MECHANISM OF ACTION OF SULINDAC AND DIINDOLYLMETHANE ANALOGS AS ANTICANCER AGENTS

A Dissertation

by

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ABSTRACT

Cancer accounts for one in eight deaths worldwide and one in four deaths in the United States. Chemotherapy is the most common treatment option for cancer; however, many chemotherapeutic drugs have toxic side effects. Our studies focused on molecular mechanisms of two groups of relatively non-toxic agents that exhibit potent anticancer activities in colon and pancreatic cancer cells.

The first group includes a nonsteroidal anti-inflammatory drug (NSAID), sulindac, and its sulfone and sulfide metabolites; among them, sulindac sulfide was the most active compound in inhibiting colon cancer cell proliferation in our studies. Sulindac sulfide induced reactive oxygen species (ROS), decreased oncogenic microRNA-27a and upregulated the transcriptional repressor, ZBTB10. As a result, sulindac sulfide downregulated Sp1, Sp3 and Sp4 transcription factors and Sp-regulated pro-oncogenic genes, including survivin, Bcl-2, epidermal growth factor receptor (EGFR), cyclin D1, NFκB-p65 and vascular endothelial growth factor (VEGF). Our results suggest that the anticancer activity of sulindac sulfide is due, in part, to downregulation of Sp-dependent gene expression.

The second group includes methylene-substituted analogs of a natural compound diindolylmethane (i.e. C-DIM). We screened a library of C-DIMs and identified several activators of NR4A2 nuclear receptor (Nurr1) by transactivation assays using GAL4-UAS system; additional assays using NBRE and NurRE response

elements confirmed that C-DIMs transactivated Nurr1 in pancreatic cancer cells. We also investigated the structure-activity relationships of C-DIM analogs/isomers and determined that C-DIMs with *para*-substituted-phenyl (DIM-C-pPh-substituent) are potent Nurr1 activators. Furthermore, DIM-C-pPhBr activated both N- and C-terminal domains of Nurr1 through site-specific phosphorylation and this activation resulted in transcriptional induction/repression of Nurr1-regulated genes in pancreatic cancer cells.

In contrast, our studies in colon cancer cells demonstrate a direct interaction between several C-DIMs and the ligand-binding domain (LBD) of NR4A1 nuclear receptor (TR3); this binding inactivated both wild type and truncated TR3 (with LBD). In addition, several C-DIMs also inactivated truncated TR3 containing transactivation domains (without LBD). Furthermore, a TR3 inactivator, DIM-C-pPhOH, downregulated survivin, induced caspase-dependent apoptosis and inhibited p53-dependent mTOR signaling in colon cancer cells. Our studies with C-DIMs suggest that their anticancer activities are due, in part, to modulation of NR4A nuclear receptors, TR3 and Nurr1.

To my parents and grandparents.

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CHAPTER I

INTRODUCTION

Cancer

Cancer is a complex disease that forms due to transformation of normal cells resulting in a neoplastic cell that exhibits uncontrolled proliferation and unregulated survival. Cancer arises from benign neoplastic cells that originate in specific tissues and organs that subsequently develop into malignant neoplasms that are capable of invading surrounding tissues, escaping into circulation and establishing foci at distal sites. Cancers can be classified into solid and non-solid types. Solid tumors are formed from benign tumors which are generally non-inflammatory masses of cells confined in a tissue such as breast, lung and gut. Many early-stage solid tumors can be surgically removed from the primary sites and after treatment patients can expect extended cancer-free survival. Benign solid tumor cells can also give rise to high-grade malignant cancer cells with accelerated proliferation rates and an invasive capacity. Non-solid tumors are usually referred to cancers of the blood and lymphatic systems such as leukemia and lymphoma and they can also be initiated from fluid-filled cystic lesions commonly associated with glands. Leukemia and lymphoma cells are dispersed and circulate in the body; hence, they are usually treated by radiation or chemotherapies. Malignant cancer cells derived from both solid and non-solid tumors can migrate and

invade other tissues and organs in the body. This process is termed metastasis and cancer metastasis is the major cause of cancer-related mortality.

Cancer is one of the leading causes of death worldwide and is responsible for approximately 13% of all human deaths according to the World Health Organization (WHO) 2008 statistics (1). Cancer-related deaths have continued to increase during the past decades and will continue to increase unless more effective treatments are developed for late-stage and metastasized tumors and certain highly malignant tumors. In 1990, 5.8 million people died of cancer and in 2010 this number increased to 7.98 million (2). Lung, breast and colorectal cancers are most commonly diagnosed cancers and along with stomach and liver cancers they also result in the most cancer deaths. Lung (1.38 million deaths, 18.2% of the total cancer deaths), stomach (0.74, 9.7%) and liver (0.70, 9.2%) cancers cause the highest cancer deaths while pancreatic (99% deceased in five years after diagnosis), liver (95%) and esophageal (92%) cancers have the highest mortality rates (3). In the less developed countries, where 70% of all cancer deaths occur, cancer causing viral infections, such as hepatitis B/C virus (HBV/HCV) and human papilloma virus (HPV), account for 20% of cancer deaths (1). In the United States, cancer is the second leading cause of death. There is 43.9% chance of developing some type of cancer and 22.9% chance of dying from cancer for males living in the US comparing to a 38.0 and 19.3% probability for females respectively (4). It is estimated that 1.66 million Americans will be newly diagnosed with cancer and 0.58 million Americans will die from cancer (i.e. 1.1 deaths per minute) in 2013 (Fig. 1.1) (5).

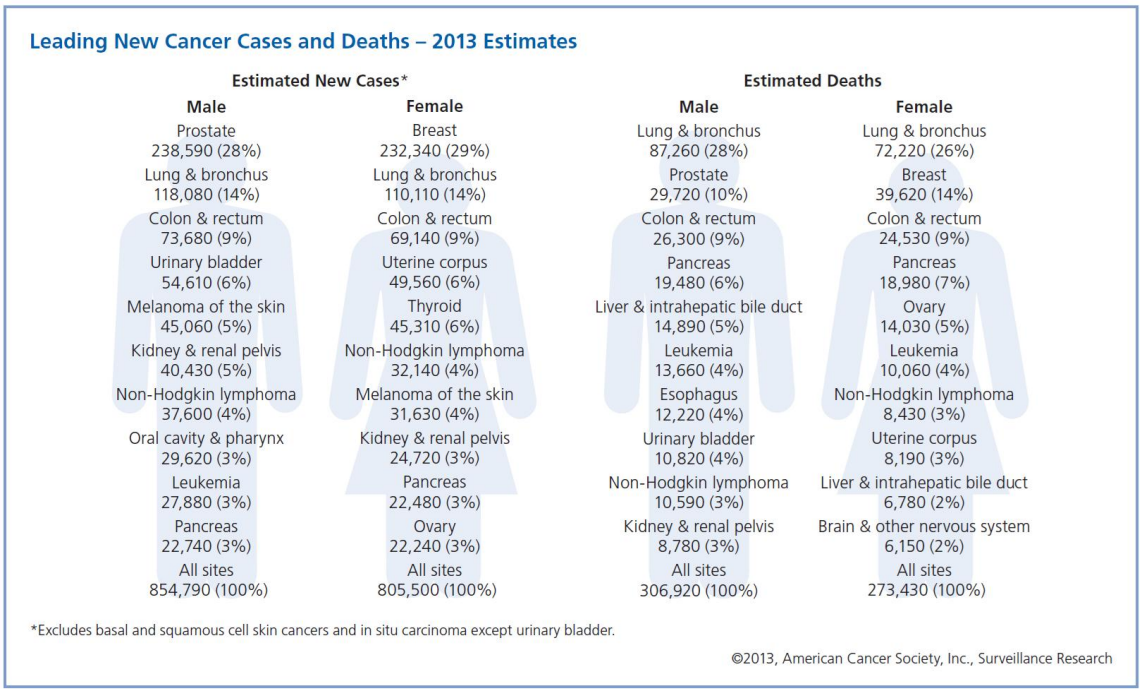


Figure 1.1. Leading new cancer cases and deaths (2013 estimate) (5).

The increasing aging population and lifestyle changes are important reasons for the increasing cancer incidence worldwide. According to the American Cancer Society (ACS) report in 2013, 77% of all cancers are diagnosed in the >55 year-old group (5). Although the risk for developing most cancers increases with age, WHO estimates that at least one-third of all cancer cases can be prevented (6). Most of these preventable cancer cases are due to lifestyle risk factors such as tobacco and alcohol use, unhealthy diets and physical inactivity. Therefore, many cancer cases can be avoided or delayed by living a healthy lifestyle and having regular screenings. Chronic infections, environmental pollution, occupational carcinogens and radiation are also causes of

cancer that can be reduced or prevented and it is generally agreed that cancer prevention is more effective than cancer treatment to reduce the overall cancer burden. However, with the large number of existing and potential cancer patients, more effective treatment regimens are needed and this requires a better understanding of the molecular biology of cancer.

The common characteristic of any cancer is uncontrolled cell division which is regulated by a complex network of genes. Mutations of these genes and their regulating elements can enhance development of the cancer phenotype. Although 5-10% of cancer cases are due to inherited gene mutations (7), the majority of the genetic damage is accumulated in a person's lifetime as a result of environmental (such as carcinogens, radiation and infection) and internal factors (such as hormones and metabolites). The most frequently mutated genes in cancer are categorized into two classes, oncogenes and tumor suppressor genes. Oncogenes are the mutated versions of the wild type proto-oncogenes and they are constitutively activated to promote pro-oncogenic pathways such as cell proliferation, migration, invasion and metastasis. Tumor suppressor genes such as p53 promote apoptosis and have repressive effects on the cell cycle. Unlike oncogenes, mutations in tumor suppressor genes usually render them defective, resulting in the loss of critical functions that inhibit cancer development. The changes in both oncogenes and tumor suppressor genes result in the transformation of normal cells into cancer cells and this process is called carcinogenesis.

Mechanism of carcinogenesis

Carcinogenesis (also oncogenesis or tumorigenesis) refers to the formation of cancer – specifically the pathways involved in the conversion or transformation of normal cells into cancer cells. Tumor transformation is a multistep process and in many cancer models it can be generalized into three stages which include cancer initiation, promotion and progression (Fig. 1.2).

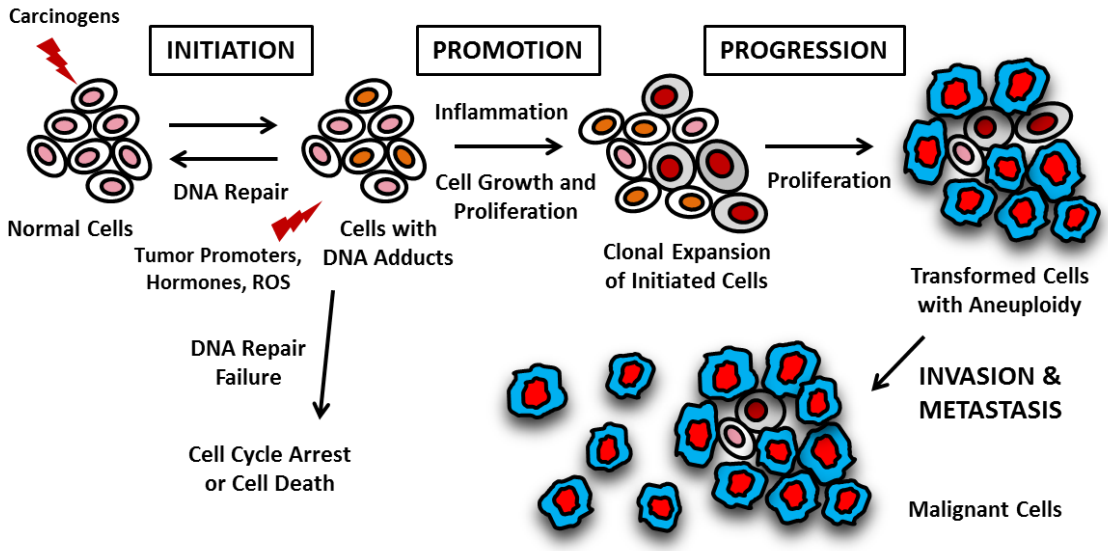


Figure 1.2. Stages of cancer cell formation.

Cancer initiation is the first stage of carcinogenesis and it involves the genetic changes induced by carcinogens. Carcinogens or mutagens are any chemical substances

or non-chemical radiation (energetic particles and waves) that induce genetic mutations that result in tumor formation. It is generally accepted by regulatory agencies that all forms of high-energy radiation that damage DNA and carcinogens, such as benzo(*a*)pyrene and aflatoxin, that bind covalently to DNA (i.e. primary genotoxic effect) may have no thresholds in their initiation of tumors (8). Some chemicals are non-carcinogenic at low doses on their own but can initiate or promote carcinogenesis when combined with other chemical carcinogens or radiation. For example, low concentrations of sodium arsenite do not cause tumors but enhance tumor formation rate and size in mice exposed to UV radiation (9). Cancer-causing viruses or oncoviruses are not usually considered carcinogens but they can initiate cancer by directly integrating their viral oncogenes into the host genome or by enhancing expression of oncogenes in the host. Viruses can also indirectly cause cancer by inducing chronic inflammation (e.g. HCV-induced liver cancer (10)) or interfering with key tumor suppressors such as p53 (e.g. HPV-induced cervical cancer (11)). Carcinogens induce cellular and DNA damage, which can occasionally provide certain cells with aberrant advantage, such as mutations that activate oncogenes and inactivate tumor suppressor genes, and this is an important component of carcinogen-induced initiation. There are many *in vivo* cancer initiation models including one developed in mouse skin (12-14). Topical administration of a carcinogen results in formation of covalent adducts between chemical carcinogens and DNA which can result in mutations of oncogenes such as Ha-ras leading to formation of benign papillomas

and eventually malignant squamous cell carcinomas (SCC) (15). However, exposure to carcinogens does not necessarily result in cancer. Most DNA damage caused by carcinogens can be efficiently repaired by DNA repair enzyme systems which also induce cell cycle arrest or death to avoid cell proliferation without DNA repair. It is believed that defects in genes that are involved in DNA synthesis, DNA repair and DNA damage assessment (during cell cycle checkpoints or apoptosis) can amplify the basal mutation rate of initiated cells hence promoting tumor formation (16).

Cancer promotion is the second stage of carcinogenesis and is characterized by the clonal expansion of initiated cells. Initiated cancer cells often have defective or decreased expression of tumor suppressor genes such as p53 and retinoblastoma (Rb) which are important for arresting cells with DNA damage and activating DNA repair or apoptosis. Initiated cells that are resistant to apoptosis may survive and this is a prerequisite for subsequent proliferation and tumor development. Although carcinogens are no longer needed at the promotion stage, chronic inflammatory conditions are required to stimulate the clonal expansion of precancerous cells. Inflammation triggers cells to produce growth factors such as the epidermal growth factor (EGF) which activate mitotic pathways and kinases such as the mitogen-activated protein kinase (MAPK) (17, 18). Inflammation also enhances the secretion of proliferative cytokines such as tumor necrosis factor-α (TNF-α) and chemokines which attract pro-inflammatory cells such as neutrophils (19-21). These cells release reactive oxygen species (ROS) that exacerbate inflammation and ROS can also cause DNA

damage (22). In addition, some hormones like 17β-estradiol (E2) can also promote initiated cancer cells to expand by activating hormone receptors which upregulate genes required for cell proliferation (23). Furthermore, some weak- or non-carcinogenic chemicals including phorbol esters, phenobarbitol and chlorinated biphenyls are called tumor promoters because they can either activate a proliferative pathway or stimulate inflammation (24). Inflammation, hormones and tumor promoters all stimulate cell proliferation and this contributes to an increase in genetic instability which drives promoted cells to a malignant stage.

Cancer progression is the third stage of carcinogenesis in which benign neoplastic cells are transformed into malignant cells that exhibit several phenotypic changes including increased growth, invasiveness and morphological changes. Malignant cancer cells also promote angiogenesis which is critical for cancer progression (25). The phenotypic changes in cancer cells are due to irreversible genomic alterations which include mutations and genetic polymorphisms that accumulate and are amplified in the first two stages of carcinogenesis; these are also accompanied by acquired chromosomal abnormalties such as aneuploidy (26, 27). Aneuploidy is unique in cancer progression and it includes the loss, gain and imbalance of chromosomes which are common karyotypic observations in many advanced cancer cells found in the skin and colon (28, 29). The degree of aneuploidy, which may include the loss of either chromosome 8p or 18q or both, is negatively correlated with the 5-year disease-free survival rate in colon cancer patients without metastasis (30). All

these genetic changes in cancer progression push cells towards a malignant phenotype in which cells are able to invade adjacent tissues and metastasize to non-adjacent organs.

In addition to the three-stage carcinogenesis model (Fig. 1.2), invasion and metastasis are also important components of cancer development and risk factors for decreased patient survival. The fundamental characteristic of malignant cancer cells is their ability to invade surrounding tissues and metastasize to other parts of the body. Tumor metastasis to other tissues represents a much more advanced phase of the disease. Malignant tumor cells can digest the encapsulating boundary as well as the basal membrane of the primary organ by using proteolytic enzymes such as matrix metalloproteinases thereby enabling them to spread or invade into surrounding tissues (31). Proteases such as urokinase are also involved in intravasation where cancer cells penetrate blood or lymphatic vessel walls and enter into circulation (32). Epithelial-mesenchymal transition (EMT) contributes to the invasive phenotype of cancer cells and migratory EMT cells are required in intravasation. However, there is evidence that non-EMT cells co-migrate with EMT cells and the adhesive non-EMT cells have greater chance than EMT cells to re-attach to vessel walls and leak or extravasate into a secondary site (33). The processes of invasion and metastasis result in tumor formation at a secondary site; and tumors that originate in one organ preferentially metastasize to certain selected organs. In 1889, Stephen Paget first discovered the non-random pattern of metastasis after examining more than 700 postmortem breast cancer cases

and proposed the “seed and soil” theory which suggested that the dynamic interaction between metastatic cancer cell and its microenvironment determines the organ-preference patterns of cancer metastases (34). The most frequently targeted organs of any metastasis are liver, lung, and bone (35). This directional dissemination is guided by distinctive chemokines in cancer cell chemotaxis which is a critical element of cancer cell invasion, intravasation, circulation, extravasation and colonization (36).

Normal cells undergo extensive changes and acquire distinctive traits and genetic changes that are required for the cancer cell phenotype. Hanahan and Weinberg have summarized six classic hallmarks of cancer which include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis (Fig. 1.3) (37). These traits are discussed below and are critical for cancer initiation, promotion, progression and metastasis.

Sustaining proliferative signaling

The most fundamental characteristic of cancer cells is their sustained proliferation which involves continuous signaling through one or several cellular pathways such as the mitogen-activated protein kinase (MAPK) pathway (38). In cancer cells, proliferation pathways are activated in either ligand-dependent or independent manner. In many cases, cancer cells secrete growth factors which act as paracrine or autocrine factors that bind membrane receptors with intracellular tyrosine kinase

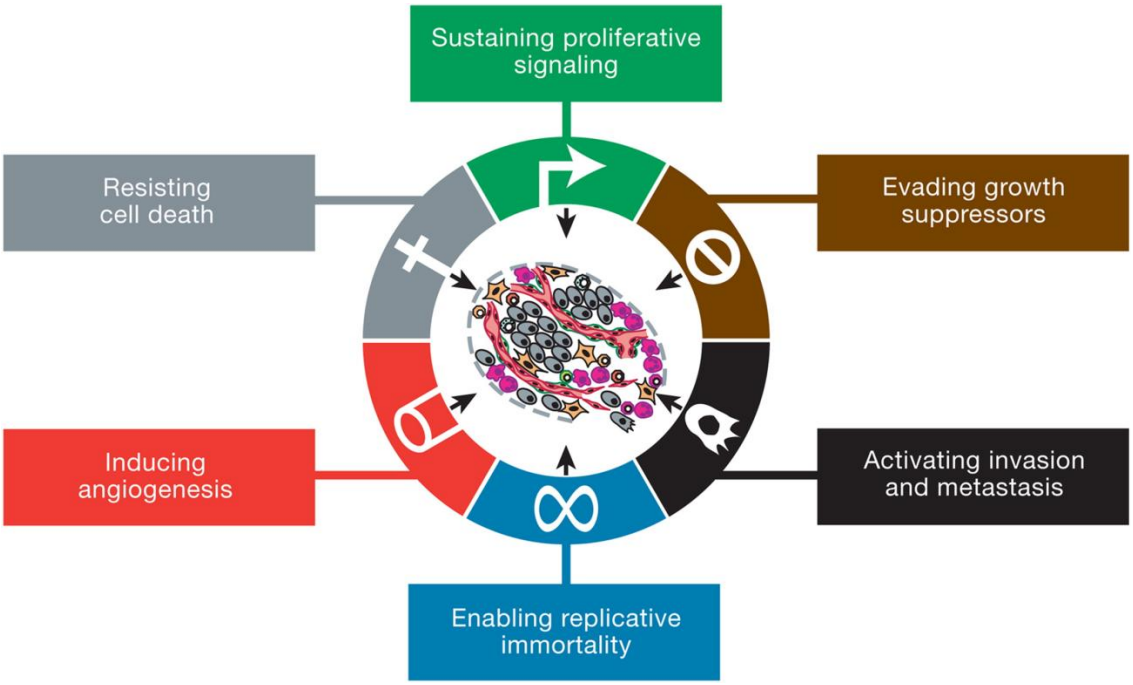


Figure 1.3. The hallmarks of cancer (37).

domains such as epidermal growth factor receptor (EGFR) (39). Cancer cells can also stimulate and acquire growth factors from nearby non-cancer cells such as stromal fibroblasts (40, 41). Many growth factor receptors are often overexpressed or mutated (into hypersensitive forms) in cancer cells and overexpressed receptors can respond to proliferation signals in cells with relatively low expression of growth factor ligands. In other cases, mutations give rise to hyperactive forms of receptors or downstream components of kinase cascades such as B-Raf (42) and phosphatidylinositide 3-kinase (PI3K) (43), rendering them constitutively active even in the absence of growth factors. In addition, negative-feedback mechanisms that normally counterbalance proliferative

signaling are often compromised in cancer cells. For example, expression of PTEN phosphatase, which works against PI3K by degrading its product, is lost due to promoter methylation in many breast (44) and lung cancer cells and tumors (45). Moreover, recent studies suggest that excessive proliferative signals also trigger cell senescence (and/or induce apoptosis) which is a characteristic of premalignant tumor cells (46). However, this senescence/apoptosis circuitry is disabled in malignant tumor cells.

Evading growth suppressors

Cancer cells must also evade powerful growth suppressors that otherwise are able to offset the effects of proliferative signals. Many growth suppressors are products of tumor suppressor genes, such as RB and TP53, which negatively regulate cell proliferation by arresting cell cycle progression, activating cell senescence and inducing cell death. Functional tumor suppressors are gatekeepers of cell cycle progression and they integrate extra- and intracellular signals. For example, Rb protein transduces inhibitory signals originating from outside of the cell whereas p53 protein gathers inputs from intracellular sensors that detect cellular stress, nutrient deficiency and DNA damage. The primary functions of activated Rb and p53 are to inhibit G1 to S phase transition during the cell cycle and recent studies indicate that Rb and p53 also regulate metabolism of critical factors required for cellular growth, such as dNTP, glutamine and glucose (47-50). Although Rb and p53 each regulate separate pathways, they are part

of a complex inhibitory network which exhibits some regulatory overlaps and functional redundancies to ensure an effective defense against uncontrolled cell proliferation. For example, RB or TP53 gene knockout mice develop normally and abnormalities such as pituitary tumors, leukemias and sarcomas are only observed in older animals (51, 52). Cancer cells often have loss-of-function mutations in one or several tumor suppressor genes. For instance, TP53 gene is mutated in more than 50% of all cancers (53, 54). Cancer cells with defective tumor suppressor genes are more readily stimulated by factors promoting cell proliferation. In addition to tumor suppressors, adhesion molecules such as E-cadherin also mediate powerful growth inhibitory pathways through cell-cell contact and expression of these cell-surface proteins are often lost in cancer cells that have undergone epithelial-mesenchymal transition (EMT) (55). Interestingly, in addition to simply shutting down growth suppressive circuitries, cancer cells also redirect some anti-proliferative mechanisms. For example, TGF-β normally inhibits proliferation but in cancer cell context TGF-β activates EMT and promotes malignancy (56).

Resisting cell death

Besides sustaining proliferative signaling and evading growth suppressors, cancer cells must also develop strategies to resist programmed cell death by apoptosis, which serves as a natural defense against cancer cell progression. Apoptosis does not cause any damage to the surrounding tissues while necrosis (unregulated cell death) is

pro-inflammatory hence cancer-promoting. Apoptosis can be triggered by extracellular death signaling, for example, through Fas ligand (FasL) binding to its receptor CD95. Extracellular apoptotic signaling pathways are often non-functional in cancer cells and facilitate the ability of cancer cells to escape from immune surveillance. For example, there is evidence that CD95 negative tumor cells overexpress FasL to induce apoptosis of infiltrating T lymphocytes (57). Apoptosis can also be induced within the cell by excessive oncogene signaling, DNA damage and oxidative stress which are consequences of cancer cell hyperproliferation. However, cancer cells maintain distinct "healthy" levels of oncoproteins, such as Myc, to avoid their pro-apoptotic potential (58) and, as mentioned above, key transmitters of cellular stress and DNA damage sensing circuits, such as p53, are often defective. In addition, cancer cells upregulate anti-apoptotic members and downregulate pro-apoptotic members of the Bcl-2 family proteins which are involved in maintaining mitochondrial membrane potential (MMP). Loss of MMP results in cytochrome c release and subsequent caspase activation; for this reason, mitochondria play an important part in regulating apoptosis. In fact, cancer cells usually shut down mitochondria and generate energy mainly by cytosolic glycolysis and this phenomenon is known as the Warburg effect (59). Moreover, caspases are executors of both mitochondria-dependent and independent apoptosis pathways and inactivating mutations of caspases are expressed in many cancers (60). In addition to apoptosis, another cellular program that can result in death is autophagy which allows the cell to degrade organelles and recycle molecules in response to nutrient deficiency

and other stressors. When operating at low levels, autophagy functions as an adaptation strategy that enhances tolerance to the stressful and nutrient-deficient environment encountered by most tumor cells. In extreme cases, activation of autophagy leads to a state of reversible dormancy (61, 62) and suppression of autophagy results in apoptosis in cancer cells (63, 64). On the other hand, excessive self-consumption can lead to cell death; and autophagy is suppressed due to the loss of expression of the essential gene beclin1 in many human tumors (65). Therefore, autophagy plays a dual role in cancer cells as either a survival strategy or a death-inducing pathway (66).

Enabling replicative immortality

Cancer cells proliferate continuously by means of the three hallmark traits mentioned above; nonetheless, in order to generate macroscopic tumors, they must also undergo unlimited proliferation. In 1961, Leonard Hayflick observed that cultured human fetal cells only divide 40 to 60 times (67) and later this phenomenon was termed the Hayflick limit (68). A common denominator for all types of cancer cells is replicative immortality which means they have the potential to divide as long as the conditions for replication are satisfied and they overcome the Hayflick limit. A good example is the HeLa cell line which was established in 1951 and is still used in research today (69). The molecular basis of the Hayflick limit is the progressive shortening of telomeres composed of hexanucleotide repeats at chromatid ends after each cell

division. Telomeres defend against terminal nucleotide loss during DNA replication and prevent end-to-end fusion during chromosome segregation. The gradual erosion of telomeres generates genomic instability and eventually induces apoptosis. Therefore, telomere renewal is absolutely vital for rapidly dividing cells. Cancer cells accomplish this restoration mission primarily by activating telomerase – a telomere-lengthening enzyme complex normally absent in somatic cells but upregulated in stem and germ cells (70). Telomerase activation has been observed in about 90% of human tumors (71) and other pathways such as alternative lengthening of telomeres (ALT) exist in tumors without telomerase activation (72, 73). Telomerase activation is often achieved by overexpression of the telomerase reverse transcriptase (TERT) mediated by oncogenic transcription factors in cancer cells (74). TERT is the catalytic component of telomerase and inhibition of TERT triggers apoptosis in cancer cells (75). TERT has also been associated with aging. Although most studies suggest that TERT immortalizes cells and promotes cancer thus reducing the lifespan of animals, one study reported that introducing TERT gene into adult and older mice increased their lifespan without increasing cancer incidence (76). Another study showed that overexpressing TERT delayed aging in cancer-resistant mice (77). In addition to telomere maintenance, TERT facilitates cancer progression by participating in other cellular activities such as gene expression, DNA repair and apoptosis (78). Paradoxically, studies on mice show that impaired telomerase function promotes carcinogenesis especially when coupled with p53 defect (79). Later analyses on human breast cancer tissues demonstrate

telomerase is inactivated in premalignant cells comparing to its activation in malignant cells (80, 81). A rational explanation for the delayed acquisition of telomerase activation is that tumor cells inactivate telomerase to generate mutations followed by activation of telomerase to stabilize the mutated genome.

Inducing angiogenesis

The above hallmarks are consistent with the cancer cell phenotype under laboratory conditions. However, the growth of tumors *in vivo* beyond the critical size of 1-2 mm requires nutrients and oxygen from dedicated blood vessels. The formation of new blood vessels from pre-existing vasculature is termed angiogenesis and this process can be transiently activated during wound healing in adults. Constant induction of angiogenesis is required in actively growing tumors and the absence of angiogenesis leads to the lack of tumor growth resulting in dormancy as evidenced by asymptomatic microscopic tumors commonly observed in clinical settings (82). In 1971, Folkman first described the interaction between tumors and vascular endothelium and predicted the involvement of angiogenic growth factors (83). Although many growth factors such as fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) can induce neovascularization (84), the angiogenic phenotype of cancer is mainly attributed to vascular endothelial growth factor-A (VEGF-A or simply VEGF) ligand secretion and signaling (85, 86). VEGF overexpression in cancer cells can be the outcome of the same signaling pathways that sustain proliferation, *i.e.* growth factor and oncogene signaling

pathways such as those mediated by EGFR, Raf, MEK and PI3K (87). VEGF expression, activation and release can also be triggered by hypoxia due to tumor metabolism and by inflammation associated with the tumor microenvironment (88). In addition, transcription factors such as NF-κB and Sp1 can directly activate VEGF gene promoters in breast, lung and pancreatic tumor cells (89-93). Tumors *in vivo* secrete VEGF ligands which bind to VEGF receptors (VEGFRs) located on the surface of vascular endothelial cells, initiating mitogenesis of these stromal cells and consequently a directional sprouting of independent vascular networks. On the other hand, endogenous angiogenesis inhibitors, such as angiostatin, endostatin and thrombospondin-1 (TSP-1), counterbalance the effect of VEGF ligand on endothelial cells (94-96) and transcription repressors, such as ZNF24, negatively regulate VEGF gene expression (97). Moreover, VEGF peptides can be sequestered by proteoglycans in the extracellular matrix (98) and release of these ligands is dependent on matrix metalloproteinases (MMPs) (99). Therefore, there exists a balance between angiogenesis activators and inhibitors in tumors. In the early 1990s, Folkman and Hanahan proposed the “angiogenic switch” concept to explain the events in tumor progression where this balance tilts towards activation of angiogenesis and the subsequent transition of tumors from dormancy to malignancy (100). The angiogenic switch can occur at different stages of the tumor progression (101) and the degree of vascularization is dependent on the tumor type and its microenvironment (102). Furthermore, many types of myeloid cells and inflammatory cells perform in concert with tumor cells to promote angiogenesis (103,

104); and certain bone marrow-derived cells can differentiate into endothelial cells and pericytes to form tumor vasculature (105-107). Inducing angiogenesis is not only important for local tumor progression but also critical for dissemination of tumor cells to distal sites.

