NEW SYNTHETIC DERIVATIVES OF TRITERPENOIDS IN THE

TREATMENT OF CANCER

A Dissertation

by

SABITHA PAPINENI

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 2008

Major Subject: Toxicology

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ABSTRACT

New Synthetic Derivatives of Triterpenoids in the

Treatment of Cancer. (December 2008)

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Chair of Advisory Committee: Dr. Stephen H. Safe

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

methyl 2-cyano-3,11-dioxo-18α-olean-1,12-dien-30-oate (α-CDODA-Me) isomers are

synthetic analogs of the naturally occurring triterpenoid glycyrrhetinic acid. The activity

of these compounds as selective peroxisome proliferator-activated receptor γ (PPARγ)

agonists and as cytotoxic anticancer agents has been investigated in colon, prostate and

pancreatic cancer cells. In colon cancer cells β-CDODA-Me arrested the growth at

G2/M and this was accompanied by decreased expression of Sp1, Sp3 and Sp4 protein

and mRNA and several Sp-dependent genes including survivin, vascular endothelial

growth factor (VEGF), and VEGF receptor 1 (VEGFR1 or Flt-1). β-CDODA-Me also

inhibited tumor growth in athymic nude mice bearing RKO cells as xenografts. β-

CDODA-Me decreased expression of microRNA-27a (miR-27a), and this was

accompanied by increased expression of two miR-27a-regulated mRNAs, namely

ZBTB10 (an Sp repressor) and Myt-1 which catalyzes phosphorylation of cdc2 to inhibit

progression of cells through G_2/M.

In LNCaP prostate cancer cells induction of two proapoptotic proteins namely

nonsteroidal anti-inflammatory drug- activated gene-1 (NAG-1) and activating transcription factor-3 (ATF-3) was PPARγ independent and required activation of kinases. β-CDODA-Me also decreased the levels of androgen receptor (AR) and prostate-specific antigen (PSA) mRNA and protein levels. Thus the cytotoxicity of β-CDODA-Me involved multiple pathways that selectively activate growth inhibitory and proapoptotic responses.

Betulinic acid (BA), an inhibitor of melanoma is a pentacyclic triterpenoid natural product that induces apoptosis and antiangiogenic responses in tumors derived from multiple tissues. However, the underlying mechanism of action of BA is unknown. In LNCaP prostate cancer cells, BA acts as a novel anticancer agent by inducing proteasome-dependent repression of Sp proteins and Sp- dependent genes. The anticancer activity of the 2-cyano substituted analogs of BA, CN-BA and its methyl ester, CN-BA-Me was also investigated in colon and pancreatic cancer cells. Both CN-BA and CN-BA-Me were highly cytotoxic and activated PPARγ and induced several receptor-mediated responses. The results clearly demonstrated that both the PPARγ agonist activities of CN-BA and CN-BA-Me were structure-, response-/gene- and cell context-dependent suggesting that these compounds are a novel class of selective PPARγ modulators with potential for clinical treatment of prostate, colon and pancreatic cancer.

DEDICATION

      I dedicate my dissertation to my lovely and adorable husband, Dr. Sudhakar Reddy Chintharlapalli and my dearest parents, Papineni Penchal Reddy and Papineni Venkata Subbamma. They are my strength and without their love and support I wouldn't stand here.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

CANCER

Cancer is a devastating disease and Celsus, a Roman encyclopedist translated the term *"carcinos"* described by Greek Physician Hippocrates, the father of Medicine into a Latin term *"cancer"*. Over 565,650 Americans are expected to die of cancer and about 1,437,180 new cancer cases will be diagnosed this year (1). Under normal conditions, cells in the body divide in a controlled and regulated manner, however; cells that lose this regulation divide continuously without control and may develop into cancer. The pathogenesis of cancer is not fully characterized and depending on the type of cancer, differs widely in etiology and biology. Cancer is caused by both external factors such as exposure to chemicals, UV radiation and infectious agents and internal factors which include hormones for breast cancer and prostate cancer, inherited mutations/genetic abnormalities and a compromised immune system. Irrespective of the type of cancer, all known cancers are characterized by abnormalities in the genetic material that results in abnormal expression of genes and proteins leading to the development of cancer. Most importantly, cancer develops when genetic changes affect two classes of genes namely, oncogenes and tumor suppressor genes. The first oncogenes identified were of viral origin with cellular functions ranging from functional roles as transmembrane protein receptors to nuclear transcription factors.

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This dissertation follows the style of *Cancer Research*.

These proto-oncogenes are constituitive or normal genes that are activated by mutations and enhance cell proliferation and transformation. Tumor suppressor genes regulate cell division or apoptosis and are downregulated or repressed in cancer cells.

Carcinogenesis

Carcinogenesis, the process of cancer development, is complex and can be categorized into five main stages: initiation, promotion, progression, invasion and metastases (Fig. 1.1). The first stage of initiation involves accumulation of genetic changes or hits in a single cell as suggested by Knudson (2) and Nowell (3). The genetic changes or DNA damage can be initiated by ionizing radiation such as X-rays, chemical carcinogens such as nitrosamines or viruses like papillomavirus and Epstein-Barr virus. Epidemiological studies based on age-dependent cancer incidence suggested that rate of tumor development is proportional to the fourth to sixth power of lifetime indicating that four to six independent genetic hits or events are necessary for the development of a tumor (4).The association of lung cancer with smoking demonstrated by Cairns *et al* (5) is a well-characterized case illustrating that the frequency of the tumors in humans is proportional to the first or second power of cigarettes per day and to the sixth power of the duration of smoking. Several different models have been put forward to characterize the process of carcinogenesis including a well-characterized mouse skin tumor promotion model. A single sub-threshold dose of a carcinogen such as 7, 12-dimethylbenz[*a*]anthracene administration is followed by the repetitive application of a tumor promoter such as 12-*O*-tetradecanoylphorbol-13-acetate. Benign squamous

papillomas generally develop within 10 weeks and contain Ha-*ras* mutations (6). Only

few of these papillomas develop into malignant squamous
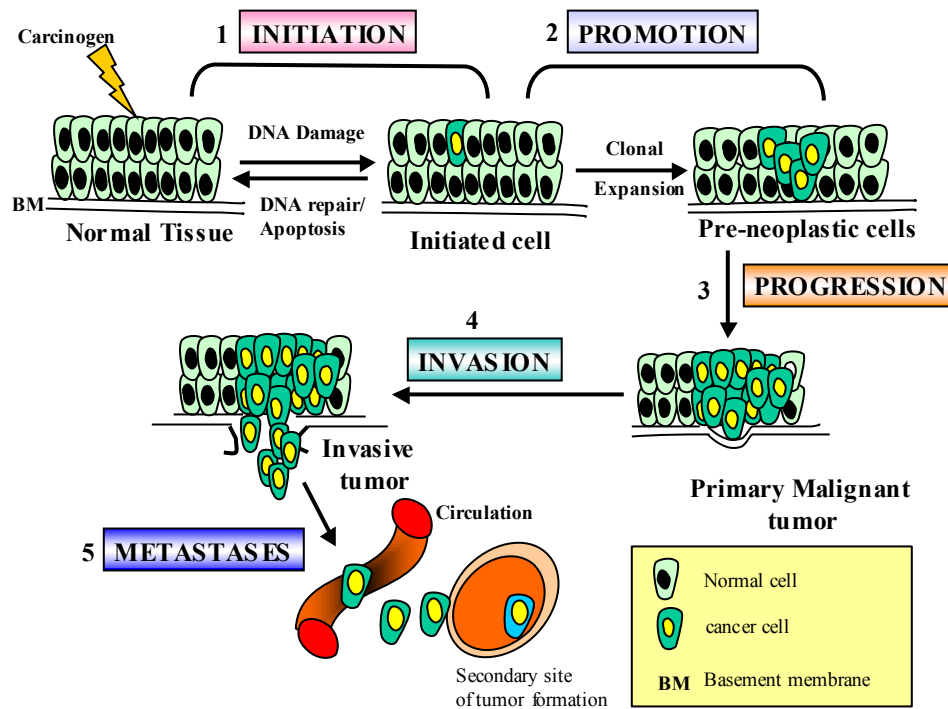
Fig. 1.1. Five stages of carcinogenesis (7).

cell carcinomas (SCC). The progression of papillomas to SCC has been characterized

phenotypically by inappropriate expression of certain membrane receptor/adhesion

molecules (8, 9), keratins (10-13), growth factors (14-16) and cyclins/cyclin-dependent

kinases(17, 18) and genotypically with respect to alterations in p53 and further

alterations in Ha-*ras* (19-23). However cancer cells contain thousands of mutations and

this finding has led to the proposal of a new hypothesis by Loeb called "mutator

phenotype hypothesis" suggesting that the normal rate of mutations doesn't correlate

with the number of mutations observed in the cancer cells (24, 25). According to this hypothesis mutations in the genes involved in the regulation of DNA synthesis, DNA repair or in the genes regulating the cell cycle or apoptosis will amplify the basal mutation rate and promote the tumor initiation/promotion model to a multihit model of carcinogenesis.

Tumor promotion is the second stage of carcinogenesis, during which a single initiatied cell with genetic changes expands clonally and is dependent on the favourable conditions for cellular growth such as interactions between tumor cells and stroma, growth factor availability, vascularization, O_2 partial pressure and many other factors. However DNA damage check points or apoptosis pathways may be induced by genetic events resulting from the initiation phase. Fifty percent of human cancers are defective in these pathways due to mutations in tumor suppressor genes or genes encoding pro-apoptotic proteins such as p53 or RB or *INK4* locus; and cancer cells continue to divide (26). There are number of tumor promoting agents such as phorbol esters, phenobarbitol, peroxisome proliferators, and biphenyls, many of which reversibly inhibit the gap junctions (27, 28).

The third stage of carcinogenesis is tumor progression and a classical example of this stage was described by Vogelstein and his colleagues in colorectal carcinogenesis which involves successive waves of clonal selection (29). According to this model, mutations occur randomly in the genome and selection of major mutated genes results from clonal proliferation; and since these cells grow rapidly even a small growth advantage will result in progeny (30).This stage is characterized by genetic alterations

that produce permanent genetic instability with a higher rate of chromosomal or base

modifications resulting in gross morphological and karyotypic changes that transform

pre-neoplastic cells into neoplastic cells (Fig. 1.1). In colorectal cancers, there is a good
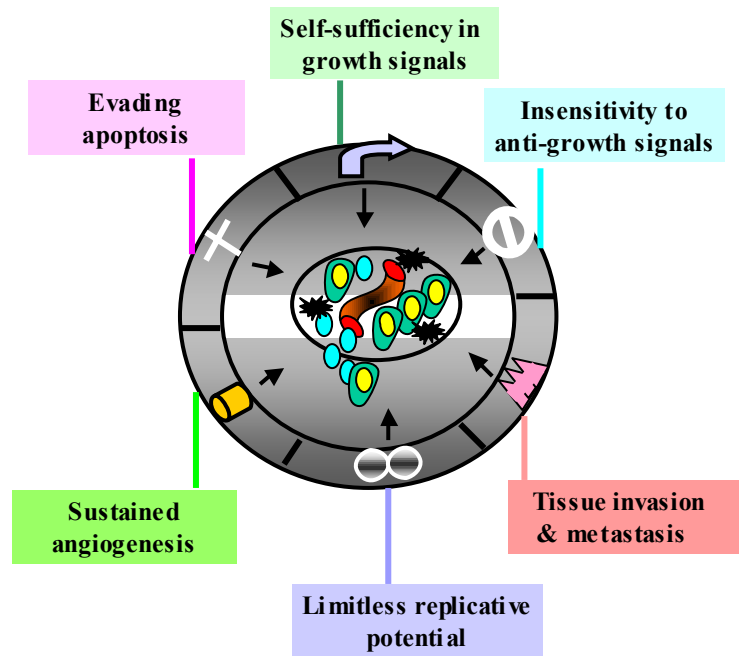
Fig. 1.2. Hallmarks of tumor cells (30).

correlation between the allelic imbalance at several loci such as loss of chromosomes 8p

and 18q and the aggressiveness of the disease measured by disease-free survival at five

years after surgery (31).

Invasion is the fourth stage of carcinogenesis and involves progression of

neoplastic cells to malignant cells; and this is associated with additional gentic and

epigenetic changes in the tumors and more aggressive characteristics with time. The

invasion and metastases phenotype of tumor cells is characterized by the ability of these

cells to attach to host cells and this may involve extracellular matrix (ECM) factors.

These cells also have the ability to secrete proteases to lyse barriers such as basement membranes in host cells and they can facilitate tumor angiogenesis in distal sites (32, 33). To date, the genes involved in this stage of tumor invasion and metastasis are not well defined, however, there is evidence that loss of E-cadherin, a tumor suppressor gene, results in rapid progression of gastric adenomas into invasive poorly differentiated metastatic carcinomas (34). According to Hanahan and Weinberg (30) there are six charactertics or hallmarks that tumor cells acquire during the process of carcinogenesis which dictate the fate of the tumor. The hallmarks include self-sufficiency in growth signals, insensitivity to anti-growth factors, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastases (Fig. 1.2).

2008 Estimated US New Cancer Deaths and Cases

Men		Women	
Cases	**Deaths**	**Cases**	**Deaths**
Prostate (25%)	Lung &Bronchus (31%)	Breast (26%)	Lung & Bronchus(26%)
Lung & Bronchus(15%)	Prostate(10%)	Lung & Bronchus(14%)	Breast(15%)
Colon & rectum (10%)	Colon & rectum (8%)	Colon & rectum(10%)	Colon & rectum(9%)
Urinary Bladder(10%)	Pancreas (6%)	Uterine corpus(6%)	Pancreas (6%)
Non-Hodgkin Lymphoma (5%)	Liver & intrahepatic bile duct (4%)	Non-Hodgkin Lymphoma (4%)	Ovary (6%)
Melanoma of skin (5%)	Leukemia (4%)	Thyroid (4%)	Non-Hodgkin Lymphoma (3%)
Kidney & renal pelvis (4%)	Esophagus (4%)	Melanoma of skin (4%)	Leukemia (3%)
Oral cavity & Pharynx (3%)	Urinary Bladder(3%)	Ovary (3%)	Uterine corpus (3%)
Leukemia (3%)	Non-Hodgkin Lymphoma (3%)	Kidney & renal pelvis (3%)	Liver & intrahepatic bile duct (2%)
Pancreas (3%)	Kidney & renal pelvis (3%)	Leukemia (3%)	Brain & other nervous system (2%)
All sites (100%)	All sites (100%)	All sites (100%)	All sites (100%)

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

Fig. 1.3. Estimated U.S cancer deaths and new cases in 2008 (1).

Tumors are classified based on the tissue of origin; in 2008 the American Cancer Society (ACS) estimates prostate cancer in men and breast cancer in women will be among the leading causes of cancer deaths, second only to lung cancer (Fig. 1.3) (ACS).

Treatment of cancer

The most common types of treatment for cancer include surgery, radiation and chemotherapy which can be used either alone or in combination with other therapies. The initial treatments given to the patient are called first-line therapy and treatments given after the first line therapy are called adjuvant therapy. Neo-adjuvant therapy is given prior to the first-line therapy and this often involves decreasing the tumor size. Surgery involves removal of the cancerous tissue and it is the primary treatment for most cancers, particularly solid tumors. It is also used as a diagnostic tool to confirm a diagnosis and determine the extent and spread of the tumor. Radiation therapy uses high energy X-rays to shrink the tumor. Radiation is mostly used in conjunction with surgery or chemotherapy or as neoadjuvant therapy to aid in surgery by reducing the size of the tumor. and is considered local treatment since it affects only the tumor region. Chemotherapy uses chemicals or drugs to kill cancer cells and the effects are systemic. There are several different classes of anticancer drugs based on the mechanism of action and these include the following: a) alkylating agents which damage DNA; b) antimetabolites that replace the normal building blocks of RNA and DNA; c) antibiotics that interfere with the enzymes involved in DNA replication; d) topoisomerase inhibitors that inhibit either topoisomerase I or II which are the enzymes involved in unwinding DNA during replication and transcription; e) mitotic inhibitors that inhibit mitosis and

cell division; f) corticosteroids which are used for the treatment of cancer and to relieve the side effects from other drugs; and g) other miscellaneous drugs such as L-asparaginase, an enzyme used in the treatment of acute lymphocytic leukemia and the proteosome inhibitor bortezomib (Velcade)(Table 1.1). Chemotherapy is also used as an adjuvant therapy alone or in combination with other treatments. Hormone therapy is used for the treatment of endocrine or reproductive related cancers like prostate, breast and testicular cancers which develop in part due to dysregulated hormone signals. For example Tamoxifen (Nolvadex) is an antiestrogen used for treatment of hormone responsive breast cancers.

Cytotoxic anticancer drugs attack all rapidly dividing cells in the body, and this includes not only cancer cells but also other dividing normal cells such as hair follicles, gastric epithelium, and blood cells. Because of these non-specific effects, chemotherapy is associated with side effects such as hair loss, nausea, vomiting, fatigue, increased sensitivity to infectious diseases and loss of appetite. Hence several new approaches are under development to increase the specificity of cancer treatment and some of these include biological therapies and targeted therapies. Immunotherapies utilize the body's defense mechanisms by stimulating the immune response against cancer cells. Monoclonal antibodies (mAbs) against antigens or specific molecules expressed on the surface of cancer cells have been developed and cancer vaccines that make immune cells

Table 1.1. Different classes of anticancer drugs

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Chemotherapeutic Drugs

Alkylating agents

Nitrogen mustards:	Cyclophosphamide (Cytoxan), melphalan, clorambucil, mechlorethamine
Nitrosoureas:	Streptozocin, carmustine (BCNU), and lomustine.
Alkyl sulfonates:	Busulfan
Triazines:	Dacarbazine (DTIC), and temozolomide (Temodar).
Ethylenimines:	Thiotepa and altretamine (hexamethylmelamine).
Platinum drugs:	Cisplatin, carboplatin, and oxaliplatin.

Antimetabolites — 5-Fluorouracil (5-FU), capecitabine (Xeloda), 6-mercaptopurine (6-MP), methotrexate, gemcitabine (Gemzar), cytarabine (Ara- C), fludarabine, and pemetrexed (Alimta).

Antitumor antibiotics

Anthracyclines:	Daunorubicin, doxorubicin (Adriamycin), epirubicin, and Idarubicin
Other antibiotic drugs:	Actinomycin-D, bleomycin, and mitomycin-C.

Topoisomerase inhibitors

Topoisomerase I inhibitors:	Topotecan and irinotecan
Topoisomerase II inhibitors:	Etoposide (VP-16) and teniposide

Table 1.1 Continued

Mitotic inhibitors

The Taxanes:	Paclitaxel (Taxol), docetaxel (Taxotere).
Epothilones:	**I**xabepilone (Ixempra).
The Vinca alkaloids:	Vinblastine (Velban), Vincristine(Oncovin), and vinorelbine (Navelbine).

Estramustine (Emcyt).

Corticosteroids	Prednisone, methylprednisone(Solumedrol), and dexamethasoneDecadron.
Others	L-asparaginase and proteosome inhibitor bortezomib(Velcade).

recognize cancer cells and kill them are examples of biological treatments. Targeted

therapies selectively target cancer cells but not normal cells, and this greatly reduces the

side effects associated with chemotherapy. Targeted therapies include antiangiogenic

drugs, various enzyme inhibitors, antisense and gene therapy. This approach has been

expanding to include drugs that are highly specialized and are only effective in treating a

narrow range of tumors. For example, the tyrosine kinase inhibitor, geftinib, which

blocks EGFR signaling, is effective only in lung cancer patients that express the

mutations in the tyrosone kinase domain of EGFR gene (35). Our laboratory has focused

on development of such specific molecules which are synthetic derivatives of natural

products and these include betulinic acid, a hydrolysed product of betulin derived from birch bark and methyl 2-cyano-3,11-dioxo-18βolean-1,12-dien-30-oate (CDODA-Me), a synthetic derivative of glycyrrhetinic acid, a triterpenoid derived from licorice root extract. In order to investigate the mechanism of action of these mechanism-based drugs in different cell and organ contexts, four different cancer cell models have been selected and are discussed in another section. The cancer models include prostate cancer, a reproductive and hormone related cancer in males, colorectal and pancreatic cancer which are gastroinstestinal related cancers and esophageal cancer, an aggressive disease in which the incidence rate has been increasing significantly in recent years.

PROSTATE CANCER

Incidence

It is estimated that there will be 186,320 new cases of prostate cancer and about 28,660 prostate cancer deaths in the US in 2008 (1). The number of cases and deaths have significantly increased from 1988-1992 and declined sharply from 1992-1995 and have been relatively constant since 1995. For unknown reasons incidence rates are higher among African American and Jamaican men when compared to Caucascians. Worldwide, the disease is common in Northern America and northwestern Europe, but less common in Asia and South America (1).

Both benign prostatic hypertrophy (BPH) and prostate cancer incidence rates are negligible or not detected in eunuchs and have been linked to the presence of testes and androgen function (36, 37). Autopsy studies indicate that approximately 70% of men

who died of other diseases had prostate cancer at the time of their death but most of these cancers were clinically insignificant (38).

Development of prostate gland

The prostate gland is a male specific reproductive organ that develops before birth and continues to grow until the end of puberty. Growth and development of the prostate gland is androgen dependent (39) and androgens elicit their effects on the prostate via binding to the androgen receptor (AR), present in the fetal urogenital sinus mesenchyme (UGM). Mesenchymal and epithelial interactions play a critical role in the prostatic development. UGM induces ductal morphogenesis, expression of the epithelial AR, regulates epithelial proliferation, and induces the expression of prostatic lobe-specific secretory proteins (40-46). Although prostate development and function are androgen dependent, several other growth factors, cytokines and hormones such as insulin like growth factor-1 (IGF-1), fibroblast growth factors (FGFs), and prostaglandins are associated with the development, growth and differentiation of the epithelium and stroma of the prostate (47).

Tumorigenesis of prostate gland

Nearly all-prostate cancers arise from glandular cells and are called adenocarcinomas. Prostate cancers can initially develop when men are 20-30 years of age or older and the incidence increases with age (48, 49) . Multiple genetic and epigenetic abnormalities have been associated with prostate cancer. Initially the process of prostate tumorigenesis begins with dysplasia associated with proliferative inflammatory atrophy (PIA), which further leads to prostatic intraepithelial neoplasia

(PIN) and then to carcinoma in few cases (50). During the process of premalignant

lesion formation and the progression to primary and metastatic cancer and to

androgen-independent cancer, genetic changes continue to accumulate in the cells and

these are illustrated in Figure 1.4 (50-52). Added to these alterations in the genome,

androgenic hormones promote further cancer cell growth and proliferation.

| Normal Epithelium | Prostatic intraepithelial neoplasia (PIN) | Invasive carcinoma | Metastasis |

| Loss of tumor suppressor genes | NKX3.1 | PTEN | Rb | p53 |
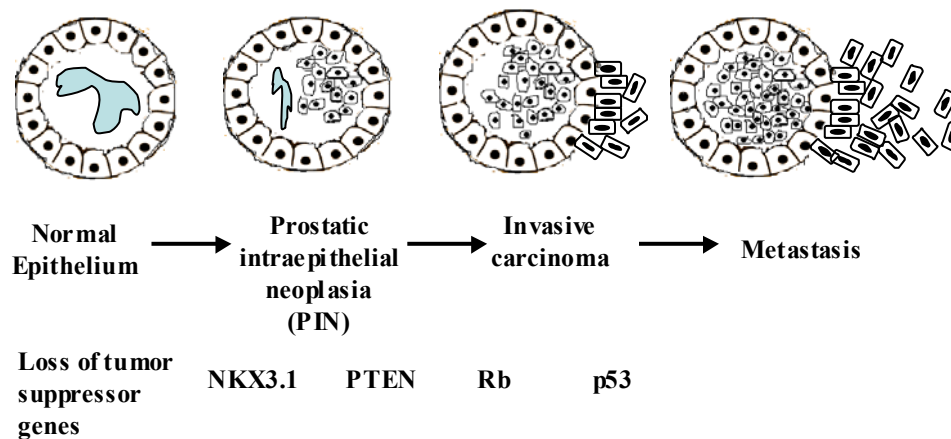
Fig. 1.4. Cellular changes in development of metastatic prostate cancer (53).

Testesterone is converted to an active metabolite, dihydrotestosterone (DHT) in

the prostate cells by the enzyme 5α-reductase. Binding of DHT to the AR results in

formation of a phosphorylated homodimer which is then translocated to the nucleus and

activates transcription of androgen-responsive genes related to growth and proliferation

(54, 55). The work of Chinnaiyan and collegues (56-59) suggests that the TMPRSS2, a

prostate-specific androgen regulated gene when fused to ETS family of transcription

factors leads to overexpression of an androgen-responsive oncoprotein. This process has

been identified as one of the most common somatic rearrangements in prostate cancer,

is associated with an invasive phenotype, and appears to be an important early step in the tumorigenesis (60). In most prostate cancers, chromosomal abnormalities with loss of chromosomes 6q, 7q, 8p, 10q, 13q, 16q, 17q, and 18q have been identified (61). NKx-3.1 is a homeobox gene located on human chromosome 8p21, a region that undergoes frequent loss of heterozygosity in prostate tumors. Loss of NKx-3.1 expression along with phosphatase and tensin homolog (PTEN) hetrozygosity, which inhibits phosphoinositol-3-kinase (PI3K) signaling has also been associated with prostate tumorigenesis (62). In addition, hypermethylation of enzymes such as glutathione S-transferase 1 and O^6-methylguanine DNA methyltransferase have also been observed (62).

Grading of prostate cancer

Grading of prostate carcinomas is based on histologic patterns which are scored using the Gleason grading system (63). Prostate cancers with a Gleason score of 5-7 are considered to be of intermediate grade or moderately differentiated and cancers with a score of 8-10 are considered to be of high grade or poorly differentiated, which predicts resistance to treatment and a low rate of patient survival.

Diagnosis of prostate cancer

Prostate cancer is diagnosed by elevated levels of prostate specific antigen (PSA) in blood and abnormal digital rectal examination (DRE). PSA is a serine protease produced by normal epithelial cells of the prostate gland and the physiological role of PSA is to liquefy seminal fluid. It is produced in small quantities in the normal prostate gland but is consistently elevated in prostate cancer and other disorders of the prostate

such as infection, inflammation and benign prostatic hyperplasia (BPH). Both for initial diagnosis of the disease and to monitor the treatment response, PSA levels are widely used as a biomarker for prostate cancer. The upper limit for normal levels of PSA has been set at 4.0 ng/ml and higher levels in patients usually results in prostate biopsies for tumors (64). With increased screening for PSA levels, most cancers are detected at early stages where they are localized to the gland itself and this can result in over-diagnosis of clinically insignificant or irrelevant prostate cancers (65-69).

Treatment of prostate cancer

Prostate cancer treatment is based on the specific type, location and stage of the cancer. Radical prostectomy is used to surgically remove the cancer when it has not spread from the gland. The major risks associated with surgery include urinary incontinence and impotence. Radiation is also used for low grade cancers that are confined to the gland itself or have spread only to the nearby tissue. Radiation is used alone or performed in combination with surgery and side effects such as bowel problems accompany radiation (1). Cryosurgery or cryotherapy which reduces the size of localized tumors by freezing is also being used for the treatment of prostate cancer.

Hormone therapy

Androgens are required for the prostate cell growth and play a role not only during prostate development but also during tumorigenesis. The major objective of hormone therapy is to lower male hormone or androgen levels and thereby minimize tumor growth. It is used in patients who are not good subjects for surgery or radiation or the combination of surgery with radiation. In most cases of prolonged hormone

treatment, tumors become resistant to hormone therapy and there is a poor prognosis for disease-free survival. In order to overcome this problem, therapy is used on an "on-again and off –again" approach (1).

Types of hormone therapy

Orchiectomy. Surgical removal of testes eliminates the source of androgen synthesis however; therapy though simple to perform is accompanied by severe side effects.

Luteinizing hormone-releasing hormone (LHRH) analogs. LHRH is a hormone produced by the hypothalamus, which stimulates the pituitary gland to produce luteinizing hormone (LH). LH then stimulates testicles to produce testosterone. Treatment with LHRH analogs initially increases levels of testosterone but due to a negative feedback loop, androgen levels decrease and slow the process of tumor growth. Leuprolide (Lupron, Viadur, Eligard), goserelin (Zoladex), and triptorelin (Trelstar) are the major LHRH analogs being used for hormone therapy (70-72). Side effects from using LHRH analogs are similar to those observed after orchiectomy and related to low testosterone levels. Abarelix (Plenaxis) is an LHRH antagonist that works like an agonist and rapidly reduces   testosterone levels but is accompanied by less severe side effects (1).

Antiandrogens. Antiandrogens work by blocking the action of androgens in the body and thus inhibit their effects. Flutamide (Eulexin), bicalutamide (Casodex), and nilutamide (Nilandron) are some antiandrogen drugs currently used for the treatment of prostate cancer (70-72). Usually antiandrogens are used in combined androgen blockade

(CAB) along with orchiectomy for total inhibition of androgen activity. Compared to orchiectomy or treatment with LHRH agonists, antiandrogens when used alone have fewer side effects on sexual competency (1).

5-alpha reductase inhibitors. Finasteride (Proscar, Propecia) or dutasteride (Avodart) inhibit enzyme 5-alpha reductase and this results in decreased conversion of testosterone to its active moiety DHT resulting in the inhibition of prostate tumor growth (73).

Others. Estrogens were once used to suppress the action of testosterone as an alternative to orchiectomy. Because of possible serious estrogenic side-effects of breast enlargement and blood clot formation estrogens have largely been replaced by antiandrogens and LHRH analogs. They are used in some situations if the patient is not responding to other treatments. Antifungal compound Ketoconazole (Nizoral) also inhibits production of androgens and is sometimes used for treatment of prostate cancer (74).

Chemotherapy for prostate cancer

Cytotoxic drugs are used for prostate cancer therapy when other treatments fail. Combination treatment with docetaxel and prednisone prolongs survival and has been extensively used for treating prostate cancer (75, 76). Other drugs used to treat prostate cancer include mitoxantrone (Novantrone), estramustine (Emcyt), doxorubicin (Adriamycin), etoposide (VP-16), vinblastine (Velban), paclitaxel (Taxol), carboplatin (Paraplatin), vinorelbine (Navelbine) (1). Mitoxantrone plus low-dose prednisone treatment showed improvement in the quality of life when compared to the prednisone

treatment alone and this finding led to a subsequent phase III study TAX 327 by Tannock *et al* (77, 78) comparing docetaxel plus daily prednisone with mitoxantrone plus prednisone given either every three weeks or weekly. In this study, docetaxel plus prednisone showed significant improvement in the survival and response rate when compared to the mitoxantrone plus prednisone treatment (75).

COLON CANCER

Incidence

Colon cancer is the third most common cancer diagnosed both in men and women in the United States excluding skin cancers. Risk factors for developing colon cancer include age, history both personal and family, inherited syndromes and lifestyle factors (1).

The risk of developing colon cancer is higher in people older than 50 years but it can be observed even in the younger population. People with history of adenomatous polyps and any bowel diseases such as inflammatory bowel disease are also at an increased risk. People with the family history of colorectal cancer are also at an increased risk of developing cancer but accounts only up to 20%. Most of the people who are diagnosed with colon cancer do not have a family history of colon cancer incidence, and only about 5 % of colon cancer patients have inherited genetic abnormalities (1).

The most common inherited syndromes that pose a risk of developing colon cancer include familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC). FAP is observed because of mutations in the adenomatous polyposis coli (APC) gene and this syndrome accounts to about 1% of of all colorectal

cancers diagnosed. This is a syndrome observed in young populations characterized by thousands of polyps in the colon and rectum (1). HNPCC, also known as Lynch syndrome accounts for about 3-4% of all the colon cancers. It is caused by mutations in genes involved in DNA repair which include hMLH1, hMLH2 and hMLH6 (79-81) When compared to FAP, HNPCC is observed in younger populations like FAP but, unlike FAP patients, have very few polyps in their gut.

Other polyposis syndromes that increase the incidence of colorectal cancer include Peutz Jegher's syndrome, familial juvenile polyposis, and hereditary mixed polyposis syndrome. These syndromes are linked to mutations in LKB1, STK11, SMAD4, PTEN, E-cadherin, cyclin D1, and transforming growth factor β receptors (82).

Racial and ethnic background

African Americans in the United States and Jews of Eastern European descent (Ashkenazi Jews) in the world have the highest colorectal cancer incidence and several mutations in genes that increase the risk of colorectal cancer have been observed in Ashkenai jews (1). The colon cancer incidence rate is very low in Asian countries especially in India (83) and the increase in incidence of this disease in migrants from Asian countries to Western countries suggests that environmental factors related to diet contribute to development of colon cancer (84, 85).

Environment and lifestyle factors

Obesity, physical inactivity, smoking, alcohol intake and diets high in red or processed meats and low in fiber (i.e. low intake of fruits and nuts) are some of the possible risk factors for colorectal cancer. Fruits, nuts and vegetables contain diverse

anticarcinogenic phytochemicals; however, epidemiological studies and particularly prospective studies do not strongly correlate high consumption of these foods with decreased incidence of colon cancer (86-88). Several reports suggest that folate intake may be protective for colon cancer (89, 90) and in a study of colorectal cancer cases and controls, there was a decrease in colon cancer risk in individuals that express a 677 T–C mutation in 5,10-methylenetetrahydrofolate reductase (91). This decreased enzyme activity results in increased levels of 5-10-methylenetetrahydrofolate that plays an integral role in DNA synthesis.

Types of colon cancers

Most colon cancers (about 90%) develop in the gland cells that form mucus to lubricate the inner lining of the gut hence called adenocarcinomas. Other type of tumors are less common and these include carcinoid tumors that develop from cells that produce hormones, gastrointestinal stromal tumors (GISTs) which develop from "interstitial cells of Cajal" and lymphomas-which develop from the immune cells of the lymph glands in the colon or rectum (1).

Diagnosis of colorectal cancer

Colorectal cancer is diagnosed as soon as the symptoms appear and this results in a complete physical examination along with blood tests to check for any tumor biomarkers such as carcinoembryonic antigen (CEA) and CA 19-9, which are produced by the cancer and released into the blood. Levels of these markers are not specific to colorectal cancer and may be high for other diseases of the gut such as ulcerative colitis; sometimes smoking increases levels of CEA. If the physical examination and symptoms

are suggestive of cancer, additional tests are carried out; and these include endoscopic tests such as sigmoidoscopy or colonoscopy or imaging such as barium enema, computed tomography (CT) colonoscopy. Once cancer is diagnosed, a biopsy is performed to confirm the diagnosis (1).

Stages of colorectal cancer

The wall of the intestine is made up of different layers. Colorectal cancer starts in the innermost lining also called epithelium. Cancer usually begins as a non-cancerous polyp on the innermost layer of the colon or rectum.
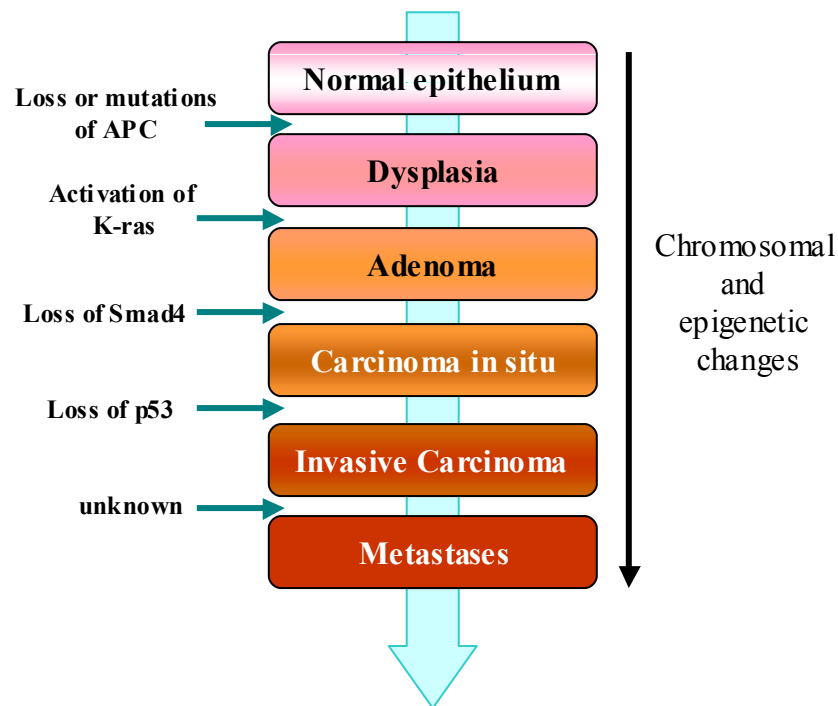
Fig. 1.5. Genetic progression model of colorectal adenocarcinoma (92).

These polyps may or may not transform into malignant tumors and their transformation depends on several other factors and the type of polyp (Fig. 1.5) (92). There are two kinds of polyps; one is adenomatous polyps that have a higher risk of developing into cancer and the second type hyperplastic polyps and inflammatory polyps, which are usually not cancerous. Dysplasia is a different type of condition where the cells of the colon and rectum look abnormal and have the potential to transform into cancer cells.

AJCC (TNM) staging system

TNM is the most common staging method used. This system is focused on three elements of the tumor: T for the extent of spreading of the primary tumor into the wall of the intestine and N for the extent of the spread of the tumor to the adjacent lymph nodes. M stands for metastases and measures the extent of tumor spreading to other organs of the body or not (1). Numbers or letters appear after T, N, and M to provide more detailed information related to the tumor and the numbers 0 through 4 indicate increasing severity. The letter "X" indicates that the tumor cannot be assessed. The letters "is" indicates "*in situ* carcinoma " indicating that the tumor is contained within the top layers of the duct and has not invaded deeper into the duct (1).

Treatment for colorectal cancer

Depending on the stage of the cancer, any one of the following treatment options are available. Colectomy or laparoscopic–assisted colectomy is performed during early stages when the cancer is limited to the colon itself. Rectal surgery is performed if the cancer is localized in the rectum itself. Sometimes high-energy radiowaves are used to destroy the cancer cells and is called radiofrequency ablation (93, 94). Percutaneous

injection of ethanol into the tumors is also an option called ethanol ablation; and cryosurgery can be used for destroying the larger tumors by freezing (95). Radiation is usually performed to destroy any cancer cells that are remaining after surgery. External-beam and internal radiation therapy or brachytherapy are the two types of radiation to destroy cancer cells after surgery, however, here are several side effects such as sexual problems, bowel incontinence, etc associated with this method (96).

Chemotherapy for colorectal cancer

For treatment of colon cancer, chemotherapy is used as an adjuvant therapy after surgery to increase patient survival rates. It is also in use as a neoadjuvant therapy prior to the surgery to shrink the tumor and facilitate its removal during surgery. Colon cancer chemotherapy usually involves drug combinations. 5-FU has been used for about 50 years and is given in combination with folinic acid drug leucovorin to increase its efficacy. 5-FU is a pyrimidine analogue which is incorporated into DNA and RNA to inhibit the cell cycle in S-phase and induces apoptosis (97). Leucovorin is metabolized in the cell into polyglutamated 5, 10 methylnetetrahydrofolate and stabilizes the binding of 5-FU with thymidylate synthase (98). Capecitabine (Xeloda) is a prodrug that is converted into 5-FU in the tissues, and Irinotecan (Camptosar) is a structural analogue of camptothecin which is a topoisomerase I inhibitor. Irinotecan is used in combination with 5-FU and leucovorin as a first line chemotherapy in the FOLFIRI regimen for treatment of advanced colorectal cancers. Topoisomerases are enzymes that catalyze DNA to unwind from its supercoiled topology and to aid in transcription or replication. Irinotecan is a topoisomerase I inhibitor which is hydrolysed into an active metabolite

SN-38 which inhibits DNA replication and transcription, tumor growth. Major side effects with this drug include severe diarrhea and immunosuppression.

Oxaliplatin (Eloxatin) is a platinum based drug used in combination with 5-FU in FOLFOX therapy and in combination with capecitabine in CapeOX therapy (99). This is a cisplatin drug with increased efficacy when compared to its counterparts; however, the mechanism of action of oxaliplatin is not known. Major side-effects include neuropathy, GI problems, neutropenia, and ototoxicity. Many of these therapies use non-specific cytotoxic agents, which induce undesirable adverse effects, and there is an increasing need to develop alternative drugs that target specific pathways to inhibit tumor growth, progression, and metastasis and to induce apoptosis.

PANCREATIC CANCER

Incidence

According to ACS, there will be about 37,680 new cases diagnosed and about 34,290 deaths because of pancreatic cancer this year in US, making it a fourth leading cause of cancer deaths (1).

Risk Factors

The incidence rates for pancreatic cancer in tobacco smokers is two times higher than in nonsmokers (100-105). Men tend to have higher risk when compared to women but the differences have decreased; and this has been attributed to increased smoking in women. Obesity, diabetes, cirrhosis and chronic pancreatitis are also risk factors for pancreatic cancer. Diets high in fat content also increase the risk for pancreatic cancer. About 10% of pancreatic cancer patients have a family history of this disease and

germline mutations in genes such as familial atypical multiple melanoma (FAMM), BRCA2, PRSS1, Lynch syndrome, Peutz-Jeghers syndrome are associated with increased risk of pancreatic cancer. In addition to these germline mutations several acquired gene mutations have also been identified in pancreatic tumors and these include mutations in oncogenes, tumor suppressors and DNA repair genes (Fig. 1.6) (106-117). The K-ras oncogene mutated in codon 12 is expressed in more than 90% of pancreatic cancers; this constitutively active form of ras increases cell proliferation (118). Mutations in the tumor suppressor gene p53, cyclin dependent kinase inhibitor p16 and SMAD4, the downstream target of transforming growth factor β (TGFβ), are also frequently observed in pancreatic tumors (107, 108); vascular endothelial growth factor (VEGF) and Erb2/neu are overexpressed in pancreatic tumors (110, 119, 120). Using microarrays, many genes that are differentially expressed in pancreatic adenocarcinoma have been identified and further research on these targets for pancreatic cancer chemoprevention and chemotherapy is needed (121, 122) .

Tumorigenesis of pancreas

The pancreas is composed mainly of two different types of glands – exocrine and endocrine. The exocrine part of pancreas secretes pancreatic juice, which contains enzymes that help in digestion of the food that enters the duodenum from the stomach. The endocrine part of pancreas accounts for a smaller portion of pancreatic mass and is arranged in the form of clusters called Islets of Langerhans, which produce two different

types of hormones, namely insulin and glucagon, which are important for maintaining glucose levels in the body. Endocrine pancreatic tumors are rare and most pancreatic tumors originate from the exocrine part and are adenocarcinomas arising in
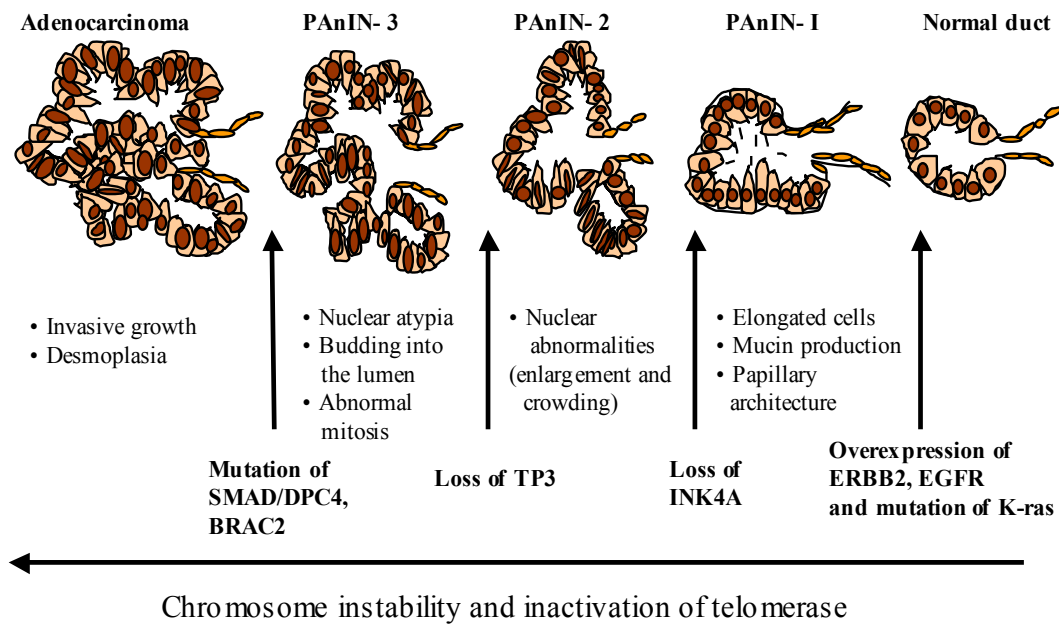
Fig. 1.6. Genetic progression model of pancreatic adenocarcinoma (123).

the ducts of glands or acinar cells and account for 85-90% of all pancreatic tumors (123, 124). A stepwise pattern of pancreatic adenocarcinoma progression has been described by studying the molecular and pathological features of pancreatic intraepithelial neoplasias (PanINs) (Fig. 1.6). Mutations in K-ras, ERBB2, and EGFR are associated with the transition from normal ductal cells to PanIN-1 stage cells which are characterized by elongated morphology and mucin production. Loss of the tumor suppressor CDKN2A due to mutations, deletions or hypermethylation of the promoter is

observed in 80-95% of the sporadic pancreatic adenocarcinomas. CDKN2A is located at

the 9q21 gene locus which encodes for two tumor suppressor genes namely INK4A and

ARF. INK4A inhibits phophorylation of retinoblastoma (RB) and prevent the entry of

cells into S phase of cell cycle and ARF inhibits MDM2-depedent proteolysis which

stabilizes p53. These genetic changes are observed in PanIN-2 lesions and are

characterized by cells with enlarged nuclei and other abnormalities. Other genetic

alterations that are associated with pancreatic cancer progression of PanIN-2 to PanIN-3

and adenocarcinoma include mutations in tumor suprressor genes, TP53,

SMAD4/DPC4, and BRAC2. Moreover chromosomal and microsatellite instability are

also frequently detected at the late stages of PanIN lesions (Fig. 1.6) (123).

Diagnosis of pancreatic cancer

Only 7% of pancreatic cancers are detected in their early stages since there are

not any effective diagnostic methods for early detection.The disease is usually

asymptomatic until it metastasizes to other organs (125, 126). CA 19-9 is a substance

produced by pancreatic cancer cells and released into the blood, however, by the time

blood levels of CA 19-9 are detectable, the disease is no longer in its early stages. Thus

the CA19-9 test is used during treatment but not for detection of pancreatic cancer .

Once the disease is advanced, patients exhibit signs and symptoms such as jaundice,

abdominal pain, weight loss, poor appetite, digestive problems, and further tests are

required to confirm the diagnosis. Imaging tests such as CT scan, positron emission

tomography (PET) scan, ultrasonography, mangnetic resonance imaging (MRI),

endoscopic Retrograde Cholangiopancreatography are used and a biopsy ultimately confirms the presence and stage of the cancer (1).

Staging of pancreatic cancer

Staging of pancreatic cancer is also done according to the AJCC –TNM system as described under colorectal cancer.

Treatment of pancreatic cancer

Although surgery is considered as a potential treatment option for pancreatic cancer, only 15% of the patients are good surgical subjects.Two types of surgeries are performed depending on the stage of cancer. Based on the imaging tests, if they suggest that the cancer is not that widespread and can be removed completely, potential curative surgery is performed. However, if the imaging tests suggest that the tumor is widespread and cannot be removed completely, palliative surgery is done to prevent the complications such as blockage of bile ducts or intestine by the cancer and to relieve the symptoms. External beam radiation therapy is the most common type of radiation used for treating pancreatic cancer (125).

Chemotherapy for pancreatic cancer

Gemcitabine is the drug most commonly used for the treatment of pancreatic cancer. Gemcitabine replaces cytidine, one of the building blocks of nucleic acids during DNA replication and this inhibits cell cycle progression. 5-FU is also used for treatment alone or in combination with Gemcitabine. Several clinical trials are underway testing the combination of 5-FU and gemcitabine with other chemotherapies that include cisplatin, oxaliplatin, docetaxel, irinotecan (125).

ESOPHAGEAL CANCER

The esophagus is a muscular tube connecting throat to the stomach, and cancer of esophagus can arise anywhere along the lining of the tube. Although the incidence of cancer is relatively rare accounting for 1% of US cancer incidence, has been increasing (1).

Risk factors

The risk of esophageal cancer increases with age with 80% diagnosed in people between 55 and 85 years old. African Americans have twice the risk as whites for developing esophageal cancer. Tobacco and obesity are major risk factors for developing esophageal cancer (127). Males have a higher risk than females and this is attributed to tobacco use. People with gastro-esophageal reflux disease (GERD) and with Barrett esophagus are also at higher risk. Diets low in fruits, vegetables and vitamins have increased risk for esophageal cancer (128, 129) . Various genetic alterations are associated with esophageal cancer progression including allelic loss of chromosomes 17p, 13q, 9p21-22 and mutations in p53 and p16 genes (130, 131). Studies using cDNA microarrays have identified differential patterns of gene expression involved in the regulation of squamous cell proliferation and differentiation. Genes involved in differentiation were downregulated and these include transglutaminase (TGM) 3 and its substrates including small proline-rich proteins (SPRRs), calcium binding S100 proteins (S100A8 and S100A9), annexin I, epithelial membrane protein (EMP) 1 and cystatin A (132-135). All these proteins are expressed in the cell envelope (CE) which functions as a barrier and results in impaired CE and accumulation of toxic substances. SPRRs and

calcium binding S100 proteins constitutes the epidermal differentiation complex (EDC) involved in the terminal differentiation of epithelial cells (134). KRT4, KRT13, and KRT15 are cytokeratins involved in epithelial differentiation and these genes were also downregulated in contrast to increased expression of KRT16 and KRT17 in esophageal cancer (136) . Studies with KRT4-/- mice demonstrated abnormal proliferation and improper terminal differention of the epithelial cells (137). Genes involved in transcription including gut enriched kruppel-like factor (GKLF), and eukaryotic translation elongation factor 1 alpha -1 (EEF1A1) which regulates the cell cycle by modulating microtubules were also downregulated (138). Genes that play a role in metastasis were differentially expressed including upregulation of proteases such as cathepsin B that degrade ECM and downregulation of protease inhibitors such as cystatin A, cystastin B and serine proteinase inhibitor. However, the role of these genes in the progression of esophageal cancer is not well characterized and needs to be investigated.

Diagnosis of esophageal cancer

Symptoms of esophageal cancer do not appear until the disease has advanced. The most common symptom as the tumor grows is difficulty in swallowing and it can also cause chest pain or burning. Endoscopy and barium swallow tests are used to diagnose esophageal cancer.

Treatment of esophageal cancer

Surgery when combined with other treatments can help ease the symptoms and improve the quality of life. Esophagectomy is the first line of treatment and is performed

if the tumor is localized in the esophagus and has not been spread to the regional lymph nodes. If the tumor spreads to other organs and regional lymph nodes, radiation and chemotherapy are used. In locally advanced cancer, concurrent cisplatin-based chemoradiotherapy or surgery result in 60-90% disease control rate and 20-40% 5-year disease free survival rate (139-145). Neoadjuvant chemoradiotherapy after esophagectomy is extensively used because of its beneficial effects in the treatment of esophageal adenocarcinoma. More than 50% of patients are diagnosed with metastatic or unresctable esophageal cancer and chemotherapy provides improvement in the quality of life relieving symptoms such as dysphagia in 60-80% of the patients (146-148).

Drugs that are used in esophageal cancer chemotherapy include 5-FU, platinum based drugs such as cisplatin, caboplatin, and antibiotics such as bleomycin, anthracycline antibiotic doxorubicin, mitotic inhibitors paclitaxel, topoisomerase inhibitors irenotecan, topotecan.

Photodynamic therapy, use drugs that make the precancerous and cancerous cells sensitive to light and when light is inserted into the esophagus using endoscope cancerous tissues are burned. This is usually used for treatment of Barrett's esophagus and has now been extended to esophageal cancer.

NEW TARGET MECHANISM BASED DRUGS

Targeted therapies for treatment of cancer derived from multiple tissues/organs are highly promising for future treatments since these agents are usually more tumor-specific and are accompanied by fewer adverse side-effects (149-153). Targeted therapies include drugs such as kinase inhibitors, antibodies that block various signaling

pathways, immunotherapies, and genomic therapies that involve re-expression of tumor suppressor genes or specific gene knockdown technologies (including RNA interference). The application of these targeted drugs may be highly tumor-specific or specific for only a sub-class of tumors such as the highly effective treatment of non-small lung cancer patients with specific EGF receptor mutations with the EGF receptor inhibitors erlotinib and gefitinib (35, 154).

Targeted therapies for prostate cancer

With the increasing knowledge of the genes that play a role in the progression of prostate cancer there is scope for development of new drugs for treatment of metastatic or hormone-refractory prostate cancer with increased specificity. Recent developments in the treatment of prostate cancer are discussed below.

Drugs that target signal transduction pathways

Several mechanisms are involved in the transformation of androgen responsive prostate cancer to an androgen refractory prostate cancer (155) and some of these pathways include i) amplification of AR and hypersensitivity to low levels of DHT; ii) decreased specificity of the AR so that it responds to other growth signals or hormones ; iii) activation of AR through kinase-dependent phosphorylation and ; iv) activation of AR independent growth pathways (Fig. 1.7) (38). Agents that target growth factors involved in androgen independent cancer include rapamycin analogs that inhibit Akt/PI3/mTOR, monoclonal antibodies and inhibitors of HER-2/3, IGF-R, IL-6R, and platelet-derived growth factor receptor (PDGFR). The Akt/PI3K pathway regulates various processes including cell proliferation, growth, apoptosis and cytosketal

rearrangement. PI3Ks are heterodimers with different regulatory and catalytic subunits.

P85 is the regulatory subunit and acts as a substrate for many cytoplasmic and receptor

tyrosine kinases that bind its SH2 domain to the phosphotyrosine residues of the kinases.

There are three classes of PI3Ks and classIA PI3Ks are strongly associated with

oncogenesis. PI3K activation by receptor tyrosine kinases results in generation of PIP3

which acts as a second messenger for downstream effects involving AKT and other
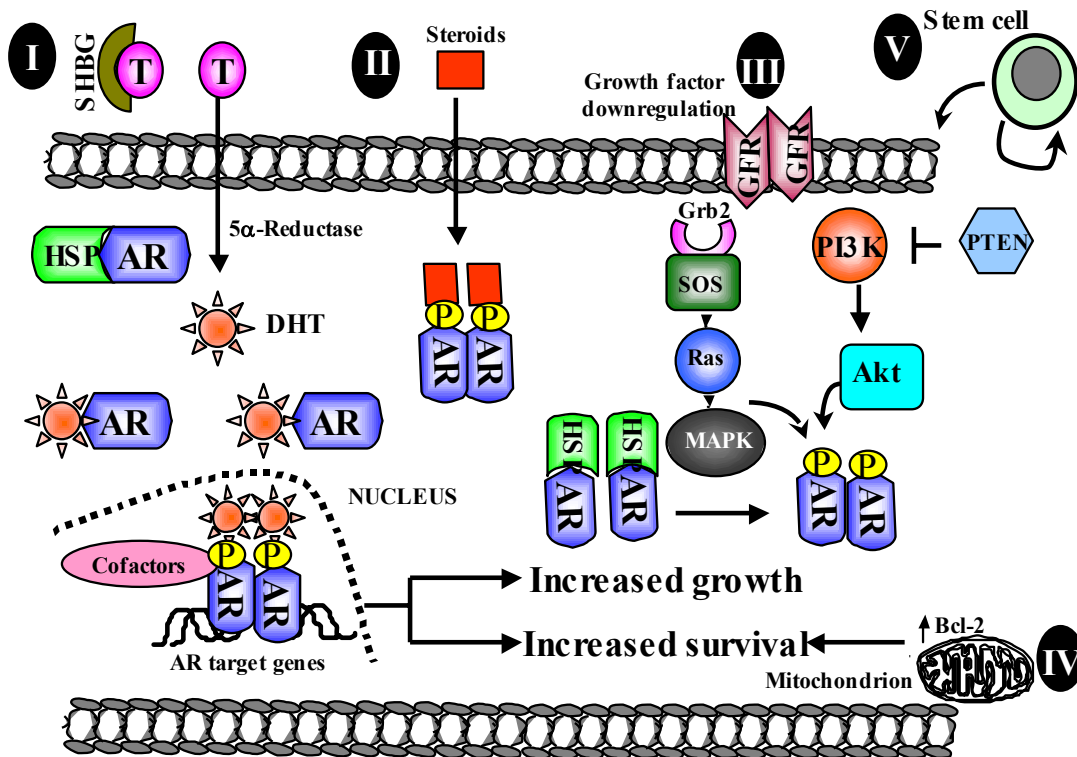
Fig. 1.7. Mechanisms of androgen independence (155).

proteins. The increasing emphasis on the role of Akt/PI3K pathway and its associated

proteins in survival, differentiation and apoptosis has lead to the identification of the

expression and alterations of these genes in the progression of many human cancers. Amplification and several mutations of the catalytic and regulatory subunits of PI3K and AKT been identified in many human cancers (117, 119).

In most prostate cancers there is a loss of tumor suppressor gene PTEN which results in activation of Akt/PI3K pathway and increased cell proliferation and tumor growth (156, 157). Loss of PTEN and activation of Akt is associated with hormonal resistance, chemoresistance and increased tumor growth (158, 159). Rapamycin analogs inhibit this pathway in mouse models of PTEN loss or Akt activation (159, 160).Three rapamycin analogs are currently being investigated, namely rapamycin, CCI-779 (Temsirolimus), and RAD001 (Everolimus) (161-163). Everolimus in combination with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib and docetaxel is in phase II clinical trials. EGFR inhibitors, geftinib and trastuzumab, have not been successful for treating metastatic prostate cancer. Recent studies showed that EGFR and HER-2 are not causal determinants in prostate cancer (164-166) and HER-3, IGF-R, TGF-β, IL-6 and other growth factors were shown to be important. Drugs that target HER- 3 include neuregulin and pertuzumab, monoclonal antibody (Omnitarg) and lapatinib, an intracellular inhibitor (GlaxoSmithkline) of HER-2/HER-3 signaling (167, 168).

Drugs that target the endothelin axis

Endothelin (ET) is an important paracrine signaling molecule that plays a role in the mediation of bone-prostate cancer crosstalk (155). ET is a vasoconstrictive agent which also regulates mitogenic pathways in prostate cancer cells and osteoblasts and is

associated with the aggressive behavior of the hormone refractory prostate cancer (169).

Atrasetan (Xinlay) is a potent antagonist of the ET-A receptor and Phase III trials using

this drug resulted in a statistically insignificant outcome (170). Another ET-A receptor

antagonist, ZD4054 (Astra Zeneca) is also in Phase II clinical trials.

