

# Docosahexaenoic acid sensitizes colon cancer cells to sulindac sulfide-induced apoptosis

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**Abstract.** Sulindac analogs represent one of the most efficacious groups of NSAIDs reducing the risk of colon cancer. Recent studies have shown that sulindac sulfide, a sulindac analog effective at lower doses compared to its parent compound, triggers the death receptor (DR)5-dependent extrinsic apoptotic pathway. Induction of apoptosis via activation of the DR-mediated pathway would be an ideal therapeutic strategy to eliminate cancer cells. In this study, we investigated the possibility that colon cancer cells are sensitized to sulindac sulfide-induced apoptosis by docosahexaenoic acid (DHA), via activation of the DR/extrinsic apoptotic pathway. Our data demonstrated that DHA combination sensitized colon cancer cells to sulindac sulfide-induced apoptosis, leading to enhanced growth suppression of human colon cancer xenografts. The combination effect was primarily attributed to increased cleavage of poly(ADP-ribose) polymerase (PARP) and caspase-8 activation. Moreover, pretreatment with z-IETD-FMK (caspase-8 inhibitor) or stable expression of dominant negative caspase-8 genes blocked DHA/sulindac sulfide cotreatment-induced apoptosis. In view of the finding that DR5 silencing abrogated the combination-stimulated apoptosis, we propose that apoptotic synergy induced by sulindac sulfide plus DHA is mediated via DR5. Our findings collectively support the utility of a combination of sulindac sulfide and DHA in the effective prevention and treatment of colon cancer.

## Introduction

Accumulating epidemiological evidence suggests that long-term use of non-steroidal anti-inflammatory drugs (NSAIDs)

reduces the risk of colorectal cancer. Sulindac is considered one of the most efficacious NSAIDs available at present, based on its ability (1) to cause regression of colonic adenomas in patients with familial adenomatous polyposis as well as its promising anticancer potential in rodents (2). However, its benefits are limited to significant, but not complete (60-70%) reduction in the number and size of polyps, and clinical trials have further disclosed various side effects, such as gastrointestinal and renal toxicity after long-term use. Combination of another chemopreventive and anticancer agents with sulindac may provide an ideal strategy to enhance the efficacy of sulindac and eliminate unwanted side effects in preclinical and clinical studies by lowering effective doses. With this regard, a recent randomized controlled clinical trial showed that a low-dose combination of sulindac and difluoromethylornithine, an inhibitor of ornithine decarboxylase, reduces the risk of recurrent colorectal adenomas and high-risk adenomas by 70 and 92%, respectively, confirming the potential of combination chemoprevention strategies including sulindac (3).

Sulindac and its analogs appears to exert chemopreventive and anticancer effects via its apoptosis-inducing capability (4), although the underlying mechanisms remain to be established. Sulindac sulfide, the major active metabolite of sulindac, induces apoptosis in a wide range of cancer cells like the parent compound sulindac, but at much lower doses compared with sulindac. Sulindac sulfide triggers apoptosis through the mitochondrial pathway involving caspase-9 (5). In addition, the compound upregulates death receptor (DR5) expression at both the mRNA and protein levels and activates caspase-8 in prostate and colon cancer cell lines. Moreover, sulindac sulfide cooperates with Apo2L/TRAIL, the ligand for DR, to induce apoptosis (6). Therefore, it appears that sulindac sulfide is a compound that triggers DR5-dependent extrinsic apoptotic pathway at least in certain types of cancer cells (2).

Induction of apoptosis via activation of the DR-mediated extrinsic apoptotic pathway may represent an ideal therapeutic strategy to eliminate cancer cells since the pathway trigger apoptosis in a wide range of cancer cells while minimally affecting normal cells (7). Activation of DR can be achieved with either ligand binding or receptor clustering and aggregation (8). Many of the currently used chemotherapeutic drugs are inducers of DR expression. Therefore, anticancer agents

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that act lower the sulindac dose to induce DR-mediated apoptosis may provide effective and safe combination regimens with sulindac.

Diets rich in omega-3 polyunsaturated fatty acids (PUFAs) reduce the incidence and growth of colorectal cancer. Consistent with these epidemiological observations, laboratory studies have shown that omega-3 PUFAs suppress the formation and growth of colorectal cancer in both established cell lines and animal models. One of the suggested underlying anticancer mechanisms is the induction of apoptosis by PUFA (9). The extensively characterized docosahexaenoic acid (DHA) is more potent at inducing apoptosis than other omega-3 PUFAs, such as eicosapentaenoic acid (10). DHA reduces viability and triggers apoptosis in colon cancer cells while increasing the viability of normal cells (11,12), and is additionally effective in enhancing the chemosensitivity of cancer cells when administered in combination with chemotherapeutic drugs, such as doxorubicin and irinotecan (13-15). Recent studies have further demonstrated that DHA modifies the expression of key molecules critical in apoptosis, including  $\beta$ -catenin, survivin and XIAP (9,16). DHA activates caspase-8 and downregulates FLIP, an endogenous inhibitor of DR-mediated apoptotic signaling, in colon cancer (16), and sensitizes colon cancer cells to Fas-mediated apoptosis (17). Moreover, DHA activates procaspase-8, but not procaspase-9 (10). The compound cooperates with TRAIL, a DR ligand, to induce apoptosis in HT-29 colon cancer cells (18). It is hypothesized that DHA may act in concert with sulindac sulfide to promote extrinsic apoptotic signaling mediated by DR. To examine this possibility, we investigated the potential of a combination of DHA and sulindac sulfide in inducing growth suppression and apoptosis in colon cancer.

