

Critical analysis of the potential for the therapeutic targeting of the Sp1 transcription factor in pancreatic cancer

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Abstract: Pancreatic ductal adenocarcinoma (PDAC) is a major cause of cancer-related deaths in developed countries and, in 2013, it is estimated that in excess of 45,220 new cases were diagnosed in the United States. PDAC is a highly aggressive disease that invariably evades early diagnosis. The mean survival time for patients with metastatic disease is only 3–6 months, and only 20%–30% of pancreatic cancer patients are alive after 12 months. Because pancreatic cancers are frequently detected at an advanced stage, treatments have provided very limited improvements in tumor regression and overall survival times after diagnosis. 5-Fluorouracil alone or in combination with other drugs has been extensively used for treatment of advanced pancreatic cancer, and gemcitabine has partially replaced 5-fluorouracil as a treatment for pancreatic cancer. Gemcitabine provides increased clinical benefits in terms of response rate; however, future studies need to focus on developing treatment modalities that will improve the survival rate for pancreatic cancer patients. Specificity protein 1 (Sp1) is overexpressed in PDAC patients, and high expression is associated with poor prognosis, lymph node metastasis, and low survival. Knockdown studies have shown that Sp1 plays an important role in cell growth, angiogenesis, inflammation, survival, and metastasis. Sp1 expression is low in normal tissue when compared to tumor tissue, which makes Sp1 a potential target for development of new mechanism-based drugs for treatment of pancreatic cancer. Several drugs such as tolfenamic acid, betulinic acid, and methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate are shown to downregulate Sp1 expression through various pathways. This review summarizes the role of Sp1 in pancreatic cancer and delineates the mechanisms of action of various drugs that downregulate expression of Sp1 and other Sp transcription factors.

Keywords: Sp transcription factors, microRNAs, ZBTB repressors, ROS

Introduction

Tumor formation is a complex, multistep process that usually takes decades, and tumor progression is defined as the evolution of normal cells into cells which exhibit an increasingly neoplastic phenotype. During tumorigenesis, many mutations and epigenetic alterations occur randomly within DNA, and this is due to gain of function of oncogenes and loss of function of tumor suppressors, which results in unrestrained growth and the spread of cancer cells to other organs and tissues.^{1,2} Cancer is the second leading cause of death in the United States following cardiovascular diseases. A total of 1,660,290 new cancer cases and 580,350 deaths are projected to occur in 2013. The 5-year relative survival rate for all cancers diagnosed between 1996 and 2004 is 66%, which is increased from 50% in 1975–1977,³ and cancer death rates decreased 24% in men and 16% in women and an overall 20% between 1990/1991 and 2009.⁴ The increase

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in survival is due to progress in diagnosing certain cancers at an earlier stage and improvements in treatment.^{3,4}

Introduction to pancreatic cancer

An estimated 45,220 new cases of pancreatic cancer and an estimated 38,460 deaths are expected to occur in the US in 2013.³ Pancreatic ductal adenocarcinoma (PDAC) is the most frequently diagnosed pancreatic cancer and is the fourth leading cause of cancer deaths. The 5-year survival rate is less than 5%. Other cancers of the pancreas are much less common and account for about 4% of all pancreatic cancers. PDAC affects both sexes almost equally and has its peak incidence in the sixth or seventh decade of life and is extremely rare before the age of 40.³⁻⁵

Risk factors for developing PDAC are cigarette smoking, previous gastric surgery, diabetes mellitus, chronic pancreatitis, familial history of pancreatic cancer, high intake of dietary polyunsaturated fats, and a diet low in fruits and vegetables.⁶⁻⁸ Familial predisposition is seen in pancreatic patients expressing BRCA1, BRCA2, p16, STK11/LKB1 germline mutations, and other genetic syndromes associated with PDAC include familial atypical multiple melanoma syndrome, telangiectasia ataxia, and Peutz–Jeghers syndrome. Germline mutations of BRCA2 increase the incidence of PDAC by 3.5-fold, and germline mutation of BRCA1 increased the incidence by 2-fold.⁹

