Identifying Novel Inhibitors of Epithelial to Mesenchymal Transition (EMT) for Targeting Pancreatic Cancer Metastasis and Cancer Stem Cells

By

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Abstract

Pancreatic cancer is the 4th leading cause of cancer related deaths in the US with a median survival of less than one year and an overall 5-year survival of less than 5%. Surgical resection is the curative option for patients with localized disease; however, less than 20% of patients have dissectible disease. Gemcitabine chemotherapy has been the standard care for nearly 20 years and it has little impact on overall survival rate. A recently developed gemcitabine-free regimen FOLFIRINOX showed a small improvement on the survival but it added significant toxicities. One of the reasons for such high mortality rate and poor treatment outcome is that pancreatic cancer is highly metastatic. The majority of the patients have locally or regionally spread and/or distant metastasis at the time of diagnosis. Moreover, these tumors are highly enriched with a cancer stem cell (CSC) population (~1%), which is highly resistant to chemotherapeutic drugs, and therefore escapes chemotherapy and promotes tumor recurrence.

Recent evidence suggests that epithelial to mesenchymal transition (EMT) is associated with metastasis, generation of CSCs, and treatment resistance in solid tumors including pancreatic cancer. Therefore, compounds inhibiting EMT hold the potential to reverse drug-resistance or inhibit metastasis and CSCs, and therefore could provide better treatment outcome for patients with pancreatic cancer. **The overall goal of this dissertation is to investigate novel EMT inhibitors for targeting pancreatic cancer metastasis and CSCs.** This study resulted in the identification of several novel EMT inhibitors that have potential clinical significance for pancreatic cancer patients.

First, we demonstrated in preclinical models that treatment with pharmacological doses of ascorbate resulted in inhibition of EMT, metastasis and CSCs. High-dose intravenous ascorbate is now in clinical testing (Chapter 4).

Next, we investigated novel derivatives of the histone deacetylase (HDAC) inhibitors SAHA and MS-275, which are known EMT inhibitors. In an effort to increase efficacy and reduce toxicities of the HDAC inhibitors, we found that the novel synthetic compounds St-1 and St-3 potently inhibited pancreatic cancer cell proliferation and CSCs. St-1 has exhibited similar potency in HDAC inhibition compared to the parent compounds (SAHA and MS-275). Surprisingly, St-3 acted via totally different mechanisms from SAHA and MS-275. St-3 exhibited anti-tumor effects by blocking the interaction of human antigen R (HuR) with its target mRNAs (Chapter 5). To date there is still a lack of highly efficient approach for discovery of CSC inhibitors. In an effort to solve the problem, we established and performed a high throughput screening (HTS) approach to identify EMT and CSC inhibitors. 1-(benzylsulfonyl) indoline (BSI) was found to be a novel EMT inhibitor. Several analogues of BSI were tested for their activities on EMT and CSC inhibition (Chapter 6).

Overall this dissertation resulted in the identification of several novel EMT inhibitors, which can be tested further in preclinical or clinical studies for their anti-tumor efficacy. These pancreatic cancer EMT and CSC inhibitors each have their own merits. A pharmacological dose of ascorbate has an outstanding safety profile. It inhibited pancreatic cancer EMT and CSCs. In addition pharmacological dose of ascorbate affected the tumor microenvironment to inhibit cancer cell metastasis. In this way, ascorbate provides a unique opportunity to target both cancer cell growth as well as tumor microenvironment. Combining with its low toxicity profile, the clinical benefit of pharmacologic ascorbate in pancreatic cancer patients is worth investigating. The compound St-1 showed better potency in inhibiting pancreatic cancer cell growth than currently used HDACs, showing the potential of having a larger therapeutic index and better efficacy than its parent compounds. The compound St-3 is a novel HuR inhibitor and provided a

novel therapeutic target for pancreatic cancer treatment. The compound BSI is structurally different from other EMT inhibitors, and thus could become a new class of EMT inhibitors. The work in this dissertation set a basis for advancing these EMT inhibitors towards drug development for pancreatic cancer treatment. It has opened the door for further preclinical, clinical and mechanistic investigations. Ultimately, we hope patients will benefit.

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List of Abbreviations

5-FU	= Five fluoro uracil
ABCG2	= ATP-Binding Cassette, Sub-Family G, Member 2
Alpha	= Amplified luminescent proximity homogeneous assay
AML	= Acute myeloid leukemia
ARE	= Adenine/uridine and U- rich elements
Asc	= Ascorbate
AU	= Adenine/uridine
bHLH	= Basic helix loop helix
BRCA2	= Breast cancer 2
BSA	= Bovine serum albumin
BSI	= 1-(benzylsulfonyl) indoline
C_2H_2	= Cys2His2
CDKN2A	= Cyclin-dependent kinase inhibitor 2A
CI	= Combination Index
CK-1	= Casein kinase-1
COX2	= Cytokine cyclooxygenase 2
CQ	= Chloroquine
CSCs	= Cancer stem cells
CTCL	= Cutaneous T-Cell Lymphoma
DPC4	= Deleted in pancreatic carcinoma 4
EGF	= Epidermal growth factor
EGFR	= Epidermal growth factor receptor

ELAV = Embryonic Lethal Abnormal Vision protein

EMT = Epithelial to mesenchymal transition

EpCAM = Epithelial cell adhesion molecule

ER = Estrogen Receptor

Erk = Extracellular signal-regulated kinase

FGF = Fibroblast growth factor

FGFR = Fibroblast growth factor receptor

FOLFIRINOX= Fluorouracil, Leucovorin, Irinotecan and Oxaliplatin

FRS2 = FGFR substrate 2

FTIs = Farnesyl transferase inhibitors

GAPs = GTPase activating proteins

GEFs = Guanine nucleotide exchange factors

GGTIs = Geranylgeranylation transferase

GSI = Gamma secretase inhibitors

GSK-3β = Glycogen synthase kinase-3β

H&E stain = Hematoxylin and eosin stain

HD = Homozygous deletion

HDAC = Histone deacetylases

HDACi = Histone deacetylases inhibitor

HH = Hedgehog

HMECs = Human mammary epithelial cells

HSP 90 = Heat shock protein 90

HTS = High-throughput screening

HuR = Human antigen R

IGF = Insulin-like growth factor

IGFR = Insulin-like growth factor receptor

IPMN = Intraductal papillary mucinous neoplasms

IVC = Intravenous ascorbate

K-Ras = Kirsten rat sarcoma viral oncogene homolog

MAPK = Mitogen-activated protein kinase

MCN = Mucinous cystic neoplasm

MET = Mesenchymal to epithelial transition

MiR200f = MicroRNA-200 family

Msi1 = Musashi1

MTT = 4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAM = Nicotinamide

NICD =Notch intra cellular domain

NMNAT =Nicotinamide mononucleotide adenylyl transferase

PanIN = Pancreatic intra epithelial neoplasia

PCNA = Proliferating cell nuclear antigen

PDAC = Pancreatic ductal adenocarcinoma

PDEδ = Phosphodiesterase delta

PDK-1 = 3-Phosphoinositide dependent protein kinase-1

PI3Ks = Phosphoinositide 3-kinases

PRC-2 = Polycomb repressive complex 2

RalGEFs = Ral guanine nucleotide exchange factors

RRMs = RNA recognition motifs

SAR = Structure activity relationship

SB = Sodium butyrate

SHH = Sonic Hedgehog

Sirt-2 = Silent information regulator 2

SMAD4 = SMAD family member 4

Smo = Smoothened

SOS = Son of Sevenless

SPR = Surface Plasmon Resonance

SUZ12 = Suppressor of zeste 12

TGF-α = Transforming growth factor- α

TSA = Trichostatin A

TβR I = TGF-β type I receptor

UTR = 5'-Untranslated regions

VEGF = Vascular endothelial growth factor

WIF-1 = Wnt inhibitory factor 1

WT = Wild Type

ZEB = Zinc-finger-enhancer binding protein

αSMA = Alpha smooth muscle actin

Chapter1. Introduction

1.1. Pancreatic cancer

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States, and it is expected to become the second-leading cause of cancer-related death by 2030. The American Cancer Society estimated that 48,960 (men=24,840, women=24,120) people will be diagnosed with pancreatic cancer in 2015 and that 40,560 (men=20,710, women=19,850) will die from the disease. Most of the pancreatic cancer cases (96%) are cancers of the exocrine pancreas. Currently, there are no early detection tests and most patients with localized disease have no recognizable symptoms. As a result, more than half of the patients with this disease are diagnosed at a stage where metastases have developed, for whom the overall 5-year survival is only 2% (8, 9).

A spectrum of distinct pancreatic malignancies have been identified that resemble normal cellular counterparts, such as pancreatic ductal adenocarcinoma (PDAC), acinar cell carcinoma, pancreato blastoma, solid pseudopapillary neoplasm, serous cystadenoma, and pancreatic endocrine tumors. Among these, PDAC, whose name is derived from its histological resemblance to ductal cells, is the most common pancreatic neoplasm and accounts for >85% of pancreatic cancer cases (4).

1.1. 1. Evolution of PDAC and its morphological characteristics

PDAC makes up over 90% of all pancreatic neoplasms (10). The majority of PDACs arises in the head region of the pancreas and exhibits a glandular pattern resembling ductal epithelial cells with varying degree of differentiation. Clinical and histological studies identified three different types of precursor lesions: pancreatic intra epithelial neoplasia (PanIN), mucinous cystic neoplasm (MCN), and intraductal papillary mucinous neoplasms (IPMN) that led to PDAC **(Fig. 1)**. Of these, PanIN is the most extensively studied precursor lesion. PanIN is graded into stages

1 to 3. PanIN 1 is characterized by columnar mucinous epithelium with slight nuclear atypia. PanIN 2 and 3 are characterized by more disorganized structural architecture and nuclear atypia. MCN and IPMN are less common precursor lesions. MCNs are large mucin producing columnar epithelial cystic lesions supported by ovarian type stroma usually found in the body and tail of the pancreas. IPMN arises in the main pancreatic duct or its major branches and resembles PanIN at cellular levels but grows into large cystic structures (11).

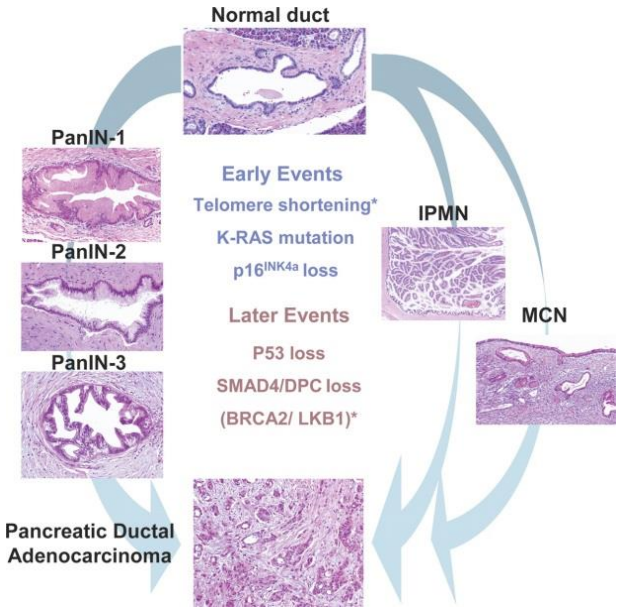


Figure 1- Pancreatic precursor lesions and genetic events involved in pancreatic adenocarcinoma progression (4). PanIN, IPMN, MCN represents three known precursor lesions of PDAC. PanINs classified into three grades: PanIN-1, 2 and 3 which then eventually develop to PDAC. Development of PDAC from IPMN and MCN shown on right side of the picture. Early genetic alterations (*K-Ras* mutations, *P16* loss) and late genetic alterations (P53 loss, *SMAD4* loss,) that occur in adenocarcinomas also occur in PanIN and to lesser extent in IPMNs and MCNs represented in the middle of the picture. Asterisks indicate events (telomere shortening, *BRCA2* mutations) that are not common to all precursor lesions.

1.1.2. Molecular biology of pancreatic cancer

Comprehensive genetic analysis revealed that pancreatic cancer contains an average of 63 genetic alterations. All these mutations are grouped into 12 different core signaling pathways that were altered in 67-100% of the tumors. The most commonly observed signature genetic lesions in pancreatic cancer are kirsten rat sarcoma viral oncogene homolog *(K-Ras)*, *P16*/ cyclin-dependent kinase inhibitor 2A *(CDKN2A)*, tumor protein P53 (*P53)*, breast cancer 2 early onset (*BRCA2)* and SMAD family

member 4 (*SMAD4)/* deleted in pancreatic carcinoma 4 (*DPC4)* (12). In patients, *K-Ras* mutations are often considered as an initiating event occurring in adult cells, soon followed by mutation to P*16* and later *P53* and *SMAD4* loss.

The K-Ras oncogene. K-Ras is activated by point mutations in more than 90% of the pancreatic cancer patients and represents the most frequent and the earliest genetic alteration, being found in low-grade PanIN lesions (12, 13). Continuous K-Ras signaling is required for pancreatic cancer cells for sustained proliferation and survival (14). Ras proteins belong to the small G protein superfamily, and their activity is regulated by guanine nucleotides such as GTP and GDP. Ras downstream signaling pathways are activated if Ras binds to GTP, and are inactivated if Ras binds to GDP. Active and inactive states of Ras signaling are regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs aid in exchange of GDP for GTP. GAPs activate intrinsic GTPase activity of Ras protein to hydrolyze GTP into GDP (15). Any mutations that inactivate the GTPase constitutively activate Ras signaling and downstream effector pathways. For example, a single point mutation of codons G12 or G13 in *K-Ras* abolishes GAP induced GTP hydrolysis, thereby making K-Ras a constitutively active form. Therefore, a pancreatic specific mutation of codons G12D or G12V is sufficient to develop acinar to ductal metaplasia and PanIN, which then progress to PDAC. PDAC development can be accelerated in the *K-Ras* mutant mouse by introducing additional mutations in tumor suppressor genes such as *p53, SMAD4,* and *P16/CDKN2A,* all of which occur frequently in precursor lesions as they progress to invasive PDAC (16).

K-Ras signaling engages various downstream effectors. In PDAC, K-Ras predominantly signals through canonical Raf/mitogen activated protein kinase (MAPK)/ extracellular signal-regulated

kinase (Erk), phosphoinositide 3-kinases (PI3Ks)/ 3-phosphoinositide dependent protein kinase-1 (PDK-1)/Akt, Ral guanine nucleotide exchange factors (RalGEFs), and phospholipase Cε (15).

Mutations or alternations in these downstream pathways complicate K-RAS driven PDAC. For example, expression of PIK3CA ^{H1047R} (codes for p110α ^{H1047R}), a constitutively active oncogenic class 1A PI3K in Ptf1a positive cells, induced acinar to ductal metaplasia and premalignant pancreatic intraepithelial neoplasia, recapitulating *K-Ras* ^{G12D} driven PDAC. Elimination of PDK-1, blocked *K-Ras*^{G12D} driven PDAC (17). RalGEF induces oncogenic activity by activating its substrate RalA (18). RalA is required for tumor initiation, whereas the other RalGEF substrate, RalB, is required for metastasis in Ras-driven pancreatic cancers (19).

Tumor suppressor genes (P16/CDKN2A, TP53, and SMAD4/DPC4). *P16/CDKN2A* is the most commonly inactivated tumor suppressor gene in pancreatic cancer. P16/CDKN2A inhibits CDK4/6 mediated phosphorylation of retinoblastoma (RB) protein, thereby blocking entry into the S phase of the cell cycle. Inactivation of *P16/CDKN2A* occurs by different mechanisms, including homozygous deletions, loss of heterozygosity and epigenetic silencing by promoter methylation (20). P16/CDKN2A cooperated with K-Ras in the development of PDAC (21). Mutations in *K-Ras* exert selective pressure for subsequent mutation in *P16/CDKN2A,* which cooperate to lead to the development of PDAC. In precursor lesions, oncogenic K-Ras expression co-exists with P16/CDKN2A and other markers of senescence. In PDAC, expression of P16/CDKN2A and senescence markers are missing (22).

P53 is inactivated in 50-75% of PDAC cases and the inactivation occurs via intragenic mutations combined with loss of the second allele (23). *P53* mutations observed in the late

PanIN stage usually lead to loss of p53 function, and subsequently provides growth and survival advantage for the cells which harbor chromosomal aberrations (24).

SMAD4 is a key signal transducer of TGF-β signaling pathway. SMAD4 is inactivated in approximately 55% of pancreatic cancer cases either by homozygous deletions or by intergenic mutations and loss of the second allele (25). Loss of SMAD4 provides growth advantage for pancreatic cancer cells by abrogating the growth inhibitory signals mediated by TGF-β (26) in late PanIN stage (PanIN-3) (27). Patients undergoing surgical resection of their pancreatic adenocarcinoma survived longer if their cancer expressed SMAD4 (28).

Growth factor receptor signaling in PDAC. PDACs overexpress multiple mitogenic growth factors and their ligands. These include: the epidermal growth factor (EGF) and its receptor (EGFR), and multiple ligands that bind to EGFR; fibroblast growth factor **(**FGF) and its receptor (FGFR) and ligands; insulin-like growth factor (IGF) and its receptor (IGFR); platelet derived growth factor; and vascular endothelial growth factor (VEGF) (29, 30).

EGFR is a transmembrane receptor tyrosine kinase, activated upon binding of its ligands, EGF and transforming growth factor-α (TGF-α). Upon activation EGFR stimulates phospholipase C gamma (PLC-γ). EGFR overexpression was detected in up to 90% of pancreatic tumors (31), and plays an important role in liver metastasis and recurrence of human pancreatic cancer (32). EGFR inhibitors decrease PDAC cell growth and tumorigenesis *in vitro* (33) and inhibited growth of orthotopic tumors when combined with chemotherapy (34). However, EGFR targeting agents in combination with gemcitabine did not provide many beneficial effects to pancreatic cancer patients in the clinic (31). Resistance to EGFR inhibitors may be due to activation of alternative receptor tyrosine kinase pathways (e.g. c-Met and IGF-1R) which bypass or evade

inhibition of EGFR signaling. Constitutive activation of downstream mediators of EGFR signaling pathway such as PTEN, K-Ras also provide resistance to EGFR targeted therapies (31, 35).

The IGFs and their receptors have been acknowledged as important players in a variety of cancers (36) by regulating cell survival, invasion, and angiogenesis (37). In PDAC patients, elevated expression of IGF-1 and its receptor IGF1R are associated with higher tumor grade and poor survival (38-41). *In vitro* experiments showed that exogenously added IGF-1 enhanced the growth of human pancreatic cancer cells and this effect was inhibited by IGF-1 neutralizing antibody (38). However, clinical trials performed with IGF1R blocking antibodies were largely disappointing (42). In 2012, Amgen announced termination of a large phase 3 clinical trial in patients with metastatic PDAC treated with the IGF1R blocking antibody ganitumab (AMG 479) and gemcitabine. The ganitumab and gemcitabine combination failed to improve overall survival compared to gemcitabine alone (http://www.amgen.com/media/media_pr_detail.jsp?releaseID=1723925).

FGFR is a transmembrane protein that triggers phosphorylation of an adaptor protein FGFR substrate 2 (FRS2) upon binding of FGF. Phosphorylated FRS2 then recruits and activates elements of Ras/MAPK and PI3K/Akt pathways. Overexpression of ligands (FGF1-7) and receptors (FGFR-1 and FGFR-2) contributed to mitogenesis and angiogenesis in a subset of pancreatic cancers (43-45). Inhibition of FGFR signaling using shRNA or dovitinib (a tyrosine-kinase inhibitor) achieved significant anti-cancer effects in preclinical pancreatic cancer models (46). Dovitinib is currently at clinical development for patients with metastatic pancreatic cancer, biliary cancers and pancreatic neuroendocrine tumors (https://clinicaltrials.gov identifier: NCT01497392, NCT01888965, NCT02108782).

Vascular endothelial growth factor (VEGF) is an angiogenic polypeptide. It promotes endothelial cell proliferation and survival by binding to its receptors VEGFR-1 and VEGFR-2 (47). Though PDAC is not a highly vascularized tumor, foci of endothelial cell proliferation are often observed in this malignancy. Patient tumor samples showed increased expression of VEGF mRNA and its expression correlated with high micro vessel density and disease progression (48, 49). TNP-40, an analog of fumagillin, which is an anti-angiogenic agent, decreased tumor growth and metastasis of pancreatic cancer cell lines in subcutaneous mice model system (50). Adenoviral vectors carrying the VEGFR tyrosine kinase inhibitor PTK 787 also inhibited the growth and metastasis of pancreatic cancer in preclinical models (51). Phase 1/2 studies of PTK787 plus gemcitabine (https://clinicaltrials.gov identifier: NCT00185588) in advanced pancreatic cancer showed promising results, and phase 2 studies are on-going (https://clinicaltrials.gov identifier: NCT00226005).

As our understanding has grown tremendously in oncogenic K-Ras, tumor suppressors, growth factors and their receptors in pancreatic cancer initiation and progression, approaches are being tested to target oncogenic K-Ras and growth factors. However, it remains challenging to improve treatment for this disease with complicated genetic and molecular alternations. It also remains challenging for researchers to further understand the molecular genetics of tumor suppressor proteins in pancreatic cancer, to restore their normal function by gene therapy, or to develop small-molecule inhibitors that reactivate tumor suppressor function (52). Novel approaches to target tumor suppressor genes need to be discovered and tested with open mind.

1.2. Epithelial to mesenchymal transition (EMT)

1.2.1. Basics of EMT

Most of the pancreatic cancer-related deaths are due to metastatic disease. Recent studies in animal models revealed that pancreatic cancer cells undergo dissemination from the primary tumor and get metastasized to liver even before frank malignancy was detected at the primary site of origin. The developmental program of epithelial to mesenchymal transition (EMT) is of virtual importance for this rapid tumor progression (53).

EMT is a multistage trans-differentiation process which allows highly polarized epithelial cells to undergo multiple biochemical changes to attain mesenchymal phenotype **(Fig.2.1)**. Epithelial cells display apical-basolateral polarity and are organized in cell layers with strong cell-cell adhesion. Mesenchymal cells are spindle shaped, exhibit anterio-posterior polarity and strong migratory potential. During EMT progression, epithelial cells lose their epithelial markers (such as E-cadherin, occludin, claudin, and laminin 1) and gain mesenchymal markers (such as N-cadherin, vimentin, and fibronectin) (6).

EMT is classified into three major types based on the context in which it occurs. Type 1 EMT is associated with implantation, embryo formation, and organ development. Mesenchymal cells generated by Type 1 EMT have potential to undergo mesenchymal to epithelial transition (MET) and generate secondary epithelia. Type 2 EMT is associated with inflammation processes and plays a major role in wound healing, tissue regeneration, and organ fibrosis. Type 3 EMT occurs in carcinoma cells **(Fig. 2.2)** and is considered important at several different stages (dissemination, invasion, intravasation and extravasation) during tumor progression (6, 54).

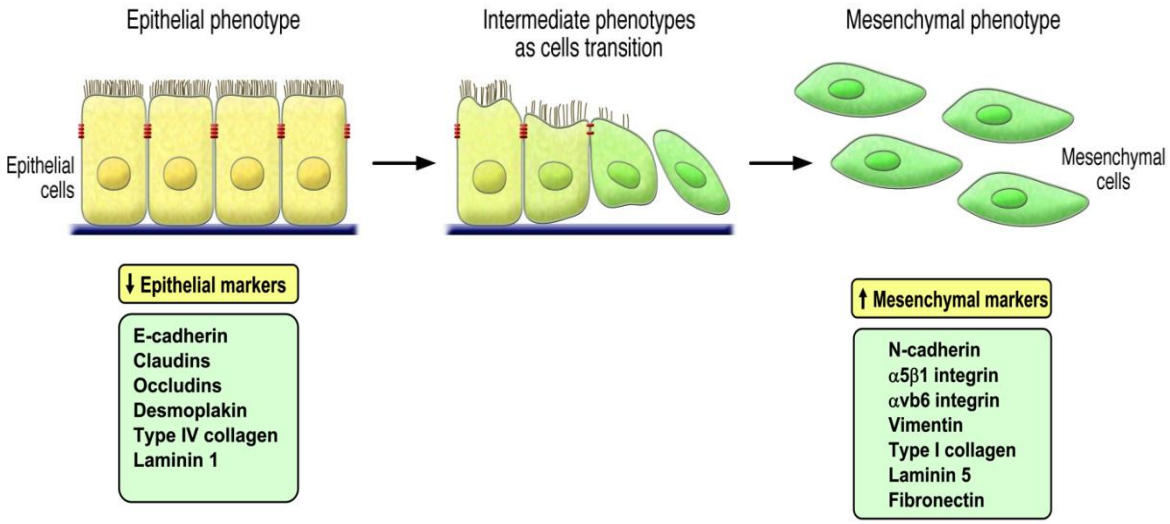


Figure 2.1- Characteristics of EMT involve a functional transition of polarized epithelial cells into mesenchymal cells (6). Epithelial cells are highly polarized in nature with apical and basolateral polarity. E-cadherin, claudins, occludins, desmoplakin, type IV collagen, and laminin-1 are markers of epithelial cells. Mesenchymal cells are spindle shaped and are highly motile in nature. N-cadherin, α5 β1 integrin, αvb6 integrin, vimentin, type-1 collagen, laminin-5 and fibronectin are the markers of mesenchymal cells. During EMT progression, epithelial cells loose epithelial markers and gain mesenchymal markers. Cells that express markers of epithelial and mesenchymal cells represent intermediate phenotype.

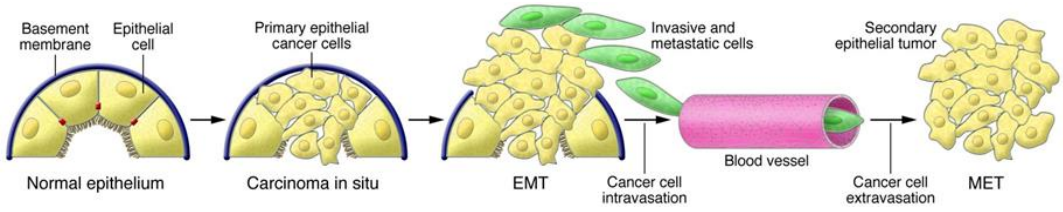


Figure 2.2- Type-3 EMT leads to cancer cell invasion and metastasis (6). Conversion of normal epithelium to invasive carcinoma is a multi-stage process. First, primary epithelial cancer cells lose their polarity and detach from the basement membrane. The next step involves basement membrane breakage, cancer cell EMT followed by intravasation into blood vessels. Cancer cells survive in circulation and exit the blood stream to form micro and macro metastasis, which may involve MET.

Metastatic tumor cells have the potential to migrate through the bloodstream and in some cases, form secondary tumors at other sites through MET (55, 56). During type 3 EMT, some cells retain epithelial traits while acquiring mesenchymal features and other cells shed most epithelial features and become fully mesenchymal (55, 57).

1.2.2 Epithelial to mesenchymal transition signaling pathways

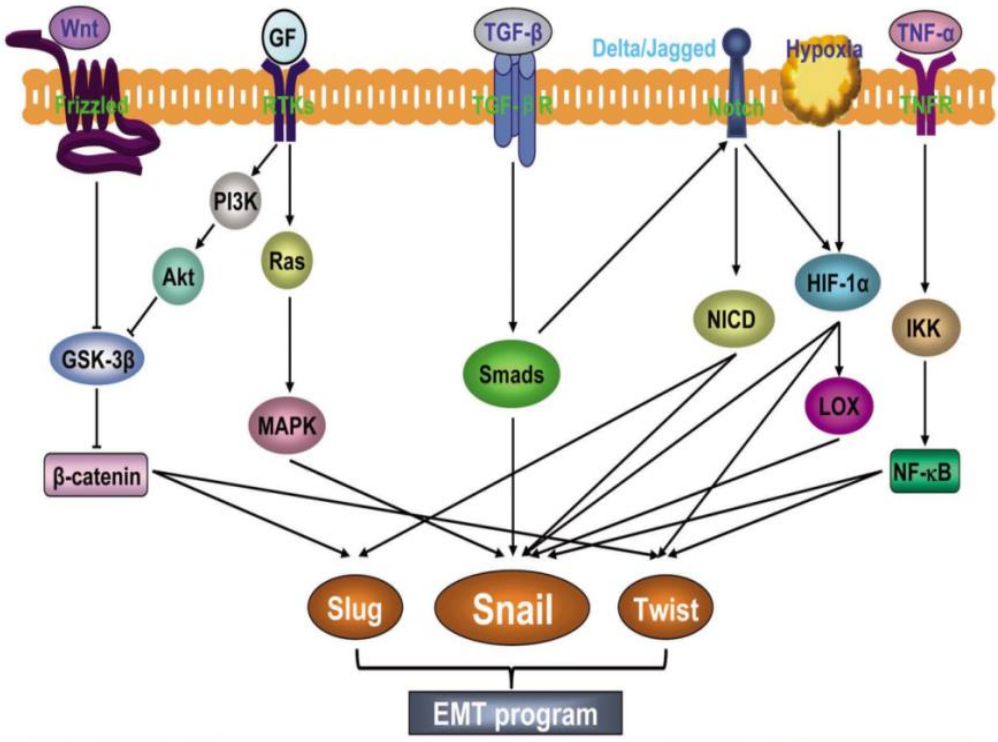


Figure 3- EMT involves an intricate interplay of multiple signaling pathways (5). Activation of different signaling pathways such as Wnt, growth factors (GF), TGF-β, Notch, HIF1α and TNF-α induces expression of transcription factors such as Slug, Snail and Twist to promote EMT.

A complex network of signaling pathways governs EMT in tumor progression. Both soluble factors (TGF-β family members, FGF, HGF, Wnt, TNFα, Notch, HIF1-α), as well as non-soluble components of the extracellular matrix (collagen and hyaluronic acid) guide epithelial mesenchymal transition during cancer progression (58, 59). These signaling events primarily induce EMT by inducing transcription factors such as Snail family of zinc finger transcription factors (Snail 1, Snail 2), zinc-finger-enhancer binding protein (Zeb-1 and 2) and basic helix loop helix (bHLH) family members (E12, E-47, and Twist) **(Fig.3)**. A common feature of these transcription factors is repression of the *CDH1* gene that encodes E-cadherin (16). Reduced E-cadherin expression is a key initial step in the trans-differentiation of epithelial to a mesenchymal phenotype, invasion and metastasis.

TGF-β signaling pathway in EMT. TGF-β is one of the most important EMT-inducing factors in a diverse range of tumor cells, including pancreatic cancer cells (60). The canonical TGF-β signaling proceeds as follows. Binding of TGF-β to a TGF-β type II receptor leads to the trans-activation of type I receptor (TβR I). TβR I is a serine/threonine kinase that subsequently phosphorylates SMAD2 and 3, which then forms a complex with SMAD4 and translocates to the nucleus to regulate the transcription of target genes (58). Transcriptional activation of Twist, Snail, Slug, and Zeb-1 has been shown to be critical for TGF-β mediated EMT induction in a majority of cancers (61).

In pancreatic cancer with SMAD4 inactivation, TGF-β might induce EMT through the non-canonical pathway (i.e. SMAD-independent pathway) (58), which involves ERK/MAPK, PI3K, p38, JNK, RhoA, and other signaling pathways (62). In some pancreatic cancer cell lines such as Colo-357, depletion of SMAD4 using RNAi did not disrupt EMT responses in these cells (63).

In other pancreatic cancer cell lines, TGF-β induced EMT was reversed by the MEK-1 inhibitor PD98059 (60).

Wnt/β-catenin signaling pathway. The canonical Wnt/β-catenin signaling pathway proceeds in the following manner. In the absence of Wnt ligand, β-catenin is kept low by a destruction complex which consists of axin, adenomatous polyposis coli, glycogen synthase kinase-3β (GSK-3β) and casein kinase (CK-1). CK-1 first phosphorylates β-catenin at Ser45, which primes β-catenin. Primed β-catenin is phosphorylated by GSK-3β, at Thr41, Ser33 and Ser37, ultimately leading to ubiquitination and proteosomal degradation by β-Trcp. This persistent removal of β-catenin prevents β-catenin nuclear accumulation. Therefore, Wnt target genes are repressed by DNA bound T cell factor (TCF)/lymphoid enhancer factor (LEF) and HDAC. Wnt ligand binding to Frizzled and LRP5/6 receptors complex leads to phosphorylation of LRP5/6. Phospho LRP5/6 binds axin leading to dissociation of the destruction complex and inactivation of GSK-3β, thereby stabilizing cytosolic β-catenin and promoting its nuclear localization. In the nucleus β-catenin forms a complex with TCF/LFE and activates expression of Wnt target genes such as cyclin D1 and c-MYC (64).

The Wnt/β-catenin pathway is associated with EMT induction (65). GSK-3β phosphorylates Snail, and promotes Snail proteosomal degradation. Wnt suppresses the activity of GSK-3β and stabilizes the protein levels of Snail, and therefore induces EMT and stem-like properties in cancer cells (66). Oncogenic K-Ras activate the Wnt/β-catenin signaling pathway, which in turn up-regulates EMT stimulators such as Snail and Zeb-1 (67). Inhibition of the Wnt/β-catenin signaling pathway blocks EMT. Restoration of the Wnt inhibitory factor 1 (WIF-1) expression resulted in increased expression of epithelial markers and decreased expression of mesenchymal markers, by decreased expression of Slug and Twist (68). Small hairpin RNA knockdown of β-

catenin resulted in elevated expression of E-cadherin, decreased expression of the mesenchymal markers vimentin, N-cadherin, MMP-2, indicating the reversal of EMT (69).

Notch signaling pathway. The Notch-signaling pathway plays an important role in the development of organs, tissue proliferation, differentiation and apoptosis (70). To date, four Notch receptors (Notch1-4) and five Notch ligands (Delta-like 1, 3, 4 and Jagged-1 and 2) have been discovered. Notch signaling is activated when the Notch ligand binds to an adjacent receptor. Upon activation, Notch is cleaved through a cascade of proteolytic cleavages by the metalloproteases, tumor necrosis factor α-converting enzyme and γ-secretase. Gamma secretase complex releases an active fragment called Notch intracellular domain (NICD). NICD translocates to the nucleus and then binds to the transcription factor CSL (CBF1, Suppressor of Hairless, and Lag-1). The CSL-NICD complex recruits a co-activator complex containing p300 and other co-activators leading to the activation of Notch target genes that are important in the regulation of cell growth, proliferation and apoptosis (eg. Akt, Cyclin D1, c-Myc, COX-2, MMP-9, ERK, mTOR, NF-kB, p21, p27, p53, VEGF) (70, 71).

Recently, the Notch signaling pathway has been found to directly upregulate Snail-1 and Slug thereby inducing EMT (72). Inhibition of Notch 1 signaling in MD-MBA-231 breast cancer cells using a monoclonal antibody resulted in EMT inhibition (73). In pancreatic cancer cells, knockdown of Notch-2 or midkine (a downstream target of Notch-2) also resulted in EMT inhibition (74). Supporting these results, NICD was found to promote EMT with increased Snail and vimentin expression, while silencing of NICD reversed this phenotype in lung cancer cells (75).

In addition to the above mentioned signaling pathways, several others, such as NF-κB(76), growth factor (77), and TNF-α (78), are also implicated in EMT induction in various carcinomas. The end result of activation of all these signaling pathways is to induce the expression of EMT transcription factors (Snail, Slug, Zeb-1, Twist), which in turn decreases expression of epithelial markers and increases the expression of mesenchymal markers.

