160
	Bound	3.3 ± 5.9	690 ± 920	980 ± 1,500	110 ± 190	6,400 ± 9,900

	Metric	Plasma	Pancreas	Liver	Kidney	Spleen
Gemcitabine	Bound/Free	N/A	14	N/A	6.4	0.40
Paclitaxel	Bound/Free	N/A	0.22	N/A	0.01	7.9



	Time of Euthanasia (Days)			
Group	**Mouse 1**	**Mouse 2**	**Mouse 3**	**Mouse 4**
IP-Free Drug	18	18	19	22
IV-DcNP	18	22	22	24
IP-DcNP	28	28	28	28
No Treatment	18	18	18	22
Control	28			

Figure 4.4 “Time-to-euthanasia” for each treatment group represents the point at which mice were terminated due to excessive tumor burden. 4 mice were used in each group, except for a control mouse that received no Pan02 nor cancer treatment. All mice, except for the control and “No Treatment” groups, were administered 20 mg/kg gemcitabine and 2 mg/kg paclitaxel as either a free or DcNP formulation through either the intravenous or intraperitoneal route. For specific details on each treatment group, please see the Figure 4.1 legend



Pan02 Inoculation	Yes	Yes	Yes	Yes	No
Treatment	None	IV GT-DcNP	IP Free Drug	IP GT-DcNP	None

Figure 4.5 Assessment of Pan02 tumor growth following treatment administration. Murine whole body and tissue images were taken using the IVIS, as described in Materials and Methods section. Mice were scanned 18 days after inoculation with Pan02 (14 days after treatment were administered). Pancreases were resected at euthanasia and were also scanned by the IVIS.

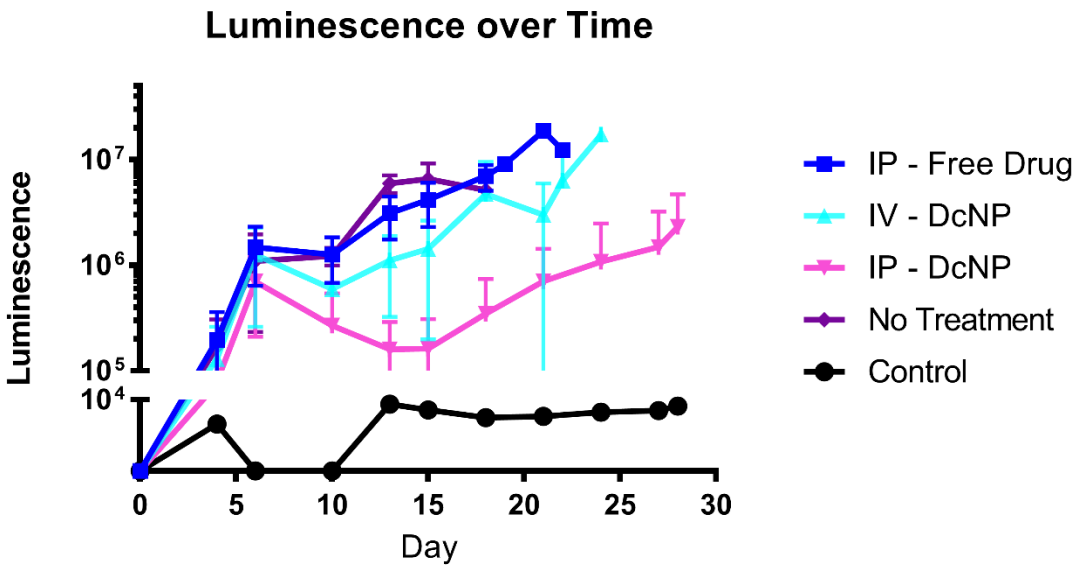


Figure 4.6 Assessment of Pan02 tumor luminescence over time following treatment administration. Murine whole body and tissue images were taken using the IVIS, as described in Materials and Methods section.

