BIOCHEMICAL PHARMACOLOGY 75 (2008) 1751–1760

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Implication of NAG-1 in synergistic induction of apoptosis by combined treatment of sodium salicylate and PI3K/MEK1/2 inhibitors in A549 human lung adenocarcinoma cells

Cho Hee Kim [a,1], *Min Young Kim* [a,1], *Ji Young Moon* [a], *Ji Won Hwang* [a], *Su Yeon Lee* [a], *Young Mi Joo* [a], *Song Iy Han* [b], *Hye Gyeong Park* [c,*], *Ho Sung Kang* [a,**]

[a] *Department of Molecular Biology, College of Natural Sciences, and Research Institute of Genetic Engineering, Pusan National University, Pusan 609-735, Republic of Korea*

[b] *Research Center for Resistant Cells, College of Medicine, Chosun University, Gwangju 501-759, Republic of Korea*

[c] *Korea Nanobiotechnology Center, Pusan National University, Pusan 609-735, Republic of Korea*

ARTICLE INFO

Article history:
Received 11 December 2007
Accepted 4 February 2008

Keywords:
Sodium salicylate
Apoptosis
PI3K
Akt/PKB
ERK1/2
NAG-1
Genistein

ABSTRACT

Aspirin is used as chemopreventive agents in a variety of human cancer cells including those of colon, lung, breast, and leukemia. Sodium salicylate (NaSal, the natural deacetylated form of aspirin) induced cell cycle arrest and apoptosis in a dose-dependent manner in A549 cells; high dose (20 mM) of NaSal-induced apoptosis, whereas low dose (2–10 mM) induced cell cycle arrest. We found that NaSal-activated Akt/PKB, ERK1/2, and p38MAPK signal cascades. Twenty micromolar of NaSal-induced apoptotic response of A549 cells was enhanced by the PI3K inhibitors (LY294002 and wortmannin) and in a less extent by the MEK1/2 inhibitors (U0126 and PD98059), whereas it was suppressed by the p38MAPK inhibitor (SB203580). Furthermore, simultaneous inhibition of the Akt/PKB and ERK1/2 signal cascades could lower the dose of NaSal to induce apoptosis to 2 mM in A549 lung cancer cells. Similar enhancement was observed in cells treated with 2 mM NaSal and 100 μM genistein, an inhibitor of receptor tyrosine kinases (RTKs) that are upstream of PI3K and MEK1/2 signaling. We further demonstrated that NAG-1 plays a key role in apoptosis by NaSal-based combined treatment. Collectively, our findings indicate that inhibition of the pro-survival Akt/PKB and ERK1/2 signaling may increase the chemopreventive effects of NaSal and combined treatment of two natural compounds (NaSal and genistein) results in a highly synergistic induction of apoptosis, thereby increasing the chemopreventive effects of NaSal against cancer.

* *Corresponding author.*
** *Corresponding author at*: Department of Molecular Biology, College of Natural Sciences, Pusan National University, Pusan 609-735, Republic of Korea. Tel.: +82 51 510 2275; fax: +82 51 513 9258.
E-mail address: hspkang@pusan.ac.kr (H.S. Kang).

[1] These authors contributed equally to this work.

Abbreviations: NaSal, sodium salicylate; RTK, receptor tyrosine kinases; NSAID, non-steroidal anti-inflammatory drugs; NAG-1, non-steroidal anti-inflammatory drug-activated gene-1; NSCLC, non-small cell lung cancer; GSK-3β, glycogen synthase kinase 3β; PBS, phosphate-buffered saline; PI, propidium iodide; FACS, fluorescence-activated cell sorter; DTT, dithiothreitol; IGF-1R, insulin-like growth factor-1 receptor; EGFR, epidermal growth factor receptor; TPA, 12-O-tetradecanoylphorbol-13-acetate; siRNA, small interference RNA; RT-PCR, reverse transcription-polymerase chain reaction.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.02.002

1. Introduction

Aspirin is one of non-steroidal anti-inflammatory drugs (NSAIDs) that exert their potent analgesic, antipyretic, and anti-inflammatory activities through their well-documented ability to inhibit prostaglandin synthesis [1,2]. Apart from the classical anti-inflammatory function, it exhibits a chemopreventive effect on a number of human cancers including those of colon, lung, and breast, and leukemia, highlighting to be a promising anti-cancer agent [3–5]. Especially, use of 300 mg or more of aspirin a day for about 5 years reduces the short-term risk of recurrent colorectal adenomas in patients with a history of adenomas or cancer. The chemopreventive activity of aspirin and sodium salicylate (NaSal), the natural deacetylated form of aspirin, is thought to be linked to their ability to inhibit cell proliferation [6,7], and to induce apoptosis [8,9]. The caspase family including caspase-3 and -8 has been shown to participate both in the initiation and execution of aspirin/NaSal-induced apoptosis in many cancer cells [8,9]. And, the pro-apoptotic activity of aspirin/NaSal is closely correlated to an increased expression of the non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1), also known as macrophage inhibitory cytokine-1 (MIC-1) and growth and differentiation factor-15 (GDF-15), a member of the TGF-β superfamily that mediates apoptosis by the agents to prevent tumor formation and development such as several NSAIDs, resveratrol, and genistein [10–14]. In addition, reactive oxygen species (ROS, a key mediator of deltapsi (m) collapse that leads to the release of cytochrome c) [15,16], an increased turnover rate of Mcl-1 (an anti-apoptotic member of the Bcl-2 family of proteins) [17], and proteasome inhibition [18] are reported to be responsible for aspirin/NaSal-triggered apoptosis. Furthermore, aspirin/NaSal-induced activation of NF-kB signaling may play a part in the apoptotic response. Although aspirin and NaSal are generally considered to inhibit this pathway [19], more recent studies have shown that they can activate NF-kB signaling and stimulate apoptosis in colorectal cancer cell lines [20]. p38MAPK has been also reported to play a critical role(s) in aspirin/NaSal-induced apoptosis [21,22].

The PI3K-PTEN-Akt/PKB and Ras-Raf-MEK1/2-ERK1/2 signaling cascades play critical roles in the transmission of signals from growth factors and oncogenes to downstream targets that control crucial elements in tumor development and prevent apoptosis [23–26]. The Akt/PKB pathway is constitutively activated in many cancer cells including non-small cell lung cancer (NSCLC) cell lines. Akt/PKB could also be activated in response to some pro-apoptotic stimuli such as UV-irradiation and DNA damage, and confers cancer cells with resistance to chemotherapy [27–29]. Furthermore, constitutive ERK1/2 activity in cancer cells is known to promote cellular survival and chemotherapeutic resistance [26]. Thus, the inhibition of the PI3K-Akt/PKB and MEK1/2-ERK1/2 pathways by pharmacological or genetic approaches is usually an effective means to induce apoptosis, implicating a potential target of chemotherapeutic and chemopreventive intervention.

In this study, we examined the pro-survival PI3K-Akt/PKB and MEK1/2-ERK1/2 signaling pathways during NaSal-induced apoptosis in A549 cells that are one of human NSCLC cell lines [30]. We found that NaSal-activated Akt/PKB via PI3K and ERK1/2. The PI3K inhibitors (LY294002 and wortmannin) and in a less extent the MEK1/2 inhibitors (U0126 and PD98059) could enhance 20 mM NaSal-induced apoptotic cell death. Furthermore, combined inhibition of the PI3K-Akt/PKB and MEK1/2-ERK1/2 signal pathways could lower the dose of NaSal to induce apoptosis to 2 mM in A549 cells. Similar results were observed when A549 cells were treated with 2 mM NaSal and 100 μM genistein, an inhibitor of receptor tyrosine kinases (RTKs) that are upstream of PI3K-Akt/PKB and Ras-MEK1/2-ERK1/2 signaling. We further found that NAG-1 plays a key role in apoptosis by NaSal-based combined treatment. These findings provide the possibility that inhibition of the pro-survival Akt/PKB and ERK1/2 signaling increases the chemopreventive effects of NaSal and combined treatment of two natural compounds (NaSal and genistein) results in a highly synergistic induction of apoptosis, thereby increasing the chemopreventive effects of NaSal against cancer.

