NEW MECHANISM BASED ANTICANCER DRUGS FOR TREATMENT OF PANCREATIC AND BLADDER CANCERS

A Dissertation

by

INDIRA DEVI JUTOORU

Submitted to Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Toxicology

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Approved by:

Chair of Committee,	Stephen H. Safe
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ABSTRACT

New Mechanism Based Anticancer Drugs for Treatment of Pancreatic and Bladder Cancers. (May 2010)

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Chair of Advisory Committee: Dr. Stephen H. Safe

Methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (CDODA-Me) is a synthetic triterpenoid that inhibits growth of Panc1 and Panc28 pancreatic cancer cell lines and activates peroxisome proliferator-activated receptor γ (PPARγ)-dependent transactivation in these cells. CDODA-Me has also induced p21 and p27 protein expression and downregulated cyclin D1; however, these responses were receptor-independent. CDODA-Me induced apoptosis, which was accompanied by receptor-independent induction of the proapoptotic proteins early growth response-1 (Egr-1), nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1), and activating transcription factor-3 (ATF3). Induction of NAG-1 in Panc28 cells was p38-mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-K)-dependent, but Egr-1-independent, whereas induction in Panc1 cells was associated with activation of p38-MAPK, PI3-K and p42-MAPK and was only partially Egr-1-dependent.

Specificity protein (Sp) transcription factors Sp1, Sp3 & Sp4 are overexpressed in multiple tumor types and negative prognostic factors for survival. Since Sp proteins regulate genes associated with survival (survivin), angiogenesis [vascular endothelial growth factor and its receptors] and growth [cyclin D1, epidermal growth factor receptor], research in this laboratory has focused on development of anticancer drugs that decrease Sp protein expression. Arsenic trioxide, curcumin, 2-cyano-3,12-dioxoleana-1,9-dien-28-oic acid (CDDO), CDDO-Me, and celastrol exhibit antiproliferative, antiangiogenic and proapoptotic activity in many cancer cells and tumors. Treatment of cancer cells derived from urologic and gastrointestinal tumors with arsenic trioxide decreased Sp1, Sp3 and Sp4 transcription factors and cotreatment with the proteosome inhibitor MG132 did not inhibit downregulation of Sp proteins in these cancer cells. Mechanistic studies suggested that compound-dependent downregulation of Sp and Sp-dependent genes was due to decreased mitochondrial membrane potential and induction of reactive oxygen species, and the role of peroxides in mediating these responses was confirmed using hydrogen peroxide, demonstrating that the mitochondriotoxic effects of these compounds are important for their anticancer activities.

Moreover, repression of Sp and Sp-dependent genes by CDDO-Me and celastrol was due to downregulation of microRNA-27a and induction of ZBTB10, an Sp repressor, and these responses were also reversed by

antioxidants. Thus, the anticancer activity of CDDO-Me and celastrol is due, in part, to activation of ROS which in turn targets the microRNA-27a:ZBTB10–Sp transcription factor axis to decrease growth inhibitory, pro-apoptotic and antiangiogenic genes and responses.

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I. INTRODUCTION

Cancer statistics

Cancer arises when cells undergo uncontrolled proliferation due to gain of function of oncogenes and loss of function of tumor suppressors (1); and cancer cells are characterized by unrestrained growth and spread to other organs and tissues. Cancer is a major public health problem in the United States and many other parts of the world and one in four deaths in the United States is due to this disease and approximately 1,479,350 new cancer cases are expected to be diagnosed in 2009 (2). This estimate does not include carcinoma in situ (noninvasive cancer) of any site except urinary bladder, and does not include basal and squamous cell skin cancers. It is estimated that more than 1 million unreported cases of basal and squamous cell skin cancers were to be diagnosed in 2009 and about 562,340 Americans died of cancer alone in 2009; that is more than 1,500 people a day. Cancer is the second most common cause of death in the US, exceeded only by heart disease. The 5-year relative survival rate for all cancers diagnosed between 1996-2004 is 66% which is increased from 50% in 1975-1977. The increase in survival is due to progress in diagnosing certain cancers at an earlier stage and improvements in treatment.

_____

This dissertation follows the style of Cancer Research.

Survival statistics vary greatly by country/region, cancer type and stage at diagnosis. The lifetime probability of being diagnosed with an invasive cancer is higher for men (44%) than for women (37%) in the United States. Among men, cancers of the prostate, lung and bronchus, colon and rectum, and urinary bladder are most common and account for about 50% of all newly diagnosed cancers. The three most commonly diagnosed types of cancer among women in 2009 are cancers of the breast, lung and bronchus, and colon and rectum which accounting for 51% of estimated cancer cases in women (Figure 1).

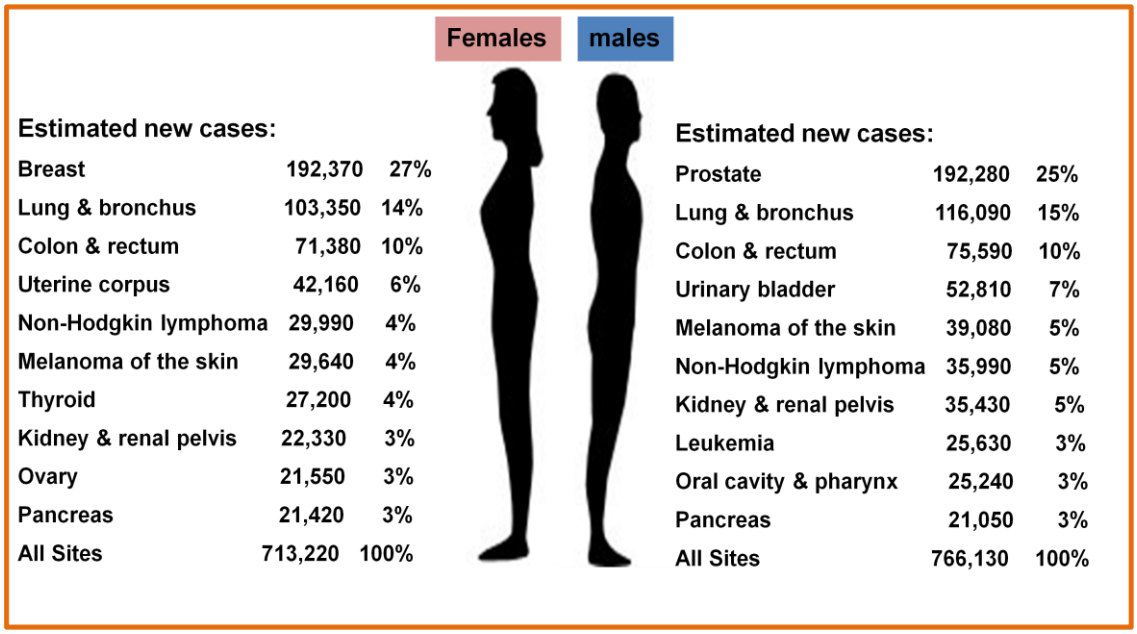

Figure 1. Estimated new cancer cases, by sex, for ten major cancer types in United States, 2009 (2).

Cancers of the lung and bronchus, prostate, colorectum and pancreas in men, and cancers of the lung and bronchus, breast, colorectum and pancreas in

women continue to be the most common fatal cancers (Figure 2) (2). The focus of this discussion will be on gastrointestinal cancers which include colon, rectum, stomach, liver, gall bladder, esophagus, small intestine, pancreas and other digestive organs and urinary system cancers which include bladder, kidney & renal pelvis and ureter and other urinary organs.

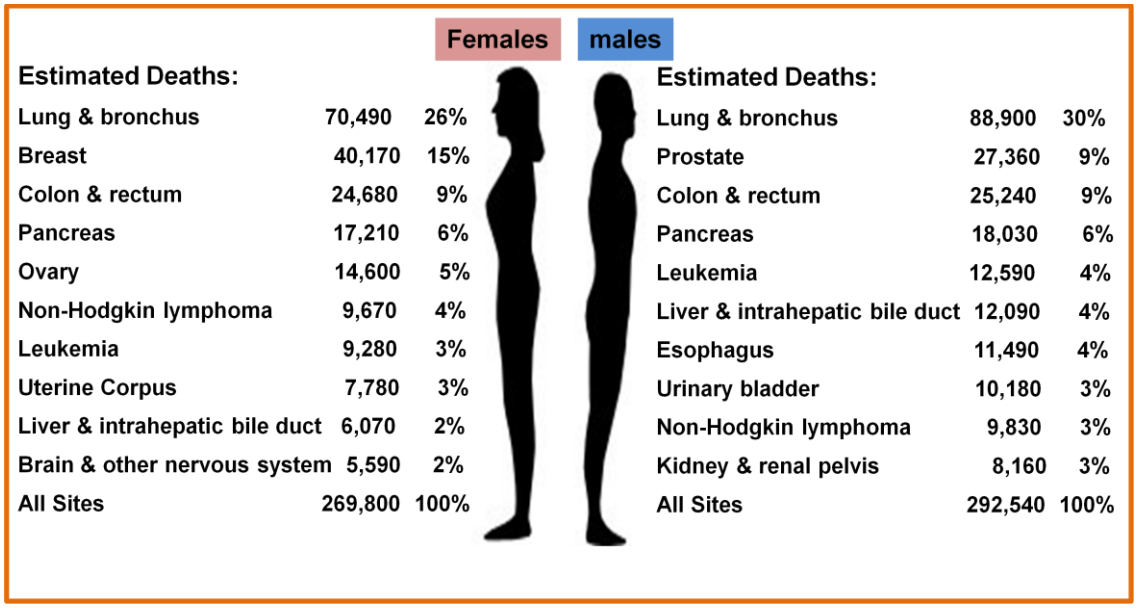

Figure 2. Estimated new cancer deaths, by sex, for ten major cancer types in United States, 2009 (2).

Gastrointestinal cancer

Major organs in digestive/gastrointestinal system include esophagus, stomach, small intestine, colon and rectum, anus, pancreas, liver and gall bladder. An estimated 275,720 new cases and about 135,830 estimated deaths from gastrointestinal cancers are expected to occur in United States in 2009 with

highest incidence and deaths due to colon cancer in both men and women followed by pancreatic cancer (Table 1).

Esophageal cancer: Esophageal cancer is the third most frequent cancer of the digestive system and has the highest rate of malignancy. An estimated 16,470 new cases and about 14,530 estimated deaths of oesophgeal cancer are expected to occur in US in 2009 alone (3).

Gastric cancer: Stomach cancer is the second most common cancer worldwide and fourth most commonly diagnosed malignancy worldwide (4). In western countries the incidence of gastric cancer is declining, however, it has been a major concern in Asia and parts of South America. It is the most common epithelial malignancy (5) and in United States an estimated 21,130 new cases and 10, 620 deaths from gastric cancer are expected to occur in 2009.

Pancreatic cancer: An estimated 42,470 new cases of pancreatic cancer and an estimated 35,240 deaths are expected to occur in the US in 2009. Incidence and death rates of pancreatic cancer have been stable in men but the incidence and death rates have been increasing in women by 0.6% and 0.1% per year.

Colorectal cancer: An estimated 106,100 cases of colon and 40,870 cases of rectal cancer are expected to occur in 2009. Colorectal cancer is the third most common cancer in both men and women. Colorectal cancer incidence rates have been decreasing for most of the past two decades (from 66.3 cases per 100,000 population in 1985 to 46.4 in 2005) due to increase in screening that allows for the detection and removal of colorectal polyps before they progress to

cancer. An estimated 49,920 deaths from colorectal cancer are expected to occur in 2009, accounting for almost 9% of all cancer deaths. Mortality rates for colorectal cancer have declined in both men and women over the past two decades, with a steeper decline since 2002 (4.3% per year from 2002 to 2005 in both men and women, compared to 2.0% per year from 1990 to 2002 in men and 1.8% per year from 1984 to 2002 in women) (2).

Liver cancer: Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world and it accounts for 5.6% of all human cancers (7.5% among men and 3.5% among women). Well established risk factors for hepatocellular carcinoma include chronic infection with hepatitis B or C virus which is present in >85% of primary liver cancers (6, 7). The estimated new cases for liver and intrahepatic bile duct cancer is 22,260 and estimated deaths from this cancer is 18,160 including both men and women in US in 2009. When it comes to cancer mortality, liver and intrahepatic bile duct cancer is the sixth and ninth most common cause of death in men and women.

Other gastrointestinal cancers: Other gastrointestinal cancers include cancers of gall bladder, small intestine, anus, anal canal, anorectum and other digestive organs. The estimated number of new cases for these cancers are 26,060 and estimated deaths are 7,360 in 2009 in United States alone (2).

Table 1. Estimated new gastrointestinal cancer cases and deaths by sex, US, 2009 (2).

	Estimated New Cases			Estimated Deaths		
	Both Sexes	Male	Female	Both Sexes	Male	Female
All sites	1,479,350	766,130	713,220	562,340	292,540	269,800
Digestive system	275,720	150,020	125,700	135,830	76,020	59,810
Esophagus	16,470	12,940	3,530	14,530	11,490	3,040
Stomach	21,130	12,820	8,310	10,620	6,320	4,300
Small intestine	6,230	3,240	2,990	1,110	580	530
Colon	106,100	52,010	54,090	49,920	25,240	24,680
Rectum	40,870	23,580	17,290			
Anus, anal canal, & anorectum	5,290	2,100	3,190	710	260	450
Liver & intrahepatic bile duct	22,620	16,410	6,210	18,160	12,090	6,070
Gallbladder & other biliary	9,760	4,320	5,440	3,370	1,250	2,120
Pancreas	42,470	21,050	21,420	35,240	18,030	17,210
Other digestive organs	4,780	1,550	3,230	2,170	760	1,410

Urinary tract

The urinary tract includes kidney, ureters, urethra, urinary bladder. An estimated 131,010 new cases and 28,100 estimated deaths from urinary tract cancer are expected to occur in 2009 with the highest incidence and deaths from bladder cancer (Table 2).

Urinary bladder: An estimated 70,980 new cases of bladder cancer and an estimated 14,330 deaths from bladder cancer are expected to occur in 2009. Over the past two decades, bladder cancer incidence and mortality rates have been stable among men. In women the incidence rates are increasing slightly (0.2% per year) but bladder cancer death rates are declining since 1975.

Bladder cancer incidence is nearly four times higher in men than in women and more than two times higher in Caucasian than in African American men (2).

Kidney and renal pelvis: There are about 57,760 cases of kidney cancer diagnosed in the US annually and nearly 13,000 deaths are caused by this disease each year. Kidney cancer is the seventh most common cancer in men and the ninth most common cancer in women and the incidence of kidney cancer, unlike that of other genitourinary malignancies, is rapidly increasing by 2.5% per year (8).

Other urinary organs: Estimated cancer cases from ureter and other renal organs is about 2,270 and about 790 cancer deaths (men and women) are reported in the United States in 2009 (2).

Table 2. Estimated new urinary tract cancer cases and deaths by sex, US, 2009 (2).

	Estimated New Cases			Estimated Deaths		
	Both Sexes	Male	Female	Both Sexes	Male	Female
All sites	1,479,350	766,130	713,220	562,340	292,540	269,800
Urinary system	131,010	89,640	41,370	28,100	18,800	9,300
Urinary bladder	70,980	52,810	18,170	14,330	10,180	4,150
Kidney & renal pelvis	57,760	35,430	22,330	12,980	8,160	4,820
Ureter & other urinary organs	2,270	1,400	870	790	460	330

Cellular and molecular mechanisms in progression of cancer

During the last ten years many important genes and gene mutations involved in development of various cancers and the pathways through which they act have been characterized. In essence, cancer is now recognized as a genetic disease that is associated with multistep processes in which a normal cell is converted into a cancer cell. In cancer cells the regulatory circuits involved in normal cell proliferation and homeostasis are defective and there are >100 distinct types of cancer and subtypes of tumors that can be found within specific organs. In general tumors are broadly classified as hematopoietic and solid tumors. Hematopoietic tumors include leukemias and lymphomas composed of neoplastic cells whose precursors are normally mobile. Solid tumors generally arise from epithelial or mesenchymal cells that are not normally mobile. It has been estimated that at least three mutations are required to develop a malignant solid tumor in adults whereas only one or two mutations are required for hematopoietic tumors since their precursors are already mobile and have invasive characteristics that solid tumors must develop to become malignant. There are many other molecular distinctions that add to cytogenetic, epidemiologic and medical evidence demonstrating that liquid and solid tumors are distinct from one another (9-11).

Unlike many genetic diseases where mutations in one gene can cause a disease, no single gene defect ‘causes’ cancer, since several mutated genes are required for development of cancer in order to circumvent multiple safeguards in

normal cells. Usually normal cells perform their functions in a tightly regulated manner and when placed in a distant tissue or organ they undergo self destruction (anoikis) whereas cancer cells grow uninterruptedly and can invade and survive in other organs. Alterations in three types of genes are responsible for tumorigenesis: oncogenes, tumor-suppressor genes and stability genes (11).

Oncogenes are mutated in ways that render the gene constitutively active and this can result from chromosomal translocations, gene amplifications or intragenic mutations affecting crucial residues that regulate activity of the gene. An activating somatic mutation in one allele of an oncogene is usually sufficient to bestow the cell with certain growth advantage (11).

Mutations in tumor suppressor genes result in decreased gene activity due to missense mutations at critical residues, or mutations that result in truncated proteins, deletions or insertions or from epigenetic silencing. For example point mutations in tumor suppressor gene p53 may result in loss of its activity to inhibit cell growth and induce cell death during stress. Amplification of MDM2 which binds to p53 and inactivates p53 by proteosomal degradation can also result in loss of p53 activity. Mutations in both maternal and paternal alleles is usually required to provide a selective growth advantage to a cell. At the physiological level both oncogene and tumor suppressor gene mutations drive a cancer cell by increasing tumor cell growth by activation of the cell cycle, inhibition of apoptosis or by increasing access to nutrients through increased angiogenesis (11).

A third class of genes involved in minimizing genetic alterations in cells are called stability genes and these include mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes which are responsible for repairing errors during normal or drug induced DNA replication. These repair genes are also important during mitotic recombination or chromosomal segregation involving large portions of the chromosomes. By inactivating stability genes mutations in other genes occur at higher rates (11).

Mutations in all three classes of genes can occur at germline or in single somatic cells resulting in hereditary predisposition to cancer or in sporadic cancers respectively. Somatic mutations in either oncogenes or tumor suppressor genes can initiate clonal expansion resulting in tumor formation. Germline mutations in any of these three classes of genes can predispose individuals to cancer but not directly induce cancer per se; however, individuals carrying these mutations develop cancer at earlier age. Interestingly, the most common forms of hereditary cancer leading to early and increased rates of breast and colon cancers are caused by inherited mutations of stability genes rather than tumor-suppressor genes or oncogenes. Tumors that become malignant must accumulate several rate-limiting mutations in cancer genes and these mutations generally occur over a period of time. Most cancers do not have a high mutation rate when compared to a normal cell (that has passed through several generations) when the mutation rate is measured at the nucleotide level where the number of mutations in a typical cancer cell is <1 per megabase of

DNA. Recent research has shown that genetic instability can contribute to cancer predisposition and chromosomal instability in cancer cells is much more common than mutations at the nucleotide level. Aneuploidy is observed in almost all solid tumors leading loss of heterozygosity (LOH) where an average of 25-30% of the alleles present in normal cells are lost in cancer cells and over 75% of the cell's alleles have been observed in some tumors. LOH gives the cancer cell an advantage due to loss of tumor suppressor genes and expression of variant gene products that facilitate rapid cell growth and adaptation to changing microenvironments. These genetic instabilities not only play a central role in cancer development but also in development of resistance to cancer chemotherapy (11, 12).

These genetic instabilities in cancer cells result in alterations in cell physiology that dictate their malignant phenotype and these are described as the six hallmarks in cancer progression (Figure 3) : self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (13).

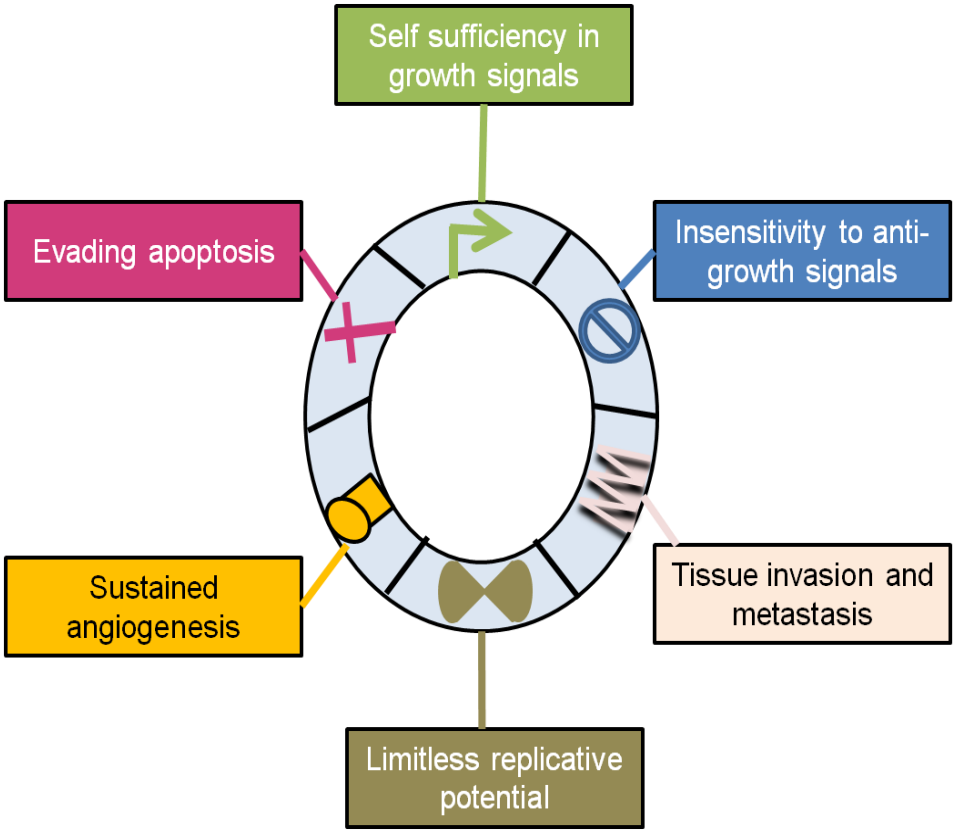

Figure 3. Hallmarks of cancer (13).

1. **Self sufficiency in growth signals:** Cancer cells unlike normal cells show reduced dependency on exogenous growth factors to proliferate and they can generate their own growth-promoting signals. In contrast to cancer cells, normal cells depend on growth factors to proliferate and these signals are transmitted through transmembrane receptors that bind various growth factors, extracellular matix components and other interacting molecules. This property of a non-transformed cell is important for maintainence of normal homeostasis in a tissue. With reduced dependency on exogenous growth factors, cancer cells become autonomous by constitutively activating

oncogenes and overexpressing growth factors such as PDGF (platelet derived growth factor), VEGF (vascular endothelial growth factor), TGF (transforming growth factor), EGF (epidermal growth factor). This creates a positive feedback signaling loop called autocrine stimulation. For example, in glioblastomas and sarcomas, cancer cells upregulate growth factors such as PDGFα and TGFα which obviates their dependency on growth factors from other cells within the tissue/organ. Cancer cells also overexpress certain cell surface receptors that transmit intracellular growth signals. These are usually transmembrane receptors whose cytoplasmic domains often carry tyrosine kinase activites; by overexpressing tyrosine kinase receptors, cells become highly responsive to physiological levels of growth signals that do not induce proliferation in normal cells. For example, the epidermal growth factor receptor family (EGFR/ErbB) includes EGFR, ErbB2/HER-2, ErbB3/HER-3 and ErbB4/HER-4. Recent studies have shown that EGFR which binds to EGF and TGFα is upregulated in stomach, brain and breast tumors and HER-2/ErbB2 which binds to heregulin is overexpressed in stomach and mammary tumors (13, 14).

2. **Insensitivity to antigrowth signals:** Cancer cells elude antiproliferative signals which inhibit proliferation by arresting cells in G0 phase or by abandoning their proliferative potential in the postmitotic state usually by acquiring differentiation-associated states. For example, Retinoblastoma (Rb), tumor suppressor gene, is mutated in childhood cancers, plays an

important role in transition from quiescent (G0 or G1) phase to replicating (S) phase of the cell cycle. Many genes are involved in Rb pathway such as cyclin D1 and p16 which activate cyclin dependent kinase 4 (CDK4) that phosphorylate Rb liberating E2Fs to allow cell proliferation. These antiproliferative signals are circumvented in the cells by disruption of Rb pathway, loss or mutation of Rb allowing cancer cells to propagate incessantly. (13, 15).

3. **Evading apoptosis:** Programmed cell death (apoptosis) is one of the major mechanisms of cell death and by evading apoptosis there is an imbalance between cell proliferation and cell death and this can result in tumor formation. Cancer cells overcome the balance between anti and pro-apoptotic signals and grow in adverse environmental conditions such as hypoxia, stress and chemotherapy which are toxic to normal cells. For example during hypoxia in normal cells, hypoxia inducible transcription factor (HIF)-1α induces apoptosis in a p53 dependent manner by promoting release of cytochrome c from mitochondria which subsequently activates apoptosis. However cancer cells evade HIF-1α induced apoptosis by upregulation of MDM2 which inhibits p53 resulting in increased resistance to hypoxia and decreased apoptosis (13, 16).
4. **Limitless replicative potential:** After a finite number of replications/cell divisions, normal cells undergo a process called senescence and stop growing. In contrast, cancer cells acquire the property of immortalization and

fail to undergo senescence by upregulating telomerase (hTERT) enzyme activity to maintain their telomere length. This property of cancer cells is essential for tumor progression and for their malignant state. For example, homozygous $p16^{INK4a}$ (cell cycle inhibitor) knockout mouse are susceptible to cancer development when exposed to carcinogens due to increased activity of telomerase; whereas in mice that are double null for both $p16^{INK4a}$ and telomerase, the cancer incidence was decreased by >50% due to shortened telomeres (13, 16) (17).

5. **Sustained angiogenesis:** Neovascularization or formation of new blood vessels is observed during early to mid-stage of tumor progression and is essential for supplying oxygen and nutrients that are crucial for survival and rapid clonal expansion. In cancer cells there is a shift in the balance between expression of angiogenesis inducers and inhibitors. For example, overexpression of vascular endothelial growth factor (VEGF) and/or fibroblast growth factors (FGF1/2) is observed in many cancers when compared to normal tissue and angiogenic inhibitors such as thrombospondin-1 or interferon β are decreased in cancer cells compared to normal cells (13).
6. **Tissue invasion and metastasis:** Metastasis of tumor cells is the major reason (>90%) for cancer deaths. Cancer cells acquire the capability of invasion and metastasis and cells migrate to distant organs resulting in outgrowth of the metastatic tumors in a new environment. This process is complex and includes activation of extracellular proteases, cell to cell

adhesion molecules such as cadherins and integrins which gives cancer cells the potential to become invasive and metastatic. In normal cells E-cadherin, belonging to family of CAMs, play an important role in bridging cell-cell and cell-surrounding environment interactions and transmit antigrowth and other signals through β-catenin to the cytoplasmic compartment of cells. This function of E-cadherin is lost in majority of epithelial cancers due to inactivating mutations, transcriptional repression or proteolysis of the extracellular domain of E-cadherin (13).

Metabolic changes in tumor development

The six hallmarks of cancer (Figure 3) noted above are also accompanied by changes in cellular metabolism. The phenomenon of altered metabolism in cancer cells was first discovered by Otto Warburg and is known as the 'Warburg phenomenon' which describes an altered increase in glycolysis and lactate production in the presence of high oxygen tension (anabolic reaction). This phenomenon is observed in many cancers which exhibit an increased uptake of glucose compared to normal cells. This increased uptake of glucose is the basis for positron emission tomography (PET) where the glucose analog 2-(18F)-fluoro-2-deoxy-D-glucose (FDG) is used to visualize/image cancers and metastasis (18).

In the presence of oxygen, normal nonproliferating differentiated cells metabolize glucose in the tricarboxylic acid (TCA) cycle to give carbon dioxide and water by oxidation of pyruvate (produced from glycolysis) and acetyl-CoA in

the mitochondria. During this reaction NADH (reduced nicotinamide adenine dinucleotide) is produced in TCA cycle and is used in mitochondrial oxidative phosphorylation to produce ATP with minimal formation of lactate. Lactate is produced in normal cells only during anaerobic glycolysis whereas large amounts of lactate are formed in cancer cells and this is independent of oxygen availability and hence called aerobic glycolysis (18, 19).

The high rate of glycolysis in cancer (also observed in normal proliferating cells) facilitates production of large amounts of ATP for energy and de novo synthesis of nucleotides, lipids and proteins necessary for rapid cell proliferation, invasion and metastasis. Hence glucose is diverted into the pentose phosphate pathway to produce NADPH (reduced nicotinamide adenine dinucleotide phosphate) and ribose-5-phosphate which is either utilized to synthesize nucleic acids or enters into glycolysis to produce ATP. Cancer cells utilized both glucose and glutamine as the two major sources of energy. Glucose is required only for lipid and nucleotide synthesis whereas glutamine replenishes TCA cycle with intermediates involved in anabolic reactions, for amino acid synthesis and for corporation of nitrogen into purines and pyrimidines required for nucleotide synthesis (Figure 4) (18-20).

In tumor cells, glycolysis appears to be aborted at either of the two steps. 1) The first aborted step is conversion of glucose into pyruvate which generates only 2 ATP molecules per glucose molecule and lactic acid. 2) In most cancers the TCA cycle appears truncated and acetyl-CoA is introduced to TCA cycle and

converted to citrate and exported to cytosol via the tricarboxylate transporter where it is cleaved by ATP citrate lyase (ACL) to generate oxaloacetate and acetyl-CoA. Acetyl-CoA serves as a building block for cell growth and proliferation and reduction of oxaloacetate gives malate which is imported into the mitochondria, reconverted to oxaloacetate and interacts with acetyl-CoA to complete the cycle. It has been proposed that enhanced glucose uptake for anabolic reactions can be advantageous for tumor cell progression for the following reasons (18):

1) Rapid growth of cancer cells is accompanied by fluctuating oxygen levels due to variable hemodynamics of distant blood vessels in a tumor environment and under these conditions cancer cells cannot survive by depending on oxidative phosphorylation to generate ATP. However, aerobic glycolysis provides the capability of producing ATP in oxygen-rich and deprived conditions. Even though aerobic glycolysis is less efficient in the production of ATP, the rate at which ATP is produced is approximately 100 times higher than in oxidative phosphorylation and the overall increased rate of glycolysis is beneficial for tumor growth. If this glycolysis-dependent energy requirement is not provided then the cells will undergo apoptosis (18, 19).

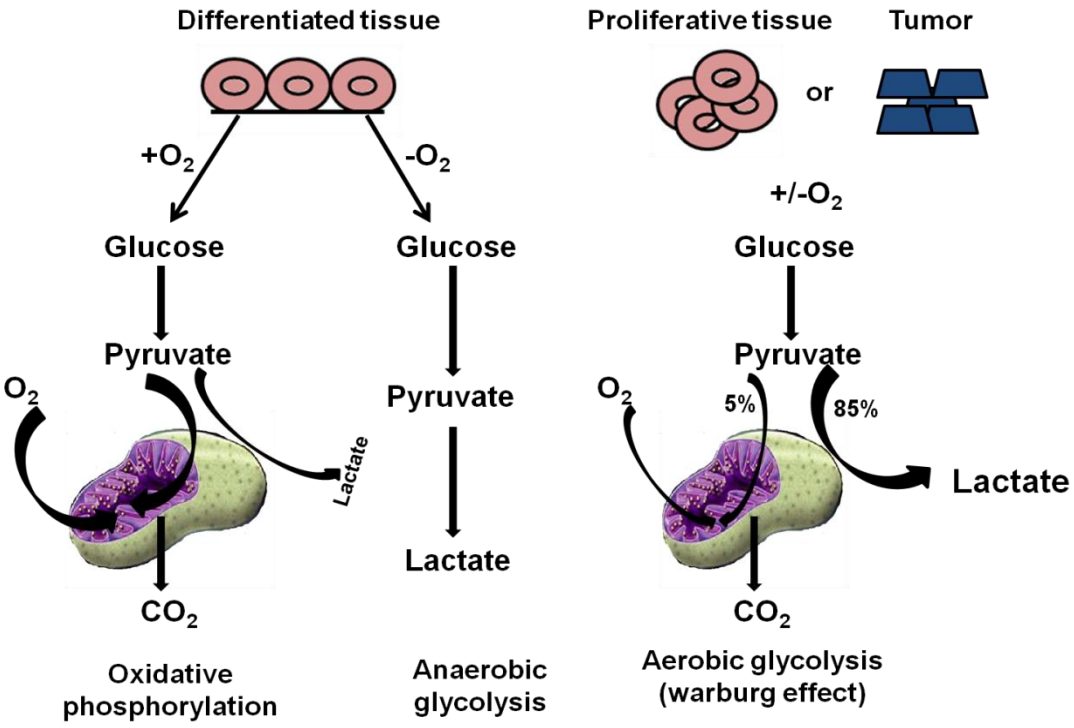

Figure 4. Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect) (19).

2) Bicarbonate and lactic acid are generated as end products of aerobic glycolysis and this favors tumor invasion since the resulting acidic environment suppresses anticancer immune effectors. Nontransformed stromal cells can also take up lactate produced by cancer cells to regenerate pyruvate that can either be used to refuel cancer cells or to enhance oxidative phosphorylation by stromal cells themselves. This creates a microenvironment that engages in complementary metabolic pathways and

products of aerobic glycolysis are recycled to sustain tumor growth and survival (18).

3) Glucose is metabolized through the pentose phosphate pathway (PPP) to provide glycolytic intermediates such as NADPH (reduced nicotinamide adenine dinucleotide phosphate) and ribose-5-phosphate which can enter into glycolysis. NADPH is not only used for generation of reduced glutathione that protects against oxidative insults and chemotherapeutic agents but also participates in lipid and cholesterol biosynthesis. For example glioblastoma cells in culture use up to 90% of glucose and 60% of glutamine to generate lactate or alanine which is transported out of the cell as waste but also facilitates formation of NADPH as a by-product (18, 19).

4) Aerobic glycolysis gives cancer cells the potential to use intermediates produced for anabolic reactions. Glycolytic intermediates such as ribose-5-phosphate can be used for nucleic acid biosynthesis, glucose-6-phosphate is used for glycogen production, dihydroxyacetone phosphate is used for triacylglyceride and phospholipid synthesis and pyruvate is utilized for alanine and malate synthesis. The embryonic isoform of pyruvate kinase which dephosphorylates phospoenolpyruvate to give pyruvate is absent from most adult tissues (except adipocytes) but is highly expressed in tumors. This isoform plays a role in the accumulation of phosphometabolites upstream of pyruvate synthesis and these are subsequently available as precursors for synthesis of amino acids, lipids and nucleic acids. In

proliferating cancer cells pyruvate enters into the truncated TCA cycle resulting in the export of acetyl-CoA from the mitochondrial matrix and pyruvate then becomes available for synthesis of fatty acids, cholesterol and isoprenoids. It has also been reported that fatty acid synthase is upregulated in numerous cancers and plays an important role in conversion of acetyl-CoA, malonyl-CoA and NADPH into long-chain fatty acids (18, 19).

During cellular metabolism increased production of reactive oxygen species (ROS) and oxidative stress is one of the hallmarks of cancer and is due to an imbalance between generation of ROS and the cell's ability to produce antioxidants. A recent report suggests that many types of cancers exhibit increased ROS (21, 22). ROS are broadly defined as oxygen containing reactive chemical species. The two main types of ROS include: 1) those which contain one or more unpaired electrons in their outer orbitals such as superoxide, nitric oxide and hydroxyl radicals and 2) non-radical ROS which do not contain unpaired electrons but are chemically reactive and can be converted to radical ROS. These ROS include hydrogen peroxide, ozone and peroxynitrate. Mitochondria are a major source of ROS generated in cells and ROS are formed through electron leakage from the electron transport system which reacts with O_2 to form superoxide and then to other ROS. ROS is also produced from phagocytic reactions and as a byproduct of many biochemical reactions such as β–oxidation in peroxisomes, prostaglandin synthesis and cytochrome P450-dependent oxidation reactions. Exposure to environmental factors such as

pollutants, tobacco smoke, radiation and iron salts can also produce ROS in cells (Figure 5) (23-25) (26).

ROS also has biological functions that regulate many signal transduction pathways either by direct interactions or by modifying the structure of proteins, transcription factors and genes. A moderate increase in ROS can induce cell proliferation and differentiation whereas excess levels of ROS can result in oxidative damage to lipids, DNA and proteins. Therefore, maintenance of ROS homeostasis is important for normal cell growth and survival. Cells are equipped with many ROS-scavenging systems such as glutathione peroxidases, peroxiredoxins, glutaredoxin, thioredoxin, superoxide dismutases and catalase (23).

Recent reports suggest that malignant cells function under higher levels of oxidative stress than normal cells (21, 22). For example freshly isolated leukemia cells from blood of chronic lymphocytic leukemia or hairy-cell leukemia patients exhibited increased ROS levels compared to normal lymphocytes. Even in solid tumors, increased oxidative damage products such as the oxidized DNA base (8OHdG), and lipid peroxidation products are seen in tumor samples, plasma and in cancer cell lines. Not only ROS but also antioxidant (SOD, glutathione peroxidase and peroxiredoxin) status is significantly altered in tumors suggesting abnormal regulation of redox homeostasis and stress adaption (23).

The precise mechanism for increased redox status in cancer cells and tumors remains unclear but activation of oncogenes (Ras, Bcr-Abl and c-Myc), aberrant metabolism, mitochondrial dysfunction and functional loss of p53 are some of the factors that may be involved (Figure 5). For example NIH3T3 fibroblast cells transformed by overexpressing H-Ras^{v12} generate large amounts of superoxide through activation of the membrane associated ROS-producing enzyme NADPH oxidase (NOX) suggesting that increased ROS is required for the oncogenic function of Ras (23, 24).

Apart from oncogenic transformations, mitochondrial dysfunction also contributes to higher levels of ROS. Mitochondrial DNA (mtDNA) encodes for 37 genes/gene products and a number of these are involved in the electron transport chain. In many cancers mRNA levels of several mtDNA encoded genes are upregulated. For example, in hepatic tumors, hexokinase II is overexpressed whereas in normal cells hexokinase IV (glucokinase) is expressed. Hexokinase II differs from hexokinase IV by having two catalytic sites and low K_{ms} ($K_{m1/4}$ 0.02-0.03 mM) value whereas hexokinase IV has one catalytic site and high K_m ($K_{m1/4}$ 5-8 mM) value. Hexokinase II binds to the mitochondrial outer membrane protein VDAC (voltage-dependent anion channel) which is a part of the mitochondrial permeability transition pore (mtPTP). The mtPTP is formed from the assembly of pro- and anti-apoptotic proteins Bax and Bcl2 family members, outer membrane protein VDAC, inner membrane protein

adenine nucleotide translocator (ANT), cyclophilin D and the benzodiazepine receptor.

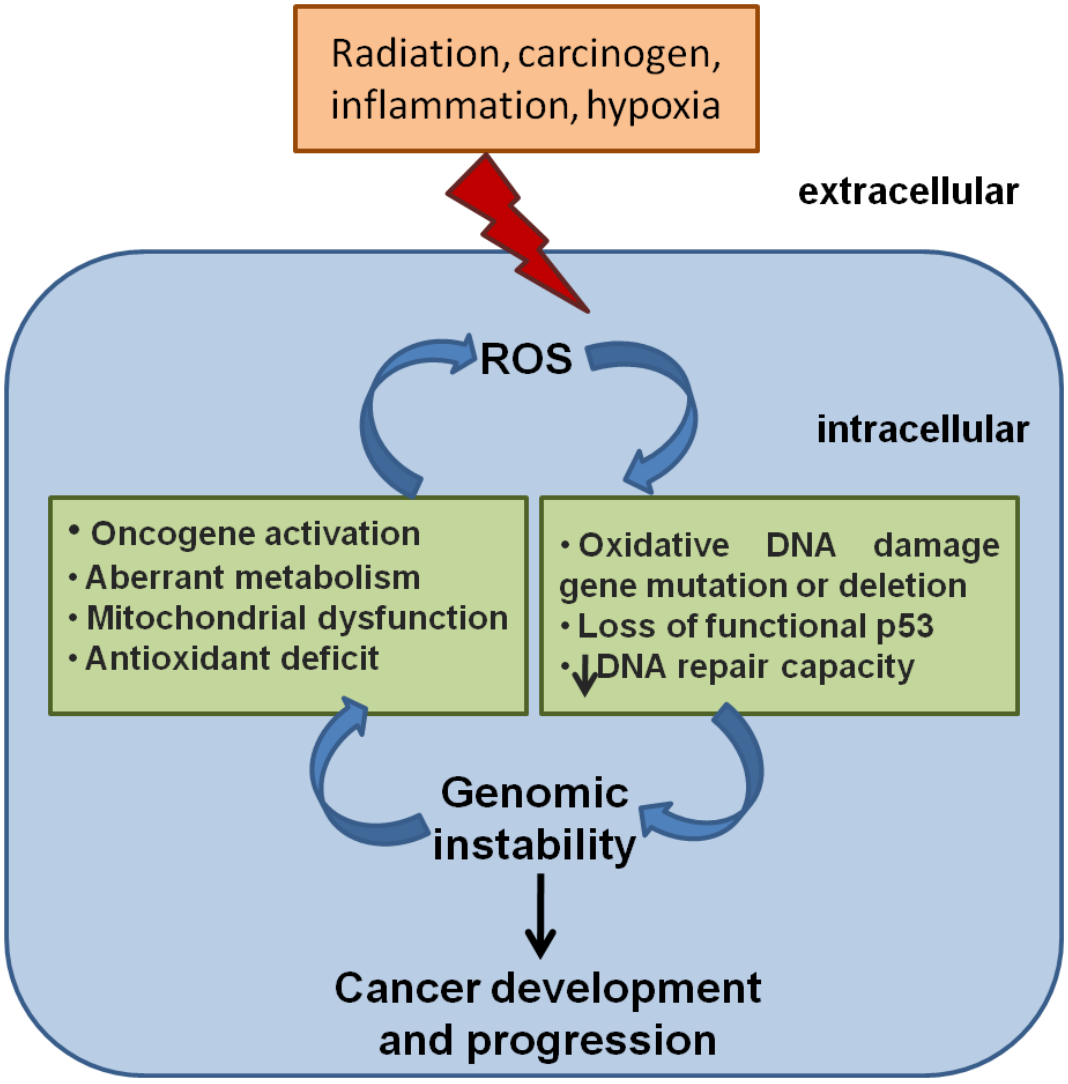

Figure 5. The vicious cycle of ROS stress in cancer (23).

Hexokinase II bound to VDAC allows solutes such as ATP to pass through the membrane and thereby catalyze rapid phosphorylation of glucose to glucose-6-phosphate which then undergoes aerobic glycolysis to generate ATP and lactate. Also the VDAC bound hexokinase II stabilizes the mitochondrial

permeability transition pore (mtPTP) and inhibits the effect of Bax and Bak and thus blocks apoptosis. Phosphorylation of hexokinase II by protein kinase D (PKB) promotes the interaction of hexokinase II with VDAC and inhibits apoptosis (27). Some germline and somatic mutations are also responsible for mitochondrial dysfunction and play an important role in cancer development. For example germline mutations causing polymorphisms on nucleotides 10398 (A>G) and 16189 (T>C) have been associated with breast cancer in African American women and endometrial cancer. Even somatic mutations ranging from severe insertion-deletion and chain termination mutations to mild missense mutations are detected in many cancers. For example in about 50% of renal adenocarcinoma patients, mtDNA contain an in-frame deletion of 294 nucleotides in the nicotinamide adenine dinucleotide dehyrogenase 1 (ND1) gene. ND1 is part of complex I in the electron transport chain which oxidizes NADH and transfers electrons to coenzyme Q (27, 28).

p53 is a tumor suppressor gene that plays an important role in protecting nuclear DNA and mtDNA by sensing and decreasing oxidative stress in order to prevent oxidative gene mutations and genomic instability. In addition p53 also regulates expression of many pro-oxidant and antioxidant genes and thus loss or inactivating mutations of p53 which is observed in >50% of cancers, is associated with redox imbalance, increased oxidative stress and aggressive tumor growth. For example p53 positively regulates the gene encoding cytochrome c oxidase 2 (SCO2) protein which is important for the assembly of

cytochrome c oxidase; and thus functional loss of p53 results in metabolic disorders and increased ROS levels (20, 23).

Multistage tumor progression

Tumor formation is a complex and multistep process that usually takes decades and tumor progression is defined as the evolution of normal cells into cells with an increasingly neoplastic phenotype. During tumorigenesis many mutations and epigenetic alterations occur randomly within DNA and this affects genes controlling cell proliferation and survival and other traits associated with malignant phenotypes (29). Epidemiological studies show that the risk of death due to colon cancer in a 70 year old man is about 1000 times higher than in a 10 year old boy. Cancer is usually a late onset disease indicating that formation of most cancer requires years to decades to develop (2). It is estimated that the onset of lung cancer due to cigarette smoking has a lag period of up to 35 years (30). Cancer incidence increases with age and with multiple mutations. A mathematical model for this process is $I=kt_{r-1}$ where 'I' is age specific incidence, 'r' is the successive mutations occurring in some cells at a constant rate 'k' k_1, k_2, $k_3 \ldots k_r$ per unit time 't'. A log-log of the equation ($\ln I = \ln k+(r-1) \ln t$) gives a linear relationship in which the slope would yield 'r-1' and it will predict the incidence and mortality rate due to various cancers as a function of I^4 to I^7, where 'I' represents the age of patients at initial diagnosis. Many cancers exhibit this relationship and for colon cancer r=6. This is based on calculations taking five or six of the slowest 'rate limiting' steps involved in the kinetics of cancer

progression and does not consider the more rapid changes that might occur in tumor development (31) (32).

The multi-hit hypothesis describes cancers that require 4-10 mutational events for development of malignant tumors. For example during the development of colorectal adenocarcinoma events include mutations in APC and p53 (two alleles) and also in one copy of the RAS oncogene (Figure 6). The multi-hit hypothesis is applicable to most cancers where preneoplastic cells and tumors grow by clonal expansion and are driven by successive mutations. The first mutation results in limited expansion and the second mutation will allow cells to form a benign growth; one of the cells may subsequently undergo a third mutation resulting in outgrowth of these cells into a malignant and invasive cancer. In vitro transformation studies in human primary cells indirectly support the multi-hit hypothesis, even though rodent cells can be easily transformed. At least five distinct cellular regulatory circuits need to be altered experimentally in “normal” human cells before they can form tumors in immunocompromised mice. These changes involve a 1) Ras mitogenic signaling pathway 2) pRb-mediated cell cycle control 3) p53 4) and telomere pathways and 5) protein phosphatase (PP2A) 2A pathway (31, 33-36).

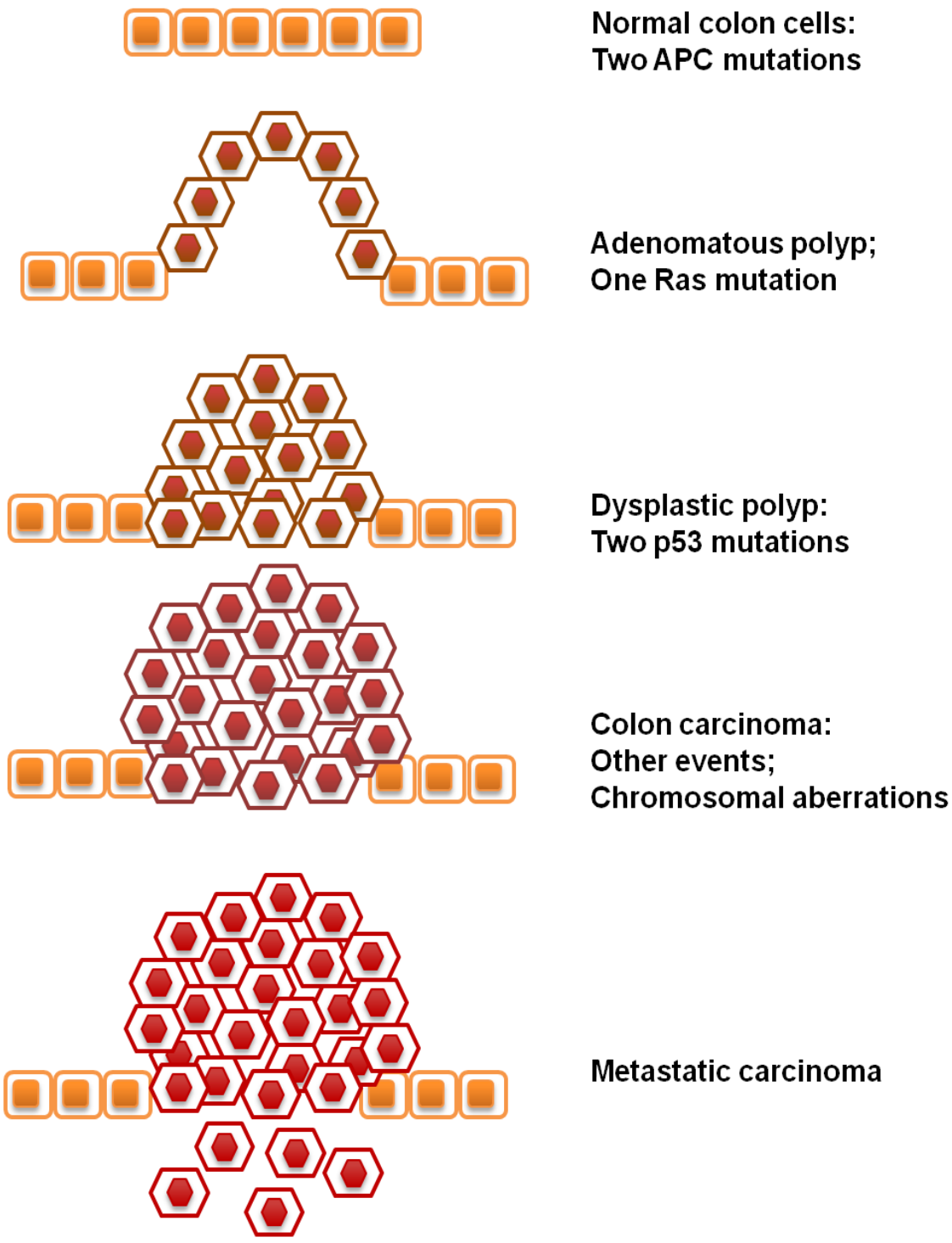

Figure 6. Five hit hypothesis for development of colorectal cancer (31).

However, this hypothesis does not fit all cancers. For example, childhood cancers occur early in life and include cancers such as osteosarcoma which has its highest incidence during adolescence when the rate of long bone growth is highest. Alveolar rhabdomyosarcoma is another pediatric cancer predominantly observed in the trunk and extremities (31, 37).

Even though formation of most tumors is a multistep process, there are some notable exceptions (Figure 7). Chronic myelogenous leukemia (CML) arises from a reciprocal translocation between chromosomes 9 and 22 resulting in the fusion of BCR-ABL oncogenes; B-cell lymphoma is the result of a translocation that activates Bcl2, an antiapoptotic gene. There are many leukemias, lymphomas and sarcomas that are characterized by single specific translocations and they are consistent with Knudson's 'One hit hypothesis' which states that a single abnormality is functionally crucial for cancer to develop (31).

Retinoblastoma is observed exclusively in children and arises from retinoblasts that normally differentiate into postmitotic retinal photoreceptor cells and neurons. Differentiation fails in these tumors and the cells continue to proliferate leading to tumor invasion and metastasis. Retinoblastoma arises from mutations in Rb1, a tumor suppressor gene (recessive mutation on both the alleles) which can be hereditary or nonhereditary in nature. In the United States, about 40% of the cases carry germline mutations in the Rb1 gene. Both hereditary and nonhereditary retinoblastoma involve two mutations and hence the term 'two hit hypothesis'. In hereditable retinoblastoma, germline mutations

are seen as deletion or chromosomal loss or recombination of chromosome band 13q14. The other notable disease consistent with the ‘two hit hypothesis’ is Li-Fraumeni syndrome (LFS) which is due to mutations in p53 tumor suppressor gene. p53 was found to be complexed with transforming proteins of certain DNA tumor viruses and p53 plays an important role in mediating repair of DNA damage induced by oncogenes or radiation by activating pathways that result in cell cycle arrest, DNA repair or apoptosis. Ataxia telangiectasia mutated (ATM) and hCHK2 and/or p14ARF proteins act as important intermediates in these p53-induced pathways. Germline mutations of p53 and in some cases hCHK2 are observed in Li-Fraumeni syndrome. Individuals carrying this mutation often develop different tumors such as osteosarcoma, brain tumors, leukemia, adrenocortical tumors and breast cancer in female carriers at an early age. In both retinoblastoma and LFS the initial somatic mutation gives rise to a mutant clone of cells and a second hit, whether it is hereditary or nonhereditary, results in tumor formation (31).

The age-dependent incidence of cancer explained by the multi-hit hypothesis may not play a major role in hormone-dependent carcinomas particularly when considering the effects of timing and dose level of various agents alone or in combination. For example breast cancer incidence is dependent in part on exposure to estrogen and is modified by other factors such as pregnancy (increased incidence), late menarche, early menopause, early first child birth and high parity (decreased incidence). Other cancers including

mesothelioma in humans are also dependent on the time and dose of asbestos exposure and lung cancer incidence depends on the duration and amount of smoking and incidence decreases when smoking stops (31, 38). These are examples of cancers where age is important but the exposure to modifying factors can also be a major contributing factor.

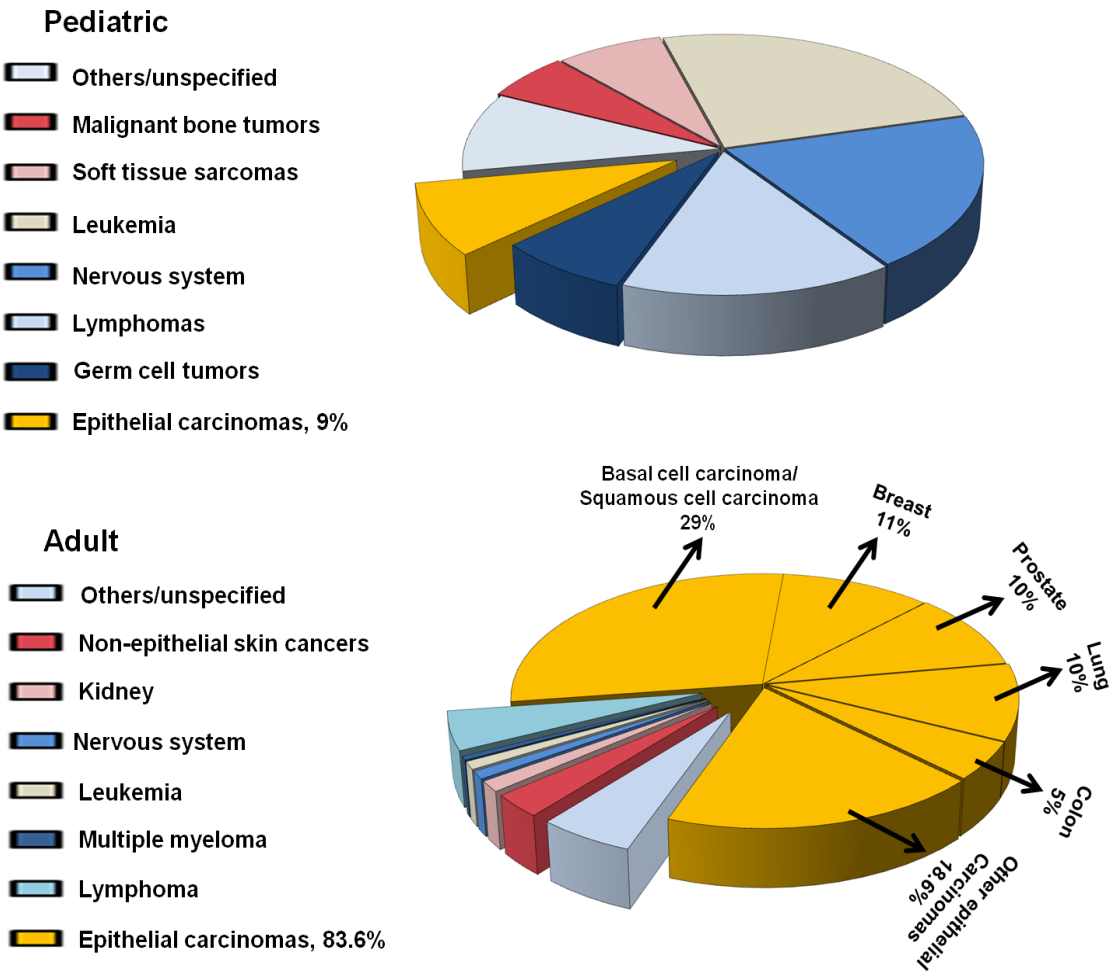

Figure 7. Tumor spectrum in pediatric and adult population (32).

Pancreatic and bladder cancer

Anatomical classification of tumors

Tumors are broadly classified based on their tissue of origin 1) epithelial, 2) nonepithelial (mesodermal) and 3) other types of cancers which do not fit either epithelial or nonepithelial cancers.

1) Majority of human cancers are epithelial in origin and account for more than 80% of all cancers reported worldwide. Epithelial tumors or carcinomas include gastrointestinal, mammary gland, lung, skin, and urogenital cancers (39). These cancers arise from epithelial cells which line the walls of cavities and channels or in the case of skin they form the outer covering of the body. Most cancers of epithelial origin fall into two major categories reflecting the biological function of the epithelium: A) cancers that arise from the cells that layer and protect the cavity or channel such as squamous cell carcinoma of the skin or esophagus. B) Epithelia which secrete substances that protect and line cavities give rise to adenocarcinoma. This includes cancers of stomach, pancreas, prostate, breast, colon and lung (39-41).

