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Biochemical and Cellular Mechanisms for the Antineoplastic Properties of Sulindac

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BIOCHEMICAL AND CELLULAR MECHANISMS FOR THE ANTINEOPLASTIC PROPERTIES OF SULINDAC

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ABSTRACT

The nonsteroidal anti-inflammatory drug (NSAID) sulindac has displayed the ability to inhibit the proliferation of colorectal cancer (CRC) cells and to increase the sensitivity of multidrug resistant (MDR) cancer cells to a wide variety of chemotherapeutic agents. The antineoplastic activity of sulindac has been attributed to inhibition of the prostaglandin producing enzyme COX-2, but the exact mechanism remains elusive and the inhibition of prostaglandin synthesis can result in gastrointestinal, hepatic, and cardiovascular toxicities. Enzymatic, cellular, and imaging assays were used to identify mechanisms that could contribute to the antiproliferative and apoptotic activity of sulindac. Screening of more than 500 sulindac derivatives revealed structural features that correlated with increased potency for growth inhibition when compared to the COX-inhibiting parent compound, sulindac sulfide (SS). An amine derivative of sulindac, sulindac benzylamine (SBA), did not inhibit prostaglandin synthesis, yet potently inhibited the growth and induced apoptosis of human colon tumor cells. This activity appeared to involve cyclic guanosine monophosphate phosphodiesterase (cGMP PDE) inhibition, activation of cGMP-dependent protein kinase G (PKG), a decrease in β-catenin mediated transcription and caspase activation.

The ability of SS to increase the sensitivity of multidrug resistant cancer cells was investigated by focusing on the two ATP-binding cassette (ABC) transport proteins that are most implicated in clinical multidrug resistant cancer, P-glycoprotein (ABCB1) and multidrug resistant protein-1(ABCC1). Cells over-expressing ABCB1 were significantly less sensitive to SS and doxorubicin in combination than ABCC1 expressing cells. SS also inhibited the efflux of LTC_4, a high affinity substrate of ABCC1, from inside-out membrane vesicles, decreased levels of reduced glutathione and increased the intracellular accumulation of calcein-AM. Using SS for comparison, two classes of compounds not previously associated with MDR inhibition were identified. The two classes, 5-quinolinones and imidazopyrimidines, contained members that selectively increased the cytotoxicity of doxorubicin in ABCC1 expressing MDR cells, in some cases potentiating the antiproliferative effect of doxorubicin treatment better than SS.

Overall, these studies demonstrate that SS can be used to identify COX-independent pathways involved in the antineoplastic activity of NSAIDs, leading to the development of novel targeted compounds for safer and more effective treatment of CRC and chemoresistance.

Keywords: ABCC1, ABCB1, β-catenin, colorectal cancer, MRP1, multi-drug resistance, NSAIDs, phosphodiesterases, sulindac

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Research Goals

Specific Aim 1: Determine if cyclooxygenase inhibition is necessary for the antineoplastic activity of sulindac in colorectal cancer (CRC) cells. Sulindac analogs will be synthesized by the medicinal chemists at Southern Research Institute and those analogs will be screened for their ability to inhibit the growth of CRC. Analogs with increased potency for growth inhibition will be investigated for cyclooxygenase (COX) inhibition. This aim will provide insight into the structural requirements for sulindac derivatives that inhibit *in vitro* CRC growth independently of COX inhibition.

Specific Aim 2: Determine whether PDE inhibition and β-catenin protein levels correlate with CRC growth inhibition by sulindac derivatives. The effect of sulindac derivatives on cGMP will be measured in both recombinant enzyme assays and in cell lysates by measuring fluorescence polarization of cyclic nucleotide substrates as an endpoint. Levels of phosphorylated vasoactivator-stimulated phosphoprotein (p-VASP), a biomarker for PKG activation, and nuclear levels of β-catenin will be measured by immunoblotting to determine the subsequent signaling events of elevated cGMP.

Specific Aim 3: Characterize the mechanism of cGMP-mediated antiproliferative or pro-apoptotic activity in colon tumor cells. Because colon cancer cells may rely on specific PDEs for survival, it is important to determine which PDE isozymes are associated with increased sensitivity to sulindac derivatives. NSAIDS in general may have several targets so this aim will include a protein pull-down assay with one of the sulindac derivatives to screen for different potential targets in CRC cell lines and normal colonocytes. This assay

will provide targets or potential biomarkers for future studies of colon cancer chemoprevention.

Previous studies have determined that sulindac and a subset of other NSAIDs can reverse the multidrug resistant (MDR) phenotype in cancer cells. In addition to the Aims listed above, studies were performed to determine the mechanism by which MDR cancer cells are sensitized to chemotherapeutic drugs. The selectivity of sulindac-mediated MDR reversal in ABCC1, ABCB1, and ABCG2 overexpressing cells was determined using cellular, enzymatic and imaging assays. Additionally, sulindac was used as a positive control by which to compare the MDR reversal activity of a library of structurally diverse compounds. The structure activity relationships determined from these experiments can be used for the design of future MDR inhibitors.

Overview of Thesis

Given the previous reports that sulindac has antineoplastic properties that are independent of COX inhibition, the studies described herein may have relevance in elucidating the importance of individual pathways in various cancer types. Sulindac derivatives that do not inhibit the COX enzymes yet still inhibit the growth of colorectal cancer cells will allow the identification of structure activity relationships (SAR) that may lead to the development of novel chemotherapeutic agents. Investigating the role of the COX-inhibitory metabolite of sulindac in MDR reversal may provide SAR data for the synthesis of selective ABCC1 inhibitors and help determine the mechanism by which it sensitizes cancer cells to other compounds. Overall, drug development strategies that focus on novel targeted agents with activity in preclinical models are likely to improve outcomes in chemoprevention regimens and combination therapies.

This thesis consists of six chapters. Chapter 1contains the necessary background, motivation, and objectives of the research efforts performed in colorectal cancer cells. Chapter 2 describes the association between NSAIDs and multidrug resistance in cancer cells and introduces the concept of selective killing of multidrug resistant cancer cells by inhibition of ABC transporters. Chapter 3 describes the effects of sulindac sulfide in multidrug resistant lung cancer cells that over-express the multidrug resistance-associated protein ABCC1. Sulindac analog screening efforts and structure-activity analysis is discussed in Chapter 4. Chapter 5 describes the inhibitory effects of a sulindac benzylamine on phosphodiesterase activity, β-catenin transcriptional activity, and colorectal cancer cell proliferation. The final chapter summarizes the previous chapters and discusses possible future directions.

CHAPTER 1

INTRODUCTION

The development of cancer can be characterized as a multistep process driven by the acquisition of activating mutations in genes that promote growth and inactivating mutations in genes that inhibit growth. One way to illustrate the process is to describe the six “hallmarks” of cancer: self-sufficient proliferation, unlimited potential for replication, evasion of apoptosis, insensitivity to anti-proliferative signals, the maintenance of vascularization, and tissue invasion [1]. Cancer can also be considered a step-wise development that consists of three phases: initiation, promotion, and progression [2]. Initiation is characterized by genomic changes such as point mutations, gene deletions or amplification, and chromosomal rearrangements which cause irreversible cellular changes. Tumor development is promoted by the survival and clonal expansion of these cells and progression encompasses the growth in tumor size and in many cases, metastasis.

In colorectal cancer, the stepwise progression from normal to dysplastic epithelium to carcinoma is referred to as the adenoma-carcinoma sequence because adenomas (polyps) are the first physical manifestation of uncontrolled cell proliferation. Although not cancerous, these polyps have the potential to develop further into cancerous lesions and current estimates are that 40 percent of the Western population will develop adenomas. In clinical studies, the distribution of adenomas and colorectal cancers is similar [3] and the removal of adenomatous polyps reduces the risk of colorectal cancer

[4]. Unfortunately, even after surgical removal one-third of patients will have adenomas again after 4 years [5-6]. Furthermore, there are no reliable criteria that predict adenoma progression or recurrence.

Although the broad use of screening methods has reduced colorectal cancer (CRC) mortality rates in recent years, it has done so only marginally[7]. Once metastatic tumors do develop, the majority are not resectable. The frontline therapeutic agent for advanced colorectal cancers has been the anti-metabolite 5-fluorouracil (5-FU) for more than 40 years. 5-FU acts by inhibiting thymidylate synthase (TS) and incorporating into DNA and RNA resulting in cell cycle arrest and apoptosis [8]. Understanding the mechanism of action has lead to combination therapies, for example with folinic acid (leucovorin) and oxaliplatin, to improve the efficacy of CRC chemotherapy. Also the development of an orally available fluoropyrimidine, capecitabine, has improved treatment as its metabolites tend to be better tolerated[9] [10]. Despite these recent advances in chemotherapy, approximately 600,000 people will die from CRC each year. Therefore, a better understanding of the molecular events underlying the development of CRC seems essential in order to address better ways to manage the disease.

Genetic Basis for Colorectal Cancer

A genetic model for colorectal tumorigenesis was proposed in 1990 by Fearon and Vogelstein [11] (**Figure 1**). They found that tumorigenesis occurs through alterations occurring primarily on chromosomes 5q, 17p, and 18q. The most commonly altered genetic pathways involved APC, p53, and K-ras. The high percentage of *ras* gene mutations in adenomas greater than 1cm in size indicated that it was an event which occurred relatively late during adenoma formation. Furthermore, it was reported that 75%

of colorectal carcinomas had chromosome 17 deletions[12] and the region lost on the chromosome contained the gene for p53[13]. Since this deletion was usually observed in either carcinomas or large adenomas, this genetic alteration was also considered a late-stage event in CRC development. Similar to p53, a region lost on chromosome 18q containing the gene for the transmembrane protein DCC (deleted in colorectal cancer) is usually observed only in carcinomas or late-stage adenomas.

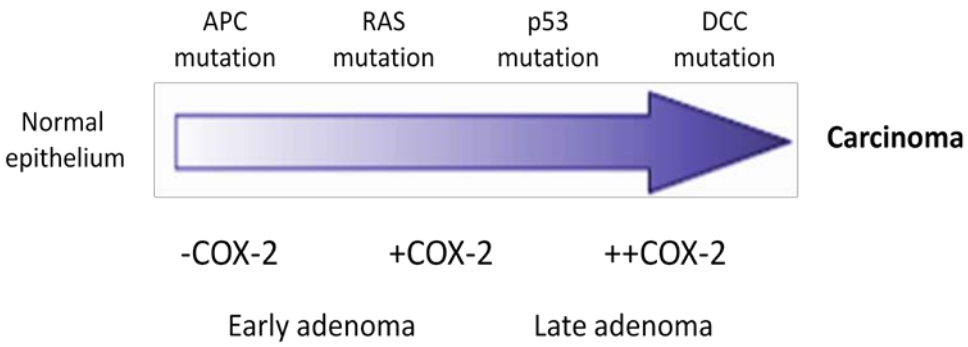


Figure 1. The adenoma-carcinoma sequence in colorectal tumorigenesis

Although these observations improved our understanding of colorectal tumorigenesis, they were unlikely candidates as the initiating events in CRC because they seemed to occur late in the process. A clue to the early stages of colorectal tumorigenesis came from observations made in individuals with familial adenomatous polyposis (FAP), an autosomal dominant condition characterized by the development of hundreds or thousands of adenomas appearing in adolescence or early adulthood. Affecting 1 in 8000 individuals and with a 95% probability of developing CRC, the disease is often fatal before individuals reach 50 years of age. Mutations of the *APC* (Adenomatous Polyposis Coli) gene on chromosome 5q were found to be responsible for this condition[14] [15]. Generally, two or more mutations are required for tumor formation and it is the

accumulation of genetic alterations rather than the order in which they occur which is important. However, the initiating event for most cases of CRC seems to be a mutation or an allelic loss (loss of heterozygosity) at this location.

Dysregulation of APC/β-catenin in CRC

The product of the *APC* gene is a large 312-kDa protein that is known to interact with several other proteins including β-catenin, glycogen synthase kinase 3β (GSK3β), end binding protein 1, and microtubules [16] [17] [18]. One important function of APC is the ability to bind to β-catenin and prevent it from moving into the nucleus where it interacts with the T-cell factor(TCF) /lymphocyte enhancer factor(Lef) family of transcription factors. In the presence of the β-catenin/ TCF-Lef transcription complex, several genes involved in cell proliferation and resistance to apoptosis are transcribed, including *c-myc, survivin, cyclin D,* and the genes for several matrix metalloproteinases. β-catenin is itself part of the Wnt signaling pathway which is involved in embryogenesis, cell differentiation, and cell polarity. Importantly, mutations in the Wnt/β-catenin signaling pathway are also associated with cancers that arise in tissues such as prostate, ovarian and medulloblastoma [19] [20] [21]. In addition to forming a complex with β-catenin and other proteins, APC is necessary for the efficient binding of spindle fibers to kinetochores during mitosis [22]. Failure to connect or disassociation of spindle fibers from the kinetochores leads to chromosome instability and the failure of daughter cells to acquire the correct number of chromosomes during cell division, a condition known as aneuploidy. In fact, aneuploidy is 10 to 100 times greater in APC-negative cells compared to normal cells. Therefore, APC mutations or deletions have a double impact in

CRC. First, the regulation of cell proliferation is impaired and cells divide continuously. Second, there is a higher probability that chromosomes with tumor suppressor genes will be lost, causing decreased susceptibility to apoptotic stimuli.

The regulation of β-catenin activity and the early role it has in tumorigenesis has attracted considerable attention from researchers who are looking to develop new anticancer therapeutics. In the absence of Wnt signal, most β-catenin is attached to the plasma membrane where it associates with E-cadherin in adherens junctions. The cytosolic β-catenin is in a multiprotein complex, consisting of the adenomatous polyposis coli (APC) protein, axin/conductin, casein kinase CK1α or CK1ε and GSK3-β. Casein kinase activity starts the serine/threonine-phosphorylation of β-catenin at residue 45 and it is subsequently phosphorylated by GSK3-β at residues 33, 37, and 41. These phosphorylation events target β-catenin for ubiquitination by the F-box-ligase β-TrCP (betatransducin repeat-containing protein) and subsequent degradation by the proteasome [23] [24].

In the event of Wnt proteins binding to membrane receptors called Frizzled (Fz), β-catenin is stabilized. Following receptor binding, Wnt signals are transmitted by the association between Fz receptors and a protein called Disheveled (Dvl). This disrupts the multiprotein complex containing APC, Axin, β-catenin, and GSK3β resulting in cytosolic accumulation of β-catenin. Truncating mutations in APC can also prevent the effective phosphorylation of β-catenin, resulting in its accumulation. The same outcome can arise through mutations in β-catenin or Axin, though these are significantly less frequent than mutations in APC[25]. Ultimately, β-catenin translocates to the nucleus and drives TCF/Lef-mediated gene transcription.

Colorectal Cancer Chemoprevention and NSAIDs

The concept that tumorigenesis is a multistep process is the foundation for chemoprevention as originally proposed in 1976 by Sporn [26]. Since then, studies have found that a large number of drugs that are in clinical use for the treatment of diseases other than cancer have shown anticancer activity and among these are the nonsteroidal anti-inflammatory drugs (NSAIDs). The association of NSAIDs with reduced risk of colorectal cancer [27-28] has been widely attributed to suppression of prostaglandin synthesis since prostaglandins are elevated in colon tumors [29].Support for this mechanism came from the observation that a significant percentage of colon tumors express high levels of COX-2[30], an enzyme responsible for the initial step in the production of prostaglandins from arachidonic acid. As further evidence for the role of prostaglandins in CRC, inhibition of COX-2 reduced the number of polyps in *APC* knockout mice [31]. Unfortunately, the long-term use of traditional NSAIDs is associated with gastrointestinal toxicity which has prevented the use of these drugs for CRC prevention.

Although the use of selective COX-2 inhibitors instead of nonselective NSAIDs reduces gastrointestinal toxicity, they are associated with an increased risk of cardiovascular side effects, including increased blood pressure, stroke, and myocardial infarction. Fortunately, there is evidence that certain NSAIDs exert their effects via COX-independent mechanisms. For example, three NSAIDs (NS-398, indomethacin, and aspirin) [32] display inhibitory activity on cell proliferation and apoptosis in colorectal cancer cells that is independent of COX-1 and COX-2 expression. Evidence obtained demonstrated that the selective COX-2 inhibitor, NS-398, had antiproliferative effects on

human colorectal cancer cells, which were not entirely dependent on COX-2 expression or associated with downregulation of β-catenin protein. Indomethacin exerted antiproliferative effects that were associated with decreased β-catenin protein expression and aspirin had activity against colorectal cancer that appeared to be a result of inactivation of NF-κB and altered expression of DNA mismatch repair proteins. Additionally, several studies have demonstrated sulindac sulfone, a metabolite of sulindac which does not inhibit the COX enzymes, inhibits azoxymethane (AOM)-induced carcinogenesis in the colons of mice and rats [33] [34]. A key demonstration of the COX-independent effects of NSAIDs, and sulindac in particular, came in 1996 when it was shown that exogenous prostaglandins could not rescue colon cancer cells from the anti-proliferative and apoptotic effects of sulindac sulfide [35]. Thus, although all NSAIDs share the ability to inhibit one or both COX isoforms, it is likely that individual NSAIDs and newer selective COX-2 inhibitors have anticancer activity via a variety of different COX-independent mechanisms.

COX-Independent Mechanisms

Previous research into the COX-independent mechanisms by which sulindac exerts its antineoplastic effects has revealed that it increases oxidative stress in cancer cells [36] and targets several signaling pathways[37] [38-39]. A pathway of interest in several tumor types is inhibition of multidrug resistance and in colorectal cancer there is compelling evidence that sulindac affects the Wnt/β-catenin signaling pathway. Previous studies have shown that sulindac treatment increases cyclic guanosine-5'-monophosphate levels[40], decreases the level of nuclear β-catenin in CRC cells[41], and enhances the

efficacy of cytotoxic agents in multidrug resistant cells[42]. The objectives of my research were to study the mechanism(s) by which sulindac suppresses the growth of colorectal cancer and multidrug resistant cancer. Sulindac sulfide was used primarily as a chemical probe to elucidate the mechanism by which dosage reduction could be achieved in multidrug resistant cancers, but also as a comparison compound for finding more potent MDR inhibitors. In CRC, sulindac analogs were screened for their ability to suppress the growth of colon cancer cells and the mechanisms associated with growth inhibition, primarily focusing on phosphodiesterase inhibition and the role it has in the disruption of β-catenin mediated, pro-proliferative transcription.

The Role of COX-2 and Inflammation in Cancer

An association between the development of cancer, inflammatory cytokines, and exogenous chemicals has been recognized for many years and these steps are dependent on chemical processes provided by the tumor environment [43]. Although the activation of oncogenes and inactivation of tumor suppressors are important initiators for uncontrolled cell growth, the proper signaling molecules are needed for tumor promotion and progression. There is evidence that the COX enzymes, which are responsible for the initial steps in the synthesis of prostaglandins, not only help to mediate the tissue repair process in the colon during inflammation [44] but may also play a critical role in tumor development [45].

Cyclooxygenases

In humans, COX-1 is found constitutively expressed in a wide range of tissues including the kidney, lung, stomach, small intestine and colon. It is considered a

housekeeping enzyme responsible for maintaining prostaglandin levels important for tissue homeostasis. In contrast, most tissues do not normally express COX-2 constitutively. The stimulation of COX-2 expression in Src-transformed fibroblasts, endothelial cells and monocytes treated with the tumor promoter tetradecanoyl-phorbol-acetate [46] led to the notion that COX-2 is an inducible enzyme that produces prostaglandins during inflammation and colon tumorigenesis [29]. The inducible nature of COX-2 was confirmed when its expression was found to be rapidly induced in several cell types by growth factors, cytokines, hormones, and tumor promoters [47-48] [49]. COX-1 and COX-2 are encoded by two separate genes located on chromosomes 9 and 1, respectively [50] [51]. The two genes are approx 60% homologous, and both COX enzymes are similar with only minor differences in their catalytic activity. However, COX-2 was found to have multiple transcriptional regulatory sequences in its promoter region.

The inducible nature and multiplicity of pathways by which its expression can be regulated has significantly increased interest in the biology of COX-2. Small differences between the NSAID binding sites of COX-1 and COX-2 have been recently exploited in the development of selective inhibitors of COX-2 [52] [53]. As mentioned above, COX-2 is responsible for the rate-limiting step in prostaglandin production. First, arachidonic acid is converted into prostaglandin G_2 (PGG_2) by the cyclooxygenase activity of the enzyme. Second, PGG_2 is reduced to prostaglandin H_2 (PGH_2) by the peroxidase enzyme (**Figure 2**). It is PGH_2 which is the precursor molecule for PGE_2 and other prostaglandins.

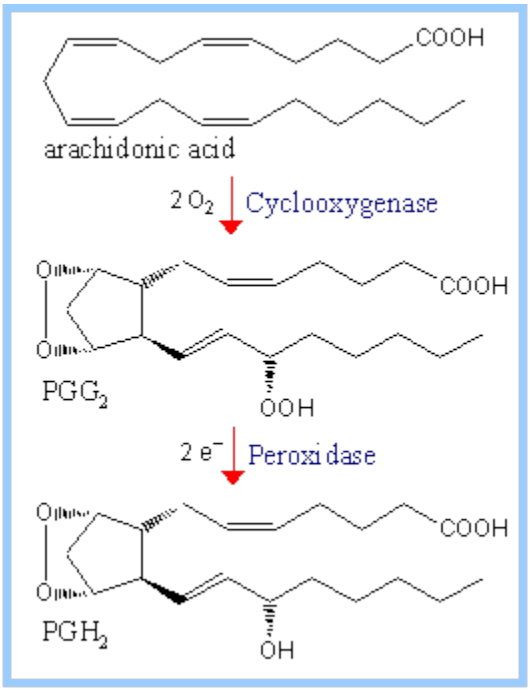


Figure 2. The prostanoid biosynthetic pathway.
Arachidonate is converted to PGG_2 by the cyclooxygenase activity of COX-1 or -2, the second catalytic activity of the COX enzymes is a peroxidase that converts PGG_2 to PGH_2. PGH_2 is then isomerized to biological active prostanoid products.

While studies on the function of specific prostaglandins in the promotion of CRC have been limited, available evidence points to a role for PGE_2 in cancer cell survival[54-55]. The DuBois lab first reported that cyclooxygenase-2 is overexpressed in colorectal adenomas $Apc^{Min/+}$ mice, an animal model that closely resembles the FAP condition in humans [56] and it has been found to result in increased incidence and number of colon tumors in rats [57]. Other studies have found increased COX-2 expression occurs in 40% to 50% of colorectal polyps and in up to 85% of CRC [58]. Bennett et al. observed higher concentrations of PGE_2 in some human colorectal cancers than in surrounding normal mucosa [59] and subsequent studies confirmed that certain human colon cancer cell lines [60] and tumor tissues [61] [62] overproduce PGE_2. The idea that tumor prostaglandins might accelerate the growth and invasion of the cancer was further supported by the

observation of Narisawa et al. [63] that PGE_2 in venous blood draining human colorectal carcinomas was higher *in vivo* when the cancers are large and locally invasive. Thus, there is good evidence that inhibition of the COX-2/PGE_2 pathway might be useful in the prevention or treatment of colorectal tumors.

NSAIDs and Chemoprevention

Pioneering studies by Narisawa and Pollard demonstrated that the NSAIDs, indomethacin and piroxicam, administered to rodents in drinking water, diet, or by intraperitoneal (ip) injection inhibited colon tumors induced by a variety of carcinogens [64] [65] [66]. Since then, a number of investigations have evaluated the chemopreventive efficacy of several NSAIDs against colon carcinogenesis. These studies have demonstrated that administration of the NSAIDs aspirin[67], ibuprofen, piroxicam,[68], ketoprofen, and sulindac[69] during the initiation and post-initiation stages of carcinogenesis suppressed the incidence and multiplicity of colon tumors.

Evidence of tumor suppression in rodents prompted Waddell and Loughy [28] to conduct uncontrolled clinical studies in which patients with FAP were treated with the prodrug sulindac to suppress polyposis. Approximately 16 case reports and uncontrolled studies, involving a total of 189 patients with FAP, reported a reduction in the number and size of adenomatous polyps in patients treated with sulindac at doses of 100–400 mg daily for up to 4 years. Later, several randomized clinical trials established that both the nonselective NSAID, sulindac [70] and the selective COX-2 inhibitor celecoxib [71] [72], suppress the development of adenomatous polyps in patients with FAP. Epidemiological studies also have shown that long-term administration of NSAIDs can

decrease CRC incidence in humans by 45% [73] or more[74]. Clearly one factor in the anticancer activity of NSAIDs is the effect they have on prostaglandins, but there may be additional mechanisms. Other studies suggest they may reduce the formation of polyps by COX-2-independent mechanisms and a number of proteins and associated signaling pathways have been implicated as targets for the NSAIDs, including 15-lipoxygenase [75], Ras [37], PPAR-δ [76], NF-κB [77], PDK-1/Akt [78] and NAG-1 [79-80].

Direct support for the idea that NSAIDs might work through the Wnt/β-catenin pathway came when a reduction in nuclear β-catenin levels was observed in the polyps of FAP patients treated with sulindac sulfide [81]. Although the majority of colon cancers display active Wnt signaling as a result of mutations in APC, increased prostaglandins may contribute to colon cancer cell proliferation by interfering with the ability of the cells to degrade β-catenin [82] [83]. In colon cancer cells, PGE_2/EP2 stimulation of the β-catenin pathway involves the G-protein subunits Gαs and Gβγ. PGE_2 causes Gαs association with the RGS (regulator of G protein signaling) domain of axin, thereby releasing GSK-3β from the complex. PGE_2 also causes release of the Gβγ subunits which activates the PI 3-kinase/Akt pathway and inhibits GSK- 3β by Akt mediated phosphorylation [82]. Therefore, GSK-3β can no longer phosphorylate and inhibit β-catenin, allowing translocation to the nucleus and activation of target genes resulting in increased proliferation of the tumor cells (**Figure 3**). It therefore appears that the reduction of prostaglandin levels by NSAIDs can account for some of their ability to inhibit the proliferation of cancer cells. However, the ability of compounds like sulindac sulfone ,which do not have COX-inhibitory capacity, to suppress the growth of cancer cells suggests other mechanisms of action [70] [40] [84].

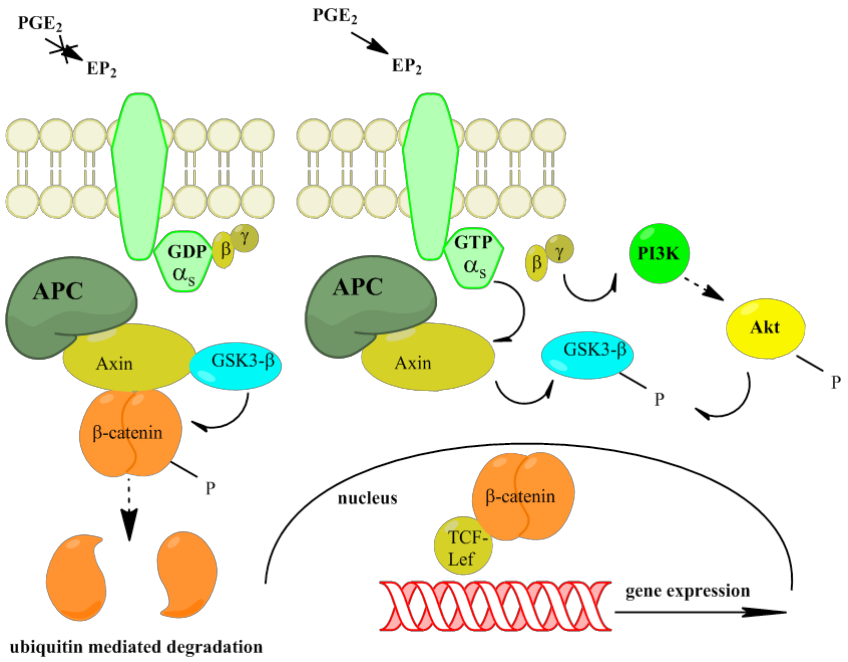


Figure 3. The role of PGE_2 in β-catenin protein stability. Activation of the EP_2 receptor by PGE_2 initiates Akt-mediated phosphorylation and separation of GSK3-β from the β-catenin degradation complex. β-catenin translocates to the nucleus and initiates the transcription of proliferative and anti-apoptotic proteins.

Cyclic Nucleotide Signaling and Colorectal Cancer

Cyclic nucleotide signaling is an important regulator of many cellular processes such as proliferation, migration, metabolism, growth and apoptosis, all of which can be altered in

cancer. The potential role of cyclic nucleotides in the growth of cultured cells was first noted in 1974 by Seifert and Rudland [85]. Numerous alterations in the content or metabolism of cAMP and cGMP have been described during normal cell growth and in neoplastic tissue [86] [87] [88]. Although no consistent pattern has emerged between changes in intracellular cyclic nucleotide levels and cellular proliferative activity, several studies have indicated an increase in cyclic GMP levels can inhibit DNA synthesis and proliferation of colon adenocarcinomas[89-91]. The mechanism or mechanisms through

which increased levels of cGMP inhibit colorectal cancer growth are still being investigated, but it is known that the changes in cGMP levels are translated into intracellular effects by a panel of cGMP-binding effector proteins, which include cyclic nucleotide-gated ion channels, cGMP-dependent protein kinase (PKG) and cGMP-regulated cyclic nucleotide phosphodiesterases (PDEs).

