THE ROLE OF SMAC IN NSAID-INDUCED APOPTOSIS

by

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Submitted to the Graduate Faculty of

School of Medicine, Department of Pharmacology and Chemical Biology

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2008

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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May 21, 2008

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ABSTRACT

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Nonsteroidal anti-inflammatory drugs (NSAIDs) are effective in cancer prevention and have been shown to suppress the formation of colorectal tumors in both humans and rodents. The chemopreventive action of NSAIDs is believed to be mediated through induction of apoptosis in preneoplastic cells. However, the precise molecular mechanisms of NSAID-induced apoptosis remain unclear. Previous studies demonstrated that second mitochondria-derived activator of caspase (SMAC) plays an important role in executing NSAID-induced apoptosis in colon cancer cells. SMAC-knockout HCT116 colon cancer cells are resistant to NSAID-induced apoptosis, and are deficient in caspase activation and cytosolic release of cytochrome *c* and apoptosis inducing factor (AIF). In this study, we tested the hypothesis that SMAC regulates the release of cytochrome *c* and activation of caspase cascade through a feed-back amplification loop. We found that the N-terminal AVPI domain

of SMAC is required for the pro-apoptotic activity of SMAC. Following NSAID treatment, SMAC promotes dissociation of caspase-3 from inhibitor of apoptosis proteins (IAPs), which in turn leads to mitochondrial dysfunction. We also studied the effects of pharmacological manipulation on NSAID-induced apoptosis by employing small molecule compounds that functionally mimic the AVPI domain of SMAC. A synergistic action of NSAIDs and SMAC mimetics was observed in inducing a robust apoptotic response in several colon cancer cell lines, as well as in NSAID-resistant *BAX*-KO and *SMAC*-KO cell lines. SMAC mimetics appear to potentiate NSAID-induced apoptosis by stimulating the release of cytochrome *c* from mitochondria and activation of caspases. Together, these results suggest that SMAC may be useful as a target for the development of more effective chemopreventive agents.

FOREWORD

I would like to acknowledge and thank the people who have made my career as a graduate student possible:

To my mentor, Lin Zhang, and his wife, Jian Yu, for unwavering support throughout my entire project.

To my thesis committee members: Dr. Robert W. Sobol, Jr., Dr. Daniel E. Johnson, Dr. Yu Jiang, and Dr. Xiao-Ming Yin, for their time and advice regarding the development of my thesis project.

To the past and present members of Zhang/Yu lab, for helpful discussions and friendly attitudes.

To my son Samuel, for helping me put all my achievements in perspective.

I dedicate this work to my wife, Irene, who stood by me from my first day as a graduate student. She has felt all of my sad moments (sometimes, sad weeks...) and cheered with me during all of my moments of success; she believes in me and always reminds me that the struggling road uphill will inevitably lead to satisfaction and pride.

PREFACE

Parts of this dissertation have been presented in an original manuscript:

Alexander Bank, Peng Wang, Chunying Du, Jian Yu, and Lin Zhang.

SMAC Mimetics Sensitize Nonsteroidal Anti-inflammatory Drug–Induced Apoptosis by Promoting Caspase-3–Mediated Cytochrome c Release.

Cancer Res. 2008. **68**(1): p. 276-284.

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1 INTRODUCTION

1.1 INTRODUCTION TO CANCER

Human bodies comprise a conglomerate of approximately 50 trillion cells, of which there are several billion cells that are actively growing, dividing, and then dying in a predictable fashion. These critical cellular mechanisms are guarded by numerous and stringent checkpoints that oversee various aspects of cellular existence and together strive to detect and eliminate any aberrant events that might compromise the existence of a whole organism. Cancer can occur when a particular cellular pathway loses its ability to effectively control cell growth, cell division or cell death, causing uncontrolled cell proliferation and growth. It is largely believed that cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis.

According to the estimates provided by the World Health Organization, there are currently 11 million new cases of cancer being diagnosed per year, and this number will increase to 16 million by 2020 [1]. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs. Most cancers arise from cells of epithelial origin that cover the body and line internal organs. Cancers of the epithelial cells are categorized as carcinomas, and together these malignancies make up about 85% of all cancers, with skin, lung, colon, breast, prostate, and uterus being the most frequent sites. Despite numerous improvements in the early detection and treatment of various forms of human cancer, the overall

mortality rates for most epithelial cancers have not declined during the last three decades [2].

1.2 CANCER INITIATION AND PROGRESSION

Tumor formation involves the progressive, multi-step transformation of normal human cells into highly malignant derivatives. Carcinogenesis can be viewed as a process that involves accelerated and abnormal cellular changes in which the genes controlling proliferation, differentiation, and cell death are transformed – mutated or deleted – in ways that endow the transformed cells with selective growth advantages among the environmental pressures of the surrounding tissue [3]. The development of a tumor from an initial transformation event follows three distinct phases: initiation, promotion, and progression [4, 5]. The initiation phase is a rapid and irreversible event that takes place when a normal cell is exposed to a biological, chemical or physical carcinogen. The initial insult to the overall integrity of the cellular genome then causes unrepairable or misrepaired DNA damage that is reiterated via continued cell division to yield a clone of mutated cells – a second step in tumor formation called the promotion phase, during which there occurs an expansion of the primary mutated cells to form an actively proliferating, premalignant lesion. During the final progression phase, an additional irreversible event occurs within the tumor mass that produces new clones with increased proliferative capacity, invasiveness, and metastatic potential [6]. Since the initiation and progression phases comprise the irreversible and relatively transient events during the process of tumor formation, the promotion phase of carcinogenesis may be most suitable for application of cancer preventive strategies [7, 8].

1.3 COLORECTAL CANCER

Colorectal cancer represents a major global health problem and is one of the leading causes of cancer mortality in the United States and other developed countries. In 2005, colorectal cancer was reported to be the third most common malignancy in the United States, with 145,000 newly diagnosed cases that account for almost 12% of all newly diagnosed cancers, and the second most lethal malignancy, with 56,000 deaths that account for about 10% of total cancer deaths [9, 10]. Worldwide, more than a million new colorectal cancer cases are diagnosed yearly. On average, the cumulative probability of developing colorectal cancer from birth to age 79 years is approximately 4% in men and 3% in women; this probability increases to 5.6% in both genders over full-life expectancy [10].

As in other systems such as skin cancer, colorectal carcinogenesis has been shown to involve many genetics steps [11-14]. The risk of colorectal cancer is much higher in patients with a strong family history or genetic mutations that result in an increased predisposition for development of colorectal cancer. The triggering events in colorectal carcinogenesis were identified through molecular genetic studies of hereditary forms of the disease. One such hereditary condition is familial adenomatous polyposis (FAP), a rare autosomal dominant disease affecting one in 13,000 births that is associated with an increased risk of colorectal cancer and is responsible for 1% of colorectal carcinomas detected in the general population [15, 16]. FAP was found to be associated with an interstitial deletion on human chromosome 5q21 and ultimately caused by a germline inactivation of one allele of

the adenomatous polyposis coli (*APC*) gene, which may be somatically mutated in up to a half of all spontaneous colorectal cancer cases [14, 17-19]. The combined activities of the APC protein and glycogen synthase kinase-3β lead to phosphorylation and subsequent degradation of cytoplasmic β-catenin, which functions in establishing cell-to-cell contact through the adherens junctions [20]. Loss of APC function through germline or somatic mutations of the *APC* gene results in subsequent stabilization of cytoplasmic β-catenin, which undergoes translocation to the nucleus and functions as a transcription factor. Nuclear β-catenin has been reported to activate the expression of a number of genes, including *c-myc*, *cyclin D1*, and *MMP-7* [21]. Patients with FAP develop hundreds or even thousands of precancerous polyps located in the colon and rectum. The evolution of a polyp from a precancerous stage to invasive cancer takes 4-11 years, and nearly all patients will develop colorectal cancer if left untreated (Figure 1.1) [22]. Current treatment options for FAP patients primarily involve colectomy – the surgical removal of sections or the entire length of the colon, followed by life-long surveillance for appearance of new premalignant polyps [23].

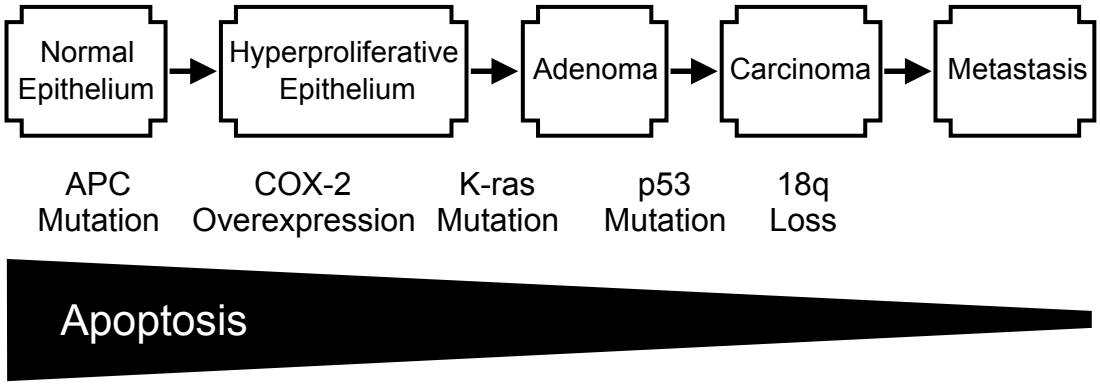


Figure 1.1 Colorectal carcinogenesis involves multiple genetic steps.
Apoptosis is gradually inhibited as precancerous cells in the colonic epithelium lose the activity of tumor suppressor genes, including APC and p53, and acquire oncogenic mutations, promoting the progression to carcinoma.

Recent years afforded increased understanding of the regulation of complex molecular events that take place during the course of cancer progression. Evidence for a potential involvement of a group of enzymes called cyclooxygenases (COXs) in colorectal cancer was derived from pharmacologic analyses of prostaglandins – unsaturated carboxylic acids consisting of a 20-carbon skeleton that also contains a five member ring. Prostaglandins play a central role in inflammation and regulate physiologic functions such as gastrointestinal (GI) cytoprotection, vascular homeostasis, renal function and blood clotting [16]. Various animal and human tumor tissues, including human colon cancer, have been reported to contain high concentrations of prostaglandins [24, 25]. Cytoplasmic phospholipase A2, activated in response to various stimuli, catalyzes the release of arachidonic acid, the most abundant polyunsaturated fatty acid in the phospholipid component of cell membranes [26]. Prostaglandins are biochemically synthesized from arachidonic acid by several isoforms of cyclooxygenase (COX). COX-1 and COX-2 are structurally distinct, membrane-associated proteins, sharing 60% sequence homology. While COX-1 is constitutively expressed in many tissues, including the gastrointestinal (GI) tract, kidneys, and central nervous system, contributing to their homeostasis and proper functions, COX-2 is induced in many inflammatory reactions by various cytokines, mitogens, growth factors and tumor promoters [27, 28].

1.4 CHEMOPREVENTION OF COLORECTAL CANCER BY NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

Early experiments that demonstrated elevated levels of prostaglandin E2 in human colorectal tumor tissue as compared to the surrounding mucosa provided the rationale for blocking prostaglandin production via the inhibition of COX enzymes as a chemoprevention strategy for colorectal cancer [24, 29]. The term chemoprevention, originally coined by Michael Sporn in 1976, refers to the use of natural or synthetic agents to slow the progression of, reverse, or inhibit the process of carcinogenesis, thereby lowering the risk of developing invasive or clinically significant disease [2, 7, 30]. Consequently, an effective cancer chemopreventive agent should intervene during the early stages of carcinogenesis in order to inhibit the initial events that lead to transformation of normal cells and eliminate premalignant cells before they reach their malignant potential and pose a health risk to the patient (Figure 1.1) [31-33].

For this purpose, a group of agents called nonsteroidal anti-inflammatory drugs (NSAIDs) were evaluated as potential chemopreventive agents due to their general or selective ability to inhibit the activity of COX-1 and/or COX-2, subsequently blocking the endogenous biosynthesis of prostaglandins. The same active sites employed by both COX enzymes for binding their natural substrates and catalyzing prostaglandin biosynthesis are used by NSAIDs to inhibit their respective activities, namely by excluding access for arachidonic acid into the active binding channel [34]. The common use of NSAIDs for the control of pain, inflammation and

prevention of vascular occlusive diseases has facilitated investigation of the effect of these drugs on GI cancer risk. The use of NSAIDs was found to affect neoplastic and non-neoplastic cellular proliferation, tumor growth, and play a role in the control of immune response [35-37]. Additionally, the inhibition of COX-1 has been related to adverse effects in the GI tract, while inhibition of COX-2 has been more directly implicated in reducing inflammation [38]. Selective COX-2 inhibitors were subsequently designed that specifically inhibit prostaglandin formation at sites of inflammation while sparing the protective effects of COX-1–mediated production of prostaglandins on the GI epithelium, reducing the occurrence of potentially dangerous and life-threatening side-effects.

Studies in a variety of rodent animal models (both genetic and carcinogen-induced) of colon cancer have indicated a significant reduction in the incidence of colorectal adenomas and tumors with treatment using NSAIDs [13, 14, 24, 39]. In experimental animals transplanted with various tumors, the NSAIDs indomethacin, aspirin, and piroxicam were shown to reduce tumor growth [40, 41]. In chemical carcinogen-induced rat and mouse tumor models for colorectal cancer, the NSAIDs indomethacin, meclofenamate, piroxicam, sulindac, and aspirin were shown to significantly decrease the incidence, multiplicity, and/or size of colon tumors in rats or mice, and this effect was sometimes observed even many weeks after carcinogen challenge [38, 42]. The *APC*$^{Min/+}$ mouse has provided an important animal model to study the molecular events in colorectal carcinogenesis by recapitulating the features of human FAP syndrome. In the *APC*$^{Min/+}$ mouse, the *APC* gene is disrupted or inactivated, leading to the development of gastrointestinal (GI) adenomas by 110 days

of age. Both traditional NSAIDs and selective COX-2 inhibitors have also been shown to induce tumor cell death and inhibit tumor development in *APC*$^{Min/+}$ mice and other murine models of FAP [43-47]. These reports suggest that NSAIDs act to suppress tumor formation in the rodents during initiation and/or progression.

On the basis of the results of the early animal experiments, a number of case series and at least 5 randomized clinical trials investigating sulindac administration were initiated in FAP patients. The majority of the clinical studies have proven the efficacy of NSAIDs in reducing tumor burden in patients with FAP; sulindac produced a statistically significant reduction in both the number and size of the polyps in these patients [16]. As in the uncontrolled trials, however, the polyps tended to increase in both number and size once sulindac treatment was discontinued. Accordingly, sulindac treatment of preoperative FAP patients was not deemed complete enough to replace colectomy, although it may be useful as an adjunct to surgery in postoperative cases [48, 49]. One of the problems often faced in sulindac treatment of FAP patients appears to be the severe side effects that are common to NSAIDs, e.g., bleeding and ulceration. Although some patients tolerate sulindac without such problems, its side effects can be serious, even fatal, in others. For this reason, there has been a strong effort to develop new therapeutic agents that lack these side effects, such as selective COX-2 inhibitors.

Another line of evidence for the inhibition of colorectal tumorigenesis by NSAIDs came from a large number of epidemiologic studies, which were encouraged by the animal and FAP studies. These epidemiologic studies, including Cancer Prevention Study II (662,424 patients) and Health Professionals Follow-Up Study

(47,900 men), have documented up to 50% reduction in colorectal adenomas, colorectal cancer, and colorectal-associated mortality in individuals regularly taking NSAIDs (ex. aspirin) [16]. Also, there was an indication of a strong protective effect of NSAIDs (primarily aspirin) on the occurrence of adenomatous polyps and the incidence of mortality from colorectal cancer [16].

In studies analyzing the effect of NSAIDs in high-risk patients with recurring colorectal cancer, the rate of recurrence of adenomas was significantly lower in the patient group taking lower-dose aspirin (81 mg/d) than in the placebo group, whereas the larger dose of aspirin (325 mg/d) did not significantly reduce the rate of recurrence, indicating that low-dose aspirin may have some benefit in reducing the risk of recurrent adenomas among persons with a history of colorectal cancer or adenomas (both doses of aspirin appeared to reduce the levels of colorectal prostaglandins to a similar extent) [50-52]. Results from these and many other observational studies strongly support the hypothesis that NSAIDs inhibit the incidence, progression, and death due to colorectal cancer. These benefits do not appear to be influenced by study design, specific NSAIDs, or patient characteristics including age, gender, and nationality.

1.5 APOPTOSIS AS A MECHANISM OF COLORECTAL CANCER CHEMOPREVENTION BY NSAIDS

During more than 30 years of pre-clinical and clinical research, NSAIDs have been shown to exert several effects in a variety of malignancies that may contribute to a broad spectrum of chemopreventive activities at different stages of carcinogenesis [53]. Numerous published reports of experiments on animal models, epidemiologic studies, and clinical trials indicate that NSAIDs can slash the incidence of colorectal cancers in human and experimental animals and can reduce the number and size of polyps in patients with FAP [54]. A number of population-based studies have detected a 40-50% decrease in the relative risk of colorectal cancer in persons who regularly use aspirin and other NSAIDs [27, 36, 55, 56]. Randomized secondary prevention and primary prevention trials in high-risk patients have clearly demonstrated that NSAID treatment caused regression of preexisting adenomas [37, 57].

Initial attempts to elucidate the molecular basis of NSAID-mediated anti-cancer action in colorectal chemoprevention led to observations that both human and animal colorectal tumors express high levels of COX-2, whereas the normal intestinal mucosa has low to undetectable levels of COX-2 expression, leading to the hypothesis that COX-2 may be playing a role in colon cancer and progression [58, 59]. Both *in vitro* and *in vivo* studies have demonstrated that proliferation of human colon cancer cell lines was inhibited by treatment with a highly selective COX-2 inhibitor in tumors that express COX-2, but not in those that lack COX-2 expression

[60, 61]. Additionally, when the COX-2 gene was inactivated in *APC*$^{Min/+}$ mice, both the number and the size of polyps were reduced dramatically, while inhibition of COX-2 expression was associated with reduced development of GI malignancy in humans and in animal models of colorectal cancer [62].

Despite a wealth of experimental and clinical data favoring the role of COX inhibition as the primary effect of NSAID-induced chemopreventive action, accumulating evidence suggests that aspirin and other traditional, non-selective NSAIDs may exert their effects by COX-independent mechanisms, such as the ability of NSAIDs to restore apoptotic cell death in colon cancer cells, inhibition of nuclear factor-κB or interference with the binding of the nuclear peroxisome-proliferator activated receptor δ to DNA [43, 63-68]. In favor of this notion, one of the major metabolites of an extensively studied chemopreventive NSAID sulindac, sulindac sulfone has been reported to be at least 5,000 times less potent than sulindac sulfide at inhibiting prostaglandin synthesis while retaining the ability to induce apoptosis in various cell types to approximately same degree [66, 69-71]. However, the specific mechanisms of these COX-independent effects and their therapeutic implications are not yet well understood.

Several animal studies have demonstrated that a number of chemopreventive agents exhibit the ability to induce apoptosis in various types of tumor cells. In the *APC*$^{Min/+}$ mouse model of human colorectal cancer, aspirin decreased the spontaneous rate of colon tumor formation by 44% and normalized the growth of enterocytes, the differentiated epithelial cells located in the lining of the intestinal mucosa that participate in absorption, by increasing apoptosis and reducing the proliferation of

preneoplastic intestinal mucosa cells [46]. The chemopreventive properties of dietary administration of sulindac were associated with a significant induction of apoptosis in colon tumor cells, combined with an overall increase in the number of apoptotic cells, in a rat model of chemical carcinogen-induced colon cancer [72].

Human chemoprevention trials have also demonstrated an association between a clinical response to NSAIDs and the induction of apoptosis in tumor cells [73-75]. Increased apoptosis in colorectal polyps was observed in a phase I clinical trial that studied the chemopreventive properties of the NSAID sulindac sulfone in FAP patients and another study examining the chemopreventive effects of the NSAID mesalazine in colorectal cancer patients [73, 75]. Both agents did not affect cellular proliferation in the malignant and normal tissue samples, indicating the apparent selectively in apoptosis induction in tumor cells by NSAIDs.

1.6 MECHANISM OF APOPTOSIS

One of the earliest observations of apoptosis induction in cancer cells came from the landmark study investigating the effects of etoposide [76]. Since then, evidence supporting the role of apoptosis in chemotherapy action continues to accumulate [77]. Apoptosis represents a regulated cell suicide program that is distinct from necrosis and is essential for organism development and for adult tissue homeostasis [78]. The initiation of apoptosis can be highly dependent on cell type and nature of the particular apoptosis stimulus. The typical morphologic markers used to identify a cell that is actively undergoing apoptosis include DNA fragmentation, chromatin condensation, cell shrinkage, plasma membrane 'blebbing', loss of cell surface features, detachment from the basement membrane and its neighbors, and externalization of phosphatidylserine to the outer leaflet of plasma membrane (signaling for phagocytic cells to engulf the dying cell). These dramatic changes are mediated through the cell-wide activation of cellular hydrolases that include a family of proteases called caspases as well as nucleases that participate in the degradation phase of apoptosis through the cleavage of proteins and DNA [79]. Caspases represent a family of cysteine-containing aspartate-specific proteases that are initially present in the cell as inactive proenzymes and are subsequently activated by cleavage at specific aspartate cleavage sites. The N-terminal pro-domain of caspases is released, while the remaining two subunits form an active heterodimer. The activation of caspases often occurs in a sequential fashion. The family of caspases can be subdivided into initiators (caspase-2, caspase-8, caspase-9) and

executioners (caspase-3, caspase-6, caspase-7). During the progression of apoptosis, the cell separates into membrane-bound fragments called apoptotic bodies without releasing cellular components into surrounding tissues, thus avoiding the initiation of an inflammatory response to the dying cell. In the colon, apoptotic cells can be sloughed into the lumen or the apoptotic bodies can be engulfed by adjacent colonocytes or macrophages in the lamina propria [80].

A wealth of research effort currently supports the notion that the mechanism of apoptotic cell death is controlled via two major pathways, one that originates at the death receptors on the plasma membrane – termed the extrinsic pathway, and another that originates at the mitochondria – termed the intrinsic pathway (Figure 1.2).

The activation of the extrinsic pathway of apoptosis takes place at the cell surface when a specific cell surface death receptor, including tumor necrosis factor receptor (TNFR), TNF-related apoptosis-inducing ligand (TRAIL) receptor, and Fas, is activated by binding to its corresponding ligand molecule(s). Following ligand binding, there is a trimerization of death receptors at the plasma membrane that subsequently promotes the recruitment of adapter proteins, including FADD and RIP1 [81]. The interaction between the adapter proteins, recruited to the activated death receptors, and the zymogen form of caspase-8 results in activation of caspase-8 and activation of downstream caspases, including caspase-3, which may or may not be sufficient to initiate the proteolytic cascade that results in apoptotic cell death, depending on the cell type [79, 81].

The intrinsic pathway, associated with most pro-apoptotic stimuli, relies solely on the permeabilization of the mitochondrial membrane and is controlled by

the carefully coordinated actions of pro- and anti-apoptotic members of the Bcl-2 family, including pro-apoptotic BAX and Bad, and anti-apoptotic Bcl-2 and Bcl-X_L. During conditions of cell stress, the ratio of pro-apoptotic Bcl-2 family members to anti-apoptotic Bcl-2 family members increases, leading to destabilization of the anti-apoptotic Bcl-2 family members present in the outer mitochondrial membrane and the formation of pores or channels in the outer mitochondrial membrane [82, 83].