2) Tumors of nonepithelial origin are broadly classified into three groups: A) Sarcomas which arises from connective tissues and include osteosarcoma (osteoblasts), liposarcoma (adipocytes), rhabdomyosarcoma (myocytes) and fibrosarcoma (fibroblasts). B) Hematopoietic malignancies which arise from blood forming tissues and include leukemia (white blood cells), and lymphoma (lymphoid tissue- B and T lymphocytes). C) Neuroectodermal

tumors arise from outer cell layers of early embryos and include glioblastomas (glial cells), neuroblastomas (cells of sympathetic ganglia of the peripheral nervous system), schwannomas (Schwann cells) and medulloblastomas (cells of granular layer of the brain) (39-41).

3) The examples of tumors which do not fit epithelial or nonepithelial origin include melanomas which are derived from melanocytes, small cell lung carcinomas and transitional cell carcinoma of urinary bladder. Melanocytes and cells in small cell lung carcinomas are derived from the neural crest and migrate during development to skin/retina and lung in order to perform their functional role and are not connected with the nervous system (39-41).

Tumor development is characterized by progressive evolution of normal cells into aggressive metastatic disease. The progression away from normal to an invasive cancer is observed in the following steps (Figure 8):

1) Hyperplastic growth is seen in which cells deviate minimally from their normal tissues and may exhibit an increase in the number of normal cells.

2) Metaplasia is seen where normal cells are displaced by different cell types that are not usually found in that particular tissue. These metaplastic cells are most often found in epithelial transition zones (where two different tissue meet) and appear normal under microscopic examination. The transition zones are seen at the junction of the cervix and uterus and also between the esophagus and stomach and are usually lined with squamous epithelium. For example, the squamous epithelium in the esophagus undergoes a

metaplastic change into mucous secreting gastric epithelium which is considered to be an early indication of premalignant change in the esophagus and is termed “Barrett’s esophagus”. This metaplastic change in patients suffering with Barrett’s esophagus increases the risk by thirty-fold for developing esophageal carcinoma.

3) Cells with dysplasia exhibit abnormal cytology and differ considerably from normal cells. The cytological changes include changes in the size of the nucleus and cytoplasm, increased mitotic activity and proliferation. This abnormal increase in number of cytologically different cells results in alterations of tissue morphology and this dysplastic growth is considered to be a transitional state between benign growths and premalignant lesions.

4) Abnormal growths, termed adenomas, polyps and papillomas, are large enough to be detected with naked eye. Cells in these growths are dysplastic and considered benign as they grow to a certain size; these cells stop growing, respect boundaries and do not penetrate the basement membrane.

5) Abnormal growths can result in neoplasias which invade underlying tissues and become malignant. Cells in primary tumors become invasive and spread to distant sites in the body through blood and lymph vessels and this process is called metastasis (33, 39-41) (42).

Pancreatic cancer

The pancreas is composed of exocrine and endocrine components. The exocrine component consists of acinar, ductal and centroacinar cells and the

endocrine portion consist of islets of langerhans. The exocrine portion of the pancreas secretes zymogen which is a digestive enzyme that is released into the duodenum. The endocrine component of the pancreas secretes insulin and other proteins which regulate glucose homeostasis.

Pancreatic ductal adenocarcinoma is the most frequently diagnosed pancreatic cancer and is the fourth leading cause of cancer deaths in the United States with 35,000 deaths reported in 2009. Other cancers of the pancreas are much less common and account for about 4% of pancreatic cancers.

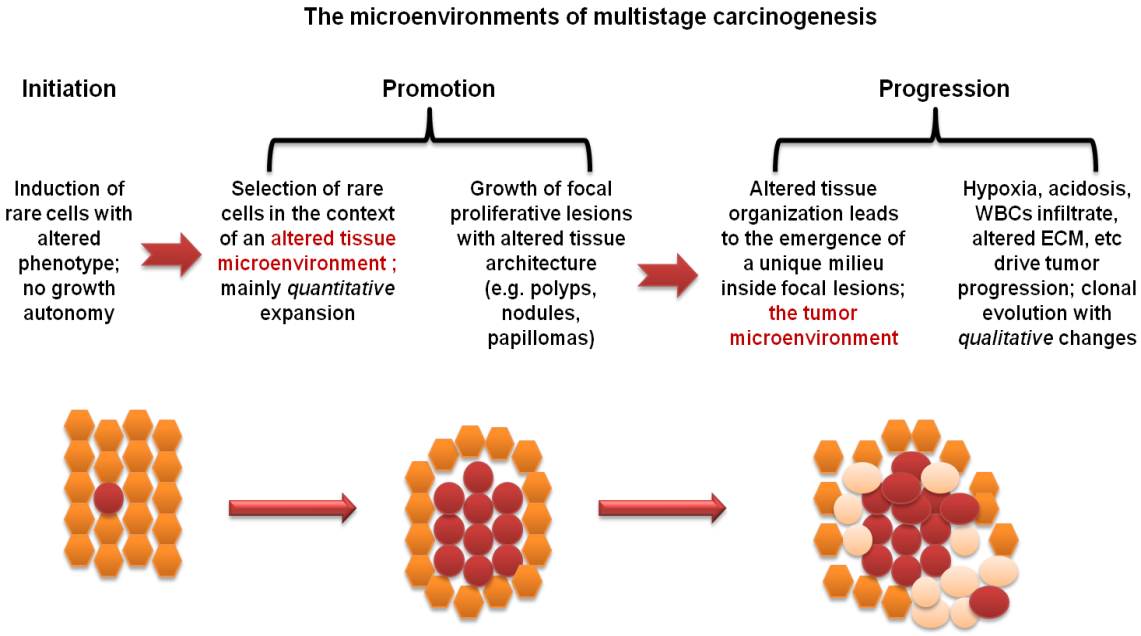

Figure 8. The microenvironments of multistage carcinogenesis (43).

Classification of pancreatic cancers

Pancreatic cancers are classified into 3 categories; a) solid non-endocrine neoplasms, b) cystic non-endocrine neoplasms and c) endocrine neoplasms, which represent the three main epithelial lineages of the pancreas: the ductal cell, the acinar cell and the endocrine cell (44) (45, 46) (47).

a) **Solid non-endocrine neoplasms:** The following cancers are included.

1) **Pancreatic ductal adenocarcinoma (PDAC) and its variants:** PDAC is commonly localized on the head of the pancreas and has infiltrating duct-like and tubular structures embedded in highly fibrous or connective stromal tissue. These growths obstruct and dilate the bile and pancreatic ducts and results in development of jaundice in many patients with PDAC. The pathological features of PDAC are described as 'pancreatic intraepithelial neoplasias or PanINs' which vary from morphological precursors to invasive cancer. PanINs arise in small ducts and ductules of pancreas and comprise mucin-producing cells that exhibit varying degrees of nuclear and architectural atypia. PanINs are considered precursors of infiltrating pancreatic ductal adenocarcinoma and studies have shown that PanINs can progress to infiltrating cancer over time. For example three patients diagnosed with high grade PanINs developed infiltrating PDAC over a span of time (months to years) (47) (48).

i) **PanIN-1A:** Flat epithelial lesions composed of tall columnar cells with basally located nuclei and abundant supranuclear mucin; nuclei are small and round to oval in shape. In normal pancreatic cells the ductal

epithelium is composed of cuboidal to low columnar epithelium with amphophilic cytoplasm. At this stage there is no considerable difference between non-neoplastic flat hyperplastic lesions and flat neoplastic lesions without atypia.

ii) **PanIN-1B:** The lesions appear similar to PanIN-1A apart from exhibiting papillary, micropapillary or basally pseudostratified architecture.

iii) **PanIN-2:** These are mucinous flat epithelial lesions with cells exhibiting nuclear abnormalities with loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism. Cells undergoing mitosis are rare.

iv) **PanIN-3:** These lesions are papillary or micropapillary but rarely flat. True cribriforming, budding off of small clusters of epithelial cells into the lumen and luminal necroses is observed. Cells are characterized by loss of polarity, dystrophic goblet cells, high abnormal mitotic figures, nuclear irregularities and large nucleoli. These lesions appear neoplastic but invasion through the basement membrane is not observed (Figure 9) (44, 49) (47) (50).

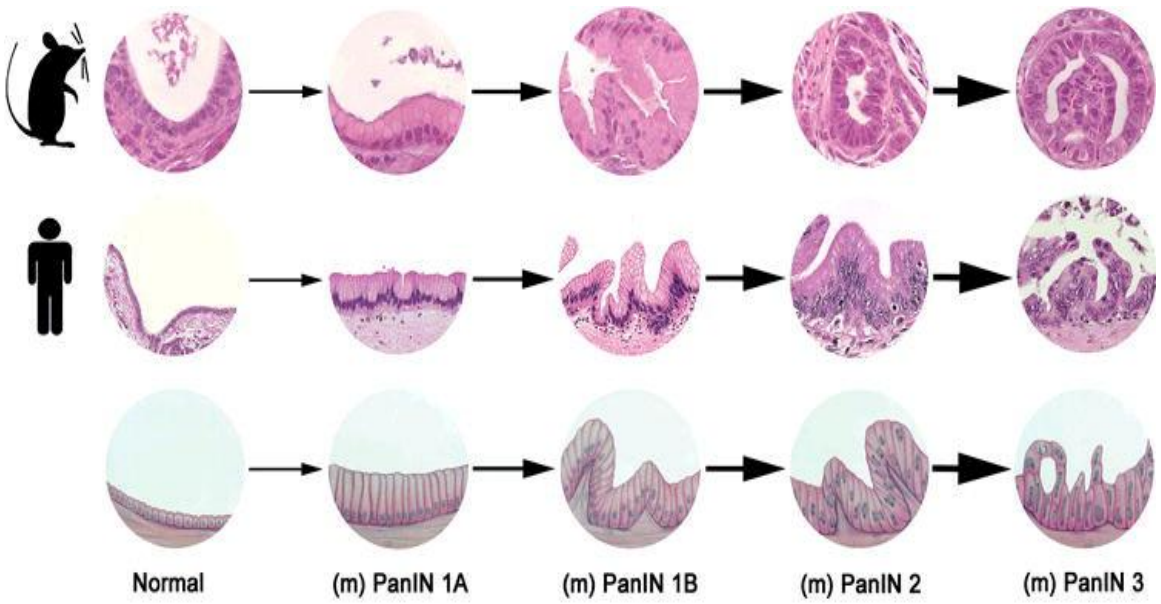

Figure 9. Tumor progression model of pancreatic carcinogenesis: bottom, schematic drawing; middle, in men; and top, in mice (44).

The pathological staging of PDAC is based on TNM classification which takes into account the size and extent of invasion of the primary tumor (pT1 – pT4) and the presence or absence of regional metastatic lymph nodes (pN1a or pN1b if multiple lymph nodes are involved) as well as distant metastases (pM) (Table 3) (47).

Table 3. TNM classification of pancreatic ductal adenocarcinoma (47).

Primary Tumor (T)				
TX	Primary tumor cannot be assessed			
T0	No evidence of primary tumor			
Tis	Carcinoma in situ			
T1	Tumor limited to the pancreas, 2cm or less in greatest dimension			
T2	Tumor limited to the pancreas, more than 2 cm in greatest dimension			
T3	Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesentric artery			
T4	Tumor involves the celiac axis or the superior mesenteric artery			
Regional Lymph nodes (N)				
NX	Regional lymph nodes cannot be assessed			
N0	No regional lymph node metastasis			
N1	Regional lymph node metastasis			
Distant Metastases (M)				
MX	Distant metastasis cannot be assessed			
M0	No distant metastasis			
MI	Distant metastasis			
Stage grouping				
Stage	0	Tis	N0	M0
Stage	IA	T1	N0	M0
	IB	T2	N0	M0
Stage	IIA	T3	N0	M0
	IIB	T1, T2, T3	N1	M0
Stage	III	T4	any N	M0
Stage	IV	any T	any N	M1

Variants of pancreatic ductal adenocarcinoma: Pancreatic carcinomas which are closely related to PDAC are categorized under this section (45, 46) (47).

i) **Adenosquamous carcinoma:** It resembles PDAC in terms of localization in the pancreas and macroscopic appearance and sex distribution. The tumor is characterized by a variable mix of neoplastic tubuloglandular and squamous (flat epithelial) cells. The squamous portion may obliterate the glandular portion resulting in squamous carcinoma. Undifferentiated spindle cells are observed in adenosquamous carcinoma (45-47).

ii) **Undifferentiated carcinoma:** Also called anaplastic, pleomorphic large cell, pleomorphic gaint cell or sarcomatoid carcinoma. It accounts for about 2-5% of exocrine pancreatic cancers and they have similar age and sex distribution as PDAC but are more aggressive. The tumors of undifferentiated carcinoma are usually soft and large with conspicuous hemorrhages, necrosis and/or cystic changes. The tumors consist of mononuclear pleomorphic (variability in shape and size) cells embedded in scanty stroma with occasional multinucleated giant cells or spindle cells. The cancer cells grow either in poorly cohesive sheets or in a sarcoma like fashion and exhibit high mitotic rates and extensive lymphatic and vascular invasion (45-47).

iii) **Undifferentiated carcinoma with osteoclast like giant cells:** These carcinomas are characterized by two different populations of mononuclear pleomorphic (spindle or polygonal) cells and osteoclast like

multinucleated giant cells. The pleomorphic cells exhibit properties of undifferentiated carcinoma with high mitotic rates whereas osteoclast like giant cells resemble normal osteoclast (bone resorption) cells with no mitotic figures. These tumors frequently exhibit point mutations in codon 12 of the K-ras gene. Recent studies showed that the patients with undifferentiated carcinoma with osteoclasts have no better prognosis than PDAC and most patients die within one year of diagnosis with the disease (45-47).

iv) **Mixed ductal-endocrine carcinoma:** It is a rare carcinoma which consists of non-neoplastic endocrine cells and is most often seen in differentiated PDAC.

2) **Acinar cell carcinoma:** These malignant epithelial neoplasms arise from the head of the pancreas and exhibit smooth borders. Cells are pink and granular and form small glands called acini. These glands usually reveal the presence of zymogen granules which hold the digestive enzymes such as trypsin, lipase, chymotrypsin and/or amylase and staining for these substances is helpful in distinguishing acinar cell carcinomas from other pancreatic neoplasms. Acinar cell carcinoma accounts for about 1-2% of pancreatic tumors and occurs mostly in adults especially in males. The carcinoma is metastatic like PDAC and spreads to lymph nodes, liver and lungs but the clinical course is not as rapid and the median survival is about 18 months. These cancers exhibit genetic mutations in APC/β-

catenin pathway but do not show any alterations in genes such as K-ras and p53 which are involved in PDAC development (50).

3) **Pancreatoblastoma:** This is a rare pancreatic cancer observed in the children of 1-15 years of age and is referred to as pancreatic carcinoma of infancy. It is observed more often in boys than girls and the survival rate is better than PDAC. The causes responsible for this cancer are still unknown and the cells are small and necrotic with a white-grey appearance of the neoplastic tissue (47).

b) Cystic non-endocrine neoplasms:

1) **Serous cystic neoplasms:** These neoplasms are usually benign and are more common in women than men. Patients with von Hippel-Lindau syndrome frequently develop benign serous cystic adenomas that can be very large (football size) but they can be cured by surgical removal of the growths. The cells in this neoplasm contain large amounts of glycogen and can be detected by staining with periodic acid-Schiff (PAS) stain.

2) **Mucinous cystic neoplasms:** These tumors are usually benign and less invasive and are seen more often in women than men and are similar to serous cystic adenomas. The tumors consist of cysts containing mucin and the inner surfaces may show papillary projections. The prognosis is better than for PDAC and patients can be cured if the tumors are surgically removed in the benign stage. Patients with invasive mucinous cystic carcinomas have median survival rates up to 5 years.

3) **Intraductal papillary mucinous neoplasm (IPMN):** Unlike mucinous cystic neoplasms, IPMN develop mucinous papillar projections in the pancreatic ductal system and are more invasive. IPMNs are most often detected in men than women. These tumors stain for MUC1, MUC2, MUC5 or CDX2 depending upon the type of papillae (intestinal type, pancreatobiliary type, oncotypic type and gastric type) present.
4) **Solid-pseudopapillary neoplasm:** These neoplasms form large cystic, haemorrhagic and necrotic masses with papillary components. They mostly occur in women in their 20's, but most patients can survive for many years after surgical resection of the tumors (45, 46) (47).

c) **Endocrine tumors:** Endocrine tumors account for about 1% of pancreatic tumors and the malignant behavior of these tumors is highly unpredictable except in poorly differentiated carcinomas. These tumors are characterized into 3 sub-categories and are summarized below (45, 46) (47).

1) **Well- and moderately-differentiated endocrine neoplasms:** This category includes benign, borderline and malignant tumors of endocrine origin. However it is hard to predict the outcome of these tumors since even benign tumors can later develop into a malignant tumor. These tumors usually stain for endocrine markers such as chromogranin, synaptophysin and neuron specific enolase. Patients with endocrine tumors can produce excessive quantities of hormones such as insulin

produced by islet of langerhans and these patients present symptoms of hypoglycemia.

2) **Multiple endocrine neoplasia syndrome, type 1 (MEN1 syndrome):** This syndrome is called as Werner's syndrome which results from a germline mutation or deletion in MEN1 gene on the long arm of chromosome 11 (11q13). About 80% of patients with MEN1 syndrome develop pancreatic tumors.
3) **Poorly differentiated endocrine neoplasms:** As the name suggests, the tumors are very poorly differentiated with high mitotic rates and exhibit invasive characteristics. Survival rates for these patients is very low (47).

Aetiology of 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 (observed primarily in cases of pancreatoblastoma). The age adjusted annual incidence of PDAC ranges from 3.1 to 20.8 cases per 100,000 people in industrialized countries. Risk factors for developing ductal adenocarcinoma are older age, cigarette smoking, previous gastric surgery, diabetes mellitus, chronic pancreatitis, familial history of pancreatic cancer and a high intake of dietary fat and low in fruits and vegetables. Smoking increases the risk of developing pancreatic cancer by 2-5 fold over non-smokers and the risk increases with the number of cigarettes smoked (51-55).

Studies showed an association between previous gastric surgery and pancreatic cancer. For example the patients who have had peptic ulcer surgery have a 2 to 5-fold increased risk of developing pancreatic cancer and the reasons for this are unknown. Some studies have also shown that diets rich in fruits and vegetable reduce the risk whereas the diets high in polyunsaturated fat increase the risk of pancreatic cancer. This has been attributed to vitamins such as folate. A nested case control in male Finnish smokers found that there is an inverse dose-response relationship between serum folate and pyridoxal-5'-phosphate concentrations and pancreatic cancer (52, 53).

Diabetes mellitus and chronic pancreatitis are also risk factors for pancreatic cancer and patients with familial pancreatitis have a 40% risk of developing pancreatic cancer and they show signs of acute pancreatitis at an early age. This eventually results in endocrine and exocrine failure of the pancreas. Familial pancreatitis is cause by an inherited mutation in the cationic trypsinogen (PRSS1) gene which results in increased autoactivation of trypsinogen or stability of trypsin causing necrosis and pancreatitis (51, 54).

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, telangiectatic ataxia and Peutz-Jeghers syndrome (PJS). Germline mutations of BRCA2 increase the incidence of PDAC by 3.5-fold and germline mutations of BRCA1 increased the incidence by 2-fold. Peutz-Jeghers syndrome is caused

by dominantly inherited mutations of the STK11/LKB1 gene. Patients with PJS develop a variety of cancers such as gastrointestinal, lung, breast and pancreatic cancers and they have >132-fold increased risk of developing pancreatic cancer. Familial atypical multiple melanoma syndrome (FAMMM) is an autosomal dominant inherited syndrome and is associated with many cancers apart from melanoma. Patients with FAMMMs that develop pancreatic cancer express a germline mutation in the p16/CDKN2A gene (51, 54, 55).

Cellular and molecular mechanisms in development of pancreatic cancer

Many inherited and somatic mutations are associated with PDAC and these include the K-ras, HER-2, BRAC2, p16, p53 and Smad4 genes and approximately 50 - 60% of the cases show mutations in p53 and Smad4.

1) **Oncogenes:** Activated or overexpressed oncogenes possess transforming properties and the oncogenes which play an important role in pancreatic cancer development include K-ras, HER2, AKT2, AIB1, BRAF and MYB genes. K-ras is activated in about 90% of pancreatic cancers by a point mutation on codon 12 and occasional mutations in codon 13 or 61 of chromosome 12p. K-ras 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 activation of

the Kras protein. The active protein is bound to GTP, and inactivation occurs through guanosine-triphosphatase-activating proteins, which promote GTP hydrolysis to the diphosphate GDP resulting in attenuation of Kras signaling. Activated Kras affects several downstream effector pathways such as RAF-mitogen activated protein kinase (RAF-MAPK), phosphoinositide-3-kinase (PI3K) and RalGDS pathways. Activating mutations impair the intrinsic GTPase activity of the K-ras gene product, resulting in a protein that is constitutively active. K-ras gene mutations are one of the earliest genetic abnormalities observed in the progression of pancreatic cancer and are observed in approximately 36%, 44% and 87% of PanIN-1A, PanIN-1B and PanIN-2/3 precursor lesions respectively. For example, mice expressing codon 12 K-ras gene mutations in the pancreas develop lesions which vary from PanINs to invasive and metastatic ductal adenocarcinoma (56) (46, 57-60) (61).

Approximately 5% of pancreatic cancers express wild type K-ras but exhibit mutations in one of the member of 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 K-ras gene mutations. For example small molecule inhibitors or antisense inactivation of the kinase suppressor of ras 1 (KSR1) which is an essential transducer of Ras signaling to RAF results in inhibition of tumor growth in athymic nude mice bearing Panc-1 cells as xenografts (60).

However in pancreatic cancers either K-ras or BRAF mutations are observed; and the occurrence of both events together appears to be mutually exclusive (46, 56, 59) (61).

The PI3-AKT pathway is an essential cell survival pathway that is constitutively activated in pancreatic cancer. The AKT2 gene is overexpressed in about 10-20% of pancreatic cancers and is located on chromosome 19q. Additional oncogenes that are overexpresed in pancreatic cancers are AIB1, C-Myc and MYB gene; these are amplified by 60%, 20-30% and 10% respectively (46, 56, 59) (61).

2) **Tumor suppressor genes:** 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. Tumor suppressor genes, as the name suggest inhibit tumor growth and the most common tumor suppressor genes that are inactivated in development of pancreatic tumor are p16/INK4A, p53 and DPC4/SMAD4 (44, 46, 56, 59, 61).

The CDKN2A gene encodes for p16/INK4A and ARF tumor suppressor genes and is located on chromosome 9p. p16/INK4A is the most frequently inactivated tumor suppressor in pancreatic cancer (~95%) and about 40% of the cases, a homozygous deletion of both alleles of the gene is observed; an intragenic mutation on one allele coupled with loss of the other allele is observed in 40% of pancreatic

cancers and inactivating hypermethylation of the promoter is observed in the remaining 15%. Loss of INK4A function is seen in early precursor lesions and is found in about 30%, 55% and 70% of PanIN-1, PanIN-2 and PanIN-3 lesions respectively. p16 regulates the cell cycle by binding to Cdk4 and Cdk6 to inhibit binding of cyclin D1 to the Cdks and phosphorylation of Rb leading to cell cycle arrest. Thus the loss of p16 deregulates the cell cycle check point leading to the development of pancreatic cancer and this is also observed in patients with germline mutations of this gene (44, 46, 56, 59, 61).

p53 gene is located on chromosome 17p and is inactivated in about 50-75% of pancreatic tumors. Loss of function of p53 occurs due to intragenic mutations in one allele coupled with loss of the other allele and inactivation of p53 appears to be a late event in the development of pancreatic cancer and is observed predominantly in high grade precursor lesions (PanIN-3) and invasive adenocarcinomas. p53 plays an important role in regulation of G1-S cell cycle checkpoint, maintenance of G2-M arrest, induction of apoptosis and inactivation of p53 leads to uninhibited cell growth in the presence of DNA damage resulting in further accumulation of genetic abnormalities (44, 46, 56, 59, 61, 62).

SMAD4/DPC4/MADH4 is located on chromosome 18q21 and is another tumor suppressor that is inactivated in about 55% of pancreatic cancers (rarely seen in extrapancreatic cancers). In about 50% of the

pancreatic patients loss of function of SMAD4 is caused by homozygous deletion and an intragenic mutation in one allele coupled with loss of the other allele is observed in the other 50%. Like p53, inactivation of SMAD4 is a late event in pancreatic tumorigenesis and SMAD4 is an intermediate protein in the transforming growth factor (TGF) signaling pathway. Upon activation of TGF-β SMAD2 and SMAD3 proteins are phosphorylated and heterodimerize with SMAD4 protein and the complex translocates into the nucleus to activate target genes involved in growth inhibition and apoptosis (44, 46, 56, 59, 61).

In addition to the above, several other tumor suppressor genes are inactivated to a lesser extent (5-10%) in pancreatic cancers and these include serine threonine kinase (LKB1/STK11), TGF-β receptors TGFBR1 and TGFBR2 and MKK4 which encodes for stress activated protein kinase (44, 56, 59, 61).

3) **DNA repair or mismatch repair genes:** Mismatch repair genes play an important role in identifying and repairing DNA damage and in maintaining the integrity of the genome. This includes the DNA mismatch repair genes MLH1 and MSH2 which encode proteins that repair small insertions, deletions and other sequence mismatches in newly replicated DNA. These mismatches are common in simple repetitive DNA sequences such as poly-A-tracts. Inactivation of either MLH1 or MSH2 results in accumulation of mutations in poly-A-tracts of various genes

(TGFBR2 and ACVR2) producing DNA changes called “microsatellite instability”. Microsatellite instability is observed in about 4% of pancreatic cancers which exhibit a specific microscopic appearance known as ‘medullary histology’. Medullary histology is characterized by pushing borders, syncytial growth pattern and poor differentiation (44, 56, 59, 61).

The Fanconi anemia family of genes is associated with genome maintenance and encodes proteins involved in repair of double-stranded DNA breaks that are induced upon the interstrand crosslinking of DNA through homologous recombination. Genes included in this family are BRCA2 on chromosome 13q; the FANCC and FANCG gene on chromosome 9q and 9p respectively are mutated in the germline (in Ashkenazi Jewish population) and are linked with familial aggregation of pancreatic cancer. The affected family members develop hematological malignancies at a young age and if they survive these malignancies they develop solid tumors at an older age (44, 56, 59, 61).

4) **Telomere length abnormalities:** Telomeres are hexameric TTAGGG repeats and form caps at the ends of chromosomes to protect the terminal sequences and prevent the ends of chromosomes from joining during cell division. Loss of telomeres or shortening of telomeres is observed very early (PanIN stage) in the development of pancreatic cancer. Shortening of telomeres promote abnormal fusion of chromosome ends which break and result in the gaining of genetic material by some

daughter cells and losing of genetic material in other daughter cells. This process is called as breakage-fusion-bridge cycles, often seen in pancreatic cancers, and this results in loss of tumor suppressor genes and gain of oncogenes leading to chromosome instability and progression of pancreatic cancer. However, in invasive pancreatic cancers, telomere reactivation is observed and this might help cancer cells to attenuate genomic instability (44, 56, 59, 61).

5) **Epigenetic abnormalities:** Tumor suppressor genes are not only inactivated by homozygous deletion and intragenic mutations but also by epigenetic events such as CpG island hypermethylation of 5' promoter regions. Epigenetic hypermethylation of CG dinucleotides (CpG islands) in the 5' regulatory regions of tumor suppressor genes abrogates RNA polymerase from binding and initiating transcription and is frequently observed in pancreatic cancer cells but not in the normal cells derived from corresponding pancreatic tissue. About 60% of the pancreatic cancers exhibit hypermethylation of genes involved in tumor suppression such as p16/CDKN2A, MLH1, E-cadherin, reprimo, TIMP3, CDH1 and these are observed in the early events (PanINs) of multistage progression of pancreatic cancers. For example, reprimo gene products are associated with p53-induced G2-M arrest and aberrant methylation leads to increased genetic instability (59, 63).

Recent studies suggest that hypomethylation of candidate genes resulting in overexpression is also important for development of pancreatic cancers. For example VAV1, a Rho-guanine exchange factor in the RAS superfamily, is overexpressed due to demethylation of the promoter resulting in activation of K-ras and overepression of VAV1 protein in primary cancers and is associated with a poor prognosis (59, 63, 64).

6) **Expression abnormalities:** The global analysis of expression profiles such as cDNA, oligonucleotide microarrays and serial analysis of gene expression in pancreatic cancer has provided insights into large number of transcripts that are differentially expressed in pancreatic cancers and precursor lesions. Genes overexpressed in pancreatic cancer are growth factors and their receptors such as HER2/NEU, EGFR (epidermal growth factor family members) and their cognate ligands EGF, TGFα, amphiregulin and also FGFR, FGF (fibroblast growth factor), IGF-1R, IGF-1 (insulin growth factor), NGF (nerve growth factor) and VEGF (vascular endothelial growth factor). For example HER2 encodes a transmembrane glycoprotein with tyrosine-kinase activity which is expressed in exocrine and endocrine cells but not in ductal epithelial cells of the pancreas and overexpression of HER2 in precursor (PanIN) lesions as well as in well differentiated PDACs correlates with the severity of dysplasia in PanIN lesions and increased tumorigenicity (59, 63).

In addition to the genes noted above, expression of microRNAs is also altered in human cancers. MicroRNAs (miRNAs) are highly conserved 18-22 nucleotide RNAs that regulate transcription of complementary target mRNAs. miRNAs regulate cellular differentiation, proliferation and apoptosis in normal cells and abnormal expression of miRNAs plays an important role in many cancers. For example human let-7 miR expression is reduced in lung cancers correlating with increased expression of RAS. Similarly upregulation of miR-216, miR-217 and lack of expression of miR-133a is observed in PDAC (63, 65).

7) **Mitochondrial DNA mutations:** Mitochondria play an important role in cellular energy metabolism and apoptosis and defects in mitochondrial function contribute to development and progression of many cancers. Southern blot analysis and direct sequencing showed that the intracellular mtDNA mass is increased by 6-8 fold in cancer cells compared to normal cells. In pancreatic cancer cell lines and xenografts, mtDNA mutations are observed in many genes encoding 12srRNA, ND1, ND2, ND5, ND6, ATPase, cytochrome oxidase I, II and XIII (63, 66, 67).

8) **Developmental signaling pathway:** Hedgehog and Notch signaling pathways play an important role in normal pancreatic organogenesis, tissue homeostasis and development of cancer. The secreted signaling proteins of Hedgehog family are Sonic, Indian and Desert Hedgehog (SHH, IHH and DHH) and they regulate the growth of pancreas during

embryogenesis. The Hedgehog pathway is negatively regulated by Patched (PTC) tumor suppressor protein which inactivates Smoothened protein (SMO). Binding of Hedgehog ligands to SMO disrupts the inhibition by PTC and SMO and activates the Gli family of transcriptional regulators. Activation of Hedgehog pathway due to loss of PTC, activating mutations of SMO or overexpression of Gli and Hedgehog ligands, has been implicated in the initiation and maintenance of pancreatic cancer (59, 68).

The notch signaling pathway is comprised of membrane bound Notch ligands (Jagged and Delta-like) which interact with Notch receptors 1-4 resulting in intramembrane proteolysis of the Notch receptor by tumor necrosis factor α-converting enzyme and γ-secretase enzyme that allows nuclear translocation of the Notch intracellular domain (NICD). Nuclear NICD mediates transcriptional activation of basic helix-loop-helix genes such as Hes1, Hey1 and HeyL which inhibit differentiation and maintain cells in an undifferentiated precursor state. For example the Notch signaling pathway maintains the pool of precursor cells required for exocrine differentiation during development of the pancreas and in the mature pancreas, Notch and its ligands are expressed at low or undetectable levels. However, in PanIN lesions and PDAC, elevation of Notch and its ligands and HES-1 is observed suggesting the role for this pathway during progression of cancer (59, 68).

Pancreatic tumor biomarkers

An ideal tumor biomarker should be highly sensitive and specific for pancreatic cancer; however, none of the current markers are sufficiently sensitive or specific for use in screening for pancreatic cancer. The carbohydrate antigen 19-9 (CA 19-9), K-ras and telomerase tumor markers have been extensively evaluated.

CA 19-9: CA 19-9 is sialylated Lewis^{a} blood group antigen and is used to follow the therapeutic response in patients treated for pancreatic cancer. CA 19-9 levels correlate well with tumor volume and response to therapy and the sensitivity of this test to about 80% in cancer patients and about 55% in small resectable cancers but is not useful for screening early pancreatic cancer patients with precursor lesions (high grade PanINs). The limitations of using CA 19-9 as a marker is that approximately 10-15% of individuals do not secrete this antigen due to their Lewis antigen blood type and these limitations are applicable to other carbohydrate antigens such as CA-125, KAM17.1, CA2.2, CA-50 and CA-242.

K-ras: K-ras mutations can be detected in pancreatic juice (preferred), duodenal fluid, stool and blood using polymerase chain reaction (PCR). Limitations for detecting K-ras include 1) K-ras gene mutations are increased in individuals with increasing age and with small non-invasive PanINs. For example during autopsy, PanINs can be found in about 10-30% of individuals, especially in smokers and patients with chronic pancreatitis. 2) Pancreatic juice is more

appropriate for detecting biomarkers but is only obtained after invasive endoscopic procedures and hence cannot be used as a general screening procedure. This assay is more applicable for high risk individuals. 3) Mutant K-ras is detected in serum of patients with advanced inoperable pancreatic cancers.

Telomerase: In about 90% of pancreatic cancer patients telomerase enzyme is measurable in pancreatic juice but the specificity and sensitivity of the enzyme to distinguish between benign and malignant lesions of the pancreas have not been determined.

DNA methylation: DNA methylation abnormalities can be detected using quantitative methylation-specific polymerase chain reaction even when they are mixed with normal copies of DNA. For example DNA methylation of the p16 gene has been found in the sputum of patients with early lung cancer and is being investigated as a possible screening tool for early detection of pancreatic cancer.

Identifying new markers: New biomarkers specific for pancreatic cancers are being investigated. One approach is serial analysis of gene expression (SAGE) which can be used to compare expression of genes in cancer and normal tissue to identify overexpressed genes. Expression of macrophage inhibitory cytokine 1 (MIC1) is overexpressed in invasive pancreatic cancers as well as in PanINs and appears to be more sensitive than CA 19-9 antigen. Other tumor markers include tissue inhibitor metalloproteinase 1 (TIMP-1), osteopontin and

mesothelin genes and preliminary studies in cohorts of pancreatic cancer and control patients indicate that these markers can be used to identify early stage pancreatic cancers (59, 61, 69, 70).

Current therapies available for pancreatic cancer

1) **Surgical management:** Pancreaticoduodenectomy is the curative resection strategy used in the management of pancreatic cancer and is performed on patients with adenocarcinoma of the head of the pancreas. Median survival rates after surgical resection for localized PDAC range from 12.7 to 17.5 months and 4- and 5-year survival rates range from 6.8% to 21% (69, 71-73).
2) **Chemotherapy:** 5-FU and gemcitabine are currently used as chemotherapeutic agents and like radiation therapy; chemotherapy for pancreatic cancer has limited effectiveness. 5-FU is a thymidylate synthase inhibitor and inhibits the synthesis of thymidine which is required for DNA replication. In many solid tumors combination therapy has been beneficial over single agent therapy. 5-FU alone or combinations with doxorubicin, mitomycin C; streptozotocin, mitomycin C and 5-FU; 5-FU, doxorubicin, cisplatin or the mallinson regimen (5-FU, cyclophosphamide, methotrexte, vincristin and mitomycin C) have improved survival rates compared to no chemotherapy or best supportive care but survival benefits among 5-FU combinations and 5-FU alone are not observed (69, 71-73).

Since 1996 gemcitabine has been the drug of choice for treating pancreatic cancer. 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% compared 4.8% to 5-FU and the overall survival was 5.65 and 4.41 months for gemcitabine and 5-FU treated patients respectively and 1-year survival rates were 18% and 2% for gemcitabine and 5-FU treated group respectively. Even with gemcitabine, the combination therapies in pancreatic cancer patients failed to produce any significant overall survival benefits. A randomized trial assigned to GemOx (gemcitabine and oxaliplatin) or Gem (gemcitabine alone) demonstrated improved response rate, progression-free survival and clinical benefits. The median overall survival rate was 9.0 and 7.1 months for GemOx and Gem respectively, although there was a higher incidence of grade 3 and 4 toxicity in patients on GemOx. The pooled analysis comparing gemcitabine alone or with combinations suggested that patients with performance status (PS) 0 had better survival outcome (8.3 months vs 6.7 months) (69, 71-74).

3) **Radiation therapy:** Radiation therapy is used for locally advanced unresectable cancer or as a palliative or adjuvant therapy when the patient is undergoing a surgical resection of the pancreas. Radiation therapy is being used as postoperative regimen along with chemotherapy

(5-FU) which improves the survival of the patients from a median of 10.9 months (for surgery alone) to 21 months (with chemoradiotherapy). Recently a study at the MD Anderson Cancer Center compared the benefits of preoperative chemoradiotherapy versus postoperative chemoradiotherapy and observed that more patients benefited from the multimodality therapy when chemoradiotherapy was delivered before pancraticoduodenectomy rather than after surgery. This was due in part to postoperative complications or delayed recoveries. However no significant difference was observed in the survival rates of the patients with both modalities. Another phase III study using gemcitabine alone or in combination with radiotherapy suggested that chemoradiotherapy significantly improved the overall survival rates form 9.2 to 11 months when compared with chemotherapy alone. However, there were no differences in progression-free survival and response rates between the treatments. Chemoradiotherapy also increased fatigue and gastrointestinal toxicities compared to chemotherapy alone (69, 71-73).

The effects of chemotherapeutic agents such as doxorubicin, and other combinations, streptozotocin, mitomycin C, cisplatin with 5-FU to enhance pancreatic cancer radiotherapy have been investigated but did not increase in patient survival rates and severe toxic side effects were observed with these combination therapies. Different radiation techniques such as radiosensitizers, particle irradiation, interstitial irradiation and

intraoperative radiation have also been investigated, but none of these have in improved therapeutic results (69, 71-73).

4) **Targeted therapies:** Research is also focused on understanding molecular pathways and factors that play an important role in pancreatic cancer progression; and therapies that target specific pathways are being developed for treatment of pancreatic cancer. VEGF is overexpressed in PDAC and inhibitors of VEGF inhibit tumor growth and metastasis. Bevacizumab (Avastin) is a recombinant humanized anti-VEGF 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 29% (71-73, 75).

The EGFR is also a target for drug development since overexpression of EGFR is a negative prognostic factor for survival of pancreatic cancer patients. Erlotinib is an oral tyrosine kinase inhibitor that inhibits EGFR activation through the phosphorylation of the intracellular tyrosine kinase domain. A phase III trial using erlotinib in combination with gemcitabine or gemcitabine alone has been conducted in pancreatic cancer patients. The overall survival was significantly prolonged in the combination therapy (median survival time of 6.24 vs 5.91 months) and the 1 year survival rate was higher with erlotinib plus gemcitabine (23% vs 17%). There was a higher incidence rate of adverse effects of grade 1 and 2 with erlotinib plus gemcitabine and patients with

good PS had a higher likelihood of developing a rash which was also associated with significantly increased survival. Another study with gemcitabine plus erlotinib plus bevacizumab compared to gemcitabine alone showed that combination improved progression free survival but not overall survival compared to gemcitabine plus erlotinib (71-73, 75).

Cetuximab is a monoclonal antibody against EGFR and a phase III trial compared cetuximab plus gemcitabine and gemcitabine alone. No significant difference in the overall survival rate was observed between the groups. However the addition of cetuximab to gemcitabine-oxaliplatin in a phase II trial showed a 38% response rate and 54% increase in 6 month survival rate; results of the phase III trial have not yet been released. Sorafenib, a small molecule inhibitor of VEGFR2 and Raf1 was evaluated in combination with gemcitabine. The combination therapy was well tolerated but ineffective for treating metastatic pancreatic cancer. Sunitinib, an inhibitor of VEGF and PDGF receptor is currently being evaluated as a second line of therapy and is in a phase II clinical trial (71-73, 75).

New agents are being evaluated for treatment of pancreatic cancer. Immunogenic telomerase peptide GV1001 is administered along with granulocyte macrophage colony stimulating factor and the treatment period was 10 weeks with monthly booster vaccinations. The vaccine was well tolerated in phase II clinical studies and the 1 year survival rate was

approximately 25%. Additionally patients with BRCA-2 germline mutations are sensitive to mitomycin C which is also being tested in pancreatic cancer patients (71-73, 75).

Animal models of pancreatic cancer

1) **Chemical carcinogen models:** Even though several chemical carcinogens induce pancreatic tumors in rodents, these tumors do not resemble human pancreatic cancer. For example, azaserine induces acinar cell hyperplasia, dysplasia and carcinomas in the rat pancreas but only minimal changes in ductal cell histology are present. Local administration of 9, 12-dimethylbenzanthracene (DMBA) in rats develop mucinous adenocarcinomas that invade bile ducts and metastasize to regional lymph nodes, peritoneum and liver similar to human PDAC. These tumors express ductal markers such as cytokeratin 19 and 20 but do not express acinar cell specific markers such as chymotrypsin. The tumors also exhibit K-ras mutations; however mutations of p16 and p53 genes that are observed in human cancers are not present in the pancreatic tumors. The most well characterized chemical carcinogen induced animal model of human pancreatic cancer is the Syrian Golden hamster treated with N-nitrosobis (2-oxopropyl) amine (BOP). The tumors exhibit mutations in K-ras and p16 gene and display perineural invasion and patterns of metastasis similar to human cancers. Unlike human PDAC which develops in the head of the pancreas, BOP-induced tumors

arise in both head and tail of pancreas. The limitations of this model include lack of knowledge of the hamster genome, lack of success in performing transgenic work in hamsters, the expense of hamsters as a tumor model. In C57BL/6J mice, implantation of cotton thread coated with 3-methylcholanthrene into the pancreas induces pancreatic ductal cancers, however, the implantation is tedious, time-consuming and costly (76).

2) **Genetically engineered mice (GEM):** Transgenic mice are created by introduction of foreign gene/genes (transgene) that undergo a stable chromosomal integration into the mouse genome. The early GEM model expressed activated oncogenes such as early region of SV40 genome (that produces large and small T antigens) in the regulatory regions of elastase I, trypsin I, amylase and glucagon genes in the pancreas. T antigen (TAg) is expressed diffusely in acinar cells and the cells undergo hyperplasia, dysplasia but very few acinar cells progress into pancreatic acinar adenocarcinoma. The TAg model exhibits a multistep process of tumor development but unlike human pancreatic cancer which expresses markers of both ductal and acinar origin, the TAg model yields neoplasms of acinar histogenesis (59, 76) (68).

A major breakthrough was achieved with the development of GEM expressing K-ras^{G12D} allele which is activated by the Pdx1-Cre transgene. Pdx1 is homeodomain protein and both exocrine and endocrine cells of

pancreas develop from Pdx1 expressing progenitor cells and Pdx1 protein expression persists postnatally in the exocrine acinar component of the pancreas. The Pdx1-Cre- K-ras^{G12D} mice developed murine PanINs (mPanIN) of various degrees and some (about 10%) develop metastatic, invasive adenocarcinomas after a long latency period of several months. The mPanINs not only resemble human lesions microscopically but also overexpress proteins such as Notch, Hedgehog and cyclooxygenase 2 (COX-2). However, the low frequency and longer latency in the development of pancreatic cancer in the K-ras model suggests that cooperating mutations in tumor suppressor genes such as INK4A/Arf or p53 are also important. K-ras mice crossed with biallelic INK4A/Arf deletions or p53 null allele or conditional knock-in mutant p53^{R172H}, develop more aggressive tumors that metastasize, they exhibit shorter latency and full penetrance. With deletion of either INK4A/Arf or p53 in the absence of K-ras^{G12D} expression, the animals do not develop pancreatic cancer (44, 59, 76-78) (68).

In addition, the presence or absence of an intact TGF-β signaling pathway in pancreatic cancer development has also been investigated. Deletion of either TGFβR2 or Smad4 in mice is not sufficient to induce pancreatic neoplasia but both genes cooperate with mutant K-ras in inducing pancreatic cancer with a shorter latency period. The pancreatic tumors that develop due to loss of TGFβR2 and mutant K-ras are well

differentiated adenocarcinomas with 100% penetrance, however, the combination of Smad4 allele loss with mutant K-ras yields cystic neoplasms that are either IPMNs or MCNs respectively (44, 59, 76-78) (68).

Bladder cancer

Classification of bladder cancers

The urinary bladder is lined by a highly specialized epithelium called the urothelium or transitional cell epithelium and it can modify the number of layers of epithelial cells depending on the level of distention of the urinary bladder wall. Approximately 95% of the bladder tumors are of epithelial in origin of which 90% are urothelial cancer and it is the fifth most common cancer and is responsible for approximately 3% of all cancer deaths in the United States (79-81).

Other types of epithelial cancers arising from bladder include squamous cell carcinomas and adenocarcinomas usually composed of malignant squamous or glandular foci and these are also typically seen in high grade urothelial tumors. Squamous cell carcinoma accounts for up to 75% of urinary bladder cases in areas where schistosomiasis is endemic and in the developed world they constitute <10% of all urinary bladder cancers. Small cell carcinoma and other neuroendocrine carcinomas of the bladder epithelium are also observed (Table 4) (79-81).

Bladder cancers of mesenchymal origin range from benign proliferations to highly malignant and aggressive tumors, however, invasive malignant tumors are less common. Leiomyoma is the most common benign mesenchymal cancer and rhabdomyosarcoma and leiomyosarcoma are the most common sarcomas in children and adults. Lymphomyoma, plastocytoma and infiltration by leukaemia are rarely present in bladder; secondary involvement of the urinary bladder by tumors is rare, however it usually occurs as an extension from adjacent organs such as rectum and uterus (Table 4) (47).

Transitional cell carcinoma is typically seen in patients over 50 years and only occasionally seen in younger adults and is a rare disease in children. Urothelial cancer occurrence is approximately 3 times more common in men than in women. The 5 year survival rate for all stages combined is 82%, however, the 5 year survival rate for localized and metastatic cancers is 94% and 6% respectively. Tumors usually occur in the lateral and posterior walls and rarely in the dome of urinary bladder. Urothelial carcinoma is broadly divided into two types 1) the papillary and 2) nonpapillary (flat sessile) type (Table 4) (47) (82) (83).

Table 4. Tumor classification of the urinary bladder (79).

Benign tumors	Malignant tumors
Epithelial tumors Urothelial papilloma Urothelial inverted papilloma Villous adenoma	**Epithelial tumors** Urothelial carcinoma (papillary noninvasive, papillary invasive, flat noninvasive, flat invasive NOS, micropapillary lymphoepithelioma-like, nested) Squamous cell carcinoma Adenocarcinoma Small cell carcinoma Undifferentiated carcinoma
Mesenchymal tumors Leiomyoma Neurofibroma Haemangioma Inflammatory myofibroblastic tumor	**Mesenchymal tumors** Rhabdomyosarcoma Leiomyosarcoma
	Miscellaneous neoplasms Lymphoma/leukemia Plasmacytoma Malignant melanoma Paraganglioma Germ cell neoplasms Adenofibromas and adenosarcomas
	Secondary Involvement Direct extension from adjacent organs (rectum, uterus, colon) Metastasis (breast)

1) **Papillary transitional cell carcinoma:** About 25% of urothelial cancers are noninvasive papillary in origin, however, 10-20% of the patients with noninvasive tumors subsequently progress to develop invasive tumors. The course of development of papillary lesions to become invasive is prolonged and may take many years before grade 2 and 3 type of tumors

are presented. Small papillary excrescences to large masses of confluent papillary tissue are observed. The large masses present a ‘cauliflower-like’ (sarcomatoid) appearance and may fill the bladder lumen. These papillae are usually soft, delicate and friable and may be creamy-white, tan, or pink to red in color. Sarcomatoid urothelial carcinoma is a high grade neoplasm that has partially or totally lost its carcinomatous morphological phenotype and differentiates into spindle-cell mesenchymal tissue. The noninvasive papillary tumors present a sharp interface between normal and tumor tissue (47).

The histological appearance of papillary transitional cell carcinoma is characterized by papillae that are tall and often branched, and may be separated or adherent to their neighbors and are covered by more than 7 cell layers whose appearance may vary from almost normal to atypical. Papillary carcinoma is graded as grade 1, 2 and 3 based on their cytological appearance. Grade 1 lesions show slightly atypical cells with an increase in the number of layers of urothelial cells but mitotic figures are rare. Grade 2 lesions show moderate atypia and occasional mitotic figures. Grade 3 lesions show high-grade atypia and mitotic figures (Figure 10) (47) (84).

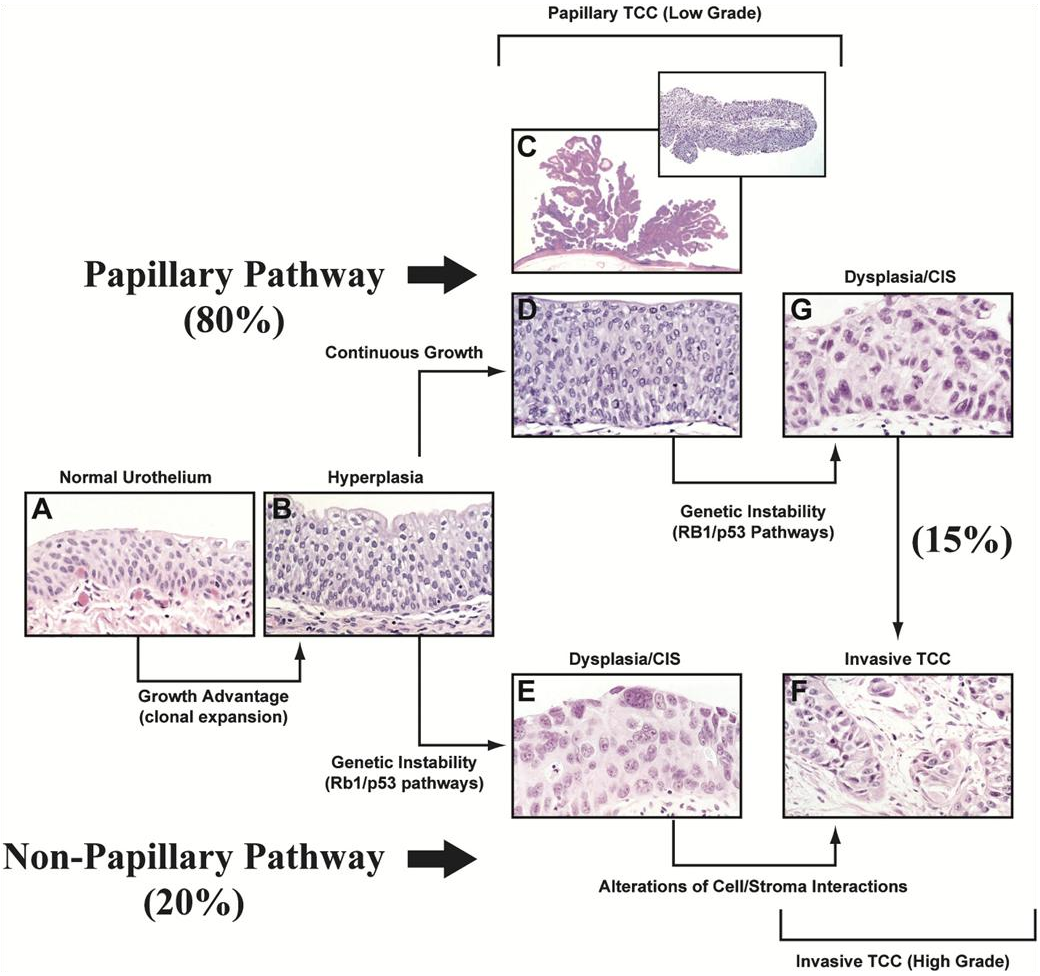

Figure 10. Dual tract concept of bladder carcinogenesis (80).

2) **Nonpapillary transitional cell carcinoma:** Grossly the bladder mucosa appears slightly granular or bulbous and is frequently associated with inflammation, edema and hypervascularization. Microscopically the abnormal urothelium may vary in thickness with cells exhibiting normal to hyperplastic appearance. Cells are usually pleomorphic and loss of intercellular cohesion and adherence to the basement membrane resulting in urothelium denudation. The tumor tissue is usually white but

may vary from tan to red and often be hemorrhagic and necrotic in appearance. The invasive tumor displays a high nuclear grade and may grow in nests, cords or trabeculae of neoplastic cells infiltrating muscular and adipose tissue of the bladder. The extent of invasion varies from microscopic tumor foci invading the lamina propria to tumors extending through the wall of the urinary bladder. In invasive neoplasms, the underlying bladder wall is replaced by firm, white tumor tissue (Figure 10) (Table 5) (47) (84).

Aetiology of bladder cancer

Several aetiological factors have been associated with bladder cancer development these include chemical and environmental exposures, radiation, chronic irritation due to bladder infections and schistosomiasis. Approximately 33% of urinary bladder tumors are associated with high exposure to tobacco smoke. The risk of developing bladder cancer increases 3-7 fold in smokers compared to non-smokers, depending upon the number of pack-years. The carcinogen present in cigarette smoke responsible for bladder cancer development is unknown; however nitrosamines, 2-naphthylamine and 4-aminobiphenyl have been associated with bladder cancer as evidenced by increased urinary tryptophan metabolites in cigarette smokers. Other forms of tobacco use are also associated with higher risk of bladder cancer (85, 86) (83).

Table 5. Stages of bladder cancer.

AJCC-TNM system	JSM system			
T0	-	No tumor		
Tis	O	Flat carcinoma in situ		
Ta	O	Papillary tumor, no invasion		
T1	A	Lamina propria invasion		
T2	B1	Muscle invasion, superficial		
T3a	B2	Muscle invasion, deep		
T3b	C	Perivesical fat invasion		
T4a	D1	Invasion of continuous viscera		
T4b	D1	Fixed to pelvic or abdominal wall		
N0	-	No lymph node involvement		
N1	D1	Single, homolateral lymph node involvement		
N2	D1	Contralateral, bilateral or multiple lymph node		
N3	D1	involvement		
N4	D2	Fixed pelvic wall mass separate from primary tumor		
		Juxtaregional lymph node involvement		
M	D2	Distant metastasis		
Stage 0a		Ta	N0	M0
Stage 0is		Tis	N0	M0
Stage I		T1	N0	M0
Stage II		T2	N0	M0
Stage III		T3	N0	M0
		T4a	N0	M0
Stage IV		T4b	N0	M0
		Any T	Any N	M0
		Any T	Any N	M1

AJC = American Joint commission on Cancer; JSM = Jewett-Strong (Marshall modification)

Occupational exposure to arylamines which are used in producing aniline dyes (coloring of fabrics) and in the rubber industry, is associated with up to one-third of urinary bladder cancers. Exposure to other chemicals such as 2-naphthylamine, 4-4-diaminobiphenyl (benzidine), 4-aminobiphenyl, and 2-amino-1-naphthol, combustion gases and soot from coal, possible chlorinated aliphatic hydrocarbons and certain aldehydes are also considered risk factors involved in bladder cancer development. Other potential sources include dietary nitrites and nitrates that are acted on by intestinal bacterial flora (85, 86) (83).

Urinary tract infection and chronic irritation are associated with increased risk of squamous cell carcinoma of the bladder. For example, paraplegic patients with long-term indwelling catheters develop bladder cancer and 80% of which are squamous cell carcinomas. Individuals with Schistosoma haematobium parasitic infection especially in endemic areas such as Egypt and other parts of Africa are at high risk of developing squamous cell carcinoma and transitional cell carcinoma. Eggs of S.haematobium are deposited in the bladder wall eliciting a chronic granulomatous inflammatory response, fibrosis, calcification and squamous or glandular metaplasia of the urothelium. High levels of nitrosamines have also been found in patients with schistosomiasis. Exposure to human papilloma virus (HPV) in individuals whose immune system is compromised also increases the risk of developing transitional cell tumors (85, 86) (83).

Women treated with radiotherapy for cervical cancers are at increased risk (2-4 fold) of developing bladder cancer compared to women who undergo surgical resection of the cancer. Bladder cancer incidence further increases if chemotherapy with cyclophosphamide is administered. Also men treated with external beam irradiation for prostate cancer are at high risk of developing bladder cancer. Similarly, individuals exposed to irradiation due to the Chernobyl nuclear reactor accident had 52% increase in incidence of carcinoma in situ (noninvasive lesions) and 6.4% increase in incidence of urothelial carcinoma compared to individuals from uncontaminated areas (85, 86) (83).

Individuals exposed to large quantities of arsenic through contaminated drinking water are at higher risk of developing bladder cancer and this is associated with RASSF1A promoter hypermethylation and with several chromosome-type breaks, gaps, exchanges and other aberrations in blood and urothelial cells. Women exposed to Aristolochia fangchi (substituted for Stephania tetrandra in weight reduction aid) developed aristolochic acid-related DNA adducts in urothelium of both upper tract and bladder resulting in an increased risk of developing urothelial carcinoma (86) (83).

Cellular and molecular mechanisms in development of bladder cancer

Bladder cancer is broadly classified into papillary and flat carcinoma in situ. Papillary carcinoma recurs locally but is rarely invasive and metastatic; however, flat carcinoma in situ is highly invasive and metastatic. Two distinct molecular pathways are activated in the development of urothelial carcinoma.

Low-grade papillary tumors usually exhibit constitutive activation of the receptor tyrosine kinase-Ras pathway with mutations in H-ras (Harvey rat sarcoma viral oncogenes homolog gene) and fibroblast growth factor receptor 3 (FGFR3) genes. Approximately 70% of noninvasive papillary tumors harbor FGFR3 mutations compared to 10-20% of invasive tumors suggesting a role for FGFR3 in development of low-grade papillary tumors. In contrast, alterations in p53 and Rb pathway are observed in flat carcinoma in situ and invasive tumors and homozygous deletion of p16/INK4A is observed in high grade papillary tumors (Figure 11) (82, 83, 87).