Drugs that target platelet-derived growth factor receptor (PDGFR)

Prostate cancer cells express higher levels of PDGFR; and this receptor

enhances PI3 kinase/Akt signaling and results in prostate cancer progression (171).The

efficacy of Imatinib (Gleevac), an inhibitor of PDGFR tyrosine kinase alone or in

combination with docetaxel, is being investigated for prostate cancer chemotherapy.

Drugs that target stem cells

Elements of the Hedgehog (Hh) signaling pathway, human telomerase and

CD133 have been identified in stem cells and are overexpressed in prostate cancer

(172-175). A prolonged period of prostate cancer relapse was observed after inhibiting

the Hh signaling pathway in xenograft models of androgen insensitive prostate cancer

(PC-3) cells (175). Cyclopamine analogs are hedgehog (Hh) antagonists that are being

investigated in pre-clinical drug development. Human telomerase is a reverse

transcriptase enzyme that maintains the stability and length of the chromosome by

protecting the ends of chromosomes also called telomeres (176). Telomeres get shorter

with each DNA replication and when they are critically short, cells stop dividing and

die, this phenomenon of limited cellular division is called the "Hayflick limit". However

some cells such as germ cells, stem cells, hair follicles and cancer cells overcome this

limit and aquire indefinite replicative ability by overexpressing telomerase which aids in

telomere repair. Normal prostate lacks telomerase activity but the enzyme is

overexpressed in prostate tumors. Drugs targeting telomerase are also underdevelopment

for treatment of prostate cancer (174).

Drugs that target angiogenesis

Angiogenesis plays an important role in physiological processes and also in

tumor growth and metastasis; inhibition of angiogenesis has become an attractive

therapeutic strategy. In 1971, Judah Folkman and his colleagues first proposed that

vascularization is essential for the growth of clinically invasive tumors (Fig. 1.8).
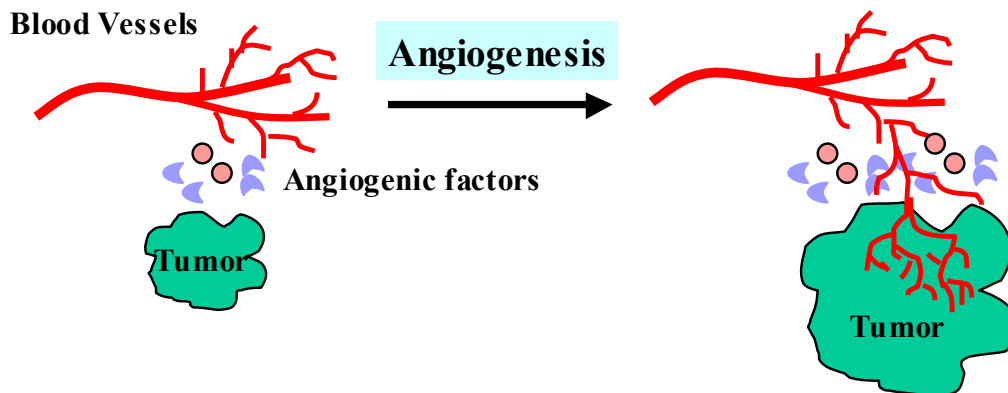
Fig. 1.8. Angiogenesis and cancer (177).

There are several pro- and anti-angiogenic factors that regulate angiogenesis, and

these include VEGF, basic FGF (bFGF), interleukin-8, placenta-like growth factor

(PlGF), angiopoeitin-2 TGF, PDEGF, pleiotrophin, and others (178, 179). VEGF is the

most potent pro-angiogenic factor and has been well characterized as an important

regulator of angiogenesis (180). The VEGF family of angiogenic factors include VEGF-

A, VEGF-B,VEGF-C,VEGF-D, and PlGF (181, 182). VEGF-A, also referred to as

VEGF, is the major mediator of tumor angiogenesis and binds to two main receptors,

namely fms like tyrosine kinase (Flt-1) or VEGFR-1and flk-1/ kinase domain region

(KDR) or VEGFR-2, which are expressed at higher levels by endothelial cells and by

circulating bone marrow-derived endothelial progenitor cells (Fig. 1.9)(180).
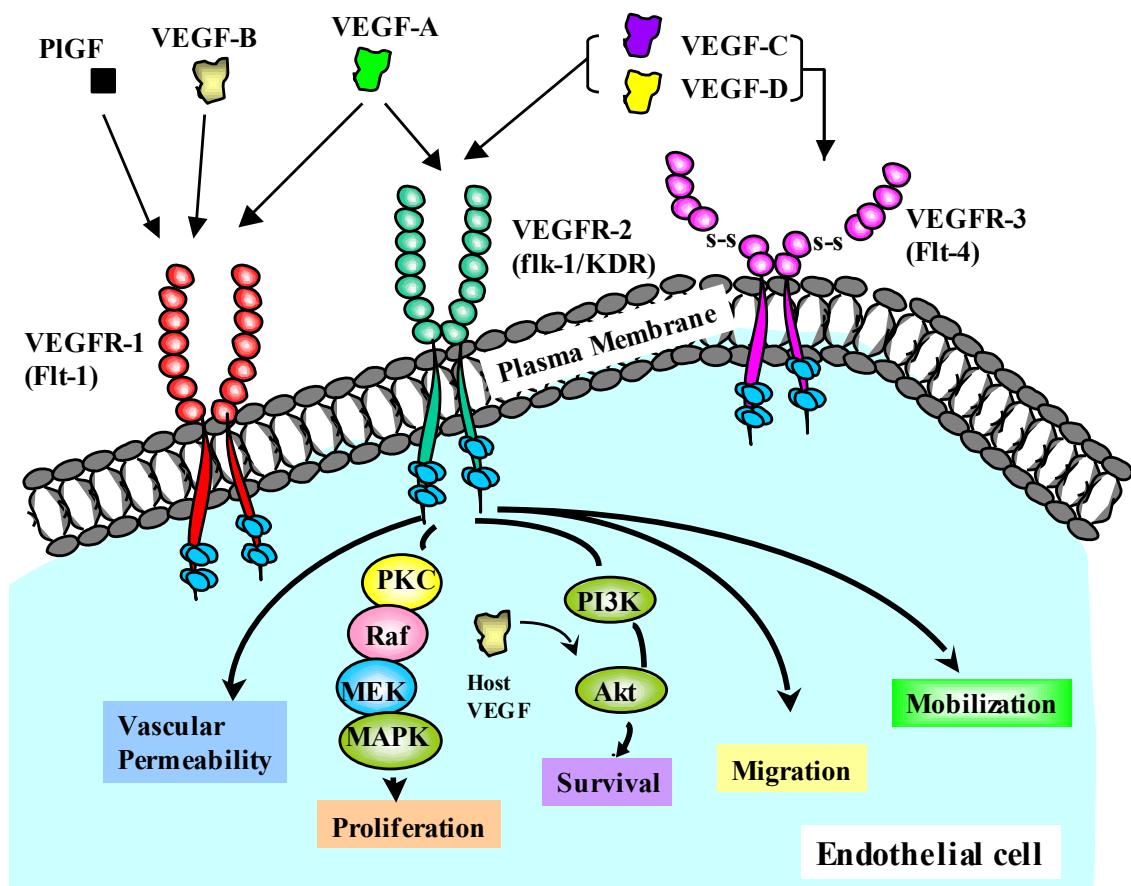
Fig. 1.9. The family of VEGF molecules and receptors (183).

Angiogenic factors stimulate angiogenesis by two mechanisms. Firstly, they bind locally to cognate receptors on the peritumoral vascular endothelial cells and this results in budding of new capillaries that supply blood to tumors from the existing host blood vessels. The second mechanism involves binding of angiogenic factors to distant receptors on bone marrow cells to mobilize them into the circulation and into the tumor to promote vacularization in cooperation with additional angiogenic factors. VEGF binding to VEGFR-2 is critical in the former mechanism since it activates endothelial cell proliferation and survival whereas PlGF binding to VEGFR-1 recruits macrophages and other proangiogenic cells derived from bone marrow and plays an important role in the latter mechanism (184). Anti PlGF antibodies inhibited tumor growth, angiogenesis and recruitment of macrophages in mouse models of cancer. In contrast antibodies against VEGFR-2 inhibited tumor growth and angiogenesis but did not inhibit the recruitment of macrophages suggesting different mechanism of actions mediated by these two receptors and their ligands (185). Combination therapy with the two antibodies resulted in greater inhibition when compared to individual treatments. This preclinical data with anti-PlGF antibodies constitutes a significant improvement that will be further evaluated in clinical trials of antiangiogenic therapy for human cancers. Hypoxia-inducible factor-1(HIF-1) mediates the transcription of angiogenic genes and is induced in response to reduced levels of oxygen; is also overexpressed in many tumors (186). Endothelial and stromal interactions are not only critical for organogenesis but also for tumorigenesis and tumor progression. Stroma with respect to tumor constitutes ECM and mesenchymal cells such as endothelial cells, fibroblasts, vascular smooth muscle cells

and pericytes (187). Interaction of endothelial cells with ECM proteins is mediated

through cell surface integrins that are expressed on the surface such as $\alpha_1\beta_1$, $\alpha_2\beta_1$ and

$\alpha_3\beta_1$, all of which bind collagen and laminin; $\alpha_6\beta_1$, which binds laminin; $\alpha_4\beta_1$ and $\alpha_5\beta_1$,

which bind fibronectin and fibrin; and $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which variously bind vitronectin,

fibronectin, fibrin and laminin (188).
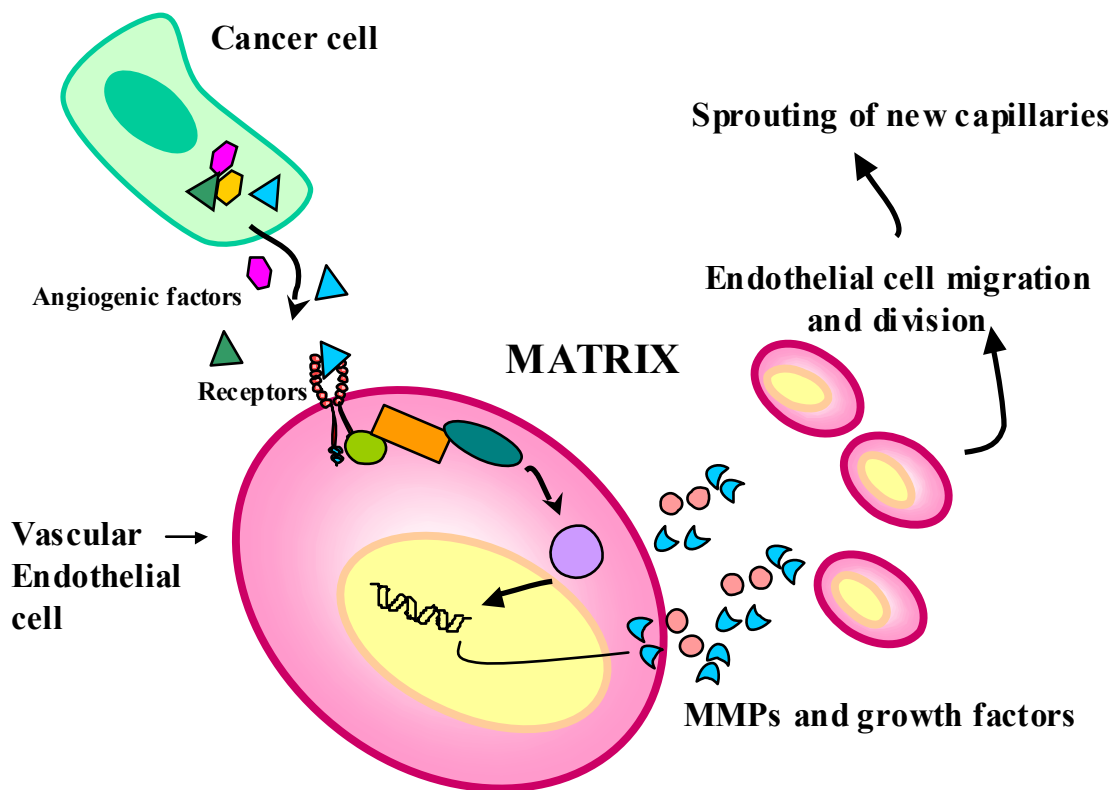
Fig. 1.10. Tumor angiogenesis signaling cascade and endothelial stromal interactions (177).

During angiogenesis endothelial cells mainly interact with integrin $\alpha_v\beta_3$ and

results in VEGFR-2/$\alpha_v\beta_3$ complex formation. VEGF-A activated VEGFR-2 recruits c-

src and PI3K, which then phosphorylates cytosolic domain of $\beta3$ integrin and thus

results in the formation of VEGFR-2/ $\alpha_v\beta_3$ complex (189-192). A recent study using

knock-in mice expressing a $\beta_3^{-/-}$ mutant was not able to form a complex with VEGFR-2

and had impaired tumor angiogenesis (191). Degradation and remodeling of the ECM is

critical in the formation of new capillaries from existing ones. Endothelial cells produce

matrix metalloproteinases (MMPs) that degrade the basement membrane and mobilize

endothelial cells and growth factors such as VEGF from the matrix towards new vessel

formation; and pericytes are recruited to form new capillary wall (Fig. 1.10). Knock

down of a membrane bound MMP, MT1-MMP using RNAinterference mechanism

impaired lumen formation in an *in vitro* assay emphasizing its importance in

angiogenesis (193). Gene deletion methods have shown that MMP2 and MMP9 play a

significant role in angiogenesis (194). MMPs also cause the release of the antiangiogenic

factors angiostatin and tumstatin. Surgical removal of a primary tumor initiates

secondary tumor development or metastasis. Systemic administration of angiostatin

inhibited secondary tumor formation and neovascularization after removal of the

primary tumor emphasizing the role of angiostatin as an antiangiogenic factor produced

by tumor cells (195, 196). VEGF has been targeted by various strategies including

monoclonal antibodies (mAbs), inhibitors of endothelial cell-receptor tyrosine kinase

activity and antisense oligonucleotides. Bevacizumab (Avastin) is the most well-

characterized recombinant humanized mAb against VEGF. It binds to all isoforms of

human VEGF and prevents its binding to cognate receptors (197). Bevacizumab is the

first anti-angiogenesis therapy approved by the U.S. Food and Drug Administration

(FDA) and is currently approved for treatment of two of the three largest cancer killers

in the U.S. (198). A Phase II study demonstrated safety and efficacy of combination therapy with drugs including docetaxel, estramustine, and bevacizumab for the treatment of hormonerefractory prostate cancer which lead to a phase III study, CALGB 90401 to compare docetaxel and bevacizumab in combination with prednisone for the treatment of hormonerefractory prostate cancer.

There are multiple receptor tyrosine kinase inhibitors (RTKIs) that target EGFR tyrosine kinase (cetuximab, erlotinib, geftinib, ZD6474,CI1033 or PKI1666), VEGFR tyrosine kinase (Sorafenib/BAY-43-9006, PTK787, ZD6474, Sutent/SU6668/SU11248), PDGF tyrosine kinase (PTK787 or SU11248), HER-2/Neu receptor (trastuzumab), and interferon (IFN)-α receptor. VEGFR tyrosine kinase inhibitors, sorafenib and sutent (SU11248), and thalidomide analogs, revlimid and actimid that inhibit VEGF, T-cell costimulatory function, TNF-α inhibition and decrease IL-6 levels, are in Phase II and Phase III studies clinical trials for the treatment of prostate cancer (199-201). A Phase II trial with docetaxel in combination with thalidomide resulted in significantly decreased PSA levels, time to disease progression and disease free survival but was accompanied by severe side effects (202).

There are number of other drugs that target angiogenesis by inhibiting basement membrane degradation, endothelial cell proliferation, migration, tube formation, neovascularization and inducing apoptosis in endothelial cells. For example, NM-3, an isocumarin inhibitor of VEGF blocks combretastatin, induces rearrangement of the actin cytoskeleton, and induces early membrane blebbing in endothelial cells (179, 203-205).

In addition, there are a number of endogenous antiangiogenic factors which include angiostatin, angiomotin, arrestan, canstatin, endostatin, tumstatin, and thrombospondin.

Agents that induce differentiation and apoptosis

Epidemiological data suggest a link between Vitamin D deficiency and prostate cancer progression indicating that the vitamin D receptor (VDR) may be a potential target for development of new anticancer drugs (206). Calcitriol, the active form of vitamin D found in the body exhibits proapoptotic, growth inhibitory and differentiation-inducing properties in prostate cancer cells and is currently being evaluated in clinical trials (207, 208). Histone deacetylase (HDAC) and DNA methyltransferase inhibitors are also being evaluated for the treatment of prostate cancer. HDAC and DNA methyltransferase enzymes play a role in epigenetic silencing of gene expression; and inhibition of these enzymes induces apoptosis, p21 signaling and inhibition of tumor growth (209). SAHA (Merck) is an orally bioavailable HDAC inhibitor currently undergoing clinical trials for treatment of androgen-independent prostate cancer. Abnormal hypermethylation and gene silencing of the antioxidant enzyme GST-II and the tumor suppressor p21 is observed in prostate cancer (210). Antisense Bcl-2, oblimersen sodium (Genasense) and the proteasome inhibitor, bortezomib (Velcade) are two drugs that target Bcl-2, an anti-apoptotic protein that is overexpressed in metastatic hormonerefractory prostate cancer (211).

Vaccination strategies

Provenge, a prostate acid posphatase (PAP)-activated dendriticcellbased vaccine, and Prosate GVAX, a wholecell allogeneic vaccine are being developed for

treatment of prostate cancer in combination with docetaxel (212). Provenge is a process of antigen delivery to activated antigen-presenting cells that are collected from patients and stimulated by fusing cells with PAP-GMCSF protein and then administered intradermally to the patients. Prostate GVAX immunotherapy uses whole–cell allogenic PC-3 and LNCaP prostate cancer cells that are virally transduced to express GM-CSF an immune adjuvant which is lethally irradiated and injected into patients intradermally (213-215). Two other vaccines that are in early phases of testing include Prostvac-VF recombinant vaccinia-fowlpox PSA vaccine (TRICOM) and the BLP25MUC1 liposomal vaccine MUC-1 (216, 217).

Novel cytotoxic drugs

The limitations of Docetaxel therapy are related to the toxicity associated with current dosing regimens and there is a need for development of novel cytotoxic drugs with fewer toxic sideeffects. Satraplatin, a platinum-based agent exhibited a favourable outcome in a Phase- III clinical trial (218). Epithilones are a new class of cytotoxic drugs that target microtubules but pose an advantage over taxanes since they are not susceptible to P-glycoprotein induced drug efflux (219). Monoclonal antibodies tagged to cytotoxic agents are also an attractive therapeutic approach, for example MLN2704 is a prostate-specific membrane antigen conjugated to a toxic substance maytansinoid and this conjugate is in clinical trials for the treatment of androgen independent prostate cancer (220). Radioimmunotherapy is also emerging as a novel therapeutic approach where specific mAbs are conjugated to radioactive molecules (220-223).

Targeted therapies for colon cancer

Several targeted therapies have already been approved for the treatment of colorectal cancer and they include bevacizumab, cetuximab (Erbitux), and panitumumab (Vectibix). Bevacizumab is given in combination with intravenous (IV) 5-fluorouracil (FU) as first- or second-line treatment in patients with metastatic carcinoma of the colon or rectum. Cetuximab (IgG1) and Panitumumab (IgG2) are the mAbs with different isotypes that target EGFR. Several other therapies are being developed, and two of these include various cyclooxygenase (COX) inhibitors and peroxisome proliferator-activated receptor γ (PPARγ) agonists. These are discussed in the following two subsections.

PPARγ Agonists

PPAR is a member of the nuclear receptor (NR) family of transcription factors (224-228), and the three members of this subfamily (Fig. 1.11) serve as regulators of lipid and carbohydrate metabolism and play a critical role in multiple diseases including diabetes, atherosclerosis and cancer. Ligand activation of PPARγ results in formation of a DNA-bound heterodimer with the retinoic acid X receptor (RXR) (Fig. 1.12); and after recruitment of the appropriate cofactors, transcriptional activation of target gene expression is observed. The assembly of a transcriptionally-active PPAR/RXR complex may be highly variable and depends on expression of coregulatory proteins; and this may dictate, in part, the tissue-specific and ligand structure-dependent activation of PPAR-mediated gene expression and responses.

PPARγ and cancer

The proliferation advantage of cancer cells over their normal counterparts is

Fig. 1.11. General structure of PPARs. Schematic representation of the domain structure of PPARs. The numbers within each domain corresponds to the percentage of aminoacid sequence identity of human PPARb and PPARg relative to PPARa. Two transcription activation functions (AFs) have been described, a constitutively active AF-1 in region A/B and a ligand inducible AF-2 in region E and a DNA binding domain in region C.
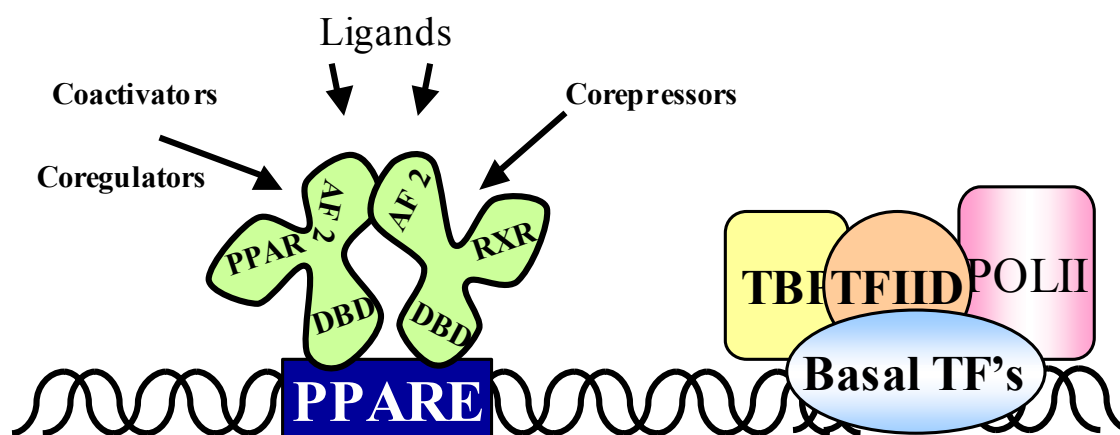
Fig. 1.12. Regulation of PPAR-mediated gene expression.

atleast, in part, due to their inability to undergo terminal differentiation, remain in a proliferative state and continue to grow. The potential uses of PPARγ agonists for cancer chemotherapy were based on results showing that PPARγ ligands inhibited cell proliferation while inducing adipocyte differentiation. Moreover PPARγ is overexpressed in most tumor samples and cancer cells lines. PPARγ agonists inhibit proliferation of transformed cells mainly by inducing cell cycle arrest or differentiation and/or apoptosis.

Cell cycle. Studies in several tumor cell lines have suggested a role for PPARγ in cell cycle arrest. It was initially observed that ligand activation of PPARγ induces cell cycle withdrawal of preadipocytes via suppression of the transcriptional activity of E2F/DP DNA-binding complex. Decreased E2F/DP activity is in part mediated by PPARγ through down-regulation of the protein phosphotase 2A (PP2A). E2F/DP activity can also be inhibited by activation of retinoblastoma protein (RB) and PPARγ ligands inhibit phosphorylation of RB in vascular smooth muscle cells, therefore maintaining RB in its hypophosphorylated form abrogating the G1 to S phase transition. Morrison and Farmer have suggested a role for PPARγ in up-regulating the cyclin-dependent kinase inhibitors (CDKIs) p18 and p21 during adipogenesis (229). Thus PPARγ ligands control genes involved in inhibition of normal cell cycle progression and similar responses were also observed in pancreatic cancer cell lines (230). However, the specific growth regulatory pathways that are affected by PPARγ agonists are highly variable even among cells derived from a common tumor type. The most widely used synthetic PPARγ agonists are the thiazolidinedione (TZD)

class of antidiabetic drugs, also referred to as glitazones and these include ciglitazone, troglitazone (TGZ) (Rezulin), pioglitazone((Actos), rosiglitazone (Avandia) and LY171.833 (Fig. 1.13). Pioglitazone, rosiglitazone and TGZ sensitize patients to insulin and lower blood glucose levels and have been used clinically to treat type 2 diabetes (231).

Troglitazone was recently removed from the market due to idiosyncratic hepatotoxicity, which resulted in liver failure in extreme cases. Several other structurally distinct ligands, such as the derivative of 2,3-disubstituted indole-5-acetic acid called GW0207 or non-thiazolidinedione tyrosine based compounds are potent and selective PPARγ agonists (232).

Non-steroidal anti-inflammatory drugs such as indomethacin, ibuprofen, fenoprofen and fulfenamic acid are also PPARγ agonists, and this might explain the anti-inflammatory effects of these drugs at concentrations that are substantially higher than those required to inhibit prostanoid synthesis (233). The synthetic triterpenoid 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid (CDDO) binds PPARγ, induces Differentiation, inhibits proliferation of a variety of cancer cells, and also exhibits anti-inflammatory activity (234).

TGZ inhibited growth of six of nine pancreatic cancer cell lines by G1 phase cell cycle arrest through the up-regulation of p21 mRNA and protein expression (230). In the Panc-1 pancreatic cancer cell line, TZD-dependent growth inhibition of G1 phase arrest

was accompanied by increased expression of p27 but not p21 (230, 235). A dose-dependent cytostatic effect of TZD and G0 to G1 cell cycle arrest was observed in different hepatoma cell lines including HLF, HuH-7, HAK-1B, and HAK-5 cells, and was related to alterations in p21 protein expression. HLF hepatoma cells, which are deficient in RB, responded more profoundly to TGZ, which induced expression of p21, p27 and p18 suggesting that these CDKIs may be involved in TGZ-induced cell cycle arrest in human hepatoma cell lines (236).

Hupfeld and Weiss reported that TZDs inhibit vascular smooth muscle cell growth by decreasing cyclin D1 and cyclin E levels, suggesting another possible mechanism for TZD action (237). MCF-7 breast cancer cell growth and G1-S phase progression was inhibited by troglitazone treatment, and cells accumulated in the G1 phase by modulating RB phosphorylation and decreasing cyclin D1 expression (238). CDK-dependent activity which was also decreased by troglitazone and overexpression of cyclin D1 and partially rescued MCF-7 cells from troglitazone mediated G1 cell cycle arrest (238). PPARγ activation inhibits proliferation of several other malignant cells from carcinoma (239-241), non-small cell lung carcinoma (242), bladder cancer cells (243) and gastric carcinoma cells (244).

Differentiation. PPARγ induces differentiation pathways beyond adipocytes, and overexpression of PPARγ induces differentiation of hepatocytes, myoblasts and several other cell types including mammary and colon epithelium (245). Furthermore, treatment of human primary liposarcoma cells with pioglitazone induced terminal differentiation (246). PPARγ agonists induce upregulation of several differentiation

markers such as carcinoembryogenic antigen, E-cadherin and alkaline phosphotase in pancreatic cancer cell lines (247). Ligand-dependent activation of PPARγ induced apoptosis in different malignant lineages such as liposarcoma (248), prostate carcinoma (249), colorectal carcinoma (239-241), non-small cell lung carcinoma (242),bladder cancer cells (243) and gastric carcinoma cells (244).

In the colon, levels of PPARγ mRNA are nearly equivalent to that found in adipocytes (250) with the highest levels of receptor expressed in the post-mitotic, differentiated epithelial cells facing the lumen (251). Consistent with this expression pattern, exposure of human colon cancer cells to PPARγ agonists inhibits growth, arrests cells in G1 phase, increase several markers of differentiation including caveolin-1 and caveolin-2, which exhibit tumor suppressor activity, and also some members of the keratin and CEA families (239, 252, 253). Levels of caveolin-1 and 2 also increase during differentiation of pre-adipocytes to adipocytes (254, 255). Breast adenocarcinoma and colon adenocarcinoma cells, where caveolin expression is normally downregulated, express a functional PPARγ with transcriptional activity (251). These cells undergo differentiation *in vitro* upon treatment with PPARγ agonists (240, 248, 256). The ability of PPARγ to promote lineage-specific differentiation and the fact that caveolins are characteristic markers for terminally differentiated cells raised the hypothesis that PPARγ transcriptionally regulates caveolin expression. Burgermeister et al. have shown that PPARγ induces caveolin gene expression in human adenocarcinoma cells and this may have important applications in the context of cancer differentiation therapy (257).
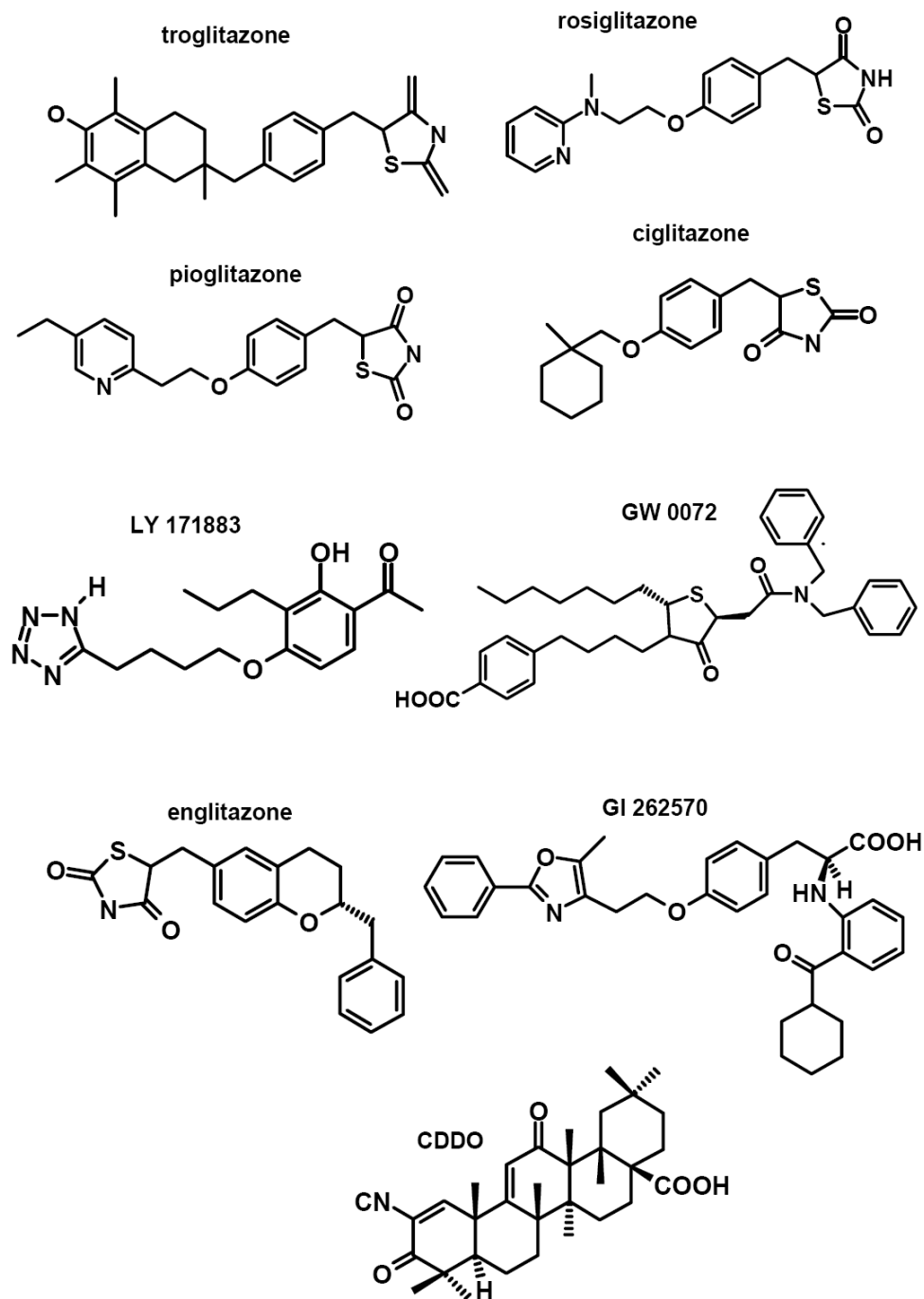
Fig. 1.13 Chemical structures of different synthetic PPARγ ligands (258).

Apoptosis. PPARγ ligands induce apoptotic cell death in a wide variety of experimental cancer models both *in vitro* and *in vivo* (259-261) including ER negative MDA-MB-231 and ER positive MCF-7 breast cancer cells (262). Treatment of MCF-7 cells with troglitazone irreversibly inhibited growth and induced apoptosis; and this was accompanied by a dramatic decrease of the anti apoptotic bcl-2 protein (263). Inhibition of RNA or protein synthesis abrogates apoptosis induced by 15d-PGJ2 in breast cancer cells. Additionally, 15d-PGJ2-induced caspase activation is inhibited by caspase inhibitors, showing that de novo gene transcription was necessary for induction of apoptosis in breast cancer cells (264).

PPARγ ligands such as 15d-PGJ2, LY171 833 and ciglitazone inhibited proliferation and induced cell death in human (U87MG and A172) and rat (C6) glioma cell lines. This cell death was characterized by DNA fragmentation and nuclear condensation which are the hallmarks of apoptosis. In contrast, primary murine astrocytes were not affected by treatment with ciglitazone (265). PPARγ ligand-induced apoptotic cell death in glioma cells was accompanied by transient upregulation of proapoptotic proteins Bax and Bad. Upregulated expression of Bad and Bax induces apoptosis by enhanced release of mitochondrial cytochrome C and subsequent activation of several effector caspases. In addition, inhibition of Bax expression by specific antisense oligonucleotides protected glioma cells against PPARγ mediated apoptosis, indicating an essential role of Bax in this process (265). PPARγ activation by troglitazone also leads to increased caspase 3 activity in human liver cancer cells (266) and in human malignant astrocytoma cell lines (267).

Shimada and coworkers noted that troglitazone-induced cell death in colon cancer cells was inhibited by pan-caspase inhibitors (268). Furthermore, ciglitazone-induced apoptosis in human pancreatic cell lines is blocked by the pan-caspase inhibitor ZVAD-FMK but not by a specific caspase-3 inhibitor (269). Moreover, treatment of human liver cancer cell lines (266) and human thyroid carcinoma cells (270) with PPARγ agonists did not increase levels of Bax protein, suggesting that PPARγ agonists induce several different apoptotic pathways. TNF-related apoptosis inducing ligand (TRAIL) is a member of TNF family of cytokines that induces apoptosis and both natural and synthetic PPARγ agonists sensitize tumor cells but not normal cells to induction of apoptosis by TRAIL. PPARγ ligands selectively decrease levels of FLICE-inhibitory protein (FLIP), an apoptosis-suppressing protein that blocks early events in TRAIL/TNF family death receptor signaling. PPARγ ligands induced ubiquitination and proteasome-dependent degradation of FLIP, without concomitant decreases in FLIP mRNA (271).

PPARγ-independent actions. PPARγ agonists inhibit proliferation of several types of cancer cells, however, the role of PPARγ remains controversial; and in many cases the compounds induce receptor-independent effects. The sensitivity of cancer cell lines growth inhibition by PPARγ agonist does not correlate with levels of PPARγ protein in these cells. For example glitazone resistance occurs even in tumors with high PPARγ concentrations (e.g., breast-tumor cells) (272). Homologous recombination was used to create embryonic stem cells with a null mutation for PPARγ and these stem cells could be differentiated into macrophages when treated with glitazones (273).

Troglitazone-induced cell cycle arrest and subsequent cell death; and this was associated with downregulation of c-myc, c-myb and cyclin D2 expression. These genes lack a PPRE in their promoter regions, indicating that these responses were not directly mediated through PPARγ (274). Similar results were observed in the human leukemia cell line (KU812) suggesting that troglitazone-mediated growth suppression was PPARγ-independent and was associated with decreased cyclin E levels and hyperphosphorylation of RB (275).

In human glioblastoma T98G cells, TZD induced cell cycle arrest and apoptosis, and the fomer response was associated with increased p27 levels, whereas apoptosis was mediated by downregulation of anti-apoptotic Bcl2 and upregulation of pro-apoptotic Bax and caspase-3 activation. None of these responses were blocked in cells treated with a specific PPARγ antagonist (276). The mechanisms by which PPARγ agonists induce glioma cell toxicity were also investigated in another study using rosiglitazone and ciglitazone. The rapidity of the mitochondrial damage caused by PPARγ agonists and the failure to reverse the cytotoxic effects using the specific PPARγ antagonist GW9662 suggested a receptor-independent action (277).

Troglitazone inhibited growth of HCT-116 colon cancer cells by induction of NAG-1, most likely via direct activation of the transcription factor early growth response-1 (EGR-1) (278). Induction was not blocked by a PPARγ antagonist, thus indicating another mechanism by which TZD can cause growth inhibition. In MCF-7 breast cancer cell lines, troglitazone- induced apoptosis was dependent upon GADD45 expression. Regulation of GADD45 by troglitazone occurred at the transcriptional level

and was associated with MAPK activation. In the same studies neither rosiglitazone nor pioglitazone induced GADD45 expression indicating a receptor-independent response (279).

The triterpenoid CDDO inhibits the growth of ovarian cancer cells that express PPARγ, but the effects of CDDO were not blocked after co-treatment with the PPARγ antagonist T007, suggesting that growth inhibition was PPARγ independent (280). Another group of PPARγ agonists, 1, 1-bis (3'-indolyl)-1-(p-substituted phenyl) methanes inhibited the G0/G1-S phase progression in MCF-7 and several cancer cell lines by downregulation of cyclin D1. The PPARγ inhibitor T007 did not affect downregulation of cyclin D1 indicating that this response was also PPARγ independent (281). More direct evidence of PPARγ independent effects comes from receptor knockout studies. Embryonic stem cell induced tumor growth was inhibited by troglitazone and ciglitazone in both PPARγ (-/-) and PPARγ (+/+) mice, confirming the receptor-independent actions of PPARγ agonists. These compounds blocked the G1/S phase transition by inhibiting translation initiation as a consequence of partial depletion of intracellular calcium stores. This resulted in activation of PKR, a kinase that phosphorylates the alpha subunit of eukaryotic initiation factor 2, thus rendering it inactive (282).

Indirect and direct evidence suggest that many of the anticancer activities of PPARγ ligands are PPARγ-independent, however the mechanisms of receptor-independent pathways warrant further study. Although PPARγ is well characterized, there are still numerous challenges before modulators of this receptor will be adopted for

therapeutic applications in treating cancer. This will require development of selective

PPARγ modulators with minimal side effects and an increased understanding of both

receptor-dependent and independent pathways.

PPARγ and colon cancer. PPARγ is overexpressed in multiple tumor-types

(283), and there is evidence that various structural classes of PPARγ agonists inhibit

growth and induce apoptosis in colon cancer cells/tumors and other cancer cells/tumors

(235, 241, 253, 284-302). There is evidence that PPARγ is overexpressed in human

colon tumors compared to non-tumor tissues (283); however, results of laboratory

animal studies have been contradictory (239, 291, 303-308). Sarrat and coworkers

reported that the TZD troglitazone decreased colon tumor growth and malignancy in

colon tumor xenografts in nude mice (239); however, two reports showed that

troglitazone and rosiglitazone enhanced colon tumorigenesis in the $APC^{min+/-}$ mouse

model which expresses a nonsense mutation in the APC gene (308, 309). In contrast,

subsequent studies demonstrate that TZDs inhibit intestinal polyp formation and

suppress hyperlipidemia in APC^{1309} mice which contain a truncation mutation in the

APC gene and is an animal model for the human genetic mutation that leads to an

increased incidence of colon cancer (291, 305). A recent study also demonstrated that

troglitazone enhances colon tumorigenesis in wild-type and mutant mice (306); and it is

possible that the effects of troglitazone may be due to the intrinsic (PPARγ-independent)

properties of the compound which has subsequently been withdrawn as a drug for

treating Type II diabetes due to toxic side-effects. Further support for the protective role

of PPARγ in colon cancer comes from a study showing that homozygous PPARγ

deficiency in APC$^{min+/-}$ mice enhances tumorigenesis (307).

Research in our laboratory has identified a series of 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes (C-DIMs) which contain *p*-trifluoromethyl (DIM-C-pPhCF$_3$), *p*-*t*-butyl (DIM-C-pPhtBu) and *p*-phenyl (DIM-C-pPhC$_6$H$_5$) and activate PPARγ (281, 297, 299, 301, 310-313). These PPARγ-active C-DIMs induce both PPARγ-dependent (induction of caveolin-1) and -independent (ER stress, NAG-1) proapoptotic and growth inhibitory responses in colon and other cancer cell lines. The antitumor activity of other PPARγ agonists is also due to their activation of receptor-dependent and -independent pathways.

Cyclooxygenase (COX) inhibitors

COX is one of the rate limiting steps in the metabolism of arachidonic acid to prostaglandins and thromboxanes, and COX inhibitors which include a broad spectrum of NSAIDs are extensively used as anti-inflammatory agents (Fig. 1.14) (314). COX-2 is also overexpressed in multiple tumor types including colon cancer; and both NSAIDs and COX inhibitors have been investigated as targets for cancer chemoprevention and chemotherapy (314-317). Several studies show that some COX inhibitors including aspirin decrease the incidence and/or mortality rate of human colorectal cancer (318-320). The COX-2 inhibitor sulindac has also been successfully used for treating patients with familial adenomatous polyposis coli (FAP), a hereditary colon cancer syndrome in which there is rapid and early development of polyps and tumors (321-323). COX-2 inhibitors are also highly effective chemotherapeutic agents for treating colon cancer in

laboratory animals including carcinogen-induced and transgenic rodent models (324-326).
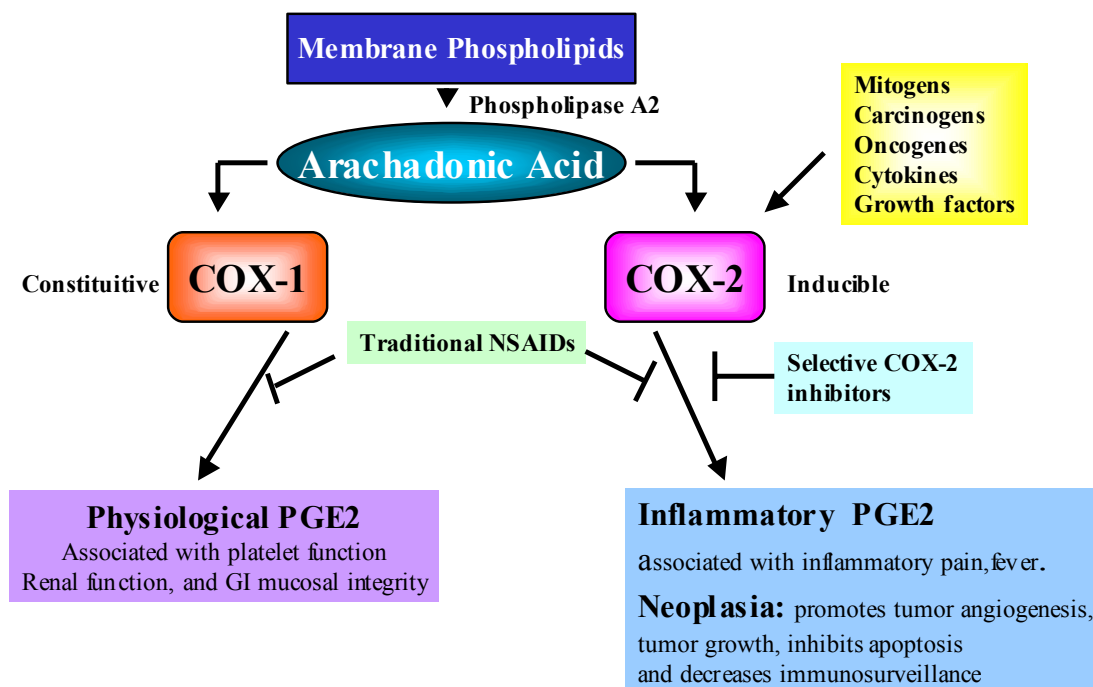
Fig. 1.14. Inhibition of prostaglandin synthesis by COX-inhibitors (327).

The mechanisms of COX-2 inhibitor-mediated cancer chemoprevention and chemotherapy are complex, and the inhibition of prostaglandin/thromboxane production (e.g. prostaglandin E2) contributes to these effects (314-317). However, it is also clear that COX-2 inhibitors can be effective in cells with minimal COX-2 expression and many cell growth inhibitory responses induced by these compounds are COX-2-independent (328-330). For example, sulindac sulfone induces apoptosis in Caco-2 cells by decreasing polyamine levels through activation of PPARγ-dependent induction

of spermidine/spermine N-acetyltransferase gene expression.

The cancer chemotherapeutic actions of COX-2 inhibitors have also been linked to their antiangiogenic activity and inhibition of VEGF expression (314, 317); and studies in this laboratory investigated the effects of the COX-2 inhibitors celecoxib (Celebrex), nimesulfide (NM) and NS-398 on colon cancer cell growth and expression of the key angiogenesis protein VEGF (331). Treatment of SW-480 colon cancer cells with Celebrex, NS-398 or NM decreased vascular endothelial growth factor (VEGF) mRNA and immunoreactive protein expression. This was also accompanied by decreased transactivation in cells transfected with constructs containing VEGF gene promoter inserts. Deletion analysis of the VEGF promoter indicated that decreased VEGF expression by COX-2 inhibitors was associated with the proximal -131 to -47 GC-rich region of the VEGF promoter which binds Sp proteins. Treatment of SW-480 cells with Cel, NM and NS also decreased Sp1 and Sp4, but not Sp2 or Sp3 protein expression. Similar results were observed in RKO, HT-29 and DLD colon cancer cells demonstrating comparable responses in COX-2 expressing and non-expressing colon cancer cell lines. COX-2 inhibitors do not affect Sp1 or Sp4 mRNA levels in SW-480 cells; however, decreased expression of both proteins was accompanied by increased protein ubiquitination and inhibited by the proteasome inhibitor gliotoxin. These results suggest that the antiangiogenic activity of COX-2 inhibitors in colon cancer cells is linked to activation of proteasome-dependent degradation of Sp1 and Sp4 protein.

Targeted therapies for esophageal cancer

Table. 1.2. Potential tumor targets for esophageal cancer (332)

Tumor targets
Growth regulation
Epidermal growth factor receptor
HER-2/Neu
Ki-67
Angiogenesis
Vascular endothelial growth factor
Inflammation
Cyclooxygenase-2
Cell cycle control
p16
p21
Cyclin D1
Apoptosis
p53
bax
bcl-2
Metastatic potential
Tissue inhibitor of metalloproteinase
E-cadherin

New drugs that target specific molecules are also being studied for treatment of esophageal cancer. Several clinical trials are in progress to test the combination of drugs with different mechanism of actions. Potential tumor markers or targets for the aggressive esophageal squamous cell carcinoma or gastroesophageal adenocarcinoma have been identified and are listed in Table 1.2 (333-335). Earlier these targets were understudied as biomarkers to predict the clinical response after chemotherapy or chemoradiotherapy but have been now identified as specific molecular targets (333-338).

MAbs and signal tranduction/tyrosine kinase inhibitors (TKIs) for EGFR, mAbs for HER-2/Neu receptor and VEGF ligand, oral COX-2 inhibitors and many other targeted therapies are in Phase I/II clinical trials.

Drugs that target epidermal growth factor receptor (EGFR)

EGFR is a member of ERBB receptor tyrosine kinase family and includes four members namely EGFR1 or ERBB1, ERBB2 (HER-2), ERBB3 and ERBB4. EGFR is a 170 kDa transmembrane glycoprotein having an extracellular domain anchored to the plasma membrane and a cytoplasmic component containing a tyrosine kinase domain. Upon ligand binding to the extracellular domain, the receptor is activated to form hetero -or homodimers resulting in activation of the cytoplasmic tyrosine kinase activity. This results in initiation of intracellular signal transduction cascades that regulate cellular growth and multiple functions depending on the substrate of the tyrosine kinase (Fig. 1.15) (339).

Known ligands for EGFR include EGF and transforming growth factor alpha (TGF-α). The Ras-Raf mitogen–activated protein kinase and PI3K/Akt are two of the important downstream signaling pathways that regulate the cellular functions for the HER /EGFR family (340-343). EGFR and mutant forms of the receptor are overexpressed in esophageal and other human cancers; and expression of this receptor is positively associated with poor prognosis and aggressiveness of the disease. In order to target this receptor monoclonal antibodies and small molecule inhibitors of the tyrosine kinase activity of the receptor have been developed.
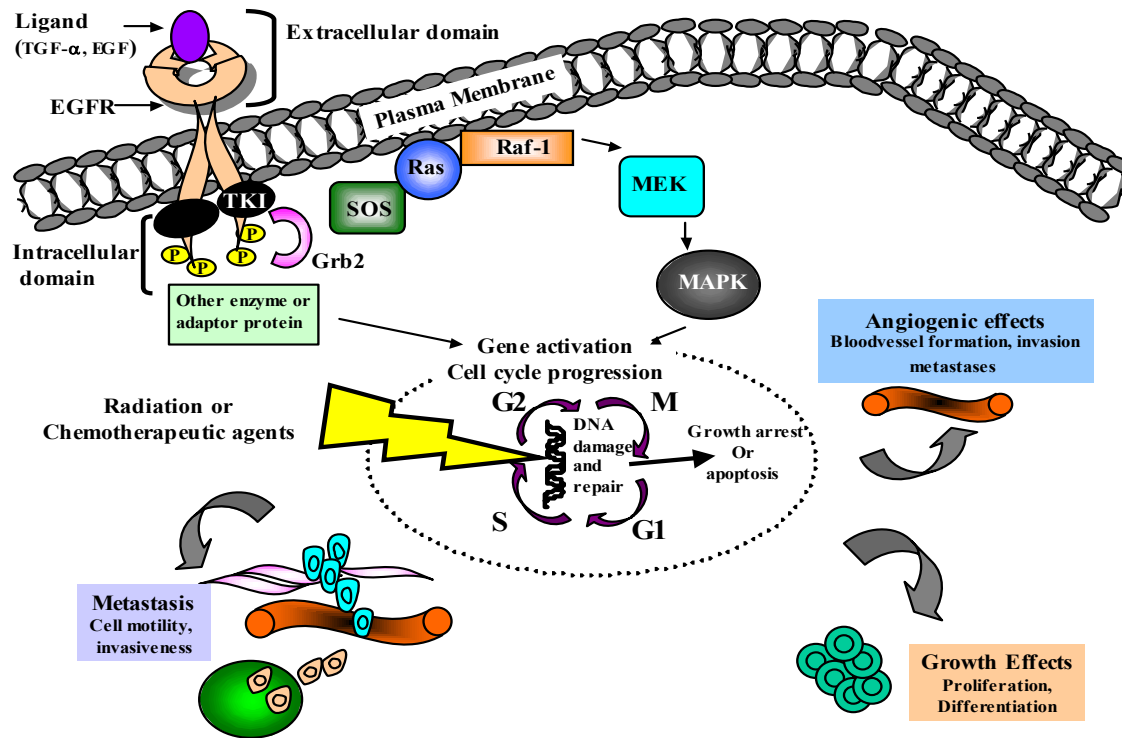
Fig. 1.15. Schematic representation of the EGFR in the transmission of signals regulating tumor growth and metastasis (344).

EGFR Antibodies

Cetuximab is a mouse-human chimeric antibody of the IgG1 subclass and binds to EGFR or HER-1. The antibody inhibits ligand binding to the EGFR and blocks phosphorylation of the tyrosine kinase domain and inhibits its activity (345). Cetuximab also decreases receptor levels by inducing internalization of the receptor which leads to receptor degradation and inhibition of activity (346). Cetuximab has been used in clinical trials for colorectal and head and neck cancers. There are several clinical trials being carried out including a South-West Oncology Group (SWOG) trial of cetuximab as second line therapy for patients with metastatic esophageal adenocarcinoma. A

Memorial Sloan-Kettering Cancer Center study of cetuximab in irinotecan/cisplatin-refractory patients with metastatic esophageal cancer and a Dana-Farber Cancer Institute preoperative trial with cisplatin, irinotecan, cetuximab and radiation are also underway. There are significant improvements in patients in Phase I studies using EGFR-directed mAbs and this is accompanied by minimal toxicity.

EGFR tyrosine kinase inhibitors

These are small molecules that inhibit the tyrosine kinase activity of EGFR by inhibiting the binding of ATP to the tyrosine kinase domain and thus inhibit its phosphorylation and signal transduction (347). Erlotinib (Tarceva) and geftinib (Iressa) have been approved as second line therapy for metastatic non-small cell lung cancer (NSCLC) and four Phase II clinical trials with these inhibitors are ongoing for treatment of esophageal and gastroesophageal adenocarcinomas. The partial response rates (PR) of these inhibitors as single agents are outlined in Table 1.3. In clinical trials for NSCLC only, erlotinib as a single agent showed a survival advantage when compared to placebo treated group, although both geftinib and erlotinib showed significant partial response rates (9-19 %), disease control (36-54%) and symptomatic improvement (35-43%) (348-350).

Drugs that target HER-2/Neu

HER-2/Neu**,** also referred to as ERBB2 is a proto-oncogene localized in chromosome 17q. Amplication of this gene is observed in up to 30% of breast cancers and is related to the aggressiveness of the tumor (351, 352). Several studies have

Table. 1.3. Tyrosine kinase inhibitors: Partial response (PR) rates in metastatic cancers (332)

Cancer type	Erlotinib (%PR)	Gefitinib (%PR)
NSCLC	9-12	9-19
Head and neck squamous cell	4	11
Gastric adenocarcinoma	0	1
Esophageal, gastroesophageal junction	9-12	12

observed overexpression of HER-2/Neu in esophageal squamous cell carcinomas and gastroesophageal adenocarcinomas; and this receptor is a potential target for treatment of esophageal cancer. Overexpression of this receptor is associated with poor response to chemotherapy and invasiveness of the disease (353). Trastuzumab (Herceptin) is a humanized IgG1 antibody that targets HER-2/Neu antigen and this drug has been approved by FDA for treatment of HER-2/Neu-positive metastatic breast carcinoma (354). Safran and colleagues have used trastuzumab, paclitaxel, cisplatin and radiation in a PhaseI/II trial in patients with locally advanced gastroesophageal cancer (355). The trial has confirmed the efficacy for addition of trastuzumab with the standard, combined modality regimen. Many other clinical trials using this mAb are underway for treatment of esophageal cancer.

Drugs that target angiogenesis

VEGF is overexpressed in 30-60% of esophageal cancers and is associated with

poor survival and clinical response and the VEGF neutralizing antibody bevacizumab has been developed as antiangiogenic therapy (356-359). As a monotherapy or in combination with other therapies, there are more than 30 clinical trials using bevacizumab for treatment of solid tumors and hematologic cancers; and for the treatment of esophageal cancer, it is still in the early stage. A Phase II study of irinotecan, cisplatin and bevacizumab in patients with metastatic gastroesophageal adenocarcinomas yielded positive encouraging results (360). VEGF antibody exhibits a radiosensitizing effect in several mouse xenograft models including lung, colon, glioma and esophageal cancers suggesting that this antibody gives an enhanced anticancer effect when combined with radiation (361-365).

Drugs that target COX-2

COX-2 affects many pathways including carcinogenesis, apoptosis, inflammation, and regulation of immune system (366, 367). There is increasing evidence to link COX-2 with carcinogenesis in Barrett's esophagus; and the use of COX-2 inhibitors such as aspirin and NSAID's reduce the risk for esophageal cancer (368, 369). With the use of these COX-2 inhibitors such as Celecoxib (Celebrex) at higher doses, an increased risk of thrombosis has been observed; and hence NCI trials with these inhibitors were sustained. Several trials are in progress using COX-2 inhibitors in combination with radiotherapy for treatment of esophageal cancer (370).

P53 gene therapy

Abnormalities or mutations in the p53 tumor suppressor gene are associated with esophageal cancer in 40-60% of the patients (371, 372). P53 genetic analysis is used as a

prognostic indicator of survival and predictor of response to the treatment for esophageal cancer (338, 373-375). Retroviral or adenoviral gene expression mediated p53 gene therapy has been evaluated in preclinical tumor models and results from these studies revealed its efficacy to maintain drug and radiation sensititvity and induces apoptosis in the tumors (376-378). Based on these results Phase I trials with adenovirus-mediated wild-type p53 gene transfer in combination with cisplatin were carried out and were revealed to be safe and efficiently induce biological effects in patients with advanced lung cancer (379-381). Similar trials in patients with esophageal cancer are in progress.

Targeted therapies for pancreatic cancer

Drugs that target epidermal growth factor receptor (EGFR)

EGFR inhibitors erlotinib and gefitinib are now being tested against pancreatic cancer along with cetuximab (35, 382). A Phase III clinical trial conducted with 569 patients with advanced pancreatic cancer randomly received gemcitabine alone or in combination with erlotinib and the increased effects of the combined therapy resulted in approval of this therapy. One-year survival rates were higher (24%) in the combination therapy group compared to the survival rate (17%) of the group receiving gemcitabine alone (383).

Drugs that target angiogenesis

Antibodies against angiogenic factors such as bevacizumab are being tested for treatment of pancreatic cancer in combination with gemcitabine. Results of a Phase II trial with this combination were encouraging and futher confirmatory studies are being implemented with gemcitabine plus bevacizumab.

Drugs that target K-ras

Since K-ras mutations are observed in most pancreatic tumors, proteins in the K-ras pathway are being investigated as drugs targets. Activation of ras and its substrates depends on farnesylation or prenylation, a process which adds hydrophobic groups that facilitates membrane association of the proteins. Farnesyl transferase is an enzyme involved in farnesylation of proteins bearing a CaaX motif, and this includes the Ras superfamily of proteins. Farnesyl transferase inhibitors such as tipifarib and lonafarnib are being evaluated for treatment of pancreatic cancer (384). Preliminary results from Phase II studies with these inhibitors alone or in combination with 5-FU or gemcitabine in patients with advanced pancreatic cancer resulted in a prolonged response. Ongoing Phase III trials will determine the clinical activity of these drugs and whether they can be used alone or in combination with other cytotoxic drugs.

Drugs that target matrix metalloproteinases (MMPs)

Matrix metalloproteinases belong to the family of zinc metalloendopeptidases that play a significant role in tissue remodeling by degrading the basement membrane and ECM. They are not only required for physiological processes but are also involved in tumor invasion, growth, metastasis and angiogenesis. MMPs are divided into five groups namely stromelysins, collagenases, gelatinases, membrane type MMPs and other MMPs, with about eighteen different subtypes and these are summarized in Table 1.4. (385-388).

