Comparison of the Apoptosis-inducing Capability of Sulforaphane Analogues in Human Colon Cancer Cells

MIN JUNG KIM[1], SO HEE KIM[2] and SOO-JEONG LIM[1]

[1]*Department of Bioscience and Biotechnology, Sejong University, Seoul, Korea;*
[2]*College of Dentistry and Research Institute of Oral Science, Gangneung-Wonju National University, Gangneung, Korea*

Abstract. *The anticancer activity of sulforaphane is known to be mediated at least partly by apoptosis induction and associated with the presence of the –N=C=S moiety. The present study explored how oxidation of sulphur in the side chain of sulforaphane affected apoptosis induction to provide the chemical basis of sulforaphane effects. Sulforaphane analogues containing oxidised sulphur (alyssin, sulforaphane, erysolin and alyssin sulfone) exerted a superior growth inhibitory effect compared with sulforaphane analogues with nonoxidised sulphur (erucin and berteroin) in human colon cancer cell lines. Furthermore, erysolin was a more potent inducer of reactive oxygen species (ROS) and apoptosis compared with erucin. Erysolin-induced ROS generation and subsequent apoptosis were inhibited by pretreatment with the antioxidant N-acetyl-cysteine. Erysolin induced caspase-8 activation, while blockade of caspsase 8 activation inhibited apoptosis induced by erysolin. Taken together, sulforaphane analogues with oxidised sulphur were the most efficient apoptosis inducers, likely due to high-level ROS induction.*

Numerous epidemiological studies have reported that potential chemopreventive agents are present in human diets or potential dietary components. A number of natural compounds with inhibitory effects on tumourigenesis have been identified in fruits and vegetables (1, 2). Among vegetables with anticarcinogenic potential, members of the cruciferous family appear to be very effective in reducing the risk of colorectal cancer (3).

Correspondence to: Soo-Jeong Lim, Department of Bioscience and Biotechnology, Sejong University, 98 Kunja-dong, Kwangjin-gu, Seoul, Korea 143-747. sjlim@sejong.ac.kr or So Hee Kim, College of Dentistry, Gangneung-Wonju National University, Gangneung, Kangwon-Do, Korea, 210-702, e-mail: shkim67@gwnu.ac.kr

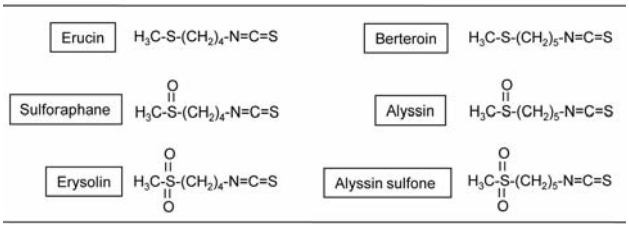
Key Words: Sulforaphane, apoptosis, colon cancer, reactive oxygen species.

The anticarcinogenic effects of cruciferous vegetables have been attributed to isothiocyanates (ITCs) that are naturally present as glucosinolates in a variety of edible plants including watercress, broccoli, and cabbage. Natural ITCs exist in many metabolic products, and include phenyl ITC, allyl ITC, and sulforaphane (4-6). The best-characterized ITC is sulforaphane, which is generally found in high concentrations in broccoli. Sulforaphane is known to exhibit the most potent anticancer activity of the various ITCs tested (7). Sulforaphane inhibited intestinal polyp formation in Apc^{min} mice (8) and blocked carcinogen-induced mammary tumour development in rats (9). The major mechanism by which sulforaphane exerts chemopreventive effects has been generally considered to reflect inhibition of carcinogen-activating enzymes and induction of detoxification enzymes that promote the removal of carcinogens (10).

In addition to chemopreventive effects, sulforaphane exerted antiproliferative activity when tested against a wide range of cancer cell lines, as well as in animal models (11). Several different mechanisms of action have been suggested to explain how sulforaphane inhibits proliferation of cancer cells. The hypothesis includes inhibition of cell cycle progress, and induction of apoptosis (5, 7, 10). Sulforaphane induced apoptosis in PC-3 human prostate cancer cells and inhibited the growth of PC-3 xenografts *in vivo* (9, 11). In HT-29 colon cancer cells, sulforaphane treatment resulted in cell cycle arrest and apoptosis, together with increased expression of the pro-apoptotic protein Bax and the proteolytic cleavage of poly(ADP-ribose)polymerase (12). Sulforaphane-induced apoptosis generally involves caspase activation, although the specific caspases involved vary depending on the cellular context (10, 12).

The several biological activities of sulforaphane appear to be mediated primarily through the reaction of the –N=C=S group with cellular nucleophilic targets (13-16). A group of naturally occurring sulforaphane analogues exist that differ in the oxidation state of sulfur, separated by several carbon atoms from the –N=C=S group (Table I), suggesting that biological

Table I. *Isothiocyante structure*.



activities of these compounds might be related. However, Zhang *et al*. demonstrated that sulforaphane and erysolin, the sulfonyl analogue of sulforaphane, more effectively induced activity of the phase II detoxification enzymes quinine reductase and glutathione *S*-transferase, in murine hepatoma cells, than did erucin, a sulforaphane thio-analogue (13). Both sulforaphane and erucin increased the expression of multidrug resistance protein (MRP)1 to a similar extent (17), although the effect of sulforaphane was much greater than that of erucin in particular cell lines. However, in the human HL-60 leukemia cells, cell cycle arrest and apoptosis induction by erucin were more potent than afforded by sulforaphane (18). Sulforaphane and erucin inhibited the growth of human A549 lung cancer cells to a similar extent (19). Sulforaphane and erucin, but not erysolin, were effective in reducing the levels of androgen receptor protein in human prostate LNCap cells (20). Collectively, these studies suggest that the biological activities of sulforaphane analogues might be affected by the oxidation state of sulphur in the side chains of such materials, and that effects might differ depending on the type of biological activity assessed. To the best of the Authors' knowledge, it is not known how the oxidation state of sulphur influences the apoptosis-inducing activity of sulforaphane analogues. This study reports that oxygen attached to sulphur potentiated the apoptosis-inducing capability of sulforaphane analogues, presumably because of increased reactive oxygen species (ROS) generation.

