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Combination Treatment with Resveratrol and Sulforaphane Induces Apoptosis in Human U251 Glioma Cells

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Abstract

Resveratrol is a naturally occurring polyphenolic compound highly enriched in grapes, peanuts, red wine, and a variety of food sources. Sulforaphane belongs to the family of isothiocyanates and is highly enriched in cruciferous vegetables. Our previous study showed that resveratrol, when used at high concentrations, inhibited cell proliferation, caused the cell cycle arrest and induced apoptotic cell death in glioma cells. In the current study, we tested the effect of combination treatment with resveratrol and sulforaphane, when both were used at low concentrations, on cell proliferation, migration and death in human U251 glioma cells. Our study shows that combination treatment with resveratrol and sulforaphane inhibits cell proliferation and migration, reduces cell viability, induces lactate dehydrogenase release, decreases pro-survival Akt phosphorylation and increases caspase-3 activation. The use of combination of bioactive food components, such as resveratrol and sulforaphane, may be a viable approach for the treatment of glioma.

Keywords

Resveratrol; Sulforaphane; Glioma; Apoptosis; Caspase-3; Akt

Introduction

Glioma is one of the most common malignant brain tumors in adults. It is difficult to treat and often resistant to conventional radiotherapy and chemotherapy [1]. Despite commonly used treatment procedures, such as surgery, radiation and chemotherapy [2], the survival of patients with such tumors has not been improved [3].

Dietary bioactive components of natural products have been extensively studied for cancer prevention and treatment [4]. Resveratrol (trans-3,4-,5-trihydroxystilbene, Res) is one of the polyphenolic compounds highly enriched in grapes, peanuts, red wine and a variety of plant and food sources [1]. Resveratrol has anti-inflammatory and antioxidant properties [5] and is a promising cancer chemopreventive agent that inhibits different stages of the carcinogenesis process, including the initiation, promotion and progression of the tumor [6]. Resveratrol induces apoptotic cell death in various cancer cell lines and experimental tumor models [7]. Most recently, resveratrol has been shown to suppress angiogenesis and intracerebral tumor growth in rats inoculated with RT-2 glioma cells [8]. Sulforaphane (1-isothiocyanate-4-methylsulfinylbutane; SFN) is an isothiocyanate highly enriched in cruciferous vegetables, such as cauliflower, cabbage, broccoli and broccoli sprouts [9]. Sulforaphane is a potent inducer of phase II detoxification enzymes [10] and an inhibitor of histone deacetylase (HDAC) [11] that has anti-tumor effects in vitro and in vivo. Sulforaphane also inhibits cancer initiation, promotion and progression [12]. Most recently, sulforaphane was reported to induce apoptosis in human glioblastoma T98G and U87MG cells [13].

In the current study, we tested the effect of combination treatment with resveratrol and sulforaphane for suppression of cell growth and induction of apoptosis in U251 glioma cells, and investigated the underlying signaling mechanisms. Our study shows that combination treatment with resveratrol and sulforaphane inhibits cell proliferation and migration, and induces apoptosis. Combination treatment changes the expression and/or activation of intracellular signaling proteins, such as proliferating cell nuclear antigen (PCNA), cyclin D1, phospho-Akt, Akt, and caspase-3 that have been implicated in cell proliferation, survival and apoptosis. The use of combination treatment with bioactive food components, such as resveratrol and sulforaphane, may be a valuable preventive and therapeutic approach for brain tumors.