1.2.3. Epithelial to mesenchymal transition transcription factor

EMT is governed by master regulators such as the Snail family of zinc finger transcription factors, the bHLH transcription factors and the zeb family transcription factors. Together with other co-repressors and co-activators, these master regulators decrease expression of epithelial markers and increase expression of mesenchymal markers during EMT in development and in cancer progression. The mechanism of such dual regulation of target genes by these transcription factors is only partially understood (79, 80).

Snail transcription factors. Snail-1 (Snail), Snail-2 (Slug), and Snail-3 (Smuc) belong to the Snail family of zinc finger transcription factors. Of these, Snail and Slug activate EMT during development and pathological conditions. All these transcription factors contain highly conserved carboxy terminal Cys2His2 (C_2H_2) type zinc finger motifs. Amino terminuses of these transcription factors are divergent in nature. The Snail1/GFI domain at the amino terminus of these transcription factors is required for protein stability and transcriptional repression of target genes (81).

Snail and Slug are key mediators of EMT in pancreatic tumor progression. Moderate to strong Snail expression was found in 78% of pancreatic cancer cases while Slug was expressed in 50% of the cases at a lower level than Snail (82). Snail expression levels in pancreatic cancers

correlated with lymph node invasion and distant metastasis (83). In an orthotopic transplantation model, Snail-transfected pancreatic cancer cell lines displayed highly metastatic and invasive abilities. Snail enabled these pancreatic cancer cell lines to undergo EMT at the invasive front of the tumor (83, 84).

Mechanisms of EMT regulation by Snail have been studied extensively. Snail acts as a repressor of genes involved in the maintenance of epithelial phenotype (E-cadherin, occludin, claudin, and cytokeratin-18) with the key target being E-cadherin. On the other hand, Snail activates the expression of mesenchymal genes such as vimentin, fibronectin, and N-cadherin. Snail also has been shown to regulate expression of genes involved in cell polarity (Crumbs3, Lgl2 and dlg3) and apoptosis (P53, BID and DFF40) (85). Among these, E-cadherin is the only known direct target of Snail (82, 86).

The mechanism of E-cadherin repression by Snail is a complex process and it involves several co-repressor proteins. Epigenetic modification of the chromatin structure at the promoter region of *CDH1* gene is the major regulatory event. The C_2H_2 type zinc finger domain allows sequence-specific binding of Snail to the E-box in the proximal promoter region of the *CDH1* gene, which encodes E-cadherin. Upon binding to the E-box region, Snail recruits co-repressor complexes such as histone deacetylases (HDAC)1/2/Sin3 (86) and polycomb repressive complex 2 (PRC-2) (87). HDAC1/2/Sin3 caused deacetylation of H3 and H4 at the *CDH1* promoter resulting in decreased expression of E-cadherin (86). The PRC2 complex catalyzes histone 3 lysine 27 trimethylation, which is a hallmark of epigenetically silenced chromatin, resulting in decreased expression of E-cadherin (87).

Pharmacological agents that target Snail have been an attractive strategy to inhibit EMT and cancer cell metastasis. A cobalt (III) Schiff base complexe ($[Co(acacen)(NH_3)_2]^+$) has been shown to inhibit C_2H_2 zinc finger transcription factors non-specifically. To enhance the specificity, an E-box DNA oligonucleotide specifically targeting Snail was attached to the acacen equatorial ligand (88, 89). This Co(III)-E-box complex was a potent inhibitor of Snail-mediated transcriptional repression in breast cancer cells and in the neural crest of *Xenopus* (90). However, this complex has not been tested on pancreatic cancer cell lines or in a mouse model system.

Zeb transcription factors. The zinc-finger-enhancer binding protein (Zeb) family of transcription factors (Zeb 1 and Zeb2) are one of the best studied EMT inducing transcription factors (91). In PDAC patients, both pancreatic cancer cells and tumor associated stroma showed high level of Zeb-1 expression, and Zeb-1 expression was associated with poor prognosis. Inverse correlation between E-cadherin and Zeb-1 expression was observed in patient tumor samples, as well as in pancreatic cancer cell lines (92, 93). Zeb-1 has been shown to regulate tumor cell invasion and migration (94). Silencing Zeb-1 has been shown to reduce cell migration, tumorigenicity and tumor dissemination (93, 95, 96).

Zeb-1 has been shown to decrease transcription of key determinants of epithelial differentiation, cell adhesion and cell polarity genes (97). E-cadherin is a well-known direct target of Zeb-1. Zeb-1 decreases E-cadherin expression by recruiting HDAC-1/2 or Switch/sucrose non-fermentable chromatin remodeling protein BRG1 to the promoter region of *CDH-1* gene (93, 98).

A negative feedback loop between Zeb-1 and the microRNA-200 family has been shown in regulating EMT (99-101). Zeb-1 is a major target of miR-200c and miR-141, members of the miR-200 family. On the other hand, Zeb-1 strongly repress the expression of miR-200 family members (102). This suggests that Zeb-1 activates microRNA mediated feed forward loop that stabilizes EMT and promotes invasion of cancer cells. Pancreatic cancer patients with a high level of miR-200c expression had better survival rates than those with a low-level of miR-200c expression. In these patients, there also existed a strong correlation between the levels of miR-200c and E-cadherin expression (100, 103, 104).

Drugs that inhibit Zeb-1 function might have clinical relevance for pancreatic cancer patients. Unfortunately, effective small-molecule inhibitors of Zeb-1 are yet to be identified.

Basic helix loop helix transcription factors (bHLH). Among all the bHLH transcription factors studied, E12, E47, Twist 1 and Twist 2 have been shown to play important roles in EMT (105). E-12 and E-47 act as a repressor of E-cadherin expression and trigger EMT. The mechanism of E-cadherin repression by E-12/47 is not well understood (106). Inhibition of differentiation 1 might be required for E-47 induced EMT (107, 108).

Twist 1 and Twist 2 are major regulators of EMT during development and pathogenesis (109). Twist expression is either absent or very weak in tissue samples from patients with PDAC. Pancreatic cancer cell lines also (MiaPaCa-2, PANC-1, Capan-1, HPAF-2 and AsPC-1) showed no expression of Twist. However, hypoxic conditions induced Twist expression in MiaPaCa-2, PANC-1, Capan-1 and HPAF-2 cell lines (82). As pancreatic tumors are often under hypoxic conditions (110), Twist may play a role in the invasive behavior of pancreatic tumors.

Like Snail, Twist interacts with several co-factors to regulate the expression of genes involved in EMT. Both E-cadherin and N-cadherin are direct targets of Twist. Twist has differential effects on expression of E-cadherin and N-cadherin. It decreases E-cadherin expression but induces N-cadherin expression. The mechanism of such dual regulation of target gene expression by Twist is not completely understood, but it is known that Twist recruits the Set-8 methyl transferase (a member of the SET domain-containing methyltransferase family) to the promoter region of E-cadherin and N-cadherin and enhances H4K20 monomethylation (111). Recently, Fu and colleagues showed that Twist interacted with several components of the Mi2/nucleosome remodeling and deacetylase complex to repress the transcription of E-cadherin (112). Twist has also been shown to specifically interact with BRD4, a bromo domain and extra terminal domain protein, to regulate the expression of target genes. Inhibition of BRD4 and Twist interaction by small molecular inhibitors such as JQ1 and MS417 suppressed tumor growth in a few animal models (113).

1.3. Cancer stem cells (CSCs)

Despite recent advances in cancer treatment, many therapies still fail. This results in disease progression, recurrence and dismal survival. Recent studies have shown that cancer stem cells (CSCs) are the predominant factors that are responsible for tumor recurrence (114). In 1997 Bonnet and colleagues discovered for the first time that a minor subpopulation of acute myeloid leukemia (AML) cells expressing markers of normal hematopoietic stem cells presented the potential to propagate AML in immune deficient mice (115). It was therefore hypothesized that malignancy originated from mutated stem cells that were transformed to generate daughter cancer cells. These tumor-generating subpopulations of cancer cells were later termed stem-like cancer cells, or CSCs. Although the biology of CSCs needs to be further understood, the

existence of CSCs has been demonstrated in almost all kinds of hematological malignancies and solid tumors (116).

1.3.1. Defining properties of CSCs

CSCs share a lot of characteristic features with normal stem cells. Normal stem cells undergo a specialized cell division termed asymmetric cell division, where each stem cell generates one daughter cell with stem cell fate (self-renewal) and one daughter cell (progenitor cell) that is destined to differentiate. It is believed that CSCs also have the features of asymmetric cell division, where self-renewal capacity helps to maintain the number of CSCs within the tumor and its descendent progeny constitute the bulk of the tumor (117). Supporting this notion it has been shown that many pathways (Wnt/β-catenin, Sonic hedgehog, and Notch) that are classically associated with cancer may also regulate stem cell self-renewal (118).

Another most common characteristic feature shared by normal and CSCs is quiescence. Mammalian adult stem cells are predominantly in a quiescent, non-dividing G_0-state in the absence of any extracellular signals (119). CSCs are also characteristically quiescent, and the dormancy of this small population protects them from chemotherapeutic drugs that are developed to target actively dividing cells, resulting in cancer relapse (117). Late relapse after initial treatment in several malignancies is explained with the existence of dormant CSCs (120).

In addition to the above two characteristic features, CSCs exhibit unique features such as drug resistance and metastasis ability.

1.3.2. EMT drives CSC formation

The exact relationship between CSCs and metastasis is not clear yet. It might lie in the close association of CSCs with cancer cell EMT. EMT and CSCs share many signaling pathways,

such as Wnt/β-catenin and Notch signaling pathway. Induction of EMT generated cells with stem-like properties in HMLER breast cancer cells , either by over expressing Twist or Snail (121), or by inhibiting the expression of E-cadherin using shRNA (122). Harvey rat sarcoma viral oncogene homolog (H-Ras) overexpression induced EMT in human mammary epithelial cells (HMECs) and concurrently induced enrichment of $CD44^{+}CD24^{-}$ HMECs, which were considered as stem-like cells (123). Conditioned media from activated fibroblasts induced EMT and increased CSCs in PC-3 prostate cancer cells by releasing MMP-2 (124). In a mouse model, it was shown that circulating pancreatic cancer cells maintained a mesenchymal phenotype and exhibited stem cell properties (53). The circulating cancer cells are considered equivalent to stem-like cancer cells. Overexpression of other EMT inducers, such as FoxM1 and Notch-1, also resulted in enrichment of pancreatic CSCs (125).

1.3.3. Pancreatic CSCs are highly metastatic and resistant to chemotherapeutic drugs

CSCs not only possess self-renewal capacity, but also have the potential and the ability to invade and metastasize to secondary sites. Herman and colleagues showed that the subset of $CXCR4^{+}$ $CD133^{+}$ pancreatic CSCs exhibited strong migratory potential and showed liver metastasis in an orthotropic model system. However, $CXCR4^{+}$ $CD133^{-}$ cells representing the non-cancer stem cell population failed to undergo liver metastasis (126). Inhibition of c-Met, a marker of pancreatic cancer CSCs, using cabozantinib (a small-molecule inhibitor of the tyrosine kinases c-Met), decreased tumor growth and metastasis (127).

CSCs play an important role in cancer drug resistance, which often impairs the successful use of chemotherapies (126, 128, 129). The mechanism by which CSCs become drug resistant is largely unknown, but it is very likely that ATP binding cassette (ABC) drug transporters are involved. Wang et al. showed that CSCs gain drug resistance partly due to over expression of ABCG2

(BCRP) (130). ABCG2 pumps cytotoxic drugs out of cancer cells resulting in protecting tumor cells from chemotherapeutic drugs (131). In another study, Hong and colleagues found that $CD44^+$ CSCs that were expanded during the acquisition of gemcitabine resistance, showed significantly elevated levels of ABCB1 (MDR1). The ABC transporter inhibitor, verapamil, resensitized the resistant cells to gemcitabine (132). It is also very likely that CSCs drug resistance is caused by both intrinsic and acquired properties such as quiescence, detoxifying enzymes, DNA repair ability and overexpression of anti-apoptotic proteins (133). Understanding the biology of drug resistance related to CSCs is necessary and would provide clues to design novel therapeutic options.

1.3.4. Markers used to study pancreatic CSCs

Several cellular markers or their combinations have been used to identify pancreatic CSCs. These include aldehyde dehydrogenase (ALDH) (134, 135), CD133 (136) and the combination of $CD133^+$ $CXCR4^+$ (CXC chemokine receptor 4) (126). Li et al. proposed usage of $CD44^+$ $CD24^+$ $EpCAM^+$ as markers for pancreatic CSC because $CD44^+$ $CD24^+$ $EpCAM^+$ pancreatic cancer cells showed properties of self-renewal and the ability to produce differentiated progeny (137). Other markers such as c-Met (127), doublecortin-like kinase 1 (138), and CD44v6 (139) were also proposed as putative pancreatic CSC markers. Although none of the proposed markers definitively identify a pure population of pancreatic CSCs, these markers are used widely because they provide consistent data for a strong enrichment of pancreatic CSCs.

1.3.5. Assays to measure CSCs

The gold standard assay to measure the self-renewal and lineage capacity of CSCs is serial transplantation in animal models. Cells are injected into the orthotopic site of NOD/SCID mice which are then assayed at different time points for tumor formation. Cells isolated from the

tumor are xenografted into a second recipient animal to measure the self-renewal capacity. However, this serial transplantation model is costly and time consuming, and is therefore not feasible to be used in drug screening (140).

An *in vitro* spheroid formation assay has been widely used to determine the self-renewal capacity of CSCs. The assay is based on the principle that only CSCs survive in suspension culture with non-stem cells die by anoikis. The ability to form several generations of spheres in serial non-adherent passages is related to the self-renewal ability of CSCs. For example, the $CD44^+CD24^+EpCAM^+$ pancreatic CSCs formed pancreatospheres *in vitro*, whereas $CD44^-$ $CD24^-EpCAM^-$ cells did not. The $CD44^+CD24^+EpCAM^+$ pancreatospheres can be passaged multiple times without loss of the tumor sphere-forming capability (141).

1.3.6. Interplay of multiple cellular signaling pathways triggers pancreatic CSCs

The understanding of the molecular mechanism by which CSCs are formed will benefit not only basic research but also clinical cancer therapy. Recent studies have demonstrated that the formation of CSCs can be triggered and maintained by the interplay of multiple cellular signaling pathways, including Notch, Hedgehog, and Wnt/β-catenin (142).

Notch-signaling pathway plays an important role in maintaining CSC population. Pancreatic CSCs expressed considerably higher levels of Notch-1 than the rest of the cancer cell population (143). Notch-1 over expression in pancreatic cancer cell lines induced EMT and increased the formation of pancreatospheres, which are indicative of CSCs (144). Activation of Notch pathway with an exogenous Notch peptide ligand increased the percentage of CSCs (145). However, further investigation is required to elucidate the molecular mechanism that explains how Notch signaling regulates pancreatic CSC self-renewal.

Hedgehog signaling pathway in maintaining CSC population. The Hedgehog (HH) pathway plays a critical role in the processes of embryonic development and maintenance of CSCs (146). The HH signaling pathway consists of HH ligands (Sonic HH, SHH; Indian HH, IHH; and Desert HH, DHH), Patched proteins (Patched-1 and 2), Smoothened (Smo), inhibitory complex (Fused, Suppressor of Fused) and the 5-zinc-finger transcription factors, Gli1, Gli2 and Gli3. In the absence of HH ligand, Patched-1 and 2 suppresses Smo. In the presence of HH ligand, inhibition of Smo by Patched is released, providing a signal for the dissociation of Gli transcription factor from the inhibitory complex. Dissociated Gli translocates to the nucleus and regulates the transcription of target genes such as Cyclin D1, N-Myc and p21, Wnt, Patched and Gli itself (147, 148).

Deregulation of HH signaling has been observed in most cancers (149). Transgenic mice with overexpression of SHH in pancreatic epithelium developed PanIN lesions, suggesting that SHH might be an early mediator of pancreatic cancer tumorigenesis (149). Two independent studies showed that pancreatic CSCs had more than 40-fold higher SHH expression than the non-CSC population (137, 150), suggesting that SHH might maintain CSC self-renewal. Slow cycling stem-like cells that exhibited higher tumorigenic potential had up-regulation of HH and TGF-β signaling pathways (151). Therefore, inhibition of HH signaling might be an alternative therapeutic approach for pancreatic cancer patients.

Wnt/β-catenin signaling pathway in maintaining CSC population. Wnt/β-catenin signaling plays an important role in CSCs of a variety of cancers. For example, constitutively activated β-catenin signaling in stem cells is essential for intestinal neoplasia (152). Wnt-1 overexpression enhanced the sphere formation capacity of the gastric cancer AGS cell line (153). The canonical β-catenin signaling pathway is the most significantly deregulated signaling pathway in

glioblastoma stem cells (154), it is also required for self-renewal of leukemia stem cells that were derived from either hematopoietic stem cells or from granulocyte-macrophage progenitors (155). The roles of Wnt/β-catenin signaling in pancreatic CSCs deserve further investigation.

1.4. Current Treatment for pancreatic cancer

Current treatment for pancreatic cancer is far from satisfactory. As advancements in molecular and targeted therapies have greatly improved survival of patients with many types of cancers, the treatment outcome for pancreatic cancer has not changed much over the past 30 years. In the last a few decades of the 20th century, five fluoro uracil (5-FU) was the standard of care for pancreatic cancer patients (156). In 1997, a randomized phase 3 study demonstrated a survival benefit for gemcitabine (2′, 2′-difluoro 2′-deoxycytidine) over 5-FU (157). Since then, gemcitabine as mono-treatment has been the standard of care for pancreatic cancer patients. Gemcitabine, is an analog of cytosine exhibited distinctive pharmacological properties (with multiple intracellular targets such as DNA polymerase, ribonucleotide reductase, cytidine triphosphate synthetase, deoxycytidylate deaminase) and a wide spectrum of anti-tumor activity (158). However, gemcitabine has little impact on median overall survival for patients with locally advanced or metastatic pancreatic cancer, who comprise the majority of cases (159, 160). Many studies used gemcitabine in combination with other chemotherapeutic drugs intending to improve the median survival in pancreatic cancer patients. However, the improvement has not been satisfactory. Gemcitabine in combination with either capacitance (prodrug of 5-FU, a pyrimidine analog) (161) or platinum drugs (cisplatin, carboplatin) (162, 163) did not result in significant improvement in overall survival. A combination of gemcitabine and nab-paclitaxel (164) improved overall survival, progression free survival and response rate compared to

gemcitabine treatment alone. However, peripheral neuropathy and myelosuppression were significantly increased (165).

Surgical resection is the only curative therapy for pancreatic cancer patients with localized disease (166). However, only 15–20% of patients have resectable disease at the time of diagnosis. Patients who undergo surgery (Whipple procedure) have a perioperative mortality of 4-18% and an additional risk of post operational complications (4, 167, 168). Furthermore, the majority of the patients have locally invasive and micro metastasis at the time of surgery. Therefore, disease recurrence following operation is very high. Adjuvant therapy (5-FU or gemcitabine-based chemoradiation) is specified to decrease the risk of loco-regional and metastatic recurrence (169).

Recently, FOLFIRINOX (oxaliplatin, irinotecan, fluorouracil, and leucovorin), as the first gemcitabine free regimen, has been shown to be more efficient than gemcitabine. The median overall survival was 11.1 months in the FOLFIRNOX group as compared with 6.8 months in the gemcitabine group. Side effects of this new combination regimen were significant, including grade 3 and 4 neutropenia, suggesting a limited use for patients with good performance status (170).

1.5. Investigational chemotherapies for pancreatic cancer

Pancreatic cancer is a multifactorial complicated disease. Conventional chemotherapies or radiation therapies often yield disappointing results and are largely ineffective in depleting CSCs, which are responsible for disease recurrence. Therefore, new innovative treatment options need to be tested. As the understanding of pancreatic cancer biology and pathophysiology increases, several novel approaches for targeted therapies are currently under investigation. In

the following paragraphs some of the investigational therapies that have potential clinical success for pancreatic cancer patients are summarized.

1.5.1. Targeting K-Ras

K-Ras is an attractive therapeutic target because it is mutated in the vast majority of PDAC cases. An ideal mechanism to prevent K-Ras signaling would be to directly block the GTP-binding site of K-Ras. However, there has not been an effective small-molecule inhibitor identified. Alternatively, other approaches have been investigated to 1) block membrane localization of K-Ras, 2) block Son of Sevenless (SOS)/K-Ras interactions, and 3) block K-Ras downstream effector targets such as PI3K, Raf, MEK 1/2 and Akt (171).

Blocking K-Ras membrane localization. Membrane localization of K-Ras brings it into contact with Ras activating protein to activate downstream signaling (172). Following translation, K-Ras undergoes a lipid modification called farnesylation and/or geranylgeranylation (together referred as prenylation). Phosphodiesterase delta (PDEδ) is a prenyl binding protein and interacts with prenylated K-Ras to aid K-Ras to translocate to the membrane (173, 174). It was hypothesized that inhibition of farnesylation and or geranylgeranylation would prevent K-Ras membrane localization, thus inhibiting its signaling pathway. A number of inhibitors for farnesyl transferase (FTIs) and geranylgeranylation transferase (GGTIs) were synthesized and tested for their anti-tumor activity (175-180). At least six FTIs have been tested in clinical trials, but unfortunately have predominantly proven unsuccessful for K-Ras driven tumors (181-183). A geranylgeranylation transferase inhibitor, GGTI-2418 is currently in phase-1 clinical evaluation (http://www.prnewswire.com/news-releases/first-patient-dosed-in-phase-i-clinical-trial-of-tigris-pharmaceuticals-ggti-2418-61683932.html). The combination of FTI and GGTI induced greater apoptotic response in cancer cells than a single agent alone, but high toxicities limited their

clinical use (184). Deltarasin, inhibited PDEδ and K-Ras interactions and K-Ras membrane localization, suppressed *in vitro* and *in vivo* proliferation of PDAC cells (185). Salirasib (S-trans, trans-farnesylthiosalycilic acid) dislodged K-Ras from the membrane thereby promoting K-Ras degradation. In a patient derived xenograft model, salirasib showed a wide range of activity and showed a heightened tumor response when combined with gemcitabine (186). Salirasib is under clinical investigation (186).

Blocking SOS/K-Ras interactions. At the membrane, K-Ras is activated by SOS (guanine nucleotide exchange factor), which aids in K-Ras binding to GTP. Blocking these interactions potentially inhibits K-Ras signaling pathway (187). Using an NMR based fragment-screening approach, Maurer and colleagues identified 4, 6-dichloro-2-methyl-3-aminoethyl-indole as a small-molecule compound that blocked the interaction between K-Ras and SOS and inhibited the nucleotide exchange (188). Later, several other compounds were discovered which were shown to bind GDP-bound K-Ras^{G12D} and to inhibit SOS catalyzed nucleotide exchange (189). Anti-cancer activities of these compounds need to be investigated in preclinical and clinical studies.

Blocking K-Ras downstream effector targets. Effector pathways are activated downstream of K-Ras in a context- and tissue-specific manner. MAPK and PI3K-Akt signaling pathways are the two most commonly activated signaling pathways in pancreatic cancer. Efforts have been made to develop pharmacological agents targeting MAPK and PI3K-Akt signaling pathways. The MAPK pathway consists of a kinase cascade, where K-Ras activates Raf kinases, which in turn activate MEK1/2 (187). In preclinical models, MEK inhibitor PD325901 reduced tumor burden and prolonged survival time and showed a synergistic effect with Akt inhibitor GSK690693 (190). In a patient derived xenograft model, another MEK inhibitor, trametinib (GSK1120212) reduced tumor mass. Trametinib (MEK inhibitor) and lapatinib (EGFR/HER2 inhibitor)

combination treatment resulted in significantly enhanced inhibition of tumor growth compared to trametinib alone (191). PI3K and Akt inhibitors were also tested in preclinical models and showed potent anti-cancer activity (17, 190). These results suggest that blocking K-Ras downstream targets is a potential therapeutic option for pancreatic cancer patients who harbor *K-Ras* mutations.

Utilizing high-throughput screening strategies, researchers have made efforts to develop drugs that specifically kill cells expressing mutant K-Ras. Several high-throughput screening assays have been performed using cells harboring *K-Ras* mutation and its isogenic cells without *K-Ras* mutation. Compounds showing selective lethality against K-Ras mutant transformed cells were identified, including sulfinyl cytidine and its derivative triphenyltetrazolium (192), oncrasin-1 (193), tolperisone and its derivative lanperisone (194), and SLI501 (195). These compounds are under preclinical and clinical testing. Success of the clinical studies on these compounds would change the shape of pancreatic cancer treatment.

1.5.2. Histone deacetylase (HDAC) inhibitors.

A growing body of literature suggests that deregulation of histone deacetylases (HDACs) could contribute to pancreatic cancer development and progression (196-199). HDACs play critical roles in the epigenetic regulation of gene expression by catalyzing the removal of acetyl groups from histones, causing compaction of the DNA/histone complex. This compaction blocks gene transcription and inhibits differentiation (200, 201). In eukaryotic cells, 18 different HDACs have been identified and are classified into 4 groups based on their homology to yeast proteins. Class I includes HDAC 1, 2, 3, and 8 and are exclusively located in the nucleus. Class IIa includes HDAC 4, 5, 7 and 9 and are localized to the cytoplasm. HDAC 6 and 10 belong to class IIb and HDAC 11 is classified in class IV. HDAC 11 is localized in both cytoplasm and nucleus.

Classes I, II, and IV HDACs are Zn-dependent enzymes. Class III HDACs include sirtuins, which are NAD^+ dependent enzymes and have homology to yeast Sir2 (201-203). Overexpression of HDAC isoenzymes has been observed in several cancers (204). In pancreatic cancer, overexpression of HDAC 1, (198), HDAC 2 (196), and HDAC7 (197), and HDAC 3 (199) were observed. As described before, HDACs also involve in Snail mediated EMT process. Thus, targeting HDACs by inhibitors could be a promising strategy for pancreatic cancer treatment.

To date, more than 15 HDAC inhibitors have been tested in preclinical and early clinical studies. Based on the chemical structure, HDAC inhibitors are classified in into 4 different classes: hydroxamates (e.g. suberanilohydroxamic acid (SAHA) and panobinostat), cyclic peptides (e.g. romidepsin), aliphatic acids (e.g. sodium butyrate, valproic acid and phenyl butyrate) and benzamides (e.g. entinostat or known as MS-275 and mocetinostat) (201). The Food and Drug Administration approved SAHA for the treatment of cutaneous T-Cell Lymphoma (CTCL). In animal models, SAHA in combination with bortezomib (proteasome inhibitor) or Zebularine (nucleoside analog of cytidine) reduced pancreatic tumor weight with minimal noted toxicity (205, 206). SAHA also reduced migration, colony formation and sphere formation ability of pancreatic CSCs. Specifically, SAHA inhibited the expression of Zeb-1, Snail, and Slug, suggesting that SAHA inhibited EMT in pancreatic CSCs. Furthermore, SAHA inhibited the Notch signaling pathway by upregulating miR-134 (207). Other HDAC inhibitors like MS-275, TSA and FK228 showed potent anti-tumor effects both *in vitro* and *in vivo* (208). However, all these HDAC inhibitors so far showed limited or no efficacy in clinical trials in solid malignancies including PDAC, when used as monotherapy (209-211), or in combination with other chemotherapeutic drugs (212-215).

The lack of effects might partially come from the non-specificity of current HDAC inhibitors. Majority of the HDAC inhibitors in clinical trials inhibit all HDAC isoforms nonspecifically (pan inhibitors). Such nonspecific inhibition leads to many toxic side effects that limited the dose that can apply (216). Selective HDAC inhibitors, which affect a single HDAC isoform, would be ideal chemical tools to elucidate the functions of each individual HDAC isoform. Also, development of class-selective HDAC inhibitors might provide more effective HDAC inhibitors than the pan HDAC inhibitors.

1.5.3. Targeting pancreatic CSCs for therapy.

Reactivation of developmental signaling pathways (Notch, SHH) is involved in the formation of CSCs (217). Inhibitors of these signaling pathways could be valuable tools to target CSCs.

Hedgehog (HH) inhibitors. Several small-molecule HH inhibitors are under preclinical and clinical development (218). For example, the HH inhibitor cyclopamine inhibited pancreatic cancer cell EMT, CSCs, and reduced metastasis in an orthotopic xenograft mouse model (219). Cyclopamine down regulated the expression of CSC markers CD44, and CD133 in gemcitabine resistant pancreatic cancer cells and restored gemcitabine sensitivity (220). In combination with the mTOR inhibitor, rapamycin, cyclopamine reduced the number of pancreatic CSCs to undetectable levels *in vitro* and *in vivo* (221). IPI-269609 and GDC-0449, two other small-molecule inhibitors of the HH signaling pathway, also effectively depleted pancreatic CSCs (222, 223). Recently, Balic and colleagues found that the anti-malarial agent chloroquine (CQ) significantly decreased pancreatic CSCs by inhibiting the HH signaling pathway (224). They further showed that CQ in combination with gemcitabine improved the overall survival of mice bearing PDAC patient derived xenografts.

However, these preclinical results were not recapitulated in clinical trials. A single arm pilot study in 25 metastatic PDAC patients was conducted to study the effect of GDC-0449 plus gemcitabine. The treatment resulted in inhibition of the HH signaling pathway, without significant changes in CSCs, fibrosis, progression free survival and overall survival. However, grade ≥3 toxicities in 56% of patients were observed. This pilot study concluded that GDC-0449 and gemcitabine were not superior to gemcitabine alone in the treatment of metastatic pancreatic cancer (225). Similarly, a double-blind, randomized phase 2 study, IPI-926 plus gemcitabine or gemcitabine plus placebo, showed no improvement in overall survival (http://www.businesswire.com/news/home/20120127005146/en/Infinity-Reports-Update-Phase-2-Study-Saridegib#.VXGcwcvwvcs). A phase 2 GDC-0449 plus gemcitabine or gemcitabine plus placebo study also showed no statistical improvement in progression free survival and overall survival (https://clinicaltrials.gov identifier: NCT01064622).

Reasons for the failure of HH inhibition in PDAC patients were explored using a genetically engineered mouse model (GEMM) (226). In this GEMM, either loss of SHH or pharmacological inhibition using IPI-926 was associated with decreased survival, higher frequency of gross metastasis. SHH deficient tumors had reduced stromal content, and increased blood vessel density (226). Therefore, SHH inhibitors warned for further clinical use in PDAC patients.

Notch inhibitors. Gamma secretase activates the Notch signaling pathway. Hence γ-secretase inhibitors (GSI) are attractive inhibitors for CSCs. The GSI MRK-003 effectively inhibited intratumoral Notch signaling and prolonged survival of tumor bearing mice when combined with gemcitabine (227). Pretreatment of PDAC cells with MRK-003 in cell culture significantly inhibited pancreatic CSCs and subsequent tumor formation in immunocompromised mice (228). PF-03084014, a selective GSI, was also shown to induce pancreatic tumor regression by

depleting CSCs (229). Inhibition of Notch singling pathway using either GSI (RO4929097) or Hes1 ShRNA reduced both *in vitro* sphere formation and *in vivo* tumor growth of orthtopic pancreatic tumors (145). A Phase 2 study was completed recently using RO4929097 in previously treated metastatic pancreatic cancer patients and showed no potential advantage over gemcitabine (https://clinicaltrials.gov identifier: NCT01232829). PF-03084014 recently entered into a phase 1/2 study in combination with gemcitabine and Nab-paclitaxel in patients with previously untreated metastatic PDAC (https://clinicaltrials.gov identifier: NCT02109445). A phase 3 study of gemcitabine plus Nab-paclitaxel combination showed slight improvement in survival (8.5 months, statistically significant) compared to gemcitabine (6.7 months) (165). Adding PF-03084014 to this combination is in hope to further improve the survival.

Other potential approaches to target pancreatic CSCs. Targeting the cell-surface antigens that are characteristic to CSCs is an attractive strategy to eliminate pancreatic CSCs. Specific monoclonal antibody targeting CD44, eliminated leukemic stem cells by inducing terminal differentiation (230). Inhibition of c-Met, a marker of highly tumorigenic CSCs has been found to inhibit tumor growth and metastasis (127). A bispecific antibody that recognizes both epithelial surface antigen (EpCAM) and CD3 has been shown to eliminate pancreatic CSCs (231). Monoclonal antibodies targeting CD44, EpCAM and c-Met needs to be tested in clinical trials.

The anti-cancer agents from diet or natural plants (e.g. genistein, curcumin, resveratrol) have been found to inhibit CSCs self-renewal through modulation of important signaling pathways, thus leading to inhibition of pancreatic cancer development and progression (232). Genistein, one of the isoflavones found in soybeans, strongly inhibited the growth of leukemic hematopoietic progenitor cells and ovarian CSCs , and inhibited the pancreatospheres *in vitro* by

down regulating the Notch signaling pathway (144, 233-235). In another study, Bao and colleagues also reported that genistein inhibited EMT, which is highly associated with CSCs (125). Curcumin, a bioactive compound found in the *Curcuma longa*, can inhibit CSCs in various human cancers (236, 237). Curcumin and 3, 4-difluoro-benzo-curcumin, a novel synthetic analog of curcumin, inhibited the sphere formation ability of pancreatic cancer cell lines and attenuated the pancreatic CSC markers CD44 and EpCAM (238). Curcumin was reported to decrease the CSC population by targeting the histone methyl transferase, enhancer of zeste homolog 2 miRNA regulatory circuit (239). Resveratrol showed a potent anti-cancer effect on various types of cancers, including pancreatic cancer (240) by modulating Src, STAT3, and FOXO (241, 242). Resveratrol inhibited the self-renewal capacity of pancreatic CSCs derived from K-Ras transgenic mice and from human primary tumors. Moreover, resveratrol decreased the expression of pluripotency maintaining factors such a Sox-2, c-Myc, Nanog and Oct-4 in pancreatic CSCs and induced CSCs apoptosis through activation of caspase-3/7 (243).

Clinical studies have been carried out on some of these dietary compounds so far showed little benefit over gemcitabine treatment.

A phase 2 trials of curcumin in patients with advanced pancreatic cancer showed good tolerance and biological activity in 2 out of 21 patients. One patient had stable disease for >18 months. Another patient had marked tumor regression (73%) (244). Two other studies investigated curcumin in combination with gemcitabine in patients with advanced pancreatic cancer. The results suggested that curcumin and gemcitabine combination did not provide survival advantage (median survival 5.3 and 6 months in the two trials, respectively) (245, 246). A phase 2 study adding genistein to gemcitabine and erlotinib combination in advanced pancreatic cancer patients, resulted in no significant improvement than gemcitabine and erlotinib combination

(247, 248). As for resveratrol, studies so far indicated it is a promising cancer preventing agent instead of chemotherapeutic agent (249).

Taken together, many efforts have been made targeting various molecular pathways using either pharmacological agents or nutraceuticals to inhibit pancreatic cancer growth, metastasis and CSCs. Despite many preclinical advancements, it remains a huge challenge to find a clinically effective therapeutic agent. In this dissertation we used several novel approaches to target pancreatic cancer metastasis and CSCs.

Chapter 2. Statement of purpose

The purpose of this study is to investigate innovative and promising inhibitors of pancreatic cancer EMT. We hypothesized that identification of novel and potent compounds that inhibit EMT holds the potential to inhibit metastasis and CSCs in pancreatic cancer. We performed studies to test the central hypothesis through the following specific aims.

