

**MOLECULAR MECHANISMS OF CANNABINOIDS AS ANTI-
CANCER AGENTS**

A Dissertation

by

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ABSTRACT

Cancer is a growing health concern world-wide and is the second most common cause of death after heart diseases. Current treatment strategies such as surgery, chemotherapy and radiation provide some relief to cancer patients but the toxic side effects associated with chemotherapy and radiation often lead to further adverse health effects. Hence there is a need for drugs with better safety profile and improved efficacy.

Cannabinoids are a group of compounds with several therapeutic properties and besides their appetite stimulant, anti-emetic and analgesic effects, cannabinoids can inhibit tumor growth, survival and metastasis. The mechanisms of action of cannabinoids as anticancer agents are highly complex and not completely understood. Studies from our laboratory indicate that the specificity protein (Sp) transcription factors, Sp1, Sp3 and Sp4 that belong to the Sp/KLF family of transcription factors are overexpressed in many tumors and regulate critical factors responsible for cancer cell proliferation, growth, angiogenesis and survival. Hence, we hypothesized that cannabinoids elicit their responses on cancer cells by downregulating the expression of Sp proteins and Sp-regulated gene products. Treatment of colon and prostate cancer cells with the cannabinoids WIN and cannabidiol (CBD) inhibited cancer cell proliferation, induced apoptosis and downregulated Sp proteins and Sp-dependent genes. Furthermore, we demonstrated that WIN and CBD-mediated induction of apoptosis and repression of Sp proteins were mediated by phosphatases and that the phosphatase involved in WIN-

dependent downregulation of Sp proteins was protein phosphatase 2A (PP2a). In addition WIN induced expression of ZBTB-10, an Sp repressor and downregulated microRNA-27a (miR27a) and these effects were PP2a-dependent indicating that WIN transcriptionally represses Sp protein expression by activating the phosphatase, PP2a.

We also investigated the effects of 1,1-bis(3'-indolyl)-1-(*p*-bromophenyl)methane (DIM-C-pPhBr) and the 2,2'-dimethyl analog (2,2'-diMeDIM-C-pPhBr), on survivin expression in colon and pancreatic cancer cells. Survivin is an anti-apoptotic protein associated with cancer cell survival and confers radiation-resistance in patients receiving radiotherapy. In addition radiation induces survivin, leading to radioresistance in tumors. In this study we demonstrated that DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr inhibit cell proliferation and induce apoptosis in colon and pancreatic cancer cells and in combination with radiotherapy, these drugs suppress radioresistance by inhibiting radiation induced survivin.

To my grandparents, Chandrika Sreenivasan and Sreenivasan C.

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CHAPTER I

INTRODUCTION

CANCER A HISTORICAL PERSPECTIVE

Paleo-oncological studies have revealed cancers in several prehistoric animals that existed before man (1). The first evidence of cancer (chondrosarcoma) was found in Utah in the fossilized remains of a theropod dinosaur (*Allosaurus fragilis*) from the Jurassic epochs (1, 2). Several ancient scriptures and manuscripts provide sufficient evidence to suggest that man had a basic knowledge of cancer at least 5000 years ago, although it was never recognized as a distinct disease entity until recently (1). Edwin Smith Papyrus, an ancient Egyptian medical text, which dates back to 3000 BC, contains one of the earliest mentions of cancers of the head and breast, which are described as bulging growths with no known remedy (3, 4). Another medical manuscript that records soft-tissue tumors and osteolytic lesions of the metastatic cancer in humans is the Ebers Papyrus written in 1500 BC (Figure 1) (4, 5). Susruta Samhitā, one of the foundational works of Indian medicine (*Ayurveda*) records tumors affecting various parts of the body and discusses the pathogenesis and management of these conditions (6). The Babylonian Talmud (central text of mainstream Judaism) mentions a 7 year headache that Titus suffered as a curse for destroying the temple of Jerusalem, which eventually after an autopsy was found to be due to a tumor in the brain (possibly a meningioma or hemangioma) (1, 7).

Although ancient manuscripts provide descriptive evidence of this long existent disease, there is no mention of the term cancer in any of these available medical documents. It was the Greek physician Hippocrates (460-370 BC), the father of medicine who used the term “karkinos”, the Greek word for crab to describe the growths found in patients that he examined (1). It was speculated that the tough surface of the tumor resembling the hard shell of the crab or the shape of the tumor with the blood vessels around it resembling a crab with claws could be the reason why he chose the term. The term “karkinos” was later translated to “cancer” derived from the Latin word “cancrum” meaning crab by the Roman encyclopaedist Aulus Cornelius Celsus (ca 25 BC - ca 50) (3, 8). The term “oncology” which is used to describe the branch of medicine which deals with the study of the development, diagnosis and treatment of cancer, is derived from the Greek word “onkos” meaning bulk mass or tumor growth and “logy” meaning study, and its coinage is credited to the Roman physician Claudius Galen (130-200 AD) (9). Galen is also known to have coined the word “sarcoma” which was used to refer to tumors with a “fleshy” appearance in cross-section (3, 10).



Figure 1. The Egyptian medical texts Edwin and Ebers papyri.

“What causes cancer?”- It is a question that has baffled physicians since the beginning of time. Several theories were put forth to explain the etiology of cancer. In ancient Egypt where magic and religion dominated medical practices, the cause of cancer was attributed to evil gods and demons and any disease was believed to be a consequence of “the will of the Gods” (3, 11). The classical period or the Greco-Roman era (500 BCE – 500 CE) witnessed a shift in the approach to understanding the cause of a disease. The influence of religion and the occult declined and made way for a naturalistic understanding of the pathology of illness (12). The humoral theory of disease proposed by Hippocrates suggested that an imbalance in the levels of 4 humors (body fluids) namely phlegm, blood, bile and urine caused diseases, with an excess of black bile being responsible for cancer. Galen who was a patron of the humoral theory performed

anatomical experiments on animals and classified tumors into different types (10, 13). The humoral theory prevailed for over 1300 years until the Renaissance, when the works of Andreas Vesalius (1514-1564) and Theophrastus Paracelsus (1493-1541), undermined the long existent Galenic principles of humoral theory. The humoral theory was superseded by the lymph theory proposed by the French physician René Descartes (1596-1650), who stated that cancer was the result of degenerating and fermenting lymph. The Age of Rationalism, brought in new proponents of the lymph theory such as Henry François le Dran (1685-1770), who further elaborated that tumors grew in localized areas and then spread to different parts of the body via the lymphatic system (3).

The eighteenth century marked the beginning of cancer epidemiology with physicians forming links between occupational and lifestyle choices and the occurrence of cancer. Bernardini Ramazzini documented an increase in the incidence of breast cancer and lower rates of cervical cancer in nuns (14). Snuff users' predisposition to developing nasal cancer observed by the English physician John Hill (1714-1775) is considered to be the first acknowledgement of carcinogenesis induced by external factors (15) while the first documented case of occupational cancer was recorded by Percival Pott (1714-1788), who observed an increased incidence of scrotal cancer in chimney-sweeps (3, 16). With the Roman law prohibiting human dissection owing to religious reasons, the field of cancer pathology suffered severely until the eighteenth century. A lift on this ban initiated a new dawn in the field cancer research. Giovanni Battista Morgagni (1682-

1771) gave birth to the field of scientific oncology with his compilations of 700 autopsies describing different cancers in his publication “*On the Sites and Causes of Disease*”. His efforts and findings disproved the Cartesian lymph theory (17, 18) and led to the establishment of the first cancer hospital, LaLutte Contre Le Cancer, in Rheims, France, and the first cancer institute in Middlesex, England (19).

Cancer began to be recognized as an independent disease with its own symptoms and treatment. With the invention of the light microscope by H. Jansen during the 19th century, scientists switched their focus to cells (20). The German physiologist Johannes Müller (1801-1858), was one of the first individuals to visualize cancerous tissue under the microscope and confirmed that cancer was not made up of lymph or black bile but instead was composed of cells. However, Müller erroneously proposed that cancer cells did not arise from normal cells and instead were a result of plastic exudates or budding elements called blastema in between normal cells (1, 3, 21). Later it was Muller’s student Rudolf Virchow (1821-1902) who discovered that cancer cells and normal cells arose from the same source. Virchow popularized the maxim *Omnis cellula e cellula* (“every cell originates from another existing cell like it.”) thus disproving the blastema theory (22, 23). He also speculated that tumors formed either due to hereditary predisposition or by chronic irritation (17). His theory of cancer spreading by a liquid was disproven in 1865 by the German surgeon, Karl Theirsch (1822-1925), who hypothesized that cancer cells reached secondary sites by cellular embolism. He studied metastasizing epithelial tumors of skin with the help of serial sectioning techniques and

established that malignant cells were ultimately responsible for the spread of the disease (24).

The foundation for the modern classification of tumors was laid due to the work of Muller, Virchow and Karl von Rokitansky (1804-1878). Rokitansky identified the two components of cancer, the parenchyma forming the cancer mass composed of cancer cells and the stroma that formed the supportive framework composed of connective tissue. This later formed the basis for classification of tumors into different types (epithelial or mesenchymal, benign or malignant) (25). During the late 19th century, the works of Robert Koch and Louis Pasteur brought to light a number of infectious agents that were responsible for a variety of diseases. The advocates of the infectious theory believed that cancer was also an infectious disease. Experiments were conducted in Russia, wherein tumors were surgically transplanted from one dog to another. The appearance of tumors in the latter after several weeks fueled the idea that cancer was infectious and transmissible (26). Similar experiments were conducted by two Danish researchers in 1908 using chicken leukemia cells. Their work was later followed up by Peyton Rous in 1910, whose experiments led to the discovery of the chicken Rous-sarcoma virus and laid the foundations for the field of tumor virology (24). Although, the infectious theory of cancer began to fade away with no evidence of tumors spreading through viruses in humans (with few exceptions), the initial discoveries of Rous and Howard Temin led to the understanding of the genetic nature of the disease. The works of these two researchers were critical in unveiling the role of certain genes that were

responsible for the cancerous phenotype, and these cancer causing genes later came to be known as “oncogenes”. The concept of genes promoting growth of cells stimulated speculation on the existence of genes that could inhibit the growth of cells as well, to balance the biological system (27). This hypothesis was confirmed by Stephen Friend in 1986, when he isolated the first “tumor suppressor” gene, Rb (retinoblastoma), associated with a familial form of eye cancer (28).

The long and tortuous route towards understanding cancer often involved long deviations and frequent mis-steps that in hindsight appear misguided or even far-fetched. However, as a wise man once said, “We can see so far because we stand on the shoulder of giants”.

CANCER STATISTICS AND GLOBAL HEALTH

Cancer is a global health problem and it is estimated that one in eight deaths worldwide are caused by cancer which is higher than deaths from infectious diseases, AIDS, tuberculosis and malaria combined. The World Cancer Report of 2008 documents 12.6 million cases of cancer, 7.5 million mortalities from cancer and 28 million individuals alive with cancer within five years from the initial diagnosis (29). The most frequently diagnosed and preeminent cause of deaths from cancer in both developed and developing countries are lung cancer in males and breast cancer in females. Prostate, colorectal, stomach and bladder cancer in men and colorectal, corpus uteri and ovarian cancers in women are the more frequently diagnosed cancers in developed nations.

Prostate cancer is observed to be the most frequently diagnosed cancer in men in the developed regions of the world with an incident rate of 21.7 %. This was followed by lung and colorectal cancer with 16.2 % and 13.2 % incident rates respectively. Lung cancer however has the highest mortality rate (26.9%) in men from the developed regions of the world. Colorectal and prostate cancers have a mortality rate of 10.9 % and 8.9 % respectively and stomach and bladder cancer have similar incidence rates in men however the mortality rate of stomach cancer is higher than bladder cancer (Figure 2). Women from economically more developed regions exhibit a higher incidence (26.7%) and mortality rate (15.5%) of breast cancer. The incidence rate of colorectal cancer in women is similar to the rates found in men from developed countries and the number of deaths caused due to lung cancer is almost equal to that of breast cancer in women from the developed nations (Figure 3).

It is estimated that less developed nations contributed over 50% of the cases of cancer in 2008 and two thirds of the cancer related deaths world-wide occur in less developed nations (29). Men in the less developed nations are commonly affected by cancers of the stomach, liver, colorectum, prostate and esophagus, while women exhibit increased incidence of cancers of the cervix uteri, stomach, colorectum and liver. Lung cancer represents 16.8% of all cancers in men but only 7.9% of all cancers in woman in less developed countries. Breast cancer and cancer of the cervix uteri are high in women from the economically less developed nations and constitute 20.0 % and 13.1% of all

cancer cases respectively. The mortality rates of breast, cervix uteri and lung cancer in women are all approximately 11.8 % (30).

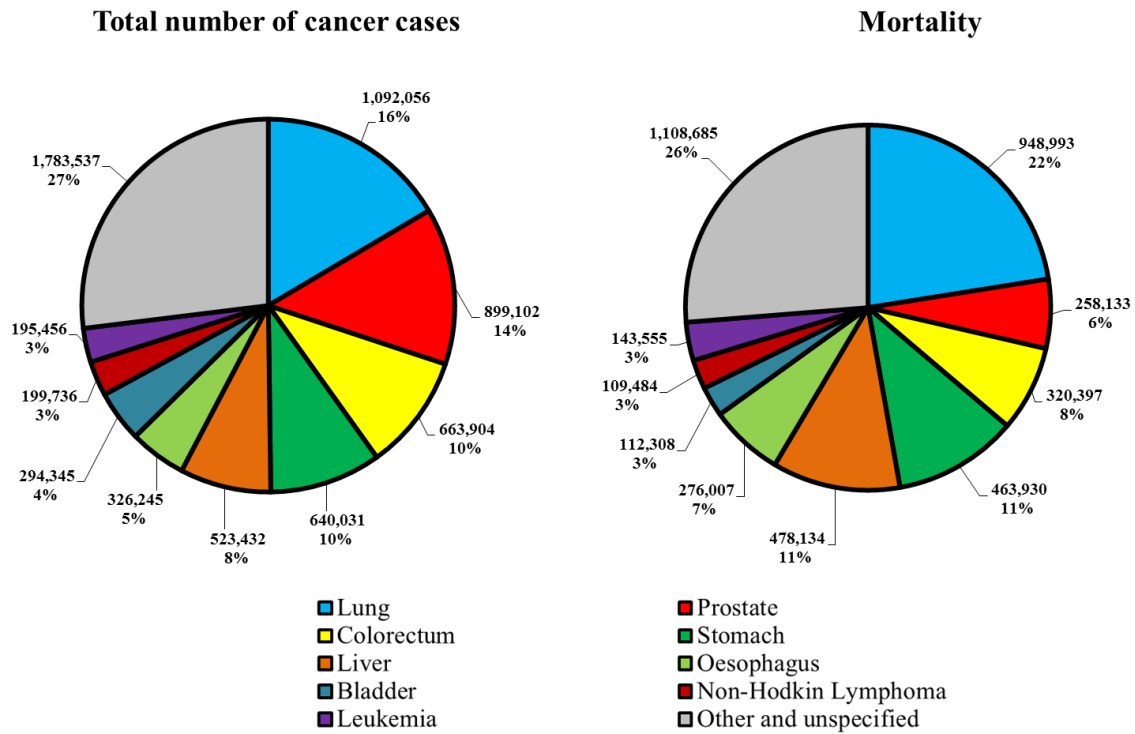


Figure 2. Total number of cancer cases and cancer mortality in men worldwide (30)

COLON CANCER

Colorectal cancer is the third most diagnosed cancer in men and second most frequently observed cancer in women worldwide according to the IARC report of 2008 (29-31). Over 1 million cases of colon cancer were diagnosed across the globe in the year 2008

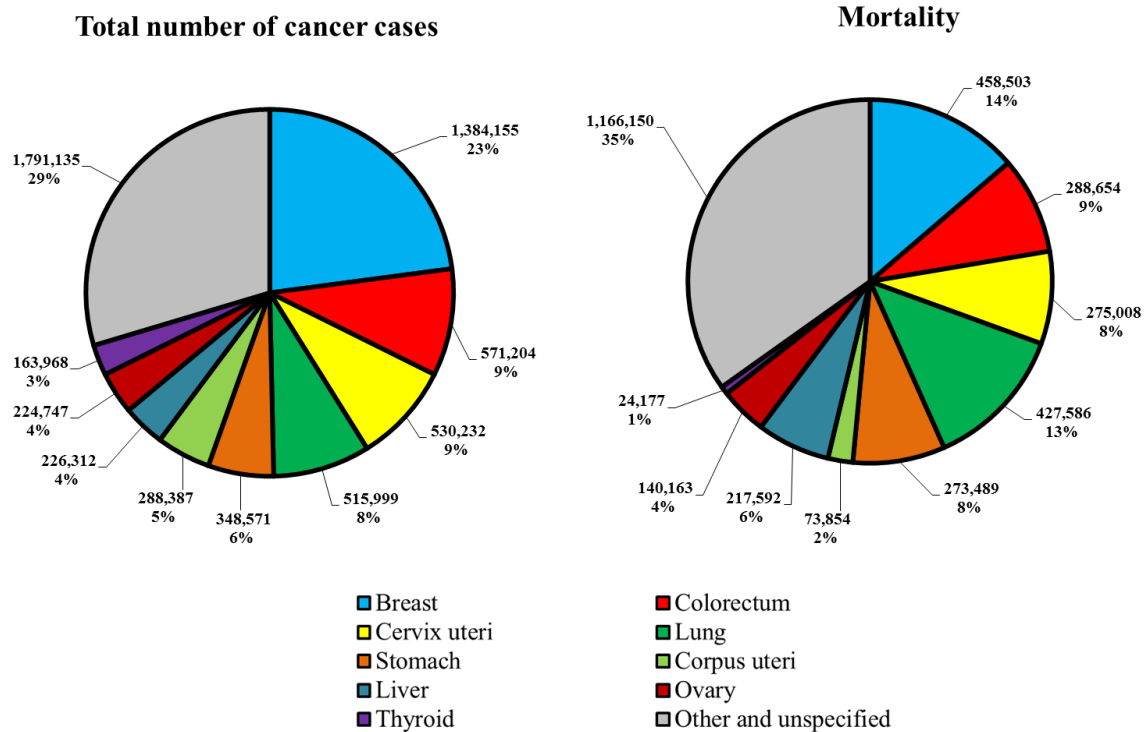


Figure 3. Total number of cancer cases and mortality in women worldwide (30)

and the number of deaths due to colorectal cancer is estimated to be more than half a million. This accounts for 8% of all cancer deaths, making it the fourth most leading cause of cancer death. Colon cancer incidence is higher in the more developed countries with 0.7 million cases compared to the less developed nations with 0.5 million cases in 2008 (30). Although, the prevalence rates for colorectal cancer are higher in individuals from the developed and industrialized nations, western lifestyles in less developed countries are resulting in increasing incidence rates (32). Individuals that migrate to an industrialized country with high incidence of colon cancer exhibit higher incidence rates compared to individuals residing in their native countries (33).

Colon cancer incidence is high in Australia, New Zealand, Europe and North America in both men and women, whereas the incidence is lower in Central, Northern, Eastern, South-Central and Middle African countries (Figure 4) (30, 31). International trends in incidence rate of colorectal cancer were examined by Center *et. al.* based on the Globocan 2008 report from IARC. Countries such as Slovenia, Slovakia and the Czech Republic exhibited the highest increase in colorectal cancer incidence from 1983-87 to 1998-2002 with more than 45% increase in men and 25% in women (34). This increase is attributed to the high prevalence of obesity owing to the increased availability and consumption of food products due to an improved economy and diets high in fat and low in fiber (35). Obesity coupled with increased exposure to tobacco (evident from high incidence of lung cancer) is thought to contribute to the increased rate of colorectal cancer in men in these parts of Europe (34, 36).

In Spain there was an, 87% increase in colon cancer rates in men and 35% increase in women and these increases also correlated with increased westernization in Spain which was delayed until the mid-1970s (34). Among other developed nations, Japan recorded a large increase in the incidence rate (>90%) of colorectal cancer during the past several decades in males and the fecal occult blood test for screening colorectal cancer that was implemented during the 1990s in Japan is believed to be a reason for the increase in the incidence rates (37, 38). In addition, an increase in obesity due to higher intakes of fat-rich food also contributed to the increase in colorectal cancer in Japan (34, 39, 40).

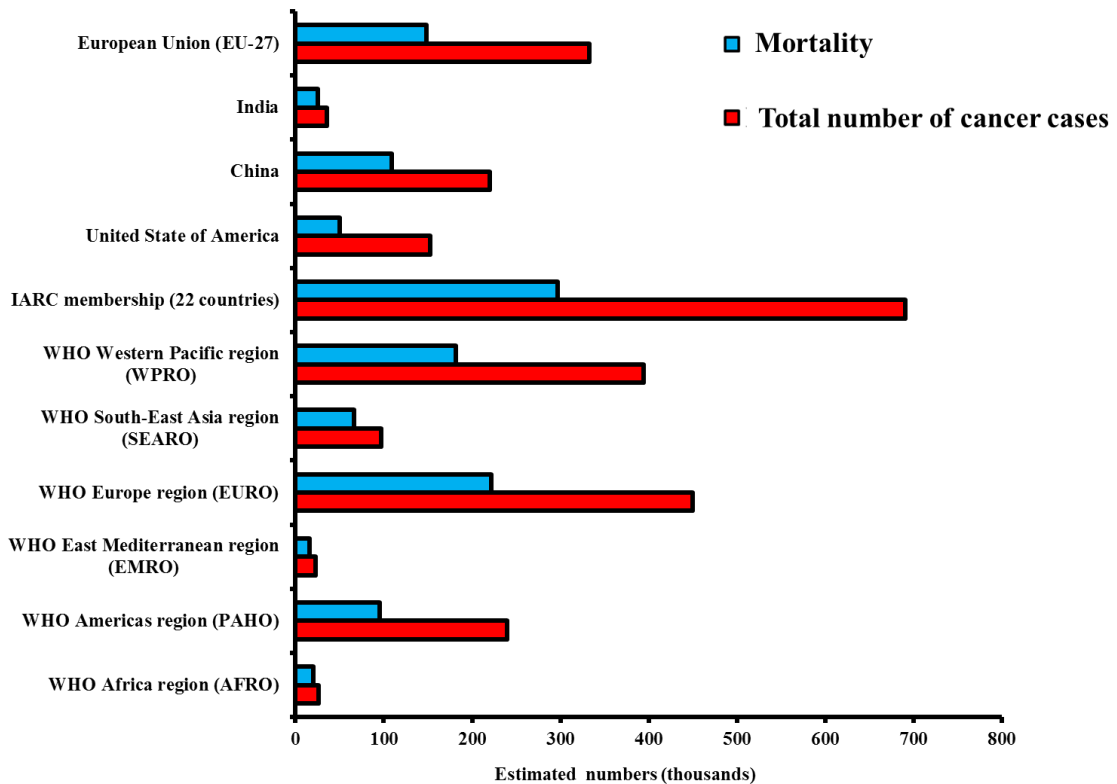


Figure 4. Colorectal cancer cases and cancer mortality worldwide in 2008 (30)

Two countries that shared changes in colorectal cancer incidence rates due to the ethnic and cultural differences in their populations were Singapore and Israel. Jews and non-Jews that comprise the Israeli population exhibited differences in the incidence of colon cancer due to a combination of environmental and genetic factors. Westernized lifestyle and other high risk factors such as genetic mutations resulting in the Lynch syndrome (HNPCC) increases colorectal cancer rates in Jews compared to non-Jews (34, 41) . In contrast, non-Jews in Israel, the majority of whom are Arabs practice a traditional lifestyle with a diet rich in fruits, vegetables and olive oil have a lower incidence of

colon cancer compared to Jews (42). The lowest rate (per 100,000 individuals) of colon cancer in the world was observed in India with an average of 5.8 and 4.35 in males and females respectively from 1998-2002, however recent trends indicates a slow rise in the rates of colon cancer (30).

The only countries that showed an exception to the increase in cancer incidence were the United States, Canada and New Zealand. There was a statistically significant decrease in the incidence of colon cancer in US men and women according to the nine SEER registries and similarly there was a decrease in the incidence rate in women from Canada and New Zealand. Canada and New Zealand showed stable incidence rates for colorectal cancer in men from 1983-87 to 1998-2002 (30, 34).

Besides the influence of diet and lifestyle choices, colorectal cancer screening can also contribute to the variation in the rates observed worldwide. Previously undetected cases can be diagnosed with the implementation of stool blood tests such as fecal occult blood test and other invasive screening tests like sigmoidoscopy and colonoscopy and this elevates the incidence rate. However, removal of precancerous polyps detected by colonoscopy can also decrease the number of colon cancer cases and this may explain the drop in the colon cancer rates in the US. However, in Canada and New Zealand national guidelines recommending colorectal cancer screening had been introduced only recently and due to lower screening rates its influence on the low cancer incidence is minimal (43-45).

Diet and Colon Cancer

Risk factors for colon cancer include obesity, increased alcohol intake, smoking, increased consumption of red meat, processed meats, highly refined grains, starch and low intakes of fruits and vegetables. The consumption of white meat such as poultry and fish, plant-based food sources of protein, substitution of saturated with unsaturated fats and incorporating legumes, sugars and unrefined grains as the major source of carbohydrates are known to reduce the risk of colon cancer (34, 46).

Fiber

The debate surrounding the fiber content in the diet and its role in reducing the risk of colon cancer has been in existence since the proposal of the dietary fiber theory by Denis Parsons Burkitt. According to Burkitt, the low incidence of colon cancer in certain African nations compared to western countries was due to the high content of fiber in their diet (46, 47). Fiber has multiple effects on the human colon such as increased fecal weight, which has been proposed to have an inverse relationship with colon cancer incidence (48). However, several studies comparing populations with similar fecal bulk and intestinal transit time have shown a difference in the incidence of colon cancer (48). In addition some studies show similar colon cancer incidence rates in populations with differences in stool weight and intestinal transit (49, 50). Fiber in the diet alters the metabolism of bile acids and increased concentration of bile acids in the feces are consistently observed in populations with high incidence of colon cancer (48, 51). The pH of the colon also contributes to colon carcinogenesis, with a low pH favoring

decreased development of colon cancer (52). Butyric acid (short-chain fatty acid), a degradation product of dietary fiber is one of the key anions contributing to a low pH environment of the colon. Butyrate is known to play a crucial role in controlling cell growth, suppressing DNA synthesis and reversing virus-induced cellular differentiation *in vitro*. Hence, butyrate may be essential for maintaining normal colonic mucosa and inhibiting transformation (48, 53, 54). The relationship between high dietary fiber and colon cancer has been examined in several case controlled and cohort studies. Although most case-control studies indicate a strong inverse correlation between intake of vegetables, fruits and fiber and risk of colorectal cancer, prospective cohort studies do not show that a high fiber diet protects against colon cancer. The inconsistencies between results from case-control and prospective cohort studies are unclear. However, according to Chan *et. al.* (46) cancer patients in case control studies are likely to recall unhealthy dietary behaviors since the information is collected after diagnosis of cancer and this can increase the chance of bias. A recent survey of 10 European populations showed that there was a 40% decrease in the risk of colorectal cancer in individuals with diets high in fiber, vegetables and fruits. This disparity may also be due to differences in levels of other nutrients in the diet such as folate, which has anti-cancer properties. High-fiber fruits and vegetables are the major source of folates in the European diet whereas folate-fortified flour and breakfast cereal are major sources in the US and consumption of large quantities of high-fiber fruits and vegetables may not prevent colorectal cancers in the US since the food products are already enriched with nutrients such as folates and other protective dietary factors (46).

Red Meat

Red meat is another dietary risk factor that has been associated with colorectal cancer. Most epidemiological studies show a clear association between increased risk of colorectal cancer and high intake of red meat (55-62). However, some studies do not show this correlation (63-65). Several theories have been postulated to explain the poorly understood association between red meat consumption and colorectal cancer. Red meat is believed to stimulate the secretion of the hormone insulin, which in turn binds to insulin receptors and insulin-like growth factor 1 (IGF-1) receptors to increase cell proliferation and inhibit apoptosis (66). Fat present in red meat is also hypothesized to contribute to colorectal carcinogenesis and studies in humans and laboratory animals show that high fat diets increase bile acid excretion (67-69). Experiments in rat and mouse models suggest that bile acids function as tumor promoters by increasing turnover of intestinal mucosal cells (55, 70-72). In addition, dietary fat also plays a role in modifying the intestinal microflora leading to transformation of bile acids into potential carcinogenic substances (69, 73). High levels of iron stores is thought to be a cancer risk factor in Western populations and heme iron is present in significant quantities in red meat and promotes growth of transformed cells and increases DNA damage by acting as a pro-oxidant and generating oxidative stress in cells (74, 75). Several studies have also examined the relationship between colorectal cancer risk and cooking of meat. Consumption of meat cooked at high temperatures for long durations leading to heavy browning of the surface of the meat increases the risk of colorectal cancer (76). Meat that is subjected to high temperatures generates mutagenic heterocyclic amines (HCA)

from creatinine, which are subsequently metabolized to N-hydroxylamines. These metabolites are further metabolized into N-OH esters by acetyltransferases and sulfonyltransferases which form DNA adducts primarily at guanine bases (77). Studies investigating the interactions between meat consumption, cooking (i.e. degree of “doneness” and genetic polymorphisms that affect the metabolism of these HCAs have shown an association between these carcinogens and colorectal neoplasia (78-80). In one such study women with genotypes that caused rapid acetylation of meat-related carcinogens had a higher risk of colorectal cancer with increased red meat intake in comparison to women who consumed large amounts of red meat and had a genotype associated with slower acetylation of meat-related HCAs (81). Avoiding the consumption of meat cooked at high temperatures for long durations and substituting red meat in the diet with alternative protein sources such as fish or poultry are effective means of reducing the risk of colon cancer (46).

Calcium

Calcium intake is hypothesized to be advantageous for reducing the risk of colon cancer. Dietary calcium is believed to form insoluble calcium soaps with toxic secondary bile acids and free ionized fatty acids in the lumen of the colon, thereby protecting the colon cells against their toxic effects (82). Calcium also plays a more direct role in reducing the risk of colorectal cancer by inhibiting proliferation and inducing apoptosis in colon cells (83, 84). Analysis of colon cancer cases from various studies evaluating the effects of dietary calcium suggests a decrease in the risk of distal colon but not proximal colon

cancer and most of the protective effects were seen with an intake of calcium within the range of 700-800 mg/day (85).

Vitamin D

Besides calcium, several studies have recorded the benefits of Vitamin D as an anti-proliferative, anti-angiogenic and pro-apoptotic agent in colon cancer cells (83, 86, 87). Results from a placebo-controlled randomized clinical trial indicate that the protective effects of calcium were observed entirely in individuals with high levels of 25-hydroxy-vitamin D in their circulation. This suggests that calcium supplementation and vitamin D levels act in conjunction and not separately in reducing the risk of colon cancer (88).

Vitamin B₉ (Folic acid)

B vitamins are essential components of the diet that affect processes such as DNA synthesis repair and methylation. Of all the B vitamins, folate has been the most studied in respect to the risk of colon cancer (46). Chronic folate deficiency is thought to result in hypomethylation of p53 and also DNA strand breaks of the p53 tumor suppressor gene, all of which can predispose a person to colon cancer (89, 90). A large prospective cohort study of women examining the effects of dietary folate on colorectal cancer showed decreased p53 mutations in subjects consuming 300-399 µg/day of folate; the beneficial effects of folate were not observed in women with tumors overexpressing wild-type p53 (91). Levels of folate and alcohol intake have also been associated with the enzyme methylenetetrahydrofolate reductase (MTHFR). MTHFR catalyzes the

conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-MTHF), which is a methyl donor for remethylation of homocysteine to methionine. A diet low in folate or depletion of methyl groups due to alcohol consumption can affect individuals expressing a functionally variant MTHFR that lacks the ability to effectively convert 5, 10-MTHF to 5-MTHF. Impaired methyl stores and insufficient dietary supplementation affect DNA methylation leading to dysregulation of gene expression (46, 92). Animal studies indicate that the dose as well as timing of folate administration can have a differential effect on colon cancer since administering high levels of folate at later stages of carcinogenesis can promote tumor growth (93). In addition, evidence from clinical studies suggests a possible detrimental effect of folate supplementation in patients with a recent history of colorectal adenoma (94).

Lifestyle and Colon Cancer

Lifestyle factors such as alcohol consumption, smoking and physical exercise also influence the risk of cancer.

Alcohol

The causal relationship between alcohol and colorectal cancer has long been debated, however most studies suggest only a modest correlation between these factors (95). The Pooling Project of Prospective Studies combined data from eight large prospective studies conducted in 5 countries and compared individuals who did not consume alcohol to those who had an alcohol intake of about 30 to <45 g/day. A multivariate relative risk

of 1.16 was associated with alcohol consumption and individuals consuming alcohol >45 g/day had a multivariate relative risk of 1.42 (96). Although, most case controlled and prospective cohort studies have indicated an increased risk of colorectal cancer in individuals consuming alcohol, some studies have failed to show this correlation (97-102). Various mechanisms have been proposed to explain how alcohol promotes colorectal carcinogenesis. Based on evidence from studies investigating folate status and colorectal cancer risk and the antagonistic effects of alcohol, it is plausible that the increased risks due to alcohol may be related to the effects on folate (92, 103). In addition, alcohol-induced immunodepression, changes in bile acid composition and activation of liver carcinogens via induction of cytochrome p450 enzymes may also contribute to the procarcinogenic effects of alcohol (46, 104).

Cigarette Smoking

Studies in the 1950s, examining the effects of cigarette smoking on colorectal cancer risk did not yield any positive correlation due to the lack of knowledge of the long gap between exposure and tumor development. However, with the discovery of colorectal adenomas and the early stages of colon cancer, a strong causal relationship was observed between smoking and increased risk of colorectal cancer. Smoking generates several genotoxic compounds including aromatic amines, polynuclear aromatic hydrocarbons, heterocyclic amines and nitrosamines. These carcinogens can irreversibly damage DNA and several meta-analyses of case-controlled and prospective cohort studies have demonstrated the relationship between tobacco smoking and increased risk of colorectal

cancer (46, 105-108). Meta-analyses revealed that past smokers were found to have a higher incidence of colorectal cancer than the current smokers, which is consistent with the time-lag between initiation and tumor development. In addition, the risk was elevated more for rectal than colon cancers and in the colon smoking increase tumors in proximal more than the distal region (106, 108, 109). Although, past smokers have a higher risk, a recent prospective study indicates that cessation of smoking for at least 31 years can reduce the risk of colorectal cancer (110).

Body Weight and Physical Inactivity

Excessive body weight or body mass index (BMI) is another risk factor for colorectal cancer in both men and women (111, 112). Meta-analysis of prospective studies showed that an increase in waist circumference (10-cm increment) elevated the risk of colorectal cancer by 33% and 16% in men and women respectively (113). Obesity related hyperinsulinemia may also promote cancer growth by increasing the levels of IGF 1, or binding to IGF- binding proteins and thus increasing the levels of free IGF1 (66). An insulin sensitizing adipokine (cytokines secreted by adipose tissue), adiponectin, exhibits an inverse correlation with adenoma and colon cancer risk in men in some studies (114, 115). Physical inactivity, which can lead to insulin resistance, is also a major colon cancer risk factor and prospective cohort and case-control studies have demonstrated an association between increased physical activity and reduced risk of colorectal cancer in both men and women. (116-118). The reasons for decreased risk of colon cancer in

physically active individuals is associated with low insulin levels, reduced systemic inflammation and decrease in abdominal adiposity due to physical exercise (46).

PROSTATE CANCER

Prostate cancer is the second most commonly diagnosed cancer accounting for 27.9 % of all cancers and sixth leading cause of cancer related deaths in men world-wide with 6.1 % of cancer mortality (30, 119). North America, Oceania and Northern and Western Europe display highest incidence rates, while the lowest rates of prostate cancer are observed in Asia and North Africa. Recent studies indicate that 23 % - 42 % of the cases of prostate cancer in high incidence areas such as Europe and U.S might be a result of over-diagnosis due to PSA (Prostate Specific Antigen) screening. Over-diagnosis could also be a confounding factor for the low mortality estimates due to prostate cancer in U.S Caucasian males and some Europeans where PSA screening is very common (120, 121). Low mortality rates from prostate cancer in most western countries including the U.S, Canada, Finland, France, Italy, Israel, the Netherlands, Norway, Portugal, Sweden and Australia have been attributed to the improved therapies and early detection (122-124). Certain Asian and East European countries such as Japan, Singapore and Poland, where PSA testing has been limited show a rise in prostate cancer incidence and mortality rates and this may be due to changing to diets rich in animal fat, physical inactivity and increased obesity (Figure 5) (122).

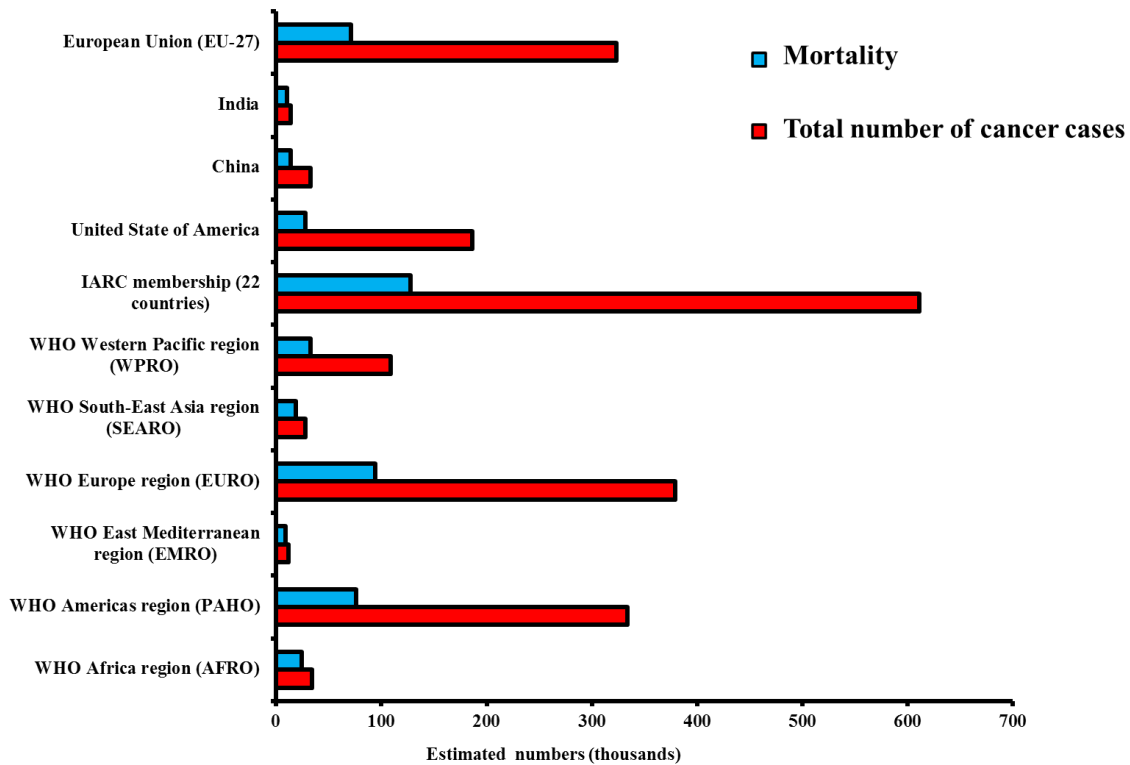


Figure 5. Prostate cancer cases and mortality worldwide in 2008 (30)

Genetic Predisposition

Risk factors associated with prostate cancer include old age, ethnicity and family history (125). Men below 40 years of age are seldom diagnosed with prostate cancer and death due to prostate cancer is a rarity in individuals who are less than 50 years of age. In the United States, African-American men are at a higher risk than individuals from other races and their mortality rate is 2.4 times higher than that of Caucasian men. Other ethnic populations such as Asian/Pacific-Islanders, Native Americans and Hispanic men have a lower than average risk for prostate cancer (125, 126). Family history of prostate cancer is an important risk factor and a 2 fold increase in risk is observed in individuals

with a paternal family history of prostate cancer and the risk increases by >3-fold if a brother has been diagnosed with prostate cancer. If both the brother and father are diagnosed with prostate cancer then the individual's risk for the disease is increased almost nine-fold (125, 127). Similarly, family history is also a contributor to prostate cancer mortality. Individuals with a father and brother who died of prostate cancer have a twofold higher risk for death resulting from prostate cancer compared to men without a family history of prostate cancer (125, 128).

Obesity

Most studies indicate that obesity in adulthood increases the risk for developing advanced stage prostate cancer and is accompanied by increased cancer recurrence and cancer-related mortality. A meta-analysis of 6 cohort studies reported that for every 5 kg m⁻² increase in BMI there was a 15 % increased risk of prostate cancer death in initially cancer-free individuals and a 20 % increase in prostate cancer-specific mortality. Similarly, a 5 kgm⁻² increase in BMI also elevated the risk for increased PSA levels by 21% (129). Evidence from several studies suggested that PSA concentrations in obese men tend to be low due to a larger blood volume, and PSA screening in obese men may be less accurate than in non-obese individuals (130, 131).

The mechanisms proposed for obesity-induced prostate cancer growth and progression are similar to that of colon carcinogenesis and include impaired functioning of multiple hormonal pathways and increasing levels of insulin and inflammatory cytokines, and

decreased levels of adiponectin, all of which can contribute to prostate cancer progression (132-134). The Physicians' Health Study reported an association between levels of C-peptide, a circulating marker for insulin secretion with the increased prostate cancer-related mortality. An increased risk was observed in obese individuals with high levels of insulin compared to obese men with lower C-peptide levels, suggesting that BMI and C-peptide levels together may be a better prognostic indicator for prostate cancer (125, 132). Results from the large (150,000 men) European Prospective Investigation into Cancer and Nutrition (EPIC) showed that risk of advanced prostate cancer was related to measures of central obesity, waist circumference (WC) and waist to hip ratio (WHR). A 6% increase risk of advanced prostate cancer was observed with a 5 cm increase in WC in men and a 21% increased risk was observed for every 0.1 units of increased WHR (135).

The effects of weight change in the time period before or after diagnosis of prostate cancer has not been extensively investigated; studies examining weight gain from early adulthood (18 or 21 years) to mid-life have produced mixed results concerning risk of prostate cancer. Some studies failed to show any significant correlation with prostate cancer incidence (136-139), while others (140, 141) such as the AARP-NIH Diet and Health Study observed that weight gain from adolescence to mid-life resulted in an increased risk for prostate cancer mortality but not for total, local or extra-prostatic disease (142). The only study to investigate effects of weight change on prostate cancer in the period shortly before and after diagnosis observed that weight gained 5 years prior

to treatment with radical prostatectomy and 1 year post-treatment increased the recurrence of prostate cancer, whereas the effects of weight loss were not significant (143). Studies on the effect of weight loss or gain before or after diagnosis of prostate cancer are important and represent a modifiable risk factor that can be used in preventing recurrence of prostate cancer in diagnosed/affected individuals (125).

Physical Activity

Several cohort studies have shown a strong correlation between physical activity and aggressive or advanced prostate cancer risk and data from the Health Professionals Follow-up Study (HPFS) found a lower risk for the incidence and mortality from advanced prostate cancer in men 65 years or older. However, no such associations were observed in younger individuals (144). Men from the American Cancer Society Cancer Prevention Study II Nutrition Cohort (CPS II) who performed the highest level of physical recreational activity and those who did not engage in any physical activity showed no difference in risk of prostate cancer. However, those individuals who were engaged in >35 metabolic equivalent-hours/week of recreational physical activity exhibited a lower risk for aggressive prostate cancer compared to subjects who were not involved in any recreational physical activity (145). The effects of post-diagnosis exercise on prostate cancer recurrence or mortality gave encouraging results; there was a 61% decrease in cancer-specific mortality in individuals exercising ≥ 3 hours per week, and brisk walking for 3 or more hours per week also decreased the incidence of prostate cancer recurrence (146, 147).

Smoking

Smoking has been consistently associated with higher risk for prostate cancer and results from a prospective cohort study showed a 1.6 relative risk for prostate cancer mortality for current smokers compared to non-smokers (148). Smoking earlier than 10 years prior to diagnosis and total life time smoking have not been linked to increased risk, however recent smoking i.e. within 10 years before diagnosis of the disease is associated with increased risk for advanced and aggressive prostate cancer (149). Smokers undergoing radiation, androgen-deprivation therapy and radical prostatectomy exhibit more severe outcomes compared to non-smokers undergoing similar treatment regimes, suggesting that smoking can also impair the beneficial therapeutic effects of prostate cancer treatment (150-152). The mechanisms of smoking-enhanced prostate cancer are not well understood but it has been suggested that cigarette smoking may increase plasma levels of total and free testosterone (153, 154) and carcinogens such as *N*-nitroso compounds and cadmium, which are present in cigarette smoke initiate prostate cancer (155, 156). Tobacco smoke may influence development of prostate cancer by enhancing cancer growth, angiogenesis and invasion which will be discussed later sections. Several studies show that nicotine enhances neovascularization, capillary growth and tumor growth associated with enhanced vascularity (157-159). Smoking may also induce certain epigenetic modifications since aberrant CpG hypermethylation of adenomatous polyposis coli (APC), glutathione S-transferase pi (GSTP1), and multidrug resistance one (MDR1) genes was observed in smokers and these genes are prognostic markers for advanced prostate cancer (160).

Oxidative Stress and Antioxidants

Oxidative stress is known to damage DNA and protein and hence contribute to carcinogenesis and the inhibitory effects of dietary antioxidants such as vitamin E, selenium and lycopenes on prostate cancer growth and progression have been investigated (125).

Vitamin E

Various cancer prevention trials such as the Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) study, have demonstrated a statistically significant reduction in the cancer risk and prostate cancer-specific mortality in men assigned to α -tocopherol (vitamin E) supplementation (161-163). In addition laboratory studies using experimental models show that vitamin E and its derivatives decrease DNA damage, inhibit malignant cellular transformation, suppress tumor growth and induce apoptosis in human prostate cancer cells (164-167). Nevertheless, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) and the Physician's Health Study II (PHS II) failed to show any significant reduction in the risk of prostate cancer in men receiving 400 IU day⁻¹ of α -tocopherol. Interestingly, a significant increase in the prostate cancer risk was observed due to vitamin E intake in the SELECT study (168, 169). The reasons for such variations observed in these studies are not clear. Examination of data from epidemiological studies indicates no overall association between prostate cancer risk and vitamin E alone but was observed in smokers with advanced prostate cancer risk (170, 171). Similarly, prospective studies demonstrate a protective effect of vitamin E and α -

tocopherol against prostate cancer in ever smokers and against lethal and aggressive prostate cancer in the general population (172-174).

Selenium

Selenium is an essential micronutrient required for the proper functioning of the antioxidant enzyme glutathione peroxidase. The amount of selenium in food depends on the soil in which it is grown in and hence can influence the amount of dietary intake of selenium. Ecological studies have indicated an inverse correlation between selenium content in the soil and prostate cancer incidence (175, 176). However, studies investigating the chemopreventive effects of selenium against prostate cancer have yielded ambiguous results. Although, some randomized trials and cohort studies indicate a decreased risk of prostate cancer among men taking selenium supplements, other studies has shown no significant correlation between selenium and prostate cancer (177-180).

Lycopene

Lycopene, a phytochemical and carotenoid pigment found in tomatoes and other fruits and vegetables has long been studied for its prevention of prostate cancer. Consumption of tomatoes, especially cooked tomatoes (processing increases the concentration of bioavailable lycopene) decreases prostate cancer risk, however this association was not observed in 11 case-control and 10 prospective cohort studies subjected to meta-analysis. A 25%-30% reduction in the risk of prostate cancer was observed in cohort

studies but dietary based case-controlled studies failed to show any correlation. (181, 182). Meta-analysis showed an inverse relation between plasma lycopene levels and prostate cancer risk with a relative risk of 0.55 in case-control studies and 0.78 for cohort studies (182). In an another nested case-control study there was a non-statistically significant inverse relationship between lycopene (OR, 0.66; 95% CI, 0.38–1.13) and risk of prostate cancer among men over 65 years, without a family history of prostate cancer (183). However, other studies show no significant association between lycopene and tomato consumption on prostate cancer risk (184-187). Most data suggest that consumption of lycopene and tomato-based products do protect against advanced stage prostate cancer if not the indolent disease (125).

Calcium

Calcium intake is another risk factor for prostate cancer due to its suppressive effects on the levels of the active metabolite of vitamin D, dihydroxyvitamin D [1,25(OH)₂D] which are involved in regulating differentiation and proliferation of the prostate epithelium (125, 188). Evidence suggesting an increased risk of aggressive prostate cancer with high intake of calcium has been reported in several studies (189-191). A recent study of African-American men in California, found a significantly lower risk of advanced prostate cancer in men who were poor absorbers of dietary calcium with the vitamin D receptor Cdx2 GG genotype. Those individuals with an AA genotype and higher amounts of calcium in the diet were at an increased risk for advanced prostate cancer (192). The association between serum calcium levels and risk of prostate cancer

has been investigated in three prospective cohort studies, the National Health and Nutrition Examination Survey (NHANES I), the NHANES Epidemiologic Follow-up Study and NHANES III. A 2.5 fold increase for cancer risk was observed for men in the highest tertile of serum calcium levels from the NHANES I and NHANES follow-up studies and similar trends were observed in the NHANES III follow-up study, suggesting that serum calcium can be used as a prospective biomarker of fatal prostate cancer (193, 194).

Coffee Intake

Lower risk of prostate cancer mortality due to coffee consumption has been reported in observational and animal studies. Improved glucose metabolism and insulin secretion is associated with long-term coffee drinking and coffee contains antioxidants that may protect against prostate cancer (195-197). Of the three studies that have examined the effects of coffee on prostate cancer mortality, two studies report no association of cancer mortality and coffee consumption. However, data from the Health Professionals Follow-up Study indicates an 18% lower risk for prostate cancer overall and 60% lower risk for prostate cancer mortality in men consuming six or more cups of coffee per day compared to non-coffee drinkers. Regular and decaffeinated coffee had a similar effect on the risk for cancer mortality in this study, indicating that active components other than caffeine might be responsible for the protective effects of coffee (198).

In summary environmental and lifestyle factors play a pivotal role in deciding the course of prostate cancer both before and after diagnosis. Well-designed prospective cohort studies and long-term follow ups can improve our understanding of the interaction between lifestyle factors and cancer outcomes and identify important prognostic risk factors, which in turn can facilitate diagnosis and improve the quality of life after prostate cancer has been diagnosed.

CANCER CLASSIFICATION

The discovery of the microscope and histopathological analyses of tumor sections coupled with the idea that cancer cells and normal cells arise from the same source led to the classification of neoplasms (27).

Classification Based on Histopathology

The human body is composed of four basic types of tissues namely, epithelial, connective, muscle and nervous tissues, and a combination of these tissues gives rise to the organs and organ systems of the body. The epithelium is made up of cells that are in close proximity to one another and rests on a thin filmy basement membrane which acts as a scaffold and a platform for their normal growth and regrowth after injury (199). Some epithelial tissues form sheets and layers that line the body cavities and protect the organs and blood vessels, while others give rise to specialized glands that secrete mucus to protect the cells and tissues from the caustic effects of acids and other substances. Epithelial tissues lack their own blood supply and hence are sustained by a supply of

essential nutrients from the underlying connective tissue also known as the stroma (200). “Carcinoma” is the term given to all cancers of epithelial origin and they account for 90% of all human cancers (201). Epithelial cells that specialize in protection give rise to “squamous cell carcinomas” and are frequently observed in locations such as the skin, nasal cavity, oropharynx, larynx, lung, esophagus and cervix. The cancers of the glandular epithelium that serve a secretory function are called “adenocarcinomas” and are commonly found at tissue sites such as the lung, colon, breast, pancreas, stomach, esophagus, prostate, endometrium and ovary (27).

Connective tissue, derived from the embryonic mesodermal layer form structures that contribute to the supportive framework of the body. Mesenchymal tissue is a type of embryonic connective tissue that gives rise to the extracellular matrix of tendons, skin, adipocytes, osteoblasts and myocytes. Cancers that arise from the mesenchymal tissues are termed “sarcomas” and account for only 10% of all cancers (202). The sarcomas are further classified into specific types depending on the site of the body that it affects. If the smooth muscle is involved then it is referred to as a leiomyosarcoma, similarly if the fat cells are affected it is termed a liposarcoma. Chondrosarcoma and osteosarcoma are the cancers of the cartilage and bone respectively (27).

Specialized connective tissues, namely blood, bone marrow and lymph nodes, give rise to hematological malignancies and are broadly divided into leukemias and lymphomas. Any myeloid or lymphoid malignancy that involves peripheral blood and bone marrow

is termed leukemia. Cells from the myeloid lineage include erythrocytes, granulocytes, platelets, macrophages and mast cells while the lymphoid lineage produces monocytes, lymphocytes and plasma cells. Leukemias are classified into acute and chronic based on the maturity of cells that are involved in the formation of the cancer. An increase in the number of immature blast cells is termed acute, whereas an elevation in levels of a more mature population of cells is referred to as a chronic condition. The terms “acute” and “chronic” can also reflect the time period required for the progression of the disease. The second criterion used to classify leukemia is the cell lineage. If the cancer forming cell population comprises of cells from the bone marrow that proceed to form lymphocytes then the term used is lymphoblastic or lymphocytic leukemia, while the term myelogenous leukemia is used when the cells involved in the malignancy are precursors of red blood cells, some types of white blood cells and platelets. Hence, this classification system gives rise to 4 major kinds of leukemia namely, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). A lymphoma on the other hand is a lymphoid malignancy that affects the lymph nodes and extramedullary sites, and is classified into Hodgkin’s and non-Hodgkin’s lymphomas (27, 29, 203, 204).

