Cancer Stem Cells And Tyrosine Kinase Receptors Directed Dual Targeted Nanoparticles For Pancreatic Ductal Adenocarcinoma Therapy And Imaging

Rami Alzhrani
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CANCER STEM CELLS AND TYROSINE KINASE RECEPTORS DIRECTED DUAL TARGETED NANOPARTICLES FOR PANCREATIC DUCTAL ADENOCARCINOMA THERAPY AND IMAGING

by

RAMI MOHAMMED ALZHRANI

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2020

MAJOR: PHARMACEUTICAL SCIENCES

Approved by:

________________________________________
Advisor

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Date

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# TABLE OF CONTENTS

Acknowledgments ................................................................................................................................. ii

List of Tables ................................................................................................................................................. ix

List of Figures ................................................................................................................................................. x

List of Abbreviations ................................................................................................................................. xiii

Chapter 1 Literature Review ......................................................................................................................... 1

1.1. Pancreatic Ductal Adenocarcinoma ............................................................................................... 1

1.2. Pancreatic Cancer Stages And Risk Factor .................................................................................. 3

1.3. Screening to Detect Pdac .............................................................................................................. 5

1.4. Challenges of Treating Pdac ........................................................................................................... 6

1.4.1. Tumor Microenvironments (TME) ............................................................................................. 7

1.4.2. Cancer Stem Cells (CSCs) In PDAC ......................................................................................... 9

1.4.3. Role of Hypoxia In PDAC ......................................................................................................... 11

1.4.4. Inflammation and Immune Cells In PDAC ................................................................................ 12

1.4.5. Role of Ras In PDAC .................................................................................................................. 13

1.5. Strategies to Overcome PDAC Barriers ............................................................................................ 14

1.6. Current PDAC Therapy and Ongoing Clinical Trials .................................................................... 17

1.6.1. First-Line Therapy ...................................................................................................................... 18

1.6.2. Second-Line Therapy ............................................................................................................... 19

1.6.3. Immunotherapy ......................................................................................................................... 20
1.7. Role of Drug Delivery In Treating PDAC ................................................................. 26

1.7.1. Advances In Nanoparticle Diagnosis and Treatment ........................................... 27

1.8. Conclusion and Future Directions ........................................................................... 32

Chapter 2 Research Objective and Specific Aims ........................................................... 34

2.1. Specific Aim 1: Synthesis, Characterization, and Optimization Hybrid Nanoparticles
As An Imaging Agent To Target Both CD44 And c-Met ............................................... 35

2.2. Specific Aim 2: Synthesizing, Characterizing, and Activity Testing of
Chemotherapeutic Agent Conjugated To HA-GE137 ................................................. 36

Chapter 3 Targeting CD44 and c-Met Overexpressed Pancreatic Cancer Cells Via Fabricating
Dual-Targeted Diagnostic Nanoparticles ...................................................................... 37

3.1. Introduction .............................................................................................................. 37

3.2. Materials and Methods .......................................................................................... 39

3.2.1. Cell Culture, Reagents and Chemicals ................................................................. 39

3.2.2. Cells Culturing Conditions .................................................................................. 40

3.2.3. Animals Husbandry ............................................................................................ 40

3.2.4. Preparation and Characterization of NIR Dye Conjugated HA-GE137 Nanoparticles
...................................................................................................................................... 40

3.2.5. Expression of CD44 and c-Met on Different PDAC Cell Lines ......................... 41

3.2.6. Rhodamine Colocalization With CD44 and c-Met Biomarkers ......................... 42

3.2.7. NIR Dye Conjugated HA-GE137 Imaging and Bio-Distribution ....................... 43

3.2.8. Tumor/ Liver Ratio Quantification ....................................................................... 43

3.2.9. Statistical Analysis ............................................................................................... 43
3.3. Results and Discussion .................................................................................................................. 43

3.3.1. Targeting CD44 In PDAC ........................................................................................................ 43

3.3.2. Targeting Tyrosine Kinase Receptor (c-Met) In PDAC ............................................................ 44

3.3.3. Rationale For Targeting CD44 and c-Met ............................................................................... 45

3.3.4. Procedure For Synthesis HA-GE137 Near-Infrared Dye ...................................................... 48

3.3.5. Specific Tumor Colocalization and Accumulation of HA-GE137 Nanoparticles .... 51

3.3.6. Conclusion ................................................................................................................................. 56

Chapter 4 Tumor Stroma Disrupting Nanoparticles For Chemo Guided Immunotherapy of Pancreatic Ductal Adenocarcinoma .................................................................................................................. 57

4.1. Introduction ...................................................................................................................................... 57

4.2. Materials And Methods .................................................................................................................. 58

4.2.1. Cell Culture, Reagents And Chemicals ..................................................................................... 58

4.2.2. Cell Culture Conditions ............................................................................................................ 59

4.2.3. Animals Husbandry .................................................................................................................. 59

4.2.4. MTT Assay of Several New Single Drugs and New Combinations .......................................... 59

4.2.5. Synthesis and Characterization of Gem Conjugated HA-GE137 ............................................. 60

4.2.6. Western Blot Analysis .............................................................................................................. 62

4.2.7. Evaluation of The Activity of HA-GE137-Gem + Everolimus In nu/nu Mice .......... 62

4.3. Results And Discussion .................................................................................................................. 63

4.3.1. Rationale of Using of Gem Conjugated HA-GE137 ................................................................. 63

4.3.2. Procedure of Synthesis HA-GE137-Gem by EDC/NHS Coupling Chemistry .......... 65
4.3.3. Preparation and Characterization of HA-GE137-Gem Nanoparticles .................. 68

4.3.4. Cell Viability Assay of Several Drugs On PDAC Cell Lines .............................. 69

4.3.5. Evaluation of The Synergistic Effect of (Gemcitabine With Everolimus) .......... 72

4.3.6. Evaluate The Synergetic Activity of HA-GE137-Gem With Everolimus ......... 75

4.3.7. Evaluation of The Antitumor Activity of HA-GE137-Gem + Everolimus In nu/nu Mice .................................................................................................................. 77

4.3.8. Conclusion ........................................................................................................... 79

References ....................................................................................................................... 80

Abstract ............................................................................................................................. 110

Autobiographical Statement ............................................................................................ 112

List of Publications: ......................................................................................................... 112

List of Poster Presentation And Meeting Attendance ..................................................... 112
LIST OF TABLES

Table 1.1: Pancreatic Cancer Stages ................................................................. 5

Table 1.2: Clinical Trials of Drug Delivery in PDAC (Gemcitabine-Based Therapies) ........ 24

Table 3: Clinical Trials of Drug Delivery in PDAC (Immunotherapy for PDAC) .............. 25
LIST OF FIGURES

FIGURE 1-1: Histological characteristics of pancreatic ductal adenocarcinoma. (A) Random arrangements of glands (B) Nuclear pleomorphism (C) Incomplete glandular lumina (D) Luminal necrosis (E) Glands adjacent to muscular vessel (F) Perineural invasion (G) Lymph vascular invasion [13]............................................................... 3

FIGURE 1-2: Pancreatic ductal adenocarcinoma (PDAC) stages and tumor microenvironments (TME). (A) different stages of PDAC and the expression of oncogenes at each stage. (B) The complexity of TME components that attenuate cytotoxic drug penetration......................... 9

FIGURE 1-3: Different classes of drugs that are being explored in the clinical trials to enhance the efficiency and the overall PDAC response. .......................................................... 23

FIGURE 1-4: Different types of nanoparticles for PDAC therapy. (A) surface decorated nanoparticles to enhance the tumor selectivity passively or actively. (B) Specific nanoparticles selectivity toward tumor cells that actively via receptor recognition or passively via EPR effect. (C) Uptake nanoparticles via the endocytosis process result in cancer cell eradication............................................................................................................ 31

FIGURE 3-1: CD44 and c-Met expression in different PDAC cell lines. (A) and (B) CD44 and c-Met expression in tumor section of Immunohistochemistry of orthotopic xenograft mice, (C) and (D) Western blot of CD44 and c-Met protein expression. (E) and (F) quantification of two normal pancreas cell lines and 5 PDAC cell lines.......................................................... 47

FIGURE 3-2: (A) CHEMICAL Schematic representation of dual-targeted diagnostic agent synthesis (HA-GE137-Rhodamine and HA-GE137-Rhodamine) (B and C) 1H NMR of HA and HA-SH respectively. The red star is an indicator of different conjugated NIR dye to HA-SH... 49

FIGURE 3-3: (A) Dynamic light scattering (DLS) of HA-SH (147 nm) and HA-GE137-Rho (196.3 nm) nanoparticles. (B) zeta potential of HA-GE137-rhodamine and HA-GE137-S0456, (C) Ellman’s assay for thiol quantification of HA-SH........................................................................................................ 50

Figure 3-4: The accumulation and colocalization of Rhodamine B and HA-GE137-Rhodamine in PDAC tumors. (A) the intensity of accumulated Rhodamine B and HA-GE137-Rhodamine in the tumor site after 96 hours. (B) the expression of CD44 and c-Met biomarkers in AsPc-1 cell line inoculated in mice bearing tumor. (C) and (D) the qualitative and quantitative colocalization of Rhodamine B and HA-GE137-Rhodamine with CD44 and c-Met overexpressed biomarkers. (E) the binding affinity of HA-GE137-Rhodamine to CD44 and c-Met receptors of AsPc-1 cell lines......................................................................................... 53

Figure 3-5: in vivo tumor accumulation of S0456 and HA-GE137-S0456 after (A) 24 hours and 48 hours. (B) bio-distribution in different body organs where HA-GE137-S0456 is mainly
accumulated in tumor sites while S0456 accumulated in the liver. (C) comparison between NIR dye conjugated HA-GE137 liver accumulation and free NIR dye. (D) and (E) evaluation the tumor/liver accumulation of HA-GE137-S0456 and S0456. Red arrows indicate the liver site, while green arrows indicate the tumor site.

Figure 4-1: Schematic representation of dual-targeted conjugated to gemcitabine.

Figure 4-2: 1H NMR characterization of the construct (HA-GE137-GEM). (A) 1H NMR of hyaluronic acid, (B) 1H NMR of thiolated hyaluronic acid. (C) 1H NMR of Gemcitabine conjugated to SPDP-Peg4-NHS (D) 1H NMR of the dual-targeted nanoparticles conjugated to gemcitabine.

Figure 4-3: Characterization of dual-targeted nanoparticles (A and B) Hydrodynamic size of thiolated dual-targeted nanoparticles and Gem conjugated dual-targeted nanoparticles. © the summary of nanoparticles diameter (nm) and the surface charge (zeta potential) of thiolated hyaluronic acid (HA-SH), thiolated dual-targeted nanoparticles (HA-GE137-SH), and Gem conjugated nanoparticles (HA-GE137-Gem).

Figure 4-4: The cytotoxicity of commercials drugs (Gemcitabine (A), Everolimus (B), and Cabazitaxel (C)) on several AsPc-1 cell lines. (D) the cytotoxicity of dual-targeted nanoparticles conjugated to Gem. (E) the summary of the half-maximal inhibitory concentration of all commercials drugs and nanoformulations on AsPc-1 cell lines. (F) western blot analysis to evaluate the pharmacological activity after exposing the AsPc-1 sequentially to Dual-targeted nanoparticles (HA-GE137-Gem + EVR) and commercial Gem + EVR.

Figure 4-5: The evaluation of the activity of Gem and HA-GE137-Gem on PDAC cell lines. (A) HA-GE137-Gem activity of AsPc-1 cell line compared to free commercial Gem. The CompuSyn® data utilized to evaluate all possible synergistic doses of combined Gem and EVR. (B) Panc-1 and (C) HPAC showed the lowest response to most of Gem + EVR doses. The other cell lines (D) MiaPaCa-2, (E) AsPc-1 and (F) BxPc-3 strongly inhibited after being treated sequentially by Gem + EVR.

Figure 4-6: Combination Index of HA-GE137-Gem and commercial Everolimus on (A) AsPc-1 and (B) BxPc-3 cell line. Combination Index of Gemcitabine and Everolimus (Commercial drugs) on (C) AsPc-1 and (D) BxPc-3 cell line. HA-GE137-Gem + EVR showed a high synergistic effect by several folds on (E) AsPc-1 and (F) BxPc-3 compared to commercial drugs.

Figure 4-7: (A) Evaluation of tumor inhibition rate of AsPc-1 bearing nude mice after treating with Vehicle, Gemcitabine (Gem) + Everolimus (EVR) and HA-GE137-Gem + EVR (the data shown as the mean ± SEM). P-value of HA-GE137-Gem + Everolimus vs control, Vehicle, and Gem + EVR (0.0008, 0.0018, and 0.0038 respectively). (B) Kaplan-Meier curves of all AsPc-1 bearing nude mice treated with Vehicle, Gemcitabine + Everolimus and
HA-GE137-Gem. (C) The mice’s body weight (g) did not show any significant changes after being exposed to several doses of VEHICLE, Gemcitabine + Everolimus and HA-GE137-Gem + EVR (the data shown as the mean ± SEM). (D) There were no significant changes in the nude mice serum level of AST, ALT, and creatinine after being exposed to Vehicle, Gemcitabine + Everolimus, and HA-GE137-Gem + EVR (the data shown as the mean ± SEM).
LIST OF ABBREVIATIONS

1. 2',2-Difluorodeox-Cytidine Triphosphate (dFdCTP) ......................................................... 8
2. Breast Cancer Gene (BRCA2) ............................................................................................ 2
3. Cancer Stem Cells (CSCs) .................................................................................................. 11
4. Cancer-Associated Fibroblasts (CAFs) .............................................................................. 32
5. Cerium Oxide Nanoparticles (CONPs) .............................................................................. 33
6. Cluster Of Differentiation 4 (CD4+) .................................................................................... 14
7. Cluster Of Differentiation 8 (CD8+) .................................................................................... 14
8. Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A) .............................................................. 2
9. Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) ................................................................. 22
10. Desmoplastic Reaction (DR) ............................................................................................ 8
11. Enhanced Permeation And Retention (EPR) ..................................................................... 30
12. Epidermal Growth Factor Receptor (EGFR) .................................................................... 2
13. Epithelial To Mesenchymal Effect (EMT) ....................................................................... 11
14. Extracellular Matrix (ECM) ............................................................................................. 9
15. Fibroblast Growth Factor, And Epidermal Growth Factor (EGF) ....................................... 9
16. Guanine Nucleotide Exchange Factors (GNEF) ............................................................... 15
17. Guanosine Diphosphate (GDP) ....................................................................................... 15
18. Guanosine Triphosphate (GTP) ........................................................................................ 15
| 19. Hepatocyte Growth Factor (HGF) | ................................................................. | 9  |
| 20. Human Relaxin-2 (RLX) | .......................................................................... | 32 |
| 21. Hyaluronan Receptor For Endocytosis (HARE) | ................................................................. | 55 |
| 22. Hypoxia-Inducible Factor (HIF) | .......................................................................... | 13 |
| 23. Immune Checkpoint Inhibitors (ICIs) | .......................................................................... | 1  |
| 24. Interstitial Fluid Pressure (IFP) | .......................................................................... | 29 |
| 25. Intraductal Papillary Mucinous Neoplasm (IPMN) | .......................................................................... | 3  |
| 26. Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) | .......................................................................... | 2  |
| 27. Magnetic Resonance Imaging MRI | .......................................................................... | 7  |
| 28. Metalloproteinase (MMPs) | .......................................................................... | 10 |
| 29. Microsatellite Instability-High (MSI-High) | .......................................................................... | 24 |
| 30. Mitogen-Activated Protein Kinase Kinase (MAP2K4) | .......................................................................... | 2  |
| 31. Mucinous Cystic Neoplasm (MCN) | .......................................................................... | 3  |
| 32. Myeloid-Derived Suppressive Cells (MDSCs) | .......................................................................... | 14 |
| 33. Natural Killers (NKs) | .......................................................................... | 14 |
| 34. Overall Response Rate (ORR) | .......................................................................... | 17 |
| 35. Overall Survival Rate (OS) | .......................................................................... | 7  |
| 36. Pancreatic Cancer (PC) | .......................................................................... | 1  |
| 37. Pancreatic Cancer Genetically Engineered Mouse Model (GEMM) | .......................................................................... | 8  |
38. Pancreatic Ductal Adenocarcinoma (PDAC) ................................................................. 1
39. Pancreatic Stellate Cells (PSCs) .................................................................................. 9
40. Patient-Derived Xenografts (PDX) .............................................................................. 29
41. Positron Emission Tomography/Magnetic Resonance Imaging (PET/MRI) ................. 30
42. Preneoplastic Pancreatic Intraepithelial Neoplasms (PANINs) ................................... 8
43. Producing Secreted Protein Acidic And Rich In Cysteine (SPARC) ....................... 9
44. Programmed Cell Death Ligand-1 (PD-L1) .................................................................. 22
45. Programmed Cell-Death-1 (PD-1) .................................................................................. 22
46. Serine/Threonine Kinase 11 (STK11) ......................................................................... 2
47. Signal Transducer And Activator Of Transcription 3 (STAT3) ................................. 17
48. Single-Photon Emission Computed Tomography (SPECT) ......................................... 39
49. Sonic Hedgehog (SHH) .............................................................................................. 9
50. Supermagnetic Iron Oxide Nanoparticle (SPION) ....................................................... 31
51. Transforming Growth Factor, Beta Receptor II (TGFBR2) ......................................... 2
52. Transforming Growth Factor-Beta (TGF-B) ................................................................. 9
53. Tumor Microenvironments (TME) ................................................................................ 8
54. Vascular Endothelial Growth Factor (VEGF) ............................................................ 13
CHAPTER 1 LITERATURE REVIEW

1.1. PANCREATIC DUCTAL ADENOCARCINOMA

In 2016, the American Cancer Society rated pancreatic cancer (PC) as the third leading cause of death in the US. Pancreatic ductal adenocarcinoma cancer (PDAC) is the most common pancreatic cancer, which accounts for 90% of all pancreatic cancer [1]. The five years survival rate of pancreatic cancer, which is 8%, is the lowest among other solid cancers. Most of the patients are diagnosed at a metastatic or an advanced stage (unresectable tumor) [2].