Activated or overexpressed oncogenes that play an important role in pancreatic cancer development include *KRAS*, *Her-2*, *AKT2*, *AIB1*, *BRAF*, *c-Myc*, and *MYB* genes. *KRAS* is activated in about 90% of pancreatic cancers by a point mutation on codon 12 and occasional mutations in codons 13 or 61 of chromosome 12p.^{5,10} *KRAS* encodes a member of RAS family of guanosine triphosphate (GTP)-binding proteins that mediate a number of important functions in cell proliferation, survival, cytoskeletal remodeling, and motility. A variety of stimuli such as binding of growth factor ligands to their cognate growth factor receptor results in signal transduction via intermediary proteins that are important in the action of the *KRAS* protein. Activated *KRAS* affects several downstream effector pathways such as Raf-mitogen activated protein kinase (RAF-MAPK), phosphoinositide-3-kinase, and RalGDS pathways. Activating mutations impair the intrinsic GTPase activity of the *KRAS* gene product, resulting in a protein that is constitutively active. *KRAS* mutations are one of the earliest genetic abnormalities observed in the progression of pancreatic cancer.¹¹⁻¹⁴ Approximately 5% of pancreatic cancers express wild-type *KRAS* but exhibit mutations in one of the members

of the RAF-MAPK signaling pathway called the *BRAF* gene, which is located on chromosome 7q. This results in activation of RAF-MAPK signaling even in the absence of *KRAS* mutations.^{15,16} Another important kinase that is constitutively activated is the phosphoinositide-3-kinase pathway, where the *AKT2* gene, located on chromosome 19q, is overexpressed in about 10%–20% of pancreatic cancers.^{11,14,16}

In contrast to dominantly acting oncogenes, tumor suppressor genes are recessive, and inhibition of their function typically requires mutations in both paternal and maternal copies. Most common tumor suppressor genes that are inactivated in development of pancreatic cancer are p16/INK4A, p53 and DPC/SMAD4. p16/INK4A is the most frequently inactivated tumor suppressor gene and about 40% of pancreatic tumors have a homozygous deletion of both alleles of the gene. p16/INK4A regulates the cell cycle by binding to CDK4 and CDK6 to inhibit binding of cyclin D1 to the CDKs and phosphorylation of retinoblastoma leading to cell cycle arrest. Thus the loss of p16/INK4A deregulates the cell cycle check point, leading to the development of pancreatic cancer, and this is also observed in patients with germline mutation of this gene.^{5,11,14,16}

In addition to these oncogenes and tumor suppressor genes, abnormalities are observed in epigenetics or telomere length mismatch repair genes or telomere length.¹⁷⁻¹⁹ During the past decade, the discovery of noncoding RNAs and their functions in both normal and cancer tissues has added to the complexity of cell biology and cell signaling but has identified another key element that regulates genes and influences cancer cell phenotype.²⁰⁻²²

Treatment options and patient outcomes

Pancreatic cancer is the tenth most common cancer and is a highly aggressive and therapy-resistant malignancy, with a 1-year survival rate of 24% and a 5-year survival rate of 6%.²³

Surgical management

Pancreaticoduodenectomy is the curative resection strategy used in the management of pancreatic cancer and is performed on patients with adenocarcinoma of the pancreas, especially of the head region. Median survival rates after resection for localized PDAC is 12.7 to 17.5 months, and 4- and 5-year survival rates range from 6.8% to 21%.²⁴⁻²⁷

Chemotherapy

5-Fluorouracil (5-FU) and gemcitabine are currently used as chemotherapeutic agents. 5-FU is a thymidylate synthase

inhibitor and inhibits the synthesis of thymidine, which is required for DNA replication. Gemcitabine has been a drug of choice for treating pancreatic cancer since 1996. Gemcitabine is a prodrug which is phosphorylated to its active metabolite that inhibits DNA chain elongation, resulting in DNA fragmentation and cell death. The clinical response to gemcitabine was 23.8% and 4.8% to 5-FU, and the overall survival was 5.56 and 4.41 months, respectively. In many solid tumors, response to combination therapy is better than single agent therapy. However, the combination therapies in patients with pancreatic cancer failed to produce any significant overall survival benefits and are associated with severe toxic side effects.^{24–28}

Radiation therapy

Radiation therapy is used for locally advanced unresectable cancer or as a palliative or adjuvant therapy when the patient is undergoing surgical resection of the pancreas. Radiation therapy is being used as a postoperative regimen along with chemotherapy, which may improve the survival rate when compared to surgery alone.^{24–27}