2.1. Specific aim 1: To investigate the mechanisms of high-dose parental ascorbate as a novel inhibitor for pancreatic cancer EMT. High dose ascorbate (Asc) is a widely used regimen in complementary and alternative medicine as a cancer treatment. The scientific basis for this use was recently revealed. In a PANC-1 orthotopic mouse model, ascorbate significantly reduced tumor progression, metastasis and final tumor weight. Consistently, a matrigel invasion assay demonstrated *in vitro* that ascorbate reduced PANC-1 cell migration and invasion. As EMT is highly associated with cancer cell metastasis, we investigated the effect of ascorbate on EMT regulation. We further studied the mechanisms of ascorbate-mediated regulation of pancreatic cancer cell metastasis, focusing on tumor microenvironment and tumor cell microtubule dynamics, which are also associated with cancer cell metastasis. Methods including western blot, real time PCR, native polyacrylamide gel electrophoresis, immunofluorescence, gelatin zymography, trichrome staining, and HPLC were used to achieve the goals of specific aim 1.

2.2. Specific aim 2: To investigate novel derivatives of HDAC inhibitors as inhibitors for pancreatic cancer EMT. HDAC inhibitors could inhibit EMT through histone modification. However, in patients with solid tumors, current HDAC inhibitors have not shown significant efficacy. Hence, the development of more efficacious and less toxic HDAC inhibitors for treating pancreatic cancer remains an important goal to improve anticancer therapy. The development of analogues and derivatives is a commonly used approach for new drug

development in order to improve potency, bioavailability, and to reduce toxicity. In the current study, we studied the efficacy and mechanism of action of novel derivatives of HDAC inhibitors SAHA and MS-275, using MTT assay, western blot, sphere formation assay, fluorescence activate cell sorter, Boyden chamber invasion assay, real time PCR, fluorescence polarization assay and amplified luminescent proximity homogeneous assay (Alpha) assay.

2.3. Specific aim 3: To establish a high-throughput screening method for identification of EMT and CSC inhibitors. Large scale screening for CSC inhibitors would be an efficient way for discovery of drugs targeting CSCs. However, to date there still lacks a highly efficient and feasible approach for such screening, because CSC only compromise a small portion of the bulk cancer cell population, and it is difficult to maintain an undifferentiated CSC culture. Loss of E-cadherin is a key initial step in the trans-differentiation of epithelial to a mesenchymal phenotype. In this study, we established an immunofluorescent high-throughput screening (HTS) assay to identify compounds that induce E-cadherin expression. . Based on the close relationship of EMT and CSC, we tested the hypothesis that this approach for identifying EMT inhibitors could be useful for discovery of CSC inhibitors. Chemical screening was conducted on PANC-1 cells utilizing 4 chemical libraries (Prestwick, MicroSource, ChemBridge and the University Of Kansas Center Of Excellence in Chemical Methodologies & Library Development) containing total of 41,472 small organic molecules with structural diversity and drug like properties to detect E-cadherin inducers. Positive hits were then validated for EMT inhibition in pancreatic cancer cell lines, using western blot, Boyden chamber invasion assay and scratch assay. Ability of the hits to inhibit pancreatic CSC was detected using spheroid formation assay.

Chapter 3. Materials and Methods

3.1. Cell culture and viability assay

Immortalized human pancreatic ductal epithelial cells (hTERT-HPNE) were provided by Dr. Shrikant Anant (University of Kansas Cancer Center, Kansas City, KS). Human pancreatic cancer cell line L3.6 was provided by Dr. Liang Xu (University of Kansas, Lawrence, KS). Mouse syngeneic pancreatic cancer cell line Pan02 was donated by Dr. Anthony Sandler (Children's National Medical Center, Washington, DC). Human lymphocytes were isolated by apheresis at Dr. Mark Levine's lab (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD). All other cell lines were obtained from the American Type Culture Collection (Manassas, VA). All cells were cultured in recommended media supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin at 37 °C in a humidified 5% CO2 atmosphere.

MTT (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used in determining cell viability as described (250). In short, Cells were plated onto 96 well plates at a starting density of 10,000 cells and treated with different chemical compounds as specified in each experiment. After incubation for indicated time in specific experiment, treatment media was subsequently removed and replaced with 100 μl of 0.5 mg/ml MTT solution in cell culture media and incubated for 2-3 hrs at 37 °C in a humidified 5% CO2 atmosphere. Cell viability was quantified by measuring the soluble purple color formazan crystals at an absorbance wavelength of 570 nm on kinetic micro-plate reader (BioTek, Winooski, VT).

3.2. Orthotropic mouse model for pancreatic cancer

All procedures were performed following the animal care and use protocol approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. PANC-1 cells were transfected with the lentivirus-expressing luciferase gene (PANC-1–Luc)

which was developed at Preclinical Proof of Concept Core Laboratory (University of Kansas Medical Center, Kansas City, KS). While under anesthesia, a small subcostal laparotomy was performed on 4-6 week old Female Ncr nu/nu mice, and then 2×10^5 PANC-1 cells injected into the tail of pancreas. Mice were imaged a week after cell implantation to monitor tumor formation. To image, each mouse was given 150 mg/kg D-luciferin by intraperitoneal injection. Animals were scanned using an IVIS imaging system (Waltham, MA). Mice were grouped based on initial tumor burden and then were treated with compounds specified. Mice were imaged longitudinally. Treatment typically lasted for 45 days. At necropsy, total tumor burden were weighed and metastatic lesions were counted. Tissue samples were fixed in formaldehyde or spot-frozen on dry ice and stored at -80^{o}C for further analysis.

3.3. RNA isolation, cDNA synthesis, and Real-Time PCR

Total RNA was extracted from cells or tissue samples by using TRIZOL reagent according to the protocol of the manufacturer (Invitrogen, Grand Island, NY). cDNA synthesis was performed with 1µg of total RNA using Omniscript RT kit according to manufacturer's protocol (Quiagen, Valencia, CA). cDNA was diluted 1:5 (or as indicated in specific experiment) in autoclaved nanopure water and used for further analysis. Real-time PCR was performed using Bio-Rad iQ iCycler detection system with iQ SYBR green supermix (Bio-Rad Laboratories Ltd, Hercules, CA). Reactions were performed in a total volume of 10 µl, including 5 µl of 2X iQ SYBR green supermix, 1µl of primers at 20 pmol/µl and 1 µl of cDNA template. All reactions were carried out in at least triplicates for every sample. Data were normalized to 18S rRNA.

3.4. Matrigel invasion assay

Cells were seeded into inserts of Boyden chambers (BD Biosciences, San Jose, CA) that were either pre or not coated with matrigel (0.1 mg/mL), at 1 x 10^4 cells per insert in 0.5% FBS

containing medium, unless otherwise described in specific experiment. Media in wells contained 10% FBS was used as chemo-attractor. After incubation for indicated time in specific experiment, cells that invaded to the bottom-side of the membrane were fixed with 4% para formaldehyde for 2 min, permeabilized with 100% methanol for 20 min, followed by staining with 0.05% crystal violet for 15 min at 37^{o}C. Non-invading cells on the top side of the membrane were removed by cotton swab. Photographs were taken from five random fields per insert. Cells in the five random fields were counted.

3.5. Masson's trichrome staining

Collagen content in the tumor and liver sections was detected by using Masson's trichrome stain kit (Sigma, St. Louis, MO) following manufacturer's protocol. The cytoplasm was stained a pink to red color and the collagen was stained blue.

3.6. Gelatin zymography

Supernatant media from cell culture was subjected to electrophoresis on 10% SDS poly acryl amide gel containing 0.2 % gelation (Sigma, St. Louis, MO). After adequate rinse in rinse buffer (1M Tris pH8.0, 1M CaCl2, 2.5% Triton X-100), the gel was equilibrated for 30 min in incubation buffer (1M Tris pH8.0, 1M CaCl2), and then was incubated in fresh incubation buffer at 37°C for 16 hrs. The gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA) for 1-2 hrs, and then destained in 10% methanol and 5% acetic acid. Areas corresponding to MMP activity appeared as clear bands against a dark blue background. The clear bands were analyzed by optical densitometry by Image J software.

3.7. Immunofluorescence and immunohistochemistry

Cells grown on 96 well plates were treated and then fixed in 4% paraformaldehyde, and blocked in blocking buffer (1X PBS+5% Goat serum+0.3%Triton X-100) at room temperature for 1hr.

Anti-acetylated α-tubulin (1:4000 dilution in 1XPBS+1% bovine serum albumin (BSA)+0.3% triton X-100) was incubated at 4^{o}C for overnight. Alexa flour 488-conjugated secondary antibody (1:500) was incubated for 2 hrs in dark. Nuclei were visualized with 1 mg/mL Hoechst33342.

Paraffin-embedded tumor sections (5 µM thick) were deparaffinized and rehydrated by serial incubation in xylene, 100%, 95% ethanol, and water. Endogenous peroxide was blocked with 3% hydrogen peroxide (H_2O_2) at room temperature for 10 min. Antigen retrieval was performed in boiling citrate buffer for 5min followed by sub boiling temperature for 10min. Anti-PCNA primary antibody (1:1000, Cell Signaling Technology, Beverly, MA) was incubated overnight at 4^{o}C. Biotinylated secondary antibody and DAB were used to develop the tissue sections (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA). All the sections were counterstained with hematoxylin.

3.8. Gene transfection

PANC-1 cells were engineered to overexpress a flag tagged HDAC6. Recombinant Plasmid pcDNA3.1+-HDAC6-flag and the empty vector pcDNA3.1+ were purchased from Addgene (Cambridge, MA). Plasmids were transfected into the PANC-1 cells with lipofectamine ^{TM} 2000 reagent (Invitrogen, Grand Island, NY) according to manufacturer instructions. G418 was used for selection of positive clones. Optimal dose of G418 was determined by MTT assay (IC_{50} = 0.625 mg/ml). Thirty-six hours after transfection, cells were passaged and were cultured in DMEM medium containing 10% FBS and 1 mg/ml G418. After 6 days, G418 was reduced to 0.5 mg/ml. G418 resistant clones were picked after two weeks and then expanded. Stable expression of HDAC6 in the clones was confirmed using western blot.

3.9. Native PAGE, SDS PAGE and western blot

Cells were lysed with RIPA buffer containing a protease inhibitor, centrifuged, and supernatant was used. BCA method was used for protein quantification (Pierce BCA protein assay kit, Waltham, MA). SDS-PAGE and Western blot was performed as routine. For native PAGE, 10 µg of protein was mixed with equal volume of 2× native sample buffer (Bio-Rad laboratories, Ltd, Hercules, CA), and were loaded onto 8% poly acryl amide gels without SDS. The electrophoresis was at 60V for 3.5-4 hour. Proteins were transferred to PVDF membrane for overnight at 4^{o}C. Dilutions for primary antibodies were anti-α-tubulin, anti-HDAC6 anti-vinculin, (1:1000, Cell Signaling Technology, Beverly, MA), anti-acetylated α-tubulin (1:5000), anti-Sirt-2 (1:500), anti-α-TAT (1:1000), anti-flag (1:1000) (Sigma Aldrich, St. Louis, MO). A goat anti-rabbit and anti-mouse polyclonal horseradish peroxidase conjugated secondary antibody (1:1000, Cell Signaling Technology, Beverly, MA) was used. Blots were established using a chemiluminescence detection kit (Pierce ECL western blotting substrate, Thermo Scientific, Rockford, IL).

3.10. Analysis of $CD44^{+}CD24^{+}EpCAM^{+}$ population in PANC-1 cells by flow cytometry

PANC-1 cells in 60 x 15mm type cell culture dish were scrapped, washed with PBS, and then blocked with staining buffer (DPBS that is supplemented with 0.2% (w/v) BSA, pH 7.4) for 10 min. Cells were triple-stained with phycoerythrin (PE)-conjugated anti-CD24, PE-Cy7 conjugated anti-CD44 and APC-conjugated anti-EpCAM antibodies (BD Biosciences, San Jose, CA) for 20 min on ice and then samples were washed with staining buffer thrice. Cells stained with individual antibodies were used as compensation controls. Live cells were analyzed

immediately with flow cytometry by using DAPI stain. PE-Cy7+ and APC+ subpopulation in the PE+ gated cells indicated $CD44^{+}CD24^{+}$ $EpCAM^{+}$ subpopulation.

3.11. Scratch assay

Confluent pancreatic cancer cells in 6-well plate were wounded manually by scraping the monolayer using a p1250 sterile pipette tip. Cells were washed immediately with media to remove debris. After taking the zero-hour time point pictures, cells were treated with compounds as indicated in specific experiments. Cells were then incubated at 37ºC in a humidified 5% CO_2 atmosphere. Wound closure was captured using an inverted microscope at 6, 12, 24, 30, 48 hrs under high-power field (100X magnification). The extent of wound closure was calculated as follows: (Distance of remaining wound gap × 100 ÷ Distance of original wound gap)-100 %.

3.12. Tumor spheroid formation assay

Single cell suspension was plated into 24 well ultra-low attachment plates (Corning Inc., Corning, NY) at a density of 4,000 cells/well in stem cell media and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Stem cell media consist of, DMEM supplemented with 1X B27 Supplement, 20 ng/ml human basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 100 units/ml penicillin/streptomycin (Invitrogen, Grand Island, NY), and 4μg/ml heparin calcium salt (Fisher Scientific, Pittsburg, PA). Primary spheroids were counted and collected after 10-14 days manually by looking under the microscope. Size of the spheroids was measured using Image J software. Primary spheroids were further trypsinized and then dissociated with 90^{0} blunt end pipetting needles (VWR, Radnor, PA) and then reseeded into ultra-low attachment plates for the secondary spheroids. After 10-14 days secondary spheroid number and sizes were measured.

3.13. Fluorescence polarization assay and Alpha assay

pTBSG-HuR and pTBSG-RRM1/2 plasmids encoding full-length human antigen R (HuR) and the RNA recognition motifs 1 and 2 (RRM1/2, residues G18-N186) of HuR, respectively, were constructed by KU COBRE-PSF Protein Purification Group. Musashi1 (MSi1) RNA oligo (5′-GCUUUUAUUUAUUUUG-3′ with 3 ′ fluorescein or 3 ′ biotin) was purchased from Dharmacon (Thermo Scientific, Lafayette, CO). RNAs were pretreated by heating at 95°C for 5 min and immediately cooling on ice for 5 min.

For the fluorescence polarization assay, experiments were performed in 96-well black plates (Corning, Corning, NY) with a final volume of 100 μl using the BioTek Synergy H4 plate reader (Biotek, Winooski, VT). Twenty-five nM full-length human HuR and 2 nM fluorescein labeled Msi1 RNA oligo were used. Compounds with five doses (2 nM-20 μM) were added to the wells prior to the protein-RNA complex. Anisotropy measurements were taken after incubation at room temperature for 2 hr. IC50, the concentration causing 50% inhibition, was calculated via sigmoid fitting of dose response curve using Prism 5.0. Ki was calculated using free online software (http://sw16.im.med.umich.edu/software/calc_ki/).

For Alpha assay, experiments were performed in 96-well white 1/2 area plate (Perkin Elmer, Waltham, MA) with a final volume of 50 μl. Multiple doses of compounds were added to the wells first, followed by pre-formed RRM1/2-Msi1 complex (100 nM RRM1/2 protein and 25 nM Msi1 RNA), donor beads and acceptor beads (Perkin Elmer, 20 μg/ml final concentration). Measurements were taken after incubation at room temperature for 2 hr. IC50 and Ki were determined as described in FP assay.

3.14. Surface Plasmon Resonance assay

The Surface Plasmon Resonance (SPR) experiments were performed using a BIACORE 3000 (GE Healthcare) at 20°C and used to study the binding interaction of St-3 with either full length HuR or RRM1/2 of HuR. The ligands, HuR, RRM1/2 were immobilized using the standard primary amine coupling reaction and followed by standard procedures. The sensor chip surface was initially activated with a 1:1 mixture of N-hydroxysuccinimide (NHS, 115 mg/ml) and N-(3-dimethyl-aminopropyl)-N'-ethyl-carbodiimide-hydrochloride (EDC, 750 mg/ml) for 7 min each with a flow rate of 5 µl/min. Full-length HuR protein and RRM1/2 protein were then applied to the flow cells in 10 mM sodium acetate, pH 4.5, and immobilized to a density of 7200 RU and 3800 RU (response units), respectively. An adjacent flow cell was left blank to serve as a reference surface. The activated carboxylic acid groups were quenched with a 7-minute injection of ethanolamine (1 M, pH 8.5). To collect kinetic binding data, compound St-3 in 20 mM HEPES pH7.4, 150 mM NaCl, 3 mM EDTA, 0.05% p20 (v/v), 5% DMSO (v/v), were injected over the flow cells at the indicated concentrations at a flow rate of 60 µl/min and at 20°C. The complex was allowed to associate for 4 min and dissociate for 3 min. Considerable care was taken to prevent contamination. Samples were carefully injected to avoid carryover effects and the system was carefully washed before injection of each new sample. The sample flow rate was set at 60 µl/min to determine the kinetic and equilibrium constant. The equipment surfaces were washed extensively with buffer solution to restore the surfaces before each binding experiment. Data analysis was performed using BIA evaluation software. Data analysis and sensor grams were automatic corrected for nonspecific bulk refractive index effects. Standard procedures for

the 1:1 Langmuir binding fit model were used for the kinetic analysis of ligand binding to the protein.

3.15. Data analysis

Combination Index (CI) were calculated using the CompuSyn software. CI value > 1.1 indicates antagonism, CI value = 0.9-1.1 indicates additive effect, and CI value < 0.9 indicates synergism. Statistical analysis was performed using SYSTAT 11 software for student T-test. A difference was considered significant at the $p < 0.05$ level. Correlation analysis used the standard Pears Tests.

Chapter 4. Mechanisms of High Dose Ascorbate Inhibiting Pancreatic Cancer Growth and Metastasis

4.1. Abstract

High-dose intravenous ascorbate (IVC) bypasses bioavailability barriers of oral ingestion and provides pharmacologic concentrations in tissues, exhibiting pro-oxidant anti-cancer activities. In pancreatic cancer pre-clinical models, pharmacologic concentrations of ascorbate has been shown to sensitize the tumor to the 1st line chemotherapeutic drug gemcitabine. In an orthotopic mouse tumor model, ascorbate treatment alone decreased pancreatic cancer growth and metastasis. Ascorbate treatment resulted in EMT inhibition, as evidenced by enhanced expressions of epithelial markers and suppressed expression of mesenchymal markers in both tumor samples as well as in pancreatic cancer cell lines. Ascorbate treatment influenced the tumor micro environment by decreasing the expression and activity of MMP-2, promoting collagen synthesis and enhancing stromal desmoplastic reaction. Furthermore, ascorbate decreased expression of HDAC6 and activity of silent information regulator-2 (Sirt-2), which are known EMT inducers. As a result of HDAC6 and Sirt-2 inhibition, ascorbate robustly increased α-tubulin acetylation in pancreatic cancer cells. Acetylation promoted tubulin polymerization and stabilization, resulted in inhibition of cell motility and mitosis. An effect mimics the cellular outcome of paclitaxel. Taken together, pharmacological concentrations of ascorbate inhibited pancreatic cancer cell EMT and affected the tumor microenvironment, led to inhibition of tumor growth and metastasis.

4.2. Introduction

Vitamin C (ascorbic acid, with its sodium salt named ascorbate) is an essential micronutrient to human beings. It is required for many enzymatic reactions catalyzed by Fe^{+2}-2-oxoglutarate dependent dioxygenases (Collagen prolyl-3-hydroxylase, prolyl-4-hydroxylase, dopamine β-hydroxylase, HIF-α hydroxylase, histone demethylases containing jumonji catalytic domain, etc.). Ascorbate maintains iron in ferrous state in these enzymes, thereby maintaining full activity of this class of enzymes (251-258).

Ascorbate has long been used in complementary and alternative medicine for treatment of cancer. Early hypothesis includes that ascorbate inhibited the enzyme hyaluronidase, which otherwise destroyed collagen so that cancers could metastasize (259). Cameron and the Nobel Prize laureate Pauling advocated the use of high-dose ascorbic acid (10 gms/day intravenous administration for up to 10days followed by 10gms/day orally indefinitely) to treat cancer. Use of such high doses improved quality of life, and prolonged survival time in terminal oncologic patients, and even resulted in complete tumor regression in a few cases (260, 261). However, two carefully designed double-blind trials using oral ascorbate carried out at Mayo Clinic showed no significant differences between ascorbate and placebo group with regard to survival time (262). Now it is known that intravenous administration of Vitamin C (IVC) achieves high systematic concentrations in milli-molar range that could not be achieved by oral ingestion. IVC bypasses the physiological "tight control" mechanisms that limit its systematic concentrations with oral ingestion, such as intestinal absorption, tissue accumulation, renal reabsorption and excretion, and achieves pharmacological concentrations (263-265). The pharmacologic concentrations of ascorbate generate ascorbate radical and H_2O_2 which induce oxidative damages selectively to tumor cells (266-269). Many laboratories have shown in rodent xenograft experiments that

ascorbate decreased the growth rate of various aggressive tumors, such as pancreatic cancer, glioblastoma, ovarian cancer, prostate cancer, hepatoma, colon cancer, sarcoma, leukemia, mesothelioma, breast cancer, and neuroblastoma (267-274).

Recently, considerable interest has been raised concerning using high dose intravenous vitamin C (IVC) as adjuvant therapy to standard chemotherapies (266). IVC is safe and free of common toxic side effects that often accompany chemotherapies. A phase 1 trial by Hoffer et al (275) with 24 terminal cancer patients found IVC of 1.5 g/kg 3x weekly was free of significant toxicity, and unexpectedly, 2 patients had stable disease. Our group reported a pilot trial in stage III-IV ovarian cancer patients (266) with participants randomized to the standard paclitaxel/carboplatin therapy or the standard chemotherapy plus IVC (75-100 g/ infusion, 2 x weekly for 1 year). IVC treatment substantially decreased chemo-associated toxicities (266). The median time for disease progress/relapse was prolonged for 8.75 months in the ascorbate + chemo group than the chemo-only group, despite that the trial was not statistically powered to detect efficacy (266). Two trials in pancreatic cancer patients were recently reported by Monti et al (276) and Cullen et al (277), both using IVC (50-100 g/infusion 2-3x weekly) together with gemcitabine or gemcitabine plus the EGFR inhibitor, erlotinib. In both trials, IVC did not increase any toxicity to the chemotherapy. In Monti's trial, 7 out of 9 patients had tumor shrinkages after only 8 weeks of treatment (276). In Cullen's trial, potential in prolongation of survival was shown compared to historical controls (277). Therefore, IVC has emerged to be a promising adjuvant treatment for pancreatic cancer with clinical benefit in low toxicity and potential improvement in efficacy.

Our previous study using a panel of 7 pancreatic cancer cell lines showed that ascorbate sensitized pancreatic cancer cells to gemcitabine treatment (278). Ascorbate inhibited metastasis

in PANC-1 orthotopic model system. Our recent studies have explored mechanisms of ascorbate mediated cell death in cancer cells (266). However, it is still an open question as to how ascorbate exerts its anti-metastatic property. A mounting body of literature suggests that EMT and tumor micro environment are both critically involved in the acquisition of metastatic potential. Led by preliminary experimental observations, we aimed to investigate the mechanisms of ascorbate on regulation of EMT and tumor environment.

4.3. Results

4.3.1. Ascorbate decreased pancreatic cancer cell viability, invasion, migration, and metastasis.

A panel of 8 human pancreatic cancer cell lines and 1 murine pancreatic cancer cell line were used to examine sensitivity to ascorbate treatment. These cell lines represent genotypes of mutations commonly found in human pancreatic cancers **(Table 1)**. Cells were exposed to 0-20 mM of ascorbate for 1-2 hrs, which are concentrations and time courses easily achievable clinically (267). Cell viability was detected 24 hrs after the treatment. Massive cell death was detected in all tested cancer cell lines, even below 5 mM ascorbate **(Fig. 4.1A)**. In contrast, the treatment of 20 mM ascorbate only minimally influenced viability of a non-tumorigenic pancreatic ductal epithelial cell hTERT-HPEN, fibroblasts (WI-38) and normal lymphocytes. Addition of catalase, an enzyme that specifically degrades H_2O_2, completely reversed ascorbate - induced cell death in cancer cells, consistent with previous studies indicating that H_2O_2 was the effective molecule when pharmacologic ascorbate was present (268, 269). Immunohistochemical analysis on tumor samples showed that proliferating cell nuclear antigen (PCNA), an indicator of cell proliferation, decreased remarkably in tumors treated with ascorbate **(Fig. 4.1B)**. Mitosis counting also decreased significantly as analyzed by hematoxylin and eosin stain (H&E stain)

Figure -4.1A

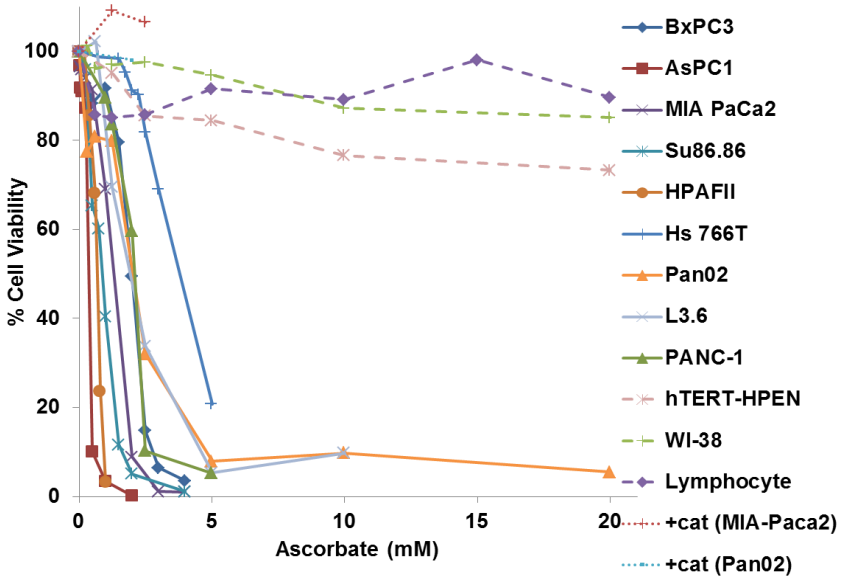


Figure -4.1B

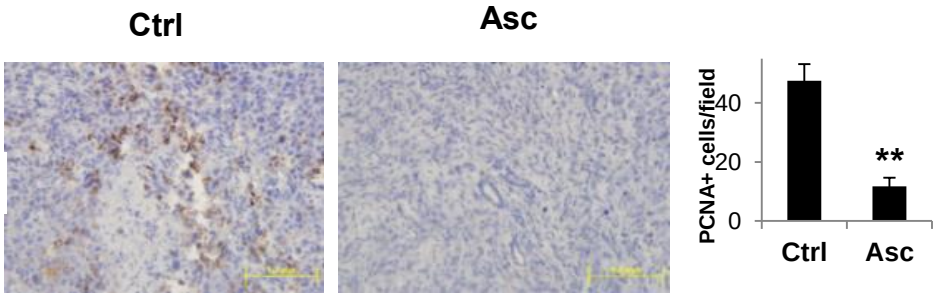


Figure -4.1C

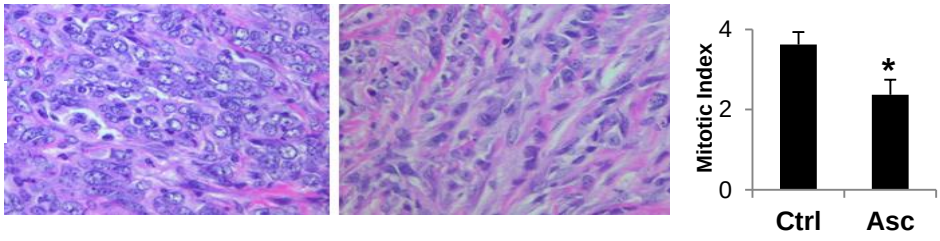


Figure 4.1- Ascorbate inhibited pancreatic cancer cell growth *in vitro* and *in vivo* *A.* Sensitivity of pancreatic cancer cells and normal cells to ascorbate. Cells were exposed to 0-20 mM ascorbate for 1-2 hrs. Cell viability was detected at 24 hrs post treatment by MTT assays. +cat, pre-incubation with 600 U/ml catalase. Data represents Mean ± SD of 3 experiments each done in triplicates. ***B.*** Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) with formalin fixed tumor samples. Bar graph (right) represents the average number of PCNA positive cells per field. 15 fields from 3 different tumors from each group were analyzed. **, P<0.001 by T-test. ***C.*** Histological analysis of mitosis on H&E stained tumor slides. Bar graph (right) shows mitotic index, which was average number of mitoses from 4 separate fields. Tumors from 4 mice in each group were examined.

Table -1

Cell line	***K-Ras***	***P53***	***CDKN2A/p16***	***SMAD4/DPC4***
BxPC-3	WT	220 Cys	WT	HD
AsPC-1	12 Asp	135 Δ1 bp	WT	WT
MIA PaCa2	12 Cys	248 Trp	HD	WT
Su86.86	12 Asp	245 Ser	HD	WT
HPAFII	12 Asp	151 Ser	Δ20–25 Δ26–27 Δ29–34	WT
HS 766T	61 His	Mut 225–282 Δexons 2–4	Intron 2 splice site	HD
PANC-1	12 Asp	273 His, 273 Cys	HD	WT

Table -1 Genotype of the pancreatic cancer cell lines used, showing the most common mutations in pancreatic cancer (7).

WT—wild type, Δ—deletion, bp—base pair, HD—homozygous deletion, Mut225-282— mutations found between codons 225-282

(Fig. 4.1C). Interestingly, at a sub-cytotoxic concentration of 1 mM, ascorbate was able to inhibit PANC-1 cell migration and invasion through matrigel gel coated Boyden chambers, without influencing viability of the cells **(Fig. 4.2A, B, and C)**. We further investigated the effects of ascorbate *in vivo* by using orthotopic pancreatic cancer model in mouse. Luciferase expressing PANC-1 cells were orthotopically injected into the pancreas of nude mice. After the tumors were formed, mice were treated with intraperitoneal doses of ascorbate at 4 g /Kg body weight/day for 45 days (equivalent to 1.3 g/Kg/day by intravenous injection) (268).

Figure -4.2A

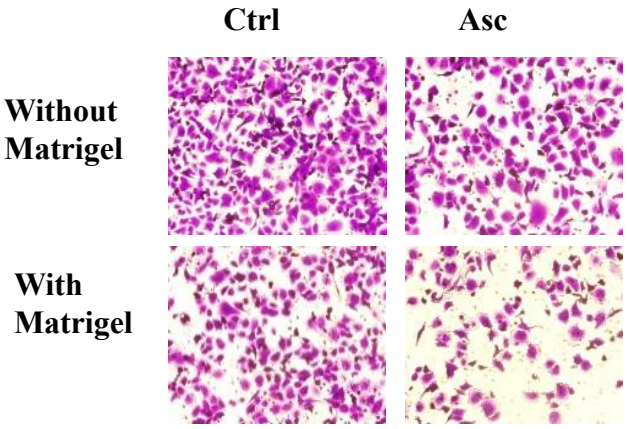


Figure -4.2B

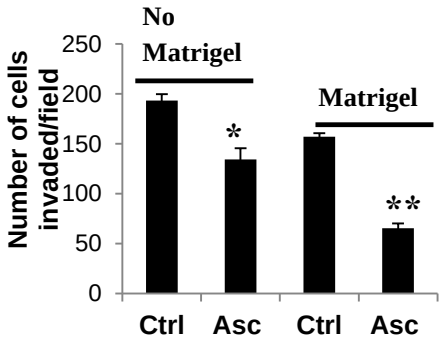


Figure -4.2C

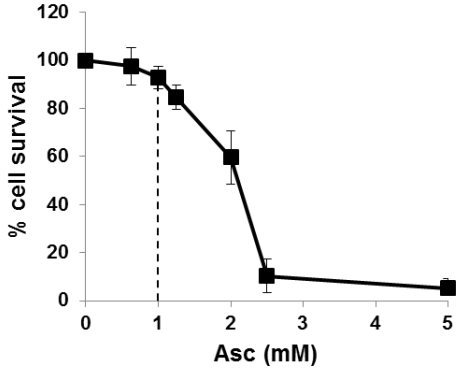


Figure 4.2- Ascorbate inhibited pancreatic cancer cell invasion and migration *in vitro* ***A.*** Matrigel invasion assays for pancreatic cancer cell migration and invasion. PANC-1 cells were exposed to a sublethal dose of ascorbate (1 mM). Cell migration (without Matrigel) and invasion (with Matrigel) were detected at 24hrs. ***B.*** Bar graph shows the average number of migrated/invaded cells per field. Data represents ≥3 experiments each done in triplicates. ***C.*** Ascorbate 1mM treatment for 24hrs had a little impact on cell viability of PANC-1 cells. *, $P<0.05$, **, $P<0.001$ by T-test.

Figure -4.3A

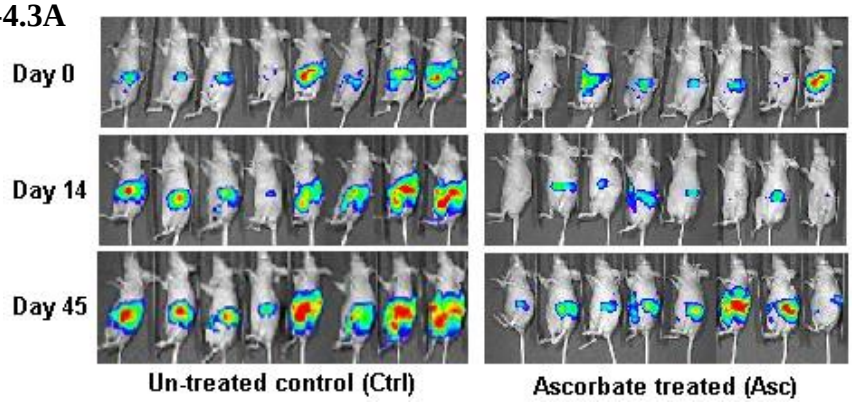


Figure -4.3B

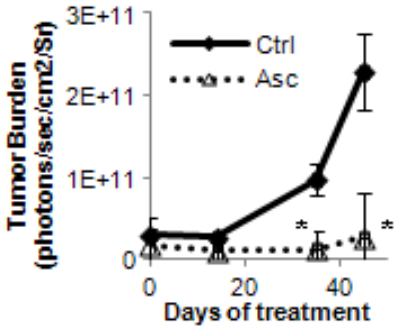


Figure -4.3C

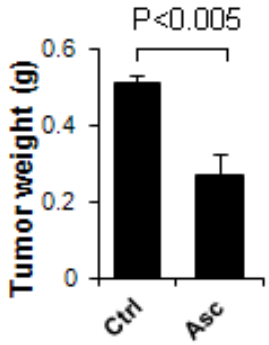


Figure -4.3D

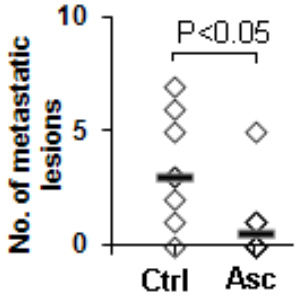


Figure -4.3E

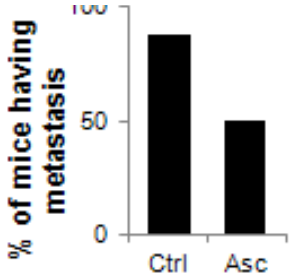


Figure 4.3- Ascorbate inhibited pancreatic cancer growth and metastasis *in vivo*. *A.* Representative bioluminescence images of mice bearing orthotopic pancreatic xenografts with and without ascorbate treatment at indicated days. Day 0 indicated the beginning of treatment, which was 2 weeks post orthotopic injection of luciferase expressing PANC-1-Leu cells into mouse pancreas. Day 45 was the end of the experiment. Asc, ascorbate treatment (n=8) at intraperitoneal dose of 4 g/kg/day. Control (Ctrl) mice (n=8) were treated with saline that had the same osmolarity as the ascorbate injections. ***B.*** Total tumor burden per mouse by imaging was quantified as photons/sec/cm^2/sr. *, P<0.05 by T-test. Total tumor weight (***C),*** number of metastatic lesions in each mouse (***D)*** and percentage of mice that had metastasis (***E)*** were determined by necropsy at Day 45.