Since cGMP can have such wide-ranging downstream effects, it is important to have some understanding of how intracellular concentrations are controlled. Tight regulation of cGMP levels is required to maintain the sensitivity of the response to the incoming signal and rapid adjustment to changes in that signal. The cellular concentration of cyclic GMP is controlled by

the activity of two enzyme classes, guanylate cyclases (GC) and phosphodiesterases. For cGMP to be produced, the guanylate cyclases must convert guanosine triphosphate to guanosine 3' 5' monophosphate [92]. Signal termination is achieved by either extrusion of cGMP from the cell [93] or by hydrolysis of cGMP to 5′GMP catalyzed by the large superfamily of cyclic nucleotide phosphodiesterases. Since the disruption of normal cyclic nucleotide levels have been observed in colon tumors[94-95], the enzymes that control them are considered potential targets for new chemotherapeutic agents.

Guanylyl Cyclases

Guanylyl cyclases are a family of enzymes that catalyze the conversion of GTP to cGMP. The family comprises both membrane-bound and soluble isoforms that are expressed in varying degrees in nearly all cell types. Two types were identified in 1969, one in the particulate fraction of cells and another in the soluble fraction. Guanylyl

cyclases may be activated by either nitric oxide (NO) or by endogenous peptide ligands, such as atrial natriuretic peptide (ANP), guanylin, and uroguanylin. The two types are comprised of different family members, differing in tissue distribution and the genes that encode them.

The guanylyl cyclase relevant to colorectal cancer is designated GC-C and is activated by the endogenous ligands, guanylin[96] , uroguanylin[97] , nitric oxide and the heat stable enterotoxins produced by bacteria[98]. This isoform is found only in the intestinal mucosa and the regenerating liver. Dysregulation of GC-C signaling may compromise intestinal mucosa homeostasis and contribute to colon carcinogenesis. For example, elimination of GC-C expression causes rapid cycling of progenitor cells and hyperplasia in the proliferating compartment of the intestine [99]. In contrast, uroguanylin treatment suppresses polyp formation by approximately 50% in $APC^{Min/+}$ mice[89], an animal model that mimics polyp formation in FAP patients. Recent evidence has linked the incidence of enteric bacterial infections with the incidence of colorectal cancer and epidemiological studies have found that regions with the lowest level of CRC have the highest incidence of these infections[90, 100]. Shailubhai *et al.* have established that the bacterial heat-stable enterotoxin (ST), which is a ligand for the GC-C receptor, has an antiproliferative effect on the growth of intestinal cancer cells[89]. Although the expression of the endogenous ligands for GC-C, uroguanylin and guanylin, are decreased in colorectal cancer, GC-C expression is maintained [101]. These data and others [88, 102] indicate that GC-C is a critical signaling molecule regulating the proliferation of intestinal epithelial cells. Since activation of GC-C leads to an increase in intracellular

cyclic GMP, the downstream effects of this nucleotide on proteins (e.g. PKG) and the possible link to pathological conditions are of increasing interest to researchers.

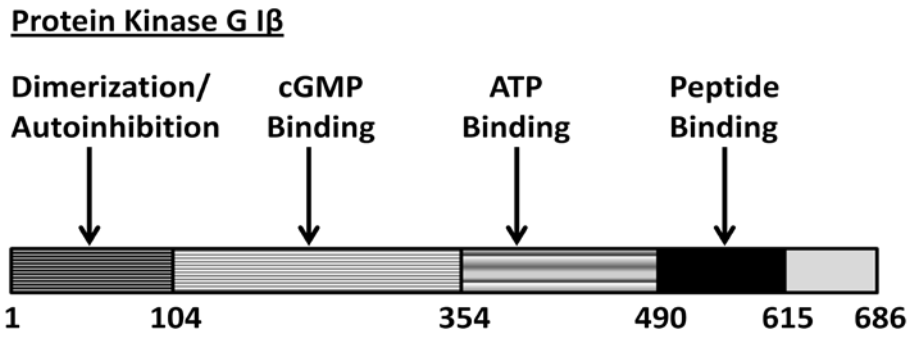


Figure 4. **The domain structure of human protein kinase G- Iβ.** Numbers represent amino acid residues.

Protein Kinase G

Most tissues contain significant amounts of protein kinase G, a serine/threonine protein kinase that is activated by cGMP. First identified as an important component in promoting vascular smooth muscle relaxation [103-104], the roles of PKG have been expanded to include processes such as gastrointestinal motility [105], cardiac protection [106],urinary tract functions [107] and endothelial permeability [108]. PKG isozymes are found in particular subcellular membrane fractions, in complex with certain cytosolic proteins, and as free proteins. They are also reported to translocate among cellular compartments after changes in cGMP levels[109]. Due to the localization effects for cGMP and PKG, a difference in affinity for cGMP may exist within cells over a broad range of concentrations. For this reason, the amount of cGMP elevation or PKG activation required to elicit specific cellular responses is still unknown. This has made studying the different PKG isozymes difficult, but the general consensus is that a decrease in PKG protein levels and/or activity allows cancer cells to proliferate.

Therefore, compounds and treatment strategies that target the cGMP/PKG pathway may provide more effective cancer treatment than what is currently available.

The enzyme is composed of three functional domains: an N-terminal domain, a regulatory domain, and a catalytic domain(**Figure 4**) [110]. The regulatory domain contains two tandem cGMP-binding sites, whereas the catalytic domain contains the Mg^{2+}- ATP- and peptide-binding pockets. The PKG isozymes are homodimers that are dimerized by leucine zipper regions located on the N-terminal end of the enzyme. The binding of cGMP to the regulatory domain increases enzyme activity by 3-to 10-fold[111]. The catalytic domain, located at the carboxy-terminus, contains the binding sites for Mg^{2+}-ATP and the target protein [112]. This domain catalyzes the transfer of a phosphate from ATP to a serine/threonine residue of the target protein.

Many extracellular signals are amplified and transduced inside cells by protein kinase cascades involving PKG. The physiological roles of PKG isozymes have been documented in many processes including gastrointestinal motility [105], blood flow [113] [114], cardiac protection [115] , and fluid homeostasis in the colon[116]. Extensive study of these PKG signaling cascades has revealed that the enzyme can be found upstream or downstream of epidemiologically relevant oncogenes or tumor suppressors [117] [118]. The possible involvement of cGMP and PKG in apoptosis is supported by several studies [40, 119-120] and has made PKG activation an attractive area for cancer treatment research.

Decreased expression of PKG occurs in most tumors compared to normal tissue and the transfection of constitutively active PKG in colon cancer cells leads to reduced tumor growth and angiogenesis [121]. This suggests the decreased PKG levels seen in

cancer cells are a survival mechanism and increased PKG expression or activation may lead to significantly decreased proliferation rates of tumor cells. This is strongly supported by studies conducted in the Browning lab where they found PKG activation led to a reduction in β-catenin expression and TCF/Lef-mediated transcription [122] .

Cyclic Nucleotide Phosphodiesterases

As previously mentioned, cyclic nucleotides are degraded by enzymes called phosphodiesterases (PDE). Cyclic nucleotide PDEs consist of 11 gene families, each having one or more isoforms. Each family of PDEs can bind and degrade cyclic AMP (cAMP) and/or cGMP, but differs in its physical and kinetic properties. Only a few PDE isoforms are expressed and used by any single type of cell or tissue to regulate cGMP or cAMP levels but they all have the same function, which is to catalyze the hydrolytic cleavage of the 3′-phosphodiester bond, resulting in formation of the corresponding inactive 5′-monophosphate [123] . There are currently 11 different PDE families comprised of 21 different proteins, but with the presence of several gene promoter regions and possibly mRNA splice variants there could be many more isoforms yet to be discovered. Although the family of PDEs is large and there is functional redundancy between members, differences in tissue distribution, cellular localization, and regulation suggests the different PDE isoforms play specific physiological roles [124]. Therefore, it has been widely believed that it should be possible to develop isoform selective inhibitors that can target specific functions and pathological conditions without a high likelihood of causing nonspecific side effects.

Structural studies on the different PDEs have revealed that the specificity of the catalytic site depends on an invariant glutamine residue (Q817) that stabilizes the purine ring in the binding pocket of the enzyme through hydrogen bonds. Free rotation of the glutamine is necessary for both cyclic nucleotides to be able to bind well, but the preferred substrate will be either cAMP or cGMP if the glutamine is constrained by neighboring residues[125]. An exception to this rule is PDE5, which may have additional interactions that are important for substrate specificity [126]. Like most other PDEs, cyclic nucleotide binding regions in the regulatory domain are important for the dimerization and stability of the enzyme but PDE5 appears to have an extra region that contributes to stabilization and one extra region that contributes to auto-inhibition. The auto-inhibitory region provides the conformational change required to block phosphorylation of Ser^{102}. Phosphorylation of PDE5 by protein kinase G (PKG) at this site augments the enzymatic activity as well as the affinity of PDE5 for cGMP [127-128]. The level of enzymatic activity has been shown to increase in parallel with phosphorylation, and the increase in activity is typically about 1.6-fold. The result is a greater degradation of cGMP, which makes less available for activating PKG. Since increased PKG expression in colon cancer cells injected into athymic mice has been shown to inhibit tumor growth and invasiveness [129], increasing PKG activation by elevating intracellular cGMP levels is thought to be a legitimate means by which to target colon cancer. The fact that increased activity of PDE5 has been detected in colon adenocarcinoma cells and this activity is associated with desensitizing the cells to stimuli that increase intracellular cGMP [130] suggests that it should be possible to develop a

PDE5 isoform selective inhibitor that can target specific functions and pathological conditions with a reduced likelihood of causing unwanted side effects.

Despite results strongly implicating the role of decreased PKG and cGMP in colorectal cancer, knowledge of the types and expression levels of protein kinases and cyclic nucleotide phosphodiesterases is critical when considering therapeutic pathways. Unless selectivity for cGMP versus cAMP is extremely high, there is typically some interaction of cGMP with sites that prefer cAMP and interaction of cAMP with sites that prefer cGMP [112] [131]. Cyclic nucleotide analogs have been useful in investigating the effects of PKG, but the relative concentrations of cyclic GMP required to elicit different cellular responses are currently unknown. Investigations have been aided by the fact that some of the analogs that have been studied are substrates for phosphodiesterases while other analogs are competitive inhibitors [132]. Comparing the structure activity relationships of these compounds will guide future attempts to develop compounds that affect cyclic nucleotide signaling.

Sulindac as a Colon Cancer Chemopreventive Drug

Sulindac is a prodrug that is reversibly reduced to the sulfide derivative in the liver or colon, or irreversibly oxidized to sulindac sulfone (exisulind) within the liver [133-134]. Sulindac sulfide is a nonselective inhibitor of COX-1 and COX-2, whereas the sulfone derivative lacks COX inhibitory activity but has chemopreventative properties independent of prostaglandins (PGs) as previously mentioned. Sulindac sulfone is an inhibitor of the cGMP phosphodiesterase PDE5, elevating intracellular levels of cGMP and inducing protein kinase G [40]. Treatment of SW480 cells (a colon cancer cell line

with mutated *APC* and elevated β-catenin) with sulindac sulfone induces apoptosis and significantly reduces β-catenin and cyclin D1 levels in a dose-dependent manner. Similar studies have consistently reinforced the evidence that exisulind and its metabolites are very effective at inducing apoptosis and reducing proliferation rates of colorectal cancer [69, 135].

Clinical studies suggest that therapeutic use of sulindac is effective in patients with familial adenomatous polyposis (FAP) [136]. Labayle et al. reported that, in a randomized, placebo-controlled, double-blind crossover study in patients with FAP, administration of sulindac at a dose of 300 mg/d for 6–12 mo caused disappearance of all colonic polyps [137]. In another study, the incidence and size of adenomas were reduced in FAP patients after long-term therapy with sulindac[138]. Although the dosage of sulindac administered in these studies varied from 150 to 400 mg/d, most of the patients treated with this drug exhibited full remission. Several studies involving sulindac found that it could inhibit the growth of several cancer types including prostate [139], lung [140], mammary [141], bladder [142],and colon [33, 143]. As previously mentioned, sulindac has the ability to increase intracellular levels of cyclic GMP [144] and reduce β-catenin mediated transcription [135] [145]. It has also previously been shown that sulindac treatment can enhance the killing of cancer cells by generating intracellular ROS[146] and can inhibit the activity of glutathione-S-transferases [147]. These observations are not inconsistent with studies reporting sulindac has the ability to inhibit the proliferation and induce apoptosis through several mechanisms of action including activation of PPARδ [38], inhibition of Ras signaling [37], induction of the N1-acetyltransferase gene [148], and inhibition of NF-κB [149].

CHAPTER 2

NSAIDS AND MULTIDRUG RESISTANCE

Exposure to environmental toxins has made it necessary for cells to develop mechanisms to avoid their damaging effects. Tumor cells use the same mechanisms to resist the cytotoxic effects of chemotherapeutics. Several mechanisms have been proposed to account for acquired drug resistance; they include increased rate of drug detoxification [1], overexpression of anti-apoptotic bcl-2 [2], and increased removal of drugs from the cell [3]. One of the most important causes of acquired drug resistance to chemotherapeutic agents is increased expression of multidrug transporter genes [4]. Increased expression of the transporters encoded by these genes presents a significant obstacle to cancer chemotherapy. Therefore, the identification of specific, selective inhibitors for drug transporters may help improve outcomes for patients with cancers that display multidrug resistance.

Two genes belonging to the ATP-binding cassette (ABC) transporter family appear to account for nearly all of the MDR in tumor cells. The first transporter to be associated with MDR was P-glycoprotein (P-gp) [5], encoded by the *ABCB1* gene. It functions as an important cellular defense mechanism found in normal tissue, but it is over-expressed in a wide variety of human tumors where it contributes to resistance to several groups of chemotherapeutic agents [6]. Encoded by the *ABCC1* gene and first identified in 1992 [7], the multidrug resistance transporter-1(MRP-1) has been shown to contribute to multidrug resistance independently of P-gp. Cells that express MRP-1 have

resistance to several anticancer drugs including vincristine, doxorubicin, and etoposide [8]. Although P-gp (ABCB1) and MRP-1 (ABCC1) are able to transport many of the same substrates, ABCC1 shows preferential transport of hydrophobic, anionic compounds such as glucoronide, glutathione, and sulfate conjugates [9-11]. Its preference for these substrates suggests the ABCC1 transporter may be a more approachable target for MDR reversal than ABCB1.

In 1998, a subset of NSAIDs, including sulindac sulfide (SS), emerged as promising candidates capable of increasing the cytotoxicity of chemotherapeutic drugs in multidrug resistance cells [15]. The MDR reversal effect of SS was found to be independent of cyclooxygenase inhibition and selective for those cells expressing the ABCC1 transporter. Although sulindac sulfide and members of several other drug classes are known ABCC1 inhibitors, there is no compound on the market that has given consistent results in efforts to treat drug resistant cancers. The development of clinically useful MDR inhibitors, which have primarily been tested only in ABCB1 over-expressing cells, has been limited by toxicity that occurs at the doses required [16] [17]. Therefore, the development of potent, highly specific inhibitors to ABCC1 could lead to the development of much more effective chemotherapy protocols.

ABCC1

An inhibitor of MRP-1(ABCC1) would have safety and efficacy advantages over inhibitors of P-gp(ABCB1). Recent studies have determined that single nucleotide polymorphisms (SNPs) in other MDR transporters can affect the chemoresistance of cancer cells[18-19] and this complicates attempts to find useful inhibitors. SNPs affecting

ABCC1 have not been found. The tissue distribution and cell membrane localization of ABCC1 indicate that a selective inhibitor of this transporter would have safety advantages over inhibitors of the other transporter. In contrast to ABCB1, ABCC1 is localized primarily to the basolateral membrane of polarized cells[20]. This distinction suggests that the pharmacokinetic (PK) profiles of drugs given in combination with an ABCC1 inhibitor will be different than the PK profile of those same drugs when given in combination with ABCB1 inhibitors [21-22]. Given the limitations and obstacles associated with ABCB1 inhibitors described above, the identification of selective ABCC1 inhibitors could be highly significant for the treatment of cancer.

The ability of NSAIDs to enhance chemotherapeutic drug toxicity was first observed with indomethacin in 1978 [23]. Several other NSAIDs have since been used in studies investigating the mechanism by which drug toxicity is increased in cancer cells[24]. In 1997, Draper et al. reported indomethacin was able to reverse ABCC1-mediated transport of doxorubicin [25], but it did not inhibit ABCB1-mediated transport. An extensive screen of NSAIDs with chemotherapeutic drugs was conducted in ABCC1 and ABCB1 overexpressing cells in 1998[15] and it revealed that sulindac sulfide (SS) was also an inhibitor of ABCC1-mediated transport. The ability of sulindac to reverse MDR was determined to be independent of cyclooxygenase inhibition because sulindac sulphone, the non-COX inhibiting metabolite of sulindac, had MDR reversal activity. Furthermore, the addition of exogenous PGE_2 was unable to abrogate the MDR reversal of SS. These results strongly suggest that sulindac can be used as a probe to investigate the mechanism by which ABCC1 cells can be sensitized to chemotherapeutic agents and as a control for determining the MDR reversal potential of other compounds.

Multidrug resistance screening

The aim of the multidrug resistance study was to identify novel ABCC1 inhibitors. Toward this goal, a high throughput screen of the NIH Small Molecule Repository of 85,200 compounds was conducted at Southern Research Molecular Libraries Screening Center using the H69AR small cell lung cancer cell line. The H69AR cells do not express P-glycoprotein (ABCB1), but do over-express ABCC1 and this renders the cells 32-fold more resistant to doxorubicin than the parental cell line[26]. From the primary screen and follow-up dose-response confirmation, 24 compounds were identified as active. The results revealed compounds from two different chemical scaffolds that were able to inhibit calcein-AM efflux and increase the sensitivity of H69AR cells to doxorubicin. A 5-quinolinone derivative decreased the IC_{50} of doxorubicin 25-fold while an imidazopyrimidine derivative caused a 16-fold increase in H69AR sensitivity to doxorubicin. By themselves, these two compounds had IC_{50} values of 46 µmol/L and 41µmol/L respectively, indicating low cytotoxicity. These same compounds had no effect on doxorubicin sensitivity in the ABCB1 over-expressing cell line, MES-SA/Dx5. Analysis of the hydrophobicity of the compounds found a correlation with the ability to reverse ABCC1-mediated multidrug resistance, consistent with the characteristics of known substrates of ABCC1. These results can be used to identify future candidates for multidrug resistance research, possibly leading to the development of clinically useful ABCC1 inhibitors.

Methods

Initial compound screening

The primary screen was conducted using the NIH MLSCN Small Molecule Repository of 85,200 compounds and was overseen by Lynn Rasmussen at the High Throughput Screening Center at Southern Research Institute. Multidrug resistant, ABCC1 over-expressing H69/AR cells were seeded at a density of 5,000 cells per/well in 96-well microtiter plates and incubated overnight at 37°C, 5% CO_2. Cells were treated with either 10 µmol/L of test compounds in the presence of 1 µmol/L doxorubicin or 10 µmol/L of test compounds without doxorubicin and incubated for an additional 72 h. At the end of the incubation, cell viability was measured using the Cell Titer Glo assay as previously described. Activity scores were assigned to compounds by subtracting the cell viability in the presence of doxorubicin from cell viability in the absence of doxorubicin (test compound alone). Compounds that reduced viability to 40% or less of controls in the presence of doxorubicin, while being non-cytotoxic themselves (≥ 75% cell viability), were considered active and warranted further testing.

Cytotoxicity assay

The parental small cell lung cancer cell line H69, the multidrug resistant variant H69/AR and MES-SA/Dx5 cells were obtained from ATCC and grown in RPMI-1640 supplemented with 4.5 g/L glucose + 4 mM glutamine, 10% fetal bovine albumin(FBS), incubated at 37ºC in 5% CO_2 and maintained at subconfluent density. For experiments, cells were seeded in tissue culture microtiter, clear bottom 96-well plates at a density of 5,000 cells/well and incubated overnight prior to treatment. Cells were treated with a 2-

log concentration range of test compounds and incubated for an additional 72 h. Viable cell number measurements using the Cell Titer Glo luminescence assay were performed according to the manufacturer's specifications using a Perkin Elmer Victor3V multi-label microplate reader. Dose response curves were generated from the luminescence measurements using the GraphPad Prism biostatistics and scientific graphing software.

Doxorubicin sensitivity assay

H69/AR or MES-SA/Dx5 cells were seeded at a density of 5,000 cells per well and incubated with test compounds at concentrations equal to their IC_{10} values for 4 h. After the incubation period, cells were treated with 3x serial dilutions of doxorubicin and incubated for a total of 72 h. At the end of the treatment period, assay plates were removed from the incubator and equilibrated to room temperature. Cell viability was measured as previously described with the Cell Titer Glo assay system.

Physicochemical descriptor calculations

The calculation of physicochemical descriptors for each test compound was performed to establish structure-activity relationships. Each of the test compounds was analyzed using the ACD/ChemSketch physicochemical properties prediction software. The ACD/ChemSketch log *P* algorithm, based on a dataset of over 18,000 log *P* measurements, was used to calculate the log *P* values for each of the test compounds. Topological polar surface area was performed using the Molinspiration™ interactive (polar surface area) PSA calculator[27].

Calcein-AM confocal imaging assay

The calcein-AM efflux assay was performed by Adam Keeton in the High Content Screening core facility at Southern Research Institute. H69/AR cells were plated in cover glass bottom 96-well plates and allowed to acclimatize overnight. On the assay day, cells were incubated for 4 h with a dilution series of test compounds followed by a 20 min incubation with 0.1 µmol/L calcein-AM, an MRP-1 substrate[28]. Sulindac sulfide was used as a positive control based on the previously published report that sulindac sulfide inhibits ABCC1-mediated efflux [15]. At the end of the incubation period, cells were washed with PBS to remove extracellular calcein-AM. Cellular fluorescence was measured using an Evotec Opera™ confocal cell imaging microscope. Fluorescent intensity was normalized to untreated cells and analyzed using GraphPad Prism™ scientific graphing software.

Drug combination assay and dose-effect calculations

The antagonistic, additive or synergistic effects of doxorubicin and test compound combinations were tested using the Chou-Talalay method of drug combination[29]. H69/AR cells were seeded in flat-bottom 96-well microtiter plates at a density of 5,000 cells per well and incubated overnight. Eight 2x serial dilutions of doxorubicin and test compound were added at a constant ratio of their individual IC_{50} values and cells were incubated for 72 h at 37 °C, 5% CO_2. Cell viability was measured by the Cell Titer Glo luminescence assay per the manufacturer's suggested protocol. The cell viability data was analyzed using the CalcuSyn™ software program from Biosoft (Cambridge, UK). This program allows calculation of a combination index(CI) from eq. 1(**Figure 1**), which was

derived from the median-effect equation (eq. 2) by Chou and Talalay in 1984[30]. Combination index values < 1, =1, and > 1 indicate synergism, additive effect, and antagonism, respectively.

$$\textbf{Eq. 1} \quad \mathrm{CI} = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \qquad\qquad \textbf{Eq. 2} \quad \frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^m$$

Figure 1. **Combination index equation.** The combination index (CI) equation (Eq.1) was derived from the median effect equation (Eq.2). D_1 is the concentration of drug 1 alone, D_2 is the concentration of drug 2 alone, x is the percent inhibition, f_a is the fraction affected by D, and f_u is the fraction unaffected. D_m is the IC_{50}, and m is the slope of the dose response curve.

Results

Primary screen/ Doxorubicin sensitivity

The initial screening found 31 compounds that met the criteria for activity. They could be clustered into four groups based on shared structural features. One group, containing a common triazole ring, was not chosen for further study. Structurally similar compounds have previously been identified as drug transporter substrates or antagonists[31], but can also be potent cell cycle inhibitors [32]. Therefore, the triazolopyrimidines identified in the initial screening are not an ideal probe for specifically investigating the structure activity relationship of ABCC1 inhibition using cell viability assays. Another group contained thiazolopyrimidine thiones, a class of compounds that have been previously identified as having inhibitory activity against CDC25B phosphatase [33] and anti-inflammatory activity [34]. Primarily because of the difficulty of synthesizing derivatives, further studies of thiazolopyrimidines in multidrug resistance were not pursued. However, two chemical scaffolds were chosen for analoging efforts based on the ease of chemically modifying the compounds and the availability of reagents. The largest group contained members that shared the 5-quinolinone scaffold

(**Figure 2A**) and the second group contained imidazo[1,2-*a*] pyrimidines (**Figure 2B**). These compounds presented novel scaffolds that were not previously associated with MDR reversal.

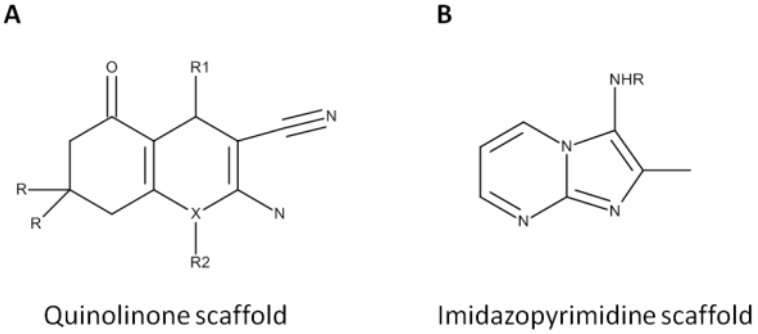


Figure 2. **Chemical scaffolds of 5-quinolinone and imidazo[1,2-a] pyrimidines.** Initial screening for inhibitors of ABCC1-mediated efflux identified compounds from the quinolinone (**A**) and imidazopyrimidine (**B**) classes possessed MDR reversal activity.

5-quinolinones and imidazopyrimidines selectively sensitize ABCC1 expressing cells to doxorubicin

The 5-quinolinone SID 4256579 and the imidazopyrimidine SID 14737887 were determined to be the most active compounds in the initial library screen. The 5-quinolinone SID 4256579 (**Figure 3A**) inhibited the growth of H69AR cells with an IC_{50} value of 45.7 µmol/L and the imidazopyrimidine SID 14737887 had an IC_{50} value of 41.0 µmol/L (**Figure 3B**). Half-maximal growth inhibition of H69AR cells occurred in the range of 5.2- 5.5 µmol/L doxorubicin, which is consistent with the previously published report of these cells being resistant to doxorubicin [26] (**Figure 3C**). In the presence of the IC_{10} concentration of the 5-quinolinone, this was reduced to 0.4 µmol/L. Similarly, treatment of H69AR cells with the IC_{10} concentration of the imidazopyrimidine reduced the IC_{50} of doxorubicin to 0.2 µmol/L (**Figure 3D**).

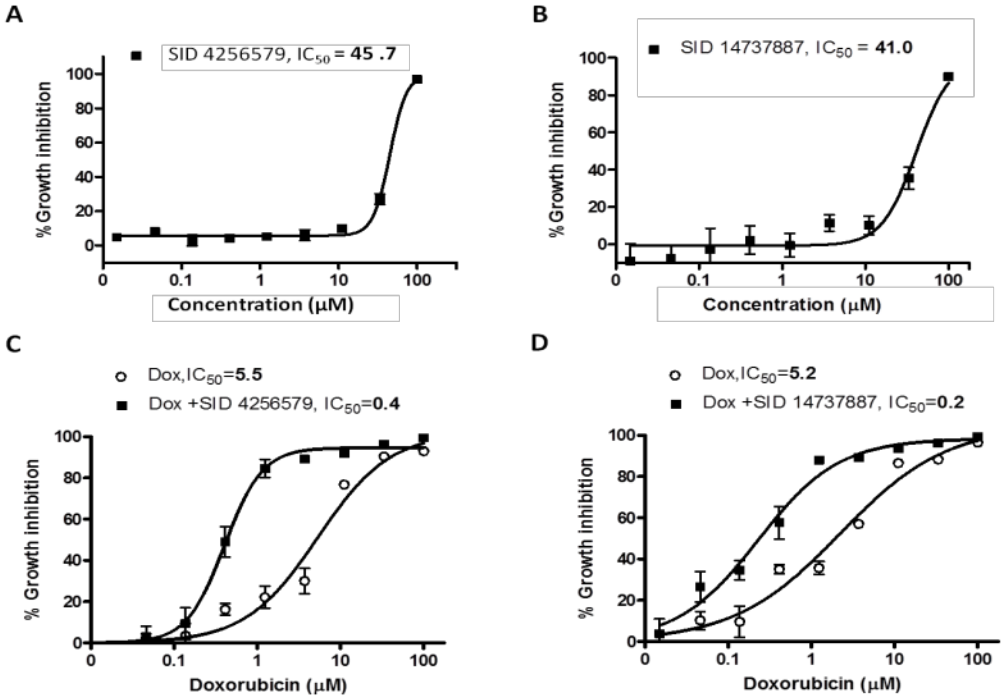


Figure 3. Cytotoxicity and doxorubicin sensitizing activity of 5-quinolinone and imidazopyrimidine compounds from initial compound screen. Half-maximal growth inhibition of H69AR cells by the most active quinolinone compound SID 4256579 (**A**) and the imidazopyrimidine SID 14737887 (**B**). Doxorubicin inhibits the growth of H69AR cells with an IC_{50} of 5.5 μmol/ L, but the addition of an IC_{10} concentration (15 μmol/L) of the quinolinone reduces the IC_{50} of doxorubicin to 0.4 μmol/L (**C**). The presence of an IC_{10} concentration (12 μmol/L) of the imidazopyrimidine reduces the IC_{50} of doxorubicin to 0.2 μmol/L (**D**).