Ras-MAPK signal transduction pathway: Even though the FGFR3-Ras-MAPK pathway is activated in noninvasive papillary tumors, other receptor tyrosine kinases such as EGFR and ERBB2/HER2 are overexpressed in invasive carcinoma and are associated with a poor prognosis. RASSF1A is a tumor suppressor gene that encodes Ras association domain family 1 protein and inhibits the function of activated Ras protein. RASSF1A gene is highly methylated in bladder cancer and is associated with increasing tumor stage (82, 83, 87).

p53 and Rb cell cycle regulation pathway: Most urothelial cancers exhibit loss of 17p allele and this is accompanied by mutations in the remaining allele resulting in inactivation of p53. $p21^{WAF1/CIP1}$, a cyclin dependent kinase inhibitor (CDKI), is located on chromosome 6p21 and p21 is regulated by p53 dependent and independent pathways. p53 inhibits G1-S phase cell cycle progression

through activation of p21 and loss of p21 expression associated with p53 alterations and this plays an important role in tumor progression. For example, patients with p21-negative and p53 mutations in tumors had a greater recurrence and lower survival rate compared to patients with p21-positive tumors. p14/ARF gene is inactivated due to promoter hypermethylation or by a homozygous deletion which transcriptionally inhibits expression of Mdm2 protein. Mdm2 protein binds p53 and activates ubiquitin-mediated degradation of p53 and the resulting lower levels of p53 results in decreased Mdm2 in an autoregulatory feed-back loop. Loss of p14 and amplification of Mdm2 is observed in urothelial cancers and correlated with poor prognosis in these patients (82, 83, 88).

Inactivating mutations of the Rb gene are observed in low to high grade and invasive bladder cancers and inactivation of Rb results in the loss of p16 (CDKI) and/or overexpression of cyclin D1 and this enhances tumor proliferation. Patients were classified into four groups taking the number altered genes (p53, p21 and Rb) and in patients with 0, 1, 2 or 3 altered genes, the 5 year recurrence rates were 23%, 32%, 57% and 93% respectively (Figure 11) (82, 83, 88).

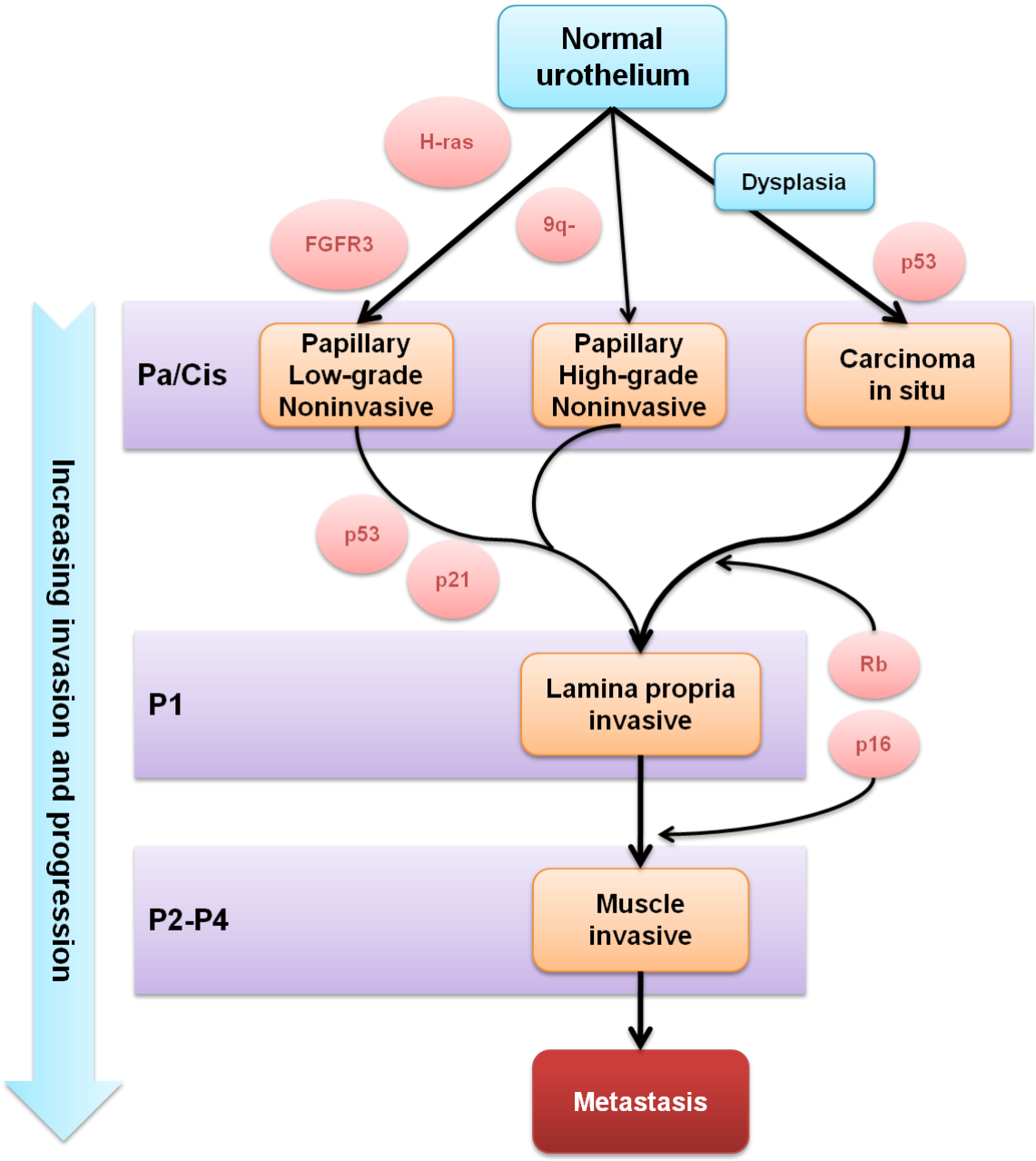

Figure 11. Proposed model for urothelial tumorigenesis and progression (83).

Tumor angiogenesis pathways: Many factors are involved in tumor angiogenesis which facilitates interactions with stroma to recruit endothelial cells

and establish a vascular system that provides nutrients for rapidly growing cancer cells. Hypoxia inducible factor (HIF)-1α regulates oxygen concentrations and also induces VEGF which stimulates tumor vascularization. Overexpression of HIF-1α and VEGF are observed in many urothelial cancers. Expression of the enzyme thymidine phosphorylase (TP) in invasive cancers is 33-fold higher than in superficial tumors and 260-fold higher than in normal bladder. TP promotes the production of interleukin-8 and MMPs. MMPs activate acidic and basic FGF which play an important role in endothelial cell migration and tumor angiogenesis (82, 83).

Cell death pathway: Antiapoptotic Bcl-2 plays an important role in the intrinsic pathway of apoptosis and overexpression of Bcl-2 correlates with poor prognosis in urothelial cancer patients treated with radiotherapy or chemoradiotherapy (82, 83).

Other pathways: Many different pathways are altered in urothelial cancers and these include activating mutations in genes involved growth signaling pathway such as FGFR3, EGFR, ERBB2, VEGR1 and VEGFR2. Kinase pathways such as Ras-MAPK pathway, phopholipase C (PLC)-protein kinase C (PKC) pathway, PI3K-Akt pathway, the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways and the nuclear factor-kappa B (NF-κB) also play a role in bladder tumor development. NF-κB is important for inflammation, autoimmune response, cell proliferation and apoptosis. Bacille Calmette-Guerin (BCG) induces interleukin-6 expression through NF-κB and is used for treatment

of urothelial cancers. Patients with homozygous deletion of NF-κB1 gene exhibit a higher risk of recurrence than those with a homozygous insertion. MicroRNA expression is altered in many cancers and in urothelial cancers microRNAs such as miR-223, miR-26b, miR-221, miR-103-1, miR-185, miR-23b, miR-17-5p, miR-23a and miR-205 are increased in urothelial tumors. For example, miR-17-5p and its cluster miR-17-92 are overexpressed in lung, breast, colon, pancreas and bladder. MiR-17-5p and miR-20a are associated with c-Myc amplification and negatively regulate the expression of E2F1 (83) (89).

Prognostic and molecular markers of bladder cancer

Clinical screening for urothelial cancer is carried out on individuals with a history of environmental or occupational exposures to potential bladder carcinogens and also in individuals with microscopic hematuria or irritative voiding symptoms. Urine cytology and cystoscopy are still considered the gold standard for detection of bladder cancers. Urine cytology is a noninvasive test which is useful for identification of high-grade bladder tumors and for monitoring patients with persistent or recurrent disease. This test has a high specificity (95-100%) but low sensitivity (66-79%) for detection of asymptomatic and low-grade bladder cancers, hence limiting its use. Cystoscopy is used for determining tumor size, location and appearance and the sensitivity of cystoscopy can be increased by using fluroscence methods and photosensitizer and 5-aminolevulinic acid or hexaminolevulinic acid. Bladder wash cytology is helpful in detecting most cases of carcinoma in situ even when the urothelium appears

grossly normal. A number of urinary tumor markers are being developed for detection of bladder cancer and some of these are indicated below (79, 90).

Bladder tumor antigen (BTA): BTA is a human complement factor H-related protein similar to human complement factor H. BTAstat test is a one step qualitative immunochromatographic assay in which two monoclonal antibodies are used to recognize two epitopes of human complement factor H-related protein. BTA TRAK is a quantitative two step enzyme-linked immunosorbent assay (ELISA) and shows higher sensitivity (52-83%) for detecting high-grade tumors (79, 83, 90, 91).

Nuclear matrix protein (NMP) 22: NMP22 is a nuclear scaffold protein that regulates mitosis and distributes chromatin to daughter cells. The intracellular concentration of the NMP22 protein is approximately 25-fold higher in bladder cancer cells than normal urothelial cells and this protein is also released in soluble form during apoptosis. The specificity and sensitivity of the test is 85.7 and 55.7% respectively (79, 83, 90, 91).

Fibrinogen degenerative product (FDP): VEGF increases vessel wall permeability for blood and plasma proteins such as plasminogen, fibrinogen and other clotting factors. Clotting factors convert plasminogen to plasmin and fibrinogen to fibrin and plasmin can further degrade fibrin into FDPs and these are released into circulation and are detected in urine using the FDP test. The sensitivity of FDP test is approximately 68-83% and FDP levels increase in

patients with cancer as the grade and stage increase, however the sensitivity of this test for detecting carcinoma in situ is not yet proven (79, 83, 90, 91).

ImmunoCyt: This is an immunofluorescence assay which uses 3 antibodies against high molecular weight forms of carcinoembryonic antigen (19A211) and mucins (M344 and LDQ10) that are expressed in bladder cancer but not in normal epithelium. The sensitivity of the test can be improved in combination with conventional cytology (79, 83, 90, 91).

Fluorescence in situ hybridization (FISH): FISH detects genetic alterations in urine sediments in bladder cancer patients using fluorescently-labeled DNA probes to assess centromeres of chromosomes 3, 7, 17 and 9q21 (p16/CDKN2A). FISH exhibits higher sensitivity when compared to cytology in detection of bladder cancer while maintaining the higher specificity of cytology (79, 83, 90, 91).

Hyaluronic acid (HA)-hyaluronidase (HAase) test: HA is a nonsulfated glycosaminoglycan with repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine that is secreted by stomal fibroblasts and HA regulates cell adhesion. HA levels are 3-4 fold higher in tumor than in normal tissue. HAase is an endoglycosidase which degrades HA into smaller fragments to promote angiogenesis and in bladder cancer patients the secretion of both HA and HAase is observed in urine. The overall sensitivity and specificity of this test is 91.2 and 84% respectively (79, 83, 90, 91).

Survivin: Survivin is a member of the inhibitor of apoptosis (IAP) family that inhibits apoptosis by binding to caspase 3 and 7. Overexpression of survivin is observed in various cancers such as lung, colon, pancreas, prostate, bladder and breast and is associated with biologically aggressive disease, resistance to therapy and poor clinical outcome in patients with various cancers. The sensitivity and specificity of identifying survivin expression in urine specimens are 64-100% and 93-100% respectively in bladder cancer patients (79, 83, 90, 91).

Telomerase: Telomeric repeat amplification protocol (TRAP) detects the activity of telomerase enzyme which catalyzes the synthesis of telomeres that maintain chromosomal ends. The overall sensitivity of the TRAP assay is about 70-86% with a false-positive rate of 21-76%. Recent studies using RT-PCR for detecting mRNA of human telomerase reverse transcriptase (hTERT) has better sensitivity (74-92%) and specificity (70-93%) than the TRAP assay (79, 83, 90, 91).

Microsatellite alterations: Microsatellites are inherited short tandem DNA repeat sequences and mutations in mismatch repair genes such as hMSH1, hMSH2 and hMSH6 results in microsatellite instability. The microsatellite assay is performed using PCR and this assay is highly sensitive and specific but is also complex and expensive. Blunt-end single strand DNA conformation polymorphism (blunt-end SSCP) analysis detects loss of heterozygosity (LOH) on chromosome 9 in urine samples of bladder cancer patients (79, 83, 90, 91).

Cytokeratins: These are intermediate filament proteins specific for epithelial cells and CK18, CK19 and CK20 proteins are overexpressed in bladder cancer. CYFRA21-1 is an ELISA assay that detects fragments of CK-19 with sensitivity and specificity of 76.2 and 84.2% respectively (79, 83, 90, 91).

Other markers including Ki-67 and p27 may predict recurrence and disease progression but are not yet clinically applicable. Ki-67 is marker for cell proliferation and correlates with grade and stage of tumor progression. p27 is a cyclin dependent kinase inhibitor regulating G1 cell cycle arrest and low levels of p27 have been associated with unfavorable prognosis (81, 91).

Current therapies for bladder cancer

Superficial bladder cancer: Bladder cancer is commonly observed as a superficial disease with 70% confined to bladder mucosa (Ta or Tis) and 30% involving submucosa (T1). These are initially treated with transurethral resection followed by close observation or intravesical chemotherapy or immunotherapy. Immunomodulators such as BCG and interferon α and chemotherapeutic agents such as mitomycin, doxorubicin, thiotepa and gemcitabine are used for intravesical therapy. BCG therapy stimulates the local immune response to induce cytokines attracting inflammatory cells and interferon α synergistically augments BCG by prolonging the T-helper type 1 immune response in leukocytes (92). Intravesical chemotherapy is used when the side-effects of BCG are intolerable. Thiotepa is a 189 d molecule with alkylating properties and inhibits nucleic acid synthesis. However, thiotepa causes toxicity from systemic

absorption and patients exhibit signs of leucopenia or thrombocytopenia. Doxorubicin is an anthracycline antibiotic that binds to DNA and interrupts DNA replication, transcription and protein synthesis. Systemic effects from doxorubicin are low due to its high molecular weight (580 d). Tumor recurrence rates with both of these treatments were similar. Mitomycin-C is another alkylating agent used for intravesical treatment of bladder cancer. Due to high molecular weight (334 d), mitomycin-C is minimally absorbed and exhibits low systemic toxicity. Repeat installation of mitomycin-C after transurethral resection decreased tumor recurrence rates and increased the duration of disease free interval (93) (94, 95) (79).

Laser treatment also allows the effective ablation of superficial tumors and is best suited for the treatment of recurrent superficial disease. Argon, potassium titanyl phosphate and holmium yttrium-aluminum-garnet lasers are being used for treatment of bladder cancer (90, 93).

Photodynamic therapy is used in patients whose conditions do not respond to intravesical therapy with BCG or mitomycin-C. A hemoporphyrin (photofrin-porfimer sodium) photosensitizer is given intravenously with subsequent activation by mercury light illuminating the whole bladder. After light exposure the photosensitizer reacts with oxygen to form cytotoxic free radicals, however, effective photosensitization is dependent on oxygen levels in the bladder (93) (90).

Invasive bladder cancer: Radical cystectomy with pelvic lymph node dissection is used for treatment of invasive bladder cancer and this includes removal of the bladder, regional pelvic lymph nodes, distal ureters as well as the prostate gland, seminal vesicles and proximal urethra in men and the urethra, uterus, fallopian tubes, anterior vaginal wall and surrounding fascia in women. Radical cystectomy is usually performed in older patients and in healthier younger patients trimodal therapy consisting of transurethral resection of the bladder tumor followed by chemoradiotherapy is used. A number of radiosensitizing drugs such as 5-FU, cisplatin, gemcitabine, paclitaxel and more recently cetuximab are used. Cisplatin is not used in patients with kidney disease and gemcitabine, as a single agent, displays minimal toxicity (81, 94, 95) (79).

Unresectable and metastatic disease: The combination of methotrexate, vinblastine, doxorubicin and cisplatin (M-VAC) or a combination of gemcitabine and cisplatin are used for the treatment of bladder cancer. Gemcitabine plus cisplatin has become a standard therapy due to significant toxicity associated with M-VAC therapy and gemcitabine plus cisplatin gives response rates and median survival rates similar to MVAC but with better safety and tolerability in bladder cancer patients (79, 81, 94, 95) (80).

Targeted therapies: A wide range of therapeutic agents are being developed against specific genes/proteins that are important in key urothelial cancer pathways. These therapies include cetuximab, an anti-EGFR monoclonal antibody; Gefitinib and erlotinib, EGFR-specific tyrosine kinase inhibitors;

trastuzumab, an anti-ERBB2 monoclonal antibody; lapatinib, a dual inhibitor of EGFR and ERBB2 associated tyrosine kinases; sorafenib, a multitarget kinase inhibitor of c-Raf-1, B-Raf, VEGFR1/2/3 and PDGFRβ; and bevacizumab, an anti-VEGF monoclonal antibody. These are all in clinical trials for treatment of bladder cancer (80, 81, 96).

Animal models of bladder cancer

Chemical carcinogen models: Rats and mice are most commonly used as in vivo models for studying urothelial cancer. N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT), N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) and N-methyl-N-nitrosourea (MNU) are the three chemicals that induce bladder cancer. FANFT can act as an initiator or promoter and is highly specific to the bladder in rat, mouse, hamster and dog. Oral administration of FANFT results in DNA adducts and requires 8-11 months to induce urothelial cancer. MNU is the only carcinogen that directly acts on the urotheilum after undergoing a spontaneous pH-dependent activation. Intravesical treatment with MNU induces lesions such as hyperplasia, atypia, carcinoma in situ, papillary carcinoma and finally large bulky muscle invasive tumors that completely fill the bladder lumen (97-99).

Genetically engineered mice (GEM): The development of transgenic mouse models has allowed researchers to recapitulate the dual track pathway of bladder cancer development. The uroplakin II gene promoter is specific to bladder and is used to drive the expression of SV-40 large T antigen which inactivates both p53 and Rb pathways resulting in development of carcinoma in

situ and highly invasive tumors. Alternatively mutant H-ras expression driven by uroplakin promoter in mice results in development of papillary lesions that become invasive when these animals are crossed with p53 null mice. Similarly overexpression of EGFR in the urothelium results in hyperplasia; the phenotype of animals expressing both mutant H-ras and EGFR is indistinguishable from animals expressing mutant H-ras alone. However, EGFR overexpression in animals with SV-40 low copy number resulted in high grade urothelial carcinomas. Loss of LoxP-flanked PTEN by Cre recombinase driven by the fatty acid binding protein 1 promoter resulted in urothelial hyperplasia and in a few case urothelial cancers were observed. This indicates that loss of PTEN alone is not sufficient for initiation of bladder cancer and requires another genetic event. Double knockout of PTEN and p53 resulted in invasive bladder cancer (80, 97-99).

Cancer chemotherapy

From 1950-1970 agents that interact with DNA or its precursors and inhibit the DNA synthesis or compounds that damageDNA were widely used in cancer chemotherapy. Recent research has focused on natural products (paclitaxel) and semisynthetic agents such as etoposide which target specific pathways involved in cancer cell proliferation, survival and angiogenesis (100). Chemotherapeutic agents can be classified into the following groups.

1) **Alkylating agents:** Alkylating agents are the oldest class of drugs and are used in treatment of leukaemia, lymphoma and solid tumors.

Alkylating agents form covalent bonds with DNA through specific sites on purine bases leading to cross-linking of DNA and induction of apoptosis. Six major types of alkylating agents are used in cancer chemotherapy (101).

a) **Nitrogen mustards:** Nitrogen mustards (N-mustard) are related to sulfur-containing mustard gases and N-mustards form DNA adducts at O^6 and N^3 sites of guanine, N^1, N^3, N^7 of adenine and N^1, N^3 of cytosine. N-mustards have two electrophilic sites and acts as bifunctional alkylating agents resulting in intrastrand and interstrand cross-linking of DNA leading to inhibition of replication and transcription. Drugs included in this class are chlormethine, chlorambucil, melphalan, cyclophosphamide, bendamustine and ifosfamide. Chlormethine is the first N-mustard to be introduced and is the most reactive of all the drugs in this class. It is primarily used for treatment of Hodgkin's disease in combination with vincristine plus procarbazine plus prednisone (MOPP regimen). Chlorambucil is used for treatment of chronic lymphocytic leukemia (CLL) and primary (Waldenstrom's) macroglobulinaemia and melphalan is used for treatment of multiple myelomas. Cyclophosphamide is the most widely used N-mustard for treatment of malignant diseases of the brain, breast, endometrium, lung, bladder, cervical, testis and ovary. It is also used as an immunosuppressive agent (101-103).

b) **Methylmelamines and ethylenimines:** This class includes thiotepa and altretamine and even though N-mustards have replaced ethylenimines, some of these agents are used for specific therapies. For example thiotepa is used as intravesical therapy for bladder cancer and in high-dose chemotherapy for solid tumors in combination with cyclophosphamide and carboplatin (CTCb regimen). Altretamine is used for treatment of ovarian cancer when the first line therapy has failed (101, 102, 104).

c) **Methylhydrazine derivatives:** Procarbazine was initially synthesized as a monoamine oxidase (MAO) inhibitor and was later found to have antineoplastic properties. It is used for treatment of Hodgkin's disease in a combination therapy (MOPP regimen) and also for the treatment of non-Hodgkin's lymphoma, small-cell lung cancer and tumors of the brain (101, 102) (105).

d) **Alkylsulfonates:** Busulfan is also a bifunctional alkylating agent which has 2 methanesulfonate groups and upon hydrolysis produces reactive carbonium ions that alkylate DNA. Busulfan, at low doses causes selective depression of granulocytopoiesis and is primarily used for treatment of chronic myelogenous leukemia (CML). At low doses cytotoxic effects are not observed on lymphoid tissue or gastrointestinal epithelium, however, at high doses it causes myelosuppression, pulmonary fibrosis, liver damage and other toxicities (101, 102).

e) **Nitrosoureas:** Nitrosoureas are used in treatment of wide range of cancers including solid and non-solid tumors. Most nitrosourea compounds are lipophilic and can cross blood-brain barrier and provide high CSF to plasma ratio of these drugs compared to other alkylating agents. Compounds included in this class are carmustine, lomustine, fotemustine, nimustine and streptozocin. The therapeutic efficiency of these drugs is limited by the development of resistance which involves multiple DNA repair pathways such as O^6-methylguanine DNA-methyltransferase (MGMT) and mismatch repair (MMR) genes (101, 102).

f) **Triazines:** This class includes dacarbazine and temozolomide which cause methylation of DNA at the N^7 position of guanine, followed by the N^3 postion of adenine and O^6 postion of guanine. Dacarbazine is used in treatment of metastatic malignant melanoma and temozolomide is used for treatment of refractory high-grade glioma (101, 102).

2) **Antimetabolites:** An antimetabolite is a chemical with a structure similar to a metabolite but is sufficiently different to interfere with the normal function of cells including cell division. Their chemical structure is similar to either folate or nucleotides that become the building blocks of DNA.

a) **Folate antagonists:** Folic acid is a water soluble member of vitamin B class that is essential for cell proliferation and tissue regeneration and plays an important role in nucleotide synthesis. Dihydrofolate reductase

(DHFR) reduces folate to dihyrofolate and tetrahydrofolate (active form). Tetrahydrofolate donates methyl groups to specific molecules in the presence of thymidylate synthase (TS) and these are used for the synthesis of purine nucleotides and thymine. This class of drugs includes methotrexate, permetrexed, ralitrexed, nolatrexed, pralatrexate and talotrexin. Methotrexate binds to DHFR and inhibits synthesis of tetrahydrofolate and thus inhibits cellular synthesis of DNA and RNA. Methotrexate is used for treatment of lymphoblastic lymphoma, osteosarcoma in children, choriocarcinoma and related trophoblastic tumors of women and carcinomas of head, neck, ovary and bladder. To mitigate the toxic side effects of methotrexate, reduced folate (leucovorin, citrovorum factor) is supplemented to patients to bypass inhibited DHFR. Permetrexed inhibits TS and is used for treatment of malignant pleural mesothelioma and non-small cell lung cancer. Ralitrexed, a quinozoline folate analog that blocks TS is used for treatment of advanced colorectal cancer. pralatrexate and talotrexin are DHFR inhibitors that are currently in clinical trials for treatment of various cancers (101, 102) (106, 107).

b) **Pyrimidine antagonists:** This class includes many drugs such as 5-FU, capecitabine, gemcitabine, cytarabine, azacitidine, CP-4055, tegafur, floxuridine, doxifluridine, sapacitabine and decitabine. 5-FU is converted to two active metabolites, 5-fluoroxyuridine monophosphate (F-UMP) and 5-5’-fluoro-2’-deoxyuridine-5’-O- monophosphate (F-dUMP). F-UMP

competes with uracil and is incorporated into RNA and thereby inhibits RNA synthesis and F-dUMP inhibits TS resulting in thymidine depletion and decreased DNA synthesis. 5-FU is used for treatment of colorectal cancer and pancreatic cancer. Capecitabine is an oral prodrug of 5-FU and the reaction is catalyzed by thymidine phosphorylase which is found at a higher level in cancer cells and is selectively activated in tumors. Capecitabine is in clinical trials for treatment of breast, colorectal cancer and other solid tumors.

Gemcitabine is converted into two active metabolites difluorodeoxycytidine di- and triphosphate (dFdCDP and dFdCTP). dFdCDP inhibits ribonucleotide reductase and decrease deoxynucleotide pool required for DNA synthesis and dFdCTP is incorporated into DNA resulting in termination of DNA strand synthesis. Gemicitabine is used widely for treatment of pancreatic, breast, bladder, lung, ovarian and renal cell carcinoma. Azacitidine, an analogue of cytidine, is incorporated in to DNA and inhibits DNA methyltransferase resulting in hypomethylation of DNA thereby activating tumor suppressor genes silenced by hypermethylation. It also is incorporated into RNA and inhibits tRNA cytosine-5-methyltransferase activity. Azacitidine is in clinical trials for treatment of myelodysplasia and AML (101, 102).

c) **Purine antagonists:** Mercaptopurine was the first synthetic purine antagonist and is an analogue of the purine bases adenine and

hypoxanthine. Mercaptopurine is incorporated into DNA and inhibits DNA synthesis. Mercaptopurine is used for treatment of ALL, AML, Hodgkin's lymphoma in children and lymphoblastic lymphoma. Other drugs included in this class are fludarabine, pentostatin, cladribine, thioguanine, nelarabine, cordycepin, clofarabine, triciribine phosphate, pelitrexol and dezaguanine. Fludarabine is fluorinated nucleotide analogue of the antiviral agent vidarabine and is used to treat acute non-lymphocytic leukaemia (ANLL) and CLL. Pentostatin is a purine analogue isolated from *Streptomyces antibioticus* that blocks adenosine deaminase which is essential for purine synthesis. Thioguanine is a synthetic guanosine analogue and cordycepin is an adenosine analogue isolated from fungus cordyceps militaris (101, 102).

3) Antimicrotubule agents:

a) Taxanes: Paclitaxel (Taxol) was the first taxane isolated from the bark of the pacific yew, *Taxus brevifolia*. Docetaxel is the semisynthetic durg derived from an inactive taxane precursor. Taxanes bind β-tubulin preferentially in microtubules rather than to soluble tubulin and they stabilize the microtubules. This inhibits microtubule polymerization and disrupts the normal dynamic reorganization of the microtubule network required for mitosis and cell proliferation resulting in cell cycle arrest in the G2/M phase. The binding affinity of docetaxel to microtubules is higher than paclitaxel and docetaxel is twice as potent as paclitaxel.

Taxanes are used in treatment of ovarian, breast, prostate and non-small cell lung cancer (101, 102) (108, 109).

b) Vinca alkaloids: Vinca alkaloids (vincristine and vinblastine) are extracted from *Catharanthus roseus* (Madagascar periwinkle). Vincrinstine and vinblastine along with semisynthetic derivatives such as vindesine, vinorelbine and vinflunine are used in treatment of various cancer including leukemia, lymphoma, melanoma, breast and lung cancers. Vinca alkaloids bind to the both microtubule and soluble portion of β-tubulin and they destabilize the microtubules by inhibiting polymerization results in the inhibition of mitosis and cell cycle progression (101, 102) (110, 111).

4) Antitumor antibiotics: Drugs that are classified as antitumor antibiotics are produced by microorganisms that interact with DNA to produce their anticancer effects.

a) Actinomycin D: Actinomycin D is extracted from *Actinomyces antibioticus* or *Streptomyces parvulus* and is used for treatment of Wilm's tumor, rhabdomyosarcoma, Ewing's sarcoma, trophoblastic neoplasms and testicular carcinomas. Actinomycin D is a phenoxazinone chromophore linked to two pentapeptide lactones which intercalate into the DNA with their lactone moieties residing in the minor groove. This binding is sequence selective for GpC base pairs by forming hydrogen

bonds between the N2 amino group of guanine and carbonyl groups in the peptide side-chains of the drug (101, 102).

b) Mitomycin C: Mitomycin C is isolated from *Streptomyces caespitosus* and is used in combination therapy for treatment of solid tumors such as adenocarcinoma of stomach or pancreas, superficial bladder cancer, epidermoid anal carcinomas and esophageal carcinomas. Mitomycin C forms interstrand and intrastrand crosslinks with DNA. In the interstrand crosslink mitomycin C links two guanines in opposite strands at 5'-CpG sequences and the intrastrand crosslinks are formed at GpG sites of DNA. The major reduced metabolite of mitomycin C, 2, 7-diaminomitosene, generates two additional monoadducts by reacting with DNA. The selective toxicity of mitomycin C towards solid tumors is primarily due to reductive activation since solid tumors are characterized by low oxygen levels and the hypoxic cells readily reduce mitomycin C to give active metabolites. Many potent synthetic analogues of mitomycin C are also less toxic and for example, apaziquone is in clinical trials for treatment of advanced colorectal, breast, gastric, pancreatic, non small cell lung cancer and noninvasive bladder cancer (101, 102) (112).

c) Bleomycin: Bleomycin A2 and B2 are linear glycosylated peptides isolated from *Streptomyces verticellus* and the clinical drug Blenoxane contains a mixture of bleomycin A2 and B2. Blenoxane is used in combination therapy for treatment of Hodgkin's and non-Hodgkin's

lymphomas, squamous cell carcinomas, testicular carcinomas and malignant pleural effusions. The clinical applications of bleomycin are limited by its secondary effects and its lack of permeability through cell membranes. The use of electroporation techniques has increased the permeability of bleomycin by approximately 1000-fold and clinical studies with bleomycin using electroportation in patients with cutaneous and subcutaneous nodes of melanoma, breast, colon cancer, squamous cell carcinoma of skin and cervix exhibited high response rates with minor-side effects and is well tolerated by the patients. Bleomycin induces RNA and DNA breaks and the cofactors involved in this process are a metal ion (usually Fe2+/Fe3+), oxygen and an electron reducing agent (101, 102) (113).

d) Anthracyclines: The major anticancer agents in this class include doxorubicin and daunorubicin which are isolated from *Streptomyces peucetius*. The only difference between daunorubicin and doxorubicin is its lack of hydroxyl group in the chromophore side chain. The activity of daunorubicin is narrow and is used for treatment of ALL, AML and CML. Doxorubicin is used for treatment of various solid tumors such as breast cancer, ovarian cancer, urothelial bladder cancer, bronchogenic lung cancer, thyroid cancer and gastric cancer. Anthracyclines mediate their anticancer effects as topoisomerase IIα inhibitors which play an important role in regulation of DNA supercoiling during replication or transcription

and these drugs induce double stranded DNA breaks leading to apoptosis. Semisynthetic analogues of doxorubicin are epirubicin and idarubicin and these compounds are also in clinic trials for treatment of solid and nonsolid tumors (101, 102) (114).

e) **Camptothecins:** Camptothecin is a pentacyclic antibiotic isolated from *Camptotheca acuminate*. The activity of camptothecin is limited due to its low water solubility and toxic side effects, however, many synthetic analogues with increased water solubility have been developed. The two analogues which are in clinic trials are topotecan and irinotecan which inhibit topoisomerase I and block religation of the cleaved DNA strand and inhibits DNA synthesis and decreases cell viability. They are used in treatment of AML, small cell lung cancer, ovarian cancer and colorectal cancers (101, 102) (115).

f) **Podophyllotoxins:** Podophyllotoxins are aryltetralinlactone lignans isolated from *Podophyllum peltatum* and despite their strong anticancer activities clinical applications are not ongoing due to their severe toxic side effects. Their synthetic analogues etoposide and teniposide are used clinically for treatment of various cancers. Podophyllotoxins inhibit assembly of microtubules by binding to tubulin, however, their analogues, etoposide and teniposide, inhibit topoisomerase II (101, 102) (116).

5) **Platinum based drugs:** Cisplatin is used in combination therapy for treatment against cancers of the lung, head-and-neck, esophagus,

stomach, colon, bladder, testis, ovaries, and cervix. Cisplatin induces DNA crosslinking, oxidative stress and stress-signaling pathways involving MAP kinase cascades. Carboplatin and oxaliplatin are analogues of cisplatin but none of them are as effective as cisplatin. Carboplatin is less toxic than cisplatin to kidneys and the nervous system and retains equivalent antitumor activity. Carboplatin is highly effective for treatment of advanced ovarian cancers in women and is also used in combination therapies for treatment of hormone-refractory prostate cancer, anaplastic astrocytomas and glioblastomas (101, 102) (117, 118).

6) **Targeted based therapies:** The rationale for designing and developing targeted- based therpies comes from the knowledge and understanding the biology of cancer cells which typically exhibit aberrant protein expression and signal transduction networks and drugs that inhibit proliferation and survival pathways are being developed (101).

a) **Hormonal therapy:** Hormonal therapy is used for treatment of several cancers that are derived from hormone-responsive tissue including breast, prostate, endometrium and adrenal cortex.

i) **Estrogen receptor targeted therapies:** The estrogen receptor (ER) is expressed as two isoforms, ERα and ERβ that exhibit distinct tissue specific expression. Estrogens induce gene expression and cellular changes through genomic and non-genomic mechanisms. Estrogen binds to ER located in the nucleus and this nuclear estrogen-ER complex binds

directly to estrogen response elements or indirectly through protein-protein interactions involving AP1 or Sp1 bound to AP1 or GC-rich sites respectively. In the non-genomic mechanism estrogen binds to ER located in or adjacent to plasma membranes, or through other non-ER plasma membrane associated factors resulting in increased levels of Ca^{2+} or NO and activation of kinases. ERs are implicated in several diseases such as breast and ovarian cancers, and the feasibility of anti-hormone endocrine therapy is determined by level of ER expression (101, 102) (119).

Selective estrogen receptor modulators (SERMs) bind with high affinity to ER and mimic the effect of estrogens in some tissues but act as estrogen antagonists in other tissues. Tamoxifen has been the most widely used SERM treating ER positive breast cancer and exhibits antiestrogenic activity in breast tumors, estrogen-like activity in bone and also decreases cholesterol. However tamoxifen increases proliferation in the endometrium and is a risk factor for endometrial cancers. Response rates in patients treated with tamoxifen ranged from 16-56% and the 5 year survival rate is approximately 26% in breast cancer patients (101, 102) (120).

Antiestrogens such as fulvestrant and GW5638 bind to ER and induce ER degradation. These drugs are in clinical trials and are being

considered as a third line of therapy where tamoxifen and aromatase inhibitors have failed (101, 102) (121).

Estrogen deprivation therapy is attained by removal of ovaries or through chemicals such as LH releasing hormone analogues and aromatase inhibitors. Aromatase inhibitors are of two types, irreversible steroidal inhibitors (exemestane) and reversible non-steroidal imidazole based inhibitors (anastrozole, letrozole). These drugs reduce aromatase activity and estradiol concentrations and are clinically used for treating breast cancer patients (101, 102) (121).

ii) **Progesterone targeted therapy:** Progestins or progestational agents are used in treatment of endometrial carcinoma and metastatic hormone dependent breast cancer; these cancers express progesterone receptor (PR). Hydroxyprogesterone caproate, medroxyprogesterone and megestrol acetate are used in progestin therapy (101, 102).

iii) **Adrenocorticosteroids:** These compounds are used for treatment of acute leukemia in children and malignant lymphoma in children and adults since they exhibit lympholytic effects and suppress mitosis in lymphocytes. Glucocorticoids such as dexamethasone and prednisone in combination with other chemotherapeutics are used (102).

b) **Tyrosine kinase inhibitors:** In many cancers, mutations or overexpression or autocrine/paracrine stimulation of tyrosine kinase pathway is observed. For example nonsmall cell lung cancers (NSCLC)

overexpress EGFR which plays an important role in tumor cell proliferation, angiogenesis and metastasis and EGFR expression correlates with poor prognosis and decreased patient survival. Monoclonal antibodies (cetuximab) against the extracellular ligand binding domain of EGFR and also small molecule inhibitors (gefitinib and erlotinib) of the intracellular tyrosine kinase domain of EGFR have been developed. Erlotinib exhibited antitumor activity and improved the quality of life of NSCLC patients (101) (122) (123).

CML patients exhibit a reciprocal chromosomal translocation between chromosome 9 and 22 which give rise to the bcr-abl gene (Philadelphia chromosome). Small molecule inhibitors (imatinib, dasatinib and nilotinib) of bcr-abl tyrosine kinase activity were developed that binds to the ATP pocket and inhibit downstream signal transduction have been developed. In phase II clinical trials with imatinib there was a 95% response rate in CML patients and imanitib also targets PDGFR and other tyrosine kinases. Nilotinib has higher binding affinity and selectivity of bcr-abl kinase than imatinib and dasatinib binds to the active and inactive confirmations of bcr-abl kinase domain. Dasatinib and nilotinib are in clinical trials for treatment of imanitib resistant CML (101) (124).

In metastatic breast cancer HER2 is overexpressed in approximately 25-30% of the patients. Patients treated with herceptin, a monoclonal antibody against the extracellular domain of HER2 protein

exhibit sustained improvement and increased-progression-free survival in clinical trials with herceptin plus doxorubicin or paclitaxel or epirubicin plus cyclophosphomide (101) (125).

c) **Angiogenic inhibitors:** The proliferation of new capillaries is known as angiogenesis and is usually short lived (1-2 weeks); however angiogenesis is upregulated in tumor cells. When the distance between a tumor cell and a blood vessel exceeds 1 mm, the supply of oxygen and nutrients diminish resulting in hypoxia and hypoxic cells that secrete VEGF which is essential for endothelial recruitment and angiogenesis. The monoclonal antibody against VEGF (Bevacizumab) in combination with chemotherapy is used as first line of therapy for treatment of metastatic colorectal cancer. The tyrosine kinase inhibitor, sunitinib also inhibits VEGFR and PDGFR and is used in clinical trials against renal cell carcinoma and AML (101) (13) (126) (127).

d) **Targeting cell cycle:** Cyclin dependent kinases (CDKs) are serine/threonine kinases that are involved in the control of cell cycle progression. Flavopiridol is a synthetic flavonoid derived from the alkaloid rohitukine that inhibits CDKs. Ispinesib inhibits kinesin spidle protein (KSP) which is essential for cell proliferation. P276-00 is also derived from rohitukine and selectively inhibits the CDK4-cyclin D1 and CDK1-cyclin B complexes. These drugs are all in clinical trials for treatment of various cancers (101) (128, 129).

e) **Targeting mTOR:** The mammalian target of rapamycin (mTOR) is a serine threonine kinase that regulates cell growth, proliferation, survival, protein translation and angiogenesis. Rapamycin, a macrolide antibiotic isolated form Streptomyces hygroscopicus, is an inhibitor of the mTOR pathway and temsirolimus is a rapamycin derivative which is in clinical trials for treatment of renal cell carcinoma (101) (130) (131).

f) **Targeting apoptosis:** Obatoclax is an inhibitor of Bcl2 and activates apoptosis and this drug is in clinical trials for treatment of myelodysplastic syndromes, NSCLC, follicular lymphoma, myelofibrosis and Hodgkin's lymphoma (101) (132).

Transcription factors as targets for cancer therapy

Peroxisome proliferator-activated receptor (PPAR) γ

Nuclear receptors (NR) are transcription factors that regulate transcriptional activation and repression in ligand-dependent or –independent manner. In contrast to the cell surface receptors such as EGFR, IGFR, which regulate gene transcription through complex intracellular signaling cascades, NRs regulate transcription by binding to specific DNA-sequences called hormone response elements (HRE). Nuclear receptors are classified into 7 subfamilies based on sequence comparision (Table 6) and they contain a N-terminal regulatory domain (activation function1-AF1), a DNA-binidng domain (DBD), a ligand binding domain (LBD) and a C-terminal domain (activation

function -2 -AF2). Even though NRs exhibit a conserved structural organization, they are functionally distinct and have been divided into two classes. Cytoplasmic NRs are present in cytoplasm as multiprotein complexes and after ligand binding they translocate into nucleus and bind to HREs as homo- or heterodimers. Some NRs reside in the nucleus in a complex with corepressors and ligand binding triggers corepressor dissociation and coactivator recruitement. Since NRs modulate several functions including cell proliferation, apoptosis, invasion and migration, they are important targets for cancer drug development (133). For example the estrogen receptor which is linked to breast cancer is antagonized by tamoxifen or raloxifene and PPARγ, a receptor important for the differentiation of cancer cells is another drug target (134).

PPARs are a family of ligand-activated transcription factors belonging to the nuclear hormone receptor family and related to retinoid, glucocorticoid and thyroid hormone receptors. There are three different PPAR subtypes, PPARα, PPARβ and PPARγ which are widely expressed and exert a wide range of effects on metabolism, cell proliferation and immune responses. PPARα is expressed predominantly in liver and skeletal muscles and plays an important role in fatty-acid catabolism. PPARβ is present in moderate levels in all human tissues exhibiting higher expression in placenta and large intestine. Very little is known about its functional role but PPARβ has been implicated as a regulator of glucose and lipid metabolism. PPARγ is the most frequently studied PPAR and is involved in glucose and lipid metabolism, modulates inflammation in immune

cells, induces adipose cell differentiation and exhibits antiproliferative activity (135-137).

Table 6. Classification of the NR superfamily into subfamilies according to sequence homology (133).

Subfamily	Thyroid hormone receptor like	Retinoid X receptor- like	Estrogen receptor- like
Nuclear receptors	Retinoic acid receptor (RAR) Peroxisome proliferator-activated receptor (PPAR) Thyroid hormone receptor (TR) Vitamin D receptor (VDR) Farnesoid X receptor (FXR) RAR-related orphan receptor (ROR) Constitutive androstane receptor (CAR)	Retinoid X receptor (RXR) Hepatocyte nuclear factor (HNF4)	Estrogen receptor (ER) Progesteron receptor (PR) Androgen receptor (AR) Glucocorticoid receptor (GR) Mineralocorticoid receptor (MR)
Orphan receptors	Liver X receptor (LXR) Rev-Erbα	Chicken ovalbumin upstream promoter transcription factor (COUP-TF) Testicular receptor 2 and 4	Estrogen-related receptor 1 and 2 (ERR)

Subfamily	Nerve growth factor IB-Like	Steroidogenic factor –like	Germ cell nuclear factor -like	Miscellaneous
Orphan receptors	Nerve growth factor IB-Like (NGFIB) Nucelar receptor related 1 (NURR1) Neuron-derived orphan receptor 1 (NOR1)	Steroidogenic factor 1 (SF-1) Liver receptor homlog 1 (LHR-1)	Germ cell nuclear facor (GCNF)	Small heterodimer partner (SHP) Dosage-sensitive sex reversal, adrenal hypoplasia crical region (DAX)

PPARs have similar structural organization that includes an A/B (ligand-independent activation domain), C (DNA binding domain-DBD), D (hinge domain) and E/F (ligand binding domain –LBD) domains (Figure 12). The N-terminal A/B domain is well conserved among PPARs and the α-helix fragment has ligand-independent activating function (AF-1) and the C-domain is the most conserved of all the functional domains and contains two zinc finger-like motifs that bind to a PPRE (PPAR response elements) in target gene promoters. Apart from the zinc finger motifs, PPAR binding is determined by amino-acid motifs present on C-domain and the C-domain also takes part in dimerization with other

NRs such as RXR. Domain D is less conserved and acts as a flexible hinge between C and E/F domains. It contains the nuclear localization signal which plays an important role in transporting the receptor from the cytoplasm to the nucleus. The D-domain takes part in dimerization and recognition of PPREs and also regulates binding of co-factors to the receptor. The LBD is the largest domain located on the C-terminus and contains a fragment (AF-2) that is responsible for ligand-dependent activation of PPARs (135-137).

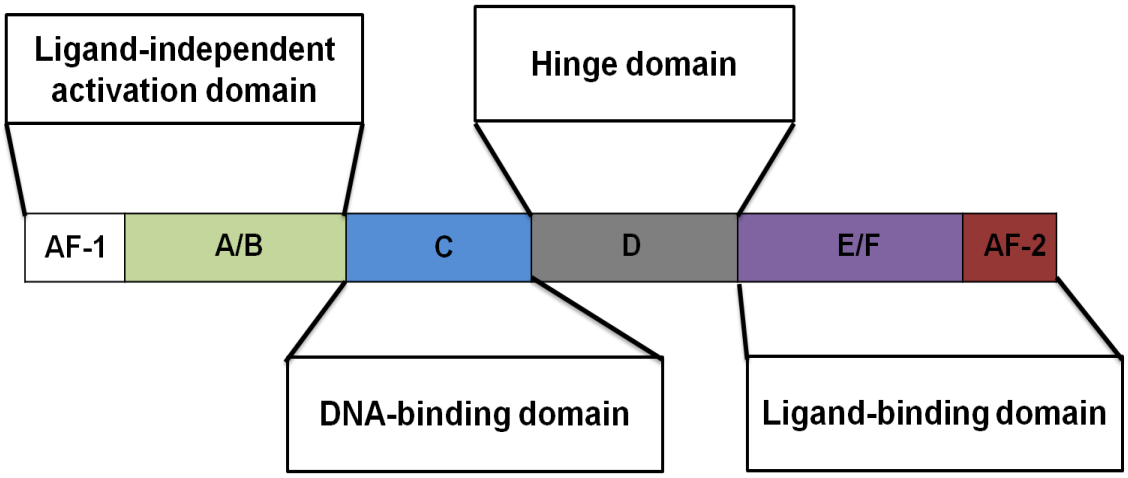

Figure 12. Schematic representation of genomic structure of PPARγ (137).

PPARγ exists as two isoforms (PPARγ1 and PPARγ2) encoded by multiple transcript variants and PPARγ1 is the major isoform expressed in humans. PPARγ undergoes heterodimerization with RXRα and is constitutively bound to PPREs within promoters of PPARγ-targeted genes. The PPRE motif contains a 13-nucleotide sequence, AGGTCA N AGGTCA (N-any nucleotide) that is composed of 2 hexanucleotides separated by one nucleotide (direct repeat-1, DR-1) which is specifically recognized by PPARγ/RXRα heterodimer.

PPARγ/RXRα heterodimer is activated by various ligands including natural and synthetic lipophilic ligands. The natural ligands include polyunsaturated fatty acids (linoleic and arachidonic acid), prostaglandin J2 (PGJ2) derivatives and oxidized fatty acids and the synthetic ligands include thiazolidinediones (TZD) such as rosiglitazone, pioglitazone and troglitazone (137).

PPARγ plays a role in adipocyte differentiation, glucose metabolism (used as antidiabetic drugs) and inflammation and PPARγ agonists such as TZDs are used for treatment of insulin-resistant type II diabetes. Recent studies have shown that PPARγ is widely expressed in many tumors and cancer cell lines. For example, PPARγ is expressed in colon, breast, lung, prostate, osteosarcomas, glioblastomas, AML, ALL, non-Hodgkin's lymphoma and myelodisplsatic syndrome clinical tumor samples (138) (139). Expression of PPARγ is associated with cellular differentiation which decreased or blocks cell growth; and PPARγ agonists (natural and synthetic) inhibit liposarcoma, breast cancer, colon cancer, bladder cancer, pancreatic cancer, prostate cancer and gastric cancer growth. For example PPARγ is expressed in pancreatic tumors and cell lines and treatment with the PPARγ agonist troglitazone results in G0/G1 arrest and increased p27 expression in Panc-1 cells (140). These growth inhibitory effects are highly variable and dependent on ligand structure and cell context and can be PPARγ-dependent or -independent. For example, PGJ2, troglitazone and GW7845 induce apoptosis, increase PPARγ-dependent activity and expression of CD36 (PPARγ-dependent) gene in breast cancer cell lines

and treatment with GW9662 (an irreversible PPARγ antagonists) decreased the PPARγ activity and expression of CD36 but did not block apoptosis (141). Troglitazone induced apoptosis in PPARγ-independent manner in colon cancer cell lines through activation of early growth respose-1 (Egr-1) and NSAID-activated gene (NAG-1) (142, 143).

Several new classes of PPARγ agonists have been developed and these also exhibit anticancer activities through PPARγ-dependent and independent mechanism in various cancer cell lines. For example, 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes containing *p*-trifluoromethyl (DIM-C-pPhCF_3), *p*-*t*-butyl (DIM-C-pPhtBu), and phenyl (DIM-C-pPhC_6H_5) substitutes inhibited bladder cancer growth in vitro and in vivo through induction of caveolin-1 and p21 and these effects were reversed with treatment of PPARγ antagonists GW9662 in a PPARγ-dependent manner. In contrast, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), CDDO-Me and CDDO-Im which are the synthetic triterpenoids derived from oleanolic acid, inhibited colon cancer growth through PPARγ-independent mechanisms (144). Methyl 2-cyano-3,11-dioxo-18beta-olean-1,12-dien-30-oate (β-CDODA-Me) which is a synthetic analogue derived from glycyrrhetinic acid, also induced PPARγ-dependent activity and inhibited colon cancer growth in a PPARγ-dependent manner, however, inhibition of prostate and pancreatic cancer growth was PPARγ-independent and involved induction of NAG-1 (145-147).

Specificity protein (Sp) transcription factors

The Sp/KLF (Kruppel-like factor) 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-(GGGCGG or GGCG) and GT-(GGTGTGGGG) boxes with different affinities due to differences in the amino acid substitutions in the zinc fingers (Figure 13). 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 (148). Sp1 and Sp7 (osterix) are on 12q13.13 (HOX C); Sp2 and Sp6 on 17q21.31/32 (HOX B); Sp3, Sp5 and Sp9 on 2q31.1 (HOX D) and Sp4 and Sp8 on 7q21.2 (HOX A). The Sp family is divided into Sp1-4 which contain N-terminal glutamine rich transactivation domains (TADs) A and B and Sp5-9 which lack the N-terminal glutamine rich TADs respectively. Sp1, 3 and 4 posses two glutamine rich TADs (A and B domain) and binds to GC-boxes whereas Sp2 has one TAD and does not bind to GC-boxes due to substitution of a critical histidine by a leucine residue in the zinc finger 1; Sp2 exhibits higher binding specificity to GT-boxes. Serine/threonine-rich sequences (C-domain) are located next to the A and B domains and are targets for post-translational modifications. All the Sp family proteins contain a buttonhead box N-terminal to the zinc finger domain and a conserved stretch of 11 amino acid residues which contribute to their transactivation potential; deletion of this region

in Sp1 results in decreased activity of Sp1 (149). The Sp box (SPLALLAATCSR/KI) is located at the N-terminus of Sp proteins and contains a endoproteolytic cleavage site that plays an important role in proteolysis of Sp proteins (figure 14) (150, 151) (152).

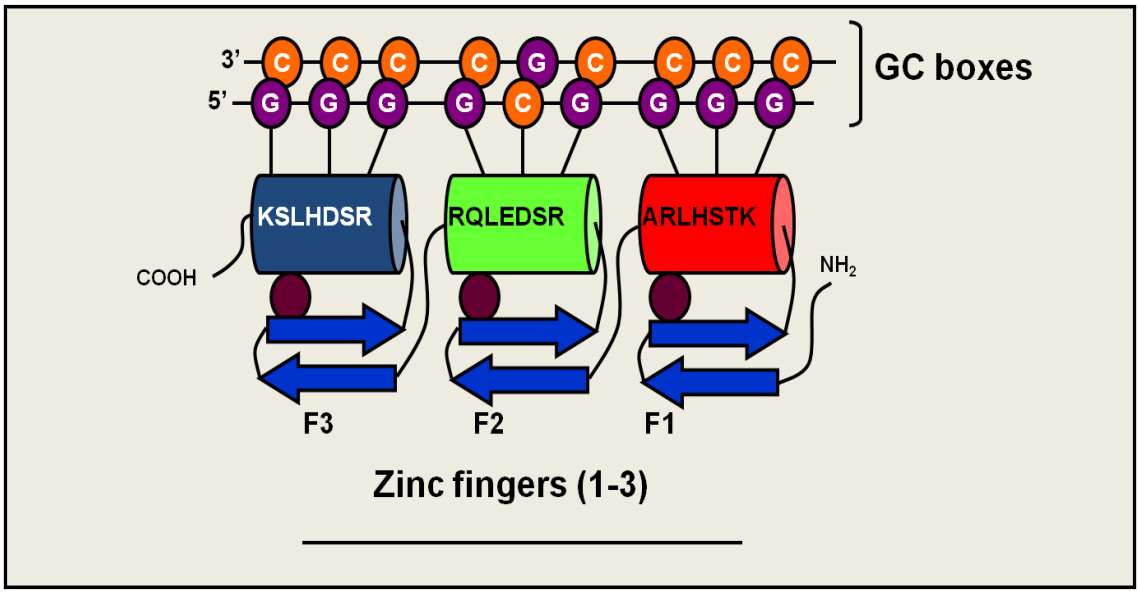

Figure 13. The zinc fingers of Sp/XKLF transcription factors (150).

Sp1 was the first transcription factor identified and binds GC-boxes and also binds to CT and GT-boxes with lower affinity. Sp1 and Sp3 are expressed ubiquitously whereas Sp4 exhibits tissue specific (brain, testis and epithelial tissues) expression. Sp1 and Sp3 exhibit similar DNA-binding affinities and compete for binding to the same GC-, GT- and CT-boxes and the Sp1 to Sp3 ratio may play an important role in regulation of some genes. On some promoters Sp3 cooperates with Sp1 whereas on other promoters Sp3 competes and represses Sp1-mediated transactivation. For example, all 3 Sp proteins

cooperatively regulate VEGF expression in Panc-1 cells (153); however, in human umbilical vein endothelial cells hypoxia enhances Sp1 proteins levels but not Sp3 levels. In general, increased Sp1/Sp3 ratios have been correlated with the increased gene expression and many of these genes are activated by Sp1 and repressed by Sp3 (150, 151).

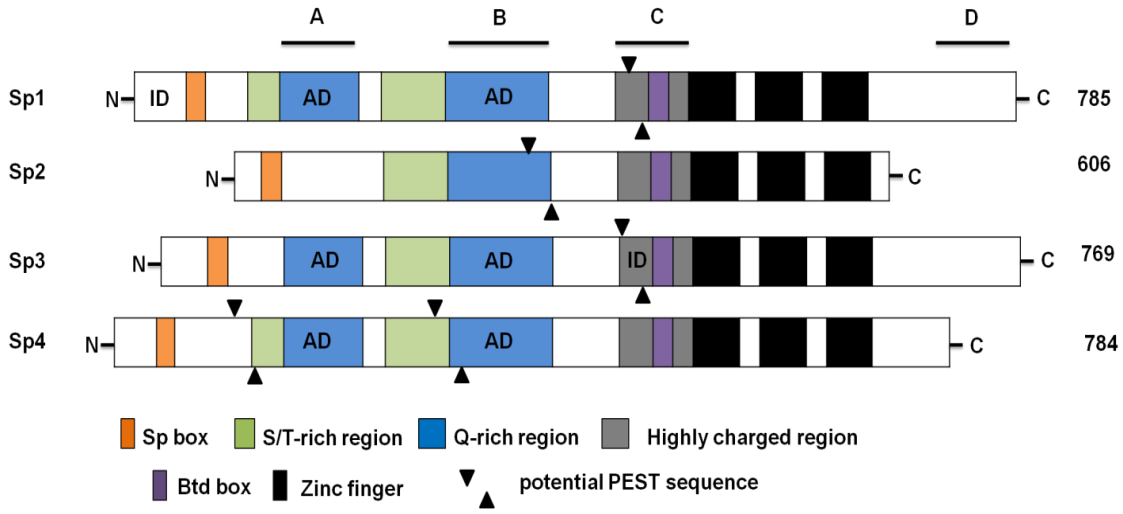

Figure 14. Structural motifs in Sp1/2/3/4 proteins (150).

Sp1-knockout embryos are severely retarded in development and die around day 11 of gestation; however Sp1 null embryos express Sp1 target genes at normal levels and only thymidine kinase and the methyl-CpG binding protein 2 (MeCP2) gene expression was decreased. MeCP2 gene is associated with maintainance of differentiated cells suggesting a role of Sp1 in regulation of differentiation (154). This demonstrates that other Sp-family members may compensate for loss of Sp1 during early embryogenesis. Sp3 loss in mouse embryos results in growth retardation and mice die at birth due to respiratory

failure (155). Approximately 2/3 of Sp4 null mice die within 4 weeks after birth and the mice which survive exhibit growth retardation. Male Sp4 null mice do not breed and females exhibit a pronounced delay in sexual maturation (150) (156).