Activating invasion and metastasis

The ability to invade and metastasize is the defining characteristic of cancer compared to other diseases and it is responsible for more than 90% of cancer mortality. New evidence shows that acquisition of this hallmark can happen early in carcinogenesis and 60 to 70% of cancer patients have overt or occult metastases at diagnosis (108). Invasion refers to the infiltration of tumor cells to adjacent tissues and body cavities (i.e. transcoelomic spreading) and metastasis refers to the dissemination of tumor cells to distant organs via circulation which is facilitated by angiogenesis as discussed above. Some tumors induce lymphangiogenesis by secreting VEGF family members VEGFC and VEGFD, which bind VEGFR3 on lymphatic endothelium, and this pathway also enhances cancer metastasis (109). Invasion and metastasis are generally considered integral components of the invasion-metastasis cascade which consists of these sequential events: local invasion, intravasation, circulation, extravasation, micrometastasis and colonization (110). Invasion and metastasis use many of the same gene expression cassettes to increase cell motility and enable cell migration (e.g. mesenchymal migration). The most important phenotypic change enabling cancer cell

migration is the loss of cell attachment. E-cadherin is a key cell-cell adhesion molecule, hence a natural suppressor of invasion and metastasis, and transcription of the E-cadherin gene is directly repressed by Snail and ZEB family members in many human carcinomas (111-113). On the other hand, genetic mutations of E-cadherin are rare in most cancer types but are present in up to 50% of breast cancers (114). In contrast, another member of the cadherin superfamily, N-cadherin, facilitates transendothelial migration and is upregulated in many invasive carcinomas (115). Transcription repressors of E-cadherin such as Snail, Slug, Twist and Zeb1/2 also orchestrate the epithelial-mesenchymal transition (EMT) which is normally activated in embryonic morphogenesis and physiological processes such as wound healing. EMT can be transiently or stably activated in both epithelial and non-epithelial tumor types, rendering tumor cells invasive and metastatic phenotypes and this is accompanied by loss of polarity and adherent junctions, a fibroblastic or amoeboid morphology, increased motility and the expression of matrix-degrading enzymes. However, activation of EMT is usually observed in cells at the margins of a malignant tumor (i.e. the invasive front) and not in cells residing in the core of tumor, suggesting microenvironment stimuli such as inflammation play a pivotal role in triggering this transition (116). Tumor cells also actively stimulate stromal cells such as mesenchymal stem cells (MSCs) and tumor-associated macrophages (TAMs); these cells foster the invasive behavior of tumor cells by secreting chemokines such as CCL5 (117) and matrix-degrading enzymes such as metalloproteinases (99). Another feature of EMT in

tumors is its reversibility where a mesenchymal-epithelial transition (MET) reverts invasive cells to a noninvasive state allowing them to colonize at secondary sites (118). However, plasticity of the invasion programming alone does not guarantee successful metastasis. It is estimated that millions of tumor cells enter the circulation daily and less than 0.01% of these circulating tumor cells can initiate metastasis (108). An important impediment is that most cells from a primary tumor adapt poorly, at least initially, to a foreign microenvironment (i.e. without supportive stroma) and this is apparent by the prevalence of dormant micrometastases that never develop into macroscopic secondary tumors in many patients (119, 120). Some primary tumors also release angiogenic inhibitors such as angiostatin and endostatin to suppress the growth of micrometastases (121, 122) and surgical removal of a primary tumor usually results in explosive growth of secondary tumors (123). Therefore, cancer metastasis is a highly selective and competitive process that only favors a fraction of the heterogenous neoplastic population. Nonetheless, certain organs are highly hospitable to metastases, at least to that of the corresponding tumor types. For example, breast cancer tends to metastasize to bones and lungs; colon cancer to liver; malignant melanoma to brain and stomach cancer to ovaries (i.e. Krukenberg tumor (124)). This non-random organotropism of cancer metastasis can be explained by Paget's "seed and soil" theory as mentioned before. In 1928, this theory was challenged by James Ewing who proposed that mechanical forces, such as blood flow and capillary diameter, and circulatory patterns between primary and secondary tumor sites resulted in the organ-

specific metastasis (125). For example, the lung is a favorable habitat of metastasis because of its dense vascular surface area; the liver is the usual metastatic destination of colorectal tumor cells due to the mesenteric circulation pattern. Ewing's hypothesis was dominant for half a century until disproven (or invalidated as inadequate) by molecular evidence in recent decades (126-128). It is now accepted that cancer metastasis is mainly regulated by crosstalk between tumor cells (i.e. "seed") and microenvironment in a specific organ (i.e. "soil"). For example, breast cancer cells express chemokine receptor CXCR4 and metastasize to the bone marrow which express its ligand CXCL12 (129) while TGF-α/EGFR signaling mediates colon cancer metastasis to the liver (130). Moreover, some of these molecular determinants are chemokines which navigate cancer cell chemotaxis such as that in the CXCL12-CXCR4 axis. These chemokines are constitutively expressed in specific tissues hence called homeostatic chemokines. It should be noted that organ-specific metastasis mediated by homeostatic chemokines are distinctive from lymph node metastasis, and therefore, the common perception that cancer cells metastasize first to regional lymph nodes then distant organs may be inaccurate (131).

Colon Cancer

Colon or colorectal cancer is the generic term for several types of cancer that arise in the colon and rectum, including adenocarcinoma, carcinoid and stromal tumors, lymphoma and sarcoma. Adenocarcinomas originate from the gland cells that make the

mucus of the colon and rectum. Carcinoid and stromal tumors originate from the specialized hormone-producing and wall-forming cells respectively and they can also be found elsewhere in the gastrointestinal (GI) tract. Lymphomas originate from cells in the lymph nodes associated with the intestinal tract and sarcomas originate from connective tissues such as blood vessels and muscles in the wall of the colon and rectum.

Adenocarcinomas are the most commonly diagnosed colon cancers (e.g. 95% of all colon cancer cases) and they arise from adenomatous polyps (i.e. adenomas) formed in the innermost lining of colon that eventually grow into the wall and invade underlying tissues. When cancer cells invade into blood and lymph vessels, they travel and spread to other parts of the body and develop into a more metastatic tumor. Colon cancer, like all other cancers, can be categorized into four stages with each stage representing an increasing aggressiveness of the disease status. After carcinogenic transformation, neoplastic cells form a high-grade dysplasia which is either a hyperplastic or inflammatory polyp. This polyp is termed carcinoma *in situ* (or Stage 0) which is a superficial non-invasive pre-cancer lesion. Stage 0 colon polyps can be removed by an endoscopic method called polypectomy and patients are likely cancer-free after this procedure. Stage I tumors are polyps that have infiltrated the inner lining into the colon wall and Stage II tumors are larger tumors that have penetrated the colon wall. Stage III refers to tumors that have spread outside the colon to lymph nodes and Stage IV indicates tumor metastasis to other tissues such as the liver or the lungs.

Surgery is the initial and most effective treatment option for early-stage colon cancers and a complete removal of early-stage cancerous tissues is often curative for patients. All colon tumors can be surgically removed except that surgery is usually not effective for managing Stage IV tumors. Chemotherapy and radiation are options for treating patients with Stage II to IV colon tumors or patients unsuitable for surgery. According to the American Cancer Society, the five-year survival rates are 93, 78, 64 and 8% for Stage I to IV colon cancers respectively (5).

Colon cancer is the second and the third most commonly diagnosed cancer in women and men respectively. More than one million people worldwide are diagnosed with tumors in the colon and rectum every year (132) and recent molecular studies demonstrate that tumors in the colon and rectum are genetically the same type (133). About 15 to 30% of these colon cancer cases may have a strong hereditary component based on genetic data from family history studies (134). Hereditary colon cancer syndromes are usually initiated by loss of heterozygosity (LOH) at genomic loci that encode or regulate a tumor-suppressive protein product. For example, familial adenomatous polyposis (FAP) and familial colorectal polyposis (Gardner syndrome) are caused by mutations in the tumor suppressor gene adenomatous polyposis coli (APC); hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) is caused by defects in DNA repair proteins encoded by genes such as MSH2, MLH1, MSH6/GTBP and PMS2 (135). In addition to familial cases, sporadic colon cancer cases are linked to inflammatory bowel diseases, old age and lifestyle factors such as alcohol, smoking,

unhealthy diet and lack of exercise. Among these causes, inflammatory bowel diseases (IBD), primarily ulcerative colitis and Crohn's disease, are important risk factors for colon cancer (136). Meta-analysis studies indicate that the risk for colon cancer increases with the duration and extent of inflammation in ulcerative colitis (137) but not in Crohn's disease (138), which is in agreement with the higher cancer incidence in patients with ulcerative colitis than Crohn's disease. More than 20% of IBD result in colon cancer within 30 years of disease onset and these cases are often referred to as colitis-associated cancer (CAC), a colon cancer subtype that has a mortality rate higher than 50% (139). CAC and non-CAC colon cancers (or spontaneous colon cancers) share the same mechanism of pathogenesis and it can be summarized as the adenoma-carcinoma sequence (Fig. 1.4).

The adenoma-carcinoma sequence

Carcinogenesis is a multistep process (140) and this concept was first applied to the development of colon cancer in 1990 (141). The adenoma-carcinoma sequence model was later proposed to describe the consecutive genetic changes (inherited or not) which progress over several years, even decades, and eventually lead to colon carcinoma (Fig. 1.4) (142). According to this classic model, environmental insults such as mutagens and reactive oxygen species (ROS) induce DNA damage and initiate the formation of aberrant crypt foci (ACF) from single cells. Mutations in APC, β-catenin (or other components of the Wnt signaling pathway) are early events that trigger

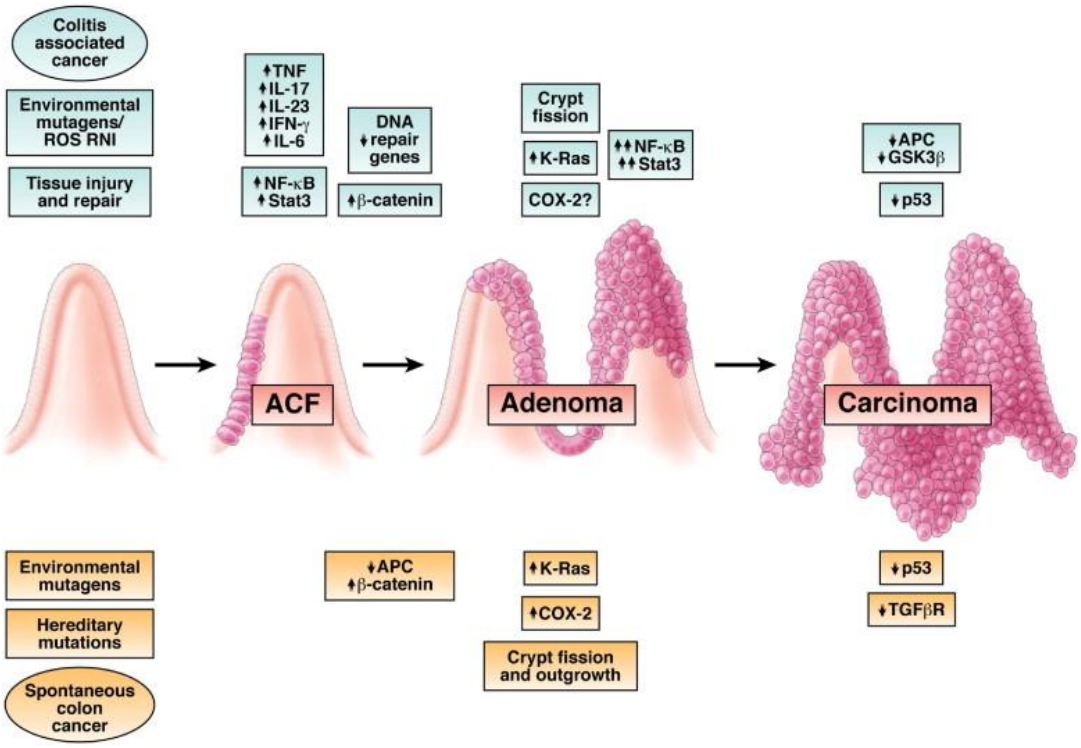


Figure 1.4. The adenoma-carcinoma sequence: development of colon cancer (142).

outgrowth of ACF to hyperplastic lesions projecting above the colorectal mucosa known as polyps. Wnt signaling regulates stem cell differentiation and shedding of crypt cells into the lumen (143, 144) and components of the Wnt pathway including APC and β-catenin are important in formation of colorectal polyps. In addition, APC is a large gene (encoding a 311.6 kDa protein) and spontaneous inactivating mutations are readily formed. Nevertheless, not all hyperplastic polyps become cancerous and only polyps from glandular epithelium or adenomatous polyps develop into adenomas (145). Adenomas are characterized by overexpression of oncoproteins, such as K-Ras and B-

Raf, and the transition from adenoma to carcinoma requires further insults such as inflammatory conditions which are mediated by cyclooxygenase 2 (COX-2) (146). On the other hand, carcinomas are characterized by additional inactivation of tumor suppressors such as p53 and TGF-β receptor II. Some of these tumor suppressors activate DNA repair mechanism at cell-cycle checkpoints and are sentinels of genome integrity. Activation of oncogenes and inactivation of tumor suppressors contribute to uncontrolled cell proliferation and genetic instability which results in an invasive and metastatic colon carcinoma.

Pancreatic Cancer

The term pancreatic cancer stands for malignancies that originate in the pancreas. The human pancreas is composed of the endocrine component or islets of Langerhans, which produces peptide hormones such as insulin, glucagon and somatostatin, and the exocrine component, which secretes digestive enzymes into the duodenum. Tumors originating in the endocrine glands are rare and they are classified as neuroendocrine tumors (NETs) which also include the previously mentioned carcinoid tumors in the colon. On the other hand, about 95% of pancreatic tumors arise from the exocrine component (5) and majority of these tumors are adenocarcinomas formed in the ductal epithelium. Therefore, they are called pancreatic ductal adenocarcinomas (PDACs) or simply referred to as pancreatic cancer.

The epithelium of pancreatic duct consists of cuboidal or low-columnar cells with amphophilic cytoplasm and occasionally this normal epithelium is replaced by mature squamous cells forming transitional metaplasias which are not necessarily precancerous. In some cases, this transition is accompanied by nuclear irregularities in cells or structural abnormalities in the epithelium (i.e. atypia) that potentially initiate cancer. The pathological features of PDACs are categorized by the pancreatic intraepithelial neoplasia (PanIN) nomenclature system (PanIN-1 to 3) (147) which depicts the consecutive changes in morphology and histology of these lesions (Fig. 1.5) (148). Different PanIN lesions exhibit varying degrees of cellular and architecture atypia and are considered precursors of infiltrating PDACs that develop over several years (149). The initial stage of these lesions is PanIN-1A, which refers to a flat epithelium

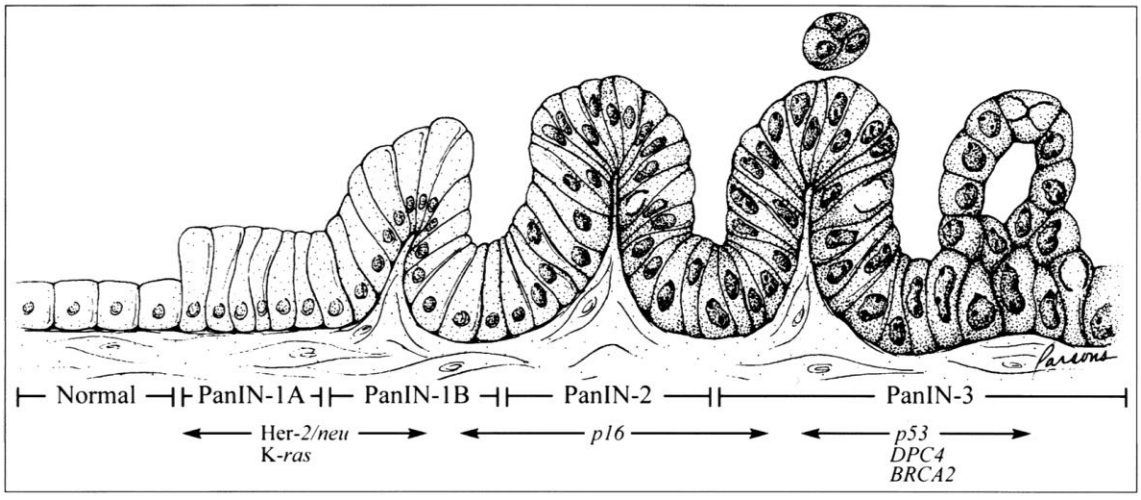


Figure 1.5. Progression of pancreatic cancer: the PanIN model (148).

histologically similar to a non-neoplastic lesion while PanIN-1B lesions are similar to PanIN-1A except for the micropapillary and basally pseudostratified architecture. PanIN-2 lesions are characterized by enlarged crowding nuclei, loss of cellular polarity and hyperchromatism but they rarely undergo mitosis. PanIN-3 lesions have papillary structure and sporadically bud off small clusters of necrotic epithelial cells into the lumen. Cells in PanIN-3 lesions are goblet-shaped and display further loss of polarity (e.g. mucinous cytoplasm oriented toward the basement membrane) and nuclear irregularities (e.g. large nucleoli). Most importantly, PanIN-3 cells are mitotic. Therefore, PanIN-3 denotes a high-grade dysplasia which can be classified as carcinoma *in situ* or Stage 0.

In addition to local advancement, pancreatic cancer is also a systemic disease and can be described by the TNM staging system. Stage 0 dysplasia is limited to the top layer of the pancreatic duct (*in situ* or Tis) and there is no spread outside the pancreas (N0, M0). Stage I refers to local growth of pancreatic tumors to less than 2 centimeters across (T1) or greater than 2 centimeters (T2) without spread (N0, M0). Stage II indicates local spread of tumors outside the pancreas (T3, N0) sometimes to nearby lymph nodes (N1) but not distant sites (M0). Stage III indicates a wider spread of pancreatic tumors into nearby blood vessels or nerves (T4) and Stage IV indicates metastasis to distant organs (M1). Stage I and II pancreatic tumors can be treated by pancreaticoduodenectomy (i.e. Whipple procedure) which involves removal of the head of pancreas, local lymph nodes, parts of the stomach and small intestine, the

gallbladder and the common bile duct. After this complex surgery, chemotherapy using a nucleoside analog gemcitabine and external radiation therapy are usually applied separately or in combination to increase the chance of patient survival. However, only 10 to 15% of pancreatic tumors are considered resectable at diagnosis (150).

It is estimated that more than 45,000 people in the United States will be diagnosed with pancreatic cancer in 2013 and this occurs primarily in individuals 50 years or older (151). Pancreatic cancer barely makes the top ten commonly diagnosed cancers; however, its nature of silent spreading (i.e. spreading without symptoms before diagnosis) results in pancreatic cancer being the fourth leading cause of cancer mortality and, in the United States, it is estimated that in 2013 more than 38,000 patients will die from this disease (5). Pancreatic cancer accounts for 7% of all cancer deaths according to the American Cancer Society; and all-stage combined five-year survival rate for pancreatic cancer patients is 6%, which is the lowest among all major cancer types, with most patients dying within the first year after diagnosis (5).

The risk factors for pancreatic cancer include smoking, old age, high fat diet, chronic pancreatitis, alcohol, obesity and physical inactivity. Among these factors, smoking contributes to at least 25% of pancreatic cancer cases (152). Men are 30% more likely to develop pancreatic cancer than women and the lifetime probability of developing pancreatic cancer is about 1.5% for both sexes (5). The relationship between pancreatic cancer and diabetes is still debatable with respect to diabetes as a cause or effect of tumors in the pancreas (153). Hereditary syndromes due to

mutations in tumor suppressor genes are also risk factors for pancreatic cancer but only account for 5% of the cases. Examples of these syndromes include: syndromes caused by BRCA2 gene germline mutations, familial atypical multiple mole melanoma syndrome (FAMMM) (CDKN2A/p16 mutations), hereditary pancreatitis (PRSS1 mutations), Lynch syndrome (MLH1, MSH2 or other DNA repair gene mutations) and Peutz-Jeghers syndrome (STK11 mutations) (153, 154). Diagnostic screenings for pancreatic cancer may be focused on individuals with specific genetic backgrounds or chronic pancreatitis and currently there is no reliable single test for early detection available to the general public.

Conventional techniques are used to diagnose pancreatic cancer and these include endoscopic ultrasound (EUS), computed tomography (CT), magnetic resonance imaging (MRI) and endoscopic retrograde cholangiopancreatography (ERCP), which are also used in combination with cytological and histological methods to confirm diagnosis. Nonetheless, early diagnosis of this disease is still a challenge largely due to the fact that early stage pancreatic cancer is usually asymptomatic. In addition, common symptoms of pancreatic cancer are difficult to distinguish from that of the pancreatitis. Alternatively, tumor markers, which are measurable diagnostic indicators, may facilitate early diagnosis of cancer and the carbohydrate antigen 19-9 (CA19-9) is the most commonly used tumor marker for pancreatic cancer. However, the American Society of Clinical Oncology does not recommend CA19-9 as screening method for pancreatic cancer due to lack of specificity and frequent false positive/negative results

(155, 156). Therefore, early detection requires better understanding of the molecular genetics and pathways that lead to development of pancreatic cancer.

Genetic changes in the pancreatic intraepithelial neoplasia model

Mutations of several genes are linked to the previously discussed pancreatic intraepithelial neoplasia (PanIN) pathogenesis model and these genetic changes involve KRAS, HER2, CDKN2A/p16, TP53, DPC4/SMAD4 and BRAC2 (Fig. 1.5). Early PanIN lesions harbor KRAS mutations and often concomitantly overexpress HER2/neu gene product. HER2 gene is rarely expressed in normal pancreatic duct epithelium but is amplified in 82 to 92% of histologically different ductal dysplasias (157). Its protein product Her2 is an epidermal growth factor receptor (EGFR/ERBB) which activates KRAS gene product, K-Ras, through the Grb2-SOS complex. Activating mutations of KRAS are also observed in early ductal lesions without atypia. The KRAS proto-oncogene encodes a RAS family enzyme that hydrolyzes guanosine triphosphate (GTP) and mediates variety of cellular functions. Most importantly, K-Ras activates B-Raf in the MAPK pathway and promotes cell proliferation (i.e. the first hallmark of cancer). Furthermore, prevalence of KRAS mutations increases with the degrees of atypia in PanIN lesions (i.e. 36%, 44%, and 87% in PanIN-1A, 1B, and 2 – 3 lesions, respectively (158)). Interestingly, Her2 is absent in poorly differentiated infiltrating carcinomas (157), suggesting that HER2 gene expression is lost during cancer dedifferentiation and survival (159). Nonetheless, both

Her2 and K-Ras are upstream in the MAPK signaling pathway and their alterations are early events that potentiate pancreatic neoplasia (Fig. 1.5).

Inactivation of tumor suppressor genes requires biallelic mutations and appears to be one of the later events in pancreatic tumor development. The tumor suppressor gene CDKN2A encodes a cyclin-dependent kinase inhibitor, p16, which prohibits the G1 to S phase transition by binding to cyclin dependent kinases Cdk4 and Cdk6. Cdk4 and 6 normally bind cyclin D1; and this protein complex phosphorylates and releases retinoblastoma protein (pRB) from E2F1 which in turn promotes cell cycle progression. The p16-pRB axis is inactivated in 95% of the invasive pancreatic carcinomas (160); and CDKN2A/p16 expression is progressively absent in 30%, 55%, and 70% of PanIN-1, PanIN-2, and PanIN-3 lesions, respectively (161). While loss of p16 is observed in some early lesions, loss of the TP53, DPC4 and BRCA2 tumor suppressor gene expression occurs late in the development of PanIN. TP53 is the most frequently mutated gene in malignant tumors and its protein product, p53, has multiple functions including cell cycle arrest, DNA damage repair and inducing apoptosis. The absence of p53 is predominantly observed in advanced precursor lesions (PanIN-3) and up to 75% of the invasive pancreatic cancer (160). Another important tumor suppressor in pancreatic cancer is DPC4/SMAD4. DPC4 stands for “deleted in pancreatic cancer locus 4” and is absent in 30 to 40% PanIN-3 lesions and 55% of pancreatic carcinomas (160). DPC4/SMAD4 protein product Smad4 is an intermediate protein in the TGF-β signaling pathway which mediates growth inhibition and apoptosis. BRCA2 is involved in the

repair of DNA-interstrand cross-links and is mutated only in PanIN-3 lesions and less than 10% of invasive pancreatic tumors; however, germline BRCA2 mutations are a risk factor for pancreatic cancer (162). Furthermore, inactivation of tumor suppressive serine/threonine kinase STK11 is less commonly (i.e. less than 7%) observed in pancreatic adenocarcinomas (163); and both tumor suppressive and oncogenic roles of the stress kinase MKK4 have been suggested in pancreatic tumors (164, 165). In contrast to oncogenes, mutations of tumor suppressor genes are observed primarily in advanced lesions with significant atypia (Fig. 1.5) or invasive pancreatic carcinomas.

Specificity Protein Transcription Factors

Specificity proteins (Sp proteins) such as Sp1 are members of a family of transcription factors which have similar modular structures to the Krüppel-like factor (KLF) type; hence, they are also referred to as the Sp/KLF family. The array of three Cys_2-His_2 (C_2H_2) zinc fingers is a common feature of Sp/KLF family members; however, most Sp family members bind more tightly to GC-boxes (5'-GGGGCGGGG) than GT-boxes (5'-GGTGTGGGG) (166) and the KLF family members preferentially bind GT-boxes (167). In 1983, Sp1 was identified as the first transcription factor (168, 169) and it was shown to bind specific DNA sequences in a broad spectrum of mammalian and viral genes (170-172). Several Sp1-like factors, namely Sp2, Sp3 and Sp4, were later discovered and cloned (173, 174); the Sp family of transcription factors now includes nine members (Sp1 to 9). The Sp family members can be further subdivided into Sp1-4

and Sp5-9 subgroups based on their protein domain characteristics. Sp1 to Sp4 all contain glutamine rich transactivation domains (TADs or ADs) A/B (175) and a zinc finger DNA binding domain (DBD) C/D, with the exception that Sp3 has an extra inhibitory domain (ID) (176) and Sp2 has only one TAD instead of two (177). Sp5 to Sp9 appear to be truncated forms of Sp1-4, lacking the N-terminal TADs (Fig. 1.6) (177). In addition, the zinc fingers of Sp1, Sp3 and Sp4 are conserved and bind GC-boxes whereas there is a histidine to leucine substitution in the first zinc finger of Sp2 resulting in a switch to a GT-box binding preference for Sp2. Based on these structural features, it has been shown that Sp1, Sp3 and Sp4 share similar functional roles apart from the other Sp family members.

The Sp family genes are located adjacent to a homeobox (HOX) gene cluster, which encodes genes that determine the anterior-posterior (head-tail) axis of the embryo (178); and Sp transcription factors are likewise essential in early embryonic development. For example, Sp1 knockout mouse embryos exhibit multiple abnormalities and die on 11th day of gestation (179). Sp3 knockout mice show impaired ossification and tooth development and die shortly after birth due to respiratory failure (180, 181). However, one third of Sp4 knockout mice are able to survive with severe growth retardation and male infertility (182). The physiological importance of Sp proteins in development is due to their molecular function. Studies show that Sp1 directly interacts with TATA-binding protein associated factors (TAFs)

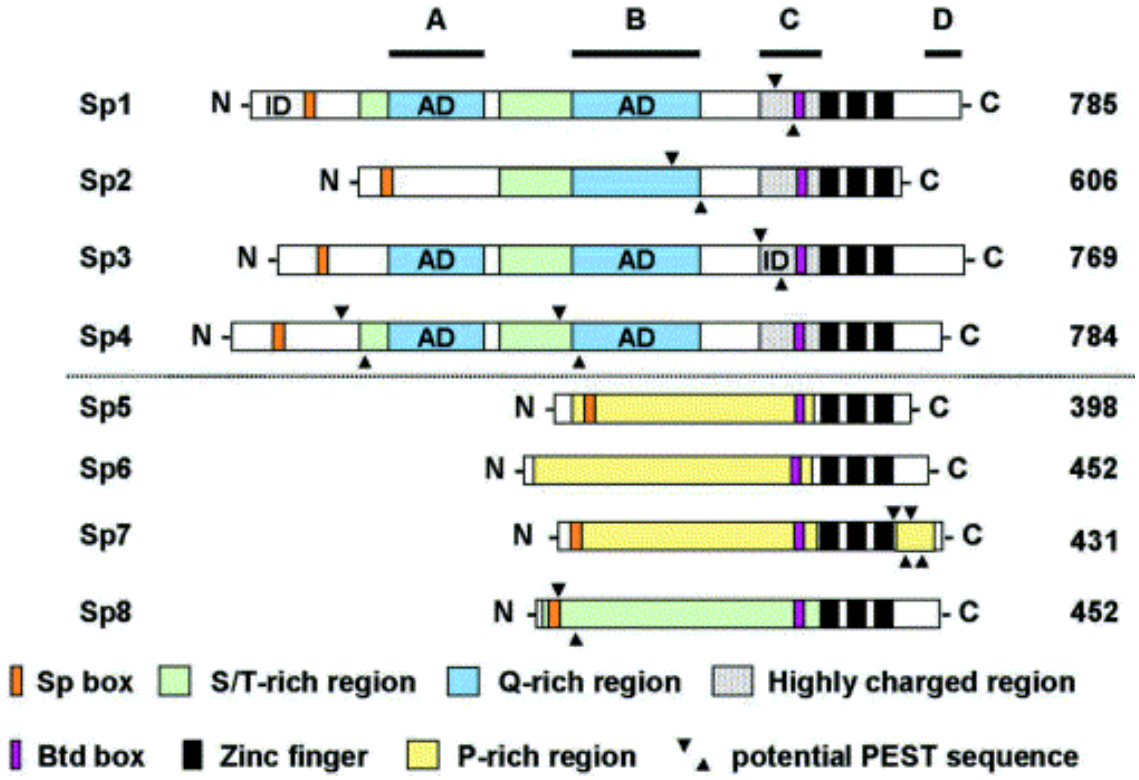


Figure 1.6. Structure motifs of Sp family proteins (177).

and other cofactors which include many basal transcription factors (183-186); and in this manner, many mammalian genes that contain GC-boxes are regulated by Sp proteins. These Sp-regulated genes are involved in a variety of physiological processes including cell growth/proliferation, angiogenesis and hormone activation.

Sp transcription factors in cancer

Sp1 and Sp3 transcription factors are expressed ubiquitously whereas Sp4 exhibits a more tissue-specific expression pattern primarily observed in central nervous

system, testis and epithelial tissues. However, their expression levels in adult tissues are relatively low and there is evidence that Sp1 expression decreases with age in rodents and humans (187-189). In contrast, several reports show that Sp1, Sp3 and Sp4 are highly expressed in tumors. For example, Sp1 is overexpressed in colon tumors compared to the adjacent normal tissues (190) and Sp1 overexpression has also been detected in pancreatic (92), gastric (191), breast (192) and thyroid tumors (193) compared to non-tumor tissues. In addition, Sp1 is also a negative prognostic factor for survival of cancer patients in these reports. The roles of Sp3 and Sp4 as cancer prognostic factors have not been reported even though their expression appears to be high in cancer cell lines. Genes that regulate cell cycle progression frequently contain GC-boxes in their proximal promoters; hence, they are induced by the overexpressed Sp transcription factors in cancer cells. Sp transcription factors contribute to the malignant cancer phenotype through upregulation of a growing list of pro-oncogenic factors including survivin, EGFR, VEGF, Bcl-2, cyclin D1, E2F1, c-fos, TGF-α and TGFβR. Therefore, it is likely that drugs targeting (or repressing) Sp transcription factors will be highly effective due to their downregulation of pro-oncogenic Sp-regulated genes. Several strategies to decrease Sp1, Sp3 and Sp4 in cancer cells and tumors have been reported including chemical- and enzyme-dependent modulations, drugs that competitively inhibit DNA binding, GC-rich oligonucleotide decoys that also decrease binding to GC-rich elements and several anticancer agents that downregulate Sp proteins through enhancing their degradation or gene repression (Fig. 1.7) (194). In the

second chapter of this thesis, the mechanism of action of the NSAID metabolite sulindac sulfide that targets Sp downregulation in colon cancer cells will be reported.

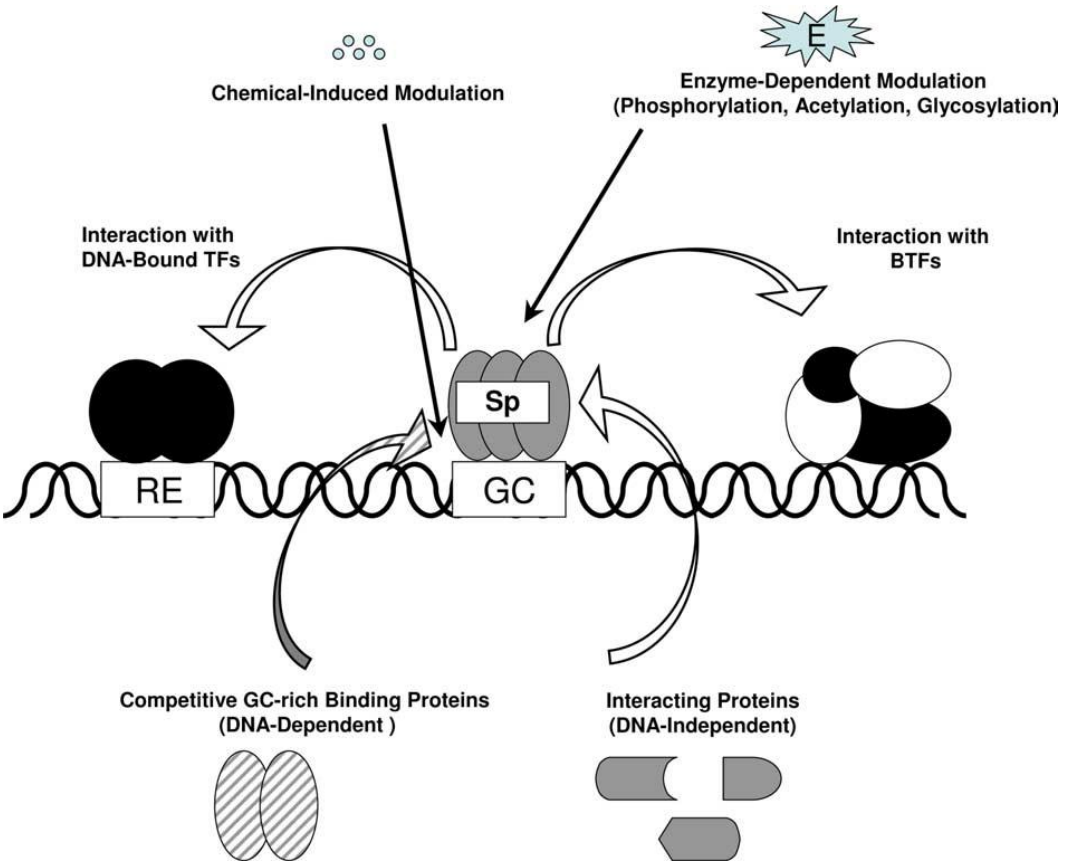


Figure 1.7. Strategies for Sp-modulation in cancer (194).