The parental H69 cell line, which is known to express relatively little ABCC1 or ABCB1 [26, 35], showed only a twofold difference in sensitivity to doxorubicin when given in combination with the 5-quinolinone (**Figure 4A**) and no significant difference in the presence of the imidazopyrimidine (**Figure 4B**). Doxorubicin sensitivity of the ABCB1 expressing MES-SA/Dx5 cells was not appreciably increased by either the 5-quinolinone (**Figure 4C**) or the imidazopyrimidine (**Figure 4D**). These data strongly suggests the two compounds have selectivity for ABCC1 expressing cells.

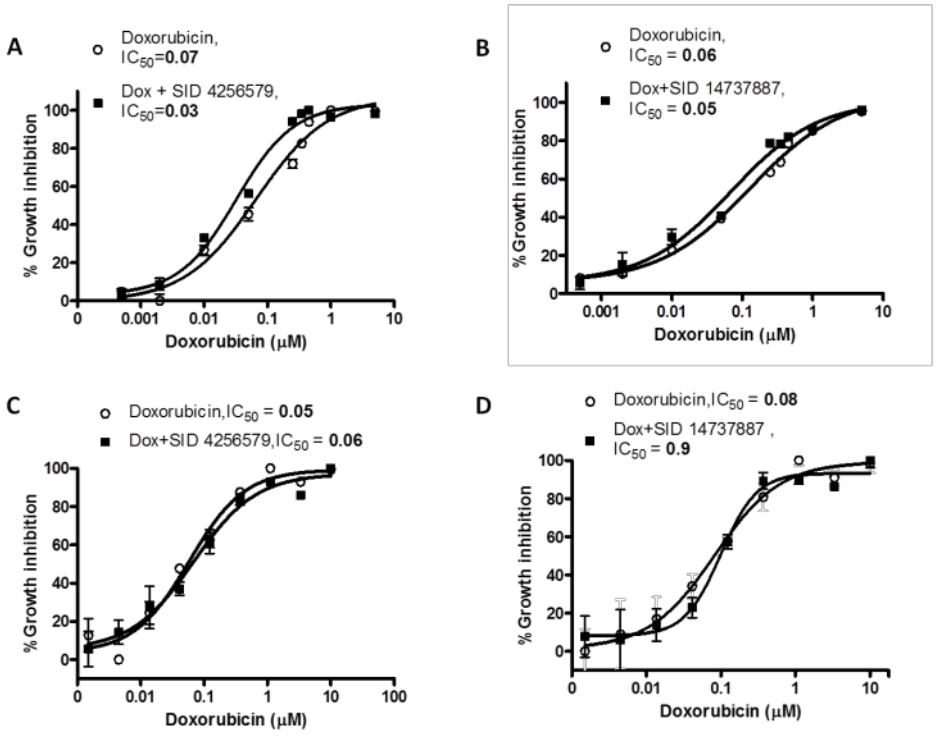


Figure 4. Cytotoxicity of 5-quinolinone and imidazo[1,2-*a*]pyrimidine compounds in H69 parental cells and ABCB1 overexpressing MES-SA/Dx-5 cells. Half-maximal growth inhibition of H69 and MES-SA/Dx5 cells in the presence of a 4-log concentration range of doxorubicin and a sub-toxic concentration of SID 4256579 or SID 14737887. The H69 parental cell line was treated with an IC_{10} concentration of SID 4256579 (**A**) or SID 14737887(**B**) and increasing concentrations of doxorubicin. ABCB1 expressing MES-SA/Dx5 cells were treated with SID 4256579 (**C**) or SID 14737887 (**D**) and increasing concentrations of doxorubicin.

Calcein-AM efflux is inhibited by 5-quinolinones and imidazopyrimidines

Cellular accumulation of the ABCC1 substrate calcein-AM was used as an indicator of ABCC1 inhibition. SID 4256579 and SID 14737887 increased cellular accumulation of calcein-AM in a dose dependent manner in H69/AR cells (**Figure 5**). Maximum accumulation was similar to that seen with the positive control, sulindac sulfide (SS). However, lower concentrations of SID 4256579 and SID 14737887 were required in comparison to SS. Half-maximal accumulation of calcein-AM occurred with

11.3 µmol/L of SS. By comparison, half- maximal accumulation of calcein-AM was achieved in the presence of the 5-quinolinone and imidazopyrimidine at 4.0 and 2.5 µmol/L, respectively. These results strongly suggest that these compounds are substrates or inhibitors of ABCC1, thereby interfering with the transport of calcein-AM from the cells. These results led to the synthesis of derivatives of both compound classes, which were then tested for MDR reversal activity in H69/AR cells.

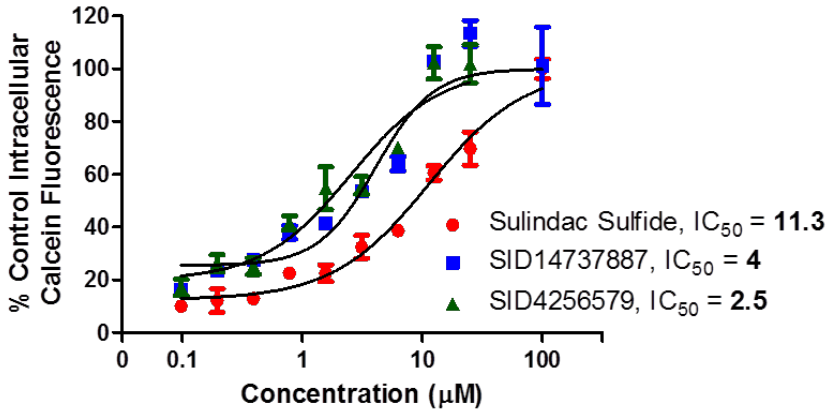


Figure 5. Comparison of calcein-AM accumulation in H69/AR cells. Intracellular fluorescence of the ABCC1 substrate, calcein-AM. Half-maximal fluorescence was measured in the presence of 11.3 µmol/L sulindac sulfide. Half-maximal calcein-AM fluorescence was detected in the presence of 4 µmol/L SID 14737887 and 2.5 µmol/L SID 4256579.

Structure-activity relationships of 5-quinolinone and imidazopyrimidines

Analogs of the 5-quinolinone and the imidazopyrimidine class were synthesized by the medicinal chemists at Southern Research Institute and tested for MDR reversal activity in H69/AR and ME-SA/Dx5 cells. The most active imidazopyrimidine increased doxorubicin sensitivity 16-fold (**Table 1**) and the most active 5-quinolinone increased sensitivity by 24-fold (**Table 2**). Selective potentiation of cytotoxicity by the 5-quinolinone SRI 22049, was also observed when used in combination with SN-38, an

active metabolite of irinotecan (**Figure 6**), indicating these compounds could be used for dosage reduction in chemotherapeutic regimens containing either doxorubicin, irinotecan and possibly other drugs. Physicochemical parameters of the compounds were measured for semi-quantitative structure activity analysis. Due to the limitations of the ACD/ChemSketch algorithm, there was considerable overlap of log *p* values. However, the most hydrophobic member of each class of compound correlated with the ability to reverse MDR. These results are consistent with the previously published report by Renes *et al*[36]. By contrast, there was no correlation with topological polar surface area. Pharmacophore modeling (**Figure 7**) by Judith Hobrath at Southern Research Institute found overlap of the ABCC1 hits with each other and with sulindac sulfide. A distance of 9.4 Â between hydrogen bond donor or acceptor sites and an overlap of the hydrophobic rings was observed. These results suggest that hydrophobicity (lipophilicity) is an important determinant of MDR reversal activity, but some rigidity of structure is necessary for optimal activity.

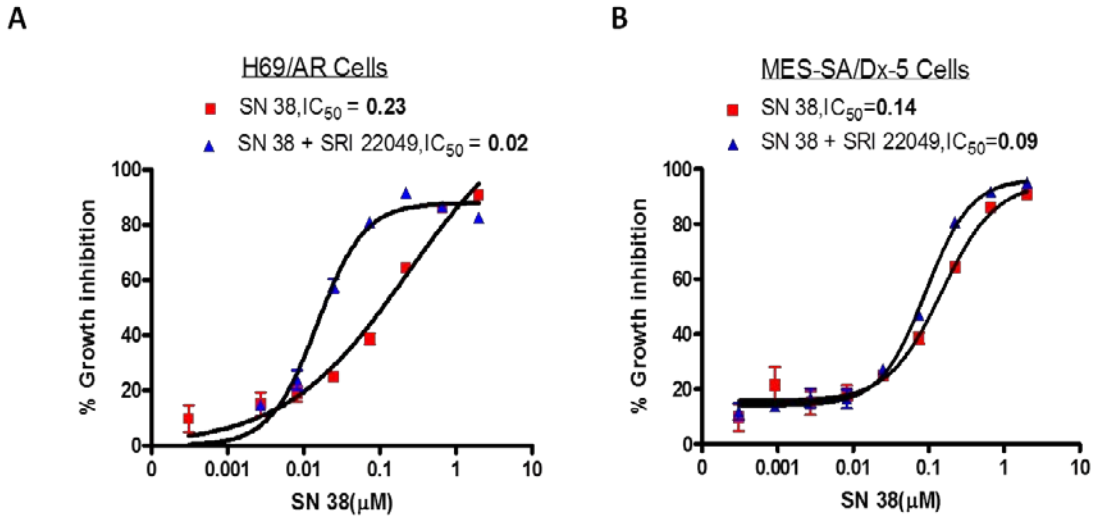


Figure 6. **Reversal of SN-38 drug resistance by SRI 22049**. (**A**) Sensitivity of H69/AR small cell lung cancer cells or (**B**) MES-SA/Dx-5 uterine sarcoma cells to SN-38 in the presence of an IC_{10} concentration of the 5-quinolinone SRI 22049.

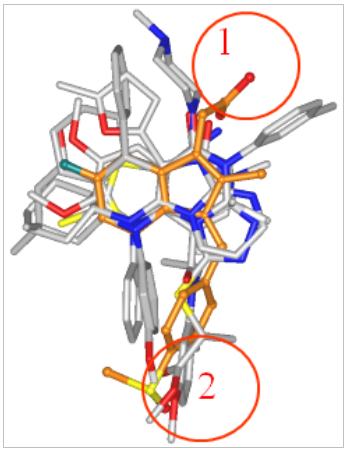
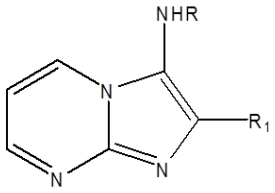


Figure 7. **Pharmacophore modeling.** Alignment of ABCC1 hits with sulindac sulfide (orange). Hydrogen bond/acceptor site 1 is 9.4 Â from site 2.

Imidazo[1,2-*a*]pyrimidine Probe Development for MRP1 Drug Resistance Reversal



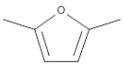
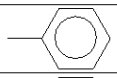
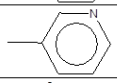
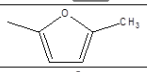
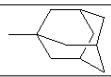
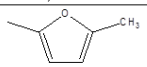
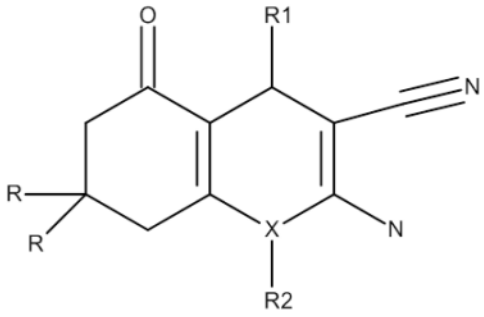
SRI #	R	R1	Dose Response (fold left shift)	Log *P*	Polar Surface Area (Å)
22159	$CH_2C_6H_5$		2.4	2.6±1.13	55.4
22155	$CH_2CH_2CH_2CH_3$		2.7	3.28±1.10	55.4
22158	$CH_2C_6H_5$		3.4	3.44±1.12	42.2
22160	C_6H_{11}		5.8	4.12±1.09	42.2
22162	C_6H_{11}		6.7	2.63±1.09	55.1
22154	C_6H_{11}		8.4	3.75±1.10	55.4
22161	C_6H_{11}		9.7	3.36±1.42	55.4
22156			16.0	4.18±1.42	55.4

Table 1. Structure, MDR reversal activity, and physicochemical descriptors of imidazo[1,2-*a*] pyrimidine compounds.

5-Quinolinone Probe Development for MRP1 Drug-Resistance Reversal

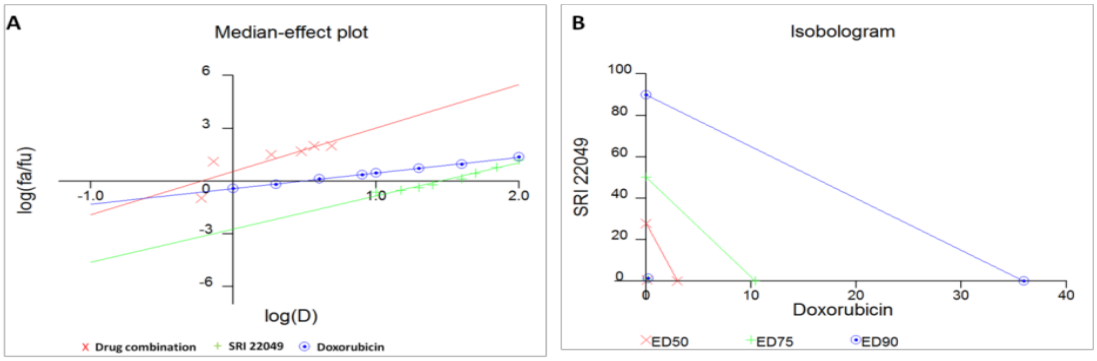


SRI #	X	R	R1	R2	Dose Response (fold left shift)	Log *P*	Polar Surface Area (Å)
22061	N	H	4-Pyridine	4-Methoxyphenyl	0	1.11±0.64	92.3
22006	O	H	Phenyl		0.9	1.35±0.50	76.1
22008	N	H	Phenyl	4-Methoxyphenyl	1.6	2.60±0.64	79.3
22054	N	H	2-Furan	4-Methoxyphenyl	2.1	1.76±0.65	92.5
22010	N	H	Phenyl	3,4-Dichlorophenyl	2.2	3.59±0.50	70.1
22009	N	H	Phenyl	4-Methylphenyl	2.3	2.98±0.50	70.1
22028	N	Methyl	3-pyridine	4-Methoxyphenyl	2.6	2.14±0.65	92.3
22007	O	Methyl	Phenyl		2.6	2.38±0.50	76.1
22062	N	H	4-Pyridine	4-Methoyphenyl	3.5	1.11±0.64	92.3
22053	N	H	3-Pyridine	4-Methoxyphenyl	3.8	1.11±0.64	92.3
22059	N	Methyl	2-Furan	4-Methoxyphenyl	3.8	2.80±0.66	92.5
22029	N	Methyl	3-Pyridine	3,5-Dimethoxyphenyl	4	1.77±1.11	101.5
22066	N	H	4-Pyridine	3,4-Dichlorophenyl	5	2.09±0.50	70.1
22011	N	Methyl	Phenyl	4-Methylphenyl	5.4	4.01±0.50	70.1
22013	N	Methyl	Phenyl	4-Methoxyphenyl	5.5	3.63±0.65	79.4
22012	N	H	Phenyl	3-Chlorophenyl	5.7	3.12±0.50	70.1
22057	N	Methyl	2-Furan	4-Methylphenyl	5.8	3.18±0.50	83.3
22056	N	H	2-Furan	4-Methylphenyl	6.5	2.14±0.50	83.3
22052	N	H	3-Pyridine	3-Chlorophenyl	8.5	1.62±0.50	83.0
22055	N	H	2-Furan	Phenyl	8.8	1.68±0.50	83.3
22051	N	H	3-Pyridine	Phenyl	9	1.03±0.50	83.0
22050	N	Methyl	3-Pyridine	3-Chlorophenyl	10.6	2.66±0.50	83.0
22031	N	Methyl	3-Pyridine	3,4-Dichlorophenyl	11.3	3.13±0.52	83.0
22063	N	Methyl	4-Pyridine	4-Methylphenyl	11.6	2.52±0.50	83.0
22064	N	Methyl	4-Pyridine	4-Methoxphenyl	11.8	2.14±0.65	92.2
22065	N	Methyl	4-Pyridine	Phenyl	11.9	2.06±0.50	83.0
22058	N	Methyl	2-Furan	Phenyl	12.3	2.72±0.50	83.3
22030	N	Methyl	Phenyl	3,4-Dichlorophenyl	15.1	4.02±0.51	70.1
22060	N	H	4-Pyridine	Phenyl	17	1.03±0.50	83.0
22032	N	Methyl	4-Pyridine	3,4-Dichlorophenyl	19.6	3.13±0.52	83.0
22049	N	Methyl	Phenyl	3-Chlorophenyl	24.7	4.15±0.50	70.1

Table 2. Structure, MDR reversal activity, and physicochemical descriptors of 5-quinolinone compounds.

Synergistic cell killing by SRI 22049/doxorubicin drug combination

The combination of doxorubicin and test compounds was tested in H69/AR cells to determine if the effect was additive, synergistic or antagonistic. A median effect plot and isobologram were generated from the dose response curves of each compound alone at the ED_{50}, ED_{75} and ED_{90} effect levels (**Figure 8A, B**). The ED_{50}, ED_{75} and ED_{90} combination index values for a 6:1 SRI 22049/doxorubicin drug combination were 0.05, 0.03 and 0.02, respectively (**Figure 8C**). By comparison, a sulindac sulfide/doxorubicin combination had CI values of 0.4, 0.7 and 1.2 at those equivalent doses (**Table 3**). These results show that the SS/doxorubicin combination becomes slightly antagonistic at higher effect levels whereas the SRI 22049/doxorubicin combination is synergistic at all effect levels.



C

Summary Table						
Drug	CI Values at					
	ED50	ED75	ED90	Dm	m	r
SRI 22049 (Not a combination)	N/A	N/A	N/A	27.9715	1.8833	0.98059
Doxorubicin (Not a combination)	N/A	N/A	N/A	3.01205	0.8859	0.99922
Drug Combination (6:1)	0.05429	0.03349	0.02294	0.59608	2.46164	0.83333

Figure 8. **SRI 22049/doxorubicin drug combination.** Median-effect (**A**) and isobologram plot (**B**) of SRI 22049 and doxorubicin drug combination. (**C**) Combination index value summary table.

Summary Table						
Drug	CI Values at					
	ED50	ED75	ED90	Dm	m	r
DOX (Not a combination)	N/A	N/A	N/A	5.35081	5.77835	0.91765
SULINDAC (Not a combination)	N/A	N/A	N/A	72.7614	26.97317	0.93164
Drug Combination (20:1)	0.4136	0.70675	1.21397	0.8957	1.73196	0.91387

Table 3. Combination index summary table for sulindac sulfide/doxorubicin drug combination.

Summary and Future Directions

Our synthesis and testing efforts have provided evidence that selective inhibitors of ABCC1 may be found in either the 5-quinolinone or imidazo[1,2-*a*]pyrimidine class of compounds. Chemical modifications to the structures resulted in a group of compounds that had significantly improved MDR reversal activity compared to the parent compounds. Dose dependent cellular accumulation of calcein-AM occurred in the presence of both compounds, but compared to sulindac sulfide these compounds inhibited calcein-AM efflux at lower concentrations. Calculation of the octanol-water coefficient (log *p*) and topological polar surface area of the compounds indicated that relatively hydrophobic members of these two classes of compounds have the highest MDR reversal activity. In addition, synergistic cell killing was found in the SRI 22049/doxorubicin drug combination assay, an appreciable improvement over what was seen with the SS/doxorubicin combination. To the best of our knowledge, these compounds represent the first identification of 5-quinolinone and imidazo[1,2-*a*] pyrimidines as ABCC1 inhibitors.

The successful development of drugs depends on getting the drug to the target and keeping it there long enough to have an effect. This is achieved by finding a balance

between the tendency to associate with lipids or with the aqueous environment, which affects the absorption, distribution, metabolism and excretion of the drug. As a general rule, a successful compound will follow Lipinski's rule of Five, which states that the molecular weight will be less than 500 daltons, there will be no more than 10 hydrogen bond acceptors or 5 hydrogen bond donors, and the log *p* should be less than 5 [37]. In addition to the molecular properties discussed by Lipinski, other properties have been discussed in regard to oral bioavailability. For example, Palm *et al*. have identified the negative impact of a polar surface area greater than 140 Â on intestinal absorption [38]. Based on Lipinski's rule and the topological polar surface area for the imidazo[1,2-*a*] pyrimidine SRI 22156 and the 5-quinolinone SRI 22049, these compounds would be predicted to be readily absorbed from the intestine. However, *in vivo* studies have not been performed to confirm this prediction.

Pharmacophore modeling conducted by Judith Hobrath at Southern Research Institute found overlap of ring structures of similar shape as well as overlap of hydrogen bonding groups with the known ABCC1 inhibitor sulindac sulfide. Molecular flexibility is considered a desirable quality for membrane permeation [39], but the overlap of hydrogen bonding groups indicates optimal ABCC1 inhibition could be hindered by flexibility in some parts of the compounds if that flexibility interferes with the alignment of hydrogen bonding groups of the molecule and the transporter. A comparison of the number of rotatable bonds, an indicator of membrane permeation, strongly suggests this is the case for 5-quinolinones and imidazopyrimidines. For example, the most active 5-quinolinone SRI 22049 only has two rotatable bonds. By comparison, the least active 5-quinolinone has three rotatable bonds. Similarly, the most active imidazo[1,2-*a*]

pyrimidine SRI 22156 has three rotatable bonds and the least active compound of the class has four rotatable bonds. There is obvious variation between this parameter and MDR reversion among the compounds, but these observations should help in the development of the next generation of ABCC1 inhibitors in regard to oral bioavailability and potency.

The observation of synergism between SRI 22049 and doxorubicin shows promise for the development of 5-quinolinones as ABCC1 inhibitors. The theoretical basis of the combination index allows the calculation of a dose reduction index (DRI) for doxorubicin [40]. Based on the experimental values, the doxorubicin concentration could be reduced by 30-fold at an effect level of 50% growth inhibition. These results are encouraging for the use of the 5-quinolinone class of compounds in chemotherapy treatment protocols, but the same cannot be said for the sulindac sulfide/ doxorubicin combination at growth inhibition levels above 90% as there appears to be slight antagonism at this level (**Table 3**). Since it is important to kill the population of cancer cells by 90% or greater, including SRI 22049 or a SRI 22049 derivative in a treatment regimen would be more relevant to therapy than sulindac sulfide.

The identification of 5-quinolinones and imidazo[1,2-*a*]pyrimidines as MDR reversal agents has provided chemical probes for the continued development of ABCC1 inhibitors. Although the *in vitro* experiments used so far indicate low cytotoxicity, a limited *in vivo* study indicated hypotension as a possible toxic effect of these compounds. If clinically useful ABCC1 inhibitors are to be developed, then properties such as potency and specificity will still need to be improved. However, these studies will provide a basis to more thoroughly explore the chemical space that includes these two classes of

compounds, the structural requirements of ABCC1 inhibition, and potentially the development of *in silico* predictive models for ABCC1 inhibition.

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CHAPTER 3

SULINDAC INCREASES THE SENSITIVITY OF ABCC1 OVER-EXPRESSING TUMOR CELLS TO DOXORUBICIN BY INHIBITING EFFLUX AND CAN CAUSE SELECTIVE KILLING BY GLUTATHIONE DEPLETION

by

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Format adapted and errata corrected for dissertation

Abstract

Purpose: The ATP-binding cassette (ABC) transporters such as ABCB1 (Pgp), ABCC1 (MRP1), and ABCG2 (BCRP) contribute to chemotherapy failure. Previous studies have shown that some nonsteroidal anti-inflammatory drugs (NSAIDs) can increase the sensitivity of ABCC1 expressing cells to various chemotherapeutic drugs, although the mechanism and interaction with other transporters is not well understood. The purpose of this study was to investigate whether the NSAIDs sulindac sulfide (SS) and indomethacin can increase the cytotoxicity of chemotherapeutic agents in ABCC1, ABCB1 and ABCG2 expressing cells and provide a mechanism by which the NSAIDs may sensitize cells to commonly used chemotherapeutic drugs.

Experimental Design: The cytotoxicity of Dox in ABCC expressing Jurkat cells, the multidrug resistant non-small lung cancer cell line H69AR, and the parental H69 cell line was measured in the presence or absence of subtoxic concentrations of either SS or indomethacin. Similarly, the effect of SS or indomethacin on Dox cytotoxicity was measured in ABCB1 expressing uterine sarcoma MES-SA/DX5 cells. The ability of SS to increase growth inhibition in the presence of vincristine or daunarubicin was measured in cell lines expressing either ABCC1, ABCB1, or ABCG2 and inhibition of transporter efflux was assessed using inside-out membrane vesicle transport of ^{3}H-LTC$_4$, flow cytometric analysis, and imaging assays. Increases in Dox sensitivity in the presence of SS were also measured in NCI-60 tumor cell lines. Imaging assays and the measurement of intracellular glutathione (GSH), which is conjugated or co-transported with the majority of ABCC1 substrates, was used to characterize the mechanism by which SS elicited a response.

Results: SS and indomethacin increase the cytotoxicity of doxorubicin (Dox) in ABCC1 expressing human H69AR lung tumor cells, but do not affect the sensitivity of cancer cells expressing ABCB1 or ABCG2. The effective concentration range of both NSAIDs was clinically achievable, while higher concentrations were cytotoxic but did not enhance sensitivity to Dox. The mechanism involved decreased efflux of Dox since SS increased intracellular levels of Dox and fluorescent substrates in ABCC1 expressing cells, but not in ABCB1 or ABCG2 expressing cells. SS also inhibited the uptake of leukotriene C_4, an ABCC1 specific substrate, into inside-out membrane vesicles. Consistent with previous studies indicating the co-transport or conjugation of glutathione (GSH) with ABCC1 substrates, SS decreased intracellular GSH levels in ABCC1 expressing cells. Moreover, the glutathione synthesis inhibitor, L-buthionine sulfoximine enhanced GSH depletion by SS and selectively increased the sensitivity of ABCC1 expressing cells to SS-induced cytotoxicity.

Conclusion: SS, and to a lesser extent indomethacin, potently and selectively increased the sensitivity of ABCC1 expressing cells to Dox indicating that there may be possible benefits of combining sulindac with certain chemotherapeutic drugs in patients with ABCC1 expressing malignancies. These studies also suggest that ABCC1 expressing cells may be more susceptible to growth inhibition by compounds that increase oxidative stress.

Introduction

Multi-drug resistance (MDR) is a major clinical obstacle that limits the efficacy of many cancer chemotherapeutic drugs. Tumors that progress following chemotherapy often contain populations of cells that express the MDR phenotype, which can make

chemotherapy less effective against recurrent tumors. An important factor that contributes to MDR is the expression of certain ATP-dependent membrane transport proteins that cause the efflux of a number of cancer chemotherapeutic drugs, thereby reducing intracellular concentrations to limit their antiproliferative and pro-apoptotic activity [1]. The cancer chemotherapeutic drugs that are most frequently effected by increased expression of transport proteins include taxanes (paclitaxel, docetaxel), vinca alkaloids (vinorelbine, vincristine, and vinblastine), anthracyclines (doxorubicin, daunorubicin, epirubicin), epipodophyllotoxins (etoposide), camptothecins (irinothecan, topotecan), dactinomycin, and mitomycin C [2].

