

**MECHANISM BASED ANTICANCER DRUGS THAT DEGRADE Sp
TRANSCRIPTION FACTORS**

A Dissertation

by

GAYATHRI CHADALAPAKA

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

DOCTOR OF PHILOSOPHY

December 2009

Major Subject: Toxicology

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ABSTRACT

Mechanism Based Anticancer Drugs That Degrade Sp Transcription Factors.

(December 2009)

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Chair of Advisory Committee: Dr. Stephen H. Safe

Curcumin is the active component of tumeric, and this polyphenolic compound has been extensively investigated as an anticancer drug that modulates multiple pathways and genes. We demonstrated that curcumin inhibited 253JB-V and KU7 bladder cancer cell growth, and this was accompanied by induction of apoptosis and decreased expression of the proapoptotic protein survivin and the angiogenic proteins vascular endothelial growth factor (VEGF) and VEGF receptor 1 (VEGFR1). Since expression of survivin, VEGF and VEGFR1 are dependent on specificity protein (Sp) transcription factors, we also investigated the effects of curcumin on downregulation of Sp protein expression as an underlying mechanism for the apoptotic and antiangiogenic activity of this compound. Curcumin decreases expression of Sp1, Sp3 and Sp4 in bladder cancer cells indicating that the cancer chemotherapeutic activity of curcumin is due, in part, to decreased expression of Sp transcription factors and Sp-dependent genes. Betulinic acid (BA) and curcumin are phytochemical anticancer agents, and we hypothesized that both compounds decrease EGFR expression in bladder cancer through downregulation of specificity protein (Sp) transcription factors. BA and curcumin decreased expression of EGFR, Sp1, Sp3, Sp4 and Sp-dependent proteins in 253JB-V and KU7 cells; EGFR was also decreased in cells transfected with a cocktail (iSp) containing small inhibitory RNAs for Sp1, Sp3 and Sp4 showing that EGFR is an Sp-regulated gene. Methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oate (CDODA-Me) is a synthetic triterpenoid derived from glycyrrhetic

acid which inhibits proliferation of KU7 and 253JB-V bladder cancer cells. CDODA-Me also decreased expression of specificity protein-1 (Sp1), Sp3 and Sp4 transcription factors. Similar results were observed for a structurally-related triterpenoid, methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), which is currently in clinical trials for treatment of leukemia. Celastrol, a naturally occurring triterpenoid acid from an ivy-like vine exhibits anticancer activity against bladder cancer cells. Celastrol decreased cell proliferation, induced apoptosis and decreased expression of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 and several Sp-dependent genes like Fibroblast growth factor receptor 3 (FGFR3). *In vivo* studies using KU7 cells as xenografts showed that celastrol represents novel class of anticancer drugs that acts, in part, through targeting downregulation of Sp transcription factors.

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I. INTRODUCTION

Cancer is a leading cause of death worldwide and cancer deaths are projected to continue rising, with an estimated 12 million deaths in 2030 according to World Health Organization statistics. In 2007, world wide cancer deaths accounted for 7.6 million deaths and more than 12 million people were diagnosed with cancer. Cancer is the second most common cause of death in the US, exceeded only by heart disease and cancer accounts for nearly 1 of every 4 deaths. It is estimated that 1,479,350 new cancer cases will be diagnosed in 2009 and about 562,340 Americans are expected to die of this disease i.e., more than 1,500 people a day. The National Cancer Institute estimates that approximately 11.1 million Americans with a history of cancer were alive in January 2005; some of these individuals were cancer-free, while others still had evidence of cancer and may have been undergoing treatment (1). Furthermore, reports suggest that autopsies of individuals who died of 'non-cancer' causes of death often reveal microscopic colonies of cancer cells, known as in situ tumors. It has been estimated that more than one-third of women aged 40 to 50, who did not have cancer-related disease in their life-time, were found at autopsy with in situ tumors in their breast. Moreover, virtually all autopsied individuals aged 50 to 70 had in situ carcinomas in their thyroid gland, whereas only 0.1% of individuals in this age group are diagnosed with thyroid cancer during this period of their life. Clearly, in the male population, prostate, lung, colorectal, urological cancers followed by melanoma of skin are the prime organ sites of cancer incidence, but estimated cancer deaths are due to lung, prostate, colorectal, pancreatic cancer and leukemia's (Table 1).

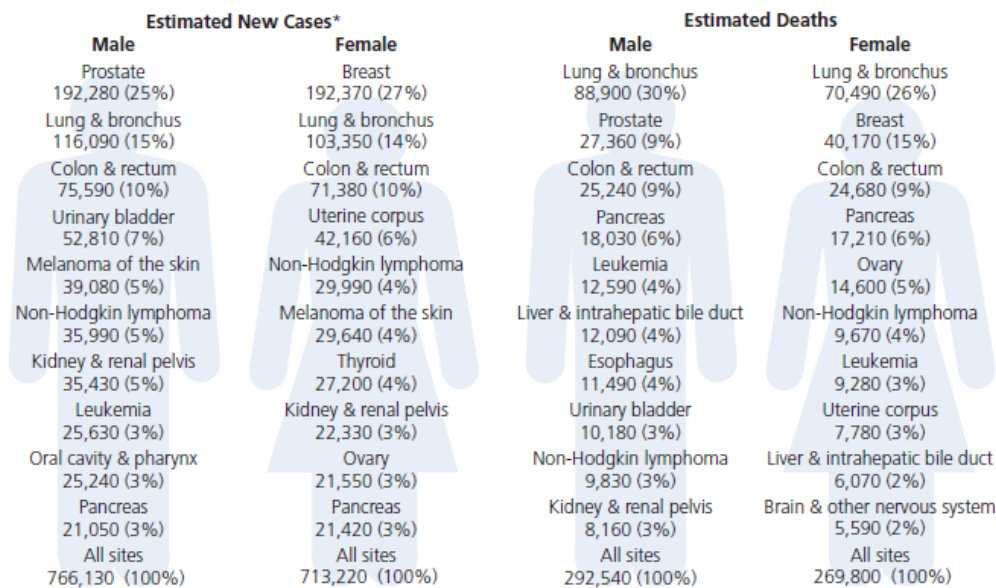
This dissertation follows the style of Cancer Research.

In females, breast, lung, colorectal, uterine and lymphomas are the major organ cancers, but the estimated deaths are primarily due to lung, breast, colorectal, pancreatic and ovarian cancers (Table 1) (2) (3). Bladder and pancreatic cancers will be the major focus of this section.

Bladder and pancreatic cancer incidence

Bladder cancer develops in tissues of the bladder (the organ that stores urine) and most bladder cancers are transitional cell carcinomas that begin in cells that normally form the inner lining of the bladder. Other types include squamous cell carcinoma (cancer that begins in thin, flat cells) and adenocarcinoma (cancer that begins in cells that produce mucus). Cells that form squamous cell carcinoma and adenocarcinoma develop in the inner lining of the bladder as a result of chronic irritation and inflammation. Bladder cancer is the fifth most common cancer in the United States (Figure 1) and, on a per capita basis, is the most expensive cancer from diagnosis to death because of disease recurrence, extended surveillance and repeated use of endoscopic and intravesical therapies (4).

In 2009 it is estimated that there will be 70,980 cases of bladder cancer in the United States and 14,330 patients will die from this disease. Bladder cancer incidence is nearly four times higher in men than in women and more than two times higher in white men than in African American men. Mortality rates have recently stabilized in men after decreasing for most of the past three decades; rates have been declining in women since 1975 (1).



*Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder.

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Figure 1. Leading sites of new cancer cases and deaths 2009 estimates (1).

An estimated 42,470 new cases and an estimated 35,240 deaths due to pancreatic cancer are expected to occur in the US in 2009. Incidence rates for pancreatic cancer have been stable in men since 1993 and have been increasing in women by 0.6% per year since 1994. Based on histological grading, pancreatic carcinomas are classified as adenocarcinomas, intraductal papillary mucinous neoplasm (IPMN) and pancreatoblastomas.

Cellular and molecular mechanisms of cancer formation

Cancer is characterized by uncontrolled growth and spread of abnormal cells. Cancer is caused by both external factors (tobacco, infectious organisms, chemicals, and radiation) and internal factors such as inherited mutations, hormones, immune conditions, and mutations that occur from metabolism (5). These causal factors may act together or in sequence to initiate or promote carcinogenesis. Ten or more years often pass between exposure to external factors and detectable cancer.

It is widely accepted that cancer arises in a multistep fashion and that environmental exposure, particularly to physical, chemical, and biological agents, is a major etiological factor. Besides chemicals, radiation, and viruses, other influences (e.g., genetic, hormonal, nutritional, and multifactor interactions) are also involved. Experimental cancer models suggest that at least three steps are important for tumor formation, namely initiation, promotion, and progression. The process of oncogenesis has been studied only indirectly in humans; measurements of age-dependent cancer incidence have shown that the rate of tumor development is proportional to the fourth to sixth power of elapsed time, suggesting that four to six independent steps are necessary (6) (7). Several well studied cancer models of human epithelial cell carcinogenesis affirm that tumor formation involves multiple steps and molecular events (8):

1. Tumor initiation. The first stage of initiation involves accumulation of genetic changes in a single cell as suggested by Knudson and Nowell (9). Initiation occurs after exposure to mutagens and results in almost no observable changes in the cellular tissue morphology but it confers an increase in susceptibility to cancer formation. Initiation corresponds to the introduction of mutation and during the initiation phase mutant cells proliferate very slowly (10). Cancer initiation results from exposure to mutagens such as X-rays and this result in heritable cellular change that do not significantly change cellular or tissue morphology but confer a long-term increase in risk of cancer development. The initiation stage of mouse skin carcinogenesis involves genetic damage in the form of covalent adducts between the initiator and DNA and these changes ultimately lead to mutations in critical target stem cell genes. The Ha-ras and to a limited extent, N-ras oncogenes have been identified as target genes for certain tumor initiators (11).

2. Tumor promotion. Tumor promotion involves non-mutagenic tissue disruption by wounding or inflammation resulting in formation of a non-malignant tumor which may regress without further stimuli. This phase is featured by

enhanced cellular proliferation and localized increases in vascular density and blood flow. The promotion step results in formation of a localized altered cell population that displays a non-malignant, self-limited growth that may regress if the promoting stimulant is withdrawn. Growth in this lesion is limited by diffusion of oxygen as the precancerous cell expansion carries proliferating cells further away from blood vessels. Tumor promoters may cause up regulation of some receptor signaling pathways such as epidermal growth factor receptor (EGFR) (12). In contrast to initiators, tumor promoting agents usually cause dramatic morphological and biochemical effects that are reversible in the absence of treatment. In this promotion phase, the initiated cell expands clonally to give rise to a population of initiated cells. When the initiated cells acquire further genetic changes required for malignant phenotype, the third phase of malignancy occurs and this includes events such as clonal evolution of tumors after malignant transformation.

Clonal selection of partially altered cells in the pathway to cancer can substantially increase the population of cells that have acquired some of the mutations critical for carcinogenesis, thus increasing the probability that a subset of these cells will acquire the remaining mutations required for malignant transformation. There is evidence that clonal expansion of premalignant cells is a feature of carcinogenesis in many tissue and organ sites (13).

3. Tumor progression and malignant transformation. Tumor progression and malignant transformation requires some additional cellular disruption although most changes in this phase do not require additional external stimulus. In the final progression step, the tumor transitions into limitless, invasive growth. A well studied colon carcinogenesis model by Vogelstein and his colleagues show that tumor progression involves of successive waves of clonal selection (14) where genetic alterations cause permanent genetic instability with a high rate of chromosomal or base modifications resulting in morphological and karyotypic changes that transform pre-neoplastic cells into neoplastic cells. Other reports

suggest that clonal expansion of partially altered cells on their way to malignancy, do not require genomic instability and this is relevant for explaining colon cancer rates in human populations (13).

4. Tumor invasion and metastasis. The distant settlement or metastasis of cancer cells from their site of origin to distal locations are the cause of 90% of human cancers deaths (15). The phase of invasion involves progression of neoplastic cells to malignant cells with the addition of genetic and epigenetic changes resulting in more aggressive characteristics. These cells acquire the ability to secrete proteases that dissolve barriers such as basement membranes in host cells and they also acquire the ability to undergo angiogenesis. Proteins that tether cells to their surroundings are altered in the phenotype possessing invasive and metastatic capabilities. These characteristics include; loss of CAM (cell-cell adhesion molecules), inactivation of E-cadherin, β -catenin, changes in integrin expression, up regulation and activation of extracellular proteases such as matrix metalloproteinases (MMP) and kallikrein (16).

Many human cancers exhibit age-dependent increase in incidence that involves four to seven rate-limiting, stochastic events. Hence the succession of genetic changes, each conferring one or another type of growth advantage leads to a progressive conversion of normal human cells into cancer cell. Overall six essential alterations in cell physiology dictate malignant growth (Figure 2); Self-sufficiency of growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis are the acquired characteristics of cancer cells (16).

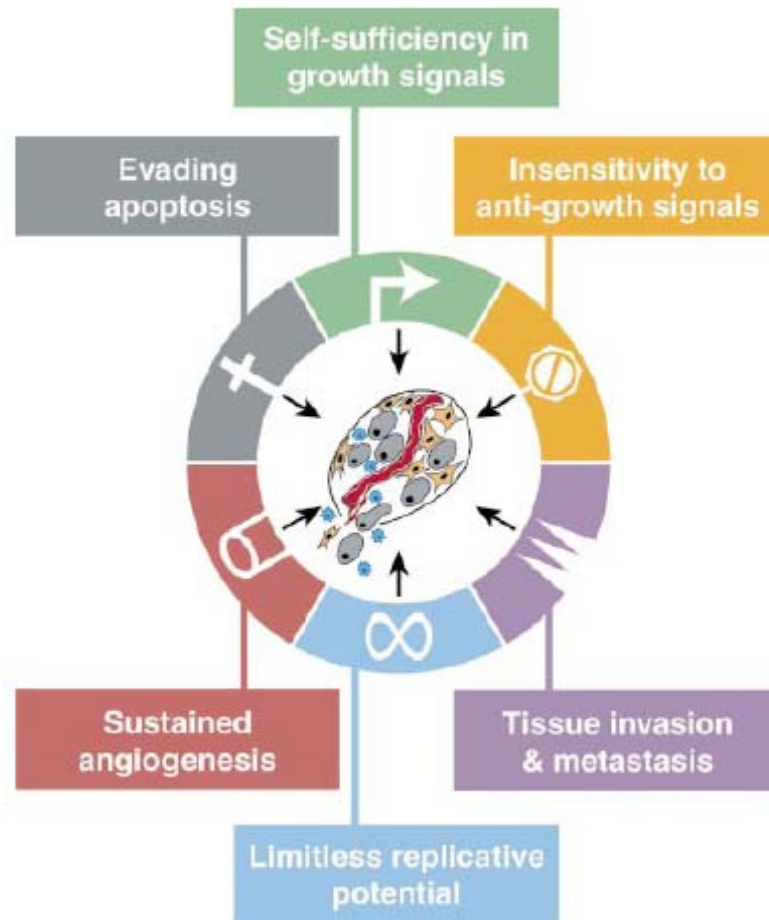


Figure 2. Acquired characteristics of cancer cells.

1. Self sufficiency of growth signals. Many oncogenes act by mimicking normal cellular growth signaling pathways and tumor cells generate their own internal growth signals that are not dependent on the surrounding environment. This autonomous functioning provides autocrine stimulation within the tumor cells. For example, receptor tyrosine kinases are overexpressed, amplified and dysregulated in many cancers. EGFR is overexpressed in many cancers of epithelial origin and this receptor is known for autocrine signaling as well as

crosstalk with other kinase signaling cascades. The frequency of overexpression of EGFR in human head and neck carcinomas is reported to be 100% (17). Heterotypic signaling within diverse tumors further explains their ability of to co-opt their neighbors to release growth-stimulating signals suggesting oncogenic crosstalk between signaling cascades.

2. Insensitivity to anti-growth signals. Anti-proliferative signals ensure cellular quiescence and tissue homeostasis that satisfies the normal physiological needs of the cell. E2F family members play a major role during the G1/S transition in the mammalian cell cycle. The Rb tumor suppressor protein (pRb) binds to the E2F-1 transcription factor to prevent interaction of this TF with the transcription machinery. In the absence of or disruption of pRb, E2F-1 (along with its binding partner DP-1) mediates the trans-activation of E2F-1 target genes that facilitate the G1/S transition and S-phase resulting in decreased response to inhibitory signals. Loss or mutation of pRb is involved in the etiology of many tumors including retinoblastomas and osteosarcomas. The Adenomatosis Polyposis Coli (APC) protein normally forms a complex with glycogen synthase kinase 3beta (GSK 3 β) and axin via interactions with the 20AA and SAMP repeats. Casein kinase 1 (CK1) catalyzes phosphorylation of β -catenin and there is subsequent phosphorylation by GSK-3 β . This targets β -catenin for ubiquitination and proteasome-dependent degradation and thereby inhibiting nuclear uptake of this protein, where it acts as a transcription factor for genes involved in cell proliferation. Inactivation of APC/ β -catenin pathway inhibits differentiation of colonic crypts and hence insensitivity to anti-growth signals forms a feature of many cancers (17).

3. Evading apoptosis. Programmed cell death is a major mechanism for maintaining homeostasis within a population of cells and resistance to apoptosis is a hallmark of most types of cancer (Figure 2). The tumor-suppressor protein p53 accumulates when DNA is damaged due to a chain of biochemical reactions. p53 prevents cells from replicating by terminating the cell cycle at G1,

or interphase for repair; however if damage is extensive and repair efforts fail p53 also induces apoptosis. Dysregulation of or p53-dependant genes may block apoptosis and lead to formation of tumors. In mammalian cells there is a balance between pro-apoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (Bcl-XI and Bcl-2) members of the Bcl-2 and this balance is proportional to the pro-apoptotic protein homodimers that form in the outer-membrane of the mitochondrion. These homodimers are required to make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c (17). Resistance to apoptosis by cancer cells is acquired after the loss of p53 tumor suppressor gene (17).

4. Limitless replicative potential. Cells in culture have a finite replicative potential and after a certain number of cell divisions they stop dividing and enter into senescence (18). However, disabling pRb or p53 tumor suppressor genes enable cells to continue multiplying for additional generations until they enter a second phase called crisis. At this stage cells undergo massive cell death, karyotypic disarray, end to end fusion of chromosomes resulting in variant immortalized cells that have acquired the ability for limitless replication (19). Telomeres are repeat sequences at the end of chromosomes that protect the genetic stability during DNA replication. Telomeres are lost during each cell division, and this increases chromosomal instability and cellular senescence. Telomere maintenance is evident in all types of malignant cells and 90% of cancers exhibit increased telomerase activity that adds hexanucleotide repeats to the ends of telomeric DNA (20).

5. Sustained angiogenesis. Once a tissue is formed, the growth of new blood vessels (angiogenesis) is transitory and carefully regulated. Vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF) are key angiogenic proteins and integrin signaling also contributes to the regulatory balance of angiogenesis. The ability to induce and sustain angiogenesis in cancer cells appears to be acquired in a discrete step during tumor development. Many

tumors overexpress VEGF and FGF proteins when compared to normal tissue and endogenous inhibitors of angiogenesis like thrombospondin, tumstatin, endostatin are downregulated (2). The VEGF gene is under complex transcriptional control and the role of specificity protein (Sp) transcription factors in the regulation of VEGF has been reported and will be discussed later in this section (21).

6. Tissue invasion and metastasis. Invasion and metastasis are hallmarks of an advanced stage of cancer and are associated with poor patient prognosis. This phase is characterized by loss of cell adhesion, gain of motility and proteolysis. Epidermal growth factor EGF-mediated downregulation of focal adhesion kinase (FAK) is necessary for early dissemination and cell detachment from the primary tumor. At secondary sites, interactions with extracellular matrix via integrins activate FAK and mediate cell attachment which is necessary for establishment of metastatic tumors. Loss of E-cadherin is also observed in invasive human tumors and its re-expression can reverse the invasive phenotype and restores epithelial morphology (22). Recruitment of PLC β 1, interaction of mitogen activated protein kinase (MAPK) with the cytoskeletal machinery and RHO family of GTPases regulate the motility of tumor cells. Proteolysis of the extracellular matrix barriers is attributed to matrix metalloproteinases (MMPs) which are enhanced and facilitate tumor invasion *in vitro* and *in vivo*. Overexpression of urokinase plasminogen activator (uPA) correlates positively with invasive potential for a variety of cancers because uPA plays a key role in tumor invasion and metastasis by its ability to initiate proteolytic cascade (23) (24). Apart from these hallmarks of cancer cells, genomic instability is an enabling characteristic that facilitates evolving populations of premalignant cells to reach these six biological endpoints.

Bladder cancer incidence and classification

Bladder cancer is the fourth most prevalent cancer in males and ninth in females. Risk factors include smoking, occupational exposure to aromatic amines, consumption of arsenic contaminated water, and chronic infection with parasite *Schistosoma* species, radiation therapy of neighboring organs and therapeutic use of alkylating agents. The term 'superficial bladder cancer' has been used to describe tumors that have not invaded into muscularis propria. This designation includes noninvasive papillary urothelial carcinoma (pTa), carcinoma in situ (CIS) (pTis), and tumor invading lamina propria (pT1). It is now recommended that the term 'superficial' be entirely eliminated from bladder cancer nomenclature (25).

The 2002 revision of the American Joint Committee on Cancer/ International Union against Cancer (AJCC/UICC) TNM systems is the most widely used staging system at this time. Seventy to eighty percent of diagnosed bladder tumors are non-muscle invasive tumors (Stage Ta, Tis- [Carcinoma in situ/CIS], T1), 25% are muscle invasive (Stage T2, T3), and 5% are metastatic. Recurrence is observed in 60-70% of muscle invasive tumors of which 20-30% progress to a higher stage or grade 4. The frequencies of nonmuscle invasive bladder cancer at stages Ta, T1 and Tis are 60, 30 and 10% respectively (26); 90% of the tumors are of transitional (urothelial) cell type and the rest are adenocarcinomas, small cell carcinomas and squamous cell carcinomas.

Table 1. The staging of urothelial carcinoma (27).

<i>Primary tumor</i>	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Noninvasive papillary carcinoma
Tis	Carcinoma <i>in situ</i>
T1	Tumor invades subepithelial connective tissue (lamina propria)
T2	Tumor invades muscularis propria bladder wall
T2a	Tumor invades superficial muscle (inner half)
T2b	Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue
T3a	Microscopically
T3b	Macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostate, uterus, vagina, pelvic wall, and abdominal wall
T4a	Tumor invades prostate, uterus, or vagina
T4b	Tumor invades pelvic or abdominal wall
 <i>Regional lymph nodes (N)</i>	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastases in a single lymph node, 2 cm or less in greatest dimension
N2	Metastases in a single lymph node, more than 2 cm but not more than 5 cm in greatest dimension, or multiple lymph nodes, none more than 5 cm in greatest dimension
N3	Metastasis in a lymph node more than 5 cm in greatest dimension
 <i>Distant Metastasis (M)</i>	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

The urinary bladder consists of a transitional epithelial layer covering the inner surface of bladder and the lamina propria is a thin layer of loose connective tissue lies beneath the epithelium. The next layer is sub-mucosa which acts as structural support for the mucosal layer. The third layer is

muscularis mucosa which is formed by interlacing three smooth muscles together known as detrusor muscle. The outermost layer of the bladder wall is called is adventitia (28).

The specific features, in brief, of various stages of bladder cancer are outlined below:

- 1. Stage pT0 carcinoma** is defined by no evidence of residual carcinoma after biopsy or transurethral resection (TUR) specimens. The incidence of pT0 is nearly 10%. In patients with pT0 carcinomas, the recurrence free, overall survival ranges from 84-88%.
- 2. Stage pTa carcinoma**, based on the 2002 TNM (tumor, lymph node and hematogenous metastasis) staging, pTa carcinoma is defined as noninvasive papillary carcinoma and is distinguished from pT1 cancer by the absence of lamina propria invasion.
- 3. Stage pT1 carcinoma** is defined by invasion into lamina propria but not muscularis propria (Table 1). pT1 carcinomas invade the underlying stroma as single cells or irregularly shaped nests of tumor cells. If the tumor invasion, in patients, is greater than 1.5 mm, the 5 year progression free survival is 67% and if the invasion is less than 1.5 mm, then the survival is 93%.
- 4. Stage pT2 carcinoma** is characterized by tumor invasion into muscularis propria. Ten year recurrence free survival rates range from 72-84% for lymph node negative bladder cancer patients.
- 5. Stage pT3 carcinoma** is defined by tumor invasion into perivascular soft tissue. (Table 1) The presence of adipose tissue is well documented.
- 6. Stage pT4** is defined by tumor invasion into an adjacent organ such as the uterus, vagina, prostate, pelvic wall or abdominal wall. Overall survival of these patients was only 10% and the overall survival for patients with prostatic stromal involvement was 38% and overall relative 5 year survival is only 15% (27).

Animal models for bladder cancer

With the exception of transgenic rodent models, carcinogen-induced bladder tumors are not observed in rodents until 8-12 months after initiation. Bladder cancer can also be induced by pellet implantation, urinary calculi, radiation and chemicals as discussed in this section (29).

Spontaneous bladder tumors are extremely rare and most wild type strains of rodents do not develop spontaneous bladder cancer, although some tumors have been observed in older animals. Exceptionally high levels of urothelial and ureteric neoplasms have been reported in two rat strains, Brown/Norway (BN/RijHsd) and Dark Agouti (DA/OlaHsd), and they were associated with the presence of calculi (30). Some spontaneous bladder tumors in rats can be explained by infection with the bladder parasite *Trichosomoides crassicauda* (31). Spontaneous bladder tumors in other rodent species including hamsters, guinea pigs, and rabbits are also rare (32). Yamagiwa and Ichikawa, in 1918, were the first to prove that cancer could be induced in experimental animals by chemical means (33). The induction of bladder cancer in dogs by 2 – naphthylamine was reported by Hueper in 1938 and this established the experimental basis of bladder carcinogenesis (34). Early attempts to induce tumors in mice by chemicals were unsuccessful until Armstrong and Bronser (1944) induced papillomas and carcinomas through the oral administration of 2 - acetylaminofluorene (AAF) in CBA strain mice (35):

1. Pellet implantation and urinary calculi. During the past three decades, numerous rodent bladder carcinogens have been identified and their activity has been associated with the appearance of urinary calculi. The presence of foreign bodies within the lumen of the bladder can cause irritation or trauma to the urothelium and stimulate mitotic activity, thereby causing nodular and papillary hyperplasia (36). This technique involves surgical implantation of pellets into the lumen of the rodent bladder. The pellets containing paraffin or cholesterol have

been used and it was hypothesized that they would remain inert in the bladder lumen and it was also assumed that the bladder epithelium was incapable of metabolizing these chemicals (37). In 1979 Jull demonstrated that insertion of pellets alone (without enclosed carcinogen) into the mouse bladder resulted in bladder cancer. In addition, surgical procedure, to implant the pellet, produced nodular and papillary hyperplasia. Chemicals used in the pellet formulation include uric acid, calcium oxalate, uracil, thymine and melamine (38).

2. Bladder Carcinogens:

i. 2 -Acetylaminoflourene (AAF) is a pluripotent carcinogen inducing tumors in liver, pancreas, breast, and skin, fore stomach and ear duct apart from urothelium. Three chemicals are particularly effective given the appropriate route, dose and appropriate strain of animal. Chemicals that produce 100% incidence of bladder tumors and are complete carcinogens include N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT), N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) and N-Methyl-N-nitrosurea (39). The nitrofurane FANFT specifically induces urinary bladder tumors in the rat, mouse, hamster and dog. FANFT is deformed in kidney and liver to give 2-amino-4-(5-nitro-2-furyl) thiazole metabolite which is subsequently excreted (40). FANFT is incorporated into the diet and induction of bladder cancer requires 8 to 11 months (41). Tumors induced by this compound are predominantly transitional cell carcinomas (TCC), with a large proportion exhibiting squamous cell differentiation.

ii. N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) is widely used as bladder carcinogen and tumors are induced in rats and mice resemble their human counterparts (42). BBN is metabolite of symmetric dibutyl nitrosamine (DBN) and is a bladder specific carcinogen in rats, mice and dogs. One hundred percent incidence of tumors can be induced by continuous administration of BBN in drinking water or by intra-vesicle instillation. BBN is a genotoxic compound and is rapidly oxidized to N-butyl-N-(3-carboxybutyl) nitrosamine (BCPN) which is also a bladder carcinogen and comes in contact with the urothelium via urinary

excretion. BCPN binds covalently to cellular macromolecules and acts as an initiator of carcinogenesis and *in vitro* studies confirm that this compound induces transformation of rat urothelial cells (29).

iii. N-Ethyl-N-(4-hydroxybutyl) nitrosamine (EHBN) is a genotoxic compound more potent than BBN. EHBN is metabolized by hepatic enzymes to give N-ethyl-N-(3-carboxypropyl) nitrosamine which is excreted in urine (45). **4-Ethylsulfonylnaphthalene-1-sulfonamide (ENS)** is a carbonic anhydrase inhibitor that produces alkaline, hypoosmolar urine with crystalluria and calculi formation. Benzidine, 3,3'-dichlorobenzidine, 2-naphthylamine, 4-aminobiphenyl, 2-acetylaminofluorene, phenacetin, and sodium o-phenylphenate are also urinary bladder carcinogens. Bracken fern (*Pteridium aquilinum*) also induces upper alimentary tract and bladder cancer in a number of species (43).

iv. Dimethylarsinic acid (DMA) is a rat urinary bladder carcinogen, but is not carcinogenic in mice. The carcinogenic mode of action involves cytotoxicity followed by regenerative cell proliferation. Dietary DMA does not produce urinary solids or significant alterations in urinary composition. The cytotoxicity is due to formation of a reactive metabolite, which may be dimethylarsinous acid (DMA), and this is concentrated and excreted in the urine (44).

3. Promoting agents. Multistage models of bladder carcinogenesis involves initiation of neoplastic change in cells by a threshold dose of carcinogen, followed by alteration of these latent tumor cells into an autonomous cancer by additional doses of the same and/or other carcinogens, and/or promoting agents. Animals exposed subsequently to promoters will develop bladder cancer (45). Urinary bladder promoters can be broadly classified into: 1) sodium and potassium salts associated with increased concentration of urinary levels of sodium and potassium ions and alkaline urine 2) urolithiasis inducing agents 3) antioxidants 4) anticancer drugs 5) amino acids 6) drugs and other compounds (46).

4. Radiation. Ionizing radiation also causes bladder cancer in humans. For example X-rays induce preneoplastic urothelial hyperplasia and development of urothelial carcinoma in rodents. A single dose of radiation generated from a linear accelerator induced hyperplasia of the urothelium in mice, 3-19 months after exposure (47).

Genetic models for bladder cancer

Transgenic or gene targeted mice, are frequently used as models to determine specific genetic changes, including oncogene overexpression/mutation or tumor suppressor gene loss, that increases the risk for neoplastic progression. These animals can be used to investigate cooperation of two or more genes during bladder carcinogenesis and how the genetic signature of a neoplasm correlates with specific aspect of tumor development (48). A tumor suppressor gene protects cells from one or more steps required for tumor formation, growth and inactivating mutations of tumor suppressor genes enhance cancer progression and this usually involves multiple genetic changes. The p53 tumor-suppressor protein, is lost in 70% of colon cancers, 30-50% of breast and 50% of lung cancers due to homologous loss (49). Wild-type p53 binds to DNA and activates several genes such as p21 which in turn binds to G1-S phase cyclin dependent kinases and inhibit their effects on cell cycle progression. Phosphatase and tensin homolog (PTEN), is another tumor suppressor that inactivates the activity of phosphoinositide 3-kinase (PI3K), which is essential for several pro-survival pathways (50). Mutations of p53 or PTEN, are among the most frequent causal events in many cancers, and their combined inactivation has profound consequences for tumor development (51) (52). The oncogene Ras encodes for small GTPases involved in cell growth, survival, differentiation and activation of Ras mutations or overexpression enhances the role of tumor formation. Some Ras mutations result in constitutive activation of signaling associated with growth and survival. Activating Ras

mutations are found in 20-25% of all humans up to 90% in gastrointestinal and colorectal cancers (53).

Transgenic mice carrying the human C-Ha-ras proto-oncogene, v-Ha-ras transgenic mice, pim-1 transgenic mice, and several knockout strains of mice deficient in tumor suppressor genes such as p53 exhibit increased susceptibility to carcinogens (54). p53 knockout mice are more sensitive to N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)- induced urinary bladder tumor formation compared to the parental C57Bl/6 mice (55). A transgenic rat line carrying three copies of the human c-Ha-ras proto-oncogene and its original promoter is highly susceptible to BBN-induced carcinogenesis and is utilized as a rat model for genetic analysis of bladder tumor development (56). Tsuda et al generated transgenic rats with the same human c-Ha-ras protooncogene used for generation of transgenic mice called Hras128 and the rat model is highly susceptible to BBN-induced bladder carcinogenesis.

Cory-Abate-Shen and colleagues recently demonstrated for the first time that the combined deletion of p53 and PTEN in bladder epithelium leads to invasive bladder cancer in a novel mouse model. Combined inactivation of p53 and PTEN were reported to be major causal factors that predicts poor outcome of invasive bladder cancer patients (57). Surgically an adenovirus expressing Cre recombinase was delivered into the bladder lumen of adult male mice to induce conditional deletion of p53 and PTEN alone or together in the epithelium. p53^{flox/flox}; PTEN^{flox/flox} mice produced large tumors with features of carcinoma in situ (CIS), as well as high-grade invasive carcinoma with areas of transitional cell, squamous, and sarcomatoid carcinoma. Furthermore, the transgenic mouse study documents synergistic interaction of p53 and PTEN deletion that lead to deregulation of mammalian target of rapamycin (mTOR) signaling, and this is correlates with the ability of rapamycin to block bladder tumorigenesis in preclinical studies.

A murine bladder cancer model in which the uroplakin II gene promoter drives the urothelial expression of EGFR in transgenic mice has also been reported (58). Three established transgenic lines express higher levels of EGFR mRNA and protein in the urothelium compared to the nontransgenic controls. The overexpressed EGFR was functionally active due to constitutive autophosphorylation and activation of downstream mitogen-activated protein kinases. The luminal surface of mammalian urothelium is covered with numerous plaques composed of semi-crystalline, hexagonal arrays of 12-nm protein particles called uroplakins (59) and the urothelial plaque associated proteins or uroplakins are excellent markers for identifying urinary carcinomas (60). Uroplakin ablation elevates urothelial permeability as uroplakins perform the permeability barrier function (61).

Phenotypically, the urinary bladders of all transgenic rodents developed simple urothelial hyperplasia. Coexpression of the EGFR and the activated Ha-ras oncogene in double transgenic mice did not enhance tumorigenicity compared to effects observed with H-ras oncogene alone. However, when H-ras was coexpressed the SV40 large T antigen, EGFR accelerated tumor growth and converted the carcinoma in situ observed in of SV40 largeT mice into high-grade bladder carcinoma (62). This study indicates that overexpression of urothelial EGFR induces proliferation but not frank carcinoma formation and that EGFR and Ha-ras, both of which act on some of the same kinase pathways, stimulate urothelial hyperplasia, but not urothelial tumorigenesis. Moreover, it was also reported that EGFR overexpression can cooperate with p53 and mutated pRB to enhance bladder carcinogenesis (63).

This same group in 2007 reported that hyperactivation of Ha-ras oncogene triggered bladder tumorigenesis suggesting that overactivation of Ha-ras is both necessary and sufficient to induce bladder tumors (64). Activation of H-ras by an activating point mutation, or intensified signaling from fibroblast growth factor receptor 3 (FGFR3) (Figure 3) is frequently observed in human

bladder tumors. These RTKs can functionally overactivate the ras pathway in the presence or absence of ras mutations (65).

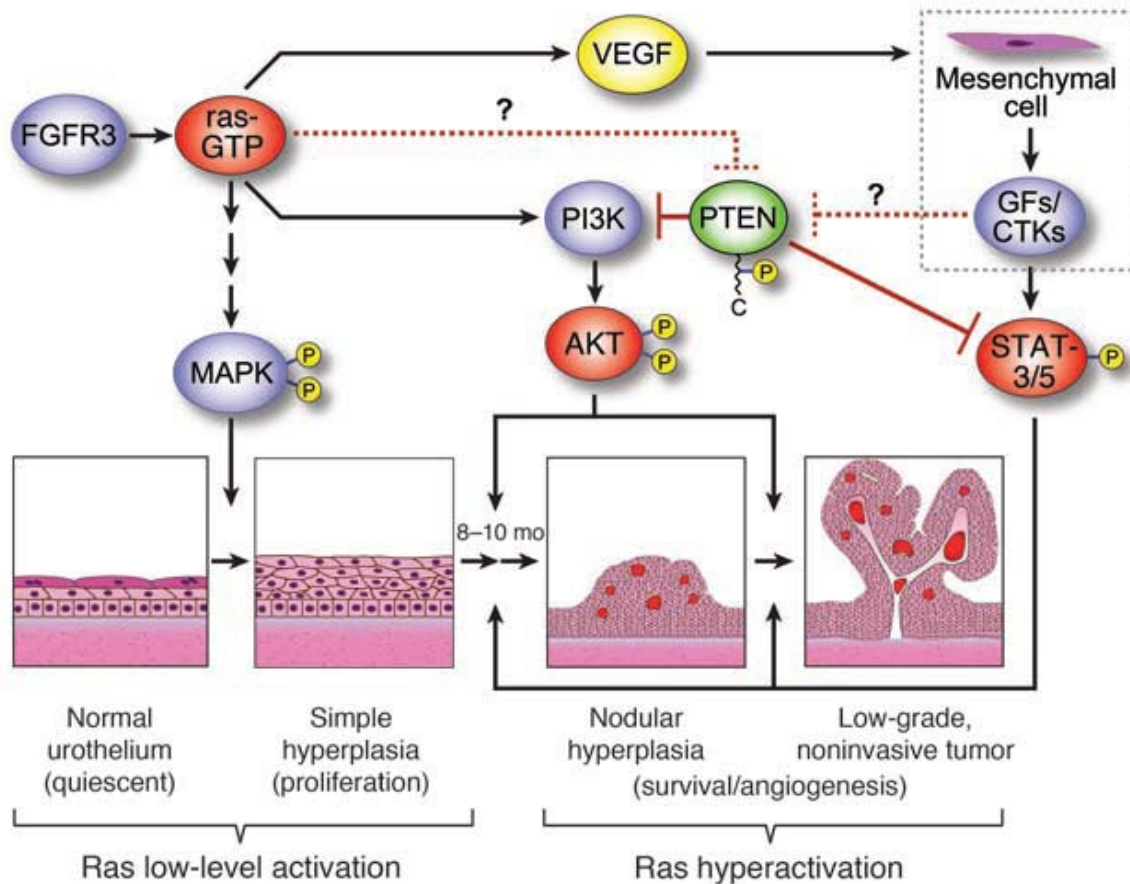


Figure 3. Schematic diagram of signaling pathway underlying the formation of low-grade, noninvasive papillary urothelial tumors (65).

Prognostic markers for bladder cancer

Prognosis is a prediction of a disease outcome. The depths of infiltration, differentiation grade and histological staging (Table 1) have been the most important prognostic parameters for predicting bladder tumor progression and survival. Patients are diagnosed and monitored with urethrocytoscopy, cytology, and imaging of the upper urinary tract. Urethrocytoscopy is the examination of interior of urinary bladder; this technique is considered the gold

standard for bladder examination however, certain lesions, particularly small areas of carcinoma in situ (CIS) are often not detected. Cytological examination of bladder tissue is also used extensively and is useful for detecting high-grade tumors and CIS; however, it has a median sensitivity of only 35% and median specificity of 94%. Predicting which invasive tumors will or will not recur or metastasize is crucial for selecting initial therapies and for counseling patients. Tumor markers when incorporated into clinical practice add prognostic information to the conventional 'Tumor, Node, Metastasis'(TNM) grading systems in terms of treatment response and prognosis (66). The limitations of cytology and the invasiveness of urethrocystoscopy for detecting bladder cancer have generated interest in developing other noninvasive diagnostic tools including urinary biomarkers that aid in detection and follow up of bladder cancer (67).

1. Fluorescence in situ hybridization (FISH). The FISH technique consists of coupled antibodies and fluorochromes to detect chromosomal anomalies in the exfoliated bladder cells. A commercially available test, the UroVysion™ Bladder Cancer Kit (Vysis Inc, Downers Grove, IL, USA), has probes for chromosome 3, 7, and 17, and a locus-specific probe for 9p21. The overall cancer detection sensitivity of FISH varies between 69 and 87% and the detection of carcinoma in situ (CIS) is close to 100%. The specificity of FISH is high (89–96%) and is comparable to cytology. Another advantage of FISH is that it is unaffected by Bacillus Calmette-Guerin (BCG) therapy and therefore this technique can be used for surveillance of patients treated with intravesical BCG (68). A disadvantage of the FISH procedure is the labor intensity and requirement of an extensive learning curve before it can be used reliably and these results in minimal use of the FISH assay.

2. Microsatellite analysis. Microsatellites are highly polymorphic, short, tandem DNA repeats found in the human genome. Two types of microsatellite alterations can be found in many cancers namely, Loss of heterozygosity (LOH), which is

an allelic deletion, and somatic alteration of microsatellite repeat length (69). In bladder cancer, most mutations are in the form of LOH. Microsatellite alterations in exfoliated cells in urine are detected by a polymerase chain reaction (PCR) using DNA primers for a panel of known microsatellite markers. This test has good overall sensitivity and specificity, but is complex and expensive and this limits its clinical usage.

3. Telomerase. Telomeres are repeat sequences at the end of chromosomes that protect genetic stability during DNA replication. Telomeres are lost during each cell division, and this increases chromosomal instability and cellular senescence. Bladder cancer cells express telomerase, an enzyme that regenerates telomeres at the end of each DNA replication and therefore sets the cellular clock to immortality. Overall sensitivity and specificity of the telomerase assay, varies between 70 to 100% and 60 to 70%, respectively (70). The telomerase assay has good sensitivity but lacks sufficient specificity and test results can be influenced by inflammation and age. These disadvantages make it a suboptimal test for detection of bladder cancer.

4. BTA-TRAKTM and BTA-statTM (Alidex Inc, Redmond, WA, USA). These are both versions of the bladder tumor antigen assay that measures complement factor H-related protein in urine. These tests exhibit sensitivity slightly higher than that of cytology, however specificity is lower due to false-positive test results in patients with benign genitourinary conditions, inflammation, infection, or haematuria (71).

5. Hyaluronic acid (HA). HA is a glycosaminoglycan and a normal component of tissue matrices and body fluids. In tumor tissues, elevated HA is primarily localized to tumor stroma, in bladder carcinoma and elevated HA levels have been detected in urinary samples of bladder cancer patients (72). The concentration of HA is also associated with tumor metastases and hyaluronidase (HA-ase); an enzyme that cleaves HA into fragments. HA-ase levels are elevated in bladder tumor tissue, and an increase is correlated with tumor grade

(73). In conclusion, the test has high sensitivity to detect both low- and high-grade/stage tumors indicating that the HA-HA-ase is a promising assay that deserves further study.

6. Nuclear matrix protein 22 (NMP22). NMP22 is a nuclear matrix protein that regulates mitosis and in tumor cells the nuclear mitotic apparatus is elevated and NMP22 is released from tumor cells. Grossman et al (74) investigated the capability of this test for detecting malignancy in 1331 patients with risk factors of bladder cancer; the assay sensitivity was 55.7 and specificity was 85%.

7. Cytokeratins. These are intermediate filaments and their main function is to enable cells to withstand mechanical stress. In humans 20 different cytokeratin isotypes have been identified. Cytokeratins 8, 18, 19, and 20 have been associated with bladder cancer (75). CYFRA 21-1 is a soluble fragment of cytokeratin 19 and is used as a urinary marker for bladder cancer. CYFRA21-1 levels are strongly influenced by benign urological diseases, intravesical instillations and do not detect early stage bladder cancer.

8. Survivin. It is a member of the inhibitor of apoptosis (IAP) family of proteins that regulate cell death. Overexpression of survivin inhibits extrinsic and intrinsic pathways of apoptosis (76). Survivin is expressed during fetal development but not in terminally differentiated adult tissues (77) and is one of the most commonly overexpressed genes in cancer. In bladder cancer, survivin is found in urine, and levels of survivin are associated with disease recurrence, stage, progression, and mortality (78) . Survivin identified 71.4 and 69.6% of the patients with long or short recurrence-free periods, respectively. Urinary survivin seems predictive for bladder cancer recurrence and can be helpful in preventing unnecessary urethroscopies.

9. Growth factors. Growth factors such as EGFR, vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF- α), type 1 insulin like growth factor receptor (IGFR), fibroblast growth factor receptor (FGFR)- 3, and heparin-binding epidermal growth factor are overexpressed in multiple cancers.

Members of the EGFR family, a type I tyrosine kinase, are involved in various forms of cancers and serve as prognostic markers and therapeutic targets (79). EGFR is considered one of the most important oncogenes in bladder cancer development and high levels of EGF were found in of certain transitional cell carcinomas (TCC)(80). Tumors with elevated expression of high-molecular-weight cytokeratin and EGFR positive cases were associated with higher metastases and shorter patient life span. Increased EGFR and HER2 also predict a poor prognosis, and HER4 and FGFR3 are favorable prognostic indicators. However, validation studies are required to answer many remaining questions on the prognostic value of growth factors and their receptors.

Current treatments for bladder cancer

Surgery, alone or in combination with other treatments, is used in more than 90% of bladder cancer cases. Superficial, localized cancers may also be treated by administering immunotherapy or chemotherapy directly into the bladder. Chemotherapy alone or combined with radiation before cystectomy (bladder removal) has improved treatment results.

1. Transurethral Resection (TUR). TUR is used to surgically remove cancerous tissue from the bladder and it remains the mainstay for the diagnosis and treatment of Ta and T1 bladder cancer. After this procedure, the 10-year disease-free specific survival for Ta tumors is 85%, and for T1 tumors it is 70%.⁵ Biopsy samples should be taken from the bladder neck and prostate to assess whether the tumor has spread within the bladder neck musculature, prostatic urethra, ducts, and stroma.

2. Intravesical therapy. This is a treatment procedure where in the therapeutic agent is deposited in bladder directly. The high recurrence rate and the unpredictability of the progression pattern of bladder cancer have resulted in the widespread use of intravesical therapy as a supplement to TUR. Superficial bladder cancer lends itself to intravesical therapy owing to direct contact of the

chemotherapeutic agent with the bladder mucosa and tumor. Furthermore, some agents can be used at high doses with minimal systemic side effects because of a lack of absorption.

3. Early radical cystectomy. The removal of bladder as well as surrounding tissues based on the extent of spread of the tumor is called cystectomy. The indications for radical cystectomy among patients with superficial bladder cancer include high-grade disease recurrence after intravesical BCG and CIS refractory to intravesical immunotherapy. The case for performing early cystectomies is strengthened by low peri-operative mortality and morbidity, improvements in surgical techniques and perioperative care, and increased acceptance of orthotopic neobladder as a choice of urinary diversion (81). Radical cystectomy remains the standard, accepted treatment for muscle-infiltrating bladder cancer (82).

4. Chemotherapeutic agents. The common chemotherapeutic agents that are used for bladder cancer treatment are briefly described:

i. Methotrexate. It inhibits folic acid reductase which is responsible for the conversion of folic acid to tetrahydrofolic acid. At two stages in the biosynthesis of purines (adenine and guanine) and at one stage in the synthesis of pyrimidines (thymine, cytosine, and uracil), one-carbon transfer reactions occur and require specific coenzymes synthesized in the cell from tetrahydrofolic acid. Tetrahydrofolic acid itself is synthesized from folic acid and requires the enzyme, folic acid reductase. Methotrexate binds the enzyme and inhibits its activity and thereby DNA synthesis (83). Methotrexate is used as part of the major chemotherapeutic treatment for bladder cancer namely the combination of Methotrexate, Vinblastine, Doxorubicin and Cisplatin (MVAC).

ii. Triethylenethiophosphoramide (thiotepa). It is an organophosphorus alkylating agent known to cross-link DNA and prevent cells from replicating. Thiotepa has a low molecular weight (189 kDa), and is readily absorbed into the systemic circulation. A review of nine randomized trials revealed a 61% average

bladder cancer recurrence rate with Transurethral Resection (TUR) compared with 49% among the patients treated with thiotepa, yielding an overall advantage of a 12% decrease in tumor recurrence (84). Thiotepa is rarely used in the US because of its high associated risk of myelosuppression (up to 54%).

iii. Doxorubicin (trade name- Adriamycin). It is an anthracycline produced by the *Streptomyces* species. Anthracyclines interact with topoisomerase II (Topo II) and inhibit its activity. Topo II enzyme controls changes in DNA structure by catalyzing the breaking and rejoining of the phosphodiester backbone of DNA strands during the normal cell cycle. Topoisomerase inhibitors block the ligation step of the cell cycle, generating single and double stranded breaks that harm the integrity of the genome (85). These strand breaks subsequently lead to apoptosis and cell death. In seven randomized trials, the average recurrence rate was 58% with transurethral resection versus 38% with doxorubicin, a 20% lowering of tumor recurrence. Toxic side-effects have hampered the use of doxorubicin; chemical cystitis has been reported in as many as 56% of patients, with a decreased bladder capacity in 16% and hematuria (blood in urine) in 40%. Also, systemic reactions, including fever and allergy, have been reported in 5% of patients (86). **Epirubicin (4'-epi-doxorubicin)**. Epirubicin is a synthetic derivative of doxorubicin, with a similar therapeutic efficacy to that of doxorubicin. However, epirubicin is accompanied by fewer toxic side-effects which are generally mild, with the most common being cystitis, hematuria, or both (87).

iv. Mitomycin C. This is an alkylating agent that causes DNA cross-linking and is produced by *Streptomyces*. The most effective dose of mitomycin C is 40 mg in 20 ml distilled water which is administered once weekly for 6 consecutive weeks (88). Dysuria, cystitis, dermatitis, rash and increased urinary frequency have been reported in 41% of the patients. Current data suggest that all current intravesical chemotherapeutic agents are similar in efficacy but differ in toxicity.

v. Cisplatin. It is platinum-based chemotherapeutic drug used to treat various

types of cancers, including sarcomas, some carcinomas (e.g. small cell lung cancer, and ovarian cancer), lymphomas, and germ cell tumors. These platinum complexes bind to and cause crosslinking of DNA which ultimately triggers apoptosis (programmed cell death). In spite of its efficacy for treating various cancers, cisplatin suffers from two major setbacks. First, it is particularly toxic against normal tissues and second; many tumors develop resistance or acquire resistance to cisplatin-induced chemotherapy. Gemcitabine and Cisplatin (GC) are used in combination therapy. Gemcitabine is a pyrimidine (nucleoside) analog in which the hydrogen atoms on the 2' carbons of deoxycytidine are replaced by fluorine atoms. The drug replaces cytidine, a building block of nucleic acid during DNA replication and causes tumor growth arrest since the new nucleosides cannot be attached to the 'anti-metabolite' nucleoside, resulting in apoptosis.