## Materials and methods

**Cell lines and cultures.** The human HCT-116 and LoVo colon cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and the human SNU-C4 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 100 units/ml each of penicillin and streptomycin. All cells were grown in incubators in a humid atmosphere of 95% air/5% CO<sub>2</sub>.

**Reagents and antibodies.** DHA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and propidium iodide were obtained from Sigma (St. Louis, MO, USA). Sulindac sulfide was obtained from LKT Laboratories (St. Paul, MN, USA). z-VAD-FMK (pan-caspase inhibitor), z-IETD-FMK (caspase-8 inhibitor) and z-LEHD-FMK (caspase-9 inhibitor) were obtained from Enzyme Systems Products (Livermore, CA, USA). Ac-IETD-AFC (caspase-8 substrate) was purchased from Alexis Biochemicals (Lausen, Switzerland). Antibodies to human poly(ADP-ribose) polymerase (PARP) and Bid were purchased from BD Pharmingen (San Diego, CA, USA) and antibodies to procaspase-8 and -9 were obtained from Cell Signaling Technologies (Beverly, MA, USA). Antibodies to  $\beta$ -actin were obtained from Sigma. Antibodies to death receptor-5 (DR-5) and TRAIL were obtained from Alexis

Biochemicals. All other chemicals were of reagent grade and used without further purification.

**Cell viability analysis.** Cells were seeded into 96-well plates. Starting 24 h later, cells were treated with various doses of sulindac sulfide or DMSO vehicle in the presence or absence of DHA. At 48 h post-incubation, the growth and viability of cells were determined by using MTT. The ability of cells to form formazan crystals by active mitochondrial respiration was determined using a microplate spectrophotometer (BioTek, Winooski, VT, USA) after dissolving the crystals in DMSO on the shaker (IKA, Staufen, Germany).

**Colony formation assay.** Four hundred cells were seeded in 5-cm diameter dishes and incubated for 16 h at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Cells were then treated with increasing concentrations of sulindac sulfide in the presence or absence of DHA. The medium containing treatments was replaced every three days. After 10 days, surviving cells forming visible colonies (containing >50 cells) were counted after staining with crystal violet in 60% methanol.

**Immunoblot analysis.** At time of harvest, treated cells were scraped from the plates, washed twice with ice-cold PBS, and pelleted at 2,400 x g for 5 min. The cell pellet was then incubated for 15-30 min on ice in the lysis buffer containing 150 mM NaCl, 10 mM Tris, 0.2% Triton X-100, 0.3% NP-40, 0.2 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitors (Roche Diagnostics, Basel, Switzerland), pH 7.4. After centrifugation at 14,000 rpm for 15 min at 4°C, the supernatants were collected, and the protein concentration in each was measured by the Bradford method. Aliquots of supernatants containing equal amounts of protein were boiled in SDS-reducing buffer for 5 min, electrophoresed on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk and probed with specific primary antibodies, followed by incubation with appropriate peroxidase-conjugated secondary antibodies. The blots were developed with ECL Plus reagent (Amersham, Arlington Heights, IL, USA) according to the manufacturer's protocol.

**Apoptosis assay.** Cytoplasmic histone-associated DNA fragments were quantified by a photometric enzyme immunoassay using Cell Death Detection ELISApplus (Roche Diagnostics) following the manufacturer's protocol. Briefly, after treatment, cells were pooled and lysed. Cytoplasmic fractions containing histone/DNA fragments were extracted and adhered to an immobilized anti-histone antibody plate. A peroxidase-conjugated anti-DNA antibody was used for detection of adhered histone/DNA fragments. A colorimetric substrate for peroxidase was then added to each well containing each sample. The net absorbances at the suggested wavelengths ( $A_{405\text{ nm}} - A_{490\text{ nm}}$ ) were obtained for the lysates derived from treated as well as control cells. Analysis was performed on a microplate spectrophotometer (BioTek).

Apoptosis induction was also assessed by flow cytometry of cells with Sub-G1 DNA content following staining with propidium iodide. For analysis of Sub-G1 DNA content cells (subdiploid cells), HCT-116 cells were plated in 60-mm cell culture dishes. After treatment, the cells were harvested

by scraping, washed, fixed in 50% ethanol for 30 min and resuspended in cold-PBS. Containing 0.1% Triton X-100, 2  $\mu$ l of 10 mg/ml propidium iodide, and 5  $\mu$ l of 10 mg/ml ribonuclease A for DNA staining to be analysed by flow cytometry (FACScalibur, BD Biosciences, CA, USA). Calculation was performed with CellQuest Pro software (Becton-Dickinson, CA, USA).

**Caspase activity assay.** Caspase-8 activity was determined by caspase-8 fluorometric protease assay. Cells were harvested by scraping, then washed with PBS and resuspended in lysis buffer [10 mM Tris (pH 7.4-7.5), 130 mM NaCl, Triton 0.5 ml, 10 mM  $\text{NaH}_2\text{PO}_4$ , 1 mM EDTA, digitonin 10  $\mu$ M] on ice for 15 min. Protein content was determined using the Bradford assay. The equal amount of protein were incubated with a 50  $\mu$ l caspase buffer [10 mM DTT, 50 mM HEPES (pH 7.4), 1 mM EDTA, 100 mM NaCl, 0.1% CHAPS] containing 100  $\mu$ M Ac-IETD-AFC for 2 h at 37°C. The fluorescence retaining samples were analyzed by fluorescent microplate reader (XFLuor4, GENios, Haymarket, VA, USA) with a 360 nm excitation wavelength and a 405 nm emission wavelength.