The two most well studied transport proteins that contribute to drug resistance are the permeability glycoprotein (P-gp or ABCB1) that was discovered in 1976 [3] and the multidrug resistance protein (MRP1 or ABCC1) that was discovered in 1992 [4]. These transporters belong to a larger family of proteins referred to as the ATP-binding cassette (ABC) family, of which there are currently 48 members. Functionally, all ABC proteins are ATPases and use energy from ATP hydrolysis to transport their substrates across cell membranes. ABCB1 is a 170 kD phospho-glycoprotein encoded by the *ABCB1* gene [5], while ABCC1 is a 190 kD polypeptide encoded by the *ABCC1* gene [4]. Although there is a relatively small degree of sequence homology between ABCB1 and the ABCC family [6], these proteins share the ability to transport a number of commonly used chemotherapeutic drugs such as the anthracyclines and vinca alkaloids [1]. In general, ABCB1 shows preferential binding to basic hydrophobic compounds, while ABCC1 transports mainly anionic hydrophobic compounds [7]. Additional ABC proteins may also be important to MDR, for example the recently characterized breast cancer

resistance protein (BCRP, ABCG2) [8], but less is known about their role in chemoresistance or substrate structural requirements.

The first generation of ABC transport inhibitors targeted ABCB1, but was non-selective and displayed low potency that led to disappointing clinical results due to unacceptable toxicity. A number of newer drugs have since been identified that inhibit ABCB1 with greater potency and selectivity, but also failed because these agents were found to alter the pharmacokinetic properties of many chemotherapeutic drugs [9]. This is generally attributed to the expression of ABCB1 in normal epithelial cells of the colon, kidney, and liver, which caused unpredictable effects on the absorption and excretion of many chemotherapeutic drugs, necessitating counterproductive dose reduction [10-11]. However, a potentially important difference between ABCB1 and ABCC1 is the role the former has in protecting normal tissues from xenobiotics. For example, ABCB1 is localized to the apical surface of normal epithelial cells of the colon, liver, and kidney and can influence the metabolism and elimination of chemotherapeutic drugs. In contrast, ABCC1 is usually localized to the basolateral surface of polarized cells except for brain capillary endothelial cells [12]. As such, it is possible that ABCC1 inhibitors may be less likely to interfere with the absorption and elimination of chemotherapeutic drugs to the same extent as ABCB1 inhibitors.

Recent reports have demonstrated the ability of certain nonsteroidal anti-inflammatory drugs (NSAIDs) to increase the sensitivity of ABCC1 overexpressing cells to chemotherapeutic drug substrates. For example, Duffy and colleagues performed an extensive series of *in vitro* experiments to evaluate the ability of various NSAIDs to increase the sensitivity of ABCC1 expressing tumor cell lines to chemotherapeutic drugs

[13]. These investigators concluded that the effect was independent of the cyclooxygenase-inhibitory activity of the NSAIDs, although the exact mechanism of action is not known. Interestingly, the effect was not observed in cell lines overexpressing ABCB1 but was only noted in lines that displayed ABCC1 overexpression, which suggests a direct inhibition of the pump and is consistent with selectivity of ABCC1 to transport anionic hydrophobic compounds such as NSAIDs [7, 14]. There is also *in vivo* evidence showing that the NSAID sulindac can increase the anticancer efficacy of epirubicin, a known ABCC1 substrate [15-16]. In addition, a recent clinical trial demonstrated that sulindac did not interfere with the absorption or excretion of epirubicin, which is consistent with the feasibility of inhibiting transport in ABCC1 expressing cells without interfering with pharmacokinetics [17]. As such, there may be advantages of combining NSAIDs with conventional chemotherapy to prevent tumor recurrence and the emergence of drug resistant tumor cells. Here we show that the sulfide metabolite of sulindac can potently and selectively enhance the sensitivity of ABCC1 expressing cells to chemotherapeutic drugs and investigate the mechanism and selectivity of this interaction.

Materials and Methods

Drugs and Reagents: SS, indomethacin and doxorubicin were purchased from Sigma-Aldrich (St. Louis, MO). ABCC1 antibody (QCRL-1, monoclonal) was purchased from Alexis Biochemicals (San Diego, CA). ABCB1 antibody was purchased from Covance (Princeton, New Jersey). Secondary antibodies were purchased from Cell Signaling Technology. All other reagents were purchased from Sigma-Aldrich unless otherwise stated.

***Cell Culture**:* Human NCI-H69 (H69), H69AR, MES-SA and MES-SA/DX5 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MES-SA and MES-SA/DX5 cells were grown in McCoy's medium containing 10% FBS under standard cell culture conditions. Jurkat and SupT1cells overexpressing either ABCC1 or ABCB1 were generated as previously described [18]. The human epidermoid KB carcinoma cells, KB-3-1, were propagated in Dulbecco's modified Eagle's medium with 10% FBS under 5% CO_2 at 37°C. The ABCC1-overexpressing cell line KB-CV60 were cloned from KB-3-1 cells and maintained in medium containing 1 µg/ml cepharanthine and 60 ng /ml vincristine. The NCI-60 panel and other tumor cell lines were seeded and incubated under conditions as previously established by the NCI Developmental Therapeutics Program [19-20].

Cytotoxicity Assays: For H69/H69AR and MES-SA/DX5 growth assays, the growth inhibitory activity of doxorubicin, SS, and indomethacin was determined by measurement of ATP levels, an indicator of viable cell number (Cell Titer Glo assay, Promega). For MDR reversal experiments, cells were seeded in tissue culture microtiter 96-well plates at a density of 5,000 cells/well and incubated 16 h prior to treatment. Cells were treated with 5 µM SS or 10 µM indomethacin for 4 h prior to treatment with doxorubicin. Once dosing was complete, cells were incubated at 37^{o}C for 72 h for dose response experiments. Viable cell number measurements using the Cell Titer Glo luminescence assay were performed according to the manufacturer's specifications using a Perkin Elmer Victor3V multi-label microplate reader. For Jurkat cells expressing either ABCC1 or ABCB1, direct compound toxicity and reversal of chemoresistance was determined as previously described [18]. In brief, a 3-log dose range of either sulindac or

cyclosporine A was added to cells in the presence of 150 nM vincristine or daunorubicin. On day 7, cell viability was determined using a hemacytometer and trypan blue staining. Dose response curves of cells treated with SS or cyclosporine A with or without the chemotherapeutic agent present were compared using GraphPad Prism software.

Glutathione Assay: Cells were plated at a density of 2,500 cells per well in 96-well plates and incubated overnight at 37^{o}C and 5% $CO_{2.}$ Cells were incubated 18 h in the presence of drug or drug combinations. At the end of the incubation period, glutathione levels were measured using the GSH-Glo kit (Promega) according to the manufacturer's instructions.

LTC_4 Transport Assay: Membrane vesicles (20 µg) were prepared from KB-3-1 and KB-CV60 cells as described previously [21]**.** For inhibition experiments, the standard incubation medium contained membrane vesicles (25 mg of protein), 137 nM ^{3}H-LTC_4, 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM ATP, 10 mM phosphocreatine and 100 mg/ml creatine phosphokinase with or without unlabeled LTC_4 in a final volume of 50 µl. Reactions were carried out at 37°C and stopped with 3 ml of ice-cold stop solution containing 0.25 M sucrose, 100 mM NaCl, and 10 mM Tris-HCl (pH 7.4). Samples were passed through 0.22-µm Dura pore membrane filters (Millipore, Bedford, MA) under vacuum. The filters were washed three times with 3 ml of ice-cold stop solution and dried at room temperature for 30 min. Incorporated radioactivity was measured by the use of liquid scintillation counter. In control experiments, ATP was replaced by an equal concentration of 5'-AMP. Rates of net ATP-dependent transport were determined by subtracting the values obtained in the presence of 4 mM AMP from those obtained in the presence of 4 mM ATP.

Immunoblotting: H69AR and MES-SA/DX5 cells were lysed using SDS lysis buffer containing 1% SDS, 10mM Tris pH 7.5, 7.5ug/ml aprotonin, 5mM benzamidine, 5mM PMSF, 50mM NaF and 1.25mM $NaVaO_4$. Whole cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 3% BSA, membranes were incubated overnight at 4°C with antibodies directed against ABCC1 or ABCB1 and subsequently with anti-mouse secondary antibody conjugated with horseradish peroxidase. β-actin antibody (Cell Signaling Technology, Danvers, MA) was used as a protein loading control. The SuperSignal West Pico Substrate kit (ThermoScientific, Waltham,MA) was used for enhanced chemiluminescence detection.

Doxorubicin/Calcein-AM Confocal Imaging Assay: H69AR cells were plated in coverglass bottom 96-well plates and allowed to acclimatize overnight. Cells were then treated overnight with MRP-1 antagonists in phenol free complete growth medium. On the assay day, cells were incubated for 2h with 10 µM Doxorubicin or 30 min with 0.1 µM Calcein-AM and 2 µM Draq5 nuclear stain. At the end of the loading period, media was aspirated and replaced with phenol free medium plus SS. Plates were immediately analyzed by high speed confocal microscopy using the Evotec Opera with a 20X water immersion objective lens. Mean intracellular intensity of doxorubicin or calcein-AM was determined using the Acapella image analysis software.

Laser Scanning Cytometry Assay: H69AR cells were plated in covered glass bottom 96-well plates and allowed to acclimatize overnight. On the assay day, cells were incubated for 3.67 h with a dilution series of SS followed by incubation for 20 min with 100 nM calcein-AM. At the end of the loading period, free calcein-AM was washed away

with PBS. Cellular fluorescence was analyzed using a Blueshift Isocyte laser scanning cytometer.

Transporter Activity Assay: Cells expressing ABCB1 (JurkatDNR), ABCC1 (SupT1-Vincristine), or ABCG2(IgMxp3) were generated as previously described [22-23]. SS was added to cell suspensions to a final concentration of 50 μM and incubated for 10 min at room temperature. Calcein-AM (250 nM) was then added and incubated for an additional 15 min at room temperature. The fluorescent signal of the cells was evaluated in the HyperCyt flow cytometry system (Intellicyt, Albuquerque, NM) as previously described [22].

Results

Characterization of tumor cell lines expressing ABCC1 and ABCB1

Initial experiments were performed to compare the expression of ABCC1 and ABCB1 in the human H69AR small cell lung and MES-SA/DX5 uterine sarcoma tumor cell lines, which were previously reported to express ABCC1 [24] and ABCB1 [25-26], respectively. As determined by Western blotting, ABCC1 was not detected in the parental cell line H69 or in MES-SA/Dx5 cells, but was highly expressed in H69AR cells (**Figure 1A**). By contrast, ABCB1 was highly abundant in MES-SA/Dx5 cells compared with the parental MES-SA cells, but was minimally detectable in H69AR cells (**Figure 1B**). Experiments were next performed to determine the potency of Dox to inhibit the growth of both pairs of sensitive and resistant cell lines. As shown in Figure 1C, Dox inhibited growth of H69 and H69AR cells with an apparent IC_{50} of 0.12 and 4.0 μM, respectively, which reflects a 33-fold difference in sensitivity. Similarly, MES-SA cells were

approximately 17 times more sensitive to Dox compared with MES-SA/Dx5 cells with IC_{50} values of 0.21 µM and 3.55 µM, respectively. These results confirm the nature and extent of MDR in our cell models.

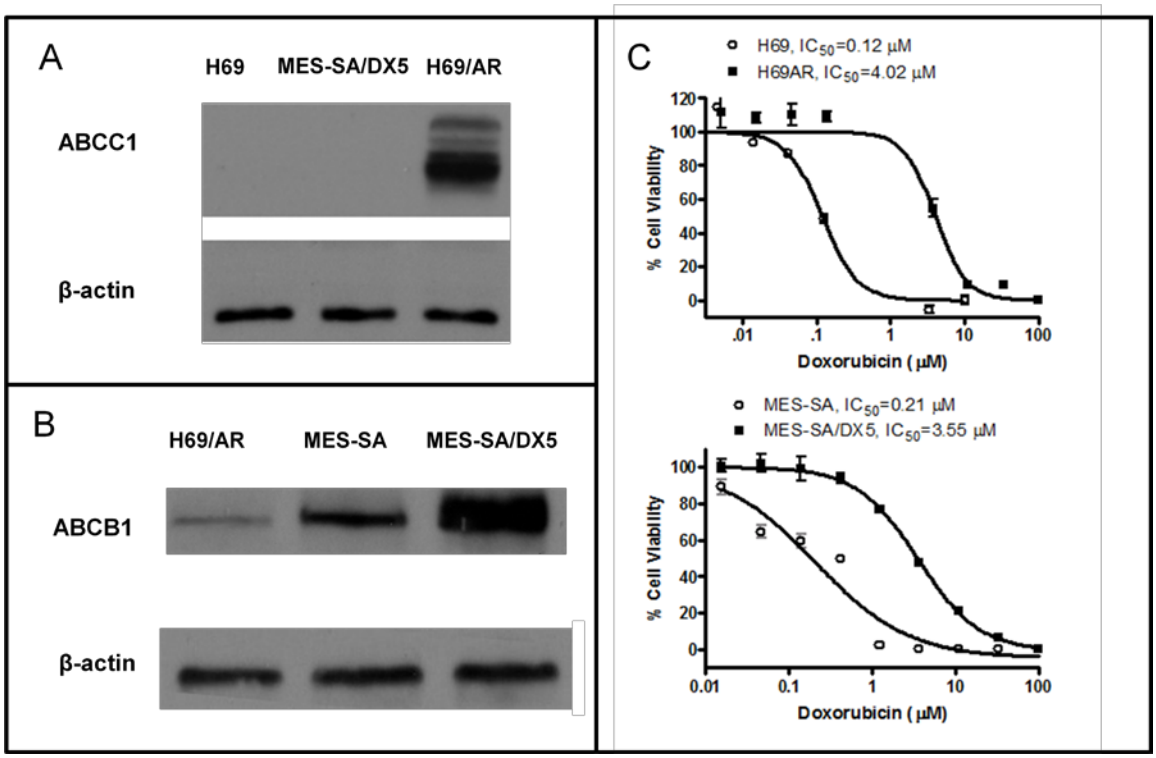

Figure 1. Characterization of MDR cell models. (A) Whole cell lysates of parental H69 cells compared to drug resistant H69AR and MES-SA/DX5 cells. **(B)** Lysates of H69AR, the uterine sarcoma cell line MES-SA and the drug resistant variant MES-SA/DX5 showing relative levels of ABCB1/P-glycoprotein expression. **(C)** MRP1 expressing(H69AR) and P-gp expressing (MES-SA/DX5) cells were grown in increasing concentrations of doxorubicin to determine drug resistance compared to their parental lines, H69 and MES-SA respectively.

SS and indomethacin increase sensitivity to Dox in ABCC1 overexpressing cells

ABCC1 overexpressing H69AR cells and the H69 parental line were treated with SS or indomethacin to determine their sensitivity to growth inhibition in the presence of either compound alone (**Figure 2A**). Subtoxic concentrations of Dox at 25 and 500 nM that correspond to their approximate IC_{20} value to inhibit the growth of H69 and H69AR

cells, respectively, were selected to distinguish between additive toxicity and ABCC1 inhibition. Both cell lines were pre-treated for 4 hours with SS or indomethacin over a concentration curve ranging from 1 - 500 µM prior to the addition of Dox. Both NSAIDs significantly increased the sensitivity of drug resistant H69AR cells to Dox (**Figure 2B**), but did not affect the sensitivity of parental H69 cells to Dox (**Figure 2B**). SS increased cytotoxicity of 500 nM Dox by 30-40% within a concentration range of 2-32 µM, while indomethacin increased cytotoxicity by 20-30% within the same concentration range. Compared to the cytotoxicity of the NSAIDs in the absence of Dox, the effective concentration range of SS and indomethacin was significantly less than the concentration range that resulted in cytotoxicity in the absence of Dox, which suggests that the mechanism is unrelated to their known tumor cell growth inhibitory activity [27-28]. In the case of SS, the effect was within the concentration range required for inhibiting COX-1 and COX-2 which can be achieved clinically with standard dosages of sulindac (**Figure 2D**) [29].

To further quantify the reversal of resistance by SS and indomethacin, we selected a single subtoxic concentration of SS and indomethacin that caused reversal and varied the concentration of Dox. Both drugs decreased the IC_{50} value of Dox in H69AR cells by approximately 18-fold (**Figure 3A**). By contrast, neither drug significantly affected the IC_{50} value of Dox in H69 cells (**Figure 3B**). SS did not significantly enhance the sensitivity of the ABCB1 expressing MES-SA/Dx5 cells to Dox (**Figure 3B**), while a known inhibitor of ABCB1, cyclosporine A, decreased the IC_{50} value from 2.2 µM to 0.1 µM.

To confirm that the effects of SS were specific for ABCC1 and not an unknown cellular change caused by selection of MDR variants, we utilized Jurkat cells that expressed either ABCC1 or ABCB1 by treatment with incrementally increasing doses of daunarubicin or vincristine [18, 22-23]. Both ABCC1 and ABCB1 overexpressing cells were grown in increasing concentrations of SS with a subtoxic dose of vincristine (150 nM) to determine the ability of the NSAID to enhance sensitivity. SS at or above 1 μM in the growth media

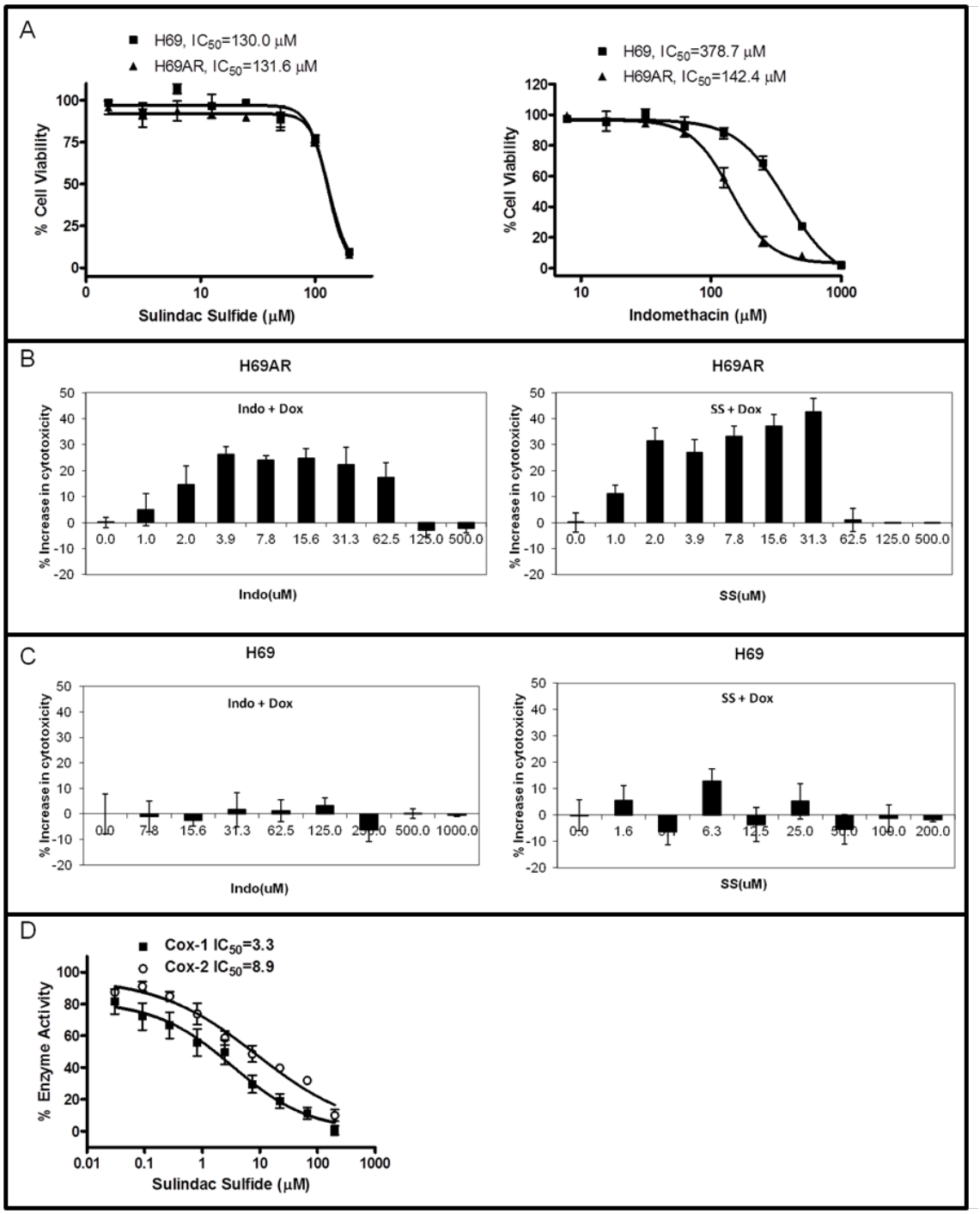

Figure 2. NSAID Cytotoxicity, drug sensitization, and COX enzyme inhibition. (A) The IC_{50} values for growth inhibition of H69AR and parental H69 cells in the presence of either SS or indomethacin. **(B)** H69AR and **(C)** H69 cells were treated with increasing concentrations of SS or indomethacin before addition of subtoxic concentrations of Dox (500 nM and 25 nM, respectively) to determine the ability of the NSAIDs to enhance sensitivity. Data is expressed as the difference between cytotoxicity of NSAID alone versus NSAID + Dox IC_{20} (Single factor ANOVA, P=<0.05). (**D**) COX-1 and COX-2 inhibition by sulindac sulfide.

reversed the ABCC1 mediated vincristine resistance (**Fig. 3C**). By contrast, SS provided no significant enhancement of vincristine cytotoxicity in Jurkat cells expressing ABCB1(**Figure 3D**). The cells were also cultured in SS alone to demonstrate that the return to chemosensitivity was not due to the toxicity of sulindac. As demonstrated with MES-SA/DX5 cells, a known inhibitor of ABCB1, cyclosporine A, reversed the chemoresistance to vincristine in ABCB1 overexpressing cells (**Figure 3E**).

To determine the prevalence of this sensitizing effect, we performed similar Dox dose-response studies with or without SS in a large panel of human tumor cell types (Table 1). Of the 47 cell lines evaluated, 24 demonstrated a significant increase in sensitivity to Dox in the presence of 5 μM SS (no overlap in 95% confidence interval of the IC_{50} value). Changes in potency ranged from over 4-fold to less than 1-fold in tumors of the various histotypes. Strikingly, all of the melanoma cell lines tested demonstrated significant sensitization to Dox following SS treatment.

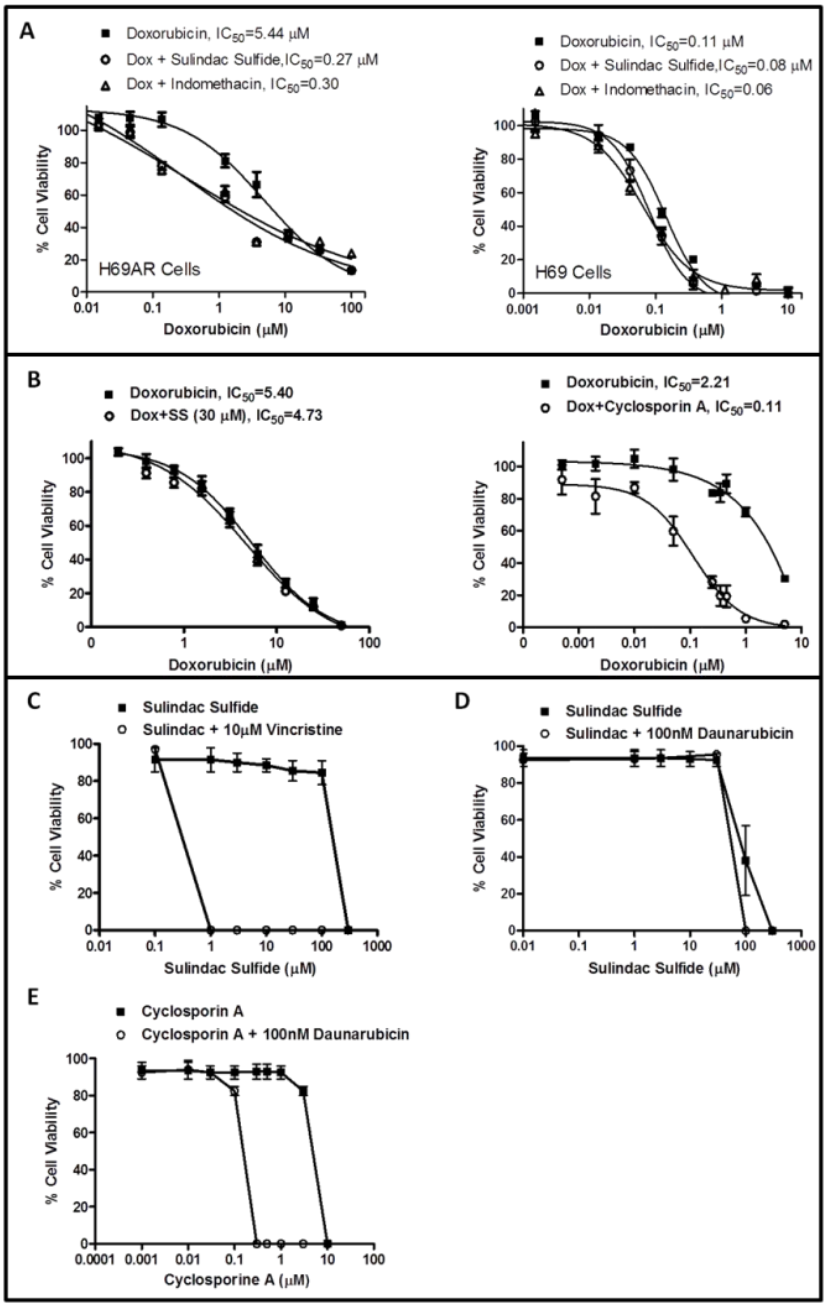

Figure 3. ABCC1 Selectivity: (**A**) H69AR and the parental H69 cells were treated with increasing concentrations of doxorubicin in the presence of the IC_{10} of either SS or indomethacin. The doses of NSAIDs used were 30 μM for sulindac sulfide and 45 μM for indomethacin. (**B**) The ABCB1 expressing uterine sarcoma cell line MES-SA/DX5 was treated with doxorubicin and either 10 μM SS or 30 μM cyclosporine A. (**C**) The ability of sulindac sulfide to sensitize cells to vincristine was tested in Jurkat cells expressing ABCC1. Cells were cultured in SS alone (squares) to show the toxicity of sulindac. The ability of sulindac sulfide **(D)** or cyclosporine A **(E)** to sensitize cells to daunarubicin in Jurkat cells expressing ABCB1. Columns, mean of triplicate determinations; bars, SD. *, $P < 0.05$; **, $P < 0.01$, versus the control group. ABCB1. Columns, mean of triplicate determinations; bars, SD. *, $P < 0.05$; **, $P < 0.01$, versus the control group.