5. Immunotherapy. Multiple clinical trials have directly compared transurethral resection (TUR) alone with TUR plus BCG for tumor prophylaxis. BCG is obtained from attenuated live bovine tuberculosis virus, *Mycobacterium bovis*. BCG elicits immune response against residing cancer cells by inducing a variety of cytokines, including interferon (interferon-inducible protein (IP-10), interferon gamma (IFN-gamma)) and interleukins (IL-12). In addition to cellular immune response, BCG also induces cytokines that mediate antiangiogenic responses that inhibit future tumor growth and progression (89). Numerous studies with BCG combination therapy have demonstrated statistically significant benefits in the reduction of bladder cancer recurrence rates and these ranges from 20 to 57%. More than 50% of complete responders remain disease free for more than 5 years from the start of therapy. The effect of BCG in reducing tumor progression is controversial due to local and systemic toxic effects (90). Intravesical valrubicin, a synthetic analog of doxorubicin, although not commonly used in clinical practice, was approved by the FDA for the treatment of BCG-refractory CIS in patients. Co-treatment of intra vesical BCG and α -2b interferon

has demonstrated activity in patients who did not respond to BCG, with a 2-year disease-free estimate of 42% in patients with prior BCG failures (91).

6. Radiotherapy. Brachytherapy is the usage of radiation placed very close to or inside the tumor. A study by Nieuwenhuijzena and colleagues compared combination radiotherapy with cystectomy for stage T1–2 bladder cancer (92). In total, 108 patients received 30 Gy external beam radiotherapy followed by 40 Gy brachytherapy; they were compared with 77 patients who were treated with cystectomy alone. Overall survival after 5 and 10 years was 62 and 50% after radiotherapy (brachytherapy), and 67 and 58% after cystectomy, respectively. There are also reports that radiation therapy tends to release radiolytic products that are stored in urinary bladder and may thereby enhance bladder carcinogenesis. Overall, these treatment options appear to be effective for certain patients with specific bladder cancer stages, but their use is limited by the acute and chronic adverse side effects elicited by chemotherapy and radiation. Patients experience more chemotherapy-related adverse effects than reported in clinical trials. Most of the toxicity is attributable to the effects on non-target tissue including, granulocytopenia, and stomatitis, hematologic toxicity (leucopenia and febrile neutropenia), nephrotoxicity and other organ toxicities (93). Adverse effects include in loss of hair, loss of appetite, diarrhoea, nausea and fatigue due to toxic effects of radiation on rapidly proliferating tissue.

Pancreatic cancer incidence and classification

Incidence. Pancreatic cancer is the fourth leading cause of cancer-related mortality, accounting for about 6% of all cancer-related deaths, in both men and women in U.S.A. The median age of diagnosis is 72 years and patients with pancreatic cancer have a mean relative 5-year survival rate of 5%, and this disease remains a major unsolved health problem (94). Predisposing etiologies for pancreatic cancer include cigarette smoking, diabetes mellitus, heavy alcohol consumption and a family history of pancreatic cancer (95).

Classification. Classification of pancreatic cancer is based on the anatomical origin and molecular signatures of the tumors. Jones et al identified more than 1500 somatic mutations in a pool of 1007 genes in 24 pancreatic cancer patients and reported that genes mutated at the highest frequency include KRAS, p16/CDKN2A, TP53, and SMAD4 (96) (97). The KRAS gene was activated by point mutations in virtually all of the 24 cancers. A number of other potentially significant (“driver”) genes were mutated at a much lower frequency:

1. Ductal adenocarcinoma. This class of pancreatic cancer is featured by small microscopic lesions, called pancreatic intraepithelial neoplasia (PanIN), as well as larger intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms. Mutations in KRAS, in TP53, in SMAD4, and in the p16/CDKN2A genes have all been reported in PanINs, as well as in infiltrating ductal adenocarcinomas (Figure 4) (98). Brune and Detlefsen et al (99) have shown that PanINs are often associated with a distinctive lobulocentric atrophy and fibrosis. PanINs may be too small to be detected using currently available imaging technologies, but focal areas of pancreatic fibrosis may serve as an indirect marker for the presence of a PanIN.

2. Medullary carcinoma. Medullary carcinoma of the pancreas is characterized by poor differentiation, a syncytial growth pattern, and pushing borders. These cancers also may have necrosis, a Crohn-like lymphocytic infiltrate and unstable microsatellites due to inherited or acquired mutations. The recognition that medullary carcinomas of the pancreas are associated with microsatellite instability has prognostic and therapeutic implications. Despite their poor differentiation, microsatellite unstable carcinomas may have a better prognosis (100).

3. Undifferentiated carcinoma. Undifferentiated carcinomas, like medullary carcinomas, lack significant differentiation, but unlike medullary carcinomas, undifferentiated carcinomas are among the most aggressive of all of the carcinomas of the pancreas. The mean survival for some groups is only 5

months (101). Undifferentiated carcinomas are highly malignant epithelial neoplasms without a more definite direction of differentiation. Winter et al have recently identified a molecular basis for the undifferentiated appearance of these neoplasms. Undifferentiated carcinomas are typically noncohesive, and these noncohesive pancreatic cancers are characterized by the loss of e-cadherin and β -catenin protein expression (102). Thus, at the poorly differentiated/undifferentiated end of the spectrum of pancreatic neoplasia, are two very different carcinomas i.e., medullary carcinomas that are associated with microsatellite instability and have a good prognosis, and undifferentiated carcinomas with the loss of E-cadherin, and that have a poor prognosis.

4. Undifferentiated carcinoma with osteoclast-like giant cells.

Undifferentiated carcinomas with osteoclast-like giant cells are a poorly differentiated/undifferentiated carcinoma of the pancreas. These histologically striking carcinomas contain a mixture of highly atypical pleomorphic cells and dramatic multinucleated giant cells with uniform nuclei. These are undifferentiated carcinomas with reactive, nonneoplastic, multinucleated giant cells. The undifferentiated cells in these neoplasms harbor the same mutations as do their associated noninvasive epithelial precursors. These distinctive neoplasms are classified as epithelial neoplasms (carcinomas) that arise from well-differentiated epithelial precursor lesions (103).

5. Colloid carcinoma. Colloid carcinoma, also known as mucinous noncystic adenocarcinoma, almost always arises in association with an Intraductal papillary mucinous neoplasm (IPMN). Colloid carcinoma is characterized by well-demarcated pools of mucin infiltrating stroma. Some of the mucin pools contain clusters of well-differentiated neoplastic cells in variable patterns including strips, stellate units, and individual signet-ring like cells. The neoplastic cells of colloid carcinoma show intestinal differentiation. CDX2, a transcription factor that regulates intestinal programming, is not normally expressed in pancreatic tissues nor is it significantly expressed in conventional ductal

adenocarcinoma of the pancreas. However, CDX2 is uniformly expressed in colloid carcinomas along with expression of MUC2, the goblet-type (or intestinal type or gel forming) mucin, not unexpectedly, colloid carcinomas of the pancreas express high-levels of MUC2 (104).

6. Intraductal papillary mucinous neoplasm (IPMN). Intraductal IPMNs are large noninvasive mucin-producing epithelial neoplasms that arise in the larger pancreatic ducts (105). IPMNs can be a precursor to invasive adenocarcinoma of the pancreas, particularly invasive colloid carcinomas. Activating mutations in the KRAS oncogene and inactivating mutations in the TP53 and p16/CDKN2A tumor suppressor genes are observed in this type of tumors. (106). In the pancreas, the diffuse/strong expression of CDX2 and MUC2 is seen only in the intestinal type of IPMNs and in the colloid carcinomas associated with intestinal type IPMNs (107).

7. Solid-pseudopapillary neoplasm. Grossly, solid-pseudopapillary neoplasms can be solid and cystic or almost completely cystic. The cysts are filled with necrotic and hemorrhagic material. By light microscopy, solid-pseudopapillary neoplasms are composed of uniform poorly cohesive cells surrounding thin delicate blood vessels composed of foam cells, clear cells, cholesterol clefts, and eosinophilic hyaline globules are often present. More than 90% of solidpseudopapillary neoplasms have mutations in exon 3 of the β -catenin gene. These mutations interfere with the degradation of the β -catenin protein, and as a result, the β -catenin protein abnormally accumulates in the neoplastic cells and is translocated to the nucleus where it increases the transcription of c-myc and cyclin D1 (108).

8. Pancreatoblastoma. Pancreatoblastomas are distinctive neoplasms characterized by the presence of neoplastic cells with acinar differentiation and squamoid nests (109). The most common genetic abnormality in pancreatoblastomas is loss of one copy of the short arm of chromosome 11 near the WT-2 gene locus (11p15.5) (110) (111). Genetic mutations in the β -

catenin/APC pathway are seen in up to 80% of pancreatoblastomas, and most of these are in the β -catenin gene. Unlike the solid-pseudopapillary neoplasms, pancreatoblastomas with β -catenin gene mutations remain cohesive.

9. Acinar. Acinar carcinomas of the pancreas are distinctive neoplasms characterized by the production of exocrine enzymes (trypsin, chymotrypsin and lipase) by the neoplastic cells (112). The cells are often pyramidal in shape, with abundant granular apical cytoplasm and minimal fibrous stroma. Most acinar cell carcinomas do not harbor KRAS gene mutations, or mutations in the TP53, p16/CDKN2A, or SMAD4 genes. Thus, these morphologically distinct neoplasms are also genetically distinct from other pancreatic carcinomas.

10. Serous cystic neoplasms. Serous cystic neoplasms are benign cyst-producing neoplasms in which the cysts are lined by cuboidal cells with clear cytoplasm. Hypoxia induced factor-1 α is expressed uniformly in serous cystic neoplasms. The accumulation HIF-1 initiates a cascade of events in glucose metabolism, via modification glucose uptake and glucose transferase-1 as well as carbonic anhydrase IX, the accumulation of glycogen and thus the characteristic clear cell appearance. HIF-1 also induces overexpression of vascular endothelial growth factor, which in turn may lead to the recruitment of capillaries immediately adjacent to the neoplastic epithelial cells (113).

Animal and genetic models for pancreatic cancer

Environmentally-induced cancers in certain strains of mice develop either spontaneously or following various 'environmental' exposures, including radiation, chemicals, pathogenic viruses and microbial flora.

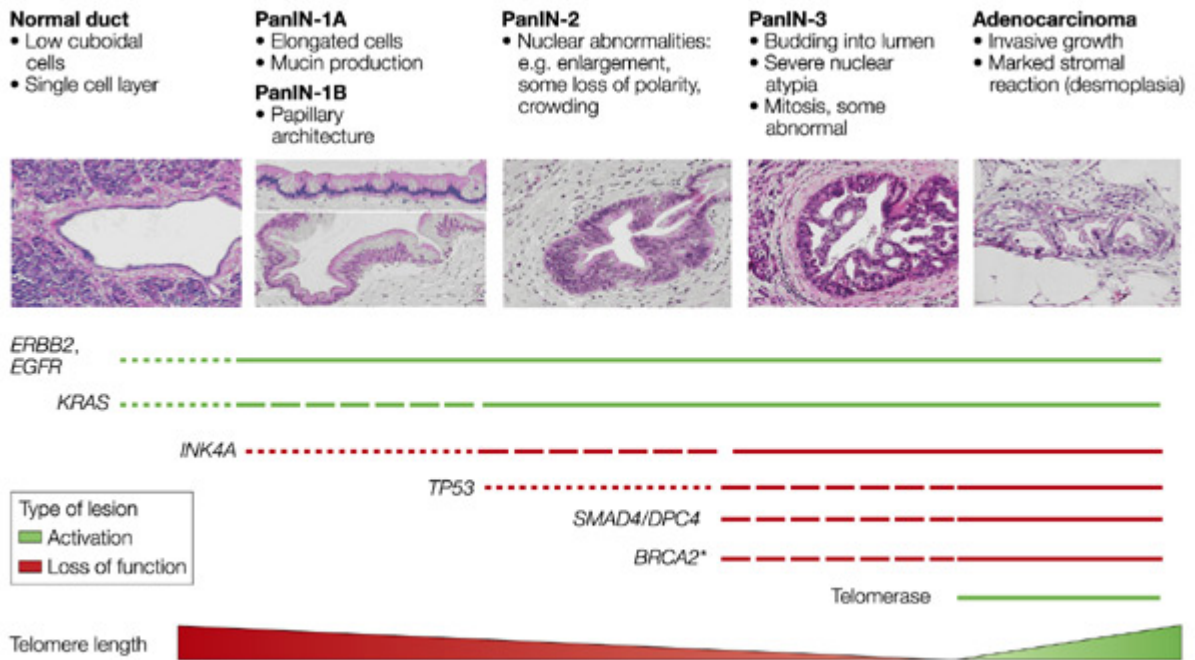


Figure 4. Genetic progression of pancreatic adenocarcinoma (114).

Development of a genetic mouse model for pancreatic cancer recapitulates the exact multistep progression of human pancreatic adenocarcinoma. In 2003, Hingorani et al. reported a mouse model of pancreatic neoplasia by conditional mis-expression of mutant KRAS in the pancreas from its endogenous promoter (115). The bitransgenic mice express a ‘knock-in’ $Kras^{G12D}$ upon Cre-mediated recombination and removal of a lox-STOP-lox allele within the Pdx1 expression domain. Pdx1 is a transcription factor that is expressed in the developing pancreas and foregut, restricting mutant KRAS expression to these organs. The Pdx1-Cre, lox-STOP-lox- $Kras^{G12D}$ mice develop the entire histologic compendium of murine PanIN (mPanIN) lesions observed in the cognate human disease, and a subset of mice develop invasive pancreatic carcinomas. Subsequent models have utilized additional mutations with Kras (for example, an oncogenic $Trp53^{R172H}$ allele or biallelic deletions of INK4a/Arf); these compound transgenic mice develop metastatic pancreatic cancers with

near-universal penetrance, and represent biologically relevant models of advanced pancreatic cancer in humans (116). K-ras (Kirsten Rat sarcoma virus oncogene) is a protooncogene located in chromosome 12p12.1. The ras pathway is a growth-promoting signal transduction from cell surface receptors to the nucleus, affecting the production and regulation of several key proteins. There are three RAS family protooncogenes namely H-ras, K-ras and N-ras and all are located in the inner plasma membrane, bind GDP and GTP and possess an intrinsic GTPase activity which cleaves the GTP to GDP (switch off position). K-ras protein is active and transmits signals by binding to GTP (turned on), but it is inactive (turned off) when GTP is converted to GDP.

Mutations of K-ras proto-oncogene lead to an inactive GTPase and therefore persistent activation (switch on position). This type of K-ras mutation is expressed in pancreatic adenocarcinoma, colorectal adenocarcinoma, lung cancer and other solid tumors. The frequency of K-Ras mutations in pancreatic cancer ranges from 74 to 100% (117). There is an absolute requirement for mutant Kras to initiate pancreatic neoplasia along the mPanIN pathway, and this is consistent with the extremely high frequency of KRAS abnormalities in human PanIN lesions and pancreatic cancer (Figure 4) (118). These mouse models have provided some insights into the putative cell-of-origin of pancreatic cancer. For example, recent studies by Guerra and colleagues have demonstrated that mPanINs and adenocarcinomas can be reproduced in the pancreas of adult mice by conditional misexpression of mutant Kras to the elastase-expressing acinar/centroacinar compartment accompanied by ongoing injury such as chronic pancreatitis (119). The development of these models has provided an unprecedented opportunity to explore preclinical diagnostic and therapeutic strategies in autochthonous models that are not afforded by short-term mouse xenograft studies.

Prognostic markers of pancreatic cancer

1. Growth factors and their receptors. The pattern and level of expression of growth factor receptors and their ligands are important prognostic factors for pancreatic cancer:

i. Epidermal growth factor receptor (EGFR) family and its ligands

The epidermal growth factor receptor family is comprised of four transmembrane tyrosine kinases that include the epidermal growth factor receptor (EGFR), c-erbB2, c-erbB3 and c-erbB4. EGFR is activated by various peptide ligands that include the EGF, TGF α , heparin-binding EGF-like growth factor, betacellulin, and amphiregulin. EGF is a polypeptide of 53 amino acids that stimulates proliferation and differentiation of a wide variety of cell types through binding the EGFR. In the normal pancreas, EGFR is expressed only in the islets of Langerhans. The EGFR gene, however, is overexpressed in 95% of ductal adenocarcinomas and human pancreatic cell lines, and this increases production of EGF and TGF α , promoting autocrine and paracrine loops that enhance cell proliferation. While the EGF is not detectable in the normal pancreas, it is found in 12% of pancreatic cancers.

ii. Transforming growth factor (TGF) receptors. Transforming growth factors are a family of cytokines that actively influence cell division, cell death, and cellular differentiation. They bind to specific cell surface receptors and downregulate transcription factors, decrease phosphorylation of target proteins, and inactivate cell-cycle regulatory enzymes such as the G1 cyclins and cyclin-dependent kinases. The three isoforms (TGF1–3) are overexpressed in pancreatic cancer.

iii. Fibroblast growth factors Fibroblast growth factors (FGFs) belong to a large group of closely homologous polypeptide growth factors which include two main members, acidic FGF (aFGF) and basic FGF (bFGF). These growth factors are highly abundant in the basement membrane and extracellular matrix of a variety of tissues. FGFs influence cell differentiation, tissue homeostasis,

tissue regeneration and repair, cell migration, and angiogenesis. Both aFGF and bFGF are overexpressed in pancreatic cancer tissue at the mRNA and protein levels (120).

2. Tumor suppressor genes:

i. p53. The p53 gene encodes a 53-kDa nuclear phosphoprotein consisting of 393 amino acids. Under normal conditions, p53 protein is found in the cell nucleus and p53 controls the entry of cells into the growth cycle at the G1/S boundary and has a critical role in initiating the repair of damaged DNA before it is replicated. The inability to affect DNA repair with accuracy may result in p53-dependent apoptosis. The p53 gene is mutated in over 50% of human cancers. In pancreatic cancer it is inactivated in approximately 65% of all cases by a missense point mutation; loss of the remaining normal allele commonly occurs in pancreatic cancer cell lines. Point mutations result in production of mutant p53 proteins which are more stable than the wild-type protein and accumulate in the nucleus. Patients with combined p53 positive and p21 negative tumors exhibited poor survival and those who have undergone chemotherapy exhibited a nonsignificant trend for improved survival (121).

ii. p21WAF1. p21WAF1, a cyclin-dependent kinase inhibitor, is a downstream target and effector of p53. The 21-kDa product of the WAF1 gene forms part of a quaternary complex, along with cyclin/CDKs and the proliferating cell nuclear antigen (PCNA) in normal cells, but not in transformed cells, and is a universal inhibitor of CDK activity. Loss of p21 activity has been observed in approximately 30–60% of pancreatic tumors (121).

3. Oncogenes.

i. K-ras. The ras family of protooncogenes includes Hras, N-ras, and K-ras, and encodes proteins with GTP-ase activity; function as molecular switches in signal transduction. The K-ras gene is mutated in 75 to 90% of pancreatic cancers and the mutations present in pancreatic cancer are almost exclusively at codon 12. The presence or the type of K-ras mutations in pancreatic tumors has not been

shown to be associated with patient survival (122). K-ras is a signature gene for initiation of pancreatic carcinogenesis and is an important prognostic marker.

ii. Cyclin D1. Cyclin D1 is a cell cycle regulator that may act as an oncoprotein. It is part of the enzyme complexes that are active in G1 phase of the cell cycle and which inactivate retinoblastoma (pRb) by phosphorylation. Overexpression of cyclin D1 leads to constitutive phosphorylation of pRb and increased expression of E2F activity. A study of 82 pancreatic cancers reported overexpression (by immunostaining) of cyclin D1 in 65% of tumors, and this was associated with decreased survival for these patients (123).

4. Apoptotic factors. Apoptosis or programmed cell death is a central regulator of homeostasis in normal tissue. The bcl-2 family of antiapoptotic genes includes bcl-2, bcl-x, bax and other related proteins, and the majority has four conserved domains. Bcl-2 is an antiapoptotic factor and has been referred to as a cooperating oncogene. By itself it is unable to transform cells, but, when activated in the presence of other oncogenes, bcl-2 is important for malignant transformation. Bcl-x is a bcl-2-related gene and exists as two subforms; bcl-xL is the longer form and functions as an apoptotic inhibitor; bcl-xS is the shorter form and functions as an apoptosis promoter. Bax is a promoter of apoptosis and bax/bcl-2 ratios can be important for determining cell survival. Several studies have shown that the expression of bcl-xL is significantly associated with poor survival in pancreatic cancer patients (124).

5. Tumor angiogenesis. Angiogenesis is essential for tumor growth and metastasis and angiogenic factors are produced by the tumor, by endothelial and stromal cells.

i. Angiogenin Angiogenin (ANG) is a polypeptide and inducer of vascularization. ANG interacts with endothelial cells via a cell surface receptor and extracellular matrix (ECM) molecules such as proteoglycans. ANG binds to actin on the endothelial cell surface, and this complex may lead to the activation of several protease cascades. In patients with pancreatic cancer, high serum

levels of ANG mRNA expression were significantly associated with poor survival (125).

ii. Vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF). VEGF is a potent and selective endothelial cell mitogen that is associated with tumor progression and metastases in a variety of gastrointestinal malignancies. VEGF is a 38 to 46-kDa dimeric N-glycoprotein that is chemotactic, as well as mitogenic, for endothelial cells *in vitro* and induces angiogenesis *in vivo* by increasing the permeability of the vascular endothelium. PD-ECGF stimulates the chemotaxis of endothelial cells and, therefore, indirectly induces angiogenesis. VEGF and PD-ECGF are frequently coexpressed in human cancers. Two studies in pancreatic cancer (a relatively hypovascular tumor) did not show prognostic value for micro vessel density (MVD); the expression of PD-ECGF, however, was associated with a significantly reduced survival. (126).

6. Stromal factors and adhesion molecules.

i. Urokinase plasminogen activator (uPA) and its receptor (uPAR) Plasminogen is an inactive proenzyme that can be converted to urinary or tissue plasminogen activator (uPA and tPA). uPA appears to play a pivotal role in pericellular proteolysis during cell migration and tissue remodelling. The resultant cellular activation and extracellular matrix proteolysis enhance the ability of pancreatic cancer cells to invade and metastasize. There is concomitant overexpression of uPA and uPAR in pancreatic cancer. Coexpression of uPA and uPAR in pancreatic cancer was associated with significantly decreased survival compared to patients with tumors that do not express one or both proteins (127).

ii. E-cadherin and matrix-metalloproteinases (MMPs) E-cadherin is a transmembrane glycoprotein that is responsible for homotypic binding and morphogenesis of epithelial tissues. E-cadherin is associated with a decrease in cellular and tissue differentiation and higher metastatic potential.

Levels of MMP enzyme activity correspond to tumor grade, regional lymph node metastases, and distant metastases. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are type IV collagenases and are overexpressed in pancreatic cancer. The expression of these MMPs directly correlates with invasion and metastasis in pancreatic cancer. Patients with cancers that express MMP/E-cad ratios of less than 3 had a significantly better prognosis compared to those with ratio > 3 (128).

7. Cytokeratins. Cytokeratins comprise a multigene family of 20 related polypeptides (CKs 1-20), are constituents of the intermediate filaments of epithelial cells, and are expressed in various combinations depending on the epithelial type and the degree of differentiation. The differential expression of individual CKs in various types of carcinomas provides relevant information concerning the differentiation and origin of carcinomas, especially when tumors initially undergo metastases. The CKs that are of particular value for differential diagnosis include CK 20, since it is mainly expressed in carcinomas derived from CK 20-positive epithelia; it is also found in bile-tract and pancreatic adenocarcinomas, and is not detected in most other carcinomas. In certain carcinoma types, changes in expression of individual CKs that occur during tumor progression may be of prognostic relevance (129).

8. Micro-RNAs (miRNAs). MiRNAs, a novel class of 18–23 nucleotide noncoding RNAs, have gained attention as another family of molecules involved in cancer development. There is evidence that miRNAs are misexpressed in various human cancers, suggesting that miRNAs can function as tumor suppressors ('TSGmiRs') or oncogenes ('oncomiRs')'. Upon binding to their target RNAs, miRNAs cause posttranscriptional gene silencing by either cleaving the target mRNA or by inhibiting the translation process (130). Several studies have shown that, miRNA expression is deregulated in pancreatic cancer. A miRNA signature of pancreatic cancer has been elucidated, and it includes the up regulation of miR-21, miR-155, miR-221 and miR-222 (131) Moreover, Chang

et al. found that miR-34a is frequently lost in pancreatic cancer cell lines (132). These studies demonstrate that miRNAs may become useful biomarkers and diagnostic factors for pancreatic cancer. In addition, these aberrantly expressed miRNAs might be useful as potential therapeutic targets, with the recent availability of *in vivo* miRNA knockdown strategies ('antagomirs').

Pancreatic cancer therapy

Surgery, radiation therapy, and chemotherapy are treatment options that may extend survival and/ or relieve symptoms in many patients, but rarely produce a cure. The drug erlotinib, that inhibits EGFR, blocks tumor cell growth but only has minimal effects on survival of pancreatic cancer patients. It has been approved by the FDA for the treatment of advanced pancreatic cancer. Clinical trials with several new agents, combined with radiation and surgery, may offer improved survival and should be considered as a treatment option. Interestingly, the cross-talk between RAS/MAPK and Hedgehog signaling pathways in pancreatic carcinomas also suggest that targeting the RAS and Hedgehog pathways synergistically may represent a new therapeutic strategy. Additionally, there are a few promising agents undergoing clinical trials that include, bevacizumab, the monoclonal antibody against VEGF, which targets tumor vascularization and cetuximab, the monoclonal antibody against the EGFR. Trastuzumab (Herceptin) is a humanized monoclonal antibody that acts on the HER2/neu (erbB2), a member of the EGFR family that shows profound beneficial results with breast cancer patients whose tumors overexpress this receptor (Table 2) (133). Gemcitabine (2',2'-difluorodeoxycytidine) is a deoxycytidine-analog antimetabolite with broad activity against a variety of solid tumors and lymphoid malignancies. It was approved as standard of care in patients with pancreatic cancer one decade ago, based primarily on improved clinical benefits such as pain reduction, improvement in Karnofsky performance status and increase in body weight (134).

Table 2. Ongoing Phase III clinical trials for targeted therapies in pancreatic cancer (135).

Treatment	Target	Disease stage
Erlotinib, capecitabine and gemcitabine	EGFR	Locally advanced or metastatic
Curcumin, celecoxib and gemcitabine	NFκB and COX2	Locally advanced or metastatic
Axitinib and gemcitabine	VEGF receptor and other tyrosine kinases	Locally advanced or metastatic
Sorafenib and gemcitabine	VEGF receptor and other tyrosine kinases	Locally advanced or metastatic
GV1001, capecitabine and gemcitabine	Telomerase	Locally advanced or metastatic
Aflibercept and gemcitabine	VEGF	Metastatic

Chemotherapy based on 5-fluorouracil (5-FU) prolongs survival of patients with advanced pancreatic cancer and Gemcitabine improves major symptoms and survival outcomes compared with bolus 5-FU. Many novel small molecule anticancer drugs are being investigated and include mechanism-based compounds and biological therapies targeting novel cellular survival pathways. Some examples include fluoropyrimidines, nucleoside cytidine analogues, platinum analogues, topoisomerase inhibitors, antimicrotubule agents, proteasome inhibitors, vitamin D analogues, arachidonic acid pathway inhibitors, histone deacetylase inhibitors, farnesyltransferase inhibitors and epidermal growth factor receptor therapies which are discussed elsewhere in this section. A recent randomized trial compared chemoradiotherapy with best supportive care in 31 patients with advance pancreatic carcinoma. Chemoradiotherapy was delivered in 16 patients using a standard fractionation scheme to a planned total dose of 50.4 Gy concurrently with a continuous infusion of fluorouracil (FU) at 200 mg/m²/d and results demonstrated a significant benefit of chemoradiotherapy (136).

Cancer chemotherapy

Historically anti cancer drugs have originated from all available chemical sources and include synthetic and natural products from plants, microbes and fungi and currently used drugs for cancer chemotherapy can be classified into the following groups:

1. Nitrogen mustards. Nitrogen mustards are highly reactive alkylating agents that dissociate to give positively charged carbonium ion intermediates. These electrophilic alkyl groups form covalent bonds with electron rich nucleophiles, such as sulfhydryl, hydroxyl, acetyl, phosphoryl, amino and imidazole groups present in normal and neoplastic cells. Thus they interact with a large variety of biological molecules including DNA, RNA and proteins. There is evidence linking alkylation of DNA to the cytotoxic carcinogenic and mutagenic effects of these agents. Formation of interstrand-DNA cross links inhibits DNA replication, repair and transcription and they also inhibit protein synthesis (137). Chlorambucil, Melphalan and Myleran (Busulfan) are phenylbutyric acid derivatives of nitrogen mustards. Chlorambucil is commonly used in the treatment of low-grade lymphomas and leukemias as well as in some immunological diseases such as glomerulonephritis and rheumatoid arthritis (138). Long term usage of these drugs causes permanent gonadal dysfunction and carcinogenicity. The nitrogen mustards damage DNA and their use as anti-cancer agents has declined due to these side effects.

2. Phosphoramidate and Oxazaphosphorine mustards. These compounds are also alkylating agents and phosphoramidate and oxazaphosphorine mustards are the only phosphorylated mustard compounds currently prescribed for cancer treatment (139). Cyclophosphamide is sulfur mustard that causes delayed bone marrow suppression and low doses of this compound are used for treatment of cancer even though the *in vivo* half-life of this compound is short. Tris (2-chloroethyl) amine is a nitrogen mustard agent used against Hodkin's lymphoma

and this compound disrupts cell renewal of lymphatics, bone marrow and gastrointestinal epithelia.

3. Nitrosoureas. In the 1950s National Cancer Institute organized a comprehensive screening program to identify new compounds with anti-cancer activities against malignant gliomas (140). One of the compounds, N-methyl-N-nitrosourea (MNU) was effective against intra-cerebrally implanted tumor cells (141). Nitrosoureas decompose rapidly in aqueous solution and alkylate both DNA and proteins (142). Lijinsky et al. showed that fully deuterated methyl groups were transferred intact from MNU to DNA. Kohn then showed that DNA modified by MNU underwent reversible denaturation indicating that interstrand crosslinks had been formed (143). Nitrosoureas cause adverse effects similar to that of other alkylating agents.

4. Platinum complexes. The platinum based drug Cisplatin (cis-diamminedichloro platinum [II]) was approved as an anti-cancer drug over 25 years ago. Cisplatin crosslinks DNA causing 1,2-intrastrand cross-links with purine bases. These include 1,2-intrastrand d(GpG) adducts which form nearly 90% of the adducts and the less common 1,2-intrastrand d(ApG) adducts. 1,3-Intrastrand d(GpXpG) adducts occur but are readily excised by nucleotide excision repair (NER). Other adducts include inter-strand crosslinks and nonfunctional adducts that may also contribute to activity of cisplatin. Cisplatin also interacts with cellular proteins, particularly high mobility group (HMG) domain proteins resulting in interference with mitosis. Although cisplatin is frequently designated as an alkylating agent, it has no alkyl group and does not undergo alkylating reactions. There was dramatic increase in long term survival of patients with small-cell lung, bladder, cervical, head and neck carcinomas after treatment with cisplatin. Carboplatin is a less toxic analog and exhibits lower nephrotoxicity, neurotoxicity and nausea however myelosuppression is dose-limiting for this compound (144). Oxaliplatin, another analog, exhibited neurotoxicity during phase I clinical trials (145). In spite of the efficacy of

cisplatin against cancers, this compound suffers from two major drawbacks. First, it is highly toxic against normal tissues and second many tumors develop resistance to the tumor inhibitory properties of cisplatin.

5. Anthracyclins. Actinomycin D was the first anthracyclin to be discovered during its application in treatment of Wilm's kidney tumor in 1950. The drug is produced by *Streptomyces* soil mold and is effective against blood leukemias. Actinomycin D inhibits DNA topoisomerase I and II, and helicases and induces formation of toxic free radicals that alter membrane structure and function and endonucleolytic cleavage (146). Doxorubicin, another anthracyclin analog is employed as single agent as well as in combination therapies for treatment of lymphomas, breast and small cell lung carcinoma and sarcomas but is ineffective against colon, neuronal cancers and renal cancer (147).

Anthracyclins have a narrow therapeutic index and documented toxicities include myelosuppression, gastrointestinal toxicity, alopecia and stomatitis. Leucopenia is the dose-limiting step. The acute and chronic effects of doxorubicin and daunorubicin on the heart can lead to cardiotoxicity and congestive heart failure which is a major adverse health effect (148). Another major drawback is drug resistance which is due to elevation of the multidrug resistance phenotype (MDR) and multi-drug resistance protein (MRP). The MDR phenotype which is characterized by increased membrane associated P-glycoprotein confers cross-resistance to a broad spectrum of natural product-derived drugs including anthracyclins.

6. Topoisomerase inhibitors. The helical structure of DNA was proposed by Watson and Crick in 1953 and Vinograd et al found that the helical axis can also be coiled in circular DNA called supercoiling. Since most of the functions of DNA require untwisting, the importance of topoisomerases are self-evident. The first enzyme identified as topoisomerase I, is ubiquitous, and breaks only one of the two strands as it untwists the DNA. Topoisomerase II, also known as gyrase, breaks both strands (149). Topoisomerase I inhibition is an important target for

anticancer drugs and camptothecin (CAM), obtained from Chinese tree *Camptotheca acuminata* is a potent Topo I inhibitor and a potent anti-cancer drug. The topoisomerase I enzyme forms a complex with DNA and then rolls along the DNA to release the super coiling. CAM derivatives stabilize and prolong this process rendering the cleavable complex (Topo I+DNA+CAM) vulnerable to degradation by other nucleases that first produce reversible and then irreversible DNA damage (150). Cells in S phase are 1000 times more sensitive to CAM than cells in other phases of cell cycle (151). Resistance to these drugs is explained by the presence of Topo I mutated genes and loss of topoisomerase activity (152). Combinations of Topotecan and cisplatin has been investigated *in vitro* but Topo I inhibitors can interfere with cisplatin-induced DNA damage (153).

7. DNA Topoisomerase II inhibitors. DNA topoisomerases represent a major focus for not only cancer research but also for gene regulation, cell cycle progression, and mitosis and chromosome structure. DNA gyrase is a bacterial equivalent of Topo II. Antibacterial quinolones (nalidixic acid, ciprofloxacin, norfloxacin and derivatives) are gyrase inhibitors with limited effects on host human Topo II. Topoisomerase mediated DNA strand breakage corresponds to transesterification reactions where a DNA phosphoester bond is transferred to a specific enzyme tyrosine residue. Topo I relaxes DNA by forming a covalent bond with the 3'-terminus of the DNA single stranded break (154). Topo II forms a dimer and a double stranded break as it binds covalently to the 5'-terminus of the double stranded DNA break (155). Topo II suppressors inhibit DNA cleavage steps by trapping the enzyme in the form of a closed protein clamp and inhibit their function either before or after DNA strand passage depending on the inhibitor (156).

Topo I poisons like Camptothecin do not affect Topo II and Topo II poisons (etoposide, doxorubicin, amsarcine) do not trap Topo I cleavable complexes. Enzyme deletion mutants may prove useful for understanding

interactions of the Topo II domain with drugs and other cellular proteins during the sub-cellular distribution and phosphorylation of the enzyme (157). The epipodophyllotoxins are Topo II inhibitors extracted from plants and they act as antimetabolic agents that bind tubulin at a site distinct from those occupied by the Vinca alkaloids. Anthracyclins like anthraquinones (Mitoxantrone) inhibit Topo II activity and also cause DNA intercalation (158). Ellipticines derived from alkaloids of Apocyanaceae plant family are also potent DNA intercalators as well as Topo II inhibitors.

8. The taxoids. Paclitaxel and docetaxel belong to the taxoid family, a relatively new class of antineoplastic drugs. The name taxoids refer to compounds, natural or modified that have a taxane skeleton. Paclitaxel was extracted from the bark of the Pacific Yew, *Taxus brevifolia*. Because of the scarcity of the drug and difficulty in formulation, initial development was slow. Preliminary studies indicated that taxoids act as a spindle poison that inhibit cell proliferation at the G2-M phase in cell cycle and thereby block mitosis (159).

Together with actin microfilaments and intermediate filaments microtubules form the cytoskeleton of eukaryotic cells. The microtubules are involved in a variety of cell functions including chromosome movement, regulation of cell shape and motility (160). The depolymerization of mitotic spindle microtubules is essential for specific mitotic events like movement of chromosomes to the metaphase plate and their correct segregation during anaphase (161). Microtubules are long hollow cylinders assembled from a heterodimeric globular protein called tubulin. They consist of 13 aligned proto-filaments within the tubulin subunits that interact with longitudinal and lateral bonds (162). Paclitaxel stabilizes microtubules and inhibits depolymerization back to tubulin. This differs from the mechanism of action of other poisons such as vinca alkaloids that bind tubulin and inhibit its polymerization (Figure 5) (163).

The dose-limiting toxicity of paclitaxel was neutropenia in seven out of nine Phase I trials (164). The mechanism of action of taxoids is unique, since all

other known mitiotic spindle poisons in particular the vinca alkaloids shift the tubulin-microtubule equilibrium towards tubulin.

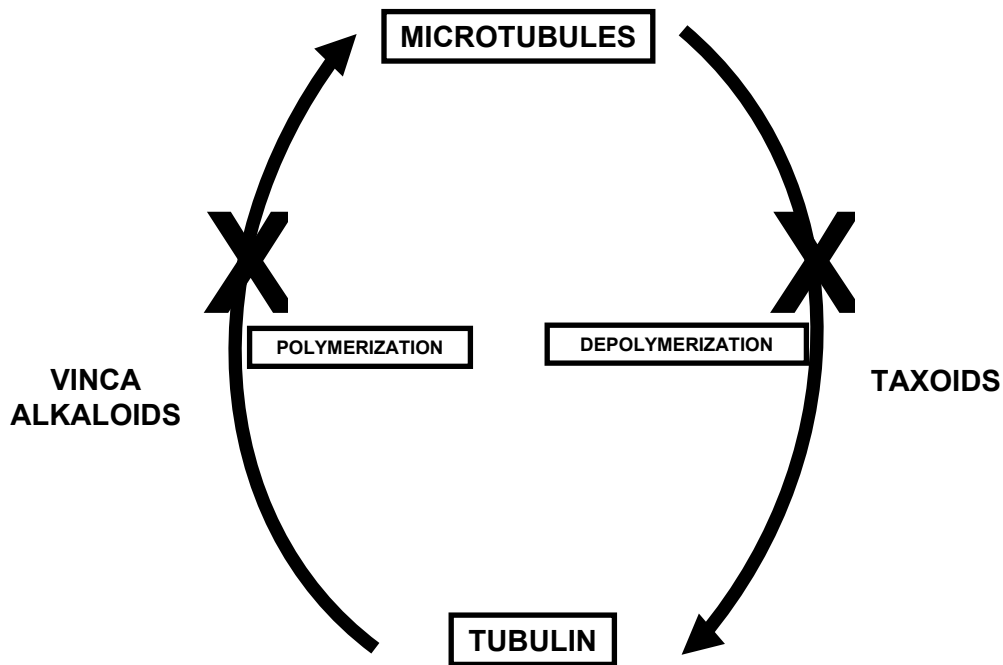


Figure 5. Mechanism of action of Vinca alkaloids and taxoids.

9. Sequence selective groove binders. A large number of agents bind DNA and interfere with multiple DNA functions in living cells. The ability to interact with DNA is associated with several biological effects including anti-viral antibacterial anti-protozoal and anti-tumor activities. From a pharmacological viewpoint, the most relevant DNA binding agents are antitumor drugs. They exert their cytotoxic effects as a consequence of random DNA damage and these drugs often have a low therapeutic index since they cause DNA lesions not only in tumors, but also in normal cells. However, certain selectivity toward specific tumor types has been recognized for some agents as documented by the efficacy of platinum compounds in the treatment of testicular and ovarian cancers.

i. Major groove binders. Although the major groove has a greater recognition potential based on the number of hydrogen bonds, only a few DNA intercalating agents are major-groove specific. The bi-functional alkylating agents, methylating agents and cisplatin covalently interact with the N-7 position of guanine located in the major groove.

ii. Intercalating anti-tumor agents. Doxorubicin is the widely used anthracycline and DNA binding and intercalation are necessary but not sufficient for optimal antitumor activity of this drug (165). Indeed strong intercalating agents like ethidium bromide have minimal anti-tumor activity and it is likely that the mode and site of binding are more critical than the binding affinity. The mechanism of cytotoxic and antitumor activity of intercalating agents is related to their ability to interfere with the function of Topo II (166), a nuclear enzyme that regulates DNA topology during multiple metabolic DNA processes.

iii. Noncovalent DNA minor groove binders. Non-intercalating drugs have been extensively studied for their sequence-specific in DNA binding (1). Anti-viral antibiotics like distamycin and netropsin bind non-covalently to the minor groove with an AT preference and cause widening of the minor groove (167). These agents are incorrectly identified as antitumor agents since they have low cytotoxicity and negligible antitumor activity.

iv. Minor groove alkylating agents. Mitomycin C is well known for its antibiotic and antitumor activities and it binds covalently to the minor groove of DNA resulting in mono and bi functional adducts. Mitomycin C preferentially crosslinks a 4 bp CG sequence. Groove binding drugs exhibit sequence-specific DNA interaction however the sequence-specific recognition potential of DNA-intercalating drugs is not sufficient to provide selective cytotoxicity against tumor cells. Many of the lesions produced by these intercalating are not required for the therapeutic activity (168). Other disadvantage includes chemical and pharmacological problems associated with stability of carriers, poor cellular penetration and synthetic difficulties.

10. Bis-Naphthalimides. Naphthalamides were synthesized as a new series of antitumor compounds in the late 1970s as bis-intercalating agents with a high binding affinity for DNA (169). Amonfide and Mitonafide bind to double stranded DNA by intercalation. These drugs stabilize DNA against thermal denaturing, inhibit RNA and DNA synthesis, and initiate DNA cleavage by activation of topoisomerase II. Amonfide and Mitonafide specifically induce DNA cleavage near nucleotide No. 1830 on pBR322 DNA. However it is unclear if the antitumor effects are due to alterations of topoisomerase II activity. These drugs also have anti-viral activity specifically against DNA viruses and their dose-limiting toxicities are myelosuppression and stomatitis (170).

11. Radiation. Radiation therapy uses ionizing radiation to kill cancer cells and shrink tumors. Radiation therapy is used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, spine, stomach, uterus, or soft tissue sarcomas (171). Radiation can also be used to treat leukemia and lymphoma (cancers of the blood-forming cells and lymphatic system, respectively). The dose of radiation to each site depends on a number of factors, including the type of cancer and whether there are tissues and organs nearby that may be damaged by radiation.

Approximately 50% of all patients are treated with radiation therapy, either alone or in combination with other types of cancer treatments (172). The amount of radiation absorbed by the tissues is called the radiation dose and the unit is called a gray (abbreviated as Gy). **External radiation therapy** usually is given on an outpatient basis and most patients do not need to stay in the hospital. External radiation therapy is used to treat most types of cancer, including cancer of the bladder, brain, breast, cervix, larynx, lung, prostate, and vagina (173).

Internal radiation therapy (brachytherapy) uses radiation that is placed very close to or inside the tumor. The radiation source is usually sealed in a small holder called an implant which are in the form of thin wires, plastic tubes called catheters, ribbons, capsules, or seeds (174) and placed directly into the body.

Internal radiation sources are iodine 125 or 131, strontium 89, phosphorus, palladium, cesium, iridium and cobalt isotopes.

Target based anti cancer drugs

1. Matrix Metalloproteinase (MMP) inhibitors. Members of this family selectively degrade collagen triple helix. Liotta et al showed that destruction of the basement membrane is often associated with tumor invasion, and that tumor cells selectively degrade components of the basement membrane ((175). Type IV collagen is a major structural component of the basement membrane. A direct correlation exists between MMP expression and the invasive phenotype of human lung, prostate, stomach, colon, breast, ovary and thyroid tumors and squamous cell carcinomas. MMP family members are overexpressed in cancers and they are Stromelysin-3, gelatinase A and gelatinase B and they cooperate with other proteases to facilitate matrix turnover. The relative expression of MMPs varies with tumor heterogeneity, progression as well as differential expression in response to changing extracellular matrices encountered during tumor progression. Substrate analog inhibitors of MMPs are used as anticancer agents. **Batimastat (BB-94)** is a hydroxamic acid analog with broad spectrum MMP inhibitory activity but little activity against other unrelated metalloproteinases. The low solubility and poor oral bioavailability of BB-94 are severe limitations for the formulation and delivery of this compound. Malignant tumor cell invasion is a dysregulated physiologic process similar to angiogenesis, wound healing, embryonic development and tumor invasion (176). These pathways are regulated spatially and temporally and are responsive to negative regulatory signals in non tumor tissues and MMP's activity is a common denominator for these processes. Abrogation of MMP activity through use of synthetic, low molecular weight substrate inhibitors can effectively block tumor dissemination and growth. The suppression of tumor growth is indirectly linked to suppression of tumor-induced angiogenesis by the MMP inhibitors. **Neovastat**

obtained from shark cartilage extract exhibits antitumor and anti-metastatic properties in experimental tumor models. It targets several angiogenic pathways including VEGF signaling, MMP signaling and endothelial apoptosis. Neovastat is well tolerated in phase I studies with no dose limiting toxicities, patient survival was significantly increased and there was a 50% decrease in relative risk of death in patients with non small cell lung carcinoma (NSCLC).

2. Interferons and other cytokines. Cytokines are proteins that regulate cell behavior in a paracrine or autocrine manner and when administered exogenously these proteins often possess biological activities that make them attractive for cancer therapy. IFN- γ , an antiviral agent effects cell growth and differentiation and in combination with other cytotoxic agents such as, doxorubicin, cisplatin, vinblastine and methotrexate and synergistic activities have been reported (177). IFN- γ slows growth of tumor cells by increasing the length of their cell cycle in G0/G1 transition and suppressing cellular oncogenes like c-myc and c-fos oncogene expression (178). IFN- γ increases expression of class I major histocompatibility complex (MHC) antigens (179) on tumor cells resulting in efficient recognition and killing of tumor cells by cytotoxic T-cells and natural killer cells. IFN also inhibits angiogenesis due to degeneration of endothelial cells in tumor vessels (180) and leucocyte IFN inhibits capillary endothelial cell motility *in vitro* (181). Gresser and others showed that IFN not only inhibits virally induced tumors but also spontaneous and transplantable tumors (182). Limitations of interferons include species-specific effects on some cytokines, and the immunogenicity of some synergistic tumors, tumor growth inhibition and decreased metastasis are often not observed in patients.

3. Angiogenesis inhibitors. Most current chemotherapies are cytotoxic and are designed to kill rapidly growing tumor cells. Because many normal tissues contain stem cells that also proliferate rapidly (bone marrow, hair follicles, intestines), the effectiveness of these agents is limited because of toxic side effects. One of the novel approaches to cancer therapy does not target the

tumor cells directly but inhibits growth of new capillary blood vessels that feed the growing tumor, and thereby inhibits angiogenesis. Tumors that grow larger than approximately 1-2 mm³ in size, must stimulate growth of new capillaries (183) in order to obtain a continuous supply of oxygen and nutrients for growth. TNP-470, a compound isolated from *Aspergillus fumigatus* Fresenius fungus inhibited endothelial cell proliferation *in vivo*, embryonic angiogenesis, and tumor induced neo-vascularization in a mouse model (184). A woman with metastatic cervical carcinoma treated with TNP-470 continuously remained free of tumors and toxicity for over a year (185).

4. Antisense oligonucleotides. Antisense therapy that interferes with signaling pathways involved in cell proliferation and apoptosis are promising treatments in combination with conventional anticancer drugs (186). Aptamers, also termed as decoys or “chemical antibodies,” represent an emerging class of therapeutics. They are short DNA or RNA oligonucleotides or peptides that assume a specific and stable three-dimensional shape *in vivo*, thereby providing specific tight binding to protein targets. In contrast to antisense oligonucleotides, effects aptamers can be used against extracellular targets and thereby circumvent the need for intracellular transportation. The most advanced aptamer in the cancer setting is AS1411, formerly known as AGRO100, which is being administered systemically in clinical trials. AS1411 is a 26-mer unmodified guanosine-rich oligonucleotide, which induces growth inhibition *in vitro*, and exhibited activity against human tumor xenografts *in vivo*. In a dose escalation phase I study in patients with advanced solid tumors, doses up to 10 mg/kg/d have been studied. Promising signs of activity have been reported and this includes multiple cases of stable disease and one near complete response in a patient with renal cancer in the absence of any significant adverse side effects (187). AS1411 represents the first nucleic acid-based aptamer to be tested in humans for the treatment of cancer.

5. Growth factor receptors and their inhibitors. Members of the EGF receptor family are overexpressed in many cancers and this often correlates with increased tumor grade, increased metastatic potential, and poor prognosis in bladder breast and gastric cancers (188). EGFR was originally cloned in 1984 and belongs to a family of transmembrane receptor tyrosine kinases involved in cell growth and differentiation. EGFR tyrosine kinase inhibitors inhibit the EGF receptor function by binding to its ATP binding pocket and inhibiting auto phosphorylation. The adverse toxic effects of gefitinib, an EGFR inhibitor, are skin rashes due to inhibition of EGFR in the basal epidermal layer that expresses high levels of EGFR. Phase III trials with gefitinib have been reported and neither the addition of gefitinib to paclitaxel/carboplatin or gemcitabine/cisplatin improved the outcome of patients with NSCLC. It is currently approved for use in Japan and cancer-organ specific growth factor receptors and their inhibitors are discussed elsewhere in this section.

6. Immuno-conjugates. Bevacizumab is a humanized antibody against VEGF and is generated by engineering the VEGF binding sites of murine neutralizing antibody into the framework of a normal human immunoglobulin G (IgG). This antibody binds all biologically active forms of VEGF and In phase II clinical trials, in breast cancer there was complete response and prolonged time-to-disease progression compared to chemotherapy alone. The primary toxicity concern is hemorrhaging which was observed in squamous cell carcinoma patients in a phase II trials.

Transcription factors as drug targets

Pro-cancer signals are controlled by the expression and transcription of oncogenes. Gene transcription is dependent on the spatially and temporally coordinated interactions between the transcriptional machinery (RNA polymerase II, transcription factors (TFs) and transcriptional regulatory components (gene promoter elements, enhancers, silencers and locus control

regions). Several TFs have been associated with cancer. In one study, it was reported that among the 50% of promoter sequence variants that alter gene expression, more than 1.5-fold were located within 50-100 bases from the transcription start site. Gene regulation at the level of transcription initiation is important and this has encouraged attempts to establish topologies of transcriptional regulatory networks to facilitate analysis of regulatory links (transcription factor (TF)-gene) in addition to changes in expression and co-expression of specific genes (189). TFs, the transcriptional machinery and transcriptional regulatory elements are novel therapeutic targets and understanding transcriptional regulatory networks has revealed their critical roles in carcinogenesis (Table 3). Transcription factors form an integral part of each of the hallmarks of cancer and contribute to the overall tumor-specific phenotypes.