Table. 1.4. Different classes of MMPs (389)

Family of Matrix Metalloproteinases (MMPs)

Stromelysins

    MMP-3

    MMP-7 (Matrilysin)

    MMP-10

    MMP-12 (Metalloelastase

Collagenases

    MMP-1

    MMP-8

    MMP-13

Gelatinases

    MMP-2

    MMP-9

Membrane-type MMPs

    MMP-14 (MT-MMP1)

    MMP-15 (MT-MMP2)

    MMP-16 (MT-MMP3)

    MMP-17 (MT-MMP4)

    MMP-24 (MT-MMP5)

Other MMPs

    MMP-18 (Xenopus MMP)

    MMP-19

    MMP-20 (Enamelysin)

MMPs are produced as propeptides and are autocatalytic cleaved to form active MMPs. This activation can be induced using organomercurials such as p-aminophenylmercuric acetate which binds *in vitro* to the conserved cysteine residue in the propeptide and it is then released from the covalently bound catalytic zinc iron. *In vivo,* MMPs are cleaved by a wide range of different extracellular proteinases such as plasmin, serine proteases and others (390). MMPs are regulated in tissues by endogenous tissue inhibitor of metalloproteinases (TIMPs). The specific role of TIMPs is unclear and they may promote tumorigenesis by activating MMPs or inhibit MMP activity and exhibit antitumor activity. MMPs are upregulated in many tumors including thyroid, prostate,

ovarian, gastric, lung, head and neck, pancreatic and colorectal carcinomas; and this increased expression correlates with tumor invasiveness of the disease (386, 387). MMP-2 and MMP-9 play a major role in tumor metastases; and a study by Bramhall and his colleagues showed that MMP-2 mRNA was expressed in approximately 93% of tumors from pancreatic cancer patients. Northern blot analysis and *in situ* the hybridization did not detect MMP expression in normal pancreatic tissue specimens (391). MMPs and particularly MMP2 and to a lesser extent MMP-9 play an important role in the pathogenesis of pancreatic cancer as indicated in Table 1.5, however, the precise function of these protein has not yet been identified (392-394). BB-94 (Batimastat), inhibited MMP-2 activity in pancreatic cancer cells and inhibited pancreatic cancer cell invasion without affecting cell proliferation and in an orthotopic nude mouse model BB-94 treated animals formed fewer tumors and exhibited decreased metastases (395-397). An enhanced protective effect was observed in animals cotreated with BB-94 plus gemcitabine, the standard drug used as a first line therapy for pancreatic cancer (398). BAY12-9566 and BB-2516 (Marimastat) are orally active synthetic inhibitors of matrix metalloproteinases and are being investigated in clinical trials for treatment of pancreatic cancer (399, 400). A Phase I study with marimastat determined that 100 mg was the highest dose that could be used with minimal toxic side- effects and this drug which is tumorostatic was evaluated in a chronic dosing study and a series of six subsequent Phase I/II trials were carried out in pancreatic, prostate, ovarian, and colorectal cancers.

Table. 1.5. MMP expression in pancreatic cancer (389)

Year	Study	No. Patients	MMP	Findings
1995	Gress et al.	8	2, 9	Correlation with degree of desmoplasia
1998	Koshiba et al.	33	2, 9	Correlation with tumor extent, nodal status, metastases, and recurrence rate (MMP-2 only)
1999	Kuniyasu et al.	22	2, 9	No correlations
1999	Ito et al.	46	1	Correlation with survival
2000	Gong et al.	15	2, 9	Correlation with nodal status
2000	Maatta et al.	35	2, 9, MT-1	No correlations
2000	Ellenrieder et al.	18	2, 9, MT-1, MT-2, MT-3	Correlation with degree of desmoplasia
2001	Fukushima et al.	70	7	Correlation with tumor extent, nodal status, and TNM classification
2001	Yamamoto et al.	70	7	Correlation with tumor extent, nodal status, and TNM Classification and survival

MMP, matrix metalloproteinases; MT, membrane-bound; TNM, tumor, node, metastasis.

Changes in levels of tumor markers which include cancer antigen (CA) 19-9 , PSA, CA125 and CEA, respectively, were used as determinants of tumor response over a 28-day period (401). The studies demonstrated a dose-dependent decrease in levels of serum tumor markers and this was accompanied by musculoskeletal side effects in patients who received the treatment beyond 28 days (402). Another Phase II study was conducted in 113 patients with advanced pancreatic cancer using radiological/computed tomography response and changes in CA-19-9 levels as tumor response determinants. Approximately 49% of the patients had stable disease by computed tomography over a 28-day treatment period with no difference in disease-free survival whereas decreased levels of serum CA 19-9 were observed in 30% of the patients with a significant improvement in disease-free survival. Based on these reports a large multiinstitutional prospective randomized trial was conducted with three different doses of marimastat 5, 10, or 25 mg BID or

gemcitabine with survival as a primary end point (400). Survival was not significantly different between the groups, but was longer for patients with nonmetastatic disease when compared to metastatic disease in the group receiving marimastat alone, and slightly longer when compared to the group treated with gemcitabine alone. The National Cancer Institute of Canada sponsored a similar trial and compared gemcitabine with another oral synthetic MMP inhibitor, BAY12-9566 in patients with advanced pancreatic cancer. There was lower survival in the BAY12-9566 treated group compared to treatment with gemcitabine alone, the median progression-free survival in the gemcitabine treated group was 3.5 months but only 1.8 months for BAY12-9566 treatment and the trial was terminated (403).

Additional studies with these inhibitors has focused on localized disease or adjuvant therapy; for example, a Phase III study by the British Biotech in patients with resected pancreatic cancer observed a potential benefit using marimastat; and this study is still ongoing.

NATURAL PRODUCTS IN THE TREATMENT OF CANCER

Phytochemicals have been extensively used in traditional medicine by most cultures for thousands of years. Various diseases are treated with crude or refined extracts derived from many natural sources, and the current widespread use of many nutriceuticals, herbal extracts, and other non-traditional medicines stems from the long history of their therapeutic applications (404-410). Individual natural products from various sources have proven to be highly effective for treating many diseases and some of the earliest medicines including aspirin, morphine, quinine, digitoxin and pilocarpine

were derived from plants (405, 406, 411). The discovery of penicillin (412, 413)

spurred research on natural products or "secondary metabolites" from various

microorganisms resulting in the discovery and applications of several different structural

classes of drugs including antibiotics such as penicillins, cephalosporines, streptomycin,

tetracyclines, and their synthetic analogs (413-416); HMG-CoA reductase inhibitors

lovastatin and mevastatin and their analogs are now extensively used as antilipidemic

drugs for preventing and treating cardiovascular disease (417-419). Natural products

and their synthetic derivatives are also important drugs for cancer chemotherapy and are

further discussed in this section. Although newer high-throughput screening techniques

and the development of chemical libraries have facilitated discovery of many new

synthetic drugs (410), the overall percentage of new drugs derived from natural products

or their synthetic analogs is approximately 25% (420-422).

The root extract of mayapple, *Podophyllum peltatum* has been used for treating

skin cancers and venereal warts by American Indians. The active ingredient in that root

extract was identified to be podophyllotoxin which is the forerunner of today's

anticancer drugs etoposide and teniposide. *Catharanthus roseus* also called vinca rosea

was used in Asian countries as a hypoglycemic agent, but not until 1958 was it found to

have cytotoxic properties. The cytotoxicity of the plant has been attributed to two main

ingredients, namely vinblastine and vincristine. In the modern world these compounds

play a major role as anticancer drugs in treating childhood leukemias, testicular cancer,

hodgkin's lymphoma. Several structural analogs of these compounds are also in clinical

use. Paclitaxel (Taxol), a compound purified from the bark of the plant Pacific yew

Taxus brevifolia has become one of the most important anticancer drugs and in contrast to the vinca alkaloids paclitaxel stabilizes the microtubules during cell division (423).

Camptothecin, a specific inhibitor of topoisomerase I enzyme is derived from Chinese ornamental tree *Camptotheca accuminata*. In order to overcome the bladder toxicity observed with camptothecin, two structural analogs namely topotecan (Hycamptin) and irinotecan (Camptosar) were synthesized (424). Topoisomerase I is an enzyme that unwinds the DNA double helix during transcription and replication and camptothecin and its structural analogs inhibit this enzyme which results in inhibition of transcription and replication leading to cell death (425).

There are several molecules derived from plants that are under investigation and show promise for treating different cancers. Polyphenols are antioxidant molecules found in the skin and seeds of grapes and also from tea leaves. These compounds are usually produced by plants as a defense mechanism. Polyphenols have antioxidant properties and thus counteract the effects of free radicals which are implicated as a causal factor in cancer progression. Resveratrol is a phytoalexin found abundantly in red wine and also in grapes, raspberries and other plants. It has also been shown to reduce tumor growth and decrease inflammation by inhibiting nuclear factor κB (NFκB) (426-429).

 Epidemiological studies have indicated that nutrition plays a major role in carcinogensis. Adjustment of diets can reduce tumor formation (327, 430, 431) and epidemiological studies indicate that fruits, vegetables, herbs and spices contain compounds associated with cancer chemoprevention (432-435). Tea which is an

important source of anticarcinogenic polyphenols is the second largest drink consumed in the world. The major polyphenolic group of compounds in tea leaves is catechins which induce apoptosis and cell cycle arrest in cancer cells and tumors. Other chemopreventive and chemotherapeutic properties of polyphenolics include their antioxidant activity (436), inhibition of cyclooxygenase and lipooxygenase enzymes (437), inhibition of angiogenesis (438), and VEGF receptor phosphorylation (439), activation of tumor suppressor protein p53 (440) inhibition of telomerase enzyme (441) and also activation of NAG-1, a pro apoptotic member of TGF-β superfamily (442).

Curcumin (diferuloylmethane) is a polyphenolic natural product and the active component of tumeric (*Curcuma* species) which is used in cooking and in traditional medicines (443-445). Curcumin has been extensively investigated as an anticancer drug in various cancer cells and laboratory animal models. Curcumin has been evaluated in humans, for cancer chemotherapy; and one of the major problems associated with clinical applications of curcumin is its low bioavailability (446-449). 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 (450-453). 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 NFκB.

Anticancer drugs have been derived not only from plants but also from microbes (bleomycins,doxorubicin) (454) and marine sources (bryostatin I, eleutherobin) (455,

456). Nature has already provided scientists with highly complex mixtures of medicinal compounds which need to be isolated and identified and subsequently modified to make them more effective for treating different types of cancers.

Triterpenoids

A large number of structural classes of natural products and their derivatives have been developed as chemopreventive and chemotherapeutic agents, and these include triterpenoids. Lanosterol, a C-30 triterpenol, is degraded in several steps to cholesterol which, in turn, serves as a precursor for the biosynthesis of androgens, estrogens, progestins, glucocorticoids and mineralocorticoids. Lanosterol and other triterpenoids are derived from squalene which undergoes 2, 3-epoxidation and cyclization to give tetracyclic triterpenes such as lanosterol and many other tetra- and pentacyclic triterpenene compounds (Fig. 1.16). In addition to the differences in the overall ring or skeletal structure of triterpenoids, there are multiple sites within each ring that can undergo site and stereoselective oxidation or dehydrogenation along with oxidation of the exocyclic methyl groups. Not surprisingly, there are thousands of triterpenoid natural products, and these compounds along with their synthetic derivatives exhibit a wide spectrum of biological activities.

Oleanolic and ursolic acids are oleane-derived triterpenes that contain a carboxyl group at C-28 (methyl oxidation), and they have been used as traditional medicines in Asian countries due to their anti-inflammatory and potential anticancer activities (457-460) (Fig. 1.16). Honda, Sporn and coworkers have synthesized several hundred
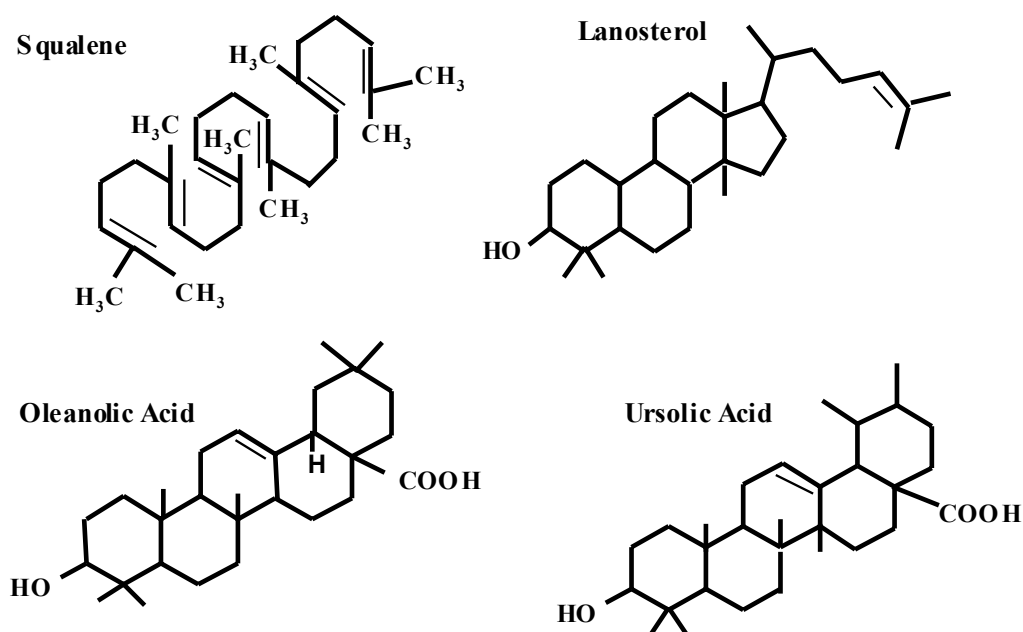
Fig. 1.16. Chemical structures of Squalene and different triterpenoids.

derivatives of oleanolic and ursolic acid, and evaluated their potential anti-inflammatory

activities as inhibitors of inducible nitric oxide synthase (iNOS). The IC_{50} values of

these analogs ranged over at least 4 orders of magnitude (461-466) and they identified a

lead compound, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), and an

imidazole derivative (CDDO-Im) as a group of highly potent anti-inflammatory and

anticancer drugs (Fig. 1.17) (234, 280, 302, 466-483). Initial studies showed that CDDO

was a peroxisome proliferator-activated receptor γ (PPARγ) agonist that bound and

activated receptor-dependent responses; however, many of the activities of CDDO and

related compounds are PPARγ-independent and highly dependent on cell context.
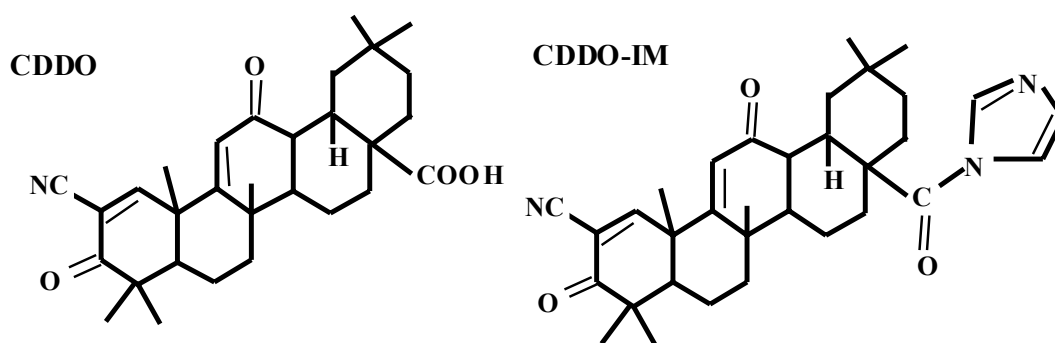
Fig. 1.17. Chemical structures of synthetic derivatives of oleanolinic acid, CDDO and CDDO-IM.

These compounds typically inhibit cancer cell proliferation and induce apoptosis through a variety of mechanisms including induction of endoplasmic reticulum stress, cellular redox, and direct perturbations of mitochondrial pathways. A recent report showed that CDDO-Im inhibited aflatoxin-induced hepatocarcinogenesis through induction of a battery NrF2-dependent cytoprotective genes that alter the metabolism of aflatoxin (484, 485). Research in this laboratory showed that CDDO compounds induce PPARγ-dependent transactivation in colon cancer cells; and this is accompanied by receptor-dependent induction of the tumor suppressor gene caveolin-1. In contrast, induction of apoptosis and the proapoptotic NSAID-activated gene-1 (NAG-1) by CDDO was receptor-independent (302). Currently CDDO is undergoing clinical trials at the M. D. Anderson Cancer Center for treatment of patients with leukemia.

Glycyrrhetinic acid and its derivatives
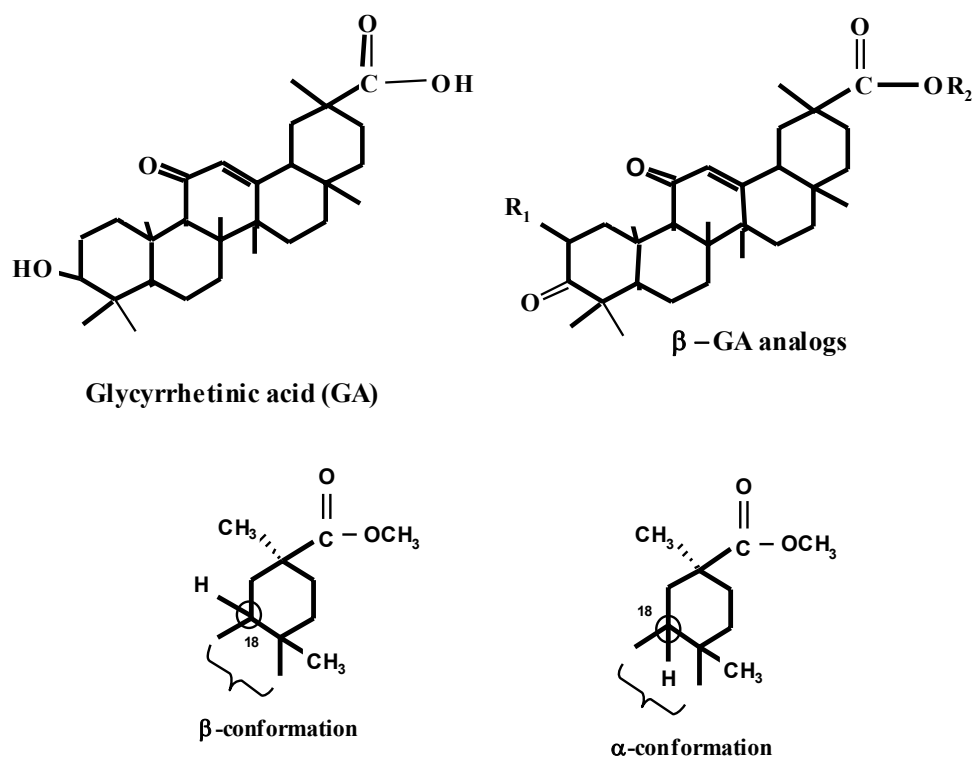
Fig. 1.18. Chemical Structures of GA and the synthetic CDODA-Me analogs.

Licorice root extracts have been extensively used for their therapeutic properties which include the potentiation of cortisol action, inhibition of testosterone biosynthesis, reduction in body fat mass and other endocrine effects (486-489). The activities of these extracts are linked to different classes of phytochemicals particularly the major water-soluble constituent glycyrrhizin and its hydrolysis product glycyrrhetinic acid (GA) (Fig. 1.18).

Many of the properties of licorice root can be attributed to GA; for example, GA inhibits 11β-hydroxysteroid dehydrogenase activity increasing corticosterone levels, and

this has been linked to apoptosis in murine thymocytes, splenocytes and decreased body fat index in human studies (489-492). GA directly acts on mitochondria to induce apoptosis through increased mitochondrial swelling, loss of mitochondrial membrane potential, and release of cytochrome c. GA has also been used as a template to synthesize bioactive drugs such as the carbenoxolone, the 3-hemisuccinate derivative of GA, which is used in treatment of gastritis and ulcers (493). Some of the activity of carbenoxolone may be due to hydrolysis to GA; however, carbenoxolone itself induced oxidative stress in liver mitochondria and decreased mitochondrial membrane potential. Other carboxyl and hydroxyl derivatives of glycyrrhizic acid inhibit HIV and exhibit anti-inflammatory and immunomodulatory activities (494). In addition, GA derivatives containing a reduced carboxylic acid group at C-30 (CH_2OH) and some additional functional changes exhibited strong antioxidant activity (495).

Ongoing studies in this laboratory are developing GA derivatives for cancer chemotherapy and as ligands for some nuclear orphan receptors. Introduction of a cyano group at C-2 position of oleanolic and ursolic acid increased the cytotoxicity of the analogues (464). Methyl 2-cyano-3,11-dioxo-18βolean-1,12-dien-30-oate (βCDODA-Me), a synthetic derivative of GA is isomeric with 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid CDDO-Me (βCDDO-Me), a 2-cyano derivative of oleanolic acid but differs with respect to the position of carboxylic acid group in the E-ring, the stereochemistry at C-18 (β versus α) in the E/D ring junction, and the enone function in the C ring (Fig1.18).

To investigate the importance of the stereochemistry at the C-18 position methyl 2-cyano-3, 11-dioxo-18αolean-1, 12-dien-30-oate (αCDODA-Me) was also synthesized in our laboratory. Introduction of the 2-cyano group into α-glycyrrhetinic acid or β-glycyrrhetinic acid enhanced their cytotoxicity; both compounds induced PPARγ–dependent transactivation in colon cancer cells and act as selective receptor modulators based on their tissue selective induction of caveolin-1 and KLF-4. These genes are associated with growth inhibitory and pro-apoptotic responses and the role of these responses on the anticarcinogenic activity of these compounds as new class of anticancer drugs will be investigated.

Betulin and derived lupane triterpenoids

Lupeol and betulin are pentacylic triterpenoids that contain a five-membered E ring and an exocyclic propylidene group and triterpenoids with a lupane skeleton have been identified as highly promising anticancer drugs (Fig. 1.19) (496-503). Lupeol is found in medicinal plants and various fruits and exhibits multiple biochemical activities including inhibition of protein kinases, serine proteases, and topoisomerase II (504-506). Lupeol induces differentiation and exhibits a broad spectrum of anti-inflammatory and anticarcinogenic activities (502, 503, 507). A recent study showed that lupeol inhibited proliferation and induced apoptosis in pancreatic cancer cells; this was due, in part, to inhibition of Ras signaling which is frequently mutated (and constitutively active) in pancreatic cancer (502).

Lupeol

Betulin

CH$_2$OH

HO

HO

Betulonic acid

COOH

O

Betulinic acid

COOH

HO

BA derivative

R$_1$

COOR$_2$

O

R$_1$= CN; R$_2$= H (CN-BA)
R$_1$= CN; R$_2$= CH$_3$ (CN-BA-Me)
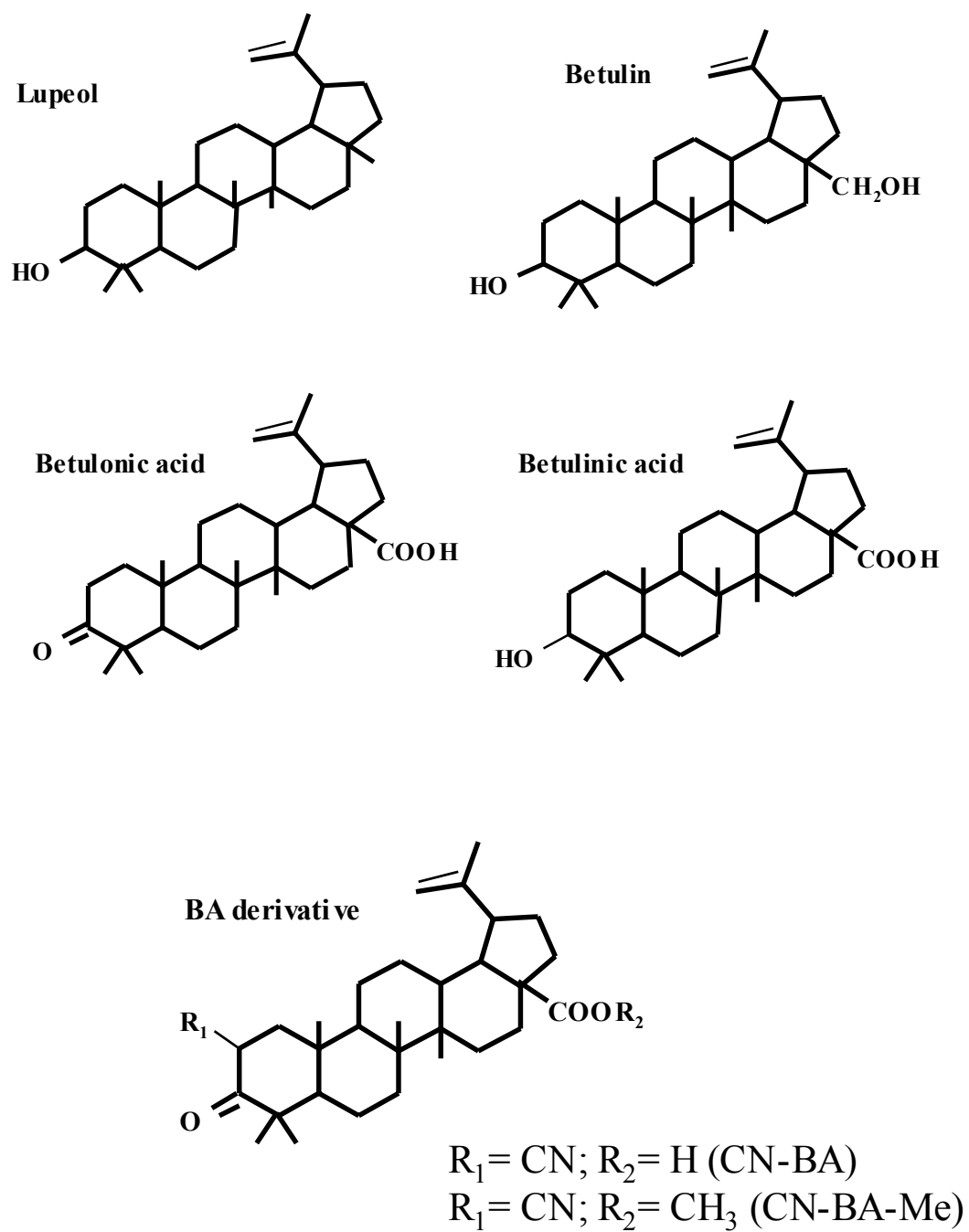
Fig. 1.19. Chemical structures of lupane derivatives.

Betulin or lup-20(29)-ene-3β,28-diol is found in concentrations as high as 30% (dry weight) in birch bark (508), and extracts containing betulin have been used in traditional medicine for treating skin diseases (509). Betulin can be chemically modified to give compounds with anti-inflammatory, antibacterial, antiviral and anticarcinogenic activities, and many studies within this class of lupane derivatives have focused on the anticancer activity of betulinic acid (BA) (Fig. 1.19) (499). BA has been identified in bark extracts but can also be readily synthesized in 75% yield from commercially-available betulin by oxidation to give betulonic acid which can then be reduced to BA; these reactions are routinely carried out in this laboratory. Initial studies with BA showed that it was a highly selective inhibitor of human melanoma cell proliferation through induction of apoptosis, and at doses of 50 - 500 mg/kg every 4 days, BA inhibited tumor growth in nude mice bearing MEL-2 melanoma cells as xenografts (510). In parallel xenograft studies using MEL-1 cells, doses of BA as low as 5 mg/kg inhibited tumor growth, whereas at doses as high as 500 mg/kg, minimal toxicity was observed. In a human neuroblastoma cell line (SHEP), BA decreased mitochondrial membrane potential resulting in the release of cytochrome c and activation of multiple caspases (511). Treatment of human melanoma cells with BA also decreases mitochondrial membrane potential, and this is accompanied by modulation of other proapoptotic pathways including activation of p38 and JNK and induction of reactive oxygen species (512).

Subsequent studies have reported that BA or its derivatives exhibit antitumorigenic activity, not only in melanoma cells (500, 510, 513-517) but in other

tumor-derived lung, colon, prostate, leukemia, ovarian, endothelial carcinoma, breast, cervical, medulloblastoma, neuroblastoma, glioblastoma, liver, osteosarcoma, oral epidermoid carcinoma, and rhabdomyosarcoma cell lines (500, 510, 517-540). Other lupane derivatives similar in structure to BA exhibit comparable activities in cancer cells (536, 541). In most of these cell lines, the mechanisms of action of BA are variable; however, induction of apoptosis is clearly a predominant pathway. Studies in this laboratory have demonstrated that like BA, CDDO and related compounds induce apoptosis in several cancer cell lines; and we have observed similar responses using a series of new 2-cyano-substituted analogs of glycyrrhetinic acid (GA) that are isomeric with CDDO (302) . However, results obtained with GA derivatives showed that the unsubstituted compounds exhibited minimal activity as inhibitors of cancer cell proliferation, whereas BA was active in several cell lines at 1 - 5 μM concentrations. The high cytotoxicity of 2-cyano substituted analogs of GA was due, in part, to their PPARγ activity which is not observed with BA and thus we hypothesized that introduction of 2-cyano group into the lupine skeleton of BA would generate a new class of PPARγ agonists. Studies to compare the effects of BA, CN-BA, and the corresponding methyl ester (CN-BA-Me) in pancreatic and colon cancer cells are carried out (Fig. 1.19). These data suggest that among the naturally-occurring triterpenoids which contain a carboxylic acid moiety in the E-ring derived from oleane (GA and oleanolic acid), ursane (ursolic acid), and lupane (BA), BA was clearly the most potent antitumorigenic compound. Based on the reported low toxicity of BA (510) coupled with its high anticancer potency, we initiated both *in vitro* and *in vivo* studies to probe

the antiproliferative, antiangiogenic and proapoptotic mechanisms of action of BA and

its cyano analogs in models for prostate, bladder and other cancers. Our preliminary

results now show a novel mechanism of action for BA which involves degradation of Sp

proteins in tumors, whereas minimal effects on Sp protein expression were observed in

non-tumor tissue. These results are consistent with the low toxicity of BA in rodent

models (510). Moreover, we also show that BA-induced degradation of Sp proteins

inhibits Sp-dependent antiapoptotic, angiogenic and proliferative pathways in prostate

and colon cancer cells.

SPECIFICITY PROTEINS AS NEW DRUG TARGETS

Specificity protein 1 (Sp1) was the first transcription factor identified in 1983 by

its ability to selectively bind and activate transcription of the viral SV40 promoter (542) ,

and the Sp/Krüppel-like factor (KLF) family of zinc finger transcription factors exhibit a

broad range of tissue-specific and overlapping functions (543-547). Subsequent studies

revealed that multiple genes that contain GC-boxes are activated by Sp1 and related Sp

proteins, including genes such as thymidine kinase (548, 549) , insulin-like growth

factor- receptor (550)(96), dihydrofolate reductase (DHFR) (551), growth hormone (GH)

receptor (552)(98), and alcohol dehydrogenase 5 (ADH5) (553). Kadonaga et.al

determined various functional domains by cloning Sp1 cDNA from HeLa cell RNA

using *in vitro* and whole cell assays (554). These proteins are characterized by their

carboxy-terminal domains C and D, which contain three C2H2-type zinc fingers that

recognize GC/GT boxes in promoters of target genes. The amino-terminal domains A

and B of Sp/KLF proteins are highly variable in both structure and function (Fig. 1.20).
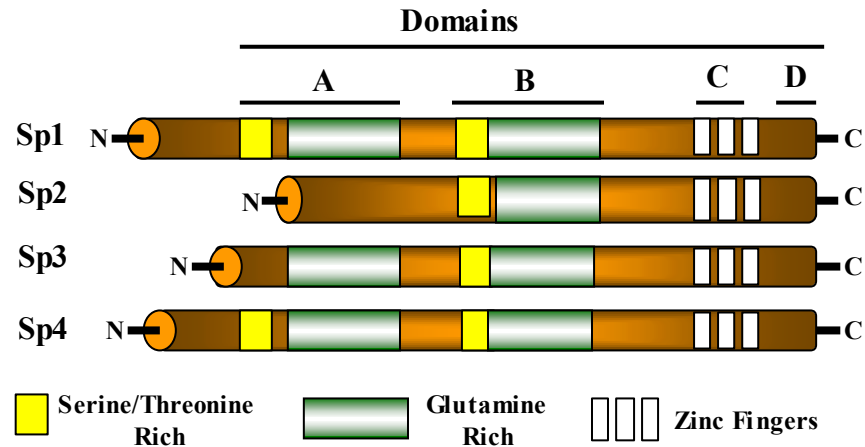
Fig. 1.20. Structural motifs in Sp family of proteins (554).

Sp proteins interact with KLF proteins and also with other transcription factors on GC-rich promoters to activate or repress different classes of genes that are important in the regulation of cellular homeostasis (555). Sp1 and Sp3 proteins are ubiquitously expressed and have been extensively investigated. For example, $Sp1^{-/-}$ embryos exhibit multiple abnormalities, retarded development and embryolethality on day 11 of gestation (556). $Sp3^{-/-}$ mice also exhibit growth retardation, defects in late tooth development, and the animals die at birth (557, 558).

Other members of the Sp/KLF family such as Sp5, Sp6, Sp7, Sp8, and the Krüppel-like factors differ from the first four members Sp1,Sp2,Sp3 and Sp4 because they are generally of lower molecular weight and do not contain the glutamine-rich N-terminal activation domain Sp1-dependent activation of gene expression requires interaction with a coactivator complex called CRSP (cofactors required for activation of Sp1). CRSP is a multisubunit complex consisting of six to eight polypeptides and is also

involved in Sp1-mediated activation of GC-rich genes with TATA boxes or initiator

sequences and interacts with proteins of the RNA polymerase preinitiation complex

(559, 560). Sp1 directly interacts with the TBP (561), TATA-binding protein associated

factors (TAFs) such as TAF130 (562) and TAF110 (563, 564) and other members of the

preinitiation complex through the glutamine-rich activation domains A and B and with

TAF55 through the C-terminal domain (565). Sp1 also interacts with proteins that are

not involved directly with the transcription machinery and these interactions may

activate or repress transcription. Most of the proteins that functionally interact with Sp1

are other sequence-specific transcription factors and these include Oct-1 (566), NF-κB

(567, 568), and E2F-1 (549, 569). Sp1 and E2F interact cooperatively on the

dihydrofolate reductase (DHFR) and thymidine kinase promoters resulting in a

synergistic activation of transcription. Sp1 also functionally and physically interacts with

GATA-1, an erythroid transcription factor and this interaction activates the

erythropoietin receptor (EpoR) and chicken α-globin promoters (570).

Sp proteins and cancer

The critical requirement for Sp proteins during embryonic and postnatal

development is in contrast to decreased expression in mature tissue/organs, which are

relatively quiescent. In contrast, there is increasing evidence that Sp1 and other Sp

proteins such as Sp3 and Sp4 are overexpressed in tumors compared to most other

tissues/organs (571-576). In gastric cancer, expression of Sp1 was high in the tumor cells

whereas the expression was weak in the stromal and normal glandular cells (576). Lou

and coworkers (577) reported that transformation of fibroblasts resulted in an 8- to 18-

fold increase in Sp1 expression; and these transformed cells formed highly malignant tumors in athymic nude mouse xenograft models, whereas untransformed fibroblasts expressing low levels of Sp1 did not form tumors. In addition, ribozyme-dependent knockdown of Sp1 in the transformed cells decreased VEGF expression and increased apoptosis. Sp1 knockdown by using small inhibitory RNA against Sp1 was shown to inhibit G0-G1 to S-phase progression in MCF-7 breast cancer cells (578). Knockdown of Sp3 and Sp4 along with Sp1 in pancreatic cancer cells show that these proteins regulate expression of VEGF, VEGF receptor 1(VEGFR1 or Flt), and VEGFR2 (KDR) (579-581). In addition, Sp3 has been shown to act as a repressor of p27, a CDK inhibitor in pancreatic cancer cells showing that Sp1 and Sp3 play a role in cancer cell proliferation and angiogenesis.

Since Sp proteins are overexpressed in tumors/cancer cells and regulate expression of growth, angiogenic and survival genes, then agents that target Sp protein degradation should be highly effective as anticancer drugs. Different strategies to degrade or repress Sp protein-dependent transactivation are illustrated in Figure 1.21. Sp proteins can be targeted by nucleic acid therapy by designing oligos that bind and sequester Sp proteins or develop chemical intercalators that inactivate or block Sp binding to GC rich motifs. In addition, small molecules that selectively inhibit Sp protein activation or expression would also block both Sp-dependent genes or pathways. The COX-2 inhibitor celecoxib decreases expression of Sp1 and VEGF by downregulating the expression of Sp1 in pancreatic cancer cells (582). The Cox-2 inhibitor Celecoxib, the NSAID tolfenamic acid (and related anthranilic acids), and the phytochemical
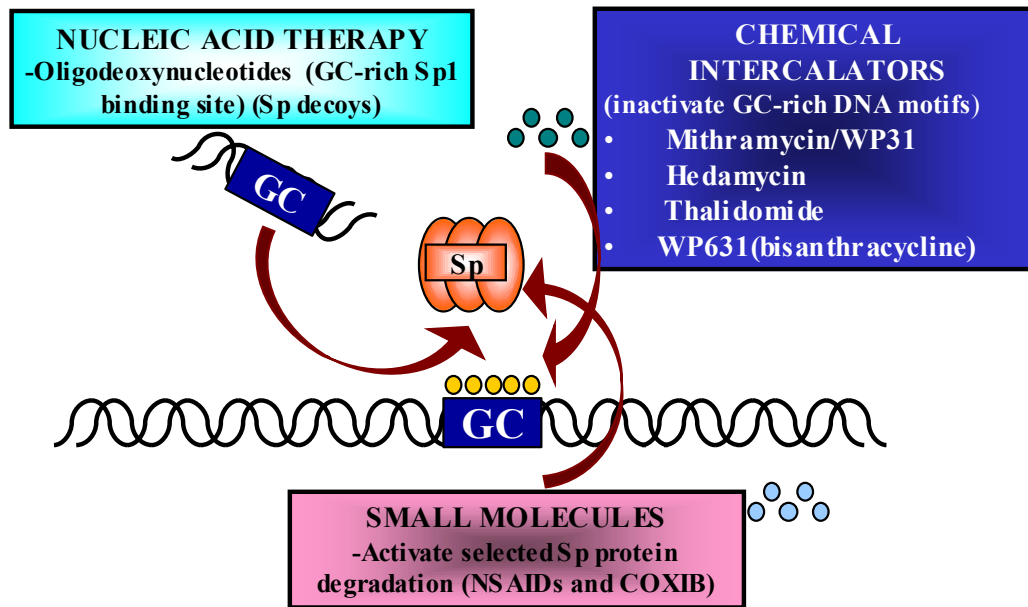
Fig. 1.21. Strategies for targeting Sp proteins in cancer cells.

betulinic acid also represses Sp protein expression in cancer cells and tumors but not in non-tumor tissue (331, 547, 578-581, 583-585). Moreover, these chemicals are potent inhibitors of tumor growth and metastasis and exhibit minimal toxic side-effects. Previous studies on these compounds demonstrated their cytotoxicity, proapoptotic and antiangiogenic activities but did not show the important contributions of Sp protein repression in mediating these responses. Many other anticancer drugs including phytochemicals are also cytotoxic and induce antiangiogenic and proapoptotic effects in cancer cells. We hypothesize that the underlying mechanism of action may be through repression of Sp proteins.

MICRORNA'S AS NEW DRUG TARGETS

MicroRNA's(miRNAs) are small noncoding single stranded RNA molecules about 18-24 nucleotides in length that play a role in posttranscriptional gene regulation (586, 587). The first miRNA discovered was lin-4 from Caenorhabditis elegans (588). In mammalian cells, miRNAs are processed from primary transcripts known as pri-miRNA which are generated by type II RNA polymerase in the form of long, polycystronic messages (589, 590). These pri-miRNA's are then processed into short hairpin precursors of about 60-110 basepair pre-miRNA's by a microprocessor complex comprised of an RNase III endonuclease enzyme Drosha and a double stranded RNA binding protein DGCR8 in the nucleus. These pre-miRNA's are again transported back to cytoplasm by exportin 5 where they are processed by Dicer into mature miRNA's of about 18-24 base pair length with 5' phosphates and a 2-nucleotide 3' overhang (591-593). Mature miRNA's are partially complementary to multiple mRNA molecules and hybridization results in downregulation of their gene expression. The antisense strand of the miRNA is incorporated into the ribonucleoprotein complex called RNA-induced silencing complex (RISC) and this complex binds to the target mRNA at the 3'UTR through complementary base pairing as shown in the Figure 1.22 (594).

Each miRNA interacts with multiple mRNAs by perfect base pairing and this results in translational inhibition and silencing of multiple transcripts (595). MiRNAs
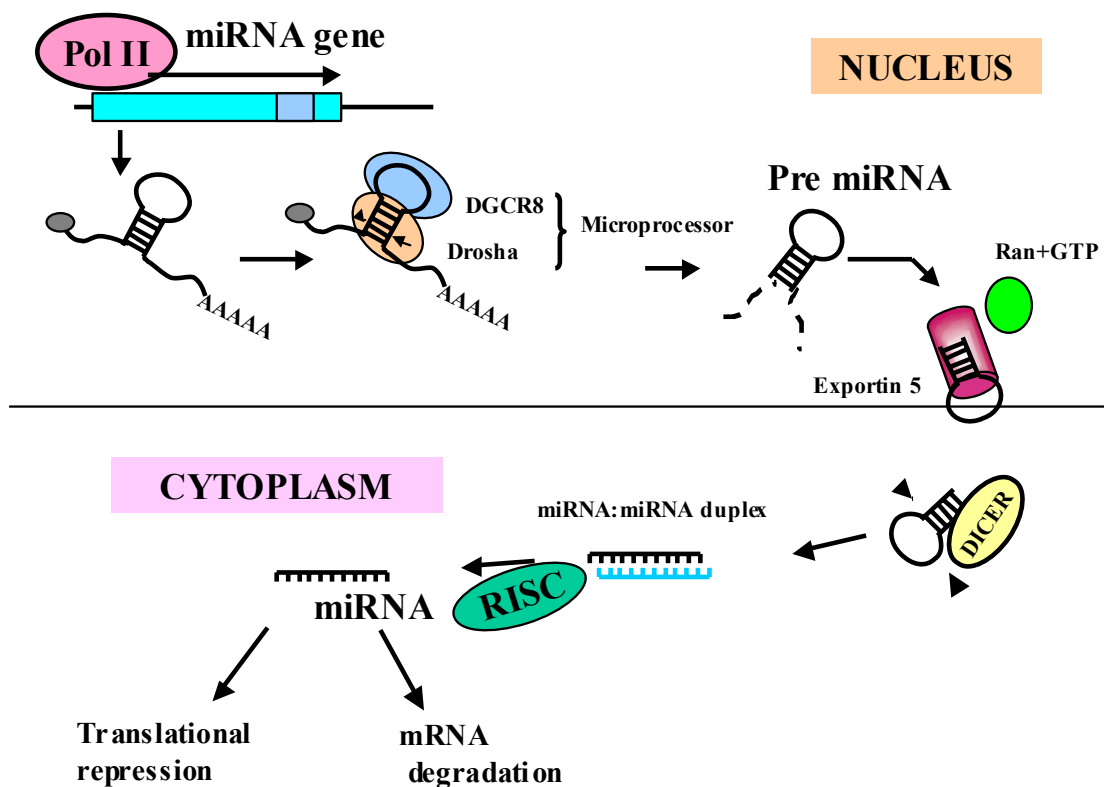
Fig. 1.22. Schematic representation of miRNA biogenesis (596).

have a wide range of functions which include regulation of cell proliferation,

differentiation, death, stress resistance, and fat metabolism (597). The number and tissue

distribution of miRNAs is not yet been determined but some miRNAs are present in all

tissues and others are tissue specific (598). The function of miRNAs in normal and

diseased tissues is being extensively studied and transgenic mice deficient in specific

miRNAs have clarified the physiologic roles of specific miRNAs (599-602). For

example, miR-208 is a cardiac-specific miRNA and knockdown of this miR in mice

resulted in modulation of both a and b myosin heavy chain expression of the muscle

fibers and their response to both stress and hypothyroidism (602).

MicroRNA's and cancer

The role of microRNA's in the development of cancer is being extensively studied and includes their use as biomarkers for cancer diagnosis and prognosis (603-606). The technology of miRNA microarrays is an important element for high throughput screening of miRNA fingerprints in normal and cancer cells (605). Other miRNA technologies have also been developed and these include macroarrays (607), bead-based flow cytometric miRNA expression (606) and quantitative reverse transcription-PCR (608) for determining microRNA expression profiles in different types of cancers. Cancer-specific miRNA's have been identified in almost every tumor type using these technologies. The identification of miRNA's that are altered in different types of solid tumors have lead to identification of the their role in pathways associated with the development of cancer (609). MicroRNA's are located in regions of DNA that are involved in cancer and these include minimal regions of loss of heterozygosity (LOH), minimal amplicons or break point cluster regions leading to the widespread misexpression in cancers (588). MicroRNA's are contributors to oncogenesis and are involved both in tumor-suppression (e.g. miR-15a and miR-16-1) and oncogenic (e.g. miR-155 or miR-17-92 cluster) activities. Repression of the tumor suppressor miR-15a/miR-16-1 leads to overexpression of the antiapoptotic protein, Bcl-2, whereas overexpression of oncogenic miR-17-92 activates c-myc and leads to cell proliferation. Overexpressed oncogenic miRNAs are located in the amplified regions of DNA and dowregulated tumor suppressor miRNAs are located in the deleted regions in cancer. For example, miR-21 interacts with 3'UTR of tropomyosin (TPM1), a tumor

suppressor in breast cancer cells and thus plays a role as an oncogenic miR by downregulating a tumor suppressor (610).

MiRNA's are located in all regions of the human genome but several are concentrated in regions that are altered in many cancers (611) resulting in changes in miR expression. For example, miR-15 and −16 are downregulated in B-cell chronic lymphocytic leukemia (CLL) and miR-155 is upregulated in Burkitts lymphoma, nonsmall cell lung cancer and breast cancer (612-614) (615, 616); miR-143 and −145 are downregulated in colon cancer whereas let-7, which targets RAS, is downregulated in nonsmall cell lung cancers (617) and is indicative of a poor prognosis. MiRNA alterations that are observed in somatic cells initiate tumorigenesis, and expression of specific miRNAs in germ line cells may predispose these individuals to cancer.

MicroRNA's and Sp proteins

Scott and coworkers (618) reported that miR-27a suppressed ZBTB10/RINZF expression in SKBR3 breast cancer cells and previous studies showed that ZBTB10 is a novel zinc finger protein that inhibits Sp-1 dependent activation of the gastrin gene promoter (619). This indicates that miR-27a supressess a Sp repressor ZBTB10 and could lead to overexpression of Sp proteins in cancer cells. The role of miR-27a in regulation of Sp proteins in MDA-MB-231 breast cancer cells was investigated by transfecting cells with antisense miR-27a (as-miR-27a) or ZBTB10 expression plasmid. As-miR-27a and ZBTB10 decreased levels of Sp proteins and as-miR27a increased ZBTB10 mRNA levels. In contrast, the effects of as-miR-27a and ZBTB10 overexpression on the cell cycle were different. ZBTB-10 overexpression blocked G0-

G1 to S phase progression and this was consistent with previous studies in MCF-7 cells using RNA interference for Sp1. In contrast, transfection of as-miR-27a resulted in accumulation of MDA-MB-231 breast cancer cells in G2-M (585). The failure of cells to undergo mitosis is linked to decreased activity of cdc2/cyclin B and both Wee-1 and Myt-1 are two genes that are important in the downregulation of cdc2/cyclin B activity and are also potential targets of miR-27a since their 3'UTRs have miR-27a binding sites. In MDA-MB-231 cells, as-miR-27a induces Myt-1and but not Wee-1gene expression, and this results in phosphorylation of cdc-2 and cell cycle arrest at G2/M (585).

EIF-4E AS A NEW ANTICANCER DRUG TARGET

The eukaryotic translation initiation factor eIF-4E is dysregulated in most human cancers including breast cancer and acute myeloid leukemia (620) where it is used as a prognostic indicator for cancer survival (621). In experimental mouse models increased levels of eIF-4E results in increased tumor number and malignancy (622). Translation is a critical step in many important cellular processes and this requires regulation of the rate of translation. Translation has been described as a housekeeping mechanism but is now recognized as a critical step in different pathophysiological processes such as apoptosis and cell proliferation, and dysregulation of this step has been associated with malignant transformation and development of multiple cancers (623, 624). Eukaryotic mRNA is monocistronic and has a 5'-7-methylguanosine cap and 3' poly (A) tail. Cap-dependent translation is the major translation mechanism in eukaryotes. Translation involves three different steps namely initiation, elongation and termination and translation initiation has always been the rate limiting step in the process

of protein synthesis. This rate limiting step is primarily controlled by two multiprotein complexes and their association with mRNA and includes the ternary and eIF-4F complexes (624). The convergence of these complexes on mRNA allows scanning of ribosomes and the initiation of translation. EIF-4F complex is comprised of the scaffold eIF4G, the ATP-dependent RNA-helicase eIF4A, and the cap binding protein eIF-4E. EIF-4E binds the 5'cap of mRNA and helps deliver mRNAs to the eIF-4F initiation complex. Elevated levels of eIF-4E result either from increased expression or release of eIF-4E from 4EBPs (eukaryotic binding proteins) after their phosphorylation by AKT/mTOR and ras signaling.

The availability of eIF-4E is the rate limiting factor in the initiation process. The availability of eIF-4E and the structure of mRNA 5' untranslated region (5'UTR) dictate the efficiency of translation (625). Highly complex 5'UTR structured mRNA also called weak mRNAs requires eIF-4E in excess and the translation of these mRNAs is restricted whereas strong mRNAs those with simple 5'UTRs do not depend on the availability of eIF-4E and are translated. Weak mRNAs are those that are involved in cell proliferation (cyclin D1, c-Myc, and ornithine decarboxylase), survival(Bcl-xl) and angiogenesis (VEGF, basic FGF, and HIF-1α) and malignancy (MMP9) (Fig. 1.23) (625). Increased levels of free eIF-4E have been identified in multiple cancers including cancers of breast, colon, bladder, lung, prostate and head and neck, leukemias, and lymphomas and are associated with their progression to malignancy (625, 626). Overexpression and dysregulation of eIF-4E results in an increased number of tumors, invasion and
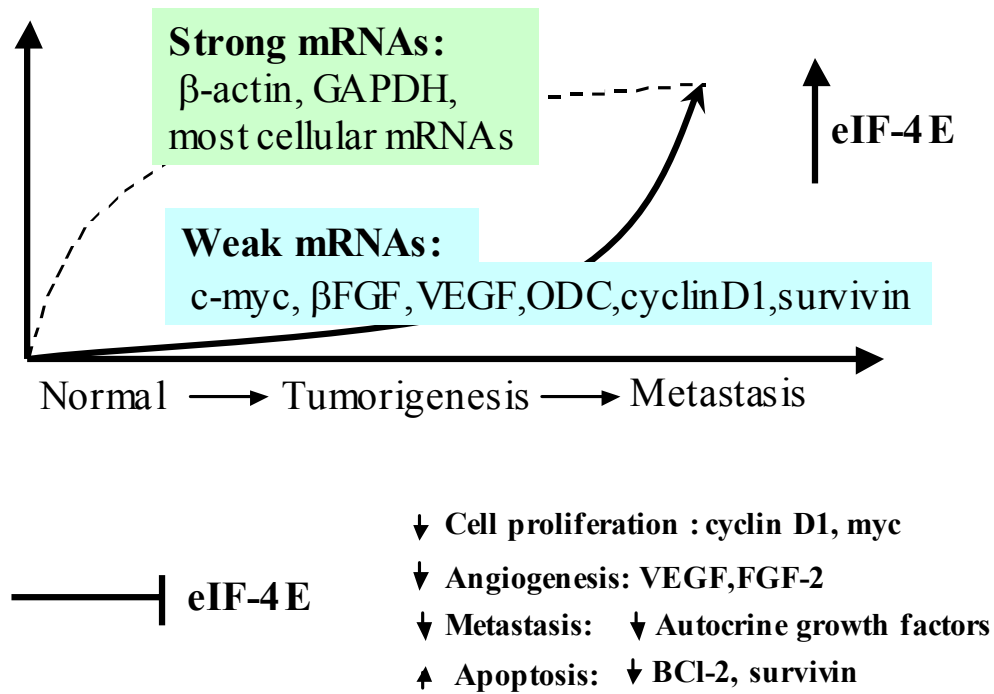
Fig. 1.23. Reducing translation initiation factor supresses malignancy (627).

metastases in mouse models (628). Therefore eIF-4E is an attractive target for anticancer

drugs since multiple genes can be affected (Fig. 1.23).

At present there are no specific inhibitors of eIF-4E or its association with

mRNA has not been identified. Ribavarin is an antimetabolite drug, used in the

treatment of viral diseases that inhibits the interaction of eIF-4E with the 5'cap of

mRNA resulting in decreased expression of cyclin D1 and suppressesion of tumor

growth (629). Inhibitors of eIF-4E-eIF4G interactions were developed by Wagner and

his colleagues; and these inhibitors decreased the expression of c-myc and cyclinD1 in

cell cultures (630).

Antisense oligonucleotide chemistry (ASO) has been developed to decrease eIF-4E expression. Several modifications were made to increase the tissue stability and to avoid the immunostimulatory effects of a 20-mer ASO which suppresses the expression of eIF-4E (631). Graff and his colleagues used second generation eIF4E ASOs to treat mice with subcutaneous xenograft tumors and the results showed that there was approximately 50% decrease in eIF4E levels within the tumors and almost complete reduction in the tumor growth. EIF4E ASO treatment also reduced VEGF levels and vessel number in the tumors with minimal or negligible effect on liver transaminase levels (631). Unlike cycloheximide which blocks translation elongation and reduces all new protein synthesis, ASO treatment decreases protein synthesis by only 20% suggesting some specificity. Clinical trials are underway with eIF4E ASO to determine the efficacy of targeting eIF4E in human cancers.

EIF-4E is phosphorylated by agents that aid in the activation of translation (632, 633). In normal tissues eIF-4E is sequestered by hypophophorylated 4EBPs and thereby restrict the translation to housekeeping genes whereas in tumors, 4EBPs are phosphorylated and release eIF-4E resulting in increased translation of genes involved in cell proliferation, survival, and metastases (Fig. 1.24). EIF-4E in mammals is phosphorylated on serine209 by the Mnk1 and Mnk2 kinases which are bound to eIF4G. The role of eIF-4E phosphorylation has not been determined; however, some studies indicate that phosphorylated eIF-4E aids in growth by increasing affinity for cap analogs (634).
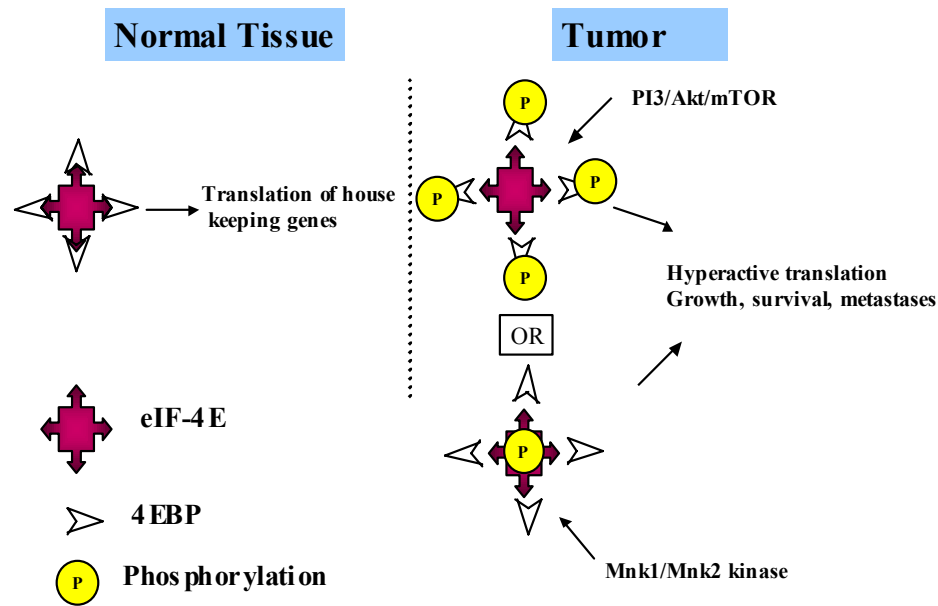
Fig. 1.24. Availability and phosphorylation of eIF-4E playing a role in differential translation in normal tissues and tumors (635).

In contrast, some studies have reported that there is decreased affinity of cap

analogs for phospho- eIF-4E (636). A correlation between eIF-4E phosphorylation and

enhanced protein synthesis has been reported; and two models have been proposed to

describe the regulation of eIF-4E activity by phosphorylation at Ser209 (637). Based on

the crystallographic structure of the mouse eIF-4E complex with m7GDP, Marcotrigiano

et al (638) have proposed a clamping model in which there is formation of salt bridge

between the phosphorylated Ser 209 and Lys 159 situated across the entry to the cap

binding slot and these are separated by 7 A^0 . This clamp stabilizes the mRNA chain at

the protein surface. Phosphorylation and clamping may occur only after mRNA binding.

The second model was proposed by Scheper who observed 10-fold increase in the

dissociation rate of eIF-4E with cap analogs after phosphorylation and this is contradictory to the clamping model. With decreasing binding affinity for cap it is possible that the tethered eIF4F is released from the 5'end of mRNA to enhance ribosome scanning. Another explanation could be the reprogramming of the translational machinery by releasing initiation factors from the existing complexes to allow other mRNAs to be translated (639). A recent study by Topisirovic and his colleagues demonstrated that phosphorylation of eIF-4E though not necessary enhances its ability to transform cells and eIF-4E dependent nucleocytoplasmic transport of some mRNAs including cyclin D1 (640). Therefore inhibiting the activity of eIF-4E by decreasing the phsophorylation also stands as an exciting novel approach in the treatment of cancer.

The potential for eliciting toxicity with a treatment that inhibits a general protein synthesis factor was of concern but surprisingly results from the ASO studies that target eIF4E show that there was minimal toxicity associated with this approach. Moreover, the ASO significantly decreased the tumor growth suggesting that this may be an effective strategy for treatment of human malignancies that should be further investigated.

CHAPTER II

**STRUCTURE-DEPENDENT ACTIVITY OF GLYCYRRHETINIC ACID
DERIVATIVES AS PEROXISOME PROLIFERATOR-ACTIVATED
RECEPTOR γ (PPARγ)
AGONISTS IN COLON CANCER CELLS***

INTRODUCTION

Licorice root extracts have been extensively used for their therapeutic properties

which include the potentiation of cortisol action, inhibition of testosterone biosynthesis,

reduction in body fat mass and other endocrine effects (487, 641-643). The activities of

these extracts are linked to different classes of phytochemicals particularly the major

water soluble constituent glycyrrhizin and its hydrolysis product 18β-glycyrrhetinic acid

(GA) (Fig. 1.18). Glycyrrhizin is a pentacyclic triterpenoid glycoside which is

hydrolyzed in the gut to GA and many of the properties of licorice root can be attributed

to GA. For example, GA inhibits 11β-hydroxysteroid dehydrogenase activity increasing

corticosterone levels and this has been linked to apoptosis in murine thymocytes,

splenocytes and decreased body fat index in human studies (489, 644-647). GA also

directly acts on mitochondria to induce apoptosis through increased mitochondrial

swelling, loss of mitochondrial membrane potential and release of cytochrome C (648,

649).