Chapter 5. Conclusions and Future Directions

5.1 Summary

Leukemia and pancreatic cancer therapies have both been limited by a high degree of drug clearance rates, limiting the ability of native drug molecules to reach, uptake, and accumulate in tissues and cells of interest for the purpose of controlling cancer growth and disease progression. Advancements in the discovery of cancer-associated growth/survival checkpoints controlled by select kinase enzyme that can be targeted with specific kinase inhibitors (TKI) and other forms of small molecule therapy have transformed many forms of leukemia from a deadly disease into a manageable, chronic disease with the possibility of treatment-free remission (TFR) in many patients. However, these modern and molecularly targeted treatments continue to be reliant on self-administered, high daily doses of chemotherapeutic drugs that can be burdensome to patients. Certain drugs, including the groundbreaking zanubrutinib (a kinase inhibitor), even require multiple daily administrations to achieve adequate drug concentrations at the desired sites of action due to their high clearance rates, which can lead to poor patient dose adherence. Similarly, treatments for pancreatic cancer also require frequent weekly intravenous administrations due to the high clearance rates and limited efficacy of their highly toxic and nonspecific small molecule components. Synchronizing the delivery of multi-drug regimens is also a problem due to the disparate pharmacokinetics of the variety of small molecule drugs utilized against the disease. Unlike leukemia, however, virtually all existing treatments for pancreatic cancer require some form of multiple drugs, given in sequential high-dose through intravenous infusion, making these complex treatments extremely taxing and intolerable for patients. The specific aim of this dissertation was to explore the use of a lipid-based nanoformulation to improve the pharmacokinetics of first-line small molecule drugs used in leukemia and pancreatic cancer treatment through the extension of constituent drug half-lives and enhanced uptake and exposure

in tissue and cells of interests. This nanoformulation, DcNP, has been explored for its applications in HIV treatment and prevention in humans as well as against metastatic breast cancer due to its ability to incorporate multiple drugs across a range of LogP values (i.e., both hydrophilic drugs and hydrophobic drugs in a single formulation). We found that VZ-DcNP (venetoclax and zanubrutinib) and GT-DcNP (gemcitabine and paclitaxel) results were promising, and with additional validation, they may be provide the possibility to eliminate both blood and pancreatic cancers.

Due to the lack of appropriate and robust pre-clinical pancreatic tumor models that consistent provide pancreatic cancer growth in the mouse pancreas, we investigated and discovered that only intraperitoneal inoculation of Pan02 (ductal pancreatic adenocarcinoma cells) led to the initial development of pancreatic tumors before spreading to other major organs; this was not observed when the cells were administered intravenously. These results, described in detail as part of Chapter 2, provide a foundation for a simple and consistent Pan02 pancreatic adenocarcinoma that forms tumors in the pancreas of C57/BL6 mouse. These findings along with the discovery that Pan02 orthotopic pancreatic tumors consistently develop over time in C57BL/6 mice, may enable the future evaluation of promising pancreatic treatment modalities. This pre-clinical model is reproducible, time-efficient, and operates in an immunocompetent environment. With the establishment of this validated pancreatic tumor model, we then evaluated the therapeutic impacts of DcNP composed of gemcitabine and paclitaxel.

In Chapter 3, we successfully developed a leukemia drug-combination nanoparticle that is stabilized by two lipid excipients, referred to as VZ-DcNP, that is suitable for testing in mice. The DcNP is capable of extending the half-lives of antileukemic drugs, venetoclax (V) and

zanubrutinib (Z), by 43- and 50-fold, respectively, compared to an equivalent dose of free drug in mice. VZ, an orally active drug regimen, is a next-generation small molecule combination therapy for leukemia; venetoclax, a Bcl-2 inhibitor has shown strong anticancer effects when paired with ibrutinib, a TKI of Bruton's Tyrosine Kinase (BTK). Zanubrutinib is a second-generation BTK inhibitor with an improved safety profile compared to ibrutinib; though research is early and zanubrutinib is currently only FDA-approved as a second-line monotherapy for refractory leukemia, zanubrutinib will likely replace ibrutinib in the coming years as seen in the ongoing SEQUOIA trial. Unlike the contemporary orally active regimen, our VZ-DcNP could represent an alternative delivery mechanism using a once-per-month injection as opposed to the multiple daily treatments required for the current regimen, which could alleviate patient burdens and reduce patient infidelity to strict daily regimens.