Materials and Methods

Cell lines and reagents. The human HCT116, LoVo, CaCo-2 and HT-29 colon cancer cell lines were obtained from the ATCC (Manassas, VA, USA). HCT116, LoVo and HT-29 were cultured in RPMI-1640 medium (Hycolon, Logan, UT, USA), Caco-2 was cultured in DMEM (Hycolon). Culture mediums were supplemented with 100 μg/ml Penicillin/ streptomycin and 10% heat-incubated foetal bovine serum (Hycolon, Logan, UT, USA). The cells were incubated in 5% CO_2 at 37˚C.

Sulforaphane, erysolin, erucin, berteroin, alyssin and alyssin sulfone were purchased from LKT Laboratories (Minneapolis, MN, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). 3-(4,5-Dimethylthiazol-z-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) solution, *N*-Acetyl-L-cystein (NAC) and buthionine sulfoximine (BSO) were purchased from Sigma (St. Louis, MO, USA). Antibodies to procaspase 8 were obtained from BD Bioscience PharMingen (San Diego, CA, USA). Antibodies against procaspase 9 were obtained from Cell Signaling Technology (Beverly, MA, USA). β-actin antibody was purchased from Sigma. Anti-mouse- and anti-rabbit secondary antibodies were obtained from Amersham (Arlington Heights, IL, USA).

Stable cell transfection. HCT116 cells were transfected with the control pcDNA3 empty vector or with the vector containing FLAG-tagged FLICED-DN by use of lipofectamine (Life Technologies, Inc, USA), according to the manufacturer's instructions. The pcDNA3 expression vectors expressing FLAG-tagged dominant-negative mutant FLICE (FLICE-DN) was a kind gift from Dr. Dixit VM (University of Michigan, MI, USA). After selection in culture medium containing G418 (Gibco-BRL, Rockville, MD, USA) until individual colonies appeared, clones were collected separately and analysed by western blot to confirm the FLICE-DN expression. Cells transfected with the empty vector were designated HCT116/CTL, and cells transfected with vector containing FLICE-DN were designated HCT116/DN.

Cell growth and viability assay. Cells were seeded in 96-well plates in 0.1 ml of culture media supplemented with 10% FBS. On the following day cells were treated with varying concentrations of sulforaphane, erysolin, erucin, berteroin, alyssin and alyssin sulfone reagents. Cell growth and viability were measured using MTT. The formation of formazan crystals by active mitochondrial respiration in cells was determined using a microplate spectrophotometer (BioTek, Winooski, VT, USA) after dissolving the crystals in DMSO.

Immunoblotting. Cells were collected from the plates by scraping, washed twice with phosphate-buffered saline (PBS), and suspended in lysis buffer (150 mM NaCl, 10 mM Tris, 0.2% Triton X-100, 0.3% NP-40, 0.2 mM Na3VO4, and protease inhibitor, pH 7.4] on ice for 20~30 min. Protein content was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Aliquots of cell lysates containing equal amounts of protein were denatured in SDS-reducing buffer by boiling for 5 min, resolved on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The resulting blots were blocked with 5% nonfat dry milk (Amresco Inc. Cochran Road Solon, OH, USA) and incubated with specific primary antibodies. The blots were subsequently incubated with appropriate peroxidase-conjugated secondary antibodies and developed with ECL Plus reagent (Amersham, Arlington Heights, IL, USA) according to the manufacturer's protocol.

Apoptosis assay. Cells were plated and allowed to attach overnight. The cells were treated with SFN analogue and control received the same amount of dimethyl sulfoxide (DMSO). When pretreatment with caspase inhibitors was required, cells were incubated with 25 μM of the appropriate caspase inhibitor for 2-h and then treated with SFN analogue. Following treatment, histone-associated DNA fragment were quantified using a photometric enzyme immunoassay using Cell Death ELISA Plus (Roche Applied Bioscience, Palo Alto, CA, USA), following the manufacturer's protocol. Analysis was performed on a EL800 microplate reader (measured at 405 nm).

