

Sedanolidide induces autophagy through the PI3K, p53 and NF- κ B signaling pathways in human liver cancer cells

SHU-LING HSIEH¹, CHI-TSAI CHEN², JYH-JYE WANG³, YU-HAO KUO¹,
CHIH-CHUN LI⁴, LAN-CHI HSIEH⁵ and CHIH-CHUNG WU⁶

¹Department of Seafood Sciences, National Kaohsiung Marine University, Kaohsiung 81143; ²Department of Restaurant and Hospitality Management, Chung Hwa University of Medical Technology, Tainan 71703; ³Department of Nutrition and Health Science, Fooyin University, Kaohsiung 83111; ⁴School of Nutrition, Chung Shan Medical University, Taichung 40201; ⁵Department of Dietetics Kaohsiung Municipal United Hospital, Kaohsiung 80457; ⁶Department of Nutrition and Health Sciences, Chang Jung Christian University, Tainan 71101, Taiwan, R.O.C.

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Abstract. Sedanolidide (SN), a phthalide-like compound from celery seed oil, possesses antioxidant effects. However, the effect of SN on cell death in human liver cancer cells has yet to be determined. In this study, cell viability determination, monodansylcadaverine (MDC) fluorescent staining and immunoblot analysis were performed to determine autophagy induction and autophagy-induced protein expression changes via molecular examination after human liver cancer (J5) cells were treated with SN. Our studies demonstrate that SN suppressed J5 cell viability by inducing autophagy. Phosphoinositide 3-kinase (PI3K)-I, mammalian target of rapamycin (mTOR) and Akt protein levels decreased, whereas PI3K-III, LC3-II and Beclin-1 protein levels increased following SN treatment in J5 cells. In addition, SN treatment upregulated nuclear p53 and damage-regulated autophagy modulator (DRAM) and downregulated cytosolic p53 and Tp53-induced glycolysis and apoptosis regulator (TIGAR) expression in J5 cells. Furthermore, the cytosolic phosphorylation of inhibitor of kappa B (I κ B) and nuclear p65 and the DNA-binding activity of NF- κ B increased after SN treatment.

These results suggest that SN induces J5 cell autophagy by regulating PI3K, p53 and NF- κ B autophagy-associated signaling pathways in J5 cells.

Introduction

Human hepatocellular carcinoma (HCC) is one of the most common malignant tumors and a significant cause of mortality in several regions of Africa and Asia (1,2). Triggering cancer cell death to reduce cancer cell number and inhibiting cancer cell proliferation by phytochemicals or chemotherapeutic agents represent some of the most effective current anticancer strategies (3). Cell death can occur through one of three pathways: necrosis, apoptosis or autophagy (4).

Autophagy is induced by various physiological conditions, such as mitochondrial damage, protein aggregation, pathogen infection and nutrient starvation (5,6). Autophagy is a multi-step cellular pathophysiological program, including initiation (pre-autophagosome formation), autophagosome formation, maturation and degradation (7). In these steps, phosphoinositide 3-kinases (PI3Ks) play key regulatory roles in many cellular processes, including cell survival, proliferation and differentiation (8-10). Both class I PI3K (PI3K-I)/Akt/mammalian target of rapamycin (mTOR) and Beclin-1/class III PI3K (PI3K-III)/LC3-II signaling pathways are involved in pre-autophagosome formation (11-14). Cytosolic and nuclear p53, damage-regulated autophagy modulator (DRAM) and the p53-induced glycolysis and apoptosis regulator (TIGAR) are involved in autophagosome formation (15). In addition, p53 initiates a cascade of starvation signals and triggers a starvation-like response by inhibiting mTOR (16). Furthermore, p53 activates both the DRAM and PI3K-III pathways upon autophagosome formation (15). Phytochemicals or chemical agents that regulate p53- and PI3K-mediated autophagy induction could serve as a potential starting point for novel methods of cancer prevention and treatment.

The transcription factor nuclear factor-kappa B (NF- κ B) plays an important role in autophagy-induced signaling pathways (17,18). Several genes involved in autophagy, such as the

Correspondence to: Dr Chih-Chung Wu, Department of Nutrition and Health Sciences, Chang Jung Christian University, 1 Changda Road, Guiren, Tainan 71101, Taiwan, R.O.C.
E-mail: ccwu@mail.cjcu.edu.tw

Abbreviations: SN, sedanolidide; MDC, monodansylcadaverine; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; DRAM, damage-regulated autophagy modulator; TIGAR, Tp53-induced glycolysis and apoptosis regulator; I κ B, inhibitor of kappa B; NF- κ B, nuclear factor-kappa B; HCC, human hepatocellular carcinoma

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genes encoding Beclin-1, LC3-II and DRAM, are regulated by NF- κ B (15,19,20). Regulation of NF- κ B activation affects autophagy-relevant gene expression, leading to the induction of autophagy.