Targeted therapies

Recent research has been focused on understanding the molecular pathways and factors that play an important role in pancreatic cancer progression, and therapies that target specific pathways are being developed. Bevacizumab (Avastin) is a recombinant humanized antivascular endothelial growth factor monoclonal antibody, which has been used in combination with gemcitabine in a Phase II study, and 6-month and 1-year survival rates were 77% and 26% respectively. Overexpression of epidermal growth factor receptor (EGFR) is a negative prognostic factor for survival of pancreatic cancer patients. Erlotinib, an oral tyrosine kinase inhibitor, and cetuximab, a monoclonal antibody against EGFR, are being used in combination with gemcitabine. Sorafenib, a small molecule inhibitor of vascular endothelial growth factor (VEGF) receptor (VEGFR)2 and RAF1 are being evaluated in combination with gemcitabine, and the combination therapy is well tolerated but ineffective in treating metastatic pancreatic cancer. Sunitinib, an inhibitor of VEGF and platelet derived growth factor receptor is also being evaluated as a second line of therapy and is in a Phase II clinical trial.^{25–27,29}

Also, new agents such as immunogenic telomerase peptide GV1001, along with granulocyte macrophage colony stimulating factor, is administered over 10 weeks with monthly booster vaccinations. Additionally, patients with

BRCA-2 mutations are sensitive to mitomycin C, which is also being tested in pancreatic cancer patients.^{25–27,29}

Sp1 transcription factor

The specificity protein (Sp)/Krüppel-like factor (KLF) family is subdivided into the Sp family, which bind GC-boxes, and the KLF family, which binds to GT-boxes, that act as activators as well as repressors of transcription. The Sp/KLF family of transcription factors contains three C2H2-type zinc finger DNA-binding domains and recognizes GC-(GGCGG or GGCG) and GT-(GGTGTGGGG) boxes with different affinities due to differences in the amino acid substitutions in the zinc finger domain of these proteins. The Sp family genes (Sp1–9) are located on the hox gene cluster, which encodes a large family of transcription factors that specify head–tail axis in embryonic development.³⁰ The Sp family is divided into Sp1–4, which contain the N-terminal glutamine-rich transactivation domains A and B, and Sp5–9, which lack the N-terminal glutamine-rich transactivation domains. All the Sp family proteins contain a buttonhead box N-terminal to the zinc finger domain and a conserved stretch of eleven amino acid residues which contribute to their transactivation potential. Deletion of this region in Sp1 results in decreased activity of Sp1. The Sp box (SPLALLAATCSR/KI) is located at the N-terminus of Sp proteins and contains an endoproteolytic cleavage site that plays an important role in proteolysis of Sp proteins.^{31–34}

Sp1 is the first transcription factor identified and is located on chromosome 12q13.13 (HOX C). It binds to GC-boxes and also to CT- and GT-boxes with lower affinity. Sp1 is ubiquitously expressed, and Sp-knockout embryos are severely retarded in development and die around day eleven of gestation. However, Sp1 null embryos express Sp1 target genes at normal levels, and only thymidine kinase and the methyl-CPG binding protein 2 gene expression was decreased. The methyl-CPG binding protein 2 gene is associated with maintenance of differentiated cells, suggesting a role of Sp1 in regulation of differentiation. This demonstrates that other Sp family members may compensate, in part, for loss of Sp1 during early embryogenesis.^{32,33,35,36}

Sp1 is an important transcription factor regulating various cellular and viral genes containing GC-boxes and plays a significant role in many signal transduction pathways linked to cancer. Carcinogen-induced transformation of human fibroblast cells or stable transfection of H-Ras^{V12} in these cells results in an 8–18-fold increase in Sp1 expression, and the malignant cells formed tumors in athymic nude mice.³⁷ Also, increased expression and binding activity of Sp1 is

observed in skin tumors compared to papillomas and is associated with increased tumor progression. The promoters of many proapoptotic and antiapoptotic genes such as Bcl-2, Bax, survivin, Fas and Fas ligand, TGF β and its receptors, TNF α , and TNF α -mediated apoptosis-inducing ligand contain GC-rich Sp binding sites. Sp1 also regulates expression of angiogenic genes VEGFR1, VEGFR2, and VEGF,^{35,38,39} and also regulates growth inhibitory genes such as p21 and caveolin. Functional GC-boxes are present on the retinoblastoma, c-Myc, c-jun, c-fos, E2F1, nuclear factor κ B (NF κ B), and Egr-1 gene promoters.³⁴

Several studies have reported that Sp1 and also Sp3 and Sp4 are highly expressed in pancreatic, breast, bladder, and colon cancer cell lines and tumors derived from these cells in xenograft models.^{35,38–44} Even though the knockout of Sp1 in mice is embryo-lethal, the expression of Sp1 is significantly decreased in rodent and human tissues with increasing age, and studies in our laboratory have shown that Sp1 expression is low in most organs when compared to tumors.^{44–47}