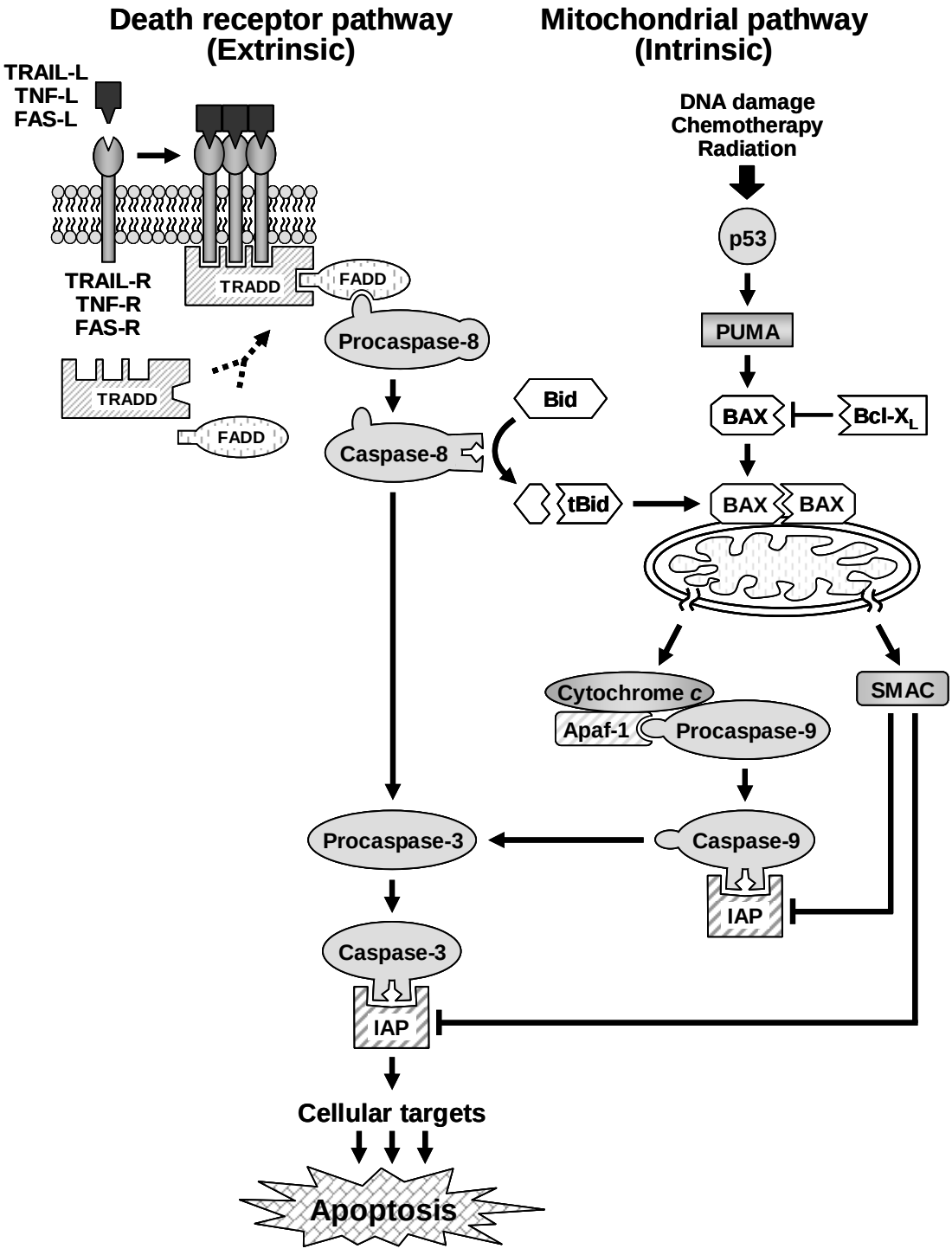


Figure 1.2 Mechanisms of apoptosis.
In the extrinsic pathway, activation of a death receptor by ligand binding leads to cleavage and activation of initiator caspase-8. In the intrinsic pathway, activation of pro-apoptotic proteins results in a release of cytochrome *c*, which interacts with Apaf-1 and procaspase-9 to form the apoptosome that cleaves and activates caspase-9. Activation of caspase-8 or -9 activates executioner caspase-3 [84].

Activation of the intrinsic apoptotic pathway leads to the cytosolic release of several mitochondrial apoptogenic proteins that normally reside in the inter-membrane space of the mitochondria prior to initiation of apoptosis. Once in the cytosol, these proteins can trigger either the activation of the caspase cascade or activation of caspase-independent cell death pathways [85, 86]. Mitochondrial proteins that cause caspase-dependent cell death include cytochrome *c*, SMAC/Diablo and HtrA2/Omi. The cytosolic form of cytochrome *c* interacts with apoptosis protease activating factor-1 (Apaf-1) and the zymogen form of caspase-9 to form a trimeric complex called an apoptosome that subsequently recruits additional pro-caspase molecules and ultimately triggers global activation of the caspase cascade [87]. SMAC and HtrA2/Omi potentiate caspase activation by binding inhibitor of apoptosis proteins (IAPs) and blocking their caspase inhibitory activity [88-91]. Mitochondrial proteins that participate in caspase-independent cell death include apoptosis-inducing factor (AIF) and endonuclease G (EndoG). Both AIF and EndoG undergo nuclear translocation after being released from the mitochondria, where they participate in inducing chromatin condensation and large-scale DNA fragmentation [92]. In addition to mitochondria, it is believed that additional subcellular organelles, including the endoplasmic reticulum, Golgi apparatus, and lysosomes, may participate in damage sensing, pro-apoptotic signaling, and caspase activation [93].

There exist several models that attempt to delineate the precise molecular events that result in the release of mitochondrial proteins. In one model, the mitochondrial permeability transition (MPT), characterized by increased permeability of the mitochondrial membranes, occurs as a result of opening of mitochondrial

permeability transition pores that are formed in the membranes of mitochondria under certain pathological conditions and leads to mitochondrial swelling and ultimately cell death [86, 94, 95]. According to another model, pro-apoptotic Bcl-2 proteins like BAX and/or Bak induce outer-membrane permeabilization through the formation of channels or pores, thus allowing the release of mitochondrial apoptogenic proteins [85, 96, 97].

Recent evidence has revealed the ability of several chemopreventive agents, including NSAIDs, to induce apoptosis in a variety of premalignant and cancer cell types. The chemopreventive effects of NSAIDs were originally envisioned to involve the inhibition of COX enzymes, resulting in modulation of cellular pro-inflammatory prostaglandin synthesis. Presently, there is a change in the prevailing view of how NSAIDs manifest their anticancer effects, with a shift towards appreciating the role of apoptogenic effects of these agents that are independent of their COX inhibitory activities – COX-2 selective celecoxib and nonselective sulindac can both induce apoptosis in COX-2–deficient cells, arguably through COX-2–independent mechanisms, and activate caspases through intrinsic effector mechanisms that are regulated by Bcl-2 family members or the mitochondrial permeability transition. At the same time, there are data suggesting that sulindac may also employ extrinsic effectors in addition to its ability to engage the intrinsic effects, necessitating further research into the precise mechanism of apoptosis induced by NSAID treatment [98].

2 MATERIALS AND METHODS

2.1 CELL CULTURE

The colorectal cancer cell lines used in the study, including HCT116, HT29, and DLD1, were obtained from American Type Culture Collection. *SMAC*-knockout (*SMAC*-KO) and *BAX*-knockout (*BAX*-KO) HCT116 cell lines were previously described [71, 99]. All cell lines were cultured in McCoy's 5A media (Invitrogen) supplemented with 10% defined fetal bovine serum (Hyclone), 100 units/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) and were maintained at 37°C in 5% CO_2. SMAC-reconstituted *SMAC*-KO cell lines were generated by transfecting *SMAC*-KO cells with wild-type (AVPI) or mutant SMAC (ΔA), followed by selection in 0.4 mg/mL G-418 (Invitrogen) [100].

2.2 DRUG TREATMENT

To induce apoptosis, cells were plated at 20% to 30% density in 12-well plates and allowed to attach overnight. DMSO stock solutions of sulindac sulfide (Merck) at 40 mmol/L and indomethacin (Sigma) at 100 mmol/L were freshly prepared and diluted into appropriate concentrations by cell culture medium. Different concentrations of sulindac and indomethacin were used for apoptosis induction in different colon cancer cell lines and were determined based on their dose responses. The SMAC mimetic and control compounds, C3 and C4, respectively, were provided by Dr. Xiaodong Wang and Dr. Patrick G. Harran at University of Texas Southwestern Medical Center. An independent set of SMAC mimetic test (GT-T) and control (GT-

C) compounds were supplied by TetraLogic Pharmaceuticals. All compounds were prepared as 100 μmol/L stock solutions in DMSO. In some cases, caspase-3 inhibitor Z-DEVD-fmk (20 μmol/L; R & D Systems) was used to treat cells in combination with the agents described above.

2.3 WESTERN BLOTTING

The antibodies used for Western blotting included those against caspase-9, Bid (Cell Signaling Technology), cytochrome *c*, α-tubulin (BD Biosciences), caspase-3 (Stressgen Bioreagents), cytochrome oxidase subunit IV (COX IV; Invitrogen), Bim, and SMAC (EMD Biosciences). Western blotting analysis was performed as previously described [101]. Briefly, cell lysates resuspended in 2 × Laemmli sample buffer were heated 95°C for 20 min and loaded onto a 10-well or a 12-well pre-cast NuPAGE® 10% Bis-Tris gel (Invitrogen). The gels were run in a Novex Xcell *Sure*Lock mini-cell gel apparatus (Invitrogen); the inner chamber was filled with 200 mL NuPAGE MES SDS running buffer (Invitrogen) containing 500 μL NuPAGE antioxidant (Invitrogen), while the outer chamber was filled with 300 mL NuPAGE MES SDS running buffer alone. The proteins were resolved at constant voltage. Prior to transfer, the Immobilon-P (pore size: 0.45μm) transfer membrane (Millipore) was briefly washed with methanol, rinsed with deionized water and equilibrated in Tris-Glycine transfer buffer (48 mmol/L Tris base, 40 mmol/L glycine, 20% methanol, 0.04% SDS). The gels were transferred at constant current.

2.4 IMMUNOPRECIPITATION

Following drug treatment, the cells were harvested by gently scraping with a rubber policeman and resuspended in 1 mL of EBC buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 0.5% Nonidet P-40) supplemented with protease inhibitor mixture (Roche Applied Sciences). The cells were disrupted by sonication and then spun at 10,000 × g for 10 min to collect the cell lysates. For immunoprecipitation (IP), 1-2 μg of IP antibodies were added to 50 μL cell lysates. The mixtures were mixed on a rocker at 4°C overnight. The immunocomplexes were captured by the addition of protein G/A-agarose (Roche Applied Sciences) mixed at 1:5 ratio, followed by incubation at 4°C for 1 hour. The beads were washed four times by EBC buffer and then collected by centrifugation at 6,000 × g. After the final wash, the beads were mixed with 50 μL of 2 × Laemmli sample buffer, heated at 95°C for 15 min, and analyzed by Western blotting.

2.5 NUCLEAR AND CYTOSOLIC FRACTIONATION

Nuclear and cytosolic fractions were isolated from treated cells using Pierce NE-PER kit (Pierce, #78833) according to the manufacturer's instructions.

2.6 APOPTOSIS ASSAYS

After treatment, attached and floating cells were harvested at various time points. The fractions of apoptotic cells were evaluated by nuclear staining and Annexin V/propidium iodide staining. For nuclear staining, cells were fixed in a solution containing 3.7% formaldehyde, 0.5% Nonidet P40, and 10 μg/mL 4',6-diamidino-2-

phenylindole dihydrochloride in PBS and assessed for apoptosis through microscopic visualization of condensed chromatin and micronucleation. For each measurement, at least three independent experiments and a minimum of 300 cells were analyzed. For Annexin V/propidium iodide staining, cells were stained by propidium iodide and then Annexin V-Alexa 594 (Invitrogen) according to the manufacturer's instructions. Flow cytometry was used to quantitate Annexin V–positive and propidium iodide–positive cells. Annexin V protein conjugated with a fluorochrome allows for the detection of phosphatidylserine that is translocated from the inner leaflet to the outer leaflet of the plasma membrane during induction of apoptosis. Propidium iodide only stains non-viable cells and becomes highly fluorescent upon binding of DNA.

2.7 COLONY FORMATION ASSAY

Long-term cell survival was evaluated by colony formation assays. In brief, cells were plated in a single 75-cm^2 flask. Following treatment, cells were harvested by trypsinization and plated in 12-well plates at appropriate dilutions. Cells were allowed to grow for 10 to 14 days before staining with Crystal Violet (Sigma).

2.8 CELL VIABILITY ASSAY

The MTS cell proliferation assay is a colorimetric method for determining the number of viable cells in proliferation. It is composed of solutions of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES). MTS is reduced by mitochondrial activity inside the cells into

formazan product that is soluble in cell culture medium. Cells were plated in 96-well tissue culture plates. Following treatment, cells were incubated with 20 µl of MTS (2.5 mg/mL: Sigma Chemical Co) stock solution in each well. After 30 min incubation under standard conditions of 5% CO_2 and 37°C, the absorbance was read on PerkinElmer Victor3 plate reader at 490 nm. The signal generated (color intensity) is directly proportional to the number of viable (metabolically active) cells in the wells.

2.9 ANALYSIS OF CYTOCHROME *C* RELEASE

Mitochondrial and cytosolic fractions were isolated from treated cells by differential centrifugation as previously described [102]. Briefly, a single 75-cm^2 flask of cells was harvested after one wash with Hanks buffer at 4°C. Whole-cell pellets were resuspended in homogenization buffer (0.25 mol/L sucrose, 10 mmol/L Hepes, pH 7.4, 1 mmol/L EGTA) and subjected to 40 strokes in a 2-ml Dounce homogenizer on ice. The homogenates were subjected to centrifugation at 1,000 × g for 15 min at 4°C to pellet nuclei and unbroken cells. The supernatant was subsequently subjected to centrifugation at 10,000 × g for 15 min at 4°C to obtain cytosolic fractions (supernatant) and mitochondrial fractions (pellet). Mitochondrial pellets were resuspended in homogenization buffer following one wash with homogenization buffer at 10,000 × g for 15 min at 4°C. The concentrations of cytosolic fractions obtained from different samples were normalized using protein assay dye reagent from Bio-Rad. All fractions were mixed with equal volumes of 2 × Laemmli sample buffer for Western blotting analysis.

2.10 RNA INTERFERENCE

RNA interference is a technique whereby protein synthesis is prevented through silencing of the gene-specific mRNA and ultimate inhibition of protein translation. Two caspase-3 small interfering RNA (siRNA) duplexes (Cas-3 986, 5'-UGA GGU AGC UUC AUA GUG Gtt-3'; Cas-3 182, 5'-TGA CAT CTC GGT CTG GTA Ctt-3') were previously characterized [103, 104] and purchased from Dharmacon, Inc., as 20 μmol/L stock solutions. Cells were transfected with siRNA at 100nmol/L final concentration by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Down-regulation of caspase-3 expression was verified by Western blotting.

2.11 STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism IV software. The averages ± 1 standard deviation were displayed in the figures.

3 THE ROLE OF THE BCL-2 FAMILY MEMBERS BAX, BCL-X_L AND MCL-1 IN NSAID-INDUCED APOPTOSIS

3.1 INTRODUCTION

Among many described activities of NSAIDs, induction of apoptosis secondary to inhibition of COX enzymes seems to play a critical role in NSAID-mediated chemoprevention [105-107]. Apoptosis is a major mechanism regulating turnover of intestinal epithelial cells from which colon tumors are derived [108]. During the formation of a colon tumor, apoptosis is progressively inhibited due to oncogenic mutations [109]. Deregulation of apoptosis is necessary for oncogenic transformation and drives neoplastic cells to gain additional tumorigenic features [110]. NSAIDs have been found to induce apoptosis in colon cancer cells *in vitro* and *in vivo* [105, 106]. The apoptotic level can be used as a biomarker for NSAID-mediated chemoprevention [72, 111]. Furthermore, it has been shown that NSAID treatment can reverse the anti-apoptotic effects of COX-2 [63].

Numerous cell line and animal studies have provided a solid foundation for the role of Bcl-2 family proteins in playing a critical and fundamental role in controlling the pivotal aspects of cellular life and death [112]. The initial discovery of mammalian regulators of apoptotic cell death emerged from the studies focused on the genetic changes that occur during the development of human follicular lymphoma. The cloning of the chromosomal breakpoint present in the chronic lymphocytic leukemia cells revealed a translocation of the long arms of chromosomes

11 and 14, resulting in an overabundant activation of the *Bcl-2* gene [113]. It was subsequently shown that the product of the *Bcl-2* gene permitted the survival of cytokine-dependent hematopoietic cells under cytokine withdrawal culture conditions [114]. The observations that followed together led to the development of a model in which cell survival and proliferation were controlled by distinct and separate genetic pathways regulated by members of the Bcl-2 family, with some members such as Bcl-2 and Bcl-X_L inhibiting cell death and others such as BAX inducing cell death.

All Bcl-2 family members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) and can be subdivided into "multidomain" and "BH3-only" groups. The multidomain proteins contain three or four BH domains, while the BH3-only proteins display sequence homology with other members only within an α-helical BH3 domain, which is essential for apoptosis induction [115]. Pro- and anti-apoptotic family members can form heterodimeric complexes through associations between their respective BH1, BH2, and BH3 homology domains. These interactions have an overall effect of regulating each other's function, suggesting that the relative abundance of pro-survival versus pro-apoptotic proteins may act as a rheostat for the suicide program [116]. The alterations in the ratio between pro-apoptotic and anti-apoptotic members of the Bcl-2 family can determine the sensitivity of a cell to induction of apoptosis [95].

It is well known that NSAIDs inhibit cyclooxygenase-2 (COX-2) and other enzymes. However, the mechanisms underlying the anti-neoplastic effects of NSAIDs are currently unclear. Experimental data has implicated the involvement of apoptotic cell death induction as a plausible mechanism behind NSAID-mediated

chemopreventive effects. In mammalian cells, apoptosis induced by anticancer agents is often mediated through mitochondria by the Bcl-2 family proteins. In this pathway, pro-apoptotic Bcl-2 family proteins, such as BAX, mediate the release of cytochrome *c* from the mitochondria, which triggers the caspase activation cascade and other events in apoptosis. Anti-apoptotic Bcl-2 proteins, including Bcl-2, Mcl-1 and Bcl-X_L, inhibit apoptosis presumably through the formation of heterodimers with pro-apoptotic proteins, thus preventing their activation and the subsequent release of cytochrome *c*.

The involvement of pro-apoptotic BAX and anti-apoptotic Bcl-X_L in anticancer effects of NSAIDs has been recently explored in human colon cancer cell lines [71]. These studies, facilitated by the development of a *BAX* knock-out cell line, initially indicated that BAX-deficient HCT116 cells displayed a dramatic resistance to the induction of apoptosis when treated with up to 200 μmol/L sulindac, as evidenced by lack of apoptotic markers, including chromatin condensation, nuclear fragmentation, and annexin-V/PI positivity, that typically occur in wild-type HCT116 cells treated with 120 μmol/L sulindac [71]. Furthermore, it was discovered that the prolonged exposure of HCT116 cells to NSAID treatment resulted in clonal selection and expansion of multiple NSAID-resistant subpopulations of cells. These NSAID-resistant cells have acquired (presumably inactivating) mutations, characterized by insertions or deletions, within the *BAX* gene, bestowing selective growth advantage to cells with a defective *BAX* gene as compared to cells that carried a wild-type *BAX* gene and hence retained the ability to initiate and carry out apoptotic cell death in response to NSAID treatment.

While NSAID treatment did not induce any changes in BAX mRNA expression or protein levels, the expression of Bcl-X_L was substantially reduced by NSAID treatment. The downregulation of Bcl-X_L by NSAIDs coincided with the appearance of apoptotic markers including cleaved caspase-3 and nuclear fragmentation, indicating the significance of the ratio between pro-apoptotic BAX and anti-apoptotic Bcl-X_L in regulating NSAID-induced apoptosis [71]. Expression of ectopic Bcl-X_L rescued HCT116 cells with an intact BAX gene from NSAID-mediated apoptosis and enhanced long-term survival and colony formation ability, indicating the role of Bcl-X_L in mediating resistance to the apoptotic cell death program. Together, the significance of BAX and Bcl-X_L during apoptosis induced by NSAIDs reinforces the overall importance of the stoichiometric ratio among pro-apoptotic proteins and anti-apoptotic proteins for proper control over the cellular propensity to either resist or undergo apoptosis following chemotherapeutic treatment.

Acquired resistance to chemotherapy is an important factor that negatively influences the success of cancer treatment and a crucial determinant of the ultimate outcome of cancer treatment [117, 118]. There are various mechanisms responsible for cancer cell chemoresistance, such as modification of drug-target interactions, decreased uptake or increased elimination of active drug molecules, and apoptosis defects. The evasion of apoptosis is a critical component of oncogenic transformation and chemotherapy resistance. Cancer cells may escape from apoptosis in response to various stimuli, such as chemotherapy and radiotherapy, through overexpression of anti-apoptotic Bcl-2 protein family members or loss of pro-

apoptotic Bcl-2 protein family members. An elevated level of anti-apoptotic proteins of the Bcl-2 protein family, including Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and Bfl-1, has been shown to negatively affect the outcome of chemotherapy in various tumor types [119].

In addition to its apparent ability to engage a BAX-dependent mitochondrial pathway of apoptosis in colon cancer cell lines, sulindac has been recently demonstrated to engage the death receptor pathway of apoptosis via the induction of death receptor 5 (DR5) expression [98]. Following ligand binding (ex. TRAIL), death receptors, including DR5, form homotrimers that signal through the adaptor protein FADD, which subsequently recruits caspase-8. Activation of caspase-8 initiates downstream caspase cleavage events. The human colon cancer cell line HCT116 shows a type II response to activation of the extrinsic apoptotic pathway and requires activation of caspase-9 for TRAIL-induced apoptosis. Previous studies have demonstrated that death receptors can be activated in a ligand-independent manner when the levels of death receptors are increased through endogenous induction or exogenous overexpression. Forced overexpression of DR5, independent of the levels of its ligand, has been shown to induce apoptosis [120]. These findings raise the possibility that sulindac may activate DR5 to induce apoptosis in colon cancer cells in a ligand-independent manner.

An additional mechanism of apoptosis resistance in BAX-deficient HCT116 cells has been recently demonstrated. Experiments using TRAIL treatment in HCT116 wild-type and *BAX*-KO cells indicated that TRAIL induces NF-κB–mediated expression of the anti-apoptotic Mcl-1 protein specifically in TRAIL

resistant *BAX*-KO cells [121]. Overexpression of Mcl-1, first identified as an 'early induction' gene during differentiation of the myeloid cell line ML-1, prolongs the survival of cells exposed to a variety of apoptosis-inducing stimuli, including cytokine withdrawal, staurosporine, etoposide, and UV irradiation, while depletion of Mcl-1 facilitates entry into apoptosis [122]. The ability of TRAIL to induce Mcl-1 appeared to necessitate the suppression of the mitochondrial pathway of apoptosis, as illustrated by inhibition of caspase-9 activity, overexpression of Bcl-X_L, or BAX deficiency [121]. Inhibition of TRAIL-induced expression of Mcl-1, through combination of TRAIL treatment with either ectopic expression of c-Myc or treatment with the multikinase inhibitor sorafenib, significantly sensitized *BAX*-KO cells to TRAIL-induced apoptosis, suggesting a possible mechanism of overcoming resistance to cell death signaling in these cells. In this study, the functional role of Bcl-X_L and its interaction with the pro-apoptotic protein BAX was investigated in apoptosis induced by NSAIDs in colon cancer cells. Additionally, the role of Mcl-1 in mediating additional resistance to NSAIDs was investigated.

3.2 RESULTS

3.2.1 NSAIDS INDUCE PROTEASOMAL DEGRADATION OF ANTI-APOPTOTIC BCL-X_L

To establish the effects of NSAIDs on the anti-apoptotic Bcl-2 family member Bcl-X_L, HCT116 cells and the BAX-knockout (*BAX*-KO) cells were subjected to treatment with 500 μmol/L indomethacin for up to 24 hours. In HCT116 cells, a time-dependent degradation of Bcl-X_L occurred that resulted in approximately 50% reduction in Bcl-X_L levels after a 24 hour indomethacin treatment (determined by densitometry analysis of protein band intensity on the developed film) (Figure 3.1), confirming the published results [71]. The same effect was not evident in the *BAX*-KO cells, suggesting that Bcl-X_L degradation induced by indomethacin requires the presence of the *BAX* gene (Figure 3.1).

A wide variety of cellular proteins undergo proteolytic degradation catalyzed by the ubiquitin-proteasome pathway. A small molecule proteasome inhibitor PS-341 was used, at non-cytotoxic concentrations, to determine whether Bcl-X_L degradation is mediated by the proteasome in HCT116 cells. A time-dependent accumulation of Bcl-X_L was observed when cells were treated with PS-341 at two different concentrations, in agreement with the proposed proteasome-mediated degradation of Bcl-X_L in healthy cells (Figure 3.2A). The process of covalent protein poly-ubiquitination serves as a critical signaling event for a multitude of cellular proteins to be degraded as a means of regulating protein half-life and stability. To determine

whether ubiquitin is conjugated onto Bcl-X_L protein during NSAID treatment, HCT116 cells were transiently co-transfected with HA-tagged ubiquitin and V5-tagged Bcl-X_L plasmids for 12 hours, and subsequently treated with sulindac for up to 24 hours. Whole cell lysates were collected at specified time points, and exogenous Bcl-X_L was immunoprecipitated with a V5 antibody. Since protein ubiquitination represents a covalent modification, the immunoprecipation lysates were analyzed on a typical denaturing SDS-PAGE gel. Immunoprecipitation of exogenously expressed V5-tagged Bcl-X_L indicated an appearance of high molecular weight bands believed to represent mono- and poly-ubiquitinated Bcl-X_L species (Figure 3.2B). Treatment with sulindac caused an accumulation of high molecular weight Bcl-X_L–ubiquitin complexes, which was enhanced by the presence of the proteasome inhibitor PS-341 (Figure 3.2B).