Materials and Methods

Reagents

Resveratrol was obtained from Sigma Aldrich (St. Louis, MO). Sulforaphane and PI3 K inhibitor LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] were obtained from EMD Biosciences (Gibbstown, NJ). Both resveratrol and sulforaphane were prepared in dimethyl sulfoxide (DMSO) at the stock solution of 100 mM and further diluted to appropriate concentration with cell culture medium immediately before use. Control experiments contain the same volume of DMSO only. Antibodies against phospho-Akt (ser473, #9271), Akt (#9272), and cleaved caspase-3 (Asp-175, #9661) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against caspase-3 (E-8, sc-7272), PCNA (PC10, sc-56), cyclin D1 (C-20, sc-717), Bax (B-9, sc-7480), and β -actin (I-19, sc-1616) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture

Human U251 and U87 glioma cells, as well as HEK293 cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml of penicillin and 100 μ g/ml streptomycin

(Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator containing 5% CO₂ and 95% air, as previously described [14]. U251-GFP cells were obtained by transfection with GFP expressing plasmid and stably transfected cells were selected with G418. One of the U251-GFP clones was used for colony formation and in vitro scratch assays only.

Colony Formation Assay

Two ml of 0.5% low melt agarose (Bio-Rad, Hercules, CA) in DMEM supplemented with 10% FBS was poured into each well of 6-well plates and cooled down at 4°C for 1 h. Two ml of 0.35% agarose in DMEM + 10% FBS containing 5000 U251-GFP cells were then poured onto the bottom agarose layer and left at room temperature for 30 min. Finally, two ml of DMEM + 10% FBS containing DMSO, 25 µM of Res, 25 µM of SFN, or the combination of Res and SFN were added on the top of the second agarose layer. Plates were incubated at 37°C in a humidified incubator and medium was changed twice a week with fresh reagents. After 19 days of incubation, colonies were stained with 0.5 ml of 0.005% crystal violet for 2 h at room temperature. The number of colonies was counted in twenty randomly selected fields from each well using a light microscope. The average number of colonies per field was calculated.

In vitro Scratch Assay

U251-GFP cells were seeded in two 4-well chambers until confluence. In vitro scratch assay was performed using a sterile 200 µl pipette tip to scratch several straight lines on the cell monolayer. Immediately after scratching, one 4-well chamber was washed with 1X PBS and fixed in 4% paraformaldehyde for 20 min and used as a baseline control at 0 h. The cells in the other 4-well chamber were treated with DMSO, 25 µM Res, 25 µM SFN or the combination of Res and SFN for 24 h. Cells were fixed and images were captured by a fluorescence microscope. The distance between the gaps was measured and the average distance was calculated.

MTS Assay

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay was performed using a CellTiter 96® Aqueous non-radioactive cell proliferation colorimetric assay kit from Promega (Madison, WI). Cells (1×10^4 cells per well in 100 µl of medium) were plated in a 96-well plate the day before the experiment. After various treatments for 24 h, 20 µl of MTS and phenazine methosulfate (PMS) mixture solution was added to each well and incubated further for 1 h at 37°C. Optical density was measured at a wavelength of 490 nm by a BioTek EL-340 microplate reader. Data were presented as the percentages of control.

Sulforhodamine B (SRB) assay

SRB assay is used to determine the cell density before and after treatment based on the measurement of cellular protein content. Cells (1×10^4 cells per well in 100 µl of growth medium) were plated in a 96-well plate the day before the experiment. After various treatments for 24 h, culture medium were removed and cells were fixed with 12.5% (wt/vol) trichloroacetic acid (TCA) for 1 h. After washing with distilled H₂O to remove TCA, cells were stained with 0.4% (wt/vol) SRB in 1% acetic acid for 30 min. After extensive washing with 1% (vol/vol) acetic acid, the protein-bound dye was dissolved in 10 mM Tris base solution and optical density was determined at 570 nm. The decrease of OD₅₇₀ nm reflects the increase of cell death.

Lactate Dehydrogenase (LDH) Release Assay

A CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI) was used to measure LDH release. Cells (2×10^5 cells per well) were plated in 24-well plates the day before

the experiments. After various treatments for 24 h, medium from each well was collected to measure the amount of released LDH. Cells in separate sister wells were exposed to lysis buffer (9% Triton X100) for 30 min at 37°C and media were collected to measure the total amount of cellular LDH. Optical density was measured at a wavelength of 490 nm and the percentage of released LDH vs. total intracellular LDH was calculated.