Sp proteins are involved in many signal transduction pathways linked to cancer and carcinogen or stable transfection of H-Ras^{V12} induced transformation of human fibroblast cells results in a 8-18 fold incresase in Sp1. The malignant cells formed tumors in athymic mice (157). Also Sp1 mRNA and Sp1 and Sp3 DNA binding activity is increased in skin tumors compared to papillomas and the increased expression of these proteins correlates with increased tumor progression. The promoters of many pro- and anti-apoptotic genes such as Bcl-2, Bak, Bax, survivin, Fas and Fas ligand, TGFβ and its receptors, TNFα and TRAIL contain GC-rich Sp binding sites and Sp proteins also regulate expression of angiogenic genes, VEGFR1, VEGFR2 and VEGF (153, 158, 159). GC-rich sites on the wild type p53 gene are also involved in upregulation of growth inhibitory genes such as p21 and caveolin and functional GC-boxes are present on the Rb, c-Myc, c-jun, c-fos, E2F1, NFκB and Egr-1 gene promoters (Table 7). Sp1 overexpression in patients with pancreatic ductal adenocarcinoma is associated with metastasis and the 5-year survival rate was 19% in patients with Sp1 overexpression compared with 55% in patients without Sp1 overexpression (160) (161). Several studies in this laboratory have reported that Sp1, Sp3 and Sp4 are highly expressed in pancreatic, breast, bladder and colon cancer cell lines (153, 158, 159, 162-164) (165) and tumors derived from

these cells in xenograft models (162, 163) (165). Even though the knockout of Sp proteins in mice is embryolethal, the expression of these proteins is significantly decreased in rodent and human tissues with increasing age (166, 167) and studies in this laboratory have shown that Sp1, Sp3 and Sp4 expression is low in most organs when compared to tumors (168).

Table 7. Functions of Sp1 target genes (152).

Self-sufficiency in growth signals	Insensitivity to anti-growth signals	Evading apoptosis	Limitless replicative potential	Sustained angiogenesis	Tissue invasion & metastasis
Receptor tyrosine kinases and their growth factors PDGFRα PDGFRβ PDGFα PDGFβ TGFα EGFR c-Met **Non-receptor tyrosine kinases** c-Src c-Myc	**Transcription factors** p53 **CDKIs** p15 p16 p21 p27 **Cyclins** Cyclin D1 Cyclin D2 Cyclin D3 Cyclin E Cdc25c	**Cell survival** IGF-II IGF-1R XIAP survivin MCL-1 **Apoptosis** FasL PUMA TNF-α DR5	hTERT	**Angiogenesis** VEGF VEGFR1 VEGFR2 FGFR1 FGFR3 **Anti-angiogenesis** TIMP-1	**Metastasis suppressor** KiSS-1 SSeCKS **Cell adhesion** COL1A2 COL2A1 VCAM-1 ICAM-1 **Invasion & metastasis** MMP-2 MMP-9 vimentin

Several compounds decrease Sp protein expression and this contributes to their inhibition of tumor growth. COX-2 inhibitors/NSAIDS such as celecoxib, nimesulide or NS-398 decrease VEGF expression through degradation of Sp1 and Sp4 but not Sp3 in colon cancer cell lines. In contrast, the NSAID tolfenamic acid inhibited VEGF expression in pancreatic cancer cells and this was associated with decreased Sp1, Sp3 and Sp4 expression (162, 169). Betulinic acid, a pentacyclic triterpenoid isolated from birch bark induces proteosome-

dependent degradation of all 3 Sp proteins in prostate cancer cells and in tumors (xenografts) (168). Curcumin, a polyphenolic anticancer agent from rhizome of Curcuma longa also induced proteosome-dependent degradation of Sp1/3/4 proteins in bladder cancer cells; however, β-CDODA-Me decreased expression of Sp proteins in a proteosome independent manner in colon cancer cells and downregulation of Sp proteins was due to decreased expression of miR-27a which in turn increased expression of ZBTB10 (an Sp repressor) (163) (170).

The proposed studies in this thesis research are focused on curcumin, arsenic trioxide, CDDO and celastrol and their mechanism of action as anticancer drugs in pancreatic and bladder cancer cells and in mouse tumor xenograft or orthotopic models. The research will also investigate the effects of these compounds on Sp1, Sp3 and Sp4 expression and the role of these proteins in mediating genes that are important for cancer cell proliferation (cyclin D1), survivial (survivin, NFκB) and angiogenesis (VEGF, VEGFR1, VEGFR2).

II. INDUCTION OF APOPTOSIS AND NONSTEROIDAL ANTIINFLAMMATORY DRUG-ACTIVATED GENE 1 IN PANCREATIC CANCER CELLS BY A GLYCYRRHETINIC ACID DERIVATIVE*

Methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (CDODA-Me) is a synthetic triterpenoid derived from glycyrrhetinic acid, a bioactive phytochemical in licorice, CDODA-Me inhibits growth of Panc1 and Panc28 pancreatic cancer cell lines and activates peroxisome proliferator-activated receptor γ (PPARγ)-dependent transactivation in these cells. CDODA-Me also induced p21 and p27 protein expression and downregulates cyclin D1; however, these responses were receptor-independent. CDODA-Me induced apoptosis in Panc1 and Panc28 cells, and this was accompanied by receptor-independent induction of the proapoptotic proteins early growth response-1 (Egr-1), nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1), and activating transcription factor-3 (ATF3). Induction of NAG-1 and Egr-1 by CDODA-Me was dependent on activation of phosphatidylinositol-3-kinase (PI3-K) and/or p42 and p38 mitogen-activated protein kinase (MAPK) pathways but there were differences between Panc28 and Panc1 cells. Induction of NAG-1 in Panc28 cells was p38-MAPK- and PI3-K-dependent but Egr-1-independent, whereas

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* Reprinted with permission from “Induction of Apoptosis and Nonsteroidal Antiinflammatory Drug-Activated Gene 1 in Pancreatic Cancer Cells by A Glycyrrhetinic Acid Derivative” by Jutooru I, Chadalapaka G, Chintharlapalli S, Papineni S and Safe S. Mol. Carcinogenesis. 48(8):692-702, 2009.

induction in Panc1 cells was associated with activation of p38-MAPK, PI3-K and p42-MAPK and was only partially Egr-1-dependent.

Introduction

Glycyrrhetinic acid (GA) is a pentacyclic triterpenoid acid that is found as a conjugate (glycyrrhizin) in licorice extracts (171) (172). GA is one of the medicinally active compounds of licorice and exhibits multiple activities which include the enhancement of corticosterone levels which contributes to decreased body fat index in human studies with GA (173) (174). In addition, several derivatives of GA are also biologically active, and carbenoxolone, a 3-hemisuccinate of GA, has been used for the treatment of ulcers and arthritis (171) (172) (175). Previous studies with closely related triterpenoid acids, ursolic acid and oleanolic acid, have demonstrated that introduction of a 2-cyano-1-en-3-one function in their A ring greatly enhances their anti-inflammatory activity in a mouse macrophage model (176, 177), and one of these compounds, 2-cyano-3,12-dioxo-18β-olean-1,9(11)-dien-28-oic acid (CDDO), its methyl ester (CDDO-Me), and imidazole derivatives exhibit antitumorigenic activity (178, 179). We have synthesized 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oic acid (CDODA) and its methyl ester (CDODA-Me) from GA and have demonstrated that these compounds are highly cytotoxic in colon, prostate, bladder and pancreatic cancer cells (145, 147, 180). The most active member of these GA derivatives is CDODA-Me (18β isomer) which activates peroxisome proliferator-activator receptor γ (PPARγ) and induces both

receptor-dependent and -independent responses in colon and prostate cancer cells. For example, in colon cancer cells, β-CDODA-Me induced receptor-dependent caveolin-1 expression in HT-29 and HCT-15 but not SW480 colon cancer cells, whereas α-CDODA-Me induced receptor-mediated caveolin-1 protein levels in all three cell lines (145). In contrast, the pattern of receptor-dependent induction of Krüppel-like factor-4 (KLF-4) in HT-29, HCT-15 and SW480 colon cancer cells was similar for both β- and α-CDODA-Me. β-CDODA-Me induced apoptosis and several proapoptotic proteins in LNCaP prostate cancer cells and these included nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) and activating transcription factor 3 (ATF3), and activation of these pathways was not inhibited by PPARγ antagonists (147).

In this study, we demonstrate that β-CDODA-Me induced PPARγ-dependent transactivation in Panc28 and Panc1 pancreatic cancer cells and β-CDODA-Me induced the characteristic PPARγ-dependent differentiation of 3T3-L preadipocytes. β-CDODA-Me induced expression of several growth inhibitory and proapoptotic proteins including p21, p27, NAG-1 and ATF3 and downregulated cyclin D1 proteins, and effects on these growth inhibitory responses were receptor-independent. β-CDODA-Me also activated multiple kinases in pancreatic cancer cells including p38 and p42 mitogen-activated protein kinase (MAPK), phosphotidylinositol-3-kinase (PI3-K), and c-jun N-terminal kinase (JNK) pathways, and the role of these kinases in the induction of NAG-1 and apoptosis was cell context-dependent.

Materials and methods

Cell lines

The Panc28 cell line was a generous gift from Paul Chiao (University of Texas M.D. Anderson Cancer Center, Houston, TX) and Panc1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Antibodies and reagents

Both pancreatic cancer cell lines were maintained in DMEM-F12 supplemented with 5% FBS, 0.22% sodium bicarbonate, and 10 ml/L of 100X antibiotic/antimycotic cocktail solution (Sigma Aldrich Co., St. Louis, MO). Cells were grown in 150 cm^2 culture plates in an air/CO_2 (95:5) atmosphere at 37°C. Cyclin D1, p21, p27, ATF3, p-c-jun, c-jun, p-Akt 1/2/3, Akt 1/2, p-Erk, Erk and p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP, Egr-1 and p-p38 antibody were purchased from Cell Signaling Technology (Danvers, MA) and NAG-1 antibody was purchased from Upstate USA, Inc. (Lake Placid, NY). Monoclonal □-actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase substrate for western blot analysis was obtained from NEN Life Science Products (Boston, MA). Proteinase K was obtained from Sigma Aldrich. Lipofectamine was purchased from Invitrogen (Carlsbad, CA). β-Galactosidase reagent was obtained from Tropix (Bedford, MA). LY294002, PD98059 and SB203580 were purchased from EMD Chemicals, Inc (Gibbstown, NJ).

Cell proliferation assay

Pancreatic cancer cells (3 x 10^4 per well) were plated in 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or CDODA-Me were added. Fresh medium and test compounds were added every 48 hr, and cells were then trypsinized and counted at the indicated times using a Coulter Z1 particle counter. Each experiment was done in triplicate and results are expressed as means $\pm$ SE for each treatment group.

Transfection and luciferase assay

The pancreatic cancer cells (1 x 10^5 per well) were plated in 12-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 24 hr, various amounts of DNA (i.e., 0.4 µg pGal4, 0.04 µg β-galactosidase, and 0.04 µg PPARγ-GAL4 or 0.4 µg of PPRE3-Luc) were transfected using Lipofectamine reagent according to the manufacturer's protocol. Five hours post-transfection, the transfection mix was replaced with complete medium containing either vehicle (DMSO) or the indicated compound in DMSO. After 22 hr, cells were then lysed with 100 µL of 1x reporter lysis buffer, and cell extracts (30 µL) were used for luciferase and β-galactosidase assays. A Lumicount luminometer was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

Western blots

Pancreatic cancer cells were initially seeded in DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS and, after 24 hr, cells were treated with either vehicle (DMSO) or the indicated compounds. Cells were collected using high-salt buffer [50 mmol/L HEPES, 0.5 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, and 1% Triton-X-100] and 10 □L/mL of Protease Inhibitor Cocktail. Protein lysates were incubated for 3 min at 100°C before electrophoresis, and then separated on 10% SDS-PAGE 120 V for 3 to 4 hr. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by wet electroblotting in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol for 1.5 hr at 180 mA. Membranes were blocked for 30 min with 5% TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, and 5% nonfat dry milk] and incubated in fresh 5% TBST-Blotto with 1:500 primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody (1:5000) in 5% TBST-Blotto for 2 hr by gentle shaking. The membrane was washed with TBST for 10 min, incubated with 6 mL of chemiluminescence substrate for 1 min, and exposed to Kodak X-OMAT AR autoradiography film.

DNA fragmentation

The isolation of DNA was performed according to the protocol 6.2 "Rapid Isolation of Mammalian DNA". Extracted DNA was run on 0.9% agarose gel and

stained with 0.5 µg/mL ethdium bromide and the fragmented DNA was visualized using a Transilluminator on an ultraviolet light.

Fluorescence-activated cell-sorting assays (FACS)

Both Panc1 and Panc28 pancreatic cancer cells were treated with either the vehicle (DMSO) or the indicated compounds for 48 hr. Cells were trypsinized, centrifuged and re-suspended in staining solution containing 50 mg/ml propidium iodide, 4 mM sodium citrate, 30 units/ml RNase and 0.1% Triton X-100. After incubation at 37°C for 10 min, sodium chloride was added to give a final concentration of 0.15 M. Cells were analysed on a FACS Calibur flow cytometer using CellQuest acquisition software (Becton Dickinson Immunocytometry Systems, Franklin Lakes, NJ). PI fluorescence was collected through a 585/42 nm band pass filter, and list mode data were acquired on a minimum of 20,000 single cells defined by a dot plot of PI width versus PI area. Data analysis was performed in Modfit LT using PI width versus PI to exclude cell aggregates.

Differentiation and oil red O staining

3T3-L1 preadipocytes were cultured on Lab-Tek Chamber 4-well Slide with DMEM-F12 and 10% FBS at 5% CO_2. At 2 days postconfluence, fresh media supplemented with 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (1 mM), insulin (1.7 mM), and DMSO or CDODA-Me (0.25 µM) was added. After 48 hr, fresh media was added and cells were treated with

DMSO and CDODA-Me for 5 days. Cells without any treatment for the entire 7 days were used as controls. Cells were then fixed with 10% formalin, washed with 60% isopropanol and stained with filtered 60% Oil Red O in deionized water. After staining, cells were washed with water and photographed to visualize the staining.

Results

Effects on cell proliferation and apoptosis

Previous studies in colon and prostate cancer cells show that the cytotoxicity of a series of glycyrrhetinic acid derivatives is dependent on the introduction of a 2-cyano group and a 1-en-3-one functionality in the A ring (145, 147, 180). Figure 15 compares the cytotoxicities of DODA-Me, which contain the A-ring 1-en-3-one function, to CDODA-Me, which contains both the 1-en-3-one and 2-cyano substituents. At concentrations of 1 - 15 μM, DODA-Me had minimal effects on Panc1 and Panc28 pancreatic cancer cell growth (Figures 15A and 15B) with growth inhibitory IC_{50} values > 15 μM. In contrast, CDODA-Me was a potent inhibitor of pancreatic cancer cell growth. The IC_{50} values for CDODA-Me in Panc1 and Panc28 cells were 1.21 and 1.79 μM, respectively. We also investigated the cytotoxicities of the free acids (DODA and CDODA); IC_{50} values for DODA were > 15 μM and the values for CDODA were 7.31 and 3.8 μM in Panc28 and Panc1 cells, respectively (data not shown). Thus, the

methyl ester was the most cytotoxic of the 2-cyano derivatives and was used as a model compound for subsequent studies in pancreatic cancer cells.

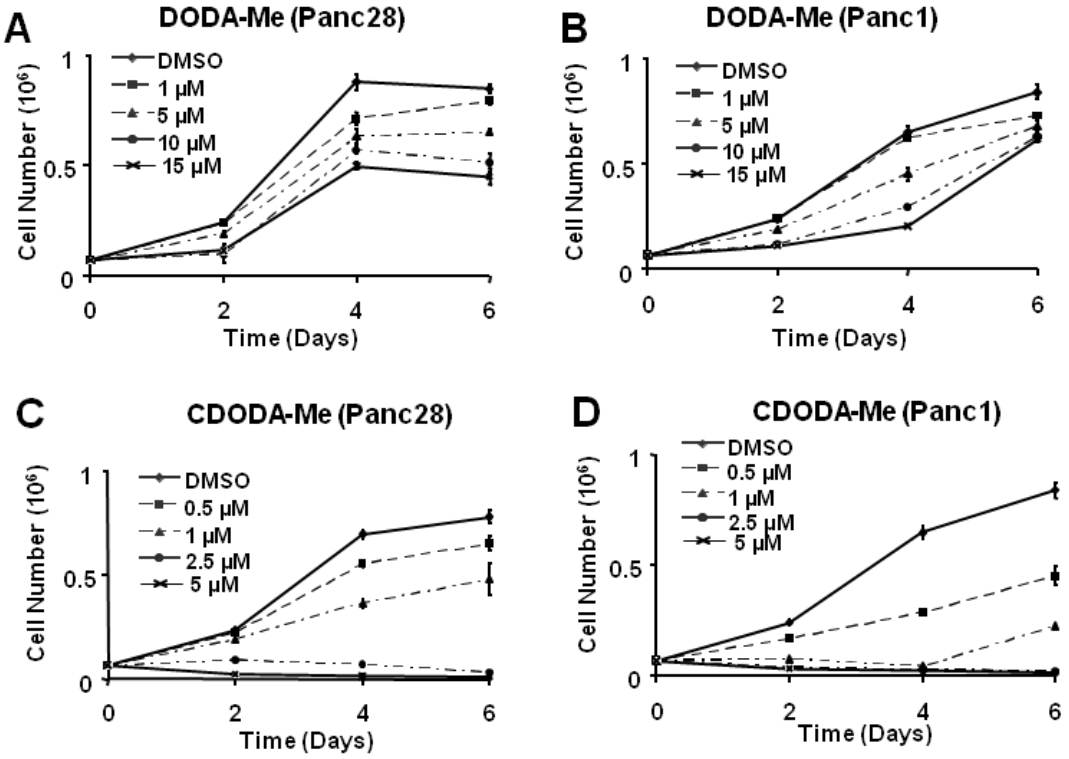

Figure 15. Cytotoxicity of glycyrrhetinic acid derivatives in pancreatic cancer cells.

Effects of DODA-Me on Panc28 (A) and Panc1 (B) cell proliferation. Cells were treated with different concentrations of DODA-Me for up to 6 d and cell numbers were determined as described in the Materials and Methods. Growth inhibitory IC_{50} values were > 15 μM in both cell lines. Effects of CDODA-Me on Panc28 (C) and Panc1 (D) cell proliferation. Cells were treated with different concentrations of CDODA-Me for up to 6 d and cell numbers were

determined as described in the Materials and Methods. Growth inhibitory IC_{50} values were > 1.79 and 1.21 μM in Panc28 and Panc1 cell lines, respectively. Results are expressed as means ± SE for 3 replicate determinations for each treatment group. Similar studies were carried out for the corresponding free acids (DODA and CDODA) (data not shown) which exhibited lower cytotoxicity in both cell lines as previously described for these compounds in colon and pancreatic cancer cells (145, 147).

Figures 16A and 16B summarize the concentration-dependent effects of CDODA-Me on percent distribution of Panc28 and Panc1 cells in G_0/G_1, S and G_2/M phases of the cell cycle after treatment for 48 hr. In Panc28 cells, CDODA-Me decreased the percentage of cells in G_0/G_1 and increased the percentage in S and G_2/M phases, whereas in Panc1 cells, CDODA-Me induced cell cycle arrest in G_0/G_1 and inhibited G_0/G_1 to S phase progression. Thus, the effects of CDODA-Me on the cell cycle are cell context-dependent in the two pancreatic cancer cell lines. Treatment of Panc28 and Panc1 cells with CDODA-Me (5.0 and 7.5 μM) for 24 hr induced a DNA ladder indicative of apoptosis (Figure 16), and similar effects were observed in previous studies with this compound in prostate cancer cells (147).

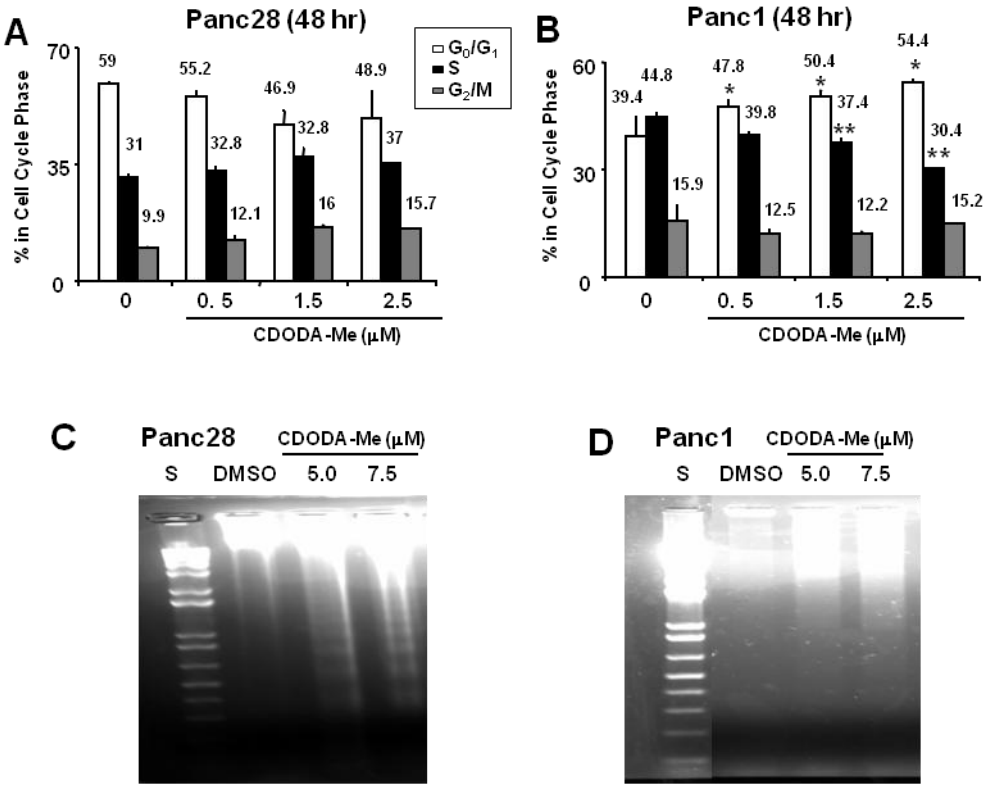

Figure 16. Effects of CDODA-Me on cell cycle progression and apoptosis. Cell cycle progression in Panc28 (A) and Panc1 (B) cells.

Cells were treated with DMSO or different concentrations of CDODA-Me for 48 hr, and % distribution of cells in G_0/G_1, S and G_2/M phases were determined by FACS analysis as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group, and significant ($p < 0.05$) CDODA-Me-induced increases (*) or decreases (**) in percentages of a phase compared to the solvent (DMSO) control are indicated. Induction of DNA laddering in Panc28 (C) and Panc1 (D) cells. Cells were treated with DMSO or CDODA-Me for 24 hr and DNA

laddering was determined as described in the Materials and Methods. The standard (S) of 1 kb was used as a marker for DNA laddering.

Activation of PPARγ by CDODA-Me

CDODA-Me activates the nuclear receptor PPARγ in colon and prostate cancer cells (145, 147). Results in Figure 17A show that CDODA-Me induced transactivation in Panc28 and Panc1 cells transfected with a GAL4-PPARγ chimera and $pGAL4_5$ which contains 5 tandem GAL4 response elements linked to the luciferase gene. Similar results were obtained in the pancreatic cancer cells transfected with a $PPRE_3$-luc construct which relies on activation of endogenous PPARγ and RXR (Figure 17B). Using this same construct, we also showed that induction of luciferase activity in Panc28 and Panc1 (Figure 17C) cells by CDODA-Me was inhibited after cotreatment with the PPARγ antagonist T007. These results confirm that CDODA-Me exhibits PPARγ agonist activity in pancreatic cancer cells, and the PPARγ activity of CDODA-Me was confirmed using 3T3-L1 preadipocytes in which treatment with concentrations as low as 0.25 μM induced differentiation of these cells and formation of highly characteristic oil-Red-O droplets (Figure 17D). In contrast, these droplets were not observed in cells treated with solvent control (DMSO); however, these cells were also weakly stained due to endogenous triglycerides.

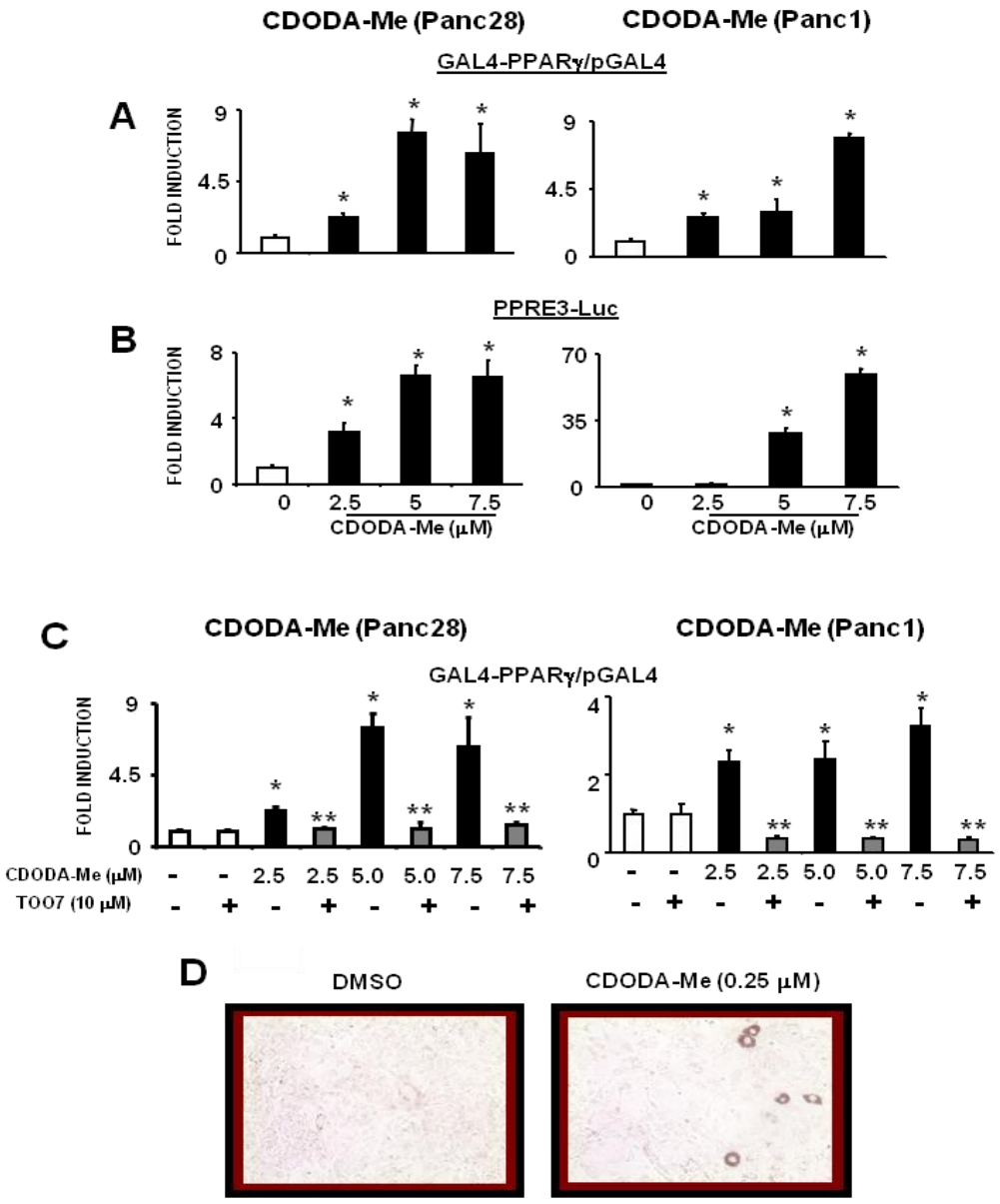

Figure 17. Activation of PPARγ by CDODA-Me. Transfection with PPARγ-GAL4/pGAL4 (A) and PPRE-luc (B).

Panc1 or Panc28 cells were transfected with PPARγ-GAL4/pGAL4 or PPRE-luc constructs, treated with DMSO or different concentrations of CDODA-Me, and luciferase activity was determined as described in the Materials and

Methods. Inhibition of transactivation in Panc28 or Panc1 cells (C) by T007. Cells were transfected with PPARγ-GAL4/pGAL4, treated with DMSO, CDODA-Me, T007 or their combination, and luciferase activity was determined as described in the Materials and Methods. Results in (A) - (C) are expressed as means ± SE for 3 replicate determinations for each treatment group, and significant ($p < 0.05$) induction by CDODA-Me (*) or inhibition after cotreatment with T007 (**) is indicated. (D) CDODA-Me induces differentiation. 3T3-L1 preadipocytes were treated with DMSO and CDODA-Me, and differentiation was detected by oil-red-O staining as described in the Materials and Methods.

Modulation of cell cycle proteins

Several studies report the growth inhibitory and proapoptotic effects of PPARγ agonists in pancreatic cancer cells (140, 181-190). The effects of CDODA-Me on expression of the key cell cycle genes p21, p27 and cyclin D1 that are often affected by different classes of PPARγ agonists that inhibit growth of pancreatic cancer cells were also investigated in the Panc28 and Panc1 cell lines. In Panc28 cells, treatment with 0.5 - 7.5 μM CDODA-Me induced expression of both p21 and p27 but downregulated cyclin D1 protein (Figure 18A). p21 and cyclin D1 were induced and repressed, respectively, in Panc1 cells treated with CDODA-Me, whereas p27 protein was unchanged (Figure 18B). This may be due, in part, to the high constitutive levels of p27 in Panc1 cells. There were also response-specific differences in the sensitivity of Panc1

and Panc28 cells to CDODA-Me. Induction of p21 in Panc1 and Panc28 cells was observed after treatment with 2.5 and 5.0 μM CDODA-Me, respectively, whereas 7.5 and 1.0 μM CDODA-Me were required for cyclin D1 downregulation, respectively.

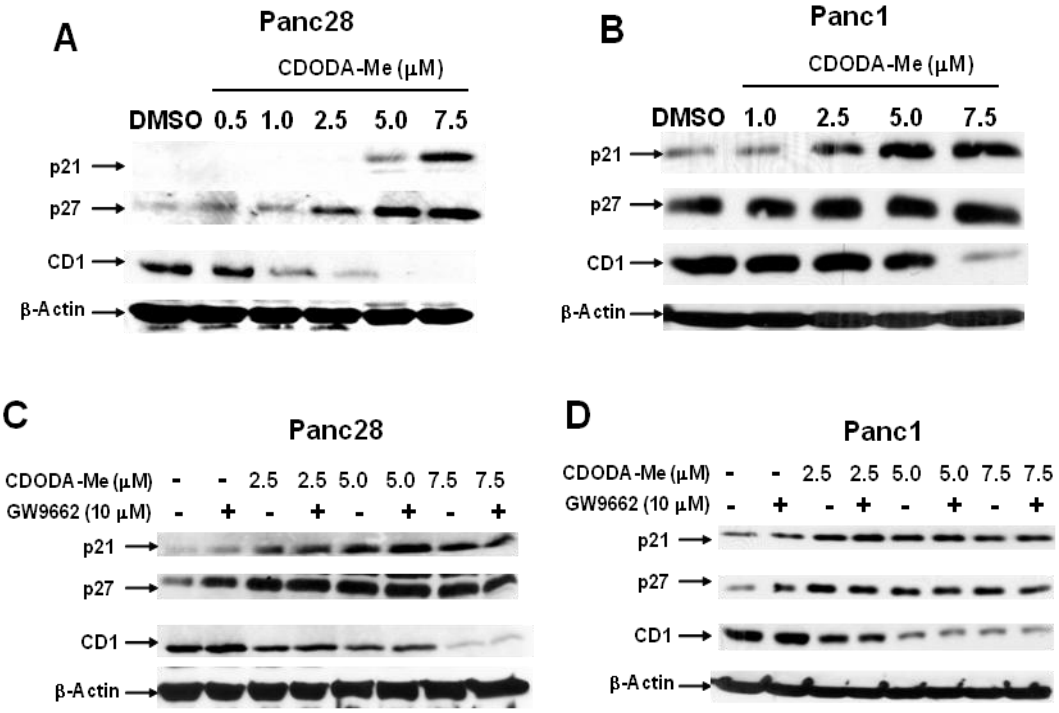

Figure 18. Effects of CDODA-Me on cell cycle proteins.

Induction of p21, p27, cyclin D1 in Panc28 (A) or Panc1 (B) cells by CDODA-Me, and the effects of PPARγ antagonists on induction of these proteins in Panc28 (C) and Panc1 (D) cells. Cells were treated for 24 hr with DMSO, different concentrations of CDODA-Me alone, or in combination with

GW9662, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. β-Actin served as a protein loading control.

Thus, CDODA-Me differentially modulated expression of cell cycle genes in Panc1 and Panc28 cells. These effects correlated with the G_0/G_1 arrest in Panc1 but not in Panc28 cells (Figures 16A and 16B) and the reason for these cell context-dependent differences are currently being investigated. Previous studies on PPARγ-active 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methane (C-DIM) compounds show that induction of p21 is receptor-dependent in Panc28 cells and inhibited by the PPARγ antagonist GW9662 (181). Therefore, we examined the effects of the PPARγ antagonist GW9662 on CDODA-Me-induced p21, p27 and cyclin D1 in Panc28 (Figure 18C) and Panc1 (Figure 18D) cells. The results show that cotreatment of these cells with GW9662 plus CDODA-Me did not modulate the activity of the latter compound and similar results were obtained for other PPARγ antagonists (data not shown) and GW9662 alone did not affect cyclin D1, p27 or p21 protein expression in these cells (Figure 18). Previous studies showed that CDODA-Me induces receptor-dependent activation of caveolin-1 and KLF-4 in colon cancer cells (145); however, in Panc1 and Panc28 cells, CDODA-Me did not induce expression of either protein (data not shown) demonstrating that CDODA-Me is a selective PPARγ modulator in pancreatic cancer cells.

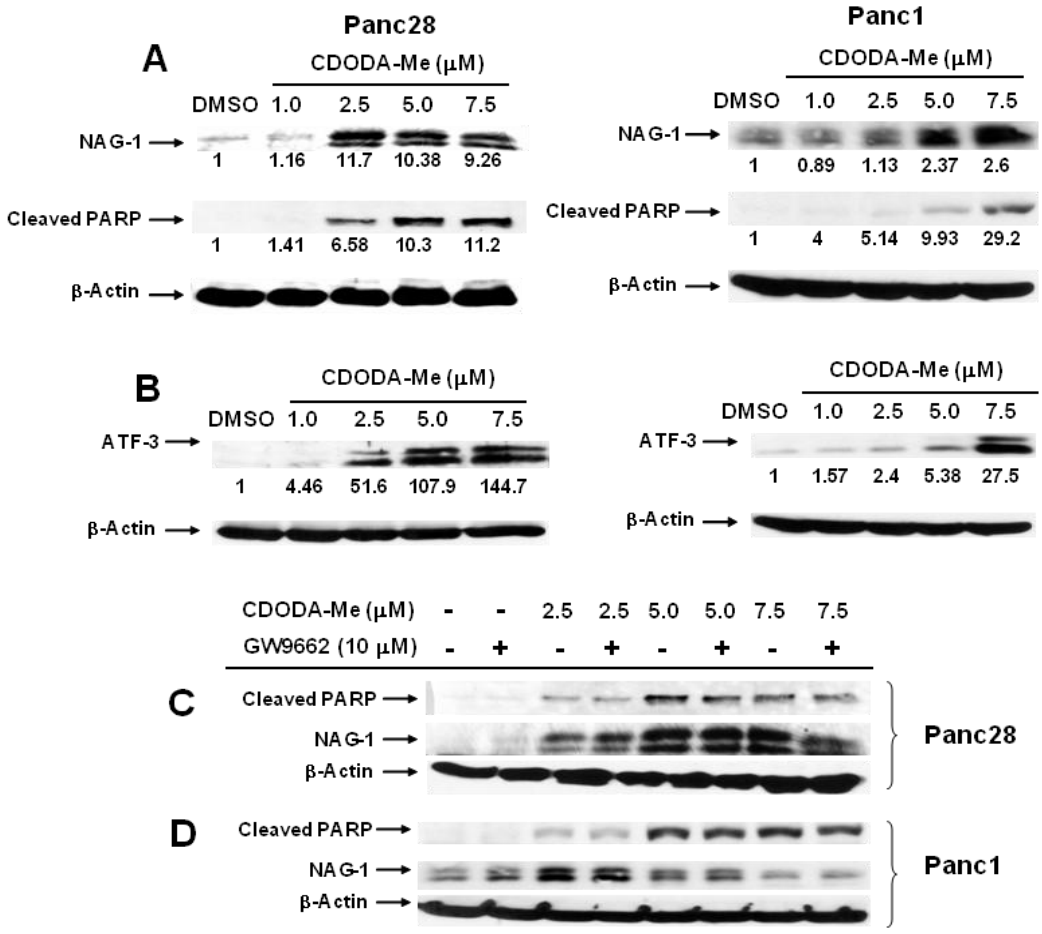

Figure 19. Induction of apoptosis and proapoptotic NAG-1 and ATF3 proteins.

Concentration-dependent induction of NAG-1/PARP cleavage (A) and ATF3 (B) by CDODA-Me. Panc28 and Panc1 cells were treated with DMSO and CDODA-Me for 24 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. Band intensities in (A) and (B) were quantitated relative to β-actin from 3 replicate determinations, and DMSO values were set at 1.0. Significant ($p < 0.05$) induction of NAG-1, PARP cleavage and

ATF3 in Panc28 cells were observed at concentrations of CDODA-Me ≥ 2.5, 2.5 and 1.0 μM, respectively. The induction responses in Panc1 cells were observed at concentrations of ≥ 5.0, 2.5 and 2.5 μM, respectively. Effects of GW9662 on induction of NAG-1/PARP cleavage in Panc28 (C) and Panc1 (D) cells. Cells were treated and analyzed as described above in (A) and (B). β-Actin served as a protein loading control.

Induction of NAG-1 and other responses

CDODA-Me induced DNA fragmentation in pancreatic cancer cells (Figures 16C and 16D) and, in prostate cancer cells, CDODA-Me also induced apoptosis and the proapoptotic proteins NAG-1, ATF3 and early growth response-1 (Egr-1) (147) and the effects of CDODA-Me on these proapoptotic responses was also investigated in pancreatic cancer cells. Figures 19A and 19B show that CDODA-Me induced NAG-1 and ATF3 in Panc28 and Panc1 cells, respectively. Significant induction of these proteins was observed at 2.5 or 5.0 μM concentrations of CDODA-Me, and Panc28 cells were more sensitive than Panc1 cells for induction of these proteins (Figure 19). NAG-1 is induced by a variety of anticancer agents in colon and other cancer cell lines (142, 143, 191-200), and this includes induction by PPARγ agonists such as PGJ2, troglitazone and PPARγ-active C-DIMs (142, 143, 200); however, among these compounds, PPARγ-dependent induction is only observed for PGJ2 in colon cancer cells (143). Results in Figures 19C and 19D show that different

concentrations of CDODA-Me induced both NAG-1 and caspase-dependent PARP cleavage and cotreatment with the PPARγ antagonist GW9662 did not affect the magnitude of these responses, suggesting induction of these responses was also PPARγ-independent. Similar results were observed for ATF3 (data not shown).

NAG-1 induction is complex and dependent on the chemical agent and cell context (142, 143, 147, 192-200), and the effects of CDODA-Me on induction of the protein in Panc1 and Panc28 cells was further investigated. In a time course study (Figure 20A), NAG-1 protein levels were increased in Panc28 cells after treatment for 8, 16 and 24 hr, whereas induction in Panc1 cells was observed at later time points (18 - 24 hr). ATF3 was induced 4 - 6 hr after treatment in both cell lines. Previous studies have shown that prior induction of Egr-1 is involved in enhanced expression of NAG-1 in some cell lines (142, 143, 147, 192, 193, 199, 200), and Figure 20A illustrates that Egr-1 protein was increased in Panc28 cells within 2 hr after treatment with CDODA-Me and was rapidly induced in Panc1 cells within 1 hr and declined thereafter.

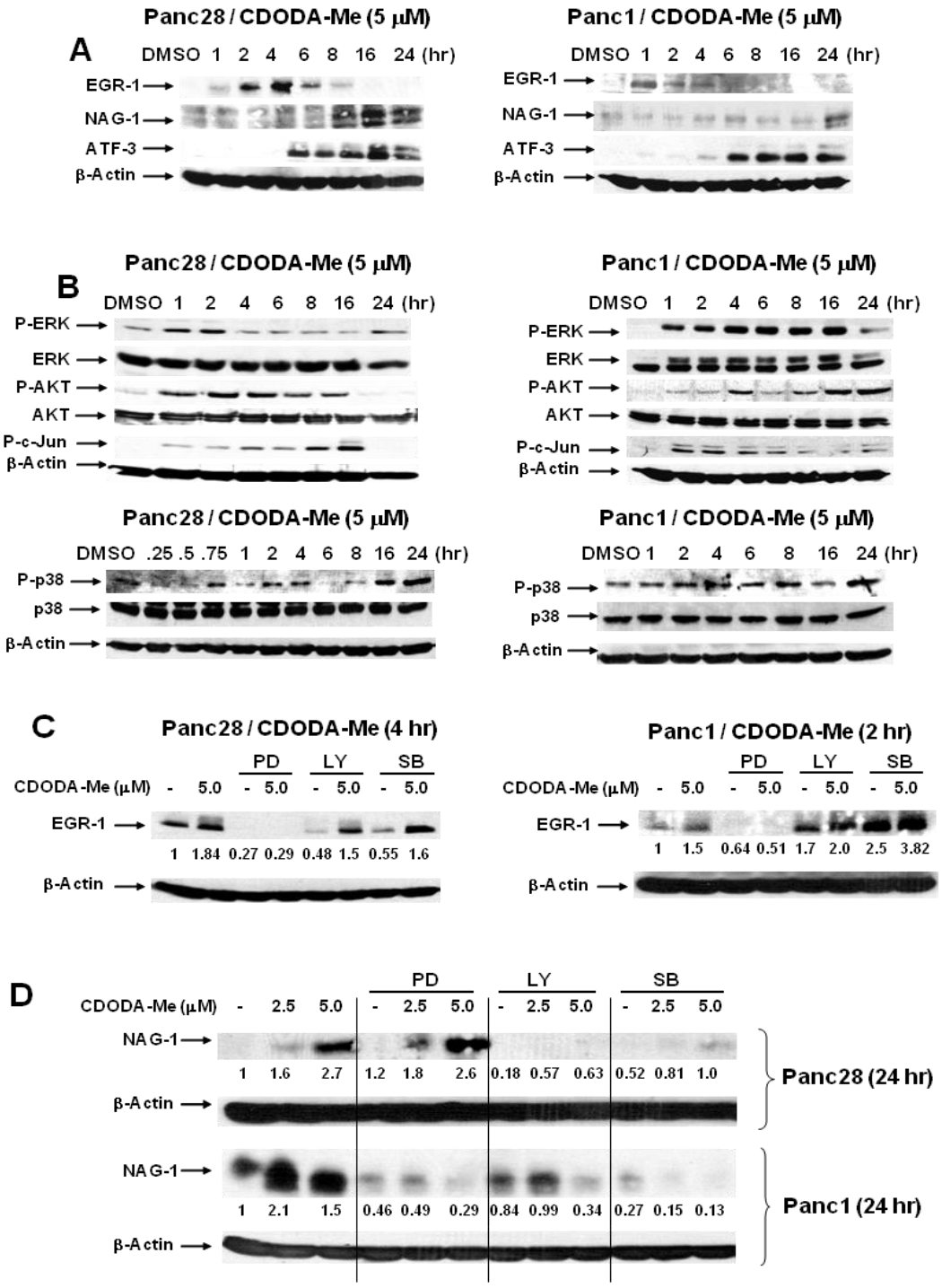

Figure 20. Kinase-dependent activation of NAG-1, Egr-1, PARP cleavage and ATF3 proteins induced by CDODA-Me.

Time-dependent induction of NAG-1, Egr-1 and ATF3 (A) and kinases (B) by CDODA-Me in Panc1 and Panc28 cells. Cells were treated with DMSO or 5 μM CDODA-Me for 1, 2, 4, 6, 8, 16 or 24 hr, and whole cell lysates from each treatment group were analyzed by western blots as described in the Materials and Methods. Effects of kinase inhibitors on induction of Egr-1 (C) and NAG-1 (D) in Panc1 and Panc28 cells. Cells were treated with DMSO, CDODA-Me alone or in the presence of kinase inhibitors, and whole cell lysates from each treatment group were analyzed by western blots as described in the Materials and Methods. β-Actin served as a protein loading control. The concentrations of kinase inhibitors were 20 μM LY294002 (LY), 20 μM PD98059 (PD), and 20 μM SB203580 and similar results we observed in duplicate studies. LY294002 and SB203580 inhibited Akt and p38 phosphorylation (positive control, data not shown). Band intensities in (C) and (D) were quantitated relative to β-actin from 3 replicate determinations and mean values are given relative to DMSO (set at 1.0). Significant ($p < 0.05$) inhibition of Egr-1 induction was observed for PD98059 in both cell lines. LY294002 and SB20358 significantly ($p < 0.05$) inhibited induction of NAG-1 in Panc1 and Panc28 cells, and PD98059 significantly ($p < 0.05$) inhibited induction of NAG-1 only in Panc1 cells.

The temporal pattern of Egr-1 induction prior to NAG-1 induction in both cell lines is similar to that previously reported for other NAG-1 inducers including CDODA-Me in prostate cancer cells (147) in which CDODA-Me-dependent activation of PI3-K- and/or MAPK-dependent pathways was important for

induction of Egr-1 and NAG-1 (Figure 20). Results in Figure 20B were determined as part of the time course study shown in Figure 20A and show that CDODA-Me induced phosphorylation of Akt, c-jun and p38- and p42-MAPK after treatment of Panc28 and Panc1 cells for 1 hr, whereas levels of Akt, c-jun and MAPK proteins were unchanged. The temporal patterns of increased kinase phosphorylation were cell context-dependent (Figure 20B); in Panc28 cells, increased phosphorylation of Akt and p42-MAPK was observed 1 - 8 and 1 - 2 hr, respectively, after treatment with CDODA-Me, whereas in Panc1 cells, enhanced phosphorylation persisted for at least 24 hr. In contrast, p38-MAPK phosphorylation was variable but maximally induced after treatment for 24 hr.

Results of kinase inhibitor studies show that induction of Egr-1 by CDODA-Me was inhibited by PD98059 but not by LY294002 or SB203580, suggesting that induction of Egr-1 was p42-MAPK-dependent in both cell lines (Figure 20C). PD98059 also inhibited induction by NAG-1 by CDODA-Me in Panc1 cells, suggesting that this response may be, in part, Egr-1-dependent (Figure 20D). However, even in Panc1 cells, NAG-1 induction was also inhibited by LY294002 and SB203580 and since these compounds did not affect Egr-1 expression, the induction of NAG-1 in Panc1 cells was Egr-1-independent. Moreover, in Panc28 cells NAG-1 induction was inhibited by LY294002 and SB205380 but not PD98059 (Figure 20D), confirming an Egr-1-independent pathway in this cell line.

Discussion

PPARγ is an orphan nuclear receptor that is overexpressed in multiple tumor types and cancer cell lines, and this receptor is a potential target for cancer chemotherapy (201, 202). Different structural classes of PPARγ agonists, including the thiazolidinediones (TZDs), 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PGJ2), CDDO-Me and PPARγ-active 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methanes (C-DIMs), activate overlapping and compound-specific growth inhibitory and proapoptotic responses in pancreatic cancer cells (183-190). Many of these compounds induce p21 and/or p27 expression, downregulate cyclin D1 protein, and cause G_0/G_1 to S phase arrest and these effects are both cell context- and structure-dependent. For example, troglitazone, a TZD, induces p27 and not p21 protein expression in several pancreatic cancer cell lines, whereas in another study TZD induces p21 and differentiation markers in a number of pancreatic cancer cells (140, 181-183). PPARγ-active C-DIMs also induce p21 but not p27 in Panc28 cancer cell lines and this is accompanied by a significantly higher percentage of cells in G_0/G_1 (28%) and a decreased percentage in S phase (21%) after treatment for 24 hr (183). The induction of p21 in Panc28 cells by PPARγ-active C-DIMs is inhibited by PPARγ agonists; however, most other studies in pancreatic cancer cells have not investigated the role of this receptor in mediating these responses.

In this study, CDODA-Me inhibited Panc1 and Panc28 cell proliferation (Fig. 15), and comparative studies with DODA-Me demonstrated the importance

of the 2-cyano group which markedly enhanced antiproliferative activity. The 2-cyano group was necessary for the PPARγ agonist activities of the glycyrrhetinic acid derivatives (145, 147) and structurally related oleane and lupane derivatives (177, 203). CDODA-Me induced PPARγ-dependent transactivation and activity (Figure 17), whereas DODA-Me which does not contain a 2-cyano substituent exhibited decreased antiproliferative activity (Fig. 15) and did not exhibit PPARγ agonist activity (data not shown). In addition, CDODA-Me induced differentiation of 3T3-L pre-adipocytes and this is a highly prototypical PPARγ-dependent response (Figure 17D). These results on activation of PPARγ by DODA-Me and CDODA-Me in Panc1 and Panc28 cells are similar to those observed in prostate and colon cancer cells (145, 147).

Like other PPARγ agonists, CDODA-Me induced p21 and p27 and decreased cyclin D1 expression in Panc1 and Panc28 cells (Figure 18) and, in Panc1 cells (Fig. 16B), this was accompanied by a G_0/G_1 to S phase arrest. However, studies with the PPARγ antagonist GW9662 indicated that these responses were receptor-independent, and this contrasted to the receptor-dependent induction of p21 in Panc28 cells by PPARγ-active C-DIMs and the 2-cyano derivative of betulinic acid which, like CDODA-Me, contains a 1-en-3-one function in the A-ring 0 (183, 202). Thus, among the three PPARγ agonists, there was a structure-dependent induction of p21 and similar results have been observed for induction of KLF4, suggesting that CDODA-Me, PPARγ-active C-

DIMs, and the cyano derivative of betulinic acid are selective PPARγ modulators.

CDODA-Me also induces apoptosis (DNA laddering) in Panc1 and Panc28 cells (Figure 16C) and previous studies indicate that CDODA-Me, other PPARγ agonists, and anticancer drugs induce the proapoptotic proteins NAG-1 and ATF3 in colon, prostate and other cancer cells (142, 143, 191-200); however, induction of these proteins has not previously been investigated in pancreatic cancer cells. Results in Figures 19A and 19B demonstrate that CDODA-Me induced NAG-1 and ATF3 proteins in Panc28 and Panc1 cells and cotreatment with the PPARγ antagonist GW9662 did not affect the induction responses or activation of caspase-dependent PARP cleavage (Figures 19C and 19D). Previously, we also observed receptor-independent induction of the proapoptotic proteins NAG-1 and ATF3 by CDODA-Me in LNCaP prostate cancer cells (147), and these responses were kinase-dependent and the prior induction of Egr-1 was associated with induction of NAG-1. In prostate cancer cells, induction of ATF3 by CDODA-Me was JNK-dependent (147); however, in pancreatic cancer cells, JNK and other kinase inhibitors had no effect on ATF3 induction (data not shown) which was not further investigated.

Egr-1 was induced in Panc1 and Panc28 cells within 1 - 2 hr after treatment with CDODA-Me, whereas NAG-1 was induced at later time points in Panc28 (6 - 24 hr) and Panc1 (24 hr) cells (Figure 20A). This temporal pattern of NAG-1 and Egr-1 induction is similar to that observed in other studies where

Egr-1 activates NAG-1 through interactions with the GC-rich proximal region of the NAG-1 promoter (142, 143, 192, 195, 199, 200). CDODA-Me induces phosphorylation of several kinases (PI3K, p38/p42MAPK and JNK) in both Panc28 and Panc1 cells (Figure 20B) as previously observed in prostate cancer cells (147); however, induction of Egr-1 was p42MAPK-dependent (Figure 20C). In Panc1 cells, the p42MAPK inhibitor PD98059 also inhibited induction of NAG-1 which is consistent with a role for Egr-1 in mediating the induction of NAG-1 by CDODA-Me. However, the PI3-K and p38 MAPK inhibitors LY294002 and SB203580, respectively, also decreased induction of NAG-1, demonstrating the contributions of Egr-1-independent pathways in Panc1 cells. Kinase inhibitor studies in Panc28 showed that induction of NAG-1 by CDODA-Me was primarily Egr-1-independent and was inhibited by LY294002 and SB20358 which had no effect on induction of Egr-1 in this cell line. This is one of the first examples of drug-dependent activation of both Egr-1 and NAG-1 in which induction of the latter gene is Egr-1-independent in one cell line (Panc28) and partially Egr-1-independent in another (Panc1 cells).

In summary, results of this study demonstrate for the first time that CDODA-Me inhibits growth and induces apoptosis in pancreatic cancer cells. Although CDODA-Me activates PPARγ in Panc28 and Panc1 cells, induction of growth inhibitory and proapoptotic proteins and activation of multiple kinase activities is receptor-independent. This is the first report of the induction of the proapoptotic protein NAG-1 in pancreatic cancer cells; however, it was evident

from studies with kinase inhibitors that the mechanisms of NAG-1 induction and the role of Egr-1 is cell context-dependent in Panc28 and Panc1 cells and differs from results of previous studies on NAG-1 induction (142, 143, 192, 195, 199, 200). Current studies are investigating the interplay between kinase activation, induction of proapoptotic proteins, and apoptosis by CDODA-Me in pancreatic cancer cells and the contributions of other pathways in mediating the proapoptotic effects of CDODA-Me in pancreatic cancer cells and tumors.

III. ARSENIC TRIOXIDE DOWNREGULATION OF SPECIFICITY PROTEIN (Sp) TRANSCRIPTION FACTORS IN BLADDER CANCER CELLS IS DEPENDENT ON REACTIVE OXYGEN SPECIES (ROS)

Arsenic trioxide exhibits antiproliferative, antiangiogenic and proapoptotic activity in cancer cells, and many genes associated with these responses are regulated by specificity protein (Sp) transcription factors. Treatment of cancer cells derived from urologic (bladder and prostate) and gastrointestinal (pancreas and colon) tumors with arsenic trioxide demonstrated that these cells exhibited differential responsiveness to the antiproliferative effects of this agent and this paralleled their differential repression of Sp1, Sp3 and Sp4 proteins in the same cell lines. Using arsenic trioxide responsive KU7 and non-responsive 253JB-V bladder cancer cells as models, we show that in KU7 cells, $\leq$ 5 μM arsenic trioxide decreased Sp1, Sp3 and Sp4 and several Sp-dependent genes and responses including cyclin D1, epidermal growth factor receptor, bcl-2, survivin and vascular endothelial growth factor, whereas at concentrations up to 15 μM, minimal effects were observed in 253JB-V cells. Arsenic trioxide also inhibited tumor growth in athymic mice bearing KU7 cells as xenografts, and expression of Sp1, Sp3 and Sp4 was significantly decreased. Inhibitors of oxidative stress such as glutathione or dithiothreitol protected KU7 cells from arsenic trioxide-induced antiproliferative activity and Sp repression, whereas glutathione depletion sensitized 253JB-V cells to arsenic trioxide. Mechanistic studies suggested that arsenic trioxide-dependent downregulation of Sp and Sp-

dependent genes was due to decreased mitochondrial membrane potential and induction of reactive oxygen species, and the role of peroxides in mediating these responses was confirmed using hydrogen peroxide.

Introduction

Arsenical compounds alone or in combination with other agents have been used widely in medicine to treat a variety of conditions and diseases including syphilis, psoriasis, trypanosomiasis, pernicious anemia and Hodgkin's disease (204, 205). Moreover, until the mid-1990s, arsenic trioxide was used as the major drug for treatment of chronic myeloid leukemia (CML) and other leukemias (204, 205). Arsenic trioxide has been associated with adverse side-effects and studies on individuals occupationally or environmentally exposed to high levels of arsenicals exhibit increased incidence of skin cancer. These observations contributed to a temporary decrease in the medicinal use of arsenicals until studies from China reported that arsenic trioxide was remarkably effective for treating patients with acute promyelocytic leukemia (APL) (206-208). Subsequent clinical studies have confirmed the effectiveness of this compound which is now routinely used for treating APL (204, 205). Based on the success of arsenic trioxide for treating APL, the clinical applications of this drug alone or in combination with other agents for treatment of solid tumors is currently being investigated (204, 209, 210).

The chemotherapeutic effectiveness of arsenic trioxide is due to modulation of several pathways in cancer cells leading to increased apoptosis,

enhanced differentiation, inhibition of cell proliferation, and angiogenesis (204, 205). Most cases of APL overexpress the PML-RARα fusion gene resulting from t(15;17) chromosome translocation and PML-RARα inhibits expression of genes involved in myeloid differentiation (211, 212). Arsenic trioxide induces PML-RARα inactivation or degradation by multiple pathways resulting in the subsequent activation of myeloid differentiation pathways (213, 214). Arsenic trioxide also targets the mitochondria in cancer cell lines resulting in decreased mitochondrial membrane potential, induction of reactive oxygen species (ROS), release of cytochrome c, and activation of several cell death pathways (215-219).

Previous studies indicate that arsenic trioxide decreases expression of genes associated with angiogenesis (VEGF), survival (bcl-2 and NFκB), and cell proliferation (cyclin D1) in cancer cell lines (220-228). Recent studies in this laboratory show that anticancer drugs such as curcumin, tolfenamic acid and betulinic acid also decrease expression of these same genes (153, 159, 165, 169, 229) and this was due, in part, to decreased expression of specificity proteins (Sp), Sp1, Sp3 and Sp4 which are overexpressed in many tumors (164, 230-233). These genes and others are also decreased in cancer cells transfected with a mixture (iSp) of small inhibitory RNAs for Sp1, Sp3 and Sp4 (153, 159, 165, 169, 229). Since arsenic trioxide decreases expression of several Sp-dependent genes, we hypothesized that the anticancer activity of this compound may be due, in part, to repression of Sp transcription factors Sp1,

Sp3 and Sp4. Results of this study show that arsenic trioxide does indeed decrease expression of Sp1, Sp3 and Sp4 proteins and Sp-dependent genes in several cancer cell lines derived from solid tumors and the mechanism of this response is related to decreased mitochondrial membrane potential (MMP) and induction of ROS. This demonstrates for the first time that mitochondrial-induced ROS also results in repression of Sp1, Sp3 and Sp4 and several Sp-dependent genes and these effects contribute to the anticancer activity of arsenic trioxide.