2. Materials and methods

2.1. Cell culture and drug treatment

Human lung adenocarcinoma cells A549 were obtained from American Type Culture Collection and grown in RPMI 1640 media (Gibco) supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin–streptomycin in a 37 °C humidified incubator with 5% CO_2. For the studies concerning the effects of inhibitors, A549 cells were pretreated with inhibitors including LY294002 (Sigma, 20 μM), wortmannin (Sigma, 0.2 μM), U0126 (Sigma, 20 μM), PD98059 (Calbiochem, 30 μM), SB203580 (Calbiochem, 10 μM), z-VAD-fmk (Calbiochem, 20 μM), z-DEVD-fmk (Calbiochem, 20 μM), N-acetyl-L-cysteine (Sigma, 10 mM), genistein (Calbiochem, 100 μM), AG1478 (Calbiochem, 10 μM), and AG1024 (Calbiochem, 10 μM) for 1 h, and then treated with NaSal (Sigma, S-3007) in the presence of the inhibitors.

2.2. SDS-PAGE and Western blot analysis

SDS-PAGE and Western blot analysis were performed as described previously [31]. Briefly, cells were prepared by washing with cold-phosphate-buffered saline (PBS) and lysed. The protein concentration was determined using the Bio-Rad protein assay kit. Equal amount of proteins was loaded, and separated by SDS-PAGE and then transferred to nitrocellulose membrane. After blocking with skim milk, the membrane was incubated for overnight at 4 °C with primary antibodies to PARP, procaspase-3, ERK1/2, phospho-ERK1/2 (Cell Signaling Technology) p38MAPK, phospho-p38MAPK, Akt/PKB. phospho-Akt/PKB (Cell Signaling Technology), phospho-GSK-3β (Calbiochem) and NAG-1 (Santa Cruz). After washing in 1× PBS with 0.1% Tween 20, primary antibody was detected using 1:1000 diluted HRP-conjugated secondary antibodies and visualized with the enhanced chemiluminescence detection system (Amersham-Pharmacia Biotech, Buckinghamshire, England).

2.3. Cell viability assay

For cell viability assay, A549 cells (1×10^4) were seeded in a 96-well tissue culture plate and incubated for 24 h. After

treatment of NaSal, 5 μl of MTT solution (5 mg/ml) was added to each well. After incubation for 3 h at 37 °C, formazan crystals in viable cells were solubilized with 150 μl of DMSO. The absorbance of each well was then read at 570 nm using microplate reader (Molecular devices, Palo Alto, CA).

2.4. Flow cytometric analysis of apoptosis

Exponentially growing cells were treated with NaSal, and then collected and fixed with chilled 70% EtOH. Ten thousand cells stained with propidium iodide (PI) were analyzed on a fluorescence-activated cell sorter (FACStarPLUS, Becton-Dickinson, San Jose, CA, USA), and the resulting DNA histogram were converted to proportions of each cell cycle phase by the ModiFit LT software (Becton-Dickinson).

2.5. Morphological detection of apoptosis by HO-33342 assay

A549 cells were grown on coverslips in multiwell culture plates and treated with NaSal. The cells were harvested and fixed by adding 3.7% formaldehyde in PBS for 30 min on ice and then attached to slide glass by using CytoSpin. After washing with cold-PBS three times, the cells were stained with Hoechst 33342 (Molecular Probe, 10 μg/ml in D.W.) for 10–30 min and then observed by fluorescence microscopy. Percentage of apoptosis was calculated by counting the condensed and fragmented nuclei in cells.

2.6. In vitro caspase-3 activity assay

A549 cells were treated with NaSal, and the enzymatic activity of caspase-3 was determined using ApoAlert cpp32/caspase-3 assay kit (Clontech, Palo Alto, CA) as recommended by the manufacturer. Briefly, 2×10^6 cells were suspended in 50 μl of lysis buffer, the supernatants were collected, and then reaction buffer containing dithiothreitol (DTT) and a chromogenic caspase-3 substrate DEVD-*p*-nitroanilide (DEVD-pNA) was added. Reactions were incubated at 37 °C for 1 h and samples were measured at 405 nm using the VERSAmax tunable microplate reader (Molecular devices, Palo Alto, CA).

2.7. Oligonucleotide microarray analysis

The total RNA from A549 lung cancer cells was used to prepare fluorescence labeled cDNA probes for microarray analysis. Briefly, 50 μg of total RNA was converted to double-stranded cDNAs using an oligo $(dT)_{18}$-primed polymerization using SuperScript II reverse transcriptase (Invitrogen, NY). The reverse transcription mixture included 400 U SuperScript RNase H-reverse transcriptase (Invitrogen), 0.5 mM dATP, dTTP and dGTP, 0.2 mM dCTP and 0.1 mM Cy3 or Cy5 labeled dCTP (NEN Life Science Product Inc.). Fluorescence labeled cDNA probes were fragmented and applied to the GeneChip Human Apoptosis 0.4 K (Genocheck, Korea), which contain probe sets for more than 3000 human genes. Two chips were used for each group. The signal intensities from hybridized cDNA were quantified, and the GeneChip analysis software was used to identify differentially expressed genes.

2.8. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNAs were extracted from cell using TRIzol reagent (Invitrogen). Two micrograms total RNA were converted into cDNA with M-MLV reverse transcriptase and oligo $(dT)_{15}$ (Bioneer Co., Korea) according to the manufacture's instruction. Equal amounts of cDNA were subsequently amplified by PCR in a reaction mixture containing 10 mM Tris–HCl (pH 8.3), 200 μM dNTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq polymerase (Promega Co., USA). The primers were: NAG-1, sense-TCT CAG ATG CTC CTG GTG TT, antisense-AAT CTG GGT CTT CGG AGT G; GAPDH, sense-TTC ACC ACC ATG GAG AAG GCT, antisense-A GCC TTG GCA GCA CCA GT. The thermal cycles were: NAG-1, GAPDH, 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 25 s for 27 cycles, and a final extension for 5 min at 72 °C. The final PCR-products were electrophoresed on a 1.2% agarose geland visualized by ethidium bromide staining.