Live animal tumor imaging showed that ascorbate treatment significantly reduced tumor progress longitudinally **(Fig. 4.3A, B)**. Number of mice with metastasis decreased **(Fig. 4.3E)**, and number of metastatic lesions in each mouse were significantly decreased in the ascorbate group **(Fig. 4.3D)**. At the end of the experiment, mice were euthanized and total tumor weight was measured. A significant decrease in average tumor weight was found with ascorbate treatment **(Fig. 4.3C)**.

4.3.2. Ascorbate influenced pancreatic cancer cell EMT, inhibited MMPs, and increased collagen content and desmoplasia in tumor stroma.

We have published previously that the oxidative action of high ascorbate concentrations could directly induce cancer cell death (266). Here, our data showed that even at sub-cytotoxic concentrations, ascorbate (1 mM) decreased PANC-1 cell invasion and metastasis (**Fig. 4.2 A, B, and C**). To investigate the mechanisms involved, markers in pancreatic cancer EMT were examined, because EMT has been suggested to be the initial step for the complicated process of cancer cell dissemination and metastasis (5). Loss of E-cadherin, a cell surface protein, is the hallmark of EMT (279). In pancreatic cancer cells treated with ascorbate, E-cadherin expression was enhanced at both mRNA level (**Fig. 4.4A**) and protein level (**Fig. 4.4B**). Consistently, Snail, the E-cadherin repressor, was inhibited (**Fig. 4.4A, 4.4B**). The mesenchymal marker vimentin was decreased at both mRNA and protein levels **(Fig. 4.4A, B**). Other markers of EMT also showed a pattern supporting the inhibition of EMT, which meant increase in epithelial markers and decrease in mesenchymal markers with ascorbate treatment (**Fig. 4.4A-C**). Decrease of Snail was confirmed in mouse tumors treated with ascorbate (**Fig. 4.4C**). Moreover, the epithelial molecule CK-19 showed a robust increase in tumor samples from ascorbate-treated mice (**Fig. 4.4C**). As EMT is highly associated with CSC, we examined the effect of ascorbate on

pancreatic CSCs. Indeed, spheroids formation assays showed that ascorbate of 2.5 mM reduced the number of tumor spheroids in both PANC-1 and BxPC-3 cells (**Fig. 4.4D**).

Remarkably, mRNA of collagens was greatly increased in ascorbate treated pancreatic cancer cells *in vitro* (**Fig. 4.4A**), accordingly collagen content was increased in tumor stroma of ascorbate treated mice (**Fig. 4.5A**). Desmoplasia in tumor stroma was significantly enhanced in ascorbate treated mice (**Fig. 4.5B**). These changes in the tumor microenvironment may contribute to the reduction of invasiveness of the tumor (280). In contrast, no such increase in collagen or fibrosis was observed in the livers of the ascorbate treated mice (**Fig. 4.5C**).

Ascorbate decreased mRNA levels of multiple MMPs **(Fig. 4.4A)**. We particularly examined MMP-2 expression and activity, because MMP-2 was suggested to be important in pancreatic cancer cell invasion (281). Ascorbate treatment decreased MMP-2 expression in a dose-dependent manner in pancreatic cancer cells **(Fig. 4.6A)**. Activity of secreted MMP-2 was determined using gelatin zymography assay. Significant decrease was found in gelatinolytic activity of MMP-2 in the supernatant media of PANC-1 cells treated with ascorbate **(Fig. 4.6B)**.

4.3.3. Ascorbate enhanced α-tubulin acetylation and increased tubulin polymerization

In addition to the EMT inhibition and changes in tumor microenvironment, robust α-tubulin acetylation was found in PANC-1 and BxPC-3 pancreatic cancer cells and the acetylation was dose dependent to ascorbate treatment (**Fig. 4.7A**). At the same concentration, ascorbate only minimally increased α-tubulin acetylation in the immortalized pancreatic ductal epithelial cell hTERT-HPEN. Treatment with H_2O_2 mimicked the effects of ascorbate, whereas catalase completely eradicated α-tubulin acetylation induced by ascorbate **(Fig. 4.7B**), reconfirming that formation of H_2O_2 is the mechanism of ascorbate action (269). The microtubule dynamics

Figure -4.4A

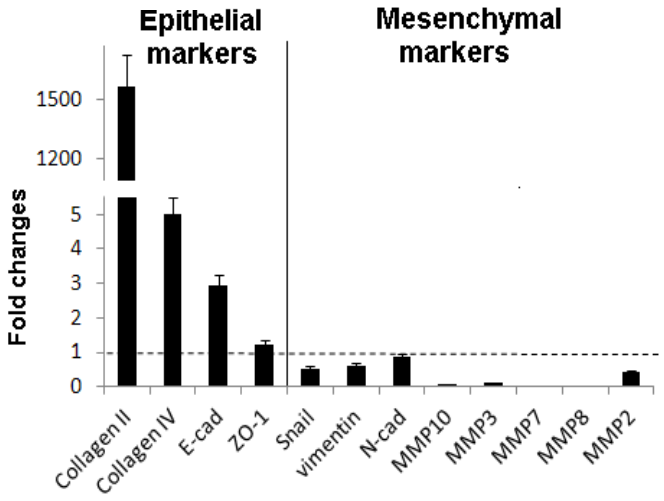


Figure -4.4B

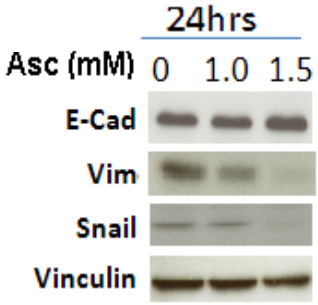


Figure -4.4C

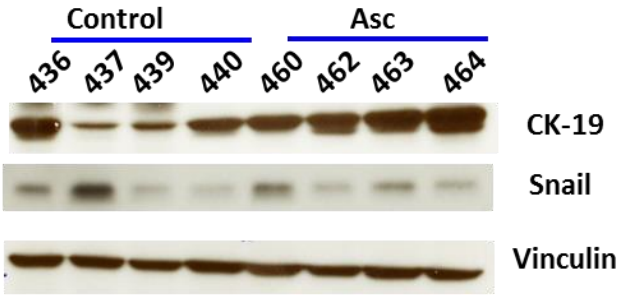
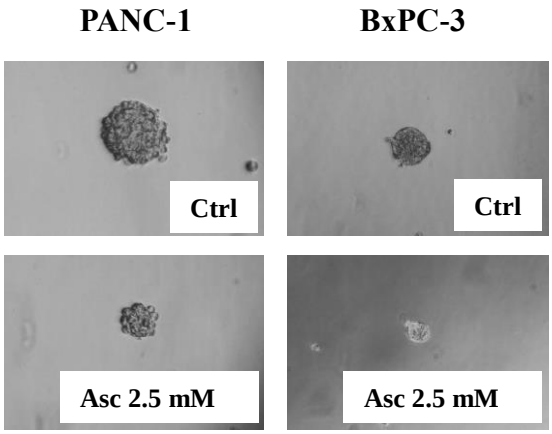


Figure -4.4D



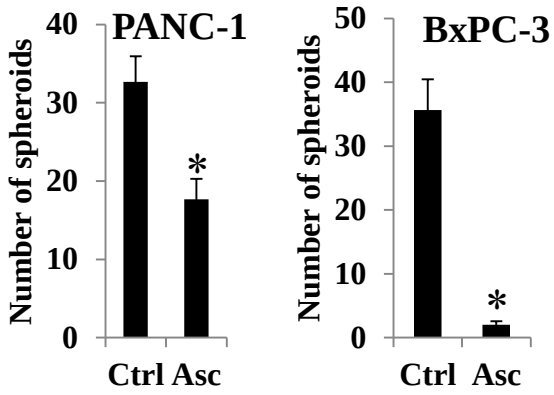


Figure 4.4- Ascorbate affected pancreatic cancer EMT markers. *A.* qRT-PCR for changes in EMT markers. PANC-1 cells were treated with 2.5 mM ascorbate. Data represent Mean ± SD of 2-6 independent experiments. ***B.*** Western blot in PANC-1 cells showing expression of E-cadherin (E-Cad), vimentin (Vim) and Snail after ascorbate treatment. Vinculin was a loading control. ***C.*** Western blot in mouse tumor samples from ascorbate treated and saline treated (Control) mice showing changes in CK-19 and Snail. D. CSCs population was measured by sphere formation assay after ascorbate treatment. *, P<0.05 by T-test.

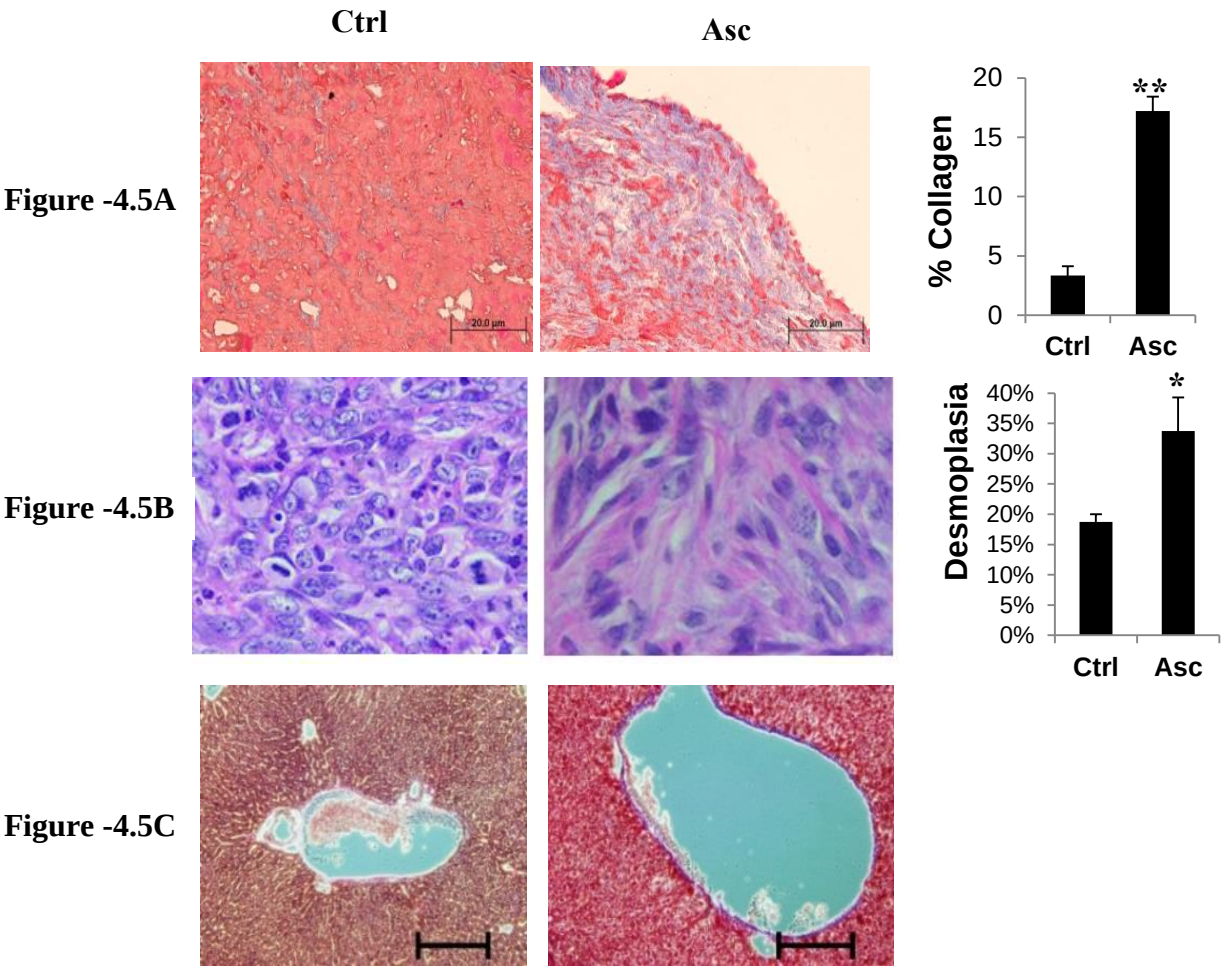


Figure 4.5- Ascorbate increased collagen content and desmoplasia in tumor stroma. ***A***. Histological analysis of tumor samples for collagen. Formalin fixed tumor samples were sliced and subjected to Masson's trichrome staining. Collagen was stained blue, while cytoplasm was stained pink. Bar graph representing the average of % area collagen/cross section. 15 fields from 3 different tumors from each group were analyzed. ***B.*** Histological analysis of desmoplasia on H&E stained tumor slides. Bar graph shows desmoplasia, which was represented as % of area contains desmoplastic response. Tumors from 4 mice in each group were examined. ***C.*** Masson's trichrome staining for collagen and fibrosis of livers from control and ascorbate treated mice. *, $P<0.05$, **, $P<0.001$ by T-test.

Figure -4.6A

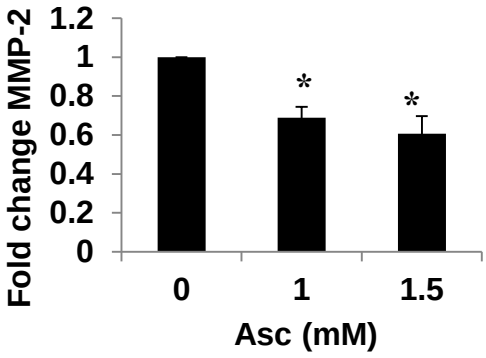


Figure -4.6B

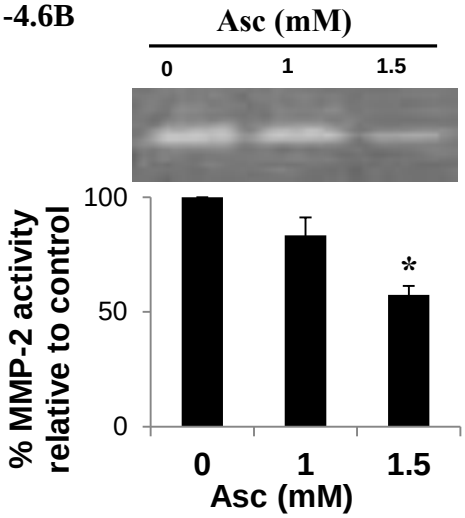


Figure 4.6- Ascorbate affected MMP-2 expression and activity. ***A.*** qRT-PCR for MMP-2 mRNA expression in PANC-1 cells treated with ascorbate. ***B.*** Gelatin zymography assay for MMP-2 enzymatic activity after ascorbate treatment. Supernatant media was used for detection of MMP-2 activity using gelatin zymography. Data represents Mean ± SEM of 3 experiments each done in triplicates. *, P<0.05 by T-test.

(assembly and disassembly) is important for general cellular functions such as cell division, intracellular trafficking, and ciliary beating (282). Acetylated α-tubulin is associated with stable microtubules (283). Overstabilized microtubules could result in malfunction of cell division and movement (283). The chemotherapeutic drug paclitaxel works to bind and stabilize microtubules. Here, we found overstabilization of microtubules induced by ascorbate treatment. Four hours after ascorbate treatment, there was an enrichment of high molecular weight fractions of acetylated α-tubulin, indicating microtubule polymerization, mimicking the effect of paclitaxel **(Fig. 4.8)**. As cold temperature is known to induce depolymerization of tubulin, cell lysates were put on ice (4°C) and the ascorbate-induced tubulin polymerization was found stable over time at either 37 °C or 4 °C (**Fig. 4.9**). The degree of α-tubulin acetylation was inversely correlated to the viability of pancreatic cancer cells after ascorbate treatment (BxPC-3, r = -0.88609, PANC-1, r = -0.98287 by Pears test) (**Fig. 4.12A, B**). These results were consistent

with the mitosis and metastasis inhibition we found in tumors from ascorbate-treated mice (**Fig. 4.1, 4.2, and 4.3**).

Figure -4.7A

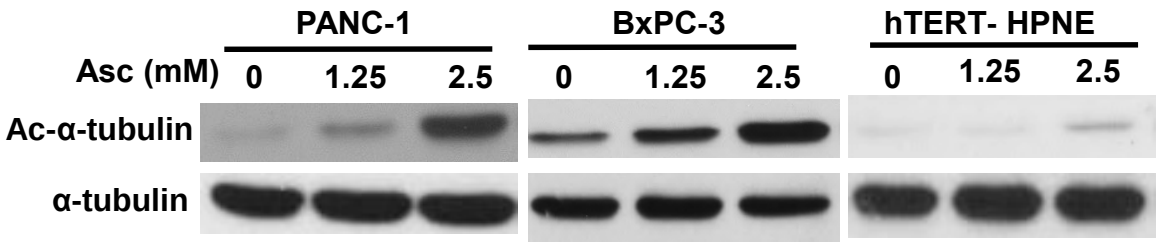


Figure -4.7B

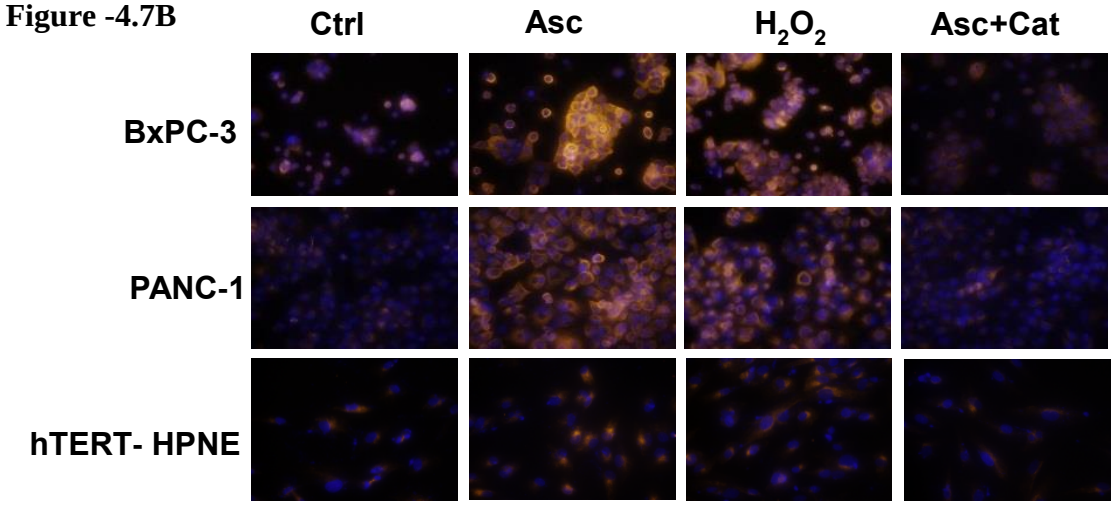


Figure 4.7-Ascorbate promoted α-tubulin acetylation. ***A.*** Western blot analysis of acetylated α-tubulin in pancreatic cancer cells (BxPC-3 and PANC-1), and an immortalized non-cancerous pancreatic epithelial cell line hTERT-HPNE. Cells were treated with 0, 1.25, 2.5 mM ascorbate for 4 hrs. ***B.*** Immunofluorescence showing acetylated α-tubulin in cells after ascorbate 2.5 mM and H_2O_2 500 µM for 4 hrs. Ascorbate +Cat, co-treatment of ascorbate and 600 U/ml catalase for 4hrs. Cell nuclei were counter-stained blue with hoechst33342 (1 mg/mL).

Figure -4.8

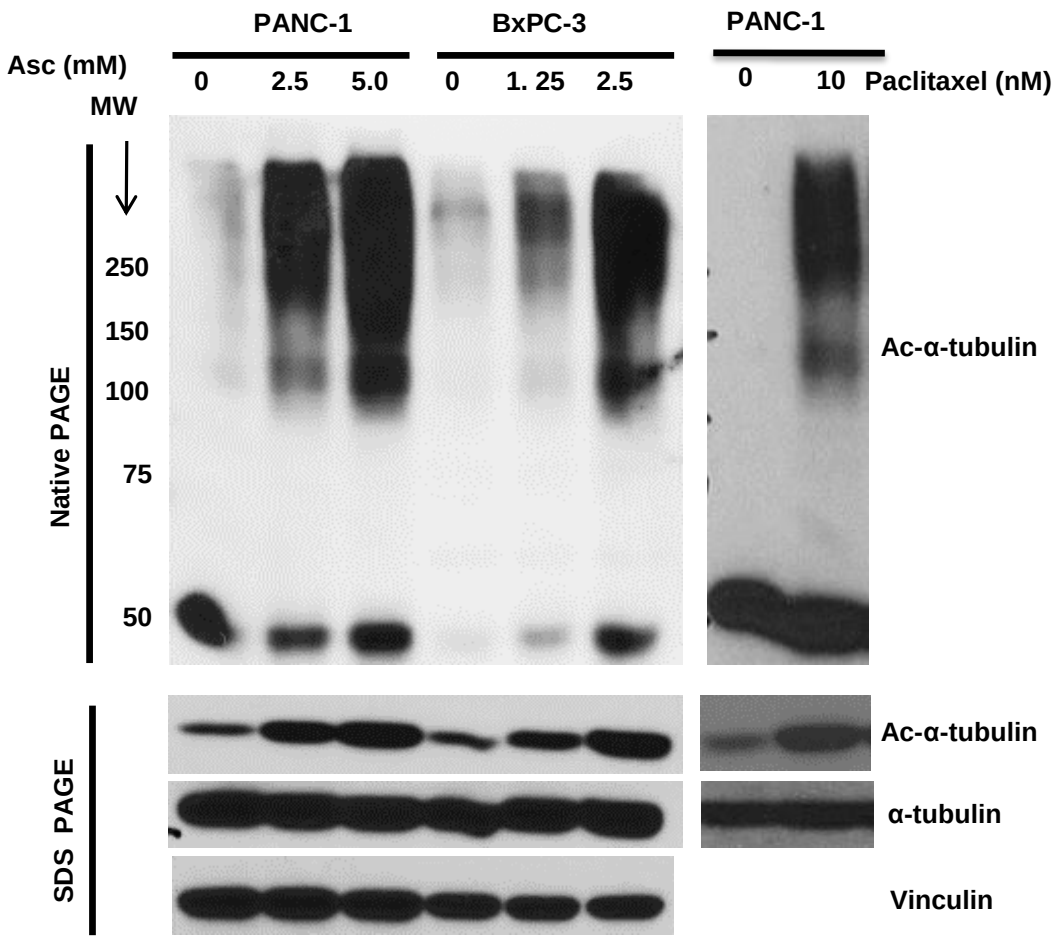


Figure 4.8- Ascorbate promoted tubulin polymerization. Tubulin Polymerization was detected by native PAGE. After 4hrs of ascorbate or paclitaxel treatment, cells were lysed in RIPA buffer. For native PAGE 10 µg cytosolic fraction was loaded in β-mercaptoethanol free buffer without boiling and the electrophoresis was performed on 8% poly acryl amide gel. α-tubulin acetylation was confirmed by SDS PAGE and western blot as shown in the bottom panels. MW, molecular weight in kDa.

Figure -4.9

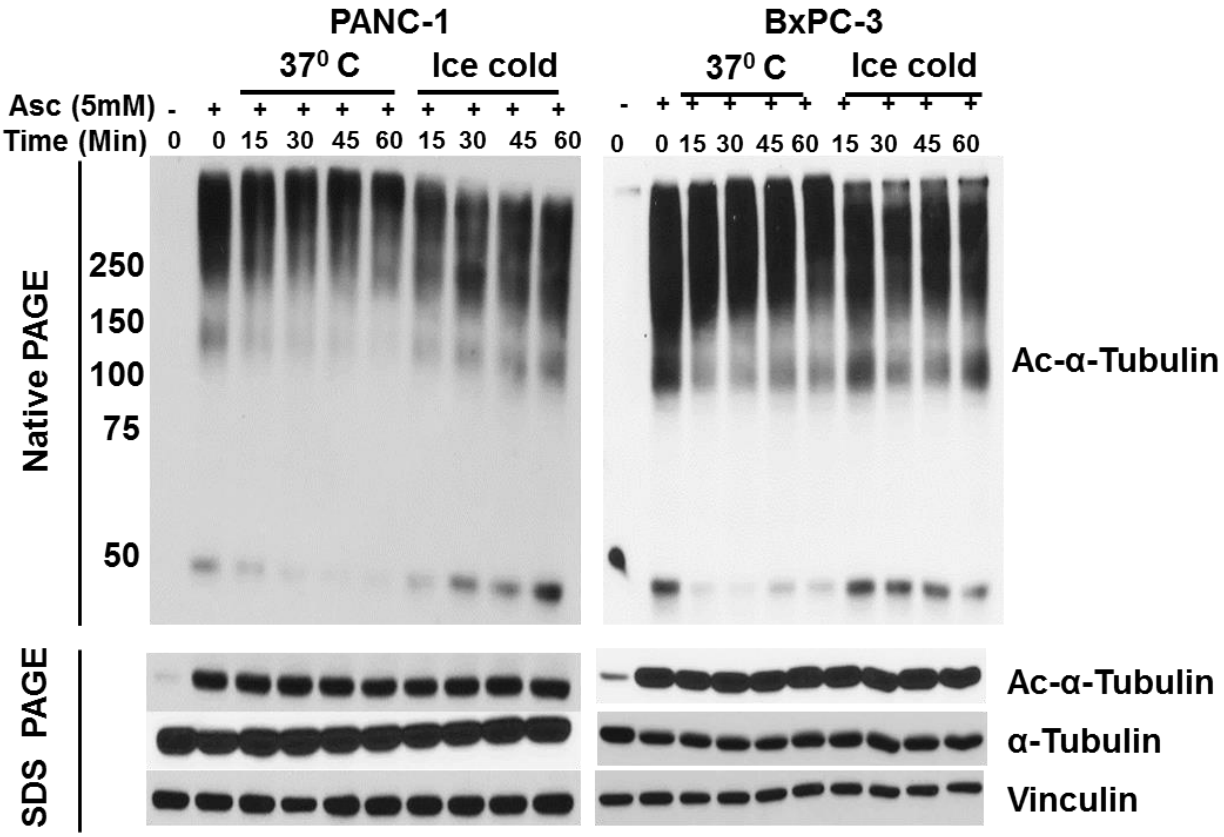


Figure 4.9-Ascorbate stabilized microtubule polymers. Cold induced microtubule depolymerization assay in PANC-1 and BxPC-3 pancreatic cancer cells. After ascorbate treatment for 4hrs cell lysates were exposed to either 37^0C or sit on ice (4 ^{0}C). At indicated time points, samples were analyzed by native PAGE analysis for Ac-α-tubulin polymers. The same samples were run on SDS PAGE to confirm α-tubulin acetylation.

4.3.4. Ascorbate enhanced α-tubulin acetylation through inhibition of Sirt-2 and HDAC6

Alpha-tubulin acetylation is under the balanced control of enzymatic activities of acetyl transferase (alpha tubulin acetyltransferase(α-TAT)) and deacetylases (Sirt-2 and HDAC6) (282). In both PANC-1 and BxPC-3 cells **(Fig. 4.10A**), and in tumors from ascorbate treated mice (**Fig. 4.10B**), ascorbate decreased protein levels of HDAC6. No change in Sirt-2 protein

levels was detected either in cell lines or in tumor samples (**Fig. 4.10A, B**). No change in α-TAT expression observed in PANC-1 and BxPC-3 cells after ascorbate treatment (**Fig. 4.10A**).

To examine whether HDAC6 plays a role in ascorbate-mediated α-tubulin acetylation, we first overexpressed HDAC6 in PANC-1 cells using a Flag-tagged HDAC-6 plasmid (pcDNA3.1 + HDAC6-Flag) (**Fig. 4.10C**). The overexpression only partially counteracted with ascorbate and partially decreased the level of α-tubulin acetylation caused by ascorbate (**Fig. 4.10C**). We further inhibited HDAC6 activity by using an HDAC inhibitor trichostatin A (TSA). TSA markedly enhanced α-tubulin acetylation in both PANC-1 and BxPC-3 cells, despite the fact that the two cell lines had different sensitivity to the treatment **(Fig. 4.13A).** Co-treatment of ascorbate and TSA were evaluated against cell viability. Results showed additive to synergistic effect in inducing PANC-1 and BxPC3 cell death, as shown by the "heat map" of cell viability and the combination indices (CIs) **(Fig. 4.13B)**. These data indicated that by manipulating activity of HDACs, ascorbate induced α-tubulin acetylation and cell viability were affected.

Activity of enzymes can be inhibited at either expression level or enzyme activity level. Although there was no change in protein levels of Sirt-2 after ascorbate treatment, we found a decreased NAD^+ levels in pancreatic cancer cells treated with ascorbate (**Fig. 4.11A**). Because NAD^+ is an essential co-factor for Sirt-2 activity (284), ascorbate may affect Sirt-2 deacetalyzing activity even though Sirt-2 protein levels were not changed. To investigate this possibility, NAD^+ was supplemented to the cell culture media. With 2 mM of NAD^+ added to the culture media, intracellular NAD^+ was partially rescued (**Fig. 4.11A**), and ascorbate mediated α-tubulin acetylation was partially reversed (**Fig. 4.11B**). A more complete rescue of NAD^+ by adding 8 mM (**Fig. 4.11A**), almost completely reversed ascorbate mediated α-tubulin acetylation (**Fig. 4.11B**). As the tubulin acetylation was prevented, cell death was prevented (**Fig. 4.12C**).

Figure -4.10A

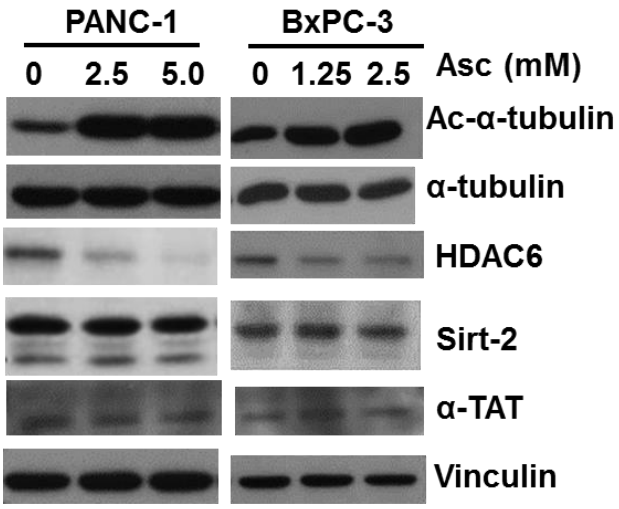


Figure -4.10B

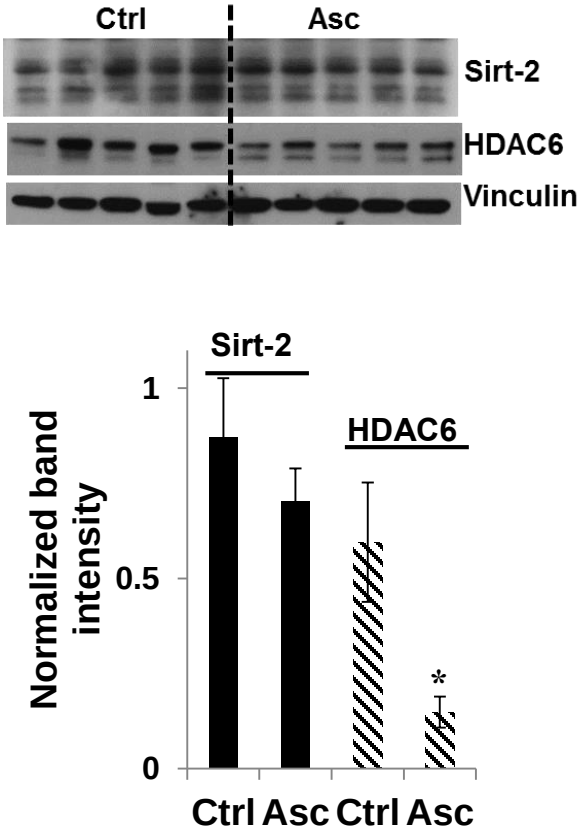


Figure -4.10C

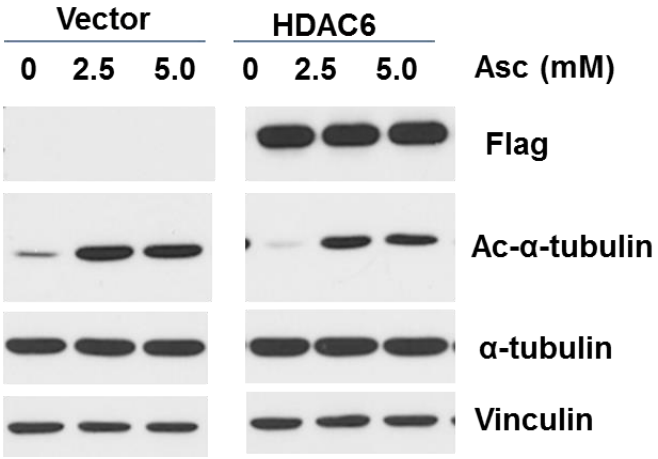


Figure 4.10- Ascorbate decreased expression of HDAC6 to regulate α-tubulin acetylation. ***A***. Western blot analysis for Sirt-2, HDAC6 and α-TAT. PANC-1 and BxPC-3 cells were treated with ascorbate for 4 hrs. Vinculin was a loading control. ***B.*** Western blot analysis of tumor samples from control and ascorbate treated mice, probed for HDAC6, Sirt-2, and vinculin antibodies. Bar graph (right) represents the average Sirt-2/vinculin and HDAC-6/vinculin band intensities in the tumor samples (n=5). Data represents Mean ± SEM. ***C.*** Overexpression of Flag tagged HDAC6 in PANC-1 cells and α-tubulin acetylation induced by ascorbate (4 hr treatment). *, $P<0.05$ by T-test.

Figure -4.11A

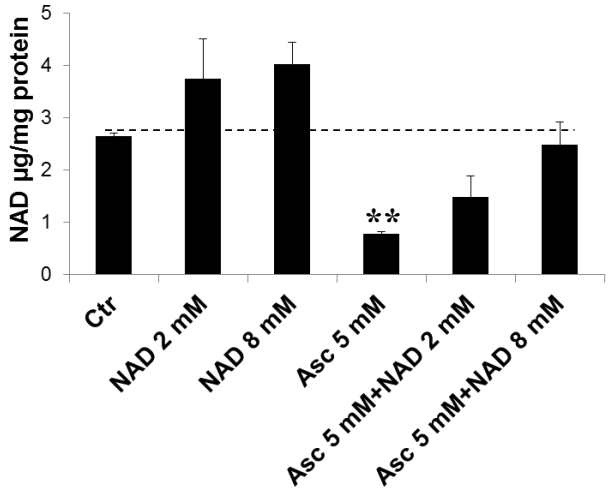


Figure -4.11B

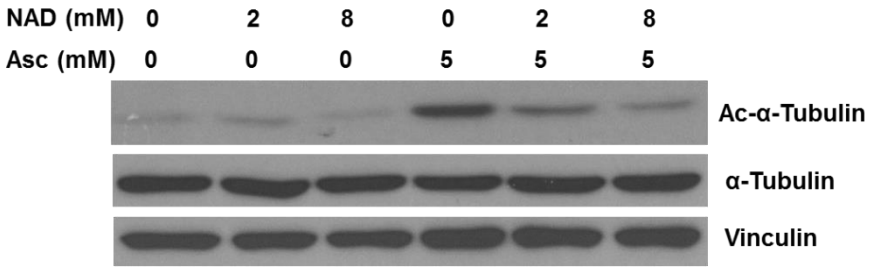


Figure 4.11-Ascorbate regulates Sirt-2 activity by depleting NAD^+ levels. ***A***. NAD^+ levels in PANC-1 cells with ascorbate treatment (5 mM, 4 hrs) and NAD^+ supplementation (8 mM, 6 hrs prior to ascorbate treatment). ***B.*** Western blot for α-tubulin acetylation after NAD^+ supplementation. **, P<0.001 by T-test.