Histotype	Cell Line	IC_{50} (Dox only)	95% CI	IC_{50} (+5 uM SS)	95% CI
Breast	T47D	0.080	0.002592 to 2.444	0.045	0.02520 to 0.08156
Breast	MCF-7	0.025	0.01486 to 0.04201	0.020	0.006678 to 0.05964
Breast	HS578T	0.421	0.3602 to 0.4911	0.217	0.1743 to 0.2693
Breast	MDA-MB-468	0.128	0.1129 to 0.1440	0.140	0.1057 to 0.1854
Breast	NCI-ADR-RES	6.337	1.326 to 30.29	5.884	0.2930 to 118.2
Breast	BT-549	0.201	0.1582 to 0.2548	0.112	0.09033 to 0.1383
CNS	U-251	0.081	0.06062 to 0.1073	0.040	0.02600 to 0.06072
CNS	SNB75	0.206	0.1789 to 0.2379	0.080	0.06390 to 0.09990
CNS	SF-295	0.097	0.06996 to 0.1333	0.043	0.02875 to 0.06375
CNS	SF-268	0.204	0.1348 to 0.3089	0.092	0.06428 to 0.1317
CNS	SNB-19	0.035	0.02804 to 0.04343	0.015	0.008425 to 0.02638
CNS	SF-539	0.142	0.1117 to 0.1810	0.091	0.06602 to 0.1264
Colon	SW620	0.085	0.06697 to 0.1083	0.048	0.03155 to 0.07170
Colon	COLO-205	0.031	0.009826 to 0.09668	0.022	0.009727 to 0.04938
Colon	HCC-2998	0.186	0.1321 to 0.2607	0.127	0.09792 to 0.1647
Colon	HCT-15	0.692	0.5059 to 0.9453	0.737	0.5131 to 1.058
Hematopoietic	K562	0.102	0.08417 to 0.1247	0.069	0.05797 to 0.08182
Hematopoietic	SR	0.003	0.002784 to 0.003638	0.002	0.0002999 to 0.009004
Hematopoietic	MOLT-4	0.005	0.004663 to 0.005014	0.004	0.001509 to 0.01221
Hematopoietic	CCRF-CEM	0.015	0.01192 to 0.01934	0.013	0.01235 to 0.01441
Lung	HOP92	0.108	0.08602 to 0.1362	0.038	0.02974 to 0.04819
Lung	NCI-H522	0.066	0.05279 to 0.08313	0.049	0.03466 to 0.06996
Lung	NCI-H460	0.041	0.02129 to 0.08052	0.016	0.004571 to 0.05433
Lung	NCI-H322M	0.592	0.4865 to 0.7210	0.202	0.1470 to 0.2786
Lung	A-549	0.065	0.03443 to 0.1212	0.029	0.01534 to 0.05355
Lung	EKVX	0.646	0.5773 to 0.7229	0.827	0.5662 to 1.209
Lung	NCI-H23	0.085	0.06888 to 0.1038	0.041	0.03191 to 0.05177
Melanoma	SKMEL5	0.150	0.1301 to 0.1736	0.087	0.07548 to 0.1003
Melanoma	MALME3M	0.220	0.1972 to 0.2454	0.096	0.08418 to 0.1085
Melanoma	LOX1-MV1	0.098	0.08694 to 0.1099	0.071	0.06090 to 0.08359
Melanoma	M-14	0.241	0.1992 to 0.2909	0.113	0.09592 to 0.1326
Melanoma	UACC-257	0.388	0.3462 to 0.4354	0.222	0.1903 to 0.2590
Melanoma	UACC-62	0.163	0.1473 to 0.1814	0.086	0.07628 to 0.09676
Melanoma	MDA-MB-435	0.378	0.3186 to 0.4494	0.135	0.1151 to 0.1582
Ovarian	IGR-OV1	0.073	0.04980 to 0.1071	0.031	0.01580 to 0.06229
Ovarian	OVCAR-3	0.299	0.03948 to 2.268	0.121	0.08684 to 0.1686
Ovarian	OVCAR-8	0.116	0.07669 to 0.1738	0.064	0.03984 to 0.1040
Ovarian	OVCAR-4	0.984	0.8053 to 1.203	0.439	0.2930 to 0.6574
Ovarian	OVCAR-5	0.542	0.3245 to 0.9039	0.134	0.08166 to 0.2200
Prostate	PC-3	0.429	0.3572 to 0.5159	0.162	0.1227 to 0.2128
Prostate	DU-145	0.077	0.06209 to 0.09448	0.029	0.02328 to 0.03729
Renal	TK10	0.183	0.1053 to 0.3169	0.093	0.06495 to 0.1319
Renal	786-0	0.086	0.06969 to 0.1071	0.046	0.03371 to 0.06326
Renal	RXF-393	0.345	0.2841 to 0.4190	0.187	0.1678 to 0.2081
Renal	U-031	0.592	0.4489 to 0.7819	0.393	0.3120 to 0.4943
Renal	A-498	0.116	0.08538 to 0.1562	0.064	0.04588 to 0.08837
Renal	ACHN	0.042	0.03147 to 0.05536	0.018	0.01253 to 0.02652

Table 1. Reversal of doxorubicin chemosensitivity in the NCI-60 tumor cell lines treated with 5 μM SS

SS inhibits ABCC1-mediated efflux

We next performed studies to determine the mechanism by which SS increases sensitivity to ABCC1 expressing tumor cells. Increased intracellular autofluorescence of Dox or the control substrate calcein was demonstrated in H69AR cells in the presence of SS by confocal microscopy (**Figure 4A**). Next, adherent cultures of H69AR cells were pretreated with a range of concentrations of SS, followed by a 30 minute incubation with the fluorogenic ABCC1 substrate, calcein AM. After cells were washed to remove free calcein AM, the fluorescence intensity of retained intracellular calcein was measured using a laser scanning fluorimeter (**Figure 4B**). These studies indicated that SS inhibited ABCC1-mediated efflux with an IC_{50} value of 5.6 μM. This is consistent with the concentration required to enhance sensitivity of H69AR cells to inhibition of growth by Dox.

Population analysis of transporter activity was next evaluated using fluorescent substrates for three different ABC transporters, calcein-AM for ABCC1 and JC1 for ABCB1 and ABCG2. The distribution of fluorescent intensity of ABCC1 overexpressing cells (SupT1-Vin) was significantly increased in the presence of SS with the mean cellular fluorescence (MCF) increasing from 234 ± 32.9 in untreated cells to 2445 ± 73.8 in the presence of SS. In contrast, SS treatment caused very little change in fluorescent intensity in cells overexpressing either ABCB1 or ABCG2 (**Figure 4C**).

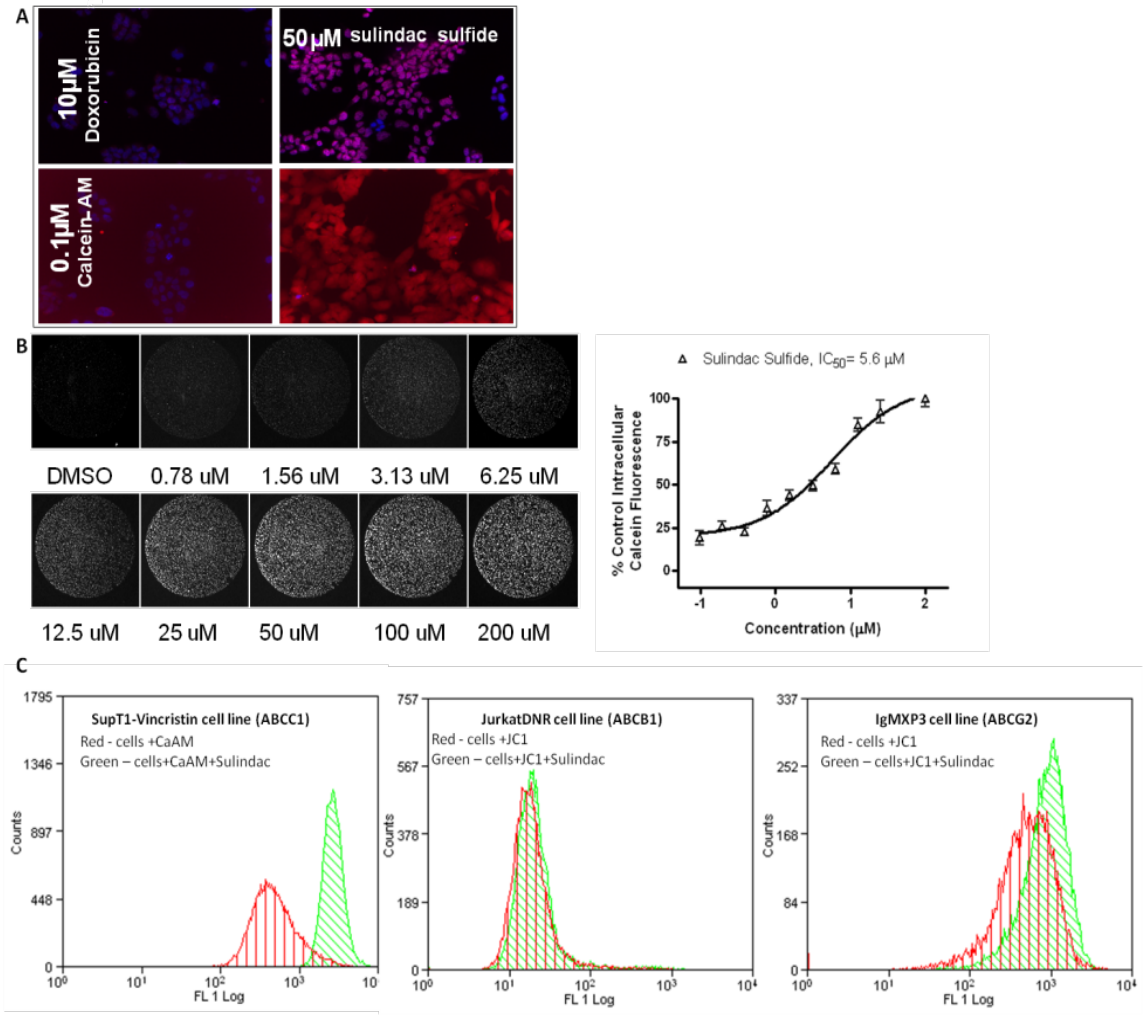

Figure 4. ABCC1 Efflux Inhibition: (A) Intracellular accumulation of calcein-AM and doxorubicin in H69AR cells pretreated with 50 μM sulindac sulfide measured by confocal laser microscopy. **(B)** Intracellular fluorescence in H69AR cells measured with increasing concentrations of sulindac sulfide fluorescent substrate measured by laser scanning cytometer. **(C)** Fluorescent intensity of calcein-AM (ABCC1 substrate) or JC1 (ABCB1, ABCG2 substrate) in the presence of sulindac (50uM).

Effect of SS on ABCC1 mediated transport of LTC_4

In order to determine if the observed cellular effects of SS occur directly on the ABCC1 protein, the transport of LTC_4 was measured. The leukotriene LTC_4 is a high affinity physiological substrate of ABCC1 [30]. Inside out membrane vesicles were isolated from the ABCC1 expressing clone KB-CV60. The transport of LTC_4 into the

vesicles was measured in the presence of SS or the pyridine analog PAK-104P, which has been previously shown to reverse both ABCC1 and ABCB1 mediated drug resistance [31]. SS inhibited [H]LTC_4 transport significantly at 10 μM and in a dose dependent manner at concentrations similar to those which reversed resistance to chemotherapeutics (**Figure 5A**). By contrast, the transport of LTC_4 into membrane vesicles from the parental line KB-3-1 was unaffected by SS. This suggests SS selectively inhibits ABCC1-mediated substrate transport.

Glutathione depletion in H69AR cells

Previous studies have shown that reduced glutathione (GSH) is either co-transported with or stimulates the transport of some substrates in cells overexpressing ABCC1 [32]. We therefore measured intracellular glutathione to determine if SS transport by ABCC1 induced glutathione depletion. As shown in **Figure 5B**, SS significantly reduced glutathione levels in a concentration dependent manner at levels that paralleled those that were effective for enhancing sensitivity to Dox. In addition, glutathione depletion by either the glutathione synthetase inhibitor BSO or Dox was significantly increased after 18 h in the presence of 10 μM SS (**Figure 5C**). Combined treatment of SS and BSO resulted in highly potent suppression of the growth of H69AR cells (**Figure 5D**). This combined effect appeared to be specific for cells expressing ABCC1 since the growth inhibitory activity of SS in HT-29 colon tumor cells, which express either low or no ABCC1 (or ABCB1), was not affected by combined treatment with BSO(**Figure 5E**). These data suggest that SS reduces the levels of intracellular glutathione and cells overexpressing ABCC1may be more susceptible to growth inhibition by this mechanism.

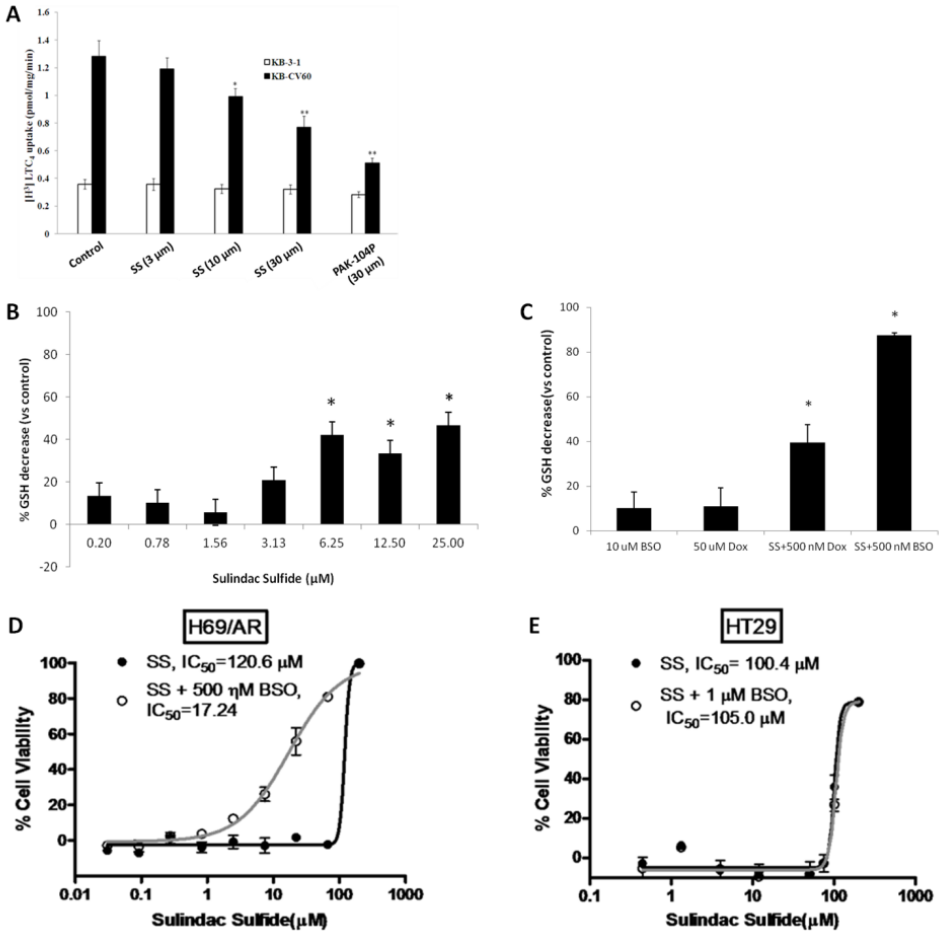

Figure 5. LTC_4 Transport and Glutathione Depletion. (A) ATP-dependent transport of [^{3}H]-LTC4 into ABCC1-positive(KB-CV60) and ABCC1-negative(KB-3-1) membrane vesicles and its inhibition by sulindac sulfide and the non-specific inhibitor PAK-104P.**(B)** Intracellular glutathione levels in H69AR cells treated with non-cytotoxic concentrations of sulindac sulfide for 18 hrs.(ANOVA , *P=< 0.05). **(C)** Intracellular GSH in H69AR cells treated with sulindac sulfide or Dox in the presence of a subtoxic dose of BSO(500 nM) **(D)** The affect on intracellular glutathione levels by SS alone and in the presence of a non-cytotoxic concentration of Dox or BSO (ANOVA, $P < 0.05$) ***Columns***, mean of three experiments; *bars*, SEM; *, significant, P<0.05**) (E)** Cell viability measured of H69AR cells or the colon cancer cell line HT-29 to SS or SS and BSO(1 μM) combination treatment.

Discussion

Here we show that, like indomethacin, the NSAID sulindac sulfide can increase the sensitivity of ABCC1 overexpressing cells to chemotherapeutic agents at concentrations that can be achieved in the plasma with clinically relevant dosages of

sulindac [29]. In fact, SS was able to affect ABCC1 mediated MDR and substrate transport at concentrations below its IC_{50} for either COX-1 or COX-2. SS also significantly increased the intracellular accumulation and retention of Dox *in vitro.* SS significantly decreased the accumulation of LTC_4 in inside-out membranes harboring ABCC1 and increased the fluorescent intensity of calcein-AM and Dox in ABCC1 overexpressing cells. Moreover, we show that SS increased glutathione depletion in ABCC1 expressing cells in a dose dependent manner and further sensitized the cells to BSO and Dox treatment.

Previous studies have shown that certain NSAIDs are able to enhance the effects of some chemotherapeutic agents *in vitro* [13]. These effects appear to be independent of COX-2 inhibition as the non-COX inhibitory sulfone metabolite also inhibited ABCC1 transport. In support of this, we found that pretreatment of human lung cancer cells with SS enhanced their sensitivity to growth inhibition by Dox. The enhanced sensitivity to Dox was not observed in lung cancer cells expressing little or no ABCC1. Similar effects were apparent with a more potent COX-2 inhibitor, indomethacin, although sulindac had a more pronounced effect on MDR reversal. The mechanism by which SS enhanced the action of Dox is most likely independent of the suppression of COX as there is no correlation between the potency of COX inhibition and the sensitization to growth inhibition. In contrast, there was a good correlation between SS potency on acute effects of efflux compared with longer term effects on cell growth.

SS displayed selectivity for ABCC1 compared to ABCB1 and ABCG2 as shown by the flow cytometry and the LTC_4 uptake data. This may have important implications for SS as a MDR reversal agent. While previous generations of ABC transport inhibitors

have demonstrated toxicity, sulindac may have a better safety profile. The toxicity associated with previous MDR reversal agents has been attributed to the tissue distribution of ABCB1 and the effects of ABCB1 inhibitors on cytochrome P450 enzymes. Sulindac and its metabolites appear not to interfere with the cytochrome P450 enzymes or increase the toxicity among patients receiving epirubicin and sulindac in combination [29]. Although ABCC1 is found in tissues throughout the body, it is generally localized to the basolateral membrane. In comparison, both ABCB1 and ABCG2 are located in the apical membrane of cells such as colon epithelium and bile canalicular membranes [33]. Further complicating matters is the evidence that polymorphisms in ABCG2 can lead to unexpected anticancer drug interactions [34]. In contrast, transport mediated by ABCC1 seems relatively unaffected by such polymorphisms [35]. Thus, the selectivity of sulindac for ABCC1 indicates it may have reduced toxicities when used in drug combinations.

ABCC1 is capable of transporting numerous substrate types, and several model systems are available to assay ABCC1 activity. In the present work, SS inhibited ABCC1-mediated transport of a variety of endogenous and xenobiotic substrates. For example, the endogenous ABCC1 substrate, LTC4, is incorporated into isolated membrane vesicles isolated from KB-CV60 cells and this activity was potently inhibited by SS. Consistent with previous reports in which calcein-AM efflux can be strongly correlated with ABCC1 expression and activity [36-37], we found that calcein-AM was excluded from ABCC1 expressing cells by both imaging and flow cytometry assays, and that this activity was also potently inhibited by SS. With the range of clinically important substrates for ABCC1 it is likely that compounds such as SS or derivatives have the

potential to be used in different chemotherapeutic regimens to improve response to these drugs.

Although there is partial overlap of substrate specificities between ABCB1 and ABCC1, GSH conjugation or co-transport seems to be a requirement only for ABCC1 mediated transport. In contrast, GSH-conjugated organic anions are transported much less efficiently, if at all, by ABCB1. Consistent with a requirement for GSH to transport xenobiotics, growth inhibition of ABCC1 expressing cells by SS was increased nearly 7-fold in the presence of BSO, an inhibitor of the enzyme responsible for the rate-limiting step in GSH synthesis, gamma-glutamylcysteine synthetase. In contrast, sensitization to SS by BSO was not observed in cells expressing little or no ABCC1. Our data suggest that SS can sensitize ABCC1 expressing cells to further oxidative stress by decreasing intracellular glutathione levels. Although the interaction between ABCC1, anticancer drugs, and glutathione is not completely understood, it seems that most of the anticancer drugs to which ABCC1 confers resistance are not conjugated to GSH *in vivo* [38]. Instead, some of them are co-transported from cells with the reduced form of glutathione by ABCC1. Exploiting this distinction may lead to the development of selective inhibitors of MDR, especially for malignancies where ABCC1 seems to be the dominant cause of multidrug resistance, such as melanoma, gliomas, and chronic lymphocytic leukemia [39-41].

Our data indicate that SS has a mechanism of action where it not only inhibits ABCC1 mediated efflux of doxorubicin and other substrates leading to the intracellular accumulation of those substrates, it also depletes cells of GSH. The sensitivity of ABCC1 expressing cells to oxidative stress as seen in our experiments is in agreement with

previously published data where either inhibition of GSH synthesis or increased GSH export preceded tumor cell apoptosis[42]. Based on the previous research of others and the data we have presented above, we believe the data is significant for the addition of sulindac to certain chemotherapeutic regimens, for the design of novel ABCC1 inhibitors, and the potential to chemically modify SS to block COX inhibitory activity, while retaining the selective ability to inhibit ABCC1 mediated drug resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Chapter 4

SULINDAC ANALOG STRUCTURE ACTIVITY ANALYSIS AND IDENTIFICATION OF PROTEIN TARGETS IN COLORECTAL CANCER CELLS

The Wnt/β-catenin signaling cascade increasingly attracts the attention of cancer researchers and pharmacologists. As mentioned previously, inappropriate activation of this pathway is considered to be the initiating event in transformation of intestinal epithelial cells. Studies with sulindac and metabolites have found that the degradation of β-catenin precedes apoptosis in CRC cell lines [1] [2].The degradation of β-catenin is associated with increased intracellular cGMP accumulation and PKG activation, likely due to the inhibition of PDE5 by sulindac sulfide (**Figure 1**). Unfortunately, the required dosage for anticancer activity also inhibits prostaglandin synthesis which is associated with gastrointestinal, hepatic and renal toxicity. Clinical outcome could be improved through the development of sulindac analogs that target novel molecular targets without inhibiting prostaglandin synthesis.

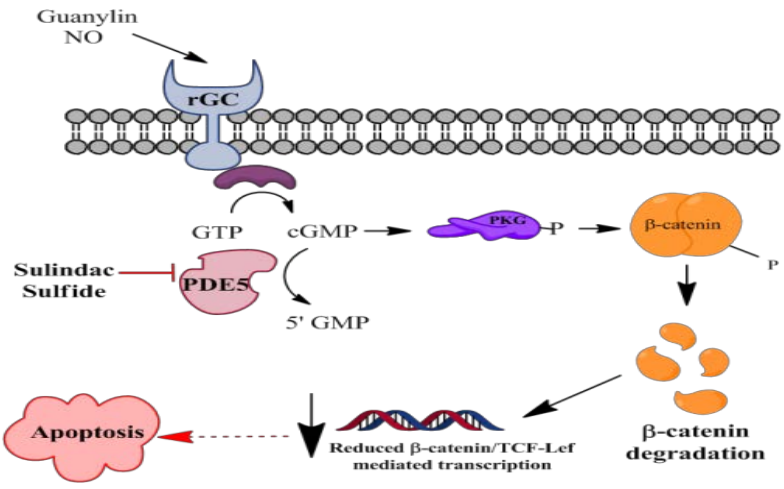


Figure 1. Antitumor mechanism proposed for sulindac sulfide. Sulindac sulfide prevents the conversion of cGMP to 5'GMP by PDE5. Activation of PKG by cGMP initiates the phosphorylation and subsequent degradation of β-catenin. Reduced transcription of pro-proliferative and anti-apoptotic genes by the β-catenin/TCF-Lef transcription complex leads to growth suppression and apoptosis.

The major aim of the studies carried out in the Piazza lab at Southern Research Institute was to develop a sulindac derivative that retains antineoplastic activity without inhibiting prostaglandin synthesis. The first step toward this goal was the identification of the negatively charged carboxylic acid moiety of sulindac sulfide as an important structural component for COX inhibition [3]. These results indicated that replacing the negatively charged group with a positively charged group could remove the COX-inhibitory activity of sulindac analogs. The second step was the synthesis of 536 sulindac analogs that were screened for COX-inhibition and colorectal cancer (CRC) cell growth suppression. This process identified several compounds that inhibited the growth of CRC cell lines more potently than the parent compound, sulindac sulfide. The activity was independent of an effect on prostaglandins since the compounds were substantially less effective at inhibiting the COX enzymes. The ability of SS and a benzylamine analog to inhibit growth and induce apoptosis correlated with the ability to inhibit cGMP-specific

phosphodiesterase activity. Elevated expression of the cGMP-specific phosphodiesterase PDE5 was found in the CRC cell lines tested, indicating this PDE isozyme may be necessary for the proliferation of CRC cells. Using a novel sulindac capture compound approach, several other proteins were also identified as possible targets of SS, including cellular apoptosis susceptibility protein, poly (ADP-ribose) polymerase (PARP-1), and 3-phosphoglycerate dehydrogenase. Additionally, structure activity relationships have identified structural features such as a molar refractivity in the 120-135 cm^3 range and a polar surface area less than 50 Â may increase the potency of sulindac analogs. These data strongly suggest that low levels of cGMP in CRC cells are important for their proliferation, that targeting PDE5 will lead to the development of a novel class of chemotherapeutic agents with higher potency, and structural features of sulindac sulfide such as molar refractivity and polar surface area may be modified to increase the potency of sulindac analogs in CRC cells.

Methods

Reagents

Sulindac sulfide was purchased from Sigma-Aldrich (St.Louis, MO). NOR-3 was purchased from Cayman Chemical (Ann Arbor, MI). Sulindac analogs were synthesized by Dr. Robert Reynolds and Bini Mathew at Southern Research Institute. The sulindac capture compound was synthesized by Caprotec Bioanalytics (Berlin, DE). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were also obtained from Cell Signaling Technologies. Compounds were solubilized in DMSO and diluted to a final concentration of 4.5% in enzyme- and 0.1% in cell-based experiments, which did not interfere with the assays. Unless otherwise specified, all other reagents

were purchased from Sigma-Aldrich. PCR tubes and desalting spin columns were purchased from Thermo Scientific (Rockford, IL).

Growth inhibition assay

HT 29 adenocarcinoma cells were plated in 96-well plates at a density of 5,000 cells per well and allowed to acclimatize overnight. Cells were treated with a 2-log dose range of compounds and incubated for an additional 72 hours. At the end of the incubation period, cell viability was determined by measuring luminescence using the Promega Cell Titer Glo Assay, which measures viable cells based on ATP content. The assay was done according to the manufacturer's specifications.

Western blotting

Cell lysates were obtained by harvesting cells at 70-90% confluency and vortexing in either ice-cold membrane lysis buffer(1.0% Triton X-100, 1.5 mM $MgCl_2$,10 mM KCL, 1 mM DTT, 10 mM HEPES, 50mM NaF, and protease inhibitor cocktail at pH 7.4), or nuclear lysis buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 200 µM EDTA, 1 mM DTT, 400 mM NaCl , 25% glycerol and protease inhibitor cocktail at pH 7.4) and centrifuging at 10,000g for 10 minutes at 4^oC. Western blots were performed by separating 30-50 µg of protein by SDS-PAGE on 12% acrylamide gels prior to electrophoretic transfer to nitrocellulose membranes. Tris-buffered saline (TBS) containing 0.05% Tween-20 and 5% BSA was used as a blocking agent. Primary and horseradish peroxidase conjugated secondary antibodies were used according to the manufacturers' specifications. Protein bands were visualized using Millipore Chemiluminescent HRP substrate ECL reagent.

PDE inhibition assay

Cyclic nucleotide hydrolysis was measured in cell lysates using the IMAP fluorescence polarization (FP) PDE assay from Molecular Devices (Sunnyvale, CA). A 2x serial dilution series of sulindac sulfide, analogs and known PDE inhibitors were incubated in the dark with either cell lysate or purified PDE isozyme for 30 min at 30°C. After incubation, a final concentration of 50 nmol/L TAMRA-cGMP was added to the reaction solution and incubated for an additional 1.5 h at 30°C.

The reaction was stopped by the addition of 60 μl IMAP binding solution and incubated for 30 min at 30°C. TAMRA fluorescence polarization was measured using a Biotek Synergy 4 (Winooski, VT) microplate reader and analysis was performed with GraphPad Prism scientific graphing software.

Structure activity analysis

The structural features of a subset of the sulindac analogs were analyzed to determine the role they have in modifying the biological activity of the compounds. The octanol-water coefficient (log *p*) and molar refractivity parameters of the compounds were determined using ACD/ChemSketch (ACD/Labs) software. Topological polar surface area was calculated using the Molinspiration online cheminformatics service. The relationship between experimentally determined IC_{50} values and the calculated physicochemical parameters was assessed by multiple linear regressions using the data analysis module in Microsoft® Excel.

Capture compound protein isolation

Isolation of target proteins from cell lysates and tissues was achieved by using a sulindac capture compound (**Figure 2A**) synthesized by Caprotec Bioanalytics. The capture compound allows the selection and isolation of a subset of the proteome from a complex protein mixture without the need for a solid phase support (**Figure 2B**). HT-29 cells were lysed in 0.5% CHAPS lysis buffer(250 mM sucrose, 20 mM HEPES, 150 NaCl, 5 $MgCl_2$, 2 mM β-mercaptoethanol and 0.5% CHAPS, pH 7.5). Lysates were centrifuged at 1000 x *g* for 2 min through ZEBA™ desalting spin columns to remove small molecules. Sulindac capture compound, cell lysates and buffers were added to 200 µl PCR tubes in the order indicated in **Table 1** and maintained at 4° C. Solutions were irradiated with 312 nm UV light for 10 min and resuspended every 2 min.

Component	Stock concentration	Assay concentration	Assay without competitor	Assay with competitor
H_2O	NA	NA	40 µl	35 µl
Capture Buffer	5x	1x	20 µl	20 µl
Competitor	2 mM	100 µM	NA	5µl
Cell Lysate	7 mg/ml	2.73 mg/ml	39 µl	39 µl
10 min incubation				
Capture Compound	10 mM	100 µM	1 µl	1 µl
Final volume			100 µl	100 µl

Table 1. Capture compound reaction solution pipetting scheme.