Materials and methods

Cell lines, reagents and antibodies

KU-7 and 253JB-V human bladder cancer cells were provided by Dr. A. Kamat (M.D. Anderson Cancer Center, Houston, TX). LNCaP and PC3 human prostate carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). RKO and SW480 human colon carcinoma cell lines were provided by Dr. Stanley Hamilton (University of Texas M.D. Anderson Cancer Center, Houston, TX). The Panc28 cell line was a generous gift from Dr. Paul Chiao (University of Texas M.D. Anderson Cancer Center, Houston, TX). L3.6pL cell line was developed at the M.D. Anderson Cancer Center (Houston, TX) and kindly provided by Dr. I.J. Fidler. SW480, L3.6pL, RKO and Panc28 cells were maintained in Dulbecco's modified/Ham's F-12 (Sigma-Aldrich, St. Louis, MO) with phenol red supplemented with 0.22% sodium bicarbonate, 5%

fetal bovine serum and 10 ml/L 100x antibiotic anti-mycotic solution (Sigma). 253JB-V, KU7, LNCaP and PC3 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 0.15% sodium bicarbonate, 0.011% sodium pyruvate, 0.24% Hepes and 10 ml/L of antibiotic/antimycotic cocktail solution. The cells were grown in 150 cm^2 culture plates in an air/CO_2 (95:5) atmosphere at 37°C and passaged approximately every 3-5 days. With the exception of cleaved poly (ADP) ribose polymerase (PARP) (Cell Signaling Technology, Danvers, MA), Sp1 (Millipore, Temecula, CA), survivin (R&D Systems, Minneapolis, MN) and β-actin antibodies (Sigma-Aldrich), all remaining antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Diethyl maleate (DEM), glutathione, 98% (γ-glu-cys-Gly, GSH), butylated hydroxyanisole (BHA), proline, N-acetylcysteine (NAC), catalase, and arsenic trioxide (99.995% pure) were purchased from Sigma-Aldrich. Dithiothreitol (DTT, 98%) was obtained from Boehringer Mannheim Corp, (Indianpolis, IN).

Cell proliferation assays

All the above-mentioned cancer cell lines were plated (3×10^4 per well) using DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped fetal bovine serum (FBS) in 12-well plates and left to attach for 24 hr. Cells were then treated with either vehicle or the indicated concentrations of arsenic trioxide. After 24 hr of treatment, cells were counted using a Coulter Z1 particle counter. RNA interference studies using a small inhibitory RNA for Sp1 as a prototype

was carried out essentially as described (28; 30). Each experiment was done in triplicate and results are expressed as means ± SE for each determination.

Western blot assays

All the above-mentioned cancer cells were seeded in DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS. Twenty-four hours later, cells were treated with either vehicle or the indicated compounds for 24 hr and western blot analysis was performed. The tumor tissues from the KU7 bladder cancer xenograft study were also processed similarly and probed for proteins of interest and β-actin served as loading control. Pretreatment with diethyl maleate for 60 min was carried out to deplete the GSH levels, and 253JB-V cells were then treated with solvent control or arsenic trioxide. Cells were also co-treated with dithiothreitol (DTT) and glutathione (GSH) in the presence or absence of arsenic trioxide for a time interval of 24 hr. Protein quantification used Image J software, and optical densities for each protein were normalized to β-actin and the solvent control group.

Terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) assay

253JB-V and KU7 cells (7×10^4) were seeded in four-chambered glass slides and left overnight to attach. After treatment with indicated compounds for 18 hr, the in situ cell death detection POD kit was used for the terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL) assay

according to the instructions in the protocol manual for fixed cells. The percentage of apoptotic cells was calculated by counting the stained cells in eight fields, each containing 50 cells. The total number of apoptotic cells was plotted as a percentage in both cell lines.

Xenograft tumor study

Athymic female nude mice, age 3 to 5 weeks, were purchased from Harlan Laboratories (Indianapolis, IN). KU7 cells (1×10^6) in 1:1 ratio of Matrigel (BD Biosciences, San Jose, CA) were injected into both the sides of the flank area of nude mice. A week after the tumor cell inoculation, mice were divided into two groups of six animals each. The first group received 100 μL of vehicle(PBS/KOH) by i.p. injection, and the second group of animals received 5 mg/kg/d injection of arsenic trioxide in PBS/KOH every other day for 24 days (12 doses) by i.p. injection. Mice were weighed, and tumor areas were measured throughout the study. After 24 days, the animals were sacrificed; final body and tumor weights were determined and plotted. At the end of the experiment, major visceral organs were collected and analyzed for Sp protein expression levels using western blotting as described earlier.

GSH estimation

The Stallion Imaging workstation, equipped with a Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging, Thornwood, NY) and slidebook software (Intelligent Imaging Innovations Inc., Denver, Co), was used with a 20X objective

0.75NA for acquiring images. Cellular GSH levels were evaluated with the cell permeant probe mBCl. mBCl is nonfluorescent, but forms a fluorescent adduct with GSH in a reaction catalyzed by glutathione 5-transferase. Following 20-22 hr treatment, kinetic analysis of mBCl-GSH conjugation was performed at room temperature by exciting the cells at 365 nm wavelength and recording changes in fluorescence intensity with a BP 445/50 nm filter at 1-min intervals for up to 15 min. GSH level per cell was then determined by applying non linear regression analysis to acquire data. Two experiments were preformed on different days. At least 50 cells per treatment group were collected in these studies.

ROS estimation

Cellular reactive oxygen species (ROS) levels were evaluated with the cell permanent probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2',7' dichlorodihydrofluorescein diacetate acetyl ester). CM-H_2DCFDA is nonfluorescent until removal of the acetate groups by intracellular esterases and oxidation occurs within the cell. Following 20-24 hr treatment, cells plated on 96 well cell culture plate were loaded with 10 μM CM-H_2DCFDA for 30 min, washed once with serum free medium, and analyzed for ROS levels using the BioTek Synergy 4 plate reader (BioTek Instruments, Inc., Winooski, VT) set at 480 nm and 525 nm excitation and emission wavelengths respectively. Following reading of ROS, cultures were washed twice with PBS and fixed with methanol for 3 min at room temperature. Methanol was then completely removed and 1 mg/ml Janus green was added to the cultures for 3 min. Following removal of

Janus green, cultures were washed twice with PBS and 100 μl of 50% methanol was added to each well. Cell counts were then determined with the plate reader set to an absorbance of 654 nm and ROS intensities were then corrected accordingly. Two experiments were preformed on different days. At least 16 wells per treatment were analyzed for each experiment.

Measurement of mitochondrial membrane potential (MMP)

The MMP was measured with Mitochondrial Membrane Potential Detection Kit (Stratagene, Cedar Creek, TX). Briefly, cells were seeded on Lab-Tek Coverglass system (NUNC, NY) and treated with compounds alone or with inhibitors for 24 hr. They were then incubated with 1X JC-1 cationic dye at 37°C for 30 min according to the manufacturer's instruction. After washing with 1X JC-1 assay buffer twice, cells were subjected to microscopic analysis using a confocal instrument (Zeiss LSM510, Germany). The mitochondrial potential shift was also measured by flow cytometry analysis (Beckman Coulter, Miami, FL). Cells were seeded in cell culture plates and treated with indicated compounds for 24 hr. They were then incubated with JC-1 dye for a further 30 min. After washing with JC-1 assay buffer twice, cells were trypsinized and suspended in cell culture medium. J-aggregates are detected as red fluorescence and J-monomers are detected as green fluorescence.

Statistical analysis

Statistical significance of differences was determined by an analysis of variance and student t-test, and the levels of probability were noted. IC_{50} values were calculated using non-linear regression analysis and expressed in μM, at 95% confidence intervals.

Results

Arsenic trioxide inhibits cancer cell and tumor growth and decreases Sp1, Sp3 and Sp4 proteins

KU7 and 253JB-V bladder cancer cells were used as models for investigating the molecular mechanisms associated with the anticarcinogenic activity of arsenic trioxide and Figure 21A shows that treatment of KU7 cells with 2.5, 5.0 and 10 μM arsenic trioxide for 24 hr resulted in decreased proliferation. IC_{50} values were 2.3 and 1.4 μM after treatment for 24 or 72 hr, respectively. In contrast, 253JB-V cells were more resistant to the growth inhibitory effects of arsenic trioxide and IC_{50} values were 14.1 and 5.1 μM after treatment for 24 and 72 hr, respectively. The anticancer activity of arsenic trioxide in different cancer cell lines is accompanied by decreased expression of genes/responses such as bcl-2, VEGF and angiogenesis, cyclin D1, and NFκB (220-228). Studies in this laboratory show that expression of these genes/responses is dependent on Sp transcription factors (153, 159, 162, 163, 165, 168, 169, 229), and we hypothesized that the anticarcinogenic activity of

arsenic trioxide may be due, in part, to downregulation of Sp1, Sp3 and Sp4. Figure 21A shows that arsenic trioxide induces a concentration-dependent downregulation of Sp1, Sp3 and Sp4 proteins in KU7 cells which are also sensitive to the growth inhibitory effects of this compound. In addition, we also observed a parallel decrease in Sp1, Sp3 and Sp4 mRNA levels in KU7 cells treated with arsenic trioxide for 24 hr (data not shown). In contrast, arsenic trioxide only minimally decreased Sp1, Sp3 and Sp4 in 253JB-V cells after treatment for 24 hr and there was a correlation between resistance if this cell line to arsenic trioxide-induced Sp degradation and growth inhibition. Treatment of 253JB-V cells with arsenic trioxide for 72 hr resulted in more pronounced repression of Sp1, Sp3 and Sp4 (data not shown). Previous studies reported that treatment of NB4 leukemia cells for 10 days with 0.75 μM arsenic trioxide resulted in oxidation of Sp1 but changes in Sp1 levels were not observed (234). Figure 21B shows that treatment of KU7 cells with 1.0 or 0.75 μM arsenic trioxide decreased both cell growth and expression of Sp1, Sp3 and Sp4 proteins. This corresponded with the 24 hr studies (Figure 21A) which required higher concentrations of arsenic trioxide to inhibit growth and induce Sp downregulation.

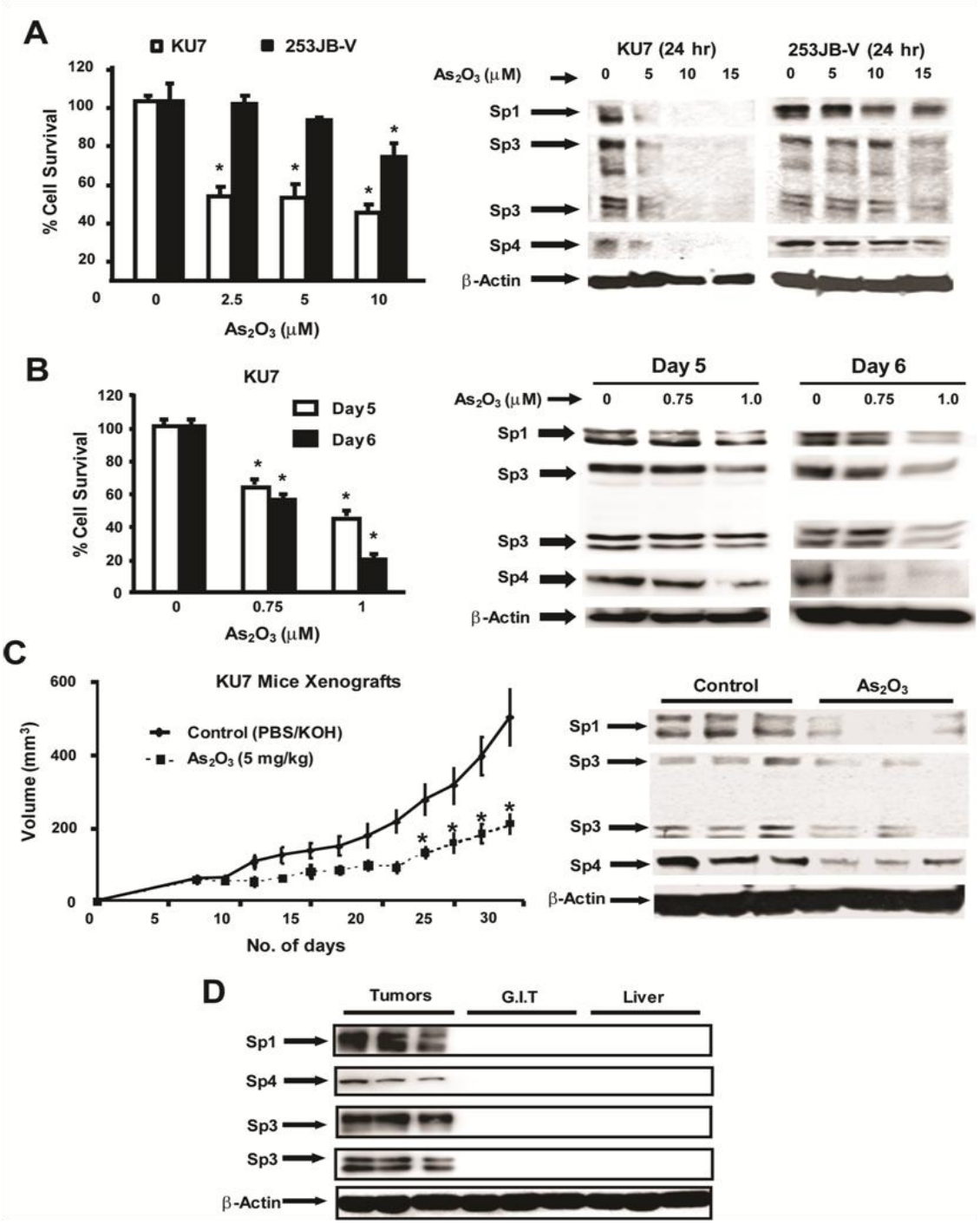

Figure 21. Arsenic trioxide inhibits bladder cancer cell and tumor growth and downregulates Sp1, Sp3 and Sp4.

(A) Inhibition of cell growth and Sp downregulation. Cells were treated with arsenic trioxide for 24 hr and cell growth and Sp proteins were determined as outlined in the Materials and Methods. (B) Prolonged treatment of KU7 cells. Cells were treated with 0.75 or 1.0 μM arsenic trioxide for 120 or 144 hr and cell proliferation and Sp protein were determined as described in the Materials and Methods. (C) Arsenic trioxide inhibits bladder tumor growth and downregulates Sp proteins. Athymic nude mice were treated with PBS/KOH (solvent control) or arsenic trioxide (5 mg/kg/day) in PBS/KOH for 24 days. Tumor volumes were determined every second day and tumor weights were determined at sacrifice as described in the Materials and Methods. Expression of Sp proteins was determined in triplicate (3 animals/group) by western blot analysis of tumor lysates as described in the Materials and Methods, and significant ($p < 0.05$) decreases in Sp1, Sp3 and Sp4 proteins were observed in the arsenic trioxide-treated tumors. (D) Western blot analysis. Lysates from tumors, the gastrointestinal tract (GIT) and liver from control (untreated) animals were analyzed by western blots as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate experiments (A and B) or 3 animals per treatment group, and significant ($p < 0.05$) decreases in cell proliferation (A and B) and in tumor growth (B) are indicated (*).

The effectiveness of arsenic trioxide as a tumor growth inhibitor was investigated in athymic nude mice bearing KU7 bladder cancer cells as xenografts. After the initial appearance of palpable tumors, mice were treated

with buffered arsenic trioxide (5 mg/kg/d) or buffer alone, and tumor volumes were measured over the 24 day treatment period. The results (Figure 21C) show that arsenic trioxide significantly inhibited tumor volume, and tumor weights (data not shown) were also significantly decreased in the arsenic trioxide-treated animals. Lysates from tumors of treated and control mice were analyzed by western blots and there was a significant ($p < 0.05$) decrease in expression of Sp1, Sp3 and Sp4 proteins in tumors from arsenic trioxide-treated animals (Figure 21C) and this has previously been observed with other anticancer agents that also decrease Sp1, Sp3 and Sp4 expression in cancer cell culture and tumors in athymic nude mice (162, 165, 168). Figure 21D illustrates the relatively high expression of Sp1, Sp3 and Sp4 in tumors, whereas relatively low to non-detectable levels of these proteins were observed in the gastrointestinal tract and liver lysates, confirming the high expression of these transcription factors in tumor vs. non-tumor tissue.

The comparative effects of arsenic trioxide on growth inhibition and downregulation of Sp proteins was also examined in pancreatic (L3.6pL and Panc28), colon (SW480 and RKO), and prostate (LNCaP and PC3) cancer cell lines (Figures 22A - 22C). L3.6pL and Panc28 exhibited differential arsenic trioxide responsiveness as observed for KU7 and 253JB-V cells, whereas in the other four cell lines, there was a similar concentration-dependent decrease in growth inhibition and Sp1, Sp3 and Sp4 proteins (Figure 22). These results suggest that arsenic trioxide induces downregulation Sp transcription factors in

multiple cancer cell lines and tumors and this effect may contribute to the anticarcinogenic activity of this compound.

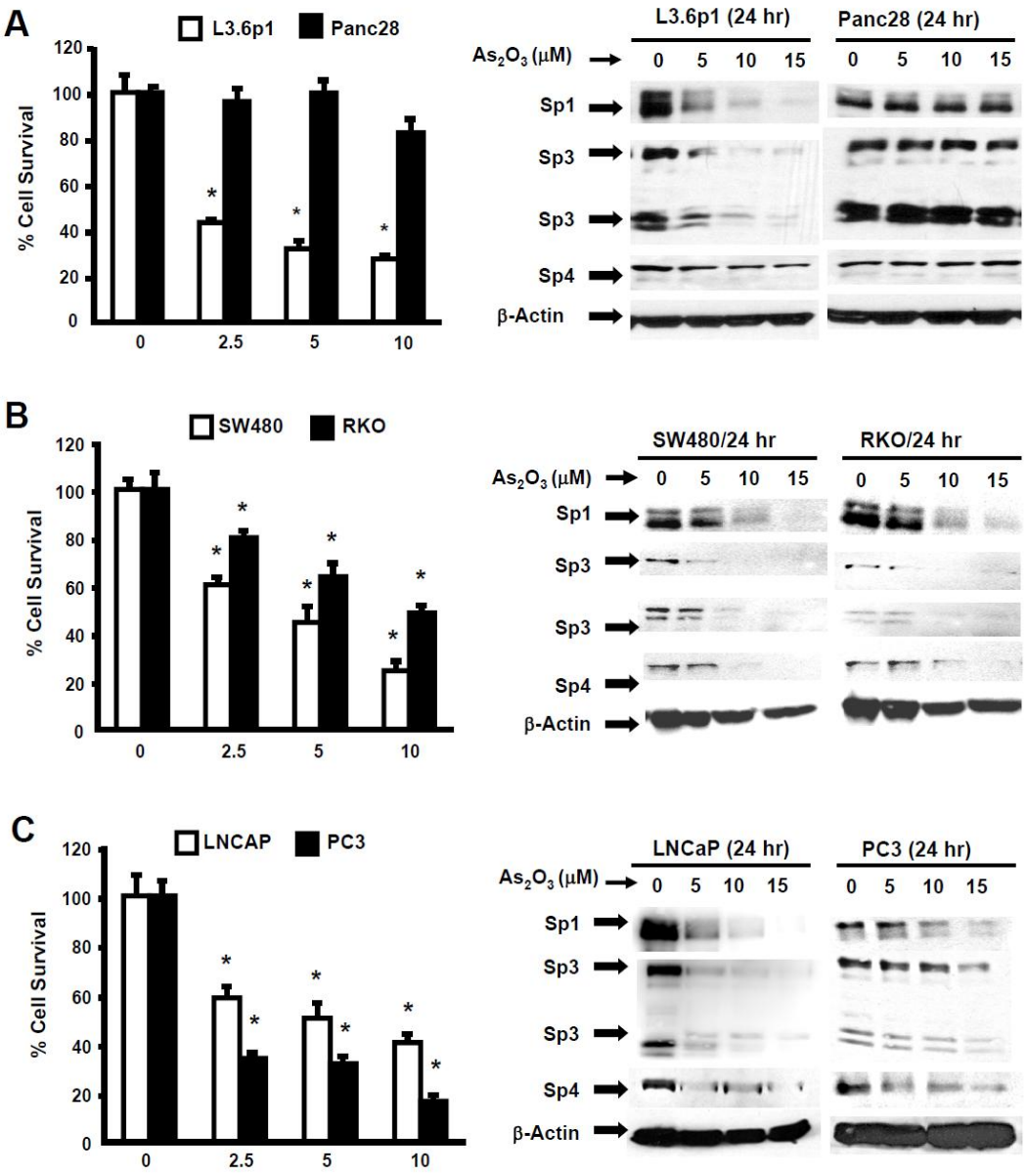

Figure 22. Arsenic trioxide decreases cell growth and Sp1, Sp3 and Sp4 expression in cancer cell lines.

Pancreatic (A), colon (B), and prostate (C) cancer cells were treated with solvent (control) and arsenic trioxide in PBS/KOH for 24 hr; cell growth was determined, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. Western blot results illustrated in the right panels were typical of duplicate (or more) determinations. Results of cell proliferation studies are expressed as means ± SE for 3 replicate determinations for each treatment group, and significant ($p < 0.05$) inhibition of cell growth is indicated (*).

Arsenic trioxide decreases Sp-dependent genes and responses

Previous RNA interference studies in bladder cancer cells demonstrated that several genes associated with cell proliferation (EGFR, CD1), survival (survivin and bcl-2), and angiogenesis (VEGF) were regulated by Sp1, Sp3 and Sp4 (165). Results in Figures 23A and 23B confirm that arsenic trioxide decreased expression of these proteins in KU7 cells. In contrast, arsenic trioxide-dependent downregulation of these proteins was minimal in 253JB-V cells after treatment for 24 hr and there was a correlation between responsiveness to arsenic trioxide-induced downregulation of Sp1, Sp3 and Sp4 and the parallel decrease of Sp-dependent genes in the two bladder cancer cell lines. In addition, the relative responsiveness of KU7 vs. 253JB-V cells to arsenic trioxide was also observed with respect to induction of PARP cleavage (Figure 23B) and in the apoptotic TUNEL assay where increased TUNEL staining was observed for KU7 but not 253JB-V cells (Figure 23C).

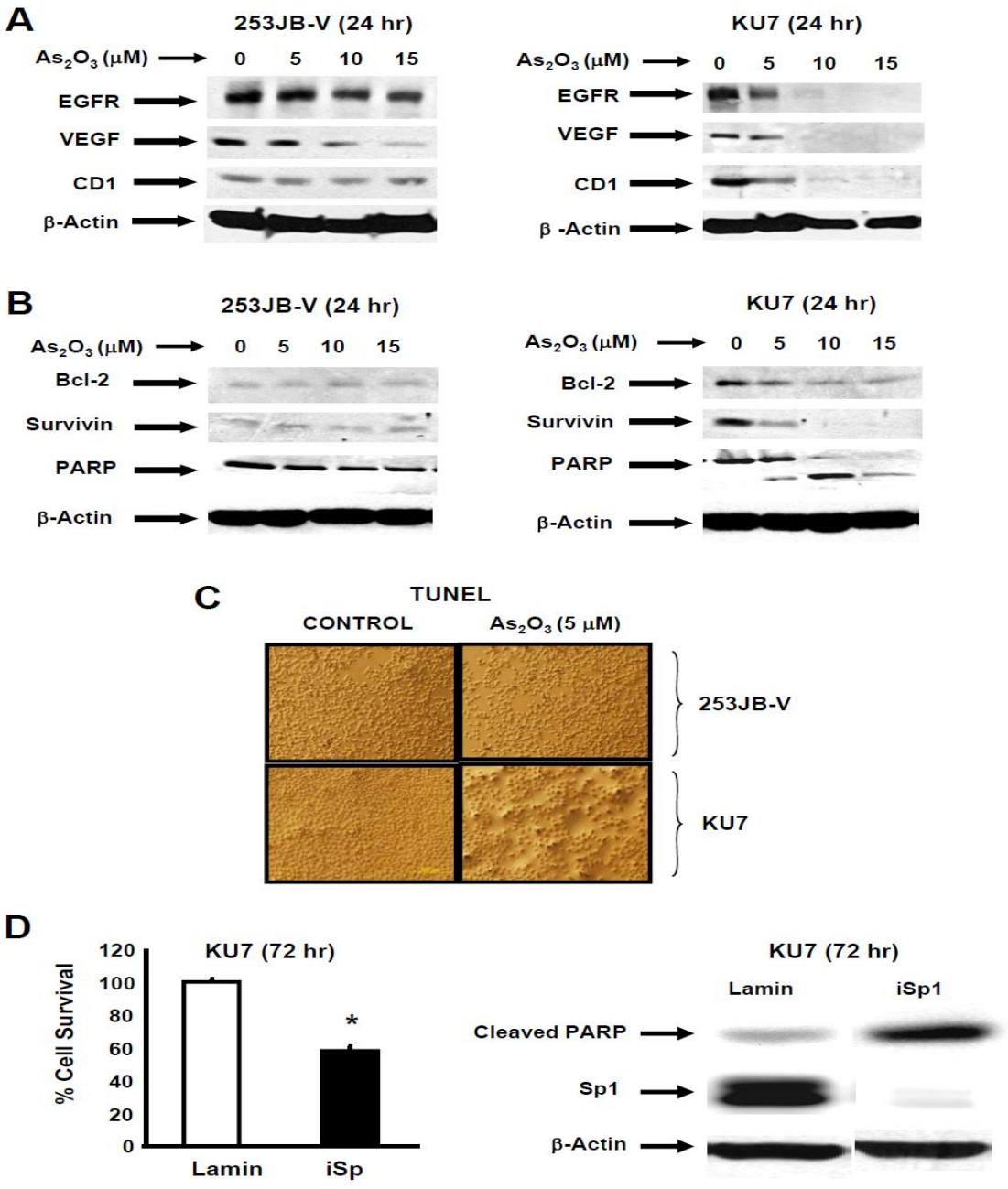

Figure 23. Arsenic trioxide differentially affects Sp-dependent responses in KU7 and 253JB-V cancer cells.

Effects on (A) EGFR, cyclin D1 (CD1), and VEGF and (B) proapoptotic proteins/responses. Cells were treated with 5, 10 or 15 μM arsenic trioxide for 24 hr and whole cell lysates were analyzed by western blots as described in the Materials and Methods. Similar results were observed in duplicate experiments. (C) TUNEL staining. KU7 and 253JB-V cells were treated with 5 μM arsenic trioxide for 24 hr and cells were examined for TUNEL staining as described in the Materials and Methods. Significant TUNEL staining was observed only in KU7 cells in replicate experiments. (D) RNA interference studies. KU7 cells were transfected with siRNA for Sp1 as indicated and the effects of Sp1 knockdown on cell proliferation and PARP cleavage were determined by cell counting and western blot analysis, respectively as described in the Materials and Methods. Significant ($p < 0.05$) effects on cell proliferation are indicated (*) from replicate (3) determinations.

Confirmation that downregulation of Sp proteins by arsenic trioxide contributes to the growth inhibitory and proapoptotic effects of this compound was determined in KU7 cells transfected with a small inhibitory RNA for Sp1 (iSp1) as a prototype for the Sp proteins. Results in Figure 23D confirm that knockdown of Sp1resulted in inhibition of KU7 cell growth and induction of caspase-dependent PARP cleavage. Thus, downregulation of Sp proteins resulted in growth inhibition and apoptosis which was comparable to the effects of arsenic trioxide in this cell line (Figures 21B, 23B and 23C). Attempts to reverse the effects of arsenic trioxide on Sp proteins by overexpression of Sp1

(as a model) were unsuccessful (data not shown). This may be due to the sensitivity of cells to levels of Sp1 and this is supported, in part, by the proapoptotic effects observed after overexpression of Sp1 in cancer cells (235).

Arsenic trioxide induces ROS; and ROS inhibitors block downregulation of Sp transcription factors

Several reports indicate that the proapoptotic and growth inhibitory effects of arsenic trioxide were associated with decreased MMP and induction of reactive oxygen species (ROS) (216-219). Moreover, the susceptibility of several cancer cell lines to the cytotoxicity of arsenic trioxide correlated with the relative expression of the antioxidant GSH (236). Constitutive GSH(253JB-V)/GSH(KU7) ratios were 1.47 and the corresponding ROS ratio was approximately 1.0 in the two cell lines indicating that enhanced expression of GSH in 253JB-V cells may contribute to the differences in the arsenic trioxide-responsive and -nonresponsive KU7 and 253JB-V bladder cancer cells. Treatment of KU7 cells with arsenic trioxide significantly decreased GSH and this response was blocked after treatment with the antioxidants GSH or DTT (Figure 24A). Moreover, arsenic trioxide also induced ROS in KU7 cells and this effect was inhibited after cotreatment with DTT (Figure 24A). Since GSH and DTT inhibited arsenic trioxide-induced ROS, we investigated the role of ROS in mediating the effects of arsenic trioxide on KU7 cell proliferation and Sp protein expression. Arsenic trioxide alone inhibited KU7 cell proliferation and decreased Sp1, Sp3 and Sp4 protein expression (Figures 24B and 24C, respectively).

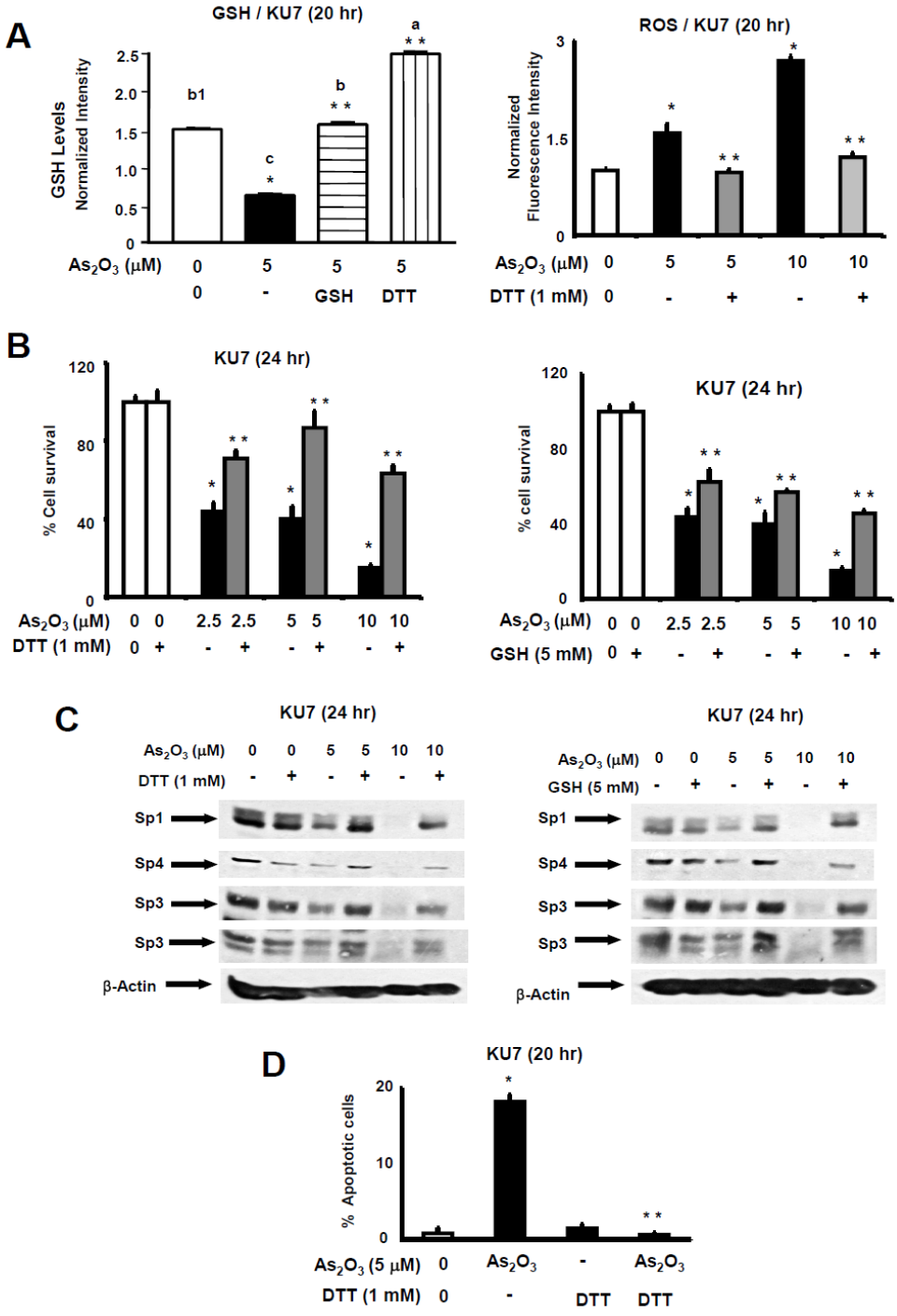

Figure 24. Role of glutathione/oxidative stress in mediating the anticancer activity of arsenic trioxide in KU7 cells.

(A) Effects of arsenic trioxide and ROS inhibitors on GSH and ROS. KU7 cells were treated with aqueous solvent, arsenic trioxide alone, or in combination with GSH or DTT. Cellular GSH levels and ROS were determined as described in the Materials and Methods. Results are expressed at means ± SE for 3 separate experiments for each treatment group and significant ($p < 0.05$) effects by arsenic trioxide (*) and reversal by DTT or GSH (**) are indicated. GSH values in control (100%), arsenic trioxide, arsenic trioxide + GSH or DTT groups were 100±0.7, 40.0±0.1, 101.5±1.9 and 157.6±1.2, respectively. ROS values in control (100%), arsenic trioxide alone (5 or 10 μM), and DTT + arsenic trioxide (5 or 10 μM) were 100±2, 156±13, 269±6, 97±4 and 120±6, respectively. (B) GSH and DTT inhibit the antiproliferative activity of arsenic trioxide. KU7 cells were treated with solvent control, 2.5, 5 or 10 μM arsenic trioxide alone, or in combination with DTT (left) or GSH (right). After 24 hr, cells were counted as described in the Materials and Methods. Results are means ± SE for 3 determinations for each treatment group and significant ($p < 0.05$) inhibition by arsenic trioxide (*) and reversal by DTT or GSH (**) are indicated. (C) Sp expression levels. KU7 cells were treated with arsenic trioxide alone or in the presence of DTT (left) or GSH (right) for 24 hr and whole cell lysates were examined by western blot analysis as described in the Materials and Methods. (D) TUNEL assay. KU7 cells were treated with solvent (control), 5 μM arsenic trioxide alone, or in combination with DTT and the TUNEL assay was determined and quantified as described in the Materials and Methods. The

percent of apoptotic cells in control (100%), arsenic trioxide alone, DTT alone, and arsenic trioxide + DTT were 100±46, 2234±58, 182±35 and 77±14, respectively. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($p < 0.05$) induction of TUNEL staining (*) and inhibition of this response by DTT (**) are indicated.

However, after cotreatment with GSH or DTT, there was significant reversal of the arsenic trioxide-induced responses (Figure 24). Moreover, in gel mobility shift assays, we also observed that nuclear extracts from KU7 cells treated with arsenic trioxide exhibited decreased binding to a GC-rich oligonucleotide that binds Sp proteins, and cotreatment of KU7 cells with DTT restored binding to this oligonucleotide (Supplement Figure 1). In addition, we also used the TUNEL assay and the quantitative results in Figure 24D shows that the increased TUNEL staining observed in KU7 cells after treatment with arsenic trioxide was significantly inhibited after cotreatment with DTT.

253JB-V cells were resistant to the cytotoxic effects of arsenic trioxide and the role of GSH and ROS in regulating resistance was investigated using diethylmaleate (DEM), a reagent that depletes intracellular GSH. Figure 25A shows that treatment of 253JB-V cells with arsenic trioxide or DEM resulted in a 10.2% decrease in GSH levels; however, in cells cotreated with DEM plus arsenic trioxide, a 55.6% decrease in GSH was observed. Using the same treatment protocol, arsenic trioxide or DEM alone had minimal effects on ROS levels, whereas in 253JB-V cells treated with DEM plus arsenic trioxide, there

was a 2.2- or 4.9-fold increase in ROS in cells treated with 5 or 10 μM arsenic trioxide (compared to untreated cells). Treatment of 253JB-V cells with arsenic trioxide alone or in combination with DEM showed that DEM-dependent depletion of GSH sensitized these cells to arsenic trioxide-induced inhibition of cell proliferation (Figure 25B) and repression of Sp1, Sp3 and Sp4 proteins (Figure 25C). Moreover, quantitation of the TUNEL staining results (Figure 25D) show that minimal staining was observed in 253JB-V cells treated with arsenic trioxide; however, in cells cotreated with DEM plus arsenic trioxide, there was a significant increase in TUNEL staining. Thus, GSH expression and induction of ROS are critical factors that regulate arsenic trioxide-induced anticarcinogenic responses in bladder cancer cells and this includes downregulation of Sp proteins and Sp-dependent gene products and induction of apoptosis.

Antioxidants block hydrogen peroxide and arsenic trioxide-dependent growth inhibition and Sp downregulation

These results in bladder cancer cells suggest that induction of ROS by arsenic trioxide appears to be a critical common factor in mediating growth inhibition and Sp protein downregulation, and therefore, we investigated the effects of hydrogen peroxide alone on expression of Sp1, Sp3 and Sp4 in KU7 cells (Figure 26).

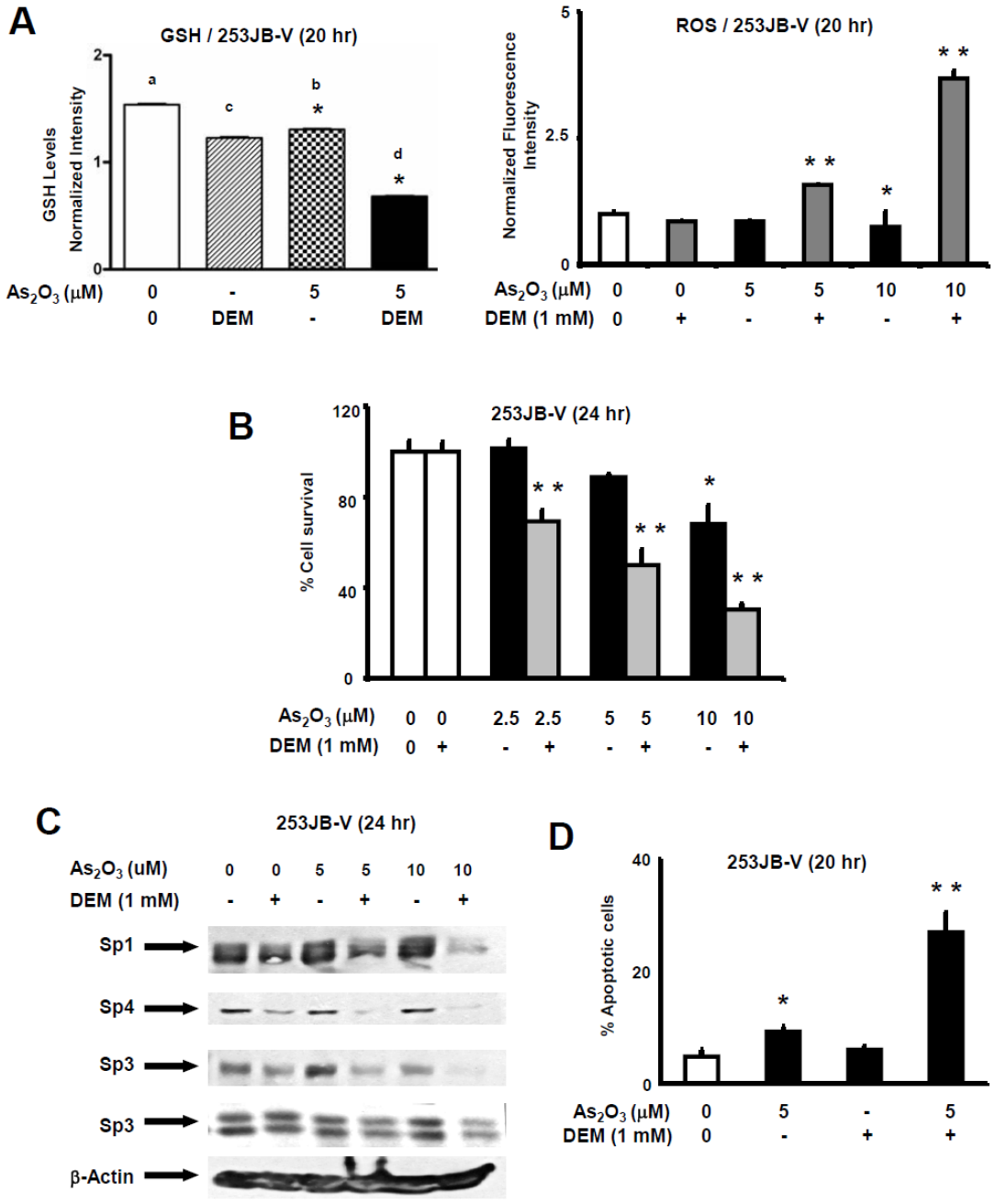

Figure 25. Role of glutathione/oxidative stress in mediating the anticancer activity of arsenic trioxide in 253JB-V cells.

(A) Effects of arsenic trioxide and DEM on GSH and ROS. 253JB-V cells were treated with aqueous solvent, arsenic trioxide alone, or in combination with DEM. Cellular GSH levels and ROS were determined as described in the Materials and Methods. Results are expressed at means ± SE and significant ($p < 0.05$) effects by arsenic trioxide (*) and enhancement by DEM (**) are indicated. GSH values in control (100%), DEM, arsenic trioxide alone or in combination with DEM were 100±0.7, 79.8±0.5, 85.0±0.3 and 44.4±0.4, respectively. ROS values in control (100%), arsenic trioxide alone (5 or 10 μM), DEM alone, and DEM + arsenic trioxide (5 or 10 μM) were 100±2, 85±0.7, 75±1.2, 85±1.7, 163±30 and 368±15, respectively. (B) DEM enhances the antiproliferative activity of arsenic trioxide. 253JB-V cells were treated with solvent control, 2.5, 5 or 10 μM arsenic trioxide alone, or in combination with DEM. After 24 hr, cells were counted as described in the Materials and Methods. Results are means ± SE for 3 determinations for each treatment group and significant ($p < 0.05$) inhibition by arsenic trioxide (*) and enhancement by DEM (**) are indicated. (C) Sp expression levels. 253JB-V cells were treated with arsenic trioxide alone or in the presence of DEM for 24 hr and whole cell lysates were examined by western blot analysis as described in the Materials and Methods. (D) TUNEL assay. 253JB-V cells were treated with solvent (control), 5 μM arsenic trioxide alone, or in combination with DEM and the TUNEL assay was determined and quantitated as described in the Materials and Methods. The percent of apoptotic cells in control (100%), arsenic trioxide

alone, DEM alone, and arsenic trioxide + DEM were 100±25, 193±18, 125±12 and 566±105, respectively. Results are expressed as means ± SE for 2 replicate determinations for each treatment group and significant ($p < 0.05$) induction of TUNEL staining (*) is indicated.

The results show that treatment of KU7 cells with hydrogen peroxide (500 μM) for 24 hr decreased expression of Sp1, Sp3 and Sp4 proteins and cotreatment with hydrogen peroxide plus GSH blocked downregulation of Sp proteins, whereas only minimal effects were observed for DTT (Figure 26A). In addition, hydrogen peroxide (500 μM) inhibited KU7 cell growth and this response was also inhibited after cotreatment with GSH. Downregulation of Sp proteins and growth inhibition was also observed using t-butylhydroperoxide (data not shown). In some cancer cell lines, arsenic trioxide-induced ROS has been associated with mitochondriotoxicity and loss of MMP (215, 216, 237).

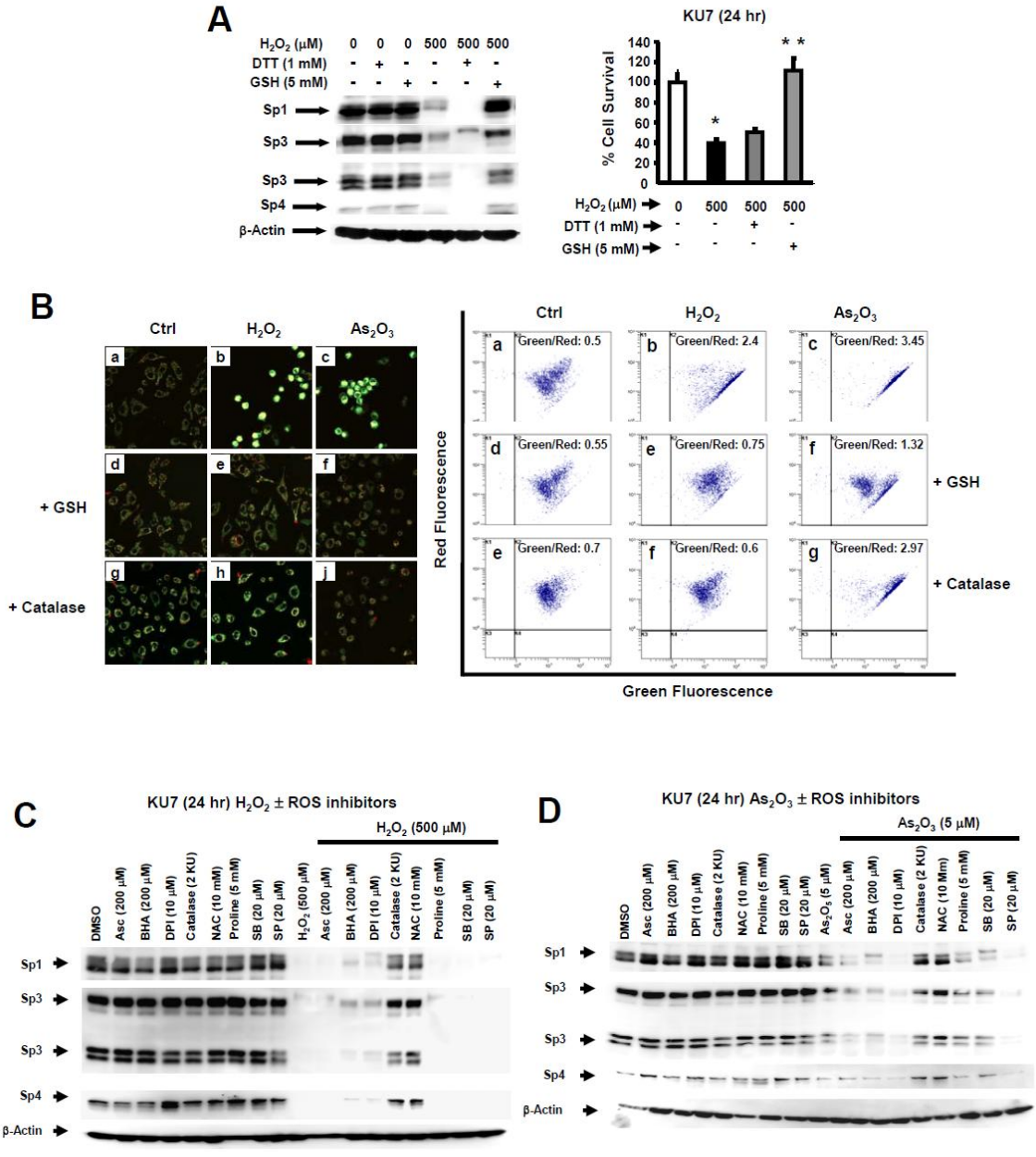

Figure 26. Comparative effects of hydrogen peroxide and arsenic trioxide on Sp proteins and MMP in KU7 cells.

(A) Hydrogen peroxide downregulates Sp proteins and inhibits proliferation. KU7 cells were treated with 500 μM hydrogen peroxide alone or in

combination with GSH or DTT, and Sp protein expression and cell growth were determined by western blot analysis and cell counting as described in the Materials and Methods. Cell proliferation results are given as means ± SE for replicate (3) determinations, and significant ($p < 0.05$) growth inhibition by hydrogen peroxide (*) and reversal of this effect by GSH (**) are indicated. (B) Effects of arsenic trioxide and hydrogen peroxide on MMP. KU7 cells were treated with the compounds or catalase, and MMP was determined by confocal microscopy or FACS analysis as described in the Materials and Methods. Effects of various inhibitors on hydrogen peroxide (C)- or arsenic trioxide (D)-induced downregulation of Sp1, Sp3 and Sp4. KU7 cells were treated with the compounds alone or in combination with various inhibitors, and Sp proteins were examined by western blot analysis as described in the Materials and Methods. β-Actin served as a loading control for the western blots. The following concentrations of inhibitors were used: SB203580 (20 μM); SP6000125 (20 μM); NAC (10 mM), ascorbic acid (200 μM); BHA (200 μM); DPI (10 μM), and catalase (2 KU).

The comparative effects of arsenic trioxide and hydrogen peroxide on MMP was determined by confocal microscopy in KU7 cells (Figure 26B) using JC-1, a red fluorescent dye that accumulates and forms oligomers in the mitochondrial matrix. In solvent-treated cells, the green (cytosol) and red (mitochondrial) staining was evident using confocal microscopy, whereas after treatment with 10 μM arsenic trioxide or 500 μM hydrogen peroxide, there was a

decrease in red and an increase in green staining consistent with decreased MMP (Figure 26B). Similar results were observed in FACS analysis where there was an increase in the ratio of green/red stained cells. Moreover, the effects of arsenic trioxide and hydrogen peroxide on decreased MMP were partially reversed in KU7 cells cotreated with the thiol antioxidant GSH; catalase, which catalyzes the detoxication of peroxides, blocked the effects of hydrogen peroxide but was minimally effective against arsenic trioxide in this assay (Figure 26B). These results are consistent with a comparable mechanism of action for both arsenic trioxide and hydrogen peroxide which includes decreased MMP and the subsequent induction of ROS which has previously been reported for arsenic trioxide and also observed in this study (Figure 24).

The effects of the antioxidant BHA on arsenic trioxide- and hydrogen peroxide-dependent decrease in MMP were investigated. BHA did not ameliorate the effects of these compounds on MMP and was minimally effective as an inhibitor of ROS in the bladder cancer cell lines (data not shown). The linkage between arsenic trioxide- and hydrogen peroxide-induced ROS and downregulation of Sp proteins was further investigated using several inhibitors including catalase, proline, an inhibitor of oxidative stress, N-acetylcysteine (NAC), BHA, diphenyliodonium (DPI), an inhibitor of NADPH oxidase, and inhibitors of p38 (SB203580) and JNK (SP6000125) kinase pathways. Catalase and NAC blocked hydrogen peroxide-induced downregulation of Sp1, Sp3 and Sp4 proteins (Figure 26C), whereas minimal (DPI and BHA) or no inhibition was

observed with the other compounds. The same set of inhibitors was used to investigate arsenic trioxide-dependent downregulation of Sp proteins (Figure 26D) and similar results were observed except that minimal inhibition was observed for ascorbate, proline and SB203580. Thus, thiol reducing agents, such as NAC, GSH and DTT that block arsenic trioxide-dependent decrease in MMP and formation of ROS, or catalase, which decreases ROS, also inhibited downregulation of Sp proteins. This suggests that, in KU7 cells, the anticarcinogenic activity of arsenic trioxide is associated, in part, with Sp downregulation which is due to decreased MMP and induction of ROS.

Discussion

Sp transcription factors are members of the Sp/Krüppel-like family (KLF) of 25 transcription factors that bind GC-rich promoter sequences and regulate basal expression of multiple mammalian and viral genes (150). Although knockout of many of the Sp genes in mice is embryolethal or induces serious defects in the neonates, the expression of Sp1, the most widely distributed Sp/KLF member, is significantly decreased in rodent and human tissues with increasing age (166, 167). Studies in this laboratory show that in mouse xenograft studies, Sp1, Sp3 and Sp4 expression is low in liver (168) and also in more proliferative tissues such as the gastrointestinal tract (Figure 21D). In contrast, expression of Sp1, Sp3 and Sp4 is high in breast, colon, pancreatic, prostate and bladder cancer cells (153, 159, 162-165, 168, 169, 229) and in other cancer cell lines (data not shown). RNA interference studies in which Sp1,

Sp3 and Sp4 are knocked down simultaneously demonstrate that these transcription factors regulate several genes critical for cancer cell survival, angiogenesis and proliferation (153, 165, 168, 169, 229). For example, in bladder cancer cells, results of RNA interference studies show that Sp1, Sp3 and Sp4 regulate expression of EGFR, cyclin D1, survivin, bcl-2, VEGF and NFκB-dependent activity and this is only a partial list of Sp-regulated genes (165).

Therefore, based on the overexpression of Sp proteins in cancer cells and tumors (153, 159, 162-165, 168, 169, 229) and the fact that Sp1 is a negative prognostic factor for survival of patients with some solid tumors (230, 231), we have been investigating the mechanism of action of several anticancer agents that may act, in part, through decreasing expression of Sp transcription factors in tumors. Tolfenamic acid and betulinic acid induce proteasome-dependent degradation of Sp1, Sp3 and Sp4 in pancreatic and prostate cancer cells and tumors (162, 163, 168) and curcumin also induces proteasome-dependent degradation of these proteins in bladder cancer cells (165). The remarkable anticancer activity of arsenic trioxide for treating leukemia and the potential of this drug for treatment of solid tumors coupled with the reported effects of arsenic trioxide on downregulation of several Sp-dependent genes and responses (VEGF, angiogenesis, survivin, bcl-2, NFκB activity) (221-228) prompted us to examine the effects of arsenic trioxide on Sp expression. A previous study with promyeolcytic leukemia cells treated with arsenic trioxide for

10 days showed that Sp1-DNA binding was decreased and this was correlated with increased Sp1 oxidation and not decreased expression of this transcription factor (234). In contrast, we observed that, in KU7 bladder cancer cells, arsenic trioxide (1 μM) decreased expression of Sp1, Sp3 and Sp4 after treatment for 6 days indicating comparable sensitivity in leukemia vs. bladder cancer cells but clearly different effects on Sp1, Sp3 and Sp4 protein expression. The effects of arsenic trioxide on Sp1 (downregulation) observed in this study were also reported in gall bladder carcinoma (227); however, the mechanism of this response and the effects on Sp3 and Sp4 were not determined.

Figures 21 and 22 demonstrate that in a series of urological- and gastrointestinal-derived cancer cell lines, there was a correspondence between their responsiveness to the antiproliferative effects of arsenic trioxide and their downregulation of Sp1, Sp3 and Sp4 proteins. Using KU7 and 253JB-V bladder cancer cells as prototypical models of arsenic trioxide responsive and non-responsive cells, respectively, it was apparent that relatively low concentrations ($\leq$ 5 μM) inhibited KU7 cell proliferation (Fig. 21A) and KU7 tumor growth in athymic nude mice (Figure 21C). Arsenic trioxide also downregulated Sp1, Sp3 and Sp4 in KU7 cells and tumors (Figures 21A and 21C), whereas at comparable concentrations, only minimal effects on growth and Sp downregulation were observed in 253JB-V cells. Results in Figure 22 confirm that arsenic trioxide inhibits growth and induces Sp downregulation at similar concentrations in pancreatic, colon and prostate cancer cell lines. A comparison

of arsenic trioxide induced antiproliferative, antiangiogenic and proapoptotic genes/responses in KU7 and 253JB-V cells (Figure 23) also demonstrated comparable cell context-dependent responsiveness and non-responsiveness to the effects of arsenic trioxide, respectively. Interestingly, unlike betulinic acid, tolfenamic acid and curcumin (162, 165, 168) (in the same bladder cancer cells), arsenic trioxide did not induce proteasome-dependent degradation of Sp1, Sp3 and Sp4 proteins but decreased Sp1, Sp3 and Sp4 mRNA levels (data not shown). In summary, our results show that arsenic trioxide decreases Sp transcription factors and Sp-dependent genes and results of Sp protein knockdown studies in bladder cancer cells (165) (Figure 24D) indicate that the effects of arsenic trioxide on Sp proteins contribute to the growth inhibitory, proapoptotic and antiangiogenic activity of this compound.

Previous studies in 19 different cancer cell lines (including bladder cancer cells) reported that their differential responsiveness to the antiproliferative effects of arsenic trioxide were related, in part, to their expression of GSH (236). Moreover, since intracellular GSH is an important buffer against mitochondrial disruption and ROS which is rapidly induced by arsenic trioxide in cancer cell lines (204, 205, 211, 213-218), we hypothesized that ROS/GSH levels may be responsible for the cytotoxic/proapoptotic effects of arsenic trioxide and be an important determinant for regulating Sp expression. Absolute levels of ROS were similar in KU7 and 253JB-V cells, but there was a 47% higher level of GSH in 253JB-V compared to KU7 cells and this may contribute to their

responsiveness to arsenic trioxide. It was also apparent that arsenic trioxide induced ROS and decreased GSH in KU7 cells and these responses were ameliorated after cotreatment with the thiol antioxidants GSH or DTT (Figure 24A). GSH and DTT also significantly protected against the antiproliferative effects of arsenic trioxide in KU7 cells (Figure 24B) and inhibited downregulation of Sp1, Sp3 and Sp4 proteins in KU7 cells treated with arsenic trioxide (Figure 24C). In contrast, the decrease in GSH levels in 253JB-V cells treated with arsenic trioxide (Fig. 25A) was much less than observed in KU7 cells and, in the former cell line, this was not accompanied by changes in ROS. However, depletion of GSH by diethyl maleate in 253JB-V cells sensitized this "non-responsive" cell line to arsenic trioxide-mediated antiproliferative and proapoptotic activity and downregulation of Sp proteins (Figure 25). These results in KU7 and 253JB-V cells suggest that induction of ROS by arsenic trioxide is a key element in the subsequent downregulation of Sp proteins and ROS has been directly linked to the cytotoxicity of arsenic trioxide and other mitochondriotoxic anticancer drugs (205, 215, 216, 237).