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

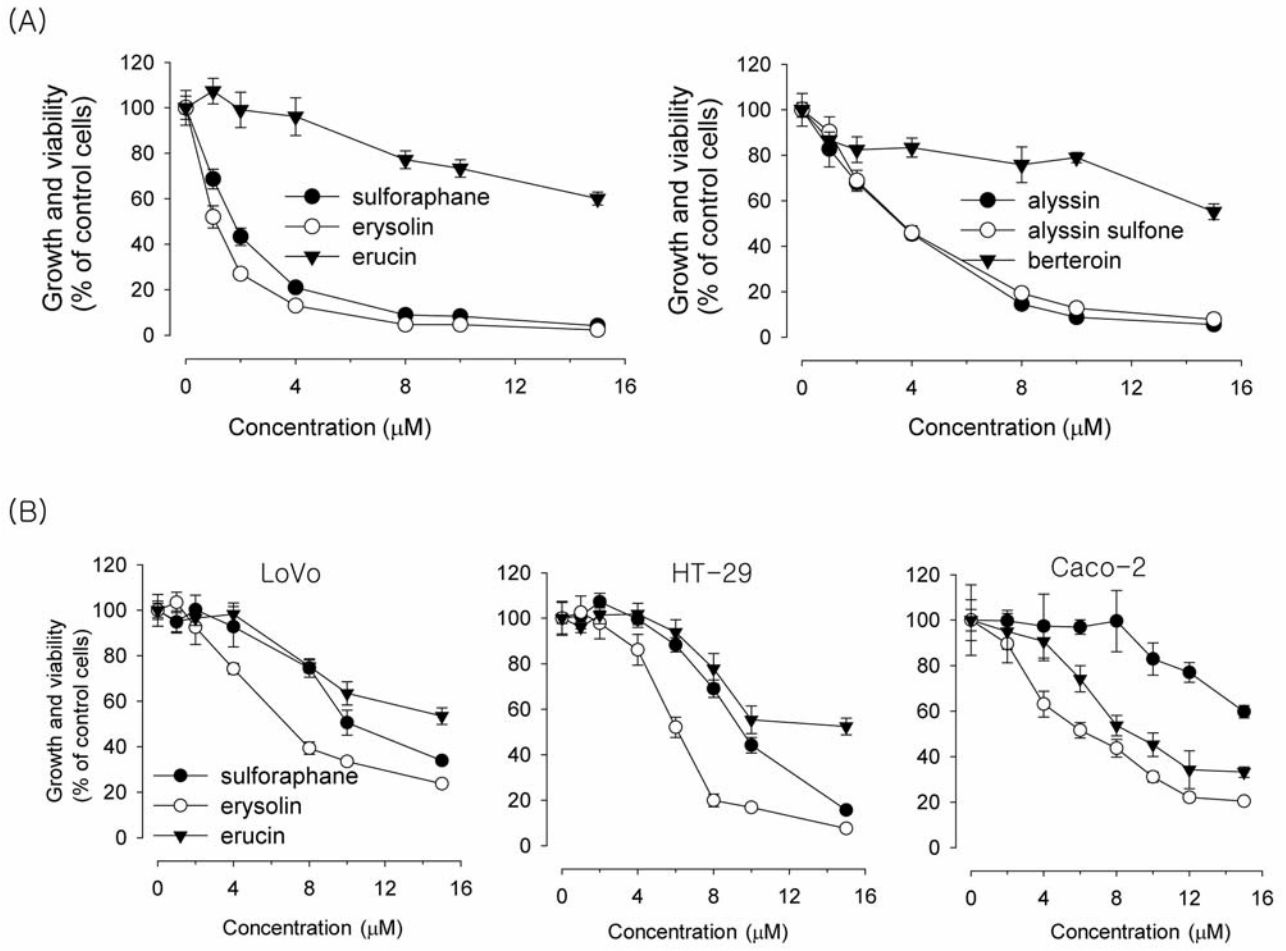


Figure 1. *Effect of Sulforaphane (SFN) analogues on the growth and viability of colon cancer cells. A: HCT116 and B: LoVo, HT29 and Caco-2 cells. At 72 h post-incubation with different SFN analogues, the growth and viability of cells was determined by MTT assay. Results are expressed as the percentage growth (mean±S.D. of triplicate wells) relative to control (DMSO-treated) cells.*

mM Tris (pH 7.4-7.5), 130 mM NaCl, Triton 0.5 ml, 10 mM NaH_2PO_4, 1 mM EDTA, digitonin 10 μM] on ice for 15min. Protein content was determined using the Bradford assay. The equal amount of protein were incubated with a 50 μl caspase buffer (10 mM DTT, 50 mM HEPES (pH 7.4), 1 mM EDTA, 100 mM NaCl, 0.1% CHAPS] containing 100 μM Ac-IETD-AFC for 2 h at 37˚C. The fluorescence retaining samples were analysed by fluorescent microplate reader (XFLUOR4, GENios) with a 360 nm excitation wavelength and a 405 nm emission wavelength.

ROS-generation assay. Intracellular ROS generation was measured by flow cytometry following staining with DCFH-DA. Non fluorescent DCFH-DA, hydrolysed to DCFH inside cells, yields highly fluorescent DCF in the presence of intracellular H_2O_2 and related peroxides (21). Cells were plated in 6-well plates, allowed to attach overnight, and treated. After treatment, the cells were harvested by scraping, washed twice with PBS, resuspended in serum-free medium for 1 h, and incubated with 5 μM DCFH-DA for 15-20 min at 37˚C. The cells were washed with ice-cold PBS and resuspended in cold-PBS, and cell fluorescence was measured by flow cytometry (FACScalibur; BD Biosciences, San Jose, CA, USA). DCF fluorescence was analysed using the FL1 channel. Calculation was performed with CellQuestPro software (Becton Dickinson, San Jose, CA, USA).

Cell cycle analysis. Apoptosis induction by SFN analogues was assessed by flow cytometry of cells with sub-G_1 DNA content following staining with propidium iodide (Sigma). For analysis of sub-G_1 DNA content cells (subdiploid cells), HCT116 cells were plated in 60-mm cell culture dishes. After treatment, 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 μl of 10 mg/ml propidium iodide, and 5 μl of 10 mg/ml ribonuclease A for DNA staining to be analysed by flow cytometry (FACScalibur). Calculation was performed with CellQuest Pro software (Becton Dickinson).