NR4A Nuclear Receptors

Nuclear receptors are a large family of intracellular transcription factors that are involved in diverse developmental and physiological processes such as organogenesis,

homeostasis, metabolism and reproduction (195-199). There are 48 human nuclear receptors and based on sequence homology they have been categorized into seven subfamilies which include the thyroid hormone receptor-like (NR1), the retinoid X receptor-like (NR2), the estrogen receptor-like (NR3), nerve growth factor IB-like (NR4), steroidogenic factor-like (NR5), germ cell nuclear factor-like (NR6) and miscellaneous (NR0) receptors (200).

The nerve growth factor IB-like nuclear receptor subfamily is consist of three members that are subcategorized as group A (NR4A) and include TR3 (testicular receptor 3; nerve growth factor IB, NGFIB; Nur77), Nurr1 (nuclear receptor related 1) and Nor1 (neuron-derived orphan receptor 1), which are encoded by NR4A1, NR4A2 and NR4A3 genes respectively. The first NR4A member, TR3, was initially identified as nerve growth factor-induced early immediate genes in rat pheochromocytoma PC12 cells (201, 202). NR4A receptors exhibit a wide range of functions in humans. For example, Nurr1 is important for neuron development and maintenance in the central nervous system (203); TR3 and Nurr1 both mediate neuroendocrine regulation in the hypothalamic pituitary adrenal axis (204); TR3, Nurr1 and Nor1 are involved in glucose metabolism and modulation of adipocyte differentiation (205); Nor1 is involved in regulation of metabolism in the skeletal muscle (206). There is also increasing evidence that demonstrates a role for NR4A receptors in cancer. Several studies show that NR4A receptors are involved in tumor and cancer cell apoptosis, proliferation, angiogenesis, DNA repair and fatty acid metabolism (Fig. 1.8) (207).

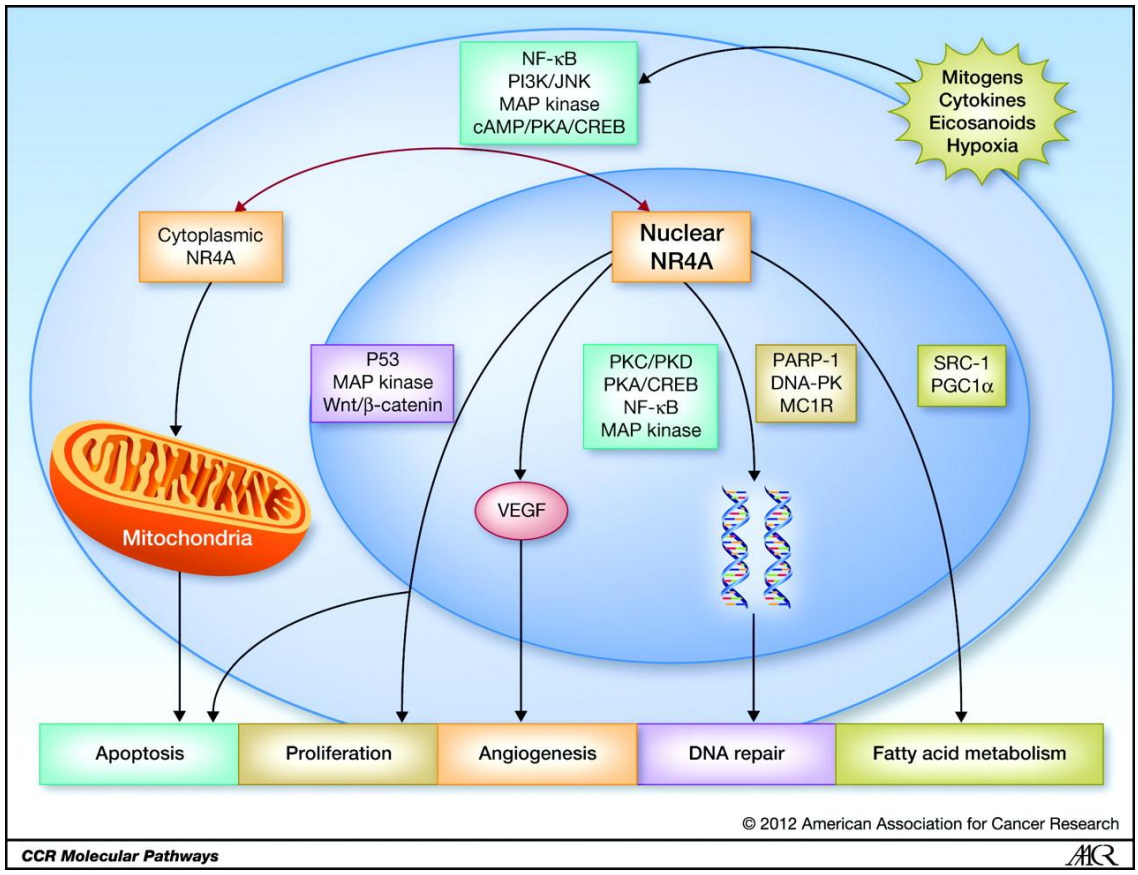


Figure 1.8. Current and potential roles of NR4A receptors in cancer (207).

NR4A1 receptor in cancer

TR3/NR4A1 exhibits tumor-suppressive functions in some cancer cells largely based on its cytosolic and not on its nuclear transcriptional functions and this tumor suppressive activity of TR3 is cell context dependent. Upon stimulation by phorbol esters and some pro-apoptotic agents and drugs, TR3 expression is induced and nuclear TR3 is exported and translocated to mitochondria where TR3 directly converts the anti-

apoptotic Bcl-2 into a pro-apoptotic complex by exposing its BH3 domain (208-210). This apoptosis pathway mediated by TR3 has been reported in several types of cancer cells and a similar mechanism is observed in the negative selection of T cells (209). In colon cancer cells, TR3 indirectly activates Bax resulting in cytochrome c release from mitochondria and apoptosis (211). In gastric cancer cells, TR3 translocation and induction of apoptosis is mediated by protein kinase C signaling pathway and, when translocation is blocked, TR3 inhibits cancer cell proliferation in the nucleus (212). TR3 also suppresses colon tumorigenesis in mice by inhibiting Wnt signaling pathway (213) and TR3-mediated β-catenin degradation is observed in human colon cancer cell lines (214).

Nuclear/transcriptional functions of TR3 in cancer have been investigated in several human cancer cell lines in this laboratory. Transactivation of TR3 by several 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl)methane analogs (C-DIMs) results in activation of caspase-dependent apoptosis mediated by TRAIL and PARP cleavage in pancreatic (215) and colon cancer cells (216). Further studies indicate that TR3-induced apoptosis is mediated by induction of pro-apoptotic genes such as ATF3 and p21 in pancreatic cancer cells and tumors (217). Similarly, activation of TR3 induces apoptosis in bladder cancer cells and inhibits the growth of bladder tumor xenografts (218). In contrast, inactivation of nuclear TR3 by C-DIM analogs results in apoptosis in pancreatic cancer cells and growth inhibition in pancreatic tumors in an orthotopic mouse model. In this case, TR3 and the co-regulator p300 interact with Sp1 which binds survivin promoter

and induces its expression. Inactivation of TR3 disrupts this regulatory complex and decreases transcription of the pro-survival factor survivin (219). Thus, both activation and inactivation of TR3 induce anticancer activity which depends on the drug/ligand and specific cell context.

Drug-induced nuclear export or transactivation of TR3 results in the induction of apoptosis, however, silencing of TR3 by RNA interference results in decreased cell proliferation and migration in colon, pancreatic, gastric, lung, lymphoma, melanoma and cervical cancer cells (219-225). This indicates that the role for TR3 in cancer is complex and depends, in part, on the cytosolic vs. nuclear location of the receptor.

NR4A2 receptor in cancer

Nurr1/NR4A2 expression is upregulated and associated with cell proliferation, migration/invasion and drug resistance in several cancer cell lines (224, 226-228). Nurr1 exhibits anti-apoptotic and pro-survival activities in some cancer cell lines. Normally, Nurr1 inhibits apoptosis through Wnt and MAPK pathways in dopaminergic neurons (229, 230). However, in breast cancer cells, Nurr1 inhibits p53-mediated induction of Bax, resulting in inhibition of apoptosis (231). In colon cancer cells, Nurr1 expression is associated with inflammatory responses where prostaglandin E_2 (PGE_2) induces Nurr1 through cAMP/CREB and NF-κB signaling and, in turn, Nurr1 induces expression of genes that promote cell proliferation (232). Inhibition of this PGE_2-Nurr1-mediated pathway by COX inhibitors or NSAIDs may reduce the risk for colon cancer. Also in colon

cancer cells, Nurr1 interacts with SRC-1 and PGC1α and induces the expression of fatty acid oxidation enzymes which promote cancer cell survival (233). Furthermore, Nurr1 expression is a negative prognostic factor for bladder cancer patients (234) and, interestingly, transactivation of this nuclear receptor inhibits growth of bladder cancer cells and tumors in mouse xenograft models (227).

The tumor suppressive functions of Nurr1 have also been reported. For example, a breast cancer study demonstrates that silencing Nurr1 is associated with decreased expression of p53 and increased metastasis to the lymph nodes (i.e. lymph node metastases do not express Nurr1) (226).

NR4A3 receptor in cancer

Nor1/NR4A3 appears to be functionally redundant and has an expression pattern similar to TR3 (235). Although Nor1 is overexpressed in many human cancer cells, its role has not been fully investigated. It is reported that Nor1 and TR3 double knockout mice rapidly develop acute myeloid leukemia (AML) and die within 2 to 4 weeks after birth (236). It is also reported in the same study that decreased expression of both Nor1 and TR3 is commonly observed in leukemia patients, suggesting a tumor suppressor role for these two receptors in AML. In addition, chromosomal translocation between Nor1 gene and Ewing sarcoma region-1 (EWS) gene is common in extraskeletal myxoid chondrosarcomas and this translocation results in a hyperactive oncogenic fusion protein (237-239).

In addition, CREB binding sites are present in the promoters of all NR4A genes; hence, NR4A receptors are potential downstream targets of VEGF signaling. There is evidence that TR3, Nurr1 and Nor1 mediate angiogenesis in endothelial cells (240-242). Furthermore, results remain contradictory for NR4A-mediated DNA damage repair in cancer cells. Two studies suggest a role for TR3 and Nurr1 in promoting DNA repair through PARP-1 and the melanocortin-1 receptor (243, 244) while one study reports that TR3 inhibits DNA repair and induces p53-mediated apoptosis in hepatoma cells (245).

TR3 and Nurr1 are overexpressed in many human cancer cell lines and tumors from cancer patients (215-219, 227, 234) despite the fact that their roles are complex and often confusing in cancer. Previous studies show that cytosporone B binds and activates TR3 (246) and 6-mercaptopurine activates the N-terminal domain of Nurr1 (247). Studies in this laboratory also show that a group of C-DIM analogs activate or inactivate NR4A receptors depending on the cell context (215-219, 227). Small molecules that modulate NR4A receptor activities (Fig. 1.9) are emerging as new therapeutic options for cancer patients (and for other diseases) and it is important to characterize these molecules and investigate their mechanisms of action. In Chapter III, we will discuss the activation of Nurr1 by synthetic C-DIM analogs in pancreatic cancer cells. In Chapter IV, we will report the identification of novel ligands/antagonists that bind and inactivate TR3 in colon cancer cells.

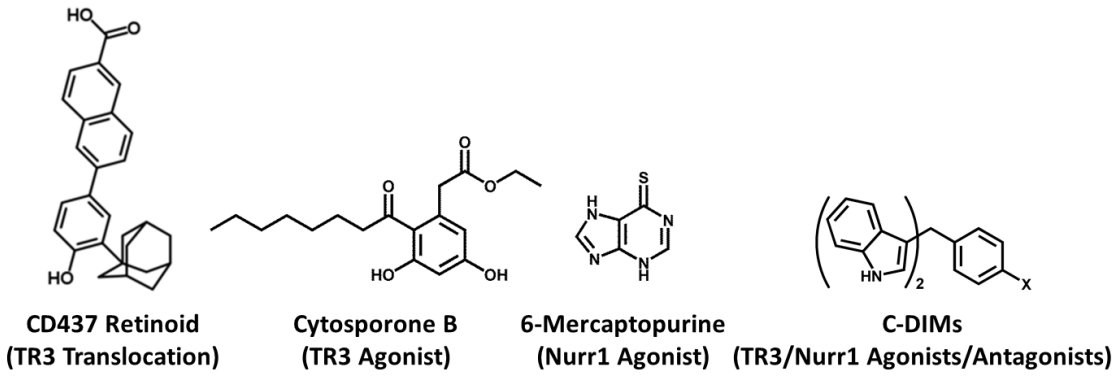


Figure 1.9. Examples of small molecules that modulate NR4A receptors.

CHAPTER II

SULINDAC SULFIDE INHIBITS COLON CANCER CELL GROWTH AND DOWNREGULATES SPECIFICITY PROTEIN TRANSCRIPTION FACTORS: A MECHANISTIC STUDY

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase (COX) inhibitors are widely used as analgesics and treatment of diseases associated with an inflammatory response, such as arthritis and cardiovascular diseases. Cancer has been associated with inflammation and there is epidemiologic evidence that NSAIDs decrease the risk for development of several cancers (248-254). Several studies show that the use of aspirin and other NSAIDs is associated with decreased incidence of colon cancer and aspirin use and treatment is also associated with a decrease in colon polyp formation (255-259). Aspirin and NSAIDs such as sulindac decrease colon polyp formation and the latter compound has been used in several clinical studies for inhibition of polyp formation in colon cancer patients and genetically susceptible individuals (260-264).

Sulindac, a COX-1 and COX-2 inhibitor, has been extensively investigated as a potent chemotherapeutic drug for treatment of colon and other cancers; however, due to the metabolism of sulindac (sulfoxide) to its sulfone (oxidation) or sulfide (reduction) metabolites, the mechanisms of action and identity of the active compound(s) are

uncertain. Several reports show that sulindac and its metabolites exhibit pronounced pro-apoptotic activity in cancer cells and animal models and this includes activation of both extrinsic and intrinsic apoptosis pathways (265-274). Sulindac/sulindac metabolites induce pro-apoptotic pathways in cancer cell lines derived from different tumors and several studies also report downregulation of the anti-apoptotic protein survivin (266-271). For example, sulindac-induced apoptosis in HT-29 cells is related to downregulation of survivin which in turn is due to decreased expression of β-catenin which regulates survivin expression through the transcription factor TCF-4 (270). Other studies also show downregulation of β-catenin and/or survivin in cancer cells and tumors treated with sulindac or its metabolites (272-274). However, it should be pointed out that the growth inhibitory and pro-apoptotic effects of survivin downregulation in head and neck sarcoma and carcinoma cells are STAT2-dependent (267, 268).

In addition, it has also been reported that sulindac and its metabolites exhibit growth inhibitory activity and this was associated, in part, not only with downregulation of survivin but also decreased expression of the epidermal growth factor receptor (EGFR) (275, 276) and vascular endothelial growth factor (VEGF) (277, 278). Studies in this laboratory have demonstrated that basal expression of these gene products in various cancer cell lines is dependent on specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 (279-281) which are highly expressed in cancer cells and tumors (190, 192, 193, 282). In this study, we initially compared the growth

inhibitory effects of sulindac and its metabolites in SW480 and RKO colon cancer cells and their order of activity was sulindac sulfide > sulindac sulfone > sulindac after treatment for 24, 48 or 72 hr. At concentrations of sulindac and its metabolites that inhibited cell growth, we observed that only sulindac sulfide decreased levels of Sp1, Sp3 and Sp4 proteins and this was accompanied by decreased expression of Sp1-dependent genes such as VEGF, survivin, EGFR and Bcl-2. Mechanistic studies suggest that sulindac sulfide induces reactive oxygen species (ROS) which in turn downregulates of Sp1, Sp3 and Sp4 in colon cancer cells (283).

Materials and Methods

Cell lines and cell culture

SW480 and RKO colon cancer cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium nutrient mixture with Ham's F-12 (DMEM/Ham's F-12, Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS, Sigma-Aldrich), 0.22% sodium bicarbonate and 1% antibiotic antimycotic solution (Gibco). In medium for drug treatment experiments, 2.5% charcoal-stripped FBS was used. Cells were maintained at 37°C in 5% CO_2.

Chemicals, oligonucleotides, siRNAs, plasmids and antibodies

Sulindac, sulindac sulfone and sulindac sulfide were purchased from LKT Laboratories. Real-time PCR primer sequences for ZBTB10 are forward: 5'-GCT GGA

TAG TAG TTA TGT TGC and reverse: 5'-CTG AGT GGT TTG ATG GAC AGA. The siRNA targeting Sp1 was purchased from Sigma-Aldrich (SASI_Hs02_00363664) and the sequences for Sp3 and Sp4 siRNAs are 5'-GCG GCA GGU GGA GCC UUC ACU dTdT and 5'-GCA GUG ACA CAU UAG UGA GC dTdT, respectively.

Sp1 construct (pSp1-Luc) contains -751 bp promoter insert linked to a luciferase reporter gene and Sp3 construct (pSp3-Luc) contains -417 bp promoter insert (284). Survivin construct (pSurvivin-Luc) contains -269 bp promoter insert and VEGF construct (pVEGF-Luc) contains -2018 bp promoter insert (279). MiR-27a construct (pMir27a-Luc) contains -639 bp promoter insert (285). The empty luciferase vectors pGL2 and pGL3 were also used in parallel as a negative control in luciferase assays.

Sp1, survivin, cleaved PARP and β-actin antibodies were purchased from Upstate/Millipore, R&D Systems, Cell Signaling Technology and Sigma-Aldrich, respectively. Sp3, Sp4, EGFR, p65 and VEGF antibodies were purchased from Santa Cruz Biotechnology.

Cell proliferation assay

Cells were plated in 12-well plates (3×10^4 per well) and allowed to attach for 24 hr. Cells were then treated with solvent control (DMSO) or varying concentrations of compounds or transfected with different siRNAs. Cells were trypsinized and counted at 24, 48 and 72 hr using a Z1 particle counter (Beckman Coulter).

Western blot analysis

Cells were plated in 6-well plates (3×10^5 per well), allowed to attach for 24 hr, and then treated with DMSO or varying concentrations of indicated compounds. Cells were lysed after 24 and 48 hr and whole cell lysates were resolved on 7.5% or 12% SDS-PAGE gels and proteins were transferred onto polyvinylidene difluoride membranes by wet electroblotting. Membranes were probed for indicated proteins by antibodies and β-actin was used as a loading control.

Flow cytometry

Cells were treated with 100 μM sulindac sulfide for indicated time and the general oxidative stress indicator CM-H_2DCFDA (Invitrogen) was used according to the manufacturer's protocol. Fluorescence was measured by Accuri C6 flow cytometer (BD Biosciences) and data was analyzed according to the manufacturer's guide.

Transfection and luciferase assay

Cells were plated in 12-well plates (1.5×10^5 per well), allowed to attach for 24 hr, and 400 ng of luciferase constructs (pSp1, pSp3, pSurvivin, pVEGF or pMir27a-Luc) and 40 ng of β-galactosidase constructs (β-gal) with a constitutively active promoter were cotransfected into each well by Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After 6 hr, transfection mixture was replaced with treatment medium containing either DMSO or varying concentrations of sulindac

sulfide for indicated time. Cells were then lysed using a freeze-thaw protocol and 30 μL of cell extract was used for luciferase and β-gal assays. LumiCount (Packard) was used to quantify luciferase and β-gal activities. Luciferase activity values were normalized against corresponding β-gal activity values and protein concentration values determined by Bradford assay. For RNA interference experiments, cells were transfected with either indicated siRNA (100 pmol per well in 12-well plate) or its scrambled control using Lipofectamine 2000 reagent.

Quantitative real-time PCR and TaqMan assay

Cells were treated as indicated and total RNA was extracted using RNeasy kit (Qiagen) then reverse transcribed using SuperScript reverse transcriptase (Invitrogen). Real-time PCR was carried out using a SYBR Green method (Applied Biosystems) and messenger RNA (mRNA) levels of target genes were normalized to that of the TATA-binding protein (TBP). Total miRNA was extracted using mirVana isolation kit (Ambion) and TaqMan probe for miR-27a was purchased from the same company. TaqMan assay and analysis were carried out according to the manufacturer's protocol.

Statistical analysis

Statistical significance of differences between experiment groups in cell proliferation, luciferase, flow cytometry, real-time PCR and TaqMan assays was analyzed using unpaired Student's t-test and *P* value of <0.05 was considered

statistically significant. Experiments were carried out in triplicate and all results are expressed as mean ± standard deviation (S.D.) for at least three independent determinations for each group.

Results

Results illustrated in Figures 2.1A and 2.1B show that sulindac and sulindac sulfone inhibited growth of SW480 and RKO cells at cytostatic concentrations between 600 – 900 and 225 – 300 μM respectively. Western blot analysis of whole cell lysates from these cells indicated that 600 to 1200 μM concentrations of sulindac did not affect expression of Sp1, Sp3 and Sp4 proteins in SW480 and RKO cells after treatment for 24 and 48 hr (Fig. 2.1C). Similar results were observed in these cells after treatment with 225 or 300 μM sulindac sulfone for 24 and 48 hr (Fig. 2.1D). Treatment of SW480 cells with 50 or 75 μM sulindac sulfide for 24 hr inhibited cell proliferation (Fig. 2.2A) and also slightly decreased expression of Sp1, Sp3 and Sp4 proteins (Fig. 2.2B). Sulindac sulfide induced similar responses after treatment for 48 hr; however, at this time point, there was a pronounced downregulation of Sp1, Sp3 and Sp4 proteins (Fig. 2.2B). Similarly, sulindac sulfide also decreased RKO cell growth after treatment for 24 or 48 hr (Fig. 2.2C) and this was accompanied by time-dependent downregulation of Sp1, Sp3 and Sp4 proteins (Fig. 2.2D). Thus, among sulindac and its two metabolites, it was apparent that sulindac sulfide was the most active compound and this has been observed in previous studies (286, 287). Moreover, the results suggest that the anti-

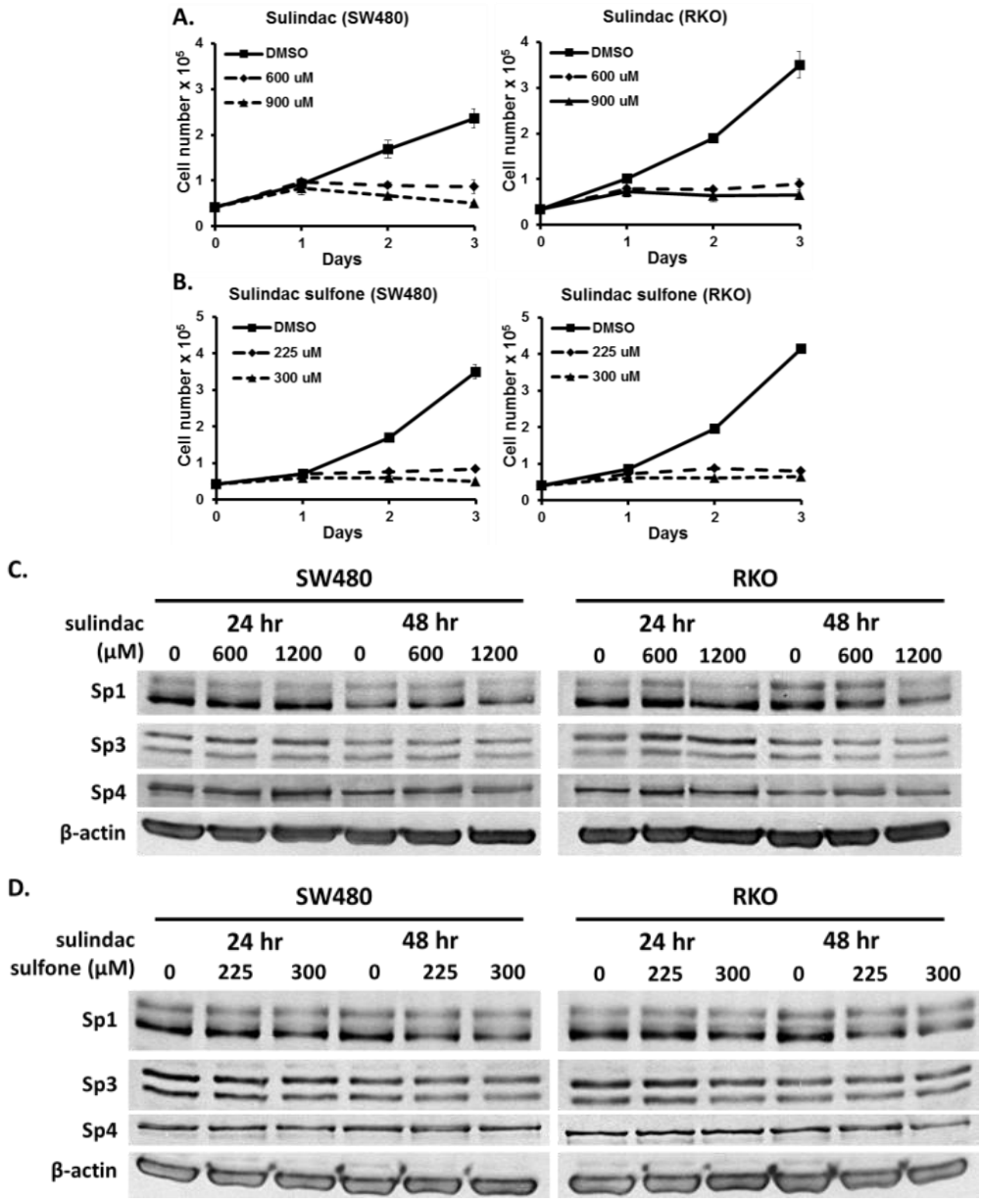


Figure 2.1. Sulindac and sulindac sulfone inhibit colon cancer cell growth without decreasing expression of Sp1, Sp3 and Sp4 proteins. *A*, *B*, sulindac and sulindac sulfone inhibit SW480 and RKO cell proliferation. Cells were treated with solvent control (DMSO), 600, 900 μM sulindac or 225, 300 μM sulindac sulfone for 24, 48 and 72 hr. Cell numbers were determined as described under *Materials and Methods*. Experiments were carried out in triplicate and results are expressed as mean ± S.D. for each determination. *C*, *D*, sulindac and sulindac sulfone have no effect on expression of Sp proteins in SW480 and RKO cells. Cells were treated with DMSO, 600, 1200 μM sulindac or 225, 300 μM sulindac sulfone for 24 and 48 hr. Levels of Sp1, Sp3 and Sp4 proteins were determined by western blot analysis as described under *Materials and Methods* and β-actin was used as loading control.

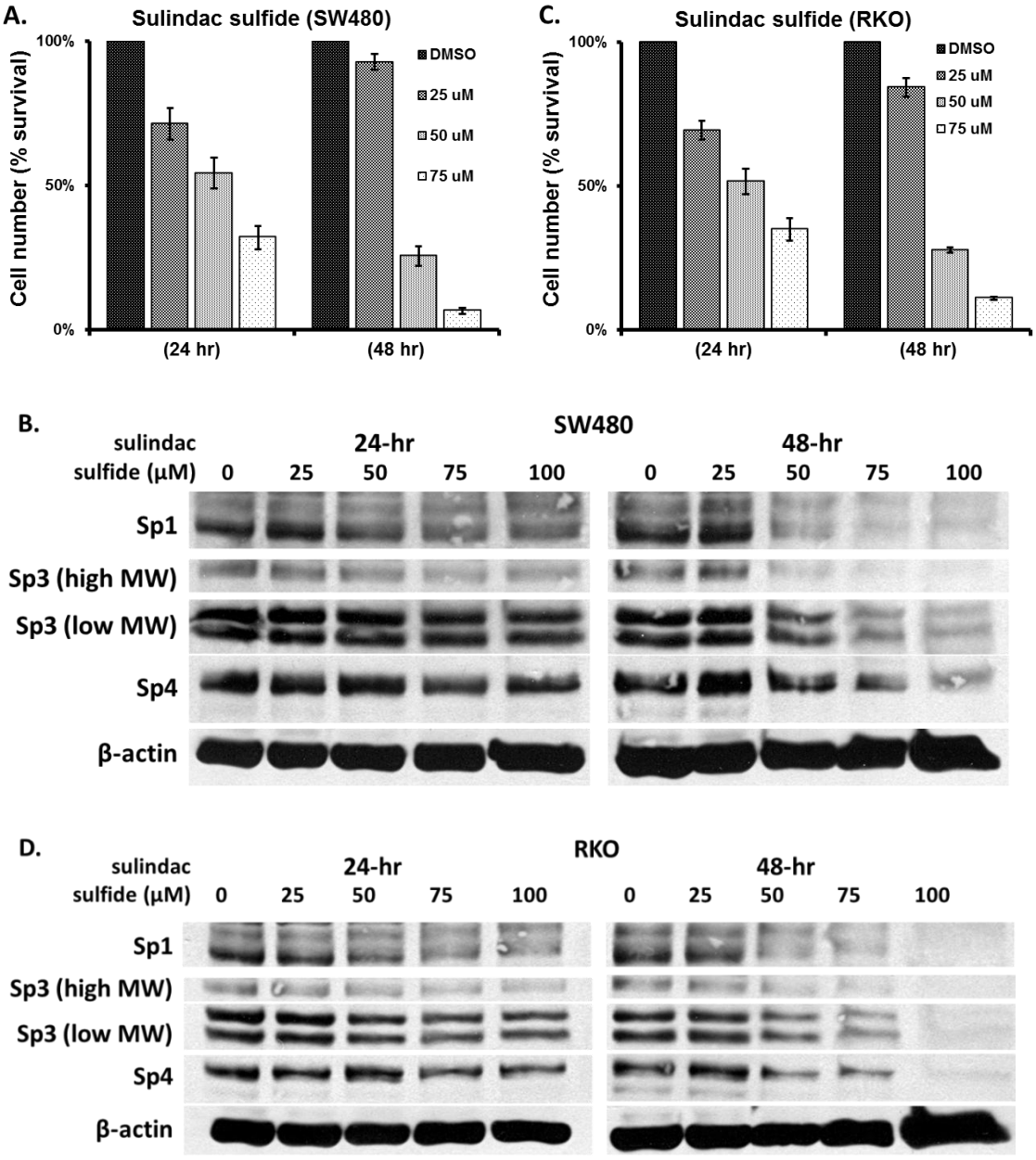


Figure 2.2. Sulindac sulfide inhibits colon cancer cell growth and decreases expression of Sp1, Sp3 and Sp4 proteins. *A*, *C*, sulindac sulfide inhibits SW480 and RKO cell proliferation. Cells were treated with DMSO, 25, 50, and 75 μM sulindac sulfide for 24 and 48 hr. Cell numbers were determined as described under *Materials and Methods*. Experiments were carried out in triplicate and results are expressed as percentage of control (mean ± S.D.). *B*, *D*, sulindac sulfide decreases expression of Sp1, Sp3 and Sp4 proteins in SW480 and RKO cells. Cells were treated with DMSO, 25, 50, 75 and 100 μM sulindac sulfide for 24 and 48 hr. Levels of Sp1, Sp3 and Sp4 proteins were determined by western blot analysis as described under *Materials and Methods* and β-actin was used as loading control.

neoplastic effects of sulindac sulfide are due, in part, to downregulation of pro-oncogenic Sp proteins.