Hydrogen peroxide and other pro-oxidants are cytotoxic to various transformed cell lines (234). The linkage between ROS and downregulation of Sp proteins was investigated in KU7 cells treated with 500 μM hydrogen peroxide for 24 hr (Figure 26A). Like arsenic trioxide, hydrogen peroxide inhibited growth and decreased expression of Sp1, Sp3 and Sp4 proteins in KU7 cells and these responses were blocked after cotreatment with the antioxidant

glutathione. Arsenic trioxide induces ROS by several pathways and this includes direct effects on mitochondria and thiol-containing mitochondrial proteins which leads to decreased MMP, release of proapoptotic factors such as cytochrome c, and induction of ROS (205, 237). Using confocal microscopy and FACS analysis, we showed that both arsenic trioxide and hydrogen peroxide decreased MMP in KU7 cells and this response was partially blocked after cotreatment with GSH (Fig. 26B). Catalase blocked hydrogen peroxide-dependent decrease in MMP in KU7 cells (Figure 26B) and induction of ROS by hydrogen peroxide was also inhibited by catalase (data not shown). Catalase also inhibited induction of ROS by arsenic trioxide (data not shown) but had minimal effects on decreased MMP in KU7 cells treated with arsenic trioxide (Figure 26B). These results suggest that induction of extramitochondrial ROS by arsenic trioxide in KU7 cells has a minimal effect on MMP, indicating that arsenic trioxide-dependent decrease in MMP and induction of ROS are due to direct effects on the mitochondria. This data is also consistent with the effectiveness of thiol reducing agents such as GSH in ameliorating the activity of arsenic trioxide in cancer cells since these agents act not only as antioxidants but also counteract interactions of arsenic trioxide on thiol-containing mitochondrial proteins (205, 237). Arsenic trioxide-mediated induction of ROS and downstream effects have also been linked to activation of the flavoprotein-dependent NADPH oxidase enzyme (219) or inhibition of thioredoxin reductase which can result in activation of downstream stress kinase pathways such as

p38MAPK and JNK (238, 239). A recent report also showed that inhibition of arsenic trioxide-induced ROS by BHA in some leukemia cell lines did not affect induction of apoptosis, suggesting an ROS-independent pathway (240). We therefore compared the effects of catalase and NAC, an additional thiol antioxidant, with BHA, stress kinase inhibitors of p38MAPK and JNK (SB203580 and SP600125), and the NADPH oxidase inhibitor DPI on hydrogen peroxide- and arsenic trioxide-mediated downregulation of Sp1, Sp3 and Sp4 in KU7 cells (Figures 26C and 26D). The results showed that SB203580, SP6000125, DPI and BHA had minimal to non-detectable effects on arsenic trioxide-induced downregulation of Sp1, Sp3 and Sp4 proteins, suggesting that the major pathway targeting these transcription factors involves mitochondria and induction of ROS.

Low dose toxicity of arsenic trioxide in endothelial cells and increased growth of some tumors has been associated with increased angiogenesis (241-243). In contrast, this study shows that 1.0 μM arsenic trioxide decreased KU7 cell growth and expression of Sp1, Sp3 and Sp4 and this corresponded to comparable effects of higher concentrations of arsenic trioxide in several different cancer cell lines (Figures 21 and 22). However, the overall contributions of downregulation of Sp transcription factors to the anticancer activity of arsenic trioxide will be variable and dependent on cancer cell and tumor type and other activities and/or pathways activated by this compound. Currently, we are investigating the role of drug-induced ROS and specific

oxidative stress pathways that are important for downregulation of Sp transcription factors in cancer cells and the mechanism of oxidative stress-Sp1/Sp3/Sp4 interactions. In contrast to results of a recent study in colon cancer cells (163), arsenic trioxide did not affect expression of microRNA-27a and ZBTB10, an Sp-repressor (data not shown) in KU7 cells; however, the role of other microRNAs as proximal regulators of other Sp repressor proteins is currently being investigated in bladder and other cancer cell lines and tumors.

IV. INHIBITION OF NFκB AND PANCREATIC CANCER CELL AND TUMOR GROWTH BY CURCUMIN IS DEPENDENT ON SPECIFICITY PROTEIN DOWNREGULATION

Curcumin activates diverse anticancer activities that lead to inhibition of cancer cell and tumor growth, induction of apoptosis, and antiangiogenic responses. In this study, we observed that curcumin inhibits Panc28 and L3.6pL pancreatic cancer cell and tumor growth in nude mice bearing L3.6pL cells as xenografts. In addition, curcumin decreased expression of p50 and p65 proteins and NFκB-dependent transactivation and also decreased Sp1, Sp3 and Sp4 transcription factors which are overexpressed in pancreatic cancer cells. Since both Sp transcription factors and NFκB regulate several common genes such as cyclin D1, survivin and vascular endothelial growth factor that contribute to the cancer phenotype, we also investigated interactions between Sp and NFκB transcription factors. Results of Sp1, Sp3 and Sp4 knockdown by RNA interference demonstrate that both p50 and p65 are Sp-regulated genes and that inhibition of constitutive or tumor necrosis factor-induced NFκB by curcumin is dependent on downregulation of Sp1, Sp3 and Sp4 proteins by this compound. Curcumin also decreased mitochondrial membrane potential and induced reactive oxygen species in pancreatic cancer cells, and this pathway is required for downregulation of Sp proteins in these cells, demonstrating that the mitochondriotoxic effects of curcumin are important for its anticancer activities.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a major cause of cancer-related deaths in developed countries and, in 2009, it is estimated that in excess of 34,000 new cases will be diagnosed in the United States (2). PDAC is a highly aggressive disease that invariably evades early diagnosis (244). The mean survival time for patients with metastatic disease is only 3 – 6 months, and only 20-30% of pancreatic cancer cases are alive after 12 months. Several factors are associated with increased risk for pancreatic cancer and these include chronic pancreatitis, prior gastric surgery, smoking, diabetes, exposure to certain classes of organic solvents, radiation, and specific gene polymorphisms (245, 246). In addition to heritable mutations, several acquired gene mutations have been identified in sporadic pancreatic tumors (247, 248). The K-ras oncogene is primarily mutated in codon 12 in >90% of pancreatic tumors and the mutation results in a constitutively active form of ras which can lead to increased cell proliferation. Mutations in the cyclin-dependent kinase inhibitor p16, the tumor suppressor gene p53, and SMAD4, a downstream target of transforming growth factor β (TGFβ) also exhibit high mutation frequencies in pancreatic tumors.

Since pancreatic cancers are frequently detected at an advanced stage, treatments have provided very limited improvements in tumor regression and overall survival times after diagnosis (249, 250). 5-Fluorouracil (5-FU) alone or in combination with other drugs has been extensively used for treatment of

advanced pancreatic cancer, and gemcitabine, a deoxycytidine analog (or antimetabolite), has partially replaced 5-FU as a treatment for pancreatic cancer. Gemcitabine provides increased clinical benefits in terms of response rate, time to progression, and median survival and several other drugs for treatment of pancreatic cancer are also being investigated (251-254). Curcumin (diferuloylmethane) is a polyphenolic phytochemical that exhibits a broad spectrum of anticancer activities against multiple tumor types (255-257), including pancreatic cancer (258-262). Curcumin decreased survival and induced apoptosis in pancreatic cancer cells and, in the same cells, curcumin also decreased the pro-survival nuclear factor κB (NFκB) DNA binding in a gel mobility shift assay (258). Treatment of athymic nude mice with orthotopically implanted tumors with 1 g/kg curcumin daily did not inhibit tumor volume but in combination studies, curcumin enhanced the activity of gemcitabine as an inhibitor of pancreatic tumor growth (260). Curcumin also decreased several NFκB-regulated genes in tumors and these include cyclin D1, c-myc, bcl-2, cyclooxygenase-2 (COX-2), and vascular endothelial growth factor (VEGF) (260).

Recent studies in this laboratory demonstrated that the anticancer activity of curcumin in bladder cancer cells and tumors was associated with repression of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 which was accompanied by decreased expression of Sp-regulated survival, angiogenic and growth promoting genes (165). In this study, we show that curcumin also

decreased expression of Sp proteins and Sp-dependent gene products in pancreatic cancer cells and in mouse tumors (xenograft). Moreover, in pancreatic cancer cells, the p65 and p50 subunits of NFκB are also Sp-regulated genes and inhibition of constitutive and induced NFκB expression by curcumin is also due, in part, to downregulation of Sp transcription factors. Moreover, the mechanism of Sp downregulation by curcumin is due to the mitochondriotoxicity of this compound and the subsequent induction of reactive oxygen species (ROS).

Materials and methods

Cell lines

The Panc28 cell line was a generous gift from Dr. Paul Chiao and L3.6pL cells were kindly provided by Dr. Isaiah Fidler (Univesity of Texas M.D. Anderson Cancer Center, Houston, TX).

Antibodies and reagents

Both pancreatic cancer cell lines were maintained in DMEM-F12 supplemented with 5% FBS, 0.22% sodium bicarbonate, and 10 mL/L of 100X antibiotic/antimycotic cocktail solution (Sigma-Aldrich Co., St. Louis, MO). Cells were grown in 150 cm^2 culture plates in an air/CO_2 (95:5) atmosphere at 37°C. Cyclin D1, Sp3, Sp4, VEGF and p50 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP and COX-2 antibody were purchased from Cell Signaling Technology (Danvers, MA) and Sp1

antibody was purchased from Millipore (Billerica, MA). Survivin antibody was purchased from R&D systems (Minneapolis, MN). NFκB-p65 antibody was ordered from Abcam (Cambridge, MA). Monoclonal β-actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase substrate for western blot analysis was obtained from Millipore. Dithiothreitol and γ-L-glutamyl-L-cysteinyl-glycine (GSH) were obtained from Sigma-Aldrich. TNFα was purchased from R&D systems. Curcumin (98% pure) was purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). LipofectAMINE and LipofectAMINE 2000 were purchased from Invitrogen (Carlsbad, CA). Luciferase reagent was from Promega (Madison, WI). β-Galactosidase reagent was obtained from Tropix (Bedford, MA). The VEGF and survivin promoter constructs were provided by Drs. Gerhard Siemeister and Gunter Finkenzeller (Institute of Molecular Medicine, Tumor Biology Center, Freiburg, Germany) and Dr. M. Zhou (Emory University, Atlanta, GA). Sp1 and Sp3 promoter constructs were kindly provided by Drs. Carlos Cuidad and Veronique Noe (University of Barcelona, Barcelona, Spain). NFκB promoter construct was purchased from Stratagene (Cedar Creek, TX).

Cell proliferation assay

Pancreatic cancer cells ($1X10^5$ per well) were plated in 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO)

or GSH or DTT and/or curcumin were added. Cells were then trypsinized and counted at the indicated times using a Coulter Z1 particle counter. Each experiment was done in triplicate and results are expressed as means ± SE for each treatment group.

Transfection and luciferase assay

The pancreatic cancer cells ($1x10^5$ per well) were plated in 12-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 24 hr, various amounts of DNA [i.e., 0.4 μg PGL2-Luc, 0.4 μg PGL2-Luc, 0.04 μg β-galactosidase, and 0.4 μg pSp1 (4)-Luc or 0.4 μg pSp3-Luc or 0.4 μg VEGF (2068)-Luc or 0.4 μg pSurvivin (269)-Luc] were transfected using Lipofectamine reagent according to the manufacturer's protocol. Five hr post-transfection, the transfection mix was replaced with complete medium containing either vehicle (DMSO) or the indicated compound in DMSO. After 22 hr, cells were then lysed with 100 μL of 1X reporter lysis buffer, and cell extracts (30 mL) were used for luciferase and β-galactosidase assays. A Lumicount luminometer was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

Western blots

Pancreatic cancer cells were seeded in DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped FBS and after 24 hr, cells were treated with either vehicle (DMSO) or the indicated compounds. Cells were collected using

high-salt buffer [50 mmol/L HEPES, 0.5 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, and 1% Triton-X-100] and 10 μL/mL of Protease Inhibitor Cocktail (Sigma-Aldrich). Protein lysates were incubated for 3 min at 100°C before electrophoresis, and then separated on 10% SDS–PAGE 120 V for 3 to 4 hr. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by wet electroblotting in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol for 1.5 hr at 180 mA. Membranes were blocked for 30 min with 5% TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, and 5% nonfat dry milk] and incubated in fresh 5% TBST-Blotto with 1:500 primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody (1:5000) in 5% TBST-Blotto for 2 hr by gentle shaking. The membrane was washed with TBST for 10 min, incubated with 6 mL of chemiluminescence substrate for 1 min, and exposed to Kodak image station 4000mm Pro (Carestreamhealth, Woodbridge, Connecticut).

Electrophoretic mobility shift assay

Cells were rinsed in cold PBS buffer and harvested in reporter lysis buffer (Promega). After 15-min incubation on ice and 10-min centrifugation at 16,000 x g, 4°C, the pellet was resuspended in 1X reporter lysis buffer (Promega) supplemented with 0.5 mol/L KCl and incubated on ice for 30 min. The supernatant containing nuclear proteins was collected after centrifugation for 10 min at 16,000 x g, 4°C and quantified for protein concentrations by Bradford

method. The NFκB probe was prepared by annealing the two complementary polynucleotides and NFκB sense strand probe was 5'-AGT TGA GGG GAC TTT CCC AGG C-3'. The annealed probe was 5'-end–labeled using T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P] ATP (Perkin-Elmer). The labeled probe was purified with the Chroma Spin TE-10 column (BD Biosciences, San Jose, CA). The electrophoretic mobility shift assay reaction was carried out in the reporter lysis buffer supplemented with 0.1 mol/L KCl. Each reaction contained 2 μg nuclear protein, 1 μg of poly(dI-dC) (Roche Molecular Biochemicals) with or without unlabeled competitor oligonucleotides, and 10 fmol of labeled probe; the mixture was incubated for 15 min on ice. Protein-DNA complexes were resolved by 5% native PAGE at 160 V at room temperature for 1.5 hr and visualized after exposing it to ImageTek-H autoradiography X-Ray film.

siRNA interference assays

SiRNAs for Sp1, Sp3, Sp4, p65, p50 and LMN were purchased from Sigma-Aldrich. The siRNA complexes used in this study are indicated as follows:

LMN SASI_Hs02_00367643

Sp1 SASI_Hs02_00363664

Sp3 5'-GCG GCA GGU GGA GCC UUC ACU TT

Sp4 5'-GCA GUG ACA CAU UAG UGA GCT T

p65 (REL1096) 5'-GAT TGA GGA GAA ACG TAA ATT

p50 (REL 1911) 5'-GTC ACT CTA ACG TAT GCA ATT

The Panc28 and L3.6pL pancreatic cancer cell lines were seeded (6×10^4 per well) in 12-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotic and left to attach for 1 d. The triple Sp siRNA knockdown (iSp1, iSp3, iSp4 complex) along with iLamin as control was performed using LipofectAMINE 2000 transfection reagent as per the manufacturer's instructions.

Xenograft study

Female athymic nude mice, age 4 to 6 weeks, were purchased from Harlan. L3.6pL cells (3×10^5) in 1:1 ratio of Matrigel (BD Biosciences) were injected into the either side of the flank area of nude mice. Seven days after the tumor cell inoculation, mice were divided into two groups of 10 animals each. The first group received 100 μL vehicle (corn oil) by i.p. injection, and the second group of animals received 100 mg/kg/d injection of curcumin in corn oil every 2nd day for 18 d (9 doses) by i.p. injection. The mice were weighed, and tumor areas were measured throughout the study. After 20 d, the animals were sacrificed; final body and tumor weights were determined and plotted.

GSH estimation

GSH-Glo Glutathione assay kit (Promega) was used to estimate GSH levels according to manufacturer's protocol using a 96 well cell culture plate and luminescence is measured using a Lumicount luminometer.

ROS estimation

Cellular ROS levels were evaluated with the cell permeant probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen. Following 20-24 hr treatment, cells plated on 96 well cell culture plate were loaded with 10 μM CM-H_2DCFDA for 30 min, washed once with serum free medium, and analyzed for ROS levels using the BioTek Synergy 4 plate reader (Winooski, VT) set at 480 nm and 525 nm excitation and emission wavelengths, respectively. Following reading of ROS, cultures were then treated with Janus green and cell counts were determined with the plate reader set to an absorbance of 610 nm, and ROS intensities were then corrected accordingly. Each experiment was done in triplicate and results are expressed as means ± SE for each treatment group.

Measurement of mitochondrial membrane potential (MMP)

MMP was measured with Mitochondrial Membrane Potential Detection Kit (Stratagene) according to manufacturer's protocol using JC-1 dye and mitochondrial membrane potential shift was measured using FACS Calibur flow cytometer using CellQuest acquisition software (Becton Dickinson

Immunocytometry Systems). J-aggregates are detected as red fluorescence and J-monomers are detected as green fluorescence.

Statistical analysis

Statistical significance of differences between the treatment groups was determined by an analysis of variance and Student's t-test, and levels of probability were noted. IC_{50} values were calculated using linear regression analysis and expressed in μM, at 95% confidence intervals.

Results

Curcumin inhibits constitutive NFκB

Figure 27 illustrates the concentration-dependent inhibition of Panc28 and L3.6pL pancreatic cancer cell proliferation after treatment with 10 - 50 μM curcumin for 24 hr, and IC_{50} values for this response were 34.0 and 28.8 μM, respectively. After prolonged treatment of these cells for 96 and 144 hr, IC_{50} values were 12.4 and 11.2 μM and 11.8 and 9.9 μM in Panc28 and L3.6pL cells, respectively, and concentrations of curcumin required for growth inhibition decreased with increasing treatment times as observed for many anticancer drugs. Interactions between curcumin and NFκB signaling have been extensively reported (255-257). Figure 27B illustrates western blots of nuclear extracts from DMSO- and curcumin (35 and 50 μM)-treated L3.6pL and Panc28 cells; 35 μM decreased p65 and p50 protein levels in L3.6pL but not Panc28 cells, whereas 50 μM curcumin decreased expression of both proteins in both

cell lines. It has also been reported that curcumin decreases NFκB binding to its cognate response element in gel mobility shift assays (258), and Figure 27C summarizes binding nuclear extracts from L3.6pL cells treated with DMSO (solvent) or curcumin (25 and 50 μM) to an oligonucleotide containing a consensus NFκB site. The free probe in the absence of nuclear extract (lane 1) did not form a retarded band; however, nuclear extracts from solvent-treated cells formed an NFκB retarded band (lane 2, indicated by an arrow). The retarded band intensity was decreased when nuclear extracts from cells treated with 25 or 50 μM curcumin were used (lanes 3 and 4). The intensity of the NFκB-DNA complex (lane 2) was decreased after competition with 100-fold excess of unlabeled wild-type (lane 5) but not mutant (lane 6) NFκB oligonucleotide. In addition, the intensity of the NFκB-DNA complex was also decreased after incubation with p50/p65 (combined) antibodies due to immunodepletion (lane 7); however, we did not observe a supershift complex with these antibodies. We also investigated the effects of curcumin on NFκB-dependent transactivation in pancreatic cancer cells transfected with pNFκB-luc, a construct containing 5 tandem NFκB response elements linked to a luciferase reporter gene. The results show that curcumin decreased luciferase activity (Figure 27D) and this was consistent with results in Figures 27A - 27C showing that curcumin repressed constitutive NFκB primarily through downregulation of p50 and p65 proteins.

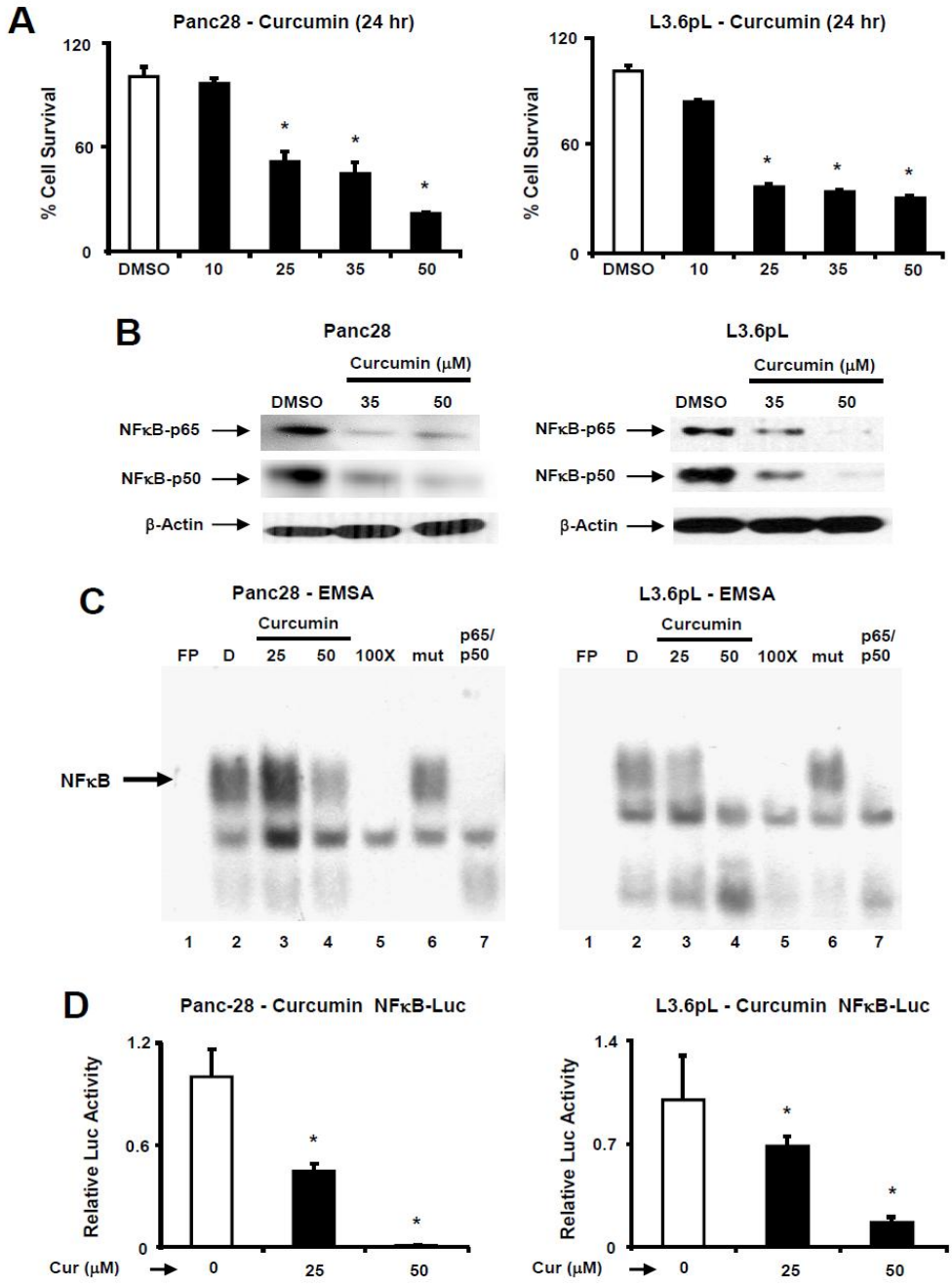

Figure 27. Curcumin inhibits pancreatic cancer cell growth, decreases expression of p65, p50 proteins, NFκB-DNA binding and transactivation of the NFκB promoter.

(A) Inhibition of Panc28 and L3.6pL cell growth. Cells were treated with DMSO (solvent control) or 10, 25, 35 or 50 μmol/L curcumin, and effects on cell growth were determined after treatment for 24 hr as described in the Materials and Methods. (B) Effects of curcumin on p65 and p50 subunits of NFκB in Panc28 and L3.6pL cells. Cells were treated with DMSO (0), 35 or 50 μmol/L curcumin for 24 hr, and p65 and p50 protein levels in nuclear extracts were determined as described in Materials and Methods. β-Actin served as loading control. (C) Gel mobility shift assay. Panc28 and L3.6pL cells were treated with DMSO or 25 or 50 μmol/L curcumin for 24 hr, and nuclear lysates were incubated with ^{32}P-labelled GC rich oligonucleotide alone or in the presence of other factors. Retarded bands were analyzed by electrophoretic mobility shift assay as described in Materials and Methods. (D) Decrease in transactivation of NFκB promoter. Panc28 and L3.6pL cells were transfected with NFκB-luc construct, then treated with DMSO or 25 and 50 μM of curcumin, and luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group, and significant (P<0.05) compared to the solvent (DMSO) control are indicated by an asterisk.

Curcumin decreases Sp transcription factors and Sp-dependent responses

Recent studies in this laboratory showed that curcumin decreased expression of the Sp transcription factors Sp1, Sp3 and Sp4 in bladder cancer

cells (165). We also observed that these proteins were overexpressed in L3.6pL (Figure 28A) and Panc28 (Figure 28B) cells, and curcumin induced a concentration-dependent decrease of these proteins in both cell lines. In bladder cancer cells, curcumin-induced Sp downregulation was blocked by proteasome inhibitors; however, results in Figure 28B show that the proteasome inhibitor MG132 did not alter the effects of curcumin on Sp1, Sp3 and Sp4 protein expression in pancreatic cancer cells. Curcumin also decreased expression of several Sp-dependent genes in Panc28 and L3.6pL cells and these included VEGF, VEGFR1, cyclin D1 and survivin (Figure 28C). This was accompanied by increased PARP cleavage, a marker of apoptosis. Using Panc28 cells as a model, curcumin decreased luciferase activity in cells transfected with constructs containing GC-rich promoter inserts from the Sp1 (pSp1For4), Sp3 (pSp3For5), VEGF (pVEGF) and survivin (pSurvivin) genes linked to a luciferase reporter gene (Figure 28D). Thus, curcumin decreased expression of Sp1, Sp3, Sp4 and several Sp-dependent gene products in pancreatic cancer cells.

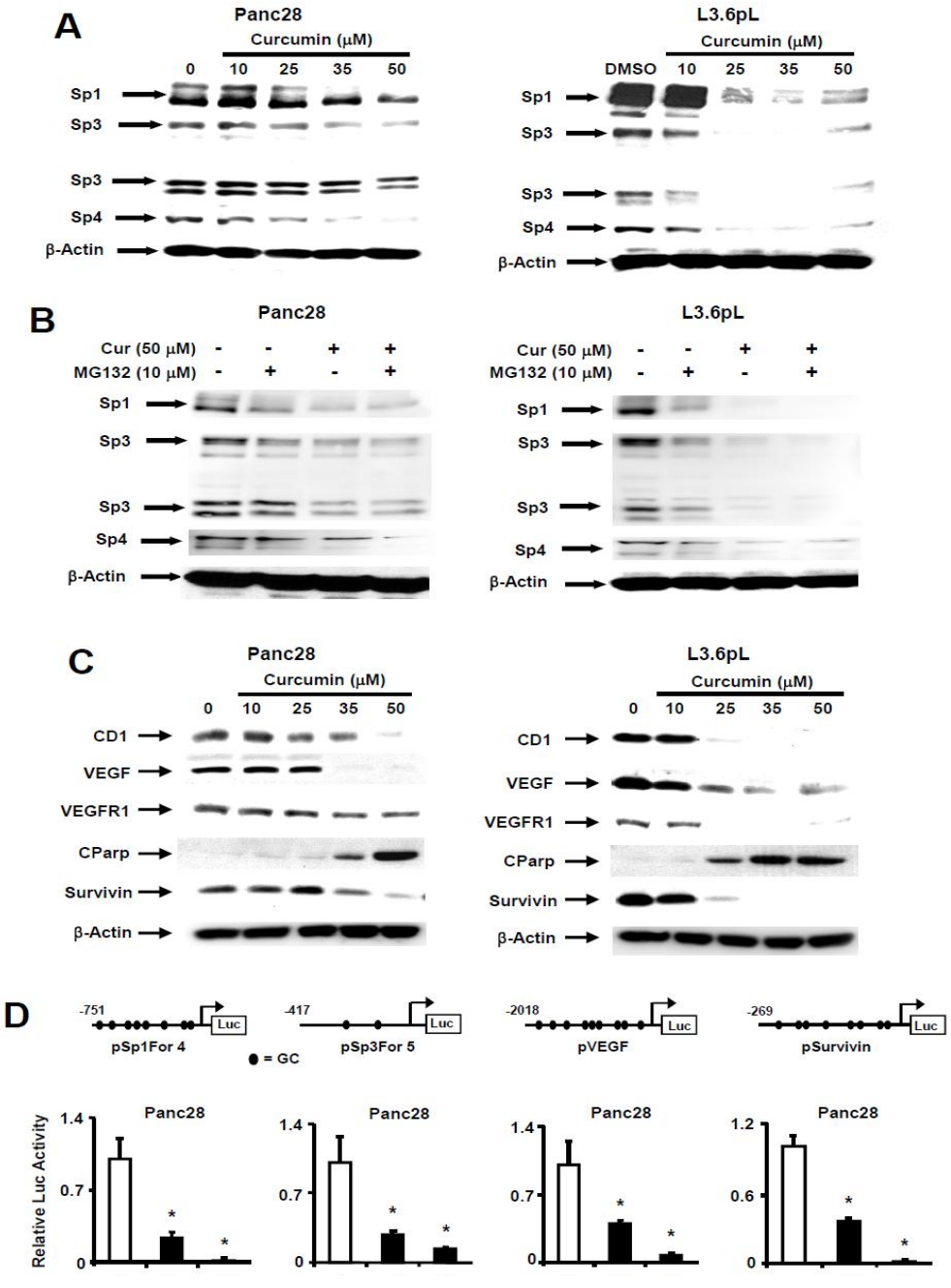

Figure 28. Curcumin activates proteosome-independent downregulation of Sp proteins, decrease cell growth, angiogenic and apoptotic proteins and their promoters.

(A) Decreased Sp proteins Panc28 and L3.6pL cells were treated with DMSO or 10, 25, 35 and 50 μmol/L curcumin for 24 hr and whole-cell lysates were analyzed by western blot analysis as described in Materials and Methods. (B) Curcumin causes proteasome-independent Sp degradation. Cells were treated with DMSO or 50 μmol/L curcumin in the presence or absence of proteasome inhibitor MG132, and the effects on Sp protein degradation were determined after treatment for 24 hr by western blot as described in Materials and Methods. (C) Curcumin decreases expression of Sp-dependent gene products. Panc28 and L3.6pL cells were treated with DMSO or 10, 25, 35 or 50 μmol/L curcumin for 24 hr, and whole-cell lysates were analyzed by western blot analysis as described in Materials and Methods. β-Actin served as a loading control. (D) Curcumin decreases transactivation in cells transfected with Sp1, Sp3, VEGF and survivin promoter constructs. Cells were treated with DMSO (solvent control) or 25 or 40 μmol/L curcumin, and the effects on transactivation of promoters were determined after treatment for 24 hr as described in Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group, and significant (P<0.05) decreases in luciferase activity compared to the solvent (DMSO) control are indicated (*).

Sp transcription factors regulate NFκB

The role of Sp transcription factors in regulating p65 and p50 proteins that form the NFκB complex was investigated in Panc28 and L3.6pL cells transfected

with a cocktail (iSp) containing small inhibitory RNAs for Sp1 (iSp1), Sp3 (iSp3) and Sp4 (iSp4) as previously described (165). Figure 29A illustrates that after transfection of these cells with iSp, there was a decrease in p65 and p50 expression and Sp proteins were also downregulated. Similarly, transfection of cells with siRNA for p65 plus p50 (combined) (ip65-50) decreased expression of p65 and p50 proteins (Figure 29B); however, Sp1, Sp3 and Sp4 protein expression was unchanged (data not shown). These results demonstrate that Sp transcription factors regulate expression of p65 and p50. Results in Figure 29C compare the effects of transfection of iSp and ip65-50 on expression of putative NFκB- and Sp-regulated gene products, cyclin D1, VEGF and surviving (153, 158, 162, 165, 168, 260, 263-267) and iSp > ip65-50 in decreasing expression of all three proteins. In these studies, cells were transfected with siRNAs and whole cell lysates were analyzed by western blots and, therefore, the observed extent of downregulation was limited not only by the effectiveness of the siRNAs but also by transfection efficiencies. We also investigated the effects of iSp and individual siRNAs for Sp1, Sp3 and Sp4 on luciferase activity in Panc28 and L3.6pL cells transfected with pNFκB-luc, and luciferase activity was significantly decreased in all treatment groups (Figure 29D).

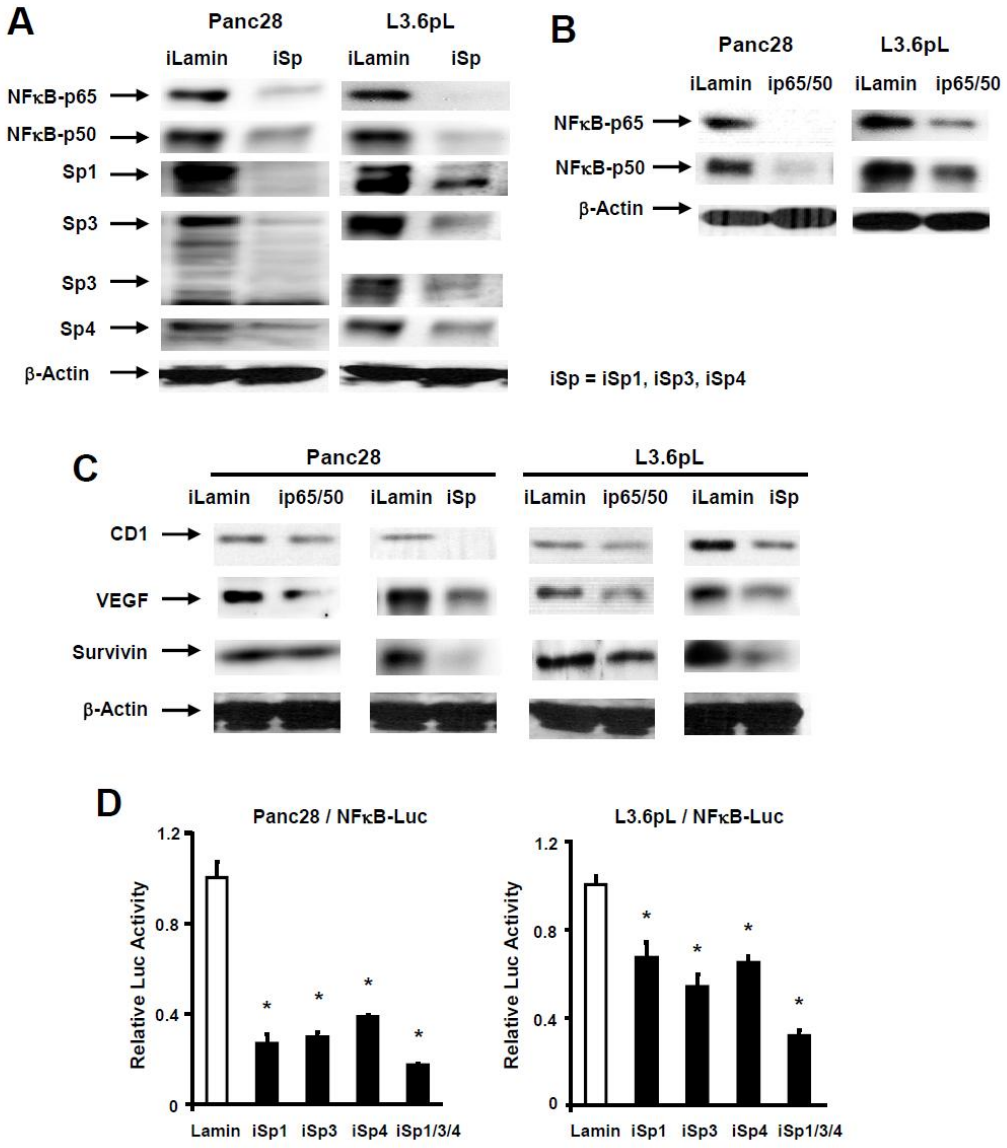

Figure 29. Sp and NFκB knockdown and effects on NFκB subunits, angiogenic and survival proteins.

Sp (A) and NFκB (B) knockdown by RNA interference. Panc28 and L3.6pL were transfected with iSp (A) or ip65/p50 (B) and effects on Sp proteins, and p65 and p50 subunits of NFκB were determined by western blot analysis as

described in Materials and Methods. (C) Effect of Sp and NFκB knockdown on expression of CD1, VEGF and survivin proteins. Protein lysates from Panc28 and L3.6pL cells transfected with iSp or ip65/p50 were analyzed for CD1, VEGF and survivin proteins by western blot analysis as described in Materials and Methods. (D) Effects of iSp on transactivation of NFκB promoter. Cells were transfected iSp and NFκB-luc, and luciferase activity was estimated as described in Materials and Methods. β-Actin and Lamin served as a loading control and similar results were observed in duplicate experiments (A - C). Luciferase activity (D) in transfection experiment was expressed as means ± SE for 3 replicated experiment a significant ($P<0.05$) decreases are indicated (*).

Stressors such as TNFα induce NFκB-dependent responses through increased formation of the nuclear NFκB complex and this was observed in L3.6pL cells, whereas minimal induction by TNFα was observed in Panc28 cells. Using L3.6pL cells as a model, we show that 1 and 10 ng/ml TNFα increased nuclear p65/p50 levels and cotreatment with curcumin decreased TNFα-induced nuclear accumulation of p65/p50 (Figure 30A). Similar results were observed in a gel mobility shift assay (Figure 30B). Nuclear extracts from cells treated with solvent (control) or 1 and 10 ng/ml TNFα formed an NFκB-DNA retarded band using a ^{32}P-labeled consensus NFκB oligonucleotide and TNFα increased retarded band intensity, whereas band intensities decreased using extracts from cells cotreated with TNFα plus curcumin. Retarded band intensities decreased

after cotreatment with unlabeled wild-type but not with mutant NFκB oligonucleotide, and after coincubation with p65 plus p50 antibodies (combined). Figure 30C shows that TNFα had minimal effect on expression of Sp1, Sp3, Sp4 and Sp-dependent gene products VEGF; cyclin D1 was induced and COX-2 expression was the most highly induced by TNFα. Curcumin alone (50 μM) or in combination with TNFα resulted in decreased expression of all of these proteins. Results in Figure 29A show that iSp inhibited basal expression of p65 and p50, and this is confirmed in Figure 30D which also shows that iSp inhibited TNFα-induced p65 and p50 expression in L3.6pL cells. TNFα had minimal effects on Sp expression but enhanced levels of cyclin D1 and COX-2; however, in cells cotransfected with iSp, there was a significant decrease in Sp1, Sp3 and Sp4, and TNFα-induced expression of COX-2 and cyclin D1 was also decreased. These results suggest that although TNFα-dependent induction of cyclin D1 and COX-2 correlated with induction of p65/p50, these responses were blocked after downregulation of Sp transcription factors.

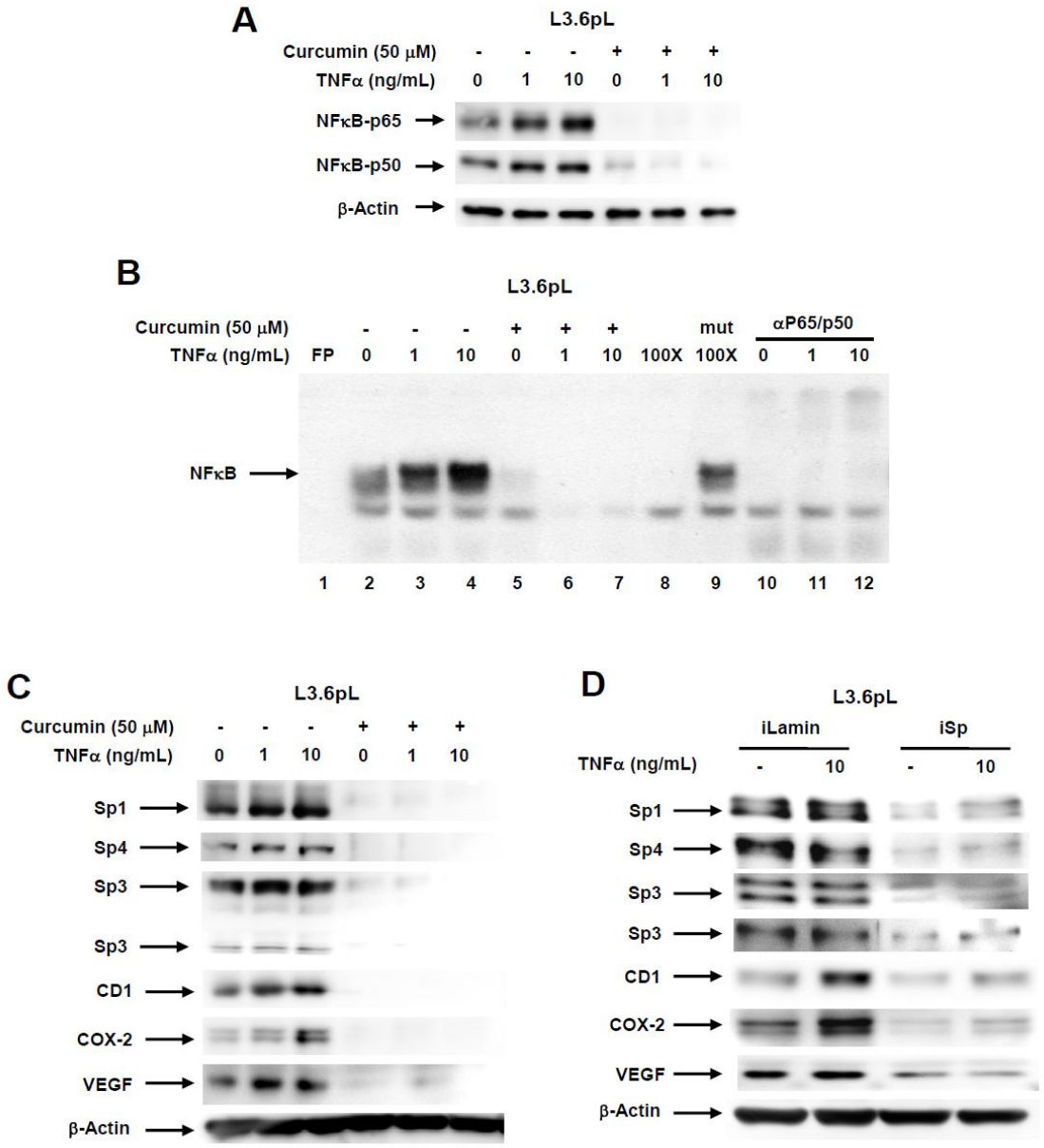

Figure 30. Role of Sp proteins in curcumin-dependent inhibition of TNFα inducible responses in L3.6pL pancreatic cancer cells.

(A) Curcumin decreases TNFα induced expression of p65 and p50 proteins. Cells were treated with TNFα in the presence or absence of 50 μM

curcumin, and the nuclear lysates were examined for expression of p65 and p50 proteins by western blots as described in Materials and Methods. (B) Curcumin decreased TNFα induced NFκB oligonucleotide-protein binding. L3.6pL cells were treated with DMSO or 50 μmol/L curcumin in the presence or absence of TNFα for 24 hr, and nuclear extracts were incubated with ^{32}P-labelled NFκB oligonucleotide alone or in the presence of other factors. Retarded bands were analyzed by electrophoretic mobility shift assay as described in Materials and Methods. Effects of curcumin (C) and iSp (D) on Sp/ NFκB-dependent protein expression. L3.6pL cells were treated with 50 μM curcumin (C) or transfected with iSp (D) in the presence or absence of TNFα, and the whole cell and nuclear lysates were analyzed for Sp1, Sp3, Sp4, p65, p50, CD1, COX-2 and VEGF proteins by western blot analysis as described in Materials and Methods. The gels were typical of results of at least two replicate determinations per treatment group.

Ongoing studies in this laboratory indicate that drug-induced downregulation of mitochondrial membrane potential (MMP) and induction of ROS in cancer cell lines leads to decreased expression of Sp1, Sp3 and Sp4 and this is also observed after treatment with hydrogen peroxide (Unpublished data). Results in Figure 31A show that after treatment of L3.6pL and Panc28 cells with 40 μM curcumin for 20 hr, there was a significant loss of MMP, respectively (also see Supplemental Figure 1). Moreover, in these same cells, cotreatment with curcumin and the thiol antioxidants dithiothreitol (DTT) and

glutathione (GSH), the curcumin-induced loss of MMP was significantly inhibited. In addition, the loss of MMP in L3.6pL and Panc28 cells treated with curcumin was accompanied by induction of ROS which was also significantly attenuated after cotreatment with DTT or GSH (Figure 31B). The role of antioxidants in protecting against curcumin-induced downregulation of Sp1, Sp3 and Sp4 was also investigated in Panc28 and L3.6pL cells. Treatment with curcumin alone decreased expression of Sp1, Sp3 and Sp4 proteins in L3.6pL and Panc28 cells; however, cotreatment with the antioxidants GSH or DTT blocked downregulation of these transcription factors (Figure 31C). The direct effects of ROS on expression of Sp1, Sp3 and Sp4 proteins was determined by treatment of Panc28 and L3.6pL cells with 500 μM hydrogen peroxide and this resulted in decreased expression of Sp1, Sp3 and Sp4 proteins in both cell lines. These effects were inhibited after cotreatment with glutathione (Supplemental Figure 2). It was evident that L3.6pL cells were more resistant to the effects of hydrogen peroxide than Panc28 cells in terms of Sp downregulation. Curcumin-dependent inhibition of Panc28 and L3.6pL cell growth was also reversed in cells cotreated with GSH or DTT and the effects of the antioxidants were more pronounced in Panc28 than L3.6pL cells (Figure 29D).

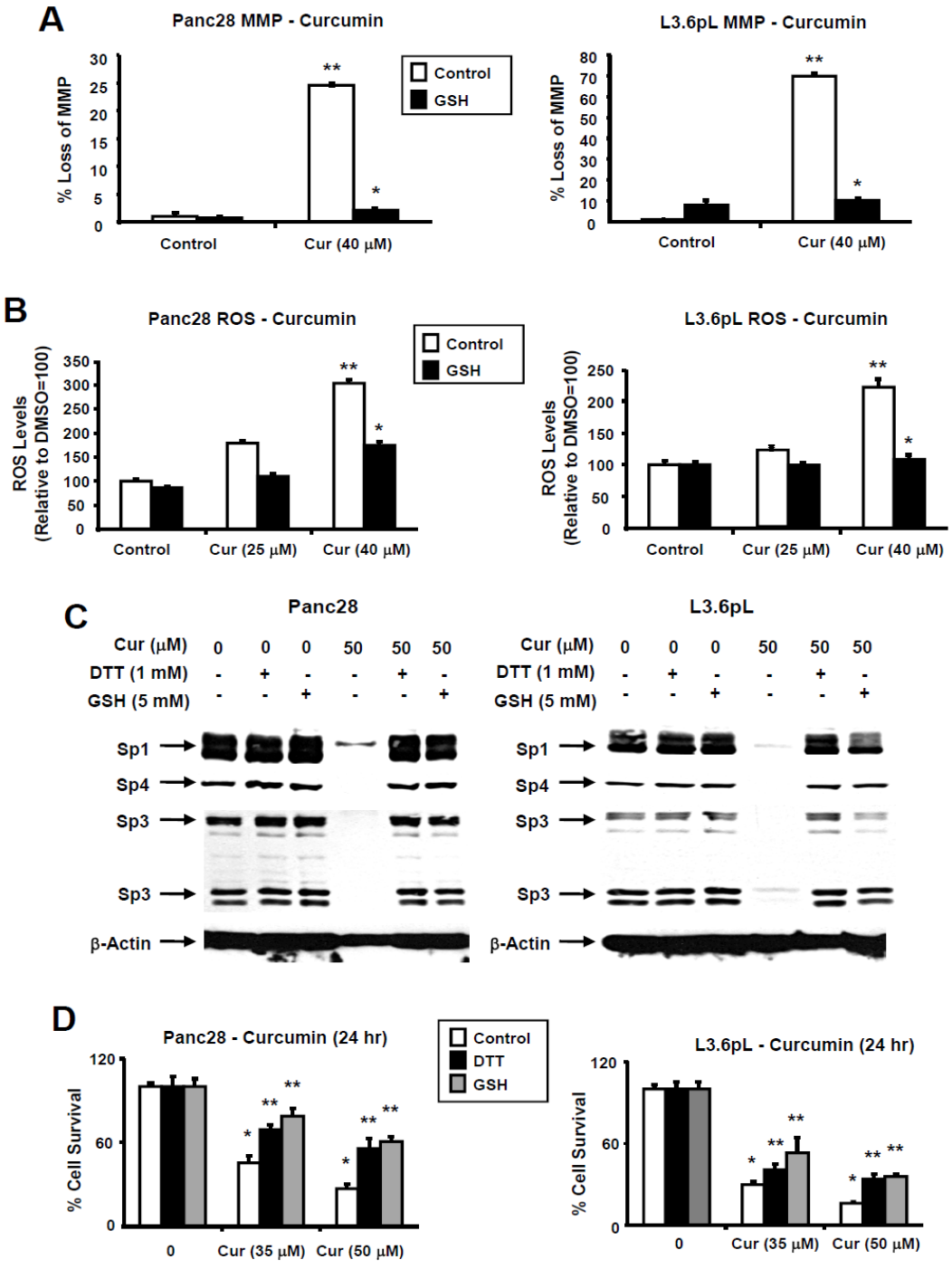

Figure 31. Effects of curcumin on mitochondrial membrane potential (MMP) and ROS and related responses.

Induction of changes in loss of MMP (A) and ROS (B) by curcumin. Panc28 and L3.6pL cells were treated with DMSO or 25 or 40 μmol/L curcumin for 24 hr, in the presence or absence of antioxidant GSH, and mitochondrial membrane potential and ROS were determined as described in Materials and Methods. ROS-mediated Sp degradation (C) cell growth inhibition (D) in the presence or absence of antioxidants. Cells were treated with DMSO or 35 or 50 μmol/L curcumin in the presence or absence of thiol antioxidants for 24 hr, and cells were then counted or the whole cell lysates were analyzed by western blots as described in Materials and Methods. β-Actin served as a loading control. Results in A, B and D are expressed as means ± SE for three replicate determinations for each treatment group, and significant (P<0.05). Curcumin-mediated decreases (*) or increases after cotreatment with antioxidants (**) compared to the solvent (DMSO) control are indicated. GSH levels in Panc28 (4.33 μM) and L3.6pL (2.64 μM) cells were also determined as described in the Materials and Methods.

Curcumin inhibits tumor growth and downregulates Sp transcription factors

The in vivo antitumorigenic activity of curcumin was investigated in athymic nude bearing L3.6pL cells as xenografts. At a dose of 100 mg/kg/d, curcumin inhibited tumor weights (Figure 32A) and growth (Figure 32B) over an 18-day treatment period. We also examined Sp1, Sp3 and Sp4 protein

expression in tumors from the corn oil (control)- and curcumin-treated mice (Figures 32C and 32D). All three transcription factors were decreased after treatment with curcumin; however, only Sp1 and Sp4 were significantly ($p < 0.05$) lower due to the inter-animal variability. Curcumin treatment also decreased cyclin D1 protein expression in tumors. Thus, curcumin-dependent downregulation of Sp transcription factors correlated with the growth inhibitory effects of this compound in both in vitro and in vivo pancreatic cancer cells, suggesting that targeting these transcription factors play a role in the antitumorigenic activity of curcumin.

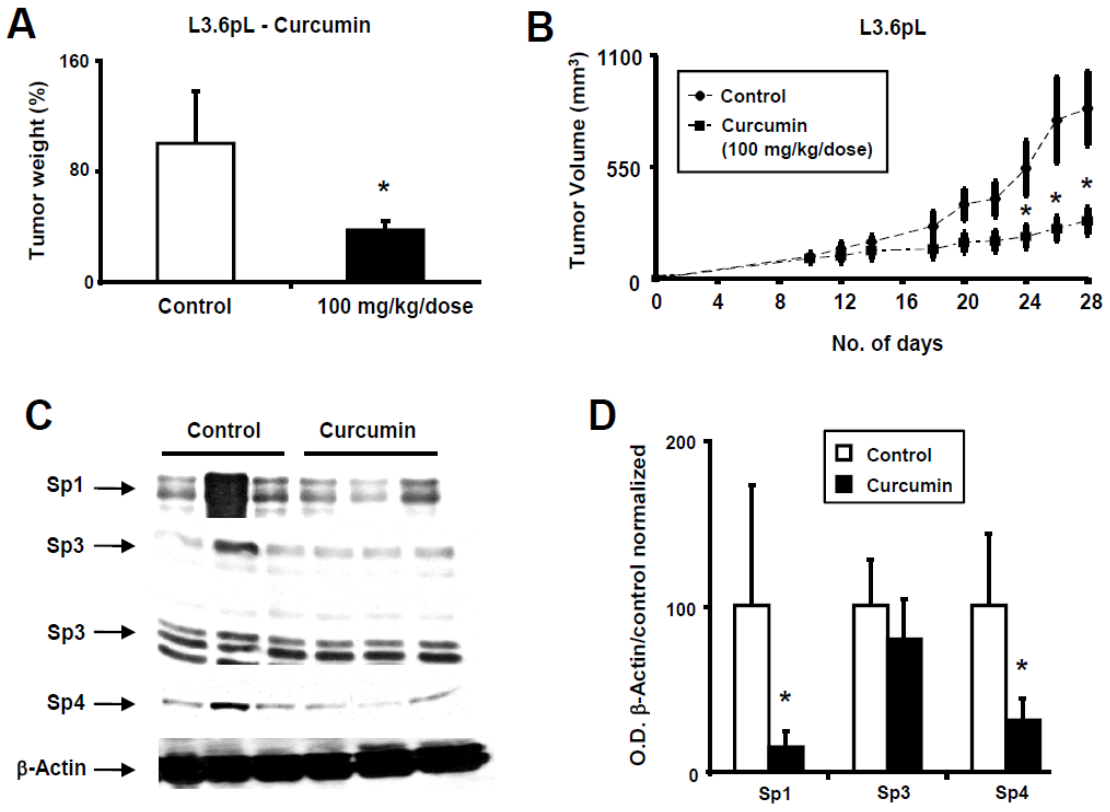

Figure 32. Curcumin inhibits pancreatic cancer xenograft tumor growth.

Tumor weights (A) and volume (B). Athymic nude mice bearing L3.6pL xenografts were treated with corn oil or curcumin (100 mg/Kg/d), and tumor weights and tumor volumes (mm^3) were determined as described in Materials and Methods. (C) Western blot analysis of tumor lysates. Lysates from three mice in the treated and control groups were analyzed by western blots as described in Materials and Methods. β-Actin served as loading control and for standardizing quantitative protein determinations, Sp proteins levels of control animals were set at 100%. Columns, means for three separate determinations; bars, SE. *, significantly (P<0.05) decreased protein levels.

Discussion

The nuclear NFκB complex containing p65 (Rel A) and p50 (NFκB1) or closely related proteins is a multifunctional nuclear transcription factor that regulates expression of multiple genes that promote inflammation and carcinogenesis (263, 265, 267). The inactive cytosolic NFκB-IκB complex is activated and processed through phosphorylation and proteasome-dependent degradation of IκB and this results in enhanced accumulation of nuclear NFκB and modulation of NFκB-dependent gene expression. Upstream activators of nuclear NFκB include various cellular stressors such as cytokines, apoptosis inducers, carcinogens and tumor promoters, ROS, endotoxins, and bacterial and viral infections (263, 265, 267). Activation of NFκB in a cancer cell context results in the induction of cancer cell proliferation, survival, angiogenesis and

metastasis, epithelial to mesenchymal transition (EMT), and inflammation, and this is accompanied by induction of genes such as cyclin D1, survivin, VEGF, bcl-2, and COX-2 that contribute to these responses (263, 265, 267). Curcumin has been extensively characterized as an anti-inflammatory and anticancer agent and these effects have been linked to modulation of several pathways and genes in different cancer cell lines (255-257). In addition, curcumin has been extensively investigated as an inhibitor of basal and induced NFκB-dependent responses and this plays an important role in the remarkable anticancer activities of this compound (255-257).

Studies in this laboratory have been focused on drugs such as tolfenamic acid, betulinic acid and the synthetic triterpenoid methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (CDODA-Me) that also inhibit tumor growth and this is due, in part, to downregulation of Sp1, Sp3 and Sp4 transcription factors (158, 162, 163, 168). These agents also decrease expression of several Sp-dependent genes including survivin, VEGF and its receptors, cyclin D1, bcl-2, EGFR and several other genes. Initial studies showed that Sp1 was highly expressed in many pancreatic cancer cell lines and was required for VEGF expression (268), and it has subsequently been shown that Sp1, Sp3 and Sp4 are highly expressed in pancreatic and other cancer cell lines (Unpublished data) (158, 162, 163, 168, 268). Moreover, a recent report showed that Sp1 was a negative prognostic factor for pancreatic cancer patient survival (160). Many Sp-dependent genes are also co-regulated in some cells by NFκB and not

surprisingly, there is also a striking similarity between Sp- and NFκB-dependent growth inhibitory, angiogenic and survival responses and genes. Moreover, our recent studies with curcumin in bladder cancer cells (165) showed that this compound also decreased expression of Sp transcription factors and Sp-dependent genes, and there was evidence in 253JB-V cells that p65 was also an Sp-regulated gene. Curcumin is currently in clinical trials for pancreatic cancer (262) and we used this tumor type as a model for investigating the effects of this compound on Sp1, Sp3, Sp4 and NFκB and also Sp-NFκB interactions.

Curcumin inhibited Panc28 and L3.6pL cell proliferation (Figure 27A) and decreased expression of both p65 and p50 and their DNA binding activity (Figures 27B and 27C) and luciferase activity in cells transfected with an NFκB-luc construct (Figure 27D). Thus, basal NFκB which is overexpressed in many cancer cell lines and tumors including pancreatic cancer (258), is also inhibited in Panc28 and L3.6pL cells treated with curcumin and this is related, in part, to decreased expression of p65 and p50. In parallel experiments, we also demonstrated that curcumin decreased expression of Sp1, Sp3 and Sp4 (Figure 28A) and several Sp-dependent genes (Figure 28C), and similar results were previously observed in bladder cancer cells (165). However, in bladder cancer cells, Sp downregulation after treatment with curcumin was blocked by the proteasome inhibitor MG-132, whereas in pancreatic cancer cells, MG-132 did not affect curcumin-dependent repression of Sp1, Sp3 and Sp4 (Fig. 28B).

Curcumin decreased pancreatic tumor growth in athymic nude mice bearing L3.6pL cells as xenografts and this was also accompanied by Sp downregulation (Figure 32) and parallels the in vitro effects of curcumin in pancreatic cancer cells (Figure 28). Thus, curcumin decreases both Sp and NFκB transcription factors in pancreatic cancer cells and this is accompanied by decreased expression of several genes that may be regulated by both NFκB and Sp transcription factors, depending on the cell context.