In PDAC, poor prognosis is the main challenge due to the propensity of tumors to metastasize and develop resistance against chemo and radiation therapy [2]. The incidence of pancreatic cancer in the US is high among African Americans than other races; and the reason is attributed to several risk factors such as smoking and diabetes [3,4]. The incidence of pancreatic cancer differs between sexes; men are 50% more at risk than women [5]. Also, the most occurring incidence is in older adults (60-80 years old). Genetic factors play a role in causing pancreatic cancer; for instance, germline mutation in breast cancer gene (BRCA2) induces several cancers (ovarian, breast, and pancreatic cancer) [6]. The most common genetic abnormalities in pancreatic ductal adenocarcinomas are mutations in kirsten rat sarcoma viral oncogene homolog (KRAS) oncogene activator, inactivation of tumor-suppressor genes such as cyclin-dependent kinase inhibitor 2A (CDKN2A), TP53, and BCRA2 [7]. It has been found that KRAS mutation and telomere shortening are one of the earliest genetic abnormalities in PDAC. Moreover, genetic mutations may happen in pancreatic cancer oncogenes such as BRAF, AKT2, epidermal growth factor receptor (EGFR), or might occur in pancreatic tumor suppressor genes like mitogen-activated protein kinase kinase (MAP2K4), serine/threonine kinase 11 (STK11), and transforming growth factor, beta receptor II (TGFBR2) [8]. In 90% of KRAS, which is responsible for encoding GTPase that mediates
downstream pathway via growth factor receptor, mutations occurs in codon 12 [9]. 90% of CDKN2A, which is responsible for encoding essential cell-cycle regulator, is one of the suppressor genes that is mutated in PDAC. TP53 is another suppressor gene, which plays a role in cellular stress response, is mutated in a wide range of cancers [9]. Finally, SMAD4, which plays a significant role in mediating downstream pathways of TGFβ, is another suppressor gene that is inactivated in 50% of cancer types [9].

The signs of genetic alteration in each PDAC has been studied; KRAS mutations and CDKN2A alterations are found to be one of the early events in low-grade pancreatic intraepithelial neoplasias [10,11]. On the other hand, alteration of P53 and loss of Smad4 are found to be two of the late events that occur in intraepithelial neoplasias grade 3 [10]. PDAC is the most common pancreatic cancer characterized by the presence of dense stroma produced by the mucin-producing gland [5]. Several histological characteristics can help in identifying PDAC, as it is shown in Figure 1.

Some pancreatic ductal adenocarcinomas originate form intraductal papillary mucinous neoplasm (IPMNs) and mucinous cystic neoplasm (MCNs). IPMNs are composed of a mucinous cyst that plays a role in forming the pancreatic duct system. IPMNs are further categorized based on dysplasia that differentiates the epithelium lining into low, intermediate, and high grade [12]. In recent years, researchers began to focus on finding new ways to better understand the genetics and biology of PDAC to improve therapeutic outcomes. Stromal compartment is one of the significant areas that draws attention to be evaluated due to its role in patients’ poor prognosis. In the following section, PDAC stages, the major PDAC challenges, the current therapy as well as the recent clinical trials will be covered in detail.
Figure 1-1: Histological characteristics of pancreatic ductal adenocarcinoma. (A) Random arrangements of glands (B) Nuclear pleomorphism (C) Incomplete glandular lumina (D) Luminal necrosis (E) Glands adjacent to muscular vessel (F) Perineural invasion (G) Lymph vascular invasion [13]

1.2. PANCREATIC CANCER STAGES AND RISK FACTOR

American Cancer Association categories pancreatic cancer to multiple stages based on its spreading out to adjacent organs and its size, as shown in Table 1. Many risk factors are associated with PDAC. Smoking is one of the major risk factors estimated to cause 25-29% of pancreatic cancer incidence. Family history is another risk factor that might increase the risk of getting a PC. Genetic diseases account for 5-10% of pancreatic cancer disease. BRAC2 is one of the significant genes that cause pancreatic cancer, where BRAC2 is a suppressor gene that helps in DNA repair mechanisms [14,15]. Other examples of hereditary diseases linked to pancreatic cancer are
hereditary pancreatitis, familial breast cancer, and multiple-mole melanoma increase the risk of PDAC [16]. Type I diabetes is not linked with PC, while type II diabetes is one of PC risk factors. Many studies have shown that type II diabetes accounts for a two-fold increase in PC [17]. Exposure to a carcinogenic agent is another risk factor that has been believed to participate in PC, but the evidence in this regard is inadequate. Age is one of the players that increases the incidence of PC; it has been found that PC incidence developed at around 65 years, but some patients develop PC early (at or before age 50). The exact etiology of the disease is still ambiguous, but studies show that patients might develop PC faster if they expose to several risk factors at the same time [18]. Up to date, insufficient knowledge about the exact causes of PC increase the challenges of getting therapeutic improvement. Therefore, there is an unmet need to know more about the causes of PC, and only then PC therapy would be more directed and efficient.
Table 1.1: Pancreatic Cancer Stages

<table>
<thead>
<tr>
<th>Stages</th>
<th>Keyword definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tis</td>
<td>The cancer presence on the top layer of the pancreas; no deep tissue involvement</td>
</tr>
<tr>
<td>T1</td>
<td>Cancer localized in the pancreas (its size is less than 2 cm)</td>
</tr>
<tr>
<td>T2</td>
<td>Cancer localized in the pancreas (its size is bigger than 2 cm but smaller than 4 cm)</td>
</tr>
<tr>
<td>T3</td>
<td>Cancer localized in the pancreas (its size is bigger than 4 cm)</td>
</tr>
<tr>
<td>T4</td>
<td>Cancer starts growing outside the pancreas and major blood vessel</td>
</tr>
<tr>
<td>Any T</td>
<td>It can be any size</td>
</tr>
<tr>
<td>N0</td>
<td>There is no lymph node involvement</td>
</tr>
<tr>
<td>N1</td>
<td>It has spread out to no more than three nearby lymph nodes</td>
</tr>
<tr>
<td>N2</td>
<td>It has spread out to no more than three nearby lymph nodes</td>
</tr>
<tr>
<td>Any N</td>
<td>Cancer may or may not spread out to the nearby lymph nodes</td>
</tr>
<tr>
<td>M0</td>
<td>No distant sites involvement</td>
</tr>
<tr>
<td>M1</td>
<td>Cancer starts spreading out to other organs such as liver and peritoneum, lungs or bones</td>
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</table>

<table>
<thead>
<tr>
<th>Stage</th>
<th>Stage grouping</th>
</tr>
</thead>
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1.3. SCREENING TO DETECT PDAC

The challenge of PDAC is that most of the patients are presented at the clinic at an advanced stage where the tumor starts to metastasize to different organs [19]. Therefore, detecting PDAC tumors in the early stage is crucial. Family history is used as one of the quantitative parameters in detecting PDAC; followed by screening individuals for inherited predispositions of early PDAC signs such as non-invasive intraductal papillary neoplasm and pancreatic intraepithelial neoplasm
There is an effort going by researchers to find an ideal screening test to diagnose those who are at risk of pancreatic neoplasia. The perfect test should be non-invasive and easy to do, like a blood test; unfortunately, none has been discovered so far [22]. The individual screening successfully identified silent pancreatic neoplasia in several patients whose families have a history of PC [23]. The effort now is to discover preinvasive lesions in PC instead of early PC, since finding and resection of preinvasive lesions stop developing invasive PC [24]. Several endoscopic screening tests can detect preinvasive lesions in pancreatic cancer. The CAPS3 multicenter screening trial showed that pancreatic cystic lesions were able to be identified using endoscopic ultrasound (93% success), magnetic resonance imaging MRI (81% success) [25]. Due to the impossibility of detecting pancreatic intraepithelial neoplasias microscopically, the researchers are attempting to find fluidic pancreatic markers that might help in identifying high-risk patients [26].

1.4. CHALLENGES OF TREATING PDAC

Pancreatic ductal adenocarcinoma (PDAC) prognosis is the poorest among all solid tumors. Even though the new advancements in finding the initial sign of PDAC, poor response to current therapies is still evident. One of the hypotheses that has emerged in the last few years is that the PDAC microenvironment is responsible for increasing both carcinogenesis and drug resistance. PDAC has an enriched microenvironment of the stromal barrier, which composes of fibroblasts, immune cells, blood vessels, neural cells, and cellular proteins (Figure 2, B) [27]. Stromal barrier decreases drug penetration to pancreatic cancer cells; consequently, the drug resistance increases [28]. Because of the stromal barrier, most of the combination therapies failed to achieve a significant increase in the survival rate [29]. In resectable tumors, the overall survival rate (OS) that has been achieved is ranging from 25.5 months using gemcitabine alone to 28 months when combined with capecitabine [30]. While in local advanced PDAC tumors, the OS ranged from 6.8
months using gemcitabine alone to 11.1 months after using FOLFIRINOX (fluorouracil (5-FU), irinotecan, and oxaliplatin) [31]. The following factors are believed to play a role in attenuating current therapeutic efficiency:

1.4.1. **Tumor microenvironments (TME)**

One of the main challenges in PDAC therapy is the presence of heterogeneous tumor microenvironments (TME). Pancreatic cancer genetically engineered mouse model (GEMM) has enabled researchers to mimic human pancreatic cancers in many aspects such as resistance to gemcitabine, and that opens new avenues in understanding pancreatic cancer microenvironments [32–34]. It has been discovered that PDAC is a stroma enriched TME which keeps changing its composition especially during progression from preneoplastic pancreatic intraepithelial neoplasms (PanINs) to invasive pancreatic cancer (Figure 2, A). Thus, stroma has a major role in increasing proliferation, metastasis, immune escape, and drug resistance [27]. Desmoplastic reaction (DR) is one of the hallmarks of PDAC that contributes to PDAC's poor prognosis. The dense desmoplasia plays a major role in distorting the normal architecture of pancreatic tissue and hinder blood perfusion; thus, drug penetration decreases, and disease resistance increases. Olive, K. et.al have reported that accumulation of active gemcitabine metabolites (2’,2-difluorodeox-cytidine triphosphate (dFdCTP)) was high in poor stromal cancer in subcutaneous and orthotopic mice model while it was barely detectible in high dense stroma cancer such as PDAC [35].

One of the pathways that participates in enhancing PDAC desmoplastic reaction is sonic hedgehog (SHH) signaling. Importantly, DR consists of multiple components such as fibroblasts, pancreatic stellate cells and extracellular matrix (collagens I, collagen II, and fibronectin) that all react together and worsen the clinical outcomes of PDAC patients [36]. The major two components of DR are pancreatic stellate cells (PSCs) and fibroblasts. It has been found that PSCs are involved
in secreting many cytokines that are contributing in proliferating, migrating, and producing extracellular matrix (ECM) protein [37]. Collagen is believed to be one of the factors that plays a role in enhancing and forming PSCs within pancreatic microenvironments [38]. Cross talk between the pancreatic cell and PSCs is a trigger of PDAC cells to grow and migrate via releasing some growth factors and cytokines [39]. Several pathways are believed to play a significant role in PSCs processes such as TGF-β, hepatocyte growth factor (HGF), fibroblast growth factor, and epidermal growth factor (EGF). Lohr et al. found that co-culture of transforming growth factor-beta (TGF-β) expressing Panc-1 is more proliferative and induce more production of collagen I and fibronectin [37]. Armstrong et al. have shown that PSC incubated with TGF-β enhanced [3H] thymidine uptake and induce collagen production [40]. Fibroblasts play a role in producing secreted protein acidic and rich in cysteine (SPARC) that enhances cell migration and proliferation and worsens prognosis in PDAC patients [2,41]. The activated factors of pancreatic cells promote activation of stroma cells which consequently affect other epithelial tumor components of pancreatic cancer [2]. Therefore, PSCs, fibroblasts and epithelial cells play a significant role in controlling extracellular matrix (ECM) through proteolytic enzyme of metalloproteinase (MMPs) which expresses in pancreatic cancer cells. It has been found that several enzymes of MMPs such as MMp-1, MMp-2, and MMp-9 are overexpressed when they come in contact with specific ECM proteins [42,43]. ECM is one of the major factors that enhance tumor aggressiveness and invasiveness; however, several studies proved that presence of PSCs and type I collagen in the pancreatic cancer environment leads to development of resistance against gemcitabine and radiation therapy [40,44]. Further understanding of activated pathway of PSCs- PDAC cross-talk is needed to develop PSCs targeted therapy [45,46].
**Figure 1-2:** Pancreatic ductal adenocarcinoma (PDAC) stages and tumor microenvironments (TME). (A) different stages of PDAC and the expression of oncogenes at each stage. (B) The complexity of TME components that attenuate cytotoxic drug penetration.

### 1.4.2. Cancer stem cells (CSCs) in PDAC

It is well known that TME is characterized by its heterogeneity which makes its pathological and physiological effects on tumor therapy not well understood. Epithelial to mesenchymal effect (EMT) is a stem cell characteristic that is believed to have a significant role in promoting tumor heterogeneity and cell metastasis [47]. Within TME, there is a subpopulation of the cells called cancer stem cells (CSCs) that are capable of self-renewal [48], and that can justify why many of tumors regenerate after being collapsed during chemotherapeutic treatment [49]. CSCs were firstly found in hematopoietic system. However, researchers found CSCs to be present in solid tumors such as breast [50], colon [51], brain [52], and pancreatic cancer [49]. Breast CSCs are found to be characterized by CD$_{44}^{\text{high}}$/CD$_{24}^{\text{low}}$ antigenic phenotypes which promote initiation of the tumor compared to other carcinomas that have CD$_{44}^{\text{low}}$/CD$_{24}^{\text{high}}$ [50].
However, in xenograft animal model of pancreatic cancer, it has been identified that a subpopulation of cells have CSCs properties and have CD44+, CD24+, and ESA+. In this study, the authors found that CD44+, CD24+, and ESA+ pancreatic cells have high potential to form tumors when injected at a concentration as low as 100 cells per mouse compared to CD44-, CD24-, and ESA- [49].

CD44 is a non-kinase, transmembrane glycoprotein (P-glycoprotein) that expresses on several cells and tissues such as embryonic stem cells and bone marrow. It has been found that CD44 is overexpressed in various tumor cells, and it is known as the biomarker of CSCs [53]. Furthermore, CD44 has a significant role in cancer stemness and promoting cancer tumorigenicity [54]. Hyaluronic acid binds to CD44; thus, many publications discussing drug delivery have reported the efficiency of using hyaluronic acid as CD44 binding ligand to improve the efficiency of PDAC first-line therapy [55–57].

In addition, hepatocyte growth factor receptor (c-Met), which presents in both normal and tumor cells, is essential for embryonic development and tissue repair [58]. c-Met receptor is a unique receptor that has only one ligand that can bind to it which is HGF [58]. Upon HGF binding to c-Met, a series of downstream signaling pathway events are mediated leading to enhanced normal cell growth, promote cell motility, and protect normal cells from apoptosis [59]. In cancer cells, c-Met functions differently than normal cells due to c-Met being overexpressed or mutated which ultimately enhances pancreatic carcinoma [60]. It has been found that exposing cells to chemotherapeutic agents such as gemcitabine enhances EMT, increases c-Met phosphorylation which promotes expression of CSCs biomarkers such as CD44 and CD24 [61]. Interestingly enough, CD44 plays a role in regulating HGF/c-Met signaling pathway which leads to maintaining CSCs function. Overexpression of c-Met in pancreatic cancer is a reason for poor prognosis which
elevate EMT phenotype. Li et al. have studied the role of c-Met forming tumorsphere. They found that c-Met+ cells had the ability to form spheres while c-Met− cells were not. Moreover, treating pancreatic xenografts in NOD-SCID mice with combination of c-Met inhibitor (XL184) and gemcitabine showed significant tumor growth regression even after 32 days of therapy cessation [62]. CSCs are one of the main players of PDAC distal metastasis as shown by Herman et al. who proved that PDAC distal metastasis is correlated with presence of CD133+ of pancreatic CSCs and CXCR4+ expression. [63]. Based on the above-mentioned findings, PDAC has a subpopulation of cells (CSCs) which play a crucial role in developing metastasis and chemotherapeutic resistance. Moreover, c-Met is another PDAC biomarker that enhances tumorigenicity of CSCs subpopulation. Together, these studies suggest that CSCs biomarkers such as (CD44+ and CD133+ cells) and c-Met+ cells can be used as new target therapy to minimize the tumor growth and enhance the tumor response to current therapy.