Sedanolid (SN; 3-butyl-3a,4,5,6-tetrahydro-1(3H)-isobenzofuranone), is a phthalide-like compound from celery (*Apium graveolens* L.) seed oil (21,22). Celery seed contains 2% volatile oil. The typical aroma of celery seed essential oils arises due to SN, which is present at very low levels (1-3%) in the essential oil (23). Previous studies have demonstrated that SN protects against hydrogen peroxide- and tert-butyl hydroperoxide-induced toxicity in HepG2 and CaCo-2 cells (22). SN exhibits prostaglandin H endoperoxide synthase-I (COX-I) and prostaglandin H endoperoxide synthase-II (COX-II) inhibitory activities and topoisomerase-I and -II enzyme inhibitory activities (24). In benzo[α]pyrene (BP) induced forestomach cancer in mice, SN reduced tumor incidence by 57% and tumor multiplicity by 83% by increasing glutathione S-transferase (GST) activity of mice (21). However, the role that SN plays in cancer prevention remains unclear, and research regarding its molecular mechanism through a role in cell death induction is limited. If SN is cytotoxic or induces cell death, its chemopreventative properties would be more favorable.

The aim of this study was to determine whether SN exhibits cytotoxic effects and whether these effects involve autophagy induction in human liver cancer cells. To investigate the effects of SN on cell viability and autophagy, an MTT assay and monodansylcadaverine (MDC) staining of J5 cells were performed. To understand the mechanism by which SN regulates pre-autophagosome and autophagosome formation in autophagy induction, Beclin-1, PI3K-III, LC3-II, PI3K-I, Akt and mTOR expression were investigated using an immunoblot assay. In addition, the roles of p53 and NF- κ B on SN-induced autophagy were also investigated. These experimental results will be helpful to determine the mechanism by which SN induces human liver tumor cell death and its potential effects on chemoprevention and/or cancer therapy.

Materials and methods

Chemicals and reagents. SN and MDC stain were purchased from Sigma Chemical Co. (St Louis, MO, USA). Antiserum against p53, DRAM, phosphorylated (p)-I κ B, I κ B or NF- κ B (P65) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antiserum against TIGAR was obtained from Abcam, Inc. (Cambridge, MA, USA). Antiserum against PI3K-I, PI3K-III, LC3-II, Beclin-1 and mTOR were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and SN treatment. The human hepatocellular carcinoma HepJ5 (J5) cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). J5 cells (2.5×10^6) were plated on 60-mm plastic tissue culture dishes (Becton-Dickinson Labware, Franklin Lakes, NJ, USA) and incubated at 37°C (in humidified 5% CO₂ and 95% air at 1 atm) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin (100 U/ml), and 1% L-glutamine (0.33%, w/v).

After primary plating for 24 h, cells were treated with 100, 250 or 500 μ M of SN for 24 h. All SN was diluted in

dimethyl sulfoxide (DMSO). Cells treated with DMSO alone were regarded as the control group.

Cell viability and morphology examination. Cell viability was measured using an MTT (3-(4, 5-dimethyl-2-yl)-2, 5-diphenyl tetrazolium bromide) assay and morphological examination. The MTT assay was performed as described by Debizot and Lang (1986) (25). Briefly, J5 cells were incubated in 3-cm plates (1×10^6 cells) for 24 h and treated with 100, 250 or 500 μ M of SN for 24 h. Then, MTT reagent (5 mg/ml) was added, and cell viability was determined by measuring the optical density (OD) at 570 nm. A phase-contrast microscope was used to examine morphological changes (Olympus IX 51, Olympus, Tokyo, Japan).

Monodansylcadaverine staining. To determine whether J5 cell death induced by SN was attributed to autophagy, the autophagic vacuole staining dye MDC was used as an autophagy indicator (26). Cells were grown on cover slips and treated with 100, 250 or 500 μ M of SN for 24 h. After SN treatment, cells were incubated with 0.1 mM MDC for 15 min. Micrographs were acquired at x400 magnification on an inverted fluorescence microscope (Olympus IX 51, Olympus). MDC stained cells were measured fluorometrically at an excitation wavelength of 360 nm and an emission wavelength of 530 nm.