We also investigated the effects of sulindac sulfide on Sp-dependent pro-apoptotic, growth inhibitory and anti-angiogenic responses in colon cancer cells. Results illustrated in Figures 2.3A and 2.3B show that sulindac sulfide decreased EGFR expression in SW480 and RKO cells after treatment for 24 and 48 hr and this is consistent with a decrease of EGFR transcripts (qPCR data not shown). We also examined the effects of sulindac sulfide on the p65 subunit of NF-κB, which is an Sp-dependent gene product in some cancer cell lines (284, 288, 289), and sulindac sulfide also decreased p65 expression in SW480 and RKO cells (Figs. 3A and 3B). In addition, sulindac sulfide also decreased expression of NF-κB subunit p105 and upregulated expression of the NF-κB inhibitor IκBα in SW480 and RKO cells (qPCR data not shown). Thus, sulindac sulfide-induced inhibition of SW480 and RKO cell proliferation was accompanied by inhibition of Sp1, Sp3, Sp4 and the Sp-dependent gene products, EGFR and p65. Treatment of SW480 cells with sulindac sulfide also decreased survivin expression and this was accompanied by caspase-dependent PARP cleavage which was observed after treatment for 24 and 48 hr (Fig. 2.3C). Similar results were observed in RKO cells (Fig. 2.3D) and western blot data are in agreement with the decrease of survivin mRNA levels in SW480 and RKO cells treated with sulindac sulfide (qPCR data not shown). It should be pointed out that the pro-apoptotic concentrations of sulindac sulfide were >25 or >50 μM in both cell lines with effective concentrations decreasing

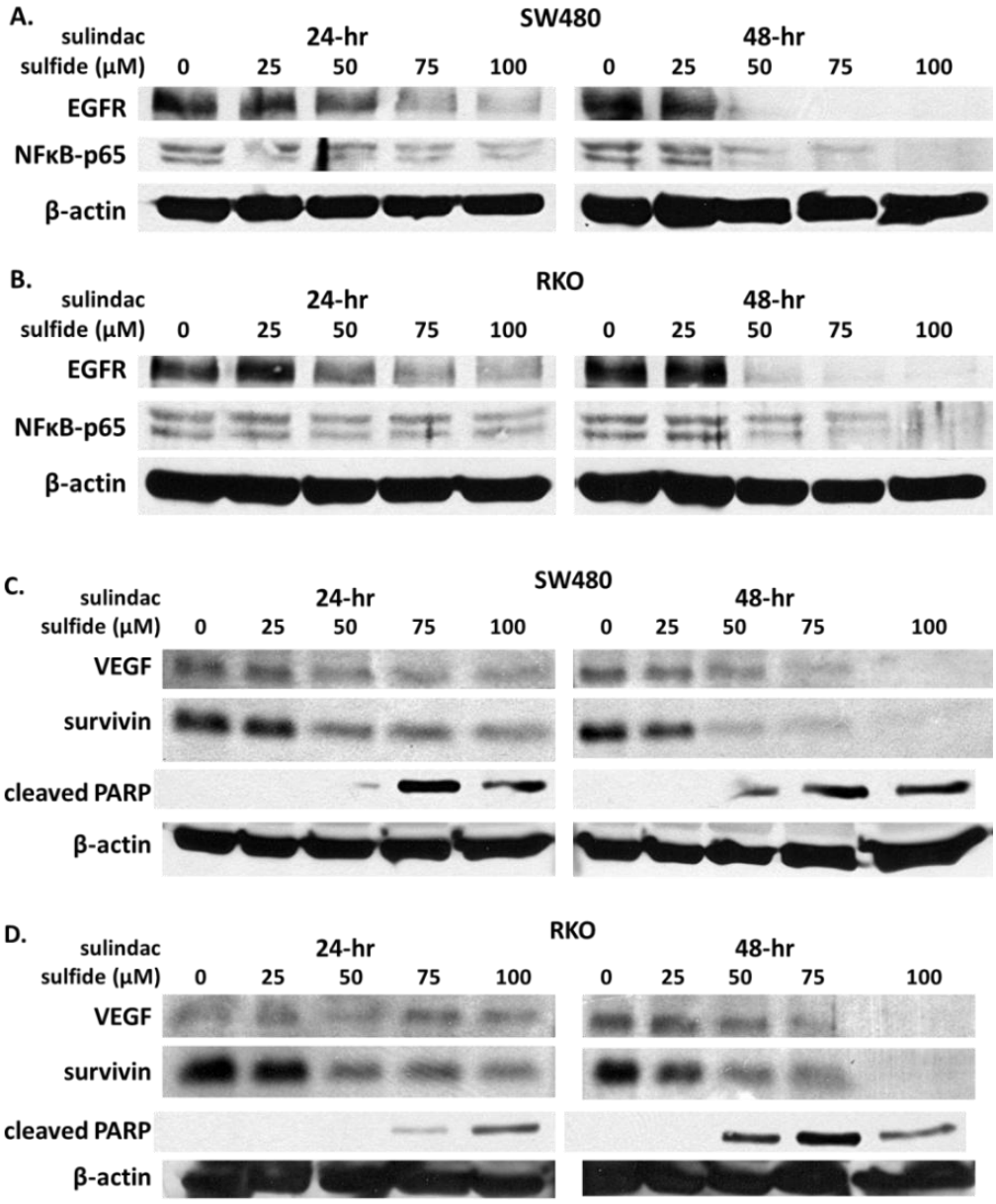


Figure 2.3. Sulindac sulfide decreases expression of EGFR, p65, VEGF and survivin and induces PARP cleavage in colon cancer cells. *A*, *B*, sulindac sulfide decreases expression of EGFR and the p65 subunit of NF-κB in SW480 and RKO cells. Cells were treated with DMSO, 25, 50, 75 and 100 μM sulindac sulfide for 24 and 48 hr. Levels of EGFR and p65 proteins were determined by western blot analysis as described under *Materials and Methods* and β-actin was used as loading control. *C*, *D*, sulindac sulfide decreases expression of VEGF and survivin and induces PARP cleavage in SW480 and RKO cells. Cells were treated with DMSO, 25, 50, 75 and 100 μM sulindac sulfide for 24 and 48 hr. Levels of VEGF, survivin and cleaved PARP proteins were determined by western blot analysis as described under *Materials and Methods* and β-actin was used as loading control.

with increasing treatment times which is similar to that observed for sulindac sulfide-dependent downregulation of Sp1, Sp3 and Sp4. In addition, sulindac sulfide also decreased expression of the Sp-dependent angiogenic VEGF gene product in SW480 (Fig. 2.3C) and RKO (Fig. 2.3D) cells, demonstrating that sulindac sulfide-dependent downregulation of Sp1, Sp3 and Sp4 is accompanied by decreased expression of Sp-dependent growth promoting (EGFR), inflammatory (p65), survival (survivin) and angiogenic (VEGF) gene products. The functional importance of sulindac sulfide-mediated downregulation of Sp1 was further investigated in SW480 and RKO cells by RNA interference. Knockdown of Sp1 decreased proliferation of both SW480 and RKO cells (ca. 60%, Fig. 2.4) whereas minimal growth inhibitory effects were observed after

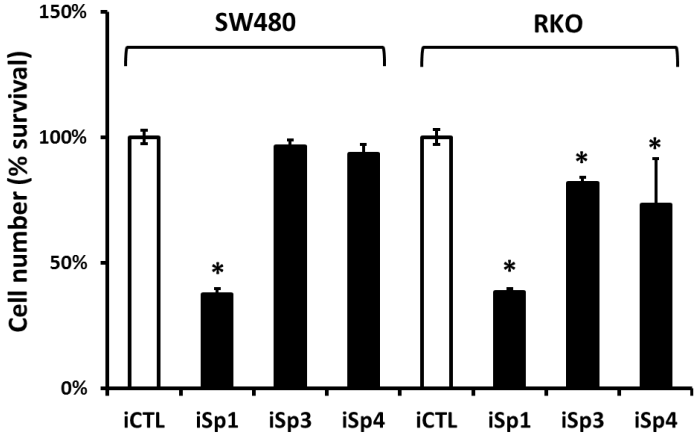


Figure 2.4. Effects of Sp knockdown on colon cancer cell proliferation. Knockdown of Sp1 decreases cell proliferation by >60% in SW480 and RKO cells and knockdown of Sp3 and Sp4 result in no inhibition of cell proliferation in SW480 cells and only minimal inhibition in RKO cells. Cells were transfected with scrambled control (iCTL) and Sp1, Sp3 or Sp4 siRNA (iSp1, iSp3, iSp4). Cell numbers were determined as described under *Materials and Methods*. Experiments were carried out in triplicate and results are expressed as mean ± S.D. for each determination.

Sp3 or Sp4 knockdown. These results suggest that Sp1 plays a pivotal role in colon cancer cell proliferation.

There is evidence that Sp1 and Sp3 are transcriptionally self-regulated through GC-rich elements that are presented in their promoters (194). We also examined the effects of sulindac sulfide on luciferase activity in cells transfected with reporter constructs containing GC-rich sequences from the Sp1 (pSp1-Luc) and Sp3 (pSp3-Luc) gene promoters. Sulindac sulfide decreased luciferase activity in both cell lines (Fig. 2.5A). Similar results were observed in cells transfected with the GC-rich survivin (pSurvivin-Luc) and VEGF (pVEGF-Luc) promoter constructs (Fig. 2.5B), demonstrating that sulindac sulfide also decreased expression of Sp-regulated genes with GC-rich promoters. These results suggest a mechanism that involves transcriptional repression of GC-box-driven genes through sulindac sulfide-induced Sp downregulation.

Previous studies show that betulinic acid, tolfenamic acid and curcumin induce proteasome-dependent degradation of Sp proteins in prostate, pancreatic and bladder cancer cells respectively (279, 290, 291); however, proteasome inhibitors did not block downregulation of Sp1, Sp3 or Sp4 by sulindac sulfide in SW480 and RKO cells (data not shown). Recent studies have identified a role for ROS in mediating repression of Sp proteins (292) and the nitro-NSAID GT-094 and curcumin induce ROS-dependent downregulation of Sp proteins and Sp-regulated gene products in colon cancer cells (283, 293). In Figure 2.6A, we observed that sulindac sulfide induced ROS in SW480 and RKO cells and the induction of ROS was consistently observed by using different

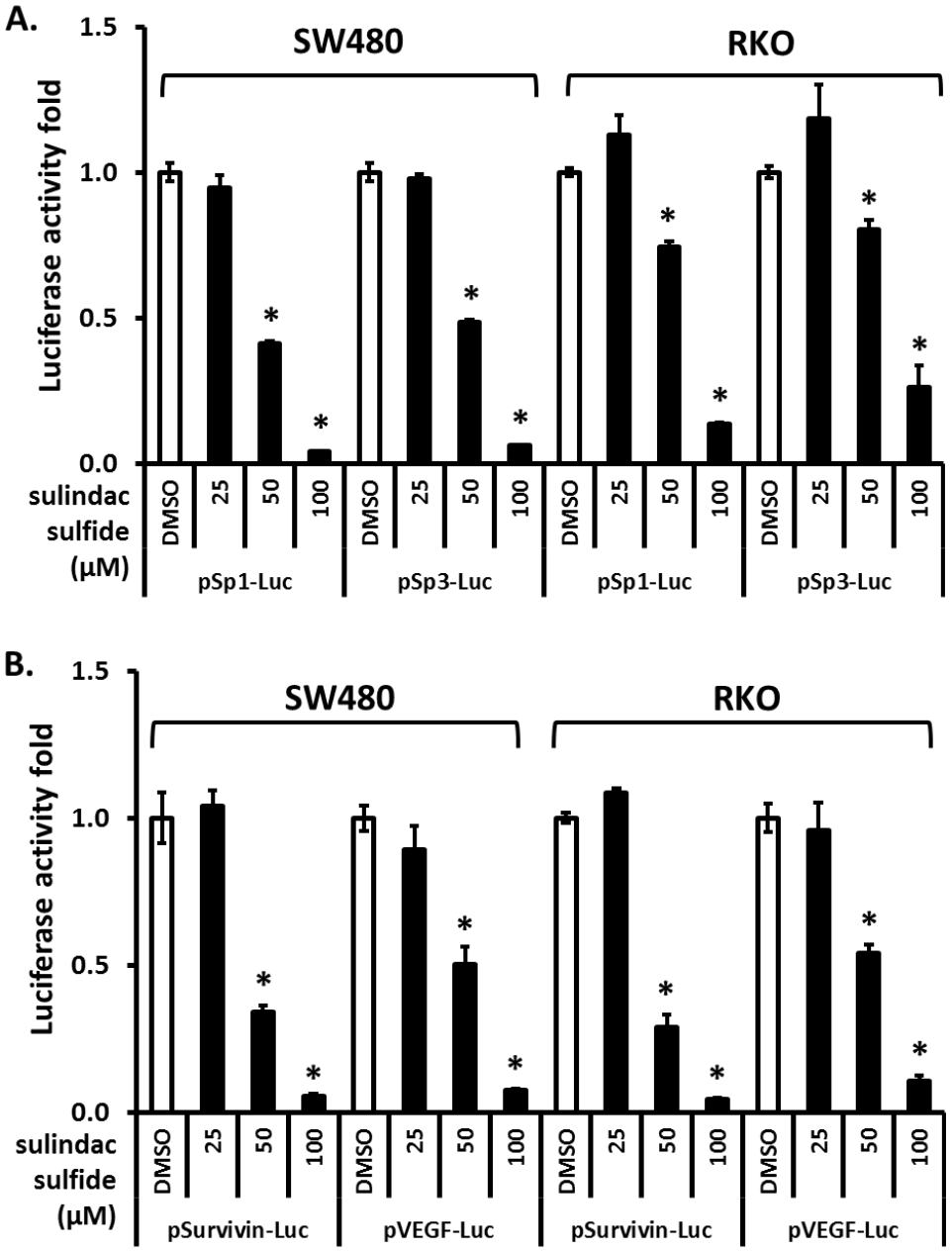


Figure 2.5. Sulindac sulfide decreases promoter gene activity of Sp1, Sp3, survivin and VEGF in colon cancer cells. *A*, cells were transfected with promoter-luciferase reporter constructs containing -751 bp promoter sequence of *SP1* gene (pSp1-Luc) or -417 bp of *SP3* gene (pSp3-Luc). *B*, cells were transfected with constructs containing -269 bp of *BIRC5* gene (pSurvivin-Luc) or -2018 bp of *VEGFA* gene (pVEGF-Luc). After 6 hr of transfection, cells were treated with DMSO, 25, 50 and 100 μM sulindac sulfide for 13 hr. Luciferase activity was determined as described under *Materials and Methods*. Experiments were carried out in triplicate and results are expressed as fold of control (mean ± S.D.). Asterisk (*) indicates statistical difference between control (DMSO) and treatment ($P < 0.05$).

methods including flow cytometry, fluorescence plate-reading and fluorescent microscopy (data not shown). Figure 2.6B shows that sulindac sulfide-induced downregulation of Sp1, Sp3 and Sp4 proteins was partially reversed in SW480 cells co-treated with sulindac sulfide plus the antioxidant dithiothreitol (DTT). The other thiol antioxidants glutathione (GSH) and N-acetylcysteine (NAC) were less active than DTT as inhibitors of Sp downregulation in SW480 cells. However, in parallel experiments in RKO cells (Fig. 2.6C), DTT, GSH and NAC exhibited comparable activity as inhibitors of sulindac sulfide-induced downregulation of Sp1, Sp3 and Sp4. We also investigated the effects of thiol antioxidants on sulindac sulfide-induced downregulation of Sp-dependent genes, VEGF and survivin, and the results show that co-treatment with the thiol antioxidants inhibited the effects of sulindac sulfide on both VEGF and survivin proteins in SW480 (Fig. 2.6D) and RKO (Fig. 2.6E) cells. A similar interaction between sulindac sulfide and thiol antioxidants was observed for Bcl-2 expression in both cell lines (Figs. 2.6D and 2.6E) and this is consistent with previous RNA interference studies showing that Bcl-2 is an Sp-regulated protein (291). In contrast, the expression of pro-apoptotic protein Bax was decreased by sulindac sulfide (and reversed by antioxidants) in SW480 cells (Fig. 2.6D) but not in RKO cells (Fig. 2.6E). It should be noted that RKO cells express wild type p53, which induces Bax in some studies, whereas p53 is mutated in SW480 cells. These results indicate that the effect of sulindac sulfide on Bax is cell context-dependent. Furthermore, cyclin D1 expression was decreased in SW480 (Fig. 2.6D) and RKO (Fig. 2.6E) cells by sulindac sulfide and cyclin D1 transcript was also

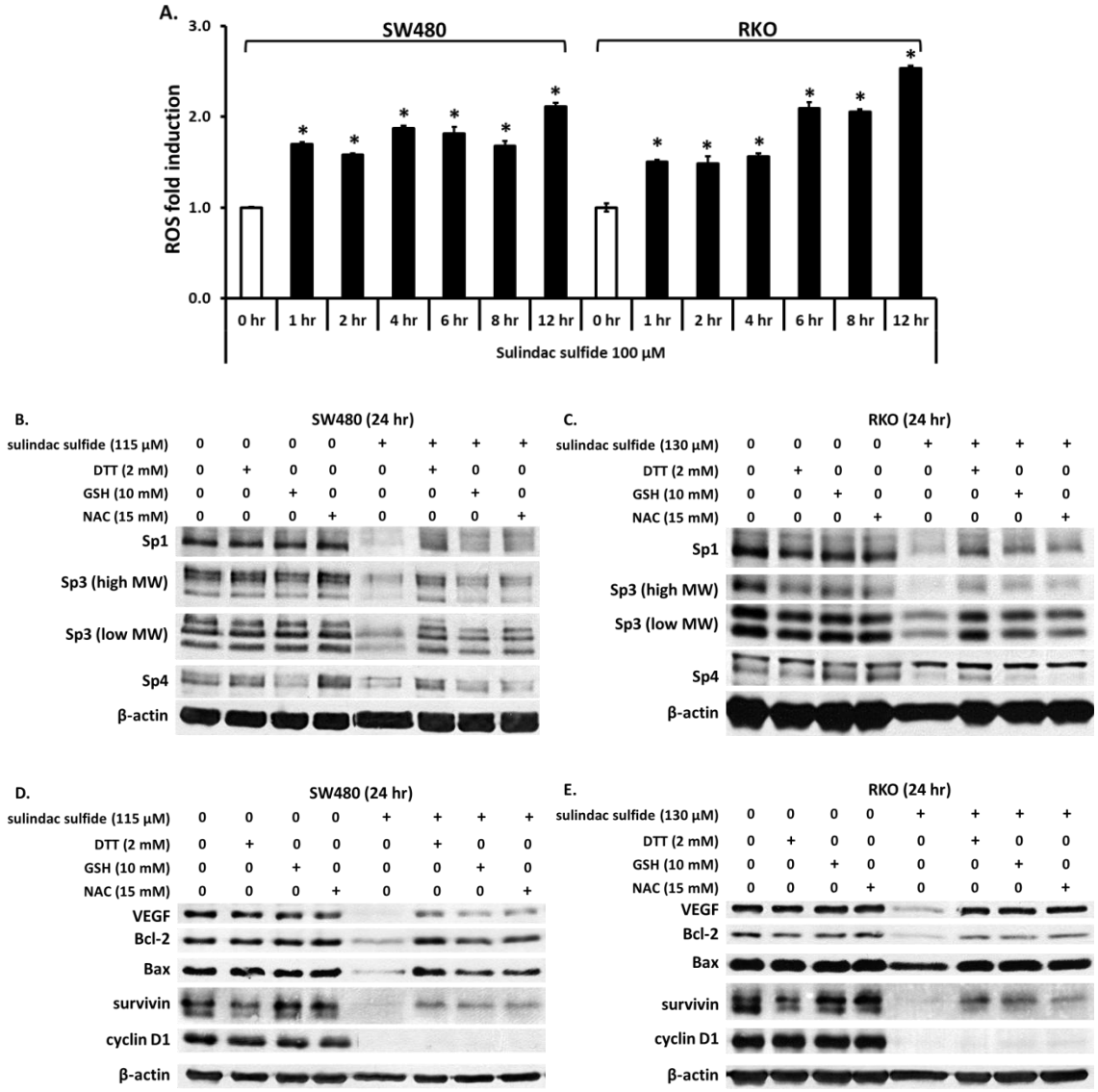


Figure 2.6. Sulindac sulfide induces ROS and ROS-dependent effects on Sp proteins and Sp-regulated gene products in colon cancer cells. *A*, cells were treated with 100 µM sulindac sulfide for indicated time and cellular ROS levels were measured by flow cytometry as described under *Materials and Methods*. Experiments were carried out in triplicate and results are expressed as fold of control (mean ± S.D.). Asterisk (*) indicates statistical difference between control (DMSO) and treatment ($P < 0.05$). *B*, *C*, cells were treated with DMSO, sulindac sulfide alone or in combination with antioxidants as indicated for 24 hr. Cells were treated with antioxidants for 45 min before treatment with sulindac sulfide in combination. Levels of Sp1, Sp3 and Sp4 proteins were determined by western blot analysis. *D*, *E*, Levels of VEGF, Bcl-2, Bax, survivin and cyclin D1 proteins were determined by western blot analysis as described under *Materials and Methods* and β-actin was used as loading control.

decreased in both cell lines after sulindac sulfide treatment (qPCR data not shown). However, thiol antioxidants did not block the effects of sulindac sulfide on cyclin D1, suggesting cyclin D1 is regulated in an ROS/Sp-independent manner in SW480 and RKO cells. Previous studies with ROS inducers suggest that the mechanism of Sp downregulation is associated with ROS-dependent repression of microRNA-27a (miR-27a) which results in the induction of the Sp repressor ZBTB10 (294). Treatment of SW480 and RKO cells with sulindac sulfide decreased both miR-27a promoter activity (Fig. 2.7A) and cellular level of miR-27a transcript and this response was attenuated after co-treatment with antioxidant NAC (Fig. 2.7B). In a parallel experiment, we observed that sulindac sulfide induced miR-27a-targeted ZBTB10 gene expression in SW480 and RKO cells (Fig. 2.7C). Thus, like other ROS-inducing anticancer agents (283-285, 292, 295), sulindac sulfide disrupts the miR-27a:ZBTB10 axis and the transcriptional repressor ZBTB10 competitively binds GC-rich *cis*-element to decrease expression of Sp1, Sp3, Sp4 and Sp-regulated genes (Fig. 2.8).

Discussion

Sp transcription factors Sp1, Sp3 and Sp4 are highly expressed in cancer cells/tumors and Sp1 is a negative prognostic factor for survival of gastric and pancreatic cancer and glioma patients (191, 296-298). Although Sp1 and other Sp proteins are important for early embryonic and postnatal development in mice, their expression is relatively low in adult tissues and there is evidence that Sp1 expression

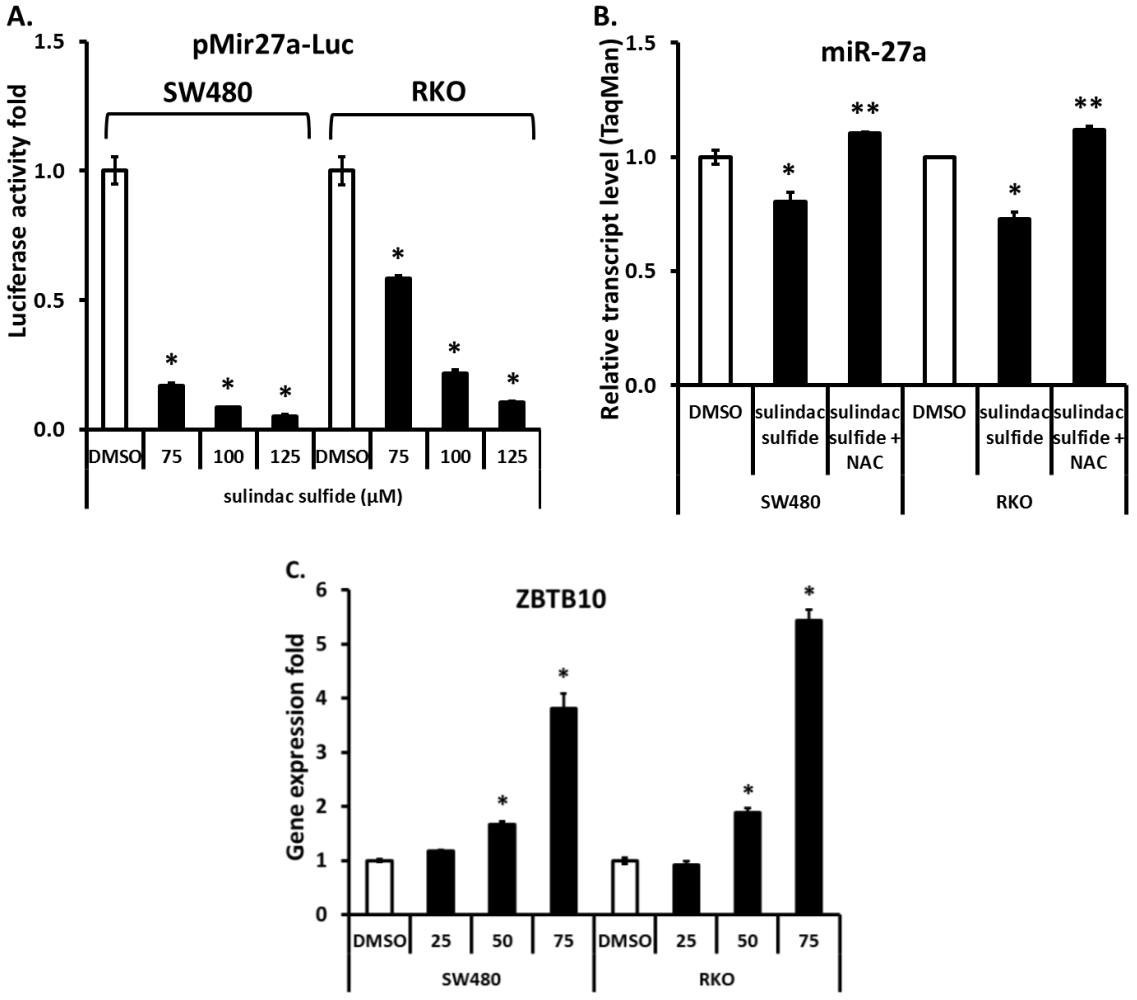


Figure 2.7. Effects of sulindac sulfide on miR-27a and ZBTB10 in colon cancer cells. *A*, sulindac sulfide decreases miR-27a promoter activity in SW480 and RKO cells. Cells were transfected with luciferase construct containing -639 bp promoter sequence of *mir-27a* gene (pMir27a-Luc). After 6 hr, cells were treated with DMSO, 75, 100 and 125 μM sulindac sulfide for 18 hr and luciferase activity was determined as described under *Materials and Methods*. *B*, effects of antioxidants on sulindac sulfide-mediated downregulation of miR-27a. Cells were treated with DMSO, 100 μM sulindac sulfide alone or in combination with 10 mM NAC for 24 hr and expression of miR-27a transcript were determined by TaqMan PCR analysis as described under *Materials and Methods*. *C*, sulindac sulfide increases gene expression of ZBTB10 in SW480 and RKO cells. Cells were treated with DMSO, 25, 50 and 75 μM sulindac sulfide for 24 hr and levels of ZBTB10 mRNA were determined by real-time PCR analysis as described under *Materials and Methods*. All experiments were carried out in triplicate and results are expressed as fold of control (mean ± S.D.). Asterisk (*) indicates statistical difference between control (DMSO) and treatment and double-asterisk (**) indicates statistical difference between single treatment and combination treatment ($P < 0.05$).

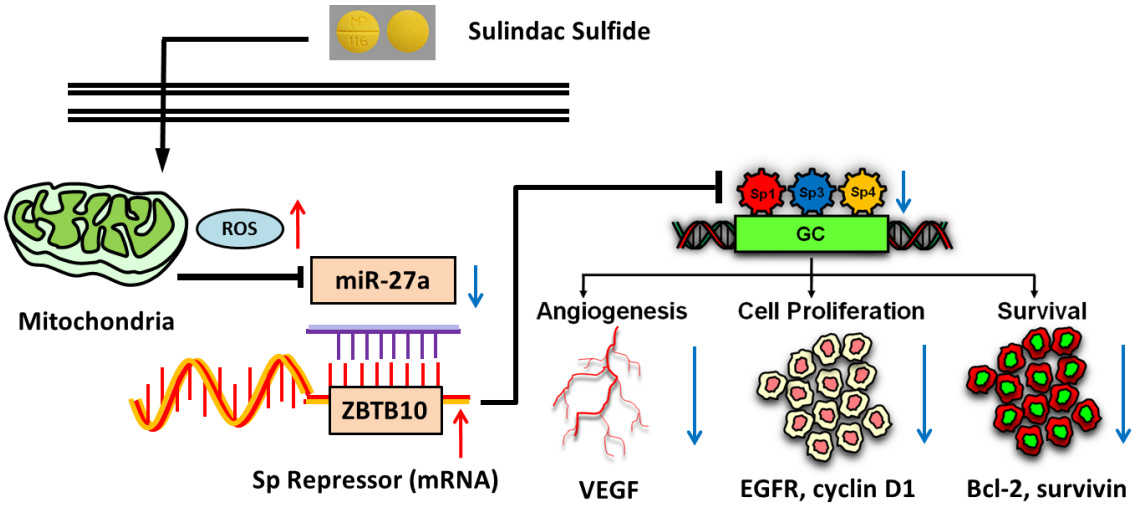


Figure 2.8. Effects of sulindac sulfide on the ROS-miR-27a-ZBTB10-Sp axis. Sulindac sulfide induces ROS; downregulates miR-27a; upregulates ZBTB10; downregulates Sp proteins and Sp-dependent survival/proliferative, inflammatory and angiogenic protein products; results in growth inhibition and apoptosis.

decreases with age in rodents and humans (187-189). The functional importance of Sp1, Sp3 and Sp4 in cancer cells has been confirmed by RNA interference (RNAi) showing that knockdown of Sp (singly or combined) decreases cell proliferation, survival, angiogenesis and inflammation (284, 291, 292). These results are consistent with identification (by RNAi) of several pro-oncogenic Sp-regulated genes important for cell growth (cyclin D1, EGFR, c-Met), survival (Bcl-2, survivin), angiogenesis (VEGF and VEGF receptors), and inflammation (p65 subunit of NF-κB) (279, 280, 284, 291, 292). Thus, Sp transcription factors clearly contribute to the transformed cell phenotype and represent an example of non-oncogene addiction by cancer cells. Studies in this laboratory show that several structurally-diverse anticancer drugs downregulate Sp

transcription factors through two major pathways, namely, degradation (activation of proteasomes or caspases) or by ROS-dependent transcriptional repression (Fig. 2.8) and activation of one or both pathways is dependent on the agent and cell context. For example, previous studies with NSAIDs show that tolfenamic acid induced proteasome-dependent degradation of Sp proteins in pancreatic cancer (290), the nitro-NSAID GT-094 induced ROS-dependent repression (283) and aspirin induced caspase-dependent degradation of Sp1, Sp3 and Sp4 (288).

In this study, we also observed that sulindac and its metabolites inhibited proliferation of SW480 and RKO colon cancer cells and sulindac sulfide was the most active compound (Figs. 2.1A, 2.1B, 2.2A and 2.2C) and this was consistent with their relative growth-inhibitory effects in other studies (286, 287). At concentrations of sulindac or sulindac sulfone that inhibited SW480 and RKO cell proliferation, the levels of Sp1, Sp3 or Sp4 proteins were unchanged (Figs. 2.1C and 2.1D) whereas sulindac sulfide-dependent growth inhibition was accompanied by decreased expression of Sp1, Sp3 and Sp4 (Figs. 2.2B and 2.2D). These results clearly distinguish between sulindac sulfide and sulindac/sulindac sulfone and indicate that the anticancer activity of sulindac sulfide is due, in part, to downregulation of Sp transcription factors.

Like tolfenamic acid and other compounds that induce Sp downregulation, sulindac sulfide also decreased expression of EGFR, survivin, VEGF and Bcl-2 and also decreased the p65 subunit of NF-κB which is Sp-regulated in only some cancer cell lines (284, 288, 289). Tolfenamic acid induces proteasome-dependent downregulation of

Sp1, Sp3 and Sp4 in pancreatic cancer cells (290). However, proteasome inhibitors did not block sulindac sulfide-mediated repression of these transcription factors (data not shown).

Studies with curcumin, celastrol, betulinic acid, synthetic triterpenoids and NSAID analogs show that ROS-dependent transcriptional repression of Sp1, Sp3 and Sp4 (and Sp-regulated genes) is due to downregulation of miR-27a and/or miR-20a/17-5p which are overexpressed in multiple tumors (294, 299). Decreased expression of these microRNAs results in induction of miR-targeted ZBTB10 and/or ZBTB4, which have been characterized as "Sp-repressors" that competitively displace Sp transcription factors from GC-rich promoter sites to decrease gene expression. The role of ROS in targeting repression of Sp transcription factors has been confirmed by studies showing that arsenic trioxide, pharmacologic doses of ascorbate (which induces H_2O_2), *t*-butylhydroperoxide and H_2O_2 decreased expression of Sp1, Sp3, Sp4 and Sp-regulated gene products in cancer cell lines (284, 295, 300).