Transcription factors have been successfully targeted in several clinical trials (Table 4) and these outcomes support the rationale for their selection as drug targets.

Sp proteins as targets for anti-cancer drugs. Studies from this laboratory have identified the importance of Sp transcription factors as potential targets for cancer chemotherapy. Specificity protein 1 (Sp1) and other Sp and Krüppel-like factor (KLF) proteins are members of the Sp/KLF family of transcription factors that bind GC/GT-rich promoter elements through three C₂H₂-type zinc fingers present in their C-terminal domains.

Table 3. Hallmark traits of oncogenic transcription factors (190).

Transcription factor	Hallmark traits					
	Self-sufficiency in growth signals	Insensitivity to growth-inhibitory signal	Evasion of programmed cell death	Limitless replicative potential	Sustained angiogenesis	Tissue invasion and metastasis
AP-1	X		X		X	X
AR	X					
ATF-1						X
BRN-3b		X				
C/EBP α		X				
CREB			X		X	
E2F-1				X		
ETS-1					X	X
EWS/ETS				X		
FOXO3a			X			
HIF-1 α /HIF-1 β (ARNT)					X	X
Myc	X				X	X
NF- κ B	X		X		X	X
PEA3						X
RAR α	X					
RB1				X		
SP-1					X	
STAT3		X	X		X	
STAT5		X	X			
TP53			X			

Table 4. Preclinical studies that have focus on modulation of the TF expression and/or function (190).

Transcription factor	Justification summary
AP-1 (c-Jun)	Inhibition of AP-1 reduced breast cancer cell proliferation in mice (191)
AR	Downregulation of AR resulted in prostate tumor size reduction in mouse xenograft (192)
ATF-1/CREB	Knockdown of ATF-1/CREB in mice resulted in a decrease in size of subcutaneously transplanted tumors via induction of apoptosis (193)
BRN-3b (POU)	Results suggest that Brn-3b elevation in breast cancer is a significant transcription factor in regulating mammary cell growth (194)

Table 4 continued.

CREB	Mice transfected with CREB300/310 dramatically enhanced tumor growth, whereas CREB300/310/133 inhibited hepatoma growth (195)
E2F-1/RB1	Knockdown of E2F in mouse embryonic fibroblast leads to phosphorylation of RB1 and increased cell proliferation (196)
Myc	Overexpression of Myc in mice resulted in downregulation of IL-6- and VEGF-induced rabbit corneal angiogenesis (197)
NF-kB	Bortezomib-induced downregulation of NF-kB pathway has been observed in refractory multiple myeloma and relapsed myeloma patients in phase II clinical trials (198)
PEA3	Use of a dominant-negative PEA3 delayed onset, reduced number and size of mammary tumors in mice (199)
RAR α	Downregulation of RAR α resulted in lymphoma in 44% of homozygous transgenic mice (200)
SP-1	Celecoxib-affected Sp1-binding sites on VEGF gene expression and limited metastasis in nude mice (201)
STAT5	Downregulation of STAT5b mediates proliferation of SCCHN cancer cells (202)
p53	Adenovirus-mediated wild-type p53 gene transfer with chemotherapy and radiation inhibits progression of lung cancer growth in animal models with minimal toxicity (203)

Each of the three zinc fingers in Sp1 recognizes three bases in one strand, and a single base in the complementary strand of the GC-rich elements and the consensus Sp1 binding site is 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3'. Sp/KLF transcription factors have similar modular structures. Sp1–Sp4 form a subgroup (Figure 6) containing several distinct overlapping features/regions which include activation domains (AD), the C-terminal zinc finger DNA-binding

region, and an inhibitory domain (ID) in Sp3 that is involved in the suppressive activity of Sp3. Sp5–Sp8 are structurally similar and appear to be truncated forms of Sp1–Sp4 in which portions of the N-terminal regions have been deleted.

Sp1–Sp6 proteins contain several common domains in their C-terminal region, whereas Sp5 and Sp6 exhibit a truncated N-terminal structure. Buttonhead (Btd) and Sp boxes are conserved regions in all Sp proteins (204). Sp1–Sp4 proteins regulate expression of multiple genes in normal tissues and tumors. Sp1 directly interacts with TATA-binding protein associated factors (TAFs) and other basal transcription factors.

Sp1^{-/-} embryos exhibit multiple abnormalities and retarded development and embryolethality on day 11 of gestation. Sp3^{-/-} mice exhibit growth retardation, defects in late tooth formation, and the animals die at birth and Sp4^{-/-} mice either die shortly after birth or survive with significant growth retardation (205).

Mechanisms of Sp-mediated gene expression. The primary mechanism of Sp protein-dependent transactivation in cancer and non-cancer cell lines involves initial binding to GC-rich promoter sequences and subsequent interactions with components of the basal transcription machinery to activate gene expression. Sp-dependent activation of genes has been extensively investigated primarily using Sp1 and Sp3 proteins as models. Courey and coworkers first reported synergistic interactions of Sp1 on GC-rich promoters where it has been hypothesised that four Sp1 proteins cooperatively bind to form a homooligomeric complex (206).

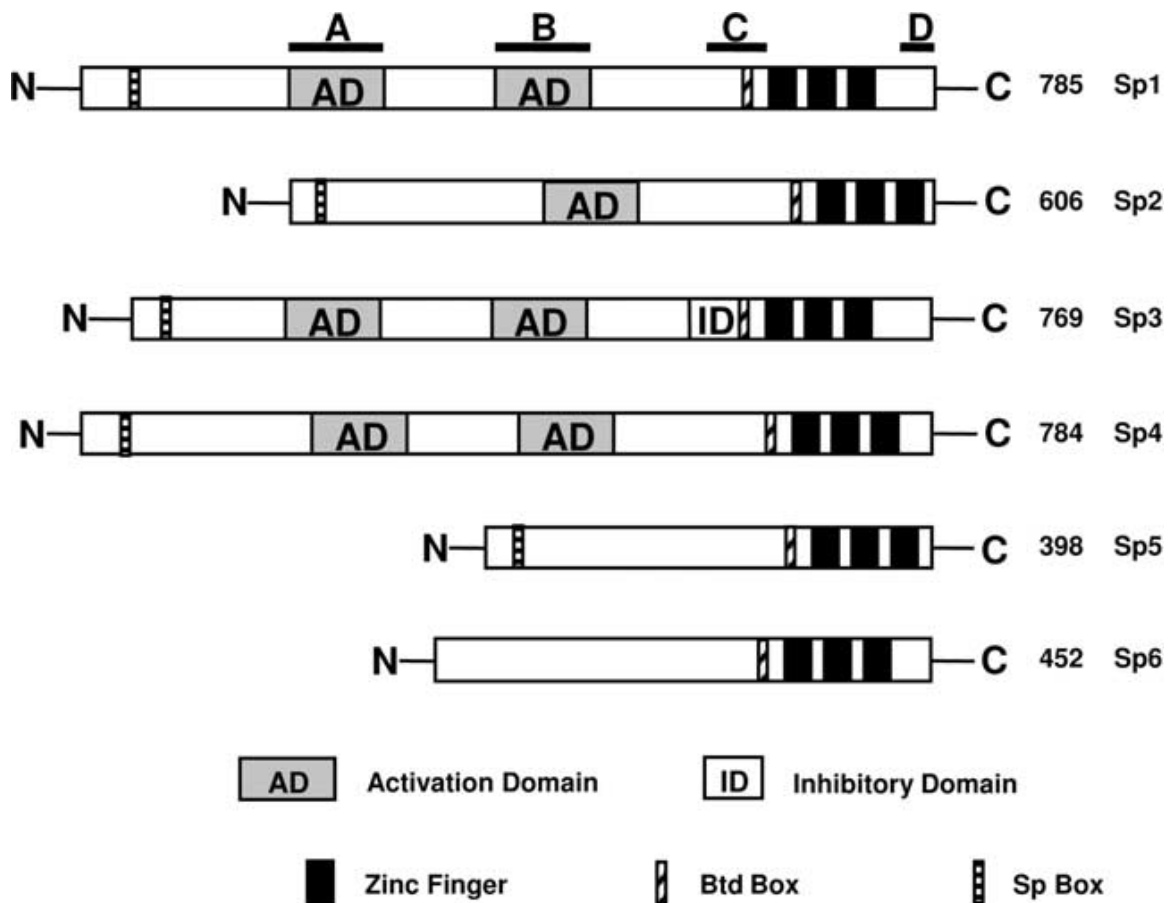


Figure 6. Structural features of Sp proteins.

Sp1 and Sp3 both bind GC-rich sequences, and these interactions can be cooperative or Sp3 can decrease Sp1-dependent transactivation. Genes regulated by Sp1 and other Sp transcription factors may contain their respective cis-element but transactivation may also be due to direct protein–protein interactions with other nuclear factors in which only the Sp protein is bound to promoter DNA. Sp1-dependent transactivation is dependent on additional protein complexes designated as cofactors required for Sp1 coactivation (CRSP). The precise role of these proteins in mediating Sp-dependent transactivation is not completely defined (207). Sp-dependent transactivation through interactions with GC-rich sites is also modulated by Sp1 interactions

with other proteins like the AhR, Arnt, several GATA transcription factors, p53, MEF2C, SMAD2, SMAD3, SMAD4, Msx1, cyclin A, Oct-1, TBP, HNF3, BRCA1, and others.

Sp1 also binds to several ligand-activated nuclear and orphan nuclear receptors, and these include the estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR), retinoic acid receptor (RAR), retinoic X receptor (RXR), peroxisome proliferator-activated receptor γ (PPAR γ), vitamin D receptor (VDR), steroidogenic factor-1 (SF-1), chicken ovalbumin upstream promoter transcription factor-II (COUP-TFII) and HNF-4 (208). The C-terminal C/D domain of Sp1 is the major site for protein interactions. Thus, Sp1 and possibly other Sp proteins act as multifunctional modulators of gene expression through direct interactions with different nuclear proteins (DNA-independent) or by interacting with DNA-bound transcription factors.

Several reports indicate that phosphorylation of Sp1 by various kinase pathways is important for Sp1-dependent activation of some genes, and this adds to the functional complexity of this transcription factor. Regulation of VEGF in several prostate cancer cell lines is dependent on phosphatidylinositol-3-kinase (PI3-K) activity, and this is linked to increased phosphorylation of Sp1 and enhanced binding to the proximal GC-rich -88/-66 VEGF promoter sequence. In contrast, mitogen-activated protein kinase (MAPK)-dependent phosphorylation of Sp1 is important for induction of α 6-integrin gene expression in prostate cancer cells (209). Acetylation of Sp1 and Sp3 have also been linked to increased transactivation, for example topoisomerase II inhibitors activate the SV40 promoter in cancer cell lines through acetylation of Sp1 (210) and acetylation of Sp1 and Sp3 is dependent on the coregulator p300 which exhibits histone acetyltransferase activity.

Sp proteins and critical Sp-dependent tumor genes as targets for therapeutic intervention. Sp family proteins regulate basal/constitutive expression of genes in normal and tumor tissues. Initially, the ubiquitous

transcription factor Sp1 was considered a constitutive activator of housekeeping genes and other TATA-less genes. Genes that regulate growth and cell cycle progression frequently contain proximal GC-rich promoter sequences, and their interactions with Sp proteins and other transcription factors are critical for their expression. Several studies show that VEGF expression in cancer cell lines is regulated through Sp protein interactions with several proximal GC-rich motifs and this suggests that Sp transcription factors may contribute to cancer cell proliferation, survival and angiogenesis. (211). Mapping of GC-rich Sp binding sites on chromosomes 21 and 22 indicates that the full genome has at least 12000 GC-rich Sp binding sites in the full human genome. (212).

Among Sp1 target genes in cancer cells (Figure 7) are many key players in cell proliferation, survival and metastasis including prominent oncogenes and tumor suppressors. Several Sp-regulated genes are important for phenotypes associated with the six hallmarks of cancer (16), namely self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis genomic instability and cell growth/metabolism (mobilization of resources) (213) are also regulated by Sp transcription factors. With respect to cell cycle progression Sp1 activates transcription of several genes importantly for cell proliferation and G1 to S phase cell cycle progression and these include D-type cyclins, cyclin E, Cdk2, E2F-1 and c-Myc (Figure 7). Thus, Sp transcription factors promote the G1/S-transition because cyclin D/Cdk4 and cyclin E/Cdk2 induce the entry of cells into S-phase and because E2F-1 and c-Myc are the two transcription factors, that induce S-phase entry of quiescent cells (214). In some cells Sp1 can be phosphorylated specifically in mid-late G1-phase (215) and expression of a dominant-negative form of Sp1 prolonged S-phase and decreased the growth of fibroblast cells. Sp1 is versatile and also activates transcription of genes encoding all seven cyclin-dependent inhibitors, i.e. p15^{INK4B}, p16^{INK4A}, p18^{INK4C}, p19^{INK4D}, p21^{WAF1/CIP1}, p27^{KIP1} and p57^{KIP2} indicating

that Sp1 also has the potential to contribute to cell cycle arrest. In addition, p53 synergizes with Sp1 in transactivation of the p21 promoter (216). Sp1 target genes include both pro- and anti-angiogenic factors/proteins that promote and inhibit invasion and metastasis. Depletion of Sp1 by siRNA impaired the angiogenic potential of PANC-1 pancreatic adenocarcinoma cells (217) and in another study, Sp1 U1snRNA/ribozyme inhibited the anchorage-independent growth on soft agar of malignantly transformed PH2MT and γ 2-3A/SB1 fibrosarcoma cells (218). Sp1 is overexpressed in several cancers and the Sp1 levels correlated with tumor grade/stage and poor prognosis (204) (219) (220) (221). Thus, the outcome of Sp1 action is highly cell context-dependent underscoring the versatility of this transcription factor. Studies in this laboratory have demonstrated that an underlying mechanism of action of anticancer agents such as tolfenamic acid (TA), betulinic acid and curcumin is due to repression of Sp proteins in cancer cells (222).

Genes regulated by Sp1 and other Sp transcription factors promote cell proliferation, self-sufficiency of growth signals, limitless replicative potential, cell survival, angiogenesis, invasion and metastasis and cell growth (223). Sp-regulated gene products are also important for maintaining and perturbing genomic stability. Sp1 overexpression induced apoptosis whereas a dominant-negative form of Sp1 suppressed apoptosis (224). In contrast, siRNA-mediated knockdown of Sp1 enhanced the H₂O₂-induced apoptosis of U2OS osteosarcoma cells, but had no effect in untreated cells. Depletion of Sp1 in normal human dermal fibroblast (NHDF) cells by siRNA increased the number of DNA double-strand breaks after exposure to ionizing radiation, but had no effect in untreated cells (225). The Sp1 target gene c-Myc (226) (227) is a potent oncogene, which stimulates every aspect of cell proliferation, cell growth and oncogenesis and hence Sp1 has the potential to support tumorigenesis. Moreover, depletion of Sp1 by siRNA decreased tumor growth and metastasis of N67 gastric cancer cells in nude mice (228). Sp/KLF gene knockout studies

emphasized the critical role of these transcription factors in normal development of tissues/organs. For many human cancers, Sp protein overexpression is a negative prognostic factor for survival and these transcription factors contribute to the proliferative and metastatic tumor phenotype. Strategies for inhibiting Sp-dependent pathways have focused on several approaches which include drugs that inactivate GC-rich DNA motifs, oligonucleotides and peptide nucleic acid–DNA chimeras that specifically interact with Sp1 binding motifs (decoys), and chemicals that modulate Sp protein expression. The DNA binding antitumor drug hedamycin complexes with G-rich DNA, and this compound inhibits survivin transcription through interactions with a proximal GC-rich Sp1 binding site (–115 to –95) in the survivin promoter. Double stranded oligodeoxynucleotides containing a consensus GC-rich Sp1 binding site (Sp1 decoys) have been developed for inhibiting Sp1-dependent gene expression (229).

Transcription factors are now recognized as targets for development of new anticancer drugs (230), and the important role of Sp-dependent gene expression in tumor development, growth and metastasis has been established (204) (204). The complexity of Sp-dependent regulation of genes in cancer has primarily been reported for Sp1 and to a lesser extent Sp3; however, based on recent reports it is conceivable that Sp4 protein may also be important. The complexity of Sp protein-dependent regulation of genes is illustrated in Figure 7 and regulation of these genes is dependent on domain-specific interactions of Sp1 with other nuclear factors (DNA-independent), DNA-bound transcription factors, and chemical- and enzyme-induced modifications. Elevated Sp protein expression in tumors is correlated to up regulation of multiple genes that are involved in tumor growth and metastasis. Targeting transcription factors as a therapeutic strategy in cancer patients raises three major concerns: Selectivity, specificity and differential sensitivity. The global inhibition of a specific TF is likely to result in undesirable side effects in non-target tissues. The requirement for selective targeting necessitates optimization through cancer-directed delivery

and cancer-specific effector activation (that is, using cancer-specific promoters, such as hTERT). Transcription factors in the same family also have similar or shared motifs, or structures, but different functions. This presents the second challenge. For treatments to be effective, the targeting moieties will have to be able to distinguish between TFs of the same family.

Finally, quantitative rather than qualitative differences between cancer and normal tissues may be important for differential sensitivity to drugs targeting Sp proteins. Recent developments in delivery vehicles and directed targeting (for example, liposomes, nanoparticles and aptamers) along with more accurate and potent effectors (that is, shRNA, siRNA, ribozymes, antisense oligonucleotides, small-molecule inhibitors and zinc finger proteins) may be important for enhancing the effectiveness and selectivity of agents that target Sp and other transcription factors.

Natural products and their use as ancient medicinals

Phytochemicals from plant extracts have been used throughout human history for health benefits. Medicines derived from plants have played a pivotal role in the health care of many cultures, both ancient and modern (231) (232) (233). Of the approximately 877 small molecule drugs introduced worldwide between 1981 and 2002, approximately 60% can be traced back to their origins as natural products (231). Examples of natural products that provide frontline pharmacotherapy include steroids, cardiotonic glycosides (Digitalis glycosides), anticholinergics (belladonna type atropine alkaloids), analgesics and antitussives (opium alkaloids), antihypertensives (reserpine), cholinergics (physostigmine, pilocarpine), antimalarials (Cinchona alkaloids), antigout (colchicine), anesthetic (cocaine), skeletal muscle relaxant (tubocurarine), and anticancer agents. It has been estimated that plant-derived drugs represent about 25% of the entire prescription drug market in the United States and over one-half of the top 25 prescription products are plant-derived (234).

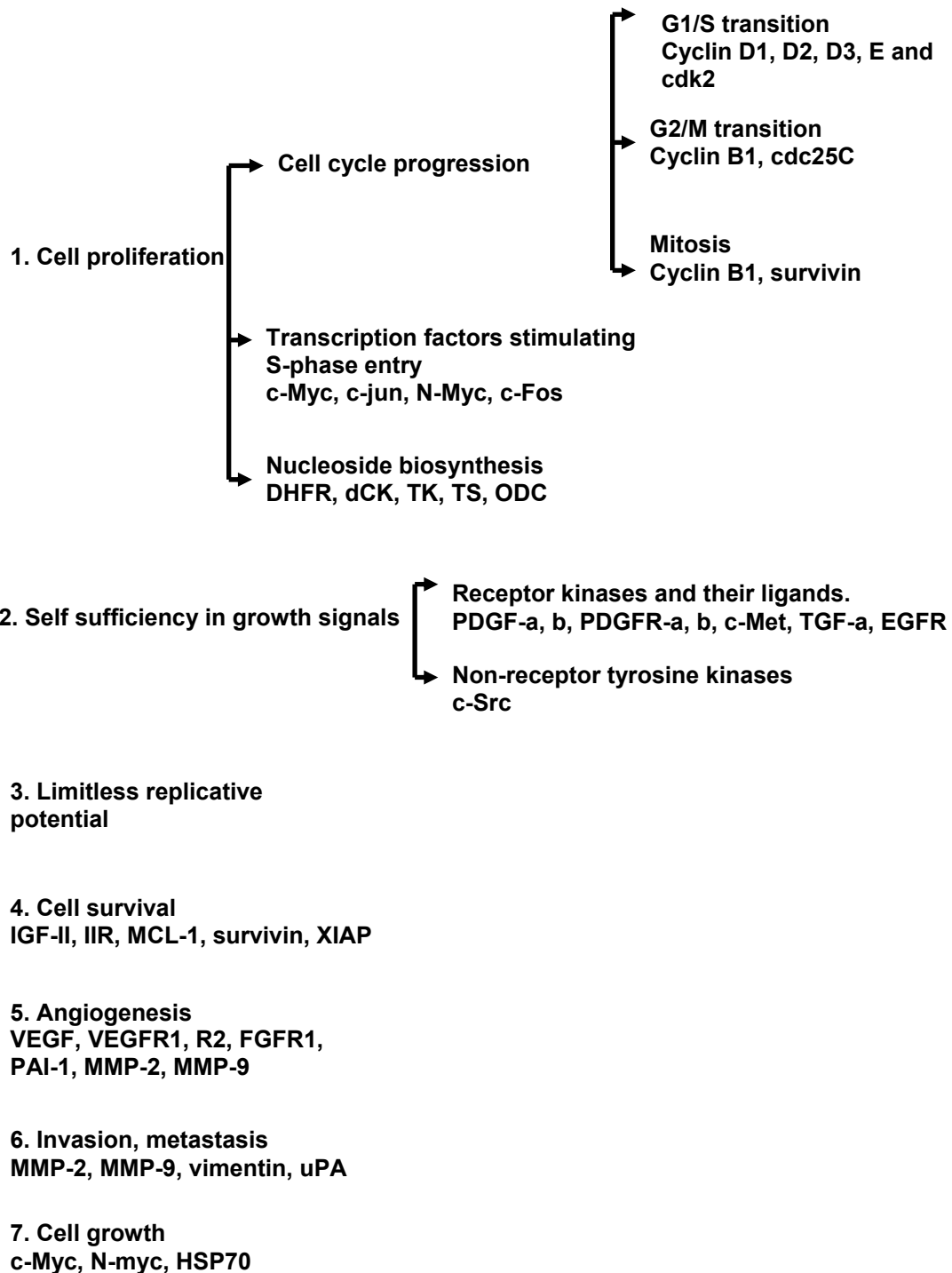


Figure 7. Functions of Sp target genes in regulating hallmarks of cancer cells.

Moreover certain natural compounds polyphenols, flavonoids, and isothiocyanates have multiple pharmaceutical effects including their antioxidant, antiviral, antibacterial, anti-inflammatory, antifungal, immunomodulatory, and cancer preventive and anti-cancerous activities (235).

Cancer chemoprevention

During the 1960s and 1970s, studies by Wattenberg et al demonstrated that various compounds, especially those associated with fruits and vegetables such as indoles and isothiocyanates; inhibit chemically-induced tumor formation and growth in laboratory animals. This was the advent of “chemoprophylaxis of carcinogenesis” (236), and the implications of these observations in terms of human health maintenance were immediately apparent. Subsequently based on certain hallmark studies performed with a variety of retinoids, Sporn coined the term “cancer chemoprevention” (237) to further define the phenomenon. Cancer chemoprevention in human populations is defined as prevention of cancer by ingestion of chemical agents that prevent carcinogenesis. It is important to differentiate the concept of cancer chemoprevention from primary cancer prevention, which is exemplified by the cessation of cigarette smoking, or cancer chemotherapy, which may enhance or initiate cancers after treatment. Cancer chemoprevention has developed as a well-defined and distinct discipline of science. Epidemiological studies show that dietary factors decrease the incidence of some cancers (238) presumably through chemopreventive mechanisms and various prospective studies are currently underway (239) (240). It is likely that dietary constituents such as garlic, ginger, soy, curcumin, onion, tomatoes, cruciferous vegetables, chillies and green tea exhibit chemopreventive activity against some cancers. There is a diverse structural array of compounds that may be considered “chemopreventive,” and over 600 agents may be considered in this category (241). Examples include inhibitors of initiation such as phenols, flavones, aromatic isothiocyanates, diallyldisulfide,

ellagic acid, antioxidants and inhibitors of post-initiation events such as carotene, retinoids, terpenes, protease inhibitors, prostaglandin inhibitors, and nerolidol. Natural products have been classified as chemopreventive agents due to their ability to delay the onset of the carcinogenic process and since these chemopreventive agents are derived from natural sources, they are considered pharmacologically safe. Some dietary agents suppress the transformative, hyperproliferative and inflammatory processes that initiate carcinogenesis (Figure 8) and thus may ultimately suppress the final steps of carcinogenesis, i.e., angiogenesis and metastasis.

Natural products and their synthetic analogs as anti-cancer agents

It has been estimated that genetic factors cause only 5–10% of all human cancers, while the remainder is caused by lifestyle. In spite of extensive research on development of safe and efficacious drugs for cancer chemotherapy, most agents are also toxic and associated with adverse side effects, suggesting a need for mechanism based, less toxic alternatives.

Interestingly, more than 70% of the FDA approved anticancer drugs can be traced back to their origin as plant-derived natural products, which were traditionally used as ancient remedies for various ailments (94). Vinblastine from *Vinca rosea* is one of the earliest example of an anticancer drug from an oriental medicine and paclitaxel is a recent example of a compound that originated from the Chinese pacific yew plant (242).

There is increasing evidence that natural products are important as pharmaceuticals particularly for antitumor drugs such as paclitaxel (Taxol), vincristine (Oncovinm), vinorelbine (Na, velbine), teniposide (Vumon), and various water-soluble analogs of camptothecin (e.g., Hycamtin) (234). Cancer chemopreventive agents, many of which are natural products, can also inhibit and prevent carcinogenesis.

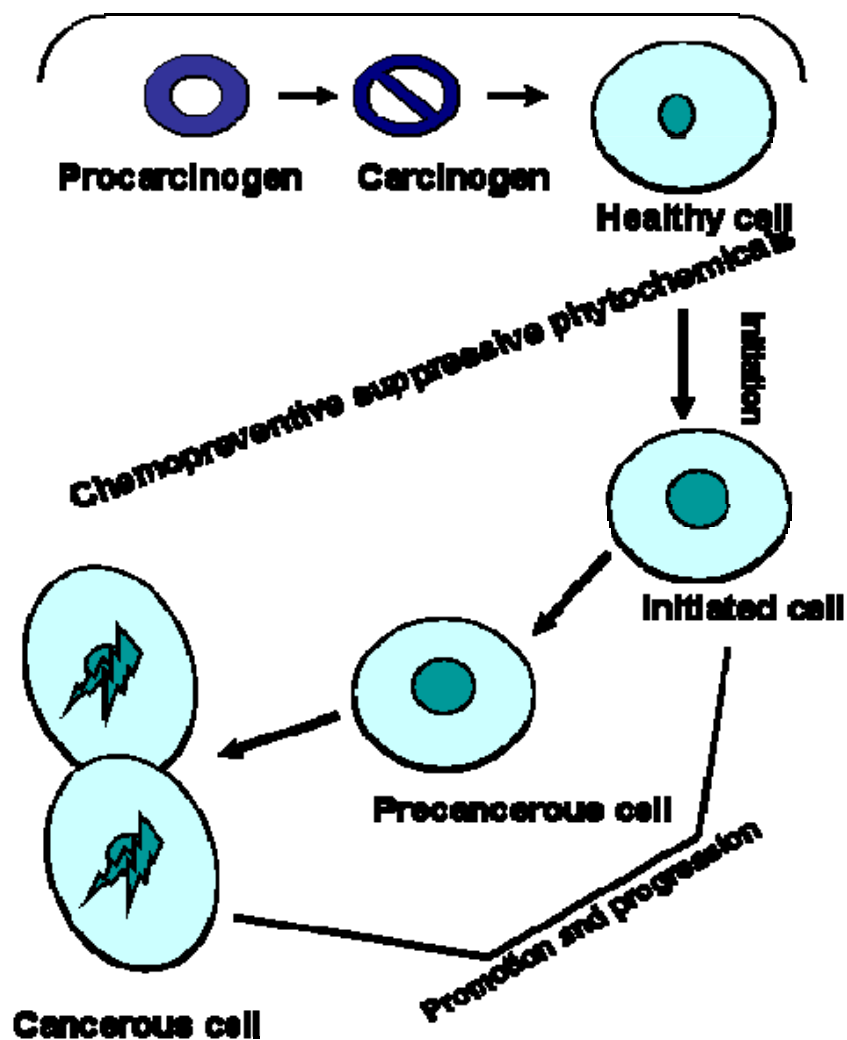


Figure 8. The multistep process of carcinogenesis showing steps of intervention by chemopreventive agents.

Cancer rates increase with advancing age and it is estimated that tumorigenesis begins at a young age (around 20 years) and tumors are not observed until 20–30 years after initiation (243) (94). Recent studies indicate

that in any given type of cancer 300–500 normal genes have been modified (dysregulated) to give the cancer phenotype. Although cancers are characterized by dysregulation of cell signaling pathways, most current anticancer therapies are directed against a single target and these are ineffective when compared to multitargeted therapies. Several phytochemical-derived drugs including curcumin, triterpenoids and betulinic acid modulate several pathways in cancer cells and are discussed in the following section.

Curcumin

Curcumin (diferuloylmethane), a polyphenol, is a yellow colored active principle of the perennial herb *Curcuma longa* (commonly known as turmeric) that belongs to the ginger (*Zingiberaceae*) family (235). Curcumin was first isolated in 1815, obtained in crystalline form in 1870, and ultimately identified as 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E) or diferuloylmethane. In 1910, the feruloylmethane skeleton of curcumin was confirmed and synthesized by Lampe (244). Curcumin is insoluble in water and ether but soluble in ethanol, dimethylsulfoxide, and acetone. Curcumin has a melting point of 183 °C, a molecular formula of $C_{21}H_{20}O_6$, and a molecular weight of 368.37 g/mol. Curcumin exists in enolic and β -diketonic forms. The fact that curcumin in solution exists primarily in its enolic form has an important influence on the radical-scavenging ability of curcumin.

Curcumin, the active component of turmeric and is now recognized as being responsible for most of the therapeutic effects. It is estimated that 2–5% of turmeric is curcumin. Turmeric contains three different analogues of curcumin (i.e., diferuloylmethane, also called curcumin, demethoxycurcumin, and bisdemethoxycurcumin) (Figure 9). Whether all three analogues exhibit equal activity is not clear.

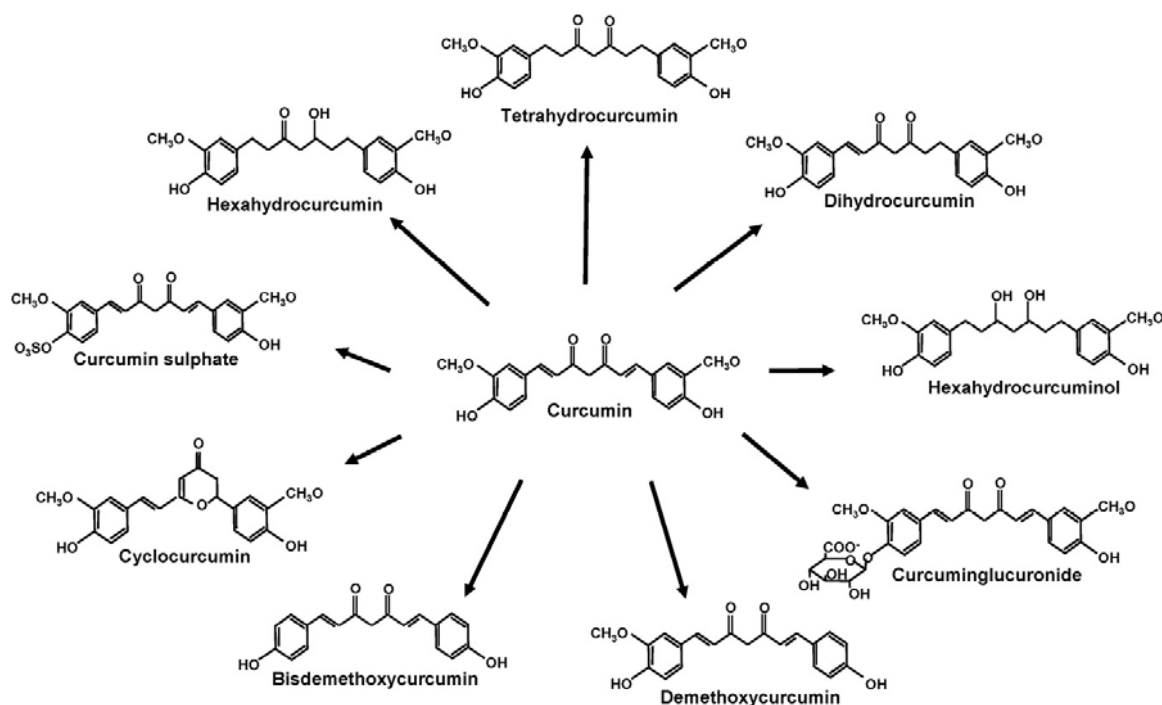


Figure 9. Natural analogs of curcumin and curcumin metabolites.

When administered orally, curcumin is metabolized into curcumin glucuronide and curcumin sulfate. In contrast, when administered systemically or intraperitoneally, it is metabolized into tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol (Figure 9) (245). Curcumin can act as a food preservative, a coloring agent and enhances the taste of various foods and in recent years the health promoting affects of curcumin have been recognized.

Curcumin was once considered a cure for jaundice, an appetite suppressant, and a digestive. This spice was also used for alleviating stomach and liver problems, to heal wound healing and as a cosmetic (246). Curcumin exhibits antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, and anticancer activities and has potential for treating several malignant diseases, diabetes, allergies, arthritis, Alzheimer's disease and other chronic illnesses. The effects of curcumin are mediated through regulation of various transcription

factors, growth factors, inflammatory cytokines, protein kinases, and other enzymes.

Turmeric's pharmacological safety is accepted, since it has been consumed as a dietary spice, at doses up to 100 mg/d, for centuries (247). Research over the last 20 years has shown that curcumin is a potent anti-inflammatory agent with strong therapeutic potential against a variety of cancers. Curcumin suppresses transformation, proliferation, and metastasis of tumors. It also inhibits proliferation of cancer cells by arresting cancer cell cycle progression and by inducing apoptosis. Curcumin inhibits carcinogen bioactivation via suppression of specific cytochrome P450 isozymes, and induces expression of phase II carcinogen detoxifying enzymes, and this may account for the cancer chemopreventive effects of this compound. Curcumin exhibits therapeutic effects against cancers of the blood, skin, oral cavity, lung, pancreas, and intestinal tract, and suppresses angiogenesis and metastasis in rodent tumor models (248). Curcumin inhibits proliferation of various tumor cells in culture, prevents carcinogen-induced cancers in rodents and inhibits growth of human tumors in xenograft or orthotopic animal models either alone or in combination with other chemotherapeutic agents or radiation. Several phase I and phase II clinical trials indicate that curcumin is quite safe and exhibits therapeutic efficacy. Phase I clinical trials indicate that doses as high as 12 g of curcumin per day for over 3 months is well tolerated in humans (242). Curcumin modulates expression of several critical genes in cancer and these are discussed in this section:

1. Transcription Factors. Curcumin regulates the expression of various transcription factors as discussed:

a. NF- κ B. NF- κ B is a family of five closely related proteins which are found in several dimeric combinations and bind to the κ B sites on DNA. Under resting conditions, NF- κ B dimers reside in the cytoplasm. On activation by free radicals, inflammatory stimuli, cytokines, carcinogens, tumor promoters, endotoxins,

gamma-radiation, ultraviolet (UV) light, or x-rays, NF- κ B is translocated to the nucleus, where it induces the expression of more than 200 genes that suppress apoptosis and induce cellular transformation, proliferation, invasion, metastasis, chemoresistance, radioresistance, and/or inflammation (235). Curcumin suppresses activation of NF- κ B induced by various tumor promoters, including phorbol ester, TNF, and hydrogen peroxide. Recently, it was shown that curcumin down-regulated cigarette smoke-induced NF- κ B activation through inhibition of IKK in human lung epithelial cells and curcumin also suppresses constitutively active NF- κ B in multiple myeloma, head and neck cancers, pancreatic cancers, and mantle cell lymphoma. NF- κ B plays important roles in inflammation, cell proliferation, apoptosis, and oncogenesis. Suppression of NF- κ B activation by curcumin is important for its anticancer activity (249).

b. STAT. signal transducers and activators of transcription (STAT) proteins are signaling molecules with dual functions that are activated by phosphorylation through janus kinase (JAK) or cytokine receptors, G-protein-coupled receptors, or growth factor receptors. Of the seven STAT proteins constitutively activated STAT3 and STAT5 have been implicated in multiple myeloma, lymphomas, leukemias, and several solid tumors, making these proteins logical targets for cancer therapy. STAT proteins contribute to cell survival and growth by preventing apoptosis through increased expression of antiapoptotic proteins, such as bcl-2 and bcl-X_L. Recently, STAT3 was shown to be a direct activator of the angiogenic VEGF gene. Bharti et al demonstrated that curcumin inhibited interleukin (IL) 6-induced STAT3 phosphorylation and subsequent STAT3 nuclear translocation (250). The constitutive phosphorylation of STAT3 found in multiple myelomas was decreased after treatment with curcumin (251) (Figure 10).

c. AP-1. AP-1 is a transcription factor that is frequently associated with activation of NF- κ B and has been closely linked with proliferation and transformation of tumor cells. Curcumin inhibits activation of AP-1 induced by

tumor promoters. Activation of AP-1 requires the phosphorylation of c-jun through activation of stress-activated kinase JNK, and curcumin suppresses carcinogen-induced JNK (25). Hydrogen peroxide stimulates proliferation and migration of human prostate cancer cells through activation of AP-1 and up-regulation of the heparin affinity regulatory peptide (HARP) gene. Curcumin abrogated both hydrogen peroxide-induced HARP expression and LNCaP cell proliferation and migration (252).

d. PPAR- γ . Peroxisome proliferator-activated receptors (PPARs) are a subfamily of the nuclear receptor superfamily of ligand-activated receptors; PPAR α , PPAR γ , and PPAR β/δ , are lipid sensors and play a key role in regulating tissue-specific lipid homeostasis and metabolism. PPARs also play an important role in many diseases, particularly those related to obesity, metabolic disorders, cancer, and atherogenesis. Endogenous ligands for PPARs include fatty acid-derived compounds and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 (PGJ2), which exhibits high affinity for PPAR γ ; however, PGJ2 might not be the endogenous ligand for this receptor because of the low cellular expression of this metabolite. Synthetic PPAR γ agonists, such as the thiazolidinediones rosiglitazone and pioglitazone, are insulin-sensitizing drugs that are widely used for clinical treatment of type II diabetes. PPAR γ is overexpressed in many tumor types and cancer cell lines (253), and PPAR γ agonists show promise for clinical treatment of various types of tumors (254). Ligands for this receptor typically inhibit G₀/G₁-to S-phase progression, and this is accompanied by downregulation of cyclin D1 expression and induction of the cyclin-dependent kinase inhibitors p27 or p21. Activation of PPAR- γ inhibits the proliferation of cancer cells and nonadipocytes (255). Inhibition of PPAR γ dependent transactivation by PPAR γ antagonists markedly decreases the growth-inhibitory effects of curcumin. Chen and coworkers recently reported that curcumin-dependent activation of PPAR γ inhibited Moser cell growth and mediated suppression of cyclin D1 and EGFR gene expression (256).

e. CAMP Response Element-Binding Protein. p300/CBP, along with other histone acetyltransferases (HATs), have been implicated in cancer cell growth and survival. HAT-dependent acetylation of specific lysine residues on the N-terminal tail of core histones results in uncoiling of DNA and increased accessibility for binding transcription factors. In contrast, histone deacetylation by histone deacetylase represses gene transcription by promoting DNA winding, thereby limiting access to transcription factors. Curcumin is a selective HAT inhibitor (257). The α and β unsaturated carbonyl groups in the curcumin side chain function as Michael reaction sites, which are required for its HAT-inhibitory activity. Balasubramanyam and coworkers found that curcumin is a specific inhibitor of the p300/CBP HAT activity but not of p300/CBP-associated factor, *in vitro* and *in vivo* (258).

f. β -Catenin. β -Catenin is a central component of the cadherin cell adhesion complex and plays an essential role in the Wingless/Wnt signaling pathway. In the nucleus, β -catenin interacts with members of the TCF/LEF family of transcription factors to stimulate expression of target genes. Curcumin treatment impairs both Wnt signaling and cell–cell adhesion pathways, resulting in cell cycle arrest at the G₂/M phase and induction of apoptosis in HCT-116 colon cancer cells. Mahmoud and coworkers, while investigating the efficacy of curcumin for prevention of tumors in C57BL/6J-*Min*⁺ (*Min*⁺) mice, found that curcumin decreased expression of the oncoprotein β -catenin in the enterocytes of the *Min*⁺ mouse, and this was important for the antitumor effect of curcumin (259).

g. Tumor Suppressor Gene p53. p53 is a tumor suppressor and a transcription factor. It is a critical regulator of many cellular processes, including cell signaling, the cellular response to DNA damage, genomic stability, cell cycle control, and apoptosis. P53 activates transcription of downstream genes such as p21^{WAF1} and Bax to induce the apoptosis and inhibit the growth of cells with damaged DNA (260). In neuroblastoma, curcumin up-regulated p53 expression and

induced nuclear translocation of p53, followed by induction of p21^{WAF-1/CIP-1} and Bax expression (261).

h. Sp. Several reports show that Sp1 protein is overexpressed in various cancer types including gastric, colorectal, pancreatic, epidermal, thyroid and breast cancers and recent studies in this laboratory clearly show overexpression of the Sp1, Sp3 and Sp4 proteins in cancer versus non-cancer cells (220) (262). Lou et al. 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 (218). In contrast, Sp1 knockdown gave cells that were non-tumorigenic in the same mouse xenograft model. This suggests that Sp transcription factors may contribute to tumor phenotype and studies in this laboratory have demonstrated that an underlying mechanism of action of anticancer agents such curcumin is the targeted repression of Sps in cancer cells (263).

2. Tumor necrosis factor (TNF). TNF mediates tumor initiation, promotion, and metastasis and the pro-inflammatory effects of TNF are due primarily to its ability to activate NF- κ B (264). TNF activates NF- κ B in most cells, leading to expression of inflammatory genes such as COX-2, LOX-2, cell adhesion molecules, inflammatory cytokines, chemokines, and inducible nitric oxide synthase. Curcumin suppresses expression of TNF at both the transcriptional and posttranscriptional levels (265).

3. Inflammatory Enzymes.

a. Cyclooxygenase-2 . Cyclooxygenases are forms of prostaglandin H synthase, which converts arachidonic acid released by membrane phospholipids into prostaglandins. COX-2 is regulated by mitogens, tumor promoters, cytokines, and growth factors. It is overexpressed in practically every premalignant and malignant condition involving the colon, liver, pancreas, breast, lung, bladder, skin, stomach, head and neck, and esophagus (266).

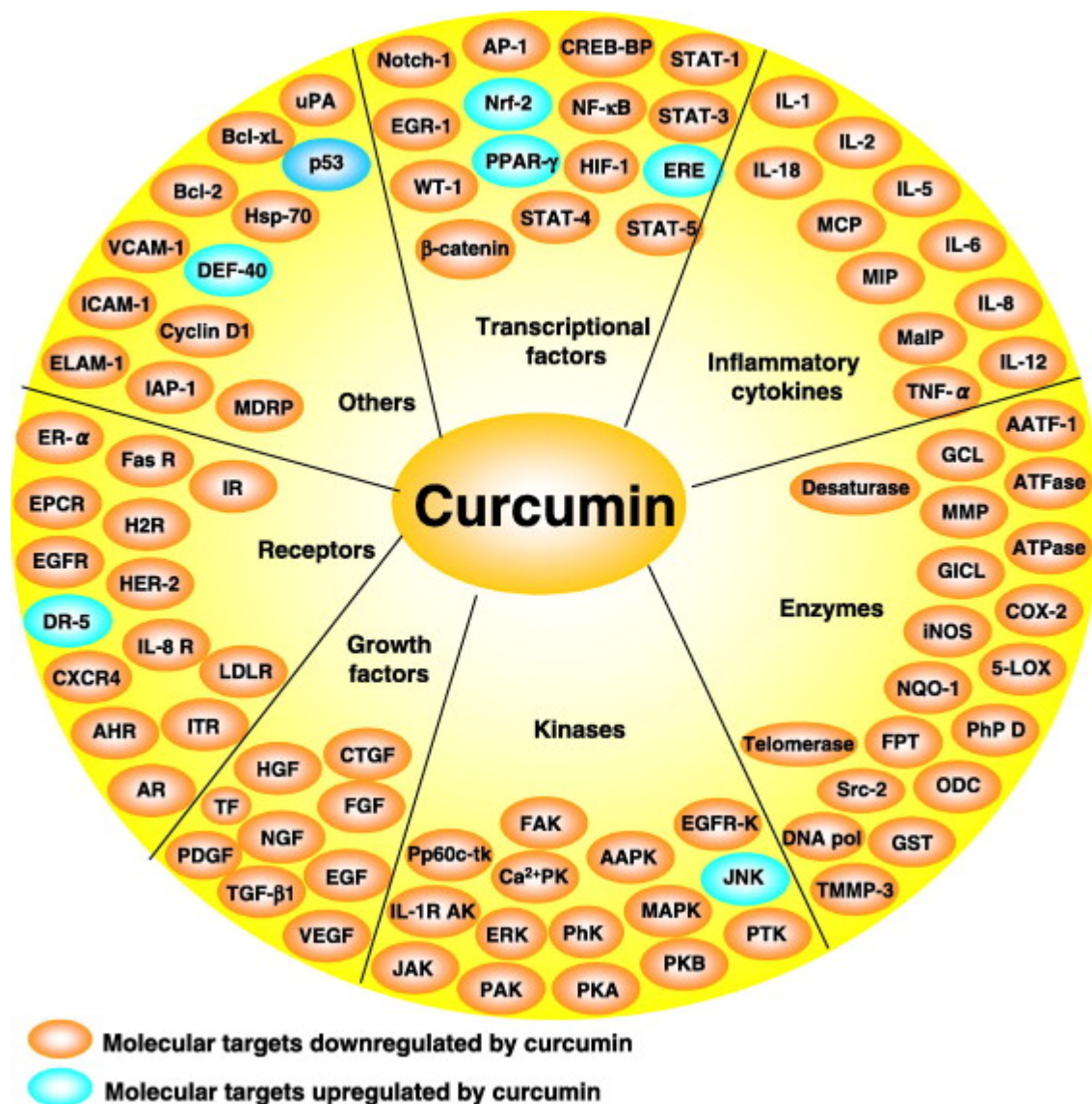


Figure 10. Molecular targets of curcumin (94).

Preclinical studies have shown that curcumin suppresses COX-2 activity through suppression of the NF-κB-inducing kinase and IKK enzymes. Plummer

et al showed that inhibition of COX-2 is a biomarker of drug efficacy and is used in clinical trials of many chemopreventive drugs that inhibit this enzyme. When 1 μ M curcumin was added *in vitro* to blood from healthy volunteers, LPS-induced COX-2 protein levels and concomitant prostaglandin E2 production was reduced by 24 and 41%, respectively (267).

b. Lipoygenase . Lipoygenases (LOX) are enzymes responsible for generating leukotrienes from arachidonic acid. There are three types of LOX isozymes, namely, 15-LOX which is required for the synthesis of anti-inflammatory 15-HETE; 12-LOX which is involved in provoking inflammatory/allergic disorders; and 5-LOX produces 5-HETE and leukotrienes which are potent chemo- attractants that promote development of asthma. Aberrant arachidonic acid metabolism is involved in the inflammatory and carcinogenic processes. Curcumin and its metabolite tetrahydrocurcumin effectively inhibited the release of arachidonic acid and its metabolites in LPS-stimulated RAW cells and A23187-stimulated HT-29 colon cancer cells. They were potent inhibitors of prostaglandin E2 formation in LPS-stimulated RAW cells. Curcumin affects arachidonic acid metabolism by blocking phosphorylation of cytosolic phospholipase A2, decreasing the expression of COX-2, and inhibiting the catalytic activities of 5-LOX. These activities may contribute to the antiinflammatory and anticarcinogenic actions of curcumin and its analogs (Figure 10) (268).

4. Cyclin D1. Cyclin D1 is a component subunit of the Cdk4 and Cdk6 complexes which are rate-limiting for progression of cells through the G₁ phase of the cell cycle. The loss of this regulation is a hallmark of cancer. (269). Cyclin D1 is overexpressed in many cancers, including those of the breast, esophagus, head and neck, and prostate, and mantle cell lymphoma (247). Curcumin down-regulates expression of cyclin D1 in bladder cancer cells (Figure 10) (263). Suppression of NF- κ B in mantle cell lymphoma by curcumin also decreased cyclin D1 levels and formation of the cyclin D1-Cdk4 holoenzyme complex

resulting in inhibition of cell proliferation and induction of apoptosis. In another study, curcumin induced G₀/G₁ and/or G₂/M phase cell cycle arrest, up-regulated cdk inhibitors such as p21^{Cip1/waf1} and p27^{Kip1}, and down-regulated cyclin B1 and cdc2 (270).

5. Protein Kinases.

i. EGFR/HER2/neu . HER2/neu (also known as ErbB-2, avian erythroblastosis oncogene B) is a member of the EGFR family and is notable for its role in the pathogenesis of breast cancer. HER2/neu is a cell membrane surface-bound tyrosine kinase and is involved in the signal transduction pathways leading to cell growth and differentiation. Almost 30% of breast cancers overexpress the HER2/neu protooncogene, and both HER2 and EGF receptors stimulate proliferation of breast cancer cells. Overexpression of these two proteins correlates with progression of human breast cancer and poor patient prognosis (271). Curcumin downregulates the activity of EGFR and HER2/neu and depletes cells of HER2/neu protein (272) (273). EGFR is expressed at high levels in colorectal, bladder, prostate and other cancers. Curcumin down-regulates EGFR signaling in prostate cancer cells by down-regulating levels of EGFR protein; curcumin also inhibits the intrinsic EGFR tyrosine kinase activity, by blocking ligand-induced activation of EGFR (274).

ii. Other Protein Kinases. Curcumin can mediate its effects through modulation of other protein kinases involved in carcinogenesis including protein kinase A (PKA), protein kinase C (PKC), protein kinase (cPK), phosphorylase kinase (PhK), autophosphorylation-activated protein kinase (AK), and pp60c-src tyrosine kinase. Approximately 0.1 mmol/L curcumin inhibited PhK, pp60c-src, PKC, PKA, AK, and cPK by 98, 40, 15, 10, 1, and 0.5%, respectively. The ubiquitously expressed nonreceptor tyrosine kinase c-Abl regulates stress responses induced by oxidative agents such as ionizing radiation and hydrogen peroxide and curcumin activates c-Abl, which in turn mediates the cell death response, in part through activation of JNK (275).