Immunoblot analysis of autophagy-associated protein expression. To elucidate the signal transduction pathways by which autophagy was induced by SN in J5 cells, we analyzed the protein expression of autophagy regulators of pre-autophagosome and autophagosome formation. J5 cells were treated with 100, 250 or 500 μ M of SN for 24 h. Total protein was collected and prepared from cells. Nuclear extracts were obtained from cell pellets using an NE-PER extraction kit (Thermo Scientific, Rockford, IL, USA) as per the manufacturer's instructions.

Cellular protein concentration of cells was assayed by the method of Lowry *et al.* (27). Cytoplasmic PI3K-I, PI3K-III, Akt, mTOR, Beclin-1, LC3-II, p53, DRAM and TIGAR as well as nuclear p53 expression were determined using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot assay (28,29). Bands were visualized using hydrogen peroxide/tetrahydrochloride diaminobenzidine or an enhanced chemiluminescent detection kit (Amersham Life Science, Buckinghamshire, UK) and were quantitated with an AlphaImager 2000 (Alpha Innotech, San Leandro, CA, USA).

Analysis of NF- κ B activation and NF- κ B-DNA binding activation. To determine whether autophagy-related molecules are regulated by the NF- κ B pathway after SN treatment, J5 cells were treated with 100, 250 or 500 μ M of SN for 24 h. Total protein was collected and prepared from cells. Cytosolic p-I κ B, I κ B, and NF- κ B as well as nuclear NF- κ B (p65) expression were determined using SDS-PAGE and immunoblot assay (28,29). Bands were visualized using hydrogen peroxide/tetrahydrochloride diaminobenzidine or an enhanced chemiluminescent detection kit (Amersham Life Science) and were quantitated with an AlphaImager 2000 (Alpha Innotech).

For the NF- κ B-DNA binding activity assay, nuclear extracts were obtained from cell pellets using an NE-PER

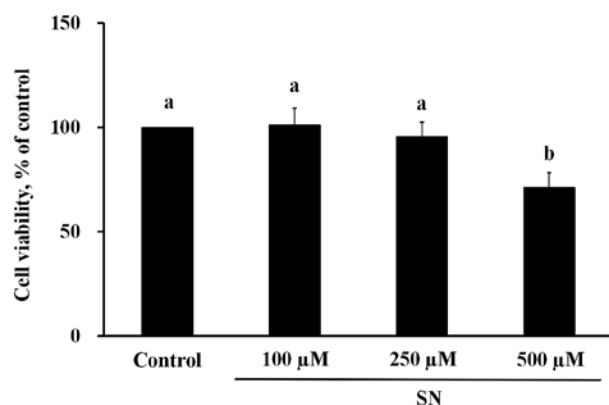


Figure 1. Effects of SN on J5 cell viability. J5 cells (5×10^5 cells/ml) were treated with 0, 100, 250 or 500 μ M SN for 24 h. The 0 μ M concentration was defined as the control group in this study. Values are shown as the mean \pm SD (n=3). a,b, Groups with different letters differ significantly based on Duncan's test ($p < 0.05$).

extraction kit (Thermo Scientific) as per the manufacturer's instructions. NF- κ B-DNA binding activity within the nuclear fraction was determined using an NF- κ B (p65) transcription factor activity assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) as per the manufacturer's instructions.

Statistical analysis. Statistical analyses were performed using SAS software (SAS Institute). Analysis of variance (ANOVA) and Duncan's multiple-range test were used to identify significant differences among the means ($p < 0.05$).

Results

SN induces cytotoxicity in human liver tumor cells. According to the results of the MTT assay (Fig. 1), when J5 cells were treated with 100, 250 or 500 μ M SN for 24 h, the cell viability measurements were 101 ± 8 , 96 ± 7 and $71 \pm 7\%$, respectively. The cell viability of the 500 μ M SN group was significantly reduced compared with the control group (100%) ($p < 0.05$). From the microscopic observation results, no significant changes in the morphology of J5 cells treated with 100 or 250 μ M SN were noted compared with the controls (data not shown). However, significant changes in J5 cell morphology were observed upon treatment with 500 μ M SN after 24-h incubation compared with controls.

SN induces autophagy in human liver tumor cells. As shown in Fig. 2A, J5 cells were stained by MDC after various concentrations of SN treatment for 24 h, MDC-stained cell levels significantly increased ($p < 0.05$), as measured fluorometrically. After J5 cells were treated with 100, 250 or 500 μ M SN for 24 h, MDC fluorescence (35 ± 5 , 46 ± 8 and $83 \pm 9\%$, respectively) also significantly increased in a dose-dependent manner compared with the control group ($6 \pm 3\%$) (Fig. 2B). These results demonstrate that SN induces autophagy in J5 cells.