In this study, sulindac sulfide was observed to induce ROS in SW480 and RKO cells in a time-dependent manner in flow cytometry experiments using an ROS indicator (Fig. 2.4). Results of our studies with sulindac sulfide are consistent with the ROS-dependent gene repression pathway where induction of ROS disrupts miR-27a-ZBTB10 interaction to decrease miR-27a and induce ZBTB10 (Fig. 2.7). ZBTB10 overexpression or miR-27a antagonism decreases Sp protein expression (294) and this

is also consistent with sulindac sulfide-induced downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes (Figs. 2.2 and 2.3).

This study demonstrates that sulindac sulfide is the active metabolite of sulindac which is known to exhibit anti-neoplastic activity in human and experimental models of colon cancer. Results of this study suggest that induction of ROS and downregulation of Sp transcription factors contribute to the anticancer activity of sulindac and also suggest that other drugs that activate the same pathway in colon cancer may also be effective for treatment of this disease.

CHAPTER III

STRUCTURE-DEPENDENT ACTIVATION OF NR4A2 (NURR1) BY 1,1-BIS(3'-INDOLYL)-1-(AROMATIC)METHANE ANALOGS IN PANCREATIC CANCER CELLS*

Introduction

The nuclear receptor (NR) superfamily of transcription factors are characterized by their structural homology which includes N- and C-terminal domains (A/B and E/F, respectively), a DNA binding domain (C), and an adjacent hinge region (D) (301-303). Both N- and C-terminal regions may contain activation functions (AFs), and the ligand binding domain (LBD) resides in the C-terminus of NRs (301-304). The 48 members of the NR superfamily can be subdivided into three broad categories, namely, the endocrine nuclear receptors, adopted orphan receptors, and orphan receptors such as NR4A for which cognate ligands have not yet been identified (301-304). The NR4A orphan receptor subfamily includes NR4A1 (Nur77, NGFI-B, TR3), NR4A2 (Nurr1, NOT), and NR4A3 (Nor-1, MINOR) (305-308), and it has been suggested that the failure to identify an endogenous ligand may be due to the lack of a typical NR ligand binding pocket in the LBD domain of NR4A receptors (309).

NR4A receptors are immediate-early genes that are induced by diverse stimuli in multiple tissues, and there is increasing evidence that these receptors play important roles in maintaining tissue homeostasis and in pathophysiological processes including cancer (305-308). NR4A receptors have specific functions in the brain, T-cells/thymocytes, adipose tissue, steroidgenesis, muscle, blood vessels, macrophages, and cardiovascular system; however, the role of NR4A receptors in carcinogenesis is less well understood (307, 308). Knockdown of NR4A1 by RNA interference in cancer cell lines induced apoptosis or inhibited growth in multiple cell lines (reviewed in 308) and NR4A2 knockdown also induced apoptosis and decreased metastasis in cancer cells lines (225, 234, 310).

The effects and mechanisms of action of drug-induced activation or inactivation of NR4A receptors are complex and are dependent on both cell context and structure. Studies with phorbol esters, retinoids and other apoptosis-inducing agents have unraveled a novel NR4A1-dependent pro-apoptotic pathway that involves nuclear export of the receptor which in some cell lines forms a mitochondrial pro-apoptotic Bcl-2-NR4A1 complex (311, 312). Studies in this laboratory have demonstrated that among 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl) methanes (C-DIMs), the *p*-methoxy (DIM-C-pPhOCH_3) and *p*-hydroxy (DIM-C-pPhOH) derivatives induce nuclear NR4A1-dependent apoptosis and growth inhibition of colon, pancreatic and bladder cancer cells through activation or inactivation of the receptor (215, 218, 219, 313). Cytosporone B and

related compounds bind directly the receptor and appear to activate nuclear NR4A1-mediated transcription; these agonists also induce nuclear NR4A1 export (246, 314).

6-Mercaptopurine (6-MP) activates NR4A2 in CV1 and HEK293 cells through the N-terminal A/B domain and this pathway involves metabolic activation of 6-MP (247). Several benzimidazoles also induce NR4A2-dependent transactivation; and a *p*-chloro-substituted C-DIM analog (DIM-C-pPhCl) also activates Nurr1 in bladder cancer cells (227, 315). In this study, we have investigated the structure-dependent activation of NR4A2 in pancreatic cancer cells by twenty-three C-DIM analogs containing various *p*-substituted phenyl and heteroaromatic substituents and their activity was compared to 6-MP. Among the most active compounds were the *p*-trifluoromethyl (DIM-C-pPhCF_3), *p*-bromo (DIM-C-pPhBr), *p*-*t*-butyl (DIM-C-pPhtBu), *p*-cyano (DIM-C-pPhCN), *p*-iodo (DIM-C-pPhI), and *p*-trifluoromethoxy (DIM-C-phOCF_3) analogs. Using one or more NR4A2-active C-DIMs as models, these compounds also induced transactivation in cells transfected with constructs containing three copies of an NGFI-B response element ($NBRE_{x3}$-Luc) and three copies of a Nur response element ($NurRE_{x3}$-Luc). Nurr1-active C-DIMs also activated a wild type GAL4-Nurr1 variant and GAL4-Nurr1-(A/B) and GAL4-Nurr1-(C-F) chimeras containing N- and C-terminal regions of NR4A2, respectively; and the prototypical model NR4A2 activator DIM-C-pPhBr induced expression of several NR4A2-dependent genes in Panc1 and Panc28 that were confirmed by RNA interference. These studies demonstrate that the C-DIM structure is an excellent scaffold for developing NR4A2-active compounds.

Materials and Methods

Cell lines and cell culture

Panc1 and Panc28 pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium nutrient mixture with Ham's F-12 (DMEM/Ham's F-12, Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS, Sigma-Aldrich), 0.22% sodium bicarbonate and 10 mL/L 100× antibiotic antimycotic solution (Invitrogen). Cells were maintained at 37°C in the presence of 5% CO_2 and the solvent [dimethyl sulfoxide (DMSO)] used in the experiments was $\leq$ 0.15%.

Plasmids

The GAL4-Nurr1 chimeras GAL4-Nurr1 (full length, amino acid 1 to 598), GAL4-Nurr1-AB (amino acid 1 to 259), and GAL4-Nurr1-(C-F) (amino acid 260 to 598) were constructed by inserting PCR-amplified each fragment into the BamHI/HindIII site of pM vector (Clontech). The FLAG-tagged full-length Nurr1 (FLAG-Nurr1) was constructed by inserting PCR-amplified full-length Nurr1 fragment into the HindIII/BamHI site of p3XFLAG-CMV-10 expression vector (Sigma-Aldrich). The $NBRE_{x3}$-Luc was generously provided by Dr. Jacques Drouin (University of Montreal, Quebec, Canada). All other reporter constructs have been previously described (219).

Antibodies, chemicals, reagents, siRNAs and oligonucleotides

The NR4A2 antibody (sc-991) was purchased from Santa Cruz Biotechnology and the TR3/Nur77 antibody (IMG-528) was purchased from Imgenex. The FLAG (F3165) and β-actin antibodies were purchased from Sigma-Aldrich.

The methylene-substituted diindolylmethanes were synthesized in this laboratory by condensation of indole or substituted indoles with corresponding aromatic aldehyde (2:1 indole/aldehydes) at 80°C in a pH5 buffer essentially as described (215, 316, 317). Compounds were crystallized from benzene/hexane ($\geq$ 1 time) and purities were $>$ 97% as determined by gas chromatography or gas chromatography-mass spectrometry (215, 316, 317). Indole was purchased from Sigma-Aldrich and the following benzaldehyde derivatives were used to make a series of DIM-C-pPhX analogs where X represents the *para*-substituent: *p*-trifluoromethylbenzaldehyde, *p*-bromobenzaldehyde, *p*-fluorobenzaldehyde, *p*-*t*-butylbenzaldehyde, *p*-*N*-dimethylamino, benzaldehyde (no substituent), *p*-hydroxybenzaldehyde, *p*-phenylbenzaldehyde, *p*-cyanobenzaldehyde, *p*-tolualdehyde, *p*-chlorobenzaldehyde, *p*-iodobenzaldehyde, *p*-carboxymethylbenzaldehyde, *p*-methoxybenzaldehyde, *p*-butoxybenzaldehyde, and *p*-trifluromethoxybenzaldehyde. The *meta*- and *ortho*-bromo substituted isomers were prepared by condensing indole with *meta*- and *ortho*-bromobenzaldehye, respectively. The *N*-methyl and 2-methyl analogs of DIM-C-pPhBr were prepared by condensing *p*-bromobenzaldehyde with *N*-methylindole and 2-methylindole, respectively as described (317). The C-DIM analogs

containing heteroaromatic substituents were synthesized by condensing indole with the following aldehydes: 2-furaldehyde, 2-thiophene-carboxaldehyde, 3-thiophenecarboxaldehyde, pyrrole-2-carboxaldehyde, piperonal, 4-pyridine-carboxyaldehyde, *N*-*O*-4-pyridine-carboxaldehyde, and indole-3-carboxaldehyde. All indole derivatives, aldehydes and 6-mercaptopurine (MP) were purchased from Sigma-Aldrich.

Reporter lysis buffer, luciferase and β-galactosidase (β-gal) reagents were purchased from Promega and Applied Biosystems. Plasmid and total-RNA extraction kits were purchased from Qiagen. SYBR Green (Applied Biosystems) was used for triplicate real-time PCR reaction.

All the primers and the small inhibitory RNAs were prepared by Sigma-Aldrich. Two siRNA oligonucleotides were used in combination to target NR4A2 (Nurr1): 5'- CAG UUA CCA CUC UUC GGG A dTdT and 5'- CGU GUG UUU AGC AAA UAA A dTdT. The sequences of the primers used for real-time PCR were as follows: NR4A2 forward 5'-AGT CTG ATC AGT GCC CTC GT, reverse 5'-TAT GCT GGG TGT CAT CTC CA; VIP forward 5'-TCA GGT TCA TTT GCT CCC TC, reverse 5'-TCT TCT CAC AGA CTT CGG CA. SPP1 (osteopontin) forward 5'-TTG CAG TGA TTT GCT TTT GC, reverse 5'-GCC ACA GCA TCT GGG TAT TT; NRP1 forward 5'-AAG GTT TCT CAG CAA ACT ACA GTG, reverse 5'-GGG AAG AAG CTG TGA TCT GGT C. NRP2 forward 5'-GAT TCG GGA TGG GGA CAG TGA, reverse 5'-GGT GAA CTT GAT GTA GAG CAT GGA.

Transfection, luciferase assay and quantitative real-time PCR

Cells were plated on 12-well plates at 7×10^4 per well in DMEM/F12 supplemented with 2.5% charcoal-stripped FBS and 0.22% sodium bicarbonate. After 24 hr growth, various amounts of DNA [i.e. UAS_{x5}-Luc (400 ng), GAL4-Nurr1 (40 ng) and β-gal (40 ng)] were cotransfected into each well by Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After 5-6 hr of transfection, cells were treated with plating media (as above) containing either solvent (DMSO) or the indicated concentration of compound for 18 hr. Cells were then lysed using a freeze-thaw protocol and 30 µL of cell extract was used for luciferase and β-gal assays. LumiCount (Packard) was used to quantify luciferase and β-gal activities. Luciferase activity values were normalized against corresponding β-gal activity values as well as protein concentrations determined by Bradford assay. For RNA interference experiment, cells were transfected with equal amount of both siRNA duplex (i.e. 75 pmol each/well for 6-well plate) using Lipofectamine 2000 reagent for 24 hr prior to treatment. Total RNA was extracted, reverse transcription and real-time PCR were carried out as described previously (219), and messenger RNA (mRNA) levels were normalized to the expression of TATA-binding protein (TBP).

Subcellular localization assay and western blot analysis

Cells were seeded on cover glass and transfected with adenovirus expressing FLAG-Nurr1 (5 MOI) for 4 hr. At 18 hr after transfection, cells were treated with DIM-C-

pPhBr for 12 hr and immunostained with anti-FLAG antibody. Cells were then mounted in mounting medium including DAPI (Vector Laboratories) and the fluorescent images were obtained using a Zeiss Axioplan2 fluorescence microscope (Carl Zeiss).

For western blotting, cells (2×10^5) were plated on 6-well plates in DMEM/Ham's F-12 media containing 10% charcoal-stripped FBS for 16 hr and then treated with indicated concentrations of compounds. Cellular lysates and their subsequent separation by electrophoresis were carried out as described previously using β-actin as loading control (219) and Sp1 as the representative nuclear protein (215).

Statistical analysis

Statistical significance of differences in luciferase activities and gene expression levels between groups was analyzed using unpaired Student's t-test. A *P* value of <0.05 was considered statistically significant. Results are expressed as mean $\pm$ standard deviation (S.D.) for at least three independent determinations for each treatment group.

Results

Panc1 and Panc28 pancreatic cancer cell lines were used in these studies for screening a series of C-DIM compounds as activators of NR4A2. Transfection of Panc28 cells with a UAS_{x5}-Luc construct containing 5 tandem GAL4 response elements gave relatively low basal luciferase activity compared to Panc1 cells. For the C-DIM screening

assay, Panc28 cells were transfected with UAS_{x5}-Luc and the GAL4-Nurr1 chimera (containing the yeast GAL4 DNA binding domain fused to wild type NR4A2) and treated with DMSO, 7.5 or 15 μM concentrations of various C-DIMs containing *p*-substituted phenyl or heteroaromatic groups (Figs. 3.1A and 3.1B). Compounds containing *p*-substituted CF_3, Br, *t*-Bu, CN, I and OCF_3 groups induced the highest activity at the 15 μM concentration. Among the heteroaromatics, only 1,1-bis(3'-indolyl)-1-[2,4-(methylendioxy)benzaldehyde]methane, the piperonal condensation product (C-DIM-pip), induced activity > 5-fold. A similar approach was used in Panc1 cells and the fold-induction of luciferase activity by C-DIMs was significantly lower than observed in Panc28 cells (Figs. 3.1C and 3.1D) and this may be due, in part, to the relatively high basal luciferase activity in this cell line transfected with UAS_{x5}-Luc. Despite the compression of induced luciferase activities, most of the *p*-substituted phenyl and heteroaromatic compounds that activated GAL4-Nurr1 in Panc28 cells were also active in Panc1 cells.

The subcellular location of NR4A2 was determined in Panc1 cells transfected with FLAG-Nurr1 (Fig. 3.2A). Immunostaining showed only nuclear FLAG, and treatment with 15 μM DIM-C-pPhBr for 12 hr did not induce any changes in nuclear NR4A2 staining. Immunostaining with NR4A2 antibodies also gave a nuclear signal which was weak (data not shown) and this necessitated the use of FLAG-tagged NR4A2. A similar immunostaining pattern was observed in Panc28 cells treated with solvent control (DMSO) or 15 μM DIM-C-pPhBr for 12 hr (Fig. 3.2B)**;** and the western blot in Figure 3.2C

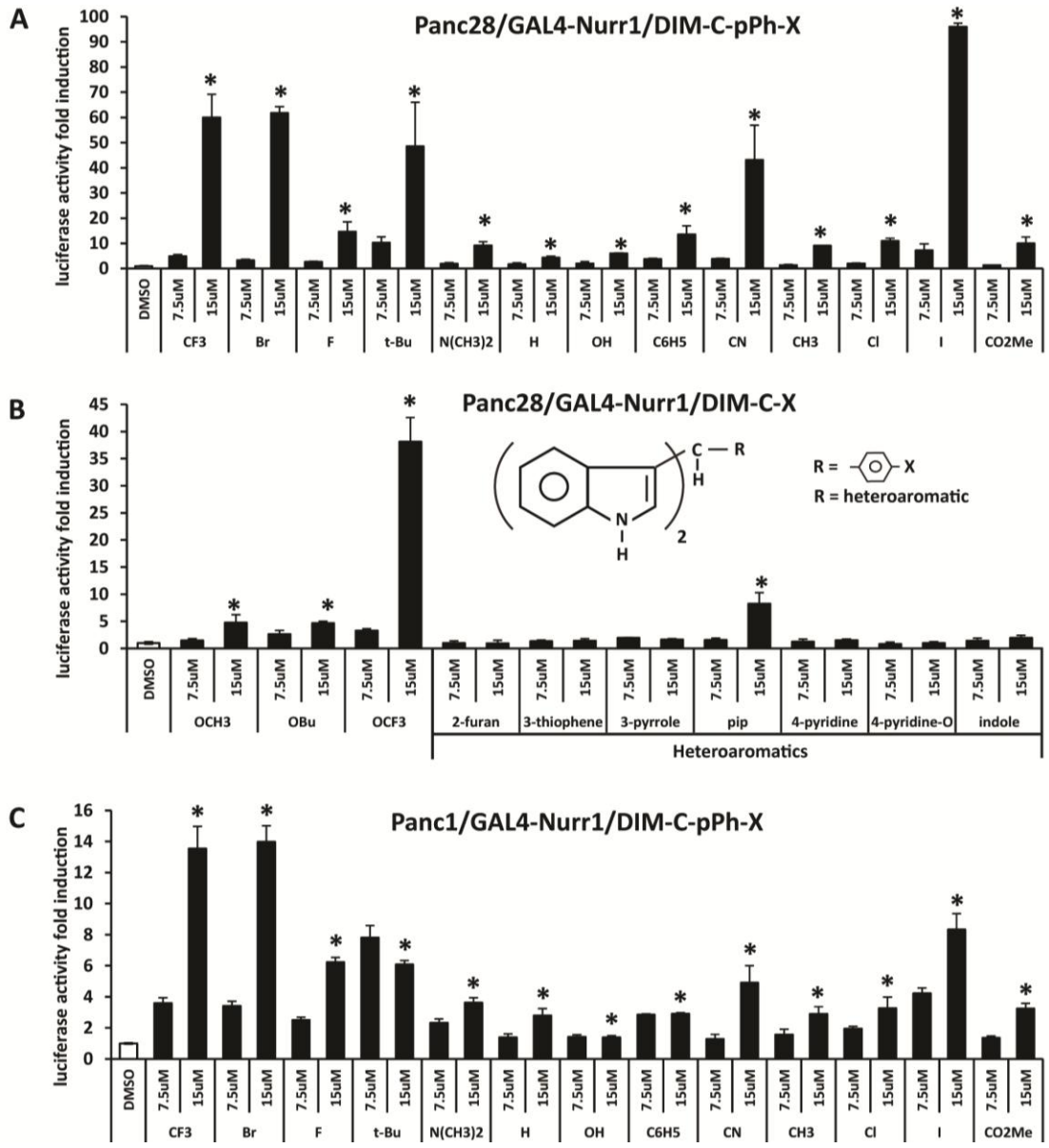


Figure 3.1. Activation of GAL4-Nurr1 chimeras by C-DIMs. UAS_{x5}-Luc (400 ng) and GAL4-Nurr1 (40 ng) were cotransfected into Panc28 (*A, B*) and Panc1 (*C, D*) cells for 6 hr and then treated with 7.5 and 15 μM phenyl-substituted C-DIMs including trifluoromethyl (CF_3), bromo (Br), fluoro (F), *tert*-butyl (*t*-Bu), dimethylamino ($N(CH_3)_2$), hydrogen (H), hydroxy (OH), phenyl (C_6H_5), cyano (CN), methyl (CH_3), chloro (Cl), iodo (I), carboxymethyl (CO_2Me) (*A, C*), methoxy (OCH_3), *tert*-butoxy (OBu) or trifluoromethoxy (OCF_3) (*B, D*) group on the *para* position, or the heterocyclic C-DIMs including 2-furan, 3-thiophene, 3-pyrrole, piperonal, 4-pyridine, 4-pyridine-N-oxide or indole ring (*B, D*) for 18 hr. Luciferase activity was determined as described in *Materials and Methods*. Results are expressed as mean ± S.D. for at least three separate determinations for each treatment. *, $P < 0.05$, high concentration treatment (15 μM) vs. solvent control (DMSO).

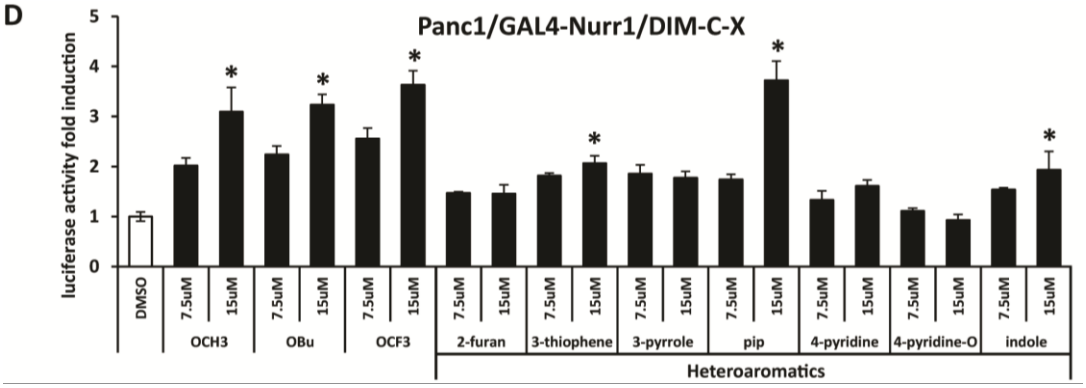


Figure 3.1. Continued

shows that both NR4A2 and NR4A1 proteins were expressed in Panc1 and Panc28 cells. Moreover, after treatment of Panc1 cells with DMSO or 15 µM DIM-C-pPhBr for 24 hr, Nurr1 protein was isolated in nuclear (NE) but not cytosolic (CE) extracts, and expression was not changed by the treatment (Fig. 3.2D). Sp1 protein served as a nuclear protein control for this experiment. Preliminary studies showed that transfection of Panc1 and Panc28 cells with $NBRE_{x3}$-Luc or $NurRE_{x3}$-Luc constructs resulted in low basal activity and inducibility; however, higher activities and inducibility were observed only after transfection with FLAG-Nurr1 expression plasmid (data not shown), suggesting endogenous levels of NR4A2 were limiting in cells transfected with the response element constructs. Based on these results, 10 ng FLAG-Nurr1 construct was transfected for the structure-activity studies in Panc1 and Panc28 cells. Results in Figures 3.3A and 3.3B show that in Panc28 cells transfected with $NBRE_{X3}$-Luc or $NurRE_{x3}$-Luc constructs respectively, the most active C-DIM compounds identified in the

GAL4-Nurr1/UAS_{x5}-Luc screening assays were also active in Panc28 cells; and similar results were observed in Panc1 cells (Figs. 3.3C and 3.3D). The compounds exhibited similar potencies and some cell context- and construct ($NBRE_{x3}$-Luc vs. $NurRE_{x3}$-Luc)-dependent differences.

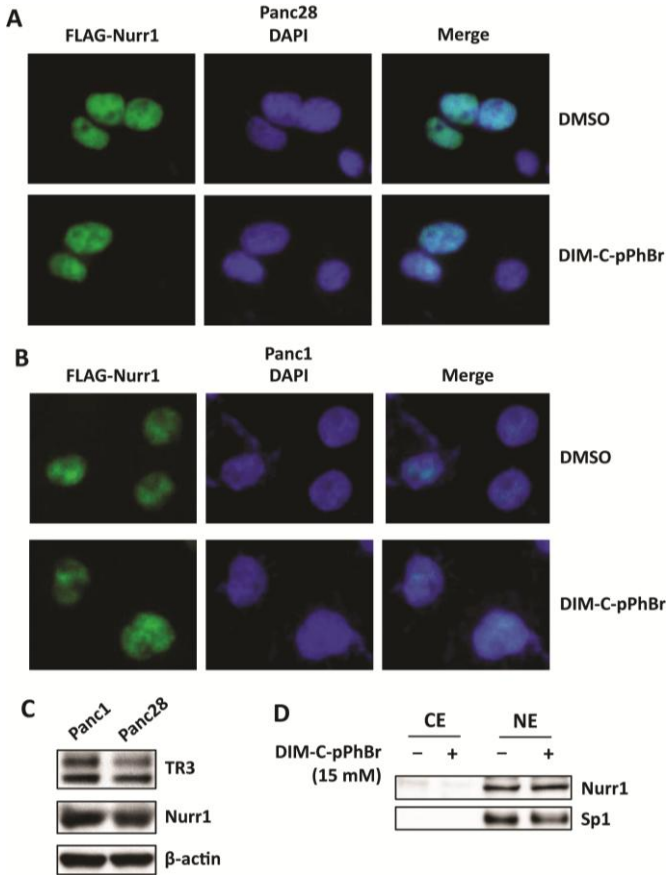


Figure 3.2. Expression and subcellular localization of NR4A2 in Panc28 and Panc1 cells. Panc28 (*A*) and Panc1 (*B*) cells were transfected with adenovirus expressing FLAG-Nurr1 for 4 hr; media was changed and after 18 hr, cells were treated with 15 µM of DIM-C-pPhBr for 12 hr and immunostained with anti-FLAG antibody. Fluorescent images were obtained as described in *Materials and Methods*. *C*, whole cell lysates from Panc1 and Panc28 cells were analyzed by western blotting and β-actin was used as loading control. *D*, cells were treated with DMSO or 15 µM DIM-C-pPhBr for 24 hr, and cytosolic (CE) or nuclear (NE) extracts were analyzed by western blots (Sp1 as nuclear protein control).

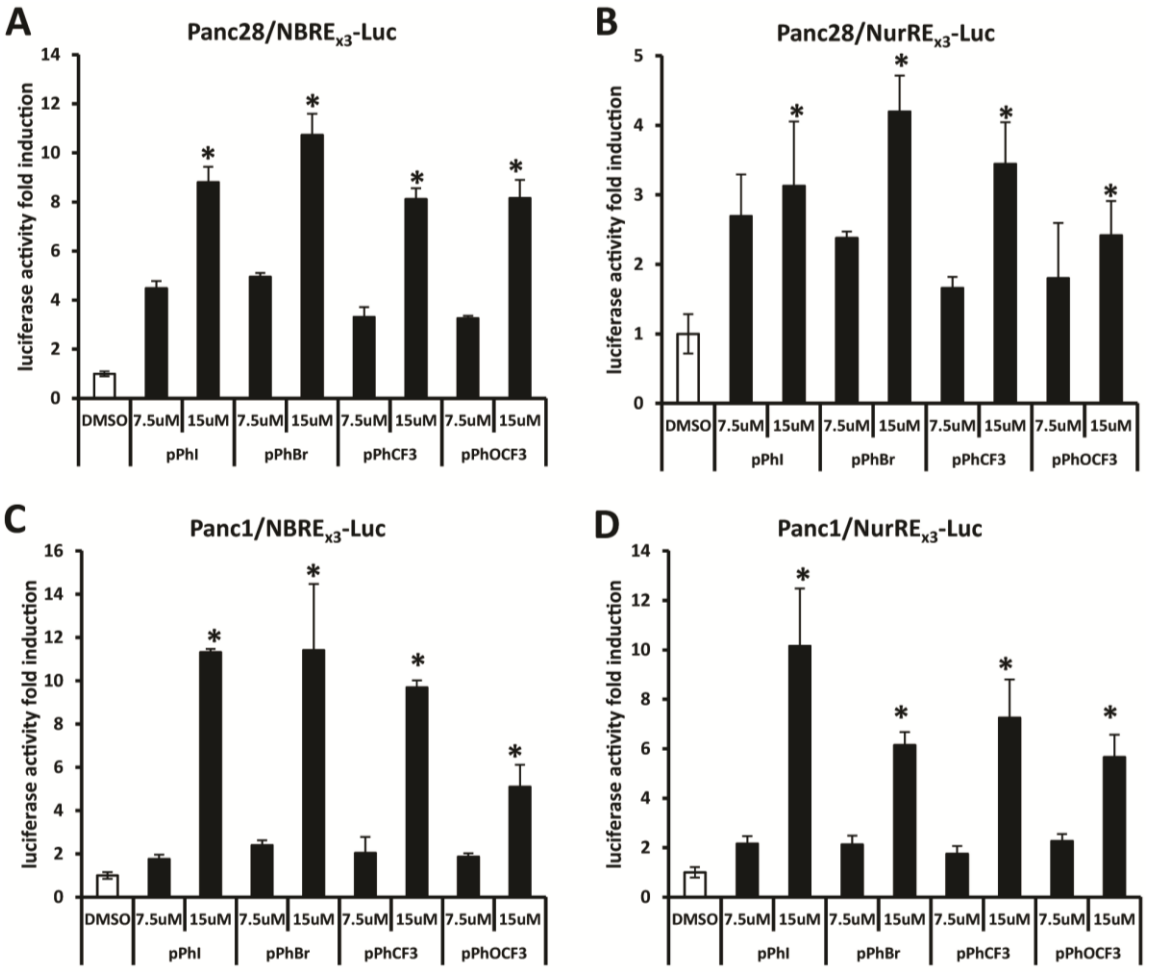


Figure 3.3. Selected NR4A2 activators increase activities of Nur response elements containing $NBRE_{x3}$-Luc and $NurRE_{x3}$-Luc luciferase genes. $NBRE_{x3}$-Luc (200 ng) (*A, C*) or $NurRE_{x3}$-Luc (200 ng) (*B, D*) was cotransfected with FLAG-Nurr1 (10 ng) into Panc28 (*A, B*) or Panc1 (*C, D*) cells for 6 hr and then treated with 7.5 and 15 μM *p*-substituted phenyl-C-DIMs DIM-C-pPhI, -pPhBr, -$pPhCF_3$, and -$pPhOCF_3$ for 18 hr. Luciferase activity was determined as described in *Materials and Methods*. Results are expressed as mean ± S.D. for at least three separate determinations for each treatment. *, $P < 0.05$, high concentration treatment (15 μM) vs. solvent control (DMSO).

Among the Nurr1-active C-DIMs, previous studies showed that DIM-C-pPhBr exhibited minimal activation of PPARγ or TR3 (215, 316). Therefore, this compound was used as a model for further investigating the structure-dependent activation of NR4A2;

and results obtained for the C-DIM analogs were compared to 6-MP, an activator of Nurr1 in CV-1 and HEK293 cells (247). Results summarized in Figures 3.4A – 3.4C compare the activity of DIM-C-pPhBr with the corresponding *ortho*- and *meta*-bromo-substituted analogs (DIM-C-oPhBr and DIM-C-mPhBr) on activation of GAL4-Nurr1/UAS_{x5}-Luc, $NRBE_{x3}$-Luc and $NurRE_{x3}$-Luc (cotransfected with 10 ng FLAG-Nurr1), respectively, and the results show that the order of potency was *para*- ≥ *meta*- > *ortho*-bromo-substituted analogs for all three reporter constructs. The 2-methylindole-

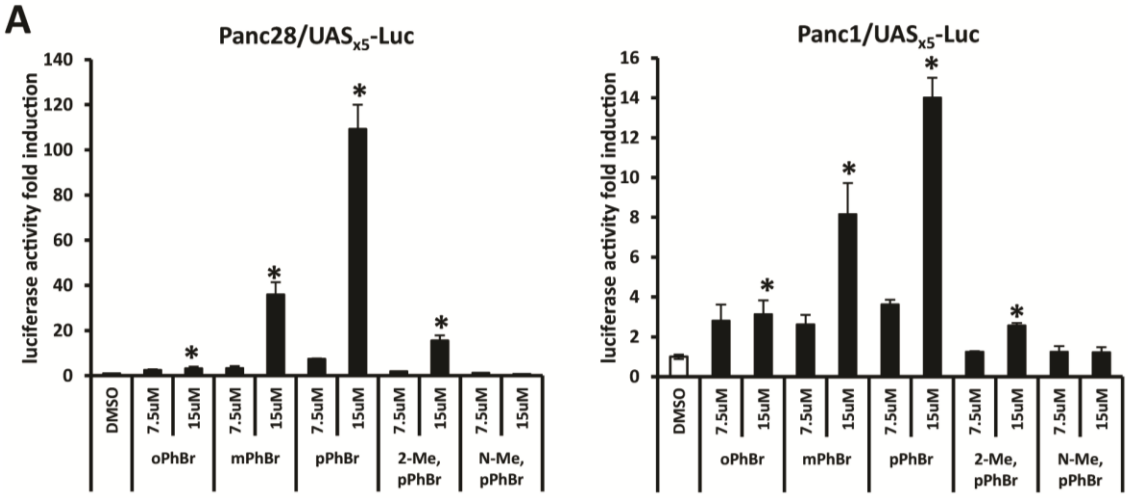


Figure 3.4. Differential NR4A2 activation by DIM-C-PhBr analogs and 6-mercaptopurine. UAS_{x5}-Luc (400 ng) (*A*) was cotransfected with GAL4-Nurr1 (40 ng); $NBRE_{x3}$-Luc (200 ng) (*B*) or $NurRE_{x3}$-Luc (200 ng) (*C*) was cotransfected with FLAG-Nurr1 (10 ng) into Panc28 or Panc1 cells for 6 hr and then treated with 7.5 and 15 μM *ortho*-, *meta*-, *para*-substituted or indole ring-substituted bromo-phenyl-C-DIMs for 18 hr. A gradient of 15 μM, 50 μM and 100 μM 6-mercaptopurine was included in the treatment and compared with the DIM-C-PhBr analogs (*B, C*). Luciferase activity was determined as described in *Materials and Methods*. Results are expressed as mean ± S.D. for at least three separate determinations for each treatment. *, $P < 0.05$, high concentration treatment (15 μM or 100 μM) vs. solvent control (DMSO).