**Silencing of DR5 gene expression with small interfering RNA (siRNA).** Gene silencing was achieved by transfecting cells with siRNAs by use of the RNAiFect transfection reagent (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. DR5 siRNA duplexes that target the sequence 5'-AAGACCCTTGTGCTCGTTGTC-3', which has been reported to silence the DR5 expression in other studies were purchased from Qiagen. Control siRNA oligonucleotides that target the sequence 5'-AATTCTCCGAACGTGTCACGT-3', were also employed (19). Cells plated at a density of  $2.5 \times 10^5$  cells/3 ml of 60 cm<sup>2</sup> dishes were transfected with DR5 siRNA or control siRNA and 48 h after the transfection, cells, washed with PBS, were treated with DHA/sulindac sulfide combination. On the second day after treatment, cells were harvested and subjected to apoptosis analysis. Gene silencing effects were also separately evaluated by immunoblot analysis.

**Animal studies.** Five to six weeks-old female BALB/c nude mice were housed and cared for according to the standards of the Sejong University (Seoul, Korea) for animal care and were used under a protocol approved by the Sejong University on animal care. The animals were injected subcutaneously on the right flank with  $1 \times 10^7$  human HCT-116 colon cancer cells suspended in 0.2 ml PBS. When the tumor reached an average volume of about 25 mm<sup>3</sup>, mice were randomly divided into four groups (10-11 animals per group) and treated intraperitoneally three times a week with 500  $\mu$ l of vehicle, DHA alone, sulindac sulfide alone or DHA/sulindac sulfide combination. Tumors were measured using an external caliper and volume was calculated by the formula:  $1/2 \times \text{length} \times (\text{width})^2$ . All studies were performed in accordance with the approved animal protocol.

**Immunohistochemistry.** At the end of the experiments mice were sacrificed and the tumors were excised, fixed in 4% neutral buffered formalin and then processed for paraffin embedding and sectioning using standard histological procedures. Tumor sections were stained with hematoxylin and eosin (H&E) for

general morphologic evaluation, with anti-Ki67 antibodies (MIB-1, Dako Corp., Carpinteria, CA, USA) for proliferating cells. Briefly, the paraffin-embedded formalin-fixed tissue blocks were cut in 5  $\mu$ m thickness and then deparaffinized in xylene. Following a rinse with water, 3% hydrogen peroxide was applied to the slides for 4 min at room temperature. Another rinse with Tris-buffer was performed before the primary Ki67 ready to use antibody was overlaid for 32 min at 42°C. The slides were again rinsed in Tris-buffer twice, then incubated with Biotin (iView DAB Detection Kit, Ventana Medical Systems, Inc., Tucson, AZ, USA) for 10 min, rinsed again, and incubated with streptavidin. After a final rinse with Tris-buffer, chromogen (DAB) was applied for 8 min and copper solution for 4 min. Counterstaining was then performed with commercially prepared hematoxylin for 4 min. After the post counterstaining performed with bluing solution, the slides were dehydrated and coverslipped with Permount. Benchmark XT autostainer (Ventana Medical Systemes, Inc.) was used for all staining. Finally, the slides were observed under a light microscope at a magnification of x400, and photographed.

**Statistical analysis.** Statistically significant differences between values obtained under different experimental conditions were determined using two-tailed unpaired Student's t-tests.

## Results

**DHA effects on the sulindac sulfide-mediated growth inhibition.** To establish the effects of DHA on sulindac sulfide-induced growth inhibition of human colon cancer cells, we treated three colon cancer cell lines, HCT-116, SNU-C4 and LoVo, with increasing doses of sulindac sulfide in the presence or absence of fixed doses of DHA. Incubation of HCT-116 and LoVo cells with 100  $\mu$ M sulindac sulfide alone for 2 days inhibited cell growth by 8.2 and 28.7%, respectively (Fig. 1A). Notably, in the presence of 15  $\mu$ M DHA, 100  $\mu$ M sulindac sulfide inhibited HCT-116 and LoVo cell growth by 55.6 and 60.6%, respectively (Fig. 1A). SNU-C4 was more resistant to the effects of sulindac sulfide than the other cell lines. Specifically, incubation of this cell line with 125  $\mu$ M sulindac sulfide for 2 days inhibited growth by only 0.5%. In contrast, a combination of 125  $\mu$ M sulindac sulfide and 20 M DHA inhibited SNU-C4 cell growth by 65.0% (Fig. 1A). Incubation with DHA alone affected the growth of HCT-116, LoVo and SNU-C4 cells by 18.3, 7.8 and 16.4%, respectively.

Next, we performed the colony formation assay to evaluate the combination effects of DHA and sulindac sulfide after prolonged treatment. DHA (5  $\mu$ M) enhanced the sulindac sulfide-induced inhibition of colony formation in HCT116 cells by 3.7- and 2.7-fold, compared with 50 and 75  $\mu$ M sulindac sulfide alone (Fig. 1B). Our findings collectively demonstrate that DHA sensitizes human colon cancer cells to sulindac sulfide-induced growth inhibition.