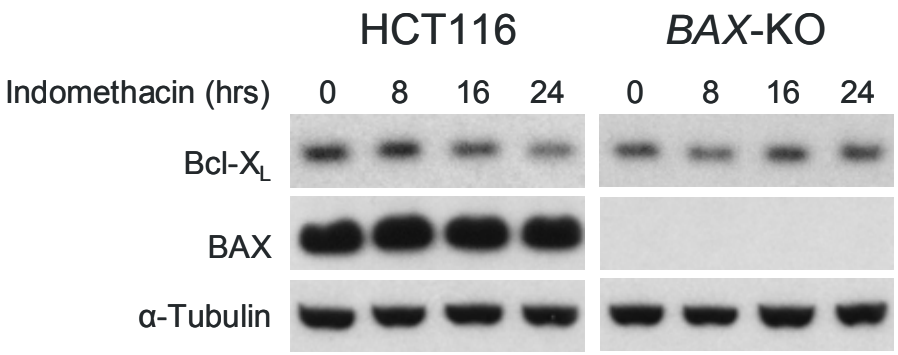


Figure 3.1 NSAIDs induce Bcl-X_L degradation.
Time course of Bcl-X_L degradation. HCT116 and *BAX*-KO cells were treated with 500 μmol/L indomethacin. Changes in Bcl-X_L levels were analyzed by Western blotting at the indicated time points after indomethacin treatment.

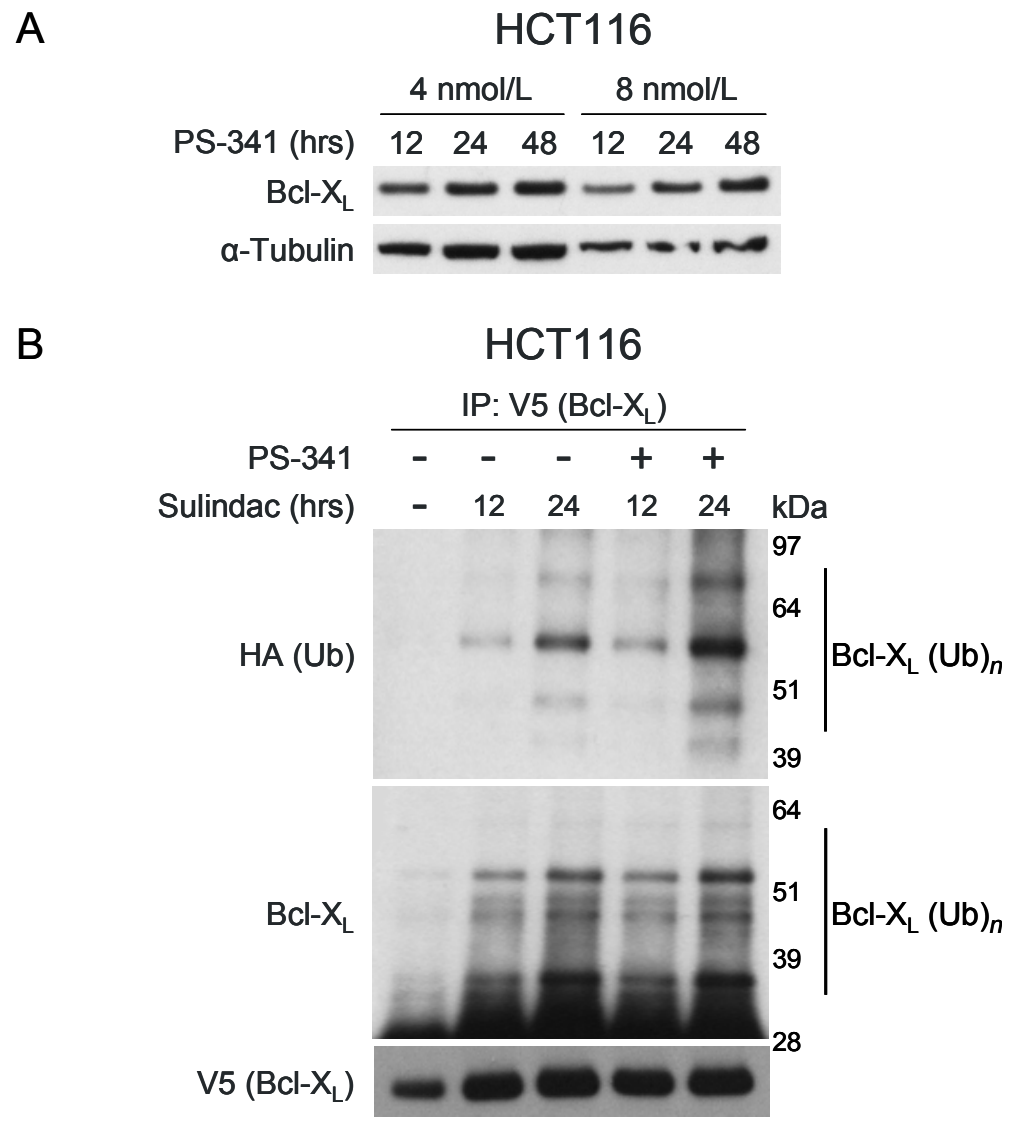


Figure 3.2 NSAIDs induce proteasome-mediated Bcl-X_L degradation.
A, Effect of proteasome inhibitor PS-341 on Bcl-X_L level. HCT116 cells were treated with two sub-cytotoxic doses of PS-341. Changes in Bcl-X_L levels were analyzed by Western blotting at the indicated time points after treatment.
B, Analysis of Bcl-X_L degradation. HCT116 cells were transiently co-transfected with HA-tagged ubiquitin and V5-tagged Bcl-X_L plasmids for 12 hours. After transfection, HCT116 cells were treated with 120 μmol/L sulindac alone or in combination with the proteasome inhibitor PS-341. The appearance of poly-ubiquitinated Bcl-X_L at indicated time points was analyzed by immunoprecipitation (IP) with a V5 antibody.

3.2.2 NSAIDS DISRUPT INTERACTION BETWEEN BAX AND BCL-X_L

Treatment of HCT116 cells with NSAIDs caused proteasomal degradation of Bcl-X_L; however the degradation of Bcl-X_L appeared to be incomplete following a 24 hour treatment with NSAIDs, which is sufficient in inducing the appearance of apoptotic markers such as chromatin condensation, nuclear fragmentation, and activation of caspases (data not shown). The partial persistence of Bcl-X_L in NSAID-treated cells coincided with a stable level of BAX throughout the progression of apoptosis, suggesting that the apoptosis induced by sulindac and indomethacin was dependent on the continuous presence of functional BAX protein in colon cancer cells. Since the remaining Bcl-X_L could potentially neutralize the pro-apoptotic function of BAX through heterodimerization, the level of interaction between endogenous anti-apoptotic Bcl-X_L and pro-apoptotic BAX proteins was analyzed during NSAID treatment. HCT116 cells were treated with 500 μmol/L indomethacin for up to 16 hours, and whole cell lysates were collected. Purification of endogenous BAX–Bcl-X_L complexes by immunoprecipitating with anti-BAX antibody indicated a time-dependent reduction in BAX–Bcl-X_L complexes in NSAID-treated cells (greater than 60% reduction by 16 hours of treatment as determined by densitometry analysis of protein band intensity on the developed film) (Figure 3.3). These findings suggest that in addition to depletion of the Bcl-X_L pool within the cell, the disruption of BAX–Bcl-X_L interactions was important for initiating an NSAID-induced apoptotic response in colon cancer cells.

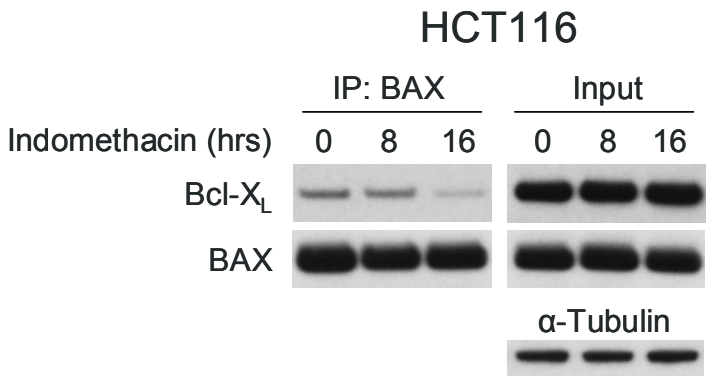


Figure 3.3 Dissociation of BAX and Bcl-X_L during NSAID treatment.
The association between endogenous BAX and Bcl-X_L following treatment with 120 μmol/L sulindac was analyzed at indicated time points by immunoprecipitation (IP) with anti-BAX antibody.

3.2.3 NSAIDS INDUCE BAX TRANSLOCATION FROM CYTOSOL TO MITOCHONDRIA

Since BAX plays an important pro-apoptotic role at the mitochondrial membrane of cells that have been challenged with an apoptosis-inducing stimulus, the subcellular distribution of BAX was analyzed during NSAID treatment in HCT116 cells. Following the isolation of mitochondrial and cytosolic fractions, BAX was initially found to be localized in both fractions of untreated cells. Sulindac-treated cells displayed a time-dependent accumulation of BAX in the mitochondrial fraction (greater than 40% increase by 32 hours of treatment as determined by densitometry analysis of protein band intensity on the developed film), which coincided with a reduction of BAX present in the cytosolic fraction (Figure 3.4), indicating that a translocation of BAX from the cytosolic pool to the mitochondrial pool may play a role in mediating the initiation of apoptotic cascade in cells treated with NSAIDs.

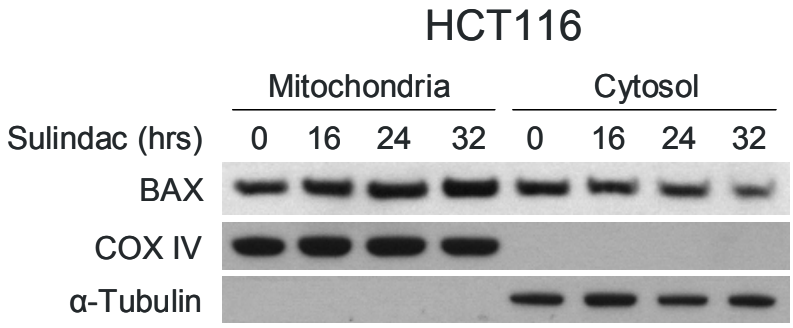


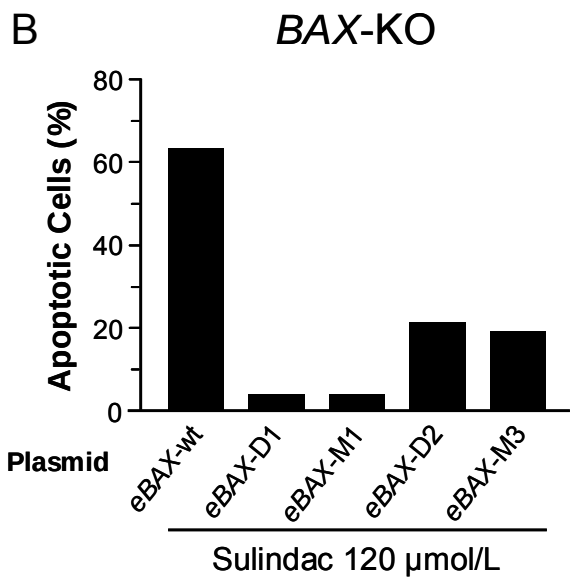
Figure 3.4 NSAIDs induce BAX accumulation in the mitochondria.
Analysis of BAX localization during a time course of sulindac treatment. Following treatment with 120 μmol/L sulindac, the mitochondrial and cytosolic fractions were isolated at indicated time points. The expression of BAX was analyzed by Western blotting. COX IV and α-tubulin, which are exclusively expressed in the mitochondria and cytosol, respectively, were used as controls for loading and fractionation.

3.2.4 MULTIMERIZATION AND TRANSMEMBRANE DOMAINS OF BAX ARE REQUIRED FOR NSAID-INDUCED APOPTOSIS

To further elucidate the importance of dissociation of BAX from Bcl-X_L and translocation of BAX to the mitochondria in NSAID-treated cells, the effects of NSAID treatment were studied in previously established *BAX*-KO cell lines with stable expression of various *eGFP-BAX* (*eBAX*) constructs (Figure 3.5A) [123]. The *BAX* gene encodes a protein that contains three BH domains (BH1-3) and a transmembrane domain (TM). The domains and amino acid sequence that are required for its interactions with other Bcl-2 family proteins and for mitochondrial translocation were previously described [123]. The engineered BAX constructs contained a predicted defect in either the ability of BAX to interact with other BH3 family proteins (*eBAX*-D1 and *eBAX*-M1), or the ability of BAX to localize to mitochondria (*eBAX*-D2 and *eBAX*-M3), corresponding to a mutation or deletion of the transmembrane (TM) domain (Figure 3.5A) [123]. Plasmids *eBAX*-D1 and *eBAX*-M1 encoded full-length BAX proteins containing a deletion of the BH3 domain or a single-point mutation of the BH3 domain, respectively, with a predicted deficiency in protein-protein interactions. Plasmids *eBAX*-D2 and *eBAX*-M3 encoded full-length BAX proteins containing a deletion of the TM domain or a point mutation of the TM domain, respectively (Figure 3.5A). All constructs were experimentally verified by immunoprecipitation and immunostaining to exhibit the predicted loss of function [123].

A

BAX constructs	Predicted loss of function
eBAX-D1	Cannot heterodimerize or homodimerize
eBAX-M1	Cannot heterodimerize or homodimerize
eBAX-D2	Cannot localize to mitochondria
eBAX-M3	Cannot localize to mitochondria



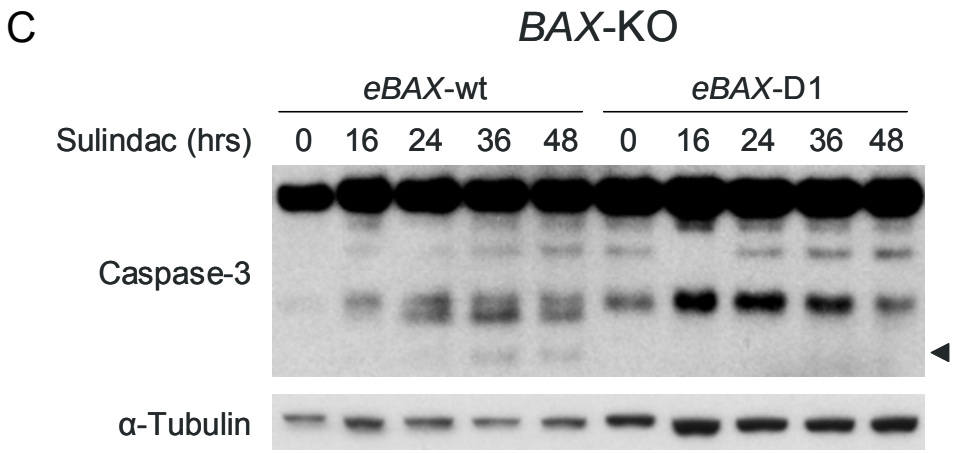


Figure 3.5 BAX multimerization and mitochondrial localization domains are required for mediating NSAID-induced apoptosis.

A, Previously, *BAX*-KO cells were stably transfected with BAX vectors containing deletions or mutations in multimerization or transmembrane domains [123].

B, BAX multimerization and mitochondrial localization domains are required for mediating NSAID-induced apoptosis. Stable cell lines were treated with 120 µmol/L sulindac for 48 hours. Apoptosis was analyzed by nuclear staining.

C, Time course of caspase-3 activation. Representative stable cell lines expressing wild-type or mutant BAX were treated with 120 µmol/L sulindac. Caspase-3 activation was analyzed by Western blotting at the indicated time points after sulindac treatment. Arrow indicates active caspase-3 fragment.

BAX-KO cells with stable expression of *eBAX* constructs described above were treated with 120 μmol/L sulindac for 48 hours, at which point floating and attached cells were collected and analyzed for chromatin condensation and nuclear fragmentation to score apoptotic induction. Apoptosis was significantly induced in cells expressing wildtype BAX (*eBAX*-wt), at a level similar to the parental HCT116 cells (Figure 3.5B). Expression of BAX constructs with a defective BH3 domain (*eBAX*-D1 and *eBAX*-M1) failed to rescue the resistance to NSAID-induced apoptosis in *BAX*-KO cells, while expression of BAX constructs with a defective TM domain resulted in an intermediate phenotype, characterized by an incomplete but significant resistance to apoptosis induced by NSAIDs (Figure 3.5B). *BAX*-KO cells with stable expression of *eBAX*-wt and *eBAX*-D1 constructs were analyzed for caspase-3 activation during treatment with 120 μmol/L sulindac for up to 48 hours. Lack of NSAID-induced caspase-3 activation that was previously observed in *BAX*-KO cells was rescued by the expression of the *eBAX*-wt construct, but not by the *eBAX*-D1 construct (Figure 3.5C). Together, these results demonstrated the importance of the ability to form multimeric protein interactions mediated by the BH3 domain and to undergo mitochondrial localization mediated by the TM domain for BAX-mediated apoptotic response.

3.2.5 NSAIDS INDUCE MCL-1 EXPRESSION IN *BAX*-KO CELLS

BAX-deficient HCT116 cells are resistant to apoptosis induced by a variety of different agents, including TRAIL and NSAIDs. However, despite this block in apoptosis initiation upstream of mitochondria-driven changes, *BAX*-KO cells still display activation of caspase-8 and cleavage of Bid protein when treated with TRAIL or NSAIDs, suggesting a common pathway for the induction of apoptosis for these distinct agents. Previously, the expression of the anti-apoptotic protein Mcl-1 has been shown to become upregulated in TRAIL-treated BAX-deficient HCT116 cells. Human Mcl-1 belongs to the Bcl-2 protein family of anti-apoptotic regulators and shares similar BH-multidomain structures as Bcl-2 and Bcl-X_L [83]. It has been proposed that the increase in Mcl-1 expression likely confers additional protection against tBid in TRAIL-treated BAX-deficient cells [121]. Due to the similarity between TRAIL and NSAIDs in generating active tBid protein, the expression of several anti-apoptotic proteins, including Mcl-1 and members of the IAP family (data not shown), was analyzed in sulindac-treated HCT116 cells and *BAX*-KO cells. The basal level of Mcl-1 protein was similar in untreated BAX-proficient and BAX-deficient cells (Figure 3.6A). However, unlike NSAID-sensitive HCT116 cells, NSAID-resistant *BAX*-KO cells showed significant increase in expression of Mcl-1 and cIAP-1 (data not shown) proteins when treated with sulindac (Figure 3.6A).

The upregulation of Mcl-1 expression in TRAIL-treated *BAX*-KO cells has been previously shown to depend on the activity of nuclear factor-kappa B (NF-κB) [121]. NF-κB is a transcription factor composed of two subunits, p50 and p65, which

can function independently or as a duo. It is typically found in the cytoplasm in an inactive form bound to I-κB. Phosphorylation of I-κB leads to its dissociation from NF-κB, followed by proteasomal degradation. Uncoupling of I-κB and NF-κB induces activation of NFκB by allowing translocation into the nucleus where it promotes transcription. We studied the activation of NF-κB signaling in NSAID-treated cells by analyzing the phosphorylation of the NF-κB p65 subunit, phosphorylation of I-κB, an endogenous inhibitor of NF-κB, and NF-κB nuclear translocation. A 24 hour treatment with sulindac induced a reduction of NF-κB phosphorylation and no change in I-κB phosphorylation in HCT116 and *BAX*-KO cell lines (Figure 3.6B). Additionally, both cell lines indicated a minor increase in NF-κB nuclear levels following sulindac treatment (Figure 3.6C). These results suggest that the apparent increase in Mcl-1 levels following sulindac treatment in *BAX*-KO cells occurred via an NF-κB–independent mechanism, since no difference between the levels of active NF-κB and inactive I-κB were observed between HCT116 cells, which did not show increased Mcl-1 expression, and *BAX*-KO cells.

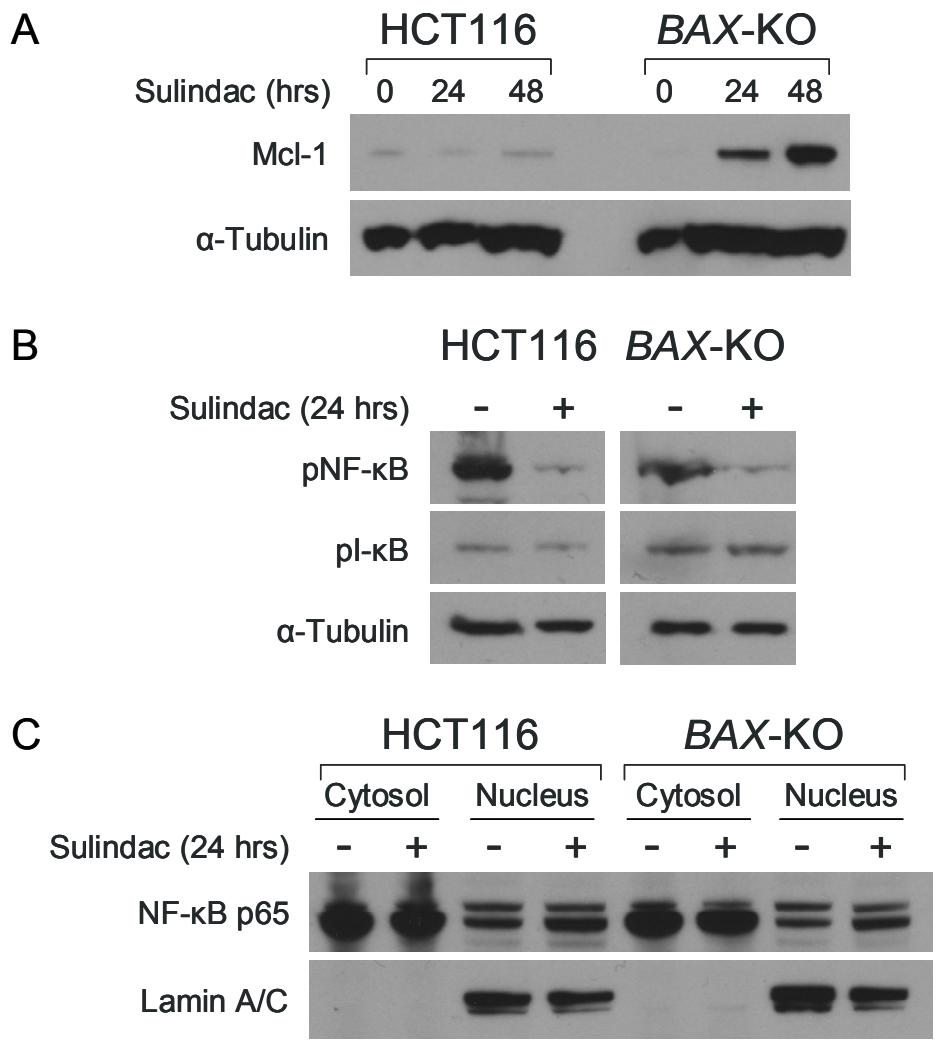


Figure 3.6 Sulindac induces Mcl-1 expression in *BAX*-KO cells.
A, Time course of Mcl-1 induction. HCT116 and *BAX*-KO cells were treated with 120 μmol/L sulindac. Changes in Mcl-1 expression were analyzed by Western blotting at the indicated time points after sulindac treatment.
B, Detection of NF-κB and I-κB phosphorylation. Phospho-specific antibodies were used to study changes in activation of NF-κB and I-κB following sulindac treatment.
C, Analysis of NF-κB localization. HCT116 and *BAX*-KO cells were treated with 120 μmol/L sulindac for 24 hours. Cytosolic and nuclear fractions were isolated, and the expression of NF-κB was analyzed by Western blotting. Lamin A/C, which is exclusively expressed in the nucleus, was used as controls for loading and fractionation.