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GA has also been used as a template to synthesize bioactive drugs. For example carbenoxolone, the 3-hemisuccinate derivative of GA, has been used for the treatment of gastritis and ulcers (650). Some of the activity of carbenoxolone may be due to hydrolysis to GA however carbenoxolone itself induced oxidative stress in liver mitochondria and decreased mitochondrial membrane potential. Other carboxyl and hydroxyl derivatives of glycyrrhizic acid inhibit HIV and exhibit anti-inflammatory and immunomodulatory activities (651). In addition, GA derivatives containing a reduced carboxylic acid group at C-30 (CH_2OH) and some additional functional changes exhibited strong antioxidant activity (652).

Structure-activity studies on the anti-inflammatory activities and cytotoxicity of several oleanolic and ursolic acid derivatives demonstrated that addition of a 2-cyano substituent greatly enhanced their activity (463, 464, 653-655). Moreover, one of the 2-cyano analogs of oleanolic acid, namely 2-cyano-3,12-dioxo-17α-olean-1,9(11)-diene-28-oic acid (CDDO) and its methyl ester (CDDO-Me) exhibited PPARγ agonist activity (234, 302, 656). Although GA also has an oleanolane triterpenoid backbone, there are major structural differences between GA and oleanolic acid and between CDDO-Me and the synthetic GA analog methyl 2-cyano-3, 11-dioxo-18β-olean-1, 12-dien-30-oate (β-CDODA-Me). CDODA-Me (β-CDODA-Me) is isomeric with CDDO-Me (α-CDDO-Me) but differs with respect to the carboxy substitution in the E ring, the stereochemistry at C-18 (β vs. α) in the E/D ring junction, and the enone function in the C ring (Fig. 1.18). In order to more fully investigate the importance of the stereochemistry at C-18 in modulating cytotoxicity and PPARγ agonist activity of triterpenoid acids, we also

synthesized methyl 2-cyano-3, 11-dioxo-18α-olean-1,12-dien-30-oate (α-CDODA-Me).

Our results show that introduction of the 2-cyano group into α- or β-GA resulted in

enhanced cytotoxicity, and both compounds induced PPARγ-dependent transactivation

in colon cancer cells, including receptor and cell context-dependent activation of

caveolin-1 and Krüppel-like factor-4 (KLF-4), two genes associated with growth

inhibitory responses in colon cancer. However, it was also apparent that the different

stereochemistries at C18 and the altered confirmation of the E-ring resulted in different

PPARγ-dependent effects in colon cancer cells, suggesting that the α-CDODA-Me and

β-CDODA-Me isomers are selective receptor modulators (SRMs).

MATERIALS AND METHODS

Cell lines

Human colon carcinoma cell lines SW480, HCT-15 and HT29 were provided by

Dr. Stan Hamilton, M.D. Anderson Cancer Center (Houston, TX); SW-480 and HT-29

cells were maintained in Dulbecco's modified Eagle's medium nutrient mixture with

Ham's F-12 (DMEM/Ham's F-12; Sigma-Aldrich, St. Louis, MO) with phenol red

supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, and 5% fetal

bovine serum and 10 ml/L 100x antibiotic antimycotic solution (Sigma-Aldrich). HCT-

15 cells were maintained in RPMI 1640 (Sigma) supplemented with 0.22% sodium

bicarbonate, 0.11% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% fetal bovine

serum, and 10 ml/L of 100X antibiotic antimycotic solution. Cells were maintained at 37

°C in the presence of 5% CO_2.

Synthesis

3, 11-Dioxo-18β-oleana-1,12-dien-30-oic acid (β-DODA) and α-DODA. A

mixture of 18β-glycyrrhetinic acid (157 mg, 0.3333 mmol) (Sigma-Aldrich) and 2-

iodoxybenzoic acid[1] (IBX) (373.4 mg, 1.333 mmol, 4 equiv) in 7 ml DMSO was stirred

with heating at 85 °C for 21 h. After cooling, the solution was poured into water (100

ml) giving a white precipitate which was filtered and washed with methanol/methylene

chloride (1:9). This material (381 mg) was triturated with ethyl acetate (5 ml), washed

several times with this solvent, and the dissolved material recovered by evaporation and

purified by preparative scale TLC using $MeOH/CH_2Cl_2$ (1:19) as eluant. The main band

gave β-DODA as a white solid (133 mg, 85.5%) which was crystallized from methanol

(104 mg), mp 270-5 °C. ^1H NMR δ 7.746 (1H, d, J = 10.4 Hz, C1-H), 5.816 (1H, d, J =

10.4 Hz, C2-H), 5.817 (1H, s, C12-H), 2.691 (1H, s, C9-H), 1.422, 1.401, 1.245, 1.191,

1.169, 1.118, 0.872 (all 3H, s, CMe). A similar procedure was used for the synthesis of

α-DODA from 18α-glycyrrhetinic acid (Sigma-Aldrich).

Methyl 3, 11-dioxo-18β-oleana-1,12-dien-30-oate (β-DODA-Me) and α-DODA-

Me. Methyl 18β-glycyrrhetinate was prepared by diazomethylation of 18β-

glycyrrhetinic acid and a sample (161 mg, 0.3333 mmol) reacted with the IBX reagent

(373 mg, 1.333 mmol, 4 equiv) as above for the parent acid. After a similar work-up,

the recovered product was purified by preparative TLC ($MeOH/CH_2Cl_2$; 1:19) to give a

colorless solid (155.3 mg, 96.9%) which on crystallization, gave needles (140 mg), mp

192-4 °C. ^1H NMR δ 7.745 (1H, d, J = 10.0 Hz, C1-H), 5.812 (1H, d, J = 10.0 Hz, C2-

H), 5.770 (1H, s, C12-H), 3.078 (3H, s, OMe), 2.681 (1H, s, C9-H), 1.419, 1.390, 1.184,

1.166, 1.159, 1.118, 0.833 (all 3H, s, CMe). A similar procedure was used for the synthesis of α-DODA-Me from α-DODA.

2-Cyano-3,11-dioxo-18β-oleana-1,12-dien-30-oic acid (β-CDODA) and α-CDODA. The two cyano derivative of 18β-glycyrrhetinic acid was synthesized as previously described (657), and a sample (422 mg, 0.8961 mmol) of this compound and DDQ[3] (247 mg, 1.088 mmol) in dry benzene (55 ml) was heated to reflux, with stirring, for 6 h. The reaction mixture was filtered, washed with benzene, and the filtrate plus washings were combined, evaporated and purified by preparative TLC (ethyl acetate/hexane, 1:1) to give β-CDODA (149 mg, 33.7%). This material was crystallized twice from ethyl acetate/hexane to give a yellow solid (55.5 mg), mp 195-7 °C. ^{1}H NMR δ 8.550 (1H, s, C1-H), 5.846 s, C12-H), 2.2.715 (1H, s, C9-H), 1.455, 1.404, 1.255, 1.225, 1.200, 1.162, 0.876 (all 3H, s, CMe). A similar procedure was used for the synthesis of α-CDODA from 18α-glycyrrhetinic acid.

Methyl 2-cyano-3, 11-dioxo-18β-oleana-1,12-dien-30-oate (β-CDODA-Me) and α-CDODA-Me. The nitrile was also prepared from methyl 18β-glycyrrhetinate, and the resulting ester (246 mg, 0.4863 mmol) and DDQ (134 mg, 0.5905 mmol) in dry benzene (20 mL) was refluxed for 5 h to give β-CDODA-Me. The compound was purified by TLC (ethyl acetate/hexane; 1:3) to give β-CDODA-Me and crystallized from ethyl acetate/hexane (138 mg), mp 243-5 °C. ^{1}H NMR δ 8.553 (1H, s, C1-H), 5.805 s, C12-H), 3.716(3H, s, OMe), 2.706 (1H, s, C9-H), 1.454, 1.393, 1.223, 1.194, 1.168, 1.161,

0.834 (all 3H, s, CMe). A similar procedure was used for the synthesis of α-CDODA-Me from α-DODA.

Antibodies and reagents

Caveolin 1 antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal β-actin antibody and 18α- and 18β-glycyrretinic acid (GA) were purchased from Sigma-Aldrich. Reporter lysis buffer and luciferase reagent for luciferase studies were supplied by Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA), and LipofectAMINE reagent was purchased from Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagent was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). The PPARγ antagonists 2-chloro-5-nitro-N-phenylbenzamide (GW9662) and N-(4'-aminopyridyl)-2-chloro-5-nitrobenzamide (T007) were synthesized in this laboratory, and their identities and purity (>98%) were confirmed by gas chromatography-mass spectrometry. Melting points were determined with a Kofler hot-stage apparatus. ^{1}H NMR spectra were run in CDCl$_3$ on a Bruker Avance-400 spectrometer using Me$_4$Si as an internal standard. For analytical and preparative use, TLC plates were spread with Silica Gel 60 GF (Merck). Elemental microanalyses were carried out by Guelph Chemical Laboratories Ltd.

Plasmids

The Gal4 reporter containing 5x Gal4 response elements (pGal4) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). Gal4DBD-PPARγ construct (gPPARγ) was a gift of Dr. Jennifer L. Oberfield

(GlaxoSmithKline Research and Development, Research Triangle Park, NC). $PPRE_3$-luc construct contains three tandem PPREs with a minimal TATA sequence in pGL2. The GAL4 coactivator (PM coactivator) and VP-PPARγ chimeras were provided by Dr. S. Kato, University of Tokyo (Tokyo, Japan) (658).

Transfection and luciferase assay

Colon cancer cell lines SW480 and HT29 (1 x 10^5 cells/well) were plated in 12-well plates in DMEM/Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After 16 h, various amounts of DNA [i.e., Gal4Luc (0.4 μg), β-Gal (0.04 μg), Gal4PPARγ and PPRE-Luc (0.04 μg)] were transfected using Lipofectamine reagent (Invitrogen) following the manufacturer's protocol. Five h after transfection, the transfection mix was replaced with complete media containing either vehicle (DMSO) or the indicated ligand for 20 to 22 h. Cells were then lysed with 100 μL of 1x reporter lysis buffer, and 30 μL of cell extract was used for luciferase and β-Gal assays. A LumiCount luminometer (PerkinElmer Life and Analytical Sciences) was used to quantitate luciferase and β-Gal activities, and the luciferase activities were normalized to β-Gal activity. Results are expressed as means ± S.E. for at least three replicate determinations for each treatment group.

Mammalian two-hybrid assay

SW480 cells were plated in 12-well plates at 1 x 10^5 cells/well in DMEM/F-12 media supplemented with 2.5% charcoal-stripped fetal bovine serum. After growth for 16 h, various amounts of DNA, *i.e.* Gal4Luc (0.4 μg), β-gal (0.04 μg), VP-PPARγ (0.04 μg), pMSRC1 (0.04 μg), pMSRC2 (0.04 μg), pMSRC3 (0.04 μg), pMPGC-1 (0.04 μg),

pMDRIP205 (0.04 μg), and pMCARM-1 (0.04 μg) were transfected by Lipofectamine (Invitrogen) according to the manufacturer's protocol. After 5 h, the transfection mix was replaced with complete media containing either vehicle (DMSO) or the indicated ligand for 20 - 22 h. Cells were then lysed with 100 ml of 1x reporter lysis buffer, and 30 μL of cell extract was used for luciferase and β-galactosidase assays. Lumicount was used to quantitate luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

Cell proliferation assay

SW480, HCT-15 and HT 29 cells (2×10^4) were plated in 12-well plates, and media was replaced the next day with DMEM/Ham's F-12 media containing 2.5% charcoal-stripped FBS and either vehicle (DMSO) or the indicated ligand and dissolved in DMSO. Fresh media and compounds were added every 48 h. Cells were counted at the indicated times using a Coulter Z1 cell counter. Each experiment was done in triplicate, and results are expressed as means ± S.E. for each determination.

Western blot analysis

SW-480, HCT-15 and HT-29 (3×10^5) cells were seeded in six-well plates in DMEM/Ham's F-12 media containing 2.5% charcoal-stripped FBS for 24 h and then treated with either the vehicle (DMSO) or the indicated compounds. Whole-cell lysates were obtained using high-salt buffer [50 mM HEPES, 500 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, and 1% Triton X-100, pH 7.5, and 5 μL/ml Protease Inhibitor Cocktail (Sigma-Aldrich)]. Protein samples were incubated at 100 °C for 2 min, separated on 10% SDS-PAGE at 120 V for 3 to 4 h in 1x running buffer (25 mM Tris-

base, 192 mM glycine, and 0.1% SDS, pH 8.3), and transferred to polyvinylidene difluoride membrane (PVDF; Bio-Rad, Hercules, CA) at 0.1 V for 16 h at 4 °C in 1x transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 0.025% SDS). The PVDF membrane was blocked in 5% TBST-Blotto (10 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.05% Triton X-100, and 5% nonfat dry milk) with gentle shaking for 30 min and was incubated in fresh 5% TBST-Blotto with 1:1000 (for caveolin-1), and 1:5000 (for β-actin) 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 90 min. The membrane was washed with TBST for 10 min, incubated with 10 ml of chemiluminescence substrate (PerkinElmer) for 1.0 min, and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

Semi quantitative reverse transcription-PCR analysis

SW480, HT-29 and HCT-15 cells were treated with either DMSO (control) or with the indicated concentration of the compound for 12 h. Total RNA was extracted using RNeasy Mini Kit (Qiagen Inc., Valencia, CA), and one microgram of RNA was used to synthesize cDNA using Reverse Transcription System (Promega). The PCR conditions were as follows: initial denaturation at 94 °C (1 min) followed by 28 cycles of denaturation for 30 sec at 94 °C, annealing for 60 sec at 55 °C and extension at 72 °C for 60 sec, and a final extension step at 72 °C for 5 min. The mRNA levels were normalized using GAPDH as an internal housekeeping gene. Primers were obtained from IDT (Coralville, IA) and used for amplification were: KLF4 (sense 5'-CTA TGG

CAG GGA GTC CGC TCC-3') (antisense 5'-ATG ACC GAC GGG CTG CCG TAC - 3'); GAPDH (sense 5'-ACG GAT TTG GTC GTA TTG GGC G-3') (antisense 5'-CTC CTG GAA GAT GGT GAT GG-3'). PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and visualized under UV transillumination.

Statistical analysis

Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean ± standard deviation for at least three separate determinations for each treatment.

RESULTS

Growth inhibitory effects of isomeric GA derivatives

This study compares the cytotoxicity of 18β-GA and 18α-GA derivatives and results in Figure 2.1 summarize the cytotoxicity of β-DODA-Me, β-CDODA-Me, and the corresponding 18α-isomers. Initial studies showed that GA and its methyl esters exhibit minimal cytotoxicity and the methyl esters were more potent than the corresponding free triterpenoid acids (data not shown). The α-DODA and β-DODA methyl esters exhibited growth inhibitory IC_{50} values of 10-20 and 10-15 μM, respectively, whereas introduction of the 2-cyano substituents into the α- and β-isomers greatly enhanced cytotoxicity. The IC_{50} values for α-CDODA-Me and β-CDODA-Me were 0.5 and 0.2-0.5 μM, respectively, demonstrating the greatly enhanced cytotoxicity of the GA derivatives containing the 2-cyano substituents. Similar results were observed in HT-29 and HCT-15 colon cancer cells (data not shown).

α-CDODA-Me and β-CDODA-Me activate PPARγ

Previous studies have demonstrated that introduction of 2-cyano substituents into

oleanolic acid and ursolic acid derivatives enhances cytotoxicity of these triterpenoid
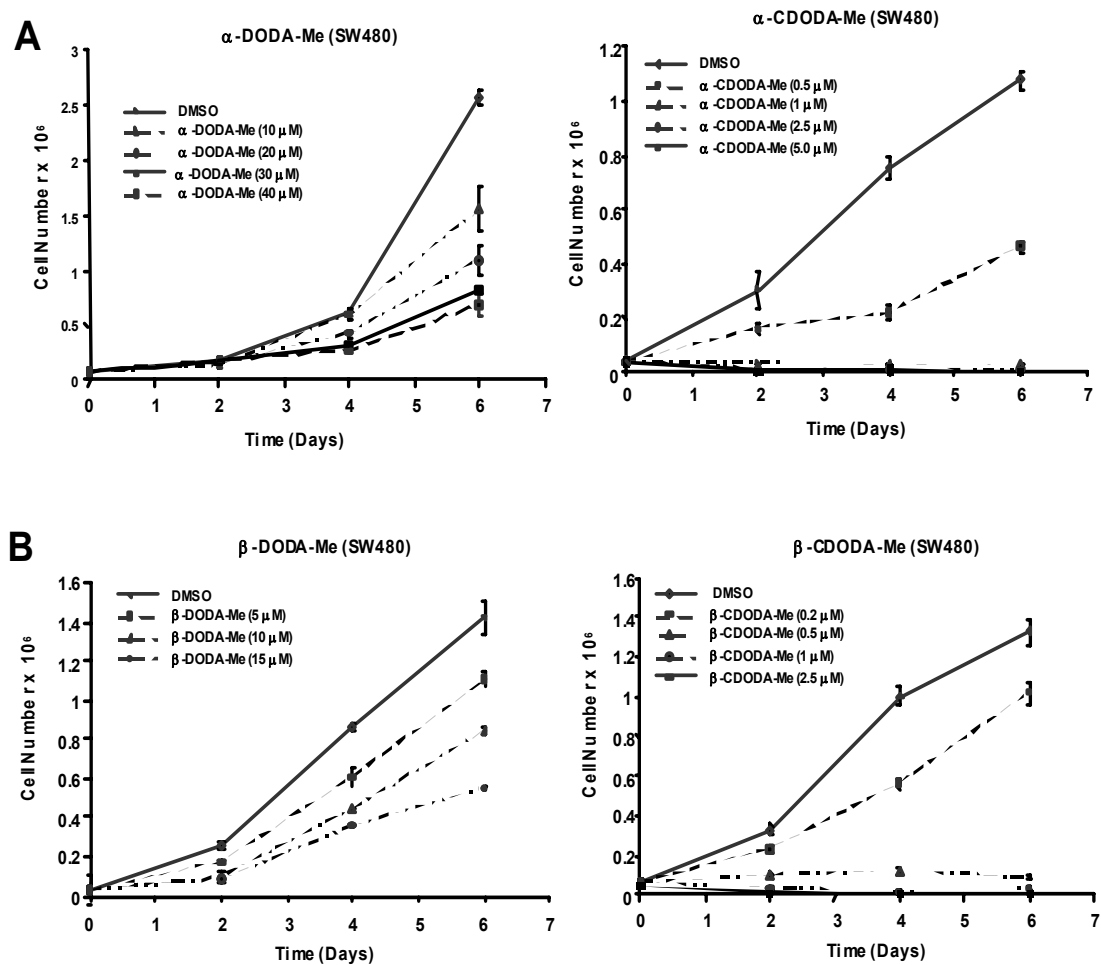
Fig. 2.1. Growth inhibition studies. SW480 colon cancer cells were treated with different concentrations of α-DODA-Me , α-CDODA-Me (A), β-DODA-Me and β-CDODA-Me (B) for 6 days, and cell numbers were determined using a Coulter Counter as described in the Materials and Methods. Results are expressed as means ± SE for 3 separate determinations at each time point. Significant (p<0.05) inhibition of cell growth was observed for β-DODA-Me (≤ 5 μM), β-CDODA-Me (≤ 0.2 μM), α-DODA-Me (≤ 10 μM), and α-CDODA-Me (≤ 0.5 μM).

acids (463, 464, 655) as observed in this study for the α- and β-GA derivatives (Fig.

2.1). 2-Cyano derivatives of oleanolic acid also exhibit PPARγ agonist activity (234)

and in this study, we have investigated the PPARγ agonist activity of α- and β-CDODA-

Me isomers which exhibit major structural differences in the E-ring of GA. Results in

Figure 2.2 A compare activation of PPARγ by α-CDODA-Me and α-DODA-Me in

SW480 cells transfected with PPARγ-GAL4/pGAL4; 5 μM α-CDODA-Me induces a

>10-fold increase in activity, whereas α-DODA-Me was inactive at concentrations as

high as 20 μM. In a separate experiment, similar results were obtained for β-CDODA-

Me and β-DODA-Me. The former compound (5 μM) induced a >18-fold increase in

luciferase activity, whereas the latter compound (30 μM) was inactive (Fig. 2.2 B). A

direct comparison of both α-CDODA-Me and β-CDODA-Me is illustrated in Figure 2.2

C where 5 μM of both compounds induced an (12- to 16- fold) increase in luciferase

activity in SW480 cells transfected with PPARγ-GAL4/pGAL4. Cotreatment with 10

μM of the PPARγ antagonist T007 significantly decreased α-CDODA-Me/β-CDODA-

Me-induced transactivation. Both α-CDODA-Me and β-CDODA-Me also induced

transactivation (Figs. 2.2 D and 2.2 E) in SW480 cells transfected with PPRE3-luc, a

construct which contains three tandem PPARγ response elements linked to luciferase

and which relies on activation of the endogenous PPARγ-RXR complex expressed in

this cell line (302). In addition, both PPARγ antagonists T007 and GW9662 inhibited α-

CDODA-Me/β-CDODA-Me-induced transactivation. These results demonstrate for the

first time that introduction of a 2-cyano group into the GA triterpenoid acid structure is
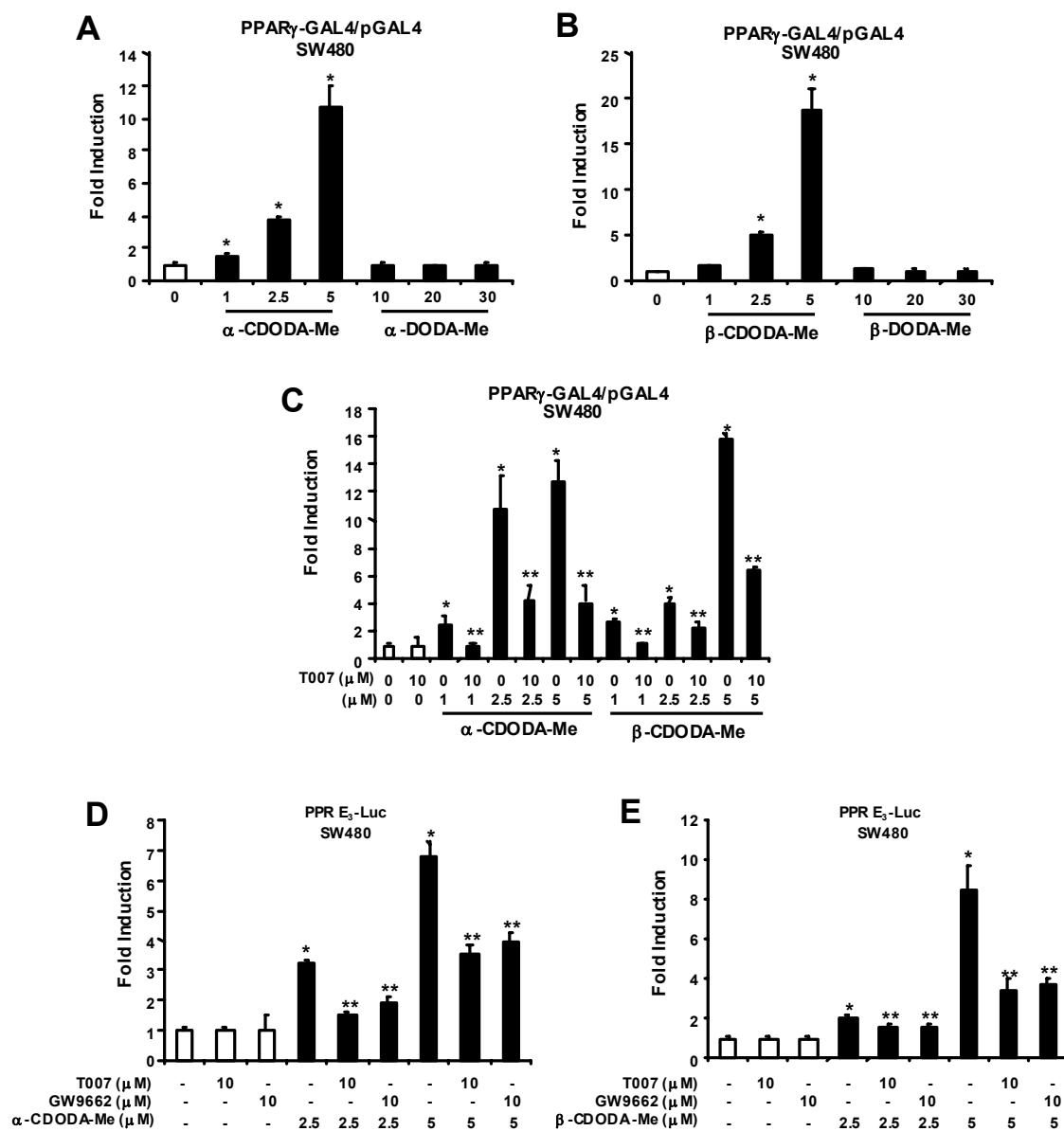
Fig. 2.2. Ligand-induced activation of PPARγ, and effects of PPARγ antagonists. Activation of PPARγ-GAL4/pGAL4 in SW480 cells treated with α-DODA-Me/α-CDODA-Me (A), β-DODA-Me/β-CDODA-Me (B), and both isomers plus T007 (C). Cells were transfected with PPARγ-GAL4/pGAL4, treated with different concentrations of the triterpenoids alone or in combination with T007, and luciferase activity was determined as described in the Materials and Methods. Results of all transactivation studies in this Figure are presented as means ± SE for at least 3 separate determinations for each treatment group and significant (p < 0.05) induction compared to solvent (DMSO) control (*) and inhibition by cotreatment with T007 (**) is indicated. Activation of PPRE-luc in SW480 cells treated with α-CDODA-Me (D) or β-CDODA-Me (E) alone or in combination with PPARγ antagonists.   SW480 cells were transfected with PPRE-Luc, treated with different concentrations of CDODA-Me isomers alone or in combination with 10 μM GW9662 and/or T007, and luciferase activities were determined as described in Figure 3A. Significant (p < 0.05) induction of luciferase activity (*) and inhibition of induced transactivation by GW9662 or T007 (**) is indicated.

sufficient for conferring PPARγ agonist activity on the resulting compound. Moreover, both the α-and β-CDODA-Me isomers exhibit similar potencies as PPARγ agonists (β-CDODA-Me ≥ α-CDODA-Me), suggesting that the conformational differences in the E-ring which are observed for the 18α- and 18β-isomers do not affect their PPARγ agonist activities in the PPARγ-GAL4/pGAL4 and PPRE-luc transactivation assays.

α-CDODA-Me and β-CDODA-Me as Selective Receptor Modulators (SRMs)

PPARγ agonists are structurally diverse (658-661) and there is evidence that many of these compounds are selective PPARγ modulators that exhibit tissue-specific differences in their activation of receptor-dependent genes/protein. The selectivity of various structural classes of PPARγ ligands is due, in part, to differential interactions within the ligand binding domain of PPARγ which can lead to different conformations of the receptor. This can result in differential interactions of the ligand-bound PPARγ with nuclear receptor coactivators (658), and results in Figure 2.3 A summarize β-CDODA-Me-induced transactivation in SW480 cells transfected with GAL4-coactivator and VP-PPARγ (ligand binding domain) chimeras and a pGAL4 reporter gene. In this mammalian two-hybrid assay, β-CDODA-Me induced PPARγ interactions only with PGC-1 and SRC-1 but not with AIB1 (SRC-3), TIFII (SRC-2), CARM1, TRAP220 and the corepressor SMRT. α-CDODA-Me also induced PPARγ-PGC-1 interactions (Fig. 2.3). These results suggest that the two α- and β-CDODA-Me isomers, which differ only in the conformations of their E-rings, are selective receptor modulators (SRMs) and
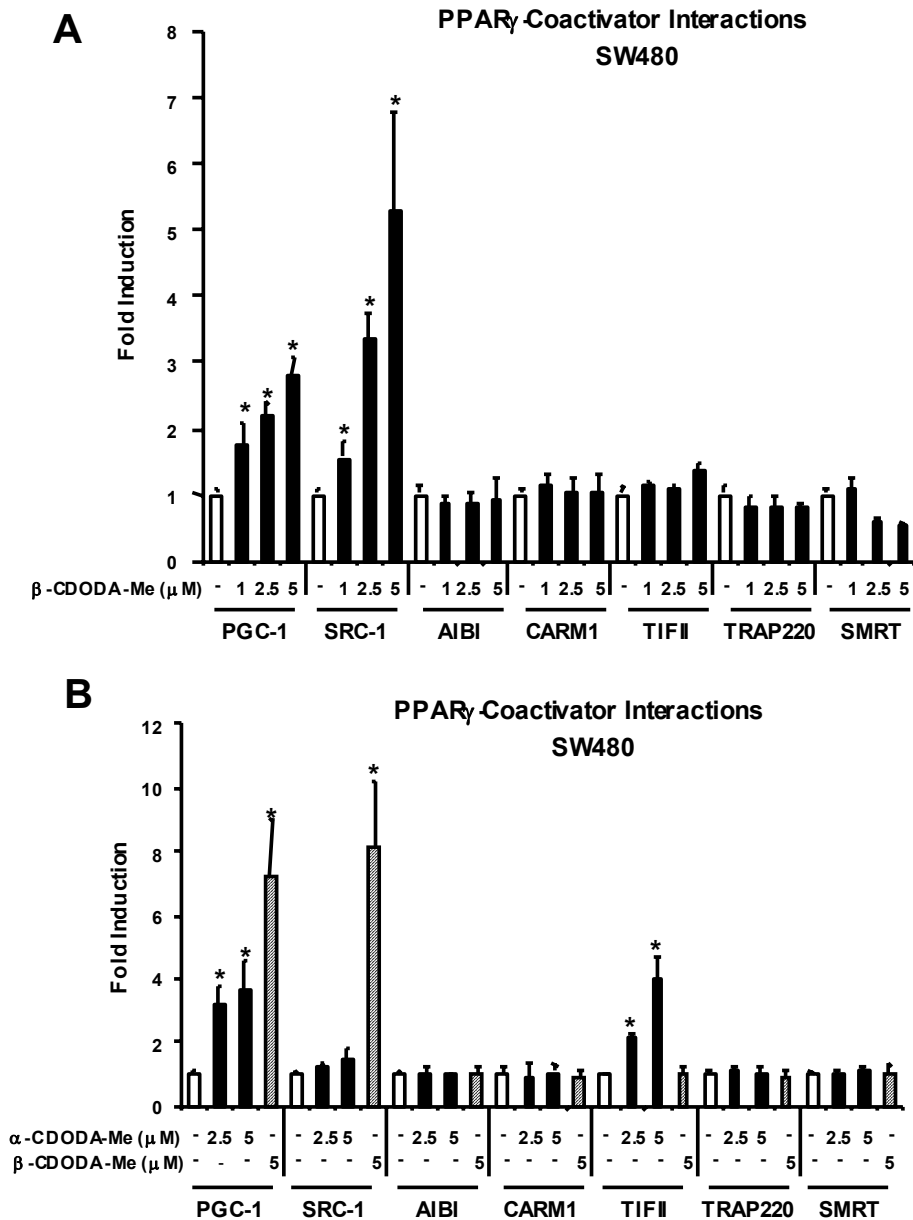
Fig. 2.3. Ligand-induced PPARγ-coactivator interactions. SW480 cells were transfected with VP-PPARγ, coactivator-GAL4/pGAL4, treated with different concentrations of α-CDODA-Me (A) or both α- and β-CDODA-Me (B), and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations for each treatment group, and significant ($p < 0.05$) induction is indicated by an asterisk.

induce different patterns of coactivator-receptor interactions in a mammalian two-hybrid assay. These results also suggest that the α- and β-CDODA-Me isomers should exhibit some tissue/cell or response-specific differences in their activation of receptor-dependent genes.

PPARγ agonists induce caveolin-1 in colon cancer cells through a receptor-dependent mechanism (257, 300). The effects of α-CDODA-Me on caveolin-1 expression in HT-29, HCT-15 and SW480 cells is summarized in Figure 2.4 A, and induction was observed in all three cell lines. In contrast, β-CDODA-Me induced caveolin-1 in HT-29 and HCT-15 but not SW480 colon cancer cells (Fig. 2.4 B), and the failure to observe induction of caveolin-1 in SW480 cells was noted in several replicate experiments. Figures 2.4 C and 2.4 D show that induction of caveolin-1 by α- and β-CDODA-Me isomers was inhibited in HT-29 and HCT-15 cells cotreated with the PPARγ antagonist T007, and similar results were observed for α-CDODA-Me in SW480 cells (data not shown). These results demonstrate the tissue-selective induction of caveolin-1 expression by β-CDODA-Me and this is consistent with the activity of α- and β-CDODA-Me isomers as SRMs.

Based on results of preliminary studies on growth inhibitory/proapoptotic genes induced by CDODA-Me isomers, we investigated the induction of the tumor suppressor gene KLF-4 in colon cancer cells. Results in Figure 2.5 A show that 1-5 μM
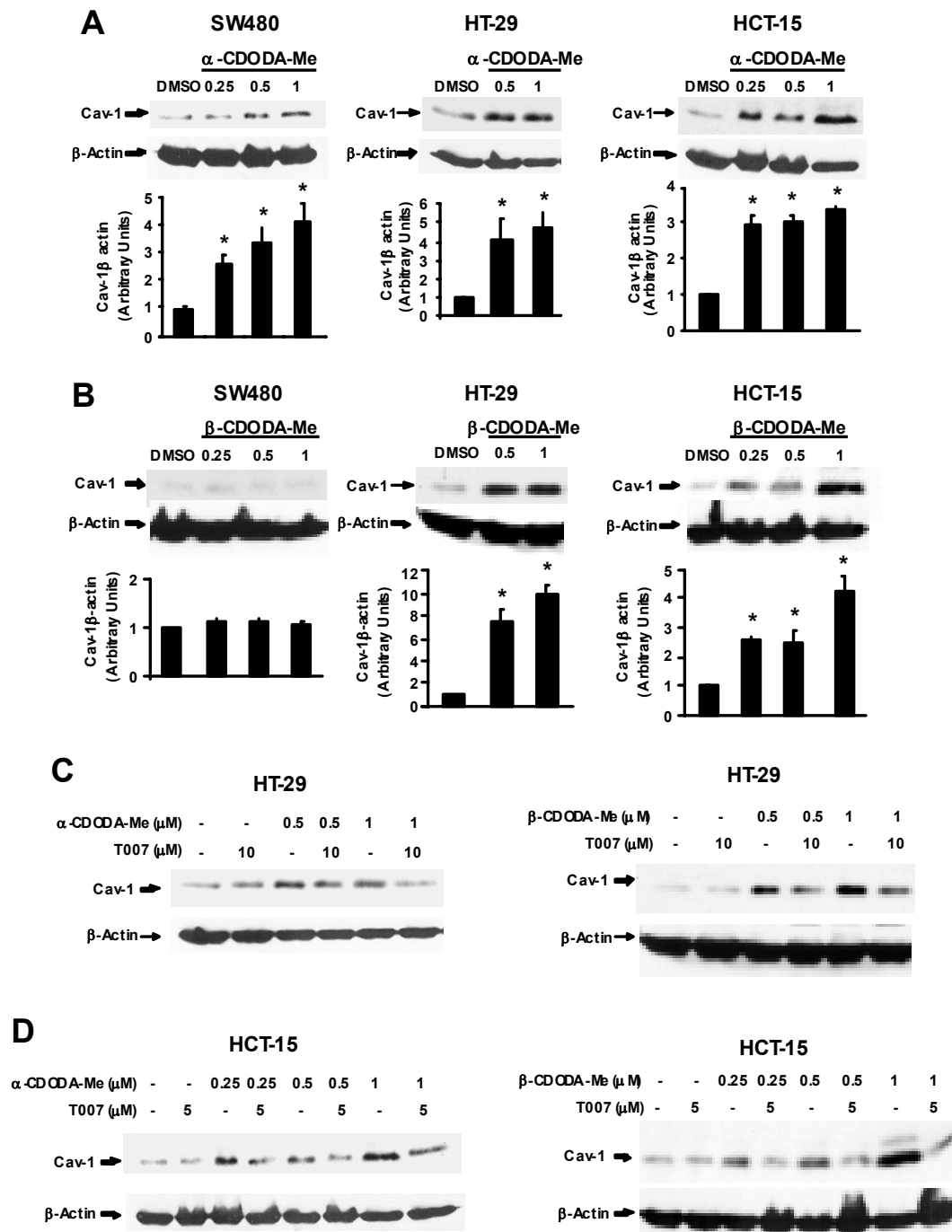
Fig. 2.4. Induction of caveolin-1 in colon cancer cells. SW480, HT-29 or HCT-15 colon cancer cells were treated with α-CDODA-Me (A) or β-CDODA-Me (B) for 72 hr as previously described (302), and whole cell lysates were analyzed by Western immunoblot analysis as described in the Materials and Methods. Inhibition of caveolin-1 induction by α- or β-CDODA-Me by T007 in HT-29 (C) or HCT-15 (D) cells. Cells were treated with 5 μM T007, 0.5 - 1.0 μM α- or β-CDODA-Me or combinations (as indicated) for 72 hr, and whole cell lysates were analyzed by Western immunoblot analysis as described in the Materials and Methods. Similar results were observed for α-CDODA-Me in SW480 cells (data not shown). Caveolin-1 protein expression (relative to β-actin) in the DMSO-treated cells (A and B) was set at 1.0, and significant (p < 0.05) induction is indicated (*). Results are means ± SE for three replicate determinations for each treatment group.

concentrations of both α- and β-CDODA-Me isomers induced KLF-4 mRNA levels in SW480 cells. The PPARγ antagonist T007 (10 μM) alone did not induce KLF-4. In SW480 cells cotreated with T007 and the CDODA-Me isomers, there was a significant inhibition of the induced response. A similar experiment was carried out in HT-29 cells (Fig. 2.5 B), and both CDODA-Me isomers induced KLF-4 mRNA levels which were inhibited after cotreatment with T007. In contrast, α- and β-CDODA-Me isomers did not consistently alter expression of KLF-4 mRNA levels in HCT-15 cells (< 2-fold and variable) (Fig. 2.5 C). These results demonstrate that CDODA-Me isomers exhibited similar activities as PPARγ agonists in HT-29 and SW480 colon cancer cells; however, induction of KLF-4 mRNA was cell context-dependent and, over several experiments, we did not observe significant induction of KLF-4 in HCT-15 cells. These results on the receptor-dependent induction of KLF-4 gene expression by CDODA-Me isomers contrasts to the reported receptor-independent induction of KLF-4 gene expression by the PPARγ agonist 15-deoxy-Δ12,14-prostaglandin J2 (PGJ2) in HT-29 cells (662).

DISCUSSION

PPARγ and other members of the nuclear receptor superfamily are characterized by their modular structure which contains several regions and domains that are required for critical receptor-protein and receptor-DNA interactions (659-661). Nuclear receptors typically contain N- and C- terminal activation functions (AF1 and AF2, respectively), a
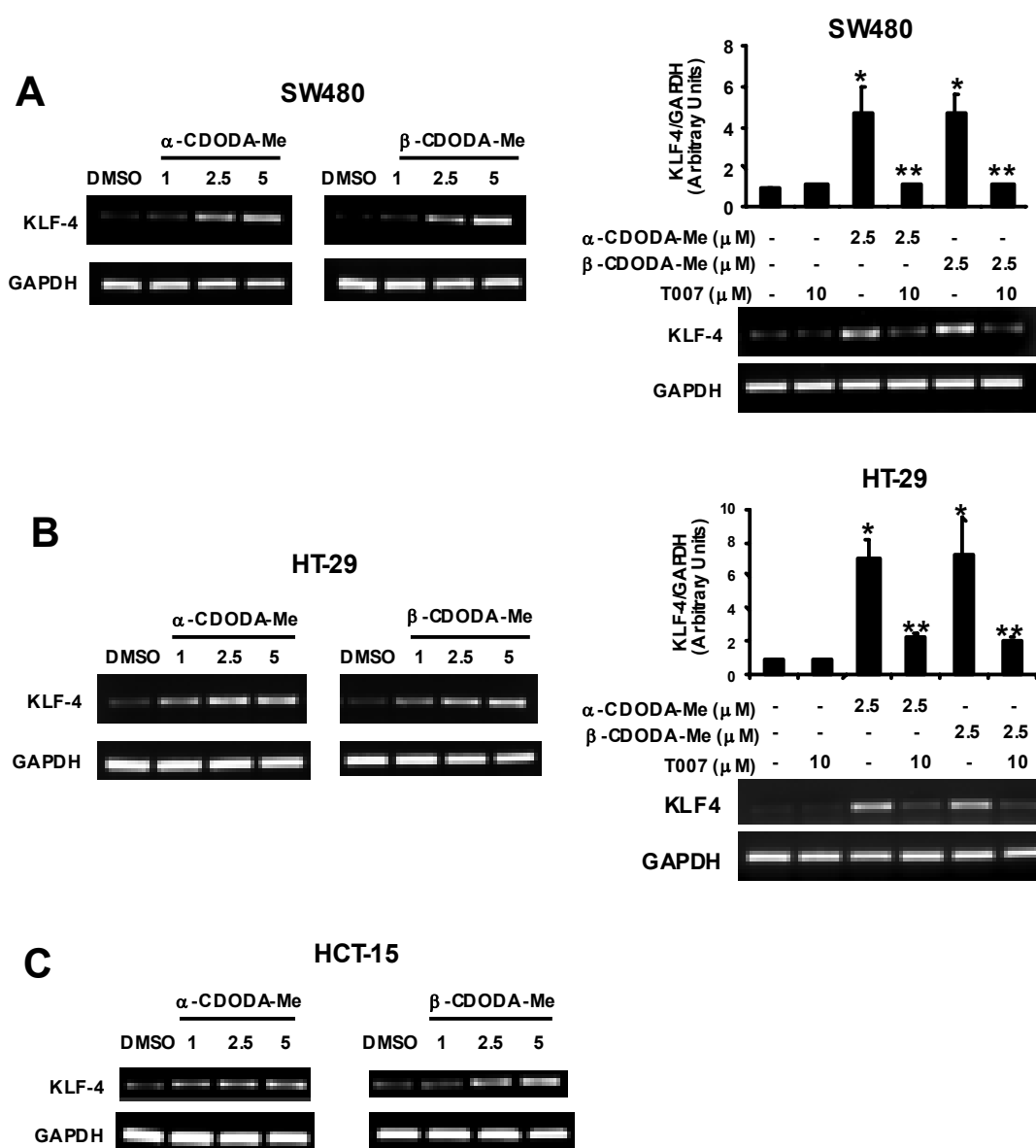
Fig. 2.5. Induction of KLF-4 gene expression by α- and β-CDODA-Me. Induction of KLF-4 in SW480 (A), HT-29 (B) and HCT-15 (C) cells. Cells were treated with different concentrations of CDODA isomers or T007 alone or in combination and KLF-4 mRNA levels were determined by real time PCR as described in the Materials and Methods. Quantitated results(A, B) are means ± SE from three replicate experiments, and significant ($p < 0.05$) induction or inhibition of KLF-4 mRNA levels (*) and inhibition of these responses after cotreatment with T007 (**) are indicated. Induction of KLF-4 in HCT-15 cells was highly variable (< 2-fold) and was not further quantitated

DNA binding domain and a flexible hinge region. The addition of receptor ligand usually results in formation of a transcriptionally active nuclear receptor complex which binds cognate response elements in promoter regions of target genes and activates transcription. However, receptor-mediated transactivation is dependent on several factors including cell context-specific expression of coregulatory proteins (e.g. coactivators), gene promoter accessibility and ligand structure (663). The complex pharmacology of receptor ligands is due, in part, to the ligand structure-dependent conformational changes in the bound receptor complex which may differentially interact with coregulatory factors and exhibit tissue-specific agonist and/or antagonist activity (663, 664). This has led to development of selective receptor modulators (SRMs) for several nuclear receptors which selectively activate or block specific receptor-mediated responses in different tissues/cells.

There is evidence that different structural classes of PPARγ agonists are also SRMs and induce tissue-specific receptor-dependent and independent responses. For example, induction of NAG-1 in HCT116 colon cancer cells by PGJ2 was PPARγ-dependent, whereas both troglitazone and PPARγ-active 1,1-bis(3'-indolyl)-1-(*p*-substitutedphenyl) methanes (C-DIMs) also enhanced NAG-1 expression through receptor-independent pathways in the same cell line (278, 299, 665). Evidence that different structural classes of PPARγ agonists are SRMs has been reported in mammalian two-hybrid assays in which cells have been transfected with VP-PPARγ and GAL4-coactivator constructs. For example, PGJ2 and rosiglitazone differentially

induced coactivator-PPARγ interactions in COS-1 cells (658), and differences in ligand-

dependent coactivator-receptor interactions were also observed for rosiglitazone and

PPARγ-active C-DIMs in colon cancer cells (300).

Previous studies demonstrated that introduction of a cyano group at C-2 of

oleanolic acid or ursolic acid enhanced the cytotoxicity of the resulting synthetic analogs

(464). Moreover, the oleanolic acid derivatives CDDO and CDDO-Me exhibited

PPARγ agonist activities (234, 302, 666). Results of this study also demonstrated that 2-

cyano analogs of the α- and β-GA methyl esters also exhibited increased cytotoxicity

(Fig. 2.1) and PPARγ agonist activity. Similar results were observed for the

corresponding acid derivatives which were less active than α-CDODA-Me or β-

CDODA-Me (data not shown). Thus, introduction of the 2-cyano group into the

oleanolic acid and GA backbone is necessary for their PPARγ agonist activities and

differences in their substitution in the C-ring and the position of carboxymethyl groups

at C-30 (in GA) or C-28 (in oleanolic acid) did not affect PPARγ agonist activity. GA

and oleanolic acid are 18β- and 18α-isomers, respectively (e.g. Fig. 1.18), and their

different sterochemistries at C-18 results in conformational differences in the E-ring of

these triterpenoids. Therefore, in order to directly compare the effects of different E-ring

conformations on cytotoxicity and PPARγ agonist activity, we investigated the

comparative effects of α- and β-CDODA-Me. Both isomers exhibited similar

cytoxtoxicities and PPARγ agonist activities, suggesting that the sterochemical

differences at C-18 do not affect PPARγ-dependent transactivation in reporter gene

assays (Fig. 2.2), indicating that the PPARγ agonist activity in this assay was primarily

governed by the 2-cyano substituents.

However, results of the mammalian two-hybrid assay (Fig. 2.3) show that α-

CDODA-Me induces interactions between PGC-1 and TIFII (SRC-2), whereas β-

CDODA-Me induces interactions between PGC-1 and SRC-1. These differences must

be due to the unique conformations of the E-ring of these isomeric triterpenoids which is

dependent on the different sterochemistries (α and β) at C-18 located at the E/D ring

junction (Fig. 1.18). The mammalian two-hybrid assay uses the GAL4-coactivator

chimeras as probes for investigating differences in ligand-dependent conformational

changes in PPARγ. These results do not necessarily identify which coactivators are

important for activation of PPARγ since this will also depend on tissue-specific

expression of coactivators and other important coregulatory proteins. However, data

from the two-hybrid assay suggest that, like other structural classes of PPARγ agonists,

α-CDODA-Me and β-CDODA-Me are selective receptor modulators and this selectivity

was further investigated using induction of caveolin-1 and KLF-4 as end-points. Both

caveolin-1 and KLF-4 were selected as potential PPARγ-dependent responses based on

results of previous studies showing that both genes are induced by one or more structural

classes of PPARγ agonists (302, 667-669). Caveolin-1 expression in colon cancer and

some other cancer cell lines is associated with reduced rates of cancer cell proliferation

and anchorage-independent growth (670-673). KLF-4 is a member of the Sp/KLF

family of zinc finger transcription factors (674, 675), and KLF-4 expression is also

correlated with tumor/cancer cell growth inhibition in gastric and colon cancer

suggesting that KLF-4 acts as a tumor suppressor gene (676-679). Previous studies have

shown that caveolin-1 is induced by thiazolidinediones, CDDO/CDDO-Me, and 1,1-

bis(3'-indolyl)-1-(p-substitutedphenyl)methanes (C-DIMs) in HT-29 and other colon

cancer cell lines. However, PPARγ-active C-DIMs, but not rosiglitazone, induced

caveolin-1 in HCT-15 cells (300, 302) and this was related, in part, to a mutation in

PPARγ expressed in the HCT-15 cell line. The differences in caveolin-1 induction

between the two structurally unrelated PPARγ agonists in HCT-15 cells is an example of

the SRM activity of different structural classes of PPARγ agonists. We also observed

cell-specificity differences between α-CDODA-Me and β-CDODA-Me with respect to

their induction of caveolin-1 in colon cancer cells (Fig. 2.4). Although α- and β-

CDODA-Me induced caveolin-1 in HT-29 and HCT-15 cells, only the former isomer

induced this response in SW480 cells and this was observed in replicate experiments.

Since α-CDODA-Me and CDDO-Me contain the 18α configuration and both

compounds also induce caveolin-1 (Fig. 2.4) (302), this suggests that differences in

caveolin-1 induction by α- and β-CDODA-Me are due to their different E-ring

conformations (Fig. 1.18) which also affects ligand-induced PPARγ-coactivator

interactions (Fig. 2.3).

A previous report (680) showed that KLF-4 induction by PGJ2 was PPARγ-

independent and this response was used as a model to investigate mechanistic

differences in KLF-4 induction by α and β-CDODA-Me and PGJ2. α- and β-CDODA-

Me induce KLF-4 mRNA levels in HT-29 and SW480 cells (Fig. 2.5A and 2.5B), and

cotreatment of these cells with the PPARγ antagonist T007 inhibits induction of KLF-4. Similar results were observed for induction of KLF-4 protein (data not shown) demonstrating receptor-dependent (α- and β-CDODA-Me) and receptor-independent (PGJ2) induction of KLF-4 in HT-29 cells and that the two different structural classes of PPARγ agonists exhibit SRM-like activity.

Results of this study demonstrate for the first time that introduction of 2-cyano substituents into the A ring of α- and β-GA significantly enhances their cytotoxicity and is necessary for their activity as PPARγ agonists. This represents an important extension of the potential therapeutic applications of synthetic analogs of GA, a major component of licorice extracts. In addition, we also demonstrate that both α- and β-CDODA-Me are SRMs based on their tissue-selective induction of caveolin-1 and KLF-4 in colon cancer cells. These differences in activity are consistent with their structure-dependent induction of PPARγ interactions with different coactivators in SW480 cells. Thus, synthetic analogs of GA exhibit potent anticancer activity in colon cancer cells and mechanisms of their induction of KLF-4 and other receptor-dependent and -independent responses and *in vivo* applications of these compounds as a new class of anticancer drugs is currently being investigated.

CHAPTER III

METHYL 2-CYANO-3,11-DIOXO-18 β-OLEAN-1,12-DIEN-30-OATE IS A PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ AGONIST THAT INDUCES RECEPTOR-INDEPENDENT APOPTOSIS IN LNCaP PROSTATE CANCER CELLS*

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are a sub-family of the nuclear receptor superfamily of ligand-activated receptors (681, 682). The three members of this family, PPARα, PPARγ and PPARβ/σ are lipid sensors and play a key role in regulating tissue-specific lipid homeostasis and metabolism (683). PPARs also play an important role in many diseases particularly those related to obesity, metabolic disorders, cancer and atherogenesis (659, 684, 685). Endogenous ligands for PPARs include fatty acid derived compounds and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (PGJ2) which exhibits high affinity for PPARγ; however, PGJ2 may not be the endogenous ligand for this receptor due to the low cellular expression of this metabolite. Synthetic PPARγ agonists, such as the thiazolidinediones (TZDs) rosiglitazone and pioglitazone, are insulin-sensitizing drugs that are widely used for clinical treatment of type II diabetes. Several different structural classes of PPARγ agonists have been characterized and these include flavones,

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*Reprinted with permission from "Methyl 2-cyano-3,11-dioxo-18 β-olean-1,12-dien-30-oate is a peroxisome proliferator-activated receptor γ agonist that induces receptor-independent apoptosis in Lncap prostate cancer cells" by Papineni S, Chintharlapalli S, Safe S. Mol Pharmacol 2008;73:553-65. Copyright 2008 by American Society for Pharmacology and Experimental Therapeutics.

 various indole derivatives, chromane carboxylic acids, phosphonophosphates, and triterpenoids such as 2-cyano-3,12-dioxo-17α-olean-1,9-dien-28-oic acid (CDDO) and related compounds (281, 463, 482, 686-691). PPARγ is overexpressed in many tumor types and cancer cell lines (283) and PPARγ agonists show promise for the clinical treatment of various types of tumors (242, 253, 692-694). Ligands for this receptor typically inhibit G_0/G_1 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. Research from our laboratory has identified a series of 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methanes [methylene-substituted diindolyl-methanes (C-DIMs)] which inhibit cancer cell and tumor growth (281, 296, 297, 299, 300, 313, 695) (301)through both receptor-dependent and independent pathways and similar observations have been reported for other PPARγ agonists (262, 696-699).

A new class of synthetic PPARγ agonists has been derived from glycyrrhetinic acid (GA), a major triterpenoid acid found in licorice extracts. Methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (β-CDODA-Me) is a 2-cyano derivative of GA and has the same oleanolic acid pentacyclic triterpene backbone structure as CDDO which is also a 2-cyano derivative of oleanolic acid (482). However, there are major structural differences between CDODA and CDDO with respect to the position of the carboxylic acid group in the E ring, the position of the double bonds and keto group in the C-ring. We recently reported that the 18α and 18β isomers of CDODA-Me activate PPARγ in colon cancer cells and induced both caveolin-1 and Krüppel-like Factor-4 (KLF4) through receptor-dependent pathways (312). In this study, we have investigated the

effects of β-CDODA-Me on the proliferation of LNCaP prostate cancer cells and the

IC_{50} value for growth inhibition was approximately 1 μM. In contrast to studies in colon

cancer cells, β-CDODA-Me had minimal effects on caveolin-1 or KLF4 expression in

LNCaP cells. The proapototic and growth inhibitory effects of β-CDODA-Me in

LNCaP cells were associated with induction of p21 and p27 expression, downregulation

of cyclin D1, and induction of NAG-1. β-CDODA-Me also decreased androgen

receptor (AR) and prostate specific antigen (PSA) protein and RNA expression and all of

these responses were PPARγ-independent. Thus, β-CDODA-Me, a PPARγ agonist,

inhibited growth and induced apoptosis in LNCaP cells through activation of multiple

receptor-independent pathways including ablation of AR gene expression.

MATERIALS AND METHODS

Cell lines

LNCaP human prostate carcinoma cells were obtained from American Type

Culture Collection (Manassas, VA). Fetal bovine serum was obtained from JRH

Biosciences, Lenexa, KS. LNCaP cells were maintained in RPMI 1640 (Sigma

Chemical, St. Louis, MO) supplemented with 0.22% sodium bicarbonate, 0.011%

sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 mL/L of 100X

antibiotic/antimycotic solution (Sigma). Cells were maintained at 37°C in the presence

of 5% CO_2.

Antibodies and reagents

Antibodies for poly(ADP-ribose) polymerase, cyclin D1, p27, FKBP51, AR,

ATF3, Akt and caveolin-1 were purchased from Santa Cruz Biotechnology, Inc. (Santa

Cruz, CA). PSA was obtained from Dako Denmark A/S (Glostrup, Denmark); NAG-1 was purchased from Upstate Biotechnology (Charlottesville, VA); and EGR-1, pAKT, pERK, ERK, pJNK, JNK were obtained from Cell Signaling Technology Inc. (Danvers, MA). Monoclonal β-actin antibody and dihydrotesterone were purchased from Sigma-Aldrich. Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA), and lipofectamine reagents were supplied by Invitrogen (Carlsbad, CA). Western blotting chemiluminescence reagents were from Perkin-Elmer Life Sciences (Boston, MA). The PPARγ antagonist N-(4'-aminopyridyl)-2-chloro-5-nitrobenzamide (T007) was prepared in this laboratory and the synthesis of the GA derivatives has been previously described (312).

Cell proliferation and DNA fragmentation assays

LNCaP prostate cancer cells (2×10^4 per well) were added to 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM/Ham's F-12 media containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or the indicated C-DIMs were added. Fresh medium and indicated compounds were added every 48 hr, and cells were then trypsinized and counted after 2, 4, and 6 days using a Coulter Z1 cell counter (Beckman Coulter, Fullerton, CA). Each experiment was done in triplicate, and results are expressed as means ± S.E. for each set of three experiments. The DNA fragmentation assay was performed using a BioVision Apoptotic DNA ladder extraction kit (BioVision, Mountain View, CA) according to the manufacturer's protocol.

Transfections

The Gal4 reporter construct containing 5X Gal4 response elements (pGal4) was kindly provided by Dr. Marty Mayo (University of North Carolina, Chapel Hill, NC). The Gal4DBD-PPARγ construct was a gift of Dr. Jennifer L. Oberfield (Glaxo Wellcome Research and Development, Research Triangle Park, NC). The PPRE-luc construct contains three tandem PPREs with a minimal TATA sequence linked to the luciferase gene in pGL2. The AR-luc construct containing the -5400 to +580 region of the androgen receptor promoter was provided by Dr. Donald J. Tindall (Mayo Clinic, Rochester, MN), and the PSA-luc construct containing the 5.8-kilobase region of the PSA promoter was provided by Dr. Hong-Wu Cheng (University of California, Davis, CA). LNCaP cells (1×10^5) were seeded in 12-well plates in DMEM/Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS and grown overnight. Transient transfections were performed using Lipofectamine reagent (Invitrogen) according to the protocol provided by the manufacturer. Transfection studies were performed using 0.4 μg of Gal4Luc, 0.04 μg of β-galactosidase, 0.04 μg of Gal4DBD-PPARγ, 0.4 μg of AR-luc, and 0.3 μg of PSA-luc. Six hr after transfection, the transfection mix was replaced with complete media containing either vehicle (DMSO) or the indicated ligand for 20 to 22 hr. Cells were then lysed with 100 μl of 1 x reporter lysis buffer, and 30 μl of cell extract was used for luciferase and β-galactosidase assays. A Lumicount luminometer (PerkinElmer Life and Analytical Sciences) was used to quantify luciferase and β-galactosidase activities, and the luciferase activities were normalized to β-galactosidase activity.

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 μl of RNasefree 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 LNCaP cell line 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-μl 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 15 s and 60°C 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. Values for each gene were normalized to expression levels of TATA-binding protein. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of the primers used for reverse transcription-PCR were as follows: AR forward, 5'-GTA CCC TGG CGG CAT GGT-3' and AR reverse, 5'-CCC ATT TCG CTT TTG ACA CA-3'; PSA forward, 5'-GCA TTG AAC CAG AGG AGT TCT TG-3' and PSA reverse, 5'-TTG CGC ACA CAC GTC ATT G-3'; and TATA-binding protein forward, 5'-TGC ACA GGA GCC AAG AGT GAA-3' and reverse, 5'-CAC ATC ACA GCT CCC CAC CA-3'.