**DHA effects on the sulindac sulfide-mediated apoptosis.** Since the growth inhibitory effects of sulindac sulfide are known to be mediated via apoptosis, we further investigated whether DHA sensitization is associated with enhanced apop-

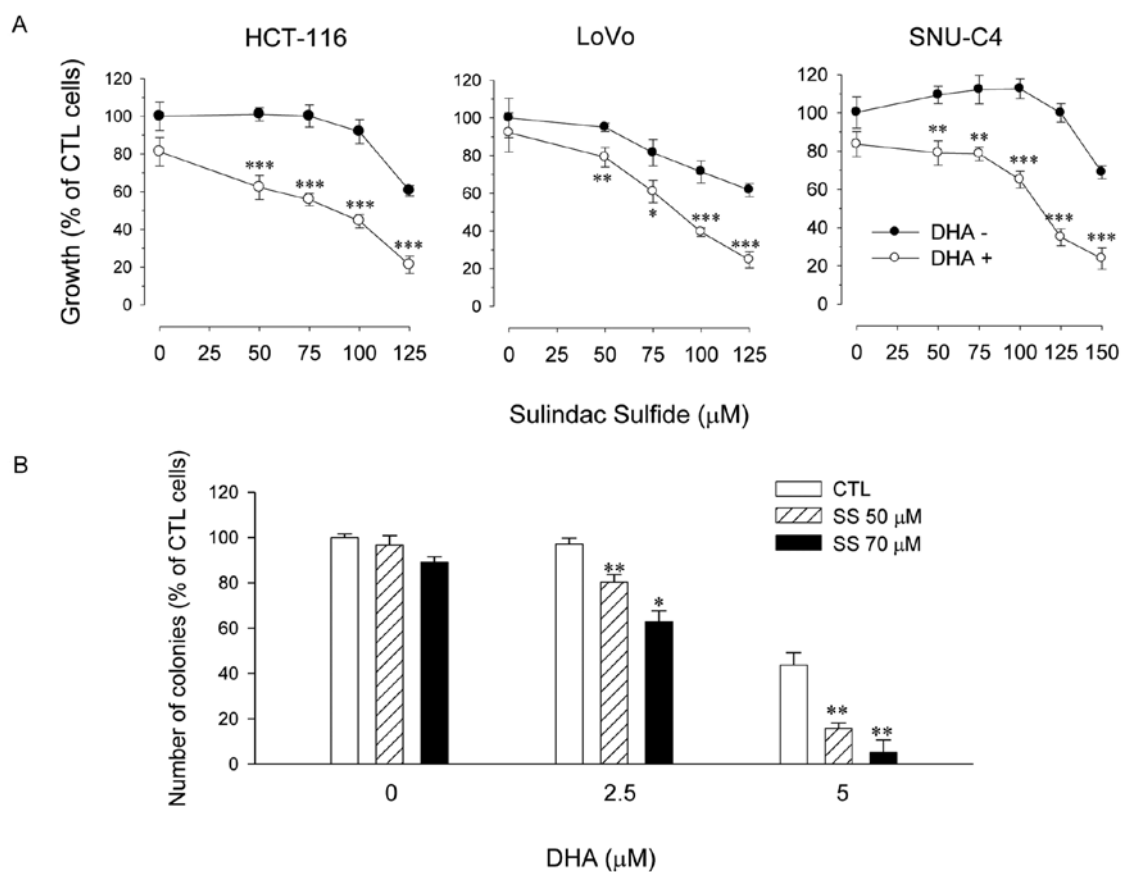


Figure 1. DHA effect on sulindac sulfide-induced growth suppression in colon cancer cells. (A) HCT-116, LoVo and SNU-C4 cells were seeded at densities of  $4\text{-}8 \times 10^3$ /well in 96-well plates and, starting 24 h later, treated with increasing doses of sulindac sulfide or DMSO vehicle in the presence (●) or absence (○) of DHA ( $15 \mu\text{M}$  for HCT-116 and LoVo Cells and  $20 \mu\text{M}$  for SNU-C4 cells). At 48 h post-incubation, the growth and viability of cells were determined by using MTT. Results are expressed as percentage growth (mean  $\pm$  SD of triplicate wells) relative to DMSO-treated cells. (B) Effect of combination treatment on HCT-116 colony formation. Colony formation assay was performed as described in the text. The number of colonies in the dish treated with vehicle alone was used as an index for a 100% survival rate (control) and this value was used to obtain the percentage survival rates for other dishes. Data are mean  $\pm$  SD of two independent experiments. Significant differences are indicated by asterisks: \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$  compared with cells treated with sulindac sulfide alone. SS, Sulindac sulfide treatment.

totis. When we looked for apoptosis by the ELISA assay after treatment of HCT116 cells with  $100 \mu\text{M}$  sulindac sulfide in combination with  $15 \mu\text{M}$  DHA for 30 h, DNA fragmentation was enhanced 2.4-fold, compared to that in cells treated with sulindac sulfide alone (Fig. 2A). DHA alone ( $15 \mu\text{M}$ ) did not increase DNA fragmentation in cells. Consistent with ELISA data, cell cycle analysis also revealed that the DHA/sulindac sulfide combination significantly enhanced the apoptotic cell content. Specifically, the proportion of cells accumulating at the sub-G1 phase was increased by 0.98-, 3.3- and 5.9-fold in groups treated with DHA, sulindac sulfide and DHA/sulindac sulfide, respectively (Fig. 2B). Moreover, we observed increased apoptotic morphological changes, including cell shrinkage and chromatin condensation, in cells treated with both DHA and sulindac sulfide (data not shown). Apoptosis was additionally evaluated by assaying for cleaved PARP, a general marker of apoptosis. The cleaved 85-kDa PARP fragment was evident only when cells were treated with DHA and sulindac sulfide together for 24 h (Fig. 2C). Clearly, DHA enhances apoptosis induced by sulindac sulfide in human colon cancer cells.