Since curcumin decreased p65 and p50 proteins in Panc28 and L3.6pL cells (Figure 27B), we hypothesized that this response may be dependent, in part, on downregulation of Sp1, Sp3 and Sp4 in pancreatic cancer cells (Figure 28A). Direct evidence for the role of Sp transcription factors in regulating NFκB was obtained by RNA interference in which cells were transfected with iSp, which contained siRNAs for Sp1, Sp3 and Sp4 (in combination). The results showed that knockdown of Sp transcription factors decreased expression of both p65 and p50 proteins (Figure 29A); combined knockdown of p65 and p50 (ip65-p50) by RNA interference decreased expression of both proteins (Figure 29B). Moreover, a comparison of the effects of iSp vs. ip65-p50 on several putative Sp- and NFκB-regulated genes [cyclin D1, VEGF and survivin (Figure 29C)] confirmed that expression of these genes was primarily dependent on Sp transcription factors, and luciferase activity in cells transfected with NFκB-luc was also decreased by Sp knockdown (Fig. 29D). Moreover, while TNFα induced levels of nuclear p65 and p50 proteins (Figs. 30A and 30B) and this

resulted in induction of some NFκB-dependent gene products such as COX-2 (Figure 30C), both curcumin and iSp inhibited not only basal (Figure 30) but TNFα-induced responses (Figure 31) in pancreatic cancer cells. Thus, curcumin-dependent inhibition of NFκB is due, in part, to downregulation of Sp transcription factors and these results are consistent with previous reports showing that the p65 and p50 promoters contain functional GC-rich Sp binding sites and both genes are regulated by Sp1 (269, 270). However, the role of Sp1, Sp3 and Sp4 in regulation of p65 and p50 will also be dependent on cell context since we previously observed that knockdown of Sp transcription factors in bladder cancer cells decreased p65 but not p50 proteins (165).

Ongoing studies in this laboratory have been investigating the mechanisms associated with drug-induced Sp downregulation in cancer cells (Unpublished data) (163), and recently we have shown that induction of ROS is a critical element for this response (163). For example, arsenic trioxide decreased MMP and induced ROS and this resulted in downregulation of Sp1, Sp3 and Sp4 in bladder and pancreatic cancer cells. Treatment with hydrogen peroxide also induced Sp downregulation and in cells cotreated with arsenic trioxide or hydrogen peroxide and the thiol antioxidants DTT or GSH, the effects of Sp protein and cell growth inhibition were reversed (Unpublished data). Previous reports show that curcumin decreases MMP and induces ROS in some cancer cell lines (271-273), and this was also observed in pancreatic cancer cells (Figures 31A and 31B). Moreover, in cells cotreated with curcumin and

GSH or DTT, the effects of curcumin on downregulation of Sp1, Sp3 and Sp4 and growth inhibition were significantly reversed by the antioxidants (Figures 31C and 31D). In addition, hydrogen peroxide also decreased expression of Sp1, Sp3 and Sp4 proteins in Panc28 and L3.6pL cells and cotreatment with glutathione attenuated these effects (Supplemental Figure 2). Interaction of curcumin with pancreatic cancer cell mitochondria, induction of ROS, and the attenuation of curcumin-induced Sp downregulation by antioxidants is also consistent with a role for ROS in regulating expression of Sp1, Sp3 and Sp4. Previous studies showed that among several cancer cell lines, their sensitivity to arsenic trioxide was dependent, in part, on constitutive glutathione levels (236), and the higher levels of glutathione in Panc28 (4.33 μM) vs. L3.6pL (2.64 μM) cells may explain the increased resistance of the former cell line to curcumin-mediated repression of Sp1, Sp3 and Sp4 proteins (Figure 28A). In contrast, we also observed that antioxidants were less effective in reversing curcumin-mediated inhibition of cell proliferation in L3.6pL compared to Panc28 cells (Figure 31D) and this is an example of cell context-dependent differences in the contribution of the ROS-Sp degradation pathway to pancreatic cancer cell growth inhibition.

Thus, like arsenic trioxide and other mitochondriotoxic drugs, curcumin induces ROS in pancreatic cancer cells and this results in downregulation of Sp and Sp-dependent gene products which includes NFκB. These results highlight a novel mechanism of action for curcumin which includes ROS-Sp and Sp-NFκB

interactions and further demonstrates that in pancreatic cancer cells, Sp transcription factors are an important drug target. The downstream targets of curcumin-induced ROS are also being investigated and these include microRNAs such as miR-27a that inhibit expression of the Sp repressor, ZBTB10 (163). Preliminary studies indicated that only minimal induction of ZBTB10 by curcumin is observed in pancreatic cancer cells and a search for activation of other Sp repressor genes is ongoing. Current studies are also focused on development of new agents that repress expression of Sp transcription factors in pancreatic cancer and their applications as stand-alone drugs or in combination with other agents such as gemcitabine for treatment of this devastating disease.

V. METHYL 2-CYANO-3,12-DIOXOOLEANA-1,9-DIEN-28-OATE (CDDO-Me) DECREASES SPECIFICITY PROTEIN (Sp) TRANSCRIPTION FACTORS AND INHIBITS PANCREATIC TUMOR GROWTH

The anticancer agent 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me) typically induce a broad spectrum of growth inhibitory, proapototic and antiangiogenic responses. Treatment of Panc1, Panc28 and L3.6pL pancreatic cancer cells with low μM concentrations of CDDO or CDDO-Me resulted in growth inhibition, induction of apoptosis, and downregulation of cyclin D1, survivin, vascular endothelial growth factor (VEGF) and its receptor (VEGFR2). RNA interference studies indicate that these repressed genes are regulated by specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4, and western blot analysis of lysates from pancreatic cancer cells treated with CDDO and CDDO-Me shows for the first time that both compounds decreased expression of Sp1, Sp3 and Sp4. Moreover, CDDO-Me (7.5 mg/kg/day) also inhibited pancreatic human L3.6pL tumor growth and downregulated Sp1, Sp3 and Sp4 in tumors using an orthotopic pancreatic cancer model. CDDO-Me also induced reactive oxygen species (ROS) and decreased mitochondrial membrane potential (MMP) in Panc1 and L3.6pL cells, and cotreatment with antioxidants (glutathione and dithiothreitol) blocked formation of ROS, reversed the loss of MMP, and also inhibited downregulation of Sp1, Sp3 and Sp4. Repression of Sp and Sp-dependent genes by CDDO-Me was due to downregulation of microRNA-27a and induction of ZBTB10, an Sp

repressor, and these responses were also reversed by antioxidants. Thus, the anticancer activity of CDDO-Me is due, in part, to activation of ROS which in turn targets the microRNA-27a:ZBTB10–Sp transcription factor axis to decrease growth inhibitory, pro-apoptotic and antiangiogenic genes and responses.

Introduction

Extracts of plants and microorganisms and individual natural products have been extensively used as traditional medicines for treatment of several diseases including cancer. Individual natural products including aspirin, morphine, quinine, statins, penicillins, taxanes and many other compounds are widely used pharmaceutical agents and also serve as templates for the synthesis of more potent analogs (274-276). Triterpenoid are derived from cyclization of oxidosqualene, and different cyclization pathways coupled with post-cyclization modifications can give several thousand possible analogs including oleanolic acid which contains a pentacyclic oleanane skeleton and a C28 carboxyl group (277, 278). Oleanolic acid has been used by Sporn, Honda and their collaborators as a template for extensive structure-activity studies to identify antiinflammatory drugs, and the most active compounds identified were 2-cyano-3,12-dioxoleana-1,9-dien-28-oic acid (CDDO) and its corresponding methyl (CDDO-Me) and imidazole (CDDO-Im) esters (176, 177, 279, 280).

The anticancer activities of CDDO and related compounds have been extensively investigated in several different cancer cell lines and *in vivo* and their remarkable potency is due to modulation of several important pathways

[reviewed in (280)]. Initial studies showed that CDDO was a peroxisome proliferator-activated receptor γ (PPARγ) agonist (281); however, most subsequent studies indicate that the anticancer activities of CDDO and related compounds were PPARγ-independent (203, 282-284). The effects of CDDO, CDDO-Me and CDDO-Im vary among different cell lines and are dependent on the specific parameters measured; however, treatment with these compounds invariably resulted in growth inhibition, antiangiogenic activity and induction of apoptosis (280). Induction of these responses is associated with modulation of several pathways and genes including activation of endoplasmic reticulum (ER) stress, microtubule disruption, inhibition of NFκB signaling, and mitochondriotoxicity, resulting in decreased mitochondrial membrane potential (MMP) (185, 285-290). For example, in pancreatic cancer cells, CDDO-Im inhibits cell growth and induces apoptosis and this is associated with decreased MMP and mitochondrial glutathione (GSH) and induction of reactive oxygen species (ROS) (185).

Studies in this laboratory have characterized the anticancer activity of 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oic acid (CDODA) and its methyl ester (CDODA-Me) which are structurally similar to CDDO and CDDO-Me but are derived from the triterpenoid glycyrrhetinic acid, a bioactive component of licorice (145-147, 163, 180). A recent study reported that one of the underlying mechanism of action of CDODA-Me in colon cancer cells was due to downregulation of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4

and Sp-dependent genes (163). In this study, we demonstrate for the first time that CDDO and CDDO-Me also decrease expression of Sp1, Sp3 and Sp4 and Sp-dependent gene products (VEGF, cyclin D1, survivin and VEGFR2) in pancreatic cancer cells and tumors in an orthotopic mouse model. The mitochondriotoxicity of CDDO-Me results in decreased mitochondrial membrane potential (MMP), induction of reactive oxygen species (ROS), ROS-dependent downregulation of microRNA-27a, and induction of ZBTB10 (an Sp repressor protein) which in turn downregulates Sp transcription factors and Sp-dependent genes. Thus, CDDO-Me-dependent repression of Sp1, Sp3 and Sp4 contributes to the potent anticancer activity of CDDO and related compounds.

Materials and methods

Cell lines

Panc28 cell line was a generous gift from Dr. Paul Chiao and L3.6pL cells were kindly provided by Dr. Isaiah Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX) and Panc1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Antibodies and reagents

All three pancreatic cancer cell lines were maintained in DMEM-F12 supplemented with 5% FBS, 0.22% sodium bicarbonate, and 10 mL/L of 100X antibiotic/antimycotic cocktail solution (Sigma-Aldrich Co., St. Louis, MO). Cells were grown in 150 cm^2 culture plates in an air/CO_2 (95:5) atmosphere at 37°C.

Cyclin D1, Sp3, Sp4, VEGF and VEGFR2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP antibody was purchased from Cell Signaling Technology (Danvers, MA) and Sp1 antibody was purchased from Millipore (Billerica, MA). Survivin antibody was purchased from R&D systems (Minneapolis, MN). Monoclonal β-actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase substrate for western blot analysis was obtained from Millipore. Dithiothreitol and γ-L-glutamyl-L-cysteinyl-glycine (GSH) were obtained from Sigma-Aldrich. Superscript II, LipofectAMINE and LipofectAMINE 2000 was purchased from Invitrogen (Carlsbad, CA). Reporter lysis buffer and luciferase reagent were purchased from Promega (Madison, WI). β-Galactosidase reagent was obtained from Tropix (Bedford, MA). Primers for TBP and ZBTB10 were purchased from Integrated DNA Technologies Technologies (Coralville, IA). Primers for Sp3 and Sp4 were obtained from Qiagen (Valencia, CA); ZBTB10 expression vector and empty vector (pCMV6-XL4) were from Origene (Rockville, MD). MiRNA mirvaRNA extraction kits and the reverse transcription (RT) and real-time PCR amplification kits were purchased from Applied Biosystems (Foster City, CA). The VEGF and survivin promoter constructs were provided by Drs. Gerhard Siemeister and Gunter Finkenzeller (Institute of Molecular Medicine, Tumor Biology Center, Freiburg, Germany) and Dr. M. Zhou (Emory University, Atlanta, GA). Sp1 and Sp3 promoter constructs were kindly provided by Drs. Carlos Cuidad and Veronique Noe (University of Barcelona, Barcelona, Spain).

Cell proliferation assay

Pancreatic cancer cells ($3x10^4$ per well) were plated in 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or different doses of CDDO or CDDO-Me were added. Cells were trypsinized and counted every 48 hr using a Coulter Z1 particle counter for 6 days. Fresh medium and test compounds were added every 48 hr. Each experiment was done in triplicate and results are expressed as means ± SE for each treatment group.

Transfection and luciferase assay

Pancreatic cancer cells ($1x10^5$ per well) were plated in 12-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 24 hr, various amounts of DNA [i.e., 0.4 μg PGL2-Luc, 0.4 μg PGL3-Luc, 0.04 μg β-galactosidase, and 0.4 μg pSp1 (4)-Luc or 0.4 μg pSp3-Luc or 0.4 μg VEGF (2068)-Luc or 0.4 μg pSurvivin (269)-Luc] were transfected using Lipofectamine reagent according to the manufacturer's protocol. Five hr posttransfection, the transfection mix was replaced with complete medium containing either vehicle (DMSO) or the indicated compound in DMSO. After 22 hr, cells were then lysed with 100 μL of 1X reporter lysis buffer, and cell extracts (30 mL) were used for luciferase and β-galactosidase assays. A Lumicount

luminometer was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

Transfection with ZBTB10 expression vector

Pancreatic cancer cells ($1x10^5$ per well) were plated in 12-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 24 hr, cells were transfected with empty vector (pCMV6-XL4) or 4 μg/well ZBTB10 expression plasmid pCMV6-XL4 vector using Lipofectamine 2000 reagent according to the manufacturer's protocol. After transfection for 5 hr, the transfection mix was replaced with complete medium and incubated for 48 hr.

Western blots

Pancreatic cancer cells ($3x10^5$ per well) were seeded in 6-well plates in DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped FBS and after 24 hr, cells were treated with either vehicle (DMSO) or the indicated compounds. Cells were collected using high-salt buffer [50 mmol/L HEPES, 0.5 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, and 1% Triton-X-100] and 10 μL/mL of Protease Inhibitor Cocktail (Sigma-Aldrich). Protein lysates were incubated for 3 min at 100°C before electrophoresis, and then separated on 10% SDS–PAGE 120 V for 3 to 4 hr. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by wet electroblotting in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol for 1.5 hr at 180 mA. Membranes were blocked for 30 min with 5% TBST-Blotto [10 mmol/L

Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, and 5% nonfat dry milk] and incubated in fresh 5% TBST-Blotto with 1:500 primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody (1:5000) in 5% TBST-Blotto for 2 hr by gentle shaking. The membrane was washed with TBST for 10 min, incubated with 6 mL of chemiluminescence substrate for 1 min, and exposed to Kodak image station 4000 mm Pro (Carestreamhealth, Woodbridge, Connecticut).

Animals and orthotopic implantation of tumor cells

Male athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). Mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institutes of Health. Mice with 8-12 wk of age were used for the current study. L3.6pL cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in HBSS. Only suspensions consisting of single cells with >90% viability were used for the injections. Injection of cells into

the pancreas was performed as described previously (27). One week after the injection of the cells, 4-5 mice were randomly selected and sacrificed, and the pancreas was isolated from all the animals and tested under microscope to confirm the initiation of tumor growth. The remaining mice were divided into two groups (at least 5 animals/group) and treated with vehicle (control) or 7.5 mg/kg CDDO-Me daily for 4 weeks. Animals were sacrificed and the primary pancreatic tumors were isolated. All the tumors were measured and their weights were recorded. The tumor tissues were properly isolated into 3 portions for preparing (1) protein extracts (snap frozen in liquid nitrogen and stored at -80°C); (2) RNA [treated with RNA stabilization solution (RNAlater) and then stored at -80°C]; and (3) paraffin sections for immunohistochemistry (fixed in formaldehyde).

ROS estimation

Cellular ROS levels were evaluated with the cell-permeable probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen. Following treatment for 20-24 hr, cells plated on 96-well cell culture plates were loaded with 10 μM CM-H_2DCFDA for 30 min, washed once with serum free medium, and analyzed for ROS levels using the BioTek Synergy 4 plate reader (Winooski, VT) set at 480 nm and 525 nm excitation and emission wavelengths, respectively. Following reading of ROS, cultures were then treated with Janus green and cell counts were determined with the plate reader set to an absorbance of 610 nm, and ROS intensities were

then corrected accordingly. Each experiment was done in triplicate and results are expressed as means ± SE for each treatment group.

Measurement of mitochondrial membrane potential (MMP)

MMP was measured with Mitochondrial Membrane Potential Detection Kit (Stratagene Cedar Creek, TX) according to manufacturer's protocol using JC-1 dye. Pancreatic cancer cells were plated on 2-well Lab-Tex Coverglass slides (NUNC, NY) and, after 24 hr, cells were treated with DMSO or CDDO-Me alone or with GSH for 16 hr. Cells were then incubated with 1X JC-1 dye at 37°C for 15 min and washed twice with assay buffer according to manufacturer's protocol and then cells were subjected to microscopic analysis using Zeiss Stallion Dual Detector Imaging System (Carl Zeiss Microimaging Inc., Thornwood, NY) using a C-Apochromat 63X, 1.2 NA water immersion lens. J-aggregates are detected as red fluorescence and J-monomers are detected as green fluorescence. The ratio of red fluorescence to green fluorescence was measured using Image J Software. Cells were examined in more than ten fields per slide on multiple slides. Data represent the average of all the fields.

Quantitative real-time PCR of mRNA and miRNA

cDNA was prepared from Panc1 and L3.6pL cell lines using Superscript II reverse transcriptase (Invitrogen) according to manufacturer's protocol. Each polymerase chain reaction (PCR) was carried out in triplicate in a 20-μl volume using SYBR GreenER (Invitrogen) for of 95°C for 10 min, then 40 cycles of 95°C

for 15 s and 60°C for 1 min in the Applied Biosystems 7500 Fast Real-time PCR System. The following primers were used.

TBP (F): 5'-TGCACAGGAGCCAAGAGTGAA-3'

TBP (R): 5'-CACATCACAGCTCCCCACCA-3'

ZBTB10 (F): 5'-GCTGGATAGTAGTTATGTTGC-3'

ZBTB10 (R): 5'-CTGAGTGGTTTGATGGACAGA-3'

mirVana miRNA extraction kit (Applied Biosystems) was used for extraction of miRNA according to manufacturer's protocol. Quantification of miRNA (RNU6B and miRNA-27a) was done using the Taqman miRNA kit (Applied Biosystems) according to the manufacturer's protocol with real-time PCR. U6 small nuclear RNA was used as a control to determine relative miRNA expression.

Statistical analysis

Statistical significance of differences between the treatment groups was determined by an analysis of variance and/or Student's t-test, and levels of probability were noted. IC_{50} values were calculated using linear regression analysis and expressed in μM, at 95% confidence intervals.

Results

The synthetic oleanolic acid derivatives CDDO, CDDO-Me and CDDO-Im are cytotoxic to several different cancer cell lines and the latter derivative was a

potent inhibitor of pancreatic cancer cell proliferation (185). In this study, we initially compared the effects of CDDO and CDDO-Me on proliferation of Panc1, Panc28 and L3.6pL pancreatic cancer cell lines for up to 144 hr (Figures 33A and 33B). IC_{50} values for growth inhibition by CDDO-Me and CDDO after treatment for 48 hr were 5.2 and 0.37 (Panc1), 5.3 and 0.37 (Panc28), and 3.0 and 0.4 μM (L3.6pL), respectively. CDDO-Me was more active than CDDO in all three pancreatic cancer cell lines and there were minimal differences between cell lines to the cytotoxic effects of both compounds. After prolonged treatment (144 hr) with CDDO-Me and CDDO, IC_{50} values were 0.25 and 1.8 (Panc1), 0.30 and 2.3 (Panc28), and 0.28 and 1.4 μM (L3.6pL), respectively, and the relative potencies of both compounds and cellular responsiveness were similar to that observed after 48 hr (Figure 33). Inhibition of Panc1, Panc28 and L3.6pL cell growth by CDDO and CDDO-Me was also accompanied by apoptosis, and Figures 33C and 33D show that both compounds induced caspase-dependent PARP cleavage in the pancreatic cancer cell lines.

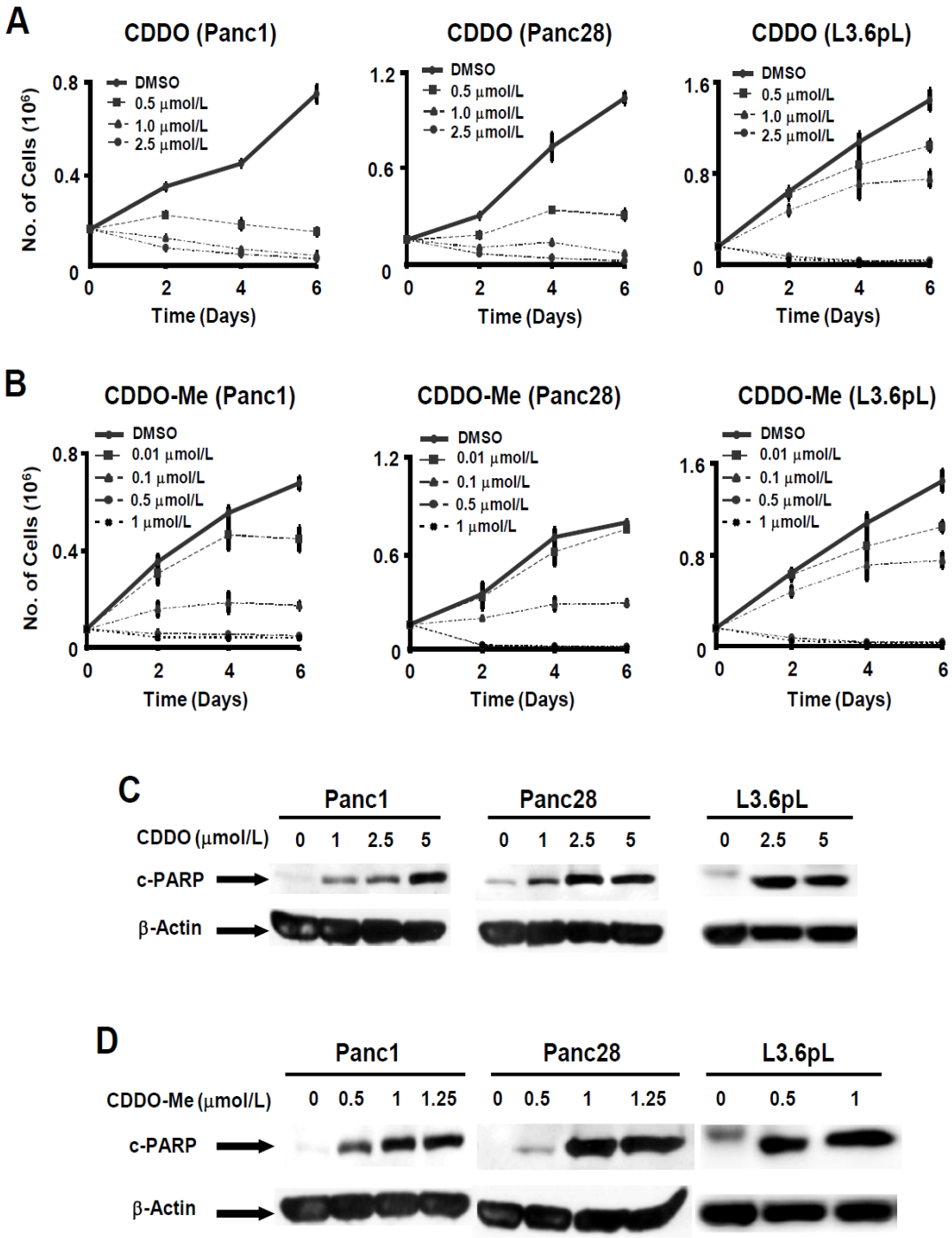

Figure 33. CDDO and CDDO-Me inhibit cell growth and induce apoptosis in pancreatic cancer cell lines.

Inhibition of Panc1, Panc28 and L3.6pL cell growth by CDDO (A) and CDDO-Me (B). Cells were treated with DMSO (solvent control), CDDO (0.5, 1.0 or 2.5 μmol/L), or CDDO-Me (0.01, 0.1, 0.5 or 1.0 μmol/L), and effects on cell growth were determined over a period of 6 days as described in the Materials and Methods. Induction of PARP cleavage by CDDO (C) and CDDO-Me (D). Panc1, Panc28 and L3.6pL cells were treated with DMSO, CDDO (1.0, 2.5 or 5.0 μmol/L), or CDDO-Me (0.5, 1.0 or 1.25 μmol/L) for 24 hr, and whole-cell lysates were analyzed by western blot analysis as described in Materials and Methods. β-Actin served as a loading control.

CDDO and related compounds inhibit growth, induce apoptosis, and exhibit antiangiogenic activity in cancer cells derived from multiple tumor types [reviewed in (280)], and the effects of CDDO-Me and CDDO on expression of prototypical gene products representing these activities were investigated in the three pancreatic cancer cell lines. Results in Figure 34A show that 0 - 1.25 μM CDDO-Me and 0 - 5 μM CDDO decrease expression of cyclin D1, survivin, VEGF and VEGFR2 proteins in Panc1 cells. Moreover, similar results were observed in Panc28 (Figure 34B) and L3.6pL (Figure 34C) cells, and results in Figures 33 and 34 are consistent with previous studies on CDDO and related compounds in cancer cells (176, 177, 185, 279-290).

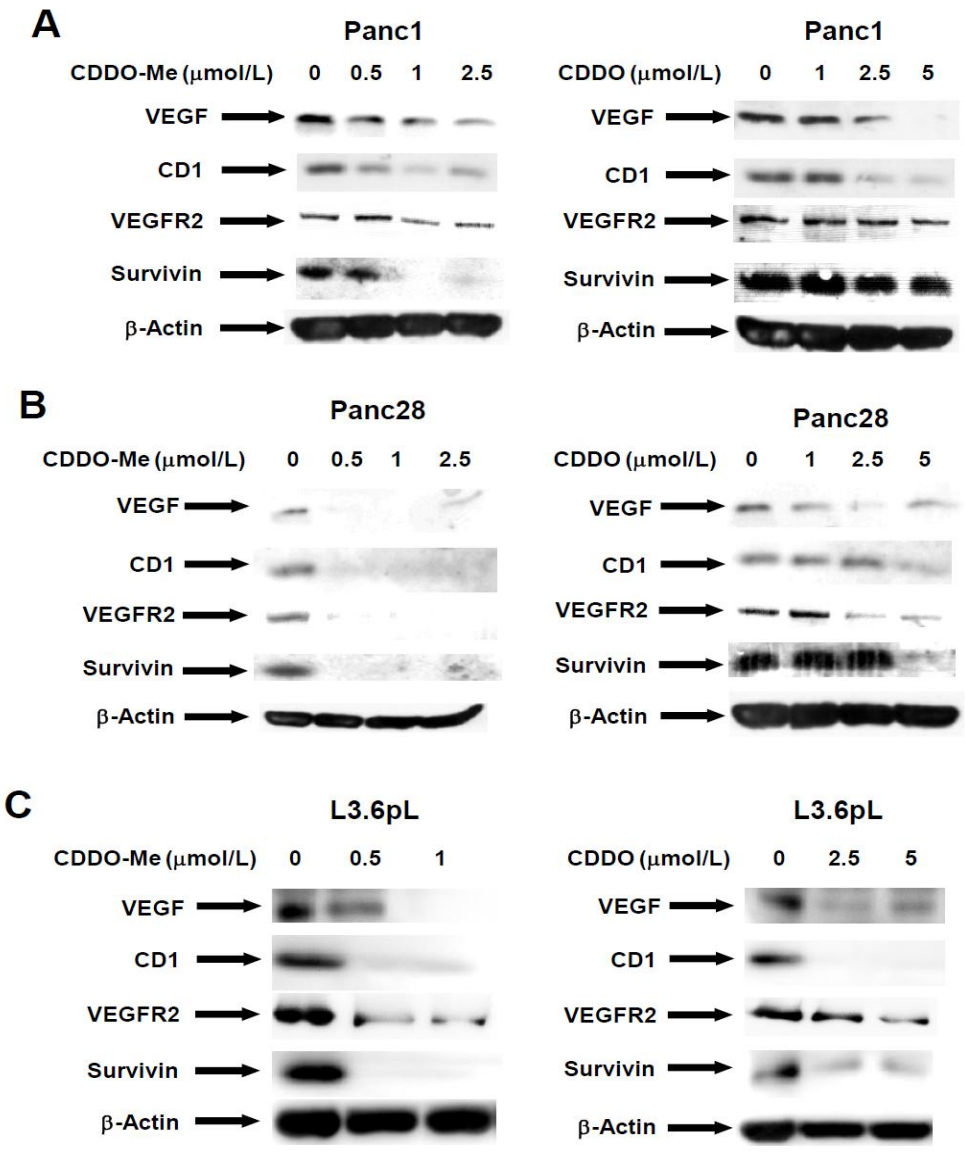

Figure 34. CDDO and CDDO-Me decrease expression of VEGF, VEGFR2, cyclin D1 (CD1) and survivin proteins in Panc1 (A), Panc28 (B) and L3.6pL (C) pancreatic cancer cell lines.

Cells were treated with DMSO, CDDO (1.0, 2.5 or 5.0 μmol/L), or CDDO-Me (0.5, 1.0 or 1.25 μmol/L) for 24 hr, and whole-cell lysates were analyzed by

western blot analysis as described in Materials and Methods. β-Actin served as a loading control. The gels were typical of results of at least two replicate determinations per treatment group.

Knockdown of Sp1, Sp3 and Sp4 transcription factors by RNA interference in pancreatic cancer cells showed that VEGF, VEGFR1 and VEGFR2 expression were regulated by Sp1, Sp3 and Sp4 (153, 159, 162), suggesting that an underlying mechanism of action of CDDO and CDDO-Me may involve downregulation of Sp proteins as previously described for other bioactive triterpenoids in cancer cells (163, 168). Results in Figure 35A demonstrate that 0 - 1.25 μM CDDO-Me and 0 - 5.0 μM CDDO decreased expression of Sp1, Sp3 and Sp4 proteins in Panc1 cells and similar results were observed in Panc28 (Figure 35B) and L3.6pL (Figure 35C) cells. The observed CDDO-/CDDO-Me-dependent downregulation of Sp transcription factors (Figures 35A - 35C) is consistent with their parallel decrease in Sp-dependent genes as illustrated in Figure 34. Previous studies show that the NSAID tolfenamic acid induced proteasome-dependent downregulation of Sp1, Sp3 and Sp4 in Panc1 cells that was blocked by the proteasome inhibitor MG132 (162); however, results in Figure 35D using CDDO-Me, indicate that downregulation of Sp1, Sp3 and Sp4 by this compound in pancreatic cancer cells was not blocked by the proteasome inhibitor MG132, indicating a proteasome-independent pathway.

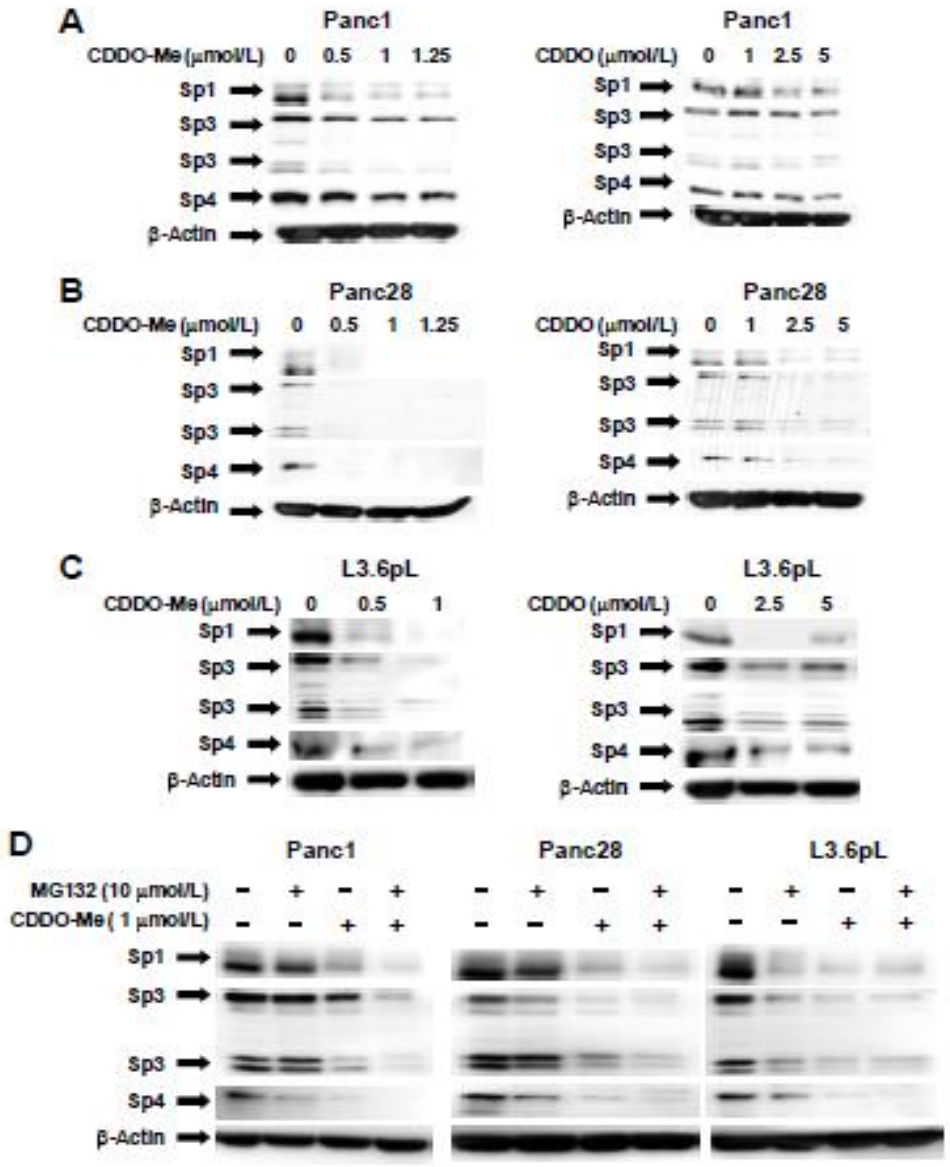

Figure 35. CDDO-Me downregulates Sp proteins in a proteosome-independent manner. CDDO-Me decreases Sp protein expression in Panc1 (A), Panc28 (B), and L3.6pL (C).

Cells were with DMSO, CDDO (1.0, 2.5 or 5.0 μM), or CDDO-Me (0.5, 1.0 or 1.25 μM) for 24 hr, and whole-cell lysates were analyzed for Sp1, Sp3 and

Sp4 by western blot analysis as described in Materials and Methods. D. Proteosome-independent downregulation of Sp proteins by CDDO-Me. Cells were treated with DMSO and CDDO-Me (1.0 μmol/L) in the presence or absence of proteasome inhibitor MG132 (10 μM), and the effects on Sp protein degradation were determined after treatment for 24 hr by western blot as described in Materials and Methods. β-Actin served as a loading control.

The proximal regions of the Sp1, Sp3, VEGF and survivin promoters are GC-rich and the effects of CDODA-Me and CDDO on promoter activity were investigated in Panc1 cells transfected with pSp1For4, pSp3For5, pVEGF and pSurvivn constructs which contain -751 to -20 (Sp1), -417 to -38 (Sp3), -2018 to +50 (VEGF), and -269 to +49 (survivin) promoter inserts, respectively. CDDO and CDDO-Me significantly decreased luciferase (reporter gene) activity in Panc1 cells transfected with these constructs (Supplement Figure 1) indicating that CDDO and CDDO-Me act at the transcriptional levels and this is consistent with the observed proteasome-independent downregulation results shown in Figure 35D. We also observed that CDDO-Me decreased Sp1 mRNA levels (Supplement Figure 2) which is consistent with the inhibitory effects of this compound on transcription.

Previous reports showed that the imidazole derivative of CDDO was mitochondriotoxic in pancreatic cancer cells and this was characterized by decreased MMP and accompanied by induction of ROS (185). Treatment of Panc1 or L3.6pL cells with CDDO-Me also increased total ROS levels as

determined by CM-H_2DCFDA fluorescent dye and this response was also attenuated after cotreatment with the antioxidant glutathione (Figure 36A). Panc1 and L3.6pL cells were incubated with the fluorescent dye JC-1 and after treatment with DMSO or GSH, the typical orange/red fluorescent staining of mitochondria was observed (Figure 36A). However, in Panc1 or L3.6pL cells treated with 1.25 or 1.0 μM CDDO-Me, respectively, there was a significant decrease in orange/red staining and an increase in green fluorescence indicative of decreased MMP and this response was attenuated after cotreatment with GSH in both cell lines (Figure 36B). These results demonstrate that CDDO-Me was mitochondriotoxic in pancreatic cancer cells and we therefore investigated the possible connection between CDDO-Me-induced ROS and mitochondrial effects and CDDO-Me-dependent downregulation of Sp1, Sp3 and Sp4 proteins. Results in Figure 36C demonstrate that Sp1, Sp3 and Sp4 are downregulated in Panc1 and L3.6pL cells after treatment with CDDO-Me and decreased expression of Sp1, Sp3 and Sp4 proteins was inhibited after cotreatment with thiol antioxidants (DTT and/or GSH) and similar results were observed in Panc28 cells (data not shown). Hydrogen peroxide or *t*-butyl hydroperoxide were used as prototypical model ROS and both compounds decreased expression of Sp1, Sp3 and Sp4 proteins (data not shown), confirming that induction of ROS in pancreatic cancer cells is a critical upstream response in targeting repression of Sp transcription factors, and comparable results have been observed in other cancer cell lines.

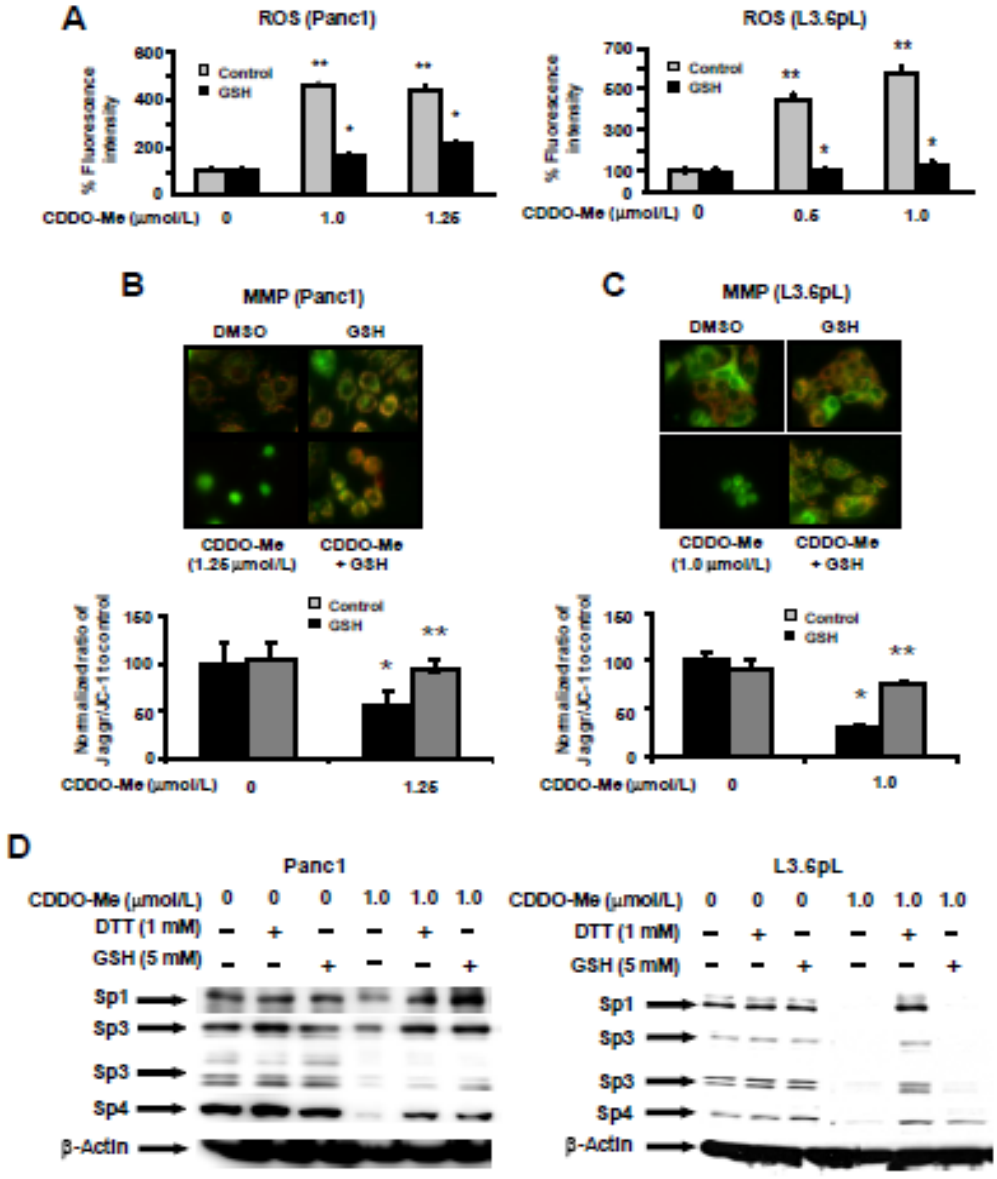

Figure 36. Role of oxidative stress and mitochondrial membrane potential (MMP) in mediating the effects of CDDO-Me on Sp proteins in pancreatic cancer cells. Effect of CDDO-Me on ROS (A) and MMP (B, C).

Panc1 and L3.6pL cells were treated with DMSO and CDDO-Me (0.5, 1.0 or 1.25 μmol/L) for 24 hr in the presence or absence of antioxidant GSH. ROS

was measured using BioTek Synergy 4 plate reader using CM-H_2DCFDA (10 μM) dye as described in Materials and Methods, and normalized fluorescence intensity against control is plotted as a bar diagram. MMP was determined using JC-1 dye and quantitation of the ratio of red to green fluorescence was measured using Image J Software as described in Materials and Methods. D. Reversal of CDDO-Me-mediated downregulation of Sp proteins by thiol antioxidants. Cells were treated with DMSO or CDDO-Me (1.0 μmol/L) in the presence or absence of DTT or GSH for 24 hr, and whole cell lysates were analyzed by western blots as described in Materials and Methods. β-Actin served as a loading control. Results in A and C are expressed as means ± SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) CDDO-Me-mediated decreases (*) or increases (**) after cotreatment with antioxidants compared to the solvent (DMSO) control are indicated.

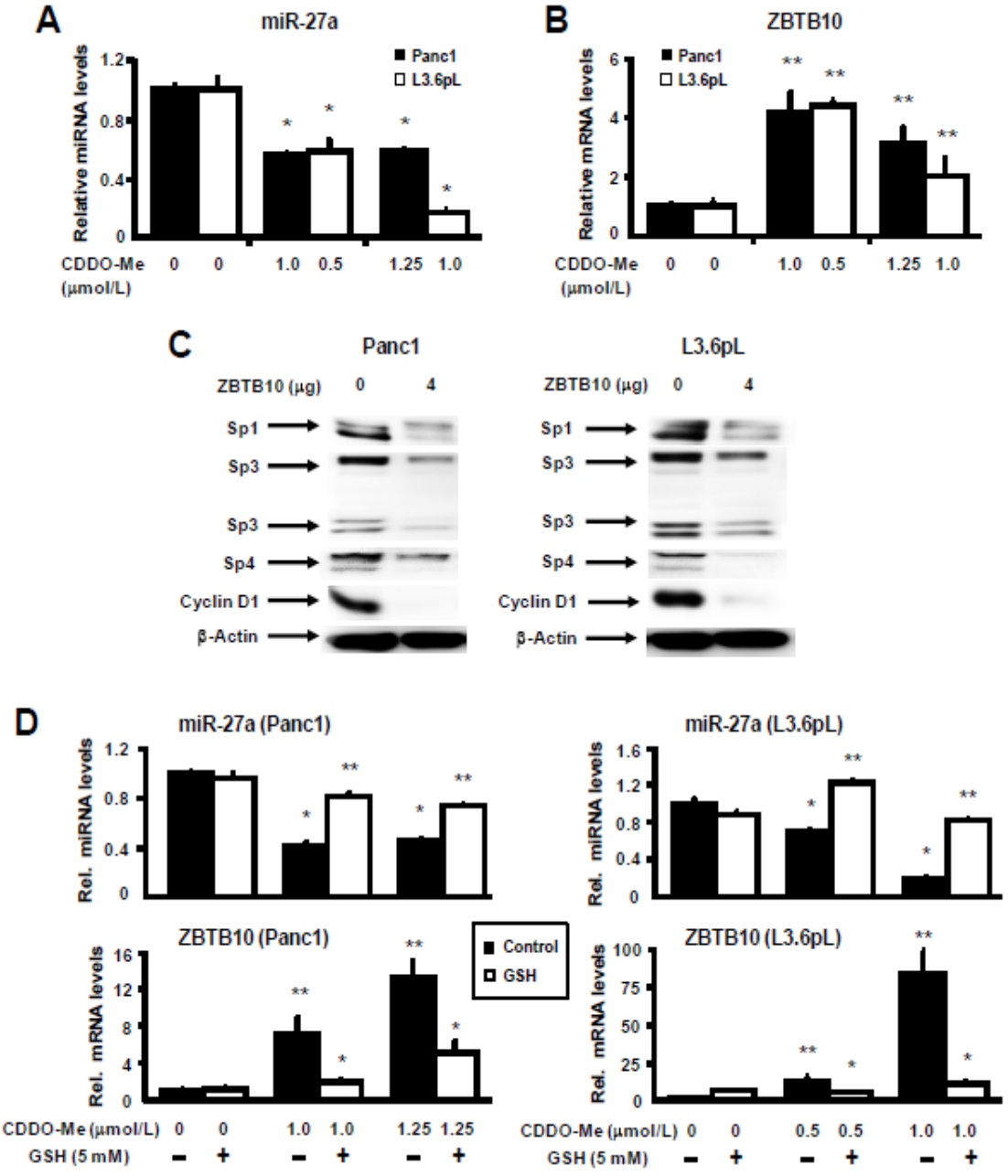

Figure 37. Effect of CDDO-Me on expression miR-27a and ZBTB10 mRNA and role of ZBTB10 overexpression on Sp proteins in pancreatic cancer cells.

CDDO-Me decreases miR-27a (A) and induces ZBTB10 mRNA (B). Panc1 and L3.6pL cells were treated with the indicated doses of CDDO-Me for 24 hr, and miR-27a and ZBTB10 levels were analyzed by real time PCR as described in Materials and Methods. C. Effect of ZBTB10 overexpression on Sp proteins and Sp-dependent genes. Panc1 and L3.6pL cells were transfected with empty vector (pCMV6-XL4) or 4 μg/well ZBTB10 expression plasmid pCMV6-XL4 vector, and whole cell lysates were analyzed by western blots as described in Materials and Methods. D. Effect of GSH on CDDO-Me-mediated miR-27a and ZBTB10 mRNA expression. Panc1 and L3.6pL cells were treated with indicated doses of CDDO-Me in the presence or absence of GSH for 24 hr, and miR-27a and ZBTB10 mRNA levels were analyzed by real time PCR as described in Materials and Methods. Results in A, B and D are expressed as means ± SE for three replicate determinations for each treatment group and significant ($P < 0.05$) inhibition (*) or induction (**) of responses are indicated.

CDODA-Me, a triterpenoid structurally-related to CDDO-Me, decreased Sp proteins in colon cancer cells through downregulation of microRNA-27a (miR-27a) and induction of ZBTB10, a zinc finger Sp-repressor protein (163). After treatment of Panc1 and L3.6pL cells with CDDO-Me, there was a decrease in expression of miR-27a (Fig. 37A) and this was accompanied by increased ZBTB10 mRNA levels (Fig. 37B). Since ZBTB10 suppresses expression of Sp-regulated genes through competitive interactions with GC-rich promoter elements (163, 164, 291), we investigated the effects of ZBTB10 overexpression

in Panc1 and L3.6pL cells (Fig. 37C). Overexpression of ZBTB10 in pancreatic cancer cells decreased expression of Sp1, Sp3 and Sp4 proteins and similar results have previously been observed in breast and colon cancer cells (163, 164). CDDO-Me-dependent induction of ROS and downregulation of Sp proteins is inhibited after cotreatment with antioxidants (Fig. 36) and, therefore, we investigated the role of ROS induction and the effects of antioxidants on expression of ZBTB10 and miR-27a. CDDO-Me decreased miR-27a and induced ZBTB10 in Panc1 and L3.6pL cells and, in cells cotreated with CDDO-Me plus glutathione, these responses were significantly reversed (Fig. 37D). This demonstrates that CDDO-Me-induced ROS is a common upstream factor regulating disruption of miR-27a:ZBTB10–Sp axis.

The *in vivo* anticancer activity of CDDO-Me was also investigated in an orthotopic model of pancreatic cancer in which L3.6pL cells were injected directly into the pancreas of 8 - 12 week old male thymic nude mice (162, 292). Treatment with CDDO-Me (7.5 mg/kg daily) was initiated 7 days after injection of the cells and continued for an additional 28 days. Treatment with CDDO-Me significantly decreased pancreatic tumor volume and weight (Figs. 38A and 38B) compared to the vehicle control group. In addition, lysate from tumors treated with the vehicle or CDDO-Me (from 3 different animals/group) were also analyzed by western blots and there was a marked decrease in expression of Sp1, Sp3 and Sp4 proteins in tumors from mice treated with CDDO-Me compared to the control group (Fig. 38C).

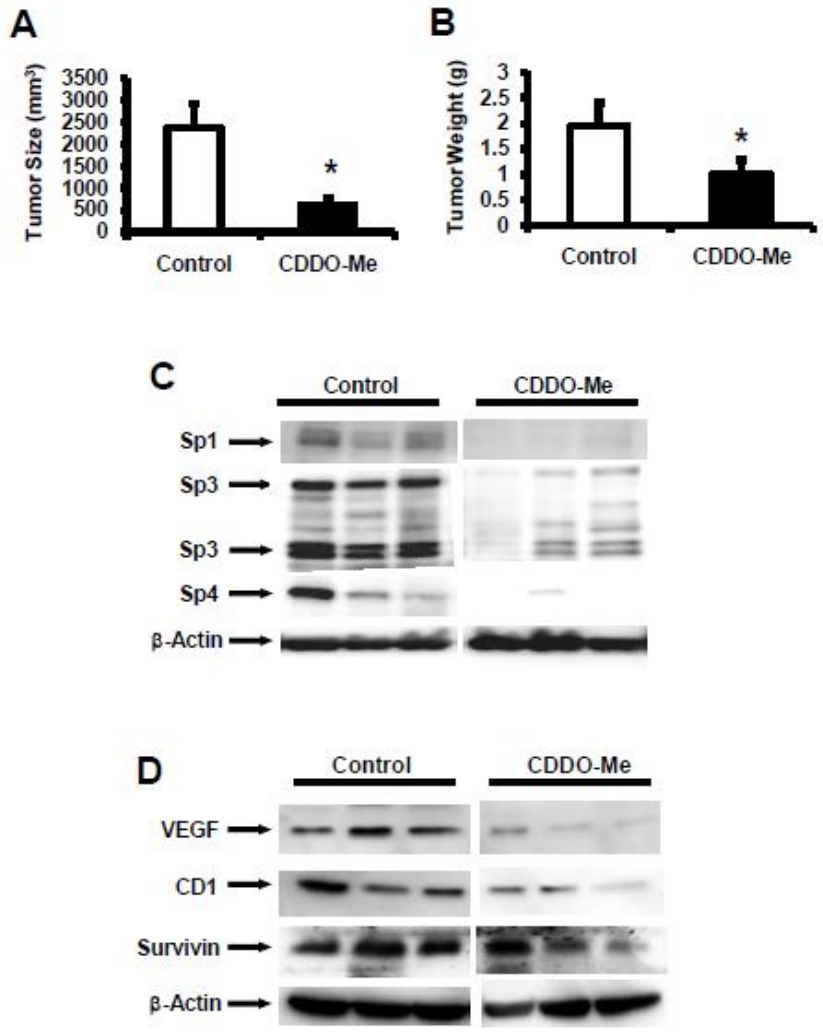

Figure 38. CDDO-Me inhibits pancreatic tumor growth and downregulates Sp proteins and Sp dependent genes.

Tumor weights (A) and volume (B). Male athymic nude mice bearing orthotopic pancreatic (L3.6pL) tumors were treated with corn oil or CDDO-Me (7.5 mg/Kg) for 4 weeks and tumor weights and tumor volumes (mm^3) were determined as described in Materials and Methods. Significant ($P < 0.05$) inhibition (*) is indicated in results as means ± SE for five animals per treatment.

Western blot analysis of tumor lysates for Sp proteins (C) and Sp-dependent proteins (D). Lysates from three mice in the treated and control groups were analyzed by western blots as described in Materials and Methods. β-Actin served as loading control.

Moreover, we also observed decreased expression of VEGF, cyclin D1 and survivin in tumors from CDDO-Me-treated mice compared to animals receiving vehicle control (Fig. 38D). These *in vivo* results are consistent with cell culture studies (Figs. 34 and 35) demonstrating for the first time that CDDO-Me represses expression of Sp1, Sp3 and Sp4 transcription factors and Sp-dependent gene products, suggesting that this hitherto unrecognized pathway also contributes to the anticancer activity of CDDO-Me and related compounds.

Discussion

Sp1 was the first transcription factor identified and is a member of the Sp/Krüppel-like family (KLF) of zinc finger transcription factors that exhibit a broad range of tissue-specific and overlapping functions (150, 170). Although Sp transcription factors are important during embryonic development, there is evidence that in humans and laboratory animals of a marked decrease in Sp1 with aging (166, 167) and studies in this laboratory show that Sp1, Sp3 and Sp4 levels in non-tumor tissue of mice are minimal to non-detectable (158, 162, 168). Several reports show that Sp1 protein is overexpressed in different human tumor types including gastric, colorectal, pancreatic, epidermal, thyroid and breast cancers (160, 164, 230-233, 268, 293). In gastric cancer patients, there was an

association between Sp1 expression with advanced stage of the tumor, VEGF expression, and poor survival (231). Although the mechanism of Sp overexpression has not been determined, Lou and coworkers (157) have shown that malignant transformation of human fibroblasts resulted in an 8- to 18-fold increase in Sp1 expression and the transformed cells formed tumors in athymic nude mouse xenografts. In contrast, these transformed cells were not tumorigenic after knockdown of Sp1. Sp1 is overexpressed in pancreatic cancer cells (268) and there is a correlation between expression of Sp1 and the angiogenic factor vascular endothelial growth factor (VEGF). Moreover, it was recently reported that Sp1 was a biomarker that identifies patients with a highly aggressive sub-type of pancreatic ductal adenocarcinomas (160). Studies in this laboratory show that Sp1, Sp3 and Sp4 are overexpressed in pancreatic and other cancer lines and knockdown of Sp1, Sp3 and Sp4 by RNA interference indicates that expression of several growth promoting, pro-survival and angiogenic genes/responses are regulated by these transcription factors (153, 162, 164, 165, 168).

The high expression of Sp1, Sp3 and Sp4 proteins in cancer cells and tumors coupled with their regulation of several critical pro-oncogenic genes suggests that these transcription factors are potentially important drug targets. The non-steroidal antiinflammatory drug tolfenamic acid, the triterpenoid betulinic acid, curcumin and CDODA-Me have previously been characterized as agents that inhibit cancer cell and tumor growth in rodent models and induce

downregulation of Sp1, Sp3 and Sp4 through proteasome-dependent and -independent pathways (162, 163, 165, 168). CDDO/CDDO-Me and CDODA/CDODA-Me activate PPARγ and exhibit some common and also different activities in colon and pancreatic cancer cells (145, 203, 281). A recent study in colon cancer cells showed that CDODA-Me downregulated expression of Sp1, Sp3 and Sp4 and Sp-regulated gene products (163). Results in Figures 34 and 35 show that CDDO and CDDO-Me also decreased expression of Sp1, Sp3 and Sp4 and Sp-regulated genes such as cyclin D1, VEGF, VEGFR2 and survivin in Panc1, Panc28 and L3.6pL pancreatic cancer cells. Moreover, repression of these gene products by CDDO and CDDO-Me were observed at concentrations that were comparable to those required for inhibition of Panc1, Panc28 and L3.6pL cell growth and induction of apoptosis (Figure 33). In contrast to previous reports with tolfenamic acid in pancreatic cancer cells (158, 162), CDDO-Me induced proteasome-independent downregulation of Sp1, Sp3 and Sp4 in pancreatic cancer cells (Figure 35D). CDDO-Me also decreased luciferase activity in cells transfected with constructs containing Sp1, Sp4, VEGF and survivin gene promoter inserts and decreased Sp1 mRNA expression (Supplement Figures 33 and 34) which is consistent with the effects of CDDO-Me on transcription. These *in vitro* cell culture studies confirm the potency of CDDO-Me as an inhibitor of pancreatic cancer cell growth and demonstrate for the first time that that anticancer activity is due, in part, to targeting of Sp transcription factors and Sp-regulated genes. Further evidence for the role of

this pathway in the activity of CDDO-Me was observed in an orthotopic model for pancreatic cancer where CDDO-Me (7.5 mg/kg/day) not only inhibited tumor growth but also decreased expression of Sp1, Sp3 and Sp4 proteins in pancreatic tumors (Figure 38). These results suggest that CDDO-Me activates comparable pathways both *in vitro* and *in vivo* and this has previously been observed for other agents that target Sp transcription factors (158, 162, 163, 165).

CDDO-Im-dependent anticarcinogenic activity in pancreatic cancer cells has been linked to its mitochondriotoxic activity (185) which was characterized by decreased MMP and GSH and induction of ROS and these responses were blocked in cells cotreated with thiol antioxidants. Not surprisingly, CDDO-Me also induced a similar pattern of mitochondriotoxic responses in pancreatic cancer cells which were inhibited after cotreatment with thiol antioxidants (Figure 36). A recent study in prostate cancer cells also linked the pro-apoptotic activity of CDDO-Me to induction of ROS (294) Since induction of ROS and ROS-dependent responses play an important role in the anticancer activity of mitochondriotoxic anticancer drugs such as arsenic trioxide (205), we hypothesized that repression of Sp transcription factors may be an ROS-dependent response. Results in Figure 36D show that CDDO-Me-dependent downregulation of Sp1, Sp3 and Sp4 proteins is significantly inhibited in Panc1 and L3.6pL cells after cotreatment with GSH and DTT. Moreover, using hydrogen peroxide and *t*-butyl hydroperoxide as prototypical ROS, both oxidants

also decreased expression of Sp1, Sp3 and Sp proteins in pancreatic cancer cells (data not shown). These results demonstrate that the effects of CDDO-Me on mitochondria are linked to ROS-dependent downregulation of Sp1, Sp3 and Sp4 and we have also observed similar effects with arsenic trioxide in pancreatic cancer cells (205).