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

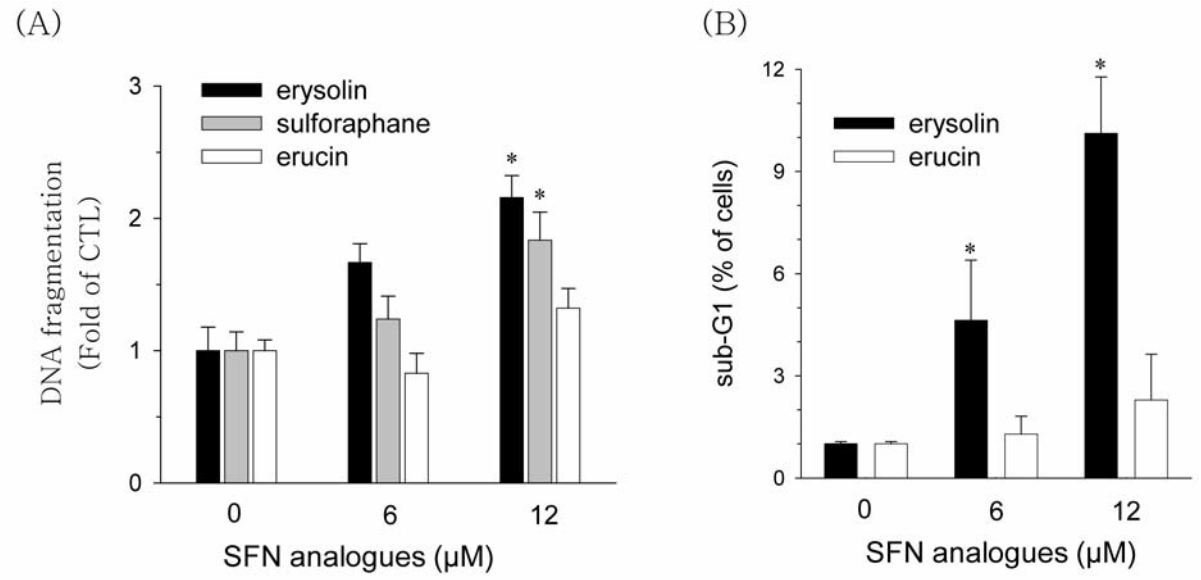


Figure 2. *Differential degree of apoptosis induction by SFN analogues in HCT116 cells. At 30 h post-incubation with DMSO or the indicated doses of SFN analogues, A: Apoptosis quantified by an ELISA. The bar represents the ratio of the absorbance at 405 nm in cells incubated with SFN analogue and in vehicle-treated control (CTL) cells (mean±SD of two experiments performed in duplicate). B: Percentages of cells accumulated in the sub-G_1 phase analysed as described in the text. Significant differences are indicated by asterisks: *p<0.05, compared with control cells.*

Results

Growth inhibitory effects of sulforaphane analogues. First the effects of sulforaphane analogues (Table I) on the growth of human HCT116 colon cancer cell lines were compared. After 72 h incubation with each tested analogue at concentrations ranging from 1 to 15 μM, the MTT assay was performed. As shown in Figure 1A, erucin and berteroin were much less effective as growth inhibitors compared with other sulforaphane analogs: the IC_{50} value of erucin and berteroin were no lower than 15 μM, whereas those of other sulforaphane analogues were lower than 4 μM. These data suggest that sulforaphane analogues containing oxidised sulphur (erysolin, sulforaphane, alyssin and alyssin sulfone) are likely to be more potent antiproliferative agents than analogues containing non-oxidised sulphur (erucin and berteroin). Sulforaphane analogues containing four atoms of carbon between oxidised sulphur and the –N=C=S groups (sulforaphane and erysolin) were slightly more potent compared with those containing five atoms of carbon (alyssin and alyssin sulfone) (Figure 1A). These data suggest that the number of carbon separating the sulphur atom from the –N=C=S groups is a less important factor determining antiproliferative potency of sulforaphane analogs.

Other colon cancer cell lines were then tested with each sulforaphane analogue, and a similar potency was found in these cell lines: The IC_{50} value of erucin was no lower than 15 μM in any cell line. In contrast, both erysolin and sulforaphane exerted higher growth inhibitory effects than did erucin, in all cell lines (Figure 1B, data not shown for alyssin, alyssin sulfone and berteroin). In HCT116, HT-29, LoVo, and Caco-2 cells, the IC_{50} values of erysolin were approximately 1.1 μM, 6.1 μM, 6.6 μM, and 8.7 μM, respectively, and the corresponding IC_{50} concentrations of sulforaphane were approximately 1.8 μM, 9.5 μM, 10.1 μM, and 6.3 μM. When erysolin and sulforaphane were compared, the growth inhibitory effect of erysolin was higher than that of sulforaphane (HT-29 and LoVo cells), similar (HCT116 cells), or lower (CaCo-2 cells), suggesting that IC_{50} values depended on the cell type under test. Collectively, these data showed that attachment of oxygen to sulphur played an important role in mediating the growth inhibitory effect of sulforaphane analogues in colon cancer cells. In other words, sulforaphane analogues containing oxidised sulphur appeared likely to be more potent antiproliferative agents than were analogues containing non-oxidised sulfur.

Induction of apoptosis by sulforaphane analogues. Earlier studies showed that sulforaphane inhibited cell proliferation by inducing apoptosis in a wide range of cancer cell types including colon cancer cells (1, 22). To investigate whether SFN analogue-induced growth inhibition was associated with a capability to induce apoptosis, an ELISA test was used to measure DNA fragmentation, and flow cytometry was used to assess accumulation of cells at specific phases of the cell