*Involvement of caspase-8 in DHA-augmented sulindac sulfide-induced apoptosis.* Apoptosis is generally associated

with caspase activation. Sulindac sulfide-induced apoptosis involves the caspase-8 or -9 activation cascade, depending on the cellular context (2,5). To determine whether DHA sensitization is mediated via enforced caspase activation, we examined the changes in the expression patterns of procaspase-8 and -9, two major initiator caspases. Expression of procaspase-8 protein was decreased only upon DHA/sulindac sulfide co-treatment for 24 h. On the other hand, procaspase-9 protein expression was not affected upon sulindac sulfide treatment, regardless of the presence or absence of DHA (Fig. 3A). Caspase-8 activity was significantly increased in cells treated with the DHA/sulindac sulfide combination, as shown in Fig. 3B, consistent with immunoblot analysis data.

Since caspase-8 activation and PARP cleavage were observed in these cell lines by DHA combination, we next examined whether pretreatment of cells with z-IETD-FMK (caspase-8 inhibitor), z-LEHD-FMK (caspase-9 inhibitor) or z-VAD-FMK (pan-caspase inhibitor) suppressed apoptotic cell death induced by DHA/sulindac sulfide. Inhibition of pan-caspase and caspase-8 blocked cotreatment-induced apoptosis to a significant extent, as evident from the marked decrease in DNA fragmentation, whereas caspase-9 inhibition by z-LEHD-FMK pretreatment suppressed DNA fragmentation

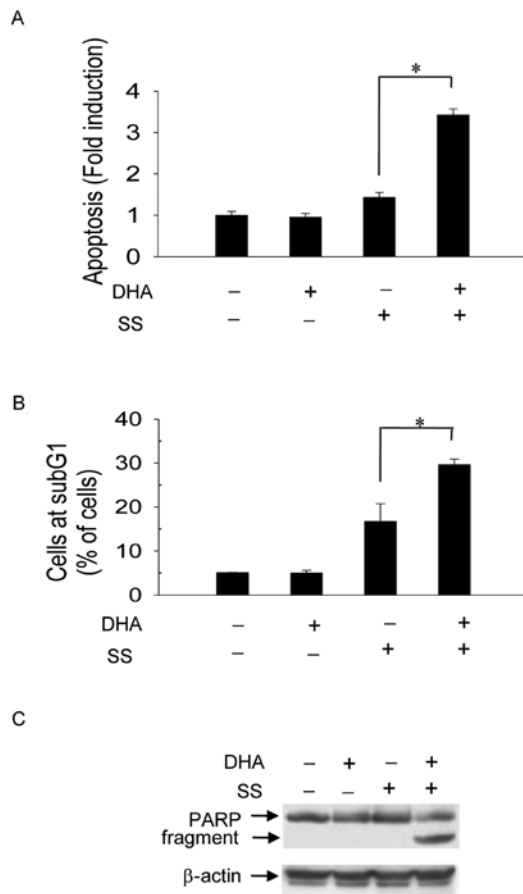


Figure 2. DHA effects on sulindac sulfide-induced apoptosis. Exponentially growing HCT-116 cells were treated with 100  $\mu$ M of SS in the absence or presence of DHA (15  $\mu$ M) for 30 h and then subjected to apoptosis analysis. (A) Apoptosis quantified by an ELISA that specifically detected histone-associated DNA. Bar represents the ratio of net absorbances at the suggested wavelengths ( $A_{405\text{ nm}} - A_{490\text{ nm}}$ ) in cells treated as indicated and in control (DMSO) cells (mean  $\pm$  SD of three experiments performed in duplicate). (B) Percentages of cells accumulated in the sub-G1 phase analyzed as described in the text. Significant differences are indicated by asterisks: \* $p < 0.05$  compared with cells treated with SS alone. (C) PARP cleavage determined by immunoblot analysis. Cell lysates obtained after 24 h of treatment were subjected to immunoblotting. Immunoblotting with an antibody to  $\beta$ -actin was used to control for equal loading of proteins per lane.

induced by the combination treatment only slightly (Fig. 3C). Similarly, in HCT116/FLICE-DN cells stably transfected with dominant-negative caspase-8 genes (20), the extent of DNA fragmentation (Fig. 3D) and PARP fragmentation (Fig. 3E) induced by DHA/sulindac sulfide combination was significantly lower than that in HCT116/CTL cell lines. These results collectively suggest that DHA/sulindac sulfide-induced apoptosis is mediated by and dependent on caspase-8 activation in HCT-116 cells.

**DR5 involvement in the DHA/sulindac sulfide-mediated apoptosis.** Activation of the DR with either ligand binding or receptor aggregation triggers the caspase 8-dependent extrinsic apoptotic pathway. Since sulindac sulfide appears to upregulate DR5 while having no effect on the levels of other DRs in several colon cancer cells (2,4), we further examined the role of DR5 in cotreatment-induced apoptosis. At the examined doses, sulindac sulfide or DHA alone did not cause

DR5 upregulation, while a combination of sulindac sulfide and DHA enhanced DR5 expression significantly at 16 h post-treatment (Fig. 4A). In contrast, expression of TRAIL, the ligand for DR5, was not altered upon co-treatment. When HCT-116 cells were transfected with DR5 siRNA prior to co-treatment, DNA fragmentation was reduced by 64%, compared with that in control siRNA-transfected cells (Fig. 4B). Based on these results, we propose that the DHA and sulindac sulfide combination promotes DR5 upregulation, in turn, activating caspase 8-mediated apoptotic pathways.