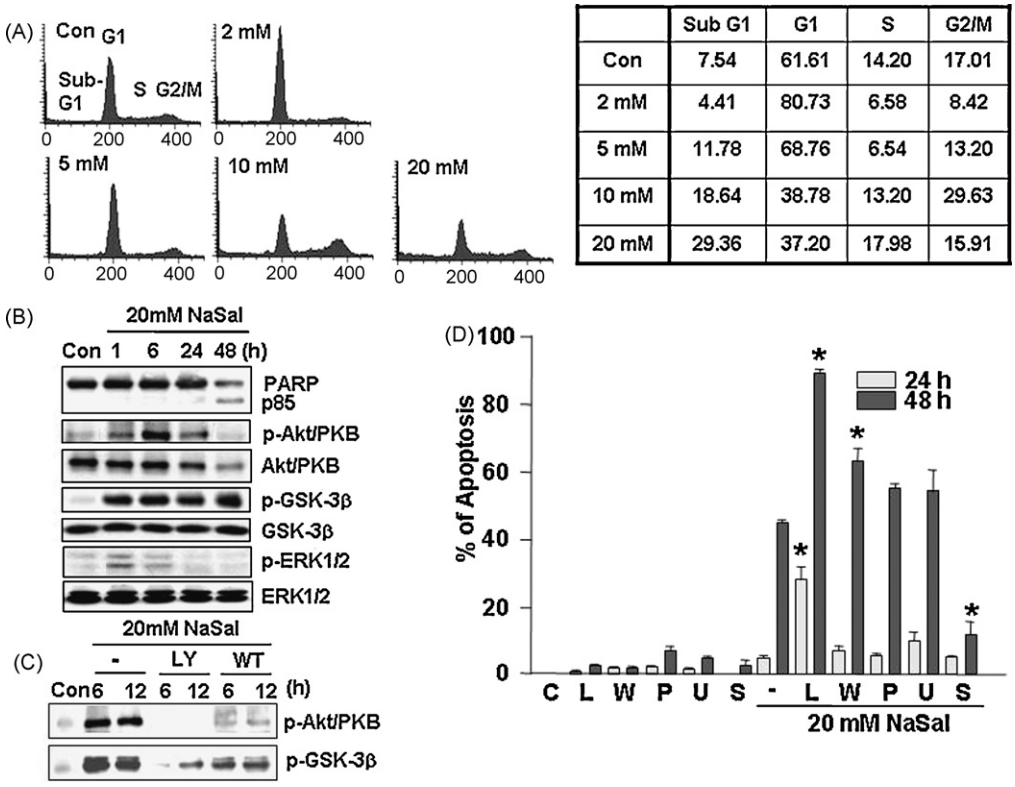
2.9. NAG-1 RNA interference

The NAG-1 small interference RNA (siRNA) vector (pSuper-neo-gfp-Si NAG-1) was constructed using a pSuper-neo-gfp and a synthetic oligonucleotide targeting 5′-ACATGCACGCG CAGATCAA-3′ corresponding to positions 780–798 on NAG-1 mRNA. A549 cells at 50% confluence were transfected with pSuper-neo-gfp NAG-1 siRNA(1 μg/well) for 3 h using Gene-Juice (Novagen) according to manufacturer's protocol. After incubation for 48 h at 37 °C (5% CO2), the transfected cells were selected by using 1 mg/ml G418, and were maintained in the presence of 0.2 mg/ml G418 (Invitrogen). G418-resistant A549 cells were pooled and used for RT-PCR analysis and for apoptosis analysis.

3. Results

3.1. Inhibition of PI3K-Akt/PKB and ERK1/2 pathways promotes NaSal-induced apoptosis in A549 lung cancer cells

NaSal has been shown to induce cell cycle arrest and apoptosis depending on its concentrations in a variety of cancer cells [6–9]. In A549 cells, low concentration of NaSal (2–10 mM) induces cell cycle arrest, whereas it induces apoptosis at higher concentration of 20 mM (Fig. 1A). NaSal at concentrations higher than 20 mM activated caspase-3 and induced degradation of its substrates, PARP, β-catenin and Rb [22], data not shown]. Similar results were obtained in colon cancer cells such as HCT116 and HT29 cells (data not shown). In most cases, the chemopreventive and therapeutic agents that induce apoptosis are known to activate an antagonistic anti-apoptotic program such as the PI3K-Akt/PKB and ERK1/2 pathways that interferes with their intended action [23–29]. Therefore, we examined the impacts of NaSal on the PI3K-Akt/PKB and Raf-ERK1/2 signal pathways that are known to be the pro-survival signal pathways in most NSCLC cell lines including A549 cells. NaSal-activated Akt/PKB through phosphorylation of Ser-473, ERK1/2 through phosphorylation of Thr 202/Tyr 204 (Fig. 1B), and p38MAPK through phosphorylation of



	Sub G1	G1	S	G2/M
Con	7.54	61.61	14.20	17.01
2 mM	4.41	80.73	6.58	8.42
5 mM	11.78	68.76	6.54	13.20
10 mM	18.64	38.78	13.20	29.63
20 mM	29.36	37.20	17.98	15.91

Fig. 1 – (A) NaSal induces cell cycle arrest and apoptosis depending on its concentration in A549 human lung cancer cell line. A549 cells were treated with 2–20 mM NaSal for 48 h and stained with PI and analyzed by FACS as described in Section 2. Con, cells treated with vehicle 48 h. (B) NaSal activates Akt/PKB-GSK-3β and ERK1/2 signaling pathway. A549 cells were treated with 20 mM NaSal for the indicated times and the cellular proteins were analyzed by SDS-PAGE and Western blotting with antibodies to PARP, phospho-Akt/PKB (Ser-473), Akt/PKB, phospho-GSK-3β (Ser-9), GSK-3β, phospho-ERK (Thr-202/Tyr-204), and ERK1/2. Con, cells treated with vehicle for 48 h. (C) A549 cells were pretreated with LY294002 (LY, 20 μM) or wortmannin (WT, 0.2 μM) for 1 h and treated with 20 mM NaSal for the indicated times. The cellular proteins were analyzed by SDS-PAGE and Western blotting with antibodies to phospho-Akt/PKB and phospho-GSK-3β. Con, cells treated with vehicle for 12 h. (D) A549 cells were pretreated with LY294002 (L, 20 μM), wortmannin (W, 0.2 μM), U0126 (U, 20 μM), PD98059 (P, 30 μM), or SB203580 (S, 10 μM) for 1 h and then treated with 20 mM NaSal for 24 and 48 h. Con, cells treated with vehicle for 24 and 48 h. For morphological apoptosis analysis, the cells were stained with Hoechst 33342 and the apoptotic cells with condensed/fragmented nuclei were scored under a fluorescence microscope. Results (500–800 cells in each group) are expressed as the mean ± S.E.M. from three independent experiments. *$p < 0.01$, compared with corresponding value for cells treated with 20 mM NaSal.

Thr 180/Tyr 182 (data not shown). Phosphorylation of GSK-3β at Ser-9, a well-documented Akt/PKB phosphorylation site that is inactivating modification of GSK-3β, was also increased in response to NaSal treatment. The PI3K inhibitors, LY294002 and wortmannin, completely suppressed phosphorylation of Akt/PKB and GSK-3β in response to NaSal, indicating PI3K-dependent activation of Akt/PKB and inactivation of GSK-3β (Fig. 1C).