Western blot analysis

Cells were seeded in DMEM:Ham's F-12 media containing 2.5% charcoal-stripped FBS for 24 hr and then treated with either the vehicle (DMSO) or the indicated compounds. Cells were collected by scraping in 150 µl high salt lysis buffer (50 mM HEPES, 0.5 M NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton-X-100 and 5 µL/ml of Protease Inhibitor Cocktail (Sigma). The lysates were incubated on ice for 1 hr with intermittent vortexing followed by centrifugation at 20,000 g for 10 min at 4°C. Before electrophoresis, samples were boiled for 3 min at 100°C; the amount of protein was determined and 60 µg protein applied per lane. Samples were subjected to SDS-PAGE on 10% gel at 120 V for 3 to 4 hr. Proteins were transferred on to polyvinylidene difluoride membrane (PVDF; Bio-Rad, Hercules, CA) at 0.9 amp for 90 min at 4°C in 1x transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 0.025% SDS). The membranes were blocked for 30 min with 5% TBST-Blotto (10 mM Tris-HCl, 150 mM NaCl (pH 8.0), 0.05% Triton X-100 and 5% non-fat dry milk) and incubated in fresh 5% TBST-Blotto with 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 2-3 hr. The membrane was washed with TBST for 10 min and incubated with 10 ml of chemiluminiscence substrate (PerkinElmer Life Sciences) for 1.0 min and exposed to ImageTeK-H medical imaging film (Eastman American X-ray Supply, Inc.).

Statistical analysis

Statistical differences between different groups were determined by ANOVA and Scheffe's test for significance. The data are presented as mean ± S.E. for at least three separate determinations for each treatment group.

RESULTS

Cell proliferation and activation of PPARγ

β-DODA or 1, 2-dehydro-GA exhibited minimal inhibition of LNCaP cell growth with a IC_{50} value > 15 μM whereas the IC_{50} for the corresponding methyl ester derivative was between 10-15 μM (Fig. 3.1). Introduction of a 2-cyano group to give β-CDODA-Me increased the cytotoxicity by at least an order of magnitude and the IC_{50} was approximately 1 μM in LNCaP cells (Fig. 3.1A). These results were similar to those observed in colon cancer cells (312) and demonstrate the importance of 2-cyano substituents in mediating the cytotoxicity of GA derivatives. The induction of PPARγ-dependent transactivation by β-CDODA-Me was also investigated in LNCaP cells transfected with PPARγ-GAL4/GAL4-Luc or $PPRE_3$-Luc constructs and treated with 1-5 μM concentrations. β-CDODA-Me significantly induced luciferase activity (Fig. 3.1B) and in cells cotreated with β-CDODA-Me plus 10 μM T007 (a PPARγ antagonist), there was significant inhibition of induced transactivation. In contrast, β-DODA-Me did not activate PPARγ (data not shown) demonstrating the requirement for the 2-cyano substituent to confer PPARγ agonist activity on the GA derivative. PPARγ agonists typically modulate expression of one or more of the cell cycle proteins p27, p21
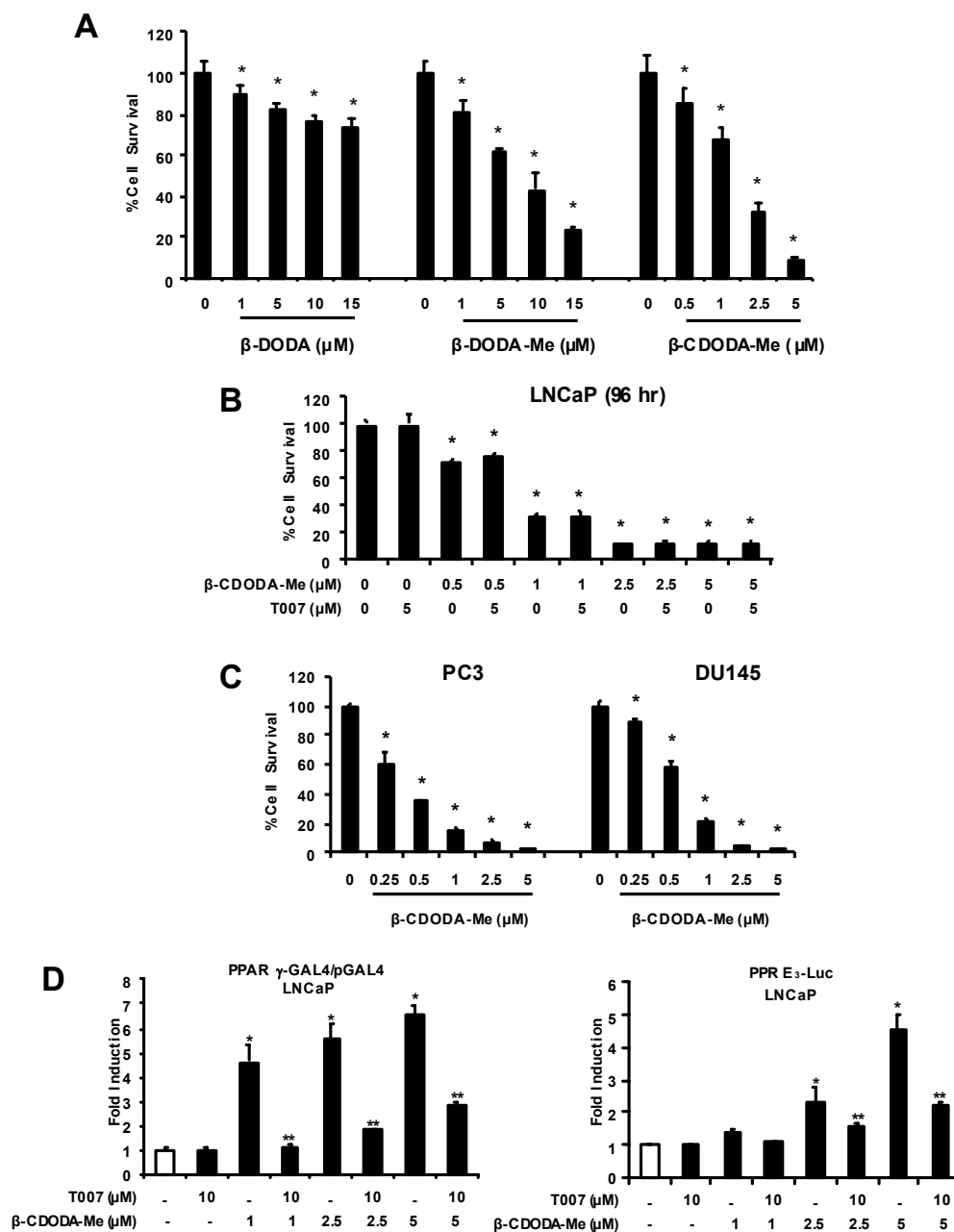
Fig. 3.1. Effects of β-CDODA-Me and related compounds on LNCaP cell survival and activation of PPAR. Cell survival of LNCaP (A and B), PC3, and DU145 (C) cells. Prostate cancer cells were treated with different concentrations of β-DODA, β-DODA-Me, or β-CDODA-Me alone or in combination with 5 μM T007 (C) for 96 h, and the percentage of cell survival relative to DMSO (solvent control set at 100%) was determined as described under Materials and Methods. Results are expressed as means ± S.E. for three separate determinations for each treatment group, and significantly ($p < 0.05$) decreased survival is indicated ([*]). D, β-CDODA-Me activates PPAR. LNCaP cells were treated with β-CDODA, T007, or their combination, transfected with PPAR-GAL4/pGAL4 or PPRE-luc, and luciferase activity determined as described under Materials and Methods. Results are expressed as means ± S.E. for three replicate determinations for each treatment group, and significant ($p < 0.05$) induction by β-CDODA-Me ([*]) and inhibition after cotreatment with T007 ([**]) are indicated.

and cyclin D1, and Figure 3.1C illustrates the effects of 1-5 µM β-CDODA-Me on expression of these proteins in LNCaP cells. There was a concentration-dependent induction of p27 and p21 and a decrease in cyclin D1 proteins and Rb phosphorylation in cells treated with β-CDODA-Me alone, and similar results were observed in cells cotreated with the PPARγ antagonist T007 and β-CDODA-Me (Fig. 3.1 D) suggesting that these responses were PPARγ-independent.

Induction of proapoptotic responses by β-CDODA-Me

NAG-1 and ATF-3 are proapoptotic proteins induced by PPARγ agonists and results in Figure 3.2 A show that 1-5 µM β-CDODA-Me induced NAG-1 and ATF-3 which are often co-induced and this was accompanied by caspase-dependent PARP cleavage, DNA fragmentation, and decreased bcl2 expression in LNCaP cells. In LNCaP cells cotreated with β-CDODA-Me plus T007 (Fig. 3.2 B), the induced responses were not inhibited by the PPARγ antagonist indicating that induction of these proapototic responses was receptor-independent. Previous studies show that different structural classes of PPARγ agonists downregulate AR expression in LNCaP cells and this response can also result in activation of apoptosis (695, 699). Figure 2C summarizes the effects of β-CDODA-Me on AR expression in the presence or absence of 10 nM DHT and also on the expression of FKBP51 and PSA, two androgen-responsive genes in LNCaP cells. DHT increases expression of AR due to stabilization
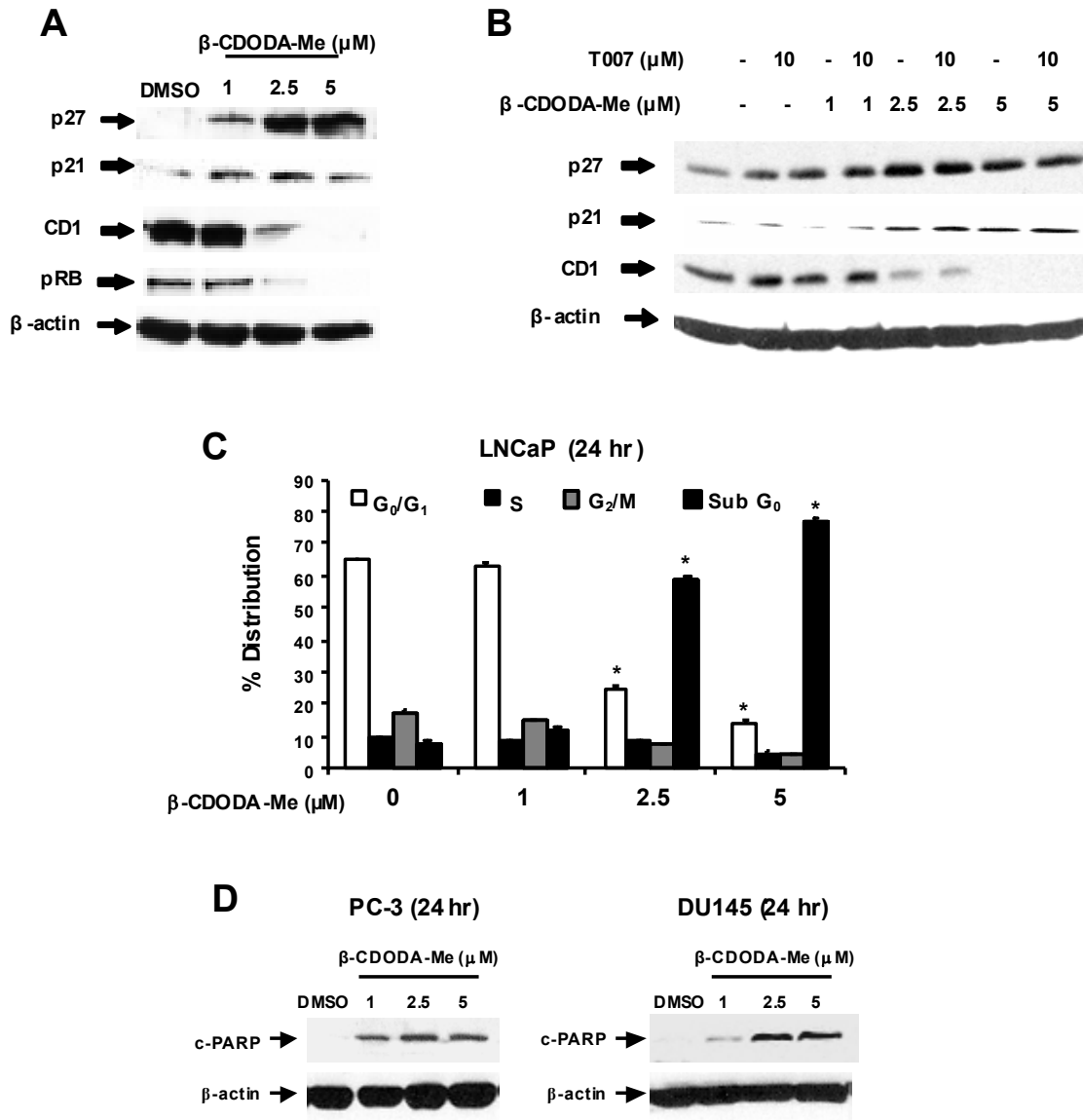
Fig. 3.2. β-CDODA-Me modulates the cell cycle and cell cycle genes and induces apoptosis in prostate cancer cells. Modulation of cell cycle genes by β-CDODA-Me alone (A) and in combination with T007 (B). Cells were treated as indicated for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described under Materials and Methods. C, cell cycle progression. LNCaP cells were treated with DMSO and different concentrations of β-CDODA-Me for 24 h and analyzed for the percentage of distribution of cells in different phases of the cell cycle by FACS analysis as described under Materials and Methods. Results are expressed at means ± S.E. for three replicate determinations, and significant ($p < 0.05$) changes (compared with DMSO group) are indicated by an asterisk. D, induction of PARP cleavage. PC3 and DU145 cells were treated for 24 h with different concentrations of β-CDODA-Me, and whole-cell lysates were analyzed by Western blots as described under Materials and Methods

of the receiver and also induces both androgen-responsive FKBP51 and PSA genes and, in cells treated with 1-5 μM β-CDODA-Me, there was a concentration-dependent decrease in AR, PSA and FKBP51 expression in the presence or absence of DHT. In addition, downregulation of AR, PSA and FKBP51 proteins in LNCaP cells treated with β-CDODA-Me was not affected by cotreatment with the PPARγ antagonist T007 (Fig. 3.2D) or the proteasome inhibitor MG132 (Fig. 3.2D). In contrast, β-CDODA-Me-dependent degradation of cyclin D1 was inhibited after cotreatment with MG132 and these observations are similar to those reported for other PPARγ agonists that induce proteasome-dependent degradation of cyclin D1 (300-302). These results clearly show that β-CDODA-Me decreases expression of androgen-responsive genes and AR through PPARγ-independent pathways. The downregulation of AR in cells treated with β-CDODA-Me is consistent with the induction of apoptosis by this compound since decreased AR expression by small inhibitory RNAs in LNCaP cells also induces apoptosis (700).

β-CDODA-Me induces kinase-dependent activation of proapoptotic/growth inhibitory pathways

Previous studies show that NAG-1 is induced by some PPARγ agonists and other cytotoxic compounds in colon cancer cells (278, 299, 313, 442, 665) through PI3K-dependent activation of EGR-1 which acts as a *trans*-acting factor to induce NAG-1 expression. Figure 3.3A summarizes the time-dependent induction of EGR-1, ATF-3 and NAG-1 by 2.5 μM β-CDODA-Me and the induction responses followed a similar time course, whereas EGR-1 dependent induction of NAG-1 in colon cancer cells is

associated with the increased expression of EGR-1 prior to induction of NAG-1 (299,

442). Previous studies show that NAG-1 induction is kinase-dependent (299, 442), and

results in Figure 3.3B show that 2.5 μM β-CDODA-Me induces activation of the JNK

(p-JNK), PI3K (p-Akt) and MAPK (p-Erk) pathways. Maximal activation of JNK and

PI3K was observed after 8 and 8-12 hr, respectively, whereas p-Erk activation remained

elevated for 24 hr. The effects of inhibitors of MAPK (PD98059), PI3K (LY294002),

protein kinase C (GF109203X) and JNK (SP600125) on induction of NAG-1 and ATF3

and decreased expression of AR, PSA and FKBP51 was also investigated in LNCaP

cells treated with 2.5 μM β-CDODA-Me (Fig. 3.3C). Both PD98059 and LY294002

inhibited induction of NAG-1 by β-CDODA-Me. However, the JNK inhibitor

SP600125 was the most potent inhibitor of ATF-3 induction (Figs. 3.3C and 3.3D). In

contrast, decreased expression of AR, PSA and FKBP51 in LNCaP cells treated with β-

CDODA-Me was unaffected by kinase inhibitors. These results suggest that the

underlying pathways associated with the growth inhibitory/proapoptotic pathways

induced by β-CDODA-Me in LNCaP cells are due in part to activation of kinases.

Therefore, the effects of kinase inhibitors on modulation of cell cycle proteins

 by β-CDODA-Me were also investigated and the downregulation of cyclin D1 and

induction of p21 were partially blocked in cells cotreated with the MAPK inhibitor

PD98059 (Fig. 3.4A), and MAPK-dependent activation of p21 has previously been

observed in embryonal rhabdomyosarcoma cell lines treated with TPA (701). Results in

Figure 4B show that the 1-5 μM β-CDODA-Me also induces luciferase activity in

LNCaP cells transfected with constructs containing -2325 to +8 [p21-Luc (Fl)], -124 to
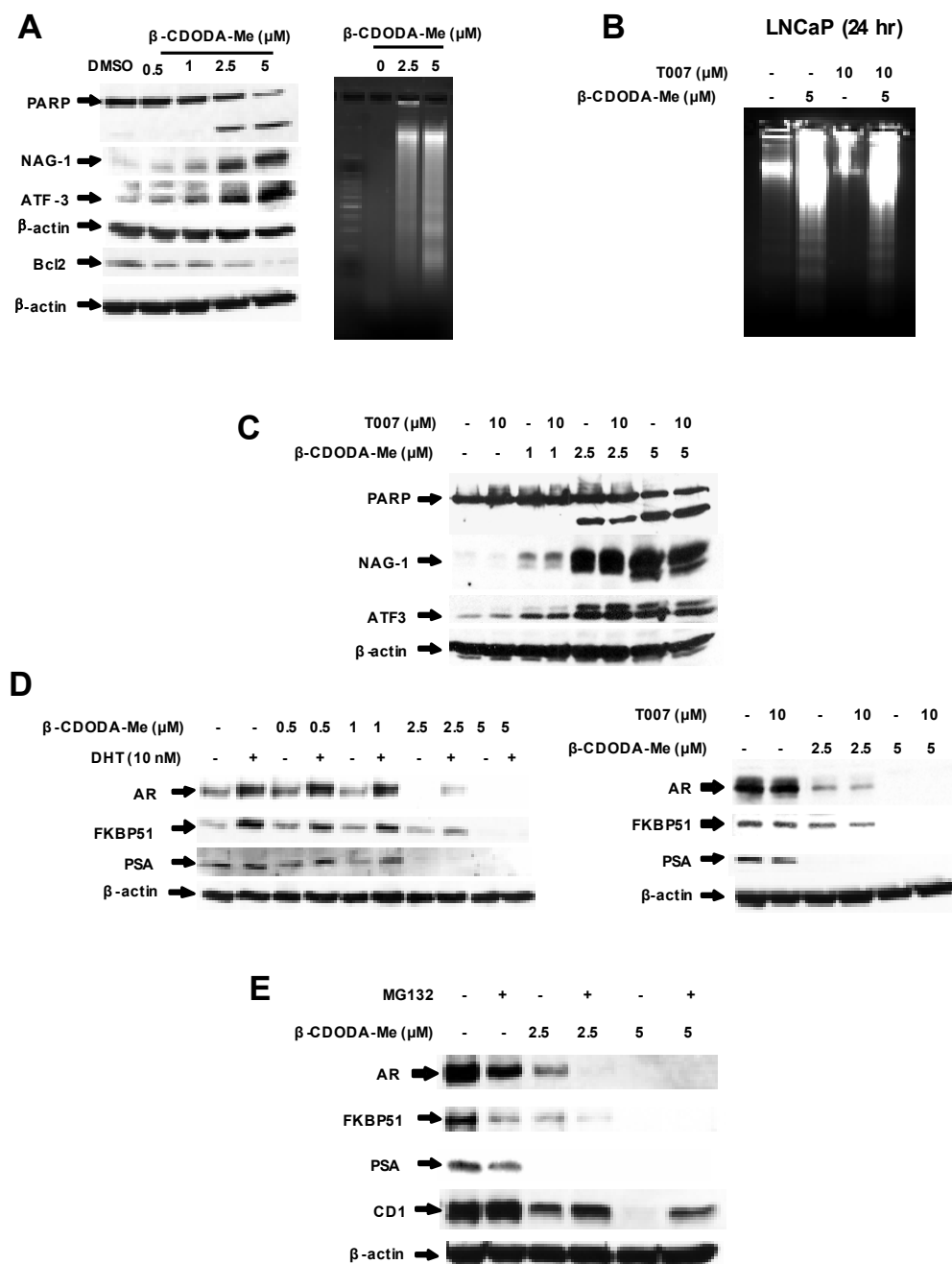
Fig. 3.3. β-CDODA induces apoptotic pathways and decreases androgen-responsiveness in LNCaP cells. β-CDODA-Me alone (A) and in combination with T007 (B and C) induces proapoptotic pathways. LNCaP cells were treated as indicated for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described under Materials and Methods. β-CDODA-Me-induced DNA fragmentation (A and B) was also determined as described. Effects of β-CDODA-Me alone and in combination with DHT or T007 (D) or MG132 (E) on AR and androgen-responsive proteins. LNCaP cells were treated with DMSO or the various compounds for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described under Materials and Methods.

+8 [p21-Luc (-124)], -101 to +8 [p21-Luc (-101)], and -60 to +8 [p21-Luc (-60)] p21 promoter inserts. The latter 3 constructs contain the 6 proximal GC rich site (VI - I) and the results of the transfection studies suggest that these GC-rich sites are necessary for β-CDODA-Me-induced transactivation. Deletion analysis of the p21 promoter indicates that loss of inducibility [i.e. p21-luc(60)] is associated with loss of GC-rich sites IV and III which are essential for MAPK-dependent activation of p21 by

β-CDODA-Me. The role of MAPK in activation of the p21 promoter was confirmed in LNCaP cells transfected with p21-luc (101); β-CDODA-Me induced luciferase activity and cotreatment with the MAPK inhibitor PD98059 inhibited this response (Fig. 3.4C). These results show that the induction of p21 and the proapototic NAG-1 protein by β-CDODA-Me were related to the activation of MAPK and PI3K but were independent of PPARγ (Figs. 3.1 D and 3.2 B).

β-CDODA-Me differentially decreases AR and PSA gene expression in LNCaP cells

β-CDODA-Me decreases expression of AR, PSA and FKBP51 protein levels through proteasome and PPARγ-independent pathways (Fig 3.2 C-3.2 E) and these responses are also not modulated by kinase inhibitors (Fig. 3.3 B). The results in Figure 3.5 A show that β-CDODA-Me also decreases AR mRNA levels after treatment for 12 and 18 hr, and cotreatment with the PPARγ antagonist T007 did not affect mRNA levels confirming the β-CDODA-Me-induced downregulation of AR mRNA levels was also PPARγ-independent. Similar results were obtained in LNCaP cells treated with β-
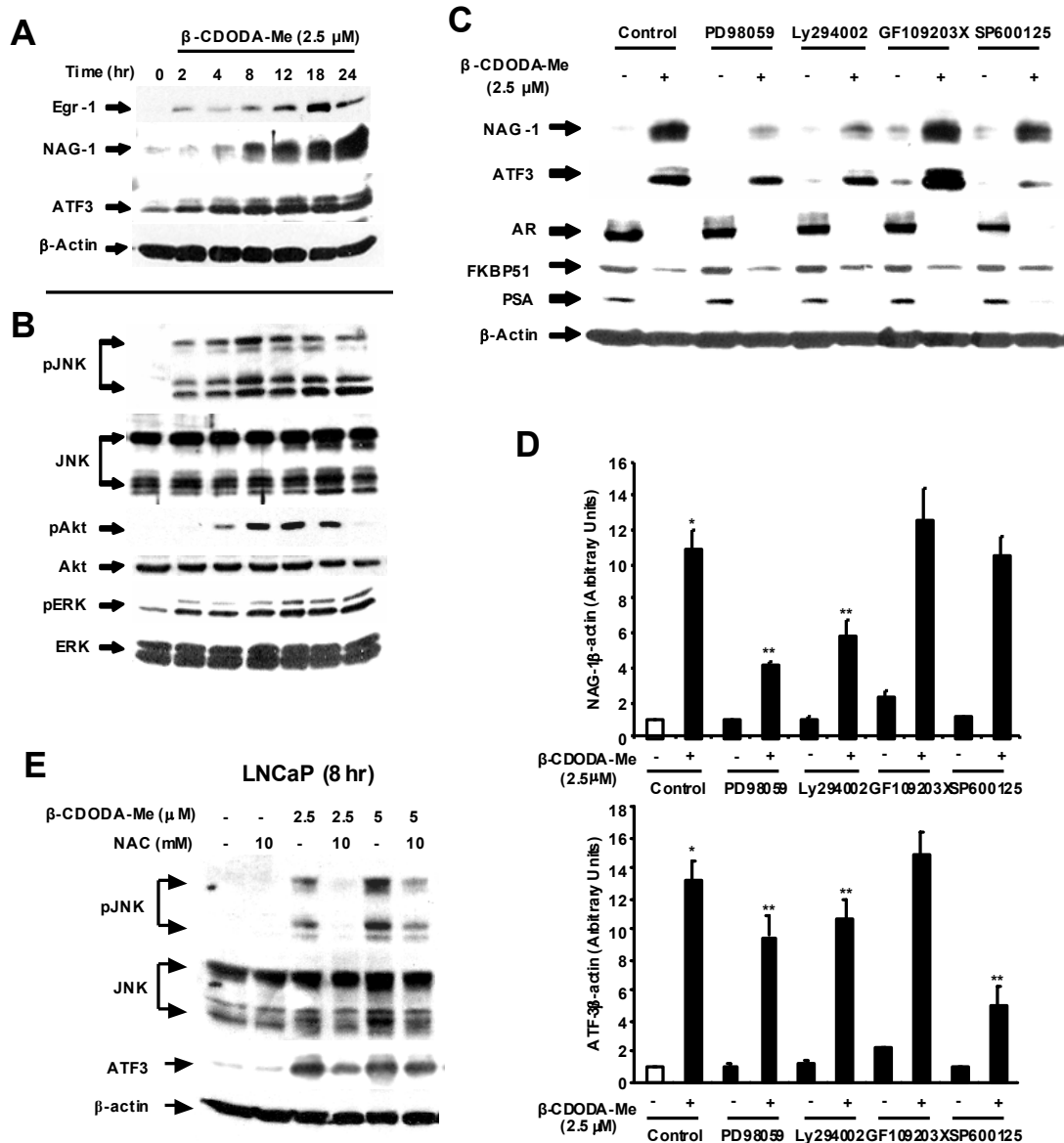
Fig. 3.4. β-CDODA-Me induces proapoptotic proteins and kinases. Induction of NAG-1, ATF3, and Egr-1 (A) and kinases (B) by β-CDODA-Me. LNCaP cells were treated with 2.5 μM β-CDODA-Me, and whole-cell lysates isolated at different times after treatment were analyzed by Western blot analysis as described under *Materials and Methods*. Effects of kinase inhibitors on proapoptotic responses (C) and quantitation of NAG-1 and ATF3 expression (D). LNCaP cells were treated with 2.5 μM β-CDODA alone or in combination with various kinase inhibitors, and after 24 h, whole-cell lysates were analyzed by Western blot analysis. Levels of NAG-1 and ATF3 proteins (normalized to β-actin) (D) are means ± S.E. for three separate determinations for each treatment group, and significantly ($p < 0.05$) decreased levels after cotreatment with a kinase inhibitor are indicated ([**]).
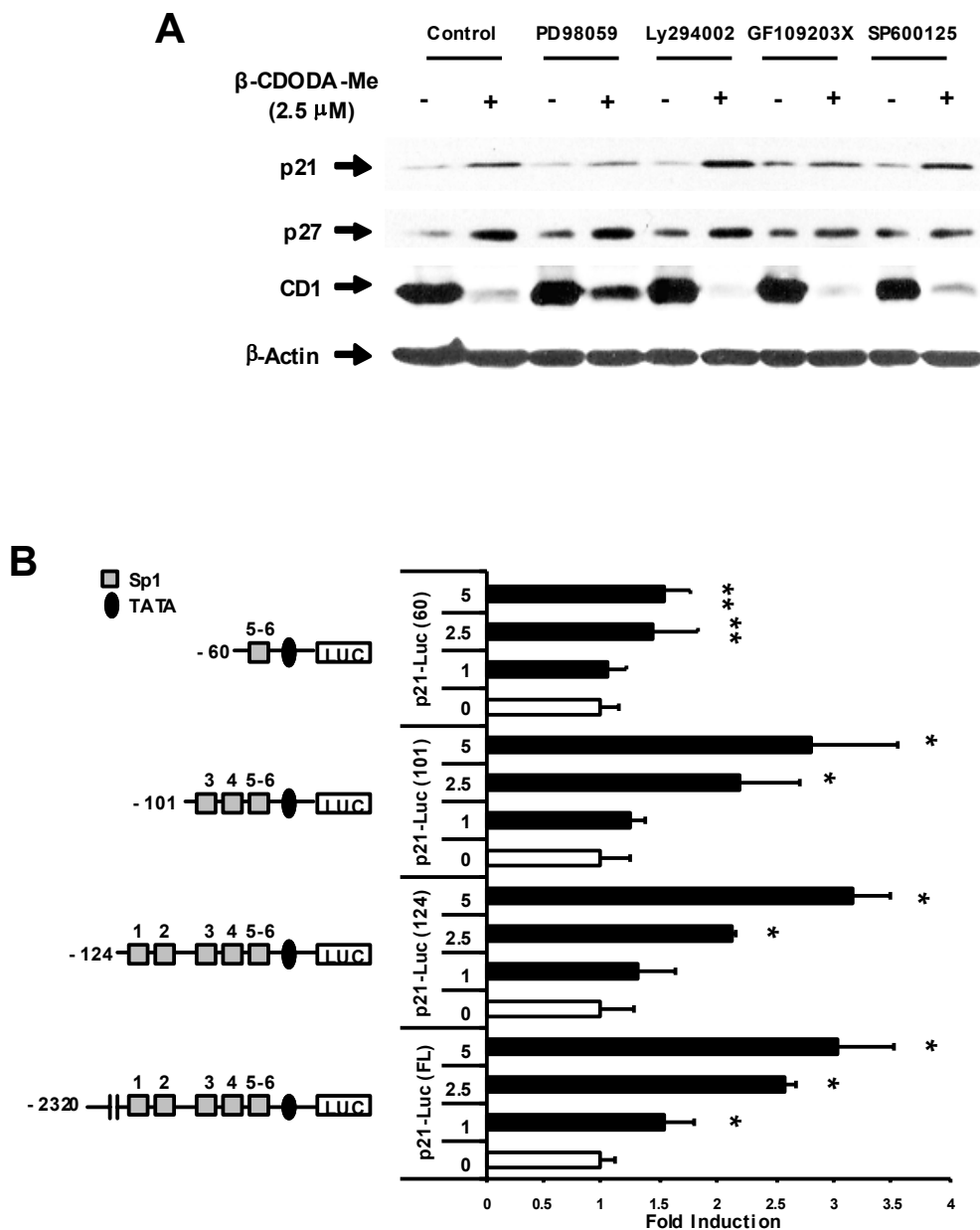
Fig. 3.5. β-CDODA-Me induction of p21 is MAPK-dependent. A, effects of kinase inhibitors on induction of p21. LNCaP cells were treated with DMSO, 2.5 μM β-CDODA-Me alone or in combination with kinase inhibitors for 24 h, and whole-cell lysates were analyzed by Western blot analysis as described under Materials and Methods. B, β-CDODA-Me activates p21 promoter constructs. LNCaP cells were transfected with p21 promoter constructs, treated with DMSO or different concentrations of β-CDODA-Me, and luciferase activity was determined as described under Materials and Methods. Results are means ± S.E. for three separate determinations for each treatment group, and significant (*p* < 0.05) induction of activity is indicated (*). C, inhibition by PD98059. Cells were transfected with p21-luc(101), treated with DMSO or β-CDODA-Me alone or in combination with 10 μM PD98059. Results are expressed as means ± S.E. for three separate determinations for each treatment group, and significant (*p* < 0.05) induction by β-CDODA-Me (*) and inhibition after cotreatment with PD98059 (**) are indicated.
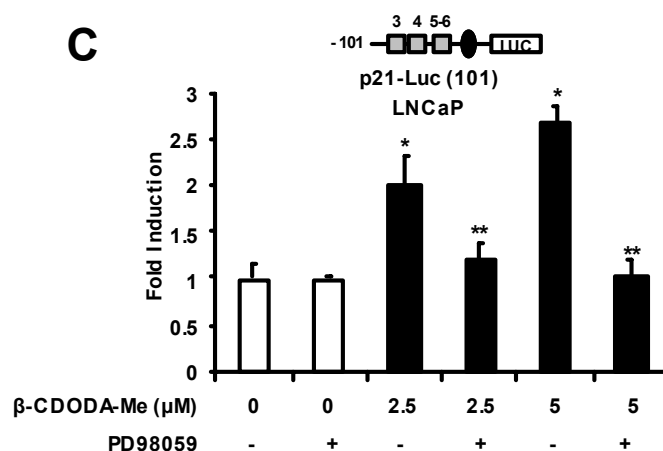
Fig 3.5 Continued

CDODA-Me alone or in the presence of the protein synthesis inhibitor cycloheximide (10 µg/ml) (Fig. 3.5 B); cycloheximide did not modulate the effects of β-CDODA-Me, suggesting that an induced inhibitory protein(s) does not mediate the effects of β-CDODA-Me on AR mRNA expression. β-CDODA-Me also decreased luciferase activity in LNCaP cells transfected with the AR-Luc construct that contains the -5400 to +580 region of the AR promoter linked to the luciferase genes (Fig. 3.5 C). The results indicate that β-CDODA-Me inhibits AR transcription without the parallel induction of inhibitory *trans*-acting factors. Recent studies suggest that AR downregulation of a PPARγ-inactive thiazolidinedione analog was due to downregulation of Sp protein (702). Results in Figure 3.5 D show that β-CDODA-Me induces a time-dependent induction of PARP cleavage and a decrease of both AR and Sp1, suggesting that decreased expression of AR may be Sp1-dependent as previously reported (702).

PSA protein expression is also decreased in LNCaP cells treated with β-

CDODA-Me (Fig. 3. 2 C) and similar effects were observed for PSA mRNA levels after
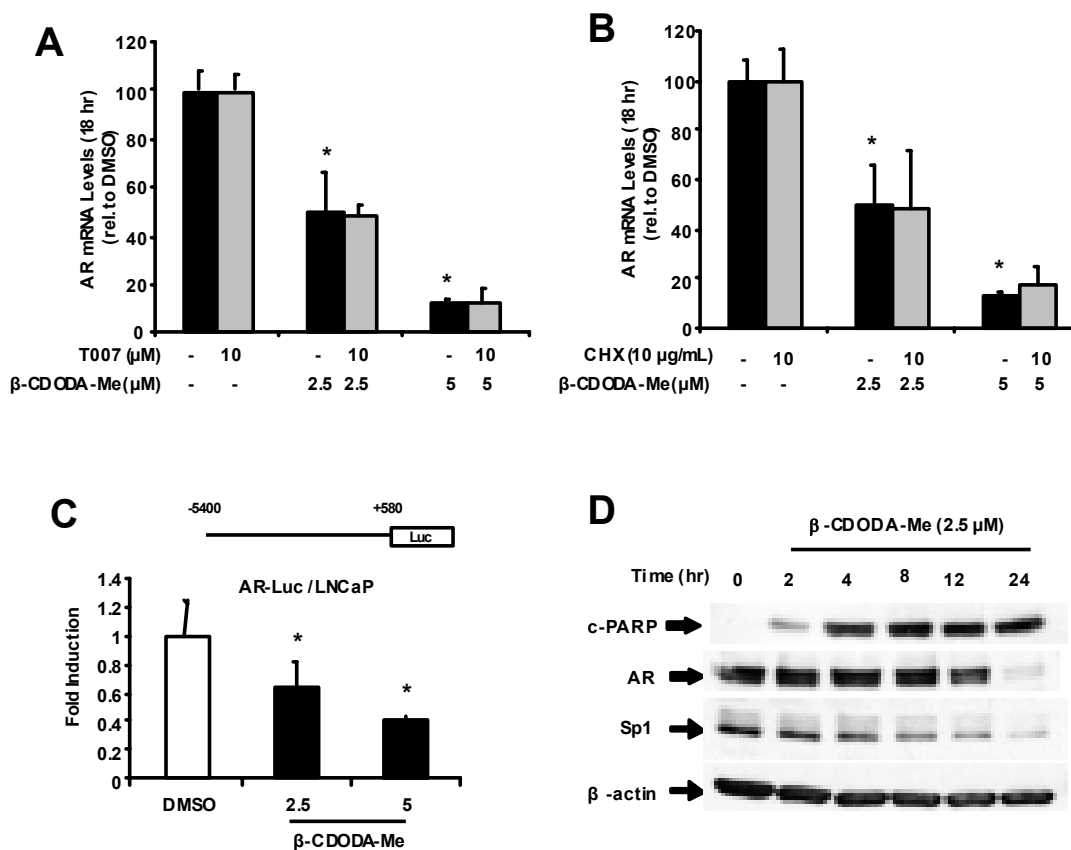
Fig. 3.6. β-CDODA-Me decreases AR gene expression. Effects of T007 (A) and cycloheximide (B) on β-CDODA-Me-dependent effects on AR gene expression. LNCaP cells were treated with β-CDODA-Me alone or in combination with T007 or cycloheximide for 18 h, and AR mRNA levels were determined by real-time PCR as described under Materials and Methods. Similar results were observed after treatment for 12 h (data not shown). C, β-CDODA-Me decreases AR promoter activity. LNCaP cells were transfected with AR-luc, treated with DMSO or β-CDODA-Me, and luciferase activity was determined as described under Materials and Methods. Results are means ± S.E. for three separate experiments for each treatment group, and a significant ($p < 0.05$) decrease in activity is indicated (*). D, time-dependent effects of β-CDODA-Me on AR, Sp1, and PARP (cleaved). LNCaP cells were treated with DMSO or β-CDODA-Me for up to 24 h, and whole-cell lysates were analyzed by Western blot analysis as described under Materials and Methods.

treatment for 12 or 18 hr, and these responses were not inhibited after cotreatment with the PPARγ antagonist T007 (Fig.3.6A). However, β-CDODA-Me-induced downregulation of PSA mRNA levels after treatment for 12 or 18 hr was significantly inhibited after cotreatment with cycloheximide (Fig. 3.6 B). In addition, β-CDODA-Me inhibited transactivation in LNCaP cells transfected with the PSA-Luc construct (contains 5.85 kb of the PSA promoter insert) (Fig. 3.6 C) and similar results were obtained for DHT-induced luciferase activity (Fig. 3.6 D). Thus, in contrast to results obtained for AR, β-CDODA-Me inhibits PSA expression through induction of inhibitory *trans*-acting factors and the mechanisms associated with the decreased PSA expression and the *cis*-elements important for this response are currently being investigated.

DISCUSSION

PPARγ agonists have been extensively investigated in both *in vitro* and *in vivo* cancer models for their potential applications in cancer chemotherapy (281, 463, 482, 659, 685-691). PPARγ agonists inhibit prostate cancer cell and tumor growth (249, 695, 703, 704) and the fact that approximately 40% of prostate cancer patients carry hemizygous deletions of PPARγ (705) suggests that this receptor may serve as a tumor suppressor gene for prostate cancer. However, in animal studies using the transgenic adenocarcinoma mouse prostate (TRAMP) model with hemizygous deletion in PPARγ, it was shown that the loss of receptor expression did not enhance or inhibit prostate tumor development in these animals (706). Thus, at least in the TRAMP mouse model, PPARγ does not appear to act as a tumor suppressor gene.

One of the perplexing problems with PPARγ agonists is that although these compounds inhibit cancer cell and tumor growth, their mechanisms of action are both receptor-dependent and -independent in different cancer cell lines. For example, PGJ2, troglitazone and PPARγ-active C-DIMS induce NAG-1 in HCT-116 colon cancer cells; however, only induction by PGJ2 is inhibited by a PPARγ antagonist (278, 299, 665). Caveolin-1 is induced by C-DIM compounds and CDDO in colon cancer cell lines, and this response is inhibited after cotreatment with PPARγ antagonists (300, 302). In contrast, C-DIMs decreased caveolin-1 expression in LNCaP cells and this response was PPARγ-independent (695).

β-CDODA-Me is a triterpenoid acid that contains an oleanolic acid backbone structure similar to that of CDDO and CDDO-Me (methyl ester) (463, 482), but there are important structural differences in the C, D and E rings that differentiate between these compounds; however, for both compounds the 2-cyano group was necessary for activation of PPARγ.

In this study, we investigated the growth inhibitory and proapoptotic effects of β-CDODA-Me in LNCaP cells and the role of PPARγ in mediating these responses. β-CDODA-Me was a more potent inhibitor of LNCaP cell growth than analogs (β-DODA and β-DODA-Me) that did not contain a 2-cyano substituent (Fig. 3.1A). Moreover, β-CDODA-Me also activated PPARγ-dependent transactivation in transient transfection studies in LNCaP cells (Fig. 3.1B), and compounds without the CN-group were inactive (data not shown) as previously reported for these analogs in colon cancer cells (312). β-

CDODA-Me induced p27 expression and downregulated levels of cyclin D1 protein

(Figs. 3.1C and 3.1D). Similar effects were previously reported for C-DIMs (695) and

the effects of both compounds were receptor-independent; however, β-CDODA-Me-

induced responses were observed at lower concentrations (1 - 2.5 μM) than the C-DIM

compounds (7.5 - 10 μM). C-DIMs did not induce p21 in LNCaP cells (695), whereas

β-CDODA-Me induced p21 protein and this response was not inhibited after cotreatment

with PPARγ antagonist T007 (Fig. 3.1D). Differences between PPARγ-active C-DIMs

and β-CDODA-Me in their induction of p21 in LNCaP cells was due to activation of

MAPK signaling by the latter compound (Fig. 3.3 C) which was required for induction

of p21 protein (Fig. 3.4A) and activation of the p21 promoter (Fig. 3.4 C). This is a

novel pathway for induction of p21 in LNCaP cells; however, previous studies in other

cell lines also demonstrated MAPK-dependent induction of p21 expression (701, 707,

708).

NAG-1 and ATF3 are growth inhibitory and proapoptotic proteins (709, 710),

and previous studies with PPARγ agonists report both receptor-dependent and -

independent induction of NAG-1 (278, 299, 302, 665). Induction of NAG-1 and ATF3

by β-CDODA-Me in LNCaP cells was also PPARγ-independent. Both PI3K and MAPK

inhibitors blocked induction of NAG-1; however, the JNK inhibitor SP600125 was the

most potent inhibitor of ATF-3 (but not NAG-1) induction. The inhibitory effects of

SP600125 are consistent with previous studies showing that homocysteine also induces

ATF3 in vascular cells through activation of JNK which activates c-*jun* and ATF-3

through an AP-1 site in the ATF-3 promoter (711). The kinase-dependent induction of

NAG-1 has previously been reported and these effects are both structure and cell context-dependent. For example, troglitazone and PPARγ-active C-DIMs induce NAG-1 in HCT116 colon cancer cells through rapid activation of Egr-1 which subsequently activates NAG-1 through direct interaction with the proximal region of the NAG-1 promoter (278, 299). However, this induction response is MAPK-dependent for troglitazone and PI3-K-dependent for the C-DIM compound. In this study, the time-dependent induction of both EGR-1 and NAG-1 are similar in LNCaP cells (Fig. 3.3 A), and inhibition of NAG-1 expression is observed with both PI3K and MAPK inhibitors (Fig. 3.3 B). This may involve cooperative interactions of both kinase pathways for induction of NAG-1 by β-CDODA-Me in LNCaP cells, and mechanisms for these responses are currently being investigated. Interestingly, induction of NAG-1 by PPARγ-active C-DIMs in LNCaP cells was inhibited only by the MAPK inhibitor PD98059 (695), suggesting differences between β-CDODA-Me and C-DIMs in the same cell line. Thus, induction of both NAG-1 and ATF3 in LNCaP cells is differentially induced by two PPARγ agonists, C-DIMs and β-CDODA-Me through receptor-independent activation of different kinase pathways.

Two recent reports show that in LNCaP cells AR knockdown by RNA interference results in apoptosis (700) and stable knockdown using short hairpin RNAs for AR results in decreased AR and PSA expression and inhibition of tumor growth *in vivo* (712). Like β-CDODA-Me, AR and PSA expressions are also decreased by C-DIMs and troglitazone in LNCaP cells, and 3,3'-diindolylmethane (DIM) also decreases expression of both genes and proteins (713, 714). Troglitazone differentially decreases

PSA and AR expression at relatively low ($IC_{50} \leq 10$ µM) and high ($IC_{50} \sim 40$ µM)

concentrations, respectively (699) . In contrast, C-DIMs decreased AR and PSA mRNA,

protein and reporter gene activity in cells transfected with PSA-Luc and AR-Luc

constructs over a narrow range of concentrations (7.5 - 10 µM) (695) and similar results

were observed for β-CDODA-Me (1 - 2.5 µM) in this study (Figs 3.5 and 3.6).

Moreover, cycloheximide reversed the β-CDODA-Me- and C-DIM-dependent

downregulation of PSA but not AR mRNA levels, suggesting a similar mechanism of

action for both compounds. One study reported that DIM inhibited nuclear uptake of

AR in LNCaP cells (715), and like β-CDODA-Me, DIM also decreased AR and PSA

expression in LNCaP and androgen-insensitive C4-2B cells (713, 714). However, there

are several differences between the pathways associated with downregulation of these

genes by β-CDODA-Me and DIM and this includes the pivotal role for DIM as an

inhibitor of phospho-Akt (713, 714), whereas β-CDODA-Me induces phospho-Akt (Fig.

3.3B) and the PI3K inhibitor LY294002 does not affect β-CDODA-dependent

downregulation of AR, PSA or FKBP51 (Fig. 3.3C) or induction of p21 or p27 (Fig. 3.4

A).

A recent report indicated that decreased AR expression in LNCaP cells treated

with a PPARγ-inactive thiazolidinedione derivative was due to proteasome-dependent

degradation of Sp1 (702) and our results also show a parallel decrease in AR and Sp1 in

LNCaP cells treated with β-CDODA-Me (Fig. 3.5 D). However, in contrast to the

previous report, this effect on AR was not reversed by a proteasome inhibitor (Fig. 3.2

D). Loss of AR by RNA interference results in the induction of apoptosis in LNCaP

cells (700). In contrast, 2.5 µM β-CDODA-Me rapidly induces PARP cleavage and

apoptosis in LNCaP cells prior to decreased AR expression (Fig. 3.5D) demonstrating

that apoptotic pathways activated by β-CDODA-Me in LNCaP cells are not associated

with loss of AR, and the proapoptotic mechanisms are currently being investigated.

Results of this study demonstrate that β-CDODA-Me is a potent inhibitor of

LNCaP cell growth and induces proapoptotic responses through activation of kinases

which differentially activate ATF3, NAG-1 and p21. In contrast, decreased expression

of AR and PSA are kinase independent and occur through different pathways (Fig. 3.7).
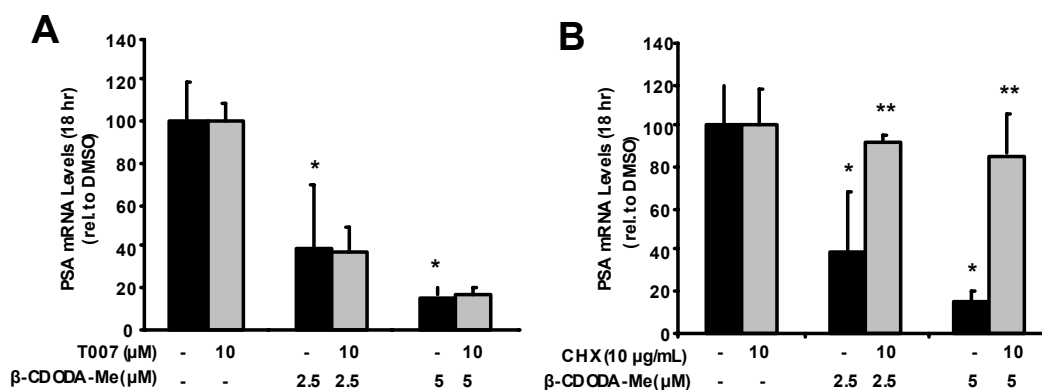
Fig. 3.7. β-CDODA-Me decreases PSA expression. Effects of T007 (A) and cycloheximide (B) on β-CDODA-Me-dependent effects on PSA gene expression. LNCaP cells were treated with β-CDODA-Me alone or in combination with T007 or cycloheximide for 12 or 18 hr, and PSA mRNA levels were determined by real time PCR as described in the Materials and Methods. β-CDODA-Me decreases PSA promoter (C) and DHT-induced (D) PSA promoter activity. LNCaP cells were transfected with PSA-luc, treated with DMSO, β-CDODA-Me, DHT and β-CDODA-Me plus DHT (combined), and luciferase activity determined as described in the Materials and Methods. Results are means ± SE for three replicate determinations for each treatment group, and significantly (p < 0.05) decreased basal or DHT-induced luciferase activity by β-CDODA-Me is indicated (*).
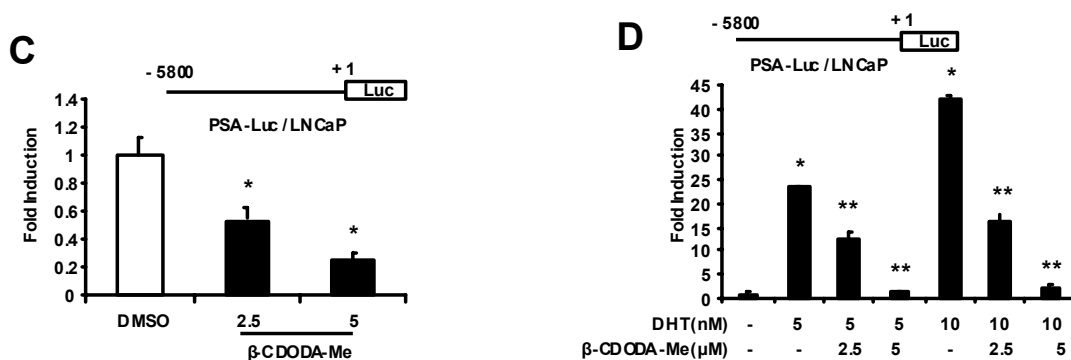
Fig. 3.7 Continued

β-CDODA-Me, C-DIMs, DIM and troglitazone exhibited both differences and similarities in their modes of action in LNCaP cells, although all of these compounds decreased expression of AR and PSA. The growth inhibitory and proapoptotic effects of β-CDODA-Me were primarily receptor-independent in LNCaP cells, and similar results have been observed for other PPARγ agonists such as the C-DIMs and troglitazone. Thus, each agent activates multiple pathways, and the successful use of β-CDODA-Me and other such compounds for single or combined prostate cancer chemotherapies requires detailed insights on their mechanisms of action and prostate cancer cell context-dependent differences in activating critical pathways such as those illustrated in Figure 3.8.
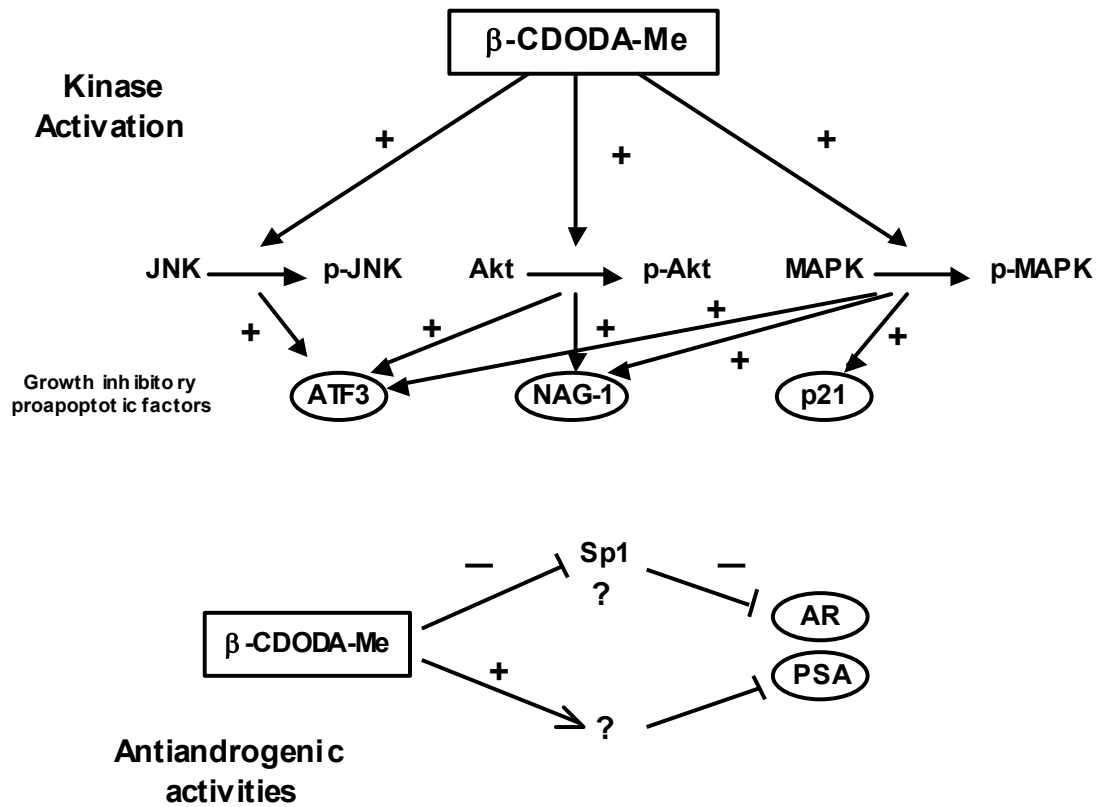
Fig. 3.8. β-CDODA-Me-dependent activation of kinases and kinase-dependent genes and repression of AR and PSA.

CHAPTER IV

BETULINIC ACID INHIBITS PROSTATE CANCER GROWTH THROUGH INHIBITION OF SPECIFICITY PROTEIN TRANSCRIPTION FACTORS*

INTRODUCTION

Natural products derived from plant sources have been used extensively in traditional medicine for treatment of a myriad of diseases including various types of cancers (410, 716, 717). Several individual phytochemicals or their synthetic analogs are among the most widely used drugs for cancer chemotherapy and these include taxane microtubule inhibitors such as paclitaxel and synthetic analogs that are now widely used in cancer chemotherapy (718, 719). Phytochemical and microbial extracts from various sources are routinely screened for biological activities, and it is estimated that 20-25% of new drugs are derived from natural products or their synthetic analogs (720-722). The triterpenoids oleanolic acid, ursolic acid and their derivatives, exhibit anti-inflammatory and anticarcinogenic activities (459, 723, 724). Structure-activity studies among several oleanolic and ursolic acid derivatives (462, 463, 465) have identified 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and related compounds as highly potent anti-inflammatory compounds that inhibit growth and induce apoptosis in several

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cancer cells and tumor types (302, 482, 483, 725-727). CDDO-like compounds act through multiple pathways and are currently undergoing clinical trials for treatment of leukemia.

Betulin is a lupane-derived triterpene which is present in high concentrations in birch bark and betulinic acid (BA), an oxidation product of betulin has also been detected in bark extracts (499). BA was initially characterized as a highly selective inhibitor of human melanoma cell and tumor growth through induction of apoptosis (510). Subsequent research has shown that BA and other derivatives are effective inhibitors of cell proliferation and induce apoptosis in many different cancer cells. However, the underlying mechanisms for these responses are not well understood.

In this study, we show that BA decreased proliferation of several cancer cell lines including SK-MEL2 melanoma cells and, using LNCaP prostate cancer cells as a model, we determined a unique mechanism of action of this compound. BA modulates expression of several growth-related proteins and decreases expression of the proangiogenic and proapoptotic proteins vascular endothelial growth factor (VEGF) and survivin, respectively. Previous reports show that in some cancer cell lines, VEGF and survivin expression is dependent, in part, on Sp proteins (331, 547, 580, 583, 728-732), and Sp1 is overexpressed in cancer cells and tumors (576, 733-736). Results of this study now show that BA induces proteasome-dependent degradation of Sp1, Sp3 and Sp4 in LNCaP cells. The proteasome inhibitor MG132 not only blocked BA-induced degradation of Sp proteins but also inhibited the decreased expression of VEGF and survivin in cells treated with BA. *In vivo* studies showed that BA inhibited tumor

growth in athymic nude mice bearing LNCaP cell xenografts, and this was accompanied by decreased expression of Sp1, Sp3 and Sp4 proteins and VEGF and increased apoptosis in tumors from BA-treated mice. The results indicate that the antitumorigenic effects of BA are associated with targeted degradation of Sp transcription factors which are overexpressed in many tumors and this results in activation of proapoptotic and antiangiogenic responses in tumor but not in non-target tissues (e.g. liver) which exhibit low Sp protein expression.

MATERIALS AND METHODS

Chemicals, antibodies, plasmids and reagents

BA and β-actin antibody were purchased from Sigma Aldrich (St. Louis, MO) and proteasome inhibitor MG132 was purchased from Calbiochem (San Diego, CA). Antibodies against Sp1, Sp4, Sp3, VEGF, CD1, AR, KLF6, survivin and PARP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and CD31 antibody from DakoCytomation (Glostrup, Denmark). The pVEGF-2018 and pVEGF-133 constructs contain VEGF promoter inserts (positions -2018 to +50 and positions -131 to +54, respectively) linked to luciferase reporter gene (580). The pSurvivin-269 and pSurvivin-150 were kindly provided by Dr. M. Zhou (Emory University, Atlanta, GA). Reporter lysis buffer and luciferase reagent for luciferase studies were purchased from Promega (Madison, WI). β-Galactosidase (β-gal) reagent was obtained from Tropix (Bedford, MA). Lipofectamine reagent was supplied by Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagent was from Perkin-Elmer Life Sciences (Boston, MA).

Cell lines

Human carcinoma cell lines LNCaP (prostate) and SK-MEL2 (melanoma) were obtained from American Type Culture Collection (Manassas, VA). Cell lines were maintained in RPMI 1640 (Sigma) supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% FBS, and 10 mL/L of 100x Antibiotic Antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO_2.