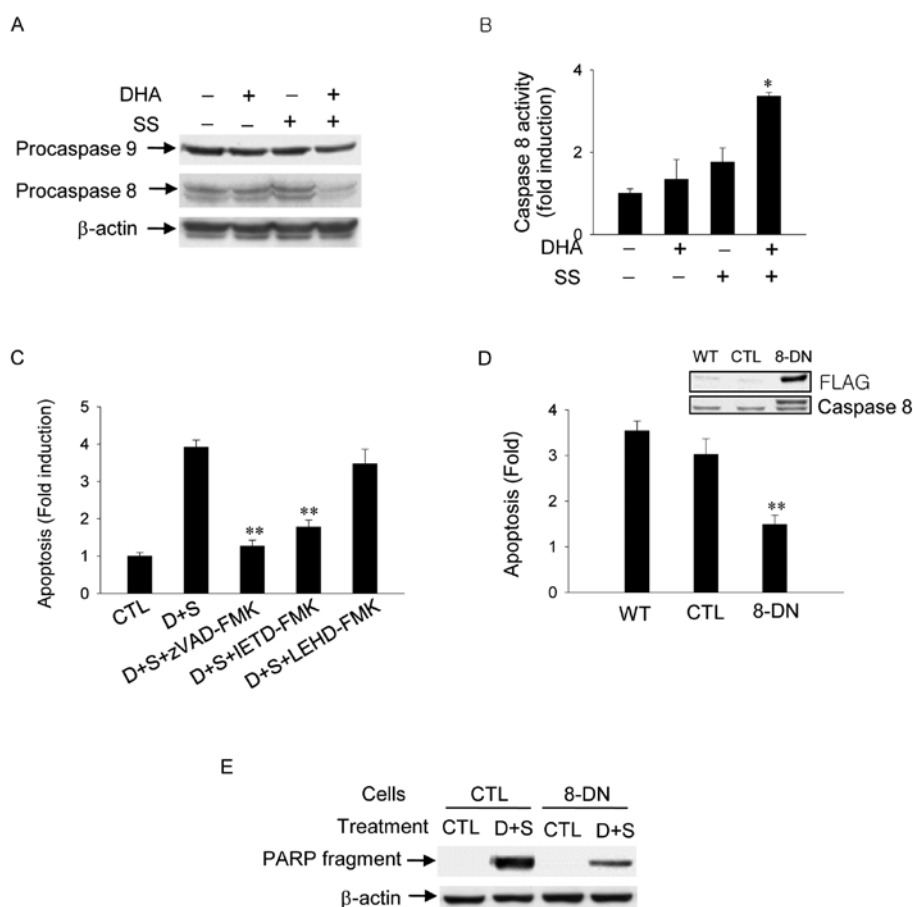
**Treatment of HCT-116 cancer cell xenografts with the DHA-sulindac sulfide combination.** Since DHA sensitized colon cancer cells to sulindac sulfide-induced growth suppression *in vitro*, we further examined whether DHA alters the effects of sulindac sulfide *in vivo* using a xenograft tumor model. For administration, DHA and/or sulindac sulfide were dispersed in distilled water using carboxymethyl cellulose and Tween-80 as the detergent mixture. Intraperitoneal administration of DHA or sulindac sulfide alone partially suppressed tumor growth, compared with that in vehicle-treated groups. Tumor size increased up to 18.6-fold from the initial day of treatment in control mice, and up to 12.0- and 10.9-fold in DHA- or sulindac sulfide-treated groups, respectively. Combined administration of DHA plus sulindac sulfide suppressed tumor growth to a greater extent. The average tumor size in DHA/sulindac sulfide-treated mice increased only by 6.9-fold after three weeks (Fig. 5A). Consequently, the average tumor sizes in DHA-, sulindac sulfide- and DHA/sulindac sulfide-treated mice after three weeks were 0.64-, 0.57- and 0.36-fold those of vehicle-treated mice (Fig. 5B).

Immunohistochemical investigations performed at the end of the experiments revealed changes in the patterns of Ki-67 protein, a marker for cancer cell proliferation. The numbers of Ki67-positive cells were greatly reduced in sections of DHA/sulindac sulfide-treated tumors, compared with those in groups treated with DHA or sulindac sulfide alone (Fig. 2C). Together with tumor size data, the results indicate that DHA augmented sulindac sulfide-mediated inhibition of cellular proliferation in HCT-116 tumor xenografts.

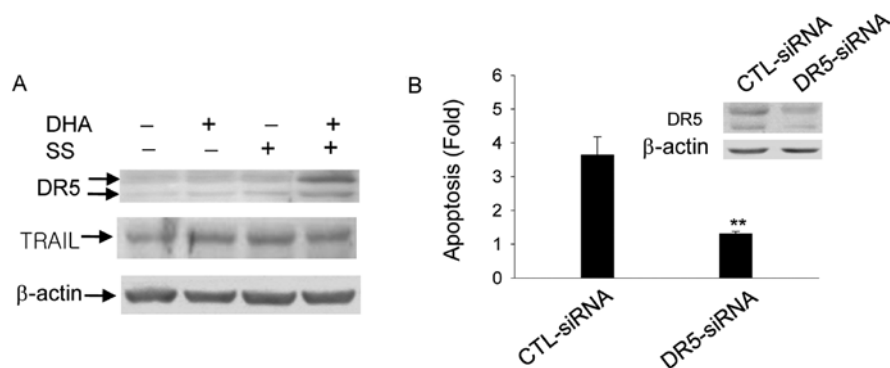
We observed slight loss of body weight after 21 days of treatment in both DHA- and DHA/sulindac sulfide-treated groups, compared with vehicle- and sulindac sulfide-treated groups, but the differences were not statistically significant (data not shown).