We hypothesized that inhibition of PI3K-Akt/PKB and Raf-ERK1/2 signal pathways makes cancer cells susceptible to the effects of NaSal-induced apoptosis. We treated A549 cells with NaSal in combination with either the PI3K inhibitors or MEK1/2 inhibitors. The PI3K inhibitors, LY294002 and wortmannin, could inhibit PI3K-releated kinases as well as PI3K, LY294002 (at concentration of 200 μM) is used to inhibit DNA-activated protein kinase (DNA-PK) and wortmannin (at high concentration of > 3 μM) is used to inhibit ataxia telangiectasia mutated (ATM) and DNA-PK [32]. Therefore, to minimize the other effects of the inhibitors, we used these PI3K inhibitors at low concentration of 20 μM LY294002 and 0.2 μM wortmannin. Treatment of A549 cells with either the PI3K inhibitors (LY294002 and wortmannin) or MEK1/2 inhibitors (U0126 and PD98059) enhanced 20 mM NaSal-induced apoptosis, although the inhibitors alone did not induce apoptosis for up to 48 h (Fig. 1D). Similar stimulatory effects of the PI3K inhibitors and MEK1/2 inhibitors were obtained for PARP degradation (Fig. 1D). The PI3K inhibitors (LY294002 > wortmannin) were more effective than the MEK1/2 inhibitors (U0126 > PD98059) to enhance NaSal-triggered apoptosis. In contrast, the p38MAPK inhibitor SB203580 reduced the NaSal-induced apoptosis morphologically and biochemically (data not shown). A significant role of p38MAPK in NaSal-induced apoptosis has been reported in a number of other cells including normal human fibroblasts cells and cancer cells,

possibly regulating both caspase-dependent and caspase-independent nuclear condensation/fragmentation [21,22]. These results implicate that NaSal may not only exert cytotoxicity by activating p38MAPK but also intrinsic chemoresistance by activating the PI3K-Akt/PKB and MEK1/2-ERK1/2 cytoprotective pathways and that the Akt/PKB and MEK1/2-ERK1/2 signaling pathways may play a role in resistance to NaSal treatment.

3.2. *Simultaneous inhibition of PI3K-Akt/PKB and MEK1/2-ERK1/2 confer apoptotic ability to low dose of sodium salicylate*

We also found that Akt/PKB and ERK1/2 were activated in response to low dose (2 mM) of NaSal and Akt/PKB and ERK1/2 activation were markedly prevented by LY294002 and U0126, respectively (Fig. 2A). Interestingly, combined treatment of NaSal and U0126 significantly activated the pro-survival kinase Akt/PKB (Fig. 2A). To determine whether the PI3K inhibitors and the MEK1/2 inhibitors could affect the potential apoptotic activity of low dose of NaSal, A549 cells were treated with NaSal and the inhibitors in different combinations. In contrast to 20 mM NaSal, we could not detect a significant apoptotic response when cells were treated with 2 mM NaSal combined with either the PI3K inhibitors or the MEK1/2 inhibitors except for group of combined treatment of NaSal and U0126 (apoptosis about 20%). However, we observed an impressive synergistic effect when NaSal was combined with both the PI3K inhibitors (LY294002 or wortmannin) and MEK1/2 inhibitors (U0126 or PD98059) at 48 h (Fig. 2B). Although no prominent apoptosis was detected in cells treated with LY294002 + U0126 (3% at 24 h and 5% at 48 h), LY294002 + PD98059 (3% at 24 and 48 h), wortmannin + U0126 (1% at 24 and 48 h), or wortmannin + PD98059 (1% at 24 h and 2% at 48 h), apoptosis was prominently increased by combined treatment of NaSal and the PI3K/MEK inhibitors: NaSal + -LY294002 + U0126 (10% at 24 h and 83% at 48 h), NaSal + -LY294002 + PD98059 (6% at 24 h and 43% at 48 h), NaSal + wortmannin + U0126 (6% at 24 h and 24% at 48 h), and NaSal + wortmannin + PD98059 (4% at 24 h and 22% at 48 h) (Fig. 2B). These results clearly indicate a synergistic effect of combined treatment of NaSal with both the PI3K inhibitors (LY294002 or wortmannin) and MEK1/2 inhibitors (U0126 or PD98059) on apoptosis. The synergistic effect between NaSal and the PI3K inhibitors/MEK1/2 inhibitors was more prominent when NaSal + LY294002 + U012 were used. Similar results were obtained in HCT-116 and HT-29 colorectal cancer cells (data not shown), implicating that NaSal-based combinatorial

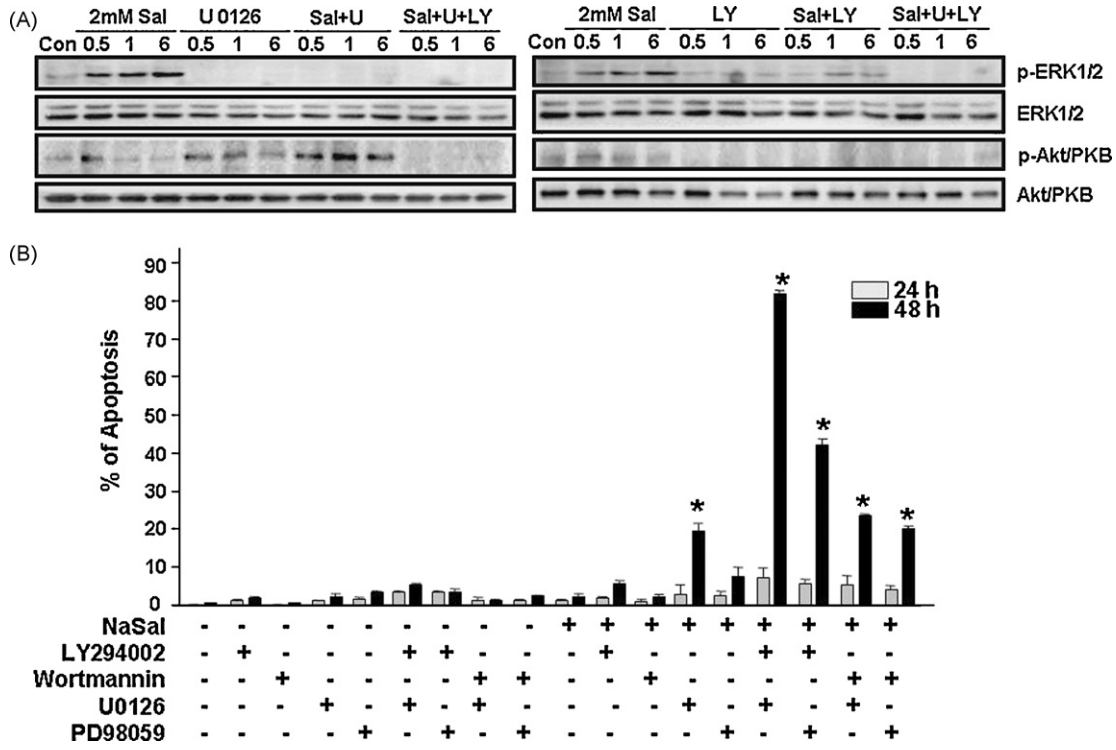


Fig. 2 – (A) Low dose (2 mM) of NaSal activates PI3K-Akt/PKB-GSK-3β and MEK1/2-ERK1/2 signaling pathways through PI3K. A549 cells were pretreated with LY294002 (L, 20 μM), or U0126 (U, 20 μM) for 1 h and then treated with 2 mM NaSal (Sal) for the indicated times and the cellular proteins were analyzed by SDS-PAGE and Western blotting with antibodies to phospho-Akt/PKB (Ser-473), Akt/PKB, phospho-ERK (Thr-202/Tyr-204), and ERK1/2. (B) A549 cells were pretreated with LY294002 (20 μM), wortmannin (0.2 μM), PD98059 (30 μM), and U0126 (20 μM) in different combinations for 1 h and then treated with 2 mM NaSal for 24 and 48 h. For morphological apoptosis analysis, the cells were stained with Hoechst 33342 and the apoptotic cells with condensed/fragmented nuclei were scored under a fluorescence microscope. Results (500–800 cells in each group) are expressed as the mean ± S.E.M. from three independent experiments. *$p < 0.01$, compared with corresponding value for 2 mM NaSal-treated cells.

treatment is applicable to various cell types. Pre-treatment of SB203580 suppressed combined treatment-induced apoptosis of A549 cells with abolishing p38MAPK activation (data not shown). Thus, inhibition of both PI3K-Akt/PKB and MEK1/2-ERK1/2 signaling highly increased the sensitivity of A549 cells to the potential cytotoxic effects of NaSal and switched the cell fate in response to low dose (2 mM) of NaSal from cell cycle arrest to apoptosis.