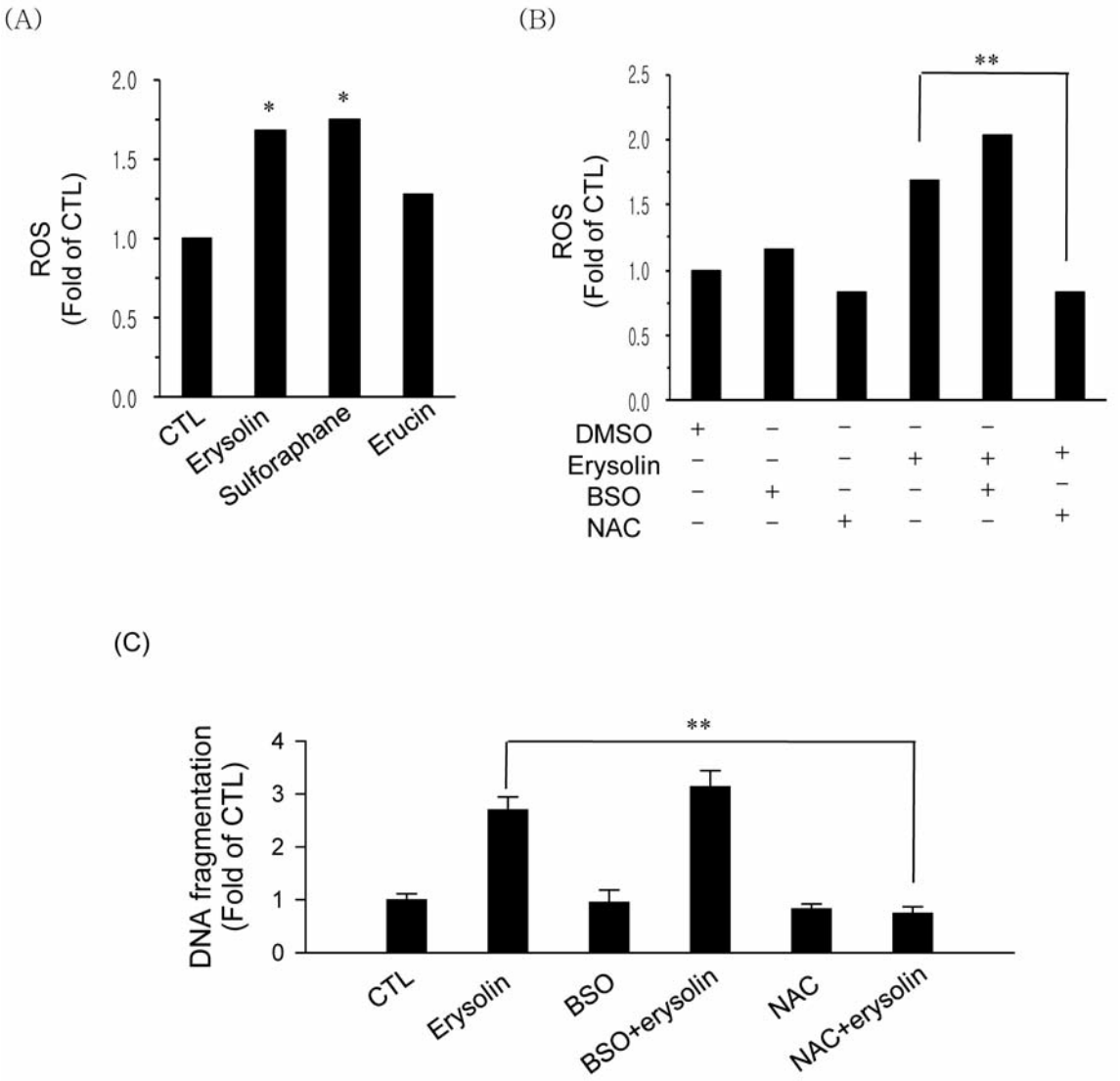


Figure 3. *Effect of SFN analogues on ROS generation (A, B) and apoptosis induction (C). A: ROS generation by SFN analogues. HCT116 cells were treated with 12 μM of erysolin, SFN or erucin overnight prior to ROS generation. ROS data are expressed as the increase in channel fluorescence of treated cells relative to vehicle-treated cells. Effect of pretreatment with antioxidant NAC or GSH-depletion agent BSO on the ROS generation (B) and apoptosis induction (C) by erysolin. HCT116 cells were pretreated with BSO or NAC for 2 h before treating cells with 12 μM erysolin overnight and subjected to ROS analysis or apoptosis analysis. Results are from duplicate assays in each of at least two independent experiments (mean±SD) **p<0.01 by unpaired t-test.*

cycle, employing HCT116 cells, as such cells appeared to be particularly sensitive to treatment with sulforaphane analogues.

The ELISA test, performed at 30 h post-incubation with 12 μM of each sulforaphane analog, showed that DNA fragmentation was significantly higher in erysolin- or sulforaphane-treated cells, but not in erucin-treated cells, compared with cells exposed to DMSO. These data suggested that growth inhibition observed at this time point was preceded by apoptosis at an earlier stage. Moreover, the extent of DNA fragmentation induced by sulforaphane analogues was in the same order as observed when growth inhibitory effects were studied, namely erysolin>sulforaphane>erucin (Figure 2A). When a cell cycle analysis was performed after 30 h of treatment with either 6 μM or 12 μM of erysolin or erucin, it was found that erysolin dose-dependently increased accumulation of cells in the sub-G_1 phase, whereas treatment with erucin afforded only a minimal increase in such accumulation. At doses of 12 μM, the percentages of cells accumulated in sub-G_1 were 10% or 2% in cells treated with