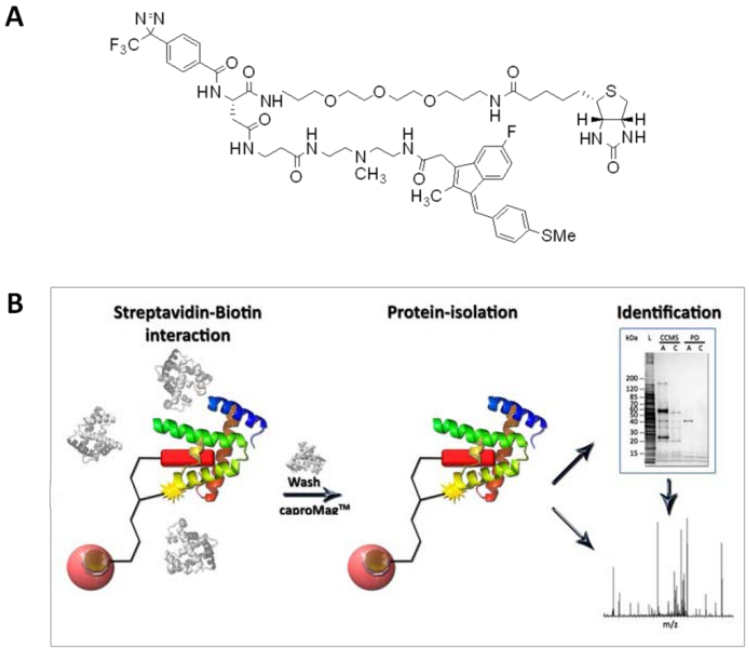


Figure 2. Sulindac capture compound protein isolation. A) Sulindac capture compound(SCC), **B**) Target protein isolation using magnetic bead/SCC-protein complexes followed by SDS-PAGE and mass spectrometry.

Invitrogen streptavidin-coated magnetic beads were washed twice with PBS and 50 µl added to each capture compound reaction tube and mixed thoroughly. Solutions were never vortexed in order to avoid denaturing the proteins. Reaction mixtures were incubated overnight at 4° C on a rotating wheel.

Streptavidin coated beads were collected in the lids of the PCR tubes using a Neodymium magnet. PCR tubes and supernatant were discarded and replaced with new PCR tubes containing 200 µl Caprotec wash buffer containing 0.5 mM NaCl. Beads were gently resuspended in the PCR tubes. PCR tube lids were replaced and wash steps were repeated 5 times. Bead complexes were washed three times with 80% acetonitrile and twice with 200 µl ultrapure water.

Bead protein complexes were resuspended in 15 µl Laemmli buffer, heated to 95° C for 5 min and electrophoresed on 8-16% linear gradient pre-cast polyacrylamide gels (Bio-Rad

Life Sciences). Bands were visualized by SYPRO Ruby protein gel stain. Mass spectrometry was performed by Landon Wilson at the Targeted Metabolomics andProteomics Laboratory at UAB under the direction ofDr. Stephen Barnes. Proteins were identified using the MOWSE algorithm and database searching [4-5].

Results

Quantitative structure activity relationships for sulindac analogs

The hydrophobicity (log *p),* molar refractivity (MR) and topological polar surface area (TPSA) were calculated for sulindac sulfide and ten analogs (**Table 2**). Molar refractivity represents the volume of the molecule and is also directly proportional to the polarizability of the molecule. Polar surface area is the sum of the surfaces of polar atoms in a molecule and is a useful predictor of drug transport [6]. Linear regression was used to derive the best equation that can be used to predict the growth inhibitory activity of a compound based on log *p,* molar refractivity and topological polar surface area. In 1975, Corwin Hansch was the first to use this approach in his analysis of 256 4,6-diamino-1,2-dihydro-2,3-dimethyl-1-phenyl-s-triazines which were active against dihydrofolate reductase [7]. The quantitative structure activity relationship (QSAR) equation that is derived from this approach relates the variations in biological activity to variations in the physicochemical descriptors of the molecules. The relationship between the growth inhibitory activity of the ten sulindac analogs and these three parameters can best be described by the equation:

$$\boldsymbol{Predicted\ IC_{50} = 319.5 + 17.2(Log\ P) - 3.7(MR) + 0.04(TPSA)^2}$$

This should be considered the most basic QSAR equation for the prediction of sulindac analog activity. The predictive power of the equation is expected to increase as the number and diversity of compounds increases, since traditional regression methods require that the number of parameters be considerably smaller than the number of compounds in the data set. It is likely that other physical descriptors of the compounds will also lead to the derivation of a more predictive equation. Unfortunately, the lack of suitable structure files prevents analysis of all the sulindac analogs. However, as the equation stands 75% of the variability in compound activity (the R^2 value of the regression analysis) can be attributed to the molar refractivity, TPSA and log *p* values. Statistically significant contributions to activity come from TPSA and MR, but not from log *p*. These calculations indicate that the effects of the smaller dipole moment of the sulfide group in comparison to the sulfoxide groups do not have a significant impact on the potency of the sulindac analogs despite the fact they would be expected to partition through the cell membrane more easily [8]. Surprisingly, the nonlinear relationship observed between TPSA and the predicted activity is normally observed between log *p* and activity [9]. These results suggest that the activity of sulindac analogs may be improved by synthesizing compounds with a polar surface area not exceeding 50 Â ^{2} (5.0 nm^2) and possessing a molar refractivity in the 120-135 cm^3 range.

Resistance to cGMP-mediated growth suppression is associated with increased PDE5 protein expression

One mechanism of action associated with the antineoplastic activity of sulindac sulfone and sulindac analogs is the inhibition of cGMP-selective PDE isozymes [10]. We determined the relative sensitivity of the HT-29 colorectal cancer cell line and the LT-97

adenoma cell line to NOR-3, an NO donor which increases cGMP levels (**Figure 3A**). The LT-97 adenoma cells were approximately two-fold more sensitive to growth inhibition by NOR-3 than the adenoma cells, results that suggest the adenoma cells are more sensitive to increases in intracellular cGMP levels than the HT-29 cells. Increased expression of the cGMP-selective PDE5 protein was found in HT-29 cells in comparison to the LT-97 cells (**Figure 3B**). By comparison, cGMP selective PDE2 protein levels were not elevated in either HT-29 or LT-97 cells (**Figure 3C**). These results suggest that PDE5 plays an important role in maintaining proliferation and controlling cGMP levels in CRC cells.

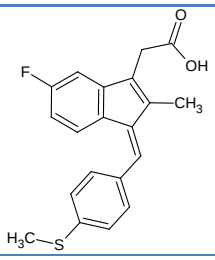
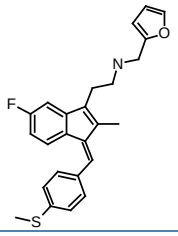
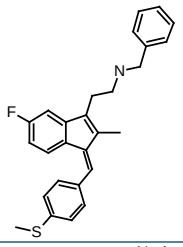
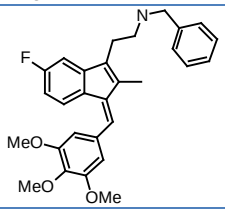
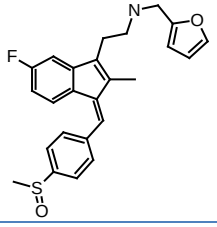
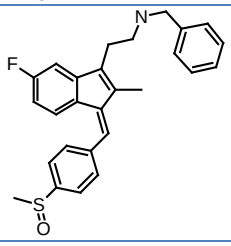
Compound	Structure	IC_{50}(μM)	COX-2 Inhibition(μM)	Polar Surface Area	Log *P*	Molar Refractivity(cm^3)
Sulindac sulfide		110	9	37.3	3.59±0.55	95.36±0.4
21926		5.8	>100	25.2	7.1±0.54	119.19±0.4
21925		8.8	>100	12.0	7.93±0.53	126.7±0.4
21878		3.2	>100	39.7	7.04±0.54	135.44±0.3
21879		2.3	>100	42.2	4.94±0.57	120.05±0.4
21882		3.4	>100	29.9	5.78±0.56	127.56±0.4

Table 2. Sulindac analog structures, IC_{50} values and physicochemical descriptors.

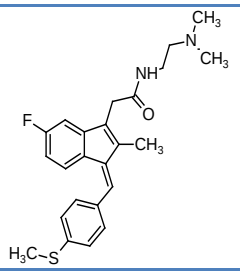
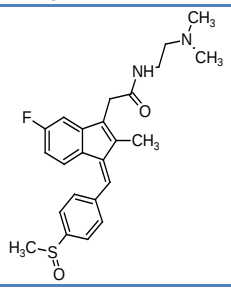
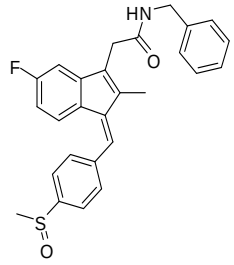
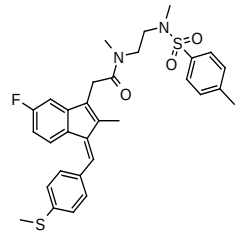
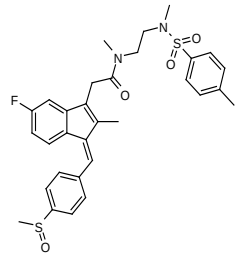
Compound	Structure	IC_{50}(μM)	COX-2 Inhibition(μM)	Polar Surface Area	Log *P*	Molar Refractivity(cm^3)
21009		1.5	>100	32.3	5.13±0.62	119.88±0.4
21004		25.1	>100	49.9	2.98±0.65	120.74±0.4
21211		7.3	>100	46.2	4.64±0.64	127.68±0.4
21618		5.8	>100	57.7	7.42±0.65	158.27±0.4
21621		51.9	>100	74.8	5.26±0.68	159.13±0.4

Table 2. Sulindac analog structures, IC_{50} values and physicochemical descriptors (continued).

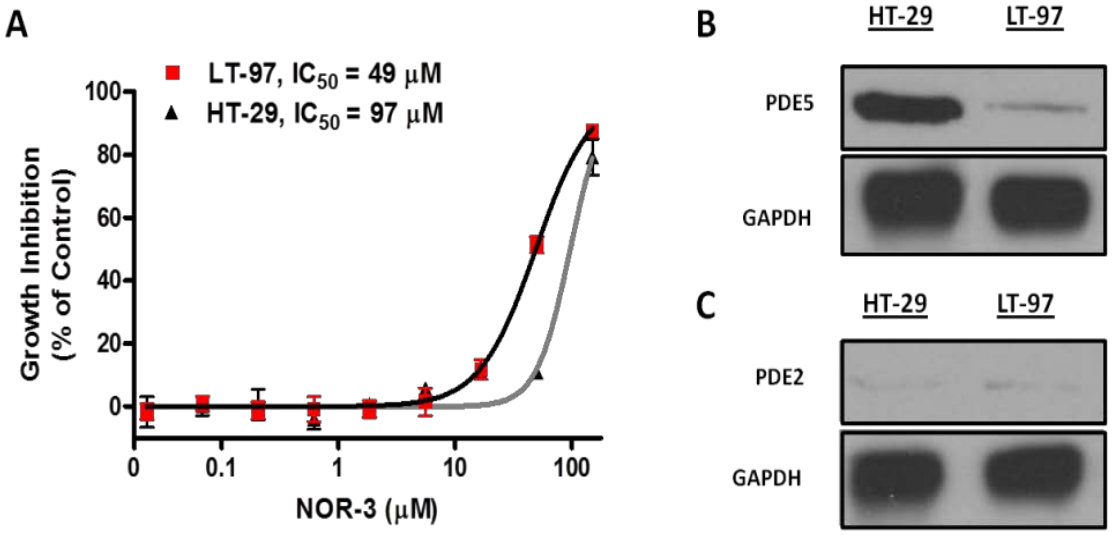


Figure 3. NOR-3 growth suppression and PDE5 expression. A) Growth inhibition by NOR-3 in HT-29 CRC and LT-97 adenoma cell lines, **B**) Expression of PDE5 and PDE2 (**C**) in HT-29 and LT-97 cells.

Increased CRC growth suppression is associated with inhibition of cGMP degradation

The effect of SS on cGMP and cAMP levels was measured in HT-29 cell lysate to determine whether changes in cyclic nucleotide levels were associated with growth inhibition. As shown in **Figure 4A**, SS inhibited cGMP hydrolysis in HT-29 cells with an IC_{50} value of 49 µmol/L. By contrast, the IC_{50} values for inhibition of cAMP hydrolysis were greater than 100 µmol/L. Cyclic GMP levels were measured in the presence of purified, recombinant PDE5 and several sulindac analogs that showed increased potency to inhibit the growth of CRC cells. The benzylamine sulindac analog SRI-21882 showed increased potency for inhibition of PDE5 compared to SS, with an IC_{50} of 8.7 µmol/L (**Figure 4B**). Despite inhibiting the growth of CRC cells with greater potency than SS, other sulindac derivatives such as SRI 21009 and SRI 21925 did not appreciably inhibit cGMP hydrolysis at concentrations below 100 µmol/L. This indicates that SS can increase intracellular levels of cGMP by inhibition of PDE activity and that it can be

modified to selectively inhibit cGMP-selective PDE5 to improve potency for CRC growth suppression.

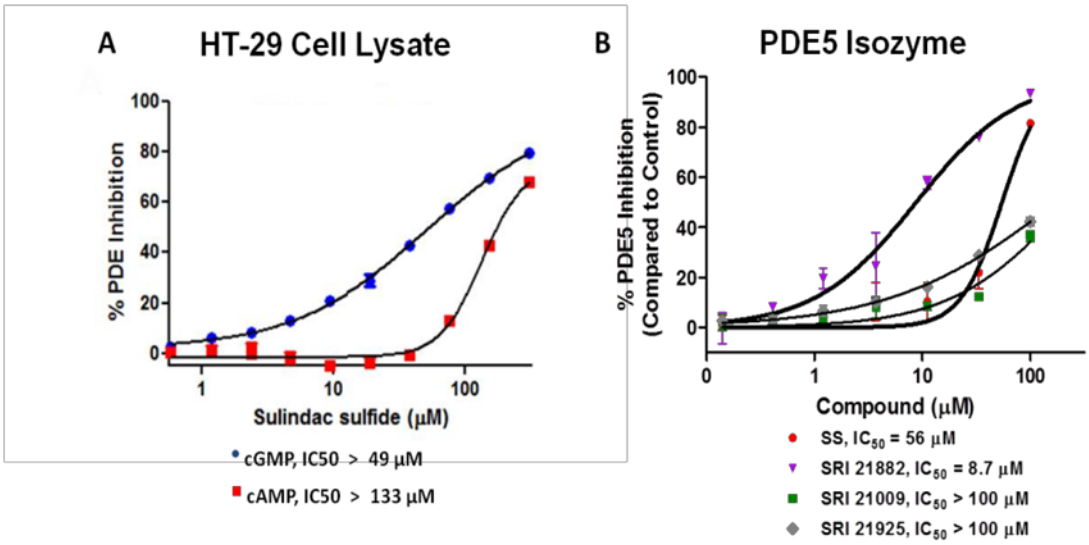
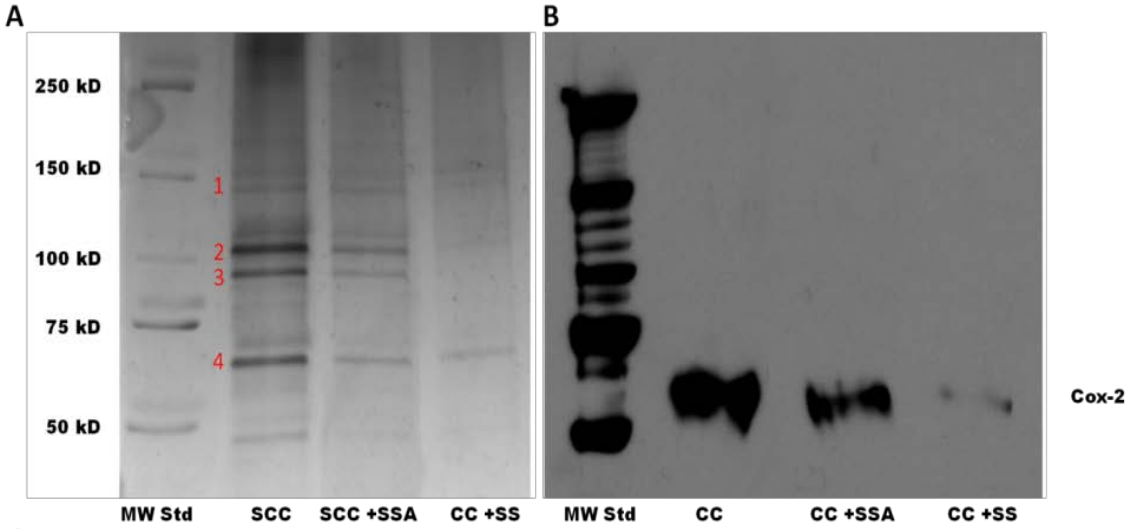


Figure 4. **Inhibition of phosphodiesterase activity by SS and sulindac analogs.** **A)**Half-maximal inhibition of cGMP hydrolysis occurs at a concentration of 49 μmol/L SS. Half-maximal inhibition of cAMP hydrolysis occurs at a concentration of 133 μmol/L. **B)** The benzylamine sulindac analog SRI 21882 inhibits the cGMP selective PDE5 isozyme with an IC_{50} value of 8.7 μmol/L. By comparison, the parent compound inhibits PDE5 with an IC_{50} value of 56 μmol/L while other sulindac analogs have IC_{50} values greater than 100 μmol/L.

Sulindac interacts with proteins having increased expression and activity in cancer cells

In an attempt to identify possible targets of SS, a protein pull-down assay using a sulindac capture compound (SCC) with HT-29 cell lysate was developed. The SCC is comprised of SS attached by a linker to a biotin and azido group. The azido group allows the SCC to be covalently attached to target proteins when activated by UV radiation, but is unaffected by normal fluorescent lighting. The biotin group allows for the isolation of SSC protein complexes using streptavidin-coated magnetic beads. The SSC was able to isolate several proteins as seen in **Figure 5A**. Competition with 100 μmol/L free SS was able to effectively reduce the intensity of the protein bands seen in the sample without

competitor. By comparison, the sulindac sulfide amide analog (SSA) was appreciably less effective at reducing protein band intensity. In addition, COX-2 was identified as a target by SDS-PAGE and immunoblotting after protein pull-down (**Figure 5B**). Competition with 100 μmol/L SSA or with SS resulted in a decrease in COX-2 band intensity of 43 and 97 percent, respectively. These results demonstrated the ability of SSC to interact with the known target of SS, COX-2 and validated the principal behind the SSC-protein isolation approach. Proteins with increased activity and expression in cancer cells were identified, including 3-phosphoglycerate dehydrogenase [11], cellular apoptosis susceptibility protein(CAS) [12-13] and poly(ADP-ribose) polymerase [14](**Figure 5C**). The proteins identified with the highest MOWSE scores were poly(ADP-ribose) polymerase (PARP-1), CAS, beta-tubulin, 3-phopsphoglycerate dehydrogenase, and heat shock protein 90. These data appear to be the first indication that sulindac may directly interact with these proteins, suggests the SS capture compound has the potential to be used for the identification of new targets in cancer cells, and that the SSC-mass spectrometry approach may aid in toxicity risk assessment for sulindac analogs.



C

Band 1	MOWSE Score	Band 2	MOWSE Score	Band 3	MOWSE Score	Band 4	MOWSE Score
poly(ADP-ribose) polymerase	358	CAS	203	heat shock 90kDa protein 1, beta	528	beta-tubulin	474
beta-tubulin	326	heat shock protein	90			3-phosphoglycerate dehydrogenase	365
3-phosphoglycerate dehydrogenase	133					chaperonin containing TCP1, subunit 2	166
tubulin, beta polypeptide 4, member Q	98					Tubulin alpha-1A	152
alpha-tubulin	67					Serine hydroxy-methyltransferase	98
cullin-associated and neddylation-dissociated 1, isoform CRA_a	58					phospholipase C-alpha	98

Figure 5. Sulindac capture compound-mass spectrometry approach for the identification of sulindac protein targets. A) SYPRO Ruby stained gel of SSC-HT 29 cell lysate samples in the presence or absence of free competitors, sulindac sulfide amide SSA or sulindac sulfide SS. **B**) Western blot of SSC-HT 29 lysate samples with either 100 μmol/L SSA or SS as competitor. **C**) Identification and MOWSE scores of labeled protein bands in panel **A**.

Summary and Future Directions

The growth inhibition of sulindac and structurally similar compounds has been linked to interactions with NF-κB [15], 15-lipoxygenase [16], PPAR-δ [17], Ras [18],

phosphodiesterase[10]and others [19-20]. The present studies have identified several sulindac analogs that have greater potency for CRC cell growth inhibition than SS, determined the mechanism is independent of COX-2 inhibition, and that there is a positive correlation between potency and the polar surface area of the compounds. Although the activity of most of the sulindac analogs has not been associated with a particular mechanism, there is a correlation between increased intracellular cGMP levels and growth suppression. Evidence that high levels of the phosphodiesterase isozyme PDE5 conveys resistance to growth suppression by NOR-3 and that SS can inhibit PDE5 is consistent with previous reports of phosphodiesterase inhibition by sulindac metabolites [10] and analogs [21]. Identification of a potent sulindac benzylamine derivative that inhibits PDE5 activity is strongly supportive of the pursuit of PDE inhibition for the treatment or prevention of CRC.

Although the discovery of PDE5 as a target for the next generation of sulindac analogs may someday improve clinical outcome in CRC, there is always the potential for unforeseen side effects and the assessment of human toxicities from animal studies is only about 71% reliable [22]. Drug developers are therefore in need of a method for identifying drug-protein interactions for adequate risk assessment and the capture compound seems to be well-suited for this purpose. The capture compound-mass spectrometry approach presents a new method for the identification of sulindac target proteins and the specificity of the capture experiments can be validated through the use of samples to which an excess of free SS has been added. Even though the capture compound experiments did not identify PDE5, this approach requires relatively large amounts of protein input and considerable effort is required to prepare samples, which

may prevent the identification of all target proteins. Furthermore, cyclic nucleotide concentrations as high as 2mM have been required to confirm non-specific binding to a cAMP-capture compound [23]. Such high concentrations of free SS are not achievable under the experimental conditions, so it may have been difficult to confirm non-specific binding if PDE5 had been isolated from the samples. However, the sulindac capture compound did isolate a key enzyme in the serine biosynthetic pathway, 3-phosphoglycerate dehydrogenase, an enzyme associated with neoplastic tissues [24]. The capture compound also isolated the molecular chaperone HSP90 [25] and the cellular apoptosis susceptibility(CAS) protein, which has been linked to increased metastatic potential in CRC cells [26-27]. This is the first time these proteins have been identified as possible targets of sulindac. Additionally, the isolation of β-tubulin in the capture compound assay is consistent with the previous discovery that sulindac derivatives can bind to and prevent the assembly of microtubules [28]. These results demonstrate that the sulindac capture compound is a powerful tool for the investigation of sulindac-protein interactions.

The details of an interaction between a compound and putative target are unknown in most cases, requiring inferences to be made based on molecular properties that can be discovered or calculated. Quantitative relationships that can be made between the activity of a compound and its chemical properties can help explain these interactions and predict the activity of new compounds. The quantitative structure-activity relationship (QSAR) derived for the sulindac analogs will allow medicinal chemists to focus on synthesizing compounds more likely to have greater potency for growth inhibition than SS. While all sulindac analogs synthesized so far could not be analyzed,

the inclusion of some sulindac analogs in the QSAR analysis appeared to decrease the fit of the equation, a problem similar to one encountered by Silipo and Hansch with inhibitors of dihydrofolate reductase [29]. For the sulindac analogs, the inclusion of more compounds with tri-methoxy and sulfonamide groups decreased the predictive ability of the Hansch equation. This is possibly due to the increased nucleophilicity of the tri-methoxy substituted benzene ring compound compared to an unsubstituted ring [30], which could affect the stability of the compound. It may also be due to increased steric hindrance caused by the methoxy and sulfonamide groups, which may require the use of a Taft parameter [31] in future sulindac QSAR equations to account for steric effects [32].

These studies have allowed us to identify PDE5 and possibly other proteins as targets for CRC cell growth inhibition and provided data for the development of sulindac analogs with improved antineoplastic activity. The protein pull-down method should be further refined to identify both on target and off-target proteins, a goal that may be accomplished by changing the attachment point of sulindac to the linker, azido and biotin groups of the capture compound (**Figure 2A**). QSAR analysis should provide a basis from which to synthesize future sulindac analogs that have physicochemical characteristics that optimize their potency. These studies have also provided a sulindac benzylamine analog that appears to inhibit CRC growth by antagonism of PDE5 activity. The downstream effects of PDE5 inhibition in CRC cells will be the focus of future studies.

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CHAPTER 5

A NOVEL SULINDAC DERIVATIVE THAT POTENTLY SUPPRESSES COLON TUMOR CELL GROWTH BY INHIBITING cGMP PHOSPHODIESTERASE AND β-CATENIN TRANSCRIPTIONAL ACTIVITY

by

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Cancer Prevention Research

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been widely reported to inhibit tumor growth by a cyclooxygenase (COX) independent mechanism, although alternative targets have not been well defined or used to develop improved drugs for cancer chemoprevention. Here we characterize a novel sulindac derivative referred to as sulindac benzylamine (SBA) that does not inhibit COX-1 or COX-2, yet potently inhibits the growth and induces the apoptosis of human colon tumor cells. The basis for this activity appears to involve cyclic guanosine monophosphate phosphodiesterase (cGMP PDE) inhibition as evident by its ability to inhibit cGMP hydrolysis in colon tumor cell lysates and purified cGMP-specific PDE5, increase intracellular cGMP levels, and activate cGMP-dependent protein kinase G at concentrations that suppress tumor cell growth. PDE5 was found to be essential for colon tumor cell growth as determined by siRNA knockdown studies, elevated in colon tumor cells as compared with normal colonocytes, and associated with the tumor selectivity of SBA. SBA activation of PKG may suppress the oncogenic activity of β-catenin as evident by its ability to reduce β-catenin nuclear levels, Tcf transcriptional activity, and survivin levels. These events preceded apoptosis induction and appear to result from a rapid elevation of intracellular cGMP levels following cGMP PDE inhibition. We conclude that PDE5 and possibly other cGMP degrading isozymes can be targeted to develop safer and more efficacious NSAID derivatives for colorectal cancer chemoprevention.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world that accounts for approximately 600,000 deaths per year. While colonoscopy allows for the early detection of disease and the identification of individuals who are at high risk of disease progression, the mortality rate from CRC has decreased only marginally in the last two decades [1]. Additionally, certain lesions such as flat adenomas cannot be readily detected by colonoscopy [2] and surgical management of adenomas in high risk individuals, such as with familial adenomatous polyposis (FAP) often requires complete or segmental removal of the colon [3]. Given the slow progression of carcinogenesis and the limitations of colonoscopy, much research has focused on cancer chemoprevention to reduce the development and progression of CRC.

One class of drugs that has shown promise for chemoprevention is the nonsteroidal anti-inflammatory drugs (NSAIDs), a chemically diverse family of drugs commonly used for the treatment of pain, fever, and inflammation. Epidemiological studies have shown that long-term use of NSAIDs such as aspirin can significantly reduce the incidence and risk of death from CRC [4]. In addition, certain prescription strength NSAIDs, such as sulindac can cause the regression and prevent recurrence of adenomas in individuals with FAP [5]. The antineoplastic activity of NSAIDs is widely attributed to their cyclooxygenase (COX) inhibitory activity because prostaglandins are elevated in colon tumors [6] and a significant percentage of colon tumors express high levels of the inducible COX-2 isozyme [7]. However, there is evidence that alternative mechanisms either contribute to or fully account for the CRC chemopreventive activity of NSAIDs [8-10]. For example, the non-COX inhibitory sulfone metabolite of sulindac has

been reported to inhibit the growth and induce apoptosis of colon tumor cells *in vitro* [11-12] and suppress colon tumorigenesis in animal models [13-15]. Sulindac sulfone (exisulind) was also shown to suppress adenoma formation in individuals with FAP or sporadic adenomas [16-17], but did not receive FDA approval due to hepatotoxicity. Nonetheless, because the use of NSAIDs is associated with gastrointestinal, renal and cardiovascular toxicities from suppressing prostaglandin synthesis [18-19], the investigation of COX-independent mechanisms may provide insight that could lead to new drug candidates that are potentially safer and more efficacious for CRC chemoprevention.

Previous studies have suggested that there is a close association between the antineoplastic activity of NSAIDs and their ability to suppress Wnt/β-catenin signaling in colon tumor cells. For example, studies have shown that certain NSAIDs can decrease nuclear levels of β-catenin to inhibit the transcription of genes (e.g. cyclin D, survivin) that provide a survival advantage to allow for clonal expansion of neoplastic cells [20-22]. Several groups have reported that sulindac sulfone can also induce proteasomal degradation of oncogenic β-catenin, which suggests that the underlying biochemical mechanism by which NSAIDs suppress β-catenin signaling may not require COX inhibition [22-24].