3.2.6 ABLATION OF MCL-1 DOES NOT SENSITIZE *BAX*-KO CELLS TO NSAID-INDUCED APOPTOSIS

Previously, reduction of Mcl-1 expression by c-Myc or the kinase inhibitor sorafenib was shown to sensitize *BAX*-KO cells to TRAIL-induced apoptosis [121]. Since treatment with NSAIDs induced an upregulation of Mcl-1 in *BAX*-KO cells similar to TRAIL-treated cells, the significance of Mcl-1 expression in establishing resistance to NSAIDs in *BAX*-KO cells was analyzed. Cells were transiently transfected with Mcl-1 siRNA before sulindac treatment. Induction of Mcl-1 by sulindac was significantly blocked by Mcl-1 siRNA (Figure 3.7A). However, in contrast to apoptosis induced by TRAIL, ablation of Mcl-1 expression failed to restore sensitivity to NSAID-induced apoptosis in *BAX*-KO cells (Figure 3.7B). Inhibition of NF-κB expression by siRNA transfection similarly had no effect on apoptosis induced by sulindac (Figure 3.7B). These results indicate that Mcl-1 induction is not responsible for imparting an additional level of resistance to apoptosis induced by NSAIDs, while the mechanism of NSAID-induced apoptosis appears to be independent of gene transcription regulated by NF-κB transcription factor in HCT116 colon cancer cells.

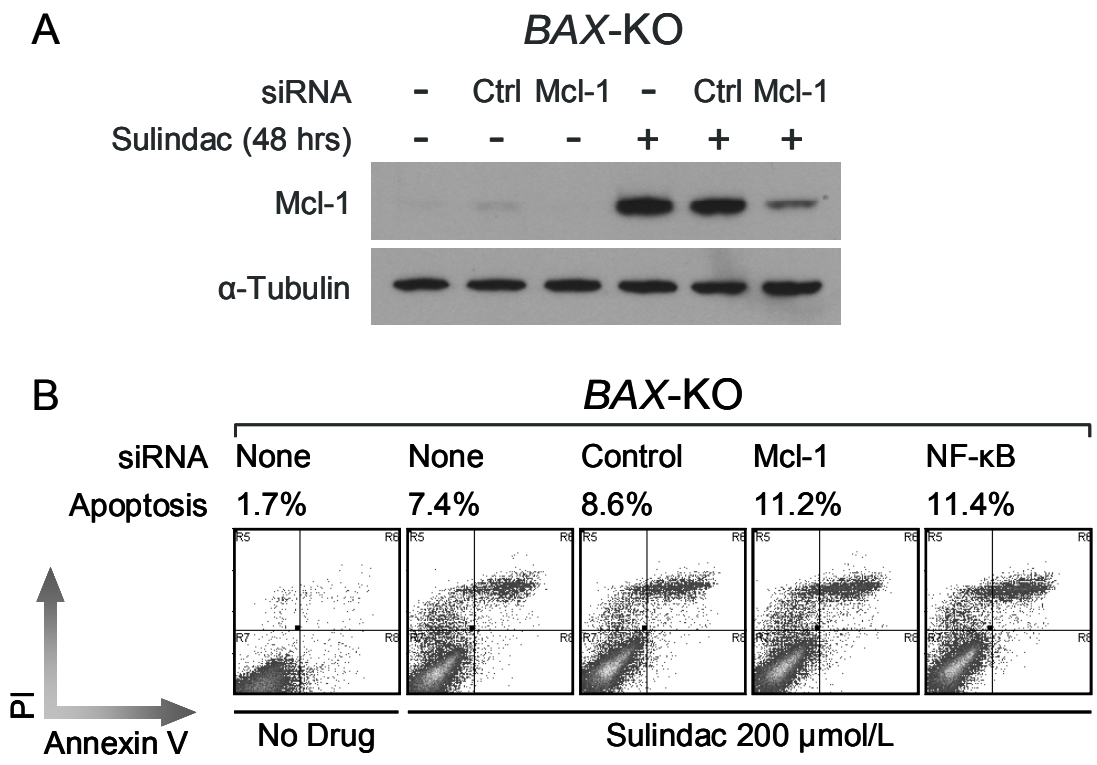


Figure 3.7 Mcl-1 induction is not necessary for resistance to NSAID-induced apoptosis in *BAX*-KO cells.

A, Inhibition of Mcl-1 by siRNA knockdown. *BAX*-KO cells were transiently transfected with indicated siRNA for 12 hours followed by 120 μmol/L sulindac treatment. Mcl-1 expression was probed by Western blotting 48 hours after treatment. Ctrl, control scrambled siRNA.

B, Effects of Mcl-1 ablation on sulindac-induced apoptosis. *BAX*-KO cells were transiently transfected with indicated siRNA for 12 hours followed by 200 μmol/L sulindac treatment. Apoptosis induction was analyzed by Annexin V/PI staining. The percentage of apoptosis represents Annexin V/PI double-positive population. Ctrl, control scrambled siRNA.

3.3 DISCUSSION

The induction of apoptosis by NSAIDs in human cancer cell lines has been well characterized. However, the exact mechanisms underlying the anticancerous effects of NSAIDs remain unclear. The initial role of NSAID-mediated inhibition of cyclooxygenase enzymes failed to provide a definitive answer, since the effects of NSAIDs have been shown to be equally potent in cell lines that expressed COX enzymes and those that did not. In addition to COX-2 inhibition, recent evidence has indicated that NSAIDs-mediated anti-neoplastic effects are exerted through the activation of an apoptotic pathway [62]. This process involves activation and translocation of several key molecules and ultimately results in caspase activation in the malignant tissues. Several novel COX-independent mechanisms for NSAIDs have been described that involve inhibition of NF-κB activation and downregulation of the anti-apoptotic protein Bcl-X_L [124]. The present study aimed at elucidating the importance of several key apoptotic players, and their role in NSAID-induced apoptosis. We analyzed the role of BAX and Bcl-X_L, two functionally opposing members of Bcl-2 family of apoptotic regulators, in mediating the response of cancer cells to NSAIDs. The availability of NSAID sensitive and isogenic NSAID resistant cell lines allowed the investigation of molecular events that followed the induction of apoptosis in sensitive cells and resistance to apoptosis in resistant cells.

Initially, the reduction of Bcl-X_L protein level was observed in HCT116 cells with wild-type BAX, an effect that was previously described [71]. Under similar treatment, there was no noticeable decrease in Bcl-X_L in BAX-deficient HCT116

cells. Since *BAX*-KO cells showed remarkable resistance to apoptosis induced by NSAIDs, which coincided with a lack of Bcl-X_L degradation, the active degradation of Bcl-X_L was proposed to act as a prerequisite for apoptosis initiation. It was enticing to hypothesize that the degradation of anti-apoptotic Bcl-X_L during early stages of apoptosis served as a possible mechanism by which cells generate a pool of active BAX protein by liberating BAX from the inhibitory interaction with Bcl-X_L. Inhibition of cellular ubiquitin-proteasome machinery, which serves as a major pathway that regulates protein degradation and turn-over, indicated that Bcl-X_L was indeed poly-ubiquitinated and degraded by the proteasome during NSAID treatment.

The dissociation of BAX and Bcl-X_L by NSAIDs presumably activates the pro-apoptotic function of BAX by promoting its mitochondrial translocation and multimerization. The interaction between BAX and Bcl-X_L was markedly inhibited by 16 hours of NSAID treatment. At the same time, there was a noticeable accumulation of BAX in the mitochondria with concomitant reduction of BAX levels in the cytosol of NSAID-treated cells, favoring the model of the pro-apoptotic action of BAX at the mitochondria. A recent study has indicated that NSAIDs celecoxib and indomethacin can induce a significant increase in the expression of PUMA, both at the RNA and protein levels, in AGS and Kato III human stomach carcinoma cell lines [125]. PUMA is a member of the BH3-only group of Bcl-2 family of apoptotic regulator proteins, while also being a downstream target of the p53-mediated transcriptional activity. The increase in PUMA levels by NSAIDs occurred through a COX-independent mechanism and coincided temporally with the dissociation between BAX and Bcl-X_L observed in the present study, taking place between 12 and

24 hours of NSAID treatment. Additionally, PUMA has been demonstrated to mediate the disruption of BAX–Bcl-X_L complexes by actively competing with BAX for binding to Bcl-X_L [123]. Such an effect of PUMA was dependent on the BH3 domain, since PUMA containing a deletion of the BH3 domain was unable to dissociate BAX and Bcl-X_L [123]. Taken together, the presently observed dissociation of BAX from Bcl-X_L during NSAID treatment, combined with the previously published data outlining the NSAID-induced upregulation of PUMA and the role of PUMA in disrupting the interaction between BAX and Bcl-X_L, suggest a possible mechanism by which NSAIDs promote apoptosis: upregulation of PUMA expression by NSAIDs in turn promotes BAX-mediated release of mitochondrial apoptogenic factors through competitive binding to Bcl-X_L.

The findings illustrated above are based on experiments performed in BAX-proficient cells undergoing NSAID-induced apoptosis, suggesting that the induction of cell death occurs as a result of displacement of BAX from Bcl-X_L. Previously established stable cell lines expressing BAX mutants that are unable to form multimers (e*BAX*-D1 and e*BAX*-M1) did not undergo NSAID-induced apoptosis, suggesting that the ability of BAX to form protein-protein complexes is critical for its function in NSAID-induced apoptosis. Stable cell lines expressing BAX mutants in the TM domain (e*BAX*-D2 and e*BAX*-M3), which disrupt its mitochondrial localization, were resistant to NSAID-induced apoptosis, suggesting that the mitochondrial localization of BAX plays an important role in mediating its pro-apoptotic function. NSAID-induced caspase activation also appeared to be dependent on these BAX-related changes, as illustrated by a representative cell line with a

multimerization-defective BAX mutant. Together, these results support the model in which the multimerization of BAX at the mitochondrial outer membrane facilitates the initiation of the apoptotic response in cells treated with NSAIDs.

The role of another anti-apoptotic member of Bcl-2 family of apoptotic regulators, Mcl-1, has been recently explored in TRAIL-induced apoptosis in BAX-proficient and BAX-deficient HCT116 cells [121]. Despite being resistant to apoptosis induced by TRAIL, *BAX*-KO cells seemingly developed an additional strategy of inhibiting the apoptotic machinery by inducing the expression of Mcl-1, which acts similar to Bcl-X_L by interacting with pro-apoptotic BH3-only proteins and sequestering their activity. A recent study has indicated a similarity between the engagement of the death receptor pathway by TRAIL and sulindac. While treatment of *BAX*-KO cells with sulindac resulted in a similar induction of Mcl-1, inhibition of Mcl-1 expression failed to sensitize *BAX*-KO cells to apoptosis induced by NSAIDs, as opposed to restoration to TRAIL-mediated apoptosis [121].

In summary, these results demonstrated that NSAIDs induce apoptosis by inducing proteasome-mediated degradation of Bcl-X_L and displacing BAX from Bcl-X_L, thereby promoting BAX multimerization and mitochondrial translocation.

3.4 CONCLUSION

In this study, we investigated the mechanisms by which nonsteroidal anti-inflammatory drugs (NSADs) modulate the intrinsic pathway of apoptosis. We focused on analyzing the events upstream of mitochondria and studied the roles of Bcl-2 family members BAX, Bcl-X_L and Mcl-1 in mediating an apoptotic response in human colon cancer cells treated with sulindac and indomethacin, two members of the NSAID family. Previously observed degradation of Bcl-X_L in NSAID-treated cells was shown to depend on the ubiquitin-proteasome pathway that serves as a common mechanism of protein turn-over. Treatment with the small molecule proteasomal inhibitor PS-341 induced a time-dependent accumulation of Bcl-X_L in untreated cells, while treatment with NSAIDs combined with PS-341 resulted in accumulation of ubiquitin-conjugated Bcl-X_L species. Degradation of Bcl-X_L coincided with dissociation of BAX–Bcl-X_L complexes and translocation of BAX from the cytosol to the mitochondria of NSAID-treated cells, in agreement with a previously suggested role for BAX in mediating the pore-forming function at the mitochondria during initiation of apoptosis. Analysis of Mcl-1 expression indicated a time-dependent induction in Mcl-1 levels in BAX-deficient cells following treatment with NSAIDs; the induction of Mcl-1 was not observed in wild-type HCT116 cells. Inhibition of Mcl-1 expression using RNA interference failed to sensitize *BAX*-KO cells to NSAID-induced apoptosis, suggesting the involvement of different pathways of apoptosis regulation in NSAID-treated cells and TRAIL-treated cells.

4 THE ROLE OF SMAC IN MEDIATING NSAID-INDUCED APOPTOSIS

4.1 INTRODUCTION

Prevention of human cancers through the use of chemical agents or dietary manipulation has emerged as a promising approach to reduce morbidity and mortality of cancer [14]. A number of epidemiologic studies, clinical trials, and animal studies have shown that nonsteroidal anti-inflammatory drugs (NSAID), such as sulindac and aspirin, are effective against colorectal cancer, the second leading cause of cancer-related death [105, 106]. However, the mechanisms underlying the anti-neoplastic effects of NSAIDs remain unclear. The effects of NSAIDs are incomplete, and resistance to NSAIDs is often developed [105, 106]. Furthermore, the side effects associated with high dose of NSAIDs, such as the cardiovascular toxicity of the cyclooxygenase 2 (COX-2)–specific inhibitors, have presented a significant obstacle for general use of these agents for cancer prevention [126].

Among many described activities of NSAIDs, induction of apoptosis secondary to inhibition of COX enzymes seems to play a critical role in NSAID-mediated chemoprevention [105-107]. Apoptosis is a major mechanism regulating turnover of intestinal epithelial cells from which colon tumors are derived [108]. During the formation of a colon tumor, apoptosis is progressively inhibited due to oncogenic mutations [109]. Deregulation of apoptosis is necessary for oncogenic transformation and drives neoplastic cells to gain additional tumorigenic features

[110]. NSAIDs have been found to induce apoptosis in colon cancer cells *in vitro* and *in vivo* [105, 106]. The apoptotic level can be used as a biomarker for NSAID-mediated chemoprevention [72, 111]. Furthermore, it has been shown that NSAID treatment can reverse the anti-apoptotic effects of COX-2 [63].

In mammalian cells, apoptotic cell death is executed through a cascade of events, among which a key event is the translocation of apoptogenic proteins from the mitochondria into the cytosol to trigger caspase activation. Cytochrome *c* is released from the mitochondria into the cytosol, where it binds to other proteins to form an 'apoptosome' multi-protein complex [101]. SMAC is the second mitochondrial protein found to be released into the cytosol during apoptosis [88] [127]. Under non-apoptotic cellular conditions, the BIR-2 and BIR-3 domains of IAPs participate in an inhibitory interaction with initiator caspases, including caspase-3, and executioner caspases, including caspase-9, respectively (Figure 4.1) [79, 128, 129]. After its release, SMAC binds to the BIR-2 and BIR-3 domains of IAPs through its N-terminal AVPI domain and relieves their inhibition of caspases (Figure 4.1) [129].

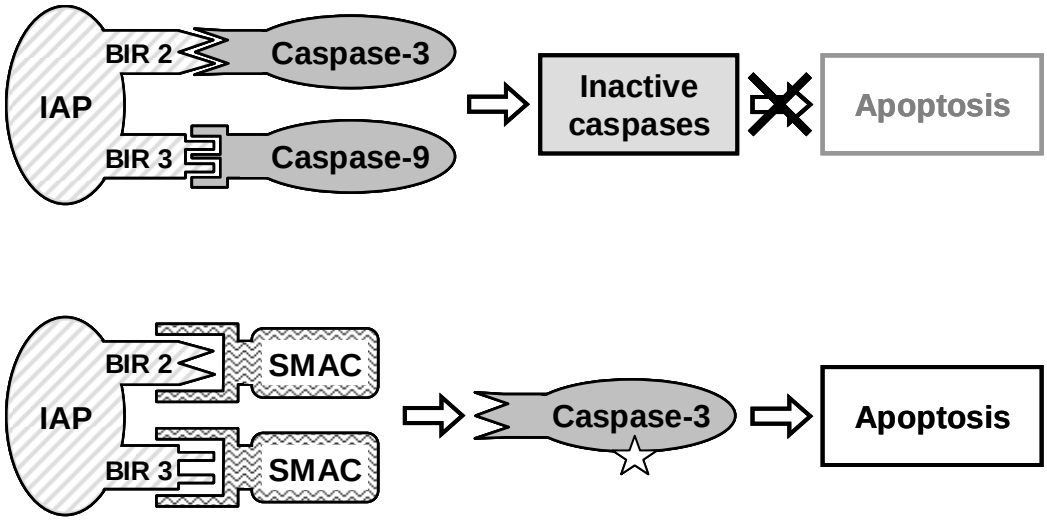


Figure 4.1 Role of SMAC in promoting apoptosis.
Activation of caspases is inhibited by interaction with IAPs. Cytosolic SMAC promotes caspase activation by releasing caspases from IAPs, leading to apoptosis.

Previous studies showed that SMAC plays an essential role in NSAID-induced apoptosis in colon cancer cells [99]. SMAC is consistently released from the mitochondria into the cytosol during NSAID-induced apoptosis. Deletion of SMAC by homologous recombination or knockdown of SMAC by RNA interference abrogates NSAID-induced caspase activation and apoptosis [99]. Unexpectedly, NSAID-induced cytochrome *c* release was also found to be compromised in the SMAC-deficient cells [99]. This finding suggested that SMAC had the potential to influence the release of cytochrome *c* (Figure 4.2), a surprising effect that could not be deduced from the canonical pathway detailing the release of mitochondrial apoptogenic factors, such as SMAC and cytochrome *c*, occurring as independent events. In this study, we further investigated how SMAC functions in NSAID-induced apoptosis.

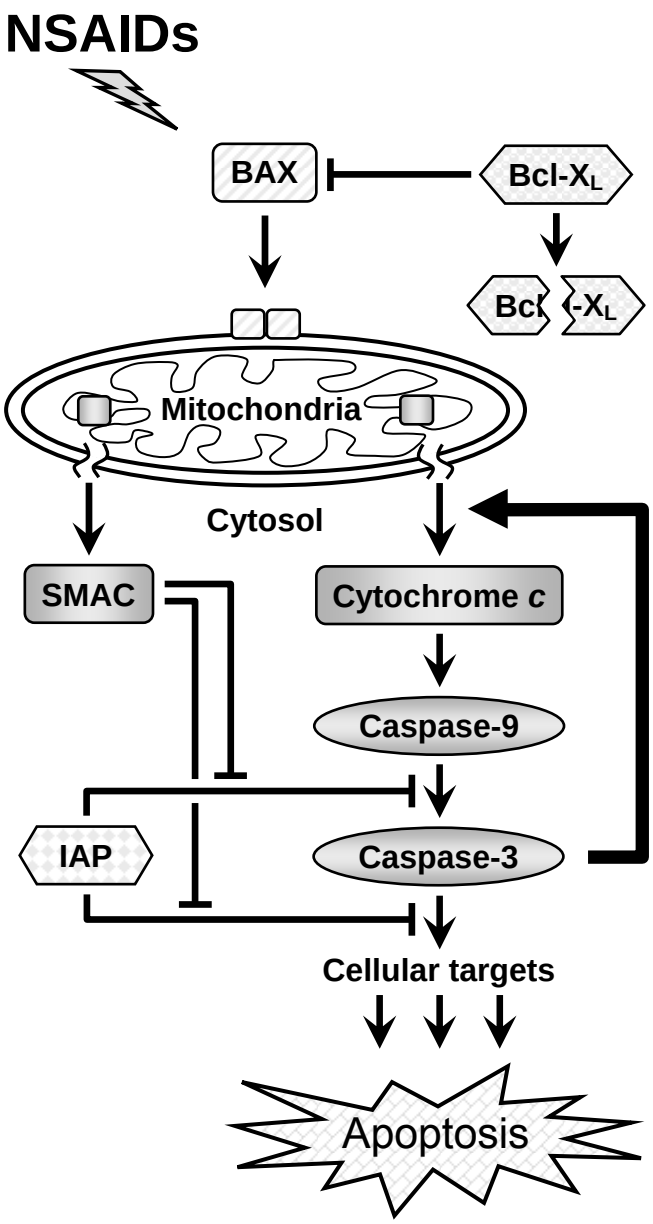


Figure 4.2 Proposed role of SMAC in regulating caspase activation and cytochrome *c* release through a feed-back mechanism.
Based on previous observations that SMAC deficiency inhibits cytochrome *c* release during NSAID treatment, a feed-back mechanism was proposed through which release of SMAC into the cytosol leads to release of cytochrome *c* by promoting activation of caspase cascade.

4.2 RESULTS

4.2.1 NSAID-INDUCED RELEASE OF CYTOCHROME *C* IS DELAYED AND ATTENUATED IN *SMAC*-KO CELLS

Previously, SMAC deficiency was shown to perturb NSAID-induced release of cytochrome *c* and apoptosis-inducing factor in HCT116 colon cancer cells [99]. To further characterize how SMAC mediates NSAID-induced apoptosis, the time courses of apoptotic events were analyzed in HCT116 cells treated with sulindac at 120 μmol/L, which is the minimum concentration that is necessary to induce a high level (>70%) of apoptosis in these cells [99]. A burst of SMAC release was detected as early as 8 hours after the treatment (Figure 4.3A), long before cells started to show morphologic signs of apoptosis. Caspase-3 was dissociated from IAP family members cIAP-1 and cIAP-2 as early as 8 hours after sulindac treatment (Figure 4.3B). At 12 to 16 hours, caspase-3 was found to be activated (Figure 4.3C), but only a very low level (<5%) of nuclear fragmentation could be detected (data not shown).

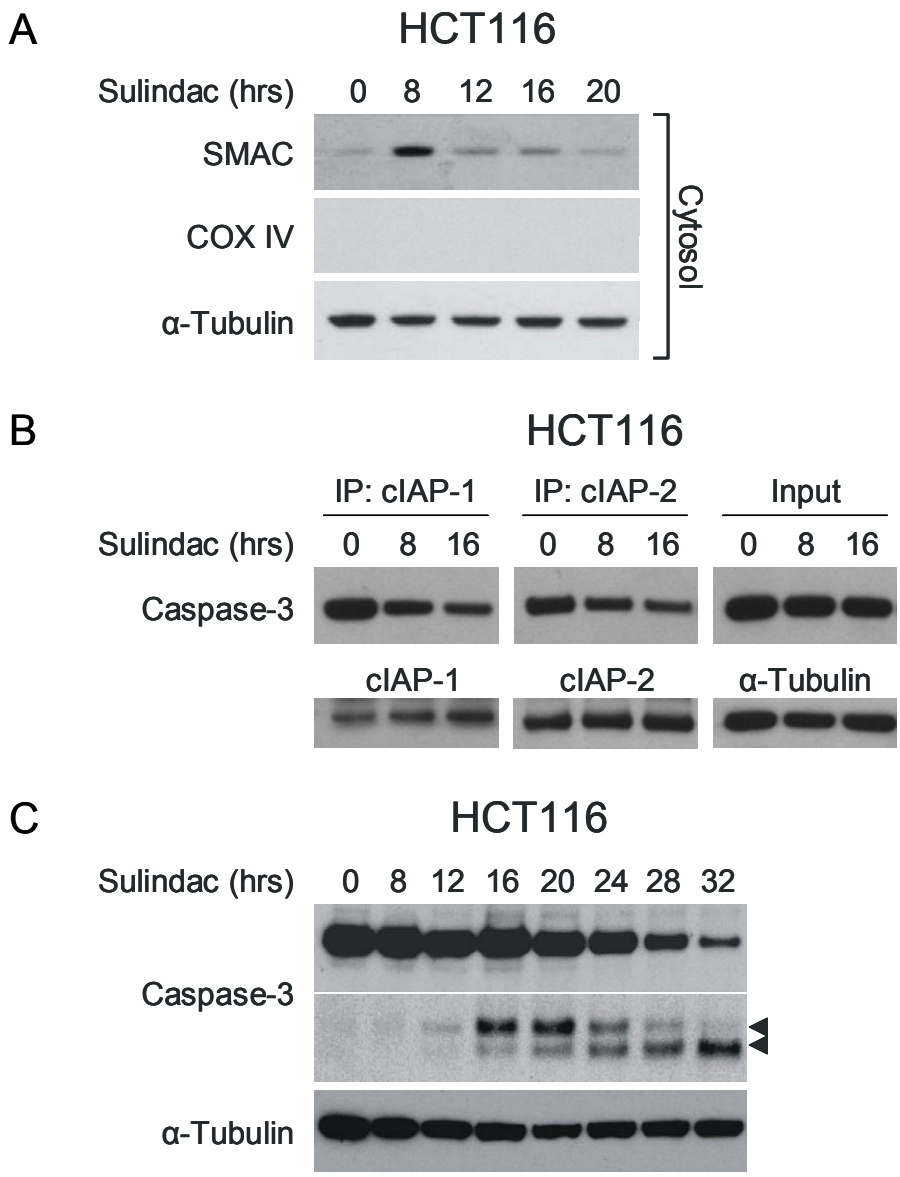


Figure 4.3 SMAC release and caspase-3 activation in NSAID-treated cells.
A, Time course of SMAC release. HCT116 cells were treated with 120 μmol/L sulindac. Cytosolic fractions at the indicated time points were isolated, and the expression of SMAC was analyzed by Western blotting. COX IV and α-tubulin, which are exclusively expressed in the mitochondria and cytosol, respectively, were used as controls for loading and fractionation.
B, After HCT116 cells were treated with 120 μmol/L sulindac, the association between caspase-3 and cIAP-1 or cIAP-2 at indicated time points was analyzed by immunoprecipitation (IP) with cIAP-1 or cIAP-2 antibodies, respectively.
C, Time course of caspase-3 activation. Caspase-3 activation was analyzed by Western blotting at the indicated time points after sulindac treatment. Arrows indicate caspase-3 cleavage fragments.