The role of miR-27a and its suppression of the zinc finger protein ZBTB10 in regulating expression of Sp1, Sp3 and Sp4 has previously been characterized in breast and colon cancer cells (27, 31). Both antisense miR-27a and ZBTB10 overexpression decrease Sp and Sp-dependent genes in these cell lines (162, 164), and this is related to the effects of ZBTB10 which binds GC-rich sites and acts as an transcriptional repressor (291). Treatment of Panc1 and L3.6pL cells with CDDO-Me decreased expression of miR-27a (Figure 37A) and this was accompanied by induction of ZBTB10 (Figure 37B). These results are comparable to those observed for the structurally-related triterpenoid CDODA-Me in colon cancer cells (162, 164). We also confirmed that ZBTB10 overexpression in Panc1 and L3.6pL cells decreased levels Sp1, Sp3 and Sp4 proteins as observed in colon and breast cancer cells (162, 164). These studies also demonstrate for the first time that CDDO-Me-dependent induction of ROS is critical for downstream events since antioxidants inhibit downregulation of miR-27a, induction of ZBTB10 (Figure 38C), and repression of Sp1, Sp3 and Sp4 (Figure 36D) in Panc1 and L3.6pL cells treated with CDDO-Me.

In summary, we have shown that CDDO-Me is highly cytotoxic to pancreatic cancer cells and tumors and this is consistent with previous reports on the potent anticancer activity of CDDO and related compounds (176, 177, 185, 203, 234, 279-289). Our results demonstrate for the first time that CDDO and CDDO-Me induce downregulation of Sp1, Sp3 and Sp4 transcription factors as well as several Sp-dependent genes that are associated with cancer cell survival, growth and angiogenesis. These observations suggest that this pathway contributes to the potent antitumorigenic activity of CDDO and related compounds and may also explain, in part, the broad spectrum of activities of these drugs in cancer cell lines. CDDO-Me, like its imidazole derivative (185), also induces ROS and decreases MMP in pancreatic cancer cells. We show that the induction of ROS leads to a cascade of events in which miR-27a is decreased and this is accompanied by induction of ZBTB10, a suppressor of Sp transcription factors and Sp-dependent genes (158, 162, 164). Activation of the ROS-miR-27a:ZBTB10–Sp axis by CDDO-Me represents a novel and highly practical route for targeting several Sp-regulated genes that play an important role in carcinogenesis. Current research is focused on improving the efficacy of drugs that target Sp transcription factors and also determining the specific ROS-induced factors and other pathways that lead to downregulation of miR-27a and other miRs that regulate potential Sp-repressor genes.

VI. CELASTROL INHIBITS PANCREATIC CANCER CELL AND TUMOR GROWTH AND DECREASES SPECIFICITY PROTEIN (Sp) TRANSCRIPTION FACTORS

Specificity protein (Sp) transcription factors Sp1, Sp3 & Sp4 are overexpressed in multiple tumor types and negative prognostic factors for survival. Since Sp transcription factors regulate genes associated with survival (survivin), angiogenesis [vascular endothelial growth factor (VEGF) and its receptors] and growth (cyclin D1), research in this laboratory has focused on development of anticancer drugs that decrease Sp protein expression. Celastrol, a naturally occurring triterpenoid acid from an ivy-like vine exhibits anticancer activity and treatment of Panc-28 and L3.6pl pancreatic cancer cells with 1.0 – 5.0 μM celastrol decreased cell survival and IC_{50} values for inhibition of cell proliferation were 3.2 & 3.6 μM respectively. Celastrol also induced apoptosis and decreased expression of survivin, cyclin D1, epidermal growth factor (EGFR), insulin growth factor receptor (IGFR)-1β and VEGF in Panc-28 and L3.6pl cells suggesting that decreased expression of these genes may be due to downregulation of Sp proteins. Treatment of Panc-28 and L3.6pl cells with 1 – 5 μM celastrol decreased Sp1, Sp3 and Sp4 protein expression in both cell lines and cotreatment with the proteosome inhibitor MG132 did not inhibit down-regulation of Sp proteins. The mechanism of celastrol-induced repression of Sp proteins was associated with down regulation of microRNA-27a (mir-27a). Decreased mir27a results in the induction of ZBTB10, a zinc finger protein that

acts as a Sp-repressor by competitive interaction with Sp proteins bound to GC-rich promoter elements. At a dose of 4 mg/kg every second day celastrol also inhibited pancreatic tumor growth in athymic nude mice bearing L3.6pl cells as xenografts and Sp proteins were also decreased in tumors from treated mice. This study demonstrates that the anticancer activity of celastrol in pancreatic cancer due to celastrol-mir27a interactions resulting in ZBTB10-dependent repression of Sp and Sp-dependent genes

Introduction

Traditional medicines have been extensively used for treating a wide variety of diseases including cancer and many individual natural products identified in plant and microbial-derived medicinal extracts have become important pharmaceuticals (274-276). Extracts of Taxus species have been used by Native American tribes and Indians for various health problems (295) and one of the active constituents (taxol, paclitaxel) and structurally related taxanes are widely used anticancer drugs (296). Extracts of the Chinese plant *Artemisia annua* have been used for several centuries to treat fever and artemisinin, the active component is used for clinical management of malaria and shows promise as an anticancer drug (297-299). Extracts from *Tripterygium wilfordii* (“Thunder of God Vine”) have been used in traditional Chinese medicine for treating various health problems and its efficacy is due, in part to celastrol, one of the bioactive components. Celastrol is a quinone methide derived from the triterpenoid glycyrrhetinic acid which is also a bioactive compound. Celastrol

exhibits anti-inflammatory activity and has been used for treatment of asthma, chronic inflammation autoimmune and neurodegenerative diseases (300-306).

Several studies show that celastrol exhibits anticarcinogenic activity in various cancer models. For example, celastrol inhibit human prostate cancer cell and tumor growth and this has been associated with its activity as a proteasome inhibitor which blocks chymotrypsin-like activity of a purified 20S proteasome (307). Celastrol also inhibits induced NFκB-dependent responses in several different cell lines (308), modulates expression and function of heat shock responses (309, 310) and induces apoptosis and activation of c-jun N-terminal kinase (JNK) in melanoma (311). Celastrol also inhibited growth, angiogenesis and expression of angiogenic genes including vascular endothelial growth factor receptor 1 (VEGFR1) and VEGFR2 in glioma tumors in a mouse xenograft model (312). RNA interference studies in this laboratory have shown that knockdown of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 that are overexpressed in pancreatic cancer cells is accompanied by downregulation of several Sp-dependent genes including VEGF, VEGFR1 and VEGFR2 (153, 158, 159, 162). In previous studies we have identified several anticancer drugs including the non-steroidal anti-inflammatory drug (NSAID) tolfenamic acid, curcumin and two triterpenoids, betulinic acid and methyl 2-cyano-3,11-dioxo-18βolean-1,12-dien-30-oate (CDODA-Me) that also decreased expression of angiogenic genes and other Sp-regulated genes through their downregulation of

Sp1, Sp3 and Sp4 proteins in pancreatic and other cancer cell lines (147, 162-165, 168).

Therefore we hypothesized that the anticancer activity of celastrol in pancreatic cancer may also be due, in part, to downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes in this study and pancreatic cancer cells and a mouse xenograft model was used to test the hypothesis. Celastrol inhibited Panc28 and L3.6pL pancreatic cancer cell growth and induced apoptosis in these cells. Celastrol also decreased expression of several Sp-regulated genes including VEGF and NFκB and we also observed downregulation of Sp1, Sp3 and Sp4 proteins. Celastrol also decreased microRNA-27A (miR-27a) and increased expression of ZBTB10, a zinc finger Sp repressor protein that interacts with GC-rich Sp binding sites to inhibit gene expression (163, 164, 291). This mechanism of action for celastrol was similar to that previously reported for CDODA-Me in colon cancer cells (163) however, we also show that Sp downregulation due to the induction of reactive oxygen species (ROS), and current studies are focus on mitochondria as an initial cellular target for celastrol and the role of ROS on miR-27a and ZBTB10.

Materials and methods

Cell lines

Panc28 cell line was a generous gift from Dr. Paul Chiao and L3.6pL cells were kindly provided by Dr. Isaiah Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX).

Antibodies and reagents

Both pancreatic cancer cell lines were maintained in DMEM-F12 supplemented with 5% FBS, 0.22% sodium bicarbonate, and 10 mL/L of 100X antibiotic/antimycotic cocktail solution (Sigma-Aldrich Co., St. Louis, MO). Cells were grown in 150 cm^2 culture plates in an air/CO_2 (95:5) atmosphere at 37°C. Cyclin D1, IGFR1β, p65, p50, Sp3, Sp4 and VEGF antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP antibody was purchased from Cell Signaling Technology (Danvers, MA) and Sp1 antibody was purchased from Millipore (Billerica, MA). Survivin antibody was purchased from R&D systems (Minneapolis, MN). Monoclonal β-actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase substrate for western blot analysis was obtained from Millipore. Dithiothreitol and γ-L-glutamyl-L-cysteinyl-glycine (GSH) were obtained from Sigma-Aldrich. Superscript II, LipofectAMINE and LipofectAMINE 2000 was purchased from Invitrogen (Carlsbad, CA). Reporter lysis buffer and luciferase reagent were purchased from Promega (Madison, WI). β-Galactosidase reagent was obtained from Tropix (Bedford, MA). Primers

for TBP and ZBTB10 were purchased from Integrated DNA Technologies Technologies (Coralville, IA). Primers for Sp3 and Sp4 were obtained from Qiagen (Valencia, CA); and ZBTB10 expression vector and empty vector (pCMV6-XL4) were from Origene (Rockville, MD). MiRNA mirvaRNA extraction kits and the reverse transcription (RT) and real-time PCR amplification kits were purchased from Applied Biosystems (Foster City, CA). The VEGF and survivin promoter constructs were provided by Drs. Gerhard Siemeister and Gunter Finkenzeller (Institute of Molecular Medicine, Tumor Biology Center, Freiburg, Germany) and Dr. M. Zhou (Emory University, Atlanta, GA). Sp1 and Sp3 promoter constructs were kindly provided by Drs. Carlos Cuidad and Veronique Noe (University of Barcelona, Barcelona, Spain).

Cell proliferation assay

Pancreatic cancer cells ($3X10^4$ per well) were plated in 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or different doses of celastrol in the presence or absence of ROS inhibitors was undertaken. Cells were trypsinized and counted after 24 hr using a Coulter Z1 particle counter. Each experiment was done in triplicate and results are expressed as means ± SE for each treatment group.

Transfection and luciferase assay

Pancreatic cancer cells ($1x10^5$ per well) were plated in 12-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 24 hr, various amounts of DNA [i.e., 0.4 μg PGL2-Luc, 0.4 μg PGL3-Luc, 0.04 μg β-galactosidase, and 0.4 μg pSp1 (4)-Luc or 0.4 μg pSp3-Luc or 0.4 μg VEGF (2068)-Luc or 0.4 μg pSurvivin (269)-Luc or 0.4 μg pNFκB-Luc] were transfected using Lipofectamine reagent according to the manufacturer's protocol. Five hr post-transfection, the transfection mix was replaced with complete medium containing either vehicle (DMSO) or the indicated compound in DMSO. After 22 hr, cells were then lysed with 100 μL of 1X reporter lysis buffer, and cell extracts (30 mL) were used for luciferase and β-galactosidase assays. A Lumicount luminometer was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

Western blots

Pancreatic cancer cells ($3x10^5$ per well) were seeded in 6 well plate in DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped FBS and after 24 hr, cells were treated with either vehicle (DMSO) or the indicated compounds. Cells were collected using high-salt buffer [50 mmol/L HEPES, 0.5 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, and 1% Triton-X-100] and 10 μL/mL of Protease Inhibitor Cocktail (Sigma-Aldrich). Protein lysates were

incubated for 3 min at 100°C before electrophoresis, and then separated on 10% SDS–PAGE 120 V for 3 to 4 hr. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by wet electroblotting in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol for 1.5 hr at 180 mA. Membranes were blocked for 30 min with 5% TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, and 5% nonfat dry milk] and incubated in fresh 5% TBST-Blotto with 1:500 primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the PVDF membrane was incubated with secondary antibody (1:5000) in 5% TBST-Blotto for 2 hr by gentle shaking. The membrane was washed with TBST for 10 min, incubated with 6 mL of chemiluminescence substrate for 1 min, and exposed to Kodak image station 4000 mm Pro (Carestreamhealth, Woodbridge, Connecticut).

Xenograft study

Female athymic nude mice, age 4 to 6 weeks, were purchased from Harlan. L3.6pL cells (3×10^5) in 1:1 ratio of Matrigel (BD Biosciences) were injected into the either side of the flank area of nude mice. Seven days after the tumor cell inoculation, mice were divided into two groups of 10 animals each. The first group received 100 μL vehicle (corn oil) by i.p. injection, and the second group of animals received 4 mg/kg/d injection of celastrol in corn oil every 2nd day for 16 d (8 doses) by i.p. injection. The mice were weighed, and tumor

areas were measured throughout the study. After 18 d, the animals were sacrificed; final body and tumor weights were determined and plotted.

ROS estimation

Cellular ROS levels were evaluated with the cell-permeable probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester) from Invitrogen. Following treatment for 20-24 hr, cells plated on 96 well cell culture plate were loaded with 10 μM CM-H_2DCFDA for 30 min, washed once with serum free medium, and analyzed for ROS levels using the BioTek Synergy 4 plate reader (Winooski, VT) set at 480 nm and 525 nm excitation and emission wavelengths, respectively. Following reading of ROS, cultures were then treated with Janus green and cell counts were determined with the plate reader set to an absorbance of 610 nm, and ROS intensities were then corrected accordingly. Each experiment was done in triplicate and results are expressed as means ± SE for each treatment group.

Statistical analysis

Statistical significance of differences between the treatment groups was determined by an analysis of variance and/or Student's t-test, and levels of probability were noted. IC_{50} values were calculated using linear regression analysis and expressed in μM, at 95% confidence intervals.

Results

Panc28 and L3.6pL pancreatic cancer cells were treated with 1.0 – 5.0 μM celastrol for 24 hr and this resulted in significant inhibition of cell proliferation with IC_{50} values of 3.2 and 3.6 μM respectively (Figure 39A). After treatment for longer time periods (144 hr) IC_{50} values decreased (data not shown) demonstrating that celastrol was a potent inhibitor of pancreatic cancer cell growth. We also examined the effects of celastrol on expression of several gene products associated with cell growth [cyclin D1, epidermal growth factor receptor (EGFR), insulin-like growth factor receptor 1β (IGFR1β), vascular endothelial growth factor (VEGF)] and apoptosis (survivin, PARP cleavage) (Figure 39). The results show that celastrol decreased expression of cyclin D1, EGFR, IGFR1β and the angiogenic growth factor VEGF and decreased expression of the antiapoptotic protein survivin and this was accompanied by induction of caspase-dependent PARP cleavage (Figures 39B & 39C).

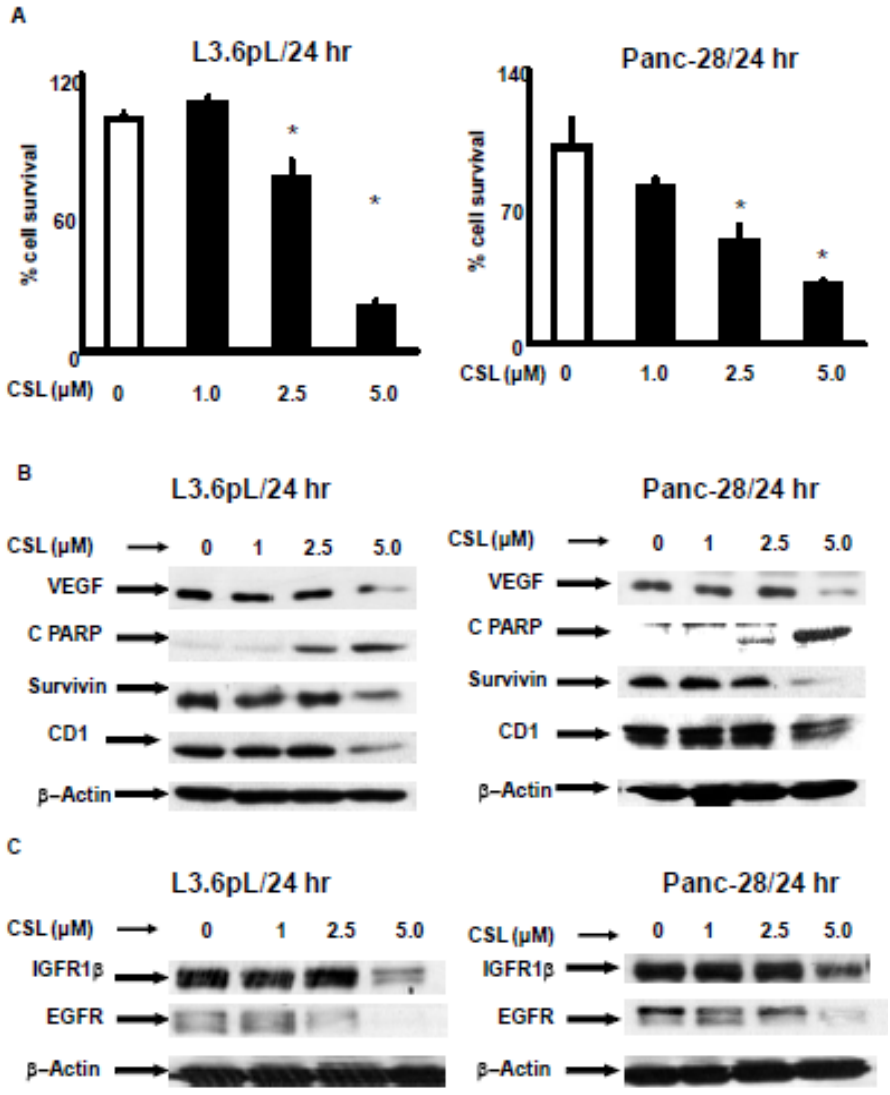

Figure 39. Celastrol inhibits cell growth, induces apoptosis and downregulates VEGF, survivin, CD1, IGFR1β and EGFR in pancreatic cancer cell lines.

Inhibition of L3.6pL and Panc-28 cell growth by celastrol (A). Cells were treated with DMSO (solvent control), 1.0, 2.5 or 5.0 μM celastrol and effects on cell growth were determined over a period of 24 hr as described in the Materials

and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group and significant ($P < 0.05$) inhibition (*) of responses are indicated. Downregulation of VEGF, survivin, CD1 (B), IGFR1β and EGFR (C) proteins in L3.6pL and Panc-28 cell lines by celastrol. L3.6pL and Panc-28 cells were treated with DMSO and 1.0, 2.5 or 5.0 μM celastrol for 24 hr and whole-cell lysates were analyzed by western blot analysis as described in Materials and Methods. β-Actin served as a loading control.

Previous studies in this laboratory show that genes such as survivin, VEGF, cyclin D1 and EGFR are regulated by Sp1, Sp3 and Sp4 transcription factors which are overexpressed in pancreatic and other cancer cell lines (147, 162-165, 168). Treatment of L3.6pL and Panc28 cells with 1.0 – 5.0 μM celastrol decreased expression of Sp1, Sp3 and Sp4 proteins in both cell lines (Figure 40A) and this response was not reversed after cotreatment with the proteasome inhibitor MG132 (Figure 40B). These results are in contrast to previous studies with tolfenamic acid which induces proteasome-dependent downregulation of Sp1, Sp3 and Sp4 in pancreatic cancer cells and tumors (Figure 40) (162).

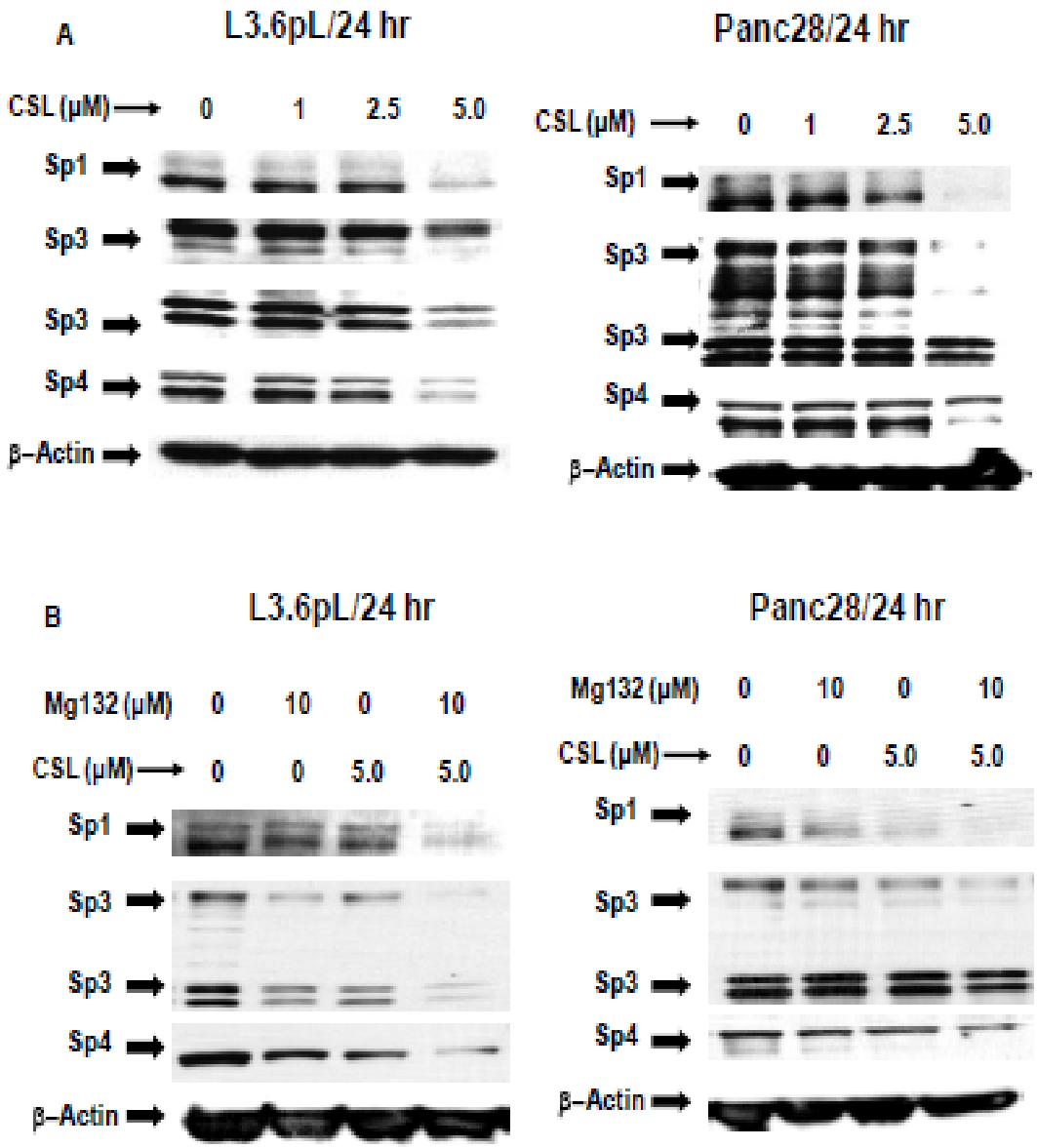

Figure 40. Celastrol downregulates Sp proteins in a proteosome-independent manner.

Celastrol decreases Sp protein expression in L3.6pL and Panc-28 cells (A). Cells were treated with DMSO and 1.0, 2.5 or 5.0 μM celastrol for 24 hr and whole-cell lysates were analyzed for Sp1, Sp3 and Sp4 by western blot analysis

as described in Materials and Methods. B. Proteosome-independent downregulation of Sp proteins by celastrol. L3.6pL and Panc-28 Cells were treated with DMSO and 5.0 μM celastrol in the presence or absence of proteasome inhibitor MG132 (10 μM) and the effects on Sp protein degradation were determined after treatment for 24 hr by western blot as described in Materials and Methods. β-Actin served as a loading control.

Recent studies in this laboratory demonstrated the p65 and p60 subunits of NFκB were also regulated by Sp transcription in bladder and pancreatic cancer cells (146, 147) (Curcumin-In revision) and results in Figure 41A show that treatment of L3.6pL and Panc28 cells with celastrol decreased expression of p65 and p50 proteins. Moreover, celastrol also decreased luciferase activity in cells transfected with a construct (NFκB-luc) containing 5 tandem NFκB response element linked to luciferase (Figure 41B). The effects of celastrol on constructs containing GC-rich promoter inserts from the Sp1, Sp3, survivin and the VEGF genes were also investigated using L3.6L cells (Figures 41C & 41D) and celastrol decreased luciferase activity in cells transfected with all of these constructs (Figure 41).

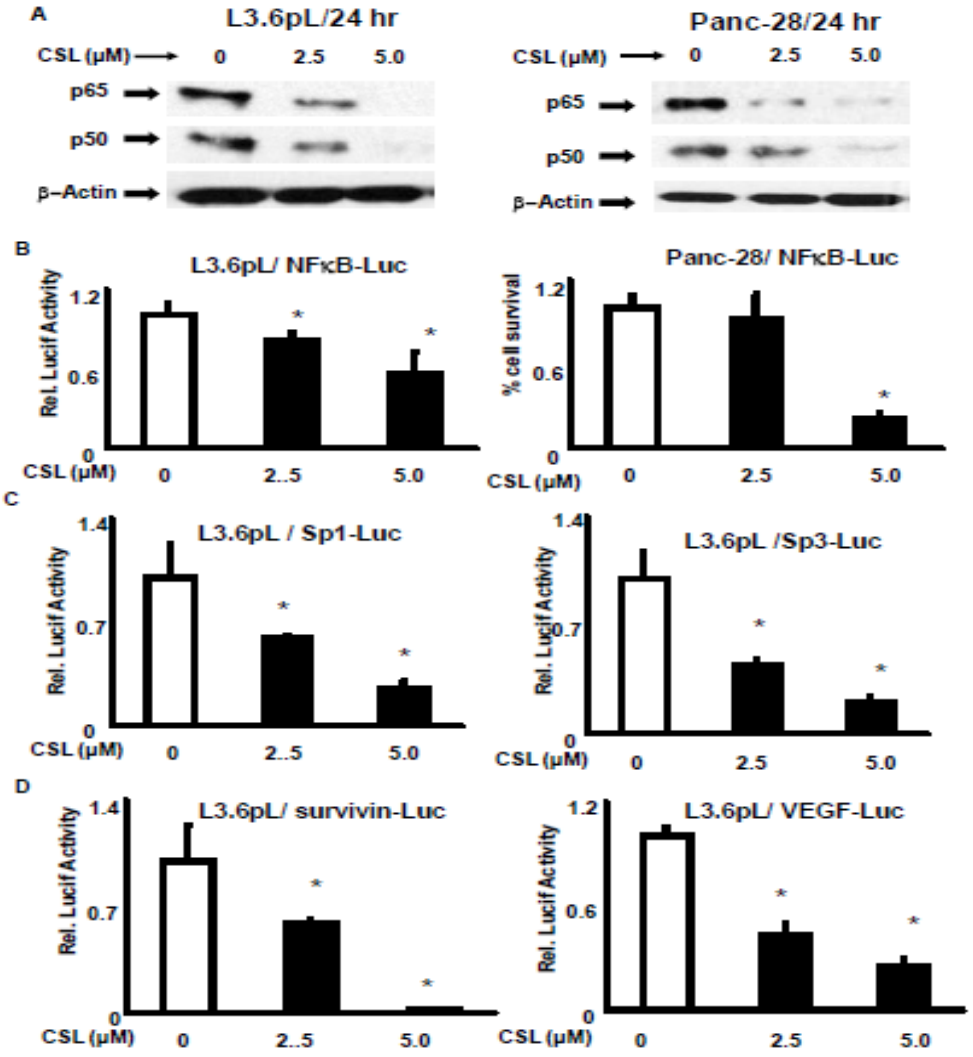

Figure 41. Celastrol downregulates p65 and p50 protein subunits of NFkB and decreases the activity of NFkB, Sp1, Sp3, VEGF and survivin promoters in L3.6pL and Panc-28 cell lines.

Effect of celastrol on p65 and p50 proteins (A). L3.6pL and Panc-28 cells were treated with DMSO and 2.5 or 5.0 μM Celastrol for 24 hr and the nuclear extracts were analysed by western blot as described in materials and methods. L3.6pL and Panc-28 cells were transfected with pNFkB (B), pSp1, pSp3 (C),

psurvivin or pVEGF-Luc (D), and treated with DMSO and 2.5 or 5.0 μM Celastrol for 22 hr and the luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group and significant ($P < 0.05$) inhibition (*) of responses are indicated.

In ongoing studies with the synthetic triterpenoid anticancer drug methyl –cyano-3,12-dioxooleana-1,9(11)-dien-29-oate (CDOD-Me) we observed that this compound also decreased expression of Sp1 proteins and that this response was dependent on induction of ROS and reversed by thiol antioxidants GSH and/or DTT (In review). Results in Figure 42A shows that celastrol induced ROS in L3.6pL and Panc28 cells and this response was significantly inhibited after cotreatment with celastrol plus GSH. In addition, we also observed that celastrol-dependent downregulation of Sp1, Sp3 and Sp4 proteins in pancreatic cancer cells was also reversed after cotreatment with DTT or GSH (Figure 42B). The role of ROS in mediating the growth inhibitory activity of celastrol was also investigated in pancreatic cancer cells (Figure 42C). Celastrol decreased proliferation of L3.6pL and Panc28 cells however cotreatment with GSH or DTT completely reversed this response demonstrating that induction of ROS was critical for both celastrol-induced growth inhibition and Sp downregulation (Figure 42). Currently we are investigating the role of ROS in mediating downregulation of microRNA-27a and induction of the SP-repressor ZBTB10 by celastrol in pancreatic cancer cells.

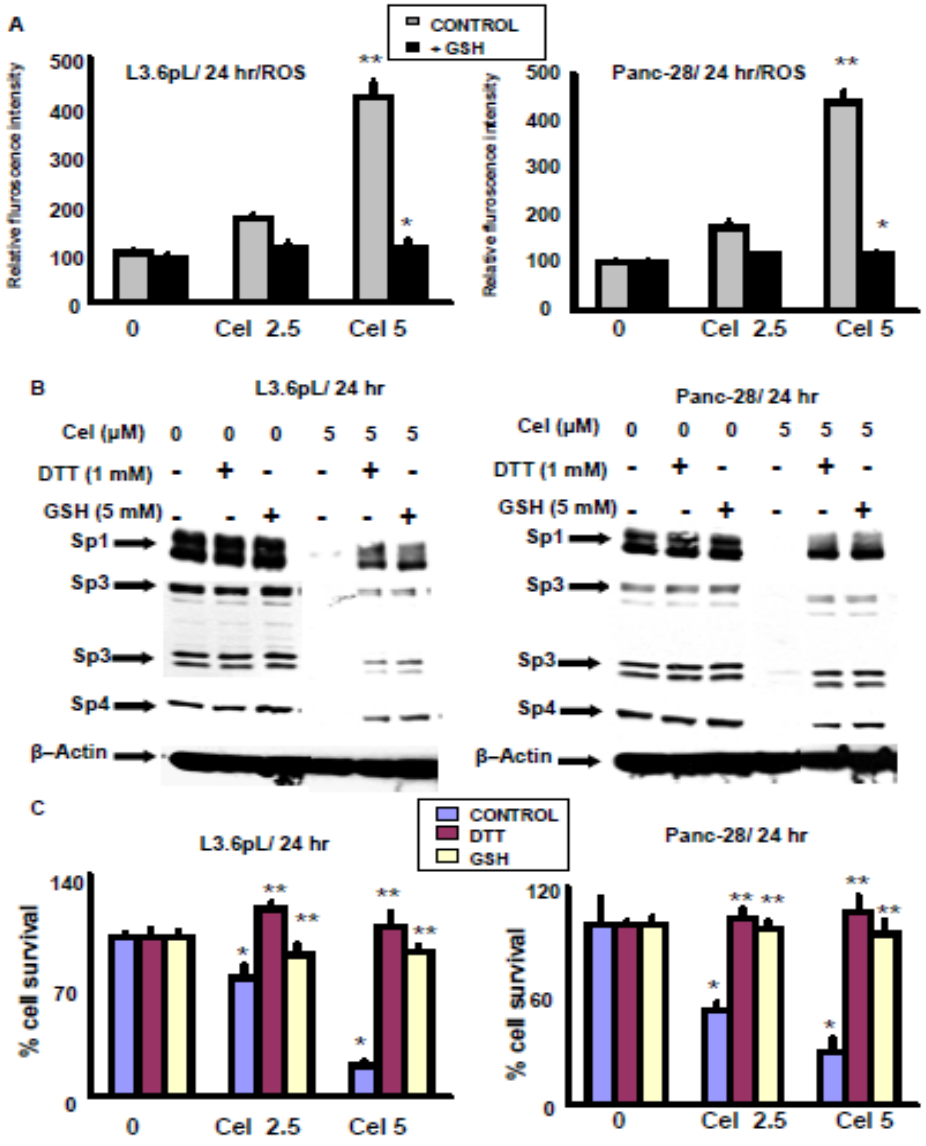

Figure 42. Role of oxidative stress in mediating the effects of celastrol on Sp proteins and cell growth in pancreatic cancer cells.

Reversal of celastrol mediated downregulation of Sp proteins by thiol antioxidants (A) and cell growth (B). Cells were treated with DMSO, 2.5 or 5.0 μmol/L celastrol in the presence or absence of DTT or GSH for 24 hr and the cells were either counted or whole cell lysates were analyzed by western blots

as described in Materials and Methods. β-Actin served as a loading control. C. Effect of celastrol on ROS. Cells were treated with DMSO, 2.5 or 5.0 μmol/L celastrol alone or in combination with GSH. ROS was measured using BioTek Synergy 4 plate reader using 10 μM CM-H_2DCFDA dye as described in Materials and Methods and normalized fluorescence intensity against control is plotted as a bar diagram. Results are expressed as means ± SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) CSL-induced increases (**) or decreases (*) compared to the solvent (DMSO) control are indicated.

The in vivo anticancer activity of celastrol was determined in athymic nude mice bearing L3.6pL cells as xenografts. Celastrol at a dose of 4 mg/kg/d decreased tumor growth and tumor weights (Figures 43A & 43B), decreased expression of Sp1, Sp3 and Sp4 proteins and also decreased survivin in VEGF levels in tumors compared to corn oil-treated animals. The in vivo studies were carried out only over a relatively short period due to the rapid growth of the L3.6pL-derived tumors. The in vivo data complemented the cell culture results and show that the potent anticancer activity of celastrol is due, in part, to downregulation of Sp1, Sp3 and Sp4 transcription factor which are overexpressed in pancreatic cancer cells and in human tumors (Figure 43) (158, 162).

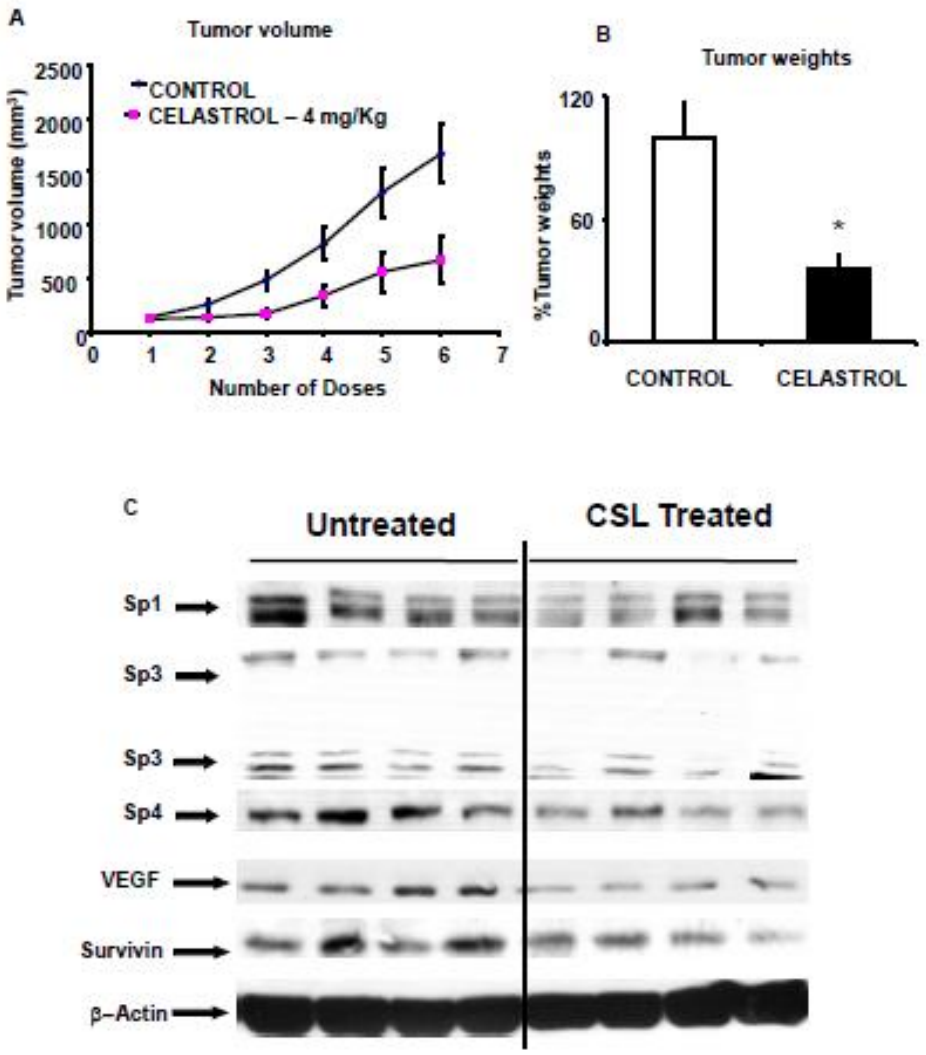

Figure 43. Celastrol inhibits pancreatic tumor growth and downregulates Sp proteins and Sp dependent genes.

Tumor Volume (A) and weights (B). Female athymic nude mice bearing pancreatic (L3.6pL) tumors were treated with corn oil or celastrol (4 mg/Kg) for 14 days after tumors have reached palpabale size and tumor weights and tumor volumes (mm^3) were determined as described in Materials and Methods.

Significant ($P < 0.05$) inhibition (*) is indicated in results as means ± SE for six animal per treatment. Western blot analysis of tumor lysates for Sp proteins and Sp dependent proteins (C). Lysates from fours mice in the treated and control groups were analyzed by western blots as described in Materials and Methods. β-Actin served as loading control.

Discussion

Pancreatic ductal adenocarcinoma (PDAC) is a major cause of cancer-related deaths in developed countries and, in 2009, it is estimated that in excess of 34,000 new cases will be diagnosed in the United States (2). PDAC is a highly aggressive disease that invariably evades early diagnosis (313). The mean survival time for patients with metastatic disease is only 3 – 6 months, and the 1-year survival time for all pancreatic cancers cases is approximately 20-30%. Moreover, current therapies for pancreatic cancer use cytotoxic drugs which are accompanied by toxic side-effects and are largely ineffective. Therefore, research in this laboratory has focused on development of new mechanism-based drugs that target Sp transcription factors and Sp-dependent genes in pancreatic tumors but are accompanied by minimal toxic side-effects (146, 153, 158, 159, 162) (curcumin-In revision). Although Sp transcription factors are important during embryonic development, there is evidence that in humans and laboratory animals that there is a marked decrease in Sp1 with aging (166, 167) and studies in this laboratory show that Sp1, Sp3 and Sp4 levels in non-tumor tissue of mice are minimal to non-detectable (163, 165). Several reports show

that Sp1 protein is overexpressed in different tumor types including gastric, colorectal, pancreatic, epidermal, thyroid and breast cancers (160, 230-232, 268). Lou and coworkers (157) have shown that malignant transformation of human fibroblasts resulted in an 8- to 18-fold increase in Sp1 expression and the transformed cells formed tumors in athymic nude mouse xenografts. In contrast, Sp1 knockdown gave cells that were non-tumorigenic in the same mouse xenograft model. Sp1 is overexpressed in pancreatic cancer cells (153, 162, 268) and there is a correlation between expression of Sp1 and the angiogenic factor vascular endothelial growth factor (VEGF). Moreover, it was recently reported that Sp1 was a biomarker that identifies patients with a highly aggressive sub-type of pancreatic ductal adenocarcinomas (160).

Tolfenamic acid induces proteasome-dependent degradation of Sp1, Sp3 and Sp4 in pancreatic cancer cells (162); however, results of preliminary studies show that although both BA and CDODA-Me decrease Sp1, Sp3 and Sp4 in pancreatic cancer cells, these effects are proteasome-independent. The triterpenoid betulinic acid in LNCaP prostate cancer cells (168) and curcumin in bladder cancer cells (165) also induced proteasome-dependent degradation of Sp proteins, however a new synthetic pentacyclic is triterpenoid, CDODA-Me decreased Sp1, Sp3 and Sp4 expression in colon cancer cells and this response was proteasome-independent (163). The cytotoxic and antiangiogenic activity of celastrol is similar to that of other triterpenoids, however, based on a report that celastrol decreased VEGFR1 and VEGFR2 expression in glioma tumors (312)

we hypothesized that these and possibly other responses may be due, in part, to downregulation of Sp transcription factors. Results in Figure 39 show that celastrol inhibited growth of L3.6pL and Panc28 cells and this was accompanied by downregulation of several proteins associated with the growth, survival and angiogenic activity of these cells. Previous studies in pancreatic and other cancer cell lines how that cyclin D1, p65/p50 (NFκB), survivin, EGFR and VEGF are regulated by Sp transcription factors (147, 153, 158, 162-165, 168) (curcumin – In revision). Moreover, the IGFR1β, gene is also an Sp regulated gene (314) and we observed that celastrol also decreased expression of this receptor (Figure 39C). Not surprisingly, decreased expression of these proteins was paralleled by celastrol-induced downregulation of Sp1, Sp3 and Sp4 proteins in L3.6pL and Panc28 cells (Figure 40) and in tumors from athymic nude mice bearing L3.6pL cells as xenografts (Figure 43). The NSAID tolfenamic acid was the first compound identified that decreased all 3 Sp proteins in pancreatic cancer cells (162) and this effect was due to activation of proteasomes and the subsequent proteasome-dependent degradation of Sp1, Sp3 and Sp4. In contrast, celastrol did not induce proteasome-dependent degradation of Sp transcription factors (Figure 40B) and studies with various promoter constructs suggest that celastrol decreased transcription of Sp1, Sp3 and Sp4 (Figure 41).

Recent studies with the synthetic triterpenoid CDDO-Me (In review) in pancreatic cancer cells indicate that this compound was mitochondrio toxic and

induced ROS and this has previously been reported for this compound in other cell lines (294, 314). Treatment of Panc28 and L3.6pL cells with celastrol also induced ROS, however, after cotreatment with the thiol antioxidant GSH there was a significant decrease in ROS levels (Figure 42A). Moreover celastrol dependent growth inhibition (Figure 42C) and downregulation of Sp1, Sp3 and Sp4 proteins (Figure 42B) was also inhibited after cotreatment with the cellular antioxidants GSH or DTT. Previous studies indicate that at least one mechanism of transcriptional repression of Sp transcription factors involves drug-induced downregulation of miR-27a with the subsequent upregulation of the miR-27a regulated ZBTB10 gene (291) which is an Sp-repressor (163, 164, 291). Celastrol downregulates miR-27a and induces ZBTB10 expression in L3.6pL cells (data not shown) and we are currently investigating the role of antioxidants in modulating the effects of celastrol of miR-27a:ZBTB10.

In summary our results confirm that celastrol, like other triterpenoids inhibits cancer cell and tumor growth and in pancreatic cancer cells these effects are accompanied by ROS-dependent downregulation of Sp1, Sp3, Sp4 and Sp-dependent genes and we are currently investigating the ROS:miR-27a:ZBT10-Sp axis as a mechanism for these responses. The results also show that induction of ROS is a critical determinant in the cytotoxicity of celastrol (Figure 42C) and this is also accompanied by ROS-dependent downregulation of Sp transcription factors suggesting that celastrol-induced downregulation of Sp1, Sp3 and Sp4 contributes to the cytotoxicity. These results expand the reported

anticancer activities of celastrol and the relative contributions of celastrol-induced repression of Sp1, Sp3 and Sp4 to the overall effects of this drug will be dependent on cancer cell context. However, for those pancreatic cancer patients that overexpress Sp1 and have tumors that exhibit aggressive behavior (160) it is possible that drugs such as celastrol and other compounds that target Sp downregulation may be a treatment option for this devastating disease.

VII. SUMMARY

PPARγ is a ligand-activated transcription factor belonging to the nuclear hormone receptor family and related to retinoid, glucocorticoid and thyroid hormone receptors. PPARγ is overexpressed in multiple tumor types and cancer cell lines suggesting that this receptor may be a potential target for cancer chemotherapy. CDODA-Me activated PPARγ in Panc28 and Panc1 cells and induced PPARγ-dependent transactivation and differentiation of 3T3-L1 pre-adipocytes which is a prototypical PPARγ-dependent response. CDODA-Me also induced p21 and p27 and decreased cyclin D1 expression in Panc1 and Panc28 cells, however, studies with the PPARγ antagonist GW9662 indicated that these responses were receptor-independent. CDODA-Me inhibited growth and induced apoptosis through induction of proapoptotic proteins NAG-1 and ATF3 in Panc28 and Panc1 cells and cotreatment with the PPARγ antagonist GW9662 did not reverse these responses. Previous studies show that CDODA-Me induced proapoptotic proteins NAG-1 and ATF3 in a receptor independent manner in LNCaP prostate cancer cells, and these responses were dependent on induction of Egr-1 (147). In pancreatic cancer cells, Egr-1 was induced prior to induction of NAG-1 and this temporal pattern of NAG-1 and Egr-1 induction is similar to that observed in other studies where Egr-1 activates NAG-1 through interactions with the GC-rich proximal region of the NAG-1 promoter. CDODA-Me induces phosphorylation of several kinases (PI3K, p38/p42MAPK and JNK) in both Panc28 and Panc1 cells, however, induction of Egr-1 was p42MAPK-

dependent. In Panc1 cells, the PI3-K and p38 MAPK inhibitors LY294002 and SB203580 also blocked induction of NAG-1 demonstrating the contributions of Egr-1-dependent and -independent pathways for induction of NAG-1 in Panc1 cells. Kinase inhibitor studies in Panc28 showed that induction of NAG-1 by CDODA-Me was primarily Egr-1-independent and was inhibited by LY294002 and SB20358 which did not affect induction of Egr-1 in this cell line. In summary, results of this study demonstrate that CDODA-Me-mediated induction of NAG-1 and Egr-1 is cell context-dependent in Panc28 and Panc1 cells and current studies are investigating the interplay between kinase activation, induction of proapoptotic proteins, and apoptosis by CDODA-Me in pancreatic cancer cells and the contributions of other pathways in mediating the proapoptotic effects of CDODA-Me in pancreatic cancer cells and tumors (146).

Sp transcription factors are members of the Sp/Krüppel-like family (KLF) of 25 transcription factors that bind GC-rich promoter sequences and regulate basal expression of multiple mammalian and viral genes. Knockout of most Sp genes in mice is embryolethal or induces serious defects in the neonates. However, expression of Sp1 is significantly decreased in rodent and human tissues with increasing age (170). Studies in this laboratory show that in mouse xenograft studies, Sp1, Sp3 and Sp4 expression is low in liver, kidney and also in more proliferative tissues such as the gastrointestinal tract (168). In contrast, expression of Sp1, Sp3 and Sp4 is high in breast, colon, pancreatic, prostate and bladder tumor xenografts and their derived cancer cell lines. RNA

interference studies in which Sp1, Sp3 and Sp4 are knocked down simultaneously demonstrate that Sp transcription factors regulate several genes involved in cancer cell survival (survivin, bcl-2, NFκB), angiogenesis (VEGF and its receptors) and proliferation (cyclin D1, EGFR) (153, 158, 159, 165, 229). Malignant transformation of human fibroblasts resulted in an 8- to 18-fold increase in Sp1 expression and the transformed cells formed tumors in athymic nude mouse xenografts whereas cells were not tumorigenic after knockdown of Sp1 (157). (158, 162, 163, 168, 268)(158, 162, 163, 168, 268)Moreover, a recent report showed that Sp1 was a negative prognostic factor for pancreatic cancer patient survival (160) and therefore we have been investigating the mechanism of action of several anticancer agents that may act, in part, through decreasing expression of Sp transcription factors in tumors. Previous studies showed that tolfenamic acid and betulinic acid induced proteasome-dependent degradation of Sp1, Sp3 and Sp4 in pancreatic and prostate cancer cells and tumors respectively and curcumin also induced proteasome-dependent degradation of Sp proteins in bladder cancer cells.

Arsenic trioxide is currently being used for treatment of leukemia and is also being investigated for treating solid tumors. Previous studies reported that arsenic trioxide downregulation of several Sp-dependent genes and responses (VEGF, angiogenesis, survivin, bcl-2, NFκB activity) and in gall bladder carcinoma arsenic trioxide decreased Sp1 protein expression (227). However, the mechanism of this response and the effects on Sp3 and Sp4 were not

determined. In this study we demonstrated that arsenic trioxide downregulated expression of Sp proteins in urological and gastrointestinal-derived cell lines and KU7 and 253JB-V bladder cancer cells were used as models for investigating the mechanisms of Sp downregulation. Arsenic trioxide inhibited growth of KU7 cells at low concentrations ($\leq$ 5 μM) and downregulated Sp1, Sp3 and Sp4 proteins in KU7 cells and tumors whereas only minimal effects on growth and Sp downregulation were observed in 253JB-V cells. In this study we demonstrated that differential expression of GSH contributed to the responsiveness of KU7 and 253JB-V to the effects of arsenic trioxide on cell growth and Sp protein downregulation. Arsenic trioxide also induced ROS and decreased GSH and MMP in KU7 cells and these responses, in addition to Sp protein downregulation were ameliorated after cotreatment with the thiol antioxidants GSH or DTT. Induction of ROS by arsenic trioxide and the subsequent downregulation of Sp proteins has been directly linked to the cytotoxicity of this compound and other mitochondriotoxic anticancer drugs. Like arsenic trioxide, hydrogen peroxide is a pro-oxidants and inhibited growth and decreased expression of Sp proteins in KU7 cells and these responses were blocked after cotreatment with the antioxidant glutathione. Catalase blocked hydrogen peroxide-dependent decrease in MMP but had minimal effects on decreased MMP in KU7 cells treated with arsenic trioxide. These results suggest that induction of extramitochondrial ROS by arsenic trioxide in KU7 cells has a minimal effect on MMP, indicating that arsenic trioxide-dependent decrease in MMP and induction

of ROS are due to direct effects on the mitochondria. We also investigated the effects of catalase, NAC, and BHA, stress kinase inhibitors of p38MAPK and JNK (SB203580 and SP600125), and the NADPH oxidase inhibitor, DPI on hydrogen peroxide- and arsenic trioxide-mediated downregulation of Sp1, Sp3 and Sp4 in KU7 cells. The results showed that SB203580, SP6000125, DPI and BHA had minimal to non-detectable effects on arsenic trioxide-induced downregulation of Sp1, Sp3 and Sp4 proteins, suggesting that the major pathway targeting these transcription factors involves mitochondria and induction of ROS.

Curcumin is an anti-inflammatory drug and an inhibitor of basal and induced NFκB-dependent-responses and these activies are important for the anticancer effects of curcumin. The nuclear NFκB complex containing p65 (Rel A) and p50 (NFκB1), is a multifunctional nuclear transcription factor that regulates expression of multiple genes in a cancer cell context and this results in cancer cell proliferation, survival, angiogenesis and metastasis, and promotes epithelial to mesenchymal transition (EMT), and inflammation. These responses are accompanied by induction of genes such as cyclin D1, survivin, VEGF, bcl-2, and COX-2 that contribute to the NFκB-regulated effects. Studies in our laboratory with curcumin in bladder cancer cells showed that curcumin decreased expression of Sp proteins and Sp-dependent genes and in 253JB-V cells and it was shown that p65; a subunit of NFκB was also an Sp-regulated gene (165). Curcumin is currently in clinical trials for treatment of pancreatic

cancer and we hypothesized that effects of this compound on NFκB were due, in part to Sp1, Sp3 and Sp4 downregulation in pancreatic cancer cells (Panc28 and L3.6pL). Curcumin inhibited proliferation of Panc28 and L3.6pL pancreatic cancer cells and decreased basal and TNFα-induced expression of both p65 and p50. Curcumin also downregulated expression of Sp1, Sp3 and Sp4 and Sp-dependent genes in Panc28 and L3.6pL pancreatic cancer cells as previously observed in bladder cancer cells (165). However, in bladder cancer cells, curcumin-mediated-Sp downregulation was blocked by the proteasome inhibitor MG-132, whereas in pancreatic cancer cells, MG-132 did not affect curcumin-dependent repression of Sp proteins. This study also showed that after knockdown of Sp proteins using siRNAs for Sp1, Sp3 and Sp4 (in combination) decreased expression of both basal and TNFα induced levels of nuclear p65 and p50 proteins was observed indicating that curcumin-dependent inhibition of NFκB is due, in part, to downregulation of Sp transcription factors. Curcumin also decreased MMP and induced ROS in pancreatic cancer cells and cotreatment with GSH or DTT reversed the effects of curcumin on downregulation of Sp1, Sp3 and Sp4 and growth inhibition.

The triterpenoids, CDDO and CDDO-Me also decreased expression of Sp1, Sp3 and Sp4 and Sp-regulated genes such as cyclin D1, VEGF, VEGFR2 and survivin in Panc1, Panc28 and L3.6pL pancreatic cancer cells. Previous studies in pancreatic cancer cells showed that CDDO-Im decreased MMP and GSH and induced ROS and these responses were blocked in cells cotreated

with thiol antioxidants (185). Not surprisingly, CDDO-Me also induced a similar pattern of mitochondriotoxic responses in pancreatic cancer cells and these were inhibited after cotreatment with thiol antioxidants. We also demonstrated that CDDO-Me-dependent downregulation of Sp proteins is inhibited in Panc1 and L3.6pL cells after cotreatment with GSH and DTT. Previous studies in breast and colon cancer cells demonstrated that miR-27a suppresses zinc finger protein ZBTB10 which regulates expression of Sp1, Sp3 and Sp4 (163, 164). Both antisense miR-27a and ZBTB10 overexpression decreased Sp and Sp-dependent genes in these cell lines, and this was related to the effects of ZBTB10 which binds GC-rich sites and acts as an transcriptional repressor. Treatment of Panc1 and L3.6pL cells with CDDO-Me decreased expression of miR-27a and this was accompanied by induction of ZBTB10 in cells cotreated with CDDO-Me plus GSH on miR-27a and ZBTB10 were reversed. These studies demonstrate that CDDO-Me-dependent induction of ROS is critical for downstream events since antioxidants inhibit the effects of CDDO-Me on the miR-27a:ZBTB10-Sp protein axis in Panc1 and L3.6pL cells.

Celastrol also inhibited growth of L3.6pL and Panc28 cells and this was accompanied by downregulation of Sp protein and Sp-dependent genes involved in growth, survival and angiogenesis. Celastrol also decreased expression of IGFR1β gene which is another an Sp regulated gene. Celastrol did not induce proteasome-dependent degradation of Sp transcription factors; however, cotreatment with thiol antioxidants reversed the effects of celastrol on

Sp protein downregulation and this was similar to the results observed with curcumin, CDDO-Me and arsenic trioxide in pancreatic cancer cells. Celastrol downregulated miR-27a and induced ZBTB10 expression in L3.6pL cells and the role of antioxidants in modulating the effects of celastrol on miR-27a:ZBTB10 is currently being investigated.

In summary, these drugs (curcumin, arsenic trioxide, CDDO-Me and celastrol) inhibit growth of pancreatic and bladder cancer through downregulation of Sp proteins and Sp-dependent genes. These compounds induce ROS, decrease MMP and this is accompanied by decreased expression of miR-27a and induction of ZBTB10 (a Sp repressor) and mechanism for these effects is illustrated in Figure 44.

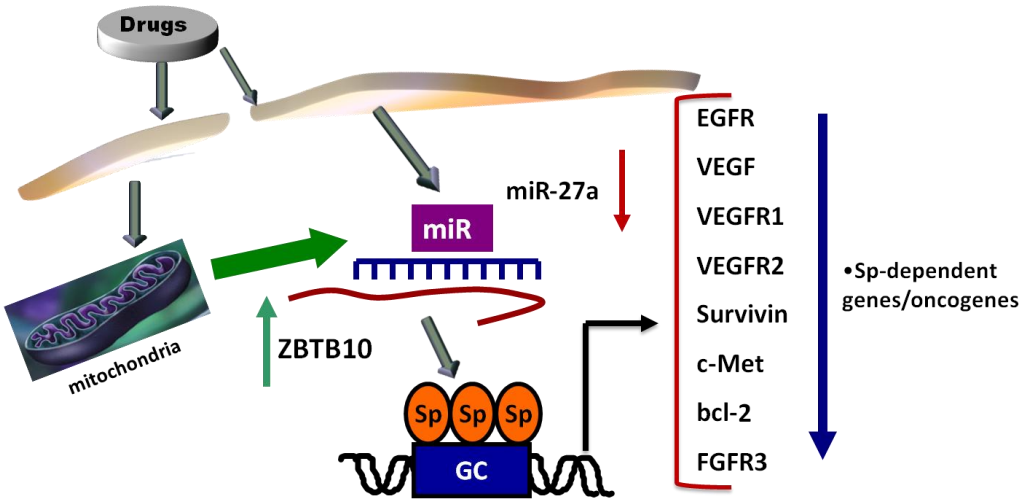

Figure 44. Mechanisms involved in downregulation of Sp transcription factors.

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