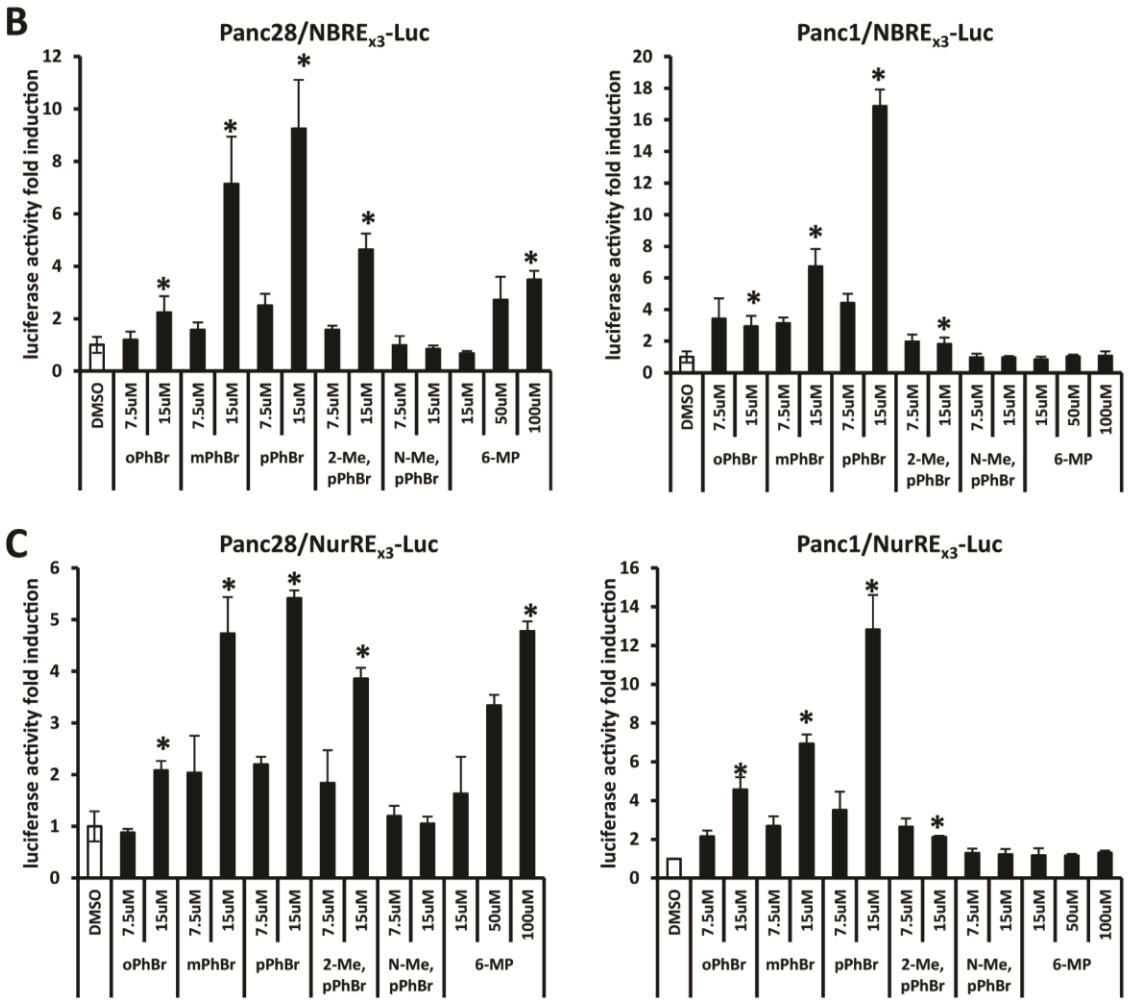


Figure 3.4. Continued

substituted derivative of DIM-C-pPhBr was also active; however, the 1-*N*-methyl indole analog was inactive in both cell lines. In contrast, 6-MP activated NBRE$_{X3}$-Luc and NurRE$_{x3}$-Luc in Panc28 cells but not Panc1 cells (Fig. 3.4C). The structure-activity study for activation of Nurr1 showed that among the bromophenyl analogs, DIM-C-pPhBr was the most potent compound and a free indole group was necessary for activation.

Activation of Nurr1 by 6-MP in CV-1 and HEK293 cells was dependent on the N-terminal A/B domain of the receptor (318) and therefore, we further investigated activation of wild type GAL4-Nurr1 and truncated GAL4-Nurr1 (A/B) and GAL4-Nurr1 (C-F) chimeras expressing the N- and C-terminal regions of the receptor, respectively. In Panc28 and Panc1 cells (Figs. 3.5A and 3.5B), DIM-C-pPhBr and the *p*-iodo, *p*-trifluoromethyl and *p*-trifluoromethoxy analogs significantly induced transactivation in cells transfected with wild type or variant GAL4-Nurr1 constructs. Similar results were observed for 6-MP (Figs. 3.5C and 3.5D); however, the fold induction in Panc1 cells was $<$ 2-fold and this was consistent with the failure of 6-MP to activate $NBRE_{x3}$-Luc or $NurRE_{x3}$-Luc in this cell line. Previous studies showed that multiple kinase inhibitors block activation of NR4A2 in different cell lines (229, 318, 319), and preliminary inhibitor screening studies (Fig. 3.6) in pancreatic cancer cells showed that inhibition of mitogen-activated protein kinase (MAPK) (PD98059) and phosphatidylinositol-3-kinase (PI3K) (LY294002) were among the most active and least active inhibitors, respectively, using wild type GAL4-Nurr1/UAS_{x5}-Luc. In Panc28 cells transfected with wild type or variant GAL4-Nurr1 chimeras, PD but not LY inhibited DIM-C-pPhBr-induced transactivation and, in cells transfected GAL4-Nurr1-(C-F), PD also decreased DIM-C-pPhBr-induced transactivation (Fig. 3.5E). The inhibitors alone had minimal effects on luciferase activity compared to DMSO (control). The pattern of inhibition by PD was similar in Panc1 cells (Fig. 3.5F); however, LY slightly inhibited and enhanced DIM-C-pPhBr-induced transactivation in cells transfected with GAL4-Nurr1 and GAL4-Nurr-(C-

F), respectively, but exhibited inhibitory activity comparable to PD in cells transfected with GAL4-Nurr-AB. These results clearly demonstrate the complex effects of just two

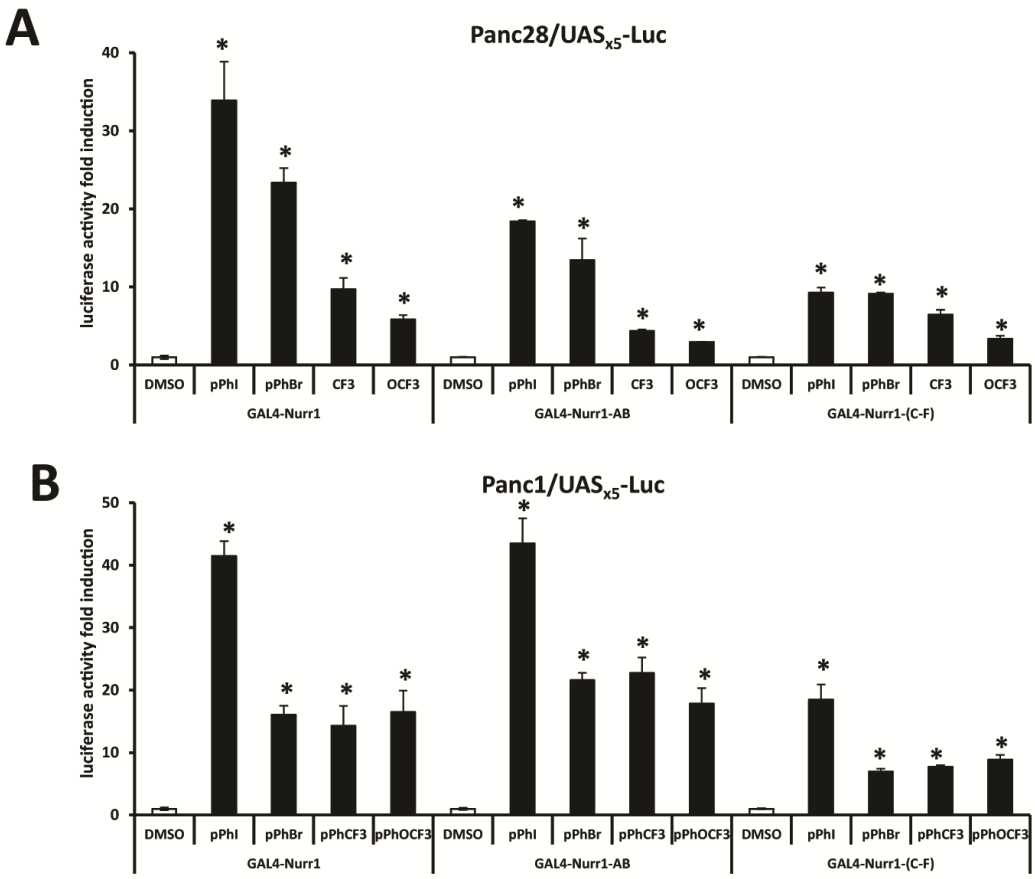


Figure 3.5. Activation of NR4A2 and different domains of NR4A2 by C-DIMs and 6-mercaptopurine. Full length (GAL4-Nurr1, 20 ng) or truncated GAL4-Nurr1 containing the A/B domain (GAL4-Nurr1-AB, 20 ng) or C to F domains [GAL4-Nurr1-(C-F), 20 ng] was cotransfected with UAS$_{x5}$-Luc (200 ng) into Panc28 (*A, C*) or Panc1 (*B, D*) cells for 6 hr and then treated with 10 μM DIM-C-pPhI, -pPhBr, -pPhCF$_3$, -pPhOCF$_3$ or 50 μM 6-mercaptopurine (*C, D*) for 18 hr. Panc28 (*E*) or Panc1 (*F*) cells were transfected with 10 ng full/truncated GAL4-Nurr1 and 200 ng UAS$_{x5}$-Luc for 6 hr and pre-incubated with 20 μM kinase inhibitors PD98059 or LY294002 for 45 min and treated with 10 μM DIM-C-pPhBr for 18 hr. Luciferase activity was determined as described in *Materials and Methods*. Results are expressed as mean ± S.D. for at least three separate determinations for each treatment. *, $P < 0.05$, treatment vs. solvent control (DMSO). **, $P < 0.05$, kinase inhibitor and DIM-C-pPhBr cotreatment vs. DIM-C-pPhBr treatment.

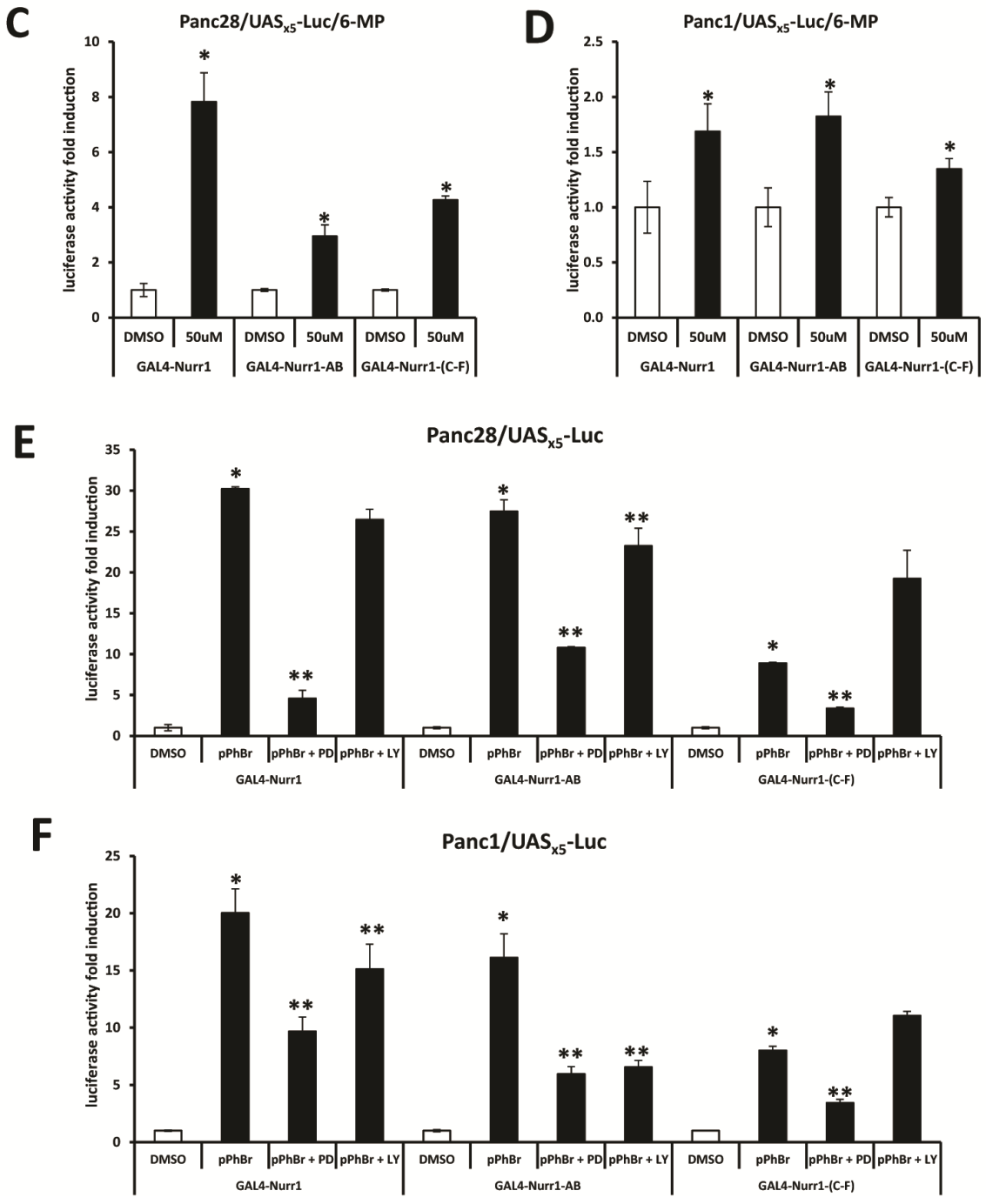


Figure 3.5. Continued

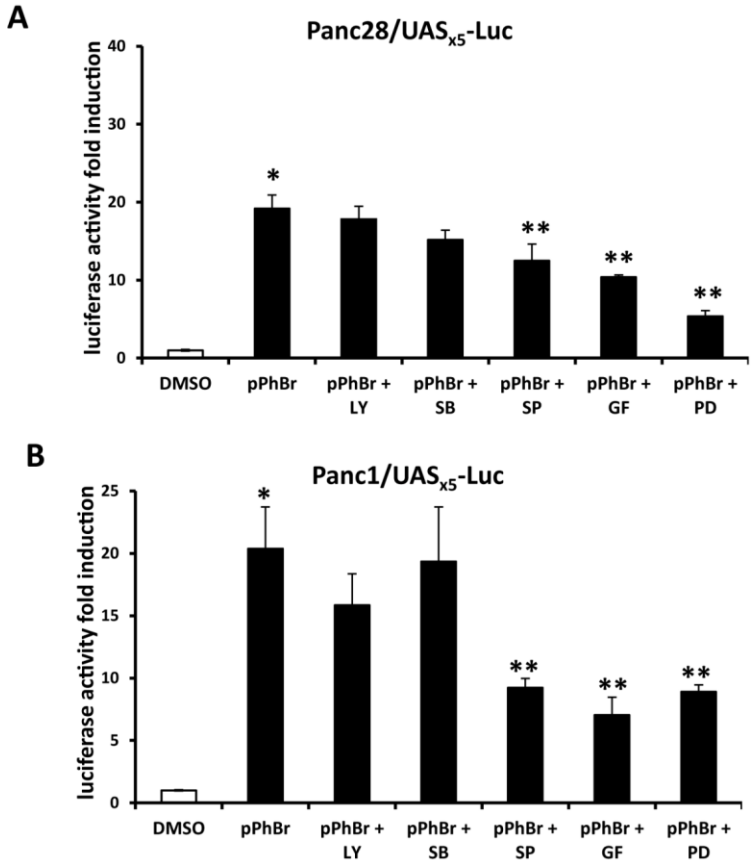


Figure 3.6. Effects of kinase inhibitors on Nurr1 activation by DIM-C-pPhBr. Panc28 (*A*) and Panc1 (*B*) cells were transfected with UAS_{x5}-Luc (200 ng) and GAL4-Nurr1 (10 ng) for 6 hr and pre-incubated with 20 μM kinase inhibitors LY294002 (LY), SB203580 (SB), SP600125 (SP), GF109203X (GF) or PD98059 (PD) for 1 hr and treated with 10 μM DIM-C-pPhBr for 18 hr. Luciferase activity was determined as described in *Materials and Methods*. Results are expressed as mean ± S.D. for at least three separate determinations for each treatment. *, $P < 0.05$, treatment vs. solvent control (DMSO). **, $P < 0.05$, kinase inhibitor and DIM-C-pPhBr cotreatment vs. DIM-C-pPhBr treatment.

kinase inhibitors on activation of NR4A2 by DIM-C-pPhBr, suggesting that multiple phosphorylation sites in different domains of NR4A2 are involved.

Previous studies have identified several NR4A2-regulated genes in different cell lines and these include vasoactive intestinal peptide (VIP), osteopontin (OPN) and

neuropilin 1 (NRP1) (229, 315, 316, 318-322). The effects of DIM-C-pPhBr on NR4A2-dependent expression of these genes was investigated in Panc28 and Panc1 cells treated with 12 μM DIM-C-pPhBr and transfected with iNurr1 or iCTL (non-specific) oligonucleotides for RNA knockdown. Figure 3.7A summarizes the effects of iNurr1 vs. iCTL on knockdown of NR4A2 which was highly efficient in both cell lines. Treatment with DIM-C-pPhBr for 18 or 24 hr induced VIP in both Panc28 and Panc1 cells, and knockdown of NR4A2 significantly decreased both basal and induced activity (Fig. 3.7B). OPN induction after treatment of Panc28 (18 hr) or Panc1 (18 and 24) cells with DIM-C-pPhBr was also Nurr1-dependent (Fig. 3.7C), whereas neuropilin 1 (NRP1) was induced by DIM-C-pPhBr in Panc28 but not in Panc1 cells (Fig. 3.7D). NRP2 is also coexpressed

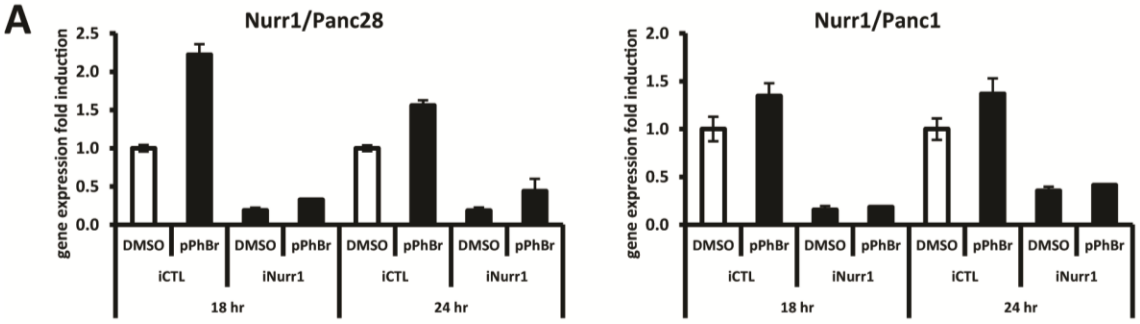
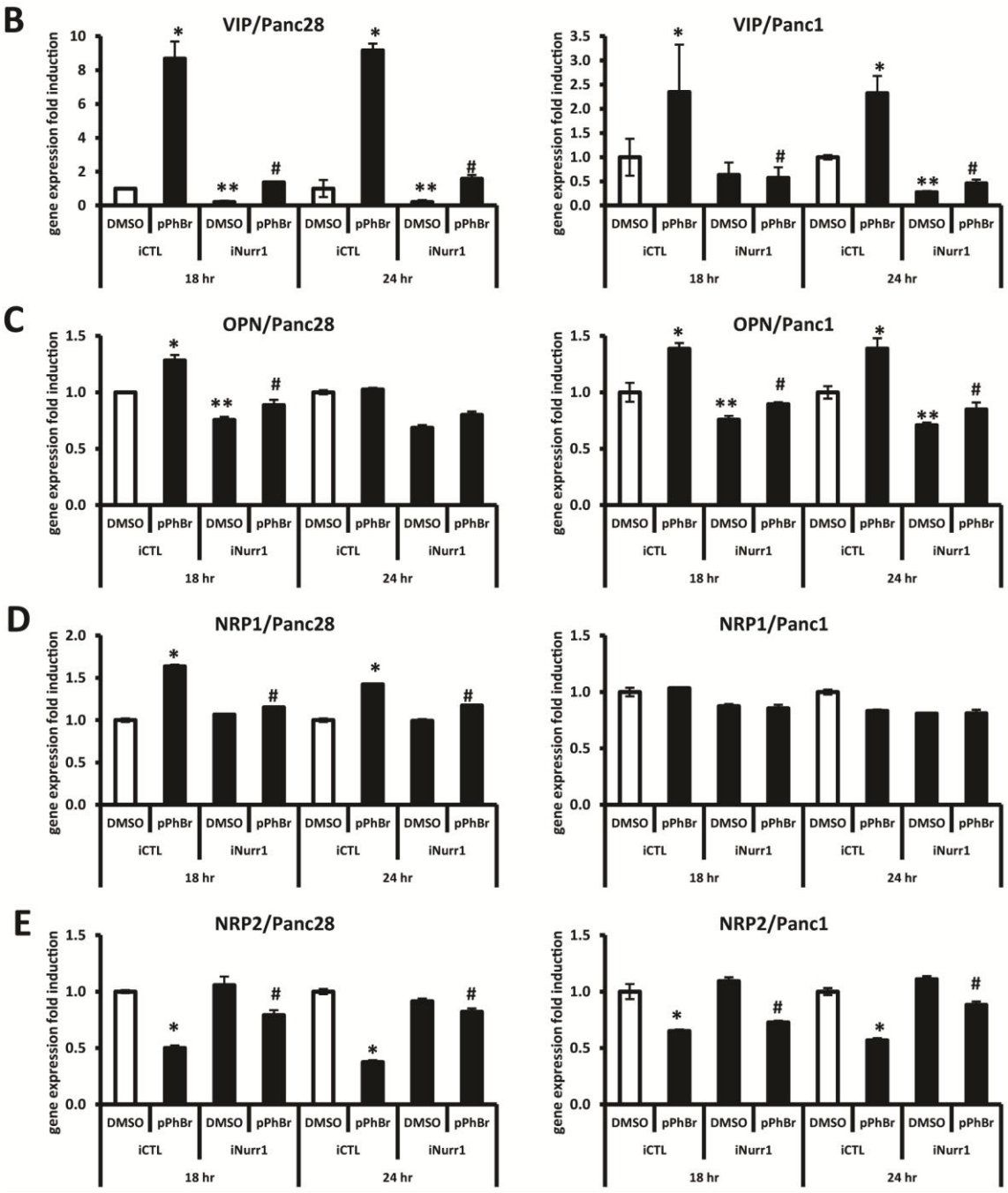


Figure 3.7. Effects of DIM-C-pPhBr on expression of several genes with or without NR4A2 knockdown. Panc28 and Panc1 cells were transfected with siRNAs targeting NR4A2 transcripts (iNurr1) or non-specific control oligonucleotides (iCTL). At 24 hr after transfection, cells were treated with 12 μM DIM-C-pPhBr for 18 and 24 hr. Relative expression levels of NR4A2 (*A*) and NR4A2-dependent genes vasoactive intestinal peptide (*B*), osteopontin (*C*), neuropilin-1 (*D*) and -2 (*E*) were determined by real-time PCR analysis as described in *Materials and Methods*. Results are expressed as mean ± S.D. for at least three separate determinations for each treatment. *, $P < 0.05$, treatment vs. solvent control (DMSO). **, $P < 0.05$, iNurr1 vs. iCTL. #, $P < 0.05$, knockdown and treatment combination vs. treatment only.



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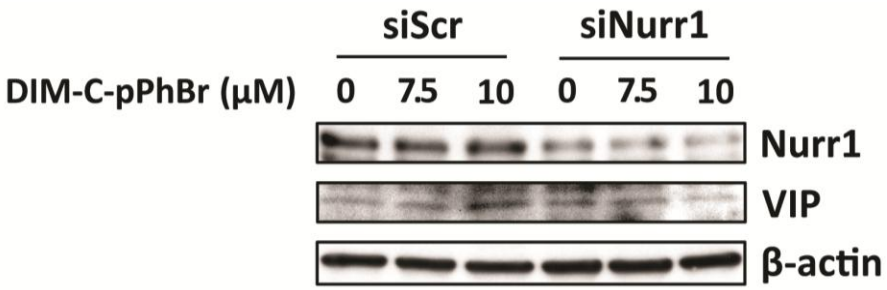


Figure 3.8. Effects of NR4A2 knockdown on DIM-C-pPhBr-induced protein expression. Panc1 cells were transfected with siRNAs targeting NR4A2 transcripts (siNurr1) or non-specific control oligonucleotides (siScr). Cells were treated with DIM-C-pPhBr at 48 hr after transfection. After treatment for 24 hr, whole cell lysates were analyzed by western blots as described in *Materials and Methods* and β-actin was used as loading control.

with NRP1 in pancreatic cancer cells (323, 324), and Figure 3.7E shows that DIM-C-pPhBr decreased NRP2 expression in Panc28 and Panc1 cells; however, based on knockdown studies, the effects were NRR4A2-dependent and -independent, respectively. Figure 3.8 shows the effectiveness of NR4A2 knockdown on the expression of Nurr1 protein in Panc1 cells, and the results also show that VIP protein was induced by DIM-C-pPhBr (NR4A2-dependent). These results demonstrate that DIM-C-pPhBr not only activates NR4A2 in pancreatic cancer cells but also induces NR4A2-dependent genes, and current studies are focused on the functional role of activated NR4A2 in mediating gene/protein expression and the anticancer activities of NR4A2-active C-DIMs.

Discussion

Although endogenous ligands for the NR4A orphan receptors have not been identified, there is increasing evidence that several agents can modulate nuclear NR4A-dependent transactivation (215, 218, 219, 234, 246, 308-314). Cytosporone B and related analogs have been extensively investigated as NR4A1 (TR3) agonists and they activate nuclear NR4A1 and induce nuclear export of this receptor which acts directly on mitochondria to induce apoptosis (312, 314). Cytosporone B analogs activate both wild type and the LBD of NR4A1 (using GAL4-receptor chimeras), and direct binding of cytosporone B and related compounds to NR4A1 was confirmed using a BIAcore surface plasmon resonance-based instrument. DIM-C-pPhOCH_3 was initially identified as a NR4A1-active compound that induces apoptosis and inhibits cancer cell growth through activation of nuclear NR4A1, and this has been linked to induction of several genes associated with these cellular responses (215, 218, 219, 309, 313). However, the effects of DIM-C-pPhOCH_3 were due to activation of nuclear NR4A1 and nuclear export of NR4A1 was not induced.

Another C-DIM analog, namely DIM-C-pPhCl, was characterized as an activator of Nurr1 in bladder cancer cells, and knockdown of NR4A2 by RNA interference inhibited DIM-C-pPhCl-induced apoptosis (227). In this study, we investigated the structure-activity relationships among a series of triarylmethane C-DIM analogs that contain a bis(3'-indolyl) moiety and either *p*-substituted phenyl or heteroaromatic groups in Panc28 and Panc1 pancreatic cell lines. Several *p*-substituted phenyl (CF_3, Br,

t-Bu, CN, I and OCF_3) and the piperonal (heteroaromatic) compounds were potent activators of wild type GAL4-Nurr1 in Panc28 and Panc1 cells with fold-inducibility higher in the former cell line. Interestingly, DIM-C-pPhCl which was characterized as a Nurr1 activator in bladder cancer cells (320) was also active in pancreatic cancer cells but was much less active than several other C-DIM analogs.

Previous studies show that DIM-C-pPhBr did not activate PPARγ or NR4A1 (215, 316) and this compound was used as a model to determine the role of *ortho*-, *meta*- and *para*-bromophenyl ring substitution and the free indole NH group on activation of NR4A2 (Fig. 3.4). There were some cell context-dependent differences in Panc28 and Panc1 cells; however, DIM-C-pPhBr (*p*-bromo substituent) was more active that the corresponding *ortho*- and *meta*-bromo isomers and methylation of the indole NH group resulted in complete loss of activity, and similar results were observed for activation of TR3 (215). In contrast, the *N*-methyl derivative of DIM-C-pPhCl activated GAL4-NR4A2 in bladder cancer cells (227); however, most other reports demonstrate the importance of a free NH group for induction of apoptosis or for activation of other nuclear receptors by C-DIM compounds (215, 316).

6-MP was previously identified as an activator of NR4A2 and NR4A3 (but not NR4A1) in CV1 and HEK293 cells (247, 318) and, using various GAL4-chimeras, it was shown that activation of NR4A2 by 6-MP in these cells was dependent on the N-terminal A/B domain of the receptor. In this study, 6-MP induced transactivation in Panc28 cells but exhibited minimal to non-detectable activation of $NurRE_{x3}$-Luc or

$NBRE_{x3}$-Luc in Panc1 cells (Figs. 3.4B and 3.4C). 6-MP also activated all domains of NR4A2 using the GAL-Nurr1, GAL4-Nurr1-(A/B) and GAL4-Nurr-(C-F) constructs in Panc28 cells and only exhibited minimal (but significant) activity in Panc1 cells (Figs. 3.5C and 3.5D). These data indicate that, in contrast to previous results indicating that 6-MP activates Nurr1 through the N-terminal domain, the effects of this compound on Nurr1 and domains of Nurr1 are more complex and highly dependent on cell context. DIM-C-pPhBr also induced transactivation in cells transfected wild type and variant GAL4-Nurr1 constructs, indicating that both N- and C-terminal domains of NR4A2 were activated by DIM-C-pPhBr. Preliminary studies showed that several kinase inhibitors blocked activation of Nurr1 by DIM-C-pPhBr (Fig. 3.6); however, a direct comparison of MAPK and PI3K inhibitors shows that MAPK played an important role in DIM-C-pPhBr-dependent activation of NR4A2 through multiple domains (Figs. 3.5E and 3.5F). Ongoing studies are investigating specific regions and amino acids that are important for kinase-mediated activation of NR4A2.

NR4A2-regulated genes are variable among different cell lines but include OPN and NRP1 in cancer cell lines and VIP in dopaminergic cells (320-322). We also observed induction of VIP, NRP1 and OPN mRNA levels in Panc28 cells and VIP and OPN in Panc1 cells treated with DIM-C-pPhBr, and this induction was abrogated after knockdown of NR4A2 by RNA interference (Fig. 3.7). In contrast, DIM-C-pPhBr decreased NRP2 mRNA levels in both cell lines (Fig. 3.7E); and since NRP1 and NRP2 may be involved in the pathogenesis of pancreatic cancer (323, 324), it is possible that

differential modulation of NRP1 and NRP2 by DIM-C-pPhBr may be important for the anti-carcinogenic activity of this compound and this is currently being investigated.

In summary, this study shows that specific C-DIMs activate NR4A2, and using DIM-C-pPhBr as a model, it was shown that activation of NR4A2 was enhanced using a *p*-bromo substituent and a free indole NH group was required for activity. DIM-C-pPhBr and related compounds activated nuclear NR4A2 and both N- and C-terminal domains of the receptor were involved. The identification of NR4A2-active C-DIMs that activate the nuclear receptor will be important for future studies on identification of specific NR4A2 sites and pathways required for receptor activation and for determining the role of NR4A2 in mediating the anticancer activities of these agents. Previous studies also reported that DIM-C-pPhBr induces apoptosis and endoplasmic reticulum stress in pancreatic and colon cancer cells (325, 326), and these and other Nurr1-independent responses also contribute to the activity of this compound. Relative contributions of receptor-mediated and -independent activities are being investigated.