BrdU Cell Proliferation Assay

Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) is a synthetic thymidine analog that can be incorporated into newly synthesized DNA of proliferating cells and used as a detection for cell proliferation. After various treatments for 24 h, cell proliferation was performed using a BrdU cell proliferation assay kit from Roche (San Diego, CA). Optical density was measured at a wavelength of 450 nm. The decrease of OD450 nm reflects the decrease of cell proliferation.

Western Blot Analysis

Western blot analysis was performed, as previously described [15]. After treatment, cells were lysed in lysis buffer [20 mM HEPES, pH 7.4, 100 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholic acid, 10% glycerol, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM NaVO₃, 50 mM NaF, and cocktail I of protease inhibitors from Calbiochem] for 30 min on ice and briefly sonicated. Soluble proteins were obtained by centrifugation at 13,000 rpm for 10 min at 4°C and protein concentration was determined by using a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL). Equal amounts of cell lysate were subjected to SDS-polyacrylamide electro-phoresis. Specific proteins were detected using a Super-Signal West Pico chemiluminescence substrate system (Pierce). The band intensity was measured and analyzed using Scion image software (Frederick, MD).

Isolation of Mitochondrial and Cytosolic Fractions

After various treatments, cells were collected and mitochondrial and cytosolic fractions were isolated by using a Mitochondrial/Cytosol Fractionation kit (Biovision). Protein concentration was determined and equal amounts of lysates were used for Western blot analysis of cytochrome C or Bax expression.

Caspase-3 Activity Assay

After various treatments, cells were collected and cell lysates were prepared. Protein concentration was determined and equal amounts of cell lysate from each sample were subjected to caspase-3 activity assay using colorimetric caspase-3 substrate I (Ac-DEVD-pNA) from Calbiochem and 2X caspase assay buffer from Biovision (Mountain View, CA). Samples were measured at 405 nm using the detection of chromophore p-nitroaniline (pNA) after its cleavage by caspase-3 from the labeled caspase-3 specific substrate, DEVD-pNA. The data were presented as pmoles of pNA per µg of cell lysate per hour of incubation.

Statistical Analysis

Data were presented as means ± SD. Differences between different treatment groups were analyzed by using student's *t*-test and a *p*-value < 0.05 was considered statistically significant.

Results

Combination Treatment with Res and SFN Inhibits Colony Formation

To examine the effect of combination treatment with Res and SFN on cell proliferation, colony formation assay was performed. The size of colony after Res, SFN or the combination treatment

was much smaller than control (Fig. 1a). The number of colonies was counted from 20 random fields for each treatment. In control, there was an average of 15 colonies per field, Res and SFN treatment alone reduced the average number of colonies to 10 and 8, respectively, (Fig. 1b). Combination treatment with Res and SFN further decreased the number to 6, which represented a 60% reduction as compared to control. These results suggest that combination treatment with Res and SFN inhibits the cell proliferation.

Combination Treatment with Res and SFN Inhibits Cell Migration

To examine the effect of combination treatment of Res and SFN on cell migration, an in vitro scratch assay was performed. Cell migration was clearly inhibited by Res, SFN or the combination treatment, as examined by either light or fluorescence microscopy (Fig. 2a). After scratching the straight lines on the cell monolayer, the gap distance was 370 μm at 0 h. After 24 h of incubation, the gap distance was reduced to 113 μm under control conditions (Fig. 2b), indicating the cell migration. The gap distance after 24 h of Res and SFN treatment alone was 180 and 305 μm , respectively, indicating the partial inhibition of cell migration. The gap distance after combination treatment with Res and SFN was 338 μm , which was 91% of the control distance at 0 h, indicating that cell migration was almost completely inhibited. These results suggest that Res or SFN treatment alone is able to partially inhibit cell migration and combination treatment with Res and SFN has an additive inhibitory effect on cell migration.