Effects of SN on protein levels of autophagy regulators. Fig. 3 indicates that in J5 cells treated with 250 or 500 μ M SN for 24 h, PI3K-I protein levels (89 ± 7 and $78 \pm 4\%$, respectively) significantly decreased following 24 h incubation compared

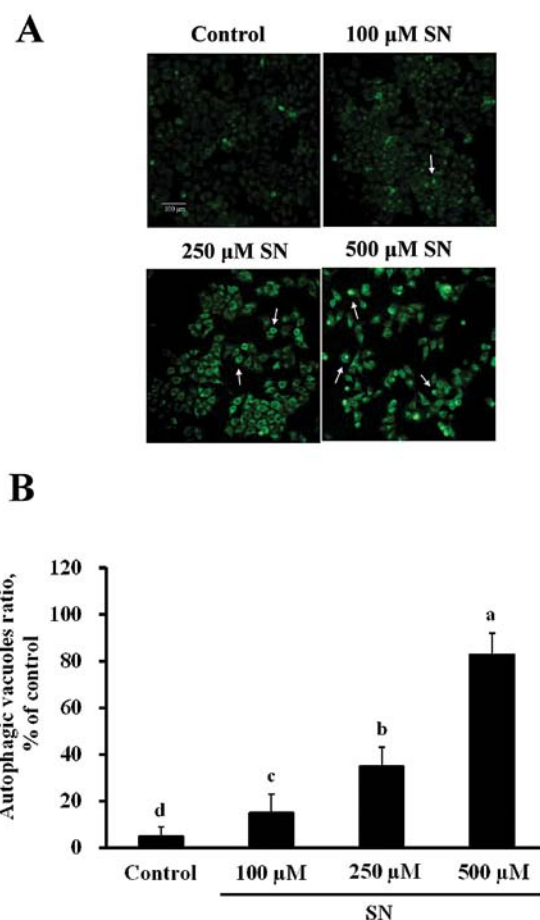


Figure 2. Effects of SN on autophagic vacuole formation in J5 cells. J5 cells (5×10^5 cells/ml) were treated with 0, 100, 250 or 500 μ M SN for 24 h. The 0 μ M concentration was defined as the control group in this study. Total autophagic cells were collected and stained with MDC dye (A). Quantitative analytical results are provided (B). Values are presented as the mean \pm SD (n=3). a-d, Groups in the same cell phase with different letters differ significantly based on Duncan's test ($p < 0.05$).

with the control group (100%) ($p < 0.05$). After J5 cell treatment with 250 or 500 μ M SN for 24 h, the Akt protein levels were decreased (86 ± 5 and $62 \pm 3\%$, respectively) compared with the control group (100%) ($p < 0.05$). mTOR protein levels of the 100, 250 or 500 μ M SN-incubated groups were significantly decreased (75 ± 4 , 65 ± 3 and $53 \pm 4\%$, respectively) compared with the control group (100%) ($p < 0.05$). These results demonstrate that SN suppresses the PI3K/Akt/mTOR pathways. Thus, this compound may play an important role in pre-autophagosome formation.

Conversely, Fig. 4 indicates that when J5 cells are treated with 500 μ M SN for 24 h, Beclin-1 protein levels were increased ($144 \pm 14\%$) compared with the control group (100%) ($p < 0.05$). When J5 cells were treated with 250 or 500 μ M SN, PI3K-III (124 ± 12 and $164 \pm 18\%$, respectively) and LC3-II (167 ± 13 and $185 \pm 15\%$, respectively) protein levels were also significantly increased compared with the control group (100%) ($p < 0.05$). These results suggest that SN regulates the PI3K-I/Akt/mTOR and Beclin-1/PI3K-III/LC3-II pathways that induce autophagy in J5 cells.