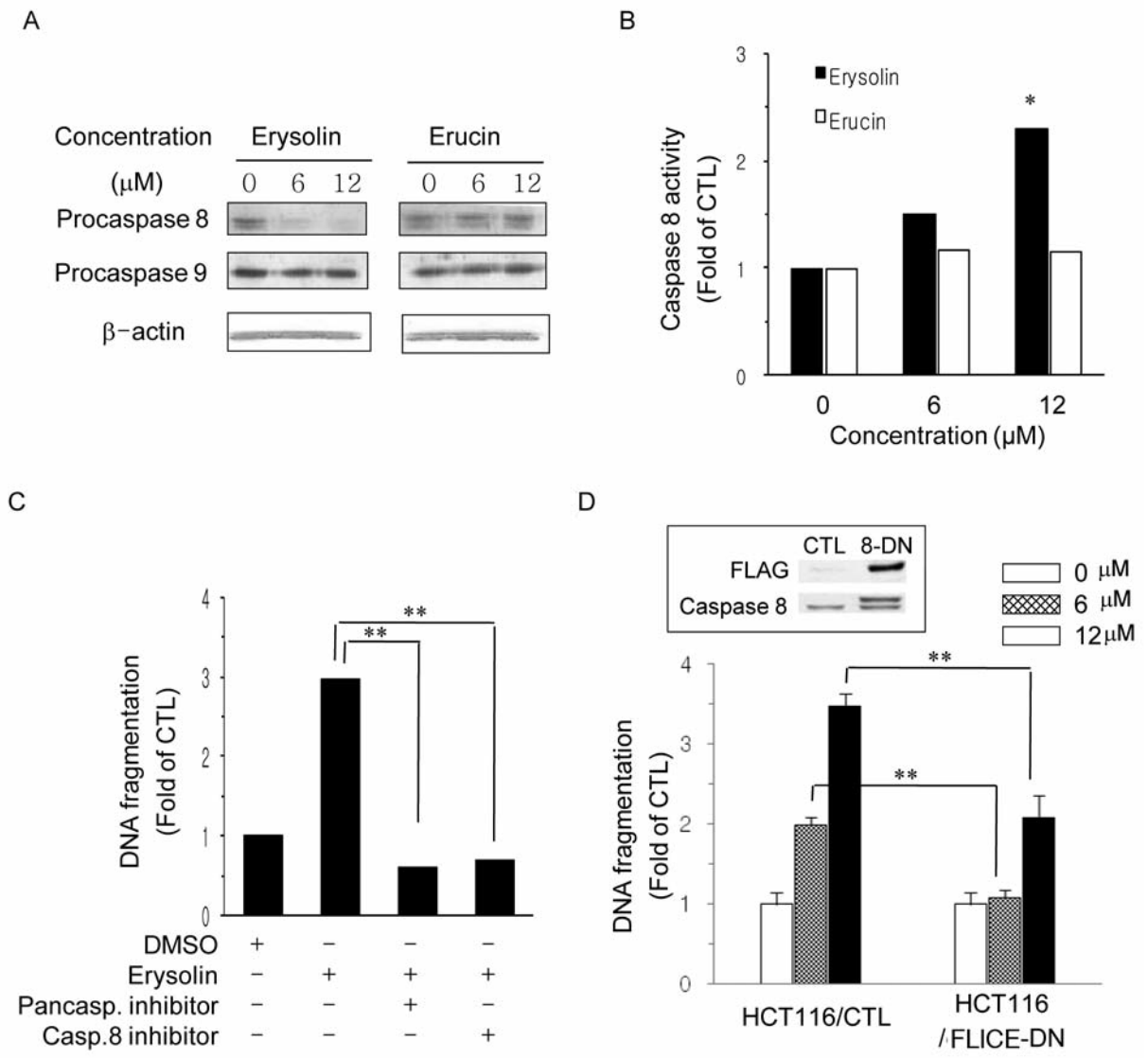


Figure 4. *Erysolin-induced apoptosis was caspase 8 dependent. A: Effect of erysolin or erucin on the expression of procaspase 8 and 9. At 24 h post-incubation of HCT116 cells with each SFN analogue, the procaspase expression was determined by immunoblot analysis. B: Effect of erysolin or erucin on the activity of caspase 8. Caspase 8 activity assay was performed after 24-h treatment. C: Effect of pretreatment with a general caspase inhibitor z-VAD-fmk or caspase 8 inhibitor z-IETD-FMK on the apoptotic cell death induced by erysolin. After 2-h pretreatment with 25 μM of the appropriate caspase inhibitor, cells were treated with 12 μM erysolin for for 24 h prior to apoptosis analysis. D: Effect of erysolin on the apoptosis induction in HCT116 cells stably transfected with empty plasmid vector (HCT116/CTL) or with plasmid containing dominant negative caspase 8 gene (HCT116/FLICE-DN). Apoptosis assay was performed after incubation with erysolin for 24-h (mean±SD). **p<0.01 by unpaired t-test. Insets, (D) Immunoblots for FLAG and procaspase-8 in HCT116/CTL and HCT116/FLICE-DN cells to determine the expression level of dominant negative caspase 8 protein.*

erysolin or erucin, respectively (Figure 2B). These results suggested that the superior growth inhibitory effect of erysolin was attributable to induction of more extensive apoptosis, compared to that seen after erucin exposure.

Induction of ROS generation and subsequent apoptosis affected by sulforaphane analogues. Oxidative stress may be linked to apoptosis and cell cycle repression in various cell lines (23), and earlier studies have demonstrated that sulforaphane-induced apoptosis is triggered by an increase in ROS levels in several cancer cell lines (24, 25). To investigate whether differences in apoptosis-inducing capabilities shown by sulforaphane analogues were caused by variation in ROS-generating ability, ROS levels were analysed after treatment with such analogues. Intracellular ROS generation in control (DMSO-treated) and test cells was assessed by flow cytometry,

after staining with DCFH-DA. Sulforaphane and erysolin (both at 12 μM) significantly increased DCF fluorescence compared to that of vehicle-treated control HCT116 cells, whereas erucin, also at 12 μM, had no such effect, thus suggesting that sulforaphane and erysolin were more efficient ROS inducers than was erucin (Figure 3A). Furthermore, pretreatment with NAC, a general ROS scavenger, significantly inhibited erysolin-induced ROS generation. In contrast, pretreatment with BSO, a general promoter of ROS production, enhanced erysolin-induced ROS generation (Figure 3B). These results indicate that sulforaphane and erysolin are more efficient ROS inducers than erucin.

It was sought to determine whether erysolin-induced ROS generation was involved in mediation of apoptosis in HCT116 cells. As shown in Figure 3C, such apoptosis were blocked by pretreatment with NAC and increased by prior exposure to BSO. This suggested that erysolin-induced ROS generation played a major role in mediating apoptotic cell death.

Activation of caspases by SFN analogues. Caspase cleavage is a general feature of apoptosis, and earlier studies showed that sulforaphane-induced apoptosis involved caspase activation (11, 26). In this study, when immunoblotting analysis was performed to determine changes in the expression of procaspase 8 and 9, it was found that apoptosis-inducing concentrations of erysolin or sulforaphane decreased expression of procaspase 8, but not that of procaspase 9 (data not shown for sulforaphane). In contrast, decrease in expression of neither procaspase 8 nor 9 was observed in erucin-treated cells. In addition, caspase 8 activity was increased in a dose-dependent manner by erysolin, but not by erucin (Figure 4B). These results indicated that caspase 8 activation was involved in erysolin-induced apoptosis of HCT116 cells.

It was next examined whether pretreatment of cells with z-IETD-FMK (a caspase 8 inhibitor) or z-VAD-FMK (a pan-caspase inhibitor) could inhibit apoptotic cell death induced by erysolin. Pretreatment with these inhibitors completely blocked erysolin-induced apoptosis (Figure 4C). Likewise, in HCT116/FLICE-DN cells established by stable transfection of dominant-negative caspase-8 genes, the extent of apoptosis induced by erysolin was significantly less than that seen in HCT116/CTL cell lines (Figure 4D). Taken together, these results suggested that erysolin induced caspase 8-dependent apoptosis in HCT116 cells.

Discussion

This study explored whether the oxidation state of sulphur separated by several carbon atoms from the –N=C=S group affected the growth inhibitory effect of sulforaphane in human colon cancer cells. The data demonstrated that the antiproliferative activity of sulforaphane analogues was in the order erysolin>sulforaphane>erucin in all colon cancer cell lines tested, except CaCo-2 cells, where the potency was ranked sulforaphane>erysolin>erucin. The fact that erucin and berteroin, isothiocynate analogues containing nonoxidised sulphur, did not inhibit the growth of cells by 50% even at the highest concentration tested (15 μM), whereas four other analogues with mono- (sulforaphane and alyssin) or di-oxidised (erysolin and alyssin sulfone) sulphur did show inhibitory activity, suggested that the oxidation of sulphur in sulforaphane may positively affect the ability to inhibit the growth of cancer cells. As the anticancer effects of ITCs such as sulforaphane appear to be at least partly associated with an ability to block proliferation of cancer cells, these data imply that chemical modifications in sulforaphane analogues, such as attachment of oxygen to sulfur, may lead to the development of more potent, sulforaphane-based anticancer agents.