In Chapter 4, we tested the effects and applications of GT-DcNP on bound-drug pharmacokinetics, bound-drug distribution, and as an anticancer agent against used the previously established Pan02 murine model of metastatic pancreatic cancer. Following a single intraperitoneal administration of GT-DcNP, we found that the DcNP nanoformulation was able to extend the plasma and tissue half-lives of both gemcitabine and paclitaxel. In DcNP dosage form, gemcitabine was detectable up to 1 week after in mouse plasma (compared to ~30 minutes for free drug treated mice). Similarly, in GT-DcNP mice, paclitaxel was detectable up to 3 hours after injection as opposed to ~5 minutes in the free dosage form. The ability of DcNP to extend plasma half-life was also noted in the enhanced tissue drug exposure, including the ability to detect gemcitabine and paclitaxel in the pancreas and kidneys for 1 week (at G:T, 20:2 mg/kg single dose). When administered to Pan02-burdened mice, intraperitoneal GT-DcNP (G:T, 20:2 mg/kg single dose) was able to drastically reduce and even eliminate pancreatic Pan02 tumors whereas an equivalent

free drug formulation or equivalent GT-DcNP dose delivered intravenously were unable to reduce the Pan02 tumor burden as seen through both *in vivo* luminescent imaging and post-mortem autopsy assessment. Notably, with a yet to be optimized G:T drug ratio, G:T at a fixed dose combination of 20:2 mg/kg single dose given through intraperitoneal dosing allowed all treated mice to survive the study window while all placebo treated mice succumbed to disease and death.

5.2. Future Prospects

This dissertation research was developed to test the hypothesis that "an anti-cancer drug-combination nanoparticle product can be made and demonstrated to exhibit long-acting pharmacokinetics that is also suitable for the treatment of currently uncurable pancreatic cancer." The overarching goal has been to develop and characterize a drug combination dosage form that will be stable, effective, and safe to provide next-generation treatment for pancreatic and blood cancers. As presented in this body of literature and experimental research, we were not only able to develop a pivotal research tool (an orthotopic pancreatic tumor model suitable for drug evaluation), but also develop and use the DcNP platform to stabilize cancer drugs with disparate physiochemical characteristics to form GT-DcNP nanoparticles suitable for testing anticancer effects and safety in mice.

Further development and evaluation of the drug-combination nanoparticle system's ability to treat both chronic leukemia and metastatic pancreatic cancer through the nanoformulation's ability to protect the associated drugs from systemic clearance, enzymatic or otherwise, demonstrated prolonged plasma half-lives and tissue distribution of drugs. The nanoparticles for pancreatic cancer and leukemia have proven useful for the extension of DcNP formulated drugs' pharmacokinetics. As GT-DcNP and VZ-DcNP are composed of a single fixed-dose combination,

the effects of varying ratios of GT or VZ that may influence drug pharmacokinetics remain unknown and require further investigation. In addition, DcNP formulated drugs' stability and safety is apparent for the current composition in mice, and their stability in humans may be of translational research interest. Although there remains additional research and develop in translation of these significant findings in DcNP platform as well as the GT-DcNP and VZ-DcNP effects on pancreatic cancer and leukemia, the results and findings described in this dissertation may serve as a basis for future work in developing the next generation drug combination nanoparticles. With the ability to enhance tissue and cell selective exposure over extended time in a synchronized manner, these DcNP dosage forms may allow development of safer and more effective drug-combination therapies against leukemic and pancreatic cancers.