3.3. *NAG-1 plays a critical role(s) in apoptosis by the combined treatment of NaSal, LY294002, and U0126*

To elucidate the mechanism of the combined treatment-induced apoptosis, we performed cDNA microarray analysis and identified that several genes were differentially up-regulated during apoptosis by combined treatment. One of them was NAG-1 that has been shown to be a

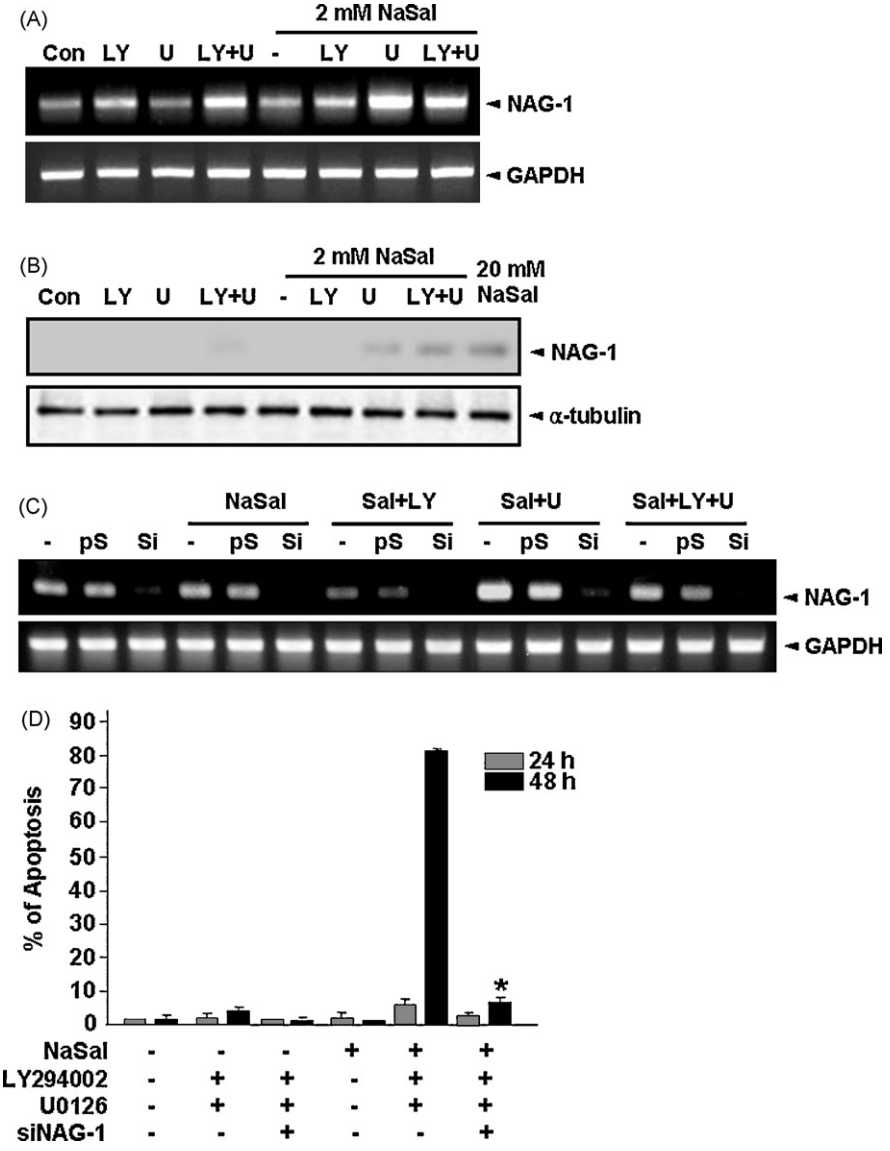


Fig. 3 – (A) A549 cells were pretreated with LY294002 (LY, 20 μM) and/or U0126 (U, 20 μM) for 1 h and then treated with 2 mM NaSal for 36 h and analyzed by RT-PCR using specific probes for NAG-mRNA. GAPDH served as a loading control. (B) A549 cells were pretreated with LY294002 (LY, 20 μM) and/or U0126 (U, 20 μM) for 1 h and then treated with 2 mM NaSal for 48 h and the cellular proteins were analyzed by SDS-PAGE and Western blotting with antibodies to NAG-1 and α-tubulin. 20 mM NaSal that could induce NAG-1 and apoptosis was used for a positive control for the NAG-1. (C) Transcript knockdown by NAG-1 siRNA in A549 cells. A549 cells were pretreated with LY294002 (20 μM) and/or U0126 (20 μM) for 1 h and then treated with 2 mM NaSal for 36 h and analyzed by RT-PCR using specific probes for NAG-mRNA. GAPDH served as a loading control. (D). A549 cells were pretreated with LY294002 (20 μM) and/or U0126 (20 μM) for 1 h and then treated with 2 mM NaSal for 24 and 48 h. For morphological apoptosis analysis, the cells were stained with Hoechst 33342 and the apoptotic cells with condensed/fragmented nuclei were scored by fluorescence microscopy. Results (500–800 cells in each group) are expressed as the mean ± S.E.M. from three independent experiments. *$p < 0.01$, compared with corresponding value for cells treated with NaSal + LY294002 + U0126.

member of the TGF-β superfamily that mediates apoptosis by the agents to prevent tumor formation and development such as several NSAIDs, resveratrol, and genistein [10–14]. Induction of NAG-1 mRNA and protein by combined treatment of 2 mM NaSal, 20 μM LY294002, and 20 μM U0126 was confirmed by RT-PCR and Western blot analysis, respectively (Fig. 3A and B). Interestingly, a significant increase of NAG-1 mRNA and protein levels was also observed by combined treatment of NaSal and U0126 as well as by treatment of NaSal combined with U0126 and LY294002, although combined treatment of NaSal- and U0126-induced apoptosis less than treatment of NaSal combined with LY294002 and U0126.

To determine whether apoptosis by NaSal-based combined treatment is linked to expression NAG-1, we examine the effects of NAG-1 siRNA on apoptosis by treatment of NaSal combined with LY294002 and U0126 (Fig. 3C). As shown in Fig. 3 D, NAG-1 siRNA reduced NAG-1 expression and apoptosis in response to combined treatment. These data suggest that NAG-1 may play a critical role(s) in apoptosis by combined treatment of NaSal and the PI3K/MEK1/2 inhibitors and selective inhibitors of the PI3K-Akt/PKB and MEK1/2-ERK1/2 pathways enhance NaSal-induced apoptosis through increasing NAG-1 expression.