The mechanism responsible for the antineoplastic activity of sulindac sulfone has been previously reported to involve cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) inhibition, although the specific isozymes involved were not identified [23, 25]. More recently, we reported that the COX inhibitory sulfide metabolite of sulindac and certain other NSAIDs also inhibit cGMP PDE, and that this activity is

closely associated with PDE5 inhibition and their tumor cell growth inhibitory and apoptosis-inducing properties [26-28]. Cyclic nucleotide PDEs are a large superfamily of enzymes responsible for regulating second messenger signaling by hydrolyzing the 3',5'-phosphodiester bond in cGMP and/or cAMP. There are at least eleven PDE isozyme family members having different substrate specificity, regulatory properties, tissue localization, and inhibitor sensitivity [29]. PDE1, 2, 3, 10 and 11 are dual substrate-degrading isozymes, while PDE5, 6, and 9 are selective for cGMP and PDE4, 7, and 8 are cAMP selective. In addition, each isozyme family contains multiple isoforms or splice variants. Depending on the PDE isozyme content of the target cell population and inhibitor selectivity, PDE inhibitors can increase the magnitude and/or the duration of the cAMP and/or cGMP intracellular signal(s). Increasing cyclic nucleotide levels can induce specific signaling pathways, which, in the case of cGMP, can activate protein kinase G (PKG) to regulate cellular activity [30].

Here we characterize the anticancer activity of a novel benzylamine derivative of sulindac that does not inhibit COX-1 or COX-2, yet can potently inhibit the growth of colon tumor cells by inhibiting proliferation and inducing apoptosis. The underlying biochemical mechanism appears to involve cGMP PDE inhibition as evident by its ability to selectivity inhibit cGMP hydrolysis in whole cell lysates, as well as purified PDE5. Moreover, treatment of colon tumor cells with sulindac benzylamine increased intracellular cGMP levels and activated cGMP-dependent protein kinase (PKG) in colon tumor cells at concentrations that paralleled those required for inhibiting cGMP PDE/PDE5 and colon tumor cell growth. PKG activation by SBA was also found to be associated with decreased nuclear levels of β-catenin, Tcf transcriptional activity, and the

suppression of the apoptosis regulatory protein, survivin; all of which preceded apoptosis induction.

Materials and Methods

Drugs and reagents

Sulindac sulfide (SS) was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant PDE isozymes were purchased from BPS Biosciences (San Diego, CA). Isozyme specific PDE antibodies were purchased from GeneTex (San Antonio, TX), while VASP antibodies were purchased from BD Transduction Laboratories (San Jose, CA). All other antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Non-targeting control siRNA, PDE5 specific siRNAs, and the SureFECT transfection reagent were purchased from SA Biosciences (Frederick, MD). All other reagents were purchased from Sigma unless otherwise specified.

Sulindac benzylamine synthesis

Sulindac benzylamine (SBA) or [(Z)-N-benzyl-2-(5-fluoro-2-methyl-1-(4-(methylsulfinyl) benzylidene)-1H-inden-3-yl)ethanamine was synthesized by converting sulindac to sulindac methyl ester. Refluxing sulindac acid in methanol in the presence of concentrated sulfuric acid, followed by recrystallization from ethyl ether gave the methyl ester as a yellow solid in quantitative yield. The ester was dissolved in methylene chloride, and then treated with diisobutylaluminum hydride (2M in toluene) at -77^{o}C. After stirring for 4 h, the reaction was quenched by methanol. The temperature was slowly raised to 0^{o}C, and the reaction solution was washed with aqueous solution of acetic acid. The organic layer was concentrated and dried in vacuum overnight to give the

aldehyde as a yellow syrup. The aldehyde solution in ethanol was treated with benzylamine at -77^{o}C in for 3 h, followed by sodium borohydride for 30 minutes. Methanol was added and the temperature was slowly brought up to -40^{o}C and stirred for 2 h. Acetic acid was added slowly to quench the reaction. The reaction mixture was concentrated, purified with a silica gel column, and recrystallized from chloroform/acetone/ether three times, which resulted in a yellow solid. The final product was characterized by high resolution mass, NMR, and elemental analysis.

Cell culture

The human colon cancer cell lines, HT-29, SW480, and HCT 116 and normal fetal human colonocytes (FHC) were purchased from the American Type Culture Collection (Mansassas, VA). HT-29, SW480, and HCT 116 cells were maintained in RPMI-1640 + 2.0 g/L glucose pH 7.4 + 5% FBS + 4 mM glutamine (complete growth medium), incubated at 37°C in 5% CO_2, and passaged at subconfluent density. The human fetal colon FHC cells were cultured in DMEM:F12 medium supplemented with 10% FBS, cholera toxin (10 ng/mL) ,5 µg/ml transferrin, 5 µg/ml insulin, and 100 ng/ml hydrocortisone. The passage number was routinely limited to approximately 20 and morphology monitored with each passage, but no additional authentication of the cell lines was performed.

COX activity

COX-1 and COX-2 activities were measured using purified ovine COX-1 and COX-2 with colorimetric assay kits obtained from Cayman Chemical Co. (Ann Arbor, Michigan) as previously reported [31]. The activities of COX-1 and COX-2 were

measured after the addition of arachidonic acid and incubation at 25°C for 5 min by absorbance at 590 nm as specified by the manufacturer.

PGE_2 assay

U937 promonocytic cells [32] were differentiated into macrophage-like adherent cells by culture in 10 nM phorbol 12-myristate 13-acetate (PMA) for 48 h. Differentiated cells were washed with fresh media and plated at a density of 1.0×10^6 cells per well in 96-well half-area plates. Cells were allowed to adhere overnight and stimulated with 10 µg/ml lipopolysaccharide (LPS). Cells were treated with SS or SBA for 24 h. Prostaglandin PGE_2 levels in supernatants were measured using the HTRF PGE_2 assay from Cisbio (Bedford, MA). This detection method utilizes the binding of exogenous PGE_2 to disrupt the fluorescence resonance energy transfer (FRET) between a PGE_2 –d2 conjugate (acceptor) and a PGE_2 antibody (donor).

PDE activity

PDE activity was measured using the IMAP fluorescence polarization assay (Molecular Devices) in which binding of hydrolyzed cyclic nucleotide substrate to immobilized metal coordination complexes increases fluorescence polarization (FP) as previously described [26]. For studies involving tumor cell lysates, human colon tumor cells were harvested and lysed with PDE activity buffer (20 mmol/L Tris-acetate, 5mmol/L magnesium acetate, 1 mmol/L EGTA, 1.0% Triton X-100, 50 mmol/L NaF, and protease inhibitor cocktail at pH 7.4). Tetramethylrhodamine (TAMRA)-cGMP and fluorescein-cAMP were used as substrates, each at final concentration of 50 nmol/L. Cell

lysates were titrated to identify a suitable protein concentration that was in the mid portion of a concentration vs. PDE activity curve. The PDE assay was done according to the manufacturer's specifications using either whole cell lysates or recombinant enzymes. FP was measured at excitation, emission wavelengths of either 530,590 nm for TAMRA-cGMP or 485,530 nm for fluorescein-cAMP using a Synergy4 (Biotek) microplate reader.

Growth assays

Cells were plated in 96-well microtiter plates at a density of 5,000 cells per well and allowed to adhere overnight prior to treatment. Cells were treated with a 2-log concentration range of either SS or SBA and incubated for an additional 72 hours. For siRNA assays, cells were transfected in OptiMEM media with 0.5% SureFECT transfection reagent and 200 nmol/L of either negative control or PDE5 siRNA and incubated at 37°C for 24 h prior to treatment. At the end of the incubation period, relative cell viability was compared to vehicle controls using the Cell Titer Glo Assay (Promega, Madison WI), which measures viable cells based on ATP content, according to the manufacturer's specifications.

Proliferation assays

The antiproliferative activity of SS and SBA was determined by measuring EdU (5-ethynyl-2'-deoxyuridine) incorporation during DNA synthesis. Cells were seeded at a density of 1.5×10^6 cells per 10-cm tissue culture dish and incubated overnight at 37°C in 5% CO_2. After growing the cells in serum-free medium for 24 h, the cultures were treated

with SS, SBA, or vehicle (0.1% DMSO) in RPMI-1640 media with 5% serum for 4 h. A final concentration of 16 μmol/L EdU was added to each dish and incubated for an additional 18 h. Cells were harvested and analyzed using the Click-iT EdU Alexa Fluor 488 flow cytometry assay kit (Invitrogen) according to the manufacturer's specifications. Proliferating cells were quantified using a Guava EasyCyte Plus flow cytometer. A minimum of 5,000 events were collected in triplicate for each treatment group.

Apoptosis assays

HCT 116 cells were seeded at a density of $1x10^6$ cells per 10cm tissue culture dish, incubated for 48 hours, and treated with the specified compound or vehicle control. After 24 hours of treatment, cells were harvested from the treatment media and dish and fixed with 10% neutral buffered formalin (4% formaldehyde) on ice for 15 min. Samples were stained for DNA strand breaks using the APO-BrdU deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (Invitrogen), which labels BrdU incorporation into DNA strand breaks with AlexaFluor-488. The assay was performed according to the manufacturer's specifications. The percentage of TUNEL positive cells was quantified using a Guava EasyCyte Plus flow cytometer. A minimum of 5,000 events were collected in triplicate for each treatment group with minimal electronic compensation. Data were analyzed using CytoSoft 5.0 Software (Guava Technologies).

Intracellular cGMP levels

Intracellular cGMP levels were measured using the Promega GloSensor™ cAMP Assay modified to use a firefly luciferase fused to the human PDE5 GAF-A cGMP

binding domain (GloSensor cGMP-40F plasmid kindly provided by Promega Corporation). HEK293 cells were plated in 96-well white, clear bottom plates at a density of 25,000 cells per well and incubated overnight at 37° C, 5% CO_2, 95% relative humidity. For each well, a final concentration of 100 ng of DNA and 0.25 µl PLUS reagent were added to 19.75 µl of Opti-MEM© media and incubated at room temperature for 5 min; then 0.35 µl of Lipofectamine LTX™ reagent was added to the solution, mixed gently, and incubated for 30 min at room temperature. To each well 20 µl of DNA-Lipofectamine LTX reagent was added and incubated overnight under standard cell culture conditions. After incubation, the media was replaced with 100 µl of equilibration solution (88 % CO_2-independent media, 10% FBS, 2% GloSensor cAMP reagent) and incubated for 2 h at room temperature protected from light. Background measurements were obtained 15 min prior to the end of the equilibration incubation by reading on a PerkinElmer Victor 3 luminometer. After the background measurements, sodium nitroprusside (SNP) or SNP plus SBA was added to the wells in 10 µl of CO_2-independent media. The final concentration of SNP was 50 µmol/L. Plates were read every 2 min for 1 h on the Victor 3 luminometer.

β-catenin-mediated Tcf transcriptional activity

Experiments to determine the effects of SBA on β-catenin mediated Tcf transcriptional activity were performed using the TOP/FOP-flash Tcf reporter constructs as described previously [33]. The TOP-flash plasmid contains Tcf binding sites for β-catenin, while the FOP-flash plasmid has mutated Tcf binding sites, which serves as a control for measuring nonspecific activation of the reporter. In brief, HCT116 cells were plated in 24 well plates and co-transfected with 0.1µg TOP-flash or FOP-flash plasmids

and 0.1 μg β-galactosidase–expressing vector. Following treatment with SBA for 24 h, luciferase activity was determined and normalized with activity of β-galactosidase. These data are expressed as the mean and SEM of triplicate values of the normalized TOP-flash and FOP-flash activity.

Western blotting

For Western blots, cells were lysed in either ice-cold membrane lysis buffer (1.0% Triton X-100, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 10 mM HEPES, 50mM NaF, and protease inhibitor cocktail), or nuclear lysis buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 200 μM EDTA, 1 mM DTT, 400 mM NaCl, 25% glycerol and protease inhibitor cocktail).For nuclear fractionation, cell lysate was maintained at 4° and vortexed for 30 seconds every 5 min for 1 h, followed by centrifugation for 15 min at 15,000 rpm. Protein concentrations were determined by the Lowry method. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels prior to transfer to nitrocellulose membranes. Membranes were blocked for 1 hour in 5% bovine serum albumin, 0.05% Tween 20, and incubated with primary antibodies overnight at 4°C. Membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h followed by incubation with Super Signal West Pico Enhanced Chemiluminescence Reagent (Pierce). Protein bands were visualized on Hyblot CL (Denville Scientific) autoradiography film.

Molecular modeling

Molecular modeling was performed using Schrödinger Suite 2010 (Schrödinger, LLC). The structural model of PDE5 catalytic domain was derived from the crystal

structure of the PDE5-GMP complex from the protein databank (PDB ID: 1T9S). The Induced Fit Docking (IFD) protocol, which takes into consideration the ligand-induced receptor conformational change, was used for all docking studies. Specifically, residues within 6 Å from ligands were allowed to be flexible; docking results were scored using the extra-precision (XP) mode of Glide® version 5.6 (Schrödinger, LLC). IDF docking protocol and parameters were first validated by docking GMP in the PDE5 catalytic site, which excellently reproduced the PDE5-GMP crystal complex conformation. The same protocol and parameters were then applied to the docking studies of SBA.

Experimental design and data analysis

For growth assays, the IC_{50} values were determined by testing a range of eight concentrations with a minimum of four replicates per dose. COX and PDE inhibition assays used a minimum of three replicates. Statistical analysis was done employing the unpaired two-tailed Student's *t* test. Significance was assumed for $P < 0.05$. Error bars represent standard deviation.

Results

SBA inhibits tumor cell growth without COX inhibition

We previously reported that the carboxylic acid moiety of SS is essential for inhibiting COX-1 and COX-2, which could be chemically modified by substituting with a positively charged amide group to effectively block COX binding [31]. Such derivatives were unexpectedly found to display enhanced potency to inhibit colon tumor cell growth compared with SS. To chemically optimize for COX-independent tumor cell growth inhibitory activity, the chemists synthesized a large series of sulindac derivatives with

various chemical modifications which the Piazza lab screened for tumor cell growth and COX inhibitory activity. A benzylamine derivative was identified as shown in **Figure 1A** that displayed high potency for inhibiting tumor cell growth, but did not inhibit COX-1 or COX-2.

As shown in **Figure 1B**, SBA treatment for 72 hours inhibited the growth of human HT-29, SW480 and HCT116 colon tumor cell lines with IC_{50} values ranging from 3-5 μmol/L, while SS was appreciably less potent with IC_{50} values of 90-120 μmol/L. SBA completely lacked inhibitory activity for COX-1 and COX-2 at concentrations up to 200 μmol/L (**Figure 1C**). By comparison, SS inhibited COX-1 and COX-2 with IC_{50} values of 2 and 9 μmol/L, respectively. To determine if SBA inhibited other enzymes involved in arachidonic acid metabolism, we measured treatments effects on the production of PGE_2, which is the primary product in most tissues. Although we used a highly sensitive fluorescence-based ELISA assay for measuring PGE_2 production, the levels produced by colon tumor cells (e.g. HT-29) did not result in a sufficient signal above noise to allow for inhibition studies. As an alternative method, we used human U937 promonocytic cells treated with LPS, which are commonly used for such assays. As shown in **Figure 1D**, SS potently suppressed PGE_2 production by these cells with an IC_{50} of 0.7 μM, while SBA was ineffective, which is consistent with its lack of COX-1 or COX-2 inhibitory activity. We also determined if the sensitivity of the colon tumor cell lines to SS and SBA was related to the expression of COX-2. As shown in Figure 1E, HT-29 cells expressed high levels of COX-2 as determined by Western blotting, but COX-2 was expressed to a lesser extent in SW480 or HCT 116 cells, despite no difference in their sensitivity to SS and SBA.

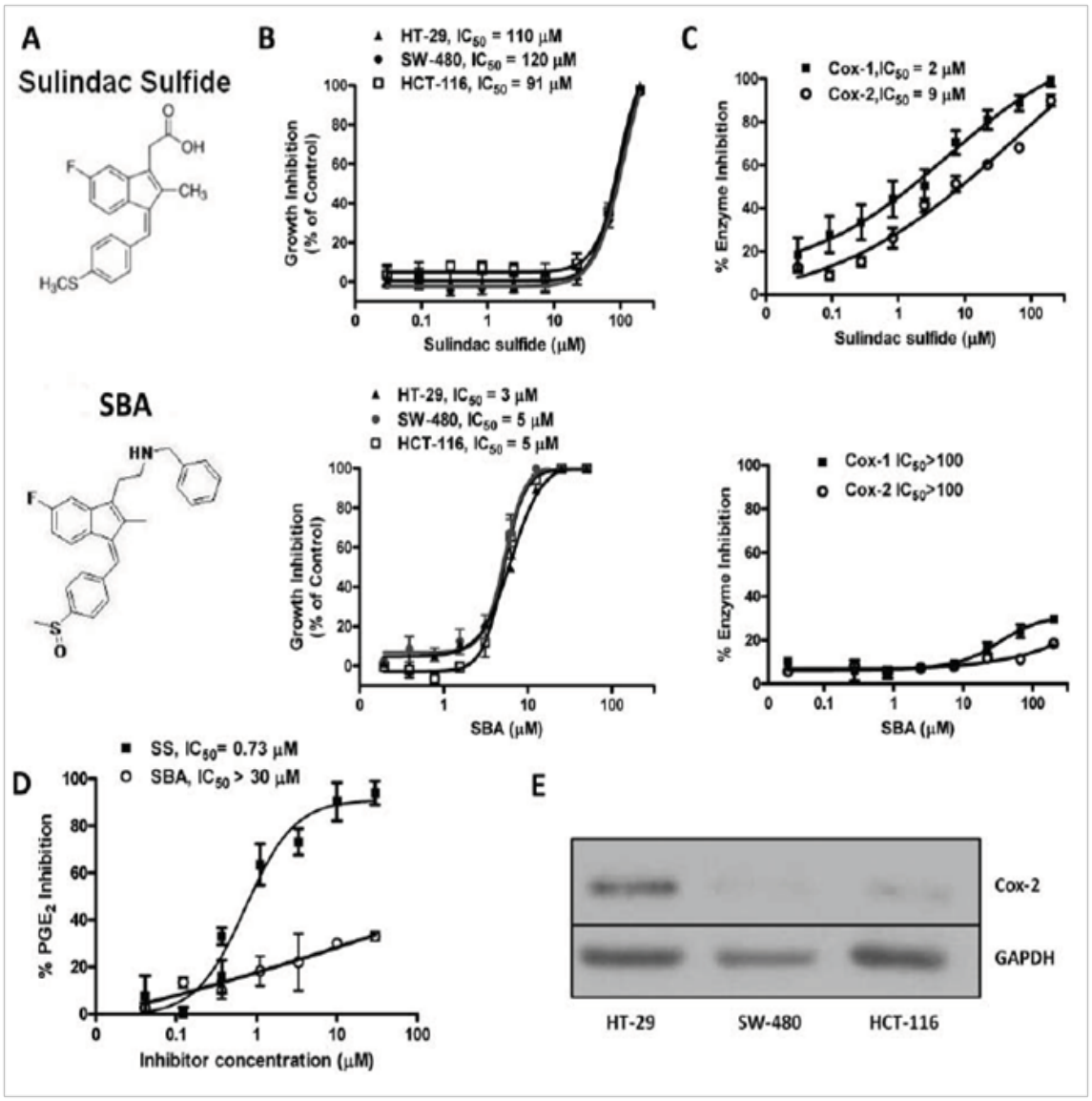

Figure 1. **CRC cell growth and COX enzyme inhibition by SS and SBA. A**, chemical structures of SS and SBA. **B**, tumor cell growth inhibitory activity of SS and SBA as measured by luciferase-based ATP assay after 72 h of treatment. **C**, COX-1 and COX-2 inhibitory activity of SS, but not SBA. **D**, inhibition of PGE_2 production by SS, but not SBA in LPS-stimulated U937 promonocytic cells. **E**, COX-2 protein expression in human HT-29, HCT 116, and SW480 colon cancer cells.

SBA inhibits colon tumor cell growth by inhibiting proliferating and inducing apoptosis

To determine the cellular basis for the tumor cell growth inhibitory activity of SBA, treatment effects on proliferation and apoptosis were measured and compared with SS. As shown in **Figure 2A**, treatment of HCT 116 colon tumor cells with SS for 24 hours reduced the number of proliferating cells by approximately 12% relative to vehicle treated cells. By comparison, treatment with SBA inhibited cell proliferation by 86% after the same treatment period. In addition, treatment with SS increased the number of apoptotic cells from 3% in the vehicle group to 12%, while SBA increased apoptotic cells to 75%, respectively (**Figure 2B**). These results show that the enhanced potency of SBA compared with SS is associated with increased effectiveness to inhibit proliferation and induce apoptosis.

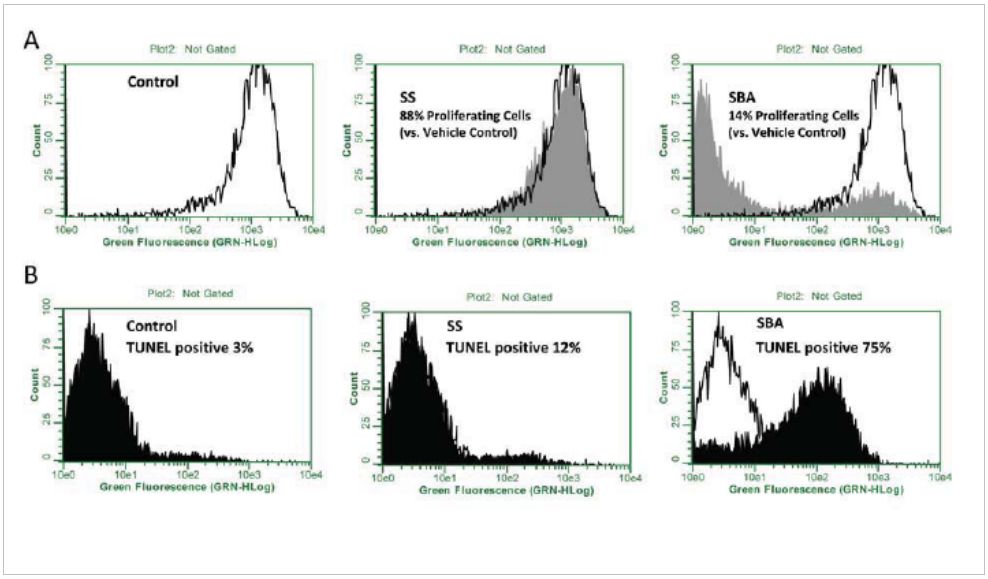

Figure 2. Comparison of apoptosis induction by SS and SBA. A, inhibition of HCT 116 colon tumor cell proliferation after 24 h treatment with 100 µmol/L SS (middle panel) or 50 µmol/L SBA (right panel) as determined by EdU incorporation and flow cytometry. **B**, apoptosis induction of HCT116 colon tumor cells after 24 h treatment with 100 µmol/L SS (middle panel) or 50 µmol/L SBA (right panel) as determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (*TUNEL*) and flow cytometry.

SBA selectively inhibits cGMP PDE

Based on previous studies that showed a close association between tumor cell growth inhibitory activity of SS and its ability to inhibit PDE5 [26-28, 34], we determined if SBA has PDE inhibitory activity. For the initial studies we measured total cGMP and cAMP hydrolysis in whole cell lysates from HT29 cells using a dual substrate assay of PDE activity as previously described [26]. As shown in **Figure 3A**, SS inhibited total cGMP hydrolysis with an IC_{50} of 49 µmol/L, but appreciably higher concentrations were required to inhibit cAMP hydrolysis with an IC_{50} of 133 µmol/L. Corresponding with its improved potency to inhibit tumor cell growth, SBA more potently inhibited cGMP hydrolysis with an IC_{50} of 8 µmol/L as shown in **Figure 3B**. In contrast with SS, SBA did not inhibit cAMP hydrolysis at concentrations as high as 200 µmol/L, which suggests the potential for improved isozyme selectivity compared with SS. The PDE5 inhibitory activity of SBA was next determined by performing similar assays except using purified recombinant PDE5. As shown in **Figure 3C**, SS inhibited PDE5 with an IC_{50} of 38 µM. SBA was appreciably more potent with an IC_{50} of 9 µM as shown in **Figure 3D**. The potency values as determined using purified PDE5 were comparable to values as measured using whole tumor cell lysates, although less inhibition was observed in the latter, which is likely attributed to the presence of insensitive cGMP PDE degrading isozymes as described below.

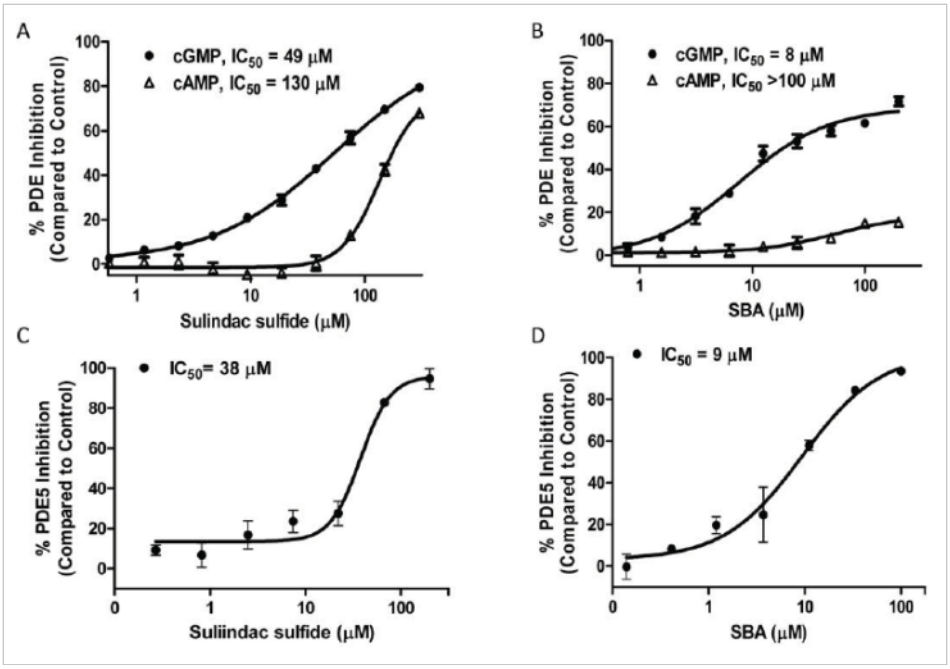

Figure 3. Inhibition of cyclic nucleotide hydrolysis by SS and SBA. A, inhibition of cAMP and cGMP PDE activity by SS in HT-29 tumor cell lysate. **B**, inhibition of cAMP and cGMP PDE activity by SBA in HT-29 cell lysate. **C**, PDE5 inhibition by SS. **D**, PDE5 inhibition by SBA.

The PDE isozyme selectivity of SS and SBA was next measured using a panel of recombinant PDE isozymes. As summarized in **Table 1**, SBA was highly selective for PDE5. All other PDE isozymes were either insensitive or resulted in IC_{50} values that appreciably exceeded the concentration range required to inhibit colon tumor cell growth. PDE5 was also the most sensitive isozyme to SS, although SS also inhibited PDE2, PDE3, and PDE10 within the same concentration range as required for tumor cell growth inhibition, which suggests that the increased potency of SBA to inhibit tumor cell growth is associated with increased potency and selectivity to inhibit PDE5. However, because these studies are limited to a group of PDE isoforms that were commercially available, we cannot rule out the potential involvement of additional isozymes that may be expressed in colon tumor cells and sensitive to SBA.

PDE Family	Substrate	SBA Sensitivity		SS Sensitivity	
		cGMP IC50(μM)	cAMP IC50(μM)	cGMP IC50(μM)	cAMP IC50(μM)
1A	cAMP/cGMP	>100	>100	>100	>100
1B	cAMP/cGMP	>100	>100	>100	>100
1C	cAMP/cGMP	>100	>100	>100	>100
2A	cAMP/cGMP	>100	>100	97	>100
3A	cAMP/cGMP	>100	>100	84	>100
3B	cAMP/cGMP	>100	>100	>100	>100
4B	cAMP	ND	75	ND	>100
5A	cGMP	9	ND	38	ND
6C	cGMP	>100	ND	>100	ND
7A	cAMP	ND	>100	ND	>100
8A	cAMP	ND	>100	ND	>100
9A	cGMP	>100	ND	>100	ND
10A	cAMP/cGMP	62	77	70	>100
11A	cAMP/cGMP	>100	>100	>100	>100

Table 1. Potency of SS and SBA to inhibit cAMP and cGMP hydrolysis by PDE isozymes.

Elevation of intracellular cGMP by SBA

We previously reported that SS can increase intracellular cGMP levels as measured by a standard immunoassay method [26]. To determine if SBA can increase intracellular cGMP levels, we used HEK293 cells transfected with a construct of cGMP binding (GAF A) protein fused to firefly luciferase. This luminescence assay in live cells allowed for kinetic measurements of intracellular cGMP levels in response to treatment. As shown in **Figure 4A**, SBA treatment caused a rapid and sustained increase in luminescence that occurred within the same concentration range as required for cGMP

PDE inhibition in cell lysates. Others studies confirmed the expression of PDE5 in HEK293 cells (data not shown), which suggest that cGMP elevation by SBA in this cell model results from PDE5 inhibition.