Neurons are involved in transmitting signals and impulses while the neuroglial cells protect, repair and support neurons. The nervous tissue gives rise to neuroectodermal tumors and account for less than 2% of all malignancies world-wide. Cancers of the glial

cells are called gliomas and are further categorized into low grade tumors called astrocytomas and high grade tumors termed as glioblastomas. The nerve sheath is lined by myelin producing cells known as Schwann cells and the neoplasms affecting them are termed schwannomas or neurilemmomas (27, 29).

Classification Based on Invasive Ability

Besides tissue of origin, invasive ability is another measure used to classify neoplasms. Neoplasms that exhibit a slow and localized growth are called benign (Figure 6). They are commonly encapsulated by a fibrous connective tissue capsule and do not infiltrate adjacent tissues. Benign neoplasms are composed of cells that are well differentiated with low numbers of mitotic figures (205). A large proportion of neoplasms that occur in the body are benign and do not pose a threat to the individual. However, in certain cases when the growth is on a vital organ, the resulting pressure on the organ can impair its normal functioning (27). Infarctions and obstructions caused as a result of benign growths on organs depending on the location can be life threatening to the host. Benign tumors may also have adverse consequences when present on endocrine glands due to secretion of enormous amounts of hormones into the circulation (27, 205).

Tumors that are not self-limiting in their growth and are potentially a threat to an individual's life are referred to as malignant neoplasms. A distinct feature of malignant tumors that sets it apart from benign growths is the ability to invade adjacent tissues and vasculature, and spread to distant sites, a process known as metastasis. Malignant

neoplasms grow rapidly and contain large numbers of cells with varying degrees of loss of differentiation (Figure 6) (27, 205). In contrast to benign tumors, they often outgrow their own blood supply causing tissue necrosis and host tissue damage (206). Their ability to metastasize to distant tissues also makes them sites of ectopic hormone production (205).

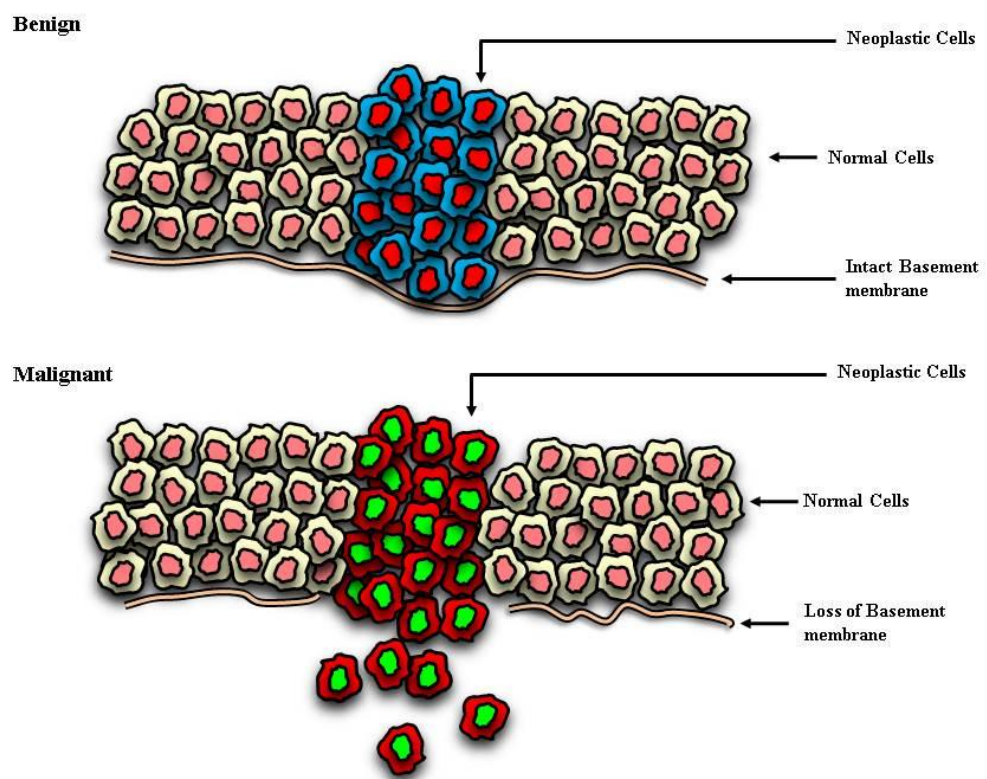


Figure 6. Histopathological representation of benign and malignant neoplasia (27)

Benign tumors are usually named using a prefix designating the specific tissue where the tumor originated followed by a suffix “-oma”, for example Lipoma, chondroma etc. The

term malignant neoplasm is synonymous with cancer and the specific designation is based on the tissue of origin followed by addition of the term “carcin” (for epithelial tissues) or “sarc” (for mesenchymal tissues) to the suffix “-oma”. Hence, a neoplasia of the bone is termed osteoma if it is benign and osteosarcoma if it is malignant. Similarly, a benign tumor of the glandular epithelium is called adenoma and its malignant counterpart adenocarcinoma (27, 205).

Classification Based on Morphology and Behavior

Neoplasms can be grouped into various categories based on the morphological and behavioral alterations they undergo prior to achieving a state of malignancy (Figure 7).

Hyperplasia

Cells that replicate at a far greater rate than the normal cells are termed hyperplastic. These cells have an abnormal proliferation rate; however, they resemble normal cells in morphology and are capable of assembling themselves into tissues like normal cells (27, 205). Besides physiological liver hyperplasia that is observed after partial hepatectomy, pathological increases in hormonal levels such as estrogen and progesterone can result in endometrial hyperplasia; similarly excessive production of adrenocorticotrophic hormone (ACTH) can lead to bilateral adrenocortical hyperplasia (205).

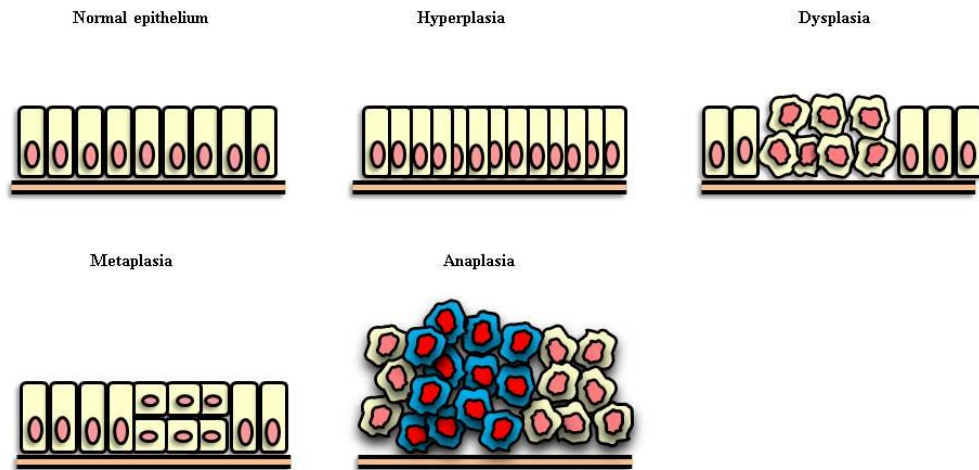


Figure 7. Histopathological representation of hyperplasia, dysplasia, metaplasia and anaplasia (205)

Metaplasia

Metaplasia is a condition where the cells of a particular region are replaced by another set of adult differentiated cells that are not normally encountered in that area. This is usually triggered in response to an adverse condition, as a protective mechanism. Metaplasia is frequently observed in the epithelial transition zones such as the regions between the cervix and the uterus or the esophagus and the stomach where the squamous epithelial cells get replaced by mucus secreting columnar epithelium (27, 205). Another example is the bronchial epithelium which is, lined by ciliated pseudostratified columnar epithelium that changes to stratified squamous epithelium as a result of chronic cigarette smoking. Similarly, squamous metaplasia is also encountered in the gall bladder due to chronic irritation from gall stones leading to a shift in the columnar or cuboidal epithelium to stratified squamous epithelium. Besides, epithelial tissues, connective

tissues such as tendons also undergo metaplastic changes and transform into cartilage or bone tissue (205).

Dysplasia

In case of dysplasia cells undergo atypical or irregular proliferative changes as a result of chronic irritation or inflammation (207). Some of the changes observed are variations in the size and shape of the nucleus, increased nuclear staining by dyes, increased ratios of nuclear versus cytoplasmic size, increased mitotic activity and absence of characteristics associated with the normal differentiated cells (27). Dysplasia is commonly encountered in the squamous epithelium of the bronchi of chronic cigarette smokers and in post-hepatic form of human liver cirrhosis associated with hepatitis B virus (HBV) (208, 209). Although these growth adaptive changes (hyperplasia, metaplasia and anaplasia) generally disappear when the stimulus is removed, they can be highly significant since these changes are the early steps in the development of neoplasia.

Anaplasia

Anaplasia is characteristic of a malignant neoplasm and literally means “to form backward” or “without form”. It is a term describing cells that have shed all their tissue-specific properties and are de-differentiated, making it impossible to trace them back to their tissue of origin. Anaplasia is characterized by two levels of disorganization namely cytologic and positional. The cytologic alterations include changes in the nucleus to cytoplasm ratio, an increase in the size of the nuclei and nucleoli, hyperchromatic nuclei,

increased cytoplasmic basophilia, an increase in the number of mitosis, atypical mitotic figures, formation of tumor giant cells and cellular pleomorphism. Positional anaplasia is characterized by loss of architecture and disordered cellular interrelations between the component cells of the neoplasm (27, 205).

CARCINOGENESIS

Cancer can be best defined as normal cells that have gone awry. The replicative potential of the cellular machinery that the metazoans are endowed with is highly complex and designed for highly ordered development and maintenance of adult tissue by repair of wounds and replacement of worn out cells. However, this finely tuned system is vulnerable to dysregulation by alterations of genes that maintain homeostasis and this results in uncontrolled cell growth and division. Oncogenes and tumor-suppressor genes are two classes of genes that are subjected to genetic alterations and this facilitates the process of carcinogenesis.

Oncogenes and Mechanisms of Activation

Genes that regulate cell proliferation, growth and differentiation in all multicellular organisms have an inherent potential to induce cancer and are called proto-oncogenes. These proto-oncogenes are precursors of oncogenes and are activated by mutations, gene translocations and gene amplifications (210, 211).

Mutations

Single point mutations of genes can often lead to alterations in the encoded protein structure as a result of amino acid substitution. These modified proteins in turn can contribute to the transformation of normal cells into a malignant phenotype (27, 205, 212). Structural alteration of the ras proteins (HRAS, NRAS and 2 types of KRAS species) encoded by the members of the ras family of oncogenes is a classic example of oncogene activation through mutations. Ras, a G-protein, is an important regulator of cell shape, motility and growth with an intrinsic guanosine-nucleotide binding activity and guanosine triphosphatase activity (213). Mis-sense mutations in the 12th, 61st and less frequently 13th codon leads to the substitution of amino acid residues glycine (G12) and glutamine (Q61) to valine resulting in an oncogenic form of ras protein that has an enhanced GTP binding and GTPase activity essential for its transforming ability (214-216). Twenty to thirty percent of all human tumors have mutations in the ras family of oncogenes, with the highest incidence in adenocarcinomas of the pancreas, colon, lung, thyroid and myeloid leukemia (217). *KRAS* mutations are commonly found in carcinomas of the lung, colon and pancreas, while *NRAS* mutations are frequently encountered in acute myelogenous leukemia and myelodysplastic syndrome (212, 218, 219). Mutations in another gene, *BRAF*, results in a structurally altered, constitutively active serine threonine kinase, B-Raf (220). The amino acid substitution of valine at position 599 to glutamic acid modifies the kinase domain of the protein that uncontrollably triggers the MAP kinase signaling cascade, which regulates cell proliferation, differentiation and survival. *BRAF* mutations are frequently observed in

melanomas, colorectal cancers, hepatocellular carcinomas and gliomas (221, 222). Similarly, Src, a tyrosine kinase, encoded by the SRC gene is another oncoprotein that is constitutively active due to a mutation in the 527th codon, which replaces tyrosine with an unrelated amino acid (27, 223).

Chromosomal Translocations

Chromosomal translocations are commonly observed in hematological malignancies such as Burkitt's lymphomas and chronic myelogenous leukemias (224). In some cases, certain sections of the chromosome carrying an oncogene break off and get juxtapositioned adjacent to a highly active transcriptional promoter or otherwise get fused with a section of a chromosome containing another oncogene. Activation of the c-myc proto-oncogene proceeds via insertional mutagenesis. A section of chromosome 8 containing the myc gene is reciprocally translocated to a region on chromosome 14 containing the transcriptional regulator for the immunoglobulin gene, thereby producing enormous amounts of growth promoting signals under the influence of a highly active promoter. Besides the t(8;14) translocation encountered in Burkitt's lymphoma, chromosomes 2 and 22 also undergo reciprocal translocation with c-myc containing chromosome 8 (27, 225-227). Philadelphia chromosome is a condition that is invariably observed in all cases of chronic myelogenous leukemia (205). Unlike Burkitt's lymphoma reciprocal translocation in CML results in the fusion of two sections of chromosome 9 and 22 each containing the genes *abl* and *bcr* respectively (224, 228,

229). The resulting fused genes synthesize a hybrid Bcr-Abl protein that exhibits elevated tyrosine kinase activity that enhances cell transformation (230).

Gene Amplifications

Growth factors and cell cycle related proteins are highly regulated under normal conditions. However, during cancer progression the increasing demand for these growth promoting factors is met by producing multiple copies of their corresponding genes via gene amplification. Cytogenetic findings from examination of tumors provide evidence for gene amplification in the form of abnormal karyotypic markers such as homogeneously staining regions, double minutes, and abnormally banded regions (231-234). Oncogenes from the MYC, cyclin D1, EGFR and RAS families are frequently amplified (212). The first evidence of gene amplification was found in the human promyelocytic leukemia cell line HL-60, with a 16-32 fold amplification of the *c-myc* locus (235, 236). C-myc amplification is also observed in colon carcinoma, small cell lung carcinoma and non-small cell lung carcinoma (237-239). A closely related gene, N-*myc* is frequently amplified in neuroblastomas and is associated with advanced tumor stage, aggressive tumor histology and clinical behavior (237, 240, 241). Amplification of EGFR (*ERBB1*) is observed in glioblastomas and cancer of the head and neck. ERBB2/HER2/neu is most often amplified in primary breast cancer and is associated with poor prognosis (242). Similarly, the *abl* proto-oncogene is also amplified in cell lines derived from patients with chronic myelogenous leukemia (243).

Oncogene Products

Products of oncogenes can be grouped into transcription factors, chromatin remodelers, signal transducers, growth factors, growth factor receptors and apoptosis regulators (212).

Transcription Factors

These are proteins that bind to short DNA sequences (cis-elements) to regulate transcription of genes positively or negatively (244). Most transcription factors share common structural domains such as, basic DNA binding domains, zinc fingers, homeobox etc. and interact with each other to regulate gene expression. Products of immediate early genes *myc*, *fos* and *jun* are frequently overexpressed in cancers (235, 239, 240, 245-247). The transcription factors Fos and Jun dimerize to form the AP1, which is a heterodimeric transcription factor often found in a hyperactivated state in cancer cells and regulates processes such as cell proliferation, differentiation and apoptosis (248). Stat3 of the STAT (signal transducers and activators of transcription) family of transcription factors is also constitutively activated in a number of human cancers. Stat 3 homodimerizes by binding to phosphotyrosine residues within their SH2 domains and migrate to the nucleus to regulate gene expression. Deactivation of Stat3 in breast cancer and melanoma cells triggers apoptosis and growth inhibition indicating its significance in cancer cell survival (249, 250). Prostate cancers commonly show fusion of an androgen responsive promoter element containing gene, TMPRSS2 with ERG1 or ETV1 genes from the ETS family of transcription factors that regulate cell proliferation,

differentiation and apoptosis. The resulting fusion products activate transcription factors that facilitate transformation of cells in the prostate gland into cancer cells by increasing proliferation and inhibiting apoptosis (251). Gli, Notch and NF κ -B are other transcription factors that function as oncoproteins (27).

Growth Factors

These are a group of polypeptides essential for cell growth and proliferation. However, a surge in levels of growth factors through constitutive activation of a growth factor gene can facilitate malignant transformation of cells (212). Under normal conditions growth factors are rate limiting components for cell proliferation. However, cancer cells have a diminished requirement for specific growth factors, which is achieved by autocrine activation, synthesis of essential growth factors, structural alterations in the growth factor receptors or switching to alternate pathways that obviate the need for a growth factor receptor (252-254). Members of the epidermal growth factor (EGF) family bind to receptor tyrosine kinases ErbB-1-4 to initiate various signal transduction cascades. Cancer cells hijack these signaling pathways to promote processes such as cell proliferation, differentiation and survival (255, 256). Transforming growth factor beta (TGF- β) is a cytokine that under normal conditions stimulates apoptosis and differentiation. However, during cancer TGF- β can act as a tumor promoter and increases epithelia-mesenchymal transitioning, which contributes to metastasis, stimulates angiogenesis resulting in increased vasculature in the tumor tissue and uncontrolled proliferation of cancer cells (256, 257). The VEGF (vascular endothelial

growth factor) family of growth factors consists of five proteins, VEGFA, VEGFB, VEGFC, VEGFD and PIGF (placenta growth factor) that bind to one of the three receptors VEGFR-1-3 to regulate angiogenesis and vasculogenesis. In addition, VEGF exerts certain autocrine responses on tumor cells and these include survival, migration and invasion (256). PDGF (platelet derived growth factor) is another growth factor that is involved in coagulation and wound healing and is capable of transforming fibroblasts containing PDGF receptors in vitro and inducing proliferation of various cancer cell types (212).

Growth Factor Receptors

Modified growth factor receptors are commonly observed in various types of cancers and contribute to pro-oncogenic pathways (258). Deletion of the ligand binding domain of EGFR can result in a constitutively active tyrosine kinase receptor that undergoes phosphorylation and transfers signals to downstream effectors even in the absence of a ligand bound to the receptor (259). Activating mutations of EGFR are found in at least 10% of non-small cell lung carcinoma patients (260, 261). Similarly, activating mutations are also observed in other members of EGR family- ERBB3, ERBB4 and less frequently in ERBB2 (212, 262). ErbB-2/HER2 is more often amplified at the corresponding portion of chromosome 17 to produce multiple copies of the receptors, a common feature observed in 20-25% of metastatic breast cancers (263). ErbB-2 is also overexpressed in stomach, ovarian and aggressive forms of endometrial tumors and ErbB-4 mutations are commonly observed in 19% of melanomas. IGF1R signaling

activates pro-survival pathways and is frequently amplified in breast cancer, melanoma and pediatric cancers. Translocations, mutations and amplifications of the PDGFR α gene are observed in gastrointestinal stromal tumors, glioblastoma, and chronic myelomonocytic leukemia (256).

Signal Transducers

Non receptor protein kinases and guanosine-triphosphate-binding proteins are two classes of signal transducers that participate in several cell signaling pathways essential for various physiological processes. Non receptor protein kinases are categorized into tyrosine kinases and serine threonine kinases depending on the amino acid residues phosphorylated by these kinases (264, 265). Mutations of signaling molecules including AKT, SRC, ABL, and RAF1, can also dysregulate the pathways and facilitate cancer progression and survival.

Apoptosis Regulators

Programmed cell death is a process by which, unhealthy or unnecessary cells are cleared from the body and it plays a vital role in development and maintenance of health. However, cancer cells express mutated genes that encode for proteins that inhibit the process of apoptosis thus providing a favorable environment for cancer cells to survive. The inhibitors of apoptosis (IAP) family of proteins are one such group of molecules that interfere with the process of programmed cell death and are frequently overexpressed in cancers. Members of IAP suppress apoptosis in cancer cells by inhibition of cysteine

proteases called caspases and pro-caspases. (266). Survivin, the key player of this family, is overexpressed in cancers of the lung, colon, breast, prostate, pancreas, stomach, high-grade lymphomas and neuroblastomas (267-270) and confers drug resistance in multiple cancer types (271, 272) Bcl2 family proteins are another group of apoptosis regulators with pro-apoptotic and anti-apoptotic abilities. Anti-apoptotic members of this family include Bcl2, Bcl-xL, Bcl-wl proteins and are commonly overexpressed in large B-cell lymphoma, chronic lymphocytic leukemia and lung cancer (212, 273-275).

MicroRNAs

In contrast to other oncogenes, genes that encode microRNAs give rise to single stranded non-coding RNAs 21-23 nucleotide in length. They act as negative regulators of protein expression at the post-transcriptional level by binding to 3'UTRs of the target mRNA to inhibit translation. MicroRNAs can act as oncogenes as well as tumor suppressors (276-278)

Tumor Suppressors

Tumor suppressor genes (TSG) encode a class of proteins that safeguard the cells from mutations and maintain the integrity of the genome thus preventing development of cancer. In contrast to oncogenes that are distinguished by their gain-of-function mutations, tumor suppressor genes acquire mutations that result in loss-of-function (213).

The existence of TSGs was not known until cell fusion experiments conducted by Harris and colleagues using normal and malignant cells demonstrated growth inhibition of murine tumor cells in syngeneic animals (279, 280). Results from this experiment suggested that malignancy was a recessive trait that can be suppressed by the presence of hypothetical wild-type alleles on a pair of chromosomes from the normal cells (280-282). In addition, tumorigenicity in the hybrid cells could be abolished despite the presence of activated oncogenes such as RAS (283). Since, these wild-type genes inhibited the tumor forming abilities in the normal-cancer hybrid cells they were referred to as tumor suppressor genes.

In spite of the evidence from the cell fusion experiments, the existence of TSGs was questioned by many for several reasons. Normal cell phenotype was considered to be a dominant trait and malignancy could be achieved only by mutating both the alleles of a tumor suppressor gene. The probability of inactivating both the copies of a gene per cell generation was estimated to be around 10^{-12} which was considered to be highly improbable. However, this conundrum was solved by the combined efforts of Carl O. Nordling and Alfred G. Knudson, who proposed the “two-hit hypothesis” (27, 284, 285).

Knudson’s “Two Hit Hypothesis”

The proof that cancer can arise in two steps with each step occurring at a rate within the accepted range of mutation rates came from the epidemiological studies conducted on a rare childhood eye tumor, retinoblastoma. Knudson observed two forms of

retinoblastoma, a familial form, characterized by bilateral lesions (presence of tumors in both eyes) with an early age of onset and a sporadic form of the disease with tumors affecting only one eye. Based on these observations he proposed the two-hit model for development of retinoblastoma. In case of the hereditary form of the disease, the first hit (mutation) is already present in the germ line, inherited from one of the parents. The germ line mutation on one allele is not sufficient for tumor development; hence a subsequent hit is required to inactivate the second copy of the wild-type gene. The second hit, which is invariably a somatic mutation, inactivates the remaining copy of the gene driving the retinal cells to proliferate uncontrollably. Since, all the cells have the germ line mutation in the retinoblastoma gene (Rb), retinal cells are highly vulnerable, and a single somatic mutation can result in tumor development, this explains the increased frequency of bilateral tumors in the familial form of the disease. On the other hand the sporadic form of the disease requires two successive somatic mutations in the retinal cells for the formation of retinoblastoma. Individuals affected with the sporadic form of the disease initially inherit two copies of wild-type alleles from their parents and hence require two sets of mutations to inactivate both the copies of the genes. The rarity with which two successive mutations occur at the same locus inactivating both the alleles explains the unilateral occurrence of the tumors in case of the sporadic form of the disease (284, 285).

Although, the two-hit hypothesis was successful in elucidating the mechanism by which inherited and somatic mutations cooperatively initiate the cancer development process it

failed to explain the highly improbable successive mutation events that are required for causing the non-inherited form of retinoblastoma (27).

“Loss of Heterozygosity”

Homologous recombination of chromosomal arms occurs during the process of gamete formation (meiosis). Chromosomal crossover is observed between the sister chromatids of the homologous chromosomes and is a process that ensures genetic diversity. Besides meiosis, sharing of genetic information between the chromosomes also occurs during active somatic cell division and is termed mitotic recombination (286-289). The inactivation of the second Rb allele was proposed to occur through the process of mitotic recombination.

It was hypothesized that inactivation of the first copy of the Rb gene takes place through a somatic mutation at a frequency (10^{-6} per generation) that was considered well within the limits of accepted values of mutation rates. This mutation results in a heterozygous condition ($Rb^{+/-}$) with one copy of the wild type *Rb* gene and a mutated copy of the inactive *Rb* allele. The second copy of the gene with a wild type configuration however is not inactivated by a mutational event but is replaced by a defective allele from the homologous chromosome that is formed during replication. Segregation of the chromatids at the end of mitosis can result in either retention of heterozygosity ($Rb^{+/-}$ and $Rb^{-/+}$) with one defective allele and one normal allele in the chromosome pair or loss of heterozygosity (LOH) resulting in an $Rb^{-/-}$ and $Rb^{+/+}$ genotype in the daughter cells.

LOH occurs at a frequency of 10^{-5} to 10^{-4} per cell generation, a far more probable event than somatic mutation, and is a more plausible explanation for the inactivation of the second copy of the gene in retinoblastoma. Since, the probability of occurrence of LOH is greater than somatic mutations, it is suggested that inactivation of the first copy of the gene might also go through LOH (27, 290). These postulations were later confirmed through LOH studies on the gene for *esterase D* that is closely located to the RB1 locus (291).

Most tumor suppressor genes can be classified into three groups: Caretakers, Gatekeepers and Landscapers.

Caretaker Genes

As discussed above the probability of multiple mutations occurring in a single cancer cell is low; however inactivation of certain specific genes called the caretaker genes can breach the genome's defense making it vulnerable to mutations. (292). The caretaker genes do not directly suppress tumor growth, instead they maintain the integrity of the genome by keeping it stable and free from mutations. Silencing of caretaker genes alone is not sufficient for tumor development instead the initiating event has to be followed by subsequent mutations in other tumor suppressor genes and oncogenes that precipitate transformation of a normal cell into a neoplastic state. In autosomal dominant diseases such as hereditary nonpolyposis colorectal cancer (HNPCC) only a single mutant allele of the caretaker gene is inherited and a somatic mutation of the second allele is required

to increase the mutation rate to inactivate the gatekeeper genes. However, in case of autosomal recessive syndromes such as xeroderma pigmentosum (XP), a condition that predisposes an individual to sunlight induced cancer, both alleles of the caretaker gene must be inherited in the mutated form (213). Germ line mutations of caretaker genes such as MLH1, MLH2, PMS1 or PMS2 that are involved in DNA mismatch repair are frequently observed in HNPCC. Similarly, nucleotide-excision repair genes, human excision repair cross complementing (ERCC) genes ERCC2 and 5 undergo mutations in case of xeroderma pigmentosum (293). Ataxia telangiectasia is a neurodegenerative disorder that is associated with a mutation in the caretaker gene, *ATM*, which plays a role in DNA repair. Under normal conditions the ATM protein senses double-stranded DNA breaks and relays signals by activating p53 to arrest cell cycle for the repair of damaged DNA, however the defective protein fails to halt the cell cycle leading to faulty repair of DNA and increased cancer risk (213, 293). Although inactivation of caretaker genes ultimately lead to cancer, restoration of these gene functions in a cancer cell will not affect its growth. Since these indirectly acting caretaker genes are not required for neoplasia, most nonhereditary, sporadically occurring tumors will develop without them (294)

Gatekeeper Genes

Unlike caretakers, gatekeepers are directly involved in control of tumor growth. These genes protect the cells from malignancy by inhibiting undue cell proliferation and inducing apoptosis. The gatekeeper genes ensure an added level of protection against

cancer in cells. When the genetic material is subjected to an insult that results in DNA damage, the caretaker genes are responsible for initiating the DNA repair process. However, in scenarios where the caretaker genes fail to carry out the DNA repair process, the gatekeepers trigger events to either stop further cell proliferation, to prevent mis-repaired DNA from replicating or to activate the programmed cell death machinery which eliminates the precancerous cells. If the gatekeepers fail to prevent damaged DNA from replicating, defective genetic material accumulates leading to malignant transformation of cells (295). The adenomatous polyposis coli (*APC*) gene functions as a gatekeeper gene in colon tissue. Truncating mutations of the *APC* gene leads to a condition called familial adenomatous polyposis (*FAP*) which is characterized by the presence of benign colorectal growths in the colon, which can progress to form malignant tumors. The gatekeeper genes have tissue-dependent functions and inactivation of a certain gene results in susceptibility to a specific cancer type. For example *APC* mutation predisposes an individual to tumors in the colon but not kidneys. Similarly, mutations in the Von Hippel–Lindau (*VHL*) gene lead to cancers of the kidney and not colon (213).

Some TSGs exhibit overlapping roles as both a caretaker and gatekeeper. For example the breast cancer susceptibility genes *BRCA1* and *BRCA2*, encode proteins that are involved in DNA repair and clearly conform to the class of caretakers. Several model systems exhibit global genome instability when *BRCA1/2* genes are functionally inactivated. It has been reported that *BRCA1/2* also regulates a wide range of genes

required for cell cycle control, cell proliferation and differentiation. These results indicate that BRCA1/2 can take on the role as a gatekeeper gene in addition their involvement in maintaining genomic stability as a caretaker (296). Similarly, the *P53* gene exhibits some level of duality in its function as a tumor suppressor. Silencing p53 leads to dysregulation of several genes controlling pathways involved in cell proliferation, growth and survival (297-299). Although p53 functions as a classical gatekeeper, evidence suggests a caretaker role with germ line mutations in the *P53* gene resulting in genome instability and tumor formation (300).

Landscaper Genes

This class of indirectly acting tumor suppressor genes was discovered following the histopathological analysis and observation of initiating lesions in juvenile polyposis syndrome (JPS). JPS is characterized by the presence of hamaratomatous polyps in the colon and have a low potential for developing into colorectal cancer. These polyps are composed of mesenchymal and inflammatory cells unlike the adenomatous polyps that give rise to colorectal cancers, which are rich in epithelial cells. The few epithelial cells within and around the hamaratomatous polyps do not exhibit any signs of neoplasia and are not at an increased risk of progressing towards malignancy. JPS can advance to a state of neoplasia by mutations in the mesenchymal cells that give rise to a defective stromal environment which in turn drives proliferation of epithelial cells and this is described as a landscaper effect. The mutations in the landscaper genes are predicted to affect various components of the tumor microenvironment including extracellular matrix

proteins, adhesion proteins, or secreted growth/survival factors, unlike the mutations in the other two classes of genes which directly influence the tumor cells (301). Recent studies investigating the molecular genetic basis of JPS, have localized the genetic defects to *PTEN* and *SMAD4* genes (302, 303). *PTEN* gene gives rise to PTEN (phosphatase and tensin homolog) a dual specificity phosphatase that regulates the cell cycle and controls growth while *SMAD4* codes for a protein that is involved in the TGF- β signaling pathway. Further genetic evaluation of the stromal and epithelial cell population of the JPS polyps has revealed that the clonal alterations of *PTEN* and *SMAD4* are located in the stromal cells and not the epithelial cells (304). These observations in addition to the presence of a significant population of mesenchymal cells in polyps concur with the idea that the stromal cells are neoplastic and exert a landscape effect on the adjacent epithelial cells (305).

The Non-oncogenic Addiction Phenomenon (NOA)

In addition to oncogenes, there are many other genes that are essential for the maintenance of the cancer phenotype in cells; however they do not undergo any oncogenic mutations or alterations to their function. This heightened dependence of tumors on several pathways driven by a set of genes that are not classical oncogenes is termed non-oncogenic addiction. Since these genes are rate-limiting for oncogenic pathways, they can be potential targets for cancer therapy (306). The genes of the NOA can be classified into two groups namely, intrinsic and extrinsic. The tumor-intrinsic NOA genes function within the tumor cell in a cell-autonomous manner while the

extrinsic NOA genes operate in the stromal and vascular cells that support the tumor (307). The heat shock factor 1 (HSF1) is a well-studied non-oncogene and a definitive example of the non-oncogene addiction phenomenon. HSF1 is a transcription factor that is activated in response to proteotoxic stresses such as heat and hypoxia. HSF1 mediates its effects via heat-shock proteins that are involved in refolding of proteins, inhibiting protein aggregation and directing misfolded proteins to the ubiquitin proteasome pathway for degradation. Studies conducted in HSF1 deficient mice demonstrate a lower incidence of tumors, reduced tumor burden, and increased survival when compared to their wild-type littermates. Similarly, knockdown of HSF1 employing small hairpin RNAs (shRNAs) decreased the viability of multiple cancer cell lines with minimal effects on normal cells, suggesting a higher dependence of cancer cells on HSF-1 compared to normal cells (307, 308). However, HSF1 does not show any somatic mutations and overexpression of HSF1 fails to transform immortalized mouse embryonic fibroblasts (MEF). In addition, MEFs devoid of HSF1 are resistant to transformation by either oncogenic *H-RAS^{V12D}* or *PDGF-B*. It is hypothesized that HSF1 induces heat-shock protein 90 (HSP90) which forms a complex with its substrate targeting unit, Cdc37 and this complex stabilizes numerous proteins such as Cdk4, HER2/ErbB2, Akt that are involved in cell proliferation and survival (306, 309).

Other examples of non-oncogenic addiction include increased dependency of BRCA-2 deficient cancer cells on the DNA stress protein poly ADP-ribose polymerase (PARP), tumor cell mediated suppression of immune surveillance by downregulation of major

histocompatibility complex (MHC) and synthesis of chemokines (e.g., CCL2), cytokines (e.g., IL6 and IL10), and prostaglandins to suppress activation of cytotoxic T cells (307, 310, 311).

Normalcy to Malignancy

Genetic changes caused by the action of carcinogens in normal cells leads to transformation of the cell into a cancerous phenotype. In addition recent studies indicate that epigenetic changes, such as those caused by methylation of gene promoters and histones are also crucial events required for development of cancer. However, it is still not clear if epigenetic changes are caused by chemical carcinogens or occur spontaneously due to dysregulation of proteins involved in promoter and histone methylation. The stages involved in the transition of a normal cell into a cancer cell include initiation, promotion, malignant conversion and tumor progression (Figure 8).

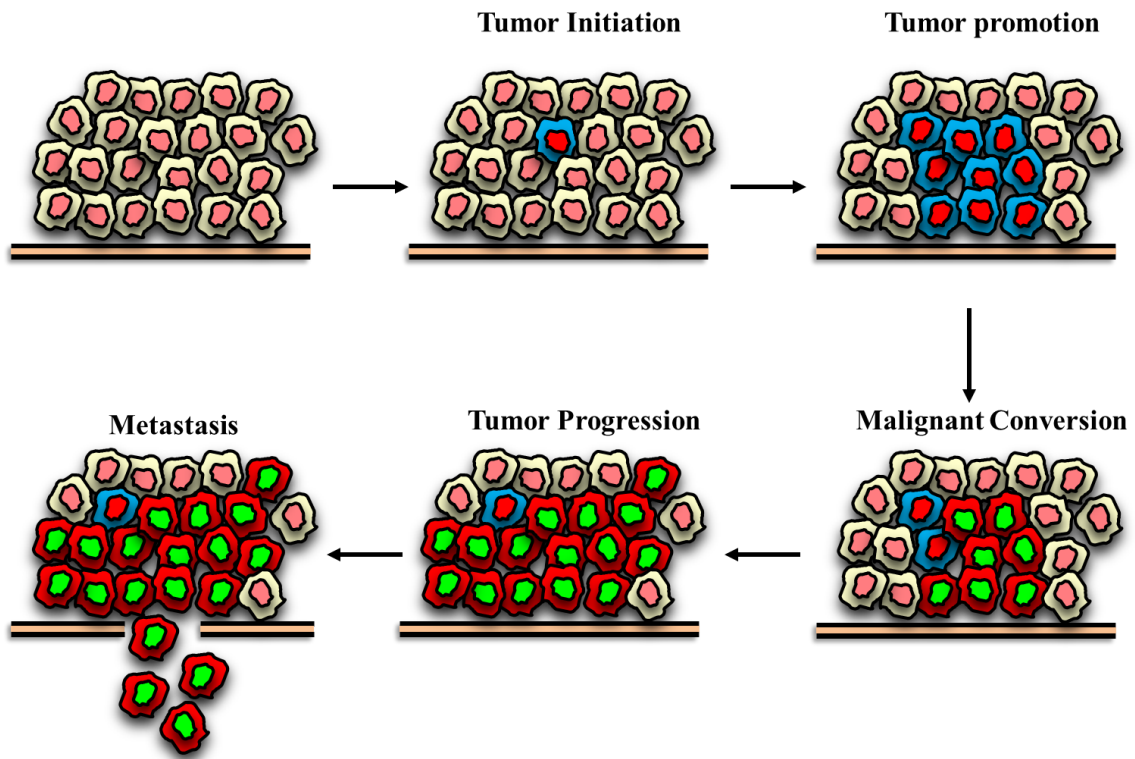


Figure 8. Schematic representation of processes involved in tumor development

Tumor Initiation

Tumor initiation is the first step in multistage carcinogenesis and is characterized by genetic alterations that result in dysregulation of the physiological control mechanisms in an initiated cell. Chemical carcinogens damage genetic material by forming adducts with nucleotides of DNA which can eventually lead to mutations, if left unrepaired (312). Most of these chemicals do not readily interact with biological molecules, but are metabolized into mutagenic electrophiles by drug metabolizing enzymes that are involved in metabolizing and eliminating endogenous and exogenous substances. The

electrophilic metabolites react with nucleophiles such as DNA and protein to form covalent adducts which can lead to irreversible damage. The ability to detoxify carcinogens or repair the damaged DNA depends on the gene-environment interactions which in turn determines the cancer risk of the exposed individual (313). Carcinogens such as X-rays can ionize water molecules in the cell to generate aqueous free radicals, which react with DNA strands to form double-strand breaks leading to loss of genetic material (314). Changes which include genetic damage that activates proto-oncogenes and inactivate tumor suppressor genes result in an undue growth and survival advantage for these cells and this can be categorized as a tumor-initiating event (315). Recent evidence suggests that epigenetic changes such as DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs may also play a key role in initiating tumorigenesis in some forms of cancer (316, 317). For example colon, lung, lymphoid and other tumors display *de novo* hypermethylation of the CpG islands in the promoter of the DNA repair gene MGMT leading to aberrant DNA repair and increased G to A mutations (318-320). Similarly, spontaneous hydrolytic deamination of hypermethylated cytosine can cause C to T transition mutations (321).

Tumor Promotion

Normal cells undergo a series of mutations in order to achieve a state of complete malignancy. Since the accumulation of mutations is dependent on the rate of cell proliferation, the initiated cells undergo rigorous cell division with no effect on the adjacent uninitiated cells. This process known as clonal expansion is dependent on

promoting agents that generate a population of cells that are predisposed to further genetic alterations and malignant transformation (322, 323).

Most tumor promoters elicit their biological responses without metabolic activation and they are primarily involved in facilitating formation of tumors in initiated cells. Promoters are generally not mutagenic and cannot function alone as a carcinogen but act in concert with the initiator. Chemicals that exhibit tumor-promoting effects include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzoyl peroxide, macrocyclic lactones, bromomethylbenzanthracene, anthralin, phenol, tryptophan, dichlorodiphenyltrichloroethane (DDT), phenobarbital, cigarette smoking condensate, polychlorinated biphenyls (PCBs), teleocidins, cyclamates, estrogens and other hormones, bile acids, ultraviolet light, wounding, abrasion, and other chronic irritation (312). Some agents such as benzo[a]pyrene and 4 aminobiphenyl function as both tumor initiators and tumor promoters and hence are termed complete carcinogens.

Malignant Conversion

Cells can undergo further genetic changes which transform them from a preneoplastic state to a malignant phenotype. Conversion of cells into a malignant form is achieved by repeated exposures to a tumor promoter. Since the rate of cell division influences the risk of conversion into malignant cells, clonal expansion facilitated by tumor promoters enhances conversion of cells into a malignant phenotype which is also paralleled by further genetic changes. The genetic alterations that occur can be attributed, in part, to

faulty DNA synthesis and repair mechanisms leading to activation of oncogenes and inactivation of tumor suppressors (324). In addition exposure of preneoplastic cells to DNA-damaging agents greatly increase the odds of malignant conversion (312).

Tumor Progression

The malignant cells acquire more aggressive traits such as the ability to invade adjacent cells and tissues. Further genetic and epigenetic changes lead to increased genomic instability and uncontrolled cell proliferation favoring tumor progression (325). Some of the changes observed during this stage are activation of proto-oncogenes such as *ras*, *myc*, *raf*, *HER2* and *jun* through point mutations, gene amplifications and chromosomal translocations. Similarly, loss of functional tumor suppressors occurs via deletion, chromosomal recombination and disjunction. These changes enhance the ability of cells to metastasize into distant tissues and establish secondary tumors at these sites by uncontrolled proliferation (315). Although there is evidence for different stages of cancer cell progression, the corresponding mutations acquired by cells to achieve a state of malignancy results from the accumulation of these genetic changes and not the order or the stage in which they occur.

The effects of tumor initiators and promoters on normal cells and the various steps involved in multistage carcinogenesis have been observed in a skin cancer model. The role of non-mutagenic agents in tumor formation and growth came to light during attempts to effectively induce skin tumors in mice (326, 327). The mouse skin tumor

model involves application of chemicals such as benzo[*a*]pyrene (BP), 7, 12-dimethylbenz[*a*]thracene (DMBA), or 3-methylcholanthracene (3-MC) on a patch of skin and after several months of treatment skin carcinomas are formed. Modifying this approach to a single application of DMBA and repeated administration of a skin irritant TPA (12-*O*-tetradecanoylphorbol-13-acetate), active ingredient of croton oil, to the same area of skin led to growth of papillomas after 4-8 weeks (328, 329). TPA application was essential for the formation of papillomas and discontinuing the application of TPA caused the papillomas to regress. DMBA application was critical for papilloma formation and repeated treatment with TPA alone did not result in papilloma formation. In addition treatment with DMBA followed by delayed (one year) administration of TPA resulted in formation of skin tumors. These observations lead to the conclusion that DMBA serves as a tumor initiator that results in an irreversible long-lived genetic alteration, evident from the fact that skin tumors form even if promotion with TPA is begun one year post DMBA treatment. TPA, on the other hand functions as a non-genotoxic tumor promoting agent. Thus DMBA initiated cells when subjected to repeated exposure to a promoter (TPA) undergoes clonal expansion essential for transforming initiated cells into a preneoplastic state. Certain papillomas exhibit TPA independence following several TPA treatments and these tumors continue to grow and develop into carcinomas even after TPA treatments are discontinued. This phenomenon suggests a second genetic alteration which facilitates acquisition of aggressive traits resulting in cell transformation. An identical effect is observed when the cells are subjected to a second application of the initiator. This phenomenon involving a second

genetic event pushing the benign growth to an aggressive phenotype is called tumor progression (330-332).

DMBA is a known mutagen and generates mutations in exposed cells and skin tumors invariably contain point mutations in the *H-ras* oncogenes and experimentally transfected skin cells expressing *H-ras* oncogenes closely resemble DMBA initiated cells. DMBA treatment also converts the *ras* proto-oncogene to an active oncogene (333). However, this mutation alone does not enhance cell proliferation and promotion with TPA gives rise to a papilloma. Exposing this papilloma to a mutagenic agent results in mutation of the *p53* tumor suppressor gene, which functions in concert with the *ras* oncogene to form carcinomas (334). TPA activates the serine threonine kinase, protein kinase-C α (PKC α), which subsequently activates several downstream genes such as NF- κ B, Fos, Jun and MAPK which cooperate with *H-ras* oncogene to enhance proliferation (335-337).

Cytotoxic agents can also function as tumor promoters. For example cigarette smoking and frequent consumption of alcohol increase the risk of certain head and neck cancers (338, 339). Various mutagenic carcinogens that are present in cigarette smoke can initiate cells present in the epithelium lining throat and mouth. Consumption of large quantities of alcohol can cause cellular toxicity and although alcohol is a weak mutagen it is capable of causing cell death due to its toxic effects. This toxic effect on the mucosal cells in turn stimulates the underlying stem cells to divide in order to restore the

epithelial cell layers. Under normal conditions these stem cells proliferate at a slow and steady rate, however, to compensate for the damage sustained the cells divide at a faster pace resulting in clonal expansion of the initiated cells. This rapid proliferation of cells helps in gaining other mutations that eventually lead to head-and-neck cancers (27, 338, 340).

Molecular Genetics of Colon Cancer

Studies on the development and progression of colorectal cancer suggest that colon cells pass through various stages prior to formation of a carcinoma; the individual stages are not readily observed in every tissue however, sequencing experiments reveal genetic alterations corresponding to all stages of tumor development (Figure 9). Human aberrant crypt foci (ACF) are flat colonic epithelial lesions characterized by altered glandular architecture with the absence of dysplasia (341). ACF are considered to be the precursor lesion which gives rise to colon cancer. Any growth that is found above the mucosal layers is termed a polyp and colorectal polyps that measure less than 5 mm in size are hyperplastic in nature and hyperplastic polyps are not precursors of colorectal cancers (342). In contrast adenomatous polyps that are formed from the glandular epithelium exhibit high degrees of dysplasia with varying levels of differentiation in the epithelial cells and there is evidence that adenomatous polyps develop into colorectal carcinomas (343).

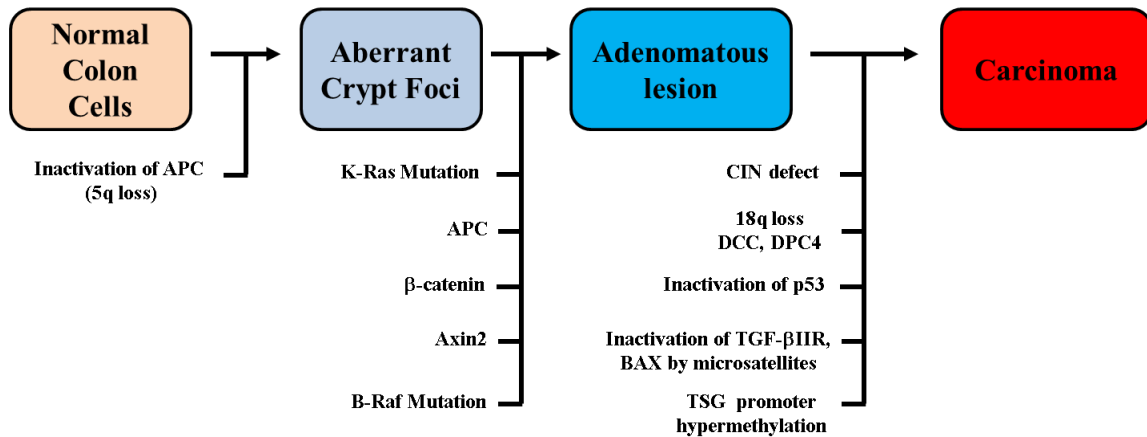


Figure 9. Genetic model of colorectal cancer (344)

Adenomatous Polyposis Coli Gene (APC), Loss of 5q

The adenomatous polyposis coli tumor suppressor gene (*APC*) is frequently found defective in various familial syndromes such as familial adenomatous polyposis (FAP), Gardner syndrome and Turcort's syndrome that predispose individuals to colorectal cancer. Besides familial syndromes *APC* is inactivated by somatic mutations in 70-80% of sporadic colorectal adenomas and carcinomas and are considered to be an early event in the formation of adenomas (345-347). Studies show that the frequency of *APC* gene mutations does not vary among small adenomas, advanced adenomas and carcinomas, unlike other somatically mutated genes found in colon cancer (347, 348). In addition the earliest lesions such as microscopic adenomas exhibit somatic mutations in the *APC* gene and both the alleles of the *APC* gene are inactivated in adenomas and carcinomas found in FAP patients and sporadic colorectal cancer patients in accordance to the Knudson's two hit model (344, 346). Hence, it is hypothesized that loss or inactivation

of the APC gene located on chromosome 5q induces proliferation of colon cells and these cells may then undergo clonal expansion to give rise to an adenoma (348). The most well studied function of the APC protein is regulation of β -catenin in the canonical Wnt signaling pathway. Wnt ligands bind to their cognate receptor complex of Frizzled proteins and inhibit the function of proteins such as glycogen synthase kinase 3 β (GSK3 β) and Axin thus stabilizing the β -catenin levels in the cell (347, 349). β -Catenin interacts with certain DNA-binding proteins such as the TCF/LEF (T-cell factor/lymphoid enhancer family) in the nucleus where it serves as transcriptional coactivator of several genes including *cyclin D1*, *CMYC*, *matrix metalloproteinase 7* (MMP7) and growth factors FGF20 and FGF9 (350). In the absence of the Wnt ligand APC binds to Axin and facilitates phosphorylation of β -catenin by two kinases namely casein kinase 1 and GSK3 β . Phosphorylation of β -catenin targets it for ubiquitination and subsequent proteasomal degradation. However, inactivation or loss of APC increases β -catenin levels, which transcriptionally activate several proto-oncogenes that encode for cell cycle proteins and growth factors (347, 349).

DNA Hypomethylation

An overall decrease in DNA methylation is observed in colon cancer cells compared to adjacent normal cells and this is also observed in early stages of colorectal cancer. DNA from small adenomas indicate hypomethylation in one-third of the DNA that was analyzed (351, 352). The loss of DNA methylation especially in pericentromeric sequences prevents chromosome condensation and can result in mitotic non-disjunction

leading to loss or gain of chromosomes (353). Sequences such as the long interspersed nucleotide element 1 are affected by the global decrease in methylation which can potentially activate some elements to transpose (354-356). In addition hypomethylation also alters genomic imprinting resulting in the loss of imprinting of regulators of cell proliferation and apoptosis (357). Lastly, epigenetic changes such as hypomethylation can predispose cells to aneuploidy causing instability in the genome leading to genetic alterations such as allelic losses of tumor suppressor genes (348).

KRAS Mutations

Mutations in the ras gene in an early adenoma triggers clonal expansion resulting in the formation of dysplastic tumors (348). The 3 important members of the ras sub-family *KRAS*, *HRAS* and *NRAS* are frequently found mutated in several human cancers with 40% of colon cancer cases containing *KRAS* somatic mutation (358). The *ras* genes encode a family of small-G proteins that operate downstream of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR). Ras plays a role in activating mitogen activated protein kinase (MAPK) and PI3K signaling pathways involved in cell growth, proliferation and survival. Mutations affecting *KRAS* are commonly found on codons 12 and 13, while mutations affecting codon 61 are rare (344). These mutations in the *KRAS* alleles are believed to be responsible for the tumorigenic properties of advanced colon cancer cells. Moreover, inactivation of the mutated *KRAS* alleles in advanced cancer cells inhibits their tumor forming abilities both *in vitro* and *in vivo* (359). Although, *KRAS* mutations are considered to be an event that transitions an

adenomatous polyp into a state of dysplasia, evidence from several studies indicate the presence of KRAS mutations in colon polyps that do not exhibit any malignant potential. In addition KRAS mutations are frequently observed in non-dysplastic lesions such as the aberrant crypt foci and hyperplastic polyps that may not develop into a carcinoma (341, 360, 361).

LOH of Chromosome 18q

A majority of colon carcinomas (70%) exhibit loss of heterozygosity on the long arm of chromosome 18. Of the 70% of colon cancers exhibiting LOH at chromosome 18, 50% are large and late-stage adenomas and less than 10% are early and small adenomas suggesting that loss of 18q may be responsible for progression rather than initiation of colorectal carcinomas. (348, 360). *SMAD2* and *SMAD4* are two tumor suppressor genes that are located on chromosome 18q and these genes are found mutated in 5% and 10-15% of colon carcinomas respectively (344, 362, 363). In addition, *SMAD4* undergoes biallelic loss in one-third of metastatic colon tumors. These genes encode proteins that play a vital role in the transforming growth factor-beta (TGF- β) receptor dependent growth inhibitory signaling pathways. Binding of TGF- β ligand to the TGF- β type II receptors leads to phosphorylation of the type I TGF- β receptors, which in turn phosphorylates members of the Smad family of transcription factors. Phosphorylated Smad 2/3 complexes interact with Smad4 to give rise to hetero-oligomeric complexes which translocate into the nucleus to modulate transcription of genes such as such as p300/CBP, TFE3, Ski and c-jun (364-366). TGF- β mediated growth arrest is

accomplished by two mechanisms namely, induction of p15 and p21, inhibition of cdc25A and downregulation of *c-myc* (366). *SMAD2* and *SMAD4* genes are inactivated by mutations (frameshift, missense or non-sense) or loss of an entire region of a chromosome or small deletions (366). *Deleted in colorectal cancer (DCC)* is another gene found on chromosome 18 and DCC is deleted in 70% of colorectal cancers. It is present in the region of allelic loss of more than 90% of colon cancers with LOH of chromosome 18q (367, 368). DCC functions as a part of a receptor complex for netrin-1, which is a laminin-related secreted protein that plays a critical role in cell migration and axonal outgrowth in developing nervous tissues (369-371). DCC exerts pro-apoptotic effects by activation of cysteine aspartic protease caspases. When DCC is unbound to its ligand, netrin-1, it is cleaved at its intracellular domain by an unknown caspase (372). The cleavage exposes an upstream addiction/dependence domain (ADD) and releases the inhibitory C-terminal domain. The ADD then interacts with the initiator caspase-9 and triggers the apoptosis cascade (373). However, when DCC is bound to netrin-1 ERK1/2 MAPK and small GTPases are activated (374, 375). In mice overexpression of netrin-1 generates hyperplastic and neoplastic lesions and inhibition of cell death in the gastrointestinal tract. The dual functions of DCC classify this gene as a conditional tumor suppressor (376).