## Discussion

Recently, considerable attention has focused on the use of combinations of anticancer agents, each exerting toxicity at the optimal dose. Combination treatments may enhance the preventive or therapeutic efficacy of individual constituents and thus require lower doses of each agent. Here, we have shown for the first time that low concentrations of DHA significantly enhance growth suppression and apoptosis induced by sulindac sulfide in colon cancer cells, both *in vitro* and *in vivo*. Since pharmacologically acceptable compound DHA exerts this sensitization effect at a significantly lower dose, compared with the reported plasma concentrations (400  $\mu$ M) in animals fed a diet supplemented with a 5% fish oil (12), the sensiti-



**Figure 3.** Involvement of caspase-8 in DHA-augmented sulindac sulfide-induced apoptosis. HCT-116 cells were treated with 100  $\mu$ M SS with or without 15  $\mu$ M DHA combination. (A) Changes in the procaspase-8 and -9 expression determined by immunoblot analysis performed after 24-h treatment. (B) Effect of DHA/SS combination on the activity of caspase-8. With cell lysates obtained after 24-h treatment, the activity of caspase-8 was measured as described in the text. Bar represents the ratio of the fluorescence in cells treated as indicated and in control cells. Data represent the average of at least three separate experiments. Significant differences are indicated by asterisks: \* $p$ <0.05 compared with cells treated with SS alone. (C) Effect of pretreatment with z-VAD-fmk, z-IETD-FMK or z-LEHD-FMK on the apoptotic cell death induced by DHA and sulindac sulfide combination. After 2-h pretreatment with 25  $\mu$ M of the appropriate caspase inhibitor, cells were treated with DHA/SS for 30 h prior to apoptosis analysis. Significant differences are indicated by asterisks: \* $p$ <0.05 compared with cells treated with DHA/SS combination. (D) Apoptosis induction by DHA/SS treatment in HCT116 cells stably transfected with empty plasmid vector (HCT116/CTL) or with plasmid containing dominant negative caspase-8 gene (HCT116/FLICE-DN). \* $p$ <0.05 by unpaired t-test. (E) PARP fragmentation by DHA/SS combination in HCT116/CTL and HCT116/FLICE-DN cells.



**Figure 4.** DR5 induction was involved in DHA/SS combination-mediated apoptosis. (A) Effect of DHA/SS combination treatment on the DR5 and TRAIL expression in HCT-116 cells. Immunoblot analysis was performed with cell lysate obtained after 16-h treatment. (B) DNA fragmentation in cells subjected to DR5 siRNA transfection prior to treatment with DHA/SS combination. Forty eight hours later after transfection with control or DR5 small interfering RNA, HCT-116 cells were treated with DMSO or 100  $\mu$ M sulindac sulfide/15  $\mu$ M DHA for 30 h. Bar represents the fold induction of DNA fragmentation, compared with DMSO-treated cells. Data are the mean value of two identical wells. \* $p$ <0.05 compared with that of control siRNA-transfected cells treated with DHA/SS.

zation is plausible *in vivo*. Accordingly, the DHA/sulindac sulfide combination may provide a basis for the rational design

of combinatorial strategies for the effective prevention and treatment of colon cancer and possibly other malignancies.