6. Adhesion molecules. Cell adhesion molecules are transmembrane proteins required for cell-cell interactions and for binding of other extracellular molecules. Expression of various cell surface adhesion molecules, such as intercellular cell adhesion molecule-1, vascular cell adhesion molecule-1, and endothelial leukocyte adhesion molecule-1, on endothelial cells is critical for tumor metastasis (276). Curcumin blocks the cell surface expression of adhesion molecules in endothelial cells (Figure 10), and this is accompanied by suppression of tumor cell adhesion to endothelial cells by modifying cell receptor binding. Curcumin inhibits binding of fibronectin, vitronectin, and collagen IV to the extracellular matrix (ECM) proteins. It also suppresses the expression of $\alpha 5\beta 1$ and $\alpha 5\beta 3$ integrin receptors, pp125 focal adhesion kinase (FAK) and collagenase activity. Curcumin also enhances expression of antimetastatic proteins, TIMP-2, nonmetastatic gene 23 (Nm23), and E-cadherin (259) (277).

In summary, curcumin exhibits antiproliferative effects by suppressing the cell cycle regulatory proteins by blocking entry to the cell cycle from G2 to M by inhibiting expression of cdc2/cyclin B (278), inhibiting growth of transplantable tumors in different animal models and increasing the life span of tumor-harboring animals. Curcumin decreases repression of antiapoptotic members of the Bcl-2 family and increases expression of p53, Bax, and procaspases-3, -8, and -9 (279). Inhibition of metastasis by curcumin is due to up regulation of anti-metastatic proteins TIMP-2, Nm23, and E-cadherin, which reduce the metastatic tendency of the melanoma cells (277). Curcumin inhibits angiogenesis by downregulation of VEGF and its receptors as well as MMPs involved in aggressive tumor phenotypes (280). Furthermore, curcumin enhances the cytotoxic effects of chemotherapeutic drugs, including doxorubicin, tamoxifen, cisplatin, camptothecin, daunorubicin, vincristine, and melphalan (281) (282) and radiation. Curcumin is a radio-sensitizer that overcomes the radio-induced prosurvival gene expression. Curcumin decreases the dose of chemotherapeutic agents; helps to enhance the effect at lower doses and thus minimize

chemotherapy-induced toxicity (283).

7. Bladder cancer. Many deaths due to bladder cancer involve advanced, unresectable, chemotherapy-resistant tumors. Numerous reports indicate that curcumin has activity against bladder cancer; for example, curcumin decreases proliferation of bladder cancer cells in culture through modulation of several genes/gene products and some synthetic curcumin analogs are also active against bladder cancer cell lines (284). Curcumin effectively inhibits tumor implantation and growth in a murine bladder tumor model and a phase I clinical trial in patients with resected bladder cancer indicates that up to 12 g per day of curcumin for 3 months is pharmacologically safe. Moreover the investigators noted an indication of histologic improvement of precancerous lesions in one out of two patients (242).

8. Pancreatic cancer. Research over the past decade has indicated that curcumin has an anticarcinogenic effect in various pancreatic cell lines (242) and in combination with gemcitabine, curcumin exhibited synergistic antiproliferative effects in pancreatic cancer cell lines (285). A polymeric nanocurcumin formulation demonstrated therapeutic efficacy comparable to that of free curcumin in a panel of human pancreatic cancer cell lines *in vitro* (286). Curcumin in combination with gemcitabine significantly down-regulated expression of the cell proliferation marker Ki-67 in tumor tissues compared with the control group and curcumin alone significantly suppressed expression of microvessel density marker CD31 and the presence of gemcitabine further enhanced the down-regulation of CD31 (286). In a clinical trial, researchers evaluated the effect of oral curcumin with piperine on markers of oxidative stress in patients with tropical pancreatitis (TP). Twenty patients with pancreatitis were randomized to receive 500 mg of curcumin with 5 mg of piperine, or placebo for 6 weeks, and the effects on the pattern of pain, and in red blood cell levels of malonyldialdehyde (MDA) and glutathione (GSH) in red blood cells were assessed. There was a significant reduction in the erythrocyte MDA levels

following curcumin therapy compared with the placebo there was a significant increase in GSH levels, but not a corresponding improvement in pain. In another clinical trial, 25 patients received 8 grams of curcumin (orally) every day until disease progression, with restaging every 2 months. Serum cytokine levels for interleukin IL-6, IL-8, IL-10, and IL-1 receptor antagonists and peripheral blood mononuclear cells (PBMC) expression of NF- κ B and COX-2 were monitored. In the 21 patients evaluated, circulating curcumin was detectable as glucuronide and sulfate conjugates suggesting poor oral bioavailability. Two of the patients demonstrated clinical biologic activity. One had ongoing stable disease for more than 18 months and one additional patient had a brief, but marked, tumor regression (73%), accompanied by significant increases (4- to 35-fold) in serum cytokine levels (IL-6, IL-8, IL-10, and IL-1 receptor antagonists). No toxicities were observed. Curcumin down-regulated expression of NF- κ B, COX-2 and phosphorylated STAT3 in PBMC in patients (287).

In spite of the above mentioned array of anti-cancer properties of curcumin, there are additional undefined targets and also disadvantages associated with the use of this compound (286). Orally administered curcumin has poor bioavailability and low tissue accumulation, yet it has been found to be effective. The low levels of curcumin in the serum and tissue may account for its safety; however, it is doubtful if a therapeutic dose can be attained. Several other agents such as piperine and ginger are known to improve the bioavailability of curcumin, but, they interfere with drug metabolism by suppressing glucuronidation in the liver, this may affect drug safety for combination therapies. Adjuvants, nanoparticles, liposomal preparations, micelles, phospholipid- conjugates and other potent analogs of curcumin may overcome the problems of low bioavailability, low tissue distribution and low half-life (286), however several significant concerns remain.

Triterpenoid acids

Triterpenoids are structurally diverse organic chemicals derived from six isoprene units that are assembled in plants by cyclization of squalene, the linear precursor of steroids. The basic triterpenoid backbone can be modified in numerous ways and more than 20000 different compounds are naturally occurring (288). Several triterpenoids, including ursolic and oleanolic acid, betulinic acid, celastrol, lupeol, and the triterpenoids of ginsenosides, possess modest antitumorigenic or anti-inflammatory properties. Triterpenoids are also studied for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory and tonic effects. They are used in the prevention and treatment of hepatitis, parasitic and protozoal infections and above all, for their cytostatic effects. In addition, several triterpenoid compounds structurally related to oleanolic and ursolic acids exhibit anti-inflammatory and anticarcinogenic activities and synthetic analogs with enhanced antitumor activity have been developed and these include 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), its methyl ester (CDDO-Me), and imidazolide (CDDO-Im), methyl (CDDO-MA), ethyl amides (CDDO-EA), and dinitrile (Di-CDDO) derivatives. Of these CDDO, CDDO-Me, betulinic acid, and the ginsenosides have shown promising anticancer activities and are presently being evaluated in phase 1 clinical trials for leukemia, lymphoma, and solid tumors (Figure 10) (289). The major disadvantage of using triterpenoids is the toxicity associated with their haemolytic and cytostatic properties (290). Triterpenoids such as CDDO inhibit multiple pathways and this may be mediated, in part, by reversible Michael addition of these compounds to biological nucleophilic groups such as accessible cysteine sulfides. Synthetic oleanane triterpenoids have profound effects on inflammation and the redox state of cells and tissues, and also inhibit growth and induced apoptosis in rodent cancer models. These drugs have unique molecular and cellular mechanisms of action and may act synergistically in combination cancer

chemotherapy regimens (288). CDDO, a synthetic oleanane triterpenoid, inhibits proliferation of various malignant p53 dependent and independent cells at nanomolar concentrations (291). CDDO, CDDO-Me, or CDDO-Im alter the expression of several key cell-cycle proteins, including cyclin D1, p21, p27, caveolin and Myc (292). CDDO and related compounds activate PPAR γ and induce several receptor-dependent genes including caveolin 1 (293) ; however in most cancer cell lines the anticancer activities of these compounds are PPAR γ independent. Triterpenoids suppress activation of NF κ B by directly binding to and inhibiting its kinase activator, IKK β (294). STAT transcription factors are constitutively activated in many cancers, and CDDO-Im suppresses both constitutive and interleukin 6 (IL6) inducible STAT3 and STAT5 phosphorylation in myeloma and lung cancer cells, resulting in the cell growth arrest and apoptosis (295).

In another study, using human umbilical vein endothelial (HUVE) cells, CDDO-Me and CDDO-Imm inhibited activation of the extracellular signal-regulated kinase ERK1/2 pathway after stimulation with vascular endothelial growth factor (VEGF). CDDO also induced apoptosis in leukemia cells through enhanced oxidative stress and loss of mitochondrial membrane potential (296). CDDO inhibited growth of ER-positive and -negative breast cancer cells and tumor growth in athymic nude mouse models, and this correlated with the modulation of genes associated with cell-cycle progression, apoptosis, and ER stress (291) In COLO 16 human skin cancer cells, CDDO induced apoptosis, and this was in part caused by ER stress and direct mitochondrial effects that disrupted calcium homeostasis. CDDO-Me induced both the intrinsic and extrinsic apoptosis pathways in lung cancer cell lines and a caspase-8–dependent apoptotic pathway was activated by CDDO in human osteosarcoma cells (297). These triterpenoid compounds are potent inducers of differentiation and apoptosis in leukemia cells; however, their proapoptotic effects were somewhat variable among different cell lines.

Methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-diene-30-oate (CDODA-Me)

CDODA-Me is a synthetic analog of the naturally occurring triterpenoid glycyrrhethinic acid; the major bioactive phytochemical in licorice, CDODA-Me contains a 2-cyano substituent in the A-ring (Figure on page 86) and is a potent inhibitor of LNCaP prostate cancer cell growth and activated peroxisome proliferator-activated receptor γ (PPAR γ). CDODA-Me induced cyclin dependent kinase inhibitors, p21 and p27, down-regulated cyclin D1 protein expression, and induced two other proapoptotic proteins, namely nonsteroidal anti-inflammatory drug-activated gene-1 (NAG-1) and activating transcription factor-3 (ATF-3) in pancreatic cancer cells. However, induction of these responses by CDODA-Me was PPAR γ -independent and due to activation of phosphatidylinositol-3-kinase, mitogen-activated protein kinase, and jun N-terminal kinase pathways. CDODA-Me also decreased androgen receptor (AR) and prostate-specific antigen (PSA) mRNA and protein levels through kinase-independent pathways. Potent inhibition of LNCaP cell survival by CDODA-Me is due to PPAR γ -independent activation of multiple pathways that selectively activate growth-inhibitory and proapoptotic responses. A recent study demonstrated that CDODA-Me induced PPAR γ -dependent transactivation in Panc28 and Panc1 pancreatic cancer cells and 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. CDODA-Me also activated multiple kinases in pancreatic cancer cells including p38 and p42 MAPK, PI3-K, and JNK pathways, and the role of these kinases in the induction of NAG-1 and apoptosis was cell context-dependent (298). Recent study indicates that in colon cancer cell lines, the anti-cancer activity of β -CDODA-Me is mediated by the oncogenic microRNA 27-a (miR27-a). MicroRNAs (miRNAs) are 20-25 basepair oligonucleotides that interact with complementary binding sites in 3'-untranslated regions of target mRNAs to inhibit their expression by blocking translation or by decreasing mRNA stability. Reports show that miR-

miR-27a targets ZBTB10 mRNA, a putative zinc finger protein that suppresses specificity protein (Sp) transcription factors and Sp-dependent gene expression. It has been reported that Sp transcription factors Sp1, Sp3 and Sp4 are highly expressed in cancer cell lines (299) (263) (262), and results of RNA interference studies show that Sp proteins regulate expression of angiogenic genes such as vascular endothelial growth factor (VEGF), VEGF receptor 1 (VEGFR1, Flt-1), VEGFR2 (KDR) and the antiapoptotic gene survivin (262) (263). CDODA-Me acts through downregulation of miR-27a and this is accompanied by enhanced expression of ZBTB10 and Myt-1 which arrests colon cancer cells at G₂/M phase. The cell culture studies were complemented by inhibition of tumor growth and decreased miR-27a expression in tumors from athymic nude mice bearing RKO cells as xenografts and treated with CDODA-Me (15 mg/kg/d) (300).

Betulinic acid

Lup-20(29)-ene-3 β ,28-diol (betulin) is a lupane-derived triterpenol that is present in high concentrations in birch bark and betulinic acid (BA), an oxidation product of betulin, has also been detected in bark extracts (Figure 11) (301). Betulin has been used in folk medicine for treating skin diseases; however, betulinic acid (BA), induces a broad range of pharmacological activities. BA and several derivatives exhibit anticancer activity, inhibit HIV and other viruses through multiple pathways, are effective antibacterial and antimalarial drugs, and exhibit anti-inflammatory activity. Betulinic acid was initially identified as a melanoma-specific cytotoxic agent that exhibits low toxicity in animal models. Subsequent studies show that betulinic acid induces apoptosis and antiangiogenic responses in tumors derived from multiple tissues (Figure 11) (302). The antitumorigenic activities of BA have been extensively investigated and this compound inhibits tumor growth through multiple pathways and these responses are also cancer cell/tumor-dependent. BA is shown to induce apoptosis through decreased mitochondrial membrane potential, activation of mitogen-activated protein kinase, and modulation of nuclear factor κ B (NF κ B)

(303, 304). Using LNCaP prostate cancer cells as a model, betulinic acid decreased expression of vascular endothelial growth (VEGF) and the antiapoptotic protein survivin. The mechanism of betulinic acid–induced antiangiogenic and proapoptotic responses in prostate cancer cells and xenograft tumors was due to activation of proteasome-dependent degradation of Sp1, Sp3 and Sp4 transcription factors, which regulate VEGF and survivin expression. Betulinic acid, thus, acts as a novel anticancer agent through targeted degradation of Sp proteins that are highly overexpressed in tumors (305).

Rzeski and coworkers demonstrated the involvement of bcl-2 and cyclin D1 in the induction of apoptosis and growth inhibition mediated by BA in HT-29 colon cancer cells; overexpression of Bcl-2 correlates with chemoresistant phenotype and bcl-2 is a negative prognostic marker for cancer survival (306). BA decreased Bcl-2 genes expression and significantly changed the bax/Bcl-2 ratios in the treated cells. The combined antitumor activity of BA with Vincristine (VCR) was evaluated in murine melanoma B16F10 cells *in vitro* and *in vivo* and the results showed synergistic cytotoxic effects. The drug combination induced cell cycle arrest at different points (BA at G1 phase and VCR at G2/M phase) and caused apoptosis in B16F10 melanoma cells. In the *in vivo* study, VCR inhibited metastasis of tumor cells to the lung. The addition of BA to VCR also augmented suppression of the experimental lung metastasis of melanoma tumor cells in C57BL/6 mice suggesting that BA enhanced the chemotherapeutic effect of VCR on malignant melanoma (307).

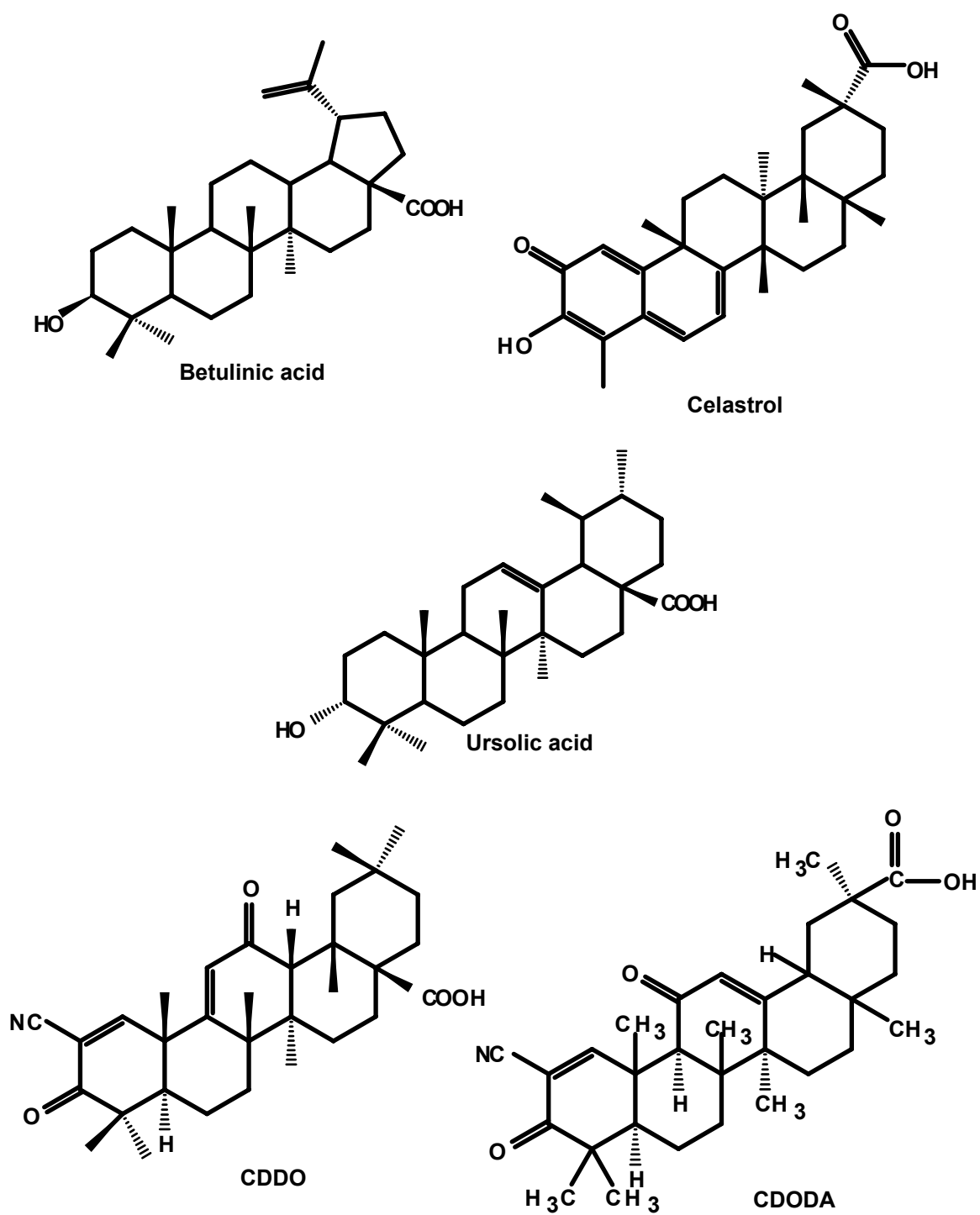


Figure 11. Structures of synthetic and natural triterpenoids (288).

Structural modifications of BA and other lupane-derived triterpenoids differentially affect their pharmacologic activities. Modification of the C-20 exocyclic position of BA did not affect the cytotoxicity of these derivatives to a panel of prostate and colon cancer and melanoma cell lines (308). In contrast, A-ring modifications of betulinic acid containing a 1-ene-3-oxo moiety substituted at C-2 with electron withdrawing groups were highly cytotoxic (309). These results were similar to ursane and oleanane triterpenoid acids where analogs containing electron-withdrawing substituents at C-2 within a 1-ene-3-one functionality were also highly cytotoxic to cancer cells compared to the parent acids (310). Chintharlapalli et al reported that introduction of a 2-cyano group into the lupane skeleton of BA gave a new class of PPAR γ agonists (305).

Celastrol

Celastrol, a quinone methide triterpenoid isolated from *Tripterygium wilfordii* Hook. f. (Celastraceae), also called Thunder God's Vine, is a woody vine distributed widely in the Orient. Its debarked root has long been used in traditional Chinese medicine to treat immune-inflammatory diseases such as rheumatoid arthritis, chronic nephritis, chronic hepatitis and lupus erythematosus. An ethyl acetate (EA) partition fraction from an ethanol extract of *T. wilfordii* has been evaluated in a phase II double blind clinical trial. Patients with rheumatoid arthritis that received the EA partition showed improvement in both clinical manifestations and laboratory findings. Over 200 chemical constituents are found in this plant (311) and this may account for its multiple uses in China for treating immune-inflammatory diseases, cancer and neurodegenerative diseases. Ro and coworkers reported that oral administration of celastrol suppressed ovalbumin-induced airway inflammation, hyperresponsiveness, and tissue remodeling by regulating the imbalance of MMP-2/-9 and TIMP-1/-2 induced by inflammatory cytokines via NF- κ B-dependent manner. Hence celastrol is a useful therapeutic agent for treating allergy-induced asthma (312). In low nanomolar concentrations celastrol

suppressed production of pro-inflammatory cytokines TNF- α and IL-1 β by human monocytes and macrophages. Celastrol suppressed adjuvant arthritis in the rat, demonstrating an *in vivo* anti-inflammatory activity. Low doses of celastrol administered to rats significantly improved their performance in memory, learning and psychomotor activity tests. The potent antioxidant and anti-inflammatory activities of celastrol, and its effects on cognitive functions, suggested a use for this compound in treating neurodegenerative diseases such as Alzheimer's disease (AD) (313).

Celastrol exhibited potent anticancer activity against pancreatic cancer cells (Panc-1) *in vitro* and *in vivo* in xenografts and also inhibited the tumor metastasis in the RIP1-Tag2 transgenic mice model of pancreatic islet carcinomas. Yang and coworkers demonstrated that celastrol preferentially inhibits the chymotrypsin-like activity of a purified 20S proteasome and human prostate cancer cellular 26S proteasome. Inhibition of the proteasome activity by celastrol in PC-3 (androgen insensitive) or LNCaP (androgen sensitive) cells resulted in the accumulation of ubiquitinated proteins of three natural proteasome substrates (I κ B- α , Bax, and p27), and this was accompanied by suppression of AR protein expression (in LNCaP cells) and induction of apoptosis. Treatment of PC-3 tumor-bearing nude mice with celastrol resulted in significant inhibition of the tumor growth (314). Celastrol activates heat shock gene transcription synergistically with other stressors and exhibits cytoprotection against subsequent exposures to other forms of lethal cell stress. The structure of celastrol is remarkably specific (Figure 11) and activates heat shock transcription factor 1 (HSF1) with kinetics similar to those of heat stress, as determined by the induction of HSF1 DNA binding and expression of chaperone genes. These results suggest that celastrol exhibits promise as a new class of pharmacologically active regulators of the heat shock response as well as an anti-cancer agent (315).

II. CURCUMIN DECREASES SPECIFICITY PROTEIN (Sp) EXPRESSION IN BLADDER CANCER CELLS *

Curcumin is the active component of tumeric, and this polyphenolic compound has been extensively investigated as an anticancer drug that modulates multiple pathways and genes. In this study, 10 - 25 μ M curcumin inhibited 253JB-V and KU7 bladder cancer cell growth, and this was accompanied by induction of apoptosis and decreased expression of the proapoptotic protein survivin and the angiogenic proteins vascular endothelial growth factor (VEGF) and VEGF receptor 1 (VEGFR1). Since expression of survivin, VEGF and VEGFR1 are dependent on specificity protein (Sp) transcription factors; we also investigated the effects of curcumin on Sp protein expression as an underlying mechanism for the apoptotic and antiangiogenic activity of this compound. The results show that curcumin induced proteasome-dependent downregulation of Sp1, Sp3 and Sp4 in 253JB-V and KU7 cells. Moreover, using RNA interference with small inhibitory RNAs for Sp1, Sp3 and Sp4, we observed that curcumin-dependent inhibition of nuclear factor κ B (NF κ B)-dependent genes such as bcl-2, survivin and cyclin D1, was also due, in part, to loss of Sp proteins. Curcumin also decreased bladder tumor growth in athymic nude mice bearing KU7 cells as xenografts and this was accompanied by decreased Sp1, Sp3 and Sp4 protein levels in tumors. These results demonstrate for the first time that one of the underlying mechanisms of action of curcumin as a cancer chemotherapeutic agent is due, in part, to decreased expression of Sp transcription factors in bladder cancer cells.

*Reprinted with permission from "Curcumin decreases Specificity protein expression in bladder cancer cells" by Chadalapaka G, Jutooru I, Chintharlapalli S, Papineni S, Smith R, Li X and Safe S. *Cancer Res.* 68:5345-5354, 2008. Copyright 2009 by American Association for Cancer Research, Inc.

Introduction

Phytochemicals, microbial metabolites, and other natural products and their synthetic analogs have been extensively used for drug development and treatment of various diseases including cancer (316, 317). Curcumin (diferuloylmethane) is a polyphenolic natural product and the active component of tumeric (*Curcuma* species) which is used in cooking and in traditional medicines (318) (283) (319). Curcumin has been extensively investigated as an anticancer drug in various cancer cells and laboratory animal models. Curcumin has also been evaluated in humans, and one of the major problems associated with clinical applications of curcumin is the low bioavailability (320) (321) (322) (323). The effects of curcumin in various tumor models are highly variable and dependent on both tumor type and cell context. In many studies, curcumin inhibits cancer cell proliferation, induces apoptosis, and inhibits angiogenesis (324) (325) (326) (327) (328) (329) (330) (331) (332). Mechanisms associated with these effects are variable and may involve direct effects on mitochondria, activation of endoplasmic reticulum (ER) stress, and modulation of kinase pathways including the inhibition of nuclear factor κ B (NF κ B).

Recent studies reported that curcumin decreased survival of RT4V6 and KU7 bladder cancer cells, and this was accompanied by increased DNA fragmentation and other parameters associated with apoptosis (333). In addition, there was also evidence that curcumin potentiated the effects of other drugs and cytokines in bladder cancer cells, and this has been observed in other studies (329) (333) (332) (334). Curcumin alone had minimal effects on NF κ B in RT4V6 or KU7 cells; however, in cells treated with agents such as gemcitabine, tumor necrosis factor (TNF), and cigarette smoke that induce NF κ B, cotreatment with curcumin inhibited NF κ B activation. It was concluded that suppression of induced NF κ B by curcumin may play a role in sensitizing bladder cancer cells and other cancer cell lines to various chemotherapeutic agents (333).

Betulinic acid (BA) is a triterpenoid natural product that inhibits growth of multiple cancer cell lines, and this compound induces apoptosis and inhibits angiogenesis (301). Studies in this laboratory showed that one of the underlying mechanisms of action of this compound in prostate cancer cells was the targeted degradation of specificity protein 1 (Sp1), Sp3 and Sp4 and Sp-dependent genes involved in cell growth, survival and angiogenesis. We also observed similar effects for the analgesic tolfenamic acid (299) (21), and RNA interference studies with small inhibitory RNAs for Sp1, Sp3 and Sp4 confirmed the role of these proteins in regulating expression of vascular endothelial growth factor (VEGF), VEGF receptor 1 (VEGFR1), VEGFR2, and survivin and repression of p27 by Sp3 (335) (336) (337) (204).

Curcumin induced apoptosis and inhibited KU7 and 253JB-V bladder cancer cell proliferation, and we hypothesized that curcumin may also affect Sp protein expression in these cells. Curcumin decreased Sp1, Sp3 and Sp4 protein expression in the bladder cancer cell lines and similar results were observed in tumors in a xenograft study. These results demonstrate that the curcumin-induced growth inhibitory, proapoptotic and angiogenic responses in bladder cancer cells and tumors are due, in part, to decreased expression of Sp1, Sp3 and Sp4. Moreover, curcumin-induced downregulation of Sp proteins also plays a role in the effects of curcumin as an inhibitor of NF κ B-dependent genes.

Materials and methods

Cell lines, antibodies, chemicals and other materials. KU-7 human bladder cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and 253JB-V cells were provided by Dr. A. Kamat (M.D. Anderson Cancer Center, Houston, TX) and maintained essentially as described (17). With the exception of cleaved PARP (Cell Signaling Technology, Danvers, MA), NF κ B-p65 (Abcam Inc., Cambridge, MA), and β -actin antibodies (Sigma-

Aldrich), all remaining antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Curcumin (98% pure) was purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). Lipofectamine and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). β -Galactosidase reagent was obtained from Tropix (Bedford, MA).

Cell proliferation assays. Bladder cancer cells (3×10^4 cells per well) were plated using DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS in 12-well plates and left to attach for 24 hr. Cells were then treated with either vehicle (DMSO) or the indicated concentrations of curcumin. Fresh medium and test compounds were added every 48 hr, and cells were then 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 determination.

TUNEL assay. 253JB-V and KU7 cells (7×10^4) were seeded in four-chambered glass slides and left overnight to attach. After curcumin treatment for 12 hr, the in situ cell death detection POD kit was used for the 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 8 fields, each containing 50 cells. The total number of apoptotic cells was plotted as a percentage in both the cell lines.

Transfection and luciferase assays. Bladder cancer cells (1×10^5 per well) were plated in 12-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 16 hr, various amounts of DNA (i.e., 0.4 μ g pGL3/pGL2-Luc, 0.04 μ g β -galactosidase, and 0.4 μ g pSp1For4-Luc, pSp3For5-Luc, pVEGF-Luc, pNF κ B-Luc and pSurvivin-Luc) were transfected using Lipofectamine reagent according to the manufacturer's protocol and luciferase activity (normalized to β -galactosidase) was determined essentially as described (24-29).

Western blot assays. Bladder cancer cells were seeded in DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS. Twenty-four hr later, cells were treated with either vehicle (DMSO) or the indicated compounds for 24 hr and Western blot analysis was determined as described (26-29). Nuclear and cytoplasmic extracts were separated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). The tumor tissues were also processed similarly and probed for Sp1, Sp3 and Sp4 proteins and β -actin served as loading control. Protein quantification used Image J software, and optical densities for each protein was normalized to β -actin in the iLamin-treated group.

Fluorescence-activated cell-sorting assays (FACS). Both 253JB-V and KU7 bladder cancer cells were treated with either the vehicle (DMSO) or the indicated compounds for 24 hr. Cells were analysed on a FACS Calibur flow cytometer using CellQuest acquisition software (Becton Dickinson Immunocytometry Systems). 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.

SiRNA interference assays. Small inhibitory RNAs for Sp1, Sp3 and Sp4 were prepared by Dharmacon RNA Technologies (Chicago, IL). The iRNA complexes used in this study are indicated as follows:

LMN 5'-CUG GAC UUC CAG AAG AAC ATT

Sp1 SMARTpool L-026959-00-0005, Human Sp1, NM_138473ss

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

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

p65 (REL1096) 5'-GATTGAGGAGAAACGTAAATT

p50 (REL 1911) 5'- GTCACTCTAACGTATGCAATT

The two bladder cancer cell lines, 253JB-V and KU7, were seeded (1×10^5 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 one day. 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. SiRNA for p65 and p50 and NC were purchased from Integrated DNA Technologies (Coralville, IA).

EMSA 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 $16,000 \times g$, 4°C , the pellet was resuspended in reporter lysis buffer supplemented with 0.5 M KCl and incubated on ice for 30 min. The supernatant containing nuclear proteins was collected after centrifugation for 10 min at $16,000 \times g$, 4°C and quantified for protein concentrations by Bradford method. The GC-rich probe was prepared by annealing the two complementary polynucleotides: 5'- CTC GTC GGC CCC CGC CCC TCT -3' and 5'- AGA GGG GCG GGG GCC GAC GAG -3'. The NF κ B 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 (PerkinElmer). The labeled probe was purified with the Chroma Spin TE-10 column (BD Biosciences). The EMSA reaction was carried out in the reporter lysis buffer (Promega) supplemented with 0.1 M KCl. Each reaction contained 2 μg nuclear protein, 500-1000 ng 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 using a Storm A60 PhosphorImager system (Molecular Dynamics).

Xenograft study. Female athymic nude mice, age 4-6 weeks were purchased from Harlan (Indianapolis, CA). KU7 cells (1×10^6 cells) in 1:1 ratio of Matrigel (BD Biosciences, San Jose, CA) 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 ten animals each. The first group received 50 μ L vehicle (corn oil) by intraperitoneal injection, and the second group of animals received 50 mg/kg/d injection of curcumin in corn oil every second day for 18 days (9 doses) by intraperitoneal injection. The mice were weighed, and tumor areas were measured throughout the study. After 20 days, the animals were sacrificed; final body and tumor weights were determined and plotted.

Results

Figures 12A and 12B illustrate the concentration-dependent effects of 5, 10 and 25 μ M curcumin on proliferation of 253JB-V and KU7 bladder cancer cells over a 6 day period with change of media and treatment with DMSO (control) or curcumin every 48 hr. Proliferation of both 253JB-V and KU7 cells was inhibited by curcumin. However, the pattern of inhibition was slightly different; 5 and 10 μ M curcumin significantly inhibited 253JB-V cells after treatment for 4 and 6 days, whereas 10 μ M (but not 5 μ M) curcumin significantly inhibited KU7 cell proliferation after treatment for 2, 4 and 6 days.

The highest concentration of curcumin (25 μ M) was cytotoxic to both cell lines, and similar results were previously reported on the cytotoxicity of curcumin in KU7 and RT4V6 bladder cancer cells (338). The effects of curcumin on the distribution of 253JB-V and KU7 cells in G_0/G_1 , S and M phases of the cell cycle are illustrated in Figures 12C and 12D, respectively. Curcumin (5 - 25 μ M) increased the percentage of 253JB-V cells in G_0/G_1 and decreased the percentage in S and G_2/M phases, although the effects on G_2/M were concentration-dependent and variable.

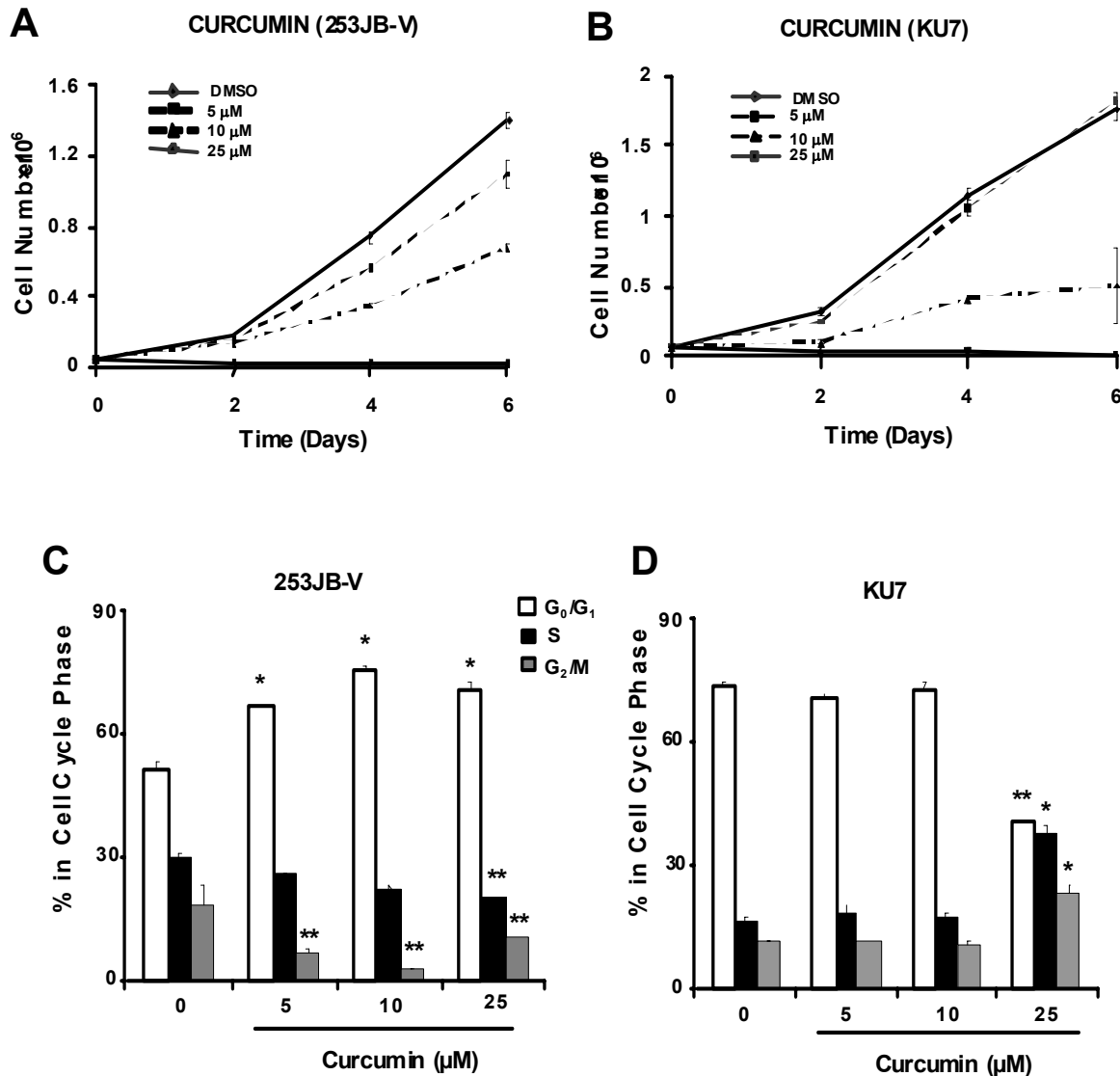


Figure 12. Curcumin inhibits bladder cancer cell growth and modulates the cell cycle.

Inhibition of 253JB-V (A) and KU7 (B) cell growth. Cells were treated with DMSO (solvent control), 5, 10 or 25 μ M curcumin and the effects of cell growth were determined after treatment for 2, 4 or 6 days as described in the Materials and Methods. Effects of curcumin on distribution of 253JB-V (C) and KU7 (D) cells in G₀/G₁, S and G₂/M phases. Cells were treated with DMSO (0), 5, 10 or

25 μ M curcumin for 24 hr, and the distribution of cells in G₀/G₁, S and G₂/M phases was determined by FACS analysis as described in the Materials and Methods. Results are expressed as means \pm SE for each data point in (A) - (D), and significantly ($p < 0.05$) increased (*) or decreased (**) responses compared to DMSO (0, control) are indicated.

In contrast, in KU7 cells, minimal effects were observed after treatment with 5 or 10 μ M curcumin, whereas 25 μ M curcumin decreased the percentage of cells in G₀/G₁ and increased the percentage in S and G₂/M phases (Figure 12D). These results demonstrate that curcumin-induced changes in the distribution of 253JB-V and KU7 cells in different phases of the cycle were dependent on cell context.

The effects of curcumin on selected proteins involved in cell cycle progression are summarized in Figure 13A. The cyclin-dependent kinase inhibitors p21 and p27 were decreased in both cell lines by curcumin, but at different concentrations. p21 was decreased by 25 - 50 and 10 - 50 μ M curcumin in 253JB-V and KU7 cells, respectively, whereas 25 - 50 μ M curcumin decreased p27 expression in both cell lines. The role of these responses in mediating the distribution of bladder cancer cells in different phases of the cell cycle is unclear.

Previous studies showed that curcumin induced apoptosis in bladder cancer cells (333) and, in pancreatic tumors; curcumin suppressed proliferation and inhibited angiogenesis (326). Results in Figure 13B demonstrate that curcumin induced PARP cleavage in 253JB-V and KU7 cells, and this was also accompanied by downregulation of the antiapoptotic gene survivin. Decreased survivin and increased PARP cleavage were initially observed at curcumin concentrations of 10 and 25 μ M in 253JB-V and KU7 cells, respectively, and maximal responses were observed in cells treated with 25 and 50 μ M curcumin. In addition, curcumin also decreased expression of two angiogenic proteins, VEGF and VEGFR1, in both cell lines (Figure 13B).

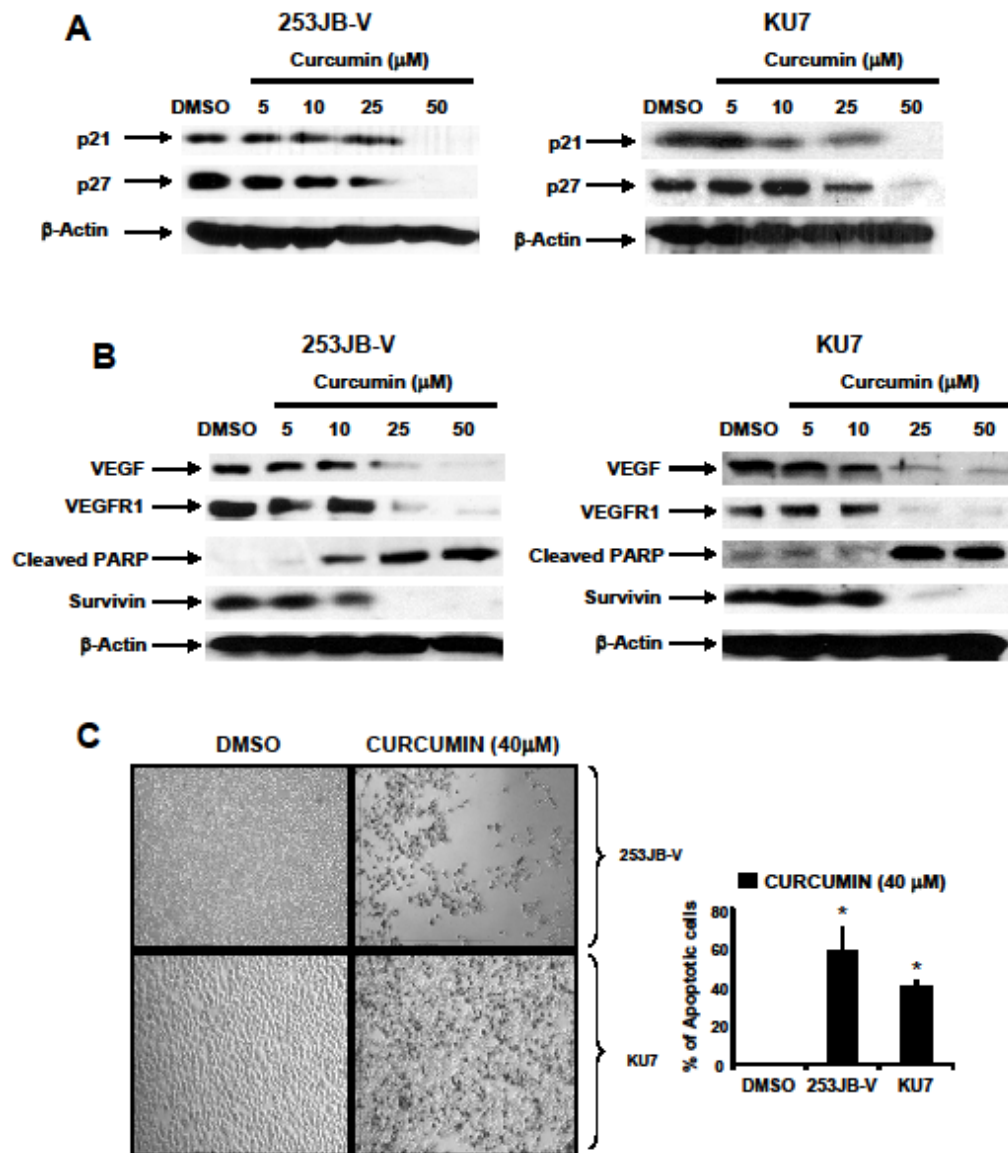


Figure 13. Curcumin modulates expression of cell cycle, survival and angiogenic proteins and induces apoptosis.

Decreased expression of p27 and p21 proteins. 253JB-V and KU7 cells were treated with DMSO, 5, 10, 25, or 50 μM curcumin for 24 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. (B) Decreased expression of survivin, VEGF and VEGFR1, and

increased PARP cleavage. 253JB-V and KU7 cells were treated with DMSO, 5, 10, 25 or 50 μ M curcumin for 24 hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. β -Actin served as a loading control. (C) TUNEL assay.

253JB-V and KU7 cells were treated with DMSO, 40 μ M curcumin for 12 hr, and TUNEL staining was determined as described in the Materials and Methods. Results of the Western blot (A and B) and TUNEL staining experiments were similar in replicate experiments in both cell lines and, for TUNEL staining, significant ($p < 0.05$) increases are indicated by an asterisk.

The proapoptotic activity of curcumin was also confirmed in a TUNEL assay (Figure 13C) where 40 μ M curcumin induced increased TUNEL staining in 253JB-V and KU7 cells, whereas minimal effects were observed in the solvent (DMSO)-treated cells.

Results in Figures 14A and 14B show that after treatment of 253JB-V and KU7 cells with curcumin (5 - 50 μ M) for 24 hr, there was a concentration-dependent decrease in the expression of Sp1, Sp3 and Sp4 proteins. Curcumin-induced downregulation of Sp proteins was dependent on the concentration of curcumin, Sp protein (i.e. Sp1, Sp3 or Sp4), and cell context. However, in both 253JB-V and KU7 cells, decreased expression of all three proteins was observed after treatment with 10 μ M and higher concentrations of curcumin for 24 hr.

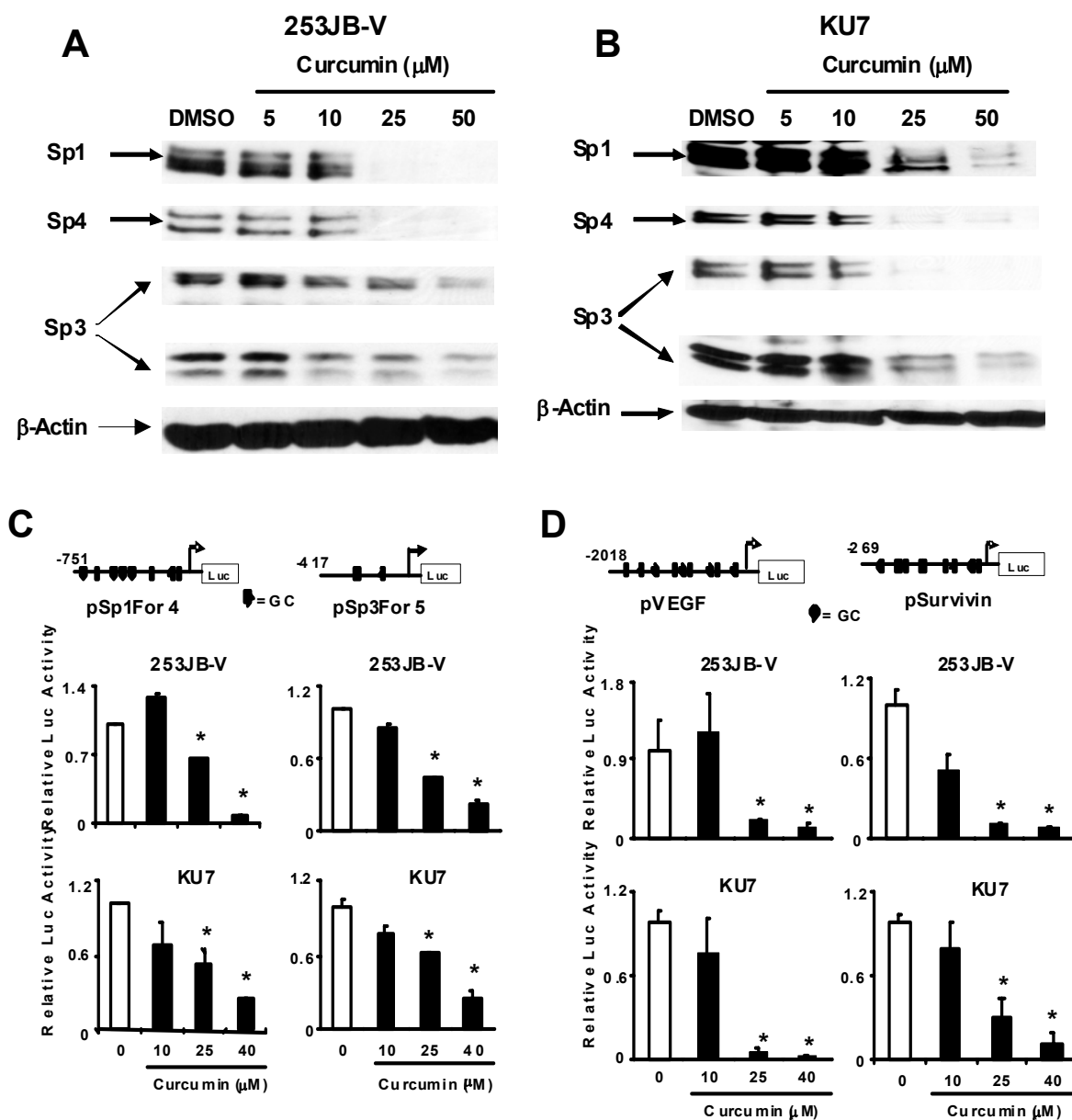


Figure 14. Effects of curcumin on Sp proteins and Sp-dependent transactivation.

Curcumin decreases Sp proteins in 253JB-V (A) and KU7 (B) bladder cancer cells. Cells were treated with DMSO, 5, 10, 25 or 50 μ M curcumin for 24 hr and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods. β -Actin served as a loading control and similar results were observed in duplicate experiments. Curcumin decreases luciferase activity in cells transfected with Sp1/Sp3 promoter (C) and Sp-regulated promoter (D) constructs. Bladder cancer cells were transfected with the indicated constructs, treated with DMSO or different concentrations of curcumin, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means \pm SE for at least three separate determinations for each treatment group and significantly ($p < 0.05$) decreased activity is indicated by an asterisk.

Since curcumin decreased expression of Sp1, Sp3, Sp4 and Sp-dependent survivin, VEGF and VEGFR1 proteins, we also investigated the effects of curcumin on luciferase activity in 253JB-V and KU7 cells transfected with constructs containing GC-rich promoters that bind Sp proteins. Luciferase activity was decreased in 253JB-V and KU7 cells treated with 10 - 40 μ M curcumin and transfected with GC-rich Sp1For4 and Sp3For5 constructs containing the -751 to -20 and -417 to -38 regions of the Sp1 and Sp3 gene promoters, respectively (339) (340) (linked to the luciferase gene) (Figure 14C). In addition, curcumin (10 - 40 μ M) also decreased luciferase activity in 253JB-V and KU7 cells transfected with GC-rich pVEGF and pSurvivin constructs that contain the -2018 to +50 and -269 to +49 GC-rich regions of the VEGF and survivin gene promoters, respectively (Figure 14D). Thus, like tolfenamic acid and betulinic acid, curcumin-induced downregulation of Sp1, Sp3 and Sp4 not only decreased expression of Sp-regulated proteins such as VEGF and survivin, but also decreased transactivation in 253JB-V and KU7 cells transfected with pVEGF and pSurvivin constructs containing GC-rich promoter inserts.