Specific role of Sp1 transcription factor in pancreatic cancer

Normal pancreatic ductal epithelial cells express very low Sp1; however, overexpression of Sp1 in patients with PDAC is associated with increased metastasis and poor prognosis. Sp1 overexpression is associated with TNM tumor staging grade IIB and higher and lymph node metastasis. In patients with metastasis, 77% of the primary tumors were Sp1 positive. The 5-year survival rate is 19% and median survival is 13 months in patients with positive Sp1 expression compared to 55% and 63 months in patients without Sp1 expression in their primary tumors.^{48,49} Sp1 could be used as a prognostic marker to predict the clinical outcome in pancreatic cancer patients, and understanding the role of Sp1 in pancreatic cancer will provide a tool with which to develop drugs that can target Sp1 in these patients.

Previous studies have shown that Sp1 is essential for angiogenesis through regulation of VEGF and its receptors VEGFR1 and 2.^{35,38,39} Sp1 and VEGF are constitutively overexpressed in pancreatic cancer, and there is a positive correlation between microvessel density and pancreatic cancer progression, even though pancreatic cancer is not a grossly vascular tumor. Sp1 plays a central role in multiple aspects of angiogenesis by interacting with various tumor suppressor and oncogenes, and also by binding to VEGF promoter and on promoters of various genes encoding angiogenic molecules such as fibroblast growth factors, EGFR, and insulin-like growth factor 1 receptor.^{50,51} VEGF neutralization with

Avastin leads to a feedback activation of Sp1 and subsequent upregulation of VEGF expression leading to Avastin resistance; however, blocking Sp1 function results in reversal of Avastin resistance and in sensitization of tumors.⁵² Sp1 plays an important role in TGF β -mediated epithelial to mesenchymal transformation leading to pancreatic cancer resistance to chemotherapy.^{53,54}

Studies in our laboratory have shown that Sp1, Sp3, and Sp4 are highly expressed in various pancreatic cancer cell lines, and knockdown of Sp1 using RNA interference in Panc1, Panc28, or L3.6pL cells decreased cell proliferation and induced apoptosis through induction of cleaved PARP and annexin V staining. Transfection of PDAC cells with siSp1 (small interfering RNA [siRNA] oligonucleotide against Sp1) significantly decreased expression of various genes involved in cell growth (cyclin D1, EGFR, c-Met), angiogenesis (VEGF, VEGFR1, VEGFR2), survival (survivin, insulin-like growth factor 1 receptor, bcl-2), inflammation (NF κ B, STAT3), and metastasis.^{35,38,39,55,56} Some of these same responses are observed after knockdown of Sp3 and Sp4.

Critical analysis of the potential for targeting Sp1 in pancreatic cancer

To identify new therapeutic targets for pancreatic cancer, a complete understanding of cellular and molecular mechanisms of tumor development and progression is needed. Among the potential targets, Sp1 plays an important role in regulation of cell growth, angiogenesis, and metastasis, and differential expression of Sp1 with higher expression in tumors when compared to normal tissues makes Sp1 an important target for drug development for pancreatic cancer. In this review, we will focus on Sp1 as a molecular target for various therapeutic strategies (Figure 1) for treatment of pancreatic cancer.

Mithramycin A (MTA) is an aureolic acid type polyketide first isolated from soil bacterium, *Streptomyces argillaceus*. MTA binds to guanosine–cytosine (GC) regions on double stranded DNA and inhibit different genes with GC-rich promoter regions. MTA selectively inhibits Sp1-mediated transcription, and the interaction is reversible,^{57,58} MTA downregulates X-linked inhibitor of apoptosis through inhibition of Sp1 and renders the cells sensitive to TNF α -mediated apoptosis-inducing ligand in prostate and pancreatic cancer cells.^{59,60}

Tolfenamic acid (TA), a nonsteroidal anti-inflammatory drug, inhibits prostaglandin biosynthesis and leukotriene synthesis. TA downregulated Sp1, Sp3, and Sp4 in Panc1, Panc28, and L3.6pL pancreatic cancer cells, both in vitro

and in vivo, and the effects were reversed when treated with lactacystin in a proteasome-dependent pathway.⁴⁰ Also, TA sensitized pancreatic cancer cells and tumors to radiation therapy by downregulating survivin,⁶¹ and combined treatment of TA with MTA synergistically decreases Sp1 activity and tumor growth in an in vivo pancreatic model.⁶²