Loss of 17p, p53

Loss of p53 function is considered to be a key step in the transformation from an adenoma to a carcinoma and. 70% of all colon cancers show LOH on 17p and most

adenomas lack 17p LOH and exhibit *p53* mutations on the remaining *p53* allele (348, 360) (377). *p53* has an active role in transcriptional regulation of genes that encode factors that participate in cell-cycle checkpoints, promote apoptosis and inhibit angiogenesis (378). The adenoma-carcinoma transition process can impose tremendous amounts of stress on cells which may be counter-productive to cancer cells. During conversion from adenoma to carcinoma stresses such as DNA strand breakage, telomere erosion, hypoxia and reduced nutrient exposure can activate cell-cycle arrest and apoptosis by means of wild-type *p53* functions. However, *p53* mutations enable tumor cells to survive unfavorable environments and facilitate cancer cells to progress to the carcinoma stage (344). In addition *p53* mutants with missense mutations retain the ability to inhibit autophagy in the cytoplasm similar to its wild-type counterpart but are unable to transcriptionally activate autophagy in the nucleus thus deregulating this cell death pathway (379-381).

In addition to these changes other gene defects including mutations in *TCF4*, *AXIN1* & 2, *PI3K*, *BRAF*, *CDK8*, *PTEN* are also observed in different subsets of colon cancers (344). *KRAS* mutations and the absence of *APC* mutations have been reported in most ACF from sporadic cancers while ACF from familial adenomatous polyposis patients exhibit *APC* mutations with no *KRAS* mutations indicating that the molecular mechanisms operating in sporadic and familial forms of colon carcinomas are different (382). Similarly, demonstration of mutational events such as loss of 17p and other genes in both carcinomas and in some very small adenomas suggest that although there is a

preferred sequence for genetic events during colon carcinogenesis, the accumulation of these events may be more critical than their order of occurrence (348).

Molecular Mechanisms: Prostate Cancer

Prostatic intraepithelial neoplasia (PIN) is considered to be the first step in development of prostate cancer and the precursor lesions are localized in the pre-existing duct acinar system (383). Based on the extent of cytological abnormalities PIN is classified from low to high grade. The distinguishing features of PIN include increased cell crowding, luminal epithelial hyperplasia, fewer numbers of basal cells, enlarged and atypical nuclei, anisonucleosis and cytoplasmic hyperchromasia with minimal basal cell layer disruption (384). Although, PIN is widely accepted as the precursor for prostate cancer, recent evidence suggest an early inflammatory lesion that precedes PIN as the precursor for prostate cancer. These studies propose that infectious agents, dietary carcinogens and hormonal imbalances lead to prostate tissue damage, chronic inflammation and regenerative lesions termed as proliferative inflammatory atrophy (PIA). Such areas of focal atrophic prostate epithelium characterized by increased epithelial proliferation are often observed in ageing men and are frequently found near PIN and adenocarcinoma. In addition some evidence indicates that certain high-grade prostatic intraepithelial neoplasias (PIN) and early adenocarcinomas appear to arise from PIA, however, further studies employing molecular pathological techniques and animal models are required to determine if PIA is indeed the driving force for prostate cancer (385-389). PIN then progresses to latent adenocarcinoma, clinical adenocarcinoma and eventually

metastasizes to lung, liver, pleura and invariably the bone where it results in characteristic osteoblastic lesions (390-392).

Prostate cancer development is associated with a series of genetic changes that facilitate cells to progress through different stages of prostate cancer. The basis for the sequence of these molecular events is derived from patient studies however; it is unclear if the genetic alterations need to occur in a well-defined temporal order (Figure 10).

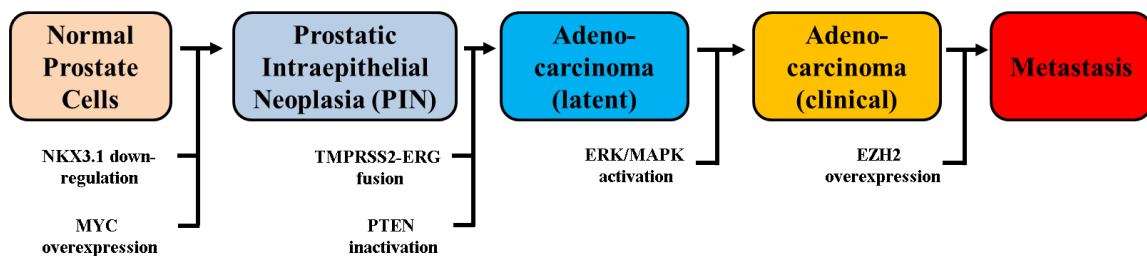


Figure 10. Genetic model of prostate cancer (392)

NKX3.1, 8p21 LOH

Prostate cancer initiation is strongly associated with downregulation of *NKX3.1* homeobox gene and is proposed to occur through multiple mechanisms (393). LOH of 8p21 is a common occurrence in 85% of high-grade PIN and adenocarcinomas and *NKX3.1* gene is localized within a 150-Mb minimal deleted region of chromosome 8p21.2 (394-397). Downregulation of *NKX3.1* occurs through epigenetic mechanisms,

possibly due to promoter methylation, in cases where 8p21 LOH is not observed (398). Contrary to the earlier belief that all advanced cancers lose NKX3.1 expression, recent studies demonstrate low levels of NKX3.1 expression in all types of prostate cancer suggesting that decreased and not total loss of NKX3.1 expression occurs during prostate cancer progression (392, 399, 400). NKX3.1 is the earliest known marker for prostate epithelium and the absence of NKX3.1 during development can decrease synthesis of secretory proteins and prostatic ductal branching (401-403). Mice that are heterozygous and homozygous mutants of NKX3.1 display prostatic hyperplasia and dysplasia and tend to develop PIN (401, 404). In addition mice with inactivated NKX3.1 exhibit deregulated expression of several antioxidant and prooxidant enzymes, including glutathione peroxidase 2 and 3 (GPx2 and GPx3), peroxiredoxin 6 (Prdx6), and sulfhydryl oxidase Q6 (Qscn6) making them susceptible to oxidative damage (405). Restoration of NKX3.1 function in human prostate cancer cell lines promotes phosphorylation of H2AX and recruitment of phosphorylated ATM for activation of DNA damage checkpoints, cell cycle arrest, DNA repair or apoptosis (406).

Up-regulation of Myc

The transcription factor Myc is an oncoprotein involved in processes such as cell proliferation, protein synthesis, metabolism and stem cell renewal. The *MYC* oncogene is localized on the 8p24 chromosomal region and since a subset of advanced prostate cancers display amplification of this region it was assumed that Myc played a role only in advanced stage prostate cancer (392, 407). However results from overexpression of

Myc protein in luminal cells of PIN, primary carcinomas and metastatic disease suggesting that Myc alterations might be an early event in prostate carcinogenesis (407). Forced expression of *MYC* immortalize nontumorigenic human prostate cells and transgenic expression of human c-Myc in the mouse prostate can give rise to PIN followed by invasive adenocarcinoma demonstrating the oncogenic potential of Myc in initiating prostate cancer (408, 409). In addition downregulation of NKX3.1 was observed in Myc-induced prostate tumors in mice implying that loss of NKX3.1 complements elevated Myc expression to facilitate the PIN to cancer progression (408).

TMPRSS2-ERG Fusion Gene

Activation of the ETS (E26 transformation-specific) family of transcription factors ERG, ETV1 by chromosomal rearrangements is frequently observed in most prostate cancers. The *TMPRSS2-ERG* created as a result of chromosomal rearrangement expresses an N-terminally truncated ERG protein under the control of an androgen-responsive promoter of *TMPRSS2* (251, 410). The chromosomal rearrangement occurs either through an interstitial deletion due to the close proximity of both genes on chromosome 21q or through a less frequent unbalanced chromosomal translocation (411, 412). Chromosomal rearrangements due to interstitial deletion are hypothesized to be an indirect effect of androgen receptor (AR) binding resulting in chromosomal proximity between *TMPRSS2* and *ERG* loci leading to formation of the fusion gene following DNA strand breakage (413, 414). *TMPRSS2-ERG* fusions occur at 15% rate in high-grade PIN and 50% rate in prostate cancer indicating that the chromosomal rearrangement might be an event that

occurs after cancer initiation (415, 416). Evidence from whole-genome chromatin immunoprecipitation assays and massively parallel sequencing studies reveal that ERG can block AR signaling by binding to AR target genes and inducing repressive epigenetic programs via activation of H3K27 methyltransferase EZH2, a Polycomb group protein which in turn disrupt lineage-specific differentiation of prostate epithelial cells (417). Cell culture assays and studies in transgenic mice demonstrate that activation of the *ETS* gene has a moderate effect on promotion of epithelial-mesenchymal transition and tumor-invasive properties (418, 419). In addition, expression of truncated ERG cooperates with loss of *PTEN* synergistically to facilitate development of high-grade PIN and carcinoma in mice. Evidence from these studies indicates that ETS chromosomal rearrangements inhibit differentiation programs and promote prostate carcinogenesis by cooperating with other transforming events (420, 421).

PTEN Inactivation

PTEN, encodes a dual-specificity phosphatase localized on chromosome 10q23, which frequently displays allelic loss in prostate cancer (422, 423). *PTEN* copy number loss is observed as an early event in cancer and is associated with progression to advanced or aggressive, castration-resistant disease. Studies investigating the loss of *PTEN* also suggest that complete loss of *PTEN* does not occur in prostate cancer, and that low levels of *PTEN* activity are retained (393). Conditional deletion of *PTEN* in the prostate epithelium or germline loss of *PTEN* in heterozygous mutants results in PIN or

adenocarcinoma (424-426). *PTEN* loss along with inactivation of *NKX3.1*, upregulation of *MYC* and the *TMPRSS-EGR* fusion gene are required for cancer progression (420, 421). Cellular senescence is observed in prostate tumors due to complete loss of *PTEN* in mouse models indicating avenues for therapeutic intervention by selective knockdown of *PTEN* in prostate cancer cells to promote senescence (427, 428). Data from cell culture and mouse studies show that decrease or loss of *PTEN* predisposes to castration-resistant form of prostate cancer however, the mechanism of this response is not clear, but suggests a possible interaction between *PTEN* and androgen receptor (429-431).

ERK/MAPK Activation

ERK/(p42/44) MAPK is often activated along with Akt signaling in advanced prostate cancer. These pathways jointly promote tumor progression and result in castration resistance in prostate cancer cell lines and mouse models (431-434). However, the events leading to activation of ERK/MAPK signaling are not well defined and defective growth factor signaling may play a role (435). *RAS* and *RAF* mutations are uncommon in early stage prostate cancer whereas deregulation of these oncogenes are more frequently observed in advanced prostate cancer (436). Expression of activated forms of *RAF* (*BRAF*^{V600E}) or *RAS* (K-ras [+V12]) in mice leads to development of prostate gland hyperplasia which progresses to rapidly growing invasive adenocarcinomas (437, 438). Dysregulation of the *RAS* and *RAF* signaling pathways can also occur since a small subset of aggressive prostate cancers exhibit translocation of *B-RAF* or *C-RAF* (439).

Ezh2

The gene enhancer of zeste homologue 2 (EZH2) gene is part of the Polycomb repressor complex and encodes for a histone lysine methyltransferase that is upregulated in hormone-refractory and metastatic prostate cancer (440, 441) . EZH2 represses the prostate-specific tumor suppressor gene *NKX3.1* through ERG (442). In addition EZH2 targets other genes associated with metastasis such as *E-cadherin* and *DAB2IP* that actively promote prostate cancer metastasis through NF- κ B and Ras signaling pathways (443-445).

Oncogenic tyrosine kinases such as Her2/Neu and Src tyrosine kinases play important roles in aggressive disease, progression to metastasis and castration resistance and are as therapeutic targets in patients with advanced disease (446, 447). AR signaling results in Src activation which in turn leads to AR phosphorylation, castration-resistance, increased cell proliferation and heightened invasive properties (448-450). Similarly, developmental signaling such as Hedgehog pathway has a substantial role in prostate cancer progression through autocrine and paracrine mechanisms in epithelial and stromal cells respectively (451-454). FGF signaling through activation of FGFR1 or overexpression of FGF10 to drive the ERK MAPK pathway is also involved in prostate carcinogenesis. MicroRNAs have specific roles in pathogenic processes concerned with cancer growth and development and miR profiling studies of human prostate tumors and xenografts indicate distinct microRNA expression patterns for indolent and aggressive prostate tumors (455, 456) (457). Tumor suppressor microRNAs such as miR-101 can

suffer loss of alleles and upregulate oncogene EZH2 while the oncogenic microRNAs such as miR-106b-25 cluster can negatively regulate the tumor suppressor PTEN (458, 459).

Hallmarks of Cancer

During the process of multistage carcinogenesis cancer cells acquire certain traits that allow them to function autonomously and progress to a state of malignancy. The six traits listed in the seminal article “*The Hallmarks of Cancer*” published by Hanahan and Weinberg include sustaining proliferative signaling, resistance to anti-growth signals, evading cell death, unlimited replicative potential, sustained angiogenesis and activation of tissue invasion and metastasis (Figure 11) (460). Advances in the field of molecular biology and development of better tools in the past decade have improved our understanding of cancer biology. The “Hallmarks of cancer” since has had four more additions that are categorized as “emerging hallmarks”- namely deregulation of cellular energetics and evasion of the immune system and “enabling characteristics”- which include genomic instability and tumor-promoting inflammation (461).

Sustaining Proliferative Signaling

The production and secretion of growth signals that facilitate cell growth and division in normal cells is highly regulated and tightly controlled whereas cancer cells can circumvent these finely tuned mitogenic signaling pathways to gain control over the proliferative machinery. The mechanisms governing these signaling cascades are highly

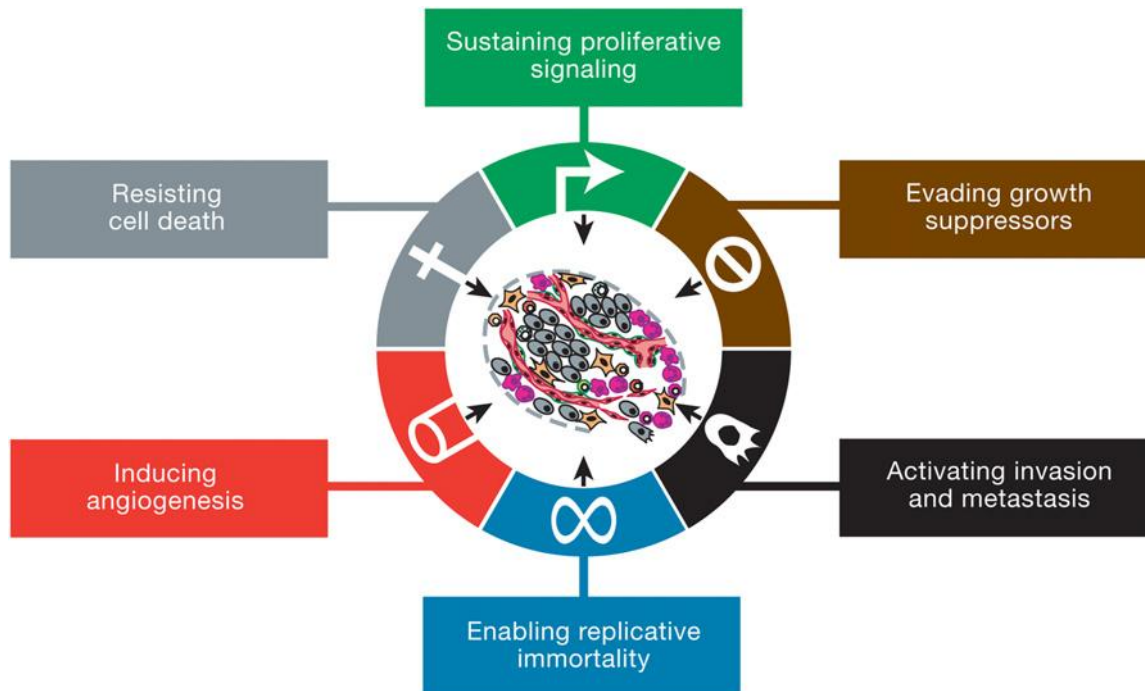


Figure 11. Hallmarks of cancer. Reprinted from “Hallmarks of Cancer: The Next Generation”, Vol. 144/ Issue 5, Douglas Hanahan, Robert A. Weinberg, Copyright (2013), with permission from Elsevier (461).

complex in normal cells and are poorly understood on the other hand these signaling pathways in cancer cells are more defined (256, 462-464). Cancer cells acquire the ability to sustain proliferative signaling in multiple ways. They synthesize growth factor receptor ligands to activate cognate receptors expressed on the cell surface initiating autocrine signaling loops to stimulate proliferation. Growth factors from normal cells in the tumor associated stroma also enhance tumor cell growth. In addition, increased expression of cell surface receptors sensitize cancer cells to limiting amounts of growth factor ligands present in the environment. Cancer cells also deregulate growth signaling

pathways by expressing constitutively active receptors that are capable of conveying signals downstream in the absence of ligand (461). The B-Raf protein is constitutively activated by a mutation in 40% of melanoma patients resulting in stimulation of the downstream MAPK pathway (465). Negative feedback loops serve as an effective mechanism to ensure that growth signal transmission is transitory. An example of a defective negative feedback loop is the Ras oncoprotein signaling. The oncogenic mutation compromises the Ras GTPase binary signaling activity thus preventing the ability of the protein from switching to an “off” state to arrest the signaling process. Similarly, the effector of PI3K signaling, phosphatidylinositol (3,4,5) trisphosphate (PIP3) is degraded by the phosphatase PTEN as a part of the regulatory feedback mechanism. However, PTEN is absent in most tumors due to promoter methylation resulting in increased PI3K signaling which contributes to tumorigenesis (466, 467). Although, cancer cells elevate growth factor levels to boost their replicative potential recent studies conclude that excessive growth factor signaling can be detrimental to cells due to triggering of cellular senescence or induction of apoptosis and these responses represent an intrinsic cellular defense mechanism against excessive signaling.(468-470).

Resistance to Anti-growth Signals

Growth inhibitory effects in cells are mediated by tumor suppressor genes and Rb (retinoblastoma-associated) and p53 are two tumor suppressors that are frequently found inactivated in several cancers. The Rb protein serves as a gatekeeper by preventing or

permitting cells to progress through its growth and division cycle based on the extracellular and intracellular stimuli that it receives (471-473).

The absence of Rb permits cells to proceed through various phases of the cell cycle without any checks and facilitates their proliferation. In contrast p53 functions as an internal sensor to various stresses such as genomic damage, suboptimal levels of nucleotides, growth promoting signals, glucose or oxygenation. In such scenarios p53 transmits signals to halt cell-cycle progression until cells attain homeostasis (461). Contact inhibition is another mechanism that exists in the cells to limit unnecessary cell proliferation and maintain tissue homeostasis. Loss of contact inhibition is a common phenomenon observed in many cancer cells and facilitates their ability to maintain uncontrolled growth. Merlin, the product of a tumor suppressor gene *NF2* mediates contact inhibition by tethering cell-surface adhesion molecules such as E-cadherin to transmembrane receptor tyrosine kinases such as the EGF receptor. Coupling E-cadherin to EGF receptors not only reinforces cell-cell adhesion but also restricts growth factor signaling due to sequestration of the receptor tyrosine kinases (461, 474, 475). Another well-known antiproliferative factor is TGF- β which in early stages of cancer acts as a tumor suppressor by inhibiting cell proliferation, but can function as a tumor promoter by activating the epithelia-mesenchymal transmission (EMT) in the late-stage cancers (476-478).

Evading Cell Death

Apoptosis or programmed cell death is a natural barrier to cancer formation and is disabled in several cancers (470, 479). Apoptosis can proceed through intrinsic and extrinsic pathways and what sets these pathways apart are the regulators involved in each of these mechanisms. The extrinsic pathway receives and processes extracellular death-inducing signals through the extracellular Fas ligand and Fas receptor and other death receptors and their ligands. The intrinsic pathway consists of regulators that respond to intracellular signals and both these pathways converge on common effectors termed as caspases, which are cysteine aspartic acid dependent proteases. The signals from the intrinsic and extrinsic pathways travel through a group of initiator caspases (caspases 2, 8, 9 and 10) that in turn relay signals to executioner caspases (caspases 3, 6 and 7). These executioner caspases are the final effectors of apoptosis leading to digestion and disassembly of cellular components and phagocytosis by neighboring cells. Cancer cells employ several schemes to evade apoptosis. The “guardian of the genome”, p53 can sense DNA strand breaks and chromosomal abnormalities in cells and induces apoptosis by increasing the expression of proteins Noxa and Puma. Loss of p53 activity renders the cells ineffective in checking for DNA damage and the ability to filter defective cells through apoptosis (480). Tumors can also resist cell death by upregulating antiapoptotic factors such as Bcl-2 and Bcl-xL. These proteins bind the pro-apoptotic factors Bax and Bak localized in the mitochondrial membrane and prevent them from disrupting the integrity of the mitochondrial membrane. Disruption of the mitochondrial membrane is essential for the release of pro-apoptotic factors such as cytochrome-*c* into

the cytoplasm to activate caspases and induce apoptosis. Alternatively, cancer cells can downregulate expression of pro-apoptotic factors such as Bax, Bak and Bim to inhibit apoptosis (479).

Autophagy is a physiological reaction that occurs in the cell in response to cellular stress (e.g. deprivation of nutrients) and mediates both tumor cell survival and death. During autophagy cellular structures and organelles are broken down and engulfed by intracellular vesicles termed autophagosomes which fuse with lysosomes for activating degradation. The catabolic products formed as a result are used for biosynthesis and energy metabolism to support the cells under extremely stressful conditions (481, 482). Regulators that govern apoptosis also play a role in autophagy. PI3K, Akt and mTOR (mammalian target of rapamycin) kinases inhibit autophagy when activated by survival signals and the absence of survival signals can decrease PI3K signaling and initiate apoptosis or autophagy (481-484). In contrast several studies demonstrate that conditions such as nutrient deprivation, radiotherapy and chemotherapy with certain cytotoxic drugs that induce autophagy can be cytoprotective to cancer cells (484-487). In addition, cancer cells that are subjected to stress can shrink by means of autophagy to a dormant state and return to full functionality when the environment is more favorable (485, 488). These contradictory findings demonstrate that autophagy can serve as a tumor suppressor and a tumor promoter and function “as a double-edged sword”.

Unlimited Replicative Potential

Cancer cells are able to undergo limitless replication cycles unlike normal cells which cease to divide and grow after a limited number of cell growth and division cycles. This unlimited replicative potential of cancer cells is associated with structures called telomeres that are hexanucleotide repeats present in tandem on the ends of every chromosome. Every cell growth and division cycle progressively erodes and shortens the telomeres on the chromosome. Complete loss of telomeres after several proliferation cycles leaves the ends of chromosomes unprotected predisposing the DNA to form end-to-end fusions with other chromosomes leading to genomic instability and cell death. Hence, the length of the telomeres on the chromosome dictates the number of cell divisions the cells can pass through before entering a state of senescence or crisis (489, 490). On the other hand cancer cells are not restricted by the limited replication cycles resulting from telomere shortening. Cancer cells express high levels of a DNA polymerase, telomerase that adds telomere repeat segments to the ends of DNA after each replicative cycle gifting the cells with the power of immortality. Studies in telomerase null mice demonstrate that telomere shortening induces senescence or apoptosis in premalignant cells which otherwise would have developed to form tumors (491). In addition evidence from studies on mice indicate that absence of telomerase function in certain cases can be beneficial for cancer progression and development. Loss of p53 can allow cells to undergo unchecked replication even after complete telomere loss which can lead to breakage-fusion-bridge (BFB) cycles. BFB cycles can accelerate the rate at which cells acquire mutations or undergo genomic alterations such as

activation of oncogenes or inactivation of tumor suppressors. The presence of telomerase function in this scenario might prove to be disadvantageous since restoring telomere length can prevent BFB cycles limiting the rate at which the cells acquire mutations (491, 492). However there is no conclusive evidence to suggest the possibility of p53 and telomerase loss cooperating to promote tumor formation in humans (461).

Sustained Angiogenesis

Angiogenesis is the process of sprouting new vessels required for the supply of nutrients and oxygen and in normal cells angiogenesis is typically functional only during embryogenesis, wound healing and female reproductive cycling. However, cancer cells demand a greater supply of oxygen and nutrients which is met by processes such as angiogenesis and vasculogenesis. In contrast to normal cells where angiogenic signaling pathways operate transiently, the “angiogenic switch” in cancer cells is always turned on (493). Factors regulating angiogenesis can be stimulatory or inhibitory. Vascular endothelial growth factor (VEGF) is a well-studied angiogenesis inducer that is involved in blood vessel formation during embryonic and post natal development as well as in physiological and pathological conditions. The VEGF family of growth factors include VEGF ligands A, B, C and D and Placental Growth Factor (PlGF) which form homo- or hetero-dimers with each other and interact with receptor tyrosine kinases VEGFR 1-3 and co-receptors Neutrophilin1 and 2 to elicit their responses in cells (494). Regulation of VEGF gene expression is controlled by many factors such as fibroblast growth factor, interleukin-1 and TGF- β in both normal and tumor cells (495-497). In addition, muscle

contraction, hypoxia and inflammation contribute to regulation of VEGF gene expression (498-500). Cells that are transformed by oncogenes may express VEGF family members and control expression of VEGF through transcription factors such as hypoxia inducible factors (HIF1 and HIF2), Ap-1, NF- κ B, E2F1 and ZNF24 (501-503). Moreover, the extracellular matrix serves as a storehouse for VEGF ligands that are sequestered in its inactive forms and are released following proteolytic cleavage by extracellular matrix degrading proteases (504). The angiogenic machinery in the cell is balanced by endogenous angiogenesis inhibitors that serve as a natural barrier for tumorigenesis. Thrombospondins are a family of proteins that play a role in tissue remodeling, embryonic development, wound healing, synaptogenesis and neoplasia. Thrombospondin-1 (TSP-1), is a well characterized member of the family and inhibits angiogenesis by affecting endothelial cell migration, survival and VEGF bioavailability. It can also suppress tumor growth by activating TGF- β , interacting with cell surface receptors and regulation of extracellular proteases (505). In addition to TSP-1, endostatin, a type 18 collagen, and angiostatin also function as endogenous angiogenesis inhibitors (505-507).

Activation of Tissue Invasion and Metastasis

The invasion-metastasis cascade is a multistep process involving a sequence of events that is initiated when cancer cells invade local tissues (508, 509). Epithelial-Mesenchymal transition (EMT) is a phenomenon that is closely associated with cancer cell invasion and metastasis and is commonly observed during embryogenesis and

wound healing. EMT enables transformed epithelial cells to invade and metastasize to distant tissues and at the same time resist cell death by evading the apoptotic machinery (510, 511). Transcription factors such as Snail, Slug, Twist and Zeb1/2 are reported to mediate EMT in cancer cells. During EMT epithelial cells undergo alterations such as conversion from a polygonal/epithelial to a spindly/fibroblastic morphology, acquire traits such as increased motility, increased expression of matrix metalloproteinases to digest extracellular matrix and the ability to resist apoptosis (508, 512). The transcription factor involved in initiating EMT can directly repress a critical component of the cell-cell junction complexes, E-cadherin (513). This cell adhesion molecule is involved in the assembly of epithelial cells into sheets and ensures that cells within tissues are bound together by forming adherens junctions. Loss or mutational inactivation of E-cadherin is often associated with a cell phenotype that is highly invasive and metastatic and overexpression of E-cadherin is reported to have tumor suppressor effects (514, 515). On the other hand cell adhesion factors such as N-cadherin that facilitate cell migration and motility in neurons and some mesenchymal cells are commonly overexpressed in several aggressive carcinomas (515). Recent studies indicate that cross talk between the neoplastic cells and stromal cells is necessary for invasion and metastasis and it is more obvious that the epithelial cells do not function autonomously (516). Cancer cells relay stimulatory signals to the adjoining tumor stroma and the mesenchymal stem cells located in the stroma secrete chemokines such as CCL5 (Chemokine [C-C motif] ligand 5) in response to signals that stimulate invasive behavior in cancer cells (517). Similarly, activation of macrophages confined to tumor periphery by IL4 secretion from cancer

cells is reciprocated by supply of metalloproteinases and cysteine cathepsin proteases from activated macrophages to allow cancer cells acquire invasive abilities (518). Following invasion cancer cells undergo intravasation in to adjacent blood and lymph vessels and are transported through the lymphatic and hematogenous routes to distant tissue sites. Experimental models of metastatic breast cancer demonstrate activation of cancer cells by EGF released from tumor associated macrophages (TAM) followed by release of colony stimulating factor-1 (CSF-1) by activated cancer cells to reciprocally stimulate TAMs, which in turn mediated cancer cell intravasation into the blood vessels and dissemination to distant sites (519). The next step in the invasion-metastasis cascade is extravasation of cancer cells from the blood and lymph vessels into tissue sites. During the process of dissemination cancer cells are drawn farther away from the source of EMT-inducing signals hence diminishing the effects of these signals on the cancer cells. As a result cancer cells that have experienced EMT and acquired invasive and metastatic properties may revert back to a form that is histopathologically identical to the primary tumor from which they arose and this phenomenon is termed as mesenchymal-epithelial transition (MET) (520). Formation of micrometastases (small nodules of cancer cells) at foreign tissue locations and successful development of macroscopic lesions from the established micrometastases is referred to as colonization (508, 509). Cancer cells that arrive at a distal tissue have poor adaptive capabilities initially and employ several strategies to thrive in the foreign tissue. In some cases the micrometastases may remain dormant due to the influence of systemic suppressors that primary tumors release and may abruptly arise as metastatic growths when the supply of

suppressors cease as in case of resection of the primary tumor (521, 522). Similarly, metastatic growths seen decades after surgical removal of primary tumors from breast cancer and melanoma patients is attributed to the ability of the micrometastases to finally adapt to the foreign environment and establish as macroscopic lesions after years of trial and error (523-525). Other mechanisms of micrometastases dormancy and survival in the new unfavorable environment may include anti-growth signals sequestered in the extracellular matrix of normal tissue and tumor suppressor effects of the immune system (523, 524, 526).

Enabling Characteristics and Emerging Hallmarks

The hallmarks of cancer described above are acquired in tumor cells through multiple mechanism, however two important characteristics that enable these cells to be transformed include, genomic instability and tumor-promoting inflammation (Figure 12) (461).

Genomic Instability

The genome maintenance system detects and corrects defects in genetic material of the cell and ensures that the mutation rates are kept low during each cell-growth-division cycle. Moreover, the surveillance system also keeps a check on the genomic integrity of cell by eliminating cells carrying any genetic defects or mutations (527-529). Defects in the maintenance machinery impairing any of its functions including ability to detect DNA damage and activate the repair mechanism or ability to directly repair damaged

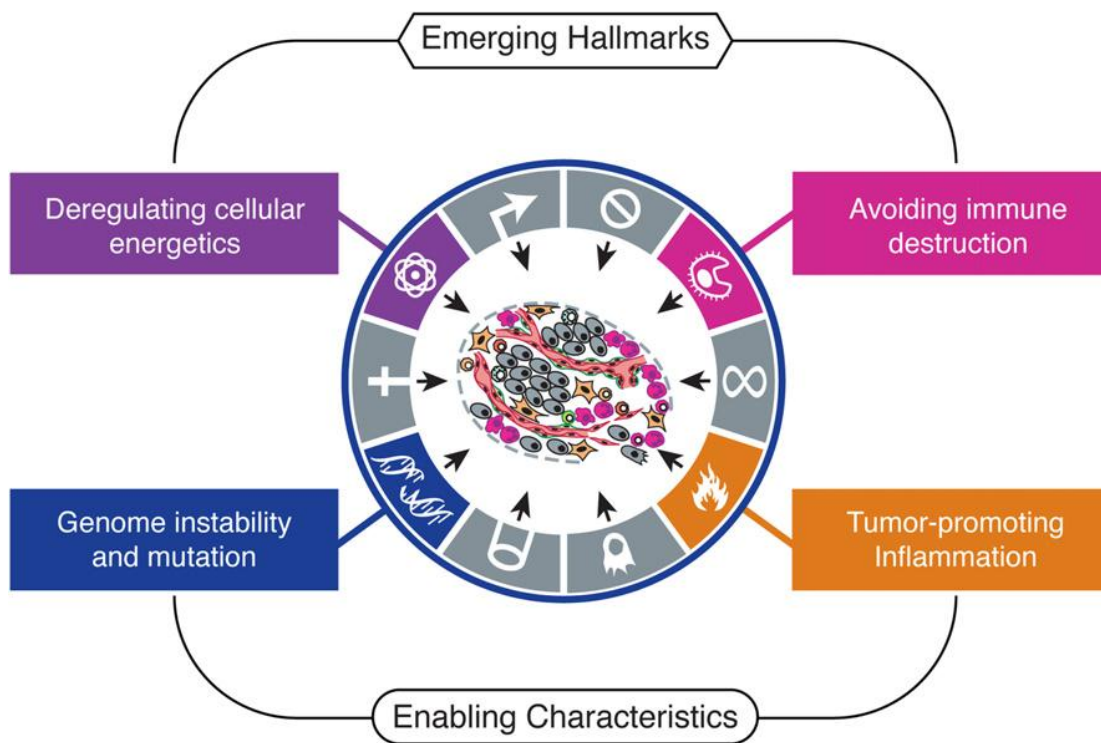


Figure 12. Emerging hallmarks and enabling characteristics of cancer. Reprinted from “Hallmarks of Cancer: The Next Generation”, Vol. 144/ Issue 5, Douglas Hanahan, Robert A. Weinberg, Copyright (2013), with permission from Elsevier (461).

DNA or ability to inactivate mutagenic molecules before it damages DNA can culminate in genomic instability and facilitate cancer development and progression (527, 528, 530-533).

Tumor-Promoting Inflammation

It is evident from several studies that inflammation also plays a crucial role in promoting tumorigenesis (534-537). Inflammation can enrich the tumor microenvironment with growth factors that can ensure continuous proliferation of tumor cells. Similarly, a constant flow of survival factors can make the cells resistant to anti-growth signals. In addition inflammation can provide cells with proangiogenic factors and matrix metalloproteinases that facilitate processes such as EMT, tumor invasion and metastasis. Activated inflammatory cells can generate reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) that are capable of inducing mutations leading to genomic instability.

The emerging hallmarks of cancer are suggested to be functionally important for the development of cancer and also include deregulation of cellular energetics and evasion of the immune system (307, 530, 537).

Deregulation of Cellular Energetics

The uncontrolled proliferation of cancer cells can result in an excessive demand for energy and nutrients and these additional requirements are typically met by modifications in metabolic pathways operating in these cancer cells. Normal cells under aerobic conditions breakdown glucose to pyruvate via glycolysis and the pyruvate subsequently enters the citric acid cycle to generate NADPH, which then undergoes oxidative phosphorylation in the mitochondria to generate ATP. However, under

anaerobic conditions the cells produce lactate via glycolysis using lactate dehydrogenase which consumes NADPH for re-use in glycolysis. Notably cancer cells even under normoxic conditions utilize the latter (lactic acid fermentation) pathway for generating energy which proceeds rapidly but results in generation of fewer ATP/cycle and this is termed as the Warburg effect, named, Otto Warburg, who first described this phenomenon (538-540). Hypoxic conditions and hypoxia regulators such as HIF1 α and HIF2 α transcription factors activate the aerobic glycolytic pathway in cancer cells and oncogenes such as RAS and MYC and mutated tumor suppressors such as TP53 are associated with aerobic glycolysis (541, 542). Ras oncoprotein and hypoxia can increase the levels of HIF1 α and HIF2 α , which can in turn upregulate aerobic glycolysis (543-545). In addition isocitrate dehydrogenase1/2 (IDH) is also reported to be activated by gain in function mutations in gliomas and other tumors (546). Glucose transporter, GLUT1 is another factor that is upregulated in cancer cells to increase the import of glucose into the cytoplasm to compensate for the 18-fold lower energy that is generated under anaerobic conditions (541, 542, 547). Although, aerobic glycolysis produces less energy compared to mitochondrial oxidative phosphorylation cancer cells prefer glycolysis even when there is sufficient quantities of oxygen. Vander Heiden et al. postulated that glycolysis generates various intermediates that can be shuttled into several pathways important for biosynthesis of macromolecules and cytoplasmic organelles (548, 549).

Evading the Immune System

Recent studies suggest an important role for the immune system in serving as a natural barrier against tumorigenesis. Genetically engineered mice devoid of CD8⁺ cytotoxic T lymphocytes (CTLs), CD4⁺ Th1 helper T cells, or natural killer (NK) cells exhibit an increased tumor incidence and higher cancer susceptibility in mice with combined deficiencies of T cells and NK cells. This suggests that both innate and adaptive immune systems have a substantial role to play in immune system mediated tumor inhibition (526, 550). Furthermore results from transplantation experiments conducted in mice corroborate the role of immune system in cancer inhibition. Cancer cells that arise in immunodeficient mice are unable to form secondary tumors in syngeneic or immunocompetent hosts; on the other hand cancer cells from tumors that are formed in immunocompetent hosts are fully capable of forming secondary tumors in both hosts. This suggests that highly immunogenic cancer cells are eradicated in the immunocompetent host leaving behind only weakly immunogenic cancer cells which can form tumors in both types of hosts. In contrast cancer cells in an immunocompromised host do not undergo the process of immunoediting to eliminate the strongly immunogenic cancer cells. As a result when these cells are transferred to an immunocompetent host the immune system eradicates the immunogenic cancer cells (526, 550, 551). Epidemiology studies on colon and ovarian cancer patients with tumors heavily infiltrated with CTLs and NK cells exhibit better prognosis than patients expressing lower levels of these lymphocytes (552-555). In addition some cancers may be held in a dormant state as a result of the immune system and these individuals do not

show any signs of cancer. However, when these “so-called” tumor-free individuals donate organs, immunosuppressed organ recipients develop donor-derived cancers confirming an important role for the immune systems in tumorigenesis (556).

CANCER THERAPY

Therapeutic measures employed to treat cancer have changed over time with increased understanding of cancer biology. Targeted therapies and sophisticated surgical techniques have replaced archaic medical practices that were once the only available methods for treatment for cancer. Clinicians now have the option to choose from a wide range of treatment methods that are best suited for the patient depending on the location, grade and stage of the tumor and the general condition of the patient. Surgery, chemotherapy and radiation are the most commonly practiced treatment interventions against cancer and more advanced treatment strategies such as targeted and immunotherapies are gaining prominence and have given encouraging results against late-stage and aggressive cancers.

Surgery

Surgical resection of tumors has been practiced since 1600 BC and it is mentioned in ancient Indian and Egyptian medical records (6). Surgical excision of entire tumors and lymph nodes were commonly performed by surgeons Bilroth, Halstead and Handley with the invention of anesthesia during the 1800s (557). Surgery is the first line of treatment for most cases of solid tumors and is performed for several reasons. Typically

it involves resection of the entire tumor mass along with some healthy tissue or even the affected organ and lymph nodes (lymphadenectomy). In some cases initial removal of part of the tumor is practiced (de-bulking or cytoreduction surgeries) in order to reduce the tumor to a manageable size for further treatment with chemotherapy or radiation. In addition surgery is required for diagnostic purposes to perform biopsies and ascertain the staging of the tumor to determine the best possible therapeutic approach. Surgery is also used for explorative purposes to assess the extent of tumor metastasis and the number of lymph nodes involved. Palliative surgeries are performed in certain cases in order to provide relief from distress caused due to tumor-related obstructions. Depending on the stage and the components involved surgical procedures for treatment of advanced colorectal cancer may vary.

Surgical intervention for rectal cancer involves low anterior resection or abdominal perineal resection in combination with radiation and chemotherapy. Stage I rectal cancer is treated with surgical resection without chemoradiation. Surgical resection in combination with adjuvant or neo-adjuvant chemoradiation is preferred for stage II and III disease. Stage IV rectal cancer is treated by surgical bypass of local bowel obstruction as a palliative measure in combination with chemoradiation. For stage I and II colon cancers, surgery alone is sufficient with some stage II individuals requiring additional chemotherapy.(558). Adjuvant chemotherapy in combination with surgery is the preferred choice for stage III patients and improves survival (559, 560). A subset of stage IV colon cancer patients with resectable bowel disease and resectable isolated

hepatic and pulmonary metastases are tractable to surgical intervention. Transarterial chemo-embolization or radiofrequency ablation may be used in cases of unresectable liver metastases (561).

Radical prostatectomy is used when prostate tumor growth is confined to the organ and the individual has a reasonable life expectancy with no other co-morbidities. Pelvic lymph node dissections (PLND) is commonly performed with radical prostatectomy however, it is not clear if removal of lymph nodes improves survival. There are several types of radical prostatectomy such as retropubic, perineal and the more recent laproscopic and robotic radical prostatectomy. Retropubic is considered to be the most efficient and since it is time-tested and there is sufficient evidence demonstrating its superiority in cancer control and potency (562, 563). The only known disadvantage of this procedure is the blood loss. The perineal approach is used less due to the inability to perform pelvic lymphadenectomy, damage to anal-sphincter and bowel-related complications that arise from the procedure. Robotic and laproscopic techniques are relatively recent techniques and result in lower blood loss and improved visualization in the depths of the pelvis. Cancers that spread beyond the margins of the organs and invade seminal vesicles with no signs of metastases are termed locally advanced. In such cases of prostate cancers radical prostatectomy is practiced in combination with adjuvant radiotherapy or neoadjuvant chemo-hormonal therapy. Local recurrence of prostate cancer following external beam radiotherapy or brachytherapy is treated by

salvage radical prostatectomy. This procedure is performed primarily for symptomatic relief and is not effective for cases of distal tumor metastasis (564).

Radiotherapy

Radiotherapy or radiation therapy is a commonly practiced treatment method that involves ionizing radiation to destroy malignant neoplastic cells. It is used for palliative and curative purposes and approximately 70% of cancer patients receive radiotherapy during their disease course (565). Radiation for therapy is used in different forms such as brachytherapy, external beam radiotherapy (EBRT) and unsealed sourced radiotherapy. Brachytherapy involves use of radioactive isotopes that are placed in the immediate vicinity of the cancerous tissue or often within the tumor tissue itself. The radioisotopes emit high doses of short range radiation which destroy the tumor tissue but have minimal effects on surrounding normal tissues (566, 567). It is a commonly employed treatment method for different kinds of tumors and is used in combination with chemotherapy and surgery. EBRT is performed by generating a beam of ionizing radiation using a linear accelerator which emits either high energy x-rays or gamma rays from radioisotopes such as cobalt-60. In order to ensure precise and effective delivery of radiation the shape, intensity, angle and direction of the beams can be altered depending on the location and size of the tumor (565-567). Unsealed source radiotherapy involves use of therapeutic radiopharmaceuticals which are radiolabeled molecules capable of transporting therapeutic doses of ionizing radiation to target sites in the body. ¹³¹I-sodium iodide for thyroid cancer and ⁸⁹Sr-strontium chloride and ³²P-sodium phosphate

for metastasis associated bone pain are commonly used radiopharmaceuticals. Due to its specificity and ability to reach distant sites, use of radiopharmaceuticals is a preferred method of treatment over EBRT for cancer metastases that have widely disseminated (568).

Radiation therapy is effective as an adjuvant treatment for individuals with advanced colon cancers that spread to neighboring organs and tissues. (565). In contrast to colon cancer which has a recurrence pattern on distant tissue sites, rectal cancer relapse occurs locally within the pelvis (569). This has led to use of radiation and chemotherapy as an adjuvant therapy after surgery for treatment of patients with stage 2-3 disease that have highest risk of locoregional recurrence. Chemotherapy operates as a radiosensitizer in the pelvis and works against metastases above the pelvis and recent studies indicate that chemotherapy before surgery gives encouraging results in a subset of patients with T3, T4 or node-positive rectal cancer in comparison to its use after surgery (570-573).

Permanent seed implant (PSI) or EBRT are suitable therapeutic options for low-risk prostate cancer patients and effective control rates are attained when a single treatment method is employed (574). EBRT is highly efficacious in controlling low-risk disease and causes minimal side-effects. The radiation doses administered through EBRT at present are higher in comparison to what was used earlier since randomized trials demonstrate better biochemical control of the disease in terms of PSA stability with increasing doses of radiation (575-578). Doses > 70 gray (Gy) was considered unsafe

due to undesirable side-effects on the bladder and rectum; however, with better delivery systems it is now possible to administer doses ranging from 76-80 Gy with minimal toxic side-effects (565). Although the effectiveness and the mild nature of side-effects of EBRT are well documented, EBRT can also be inconvenient to the patients due to the daily treatments and the high cost involved (565, 579). PSI and brachytherapy are administered as single therapy for low-risk prostate cancer patients with prostate weights ranging from 50-60 grams. The final dose delivered to patients can range up to 145 Gy and hence the resulting toxicities are of a greater magnitude than EBRT (565). Intermediate and high-risk patients are treated with a combination of surgery, radiation and androgen-deprivation therapy. Randomized trials comparing the efficacy of combination therapy and radiation alone confirm the superiority of the former approach for patients with high and intermediate-risk (580). Administration of luteinizing hormone-releasing hormone agonists such as leuprolide or goserelin in combination with radiation therapy results in a synergistic effect on radiation induced cell death by promoting apoptosis. In addition intermediate and high-risk patients are also treated with intensity modulated radiotherapy (IMRT) combined with a brachytherapy (565).

Chemotherapy

Cytotoxic chemotherapeutic drugs that are commonly used as anti-cancer agents elicit their responses by alkylating nucleophilic functional groups, inhibiting metabolism, intercalating between DNA or RNA base pairs and inhibiting synthesis, interfering with topoisomerase II function and generating free oxygen radicals. Development of more

effective systemic chemotherapeutic agents has significantly improved the mean survival time in patients with metastatic colon cancer. 5-Fluorouracil (5-FU) is the most commonly used systemic chemotherapy for treating advanced colorectal cancer. 5-FU is a fluoropyrimidine that interferes with DNA replication by inhibiting the enzyme thymidylate synthase that generates thymine, a nucleoside which is essential for DNA synthesis (581). Leucovorin, a reduced form of folate is normally administered in combination with 5-FU as a biomodulator to improve the binding between 5-FU and thymidylate synthase (582). It is employed as an adjuvant therapy for stage III colon cancer patients and stage II and III rectal cancer patients (558). The inability to detoxify fluoropyrimidines, due to dihydropyrimidine deficiency can lead to 5-FU toxicities such as neutropenia, stomatitis and diarrhea (561). There is increasing evidence to suggest that the DNA mismatch repair (MMR) function status of the tumor cells is important for the efficacy of 5-FU. DNA MMR machinery ensures the genome's stability by repairing errors committed by DNA polymerase and MMR also identifies chemotherapeutic agents that intercalate into the DNA and induce death pathways in those cells (583-585). However, dysfunctional MMR machinery that fails to recognize the intercalated chemotherapeutic agent might not trigger cell death and while 5-FU readily induces cell death in cancer cells with an intact MMR system and cells with a dysregulated DNA MMR were 28-fold more resistant to this drug.(584). Moreover, restoring the MMR function in 5-FU resistant cells enhances their susceptibility to this agent (586).

Irinotecan is currently used in combination 5-FU as a first-line therapy for metastatic colon cancer, but is not beneficial in adjuvant therapy (558). Irinotecan falls under the

class of drugs that inhibit topoisomerase I, an enzyme that is involved in uncoiling of DNA before transcription and DNA replication. Irinotecan is metabolized into SN-38 by hepatic carboxylesterases and SN-38 is the active metabolite that inhibits topoisomerase I. Irinotecan as a single agent and in combination with 5-FU is shown to increase tumor shrinkage and improve survival in patients with advanced colorectal cancer by 2-3 months (587, 588). Irinotecan is inactivated by uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) and individuals with a homozygous UGT1A1 allele unable to effectively conjugate and detoxify irinotecan exhibit severe neutropenia (587-589). In addition other side effects of irinotecan treatment include alopecia, nausea, vomiting, diarrhea and bone marrow suppression (561, 590).

Alkylating agents are another category of chemotherapeutic drugs commonly used to treat cancer. Oxaliplatin, a platinum derivative forms inter and intra DNA strand cross-links thus preventing DNA replication and this eventually leads to cell death and oxaliplatin may also induce cell death by inhibiting the enzyme thymidylate synthase (558, 591, 592). Oxaliplatin alone is not effective for treating colon cancer and hence a combination of oxaliplatin, 5-FU and leucovorin, called FOLFOX is typically used (559, 560). Acute and chronic forms of neuropathy are the most common side effects observed during treatment with oxaliplatin besides rare kidney dysfunction and ototoxicity (591).

Symptomatic metastatic prostate cancer is typically treated by androgen deprivation, using LHRH agonists such as goserelin acetate (Zoladex) or leuprorelin acetate (Prostap), which simulate the effects of castration on testosterone levels and on prostate cancer response. These agents downregulate pituitary receptors leading to inhibition of release of follicle stimulation hormone (FSH) and luteinizing hormone (LH) and decrease production of testosterone in the testicular tissue (593, 594). Palliative care for hormone-refractory prostate cancer (HRPC) patients is provided using cytotoxic chemotherapy, systemic isotope therapies and bisphosphonate therapy. Docetaxel in combination with prednisone is the most commonly used drug to treat HRPC and provides a significant survival advantage in these patients (595). Docetaxel is a semi-synthetic analogue of paclitaxel (Taxol), which is an extract from the bark of the pacific yew tree, *Taxus brevifolia*. It causes cell cycle arrest and induces apoptosis by promoting microtubular polymerization and inhibiting disassembly of microtubules (596). Besides docetaxel, a combination of paclitaxel with estramustine or capecitabine has also produced encouraging results in HRPC patients (597). Platinum-based agents such as epirubicin and carboplatin in combination with 5-FU were not effective in majority of patients due to toxic side-effects however they were considered superior to single-agent cytotoxic treatments for patients with tolerance for combination chemotherapy (598). A third generation oral platinum-containing drug Satraplatin (JM-216) is effective *in vitro* against taxane-resistant cell lines has shown significant anti-tumor activity and low toxicity in combination with prednisone in a small phase III trial (599).

Targeted Therapy

Targeted therapies unlike other conventional treatment strategies interfere with specific pathways, factors or receptors that are essential for tumor growth, survival and metastasis. In contrast to radiotherapy and chemotherapy that indiscriminately eliminate rapidly dividing cells, targeted therapy interacts with key factors that are present or highly expressed in cancer cells and absent in normal cells thus minimizing the side-effects that are encountered during general systemic chemotherapies.

Anti-Angiogenic Agents

Several antiangiogenic agents have been approved and several others are currently in clinical trials. Bevacizumab, a monoclonal antibody raised against VEGF-A, was the first anti-angiogenic drug to be approved for metastatic colorectal cancer based on results obtained from a clinical trial conducted by Hurwitz et al. Although capable of interfering with angiogenesis Bevacizumab is not effective as a single agent and is approved for use in combination with standard chemotherapy as first-line treatment for metastatic colon cancer (600). Bevacizumab elicits its responses by normalizing tumor blood vessel architecture and decreasing intratumoral hydrostatic pressure thus improving the delivery of anticancer agents to the tumor (601). Combinations of bevacizumab and chemotherapy have improved response rates and survival rates in metastatic colon cancer patients in both second-line and third-line therapy in most tested trials. According to recently reported data from the BRIT registry a significant increase in overall survival was observed in patients who continued bevacizumab throughout

treatment in comparison to those who discontinued bevacizumab (31.8 vs.19.9 months, HR 0.48, $P < 0.001$) (602). However, in patients with previously untreated metastatic colon cancer no significant difference was observed between groups receiving a combination of bevacizumab and FOLFOX and those receiving FOLFOX alone although there was an increase in progression free survival (603). Side-effects observed in patients receiving bevacizumab are gastrointestinal perforation, arterial thromboses, myocardial infarctions, hypertension and strokes (604). There are several other angiogenic inhibitors that are currently being assessed for clinical efficacy including drugs such as vatalanib, sunitinib and aflibercept (a potent inhibitor of VEGF). Aflibercept is a recombinant fusion molecule of the human VEGF receptor extracellular domain. It has shown some promise as a single agent in a phase II trial in 51 patients with refractory metastatic colorectal cancer and its efficacy in combination with chemotherapy is currently being evaluated (605).

Angiogenesis inhibitors such as bevacizumab, aflibercept and sunitinib have been investigated for their efficacy in improving the survival rate and response rates in prostate cancer patients. Some of these drugs alone and in combination with chemotherapeutic agents have provided contrasting results while others have proven to be ineffective and discontinued (606-608). In addition several other angiogenesis inhibitors operating as immune modulators and multi-tyrosine kinase inhibitors are under development for treatment of HRPC. Tasquinimod is one such drug that has demonstrated improved progression free survival (7.6 versus 3.3 months) in HRPC

patients in a placebo-controlled phase II trial (609). It is an oral quinoline-3 carboxamide derivative that binds to an immune-modulatory protein, S1000A9 expressed on myeloid-derived suppressor cells which are key promoters of angiogenesis and tumor growth (610, 611). Similarly sorafenib is another molecule that is currently under clinical development and targets oncogenes such as c-raf, BRAF, VEGFR, platelet-derived growth factor receptor, Flt-3, c-KIT, and RET.