In contrast, the majority of cytochrome *c* release occurred at 24 and 32 hours after the treatment (Figure 4.4) when high levels (>50%) of nuclear fragmentation could be detected, bolstering the notion that the initial and brief release of SMAC was sufficient to manifest its role in promoting the initiation of apoptosis, while the continued, steady release of cytochrome *c* ensured the successful progression of an apoptosis program in NSAID-treated cells [71, 99]. However, sulindac-induced cytochrome *c* release and nuclear fragmentation were significantly delayed and attenuated in the *SMAC*-KO cells compared with the parental HCT116 cells (Figure 4.4 and data not shown) [99]. These results showed that SMAC release and cytochrome *c*-independent caspase-3 activation are early events in NSAID-induced apoptosis and regulate later apoptotic events, such as cytochrome *c* release and nuclear fragmentation.

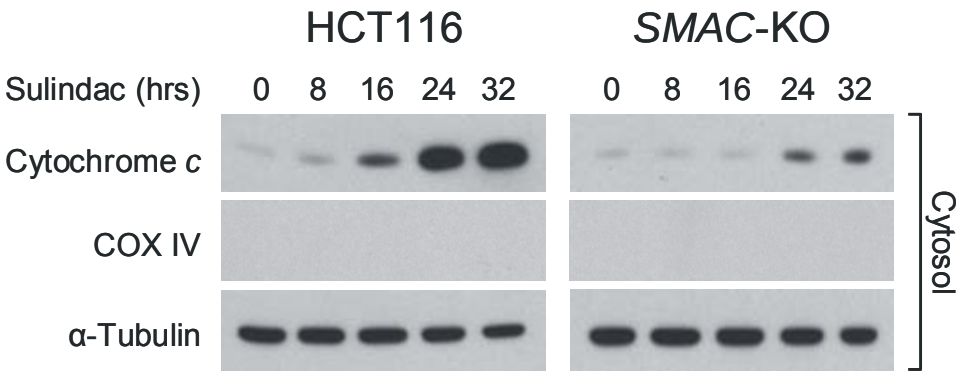


Figure 4.4 Cytochrome *c* release in HCT116 and *SMAC*-KO cells.
Cytochrome *c* release in HCT116 and *SMAC*-KO cells treated with 120 μmol/L sulindac was analyzed by Western blotting. COX IV and α-tubulin, which are exclusively expressed in the mitochondria and cytosol, respectively, were used as controls for loading and fractionation.

4.2.2 IAP BINDING IS REQUIRED FOR SMAC-MEDIATED CASPASE-3 ACTIVATION AND CYTOCHROME *C* RELEASE

To further study how SMAC mediates cytochrome *c* release, we tested whether the IAP inhibition and/or caspase-3 activation functions of SMAC, which are mediated by the N-terminal AVPI domain of cytosolic SMAC [130], are necessary for NSAID-induced apoptosis and cytochrome *c* release. The co-crystal structure of SMAC in complex with the XIAP BIR3 domain shows the SMAC N-terminus interacts with a groove formed on the BIR3 surface [131]. The four SMAC residues (AVPI) that contact BIR3 domain do so by docking a fourth strand onto an existent three-stranded antiparallel ß sheet [131]. This AVPI domain of SMAC has been demonstrated to be important and sufficient for mediating the pro-apoptotic role of SMAC in mediating a cellular apoptosis response [130, 132]. Wild-type (AVPI) or mutant SMAC containing a deletion of alanine in the AVPI domain (ΔA), which abolishes the interactions between SMAC and IAPs [130], were transfected into the *SMAC*-KO cells (Figure 4.5A). Following selection with Geneticin (G418), two independent stable cell lines expressing the wild-type or mutant SMAC were selected and analyzed for their responses to 120 μmol/L sulindac.

The deficiency in NSAID-induced apoptosis in the *SMAC*-KO cells was completely rescued by the expression of the wild-type, but not the mutant, SMAC (Figure 4.5B). Since the activation of the caspase cascade serves as a hallmark of apoptotic cell death, we analyzed the activation of caspase-3 in HCT116, *SMAC*-KO and established stable cell lines. In agreement with their resistance to NSAID-

induced apoptosis, *SMAC*-KO cells showed a strong attenuation of caspase-3 activation (Figure 4.6A). Expression of the wild-type SMAC rescued caspase-3 activation in the *SMAC*-KO cells (Figure 4.6A).

Importantly, wild-type, but not the mutant, SMAC restored sulindac-induced cytochrome *c* release to a level similar to that in the parental HCT116 cells (Figure 4.6B). These results suggest that inhibition of IAPs by cytosolic SMAC through their direct interactions promotes cytochrome *c*–independent caspase-3 activation at early stage and cytochrome *c* release at later stages in NSAID-induced apoptosis.

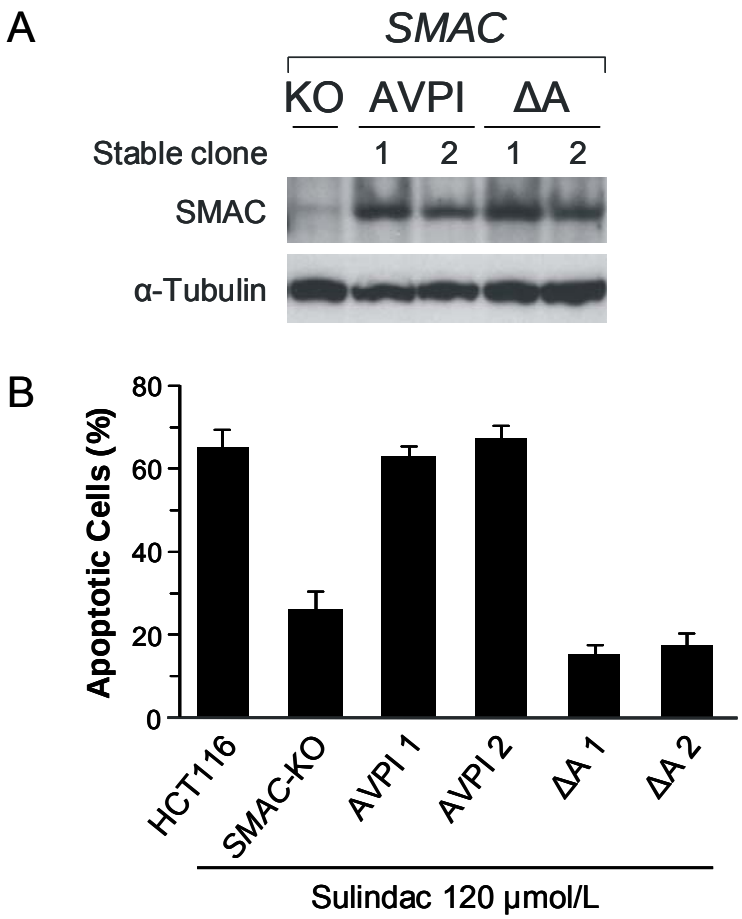


Figure 4.5 The IAP interacting function of SMAC is necessary for its activity in NSAID-induced apoptosis.

A, *SMAC*-KO cells were transfected with wild-type (AVPI) or mutant SMAC containing a deletion of alanine in the AVPI domain (ΔA). Two pairs of clones with stable expression of each SMAC construct were isolated, and SMAC expression was verified by Western blotting.

B, The indicated cell lines were treated with 120 μmol/L sulindac for 48 hours. Apoptosis was analyzed by nuclear staining.

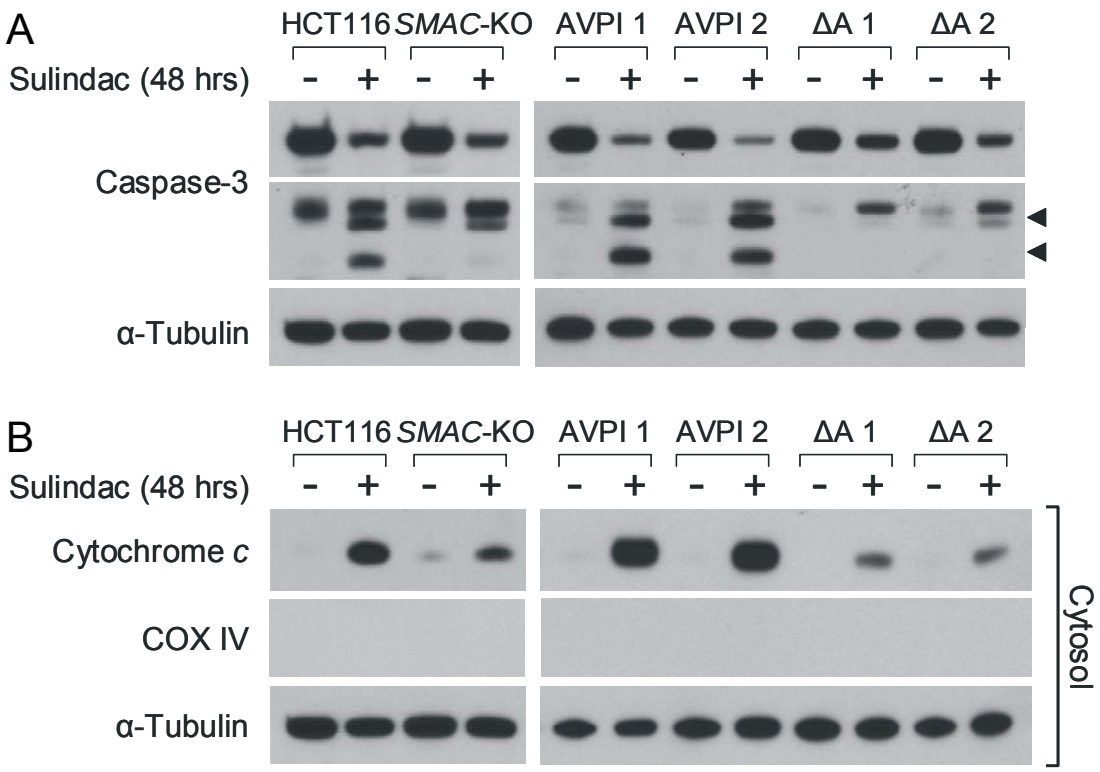


Figure 4.6 The IAP interacting function of SMAC is necessary for its activity in NSAID-induced caspase activation and cytochrome *c* release.
A, Effects of wild-type and mutant SMAC on NSAID-induced caspase activation were analyzed by Western blotting. Arrows indicate caspase-3 cleavage fragments. B, Effects of wild-type and mutant SMAC on NSAID-induced cytochrome *c* release. HCT116, *SMAC*-KO, and *SMAC*-KO cells with stable expression of wild-type SMAC (AVPI) or mutant SMAC (ΔA) were treated with 120 μmol/L sulindac. Cytochrome *c* release was analyzed by Western blotting. COX IV and α-tubulin, which are exclusively expressed in the mitochondria and cytosol, respectively, were used as controls for loading and fractionation.

4.2.3 CASPASE-3 ACTIVATION IS NECESSARY FOR SMAC-MEDIATED CYTOCHROME *C* RELEASE

Next, we determined whether caspase-3 activation is critical for NSAID-induced and SMAC-mediated apoptosis and cytochrome *c* release, since activation of caspase-3 occurs downstream of SMAC release from the mitochondria into the cytosol. Cells were transiently transfected with two non-overlapping caspase-3 siRNA constructs (Cas-3 986 and Cas-3 182) before sulindac treatment. Both caspase-3 siRNA constructs displayed a comparable efficacy in down-regulating caspase-3 expression (Figure 4.7A). Knockdown of caspase-3 expression resulted in a significant reduction of sulindac-induced apoptosis in HCT116 cells (Figure 4.7B). However, no further decrease in apoptosis was observed after knockdown of caspase-3 in the *SMAC*-KO cells (Figure 4.7B), suggesting that SMAC and caspase-3 function in a linear pathway to promote NSAID-induced apoptosis. Caspase-3 knockdown also led to significant inhibition of cytochrome *c* release (Figure 4.8). The critical role of caspase-3 in promoting sulindac-induced cytochrome *c* release was confirmed by treating HCT116 and another colon cancer cell line HT29 using the caspase-3 inhibitor Z-DEVD-fmk (Figure 4.8).

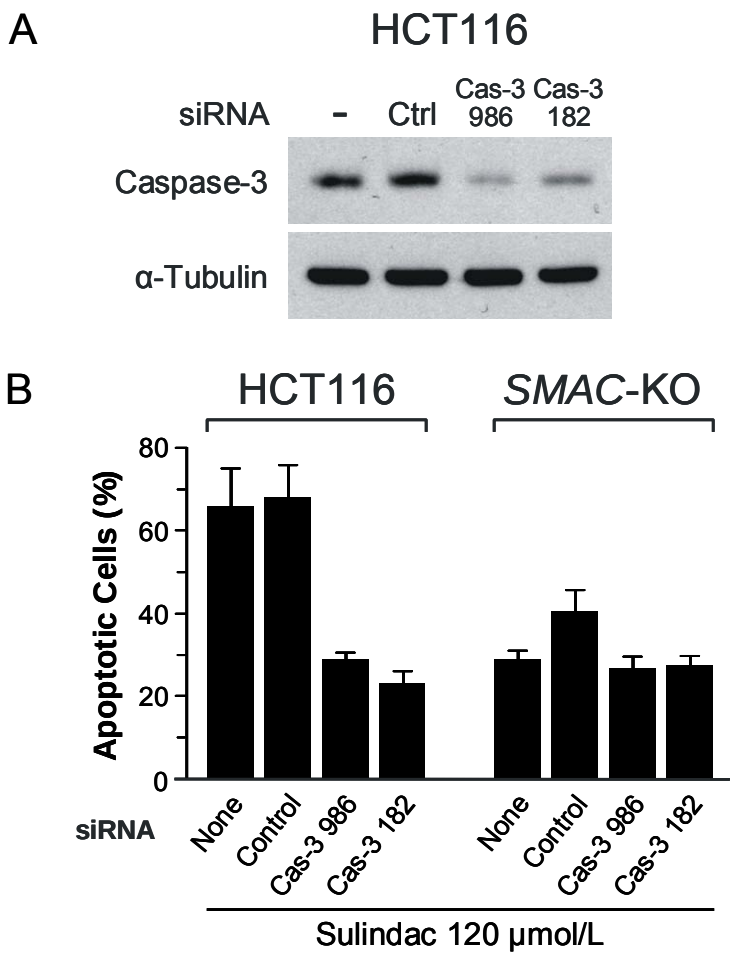


Figure 4.7 Caspase-3 is necessary for NSAID-induced apoptosis.
A, Inhibition of caspase-3 by siRNA knockdown. Caspase-3 expression was probed by Western blotting 24 hours after HCT116 cells were transiently transfected with indicated siRNA. Ctrl, control scrambled siRNA.
B, Effects of caspase-3 knockdown on sulindac-induced apoptosis. Parental and *SMAC*-KO HCT116 cells were transfected with caspase-3 or control siRNA for 24 hours followed by sulindac (120 μmol/L) treatment. Apoptosis was analyzed by nuclear staining 48 hours after sulindac treatment.

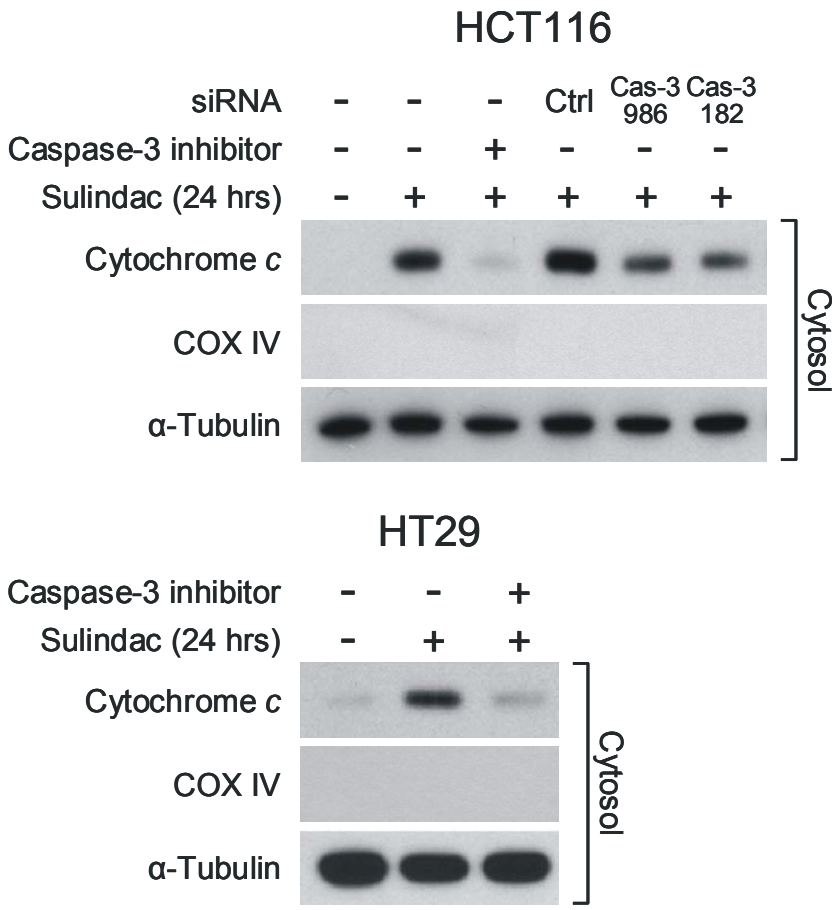


Figure 4.8 Caspase-3 is necessary for NSAID-induced cytochrome *c* release. Effects of caspase-3 inhibition on NSAID-induced cytochrome *c* release. HCT116 and HT29 cells were transfected with control scrambled or caspase-3 siRNA or treated with caspase-3 inhibitor Z-DEVD-fmk for 24 hours followed by sulindac (120 μmol/L for HCT116; 180 μmol/L for HT29) treatment. Cytochrome *c* release 24 hours after the treatment was analyzed. COX IV and α-tubulin, which are exclusively expressed in the mitochondria and cytosol, respectively, were used as controls for loading and fractionation.

4.2.4 ROLE OF BID AND BIM IN MEDIATING APOPTOSIS INDUCED BY NSAIDS

The dependence of mitochondrial cytochrome *c* release on the cytosolic actions of SMAC and caspase-3 suggested a protein, presumably a caspase or a caspase substrate, could translocate from the cytosol to the mitochondria to induce cytochrome *c* release (Figure 4.2). Initially, the direct involvement of caspase-3 itself was considered, based on reports of caspase-3 mitochondrial accumulation under specific apoptotic conditions. However, no mitochondrial accumulation of caspase-3 could be detected after sulindac treatment (data not shown). Another candidate was the BH3-only Bcl-2 family member Bid, which is cleaved by caspases in the cytoplasm [133]. In considering the role of Bid in mediating the caspase-3–dependent regulation of cytochrome *c* release, the processing to its active, truncated form and its sub-cellular localization were analyzed in wild-type HCT116 cells, as well as *BAX*-KO cells that are highly resistant to NSAID-induced apoptosis and *SMAC*-KO cells that show an intermediate level of resistance to NSAID-induced apoptosis. The truncated tBid can translocate to the mitochondria to induce cytochrome *c* release, presumably by directly interacting with BAX and Bak through its BH3 domain and inducing oligomerization of BAX and Bak [133].

It was found that Bid was indeed cleaved, and tBid was enriched in the mitochondria after sulindac treatment (Figure 4.9). However, the cleavage and translocation of Bid were also detected at the same level in the *BAX*-KO and *SMAC*-KO cells (Figure 4.9), which are deficient in NSAID-induced apoptosis and

cytochrome *c* release, excluding Bid as the candidate for regulating SMAC-mediated cytochrome *c* release [71, 99]. Bim, another BH3-only protein and a caspase substrate that can translocate to the mitochondria, underwent similar proteolytic cleavage (Figure 4.9) [134]. Although the shorter forms of Bim were enriched in the mitochondria of sulindac-treated HCT116 cells, no difference was observed in the *BAX*-KO and *SMAC*-KO cells (Figure 4.9). The apparent lack of differential processing and subcellular localization of Bid and Bim in NSAID-sensitive HCT116 versus NSAID-resistant *BAX*-KO and *SMAC*-KO cells led us to conclude that these effects were occurring by an unrelated mechanism. Therefore, Bid and Bim did not seem to play a role in NSAID-induced and SMAC-mediated cytochrome *c* release.

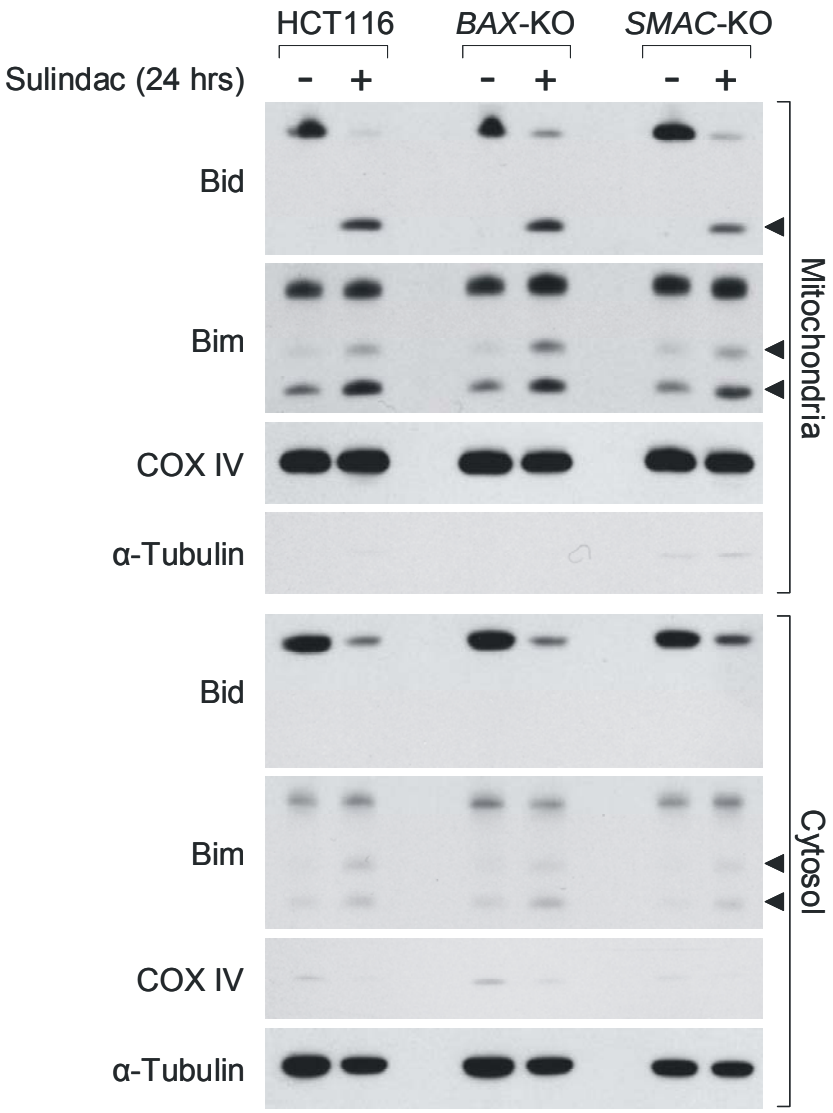


Figure 4.9 Bid and Bim do not mediate feed-back mechanism.
Expression of Bid and Bim in sulindac-treated cells. After treatment of HCT116, *BAX*-KO and *SMAC*-KO cells with 120 μmol/L sulindac for 24 hours, the cytosolic and mitochondrial fractions were analyzed for Bid and Bim expression by Western blotting. Arrows indicate Bid and Bim cleavage fragments. COX IV and α-tubulin, which are exclusively expressed in the mitochondria and cytosol, respectively, were used as controls for loading and fractionation.