Combination Treatment with Res and SFN Downregulates the Markers of Cell Proliferation and Cell Cycle Progression

Western blot analysis was performed to examine the markers of cell proliferation and cell cycle progression, e.g. proliferating cell nuclear antigen (PCNA) and cyclin D1, respectively. Both PCNA and cyclin D1 expression was further decreased after combination treatment with Res and SFN, as compared to control, Res or SFN alone (Fig. 3a, b). These results suggest that combination treatment with Res and SFN may have an additive effect on cell proliferation and cell cycle progression.

Combination Treatment with Res and SFN Decreases Cell Viability

To examine the effect of combination treatment of Res and SFN on cell viability, various assays were performed. MTS assay showed that Res and SFN treatment alone decreased cell viability to 86 and 71% of the control, respectively. Combination treatment with Res and SFN further decreased the cell viability to 59% (Fig. 4a). To further confirm the results obtained from MTS assay, SRB assay was performed under the same treatment conditions. SRB assay showed that Res and SFN treatment alone decreased cell viability to 82 and 66% of the control, respectively. Combination treatment with Res and SFN further decreased the cell viability to 52% (Fig. 4b). These results are compatible with those from MTS assay. LDH release assay showed that Res and SFN treatment alone induced an increase of LDH release (17 and 40%, respectively), as compared to control (11%). Combination treatment with Res and SFN further increased LDH release to 44% (Fig. 4c), which is also compatible with the results from MTS and SRB assays. BrdU cell proliferation assay showed that combination treatment decreased cell proliferation dose-dependently (Fig. 4d). These results suggest that combination treatment with Res and SFN decreases cell viability.

Combination Treatment with Res and SFN Induces Apoptotic Cell Death

Activation of caspase-3 is a marker of apoptotic cell death. The effect of combination treatment on the active caspase-3 expression was examined by Western blot analysis using four replicated samples. After 24 h treatment with the combination of Res and SFN, active caspase-3 levels were significantly increased by 7.5-fold (Fig. 5a), as compared to Res and SFN treatment alone (3-fold and 2-fold increase, respectively). This result indicates that combination treatment with

Res and SFN induces apoptotic cell death, probably through the activation of caspase-3. To further explore the mechanism of caspase-3 activation, translocation of Bax and cytochrome C in and out of mitochondria was examined. Both Res and SFN treatment alone induced Bax translocation to the mitochondria after 4 h of treatment and induced cytochrome C release after 4 or 24 h of treatment (Fig. 5b). Combination treatment enhanced the cytochrome C release after 4 h of treatment. These results suggest that combination treatment activates caspase-3, leading to apoptotic cell death.

Combination Treatment with Res and SFN Downregulates Pro-survival Signaling Protein Akt

The effect of combination treatment on the activation of pro-survival signaling protein Akt was examined. Both Res and SFN treatment alone reduced the phospho-Akt expression to 47 and 53% of the control levels, respectively, after normalization with the basal Akt expression. Combination treatment further decreases the phospho-Akt expression to 25% of the control levels (Fig. 6a). Res and SFN treatment alone did not change the basal Akt expression. However, combination treatment also slightly decreased the basal Akt expression (Fig. 6a). To further examine the role of Akt in combination treatment-induced caspase-3 activation, cells were pretreated with PI3 K inhibitor LY294002 for 30 min before the combination treatment. Western blot analysis showed that combination treatment decreased both phospho-Akt and basal Akt expression (Fig. 6b). LY294002 itself reduced phospho-Akt expression, but had no effect on basal Akt expression. LY294002 further enhanced the combination treatment-induced downregulation of phospho-Akt, Akt, cyclin D1 and pro-caspase-3 and upregulation of active caspase-3 expression. These results suggest that combination treatment targets both the phosphorylation and expression of Akt, leading to the caspase-3 activation and apoptotic cell death. To further examine if these results are also observed in other glioma cells, U87 glioma cells were treated under the same conditions. Both Res and SFN treatment alone reduced the phospho-Akt expression to 49 and 69% of the control levels, respectively, after normalization with the basal Akt expression. Combination treatment further decreased the phospho-Akt expression to 39% of the control levels (Fig. 6c). Caspase-3 activity was also examined in HEK293 cells under the same treatment conditions. No significant increase of caspase-3 activity was observed after treatment with Res alone, SFN alone or the combination of two reagents (Fig. 6d).