1.4.3. Role of hypoxia in PDAC

One of the significant obstacles that reduces drug delivery is tumor hypoxia that is caused due to the presence of insufficient blood vessels and high metabolic rate of cancer cells [64]. Therefore, cells that grow within these harsh conditions are known to be resistant to most chemotherapeutic agents and radiotherapy [65]. Hypoxia is an important factor in cancer. Chang Q et al. performed a study to measure the oxygen within pancreatic cancer. This experiment, which was conducted in seven patients, found a dramatic reduction of oxygen in pancreatic tumors compared to normal tissue [66]. The ability of cancer cells to survive in hypoxic conditions is attributed to activation of hypoxia-inducible factor (HIF) pathways. HIF can activate several genes that help cancer cells in controlling metabolism, survival, pH and migration as well as some angiogenic growth factors [67–69]. In PDAC, many studies have shown that pancreatic cancer
cells induce angiogenesis process via secreting vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [70,71]. Furthermore, HIF mediates several pathways such as c-Met and Hedgehog pathways that enhance cancer invasiveness and drug resistance [2,72]. Moreover, hypoxia is known to activate notch signaling pathway which plays a role in cancer cell proliferation and differentiation [73]. So far, four notch receptors have been identified which are overexpressed in both early PanIN and advanced stage of PDAC, while it is barely detectable in normal pancreatic tissue [74]. A notch signaling pathway is an early sign of PDAC which acts as a mediator to activate EGF receptors and play a role in activating PDAC precursors [74]. Furthermore, the notch pathway activates transforming growth factor-alpha (TGF-α) which functions as an activator of acinar-to-ductal metaplasia [75]. All these factors mediate cancer invasiveness, drug resistance, and increase the challenge in delivering drugs to TME of pancreatic cancer. Therefore, novel targeted therapy or delivery systems that could target hypoxic region or inhibit notch signaling pathway are needed to improve the overall therapeutic efficiency.

1.4.4. Inflammation and immune cells in PDAC

One of the major characteristics of TME is the absence of immune surveillance and presence of inflammatory cells that support aggressiveness and tumorigenesis of PDAC [76]. It is well known that several cells cooperate together to enhance PDAC proliferation [77]. Inflammatory and immune cells are components of TME that play a crucial role in PDAC aggressiveness. Within TME, the three main phases of cancer immunomodulation (elimination, equilibrium, escape) are linked with PDAC development and evolution of immune cells [78]. At the early stage, many immune cells such as anti-tumor Th1, cluster of differentiation 4 (CD4+), cluster of differentiation 8 (CD8+) and natural killers (NKs) are recruited to eliminate the cancer cells (elimination phase). So, the tumor cells start to develop an escape mechanism via recruiting
monocyte and neutrophil which acquire the anti-inflammatory phenotype (M2 and N2 respectively). Furthermore, tumor cells recruit or polarize T regulatory cells (Treg), shift anti-tumor Th1 to Th2 as well as myeloid-derived suppressive cells (MDSCs) recruitment. All these events result in deactivation of CD4+, CD8+ NKs and increase the tumor progression [79,80].

1.4.5. Role of Ras in PDAC

The Ras family belongs to small GTPase which is composed of HRAS, NRAS, and KRAS [81]. KRAS, CDKN2A, TP53, and SMAD4 are the most common genetic mutations found in pancreatic cancer, none of which is druggable. The absence of specific inhibitors for all of these genes limits the therapeutic options for PDAC patients [82]. Recent studies found that almost 95% of the patients have a mutational activation of oncogenic KRAS at codon 12 [83] that is crucial in activating and maintaining PDAC. Moreover, presence of KRAS mutation is a sign of poor prognosis in resectable and advanced PDAC patients [84]. Usually, KRAS protein has a crucial role in cell survival, differentiation and proliferation [85]. However, the mutated KRAS compromises the normal function of oncogenic KRAS; thus, the pancreatic cell growth becomes uncontrollable. The activation and inactivation of Ras proteins occur via binding and unbinding of RAS to guanosine triphosphate (GTP) and guanosine diphosphate (GDP), respectively. Guanine nucleotide exchange factors (GEFs) are responsible for regulating Ras GTP-GDP via stimulating GTPase activating protein (GAPs). GAPs play a crucial role in hydrolyzing GTP that activates Ras. The activated Ras-GTP stimulates multiple downstream effectors that regulate cell signaling and proliferation [86]. Ras mutation, which occurs in 12,13 and 61 residues, plays a crucial role in enhancing tumor proliferation in human via inhibiting GTP hydrolysis activity [87]. Ras mutation differs from one cancer to another such as HRAS mutations presented in melanoma, bladder, and mammary carcinoma; NRAS mutations found in melanoma and thyroid carcinoma; and KRAS
mutations widely found in pancreas, lung, ovary, bladder and thyroid. It is well known that KRAS 12 codon mutation is commonly present in pancreatic cancer [86]. In genetically engineered mice model, functional studies have found that KRAS switching off led to dramatic tumor inhibition [88,89]. Therefore, there are extensive pre-clinical and clinical ongoing studies to explore KRAS to design effective targeted therapy. Unfortunately, so far there is no inhibitor for KRAS that has reached the clinic [83]. Due to the undruggability of KRAS, targeting the KRAS downstream (MEK-ERK and/or PI3K) is the putative way to manage the KRAS related cancers. Using single inhibitors of downstream pathways (RAF, MEK1/2, ERK1/2 and PI3K) of RAS did not show significant clinical effect and drug resistance may induce via activating several compensatory mechanisms such as PI3K which activates several pathways such as Akt and mTOR [90]. Several inhibitors of RAS downstream pathway are being clinically tested. The role of KRAS in enhancing TME is not well understood. Mills, L et al. have shown the role of KRAS in TME. KRAS activates expression of SHH which further induce expression of GLI family zinc finger 1 (transcriptional factor) in fibroblasts. Binding of GLI1 to IL-6 promotor regulates the activation of cancer STAT3 in this study, loss of GLI family zinc finger 1 impaired the carcinogenic of KRAS in PDAC animal model [91]. Thus, targeting the crosstalk between cancer cells and TME components is one of the viable ways to promote PDAC tumor regression.

1.5. STRATEGIES TO OVERCOME PDAC BARRIERS

PDAC tumor is characterized by presence of a very dense stroma among other tumors that arise from pancreatic stellate cells (PSCs). Activation of PSCs leads to formation of extracellular matrix that enhances strength and resistance of PDAC cells to chemotherapeutic and radiation therapy [92,93]. Currently, several agents are being tested in pre-clinical and clinical studies in an attempt to enhance delivering of cytotoxic drugs to PDAC microenvironments (Figure 3). One of
the pathways that participate in enhancing PDAC desmoplastic reaction is sonic hedgehog (SHH) signaling. Using SSH signaling inhibitor is believed to be one of the effective ways to deplete stromal barriers and enhance gemcitabine delivery [94,95]. Feig et al. have combined smoothened inhibitor (IPI-926) with gemcitabine which majorly reduces tumor stroma, promotes micro-vessel density, and significantly increases intra-tumor gemcitabine active metabolite (dFdCTP); as a result, the overall survival increased [27]. Olive et al. have found that using Hh inhibitors allow chemotherapeutic agent to be delivered to the tumor site [35]. Accumulating evidence found an abundance of hyaluronan (HA) in PDAC tumors which play a significant role in enhancing tumor proliferation. A high level of HA is a sign of poor prognosis compared to those who have low level of expression. Therefore, one of the strategies in enhancing PC drug delivery is depleting HA using hyaluronidase [96]. In HALO202 clinical trial that recruited 279 patients, PEGylated hyaluronidase was combined with gem-nap in one arm and gem-nap alone on the other arm. In this study, it has been found that patients who had high level of HA responded much better than low HA with overall response rate (ORR) (45% vs. 31%) and OS (11.5 vs. 8.5 months), respectively [97].

PDAC stroma is enriched in many inflammatory cells such as mast cells, which consider the cornerstone of angiogenesis, tumor growth, and lymph node involvements [98]. Therefore, inhibition of mast cells activity is one of the current strategies to limit tumor progression. Ibrutinib, which is small molecule drug that permanently inhibits Bruton's tyrosine kinase (BTK) protein, is used to decrease fibrosis and inhibit mast cell cytokines release (IL-8, TNFα, and MPC-1) within PDAC TME. Ibrutinib has shown a reduction in tumor fibrosis in the mouse model and improve the mouse response to standard therapy [98,99].
Another target are cancer stem cells (CSC) which have a significant role in tumor proliferation and metastasis. Moreover, CSC enhances tumor resistance against chemotherapeutic agents and radiation therapy. The current hypothesis of the CSC role is CSC can tolerate standard treatment, and it is believed that CSC responsible for disease recurrence and distal metastasis [100,101]. One of the signaling pathways that promote CSC growth is Signal transducer and activator of transcription 3 (STAT3). It is well documented that STAT3 is activated in PDAC tumor and functions as tumor promotor. Therefore, inhibition of STAT3 would result in enhancing PDAC response to chemotherapeutic agents and promote tumor inhibition [102].

Another target protein that facilitate PDAC stromal depletion is secreted protein acidic and rich in cysteine (SPARC). SPARC is found to be overexpressed in human PDAC microenvironments by fibroblast and inversely correlate with survival [103]. Abraxane (albumin conjugated paclitaxel) has been hypothesized to possess ability to deplete stroma and accumulate in overexpressed SPARC pancreatic TME. There are contradictory results in this regard. One clinical trial has shown that combining gemcitabine with nab-paclitaxel increased the overall survival of high expressed SPARC patients (17.8 months) compared to those who had low SPARC expression (8.1 months) [104,105]. However, stromal depletion was not seen on a preclinical study of patient-derived xenograft mice model. The authors observed that gemcitabine activity was impaired due to activation of reactive oxygen species (ROS). This interesting observation was seen after using gemcitabine combined with nab-paclitaxel [106]. Thus, the exact role of SPARC is unknown and in-depth investigations are needed to reveal the particular role of this biomarker and its prognostic impact after treating with nab-paclitaxel or other targeted therapy.

Many methods have been utilized to target hypoxia and that includes prodrugs that are activated in hypoxic conditions, drugs that target HIF-1α active cells, and nanoparticles with
hypoxia active targeting being developed [107,108]. Evofosfamide (TH-302) is a cytotoxic prodrug composed of a mustard derivative that converted in hypoxic conditions to an active metabolite [109]. Clinically, Evofosfamide has been indicated to minimize radiotherapy resistance in PDAC. However, another clinical trial for NSCLC using combination of Evofosfamide with tarloxotonib (a hypoxia-activated tyrosine kinase inhibitor), was terminated early due to some concerns [109,110]. Another anticancer agent that was identified years ago is POP33. POP33 is a prodrug with the potential to elevate caspase-3 activity and induce apoptosis in HIF-1–active/hypoxic cells. This drug, which is a fusion protein, consists of a cleaved caspase pro-enzyme and a transduction HIF-1α dependent stabilization domain to deliver the drug into cells. Although POP33 exhibited a promise in an animal model of PDAC, there has yet to make its way for human application. While no direct inhibitors for HIF protein have reached the clinical studies for PDAC, treatment strategies that target heat shock protein (HSP) 90 have led to HIF degradation and there are many examples being tested [111,112].

1.6. CURRENT PDAC THERAPY AND ONGOING CLINICAL TRIALS

The most important challenge of the PDAC treatment is the presence of tumor microenvironment which characterized by constantly changing morphology and genetics that contributes to its aggressiveness [113]. The PDAC tumor microenvironment is also characterized by the desmoplasia (dense stromal cells) that adds to the resistance of the treatment. However, significant improvements have been achieved by using single or multiple regimes. The current ongoing clinical trials are summarized in Tables 2 and 3 which are in phase I/II. So far, the first and second-line therapies will be discussed in detail as following:
1.6.1. First-line therapy

In resectable tumors, gemcitabine so far has been the most preferred first-line drug because of its lower toxicity and good response rate [34]. This drug is used both as single therapy and in combination with other drugs such as 5-fluorouracil (5-FU) [114,115]. Using gemcitabine alone for six months after tumor resection has increased the 5-year OS to 20.7% compared to observation arm (OS of 10.4%) [116]. Other clinical trials had shown no major difference in OS and quality of life after using 5-FU/leucovorin (23.6 months) or gemcitabine (23 months) in resectable PDAC patients [30]. Therefore, gemcitabine alone or 5-FU/leucovorin was established as first-line therapy for those patients who have tumor resection. Moreover, recent clinical trial (phase III, NCT03610100) has designed a modified version of gemcitabine (Acelarin) to overcome current resistance of gemcitabine and enhance its efficiency via delivering high intracellular level of dFdCTP (gemcitabine active agent). Due to the pancreatic relapse after 6 months of gemcitabine treatment, a combination regimen should be considered as adjuvant therapy [30]. Neoptoloms et al. have investigated the efficiency of another combination of PDAC resectable using gemcitabine and capecitabine. They found that OS increased to 28 months compared to 25.5 months using gemcitabine alone. Interestingly, the adverse effects of using gemcitabine and capecitabine were tolerable [30].

For locally advanced pancreatic cancer, treatment is solely based on using combination therapy. One of the more explored combinations with some success in the metastatic PDAC is the FOLFIRINOX (fluorouracil, leucovorin, irinotecan, and oxaliplatin) [117]. Clinical trial PRODIGE 4/ACCORD 11 has shown that FLOFIRINOX was increased the OS rate up to 11.1 months compared to 6.8 months of using gemcitabine alone. FLOFIRINOX showed some adverse effects such as thrombocytopenia, neutropenia, febrile neutropenia, and diarrhea, and alopecia;
however, the patients’ quality of life was better than gemcitabine alone [31]. To minimize the FOLFIRINOX adverse effects, several retrospective studies have suggested reducing the initial chemotherapy doses which had a role in reducing side effects and maintain the regimen efficiency [118]. A phase II trial has shown that using modified FOLFIRINOX regimen enhanced treatment efficacy and reduced toxicity [119]. Also, PEFG (cisplatin, epirubicin, fluorouracil, and gemcitabine) was one of the first combinations taken to the clinical trials that showed less adverse effects and higher therapeutic efficacy [120]. Another such combination is gemcitabine with the EGFR inhibitor (erlotinib) that has been approved by FDA for PDAC [121]. Gemcitabine also has been paired with Abraxane, capecitabine alone and as gemcitabine/docetaxel/capecitabine (GTX) to give good response and better survival rates in PDAC patients. Gemcitabine with Abraxane was a successful combination [122] that was tried in phase III trials and was approved by FDA with serious adverse reactions (such as neutropenia, leukopenia, neuropathy, febrile neutropenia, or fatigue), however; its advantages outweighed them. Other combination therapies have also been known to show better results in the clinical trials. Many more combinations are put to test for PDAC constantly for increasing the success rate of therapy and to make a personalized medicine achievable. The main criteria that is taken in account in choosing the appropriate combination therapy is patient adverse effect and patient compliance [123].

1.6.2. Second-line therapy

Second-line therapy is mainly depending on the chosen first-line therapy. Thus, health care providers should take clinical trials into account for choosing the appropriate regimen that does not impact patient’s quality of life negatively and has minimal side effects. For instance, patients who received FOLFIRINOX as first-line therapy, gemcitabine-based regimen should be considered. For patients who failed to respond to FOLFIRINOX, gem-based therapy (gem-nap)
played a major role in improving OS (8.8 months) [124] with manageable side effects. On the other hand, if gem-based therapy used as first-line therapy, 5-FU can be used either as single agent or in combination with other agents such as oxaliplatin. Currently, there are many new pathways and targets are being explored such as the hedgehog pathway, KRAS pathways, JAK/STAT pathways, hyaluronic acid, angiogenesis, tyrosine kinase inhibitors, growth factor receptors and so on. Most of these trials are in phase I and phase II and are being evaluated for their safety profiles and toxicity profiles [125] (Table 3).