3.4. *Genistein promotes NaSal-potentiated apoptosis in A549 cells*

In most cases, PI3K-Akt/PKB and ERK1/2 signal pathways are activated via protein tyrosine kinases (PTKs) in response to growth factor. Thus, we hypothesized that RTK inhibitors could substitute the combined effects of the PI3K and MEK1/2 inhibitors (LY294002 + U0126 or LY294002 + PD98059) in NaSal-based combined treatment. Here, we found that combined treatment of NaSal and genistein (a general RTK inhibitor) leads to a dramatic increase in apoptotic cell death (Fig. 4A). When A549 cells were treated with 2 mM NaSal combined with 100 μM genistein for 48 h, the apoptotic rate was increased to 66%, although no significant apoptosis was detected in cells treated with 2 mM NaSal or 100 μM genistein alone (NaSal, 2% at 48 h and genistein, 5% at 48 h). In addition, no significant apoptosis was detected in A549 cells that were incubated under treatment of NaSal combined with either AG1478 (a specific epidermal growth factor receptor, EGFR, inhibitor) or AG1024 (a specific insulin-like growth factor-1 receptor, IGF-1R, inhibitor). Combined treatment of genistein and NaSal resulted in an increase of NAG-1 mRNA and protein with a massive induction of apoptosis (Fig. 4B and C). In addition, NAG-1 siRNA suppressed NAG-1 expression increased under

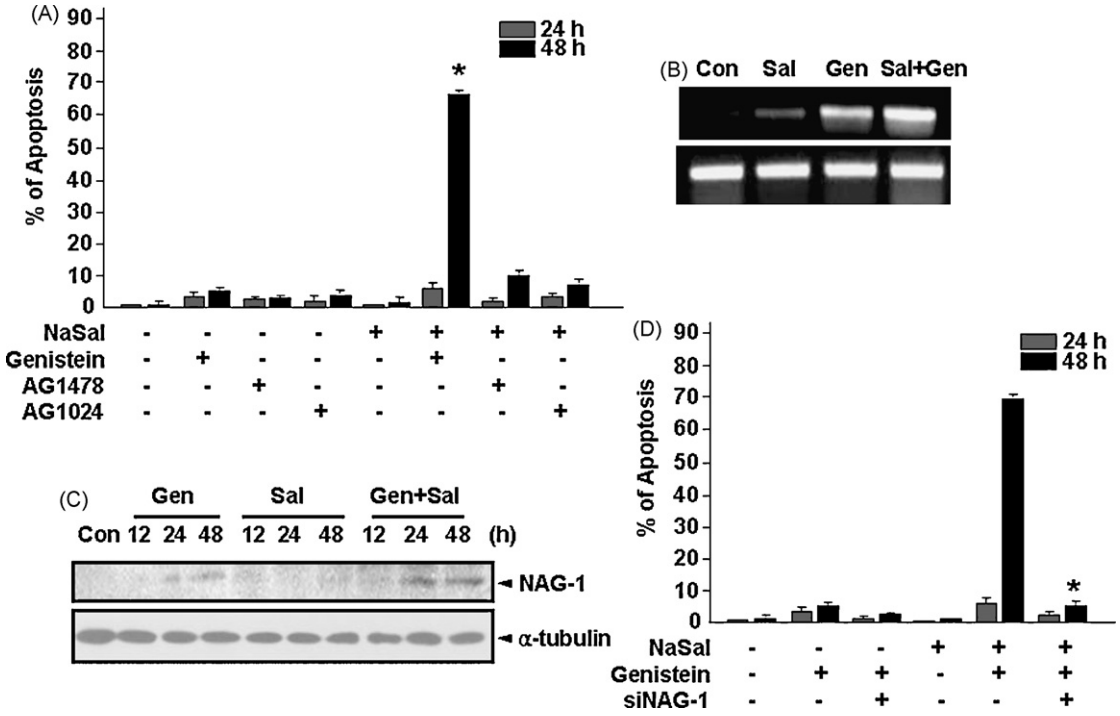


Fig. 4 – (A) A549 cells were treated with NaSal (2 mM) in combination with genistein (100 μM), AG1478 (10 μM), or A1024 (10 μM) 36 h. For morphological apoptosis analysis, the cells were stained with Hoechst 33342 and the apoptotic cells with condensed/fragmented nuclei were scored by fluorescence microscopy. Results (500–800 cells in each group) are expressed as the mean ± S.E.M. from three independent experiments. *$p < 0.01$, compared with corresponding value for 2 mM NaSal-treated cells. (B) A549 cells were treated with genistein (Gen, 100 μM) in combination with NaSal (Sal, 2 mM) for 36 h and analyzed by RT-PCR using specific probes for NAG-1 mRNA. GAPDH served as a loading control. (C) The cellular proteins were analyzed by SDS-PAGE and Western blotting with antibodies to NAG-1 and α-tubulin. (D) A549 cells were treated with NaSal (2 mM) in combination with genistein (Gen, 100 μM) for 36 h. For morphological apoptosis analysis, the cells were stained with Hoechst 33342 and the apoptotic cells with condensed/fragmented nuclei were scored under a fluorescence microscope. Results (500–800 cells in each group) are expressed as the mean ± S.E.M. from three independent experiments. *$p < 0.01$, compared with corresponding value for cells treated with NaSal + genistein.

combined treatment of genistein and NaSal and prevented the apoptotic cell death (Fig. 4D).

4. Discussion

A number of investigators have consistently shown that aspirin displayed anti-neoplastic ability in many types of cancer cells including colon, lung, and breast cancer cells [3–5]. Aspirin still appears to be an ideal candidate for chemotherapy because of its selective cytotoxicity and weak mutagenicity. The chemopreventive activity of aspirin and its natural deacetylated form NaSal is thought to be linked to their ability to inhibit cell proliferation [6,7], and to induce apoptosis [8,9]. In general, the susceptibility of tumor cells to anti-tumor drugs induced apoptosis depends on the balance between pro-apoptotic and survival (anti-apoptotic) programs. Many chemotherapeutic agents activate not only a pro-apoptotic program(s) but also an antagonistic anti-apoptotic program(s) such as transcription factor NF-kB and PI3K-Akt/PKB pathway. Strikingly, inhibition of the anti-apoptotic program that interferes with their intended action sensitizes cells to drug-mediated death. Thus, a better understanding of anti-apoptotic molecules that are activated by chemotherapeutic agents results in new, more effective therapeutic approaches. In this study, we showed that NaSal-activated PI3K-Akt/PKB, and MEK1/2-ERK1/2. NaSal-induced Akt/PKB activation resulted in phosphorylation of GSK-3β that plays a critical role(s) in apoptosis (Fig. 1B and A). Akt/PKB and ERK1/2 are known to promote cellular survival and chemotherapeutic resistance in most NSCLC cells and thus manipulating Akt/PKB and ERK1/2 activity can alter sensitivity to chemotherapy and irradiation [24,26]. We examined the effects of the PI3K inhibitors or MEK1/2 inhibitors, either alone or in combination with NaSal on NaSal-potentiated apoptosis. Inhibition of PI3K-Akt/PKB signaling by the PI3K inhibitors (LY294002 and wortmannin), and ERK1/2 by the MEK1/2 inhibitors (U0126 and PD98059) enhanced the apoptotic response of A549 cells to high dose (20 mM) of NaSal (Fig. 1). Importantly, A549 that had been incubated with the PI3K inhibitors and the MEK1/2 inhibitors exerted apoptotic response to the low dose of NaSal (2 mM) (Fig. 2). LY294002/U0126 in combination with NaSal was more effective to induce apoptosis in A549 cancer cells than other combinations. Treatment of LY294002 alone or combined treatment of the PI3K inhibitors and MEK1/2 did not induce apoptosis. These results demonstrate that the survival programs, PI3K-Akt/PKB and MEK1/2-ERK1/2 interfere with the intended action of NaSal and their inhibition synergistically sensitizes cells to NaSal-induced apoptosis.