Cell proliferation assay

Prostate and melanoma cancer cells (2×10^4 per well) were plated in 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or different concentrations of the compound were added. Fresh medium and compounds were added every 48 hr and cells were then trypsinized and counted after 48 and 96 hr using a Coulter Z1 cell counter. Each experiment was done in triplicate and results are expressed as means ± SE for each set of experiments

Transfection and luciferase assay

Prostate cancer cells were plated in 12-well plates at 1×10^5 cells/well in DMEM:Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After growth for 16 - 20 hr, various amounts of reporter gene constructs, i.e., pVEGF-2018 (0.4 μg), pVEGF-133 (0.04 μg), pSurvivin-269 (0.04 μg), pSurvivin-150 (0.04 μg) and β-gal (0.04 μg), were transfected by Lipofectamine (Invitrogen) according to the manufacturer's protocol. After 5 hr of transfection, the transfection mix was replaced

with complete media containing either vehicle (DMSO) or the indicated compound for 20 to 22 hr. Cells were then lysed with 100 μL of 1x reporter lysis buffer, and 30 μL of cell extract were used for luciferase and β-gal assays. Lumicount was used to quantitate luciferase and β-gal activities, and the luciferase activities were normalized to β-gal activity.

Western blotting

An equal amount of cell lysate (60 μg/well) was separated by 7.5% to 12% SDS-PAGE, which was followed by immunoblotting onto polyvinylidene difluoride (Bio-Rad, Hercules, CA). After blocking in TBST-Blotto (10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, 5% nonfat dry milk) for 30 min, the membranes were incubated with primary antibodies overnight at 4°C, and then with horseradish peroxidase-conjugated secondary antibody for 2 hr at room temperature. Proteins were visualized using the chemiluminescence substrate (Perkin-Elmer Life Sciences) for 1 min and exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

Xenograft study

Male athymic BALB/c nude mice (age 4 - 6 weeks) were purchased from Harlan (Indianapolis, IN). LNCaP cells (1 x 10^6) were implanted with matrigel (BD Biosciences, San Jose, CA) subcutaneously into the flank of each mouse. Ten days after cell inoculation, animals were divided into three equal groups of 10 mice each. The first group received 100 μL vehicle (1% DMSO in corn oil) by oral gavage and the second and third groups of animals received 10 and 20 mg/kg/d doses of BA in vehicle every

second day for 14 days (7 doses). The mice were weighed, and tumor areas were measured throughout the study. After 22 days, the animals were sacrificed, final body and tumor weights were determined, and selected tissues were further examined by routine hematoxylin and eosin staining and immunohistochemical analysis.

Immunohistochemistry

Tissue sections (4 - 5 μM thick) mounted on poly-L-lysine-coated slide were deparaffinized by standard methods. Endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 10 min. Antigen retrieval for VEGF and CD31 staining was done for 10 min in 10 mmol/L sodium citrate buffer (pH 6) heated at 95°C in a steamer followed by cooling for 15 min. The slides were washed with PBS and incubated for 30 min at room temperature with a protein blocking solution (VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA). Excess blocking solution was drained, and the samples were incubated overnight at 4°C with one of the following: a 1:100 dilution of VEGF antibody or a 1:40 dilution of CD31 antibody. Sections were then incubated with biotinylated secondary antibody followed by streptavidin (VECTASTAIN Elite ABC kit). The color was developed by exposing the peroxidase to diaminobenzidine reagent (Vector Laboratories), which forms a brown reaction product. The sections were then counterstained with Gill's hematoxylin. VEGF and CD31 expression was identified by the brown cytoplasmic staining. hematoxylin and eosin staining was determined as previously described (583).

RESULTS

Antiproliferative, proapoptotic and antiangiogenic effects of BA

The antiproliferative effects of BA were initially investigated in SK-MEL2 melanoma and LNCaP prostate cancer cells, and 6-day IC_{50} values for growth inhibition were 5 - 10 µM and 1 - 5 µM, respectively. Similar results were observed for pancreatic (Panc28, L3.6pl), bladder (KU7), and colon (SW480) cancer cells (data not shown). Cell survival curves for LNCaP and SK-MEL2 cells after treatment for 48 or 96 hr are illustrated in Figures 4.1A and 4.1B. At higher concentrations of BA ($\geq$ 10 µM), there was an overall decrease in the number of cells remaining compared to the number of initially seeded cells which was consistent with the cytotoxicity of BA.

We further investigated the effects of BA on growth inhibitory and proapoptotic proteins using LNCaP cells as a model. Results illustrated in Figure 4.1C show that relatively short term exposure (24 hr) to BA ($\geq$ 10 µM) induced downregulation of cyclin D1, whereas the cyclin-dependent kinase inhibitors p21 and p27 were expressed at low levels in these cells and were not affected by the treatment (data not shown). AR expression in LNCaP cells was decreased after treatment with 5 µM BA, and this protein was almost completely absent in cells treated with 10 µM concentrations. Recent studies have demonstrated that AR knockdown by RNA interference in LNCaP cells resulted in induction of apoptosis (700), suggesting that the proapoptotic effects of BA in LNCaP cells may be due, in part, to the effects on AR expression. However, this mechanism would not be applicable to melanoma and other cancer cell lines which are AR-independent. The tumor suppressor gene KLF6 was also induced by BA at
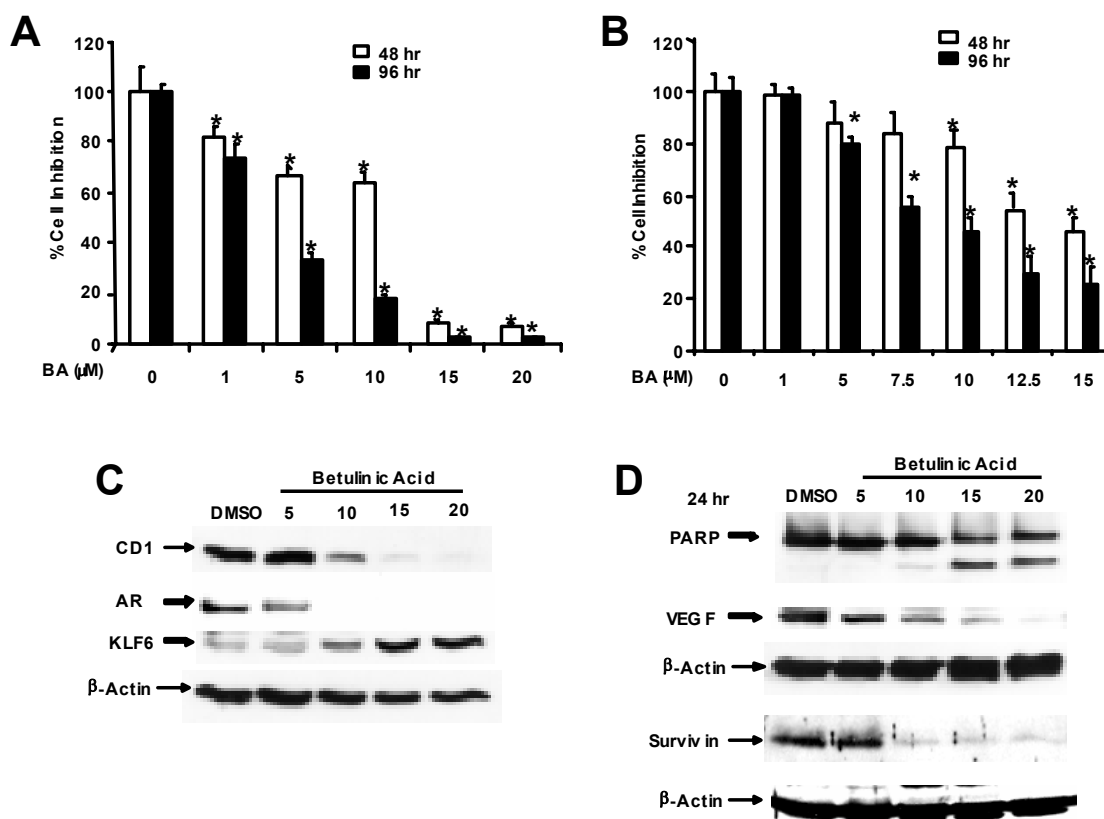
Fig. 4.1. BA inhibits growth and induces apoptosis in cancer cells. Decreased cell survival in LNCaP (A) and SK-MEL2 (B) cancer cells. Cells were seeded, treated with solvent (DMSO) or different concentrations of BA (1 - 20 μM) for 6 days as described in the Materials and Methods. Cell survival is expressed as the percentage of BA-treated cells remaining compared to DMSO (set at 100%), and significantly (p < 0.05) decreased survival is indicated by an asterisk. (C) and (D) Western blot analysis for modulation of protein expression by BA. LNCaP cells were treated with DMSO or BA (5 - 20 μM) for 24 hr and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods.

concentrations between 10 - 15 μM. BA also induced caspase-dependent PARP

cleavage in LNCaP cells, and this was accompanied by decreased expression of the

antiapoptotic protein survivin (Fig. 4.1D) and induction of DNA laddering (data not

shown) typically observed in cells undergoing apoptosis. In addition, BA also decreased expression of the angiogenic protein VEGF.

BA induces proteasome-dependent degradation of Sp1, Sp3 and Sp4 proteins

Previous studies in this laboratory have shown that both basal and hormone-induced expression of VEGF in cancer cell lines is dependent on Sp protein expression (331, 547, 580, 583), and regulation of survivin in some cells is also dependent on these transcription factors (737-740). Moreover, since Sp proteins are upregulated in many tumors/cancer cells (576, 741-743) and are associated with proliferative, angiogenic and antiapoptotic pathways, we hypothesized that the anticarcinogenic activity of BA may be due, in part, to downregulation of Sp proteins. Results in Figure 4.2A show that treatment of LNCaP cells with 5 - 10 μM BA for 24 hr induced downregulation of Sp1, Sp3 and Sp4 proteins, and this was also accompanied by decreased expression of VEGF and survivin (Fig. 4.1D). The time-dependent decrease of Sp proteins in LNCaP cells treated with 15 μM BA for 4, 8, 12, 16, 20 and 24 hr showed that lower expression of these proteins is first observed after treatment for 12 hr (Fig. 4.2B). Prolonged treatment of LNCaP cells with BA for 48 or 72 hr showed that Sp protein degradation and PARP cleavage can be observed as doses as low as 1 to 2.5 μM (Fig. 4.2C). Figure 4.2D illustrates that BA also decreased expression of Sp1, Sp3 and Sp4 proteins in SK-MEL2 melanoma cancer cells, and similar results were obtained in other cancer lines (data not shown).

Since BA induces degradation of Sp proteins within 12 hr after treatment (Fig. 4.2 B), we investigated the possible role of induced protein(s) in mediating this response
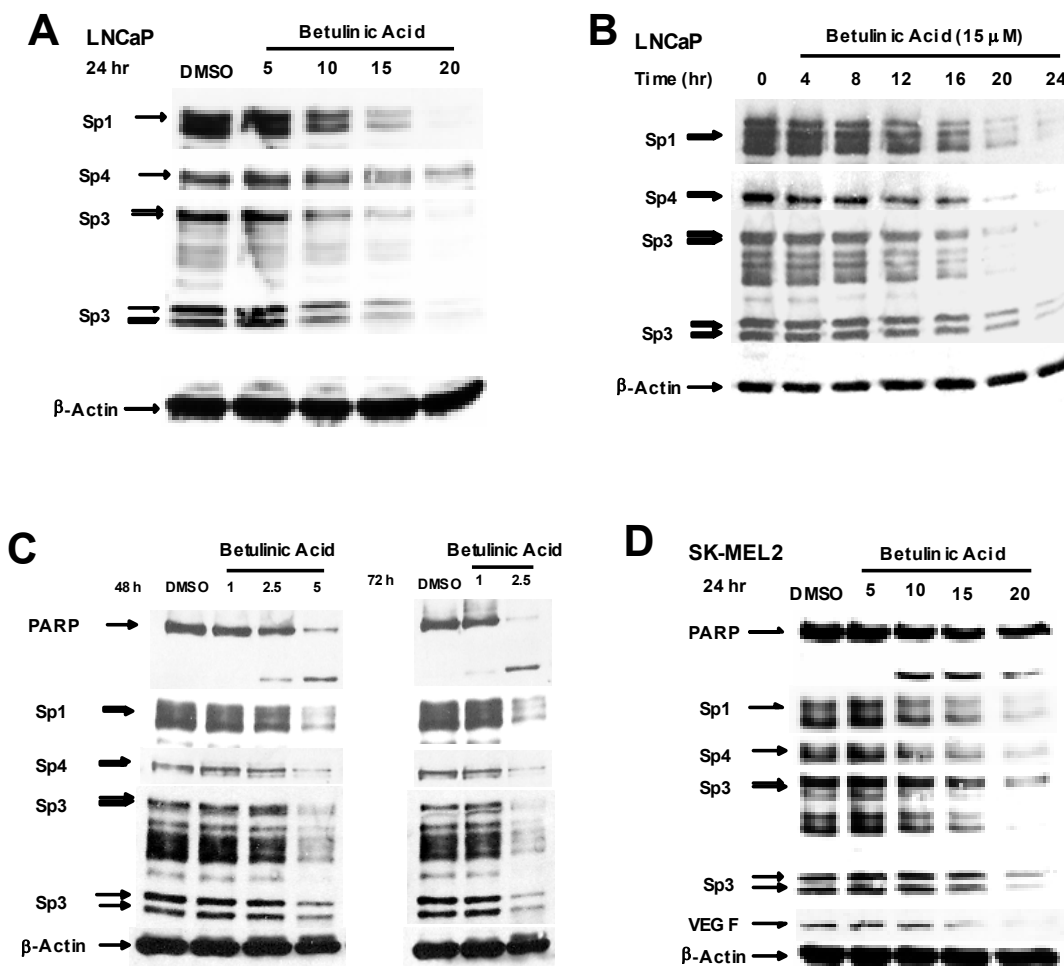
Fig. 4.2. BA induces degradation of Sp and other proteins in LNCaP. Decreased expression of Sp1, Sp3, Sp4 and VEGF in LNCaP (A - C) and SK-MEL2 (D) cells. LNCaP or SK-MEL2 cells were treated with DMSO or BA (1 - 20 µM) for 24 (A and D), 4 - 24 (B), 48 or 72 (C) hr, and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods.

by treating LNCaP cells with BA alone (10 - 20 µM) or in the presence of 10 µg/ml

cycloheximide (Fig. 4.3A). The protein synthesis inhibitor did not modulate the effects

of BA on Sp protein levels. Previous studies showed that COX-2 inhibitors, the NSAID

tolfenamic acid, and related compounds induced proteasome-dependent degradation of
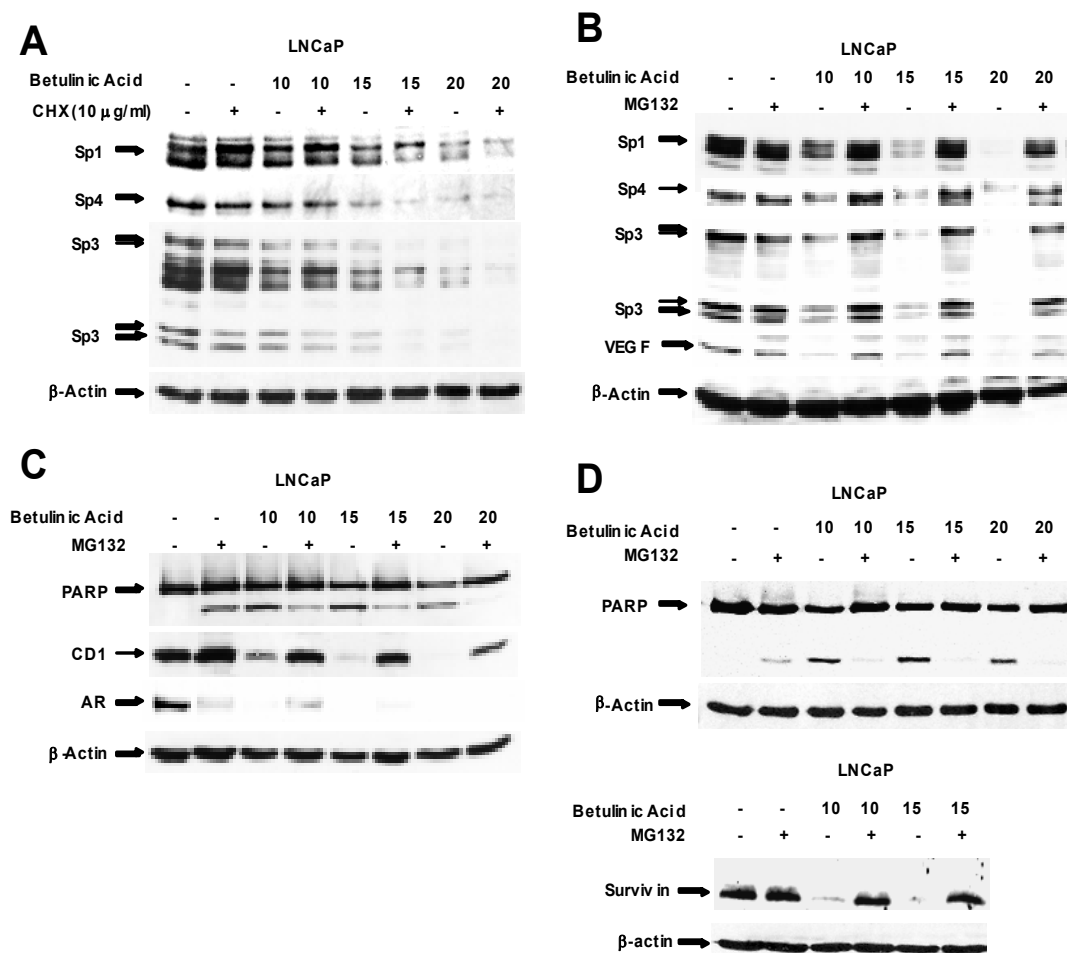
Fig. 4.3. BA induces proteasome-dependent degradation of Sp proteins in LNCaP cells. (A) Effects of cycloheximide. Cells were cotreated with 10 - 20 µM BA or 10 µg/ml cycloheximide alone or in combination, and expression of Sp proteins in whole cell lysates was determined by immunoblot analysis as described in the Materials and Methods. Effects of the proteasome inhibitor MG132 on BA-induced decrease of Sp proteins/VEGF (B), PARP cleavage, cyclin D1 and AR (C), and PARP cleavage and survivin (D). LNCaP cells were treated with DMSO or BA alone or in combination with 5 or 10 µM MG132 (pretreated for 30 min) for 24 hr, and protein expression in whole cell lysates was analyzed by Western blot analysis as described in the Materials and Methods.

Sp proteins (331, 583) and therefore we investigated the effects of the proteasome inhibitor MG132 on BA-induced downregulation of Sp1, Sp3, Sp4 and VEGF in LNCaP cells. MG132 reversed the effects of BA on expression of these transcription factors (Fig. 4.3 B). BA selectively induced proteasome-dependent degradation of Sp proteins and cyclin D1 in LNCaP cells (Fig. 4.3C); however, MG132 did not modulate expression of β-actin or reverse BA-dependent downregulation of AR which is due to decreased AR RNA expression (data not shown). MG132 (10 μM) and other proteasome inhibitors induced caspase-dependent cleavage of PARP in LNCaP cells; however, MG132 also partially inhibited BA-induced apoptosis. Moreover, using a lower concentration of MG132 (5 μM), the effects of the compound alone on PARP cleavage were decreased but in combination with BA, there was inhibition of BA-induced PARP cleavage (Fig. 4.3D). MG132 also blocked BA-induced downregulation of survivin protein (Fig. 4.3 D), suggesting that Sp protein degradation plays a role in the apoptosis-inducing effects of BA.

BA inhibits VEGF and survivin promoter expression through proteasome-dependent degradation of Sp proteins

Expression of both VEGF and survivin in some cancer cell lines is regulated by Sp protein interactions with GC-rich promoter sites (744, 745), and therefore the effects of BA on decreased expression of VEGF and survivin through Sp protein degradation was further investigated in transfection studies. The effects of BA on transactivation was investigated in LNCaP cells transfected with the pVEGF1 and pVEGF2 constructs containing the -2068 to +50 and -133 to +50 VEGF gene promoter inserts. Results in

Figures 4.4A and 4.4B show that BA decreased luciferase activity in LNCaP cells transfected with pVEGF1 and pVEGF2, and these effects were reversed by the proteasome inhibitor MG132, and similar results were observed using the proteasome inhibitor lactacystin (data not shown). These observations further confirm that BA-induced degradation of Sp proteins results in decreased VEGF expression in LNCaP cells, and this is consistent with previous RNA interference studies demonstrating that Sp1, Sp3 and Sp4 regulate VEGF expression in cancer cell lines (580).

Since the proteasome inhibitor MG132 partially blocks BA-induced PARP cleavage (Figs. 4.3C and 4.3D), we further investigated the role of Sp protein degradation on induction of apoptosis by examining the effects of BA on transactivation in LNCaP cells transfected with the GC-rich pSurvivin-269 and pSurvivin-150 constructs contain the -269 to +49 and -150 to +49 survivin promoter inserts. BA causes a concentration-dependent decrease in luciferase activity which was significantly reversed by 10 μM MG132 (Figs. 4.4C and 4.4D). In parallel studies, electrophoretic mobility shift assays also show that lysates from BA-treated LNCaP cells exhibited decreased binding to GC-rich survivin sequences (data not shown). These results complement a recent study showing that the DNA binding drug hedamycin also decreases survivin expression through inhibition of Sp protein interactions with the GC-rich survivin promoter (746).

BA inhibits tumor growth an athymic nude mice bearing LNCaP cells as xenografts

Pisha and coworkers previously reported that BA inhibits growth of tumors in athymic nude mice bearing MEL-1 melanoma cells as xenografts (510). Results in
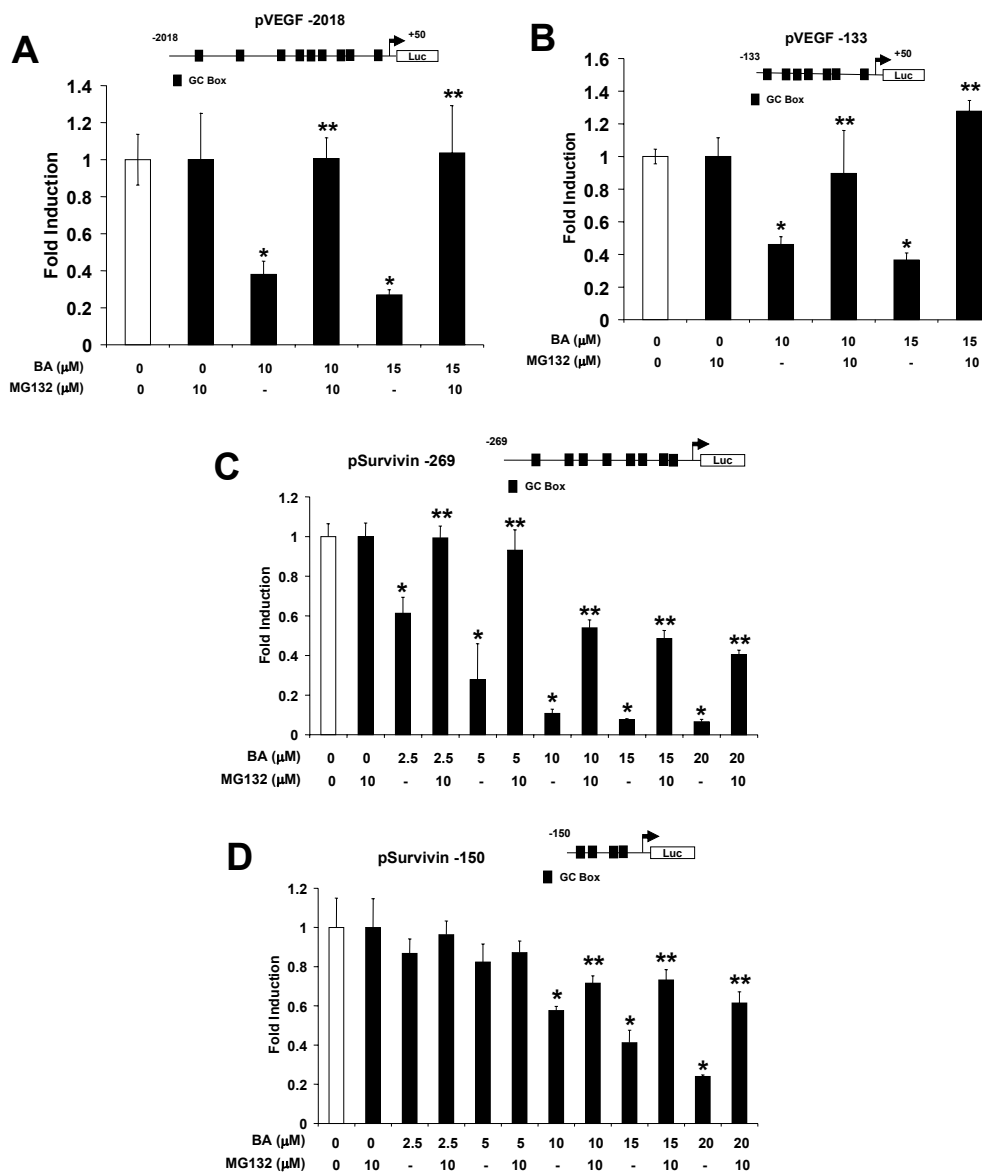
Fig. 4.4. BA decreases transactivation in LNCaP cells transfected with VEGF and survivin constructs. Transfection with pVEGF-2068 (A), pVEGF-133 (B), pSurvivin-269 (C), and pSurvivin-150 (D). LNCaP cells were transfected with the various constructs, treated with DMSO or BA (2.5 - 20 μM) alone or in combination with 10 μM MG132, and luciferase activity (relative to β-gal) was determined as described in the Materials and Methods. Luciferase activity significantly ($p < 0.05$) decreased by BA (*) and inhibition of this response by cotreatment with MG132 (**) is indicated.

Figure 4.5A show that 10 and 20 mg/kg/d betulinic acid inhibited tumor growth in mice

bearing LNCaP cell xenografts and this was accompanied by significantly decreased

tumor weights in both treatment groups (Fig. 4.5B). Examination of the mice showed

that there were no treatment-related changes in organ or body weights or in the

histopathology of liver and other tissues (data not shown), and this was consistent with

the reported low toxicity of this compounds (510). Representative hematoxylin- and

eosin-stained histopathology sections of prostate tumors from the control and treated

mice were examined. Tumors from untreated mice consisted of minimally encapsulated

[Fig. 4.5C (a)], dense expansile nests of epithelial cells with marked atypical features

such as anisocytosis, anisokaryosis, multiple variably sized nucleoli, nuclear molding,

bi- and multinucleation. Bizarre mitotic figures were frequently noted within the

neoplastic cells [Fig. 4.5C (b), arrow heads]. Abundant vascular channels were

frequently present within neoplastic cells [Fig. 4.5C (b), arrows]. Tumors from the

treated mice consisted of neoplastic cells similar to that noted from the untreated mice.

However, the mitotic activity (1-2 microfigures/hpf compared to 6-8 microfigures/hpf in

the corn oil group) and the epithelial atypia appeared to be decreased [Fig. 4.5C (c)]. In

addition, the tumor tissue was remarkably less vascular with evidence for necrosis [Fig.

4.5C (d), area within box], and this is consistent with the antiangiogenic effect of BA

through decreased expression of Sp proteins and VEGF.

BA decreases Sp protein and VEGF expression in tumors but not liver

We also compared expression of Sp proteins, AR and VEGF as well as PARP
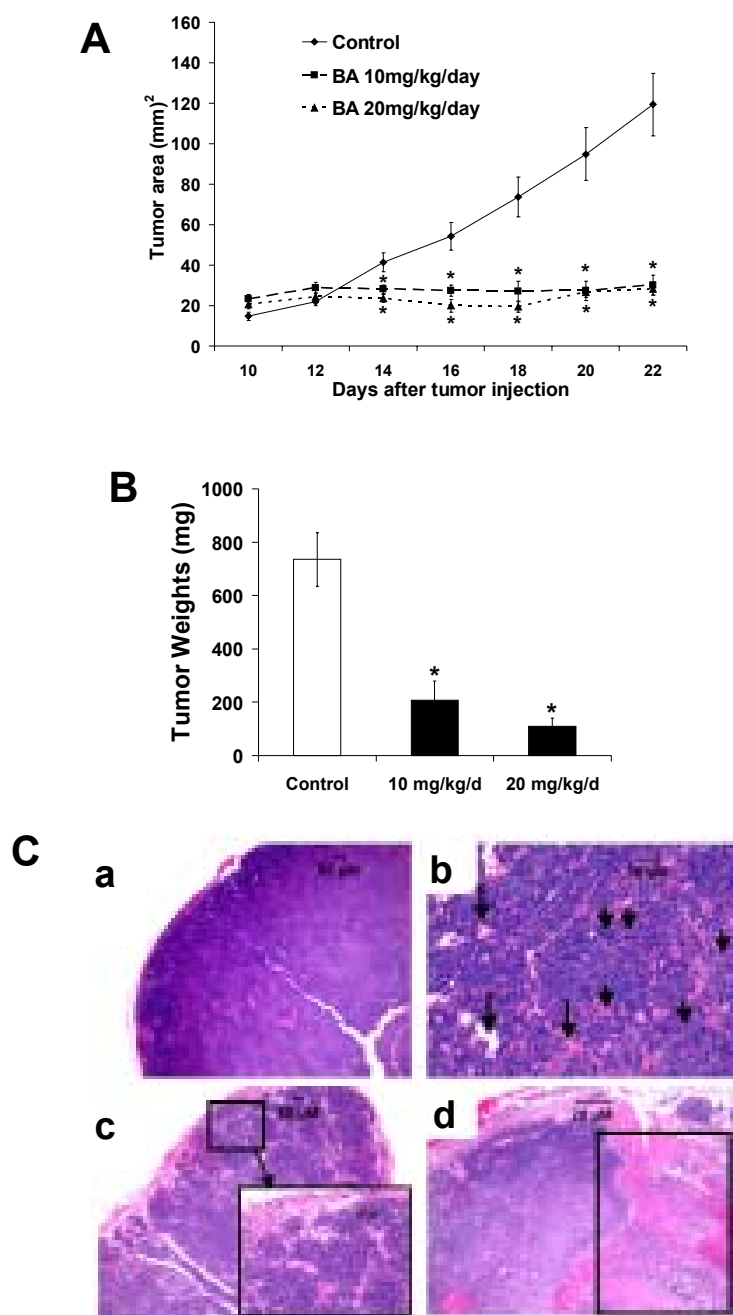
Fig. 4.5. Antitumorigenic activity by BA *in vivo*. (A) Decreased tumor area. Athymic nude mice (10 per group) bearing LNCaP cells as xenografts were treated with corn oil (control) or BA in corn oil (10 or 20 ml/kg) every second day, and tumor areas were determined as described in Materials and Methods. (B) Tumor weights. After the final treatment, animal were sacrificed and tumor weights were determined as described in the Materials and Methods. Significantly ($p < 0.05$) decreased tumor areas or volumes are indicated by an asterisk. (C) Histopathological evaluation of tumors. Tumors from corn oil (a and b) and BA (c and d) mice were fixed, stained with hematoxylin and eosin, and examined histopathologically as described in the Materials and Methods.

cleavage in tumor lysates from control and BA-treated mice (5 animals per group) by

Western blot analysis using β-actin as a loading control. Relatively high levels of Sp1,

Sp3, Sp4 and VEGF proteins were observed in the control tumors, and we did not detect

PARP cleavage in protein lysates from these tumors (Fig. 4.6A). In contrast, expression

of Sp1, Sp3, Sp4, AR and VEGF proteins was decreased in tumors from BA-treated

mice and PARP cleavage was observed. Sp protein levels in liver from untreated or BA-

treated mice could be visualized only after prolonged exposures, and the pattern of Sp1,

Sp3 or Sp4 protein expression was similar in both groups (Fig. 4.6B). A direct

comparison of Sp protein expression in tumors and liver from untreated animals (Fig. 4.6

C) indicates high levels in tumors, whereas in liver only Sp3 could be detected and

levels of Sp1 and Sp4 were very low. We also examined Sp proteins in other

tissues/organs from mice treated with corn oil or BA and observed uniformly very low to

non-detectable Sp1, Sp3 and Sp4 in both treatment groups (data not shown). Tumors

from untreated and treated mice were also stained for VEGF and CD31 (microvessel

density), and the staining for both factors was decreased in tumors from BA-treated mice

(Fig. 4.6D). This study demonstrates that BA is a potent inhibitor of prostate cancer cell

and tumor growth, and this parallels results showing that BA exhibited anticancer

activity in *in vitro* and *in vivo* models of melanoma (510). The underlying mechanisms

of proapoptotic, antiproliferative and antiangiogenic activities of BA are associated with

induction of proteasome-dependent degradation of Sp proteins in prostate tumor cells.

Moreover, Sp protein expression was decreased in SK-MEL2 (Fig. 4.2D) and other

cancer cell lines (data not shown), suggesting that BA-dependent downregulation of Sp

proteins which is accompanied by both proapoptotic and antiangiogenic responses is an

integral part of the anticarcinogenic activity of this compound.
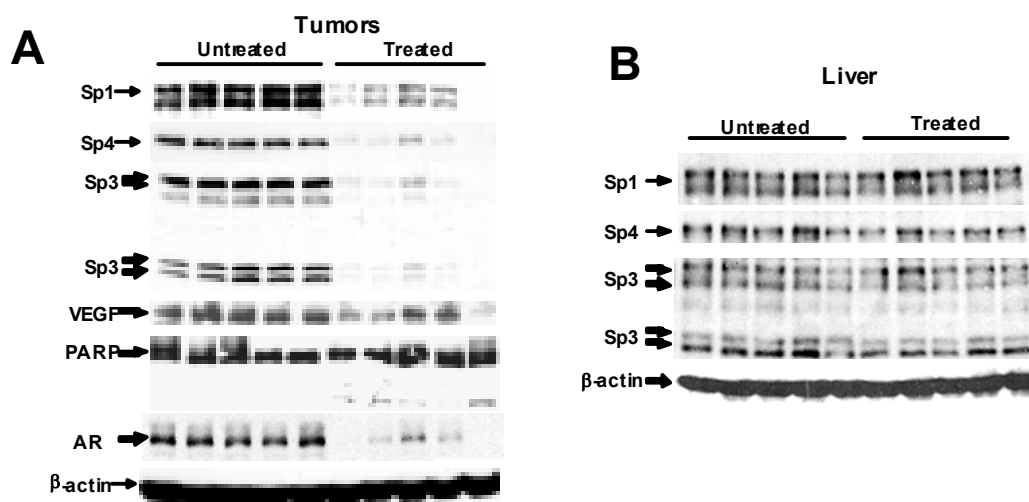
Fig.4.6. Sp and VEGF protein expression in tumors and liver. Whole cell lysates from corn oil (untreated) and BA-treated tumors (A) and liver (B) were obtained from tissue from at least 5 rats per group and analyzed by Western blot analysis as described in the Materials and Methods. (C) Comparative Sp protein expression in tumors and liver from corn oil (solvent)-treated animals. Tumor and liver lysates containing the same amount of protein were analyzed by electrophoresis and visualized as described in the Materials and Methods. (D) Immunostaining for CD31 and VEGF. Fixed tumor tissue from corn oil and BA-treated mice were stained with CD31 and VEGF antibodies as described in the Materials and Methods.

C

D
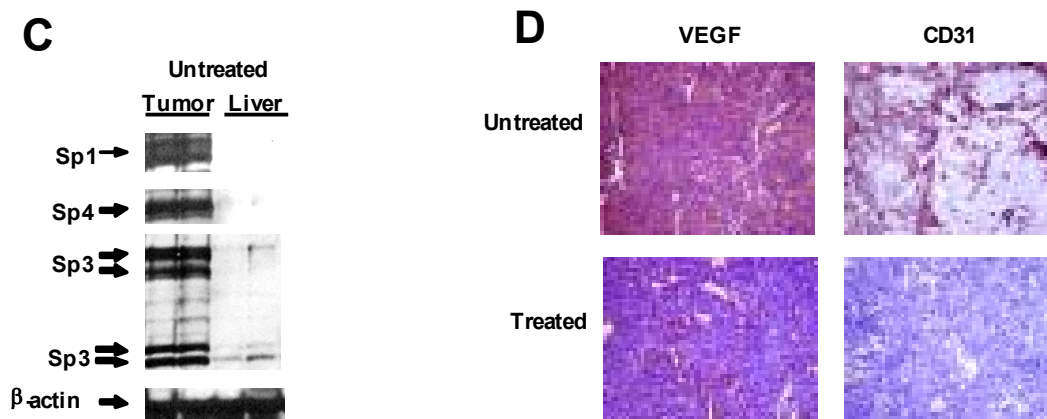
Fig. 4.6 Continued

DISCUSSION

BA is a natural product identified in various bark extracts and is readily synthesized from betulin, a major component in bark from birch trees (499). Pisha and coworkers (510) initially reported that this triterpenoid inhibited growth of several melanoma cell lines, and ED_{50} values for cytotoxicity varied from 4.8 - 1.1 μg/ml, whereas the corresponding values for a series of colon, prostate, breast, lung, squamous and glioma cancer cells were > 20 μg/ml. However, other reports also show that BA inhibits growth and induces apoptosis in several different cancer cell lines (499), and this corresponds to results of this study in which IC_{50} values (growth inhibition) for melanoma and prostate cancer cells were 5 - 10 μM and 1 - 5 μM, respectively, and similar results were obtained in other cancer cell lines. It has also been reported that BA derivatives and related lupane analogs are more cytotoxic than BA to cancer cells (499); however, a major advantage in using the latter compound for cancer chemotherapy is the

low toxicity. Doses as high as 500 mg/kg every fourth day (X6) exhibited no detectable

toxic side effects (510).

It has been reported that BA induces apoptosis in several different cancer cell

lines through multiple pathways which include direct effects on the mitochondria

accompanied by decreased mitochondrial membrane potential, upregulation of death

receptors, and interactions with other agents (511, 517, 528, 532, 537, 539, 540, 747-

750). In addition, BA exhibits antiangiogenic activity in an *in vitro* assay for tube-like

structures in ECV304 (751); however, the underlying mechanisms of action for these

responses are unknown. In this study, BA also induces proapoptotic and antiangiogenic

responses in LNCaP cells as evidenced by decreased expression of VEGF and survivin

and activation of caspase-dependent PARP cleavage (Fig. 4.1D) and DNA laddering

(data not shown). BA decreases AR expression in LNCaP cells (Fig. 4.1C) and this

response is in itself proapoptotic in this cell line since it has been reported that decreased

AR expression via RNA interference results in increased apoptosis in LNCaP cells

(700). Previous studies show that in several cancer cell lines VEGF expression is

dependent, in part, on Sp1 and other Sp proteins (331, 752), and there is also evidence

that survivin expression is Sp-dependent (753-755). Therefore, we examined the effects

of BA on expression of Sp1, Sp3 and Sp4 proteins in LNCaP cells and the results

demonstrate for the first time that BA decreases expression of Sp1, Sp3 and Sp4, and

similar results are observed in SK-MEL2 and other cancer cell lines (Figs. 4. 2A –

4.2D).

The role of BA-induced downregulation of Sp proteins in mediating the proapoptotic and antiangiogenic activities of this compound was further investigated in LNCaP cells. Proteasome inhibitors such as MG132 block Sp protein degradation induced by COX-2 inhibitors and tolfenamic acid in colon and pancreatic cancer cells (331, 511). The results in Figure 2D show that MG132 inhibited BA-induced degradation of Sp proteins. Moreover, MG132 blocked degradation of VEGF and survivin proteins in LNCaP cells treated with BA, and similar results were observed for cyclin D1 but not AR (Figs. 4.3B – 4.3D) which is decreased through proteasome-independent pathways. Moreover, in LNCaP cells transfected with constructs containing critical GC-rich sequences from the VEGF and survivin gene promoters, BA decreased transactivation which was inhibited after cotreatment with MG132 (Fig. 4.4). These results confirm the linkage between the decreased expression of survivin and VEGF in LNCaP cells treated with BA with degradation of Sp1, Sp3 and Sp4 in this cell line.

Sp proteins are overexpressed in many human tumors and cell lines (576, 756-759), and therefore these transcription factors are potential targets for development of drugs for cancer chemotherapy. Lou and coworkers (760) reported that transformation of fibroblasts resulted in an 8- to 18-fold increase in Sp1 expression and these transformed cells formed highly malignant tumors in athymic nude mouse xenograft models, whereas fibroblasts expressing low levels of Sp1 did not form tumors. In addition, ribozyme-dependent knockdown of Sp1 in the transformed cells decreased VEGF expression and increased apoptosis. Results in Figure 4.5 clearly showed that BA (10 and 20 mg/kg/d) inhibited tumor growth and final tumor weights in athymic nude

mice bearing LNCaP cells as xenografts. Moreover, examination of individual tumors clearly showed that Sp1, Sp3 and Sp4 proteins were highly expressed in tumors from control animals, whereas there was a marked decreased in expression of these proteins in tumors from mice treated with BA (Fig. 4.6A). Decreased VEGF expression also paralleled decreased Sp protein levels in tumors from BA-treated animals, although the magnitude of decreased VEGF was lower than that observed for Sp proteins in the latter treatment group. The antiangiogenic activity of BA was confirmed by decreased VEGF and CD31 staining in tumors from BA- vs. corn oil-treated mice (Fig. 4.6D), and this was consistent with the antiangiogenic activity previously reported for BA (761). Hematoxylin and eosin staining also showed that the vascularity of tumors in mice treated with BA was significantly lower than in tumors from corn oil treated mice (Fig. 4.5C).

We also examined Sp1, Sp3 and Sp4 protein expression in non-tumor tissue from control and BA-treated mice, and levels were barely detectable to non-detectable in both treatment groups; however, when gels were overexposed, it was evident that levels of Sp protein expression were similar in liver lysates from control and BA-treated mice (Figs. 4.6B and 4.6C). These results suggest that activation of proteasome-dependent degradation of Sp proteins by BA may be specific for cancer cells and tumors which overexpress these transcription factors in order to gain a growth and metastatic advantage over normal tissue. The critical role for Sp proteins in regulating expression of antiapoptotic and proangiogenic genes/proteins in tumors is supported by results of this study which also highlights the efficacy of anticancer drugs that target specific

transcription factors such as Sp proteins that are overexpressed in tumors. The effects of BA in this study were also observed for the closely related derivative betulonic acid (data not shown), and we conclude that BA and similar derivatives represent a novel class of transcription factor-targeting anticancer drugs. Moreover, since BA induces many other cytotoxic and proapoptotic effects in cancer cell lines (511, 517, 528, 532, 537-540, 762-765), it is likely that downrequlation of Sp proteins differentially contributes to the overall effect of this compound. Current studies with BA are investigating tumor-type similarities and differences in its mechanism of action and the development of more potent analogs for clinical applications in the treatment of cancer.

CHAPTER V

MICRORNA-27A IS A TARGET FOR ANTICANCER AGENT METHYL

2-CYANO-3,11-DIOXO-18β-OLEAN-1,12-DIEN-30-OATE IN COLON CANCER

CELLS

INTRODUCTION

MicroRNAs (miRNAs) are 20 to 25 bp 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 (766, 767). MiRNA

interactions with mRNA requires the overlap of 6 to 8 base pairs and, due to this

relatively low stringency, computational studies show that miRNAs can potentially

interact with several hundred mRNAs.

Despite this lack of specificity, miRNAs have a profound effect on gene

expression and cellular homeostasis and, in cancer cells, expression of several critical

oncogenes and tumor suppressor genes are regulated by miRNA expression (610, 768-

770). miR-221 and miR-222 target the cyclin-dependent kinase inhibitor p27 (770) and

miR-21 decreases expression of several mRNAs including the tumor suppressor gene

tropomyosin 1 (610).

Recent studies in this laboratory showed that miR-27a targets ZBTB10 mRNA, a

putative zinc finger protein that suppresses specificity protein (Sp) transcription factors

and Sp-dependent gene expression (585). The Sp transcription factors Sp1, Sp3 and Sp4

are highly expressed in cancer cell lines, 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* (331, 547, 579-581, 583, 584).

Betulinic acid and the non-steroidal anti-inflammatory drug tolfenamic acid inhibit prostate and pancreatic cell and tumor growth through activation of proteasome-dependent degradation of Sp1, Sp3 and Sp4 proteins (583, 584). In this study, we show that methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (β-CDODA-Me) is highly cytotoxic to colon cancer cells and also decreases Sp and Sp-dependent genes and proteins. However, these effects are proteasome-independent. We now show for the first time that β-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 are complemented by inhibition of tumor growth and decreased miR-27a expression in athymic nude mice bearing RKO cells as xenografts and treated with β-CDODA-Me 915 mg/kg/d).

MATERIALS AND METHODS

Plasmids, antibodies and reagents

Sp1 and Sp3 promoter constructs were kindly provided by Drs. Carlos Cuidad and Veronique Noe (University of Barcelona, Barcelona, Spain). The pVEGF-133 construct contain VEGF promoter insert (positions -131 to +54) linked to luciferase reporter gene. The pSurvivin-269 was kindly provided by Dr. M. Zhou (Emory University, Atlanta, GA). The pCMV6-XL4-ZBTB10 expression vector and empty

vector (pCMV6-XL4) were from Origene (Rockville, MD). Antibodies for Sp1, Sp3, Sp4, VEGF and VEGFR1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). c-PARP and survivin antibodies were from Cell Signaling Technology Inc. (Danvers, MA). Monoclonal β-actin antibody was purchased from Sigma-Aldrich. Reporter lysis buffer, and luciferase reagent for luciferase studies were supplied by Promega (Madison, WI). β-Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA), and LipofectAMINE 2000 reagent was purchased from Invitrogen (Carlsbad, CA). Western Lightning chemiluminescence reagent was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). The PPARγ antagonist N-(4'-aminopyridyl)-2-chloro-5-nitrobenzamide (T007) were synthesized in this laboratory, and their identities and purity (>98%) were confirmed by gas chromatography-mass spectrometry.

Cell proliferation and transfection assay and western blot analysis

RKO and SW480 colon cancer cells (2×10^4 per well) were plated in 12-well plates and allowed to attach for 24 hr. The medium was then changed to DMEM/Ham's F-12 medium containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or different concentrations of the compound were added. Fresh medium and compounds were added every 48 hr, and cells were then trypsinized and counted after 48 and 96 hr using a Coulter Z1 cell counter. Transfection experiments in RKO and SW480 cells used 0.4 μg of reporter gene constructs and 0.04 μg of β-Gal and LipofectAMINE 2000 reagent (Invitrogen). Results of cell proliferation and transfection studies are expressed as means ± S.E. for at least three replicate determinations for each treatment group.

Western blots were determined with whole cell lysates essentially as described (331, 579-581, 583).

Northern blot analysis

For miRNA analysis, 20 μg total RNA per lane was electrophoresed on 15% TBE urea polyacrylaminde gel (Invitrogen), electrophoretically transferred in 0.5X TBE at 300 mA for 45 minutes to GeneScreen Plus membrane (PerkinElmer, Boston, MA), UV cross-linked, and hybridized in ULTRAhyb-Oligo hybridization buffer (Ambion, Austin, TX) at 42 °C with ^{32}P end-labeled DNA oligonucleotides complementary to the miRNA under examination. Blots were washed at 42 °C in 2x SSC and 0.5% SDS for 30 min with gentle agitation.

Semiquantitative RT-PCR

RKO and SW480 colon cancer cells were transfected with either as-miRNA-27a or with pCMV6-XL4 control and pCMV6-XL4-ZBTB10 expression plasmid using Lipofectamine 2000 following manufacturer's protocol. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Inc.), and 2 μgm of RNA was used to synthesize cDNA using Reverse Transcription System (Promega). Primers were obtained from IDT and used for amplification were as follows: Sp1 (sense 5'- ATG GGG GCA ATG GTA ATG GTG G -3'; antisense 5'- TCA GAA CTT GCT GGT TCT GTA AG -3'), Sp3 (sense 5'- ATG ACT GCA GGC ATT AAT GCC G -3'; antisense 5'- TGT CTC TTC AGA AAC AGG CGA C -3'), Sp4 (sense 5'- ATG GCT ACA GAA GGA GGG AAA AC -3'; antisense 5'- TTG ACC AGG GGT GGA AGA ATT AC -3'), ZBTB10 (sense 5'- GCT GGA TAG TAG TTA TGT TGC -3'; antisense 5'- CTG AGT GGT TTG ATG GAC AGA G -3'),

VEGF (sense 5'- CCA TGA ACT TTC TGC TGT CT T -3'; antisense 5'- ATC GCA TCA GGG GCA CAC AG -3'), VEGFR1 (sense 5'- ATG GAG CGT AAG AAA GAA AAA ATG -3'; antisense 5'- TCA AGT ACC TCC TTT TCT CAC AT -3'), Survivin (sense 5'- ATG GCC GAG GCT GGC TTC ATC -3'; antisense 5'- ACG GCG CAC TTT CTT CGC AGT T -3') and GAPDH (sense 5'- ACG GAT TTG GTC GTA TTG GGC G -3'; antisense 5'- CTC CTG GAA GAT GGT GAT GG -3'). PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and visualized under UV transillumination.

Quantitative real-time PCR of mRNA and miRNA

cDNA was prepared from the RKO and SW480, cell lines using Reverse Transcription System (Promega). Each PCR was carried out in triplicate in a 20-μl volume using SYBR Green Mastermix (Applied Biosystems, Foster City, CA) 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 Applied Biosystems 7900HT Fast Real-time PCR System. The ABI Dissociation Curves software was used following a brief thermal protocol (95 °C for 15 s and 60 °C for 15 s, followed by a slow ramp to 95 °C) to control for multiple species in each PCR amplification. Values for each gene were normalized to expression levels of TATA-binding protein. The primers used for real-time PCR were Myt-1 (sense 5'- CCT TCC AAG AGT AGC TCC AAT TC -3'; antisense 5'- GCC GGT AGC TCC CAT ATG G -3') and TATA-binding protein (sense 5'- TGC ACA GGA GCC AAG AGT GAA -3'; antisense 5'- CAC ATC ACA GCT CCC CAC CA -3'). miRNA was extracted using the mirVana miRNA extraction kit (Applied Biosystems). Quantification of

miRNA (RNU6B and miRNA-27a) was done using the Taqman miRNA kit (Applied

Biosystems) according to the manufacturer's protocol with real-time PCR. U6 small

nuclear RNA was used as a control to determine relative miRNA expression.

Fluorescence-activated cell sorting analysis

RKO and SW480 cells were treated with either the vehicle (DMSO) or the

compound for 24 hr or with as-miR27a. Cells were trypsinized, centrifuged, and

resuspended in staining solution containing 50 µg/mL propidium iodide, 4 mmol/L

sodium citrate and 30 units/mL RNase. After incubation at room temperature for 1 hr,

cells were analysed on a FACS Vantage SE DiVa made by Becton Dickinson (BD),

using BD FACSDiva Software V4.1.1. Propidium iodide (PI) fluorescence was

collected through a 610SP bandpass filter, and list mode data were acquired on a

minimum of 50,000 single cells defined by a dot plot of PI width vs. PI area. Data

analysis was performed in BD FACSDiva Software V4.1.1 using PI width vs. PI area to

exclude cell aggregates.

Xenograft studies in athymic mice

Mice were used in accordance with institutional guidelines when they were 8 -

12 wk old. To produce tumors, RKO cells were harvested from subconfluent cultures by

a brief exposure to 0.25% trypsin and 0.02% EDTA and only suspensions consisting of

single cells with >90% viability were used for the injections. A xenograft was

established by s.c. injection of the cells (5×10^6) into the flanks of individual mice and,

after 6 days, mice were randomized into two groups of 5 mice per group and dosed by

oral gavage in corn oil or 15 mg/kg/d B-CDODA-Me 5 days a week for 22 days. The

mice were weighed, and tumor size was measured every third day with calipers to permit

calculation of tumor volumes: $V = LW^2/2$, where L and W were length and width,

respectively. Final tumors weights were determined at the end of the dosing regimen.

Tumor tissues and selected body organs (liver and kidney) were either stored in

RNAlater solution (per manufacture's recommendations) for later microRNA analysis,

snap frozen and stored at -80°C, or fixed in 10% formalin and embedded in paraffin.