1.6.3. Immunotherapy

Another strategy for the treatment of PDAC is immunotherapy. The presence of immunosuppressive cells in the tumor microenvironment of PDAC is one of the reasons for the development of resistance to treatments. Hence, use of immune checkpoints in designing a treatment regimen for PDAC is a good avenue. The most widely used immune checkpoint blockades (ICB) used are against the programmed cell-death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4) receptors and programmed cell death ligand-1 (PD-L1) [126]. Several clinical trials have explored the single and combination therapies for the above two receptors and PD-L1 inhibitors in PDAC treatment. Nivolumab, Ipilimumab, Pembrolizumab, Defactinib, Durvalumab, Tremelimumab, Mogamulizumab, and so on are the most common drugs used either alone or in combination with each other or the other classes of chemotherapeutic agents (Figure 3). Further, several vaccines, monoclonal antibodies, and cytokines are being deployed in this regard. Despite the success of immunotherapy in some type of cancers, the results in PDAC is not promising. The disappointing results of using ICB in PDAC are attributed to presence of dense TME which acts as a barrier for most PDAC therapy. The effectiveness of ICB mainly depends on the presence of infiltrating lymphocytes which their number is limited in PDAC [2,127]. Single-agent clinical
trials for employing ICB for PDAC have not shown promising results. Single-agent anti-CTLA-4 (Ipilimumab) dosed intravenously at 3mg/kg/dose (4 doses/course, every 3 weeks, for maximum 2 courses) was practically ineffective for treatment of advanced pancreatic cancer [128]. Monotherapy with PD-1/PD-L1 ICB Durvalumab showed partial response in 2 out of 29 patients who received the intervention and had valuable data [129]. In essence, single-agent immunotherapies for PDAC have not garnered an effective patient outcome.

Antigenicity and immunogenicity are two major factors responsible for the failure of ICB in PDAC [130]. Reduced antigenicity in PDAC refers to the inability of tumor cells to produce and present tumor-associated antigens to the effector cells of the immune system. This, in turn, negatively affects the ability of T-cells to mount an immune attack in response to antigen producing cells. Immunogenicity, which is the ability to induce an immune response in cancer, is dependent on multiple factors like composition of the stroma, infiltration of CD8+ and CD4+ T-cells, B cells, antigen presentation.

Various reports have shown the ineffectiveness of monotherapies for PDAC. Hence, it is worthwhile to explore the effect of combinations of ICB or that of chemotherapy and ICB to tackle PDAC. Patients with poor prognosis and rapidly advancing cases of metastatic PDAC were dosed with a combination of Durvalumab and Tremelimumab. Where no patients responded well to Durvalumab monotherapy, a response rate of 3.1% was seen in patients receiving combination therapy although with adverse toxicity in 22% of the cohort of patients [131]. Gemcitabine, frontline chemotherapy for PDAC, has been tested in combination with ICB to disengage PD-1/PD-L1 interaction. Attempts have been made to deliver Gemcitabine and anti-PD-L1 in a controlled manner to the tumor. Gemcitabine primes the PDAC tumor for enhanced antigen presentation, thus making anti-PD-1 therapy more effective in eliciting a robust CD8+ T-cell
response and reduction in tumor load [132]. Cisplatin, albumin-bound Paclitaxel, Nivolumab are currently under clinical trials to expand on the promising outcomes of combination of ICB with chemotherapy. So far, it is likely that moving ahead, immunotherapy for PDAC shall shift clinical focus from monotherapy to combination therapy.

Pancreatic cancer patients with microsatellite instability-high (MSI-high) have a great chance (1% to 22% of the patients) to respond to immunotherapy [133]. In 2017, pembrolizumab FDA approval has been accelerated its usage for gastrointestinal cancers that have MSI-high. Based on clinical studies, patient’s response to pembrolizumab was 39.4%. Interestingly enough, the response of 78% of patients lasted for 6 months; thus, studies now are investigating the effectiveness of pembrolizumab with PC standard therapy such as gem, nap-paclitaxel, and FOLFORINOX [99].
FIGURE 1-3: Different classes of drugs that are being explored in the clinical trials to enhance the efficiency and the overall PDAC response.
Table 1.2: Clinical Trials of Drug Delivery in PDAC (Gemcitabine-Based Therapies)

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Table 3: Clinical Trials of Drug Delivery in PDAC (Immunotherapy for PDAC)

<table>
<thead>
<tr>
<th>Study Title</th>
<th>Phase</th>
<th>NCT number</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Study of Epacadostat in Combination With Pembrolizumab and Chemotherapy in Subjects With Advanced or Metastatic Solid Tumors (ECHO-207/KEYNOTE-723)</td>
<td>Phase 1/2</td>
<td>NCT03085914</td>
<td>Active, not recruiting</td>
</tr>
<tr>
<td>Paclitaxel Protein Bound Plus Cisplatin Plus Gemcitabine and Paricalcitol for Pancreatic Adenocarcinoma (NABPLAGEMD)</td>
<td>Phase 2</td>
<td>NCT03415854</td>
<td>Recruiting</td>
</tr>
<tr>
<td>A Phase I/Ib Study of NZV930 Alone and in Combination With PDR001 and /or NIR178 in Patients With Advanced Malignancies.</td>
<td>Phase 1</td>
<td>NCT03549000</td>
<td>Recruiting</td>
</tr>
<tr>
<td>Second-line Study of PEGPH20 and Pembro for HA High Metastatic PDAC</td>
<td>Phase 2</td>
<td>NCT03634332</td>
<td>Recruiting</td>
</tr>
<tr>
<td>Study of Pembrolizumab With or Without Defactinib Following Chemotherapy as a Neoadjuvant and Adjuvant Treatment for Resectable Pancreatic Ductal Adenocarcinoma</td>
<td>Phase 2</td>
<td>NCT03727880</td>
<td>Recruiting</td>
</tr>
<tr>
<td>Trial of Neoadjuvant and Adjuvant Nivolumab and BMS-813160 With or Without GVAX for Locally Advanced Pancreatic Ductal Adenocarcinomas.</td>
<td>Phase 1/2</td>
<td>NCT03767582</td>
<td>Recruiting</td>
</tr>
<tr>
<td>A Multiple Ascending Dose Study of MEDI7247 in Advanced or Metastatic Solid Tumors</td>
<td>Phase 1</td>
<td>NCT03811652</td>
<td>Completed</td>
</tr>
<tr>
<td>Evaluation of MMR Status and PD-L1 Expression Using Specimens Obtained by EUS-FNB in Patients With Pancreatic Cancer</td>
<td>N/A</td>
<td>NCT03820921</td>
<td>Not recruiting</td>
</tr>
<tr>
<td>Study Description</td>
<td>Phase</td>
<td>NCT Number</td>
<td>Status</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Nivolumab in Combination With Chemotherapy Before Surgery in Treating Patients With Borderline Resectable Pancreatic Cancer</td>
<td>Phase 1/2</td>
<td>NCT03970252</td>
<td>Recruiting</td>
</tr>
<tr>
<td>Pooled Mutant KRAS-Targeted Long Peptide Vaccine Combined With Nivolumab and Ipilimumab for Patients With Resected MMR-p Colorectal and Pancreatic Cancer</td>
<td>Phase 1</td>
<td>NCT04117087</td>
<td>Not recruiting</td>
</tr>
<tr>
<td>Mutant KRAS G12V-specific TCR Transduced T Cell Therapy for Advanced Pancreatic Cancer</td>
<td>Phase 1/2</td>
<td>NCT04146298</td>
<td>Recruiting</td>
</tr>
<tr>
<td>A Multi-Cancer, Multi-State, Platform Study of Durvalumab (MEDI4736) and Oleclumab (MEDI9447) in Pancreatic Adenocarcinoma, Non-Small Cell Lung Cancer and Squamous Cell Carcinoma of the Head and Neck to Correlate Clinical, Molecular and Immunologic Parameters With DNA Methylation</td>
<td>Phase 2</td>
<td>NCT04262388</td>
<td>Not recruiting</td>
</tr>
</tbody>
</table>

### 1.7. ROLE OF DRUG DELIVERY IN TREATING PDAC

It is well known that PDAC stroma is the major challenge that attenuates drug delivery to the core of the tumor. Therefore, knowing the mechanism in which nab-paclitaxel works might open new avenues for targeting PDAC tumors. PDAC stroma is enriched in SPARC; thus, disruption of PDAC stroma would improve intratumor drug delivery [104]. Infante et al. have found that SPARC protein binds to albumin; therefore, developing drug delivery system that is capable of binding to albumin may improve the overall therapeutic effect. Phase I/II and III clinical trials have proved the ability of gemcitabine + nab-paclitaxel (albumin-bound paclitaxel) to improve the OS of the patients. Gemcitabine + nab-paclitaxel combination therapies have improved the median survival up to 8.5 months compared to 6.7 months of gemcitabine alone.
Von Hoff et al. have shown the ability of nab-paclitaxel in disrupting PDAC stroma which results in enhancing gemcitabine concentration in patient-derived xenografts (PDX) tumor animal model [135]. Infante et al have shown that nab-paclitaxel was able to decrease cancer-associated fibroblasts on the patients’ tissue which results in softening the tumor collagen layer and facilitate delivery of chemotherapeutic agents [136].

It is well known that hyaluronan or hyaluronic acid (HA) is one of stromal components that accumulate in tumor regions. HA creates connective tissue matrix which is believed to have a role in forming stroma. Moreover, HA is also contributing to enhance the tumor interstitial fluid pressure (IFP) which hinders the delivery of drugs to cancer region [137]. Therefore, distributing the tumor’s HA enzymatically will improve the overall therapeutic effect. Pegylated PH20 (PEGPH20) is a human recombinant hyaluronidase enzyme. PEGPH20 activity has been proved preclinically in HA_{high} expressed tumor via depleting HA interstitial which ultimately reduces tumor IFP and enhances vascular perfusion [137]. Curtis et al. have shown that depleting tumor IFP potentiates delivery of chemotherapeutic agents such as docetaxel and Doxil® in HA_{high} PC3 tumors. In summary, depleting tumor stroma via remodeling tumor interstitial fluid pressure is a novel way of using pegylated drug delivery to enhance efficiency of chemotherapeutic agents.

### 1.7.1. Advances in nanoparticle diagnosis and treatment

The currently available treatment models have limited clinical response and continuous efforts are being made to increase the treatment efficacy. Nanomedicine and gene therapy have been found to give promising preclinical outcomes in treating aggressive pancreatic tumors, a few of which also went into the clinical trial phase. Due to the complex tumor stroma composition in PDAC, many drugs failed to penetrate the stroma barrier that negatively impacted their efficiency. Nanomedicines and gene therapies have potential use in enhancing PDAC diagnosis and treatment
Moreover, nanoparticles have ability to accumulate at the tumor site passively through enhanced permeation and retention (EPR) [139] effect or actively through conjugating targeted ligand on nanoparticles surface (Figure 4) [140]. Therefore, nanoparticles provide a viable solution to improve the sensitivity of the diagnostic test and help in treating PDAC. Preclinically, several nanoparticles have been synthesized to enhance PDAC diagnosis and therapy.

Diagnostically, several research groups have used nanoparticles in an attempt to detect PDAC tumors in early stage. For instance, Dobiasch et.al. synthesized a galectin-1 targeted chitosan nanoparticle with a magnetic core that showed high affinity to the overexpressed galectin-1 in early-stage PDAC tumor of size less than 1 mm and increased the imaging sensitivity via MRI imaging [141]. Similarly, Locatelli et.al. prepared a hybrid nanocarrier composed of lipophilic core, a polymeric outer shell and 68 Ga chelating group. NPs showed better diagnostic effects and proved to be nontoxic to be used for positron emission tomography/magnetic resonance imaging (PET/MRI) imaging in vivo for early-stage PDAC imaging [142].

Biodegradable nanoparticles characterize by its ability to escape the immune system and improve in vivo circulatory time. [143]. Albumin NPs have been used widely to deliver the cytotoxic gemcitabine to PDAC. Albumin NPs enhance absorption of drugs in the circulatory system, along with being renewable and economical [144]. Many studies have revealed that gem-albumin nanoparticles enhanced the tumor regression activity compared to free gem [145–147].

Liposomal formulation is characterized by its biocompatibility, longer circulation time, passive targeting mechanism by EPR effect, and most importantly the potential of surface modulation by ligands for active targeting. Recently, NAPOLI-1 trial showed the role of using irinotecan liposomal formulation combined with 5-FU/folinic acid as second-line therapy for patients who received gem-based therapy as first-line therapy. NAPOLI-1 trial confirmed that
receiving irinotecan liposomal formulation combined with 5-FU/ folinic acid improved OS (6.1 months) compared to 5-FU/ folinic acid OS (4.2 months) [148].

Polymeric nanoparticles have several advantages such as size uniformity, good release kinetics, malleability for customization, pH responding properties, and hydrophilic shell. All these properties make it a perfect PDAC delivery system that can deliver different cytotoxic drugs, DNA, proteins and siRNA [149]. For example, using paclitaxel in its free form does not show significant activity against PDAC, but when administered via poly-lactic-co-glycolic acid (PLGA) nanoparticles, it penetrates tumor and inhibits protein synthesis. The PDAC tumor uptake paclitaxel PLGA nanoparticles was 5 folds higher than conventional therapy [150]. Furthermore, Couvreur et al. have enhanced the stability and efficiency of gem via using self-assembled nanoparticles composed of gemcitabine and the natural lipid squalene (Gem-SQ) [151]. In mice bearing Panc-1 orthotopic model, gem-SQ inhibited the tumor regression and enhance the mice survivability compared to free gem [152].

Metal nanoparticles like gold and iron oxides, that are extensively used in PDAC diagnosis are also useful in treatment. The blank nanoparticles have the capability to show cytotoxic effect which can be used synergistically with loaded chemotherapeutic drugs to enhance the overall efficacy [153]. Mardhian et.al. have designed a supermagnetic iron oxide nanoparticle (SPION) to load human relaxin-2 (RLX), an endogenic hormone, that targets and inhibits the differentiation of PSCs to cancer-associated fibroblasts (CAFs) via inhibiting pSmad2 pathway. The authors found that RLX-SPION was able to inhibit collagen deposition which results in improving gem delivery and inhibit pancreatic tumor growth [153].

Gene therapy is a promising way to enhance overall PDAC therapy, but their delivery challenges limit its usage. For example, the siRNAs’ main challenge is their stability issue within
the biological systems. Therefore, many researchers are trying to overcome this issue using drug delivery systems [154]. Khvalevsky et al. have used a biodegradable polymer matrix loaded with anti-KRASG12D siRNA. siG12D loader was able to improve siRNA stability, overcome renal clearance and most importantly knockdown KRASG12D expression which results in improving the overall tumor inhibition in xenograft animal model [155]. Zhao et al., have constructed a hybrid lipid polymer nanoparticle to co-deliver siRNA (si-HIF1α) and gemcitabine (Gem) to target the HIF1α in PDAC cells. They have successfully showed a synergistic killing effect with siRNA and Gem combination therapy in vitro and in vivo and also able to inhibit tumor metastasis in orthotopic tumor model [156]. Frederico P. et al. have designed a pegylated polymeric nanoparticles conjugated with calcium phosphate to deliver VEGF siRNA. In this work, hybrid nanoparticles enhanced serum stability and gene silencing efficiency [157].

Furthermore, an example of the inhibition of HIF1α through siRNA combined with gem release as the one proposed by Zhao et al [158]. The hypoxic TME in PDAC is producing more HIFs, which are responsible for the activation of genes that control invasion, angiogenesis, chemoresistance and proliferation [27]. The polymeric lipids coated NPs which were loaded with gem as well as siRNA, which was complexed to positively charged polylysine residues on the NPs surface, significantly inhibited the growth of subcutaneous Panc-1 tumor xenografts. Thus, it indicated a synergistic effect between HIF-1α inhibition and chemotherapy (gem). Not only that the combination treatment significantly shrunk the tumor size in an orthotopic PDAC model in comparison with un-encapsulated siRNA and gem, or with NPs loaded with gem only, but also, no peritoneal metastases were observed in combination treatment group. Also, because PDAC TME becomes resistance to chemo and radiotherapy, Wason et al. formulated one of so many examples of nanoparticles to deliver drugs using cerium oxide nanoparticles (CONPs) to regulate
production of ROS that might sensitize PDAC cells to radiotherapy (RT) [159,160]. CONPs-based pretreatment limited tumor growth in an orthotopic model nude mice, leading towards significant shrinkage in tumor weight and volume as compared to radiotherapy alone. Several nanomedicine-based strategies have been designed and tested for the treatment of PDAC and there is a need for more smart strategies to overcome these barriers and to maximize treatment accumulation in the pancreatic tumor site [161].