Figure -4.12A

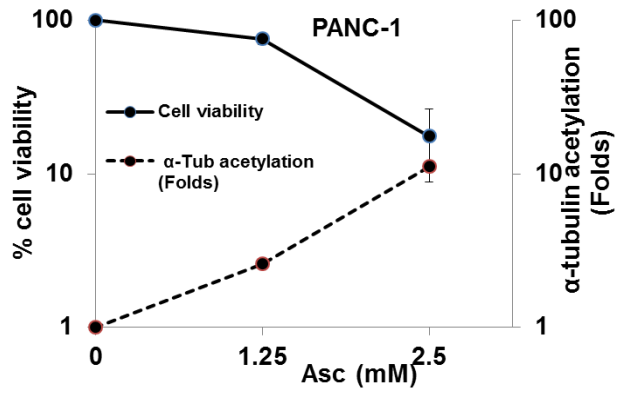


Figure -4.12B

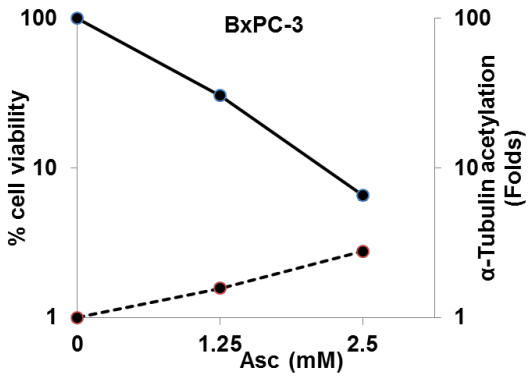


Figure -4.12C

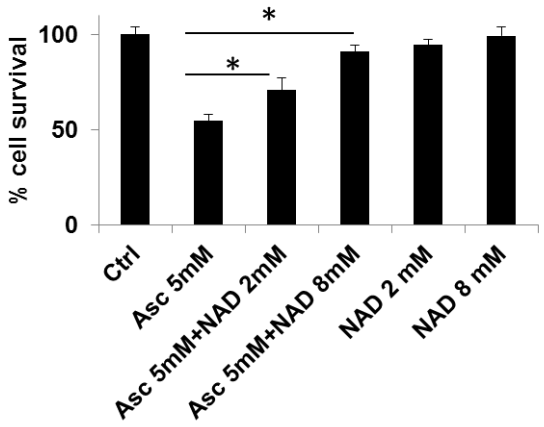


Figure 4.12- NAD$^+$ supplementation rescued cell death induced by ascorbate
A, B. Correlation between tubulin acetylation and cell death induced by ascorbate. ***C.***NAD$^+$ supplementation rescued cell death induced by ascorbate**.** *, P<0.05 by T-test.

Figure -4.13A

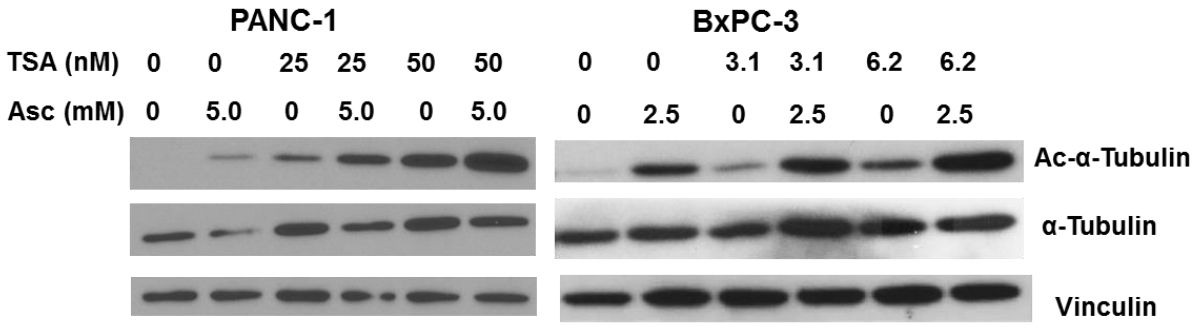


Figure -4.13B

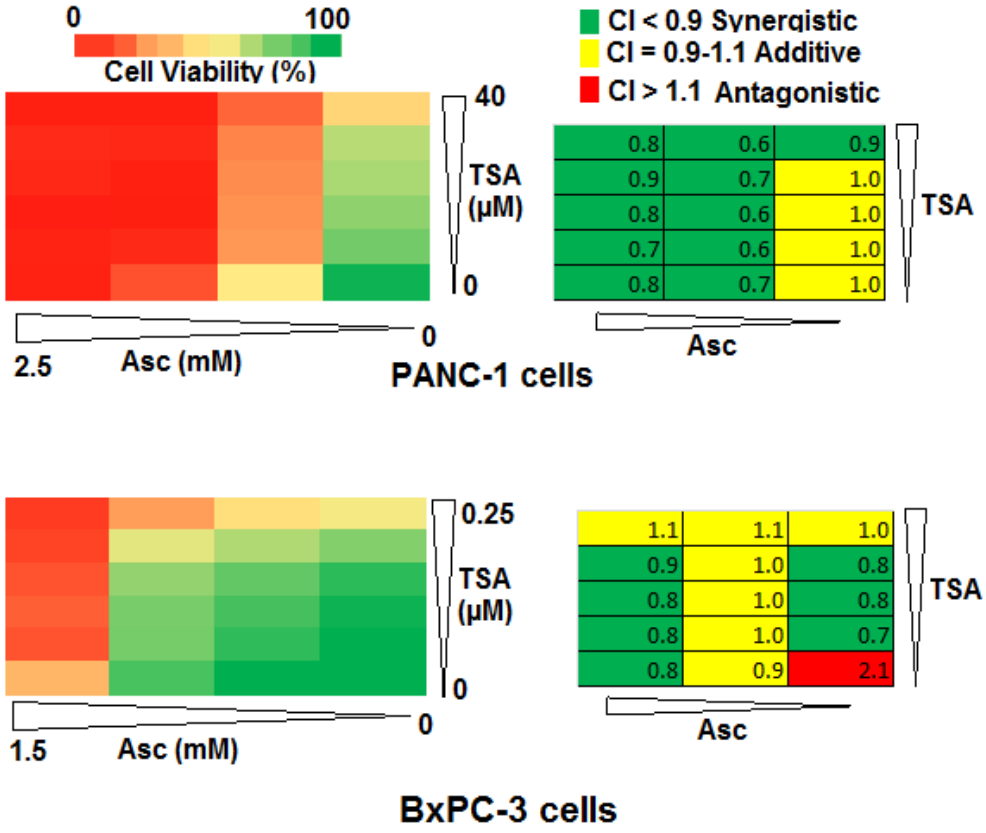


Figure 4.13 - Combination effect of ascorbate and TSA on cell viability. *A.* Combination effect of ascorbate and HDAC inhibitor, TSA on α-tubulin acetylation. PANC-1 and BxPC-3 cells were exposed to ascorbate and TSA, or combinations of ascorbate and TSA for 4 hours. ***B.*** Combination effect of ascorbate and TSA on cell viability. PANC-1 cells were exposed to ascorbate and TSA, or combinations of ascorbate and TSA for 48 hours. Cell viability was measured using MTT assay. Combination Index was calculated by the software CompuSyn.

4.4. Discussion

High-dose ascorbate had been proposed as cancer treatment by Linus Pauling and Ewan Cameron (260, 261), but was discarded as cancer therapy after two formal clinical trials showed negative results using oral ascorbate (262, 285). Later on, pharmacokinetic studies revealed that oral ascorbate could not provide concentrations high enough to induce cancer cell death. Instead, intravenous administration delivered millimolar concentrations of ascorbate into tissues which then generated H_2O_2 to induce death in cancer cells (264, 267-269). Following this rationale, many animal studies showed tumor inhibitory effects of high-dose parenteral ascorbate in various types of cancers, either as a single agent, or used in combination with chemotherapeutic drugs and radiation (267, 270-274, 286-289). Previously, our studies found that ascorbate had synergistic effects with the 1st line chemotherapeutic drug gemcitabine in inhibiting growth of 7 different pancreatic cancer cell lines (278). In the current study, our data further demonstrated that ascorbate inhibited cancer cell EMT and reinforced tumor microenvironment by enhancing collagen synthesis to inhibit metastasis of this aggressive tumor.

Lack of successful chemotherapy is partly due to heterogeneity of pancreatic tumors. Heterogeneity led to differential sensitivity of neoplastic cells to chemotherapeutic drugs leading to tumor recurrence (290). A small molecule compound that has a broad range of anti-tumor activity against a heterogeneous population of pancreatic cancer cells would be an ideal tool for treatment. High dose ascorbate has the potential to be such a tool, as it induces cell death in several different pancreatic cancer cell lines that are quite different in terms of their genomic landscape (Table 1). This suggests that ascorbate can be a potent therapeutic option for pancreatic cancer patients.

Some progress has been made in understanding the mechanism of selective toxicity to cancer cells. In previous studies, we demonstrated that pharmacologic ascorbate generated H_2O_2, induced DNA damage and ATP depletion and subsequently triggered a series of cellular responses including activation of ATM/AMPK and inhibition of mTOR, which could link to cellular outcomes such as inhibition of proliferation, cell cycle arrest, apoptosis, necrosis and autophagy (266). Known as the Warburg Effect (291), ATP production in cancer cells relies primarily on glycolysis (291-293). Since glycolysis is a much less efficient way for ATP production compared to oxidative phosphorylation that is primarily used by normal cells, cancer cells could be more sensitive to ascorbate -induced metabolic stress than normal cells. Our data here showed decrease of NAD^+ in pancreatic cancer cells treated with ascorbate, further supporting this hypothesis. Both DNA repair and ATP production require NAD^+, therefore ascorbate induced stress can be catastrophic to cancer cells.

Moreover, data here showed that depletion of NAD+ influenced the activity of a cytosolic deacetylase Sirt-2. Together with inhibition in the cytosolic histone deacetylase HDAC6, ascorbate treatment caused robust increase in α-tubulin acetylation. Acetylation of α-tubulin enhanced the stability of polymerized tubulin and thus interrupted the dynamics of microtubules. The dynamics of microtubules is important in intracellular transport, cell migration and division. Inhibition of microtubule dynamics effectively impairs cell motility and mitosis (294). An example is the chemotherapeutic drug Paclitaxel (295). Here, we found ascorbate induced α-tubulin acetylation resulted in overstabilized tubulin polymerization and subsequent reduction in mitosis and cell invasiveness.

Microtubule dynamics was also found to control cell-basement membrane interaction in the process of EMT (296). In this study, we found ascorbate inhibited parameters of cancer cell

EMT and influenced cell-stroma interaction. MMPs responsible for cancer cell dissemination were inhibited. In the ascorbate -treated tumor stroma, collagen levels and desmoplasia significantly increased. Stiffness of tumor tissues greatly increased. Increased tissue concentration of ascorbate could enhance collagen biosynthesis, as ascorbate is an essential cofactor for prolyl and lysyl hydroxylases in collagen biosynthesis (297). Increased collagen level strengthened the extra cellular matrix, which formed a stronger barrier for tumor cells to metastasize. The increased desmoplasia or fibrosis has been thought to be a host defense mechanism, similar to scar formation at healing wounds, probably to impede the invasion of carcinoma (298, 299). The function of desmoplastic tumor stroma however was thought to be dynamic during cancer progression, with a tumor-promoting role suggested by some studies (300, 301). However, a recent study convincingly demonstrated that reduction of collagen content in pancreatic cancer by depletion of alpha smooth muscle actin positive (αSMA+) myofibroblasts resulted in invasive tumor progress and poor survival (280). This indicates that fibrosis and desmoplasia in pancreatic cancer stroma constituted a protective response from the host. It is not known whether ascorbate affects the αSMA+ myofibroblasts in pancreatic tumors. Apparently, the ascorbate -induced fibrosis is specific to tumor tissues because liver pathology showed normal results without fibrosis. While the detailed tumor-stroma interaction under ascorbate treatment is worth further investigation, an inhibitory effect was seen in metastasis in ascorbate -treated tumors.

The vast majority of pancreatic cancer patients have metastatic disease at the time of diagnosis, leading to dismal prognosis and poor treatment outcomes. As there are few options in the management of this devastating cancer, the low-toxic therapy of high-dose intravenous ascorbate

holds great promise. As our mechanistic understanding advances, clinical study should be advanced to define efficacy.

Chapter 5. Novel HDAC inhibitors as inhibitors for pancreatic cancer EMT

5.1. Abstract

Inhibition of HDACs results in growth arrest, differentiation, and apoptosis of tumor cell and causes tumor regression in animal models, thereby promoting HDAC inhibitors as promising agents for anti-cancer therapy. SAHA, an HDAC inhibitor, has been approved by the FDA for cutaneous T-cell lymphomas (CTCL) treatment. SAHA and another HDAC inhibitor MS-275 are in the pipeline for clinical development in the treatment of solid tumors. However, data from clinical trials showed no significant efficacy using current HDAC inhibitors in treatment of solid malignancies (302). In order to increase efficacy, we tested a number of novel SAHA and MS-275 derivatives. Compound St-1 showed lower IC-50 values compared to the parent compounds SAHA and MS-275 against pancreatic cancer cell lines (PANC-1, and BxPC-3). Neither the parent compounds nor St-1 were toxic to immortalized non-tumorigenic pancreatic ductal epithelial cells (hTERT-HPNE). St-1 is more potent in inhibiting CSCs when compared to the parent compounds. Collectively, the above findings suggest that St-1 could be a more potent anti-cancer compound compared to SAHA and MS-275.

Surprisingly, we discovered another derivative St-3 which showed a totally different mechanism of action than the parent HDACs. St-3 potently inhibited pancreatic CSCs, cell invasion and migration and induced cytotoxicity. Unlike the parent compounds, St-3 showed poor potency in HDAC inhibition. Using fluorescence polarization assay and amplified luminescent proximity homogeneous assay, we identified that St-3 is an inhibitor for HuR, a RNA binding protein.

In this chapter, data will be described in two parts. The first part will focus on findings of the St-1 compound and the second part will discuss findings of the St-3 compound.

5.2. Part 1: St-1 as a potent HDAC inhibitor

5.2.1. Introduction

Recent studies suggested that HDACs are involved in EMT regulation. Von Burstin and colleagues demonstrated that HDAC1 and 2 together with Snail, epigenetically silenced E-cadherin expression, thereby induced EMT (303). In another study, inhibition of HDAC6 using SiRNA or a small-molecule inhibitor, tubacin, attenuated TGF-β mediated EMT induction (304). Therefore, inhibition of HDACs is an attractive strategy to inhibit EMT.

In this study, we focused on analogues of two major HDAC inhibitors, SAHA and MS-275, which are currently at different stages of clinical development for treatment of solid tumors. SAHA is a hydroxamic acid derivative that has been shown to inhibit Class1, 2, and 4 HDACs nonspecifically and was approved by FDA for CTCL treatment. However, SAHA showed only modest to no activity against breast, colorectal, non-small cell lung cancer (305) and squamous cell carcinoma of the head and neck (306) in clinical trial. The most common adverse effects of SAHA include diarrhea, fatigue, nausea, pulmonary embolism and thrombocytopenia. MS-275 is a synthetic benzamide derivative that inhibits HDAC1, 2 and 3. MS-275 enhances histone (H3 and H4) acetylation, p21 expression and caspase-3 activation in peripheral-blood mononuclear cells and therefore has been tested in phase 1 and 2 clinical trials to treat patients with leukemia and lymphoma as well as some solid tumors in phase 1 and 2 clinical trials (213, 216, 307). However, despite having a long half-life in humans (39-80 hrs) (307) it had limited antitumor activity in phase 1 clinical trials (216, 308). In pancreatic cancer patients, MS-275 had not shown objective response in a phase 1 study (213). Dose limiting toxicity of MS-275 includes infections, neurological toxicity and somnolence (201). Current HDAC inhibitors in combination with other chemotherapeutic drugs also have not provided any additional

therapeutic benefits in clinical studies (214, 215). New HDAC inhibitors with better potency and specificity are needed.

In an effort to look for more potent HDAC inhibitors, we tested 28 novel synthetic derivatives based on SAHA and MS-275 structures for their anti-cancer activity *in vitro*.

5.2.2 Results

5.2.2.1. Derivatives of SAHA and MS-275 inhibited pancreatic cancer cell proliferation

First, we compared the anti-proliferative activity of the 28 derivatives to SAHA and MS-275 against pancreatic cancer cell line PANC-1 and BxPC-3. Out of the 28 derivatives, compound St-1 showed lower IC-50 (concentration that is required to kill 50% of the cells) values compared to both of the parent compounds (**Fig. 5.1A, B**). In PANC-1 cells, IC-50 values of SAHA and MS-275 were both > 40 µM, while IC-50 of St-1 was 10.66 µM. In BxPC-3 cells, IC-50 was > 50 µM for MS-275, 16 µM for SAHA, and 6.5 µM for St-1 **(Table 2)**.

We have also used the immortalized non-tumorigenic pancreatic ductal epithelial cells (hTERT-HPNE) to examine toxicity of these compounds against normal cells *in vitro*. Results showed that neither the parent compounds nor St-1 were toxic to hTERT-HPNE cells **(Fig. 5.1C)**. St-1 might possess low *in vivo* toxicity, which is worth further investigation, given its improved cytotoxicity to pancreatic cancer cells.

5.2.2.2. St-1 potently induced apoptosis in pancreatic cancer cells

Because various HDAC inhibitors have been shown to induce apoptosis, we examined the ability of St-1 in cleaving caspase-3, a major mediator of apoptosis. PANC-1 and BxPC-3 cells were exposed to different concentrations of SAHA, MS-275 or St-1. As shown in the figure (**Fig. 5.2**),

SAHA and MS-275 only weakly induced caspase-3 cleavage in 24 hrs of treatment. However, St-1 treatment in 24 hrs resulted in strong caspase-3 cleavage in both PANC-1 and BxPC-3 cells.

Figure -5.1A

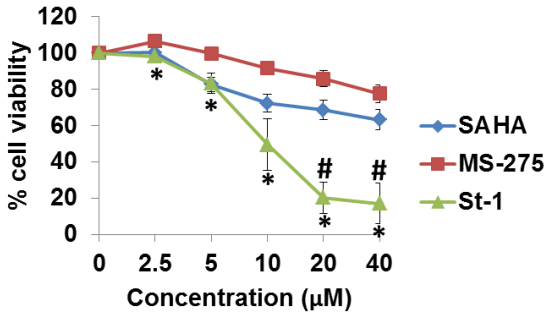


Figure -5.1B

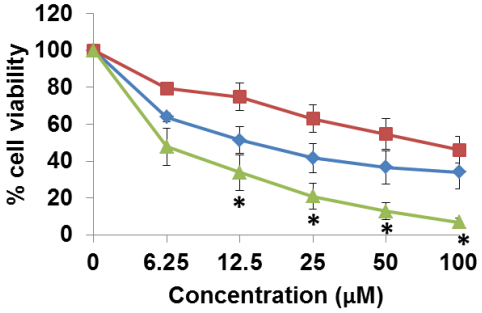


Figure -5.1C

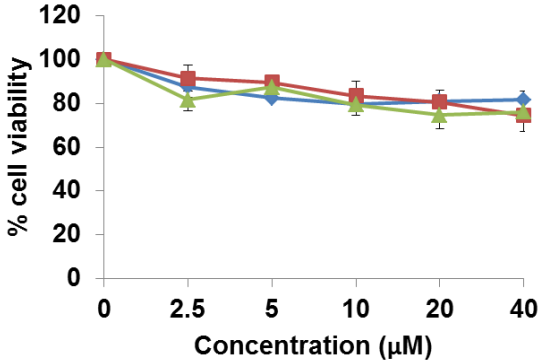


Figure 5.1- Cytotoxicity of SAHA, MS-275, and St-1 on pancreatic cancer cell lines. Pancreatic cancer cell lines PANC-1 (***A),*** BxPC-3 (***B)***, and hTERT-HPNE cells (***C)*** were treated with increasing concentrations of SAHA, MS-275, St-1. Cell viability was measured by MTT assay after 48hrs for PANC-1 and hTERT-HPNE cells, and after 24 hrs for BxPC-3 cells.*, P<0.05 versus MS-275; #, P<0.05 versus SAHA treatmentby T-test.

Table -2

IC-50 (µM)±SEM		
	PANC-1	**BxPC-3**
SAHA	**>40**	**16±4.89**
MS-275	**>40**	**>50**
St-1	**10.66±2.16**	**6.5±1.22**

Table 2- IC-50 values of SAHA, MS-275, and St-1.

Figure -5.2

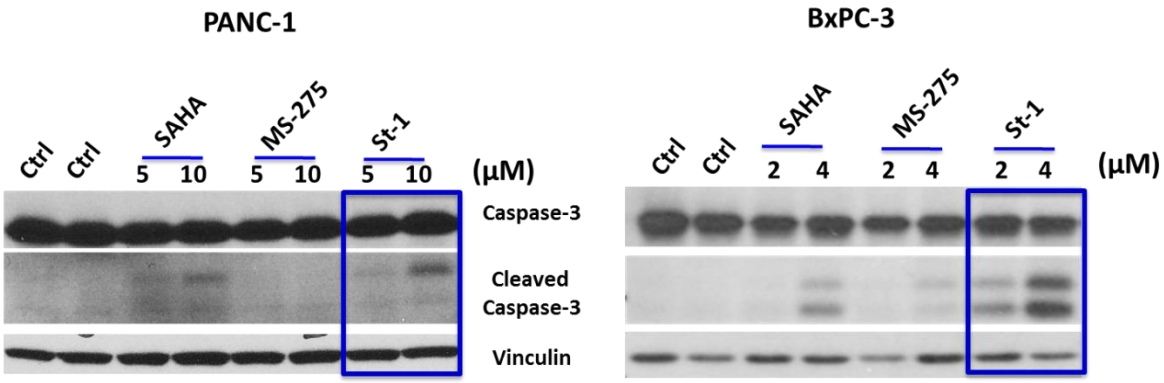


Figure 5.2-Effect of SAHA, MS-275 and St-1 on caspage-3 cleavage. PANC-1 and BxPC-3 cells were treated with various concentrations of SAHA, MS-275 and St-1 for 24hrs and then western blotting was performed to detect total and cleaved caspase 3. Vinculin was used as a loading control.

5.2.2.3. St-1 inhibited pancreatic CSCs

CSCs form spheroids in suspension culture by overcoming anoikis, a form of cell death caused by loss of adhesion. SAHA has been shown to induce morphological differentiation of PANC-1 cells (309), implying its ability to reduce the undifferentiated CSCs. Hence, we tested the effect of SAHA, MS-275 and St-1 on CSCs, using sphere formation of pancreatic cancer cells. PANC-1 cells were exposed to 10 µM, whereas BxPC-3 cells were exposed to 3 µM of SAHA, MS-275 and St-1 and spheroids were allow to form for 7 days. The results showed that SAHA and MS-275 at the concentration of 10 µM did not inhibit PANC-1 spheroid formation. St-1 under the

same condition significantly reduced both number and size of PANC-1 spheroids. In BxPC-3 cells, St-1 was more potent than SAHA in inhibiting the spheroid formation, while MS-275 did not show any effect at 3 µM (**Fig. 5.3**). The data indicated a stronger effect of St-1 in inhibiting pancreatic CSCs, compared with SAHA and MS-275.

Figure -5.3

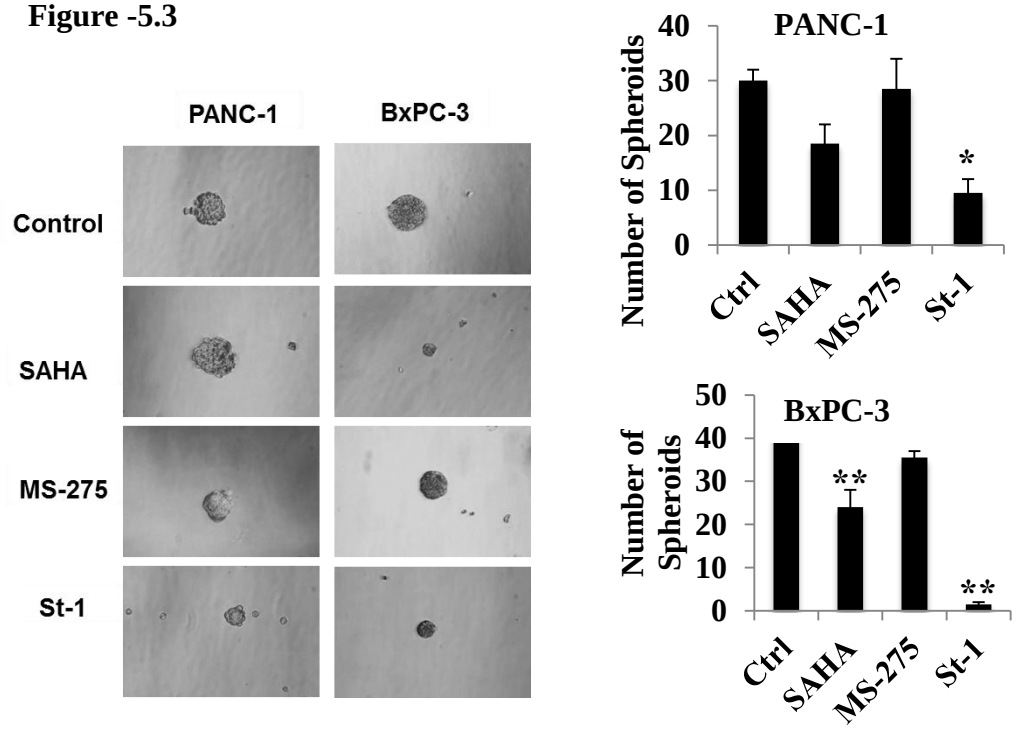


Figure 5.3- St-1 inhibits PANC-1 and BxPC3 spheroids formation. Cells were seeded at 4000 cells/well in single-cell suspension into 24-well ultra-low adherent tissue culture plates. PANC-1 was treated with 10 µM, and BxPC3 was treated with 3 µM of SAHA, MS-275 or St-3. Spheroids were counted after 7 days.*, $P<0.05$, **, $P<0.001$ by T-test.

5.2.2.4. St-1 increased histone acetylation

We then examined the activity of St-1 in inhibiting HDACs. Acetylation in all 4 histones H2A (Lys 5), H2B (Lys 5), H3 (Lys 9) and H4 (Lys 8) was detected. Western blot analysis for histone

acetylation was performed with total lysate from PANC-1 and BxPC-3 cells after treatment with SAHA, MS-275 and St-1 (**Fig. 5.4A, B**).

Figure -5.4A

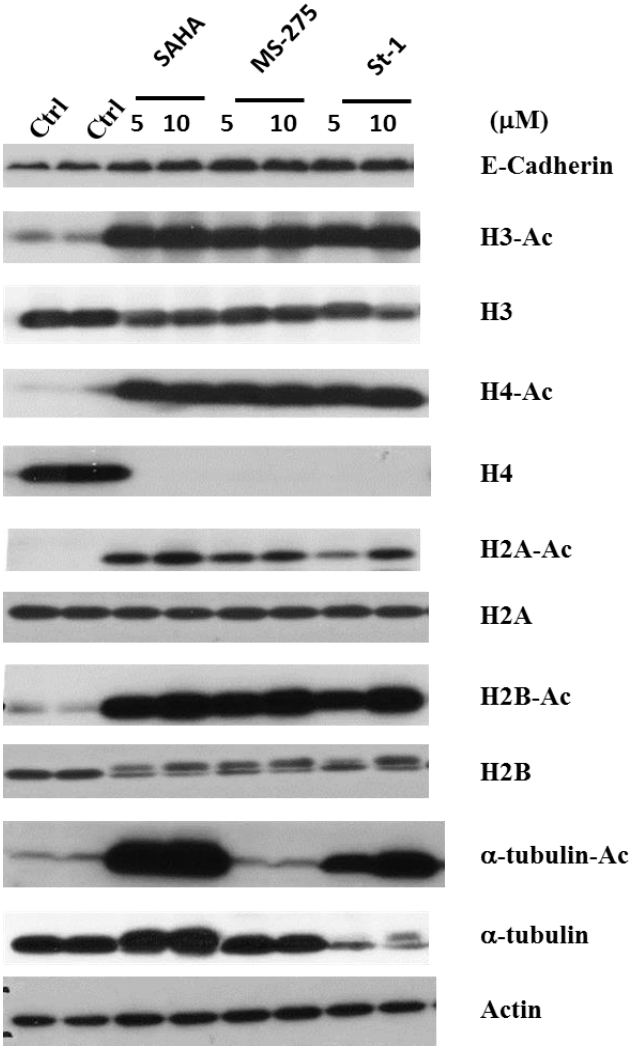


Figure -5.4B

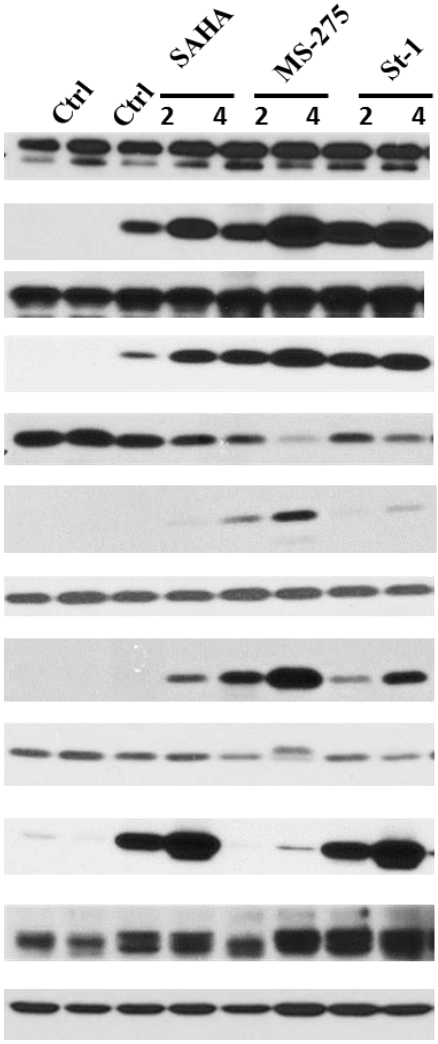


Figure 5.4- Effect of SAHA, MS-275, St-1on acetylation of histones. PANC-1***(A)*** and BxPC-3 ***(B)*** cells were exposed to SAHA, MS-275, St-1 or vehicle control for 24 hrs and acetylation of histones and α-tubulin was detected by western blot. Actin was a loading control.

As expected, SAHA and MS-275 potently increased the acetylation of all 4 histones (H2A, H2B, H3, and H4) in both cell lines. St-1 increased histone acetylation similar to SAHA and MS-275. In addition to histones, HDAC inhibitors increased acetylation of many non-histone proteins such as α-tubulin and p53. SAHA and St-1 induced tubulin acetylation, whereas MS-275 did not.

Inhibition in class I and II HDACs in theory could result in EMT inhibition by elevating E-cadherin expression. Therefore, we determined whether St-1 could inhibit EMT. Indeed, all three HDAC inhibitors increased E-cadherin expression in PANC-1 and BxPC-3 cells, indicating EMT inhibition.

5.2.3. Discussion

Epigenetic changes are associated with malignant cellular transformation. Unlike genetic changes, epigenetic changes are reversible, raising the potential for altering epigenetic changes, using HDAC inhibitors, as cancer therapy. Several HDAC inhibitors, including SAHA, are currently at various stages of clinical development for solid malignancies, however, they showed only limited efficacy so far (302). The purpose of this study was to develop novel HDAC inhibitors with improved activity and reduced toxicity. Our data showed that the novel HDAC inhibitor St-1 exhibited anti-tumor activities that involve not only cell death, but also cancer stem cell inhibition.

Like SAHA and MS-275, St-1induced cell death selectively in pancreatic cancer cells, but not in normal pancreatic ductal epithelial cells. St-1 had lower IC-50 values compared to parent compounds (210). St-1 induced caspase-3 cleavage more potently than SAHA and MS-275. The genomic land scape of PANC-1 and BXPC-3 cells is completely different (**Table 1**). Briefly speaking, BxPC-3 cells have wild type *K-Ras* and *CDKN2A/p16*, but express high level of pro

angiogenic cytokine cyclooxygenase 2 (COX2), whereas PANC-1 cells harbor mutations in *K-Ras, CDKN2A/p16* and *p53*, but have relatively low COX2 expression. The ability of St-1 to induce cell death in both pancreatic cancer cell lines that are different with respect to the status of *K-Ras, p53, CDKN2A/p16*, and COX2 (7) suggested that St-1 can potentially inhibit pancreatic tumors with heterogeneity.

There are controversies regarding whether HDAC inhibitors could inhibit CSCs. A number of small-molecule HDAC inhibitors have been reported to induce differentiation in several cancer cell lines (310). Contrary to these reports, Debeb et al. reported that SAHA and valproic acid promoted expansion of breast CSCs through dedifferentiation (311). Results from our studies showed that St-1 inhibited pancreatic CSCs with better potency than SAHA and MS-275. We did not observe an increase of sphere forming upon treatment with SAHA or MS-275. It was also reported that HDAC inhibitors desensitize breast cancer cells to chemotherapeutic drug such as taxol. Our future studies will evaluate the effect of St-1 on inducing drug resistance.

HDAC inhibition results in hyper acetylation of their target molecules such as histones. St-1 has broad specificity in HDAC inhibition and resulted in increased acetylation of all histones, just as SAHA and MS-275 (203, 312). Different isoforms of HDACs have various locations, expression levels and functions. Nonspecific inhibition of HDACs resulted in toxic side effects (313). Therefore, St-1 might have toxic side effects similar to its parent compounds. Because St-1 is more potent, it is possible that St-1 might have a larger therapeutic index (Toxic dose 50 (TD 50) ÷ Effective dose 50 (ED50)) when compared to its parent compounds. Further studies evaluating the *in vivo* activity and toxicity of St-1 will serve as a guide for structure modification on St-1 or development of new St-1 derivatives aiming for better potency and less toxicity. Some HDAC inhibitors exert their anti-tumor actions by interfering with additional targets other

than histones, such as p53 or α-tubulin (314). SAHA and St-1 induced α-tubulin acetylation, whereas MS-275 did not induce tubulin acetylation. Because HDAC6 and Sirt-2 are the predominant enzymes that regulate α-tubulin acetylation, these data suggested that HDAC6 and Sirt-2 were also targets of St-1.

Taken together, data here showed that St-1 is a novel HDAC inhibitor that showed better potency *in vitro* in inhibiting pancreatic cancer cells and CSCs, than the parent compounds SAHA and MS-275. More work is needed to assess its *in vivo* efficacy and toxicities.

5.3. Part 2: St-3 as a novel HuR inhibitor

5.3.1. Introduction

One of our 28 tested HDAC derivatives, St-3, was more cyto-toxic and more effective in inhibiting CSCs, cell migration and invasion compared to parent compounds, however, showed poor potency in HDAC inhibition. St-3 has a totally different mechanism of action. Using *in vitro* assays, we identified HuR as a target of St-3.