RESULTS

β-CDODA-Me is a PPARγ agonist in colon cancer cell lines (312). Although β-

CDODA-Me decreased proliferation (Fig. 5.1A) and induced apoptosis (Fig. 5.1B) in

RKO and SW480 cells, these responses were not affected after cotreatment with the

PPARγ agonist T007, and receptor-independent effects have been observed for other

PPAR agonists in colon cancer cells (302). Recent studies with tolfenamic acid and the

structurally-related triterpenoid betulinic acid show that many of the growth inhibitory

and proapoptotic responses in pancreatic and prostate cancer cells are due to decreased

expression of Sp proteins (581, 583, 584). Results summarized in Figure 5.1C show that

β-CDODA-Me induced a concentration- and time-dependent decrease in Sp1, Sp3 and

Sp4 proteins in RKO and SW480 cells and, in RKO cells, decreases were observed with

concentrations lower than 1.0 μM after treatment for 48 hr. The role of PPARγ and

activation of proteasomes in mediating the effects of β-CDODA-Me on Sp protein

expression was also investigated in RKO cells (Fig. 5.1D). β-CDODA-Me-induced

downregulation of Sp1, Sp3 and Sp4 in RKO cells was not affected after cotreatment

with the PPARγ antagonist T007 or the proteasome inhibitor lactacystin, and similar

results were observed in SW480 cells (data not shown). The proteasome inhibitor

MG132 also did not block Sp protein downregulation in RKO and SW480 cells treated

with β-CDODA-Me (data not shown), suggesting that β-CDODA-Me-dependent Sp

protein degradation is proteasome-independent.
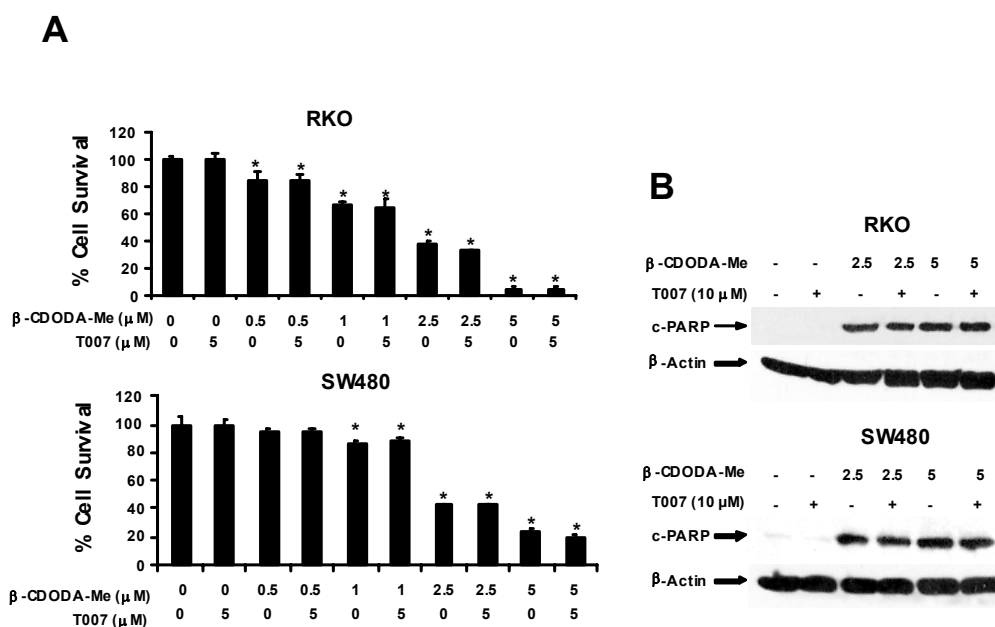
Fig. 5.1. β-CDODA-Me inhibits growth, induces apoptosis, and degradation of Sp proteins. (A) Decreased cell survival in RKO and SW480 cells. Cells were seeded and treated with solvent (DMSO) or different concentrations of β-CDODA-Me (0.5-5 μM) alone or in combination with T007 for 4 days. Cell survival is expressed as the percentage of β-CDODA-Me-treated cells remaining compared to DMSO (set at 100%). Results are expressed as means ± SE for three replicate determinations for each treatment group and significantly (p < 0.05) decreased survival is indicated by an asterisk. Induction of apoptosis (B) and decreased expression of Sp1, Sp3 and Sp4 (C, D). RKO and SW480 cells were treated with DMSO, β-CDODA-Me (1-5 μM), T007 (10 μM), Lactacystin (2 μM), or combinations as indicated for 24 hr or 48 hr and whole cell lysates were analyzed by Western blot analysis as described in the Materials and Methods.
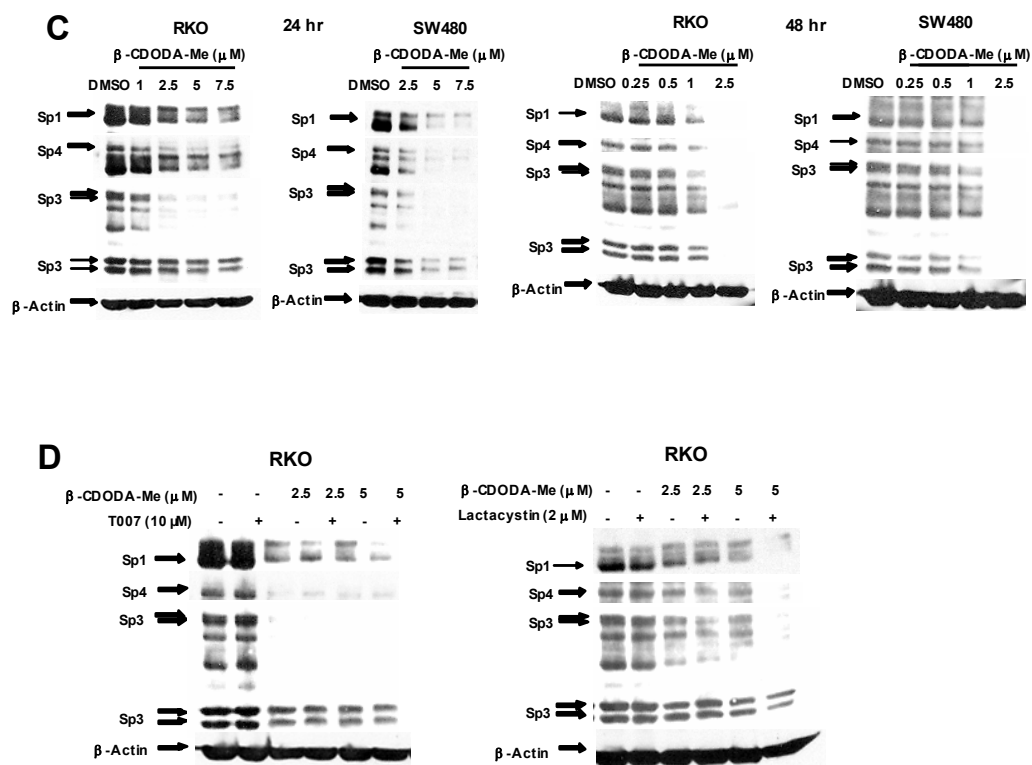
Fig. 5.1 Continued

Using RKO cells as a model, we showed that β-CDODA-Me induced caspase-
dependent PARP cleavage and decreased expression of at least three Sp-dependent
proteins including survivin, VEGFR1 (Flt-1), and VEGF (Fig. 5.2A). Figure 5.2B
shows that β-CDODA-Me also decreased expression of Sp1, Sp3 and Sp4 mRNA levels
after treatment for 24 hr, and similar effects were observed for mRNA levels of the Sp-
dependent genes *VEGFR1, VEGF and survivin* (Fig. 5.2B). Both the Sp1 and Sp3
promoters contain GC-rich sites, and Figure 5.2C shows that β-CDODA-Me decreased
luciferase activity in RKO cells transfected with pSp1For4, pSp1For2 and pSp1For1

constructs which contain the -751 to -20, -281 to -20, and -146 to -20 regions

(respectively) of the Sp1 gene promoter. Similarly, β-CDODA-Me also decreased

luciferase activity in RKO cells transfected with pSp3For5 and pSp3For2 constructs

which contain the -417 to -38 and -213 to -38 regions (respectively) of the Sp3 gene

promoter. These results demonstrate that β-CDODA-Me decreases Sp1, Sp3 and Sp4

transcription.
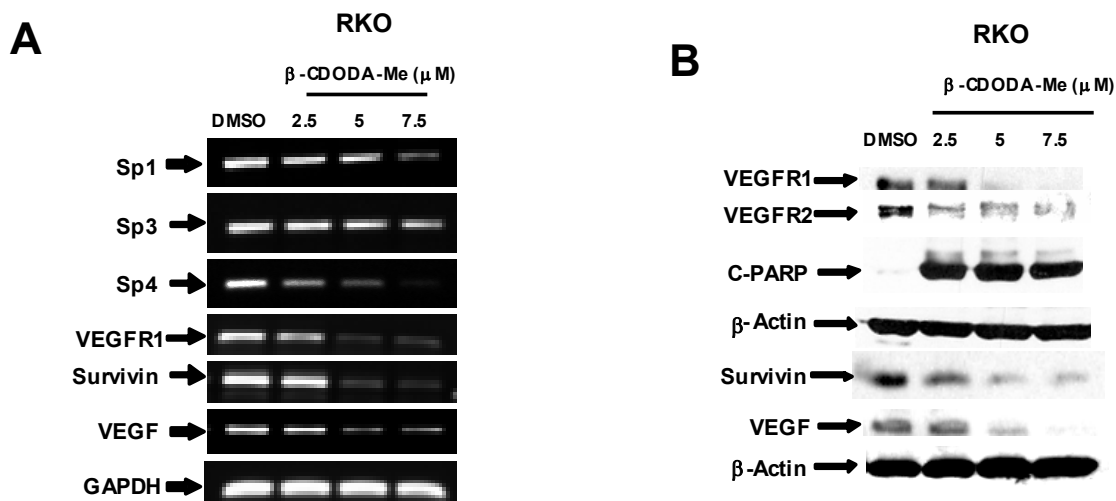
Fig. 5.2. Effects of β-CDODA-Me on Sp and Sp-dependent expression. β-CDODA-Me decreases expression of Sp1, Sp3 and Sp4 proteins (A) and expression of angiogenic/survival genes (A) and proteins (B). RKO cells were treated with different concentrations of β-CDODA-Me and after 24 hr, mRNA and protein were extracted and analyzed by semi-quantitative RT-PCR and Western blots, respectively, as described in the Materials and Methods. β-CDODA-Me decreases Sp1 (C) and Sp3 (D) promoter activity. RKO cells were transfected with various constructs, treated with different concentrations of β-CDODA-Me, and luciferase activity determined as described in the Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group and significantly (p < 0.05) decreased activity is indicated by an asterisk.
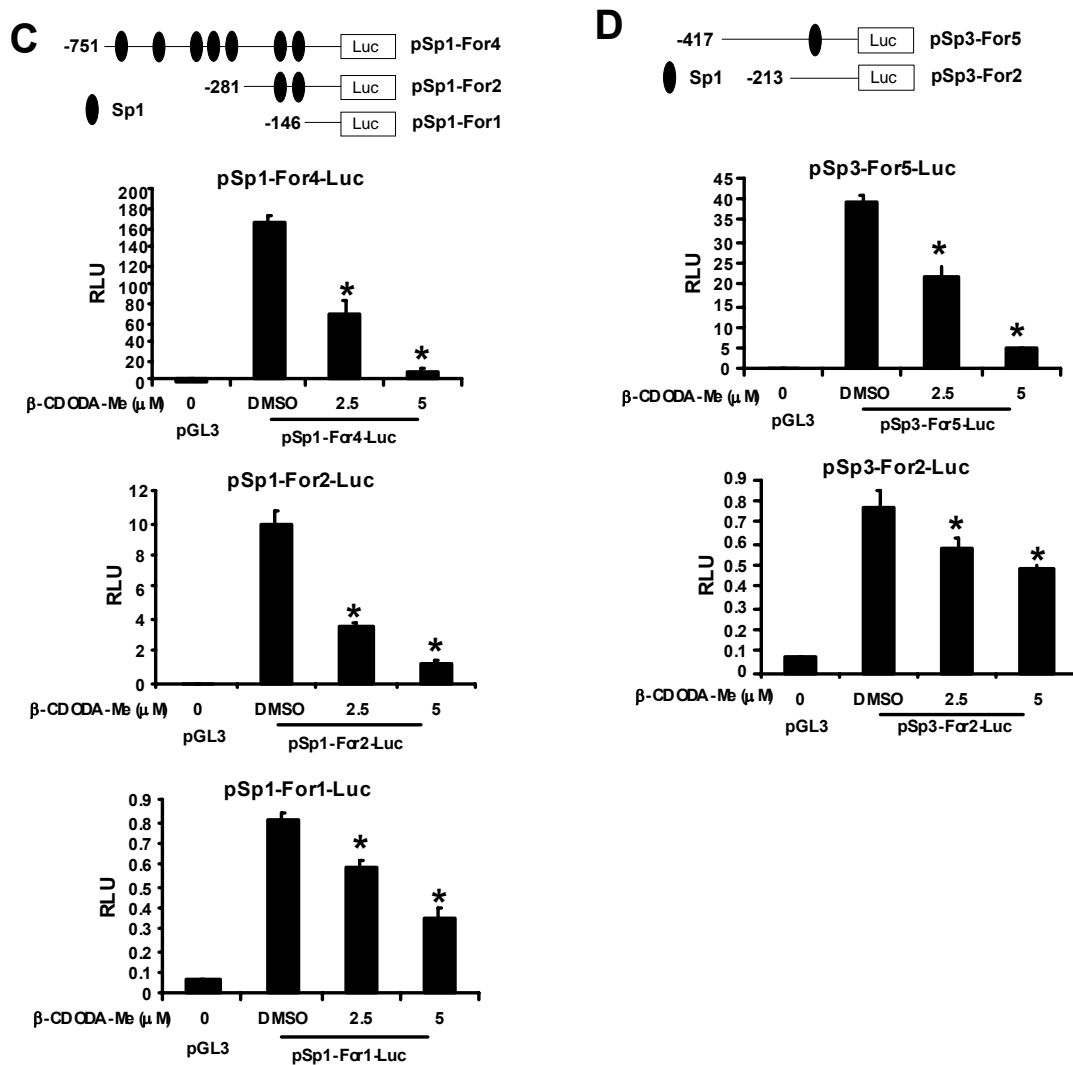
Fig. 5.2 Continued

It was recently reported that microRNA-27a (miR-27a) suppresses ZBTB10 mRNA

levels in breast cancer cells and treatment with antisense miR-27a (as-miR-27a)

increases expression of ZBTB10 and decreases expression of Sp mRNA and proteins
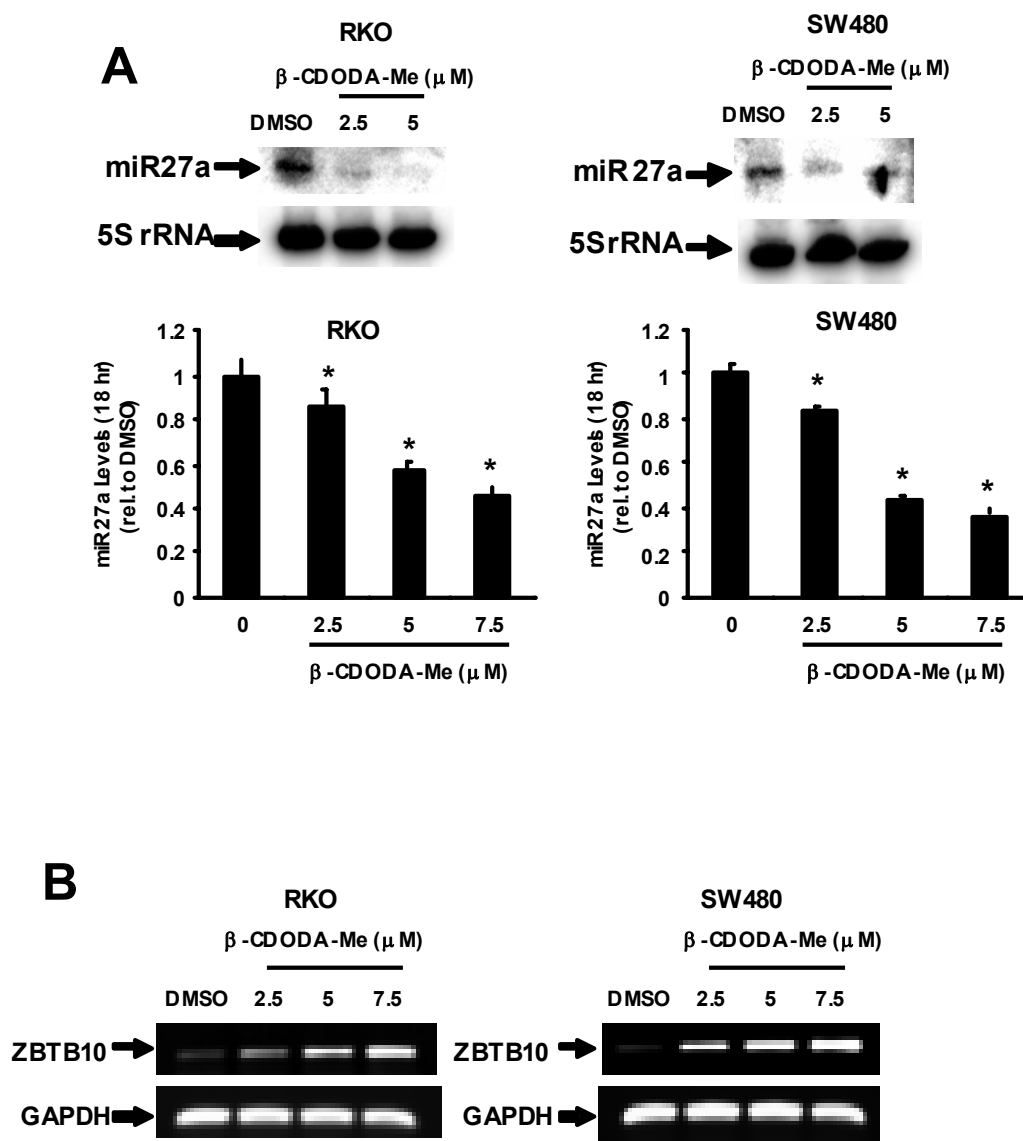
Fig. 5.3. Effects of β-CDODA-Me and as-miR27a. β-CDODA-Me decreases miR-27a (A) and increases ZBTB10 (B) expression. RKO and SW480 colon cancer cells were treated with DMSO or different concentrations of β-CDODA-Me and after 18 hr, total RNA was extracted and analyzed for miR27a by Northern blot analysis and real time PCR and ZBTB10 by semi-quantitative RT-PCR as described in the Materials and Methods. (C) as-miR27a decreases Sp1, Sp4 and Sp3 proteins. RKO cells were transfected with 50 and 100 ng as-miR27a and after 24 hr, whole cell lysates were analyzed by Western blot analysis for Sp1, Sp4 and Sp3 proteins as described in the Materials and Methods.
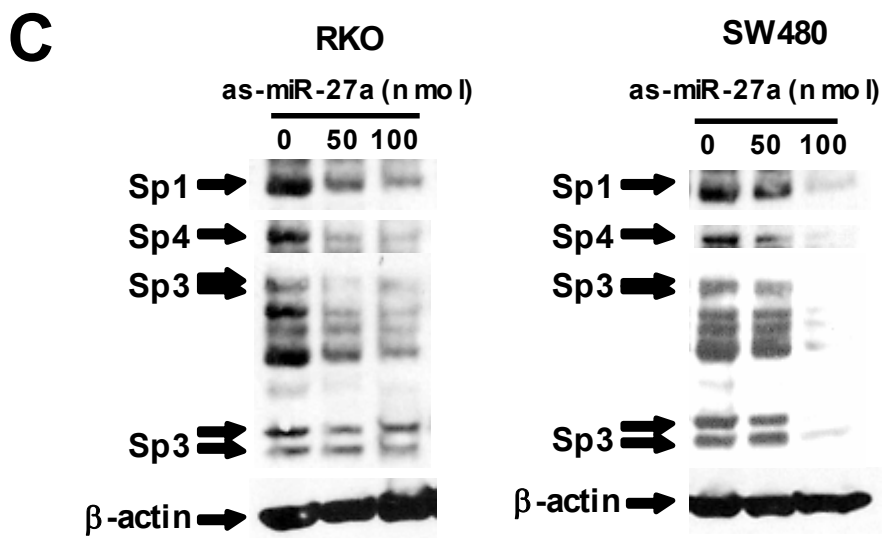
Fig. 5.3 Continued

(585). Results illustrated in Figure 5.3A show that β-CDODA-Me decreased miR-27a in

RKO and SW480 cells as determined by Northern blot analysis (top) and this was

confirmed by quantitative real time PCR (bottom). In addition, treatment of RKO or

SW480 cells with β-CDODA-Me also induced ZBTB10 levels (Fig. 5.3B). Thus, the

effects of β-CDODA-Me on miR-27a and ZBTB10 expression in colon cancer cells are

identical to those observed in breast cancer cells transfected with as-miR-27a which also

increases ZBTB10 and decreases Sp protein expression (585). Results in Figure 5.3C

confirm that as-miR-27 also decreased expression of Sp1, Sp3 and Sp4 protein levels in

RKO and SW480 colon cancer cells.

The direct effects of ZBTB10 as a "putative" Sp repressor were further

investigated in colon cancer cells transfected with ZBTB10 expression plasmid.

Transfection of SW480 and RKO cells with 2 or 4 µg ZBTB10 expression plasmid

decreased Sp1, Sp3 and Sp4 mRNA levels and decreased the Sp-dependent VEGF and

survivin mRNA levels (Fig. 5.4A). These responses were more pronounced in RKO

cells and this may be due, in part, to higher basal expression of ZBTB10 in SW480 than

in RKO cells. These differences between the two cell lines were less apparent for the

effects of ZBTB10 on Sp and Sp-dependent proteins (Fig. 5.4B). ZBTB10

overexpression decreased levels of Sp1, Sp3 and Sp4 proteins, survivin and VEGF and
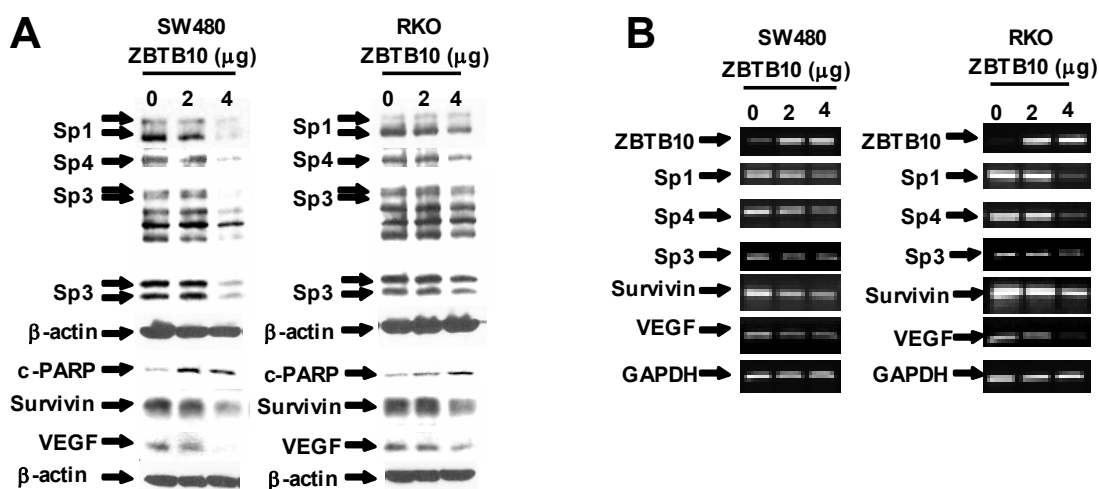
Fig. 5.4. ZBTB10 decreases expression of Sp proteins and Sp-dependent angiogenic and survival genes. ZBTB10 expression decreases expression of Sp and angiogenic/survival proteins (A) and mRNA (B). RKO and SW480 cells were transfected with ZBTB10 expression plasmid and after 24 hr, protein and mRNA were extracted and analyzed by Western blots and semi-quantitative RT-PCR, respectively, as described in the Materials and Methods. ZBTB10 expression decreases Sp1 and Sp3 (C) and VEGF and survivin (D) promoter activity. RKO cells were transfected with various constructs and ZBTB10 expression plasmid, and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group and significantly (p < 0.05) decreased activity is indicated by an asterisk.

C

pSp1-For4-Luc

pSp3-For5-Luc

D

pVEGF-131-Luc

pSVV-259-Luc
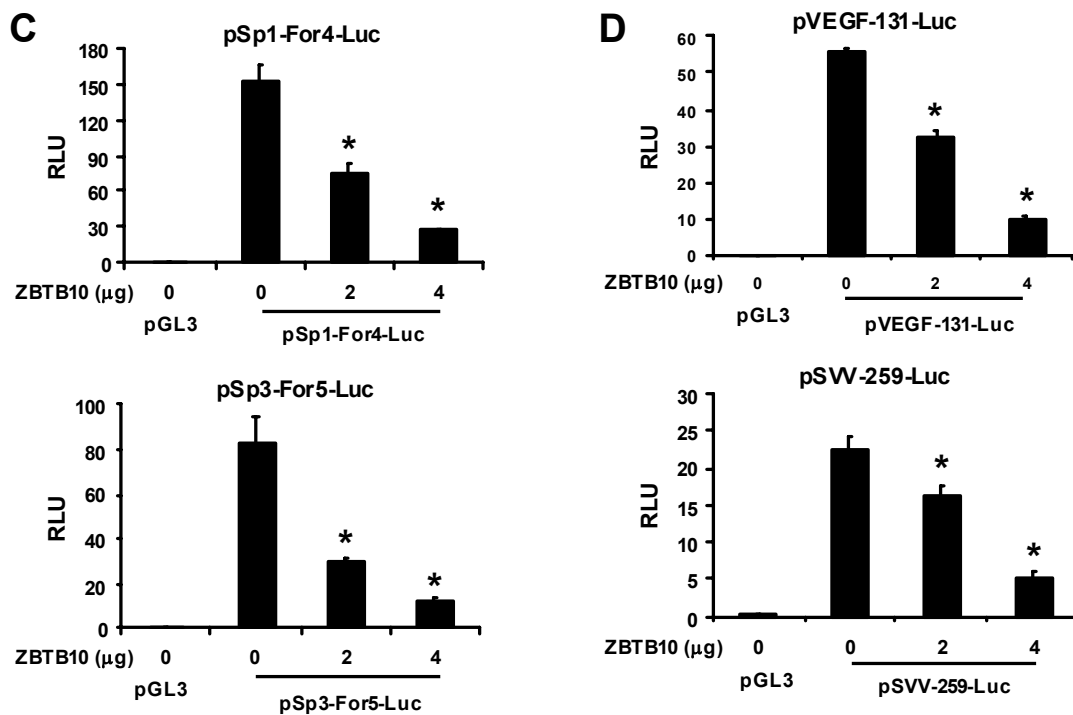
Fig. 5.4 Continued

induced PARP cleavage in these cells. The effects of ZBTB10 on luciferase activity in RKO cells transfected with constructs containing GC-rich Sp1 and Sp3 gene promoter inserts (Fig. 4C) and VEGF and survivin promoter inserts (Fig. 5.4D) complemented the effects of ZBTB10 on their respective mRNAs and proteins (Figs. 5.4A and 5.4B). Luciferase activity was decreased in RKO cells transfected with all constructs and similar results were observed in SW480 cells.

Figure 5.5A summarizes the effects of β-CDODA-Me on distribution of RKO and SW480 cells in G_0/G_1, S and G_2/M phases of the cell cycle. Compared to treatment with
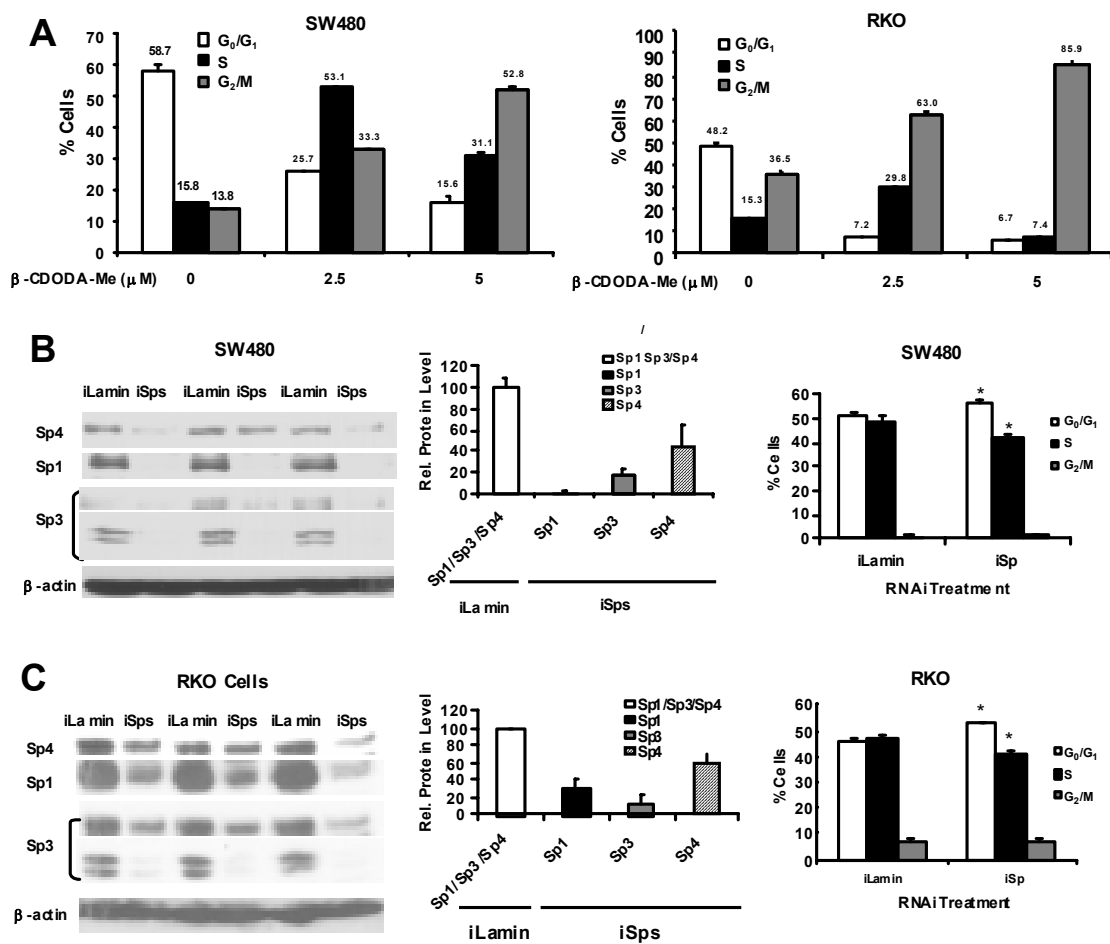
Fig. 5.5. Modulation of cell cycle progression. (A) Effects of β-CDODA-Me. RKO and SW480 cells were treated for 24 hr with DMSO (0), 2.5 and 5.0 µM β-CDODA-Me, and analyzed by FACS analysis as described in the Materials and Methods. iSp modulates Sp protein expression and the cell cycle in SW480 (B) and RKO (C) cells. Cells were transfected with iSp, a combination of small inhibitory RNAs for Sp1, Sp3 and Sp4 or a non-specific oligonucleotide (iLamin), and analyzed for Sp proteins by Western blots (to confirm Sp knockdown) and FACS analysis as described in the Materials and Methods. (D) as-miR-27a modulates the cell cycle. SW480 and RKO cells were transfected with different amounts of as-miR-27a and, after 48 hr, analyzed by FACS as described in the Materials and Methods. All experiments in this Figure [(A) - (D)] were repeated three times, and results are expressed as means ± SE. Significant (p < 0.05) changes compared to untreated (0) or iLamin-treated cells are indicated by asterisks.

D
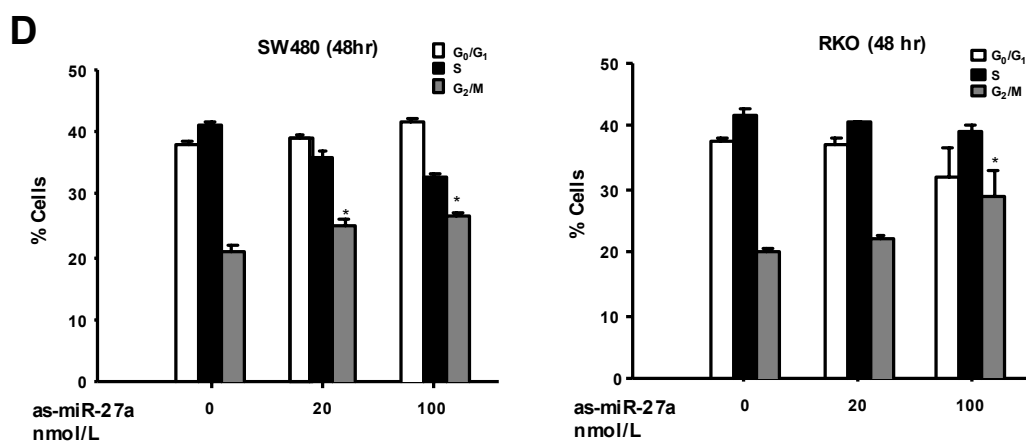
Fig. 5.5 Continued

DMSO, β-CDODA-Me induced a concentration-dependent decrease in the

percentage of cells in G_0/G_1 and an increase of cells in G_2/M. The percentage of cells in

S phase increased and then decreased after treatment with 2.5 and 5.0 μM β-CDODA-

Me, respectively; however, the dominant effects of β-CDODA-Me were associated with

a block in progression through G_2/M. The potential role of Sp protein degradation on

mediating the effects of β-CDODA-Me on distribution of cells in different phases of the

cell cycle was determined by RNA interference using a combination of small inhibitory

RNAs for Sp1 (iSp1), Sp3 (iSp3) and Sp4 (iSp4) as previously described for knockdown

of these proteins in other cancer cell lines (331, 547, 579-581, 583, 771). Transfection

of SW480 (Fig. 5.5B) and RKO (Fig. 5.5C) with iSp1/iSp3/iSp4 (combined; iSp)

significantly decreased expression of all three proteins (least efficiency observed for

Sp4) and, compared to the results for iLamin (non-specific RNA), Sp knockdown caused

a significant G_0/G_1 to S phase arrest (Figs. 55B and 5.5C). Thes.e results are comparable

to previous studies in MCF-7 breast cancer cells transfected with iSp1 (578); however,

the data were in contrast to the effects of β-CDODA-Me which induced a G_2/M arrest in

both RKO and SW480 cells (Fig. 5.5A). Since β-CDODA-Me and as-miR-27a induced

similar responses in colon cancer cells (Figs. 5.1 – 5.4), we also investigated the effects

of as-miR-27a on distribution of RKO and SW480 cells in G_0/G_1, S and G_2/M phases.

The results (Fig. 5.5D) demonstrate that like β-CDODA-Me, as-miR-27a induced a

G_2/M arrest in colon cancer cells. Transfection of as-miR-27a (100 nM RKO; 200 nM

SW480) increased accumulation of cells in G_2/M and this was accompanied by a

decrease in percentage of cells in S (SW480) and G_0/G_1 (RKO) phases. However, the

magnitude of the G_2/M arrest observed in colon cancer cells transfected with as-miR-27a

was lower than observed for β-CDODA-Me, suggesting that the compound-induced

response may also be due to other factors.

As-miR-27a also arrests MDA-MB-231 breast cancer cells in G_2/M phase and

this is due to upregulation of Myt-1 (585) which is a target for miR-27a and catalyzes

phosphorylation of cdc2 to inhibit progression through the G_2/M checkpoint enzyme

(772). Results in Figure 5.6A show that β-CDODA-Me induced Myt-1 mRNA

expression in RKO and SW480 cells, and this was accompanied by the time-dependent

induction of cdc2 phosphorylation as previously described in breast cancer cells

transfected with as-miR-27a (585). Myt-1 catalyzed inactivation of cdc2 by

phosphorylation of tyrosine-15 and this resulted in G_2/M arrest. Wee-1 is a potential
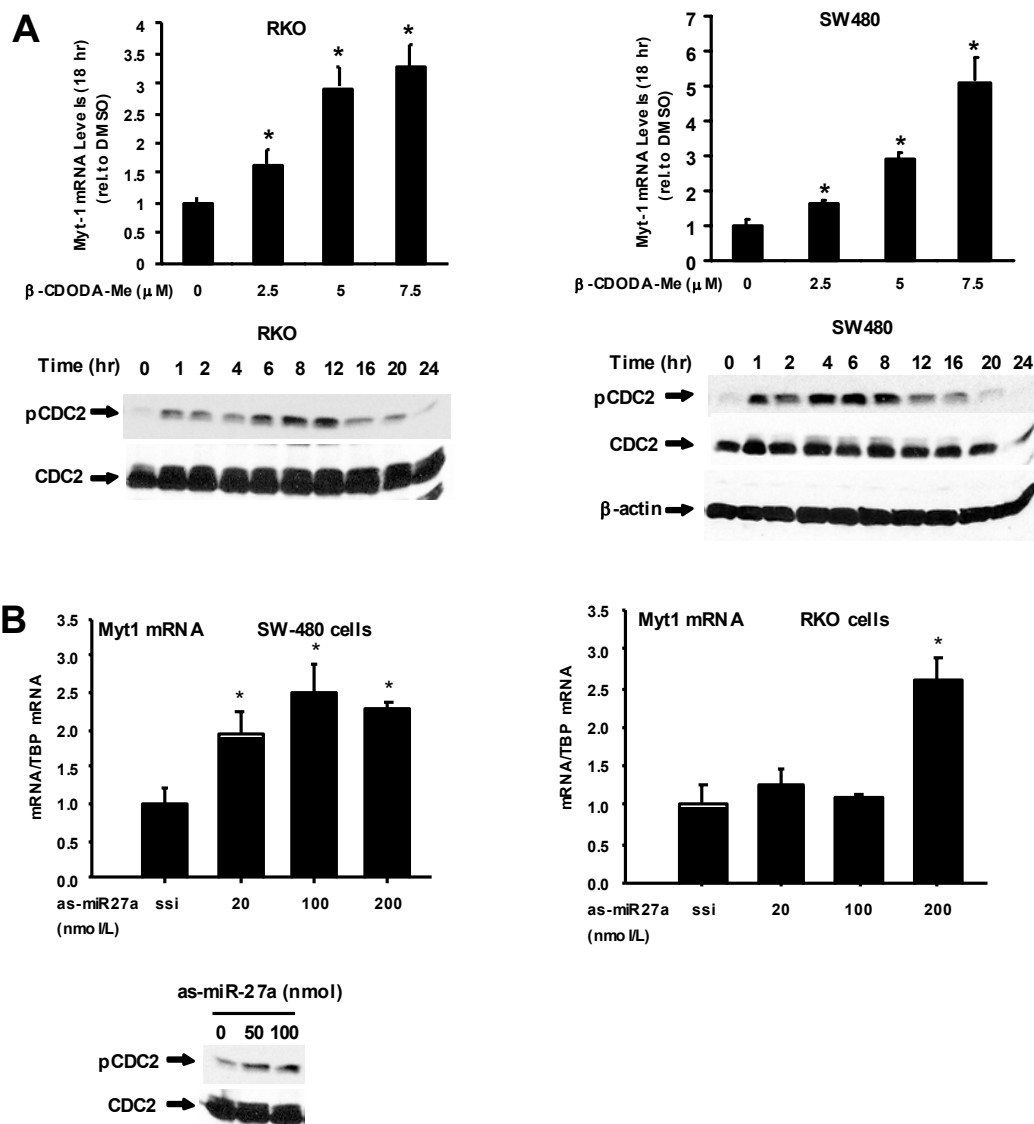
Fig. 5.6. *In vitro* and *in vivo* effects on G2/M and tumor growth. Effects of β-CDODA-Me (A) and as-miR-27a (B) on Myt-1 and cdc2 phosphorylation. Colon cancer cells were treated with different amounts of β-CDODA-Me or as-miR-27a for the indicated times, and Myt-1 expression and cdc2 phosphorylation were determined by real time PCR or Western blots, respectively, as described in the Materials and Methods. β-CDODA-Me inhibits tumor growth (volume) (C) and weight/miR-27a expression (D) in a mouse xenograft model. Nude mice bearing RKO cells as xenografts were treated with corn oil (solvent control) or β-CDODA-Me (15 mg/kg/d), and tumor volumes, tumor weights, and miR-27a expression were determined as described in the Materials and Methods. Results are expressed as means ± SE for replicate (at least three or more) determinations for each treatment group, and significantly ($p < 0.05$) decreased tumor volume or weight is indicated by an asterisk.
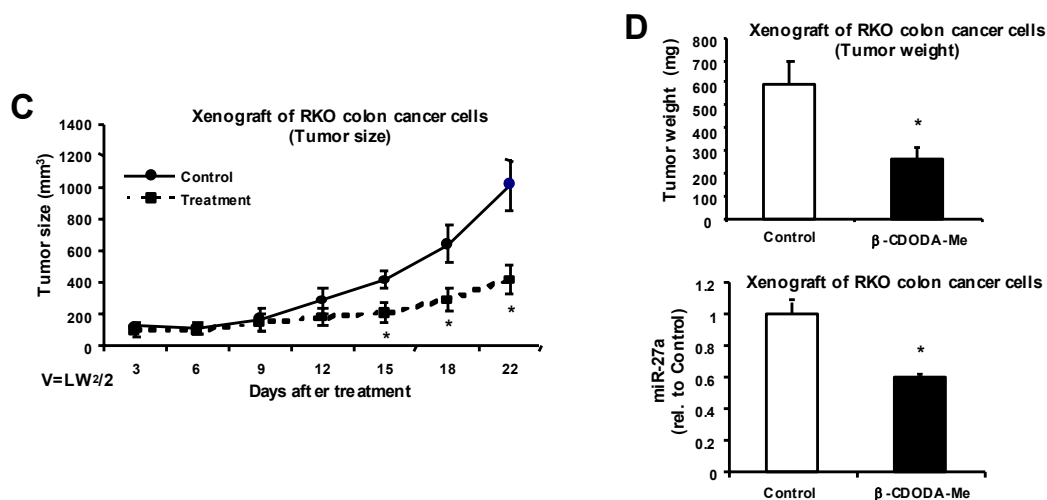
Fig. 5.6 Continued

miR-27a target that also inactivates cdc2; however, β-CDODA-Me and as-miR-27a did

not affect Wee-1 expression in SW480 or RKO cells (data not shown). We also

investigated the *in vivo* activity of β-CDODA-Me as an inhibitor of tumor growth in

 athymic nude mice bearing RKO cells as xenografts. β-CDODA-Me (15 mg/kg/d)

inhibited tumor growth (Fig. 5.6C) and tumor weight/miR-27a expression (Fig. 5.6D),

and this was not accompanied by any body or organ weight loss associated with toxic

side effects (data not shown). Thus, like other compounds such as tolfenamic and

betulinic acids that decrease Sp protein expression (583, 771), β-CDODA-Me inhibits

both colon cancer cell and tumor growth and represents a novel class of anticancer

agents that act through targeting miR-27a downregulation in colon cancer cells and

tumors.

DISCUSSION

Sp transcription factors are critical for early embryonic development and for regulating expression of multiple genes including those important for cell proliferation and differentiation (773). The age-dependent expression of Sp proteins has not been extensively investigated; however, a recent study reported that levels of these proteins decrease during cellular senescence and aging (774). Sp1 protein is overexpressed in several tumor types compared to non-tumor tissue, and Sp1 was a negative prognostic factor for cancer survival (775-780). For example, Sp1 is overexpressed in human gastric tumors compared to non-tumor tissue and overexpression of this protein in tumors is a predictor for a poor prognosis. Sp1 is also overexpressed in malignant human fibroblast cell lines and results of Sp1 overexpression or knockdown in fibroblasts and fibrosarcoma cells has established a causal linkage between Sp1 overexpression and malignant transformation (781).

Research in this laboratory has shown Sp1, Sp3 and Sp4 are highly expressed in cancer cell lines, and RNA interference studies clearly demonstrate that these transcription factors cooperatively regulate prosurvival, growth promoting, and angiogenic genes, suggesting that targeting Sp protein degradation may be a viable strategy for cancer chemotherapy (331, 547, 579-581, 583, 771, 782).

Both betulinic acid and tolfenamic acid inhibit growth of prostate and pancreatic tumors and cells, and these effects are linked to induction of proteasome-dependent degradation of Sp1, Sp3 and Sp4 proteins which is accompanied by decreased

expression of Sp-dependent genes such as survivin, VEGF and VEGFR1 (580, 581, 583, 584). However, ongoing studies with betulinic and tolfenamic acids in other cancer cell lines indicate that their effects on decreased Sp protein and mRNA levels are primarily proteasome-independent. Results in Figure 5.1 show that β-CDODA-Me induced apoptosis and inhibited SW480 and RKO colon cancer cell growth and the responses were not inhibited by the PPARγ antagonist T007 (Figs. 5.1A and 5.1B) or other PPARγ antagonists (data not shown). β-CDODA-Me also decreased Sp1, Sp3 and Sp4 protein expression in SW480 and RKO cells, and these responses were not inhibited by T007 or proteasome inhibitors (Figs. 5.1C and 5.1D) but were related to decreased Sp1, Sp3 and Sp4 transcription factors (Fig. 5.2). β-CDODA-Me decreased Sp proteins and mRNA levels and also decreased protein and mRNA levels of the Sp-dependent genes VEGF, VEGFR1 and survivin (Figs. 5.3A and 5.3B). Interestingly, β-CDODA-Me decreased transactivation in colon cancer cells transfected with pSp1For1-luc and pSp3-For2-luc (Figs. 5.3C and 5.3D) which do not contain GC-rich sequences, suggesting modulation of other trans-acting factors and these are currently being investigated.

The effects of β-CDODA-Me on Sp proteins and Sp-dependent genes in colon cancer cells were reminiscent of the effects of antisense miR-27a (as-miR-27a) in ER-negative MDA-MB-231 breast cancer cells (782). In MDA-MB-231 cells transfected with as-miR-27a, there was a parallel increase in a zinc finger transcription factor, ZBTB10, which also binds GC-rich promoter sequences (619) and inhibits expression of Sp1, Sp3 and Sp4 and Sp-dependent genes (585)f. Figures 5.3A and 5.3B show that β-CDODA-Me decreased miR-27a and increased ZBTB10 expression in RKO and SW480

colon cancer cells. Moreover, as previously reported in MDA-MB-231 cells, as-miR-27a or ZBTB10 overexpression decreased Sp proteins and mRNA levels and Sp-dependent genes (e.g. survivin and VEGF) in colon cancer cells (Figs. 5.3A and 5.3B). The effects of β-CDODA-Me on distribution of RKO and SW480 cells in the G_0/G_1, S and G_2/M phases of the cell cycle (Fig. 5.5A) showed that the dominant effect was accumulation of cells in G_2/M. Decreased Sp1 expression in MCF-7 cells by RNA interference arrests cells in G_0/G_1 (331) and, in colon cancer cells transfected with small inhibitory RNAs for Sp1, Sp3 and Sp4 (combined), we observed a significant block in G_0/G_1 to S phase progression but no effects on G_2/M (Figs. 5.5B and 5.5C). Similar results were observed in MDA-MB-231 breast cancer cells transfected with ZBTB10 (782). However, transfection of MDA-MB-231 or colon cancer cells with as-miR-27a resulted in G_2/M arrest (Fig. 5.5D), and this mimicked the effects of β-CDODA-Me (Fig. 5.5A). Growth arrest in colon cancer cells treated with β-CDODA-Me was greater than observed for as-miR-27a, suggesting that the compound may activate other pathways and these are currently being investigated.

Since miR-27a potentially targets both Myt-1 and Wee-1, two kinases that inhibit cdc2 and progression of cells through the G_2/M checkpoint, we investigated the effects of β-CDODA-Me on cdc2 and phospho-cdc2 expression. β-CDODA-Me induced Myt-1 but not Wee-1 expression in both RKO and SW480 cells (Fig. 5.6A), and this was accompanied by phosphorylation of cdc2 in RKO and SW480 cells. As-miR-27a also induced Myt-1 and cdc2 phosphorylation in these cell lines (Fig. 5.6B) as previously observed in MDA-MB-231 breast cancer cells (782). Thus, like as-miR-27a, β-

CDODA-Me acts through decreased expression of miR-27a, resulting in enhanced expression of ZBTB10 and Myt-1 which subsequently induce downstream growth inhibitory, proapoptotic and antiangiogenic genes and pathways in colon cancer cells. These *in vitro* responses induced by β-CDODA-Me were complemented by the inhibition of tumor growth and tumor weight in athymic nude mice bearing RKO cells as xenografts (Figs. 5.6C and 5.6D), and miR-27a expression was also decreased in tumors from β-CDODA-Me-treated animals compared to tumors from corn oil-treated mice.

In summary, results of this study show that β-CDODA-Me decreases expression of Sp proteins and Sp-dependent genes and induces G_2/M arrest in colon cancer cells, and these responses are due to repression of miR-27a and increased expression of ZBTB10 and Myt-1. β-CDODA-Me also decreased tumor growth and this was also accompanied by decreased miR-27a expression in the tumor and this represents one of the first *in vivo* examples of drug-miR interaction. Other compounds such as betulinic and tolfenamic acids also decrease Sp proteins in prostate and pancreatic cancer cells (581, 771), and there is evidence that a hydroxamic acid histone deacetylase inhibitor decreases expression of miR-27a and other miRs in SKBR3 cells(618). The similarities of these drugs to β-CDODA-Me and the effects of cancer cell context on their activities and their mechanisms of miR-27a downregulation are currently being investigated in this laboratory.

CHAPTER VI

2-CYANO-LUP-1-EN-3-OXO-20-OIC ACID, A CYANO DERIVATIVE OF

BETULINIC ACID, ACTIVATES PEROXISOME PROLIFERATOR-

ACTIVATED RECEPTOR γ IN COLON AND PANCREATIC CANCER CELLS*

INTRODUCTION

Lup-20(29)-ene-3β,28-diol (betulin) is a triterpene natural product found in extracts of many bushes and trees, and betulin can constitute up to 30% of the dry weight of bark from birch trees (783, 784). Betulin has been used in folk medicine for treating skin diseases; however, betulinic acid (BA), which is both a natural product and chemical oxidation product of betulin, 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 (783, 784). The antitumorigenic activities of BA has been extensively investigated. Studies show that this compound inhibits tumor growth through multiple pathways and these responses are also cancer cell/tumor-dependent (510, 511, 517, 528, 532, 533, 537-540, 783-788). Pisha and coworkers reported that BA selectively inhibited melanoma cancer cell and tumor growth and in in vivo studies, this was accompanied by minimal toxic side-effects at repeated doses of

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up to 500 mg/kg (510). Subsequent studies showed that BA was cytotoxic to many other cancer cell lines and this was associated with different activities (511, 517, 528, 532, 533, 537-540, 789-792). For example, BA induces apoptosis through decreased mitochondrial membrane potential, activation of mitogen-activated protein kinase, and modulation of nuclear factor κB (NFκB) (511, 793, 794).

Structural modifications of BA and other lupane-derived triterpenoids differentially affect their pharmacologic activities (534-536, 795-798). For example, 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 (799). 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 (534). These result 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 (234, 312, 463, 464, 655). Typical among these synthetic derivatives were 2-cyano-3,12-dioxo-18β-oleana-1,19-diene-28-oic acid (CDDO; synthesized from oleanolic acid) and 2-cyano-3,12-dioxo-18β-olean-1,12-diene-30-oic acid (β-CDODA; synthesized from glycyrrhetinic acid, a major constituent of licorice extracts). The high cytotoxicity of both β-CDODA, CDDO and related compounds was due, in part, to their peroxisome proliferator-activated receptor γ (PPARγ) agonists activity since ligands for this receptor are being developed as new anticancer drugs (659, 660).

Although there are multiple structural differences between BA and oleanolic acid (the synthetic precursor of CDDO and CDODA) , we hypothesized that introduction of a 2-cyano group into the lupane skeleton of BA would generate a new class of PPARγ agonists. Previous studies showed that 2-cyano-lup-1-en-3-oxo-20-oic acid (CN-BA), the 2-cyano derivative of 20(29)-dihydro betulinic acid, was highly cytotoxic to cancer cells, and in this study, we compared the effects of BA, CN-BA and the corresponding methyl ester (CN-BA-Me) (Fig. 1.19) in Panc-28 pancreatic and in colon cancer cell lines. Results of growth inhibition studies showed that both CN-BA and CN-BA-Me were more cytotoxic than BA in pancreatic and colon cancer cells. CN-BA and CN-BA-Me but not BA induced PPARγ-dependent transactivation; however, the receptor-dependent induction of p21, caveolin1 and Krüppel-like factor-4 expression was cell context and gene-dependent. These results demonstrate for the first time that CN-BA and CN-BA-Me are PPARγ agonists and their enhanced cytotoxicity compared to BA is due, at least in part, to activation of PPARγ. Moreover, the structure- and cell context-dependent activities of CN-BA and CN-BA-Me as PPARγ agonists suggest that these compounds are selective PPARγ modulators.

MATERIALS AND METHODS

Cell Lines and reagents

SW480, HT-29 and HCT-15 human colon cancer cells were kindly provided by Dr. Stan Hamilton (M. D. Anderson Cancer Center (Houston, TX). Panc-28 human pancreatic cancer cells and 3T3-L1 pre-adipocytes were obtained from American Type Culture Collection (Manassas, VA). SW480, HT-29 and Panc-28 cells were maintained

in Dulbecco's modified/Ham's F-12 (Sigma-Aldrich, St Louis, MO) with phenol red

supplemented with 0.22% sodium bicarbonate, 0.011% sodium pyruvate, 5% fetal

bovine serum, and 10 mL/L 100X antibiotic antimycotic solution (Sigma). HCT-15

cells were maintained in RPMI 1640 (Sigma) supplemented with 0.22% sodium

bicarbonate, 0.011% sodium pyruvate, 0.45% glucose, 0.24% HEPES, 10% fetal bovine

serum, and 10 mL/L of 100x antibiotic antimycotic solution (Sigma). Cells were

maintained at 37°C in the presence of 5% CO_2. Reporter lysis buffer and luciferase

reagent for luciferase studies were supplied by Promega (Madison, WI). β-

Galactosidase (β-Gal) reagent was obtained from Tropix (Bedford, MA), and

LipofectAMINE reagent was purchased from Invitrogen (Carlsbad, CA). The PPARγ

antagonist *N*-(4'-aminopyridyl)-2-chloro-5-nitrobenzamide (T007) (800) was synthesized

in this laboratory, and its identity and purity (>98%) was confirmed by gas

chromatography-mass spectrometry.

Synthesis of CN-BA and CN-BA-Me

CN-BA and CN-BA-Me were prepared from betulin (Sigma-Aldrich) based on

the previous methods (534). The synthesis from a key intermediate, methyl lup-2-

eno[2,3-*d*]isoxazol-28-oate, is briefly described and only definite peaks in proton NMR

are recorded. **Methyl Lup-2-eno[2,3-*d*]isoxazol-28-oate.** To a solution of methyl

lupan-2-hydroxymethylene-3-oxo-28-oate (350 mg, 0.70 mmoL) in ethanol (20 mL) and

water (1 mL), hydroxylamine hydrochloride (488 mg, 7.02 mmol) was added. The

reaction mixture was refluxed for 1 hr, cooled to room temperature and concentrated

under vacuum. Water was added to the reaction mixture and extracted with ethyl acetate

(2X). The organic layer was then washed with brine (2X), separated, and the crude

product was purified by a flash silica gel column using a solvent system of hexanes:ethyl

acetate (95:5) to yield methyl lup-2-eno[2,3-*d*]isoxazol-28-oate as a white cream colored

solid. ^{1}H NMR (400 MHz, CDCl$_3$): δ 8.00 (1H, s), 3.68 (3H, s), 1.31, 1.21, 0.99, 0.98,

0.83 (each 3H, s), 0.89 (3H, d, *J* = 6.8 Hz), 0.78 (3H, d, *J* = 6.8 Hz). ^{13}C NMR (100

MHz, CDCl$_3$): δ 177.6, 173.8, 151.1, 109.7, 57.8, 54.3, 52.0, 49.7, 49.6, 45.0, 43.4, 41.5,

39.7, 38.9, 38.1, 36.6, 35.6, 34.2, 32.8, 30.6, 30.5, 30.4, 29.5, 27.7, 23.8, 23.6, 22.2,

22.0, 19.6, 16.8, 16.5, 15.5, 15.4. **Methyl 2-Cyano-lup-3-hydroxy-2-en-28-oate.** To a

solution of lup-2-eno[2,3-*d*]isoxazol-28-oate (250 mg, 0.50 mmol) in ether (30 mL) and

methanol (15 mL) in an ice bath, 30% sodium methoxide in methanol (3071 mg, 56.87

mmol) was added drop wise. The reaction mixture was then stirred at room temperature

for 2 hr. After dilution with ether, the reaction mixture was washed with 5%

hydrochloric acid (2X). The organic layer was separated and worked up by standard

method to yield crude methyl 2-cyano-lup-3-hydroxy-2-en-28-oate (240 mg, 96%) as a

white solid, which was used for the next without further purification. ^{1}H NMR (400

MHz, CDCl$_3$): δ 3.90 (1H, m), 3.67 (3H, s), 1.27, 1.17, 0.98, 0.95, 0.83 (each 3H, s),

0.89 (3H, d, *J* = 6.8 Hz), 0.79 (3H, d, *J* = 6.8 Hz). **Methyl 2-Cyano-lup-1-en-3-oxo-20-**

oate (CN-BA-Me). A mixture of 2-cyano-lup-3-hydroxy-2-en-28-oate (230 mg, 0.46

mmol) and DDQ (117 mg, 0.52 mmol) in benzene (30 mL) was refluxed for 3 hr. The

reaction mixture was cooled in ice and filtered to remove reduced DDQ. The filtrate

was then concentrated under vacuum. The crude product was purified by a flash silica

gel column using a solvent system of benzene:acetone (98:2) to yield CN-BA-Me (170

mg, 74%) as a pale brown solid. ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.83 (1H, s), 3.66 (3H,

s), 1.25, 1.18, 1.12, 1.00, 0.95 (each 3H, s), 0.87 (3H, d, J = 6.7 Hz), 0.75 (3H, d, J = 6.6

Hz). ^{13}C NMR (100 MHz, CDCl$_3$): δ 199.0, 177.4, 171.6, 115.8, 114.6, 57.6, 53.3, 52.0,

49.4, 45.7, 44.8, 44.4, 43.6, 42.6, 41.5, 38.8, 37.9, 34.2, 32.6, 30.4, 30.2, 28.5, 27.3,

23.7, 23.5, 22.1, 22.0, 19.6, 19.1, 17.2, 15.4, 15.2. ESI-HRMS Calcd for (C$_{32}$H$_{47}$NO$_3$ +

H): 494.3634. Found: 494.3685. Anal. (C$_{32}$H$_{47}$NO$_3$) C, H. **2-Cyano-lup-1-en-3-oxo-20-**

oic acid (CN-BA). A mixture of **CN-BA-Me** (120 mg, 0.25 mmol) and lithiumiodide

(720 mg) in dimethylformamide (2.4 mL) was refluxed for 2 hr. The reaction mixture

was cooled to room temperature and 5% hydrochloric acid was added. The reaction

mixture was extracted with ethyl acetate (2X). The organic layer was then washed with

water (2X) followed by washings with brine (2X). The organic layer was separated and

worked up by standard method. The crude product was purified by a flash silica gel

column using a solvent system of hexanes:ethyl acetate (80:20) to yield CN-BA (93 mg,

80%) as a light yellow solid. ^{1}H NMR (400 MHz, CDCl$_3$): δ 10.29 (1H, broad s), 7.83

(1H, s), 1.27, 1.14, 1.04, 0.99, 0.99 (each 3H, s), 0.91 (3H, d, J = 6.8 Hz), 0.79 (3H, d, J

= 6.7 Hz). ^{13}C NMR (100 MHz, CDCl$_3$): δ 199.0, 182.1, 171.5, 115.8, 114.8, 57.5, 53.3,

49.3, 45.8, 44.8, 44.4, 43.7, 42.7, 41.5, 39.0, 38.1, 34.3, 32.7, 32.7, 30.5, 30.3, 28.6,

27.4, 23.8, 23.5, 22.2, 22.1, 19.6, 19.2, 17.3, 15.4, 15.3. ESI-HRMS Calcd for

(C$_{31}$H$_{45}$NO$_3$ + H): 480.3478. Found: 480.3540. Anal. (C$_{31}$H$_{45}$NO$_3$) C, H. CN-BA and

CN-BA-Me were > 97% pure by spectroscopic analysis.

Cell proliferation assay

This assay is performed in 12-well tissue culture plates at the concentration of 2

x 10^4 cells per well, using DMEM/Ham's F-12 media containing 2.5% charcoal stripped

FBS. The cells were counted on the initial day using Z1 cell counter (Beckman Coulter,

Fullerton, CA) and then the cells were treated either with vehicle (DMSO) or the

indicated triterpenoid compounds, each sample in triplicate. Every 48 hr, fresh medium

was added along with the indicated compounds. The count of the cells was taken after 2,

4 and 6 days. The results are expressed as means ± S.E for each set of triplicate.

Mammalian two hybrid assay

The GAL4 reporter construct containing 5 x GAL4 response elements (p GAL4),

kindly provided by Dr.Marty Mayo (University of North California, Chapel Hill, NC).

The GAL4-coactivator fusion plasmids pM-SRC1, pMSRC2, pMSRC3, pM-DRIP205,

pM-CARM-1 and PPARγ-VP16 fusion plasmid (Vp-PPARγ) containing the DEF region

of the PPARγ (amino acids 183 - 505) fused to the pVP16 expression vector were kindly

provided by Dr.Shigeaki Kato (University of Tokyo, Tokyo, Japan). SW480 colon

cancer cells were plated in 12-well tissue culture plates at 1 x 10^5 cells per well in

DMEM/Ham's F-12 medium supplemented with 2.5% charcoal stripped FBS. After

allowing them to adhere for overnight, transient transfections were carried out with

GAL4-Luc (0.4 μg), β-GAL (0.04 μg), VP-PPARγ (0.04 μg), pM-SRC1 (0.04 μg), pM-

PGC-1 (0.04 μg), pM-SMRT (0.04 μg), pM-TRAP220 (0.04 μg), pM-DRIP205 (0.04

μg), pMCARM1 (0.04 μg) using LipofectAMINE2000 (Invitrogen) following the

manufacturer's guidelines. After 6 hr of transfection, cells were treated in triplicate

either with vehicle (DMSO) or the indicated compound suspended in complete medium

for 20-24 hr. One hundred μL per well of 1x Reporter Lysis Buffer (Promega) was used

to lyse the cells and 30 μl of this lysate was used to perform the luciferase and β-GAL

assays using Lumicount (Perkin-Elmer Life and Analytical Sciences, Boston, MA). The

luciferase activities obtained were normalized to the β-gal activity.

Transfections

Cells were seeded on to the 12-well plates and 0.4 μg of GAL4-Luc, 0.04 μg of

β-GAL, 0.04 μg of GAL4DBD-PPARγ, 0.4 μg of p21-luc(FL) containing -2325 to +8

insert, 0.4 μg of p21-luc (-124) containing -124 to +8 insert and 0.4 μg of p21-LUC (-

60) containing -60 to +8 insert were transfected using LipofectAMINE reagent

(Invitrogen) following the manufacturer's protocol. Cells were treated either with

vehicle or respective compounds suspended in complete medium after 6 hr of

transfection. Cell lysate is extracted after 20-22 hr by adding 100 μl of 1x reporter lysis

buffer per well and 30 μl of this extract is used to quantitate the luciferase activity using

Lumicount (Perkin-Elmer Life and Analytical Sciences). Each experiment is done in

triplicate and the results are normalized to the β-GAL activity.

Western blot analysis

SW480, HT-29, HCT-15 and Panc-28 (3×10^5) colon cancer cells were seeded in

6-well tissue culture plates in DMEM/Ham's F-12 medium containing 2.5% charcoal-

stripped FBS. Protein is extracted from the cells treated either with vehicle or indicated

compounds suspended for 24 hr except for caveolin-1 protein which was done for 72 hr.

Samples were extracted in high salt buffer [50 mmol/L HEPES, 500 mmol/L NaCl, 1.5

mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, and 1% Triton X-100 (pH 7.5), and 5

μL/mL protease inhibitor cocktail (Sigma-Aldrich)]. Samples were incubated at 100°C

for 2 min, separated on either 10% or 12% SDS-PAGE gels and then transferred to polyvinylidene difluoride membrane (PVDF; Bio-Rad, Hercules, CA). The PVDF membrane was blocked in 5% TBST-Blotto (10mM Tris HCl, 150 mM NaCl, pH 8.0, 0.05% Triton X-100, and 5% nonfat dry milk) for about 30 min and was then incubated in fresh 5% TBST-Blotto with 1:1000 for caveolin-1 [Santa Cruz Biotechnology (Santa Cruz, CA)], 1:1000 for p21(BD Pharmingen, Frank lakes, NJ) and 1: 10000 for β-actin (Sigma) primary antibody overnight with gentle shaking at 4°C. After washing with TBST for 10 min, the membrane was incubated with respective secondary antibody (1:5000) (Santa cruz, CA) in 5% TBST-Blotto for 3 hr. The membrane is then washed with TBST for 10 min, incubated with chemiluminiscence reagent from Perkin Elmer for one min and then exposed to Kodak X-OMAT AR autoradiography film (Eastman Kodak, Rochester, NY).