4.2.5 ANALYSIS OF PROPOSED FEED-BACK MECHANISM DURING NSAID-INDUCED APOPTOSIS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

An unbiased proteomics approach was next considered as an appropriate method to identify a suitable candidate that could translate NSAID-induced activation of the caspase cascade into the release of cytochrome *c*. Mitochondrial fractions, obtained from sulindac-treated HCT116 and *BAX*-KO cells, were analyzed using 2-D gel electrophoresis followed by silver staining to identify unique protein species that underwent mitochondrial translocation exclusively in NSAID-sensitive cells. The initial attempt at 2-dimensional resolution of mitochondrial proteins indicated an abundance of high molecular weight species (vertical direction) that presumably represented the respiratory complexes responsible for mitochondrial energy generation (Figure 4.10). The dominant 'heavy' proteins had a negative effect on the proper isoelectric focusing (horizontal direction) and distribution of the less abundant 'light' proteins, which represented a suitable location for the proposed candidate molecule, resulting in a suboptimal protein visualization. To address this concern, mitochondrial fractions obtained from similarly treated HCT116 and *BAX*-KO cells were loaded onto centrifugal filter columns containing a size-exclusion membrane with 100kDa nominal molecular weight limit in order to trap and remove the abundant high molecular weight proteins while enriching the low molecular weight proteins. The resulting eluates were similarly analyzed using a 2-D SDS-PAGE gel. However, this strategy resulted in an overall reduction of both high and low

molecular weight populations and thus proved unsuccessful in improving the detection of 'light' proteins (Figure 4.10). While several candidate 'hits' were observed that were uniquely present in sulindac-treated mitochondria obtained from HCT116 cells and absent in *BAX*-KO cells (Figure 4.10), their identities have not been analyzed by mass spectrometry. These findings require further validation in additional colon cancer cell lines and with another member of NSAID family of drugs.

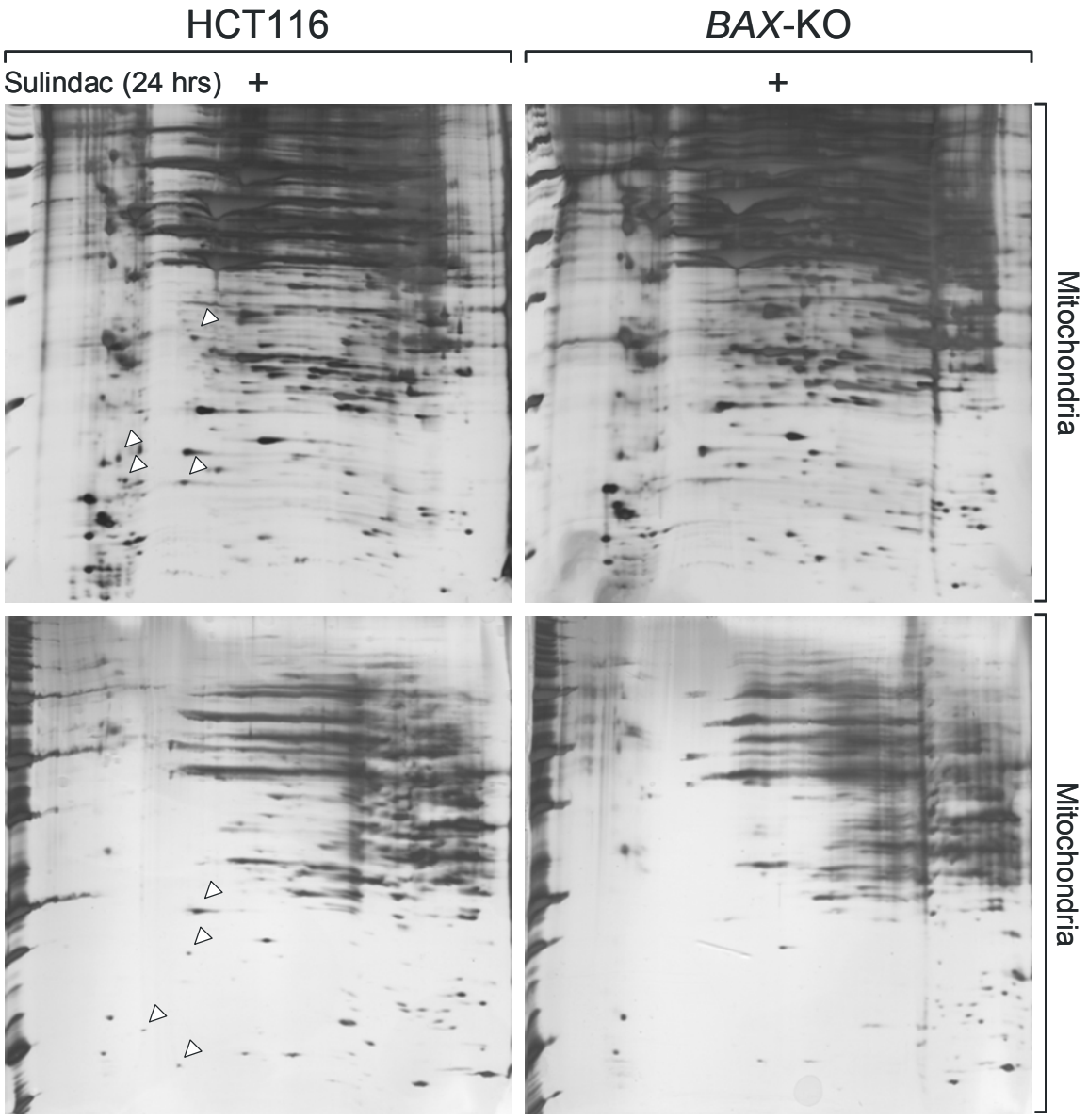


Figure 4.10 Analysis of protein population in sulindac-treated cells.
After treatment of HCT116 and *BAX*-KO cells with 120 μmol/L sulindac for 24 hours, the mitochondrial fractions were isolated and analyzed by 2-D SDS-PAGE. The gels were fixed and visualized by Silver stain. Top, analysis of entire mitochondrial proteome from sulindac-treated cells; bottom, analysis of mitochondrial proteins following purification through a 100 kDa size exclusion filtration column. Arrows indicate protein species unique to HCT116 cells.

4.3 DISCUSSION

In this study, it was found that SMAC release and cytochrome *c* release proceed through different time courses during NSAID-induced apoptosis, with the bulk of SMAC release occurring much earlier compared with that of cytochrome *c* release. The majority of cytochrome *c* release was abolished in the *SMAC*-KO cells, suggesting that SMAC plays an important role in promoting cytochrome *c* release and full execution of NSAID-induced apoptosis. Studies by other groups also showed that the release of different mitochondrial apoptogenic proteins is coordinated and occurs through different time courses [135]. The IAP-interacting activity of SMAC, which is mediated by the N-terminal AVPI domain, is critical for its ability to promote caspase activation and cytochrome *c* release during NSAID-induced apoptosis. Because IAP inhibition occurs in the cytoplasm whereas cytochrome *c* is located in the mitochondria before its release, a feed-back loop involving protein translocation seems to drive the events leading to cytochrome *c* release. A similar function of SMAC in DNA damage-induced apoptosis mediated by the BH3-only protein PUMA was recently described [136]. However, the requirement of SMAC for full execution of apoptosis does not seem to be general, as only few classes of agents among a number of stimuli tested, including NSAIDs, PUMA, and TRAIL, were found to be dependent on SMAC to induce apoptosis [99]. Under different conditions, it is possible that cytochrome *c* plays a major role in driving this feed-back amplification loop [137].

Using siRNA and inhibitor approaches, the activation of capspase-3 was shown to be a critical event in this feed-back loop. Several previous studies have also shown the functions of other caspases, including caspase-2 and caspase-7, in promoting mitochondrial events during apoptosis [138-140]. However, the link between caspase-3 activation and cytochrome *c* release remains to be identified. We did not detect any mitochondrial accumulation of caspase-3 in HCT116 cells undergoing sulindac-induced apoptosis (data not shown), excluding the possibility that caspase-3 itself directly induces cytochrome *c* release. Analysis of pro-apoptotic caspase substrates ruled out the BH3-only proteins Bid and Bim as potential candidates, because their cleavage and translocation were intact in the *BAX*-KO and *SMAC*-KO cells that are deficient in NSAID-induced apoptosis. Several other proteins that can translocate from the cytosol to the mitochondria and promote cytochrome *c* release remain to be examined. It is also possible that more than one protein mediate the intermediate steps between caspase-3 activation and cytochrome *c* release. To identify the mediators of this feed-back loop, it might be useful to compare the mitochondrial components in the wild-type and *BAX*-KO cells using an unbiased proteomic approach. While an initial look at the distribution of mitochondrial proteins in wild-type and *BAX*-KO cells indicated some unique 'hits' present in sulindac-treated wild-type cells, these candidates must withstand rigorous validation before any molecular biology experiments can be considered.

4.4 CONCLUSION

In this study, we investigated the mechanisms behind the pro-apoptotic activity of NSAIDs by analyzing the molecular events that take place following the disruption of mitochondrial membrane integrity and the subsequent release of mitochondrial apoptogenic factors. The release of SMAC into the cytosol preceded the appearance of morphological characteristics of apoptosis and coincided with release of caspases from inhibitor caspase-IAP complexes. The disruption of caspase-IAP interactions occurred shortly before the appearance of caspase-3 processing, in agreement with the predicted, chronologically accurate sequence of molecular events during apoptosis initiation. While the release of SMAC occurred as a brief burst, the release of cytochrome *c* was time-dependent, and the cytosolic accumulation of cytochrome *c* continued to steadily increase with NSAID treatment. Previously characterized resistance of SMAC-deficient HCT116 cells to NSAID-induced apoptosis was recapitulated in delayed and largely attenuated release of cytochrome *c* in these cells, suggesting an existence of a feed-back mechanism by which release of SMAC coordinated the release of cytochrome *c* in NSAID-treated cells. The apoptosis-mediating function of SMAC during NSAID treatment involved a functional IAP binding domain, as cells stably expressing mutant SMAC failed to rescue the resistant phenotype of *SMAC*-KO cells, as characterized by caspase activation and release of cytochrome *c*. Analysis of the proposed feed-back mechanism regulating cytochrome *c* release indicated the crucial role of caspase-3, as inhibition of caspase-3 activity by z-DEVD-fmk or reduction of caspase-3 expression

by RNA interference significantly blocked NSAID-induced release of cytochrome *c* in multiple cell lines. The search for molecular mediators of caspase-dependent cytochrome *c* release ruled out Bid and Bim, as their activation and mitochondrial translocation was unaffected in NSAID-resistant *BAX*-KO and *SMAC*-KO cells.

5 EFFECT OF SMAC MIMETICS ON NSAID-INDUCED APOPTOSIS

5.1 INTRODUCTION

Defects in apoptosis initiation or execution mechanisms caused by deregulated expression and/or function of anti-apoptotic or pro-apoptotic molecules are likely to contribute to tumor progression and resistance to antitumor therapies, including chemotherapy, ionizing irradiation, and immunotherapy [141]. The unfolding of the complex pathways involved in apoptosis signaling has stimulated intensive efforts to restore apoptosis in cancer cells for therapeutic purposes [142]. Inhibitor of apoptosis proteins (IAPs) inhibit the protease activity of caspases by forming direct interactions with caspases via the characteristic ~70-residue zinc-containing domain termed the baculovirus inhibitory repeat (BIR) [131]. XIAP was shown to interact with initiator caspase-9 and executioner caspase-3 and -7 through its BIR3 and BIR2 domains, respectively [101].

It has been shown that SMAC overexpression and treatment with SMAC mimetic peptides can bypass mitochondrial regulation and enhance the pro-apoptotic effects of several anticancer agents, such as tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) [88, 143-146]. Structural analysis has shown that the four N-terminal Ala-Val-Pro-Ile (AVPI) amino acids of SMAC mediate the recognition of a surface groove on the BIR3 domain of XIAP and play an essential role during binding of SMAC to members of the IAP family. SMAC peptides based

on the N-terminal AVPI domain have been previously reported to enhance caspase-3 activation in a cell-free system and sensitize tumor cells to pro-apoptotic stimuli both *in vitro* and *in vivo* [129, 144].

Several small molecules mimicking the AVPI domain of SMAC have also been developed [131, 147, 148]. For example, a compound called C3 was synthesized based on the structure of SMAC [131]. C3 is a symmetric dimer of an oxazoline-derived compound that can penetrate cell membranes and bind to the IAPs with an affinity equal to that of SMAC. The active compound, C3, differs from the inactive, control compound, C4, by substitutions at two sites [131]. The dimer form representing the C3 compound displayed a greater activity in neutralizing endogenous IAP activity in HeLa cell extract than the monomer form [131]. This observation may be attributable to bivalent interaction with adjacent BIR domains in XIAP [131]. Recent evidence suggests that SMAC, a native homodimer, binds XIAP in a similar fashion [149].

Nanomolar concentrations of C3 promote both TRAIL-induced and TNF-α–induced apoptosis in human cancer cells [131]. However, it remains unclear how SMAC mimetics potentiate apoptosis induced by anticancer agents. SMAC release occurs after mitochondrial outer membrane permeabilization, a process thought to happen after commitment to cell death [150].

Although numerous efforts have been made toward developing therapeutic agents, manipulation of apoptotic pathways for chemoprevention has not been extensively explored. In this study, we investigated the effects of small molecule SMAC mimetics on NSAID-induced apoptosis and NSAID resistance.

5.2 RESULTS

5.2.1 SMAC MIMETICS DO NOT AFFECT CELL VIABILITY

The critical role of SMAC in NSAID-induced apoptosis suggested that manipulation of the SMAC pathway may improve the effects of NSAIDs. The requirement of the AVPI domain for SMAC function prompted us to test whether pharmacologic agents that mimic this domain can enhance NSAID-induced apoptosis. Small molecule SMAC mimetic compounds have proven to be potent therapeutic agents that work either as single agents or in combination with another chemotherapeutic drug to induce a robust cellular apoptotic response at sub-micromolar concentrations.

Initial studies indicated that treatment of HCT116 cells with various doses of sulindac resulted in strong inhibition of cellular metabolic activity and growth at concentrations not sufficient to induce apoptosis (Figure 5.1A). The effects of C3, a small molecule mimic of the AVPI residues of SMAC, and its control compound C4, which differs from C3 by a single methyl group, were subsequently tested in a similar assay [131]. In agreement with the published reports indicating apoptosis-specific effects of SMAC mimetic compounds, no significant differences in cell viability between control and active compounds administered in combination with sulindac were observed (Figure 5.1B).

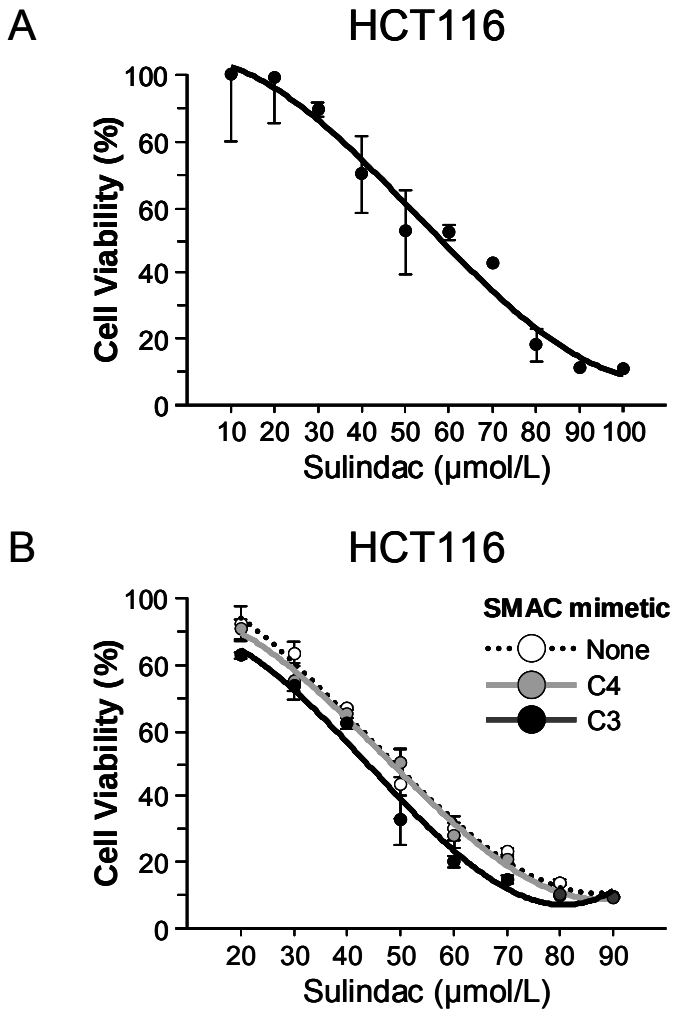


Figure 5.1 SMAC mimetics do not affect cell growth and metabolic activity.
A, Dose response to sulindac. HCT116 cells were treated with the indicated concentrations of sulindac for 48 hours. Loss of cell viability was determined by MTS colorimetric assay.
B, Effect of SMAC mimetics on cell viability. HCT116 cells were treated with NSAIDs at the indicated concentrations of sulindac alone or together with 100 nM control (C4) or test (C3) SMAC mimetic compounds for 48 hours. Loss of cell viability was determined by MTS colorimetric assay.

5.2.2 SMAC MIMETICS POTENTIATE NSAID-INDUCED APOPTOSIS IN COLON CANCER CELLS

It was previously shown that C3, but not C4, at nanomolar concentrations can sensitize human cancer cells to TRAIL or TNF-α–induced apoptosis [131]. In order to maximize the potential sensitizing effects of SMAC mimetic compounds, the response of colon cancer cells to reduced doses of NSAIDs was initially established (Figure 5.2). According to the level of apoptosis induced by various doses of sulindac and indomethacin in different colon cancer cell lines, a single dose of each drug was picked that induced approximately 30% apoptosis (Figure 5.2). SMAC mimetics have shown single agent toxicity in several cell lines. Treatment with SMAC mimetic compounds alone, even at micromolar concentrations, did not have any growth inhibitory or apoptotic effects on colon cancer cells (Figure 5.3 and data not shown). However, only 100 nmol/L of C3 was sufficient to markedly enhance NSAID-induced apoptosis in HCT116 cells, with the percentage of apoptotic cells increasing from 32% to 61% and from 22% to 59% after treatment for 48 hours with 100 μmol/L sulindac and 250 μmol/L indomethacin, respectively (Figure 5.3). In contrast, the control C4 compound did not have any effect on apoptosis (Figures 5.3 and 5.4). Similar observations were also made in HT29 and DLD1 colon cancer cells (Figure 5.3). To verify these results, the analysis of an independent set of active and control SMAC mimetic compounds, GT-T and GT-C, demonstrated similar results (Figures 5.3 and 5.4).

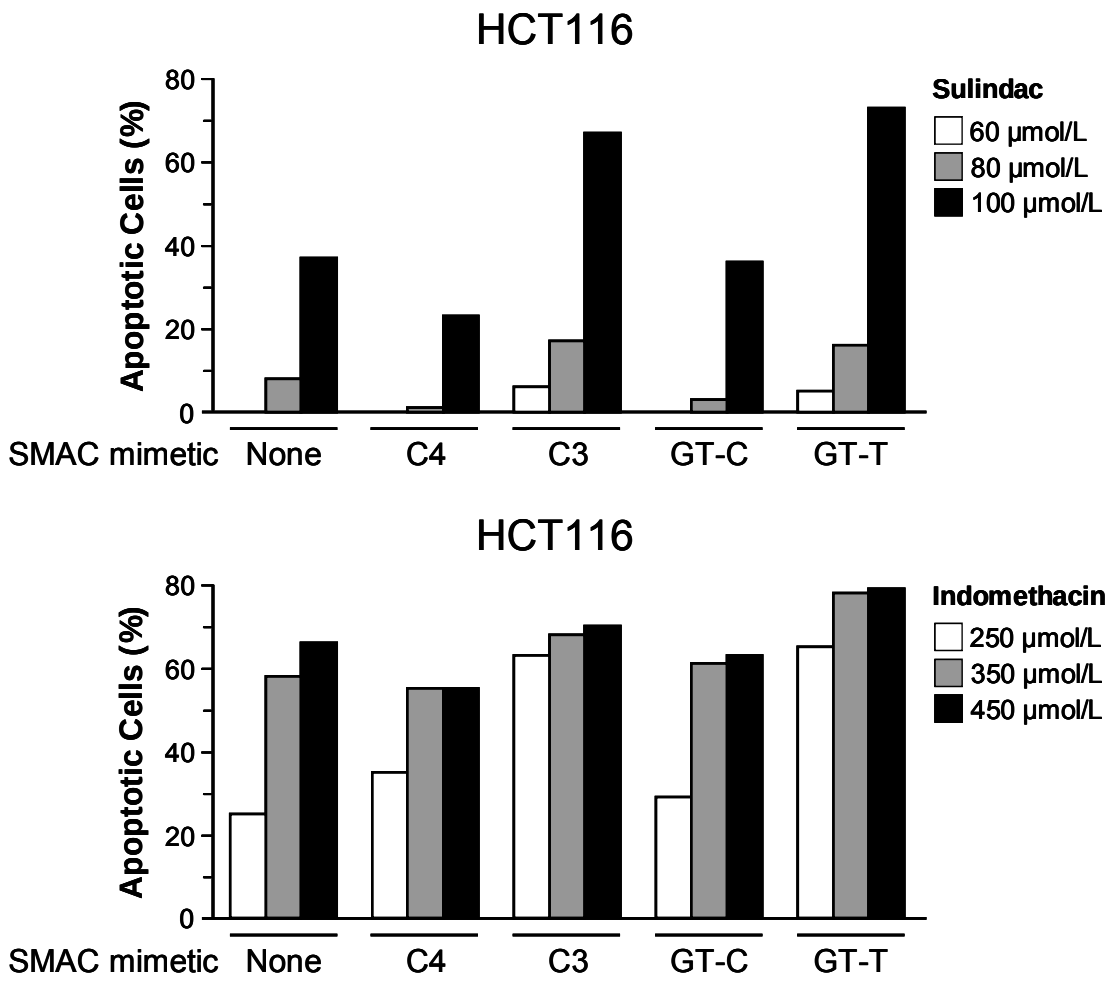


Figure 5.2 Dose response to different NSAID concentrations.
HCT116 cells were treated with NSAIDs at the indicated concentrations with or without 100 nmol/L control (C4 or GT-C) or test (C3 or GT-T) SMAC mimetic compounds for 48 hours. Apoptosis induction was determined by nuclear staining.

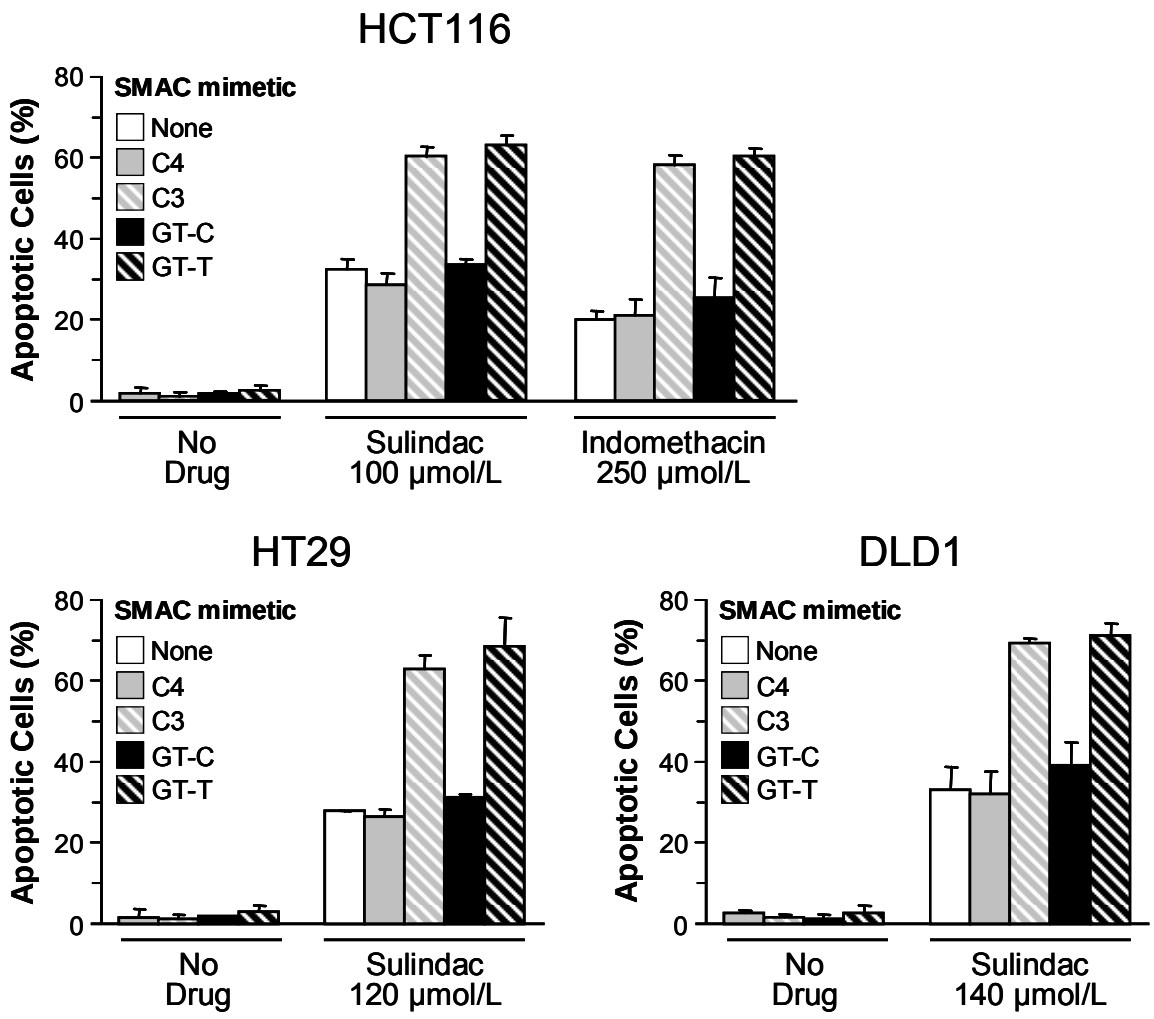


Figure 5.3 SMAC mimetics sensitize colon cancer cells to NSAID-induced apoptosis.