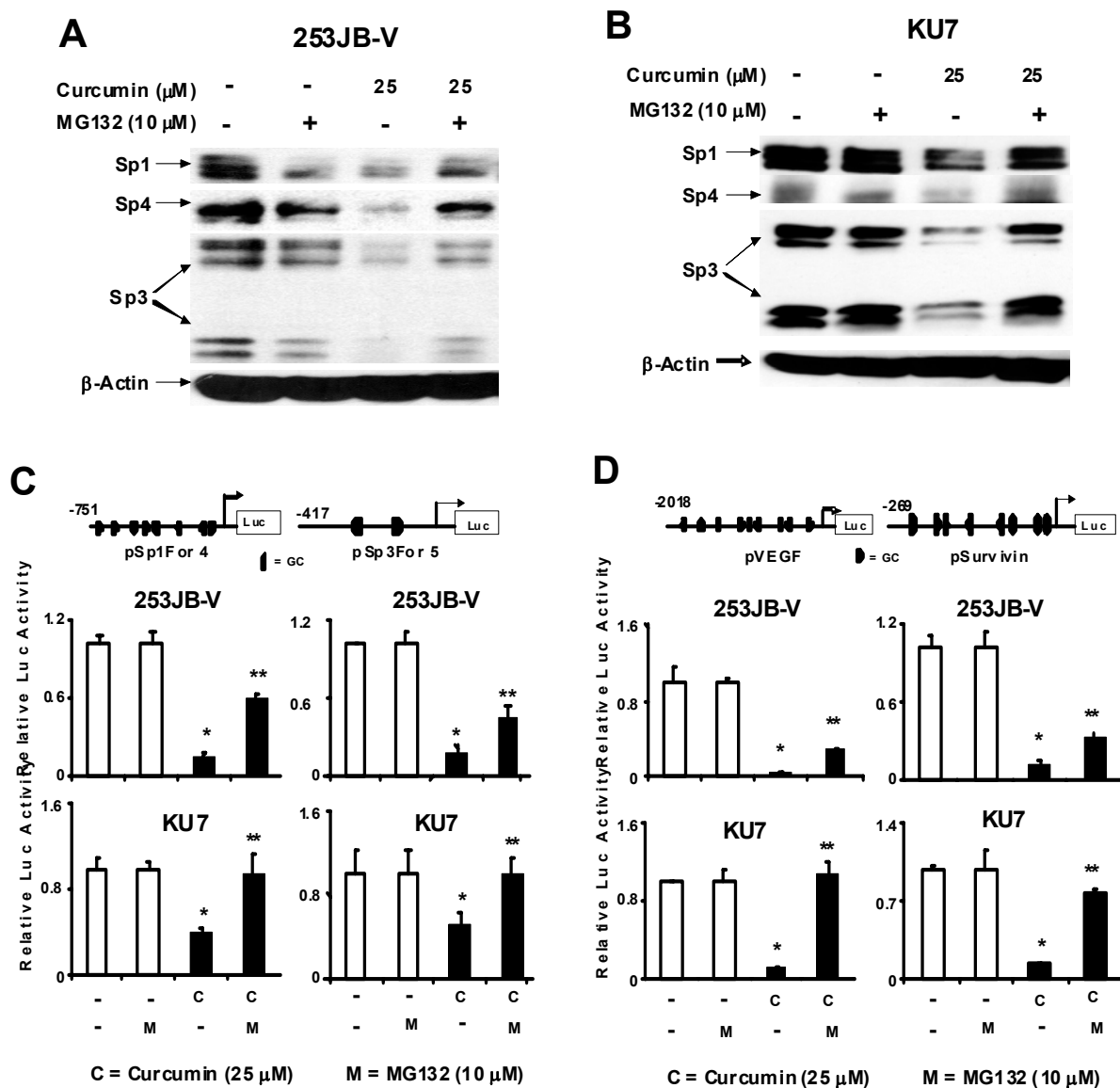


Figure 15. MG132 inhibition of curcumin-induced effects on Sp proteins and Sp-dependent transactivation.

MG132 inhibits curcumin-dependent downregulation of Sp proteins in 253JB-V (A) and KU7 (B) cells. Bladder cancer cells were treated with DMSO alone, 25 μ M curcumin, or 10 μ M MG132 in the presence or absence of 25 μ M curcumin for 24 hr, and whole cell lysates were analyzed by Western blots as described in the Materials and Methods. β -Actin served as a loading control, and similar results were observed in duplicate experiments. MG132 inhibits the effects of curcumin on luciferase activity in bladder cancer cells transfected with Sp1/Sp3 promoter (C) and Sp-regulated promoter (D) constructs. Cells were transfected with the indicated constructs, treated with DMSO, 10 μ M MG132, 25 μ M curcumin, or MG132 plus curcumin (combined), and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means \pm SE for at least three separate determinations for each treatment group, and significantly decreased activity after treatment with curcumin (*) and reversal of the effect after cotreatment with MG132 (**) are indicated.

Previous studies show that tolfenamic and betulinic acids induce proteasome-dependent degradation of Sp1, Sp3 and Sp4 in pancreatic and prostate cancer cells (299), and Figures 15A and 15B summarize the effects of the proteasome inhibitor MG132 on curcumin-induced downregulation of these proteins. In KU7 cells, MG132 inhibited curcumin-induced downregulation of Sp1, Sp3 and Sp4 proteins, and similar effects were observed in 253JB-V cells. However, in the latter bladder cancer cell line, MG132 alone also decreased Sp protein expression and this response was most pronounced for Sp1. Other proteasome inhibitors such as lactacystin and gliotoxin gave similar results and, in combination with curcumin, high cytotoxicity was observed in 253JB-V cells (data not shown). Nevertheless, it was apparent that MG132 plus curcumin blocked Sp protein downregulation in both cell lines, suggesting that curcumin, like betulinic and tolfenamic acids (299), induced proteasome-dependent downregulation of Sp1, Sp3 and Sp4 proteins in 253JB-V and KU7 cells. Figure 15 C summarizes the effects of MG132 on curcumin-dependent decreased

luciferase activity in 253JB-V and KU7 cells transfected with pSp1For4 and pSp3For5. In 253JB-V cells, MG132 only partially reversed downregulation of activity by 25 μ M curcumin, whereas in KU7 cells, MG132 completely inhibited the effects of curcumin on luciferase activity. The relative effectiveness of MG132 as an inhibitor of curcumin-dependent downregulation of luciferase activity in cells transfected with pVEGF or pSurvivin also differed in 253JB-V and KU7 cells (Figure 15D). MG132 reversed the effects of curcumin in KU7 cells (80 - 100%) but was less efficient in 253JB-V cells. This may be due, in part, to the cytotoxicity of MG132 alone and in combination with curcumin in 253JB-V cells. These results demonstrate that curcumin primarily induced proteasome-dependent degradation of Sp1, Sp3 and Sp4 in the bladder cancer cells, and similar effects have been observed for tolfenamic acid and betulinic acid in pancreatic and prostate cancer cells (299) (21).

Results illustrated in Figures 16A show that 10 - 40 μ M curcumin decreased luciferase activity in 253JB-V cells transfected with pNF κ B, a construct containing 5 tandem NF κ B response elements that regulate a luciferase reporter gene. Thus, curcumin decreases NF κ B-dependent transactivation in the 253JB-V cell line. Since curcumin also decreases expression of Sp1, Sp3 and Sp4 in bladder cancer cells, we investigated the effects of Sp protein knockdown on luciferase activity in cells transfected with pNF κ B-luc (Figure 16B). 253JB-V and KU7 cells were transfected with iSp which is a cocktail of small inhibitory RNAs for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4) (341).

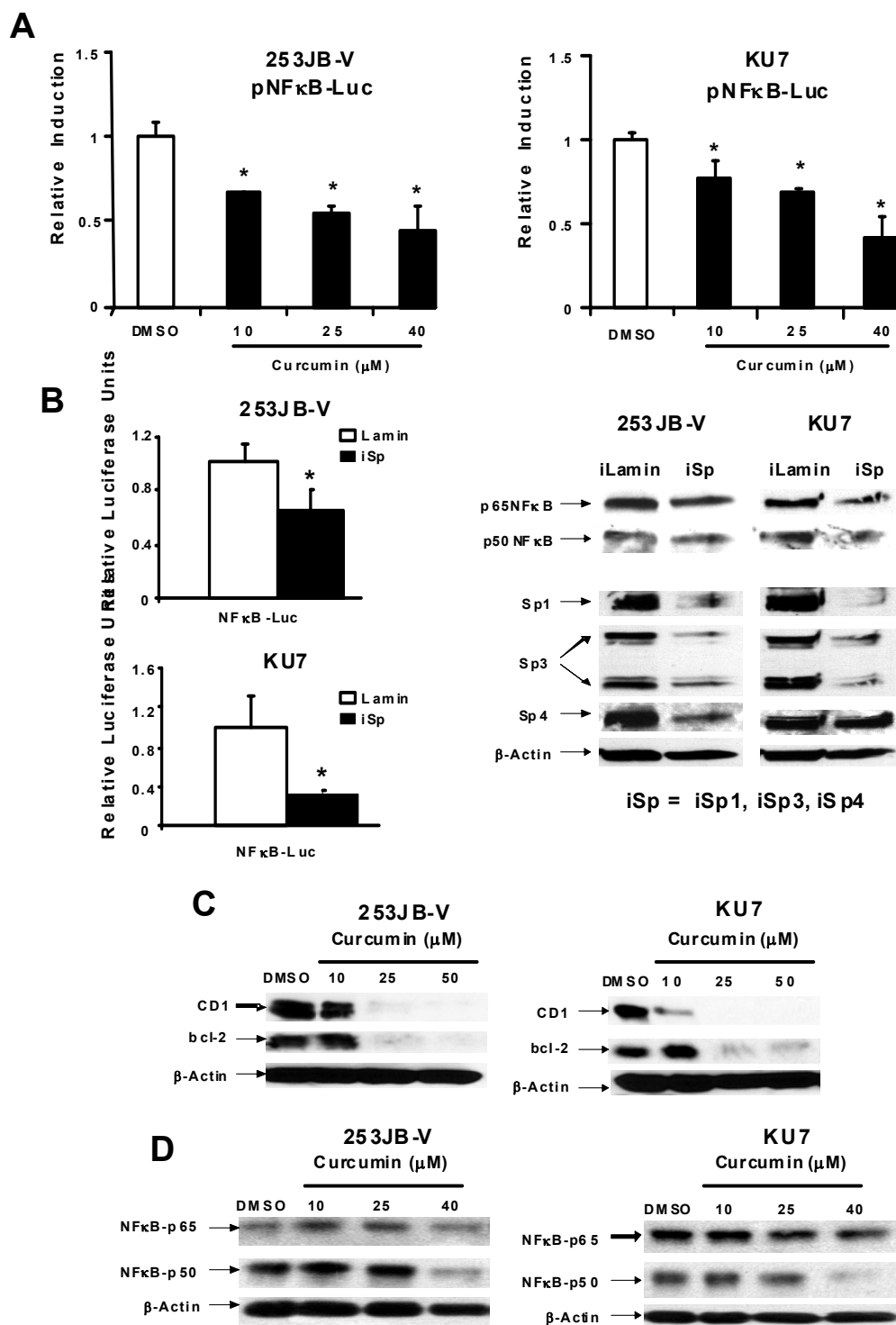


Figure 16. Effects of curcumin and Sp knockdown on NFκB.

Curcumin decreases NF κ B promoter activity in 253JB-V and KU7 cells. 253JB-V and KU7 cells were transfected with pNF κ B-luc treated with DMSO, 10, 25 or 40 μ M curcumin, and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group, and significantly ($p < 0.05$) decreased activity is indicated (*). (B) Effects of iSp on NF κ B. 253JB-V and KU7 cells were transfected with iSp (a cocktail of iSp1, iSp3 and iSp4) or iLamin, and transfected with pNF κ B-Luc; luciferase activity was determined as described in the Materials and Methods. Results are means \pm SE for three replicate determinations, and significantly ($p < 0.05$) decreased activity is indicated by an asterisk. The efficiency of Sp knockdown and the effects of p65 and p50 protein levels were determined by Western blots as described in the Materials and Methods. Effects of curcumin on bcl-2 and cyclin D1 (C) and p65 and p50 (D) proteins in 253JB-V and KU7 cells. Cells were treated with DMSO, different concentrations of curcumin, and nuclear extracts were analyzed by Western blots as described in the Materials and Methods.

The results showed that transfection with iSp significantly inhibited NF κ B-dependent activity and this was not directly related to a decrease in p65 or p50 protein levels after transfection with iSp (Figure 16B). There was a minimal but not significant decrease in these proteins over replicated experiments, suggesting that decreased NF κ B-dependent transactivation by iSp was not dependent on decreased expression of p65 or p50. We also investigated the effects of iSp1, iSp3 and iSp4 on p65 and p50 protein expression in 253JB-V and KU7 cells. In the former cell line, iSp3 and iSp4 (but not iSp1) slightly decreased p65 protein levels, whereas none of the small inhibitory RNAs affected p65 or p50 expression in KU7 cells and p50 expression in 253JB-V cells. Figure 16C shows that curcumin decreased expression of cyclin D1 and the antiapoptotic protein bcl-2 in 253JB-V and KU7 cells, and both of these genes are also regulated by NF κ B and Sp transcription factors (342, 343). It is

possible that curcumin-induced downregulation of Sp proteins may directly affect NF κ B since both p65 and p50 are Sp-dependent genes in some cell lines (344, 345). However, treatment of 253JB-V and KU7 cells with DMSO, 10, 25 or 40 μ M curcumin for 24 hr did not affect nuclear p65 expression, whereas nuclear p50 protein levels were decreased only at the higher (40 μ M) concentration (Figs. 16D) and 25 μ M curcumin, which decreases Sp protein levels, did not affect p65 or p50 expression. Thus, curcumin-mediated inhibition of NF κ B-dependent gene expression is not due to direct effects on p65 or p50.

Athymic nude mice bearing KU7 cells as xenografts were treated with corn oil (control) or curcumin in corn oil (50 mg/kg/d), and tumor volumes (mm^3) and weights were determined as described in the Material and Methods. Western blot analysis of tumor lysates from three mice in the treated and control groups were also determined as described in the Materials and Methods. Significant ($p < 0.05$) decreases are indicated by an asterisk.

A close inspection of many NF κ B-regulated genes such as cyclin D1, bcl-2, survivin and VEGF indicates that these genes also contain multiple GC-rich promoter sequences and are coregulated by Sp proteins in many cell lines (299) (346) (335) (336) (342) (343) (345, 347, 348) (349). Results in Figure 17A show that in 253JB-V and KU7 cells transfected with iLamin (control) or the iSp cocktail (containing iSp1, iSp3 and iSp4), there was a significant decrease in levels of bcl-2, cyclin D1, survivin and VEGF and this correlated with the effects of curcumin on these same proteins (Figures. 13B and 16C). In contrast, combined knockdown of p65 and p50 by RNA interference (Figure 17B) did not affect expression of all these proteins. Only VEGF and bcl-2 proteins were decreased by p65/p50 knockdown, suggesting that basal expression of cyclin D1 and survivin were NF κ B-independent in the bladder cancer cells. Figure 17C illustrates gel mobility shift assays in which nuclear extracts from 253JB-V and KU7 cells bound a ^{32}P -labeled GC-rich oligonucleotide to form Sp-DNA retarded bands as previously characterized in other cell lines (335) (336) (337).

Treatment with 25 or 40 μ M curcumin decreased retarded band intensities and coincubation with unlabeled wild-type GC-rich or mutant oligonucleotides decreased or did not affect retarded band intensities, respectively (Figure 17C). Supershift experiments with Sp1 antibodies did not give a defined supershifted (SS) antibody-protein complex but a diffuse band. However, the Sp1 band intensity was decreased due to immunodepletion. We also determined the effects of curcumin on NF κ B-DNA binding in a gel mobility shift assay using a consensus NF κ B response element and extracts from cells, and the results showed that curcumin decreased retarded band intensities associated with the NF κ B-DNA complex. These data confirm that curcumin decreases Sp proteins and Sp-DNA complex formation and the NF κ B-DNA retarded band and this correlated with decreased expression of NF κ B regulated proteins which are also coregulated by Sp transcription factors.

These factors suggest that curcumin-dependent downregulation of Sp proteins contributes to decreased expression of several NF κ B-dependent proteins which are coregulated by both Sp and NF κ B transcription factors (299) (346) (336) (342) (343) (345, 347-349). We also investigated the antitumorigenic activity of curcumin in athymic nude mice bearing KU7 cells as xenografts. At a dose of 50 mg/kg/d, curcumin significantly decreased tumor volumes and tumor weights (Figure 17D). Moreover, Western blot analysis of tumor lysates from three control and three treated mice show that Sp1, Sp3 and Sp4 protein expression is decreased in the latter group. These results complement the *in vitro* studies and demonstrate that curcumin-induced effects on Sp proteins also plays a role in the mechanism of action of this compound in bladder cancer cells.

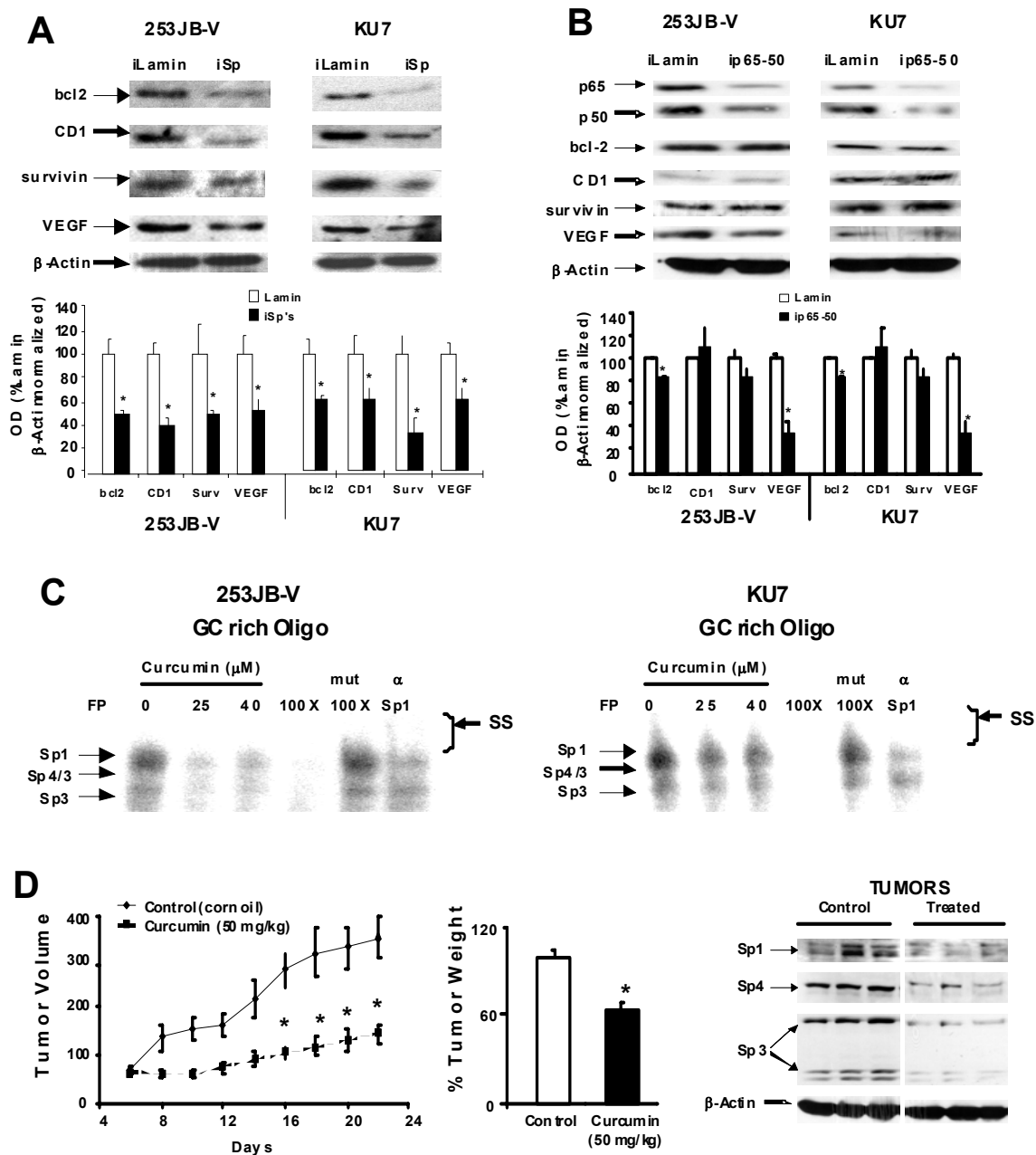


Figure 17. Role of Sp protein and NFκB on protein expression and the effects of curcumin on Sp-DNA binding and bladder tumor growth.

Effects of iSp and iLamin (A) and small inhibitory RNAs for p65 (ip65) and p50 (ip50) [combined (B)] on genes regulated by Sp and NF κ B. 253JB-V and KU7 cells were transfected with iSp (a cocktail of iSp1, iSp3 and iSp4) or iLamin, or ip65 plus ip50, and protein expression was determined by Western blots of whole cell lysates. β -Actin served as a loading control and for standardizing quantitative protein determinations where iLamin protein levels were set at 100%. Results are means \pm SE for three separate determination and significantly ($p < 0.05$) decreased protein levels are indicated by an asterisk. (C) Gel mobility shift assay. 253JB-V and KU7 cells were treated with DMSO (0), 25 or 40 μ M curcumin for 24 hr, and nuclear extracts were incubated with 32 P-labeled GC-rich oligonucleotide alone or in the presence of other factors. Retarded bands were analyzed by EMSA as described in the Materials and Methods. The identities of the retarded Sp-DNA bands and Sp1 antibody supershifted complex (SS) are indicated. (D) Curcumin inhibits bladder tumor growth.

Discussion

Curcumin has been widely used in traditional medicines, and its chemoprotective and chemotherapeutic effects are due to many different activities that are also dependent on cell context (318). One of the most predominant effects of curcumin observed in many studies is associated with inhibition of constitutive or induced NF κ B-dependent genes/proteins associated with cell survival, angiogenesis and inflammation (318) (283) (341, 350) (351) (352). However, other pathways contribute to the effects of curcumin, and these may be NF κ B-independent. For example, curcumin induces endoplasmic reticulum (ER) stress in human leukemia HL-60 cells, and this results in activation of ER stress-dependent proapoptotic pathways associated with cleavage of caspases 8, 9, 3 and 4 (338). In addition, curcumin-induced apoptosis in human leukemia U937 cells was also linked to the direct effects of

this compound on mitochondria function and subsequent activation of the intrinsic apoptosis pathway (353).

Recent studies in this laboratory have demonstrated that compounds such as celecoxib, tolfenamic acid, and betulinic acid inhibit cancer cell and tumor growth and metastasis, and exhibit both antiangiogenic and proapoptotic activity through decreased expression of Sp proteins such as Sp1, Sp3 and Sp4 (21) (346) (335). The role of Sp proteins in mediating these responses was consistent with RNA interference studies showing that Sp proteins regulate angiogenic, growth promoting, and prosurvival genes in cancer cells (336) (337) (204) (348). Results in Figure 12 illustrates that curcumin decreased proliferation of bladder cancer cells and induced apoptosis as evidenced by induction of PARP cleavage (Figure 13B) and DNA laddering (Figure 13C) in 253JB-V and KU7 bladder cancer cells. These results are consistent with a report (17) showing that curcumin exhibited growth inhibitory and proapoptotic activity in KU7 and RT4V6 bladder cancer cells. In the prior study 10 μ M curcumin slightly inhibited G₀/G₁ to S phase progression in KU7 cells, whereas our results showed that 10 μ M curcumin inhibited G₀/G₁ to S phase progression in 253JB-V but not in KU7 cells which were essentially unaffected by 10 μ M curcumin (Figures 12C and 12D). Differences in the effects of curcumin on cell cycle progression in these cell lines may be due, in part, to the effects of curcumin on p21, p27 and cyclin D1 expression (Figures 13A and 16C), and this is currently being investigated.

Curcumin also decreased expression of the angiogenic proteins VEGF and VEGFR1 in both bladder cancer cell lines, and decreased expression of the antiapoptotic protein survivin was accompanied by induction of caspase-dependent PARP cleavage (Figure 13B) and increased staining in the TUNEL assay (Figure 13C). This evidence for activation of apoptosis by curcumin is consistent with previous studies on this compound in bladder and other cancer cell lines (318) (319) (319) (325) (326) (327) (328) (329) (333). However, the

coordinate decrease in expression of survivin, VEGF and VEGFR1 in bladder cancer cells treated with curcumin was reminiscent of similar effects observed in prostate and pancreatic cancer cells treated with betulinic acid and tolfenamic acid, respectively (299) (21) (346). In those studies, it was shown that decreased expression of survivin, VEGF and VEGFR1 was due to degradation of Sp1, Sp3 and Sp4 which are known to regulate basal expression of survivin, VEGF, VEGFR1 and VEGFR2 (335) (348). Results summarized in Figure 14 illustrate that treatment of 253JB-V and KU7 cells with curcumin caused a concentration-dependent decrease in Sp1, Sp3 and Sp4 protein expression. The Sp1 and Sp3 promoters contain GC-rich motifs that bind Sp proteins (339) (340) (31, 32), and curcumin inhibited transactivation in 253JB-V and KU7 cells transfected with constructs (pSp1For4 and pSp3For5) containing Sp1 and Sp3 gene promoter inserts (Figure 14C). Sp proteins also regulate expression of survivin and VEGF (345, 347, 349) and curcumin decreased luciferase activity in bladder cancer cells transfected with pVEGF and pSurvivin.

Betulinic acid- and tolfenamic acid-induced downregulation of Sp1, Sp3 and Sp4 in prostate and pancreatic cancer cells is due to activation of proteasomes, and these are blocked by the proteasome inhibitor MG132 (299) (21) (346). This same inhibitor inhibited curcumin-induced downregulation of Sp1, Sp3 and Sp4 proteins in 253JB-V and KU7 cells (Figures. 15A and 15B), and similar effects were observed in cells transfected with pS1For4, pSp3For5, pVEGF and pSurvivin and treated with curcumin plus MG132 (Figures. 15C and 15D). MG132 and other proteasome inhibitors such as gliotoxin and lactacystin were cytotoxic to 253JB-V cells, and MG132 alone decreased Sp1 protein in this cell line. However, despite these effects, our results show that curcumin-dependent downregulation of Sp1, Sp3 and Sp4 proteins was partially reversed in 253JB-V cells cotreated with curcumin plus MG132. Moreover, similar results were observed in the transfection studies (Figures. 15C and 15D), suggesting that curcumin-dependent downregulation of Sp1, Sp3 and Sp4 in KU7 and

253JB-V cells was primarily due to activation of the proteasome pathway. These results suggest that some of the proapoptotic and antiangiogenic activities of curcumin in bladder cancer cells are due, in part, to degradation of Sp proteins and Sp-dependent survivin, VEGF and VEGFR1.

In cancer cells and tumors, NF κ B regulates expression of prosurvival and angiogenic genes, and the efficacy of several chemotherapeutic agents including curcumin has also been linked to their inhibition of NF κ B-mediated responses (318) (283) (319) (324) (329). Inhibition of NF κ B by curcumin has been associated with modulation of multiple pathways; however, the effects of curcumin-dependent downregulation of Sp proteins on NF κ B have not been determined. p65 and p50/105 are regulated by Sp1 in some cell lines (343) (344, 354), and our results show that concentrations of curcumin that decreased NF κ B-dependent activity ($\leq 25 \mu\text{M}$) did not affect nuclear p65 or p50 levels in KU7 or 253JB-V cells (Figure 16D). Moreover, in KU7 and 253JB-V cells transfected with the iSp cocktail containing small inhibitory RNAs for Sp1, Sp3 and Sp4, there was also only a minimal decrease in expression of p65 or p50 (Figure 16B). Curcumin decreased expression of several NF κ B-dependent genes including VEGF, survivin, bcl-2 and cyclin D1 (Figures. 13B and 16C) (299) (346) (336) (337) (343) (345, 347-349), and similar results were observed in bladder cancer cells transfected with iSp (Figure 17A). Since curcumin decreases both Sp proteins and NF κ B-dependent transactivation in bladder cancer cells, the role of NF κ B in mediating these responses was investigated by p65 and p50 knockdown (Figure 17B). Only VEGF and bcl-2 were decreased in both cell lines after transfection with small inhibitory RNAs for p65 and p50 (combined). This suggests that curcumin-induced effects on bcl-2, cyclin D1, VEGF and survivin expression are primarily due to Sp downregulation; however, the contribution of Sp proteins and NF κ B on regulation of these gene products are gene- and cell context-dependent even though their promoters contain both GC-rich and NF κ B elements (299) (336) (337) (342, 343, 345) (348, 349).

We also examined the *in vivo* activity of curcumin (50 mg/kg/d) in athymic nude mice bearing KU7 cells as xenografts (Figure 17D). Curcumin inhibited tumor growth and weight and this corresponded to parallel effects in 253JB-V and KU7 cells (Figures. 12A and B) where curcumin inhibited cell proliferation. Moreover, curcumin-induced effects on Sp protein expression in bladder cancer cells (Figures. 14A and B) were also observed in bladder tumors (Figure 17D) demonstrating that curcumin-dependent Sp protein degradation is an integral part of the anticancer activity of this compound in bladder cancer cells and tumors.

In summary, results of this study demonstrate for the first time that curcumin induces proteasome-dependent degradation of Sp1, Sp3 and Sp4 in bladder cancer cell lines, and this is accompanied by decreased expression of Sp-dependent survival and angiogenic proteins. The results suggest that at least some of the important anticancer activities of curcumin may be due, in part, to decreased expression of Sp proteins. A recent study (355) reported that curcumin decreased Sp1 protein expression in pancreatic cancer cells and this was related to increased microRNA-22 which directly targets Sp1 but not Sp3 or Sp4. Since curcumin induces proteasome-dependent degradation of all three Sp proteins in bladder cancer cells, the mechanism of curcumin action clearly differs in pancreatic and bladder cancer cells. Current studies are focused on the cancer cell context-dependent differences in the contributions and mechanisms of action of curcumin-dependent downregulation of Sp proteins on the overall activity of this compound as an anticancer agent.

III. EPIDERMAL GROWTH FACTOR RECEPTOR IS DOWNREGULATED BY DRUGS THAT REPRESS SPECIFICITY PROTEIN TRANSCRIPTION FACTORS

The epidermal growth factor receptor (EGFR) is an important chemotherapeutic target for tyrosine kinase inhibitors and antibodies that block the extracellular domain of EGFR. Betulinic acid (BA) and curcumin are phytochemical anticancer agents, and we hypothesized that both compounds decrease EGFR expression in bladder cancer through downregulation of specificity protein (Sp) transcription factors. The comparative effects of BA, curcumin and the tyrosine kinase inhibitor gefitinib on EGFR mRNA and protein expression were determined in 253JB-V and KU7 bladder cancer cells. The effects of curcumin and BA on Sp proteins (Sp1, Sp3 and Sp4) and their role in downregulation of EGFR and EGFR-dependent responses were confirmed by RNA interference. BA and curcumin decreased expression of EGFR, Sp and Sp-dependent proteins in 253JB-V and KU7 cells; EGFR was also decreased in cells transfected with a cocktail (iSp) containing small inhibitory RNAs for Sp1, Sp3 and Sp4. BA, curcumin and iSp also decreased phosphorylation of Akt in these cells and downregulation of EGFR by BA, curcumin and iSp was accompanied by induction of LC3 and autophagy which is consistent with recent studies showing that EGFR suppresses autophagic cell death. The results show that EGFR is an Sp-regulated gene in bladder cancer and drugs such as BA and curcumin that repress Sp proteins also ablate EGFR expression. Thus, compounds such as curcumin and BA that downregulate Sp transcription factors represent a novel class of anticancer drugs that target EGFR in bladder cancer cells and tumors by inhibiting receptor expression.

Introduction

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase and a member of the ErbB family of transmembrane receptors. Ligand-dependent activation of these receptors results in homo- or heterodimerization, autophosphorylation and induction of multiple downstream pathways required for cellular homeostasis (356). ErbB family members are frequently activated in many tumor types and this is due to several factors including activating mutations, gene amplifications, overexpression of the receptor and/or its cognate ligands and loss of inhibitory factors that regulate receptor activity (357). Enhanced EGFR activity in cancer cells and tumors is associated with increased growth, survival and angiogenesis of tumors and thereby contributes significantly to the phenotypic characteristics of cancer cells (357) (358). Not surprisingly, the EGFR has become a major target for cancer chemotherapy and development of two major classes of anti-EGFR agents, namely monoclonal antibodies against the extracellular domain of these receptors and low molecular weight drugs that competitively inhibit ATP binding to the intracellular tyrosine kinase domain. EGFR1 (ErbB1/HER1) and EGFR2 (ErbB2/HER2) are two family members that exhibit increased activity in tumors and monoclonal antibodies against EGFR1 (Cetuximab) and EGFR2 (Trastuzumab) are used alone or in combination for cancer chemotherapy, and other antibodies are also being developed.

Tyrosine kinase inhibitors have also been developed for EGFR1 and EGFR2 (ErbB2) and these include gefitinib, erlotinib, lapatinib and others which are used as single agents or in combination for treatment of multiple cancers (359). Initial studies of kinase inhibitors in non-small cell lung cancer (NSCLC) patients observed minimal treatment benefits (360) and recent reports have also shown that administration of gefitinib after radiotherapy did not improve survival of NSCLC patients (361). In contrast, treatment of NSCLC patients with tyrosine kinase inhibitors was highly successful for subsets of patients expressing EGFR

kinase domain mutations (362) and similar results were observed for gefitinib in lung cancer cell lines (363).

Bladder tumors also overexpress the EGFR, and ligands for this receptor (364) and clinical applications of EGFR blocking agents in combination with other drugs are underway or in development (365). Shrader and coworkers (366) identified gefitinib-sensitive and -insensitive bladder cancer cell lines typified by 253JB-V and KU7 bladder cancer cells, respectively, and markers of responsiveness included induction of p27 and decreased DNA synthesis after treatment with the tyrosine kinase inhibitor. Recent studies in this laboratory have demonstrated that tolfenamic acid, betulinic acid (BA) and curcumin inhibit growth of pancreatic, prostate and bladder cancer cells and tumors and this was associated with proteasome-dependent degradation of Sp1, Sp3 and Sp4 transcription factors that are overexpressed in these cells and tumors (336). The effects on Sp proteins were accompanied by a parallel decrease in several Sp-dependent genes that enhance cancer cell survival (survivin), growth (cyclin D1) and angiogenesis [vascular endothelial growth factor (VEGF) and its receptors (VEGFR1 and VEGFR2)]. Expression of EGFR is Sp-dependent in some cancer cell lines (367) and this study demonstrates that both BA and curcumin decrease EGFR expression in bladder cancer cells and tumors, demonstrating that drug-induced Sp repression represents an alternative pathway for targeting EGFR in bladder cancer cells and tumors.

Materials and methods

Cell lines. KU7 and 253JB-V human bladder cancer cells were provided by Dr. A. Kamat, (M.D. Anderson Cancer Center, Houston, TX). 253JB-V and KU7 cell lines 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 (Sigma

Aldrich, St. Louis, MO). Cells were grown in 150 cm² culture plates in an air/CO₂ (95:5) atmosphere at 37°C and passaged approximately every 3 days.

Antibodies, chemicals and other materials. Sp1 (PEP2), Sp3 (D-20), Sp4 (V-20), VEGF (147), Survivin (FL-142), AKT (sc-8312), p-AKT (sc-7985-R) and EGFR1 (1005) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP (ASP 214), p-MAPK (197G2) and MAPK (137F5) antibody was purchased from Cell Signaling Technology (Danvers, MA) and SGLT antibody was purchased from Abcam Inc, (Cambridge, MA) (ab7970-1). LC3 antibody was purchased from MBL International Corporation, (Woburn, MA). Monoclonal β -actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase substrate for western blot analysis was obtained from NEN Life Science Products (Boston, MA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). BA was purchased from Sigma-Aldrich, curcumin (98% pure) was purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ) and gefitinib (>99% pure) was obtained from LC Laboratories (Woburn, MA). The GFP-LC3 plasmid was kindly provided by Dr. Tamotsu Yoshimori (Osaka University, Osaka, Japan).

Cell proliferation assays. Bladder cancer cells (3×10^4 cells per well) were seeded using DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS in 12-well plates and left to attach for 24 hr. Cells were then treated with either vehicle (DMSO) or the indicated concentrations of BA, curcumin and gefitinib. Fresh medium and test compounds were added every 24 hr for curcumin, BA and gefitinib. Cells were then 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 determination.

Western blot assays. Bladder cancer cells were seeded in DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS. Twenty-four hr later, cells were treated with either vehicle (DMSO) or the indicated compounds for 48 hr. Cells were collected using high-salt buffer (50 mmol/L

HEPES, 0.5 mol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10% glycerol, and 1% Triton-X-100, pH 7.5) and 10 μL/mL of Protease Inhibitor Cocktail (Sigma Aldrich). The lysates were incubated on ice for 1 hr with intermittent vortexing for 90 min, followed by centrifugation at 20,000 g for 10 min at 4°C. Lysates were then incubated for 3 min at 100°C before electrophoresis, and then separated on 10% SDS-PAGE 120 V for 3 to 4 hr in 1X running buffer (25 mM Tris-base, 192 mM glycine, and 0.1% SDS). 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 at 4°C. The 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 1:200 - 1:1000 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 (PerkinElmer Life Sciences, Waltham, MA) substrate for 1.0 min, and exposed to Kodak X-OMAT AR autoradiography film (American X-ray supply Inc, Jackson, CA). The tumor tissues were also processed similarly and probed for EGFR1 protein and β-actin served as loading control. Quantification of the proteins was done using Image J software and the optical densities were plotted after normalization with lamin./β-actin.

siRNA interference assay. The two bladder cancer cell lines, 253JB-V and KU7 were seeded (1×10^5 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 one day. The triple Sp siRNA knockdown (iSp1, iSp3, iSp4 complex) along with iLamin as control was performed using Liopfectamine reagent according to the manufacturer's instructions. Small inhibitory RNAs

were prepared by Dharmacon RNA Technologies (Chicago, IL). The iRNA complexes used in this study are indicated as follows:

LMN	5' - CUG GAC UUC CAG AAG AAC ATT
Sp1	SMARTpool L-026959-00-0005
Sp3	5' - GCG GCA GGU GGA GCC UUC ACU TT
Sp4	5' - GCA GUG ACA CAU UAG UGA GCT T

Xenograft study. Female athymic nude mice, age 4-6 weeks were purchased from Harlan (Indianapolis, CA). KU7 cells (1×10^6 cells) in 1:1 ratio of Matrigel (BD Biosciences, San Jose, CA) were injected into the either side of flank area of nude mice. A week after the tumor cell inoculation, the mice were divided into two groups of 10 animals each. The first group received 100 μ L vehicle (corn oil) by intraperitoneal route, and the second group of animals received 50 mg/kg/d dose of curcumin in corn oil every second day for 18 d (9 doses) by intraperitoneal route. At the end of 21 d, the animals were sacrificed; the tumors in control and treated animals were homogenized and probed for EGFR1 protein levels using western blotting.

Real-time PCR. Total RNA was isolated using the RNeasy Protect Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 30 μ L of RNase-free water and stored at -80°C . RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA was prepared from the 253JB-V and KU7 bladder cancer cell lines at different time intervals using a combination of oligodeoxythymidylic acid and dNTP mix (Applied Biosystems, Foster City, CA) and Superscript II (Invitrogen). Each PCR was carried out in triplicate in a 25 μ L volume using SYBR Green Master mix (Applied Biosystems) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min in the ABI Prism 7700 sequence detection system (Applied Biosystems). The ABI Dissociation Curves software was used after a brief thermal protocol (95°C for 15 s and 60°C for 20 s, followed by a slow ramp to 95°C) to control for multiple

species in each PCR amplification. The comparative CT method was used for relative quantitation of samples. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of primers for EGFR1 were 5' - TTT CGA TAC CCA GGA CCA AGC CAC AGC AGG - 3' and 5' - AAT ATT CTT GCT GGA TGC GTT TCT GTA - 3'.

Fluorescence microscopy and GFP-LC3 localization. Monolayers of cells were cultured for 24 hr in 2-well coverglass chamber slides and treated as indicated. The GFP-LC3 plasmid was kindly provided by Dr. Tamotsu Yoshimori (Osaka University, Osaka, Japan). KU7 cell lines were transfected with 1 $\mu\text{g}/\text{well}$ GFP-LC3 plasmid using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Slides were examined by fluorescence microscopy using Zeiss Stallion Dual Detector Imaging System (Carl Zeiss Microimaging Inc., Thornwood, NY). The intracellular distribution of GFP-LC3 was evaluated by monitoring GFP-LC3 and DIC images throughout the entire thickness of the cell by optical slices at 0.5 μM intervals using a C-Apochromat 63X, 1.2 NA water immersion lens. Digital images were acquired using Slide Book software (Intelligent Imaging Innovations, Denver, CO). The entire z-stack was subjected to fluorescence deconvolution to remove out of plane fluorescence. Cells were examined in more than five fields per slide on multiple slides. Data represent the average of all the fields.

Staining for acridine orange. 253JB-V and KU7 bladder cancer cells were seeded in monolayers and, at 70% confluence, cells were untreated or treated with 10 μM BA and 40 μM curcumin for various time points. At the appropriate time points, cells were incubated with 1 $\mu\text{g}/\text{ml}$ acridine orange (Molecular Probes, Eugene, OR) in serum-free medium for 15 min. The acridine orange was removed and fluorescence images were obtained before and after removing the dye. The cytoplasm and nucleus of the stained cells fluoresced bright green, whereas the acidic autophagic vacuoles fluoresced bright red.

Results

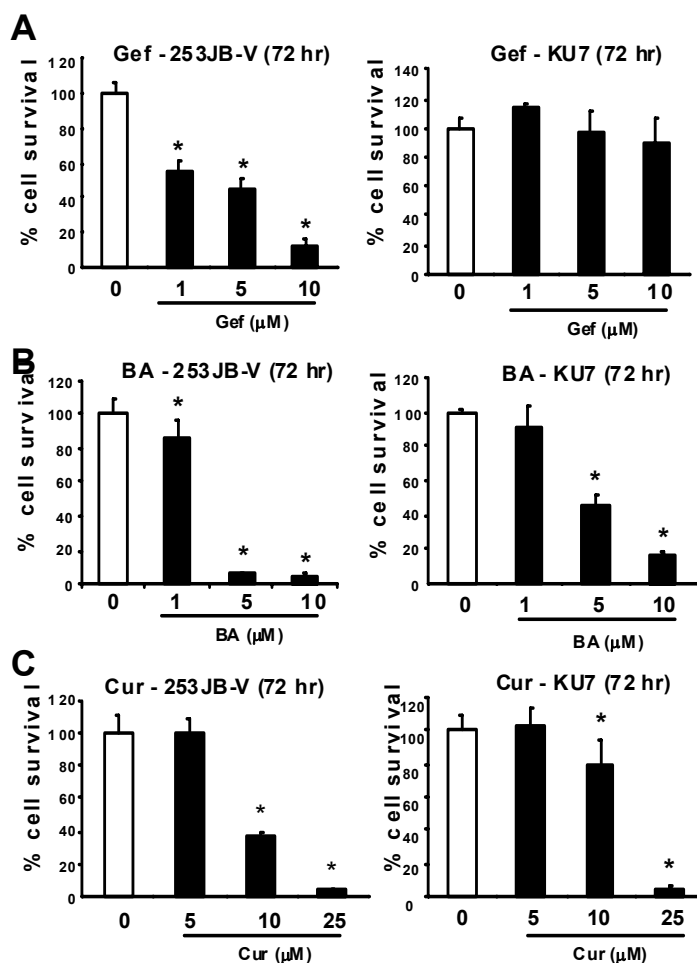


Figure 18. Effects of gefitinib (A), BA (B) and curcumin (C) on cell survival.

253JB-V and KU7 cells were treated with DMSO (solvent control) or different concentrations of gefitinib, BA and curcumin for 72 hr, and cells were counted and expressed as a percentage of DMSO as described in the Materials and Methods. Results are expressed as means \pm SE for at least 3 replicate experiments for each treatment group and significantly ($p < 0.05$) decreased survival is indicated (*).

253JB-V and KU7 cells were reported to be gefitinib-responsive and non-responsive, respectively, and results in Figure 18A demonstrate that treatment of 253JB-V and KU7 cells with 1 - 10 μM gefitinib for 72 hr significantly decreased cell survival only in the former cell line. BA and curcumin, two relatively non-toxic phytochemical anticancer agents that decrease Sp protein expression in prostate and bladder cancer cells, respectively (299), inhibited survival of both 253JB-V and KU7 bladder cancer cells (Figures. 18B and 18C). 253JB-V cells were more sensitive than KU7 cells to the antiproliferative effects of both compounds at lower concentrations but at the higher concentrations used in this study, their effects on survival were similar in both cell lines.

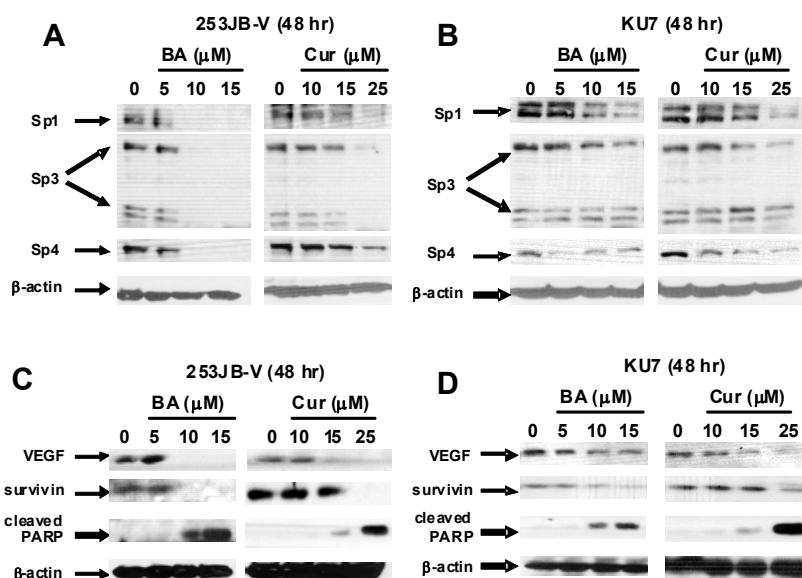


Figure 19. BA and curcumin decrease Sp proteins and Sp-dependent genes.

Compound-induced repression of Sp1, Sp3 and Sp4 in 253JB-V (A) and KU7 (B) cells. Cells were treated with DMSO or different concentrations of the compounds for 48 hr, and whole cell lysates were analyzed by western blots as described in the Materials and Methods. Compound-induced repression of Sp-dependent genes in 253JB-V (C) and KU7 (D) cells. Protein expression was

determined as outlined above in (A) and (B). Results (A - D) were observed in replicate experiments (at least 3). Results in Figures 19A and 19B show that both BA and curcumin decrease expression of Sp1, Sp3 and Sp4 proteins in 253JB-V and KU7 cells. This was accompanied by a parallel decrease in the Sp-dependent genes/proteins VEGF and survivin and induction of PARP cleavage (Figures. 19C and 19D). Thus, curcumin specifically targets Sp transcription factors in bladder cancer cells as previously reported (263), and we show for the first time that BA also exhibits comparable activity in bladder cancer cell lines. In contrast, the tyrosine kinase inhibitor gefitinib did not affect Sp protein expression in these cell lines (data not shown).

Previous studies show that EGFR1 is regulated by Sp1 in some cancer cell lines (367), and we hypothesized that BA and curcumin, which repress Sp1, Sp3 and Sp4 protein levels in bladder cancer cells (Figures. 19A and 19B), will also decrease EGFR1 and thereby provide an alternative pathway for the blockade of EGFR1 signaling. EGFR1 also enhances cancer cell survival by inhibition of autophagic cell death in breast cancer cells through stabilization of the sodium/glucose cotransporter 1 (SGLT1) and this response is independent of the kinase activity of this receptor (263). Results in Figures 21A and 21B also demonstrate that after treatment of 253JB-V and KU7 cells with BA or curcumin, there was a decrease in SGLT1 protein expression in both cell lines. Moreover, this was also accompanied by induction of LC3 which is a protein biomarker of autophagy (368). Thus, knockdown of EGFR1 in bladder cancer cells after treatment with curcumin or BA inhibited both EGFR1 kinase-dependent (PI3-K) and kinase-independent (SGLT downregulation and LC3 induction) survival pathways. Gefitinib also decreases expression of phospho-Akt and phospho-MAPK in 253JB-V cells (Figure 21C) which is consistent with inhibition of EGFR1 tyrosine kinase activity by this compound.

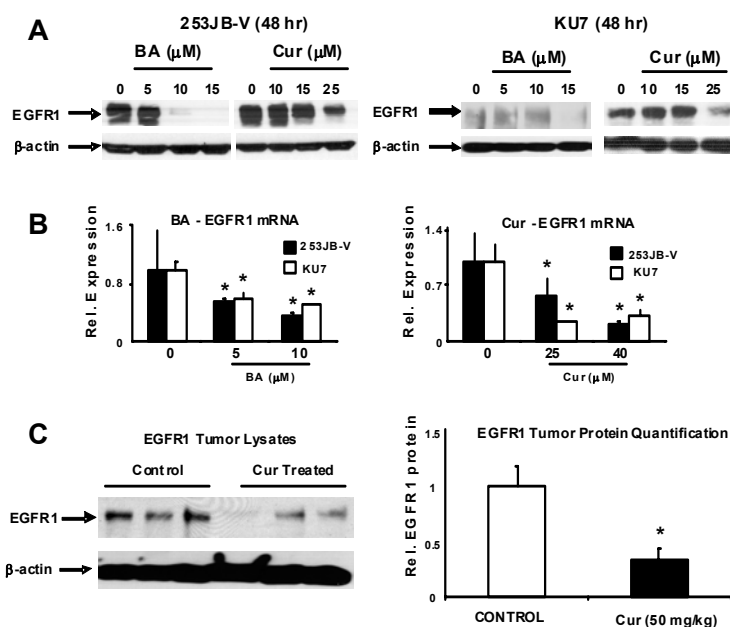


Figure 20. BA and curcumin decrease EGFR1 expression.

Downregulation of EGFR1 protein. 253JB-V and KU7 cells were treated with DMSO or different concentrations of BA and curcumin for 48 hr, and EGFR1 protein expression was determined on whole cell lysates by western blot analysis as described in the Materials and Methods. (B) BA and curcumin decrease EGFR1 mRNA levels. Cells were treated with DMSO or different concentrations of BA or curcumin for 18 hr, and EGFR1 mRNA levels were determined by RT-PCR as described in the Materials and Methods. Results are expressed as means \pm SE for 3 replicate determinations for each treatment group and significantly ($p < 0.05$) decreased expression (relative to DMSO) is indicated (*). (C) Curcumin decreases EGFR1 in tumor lysates. Tumor lysates from athymic nude mice bearing KU7 cells as xenografts were treated with curcumin (50 mg/kg/d for 18 d) and analyzed by western blot analysis as described in the Materials and Methods. Relative EGFR1 protein levels in the

curcumin treated group were significantly ($p < 0.05$) (*) lower than in tumors from corn oil-treated mice. Results were obtained from lysates from individual animals (3 per treatment group) and normalized to β -actin protein.