One proposed mechanism for how sulforaphane blocks the proliferation of cancer cells involves induction of apoptosis. The data from this study showed that sulforaphane induced apoptosis at doses inhibiting proliferation of colon cancer cells, and that the extent of growth inhibition caused by sulforaphane analogues was correlated with the capability to induce apoptosis. Therefore, a difference in the potency of inhibition of colon cancer cell proliferation was associated with a differential capability to induce apoptosis. These data, combined with those of earlier studies showing that the apoptosis-inducing potency of ITCs with the -N=C=S group was greater when an aliphatic side group was present (sulforaphane) than when the side group was aromatic (phenethyl ITC and benzyl ITC) (27), suggest that a rational design strategy based on analysis of structure-activity relationships, and the synthesis of structural sulforaphane analogues with modified side-chains, may contribute to the development of ITCs with elevated apoptosis-inducing characteristics.

These data demonstrated that increased apoptosis induction by erysolin (and sulforaphane), compared with erucin, was associated with an increase in caspase 8 activation, but not with a rise in caspase 9 activity, in HCT116 cells. Moreover, pretreatment with a caspase 8 inhibitor or stable transfection with a dominant-negative caspase 8 gene decreased erysolin-induced apoptosis, suggesting a major role for caspase 8 in apoptosis. These data are consistent with earlier studies demonstrating the involvement of the caspase 8 dependent pathway in sulforaphane-induced apoptosis in the human prostate (10, 12) and pancreatic cancer cells (24). However, as the involvement of specific caspases in sulforaphane-induced apoptosis has been shown to be cell type-specific, and to involve the caspase 9-/mitochondria-dependent pathway in human breast cancer MCF-7 and T47D cells (26), it would be of interest to investigate how the apoptotic potency of the

six sulforaphane analogues explored here differ in cell lines in which caspase 9, but not caspase 8, plays a primary role in mediating sulforaphane-induced apoptosis.

Oxidative stress is involved in a variety of different cellular processes including apoptosis (23), and sulforaphane-induced apoptotic cell death is known to be initiated by ROS generation (25, 26). These data showed that exposure of HCT116 cells to growth-inhibitory concentrations (12 μM) of erysolin or sulforaphane was preceded by ROS generation, as indicated by increased DCF fluorescence. Furthermore, apoptosis induction was significantly inhibited by antioxidant pretreatment. These results suggest that erysolin induced apoptosis by virtue of excessive ROS generation, as did sulforaphane, indicating that apoptotic pathways induced by the two drugs were similar. In contrast, ROS generation after treatment of cells with 12 μM erucin was minimal, implying that differences in the apoptosis-inducing potency of the sulforaphane analogues was attributable, at least in part, to variations in ROS-inducing ability. These results support a recent study that demonstrated that subapoptotic concentrations of erysolin strongly enhanced arsenic trioxide-mediated apoptosis in cancer cells by increasing cellular ROS levels (28).

Currently, it remains unclear how sulforaphane affects the cellular redox state. Earlier studies suggested that the –N=C=S moiety of ITCs could undergo spontaneous hydrolysis, leading to formation of hydrogen peroxide or superoxide (29). Other proposed mechanisms of sulforaphane-induced ROS generation include depletion of glutathione (GSH) by intracellular conjugation of sulforaphane, an electrophile, with intracellular nucleophiles, especially GSH, leading to increased levels of ROS (30, 31). The current data do not make it clear whether oxidation of sulphur in the side chain of structural sulforaphane analogues affects intracellular ROS generation directly or indirectly. However, it is reasonable to hypothesise that oxidation of sulphur in the side chain of sulforaphane increases the electrophilicity of the –N=C=S group, thereby elevating reactivity with particular cellular nucleophilic targets associated with ROS generation (27). This hypothesis remains to be further investigated.

In conclusion, this study demonstrated that the ROS- and apoptosis-inducing capabilities of SFN analogues were affected by the oxidation state of sulphur in the side chain. Recent studies have suggested that sulforaphane-induced oxidative stress paradoxically involves cellular resistance and/or recovery from stress, and that much of the sulforaphane-mediated chemopreventive activity is associated with this cellular response (27). Therefore, it would be of interest to investigate how other biological responses of cells are affected by structural differences in SFN analogues. An understanding of the chemical basis of these effects would contribute to the development of more effective chemopreventive and treatment strategies.

Acknowledgements

This work was supported by Grant No. R-2009-007-5055 (to S.J. Lim) and by Grant No. R-2009-006-6765 (to S.H. Kim) from the National Research Foundation of Korea.

References

1 Pappa G, Bartsch H and Gerhauser C: Biphasic modulation of cell proliferation by sulforaphane at physiologically relevant exposure times in a human colon cancer cell line. Mol Nutr Food Res *51*: 977-984, 2007.

2 Keum YS, Yu S, Chang PP, Yuan X, Kim JH, Xu C, Han J, Agarwal A and Kong AN: Mechanism of action of sulforaphane: Inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma hepg2 cells. Cancer Res *66*: 8804-8813, 2006.

3 Bonnesen C, Eggleston IM and Hayes JD: Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. Cancer Res *61*: 6120-6130, 2001.

4 Keum YS, Jeong WS and Kong AN: Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. Mutat Res *555*: 191-202, 2004.

5 Fimognari C and Hrelia P: Sulforaphane as a promising molecule for fighting cancer. Mutat Res *635*: 90-104, 2007.

6 Gerhauser C, You M, Liu J, Moriarty RM, Hawthorne M, Mehta RG, Moon RC and Pezzuto JM: Cancer chemopreventive potential of sulforamate, a novel analogue of sulforaphane that induces phase 2 drug-metabolizing enzymes. Cancer Res *57*: 272-278, 1997.