Signal Transduction Pathway Inhibitors

Tumor cell proliferation, invasion, migration and inhibition of apoptosis are regulated by numerous pathways involving key signaling molecules such as Ras-Raf-MAPK, PI3K-Akt, protein kinase C and STAT. These different pathways stem from critical tyrosine kinase receptors and EGFR is the most important member of the class. EGFR is targeted by either using monoclonal antibodies (mAb) that recognize EGFR or using small molecule inhibitors that inhibit its tyrosine kinase activity (612). Cetuximab and Panitumumab are two mAbs that are approved for therapeutic use against colon cancer (613, 614). Cetuximab inhibits cancer cell growth and survival by several mechanisms. It prevents receptor dimerization, which is an essential step for activation of the receptor and downstream signaling. It also reduces the expression of receptors on the cell surface by increasing internalization and degradation of receptors without activation. Cetuximab can also prevent any receptor-dependent DNA transcription by blocking the movement of receptor to the nucleus. In addition it also kills cells by antibody dependent cell mediated cytotoxicity (615). Evidence from recent clinical studies recommends K-Ras

testing prior to treating an individual with EGFR mAbs. Certain tumors have K-Ras mutations that enable them to remain constitutively active and be independent of EGFR signaling. In such scenarios blocking EGFR will not prevent downstream activation of Ras and its subsequent effectors and as a result the tumor might be refractory to the therapy. Hence it is recommended that the tumor be tested to determine if K-Ras is mutated or intact (616, 617). Farnesyl transferase is an enzyme that operates downstream of Ras and drugs such as tipifarnib that inhibit the enzyme are currently being tested for their effectiveness in combination with anti-EGFR and anti-angiogenic agents (618).

NOVEL GROUP OF THERAPEUTIC TARGETS

It is a known fact that cancer is a highly complex disease. The data obtained from numerous clinical studies and *in vitro* experiments show that even targeted therapies for disrupting growth and survival of cancer cells are not straightforward. Although some mechanism-based drugs show some promise their beneficial therapeutic effects until now appear to be only modest. This can be attributed to the complex labyrinth-like signaling pathways and regulatory networks that allow cancer cells to grow and survive and inhibition of individual growth/survival-promoting and angiogenic pathways are not effective. The highly complex signaling in cancer cells enable them to function by relying on alternate pathways even if an individual signal transduction pathway is abrogated. Hence there is a need for therapeutic targets that can be more effectively targeted. To this effect our laboratory has focused on specificity protein (Sp)

transcription factors regulated expression of multiple genes that are important for cancer cell growth and survival and can be effectively targeted by various anti-cancer agents.

Sp/KLF (Kruppel-like factor) Family of Transcription Factors

Members of the Sp/KLF family have a highly conserved DNA binding domain that consists of three contiguously placed C2H2-type zinc fingers that are located at the C-terminal region. Members of the family bind to GC boxes (GGGGCGGGG), GT/CACCC boxes (GGTGTGGGG) and basic transcription elements and regulate transcription of various genes (619-622). Although the DNA binding domain is conserved among members of the family their sequences in other domains are highly variable. Sp proteins exhibit a preference for GC-boxes over CACCC-boxes whereas KLFs have an increased binding affinity for GT-boxes over GC boxes (623-627). Sp/KLFs are only homologous to Sp proteins in their C-terminal DNA binding domain. Sp/KLF members are categorized into various groups based on the sequence conservation in their N-termini; For example the Sp proteins have a glutamine rich domain, whereas GKLF and UKLF contain an acidic domain in their N-terminus. Similarly, FKLF and LKLF carry a proline-rich domain while UKLF and GKLF possess a serine-rich domain (628). (624, 628-630). As a result this can lead to variable transcriptional activity between SP/KLF members, with some being transcriptionally activating, others repressive and some exhibit both activities (cell-and-gene-dependent) (631).

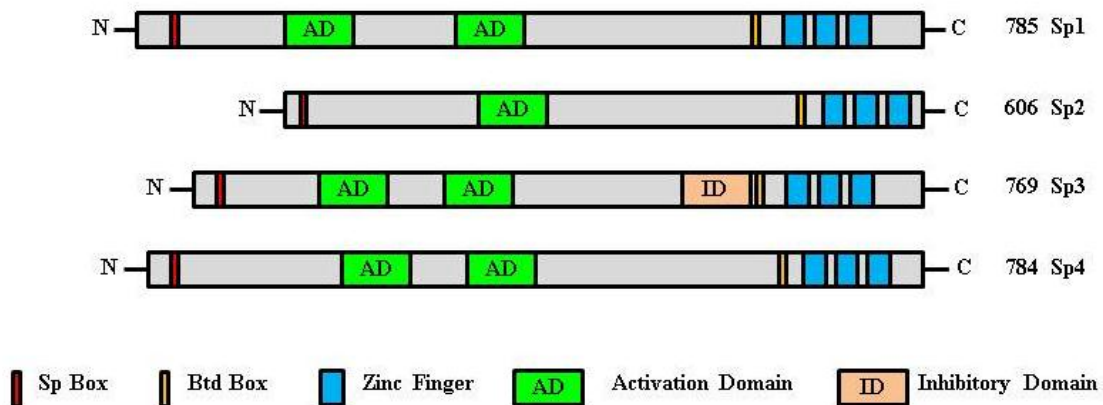


Figure 13. Structural features of Sp proteins (632)

Sp Proteins

The Sp-family consists of 9 members (Sp1-9) and all Sp transcription factors carry a hallmark buttonhead (BTD) box CXCPXC N-terminal to the zinc fingers; although the function of the BTD box is not known, its presence in *Drosophila* and *C.elegans* Sp transcription factors suggests some physiological relevance. In addition all Sp transcription factors also carry a conserved amino acid stretch called the SP box at the N-terminus (633, 634). The genes encoding Sp-proteins are evolutionarily closely related and are linked to the homeobox gene cluster. Sp family members are grouped into two subsets with Sp1-4 (Figure 13) characterized by a glutamine-rich transactivation domain (TAD) and Sp5-9 forming the other subset which although structurally similar to other Sp members lacks the TAD. The four Sp-family members Sp1-4 have similar domain structures but sequence alignment of the C₂H₂-type zinc finger domains shows that Sp1, Sp3 and Sp4 are more closely related to each other than to Sp2 (619, 634).

Structural studies on the zinc finger domains reveal that amino acids KHA, RER and RHK within the first, second and third zinc fingers respectively are all conserved in Sp1, Sp3 and Sp4 but not Sp2 proteins, where the critical histidine residue is substituted with a leucine (619, 635, 636). Hence, unlike other Sp proteins which bind GC-boxes the binding affinity of Sp2 is shifted to a GT-rich element within the T-cell receptor gene 5' flanking region. In addition Sp2 contains only 1 TAD instead of 2 activation domains that are present on Sp1, Sp3 and Sp4 proteins (627).

Sp Protein Expression in Tumor Tissue and its Role in Regulation of Critical Genes

Evidence from studies conducted in this laboratory and several others indicate that Sp transcription factors play a crucial role in tumor growth, development and metastasis besides their involvement in normal development of tissues and organs (Figure 14). Sp1, Sp3 and Sp4 proteins are overexpressed in several cancer cell lines including colon, bladder, pancreatic, prostate, breast, thyroid and esophageal cancer cell lines (331, 637-641). Wang et al. demonstrated that Sp1 levels were high in the nuclei of gastric tumor cells compared to the stroma or normal glandular epithelium within or surrounding the tumor where the levels of Sp1 protein were low or undetectable (284). Similarly, Sp1 levels are high in pancreatic tumors compared to normal pancreas and Sp1 expression decreases with increasing age (642). VEGF expression is regulated by Sp1 protein and a positive correlation between Sp1 and VEGF expression was observed in pancreatic and

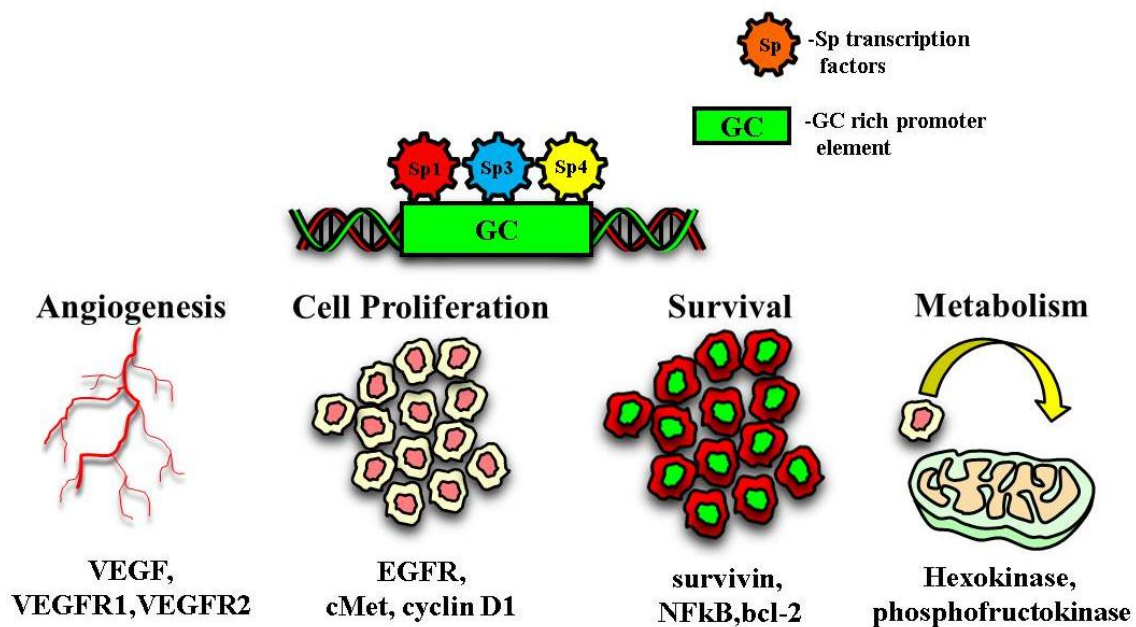


Figure 14. Transcriptional regulation of critical oncogenes by Sp transcription factors

gastric cancer patients (643, 644). In addition these studies demonstrate that Sp1 is a negative prognostic factor for survival of pancreatic and gastric cancer patients (284-286). Sp1 protein expression was also elevated in 11 out of 14 breast carcinoma while only 1 in 5 benign breast tumors displayed detectable levels of Sp1 protein (287). Similarly, Sp1 expression levels in the nuclear extracts of thyroid tumors was significantly elevated in comparison to corresponding normal tissues (288). Levels of Sp3 and Sp4 were not determined in cancer patient studies and their prognostic significance has not yet been determined.

There is growing evidence indicating that Sp family of transcription factors Sp1, Sp3 and Sp4 are critical regulators of several genes that are essential for tumor growth, development, survival and metastasis (289). Several studies demonstrate that VEGF, and its receptors VEGFR1 and VEGFR2 are important mediators of angiogenesis and these genes are regulated by Sp transcription factors through interactions with their GC-rich promoters (286, 290, 291, 335). RNA interference (RNAi) studies conducted in pancreatic cancer cells reveals that Sp1, Sp3 and Sp4 proteins are involved in VEGF, VEGFR1 and VEGFR2 expression and knockdown of Sp1, Sp3 and Sp4 by RNAi also affected pancreatic cancer cell growth and cell cycle progression with a decreased percentage of cells in G2/M and S and increased percentage of cells in G0/G1 phase. This was accompanied by increased expression of cyclin-dependent kinase inhibitor p27 with Sp3 knockdown (326). In addition several studies have demonstrated the role of Sp1 in breast cancer cells in regulating key factors associated with growth and cell cycle progression (cyclin D1, E2F1, c-fos, transforming growth factor- α), purine and pyrimidine synthesis, angiogenesis (VEGF) and survival (bcl-2) (327-330, 336, 337). RNAi studies in esophageal cancer cell lines reveal that hepatocyte growth factor receptor (c-MET) is also an Sp-regulated gene and other Sp-regulated genes important for cancer growth and development include, survivin, epidermal growth factor (EGFR), pituitary tumor-transforming gene 1 (PTTG-1) and the p65 subunit of NF κ B (331-334).

In addition to processes such as proliferation, angiogenesis and inflammation there is evidence to suggest that cancer cells manipulate mechanisms governing bioenergetics to

ensure growth and survival (549, 634). Enzymes involved in metabolism of glucose such as hexokinase, phosphofructokinase and pyruvate kinase are overexpressed in several cancer cell lines and tumors. Promoter analysis of the genes coding for these enzymes reveal that many of these genes also contain Sp binding sites and Sp proteins might be involved in the overexpression of some of the enzymes (645-649).

Evidence from RNAi studies suggest that the cancer cell phenotype is dependent, in part, on Sp transcription factors and Sp regulated genes and this represents an example of “non-oncogenic addiction”. Sp proteins do not behave as classical oncogenes since they do not exhibit any somatic gene mutations and the heightened dependence of tumors on these transcription factors for regulating several important pathways suggests that Sp proteins are ideal therapeutic targets. Expression of Sp proteins is relatively high in cancer cells whereas normal cells have lower levels of these proteins particularly in adult tissues and hence drugs that specifically inhibit expression or induce degradation of Sp proteins constitute an important class of mechanism-based anticancer drugs. Moreover drugs that act against Sp proteins will coordinately downregulate several Sp-regulated genes responsible for cell proliferation, survival and angiogenesis many of which are themselves individual drug targets. In summary it is clear that drugs targeting Sp proteins are a viable and pragmatic therapeutic approach for treating multiple-tumor types.

Targeting Sp Transcription Factors

Sp proteins and Sp-dependent pathways can be targeted by several approaches. Initial studies identified drugs that bound GC-rich DNA motifs, and these include mithramycin, hedamycin, thalidomide and daunomycin-derived bisanthracycline WP631. Drug interactions with GC-rich regions on the DNA decrease Sp1-DNA binding and blocking expression of Sp-regulated genes however these genes are accompanied by side-effects and inhibition of off-target genes (650-654). Oligodeoxynucleotides containing consensus GC-rich Sp1 binding sites (Sp1 decoys) are also promising agents that deactivate Sp-regulated genes by acting as decoys and binding Sp transcription factors and development of these reagents is in early stage (655-657).

Research in this laboratory has focused on developing anticancer drugs that target critical pathways required for cancer growth, proliferation, survival, angiogenesis and metastasis by downregulation of Sp1, Sp3 and Sp4 protein expression. Targeting Sp proteins in several cancer cell lines and xenograft animal models using anti-cancer agents such as betulinic acid (BA), curcumin, arsenic trioxide, synthetic triterpenoids and NSAIDs decrease expression of key regulators of growth (EGFR, cyclin D1, c-MET), survival (bcl-2, survivin), inflammation (NK κ B) and angiogenesis (VEGF, VEGFR1, VEGFR2) (639, 640, 658, 659). Multiple mechanistic pathways are involved in drug mediated downregulation of Sp proteins and these mechanisms are complex and dependent on the class of drugs and cancer cell line (Figure 15.). Drugs that induce

downregulation of Sp proteins mediate their responses either by inducing degradation (proteasome/caspase mediated) or transcriptional repression of Sp transcription factors.

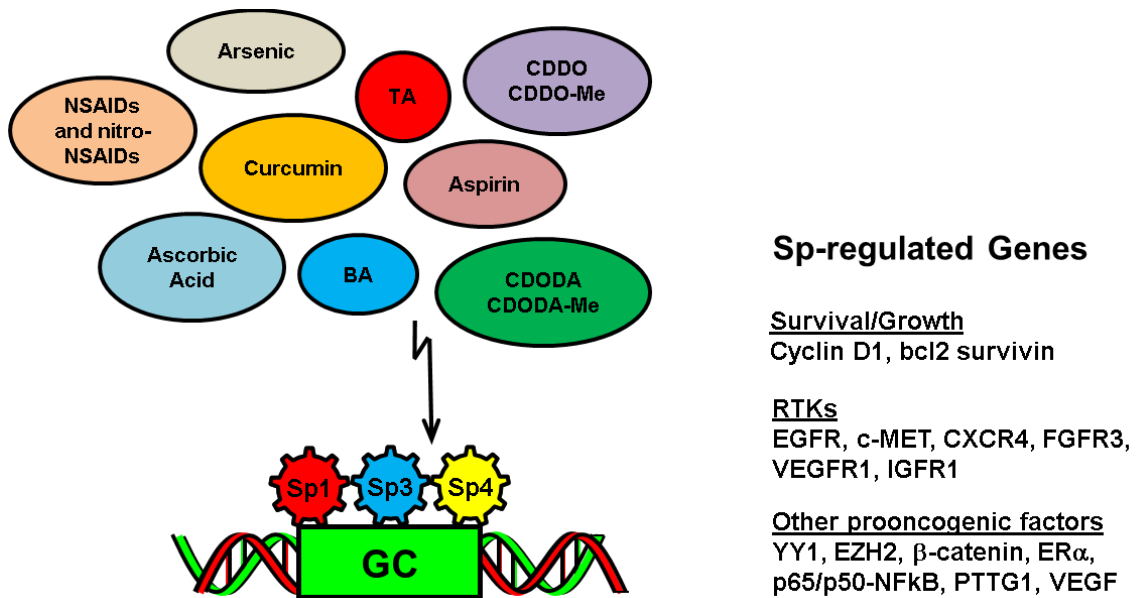


Figure 15. Downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes.

Proteasome and Caspase Mediated Degradation of Sp Proteins

Several classes of anti-cancer agents including non-steroidal anti-inflammatory drugs (NSAID), cyclooxygenase-2 (COX-2) inhibitors and triterpenoids elicit their anti-carcinogenic effects by downregulating expression of Sp transcription factors via proteasome-dependent degradation.

COX-2 Inhibitors

NSAIDs and related COX-2 inhibitors are anti-carcinogenic and their use is associated with decrease in the incidence and mortality of colon cancer (660), (661). Studies in our laboratory showed that COX-2 inhibitors such as celecoxib (Cel), nimesulide (NM) and NS-398 [NS; N-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide] decreased Sp1 and Sp4 but not Sp3 protein expression in SW480, RKO, HT-29 and DLD colon cancer cell lines. The COX-2 inhibitors did not affect Sp1 and Sp4 mRNA levels in SW480 cells however, there was increased ubiquitination of Sp1 and Sp4 and this was inhibited by proteasome inhibitor, gliotoxin suggesting that Sp1 and Sp4 protein downregulation by COX2 inhibitors was proteasome dependent (637).

Tolfenamic Acid

Similarly, the effects of tolfenamic acid (TA), an NSAID, on Sp transcription factors have been extensively investigated in a variety of cancer cell lines. Treatment of pancreatic (Panc1 and L3.6pl) and rhabdomyosarcoma (RD and RH-30) cells with TA inhibited tumor growth and decreased Sp1, Sp3 and Sp4 levels and the mechanism of action of TA was cell context dependent. In Panc1 and RD cells TA-induced degradation of Sp proteins was reversed by treatment with a proteasome inhibitor, lactacystin, indicating that TA mediated downregulation of Sp1, Sp3 and Sp4 proteins was proteasome-dependent; however, these effects were not observed in RH-30 cells co-treated with TA and proteasome inhibitors (662) (638). In contrast to pancreatic cancer cells, TA inhibited breast cancer cell proliferation and tumor growth in erbB2-

overexpressing BTB474 and SKBR3 breast cancer cells and in athymic nude mouse model respectively with minimal effects on Sp protein levels. TA downregulated expression of erbB2 and transcription factors such as YY1 and AP-2 required for basal erbB2 expression (663). In addition TA treatment decreased proliferation in lung (A549 and CRL58030), prostate (PC3) and esophageal (SEG-1 and BIC-1) cancer cell lines and inhibited c-Met expression through degradation of Sp proteins; however the mechanisms of Sp protein degradation was not fully understood (664-666).

Betulinic Acid

Betulinic acid (BA), a pentacyclic triterpene natural product also induced apoptosis and inhibited angiogenesis in cancer cells and tumors from multiple tissues. Treatment of LNCaP prostate and SW480 and RKO colon cancer cell lines with BA decreased expression of VEGF, survivin and several other oncogenic factors and these responses were mediated by downregulation of Sp1, Sp3 and Sp4 proteins. However the mechanism of action of BA on Sp proteins was cell context dependent. Co-treatment of LNCaP and SW480 cells with BA and proteasome inhibitor MG132 reversed the effects of BA, whereas the effects of BA were proteasome-independent in case of RKO cells (639, 667). A recent study investigated the effects of BA on Sp 1 transcription factor in lung tumors employing a bitransgenic mouse. BA inhibited lung tumor growth and decreased expression of Sp 1 protein levels. In the same study treatment of HeLa cells with BA increased sumoylation of Sp1 by inhibiting sentrin-specific protease-1 (SEN-

1) expression resulting in proteasome-dependent degradation of Sp1 transcription factor (668).

Celastrol

Celastrol (CSL), a naturally occurring triterpenoid acid is another anti-cancer drug that exhibits cell context dependent effects on Sp proteins. Studies conducted in our laboratory showed that CSL downregulates Sp transcription factors and Sp-dependent genes/proteins including fibroblast growth factor receptor -3 (FGFR3) by proteasome-dependent degradation in case of KU7 bladder cancer cells whereas the mechanism of Sp protein downregulation in 253JB-V cells was proteasome-independent. Degradation of Sp proteins by CSL in 253JB-V bladder cancer cells was mediated by oxidative stress, which will be discussed in detail below (669).

Curcumin

The active ingredient of turmeric, curcumin, has been extensively investigated in our laboratory. Treatment of 253JB-V and KU7 bladder cancer cell lines with curcumin inhibited cancer cell growth, induced apoptosis and inhibited expression of pro-oncogenic factors such as VEGF, VEGFR1 and survivin. These responses were accompanied by decreased levels of Sp1, Sp3 and Sp4 proteins and co-treatment with the proteasome inhibitor, MG132, inhibited curcumin induced degradation of Sp proteins and Sp dependent genes (640).

Aspirin

Recent studies conducted in our laboratory indicate that caspases also play a role in degradation of Sp proteins. Caspases are cysteine dependent aspartate-directed proteases that play a vital role in the process of apoptosis. Treatment of colon cancer RKO, SW480, HT29 and HCT116 cell lines with aspirin inhibited growth and decreased Sp1, Sp3 and Sp4 protein and Sp-dependent genes. Aspirin mediated downregulation of Sp proteins and Sp dependent genes was blocked by co-treating colon cancer cells with the pan-caspase inhibitor Z-VAD-fmk. Moreover the caspase-dependent cleavage of Sp1, Sp3 and Sp4 was mediated by zinc ion sequestration by aspirin and the effects of aspirin on caspase-dependent Sp downregulation was blocked by co-treatment with zinc sulphate (670).

Transcriptional Repression of Sp Proteins

In addition to degradation of Sp transcription factors, certain drugs transcriptionally repress Sp1, Sp3 and Sp4 proteins and mRNA to elicit their anti-carcinogenic responses.

Role of ROS in Drug Mediated Downregulation of Sp Transcription Factors

Studies in this laboratory have shown that several anti-cancer compounds mediate their effects via induction of oxidative stress and generation of reactive oxygen species (ROS). Arsenic trioxide inhibited cell proliferation and angiogenesis and induced apoptosis in several cancer cell lines including bladder, prostate, pancreas and colon cancer cells and these responses were cell context dependent (658). Treatment of KU7

bladder cancer cell lines with arsenic trioxide decreased cell proliferation and downregulated Sp1, Sp3 and Sp4 proteins and Sp-regulated gene products such as EGFR, VEGF, cyclin D1, survivin, and bcl-2. Arsenic trioxide-mediated downregulation of Sp proteins and Sp dependent genes was inhibited by co-treatment with antioxidants such as dithiothreitol (DTT) and glutathione (GSH) indicating that arsenic trioxide-mediated downregulation of Sp proteins was ROS-dependent. However, these effects were not observed in 253JB-V bladder cancer cells (658). In contrast to these findings a previous study in promyelocytic leukemia cells treated with arsenic trioxide demonstrated decreased Sp1-DNA binding and this was associated with increased Sp1 oxidation with no decrease in the levels of Sp1 protein (671).

Curcumin and synthetic triterpenoids such as 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me) also induced ROS and decreased mitochondrial membrane potential (MMP) in Panc1 and L3.6pl pancreatic cancer cells and treatment with antioxidants GSH and DTT inhibited ROS generation, prevented the loss of MMP and reversed the effects on downregulation of Sp1, Sp3 and Sp4 proteins and Sp dependent genes (672, 673). Similarly ethyl 2-((2,3-bis(nitrooxy)propyl)disulfanyl)benzoate (GT-094), a novel nitro-NSAID, ascorbic acid (vitamin C), curcumin and a synthetic analog of curcumin (3E,5E)-3,5-Bis(2,5-dimethoxybenzylidene)-1-tbutoxycarbonylpiperidin-4-one (RL197) all decreased Sp proteins in SW480 and RKO colon cancer cells via ROS activation and antioxidants

inhibited the effects on Sp proteins and other responses including cell proliferation, induction of ROS and loss of MMP (674-676)

Role of ZBTB10 and MiR27a in Drug Induced Downregulation of Sp Proteins

ROS generation and loss of MMP is only one component of the mechanistic pathway involved in drug induced transcriptional repression of Sp transcription factors. A study investigating the transcriptional regulation of gastrin, a gastrointestinal tract growth factor discovered that a novel zinc finger and BTB domain containing 10 (ZBTB-10/RIN ZF) protein that binds to the CACC *cis*-regulatory elements of the gastrin promoter and interferes with Sp1 binding and transactivation of the gastrin gene suggesting that ZBTB10 may function as an Sp1 repressor (677). Subsequent studies conducted in 2'-O-methyl RNA antisense miR-27a transfected SKBr3 cells revealed upregulation of ZBTB10 mRNA transcripts indicating that miR27a transcriptionally suppressed the expression of the Sp1 repressor, ZBTB10 (678). Hence, we hypothesized that overexpression of Sp transcription factors was due to transcriptional repression of ZBTB10 by miR27a (Figure 16).

To this effect mRNA expression of miR27a, Sp1, Sp3, Sp4 and ZBTB10 was examined in six estrogen receptor (ER)-positive (ZR75, BT474, MCF7) and ER-negative (MDA-MB435, MDA-MB231 and SKBR3) breast cancer cell lines. MiR-27a expression was observed in all the six breast cancer cells and the expression levels of Sp transcription factors were also high whereas only low levels of ZBTB10 mRNA were observed in the

breast cancer cell lines. Transfection of ER-negative MDA-MB-231 breast cancer cells with anti-sense miR-27a (as-miR-27a) increased ZBTB10 mRNA levels, decreased Sp proteins and Sp regulated proteins and also decreased the percentage of cells in S phase of the cell cycle. In addition, MDA-MB-231 cells transfected with GC-rich Sp1 and Sp3 promoter constructs showed decreased luciferase activity following co-treatment with anti-sense miR-27a. In contrast Sp1, Sp3 and Sp4 expression was enhanced when MDA-MB-231 cells were transfected with miR27a mimic. Furthermore, overexpression of ZBTB10 in these cell lines decreased Sp and Sp-dependent gene expression and increased number of cells in G₀-G₁ phase and decreased cell numbers in the S phase of the cell cycle (641). Therefore, results from these experiments established that targeting the oncogenic microRNA, miR-27a can facilitate induction of ZBTB10 expression which in turn can transcriptionally repress Sp proteins and Sp dependent genes by competitively binding to GC-rich sites on their promoter sequences (Figure 16). As mentioned above mitochondriotoxic drugs such as curcumin, CDDO-Me, CSL, BA and GT-094 induce ROS and this is accompanied by decreased miR-27a levels and induction of ZBTB10 which in turn results in downregulation of Sp proteins and Sp dependent genes and these responses can be blocked in cells co-treated with antioxidants (658, 672, 675, 679). Similarly methyl 2-cyano-3,11-dioxo-18b-olean-1,12-dien-30-oate (CDODAME), a triterpenoid also downregulates Sp proteins and Sp regulated gene products by inducing ZBTB10 and repressing miR-27a levels in SW480 and RKO colon cancer cells (680).

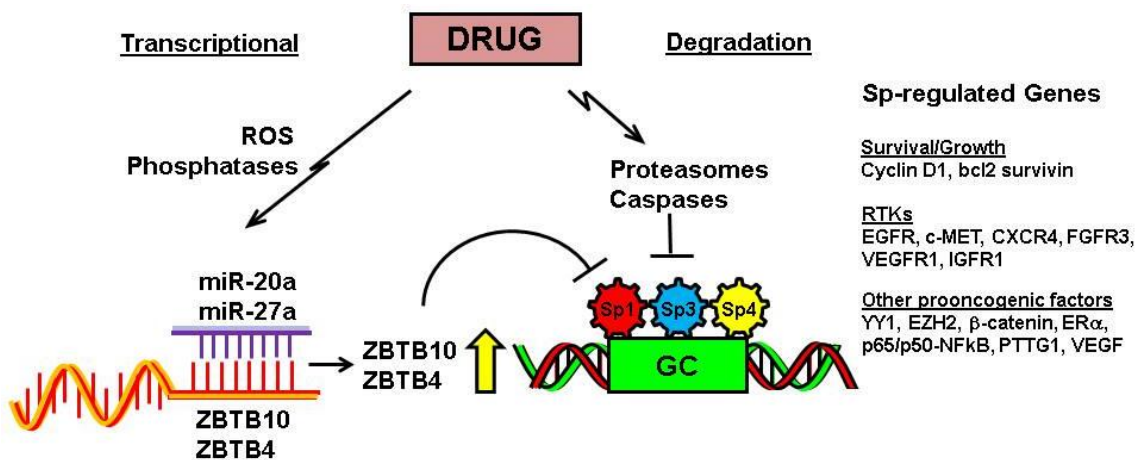


Figure 16. Mechanism of Downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes.

Role of ZBTB4/MiR-20a/MiR-17-5-p Axis in Transcriptional Repression of Sp Proteins

Recent studies investigating the role of human POK family members as transcriptional repressors showed that ZBTB4, another zinc finger and BTB domain containing protein binds to GC-rich sequences that bind Sp proteins (681). In addition, examining the Kaplan-Meier survival analysis of two publicly available breast cancer patient data sets revealed longer relapse-free survival in patients with high expression levels of ZBTB4 whereas shorter relapse-free survival was observed in patients with low ZBTB4 expression. Analysis of publicly available mRNA and miR data sets indicated that ZBTB4 can be negatively regulated by miR-17-5p and miR-20a which form part of the miR-17-92 cluster. MCF-7 cells transfected with 3'-UTR-ZBTB4-luc constructs showed

decreased luciferase activity following co-transfection with miR20a and miR17-5p mimics. Similarly, transfection with miR20a and miR17-5p mimics decreased ZBTB4 protein expression in MCF7 breast cancer cells. In addition, MCF-7 and MDA-MB-231 cells transfected with 3'-UTR-ZBTB4-luc followed by as-miR-20a and as-miR17-5p showed increased luciferase activity and increased expression of ZBTB4 protein. Furthermore, as-miR20a and ZBTB4 expression plasmid decreased luciferase activity in MCF-7 and MDA-MB231 cells transfected with Sp1 and Sp3 promoter-luciferase constructs. Similarly, knockdown of miR20a and overexpression of ZBTB4 in these breast cancer cell lines downregulated expression of Sp proteins and Sp regulated gene products. These results indicate that miR-20a enhances expression of Sp proteins by suppressing the Sp repressor ZBTB4 and targeting miR20a (antisense or drugs) enhances expression of ZBTB4 which in turn competes with Sp1 for GC-rich sites and downregulates Sp transcription factors and Sp dependent genes. Studies conducted in our laboratory show that drugs including CDODA-Me, curcumin, RL197 and CSL target Sp1, Sp3 and Sp4 proteins and Sp dependent genes by disrupting the ZBTB4/miR-20a/miR-17-5-p axis in colon, prostate and bladder cancer cells (674, 682, 683).

Besides ROS and proteasome-dependent degradation there is emerging evidence to suggest that some compounds inhibit Sp-transcription factors by inducing certain classes of phosphatases which will be discussed in detail in the following chapters. In addition recent evidence from this laboratory has indicated the involvement of cannabinoid (CB) receptors in downregulation of Sp proteins. Pre-treatment of BT474 and MDA-MB-453

breast cancer cells with CB receptor antagonists and small-interfering RNAs for CB receptors followed by treatment with BA inhibited BA mediated downregulation of Sp1, Sp3 and Sp4 proteins and other pro-oncogenic factors such as ErbB2, MAPK, Akt, YY1 and AP2 in both cell lines. Similar effects were observed in MDA-MB-453 cells pre-treated with the transient receptor potential cation channel subfamily V member 1/ Vanilloid receptor 1 antagonist, capsaizepine followed by treatment with BA. This study also demonstrated for the first time that BA bound directly to both the CB1 and CB2 receptors (684).

Compound	Cell lines	Mechanism of Action
Tolfenamic acid (638)	Panc 1	Proteasome dependent degradation
Tolfenamic acid (666)	SEG-1, BIC-1	Not Determined
Tolfenamic acid (663)	BT474, SKBR3	No effect on Sp proteins
Tolfenamic acid (664)	A549, CRL5803	Not Determined
Tolfenamic acid (665)	PC3	Not Determined
Tolfenamic acid (662)	RH-30, RD	Proteasome dependent degradation (RD)
Betulinic acid (639)	LNCaP	Proteasome dependent degradation
Betulinic acid (667)	SW480	Proteasome dependent (SW480)
Betulinic acid (667)	RKO	ROS and MMP, miR27a-ZBTB10 axis
Betulinic acid (679)	MDA-MB-231	miR27a-ZBTB10 axis
Betulinic acid (684)	BT474	CB receptor mediated, miR27a-ZBTB10 axis
Betulinic acid (684)	MDA-MB-453	CB and Vanilloid receptor, miR-27a-ZBTB10 axis
Betulinic acid (685)	HeLa	Sumoylation, Proteasome dependent degradation (Sp1)
Curcumin (640)	253JB-V, KU7	Proteasome dependent degradation

Table 1. Mechanism of action of drugs that target Sp transcription factors.

Compound	Cell lines	Mechanism of Action
Curcumin (673)	Panc28, L3.6pl	ROS and loss of MMP
Curcumin (674)	SW480, RKO	ROS, MMP, miR27a-ZBTB10/miR20a-miR-17-5p-ZBTB4
RL197 (674)	SW480, RKO	ROS, MMP, miR27a-ZBTB10/miR20a-miR-17-5p-ZBTB4
CDODA-Me (680)	SW480, RKO	miR27a-ZBTB10 axis
CDODA-Me (686)	DU-145, PC3	miR-106b/93-ZBTB4 axis
CDDO (672)	Panc 1, L3.6pl	Not Determined
CDDO-Me (672)	Panc 1, L3.6pl	ROS, MMP, miR27a-ZBTB10 axis
Celastrol (683)	KU7	Proteasome dependent degradation
Celastrol (669)	253JB-V	ROS and miR27a-ZBTB10/mir20a-17-5p-ZBTB4 axis
Nimesulfide (637)	SW480, RKO, HT-29, DLD	Proteasome dependent degradation (Sp1 and Sp4)
GT-094 (675)	SW480, RKO	ROS and loss of MMP, miR27a-ZBTB10 axis
Aspirin (670)	SW480, RKO, HT-29, HCT116	Caspase mediate cleavage (RKO and SW480)
Arsenic Trioxide (658)	253JB-V, KU7	ROS and loss of MMP in KU7
Ascorbic Acid (676)	RKO, SW480	ROS

Table 1. Continued.

CANNABINOIDS

Cytotoxic anti-cancer agents effectively kill cancer cells but also target normal cells resulting in undesirable side effects and although these treatments may be beneficial for early stage cancers their effectiveness for treating advanced cases of cancer is low. More targeted therapies that focus on inhibiting activities of single molecules produce fewer

side effects and are far more specific than chemotherapy and radiation but their efficacy is also limited and can often be by-passed. Hence Sp proteins which regulate expression of multiple genes and pro-oncogenic pathways would appear to be ideal targets for development of mechanism based drugs and as indicated above the underlying mechanisms of action of several different classes of anticancer agents.

Cannabinoids are being developed for cancer chemotherapy and have been approved for clinical trials (glioma) (687) and we hypothesize that an important underlying mechanism of action of cannabinoids is due to their downregulation of Sp transcription factors.

A Bit of History

The origin of the plant *Cannabis* is believed to be from the riverine valleys of Central Asia or from northern South Asia along the foothills of the Himalayas. It is the oldest psychotropic drug known to man and was first cultivated in China for fiber and seed production (688, 689). The medicinal properties of cannabis were first described by the Chinese emperor, Shen Nung in his compendium of Chinese medicinal herbs composed during 2737 BC (690). Cannabis then spread to India where it was referred to by the term “*bhang*” and was used for relieving anxiety. The analgesic, appetite stimulant, antiemetic, muscle relaxant and anticonvulsant properties of cannabis were discovered by the British physician and surgeon working in India, William O’ Shaughnessy in 1839. His observations introduced Europe and the Western hemisphere to the therapeutic uses

of this drug (688, 691). Later in 1854 cannabis was listed in the United State Dispensary and was available in pharmacies of Western countries (662). However, the use of cannabis was soon to be associated with causes of insanity, moral and intellectual deterioration, violence and various crimes. Hence in 1937 the US government introduced a *Marijuana Tax Act* under pressure from the Federal Bureau of Narcotics. This was followed by removal of cannabis from the United States Pharmacopeia, resulting in loss of its therapeutic legitimacy (692-694). Similarly, the 1971 Convention on Psychotropic Substances instituted by the United Nations was used by Britain and most European countries to ban cannabis (695). Currently cannabis is categorized as a Schedule I drug under the federal Controlled Substances Act of 1970 and is considered illegal for any purposes except FDA approved research programs (696). Medical cannabis is presently legal in twenty states and the District of Columbia in the US (697, 698). As of November 2012, recreational use of marijuana is legal in Washington and Colorado in the US (699).

Cannabinoids and Cannabinoid Receptors

The three main species of Cannabis are *Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis* (695). Cannabis contains more than 460 chemicals of which more than 60 of them are categorized under the class of plant-derived cannabinoids or phytocannabinoids (700). Delta-9-tetrahydrocannabinol (THC) is the main psychoactive ingredient of cannabis with delta-8-tetrahydrocannabinol (Δ^8 THC), cannabinol (CBN), cannabidiol (CBD), cannabicyclol (CBL), cannabichromene (CBC) and cannbigierol (CBG) present

in smaller quantities (688, 701). The synthetic class of cannabinoids comprises of compounds structurally similar to phytocannabinoids and analogs that are structurally different from THC (702). The third class, endocannabinoids are a group of arachidonate based lipids such as anandamide (*N*-arachidonylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG) that are synthesized within the body and are involved in various physiological processes (703). Cannabinoids elicit their responses by binding to cannabinoid receptors (CB) which are 7 transmembrane Gi/Go-coupled receptors. To this date two receptors have been identified namely CB1 and CB2 and they share 44% protein identity and exhibit different expression patterns and pharmacological properties (704-706).

CB1 Receptors

CB1 receptor is a G protein coupled receptor that contains seven transmembrane domains linked by three intracellular and extracellular loops, with the extracellular N-terminal tail and the intracellular C-terminal tail (707). The CB1 receptor is a 473 amino-acid long protein that can exist as homodimers and coexpression of other G-protein-coupled receptors can give rise to heterodimers and oligomers (708, 709). CB1 receptors are primarily expressed in areas of the central nervous system (CNS) that control motor activity (basal ganglia and cerebellum), memory and cognition (cortex and hippocampus), emotion (amygdala), sensory perception (thalamus), and autonomic and endocrine functions (hypothalamus, pons and medulla) (710). In addition they are also expressed in the peripheral nervous system and extraneural tissues such as testis,

vascular endothelium and spleen (711). They are abundantly distributed in the presynaptic terminals of the neurons, and to a lesser extent on the dendrites and soma of neurons (712, 713). Astrocytes, oligodendrocytes and neural stem cells also express low levels of CB1 receptors (714-716). CB1 receptors couple with the inhibitory Gi/o proteins and in some cases with the stimulatory Gs proteins to regulate signal transduction pathways, ion channels, enzymes generating cyclic nucleotide second messengers and kinases (706). The acute cognitive and intoxicating effects of THC are due to N-type channel blockade by CB1 receptors resulting in inhibition of synaptic transmission (717). The neuroprotective effects of cannabinoids can be attributed brain-derived neurotrophic factor (BDNF) induced by increased activity of Erk resulting from activation of CB1 receptors (713). There is also evidence demonstrating the role of CB1 receptor in stem cell fate and behavior and development of neuronal networks and functional synapses (716, 718). During neuropathological conditions the blood brain barrier gets disrupted resulting in infiltration of blood-derived leukocytes. CB1 receptors expressed in these blood cells are activated by cannabinoids that accumulate in the CNS and regulate inflammatory processes in the nervous tissue during such pathological conditions (719-721). Similarly, increased expression of cannabinoid receptors in diseases such as multiple sclerosis is considered to render a protective effect by reducing symptoms or suppressing disease progression (722). CB1 receptor levels are low in colorectal cancers due to increased DNA methylation of promoters and loss of CB1 receptor expression in mice enhanced tumor proliferation in mouse models of colorectal cancer (723, 724). Prostate cancer cells express high levels of CB1 receptors and

treatment of prostate cancer cells with cannabinoid receptor agonists inhibits cell growth and induce apoptosis (725). In contrast breast cancer cells express low levels of CB1 receptors and treatment with THC increased cell proliferation (726).

CB2 Receptors

The CB2 receptor contains 360 amino-acids with an N-terminus that is glycosylated and a C-terminus that is involved ligand-induced receptor desensitization (727). The CB2 receptor amino acid sequence is less conserved across human and rodent species compared to the CB1 receptor (728). The non-conserved residues of CB2 receptor, S3.31 and F5.46 confer CB1/CB2 receptor selectivity. Lipophilic groups bind to F5.46 residue that facilitates hydrogen bond formation with S3.31 resulting in a conformational change in the receptor structure leading to signal transduction (729). CB2 receptors are confined to the immune system in tissues of spleen, tonsils and lymph nodes and are primarily expressed in the leukocytes with B-cells displaying the highest levels and CD⁴⁺ T cells expressing the lowest levels of CB2 receptors. CB2 receptor expression is highly variable in natural killer (NK) cells, monocytes/macrophages, neutrophils and CD⁸⁺ T cells. The functional role of CB2 receptors was elucidated through experiments conducted in CB2 receptor knockout mice (705, 730). CB2 receptors are involved in modulation of immune responses and the anti-inflammatory properties of CBN such as inhibition of macrophage dependent antigen processing and T cell priming and induction of immune cell apoptosis is mediated by CB2 receptors (731-733). Most studies demonstrate absence of CB2 receptor expression in the brain (705, 719). Contrary to the

general belief that CB2 receptors are exclusive to the immune system, one study detected CB2 mRNA and protein in neurons located in the brain stem and is believed to regulate emesis (734). Pathological conditions can induce CB2 receptor expression. Prostate and colon cancer cells exhibit increased expression levels of CB2 receptor (723, 725). Activated microglial cells from mouse models of multiple sclerosis and Alzheimer's disease express high levels of CB2 receptors compared to resting microglial cells (735, 736). Cultured microglial cells, which are considered chronically activated also, express CB2 receptors on the protrusions (lamellapodia) involved in cell migrations. Like CB1 receptors, CB2 receptors are expressed in leukocytes and play a role in neuroinflammation by responding to cannabinoids that have accumulated as a result of disrupted blood brain barrier. CB2 receptors coupled with Gi/o proteins regulate secondary messengers, enzymes and signal transduction pathways. CB2 receptor activation can increase Erk activity for immune cell migration and regulation of gene expression of several genes (737).

Other Receptors

Experiments employing CB1^{-/-} mice suggest existence of non CB1/CB2 receptors in neurons and their role in regulating synaptic transmission. In one set of experiments WIN 55,212-2, (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone, (WIN) and anandamide stimulated a novel receptor while 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol (CP55,940), (6aR,10aR)- 9-(Hydroxymethyl)- 6,6-dimethyl- 3-

(2-methyloctan-2-yl)- 6a,7,10,10a-tetrahydrobenzo [c]chromen- 1-ol (HU210) and THC were inactive; the stimulatory effects of WIN and anandamide were not antagonized by CB1 receptor antagonist SR141716A indicating that the role of this receptor is distinct from CB1 and CB2 (738, 739). Studies examining the effects of WIN and CP55,940 on excitatory glutamatergic transmission and the analgesic efficacy of THC in spinal cord also suggested the expression of a novel receptor (740-743). Furthermore, certain cannabinoids induce hypotensive and vasodilatory responses in mesenteric vessels without activation of CB1, CB2 or TRPV1/vanilloid receptor (transient receptor potential cation channel subfamily V member 1) (744). The analgesic effects of palmitoylethanolamide (PEA), an AEA analog were blocked by a CB2 receptor antagonist SR144528 even though PEA does not bind to CB2 receptors, indicating the involvement of non-CB receptor-mediated anti-inflammatory effects of PEA (728, 745, 746). A G protein coupled receptor GPR55 is believed to be the third cannabinoid receptor since THC, cannabidiol, anandamide, CP55,940 and SR141716A are able to bind and activate GPR55 (747). In addition lysophosphatidylinositol and its 2-arachidonoyl derivative may be the endogenous ligands for GPR55 and activation of GPR55 results in induction of rhoA, cdc42 and rac1. It is also proposed that activation of GPR55 is responsible for the analgesic effects of PEA. GPR55 is coupled to G protein G₁₃, a subunit of the fourth class of G protein alpha subunits that are not sensitive to pertussis toxin (706, 748-750). Another G protein receptor that is considered a cannabinoid receptor is GPR119 which is activated by anandamide and PEA and is highly expressed in pancreas and gastrointestinal tract (751-753). GPR18 may also be an

abnormal cannabinoid receptor activated by AEA, abnormal cannabidiol, O-1602 and THC for processes such as microglial migration in the CNS (754, 755).

The Endocannabinoid Signaling System

CB1 and CB2 receptors are coupled to the $G_{i/o}$ subtype of G protein and when activated can inhibit adenylyl cyclase by releasing the $G_{i\alpha}$ isoforms (756). However, simultaneous activation of CB1 and dopamine D_2 receptors due to heterodimerization can stimulate 2, 4 and 7 isoforms of adenylyl cyclase by releasing $\beta\gamma$ subunits (757, 758). Receptor dimerization also facilitates CB1 receptor coupling with $G_{q/11}$ subtypes of G protein leading to increased intracellular calcium in some neuron types treated with the synthetic cannabinoid WIN (759). Normally, neurotransmitters bind to ionotropic or metabotropic receptors when there is a surge in the cytosolic free Ca^{2+} levels. Endocannabinoids are synthesized in response to increased Ca^{2+} concentrations in postsynaptic neurons as membrane-bound precursors which are cleaved and released to bind to the presynaptic CB1 receptors (760). Cannabinoids bind to CB receptors and inhibit adenylyl cyclase activity leading to decreased production of cyclic AMP (cAMP), which in turn leads to activation of K^+ channels and inhibition of Ca^{2+} channels (761). Inhibition of Ca^{2+} channels inhibits release of neurotransmitters such as glutamate, dopamine and γ -aminobutyric acid (GABA) in turn affecting processes such as learning, movement and memory respectively (Figure 17) (762). Several kinase pathways are activated by cannabinoid receptor signaling including p44/42 mitogen activated protein kinase, p38 kinase, JUN-terminal kinase and phosphatidylinositol-3-kinase pathways (763-768). In

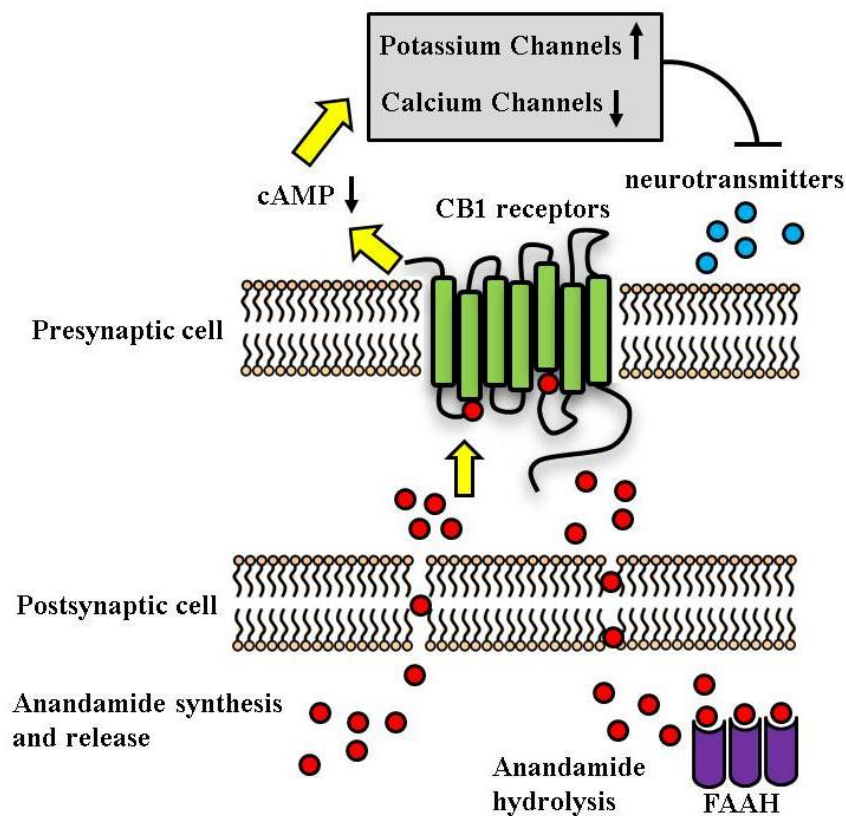


Figure 17. A model of synaptic cannabinoid signaling

addition activity of phosphatases such as protein phosphatase 2b (calcineurin), mitogen-activated protein kinase phosphatase-1 (MKP-1) are also regulated by CB1 receptors (769, 770). Some of these effects may be mediated through activation of G proteins whereas others function independently of G proteins via adaptor proteins (764, 771, 772).

Endocannabinoids

Endocannabinoids or endogenous cannabinoids are a group of eicosanoids that function as physiological ligands for cannabinoid receptors. They are arachidonate-based lipid messengers that are synthesized on demand and include compounds such as AEA, 2-AG, 2-arachidonoyl glycerol ether (noladin ether), N-arachidonoyl-dopamine (NADA) and virodhamine (OAE) (Figure 18) (703, 773). AEA and 2-AG are endocannabinoids that have been studied extensively. AEA is both a full and partial CB1 receptor agonist depending on the tissue whereas it exhibits low affinity and can function as an antagonist of CB2 receptors (774). AEA is synthesized in the cells from the membrane lipid precursor *N*-arachidonoyl phosphatidylethanolamide (NAPE), which is generated by enzymatic transfer of arachidonic acid from the *sn*-1 position of phosphatidylcholine to the amide group of PE. NAPE then undergoes hydrolysis catalyzed by the enzyme phospholipase D to give rise to AEA (775-777). In addition several studies indicate existence of parallel pathways for synthesis of AEA from NAPE. For example Sun and coworkers identified a secreted phospholipase 2 that hydrolyses *N*-acyl-PE to generate *N*-acyl-ethanolamides, including AEA in the stomach (778). Similarly, conversion of NAPE to phosphoanandamide and subsequent dephosphorylation by a phosphatase to form AEA was also reported (779).

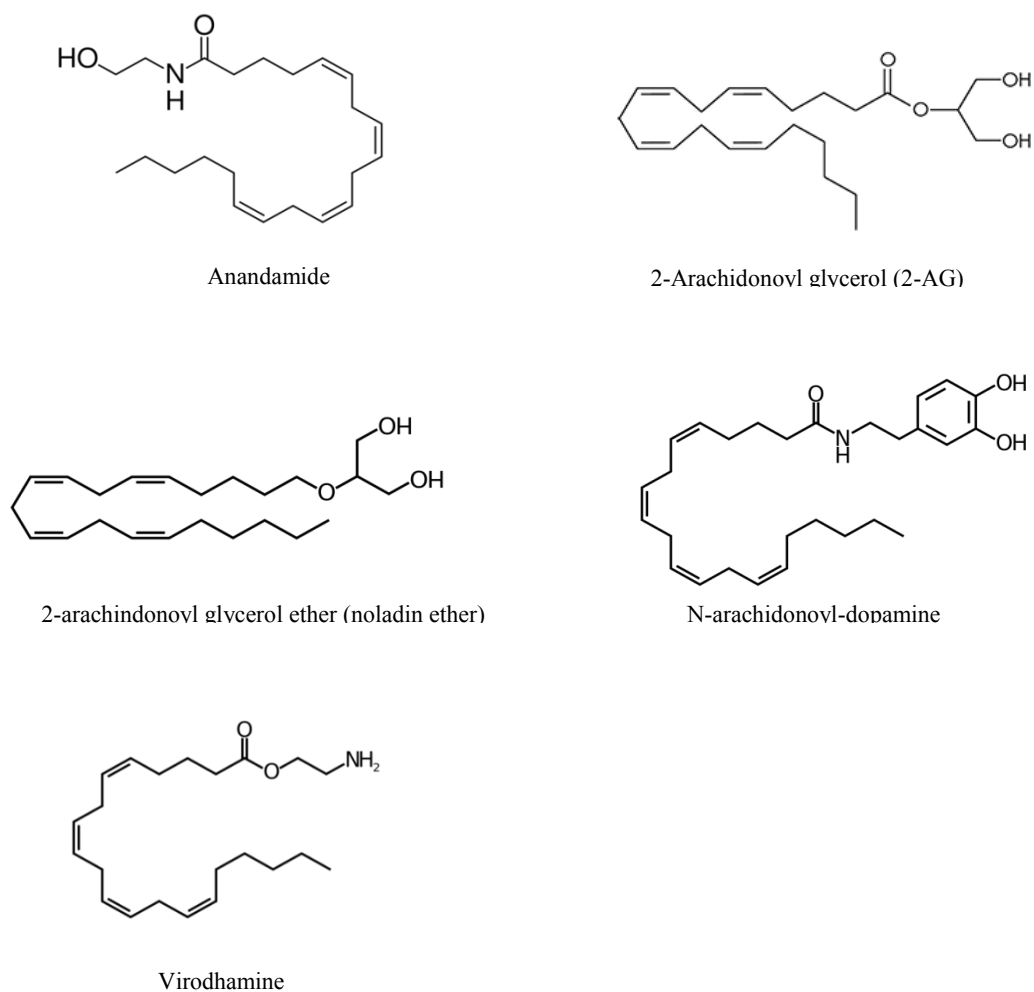


Figure 18. Endocannabinoids, lipid messengers synthesized within the body (702)

2-AG is derived from the intracellular secondary messenger diacylglycerol (DAG) which is generated from phosphoinositides or phosphatidic acid by the action of phospholipase C or phosphohydrolase respectively. DAG is then acted upon by DAG-lipase to form 2-AG (780). Studies investigating the release of endocannabinoids under physiological conditions have revealed that AEA is released by depolarized neurons both

in vivo and in vitro (776, 781). However, there is no evidence demonstrating the release of 2-AG from depolarized neurons or under conditions in which AEA was released (781). Hence, it has been suggested that AEA functions as an intercellular signaling molecule while 2-AG is not released by neurons in the brain and may play a role in intracellular cannabinoid signaling (707).