Results in Figures 20A and 20B show that after treatment of 253JB-V and KU7 cells with BA or curcumin for 48 hr, there was a decrease in EGFR1 protein expression. We also observed that BA and curcumin decreased EGFR1 mRNA levels in 253JB-V and KU7 cells (Figure 20B). Previous studies showed that in a mouse xenograft model using KU7 cells, curcumin inhibited tumor growth and decreased Sp1, Sp3 and Sp4 expression in tumors (367) and Figure 20C also shows that curcumin decreased EGFR1 protein expression in these tumors. These *in vivo* results complement the *in vitro* data showing that BA and curcumin decrease EGFR1 in bladder cancer cells.

EGFR1 regulates multiple genes and pathways through activation of downstream kinases such as PI3-K and MAPK. Figure 21A summarizes the effects of BA and curcumin on Akt/phospho-Akt and MAPK/phospho-MAPK expression in 253JB-V cells. Cells treated with 10 or 15 μ M BA and 25 μ M curcumin decreased constitutive phospho-MAPK expression; however, the same concentrations of BA also decreased levels of MAPK protein, whereas curcumin had minimal effects on MAPK protein levels. Both BA and curcumin also decreased phospho-Akt in 253JB-V cells and this was accompanied by decreased Akt protein. In KU7 cells, BA and curcumin increased levels of phospho-MAPK but did not affect MAPK protein and both compounds decreased phospho-Akt and Akt protein expression.

Thus, in the gefitinib-resistant cells, BA and curcumin inhibited the PI3-K and not the MAPK signaling pathways.

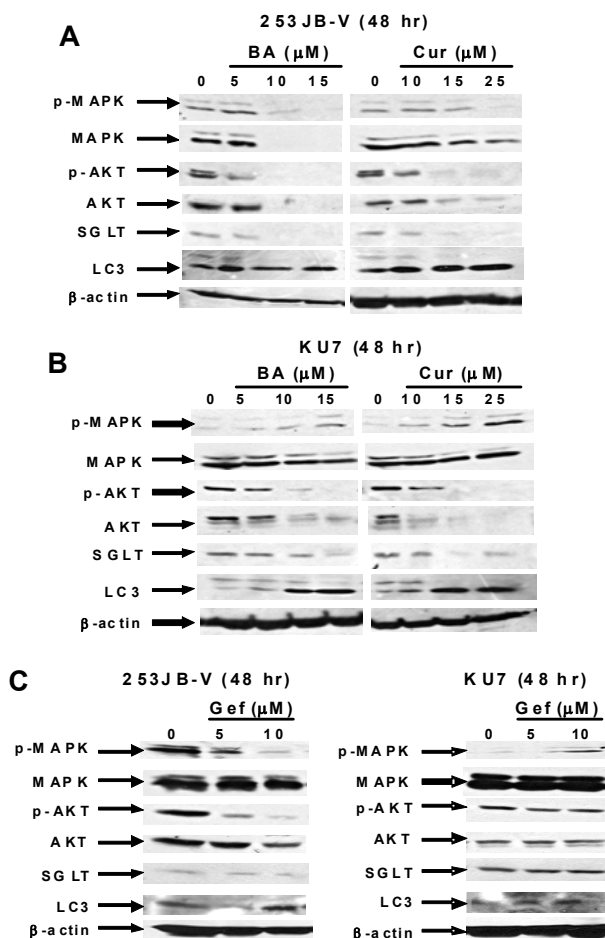


Figure 21. Modulation of putative EGFR1-dependent responses.

Effects of BA and curcumin on EGFR1-dependent effects in 253JB-V (A) and KU7 (B) cells compared to effects of gefitinib (C) in both cell lines. Cells were treated with DMSO or different concentrations of BA, curcumin or gefitinib for 48 hr, and whole cell lysates were analyzed by western blot analysis as described in the Materials and Methods. Similar results were observed in replicate (2) experiments.

However, in KU7 cells, gefitinib did not affect expression of phospho-Akt and induced phospho-MAPK (Figure 21D) which is consistent with previous studies showing that this cell line is gefitinib-resistant. Gefitinib did not affect SGLT or LC3 protein expression in either cell line which is in contrast to the effects of BA and curcumin (Figures. 21A and 21B).

Since Sp1, Sp3 and Sp4 are overexpressed in bladder and other cancer cell lines, we used a cocktail (iSp) of small inhibitory RNAs for Sp1 (iSp1), Sp3 (iSp3), and Sp4 (iSp4) for simultaneous knockdown of all three transcription factors (Figure 22A) as previously described (263). Figure 22B demonstrates that transfection of 253JB-V and KU7 cells with the iSp cocktail also decreased EGFR protein confirming that expression of EGFR1 is Sp-dependent in bladder cancer cells which is consistent with the multiple GC-rich Sp binding sites identified in the EGFR1 promoter (367).

Cells were treated with DMSO (untreated), 10 μ M BA, or 40 μ M curcumin for 18 hr, and detection of autophagic vacuoles by acridine orange staining was determined as described in the Materials and Methods. (C) Induction of punctate green fluorescence in KU7 cells transfected with GFP-LC3. Cells were transfected with the GFP-LC3 plasmid for 24 hr and treated with DMSO (untreated), BA or curcumin for 18 hr, and GFP-LC3 fluorescence was determined as described in the Materials and Methods. Observations in (A) - (C) were typical of replicate experiments. (D) Model for the effects of BA, curcumin and iSp in bladder cancer cells.

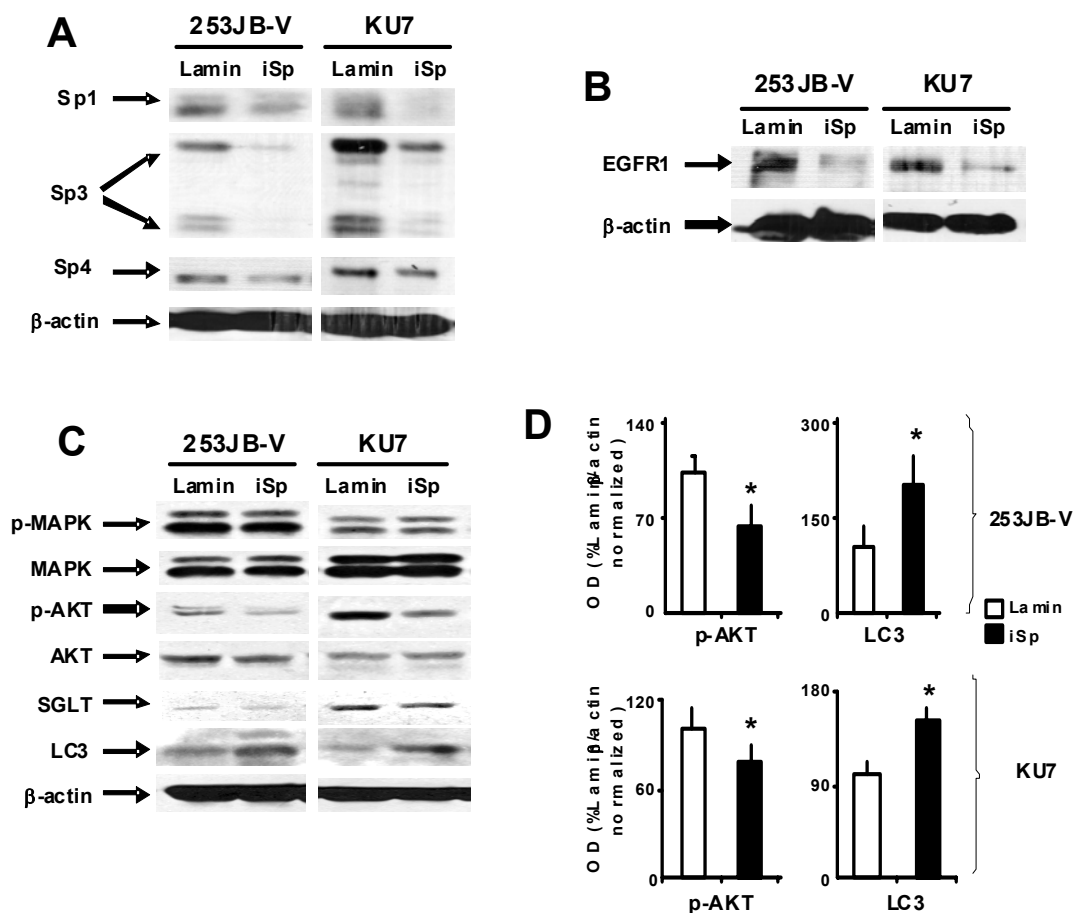


Figure 22. Effects of Sp knockdown by RNA interference on EGFR1 and EGFR1-dependent responses.

253JB-V and KU7 cells were transfected with a cocktail (iSp) containing siRNAs for Sp1, Sp3 and Sp4 or iLamin (non-specific control) and levels of Sp1, Sp3 and Sp4 (A), EGFR1 (B) and possible EGFR1-dependent responses (C) were analyzed by western blot analysis of whole cell lysates as described in the Materials and Methods. Similar results (A and B) were observed in replicate (at least 3) experiments. (D) Quantitation of phospho-Akt and LC3 proteins. The effects of iSp in phospho-Akt and LC3 proteins were determined as a % of Lamin/ β -actin protein ratios from 3 replicate western blot analyses as indicated

in (C) and described in the Materials and Methods. Results are expressed as means \pm SE and significant ($p < 0.05$) increases or decreases in the iSp transfected compared to the iLamin transfected groups are indicated (*).

Decreased EGFR expression in 253JB-V and KU7 cells transfected with iSp did not affect phospho-MAPK, MAPK or Akt expression but decreased phospho-Akt protein levels (Figures. 22C and 22D), and this was similar to the effects of curcumin and BA on phospho-Akt in these cell lines, suggesting that this response is EGFR-dependent (Figures. 21A and 21B). SGLT expression was not affected, whereas LC3 was induced in 253JB-V and KU7 cells transfected with iSp (Figures. 22C and 22D).

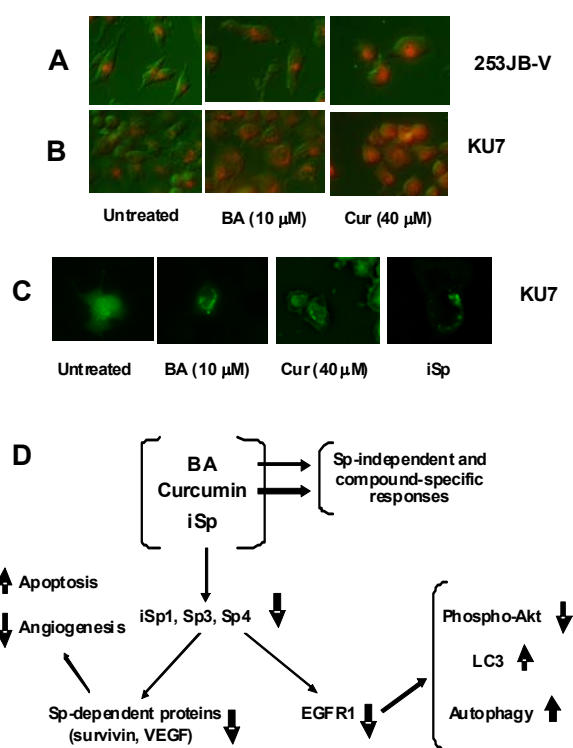


Figure 23. Induction of autophagy; Induced acridine orange staining in 253JB-V (A) and KU7 (B) cells.

These results demonstrate both similarities and differences between the effects of BA and curcumin vs. transfection with iSp in bladder cancer cells and this is due, in part, to responses induced by both compounds that are independent of Sp transcription factors and EGFR1 (301). Induction of LC3, a protein biomarker for autophagy was observed after ablation of EGFR1 by treatment of 253JB-V and KU7 cells with curcumin and BA (Figures. 21A and 21B) or transfection with iSp (Figures. 22C and 22D), and this was consistent with induction of autophagy in prostate and breast cancer cells in which EGFR1 expression was decreased directly by RNA interference (369). Further confirmation that BA, curcumin and Sp knockdown by RNA interference induced autophagy in 253JB-V and KU7 cells is illustrated in Figures 23A and 23B. Compared to the DMSO (untreated controls), BA, curcumin and iSp induced acridine orange staining which is consistent with formation of acidic autophagic vacuoles (autophagolysosomes) which are characteristically observed in autophagic cells (370). In KU7 cells transfected with the GFP-LC3 construct, there was a diffuse pattern of green fluorescence through the cells (Figure 23C); however, treatment with 10 μ M BA and 40 μ M curcumin or transfection with iSp induced a punctate fluorescent staining which is also characteristic of autophagy (371). Transfection with GFP-LC3 was unsuccessful in 253JB-V due to cytotoxicity (data not shown). Thus, BA, curcumin and iSp decrease Sp-dependent proteins including EGFR1 and also decrease EGFR1-dependent phospho-Akt and induction of autophagy in bladder cancer cells (Figure 23D).

Discussion

Sp transcription factors are critical for early embryonic development in mouse models; however, there is evidence that expression of Sp1 decreases with age in humans and laboratory animal models (372). Several different cancer cell lines overexpress Sp1, Sp3 and Sp4 proteins including breast cancer cell lines (263) (299); however, in immortalized but not transformed MCF10A

cells, expression of these proteins was significantly decreased (373). Similar differences were observed in human prostate tumors (xenografts) in athymic nude mice and mouse liver, and ongoing studies in mouse tissue/organs including proliferative gastrointestinal tissue and bone marrow confirm the low to non-detectable expression of Sp1, Sp3 and Sp4 in mature mice (data not shown). Differences in expression of Sp proteins in tumor vs. non-tumor tissue suggests that these transcription factors are potential targets for cancer chemotherapy, particularly since expression of several pro-oncogenic genes including survivin, cyclin D1, VEGF, VEGFR1 and VEGFR2 are Sp-dependent (23-25). Anticancer drugs such as curcumin and BA act, in part, by decreasing expression of Sp1, Sp3 and Sp4 in bladder and prostate tumors, respectively, and the low toxicity of these compounds suggest that their effects on Sp proteins are specific for cancer cells and tumors (263).

The EGFR1 is overexpressed in bladder tumors and tumors derived from other tissues, and wild-type EGFR is primarily expressed in bladder tumors (374). The EGFR tyrosine kinase inhibitor gefitinib is effective only on NSCLC patients that express activating kinase domain mutations of the EGFR; however, in bladder tumors and cancer cell lines, differential gefitinib responsiveness is not dependent on these mutations. Figure 18A shows that gefitinib differentially decreases survival of 253JB-V cells (gefitinib-responsive) compared to gefitinib non-responsive KU7 cells (366). In contrast, curcumin and BA decrease survival of both 253JB-V and KU7 bladder cancer cells (Figures. 18A and 18B) with only minimal differences in their responsiveness to both compounds. BA, like curcumin, also decreased Sp1, Sp3 and Sp4 expression in both cell lines and this was accompanied by decreased expression of Sp-dependent genes (survivin, VEGF and bcl-2) and induction of PARP cleavage (Figure 19). These results are typically observed in cancer cell lines following treatment with compounds that decrease Sp proteins or transfection with iSp that decreases Sp1, Sp3 and Sp4 levels by RNA interference. EGFR1 expression in some

cancer cell lines is also dependent on Sp transcription factors (375) (263). In this study, we focused on curcumin- and BA-mediated effects on EGFR1 expression in bladder cancer cells since this pathway may significantly contribute to the anticancer activity of these compounds.

Figures 20A and 20B illustrate that EGFR1 protein and mRNA levels were decreased in 253JB-V and KU7 cells after treatment with BA or curcumin, and analysis of tumor lysates from a prior mouse xenograft study with KU7 cells shows that curcumin also significantly decreased EGFR in tumors (Figure 20C) and this was accompanied by decreased expression of Sp1, Sp3 and Sp4 (299) (263). A comparison of the effects of gefitinib, BA and curcumin in gefitinib-responsive 253JB-V indicates that all three agents decreased EGFR-dependent phosphorylation of MAPK and Akt (Figures. 21A and 21C). In contrast, BA and curcumin but not gefitinib decreased phospho-Akt levels in KU7 cells, and BA and curcumin increased phospho-MAPK expression in KU7 cells, whereas minimal effects were observed for gefitinib. These responses, coupled with the downregulation of Akt protein (KU7 and 253JB-V cells) by BA and curcumin and MAPK protein by BA only in 253JB-V cells, may be associated with effects of these compounds that are independent of their downregulation of Sp or EGFR proteins (Figure 23D) and are currently being investigated.

A recent study in prostate and breast cancer cell lines investigated EGFR1 kinase-dependent and -independent responses by directly decreasing EGFR1 by RNA interference or by overexpression of wild-type and kinase domain mutant EGFR1 expression plasmids (369). One of the important observations was identification of a kinase-independent function of EGFR1 in which the wild-type and mutant (kinase domain) EGFR1 stabilized SGLT1 to prevent autophagic cell death and EGFR1 knockdown resulted in decreased SGLT1 expression and enhanced accumulation of LC3. Formation of the cleaved form of LC3 is critical for autophagosome formation and is a positive marker for autophagolysosomes (368). Both BA and curcumin induced LC3

accumulation and downregulated SGLT1 in 253JB-V and KU7 cells (Figures. 21A and 21B) and knockdown of EGFR by transfection with iSp also induced LC3 accumulation but did not decrease SGLT1 expression (Figure 22C). Not surprisingly, gefitinib did not affect expression of either SGLT1 or LC3 (Figure 21C). BA, curcumin and iSp transfection decreased EGFR1 expression (Figures. 20A and 22B) and therefore, their differences with respect to expression of SGLT1 in bladder cancer cells may be EGFR1-independent and associated with other activities of BA and curcumin (Figure 23D). The induction of autophagy by BA, curcumin and iSp was also confirmed by their induction of acridine orange staining (Figures. 23A and 23B) and induction of punctuate perinuclear green fluorescence in KU7 cells transfected with GFP-LC3 (Figure 23C). Both of these staining/fluorescent responses are characteristic of autophagy (370) (371).

In summary, results of this study demonstrate that BA- and curcumin-dependent repression of Sp1, Sp3 and Sp4 in bladder cancer cells also decrease expression of the Sp-dependent gene EGFR1 in both gefitinib-responsive 253JB-V and gefitinib-nonresponsive KU7 cells. Thus, BA and curcumin represent a new type of EGFR1 inhibitor that indirectly targets EGFR1 and EGFR1-mediated responses through repression of Sp transcription factors. This results in inhibition of EGFR1-dependent kinases and activation of autophagic cell death which is repressed by EGFR1 (kinase-independent) (369). An additional chemotherapeutic advantage of these compounds is that they also induce pro-apoptotic, antiproliferative and antiangiogenic activities through downregulation of Sp-dependent survivin, cyclin D1 and VEGF/VEGFR1 expression, respectively (263) (Figures. 2C and 2D) and several other responses that are Sp-independent (263) (Figure 23D). Relative contributions of Sp-dependent and Sp-independent pathways to the overall anticarcinogenic activity of curcumin and BA and other drugs that repress Sp proteins and Sp-regulated proteins such as EGFR1 will also vary with tumor type. Currently, we

are investigating the mechanisms of action and clinical applications of drugs such as BA and curcumin alone and in combination with other cytotoxic compounds that are used for clinical treatment of bladder cancer.

IV. CELASTROL DECREASES SPECIFICITY PROTEINS (Sp) AND FIBROBLAST GROWTH FACTOR RECEPTOR-3 (FGFR3) IN BLADDER CANCER CELLS

Bladder cancer incidence has been increasing and even after chemotherapy with highly cytotoxic drugs, the prognosis for disease-free survival in most patients is only 1 to 2 years. Celastrol, a naturally occurring triterpenoid acid from an ivy-like vine exhibits anticancer activity against 253JB-V and KU7 bladder cancer cells. Celastrol decreased cell proliferation, induced apoptosis and decreased expression of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 and several Sp-dependent genes/proteins including vascular endothelial growth factor (VEGF), survivin, and cyclin D1. Fibroblast growth factor receptor 3 (FGFR3) is overexpressed in bladder tumors and celastrol also caused a dose-dependent downregulation of this receptor in 253JB-V and KU7 bladder cancer cells. Using small inhibitory RNAs (siRNA) for Sp1, Sp3 and Sp4 (in combination) we showed that FGFR3 receptor expression in bladder cancer cells is regulated by Sp transcription factors. The mechanism of Sp downregulation by celastrol was due to induction of reactive oxygen species (ROS) and inhibitors of ROS blocked celastrol-induced growth inhibition and Sp repression. *In vivo* studies using KU7 cells as xenografts, showed that celastrol, decreased tumor growth and expression of Sp proteins confirming that celastrol represents novel class of anticancer drugs that act, in part through targeting downregulation of Sp transcription factors.

Introduction

Bladder cancer is the ninth most common cancer worldwide and ranks thirteenth as the cause of cancer deaths (376). It is estimated that in 2009 68,000 new cases will be diagnosed and 14,000 deaths will occur from this disease in the United States. At the time of detection, approximately 25% of

bladder cancer cases are already muscle-invasive and are usually treated by cystectomy. However, after removal of primary tumors by transurethral resection, multiple recurrences continue to develop in 70% of the patients (377). In spite of the initial response to intra-vesicular Bacillus Calmette-Guerin (BCG) administration, bladder tumors eventually recur to form an invasive phenotype which often leads to surgical removal of the bladder (333, 378). MVAC (Methotrexate, Vinblastine, Adriamycin and Cisplatin) chemotherapy which is extensively used for treatment of advanced bladder cancer is accompanied by toxic side effects and thus it is important to develop less toxic alternate therapies. The cytotoxic paradigm might not offer a successful means to change the outcome for metastatic bladder cancer patients (333), however epidemiological and cross-cultural studies show some success of dietary management strategies in the prevention of this disease (379).

New mechanism based drugs for bladder cancer chemotherapy have been reported and these include tyrosine kinase inhibitor against the epidermal growth factor receptor (EGFR) and antiangiogenic drugs or antibodies that block vascular endothelial growth factor (VEGF) and activation of VEGF receptors. Diindolylmethane derivatives developed in this laboratory inhibit bladder cancer cell and tumor growth through both orphan nuclear receptor-dependent and independent pathways (380). In addition, curcumin, the active component of turmeric also exhibits anticancer activity *in vitro* and *in vivo* against bladder cancer (333) and studies in this laboratory suggest that the mechanism of action of curcumin was due, in part, to downregulation of specificity (Sp) transcription factors (263) .

In this study we have investigated the activity of the triterpenoid celastrol (CSL) as an inhibitor of bladder cancer cell and tumor growth. Celastrol (CSL) is a quinone methide triterpene extracted from the root of *Tripterygium wilfordii*, (also known as Thunder of God vine) and this compound has been used in traditional Chinese medicine to treat immune-inflammatory diseases such as

rheumatoid arthritis (381), chronic nephritis, chronic hepatitis and lupus erythematosus (314) and celastrol acts as a cytokine release inhibitor (382), anti-allergic (383) (312) (384). Although CSL is in clinical trials for rheumatoid arthritis (385), the antitumor activity of this drug has not been extensively investigated. In prostate cancer cells the antitumorigenic activity of CSL was related to inhibition of VEGFR expression, induction of apoptosis and inhibition of proteasome activity (314). Our results show that CSL inhibits growth of 253JB-V and KU7 bladder cancer cells and tumor growth in athymic nude mice bearing KU7 cells as xenografts. We also observed that celastrol also induced apoptosis in both bladder cancer cell lines and decreased expression of Sp1, Sp3 and Sp4 proteins and Sp-dependent genes important for survival (survivin), growth (cyclin D1), angiogenesis (VEGF). In addition we observed that fibroblast growth factor receptor 3 (FGFR3) which is overexpressed in bladder cancer is also an Sp-regulated gene and is downregulated in bladder cancer cells after treatment with celastrol. Celastrol-induced downregulation of Sp proteins was due to induction of reactive oxygen species (ROS) which has recently been shown to decrease Sp protein expression in cancer cells.

Materials and methods

Cell lines. KU-7 and 253JB-V human bladder cancer cells were provided by Dr. A. Kamat, (M.D Anderson Cancer Center, Houston, TX). 253JB-V and KU7 cell lines 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 (Sigma Aldrich, St. Louis, MO).

Antibodies, chemicals and other materials. Sp1, Sp3, Sp4, VEGF, survivin, CD1, p65, p50 and FGFR3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP (ASP 214) antibody was purchased from Cell Signaling Technology (Danvers, MA). Monoclonal β -actin

antibody was purchased from Sigma-Aldrich. Horseradish peroxidase substrate for western blot analysis was obtained from NEN Life Science Products (Boston, MA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Celastrol, 98% pure, was purchased from Biopurify (Sichuan, China) and Calbiochem (Gibbstown, NJ). GSH is a tripeptide composed of L-glutamic acid, L-cysteine, and L-glycine. Glutathione (**GSH**), 98% pure was purchased from Aldrich (Sigma-Aldrich Inc, (St. Louis, MO). Dithiothreitol (DTT), 98% was obtained from Boehringer Mannheim Corp, (Indpls, IN).). The pVEGF-2018 construct contains VEGF promoter inserts (positions –2018 to +50) linked to luciferase reporter gene. The pSurvivin-269 contains survivin promoter inserts (positions –269 to +49) linked to luciferase reporter gene and was kindly provided by Dr. M. Zhou (Emory University, Atlanta, GA). The pSp1 and pSp3 promoter constructs (pSp1-FOR4-luc and pSp3-FOR5-luc) were provided by Drs Carlos Ciudad and Veronique Noe (University of Barcelona). Sp1-FOR4-luc (contains the –751 to –20 region of the Sp1 gene promoter) and pSp3-FOR5-luc (contains the –417 to –38 region of the Sp3 promoter) constructs respectively. YH633p(-220/-27)FR3-luc(C4) FGFR3 plasmid was a kind gift from Dr. Young-Kwon Hong (USC, CA) and Dr. David Ornitz, (WUSTL, MO).

Cell proliferation assays. Bladder cancer cells (3×10^4 cells per well) were seeded using DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS in 12-well plates and left to attach for 24 hr. Cells were then treated with either vehicle (DMSO) or the indicated concentrations of celastrol. Fresh medium and test compounds were added every 24 hr and cells were then counted at the indicated times using a Coulter Z1 particle counter. Each experiment was carried out in triplicate and results are expressed as means \pm SE for each treatment group.

Western blot assays. Bladder cancer cells were seeded in DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS. Twenty-four hr later, cells were treated with either vehicle (DMSO) or the indicated

compounds for 24 hr. Cells were collected using high-salt buffer [50 mmol/L HEPES, 0.5 mol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10% glycerol, and 1% Triton-X-100 pH 7.5] and 10 µL/mL of Protease Inhibitor Cocktail (Sigma Aldrich). The lysates were incubated on ice for 1 hr with intermittent vortexing every 10 min for a total of 90 min, followed by centrifugation at 20,000 g for 10 min at 4°C. Lysates were then incubated for 3 min at 100°C before electrophoresis, and then separated on 10% SDS-PAGE 120 V for 3 to 4 hr in 1X running buffer [25 mM Tris-base, 192 mM glycine and 0.1% SDS]. 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 0.9A at 4°C. The 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 1:200- 1:1000 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 (PerkinElmer Life Sciences, Waltham, MA) substrate for 1.0 min, and exposed to Kodak X-OMAT AR autoradiography film (American X-ray supply Inc, Jackson, CA). The tumor tissues were also processed and β-actin served as loading control. The tumor lysates were processed and probed for Sp and FGFR3 proteins and protein quantitation was determined using Image J software and the optical band intensities were plotted after normalization with lamin /β-actin. Nuclear and cytoplasmic extracts were separated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce).

SiRNA interference assay. The two bladder cancer cell lines, 253JB-V and KU7 cells were seeded (1×10^5 per well) in 6-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotic and left to attach for one day. The triple Sp SiRNA knockdown (iSp1, iSp3, iSp4

complex) along with iLamin as control was performed using Lipofectamine reagent according to the manufacturer's instructions. Small inhibitory RNAs were prepared by Dharmacon RNA Technologies (Chicago, IL) and the oligonucleotides used in this study are the same as previously described before (263).

Transfection and luciferase assays. The luciferase construct of FGFR3 containing minimal Sp binding sites on FGFR3 YH633p(-220/-27)FR3-luc(C4) was transfected into the two bladder cancer cell lines. Bladder cancer cells (1×10^5 per well) were plated in 12-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 16-24 hr, various amounts of DNA (i.e., 0.4 μ g pGL2/pGL3; 0.04 μ g β -galactosidase; and 0.4 μ g FGFR3, YH633p(-220/-27)FR3-luc(C4), pVEGF, pSurvivin, pSp1For4 and pSp1For5 constructs were transfected using Lipofectamine reagent according to the manufacturer's protocol and then treated with control solvent (DMSO) and different concentrations of celastrol. SiRNAs for lamin (control) and siSp1, siSp3 and siSp4 (combined) were also transfected into these cells and luciferase activity (normalized to β -galactosidase) was determined essentially as described.

Terminal deoxyribonucleotide transferase-mediated nick-end labeling assay. 253JB-V and KU7 cells (7×10^4) were seeded in four-chambered glass slides and left 12 hr to attach. After celastrol treatment for 20 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.

ROS estimation. Cellular reactive oxygen species (ROS) levels were evaluated with the cell-permeant probe **CM-H₂DCFDA** (5-(and-6)-chloromethyl-

2'7' dichlorodihydrofluorescein diacetate acetyl ester). **CM-H₂DCFDA** is nonfluorescent until removal of the acetate groups by intracellular esterases and oxidation occurs within the cell. Following treatment for 20-24 hr, cells were grown in a 96 well cell culture plate, then treated with 10 μ M CM-H₂DCFDA 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 determination 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 μ l 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 performed on different days. At least 16 wells per treatment were analyzed for each experiment.

Xenograft study. Female athymic nude mice, age 4-6 weeks were purchased from Harlan (Indianapolis, CA) and KU7 cells (1×10^6 cells) in Matrigel (BD Biosciences, San Jose, CA) (1:1) were injected into the either side of flank area of nude mice. When tumors were palpable, mice were divided into two groups of ten animals each. The first group received 100 μ L vehicle (corn oil) by intraperitoneal route, and the second group of animals received 4 mg/kg/d dose of celastrol in corn oil every second day for 22 days (eleven doses) by the intraperitoneal route of administration. After 23 days, the mice were sacrificed; tumors in control and treated groups were homogenized and probed for Sp proteins by western blots. Weights of animals and tumor areas were recorded every alternate day before the treatment.

Statistical analysis. Statistical significance of differences between treatment groups was determined by an analysis of variance and student t-test,

and the levels of probability were noted. IC_{50} values were calculated using linear regression methods and expressed as μM concentrations.

Results

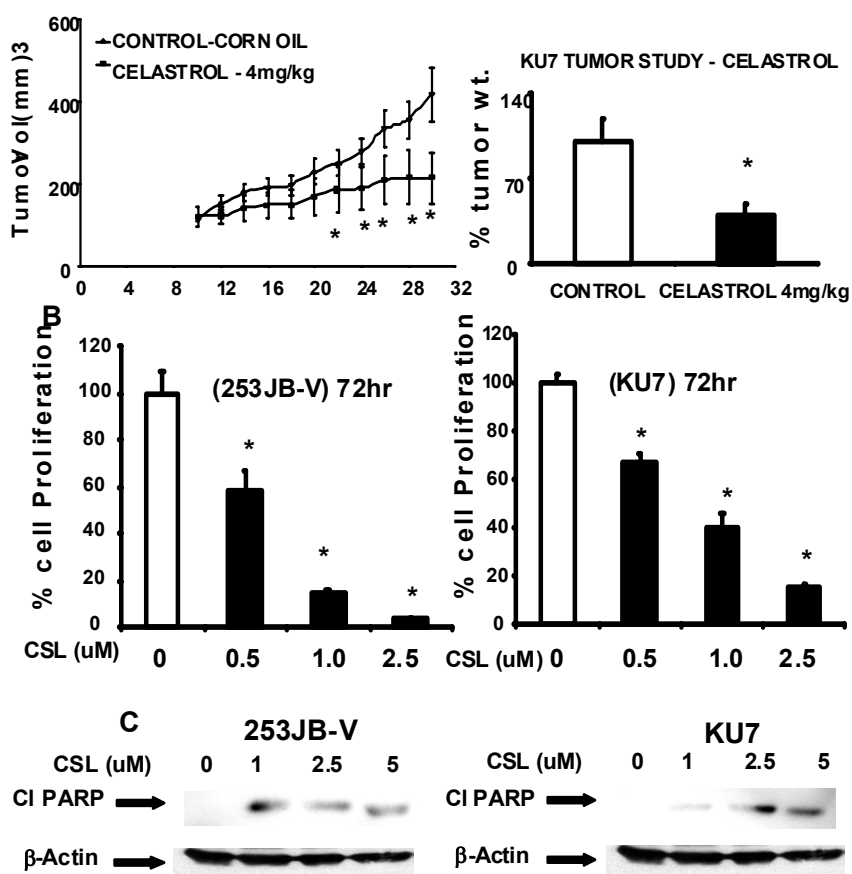


Figure 24. CSL inhibits growth of bladder tumors and bladder cancer cells and induces apoptosis.

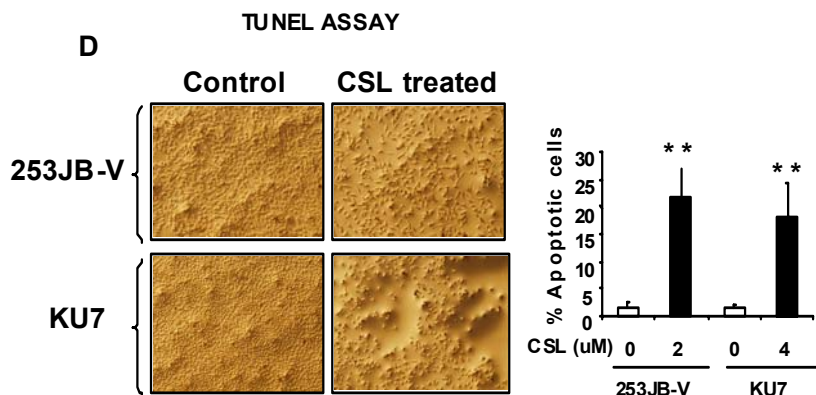


Figure 24 continued.

A. CSL decreases KU7 xenograft tumor volume and weight. Mice were injected and treated as described in the materials and methods. B. Inhibition of 253JB-V and KU7 cell growth by CSL. Cells were treated with DMSO (solvent control); 0.5, 1.0 or 2.5 $\mu\text{mol/L}$ CSL and the effects on cell growth were determined after treatment for 72 hr as described in Materials and Methods. C. Induction of apoptosis by CSL.

253JB-V and KU7 cells were treated with DMSO (0) and 1.0, 2.5 or 5.0 $\mu\text{mol/L}$ CSL for 24 hr and induction of cleaved PARP protein was determined as described in Materials and Methods. D. CSL increases TUNEL positive cells. 253JB-V and KU7 cells were treated with DMSO (0) and 2.0 or 4.0 $\mu\text{mol/L}$ CSL for 20 hr and increase in TUNEL positive cells was determined as described in Materials and Methods. Results are expressed as means \pm 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* antitumor activity of CSL (4 mg/Kg/d) was investigated using female athymic nude mice bearing KU7 bladder xenograft model. **Figure. 24A** shows that CSL significantly attenuated tumor growth in nude mice as evidenced

by decreased tumor volumes and tumor weights (**Figure. 24A**). These results are comparable to previous prostate tumor studies where growth inhibition by celastrol was observed at doses of ≤ 4 mg/kg/d (314). Celestrol (0.5-2.5 μ M) also inhibited growth of 253JB-V and KU7 bladder cancer cells (Figure 24B) and growth inhibitory IC_{50} values for 253JB-V and KU7 cells are 0.93 and 1.13 μ M respectively after treatment for 72 hr. Earlier reports show that CSL-induced apoptosis in LNCaP prostate cancer cells was associated with PARP cleavage (314) and results illustrated in **Figure. 24C** indicate that CSL also significantly induced PARP cleavage, a signature protein for apoptosis in both the cell lines. We further confirmed this finding using the TUNEL (terminal dUTP nick end labeling) assay where apoptotic TUNEL positive staining is significantly increased in both 253JB-V and KU7 bladder cancer cell lines after treatment with 4 μ M CSL for 20 hr (**Figure. 24D**). The % apoptotic cells obtained by counting apoptotic cells in various representative fields are plotted in (**Figure. 24D**), and confirm a statistically significant induction of apoptosis by CSL.

Figure 25A summarizes the effects of CSL on expression of survivin, VEGF, and cyclin D1 proteins in both 253JB-V and KU7 bladder cancer cells. We observed that concentrations of CSL ≤ 1.0 μ M decreased the expression of all three proteins in 253JB-V cells whereas higher concentration (≥ 1.0 μ M) were required to induce similar effects in KU7 cells. We further investigated the effects of CSL on luciferase activity in 253JB-V and KU7 bladder cancer cells transfected with pVEGF and pSurvivin constructs containing -2019 to +50 and -269 to +49 GC rich inserts from the VEGF and survivin gene promoters respectively.

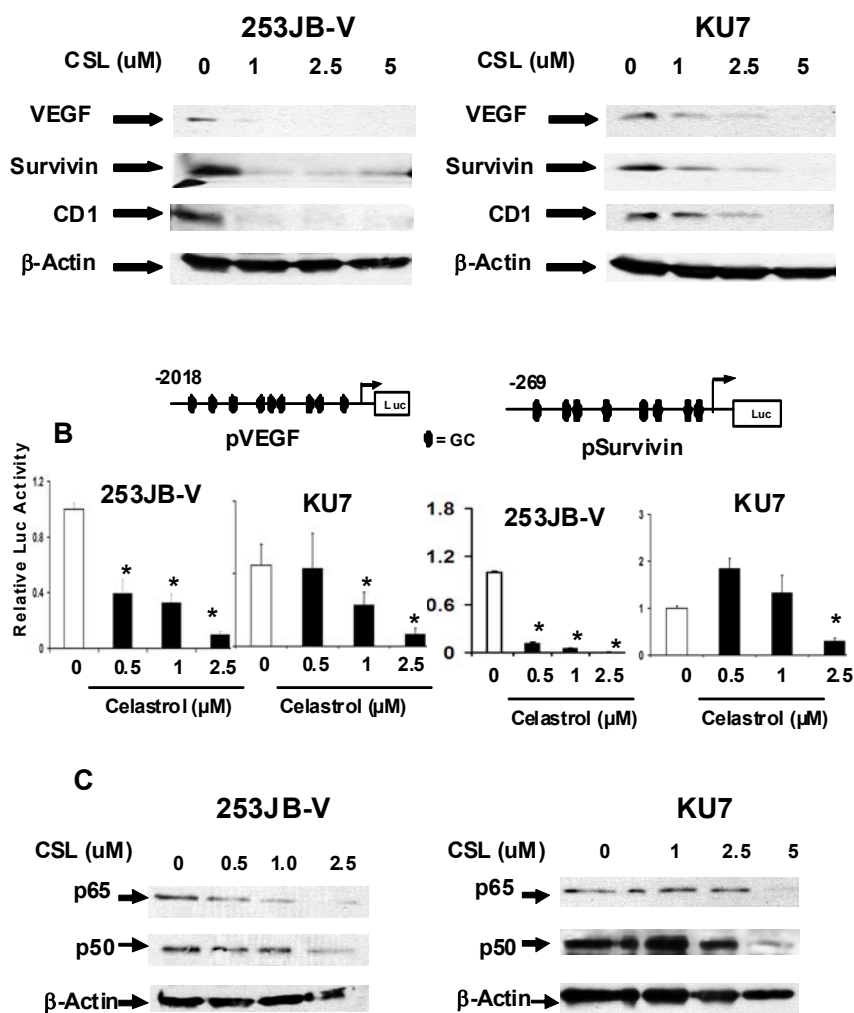


Figure 25. Effects of CSL on angiogenic, survival and cell cycle proteins.

CSL decreases VEGF, survivin and cyclin D1 proteins in 253JB-V and KU7 at 24 hr (A), and also decreases luciferase activity of the cells transfected with VEGF and survivin gene promoters (B). Bladder cancer cells were treated with DMSO (solvent control); 0.5, 1.0, 2.5 or 5.0 $\mu\text{mol/L}$ CSL and whole cell lysates were analyzed by western blots or luciferase activity was determined as indicated in Materials and Methods. C. CSL degrades NF κ B subunits. Cells were treated with DMSO (solvent control); 0.5, 1.0, 2.5 or 5.0 $\mu\text{mol/L}$ CSL and

the nuclear fraction was analyzed by Western blot analysis for p65 and p50 subunits as described in Materials and Methods. β -Actin served as a loading control and similar results were observed in duplicate experiments. Results are expressed as means \pm SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) CSL-induced decreases (*) compared to the solvent (DMSO) control are indicated.

The results show that CSL also significantly decreased luciferase activities in 253JB-V and KU7 cells transfected with these constructs suggesting that CSL also affected transcription of these genes (**Figure 25A**). The 253JB-V cells were also more responsive to the effects of CSL than KU7 cells in the transfection assays and this was similar to that observed for protein downregulation shown in **Figure 25A**.

Since CSL has been used in clinical trials to treat inflammatory conditions (383), the effects of CSL on the expression of the p65 and p50 subunits of NF κ B, in nuclear extracts of 253JB-V and KU7 bladder cancer cells were determined (**Figure 25C**). CSL, significantly decreased p65 and p50 protein subunits of NF κ B and these results are consistent with previous findings that CSL inhibits NF κ B activation in multiple cell lines (386).

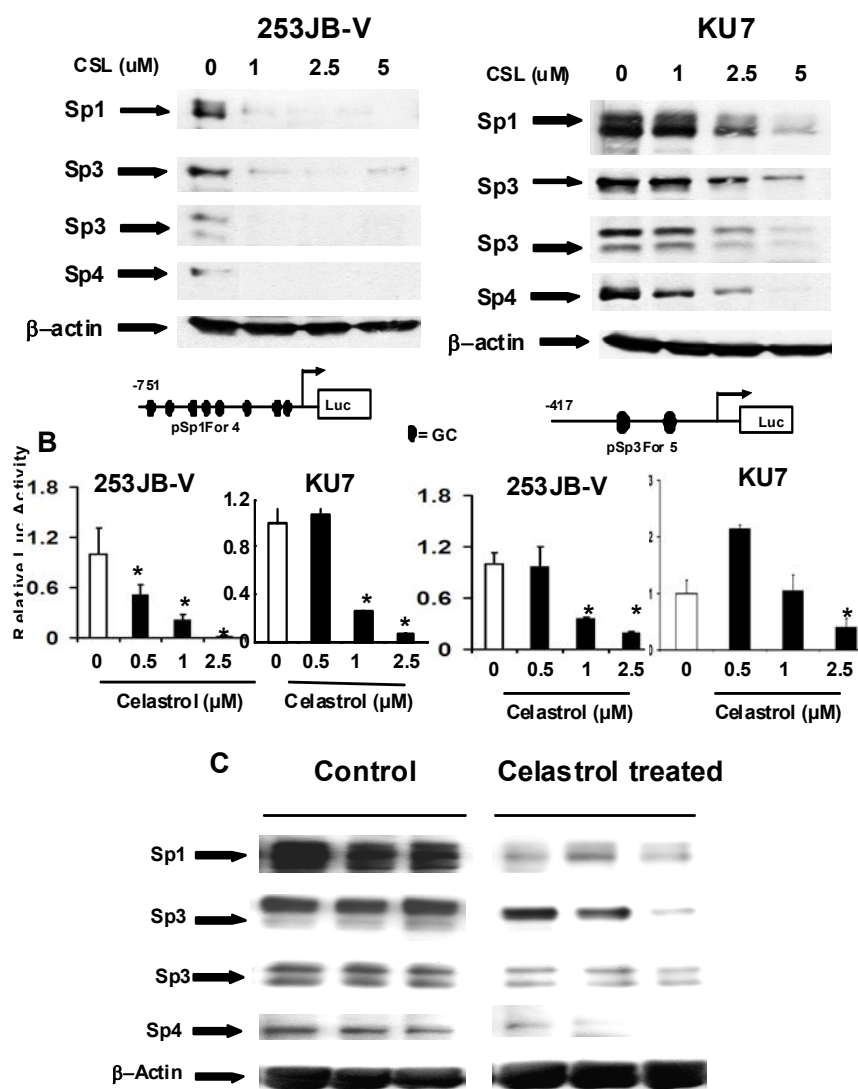


Figure 26. *In vitro* and *in vivo* effects of CSL on Sp proteins.

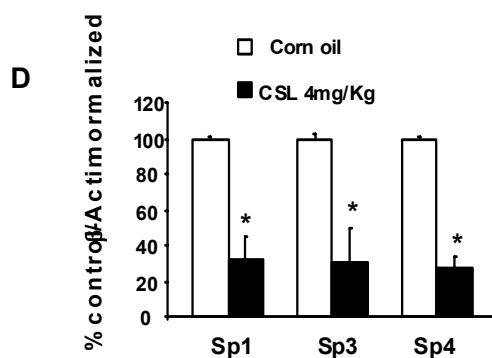


Figure 26 continued.

CSL decreases Sp proteins at 24 hr in bladder cancer cells. Cells were treated with DMSO and 1.0, 2.5 or 5.0 $\mu\text{mol/L}$ CSL 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 and similar results were observed in duplicate experiments. *B.* CSL decreases luciferase activity in cells transfected with Sp gene promoters. Bladder cancer cells were transfected with the indicated constructs, treated with DMSO and 0.5, 1.0 or 2.5 $\mu\text{mol/L}$ CSL for 24 hr and luciferase activity was determined as described in Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) CSL-induced decreases (*) compared to the solvent (DMSO) control are indicated. *C.* Sp downregulation in tumors. Western blot analysis of tumor lysates from three mice in the treated and control groups was also determined as described in Materials and Methods. *D.* Quantitation results. Relative Sp protein band intensities of control and CSL treated groups determined in western blots were quantitated as described in Materials and Methods and significant ($P < 0.05$) decreases are indicated (*). Levels of Sp proteins in tissues from CSL treated mice were compared to controls (set at 100%).

Studies in this laboratory showed that knockdown of Sp1, Sp3 and Sp4 proteins by RNA interference decreased expression of VEGF, survivin and cyclin D1 in bladder and other cancer cell lines and therefore we investigated the effects of CSL on levels of Sp1, Sp3 and Sp4 proteins which are highly expressed in bladder cancer cells (263). Results in Figure 26A shows that CSL induced a concentration-dependent decrease in expression of Sp1, Sp3 and Sp4 proteins in both the cell lines. 253JB-V cells were more sensitive to this response than KU7 cells and this was consistent with the responsiveness of these cell lines to CSL-induced growth inhibition and downregulation of CD1, survivin and VEGF (Figure 2A).

Results in **Figure 26B** show that there was a concentration-dependent decrease in luciferase activity in KU7 and 253JB-V cells transfected with pSp1 (Sp1For4) and pSp3 (Sp3For5) constructs which contain -751 to -20 and -417 to -38 regions of Sp1 and Sp3 gene promoters, respectively suggesting that CSL also affects transcription of Sp1 and Sp3 (note: the Sp4 promoter is not yet available)

The *in vitro* effects of CSL were also observed in the *in vivo* study where CSL (4 mg/kg/d) also significantly decreased Sp1, Sp3 and Sp4 protein levels in tumors compared to corn oil treated controls (Figures 26C&26D). In contrast, expression of Sp1, Sp3 and Sp4 proteins in non-tumor tissue was minimal.

Previous studies in this laboratory reported that tolfenamic acid (TA), betulinic acid (BA) and curcumin induced proteasomal degradation of Sp proteins in pancreatic, prostate and bladder cancer cells (6) (299) (346). Results summarized in Figure 27A show that the proteasome inhibitor MG132 inhibited CSL-induced Sp degradation in KU7 cells as previously reported for curcumin in the cell line, however MG132 did not inhibit the CSL-induced downregulation of Sp proteins in 253JB-V cells and similar results were obtained with other proteasome inhibitors (data not shown). Thus the role of CSL-induced proteasome activation on degradation of Sp1, Sp3 and Sp4 was cell context-

dependent. Recent studies in this laboratory show that reactive oxygen species (ROS) is a critical upstream regulator of Sp downregulation and this was typified by effects of arsenic trioxide which also decreases Sp proteins through perturbation of mitochondria and the subsequent induction of ROS in 253JB-V and KU7 cells (submitted). Results summarized in Figure 27B illustrate that treatment of 253JB-V and KU7 cells with CSL decrease mitochondrial membrane potential and this was accompanied by induction of ROS was determined using cell-permeant dye CM-H₂DCFDA.

The potential role of CSL-induced ROS on Sp proteins expression was investigated in 253JB-V and KU7 cells treated with 2.5 μ M or 5 μ M CSL alone or in combination with the antioxidants DTT or GSH (Figure 27C). CSL-induced downregulation of Sp1, Sp3 and Sp4 was inhibited after cotreatment with DTT where as the inhibitory effects of GSH were less than observed for DTT.

The contributions of ROS to growth inhibition were confirmed in both cell lines where treatment with 2.5 or 4.0 μ M CSL inhibited cell proliferation and cotreatment with 1 mM DTT significantly reversed the growth inhibitory effects of CSL (Figure 27D). We also observed that both DTT and GSH blocked the growth inhibitory and morphological changes induced by CSL in both bladder cancer cell lines as determined by differential interference contrast microscopy. FGFR3 is overexpressed and/or activated by mutations in bladder cancer and recent reports show that FGFR3 knock-down attenuated tumor progression in a mouse xenograft study indicating that FGFR3 is a potential drug target in bladder cancer (387). Since the FGFR3 promoter also contains GC-rich Sp binding sites we have investigated the effects of CSL on FGFR3 expression and the role of Sp downregulation in mediating these effects.