To determine the mechanisms underlying these effects, we examined NaSal-induced changes in gene expression using cDNA-based microarray screening of a human lung carcinoma cell line A549. We identified by microarray and confirmed by RT-PCR and Western blot analysis that NAG-1 is induced by combined treatment of NaSal and the kinase inhibitors. Considerable evidences have demonstrated that NAG-1 expression is closely linked to apoptosis [10–14]. NAG-1 transfected HCT-116 cells showed reduced tumorigenicity in athymic nude mice and NAG-1 overexpression in transgenic mice suppresses intestinal adenoma growth. Furthermore, NAG-1 siRNA suppressed apoptosis by chemotherapeutic chemicals such as 5F203-, radiation- or 12-O-tetradecanoylphorbol-13-acetate (TPA) in the cancer cells [33,34]. We showed that NAG-1 siRNA suppressed apoptosis in response to combined treatment. These data suggest that NAG-1 may play a critical role(s) in apoptosis by combined treatment of NaSal and the PI3K/MEK1/2 inhibitors and the inhibitors of the PI3K-Akt/PKB and MEK1/2-ERK1/2 pathways enhance NaSal-induced apoptosis through increasing NAG-1 expression.

We observed that combined treatment of NaSal and U0126 significantly increased NAG-1 mRNA and protein levels, but it induced apoptosis less than treatment of NaSal combined with LY294002 and U0126; NaSal + LY294002 + U0126 (10% at 24 h and 83% at 48 h) and NaSal + U0126 (3% at 24 h and 21% at 48 h) (Fig. 3). These results suggest that the MEK1/2-ERK1/2 signal pathway may exert negative effects on NaSal-potentiated NAG-1 expression and apoptosis. In contrast, the PI3K-Akt signal pathway may regulate the pro-apoptotic activities of NAG-1. NAG-1 is a member of the TGF-β superfamily that regulate cell growth, differentiation and apoptosis through activating intracellular Smad signaling that control the expression of TGF-β target genes [10–14]. The PI3K-Akt signal pathway positively or negatively regulates the functions of TGF-β depending on the cell types and the applied stimuli. For instance, LY294002 treatment resulted in Smad2 accumulation in the nuclei and an increased Smad-binding element (SBE)-luciferase activity in SK-N-SH human neuroblastoma cells, demonstrating that the PI3K pathway negatively regulates TGF-β/Smad signaling [35]. On the other hand, inhibition of PI3K and Akt kinase blocked TGF-β- and Smad 3-mediated expression of plasminogen activator inhibitor type 1 (PAI-1), which regulates degradation of extracellular matrix proteins in fibrotic diseases, indicating that TGF-β-induced PI3K/Akt signaling as a critical regulator of Smad 3-CBP interaction and Smad 3 acetylation, which cause increased PAI-1 expression [36]. In our system, the PI3K-Akt/PKB may interfere with the cell killing effects of NAG-1 induced by combine treatment of NaSal and U0126. A549 cells have the lowest levels of Akt/PKB activity that promote cellular survival and chemotherapeutic resistance, compared to other NSCLC such as H157, H1703, and H1155. We observed that combined treatment of NaSal- and U0126-activated Akt/PKB (Fig. 2A). Our results suggest that the MEK1/2-ERK1/2 signal pathway may exert negative effects on NaSal-potentiated NAG-1 expression and apoptosis, whereas the PI3K-Akt signal pathway that could be activated by combined treatment of NaSal and U0126 may interfere the pro-apoptotic activities of NAG-1. Therefore, inhibition of both PI3K-Akt and MEK1/2-ERK1/2 signal pathways could synergistically increase NaSal-potentiated apoptosis.

We also found that combined treatment of NaSal and genistein (a general RTK inhibitor) but not with AG1478 and AG1024 (specific inhibitors of EGF-R and IGF-1R, respectively) induced a synergistic enhancement in apoptosis. Genistein is an isoflavenoid found in soy that has potential chemopreventive properties and anti-tumorigenic activities with a wide variety of pharmacological effects in animal cells, and is pharmacologically safe [37,38]. Recent research has suggested that this plant polyphenol might be used to sensitize tumor cells to chemotherapeutic agents and radiation therapy by

inhibiting pathways that lead to treatment resistance and has also been found to be protective from therapy-associated toxicities [39]. Genistein is commonly used to examine intracellular signaling in concentrations of 25–100 μM. Genistein at 100 μM concentration effectively could induce apoptosis of MCF-7 cells in 24 h [39]. However, we could not detect a significant apoptotic induction in genistein-treated A549 cells. In general, NSCLC is known to be not a chemosensitive tumor, although the mechanism of resistance to the relevant anticancer drugs has not been fully elucidated. Genistein is known to induce NAG-1 in a time- and concentration-dependent manner in HCT-116 cells [12]. We could also detect a significant induction of NAG-1 in A549 cells. *Synergistic* effects of NaSal and genistein on NAG-1 expression were observed. NAG-1 siRNA prevented the apoptotic cell death. Our results demonstrate that combinatorial treatment of genistein and NaSal at non-toxic concentrations effectively induces apoptosis in A549 cells may provides novel approaches for future effective molecular cancer therapeutics using NaSal. In this study, we used 1–2 mM NaSal and aspirin. 0.5–2 mM aspirin concentrations approximates to systemic pharmacological concentrations. Although 0.5 mM aspirin is equivalent to a low therapeutic plasma concentration, 2 mM aspirin corresponds to a high therapeutic plasma concentration. Although such concentration is high to treat systemically, it can be locally achieved upon administration of aspirin during anti-inflammatory therapy, since NaSal concentrations have been suggested to increase in the mildly acidic environments that prevail at inflammatory sites [40] and tumor microenvironment that is usually acidic [41]. Consequently, these findings demonstrate that inhibition of PI3K and ERK1/2 signaling may contribute to improve apoptosis-inducing efficacy of NaSal and also provide the synergistic therapeutic interaction between NaSal and genistein, suggesting that such combinations may be effectively exploited in future human cancer clinical trials. Although combined treatment of NaSal + LY294002 + U0126 could synergistically induce apoptosis, combination of two natural compounds, NaSal and geneistein, would be more safely applicable to test clinical therapeutic effects.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korea Government (MOEHRD, Basic Research Promotion Fund, KRF-2005-217-C00008), a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A060594), and by grants from the Ministry of Science and Technology, Korea, and the Korea Science and Engineering Foundation through the Research Center for Resistant Cells (R13-2003-009).