Curcumin, also known as diferuloylmethane, is a polyphenolic phytochemical extracted from the root of *Curcuma longa*. Studies have shown that curcumin exhibits a wide range of anticancer activities in multiple tumor types, including pancreatic cancer. Curcumin inhibited NFκB subunits p65 and p50 through downregulation of Sp

transcription factors, and the mechanism of action involves a decrease in mitochondrial membrane potential and induction of reactive oxygen species (ROS). These effects were reversed when treated with antioxidants such as glutathione or dithiothreitol.⁵⁶ Similar effects were noticed when treated with synthetic analogues of curcumin.⁶³

Betulinic acid (BA), a pentacyclic triterpenoid extracted from birch bark, decreases Sp transcription factors in pancreatic cancer cells (Figure 2), and when combined with MTA, has synergistic effects on downregulation of Sp1 and tumor growth.^{47,54,64} Arsenic trioxide, which is clinically used to treat leukemia, inhibits Sp1, Sp3, and Sp4 through

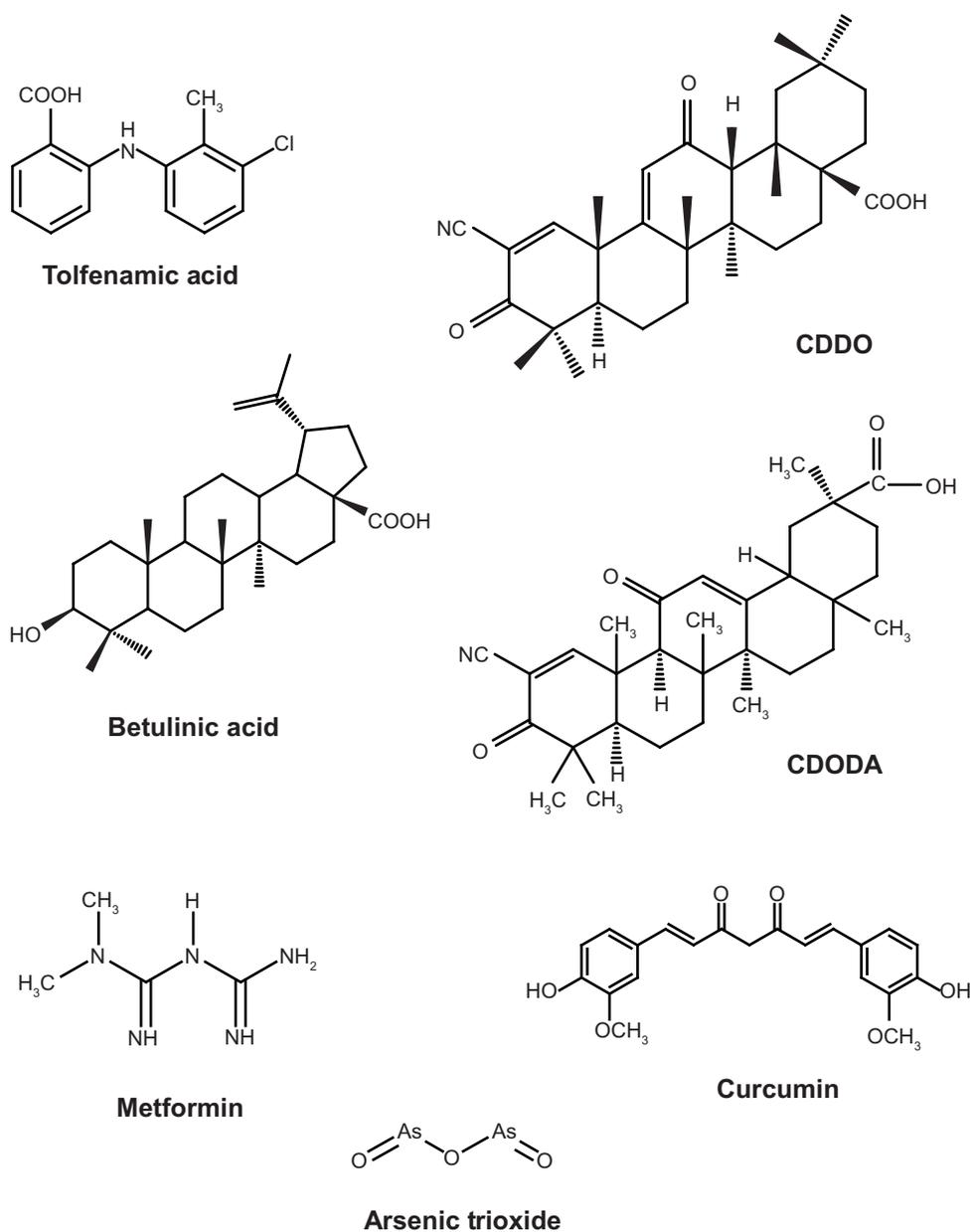


Figure 1 (Continued)