**Figure 1-4:** Different types of nanoparticles for PDAC therapy. (A) surface decorated nanoparticles to enhance the tumor selectivity passively or actively. (B) Specific nanoparticles selectivity toward tumor cells that actively via receptor recognition or passively via EPR effect. (C) Uptake nanoparticles via the endocytosis process result in cancer cell eradication.
1.8. CONCLUSION AND FUTURE DIRECTIONS

PDAC is an extraordinarily high malignancy cancer entity, particularly characterized by poor prognosis and constantly increasing patient numbers. The aggressive biology and the fact that most patients participate in advanced or disseminated disorder stages make the development of new PDAC therapeutic approaches is one of the superordinate tasks of modern oncological science. Research over the last 20 years led to a systematic multi-step model of PDAC growth and progress. While this has certainly changed our understanding of PDAC as a disease, so far, neither of these findings could be successfully converted into a medical breakthrough. It is becoming increasingly obvious that the clinical effectiveness of single-agent treatments tends to lag below acceptable estimates, and smart combinations seem to be required instead. In addition, PDAC anti-CSC therapies of next generation should be produced to attack active stroma cells and target cells such as Wnt-cell components, PSC, MSC and/or TAMs that are extremely penetrable by small molecules, nanoparticles or oligonucleotides and perhaps immunotherapy. Furthermore, the ability of nanomedicines to aggregate and target tumors could be leveraged to enhance early tumor identification, significantly improve survival and increase the extent of surgical resection. Novel compounds and nanoparticles are continuing to be developed in non-viral vector technology [162]. And a combination of gene therapy with these, along with conventional drug therapy would also prove to be beneficial.

Despite substantial advances in cancer research over the last era, PDAC seems to have very poor survival rates. The present failure to diagnose early stage prevents the use of effective treatments. Additionally, drug resistance growth is a key factor for recognizing current therapy failure in both the tumor and metastatic tissues. Hence, the improvement in survival of PDAC patients will occur not only through the identification of early serum markers, but rather through
the production of therapeutic approaches directed towards reducing pancreatic CSCs and decreasing drug resistance.

Importantly, genetic analyses have strengthened our mechanistic and translational understanding of pancreatic cancer. Genetic principles and techniques are eventually beginning to be applied to clinical practice, especially for initiatives in precision medicine. Epigenomics, however, is evolving rapidly as a promising scientific and computational model for advancing the comprehension of this disease. More significantly, recent studies have identified possible actionable mechanisms supporting the assumption that prospective pancreatic cancer trials will include rigorous epigenomic therapy research. Therefore, epigenomics aims to produce a large amount of new biologically as well as scientifically relevant information.

In this respect, all the above strategies, and especially modern techniques, represent attractive strategies for both biologically inspired utilizing PDAC TME and immunotherapy strategies might be the future for finding new cure to manage PDAC disease.
CHAPTER 2 RESEARCH OBJECTIVE AND SPECIFIC AIMS

Pancreatic ductal adenocarcinoma (PDAC) is associated with 90% of pancreatic cancers. PDAC is life-threatening cancer with more than 57,600 people (30,400 men and 27,200 women) are estimated to be diagnosed in 2020 while the expected new death is 47,050 [163]. Surgery remains the only option for curing the disease if the patient diagnosed at an early stage. However, the late-stage diagnosis is the main obstacle that makes only 20% of the patients eligible for surgery. Therefore, PDAC is one of the most challenging diseases that is ranked as the fourth leading cause of death in the US according to the American Cancer Society. The 5-year survival rate percentage of pancreatic cancer is the lowest among the other solid tumors with 7% survival rate. One challenge of PDAC therapy is PDAC microenvironments that play a significant role in increasing both carcinogenesis and drug resistance. The stromal barrier is one of the major components of PDAC microenvironment, which composes of fibroblast, immune cells, blood vessels, neural cells, and cellular proteins that participate in decreasing drug penetration to pancreatic cancer cells; consequently, the drug resistance increases. Among all epithelial tumors, PDAC has a dense stroma that contributes to chemotherapy resistance and reduces drug delivery to the core of the solid tumor. The studies found that CD44+ PDAC tumor has more tumorigenicity and more drug resistance compared to CD44-. Also, it is known that highly expressed c-Met PDAC tumor is highly tumorigenic and capable of self-renewing. Taking together, positive CD44+ and c-Met pancreatic tumors are identified to be one of the most tumorigenic tumors. Overexpressed CD44 and c-Met are novel targeting receptors that can be utilized in enhancing drug delivery and efficacy. Therefore, there is a need to develop new ways of delivering current cancer therapies to improve stromal penetration and the overall effectiveness of cancer therapy. Thus, we will synthesize a drug delivery system that will target both CD44+ and c-Met biomarkers. This
approach can be utilized for PDAC imaging and therapy. Also, this technology will overcome the significant therapy challenge and improve the tumor-stromal penetration in which Gemcitabine can be delivered into the core of the PDAC tumor.

The current work will focus on the multimodal approach, including; (a) synthesis, characterization, and optimization of CD44 and c-Met targeted dual-targeted nanoparticles; (b) in vivo tissue bio-distribution of the dye conjugated DTNPs; (c) synthesis and characterization of Gemcitabine conjugated DTNPs (HA-GE137-Gem ), followed by in vitro testing of anticancer activity of CD44 and c-Met targeted HA-GE137-Gem alone or in combination with mTOR inhibitors ;(d) in vivo testing of anticancer activity of HA-GE137-Gem combined with mTOR inhibitors.

2.1. SPECIFIC AIM 1: SYNTHESIS, CHARACTERIZATION, AND OPTIMIZATION HYBRID NANOPARTICLES AS AN IMAGING AGENT TO TARGET BOTH CD44 AND c-Met

The primary objective of AIM 1 is to synthesize and characterize dual-targeted nanoparticles (DTNPs) using amine acid coupling and copper-free “click” chemistry that allows developing a robust, selective and high yielding product. DTNPs will be conjugated to high selective, cost-effective, and high stoke shift and clinically used NIR dye (NCT02317705) for tumor imaging and to study the animal bio-distribution. The tumor bio-distribution of the optimized imaging agent will be evaluated. The bio-distribution studies include, (a) NIR dye conjugated DTNPs bio-distribution in heart, lung, liver, spleen, and tumor ;(b) the tumor core penetration of dye-conjugated DTNPs (c) the tumor/ liver accumulation of dye-conjugated DTNPs. Also in this aim, we will study the colocalization of the dual-targeted nanoparticles with overexpressed CD44 and c-Met receptors.
The biodistribution studies of imaging-guided NPs play a crucial role determining the efficiency of the delivery systems. Also, combining using imaging-guided NPs with clinically available instruments such as PET and single-photon emission computed tomography (SPECT) would improve the patients’ therapeutic outcomes. Moreover, a colocalization study will illustrate how NIR dye conjugated DTNPs interact with targeted receptors. The more colocalization, the more directly or in-directly interaction with the targeted receptors.

2.2. SPECIFIC AIM 2: SYNTHESIZING, CHARACTERIZING, AND ACTIVITY TESTING OF CHEMOTHERAPEUTIC AGENT CONJUGATED TO HA-GE137.

The main objective of AIM 2 is to synthesize and characterize a chemotherapeutic agent conjugated to DTNPs using coupling chemistry such as (EDC/NHS and HATU/DIPEA) and copper-free “click” chemistry that allows developing a powerful, selective and high yielding product.

The antitumor activity of the optimized gemcitabine conjugated DTNPs (HA-GE137-Gem) will be evaluated, and that includes (a) cell viability studies using HA-GE137-Gem alone or in combination with mTOR inhibitors; (b) testing the apoptotic effect of HA-GE137-Gem via studying cell cycle arrest biomarkers; (c) evaluation of combination index value of HA-GE137-Gem and mTOR inhibitors; and (d) in vivo pre-clinical testing of HA-GE137-Gem in combination with mTOR inhibitors using nu/nu mice and C57BL/6J syngeneic mouse.
CHAPTER 3 TARGETING CD44 AND C-MET OVEREXPRESSED PANCREATIC CANCER CELLS VIA FABRICATING DUAL-TARGETED DIAGNOSTIC NANOPARTICLES

3.1. INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most challenging cancers that is associated with 90% of pancreatic cancers [164]. It is life-threatening cancer which its 5-year survival (8%) is the lowest among the other solid tumors [165]. Surgery remains the only option for curing the disease if the patient is diagnosed at an early stage; otherwise, only 20% of the patients would be eligible for surgery at the late stage [86]. Indeed, the early detection of high-risk patients is one of the unmet needs that would increase the probability of treatment success [16]. In PDAC, tumor microenvironments play a significant role in developing both carcinogenesis and drug resistance [166]. The stromal barrier is one of the influential components of the PDAC microenvironments, which is composed of fibroblasts, immune cells, blood vessels, neural cells, and cellular proteins [27] that participate in decreasing penetrating both drugs and diagnostic agents toward pancreatic tumor [28]. Among all epithelial tumors, PDAC has a dense stroma that contributes to chemotherapy resistance and reduces drug delivery to the core of the solid tumor [167]. Also, The studies found that most pancreatic cancer cells (Panc-1, MIA-Paca-2, and AsPC-1) are overexpressing CD24 and CD44 on cancer stem cell surfaces (CSCs) [168]. Accumulating evidence established that the solid pancreatic tumor is composed of heterogenic cells that have different biological properties in which small subunit cells called cancer stem cells (CSCs) are responsible for increasing tumorigenesis, growth of the cancer cells as well as drug resistance [169]. The CSCs may interact with tumor-associated macrophages (TAMs) that may alter the composition of the stromal cells; thus, the drugs and the diagnostic agents’ penetration will decrease [170].
The cluster of differentiation-44 (CD44) is an extracellular glycoprotein that plays a role in cell adhesion and transduction [171]. CD44 presents on the surface of the normal cells, but it overexpresses on the surface of most of the solid tumors such as pancreatic, lung, and breast cancer. The CD44 biomarker, which is a stem-like receptor, has a significant role in tumor progression, disease prognosis, and drug resistance [172]. Drug resistance in pancreatic cancer is attributed to many factors; cancer stem cells is one of these factors that can detoxify drugs via its intrinsic detoxifying mechanism [173]. CSCs, which is a subpopulation of cancer cells that are characterized by the presence of specific phenotype in most of the solid tumors such as CD44+ [174], is one of the pancreatic cancer biomarkers that overexpresses on the cancer cell surface [49]. Studies have found that CD44, CD24, and ESA positive pancreatic cell lines are more drug resistance, more tumorigenic, and have CSC like properties[49]. Miyatake et al. found that leukemia T-cells aggregated and grown on a subpopulation of cells that overexpress CD44 receptor (CSCs), in which the tumor becomes more aggressive and metastasis [175]. The overexpressed CD44 receptor in pancreatic cells can be utilized as a targeting receptor to deliver many chemotherapeutic agents into the core of the solid tumor. Hyaluronic acid, which is a natural, biodegradable, and biocompatible polymer that can be utilized to target the overexpressed CD44 receptor. Because of the high affinity of HA to the CD44 overexpressed tumor cells, HA has been used widely to target cancer cells and minimize the side effects of the current chemotherapeutics agents [176,177].

Besides CD44, c-Met, which is a member of the tyrosine kinase family, is a proto-oncogene that is expressed in normal and cancer cells. In cancer cells, c- Met functions as a stimulatory factor of hepatocyte growth factor that plays a pivotal role in cancer cell invasion and metastasis [170].

Furthermore, c-Met overexpression and mutation play a significant role in cancer overgrowth and invasion [178,179]. Recent studies showed that the c-Met oncogenic receptor
interacts with the variety of receptors that enhance the cancer progression such as EGF receptor families [180] and CD44 [181]. Ebert et al. found that the expression of c-Met and HGF mRNA of pancreatic cancers are elevated 7 and 10 folds respectively, compared to the normal pancreas [179].

The precise and accurate visualizing of the presence of the tumor plays an essential role in diagnosing and optimizing the therapeutic efficacy [182,183]. To overcome the current poor penetration of the pancreatic stromal barrier, we took advantage of the overexpressed CD44 and c-MET receptors in PDAC by synthesizing a targeted ligand that can bind to CD44 and c-MET. Thus, we believe that using targeting ligand that has both hyaluronic acid and GE137 peptide to specifically attach to CD44 and c-MET receptors respectively will facilitate the penetration of the diagnostic agent to the core of the PDAC tumor.

3.2. MATERIALS AND METHODS

3.2.1. Cell culture, reagents and chemicals

Hyaluronic acid (HA) was obtained Lifecore (MN, USA), GE137 was purchased LifeTein (NJ, USA) EDC/NHS, ethylene disulfide, rhodamine B fluorescence, and N, N-Diisopropylethylamine (DIPEA) were obtained from Sigma Aldrich (MO, USA). S0456 was purchased from FEW Chemicals GmbH, Germany. All other reagents were purchased from Fisher Scientific (NH, USA) and Sigma Aldrich (MO, USA) and used without further purification. DMEM, penicillin/streptomycin, fetal bovine serum albumin (FBS) were purchased from Fisher Scientific (NH, USA). Rabbit monoclonal antibodies for β-actin, and CD44 were obtained from Cell Signaling Technology (MA, USA) while c-MET rabbit polyclonal antibody was purchased from Proteintech (IL, USA).
3.2.2. Cells culturing conditions

The human AsPC-1 cell line was obtained from ATCC, and the cells were cultured according to the ATCC protocol. AsPc-1 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 Units/ml penicillin, 100 μg/mL streptomycin, and 10% FBS. The cells were maintained at 37°C and 5% CO₂. The medium was changing every other day, and the cells were subcultured once they reached 70-90% confluency.

3.2.3. Animals husbandry

6-8 week old NOD/SCID mice were purchased from Jackson laboratories. The mice were housed in a sterile environment where the standard 12 hr dark-light cycle was maintained. Also, the mice were on the regular rodent diet and water. The mice received care according to the Institutional Animal Care and Use Committee (IACUC) guideline.

3.2.4. Preparation and characterization of NIR dye conjugated HA-GE137 nanoparticles

Firstly, HA sulfhydryl (HA-SH) was synthesized using a previously published method with slight modification [184]. In brief, 400 mg HA (5.5 KDa) was dissolved in distilled water, followed by raising pH to 9-10 using 1M NaOH. An excess of ethylene sulfide was added and stirred overnight. The product was filtered using the bed of celite® 545 (3 cm), followed by adding an excess amount of DTT and stirred overnight at pH 8.5. pH was adjusted to 3.2 using 1 N HCL, and finally, the resulted product dialyzed against acidic solution using dialysis bag MWCO 3.5 KDa; and the degree of thiolation was approved by using Ellman’s assay. The resultant product HA-SH was further conjugated with GE137 via using EDC/NHS coupling reaction. In this step, a 1:1.5 molar ratio of HA-SH and GE137 was used. HA-SH was dissolved in 2-(N-morpholine)ethane sulfonic MES buffer (pH=6), and EDC/NHS was added and stirred for one hour to activate -COOH of HA-SH followed by the gradual addition of GE137 and kept for
overnight stirring at 4 °C. The final product was dialyzed using dialysis bag MWCO 3.5 for several hours and then lyophilized and stored at -20 °C until further use. The HA-GE137 was further functionalized to NIR dye (rhodamine B or S0456) via using a 1:1 molar ratio of HA-GE137: NIR dye at pH 7.5 and stirred overnight, followed by dialyzing using cellulose dialysis membrane MWCO 3.5 for several hours. The HA-GE137-NIR dye nanoparticles size and zeta potential were performed using Beckman Coulter Delsa Nano-C-DLS Particle analyzer (Miami, FL) equipped with a 658 nm He-Ne laser. For nanoparticle size, the nanoparticles were suspended in water, and the light scattering was measured after 70 scans; the DLS data was collected as a peak average of 70 scans. The electrophoretic mobility of nanoparticles was measured after applying the electric field on nanoparticles. 1H NMR was used to confirm the chemical conjugation of NPs via measuring the functional groups' chemical shifts. The final structure of HA-SH, HA-SH-GE137, HA-SH-GE137- NIR dye was confirmed by NMR.

3.2.5. Expression of CD44 and c-Met on different PDAC cell lines

CD44 and c-Met expression on the surface of PDAC cell lines (Panc-1, AsPc-1, MiaPaCa-2, BxPc3) and normal pancreatic cell lines HPNE were evaluated through western blot. In brief, protein from cell lines was extracted on ice using whole-cell lysis buffer (RIPA buffer) supplemented with 1:100 Halt protease and phosphatase inhibitors cocktail for 15 mins. Then, the lysate was collected after centrifugation at 14,000 RPM for 15 mins. The protein quantity was determined using the BCA kit, and 20 μg of each sample was separated using 12% polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane using a wet transfer procedure. The membrane was blocked with 5% nonfat dry milk, followed by overnight incubation with primary (CD44 or c-Met) followed by 1 h incubation with an appropriate secondary antibody. The bound antibody was visualized after incubation with chemiluminescent
western blot detection reagents (Thermo Fisher Scientific, MA, USA), followed by reading out the bands using Bio-Rad ChemiDoc™ MP machine. The same membranes were re-probed with an internal control (anti-β-actin antibody). The CD44 and c-Met expression were also evaluated using immunohistochemistry. The paraffinized tumor section was deparaffinized in xylene solution, followed by section rehydration in a serious concentration of ethanol. The antigen was retrieved via incubation each section in trypsin at 37 °C. Rhodamine and HA-GE137-rhodamine sections were blocked with a 5% BSA solution containing 0.1% TritonX 100 (TBSA), followed by incubation with either CD44 or c-Met monoclonal antibody overnight. The sections were washed three times with TBSA and incubated for one hour with the FITC anti-rabbit secondary antibody. The tumor sections were observed using a confocal laser scanning microscope (CLSM).