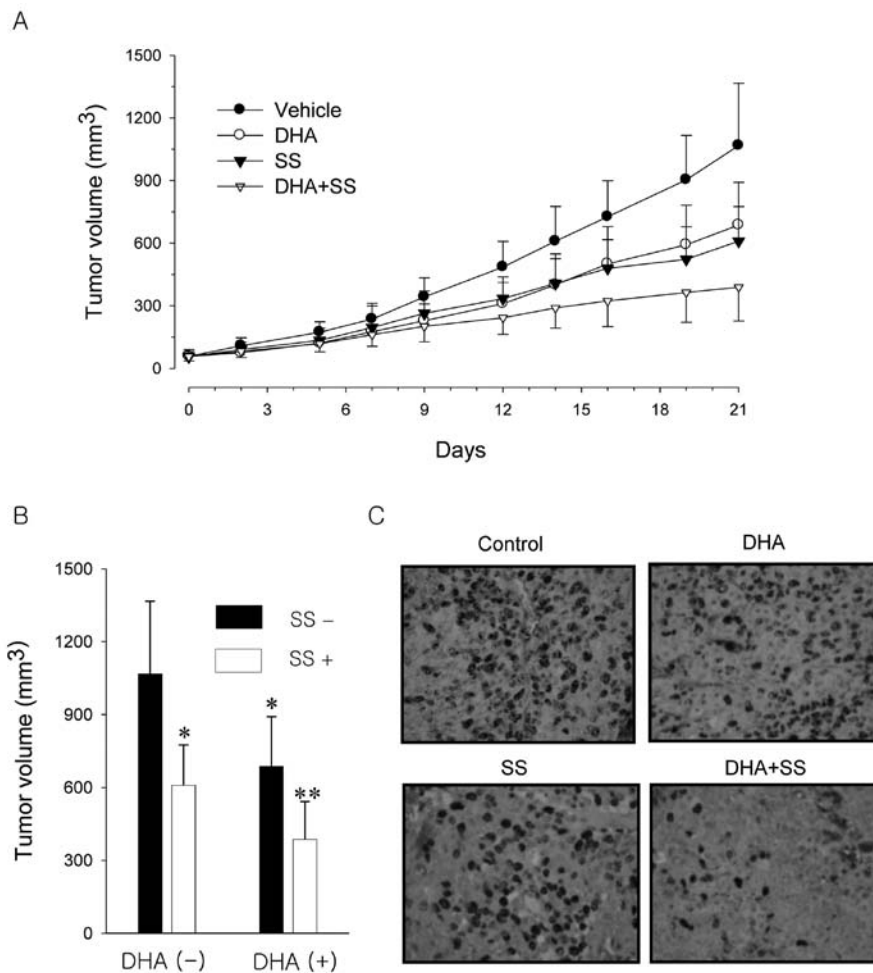


Figure 5. Sulindac sulfide in combination with DHA significantly suppressed the growth of human HCT-116 colon cancer cells implanted in nude mice, compared with those treated with sulindac sulfide or DHA alone. HCT-116 cells were xenografted on the right flank of mice as described in the text. Mice were randomly divided into four groups to receive treatment as follows: i) 500  $\mu$ l of vehicle (2% CMC and 1% Tween-80 in distilled water); ii) 500  $\mu$ l of vehicle containing 1.1 mg of DHA; iii) 500  $\mu$ l of vehicle containing 0.44 mg SS; and iv) 500  $\mu$ l of vehicle containing 1.1 mg DHA + 0.44 mg SS. (A) Time-dependent changes in the tumor volume of the vehicle-, DHA-, SS- and DHA/SS-treated mice. (B) Tumor volume was measured on day 21 in mice. \*Significant difference compared to vehicle-treated group ( $p < 0.005$ ); \*\*significant difference between SS vs. DHA/SS ( $p < 0.01$ ). (C) Representative photographs of tumor sections of the control, DHA-, SS- and DHA/SS-treated mice. At the end of the experiments, mice were sacrificed and the tumors were excised, fixed in formalin and embedded for routine wax histology. Paraffin sections were stained with hematoxylin and eosin (H&E) and Ki67 as a cell proliferation marker. Ki67-positive cells stain brown (original magnification,  $\times 400$ ).

The apoptosis-inducing capability of many anticancer agents appears to underlie their chemopreventive and/or therapeutic effects. In our experiments, co-treatment with DHA significantly enhanced apoptosis of colon cancer cells induced by sulindac sulfide, leading to cooperative growth suppression of colon cancer xenografts, suggestive of improved chemopreventive or therapeutic efficacy of the two agents in combination. Since the sulindac sulfide possesses COX inhibitory activity, we were interested in establishing whether the sensitization effect of DHA was associated with increased COX inhibition. However, DHA also enhanced apoptosis induced by exisulind (sulindac sulfone), a sulindac metabolite with no COX inhibitory activity (unpublished data). The finding that the DHA sensitization effect was common to sulindac metabolites regardless of COX inhibitory activity suggests that DHA-mediated promotion of apoptosis is not associated with COX inhibition.

Earlier studies have shown that sulindac sulfide induces DR5 expression in colon cancer cells, leading to activation of

the extrinsic DR5-mediated apoptotic pathway. In the present study, we observed DR5 upregulation following treatment with sulindac sulfide alone, consistent with previous reports. The DHA/sulindac sulfide combination caused upregulation of DR5 protein at doses at which neither agent alone causes such upregulation. In contrast, the expression of TRAIL, the DR5 ligand, appeared unchanged. However, increased caspase-8 activation were concomitantly observed upon combination treatment, suggesting the involvement of an extrinsic apoptotic pathway. In this regard, it is well known that DR is activated, not only by ligand binding but also receptor clustering and aggregation (8), and thus enforced DR5 induction may trigger ligand-independent activation (21). In contrast, DR5 silencing and caspase-8 blockade via pharmacological pretreatment with caspase-8 inhibitors or stable expression of dominant-negative caspase-8 abrogated apoptosis induced by the DHA/sulindac sulfide combination. Therefore, it appears that enhancement of DR5 expression contributes at least partially to the apoptotic synergy of DHA/sulindac sulfide.

While our data suggest that apoptotic synergy of the DHA/sulindac sulfide combination is associated with augmented DR5 induction/caspase activation, the specific mechanism underlying DR5 upregulation remains unclear with our current data. Apoptosis induced by sulindac analogs requires protein kinase G-mediated activation of JNK (c-Jun NH<sub>2</sub>-terminal kinase) (22). Additional studies have reported JNK-mediated DR5 expression by specific chemotherapeutic drugs. Therefore, it is possible that DR5 induction by DHA/sulindac sulfide combination is associated with augmented JNK activation. However, further studies by our group showed that DHA does not affect the phosphorylated JNK level. Moreover, in cells pretreated with SP600125, a JNK inhibitor, DR5 upregulation by combination treatment was not affected by pretreatment (unpublished data), while sulindac sulfide alone induced JNK activation, consistent with earlier reports.

Recent studies have shown that DR5 is regulated by CCAAT/enhancer-binding protein homologous (CHOP) protein through binding to a specific site in the 5'-flanking regions of the gene (8,23). Since CHOP is increased by DHA (15) as well as sulindac sulfide in colon cancers (24), we propose that DHA and sulindac sulfide act in concert to activate CHOP, resulting in augmentation of DR5 expression.

DHA is a pleiotropic molecule (13) that alters membrane structure and function and promotes formation and mobilization of ROS (25). DHA influences signal transduction of a number of pathways controlling apoptosis, for example, increasing the levels of the proapoptotic proteins, Bak and Bcl-xS, and reducing that of the antiapoptotic protein, Bcl-2 (26). Moreover, DHA has been shown to downregulate FLIP, an inhibitor of the death receptor-mediated apoptotic pathway (16). Therefore, although not studied herein, alterations in the expression patterns of apoptosis-related proteins by DHA may also contribute to its cell death augmentation effect.

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