Intake and Metabolism of Endocannabinoids

It is assumed that endocannabinoids are released into extracellular space from neurons through a transporter protein which is also involved in cellular uptake of cannabinoids. Evidence for this hypothesis stems from experiments that inhibit release of AEA by using transport blockers (782, 783). Endocannabinoids present in the extracellular space accumulate in neurons via Na^+ and ATP -independent facilitated diffusion that occurs due to transmembrane concentration gradient (776, 784). Involvement of a transport protein in the uptake of AEA is also implied since AEA uptake is inhibited by N-(4-hydroxy-phenyl) arachidonamide (AM404) in rat cortical neurons and astrocytes (785). However, a specific transporter protein for the uptake of anandamide has not yet been cloned. AEA is degraded in the cell by the enzyme fatty acid amide hydrolase (FAAH) into arachidonic acid and ethanolamine (786). Recent reports demonstrate inhibition of FAAH activity by AM404 in neuroblastoma cells suggesting that FAAH alone may be adequate for diffusion of AEA across the cell membrane (707). In contrast to these results experiments conducted in FAAH^{+/+} and FAAH^{-/-} cells did not show a

difference in levels of AEA uptake implying that an FAAH-independent component in AEA uptake might indeed exist (787).

2-AG is hydrolyzed by FAAH *in vitro* however, unaltered levels of 2-AG in wild-type and FAAH^{-/-} mice brain suggests that 2-AG might not be the substrate of FAAH *in vivo* (787-789). 2-AG is metabolized by monoglyceride lipase (MGL) *in vivo* (790).

Plant and Synthetic Cannabinoids

THC is considered the most important ingredient of the hemp plant *Cannabis sativa* due to its high potency and abundance (791). Δ^8 -THC is another plant-derived cannabinoid that is equally active as THC but less abundant. CBN and CBD are two other phytocannabinoids that are present in abundant amounts but have weak and no cannabimimetic activity respectively (Figure 19) (711). Besides therapeutic applications, cannabinoids are commonly used for recreational purposes and according to government reports there are currently 14 million marijuana users in the US (792). Cannabis consumption can produce a state of euphoria and anxiety and these psychoactive effects are attributed to THC, the major component of the plant (793, 794). In addition to THC, cannabis plants also contain ingredients such as CBD that do not possess psychoactive properties. The ratio of non-psychoactive to psychoactive components in the plant is responsible for the varying degrees of cerebral high and anxiety experienced by individuals smoking different species of the plant. *Cannabis indica* has a 4-5 times higher CBD:THC ratio than *Cannabis sativa* and hence causes less anxiety. THC is an

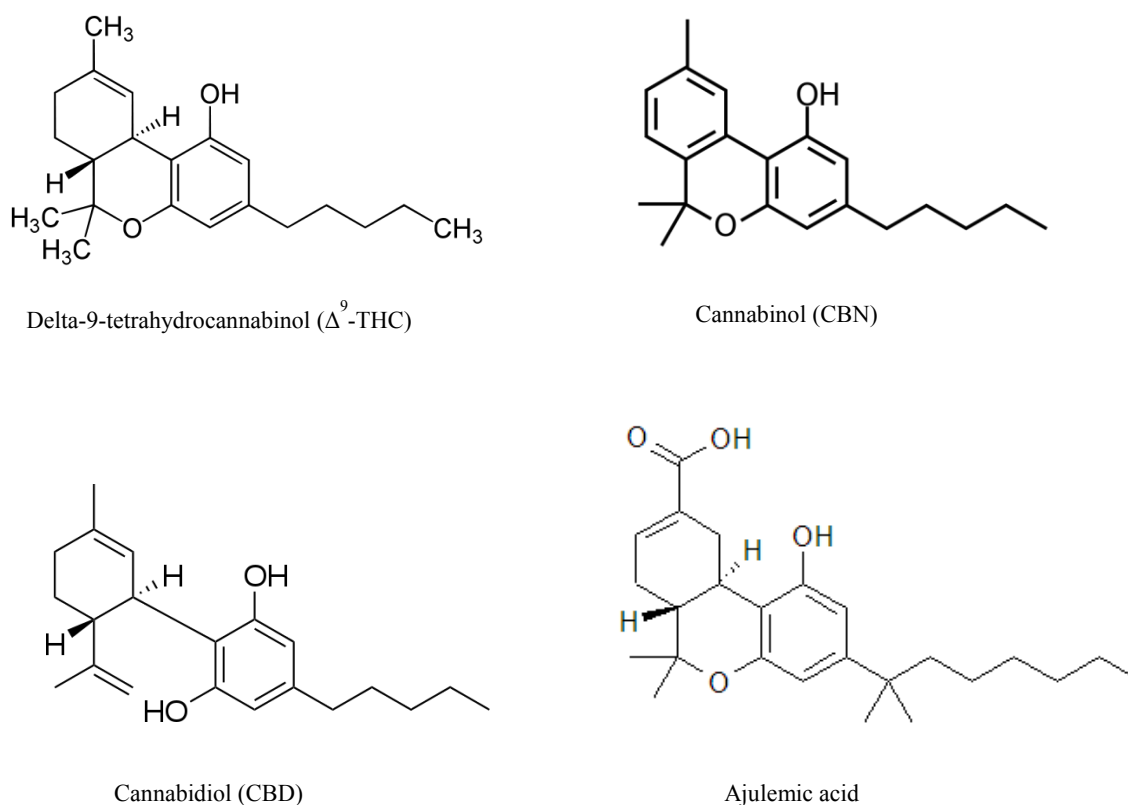


Figure 19. Phytocannabinoids, active ingredients extracted from the plant *cannabis sativa* (702)

agonist of the cannabinoid receptor and causes anxiety whereas CBD is a 5-HT_{1A} receptor agonist that elicits an anxiolytic effect and higher levels of CBD suppress the anxiogenic effects of THC. Hence, *Cannabis sativa* is preferred for daytime use as medical cannabis due to its cerebral “high” while *Cannabis indica* is favored for night time use as a therapeutic owing to its sedative effects (795).

Synthetic cannabinoids are classified into different families (Figure 20). The classical cannabinoid analogues are structurally related to THC and include (-) HU-210, Nabilone

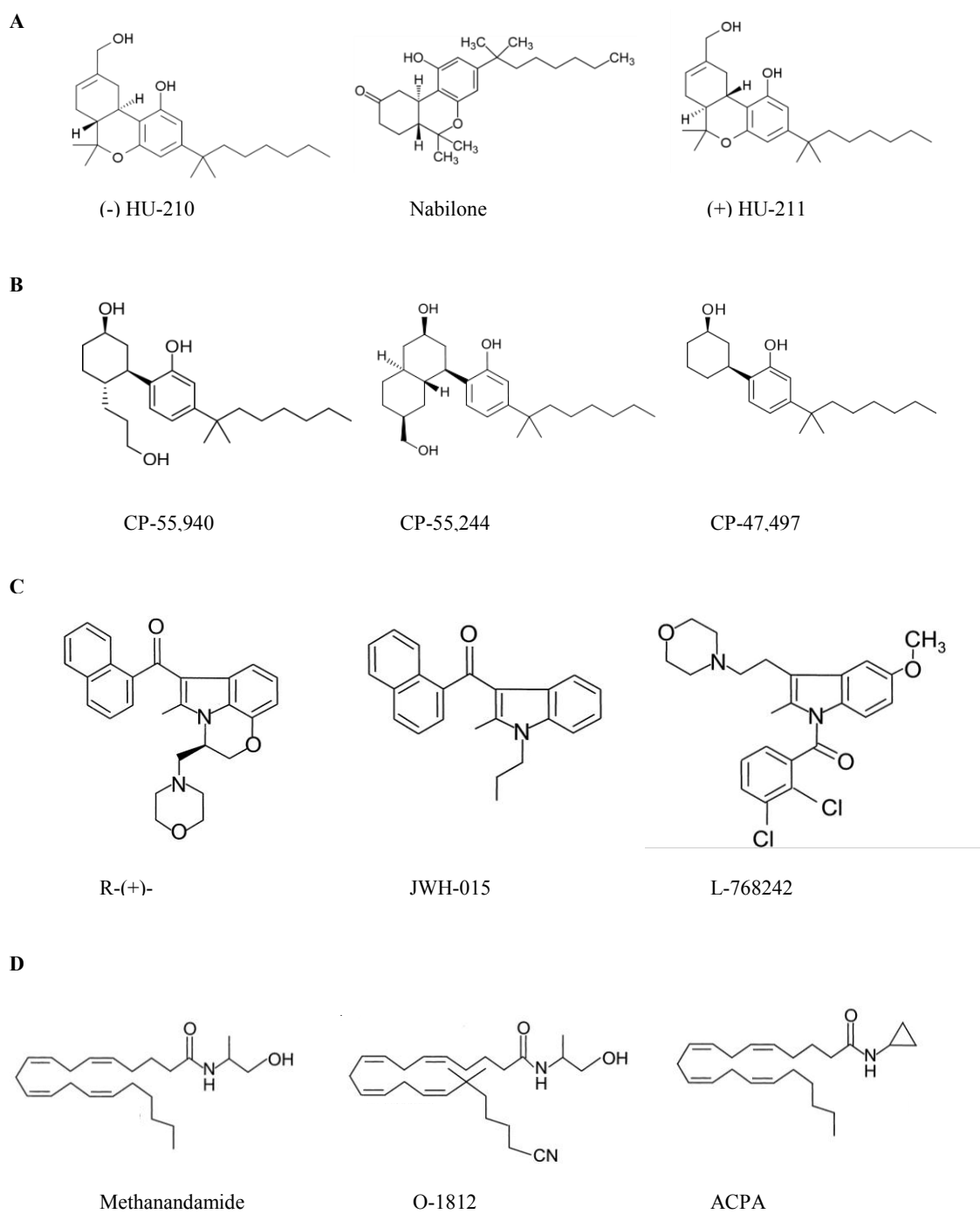


Figure 20. Synthetic cannabinoids (A) Classical, (B) Non-classical, (C) Amino-alkyl-indole derivatives and (D) Eicosanoids (702)

and (+) HU-211. While some of them display cannabinoid receptor activity others do not show any affinity to receptors. Non-classical cannabinoid analogues include compounds that are structurally related to CP-55,940. These compounds are structurally different from THC and do not possess the characteristic tetrahydropyran ring. The third class of synthetic cannabinoids is aminoalkylindole derivatives that resemble the lead compound WIN 55,212-2. Their structures are different from THC but elicit responses similar to THC and other plant-derived cannabinoids. The last set of synthetic cannabinoids constitutes eicosanoids that are structurally-related to endocannabinoids (702, 796, 797). Synthetic cannabinoids have been used to investigate the structure activity relationship among all cannabinoids and for determining various structural elements required for activity. In addition these compounds serve as templates for designing more effective drugs for various conditions (798). Some synthetic cannabinoids such as Nabilone are available as an anti-emetic, appetite stimulant and analgesic and several other synthetic cannabinoids are marketed in the form of psychoactive designer drugs for recreational purposes (670, 799) . However, the recent signing of the Synthetic Drug Abuse Prevention Act of 2012 into law has placed several cannabinoids including JWH-018, JWH-073, CP-47,497, JWH-200, and cannabicyclohexanol on Schedule I of Controlled Substances Act and they are illegal to possess or use in the US (800).

Therapeutic Applications

The therapeutic applications of cannabinoids have been known since the early 1970s and there is sufficient evidence from animal studies and clinical trials to support the

beneficial effects of cannabinoids for several conditions including cancer patients undergoing chemotherapy (711).

Anti-emetic

Nausea and vomiting are the most common side-effects observed in individuals receiving chemotherapy. Clinical trials conducted with THC, synthetic cannabinoids and cannabinoid smoking during the 1970s and 1980s demonstrated anti-emetic potencies comparable to dopamine D2-receptor antagonists prochlorperazine, domperidone and metoclopramide. Moreover patients indicated a clear preference for cannabinoids over conventional anti-emetics in this study (662, 670, 801). THC (dronabinol [Marinol]) and its synthetic analogue LY109514 (nabilone [Cesamet]) are currently available as oral medications in the form of capsules and are approved for treating nausea and emesis associated with cancer chemotherapy. In addition cannabinoids are more effective in controlling nausea and vomiting in patients during the delayed phase of chemotherapy compared to modern anti-emetics, such as 5-HT₃-receptor antagonist ondansetron and the selective substance P/neurokinin-1-receptor antagonist aprepitant (711). The anti-emetic effects of cannabinoids can be attributed to inhibition of gastrointestinal (GI) tract motility by binding to CB1 receptors located on the myentric and submucosal plexus of the GI tract and blocking the release of acetylcholine in these areas. In addition CB1 receptors are expressed on the dorsal-vagal complex of the brainstem that controls the vomiting reflex and cannabinoid receptor binding in this region may also contribute to the anti-emetic effect (802).

Appetite Stimulation

Lack of appetite (anorexia) and weight loss are commonly observed in a large proportion of patients with advanced cancer. Anorexia can result in a wasting syndrome known as cachexia and is characterized by weight loss, muscle atrophy, fatigue and weakness and this is an important risk factor for morbidity and mortality in cancer (803). Several studies have demonstrated appetite stimulatory effects of THC and related cannabinoids in AIDS and cancer patients when administered in low doses that do not result in side-effects (804). CB1 receptors and endocannabinoids present in the hypothalamus control food intake and blocking CB1 receptors or decreasing the levels of endocannabinoids can induce weight loss and decrease appetite. Dronabinol is currently prescribed for AIDS patients as an appetite stimulant at a dosage range of 2.5-5.0 mg/day. In addition several Phase II and Phase III clinical trials provide evidence supporting the appetite-stimulating effect of THC at 5.0 mg/day in advanced cancer patients (670, 805, 806).

Analgesic Effects

Patients suffering from cancer frequently experience moderate to severe pain and it worsens in cases of metastatic or advanced-stage cancer (711). Cannabinoids inhibit nociception by activating CB1 receptors in the thalamus, periaqueductal grey matter and rostral ventromedial medulla of the brain, dorsal horn of the spinal cord and dorsal root ganglia of the nerve terminals (807, 808). Similarly, peripherally located CB2 receptors elicit analgesic responses by inhibiting mediators of pain and inflammation (745, 807). Results from four Phase III clinical trials using THC and two first-generation synthetic

cannabinoid derivatives show a similar analgesic potency compared to a moderate opioid analgesic, codeine (809, 810).

Cannabinoids and Cancer

The anti-cancer effects of cannabis compounds were first reported by Munson and coworkers in 1975 showing that oral THC inhibited lung-adenocarcinoma cell growth in vitro and tumor growth in mouse tumor xenograft (811). Despite the encouraging results from these experiments, further research on the anti-tumor activities of cannabinoids was not carried out until the 1990s. Studies conducted in several laboratories demonstrate the anti-proliferative effects of various classes of cannabinoids including phytocannabinoids (THC, cannabidiol), synthetic cannabinoids (WIN, HU-210) and endocannabinoids (AEA, 2-AG) in a variety of tumor cell lines (812). Furthermore, xenograft studies in nude mice show that cannabinoids inhibit tumor growth in lung carcinomas, gliomas, thyroid epitheliomas, skin carcinomas and lymphomas (711). Several mechanisms have been proposed to explain the anti-cancer properties of cannabinoids.

The effects of cannabinoids on cancer cells are cell context dependent and the mechanisms involved are complex. As mentioned earlier cannabinoids stimulate a multitude of signaling pathways such as MAPK, ERK JNK and p38 MAPK that are involved in cell growth and differentiation. Similarly, cannabinoids also play a role in induction of PI3K-AKT survival pathways. In contrast to these findings several studies

demonstrate suppression of ERK and AKT signaling and induction of apoptosis by cannabinoids (813).

Ceramide is a lipid second messenger involved in induction of apoptosis and cell cycle arrest, formed as a result of sphingomyelin breakdown (814, 815). Cannabinoids initiate apoptosis by regulating sphingomyelin metabolism and in turn increasing levels of ceramide which is proapoptotic in cancer cells (816). This mechanism is cannabinoid receptor-dependent but does not involve G-proteins and is mediated via an adaptor protein, factor associated with neutral sphingomyelinase activation (FAN) (772). In addition to generating ceramide by breakdown of sphingomyelin, CB receptor activation can also lead to *de novo* synthesis of ceramide (817, 818). Mechanisms of cannabinoid-induced apoptosis via the ceramide pathway are best illustrated using glioma cells as the model (817, 819). Treatment of glioma cells with cannabinoids increase ceramide levels leading to activation of RAF1-MEK-ERK and inhibition of AKT signal transduction pathways (817, 818). ERK activation induces cell proliferation; however, prolonged activation of ERK results in cell-cycle arrest and cell death. Moreover, the anti-cancer effects of cannabinoids also depend on ceramide levels. Two peaks of ceramide generation with different kinetics are observed in glioma cells following cannabinoid receptor activation and the second peak of ceramide generation governs ERK activation and apoptotic effects of cannabinoids (817, 818, 820, 821). Similar effects are observed in prostate cancer cell lines where blocking *de novo* ceramide synthesis inhibits cannabinoid mediated cell death (562). Cell cycle arrest at G1-S transition due to

activation of CB1 receptors in breast cancer cell lines is attributed to inhibition of cAMP-protein kinase A (PKA) pathway. PKA inactivation results in RAF1 activation leading to prolonged ERK stimulation and inhibition of cell growth via suppression of prolactin receptor and high-affinity neurotrophin TRK receptor (563, 687, 822). Cannabinoid receptor-mediated inhibition of cell proliferation in pheochromocytoma, skin carcinoma and prostate carcinoma cells occurs via deregulation of growth factor receptor signaling (562, 663, 813). Cannabinoids inhibit angiogenesis by blocking expression of VEGF and other pro-angiogenic cytokines and also inhibit of matrix metalloproteinase-2 (MMP-2) expression in lung tumor xenografts in vivo (663, 664, 671).

Most conventional therapies invariably result in deleterious side effects since they indiscriminately target both normal and cancer cells. Cannabinoids on the other hand are toxic to cancer cells culture and tumors in mice and rats with minimal effects on normal tissues. Some studies even demonstrate a protective effect of cannabinoids on normal cells of astroglial and oligodendroglial lineages (715, 768). The reported anti-proliferative and protective effects of cannabinoids are primarily associated with ceramide levels and the AKT survival pathway (817, 818). The second peak of ceramide generation represents de novo synthesis of ceramide and this is responsible for inducing apoptosis by inhibiting AKT signaling. However, this second peak is absent in normal astrocytes and cells that are resistant to cannabinoid mediated apoptosis although they express functional CB receptors (817, 820). Hence it is apparent that in glioma cells

cannabinoids activate the CB receptor to generate ceramide to inhibit cell survival pathways while in normal cells activation of CB receptors stimulate AKT pathway and prevent ceramide mediated AKT inhibition (768, 818). Similar phenomena are observed in thyroid epithelioma and skin carcinoma cells where cannabinoids selectively inhibit tumor cells and do not affect their non-transformed counterparts (663, 676).

In contrast high concentrations of cannabinoids can induce apoptosis in non-transformed monocytes, macrophages and lymphocytes leading to defective host anti-tumor responses due to deficiency of cytokines such as interferons- γ and interleukin-12 (665, 669). Nevertheless, long-term surveys of HIV-positive patients do not show any correlation between cannabis smoking or dronabinol use and average T-cell count or AIDS progression (662, 670).

The first and only clinical trial to this date investigating the anti-tumor responses of cannabinoids was conducted by Guzman et al in 2006. The effects of THC were investigated in 9 patients suffering from recurrent glioblastoma multiforme by intratumoral administration of THC. Delivery of the drug was achieved without any apparent psychoactive effects. THC inhibited tumor cell proliferation in vitro and decreased tumor cell Ki67 immunostaining in two patients (687).

Cannabinoids are well tolerated by mice and rats in animal studies and several grams per kilograms of body weight of THC are required to cause lethality in animals. In contrast to many anticancer agents and other drugs fatalities due to cannabis consumption in

humans have not been reported (680). Prolonged administration of high doses of THC in mice and rats did not result in any marked histopathological changes in the brain or other organs and THC increased survival and lowered the incidence of primary tumors (823). Similarly, no alterations in physiological and neurological blood parameters were observed in cannabis smokers or individuals receiving long-term cannabinoid treatment (662, 670, 680). However, the most commonly associated side-effects that limit the use of cannabinoids in therapy are the psychoactive effects even though they are well within the accepted range. Employing drugs that specifically bind to CB2 receptors can significantly reduce the psychotropic effects observed due to CB1 receptor activation. Alternatively, it has been suggested that the use of non-psychotropic compounds such as CBD, dexanabinol and ajulemic acid which inhibit glioma cell growth in vitro and in vivo may also be viable cannabinoid-based anticancer agents (682, 683, 686). Drugs that inhibit degradation of endocannabinoids and significantly increase levels of endocannabinoids at tumor sites have also proven successful as inhibitors of tumor growth with minimal psychotropic effects (679).

Data from clinical trials and animal experiments indicate that cannabinoids are effective anti-cancer agents and relatively safe compared to conventional chemotherapeutics. However, their mechanisms of action are highly complex and not fully understood and there is also evidence for both receptor dependent and independent pathways. Several studies show that cannabinoids induce many of the same responses/pathways observed for anticancer agents that target degradation or suppression of Sp transcription factors.

For example, treatment of LNCaP cells with WIN decreased expression of VEGF and caused downregulation of cyclins D1, D2, E (632, 725). Similarly, WIN treated non-melanoma skin tumors displayed impaired tumor vascularization accompanied by decreased levels of VEGF and inhibition of EGFR function (663). In addition an APC (Min/+) mouse study reveals that loss or inhibition of CB1 receptor promotes intestinal adenoma growth and activation of CB1 receptors inhibit tumor growth by downregulation of the antiapoptotic protein survivin (724). Studies investigating the effects of cannabinoids such as WIN, JWH-133 and AEA on glioma cells have also shown inhibition of VEGF and the receptor VEGFR2 (824).

Therefore we hypothesize that the anticancer activity of CBs is due, in part, to downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes and this will be investigated in the proposed research. Results of these studies will facilitate more targeted clinical applications of CBs alone or in combination therapies.

CHAPTER II

INDUCTION OF APOPTOSIS BY CANNABINOIDS IN PROSTATE AND COLON CANCER CELLS IS PHOSPHATASE-DEPENDENT*

INTRODUCTION

The anticancer activities of phytochemicals and their synthetic derivatives is complex and dependent on tumor-type; however, several studies show that an important component of their activity is associated with induction of specific phosphatases which in turn inhibit kinase signaling pathways that are overexpressed in many tumors. For example, several phytochemical anticancer agents including betulinic acid and other polycyclic terpenoids inhibit STAT3 activation and this has been linked to induction of the protein tyrosine phosphatase SHP-1 (825-831). Curcumin, resveratrol and calcitriol (vitamin D analog) also induce the dual specificity phosphatase mitogen-activated protein kinase phosphatase 5 (MKP5) mRNA levels in prostate cancer cell lines (832, 833) and this response has been linked to inhibition of the p38 stress kinase activity.

Cannabinoids have long been used for ameliorating the debilitating effects of cytotoxic anticancer drugs; however, these compounds also exhibit antitumorigenic activity

*Reprinted with permission from “Induction of apoptosis by cannabinoids in prostate and colon cancer cells is phosphatase-dependent” by Sreevalsan_S, Joseph S, Jutooru I, Chadalapaka G, Safe SH, 2011. *Anticancer Res*, 31 (11) 3799-3807, Copyright [2011] by Anticancer Research: International Journal of Cancer Research and Treatment.

against multiple tumor types (711, 824, 833-835) and cannabinoids are already in clinical trials for treatment of brain tumors (e.g. gliomas). The mechanisms of action of cannabinoids are complex and dependent on ligand structure and cell context, and the effects are both receptor (CB1 and/or CB2)-dependent and -independent. Like many other phytochemical anticancer agents, cannabinoids induce growth inhibitory, pro-apoptotic and antimetastatic responses which are accompanied by modulation of several kinase activities (660, 724, 725, 836-844), and we hypothesized that cannabinoids may also induce phosphatases. This paper demonstrates that the synthetic cannabinoid WIN-55,212-2 (WIN) and cannabidiol (CBD) induced expression of several phosphatase mRNAs in LNCaP and SW480 cells and the phosphatase inhibitor sodium orthovanadate (SOV) inhibited WIN- and CBD-induced apoptosis in colon and prostate cancer cells. However, knockdown of the cannabinoid (CB) receptors (CB1 and CB2) showed that induction of apoptosis by CBD but not WIN was receptor-dependent.

MATERIALS AND METHODS

Chemicals, Antibodies, Plasmids, and Reagents

WIN was purchased from Tocris Bioscience. Cannabidiol was kindly provided by Dr. Norbert. E. Kaminski (Michigan State University, East Lansing, MI). Tyrosine phosphatase inhibitor, SOV, was purchased from Calbiochem. Cleaved poly (ADP-ribose) polymerase (PARP), phospho-c-jun N-terminal kinase (pJNK) and JNK antibodies were obtained from Cell Signaling. β -Actin antibody was purchased from Sigma-Aldrich. Antibodies for phospho-Akt, Akt, phospho-extracellular-signal-

regulated kinases (pERK), ERK, phospho-ErbB-2, ErbB-2, phospho-P38, P38, and phospho-signal transducer and activator of transcription 3 (STAT3) were purchased from Santa Cruz Biotechnology. Chemiluminescence reagents for western blot imaging were purchased from Millipore. RPMI and DMEM media were purchased from Sigma.

Cell Culture

Human LNCaP prostate carcinoma cells were obtained from American Type Culture Collection. Human SW480 colon carcinoma cell line were provided by Dr Stanley Hamilton (M.D. Anderson Cancer Center, Houston, TX). LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100X antibiotic antimycotic solution. SW480 cells were maintained in DMEM/F-12 supplemented with 5% fetal bovine serum and 100X antibiotic antimycotic solution. Cells were maintained at 37°C in the presence of 5% CO₂.

Cell Proliferation Assay

Prostate and colon cancer cells (3×10^4 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 without phenol red containing 2.5% charcoal-stripped FBS, and either vehicle (DMSO) or different concentrations of compounds were added. Cells were then trypsinized and counted after 24, 48 and 72 hr using a Coulter Z1 cell counter. Each experiment was done in triplicate, and results are expressed as means \pm SE for each set of experiments.

Western Blot Analyses

Prostate and colon cancer cells were seeded in DMEM/Ham's F-12 medium. After 24 hr, cells were treated with either vehicle (DMSO) or WIN and CBD for 48 and 24 hr, respectively, or pretreated with SOV (0.25 mM and 0.5 mM) for 40 min and then treated with WIN and CBD. Cells were lysed with high salt buffer and subjected to SDS-PAGE and immunoblotting for cleaved PARP, CB1, CB2 and cleaved caspase 3 proteins as described previously (845).

Real-Time PCR

Prostate and colon cancer cells were seeded and after 24 hr, were treated with either vehicle (DMSO) or WIN and CBD for 24 hr. Total RNA was isolated using the RNeasy Protect Mini kit according to the manufacturer's protocol. cDNA was prepared and subjected to real-time PCR analysis as described previously (845). MKP-1(forward-5'-CTCCATGCTCCTTGAGAGGAGAAATGC-3'; reverse-5'-GGTAGGTATGTCAAGCATGAAGAG-3)', MKP-2 (forward 5'-TACAAGTGCATCCCAGTGGA-3'; reverse 5'-CCCGTTTCTTCATCATCAGG-3'), c-PacP (forward 5'-TCTCAGTGGTGCCGCATCTA-3'; reverse 5'-CAGGGTGTGAGGATGGCAA-3'), s-PacP (forward 5'-GGCAGATGATGCTTTGAGAACA-3'; reverse 5'-TCATCCAAAGCCCATTTTCC-3'), PTEN (forward 5'-CGAACTGGTGTAATGATATGT-3'; reverse 5'-CATGAACTTGTCTTCCCGT-3'), DEP-1 (forward 5'-TCGTTTCGTGACTACATGAAGCA-3'; reverse 5'-CCCCAGCACTGCAATGC-3'),

SHP-1 (forward 5'-AATGCGTCCCATACTGGCCCGA-3'; reverse 5'-CCCGCAGTTGGTCACAGAGT-3'), PTP1B (forward 5'-TCCTACCTGGCTGTGATCGAG-3'; reverse 5'-CCTTCCACTGATCCTGCACTG-3'), PP2A (forward 5'-GTTGGGAGGTGGCAGTGAG-3'; reverse 5'-AAACACTGGCCTCTGGTGTC-3') primers were acquired from Sigma. MKP-5 (forward 5'-GCTCAGGACCTGGACACCAT-3'; reverse 5'-GGAAGATGAGTGGTGACGTTGAT-3'); primers were acquired from IDT. Values for each gene were normalized to expression levels of TATA-binding protein.

siRNA Interference Assay

LNCaP and SW480 cells were seeded (1×10^5 per well) in six-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal-stripped FBS without antibiotic and left to attach for 24 hr. The CB1 and CB2 knockdown (iCB1 and iCB2) along with iLamin as control was done using Lipofectamine 2000 reagent according to the manufacturer's instructions. Small inhibitory RNAs were purchased from Sigma-Aldrich.

Symbol	Name	Synonyms
DUSP1	Dual specificity phosphatase 1	MKP1
DUSP4	Dual specificity phosphatase 4	MKP2
DUSP6	Dual specificity phosphatase 6	MKP3
DUSP10	Dual specificity phosphatase 10	MKP5
ACPP	Prostatic acid phosphatase, cellular and serum	cPACP, sPACP
ERBB2	Human epiderman growth factor receptor-2	HER2
PTEN	Phosphatase and tensin homolog	PTEN
PTPRJ	Protein tyrosine phosphatase, receptor type, J	DEP1
PTPN6	Protein tyrosine phosphatase, non-receptor type 6	SHP1
PTPN1	Protein tyrosine phosphatase, non-receptor type 1	PTP1B
PPP2R4	Protein phosphatase 2A activator, regulatory subunit 4	PP2A
STAT3	Signal transducer and activator of transcription 3	STAT3

Table 2. List of the symbols, names and synonyms of genes discussed in this study.

RESULTS

WIN and CBD Induce Apoptosis and Inhibit Proliferation of LNCaP and SW480 Cells

Results summarized in Figures 21A and 21B show that WIN and CBD inhibited LNCaP cell growth, and IC_{50} values for half-maximal growth inhibition after 2 days were 5.1 and 10.0 μ M and, after 3 days, were 4.59 and 9.43 μ M, respectively. Similar results were observed in SW480 cells (Figs. 21C and 21D); IC_{50} values (for days 2 and 3) were 3.3 and 3.5 μ M for WIN and 5.95 and 5.06 μ M for CBD. The concentration-dependent effects of CBD on cell proliferation were observed within 24 hr after treatment, whereas the growth inhibitory effects of WIN were somewhat delayed in both cell lines. WIN and CBD also induced a time-dependent increase in PARP cleavage, a marker of

apoptosis, in LNCaP and SW480 (Fig. 22) cells. Maximal induction by CBD was observed within 12 hr after treatment in both cell lines, whereas WIN-induced PARP cleavage was maximal after 24 hr.

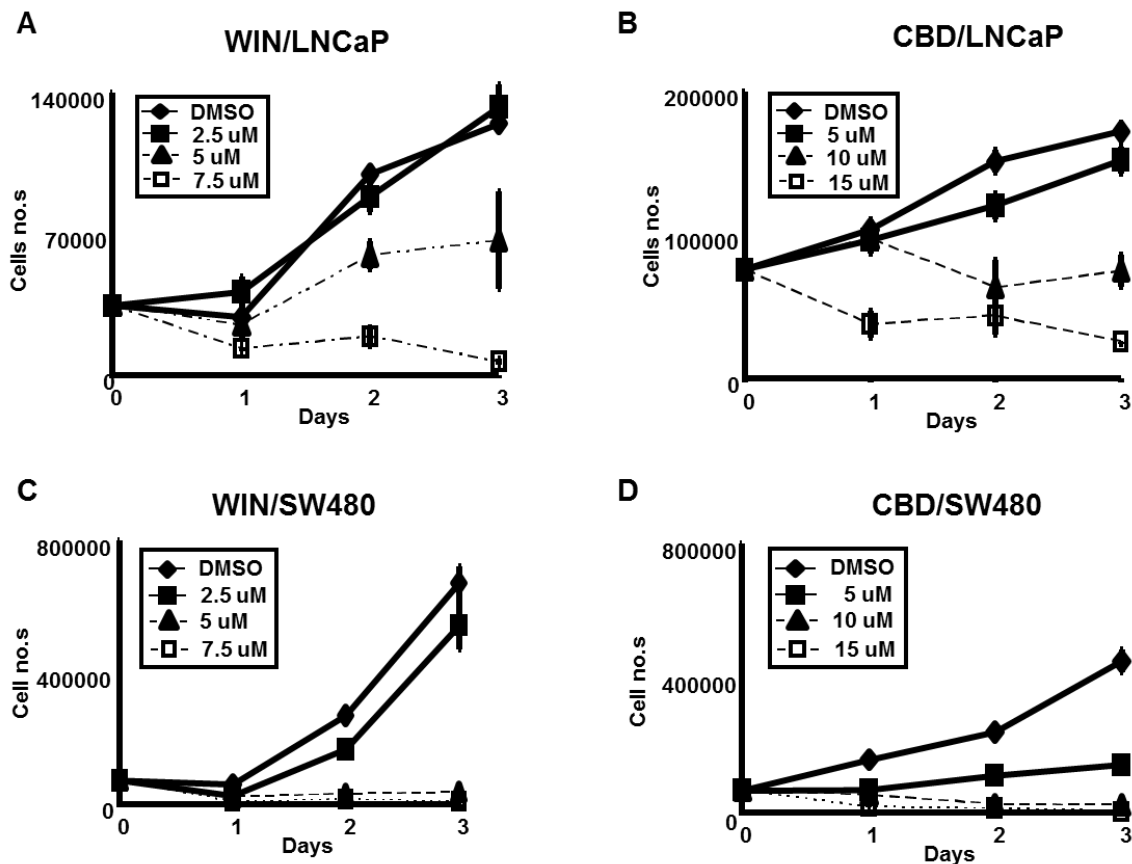


Figure 21. Effects of cannabinoids on LNCaP and SW480 cell proliferation. Concentration-dependent effects of WIN and CBD on LNCaP (A, B) and SW480 (C, D) cell proliferation. Cells were treated with DMSO and 2.5-7.5 μ M of WIN and 5-15 μ M of CBD for 72 hr. Cells were counted as described in Materials and Methods. Results are expressed as mean \pm S.E. for three replicate determinations for each treatment group, and significantly ($p < 0.05$) decreased proliferation is indicated (*). Proliferation of cells treated with DMSO (solvent control) was set at 100%. β -Actin served as a loading control for all Western blots.

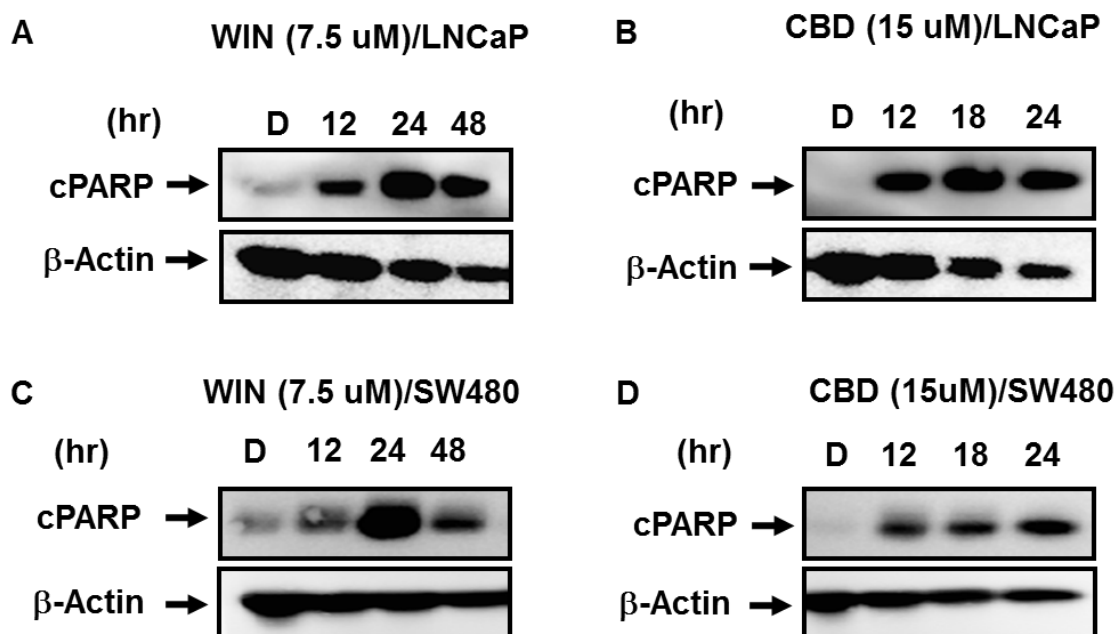


Figure 22. Induction of apoptosis by WIN and CBD. Time-dependent effects of WIN and CBD on cleaved PARP expression in LNCaP (A, B) and SW480 (C, D) cells. Cells were treated with DMSO, 7.5 μ M WIN (12, 24 and 48 hr) and 15 μ M CBD (12, 18, and 24 hr), and cPARP expression was determined by western blot analysis of whole cell lysates as described in Materials and Methods.

WIN and CBD Induce Phosphatases in LNCaP and SW480 Cells

Figure 23 summarizes the effects of WIN and CBD on induction of phosphatase mRNA levels in LNCaP cells. WIN induced MKP-1 and MKP-5 and CBD induced MKP-1, MKP-2 and MKP-5 mRNAs after 24 hr. Similar results were observed at earlier time points, and WIN also induced MKP-2 after 48 hr (data not shown). In contrast, none of the selected protein tyrosine phosphatases were induced by WIN or CBD in LNCaP

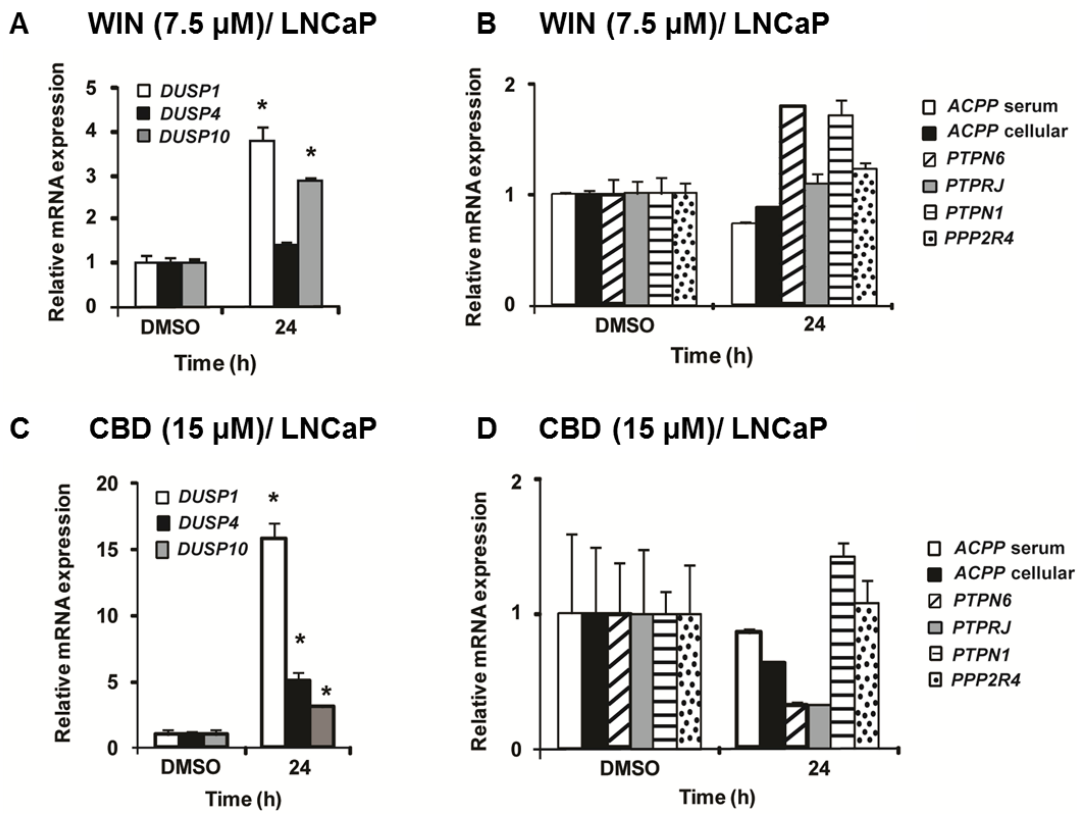


Figure 23. Effects of WIN and CBD on phosphatase mRNA levels in LNCaP cells. Induction by WIN (A, B) and CBD (C, D). Cells were treated with DMSO, 7.5 μM WIN and 15 μM CBD for 24 hr, and MKP-1, MKP-2, MKP-5, s-PacP, c-PacP, PTP1b, PP2A, DEP-1 and SHP-1 mRNA levels were determined by real time PCR as described in the Materials and Methods. Results are expressed as mean ± S.E. for 3 replicate experiments and significant (p<0.05) increases are indicated (*).

cells. In SW480 cells, WIN did not induce the dual specificity phosphatases but induced s-PacP and SHP-1 after treatment for 24 hr (Fig. 24); however, after 48 hr, induction of c-PacP, MKP-1 and MKP-5 by WIN were also observed (data not shown). CBD induced MKP-1, MKP-5, s-PacP, p-PacP and SHP-1 in SW480 cells after treatment for

24 hr. These results demonstrate that like other anticancer agents, cannabinoids induce expression of several dual specificity phosphatase and protein tyrosine phosphatase mRNAs, and current studies are focused on the induction and/or activation of individual phosphatase proteins and their functions in cancer cells.

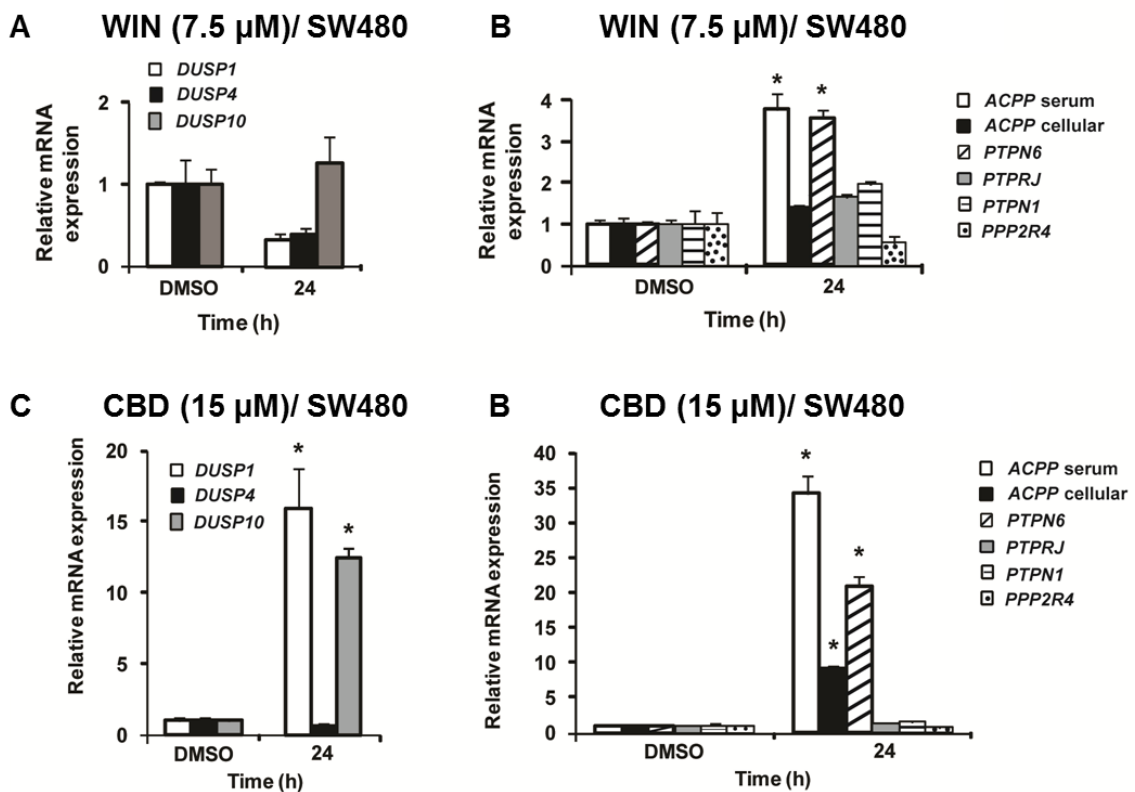


Figure 24. Effects of WIN and CBD on phosphatase mRNA levels in SW480 cells. Induction by WIN (A, B) and CBD (C, D). Cells were treated with DMSO, 7.5 μ M WIN and 15 μ M CBD for 24 hr, and MKP-1, MKP-2, MKP-5, s-PAcP, c-PAcP, PTP1b, PP2A, DEP-1 and SHP-1 mRNA levels were determined by real time PCR as described in the Materials and Methods. Results are expressed as mean \pm S.E. for 3 replicate experiments and significant ($p < 0.05$) increases are indicated (*).

The effects of WIN- and CBD-induced phosphatases on dephosphorylation of p42/44MAPK, pAKT, pSTAT3, pJNK, pErbB2, and pP38MAPK were investigated in LNCaP and SW480 cells and with few exceptions, the data were difficult to interpret since both phospho and total kinase protein levels were decreased simultaneously (Figure 25). This suggested that cannabinoid-induced phosphatases decreased multiple kinase activities but also activated other pathways that affect expression of their corresponding proteins (total). The mechanisms associated with decreased kinase proteins are currently being investigated.

WIN- and CBD-Induced Apoptosis is Phosphatase-Dependent: Role of CB Receptors is Ligand-Dependent

Preliminary studies on the effects of phosphatase inhibitor cocktails and their components on CBD- and WIN-induced growth inhibition and apoptosis showed that SOV was the most effective inhibitor and this was consistent with CBD- and WIN-dependent induction of dual-specificity and protein tyrosine phosphatases which are inhibited by SOV. WIN and CBD induced PARP cleavage as early as 12 hr after treatment, and PARP cleavage induced by WIN and CBD persisted for 48 and 24 hr, respectively (Fig. 23). Since near maximal induction of PARP cleavage by WIN and CBD was observed after treatment of SW480 and LNCaP cells for 24 or 12 hr, respectively, and these time points were used for investigating the effects of SOV on WIN- and CBD-induced PARP cleavage. Cells were treated with the cannabinoids

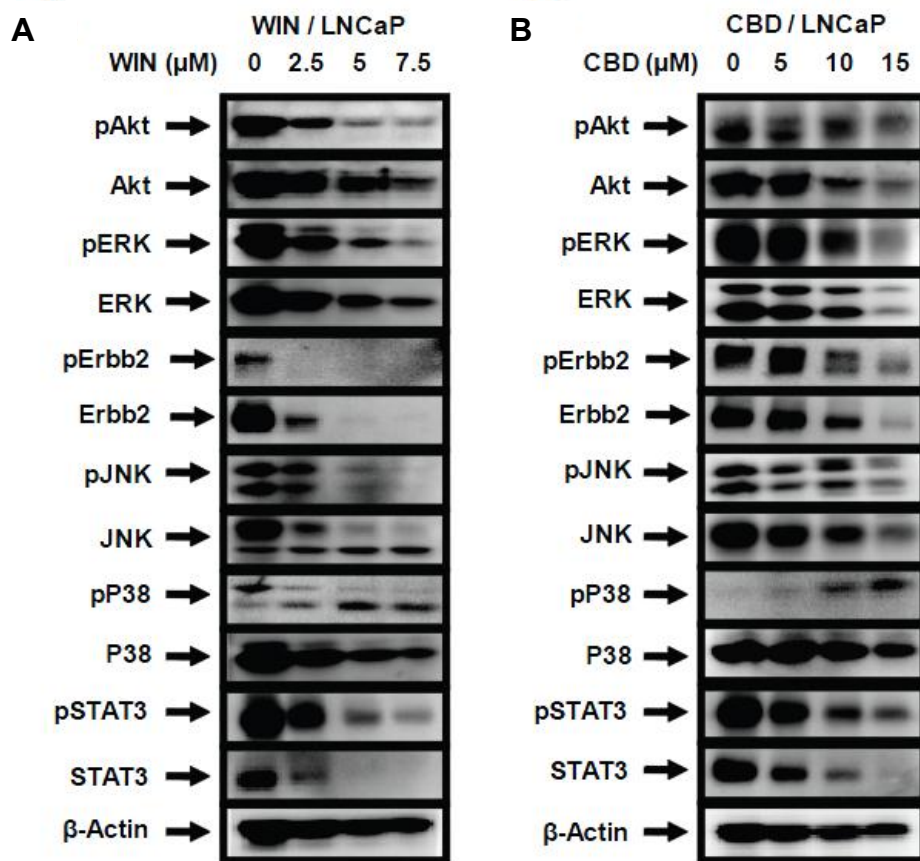


Figure 25. Effects of WIN and CBD on phospho and total kinase proteins in LNCaP (A, B) and SW480 (C, D) cells. Cells were treated with varying concentrations of WIN (2.5, 5 or 7.5 μM) and CBD (5, 10 or 15 μM) for 48 and 24 hr, respectively, and whole cell lysates were analyzed by western blot analysis as described in Materials and Methods. β-Actin served as a loading control for all western blots.

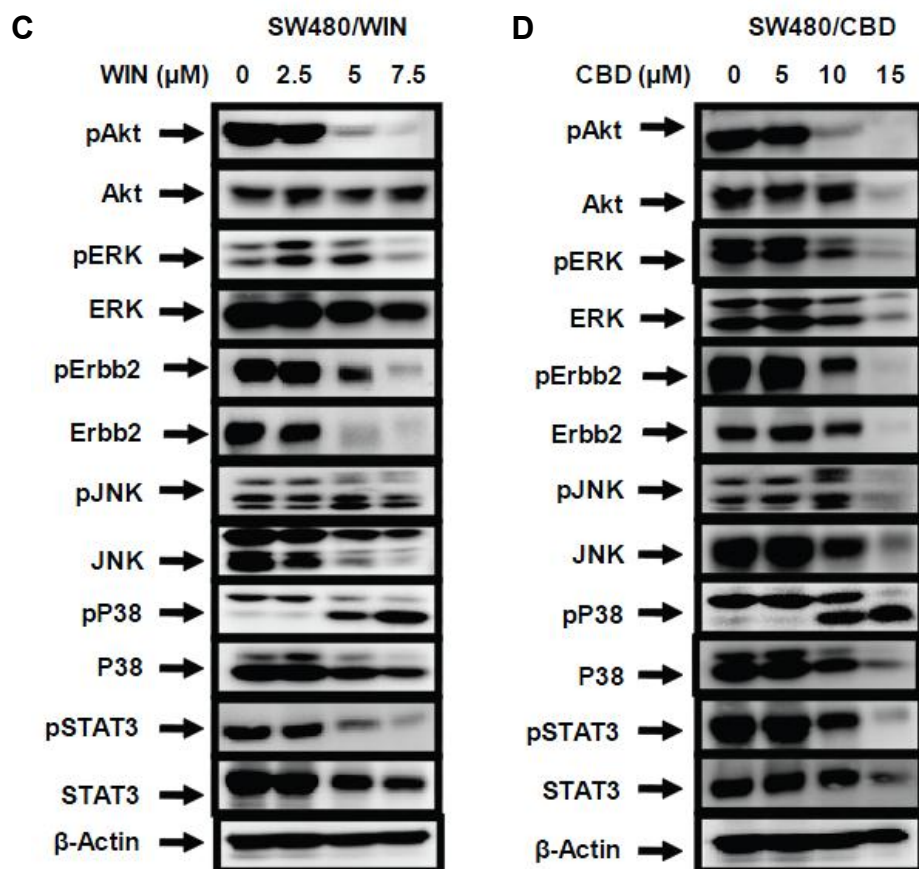


Figure 25. Continued

alone and in combination with SOV. The phosphatase inhibitor SOV significantly decreased WIN- and CBD-induced PARP cleavage in LNCaP (Fig. 26A) and SW480 (Fig. 26B) cells, demonstrating that the CBD- and WIN-induced phosphatase activities play an important role in the pro-apoptotic activity of these compounds. Receptor-dependent and -independent induction of apoptosis by WIN and CBD was investigated in LNCaP and SW480 cells treated with the cannabinoids alone or after transfection with small inhibitory RNAs (siRNAs) targeting CB1 and CB2 receptors. CBD-induced

PARP and caspase-3 cleavage in LNCaP cells was inhibited after knockdown of the CB2 receptor (Fig. 26C), whereas after CB1 receptor knockdown, CBD-induced caspase activation was partially inhibited but PARP cleavage was not

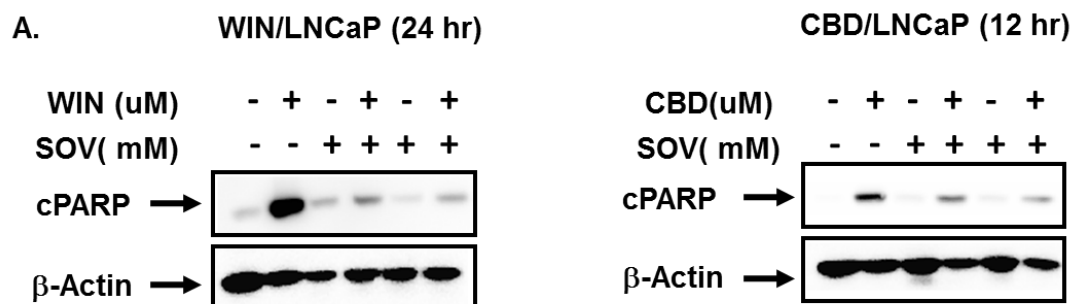


Figure 26. Effects of phosphatase inhibitor and cannabinoid receptor knockdown on cannabinoid-mediated induction of cPARP by WIN and CBD in LNCaP and SW480 cells. Effects of phosphatase inhibitor sodium orthovanadate (SOV) on cannabinoid-induced PARP cleavage in LNCaP (A) and SW480 (B) cells. Cells were pre-treated with SOV followed by treatment with WIN and CBD for 24 and 12 hr, respectively, and whole cell lysates were analyzed by western blot analysis as described in Materials and Methods. β -Actin served as a loading control for all western blots. Effects of cannabinoids and CB1 and CB2 receptor knockdown by RNA interference in LNCaP (C) and SW480 (D) cells. Cells were transfected with siRNAs against CB1 and CB2 receptors followed by treatment with CBD for 12 hr and whole cell lysates were examined for expression of cPARP, cleaved caspase3, and CB1 and CB2 receptor proteins were determined by western blot analysis as indicated in the Materials and Methods. β -Actin served as a loading control for all Western blot analyses.