CHAPTER IV

1,1-BIS-(3'-INDOLYL)-1-(P-SUBSTITUTED PHENYL)METHANES (C-DIMS) BIND NR4A1 RECEPTOR AND ACT AS ANTAGONISTS IN COLON CANCER CELLS

Introduction

The nuclear receptor (NR) superfamily are critical regulators of homeostasis at all stages of development, and many of these receptors such as steroid hormone and retinoid receptors are important drug targets for treating multiple diseases including cancer (327, 328). The 48 human NRs have been divided into three main groups, including the endocrine receptors, adopted orphan receptors, and orphan receptors; and endogenous ligands have been characterized only for the former two groups of receptors. The three members of the NR4A orphan receptor subfamily (305, 306) include NR4A1 (Nur77, TR3, NGFI-B), NR4A2 (Nurr1) and NR4A3 (Nor1), which were initially identified as immediate-early response genes induced by nerve growth factor in PC12 cells (329). The well-conserved DNA binding and C-terminal ligand binding domains of the NR4A receptors exhibit ~91-95% and ~60% homology, respectively, whereas their N-terminal A/B domains are highly divergent (330-332). NR4A receptors are induced by multiple stimuli/stressors and play essential roles in metabolic processes, inflammation, vascular function, steroidogenesis, and the central nervous system (305, 306). NR4A1 is also overexpressed in multiple tumors and cancer cell lines (308, 323, 333). In colon cancer patients, our initial studies showed that high NR4A1

expression was observed in 60% of colon tumors, whereas only 10% of normal colon tissue exhibited high staining (216) and overexpression of NR4A1 in colon tumors has been confirmed in other studies (213, 220). RNA interference (RNAi) has been used to investigate the role of NR4A1 in cancer cells; and in solid tumors NR4A1 exhibits pro-oncogenic activity and enhances both cancer cell proliferation and survival (219-224, 310, 334), whereas results from NR4A1/NR4A3 knockout mouse models suggest that NR4A is a tumor suppressor for acute myelocytic leukemia (236).

Initial studies on using NR4A1 as a drug target for inhibiting tumor growth discovered a novel pathway in which the anticancer activity of several apoptosis-inducing agents was due to induced expression and nuclear export of NR4A1 (311, 312, 323, 335). NR4A1 associated with mitochondria binds Bcl-2 and the resulting pro-apoptotic NR4A1-Bcl-2 complex decreases mitochondrial membrane potential and activates stress responses. Research in this laboratory demonstrated that among a series of synthetic 1,1-bis(3'-indolyl)-1-(substituted phenyl)methane analogs (C-DIMs), a select group of these compounds activated or inactivated nuclear NR4A1-dependent transactivation in cancer cell lines and did not induce nuclear export of NR4A1 (215, 218, 219, 222). For example, the *p*-hydroxyphenyl analog (1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH)) inhibits NR4A1-dependent transactivation and similar anti-neoplastic activities and effects on gene expression were observed in pancreatic cells after knockdown of NR4A1 (by RNAi) or after treatment with DIM-C-pPhOH (219, 222).

Cytosporone B (Csn-B) and structural analogs were first identified as NR4A1 ligands and these compounds induce NR4A1-dependent transactivation but also induce expression of NR4A1 and nuclear export of the receptor to mitochondria (246, 314). In this study, we show structure-dependent binding of C-DIMs to the ligand binding domain of NR4A1 and demonstrate that selected compounds exhibit NR4A1 antagonist activity and inactivate nuclear NR4A1 in colon cancer cell lines and exhibit anti-neoplastic activities similar to that observed after receptor knockdown.

Materials and Methods

Fluorescence quenching assay

The GST-NR4A1 (LBD) was expressed and purified on a GSH-agarose column, and the purified proteins were incubated with different concentrations of C-DIMs. C-DIM-induced quenching was determined as described (246, 314) with the excitation wave length of 280 nm and emission spectra measured between 285 and 430 nm.

Cell lines and plasmids

RKO and SW480 human colon cancer cell lines were obtained from the American Type Culture Collection (ATCC) and maintained as previously described (218). The Flag-tagged full-length FLAG-NR4A1 and mutant FLAG-NR4A1(A-D) and FLAG-NR4A1(C-F) expression plasmids were constructed by inserting PCR-amplified full-length NR4A1 (amino acid 1-598) into the EcoRI/BamHI site and C-terminal NR4A1

(amino acid 67-598) and N-terminal NR4A1 (amino acid 1-354) into the EcoRI/KpmI site of p3XFLAG-CMV-10 expression vector (Sigma-Aldrich). $NBRE_{x3}$-Luc was generously provided by Dr. Jacques Drouin (University of Montreal, Quebec, Canada). All other plasmids used in this study were previously described (219, 222).

Reagents, antibodies and western blot analysis

NR4A1 antibody was purchased from Abcam and all other antibodies were purchased from Cell Signaling Technology. The *p*-substituted phenyl C-DIMs were synthesized and purified in this laboratory as previously described (336). The *N*-methyl C-DIM analogs were prepared by condensing *N*-methylindole (Sigma-Aldrich) with the corresponding *p*-substituted benzaldehyde and the *ortho*- and *meta*-substituted isomers were prepared from condensing indole plus the corresponding substituted benzaldehydes as described (336). Purities were > 95-98% as determined by GC-MS. Reporter lysis buffer, luciferase reagent and β-galactosidase (β-gal) reagent were supplied by Promega. Western blot analysis was carried out as previously described (219, 222).

Transfection, siRNAs, cell proliferation assay and reporter gene assay

Cells were transfected with 100 nM of each siRNA duplex using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. The sequence of siNR4A1 was 5'-CAG UCC AGC CAU GCU CCU C dTdT. As a negative control, a

nonspecific scrambled small inhibitory RNA (siScr) was used (Qiagen) and all other siRNAs were purchased from Santa Cruz Biotechnology. Cell proliferation and reporter gene assays were performed as previously described (219, 222).

Computational-based molecular modeling

All molecular modeling studies were conducted using Accelrys Discovery Studio 3.5 (Accelrys Software, Inc., San Diego, CA; http://accelrys.com). The crystal structure coordinates for rat orphan nuclear receptor NR4A1 (337) was downloaded from the protein data bank (http://www.pdb.org; PDB ID: 1YJE). The crystal structure was subjected to implicit solvent-based energy minimization utilizing the conjugate gradient minimization protocol (338) with a CHARMM force field (339) and the Generalized Born implicit solvent model with simple switching (340) to an RMS convergence of <0.001 kcal/mol prior to use in the modeling studies. The flexible docking algorithm (341) was used to predict the binding orientation of the 14 compounds within the ligand binding site of NR4A1. Protein-ligand complexes underwent energy minimization *in situ* (10,000 iterations of the conjugate gradient protocol) prior to calculating the predicted binding energies. The following residue side chains were designated as flexible in the calculations: Phe442, Leu443, Arg514, His515, Arg562, Thr566, Leu569, Ile590, Asp593, and Thr594.

Statistical analysis

Statistical significance of differences between groups was analyzed using unpaired Student's t-test. The results are expressed as means with error bars representing 95% confidence intervals for three experiments for each group unless otherwise indicated, and a *P* value of less than 0.05 was considered statistically significant. All statistical tests were two-sided.

Contributions

The NR4A1 binding data was contributed by Dr. Qiao Wu (University of Xiamen, Fujian, China) and the molecular modeling data was contributed by Dr. Un-Ho Jin (Keimyung University, Daegu, Republic of Korea). All other studies were carried out in collaboration with Dr. Syng-Ook Lee in this laboratory.

Results

C-DIM binding and interactions with NR4A1

A panel of 14 *p*-substituted phenyl C-DIM analogs (Fig. 4.1A) were used to investigate their binding to the ligand binding domain (LBD) of NR4A1 using a fluorescence assay as previously described (246, 314). Figures 4.1B – 4.1D illustrates the binding curves for the 8 different C-DIMs that bound the LBD and these included the *para* trifluoromethyl (CF_3), bromo (Br), unsubstituted (H), hydroxyl (OH), cyano (CN), chloro (Cl), iodo (I) and carboxymethyl (CO_2Me) analogs. K_D values for these compounds

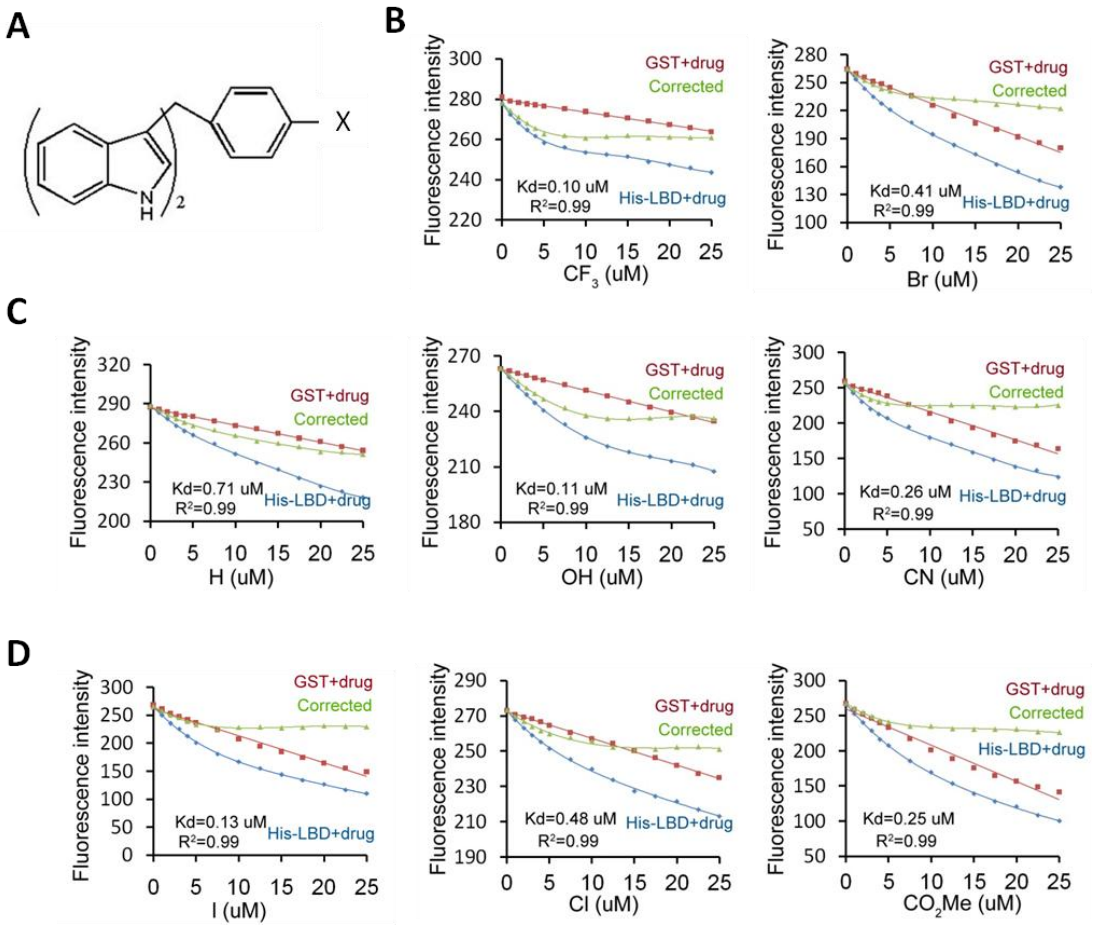
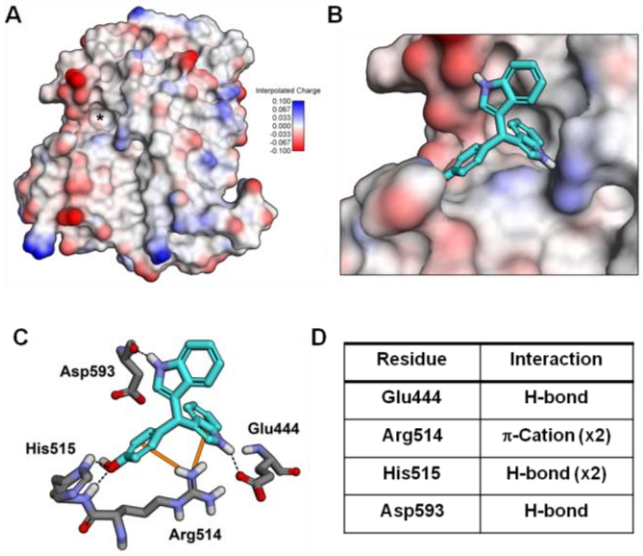


Figure 4.1. C-DIM structure and the receptor binding K_D values. *A*, composite structure of C-DIMs (DIM-C-pPh-X). *B – D*, K_D values for binding to the LBD of NR4A1 using a fluorescent binding assay as outlined in *Materials and Methods*.

ranged from 0.10 to 0.71 µM (Fig. 4.1). Binding was not observed for the fluoro (F), *t*-butyl (*t*-Bu), methoxy (OCH_3) and methyl (CH_3) compounds; the phenyl (C_6H_5) analog bound to GST and the dimethylamino ($N(CH_3)_2$) compound gave abnormal fluorescent curves that interfered with the assay (data not shown). These results demonstrate direct binding of C-DIMs with the LBD of NR4A1 and this was further investigated by molecular modeling.

In contrast to the classical NRs, the crystal structure of NR4A1 exhibits a putative ligand binding site that is not as clearly defined (Fig. 4.2A) (337). However, molecular modeling and small molecule docking studies indicate that the shallower NR4A1 binding pocket is capable of accommodating all 14 compounds and the modeling studies predict that the *p*-hydroxyphenyl (DIM-C-pPhOH) compound (Fig. 4.2B) and other C-DIMs bind with high affinity to NR4A1. The key interactions of those compounds that exhibit higher binding affinity such as DIM-C-pPhOH involves hydrogen bond interactions with His515 and π-cation interactions with Arg514 (Figs. 4.2C and 4.2D).



Residue	Interaction
Glu444	H-bond
Arg514	π-Cation (x2)
His515	H-bond (x2)
Asp593	H-bond

Figure 4.2. Predicted interactions between NR4A1 and DIM-C-pPhOH. *A*, molecular surface representation of the crystal structure of the orphan nuclear receptor NR4A1 (PDB ID: 1YJE) colored by interpolated charge from positive (blue) to neutral (white) to negative (red). Asterisk indicates location of the ligand binding pocket. *B*, predicted binding orientation of DIM-C-pPhOH within the ligand binding site. *C*, *D*, specific non-bonded interactions between DIM-C-pPhOH (cyan) and the residues of NR4A1 (gray). Dashed lines indicate predicted hydrogen bonds and solid orange lines indicate predicted π interactions.

C-DIMS inhibit NR4A1-dependent transactivation

The effects of C-DIMs on NR4A1-dependent transactivation were initially investigated in RKO cells transfected with $NBRE_{x3}$-Luc and $NurRE_{x3}$-Luc constructs containing 3 binding sites for NR4A1 monomer and homodimer, respectively (342). Basal activity was low for both constructs but significantly enhanced by cotransfection with a FLAG-TR3 expression plasmid (Fig. 4.3A), and Figure 4.4A summarizes the effects of the *p*-substituted phenyl C-DIMs on luciferase activity in RKO cells transfected with $NBRE_{x3}$-Luc. Results of this assay show that most of the compounds significantly inhibit transactivation and the effects of the *p*-substituted C_6H_5 and CH_3 analogs were minimal. Similar results were observed in cells transfected with the $NurRE_{x3}$-Luc construct (Fig. 4.3B).

The structure-dependent effects of *ortho*-, *meta*- and *para*-substituted phenyl C-DIM analogs and the importance of the free indole group on C-DIM-mediated inhibition of transactivation were also investigated for selected compounds in RKO cells (Fig. 4.4B). Treatment of RKO cells with the CN-, OH-, F- and Br-phenyl analogs and the *N*-methyl (indole) derivatives of the *para*-substituted phenyl compounds showed that the differences in the position of the phenyl ring substituents (*ortho*/*meta*/*para*) had minimal effects on antagonist activity (decreased luciferase), whereas methylation of the indole groups attenuated their NR4A1 antagonist activities in the transactivation assay. The requirements for different domains of NR4A1 for C-DIM-dependent inhibition of transactivation were investigated in RKO cells transfected with wild type

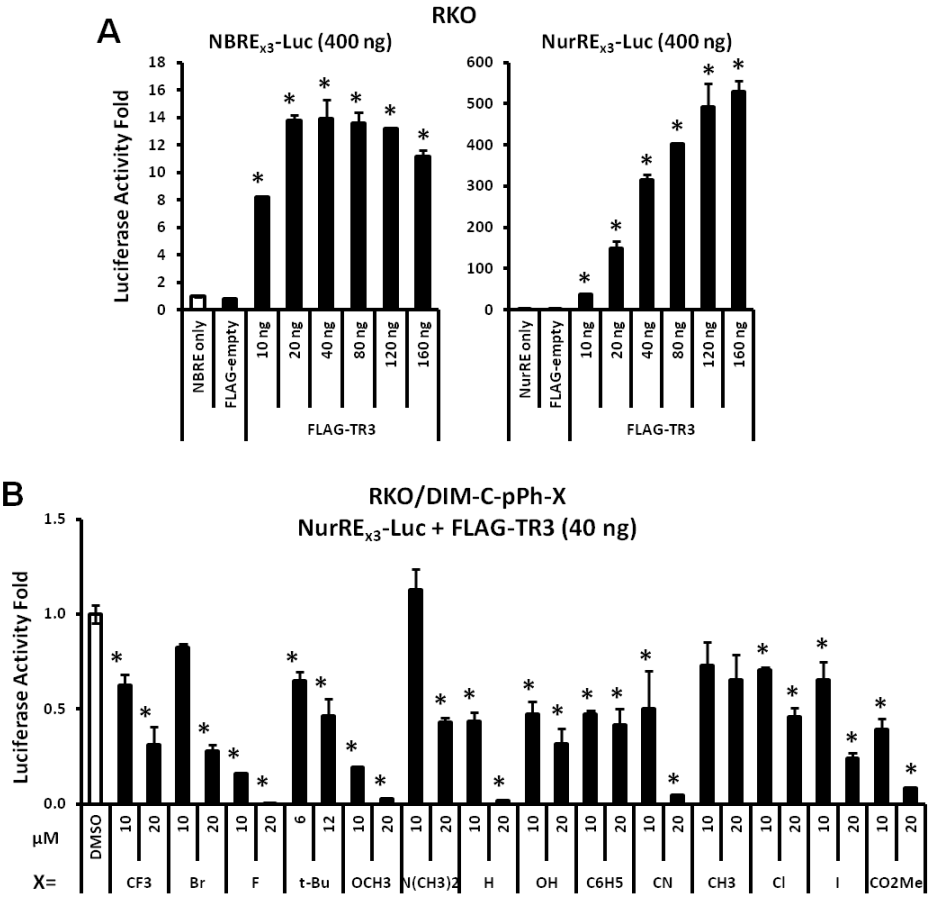


Figure 4.3. Transactivation studies. *A*, RKO cells were transfected with NurRE$_{x3}$-Luc or NBRE$_{x3}$-Luc (or empty vector) and different concentrations of FLAG-NR4A1; and luciferase activity was determined. *B*, RKO cells were transfected with NurRE$_{x3}$-Luc, treated with C-DIMs and luciferase activity was determined as described in *Material and Methods*. Results are expressed as mean ± S.D. for at least 3 separate determinations. *, $P < 0.05$.

FLAG-NR4A1 and mutants that contain the A-D (N-terminal plus DBD) and C-F (C-terminal plus DBD) domains (Fig. 4.4C). Cells were transfected with the NBRE$_{x3}$-Luc construct, the wild type and mutant NR4A1 expression plasmids and treated with the *p*-Br, *p*-OH and *p*-CO_2Me analogs. All three compounds decreased transactivation in cells transfected with wild type NR4A1 and mutant NR4A1 containing the C-terminal ligand

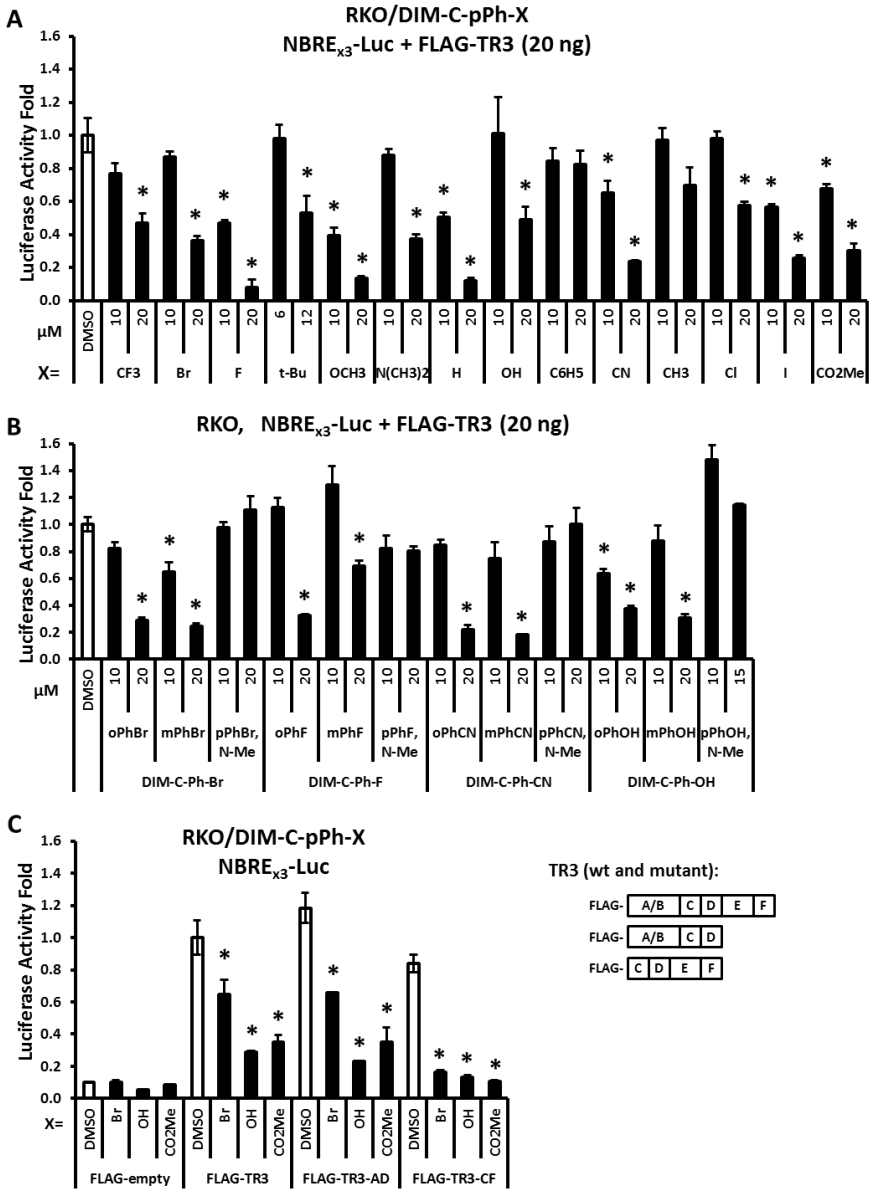


Figure 4.4. C-DIMs and NR4A1-dependent transactivation. *A*, activation of the NBRE$_{x3}$-Luc. RKO cells were transfected with FLAG-NR4A1 (342) and NBRE$_{x3}$-Luc and treated with DMSO or different concentrations of 14 *p*-substituted phenyl C-DIMs; and luciferase activity was determined as described in *Materials and Methods*. *B*, cells were transfected as outlined in *A* and treated with *ortho*- and *meta*-substituted phenyl and *N*-methyl *p*-substituted phenyl compounds; and luciferase activity was determined as described in *Materials and Methods*. *C*, RKO cells were transfected with NBRE$_{x3}$-Luc, wild type or truncated FLAG-NR4A1 expression plasmids, treated with selected C-DIMs, and luciferase activity was determined as described in *Materials and Methods*. Results are expressed as mean ± S.D. for at least 3 separate determinations for each treatment and values < 0.8 are considered significant ($P < 0.05$) decrease comparing to control (DMSO).

binding domain. However, we also observed decreased luciferase activity with RKO cells transfected with NR4A1 containing the N-terminal domain of the receptor, and we are currently investigating the underlying mechanisms for antagonism of N-terminal activation function-1 (AF-1) by C-DIMs in colon cancer cells.

Functional effects of NR4A1 antagonists

The *p*-hydroxyphenyl C-DIM analog (DIM-C-pPhOH) which exhibits high NR4A1 binding activity in both the fluorescence binding and molecular modeling assays exhibits activity similar to that observed after NR4A1 knockdown (siNR4A1) in pancreatic and lung cancer cells (219, 222) and was used as a model NR4A1 antagonist in colon cancer cells. Results in Figures 4.5A and 4.5B demonstrate that DIM-C-pPhOH inhibits growth of RKO and SW480 cells with IC_{50} values (48 hr) of 21.2 and 21.4 μM, respectively. Moreover, in RKO cells, the growth inhibitory effects of DIM-C-pPhOH were attenuated by overexpression of NR4A1 (Fig. 4.5C). DIM-C-pPhOH also induced cleavage of caspases 3 and 7 and PARP in RKO and SW480 cells (Fig. 4.5D) indicating the pro-apoptotic effects of this NR4A1 antagonist. Figures 4.7A and 4.7B demonstrate the NR4A1 antagonist activities of the *p*-bromophenyl (DIM-C-pPhBr) and *p*-carboxymethyl (DIM-C-pPhCO_2Me) which also inhibited RKO cell growth and induced apoptosis. Knockdown of NR4A1 by RNA interference also decreased growth of RKO and SW480 cells (Figs. 4.6A and 4.6B) and in RKO cells there was a marked changed in cell morphology. Knockdown of NR4A1 in RKO and SW480 cells induced markers of

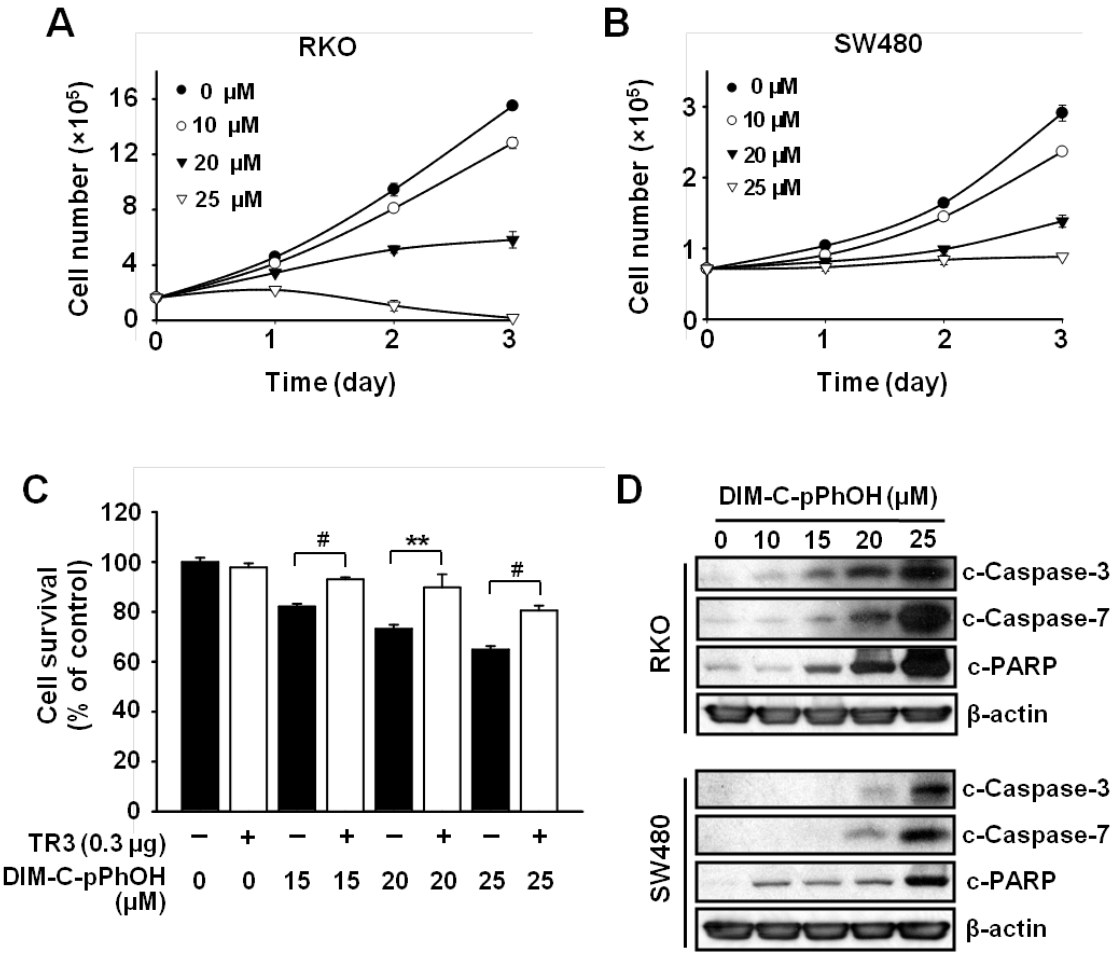


Figure 4.5. DIM-C-pPhOH inhibits cell growth and induces apoptosis in colon cancer cells. *A*, *B*, cell survival. RKO and SW480 cells were treated with various concentrations of DIM-C-pPhOH or DMSO as a control for 3 days, and cell numbers were determined on days 1, 2 and 3. *C*, RKO cells were transfected with FLAG-NR4A1, treated with DIM-C-pPhOH for 24 hr, and cell numbers were determined. *D*, RKO and SW480 cells were treated with either DMSO or various concentrations of DIM-C-pPhOH for 24 hr. Whole cell lysates were analyzed by western blot analysis and β-actin was used as loading control.

apoptosis including cleaved caspases 3 and 7 and PARP and the effects of NR4A1 knockdown overlapped with the effects observed for the C-DIM/NR4A1 antagonists (Fig. 4.5 and Fig. 4.7).

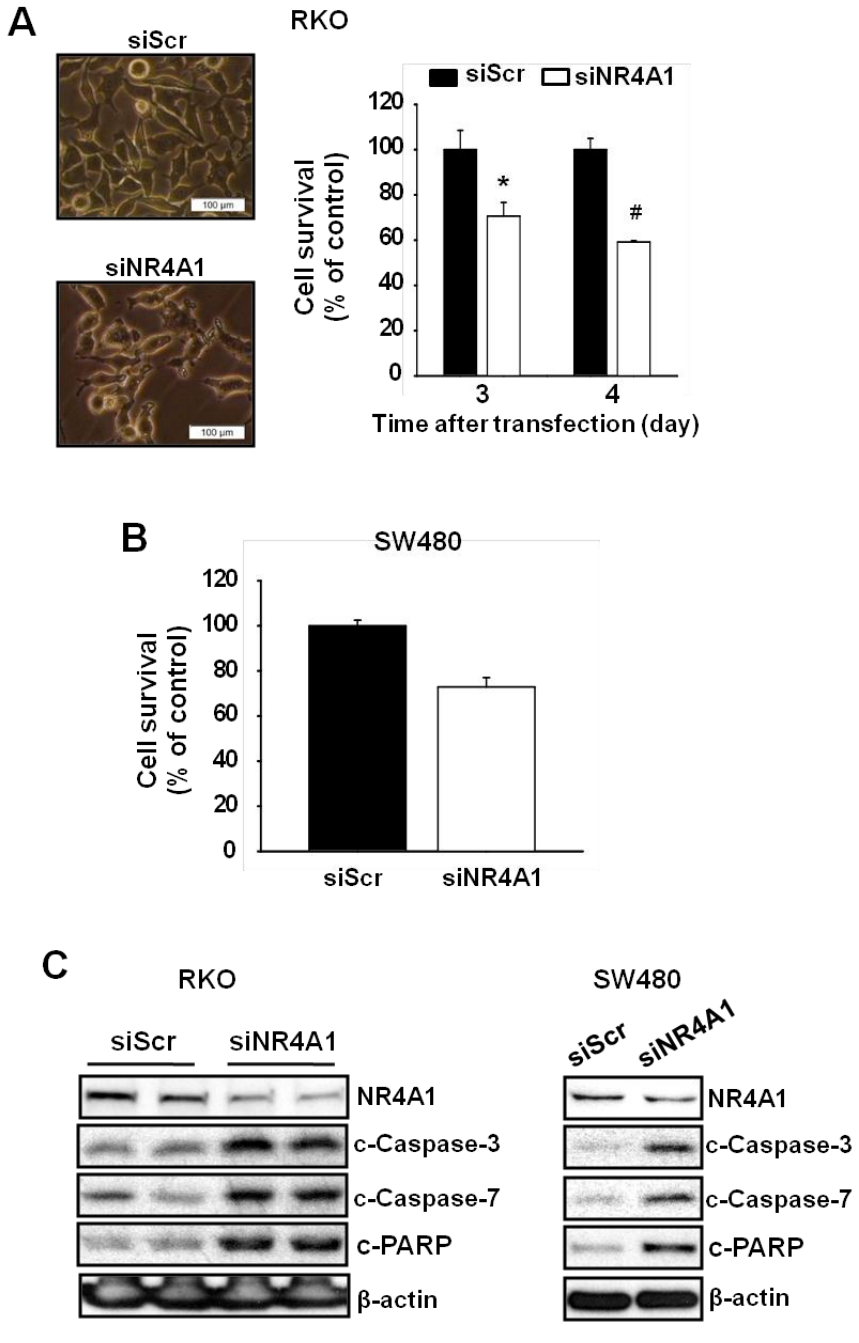


Figure 4.6. Knockdown of NR4A1 inhibits cell growth and induces apoptosis in colon cancer cells. *A*, *B*, cell morphology and survival. After transfection with either siScr or siNR4A1 for 3 or 4 days, cell numbers were determined. Images of RKO cell morphology were obtained from cells transfected with siNR4A1 for 72 hr. Results are expressed as mean ± S.D. for at least 3 separate determinations for each group. *, $P<0.05$ and $^{\#}$, $P<0.001$ vs. siScr. *C*, cells were transfected with the indicated siRNA for 72 hr. Whole cell lysates were analyzed by western blot analysis and β-actin was used as loading control.