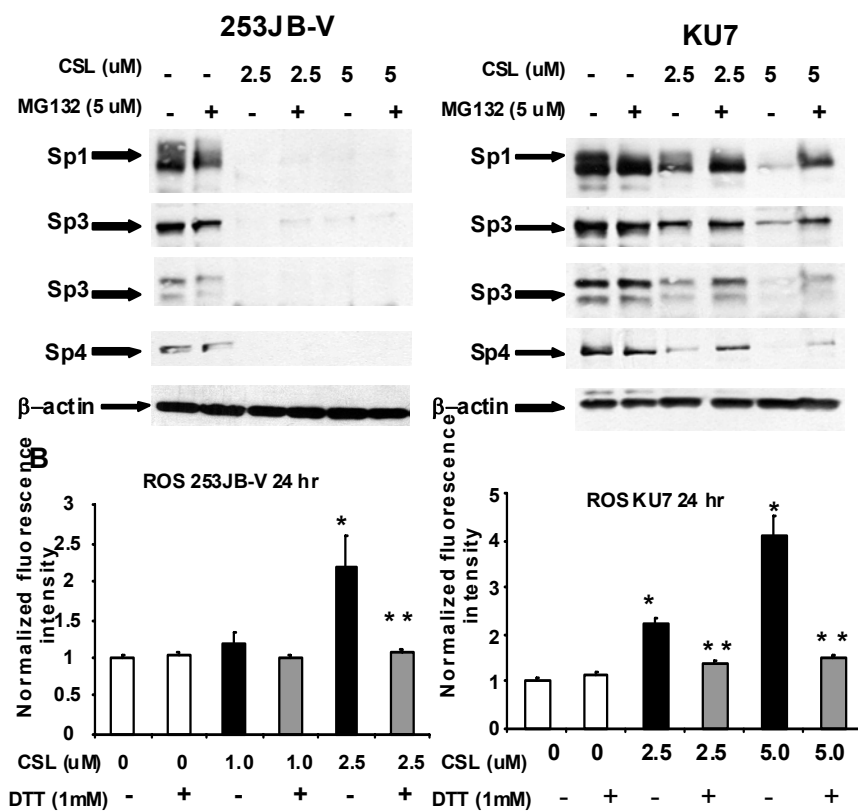


Figure 27. CSL modulates differential - proteasome dependent, ROS dependent Sp protein degradation and cell growth.

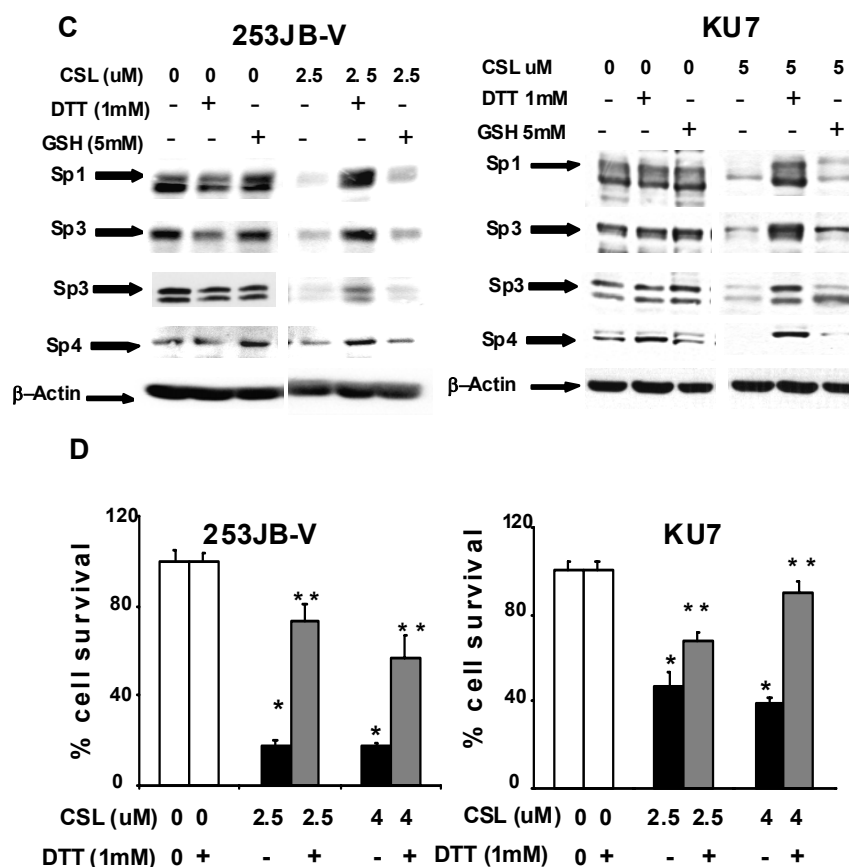


Figure 27. continued.

Cells were treated with DMSO, CSL alone or in combination with DTT and ROS levels were determined as described in the Materials and Methods. CSL-induced growth inhibition (C) and ROS mediated cell growth and Sp protein degradation (D) reversed by thiol antioxidants. 253JB-V and KU7 cells were treated with DMSO and 1.0, 2.5, 4.0 or 5 $\mu\text{mol/L}$ CSL for 24 hr, in the presence or absence of antioxidants DTT and GSH and 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. Results are expressed as means \pm 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.

Bladder cancer cells transfected with FGFR3 (C4)-luciferase promoter and then treated with DMSO and 0.5, 1.0 or 2.5 $\mu\text{mol/L}$ CSL for 24 hr and luciferase activity was determined as described in Materials and Methods. *C.* effects of iSp on FGFR3 protein. 253JB-V and KU7 cells were transfected with iSp (a cocktail of iSp1, iSp3, and iSp4) or iLamin, and whole cell lysates were analyzed for the efficiency of Sp knockdown and the effects on FGFR3 protein levels by Western blots as described in Materials and Methods. *D.* effects of iSp on FGFR3 promoter. 253JB-V and KU7 cells were transfected with iSp (a cocktail of iSp1, iSp3, and iSp4) or iLamin, and transfected with FGFR3 (C4)-Luc; luciferase activity was determined as described in Materials and Methods.

Results are expressed as means \pm SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) CSL-induced decreases (*) compared to the solvent (DMSO) or Lamin control are indicated.

Treatment of 253JB-V and KU7 cells with CSL significantly decreased FGFR3 protein levels in both bladder cancer cell lines in a dose dependent manner (Figure 28A). The western blot illustrates multiple FGFR3 bands (including the non-glycosylated, precursor and glycosylated forms) and CSL decreased expression of all forms of FGFR3 in both cell lines.

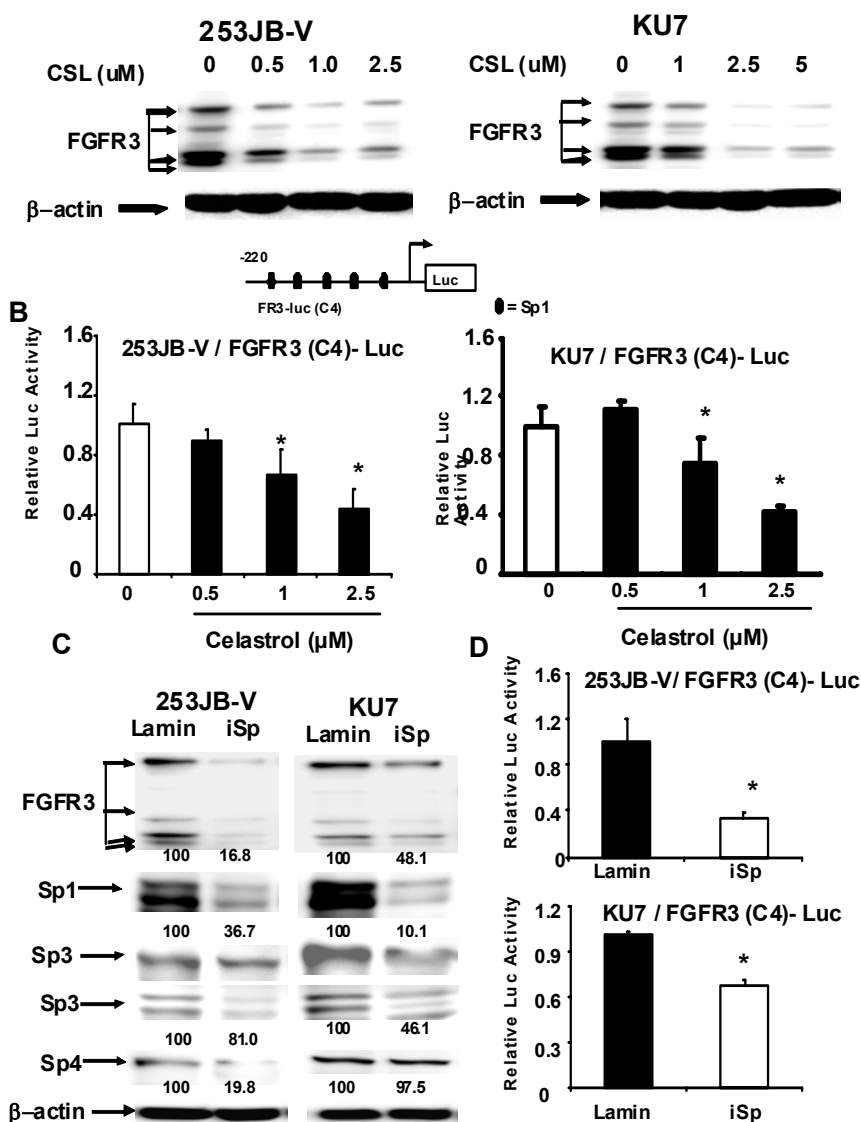


Figure 28. CSL decreases FGFR3 protein and promoter expression by reducing Sp protein expression.

A. CSL degrades FGFR3 protein in bladder cancer cells. 253JB-V and KU7 cells were treated with DMSO alone, 0.5, 1.0, 2.5 or 5.0 μmol/L CSL and whole-cell lysates were analyzed by Western blots as described in Materials and Methods. B. CSL decreases luciferase activity in cells transfected with the FGFR3 promoter luciferase construct.

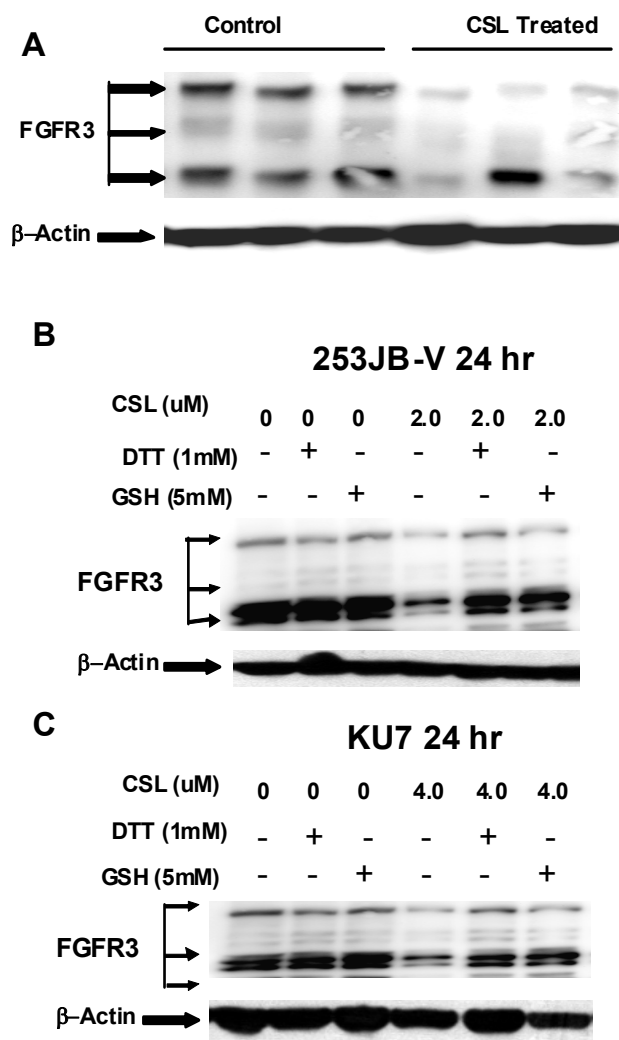


Figure 29. CSL degrades FGFR3 protein *in vivo* and induces ROS-dependent degradation of FGFR3.

A. CSL decreases FGFR3 protein expression in KU7 xenograft tumors when compared to corn oil control group. Western blot analysis of tumor lysates from three mice in the treated and control groups was determined as described in Materials and Methods. CSL modulates ROS mediated FGFR3 degradation in 253JB-V (B) and KU7 (C) bladder cancer cells. 253JB-V and KU7 cells were

treated with DMSO and 2.0 or 4.0 $\mu\text{mol/L}$ CSL for 24 hr, in the presence or absence of antioxidant DTT and whole cell lysates were analyzed for FGFR3 protein by western blots as described in Materials and Methods. β -Actin served as a loading control and similar results were observed in duplicate experiments.

Moreover, CSL also decreased the luciferase activity in bladder cancer cells transfected with a construct containing the minimal -220 to -27 GC rich region of the FGFR3 gene promoter (Figure 28B). The role of Sp proteins in mediating the expression of FGFR3 were further investigated using a small inhibitory RNA (siRNA) cocktail (iSp) containing siRNAs targeted to Sp1 (iSp1), Sp3 (iSp3) and Sp4 (iSp4) as previously described (263). Cells were transfected with iSp and whole cell lysates were examined in western blots which show that Sp1, Sp3 and Sp4 were decreased with variable overall efficiency in both cell lines (Figure 28C). We also observed that transfection of iSp also decreased FGFR3 protein levels by 16.8 and 48.1 % in 253JB-V and KU7 cells (Figure 28C).

We also investigated the effects of iSp (iSp1, iSp3 and iSp4) on the FGFR3 promoter activity and the results indicate that transfection with the iSp cocktail, significantly decreased the FGFR3 promoter activity (Figure 28D) and this was consistent with our results showing that FGFR3 is, in part, regulated by Sp transcription factors in bladder cancer cells.

Figure 29A shows that CSL also downregulated expression of FGFR3 in tumors compared to tumors from control (corn oil treated) mice and these results were similar to those observed for decreased Sp1, Sp3 and Sp4 expression in these tumors (Figure 26C). In addition we also observed that the antioxidants DTT and GSH that blocked CSL-dependent downregulation of Sp proteins (Figure 27C) and also inhibited downregulation of FGFR3 in 253JB-V (Figure 29B) and KU7 (Figure 29C) cells treated with CSL. Thus the antitumorigenic activity of CSL is due, in part to induction of ROS which in turn induces

downregulation of Sp1, Sp3 and Sp4 and Sp-dependent genes/proteins including FGFR3.

Discussion

Traditional medicines have been providing leading edge pharmaceutical agents for decades and interest in the use of traditional medicines for cancer prevention and treatment is increasing. Moreover, a large percentage of FDA approved anti-cancer drugs are obtained from plant derived natural products (94). Celastrol, a pentacyclic triterpene, from the root bark of *Tripterygium wilfordii*, the thunder god vine, has been used for treatment of several diseases including arthritis (381), lupus erythematosus (388), amyotrophic lateral sclerosis (389), Alzheimer's disease (313) and cancer. Recent studies have identified various molecular targets for the anti-tumor activity of CSL and these include inhibition of NF κ B (386, 390) proteasome activity (314), inhibition of HSP90 (391) and topoisomerase II (392), suppression of VEGFR (393) (394) and inhibition of ATF2 activation (395). The electrophilic nature of the quinone methide moiety of CSL has been linked to irreversible binding to proteins, possibly through cysteinyl residues and this is associated with the cytotoxic effects of CSL in melanoma cells (395).

Recent studies in this laboratory demonstrated that curcumin inhibited growth of 253JB-V and KU7 bladder cancer cells and tumors in athymic nude mice bearing KU7 cells as xenografts (222). Results illustrated in Figure 1 demonstrate that CSL, like curcumin also exhibited growth inhibitory and anticarcinogenic activity in bladder cancer cells and tumors. CSL induced apoptosis in bladder cancer cells (Figures 24C&24D) and decreased expression of VEGF, survivin and cyclin D1 proteins (Figure 25A) and these results were also observed for curcumin in bladder cancer cells (263). Interestingly, CSL decreased expression of the p65 and p50 proteins that form the NF κ B complex (Figure 25C) whereas curcumin only decreased p50 in 253JB-V and KU7 cells

but had minimal effects on p65 expression. The pathways associated with CSL-dependent inhibition of p65/p50 and NF κ B is currently being investigated. Research in this laboratory has previously reported that the glycyrrhetic acid derivative methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oate (CDODA-Me), NSAID tolfenamic acid, betulinic acid and curcumin inhibited growth and induced apoptosis in several different cancer cell lines and this was due, in part, to downregulation of Sp transcription factors and Sp-regulated genes (263) (299) (211). Sp proteins such as Sp1, Sp3 and Sp4 are overexpressed in cancer cell lines and tumors (21) and Sp1 is a negative prognostic factor for survival of pancreatic and gastric cancer patients (396). Moreover, we have confirmed the important causal role of Sp transcription factors in carcinogenesis since knockdown of Sp1, Sp3 and Sp4 by RNA interference decreases expression of VEGF, VEGFR1, VEGFR2, cyclin D1, bcl-2, survivin and the epidermal growth factor receptor (EGFR) in several cancer cell lines (263). Since both CSL and Sp1/Sp3/Sp4 knockdown both decrease expression of VEGF, survivin and cyclin D1, we hypothesized that CSL also decreased Sp proteins and this was confirmed in KU7 and 253JB-V bladder cancer cell lines and KU7 tumors in a xenograft experiment (Figures 26A&27A).

Several studies show that a high percentage of bladder cancers overexpress FGFR3 gene (397) (398) (399) and somatic mutations in the FGFR3 gene were identified in 60-70% papillary and 16-20% of muscle invasive bladder cancers (397) (398) (400). FGFR3 was shown to be a vital oncogenic factor due to its transforming activity and knockdown of FGFR3 arrested cell cycle progression and inhibited tumor growth in an *in vivo* model for bladder cancer demonstrating that FGFR3 is an important target for bladder cancer therapy (387). Regulation of FGFR3 expression in bladder cancer has not been previously defined; however, since the FGFR3 promoter contains GC-rich Sp binding sites (401), we investigated the effects of CSL and the role of Sp proteins in regulating FGFR3 expression in 253JB-V and KU7 cells.

Figure 28A illustrates that CSL decreased FGFR3 expression in both 253JB-V and KU7 cancer cell lines in a concentration-dependent manner and CSL also inhibited luciferase activity in these cell lines transfected with pFGFR3 (-220), a construct containing the GC-rich -220 to -27 region of the FGFR3 promoter linked to luciferase reporter gene (Figure 28B).

Furthermore, knockdown of Sp1, Sp3 and Sp4 in 253JB-V and KU7 cells by RNA interference also significantly decreased FGFR3 protein and luciferase activity in 253JB-V and KU7 cells (Figure 28C&28D). These results demonstrate for the first time that FGFR3 is an Sp-regulated gene in bladder cancer cells and that compounds such as CSL that decrease expression of Sp1, Sp3 and Sp4 in cancer cell lines also repress FGFR3 along with other Sp-regulated genes/proteins. Previous studies with drugs that decrease Sp protein levels in cancer cells indicate that downregulation is either proteasome-dependent or independent; for the latter response we have demonstrated that for CDODA-Me, this compound downregulates microRNA-27a (miR-27a) resulting in upregulation of ZBTB10, an Sp repressor (300).

Results illustrated in figure 27A show that CSL induces proteasome-dependent downregulation of Sp1, Sp3 and Sp4 proteins in KU7 cells; however, in 253JB-V cells this response was proteasome-independent. CSL did not affect miR-27a or ZBTB10 expression in bladder cancer cells (data not shown) and the mechanisms associated with downregulation of Sp proteins is currently being investigated. Recent studies with the anti-cancer drug arsenic trioxide have indicated that this compound inhibits growth of solid tumors and their derived cancer cell lines and in bladder cancer cells (data not shown) this response is associated with induction of ROS and the subsequent ROS-dependent downregulation of Sp1, Sp3 and Sp4 (submitted). Moreover arsenic trioxide-induced growth inhibition and Sp downregulation are blocked by the ROS inhibitors DTT and/or GSH and results summarized in Figures 27C and 27D

show that the growth inhibitory effects of CSL and downregulation of Sp proteins by this compound are also reversed by DTT.

In summary this study reports for the first time that celastrol decrease expression of Sp1, Sp3 and Sp4 transcription factors and Sp-dependent genes which include FGFR3, a clinically important therapeutic target for bladder cancer treatment. CSL also decreased expression of other Sp dependent genes such as survivin, cyclin D1 and VEGF which are important for cell survival, growth and angiogenesis. CSL also decreased MMP in bladder cancer cells resulting in induction of ROS which exhibits intrinsic anticancer activities and this includes ROS-dependent downregulation of Sp proteins & Sp-dependent genes. The importance of induction of ROS as a chemotherapeutic pathway has been previously reported (402) , however this study demonstrates that CSL-induced ROS leads to downregulation of Sp transcription factors which are highly overexpressed in cancer cells and tumors and are important drug targets.

V. STRUCTURE-DEPENDENT INHIBITION OF BLADDER AND PANCREATIC CANCER CELL GROWTH BY 2-SUBSTITUTED GLYCYRRHETINIC AND URSOLIC ACID DERIVATIVES *

Derivatives of oleanolic acid, ursolic acid and glycyrrhetic acid substituted with electron withdrawing groups at the 2-position in the A-ring which also contains a 1-en-3-one structure are potent inhibitors of cancer cell growth. In this study, we have compared the effects of several 2-substituted analogs of triterpenoid acid methyl esters derived from ursolic and glycyrrhetic acid on proliferation of KU7 and 253JB-V bladder and Panc-1 and Panc-28 pancreatic cancer cells. The results show that the 2-cyano and 2-trifluoromethyl derivatives were the most active compounds. The glycyrrhetic acid derivatives with the rearranged C-ring containing the 9(11)-en-12-one structure were generally more active than the corresponding 12-en-11-one isomers. However, differences in growth inhibitory IC_{50} values were highly variable and dependent on the 2-substituent (CN vs. CF_3) and cancer cell context.

Introduction

Pentacyclic triterpenoid acids such as betulinic acid, oleanolic acid, ursolic acid, and glycyrrhetic acid are phytochemicals that have been extensively used in traditional medicines for treatment of a wide variety of human ailments (403). Most of these compounds exhibit anti-inflammatory and anticarcinogenic activities as well as a large number of compound-specific effects.

* Reprinted with permission from "Structure-dependent inhibition of bladder and pancreatic cancer cell growth by 2-substituted glycyrrhetic and ursolic acid derivatives" by Chadalapaka, G., Jutooru, I., McAlees, A., Stefanac, T. and Safe, S. *Bioorg. Med. Chemistry Lett.* 18:2633-2639, 2008. Copyright 2009 by Bioorganic and Medicinal Chemistry Letters, Inc.

For example, the major bioactive component of licorice extracts is glycyrrhizic acid glycoside which is readily hydrolyzed to glycyrrhetic acid and these extracts/compounds possess anti-inflammatory, antiviral and endocrine activities (404). All of these triterpenoid acids have been used as building blocks for the synthesis of more active analogs. Oleanolic acid has been converted into 2-cyano-3,12-dioxolean-1,9-dien-28-oic acid (CDDO), the corresponding methyl ester (CDDO-Me) and other structurally-related analogs, and these compounds are potent anticancer agents (405) (310). These synthetic triterpenoids activate the nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ) (405) (406) and also induce several other responses including apoptosis in various cancer cell lines. The cytotoxicity and anti-inflammatory activity of CDDO-Me and related compounds are due to the 2-cyano group and the 1-en-3-one and 9-en-12-one functionalities in the A- and C-rings, respectively (407) (408). We also showed that the glycyrrhetic acid analog methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oate (β -CDODA-Me) and the corresponding 18 α derivative α -CDODA-Me) which contain 2-cyano-1-en-3-one and 12-en-11-one functionalities in their A- and C-rings, respectively, activated PPAR γ and were also highly cytotoxic in colon cancer cells (409). Similar results were obtained for the betulinic acid derivatives containing a 2-cyano-1-en-3-one function (305). In this study, we have further investigated the structure-dependent growth inhibitory effects of β -CDODA-Me and analogs containing different electronegative 2-substituents and a selected number of the corresponding ursolic acid 2-substituted-1-ene-3-one derivatives. In addition, we also show that for the glycyrrhetic acid derivatives growth inhibitory differences of the 9-en-12-one and 12-en-11-one isomers are lower than previously reported for anti-inflammatory activity of CDDO-Me and the 12-en-11-one isomers (408). Growth inhibition by the 2-substituted glycyrrhetic acid and ursolic acid methyl ester analogs is dependent on the nature of the 2-substituent and cancer cell

context among two bladder (KU7 and 253JB-V) and two pancreatic (Panc-1 and Panc-28) cancer cell lines.

Materials and methods

Chemicals. The ursolic acid and glycyrrhetic acid starting materials were purchased from Sigma-Aldrich (St. Louis, MO) all chemical reagents used for synthesis were purchased from Sigma-Aldrich. Dulbecco's modified/Ham's F-12 and RPMI media and the antibiotic/antimycotic solution were also obtained from Sigma Aldrich.

Cell lines, chemicals and other materials. KU7 human bladder cancer and Panc-1 pancreatic cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and 253JB-V cells were provided by Dr. A. Kamat, M.D Anderson Cancer Center, Houston, TX. The Panc-28 cell line was a generous gift from Dr. Paul Chiao, The University of Texas M.D. Anderson Cancer Center (Houston, TX).

Both 253JB-V and KU7 cell lines 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 pancreatic cancer cells were maintained in Dulbecco's modified/Ham's F-12 with phenol red supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 5% fetal bovine serum and 10 ml/L 100x antibiotic/antimycotic solution. The cells were grown in 150 cm² culture plates in an air/CO₂ (95:5) atmosphere at 37°C and passaged approximately every 3 days.

Cell proliferation assays. All four cancer cell lines (3 x 10⁴ cells per well) were plated using DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS in 12-well plates and left to attach for 24 hours. Cells were then treated with either vehicle (DMSO) or the indicated concentrations of different compounds. Fresh medium and test compounds were added every 48 hours

and cells were then 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 determination. The IC₅₀ values were calculated using linear regression method and were expressed in μ M.

Results

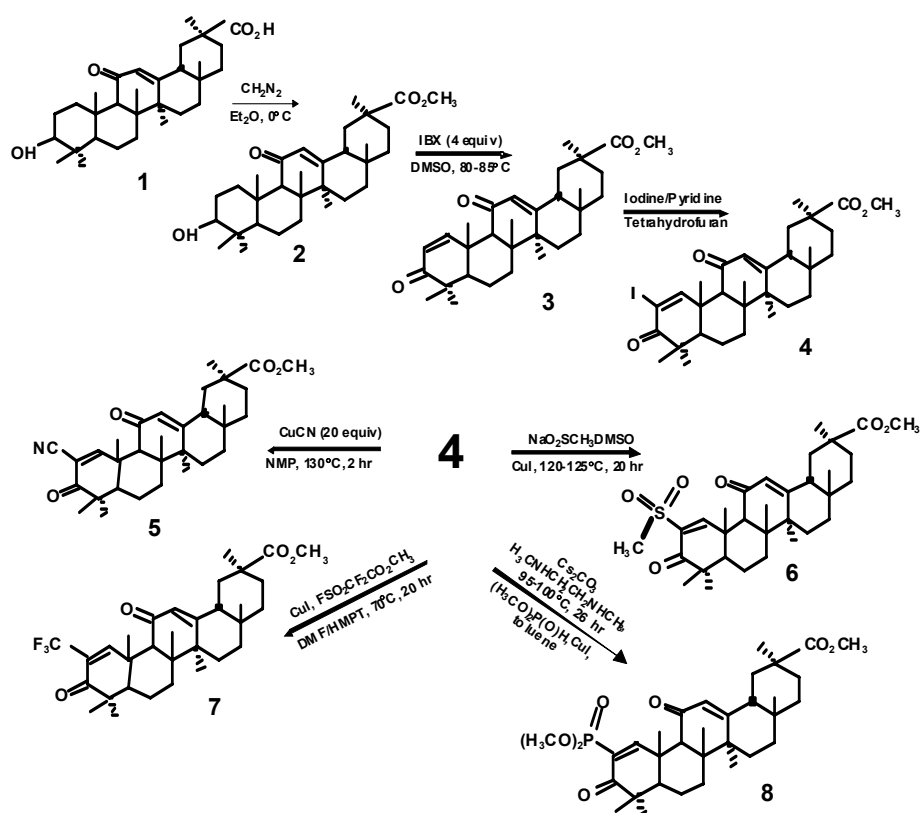


Figure 30. Synthesis of 2-substituted-1-en-3-one derivative of methyl glycyrrhetinate.

The synthesis of a series of 2-substituted glycyrrhetinic acid derivatives is summarized in Figure 30. The free acid (1) was esterified at 0°C with ethereal

diazomethane to give methyl glycyrrhetinate (**2**) which was then treated with 4 equivalents of 2-iodoxybenzoic acid (IBX) in DMSO at 85°C for 21 hours. The resulting methyl 3,11-dioxo-18 β -oleana-1,12-dien-30-oate product (**3**) was then converted into the 2-iodo derivative (**4**) by treating with 2 equivalents of iodine and 3 equivalents of pyridine in tetrahydrofuran (refluxing). The methyl 2-iodo-3,11-dioxo-18 β -oleana-1,12-dien-30-oate derivative (**4**) was used as the precursor for the synthesis of the 2-cyano (**5**), 2-methanesulfonyl (**6**), 2-trifluoromethyl (**7**) and 2-dimethylphosphonyl (**8**) analogs (Figure 30). The 2-cyano derivative was prepared by treating **4** with 2 equivalents of cuprous cyanide in N-methylpyrrolidinone (NMP) for 2 hours at 130°C. The 2-methanesulfonyl derivative was obtained by treating **4** with sodium methanesulfinate and cuprous iodide in DMSO at 120-125°C for 20 hours. The 2-dimethylphosphonyl analog was synthesized by treating **4** with dimethylphosphite, cesium carbonate, N,N-dimethylethylenediamine in toluene at 95-100°C for 26 hours. Finally the 2-trifluoromethyl derivative was obtained by treating **4** with cuprous iodide and methyl 2,2-difluoro-2-(fluorosulfonyl) acetate in dimethylformamide/HMPT at 70°C for 20 hours. The synthetic scheme used for conversion of ursolic acid (**9**) into the 2-iodo derivative (**12**) and the corresponding 2-cyano (**13**) and 2-trifluoromethyl (**14**) derivatives (Figure 31) involved intermediates (**10**) and (**11**) and was identical to that carried out for conversion of methyl glycyrrhetinate into the analogous 2-substituted compound as illustrated in Figure 30.

Previous studies with oleanolic acid derivatives reported the synthesis of several analogs including CDDO which contain the 1-en-3-one A-ring and a 9-en-12-one C-ring(407), and Figure 32 outlines an analogous route for conversion of methyl glycyrrhetinate into the rearranged C-ring analogs of CDODA-Me. Methyl glycyrrhetinate was reduced with H₂/platinum oxide catalyst in acetic acid, then acetylated with acetic anhydride/ pyridine in dimethylaminopyridine (DMAP) to give the acetylated ester (**15**) in which the 9-

oxo group had been reduced. Treatment with *m*-chloroperbenzoic acid (*m*-CPBA) in methylene chloride gave the 12,13-epoxide which was converted into the 12-oxo derivative by boron trifluoride etherate in methylene chloride. Bromination of the 12-oxo derivative followed by dehydrobromination in acetic anhydride followed by basic hydrolysis of the acetate group gave the rearranged C-ring derivative of methyl glycyrrhetinate (**16**). Subsequent treatment with 2-iodoxybenzoic acid and iodine/ pyridine in tetrahydrofuran gave methyl 2-iodo-3,12-dioxo-oleana-1,9-dien-30-oate (**17**) which is converted into the C-ring rearranged cyano (**18**) and trifluoromethyl (**19**) derivatives as outlined in Schemes 1 and 2.

Previous studies with CDDO-Me and related compounds showed that the 1-en-3-one structure containing electronegative 2-substituent groups such as cyano were highly cytotoxic (310) and similar results were observed for β -CDODA-Me, the corresponding 2-cyano analog of glycyrrhetic acid (409). In this study, we used 253JB-V and KU7 bladder and Panc-1 and Panc-28 pancreatic cancer cells to investigate the growth inhibitory effects of CDODA-Me analogs containing several electronegative 2-substituents including iodo, cyano, trifluoromethyl, dimethylphosphonyl and methanesulfonyl groups (Table 5). β -CDODA, the parent free acid derivative of β -CDODA-Me is used as a control for this group of compounds, and IC_{50} values for inhibition of bladder and pancreatic cancer cell growth varied from 5.9 – 7.3 μ M.

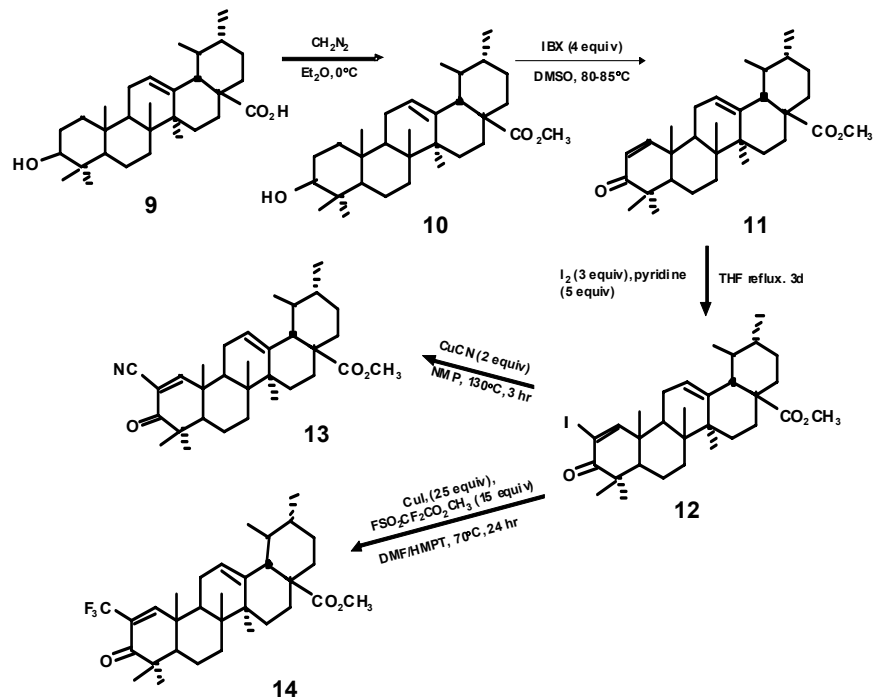


Figure 31. Synthesis of 2-substituted-1-en-3-one derivatives of methyl ursolate.

The corresponding range of IC_{50} values for β -CDODA-Me was 0.25 to $1.80 \mu\text{M}$ indicating that in cell culture studies the methyl ester derivatives were more potent than the free acid (β -CDODA) as previously reported for growth inhibition of colon cancer cells (409). The effects of compounds unsubstituted at C-2 were >7 fold less active than the 2-cyano derivatives (data not shown) and these results were similar to those observed for these compounds in colon cancer cells (409). Based on the synthetic scheme which used the 2-iodo derivative (4) as a precursor, we synthesized the 2-trifluoromethyl, dimethylphosphonyl and methanesulfonyl derivatives and determined the effects of different electronegative substituents on their inhibition of bladder and pancreatic cancer cell growth (Table 5). The IC_{50} values were lowest for 2-cyano and 2-trifluoromethyl (β - CF_3 DODA-Me) derivatives; however, their relative

potencies were dependent on cell context; β -CF₃DODA-Me was more active than β -CDODA-Me in KU7 (IC₅₀: 0.38 vs. 1.59 μ M), Panc-1 (IC₅₀: 0.82 vs. 1.22 μ M) and Panc-28 (IC₅₀: 1.14 vs. 1.80 μ M) cells, whereas β -CDODA-Me was more active in 253JB-V cells (IC₅₀: 0.25 vs. 0.67 μ M). Both the 2-dimethylphosphonyl and 2-methanesulfonyl analogs were relatively inactive with IC₅₀ values ranging from 3.34 - 12.0 μ M over the four cell lines.

Since the 2-cyano and 2-trifluoromethyl derivatives were the most active of the glycyrrhetic acid group of 2-substituted 1-en-3-one compounds, we synthesized a similar series of analogs from methyl ursolate and compared their growth inhibitory IC₅₀ values to the 2-iodo analog and methyl ursolate (Table 6).

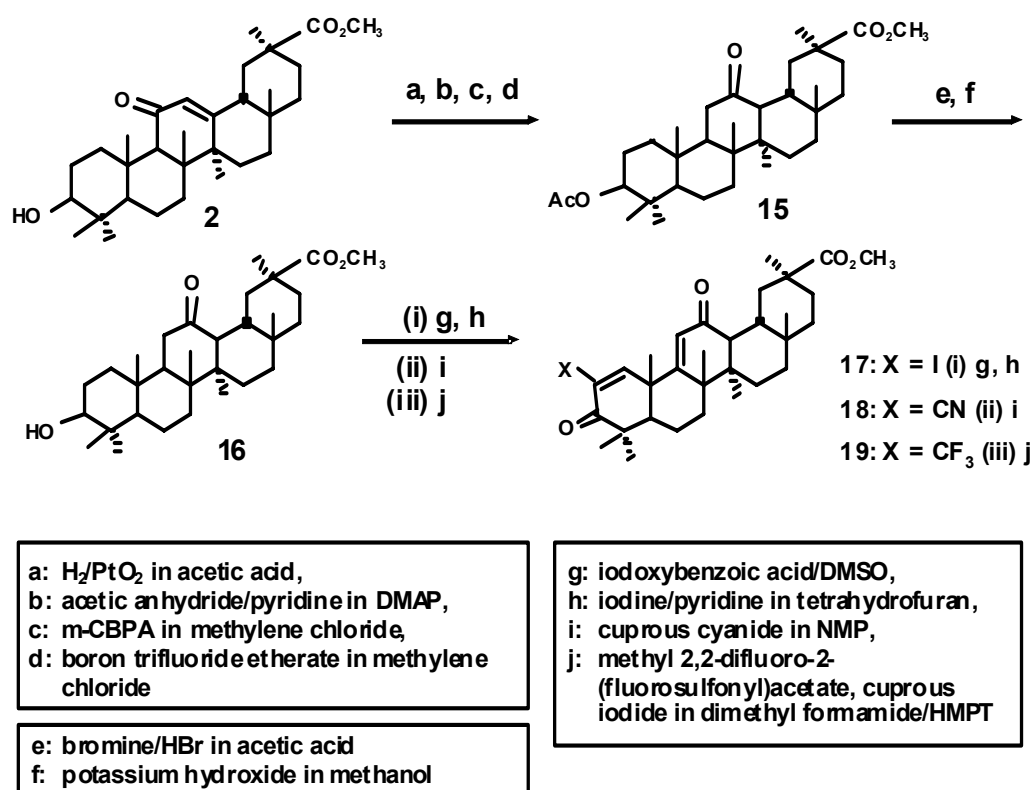
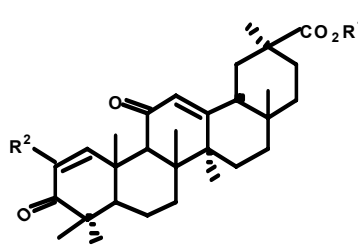


Figure 32. Synthesis of C-ring rearranged analogs of CDODA-Me.

Differences in IC_{50} values for the 2-cyano methyl-glycyrrhetinate isomers with 12-en-11-one and 9(11)-en-12-one functionalities were more dramatic and varied from a 2.2- (253JB-V cells) to a 39.1-fold (Panc-28) lower IC_{50} values for isomers containing the former C-ring structure. These differences for the 2-cyano derivatives of methyl glycyrrhetinate were significantly lower than differences observed for CDDO-Me and the corresponding 12-en-11-one isomer where IC_{50} values for inhibition of nitric oxide production in mouse macrophages was 0.1 nM and 20 nM, respectively, a 200-fold difference (406). We also directly compared the effects of the rearranged 2-cyano methyl glycyrrhetinate derivative (**18**) with CDDO-Me in the four cell lines; the former compound (**18**) was more active than CDDO-Me in Panc-1 and Panc-28 cells, whereas IC_{50} values were similar in KU7 cells and CDDO-Me ($IC_{50} = 0.03\mu\text{M}$) was more active in 253JB-V cells. Thus, a direct comparison of the 2-cyano derivative of methyl glycyrrhetinate (**18**) and CDDO-Me (derived from methyl oleanolate) on their inhibition of cancer cell proliferation indicates that both synthetic triterpenoids are highly potent and differences in their IC_{50} values in four cell lines were less than an order of magnitude.

Table 5. Cytotoxicity of 2-substituted compounds derived from methyl glycyrrhetinate.

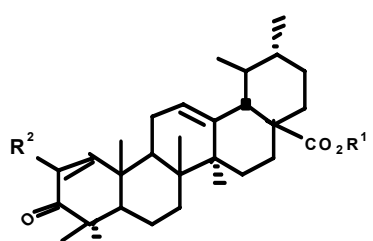


	IC_{50} (μM)			
	253JB-V	KU7	PANC 1	PANC 28
$R^1 = \text{H} : R^2 = \text{CN}$	6.10	5.88	3.81	7.32
$R^1 = \text{CH}_3 : R^2 = \text{I}$ (4)	2.67	3.04	4.08	12.75
$R^1 = \text{CH}_3 : R^2 = \text{CN}$ (5)	0.25	1.59	1.22	1.80
$R^1 = \text{CH}_3 : R^2 = \text{CF}_3$ (6)	0.67	0.38	0.82	1.14
$R^1 = \text{CH}_3 : R^2 = \text{CH}_3\text{SO}_2$ (7)	11.97	3.34	7.69	9.75
$R^1 = \text{CH}_3 : R^2 = (\text{CH}_3\text{O})_2\text{PO}$ (8)	7.90	3.73	6.11	8.14

IC₅₀ values for methyl ursolate (**9**) and the 2-iodo-1-en-3-one analog (**12**) varied from 6.13 - 11.75 and 4.90 - 13.50 μM, respectively, in the bladder and pancreatic cancer cell lines and there was less than a 2-fold difference in their IC₅₀ values. IC₅₀ values for the 2-cyano (**13**) and 2-trifluoromethyl (**14**) analogs varied from 0.17 - 0.97 and 0.17 - 1.13 μM, respectively, and were at least an order of magnitude more potent in the growth inhibition assay than the 2-iodo compound or methyl ursolate.

These data confirm that 2-cyano and 2-trifluoromethyl groups coupled with introduction of a 1-en-3-one functionality into the A-ring of methyl ursolate resulted in enhanced growth inhibition with comparable IC₅₀ values in KU7, 253JB-V, Panc-1 and Panc-28 cancer cells.

Table 6. Cytotoxicity of 2-substituted compounds derived from methyl ursolate.



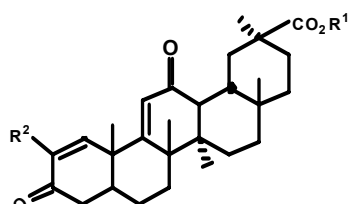
	IC ₅₀ (μM)			
	253 JB-V	KU7	PANC 1	PANC 28
Methyl Ursolate (a)	6.13	8.95	11.75	10.58
R ¹ = CH ₃ ; R ² = I (12)	4.90	6.02	6.91	13.49
R ¹ = CH ₃ ; R ² = CN (13)	0.17	0.30	0.53	0.97
R ¹ = CH ₃ ; R ² = CF ₃ (14)	0.17	0.47	0.65	1.13

Previous studies with CDDO-Me and related compounds showed that the functionality of the C-ring markedly influenced the activity of oleanolic acid derivatives with 2-cyano-1-en-3-one functionality (406). For example, CDDO-Me which contains a 9(11)-en-12-one C-ring structure is 200 times more potent as an inhibitor of nitric oxide production in mouse macrophages than the corresponding 12-en-11-one isomer (406). We therefore synthesized a series of

2-iodo, 2-cyano, and 2-trifluoromethyl-1-en-3-one analogs of glycyrrhetic acid which also contain a 9(11)-en-12-one C-ring functionality (Table 7) and compared their growth inhibitory effects to the 12-en-11-one isomers (Table 5)

The effects of the C-ring en-one functionality on the activity of these isomers was dependent not only on the 2-substituent but also on cell context. IC_{50} values were similar for both 2-iodo isomers in 253JB-V, KU7 and Panc-1 cells, whereas IC_{50} values for the 12-en-11-one and 9(11)-en-12-one isomers in Panc-28 cells were 12.75 and 3.66 μ M, respectively. Thus, for the 2-iodo derivative, the C-ring structure had minimal effects on growth inhibitory activity, except for one cell line where IC_{50} differences were < 4-fold.

Table 7. Cytotoxicity of 2-substituted compounds derived from methyl glycyrrhettinate with C-ring rearrangement.



	IC_{50} (μ M)			
	253J B-V	KU7	PANC 1	PANC 28
CDDO-Me	0.03	0.12	0.27	0.29
$R^1 = CH_3$: $R^2 = I$ (17)	3.62	2.61	4.45	3.66
$R^1 = CH_3$: $R^2 = CN$ (17)	0.11	0.12	0.07	0.05
$R^1 = CH_3$: $R^2 = CF_3$ (18)	0.36	1.37	0.68	1.13

The antiproliferative activity of the trifluoromethyl derivative (**6**) (Table 5) was <2 fold lower than the 9(11)-en-12-one isomer in 253JB-V and Panc-1 cells, whereas similar IC_{50} values were observed in Panc-28 cells. In contrast the 12-en-11-one (unrearranged) trifluoromethyl analog was more active ($IC_{50} = 0.38$ μ M) in KU7 cells than the corresponding 9(11)-en-12-one isomer ($IC_{50} = 1.37$ μ M). Figure 1 illustrates the structure-dependent differences in growth inhibitory activities in KU7 cells of the 2-cyano and 2-trifluoromethyl analogs containing the unrearranged 12-en-11-one (**5** and **7**) and rearranged 9(11)-en-12-one (**18** and **19**) C-ring in the methyl glycyrrhettinate series of isomers.

Discussion

In summary, the results of this study show that the antiproliferative activities of several 2-substituted glycyrrhetic acid and ursolic acid derivatives containing the 1-en-3-one functionality in their A-rings were maximal for both the cyano and trifluoromethyl derivatives. The corresponding 2-iodo substituted analogs were less active (Tables 5 and 6). The 2-dimethylphosphonyl and 2-methanesulfonyl derivatives of glycyrrhetic acid (Table 5) also exhibited higher IC_{50} values than the corresponding 2-cyano and 2-trifluoromethyl derivatives. The rationale for the observed differences in the activity of the different 2-substituted analogs containing electronegative substituents is unclear and is currently being investigated using other assay systems.

Compared to previous studies with oleanolic acid derivatives (406) the effects of the C-ring functionality [9(11)-en-12-one vs. 12-en-11-one] on inhibition of cancer cell growth were minimal (Tables 5 and 7) and dependent on the 2-substituent (cyano vs. trifluoromethyl) (Figure 33) and cancer cell context. This suggests that for the glycyrrhetic acid derivatives it may not be necessary to carry out the additional synthetic steps to convert the 12-en-11-one to the 9(11)-en-12-one functionality in the C-ring. This is confirmed, in part, by ongoing *in vivo* studies in mouse xenograft experiments where significant inhibition of tumor growth by β -CDODA-Me is observed at doses of 10 to 15 mg/kg/day (data not shown).

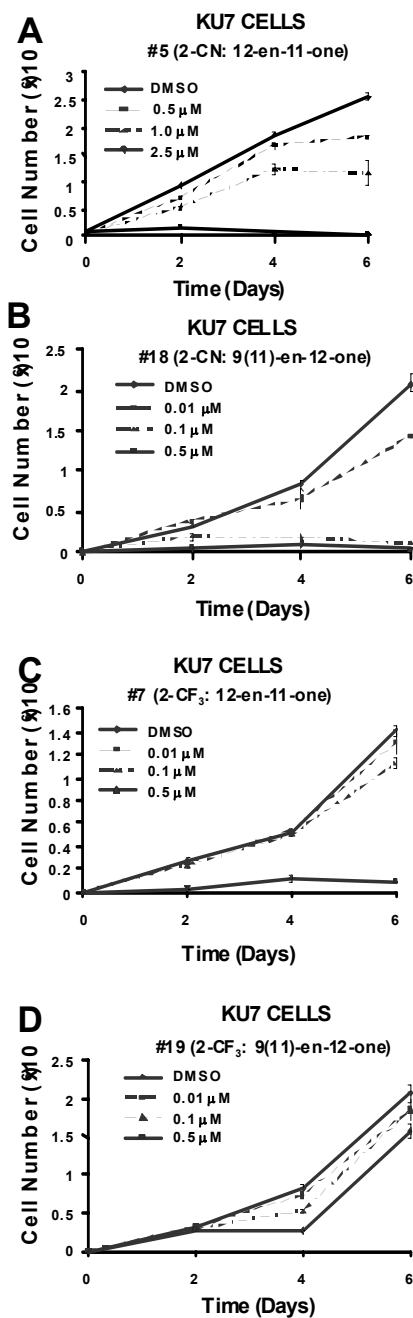


Figure 33. Effects of 2-CN- and 2-CF₃-1-en-3-one analogs containing 12-en-11-one or 9(11)-en-12-one functionality in the C-ring.

Ongoing work includes further investigation of structure-dependent anticarcinogenic potencies of the cyano and trifluoromethyl glycyrrhetic acid analogs, and studies of their underlying mechanism of action and clinical applications.

VI. SYNTHETIC OLEANOLIC ACID-DERIVED TRITERPENOID INHIBIT BLADDER CANCER CELL GROWTH AND SURVIVAL AND DOWNREGULATE SPECIFICITY PROTEIN (Sp) TRANSCRIPTION FACTORS

Methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oate (CDODA-Me) is a synthetic triterpenoid derived from glycyrrhetic acid which inhibits proliferation of KU7 and 253JB-V bladder cancer cells with inhibitory IC₅₀ values < 5 μ M. CDODA-Me-dependent growth inhibition is accompanied by caspase-dependent PARP cleavage and downregulation of survival (survivin and bcl-2) and angiogenic [vascular endothelial growth factor (VEGF) and its receptor (VEGFR1)] genes. CDODA-Me also decreased expression of specificity protein-1 (Sp1), Sp3 and Sp4 transcription factors and this was consistent with downregulation of the Sp-dependent genes survivin, bcl-2, VEGF and VEGFR1. Similar results were observed for a structurally-related triterpenoid, methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), which is currently in clinical trials. Both CDODA-Me and CDDO-Me decreased mitochondrial membrane potential and induced reactive oxygen species (ROS), and these responses were also critical for triterpenoid-induced downregulation of Sp proteins which was inhibited by the antioxidants dithiothreitol and glutathione. This demonstrates a common mechanism of action for CDODA-Me and CDDO-Me which is due, in part, to mitochondriotoxicity.

Introduction

It is estimated that in 2008, there will be 68,810 new cases of bladder cancer and 14,100 deaths from this disease in both men and women. In the United States, bladder cancer is the fourth most common cancer and ranks ninth overall in cancer deaths (410). In western countries, bladder cancer incidence increases with age and is associated with smoking, exposure to occupational carcinogens, and other lifestyle and environmental factors (411) (412). The

predominant tumors are transitional cell carcinomas in patients from industrialized countries, and 70% or more of the cancers are papillary non-invasive tumors, whereas 20-30% are non-papillary invasive bladder cancers (413) (414). The prognosis and treatment for invasive and non-invasive ("superficial") transitional cell carcinomas are different. Patients with non-invasive bladder tumors may have recurrence but their overall prognosis is good, whereas patients with invasive tumors have a poor prognosis with <50% survival after 5 years.