Discussion

Dietary agents, found in fruits, vegetables and spices, have been extensively studied and demonstrated therapeutic potential under conditions that mimic many human diseases [16]. The advantages of using such dietary agents include little or no toxic effects to normal cells, low cost, and they can be consumed orally. Dietary agents have been demonstrated to play important roles in cancer prevention and treatment [4]. It has been estimated that one third of the cancer deaths can be prevented by diet modification [17]. Multiple molecular targets of dietary agents have been identified [18], including the regulation of various cellular processes, such as cell proliferation, cell cycle, cell survival and apoptosis, impacting cancer development and progression [19].

Resveratrol and sulforaphane are two dietary agents that have been extensively studied for their roles in cancer prevention and treatment [20]. In the current study, we examine the effect of combination treatment with resveratrol and sulforaphane in human U251 glioma cells. Our results showed that combination treatment with resveratrol and sulforaphane inhibits the cell proliferation and migration, induces apoptotic cell death through the suppression of pro-survival Akt and induction of pro-apoptotic caspase-3 signaling pathways.

Resveratrol is a promising cancer chemopreventive agent [21] and induces apoptotic cell death in a number of cancer cell lines, including prostate, breast, colon, leukemia [22-24]. Loss of

mitochondrial function, altered expression of the Bcl-2 family members, and activation of caspases are involved in resveratrol-induced cell death in Bcl-2 overexpressing U937 cells [25], pancreatic carcinoma cells [26], mouse myeloid leukemia cells [27], and human Caco-2 colonic adenocarcinoma cells [28]. Resveratrol has been shown recently to suppress angiogenesis and glioma growth in rats [8] and to induce apoptosis in rat C6 glioma cells [29].

Sulforaphane has cancer chemopreventive and therapeutic potential through the inhibition of angiogenesis and endothelial function [30]. Extensive studies have demonstrated that sulforaphane has preventive and therapeutic effects in various cancer types both in vitro and in vivo [12]. Sulforaphane inhibits cancer initiation through the reduction of carcinogen formation and its metabolic activation, and induction of its detoxification capacity [31]. Sulforaphane inhibits cell proliferation and induces in G0/G1 cell cycle arrest in human colon carcinoma HT-29 cells and is associated with the increase of p21CIP1 and decrease of cyclin D1 expression [1]. Sulforaphane increases the production of reactive oxygen species, activation of caspase-3 and apoptosis in human leukemia U937 [32], cervical carcinoma HeLa, hepatocarcinoma HepG2 [33], and breast cancer cells [34]. Pro-apoptotic members of Bcl-2 family, Bax and Bak, are involved in sulforaphane-induced apoptosis [35]. Sulforaphane induces apoptosis in human glioblastoma T98G and U87MG cells through the activation of calcium-dependent calpain and caspase-3 activity [13].

Combination of two bioactive agents for cancer prevention and therapy has been used in numerous in vitro and in vivo models [20]. Each agent may have own unique targets but also share some common targets. Therefore, combination treatment may provide an advantage than the treatment with single agent only, such as lowering achievable in vivo concentration and improving preventive and therapeutic efficacy. Although the effects of resveratrol and sulforaphane have been extensively studied in various cancer cell lines and multiple targets have been identified, the effects of combination treatment using these two compounds have not been investigated.

In our current study, we elected to use 25 μ M of resveratrol and 25 μ M of sulforaphane for the combination treatment, based on our dose-finding study (data not shown) as well as reported studies by others [20,36]. For resveratrol, most of the in vitro studies use the concentrations of 1–100 μ M. However, the concentrations that show the detectable effect on cell viability are between 25–100 μ M [20]. It has been reported that sulforaphane gradually decreased viability in HepG2-C8 cells at the concentrations of 20–35 μ M, but viability is dramatically decreased at concentrations of 50–100 μ M [36], suggesting that sulforaphane concentration of 25 μ M is suboptimal. Therefore, the concentrations of resveratrol and sulforaphane used in the current study are suitable to demonstrate the additive effect of these two compounds.