REFERENCES

[1] Tegeder I, Pfeilschifter J, Geisslinger G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. FASEB J 2001;15:2057–72.
[2] Amann R, Peskar BA. Anti-inflammatory effects of aspirin and sodium salicylate. Eur J Pharmacol 2002;447:1–9.
[3] Hawk ET, Levin B. Colorectal cancer prevention. J Clin Oncol 2005;23:378–91.
[4] Ulrich CM, Bigler J, Potter JD. Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. Nat Rev Cancer 2006;6:130–40.
[5] Flossmann E, Rothwell PM. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet 2007;369:1603–13.
[6] Marra DE, Simoncini T, Liao JK. Inhibition of vascular smooth muscle cell proliferation by sodium salicylate mediated by upregulation of p21 (Waf1) and p27 (Kip1). Circulation 2000;102:2124–30.
[7] Law BK, Waltner-Law ME, Entingh AJ, Chytil A, Aakre ME, Norgaard P, et al. Salicylate-induced growth arrest is associated with inhibition of p70s6k and down-regulation of c-myc, cyclin D1, cyclin A, and proliferating cell nuclear antigen. J Biol Chem 2000;275:38261–7.
[8] Bellosillo B, Pique M, Barragan M, Castano E, Villamor N, Colomer D, et al. Aspirin and salicylate induce apoptosis and activation of caspases in B-cell chronic lymphocytic leukemia cells. Blood 1998;92:1406–14.
[9] Klampfer L, Cammenga J, Wisniewski HG, Nimer SD. Sodium salicylate activates caspases and induces apoptosis of myeloid leukemia cell lines. Blood 1999;93:2386–94.
[10] Baek SJ, Kim KS, Nixon JB, Wilson LC, Eling TE. Cyclooxygenase inhibitors regulate the expression of a TGF-beta superfamily member that has proapoptotic and antitumorigenic activities. Mol Pharmacol 2001;59:901–8.
[11] Baek SJ, Wilson LC, Eling TE. Resveratrol enhances the expression of non-steroidal anti-inflammatory drug-activated gene (NAG-1) by increasing the expression of p53. Carcinogenesis 2002;23:425–34.
[12] Wilson LC, Baek SJ, Call A, Eling TE. Nonsteroidal anti-inflammatory drug-activated gene (NAG-1) is induced by genistein through the expression of p53 in colorectal cancer cells. Int J Cancer 2003;105:747–53.
[13] Newman D, Sakaue M, Koo JS, Kim KS, Baek SJ, Eling TE, et al. Differential regulation of nonsteroidal anti-inflammatory drug-activated gene in normal human tracheobronchial epithelial and lung carcinoma cells by retinoids. Mol Pharmacol 2003;63:557–64.
[14] Baek SJ, Okazaki R, Lee SH, Martinez J, Kim JS, Yamaguchi K, et al. Nonsteroidal anti-inflammatory drug-activated gene-1 over expression in transgenic mice suppresses intestinal neoplasia. Gastroenterology 2006;131:1553–60.
[15] Chung YM, Bae YS, Lee SY. Molecular ordering of ROS production, mitochondrial changes, and caspase activation during sodium salicylate-induced apoptosis. Free Radic Biol Med 2003;34:434–42.
[16] Battaglia V, Salvi M, Toninello A. Oxidative stress is responsible for mitochondrial permeability transition induction by salicylate in liver mitochondria. J Biol Chem 2005;280:33864–72.
[17] Derouet M, Thomas L, Moulding DA, Akgul C, Cross A, Moots RJ, et al. Sodium salicylate promotes neutrophil apoptosis by stimulating caspase-dependent turnover of Mcl-1. J Immunol 2006;176:957–65.
[18] Dikshit P, Chatterjee M, Goswami A, Mishra A, Jana NR. Aspirin induces apoptosis through the inhibition of proteasome function. J Biol Chem 2006;281:29228–35.
[19] Kopp E, Ghosh S. Inhibition of NF-kB by sodium salicylate and aspirin. Science 1994;265:956–9.
[20] Stark LA, Reid K, Sansom OJ, Din FV, Guichard S, Mayer I, et al. Aspirin activates the NF-kappaB signalling pathway and induces apoptosis in intestinal neoplasia in two in vivo models of human colorectal cancer. Carcinogenesis 2007;28:968–76.

[21] Schwenger P, Bellosta P, Vietor I, Basilico C, Skolnik EY, Vilcek J. Sodium salicylate induces apoptosis via p38 mitogen-activated protein kinase but inhibits tumor necrosis factor-induced c-Jun N-terminal kinase/stress-activated protein kinase activation. Proc Natl Acad Sci USA 1997;94:2869–73.
[22] Lee EJ, Park HG, Kang HS. Sodium salicylate induces apoptosis in HCT116 colorectal cancer cells through activation of p38MAPK. Int J Oncol 2003;23:503–8.
[23] Yamaguchi H, Wang HG. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. Oncogene 2001;20:7779–86.
[24] Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 2001;61:3986–97.
[25] Crowell JA, Steele VE. AKT and the phosphatidylinositol 3-kinase/AKT pathway: important molecular targets for lung cancer prevention and treatment. J Natl Cancer Inst 2003;95:252–3.
[26] Brognard J, Dennis PA. Variable apoptotic response of NSCLC cells to inhibition of the MEK/ERK pathway by small molecules or dominant negative mutants. Cell Death Differ 2002;9:893–904.
[27] Huang C, Li J, Ding M, Leonard SS, Wang L, Castranova V, et al. UV Induces phosphorylation of protein kinase B (Akt) at Ser-473 and Thr-308 in mouse epidermal Cl 41 cells through hydrogen peroxide. J Biol Chem 2001;276:40234–40.
[28] MacKeigan JP, Taxman DJ, Hunter D, Earp III HS, Graves LM, Ting JP. Inactivation of the antiapoptotic phosphatidylinositol 3-kinase-Akt pathway by the combined treatment of taxol and mitogen-activated protein kinase inhibition. Clin Cancer Res 2002;8:2091–9.
[29] Clark AS, West K, Streicher S, Dennis PA. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. Mol Cancer Ther 2002;1:707–17.
[30] Takahashi T. Lung cancer: an ever increasing store of in-depth basic knowledge and the beginning of its clinical application. Oncogene 2002;21:6868–9.
[31] Kim CH, Han SI, Lee SY, Youk HS, Moon JY, Duong HQ, et al. Protein kinase C-ERK1/2 signal pathway switches glucose depletion-induced necrosis to apoptosis by regulating superoxide dismutases and suppressing reactive oxygen species production in A549 lung cancer cells. J Cell Phyiol 2007;211:371–85.
[32] Müller B, Blackburn J, Feijoo C, Zhao X, Smythe C. DNA-activated protein kinase functions in a newly observed S phase checkpoint that links histone mRNA abundance with DNA replication. J Cell Biol 2007;179:1385–98.
[33] Okazaki R, Moon Y, Norimura T, Eling TE. Ionizing radiation enhances the expression of the nonsteroidal anti-inflammatory drug-activated gene (NAG1) by increasing the expression of TP53 in human colon cancer cells. Radiat Res 2006;165:125–30.
[34] Martinez JM, Sali T, Okazaki R, Anna C, Hollingshead M, Hose C, et al. Drug-induced expression of nonsteroidal anti-inflammatory drug-activated gene/macrophage inhibitory cytokine-1/prostate-derived factor, a putative tumor suppressor, inhibits tumor growth. J Pharmacol Exp Ther 2006;318:899–906.
[35] Qiao J, Kang J, Ko TC, Evers BM, Chung DH. Inhibition of transforming growth factor-beta/Smad signaling by phosphatidylinositol 3-kinase pathway. Cancer Lett 2006;242:207–14.
[36] Das F, Ghosh-Choudhury N, Venkatesan B, Li X, Mahimainathan L, Choudhury GG. Akt kinase targets association of CBP with SMAD 3 to regulate TGFbeta-induced expression of plasminogen activator inhibitor-1. J Cell Physiol 2008;214:513–27.
[37] Dixon RA, Ferreira D. Genistein. Phytochemistry 2002;60:205–11.
[38] Garg AK, Buchholz TA, Aggarwal BB. Chemosensitization and radiosensitization of tumors by plant polyphenols. Antioxid Redox Signal 2005;7:1630–47.
[39] Yeh TC, Chiang PC, Li TK, Hsu JL, Lin CJ, Wang SW, et al. Genistein induces apoptosis in human hepatocellular carcinomas via interaction of endoplasmic reticulum stress and mitochondrial insult. Biochem Pharmacol 2007;73:782–92.
[40] Jurivich DA, Sistonen L, Kroes RA, Morimoto RI. Effect of sodium salicylate on the human heat shock response. Science 1992;255:1243–5.
[41] Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891–9.