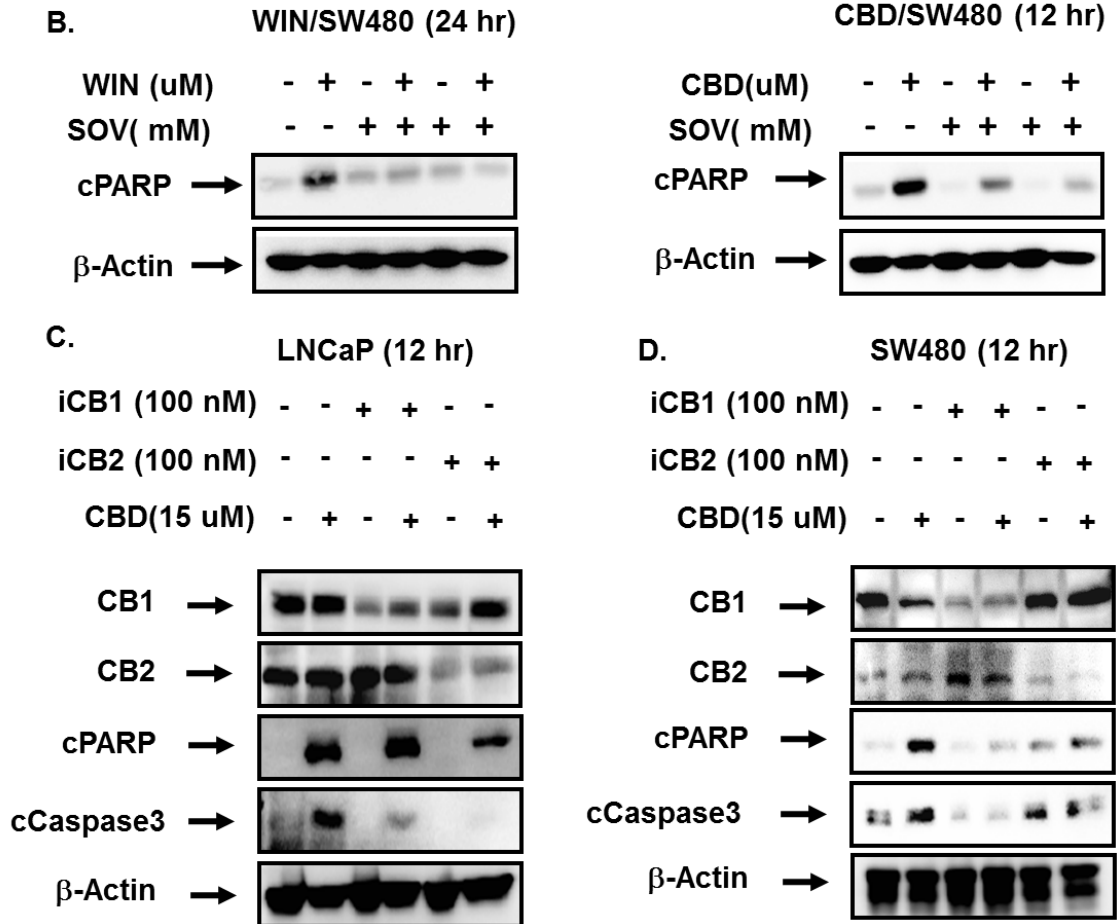


Figure 26. Continued

affected. In SW480 cells, both CB1 and CB2 receptor knockdown inhibited CBD-induced PARP cleavage and caspase-3 activation (cleavage) (Fig. 26D). In contrast, WIN-induced apoptosis and caspase-3 cleavage was unaffected by knockdown of CB1 or CB2 receptors in LNCaP or SW480 cells (data not shown). Thus, the pro-apoptotic activities of WIN and CBD were phosphatase-dependent in both cell lines but

cannabinoid receptor-independent and -dependent, respectively, in LNCaP and SW480 cells.

DISCUSSION

Cannabinoids have emerged as an important new class of anticancer drugs that bind cannabinoid receptors and activate several downstream pathways leading to inhibition of cancer cell proliferation, and induction of apoptosis (711, 824, 834, 835). Their mechanisms of action are complex and dependent on cell context and ligand structure and there are examples of cannabinoid-mediated activities that are receptor-dependent and –independent (660, 724, 725, 836-843). Several studies show that cannabinoids such as WIN, Δ THC and CBD modulate kinase activities in cancer cell lines and this includes inhibition of membrane-bound and intracellular kinases resulting in their dephosphorylation. The mechanisms associated with cannabinoid-induced inactivation of kinases is unknown; however, several different classes of anticancer drugs induce dual specificity and protein tyrosine phosphatases which selectively inactivate phospho-kinases that play a role in cancer cell growth and survival (825-832, 846-852).

Previous studies on the effects of cannabinoids on activated phospho-kinases are highly variable in various cancer cell lines and dependent on the structure of the cannabinoid and cell context. WIN inhibited growth and induced apoptosis in LNCaP cells and this was due in part to sustained phosphorylation of p42/44 MAPK and decreased p-AKT expression (842). The phytochemical Δ THC inhibited growth and induced apoptosis in

SW480 cells and this was accompanied by decreased expression of phospho-p42/p44 MAPK and phospho-AKT (840).

Although the reported effects of cannabinoids on phospho-kinases were highly variable among different cell lines, we observed that WIN and CBD induced expression of several dual specificity and protein tyrosine phosphatase mRNAs in LNCaP and SW480 cells (Figs. 23 and 24). Selection of the phosphatases was based on their putative anticarcinogenic activities and on previous reports showing that dual specificity and protein tyrosine phosphatases were induced by several phytochemical anticancer agents including curcumin, resveratrol, ursolic acid, betulinic acid, guaiane sesquiterpenoids, guggulsterone, epigallocatechin-3-gallate and retinoic acid (825-832, 846-852). Previous studies with these compounds primarily focused on induction of a single phosphatase gene, whereas results of this study showed that WIN and CBD induced multiple and overlapping phosphatase mRNAs (Figs. 23 and 24). WIN and CBD induced MKP-1 and MKP-5 after treatment for 24 hr in LNCaP cells, whereas MKP-2 induction by CBD and WIN was time-dependent (24 and 48 hr, respectively) (Fig. 23).

In SW480 cells, the pattern of phosphatase induction by WIN and CBD differed after treatment for 24 hr primarily due to induction of MKP-1 by CBD and not WIN (Fig. 24); however, after 48 hr, WIN also induced MKP-1 (data not shown). Since both CB receptor antagonists alone induced phosphatase mRNAs (data not shown), the role of the CB receptor in mediating induction of individual phosphatase mRNAs was not further

investigated. In addition, the effects of CBD- and WIN-dependent induction of dual specificity and protein tyrosine phosphatases gene expression on dephosphorylation of phospho-kinase proteins was confounded by the parallel decrease of most phospho-kinase and total kinase proteins (Fig. 25). It was apparent in SW480 cells that WIN dramatically decreased AKT phosphorylation without decreasing total AKT protein and this was comparable to the effects reported for Δ THC in SW480 cells (840). The only other consistent observation was that WIN and CBD enhanced phosphorylation of the p38 stress protein kinase in both cell lines (Fig. 25). These results demonstrate that WIN-/CBD-induced phosphatases may play a role in kinase inactivation in SW480 and RKO cells; however, these results were confounded by the parallel decrease in kinase proteins, and the mechanisms of the latter response are currently being investigated.

CBD and WIN also inhibited LNCaP and SW480 cell growth (Fig. 21) and induced apoptosis (Fig. 22) in both cell lines, and induction of apoptosis by cannabinoids is an important element of their anticancer activity (830-833). Induction of caspase-dependent PARP cleavage was used as an indicator of apoptosis and it was clear that both WIN and CBD induced PARP cleavage in LNCaP and SW480 cells (Fig. 22) and this is consistent with cannabinoid-induced apoptosis in many other cancer cell lines (660, 711, 724, 725, 824, 834-843). Moreover, we also observed that WIN-/CBD-induced PARP cleavage was dramatically decreased in cells cotreated with the phosphatase inhibitor SOV (Figs. 26A and 26B), indicating that cannabinoid-induced phosphatases played a role in the induction of SW480 and LNCaP cell death. It should

be noted that although induction of PARP cleavage by WIN is maximal after 24 hr, there was significant induction within 12 hr in LNCaP and SW480 cells (Fig. 22). In contrast, induction of phosphatase mRNA levels after treatment with WIN for 12 hr is relatively low in SW480 cells, whereas modest but significant induction was observed in LNCaP cells (data not shown). These results suggest that other “proapoptotic” phosphatases may also be induced or activated by WIN and CBD and these are currently being investigated. CB1 and CB2 receptor knockdown by RNAi was used to determine the role of the CB receptors in mediating CB- and WIN-induced proapoptotic activity (cleavage of PARP and caspase 3) and the results demonstrate that the effects of WIN were CB receptor-independent, whereas CBD-induced apoptosis was CB receptor-dependent (Fig. 26C and 26D).

In summary, this study shows for the first time that cannabinoids induced several phosphatase mRNAs in LNCaP and SW480 cells and this was consistent with induction of phosphatases by other phytochemical anticancer drugs (825-832, 846-852). The direct effects of phosphatase induction on decreased phospho-kinase proteins was confounded by a parallel decrease in most (total) kinase proteins. This response may also be important for the anticancer activities of WIN and CBD and is currently being investigated. Previous studies have reported induction of MKP-1 and MKP-3 by cannabinoids in microglial cells and this resulted in inactivation of MAPK; however, effects on apoptosis were not determined (769, 853). The results obtained using the phosphatase inhibitor SOV demonstrate that induction of apoptosis by WIN and CBD

was significantly blocked by the phosphatase inhibitor SOV, and this represents a novel proapoptotic pathway induced by cannabinoids. Current studies are focused on identification of specific proapoptotic phosphatases and their mechanisms of induction or activation by cannabinoids.

CHAPTER III

THE CANNABINOID WIN 55,212-2 DECREASES SPECIFICITY PROTEIN (SP) TRANSCRIPTION FACTORS AND THE ONCOGENIC CAP PROTEIN EIF4E IN COLON CANCER CELLS

INTRODUCTION

There are three major classes of cannabinoids which include plant-derived compounds such as $\Delta(9)$ -tetrahydrocannabinol (THC) and cannabidiol (CBD), endogenous cannabinoids (anandamide and 2-arachidonylglycerol), and synthetic compounds that mimic the effects of cannabinoids (773, 834). Cannabinoids bind the CB1 and CB2 receptors which are differentially expressed in some but not all tissues, and there is also evidence that some cannabinoids also interact with other G-protein coupled receptors (GPCR) including the receptor transient receptor potential vanilloid type 1 (TRPV1) and GPR55 (747, 854, 855). THC is the main psychoactive cannabinoid found in the marijuana plant *Cannabis sativa L*; however, in addition to the psychoactive effects of THC, endocannabinoids and synthetic cannabinoids also play a role in energy metabolism, pain and inflammation, cardiovascular, musculoskeletal and respiratory disorders, and cancer, and opportunities for cannabinoid-based pharmacotherapies are extensive (773).

The anticancer activities of cannabinoids have been known for over 3 decades, and clinical trials for treatment of gliomas with cannabinoids have been reported (674). Cannabinoids inhibit growth, induce apoptosis, and exhibit antimetastatic and antiangiogenic activities in multiple cancer cell lines and inhibit tumor growth in *in vivo* mouse models (711, 824, 835, 856). The effects of cannabinoids are complex and dependent on ligand structure and cell context, and the responses can also be CB (CB1 and/or CB2) receptor-dependent or -independent (711, 824, 835, 856). For example, the synthetic mixed CB1 and CB2 receptor agonist 2,3-dihydro-5-methyl-3-([morpholinyl]methyl)pyrrolo(1,2,3-de)-1,4-benzoxazinyl]-[1-naphthaleny]methanone [WIN 55, 212-2 (WIN)] inhibited gastric cancer cell growth and decreased VEGF expression and these responses were blocked in cells cotreated with WIN plus CB receptor antagonists (844), and similar CB receptor-dependent responses were observed in mantle cell lymphoma (857). In contrast, WIN induced phosphatase-dependent apoptosis in SW480 colon and LNCaP prostate cancer cells and these responses were inhibited by the phosphatase inhibitor sodium orthovanadate (SOV) but not by CB receptor antagonists or by CB receptor knockdown (858).

Treatment of cancer cells with cannabinoids activates or inactivates various kinases, ceramide synthesis and ceramide-mediated proapoptotic and stress-related genes, and downregulates expression of epidermal growth factor receptor (EGFR), survivin, cyclin D1, bcl-2 and vascular endothelial growth factor (VEGF) (562, 671, 725, 836, 838, 840, 844, 857-860). Previous RNA interference studies have shown that these genes are also

regulated by one or more of the specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 which are overexpressed in colon and other cancer cell lines and tumors (641, 672, 673, 861-865). Therefore, we hypothesized that the anticancer activity of cannabinoids such as WIN in colon cancer cells may also be due, in part, to downregulation of Sp proteins and this could also be related to the reported induction of phosphatases by cannabinoids in these cells (858). Results of this study show that WIN induced protein phosphatase 2A (PP2A)-dependent downregulation of Sp1, Sp3, Sp4 and Sp-regulated gene products including the pro-oncogenic cap protein eIF4e in colon cancer cells. These responses were CB receptor-independent and involved disruption of microRNA-27a (miR-27a)-mediated suppression of the zinc finger transcriptional repressor ZBTB10 which acts as an Sp repressor (641, 672, 684, 865).

MATERIALS AND METHODS

Chemicals, Antibodies, and Reagents

WIN 55,212-2 mesylate (1038), AM251 (1117) and AM630 (1120) were purchased from Tocris Bioscience (Ellisville, MO, USA). Tyrosine phosphatase inhibitor, sodium orthovanadate, was purchased from Calbiochem (La Jolla, CA, USA). Dithiothreitol (DTT) was obtained from Boehringer Mannheim Corp, (Indianapolis, IN). Gliotoxin was kindly provided by Dr. Alan Taylor (National Research Council of Canada, Halifax, NS, Canada). p-EIF4e (S209), eIF4e (9742S) and Cleaved poly (ADP-ribose) polymerase (PARP) (9541) antibodies were obtained from Cell Signaling (Danvers, MA). Lactacystin, glutathione (GSH) and antibodies for β -Actin (A1978), CB1 (C1233)

and CB2 (WH0001269M1) receptors were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for EGFR (1005), Sp3 (D-20) and Sp4 (V-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PP2A Immunoprecipitation Phosphatase Assay Kit (17-313), Immobilon Western Chemiluminescent HRP substrate (WBKLS0500) and antibodies for Sp1 (07-645) and PP2A (05421) were purchased from EMD Millipore (Billerica, MA, USA). Cyclin D1 (2261-1) and survivin (2463-1) antibodies were purchased from Epitomics (Burlingame, CA). VEGFR1 (ab32152) antibody was purchased from Abcam (Cambridge, MA). Antibody for VEGF (209-403-B99) was purchased from Rockland Antibodies and Assays (Gilbertsville, PA). ZBTB10 (A303 258A) antibody was purchased from Bethyl Laboratories (Montgomery, TX). Apoptotic, Necrotic and Healthy Cells Quantification Kit (30018) was purchased from Biotium (Hayward, CA). *In situ* cell death detection POD kit (11684817910) was obtained from Roche (Mannheim, Germany). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Sigma (St. Louis, MO).

Cell Lines

Human SW480 colon carcinoma cell line was provided by Dr. Stanley Hamilton (M.D. Anderson Cancer Center, Houston, TX). RKO and HT-29 colon carcinoma cell lines were obtained from ATCC (Manassas, VA). Cells were purchased more than 6 months ago and were not further tested or authenticated by the authors. Cells were maintained at 37°C in the presence of 5% CO₂ as described (858).

Cell Proliferation Assay

Colon cancer cells (1×10^5 per well) were seeded 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 varying concentrations of WIN was added. Cells were then trypsinized and counted after 24 and 48 hr using a Coulter Z1 cell counter (Sykesville, MD). Each experiment was performed in triplicate, and results were expressed as means \pm SE for each set of experiments.

Annexin V Staining Assay

SW480 and RKO cells were seeded in 2 well Nunc Lab-tek chambered coverglass plates from Thermo Scientific (Waltham, MA) and were 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 DMSO or WIN was added. Cells were stained after 9 hr with FITC-Annexin-V, propidium iodide and DAPI dyes according to the manufacturer's protocol and were visualized under EVOS fl, fluorescence microscope, from Advanced Microscopy Group (Bothell, WA) using a multiband filter set for FITC, rhodamine and DAPI. The proportion of apoptotic cells was determined by the amount of green fluorescence observed.

Terminal Deoxyribonucleotide Transferase-Mediated Nick-End Labeling (TUNEL) Assay

HT-29 (7×10^4) were seeded in four-chambered glass slides and left overnight to attach. After treatment with WIN for 48 hr, the in situ cell death detection POD kit was used for the terminal deoxyribonucleotide transferase-mediated nick-end labeling (TUNEL) assay according to the manufacturer's protocol. The percentage of apoptotic cells was determined by counting stained cells from eight fields, each containing 50 cells.

Western Blot Analyses

Colon cancer cells were seeded in DMEM/Ham's F-12 medium and were allowed to attach for 24 hr. Cells were treated with either DMSO or WIN for indicated time periods or pretreated with the proteasome inhibitors, antioxidants, phosphatase inhibitors and cannabinoid receptor antagonists, and then treated with WIN. Cells were lysed and analyzed by western blots as described (858).

Small Inhibitory RNAs (siRNA) Interference Assay

Colon cancer cells were seeded (2×10^5 per well) in six-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal stripped FBS without antibiotics and left to attach for 24 hr. siRNAs specific for CB1 and CB2 receptors, PP2A and ZBTB10 along with iLamin/iGL2 as control were transfected using Lipofectamine 2000 reagent according to the manufacturer's protocol. Small interfering RNAs for CB1 and CB2

receptors, PP2A and ZBTB10 were obtained from Sigma (St. Louis, MO). Lipofectamine 2000 was purchased from Life Technologies (Grand Island, NY).

Quantitative Real-Time PCR

SW480 colon cancer cells were plated (2×10^5) and left to attach for 24 hr. Cells were treated with either vehicle or CBD for 24 hr. MicroRNA was isolated using the mirVana miRNA isolation kit from Ambion-Life Technologies (Grand Island, NY) according to the manufacturer's protocol. cDNA was prepared using the Taqman MicroRNA Reverse Transcription kit and was subjected to quantitative real-time PCR with specific primers for miR27a using the Taqman Universal PCR Master Mix from Applied Biosystems. Quantification of miRNA (RNU6B and miRNA-27a) was done using the Taqman miRNA kit (Applied Biosystems) according to the manufacturer's protocol in the CFX384 Real-Time PCR Detection System from Biorad (Hercules, CA). U6 small nuclear RNA was used as a control to determine relative miRNA expression. Primers for RNU6B and miR27a were purchased from Applied Biosystems. Total RNA was isolated using the RNeasy Protect Mini kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR was carried out with the SYBR Green PCR Master Mix from Applied Biosystems on a CFX384 Real-Time PCR Detection System from Biorad (Hercules, CA) using 0.5 $\mu\text{mol/L}$ of each primer and 1 μL cDNA template in each 10 μL reaction. TATA binding protein (TBP) was used as an exogenous control to compare the relative amount of target gene in different samples. The PCR

profile was as follows: one cycle of 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The comparative C_T method was used for relative quantitation of samples. Primers were purchased from Sigma (St. Louis, MO).

The following primers were used:

TBP, forward, 5'-TGCACAGGAGCCAAGAGTGAA-3';

TBP, reverse, 5'-CACATCACAGCTCCCCACCA-3'

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

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

PP2A Phosphatase Assay

Cells were seeded (3×10^5) and left to attach for 24 hr. Cells were treated with DMSO, okadaic acid, and 5 and 7.5 μ M WIN. Cells were harvested and lysed using the high salt lysis buffer. The lysates were then subjected to buffer exchange using the Zeba Desalt Spin Columns (8981) from Thermo Scientific to eliminate any contaminating phosphates that could skew the experimental results. The lysates were then immunoprecipitated with anti-PP2A, C subunit, antibody and were incubated in a mixture of diluted phosphopeptide and serine/threonine assay buffer for 10 min at 30°C in a shaking incubator. The phosphatase activity was determined by addition of malachite green dye and comparing the absorbance between controls and treatment at 650 nm in a plate reader.

RESULTS

Treatment of SW480, RKO and HT-29 colon cancer cells with 2.5, 5.0 and 7.5 μM WIN for 24 or 48 hr significantly inhibited cell proliferation at the two higher concentrations (Fig. 27A – 27C). In addition, WIN also induced Annexin V staining in SW480 and RKO cells within 9 hr after treatment, indicating rapid induction of early apoptosis, whereas Annexin V was not induced in HT-29 cells at the early time point (data not shown). In contrast, TUNEL staining was increased in HT-29 cells treated with WIN for 48 hr, indicating differences between the timing of WIN-induced apoptosis in SW480/RKO vs. HT-29 cells. Figure 27D shows that treatment with WIN for 48 hr also induced PARP cleavage in all three colon cancer cell lines as previously reported for WIN in SW480 cells (858).

WIN-induced growth inhibition and apoptosis was also accompanied by decreased expression of growth promoting (EGFR, cyclin D1) and survival (survivin) genes products in SW480, RKO and HT29 cells (Fig. 28A). Treatment of SW480, RKO and HT29 cells with 2.5 – 7.5 μM WIN decreased levels of VEGF and VEGFR1 protein in the 3 cell lines, although the effects in HT-29 cells were less than observed in the other two cell lines (Fig. 28B). Treatment of SW480, RKO and HT-29 cells with 2.5, 5.0 or 7.5 μM WIN for 48 hr decreased expression of Sp1, Sp3 and Sp4 proteins as indicated by quantitation of the band intensities (relative to β -actin) (Fig. 28C).

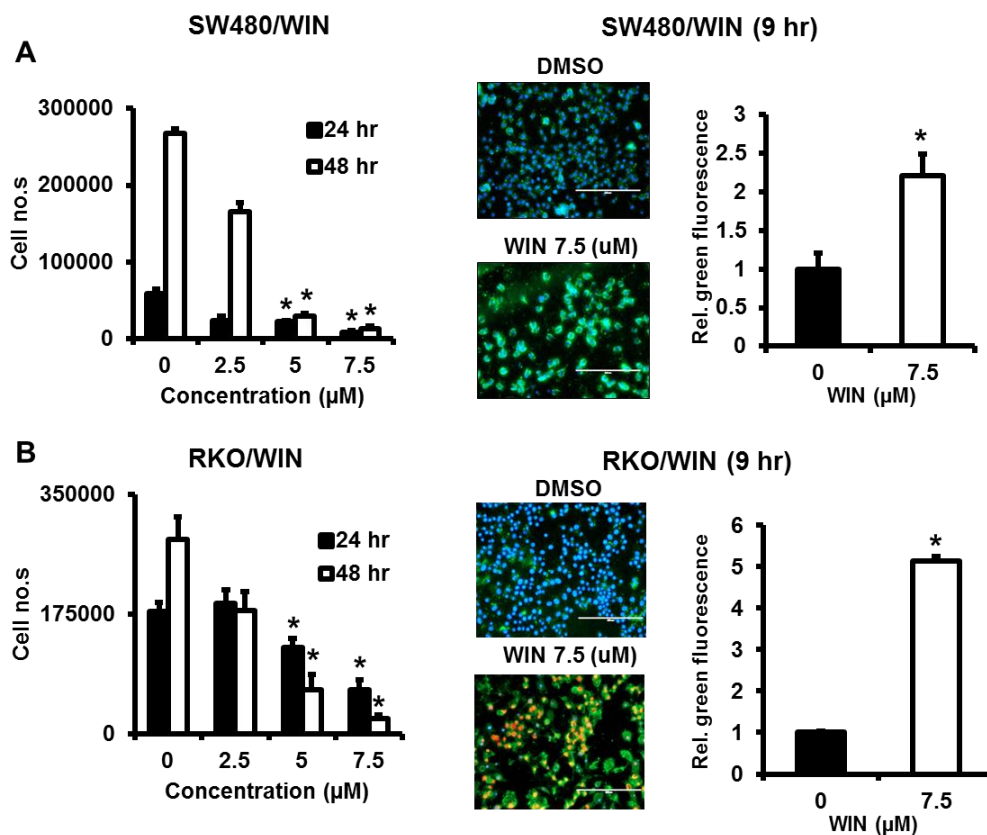


Figure 27. WIN inhibits colon cancer cell growth, induces apoptosis and PARP cleavage. SW480 (A), RKO (B) and HT-29 (C) cells were treated with DMSO and 2.5-7.5 μM WIN for 24 and 48 hr and counted, or treated with DMSO and 7.5 μM of WIN for 9 hr (A and B) and 48 hr (C) and effects on Annexin V (SW480 and RKO) and TUNEL (HT-29) staining was determined. Results are expressed as means ± SE for at least three separate determinations, and significant ($p < 0.05$) induction (*) or inhibition (**) by WIN is indicated. (D) Cells were treated with DMSO and 2.5-7.5 μM of WIN for 48 hr and whole cell lysates were examined for expression of cleaved PARP by western blots. β-Actin served as a loading control for all western blots.

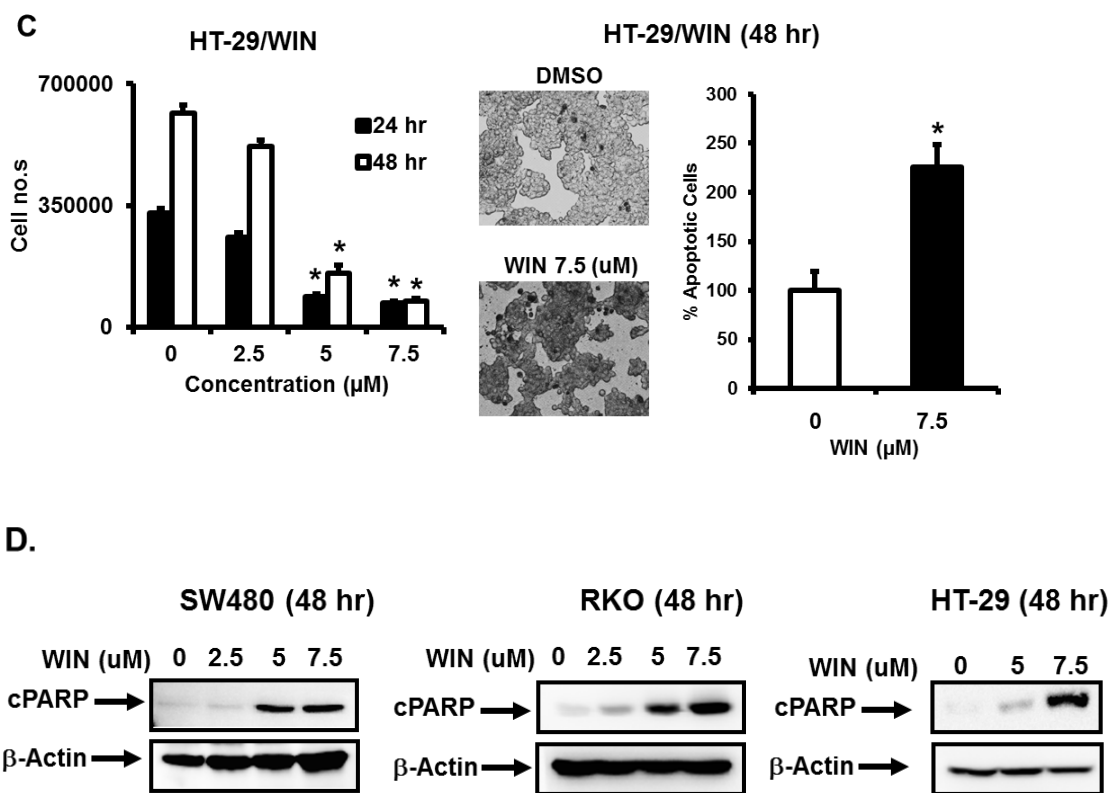


Figure 27. Continued

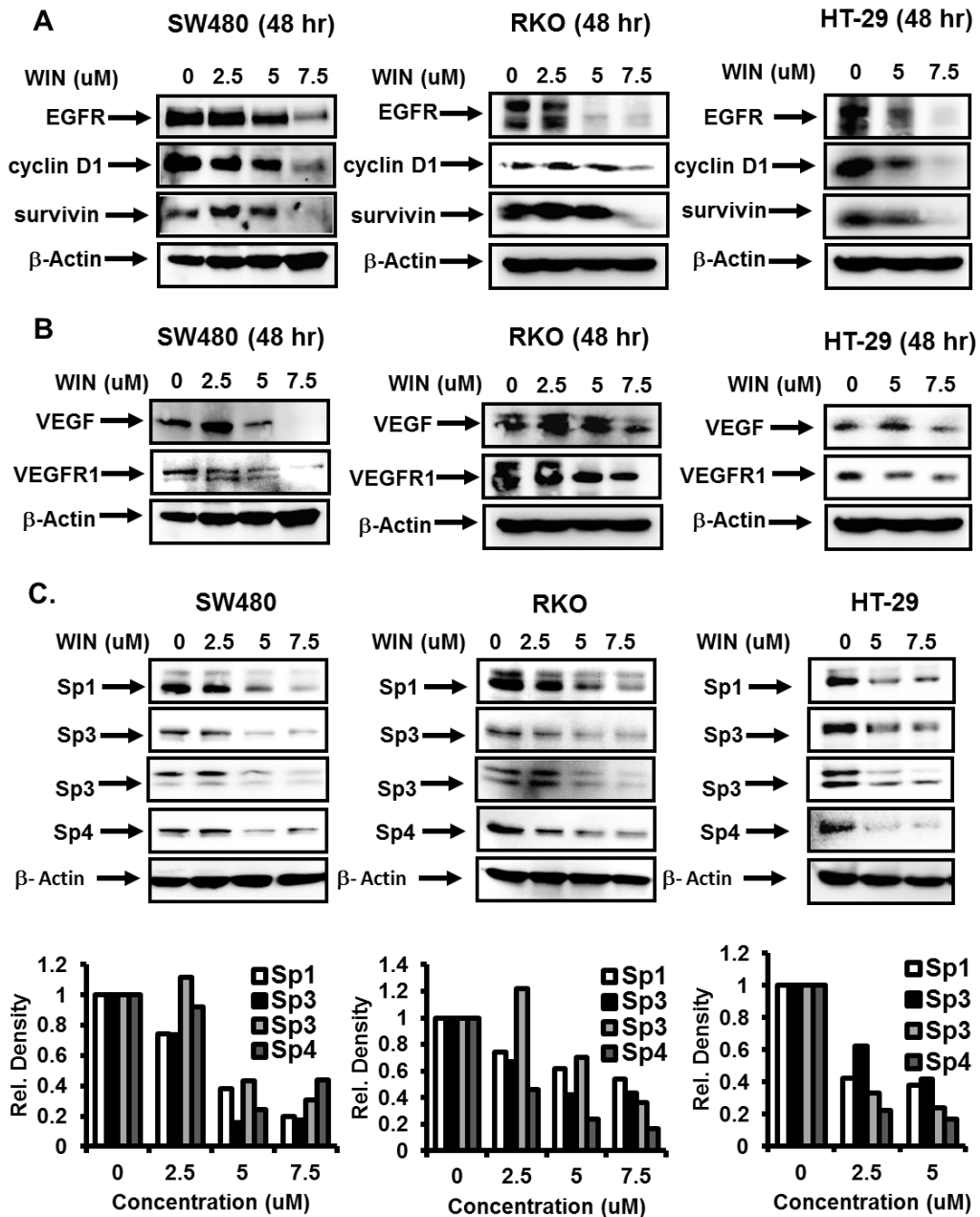


Figure 28. WIN downregulates Sp-regulated proliferative, survival (A) and angiogenic (B) gene products and Sp proteins (C) in SW480, RKO and HT-29 cells. Cells were treated with 2.5-7.5 μ M of WIN for 48 hr, whole cell lysates were analyzed by western blots, and expression of Sp1, Sp3 and Sp4 protein was quantitated (C) relative to β -actin (levels in the DMSO group were set at 1.0).

This is the first example of a synthetic cannabinoid decreasing expression of Sp proteins and Sp-regulated gene products in cancer cells; however, we have recently reported that betulinic acid (BA), a triterpenoid compound that also decreases Sp1, Sp3 and Sp4 protein levels in multiple cancer cell lines, exhibits CB receptor agonist activity (684).

Drug-induced downregulation of Sp transcription factors has been linked to activation of proteasomes or induction of ROS (638, 639, 667, 672, 675). Using SW480 cells as a model, we show that WIN-induced downregulation of Sp1, Sp3 and Sp4 was not reversed in cells cotreated with WIN plus the proteasome inhibitors lactacystin or gliotoxin (Fig. 29A). Moreover, treatment of SW480 cells with WIN in combination with the antioxidants DTT or GSH also did not inhibit WIN-induced downregulation of Sp1, Sp3 or Sp4 proteins (Fig. 29B) and this contrasted the effects of BA and GT-094 (a nitro-NSAID) in the same cell line (667, 675). The CB1 and CB2 receptor antagonists AM251 and AM560 had minimal effects on Sp protein expression and, in combination with WIN, did not inhibit WIN-induced downregulation of Sp1, Sp3 or Sp4 proteins (Fig. 29C). Moreover, similar results were observed in SW480 cells after knockdown of the CB1 (iCB1) or the CB2 (iCB2) receptor by RNA interference (Fig. 29D), demonstrating that the effects of WIN on downregulation of Sp transcription factors was CB receptor-independent.

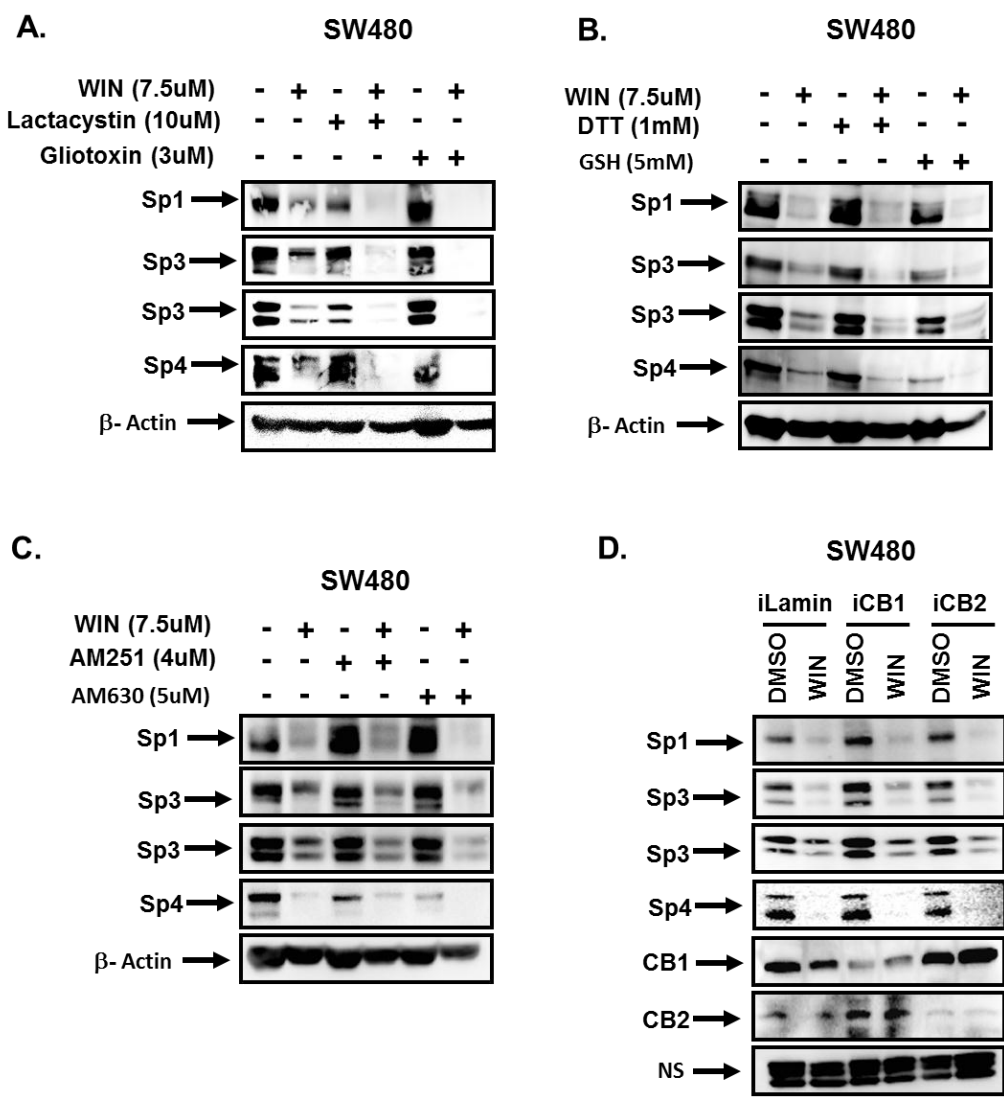


Figure 29. Effects of proteasome inhibitors, antioxidants, cannabinoid receptor antagonists, and cannabinoid receptor knockdown on WIN-mediated downregulation of Sp proteins. SW480 cells were pre-treated with lactacystin (10 μ M) or gliotoxin (3 μ M) (A), DTT (1 mM) or GSH (5 mM) (B), AM251 (4 μ M) and AM630 (5 μ M) (C) for 45 min followed by treatment with 7.5 μ M of WIN for 48 hr, and whole cell lysates were analyzed by western blots. (D) Cells were transfected with siRNAs against CB1 and CB2 receptors, followed by treatment with WIN for 48 hr, and whole cell lysates were analyzed by western blots.

WIN induced several phosphatase mRNAs in SW480 cells and the phosphatase inhibitor sodium orthovanadate (SOV) partially blocked WIN-induced PARP cleavage (858) and, therefore, we investigated the effects of SOV on WIN-induced downregulation of Sp1, Sp3 and Sp4 proteins using SW480 cells as a model. Figure 30A shows that WIN-induced downregulation of Sp1, Sp3 and Sp4 was reversed after cotreatment with SOV and these results are consistent with the inhibition of WIN-induced PARP cleavage by SOV (858) since knockdown of Sp1 (by RNAi) also enhances PARP cleavage (672). A recent study showed that α -tocopherol succinate-induced downregulation of Sp1 in prostate cancer cells was PP2A-dependent (866) and western blot analysis of whole cell lysates from SW480 cells transfected with a non-specific oligonucleotide (iCtrl) or a specific oligonucleotide that targets PP2A (iPP2A) and treated with 7.5 μ M WIN or DMSO showed that WIN-mediated downregulation of Sp1, Sp3 and Sp4 proteins was significantly attenuated in cells transfected with iPP2A (Fig. 30B). Similar results were also observed in RKO cells with some differences in the relative effectiveness of PP2A knockdown (Fig. 33A). PP2A knockdown also attenuated WIN-induced downregulation of cyclin D1 and VEGFR1 but had minimal effect on EGFR protein levels (Fig. 30C), suggesting that decreased expression of EGFR in SW480 cells treated with WIN was phosphatase- and Sp-independent.

We also used an *in vitro* assay to confirm that WIN induced PP2A activity since previous studies did not observe induction of PP2A mRNA levels in SW480 cells treated with WIN (858). PP2A activity in SW480 was measured using the PP2A

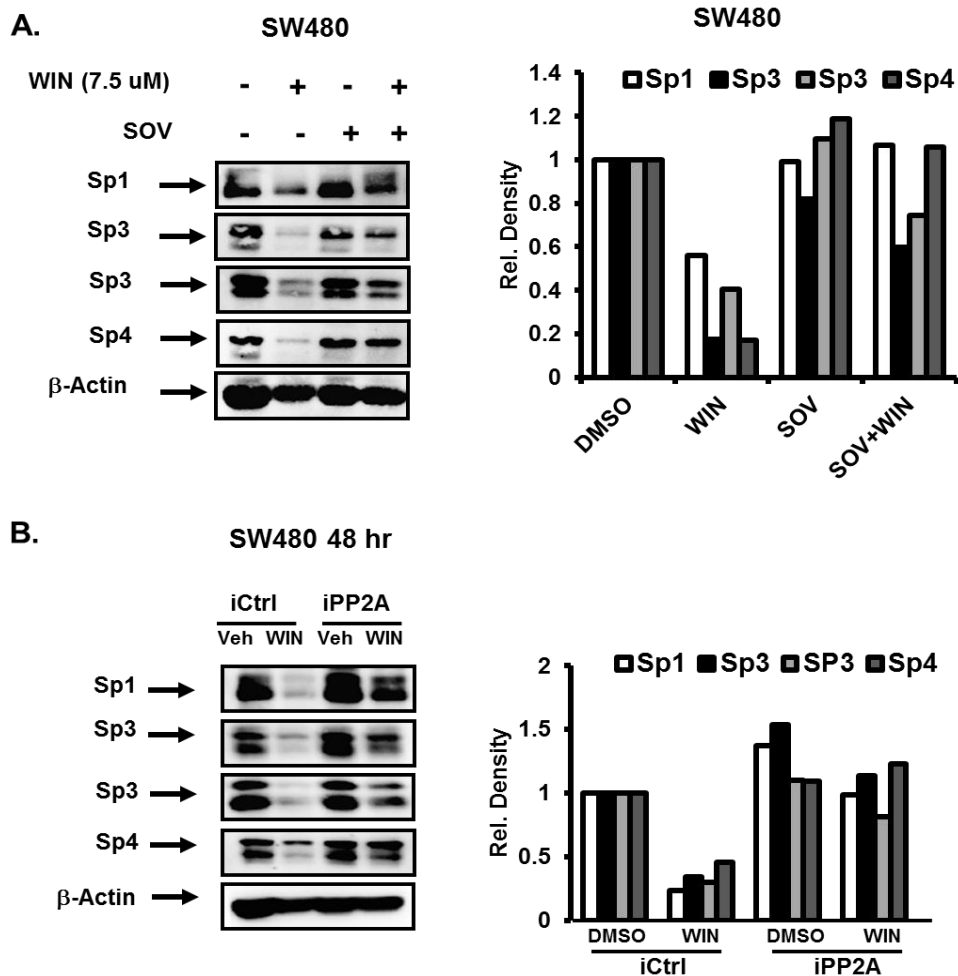
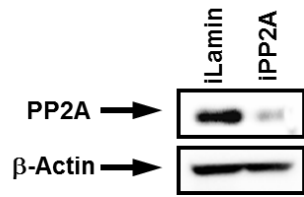
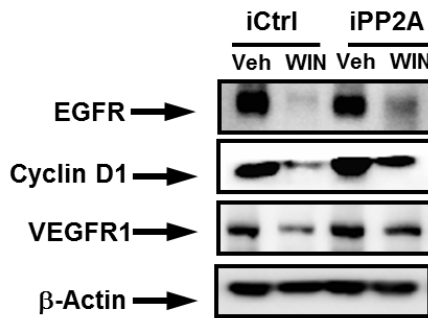


Figure 30. Effects of phosphatase inhibition on WIN-dependent downregulation of Sp proteins and Sp-regulated gene products. (A) Cells were pre-treated with 0.35 mM SOV for 45 min followed by treatment with 7.5 μ M of WIN for 48 hr, and whole cell lysates were analyzed by western blots. Cells were transfected with siRNA against phosphatase PP2A (iPP2A), followed by treatment with WIN for 48 hr, and whole cell lysates were examined for expression of Sp1, Sp3, Sp4 and PP2A proteins (B) and Sp-regulated gene products (C) by western blot analysis. (D) Cells were treated with DMSO, 5 and 7.5 μ M of WIN, and PP2A activity was determined in the absence and presence of the phosphatase inhibitor okadaic acid. Results are expressed as means \pm SE for at least three separate determinations, and significant ($p < 0.05$) induction (*) or inhibition (**) by okadaic acid is indicated. Lysates used for phosphatase assay were analyzed by western blots to determine expression of PP2A protein.

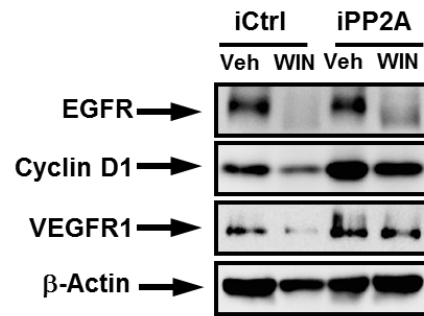
B. SW480-48 hr



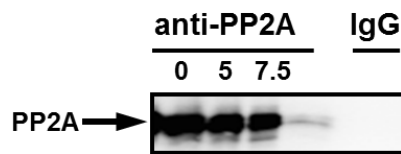
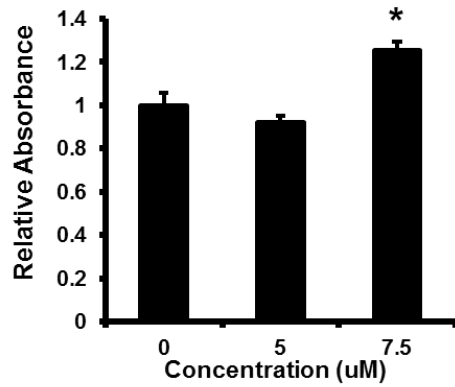
C. SW480 24 hr



SW480 48 hr



D. SW480/WIN- PP2A activity



SW480/ OA- PP2A activity

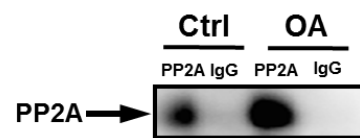
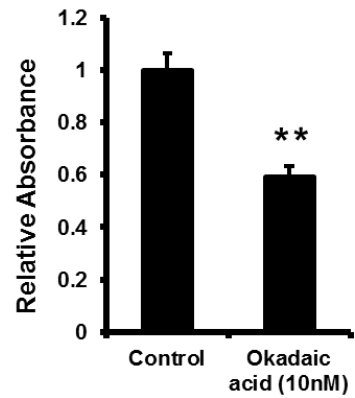


Figure 30. Continued

Immunoprecipitation Phosphatase Assay Kit from Millipore. Treatment of SW480 cells with 7.5 μ M WIN induced an approximate 30% increase in PP2A activity and this was comparable to the induction of PP2A phosphatase activity reported by Kickstein *et al.* (867). Figure 30D shows that 10 nM okadaic acid significantly inhibited PP2A activity in SW480 cells (positive control). Cell lysates used for the phosphatase activity assays were then subjected to western blot analysis and probed with PP2A antibody to confirm the presence of PP2A in the cell lysates (Fig. 30D).

PP2A decreases expression of the phosphorylated form of the pro-oncogenic cap protein eIF4E (868) (p-eIF4E) and therefore the time-dependent effects of 7.5 μ M WIN on expression of Sp1, Sp3, Sp4, p-eIF4E and eIF4E proteins were investigated (Fig. 31A). Sp1, Sp4 and Sp3 (high molecular weight forms) expression was significantly decreased after 12 hr, whereas the lower molecular weight forms of Sp3 were more slowly decreased over the 48 hr period. p-eIF4E and eIF4E proteins were significantly decreased within 12 hr after treatment with WIN and continued to decrease (over 48 hr); the decrease of eIF4E (total protein) was more gradual than observed for the phosphoprotein. Treatment of SW480 cells with WIN alone or in combination with SOV showed that the phosphatase inhibitor blocked WIN-induced effects on p-eIF4E and eIF4E, but not PP2A (Fig. 31B), demonstrating that WIN-induced downregulation of p-eIF4E, eIF4E, Sp1, Sp3 and Sp4 was phosphatase dependent. The role of PP2A in this process was investigated by RNAi, and WIN-mediated repression of p-eIF4E and eIF4E (total) proteins was significantly inhibited by knockdown of PP2A (Fig. 31C),

demonstrating a role for PP2A in mediating downregulation of eIF4E and pEIF4E and this parallels results observed for phosphatase-dependent downregulation of Sp transcription factors (Fig. 30), suggesting possible cross-regulation of eIF4E and Sp transcription factors. Knockdown of Sp1 (iSp1), Sp3 (iSp3) and Sp4 (iSp4) in SW480 cells resulted in specific knockdown of these transcription factors, and iSp1 and to a lesser extent iSp3 also decreased eIF4E protein expression (Fig. 31D), whereas Sp4 knockdown had minimal effects, suggesting that in SW480 cells eIF4E expression is primarily regulated by Sp1. In contrast, knockdown of eIF4E by RNAi decreased expression of the targeted protein and slightly increased expression of Sp1, Sp3 or Sp4 proteins, suggesting that cross-regulation between Sp transcription factors and eIF4E is unidirectional. A comparable experiment was carried out in RKO cells; iSp1, iSp3 and iSp4 decreased eIF4E protein expression and the combination of all 3 oligonucleotides caused a marked decrease of eIF4E protein (Fig. 33B), indicating that eIF4E is an Sp-regulated gene in colon cancer cells.

Drug-induced proteasome-independent downregulation of Sp1, Sp3 and Sp4 has been related to transcriptional repression due to downregulation of miR-27a and miR-20a/miR-17-5p which regulate the "Sp repressors" ZBTB10 and ZBTB4 (641, 869). Treatment of SW480 cell with 7.5 μ M WIN induced ZBTB10 protein within 12 hr and induction was high after 24 hr but markedly decreased after 36 and 48 hr (Fig. 32A). In contrast, induction of ZBTB4 protein was not observed (data not shown).