Immunoblot analysis also demonstrated that nuclear p53 protein levels of the 500 μ M SN group were significantly

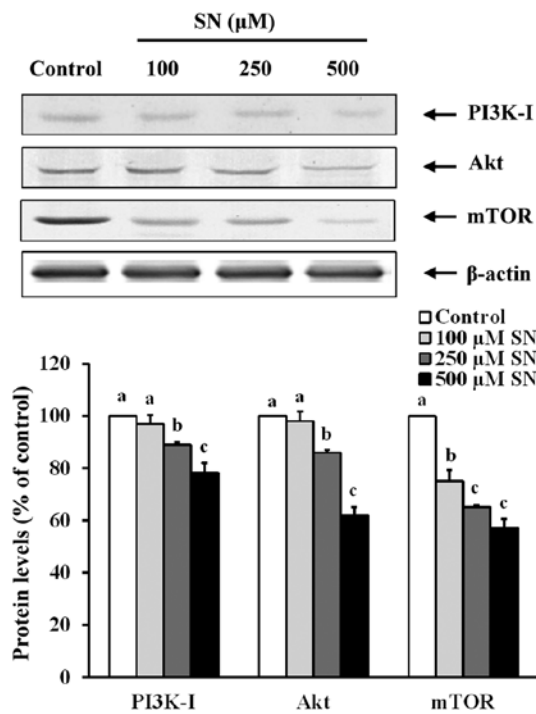


Figure 3. Expression levels of PI3K-I/Akt/mTOR after SN treatment of J5 cells. J5 cells (5×10^5 cells/ml) were treated with 0, 100, 250 or 500 μ M SN for 24 h. The 0 μ M concentration was defined as the control group, and the serum-free group was defined as the negative control group in this study. The protein levels was quantified by densitometry, and the control group level was set as 100%. Values are presented as the mean \pm SD ($n=3$). a-c, Groups in the same cell phase with different letters differ significantly based on Duncan's test ($p<0.05$).

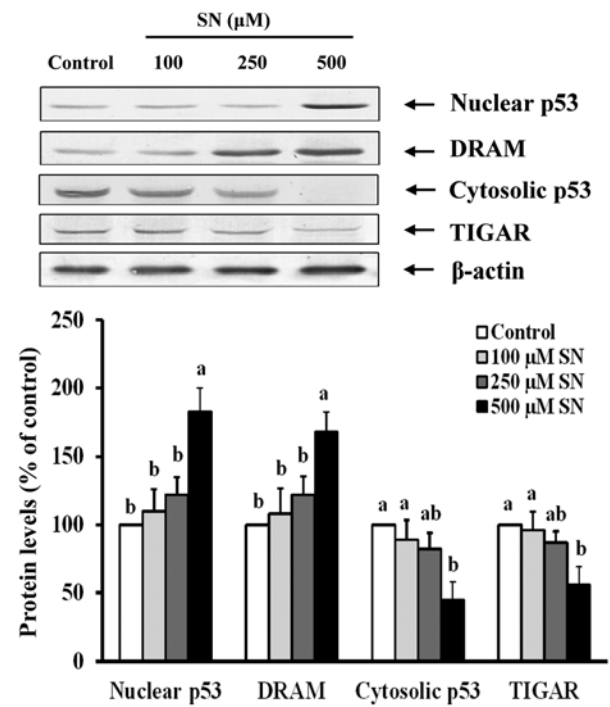


Figure 5. Expression levels of p53 after SN treatment of J5 cells. J5 cells (5×10^5 cells/ml) were treated with 0, 100, 250 or 500 μ M SN for 24 h. The 0 μ M concentration was defined as the control group, and the serum-free group was defined as the negative control group in this study. The protein level was quantified by densitometry, and the control group level was set as 100%. Values are presented as the mean \pm SD ($n=3$). a, b, Groups in the same cell phase with different letters differ significantly based on Duncan's test ($p<0.05$).

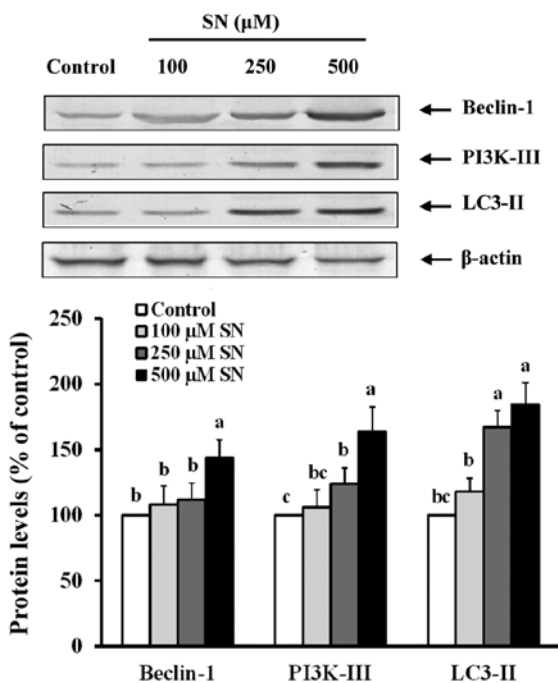


Figure 4. Beclin-1, PI3K-III, and LC3-II expression in J5 cells after SN treatment. J5 cells (5×10^5 cells/ml) were treated with 0, 100, 250 or 500 μ M SN for 24 h. The 0 μ M concentration was defined as the control group, and the serum-free group was defined as the negative control group in this study. The protein level was quantified by densitometry, and the control group level was set as 100%. Values are presented as the mean \pm SD ($n=3$). a-c, Groups in the same cell phase with different letters differ significantly based on Duncan's test ($p<0.05$).