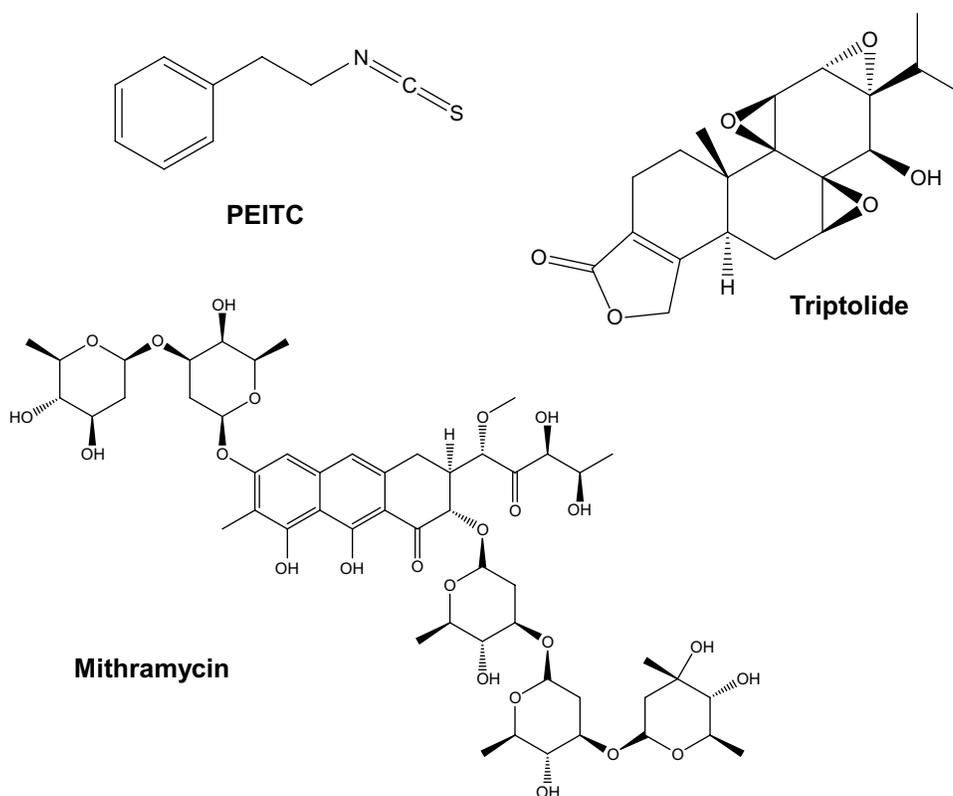


Figure 1 Structures of select compounds that downregulate Sp protein transcription factors in pancreatic cancer cells.

Abbreviations: CDDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate; CDODA, 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate; PEITC, phenethyl isothiocyanate; Sp, specificity protein.

ROS-dependent pathways in bladder, colon, prostate, and pancreatic cancer cells.⁴⁴

Methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) is a pentacyclic triterpenoid and synthetic analogue of oleanolic acid. CDDO-Me has a wide range of anticancer activity in various cancer cells and downregulates Sp transcription factors in Panc1, Panc28, and L3.6pL pancreatic cancer cells in a ROS-dependent pathway.

CDDO-Me causes mitochondriotoxicity resulting in reduction of mitochondrial membrane potential, induction of ROS and ROS-mediated downregulation of microRNA (miR)-27a, and induction of zinc finger protein ZBTB10 (an Sp repressor), which in turn downregulates Sp proteins in pancreatic cancer cells.^{55,64} CDDO-Me also downregulates telomerase (hTERT) expression and activity through inhibition of Sp1 and c-Myc in MIAPaCa-2 and Panc-1 pancreatic