HuR is a ubiquitously expressed RNA-binding protein that binds to the 5'-untranslated regions (UTR) or 3' UTR of mRNA and regulates the transcript stability and translation (315). HuR recognizes and binds to adenine/uridine (AU) and U-rich elements (ARE) in the UTR of mRNA through RNA recognition motifs (RRMs). HuR contains three highly conserved RRMs and a variable basic hinge region between its RRM2 and RRM3. RRM1 and RRM2 mediate binding of HuR to ARE. RRM3 and hinge region play a distinct role in formation of the cooperative HuR and ARE complex (316).

HuR was initially discovered to be important for the normal development and maintenance of the nervous system in *Drosophila melanogaster* (317). HuR shares common structural similarities

with RNA binding proteins of the embryonic lethal abnormal vision (ELAV) protein family. HuR expressed in many cell types including adipose, intestine, spleen and testis. Other ELAV family members HuB, HuC and HuD are exclusively localized to terminally-differentiated neurons and therefore called the neuronal Hu proteins (318).

HuR plays an important role in many pathologies, including chronic inflammation, cardio vascular diseases and cancer (319). HuR has been found to be abundant in pancreatic cancer as well as in many other types of cancers (320-323). HuR has been found to influence sensitivity of pancreatic cancer cells to gemcitabine by stabilizing the mRNA of gemcitabine metabolizing enzyme deoxycytidine kinase (324). HuR has also been found to enhance cancer cell invasion and metastasis by stabilizing mRNAs of MMPs, uPA, and Snail (325-328). HuR knockdown dramatically reduced cell growth in MCF7 breast cancer cells and invasive properties in MDA-MB-231 breast cancer cells (329). As Snail is a critical repressor of E-cadherin, and E-cadherin repression is a hallmark of EMT, a potential link between HuR and EMT is indicated. Based on the above literature evidence, inhibition of HuR function is an attractive strategy to inhibit cancer development. In this study, we tested the activity of compound St-3 as a novel HuR inhibitor, and investigated the involvement of HuR in pancreatic cancer EMT.

5.3.2. Results

5.3.2.1. St-3 inhibited pancreatic cancer cell proliferation and invasion

As a structural analogue of SAHA and MS-275, St-3 affected the growth of pancreatic cancer cells in a more potent manner than the parent compounds (**Fig. 5.5A, B**). St-3 had IC50 values of 21 μM for PANC-1 cells and 6.25 μM for BxPC-3 cells, while MS-275 had IC50 > 40 μM for both cell lines, and SAHA had IC50 of >40 μM for PANC-1 and 16 μM for BxPC-3 **(Table 3)**.

Neither the parent compounds nor St-3 affected proliferation of hTERT-HPNE cells even at 40 μM (**Fig. 5.5C**).

To test the effect of St-3 on PANC-1 cell invasion and migration, we performed Boyden chamber invasion assay. PANC-1 cells seeded into the Boyden chambers that were precoated with matrigel were used to measure cell invasion. Boyden chambers without matrigel coating were used to measure cell migration. At a sub-cytotoxic concentration of 2 μM, St-3 significantly inhibited PANC-1 cells to migrate or to invade through Matrigel (**Fig. 5.6**).

5.3.2.2. St-3 inhibited pancreatic CSCs.

Tumor spheroid formation assay was used to test the ability of St-3 in inhibiting CSCs. St-3 inhibited the growth of the spheroids from BxPC3 cells at a concentration of 3 μM and completely eradicated the spheroid formation from PANC-1 cells at 10 μM (**Fig. 5.7A, B**). These concentrations were lower than IC50s to the bulk pancreatic cancer cell population. This data suggested that St-3 inhibited CSCs preferentially than the bulk population of cancer cells. We further determined the expression of putative pancreatic CSC markers CD24, CD44 and EpCAM. Cells expressing all 3 markers are indicative for CSCs. Using immunofluorescence detection by flow cytometry, the percentage of $CD24^{+}CD44^{+}EpCAM^{+}$ triple positive cells were detected. Significant reduction was found after only 24 hrs of treatment with 4 μM St-3 for BxPC3 cells and with 10 μM St-3 for PANC-1 cells (**Fig. 5.7C**).

5.3.2.3. St-3 affected pancreatic cancer EMT markers.

St-3 potently increased epithelial marker E-cadherin and decreased mesenchymal markers Snail and N-cadherin in PANC-1 cells. SAHA on the contrary enhanced Snail and N-cadherin, while

also slightly elevated E-cadherin level. This suggested that St-3 inhibits EMT by a mechanism different than SAHA (**Fig. 5.8 A-C**).

Figure -5.5A

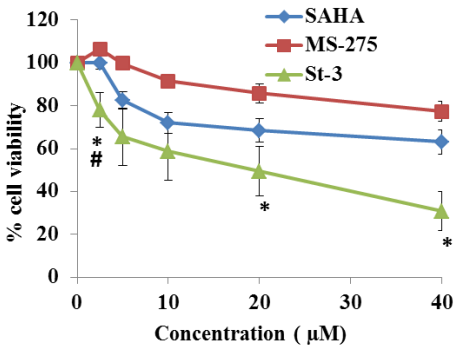


Figure -5.5B

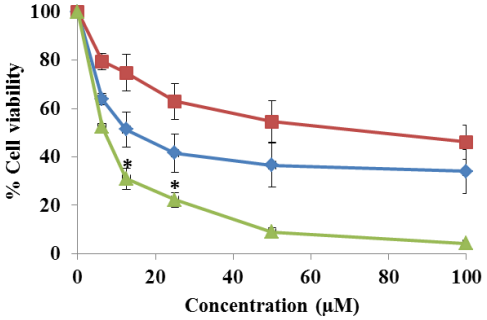


Figure -5.5C

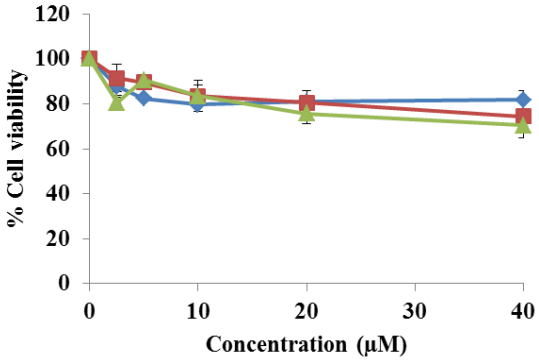


Figure 5.5- Cytotoxicity of SAHA, MS-275, St-3 on pancreatic cancer cell lines. Pancreatic cancer cell line PANC-1 ***(A),*** BxPC-3 ***(B)***, and hTERT-HPNE cells ***(C)*** were treated with increasing concentrations of SAHA, MS-275, St-3. Cell viability was measured by MTT assay after 48hrs for PANC-1 and hTERT-HPNE cells, and after 24 hrs for BxPC-3. *, P<0.05 versus MS-275; # P<0.05 versus SAHA, by T-test.

Table -3

IC-50 (µM)±SEM		
	PANC-1	**BxPC-3**
SAHA	**>40**	**16±4.89**
MS-275	**>40**	**>50**
St-3	**21±18.25**	**6.25±0.20**

Table 3- IC-50 vales of SAHA, MS-275, and St-3.

Figure -5.6

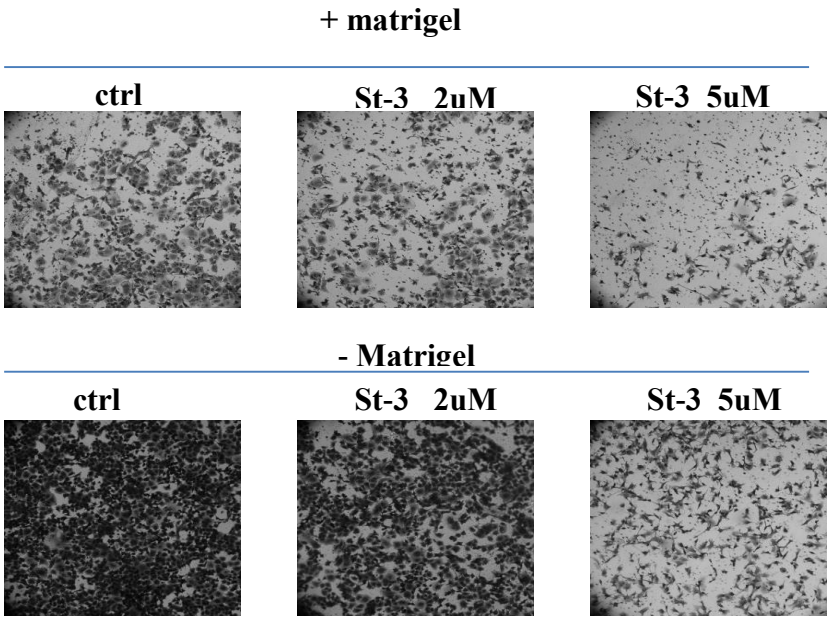


Figure 5.6- St-3 inhibited pancreatic cancer cell invasion Matrigel invasion assay. PANC-1 cells were treated with 2, 5μM of St-3 for 36 hrs.

Figure -5.7A

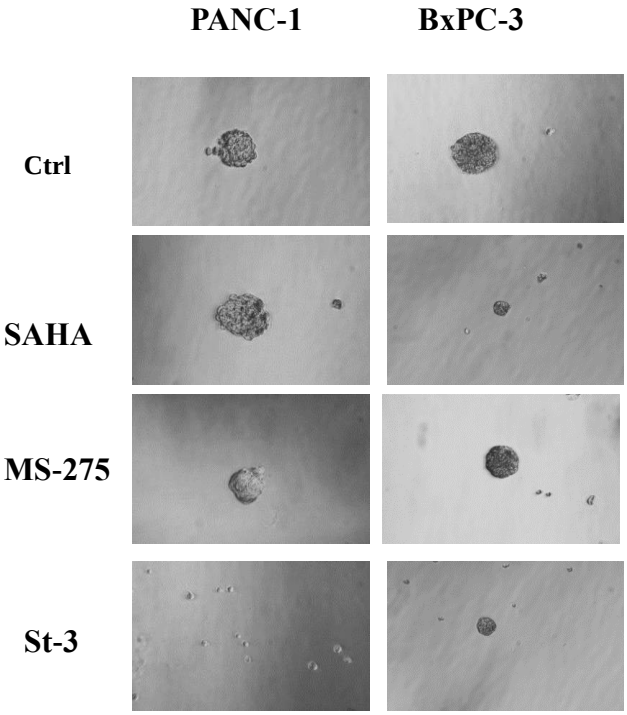


Figure -5.7B

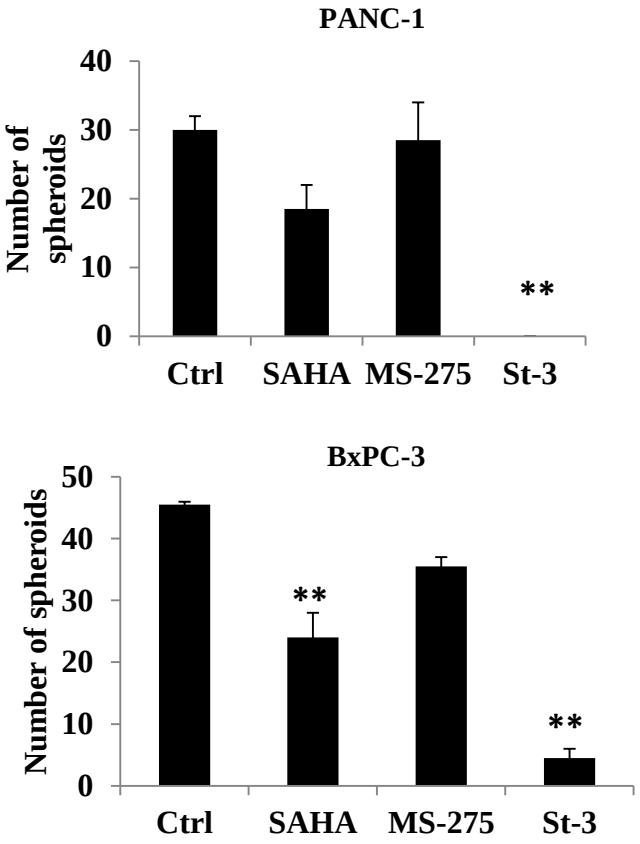


Figure -5.7C

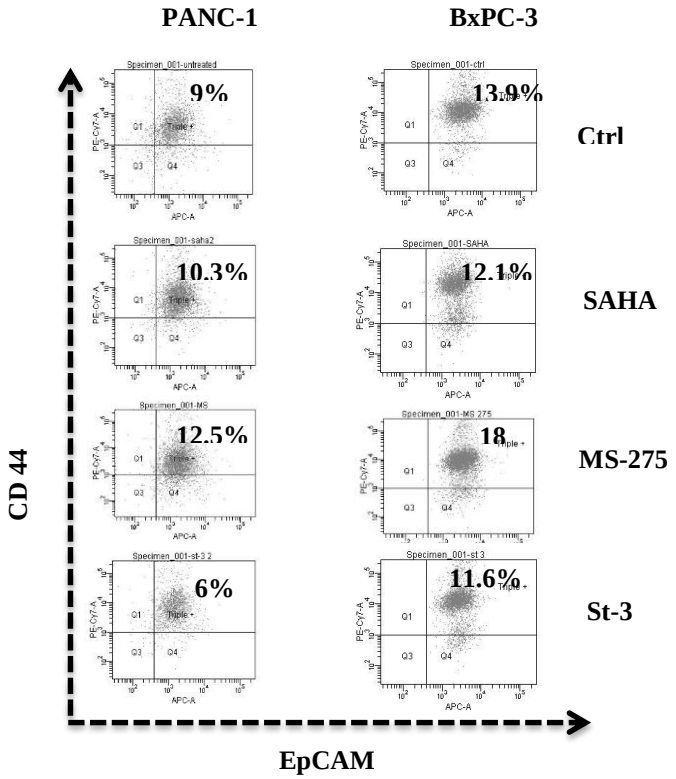


Figure 5.7- St-3 inhibits pancreatic CSCs. ***A, B*** Cells were seeded at 4000 cells/well in single-cell suspension into 24-well ultra-low adherent tissue culture plates. PANC-1 was treated with 10 µM, and BxPC3 was treated with 3 µM of SAHA or St-3. Spheroids were counted after 7 days. **, P<0.001 by T-test. ***C.*** The figures show $CD44^{+}EpCAM^{+}$ cells under the $CD24^{+}$ gate. The $CD24^{+}CD44^{+}EpCAM^{+}$ cells are putative pancreatic CSCs. St-3 treatment at 4 µM (BxPC3), or 10 µM (PANC-1) for 24 hrs already repressed the CSC subpopulation.

Figure -5.8A

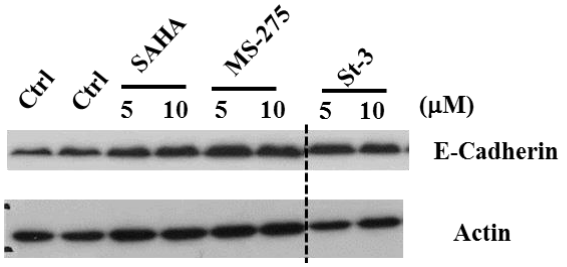


Figure -5.8B

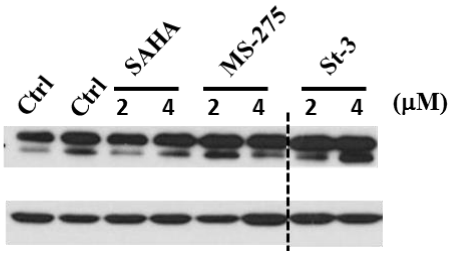


Figure -5.8C

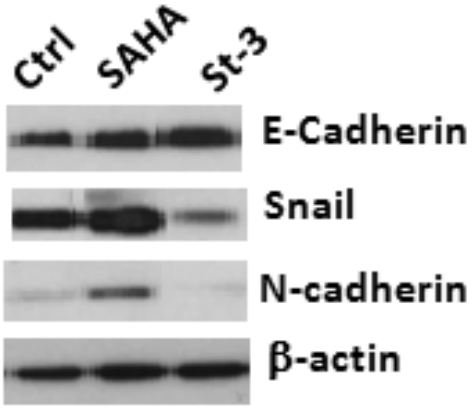


Figure 5.8- St-3 affects EMT marker proteins. PANC-1***(A)*** and BxPC-3 ***(B)*** cells were exposed to SAHA, MS-275, St-3 or vehicle control for 24 hrs and then E-cadherin expression was detected by western blot. ***C.*** PANC-1 cells were treated with 10 μM of SAHA and St-3. The pattern showing E-cadherin up and Snail and N-cadherin down indicates inhibition of EMT.

5.3.2.4. St-3 lost the ability to inhibit HDACs

We then examined the activity of St-3 in inhibiting HDACs using SAHA and MS-275 as positive controls. As expected, SAHA and MS-275 increased the acetylation of all 4 histones (H2A, H2B, H3, and H4). However, St-3 showed much weaker potency in HDAC inhibition than SAHA and MS-275. St-3 did not enhance α-tubulin acetylation **(Fig. 5.9)**. These results suggest that St-3

did not exert its anti-pancreatic cancer activity through HDAC inhibition but rather through a different mechanism(s) of action.

Figure -5.9A **Figure -5.9B**

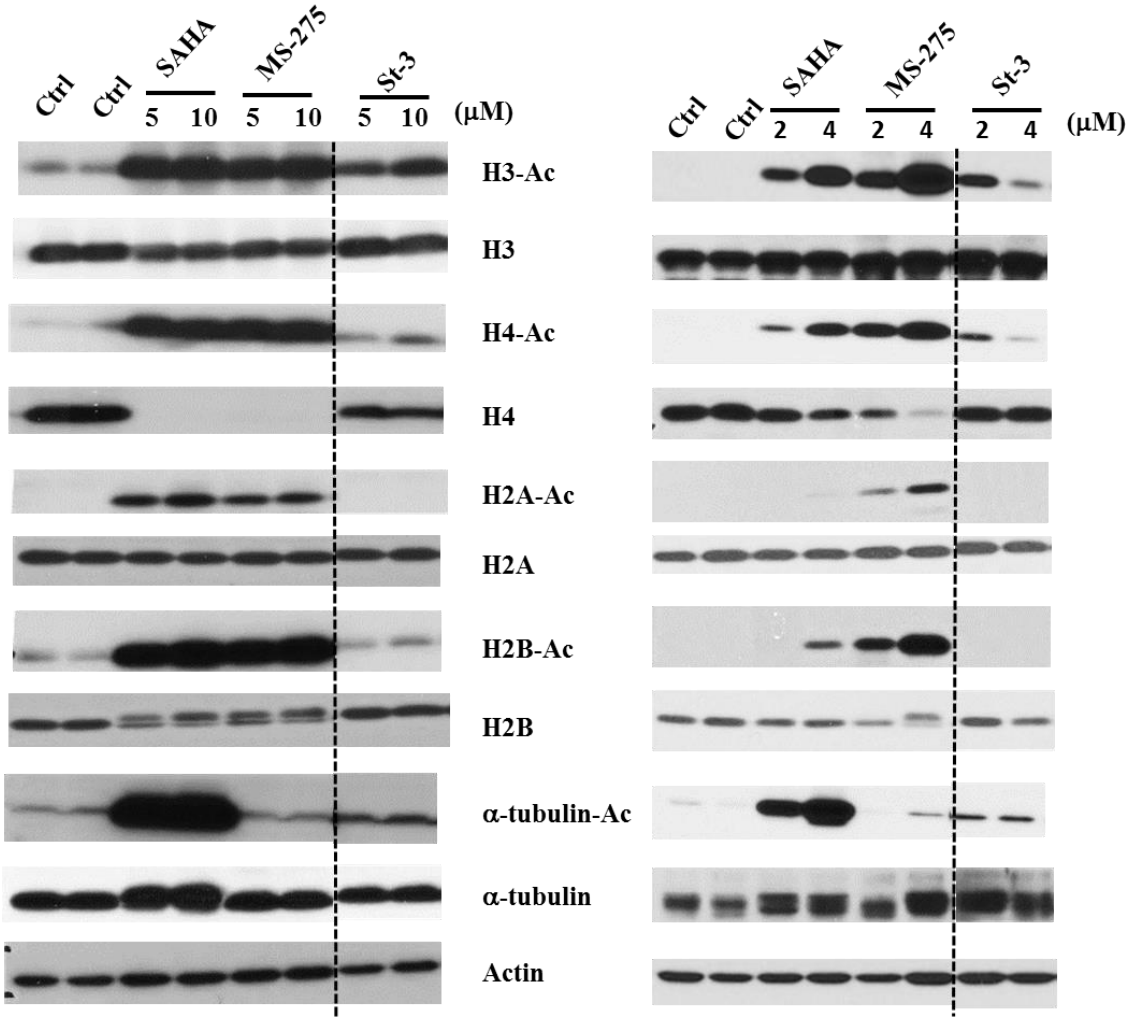
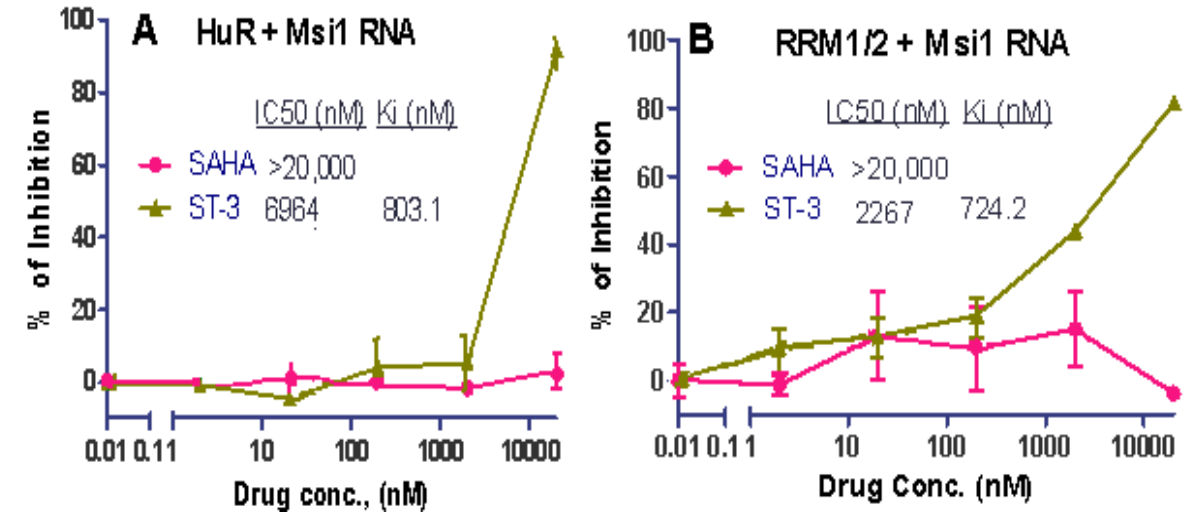


Figure 5.9- St-3 loses activity to inhibit HDAC. PANC-1***(A)*** and BxPC-3 ***(B)*** cells were exposed to SAHA, MS-275, St-3 or vehicle control for 24 hrs and acetylation of histones and α-tubulin was detected by western blot. Actin was a loading control.

Figure -5.10



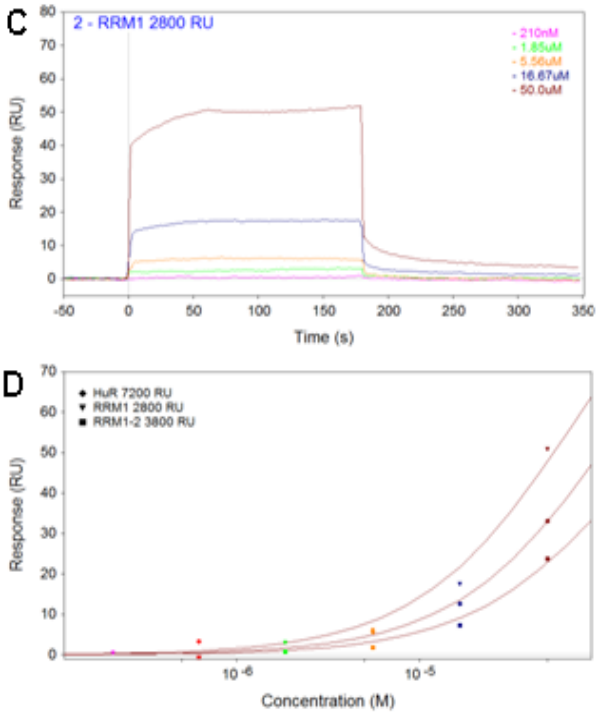


Figure 5.10- St-3 inhibits HuR binding to mRNA. ***A.*** Fluorescence polarization assay. Twenty five nM full-length human HuR and 2 nM FITC-labeled 16-nt Msi1 RNA sequence was used. ***B.*** Alpha assay. 100 nM His-tagged HuR RRM1/2 fragment and 25 nM biotinylated 16-nt Msi1 RNA sequence were used. Labeled scramble 16-nt RNA was used as control. Ki values were calculated based on the Kd and the dose-response curves. ***C-D.*** SPR analyses of St-3 binding to immobilized full length HuR, RRM1 and RRM1-2. Increase in refractive index shown by resonance (RU) indicates St-3 binding.

5.3.2.5. St-3 inhibited HuR binding with target mRNA

Instead of HDAC inhibition, St-3 potently inhibited binding of HuR to its target mRNAs. In a fluorescence polarization assay, St-3 inhibited binding of HuR protein to its known target Msi1 mRNA with IC50 and Ki of 6.9 μM and 0.8 μM respectively (**Fig. 5.10A)**. The Alpha assay, another bead-based technology widely used to analyze protein-protein interactions, and their inhibitors, was employed to monitor the interactions between RRM1/2 of HuR and its target RNA MSi1 in presence of St-3 **(Fig. 5.10B).** St-3 inhibited binding, with IC50 and Ki of 2.2 μM and 0.7 μM respectively (**Fig. 5.10A, B**). In contrast, SAHA did not show any inhibitory effect on HuR/Msi1 mRNA binding in both the assay. Data from fluorescence polarization assay or Alpha assay do not indicate whether St-3 is binding to HuR or RNA. Therefore, SPR analysis was performed to detect the direct interaction between HuR and St-3. SPR results suggested that St-3 bound to either full-length HuR or the RNA Recognition Motifs of HuR (RRM1/2) (**Fig. 5.10C, D**) as shown by increase in resonance (RU).

5.3.2.6. St-3 blocked HuR function

HuR binds to the 3'-UTR of target mRNAs, stabilizing the mRNAs and promoting translation. In order to determine the functional consequence of St-3 on downstream targets of HuR, real time PCR assay was performed on gene transcripts of putative HuR targets. HuR has a broad range of target mRNAs that involve in cell survival, proliferation, angiogenesis, anti-apoptosis, and reduced immune recognition (1). Here, we focused on a sub-set of genes related to EMT, CSC and metastasis **(Table 4)**. St-3 treatment at 5 and 10 μM, which were below IC-50, reduced β-catenin, Msi1, urokinase plasminogen activator surface receptor (UPAR), and low density lipoprotein receptor-related protein 6 (LRP 6) to different extents (**Fig. 5.11A)**. As these gene products are important in pathways regulating EMT, CSC and metastasis, the data is consistent

with inhibitions induced by St-3 in pancreatic cancer cell EMT, CSC and metastasis. Especially, the sever inhibition in β-catenin indicated an influence of St-3 on the Wnt/β-catenin pathway.

Table -4

Target mRNA	Binding Site	Influence on mRNA	Main Cancer trait
TGF-β	3′UTR	Stability ↑	Invasion, Immunity/inflammation
β-Catenin		Stability ↑	Invasion and proliferation
Snail	3′UTR	Stability ↑	Invasion
MMP-7	Not known	Not known	Invasion
MMP-9	3′UTR	Stability ↑	Invasion
uPA	3′UTR	Stability ↑	Invasion
uPAR	3′UTR	Stability ↑	Invasion
Msi1	3′UTR	Translation↑ Stability ↑	Invasion

Table 4- Putative HuR target mRNAs that play an important role in cancer cell proliferation, and invasion (1-3). ↑, HuR stabilizes or increases translation; ↓, HuR represses translation.

Figure -5.11A

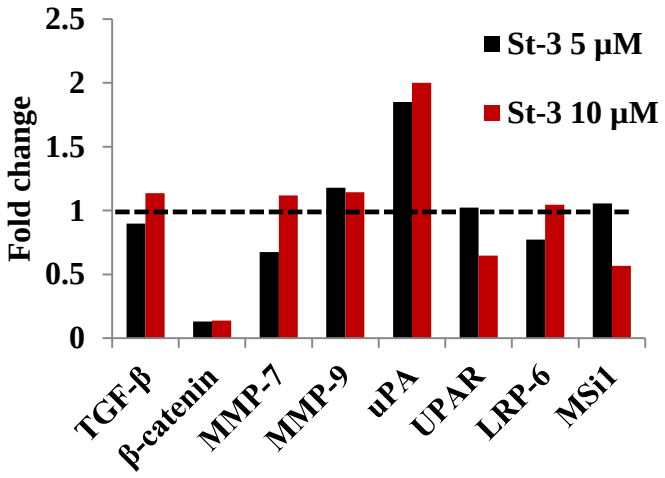


Figure -5.11B

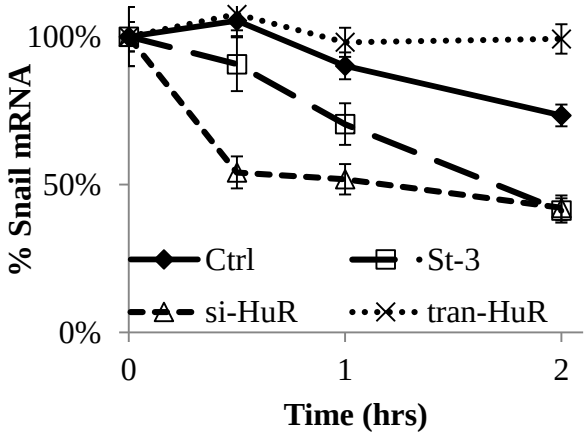


Figure 5.11- St-3 blocks HuR function and reduced HuR target mRNA stability. ***(A)*** Real time PCR determining the effect of St-3 on HuR target expression. ***(B)*** Stability of Snail mRNA after St-3 treatment, HuR knockdown (Si-HuR), HuR over expression (tran-HuR), studied in PANC-1 cells treated with actinomycin D.

Snail mRNA was reported as a direct target of HuR (328). To further confirm that St-3 disrupted HuR function on stabilizing target mRNAs, the stability of Snail mRNA was examined in PANC-1 cells. After St-3 treatment (5 µM), a transcription inhibitor, actinomycin D, was added

and RT-PCR was done at indicated time points to detect Snail mRNA. As comparisons to St-3 treatment, HuR was either knocked down using SiRNA in PNAC-1 cells or was transfected and overexpressed. The results showed that over expression of HuR stabilized Snail mRNA, while knockdown of HuR enhanced Snil mRNA degradation **(Fig. 5.11B)**. St-3 treatment mimicked HuR knockdown in enhancing Snail mRNA degradation **(Fig. 5.11B)**.

5.3.3. Discussion

In this current study, we serendipitously developed St-3 as a novel HuR inhibitor. St-3 was synthesized as a derivative of SAHA and MS-572. However, it exhibited totally a different mechanism of action. St-3 was more potent than the parent compounds in inhibiting pancreatic cancer cell proliferation, invasion, migration, as well as EMT and CSCs. Our *in vitro* data suggested that St-3 executed its function by inhibiting HuR binding to its target mRNAs.

A mounting body of literature suggests that HuR has a central role in cancer. The HuR target mRNAs encode a variety of factors required for cancer cell proliferation, survival, angiogenesis, invasion and metastasis. Both HuR expression and subcellular localization is aberrant in human tumor tissues. In response to various stimuli, HuR moves from nucleus to cytoplasm to stabilize target mRNA to promote cancer progression (323). Inhibition of HuR function by St-3 could be expanded into other cellular pathways and outcomes related to tumor progress.

Inhibitors that block the HuR and ARE interactions could be ideal tools for cancer therapy. However, due to lack of a well-defined binding pocket, limited success has been made in identifying small-molecule inhibitors that directly block the HuR and ARE interactions. Using RNA electrophoretic mobility shift assay Chae et al., identified that quercetin, b-40, b-41 strongly inhibited binding of HuR to its well-known target TNF-α mRNA *in vitro* (330). Meisner

and coworkers have shown that dehydromutactin, MS-444 and okicenone interfered with HuR function by inhibiting its homodimerization (331). More studies are needed to investigate these compounds for their anti-tumor activities. The chemical structure of St-3 has low similarity to those of quercetin, b-40, b-41, dehydromutactin, MS-444 and okicenone, suggesting that St-3 might have different binding sites on HuR or might execute a different mechanism of HuR inhibition. The previously reported compounds are all structurally independent; therefore, they cannot provide information for structure activity relationship (SAR) studies to define a binding pocket, or for rational design of potent and specific HuR inhibitors. St-3 and its structural analogues could potentially be used as a tool in the study of binding pocket of HuR.

The role of HuR in EMT is not well understood. Only one published study proposed Snail as a target of HuR in MCF-7 breast cancer cells (328). Our data here showed that in pancreatic cancer cells, overexpression of HuR stabilized Snail mRNA, whereas knockdown of HuR enhanced Snail mRNA degradation. As a HuR inhibitor, St-3 also reduced Snail mRNA. This provided additional evidence for Snail as a target for HuR. Also, it provided evidence supporting a role of HuR in EMT regulation.

By reducing Snail expression, St-3 enhanced E-cadherin expression, and decreased expression of the mesenchymal marker N-cadherin. These changes represented reversal of EMT. In contrast, SAHA enhanced both E-cadherin and N-cadherin expression in PANC-1 cells. Several studies have demonstrated that overexpression of N-cadherin promotes motility, invasion and metastasis even in the presence of E-cadherin (332, 333). This could potentially be one of the reasons for the lack of therapeutic benefit when SAHA was used to treat solid tumors.

Inhibition of HuR provides disruption in oncogenic pathways that are preferentially active in pancreatic cancer cells relative to normal cells. Therefore, St-3 showed more potent cyto-toxicity against PANC-1 and BxPC-3 cells compared to HDACs but was not toxic to hTERT-HPNE cells. However, St-3 might possess some *in vivo* toxicity. Some predictions can be made from recent studies showing that HuR depletion resulted in apoptosis of progenitor cells in the bone marrow, thymus and intestine. This is because HuR may directly regulate the expression of Mdm-2 at the post transcriptional level (334) and therefore depletion of HuR resulted in decreased Mdm2 and increased p53 and its downstream effectors critical for cell death. More research is needed to explore the mechanism of cell death caused by St-3.

Beta catenin was depleted with St-3 treatment, which plays an important role in stem cell self-renewal. Data here suggested that St-3 might block HuR interaction with β-catenin mRNA and thereby promoted β-catenin mRNA degradation. A recent study by D'Uva and colleagues showed that β-catenin stabilizes SNAI2 and carbonic anhydrase 9 mRNA in cooperation with HuR under hypoxic condition in breast cancer cell lines to induce stem cell features (335). It is possible that a similar mechanism exists in pancreatic tumor tissues under hypoxic condition. Inhibition of β-catenin mRNA and HuR interaction by St-3 warrants further investigation.

In conclusion, we investigated the link between HuR and EMT related genes, and developed a novel HuR inhibitor St-3 which exhibited inhibitory activities to pancreatic cancer cell growth, metastasis, EMT and CSCs. The novel compound St-3 could serve as a tool for study of the HuR-RNA interaction. Moreover, data in this chapter provided a basis and mechanistic evidence for developing St-3 as a new class of HuR inhibitor with potential in pancreatic cancer treatment.