3.2.6. Rhodamine colocalization with CD44 and c-Met biomarkers

AsPc-1 cell lines were incubated subcutaneously in NOD/SCID. After the tumor reached 500 mm³, the mice were divided into two groups, one group was injected intravenously with 100 nano-mole rhodamine, and the other group was injected intravenously with 100 nano-mole of HA-GE137-rhodamine. The mice were euthanized after 96 hours, and the tumors were collected and fixed in 10% paraformaldehyde. Immunohistochemistry was performed as the following: firstly, the paraffin section was deparaffinized in xylene solution, followed by section rehydration in a serious concentration of ethanol. The antigen was retrieved via incubation each section in trypsin at 37 °C. Rhodamine and HA-GE137-rhodamine sections were blocked with a 5% BSA solution containing 0.1% TritonX 100 (TBSA), followed by incubation with either CD44 or c-Met monoclonal antibody overnight. The sections were washed three times with TBSA and incubated for one hour with the FITC anti-rabbit secondary antibody. The tumor sections were observed using a confocal laser scanning microscope (CLSM).
3.2.7. NIR dye conjugated HA-GE137 imaging and bio-distribution

6-8 week-old mice were orthotopically injected with PBS contained $1 \times 10^5$ AsPC-1 cells in the pancreas. The Mice were randomly assigned into four groups which are injected with S0456, HA-GE137-S0456, rhodamine, and HA-GE137- rhodamine. The in vivo Carestream In Vivo MS FX PRO, Light Source: 400 W Xenon, Monochrome interlined, fixed lens (10x), CCD camera (13.8 x 13.8 cm / 2048 x 2048 px, 67 μmpx, 16 bit), was used to obtain the S0456 signal (Ex 760 nm, Em790 nm) fluorescence, and X-ray images were captured. Both fluorescence and X-ray images of the mouse were merged to demonstrate the localization of NPs. To evaluate the tissue and tumor bio-distribution, the S0456 and S0456 conjugated HA-GE137 were injected via vein tail, and the heart, lungs, stomach, liver, spleen, kidneys, small and large intestines were collected for imaging and quantitative bio-distribution study at 24 and 48 hrs.

3.2.8. Tumor/ liver ratio quantification

After euthanizing the mice, the tumor and liver of the injected mice were collected, and the amount of the S0456 and S0456 conjugated NPs were quantified using ImageJ software [185]. The tumor/liver ratio was calculated by dividing the region of interest (ROI) of the tumor fluorescence on the ROI of Liver fluorescence using ImageJ software [185].

3.2.9. Statistical analysis

All the reported data expressed as the mean ± standard error of the mean. Student t-test was used to determine the difference between the two groups.

3.3. RESULTS AND DISCUSSION

3.3.1. Targeting CD44 in PDAC

The cluster of differentiation-44 (CD44) is an extracellular glycoprotein that plays a role in cell adhesion and transduction [171]. CD44 presents on the surface of the normal cells, but it
overexpresses on the surface of most of the solid tumors such as pancreatic, lung, and breast cancer. The CD44 biomarker, which is a stem-like receptor, has a major role in tumor progression, disease prognosis, and drug resistance [172]. Drug resistance in pancreatic cancer is attributed to cancer stem cells that can detoxify drugs via its intrinsic detoxifying mechanism [173]. CSCs, which is a subpopulation of cancer cells that are characterized by the presence of specific phenotype in most of the solid tumors such as CD44+ [174], is one of the pancreatic cancer biomarkers that overexpresses on the cancer cell surface [186]. Studies have found that CD44, CD24, and ESA positive pancreatic cell lines are more drug resistance, more tumorigenic and have CSC like properties [186]. Miyatake et al. found that leukemia T-cells aggregated and grown on a subpopulation of cells that overexpress the CD44 receptor (CSCs), in which the tumor becomes more aggressive and metastases [175]. The overexpressed CD44 receptor in pancreatic cells can be utilized as a targeting receptor to deliver many chemotherapeutic agents into the core of the solid tumor. Hyaluronic acid, which is a natural, biodegradable, and biocompatible polymer is one of the polymers that can be utilized to target the overexpressed CD44 receptor. Because of the high affinity of HA to the CD44 overexpressed cells especially tumor cells, HA has been used widely to target cancer cells and minimize the side effects of the current chemotherapeutics agents [176,177]. Targeted therapeutics approaches should be utilized to overcome the current chemotherapeutic resistance[187,188].

3.3.2. Targeting tyrosine kinase receptor (c-Met) in PDAC

c-Met, which is a member of the tyrosine kinase family, is a proto-oncogene that expresses on both normal and cancer cells [189]. c-Met is regulated by hepatocyte growth factor (HGF) that mediates most of the biological activity of the cells such as cell invasion, motility, and metastasis [60]. in cancer cells, c-Met overexpression and mutation play a significant role in cancer
overgrowth and invasion [178,179]. Recent studies showed that c-Met oncogenic receptor interacts with the variety of receptors that enhance the cancer progression such as EGF receptor families [180] and CD44 [181]. Ebert et al. found that the expression of c-Met and HGF mRNA of pancreatic cancers are elevated 7 and 10 folds, respectively compared to the normal pancreas [179]. Also, hypoxia plays a role in increasing the expression of HGF/c-Met in tumor and stromal cells. Taking together, targeting CD44 and c-Met receptors will be an excellent approach to deliver both imaging and resistance chemotherapeutic agents efficiently to the core of pancreatic cancer and overcome most of PDAC MDR.

3.3.3. Rationale for targeting CD44 and c-Met

In this study, the expression of CD44 and c-Met biomarkers were evaluated in PDAC tumor sections and cell lines. The overexpression of CD44 on the surface of CSCs is found to be in many human tumors, including pancreatic adenocarcinoma tumors [49,190]. Immunohistochemistry of tumor section collected from the orthotopic xenograft mice model was assessed. The green intensity clearly showed the presence of CD44 and c-Met in PDAC tumors, as shown in figure (3.1, A, and B). Moreover, the western blot data proved the presence of CD44 and c-Met in various PDAC cell lines. CD44 biomarker was found to be 5 and 6 folds overexpressed in MiaPaCa-2 and Panc-1 cell lines, respectively, compared to normal pancreas cell line (HPNE) (figure 3.1, C and D). The overexpression of CD44 makes it as suggested targeted receptor for both therapy and imaging [55]. Binding of HA to CD44 receptor triggers multiple signal transduction that facilitates the internalization of HA to the cancer cells. Therefore, conjugation of a diagnostic agent to HA is a valid procedure to enhance the dye penetration to the core of the tumor [191–193]. In addition to CD44, c-Met plays a significant role in increasing tumor metastasis and development [61,62]. Moreover, our western blot data clearly showed the c-Met overexpressed 1,2,2 and 2.5 folds of
PANC-1, HPAC-1, BxPc-3, and AsPc-1, respectively, in comparison with normal pancreas cell line (HPNE) (Figure 3.1, E and F). It has been found that the gemcitabine resistant pancreatic cancer cells play a role in epithelial-to-mesenchymal transition (EMT) that increases c-Met phosphorylation; thus, the markers’ expression (CD24 and CD44) of the cancer stem cells increases. Therefore, using GE137 peptide that binds to the overexpressed c-Met facilitate directing the HA-GE137-S0456 to secure and inter to the cancer cells. Thus, we believe that combining HA and GE137 will increase the tumor specificity of the diagnostic construct and minimize liver accumulation.
Figure 0-1: CD44 and c-Met expression in different PDAC cell lines. (A) and (B) CD44 and c-Met expression in tumor section of Immunohistochemistry of orthotopic xenograft mice, (C) and
(D) Western blot of CD44 and c-Met protein expression. (E) and (F) quantification of two normal pancreas cell lines and 5 PDAC cell lines.

3.3.4. Procedure for synthesis HA-GE137 near-infrared dye

The current work aims to synthesize a dual-targeted nanoparticles agent via targeting the PDAC overexpressed CD44 and c-Met surface biomarkers. CD44 and c-Met surface biomarkers were targeted by HA-GE137 containing NPs using EDH/NHS coupling reaction, as shown in the chemical schematic in Figure 3-2, A. Under the basic condition, HA was functionalized with ethylene sulfide to arrive HA-SH (compound II). Using EDC/NHS coupling agents, HA-SH was activated for one hour, followed by adding GE137 for 24 h at 4 °C to arrive compound III which acts as a dual-targeting ligand for CD44 and c-Met biomarkers. Compound III is a universal compound that can be utilized for conjugating to multiple NIR dyes such as rhodamine and S0456 (compound IV) or chemotherapeutic agents as will be mentioned in chapter 4. HA-Ge137-rhodamine was used for both in vitro cell uptake and in vivo receptor co-localization, where HA-GE137-S046 was utilized for in vivo animal bio-distribution. All products were purified by removing unconjugated reactants via dialysis bags before lyophilization. The lyophilized product was characterized for size and charged. The size of HA-SH nanoparticles was 147 nm with a polydispersity of (PDI= 0.155) (Figure 3-3, A). After conjugating NIR dye, the size of NPs was slightly increased to be 196.3 nm with PDI = 0.150 which suggesting the successful conjugating of NIR dye to NPs (Figure 3-3, A). The current NPs size and the negative surface charge (Figure 3-3, B) are ideal for both intravenous injection and cancer delivery. The final products where chemically confirmed using 1H NMR. The chemical shift corresponding to the SH group appears to be at \( \delta = 1.1 \) ppm. Finally, the degree of thiolation was determined by colorimetric Ellman’s assay that indicated that the degree of thiolation is about 15% (Figure 3-2 B and C).
FIGURE 0-2: (A) CHEMICAL Schematic representation of dual-targeted diagnostic agent synthesis (HA-GE137-Rhodamine and HA-GE137-Rhodamine) (B and C) ¹H NMR of HA and HA-SH respectively. The red star is an indicator of different conjugated NIR dye to HA-SH.
**Figure 0-3:**

(A) Dynamic light scattering (DLS) of HA-SH (147 nm) and HA-GE137-Rho (196.3 nm) nanoparticles. (B) Zeta potential of HA-GE137-rhodamine and HA-GE137-S0456. (C) Ellman’s assay for thiol quantification of HA-SH.

**Table:**

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Diameter (nm)</th>
<th>Polydispersity (PDI)</th>
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<tbody>
<tr>
<td>HA-SH NPs</td>
<td>147</td>
<td>0.155</td>
</tr>
<tr>
<td>HA-GE137-Rhod</td>
<td>196.3</td>
<td>0.150</td>
</tr>
</tbody>
</table>
3.3.5. Specific tumor colocalization and accumulation of HA-GE137 nanoparticles

After the construct has been synthesized and characterized, NIR imaging was performed in xenograft orthotopic mice inoculated with 1X10^5 cells in the pancreas followed by injecting with HA-GE137-S0456. The colocalization study was performed to prove the ability of HA-GE137 construct to recognize and react with overexpressed CD44 and c-Met tumor surface biomarkers. The intense red fluorescence of rhodamine-conjugated HA-GE137 in the tumor section was higher than free rhodamine (Figure 3-4, A). This result is comparable with HA-GE137-S0456 tumor accumulation result that I will discussed in figure 3.5 B. Moreover, the intense green fluorescence of both CD44 and c-Met indicates the up-regulation of these two receptor biomarkers in AsPc-1 tumor microenvironments (Figure 3-4, B). We further studied the colocalization of HA-GE137-Rhod with overexpressed CD44 and c-Met biomarker using Pearson correlation coefficient (PCC) where \( r = 1 \) means a perfect positive correlation and \( r = -1 \) perfect negative correlation as shown in (Figure 3-4, C and D). The data showed that HA-GE137-Rhod is colocalize with CD44 and c-Met (\( r = 0.55 \) and \( r = 0.75 \) respectively) better than free rhodamine ((\( r = 0.38 \) and \( r = 0.58 \) respectively). Furthermore, the HA-Ge137-Rhod showed high binding affinity to CD44 and c-Met in AsPc-1 cell line (KD= 58 nM) (Figure 3-3, E).

The main aim of performing imaging with NIR dye conjugated HA-GE137 is to be used as (i) guide of therapeutic and safety outcomes in PDAC animal model, and as (ii) a guided surgery diagnostic agent that can be utilized in the clinic. Hyaluronan receptor for endocytosis (HARE) on liver sinusoidal endothelial cells is the primary receptor that regulates the excretion of high molecular weight HA from the circulation[194,195]. To minimize the HA excretion, we used small molecular weight HA (6.4 KDa) 6 to 8 saccharides length which maintains the CD44 binding affinity and reduces the circulation clearance of HA [196,197]. GE137 is a water-soluble peptide
that composed of 26 amino acid and specifically binds to c-Met tyrosine kinase and found to be really cleaned. GE137 has reached Phase I clinical trial and showed good safety in humans [198]. The results of injecting HA-GE137-S0456 intravenously in orthotopic xenograft mice clearly showed the selective accumulation of the targeted construct in the tumor site compared to free NIR dye (S0456) (Figure 3-5,A). The biodistribution study (Bio.D) in Figures 3-5, B, and C confirmed the predominant selectivity of HA-GE137-S0456 toward PDAC tumor while free dye is spreading out to liver. This suggesting that dual-targeted polymeric nanoparticles are directing the diagnostic agent to be accumulated in the tumor site and with time accumulating in the kidneys to be cleared out. Dual targeted polymeric nanoparticles will overcome the major liver toxicity of nanoparticles [199]. NIR conjugated polymeric nanoparticles have shown a significant tumor/liver accumulation with 6.5 folds higher than free dye (Figure 3-5 D and E). The tumor/liver fluorescence ratio is clearly indicating the ability of the current construct to specifically accumulate in the tumor region higher than liver. Our data was comparable to many groups that utilized the HA and GE137 as targeting ligands. Wang et al. found that the hyaluronic acid drove the nanoparticles to accumulate in the tumor site (MDA-MB-231) that has been injected in the nu/nu female mouse, while the non-targeted nanoparticles had less tumor selectivity [57]. Also, Elisa et al. found that the HA-conjugated liposomal formulations that loaded with gemcitabine showed better tumor growth inhibition than both free drug and non-targeted liposomal in the subcutaneously xenografted nude mice with MiaPaCa-2 cell line [55].
Figure 0-4: The accumulation and colocalization of Rhodamine B and HA-GE137-Rhodamine in PDAC tumors. (A) the intensity of accumulated Rhodamine B and HA-GE137-Rhodamine in the tumor site after 96 hours. (B) the expression of CD44 and c-Met biomarkers in AsPc-1 cell line.
inoculated in mice bearing tumor. (C) and (D) the qualitative and quantitative colocalization of Rhodamine B and HA-GE137-Rhodamine with CD44 and c-Met overexpressed biomarkers. (E) the binding affinity of HA-GE137-Rhodamine to CD44 and c-Met receptors of AsPc-1 cell lines.
Figure 0-5: in vivo tumor accumulation of S0456 and HA-GE137-S0456 after (A) 24 hours and 48 hours. (B) bio-distribution in different body organs where HA-GE137-S0456 is mainly
accumulated in tumor sites while S0456 accumulated in the liver. (C) comparison between NIR dye conjugated HA-GE137 liver accumulation and free NIR dye. (D) and (E) evaluation the tumor/liver accumulation of HA-GE137-S0456 and S0456. Red arrows indicate the liver site , while green arrows indicate the tumor site.