HCT116, HT29, and DLD1 colon cancer cells were treated with NSAIDs with or without 100 nmol/L control (C4 or GT-C) or test (C3 or GT-T) SMAC mimetic compounds for 48 hours. Apoptosis induction was determined by nuclear staining.

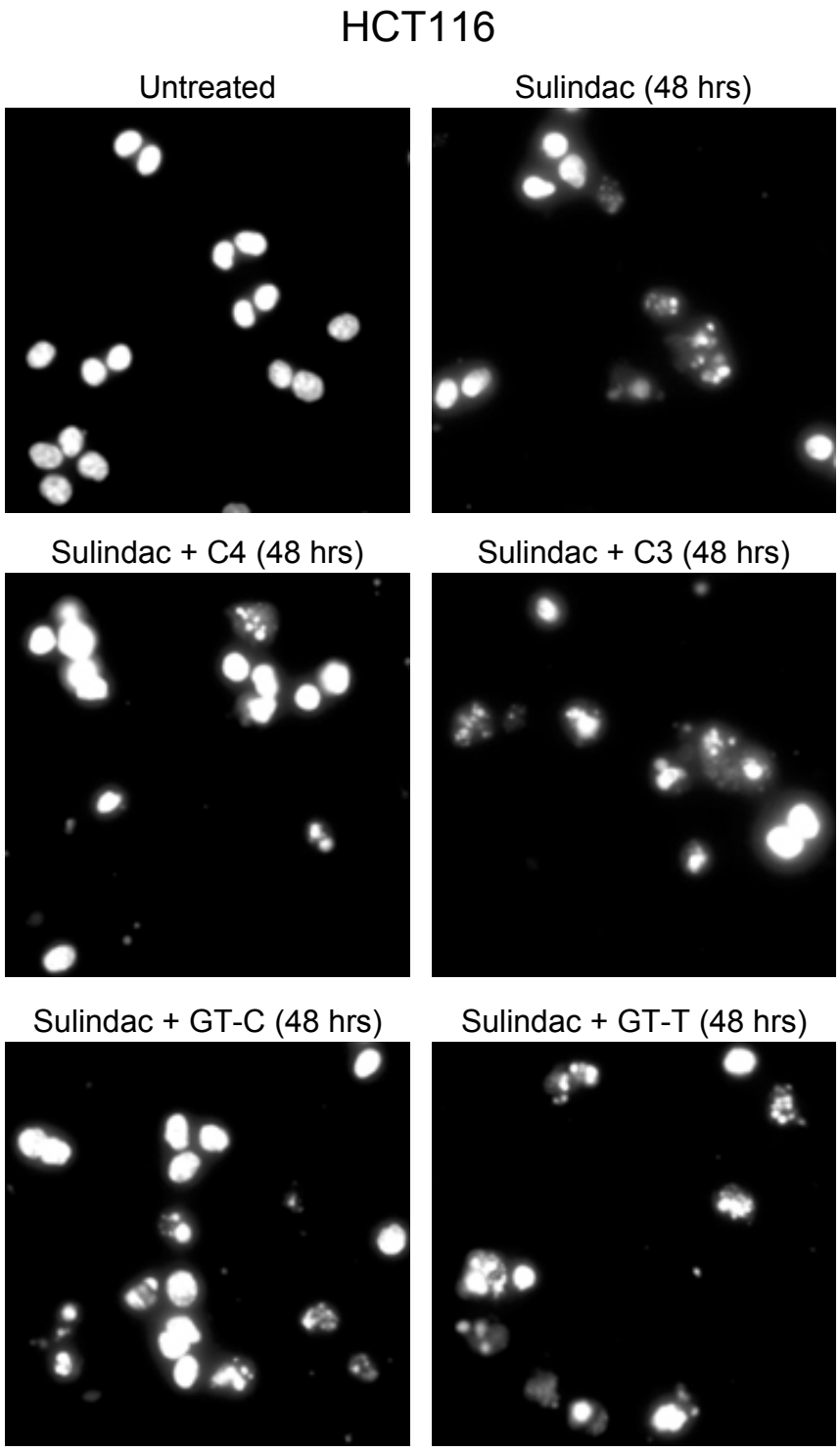


Figure 5.4 SMAC mimetics potentiate NSAID-induced apoptosis.
Representative fluorescence images of nuclei in Hoechst-stained cells. Healthy cells contain nuclei with characteristic rounded shape and uniform staining, while apoptotic cells contain fragmented nuclei with condensed chromatin.

Analysis of apoptosis using Annexin V/propidium iodide staining confirmed that NSAID-induced apoptosis is enhanced by the active C3 or GT-T compound but not by the control C4 or GT-C compound (Figure 5.5). Furthermore, colony formation assay results showed that combinations of NSAIDs with the active compounds were much more effective in inhibiting long-term survival of HCT116 and HT29 cells compared with NSAIDs alone or their combinations with the control compounds (Figure 5.6). Over 50% of the cells that could not be killed by sulindac or indomethacin alone were eliminated by the combination with C3 or GT-T (Figure 5.6). These results showed strong cooperative effects of NSAIDs and SMAC mimetics in killing colon cancer cells.

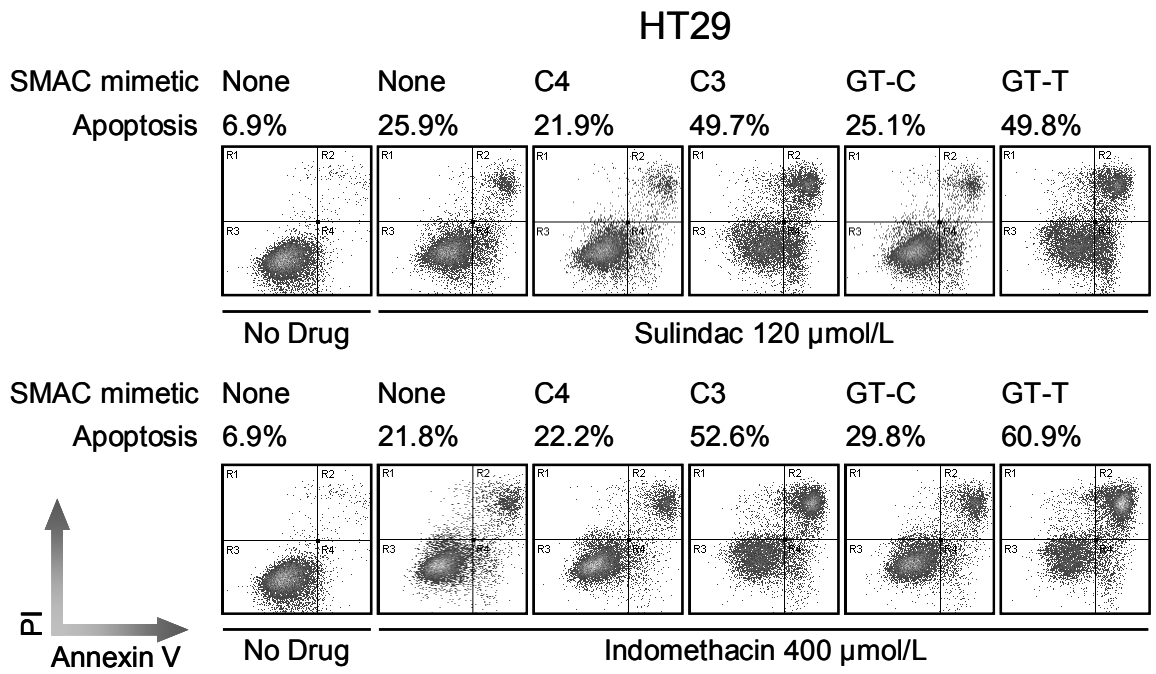


Figure 5.5 SMAC mimetics potentiate NSAID-induced apoptosis.
HT29 colon cancer cells were treated with NSAIDs at the indicated concentrations with or without 100 nmol/L control (C4 or GT-C) or test (C3 or GT-T) SMAC mimetic compounds for 48 hours. Apoptosis induction was analyzed by Annexin V/PI staining. The percentage of apoptosis represents Annexin V/PI double-positive population.

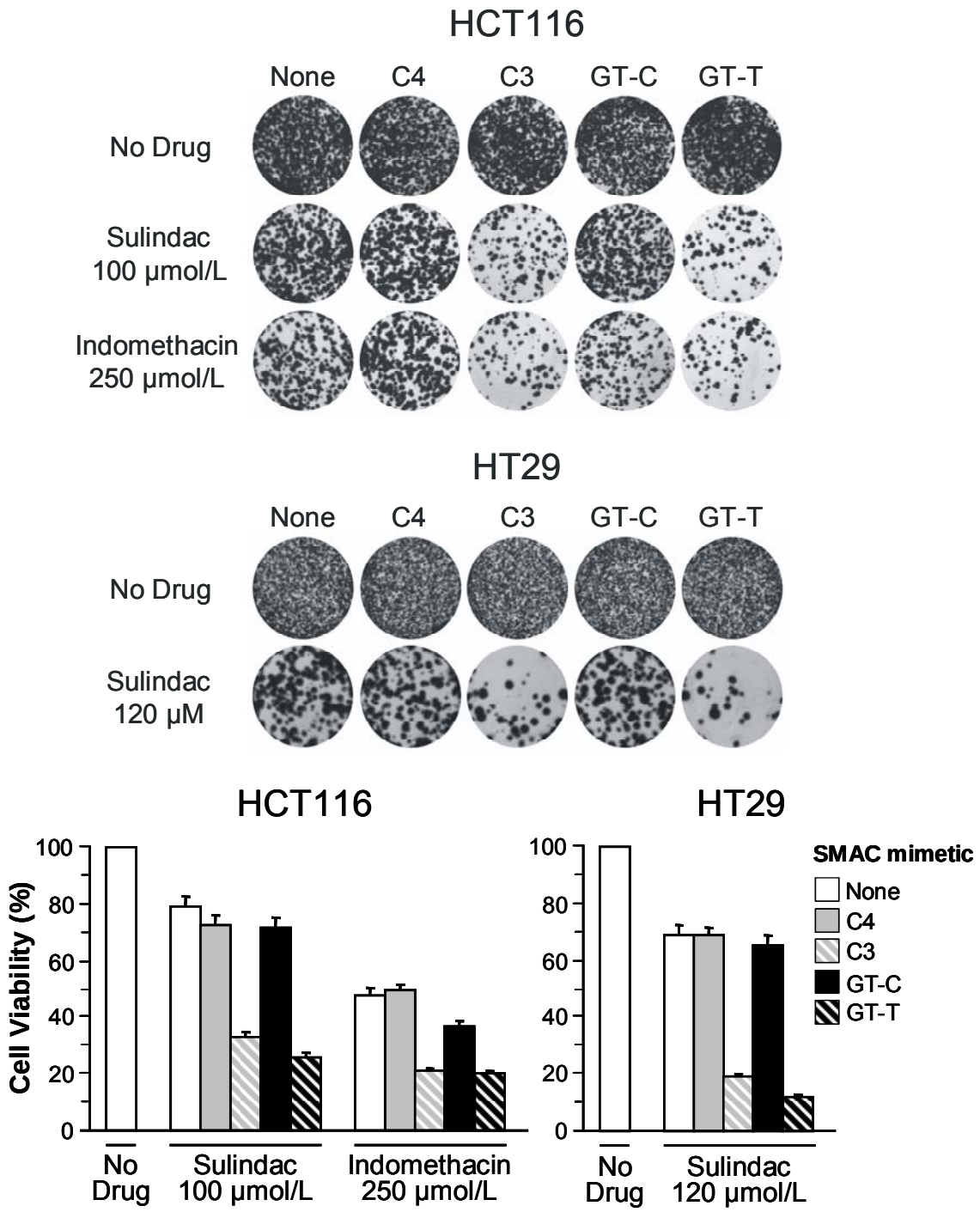


Figure 5.6 SMAC mimetics potentiate inhibition of clonogenic survival.
Long-term cell viability was assessed by colony formation assay following treatment with NSAIDs for 48 hours alone or in combination with 100 nmol/L control (C4, GT-C) or active (C3, GT-T) SMAC mimetic compounds. Top, representative pictures of cell colonies; bottom, quantitation of colony numbers.

5.2.3 SMAC MIMETICS OVERCOME NSAID RESISTANCE IN *SMAC*-KO AND *BAX*-KO CELLS

We also looked at the effect of SMAC mimetics in *SMAC*-KO and *BAX*-KO cell lines, derived from the HCT116 cell line, which exhibit a potent and nearly complete resistance to apoptosis induced by NSAIDs, respectively. The majority of HCT116 cells surviving from NSAID treatment contain BAX mutations and are defective in SMAC release [71, 99]. Acquired NSAID resistance was found to be associated with overexpression of anti-apoptotic Bcl-2 family proteins [151, 152]. Because SMAC functions downstream of BAX and other Bcl-2 family members, we asked whether manipulating SMAC can restore NSAID sensitivity in NSAID-resistant colon cancer cells that are deficient in BAX or SMAC. It was shown that cytosolic, but not mitochondrial, SMAC can bypass the requirement of activating Bcl-2 family proteins to activate caspases [144, 146]. We therefore transiently transfected a cytosolic SMAC expression construct into *BAX*-KO cells (Figure 5.7), which are completely resistant to NSAIDs even at very high concentrations [71]. To maximize the apoptotic effect so that molecular markers of apoptosis could be analyzed, we used a higher concentration of sulindac (200 μmol/L) or indomethacin (800 μmol/L) than those used for other cell lines to treat *BAX*-KO cells. We found that sulindac-induced apoptosis was indeed restored by the cytosolic SMAC in *BAX*-KO cells (Figure 5.7).

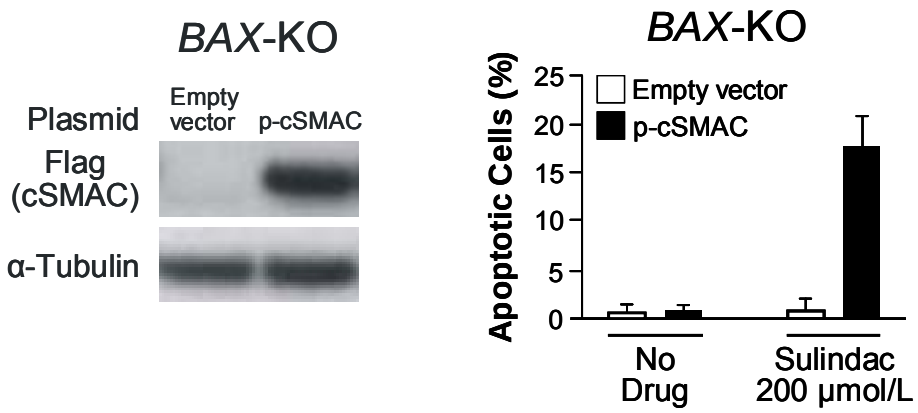


Figure 5.7 SMAC overexpression restores NSAID sensitivity in *BAX*-KO cells. *BAX*-KO cells were transfected with the cytosolic SMAC expression construct (p-cSMAC) or control empty vector. Left, the expression of Flag-tagged cytosolic SMAC 24 hours after transfection was confirmed by Western blotting. Right, cells were treated with 200 µmol/L sulindac for 48 hours. Apoptosis was analyzed by nuclear staining.

Remarkably, the active SMAC mimetic compounds C3 and GT-T, but not the control compounds C4 and GT-C, also restored apoptosis induced by sulindac or indomethacin in *BAX*-KO and *SMAC*-KO cells (Figure 5.8). These results were confirmed by analysis of apoptosis using Annexin V/propidium iodide staining (Figure 5.9). They suggest that activation of the apoptotic pathway by SMAC or SMAC mimetics can overcome NSAID resistance in colon cancer cells.

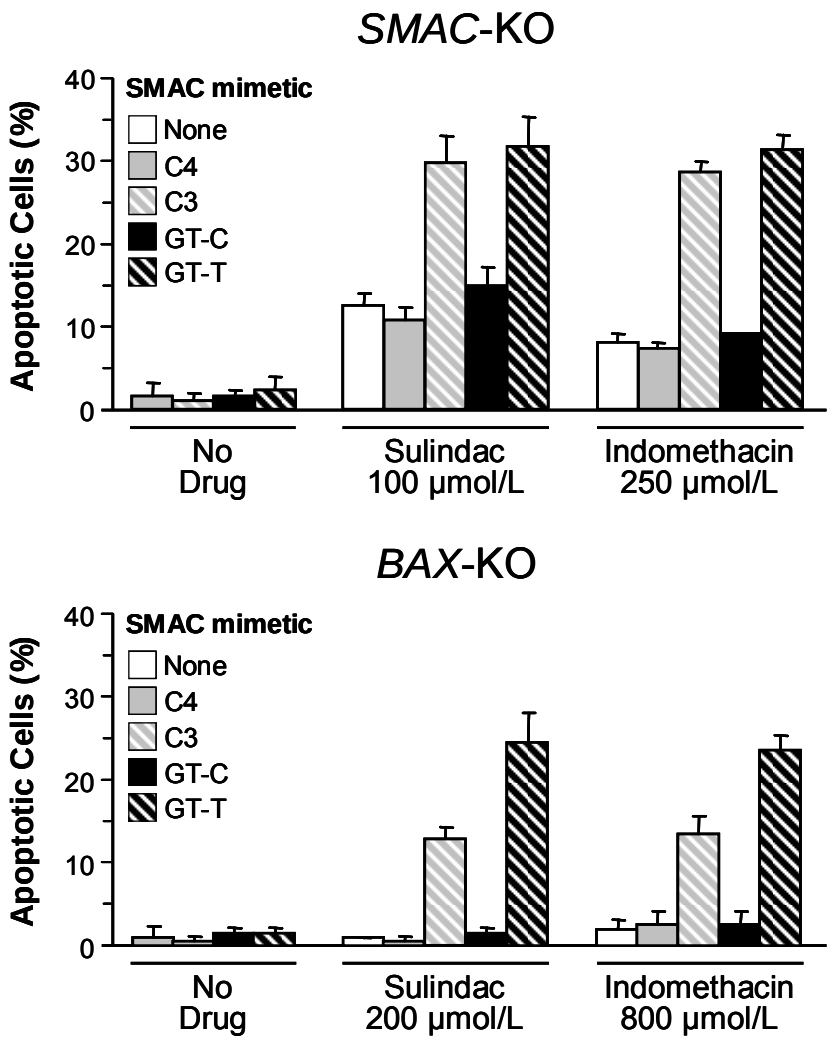


Figure 5.8 SMAC mimetics restore NSAID sensitivity in *SMAC*-KO and *BAX*-KO cells.

SMAC-KO and *BAX*-KO cells were treated with 100 nmol/L control (C4 or GT-C) or test (C3 or GT-T) SMAC mimetic compounds alone or in combination with sulindac or indomethacin at indicated concentrations for 48 hours. Apoptosis was analyzed by nuclear staining.

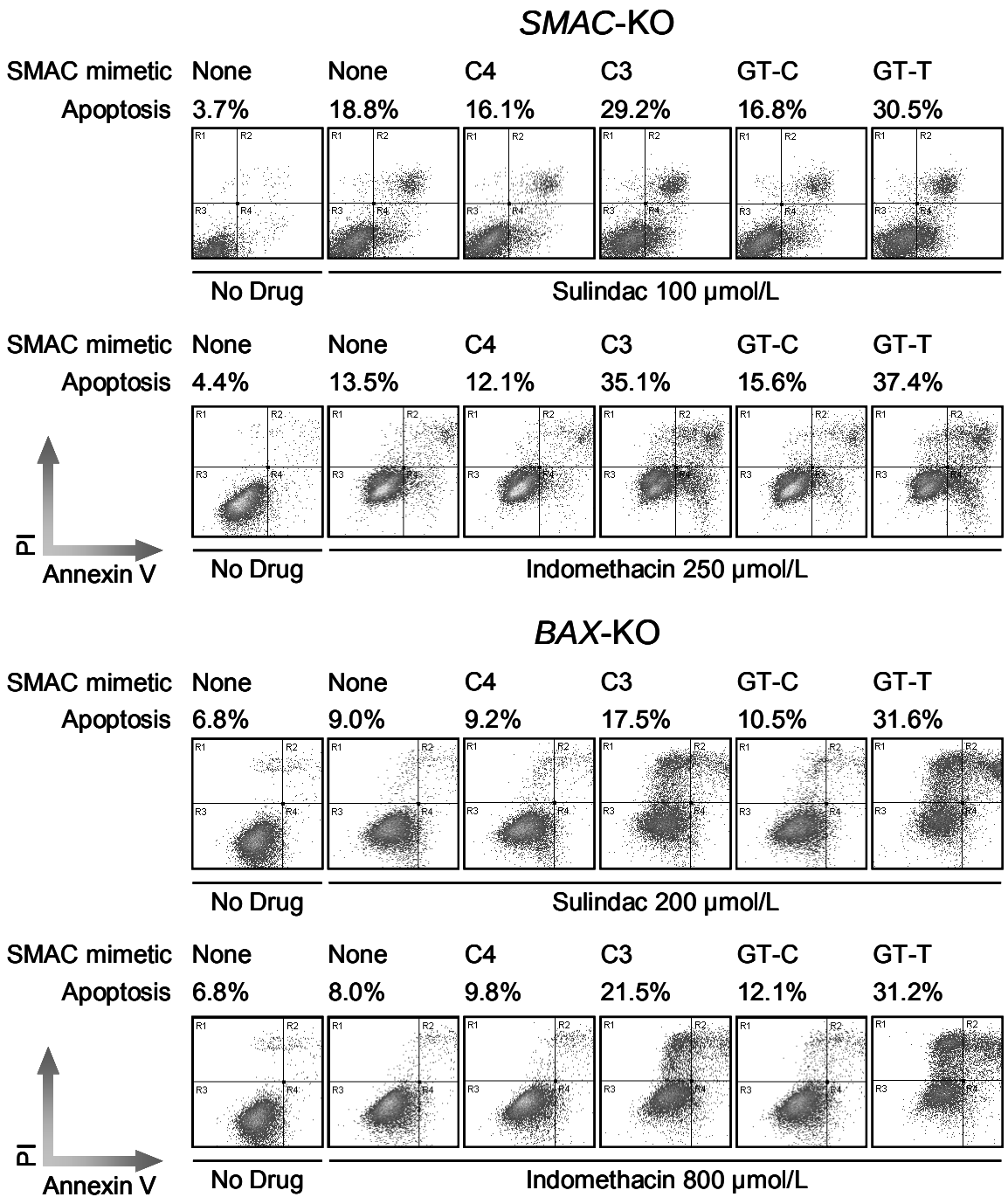


Figure 5.9 SMAC mimetics restore apoptosis in *SMAC*-KO and *BAX*-KO cells. *SMAC*-KO and *BAX*-KO cells were treated with sulindac or indomethacin at indicated concentrations alone or in combination with 100 nM control (C4 or GT-C) or test (C3 or GT-T) SMAC mimetic compounds for 48 hours. Apoptosis induction was analyzed by Annexin V/PI staining. The percentage of apoptosis represents Annexin V/PI double-positive population.