Chapter 6. Targeting EMT for Identification of Inhibitors for Pancreatic CSCs

6.1. Abstract

Drug screening assays based on cytotoxicity usually do not identify compounds that specifically target CSCs, because CSCs comprise only a small portion of the cancer cell population, and it is difficult to propagate stable CSC populations *in vitro* for HTS assays. To establish an efficient way for discovery of drugs that preferentially inhibit CSCs, we developed an immunofluorescent assay for HTS to identify inhibitiors of cancer cell EMT, which is highly associated with cancer cell metastasis and "stemness". Using this assay, 4 chemical libraries containing a total of 41,472 compounds were screened for their ability to induce E-cadherin expression in PANC-1 pancreatic cancer cell line. HTS identified 1,584 hits with the initial screening, using automated microscopy for a cell-by-cell fluorescence intensity analysis. A secondary screening among the initial hits refined the number of positive hits to 73. Each of these 73 compounds was tested at 8 concentrations ranging from 0.15 to 30 μM. Among them, 17 exhibited concentration-dependent induction of E-cadherin expression. Based on chemical structure and availability, we selected 6 compounds which belonged to 2 different chemical structural clusters for evaluation of EMT and CSC inhibition. A novel compound 1-(benzylsulfonyl) indoline (BSI, Compound #38), significantly inhibited pancreatic cancer cell migration and invasion. BSI induced Histone 3/4 acetylation resulted in E-cadherin up-regulation. Moreover, BSI effectively inhibited tumor spheroid formation, which is an indicator of CSC inhibition. Six analogues of BSI were tested but did not show superior anti-migration or anti-CSC activities compare to BSI. This study demonstrated a new and feasible approach for discovery of agents targeting EMT and CSCs using HTS, and identified a class of novel chemicals that could be developed as anti-EMT and anti-CSC drug leads.

6.2. Introduction

Few approaches have been described to directly screen for agents that are specifically cytotoxic to CSCs. One reported HTS approach involves utilizing a genetically modified breast cancer cell line that has low expression of a cell adherent protein E-cadherin and is therefore forced into a mesenchymal status (336). Other approaches use suspension cultures of tumor spheroids as an indication of CSCs (337). The challenge is likely due to two reasons: first, although CSCs can be identified and isolated by cell surface marker profiles, and tumor spheroids are cultured *in vitro*, it remains difficult to propagate a stable, undifferentiated CSC population in cell culture suitable for HTS for many solid tumors. Moreover, because CSCs comprise only a small portion of cancer cell populations, standard high-throughput cytotoxic assays applied to bulk populations of cancer cells do not identify agents with CSC-specific toxicity.

Among the biological properties of CSCs, one that is highly associated with the phenotypic characteristics identified in the induction of cancer cells is EMT. EMT is an important initial step during the complicated process of cancer cell dissemination and metastasis, characterized by progressive loss of epithelial markers, such as E-cadherin (95, 122, 338-343). Loss of cell surface E-cadherin is a classical hallmark of EMT (344). Studies have shown that loss of E-cadherin was the rate limiting step in the progression from adenoma to carcinoma and the subsequent formation of metastasis (345, 346). In this study, we aimed to establish a HTS assay to simply identify compounds that can restore E-cadherin, in the hope of discovering inhibitors for pancreatic CSCs.

6.3. Results

6.3.1. Development of HTS assay based on immunofluorescence detection of E-cadherin expression

To establish the HTS assay, we selected a pancreatic cancer cell line that possesses an "EMTed" profile and has inducible E-cadherin. Three pancreatic cancer cell lines were examined for their basal expression of epithelial (E-cadherin, ZO-1) and mesenchymal markers (N-cadherin, Vimentin), as well as transcriptional repressors of E-cadherin (Snail-1 and Zeb-1) (**Fig.6.1 A-E**). MIA PaCa-2 was highly mesenchymal in nature with undetectable levels of E-cadherin, whereas HPAF-2 is highly epithelial in nature with a high level of E-cadherin expression. PANC-1 cells were mesenchymal in nature, as shown by low levels of epithelial markers (E-cadherin and ZO-1) and relatively high levels of EMT transcription factors (Snail and Zeb), making it potentially suitable for development of the HTS assay. We then examined whether E-cadherin expression could be induced in PANC-1 cells. Indeed, 1-4 mM of sodium butyrate, a histone deacetylase inhibitor (347), induced E-cadherin in PANC-1 cells concentration dependently (**Fig.6.2**). CSC populations at baseline were detected in PANC-1 cells using multi-fluorescence labeled flow cytometry to identify $CD24^{+}CD44^{+}$ $EpCAM^{+}$ cells (150). The triple positive cells comprise ~1.3% of the PANC-1 bulk population (**Fig.6.3A**). Moreover, PANC-1 cells were resistant to gemcitabine treatment, with 100 μM gemcitabine only achieving 60% inhibition (**Fig.6.3B**). Therefore, PANC-1 was selected to be used in HTS for detection of E-cadherin induction. Immunofluorescent assay for detection of E-cadherin was established in a 96-well plate and transformed into a 384-well plate and optimized for automation with HTS, using a primary antibody for E-cadherin recognition, and a secondary antibody conjugated to a red fluorophore, Alexa Fluor 594.

Figure -6.1A

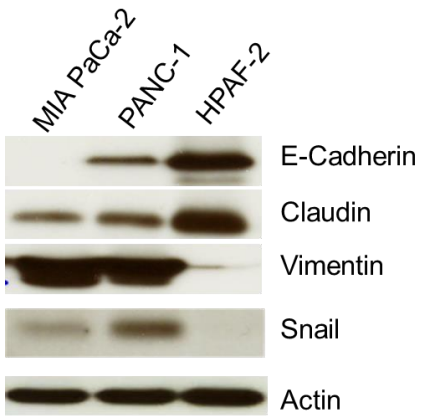


Figure -6.1B

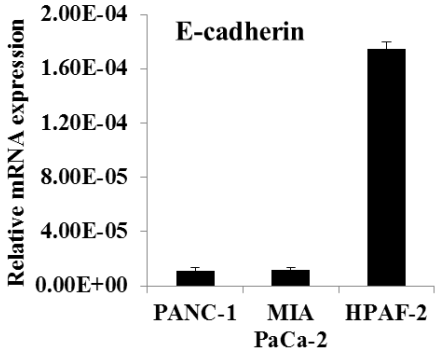
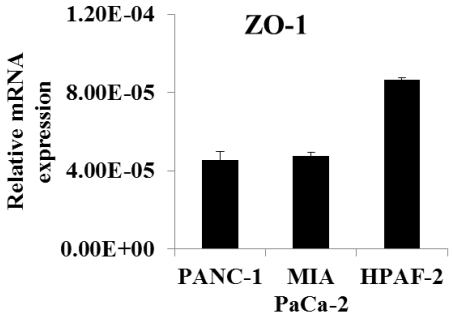


Figure -6.1C



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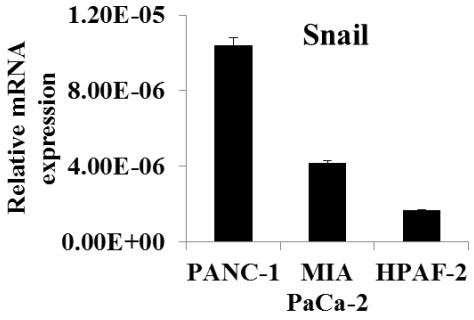

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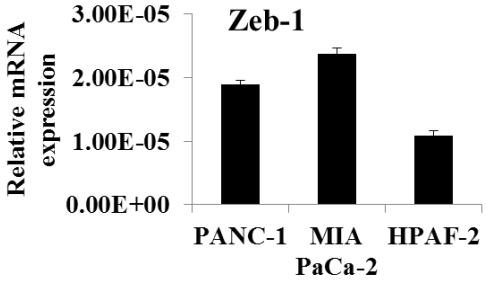


Figure 6.1- Baseline features of PANC-1, MIA PaCa2, and HPAF-2 pancreatic cancer cells in EMT related gene expression. ***A***. Western blot for E-cadherin, Claudine, vimentin, Snail expression in all 3 pancreatic cancer cell lines. ***B-E.*** mRNA levels of E-cadherin (E-Cad), Zo-1, Zeb-1 and Snail-1. RT-PCR data was normalized to 18s rRNA and represented as mean ± SD of $2^{-\Delta Ct}$ of triplicate determinations of 3 individual experiments.

Figure -6.2

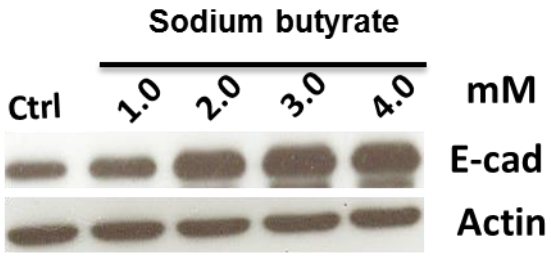


Figure 6.2- Sodium butyrate induced E-cadherin expression in PANC-1 cells. Western blot for E-cadherin expression in PANC-1 cells treated with sodium butyrate.

Figure -6.3A

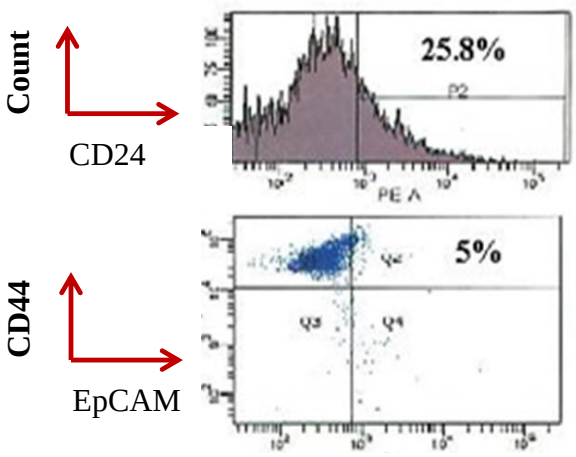


Figure -6.3B

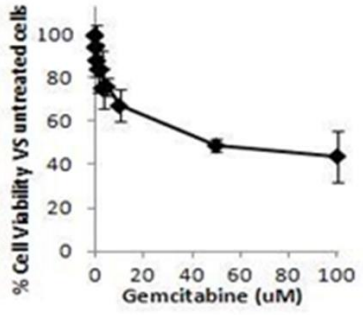


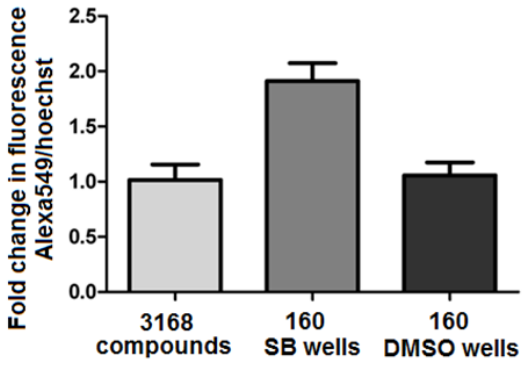
Figure 6.3- PANC-1 cells are enriched with CSC population and are resistant to gemcitabine *A.* Flow cytometry identification of CSCs in PANC-1 cells. $CD24^{+}CD44^{+}$ $EpCAM^{+}$ subpopulation were detected as pancreatic cancer CSCs. PANC-1 cells were triple stained with PE-conjugated anti-CD24, PE-Cy7-conjugated anti-CD44 and APC-conjugated anti-EpCAM. DAPI staining was used for identification of living cells. Cells were analyzed with multi-label flow cytometry. The upper panel represents $CD24^{+}$ gated population (25.8%), and the lower panel represents $CD444^{+}$ $EpCAM^{+}$ cells within the $CD24^{+}$ population. CSCs comprises about 1.29% of the bulk PANC-1 cells (25.8% × 5%). ***B***. Resistance of PANC-1 cells to gemcitabine treatment. PANC-1 cells were treated with gemcitabine and viability was determined at 72 hrs of incubation by MTT assays. Data represent Mean ± SD of triplicate measurements of 4 individual experiments. Gemcitabine concentrations up to 5 mM were used but failed to achieve 90% cell death.

To avoid a possible high rate of false positives with the whole plate fluorescence intensity detection, we used the BD Pathway automated microscopy to carry out High Content Screening (HCS), which takes into account not only fluorescence intensity, but also nuclei size and shape, to ensure fluorescence comes from living cells. The assay was able to reproducibly detect increases in E-cadherin in PANC-1 cells in response to 3.5 mM sodium butyrate (**Fig. 6.4A, B**). Using this instrument, a validation screening was carried out on 3,168 compounds at the concentration of 10 μM from Microsource and Prestwick compound libraries. Compounds in these libraries have known functions and diverse applications. The screening detected 84 compounds with low cytotoxicity (<25% cell loss) and increased fluorescence by 3 standard deviations above average, which represented a 2.65% positive rate (**Fig. 6.4C**).

6.3.2. Identification of compounds that induces E-cadherin expression by HTS

A larger compound library, Chembridge library containing 41,472 compounds with structural diversity and drug like properties, was screened using the HTS assay. HTS resulted in 1,500 positive hits at the concentration of 10 μM. To rule out auto-fluorescent compounds that have an emission overlapping with Alexa Fluor 594, we performed a secondary screening using an antibody conjugated to a green fluorophore, Alexa Fluor 488. The secondary screening was carried out over the 1,500 initial hits from Chembridge library and the previous 84 hits in the assay validation screening. The secondary screening identified 73 compounds that had fluorescence increase with both red and green fluorophore conjugated antibodies (**Fig. 6.5A, B**).

Figure -6.4A



	Compounds	SB (3.5 mM)	DMSO
Avg	1.02	1.91	1.07
Med	1	1.9	1.05
Stdev	0.14	0.17	0.14
N (#wells)	3168	160	160
Avg + 1	1.16	2.08	1.22
Avg + 2	1.3	2.25	1.36
Avg + 3	1.44	2.42	1.5

Figure -6.4B

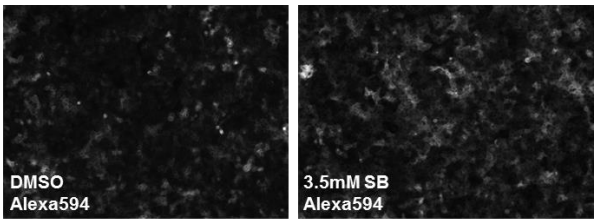


Figure -6.4C

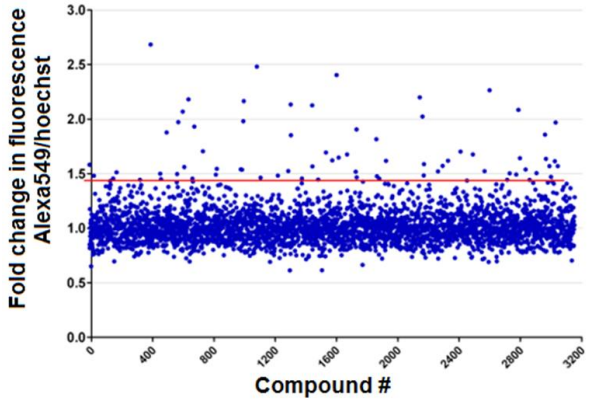


Figure 6.4- Validation of the immunofluorescent HTS assay. *A.* Average signal and standard deviation of the positive and negative controls, compared to the average of all screened compounds. A total of 3,168 compounds were from Microsource (2,320 compounds) and Prestwick (848 compounds) compound libraries. Every plate included 16 wells of vehicle (0.35% DMSO) treated cells, and 16 wells of cells treated with 3.5 mM sodium butyrate (SB). Avg +1, +2 and +3 represent signals 1, 2 or 3 standard deviations above average. ***B.*** Representative images of E-cadherin immunofluorescence of PANC-1 cells treated by 0.35% DMSO, or 3.5 mM sodium butyrate (SB). E-cadherin was detected by anti-E-cad primary antibody (1:250 dilution) followed by Alexa594 conjugated secondary antibody (1:500 dilution). ***C.*** The relative fluorescence (ratio of Alexa Fluor to Hoechst, fold over DMSO vehicle) was plotted against individual wells to visualize the data spread. The median for control wells (DMSO vehicle) was 1-fold, with a 0.1-fold standard deviation. The HCS assay cutoff was 3 standard deviations above the median (1.437-fold), marked by the red line. Eighty four compounds had readings greater than or equal to this cutoff.

These compounds were then retested at 8 concentrations in a dose response ranging from 0.15 to 30 μM. Of the 73 compounds, 17 showed dose responsive increases in both Alex594 and Alex488 fluorescence in PANC-1 cells (**Fig. 6.6**).

6.3.3. Validation of active compounds for E-cadherin restoration

Based on availability and structures, we selected 6 novel compounds for further testing, which belong to 2 different structural clusters. Compounds 150, 717, 743 are vinyl quinolone compounds (Cluster#1) and compounds 38, 353 681 are sulfonyl indoline compounds (Cluster#2) (**Fig. 6.7**). Compounds in both clusters showed low cytotoxicity (IC_{50} > 50 μM) towards PANC-1 cells except for compound 150 (IC_{50} = 2 μM, in Cluster#1) and compound 353 (IC_{50} = 22 μM, in Cluster#2). Overall, compounds in cluster#1 were more cytotoxic compared with compounds in Cluster#2. Cytotoxicity of the compounds in both clusters was also tested on BxPC-3 and L3.6pl pancreatic cancer cells and similar results were obtained (**Fig. 6.7**).

Western blotting in total cell lysate showed that at 5 and 10 μM, BSI in cluster#2, marginally increased E-cadherin expression in PANC-1 cells and showed a concentration dependence. Compound 150 in cluster#1 decreased E-cadherin protein level at 0.5 and 1 μM, probably because of cytotoxicity and other compounds did not show effects (**Fig. 6.8A**). Increased concentrations in cluster#2 compounds confirmed that BSI induced E-cadherin expression in PANC-1 cells at 25 μM, a sub-cytotoxic concentration (**Fig. 6.8B**).

Figure -6.5A

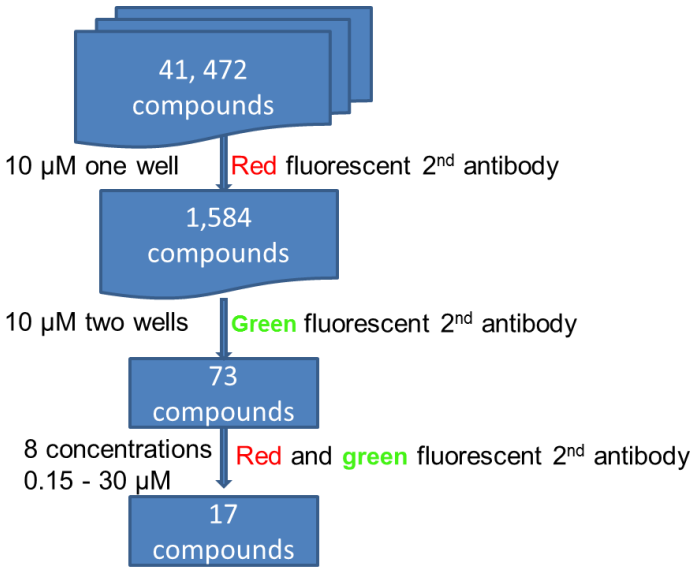


Figure -6.5B

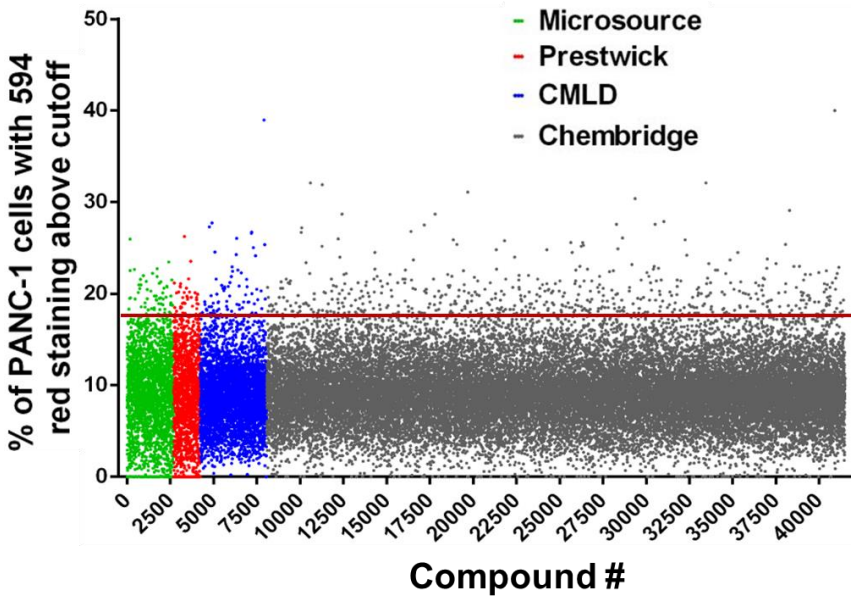
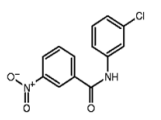
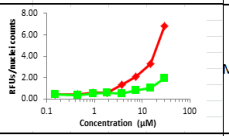
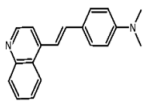
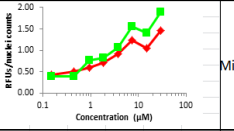
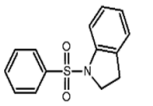
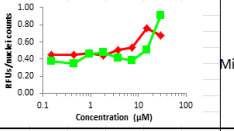
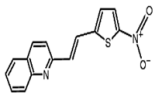
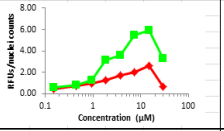
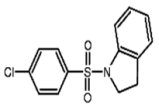
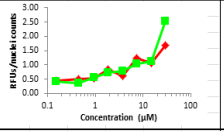
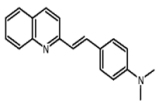
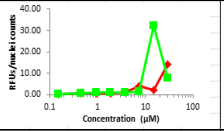
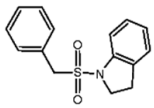
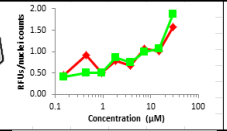
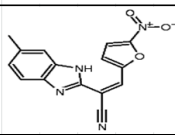
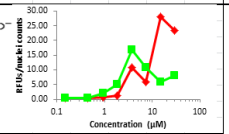
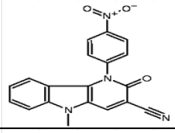
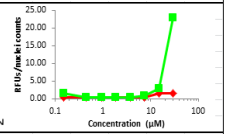
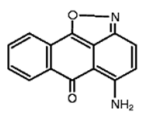
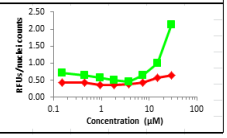


Figure 6.5- ***A***. Scheme of the HTS. ***B***. The percent of red fluorescence, relative to DMSO vehicle, was plotted against individual wells to visualize the data spread. The red line represents 3 standard deviations above average.

Figure -6.6

Library	Compound ID	Structure	RFUs concentration curve
Chembridge	367		
Chembridge	717		
Chembridge	681		
Chembridge	150		
Chembridge	353		
Chembridge	743		
Chembridge	38		
Chembridge	403		
Chembridge	69		
Chembridge	135		

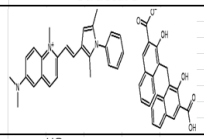
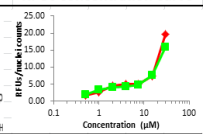
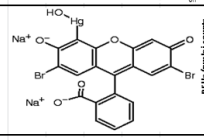
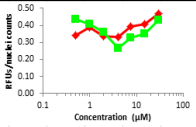
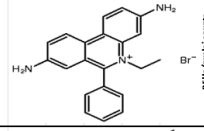
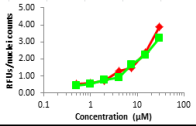
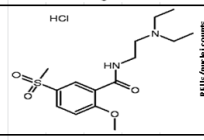
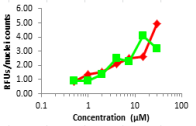
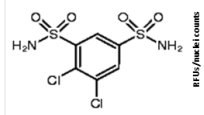
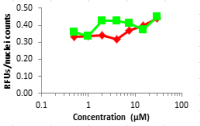
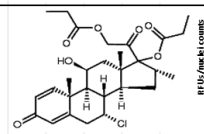
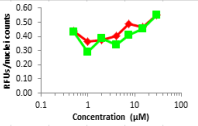
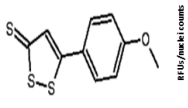
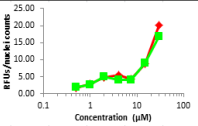
Library	Compound ID	Structure	RFUs concentration curve
Microsource	521		
Microsource	637		
Microsource	806		
Prestwick	438		
Prestwick	787		
Prestwick	792		
Prestwick	1040		

Figure 6.6- The 17 positive hits

Figure -6.7

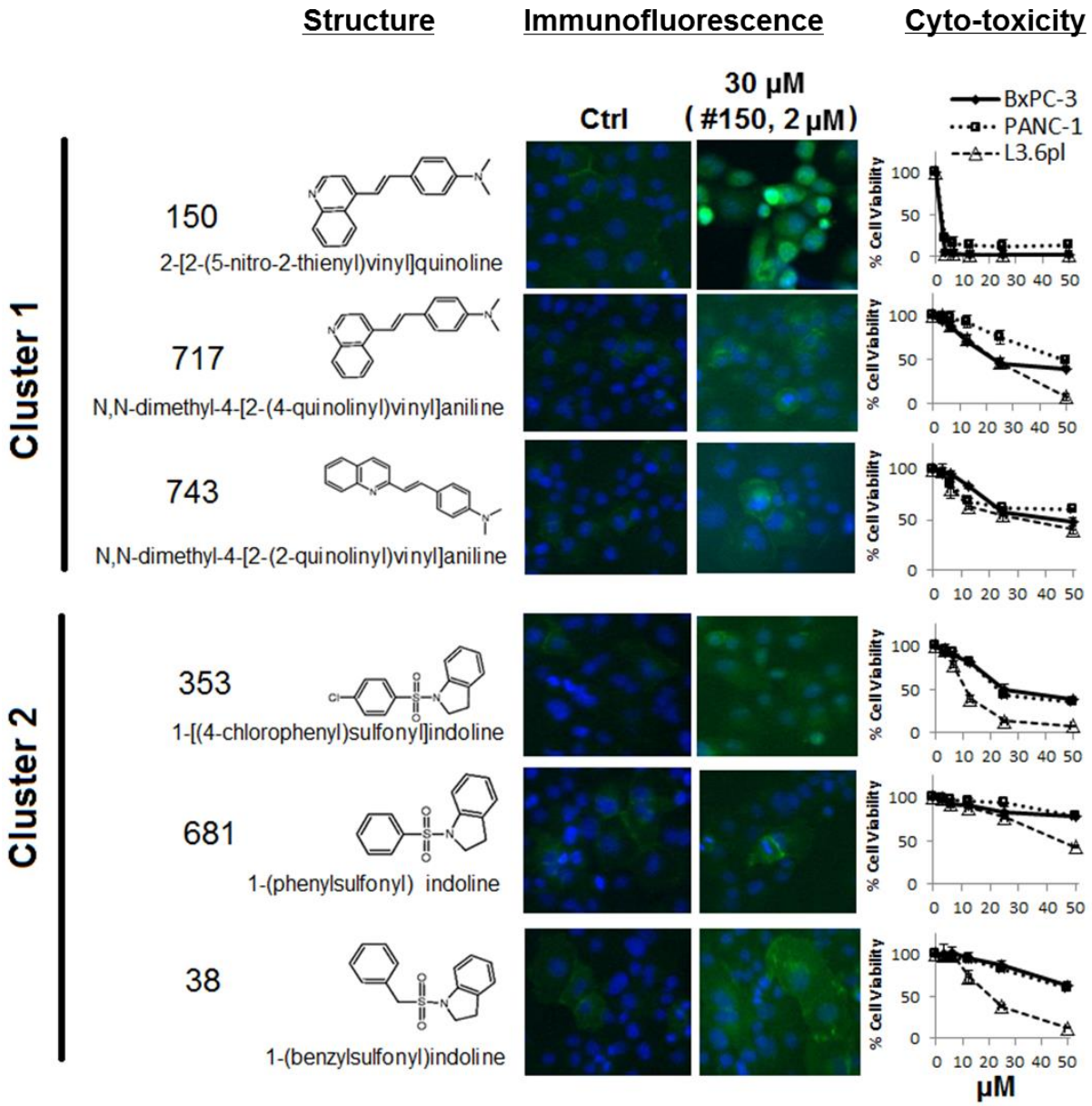


Figure 6.7- Six selected compounds structure, cytotoxicity and validation for E-cadherin induction Sensitivity of PANC-1 cells to 6 selected compounds. Cells were exposed to different concentrations of selected compounds for 48hrs. Cell viability detected at 48hrs post treatment by MTT assay. E-cadherin immunofluorescence of PANC-1 cells treated by 6 selected compounds. E-cadherin was detected by anti-E-cad primary antibody (1:250 dilution) followed by Alexa 488 conjugated secondary antibody (1:500 dilution).

Figure -6.8A

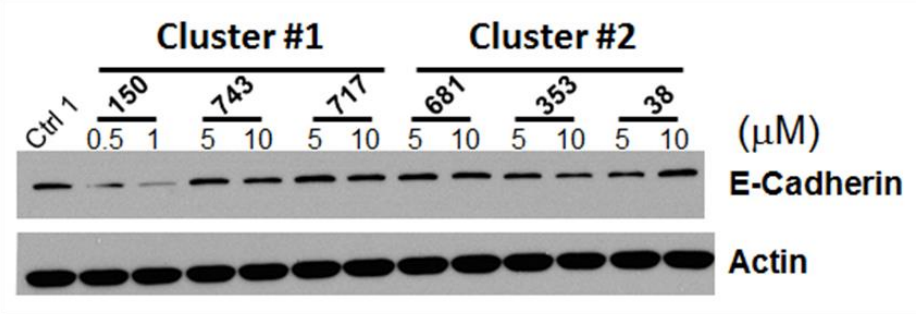


Figure -6.8B

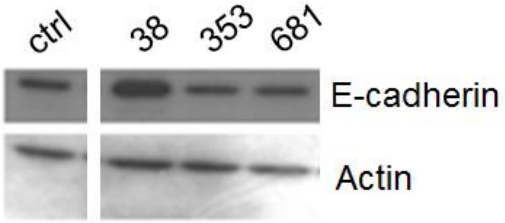


Figure 6.8- BSI increased E-cadherin expression in PANC-1 cells. ***A.*** Western blot analysis of E-cadherin in PANC-1 cell that were exposed to two different concentrations of cluster#1 and cluster#2 compounds. ***B.*** Western blot analysis of E-cadherin in PANC-1 cells that were exposed to 25 μM of cluster#2 compounds.

6.3.4. BSI increased E-cadherin expression by inhibition of Snail and HDACs

While E-cadherin was increased, decrease in the expression of Snail was detected in PANC-1 cells treated with BSI (**Fig. 6.9**). Snail is one of the major E-cadherin transcriptional repressors in pancreatic cancer cells. Snail recruits HDACs to induce epigenetic changes, resulting in a closed-chromatin configuration of DNA, and thus inhibits E-cadherin expression (200, 348). We investigated the HDAC activities by detecting acetylation of histones in PANC-1 cells. At 24 hrs of BSI treatment, there was a robust increase in H3 lysine 9 (H3-K9) acetylation and H4 (H4-K8) acetylation compared to control cells (**Fig. 6.9**). H2A acetylation increased also (**Fig. 6.9**),

indicating inhibition of HDACs. This mechanism is consistent with published data showing that H3/H4 deacetylation silenced E-cadherin (348, 349). Taken together, BSI (compound 38) was validated as an E-cadherin enhancer by inhibiting Snail and HDACs.

Figure -6.9

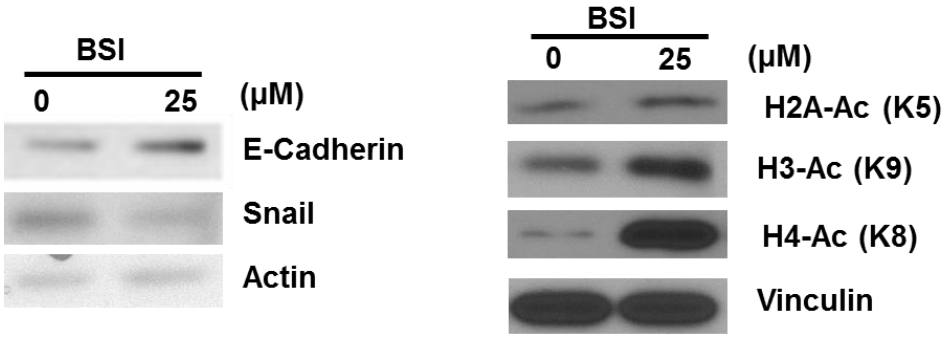


Figure 6.9- BSI increased E-cadherin expression by inhibition of Snail and HDACs. Western blot analysis of E-cadherin, Snail, H2A-Lys5, H3-Lys9, H4-Lys8 acetylation in PANC-1 cells that were exposed to BSI (25 μM) for 24hrs.

6.3.5. BSI inhibited pancreatic cancer cell invasion and migration

Boyden chambers covered with Matrigel were used to examine the ability of BSI to inhibit PANC-1 cell invasion, while chambers without Matrigel coverage were used to examine cell migrating ability. Data showed that BSI significantly decreased both invasion and migration of PANC-1 cells at 24 hrs treatment at a sub-cytotoxic concentration of 25 μM (**Fig. 6.10A**). Scratch assay was used to confirm these inhibitory effects of BSI. BSI at 25 μM significantly inhibited the ability of BxPC-3 cells to cover the scratched area (**Fig. 6.10B**).

Figure -6.10A

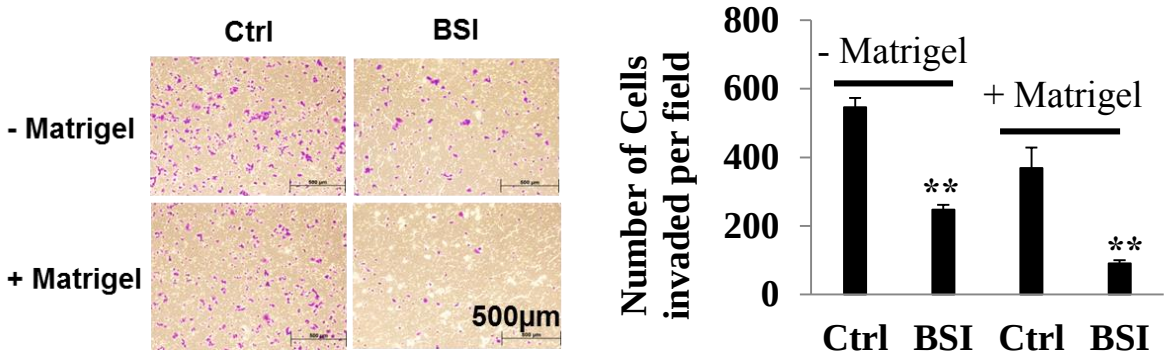


Figure -6.10B

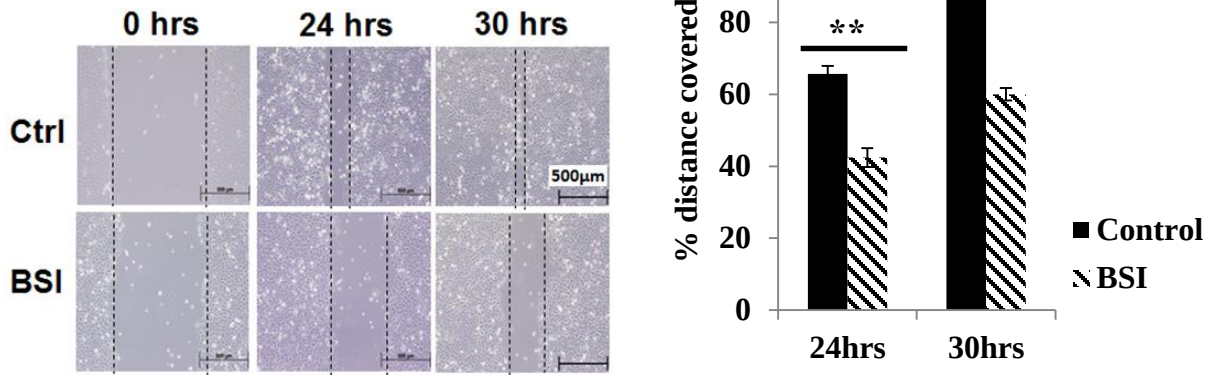


Figure 6.10- BSI inhibited cell invasion and migration of pancreatic cancer cells *A.* Matrigel invasion assays for pancreatic cancer cell migration and invasion. PANC-1 cells were exposed to 25 µM BSI. Cell migration (without Matrigel) and invasion (with Matrigel) were detected at 24hrs. Bar graph shows the average number of migrated/invaded cells per field. ***B.*** Scratch assay for pancreatic cancer cell migration. Scratch was made on confluent monolayer of BxPC-3 cells and then exposed to 25 µM BSI. Cell migration was measured at 24 and 30 hrs of post-BSI treatment. Bar graph shows the % distance covered by BxPC-3 cells.**, P<0.001 by T-test.