3.3.6. Conclusion

Early detection of PDAC increases the chances of treating options for PDAC patients. Therefore, there is unmet need to find new strategies to improve the current ways of PDAC detection. Detection of PDAC is one of the unmet needs that required to be investigated to improve the quality of life of PDAC patients. Unfortunately, the PDAC has harsh tumor microenvironments that increase its aggressiveness and reduce its response to most of the current therapies. Moreover, TME acts as a barrier that limits the penetration of most therapeutic and diagnostic agents. Therefore, in this study we are trying to find new strategies to identify PDAC using nanoparticles approach. Using dual-targeted nanoparticles enhances the accumulation of NIR-dye conjugated nanoparticles within the tumor sites. Also, our construct minimized liver accumulation and the tumor/liver ratio was 7-fold higher than free dye. Interestingly, rhodamine-conjugated nanoparticles colocalized with overexpressed CD44 and c-Met receptors which indicates that the current construct interacts with these two receptors directly or indirectly. The promising obtained imaging results pave the way forward for future therapeutic studies using the same construct conjugated to chemotherapeutic agents such as gemcitabine. Overall, the obtained imaging results portend that NIR dye conjugated dual-targeted nanoparticles would open new avenues for image guided therapy for PDAC tumors.
CHAPTER 4 TUMOR STROMA DISRUPTING NANOPARTICLES FOR CHEMO GUIDED IMMUNOTHERAPY OF PANCREATIC DUCTAL ADENOCARCINOMA

4.1. INTRODUCTION

In 2020, pancreatic cancer (PC) is estimated to be the fourth leading cause of death in the US. The 5 years survival rate percentage of pancreatic cancer is the lowest among other solid cancers, which is 7% [200]. Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive disease with the lowest survival rate among all solid tumors. The lethality of PDAC arises from late detection and propensity of the tumor to metastasize and develop resistance against chemotherapeutic and radiation therapy. One of the major barriers that significantly enhance the PDAC tumorigenesis is the presence of a complex tumor microenvironment (TME). TME composed of dense stroma, immune cells, fibroblast, and disorganized blood vessels that compromised the efficiency of the current PDAC treatment. Cancer stem cells (CSCs) are one of the components of TME that is capable of self-renewal [48] and enable many tumors to regenerate after being collapsed during a chemotherapeutic regimen [49]. CSCs were firstly recognized in the hematopoietic system; however, it has been found to be presented in multiple solid tumors such as breast [50], colon [51], brain [52], and pancreatic cancer [49]. In the xenograft animal model of pancreatic cancer, it has been identified that a subpopulation of cells has CSC properties and have CD44+, CD24+, and ESA+. CD44+, CD24+, and ESA+ pancreatic cells have a high potential to form tumors more than CD44-, CD24-, and ESA- cells [49].

In addition, hepatocyte growth factor receptor (c-Met), which presents in both normal and tumor cells, is essential for embryonic development and tissue repair [58]. In cancer cells, overexpressed c-Met functions as a tumorigenic factor that promotes pancreatic cancer formation [201]. Exposing PDAC cells to gemcitabine found to enhance EMT that promotes c-Met phosphorylation; consequently, the expression of CSCs biomarkers such as CD44 and CD24 [61].
CD44 is found to be overexpressed on the surface of CSCs in many human tumors including pancreatic adenocarcinoma tumors [190,202]. HA is a biomaterial that has a high affinity toward CD44 receptors and hyaluronan-mediated motility [203]. HA is found to have excellent physicochemical properties, high selectivity, and affinity toward overexpressed CD44 receptors; thus, it is a good candidate to be used as a targeted ligand in drug delivery [204].

On the other hand, many groups have confirmed that the c-Met receptor is overexpressed in many solid tumors including PDAC. The overexpressed c-Met on the extracellular surface of the tumor cells makes it an excellent target for drug delivery [205]. A recent study used a phage display library has discovered a small peptide (G137), which is in phase 1 clinical trial [206], which has a strong binding affinity to the c-Met receptor (~ Kd 3 nM).

In the current study, we selected GE137 conjugated to HA to develop a CD44 and c-Met dual targeting ligand to overcome many PDAC TME barriers and enhance the therapeutic outcome with improving the biosafety profile of the current front-line PDAC therapy.

4.2. MATERIALS AND METHODS

4.2.1. Cell culture, reagents and chemicals

Hyaluronic acid (HA) was obtained Lifecore (MN, USA), GE137 was purchased LifeTein (NJ, USA) EDC/NHS, ethylene disulfide, rhodamine B fluorescence, and N, N-Diisopropylethylamine (DIPEA) were obtained from Sigma Aldrich (MO, USA). Gemcitabine was purchased from LC laboratories (Woburn, MA, USA). SPDP-Peg4-NHS was obtained from TCI Chemicals (Portland, OR). All other reagents were purchased from Fisher Scientific (NH, USA) and Sigma Aldrich (MO, USA) and used without further purification. DMEM, penicillin/streptomycin, fetal bovine serum albumin (FBS) were purchased from Fisher Scientific (NH, USA). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (from
Sigma-Aldrich (St Louis, MO) Rabbit monoclonal antibodies for β–actin, and CD44 were obtained from Cell Signaling Technology (MA, USA) while c-MET rabbit polyclonal antibody was purchased from Proteintech (IL, USA).

4.2.2. Cell culture conditions

The human Panc-1, AsPC-1, BxPc3, MiaPaCa-2, and HPAC cell line was obtained from ATCC, and the cells were cultured according to the ATCC protocol. Human PANC-1, BxPc3, MiaPaCa-2, and HPAC were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 Units/ml penicillin, 100 μg/mL streptomycin and 10% FBS. While AsPc-1 was cultured in RPMI-1640 medium supplemented with 100 Units/ml penicillin, 100 μg/mL streptomycin, and 10% FBS. The cells were maintained at 37°C and 5% CO₂. The medium was changing every other day, and the cells were subcultured once they reached 70-90% confluency.

4.2.3. Animals husbandry

6-8 week-old NOD/SCID mice were purchased from Jackson laboratories. The mice were housed in a sterile environment where the standard 12 hr dark-light cycle was maintained. Also, the mice were on the regular rodent diet and water. The mice received care according to the Institutional Animal Care and Use Committee (IACUC) guideline.

4.2.4. MTT assay of several new single drugs and new combinations

MTT assay was carried out to test the cytotoxicity of Gemcitabine (Gem), Cabozantinib (Cabo) and Everolimus (Evr) on PDAC cell lines (Panc-1, AsPC-1, BxPc3, MiaPaCa-2, and HPAC). 5 x 10³ cells of each cell line were cultured in 96 well plates in the appropriate culture medium and kept overnight for attachments. After 24 h. the serial concentration of Gemcitabine, Cabazitaxel, and Everolimus was added for 24,48 and 72 h. The cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT). 20 µl of MTT was added
to each well and incubated for 3 h. the formed formazan crystals were dissolved in 100 µl DMSO and shook on an orbital shaker for 5 min. The optical density was read at 540 nm on the spectrophotometer reader (Victor3; PerkinElmer, Wellesley, MA).

After determining the IC\textsubscript{50} of every single drug, several concentrations below IC\textsubscript{50} were used for further investigation. The combination effects of (Gem, Cabo), (Gem, Evr), and (Caba, Evr) were evaluated using Compusyn\textsuperscript{\textregistered} software to calculate the combination index (CI) value. The indication of CI value is interpreted as: >1 synergistic, =1 additive and >1 antagonistic.

4.2.5. Synthesis and characterization of gem conjugated HA-GE137

This reaction was carried out in three main steps. The first step was the synthesis of HA-SH-GE137 as the following: HA sulfhydryl (HA-SH) was synthesized using a previously published method with slight modification [185]. In brief, 400 mg HA (5.5 KDa) was dissolved in distilled water, followed by raising pH to 9-10 using 1M NaOH. An excess of ethylene sulfide was added and stirred overnight. The product was filtered using the bed of celite\textsuperscript{\textregistered} 545 (3 cm), followed by adding an excess amount of DTT and stirred overnight at pH 8.5. pH was adjusted to 3.2 using 1 N HCL. Finally, the resulted product dialyzed against acidic solution using dialysis bag MWCO 3.5 KDa, and the degree of thiolation was approved by using Ellman's assay. The resultant product HA-SH was further conjugated with GE137 via using EDC/NHS coupling reaction. In this step, a 1:1.5 molar ratio of HA-SH and GE137 was used. HA-SH was dissolved in 2-(N-morpholine)ethane sulfonic acid MES buffer (pH=6), and EDC/NHS was added and stirred for one hour to activate -COOH of HA-SH followed by the gradual addition of GE137 and kept for overnight stirring at 4 °C. The final product was dialyzed using dialysis bag MWCO 3.5 for several hours and then lyophilized and stored at -20 °C until further use.
The second step was synthesizing gemcitabine. HCL SPDP thiol as the following: 460 mg of gemcitabine was dissolved in 460 ml dry tetrahydrofuran (THF), the dissolving carried out in round bottom flask under reflux 70-90 °C until all gemcitabine HCL dissolved. To enhance gemcitabine HCL solubility, DIPEA was added at 1:4 molar ratio regarding gemcitabine HCL. Once all, gemcitabine HCL dissolved, Peg4-SPDP dissolved in dry THF was added dropwise to the mixture and kept overnight at 70-80 °C. the reaction was monitored by TLC using a 2:8 ratio of methanol: DCM until confirming the consuming all Peg4-SPDP. The resultant product was separated in silica gel column chromatography using different percentages of methanol: DCM (2:98, 5:95, 10:90 and 20:90%), and the fraction of interest was collected, dried and stored until further use.

The final step was conjugating the first and second steps as the following: 30 mg of HA-SH-GE137 was dissolved in 3 ml PBS (pH 7.5). Gemcitabine-SPDP-SH that synthesized in step two, was dissolved in DMF (500 µl) and added dropwise to HA-SH-GE137 and kept stirring overnight at 4 °C. The product was dialyzed against a 2KD dialysis bag for 6 hrs. The final product was lyophilized and stored at -20 °C until further use.

HA-GE137-Gem nanoparticles size and zeta potential were performed using Beckman Coulter Delsa Nano-C-DLS Particle analyzer (Miami, FL) equipped with a 658 nm He-Ne laser. For nanoparticle size, the nanoparticles were suspended in water, and the light scattering was measured after 70 scans; the DLS data was collected as a peak average of 70 scans. The electrophoretic mobility of nanoparticles was measured after applying the electric field on nanoparticles. 1H NMR was used to confirm the chemical conjugation of NPs via measuring the functional groups' chemical shifts. The final structure of HA-SH-GE137 and HA-SH-GE137-Gem was confirmed by NMR.
4.2.6. Western blot analysis

PDAC cell lines were treated with vehicle or indicated doses of free drugs combination or nanoparticle combination. Protein from cell lines was extracted on ice using whole-cell lysis buffer (RIPA buffer) supplemented with 1:100 Halt protease and phosphatase inhibitors cocktail for 15 mins. Then, the lysate was collected after centrifugation at 14,000 RPM for 15 mins. The protein quantity was determined using the BCA kit (Thermo Fisher Scientific, Waltham, MA), and 20 μg of each sample was separated using 12% polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane using a wet transfer procedure. The membrane was blocked with 5% nonfat dry milk, followed by overnight incubation with primary antibody of protein of interest; followed by 1 h incubation with an appropriate secondary antibody. The bound antibody was visualized after incubation with chemiluminescent western blot detection reagents (Thermo Fisher Scientific, MA, USA), followed by reading out the bands using Bio-Rad ChemiDoc™ MP machine. The same membranes were re-probed with an internal control (anti-β-actin antibody).

4.2.7. Evaluation of the activity of HA-GE137-Gem + Everolimus in nu/nu mice

6 week-old female nu/nu mice using were obtained from Jackson Laboratory. PDAC subcutaneous tumor model was generated in nu/nu mice using the AsPc-1 cell line according to an approved protocol by Institutional Laboratory Animal Care & Use Committee (IACUC) at the Wayne State University. After several days of mice acclimation, 200 μl suspension of 1:1 ratio of 5 x10⁶ cells mixed with Matrigel® Matrix (Bedford, MA) was subcutaneously injected into the right flanks of nu/nu mice. Once the tumor reached 100 mm³, the mice were divided into four groups, five animals each. The first group of the mice left untreated, and the second group was treated with a vehicle. The third group was treated with a combination of intravenous (i.v.) injected
of gemcitabine (10 mg/kg/every other day) and orally (p.o.) administered Everolimus (3 mg/kg/every other day), the fourth group was treated with i.v. HA-GE137-Gem (10 mg/kg/every other day), and p.o. Everolimus (3 mg/kg/every other day). Everolimus was prepared in 10% kolliphor in PBS. The mice received five doses of gemcitabine or nanoparticles-gem and three dosed of Everolimus. The mice tumor volume was measured using the NIH formula ((tumor volume) = \( \frac{1}{2} \) (length \( \times \) width \(^2\))). Also, the body changes were monitored during the study for any sign of toxicity. Animals were euthanized once they reach the tumor endpoint. Representative tumors were collected, snap froze, and stored at \(-80^\circ\text{C}\). Histological analysis was performed by the Biobank core facility to study the safety of the current therapy on the major organs such as the liver and kidneys.

### 4.3. RESULTS AND DISCUSSION

#### 4.3.1. Rationale of using of Gem conjugated HA-GE137

The current study aims to enhance the PDAC first-line therapy efficiency via synthesizing a gemcitabine (Gem) conjugated dual-targeted nanoparticles (HA-GE137-Gem) that can actively bind to the overexpressed CD44 and c-Met surface biomarkers. The dual-targeted ligands (HA-GE137) were synthesized as illustrated in scheme 3.1. HA is a non-toxic and non-immunogenic polysaccharide. HA is composed of repeating units D-glucuronic acid and N-acetyl D-glucose amine which is abundant in hydroxyl/carboxylic acid groups. Hydroxyl/carboxylic acid groups allow doing simple coupling chemistry to form either the ester or amide group. Biologically, CD44 is found to be overexpressed on the surface of CSCs in many human tumors including pancreatic adenocarcinoma tumors [190,202]. HA is a biomaterial that has a high affinity toward CD44 receptors and hyaluronan-mediated motility [203]. HA is found to have excellent physicochemical properties, high selectivity, and affinity toward overexpressed CD44 receptor; thus, it is a good
candidate to be used as a targeted ligand in drug delivery [204].

On the other hand, many groups have confirmed that the c-Met receptor is overexpressed in many solid tumors including PDAC. The overexpressed c-Met on the extracellular surface of the tumor cells makes it an excellent target for drug delivery [205]. A recent study used a phage display library has discovered a small peptide (GE-137), which is in phase 1 clinical trial [206], which has a strong binding affinity to the c-Met receptor (~ Kd 3 nM). GE137 is 2-dicysteine containing 26 amino acid cyclic peptides that increase its in vivo stability against protease-mediated neoplasia. Thus, we selected GE137 conjugated to HA to develop a CD44 and c-Met dual targeting ligand to target several tumor microenvironments such as CSCs, cancer epithelial cells, and tumor stroma; thus, the therapeutic outcome will be improved, and toxicity will be reduced. The HA-GE137-Gem approach will (i) promote the accumulation of the targeted NPs in the tumor core (ii) decrease the drug resistance (iii) enhance drug circulation and half-life and (iv) and minimize the side effect of the chemotherapeutic agent when combined with mTOR inhibitor. The first-line therapy, Gem, was chosen to be conjugated to SPDP-Peg4-NHS as shown in scheme 4.1. Gemcitabine was chosen over other first-line therapies because it is cost-effective, well-tolerated, more effective than 5-fluorouracil (5-Flu) in terms of survival rate, and showed modest activity in the patients refracted to 5-Flu [207]. The conjugate SPDP-Peg4 grafted on the nanoparticles to (i) improve the systemic circulation time of Gem and decrease the overall immunogenicity [208], (ii) reduce the NPs aggregation and adsorption within the biological systems [209] and (iii) decrease the toxicity of nanoparticles such as erythrocytes aggregation and hemolysis via minimizing NPs – blood cells interactions [210]. Together, the targeted nanoparticles approach will be utilized to achieve better drug penetration, efficacy, solubility, and potency. Due to the nature of the PDAC, many barriers such as stroma and stem cells restrict the
penetration of first-line therapy such as gemcitabine into the tumor, and that increases the resistance of PDAC to chemotherapeutic treatments. Thus, the targeted approach will maximize the internalization of hybrid polymeric nanoparticles into the core of the tumor. Our preliminary data revealed that the dye conjugated targeted nanoparticles can bypass several tumor layers and accumulate in the tumor core. It has been reported that the pancreatic cells became more resistant when it treated with gemcitabine alone [205]; thus, we believe that the targeted polymeric nanoparticles approach will maximize tumor drug uptake and minimize the drug resistance.

4.3.2. Procedure of synthesis HA-GE137-Gem by EDC/NHS coupling chemistry

The aim of the current study is to synthesize dual-targeted polymeric nanoparticles targeting overexpressed CD44 and C-Met PDAC biomarkers. CD44 and C-Met surface biomarkers were targeted by hyaluronic acid and GE137 peptide, respectively using EDC/NHS coupling chemistry as illustrated in scheme 1 chapter 3. Second, in scheme 4-1, step1, we synthesized Gem-SPDP-NHS by adding a known amount of Gem and SPDP-Peg4-NHS in dry THF under reflex at pH 8-9 and temperature 70-80 to allow amide formation between Gem and SPDP-Peg4-NHS. Then, we conjugate GEM-SPDP-NHS with the thiol group of HA-GE137 in PBS (pH 7.5) (scheme 1, step 3). All unconjugated starting materials were removed by dialysis before lyophilization. The resultant product in each step was confirmed using 1H NMR. The characteristic peaks of Gemcitabine in Gem-SPDP-peg4-NHS was determined at the chemical shifts $\delta = 6.1$ as shown at figure 4-2, C. Also, the characterized peaks of aromatic amino acids of GE137 were identified at chemical shifts ranged from 6.8 ppm up to 7.5 ppm, indicating the success conjugation of both gemcitabine and GE137 to dual-targeted nanoparticles (Figure 4-2, D).
FIGURE 0-1: Schematic representation of dual-targeted conjugated to gemcitabine.
Figure 0-2: $^1$H NMR characterization of the construct (HA-GE137-GEM). (A) $^1$H NMR of hyaluronic acid, (B) $^1$H NMR of thiolated hyaluronic acid. (C) $^1$H NMR of Gemcitabine conjugated to SPDP-Peg4-NHS (D) $^1$H NMR of the dual-targeted nanoparticles conjugated to gemcitabine.
4.3.3. Preparation and characterization of HA-GE137-Gem nanoparticles

After synthesis of HA-GE137-Gem, the resultant product was purified through dialyzing using Spectra/Por® dialysis membrane (Rancho Dominguez, CA) followed by lyophilization. The product was further characterized for size, charge, and drug loading using DLS, zeta potential, and High-Performance Liquid Chromatography (HPLC), respectively. The data showed that the size of nanoparticles was slightly increased after conjugating a chemotherapeutic agent (Gem), suggesting the presence of Gem on the surface of nanoparticles (Figure 4-3, A and B). Also, the surface charge of nanoparticles shifted from neutral vehicle to negative six. The more negative or positive zeta potential, the more stable nanoparticles in which the negative or positive charge will increase the repulsion of the particles and minimize the aggregations. (Figure 4-3, D). Together, size and surface charge are ideal and safe to use for vein tail injection and overall drug delivery.