Early stage superficial bladder cancer is usually treated surgically (radical cystectomy) followed by adjuvant therapy with a range of cytotoxic drug combinations and the success of different regimens has been correlated with tumor histopathology or grade and molecular markers (415) (416). For example, patients with superficially early invasive bladder cancer that express mutant p53 have a higher relapse rate than those expressing the wild-type tumor suppressor gene (417) (418).

Treatment for more advanced invasive bladder cancers includes surgery and adjuvant chemotherapies and, in some cases, neoadjuvant chemotherapy is used to decrease tumor size prior to surgery. One of the most common adjuvant chemotherapies has been the combination of methotrexate, vinblastine, doxorubicin and cisplatin (M-VAC), and there has been an increase in the use of cisplatin plus gemcitabine (CG) due to lower toxicity of CG (419). The limited success of bladder cancer chemotherapies has stimulated development and applications of new targeted therapies that block or inhibit the function of critical genes and pathways that contribute to the growth, survival, angiogenesis and metastasis of bladder cancer (420) (421). These include tyrosine kinase inhibitors that block epidermal growth factor receptor (EGFR) or vascular endothelial growth factor receptors (VEGFRs) or neutralizing antibodies such as bevacizumab that interact with VEGF.

Studies in this laboratory have shown that a series of 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methane (C-DIM) compounds that activate orphan nuclear receptors inhibit bladder cancer cell growth (17, 18). We have recently reported that the triterpenoid methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oate (CDODA-Me) is a potent inhibitor of bladder, colon, pancreatic and prostate cancer cell growth (422) (423). CDODA-Me induces apoptosis, inhibited growth and expression of genes associated with cell proliferation (cyclin D1, EGFR), cell survival (survivin, bcl-2), and angiogenesis (VEGF, VEGFR1) in bladder cancer cells and these responses are due, in part, to downregulation of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4. CDODA-Me-dependent responses are due to targeting mitochondria which results in the induction of reactive oxygen species (ROS) which in turn induces downregulation of Sp proteins and Sp-dependent genes.

Materials and methods

Cell lines: KU-7 and 253JB-V human bladder cancer cells were provided by Dr. A. Kamat, (M.D Anderson Cancer Center, Houston, TX). 253JB-V and KU7 cell lines 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 (Sigma-Aldrich, St. Louis, MO).

Antibodies, chemicals and other materials: Sp1, Sp3, Sp4, VEGF, VEGFR1 survivin, CD1, bcl-2, caveolin-1 and EGFR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved PARP (ASP 214) antibody was purchased from Cell Signaling Technology (Danvers, MA). Monoclonal β -actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase substrate for western blot analysis was obtained from NEN Life Science Products (Boston, MA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). GSH is a tripeptide composed of L-glutamic acid, L-

cysteine, and L-glycine. Glutathione (GSH), 98% was purchased from Aldrich (Sigma- Aldrich Inc, (St. Louis, MO). Dithiothreitol (DTT), 98% was obtained from Boehringer Mannheim Corp, (Indpls, IN). The PPAR γ antagonist 2-chloro-5-nitro-N-phenylbenzamide (GW9662) was synthesized in this laboratory, and its identity and purity (>98%) were confirmed by gas chromatography-mass spectrometry. CDODA-Me was prepared as described previously (Chintharlapalli et al 2007). Rosiglitazone was purchased from LKT Laboratories, Inc. (St. Paul, MN). CDDO-Me was provided by Dr. Edward Sausville (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) through the Rapid Access to Intervention Developmental Program. The Gal4 reporter containing 5 \times Gal4 response elements (pGal4) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4DBD-PPAR γ construct was a gift of Dr. Jennifer L. Oberfield (GlaxoSmithKline Research and Development). The pVEGF-2018 construct contains VEGF promoter inserts (positions –2018 to +50) linked to luciferase reporter gene. The pSurvivin-269 contains survivin promoter inserts (positions –269 to +49) linked to luciferase reporter gene and was kindly provided by Dr. M. Zhou (Emory University, Atlanta, GA). The pSp1 and pSp3 promoter constructs (pSp1-FOR4-luc and pSp3-FOR5-luc) were provided by Drs Carlos Ciudad and Veronique Noe (University of Barcelona). Sp1-FOR4-luc (contains the –751 to –20 region of the Sp1 gene promoter) and pSp3-FOR5-luc (contains the –417 to –38 region of the Sp3 promoter) constructs respectively.

Cell proliferation assays : Bladder cancer cells (3×10^4 cells per well) were seeded using DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS in 12-well plates and left to attach for 24 hr. Cells were then treated with either vehicle (DMSO) or the indicated concentrations of compounds. Fresh medium and test compounds were added every 24 hr and cells were then counted at the indicated times using a Coulter Z1 particle

counter. Each experiment was carried out in triplicate and results are expressed as means \pm SE for each treatment group.

DNA fragmentation assay: 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 μ g/mL ethidium bromide and the fragmented DNA was visualized using a Transilluminator on an ultraviolet light.

Western blot assays: Bladder cancer cells were seeded in DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS. Twenty-four hr later, cells were treated with either vehicle (DMSO) or the indicated compounds for 24 hr. Cells were collected using high-salt buffer [50 mmol/L HEPES, 0.5 mol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10% glycerol, and 1% Triton-X-100 pH 7.5] and 10 μ L/mL of Protease Inhibitor Cocktail (Sigma Aldrich). The lysates were incubated on ice for 1 hr with intermittent vortexing every 10 min for a total of 90 min, followed by centrifugation at 20,000 g for 10 min at 4°C. Lysates were then incubated for 3 min at 100°C before electrophoresis, and then separated on 10% SDS-PAGE 120 V for 3 to 4 hr in 1X running buffer [25 mM Tris-base, 192 mM glycine and 0.1% SDS]. 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 0.9A at 4°C. The 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 1:200- 1:1000 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 (PerkinElmer Life Sciences, Waltham, MA) substrate for 1.0 min, and exposed to Kodak X-OMAT AR autoradiography film (American X-ray

supply Inc, Jackson, CA). Quantification of the proteins was determined using Image J software and the optical densities were plotted after normalization with laminin./ β -actin.

Transfection assays: The two bladder cancer cell lines, 253JB-V and KU7 were seeded (1×10^5 per well) in 6-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotic and left to attach for one day. For transfection assays, bladder cancer cells (1×10^5 per well) were plated in 12-well plates in DMEM:Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS. After 16-24 h, various amounts of DNA (i.e., 0.4 μ g pGL2/pGL3); 0.04 μ g β -galactosidase, pVEGF, pSurvivin, pSp1For4, pSp1For5, pGal4Luc and pGal4DBD-PPAR γ were transfected using Lipofectamine reagent according to the manufacturer's protocol and then treated with control, indicated doses of CDODA-Me and luciferase activity (normalized to β -galactosidase) was determined essentially as described.

ROS and MMP estimation: Cellular reactive oxygen species (ROS) levels were evaluated with the cell-permeant probe **CM-H₂DCFDA** (5-(and-6)-chloromethyl-2'7' dichlorodihydrofluorescein diacetate acetyl ester). **CM-H₂DCFDA** is nonfluorescent until removal of the acetate groups by intracellular esterases and oxidation occurs within the cell. Following treatment for 20-24 hr, in the cells were plated using a 96 well culture dish, 10 μ M **CM-H₂DCFDA** was added for 30 min, the cells were then 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 determination 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 μ l 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 performed on different days. At least 16 wells per treatment were analyzed for each experiment.

Statistical analysis: Statistical significance of differences was determined by an analysis of variance and student t-test, and the levels of probability were noted. IC₅₀ values were calculated using linear regression method and expressed in $\mu\text{mol/L}$ concentrations.

Results

CDODA-Me is a potent growth-inhibitory compound in colon, pancreatic and prostate cancer cells (424) (423) (222) (425), and results in Figure 34A show that CDODA-Me inhibits growth of 253JB-V and KU7 cells after treatment for 48 or 72 hr. IC₅₀ values for inhibition of cell proliferation after treatment for 24 or 72 hr were 1.79 and 1.64 μM and 1.97 and 1.71 μM for 253JB-V and KU7 cells, respectively. Figures 34B and 34C show that after treatment of 253JB-V and KU7 cells with CDODA-Me for 24 hr, there was an increase in DNA ladder formation and caspase-dependent PARP cleavage, respectively, in both cell lines confirming that CDODA-Me induces apoptosis in bladder cancer cells.

CDODA-Me is a PPAR γ agonist (20), and results in Figure 34D show that CDODA-Me induced luciferase activity in 253JB-V and KU7 cells transfected with a PPAR γ -GAL4 chimera and a pGAL4-luc reporter gene containing 5 tandem GAL4 response elements linked to a luciferase reporter gene. Rosiglitazone, a thiazolidinedione PPAR γ agonist, also activated PPAR γ -GAL4 in KU7 cells and served as a positive control for this assay. Previous studies in bladder and other cancer cell lines report that the broad spectrum of anticancer activities of curcumin have been associated, in part, with decreased NF κ B-dependent activity (318) (283).

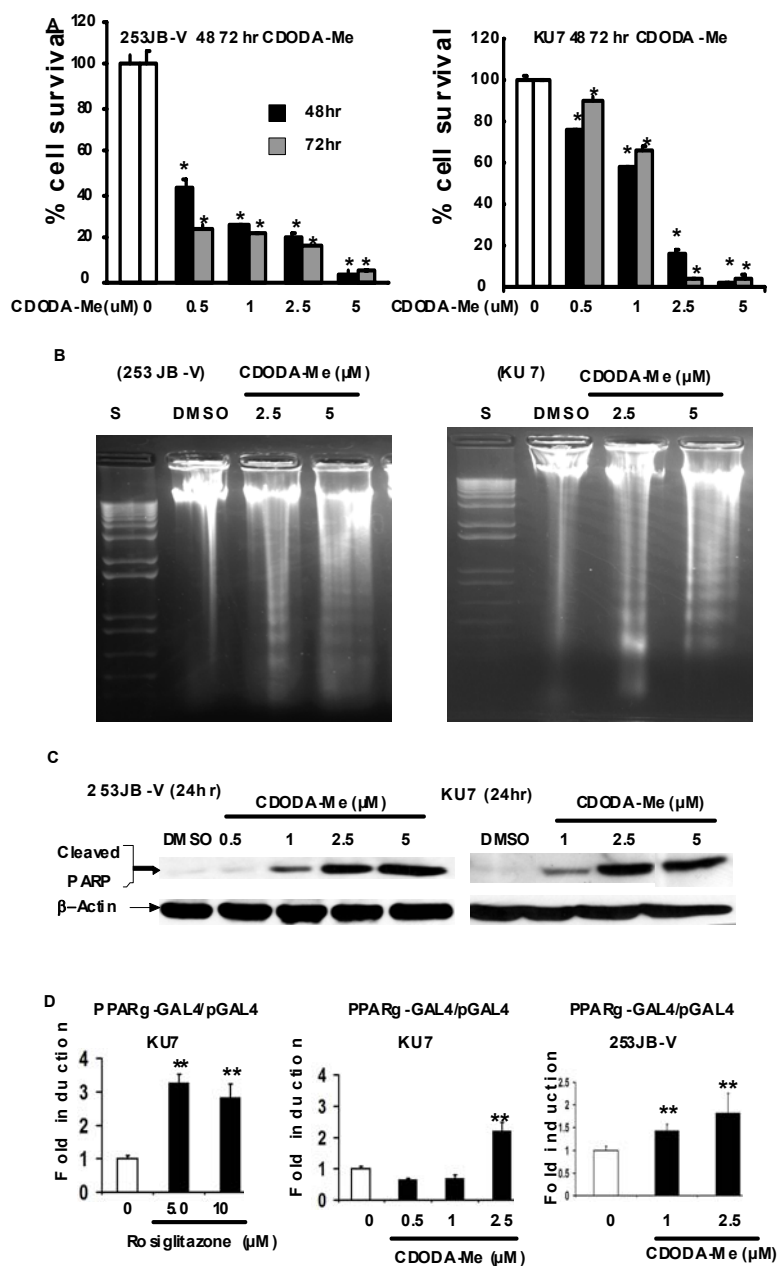


Figure 34. CDODA-Me inhibits bladder cancer cell growth, induces apoptosis and activates PPAR γ receptor.

A. Inhibition of 253JB-V and KU7 cell growth. Cells were treated with DMSO (solvent control); 0.5, 1.0, 2.5 or 5.0 $\mu\text{mol/L}$ CDODA-Me and the effects of cell growth were determined after treatment for 48 and 72 hr as described in the Materials and Methods. B. Effects of CDODA-Me on DNA laddering in 253JB-V and KU7 cells. Cells were treated with DMSO (0) and 2.5 or 5.0 $\mu\text{mol/L}$ CDODA-Me for 24 hr and induction of DNA laddering was determined as described in Materials and Methods. The standard (S) of 1 kb was used as a marker for DNA laddering. C. Induction of PARP cleavage. 253JB-V and KU7 cells were treated with DMSO and 1.0, 2.5, or 5.0 $\mu\text{mol/L}$ CDODA-Me for 24 hr, and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods. D, Activation of PPAR γ , 253JB-V and KU7 cells were transfected with PPAR γ -GAL4/pGAL4 constructs and then treated with DMSO, rosiglitazone or different concentrations of CDODA-Me, and luciferase activity was determined as described in Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) CDODA-Me-dependent increases (**) or decreases (*) in percentages of a phase compared to the solvent (DMSO) control are indicated.

Induction of the proapoptotic gene caveolin-1 by some PPAR γ agonists in bladder cancer cells is receptor-dependent, whereas rosiglitazone does not induce caveolin-1 in these cells (426); like rosiglitazone, CDODA-Me did not induce caveolin-1 in bladder cancer cells (Figure. 35A). CDODA-Me decreased expression of cyclin D1 and EGFR in 253JB-V and KU7 cells; however, cotreatment with the PPAR γ antagonist GW9662 did not block the effects of CDODA-Me on cyclin D1 or EGFR (Figure. 35B).

We also observed that the effects of CDODA-Me on PARP cleavage (Figure. 35C) and cell proliferation (Figure. 35D) were not inhibited by the PPAR γ antagonist GW9662. These results are consistent with previous studies with CDODA-Me in other cancer cell lines, indicating that the growth inhibitory

and proapoptotic activity of this compound is PPAR γ -independent (427) (428) (424).

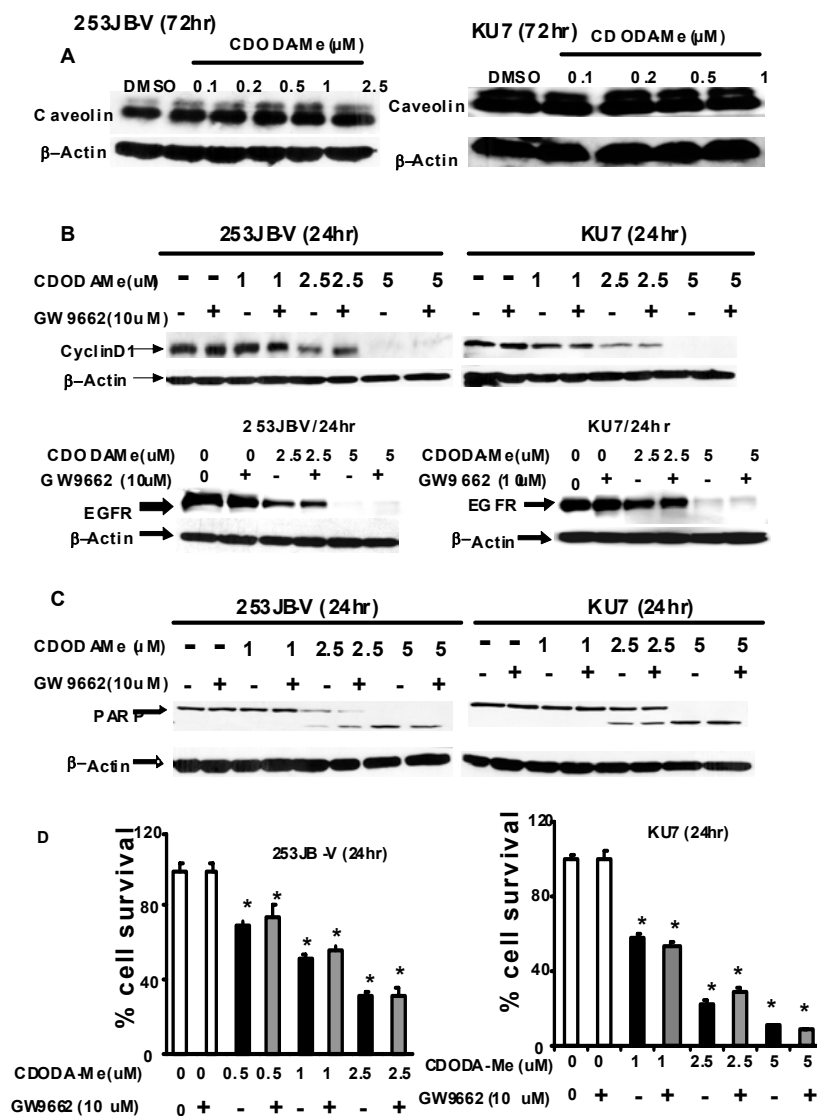


Figure 35. CDODA-Me modulates PPAR-independent cell cycle, cell growth and apoptotic proteins.

A. Caveolin-1 protein. 253JB-V and KU7 cells were treated with DMSO and 0.1, 0.25, 0.5, 1.0 and 2.5 μ mol/L CDODA-Me for 72 hr, and whole-cell

lysates were analyzed by Western blot analysis as described in Materials and Methods. Receptor-independent degradation of cyclin D1 and EGFR proteins (B) and induction of cleaved PARP (C). 253JB-V and KU7 cells were treated with DMSO and 1.0, 2.5 or 5.0 $\mu\text{mol/L}$ CDODA-Me in the presence or absence of the PPAR- γ antagonist GW9662 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, CDODA-Me causes receptor-independent growth inhibition. Cells were treated with DMSO (solvent control); 0.5, 1.0, 2.5 or 5.0 $\mu\text{mol/L}$ CDODA-Me Me in the presence or absence of PPAR γ antagonist GW9662 and the effects on cell growth were determined after treatment for 24 hr as described in Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) decrease in cell number compared to the solvent (DMSO) control are indicated (*). Treatment of 253JB-V and KU7 cells with CDODA-Me for 24 hr (Figure. 36A) or 48 hr (Figure 36B) resulted in decreased expression of survival (survivin and bcl-2) and angiogenic (VEGF and VEGFR1) proteins in 253JB-V and KU7 cells. Significant downregulation of these proteins was observed at 1 - 2.5 μM concentrations of CDODA-Me.

Cells were treated with DMSO and 0.5, 1.0, 2.5 or 5.0 $\mu\text{mol/L}$ CDODA-Me for 24 hr or 0.25, 0.5, 1.0 or 2.5 $\mu\text{mol/L}$ for 48 hr and whole-cell lysates were analyzed by Western blot analysis as described in Materials and Methods. β -Actin served as a loading control and similar results were observed in duplicate experiments. We also examined the effects of CDODA-Me on luciferase activity in 253JB-V and KU7 cells transfected with constructs containing Sp1 (-751 to -20) and Sp3 (-417 to -38) promoter inserts (Figures. 37A and 37B, respectively).

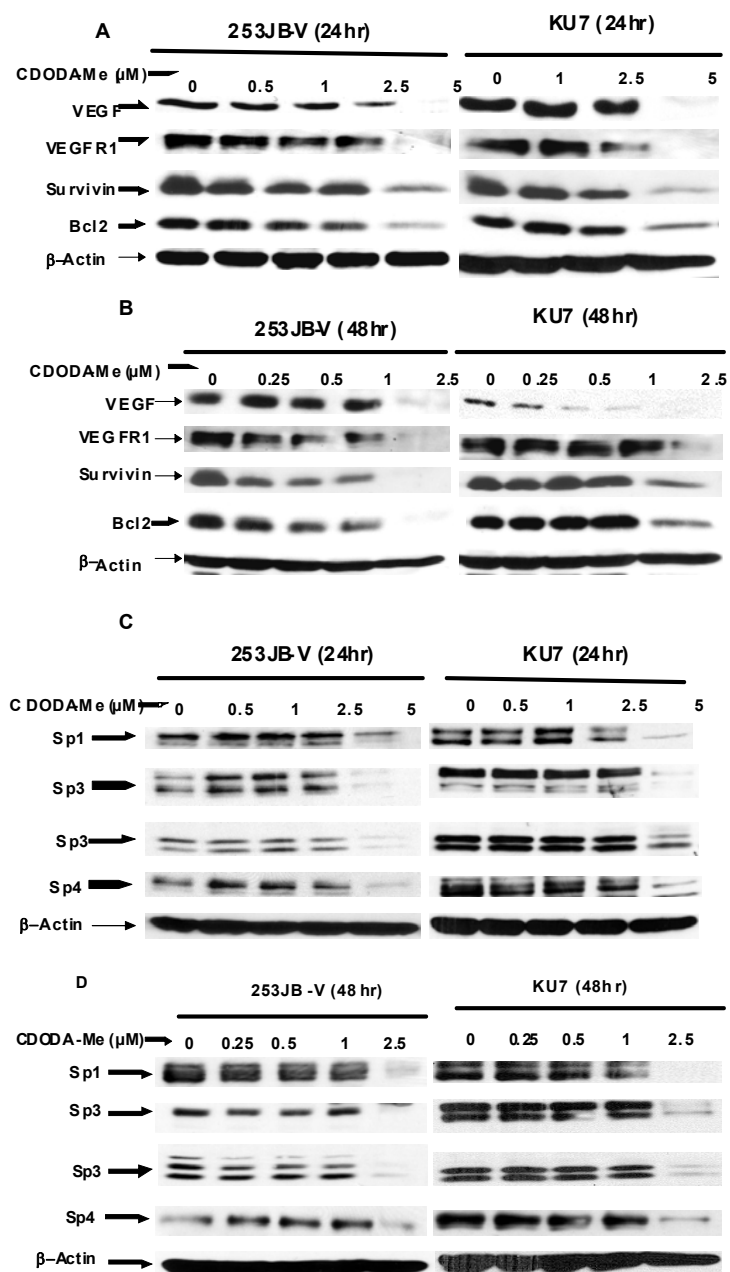


Figure 36. Effects of CDODA-Me on angiogenic, survival and Sp proteins.

CDODA-Me decreases VEGF, VEGFR1, survivin and bcl-2 proteins in 253JB-V and KU7 after treatment for 24 (A), or 48 hr (B) and also decreases Sp proteins after treatment for 24 (C), or 48 hr (D) in bladder cancer cells.

Results are expressed as means \pm SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) CDODA-Me-dependent decreases in luciferase activity compared to the solvent (DMSO) control are indicated (*). CDODA-Me significantly decreased luciferase activity in both cells transfected with pSp1For4 or pSp3For5, and this was consistent with the loss of Sp proteins which self-regulate both Sp1 and Sp3.

Ongoing studies in this laboratory show that arsenic trioxide also decreases cell proliferation and Sp protein expression in bladder and other cancer cell lines and this is related to decreased mitochondrial membrane potential (MMP) and the induction of reactive oxygen species (ROS). Figure 38A shows that after treatment of 253JB-V and KU7 cells with CDODA-Me, there was a decrease in MMP in both cell lines which was reversed after cotreatment with the antioxidant thiol reducing agent DTT.

Using the same treatment protocol, CDODA-Me also decreased GSH levels and increased ROS in both bladder cancer cell lines and these responses were also inhibited after cotreatment with the thiol reducing agent DTT (Figure 38B). The role of CDODA-Me-induced ROS in mediating inhibition of cell proliferation and Sp downregulation is supported by results in Figures 38C and 38D, respectively. CDODA-Me-mediated growth inhibition and downregulation of Sp1, Sp3 and Sp4 proteins were significantly reversed after cotreatment with DTT and GSH.

Bladder cancer cells were transfected with pSp1 (A), pSp3 (B), pVEGF (C) and pSurvivin (D) promoter constructs and treated with DMSO and 1.0, 2.5 or 5.0 $\mu\text{mol/L}$ CDODA-Me for 24 hr and luciferase activity was determined as described in Materials and Methods.

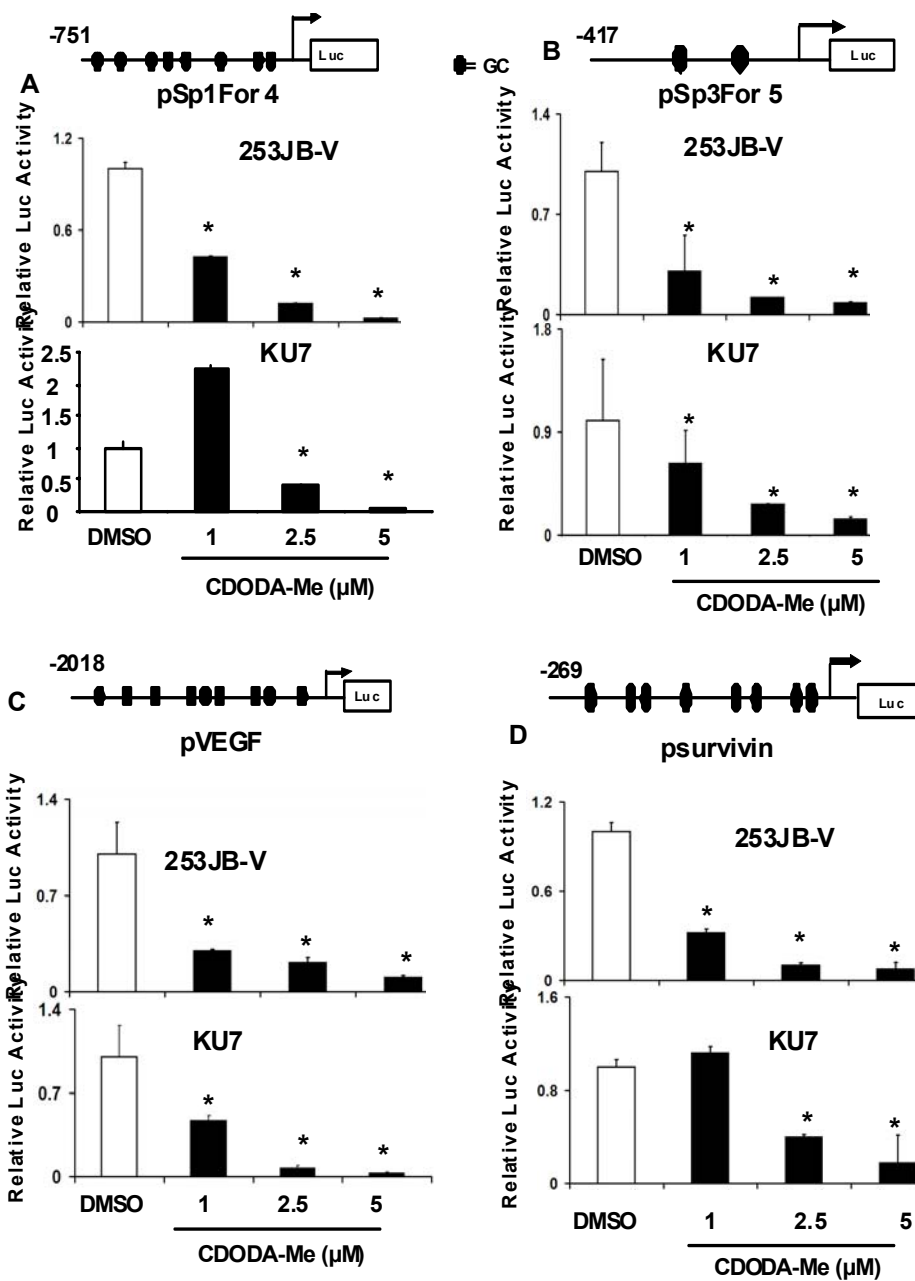


Figure 37. CDODA-Me decreases luciferase activity in cells transfected with Sp and Sp-dependent gene promoters.

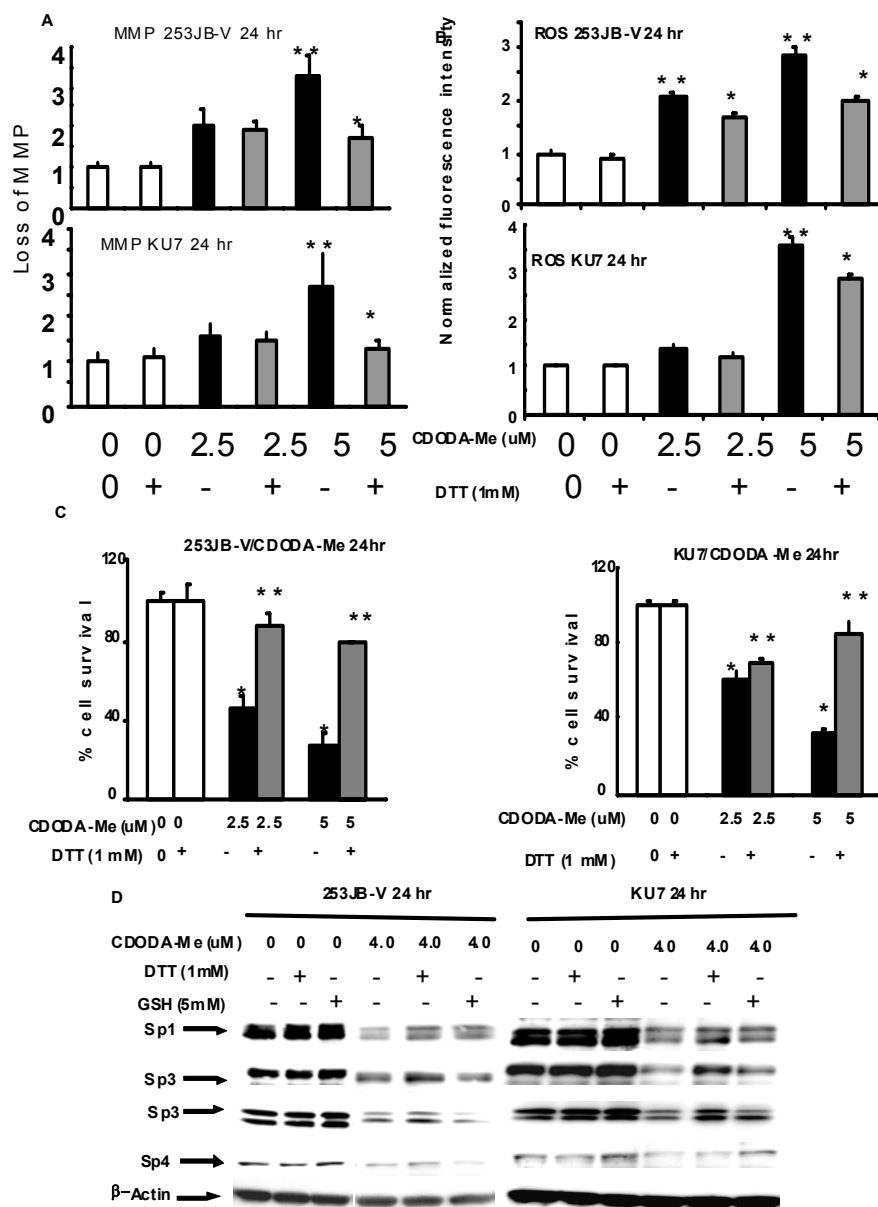


Figure 38. Effects of CDODA-Me on mitochondrial membrane potential and ROS.

Induction of changes in MMP (A) and ROS (B) by CDODA-Me. 253JB-V and KU7 cells were treated with DMSO and 2.5 or 5 $\mu\text{mol/L}$ CDODA-Me for 24 hr, in the presence or absence of antioxidant DTT and mitochondrial membrane

potential and ROS were determined as described in Materials and Methods. ROS mediated cell growth inhibition (C) and Sp degradation (D) in the presence or absence of antioxidants. Cells were treated with DMSO, 2.5, 4.0 or 5 $\mu\text{mol/L}$ CDODA-Me 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- C are expressed as means \pm SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) CDODA-Me-dependent decreases (*) or increases (**) compared to the solvent (DMSO) control are indicated. In addition, CDODA-Me also decreased luciferase activity in 253JB-V and KU7 cells transfected with the pVEGF (Figure 37C) and pSurvivin (Figure 37D) constructs which contain the -2019 to +50 to -269 to +49 regions of the VEGF and survivin gene promoters, respectively. These results are consistent with the effects of CDODA-Me on downregulation of Sp and Sp-dependent genes.

CDDO-Me is an oleanolic acid derivative and structurally related to CDODA-Me and the former compound has been extensively investigated as an anticancer agent. Figure 6A shows that CDDO-Me inhibited growth of 253JB-V and KU7 cells and cotreatment with the antioxidant DTT partially reversed growth inhibition as observed for CDODA-Me (Figure 38C). CDDO-Me also decreased expression of Sp1, Sp3 and Sp4 proteins (Figure 39B) at concentrations similar to those required for growth inhibition and these responses were also partially reversed after cotreatment with the thiol antioxidant DTT. In addition, like CDODA-Me, CDDO-Me decreased MMP in both cell lines (Figure 39C) and also induced ROS (Figure 39D), and both responses were inhibited after cotreatment with the thiol antioxidant. These results demonstrate that both CDODA-Me and CDDO-Me induce ROS-dependent downregulation of Sp1, Sp3 and Sp4 transcription factors in bladder cancer cells, and activation of these pathways contributes to their growth inhibitory and proapoptotic effects in KU7 and 253JB-V cells.

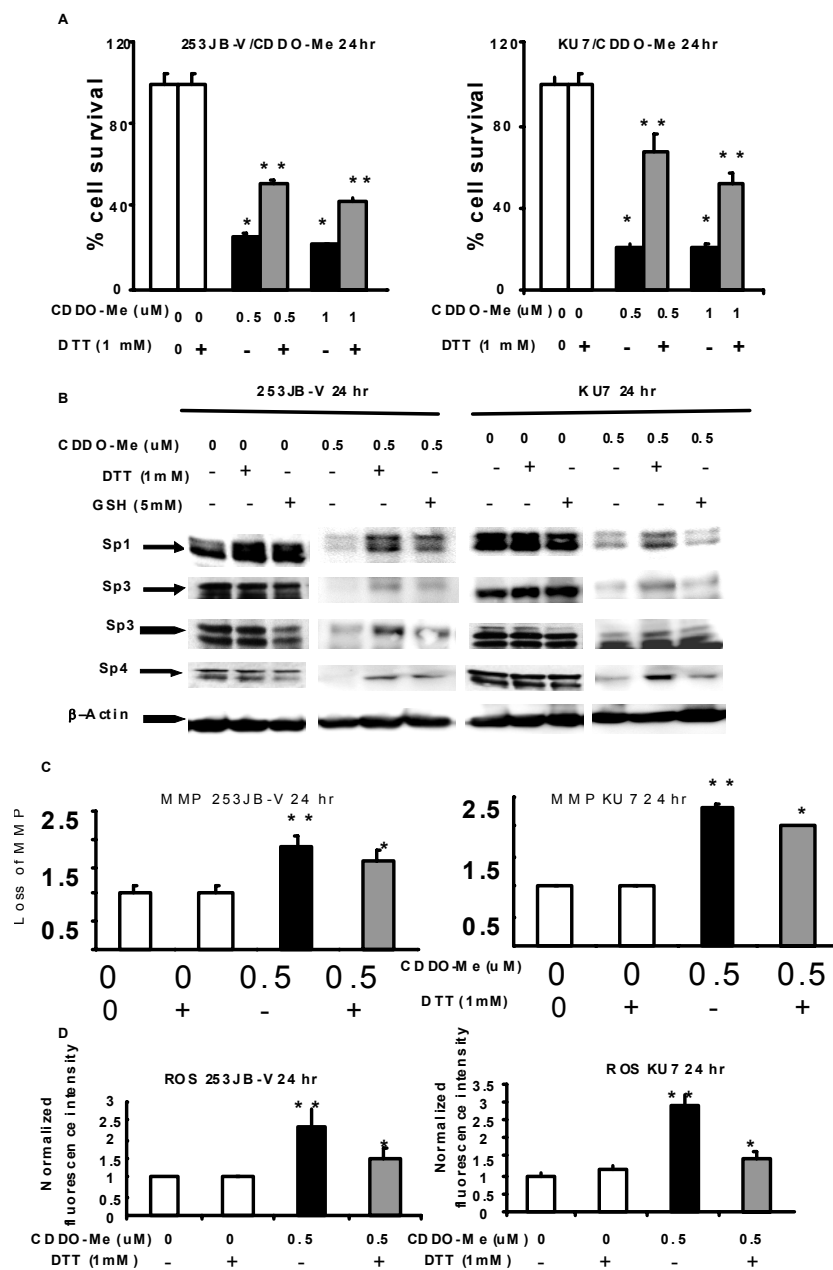


Figure 39. CDDO-Me dependent effects on cell proliferation and Sp protein degradation are ROS dependent.

ROS mediated cell growth inhibition (*A*) and Sp degradation (*B*) in the presence or absence of thiol antioxidants. Cells were treated with DMSO and 0.5 or 1 $\mu\text{mol/L}$ CDDO-Me for 24 hr and cells or whole cell lysates were analyzed by western blots as described in Materials and Methods. β -Actin served as a loading control. CDDO-Me decreases MMP (*C*) and induces ROS (*D*). 253JB-V and KU7 cells were treated with DMSO and 0.5 or 1 $\mu\text{mol/L}$ CDDO-Me for 24 hr, in the presence or absence of antioxidant DTT and mitochondrial membrane potential (*C*) and ROS (*D*) were determined as described in Materials and Methods. Results are expressed as means \pm SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) CDDO-Me-dependent decreases (*) or increases (***) compared to the solvent (DMSO) control are indicated.

Discussion

Specificity proteins are members of the Sp/KLF family of transcription factors, and Sp1 was the first transcription factor identified and has been extensively investigated (263). In transgenic mice in which Sp1 has been abrogated, embryos exhibit multiple abnormalities including retarded development, and the mice die at approximately gestation day 11 (429). The temporal changes in Sp1 and expression of other Sp/KLF proteins has not been extensively investigated; however, there is evidence that Sp1 expression decreases with age in both human and rodent tissue (430) (221). In contrast, Sp1 protein is overexpressed in multiple tumor types (220) (221), and studies in the laboratory show that Sp3 and Sp4 are also highly expressed in breast, prostate, colon, esophageal, pancreatic and bladder cancer cells (396) (428) (299) (222) (431). Sp1 overexpression in pancreatic and gastric cancer patients is a negative prognostic factor for survival (220) (396). Research in this laboratory has been investigating the effects of Sp1, Sp3 and Sp4 overexpression on the phenotype and genotype of cancer cells. Results of RNA

interference in which cancer cells are transfected with a cocktail of small inhibitory RNAs against Sp1, Sp3 and Sp4 (simultaneous knockdown) indicate that these transcription factors regulate several genes and proteins that are themselves individually targeted by anticancer drugs and neutralizing antibodies. Results of the RNA interference studies show that Sp1, Sp3 and Sp4 regulate expression of VEGF and its receptors (VEGFR1 and VEGFR2), bcl-2, survivin, epidermal growth factor receptor (EGFR) and cyclin D1 (222) (262) (335).

Since Sp transcription factors regulate expression of angiogenic, growth promoting and survival genes, research in this laboratory has been investigating small molecules that target downregulation of Sp proteins and Sp-dependent genes (335) (222). CDODA-Me, a synthetic derivative of glycyrrhetic acid, activates PPAR γ , is cytotoxic to cancer cells (222), and induces downregulation of Sp1, Sp3 and Sp4 in colon cancer cells and tumors (mouse xenografts) (222). CDODA-Me also activates PPAR γ in bladder cancer cells (Figure 34D); however, the growth inhibitory and proapoptotic effects of CDODA-Me were PPAR γ -independent (Figures. 34 and 35). However, we observed that CDODA-Me decreased expression of Sp1, Sp3 and Sp4 (Figure 36D) and this was accompanied by a parallel decrease in Sp-dependent survival (survivin), angiogenic (VEGF and VEGFR1), and growth promoting (cyclin D1 and EGFR) genes and proteins. These results, coupled with studies showing that Sp1, Sp3 and Sp4 knockdown by RNA interference decreases bladder cancer cell growth and induces apoptosis (222), indicate that the cytotoxicity of CDODA-Me in bladder cancer cells is, in part, due to Sp downregulation.

Recent studies in this laboratory indicate that arsenic trioxide is also cytotoxic to bladder cancer cells and, like CDODA-Me, this is accompanied by downregulation of Sp proteins and Sp-dependent genes. Downregulation of Sp1, Sp3 and Sp4 by arsenic trioxide was accompanied by induction of ROS and decreased MMP, and addition of antioxidant thiol reducing agents blocked all of these responses. Treatment of 253JB-V and KU7 cells with CDODA-Me

also decreased MMP as indicated by JC-1 staining and this response was reversed by DTT (Figure 38A). The same treatment protocol was also used to measure induction of ROS and GSH and depletion (Figure 38B) and inhibition of cell proliferation (Figure 38C), and all of these responses were induced by CDODA-Me and inhibited after cotreatment with DTT. Moreover, like arsenic trioxide, CDODA-Me also induced downregulation of Sp1, Sp3 and Sp4, and both DTT and GSH significantly blocked this response. The results clearly demonstrate that mitochondria are the major intracellular targets of CDODA-Me in cancer cells, and the mitochondriotoxicity of this drug plays a major role in the anticancer activity of this compound.

CDDO and its methyl ester (CDDO-Me) are oleanolic acid derivatives structurally similar to CDODA-Me, and previous studies have reported this mitochondriotoxicity of CDDO/CDDO-Me in several different cell lines (432) (433, 434). Moreover, there are also reports of antioxidants ameliorating the proapoptotic effects of CDDO and CDDO-Me, and we have observed that like CDODA-Me, CDDO-Me-dependent inhibition of bladder cancer cell growth is blocked by antioxidants. In addition, CDDO-Me downregulates Sp1, Sp3 and Sp4 and this response is also inhibited after cotreatment with GSH and DTT, demonstrating that one of the underlying mechanisms of action of CDDO-Me is associated with mitochondrial disruption and induction of ROS leading to decreased expression of Sp transcription factors.

These results demonstrate that the triterpenoid acid derivatives decrease MMP and induce ROS and these results in downregulation of Sp transcription factors and Sp-dependent genes and proteins including genes important for cell proliferation, survival and angiogenesis. The role of CDODA-Me-dependent miR-27a downregulation and induction of the Sp-repressor ZBTB10 observed in colon cancer cells (435) was also investigated in bladder cancer cells, and CDODA-Me did not significantly affect their expression (data not shown). Current studies are investigating other genes and microRNAs that may play a

role in CDODA-Me-induced downregulation of Sp1, Sp3 and Sp4 in bladder cancer cells, and CDODA-Me and related compounds are being investigated for clinical applications in bladder cancer chemotherapy.

VII. SUMMARY

Specificity proteins are members of the Sp/KLF family of transcription factors, and Sp1 was the first transcription factor identified and has been extensively investigated. Sp1 is a critical transcriptional factor for embryonic development however there is evidence in humans and rodents that Sp1 expression markedly decreases with age. In contrast, Sp1 protein is overexpressed in multiple tumor types and studies in the laboratory show that Sp3 and Sp4 are also highly expressed in breast, prostate, colon, esophageal, pancreatic and bladder cancer cells. Sp1 overexpression in pancreatic and gastric cancer patients is a negative prognostic factor for survival.

Recent studies in this laboratory have demonstrated that compounds such as celecoxib, tolfenamic acid, curcumin and betulinic acid inhibit cancer cell and tumor growth and metastasis, and exhibit both antiangiogenic and proapoptotic activity through decreased expression of Sp proteins such as Sp1, Sp3 and Sp4. Results of the RNA interference studies show that Sp1, Sp3 and Sp4 regulate expression of VEGF and its receptors (VEGFR1 and VEGFR2), bcl-2, survivin, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 3 (FGFR3) and cyclin D1. Research in this laboratory has been also investigating the effects of Sp1, Sp3 and Sp4 overexpression on the phenotype and genotype of cancer cells. Results of RNA interference in which cancer cells are transfected with a cocktail of small inhibitory RNAs against Sp1, Sp3 and Sp4 (simultaneous knockdown) indicate that these transcription factors regulate several genes and proteins that are themselves individually targeted by anticancer drugs and neutralizing antibodies.

Curcumin, an active component of tumeric has been extensively investigated as an anticancer drug. In this study, 10 - 25 μ M curcumin inhibited 253JB-V and KU7 bladder cancer cell growth, and this was accompanied by induction of apoptosis and decreased expression of the proapoptotic protein survivin and the angiogenic proteins vascular endothelial growth factor (VEGF)

and VEGF receptor 1 (VEGFR1). Since expression of survivin, VEGF and VEGFR are dependent on specificity protein (Sp) transcription factors; we also investigated the effects of curcumin on Sp protein expression as an underlying mechanism for the apoptotic and antiangiogenic activity of this compound. Moreover, using RNA interference with small inhibitory RNAs for Sp1, Sp3 and Sp4, we observed that curcumin-dependent inhibition of nuclear factor κ B (NF κ B)-dependent genes such as bcl-2, survivin and cyclin D1, was also due, in part, to loss of Sp proteins. Curcumin also decreased bladder tumor growth in athymic nude mice bearing KU7 cells as xenografts and this was accompanied by decreased Sp1, Sp3 and Sp4 protein levels in tumors. These results demonstrate for the first time that one of the underlying mechanisms of action of curcumin as a cancer chemotherapeutic agent is due, in part, to decreased expression of Sp transcription factors in bladder cancer cells.

Betulinic acid (BA) and curcumin are phytochemical anticancer agents, and we hypothesized that both compounds decrease EGFR expression in bladder cancer through downregulation of specificity protein (Sp) transcription factors. The epidermal growth factor receptor (EGFR) is an important chemotherapeutic target for tyrosine kinase inhibitors and antibodies that block the extracellular domain of EGFR. The effects of curcumin and BA on Sp proteins (Sp1, Sp3 and Sp4) and their role in downregulation of EGFR and EGFR-dependent responses were confirmed by RNA interference. BA and curcumin decreased expression of EGFR, Sp and Sp-dependent proteins in 253JB-V and KU7 cells; EGFR was also decreased in cells transfected with a cocktail (iSp) containing small inhibitory RNAs for Sp1, Sp3 and Sp4. BA, curcumin and iSp also decreased phosphorylation of Akt in these cells and downregulation of EGFR by BA, curcumin and iSp was accompanied by induction of LC3 and autophagy which is consistent with recent studies showing that EGFR suppresses autophagic cell death. In summary, results of this study demonstrate that BA- and curcumin-dependent repression of Sp1, Sp3 and Sp4

in bladder cancer cells also decrease expression of the Sp-dependent gene EGFR in both gefitinib-responsive 253JB-V and gefitinib-nonresponsive KU7 cells. Thus, BA and curcumin represent a new type of EGFR inhibitor that indirectly targets EGFR and EGFR-mediated responses through repression of Sp transcription factors. This results in inhibition of EGFR-dependent kinases and activation of autophagic cell death which is repressed by EGFR (kinase-independent). An additional chemotherapeutic advantage of these compounds is that they also induce pro-apoptotic, antiproliferative and antiangiogenic activities through downregulation of Sp-dependent survivin, cyclin D1 and VEGF/VEGFR1 expression, respectively. Relative contributions of Sp-dependent and Sp-independent pathways to the overall anticarcinogenic activity of curcumin and BA and other drugs that repress Sp proteins and Sp-regulated proteins such as EGFR will also vary with tumor type. Currently, we are investigating the mechanisms of action and clinical applications of drugs such as BA and curcumin alone and in combination with other cytotoxic compounds that are used for clinical treatment of bladder cancer.

Methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oate (CDODA-Me) is a synthetic triterpenoid obtained from glycyrrhetic acid which inhibits proliferation of KU7 and 253JB-V bladder cancer cells with inhibitory IC₅₀ values < 5 μ M. CDODA-Me-dependent growth inhibition is accompanied by induced caspase-dependent PARP cleavage and downregulation of survival (survivin and bcl-2) and angiogenic [vascular endothelial growth factor (VEGF) and its receptor (VEGFR1)] genes. CDODA-Me also decreased expression of specificity protein-1 (Sp1), Sp3 and Sp4 transcription factors and this was consistent with downregulation of the Sp-dependent genes survivin, bcl-2, VEGF and VEGFR1. Similar results were observed for a structurally-related triterpenoid, methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), which is currently in clinical trials. Both CDODA-Me and CDDO-Me decreased mitochondrial membrane potential and induced reactive oxygen species (ROS),

and these responses were also critical for triterpenoid-induced downregulation of Sp proteins which was inhibited by the antioxidants dithiothreitol and glutathione. This demonstrates a common mechanism of action for CDODA-Me and CDDO-Me which is due, in part, to mitochondriotoxicity.

Celastrol, a triterpenoid acid from an ivy-like vine exhibits anticancer activity against 253JB-V and KU7 bladder cancer cells. Celastrol decreased cell proliferation, induced apoptosis and decreased expression of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4. Fibroblast growth factor receptor 3 (FGFR3) is overexpressed in bladder tumors and celastrol also caused a dose-dependent downregulation of this receptor in 253JB-V and KU7 bladder cancer cells. Using small inhibitory RNAs (siRNA) for Sp1, Sp3 and Sp4 (in combination) we showed that FGFR3 receptor expression in bladder cancer cells is regulated by Sp transcription factors. The mechanism of Sp downregulation by celastrol was due to induction of reactive oxygen species (ROS) and inhibitors of ROS blocked celastrol-induced growth inhibition and Sp repression. *In vivo* studies using KU7 cells as xenografts, showed that celastrol, decreased tumor growth and expression of Sp proteins confirming that celastrol represents a novel class of anticancer drugs that act, in part through ROS-dependent downregulation of Sp transcription factors which are also overexpressed in bladder cancer cells. This study also confirms that Sp transcription factors which are overexpressed in bladder and other cancer cells and tumors are important drug targets for cancer chemotherapy.

Derivatives of oleanolic acid, ursolic acid and glycyrrhetic acid substituted with electron withdrawing groups at the 2-position in the A-ring which also contains a 1-en-3-one moiety are potent inhibitors of cancer cell growth. The antiproliferative activities of several 2-substituted analogs of these synthetic triterpenoid acid methyl esters derived from ursolic and glycyrrhetic acid on proliferation were investigated in KU7 and 253JB-V bladder and Panc-1 and Panc-28 pancreatic cancer cells. The results show that the 2-cyano and 2-

trifluoromethyl derivatives were the most active compounds. The glycyrrhetic acid derivatives with the rearranged C-ring containing the 9(11)-en-12-one structure were generally more active than the corresponding 12-en-11-one isomers. However, differences in growth inhibitory IC_{50} values were highly variable and dependent on the 2-substituent (CN vs. CF_3) and cancer cell context.

Current studies are investigating other genes and microRNAs that may play a role in CDODA-Me-induced downregulation of Sp1, Sp3 and Sp4 in bladder and other cancer cells, and related compounds are being investigated for clinical applications in cancer chemotherapy.

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