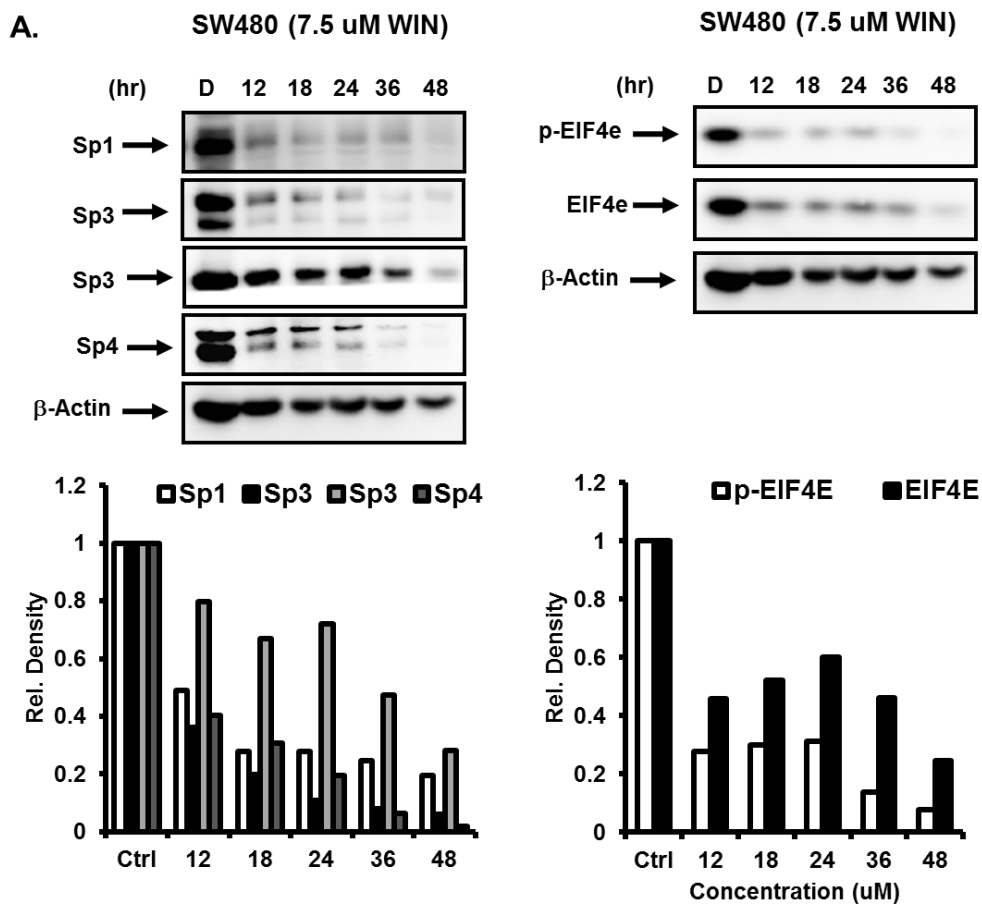


Figure 31. Effects of WIN and Sp protein knockdown on total and phospho-eIF4E in SW480 cells. (A) Cells were treated with DMSO or 7.5 μ M WIN for 12, 18, 24, 36 and 48 hr, and whole cell lysates were analyzed by western blots. Cells were pretreated with SOV for 45 min (B) or transfected with siRNA against PP2A (C), followed by treatment with WIN for 48 hr, and whole cell lysates were analyzed by western blots. (D) SW480 cells were transfected with various siRNA and whole cell lysates were analyzed for expression of Sp proteins and eIF4E by western blots.

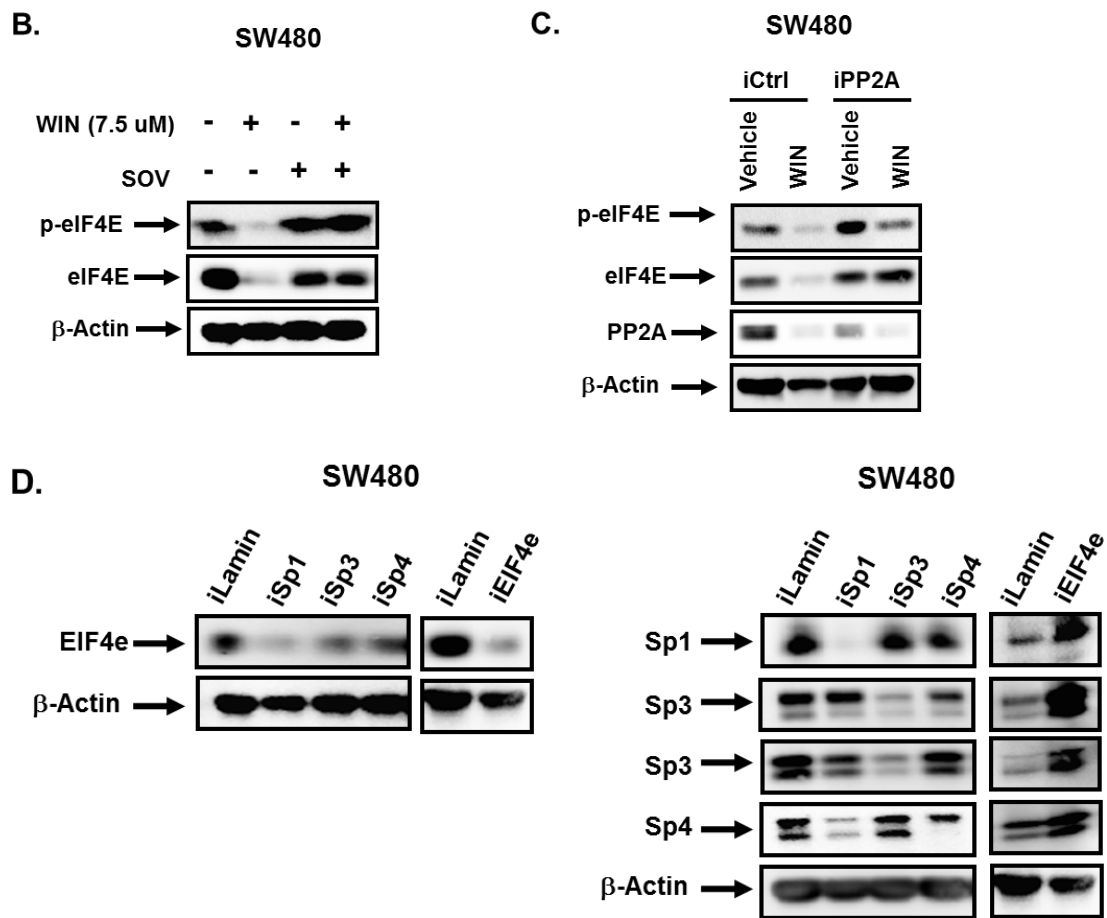


Figure 31. Continued

SW480 cells were treated with 7.5 μ M WIN and also transfected with iGL2 (control) or iPP2A oligonucleotides, and the induction of ZBTB10 in the control cells was significantly decreased after knockdown of PP2A (Fig. 32B). In a parallel experiment, WIN also decreased expression of miR-27a after treatment for 24 or 48 hr and, after knockdown of PP2A by RNAi, downregulation of miR-27a by WIN was attenuated (Fig. 32C). The role of WIN-induced ZBTB10 on suppression of Sp proteins was confirmed

in SW480 cells treated with WIN and transfected with iGL2 (control) or iZBTB10 oligonucleotides in an RNAi assay; knockdown of ZBTB10 inhibited downregulation of Sp1, Sp3 and Sp4 by WIN (Fig. 32D). We observed that WIN also induced ZBTB10 in RKO cells and this response was attenuated in cells transfected with iPP2A (RNAi), and knockdown of ZBTB10 by RNA also inhibited WIN-mediated suppression of Sp1, Sp3 and Sp4 proteins in RKO cells (Figs. 33C and 33D). These results demonstrate that WIN-mediated downregulation of Sp proteins in SW480 cells is due to induction of PP2A and PP2A-dependent disruption of the miR-27a:ZBTB10 which results in induction of ZBTB10 and ZBTB10-dependent repression of Sp proteins and Sp-regulated genes.

DISCUSSION

Sp1, Sp3 and Sp4 proteins are highly expressed in multiple cancer cell lines and tumors, whereas levels of these transcription factors are low to non-detectable in non-tumor tissue (672, 673, 863-865) and these observations are consistent with reports that Sp1 expression in rodent and human tissues decreases with age (870, 871). The pro-oncogenic activity of Sp proteins is primarily due to Sp-regulated genes which include several that play pivotal roles in cancer cell proliferation [cyclin D1, c-Met, epidermal growth factor (EGFR)], survival (survivin, bcl-2), angiogenesis [vascular endothelial

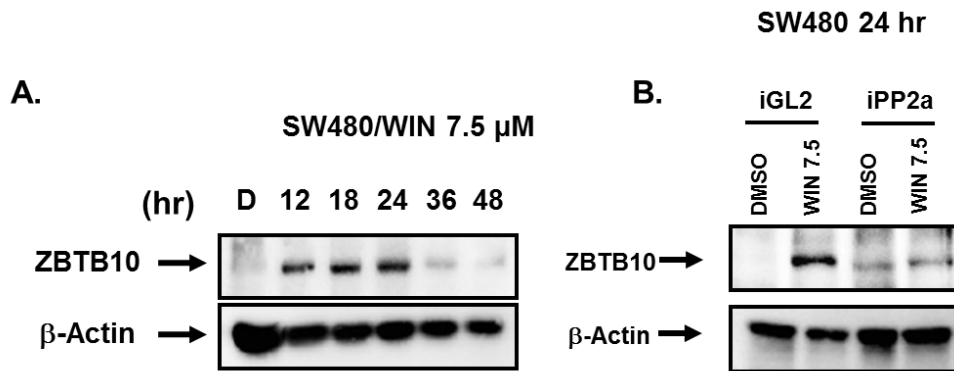


Figure 32. WIN modulates miR27a and ZBTB10 expression. (A) SW480 cells were treated with DMSO or 7.5 μ M of WIN for 12, 18, 24, 36 and 48 hr, and whole cell lysates were analyzed for ZBTB10 protein by western blots. SW480 cells were transfected with siRNA against PP2A, followed by treatment with 7.5 μ M WIN for 24 and 48 hr. Whole cell lysates were analyzed by western blot analysis for ZBTB10 expression (B) and miR27a levels (C) were determined by qPCR as described in the Materials and Methods. (D) SW480 cells were transfected with siRNA against ZBTB10 followed by treatment with 7.5 μ M WIN for 24 and 48 hr, and whole cell lysates were analyzed by western blots. Results for RNA expression are expressed as means \pm SE for at least three separate determinations, and significant ($p < 0.05$) inhibition (*) by WIN is indicated and reversal of these effects by iPP2A (**) are indicated.

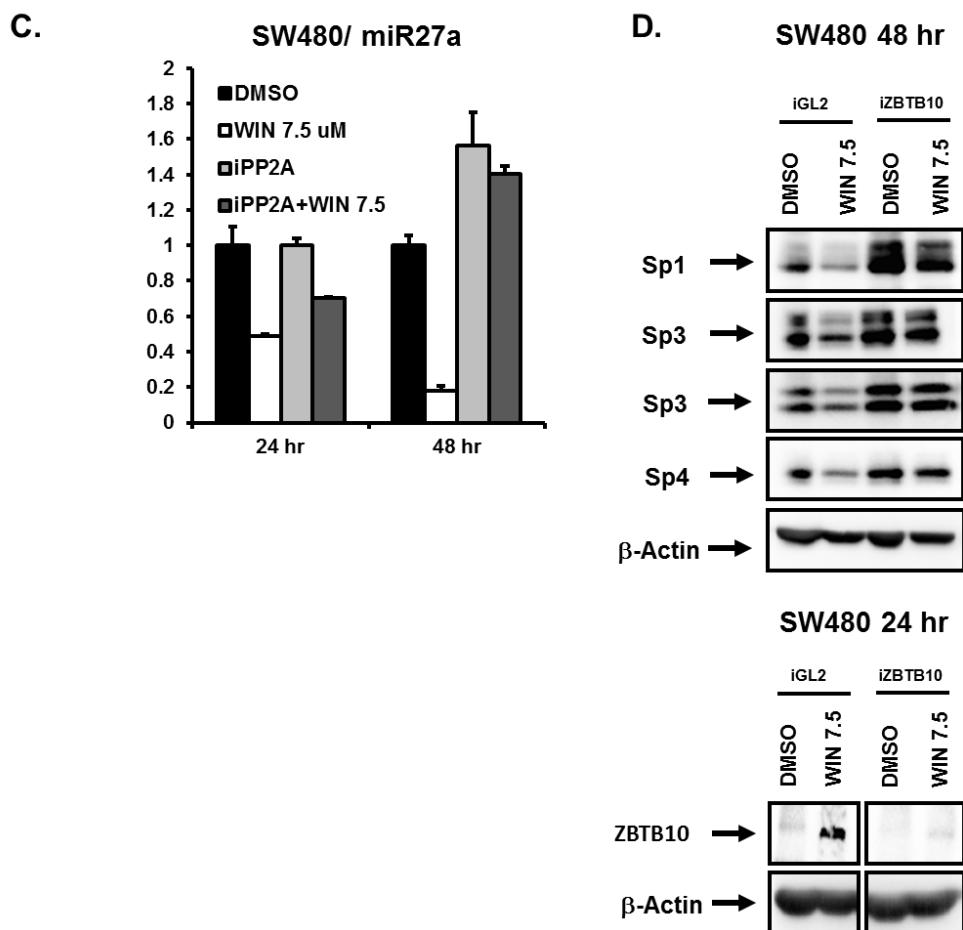


Figure 32. Continued

growth factor (VEGF) and its receptors (VEGFR1 and VEGFR2)], and inflammation (NF κ B, p65). Thus, Sp transcription factors are an excellent example of non-oncogene addiction by cancer cells (307) and therefore an important target for mechanism-based anticancer drugs. Reports from different laboratories have identified diverse agents that decrease expression of Sp transcription factors and these include various phytochemical anticancer compounds including BA and their synthetic analogs, NSAIDs, bortezimob,

α -tocopherol succinate, arsenic trioxide and other ROS inducers (638, 639, 641, 667, 672, 673, 675, 684, 863-866, 872).

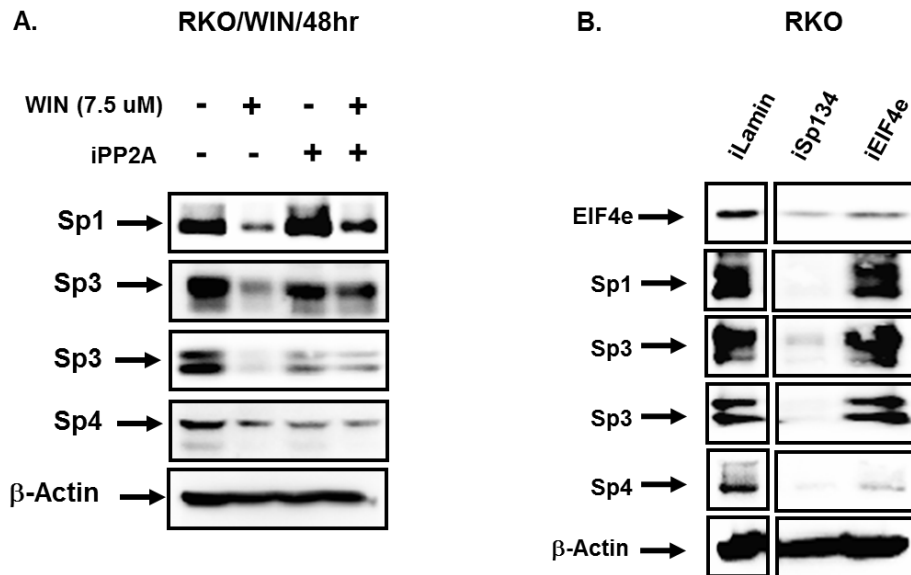


Figure 33. Effects of phosphatase inhibition in RKO cells. (A) SW480 cells were transfected with siRNA against phosphatase PP2A (iPP2A), followed by treatment with WIN for 48 hr, and whole cell lysates were examined for expression of Sp1, Sp3 and Sp4. (B) RKO cells were transfected with various siRNA and whole cell lysates were analyzed for expression of Sp proteins and eIF4E by western blots. (C) Cells were transfected with siRNA against PP2A, followed by treatment with 7.5 μ M WIN for 24 hr. Whole cell lysates were analyzed by western blot analysis for ZBTB10 expression. (D) RKO cells were transfected with shRNA against ZBTB10 followed by treatment with 7.5 μ M WIN and whole cell lysates were analyzed by western blots.

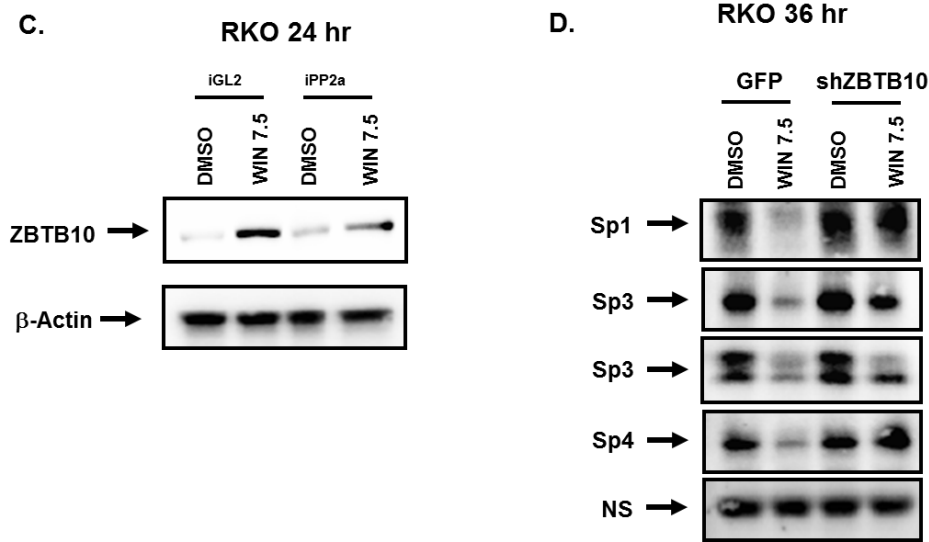


Figure 33. Continued

BA decreases expression of Sp1, Sp3 and Sp4 in prostate, colon, bladder and breast cancer cell lines (639, 667, 684, 864). Results illustrated in Figures 27 and 28 confirm that the cannabinoid WIN also inhibited growth and induced apoptosis in SW480, RKO and HT-29 colon cancer cells and downregulated Sp1, Sp3, Sp4 and Sp-regulated genes. Since knockdown of Sp1 or multiple Sp transcription factors alone in cancer cells inhibits growth and induces apoptosis (672, 673, 863, 864), then the anticancer activity of WIN is due, in part, to downregulation of Sp proteins. However, in contrast to BA and other agents that downregulate Sp transcription factors, WIN-induced Sp downregulation was proteasome- and ROS-independent and knockdown or inhibition of CB1 or CB2 receptors did not affect this response in SW480 cells (Fig. 29). The receptor-independent effects of WIN were consistent with previous studies showing that

the anticancer activity of WIN was both receptor-dependent and -independent (844, 857, 858).

We recently reported that WIN-induced apoptosis in SW480 and LNCaP cells is inhibited by the phosphatase inhibitor SOV but not by CB receptor knockdown and WIN also induces multiple phosphatase mRNAs (858). These results, coupled with a report that PP2A plays a role in α -tocopherol succinate-induced downregulation of Sp1 in prostate cancer cells (866) prompted us to investigate the effects of SOV and PP2A knockdown on WIN-induced repression of Sp proteins. Both SOV and PP2A knockdown attenuated WIN-mediated downregulation of Sp1, Sp3, Sp4 and Sp-regulated gene products (Fig. 30), confirming a role for PP2A and possibly other identified phosphatases in mediating the anticancer activities of WIN. An important proteasome-independent pathway for drug-induced downregulation of Sp1, Sp3 and Sp4 involves induction of the transcriptional repressors ZBTB10 and ZBTB4 which exhibit low expression in cancer cell lines due to their regulation (repression) by miR-27a and miR-20a/miR-17-5p, respectively (638, 639, 641, 667, 675, 684, 866-869). WIN did not induce ZBTB4 in SW480 cells; however, a time-dependent induction of ZBTB10 protein was observed (Fig. 32A) and this response was abrogated after knockdown of PP2A (Fig. 32B), and these results were paralleled by PP2A-dependent repression of miR-27a (Fig. 32C). Moreover, since knockdown of ZBTB10 by RNAi blocks WIN-mediated downregulation of Sp1, Sp3 and Sp4 proteins (Fig. 32D) and since miR-27a antagonists and ZBTB10 expression decrease Sp protein expression in colon cancer cells

(872), it is clear that disruption of miR-27a:ZBTB10 is critical for the observed responses. The mechanism of PP2A-mediated effects on miR-27a:ZBTB10 are unknown and are currently being investigated.

The induction of PP2A activity by WIN suggested that WIN may also regulate phosphorylation of the important cap protein eIF4E since it has been reported that PP2A dephosphorylates p-eIF4E which has been characterized as a pro-oncogenic phospho-protein (868). WIN clearly decreased expression of p-eIF4E; however, this was unexpectedly accompanied by downregulation of eIF4E (total protein). Since eIF4E and Sp transcription factors regulate expression of several genes in common (e.g. cyclin D1 and VEGF), we used RNAi to show for the first time that eIF4E is an Sp-regulated gene in colon cancer cells (Fig. 31D). This observation extends the number of pro-oncogenic factors that are regulated by Sp1, Sp3 and Sp4 in cancer cells emphasizing the potential clinical applications of drugs that target Sp proteins.

CHAPTER IV

CANNABIDIOL (CBD) INHIBITS GROWTH OF COLON CANCER CELLS THROUGH PHOSPHATASE DEPENDENT DOWNREGULATION OF SPECIFICITY PROTEINS

INTRODUCTION

Cannabinoids (CB) define a growing class of structurally-diverse compounds which include a large number terpenoid-phenolic phytochemicals isolated from *cannabis sativa* and related species, synthetic compounds that interact with the CB receptors and endogenous or endocannabinoids (516, 773, 873). Although the psychoactive phytochemicals such as Δ^9 -tetrahydrocannabinol (Δ^9 -THC) have been investigated for their mood-altering properties, there has been extensive research and clinical applications for the use of CBs in treating multiple diseases including cancer (685, 711, 824, 835, 856, 873). Initial clinical applications of psychoactive CBs in cancer chemotherapy has been to attenuate the emesis, wasting and pain that accompanies many different cancers; however in 1975 it was reported by Munson and coworkers that several CBs exhibit anticancer activity against Lewis lung adenocarcinoma cells (811)(10). Subsequent studies in several laboratories show that most classes of CBs exhibit anticancer activity against a wide spectrum of cancer cell lines *in vitro* and also in *in vivo* models (685, 711, 824, 835, 856).

Among the phytochemical cannabinoids one of the non-psychoactive compounds, cannabidiol (CBD) has been extensively investigated as an anticancer agent in many different cancer cell lines and shows potential for clinical applications (638, 639, 660, 661, 664, 677, 678, 681, 841-843, 858, 874-885). A recent review by Massi and coworkers (685) highlighted many of the CBD-induced responses in cancer cell lines and these included inhibition of cell growth and survival, decreased migration and/or invasion, antiangiogenic activity and cell death (apoptosis and autophagy). Not all of these responses were observed in any single study however, it was evident that the activity of CBD was consistent with that of many other phytochemical-derived anticancer agents and clinically used chemotherapeutic drugs. CBD also modulated several pathways and expression of genes which were consistent with CBD-induced anticancer activities. For example, CBD induced caspase and polyADPribose polymerase (PARP) cleavage [markers of apoptosis (858, 875, 877, 884)] induced reactive oxygen species (ROS) in some cancer cells (677, 685, 843), decrease Id-1 expression (plays a role on invasion) (881), upregulated tissue inhibitor of matrix metalloproteinases1 (TIMP-1) (invasion) which was dependent on induction of p38 and p44/42 mitogen-activated protein kinases (660). Previous studies have focused on different pathways/genes and cannot be directly compared; however, it was apparent from various CB receptor inhibition studies that the overall mechanisms of action of CBD were highly variable and cell context-dependent (685). For example in HeLa and lung cancer cells the CBD-induced downregulation of TIMP-1 or decreased invasion was significantly inhibited by CB1 and CB2 receptor antagonist, the vanilloid receptor

antagonist (capsazepine) and inhibitors of p38 and p42/42 (660). In contrast, a recent report showed that decreased viability of A549 and H460 lung cancer cells after treatment with CBD was not affected by CB1, CB2 and vanilloid receptor antagonist but was significantly blocked by cotreatment with the cyclooxygenase-2 COX-2 inhibitor NS398 and or the peroxisome proliferator-activated receptor (PPAR γ) inhibitor GW9662 (885).

Studies in the laboratory reported that CBD-induced apoptosis in LNCaP and SW480 colon cancer cells and this response was attenuated after cotreatment with the phosphatase inhibitor sodium orthovanadate (SOV). We also observed induction of several phosphatase mRNAs (858). In this study we have investigated the anticancer activity of CBD in RKO and SW480 colon cancer cell lines and demonstrate that CBD inhibits growth and induces apoptosis. We also observed that CBD decreased expression of several growth promoting [cyclin D1 and epidermal growth factor receptor (EGFR)], survival (survivin) and angiogenic [vascular endothelial growth factor (VEGF) and its receptor (VEGFR1)] gene products. In previous studies we have observed that these proteins are regulated by specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 that are overexpressed in colon and other cancer cell lines (638-641, 865, 872, 886) and in this study we also demonstrated that CBD downregulated Sp1, Sp3 and Sp4 proteins. CBD also downregulated microRNA-27a (miR27-a) induced the Sp repressor ZBTB10 however the mechanisms of this response are unknown and are currently being investigated.

MATERIALS AND METHODS

Chemicals, Antibodies, and Reagents

Cannabidiol, Lactacystin, glutathione (GSH) and antibody for β -Actin (A1978) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AM251 (1117) and AM630 (1120) were purchased from Tocris Bioscience (Ellisville, MO, USA). Tyrosine phosphatase inhibitor, sodium orthovanadate, was purchased from Calbiochem (La Jolla, CA, USA). Dithiothreitol (DTT) was obtained from Boehringer Mannheim Corp, (Indianapolis, IN). Gliotoxin was kindly provided by Dr. Alan Taylor (National Research Council of Canada, Halifax, NS, Canada). Cleaved poly (ADP-ribose) polymerase (PARP) (9541) antibody was obtained from Cell Signaling (Danvers, MA). Antibodies for EGFR (1005), Sp3 (D-20) and Sp4 (V-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immobilon Western Chemiluminescent HRP substrate (WBKLS0500) and antibodies for Sp1 (07-645) and CXCR4 (AB1846) and PP2A (05421) were purchased from EMD Millipore (Billerica, MA, USA). Cyclin D1 (2261-1) and survivin (2463-1) antibodies were purchased from Epitomics (Burlingame, CA). VEGFR1 (ab32152) antibody was purchased from Abcam (Cambridge, MA). Antibody for VEGF (209-403-B99) was purchased from Rockland Antibodies and Assays (Gilbertsville, PA). ZBTB10 (A303 258A) antibody was purchased from Bethyl Laboratories (Montgomery, TX). Apoptotic, Necrotic and Healthy Cells Quantification Kit (30018) was purchased from Biotium (Hayward, CA). Dulbecco's Modified Eagle Medium (DMEM) and real-time PCR primers for TATA binding protein (TBP) and ZBTB10 were obtained from Sigma (St. Louis, MO). MicroRNA isolation kit mirVana

was obtained from Ambion-life technologies (Grand Island, NY). Primers for RNU6B and miR27a were purchased from Applied Biosystems.

Cell Lines

Human SW480 colon carcinoma cell line was provided by Dr. Stanley Hamilton (M.D. Anderson Cancer Center, Houston, TX). RKO colon carcinoma cell line was obtained from ATCC (Manassas, VA). Cells were purchased more than 6 months ago and were not further tested or authenticated by the authors. Cells were maintained at 37°C in the presence of 5% CO₂ as described (858).

Cell Proliferation Assay

Colon cancer cells (1×10^5 per well) were seeded 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 ethanol) or varying concentrations of CBD was added. Cells were then trypsinized and counted after 24 and 48 hr using a Coulter Z1 cell counter (Sykesville, MD). Each experiment was performed in triplicate, and results were expressed as means \pm SE for each set of experiments.

Annexin V Staining Assay

SW480 and RKO cells were seeded in 2 well Nunc Lab-tek chambered coverglass plates from Thermo Scientific (Waltham, MA) and were 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 or CBD was added. Cells were stained after 9 hr with FITC-Annexin-V, propidium iodide and DAPI dyes according to the manufacturer's protocol and were visualized under EVOS fl, fluorescence microscope, from Advanced Microscopy Group (Bothell, WA) using a multiband filter set for FITC, rhodamine and DAPI. The proportion of apoptotic cells was determined by the amount of green fluorescence observed.

Western Blot Analyses

Colon cancer cells were seeded in DMEM/Ham's F-12 medium and were allowed to attach for 24 hr. Cells were treated with either vehicle or CBD for indicated time periods or pretreated with the proteasome inhibitors, antioxidants, phosphatase inhibitors ceramide synthase inhibitors and cannabinoid receptor antagonists, and then treated with CBD. Cells were lysed and analyzed by western blots as described (858).

Small Inhibitory RNAs (siRNA) Interference Assay

Colon cancer cells were seeded (2×10^5 per well) in six-well plates in DMEM/Ham's F-12 medium supplemented with 2.5% charcoal stripped FBS without antibiotics and left to attach for 24 hr. siRNAs specific for CB1 and CB2 receptors, PP2A, MKP-1 and MKP-5 along with iLamin/iGL2 as control were transfected using Lipofectamine 2000 reagent according to the manufacturer's protocol (Ref). Small interfering RNAs for CB1 and CB2 receptors, MKP-5, MKP-1 and PP2A were obtained from Sigma (St. Louis, MO). Lipofectamine 2000 was purchased from Life Technologies (Grand Island, NY).

Quantitative Real-Time PCR

SW480 colon cancer cells were plated (2×10^5) and left to attach for 24 hr. Cells were treated with either vehicle or CBD for 24 hr. MicroRNA was isolated using the mirVana miRNA isolation kit from Ambion-Life Technologies (Grand Island, NY) according to the manufacturer's protocol. cDNA was prepared using the Taqman MicroRNA Reverse Transcription kit and was subjected to quantitative real-time PCR with specific primers for miR27a using the Taqman Universal PCR Master Mix from Applied Biosystems. Quantification of miRNA (RNU6B and miRNA-27a) was done using the Taqman miRNA kit (Applied Biosystems) according to the manufacturer's protocol in the CFX384 Real-Time PCR Detection System from Biorad (Hercules, CA). U6 small nuclear RNA was used as a control to determine relative miRNA expression. Total RNA was isolated using the RNeasy Protect Mini kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR was carried out with the SYBR Green PCR Master Mix from Applied Biosystems on an ABI 7500 Fast Real-Time PCR System (PE Applied Biosystems) using 0.5 $\mu\text{mol/L}$ of each primer and 2 μL cDNA template in each 25 μL reaction. TATA binding protein (TBP) was used as an exogenous control to compare the relative amount of target gene in different samples. The PCR profile was as follows: one cycle of 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The comparative C_T method was used for relative quantitation of samples.

The following primers were used:

TBP, forward, 5'-TGCACAGGAGCCAAGAGTGAA-3';

TBP, reverse, 5'-CACATCACAGCTCCCCACCA-3'

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

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

RESULTS

RKO and SW480 colon cancer cells were initially used as models for this study, RKO cells express wild-type Ras and p53; in contrast SW480 cells express mutated forms of Ras (K12Val) and p53 (Arg 273 His). Treatment of both cell lines with (DMSO or ethanol) (control), 5, 10 or 15 μ M CBD decreased cell growth and IC₅₀ values after treatment for 24 hr were 5.08 and 13.65 μ M for SW480 and RKO cells respectively (Fig. 34A). Longer term treatment with CBD demonstrate that the minimum effective concentrations for growth inhibition were 5 and 10 μ M for SW480 and RKO cells respectively indicating the enhance sensitivity of the former cell line to the growth inhibitory effects of CBD. Subsequent studies were focused on early events induced by CBD in these cell lines and therefore higher concentrations of CBD (10 – 15 μ M) were used. Results in Figure 34B show that treatment with 15 μ M CBD for 9 hr induced annexin V staining in SW480 and RKO cells and this was also accompanied by enhanced cleaved PARP (Fig. 34C) indicating that CBD induced apoptosis in both cell lines.

We also examined the effects of CBD on prototypical gene products associated with cell growth, survival and angiogenesis and observed that CBD decreased expression of

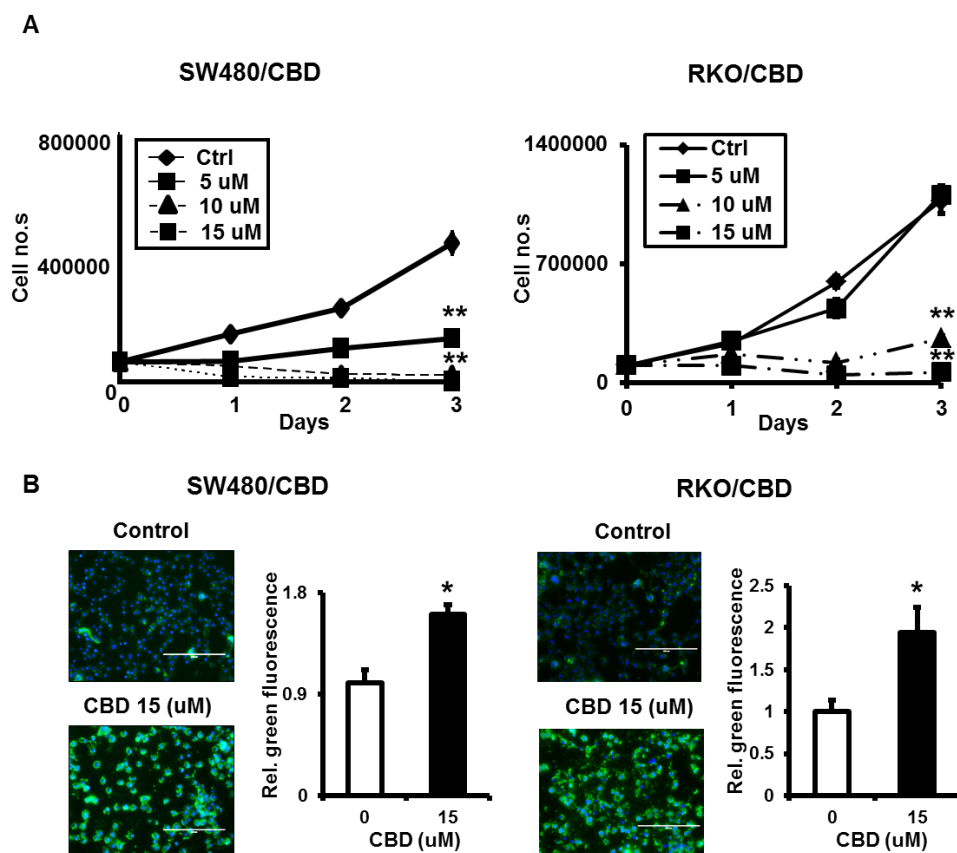


Figure 34. CBD inhibits colon cancer cell growth, induces apoptosis and PARP cleavage. (A) SW480 and RKO cells were treated with DMSO or ethanol and 5-15 μ M CBD for 24, 48 and 72 hr and counted, or (B) treated with DMSO or ethanol and 15 μ M of CBD for 9 hr and effects on Annexin V (SW480 and RKO) staining was determined. Results are expressed as means \pm SE for at least three separate determinations, and significant ($p < 0.05$) induction (*) or inhibition (**) by CBD is indicated. (C) Cells were treated with DMSO or ethanol and 5-15 μ M of CBD for 24 hr and whole cell lysates were examined for expression of cleaved PARP by western blots. β -Actin served as a loading control for all western blots.

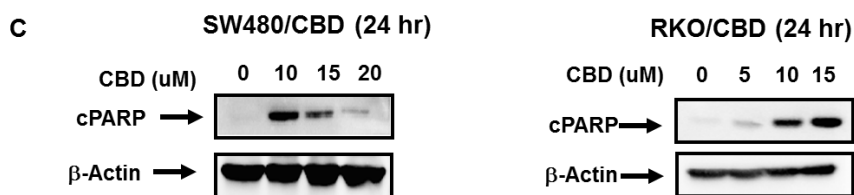


Figure 34. Continued

EGFR, cyclin D1 and survivin proteins (Fig. 35A) and also decreased expression of the angiogenic proteins VEGF and VEGFR1 proteins (Fig. 35B). Previous studies with betulinic acid (BA) which is a newly discovered cannabinoid (684) showed that BA also downregulated the same set of genes and this was due to decreased expression of Sp1, Sp3 and Sp4 proteins in SW480 and RKO cells (Fig. 35C) and this is consistent with decreased expression of Sp-regulated gene products (Figs. 35A and 35B). Moreover, silencing of Sp1, Sp3 and Sp4 by RNAi in SW480 cells (Fig. 35D) also shows that one or more of these genes regulates cyclin D1 and EGFR and also apoptosis (cleaved PARP).

Previous studies with BA demonstrated that the CB receptors were involved in repression of Sp-TFs (684); in contrast, CBD-induced downregulation of Sp1, Sp3 and Sp4 was not inhibited in SW480 (Fig. 36A) or RKO (Fig. 36B) cells cotreated with the CB1 and CB2 receptor antagonists AM251 and AM630 respectively. Moreover, we also confirmed by combined knockdown of the CB1 and CB2 receptors in SW480 (Fig. 36C) and RKO (Fig. 36D) cells that these receptor were not involved in the effects of

CBD on Sp TFs. Previous studies show that the effects of CBs may also involve other pathways and this includes induction of ceramide (proapoptotic) and ROS (677, 843); the latter response has previously been linked to downregulation of Sp1, Sp3 and Sp4

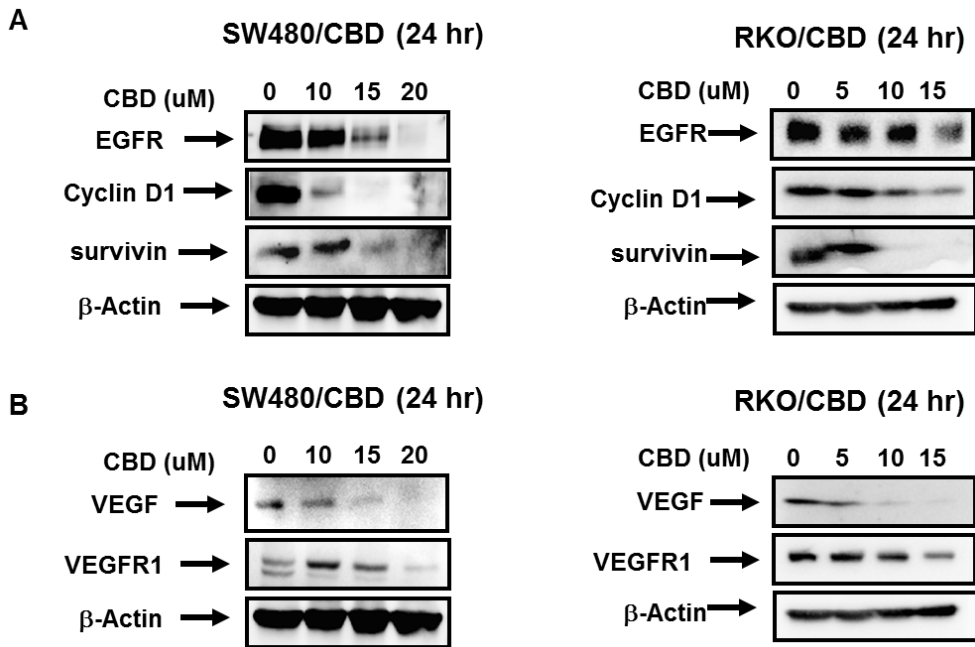


Figure 35. CBD downregulates Sp-regulated proliferative, survival (A) and angiogenic (B) gene products and Sp proteins (C) in SW480 and RKO cells. Cells were treated with 5-20 μ M of CBD for 24 hr, (D) cells were transfected with Sp1, Sp3 and Sp4 siRNAs and whole cell lysates were analyzed by western blots.

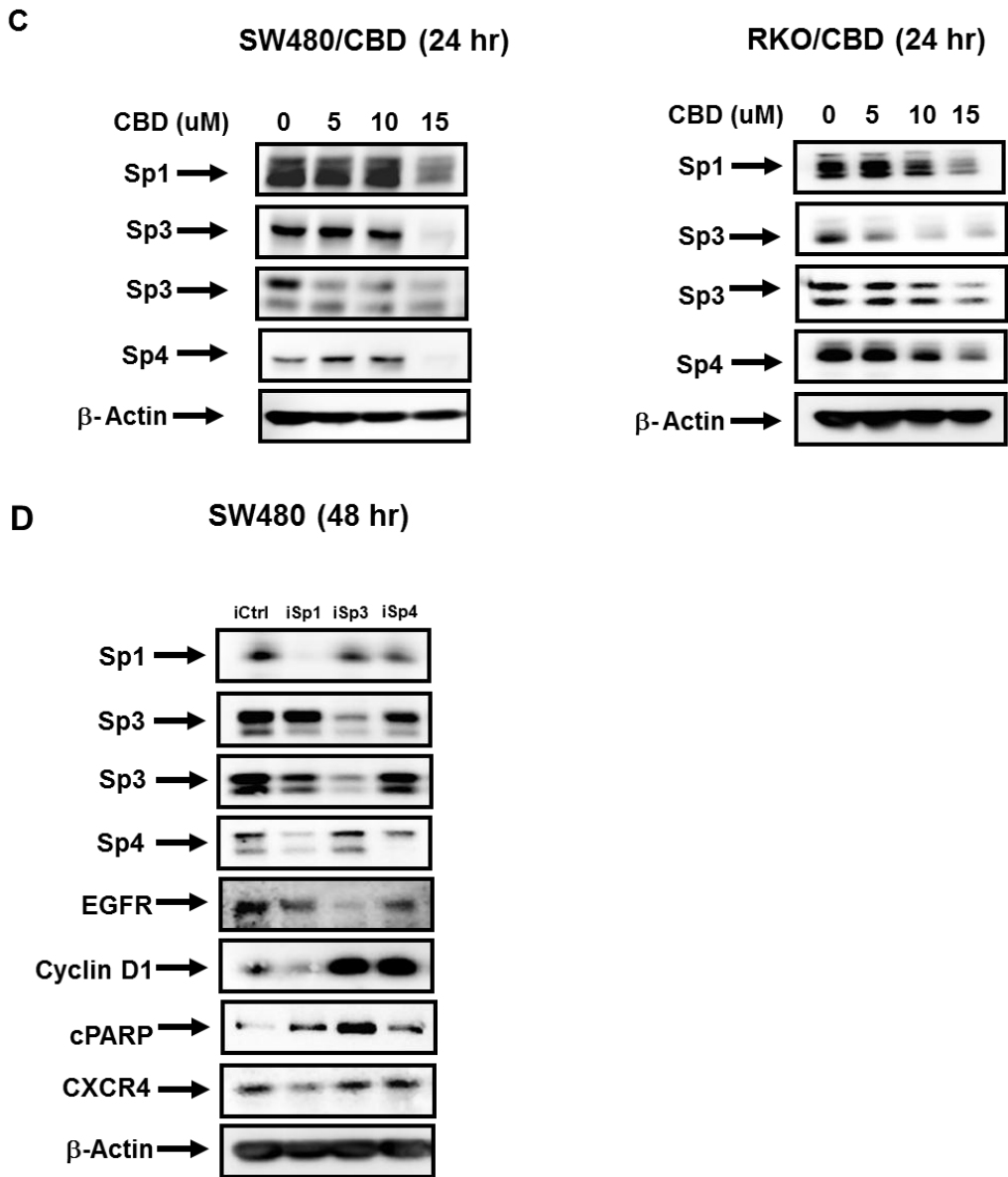


Figure 35. Continued

through ROS-dependent downregulation of miR-27a and induction of the Sp repressor, ZBTB10 (672, 675, 872, 887). Using SW480 cells as a model we observed that the ceramide synthase inhibitors fumonisin B1 or ISP1 (Fig. 37A) or the antioxidants DTT

or GSH (Fig. 37B) did not inhibit the effects of CBD on repression of Sp1, Sp3 and Sp4. Moreover, the proteasome inhibitors gliotoxin and lactacystin do not inhibit CBD-induced effects on Sp1, Sp3 and Sp4 (Fig. 37C) and therefore this degradation pathway which plays a role in drug-induced Sp degradation in some cancer cells (638-640) is not activated by CBD in SW480 cells.

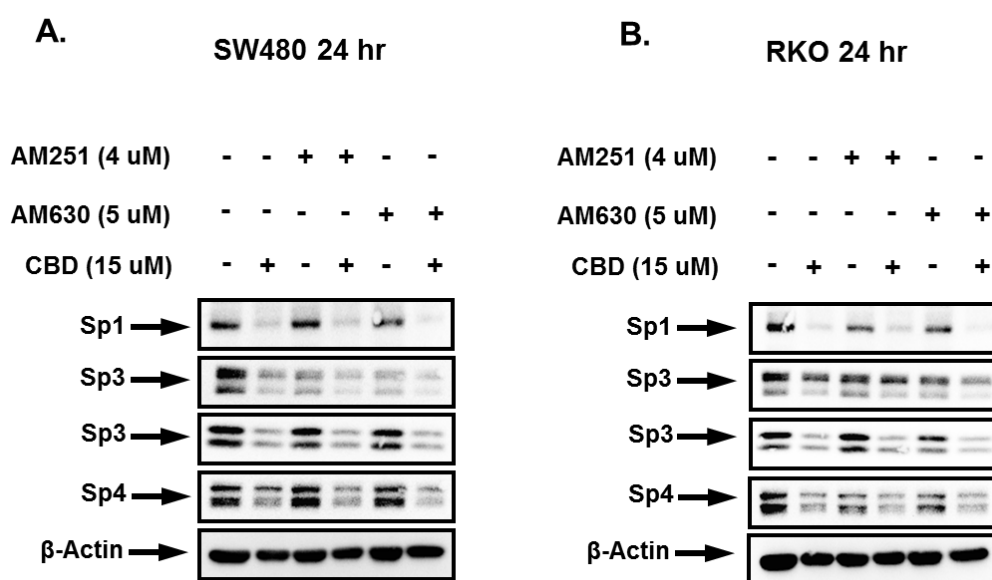


Figure 36. Effects of cannabinoid receptor antagonists, and cannabinoid receptor knockdown on CBD-mediated downregulation of Sp proteins. SW480 (A) and RKO (B) cells were pre-treated with AM251 (4 μ M) and AM630 (5 μ M) for 45 min followed by treatment with 15 μ M of CBD for 24 hr, and whole cell lysates were analyzed by western blots. SW480 (C) and RKO (D) cells were transfected with siRNAs against CB1 and CB2 receptors, followed by treatment with CBD for 24 hr, and whole cell lysates were analyzed by western blots.

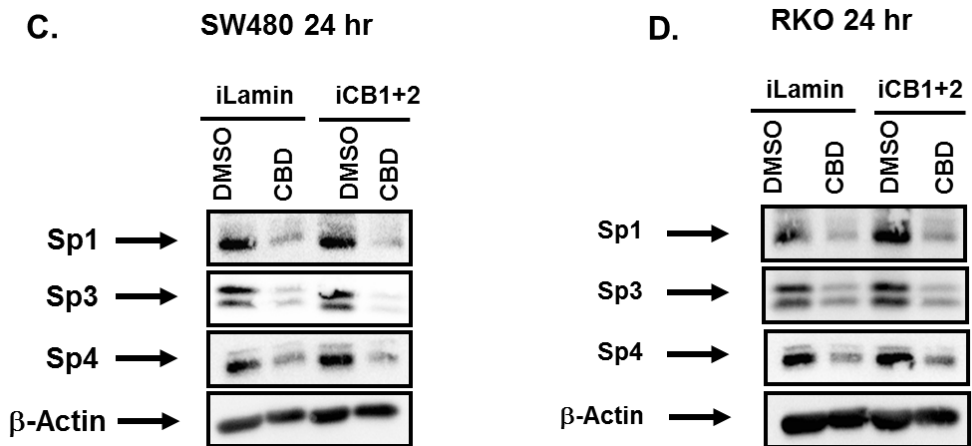


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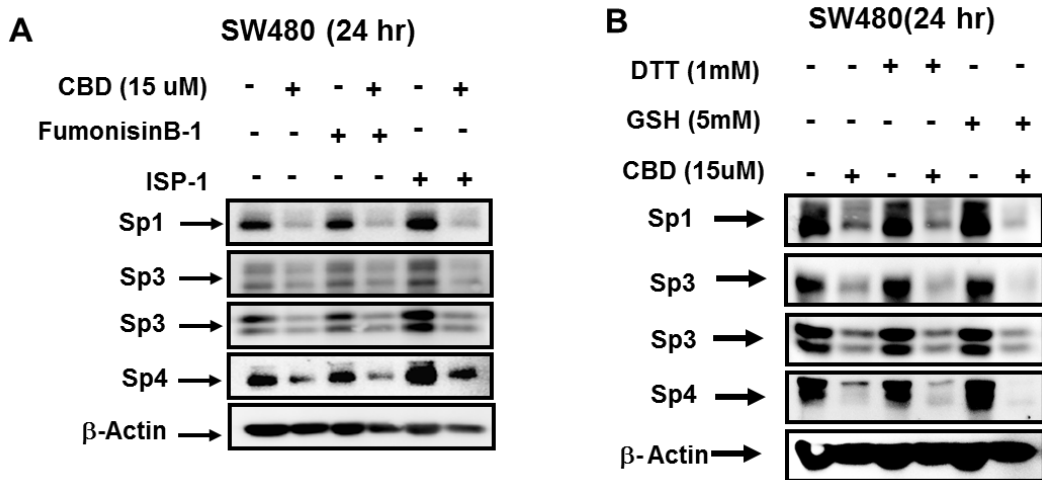


Figure 37. Effects of ceramide synthase inhibitors, antioxidants and proteasome inhibitors on CBD-mediated downregulation of Sp proteins. SW480 cells were pre-treated with fumonisin B1 (10 μ M), ISP1 (5 μ M) (A), DTT (1 mM) or GSH (5 mM) (B), lactacystin (10 μ M) or gliotoxin (3 μ M) (C) for 45 min followed by treatment with 15 μ M of CBD for 24 hr, and whole cell lysates were analyzed by western blots.

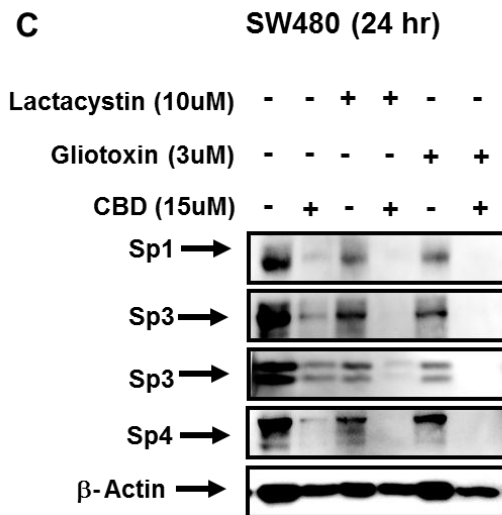


Figure 37. Continued

In previous studies we showed that CBD-induced apoptosis in LNCap and SW480 cells was attenuated by the phosphatase inhibitor SOV (858) and results in Figure 38A show that SOV significantly inhibited downregulation of Sp1, Sp3 and Sp4 by CBD in SW480 cells. CBD induces MKP-1 and MKP-5 in colon cancer cells (858) and both phosphatases have previously been linked to downregulation of Sp1 (832, 888); however, results in Figure 38B show that silencing of MKP-1 or MKP-5 does not alter CB-mediated effects on Sp1, Sp3 or Sp4 (Fig. 38B). Moreover, silencing of PP2A also does not affect this response (Fig. 38C) even though this phosphatase plays a role in WIN-mediated repression of Sp proteins in the same cell line (see Chapter III). We also observed that CBD induces expression of the Sp repressor ZBTB10 mRNA and protein (Fig. 38D) and suppresses levels of microRNA miR27a in SW480 cells and this is consistent with the effects of CBD on Sp1, Sp3 and Sp4 protein expression since previous studies show that ZBTB10 is regulated by miR27a and overexpression of

ZBTB10 or miR-27a antagonists downregulate Sp proteins in colon cancer cells (872). Current studies are focused on the pathways linking induction of phosphatases by CBD to the subsequent induction of ZBTB10.

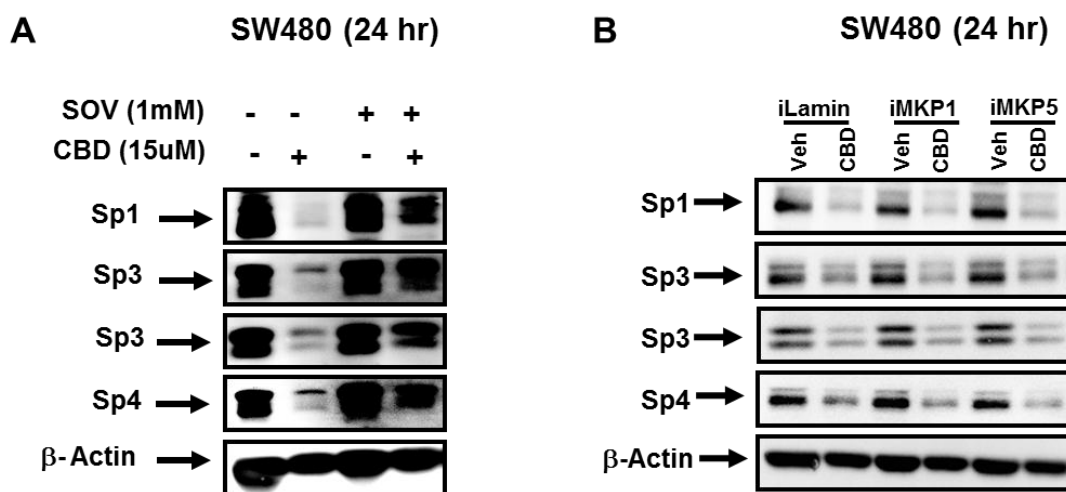


Figure 38. Effects of phosphatase inhibition on CBD-dependent downregulation of Sp proteins and CBD-dependent modulation of miR27a and ZBTB10 expression. (A) Cells were pre-treated with 0.35 mM SOV for 45 min followed by treatment with 15 μ M of CBD for 24 hr, and whole cell lysates were analyzed by western blots. Cells were transfected with siRNA against phosphatase MKP-1 (iMKP1) and MKP-5 (iMKP5) (B) and PP2A (iPP2A) (C), followed by treatment with CBD for 24 hr and whole cell lysates were examined for expression of Sp1, Sp3, Sp4 and MKP-1 and MKP-5 proteins, (D) SW480 cells were treated with DMSO or 15 μ M of CBD for 4, 8, 12, 18 and 24 hr, and whole cell lysates were analyzed for ZBTB10 protein by western blots. Cells were treated with DMSO or ethanol and 10-15 μ M of CBD for 24 hr and ZBTB10 expression and miR27a levels were determined by qPCR as described in the Materials and Methods. Results for RNA expression are expressed as means \pm SE for at least three separate determinations, and significant ($p < 0.05$) inhibition (**) and induction (*) by CBD are indicated.