6.3.6. BSI inhibited pancreatic tumor spheroids formation

The self-renewal capacity is one of the major characteristics of CSCs. Tumor spheroid formation ability in suspension culture is indicative for this feature (121). Primary and secondary tumor spheroid formation was examined in BxPC-3 cells treated with BSI. BSI significantly reduced the number and size of the primary and secondary spheroids in pancreatic cancer cell lines (**Fig. 6.11A-C**).

6.3.7. Analogues of BSI inhibited pancreatic cancer cell migration, invasion, and tumor spheroids formation

Six more analogues of BSI were purchased from Chem Bridge Chemical Store (**Fig. 6.12**) and tested for their effects on pancreatic cancer cell migration, invasion and tumor spheroid formation. All of these compounds possessed low cytotoxicity towards PANC-1 and BxPC-3 cells (**Fig. 6.12**). Compounds 16, 288, 480, 704 and 935 have comparable activities in inhibiting cell migration, as evaluated by wound healing scratching assay (**Fig. 6.13**). These compounds also inhibited spheroid formation of PANC-1 and BxPC-3 cells, with reduction in both numbers and sizes of spheroids (**Fig. 6.14, 6.15**). However, these analogues did not show superior effects compared to BSI on cell migration/invasion and tumor spheroid formation.

Figure -6.11A

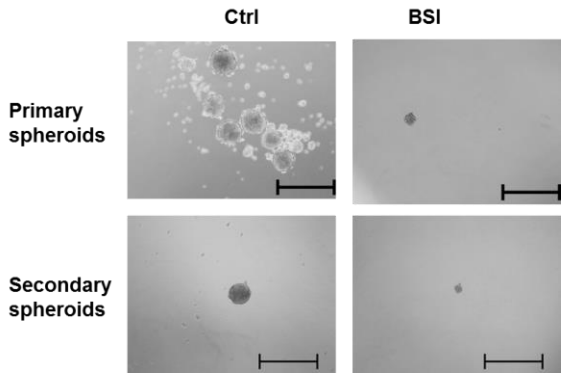


Figure -6.11B

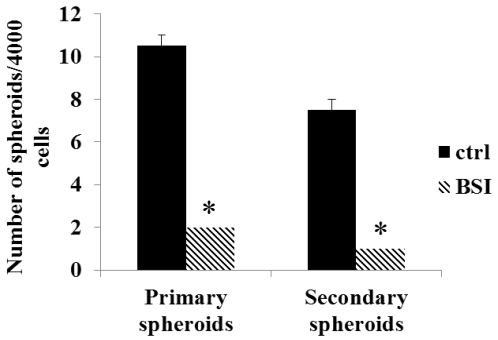


Figure -6.11C

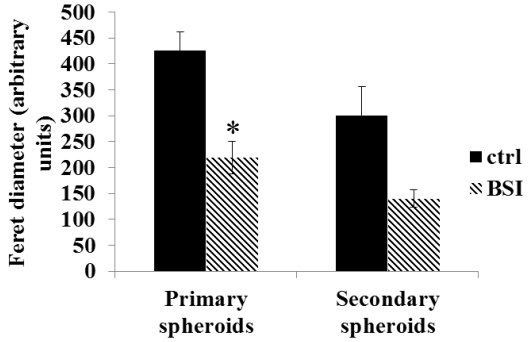


Fig. 6.11 BSI reduced the pancreato sphere formation BxPC-3 cells in ultra-low attachment plates exposed to 25 µM of BSI. ***A.*** Primary Spheroids were imaged and counted 14 days post treatment. Primary spheroids were dissociated into individual cells using trypsin and then reseeded into ultra-low attachment plates for secondary spheroids. Again cells exposed to 25 µM of BSI. Secondary Spheroids were imaged and counted 14 days post treatment. Scale bar 500 µm. Magnification of the images 100X. ***B.*** Bar graph representing the average number of spheroids. ***C.*** ferret diameter of spheroids ± SEM. *, $P<0.05$ by T-test

Figure -6.12

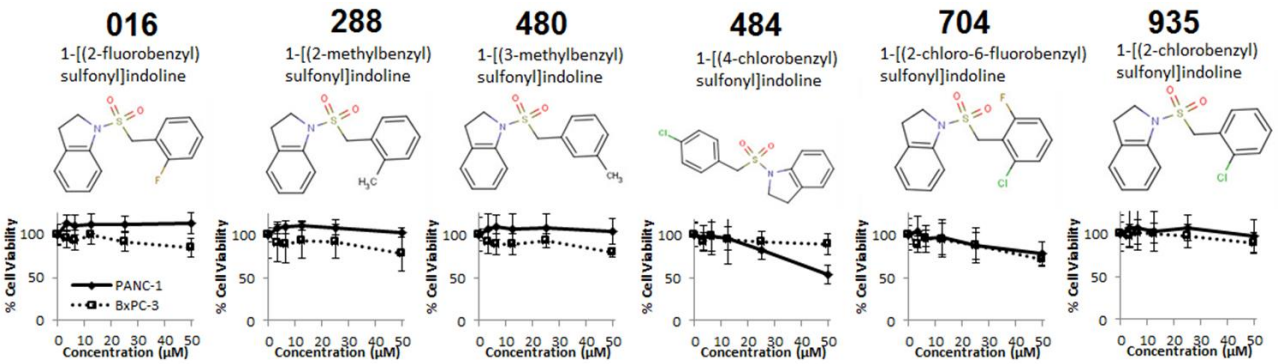


Figure 6.12- Structure and cytotoxicity of BSI analogues Sensitivity of pancreatic cancer cells (PANC-1 and BxPC-3) cells to BSI analogues (16, 288, 480, 484, 704, and 935). Cells were exposed to different concentrations of BSI analogues for 48hrs. Cell viability detected at 48hrs post treatment by MTT assay.

Figure -6.13

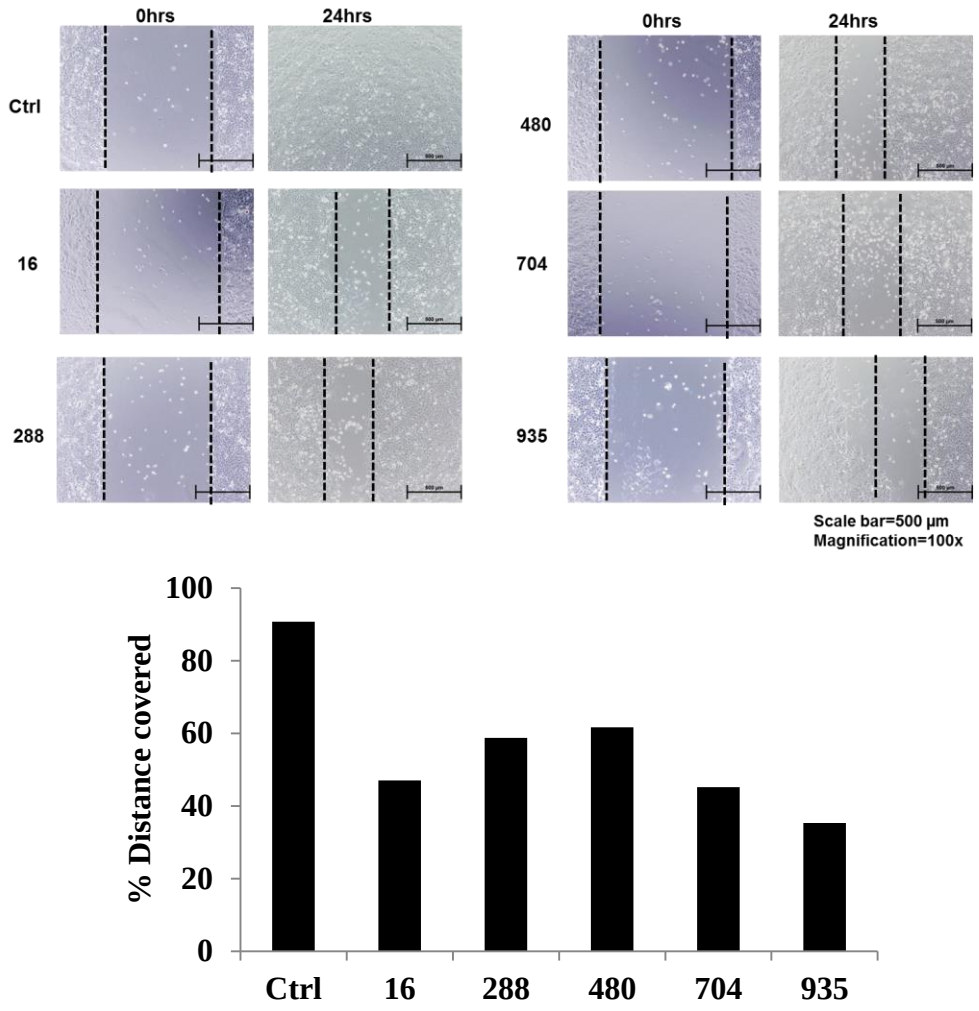


Figure 6.13- BSI analogues inhibited cell migration Scratch assay for cell migration. Scratch was made on confluent monolayer of BxPC-3 cells using 1.25 ml sterile pipette tip. After washing with media, cells were exposed to 25 µM BSI analogues (16, 288, 480, 704, and 935). Scratch was photographed at 0, 24 hrs post treatment. Bar graph shows the % distance covered by BxPC-3 cells.

Figure -6.14A

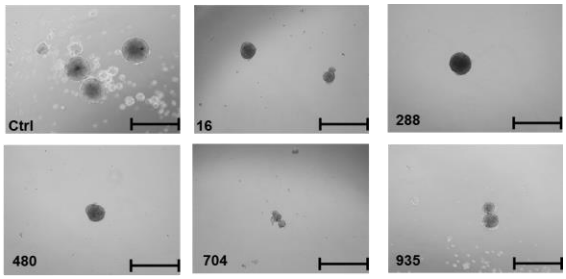


Figure -6.14D

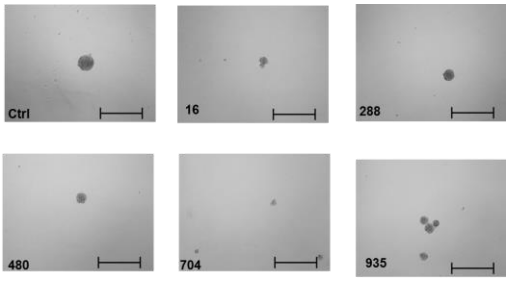


Figure -6.14B

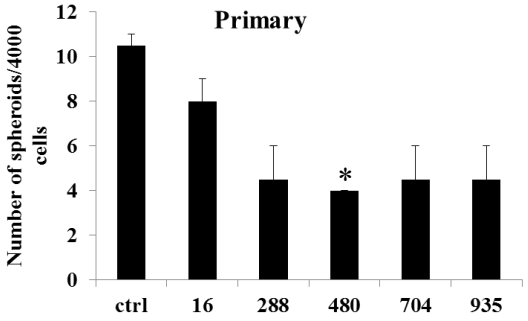


Figure -6.14E

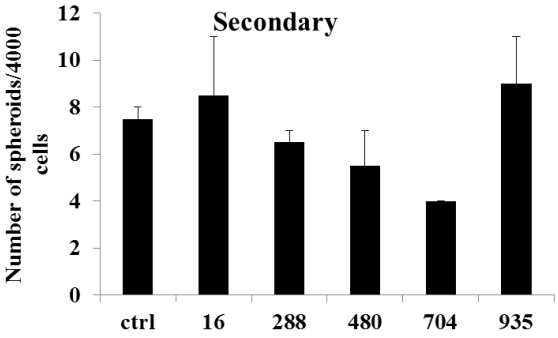


Figure -6.14C

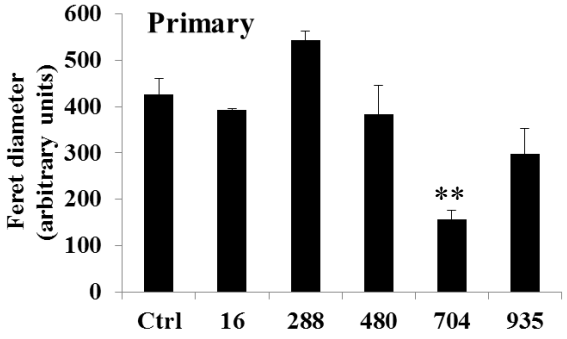


Figure -6.14F

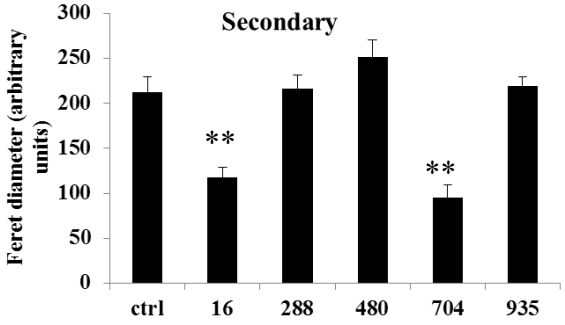


Figure -6.15A

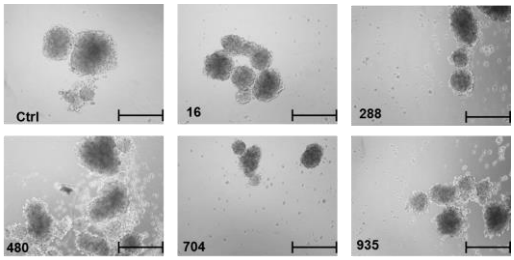


Figure -6.15D

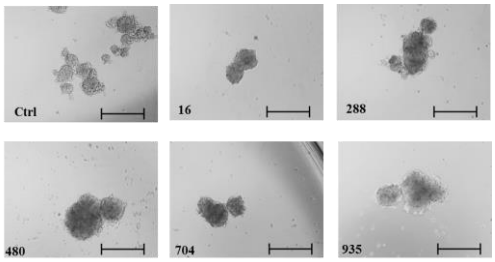


Figure -6.15B

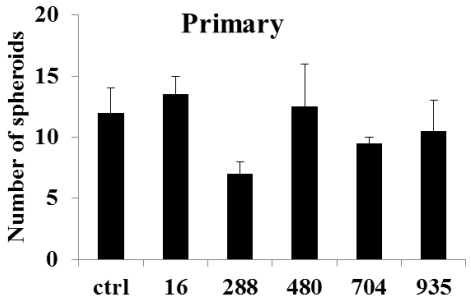


Figure -6.15E

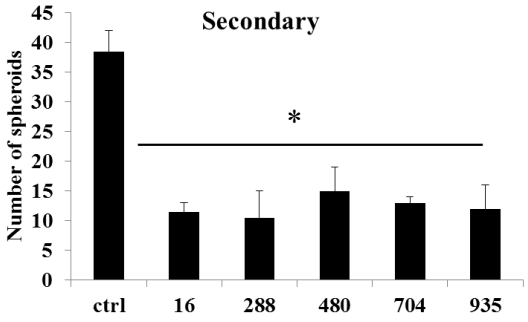


Figure -6.15C

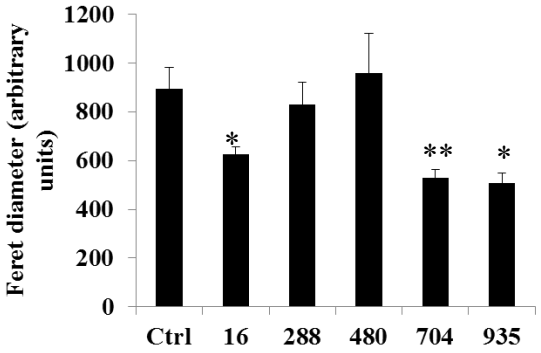


Figure -6.15F

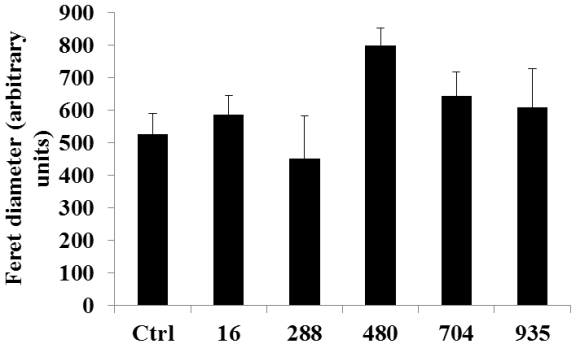


Fig. 6.14 -6.15 - BSI analogues are not as efficient as BSI in inhibiting pancreato sphere formation BxPC-3 and PANC-1 cells were seeded in ultra-low attachment plates and then cells were exposed to 25 µM of BSI analogues (16, 288, 480, 704, and 935). BxPC-3 **(Fig. 6.14A)** and PANC-1 **(Fig. 6.15A)** primary spheroids were imaged and counted 14 days post treatment. Bar graph representing the average number of primary spheroids **(Fig.6.14B, 6.15B),** feret diameter of the spheroids **(Fig. 6.14C, 6.15C)**± SEM. Primary spheroids were dissociated into individual cells using trypsin and then reseeded into ultra-low attachment plates for secondary spheroids. Again cells exposed to 25 µM of BSI analogues. BxPC-3 **(Fig. 6.14D)** and PANC-1 **(Fig. 6.15D)** Secondary Spheroids were imaged and counted 14 days post treatment. Bar graphs representing the average number of secondary spheroids **(Fig. 6.14E, 6.15E),** feret diameter of the spheroids **(Fig. 6.14D, 6.15D)** ± SEM. Scale bar 500 µm. Magnification of the images 100X. *, P<0.05, **, P<0.001 by T-test.

6.4. Discussion

The challenges in investigating CSC inhibitors are reflected in the challenges faced in the development of treatment strategies for pancreatic cancer. CSCs are proposed to be the driving force of the tumor's high metastasis rate and recurrence rate which directly result in extremely poor prognosis and treatment outcomes for patients (137, 150, 350). As direct screening is difficult for CSC-toxic compounds, our study provided an alternative screening for EMT inhibitors as an initial step for development of CSC inhibitors. The rationale has its roots in the close association between EMT and CSCs, as studies showed many genes, transcriptional factors and signaling pathways that induce EMT are also important in CSC transformation and maintenance (58, 59) . Therefore, looking for EMT inhibitors holds the hope of also discovering CSC inhibitors. Furthermore, because loss of E-cadherin expression is associated with metastasis in many neoplasms (351-354), EMT inhibitors can be extremely useful by themselves for anti-metastasis, even if they do not inhibit CSCs. As a proof of this concept, our screening for E-cadherin inducers resulted in identification of the novel compound BSI as a potent EMT inhibitor. BSI inhibited pancreatic cancer cell migration/invasion and also strongly inhibited CSCs.

Loss of E-cadherin, a classic hallmark of EMT, can occur by either genetic or epigenetic alterations (303, 355). Recent reports suggested that histone acetylation regulated E-cadherin expression. Transcriptional repressors of E-cadherin, i.e. Snail1/2 and Zeb1/2, recruit HDACs to conduct an epigenetic alternation that inhibits E-cadherin gene transcription (79, 80). Our hit compound BSI substantially increased H3 and H4 acetylation, indicating HDAC inhibition as its mechanism of action. Other mechanisms are not excluded for BSI's activity in inhibiting CSC.

Further in-depth mechanistic studies are worth carrying out, given the anti-CSC potential of this cluster of compounds.

Hydroxamic acid or benzamide groups are the functional groups in (previously) known HDAC inhibitors are either (356). BSI and its analogues studied here do not contain these structural groups but are instead, benzyl sulfonyl indoles. These data have therefore revealed a new class of potent HDAC inhibitors that possesses *in vitro* anti-EMT and anti-CSC activities, with low cytotoxicity. Additional novel drugs and drug leads can presumably be developed based on further studies using the SAR. Because these compounds were not generally cytotoxic, safety and low toxicity can probably be expected in future *in vivo* studies.

In conclusion, we have shown the development and application of novel HTS for compounds that induces E-cadherin induction in pancreatic cancer cell lines, as an approach for CSC inhibitor identification. Furthermore, our novel BSI compound inhibited pancreatic cancer EMT *in vitro* by inhibiting HDACs.

Chapter 7. Conclusions and future directions

Pancreatic cancer continues to be one of the most lethal human malignancies. By 2030, pancreatic cancer is expected to become the second-leading cause of cancer-related deaths in the United States (357). Though the oncology community has made significant progress in understanding pancreatic cancer biology, there are no effective therapies available to date, because of its multifactorial nature. At the cellular level, neoplastic cells harbor activating K-Ras mutations at high frequencies in pancreatic cancer patients. Pharmacological agents targeting K-Ras for pancreatic cancer are currently an active area of research (358). However, it is possible that blocking K-Ras function may not be sufficient to treat pancreatic cancer, because these cancer cells may adapt to K-Ras inhibition either by overexpressing or mutating genes in downstream pathways of K-Ras. Several other growth factor signaling pathways also impact disease progression and pathogenesis, which might drive growth and cell survival in the absence of K-Ras signaling (359). Layered on top of oncogenic mutations are a host of tumor suppressor gene inactivations (360). Developing effective anticancer regimens against changes of tumor suppressor pathways has emerged to be a promising area of research (52). Novel approaches need to be investigated with an open mind, as new knowledge and concepts in pancreatic cancer biology develop.

CSCs are a relatively new concept in cancer biology. A growing body of literature suggests that pancreatic tumors are enriched with CSCs (361). CSCs are frequently associated with metastatic foci and chemoresistance and are increasingly linked to an EMT phenotype (121, 362). The resistance to chemotherapy of this subpopulation is responsible for tumor recurrence. EMT in cancer cells is triggered by several different signaling pathways, which then activate a set of transcription factors to induce EMT (79, 86). Therefore, inhibitors of EMT signaling pathways or the transcription factors hold the promise to inhibit pancreatic cancer metastasis and CSCs. Due

to lack of therapeutic benefit with the currently available EMT inhibitors, this dissertation took several novel approaches to target pancreatic cancer cell EMT. The key findings can be summarized as follows.

Chapter 4 demonstrated that ascorbate at pharmacological doses was a potent inhibitor of pancreatic cancer cell EMT, cell invasion and migration. Ascorbate treatment resulted in decreased expression of several mesenchymal markers and enhanced expression of epithelial markers. Ascorbate treatment inhibited pancreatic cancer cells spheroid formation, suggesting CSC inhibition. In addition, ascorbate enhanced α-tubulin acetylation and stabilized microtubule polymers to inhibit cancer cell metastasis and mitosis, by regulating expression and activity of HDAC6 and Sirt-2. Tumor microenvironment and desmoplasia were influenced resulting in inhibited cancer cell metastasis. These data provide a basis to direct clinical research focusing on tests of efficacy of IVC in pancreatic cancer patients.

Several phase 1 and 2a studies have been carried out using high dose intravenous ascorbate in cancer treatment. These studies confirmed its safety and implied its benefit especially in combination with current chemotherapies (266, 276, 277). However, no definitive phase 2 study has been done. Given the very limited treatment options for pancreatic cancer patients, the compelling preclinical data showing effectiveness and potential benefits of high dose intravenous ascorbate, demonstrate a need for designing and conducting clinical trials to definitively test the efficacy of ascorbate.

Mechanistically, one of the most interesting findings from data presented here is that ascorbate treatment resulted in enhancement of the collagen content in tumor stroma. Tumor stroma plays an important role in tumor growth and progression. Recent studies showed that desmoplasia in

pancreatic tumor stroma restrain the tumor growth rather than supporting it (226, 280). Therefore, it is worthwhile to test how high-dose ascorbate effects the tumor growth and metastasis at different stages of the disease pathogenesis using a transgenic mouse model system such as PKCY.

The mechanism of CSC inhibition by ascorbate needs to be further studied. One possible approach is to evaluate the influence of ascorbate on Wnt/β-catenin, Notch, and SHH pathways. Understanding such basic mechanisms will provide clues for novel combination treatment approaches. Ascorbate holds the advantage of low toxicity. Assessment of such combination treatments in preclinical models will provide valuable tools for testing them in clinical settings for pancreatic cancer patients. Our *in vitro* studies showed that ascorbate worked synergistically with HDAC inhibitors. *In vivo* evaluation of this finding is another important avenue that should be studied in the future.

Another interesting finding is that ascorbate mediated regulation of HDAC6. Though HDAC6 is primarily localized to cytoplasm, recent studies have shown that HDAC6 can be localized to the nucleus to regulate histone acetylation and gene transcription (363). Future studies can be undertaken to study the effect of ascorbate on HDAC6 nuclear localization and its influence on histone acetylation. Mechanisms of HDAC6 expression regulation by ascorbate need to be characterized in detail. Saji and colleagues showed that HDAC6 expression is regulated by estrogen signaling in estrogen receptor (ER) α positive breast cancer cells (364). However, the role of ER in pancreatic cancer neoplasms remains unknown (365). Our future studies will evaluate if ascorbate regulates HDAC6 expression through estrogen signaling. In addition to α-tubulin, HDAC6 has several additional substrates such as heat shock protein 90 (Hsp90) (366), heat shock cognate protein 70, myosin heavy chain 9 (MHC9), and dnaJ homology subfamily A

member 1 (367). HSP90 chaperon activity is regulated by reversible acetylation and controlled by HDAC6. Inhibition of HDAC6 leads to hyper acetylation of HSP90 and a loss of chaperon activity (366). Future studies need to be conducted to explore HSP90 acetylation status and its chaperon function after ascorbate treatment in pancreatic cancer cells. It would also be worth testing the anti-cancer effects of ascorbate and HSP90 inhibitor combination in both *in vitro* and *in vivo*.

Data here also demonstrated that ascorbate treatment depletes NAD^+ levels in pancreatic cancer cell lines. The mechanism of NAD^+ depletion as a result of ascorbate treatment needs to be investigated in detail. We have recently showed that ascorbate caused DNA damage and PARP activation (266). PARP activation might result in NAD^+ depletion. It is also possible that ascorbate might alter NAD^+ biosynthesis (de novo and salvage) pathways. Real-time PCR or western blotting can be performed to test the effect of ascorbate on the expression of key enzymes that mediate NAD^+ biosynthesis through de novo pathway (indoleamine 2,3-dioxygenase, quinolinate phosphoribosyl transferase, nicotinamide (NAM) mononucleotide adenylyl transferase (NMNAT), NAD^+ synthase) and salvage pathway (NAM phosphoribosyl transferase, NMNAT, nicotinamide riboside kinase, nicotinic acid phosphoribosyl transferase) (368). Recently, Chini and colleagues showed that NAD^+ metabolism is essential for pancreatic cancer cell survival. Inhibition of NAD^+ synthesis via the NAM phosphoribosyl transferase pathway using FK866, resulted in reduction of NAD^+ levels, glycolytic flux, lactate production, mitochondrial function, and pancreatic tumor growth (369). It has been shown that NAD^+ intracellular shortage inhibits the mTOR signaling pathway, which is the master regulator of macromolecule synthesis (370). Future studies can be undertaken to test the combination

effect of ascorbate and mTOR inhibitor on pancreatic cancer cell survival, migration and invasion *in vitro* and *in vivo*.

Cancer cell metastasis is a complex and multifaceted process (371). Spatiotemporal reorganization of the cytoskeleton has been shown to play an important role in cell invasion and metastasis (372). In this dissertation we showed that ascorbate stabilized microtubules by promoting α-tubulin acetylation. Microtubule dynamics contribute to actin cytoskeleton regulation. It has been shown that microtubule depolymerizing agents stimulated actin polymerization in Swiss 3T3 and Rat-2 fibroblasts. However, the microtubule-stabilizing agent taxol inhibited actin polymerization induced by microtubule-disrupting agents (373). Therefore, it is more likely that ascorbate mediated tubulin stabilization might influence actin polymerization, which needs to be further investigated.

In **chapter 5**, novel derivatives of currently available EMT inhibitors (SAHA, MS-275) were evaluated for their biological activity. Of the 28 derivatives tested, St-1 and St-3 showed potent cytotoxicity against two pancreatic cancer cell lines possessing different genetic alterations. Moreover, St-1, St-3 as well as the parent compounds did not induce any cytotoxic effects on non-cancerous epithelial cells. St-1and St-3 showed potency in inhibiting pancreatic cancer CSCs. St-1 showed similar potency to the parent compounds in inducing histone acetylation. Interestingly, St-3 lost the ability to inhibit HDACs, but inhibited HuR.

Given the *in vitro* activities of St-1 and St-3 in inhibiting pancreatic cancer cell proliferation, metastasis and CSCs, a logical next step is to evaluate the anti-tumor activities of St-1 and St-3 *in vivo* using a PKCY transgenic mouse model system or the PANC-1 orthotopic model system. Metastasis of cancer cells to liver and mechanisms of metastasis regulation by St-1 and St-3

needs to be investigated. CSC population in the tumors after St-1 or St-3 treatment can be detected by isolating $CD44^{+}CD24^{+}EpCAM^{+}$ cells followed by the sphere formation assay.

As St-1 exhibited similar potency in HDAC inhibition but higher potency in inhibiting cell growth when compared to its parent compounds (SAHA, MS-275), it might have other targets in addition to HDACs to execute its function. Proteomic, genetic, and bioinformatics methods can be employed for identification of St-1 targets. For example, a few experimental approaches serving this purpose are pulldown assays, drug affinity responsive target stability (DARTS), two-dimensional (2-D) gel electrophoresis, and computational methods. Affinity based proteomics (pulldown) is one of the most widely applied methodology to identify the targets of biologically active compounds. To employ this method, St-1 has to be immobilized on a solid phase and exposed to a cell lysate to bind the target proteins. Proteins that bind nonspecifically to St-1 and matrix are removed by stringent washing prior to release of the bound protein by means of elution. Alternatively, biotin labelled St-1 incubated with cell lysates followed by pulldown of biotin-St-1-protein complex using streptavidin conjugated agarose beads can be used for St-1 target identification. Target proteins were typically validated by tryptic digestion followed by mass spectrometry. Hit validation can be performed using *in vitro* or *in silico* (molecular docking) assays. New technologies such as DARTS, can be used to discover the direct binding targets of small molecules such as St-1 on a proteome scale (374). DARTS uses label free compounds for target identification which is one of the major advantages of this method. The basic principle is that target protein bound to a small molecule might be less susceptible to proteolysis due to local or global stabilization. Two-dimensional (2-D) gel electrophoresis can also be used to identify targets of small bioactive molecules like St-1, if assumption can be made that St-1 covalently attaches to the target protein (375). Computational methods can be used for

target identification. For example, the statistics-based similarity ensemble approach compares protein targets considering similarities among their ligands (376). The limitations lie within the dependency of the method on known targets and their ligands.

Nonspecific inhibition of HDAC inhibitors is one of the reasons for their toxicity. Therefore, future studies can also be undertaken to test the specificity of HDAC inhibition by St-1.

For St-3, EMT and CSC regulation in the presence of and in the absence of HuR is an another important avenue that needs to be further investigated. Further, HuR has been shown to regulate expression of numerous mRNAs involved in cell proliferation, senescence, elevation of local angiogenesis, anti-apoptosis, and reduced immune recognition, in addition to the listed ones in Table 4 (1). Inhibition of HuR could influence these genes and pathways. Therefore, further studies need to investigate the role of St-3 in angiogenesis, cell senescence, apoptosis and immune response.

Although data here suggested that HuR is the primary target of St-3, the possibility of other targets cannot be excluded. Especially, some studies showed that HuR knockdown did not induce massive cell death in pancreatic cancer cell lines (377, 378). Identification of other targets for St-3 is another future avenue that can be undertaken. Similar approaches to that described above can be employed to identify alternative targets of St-3.

Identification of chemical compounds that preferentially kill CSCs depends on the ability to propagate stable, highly enriched populations of CSCs in vitro. However, such approaches are not feasible for the CSCs of solid tumors. For example breast CSCs are rapidly lost during in vitro culture (379). As EMT is highly associated with CSCs, in **chapter 6** we established a HTS based approach to screening for novel EMT inhibitors as an alternative approach to target

pancreatic CSCs. A novel compound BSI was identified as a novel class of EMT inhibitors. BSI showed HDAC inhibitory activities, but is structurally different from the classical signature motifs of current HDAC inhibitors. BSI is a novel EMT inhibitor, which showed low cytotoxicity towards pancreatic cancer cell lines but had inhibitory effects on cell migration, invasion, and CSCs. To improve the efficacy of BSI, we have tested 6 commercially available BSI analogues. However, they failed to show better potency when compared to BSI in inducing cell death, inhibition of cell migration and CSCs.

In vitro effects of BSI on cell migration and CSCs needs to be further validated in the PANC-1 orthotopic model system. Since BSI is not cyto-toxic to PANC-1 cells, it is possible that BSI may not have effect on tumor growth. Therefore, future studies can be undertaken to discover the suitable combination chemotherapeutic agent which works synergistically or additively with BSI.

Specificity of HDAC inhibition is another key feature that needs to be investigated. HDAC inhibition capacity of BSI might also be enhanced by modifying its chemical structure. For example, addition of an acetyl group to BSI has the potential to enhance its HDAC inhibition capacity because an acetyl group mimics the natural substrate of HDACs. This BSI derivative could potentially bind to the active site of HDACs and the rest of the molecule will traverse the tunnel of the active site to block the function of the HDACs.

The mechanism of metastasis and CSCs inhibition by BSI is another avenue of future studies one can undertake.

Identification of *in vivo* targets for BSI is also another future avenue that one can undertake. Similar approaches to that described for the identification of targets for compound St-1 can be employed to identify *in vivo* targets of BSI.

An effort needs to be made to improve the cytotoxicity of BSI by preparing its analogues with the help of medicinal chemist. Anti-cancer activities of these new BSI analogues need to be further validated both *in vitro* and *in vivo*.

Taken together, research outlined in this dissertation led to the development of novel EMT and CSC inhibitors such as high-dose vitamin C, BSI, St-1, and St-3. All of them effectively inhibited cell invasion, migration and CSCs and have therapeutic implications for pancreatic cancer treatment. Still, there are many mechanistic questions to be explored and many studies to be done. This work set a basis to develop these novel EMT inhibitors towards treatment or drug leads for pancreatic cancer.

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