PDE5 suppression by siRNA inhibits colon tumor cell growth and increases sensitivity to SBA

To assess the possibility that PDE5 is necessary for colon tumor cell growth, HCT 116 and HT-29 colon tumor cells were transfected with PDE5 siRNA to selectively suppress the expression of the enzyme. Western blot analysis as shown in Figure 4B confirmed that PDE5 siRNA reduced PDE5 protein levels. PDE5 knockdown by siRNA inhibited the growth of HCT 116 and HT-29 cells by 60% and 30%, respectively, compared with cells transfected with non-targeted siRNA. In addition, PDE5 siRNA knockdown HCT116 cells displayed increased sensitivity to SBA treatment as evident by a 2-fold decrease in its IC_{50} value compared with control cells as shown in **Figure 4C**.

Tumor cell growth inhibition by SBA is associated with PDE5 expression

To further study the possibility that PDE5 expression can influence the sensitivity of colon tumor cells to SBA, PDE5 levels were measured in normal human colonocytes (FHC) and HT-29 and HCT 116 colon tumor cells. As shown in **Figure 4D** by Western blotting, PDE5 was not be detected in FHC, while both colon tumor cell lines expressed relatively high levels of the enzyme. Consistent with these observations, SBA inhibited the growth of both tumor cell lines with an IC_{50} of 5 µmol/L, while FHC were appreciably less sensitive with greater than a 6-fold higher IC_{50} of 33 µmol/L (**Figure**

4D). By contrast, SS did not show evidence of tumor selectivity, which may be attributed to its non-selective cGMP PDE inhibitory activity.

Mechanism of PDE5 inhibition

Molecular modeling studies were performed to determine the mechanism by which SBA inhibits PDE5. For these studies we utilized a crystal structure of PDE5 complexed with the reaction product, guanosine monophosphate (GMP). Docking results as shown in Figure 4F indicate that the heterocyclic ring scaffold of SBA effectively overlays with the guanine fragment of GMP that occupies the same central hydrophobic area within the catalytic site of PDE5 located between Phe829 and Phe786. Multiple hydrogen bonds were formed in the PDE5-GMP structure between the guanine of GMP and Gln817, which is an important amino acid residue that is necessary for substrate and inhibitor binding [33]. Hydrogen-bond interactions were also observed between Gln817 and the amine group of SBA as evident by the docked SBA-PDE5 complex structure. In addition, the sulfoxide group of SBA occupied the polar area near the two metal ions, similar to the phosphate group of GMP. Likely important for enzyme inhibition, SBA occupied a hydrophobic region through its phenyl group, which is close to the surface of PDE5 that is not occupied by the GMP molecule in the GMP-PDE5 complex structure. These results indicate that SBA directly binds the catalytic domain of PDE5.

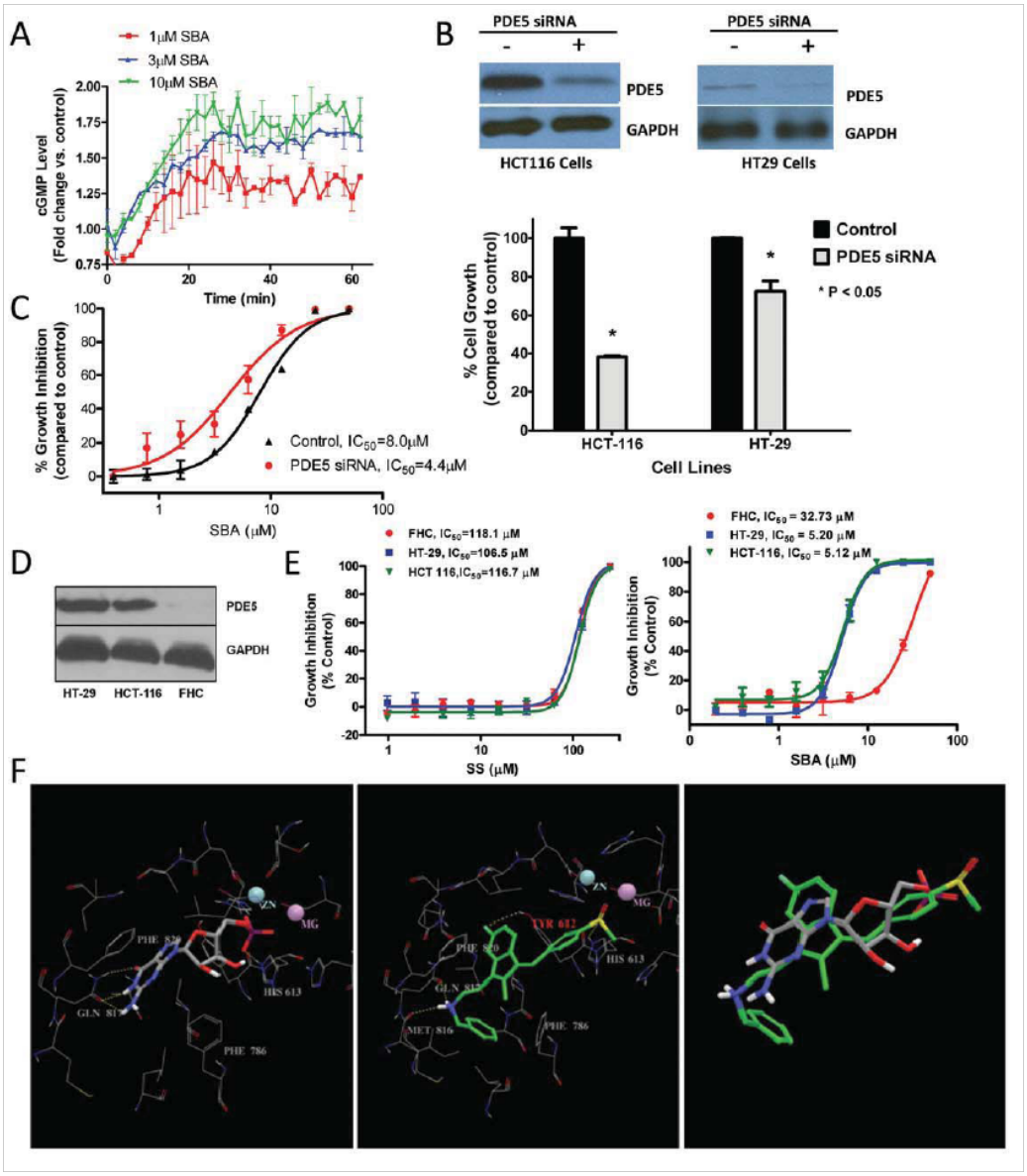

Figure 4. CRC cell growth and PDE5 inhibition by SS and SBA. A, luciferase-based cGMP biosensor assay in HEK293 cells treated with 50 μmol/L SNP in the presence of 1, 3, or 10 μmol/L SBA. **B**, siRNA suppression of PDE5 and growth in human HCT 116 and HT-29 colon tumor cells following 72 h transfection. **C**, growth inhibition by SBA of PDE5 siRNA knockdown HCT116 cells compared with controls. **D**, PDE5 expression in human HCT116 and HT-29 colon tumor cells and fetal human colonocytes (FHC). **E**, growth inhibitory activity of SBA and SS in human HCT116 and HT-29 colon tumor cells compared with fetal human colonocytes (FHC). **F**, structural representation of GMP and SBA binding to PDE5. The panels show GMP-bound PDE5 (left), docked PDE5-SBA complex structure (middle) and the aligned GMP and SBA (right). Residues within 4 Å of the bound/docked molecules are shown by lines. GMP and SBA molecules are represented in solid sticks and their carbon atoms are colored in grey and green, respectively. Hydrogen-bonds are marked in yellow dashed-lines.

PKG activation by SBA

To study the downstream events that may occur in response to cGMP PDE inhibition and cGMP elevation, we initially determined if SBA could activate PKG in colon tumor cells. For these experiments, HT-29 cells were treated with SBA and the phosphorylation of the known PKG substrate, vasoactive stimulatory protein (VASP), was measured using a phospho-specific VASP antibody that is selective for cGMP-stimulated phosphorylation at the Ser^{239} residue. As shown in **Figure 5A**, the levels of phospho-VASP increased within 1 h of SBA treatment at a concentration of 5 μmol/L and remained elevated for the duration of the experiment (5 h). These experiments provide evidence that PKG activation occurs in response to SBA treatment at concentrations and times that paralleled those required for cGMP PDE inhibition and increased intracellular cGMP levels, respectively.

Suppression of nuclear β-catenin levels and transcriptional activity by SBA

Previous studies have shown that PKG can phosphorylate β-catenin to reduce cytoplasmic and nuclear levels of β-catenin by a mechanism that appears to involve the activation of ubiquitin-mediated proteasomal degradation [23, 35]. To determine if SBA can reduce β-catenin levels within the same time period as PKG activation, nuclear β-catenin levels were measured in the same HT-29 cell lysates as used for experiments to measure PKG activation. As shown in **Figure 5A**, SBA reduced nuclear levels of β-catenin within 1 hour of treatment, which paralleled the time required for activating PKG. The expression of the apoptosis regulatory protein, survivin, in which its synthesis is under the control of the β-catenin/Tcf-Lef transcription factor [36], was decreased after a

slightly longer duration of treatment (**Figure 5B**). Levels of cleaved caspase-3 were also measured as a biochemical marker of apoptosis and found to be increased by SBA treatment at time points that matched those where survivin levels were suppressed, and occurred after the levels of nuclear β-catenin were decreased (**Figure 5C**). There were no changes in PDE5 levels during the treatment period indicating that this enzyme remained stable during apoptosis.

To determine if SBA can inhibit β-catenin mediated Tcf transcriptional activity, we used the TOP/FOP-flash Tcf luciferase assay. As shown in **Figure 5D**, SBA caused a significant decrease in β-catenin-mediated Tcf transcriptional activity. Finally, we confirmed that the PKG activator, 8-bromo-cGMP can also suppress β-catenin levels, which provides additional evidence that the cGMP and β-catenin pathways are interconnected (**Figure 5E**).

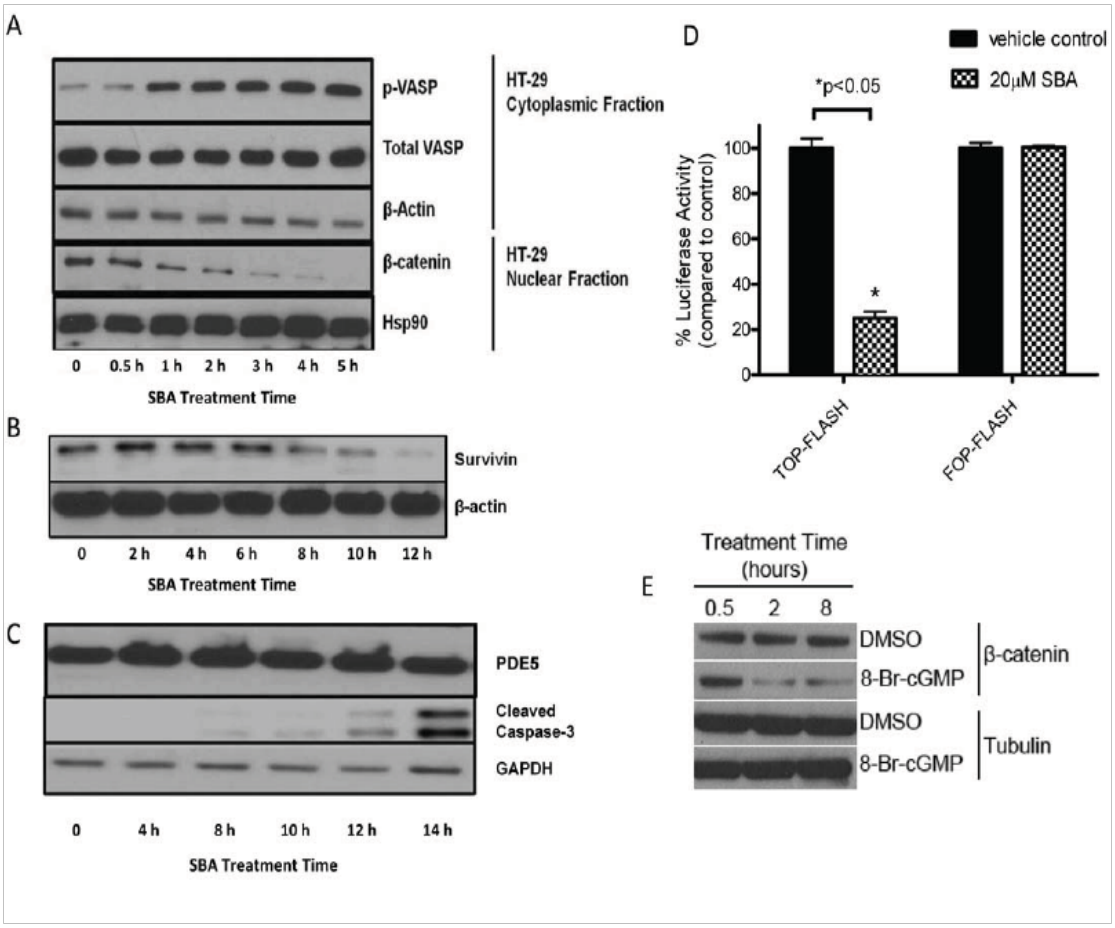

Figure 5. Activation of PKG phosphorylation and suppression of β-catenin transcriptional activity after SBA treatment. A, increased VASP phosphorylation and time-dependent decrease in nuclear β-catenin levels in HT-29 cells treated with SBA. **B**, decreasede β-catenin regulated protein, survivin after 10 μmol/L treatment with SBA. **C**, caspase-3 cleavage and PDE5 expression in HT-29 cells treated with 10 μmol/L SBA. **D**, Wnt/β-catenin signaling reporter activity in HCT116 cells treated with SBA for 24 h. **E**, suppression of β-catenin by 8-Br-cGMP (100 μM) in HCT116 cells.

Discussion

These results characterize the activity of a novel benzylamine derivative of sulindac that lack COX inhibitory activity but displays high potency to suppress the growth of colon tumor cells *in vitro*. The improved growth inhibitory potency of SBA was associated with increased effectiveness to inhibit proliferation and induce apoptosis of colon tumor cells. Similar to observations we previously reported for sulindac and

other NSAIDs, the underlying biochemical mechanism responsible for the tumor cell growth inhibitory activity of SBA appears to involve cGMP PDE inhibition, although these results are novel as they demonstrate that derivatives can be synthesized that lack COX inhibitory activity, which display higher potency to inhibit colon tumor cell growth. The cGMP PDE inhibitory activity of SBA was evident by its ability to selectively inhibit cGMP hydrolysis by lysates from colon tumor cells as well as by recombinant PDE5 within the same concentration range as required for inhibition of colon tumor cell growth. Moreover, SBA increased intracellular cGMP levels and activated PKG within the same concentration range as required for cGMP PDE and tumor cell growth inhibition. Consistent with studies showing the ability of PKG to phosphorylate β-catenin to induce degradation, SBA reduced nuclear levels of β-catenin, its transcriptional activity, and survivin levels within a treatment period that preceded the induction of apoptosis. PDE5 siRNA knockdown experiments show that PDE5 is essential for growth of colon tumor cells that can impact the sensitivity to SBA. These observations provide strong evidence that PDE5 inhibition represents an important COX-independent mechanism for the anticancer properties of NSAIDs that can be targeted to develop potentially safer and more efficacious derivatives for CRC chemoprevention.

Our observations are consistent with studies by others who have also concluded that a COX-independent mechanism is fully responsible for or can contribute to the cancer chemopreventive activity of NSAIDs [10-11, 37]. For example, NSAIDs have been shown to suppress the growth of malignant cell lines that do not express COX-2 [37] and supplementation with prostaglandins do not reverse the inhibitory activity on cellular growth [38]. In addition, the rank order potency among NSAIDs to inhibit

prostaglandin synthesis and growth of tumor cells does not correlate [39] as higher doses of NSAIDs are generally required to inhibit tumor cell growth [40]. Although the mechanism by which NSAIDs suppress tumorigenesis *in vivo* may involve both COX-dependent and independent effects, these results suggest that their intrinsic tumor cell growth inhibitory activity only involves a COX-independent mechanism.

Here we show that SBA potently and selectively inhibits cGMP hydrolysis by colon tumor cell lysates without affecting cAMP hydrolysis. This was confirmed by studies using recombinant PDE isozymes where SBA selectively inhibited the cGMP-specific PDE5 isozyme. On the other hand, SS inhibited multiple cGMP PDE isozymes, including PDE2, 3, 5, and 10. Consistent with the importance of PDE5 as a target, we demonstrated that this isozyme is essential for colon tumor cell growth by siRNA knockdown studies. PDE5 knockdown by siRNA also increased the sensitivity of colon tumor cells to SBA treatment. In addition, PDE5 was found to be elevated in human colon tumor cells compared with normal colonocytes and that the expression level was associated with the tumor selectivity of SBA. These observations are consistent with previous immunohistochemistry studies showing that PDE5 is overexpressed in human colon adenomas and adenocarcinomas as well as in bladder, lung and breast tumors [25, 28, 41-42]. However, we cannot rule out the potential involvement of additional cGMP PDE isozymes that might be expressed in tumor cells and sensitive to this class of compounds. In support of this possibility, FDA approved PDE5 inhibitors such as tadalafil used for the treatment of erectile dysfunction require high concentrations to inhibit tumor cell growth (micromolar levels) compared with concentrations required to

inhibit PDE5 in cell-free assays (nanomolar levels) [43], while others such as sildenafil are completely inactive.

Of relevance to our findings that cGMP elevation can inhibit growth and induce apoptosis of colon tumor cells, other investigators have shown an association between cGMP elevation and inhibition of colorectal tumorigenesis. Most notably, Shailubhai and colleagues demonstrated that oral administration of the enteric peptide hormone, uroguanylin, which binds a membrane associated guanylyl cyclase coupled receptor to increase intracellular cGMP levels, inhibited tumor formation in the *Apc*^{min} mouse model and increased rates of apoptosis within the tumors [44]. These experiments also reported that uroguanylin levels were reduced in both colon adenomas and adenocarcinomas, suggesting that cGMP levels may be aberrantly low in colon tumors compared to normal mucosa. Other studies have shown that human colon tumor cell lines transfected with constitutively activated mutants of PKG can undergo apoptosis and are unable to form colonies [45]. PKG is also down-regulated in many cancer types, including colorectal cancer [46], which suggests that suppression of the cGMP pathway may provide a growth or survival advantage to tumor cells.

Given that β-catenin is an important oncogenic protein involved in CRC, the observations that SBA can suppress β-catenin and its transcriptional activity support the possibility that this non-COX inhibitory derivative of sulindac (or related analogs) will be effective for CRC chemoprevention as has been established for other NSAIDs that inhibit COX-1 and/or COX-2. We found that SBA treatment can induce β-catenin degradation, which is consistent with previous studies showing that PKG can phosphorylate β-catenin to induce degradation [23, 47]. The time-dependent activation of PKG by SBA paralleled

the time required for cGMP elevation that is followed by the suppression of β-catenin nuclear and survivin levels. These events preceded caspase activation and suggest that the cGMP/PKG and Wnt/ β-catenin pathways are interconnected to trigger conditions that lead to apoptosis induction.

In conclusion, these results support further studies to evaluate the efficacy and toxicity of SBA or other non-COX inhibitory derivatives of sulindac for cancer chemoprevention in animal models. Additional studies are also warranted to better define the role of cGMP and cGMP-degrading PDE isozymes in colorectal tumorigenesis.

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CHAPTER 6

DISCUSSION

Several lines of evidence from *in vitro*, animal and clinical studies indicate the NSAID sulindac has antineoplastic activity independent of COX inhibition [69, 138, 150-151]. The goal of this dissertation was to describe some of the structural requirements for the antineoplastic activity of sulindac and the central hypothesis was that the antitumor efficacy of sulindac results, in part, from the inhibition of cGMP-specific phosphodiesterases. To address this hypothesis, three specific aims were formulated. The purpose of Aim 1 was to determine the structural requirements of sulindac derivatives that enable *in vitro* CRC growth inhibition independently of COX inhibition. Determining if there exists a correlation between the inhibition of cGMP phosphodiesterases and growth inhibition was the focus of Aim 2. The purpose of Aim 3 was to characterize the mechanism of cGMP-mediated antiproliferative and anti-apoptotic activity. These studies found that substitution of the carboxylic acid group of sulindac with a positively charged group substantially decreased the cyclooxygenase inhibitory activity of sulindac derivatives, yet some of these derivatives more potently suppressed the growth of colorectal cancer cells. I found a sulindac derivative that demonstrated a strong correlation between the ability to increase intracellular cGMP levels, growth inhibition, and apoptosis. The mechanism of cGMP-mediated growth suppression appeared to occur through protein kinase G activation, which was followed by a decrease in nuclear β-catenin levels and β-catenin-mediated Tcf transcriptional activity.

The first step in Aim 1 was the synthesis of sulindac analogs by the medicinal chemists at Southern Research Institute. Their synthesis efforts were guided by a previously published report identifying the negatively charged carboxylic acid moiety of sulindac sulfide as an important structural component for COX inhibition [151]. Those results indicated that replacing the negatively charged group with a positively charged group could remove the COX-inhibitory activity of sulindac analogs. Purified enzyme and cell based assays confirmed that substitution of the carboxylic acid moiety of sulindac sulfide with an amine or amide group resulted in a compound with significantly impaired ability to inhibit prostaglandin synthesis. Several of these compounds showed similar or greater potency for inhibiting the growth of HCT 116, HT-29 and SW-480 colorectal cancer cell lines. These results were consistent with the observation that the non-COX inhibitory sulfone metabolite of sulindac can inhibit the growth and induce apoptosis of colon tumor cells *in vitro* [150, 152]. Among the compounds synthesized, a trimethoxy sulindac derivative with greater potency was identified. The structural features of this compound were very similar to CP248, a sulindac derivative that had previously demonstrated the ability to perturb cell growth by interfering with microtubule polymerization [153]. However, a novel sulindac benzylamine (SBA) analog was identified that was 20-fold more potent than the parent compound, sulindac sulfide, in growth inhibition assays. These results confirmed that COX inhibition wasn't necessary to inhibit the growth of colorectal cancer cells and provided a unique new compound to be used in determining the pathway or pathways that are responsible for the increased antitumor activity.

The inhibition of cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) activity has been strongly implicated in the antineoplastic activity of the sulfide and sulfone metabolites of sulindac [40, 142]. The purpose of Aim 2 was to determine if SBA could inhibit cGMP PDE enzyme activity and if there was a correlation with growth inhibition and apoptosis. The measurement of cGMP in fresh HT-29 cell lysate treated with sulindac sulfide indicated that cGMP hydrolysis by phosphodiesterases was impaired with an IC_{50} of 49 µmol/L. SBA inhibited cGMP hydrolysis in HT-29 cell lysate with an IC_{50} value of 8 µmol/L, indicating an increased potency in inhibition of cGMP phosphodiesterase activity compared to the parent compound. In comparison, cAMP hydrolysis was not significantly inhibited by either sulindac sulfide or SBA (Chapter 5, figure 3A). Recombinant enzyme assays of cAMP and cGMP-specific PDE isozymes identified the cGMP-specific phosphodiesterase PDE5 as the most likely target of SBA (Chapter 5, table 1). In addition, PDE5 was found to be elevated in human colon tumor cells compared with normal colonocytes and the expression level was associated with the tumor selectivity of SBA (Chapter 5, figure 4d,e). PDE5 knockdown by siRNA increased the sensitivity of colon tumor cells to SBA treatment, confirming the connection between PDE5 and the sensitivity of CRC cells to SBA. Molecular modeling efforts found that the indene scaffold common to both sulindac and SBA can bind to the same hydrophobic area within the catalytic site of PDE5 and the sulfoxide group of SBA occupied the polar area, similar to the phosphate group of GMP (Chapter 5, figure 4f). All of these results are consistent with previously published reports linking increased cGMP levels with growth inhibition [89].

To date, no one has conclusively shown the direct target or mechanism by which sulindac exerts its antineoplastic effect, although several studies have implicated a pathway involving cGMP- dependent protein kinase(PKG) [144] [91]. Treatment of HT-29 cells with SBA increased the phosphorylation of vasoactive stimulatory protein (VASP) at the Ser^{239} residue, which is a useful marker of PKG activation [154] [155]. This firmly established the activation of PKG and the downstream effects as a mechanism of action for SBA, which would be studied further in Aim 3. Concurrent with the activation of PKG, nuclear levels of β-catenin decreased in a time-dependent manner (Chapter 5, figure 5a). Additionally, the treatment of cells with 8-bromo-cGMP, a PKG activator, reduced β-catenin levels (Chapter 5, figure 5e). SBA treatment also reduced the amount of β-catenin mediated transcription without affecting PDE5 protein levels. PKG activation, β-catenin levels, and reduced transcriptional activity occurred prior to apoptosis while PDE5 expression levels remained unchanged. These results are supported by previous reports that sulindac and sulindac metabolites induce apoptosis via a β-catenin mediated pathway [40] [156].

As previously mentioned, several mechanisms are eliciting the effects of sulindac and its metabolites in cancer cells [76], [75, 80]. Experiments using the sulindac capture compound (SCC) identified previously unreported targets for sulindac (Chapter 4). Although PDE5 was not identified with the SCC, different coupling positions and linker lengths of the SCC may display different capturing characteristics. In general, the longer the linker, the better the capture compound binds to targets [161]. The molecular modeling results of the SBA-PDE5 complex (Chapter 5, figure 4f) suggests the best linking position for an SBA capture compound would be *meta* to the sulfoxide group or

at the *para* (C-28) position of the benzylamine group. These linking positions would be the least likely to interfere with binding of the compound to the active site of PDE5, although targets other than PDE5 may still be identified with an SBA capture compound possessing a different linker position. A capture compound containing only the linker, azido, and biotin groups is available and can be used in future experiments as a control to identify non-specific binding of proteins to the capture compound. Using cell lysates from sensitive and refractory cell lines in the capture compound pull-down assay will allow us to determine the relative importance of different targets for cancer treatment and determine targets that could result in toxic effects on healthy cells. The identified targets can be prioritized for future studies of this class of compounds.

Although our studies indicated PDE5 was a low micromolar target of SBA, higher concentrations may be required in animal models of colorectal cancer, such as the *Apc* $^{Min/+}$ mouse model [157]. In support of this possibility, FDA approved PDE5 inhibitors such as tadalafil used for the treatment of erectile dysfunction require high concentrations to inhibit tumor cell growth (micromolar levels) compared with the concentrations required to inhibit PDE5 in cell-free assays (nanomolar levels) [145], while sildenafil is completely ineffective. Pharmacokinetic studies still need to be performed to determine the metabolic products of SBA *in vivo*. To be thorough, cell culture will also need to be analyzed to determine the metabolic products of SBA *in vitro.* However, the structure of SBA is likely stable in cell culture. Unlike a benzylamide group, the benzylamine group is not a substrate for amidases. Additionally, the proximity of the benzyl group decreases the basicity of the amine nitrogen of SBA through resonance interaction and this will decrease the overall reactivity of the compound. Transport out of the cell by various drug

transporters may be a more serious factor in determining whether therapeutically relevant concentrations of SBA can reach the target. Our research with the multidrug transporters suggests SBA will not be transported by either the ABCC1or ABCB1 members of the ATP binding cassette (ABC) family of proteins since they are responsible for the efflux of more hydrophobic and negatively charged substrates(Chapter 2,3). The drug combination experiments I used to determine synergism between doxorubicin and the 5-quinolinone SRI-22049 (Chapter 2, figure 8) could be used to determine if SBA is a potential substrate for the ABC transporters. If SBA is a possible substrate, vesicular transport assays can be used to directly measure the transport of SBA by ABC transporters [158]. Of the three ABC transporters most involved in multidrug resistance, ABCG2 (Breast cancer resistance protein) is the most likely candidate to alter the efficacy of SBA in CRC due to its involvement in the export of xenobiotics from the apical membrane of intestinal epithelial cells [159] and the ability to transport both positively and negatively charged compounds [160].

Overall, the results from these experiments have provided a strategy for optimizing the pharmacological effects of sulindac analogs that do not inhibit the COX enzymes. The screening of sulindac analogs found several compounds that inhibited the *in vitro* growth of colorectal cancer cells at concentrations significantly lower than the parent compound. In the absence of pharmacokinetic studies, SBA is a good lead candidate for further synthesis efforts targeting PDE5 in colorectal cancer. Due to their lack of COX inhibition, these compounds could conceivably be administered at higher doses than NSAIDs without the associated gastrointestinal and cardiovascular side effects. These compounds will form the basis for developing a structure activity

relationship (SAR) that will allow the exploration of other "off-target" effects of sulindac *in silico.* Those results will be used to guide the synthesis of more potent, selective inhibitors of PDE5 as well as other targets.

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