Moreover, the conjugated drug to nanoparticles was evaluated using HPLC. The drug efficiency was measured by dissolving nanoparticles in HPLC water supplemented with 100 mM DTT, followed by determining the drug quantity at absorbance 260 nm with respect to the standard curve of commercial gemcitabine. The drug conjugation was 6% in HA-GE137-Gem (figure 4-3, C). We established the novel conjugation method to enhance the penetration of conjugated nanoparticles within the biological system after disulfide bond being cleaved in the presence of tumor cells glutathione; the cleaved disulfide bond will lose the proton which results in forming nucleophile thiolate (S-) group that attacks the carbonyl group of the amide bond between SPDP-Peg4-NHS and Gem, leading to release the active gemcitabine [211].
**Figure 0-3:** Characterization of dual-targeted nanoparticles (A and B) Hydrodynamic size of thiolated dual-targeted nanoparticles and Gem conjugated dual-targeted nanoparticles. (C) the summary of nanoparticles diameter (nm) and the surface charge (zeta potential) of thiolated hyaluronic acid (HA-SH), thiolated dual-targeted nanoparticles (HA-GE137-SH), and Gem conjugated nanoparticles (HA-GE137-Gem)

### 4.3.4. Cell viability assay of several drugs on PDAC cell lines

PDAC is a challenging disease to treat, and the response rate to the metastatic disease is poor [13]. Thus, new strategies need to be implied to improve the overall response rate and to maximize efficiency. Hydrophobicity is one of the main obstacles that limit tumor cell penetration due to the presence of cellular lipid bilayer membranes. Mechanistically, Gem works via inhibiting DNA synthesis. Within the cells, Gem gets phosphorylated to di and triphosphate which competes with DNA synthesis; consequently, cell death occurs [207]. Everolimus is a rapamycin analog that is available as oral formulation and functions via inhibiting mTOR, which is a member of
phosphatidylinositol 3-kinases family [212]. Everolimus is an FDA approved drug to treat pancreatic cancer known as pancreatic neuroendocrine tumor (PNET) if the surgery is not an option. Cabazitaxel is a taxane anticancer drug that inhibits cell division via promoting the formation of microtubules from tubules and consequently inhibiting disassembly which results in blocking cell mitosis and cell death. Cabazitaxel in combination with prednisone, is currently FDA approved to be used in patients who have metastatic prostate cancer with hormone refractory [213,214].

The aim of this study is to screen the efficiency of multiple drugs on PDAC cell lines. The cytotoxicity of Gemcitabine, Everolimus (Evr), Cabazitaxel (Caba), and HA-GE137-Gem nanoparticles was assessed using 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. After exposing PDAC cell lines to the drugs for 72 h, the cell viability count was determined and IC$_{50}$ (half-maximal inhibitory concentration) was calculated using GraphPad Prism® dose-response curve. The IC$_{50}$ of Gem, Evr, Caba and HA-GE137-Gem on AsPc-1 were 10.01 ± 0.12, 47.6 ± 0.9, 47.25 ± 0.197 and 1.74 ± 0.09 respectively. Due to the limitation of cellular penetration of hydrophobic drugs, conjugation dual-targeted nanoparticles to Gem (HA-GE137-Gem) was an effective way that drastically improved Gem efficiency compared to commercial Gem. The improved efficiency of HA-GE137-Gem is attributed to (i) promote Gem intracellular diffusion due to its inability to diffuse via cellular lipid bilayer [215] (ii) ability of dual-targeted nanoparticle to encapsulated hydrophobic Gemcitabine and maximize Gem diffusion to the PDAC cells and (iii) our previous works have proved that decorating nanoparticles with targeting ligands such as HA enhanced the tumor accumulation and the overall chemotherapeutic efficiency [57,204]. Moreover, HA-GE137-Gem retained the activity in upregulating p-γH2AX which plays a significant role in arresting cell cycle, as shown in figure 4-4 (F).
FIGURE 0-4: The cytotoxicity of commercials drugs (Gemcitabine (A), Everolimus (B), and Cabazitaxel (C)) on several AsPc-1 cell lines. (D) the cytotoxicity of dual-targeted nanoparticles.
conjugated to Gem. (E) the summary of the half-maximal inhibitory concentration of all commercials drugs and nanoformulations on AsPc-1 cell lines. (F) western blot analysis to evaluate the pharmacological activity after exposing the AsPc-1 sequentially to Dual-targeted nanoparticles (HA-GE137-Gem + EVR) and commercial Gem + EVR.

4.3.5. Evaluation of the synergistic effect of (Gemcitabine with Everolimus)

Gemcitabine (Gem) is widely used as first-line therapy in treating pancreatic ductal adenocarcinoma. Gem mechanistically works on inhibiting the S-phase cell cycle. Gem is a centered for most of PDAC combination therapies due to in vitro and in vivo efficiency as well as improving the OS in clinical studies [216]. Recently, many classes of immunosuppressive drugs have shown an anti-tumor effect. One of these classes is mTOR inhibitors [217]. Everolimus (mTOR inhibitor) showed antitumor activity comparable to 5-fluorouracil. Moreover, Everolimus showed a dose-dependent anti-proliferative activity in the CA20948 syngeneic rat model of PDAC [218]. It well established that PDAC collagen I is one of the barriers that attenuate the delivery of most cytotoxic agents. Also, collagen I is one of the players that enhances the PDAC migratory and aggressiveness activity [219,220]. In this study, we hypothesized that combining two different classes of drugs (Gemcitabine and Everolimus) with two different mechanisms of action will enhance the overall killing activity. Furthermore, using Gemcitabine conjugated to dual-targeted nanoparticles would increase the activity of Gem as shown in table 4.5 (A). We first determined the cytotoxicity of Gemcitabine and Everolimus on different PDAC cell lines such as AsPc-1 and BxPc-3. We utilized Composyn® to evaluate the synergistic activity of free commercial drugs (Gem + EVR). The study was carried out on 5 PDAC cell lines (AsPc-1, Panc-1, BxPc-3, HPAC-1, and MiaPaCa-2). This study aimed to maximize the efficiency of Gem via combining with mTOR inhibitor. Clinically, using single EVR in treating PDAC showed disappointing results.
Also, combining EVR with other agents such as Erlotinib [221], Cetuximab + Capecitabine [222], Gemcitabine [223], and Gemcitabine + cisplatin [224] showed modest activity against PDAC. The reason for poor PDAC response to EVR combination therapies might be attributed to PDAC poor vasculature and cross-reaction between stroma and cancer cells with collagen deposition. Our in vitro studies found that different PDAC cell lines respond differently to combination therapy of Gem with EVR. Figure 4-5 indicated that increasing the dose of each drug might attenuate the cell's response toward (Gem + EVR). As shown in figure 4-5, the most combination index value of Gem + EVR on (B) Panc-1 and (C) HPAC-1 were bigger than 1, which indicates the cells develop resistance with the dose escalation. Furthermore, the same observation was seen on other cell lines in which the low doses showed maximum synergistic effect compared to high doses as shown in figure 4-5 (D, E, and F).
CI < 1 = Synergism, CI = 1 = Additive, CI > 1 = Antagonism

**Figure 0-5:** The evaluation of the activity of Gem and HA-GE137-Gem on PDAC cell lines. (A) HA-GE137-Gem activity of AsPc-1 cell line compared to free commercial Gem. The CompuSyn® data utilized to evaluate all possible synergistic doses of combined Gem and EVR. (B) Panc-1 and (C) HPAC showed the lowest response to most of Gem + EVR doses. The other cell lines (D)
MiaPaCa-2, (E) AsPc-1 and (F) BxPc-3 strongly inhibited after being treated sequentially by Gem + EVR

4.3.6. Evaluate the synergetic activity of HA-GE137-Gem with Everolimus

PDAC is a challenging disease to treat, and most of the current chemotherapeutic agents and targeted therapy failed to control the metastasized tumor [82]. Our recent on AsPc-1 cell lines showed that the HA-GE137-Gem construct is more effective than FDA approved drugs (gemcitabine) (figure 4-6, A). Moreover, combining FDA proved drugs Gemcitabine + Everolimus had shown some encouraging results which motivated us to explore and develop new drug delivery to enhance the potency of PDAC front-line therapy (gemcitabine). In this study, we utilized the dual-targeted approach plus using the mTOR inhibitor to suppress the aggressiveness of PDAC tumor proliferation. Our current data clearly indicating that using the Gemcitabine conjugating approach plays a significant role in enhancing the overall killing activity. Considering the poor cellular uptake of hydrophobic compounds, using the drug delivery system would overcome this issue and improve the cellular drug delivery of hydrophobic compounds. Thus, we observed that the killing activity of AsPc-1 using HA-GE137-Gem + EVR was enhanced. The combination index value of sequential treatment with HA-GE137-Gem + EVR was up to 90 folds higher than combining free commercials drugs (Gem + EVR). The improved killing activity is attributed to the advantages of using a sequential treatment which minimizes the cross-reactions between NPs and EVR and allows each drug to work effectively on PDAC cell lines. The same results were obtained when BxPc-3 (figure 4-6 B, D, and F) was treated with HA-GE137-Gem + EVR on one arm and Gem + EVR on another arm. Overall, the drug delivery system enhances the potency of gemcitabine and minimizes the required dose of each combined drug; consequently, the overall
toxicity will be minimized, and the safety would be improved as shown in the animal tumor regression (figure 4-7 C and D).

![Graphs showing toxicity and safety improvements](image)

**Figure 0-6:** Combination Index of HA-GE137-Gem and commercial Everolimus on (A) AsPc-1 and (B) BxPc-3 cell line. Combination Index of Gemcitabine and Everolimus (Commercial drugs)
on (C) AsPc-1 and (D) BxPc-3 cell line. HA-GE137-Gem + EVR showed a high synergistic effect by several folds on (E) AsPc-1 and (F) BxPc-3 compared to commercial drugs.

4.3.7. Evaluation of the antitumor activity of HA-GE137-Gem + Everolimus in nu/nu mice

After testing the activity of HA-GE137-Gem alone or in combination in vitro, we moved to examine its antitumor activity on in vivo tumor bearing nu/nu mice. The conjugation of Gem to nanoparticles enhance its activity nine folds potent than FDA approved Gemcitabine as shown in figure 4-5, A, and up to 90 folds when combined with EVR in comparison with free Gem + EVR. The in vivo data is matching with our in vitro results in which HA-GE137-Gem + EVR has significantly inhibited the PDAC tumor growth compared to control, single drugs, and free drugs combination as shown in figure 4-7, A. Interestingly, HA-GE137-Gem + EVR has increased the overall survival rate of the tumor bearing mice as shown on Kaplan-Meier survival curve (figure 4-7, B). Using dual-targeted ligands is a smart strategy for enhancing the efficiency of the current drug against PDAD tumors with high in vivo safety profile. Using combination therapy of HA-GE137-Gem + EVR is to improve the drug potency, target multiple signaling pathways, and minimize the toxicity of every single drug.

We successfully designed dual-targeted nanoparticles that proved its potency in both in vitro and in vivo studies and did not show any sign of toxicity either on liver and kidney organs (figure 4-7 D) or on the total body weight changes (figure 4-7 C). The current construct can function as a universal ligand for multiple anti-proliferative payloads to enhance the potency of current PDAC therapy to be utilized in a more efficient way.
**FIGURE 0-7:** (A) Evaluation of tumor inhibition rate of AsPc-1 bearing nude mice after treating with Vehicle, Gemcitabine (Gem) + Everolimus (EVR) and HA-GE137-Gem + EVR (the data shown as the mean ± SEM), P-value of HA-GE137-Gem + Everolimus vs control, Vehicle, and Gem + EVR (0.0008, 0.0018, and 0.0038 respectively). (B) Kaplan-Meier curves of all AsPc-1 bearing nude mice treated with Vehicle, Gemcitabine + Everolimus and HA-GE137-Gem. (C) The mice's body weight (g) did not show any significant changes after being exposed to several doses of VEHICLE, Gemcitabine + Everolimus and HA-GE137-Gem + EVR (the data shown as the mean ± SEM). (D) There were no significant changes in the nude mice serum level of AST, ALT, and creatinine after being exposed to Vehicle, Gemcitabine + Everolimus, and HA-GE137-Gem + EVR (the data shown as the mean ± SEM).
4.3.8. Conclusion

Despite the technological progress in most of the solid tumor therapies, there is no progress in PDAC treatment. Moreover, PDAC responses to current frontline therapies are reduced due to the complex tumor microenvironments that limit the efficacy of most current first-line drugs. Therefore, considering using the combination is actively encouraging to minimize the PDAC refractory. Many ongoing clinical trials have tried using many combination therapies with no significant success in improving the patient's OS. Thus, modifying the current front-line treatments is one of the ways that might enhance the PDAC therapy efficiency. We successfully conjugated front-line therapy (Gemcitabine) to pegylated dual-targeted nanoparticles. The current construct was more potent than commercial gemcitabine on the AspC-1 cell line.

Furthermore, combining Gemcitabine conjugated dual-targeted nanoparticles (HA-GE137-Gem) with mTOR inhibitor improved the efficiency compared to free commercial drugs up to 90 folds. The in vitro studies indicated that using the drug delivery system is a valid method to repurpose using current FDA approved medications in a more effective way. In vivo studies showed that using a combination of HA-GE137-Gem +EVR has significantly inhibited tumor regression compared to commercial Gem+ EVR. The current construct showed no significant changes in the nude mice body weight, and there were no changes in serum level of AST, ALT, and creatinine after being exposed to vehicle, Gemcitabine + Everolimus and HA-GE137-Gem + EVR. Currently, we are planning to study how the current construct modulates the immune cells to recognize and enhance the killing activity of the cancer cells within PDAC tumor microenvironments. In the future, we are planning to test the activity and efficacy of the current construct on the Patient-derived xenograft mouse model, which mimics the patients’ PDAC tumor microenvironments.
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91

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ABSTRACT

TUMOR STROMA DISRUPTING NANOPARTICLES FOR CHEMO GUIDED IMMUNOTHERAPY OF PANCREATIC DUCTAL ADENOCARCINOMA

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Pancreatic ductal adenocarcinoma is the fourth leading cause of death in the US. Its survival rate is the lowest among other solid tumors due to several factors such as tumor microenvironments, low infiltrating cytotoxic cells, and the propensity of the tumor to metastasize and develop resistance against chemo and radiation therapy. Clinically, using single agent in treating PDAC showed disappointing results. Therefore, combination therapies with different mechanisms of action are considered. Currently, gemcitabine is the centered of most combination therapies due to it is a cost-effective, well-tolerated, more effective than 5-fluorouracil (5-Flu) in terms of survival rate and showed modest activity in the patients refracted to 5-Flu. Using gemcitabine as single agent or in combination with other agents such as cisplatin or Everolimus showed modest activity against PDAC. The reason for poor PDAC response to gemcitabine combination therapies might attributed to PDAC poor vasculature and cross-reaction between stroma and cancer cells with collagen deposition. The main goal of this study is to enhance the therapeutic benefit of gemcitabine via conjugating with SPDP-Peg4 grafted on the nanoparticles to (i) improve the systemic circulation time of gem and decrease the overall immunogenicity, (ii) reduce the nanoparticles aggregation and adsorption within the biological systems and (iii)
decrease the toxicity of nanoparticles such as erythrocytes aggregation and hemolysis via minimizing nanoparticles – blood cells interactions. Also, gemcitabine anchored to targeted nanoparticles will overcome the hydrophobic drug's poor cellular penetration. The gemcitabine dual-targeted nanoparticles combined with Everolimus showed an excellently synergistic effect on PDAC cell lines and enhanced the tumor regression compared to commercials Gemcitabine + Everolimus.
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