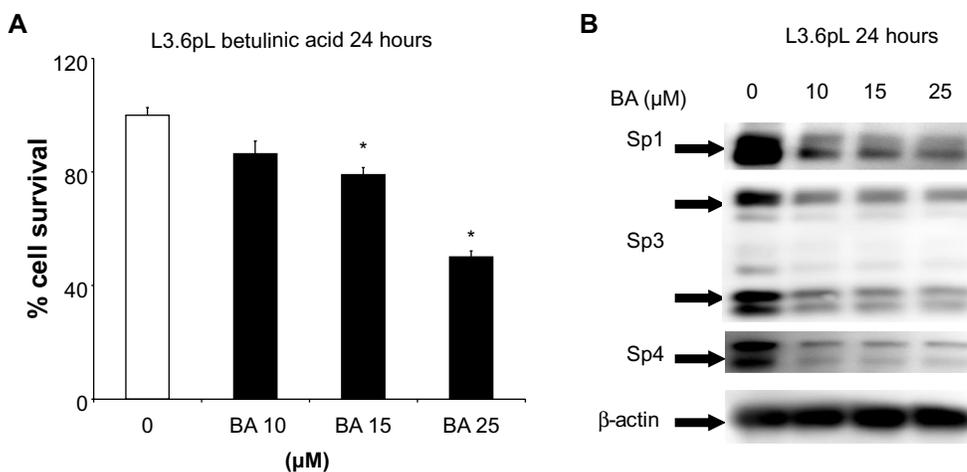


Figure 2 BA inhibits pancreatic cancer cell growth (A) and downregulates Sp1, Sp3, and Sp4 after 24 hours treatment (B).

Abbreviations: BA, betulinic acid; Sp, specificity protein.

cancer cell lines.⁶⁵ Studies in our laboratory have shown that methyl 2-cyano-3,11-dioxo-18-olean-1,12-dien-30-oate, or CDODA-Me, which is structurally similar to CDDO-Me and synthetically derived from the licorice constituent glycyrrhetic acid, also downregulates Sp1, Sp3, and Sp4 proteins in a ROS-dependent pathway in pancreatic cancer cells.⁶⁴

Metformin, also known as N, N-dimethyl biguanide, is an oral hypoglycemic drug prescribed for type 2 diabetes, and it exhibits chemopreventive effects on diabetics with pancreatic cancer.⁶⁶⁻⁶⁸ In a study by Nair et al, metformin downregulated Sp transcription factors in proteasome-dependent pathways in Panc28 and L3.6pL pancreatic cancer cells; however, in Panc1 cells, metformin decreased Sp protein through a miR-27a: ZBTB10 axis via a phosphatase-dependent pathway, which

was dependent on induction of dual specificity phosphatases MKP-1 and MKP-5. These effects were reversed upon treatment with the phosphatase inhibitor sodium orthovanadate.⁶⁹

Triptolide, a diterpenoid epoxide extracted from *Tripterygium wilfordii*, induces apoptosis through downregulation of HSP1 and HSP70 via inhibition of Sp1 activity. This is mediated by inhibition of glycosylation of Sp1 through O-GlcNAcylation, which prevents Sp1 translocation to the nucleus and thus reduces the activity.⁷⁰ Studies from our laboratory showed that celastrol, a pentacyclic triterpenoid, which is a sister compound of triptolide, decreased Sp proteins in pancreatic cancer cells.

Phenethyl isothiocyanate is a natural compound found in cruciferous vegetables and exhibits antineoplastic activity