7 Clarke JD, Dashwood RH and Ho E: Multi-targeted prevention of cancer by sulforaphane. Cancer Lett *269*: 291-304, 2008.

8 Hu R, Khor TO, Shen G, Jeong WS, Hebbar V, Chen C, Xu C, Reddy B, Chada K and Kong AN: Cancer chemoprevention of intestinal polyposis in apcmin+ mice by sulforaphane, a natural product derived from cruciferous vegetable. Carcinogenesis *27*: 2038-2046, 2006.

9 Zhang Y, Kensler TW, Cho CG, Posner GH and Talalay P: Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. Proc Natl Acad Sci USA *91*: 3147-3150, 1994.

10 Juge N, Mithen RF and Traka M: Molecular basis for chemoprevention by sulforaphane: A comprehensive review. Cell Mol Life Sci *64*: 1105-1127, 2007.

11 Singh AV, Xiao D, Lew KL, Dhir R and Singh SV: Sulforaphane induces caspase-mediated apoptosis in cultured pc-3 human prostate cancer cells and retards growth of PC-3 xenografts *in vivo*. Carcinogenesis *25*: 83-90, 2004.

12 Gamet-Payrastre L, Li P, Lumeau S, Cassar G, Dupont MA, Chevolleau S, Gasc N, Tulliez J and Terce F: Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res *60*: 1426-1433, 2000.

13 Zhang Y, Talalay P, Cho CG and Posner GH: A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. Proc Natl Acad Sci USA *89*: 2399-2403, 1992.

14 Conaway CC, Jiao D and Chung FL: Inhibition of rat liver cytochrome p450 isozymes by isothiocyanates and their conjugates: A structure activity relationship study. Carcinogenesis *17*: 2423-2427, 1996.
15 Burg D, Riepsaame J, Pont C, Mulder G and van de Water B: Peptide-bond modified glutathione conjugate analogs modulate gstpi function in GSH-conjugation, drug sensitivity and JNK signaling. Biochem Pharmacol 71: 268-277, 2006.
16 Zhang Y and Talalay P: Anticarcinogenic activities of organic isothiocyanates: Chemistry and mechanisms. Cancer Res *54*: 1976s-1981s, 1994.
17 Harris KE and Jeffery EH: Sulforaphane and erucin increase MRP1 and MRP2 in human carcinoma cell lines. J Nutr Biochem *19*: 246-254, 2008.
18 Jakubikova J, Bao Y and Sedlak J: Isothiocyanates induce cell cycle arrest, apoptosis and mitochondrial potential depolarization in HL-60 and multidrug-resistant cell lines. Anticancer Res *25*: 3375-3386, 2005.
19 Melchini A, Costa C, Traka M, Miceli N, Mithen R, De Pasquale R and Trovato A: Erucin, a new promising cancer chemopreventive agent from rocket salads, shows anti-proliferative activity on human lung carcinoma A549 cells. Food Chem Toxicol *47*: 1430-1436, 2009.
20 Kim SH and Singh SV: D,l-sulforaphane causes transcriptional repression of androgen receptor in human prostate cancer cells. Mol Cancer Ther *8*: 1946-1954, 2009.
21 Kang YH, Lee E, Choi MK, Ku JL, Kim SH, Park YG and Lim SJ: Role of reactive oxygen species in the induction of apoptosis by alpha-tocopheryl succinate. Int J Cancer *112*: 385-392, 2004.
22 Pappa G, Lichtenberg M, Iori R, Barillari J, Bartsch H and Gerhauser C: Comparison of growth inhibition profiles and mechanisms of apoptosis induction in human colon cancer cell lines by isothiocyanates and indoles from brassicaceae. Mutat Res *599*: 76-87, 2006.
23 Mates JM, Segura JA, Alonso FJ and Marquez J: Intracellular redox status and oxidative stress: Implications for cell proliferation, apoptosis, and carcinogenesis. Arch Toxicol *82*: 273-299, 2008.
24 Pham NA, Jacobberger JW, Schimmer AD, Cao P, Gronda M and Hedley DW: The dietary isothiocyanate sulforaphane targets pathways of apoptosis, cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. Mol Cancer Ther *3*: 1239-1248, 2004.
25 Kim H, Kim EH, Eom YW, Kim WH, Kwon TK, Lee SJ and Choi KS: Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand (trail)-resistant hepatoma cells to TRAIL-induced apoptosis through reactive oxygen species-mediated up-regulation of DR5. Cancer Res *66*: 1740-1750, 2006.
26 Pledgie-Tracy A, Sobolewski MD and Davidson NE: Sulforaphane induces cell type-specific apoptosis in human breast cancer cell lines. Mol Cancer Ther *6*: 1013-1021, 2007.
27 Zhang Y, Li J and Tang L: Cancer-preventive isothiocyanates: Dichotomous modulators of oxidative stress. Free Radic Biol Med *38*: 70-77, 2005.
28 Doudican NA, Bowling B and Orlow SJ: Enhancement of arsenic trioxide cytotoxicity by dietary isothiocyanates in human leukemic cells *via* a reactive oxygen species-dependent mechanism. Leuk Res 2009.
29 Murata M, Yamashita N, Inoue S and Kawanishi S: Mechanism of oxidative DNA damage induced by carcinogenic allyl isothiocyanate. Free Radic Biol Med *28*: 797-805, 2000.
30 Singh SV, Herman-Antosiewicz A, Singh AV, Lew KL, Srivastava SK, Kamath R, Brown KD, Zhang L and Baskaran R: Sulforaphane-induced G_2/M phase cell cycle arrest involves checkpoint kinase 2-mediated phosphorylation of cell division cycle 25c. J Biol Chem *279*: 25813-25822, 2004.
31 Steinkellner H, Rabot S, Freywald C, Nobis E, Scharf G, Chabicovsky M, Knasmuller S and Kassie F: Effects of cruciferous vegetables and their constituents on drug metabolizing enzymes involved in the bioactivation of DNA-reactive dietary carcinogens. Mutat Res *480-481*: 285-297, 2001.

Received May 6, 2010
Revised June 11, 2010
Accepted June 16, 2010