5.2.4 EFFECTS OF SMAC MIMETICS DEPEND ON CASPASE-3 AND INVOLVE CYTOCHROME *C* RELEASE

We then investigated the mechanisms by which SMAC mimetics potentiate NSAID-induced apoptosis. Since these compounds were designed to mimic the function of active SMAC in releasing caspases from inhibitory complexes with IAPs, we looked at whether SMAC mimetics regulated NSAID-induced activation of the caspase cascade. As previously, reduced doses of NSAIDs were used to ascertain the sensitization effect of SMAC mimetics on NSAID-induced apoptosis. Treatment with 100 μmol/L sulindac failed to induce robust caspase-3 and caspase-9 activation in HCT116 cells (Figure 5.10), consistent with the low level of apoptosis induced by sulindac at this concentration (Figures 5.2 and 5.3). However, in combination with SMAC mimetic compounds, 100 μmol/L sulindac strongly induced accumulation of active caspase-3 and caspase-9 (Figure 5.10). Although sulindac, even at 200 μmol/L concentration, could not induce caspase processing in *BAX*-KO cells, SMAC mimetics rescued caspase-3 and caspase-9 activation in these cells (Figure 5.11). In agreement with the regulation of cytochrome *c* release by SMAC, treatment of HCT116 cells with sulindac combined with the active SMAC mimetics markedly enhanced cytochrome *c* release in HCT116 cells and also restored cytochrome *c* release in *BAX*-KO cells (Figures 5.10 and 5.11).

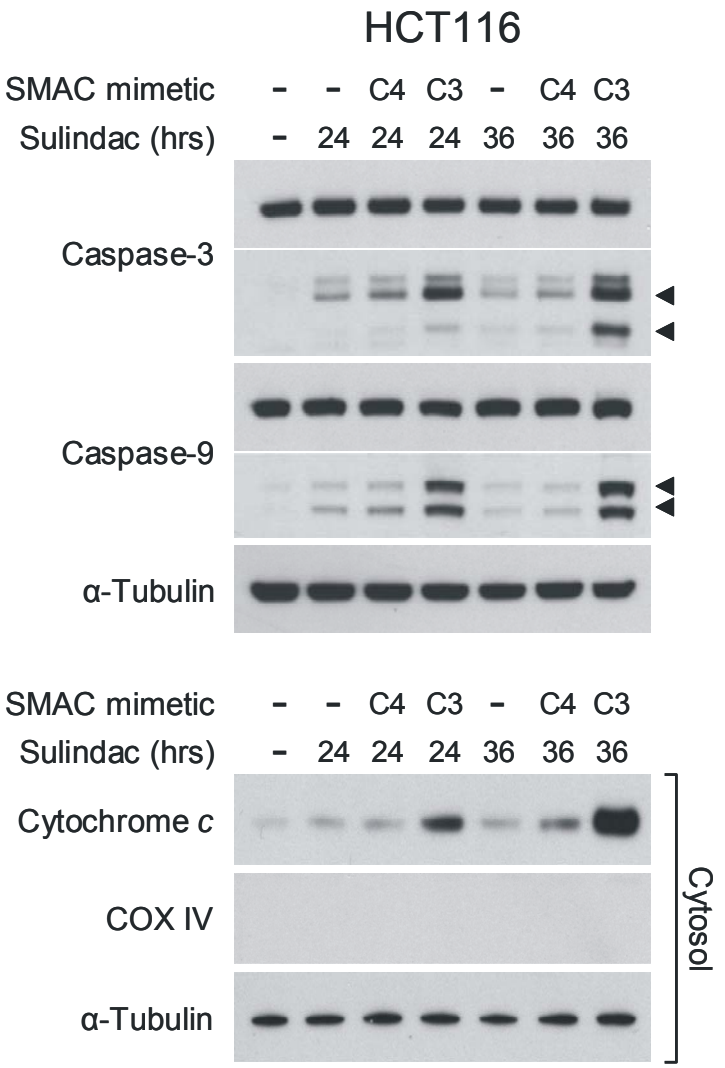


Figure 5.10 SMAC mimetics enhance caspase activation and cytochrome *c* release.

HCT116 cells were treated with 100 μmol/L sulindac alone or in combination with 100 nmol/L control (C4) or test (C3) SMAC mimetic compounds. Top, caspase-3 and caspase-9 activation was analyzed by Western blotting; bottom, cytosolic fraction was isolated from treated cells, and cytochrome *c* release was determined by Western blotting.

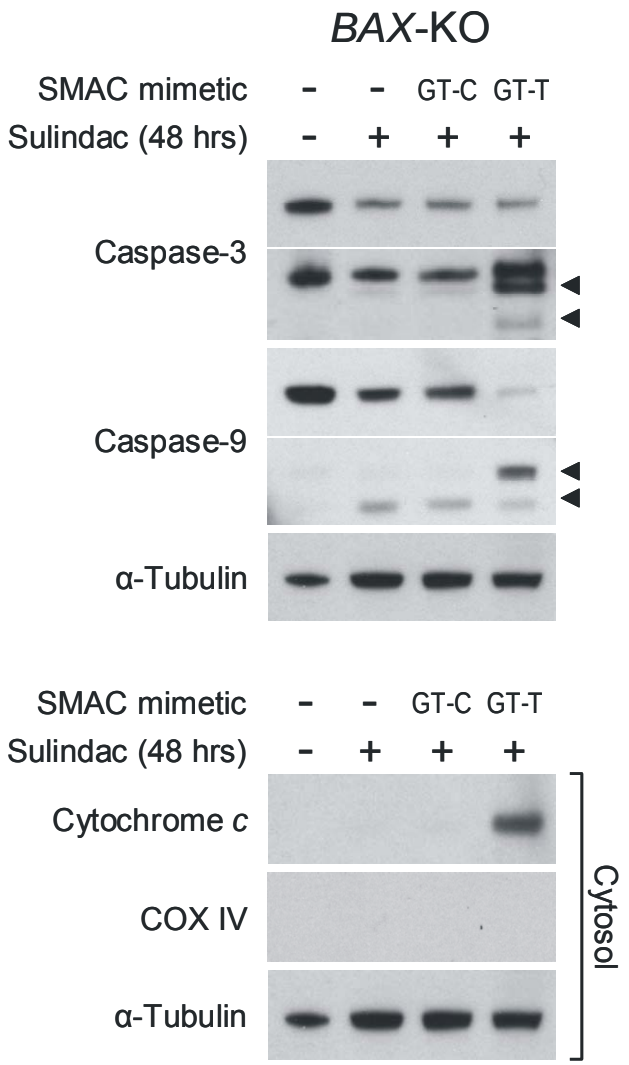


Figure 5.11 SMAC mimetics rescue caspase activation and cytochrome *c* release in *BAX*-KO cells.

BAX-KO cells were treated with 200 μmol/L sulindac alone or in combination with 100 nmol/L control (GT-C) or test (GT-T) SMAC mimetic compounds for 48 hours. Top, caspase-3 and caspase-9 activation was analyzed by Western blotting; bottom, cytosolic fraction was isolated from treated cells, and cytochrome *c* release was determined by Western blotting.

To test whether the effects of SMAC mimetics require caspase-3 activation, caspase-3 siRNA was transfected into cells before the combination treatment. Caspase-3 knockdown resulted in a significant reduction of apoptosis in the cells treated with the combinations of sulindac and SMAC mimetics, which was much more pronounced than the reduction of apoptosis seen in the cells treated with sulindac alone (Figure 5.12A), indicating that the sensitizing effects of the SMAC mimetics are at least in part mediated by caspase-3. Furthermore, caspase-3 knockdown also led to attenuation of cytochrome *c* release induced by the combination of sulindac and SMAC mimetics (Figure 5.12B). These results suggested that SMAC mimetics potentiate NSAID-induced apoptosis and overcome NSAID resistance by promoting caspase-3 activation, thereby enhancing cytochrome *c* release.

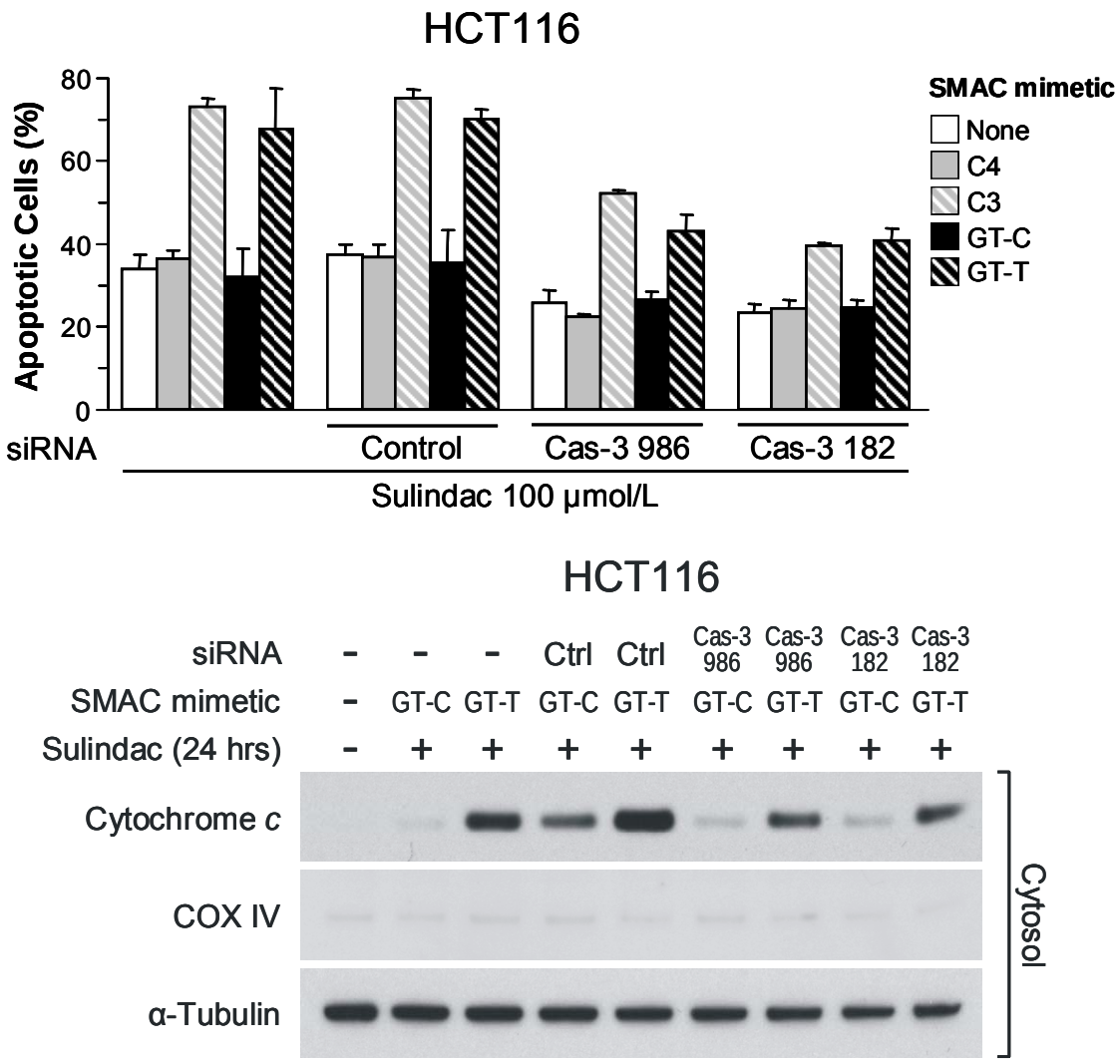


Figure 5.12 Effect of caspase-3 knockdown on sensitization by SMAC mimetics.
A, HCT116 cells were transfected with control scrambled or caspase-3 siRNA for 24 hours and treated with 100 µmol/L sulindac. Apoptosis induction was determined by nuclear staining 48 hours after sulindac treatment.
B, HCT116 cells were treated with 100 µmol/L sulindac alone or in combination with 100 nmol/L control (GT-C) or test (GT-T) SMAC mimetic compounds for 24 hours. Cytosolic fractions were isolated from treated cells, and cytochrome *c* release was determined by Western blotting

5.2.5 ESTABLISHING STABLE REPORTER COLON CANCER CELL LINES FOR FUTURE STUDIES OF SMAC MIMETICS IN ANIMAL MODELS

Having demonstrated the sensitizing effect of SMAC mimetics on apoptosis induced by NSAIDs in a panel of colon cancer cell lines, the efficacies of these compounds need to be established and validated in future animal studies. One method of studying the effect of a particular chemotherapy treatment on physiological tumor growth and development involves the establishment of xenograft tumors based on human cancer cell lines in nude mice that lack a functional immune system and therefore do not initiate a host-versus-graft response to human cells. The xenograft tumor model can be further enhanced by using cancer cells with stable expression of luciferase enzyme. Luciferases constitute a diverse group of unrelated enzymes acting upon chemically different luciferins and employ a variety of cofactors that are functionally classified as special types of oxygenases optimized for light emission [153]. These enzymes catalyze the oxidation of a luciferin substrate coupled to generation of energy-rich peroxidic intermediates, whose spontaneous decomposition results in emission of a photon of visible light with high efficiency [153]. The advantages of using bioluminescence to study tumor cells include non-invasive and real-time tumor monitoring capabilities within a living organism. Data produced is both quantitative and qualitative, and multiple events may be examined within the same host.

NSAID-sensitive and NSAID-resistant cell lines were co-transfected with linearized pGL3 control plasmid and linearized pcDNA 3.1(+) G418 selection plasmid (Figure 5.13A). The analysis of individual clones from each cell line revealed several orders of magnitude difference in luciferase activity (data not shown). Since a certain basal level of luciferase activity is required to detect the bioluminescence signal from implanted tumor cells, the activity of luciferase in representative individual clones was verified under conditions resembling the bioluminescence analysis in live animals. Detection of luciferase-mediated bioluminescence using a Xenogen IVIS 200 imaging system revealed a minimum signal strength that is required for animal studies (Figure 5.13B). Using this criteria, several confirmed stable clones were selected for future mouse xenograft studies to establish luciferase-tagged human colon cancer tumors in immuno-deficient nude mice. The response of the tumors to sulindac treatment alone or in combination with SMAC mimetic compounds will be tracked by quantifying tumor-specific luciferase activity. Whole animal bioluminescence imaging will be performed on an IVIS 200 imaging system available at the Small Animal Imaging Core.

A PerkinElmer Victor3 luminometry

HCT116				
Stable clone	38	51	57	60
Concentration (µg/ml)	36	29	38	34
Luciferase activity (units)	231859	181045	350548	1028434
***BAX*-KO**				
Stable clone	6	15	16	
Concentration (µg/ml)	42	39	48	
Luciferase activity (units)	209992	174724	752639	
***SMAC*-KO**				
Stable clone				
Concentration (µg/ml)				
Luciferase activity (units)				

B Xenogen IVIS 200 bioluminescence

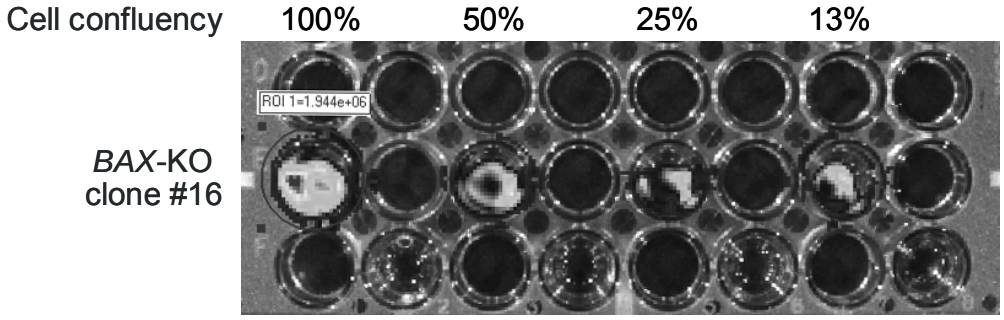


Figure 5.13 Luciferase expression in colon cancer cell lines.
A, Isolation of stable clones with high luciferase activity. HCT116 wild-type, *BAX*-KO, and *SMAC*-KO cells were co-transfected with linearized pGL3 Control plasmid and linearized pcDNA 3.1(+) G418 selection plasmid. Whole-cell lysates were collected from 24-well plates and incubated with LAR reagent. Samples were immediately analyzed for luciferase activity using PerkinElmer Victor3 plate reader. B, Detection of luciferase activity in cultured clones. Representative clones with different levels of introduced luciferase activity were incubated with luciferin and analyzed for luciferase activity using Xenogen IVIS 200 imaging system.

5.3 DISCUSSION

The results obtained from two independent sets of compounds showed for the first time that SMAC mimetics can greatly potentiate the anticancer effects of NSAIDs in both short-term apoptotic and long-term clonogenic survival assays. Other studies have described similar effects of SMAC and SMAC mimetics on chemotherapeutic drugs and irradiation [143, 144, 154, 155]. SMAC mimetics not only greatly enhanced the apoptotic effects of sulindac and indomethacin in colon cancer cells with different genetic backgrounds but also restored apoptosis in the NSAID-resistant *SMAC*-KO and *BAX*-KO cells. The data on *BAX*-KO cells were obtained using higher concentrations of NSAIDs (200 μmol/L sulindac and 800 μmol/L indomethacin) than those used for other cell lines. When SMAC mimetics were combined with NSAIDs at lower concentrations (120 μmol/L sulindac and 500 μmol/L indomethacin), we also detected ~10% apoptosis in *BAX*-KO cells (data not shown). The mechanistic studies showed that SMAC mimetics enhanced NSAID-induced caspase-3 activation and cytochrome *c* release, thereby promoting the feed-back loop for full execution of NSAID-induced apoptosis. Importantly, the effects of SMAC mimetics are caspase-3–dependent, suggesting that caspases play a major role in mediating this feed-back loop, also reinforcing that the sensitizing effects are not due to off-target activities of SMAC mimetics [131]. It will be of interest to test whether previously reported activities of SMAC mimetics also operate through similar feed-back mechanisms.

A recent study has demonstrated the possible role of TRAIL-DR5 death receptor in mediating sulindac-induced apoptosis in HCT116 cells [98]. Sulindac treated cells exhibited a marked induction of TRAIL-DR5 expression and subsequent ligand-independent death receptor signaling, ultimately resulting in activation of proximal caspase-8 and an associated caspase-8–dependent apoptosis signaling. In agreement with these findings, the present study showed that Bid, a well established caspase-8 substrate, was cleaved in sulindac treated cells to generate the active tBid fragment that showed a strong mitochondrial localization. As was noted earlier, the activation and mitochondrial translocation of Bid occurred to an equal extent in HCT116 cells as well as the BAX-deficient derivatives, supporting the observation that caspase-8 is activated in HCT116 cells and *BAX*-KO cells treated with NSAIDs (previous findings; data not shown). Active tBid fragment promotes BAX homooligomerization and BAX-dependent cytochrome *c* release by interacting with Bcl-2/Bcl-X_L at the mitochondria and relieving their inhibition of BAX, or causing the conformational change in BAX through a direct interaction. The data present in the present as well as previous studies indicate a critical role of BAX in NSAID-induced apoptosis [71]. Therefore, two hypotheses are put forth aimed at explaining the ability of SMAC overexpression and SMAC mimetics to rescue apoptosis induced by NSAIDs in *BAX*-KO cells. Since *BAX*-KO cells maintain the expression of Bak, a close relative of BAX, it can be postulated that the high doses of NSAIDs induce the hyperactivation of TRAIL-DR5 receptor, and the resulting high level of caspase-8 activity induces a strong accumulation of tBid that activates Bak, allowing Bak to drive the initial release of cytochrome *c* in place of BAX. In the concurrent presence

of a strong, cytosolic SMAC activity, caspase-9 and caspase-3 can respond to the presence of cytochrome *c* alone without the requirement for release of endogenous SMAC from mitochondria. The initial activation of caspase-3 can subsequently establish the positive feed-back loop, similar to HCT116 cells, and promote the induction of apoptosis in previously resistant cells. Alternatively, caspase-8 can directly activate caspase-3; however, caspase-3 activation is inhibited by IAPs. Addition of exogenous SMAC or SMAC mimetics results in dissociation of IAPs from caspase-3, and NSAID-activated caspase-8 can now fully activate caspase-3. Caspase-3 acts through the feed-back mechanism to induce the release of cytochrome *c*, resulting in an activation of caspase-9 and a more potent activation of caspase-3, leading to apoptosis. The validity of the proposed mechanisms behind the observed activity of SMAC mimetics in *BAX*-KO cells can be experimentally verified by studying the effects of knocking down caspase-8, Bid, and Bak expression.

Extensive efforts have been made toward developing therapeutic agents through manipulation of apoptotic pathways [156]. For example, ABT-737, a compound that mimics the BH3 domains of Bcl-2 family proteins, has shown great promise in early preclinical studies [157]. However, manipulation of apoptotic pathways for chemoprevention has not been extensively explored. Our results suggest that SMAC mimetics are potentially useful as sensitizers for NSAIDs and possibly for other chemopreventive agents by boosting their pro-apoptotic activities and also by restoring apoptosis in otherwise resistant neoplastic cells. It is worth noting that the concentrations of SMAC mimetics were lower than the concentrations of NSAIDs used in our study by three orders of magnitude. The side effects of

NSAIDs, presumably caused by high drug doses used in prevention studies, have presented a significant challenge for using these agents for cancer prevention [126]. It is possible that combinations of NSAIDs with SMAC mimetics will help to enhance chemopreventive efficacy, while reducing dose and decreasing toxicity. This possibility can be tested using animal models, such as establishing xenograft tumors in nude mice or administration of sulindac together with SMAC mimetic compounds in *APC*$^{Min/+}$ mice that spontaneously develop intestinal polyps and carcinomas.

5.4 CONCLUSION

In this study, we analyzed the effects of modulating the molecular events that occurred during NSAID-induced apoptosis by small molecule SMAC mimetics, designed to recapitulate the function of the AVPI domain of SMAC in inducing the dissociation of caspase-IAP complexes. As has been reported in the majority of human cancer cell lines derived from solid tumors, SMAC mimetics failed to inhibit cell viability and induce apoptosis as single agents. Analysis of SMAC mimetic activity in combination with NSAIDs indicated a powerful sensitizing effect that was observed in multiple colon cancer cell lines. The combination treatment of NSAIDs and SMAC mimetics invoked a potent apoptosis induction and inhibition of clonogenic potential at IC_{30} NSAID concentrations, suggesting a therapeutically relevant treatment regimen with enhanced induction of cancer cell death and reduced side effects stemming from NSAID-mediated inhibition of protective COX-1 activities. The sensitization of cancer cells to NSAID-induced apoptosis by SMAC mimetics was characterized by enhanced caspase activation and release of cytochrome *c*. The resistance to NSAID-induced apoptosis normally observed in *BAX*-KO and *SMAC*-KO cells was alleviated by SMAC mimetics, confirming the positive effect of ectopic SMAC overexpression on apoptosis induced by NSAIDs in *BAX*-KO cells. The rescue of apoptosis in NSAID-resistant cells was similarly characterized by the appearance of active caspase fragments and accumulation of cytosolic cytochrome *c*, suggesting that SMAC mimetics could bypass the apoptosis defects mediated by BAX and SMAC deficiency and act downstream of

mitochondria. The activity of these compounds relied on caspase-3, as inhibition of caspase-3 by RNA interference significantly reduced the sensitizing effect of SMAC mimetics. The validation of SMAC mimetics activity in small animals will be addressed by monitoring spontaneous cancer formation in *APC*$^{Min/+}$ mice, as well as the analysis of xenograft tumor response in nude mice, facilitated by the development of luciferase-expressing human colon cancer cell lines that allows the use of bioluminescence detection methods.

APPENDIX: GLOSSARY OF APOPTOSIS TERMS

AIF	Apoptosis-inducing factor
Apaf-1	Apoptosis activating factor-1
Bak	Bcl-2 antagonist/Killer
BAX	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma -2
BH3	Bcl-2 Homology 3
Bid	Bcl interacting domain
Bim	Bcl-2 interacting mediator of cell death
BIR	Baculoviral inhibitor of apoptosis repeat
CARD	Caspase recruitment domain
CARP	Caspase associated RING-protein
Caspase	Cysteinyl aspartate-specific protease
DED	Death effector domain
Diablo	Direct IAP binding protein with low pl
DISC	Death inducing signaling complex
FADD	Fas Associated Death Domain
IAP	Inhibitor of apoptosis protein
Mcl-1	Myeloid cell leukemia-1
NF-κB	Nuclear Factor Kappa B
SMAC	Second mitochondrial-derived activator of caspase
PUMA	p53 up-regulated modulator of apoptosis
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
XIAP	X-linked IAP

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