Differentiation and Oil Red O staining

3T3-L1 preadipocytes were cultured on poly-lysine-coated coverslips with DMEM and 10% FBS at 5% CO_2 in 24-well plates. At 2 days post-confluence, cells were incubated with fresh media supplemented with 3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (1 μM), insulin (1.7 μM), and indicated compounds (0.25 μM). After 48 hr, cells were changed to fresh media and treated with DMSO or indicated compounds for 5 days. Cells without any treatment for the entire 7 days were used as control. The cells were then fixed with 10% formalin. Fixed cells were washed with 60% isopropanol and stained with filtered 60% Oil Red O in deionized water. After staining, cells were washed with water and photographed.

Semi quantitative RT-PCR

SW480 and HT29 colon cancer cells were treated either with vehicle (DMSO) or indicated compounds and after 24 hr total RNA was extracted using RNeasy Mini kit (Qiagen, Inc., Valencia, CA). RNA concentration was measured by UV 260:280 nm absorption ratio, and 2 µg RNA was used to synthesize cDNA using Reverse Transcription System (Promega). PCR conditions were as follows: initial denaturation at 94°C (1 min) followed by 28 cycles of denaturation for 30 s at 94°C, annealing for 60 s at 55°C and extension at 72°C for 60 s, and a final extension step at 72°C for 5 min. The mRNA levels were normalized using *GAPDH* as an internal housekeeping gene. Primers obtained from IDT and used for amplification are as follows: *KLF4* (sense 5'-CTA TGG CAG GGA GTC CGC TCC-3'; antisense 5'-ATG ACC GAC GGG CTG CCG TAC-3') and *GAPDH* (sense 5'-ACG GAT TTG GTC GTA TTG GGC G-3'; antisense 5'-CTC CTG GAA GAT GGT GAT GG-3'). PCR products were electrophoresed on 1% agarose gels containing ethidium bromide and visualized under UV transillumination.

RESULTS

Figure 6.1 illustrates the effects of BA, CN-BA and the corresponding methyl ester (CN-BA-Me) on growth of SW480 and Panc-28 cells. All three compounds inhibit growth of both cell lines and IC_{50} values range from 1-5, 1-2.5 and 1-2.5 µM (Panc-28) and 1-5, 1.0 and 1-2.5 µM (SW480) were observed for BA, CN-BA and CN-BA-Me, respectively. CN-BA was the most cytotoxic compound in both cell lines and this confirms results of a previous report showing that 2-cyano derivatives of BA enhanced
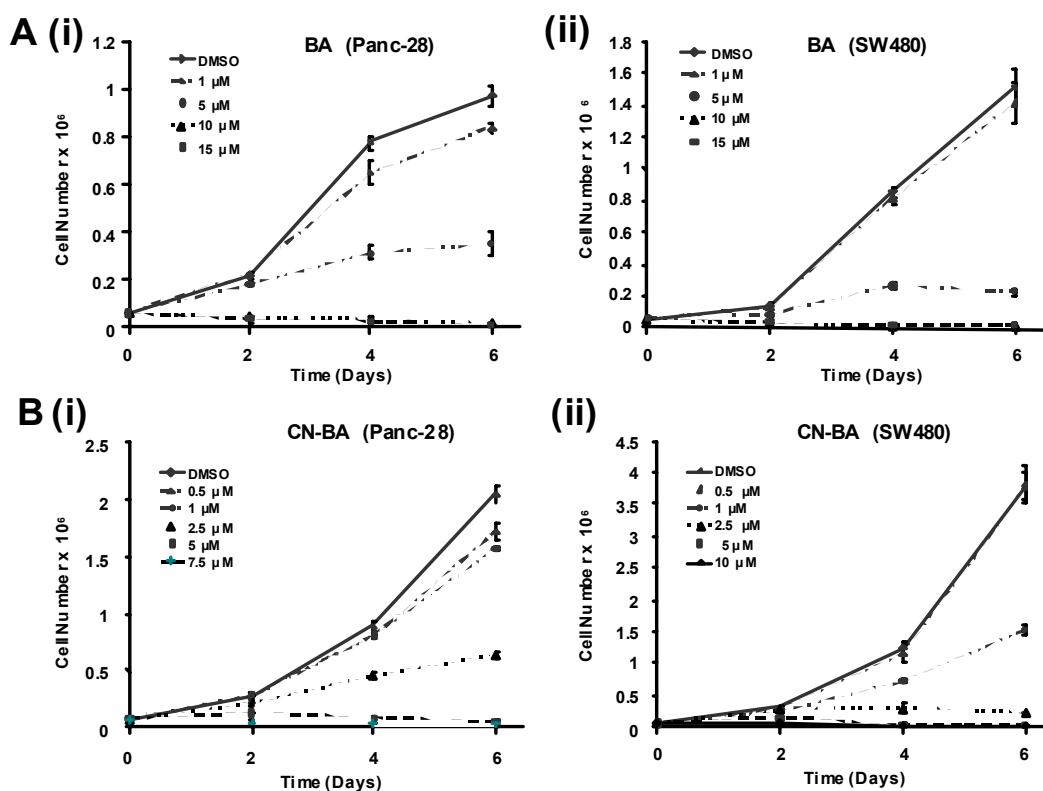
Fig. 6.1. Cell proliferation and adipocyte differentiation assays. Panc-28 and SW480 cells were treated with different concentrations of BA (A), CN-BA (B) or CN-BA-Me (C) for 6 days and the number of cells were counted after treatment for 2, 4 or 6 days as described in the Materials and Methods. Results are expressed as means ± SE for three separate determinations for each treatment group. (D) Effects of CN-BA and CN-BA-Me on differentiation of 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with 0.25 μM CN-BA, CN-BA-Me or DMSO. Induction of fat droplets by Oil-red O staining was determined as described in the Materials and Methods. Induction of intense staining for fat droplets was observed in replicate (3) experiments.
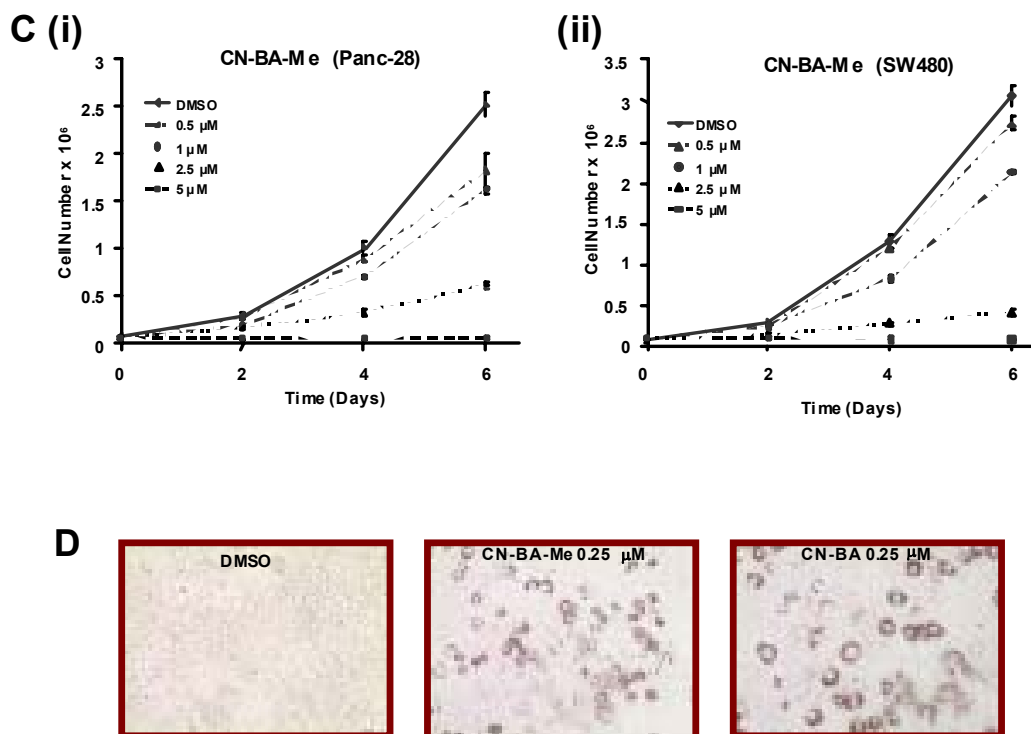
Fig. 6.1 continued

cytotoxicity (534). One of the hallmarks of PPARγ agonists is their induction of

differentiation in 3T3-L1 adipocytes which is characterized by accumulation of fat

droplets which can be detected by Oil Red O staining. Results in Figure 6.1D show that

both CN-BA and CN-BA-Me induce Oil Red O staining in this assay, whereas BA does

not induce this response (data not shown). These results suggest that these 2-cyano

derivatives of BA exhibit activity associated with PPARγ agonists.

The PPARγ agonist activity of BA and related compounds was determined in SW480 cells transfected with PPARγ-GAL4/pGAL4 and a PPRE$_3$-luc construct (Fig. 6.2A). The results show that 2.5-10 µM CN-BA and CN-BA-Me induced transactivation, whereas BA was inactive in this assay. The PPARγ agonist activities were also determined in SW480 cells using the same constructs but treated with CN-BA, CN-BA-Me alone or in combination with the PPARγ antagonist T007, and in all cases, the induced activities were inhibited by T007 (Fig. 6.2B). A similar approach was used in Panc-28 cells transfected with PPARγ-GAL4/pGAL4 and PPRE$_3$-luc (Fig. 6.2C), and CN-BA induced luciferase activity that was inhibited in cells cotreated with CN-BA plus T007. Not surprisingly, BA was inactive in these assays; however, results obtained for CN-BA-Me were highly inconsistent in Panc-28 cells compared to the colon cancer cell line (Figs. 6.2A and 6.2B). CN-BA-Me exhibited minimal induction in cells transfected with PPARγ-GAL4/pGAL4 and no induction was observed in Panc-28 cells transfected with PPRE$_3$-luc (data not shown). These results were observed in replicate experiments suggesting that there were structure-dependent differences (CN-BA vs. CN-BA-Me) for activation of the PPARγ-GAL4/pGAL4 or a PPRE$_3$-luc constructs in Panc-28 (but not SW480) cells.

PPARγ agonists are structurally-diverse and induce tissue-specific receptor-dependent responses that are typical of selective PPARγ modulators (659, 660). Similar
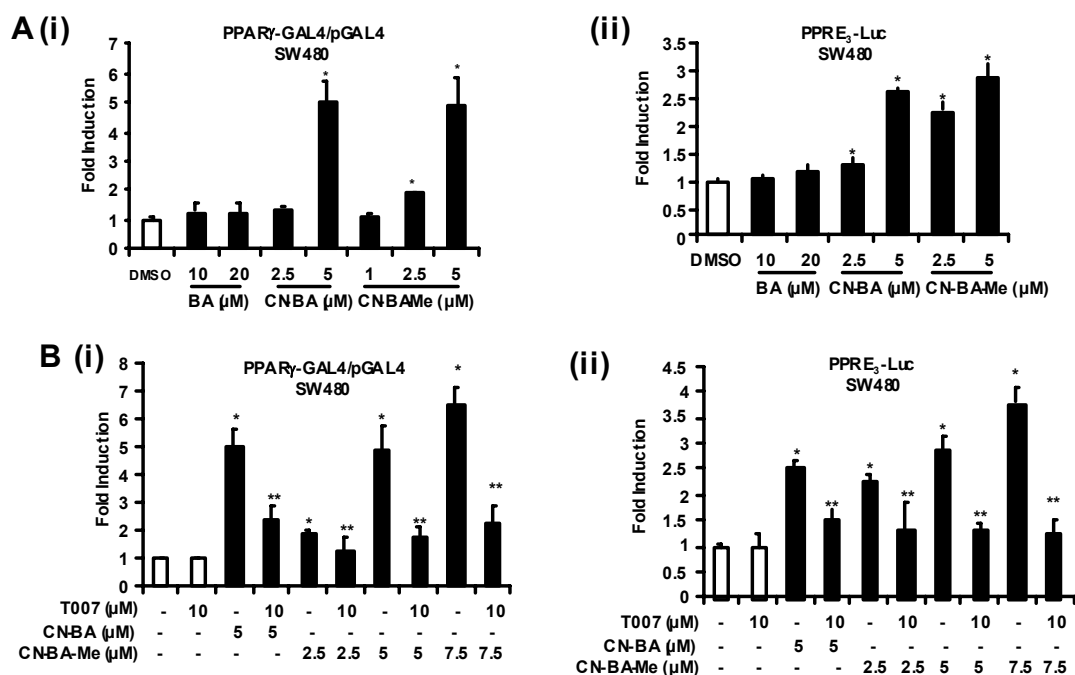
Fig. 6.2. Activation of PPARγ in SW480 and Panc-28 cells by BA, CN-BA and CN-BA-Me. SW480 cells [(A) and (B)] were transfected with PPARγ-GAL4/pGAL4 or PPRE$_3$-luc treated with DMSO (control) or different concentrations of the compounds, and luciferase activity determined as described in the Materials and Methods. (C) Activation of PPARγ in Panc-28 cells. Cells were transfected with PPARγ-GAL4/pGAL4 or PPRE$_3$-luc treated with DMSO or different concentrations of CN-BA and CN-BA-Me alone or in combination with 10 μM T007, and luciferase activity determined as described in the Materials and Methods. Results in (A) - (C) are expressed as means ± SE for three replicate determinations for each treatment group, and significant (p < 0.05) induction by the BA derivatives (*) and inhibition after cotreatment with T007 (**) are indicated. For studies in Panc-28 cells, we only observed induction of luciferase activity using CN-BA and not with CN-BA-Me or BA over several sets of experiments. (D) Mammalian two-hybrid assay in SW480 cells transfected with VP-PPARγ and GAL4-coactivator chimeras. SW480 cells were transfected with VP-PPARγ, coactivator-GAL4/pGAL4, treated with different concentrations of CN-BA or CN-BA-Me and 5 μM β-CDODA-Me, and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means ± SE for three replicate determinations for each treatment group, and significant (p < 0.05) induction is indicated by an asterisk.
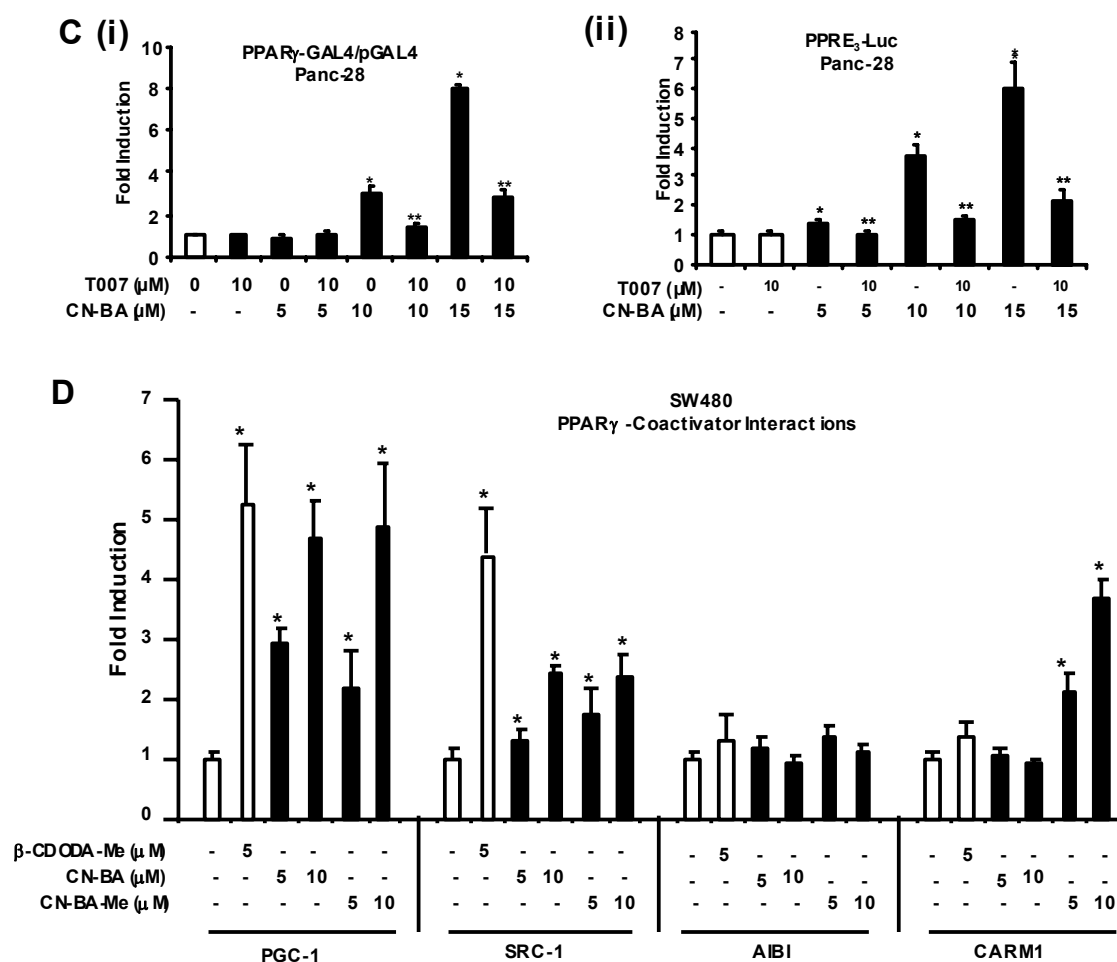
Fig 6.2 Continued

results have been observed for agonists that bind and activate other nuclear receptors and

this structure-dependent effect is due, in part, to tissue-specific expression of

coactivators and other nuclear proteins that exhibit ligand structure-dependent

interactions with receptors. Results in Figure 6.3D summarize the effects of CN-BA and

CN-BA-Me on induction of luciferase activity in SW480 cells transfected VP-PPARγ

and GAL4-co-activator and GAL4-SMRT (a co-repressor) expression plasmids. We

used β-CDODA-Me, a triterpenoid methyl ester derivative which also contains a 2-cyano-1-en-3-one function (Fig. 1.19) and activates PPARγ in colon cancer cells (312), as a comparative reference compound for the mammalian two-hybrid assay. The results show that CN-BA, CN-BA-Me and β-CDODA-Me significantly induced luciferase activity in SW480 cells transfected with VP-PPARγ and GAL4-PGC-1 and GAL4-SRC-1, but not GAL4-AIB1, GAL4-TIFII, GAL4-TRAP220 and GAL4-SMRT. In contrast, only CN-BA-Me also activated GAL4-CARM1 indicating differences between CN-BA and CN-BA-Me in the mammalian two-hybrid assay, suggesting that even among these two acid-ester analogs, some tissue-specific selective PPARγ modulator activity might be expected. The data are consistent with the differences observed for CN-BA and CN-BA-Me in activation of transfected constructs in Panc-28 cells (Fig. 6.2C).

Previous studies in this laboratory have shown that PPARγ agonists induce p21 and p27 and decrease cyclin D1 expression in Panc-28 cells, and only the former response is receptor dependent (297). Results in Figure 6.3 C show that both CN-BA and CN-BA-Me induce p21 protein expression in Panc-28 cells, and this is also accompanied by induction of p27 and downregulation of cyclin D1 (data not shown) as previously reported for a series of PPARγ-active methylene-substituted diindolylmethanes (C-DIM) analogs in this cell line (297). Cotreatment of Panc-28 cells with 5 μM CN-BA and CN-BA-Me plus the 10 μM T007 significantly inhibited induction of p21, confirming that induction of p21 was PPARγ-dependent (Fig. 6.3A). In contrast, induction of p21 by BA was not inhibited after cotreatment with T007 and this was consistent with results of transactivation studies showing that BA does not

activate PPARγ in Panc-28 or SW480 (Fig. 6.2). Figure 6.3B shows that BA, CN-BA

and CN-BA-Me induce transactivation in Panc-28 cells transfected with p21-luc(Fl)
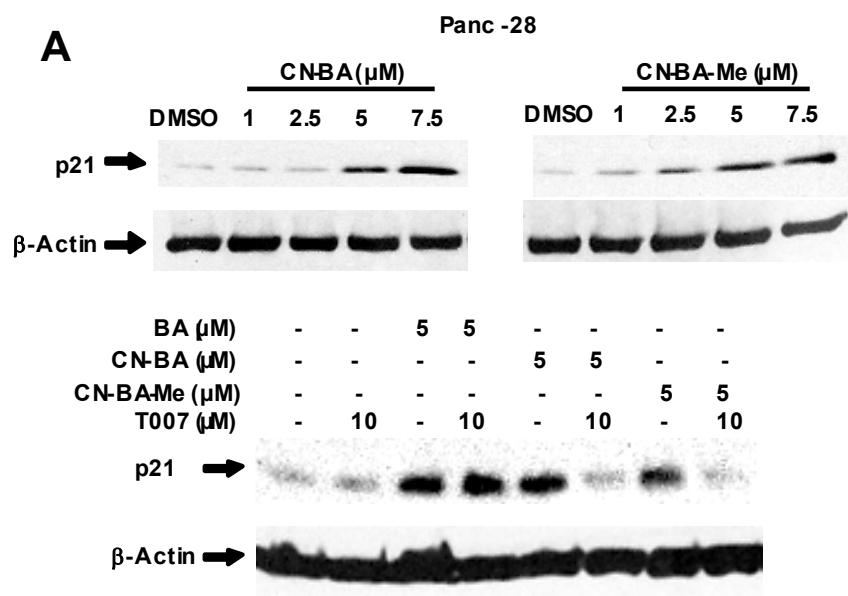
Fig. 6.3. Induction of p21 by BA, CN-BA and CN-BA-Me in Panc-28 cells. (A) Induction of p21 protein. Panc-28 cells were treated with the different compounds as indicated for 24 hr, and whole cell lysates were obtained and analyzed by immunoblots as described in the Materials and Methods. Induction of p21-luc (B) and p21 deletion constructs (C) in Panc-28 cells. Cells were transfected with the various constructs, treated with DMSO, BA, CN-BA, CN-BA-Me alone or in combination with T007, and luciferase activity determined as described in the Materials and Methods. Results of all transactivation studies in this Figure are presented as means ± SE for at least three separate determinations for each treatment group. Significant ($p < 0.05$) induction compared to solvent (DMSO) control (*) and inhibition by cotreatment with T007 (**) are indicated. (D) Chromatin immunoprecipitation assays. Primers designed for the proximal region of the p21 promoter (i) were used for a ChIP assay in Panc-28 cells (ii) treated with DMSO, 5 μM BA, 5 μM CN-BA, and 5 μM CN-BA-Me for 1 or 2 hr. Analysis of interactions of Sp1 and PPARγ with the p21 promoter were carried out in the ChIP assay as described in the Materials and Methods. The ChIP assay was also used to examine binding of TFIIB to the GAPDH promoter (positive control) (iii) and to exon 1 of CNAP1 (negative control) as described in the Materials and Methods.
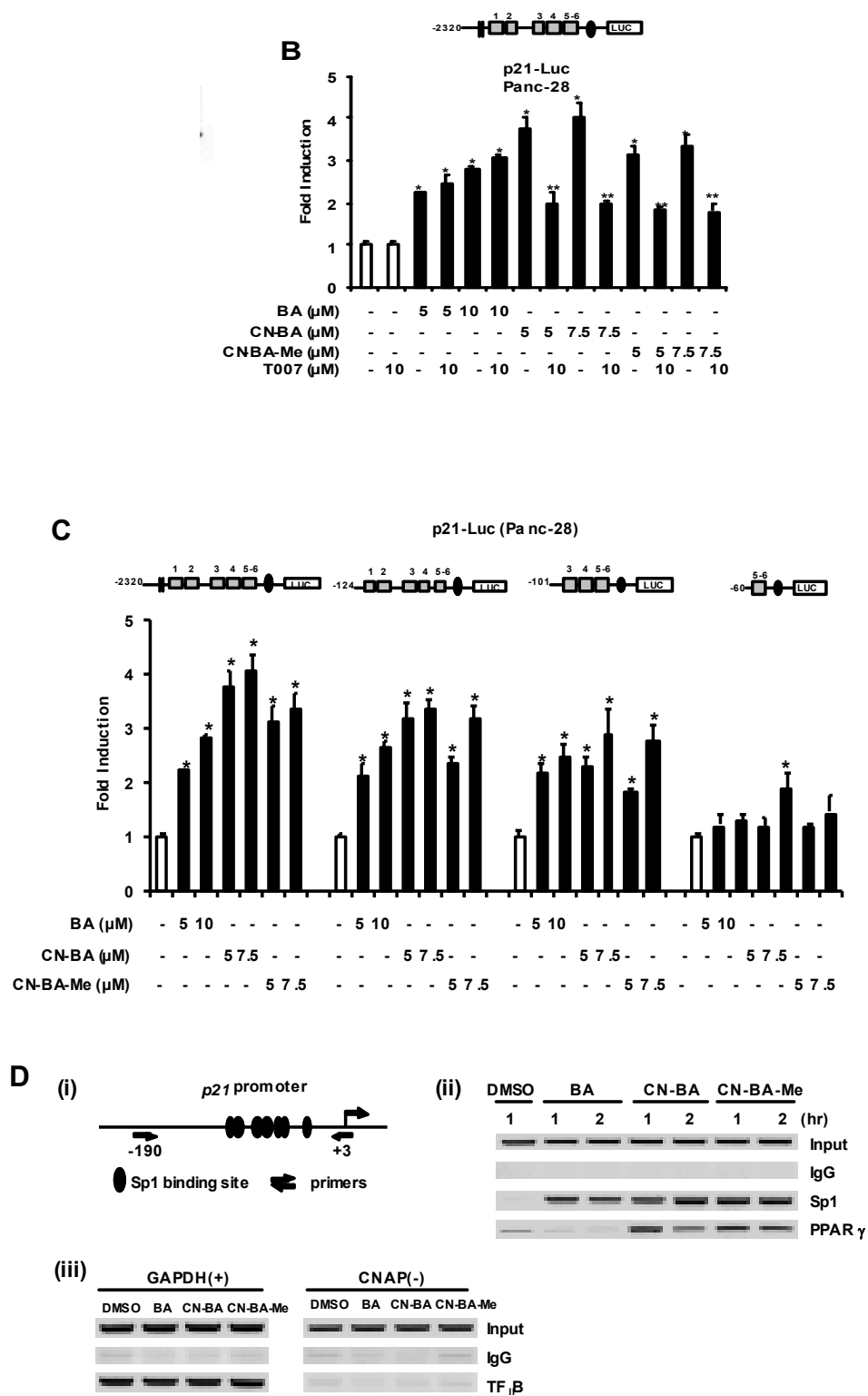
Fig. 6.3 Continued

which contains the -2325 to +8 region of the p21 promoter. In cells cotreated with BA and related compounds plus the PPARγ antagonist T007, the induction of luciferase activity by CN-BA and CN-BA-Me was inhibited, whereas BA-induced activity was unaffected by T007. The results complement the immunoblot data confirming that induction of p21 by CN-BA/CN-BA-Me was PPARγ-dependent, whereas induction of p21 by BA was PPARγ-independent. We further investigated induction of luciferase activity in Panc-28 cells transfected with constructs containing -2325 to +8 [p21-Luc (Fl)], -124 to +8 [p21-Luc (-124)], -101 to +8 [p21-Luc (-101)], and -60 to +8 [p21-Luc (-60)] p21 promoter inserts. The latter three constructs contain the 6 proximal GC rich sites (1 - 6) and the results of the transfection studies suggest that these GC-rich sites are necessary for CN-BA- and CN-BA-Me-induced transactivation. Deletion analysis of the p21 promoter indicated that loss of inducibility [i.e. p21-luc(60)] was associated with loss of GC-rich sites 3 and 4, whereas CN-BA significantly induced activity but only at the 7.5 μM concentration, suggesting sites 3 and 4 were also important for this compound but induction could also be observed using constructs containing only GC-rich sites 1 and 2. Previous studies show that PPARγ-dependent activation of p21 by other PPARγ agonists is also dependent on GC-rich sites 3 and 4 and involves PPARγ/Sp-dependent activation of p21. The ligand-dependent recruitment of PPARγ to the p21 promoter by CN-BA and CN-BA-Me was further investigated in a ChIP assay in Panc-28 cells treated with the BA, CN-BA and CN-BA-Me for 1 or 2 hr. The results

(Fig. 6.3D) show that both CN-BA and CN-BA-Me recruited PPARγ to the proximal

GC-rich region of the p21 promoter and this was also accompanied by enhanced binding

 experiment, transcription factor TFIIb bound to the proximal region of the GAPDH

gene promoter but not to exon-1 of the CNAP1 gene.

PPARγ agonists such as CDDO, β-CDODA and related esters and PPARγ-active

C-DIMs also induce receptor-dependent expression of caveolin-1 in colon cancer cells

(300-302, 312). Figure 6.4A shows that CN-BA, CN-BA-Me but not BA induce

caveolin-1 in HT-29 cells and similar results were observed in HCT-15 cells (Fig. 6.4B).

In contrast, BA, CN-BA and CN-BA-Me did not induce caveolin-1 expression in

SW480 cells, and the latter two compounds decreased expression of this protein (Fig.

6.4C). Cotreatment of HT-29 and HCT-15 cells with CN-BA/CN-BA-Me plus the

PPARγ antagonist T007 resulted in inhibition of the induced caveolin-1 response,

confirming that induction was PPARγ-dependent (Fig. 6.4D). Thus, receptor-dependent

activation of caveolin-1 by CN-BA and CN-BA-Me was dependent on cell context and

this of Sp1. In contrast, BA did not induce PPARγ interactions with the p21 promoter in

the ChIP assay. This is consistent with receptor-independent activation of p21 by BA

and the mechanism of this response is currently being investigated. As a control for this

correlated with results of previous studies with CDDO and the 18α and 18β-isomers of

CDODA-Me where CDDO and α-CDODA-Me but not β-CDODA-Me induced

caveolin-1 in HT-29 and SW480 cells, whereas like CN-BA/CN-BA-Me, β-CDODA

induced caveolin-1 in HT-29 but not in SW480 cells. Previous studies showed that α-

and β-CDODA-Me induced the tumor suppressor gene KLF-4 in HT-29 and SW480

colon cancer cells (312), and the results in Figure 6.5 summarize the effects of CN-BA

and CN-BA-Me on KLF4 expression in HT-29 and SW480 cells. In the former cell line,
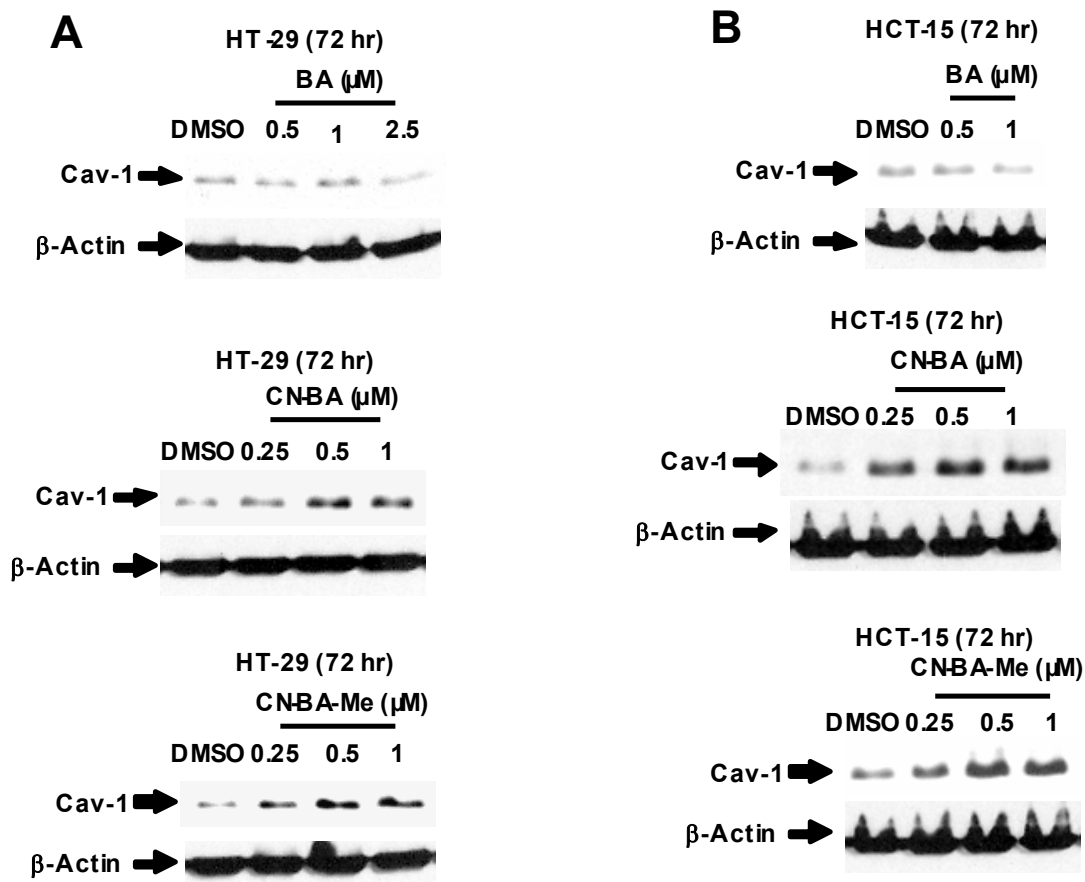
Fig. 6.4. Induction of caveolin-1 expression in colon cancer cells. HT-29 (A), HCT-15 (B) and SW480 (C) cells were treated with DMSO, different concentrations of BA, CN-BA or CN-BA-Me for 72 hr. Caveolin-1 expression was determined by Western blot analysis as described in the Materials and Methods. Similar results were observed in replicate experiments. (D) Effects of T007 on induction of caveolin-1. HCT-15 or HT-29 cells were treated with DMSO or different concentrations of CN-BA and CN-BA-Me alone or in combination with 5 μM T007 and caveolin-1 expression was determined by Western blot analysis as described in the Materials and Methods.
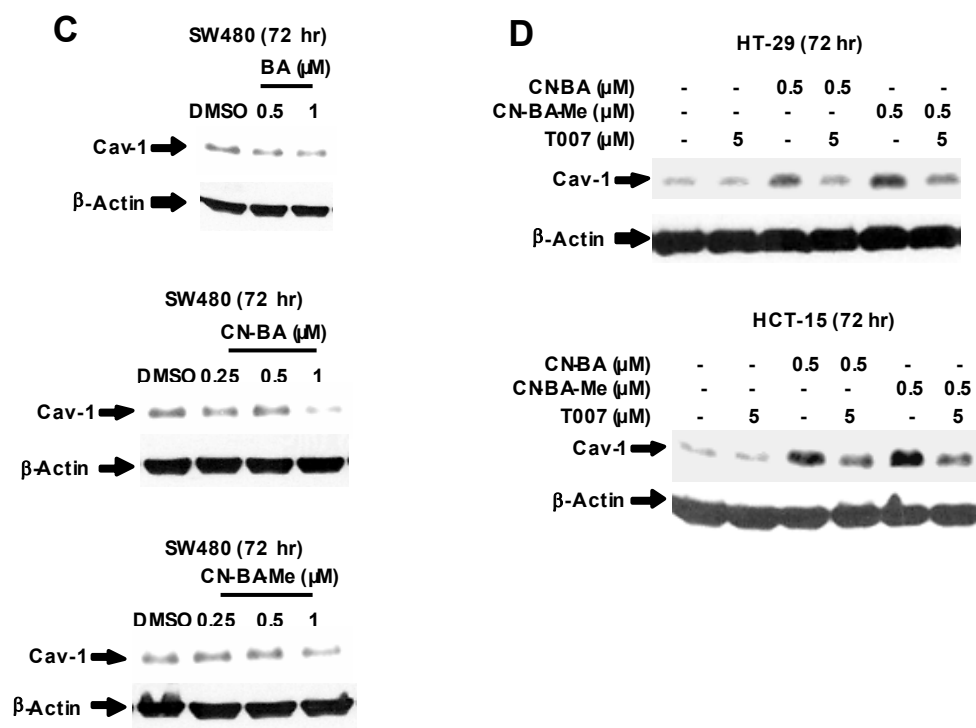
Fig. 6.4 Continued

CN-BA and CN-BA-Me induced KLF4 mRNA levels and similar results were observed

for β-CDODA-Me which was used as a positive control for this cell line. However, in

HT-29 cells cotreated with CN-BA, CN-BA-Me and β-CDODA-Me plus the PPARγ

antagonist T007, induction of KLF4 was significantly decreased only for β-CDODA-

Me. In contrast, CN-BA and CN-BA-Me did not induce KLF4 expression in SW480

cells, whereas β-CDODA-Me treatment enhanced KLF4 mRNA as previously described

(312). The differences between CN-BA/CN-BA-Me and β-CDODA-Me as inducers of

KLF4 mRNA levels in colon cancer cells clearly distinguished between two classes of

A HT-29

T007 (µM)	-	10	-	10	-	10	-	10	-	10	-	10
CN-BA-Me (µM)	-	-	-	-	-	-	5	5	10	10	-	-
CN-BA (µM)	-	-	5	5	10	10	-	-	-	-	-	-
β-CDODA-Me (µM)	-	-	-	-	-	-	-	-	-	-	5	5

KLF4

GAPDH

B SW480

	DMSO	BA (µM)			CN-BA (µM)			CN-BA-Me (µM)			β-CDODA-Me
		5	10	15	2.5	5	10	2.5	5	10	5 µM

KLF4 ➡

GAPDH ➡
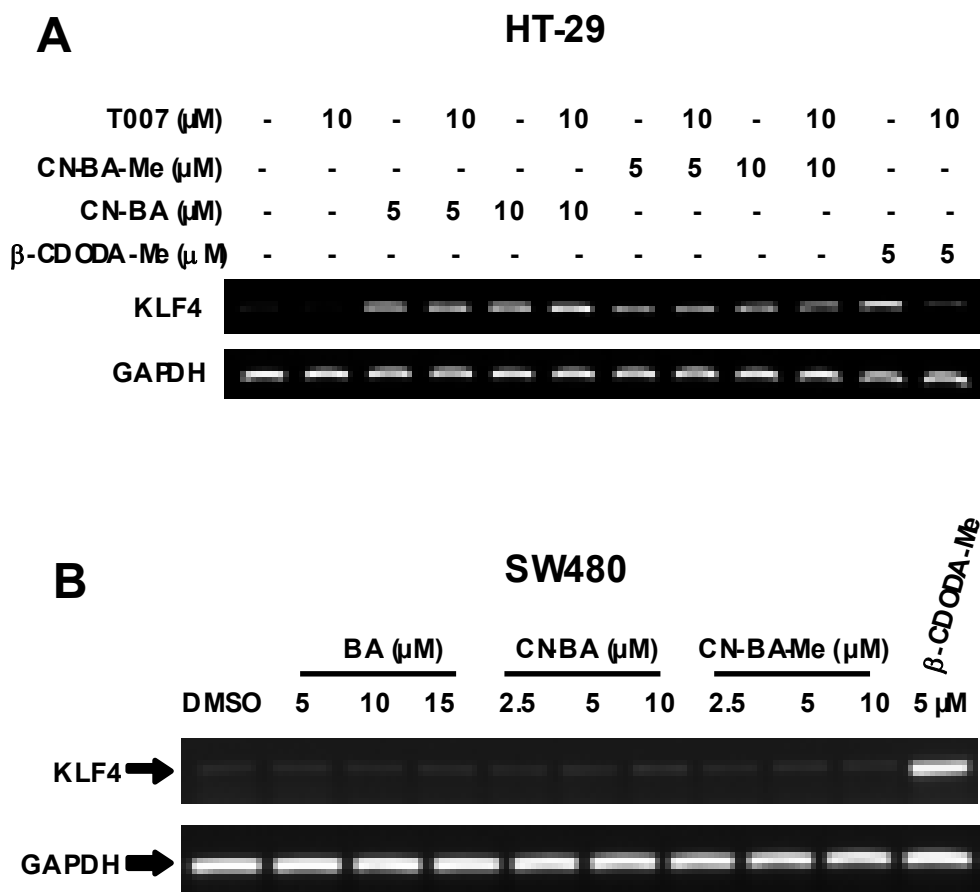
Fig. 6.5. Induction of KLF4 gene expression apoptosis by BA and related compounds. Induction of KLF4 in HT-29 (A) and SW480 (B) cells. Cells were treated with different concentrations of CDODA isomers or T007 alone or in combination, and KLF4 mRNA levels were determined by real time PCR as described in the Materials and Methods. Each experiment was replicated (> 3X) and T007 did not inhibit KLF4 mRNA induction by BA, CN-BA and CN-BA-Me, whereas 60-80% of the response induced by β-CDODA-Me was inhibited by T007. KLF4 mRNA levels were not induced in SW480 cells by BA derivatives. (C) Induction of apoptosis. Cells were treated for 24 hr with BA and related compounds, and whole cell lysates were examined by Western blot analysis as described in the Materials and Methods.
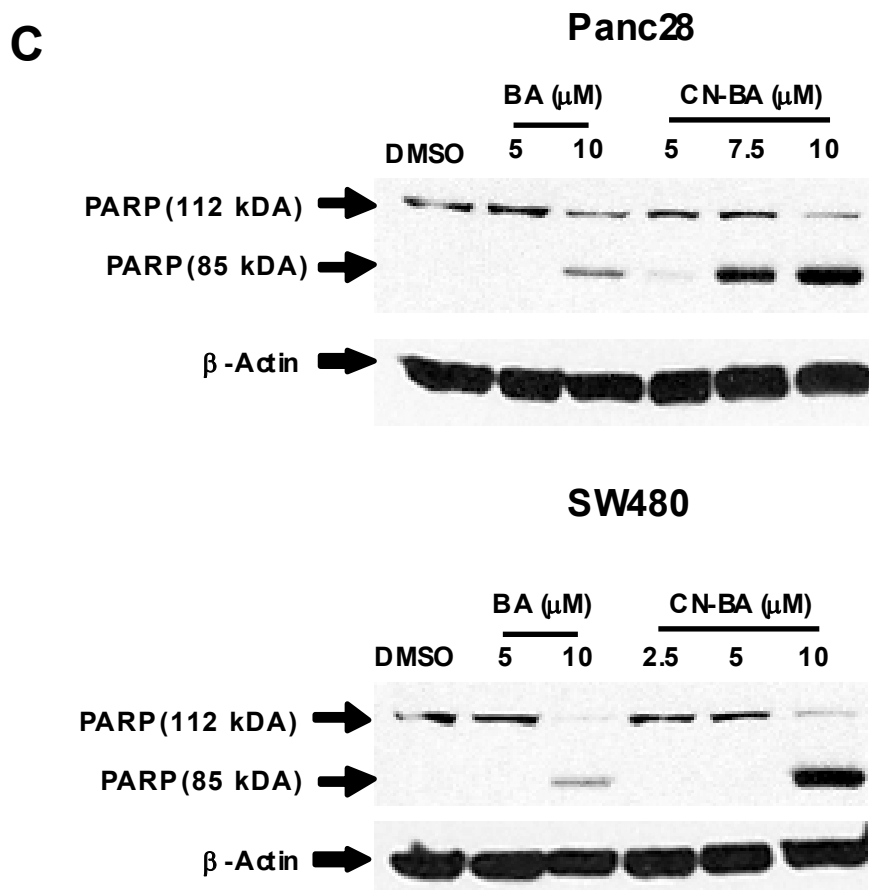
Fig. 6.5 Continued

structurally related PPARγ agonists derived from triterpenoid acids and confirm that

CN-BA/CN-BA-Me are a novel class of PPARγ antagonists. In addition, we also

confirmed that BA/CN-BA induced apoptosis in SW480 and Panc28 cells, and results in

Figure 6.5C show that both compounds induced caspase-dependent PARP cleavage in

these cell lines.

DISCUSSION

PPARγ is overexpressed in tumors from multiple tissues and cell lines (283) and PPARγ agonists are being developed as mechanism-based drugs for cancer chemotherapy. PPARγ agonists typically inhibit cancer cell growth and this is associated with induction of p21 and/or p27 and downregulation of cyclin D1 and cells treated with these compounds also exhibit morphological and biochemical features of apoptosis. The mechanisms of the growth inhibitory/pro-apoptotic responses induced by different structural classes of PPARγ agonists are cell context and gene-dependent, and induction of both receptor-dependent and -independent responses are observed. For example, the thiazolidinedione troglitazone induces non-steroidal inflammatory drug-activated gene-1 (NAG-1) in HCT-116 colon cancer cells through receptor-independent activation of kinase pathways, whereas induction of NAG-1 by 15-deoxy-Δ12,14-prostaglandin J2 in HCT-116 cells is PPARγ-dependent and inhibited by PPARγ antagonist (278, 665, 694). PPARγ-active C-DIMs induce caveolin-1 expression in HT-29 and HCT-15 colon cancer cells, whereas rosiglitazone induced caveolin-1 only in the former cell lines (300). The induction responses by both compounds were inhibited by PPARγ antagonists and cell context-dependent differences of C-DIMs and rosiglitazone in HCT-15 cells were associated with expression of mutant PPARγ (K422Q) in this cell line and the mutant receptor was insensitive to rosiglitazone (301).

CDDO and its related methyl ester and imidazole derivatives are PPARγ agonists (234, 302) and are potent anticancer drugs currently undergoing clinical trials. These triterpenoid acid derivatives of oleanolic acid, a phytochemical used in traditional

medicine induce multiple receptor-independent and some receptor-dependent responses including the receptor-dependent induction of caveolin-1 in colon cancer cells (302). Although oleanolic acid is only weakly cytotoxic to cancer cells, the introduction of the 2-cyano-substituted 1-en-3-oxo moiety into the A-ring of oleanolic acid greatly enhanced the cytotoxicity of the resulting 2-cyano derivatives including CDDO which also has an enone system in the C-ring (655) (463, 464) and similar results were observed for the corresponding 2-cyano derivatives of glycyrrhetinic acid, namely α-CDODA and β-CDODA-Me (312). Moreover, in studies with glycyrrhetinic acid analogs, it was shown that the 2-cyano group was required for PPARγ agonist activity.

The major structural differences between betulinic acid and oleanolic and glycyrrhetinic acids are their 5 and 6-member E-rings, respectively, and the position of substituents in this ring. However, despite these structural differences, introduction of the 2-cyano-1-ene-3-oxo system into the A-ring of betulinic acid gave CN-BA which activated PPARγ in both SW480 and Panc-28 cell lines (Figs. 6.1 and 6.2) and enhanced the cytotoxicity of these compounds compared to BA (1.19). Surprisingly, CN-BA-Me activated PPARγ-GAL4/pGAL4 and PPRE-luc in SW480 but was much less effective in activating these constructs in Panc-28 cells, and these cell context-dependent differences in CN-BA and CN-BA-Me suggest that these compounds may be selective PPARγ agonists.

Selective receptor modulators exhibit tissue-selective receptor agonist activities and differences between diverse structural classes of these compounds can be discerned in mammalian two-hybrid assays using VP-PPAR and GAL4-coactivator chimeras (300,

302, 312). This assay has some relevance for identifying selective receptor modulators since differences between selective receptor modulators may be due, in part, to their interaction with coactivator proteins. Induction of luciferase activity in colon cancer cells transfected with VP-PPARγ/GAL4-coactivator constructs is dependent on the coactivator and structure of the PPARγ agonist (300, 302, 312). C-DIM PPARγ agonists induce transactivation in cells transfected with GAL4-PGC-1 (300), whereas CDDO and CDDO-Me are active in cells transfected with GAL4-chimeras containing SRC-1, SRC-2 (TIFII), SRC-3 (A1B1), TRAP220, PGC-1 and CARM-1 (302). β-CDODA and α-CDODA-Me activate GAL4-chimeras containing PGC-1 and SRC-1 and PGC-1 and SRC-2, respectively (312). CN-BA activates GAL4-chimeras containing PGC-1 and SRC-1 in SW480 cells and resembles β-CDODA-Me, whereas CN-BA-Me activates PGC-1, SRC-1 and CARM-2 (Fig. 6.2D), whereas these compounds did not activate GAL4 chimeras containing SRC-2, TRAP220 or SMRT (data not shown). The unique pattern for CN-BA-Me in the mammalian two-hybrid assay highlights differences that are dependent only on methylation of the 20-carboxyl group in the E-ring, and these results are consistent with differences between CN-BA and CN-BA-Me in their activation of transfected PPARγ-responsive constructs in Panc-28 cells (Fig.6.2C).

The PPARγ agonist activities of CN-BA and CN-BA-Me and their role as selective receptor modulators were further investigated using four receptor-mediated responses, namely (i) the induced differentiation of 3T3-L1 adipocytes, (ii) induction of the cyclin-dependent kinase inhibitor p21 in Panc-28 cells, and the induction of (iii) caveolin-1 and (iv) KLF4 in colon cancer cells. Both CN-BA and CN-BA-Me induced

differentiation of 3T3-L1 adipocytes and this was characterized by accumulation of fat

droplets which are visualized by Oil-red O staining (Fig. 6.1D). Previous studies

showed that PPARγ-active C-DIMs induced p21 expression in Panc-28 cells and this

response was associated with interactions of PPARγ with the proximal GC-rich region of

the p21 promoter (297). Similar results were obtained for both CN-BA and CN-BA-Me

which induced p21 expression in Panc-28 cells and reporter gene activity in cells

transfected with p21-luc(F1), and both responses were inhibited after cotreatment with

PPARγ antagonist T007 (Fig. 6.3A). Deletion analysis of the p21 promoter suggested

that for CN-BA-Me, GC-rich sites 3 and 4 were required for activation of p21, whereas

CN-BA also induced activity with p21-luc (60) which only contained GC-rich sites 1

and 2. Nevertheless, sites 3 and 4 appear to play an important role for both CN-BA-Me

and CN-BA, and these same sites were also required for PPARγ-dependent activation of

p21 by C-DIMs (297). It has also previously been reported that progesterone receptor

and androgen receptor agonists induce p21 through receptor-Sp protein interactions with

GC-rich sites 3 and 4 and site 3, respectively (801, 802), suggesting that these GC-rich

sites in the p21 promoter are important targets for nuclear receptors. We also showed

that BA induced PPARγ-independent activation of p21, and the differences between BA

vs. CN-BA/CN-BA-Me are evident not only after treatment with the PPARγ antagonist

T007 (Figs. 6.3A and 6.3B), but also in the recruitment of PPARγ to the p21 promoter

by CN-BA/CN-BA-Me but not BA in a ChIP assay in Panc-28 cells (Fig. 6.3D).

In a recent study with the 18α and 18β isomers of CDODA, we showed that both

compounds induced caveolin-1 in HT-29 and HCT-15 cells, whereas only α-CDODA-

Me induced caveolin-1 in SW480 cells (312). These results suggested that in SW480 cells the stereochemistry at C-18 of CDODA which influences the confirmation of the E-ring also differentially affected PPARγ-dependent activation of caveolin-1. CN-BA and CN-BA-Me but not BA induced caveolin-1 in HT-29 and HCT-15 cells and cotreatment with the PPARγ antagonist T007 inhibited the induction response (Fig. 6.5). In contrast, CN-BA and CN-BA-Me do not induce caveolin-1 in SW480 cells as previously observed for 18β isomer of CDODA; however, the stereochemistry at C-18 for the BA derivatives is α, suggesting that the cell context-dependent activation of caveolin-1 by the cyano-substituted triterpenoid acids is not only dependent on the stereochemistry at C-18 but also the structure of the E-ring. The presence of the 5-membered E-ring with carboxy and isopropyl substituents (Fig. 1.19) resulted in loss of PPARγ-dependent induction of caveolin-1 in SW480 cells by CN-BA and CN-BA-Me and this cell context-dependent response was consistent with the activity of these compounds as selective receptor modulators. The cytotoxicity of BA and CN-BA derivatives (Fig. 6.1) was due not only to growth inhibition but also to induction of apoptosis (Fig. 6.5C) which was not inhibited by PPARγ antagonists (data not shown), suggesting a receptor-independent proapoptotic pathway which is currently being investigated.

The CDODA-Me compound induced the tumor suppressor gene KLF4 in SW480 and HT-29 colon cancer cells, and this response was inhibited by T007 (312). In contrast, CN-BA/CN-BA-Me did not induce KLF4 mRNA in SW480 cells and induction of this gene in HT-29 cells was receptor independent. These data, coupled with the effects of the cyano-substituted compounds on transactivation in the mammalian two-

hybrid and reporter gene assays, adipocyte differentiation, p21 and caveolin-1 expression demonstrate that CN-BA and CN-BA-Me represent a novel class of selective PPARγ agonists in colon an pancreatic cancer cells. The concentration-dependent differences in the activation of p21 and PPARγ-GAL4/PPRE$_3$-luc ($\geq 2.5\ \mu M$) and induction of Oil Red O staining and caveolin-1 ($\leq 0.5\ \mu M$) may be due, in part, to relatively short (24 hr) and longer (72 - 120 hr) treatment times, respectively. However, differences in gene-responsiveness may also be due to other nuclear proteins and competition by receptor complexes bound to response elements on different gene promoters for common nuclear cofactors. The activities of CN-BA and CN-BA-Me coupled with their cytotoxicity (Fig. 6.1) suggest that the receptor-dependent and - independent responses induced by these compounds will be advantageous for further development of these compounds for clinical applications in the treatment of colon and pancreatic cancer.

CHAPTER VII

SUMMARY

Glycyrrhizin, a pentacyclic triterpene glycoside, is the major phytochemical in licorice and this compound and its hydrolysis product glycyrrhetinic acid (GA) have been associated with the multiple therapeutic properties of licorice extracts. We have investigated the effects of 2-cyano substituted analogs of GA on their cytotoxicities and activity as selective peroxisome proliferator-activated receptor γ (PPARγ) agonists. Methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (β-CDODA-Me) and methyl 2-cyano-3,11-dioxo-18α-olean-1,12-dien-30-oate (α-CDODA-Me) were more cytotoxic to colon cancer cells than their des-cyano analogs and introduction of the 2-cyano group into the pentacyclic ring system was necessary for the PPARγ agonist activity of α- and β-CDODA-Me isomers. However, in mammalian two-hybrid assays, both compounds differentially induced interactions of PPARγ with coactivators, suggesting that these isomers, which differ only in the stereochemistry at C18 (D/E ring junction), are selective receptor modulators. This selectivity in colon cancer cells was demonstrated for the induction of two proapoptotic proteins, namely caveolin-1 and the tumor suppressor gene Krüppel-like factor-4 (KLF-4). β-CDODA-Me but not α-CDODA-Me induced caveolin-1 in SW480 colon cancer cells, whereas caveolin-1 was induced by both compounds in HT-29 and HCT-15 colon cancer cells. The CDODA-Me isomers induced KLF-4 mRNA levels in HT-29 and SW480 cells but had minimal effects on KLF-4 expression in HCT-15 cells. These induced responses were inhibited by cotreatment with a PPARγ antagonist. This demonstrates for the first time that PPARγ

agonists derived from GA induced cell-dependent caveolin-1 and KLF-4 expression through receptor-dependent pathways.

β-CDODA-Me inhibited growth of RKO and SW480 colon cancer cells and this was accompanied by decreased expression of Sp1, Sp3 and Sp4 protein and mRNA and several Sp-dependent genes including survivin, VEGF, and VEGFR receptor 1 (VEGFR1 or Flt-1). β-CDODA-Me also induced apoptosis, arrested RKO and SW480 cells at G_2/M, and inhibited tumor growth in athymic nude mice bearing RKO cells as xenografts. β-CDODA-Me decreased expression of microRNA-27a (miR-27a), and this was accompanied by increased expression of two miR-27a-regulated mRNAs, namely ZBTB10 (an Sp repressor) and Myt-1 which catalyzes phosphorylation of cdc2 to inhibit progression of cells through G_2/M. Both β-CDODA-Me and antisense miR-27a induced comparable responses in RKO and SW480 cells, suggesting that the potent anticarcinogenic activity of β-CDODA-Me is due to downregulation of oncogenic miR-27a.

β-CDODA-Me was also a potent inhibitor of LNCaP prostate cancer cell growth ($IC_{50} \sim 1$ μM) and activated peroxisome proliferator-activated receptor γ (PPARγ), β-CDODA-Me induced p21 and p27 and downregulated cyclin D1 protein expression and also induced two other proapoptotic proteins, namely NAG-1 and ATF-3. However, induction of these responses by β-CDODA-Me was PPARγ-independent and due to activation of phosphatidylinositol-3-kinase (PI3K), mitogen activated protein kinase (MAPK), and jun N-terminal kinase (JNK) pathways by this compound. In contrast, β-CDODA-Me also decreased androgen receptor (AR) and prostate specific antigen (PSA)

mRNA and protein levels through kinase-independent pathways. β-CDODA-Me repressed AR mRNA transcription, whereas decreased PSA mRNA levels were dependent on protein synthesis and was reversed by cycloheximide. Thus, potent inhibition of LNCaP cell survival by β-CDODA-Me is due to PPARγ-independent activation of multiple pathways that selectively activate growth inhibitory and proapoptotic responses.

Betulinic acid (BA) is a pentacyclic triterpene natural product initially identified as a melanoma-specific cytotoxic agent which exhibits low toxicity in animal models. Subsequent studies show that BA induces apoptosis and antiangiogenic responses in tumors derived from multiple tissues; however, the underlying mechanism of action is unknown. Using LNCaP prostate cancer cells as a model, we now show that BA decreases expression of vascular endothelial growth (VEGF) and the antiapoptotic protein survivin. The mechanism of these BA-induced antiangiogenic and proapoptotic responses in both LNCaP cells and in tumors is due to activation of selective proteasome-dependent degradation of the specificity protein 1 (Sp1), Sp3 and Sp4 transcription factors which regulate VEGF and survivin expression. Thus, BA acts as a novel anticancer agent through targeted degradation of Sp proteins which are highly overexpressed in tumors. We modified the A-ring of BA to give a 2-cyano-1-en-3-one moiety and the effects of the 2-cyano derivative of BA (CN-BA) and its methyl ester (CN-BA-Me) were investigated in colon and pancreatic cancer cells. Both CN-BA and CN-BA-Me were highly cytotoxic to Panc-28 pancreatic and SW480 colon cancer cells. CN-BA and CN-BA-Me also induced differentiation in 3T3-L1 adipocytes which

exhibited a characteristic fat droplet accumulation induced by peroxisome proliferator-activated receptor γ (PPARγ) agonists. Based on these results, we investigated the activities of CN-BA and CN-BA-Me as PPARγ agonists using several receptor-mediated responses including activation of transfected PPARγ-responsive constructs, induction of p21 in Panc-28 cells, and induction of caveolin-1 and Krüppel-like factor 4 in colon cancer cells. The results clearly demonstrated that both CN-BA and CN-BA-Me activated PPARγ-dependent responses in colon (caveolin-1) and pancreatic (p21) cells, whereas induction of KLF4 by these compounds in colon cancer cells was PPARγ-independent and also dependent on cell context. The PPARγ agonist activities of CN-BA and CN-BA-Me were structure-, response-/gene- and cell context-dependent suggesting that these compounds are a novel class of selective PPARγ modulators with potential for clinical treatment of colon and pancreatic cancer.

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