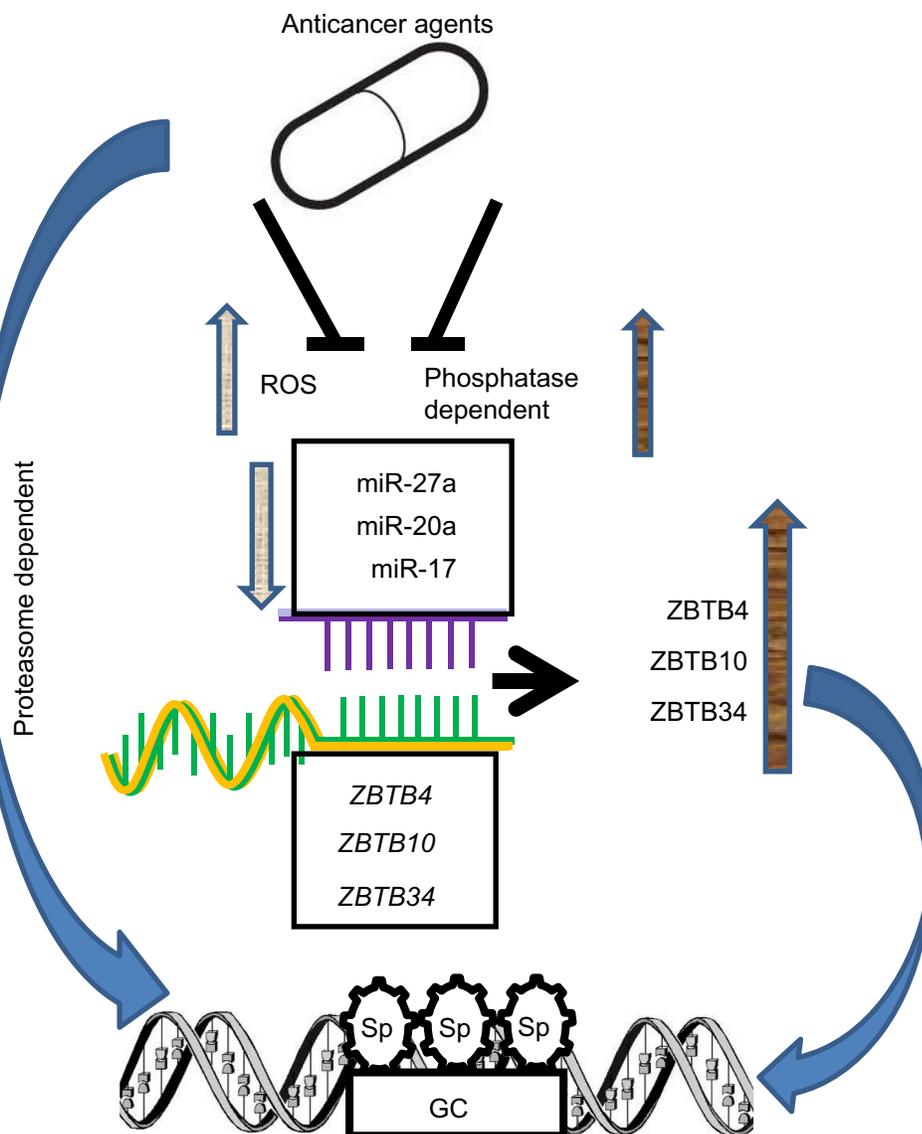


Figure 3 Proposed schematic for Sp protein downregulation in pancreatic cancer.

Abbreviations: GC, GC-box; miR, microRNA; ROS, reactive oxygen species; Sp, specificity protein.

in various cancers, including pancreatic cancer. Phenethyl isothiocyanate also downregulates Sp transcription factors through inhibition of miR-17-5p:20a, miR-27a, and induction of ZBTB4, 10, and 34 in a ROS dependent pathway.⁷¹

Conclusion

Sp transcription factors are members of the Sp/KLF of 25 transcription factors that bind GC-rich promoter sequences and regulate basal expression of multiple mammalian and viral genes. Knockout of Sp1 in mice is embryo-lethal; however, expression of Sp1 is significantly decreased in rodent and human tissues with increasing age. Studies in our laboratory show that in mouse xenograft studies, Sp1 expression is low in the liver, kidney and also in more proliferative tissues such as the gastrointestinal tract. In contrast, expression of Sp1 and also Sp3 and Sp4 are high in breast, colon, pancreatic, prostate, and bladder tumor xenografts and their derived cancer cell lines. RNA interference studies in which Sp1 is knocked down demonstrate that Sp transcription factors regulate several genes involved in cancer cell survival, angiogenesis, and proliferation, and Sp3 and Sp4 also exhibit these activities in some cancer cell lines. Moreover, it was reported that Sp1 is a biomarker that identifies patients with a highly aggressive subtype of PDAC. Targeting Sp1 can be accomplished using Sp1 inhibitors such as MTA, which bind specifically to GC-rich regions of double stranded DNA and block Sp1 binding, or by using antineoplastic drugs that downregulate expression of Sp1, Sp3, Sp4, and Sp-regulated genes. The mechanism of action through which these drugs act is variable and includes activation of proteasome-, ROS-, and phosphatase-dependent pathways (Figure 3). Combination studies with BA or TA and MTA showed that these drugs can synergistically downregulate Sp1 and enhance tumor growth inhibition, indicating there is potential for combination therapies with agents that target Sp proteins and may offer improved treatment for patients with pancreatic cancer with decreased treatment-related toxicities.

Disclosure

The authors report no conflicts of interest in this work.

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