increased ($183 \pm 17\%$) compared with the control group (100%) ($p<0.05$) (Fig. 5). DRAM protein levels were significantly increased ($168 \pm 14\%$) after 500 μ M SN treatment compared with the control group (100%) ($p<0.05$). However, cytoplasmic p53 significantly decreased ($45 \pm 13\%$) after J5 cells were treated with 500 μ M SN for 24 h compared with the control group (100%) ($p<0.05$). When J5 cells were treated with 500 μ M SN, TIGAR protein levels ($56 \pm 13\%$) were significantly reduced compared with the control group (100%) ($p<0.05$). These results indicate that SN affects autophagosome formation via p53 signaling.

SN induces NF- κ B activation. To examine whether the suppression of Beclin-1, LC3-II and DRAM expression by SN is dependent on the inhibition of NF- κ B activation, both immunoblot and NF- κ B-DNA binding activity assays were performed. Fig. 6A and B demonstrate that p-I κ B protein was significantly increased by 256 and 278% after 250 and 500 μ M SN treatment, respectively ($p<0.05$). Nuclear p65 protein levels were also significantly decreased by 334 and 345% after 250 and 500 μ M SN treatment, respectively ($p<0.05$). The changes in p65 protein levels in the nuclear fraction indicate that SN could significantly increase translocation of NF- κ B from the cytoplasm to the nucleus in J5 cells. Furthermore, Fig. 6C demonstrates that NF- κ B nuclear protein DNA-binding activity was significantly increased after 250 and 500 μ M SN treatment ($p<0.05$). These results suggest that SN regulates NF- κ B activation in J5 cells.

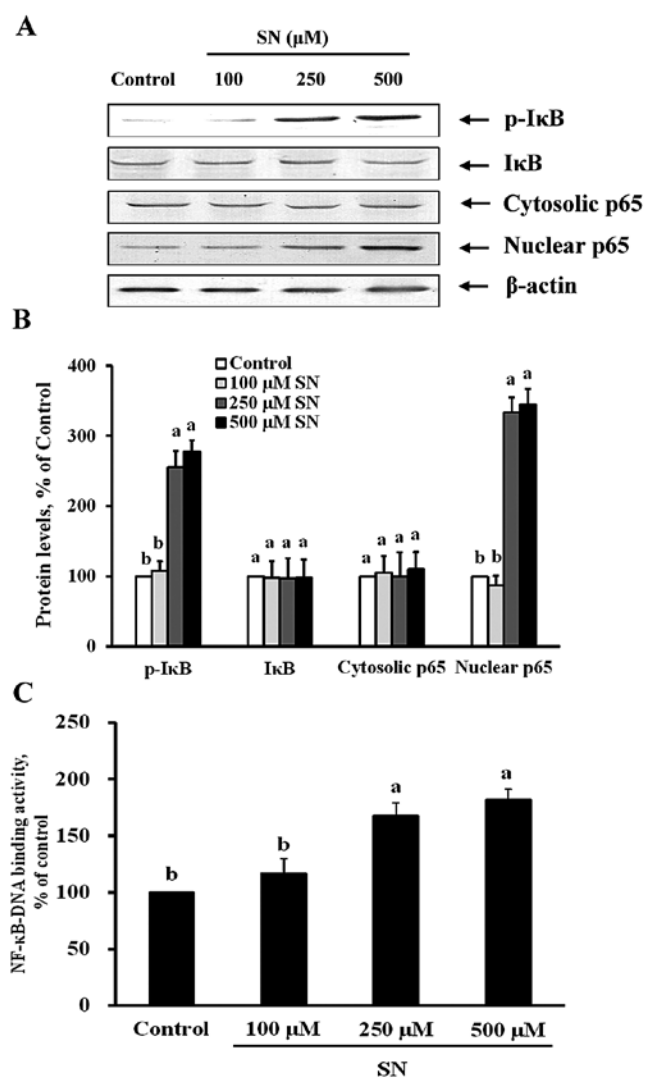


Figure 6. NF-κB expression levels in J5 cells after SN treatment. J5 cells (5×10^5 cells/ml) were treated with 0, 100, 250 or 500 μ M SN for 24 h. The 0 μ M concentration was defined as the control group, and the serum-free group was defined as the negative control group in this study. The protein level was quantified by densitometry, and the control group level was set as 100% (A and B). NF-κB-DNA binding activity (C). Values are presented as the mean \pm SD (n=3). a,b, Groups in the same cell phase with different letters differ significantly based on Duncan's test ($p < 0.05$).