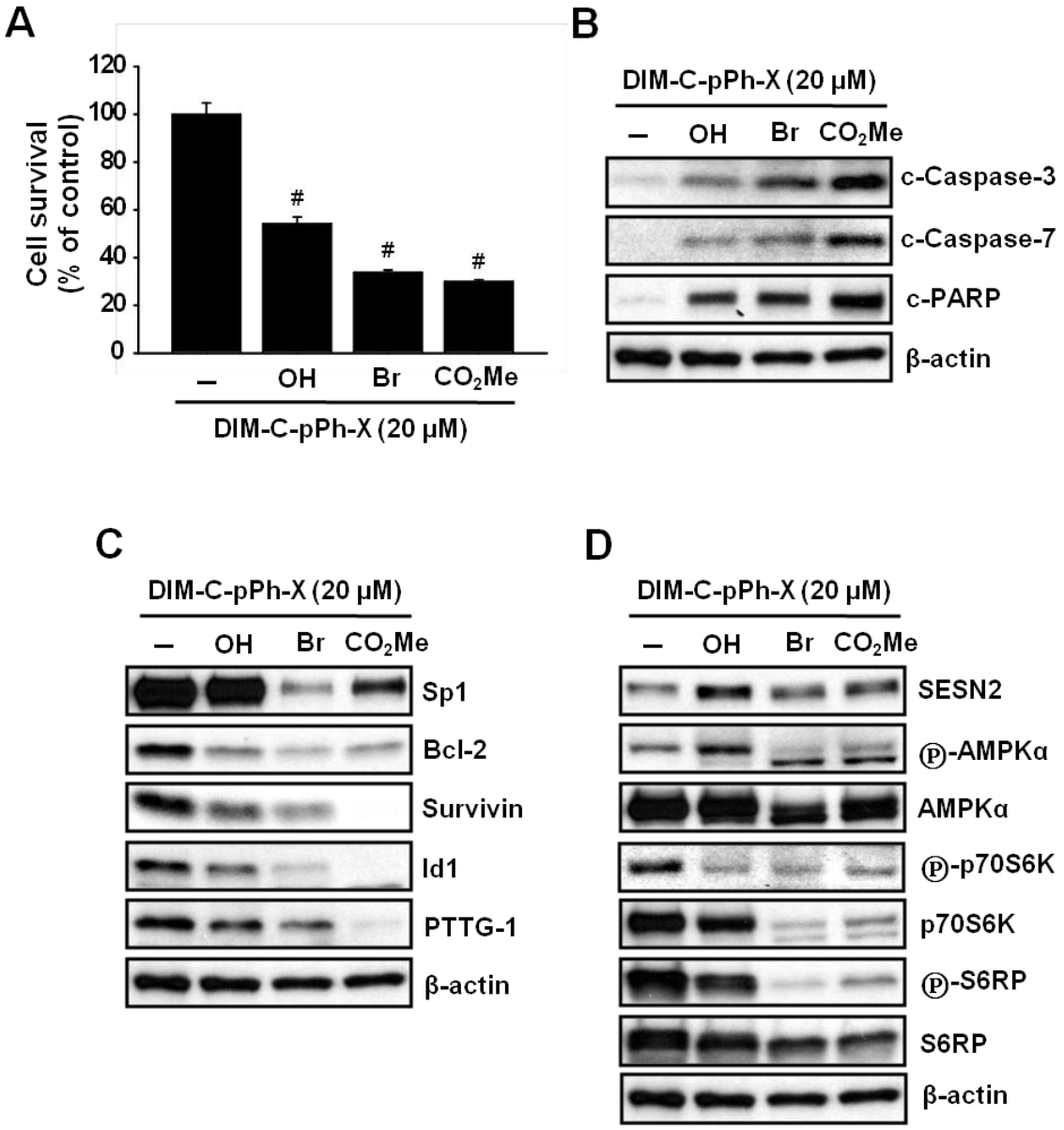


Figure 4.7. NR4A1 antagonist C-DIMs inhibit cell proliferation and induce apoptosis in RKO colon cancer cells. *A*, cell survival. RKO cells were treated with various NR4A1 antagonists for 24 hr, and cell numbers were determined. *B – D*, RKO cells were treated with various NR4A1 antagonists for 24 hr. Whole cell lysates were analyzed by western blot analysis and β-actin was used as loading control.

Previous studies show that NR4A1 expression is required for activation of Sp1-regulated genes such as survivin (219, 222). Figure 4.8 shows that treatment of RKO and SW480 cells with DIM-C-pPhOH or transfection with siNR4A1 decreased expression of three Sp-regulated genes (survivin, Id1 and PTTG) (343-345) without affecting Sp protein expression. DIM-C-pPhBr and DIM-C-pPhCO_2Me also decreased expression of these Sp-regulated genes (Fig. 4.7C); however, this was also due, in part, to downregulation of Sp1. In RKO cells transfected with GC-rich survivin promoter-reporter constructs [pGL3-SVV(-269) or pGL3-SVV(-150)] (Fig. 4.8B) or a GC-rich construct containing 3 tandem consensus GC-rich binding sites [pGL3$(GC)_3$-TK] (Fig. 4.8C), DIM-C-pPhOH or siNR4A1 significantly decreased transactivation and these effects were also observed in SW480 cells (data not shown) demonstrating that NR4A1 coregulated Sp-dependent promoters as previously observed in lung and pancreatic cancer cells (219). siNR4A1 and DIM-C-pPhOH decreased mTOR activation in p53-positive lung cancer cell lines reversing NR4A1-dependent inhibition of p53; this resulted in activation of sestrin 2 and activation of AMPKα resulting in inhibition of mTOR signaling (decreased phosphorylation of 70S6K and S6RP) (222). Figures 4.9A and 4.9B demonstrate that DIM-C-pPhOH and siNR4A1 induced sestrin 2 and phosphorylation of AMPKα and decreased phosphorylation of mTOR-regulated p70S6K and S6RP in p53-positive RKO but not in p53-negative SW480 cells. Similar results were also observed for the DIM-C-pPhBr and DIM-C-pPhCO_2Me NR4A1 antagonists in RKO cells (Fig. 4.7D). DIM-C-pPhOH and siNR4A1 also activated luciferase activity in RKO

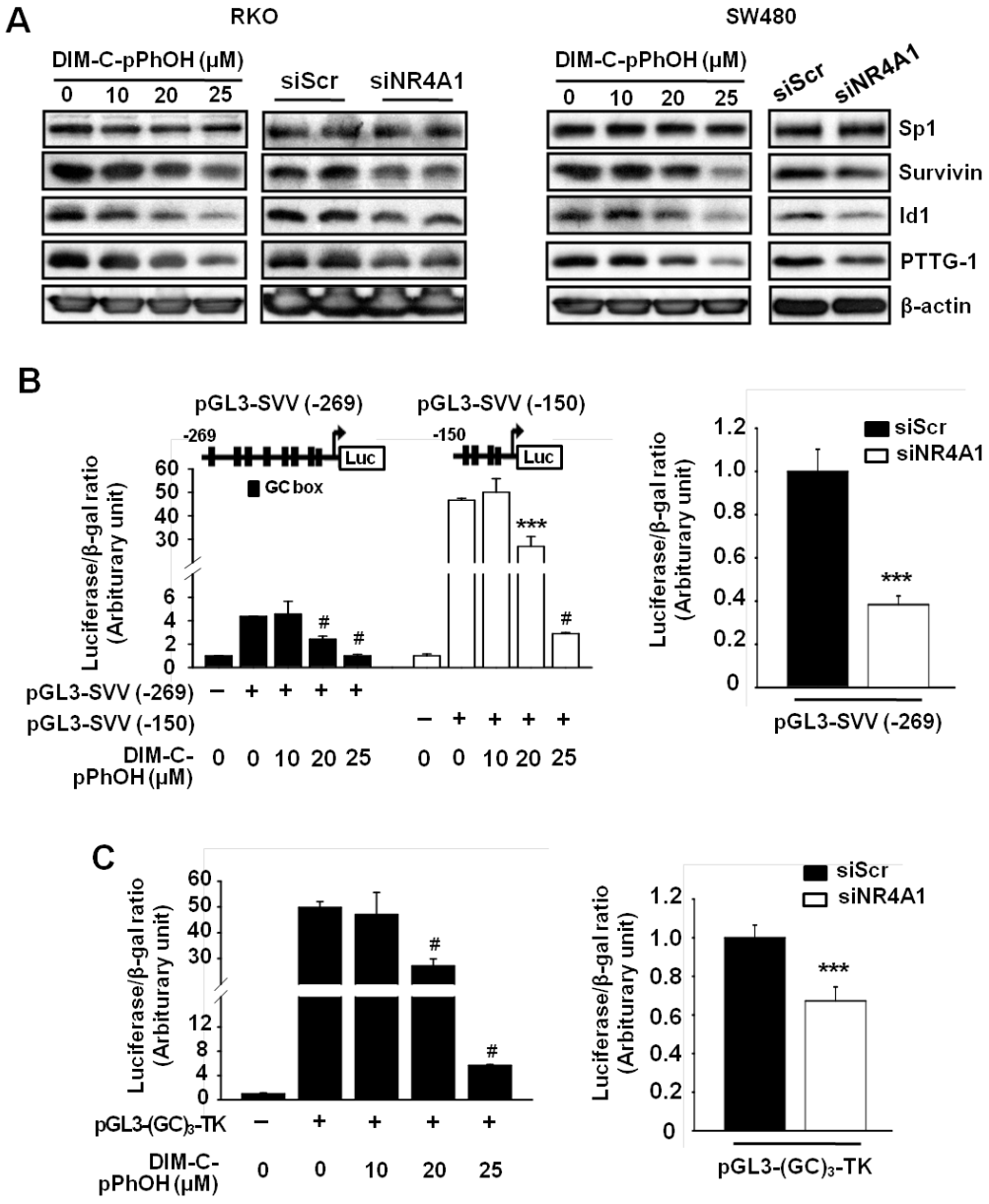


Figure 4.8. DIM-C-pPhOH and knockdown of NR4A1 inhibit Sp1-regulated gene expression through downregulation of Sp1 transactivation in colon cancer cells. *A*, western blot analysis. Cells were treated with DIM-C-pPhOH for 24 hr or transfected with siNR4A1 for 72 hr. Whole cell lysates were analyzed by western blot analysis and β-actin was used as loading control. *B*, *C*, RKO cells were transfected with 0.1 μg of either pGL3-SVV (-269), pGL3-SVV (-150) or pGL3-$(GC)_3$-TK for 4 hr and treated with various concentrations of DIM-C-pPhOH for another 18 hr or transfected with siNR4A1 and siScr. Luciferase activity (relative to β-galactosidase activity) was determined and results are expressed as mean ± S.D. for at least 3 separate determinations. ***, $P < 0.005$ and $^{\#}$, $P < 0.001$ vs. DMSO + pGL3-SVV or DMSO + pGL3-$(GC)_3$-TK. DMSO (solvent) treatment and the corresponding empty vectors were used as controls. ***, $P < 0.005$ vs. siScr.

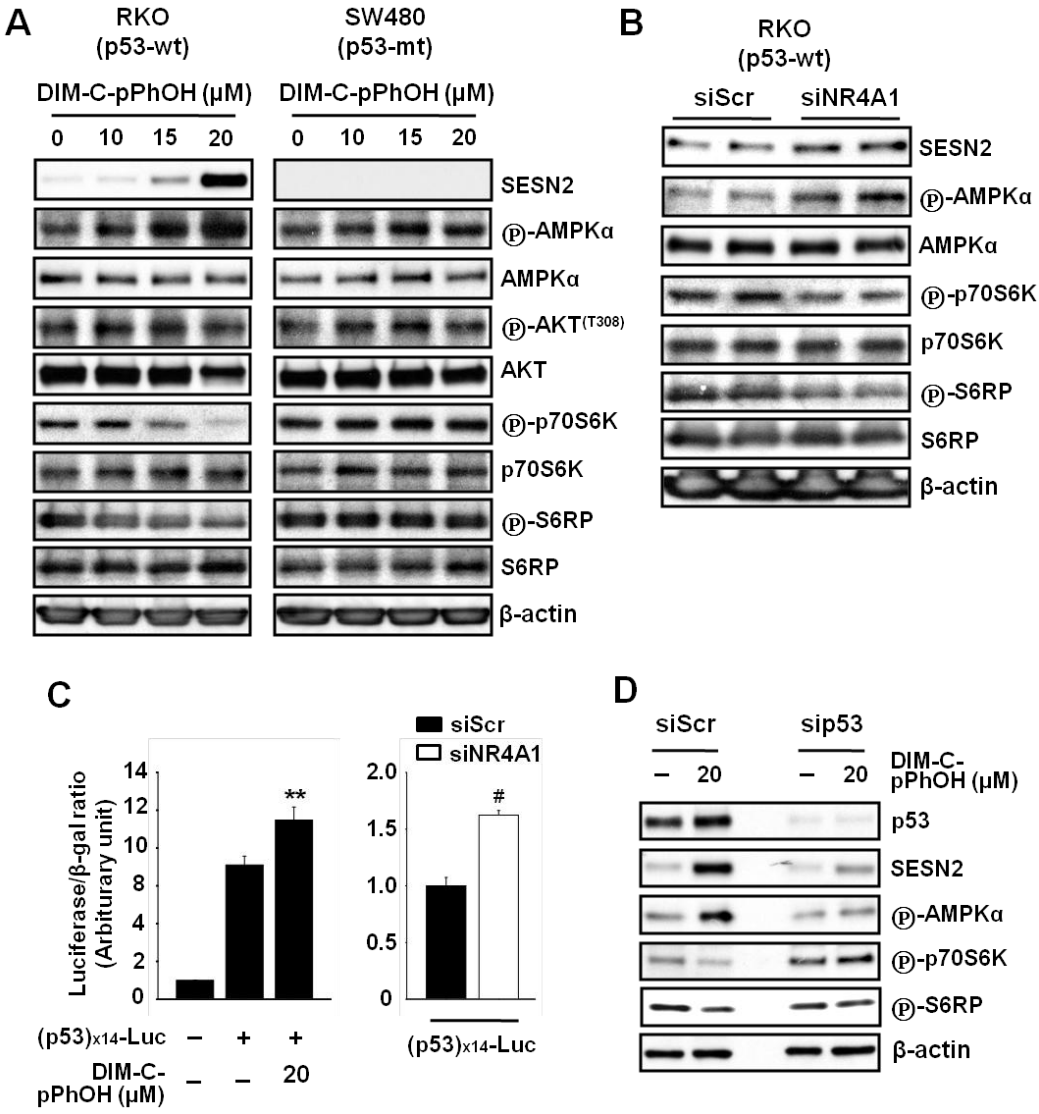


Figure 4.9. DIM-C-pPhOH and knockdown of NR4A1 inhibit mTORC1 signaling through activation of p53/sestrin2/AMPKα axis in colon cancer cells expressing wild type p53. *A*, *B*, cells were treated with DIM-C-pPhOH for 24 hr or transfected with indicated siRNA for 72 hr. Whole cell lysates were analyzed by western blot analysis and β-actin was used as loading control. *C*, RKO cells were transfected with $p53_{x14}$-Luc and treated with DIM-C-pPhOH for 18 hr (left panel) or RKO cells were cotransfected with each siRNA and $p53_{x14}$-Luc (right panel). Luciferase activity (relative to β-galactosidase activity) was determined and the corresponding empty vector was used as control. The results are expressed as mean ± S.D. for at least 3 separate determinations. **, P <0.01 vs. DMSO + $p53_{x14}$-Luc; $^{\#}$, P <0.001 vs. siScr. *D*, RKO cells were transfected with either siScr or sip53 for 48 hr and treated with DIM-C-pPhOH for another 24 hr. Whole cell lysates were analyzed by western blot analysis and β-actin was used as loading control.

cells transfected with a p53-responsive construct [$(p53)_{X14}$-Luc] (Fig. 4.9C) and the effects of DIM-C-pPhOH as an mTOR inhibitor in RKO cells were attenuated after knockdown of p53 (by sip53, Fig. 4.9D).

These results confirm that representative NR4A1 ligands, DIM-C-pPhOH, DIM-C-pPhBr and DIM-C-pPhCO_2Me, decrease NR4A1-dependent transactivation from an $NBRE_{x3}$-Luc and also antagonize two important NR4A1-regulated pro-oncogenic pathways in colon cancer cells as previously observed in lung and pancreatic cancer cell lines (219, 222). Thus, in colon cancer cells, C-DIMs such as DIM-C-pPhOH, DIM-C-pPhBr and DIM-C-pPhCO_2Me that bind NR4A1 act as NR4A1 antagonists.

Discussion

Endogenous ligands for the NR4A orphan receptors have not yet been identified; however, it is clear from knockout and transgenic mouse models that these receptors play a critical role in cellular homeostasis and disease. NR4A receptors are immediate-early genes induced by multiple stimuli and play essential roles in metabolism in the liver, pancreas and adipose tissue, neuronal and neurobehavioral functions, inflammation, cardiovascular function and steroidogenesis (305, 306). NR4A1 is also overexpressed and plays a pro-oncogenic role in multiple solid tumors; however, results from the double knockout $NR4A1^{-/-}$/$NR4A3^{-/-}$ mouse which rapidly develop leukemia suggest that NR4A1 may have tumor suppressor-like activity for acute myelocytic leukemia (236). Cytosporone B (Csn-B) was the first compound

characterized as an NR4A1 agonist which directly bound the LBD of NR4A1; and Csn-B also inhibited colon tumor growth in a mouse xenograft study and this inhibitory effect was NR4A1-dependent (246). However, Csn-B not only activated NR4A1-dependent genes through a classical ligand-activated nuclear receptor but also induced nuclear export of NR4A1 to the mitochondria to induce apoptosis as previously described for other apoptosis-inducing agents (311, 312, 323, 335).

Research in this laboratory initially identified C-DIMs that activated mouse NR4A1 in both pancreatic and colon cancer cells (215, 216). However, in pancreatic cancer cells transfected with human NR4A1-derived constructs, C-DIMs containing the *p*-substituted phenyl moiety exhibited either minimal induction or decreased transactivation in these cells (308, 333) and similar results were observed in colon cancer cells (Fig. 4.4). These results suggest that in colon cancer cells C-DIMs may act as NR4A1 antagonists; and using DIM-C-pPhOH as a model, we demonstrated that both siNR4A1 and DIM-C-pPhOH affected common set of genes and pathways (219, 222). For example, in lung and pancreatic cancer cells, nuclear NR4A1 in combination with p300 acts as a coregulator of Sp-regulated genes such as survivin; and transfection with siNR4A1 or treatment with DIM-C-pPhOH decreased expression of survivin but did not affect Sp1 expression (219). Using a similar approach, we have observed comparable responses in colon cancer cells (Fig. 4.6) demonstrating the pro-oncogenic functions of NR4A1 and the anti-neoplastic activity of DIM-C-pPhOH and other C-DIMs which antagonize nuclear NR4A1 but do not induce nuclear export of this receptor. NR4A1

binds and inactivates p53 (346) and in lung cancer cells, both siNR4A1 and DIM-C-pPhOH activate p53 which in turn induces sestrin 2 and activation of AMPKα results in mTOR inhibition (222). We used a similar approach in colon cancer cells and both siNR4A1 and DIM-C-pPhOH and related compounds inhibited mTOR signaling in p53 positive RKO but not p53-negative SW480 cells (Fig. 4.9 and Fig. 7D).

The parallel responses observed for siNR4A1 and DIM-C-pPhOH and other C-DIMs such as DIM-C-pPhBr and DIM-C-pPhCO_2Me that decrease NR4A1-dependent transactivation suggest that these compounds may be ligands or indirect inhibitors of NR4A1. Results of the transactivation studies with $NBRE_{x3}$-Luc also show that DIM-C-pPhOH and related C-DIMs decrease luciferase activity that is dependent on the N-terminal (AF-1) domain of NR4A1. There is precedent for drug-induced activation of the N-terminal domain of NR4A receptors by 6-mercaptopurine and C-DIMs (247, 342, 347) and the mechanisms of C-DIM-dependent activation of the AF-1 domains of NR4A2 and inactivation of NR4A1 in colon cancer cells are currently being investigated. However, we also observed that C-DIMs inhibited transactivation in cells transfected with the C-terminal ligand binding of NR4A1 and therefore investigated the direct binding of C-DIM analogs to the ligand binding of NR4A1 using the fluorescent assay as previously described for determining Csn-B binding to NR4A1 (246, 314). The results show that C-DIMs directly bind to the LBD of NR4A1 with variable K_D values; not all the compounds exhibited binding in the assay and some binding interferences were also observed.

In addition, we also used ligand docking studies to the ligand binding pocket of NR4A1 and results of computational-based molecular modeling show that the C-DIMs readily fit into the ligand binding pocket. DIM-C-pPhOH exhibited a low K_D in the direct binding assay and based on the results of the modeling studies this high affinity involves π-cation interactions with Arg514 and hydrogen bond interactions with His515. In addition, activation or inhibition of nuclear receptors also involves a concomitant conformational change in the protein in response to drug binding; and it may be that these interactions with Arg514 and His515 are necessary not only for optimal binding affinity but also for the requisite conformational changes required to modulate transcriptional activation. It was also apparent from the molecular docking studies with C-DIMs that the amino acid side chains and interactions that facilitate C-DIM interactions with NR4A1 are different from those previously reported for Csn-B and related compounds (246, 314) where hydrogen bonding to Tyr453 was an important interactions in the ligand binding pocket. These results are consistent with the significantly different structures of C-DIMs and Csn-B and suggest a flexible binding pocket that may influence cell context-dependent agonist or antagonist activity of C-DIMs, Csn-B and possibly other NR4A1 ligands.

In summary, results of this study demonstrate that C-DIMs which target nuclear NR4A1 also bind the receptor and function as anti-neoplastic agents in colon cancer cells by acting as NR4A1 antagonists. The results are consistent with previous studies in lung and pancreatic cancer cells where both siNR4A1 and DIM-C-pPhOH gave responses

similar to that observed in Figures 4.5 – 4.9. The interaction of C-DIM and Csn-B analogs within the NR4A1 ligand binding pockets suggests that NR4A1 may also bind other structural classes of small molecules which can be used as agonists or antagonists for treatment of cancer and other health problems where NR4A1 plays a prominent role (305, 306).

CHAPTER V

SUMMARY

Cancer is one of the major medical challenges of our time and predictions suggest that cancer will increase as a health problem as humans enjoy a longer life span. Colon cancer is the third most commonly diagnosed cancer in the United States and pancreatic cancer has the highest mortality rate due to late diagnosis (5). The main curative treatments for colon and pancreatic cancers are surgery and radiation therapies which are primarily successful for treating early stage localized tumors; once metastasized, the tumors can only be pharmacologically destroyed. During the last few decades, a myriad of natural and synthetic agents have been introduced to treat cancer patients; however, many of these agents eventually fail to effectively control cancer progression or cure the disease. Many of these agents are only effective for a short period of time or to a limited extent in patients and these failures are mainly due to acquired drug-resistance and a lack of understanding of the molecular mechanisms regulating their therapeutic actions. Thus, more mechanism-based drug discoveries have been developed in order to facilitate development of new drugs that have enhanced anticancer activity with minimal toxic side effects. Our studies in colon and pancreatic cancer cells were focused on investigating the mechanisms of action of two groups of anticancer agents, the NSAID sulindac and its metabolites and the phytochemical methylene-substituted diindolylmethane analogs (C-DIMs).

Mechanism-based Drugs That Target Sp Transcription Factors

NSAIDs inhibit cyclooxygenase (COX)-mediated prostaglandin synthesis and thereby reduce inflammation; since colon cancer is associated with chronic inflammation (142), it is not surprising that NSAIDs decrease the risk for colon cancer (348-352). In addition to their chemopreventive properties, previous studies in our laboratory show that several NSAIDs also exhibit chemotherapeutic effects in colon cancer cells and these NSAIDs include tolfenamic acid, aspirin and nitro-NSAID derivatives (283, 288, 289). In these studies, NSAIDs decrease cell proliferation, induce apoptosis and block invasion in colon cancer cell lines and xenograft mouse models and also downregulate expression of EGFR, survivin, Bcl-2, cyclin D1 and VEGF. These oncogenic and angiogenic genes are all transcriptionally regulated by Sp transcription factors which are overexpressed in colon cancer cells and low/absent in normal tissues. In addition, knockdown of Sp transcription factors by RNA interference results in anticancer activities similar to that observed after NSAID treatment. Therefore, we hypothesize that the chemotherapeutic effects of NSAIDs may be Sp-dependent (or COX-independent) and our results in Chapter II suggest that the anticancer activities of the NSAID sulindac are mediated by downregulation of Sp-dependent pathways and genes.

The NSAID sulindac inhibits colon polyp formation and is currently in clinical trials for prevention/treatment of multiple cancers including colon cancer (264, 353-358). As a sulfoxide prodrug, sulindac is converted to the active sulfide form by

methionine sulfoxide reductases (359) and, in our studies, sulindac sulfide was the most active/potent compound compared to sulindac and the other metabolite sulindac sulfone. Sulindac sulfide inhibited SW480 and RKO colon cancer cell proliferation with IC_{50} values (24 hr) of approximately 50 μM and, at growth inhibitory concentrations, sulindac sulfide downregulated Sp1, Sp3 and Sp4 proteins. Similarly, sulindac sulfide downregulated Sp-regulated pro-oncogenic proteins, including survivin, EGFR, NFκB-p65, Bcl-2 and VEGF, and concomitantly induced apoptosis in colon cancer cells. Sulindac sulfide also inhibited the GC-rich promoter activities of Sp and Sp-regulated genes, suggesting the decrease of Sp and Sp-regulated gene expression was due to transcriptional repression. Further investigations demonstrated that sulindac sulfide induced ROS in colon cancer cells and this preceded gene repression. The induction of ROS, in turn, decreased miR-27a levels through a currently unclear mechanism and, as a target of miR-27a, ZBTB10 was upregulated. ZBTB10 is a transcriptional repressor that competes with Sp transcription factors for *cis*-acting GC-rich elements; the upregulation of ZBTB10 by sulindac sulfide resulted in repression of Sp-regulated genes. Furthermore, co-treatment with antioxidants attenuated the above effects, confirming that sulindac sulfide-mediated anticancer activities were ROS/Sp-dependent. Hence, we conclude that the anticancer activities of the NSAID sulindac are due, in part, to sulindac sulfide which disrupts the ROS/miR-27a/ZBTB10/Sp axis in colon cancer cells. The detailed mechanism of action is being investigated and we believe that drugs, such as sulindac

sulfide, that target downregulation of multiple Sp-regulated genes, exhibit clear advantages over drugs that target individual oncogenes.

Mechanism-based Drugs That Modulate NR4A Receptors

Statistics show that consumption of cruciferous vegetables decreases the incidence of several cancers (360) and there is evidence that the chemopreventive effects are mediated, in part, by phytochemicals such as indole-3-carbinol (361, 362) and its dimer, diindolylmethane (DIM). Several studies have reported the chemoprotective activities of DIM, including inhibition of angiogenesis in breast cancer and hepatoma cells (363) and inhibition of invasion in colon cancer cells (364); DIM is also under clinical trials for cervical dysplasia (365, 366). Using DIM as a building block, we synthesized potent anticancer agents containing substituents on the exocyclic methylene group (-CH_2-) of DIM (hence, named C-DIM). Previous studies in our laboratory show that both DIM and C-DIM treatments induce ER or mitochondrial stress and induce apoptosis in cancer cells through different mechanisms mediated by nuclear receptors. These studies demonstrate that DIM modulates the activities of AhR, ER and AR while C-DIMs mainly modulate PPARγ and orphan receptors of the NR4A subfamily (326, 336, 367, 368). As reviewed in Chapter I, NR4A nuclear receptors play important roles in cancer and our results in Chapters III and IV suggest that the anticancer activities of C-DIMs are mediated by Nurr1/NR4A2 and TR3/NR4A1 receptors in pancreatic and colon cancer cells respectively.

Studies in Chapter III primarily focused on Nurr1-active C-DIMs in Panc1 and Panc28 pancreatic cancer cells and the structure-activity relationship (SAR) of C-DIM compounds. By using the GAL4 transactivation assay system, potent Nurr1 activators were identified, such as C-DIMs containing *p*-substituted-phenyl (pPh, or DIM-C-pPh) CF_3, Br, I and OCF_3, and these Nurr1-active C-DIMs also activated Nurr1 monomer- and dimer-binding DNA elements (NBRE and NurRE respectively) in reporter gene (luciferase) assays. In contrast to phenyl-substituted C-DIMs, heteroaromatic C-DIMs did not activate Nurr1. In addition, the effects of substituent orientation were also investigated by using compounds containing substituents on the indole rings or different positions (*ortho*-, *meta*- or *para*-) of the C-DIM phenyl ring; our comparative SAR studies demonstrated that C-DIMs with the *para*-phenyl substituents resulted in activation of Nurr1.

Using DIM-C-pPhBr as a model Nurr1 activator, we investigated the mechanism of Nurr1 activation and the results showed that Nurr1 activation was due to site-specific phosphorylation on both N- and C-terminal domains. DIM-C-pPhBr treatment did not change Nurr1 nuclear localization, indicating the effects of C-DIMs were mediated by transcriptional (nuclear) functions of Nurr1. In addition, DIM-C-pPhBr also increased/decreased the expression of several Nurr1-regulated genes, such as OPN, VIP and NRP1/2, and silencing Nurr1 by RNA interference abolished these effects, confirming the transcriptional functions of Nurr1 in pancreatic cancer cells. Furthermore, DIM-C-pPhBr exhibited higher potency than the previously identified

Nurr1 activator 6-mercaptopurine (247), suggesting potential chemotherapeutic applications for these Nurr1-active C-DIMs.

In contrast, C-DIMs inactivated TR3-dependent transactivation in SW480 and RKO colon cancer cells as reported in Chapter IV and, most importantly, this inactivation was due to a direct interaction between C-DIMs and the ligand binding domain (LBD) of TR3. By fluorescence quenching assays, we determined that the dissociation constants for C-DIMs/TR3 binding varied from 0.10 to 0.71 μM. Small molecule docking studies indicated that the TR3 ligand binding pocket was capable of accommodating C-DIM compounds; molecular modeling studies also predicted that C-DIMs such as DIM-C-pPhOH could bind TR3-LBD with high affinity. As a result of direct binding, C-DIMs inactivated wild type and mutant TR3 containing C-terminal domains (with LBD) in transactivation assays using NBRE-luciferase reporter constructs. Interestingly, selected C-DIMs also inactivated truncated TR3 containing N-terminal domains (without LBD) and kinase inhibitor studies suggested that inactivation of TR3 was also mediated by phosphorylation on the N-terminal transactivation domain. In addition, the TR3-inactivator, DIM-C-pPhOH, inhibited cell proliferation by downregulating survivin expression and also induced caspase-dependent apoptosis in colon cancer cells. Further functional studies demonstrated that DIM-C-pPhOH inhibited mTOR signaling through AMPKα phosphorylation; however, this pathway was p53-dependent and the detailed mechanism of action is currently being investigated.

In summary, we have investigated the anticancer activities of sulindac sulfide and C-DIM analogs in colon and pancreatic cancer cells. It is also possible to use these potent anticancer agents in treatment of other diseases in which Sp transcription factors and NR4A receptors play a role and this is currently being investigated.

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