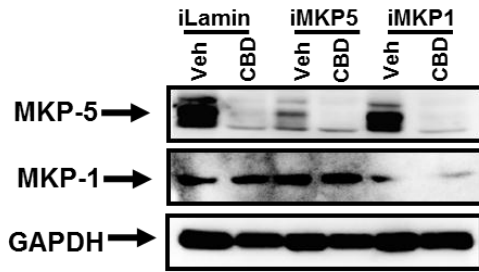
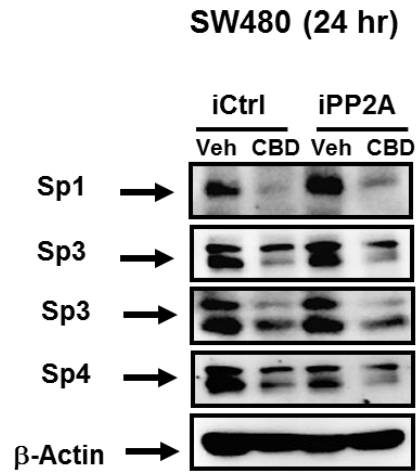
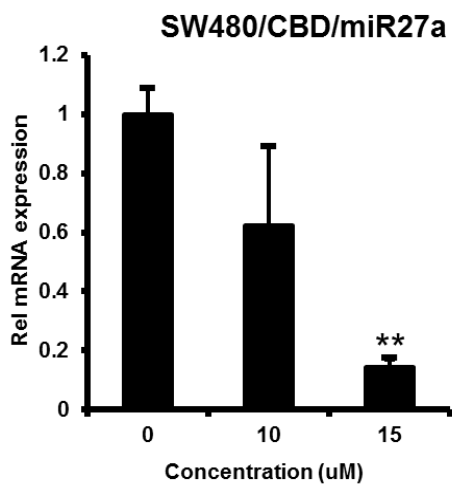
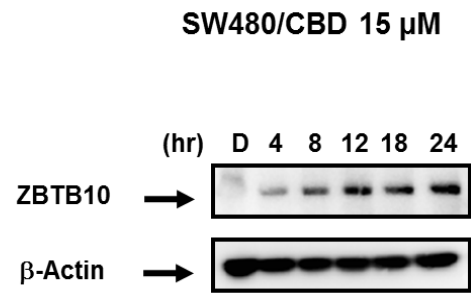
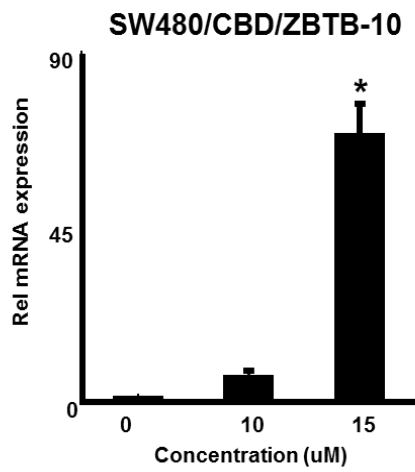
B**C****D**

Figure 38. Continued

DISCUSSION

CBs are emerging as an important class of anticancer agents that target multiple pro-oncogenic pathways. Results obtained using Δ^9 THC and other putative CB receptor agonists demonstrate that many of the critical pathways and genes that are modulated by CBs are CB1 and CB2 receptor-dependent. For example, Δ^9 THC induces stress responses in cancer cells and this includes stress-regulated proteins (p8) and other stress markers which result in the induction of apoptosis (836, 859). Induction of p8 by Δ^9 THC is partially blocked by CB1 and CB2 receptor antagonists and also by ceramide synthase inhibitors indicating an important role for ceramide in activating the stress pathways. Studies in this laboratory have been examining the anticancer activities of several phytochemicals and their derivatives as anticancer agents and one of these compounds, the naturally-occurring triterpenoid BA inhibits growth of multiple cancer cell lines in vitro and tumors in vivo (639, 667, 684, 836). Our results show that BA decreased expression of Sp1, Sp3 and Sp4 and pro-oncogenic Sp-regulated genes in prostate, colon and breast cancer cells. Subsequent studies showed that BA competitively bound the CB1 and CB2 receptors and in colon and breast cancer cells BA-mediated repression of Sp TFs was CB receptor-dependent and in colon cancer cells induction of ROS by BA also contributed to decreased expression of Sp TFs (667, 684).

These observations suggested that an important underlying mechanism of action of CBs may be due to downregulation of Sp1, Sp3, Sp4 and Sp regulated genes and we initiated studies on the synthetic CB WIN 55, 212-2 and CBD in colon cancer cells. Both

compounds induced apoptosis in LNCaP and SW480 cells and these effects were inhibited after cotreatment with the phosphatase inhibitor SOV (858). In this study we observed that like BA, CBD decreased expression of Sp1, Sp3, Sp4 and several pro-oncogenic Sp-regulated genes (Fig. 35). These results are consistent with the growth inhibitory and pro-apoptotic effects of CBD (Fig. 34); moreover silencing of Sp1, Sp3 and Sp4 by RNA interference confirmed that one or more of these proteins regulated expression of cyclin D1 and EGFR and induce expression of cleaved PARP, a marker of apoptosis (Fig. 35D).

Previous studies on the mechanisms of drug-induced downregulation of Sp TFs have identified two major pathways, namely induction of caspases or proteasomes (638, 639, 889) that degrade Sp proteins or a mechanism which involves transcriptional repression of Sp TFs and Sp-regulated genes (667, 672, 675, 887). This latter pathway has been characterized for drugs that induce ROS and this response is both drug and cell context dependent. For example, curcumin induced ROS-dependent repression of Sp proteins in pancreatic cancer cells (887) but proteasome dependent degradation in bladder cancer cells (640). Although the mechanism of ROS-mediated repression of Sp1, Sp3 and Sp4 is not fully understood, it has been shown that ROS induces ZBTB10 or another Sp repressor ZBTB4 through downregulation of miR-27a or miR-20a/miR-17-5p respectively (672, 887). Both ZBTB10 and ZBTB4 are transcriptional repressors that competitively bind and displace Sp proteins from GC-rich cis-elements and this results in decreased gene expression (641, 890). Results obtained in this study show that CBD

does not induce Sp degradation through activation of proteasomes (Fig.37C) and cotreatment of colon cancer cells with CBD plus antioxidants (DTT or GSH) does not block Sp downregulation (Fig. 37B). In contrast, we observed that CBD induced the transcriptional repressor ZBTB10 and decreased levels of miR27a (Fig. 38D) and current studies are investigating the role of CBD-induced phosphatases in mediating repression of Sp1, Sp3 and Sp4 and in the induction of ZBTB10. Thus CBD represents another cannabinoid (in addition to BA) that acts, in part, as an anticancer agent through repression of Sp proteins. These data exhibit some overlap with results of ongoing studies using the synthetic CB WIN-55, 212-2 which also induces phosphatase-dependent repression of Sp1, Sp3 and Sp4 through CB receptor-independent pathways however, the effects of WIN are PP2A-dependent. It will be important to determine the prevalence of this mechanism for other CBs in various cancer cell lines since this will facilitate clinical development of CBs for treatment of cancer in combination with other anticancer drugs.

CHAPTER V

1,1-BIS(3'-INDOLYL)-1-(*P*-BROMOPHENYL)METHANE AND RELATED COMPOUNDS REPRESS SURVIVIN AND DECREASE γ -RADIATION-INDUCED SURVIVIN IN COLON AND PANCREATIC CANCER CELLS*

INTRODUCTION

Survivin is a 16.5 kDa protein and a member of the inhibitor of apoptosis (IAP) family of proteins that suppress caspase-3, caspase-7 and caspase-9 and thereby inhibits both the extrinsic and intrinsic apoptotic pathways (891-893). In addition to the anti-apoptotic activity of survivin, this protein also acts as a subunit of the chromosomal passenger complex and plays a role in cell division and cell cycle control (894). Survivin expression in normal tissues is variable (895-899); however, several studies show that survivin is more highly expressed in precancerous and tumor tissue derived from most solid tumors and hematological malignancies (893, 895, 900, 901). Intuitively, overexpression of survivin and other IAPs is not unexpected since cancer cells and tumors typically exhibit deregulated proliferative and survival pathways.

*Reprinted with permission from “1,1-bis(3'-indolyl)-1-(*p*-bromophenyl)methane and related compounds repress survivin and decrease γ -radiation-induced survivin in colon and pancreatic cancer cells” by Sreevalsan S, Jutooru I, Chadalapaka G, Safe SH, 2009. *Int J Oncol*, 35 (5) 1191-1199, Copyright [2009] by: International Journal of Oncology.

High levels of survivin expression in cancer cells are due, in part, to several factors which regulate survivin at the transcriptional and posttranscriptional level. For example, several cancer cell lines overexpress specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 which in turn increase survivin expression through interaction with GC-rich *cis*-elements in the survivin promoter (863, 902, 903). Nuclear factor kappa B (NFκB) is overexpressed in multiple tumor types and in some leukemia cell lines, expression of survivin is regulated by NFκB (904). In other leukemia cell lines, Krüppel-like factor 5 (KLF5) upregulates survivin expression and inhibits p53 which mediates suppression of survivin (905, 906).

Several studies report that survivin overexpression is a negative prognostic factor for cancer patient survival (893, 907-911). For example, increased nuclear (but not cytosolic) survivin expression was associated with a decreased overall survival for breast cancer patients (907). Survivin expression in tumors is not only a negative prognostic factor, but expression of this gene has also been linked to drug resistance associated with chemotherapy and radiotherapy (912-919). Resistance to antiandrogen and cis-platin therapy for treatment of prostate cancer is mediated by survivin (912, 913) and taxol resistance has also been linked to induction of survivin in cancer cell lines and tumors (914, 915). Radiotherapy is important for treating several types of tumors and radioresistance is related, in part, to induction of survivin in tumors undergoing radiotherapy (916-919). These observations suggest that survivin may be an important

chemotherapeutic target for cancer chemotherapy and agents that decrease survivin expression could also serve to ameliorate drug- and radiotherapy-resistant tumors in which survivin expression is increased.

Research in this laboratory has identified a series of 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl) methanes (C-DIMs) that inhibit pancreatic, colon, prostate, bladder and breast cancer cell and tumor growth (920-925). C-DIMs containing *p*-phenyl, *p*-*t*-butyl and *p*-trifluoromethyl substituents activate peroxisome proliferator-activated receptor γ (PPAR γ) (920-923), whereas *p*-methoxy and unsubstituted C-DIMs activate the orphan receptor Nur77 (924, 925). Other receptor-inactive C-DIMs including 1,1-bis(3'-indolyl)-1-(*p*-bromophenyl)methane (DIM-C-pPhBr) and the corresponding 2,2'-dimethyl derivative (2,2'-diMeDIM-C-pPhBr) also induce apoptosis in cancer cells through activation of ER stress (926, 927). In this study, we demonstrate that DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr decrease survivin expression in pancreatic and colon cancer cells and in combination with radiotherapy, these compounds decrease radioresistance and inhibit radiation-induced survivin expression.

MATERIALS AND METHODS

Chemicals, Antibodies, Plasmids, and Reagents

C-DIMs were synthesized in this laboratory from the condensation of indole or substituted indole plus a substituted benzaldehyde derivative and confirmed by gas chromatography-mass spectrometry as described previously (920, 926, 927). Cleaved

poly (ADP-ribose) polymerase (PARP) antibody was obtained from Cell Signaling (Danvers, MA). Survivin antibody was purchased from R and D Systems (Minneapolis, MN); β -actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO); and proteasome inhibitor MG132 was purchased from Calbiochem (San Diego, CA). The pSurvivin-269 constructs containing survivin promoter inserts (positions -269 to +49) linked to luciferase reporter gene was 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

SW480 human colon carcinoma cell lines were provided by Dr. Stanley Hamilton (M.D. Anderson Cancer Center, Houston, TX). Panc28 cell line was a generous gift from Dr. Paul Chiao, The University of Texas M.D. Anderson Cancer Center (Houston, TX). Human pancreatic Panc1 cancer cell line were obtained from the American Type Culture Collection (Manassas, VA). The L3.6pl cell line was developed at The University of Texas M.D. Anderson Cancer Center. Cell lines were maintained in DMEM/F-12 (Sigma, St. Louis, MO) supplemented with 2.2% sodium bicarbonate, 2.2% bovine serum albumin, 5% fetal bovine serum, and 10 ml/L of 100X antibiotic antimycotic solution (Sigma). Cells were maintained at 37°C in the presence of 5% CO₂.

Cell Proliferation Assay

Pancreatic and colon cancer cells (3×10^4 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. Cells were then trypsinized and counted after 24 hr using a Coulter Z1 cell counter. Each experiment was done in triplicate, and results were expressed as means \pm SE for each set of experiments.

Transfection and Luciferase Assay

Colon cancer cells were plated in 12-well plates at 1×10^5 per well in DMEM/Ham's F-12 media supplemented with 2.5% charcoal-stripped FBS. After 16 to 20 hr, reporter gene constructs [i.e. pSurvivin-269 (0.04 Ag) and β -gal (0.04 Ag)] were transfected by Lipofectamine (Invitrogen) according to the manufacturer's protocol. Five hr after 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 μ L of 1X reporter lysis buffer, and 30 μ L 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 Blots

Colon and pancreatic cancer cells were seeded in DMEM:Ham's F-12 medium. Twenty-four hr later, cells were treated with either vehicle (DMSO) or the indicated compounds for 24 hr or pretreated with the proteasome inhibitor, MG132 (10 μ M) for 1 hr and then treated with the compounds. Cells were lysed using high-salt buffer and Protease Inhibitor Cocktail. Protein lysates were separated on 12% SDS-PAGE 120 V for 4 hr. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by wet electroblotting and the membranes were incubated with primary antibody. After washing with TBST, the PVDF membrane was incubated with secondary antibody in 5% TBST-Blotto and the membrane was washed with TBST for 10 min, incubated with chemiluminescence substrate for 1 min, and exposed to Kodak autoradiography film.

Irradiation

Colon and pancreatic cancer cells were plated and exposed to varying doses of γ -radiation generated from a Theratron 80 cobalt-60 teletherapy machine (Atomic Energy of Canada) with a dose rate of 80.166 cGy/minute (same as 0.8016 Gray per minute or 80.166 rads/minute), for a 30x30 cm field, at a source-surface distance of 80 cm. The irradiated cells were then treated with the indicated compounds after 8 hr, and cells were counted or lysates were obtained after the indicated treatment times.

Real-Time PCR

Total RNA was isolated using the RNeasy Protect Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 30 μ L of RNase-free water and stored at -80°C . RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. cDNA was prepared from the SW480 colon and Panc28 pancreatic cancer cell lines at different time intervals using a combination of oligodeoxythymidylic acid and dNTP mix (Applied Biosystems, Foster City, CA) and Superscript II (Invitrogen). Survivin primers (forward 5'-ATG GCC GAG GCT GGC TTC ATC-3'; reverse 5'-ACG GCG CAC TTT CTT CGC AGT T-3') were acquired from IDT (Skokie, IL). Each PCR was carried out in triplicate in a 20- μ L volume using SYBR Green Master mix (Applied Biosystems) for 15 min at 95°C for initial denaturing, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min in the ABI Prism 7500 sequence detection system (Applied Biosystems). Values for each gene were normalized to expression levels of TATA-binding protein (TBP).

RESULTS

Treatment of colon and pancreatic cancer cells with DIM-C-pPhBr or 2,2'-diMeDIM-C-pPhBr induces apoptosis and ER stress (926, 927) and results in Figure 39A show that after treatment of SW480 cells with these compounds for 24 hr, there was significant concentration-dependent decrease in cell proliferation. Both C-DIMs decreased cell numbers at concentrations of 10 or 15 μM and at a concentration of 5 μM , DIM-C-pPhBr also inhibited SW480 cell growth, whereas 2,2'-diMeDIM-C-pPhBr was inactive.

Figures 39B and 39C show that both compounds also induce caspase-dependent PARP cleavage (15 and 20 μM) and at these same concentrations there was a parallel decrease in survivin expression in SW480 cells. In addition, we also observed that both C-DIM compounds decreased survivin mRNA expression after treatment for 24 hr (Fig. 39D).

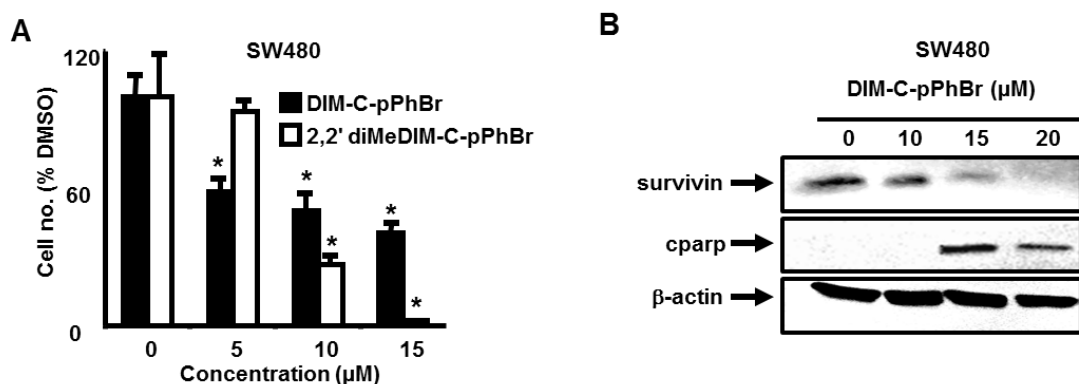


Figure 39. Effects of C-DIMs on SW480 cell proliferation and expression of survivin and cleaved PARP. Concentration-dependent effects on cell proliferation (A) and survivin and c-parp protein expression (B and C). Cells were treated with DMSO and different concentrations of DIM-C-pPhBr or 2,2'-diMeDIM-C-pPhBr for 24 hr. Cells were counted (A) or whole cell lysates were analyzed by western blots as described in the Materials and Methods. (D) Survivin mRNA levels. Cells were treated with DMSO or 10-20 μM of C-DIM compounds, and survivin mRNA levels were determined by real time PCR as described in the Materials and Methods. A significantly ($p < 0.05$) decreased response is indicated (*). β -Actin served as a loading control for all western blots in Figures 39 –27.

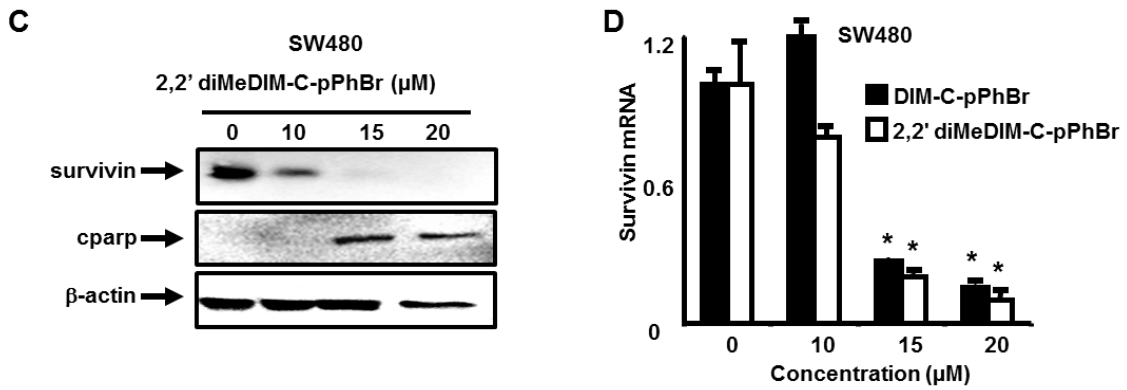


Figure 39. Continued

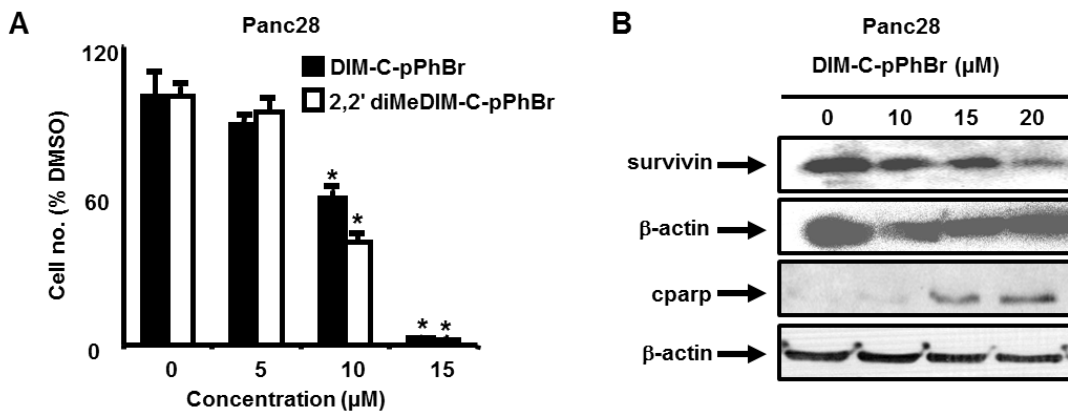


Figure 40. Effects of C-DIMs on Panc28 cell proliferation and expression of survivin and c-parp. Concentration-dependent effects on cell proliferation (A) and survivin and c-parp protein expression (B and C). Cells were treated with DMSO and different concentrations of DIM-C-pPhBr or 2,2'-diMeDIM-C-pPhBr for 24 h. Cells were counted (A) or whole cell lysates were analyzed by Western blot analyses as described in Materials and methods. (D) Survivin mRNA levels. Cells were treated with DMSO or 10-20 μM of C-DIM compounds, and survivin mRNA levels were determined by real-time PCR as described in Materials and methods. *A significantly ($p < 0.05$) decreased response is indicated.

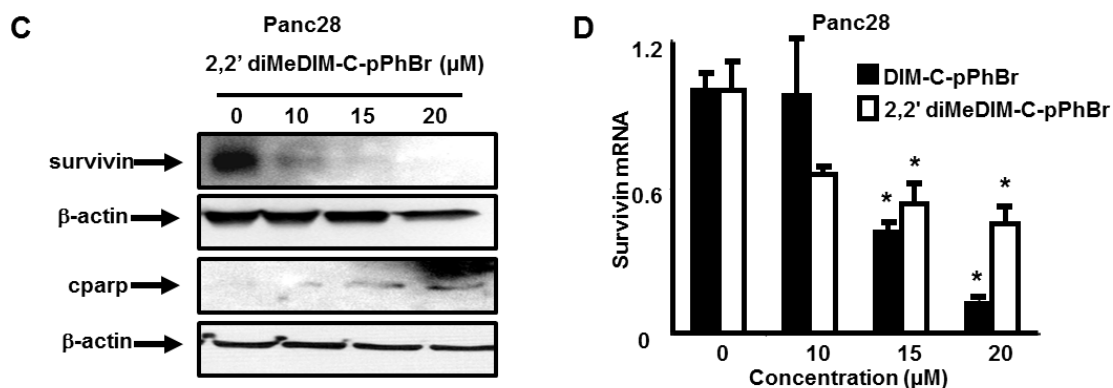


Figure 40. Continued

DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr also inhibited Panc28 pancreatic cancer cell growth (Fig. 40A) and significant inhibition was observed at concentrations of 15-20 μm. DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr also induced PARP cleavage and decreased survivin protein levels in Panc28 cells (Figs. 40B and 40C) and this was also accompanied by decreased survivin mRNA levels. All of these responses were observed after treatment of SW480 or Panc28 cells for 24 hr. IC₅₀ values for growth inhibition after treatment with DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr were 10.8 and 8.3 μM (SW480) and 9.4 and 8.8 μM (Panc28), respectively.

We also investigated the time dependent effects of DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr on survivin protein and cleaved PARP expression using a relatively high concentration (20 μM) of both compounds in order to determine differences between the temporal expression of both proteins. In SW480 cells, 20 μM DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr decreased levels of survivin protein within 2 hr after treatment,

whereas cleaved PARP protein was observed only after treatment for 12 hr (Figs. 41A and 41B). Similar results were observed in Panc28 cells (Fig. 41C and 41D); however, the extent of survivin degradation after treatment for 2 hr was lower in Panc28 compared to SW480 cells. These results show that there was a lag between the loss of survivin and induction of cleaved PARP in both cell lines.

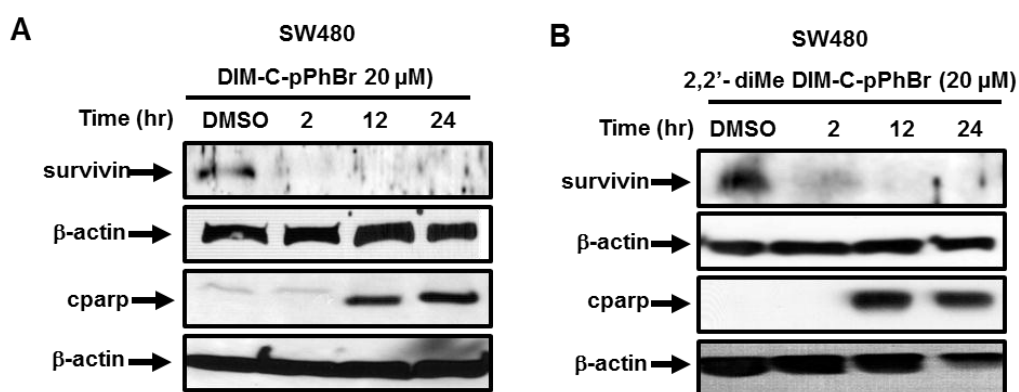


Figure 41. Time course effects of DIM-C-pPhBr (20 μM) and 2,2'-diMeDIM-C-pPhBr (20 μM) on survivin and c-parp expression. SW480 cells were treated with DIM-C-pPhBr (A) or 2,2'-diMeDIM-C-pPhBr (B) and Panc28 cells were treated with DIM-C-pPhBr (C) or 2,2'-diMeDIM-C-pPhBr (D) for 2, 12 or 24 hr. DMSO treatment served as a vehicle control. Whole cell lysates were analyzed by western blots as described in the Materials and Methods.

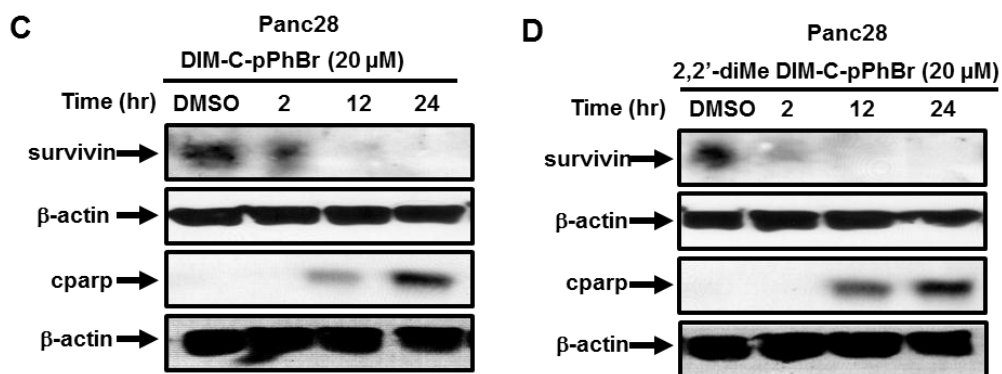


Figure 41. Continued

Figures 42A and 42B illustrate the effects of 20 μ M DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr on survivin mRNA levels in SW480 and Panc28 cells. Both compounds either had no effect or induced survivin mRNA levels after treatment of SW480 or Panc28 cells for 2 hr; significant inhibition of survivin mRNA levels was observed in SW480 and Panc28 cells after 12 hr; however, the magnitude of survivin mRNA repression was more pronounced in the colon cancer cells. Thus, the effects of DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr on survivin mRNA levels were observed at later time points compared to the rapid downregulation (within 2 hr) of survivin protein (Fig. 41).

We also confirmed that DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr decreased luciferase activity in SW480 cells transfected with pSurvivin-269, a construct containing the -269 to +49 region of the survivin promoter (Fig. 42C). Results were not obtained in Panc28 cells due to low transfection efficiencies in this cell line. Since DIM-C-pPhBr and 2,2'-diMeDim-C-pPhBr decreased survivin protein but not mRNA levels within 2 hr after treatment, we investigated the effects of these compounds alone and in combination with

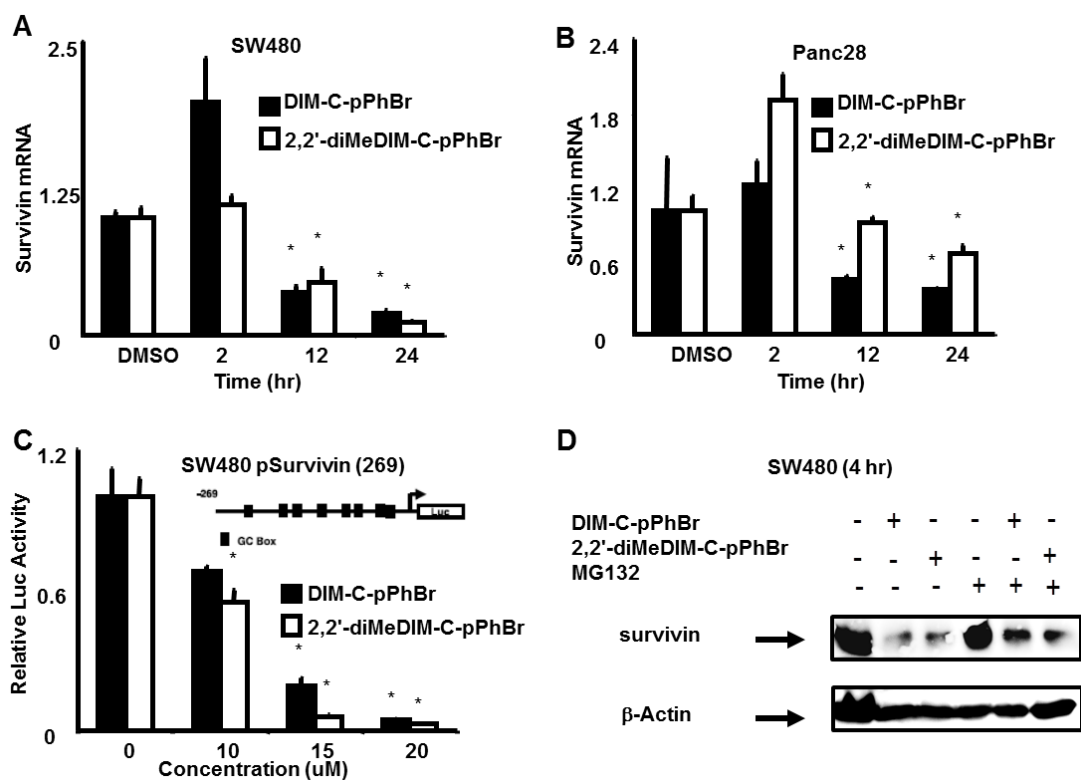


Figure 42. C-DIM-dependent effects on survivin expression. Changes in survivin mRNA expression in SW480 (A) and Panc28 (B) cells. Cells were treated with 20 μ M DIM-C-pPhBr or 2,2'-diMeDIM-C-pPhBr for 24 hr, and survivin mRNA levels were determined by real time PCR as described in the Materials and Methods. (C) Effects on the survivin promoter. SW480 cells were transfected with pSurvivin(269) and treated with DMSO or the C-DIM compounds. Luciferase activity was determined as described in the Materials and Methods. (D) Effects of MG132. SW480 cells were treated with DMSO, C-DIM compounds alone (20 μ M), 10 μ M MG132, or a combination of C-DIMs plus MG132 for 4 hr. Whole cell lysates were analyzed by western blots as described in the Materials and Methods. Significantly ($p < 0.05$) decrease mRNA levels or luciferase activity (compared to DMSO) is indicated (*).

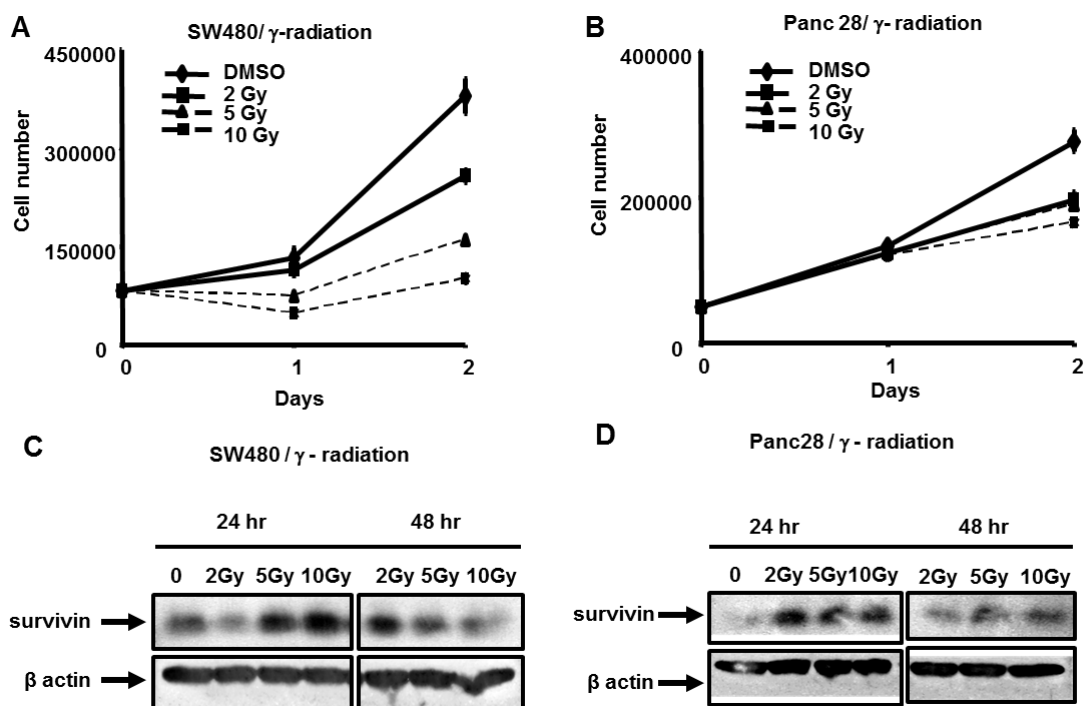


Figure 43. Effects of γ -radiation on cell proliferation and survivin expression. SW480 (A) and Panc28 (B) cell growth and expression of survivin in SW480 (C) and Panc28 (D) cells. Cells were treated with DMSO (control) and irradiated with 2, 5 or 10 Gy for 24 or 48 hr, and cell number and survivin protein expression (from whole cell lysates) were determined as described in the Materials and Methods.

the proteasome inhibitor MG132 after treatment of SW480 cells for 4 hr (Fig. 42D). The results show that C-DIM-dependent downregulation of survivin protein was partially reversed by the proteasome inhibitor, suggesting that activation of proteasomes contributed to the early decrease in survivin expression.

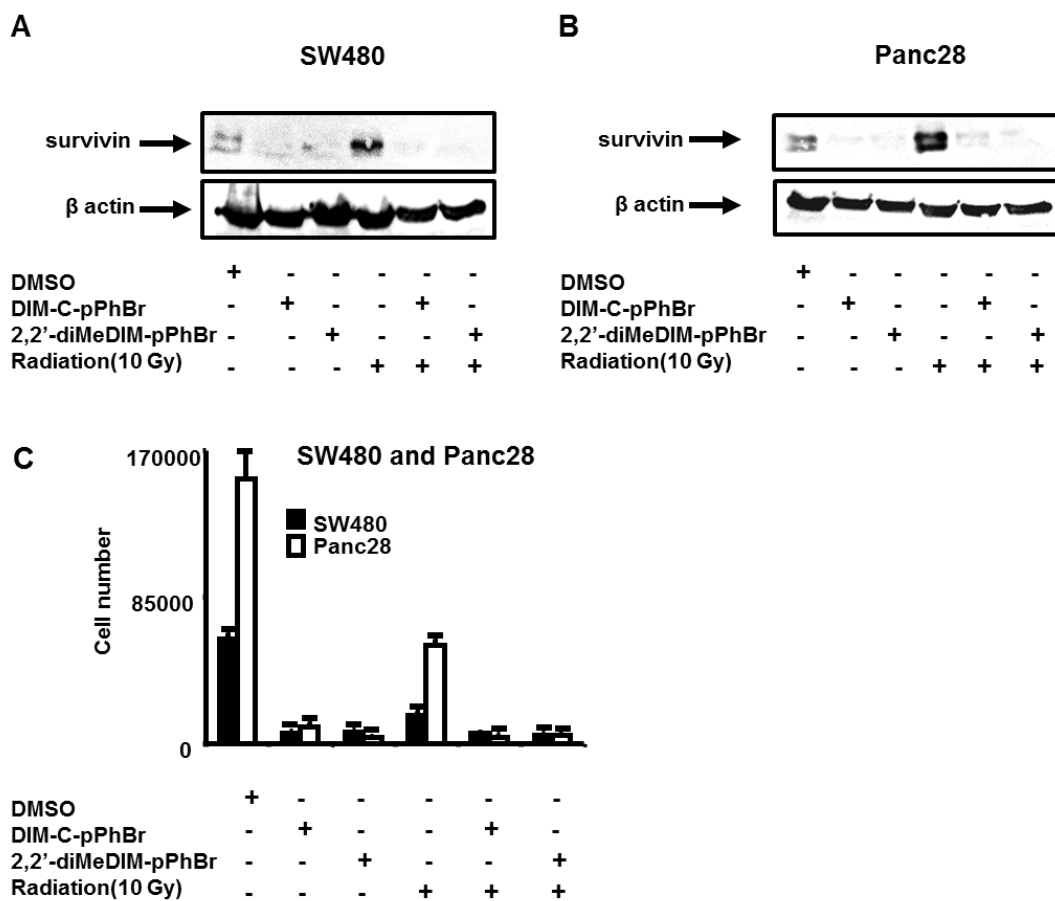


Figure 44. Interactions of γ -radiation and C-DIMs. (A) Effects of C-DIM on γ -radiation-induced survivin protein expression. Cells were treated with DMSO, C-DIMs (20 μ M), radiation (10 Gy), or C-DIMs plus radiation for 24 hr. Whole cell lysates were analyzed by western blots as described in the Materials and Methods. Effects of C-DIMs and radiation on proliferation of SW480 (B) and Panc28 cells (C). Cells were treated with DMSO, C-DIMs and radiation as described in (A), and cell numbers were determined as described in the Materials and Methods. (D) Concentration-dependent effects of C-DIMs on γ -radiation-induced survivin protein expression. SW480 cells were treated with DMSO; 5, 10 or 15 μ M DIM-C-pPhBr or 2,2'-diMeDIM-C-pPhBr alone; or in combination with γ -radiation for 24 hr. Whole cell lysates were analyzed by western blots as described in the Materials and Methods.

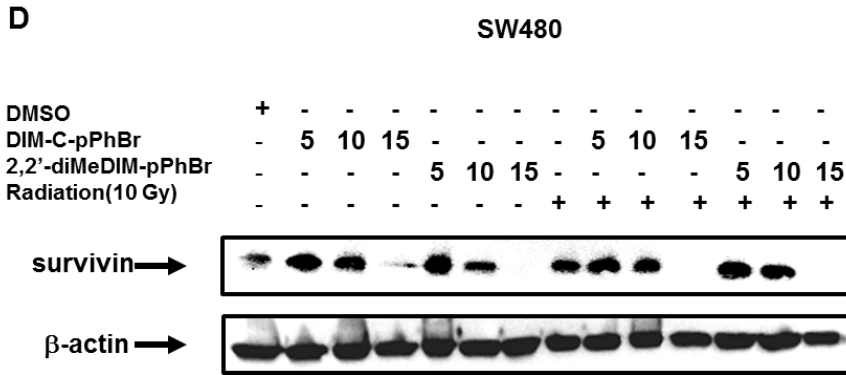


Figure 44. Continued

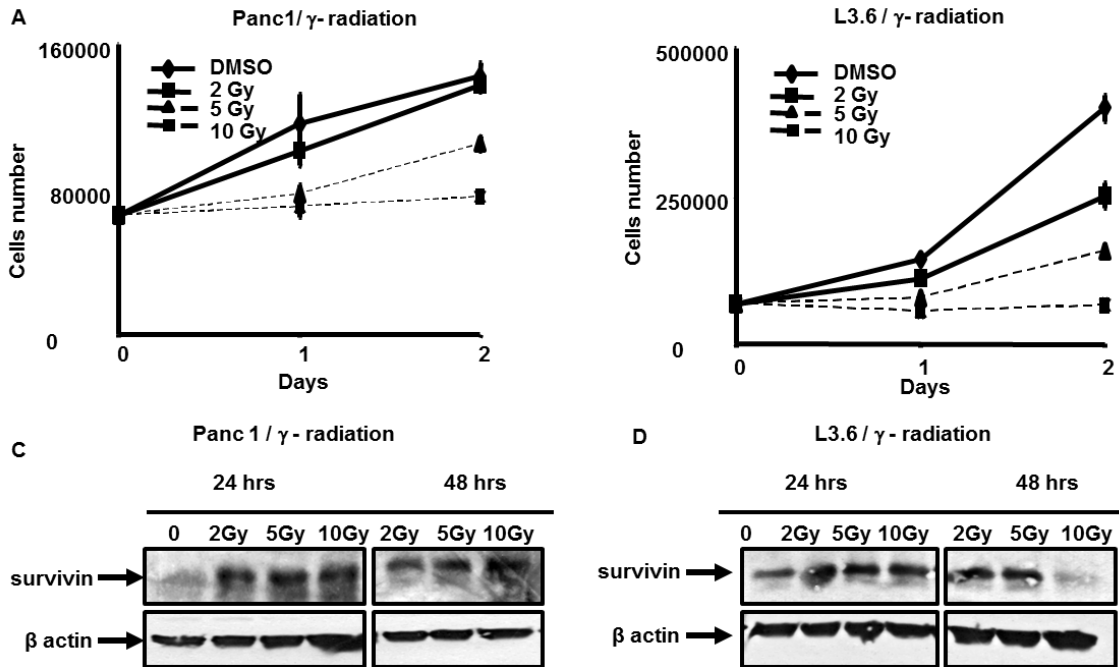


Figure 45. Effects of γ -radiation on growth of Panc1 (A) and L3.6pl (B) pancreatic cancer cells and expression of survivin in Panc1 (C) and L3.6pl (D) cells. Experiments were performed as described in the Materials and Methods. β -Actin was used as a protein loading control.

Since DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr inhibit survivin protein expression, the interactions of these compounds with radiotherapy were investigated. Figure 43A illustrates the effects of γ -radiation on proliferation of SW480 cells. Cells were administered doses of 2, 5 and 10 Gy using a Theatron 80 cobalt-60 teletherapy instrument and the effects of radiation on cell growth were determined after 24 and 48 hr. This cell line was responsive to radiation-induced inhibition of cell growth and after 24 hr, significant inhibition was observed using 5 and 10 Gy (but not 2 Gy) and all three doses of radiation inhibited cell growth after 48 hr. Using a similar protocol, γ -radiation also inhibited Panc28 cell proliferation (Fig. 43B); however, this cell line was clearly more resistant to radiotherapy than SW480 cells over this time period and significant growth inhibition was observed only after 24 hr. The effects of γ -radiation on survivin expression were also investigated in SW480 (Fig. 43C) and Panc28 (Fig. 43D) cells and in both cell lines, 5 and 10 Gy induced survivin expression after radiation for 24 hr, whereas induction of survivin was either decreased or not observed after 48 hr. γ -Radiation also induced survivin and decreased proliferation of Panc1 and L3.6pl pancreatic cells and both cell lines were more responsive than Panc28 cells to the antiproliferative activity of γ -radiation (Fig. 45).

The combined effects of γ -radiation and C-DIM compounds on survivin expression are summarized in Figures 44A and 44B. Treatment of SW480 cells with 20 μ M DIM-C-pPhBr or 2,2'-diMeDIM-C-pPhBr for 24 hr showed that the C-DIMs alone decreased survivin, γ -radiation alone increased survivin and C-DIMs in combination with radiation

decreased radiation-induced survivin expression. Similar effects were observed on cell numbers in SW480 or Panc28 cells (Fig. 44C); however, the high concentrations (20 μ M) of the C-DIM alone significantly decreased cell proliferation so that the interactions with γ -radiation were not apparent. We also examined the effects of lower concentrations of DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr (5, 10 and 15 μ M) on inhibition of γ -radiation-induced induction of survivin in SW480 cells (Fig. 44D). Inhibition was only observed using 15 μ M concentrations of C-DIMs and these concentrations alone also decreased levels of survivin protein. These results demonstrate that combinations of C-DIMs plus γ -radiation decrease radiation-induced survivin which plays a role in radioresistance.

DISCUSSION

Overexpression of survivin in cancer cell lines and tumors coupled with the negative prognostic significance of this gene for the survival of patients with some tumors has heightened interest in survivin as a potential drug target (893, 895, 901). It is also important to evaluate the effects of both old and new drugs on survivin expression since it has been demonstrated that increased expression of survivin by taxol-like drugs and radiotherapy can lead to therapy resistance (914-919). Several reports show that diverse drugs can downregulate survivin expression and these include: vitamin D3 and related analogs in leukemia and breast cancer cells (928); PPAR γ agonists in breast cancer cells (929); doxorubicin, histone deacetylase inhibitors and lovastatin (an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase) in colon cancer cells (930-932);

cyclooxygenase-2 inhibitors in glioblastoma and pancreatic cancer cells (933) and γ -tocotrienol in human embryonic kidney A93 cells (934). The mechanisms of survivin downregulation by these drugs are dependent on cell context.

Previous studies in this laboratory showed that PPAR γ -active C-DIMs downregulate survivin expression in MDA-MB-231 breast cancer cells after prolonged treatment; however, this was not accompanied by apoptosis (922). In contrast, DIM induced growth inhibition, apoptosis and downregulated survivin expression in MDA-MB-231 cells (935). DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr do not activate PPAR γ or Nur77 but induced apoptosis in colon and pancreatic cancer cells, and activation of apoptosis by these compounds was due, in part, to ER stress-dependent upregulation of death receptor 5 (926, 927). In this study, we used these same compounds to investigate their effect on survivin expression in colon and pancreatic cancer cells and their potential interactions with γ -radiation. Like many other anticancer agents, C-DIMs potentially activate multiple pathways and we focused on some of the early responses induced by these compounds within 24 hr after treatment. Concentration-dependent studies indicate that 10-20 μ M DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr decrease survivin protein expression in SW480 and Panc28 cells (Figs. 39 and 40) and this is accompanied by caspase-dependent PARP cleavage. Using a relatively high concentration (20 μ M) of the C-DIM compounds demonstrates that survivin downregulation occurs within 2 hr after treatment in both SW480 and Panc28 cells (Fig. 41), whereas in the same experiment PARP cleavage is not observed until a later time point. This does not

necessarily completely uncouple loss of survivin with induction of caspase-dependent PARP cleavage but indicates at least that this rapid downregulation of survivin is not paralleled by PARP cleavage. Interestingly, we also observed that rapid downregulation of survivin protein in SW480 and Panc28 cells treated with DIM-C-pPhBr or 2,2'-diMeDIM-C-pPhBr was not accompanied by decreased survivin mRNA levels after 2 hr since decreased transcription was not observed until 12-24 hr after treatment in both cell lines (Fig. 42). Moreover, the proteasome inhibitor MG132 blocked C-DIM-induced downregulation of survivin protein after treatment of SW480 cells for 4 hr (Fig. 42D). These results suggest that the C-DIM compounds downregulate survivin expression by both transcription-independent and -dependent pathways, and current studies are focused on the mechanisms associated with activation of these pathways by C-DIMs.

CHAPTER VI

SUMMARY

We hypothesized that the anticancer activity of cannabinoids (CBs) was linked to induction of phosphatases. The effects of cannabidiol (CBD) and the synthetic cannabinoid WIN-55,212 (WIN) on LNCaP (prostate) and SW480 (colon) cancer cell proliferation were determined by cell counting; apoptosis was determined by cleavage of poly(ADP)ribose polymerase (PARP) and caspase-3 (western blots), and phosphatase mRNAs were determined by real-time PCR. The role of phosphatases and CB receptors in mediating CBD- and WIN-induced apoptosis was determined by inhibition and receptor knockdown. CBD and WIN inhibited LNCaP and SW480 cell growth and induced expression of several phosphatase mRNAs, and the phosphatase inhibitor sodium orthovanadate significantly inhibited cannabinoid-induced PARP cleavage in both cell lines, whereas only CBD-induced apoptosis was CB1 and CB2 receptor-dependent. CB receptor agonists induce phosphatases and phosphatase-dependent apoptosis in cancer cell lines; however, the role of the CB receptor in mediating this response is ligand-dependent.

Cannabinoids (CBs) are currently in clinical trials for treatment of gliomas, and both plant-derived and synthetic CBs, such as the synthetic-mixed CB1 and CB2 receptor agonist CN1C=NC2=C1C(=O)N(C)C2 [WIN 55,212-2 (WIN)], inhibit growth of

multiple cancer cell lines via receptor-dependent and -independent pathways. In this study, WIN inhibited RKO, HT-29 and SW480 cell growth, induced apoptosis, and downregulated expression of survivin, cyclin D1, epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and its receptor (VEGFR1). WIN also decreased expression of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4, and this is consistent with the observed downregulation of the aforementioned Sp-regulated genes. In addition, we also observed by RNA interference (RNAi) that the oncogenic cap protein eIF4E was an Sp-regulated gene also downregulated by WIN in colon cancer cells. WIN-mediated repression of Sp proteins was not affected by CB receptor antagonists or by knockdown of the receptor but was attenuated by the phosphatase inhibitor sodium orthovanadate or by knockdown of protein phosphatase 2A (PP2A). WIN-mediated repression of Sp1, Sp3 and Sp4 was due to PP2A-dependent downregulation of microRNA-27a (miR-27a) and induction of miR-27a-regulated ZBTB10 which has previously been characterized as an "Sp repressor". The results demonstrate that the anticancer activity of the widely used synthetic cannabinoid WIN is due, in part, to PP2A-dependent disruption of miR-27a:ZBTB10 and ZBTB10-mediated repression of Sp transcription factors and Sp-regulated genes including eIF4E.

Cannabidiol (CBD) is a non-psychoactive phytocannabinoid present in the plant *cannabis sativa*. In addition to its use in treating cancer-related pain, nausea and reduced appetite, CBD is a promising anticancer agent that modulates kinase activities and other growth inhibitory, pro-apoptotic, antiangiogenic and antimetastatic pathways. In this

study 5-15 μ M CBD inhibited cell proliferation, induced annexin V staining and PARP cleavage in SW480 and RKO colon cancer cells. Treatment of SW480 and RKO cells with CBD also downregulated gene products associated with cell proliferation (EGFR, cyclin D1), angiogenesis (VEGF, VEGFR1) and cancer cell survival (survivin). RNA interference studies employing siRNAs for Sp1, Sp3 and Sp4 demonstrated that some of these genes such as cyclin D1 and EGFR are regulated by Sp proteins and concentrations at which CBD downregulated Sp-dependent genes also inhibited Sp1, Sp3 and Sp4. Co-treatment of SW480 cells with CB receptor antagonists AM251 and AM630 or siRNAs against CB1 and CB2 receptors did not reverse the effects of CBD on Sp transcription factors indicating that CBD-mediated repression of Sp proteins was CB receptor independent. Moreover, CBD-dependent downregulation of Sp proteins was not affected by ceramide synthase inhibitors, proteasome inhibitors or antioxidants but was reversed by the phosphatase inhibitor sodium orthovanadate. Knockdown of dual specificity phosphatases MKP-1 and MKP-5 or serine threonine phosphatase PP2a did not affect block the effects of CBD on Sp1, Sp3 and Sp4 and ongoing studies in the laboratory are focused on identifying the phosphatases involved in the anticancer effects of CBD. In addition CBD repressed levels of microRNA 27a and induced protein expression of the Sp repressor ZBTB10 which is regulated by miR-27a and these results indicate that CBD acts in part as an anti-cancer agent through downregulation of Sp proteins.

1,1-Bis(3'-indolyl)-1-(*p*-bromophenyl)methane (DIM-C-pPhBr) and the 2,2'-dimethyl analog (2,2'-diMeDIM-C-pPhBr) inhibit proliferation and induce apoptosis in SW480

colon and Panc28 pancreatic cancer cells. In this study, treatment with 10-20 μ M concentrations of these compounds for 24 hr induced cleaved PARP and decreased survivin protein and mRNA expression in both cell lines. However, results of time course studies show that DIM-C-pPhBr and 2,2'-diMeDIM-C-pPhBr decrease survivin protein within 2 hr after treatment, whereas survivin mRNA levels were decreased only at later time points indicating activation of transcription-independent and -dependent pathways for downregulation of survivin. In addition, we also observed that γ -radiation inhibited pancreatic and colon cancer cell growth and this was associated with enhanced expression of survivin after 24 (SW480) or 24 and 48 (Panc28) hr and correlated with previous studies on the role of survivin in radiation-resistance. However, in cells cotreated with γ -radiation plus DIM-C-pPhBr or 2,2'-diMeDIM-C-pPhBr, induction of survivin by γ -radiation was inhibited after cotreatment with both compounds, suggesting applications for these drugs in combination cancer chemotherapy with γ -radiation.

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