Discussion

SN is one of the major flavor compounds from celery seed oil (30). Our study demonstrates that SN suppresses human tumor cell viability through the induction of autophagy. This finding is novel in the anticancer research of SN. Autophagy plays an important role in tumor inhibition because autophagy defects are associated with metabolic stress, genomic damage, and tumorigenesis (3). By genetically or pharmacologically triggering autophagy, these autophagy inducers could act as potential therapeutic candidates to kill tumor cells (5,31). Various clinical anticancer agents, such as polyoxomolybdates, platinin, phenethyl isothiocyanate, tamoxifen, rapamycin, temozolomide, histone deacetylase inhibitors and camptothecin, involve the induction of autophagy to enhance chemotherapeutic efficacy (32-36). In this study, SN significantly reduced human liver cell viability and induced autophagy. Therefore, SN may act as a potential cancer preventative or therapeutic.

SN is a phthalide-like compound. Phthalide structures contain a lactone core with a variety of complex chemical compound substituents typically found in herbs or vegetables, such as celery, Dong quai (*Angelica sinensis*) and Chuanxiong (*Rhizoma Chuanxiong*) (37,38). SN has a similar chemical structure to butylphthalide (3-n-butylphthalide) in celery seed oil, and both compounds are primarily responsible for the aroma and taste of celery (30). Previous studies demonstrate that *Angelica sinensis* phthalides, including n-butylidene-phthalide, senkyunolide A and z-ligustilide, significantly inhibit cell proliferation of human colon cancer HT-29 cells (39). Noscapine, a phthalide isoquinoline alkaloid derived from opium, inhibits cell proliferation by inducing apoptosis in colon cancer cells (40). Furthermore, 4-(3',3'-dimethylallyloxy)-5-methyl-6-methoxyphthalide, which is isolated from *Podocarpus macrophyllus*, inhibits proliferation of HeLa tumor cell lines by inducing G1 cell cycle arrest and apoptosis (41). No study has investigated the effects of SN, a phthalide-like structure, on cancer cell death. Our results demonstrate that SN induces autophagy in human liver tumor cells, and this specific physiological effect may be attributed to its phthalide-like structure.

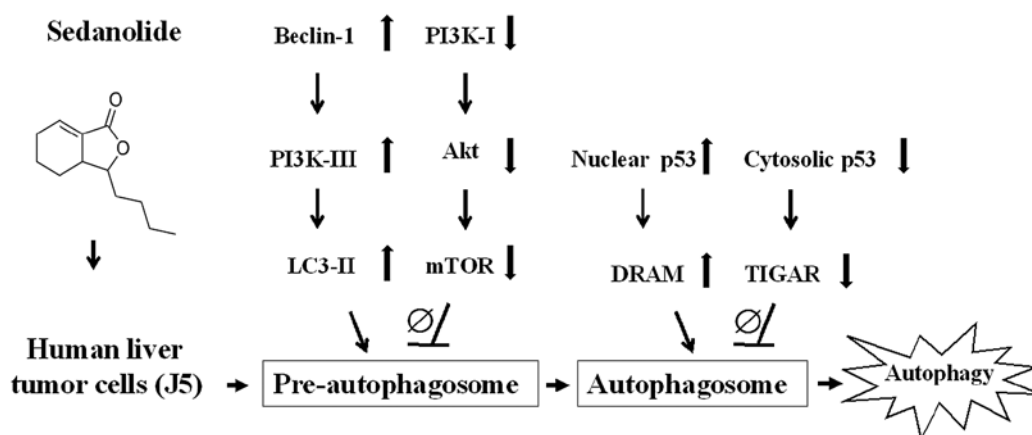


Figure 7. Proposed model of SN-induced autophagy in J5 cells. The present study proposes that SN suppresses PI3K-I/Akt/mTOR expression and enhances Beclin-1/PI3K-III/LC3-II protein levels, leading to pre-autophagosome formation. In addition, SN decreases cytosolic p53 and TIGAR protein levels but increases nuclear p53 and DRAM levels to promote autophagosome formation in J5 cells.

The PI3K pathway, a critical signal transduction system linking oncogenes and multiple receptor classes to many essential cellular functions, is perhaps the most commonly activated signaling pathway in human cancer (42). PI3K-I is highly correlated with Akt activation. Constitutive activation of the PI3K/AKT signaling pathway often causes cells to proliferate in an uncontrolled manner (10). PI3K-III is an important regulator of autophagy, a cellular response to nutrient starvation (43). Among the signaling pathways that promote autophagy induction, the PI3K-I/Akt/mTOR and Beclin-1/PI3K-III/LC3-II signaling pathways are the major transduction pathways that regulate autophagy (44). These pathways negatively and positively regulate autophagy, respectively (45,46). In this study, SN significantly suppresses PI3K-I, Akt and mTOR protein levels in J5 cells, indicating that SN is a critical suppressor of PI3K-I/Akt/mTOR signaling that leads to autophagy. In contrast, when J5 cells were treated with SN, Beclin-1 and PI3K-III expression increased, thus triggering LC-3 II activation and inducing autophagy. Clinical anticancer drugs, such as polyoxomolybdates, platinin, phenethyl isothiocyanate and rapamycin, regulate PI3K, LC-3 or mTOR expression and lead to decreased cancer cell viability (32-34,47). Previous studies demonstrate that caffeine suppresses HeLa cell growth and enhances autophagy by inhibiting the PI3K/Akt/mTOR signaling pathway (48). Wang *et al* also report that quercetin activates Akt-mTOR signaling in human gastric cancer cells, leading to increased LC3-II and Beclin-1 expression and subsequent induction of autophagy (49). Phytochemicals or chemicals that affect autophagy induction by suppressing PI3K-I/Akt/mTOR signaling and/or enhancing Beclin-1/PI3K-III/LC3-II signaling show chemopreventative or chemotherapy potential.

p53 is a tumor suppressor that regulates numerous responses, such as cell cycle arrest, apoptosis, and senescence, each of which may lead to tumor inhibition (50). Recent studies indicate a new role for p53 in autophagy induction. Two p53-associated signaling pathways are involved in autophagy induction and based on p53 cellular localization. p53 promotes autophagy as a transcription factor in the nucleus by activating genes involved in apoptosis, cell cycle arrest and autophagy. However, autophagy is suppressed when cytosolic p53 expression is inhibited (15,51,52). In addition, p53 may induce autophagy by binding to the promoter region of multiple genes that code for pro-autophagic modulators, such as DRAM and TIGAR (53,54). Cytosolic p53 inhibits autophagy by inducing TIGAR, which indirectly affects reactive oxygen species (ROS) through modulation of the glycolytic pathway (53,55). To understand the effect of SN on p53 localization and its downstream regulation of autophagy, our study demonstrated that SN induces J5 cell autophagy by upregulating nuclear p53 and DARM and downregulating cytoplasmic p53 and TIGAR in J5 cells. Furthermore, SN not only regulates cytosolic and nuclear p53 levels but also modifies the expression of mTOR and DRAM in our study. Clearly, p53 plays an important role in SN-induced J5 cell autophagy. Previous studies demonstrate a similar biochemical regulation phenomenon wherein T-47D breast cancer cells treated with sulphathiazole, a common antibacterial drug, enhanced expression of p53/DRAM and downregulated the Akt/mTOR pathway, resulting in autophagy (56). Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone;

PLB), a naturally occurring naphthoquinone isolated from the roots of *Plumbaginaceae* plants, induces autophagy in tongue squamous cell carcinoma (TSCC) through regulation of p53 and PI3K/Akt/mTOR-mediated pathways (57).

In this study, SN was significantly affected by transcript-specific autophagy genes, such as Beclin-1, LC3-II and DRAM, through upregulation of NF- κ B activation, which results in increased Beclin-1, LC3-II and DRAM protein levels. Previous studies have demonstrated that the NF- κ B pathway is activated in response to cellular starvation. When wild-type mouse embryonic fibroblasts are starved in medium, I κ B phosphorylation and degradation is triggered. Then, NF- κ B activation and increased LC3 expression is noted following starvation. These results also demonstrate that NF- κ B-DNA binding activity and target gene expression were significantly increased under starvation conditions (19).

In conclusion, 250 and 500 μ M SN induce autophagy in J5 cells, leading to human liver cancer cell death. The molecular mechanism involves regulation of PI3K, p53 and NF- κ B expression during autophagy induction.

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