Combination treatment with resveratrol and sulforaphane, when each is used at concentrations of 25 μ M, inhibits cell proliferation and migration and causes changes in the expression and activation of intracellular signaling proteins related with cell proliferation, cell cycle and apoptosis. However, sulforaphane seems to be more effective than resveratrol at the same concentration. This differential effect is likely due to the different signaling proteins that resveratrol and sulforaphane target. It requires further investigation to identify the common and unique target signaling proteins that are regulated by resveratrol and sulforaphane.

Akt signaling pathway has been implicated in various cellular processes including cell growth, differentiation, survival and apoptosis. Both resveratrol and sulforaphane have been reported separately to modulate Akt signaling pathway in various types of cancer cells. However, the effect of combination treatment with these compounds on Akt phosphorylation and expression in glioma cells has not been reported. Resveratrol has been reported to induce apoptosis in

human uterine and prostate cancer cells and decreases pro-survival Akt phosphorylation [37, 38]. Sulforaphane downregulates both total Akt and phospho-Akt expression in ovarian cancer cells [39]. Subtoxic dose of sulforaphane sensitizes the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-resistant A549 lung adenocarcinoma cells to TRAIL-induced apoptosis through the downregulation of Akt [40]. Our study shows that combination treatment with resveratrol and sulforaphane downregulates both phospho-Akt and basal Akt expression, suggesting that Akt signaling protein is an important target for the combination treatment-induced apoptotic cell death. This observation is further confirmed by using the PI3 K inhibitor LY294002 combined with resveratrol and sulforaphane, showing the additive effects on reduction of Akt phosphorylation and expression and induction of caspase-3 activation, as compared to the treatment with individual compound. Our results show that both resveratrol and sulforaphane induce Bax translocation, cytochrome C release and caspase-3 activation and the combination treatment enhances such an induction.

In summary, the current study suggests that combination treatment with resveratrol and sulforaphane is more effective than the treatment with a single compound, likely due to additive effects on their common and/or unique targets. In addition, each compound can be used at lower concentration than that is required to have a notable effect and such concentrations are more likely to be achievable in vivo. Further study of these compounds in vivo is necessary to understand the combination effect and therapeutic potential of resveratrol and sulforaphane.

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References

1. Koul D, Shen R, Bergh S, et al. Inhibition of Akt survival pathway by a small-molecule inhibitor in human glioblastoma. *Mol Cancer Ther* 2006;5:637–644. [PubMed: 16546978]
2. Boiardi A, Silvani A, Milanesi I, et al. Primary glial tumor patients treated by combining cis-platin and etoposide. *J Neurooncol* 1991;11:165–170. [PubMed: 1744684]
3. Kondo Y, Hollingsworth EF, Kondo S. Molecular targeting for malignant gliomas (Review). *Int J Oncol* 2004;24:1101–1109. [PubMed: 15067331]
4. Lambert JD, Hong J, Yang GY, et al. Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. *Am J Clin Nutr* 2005;81:284S–291S. [PubMed: 15640492]
5. Harikumar KB, Aggarwal BB. Resveratrol: a multitargeted agent for age-associated chronic diseases. *Cell Cycle* 2008;7:1020–1035. [PubMed: 18414053]
6. Delmas D, Lancon A, Colin D, et al. Resveratrol as a chemopreventive agent: a promising molecule for fighting cancer. *Curr Drug Targets* 2006;7:423–442. [PubMed: 16611030]
7. Kundu JK, Surh YJ. Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. *Cancer Lett* 2008;269:243–261. [PubMed: 18550275]
8. Tseng SH, Lin SM, Chen JC, et al. Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. *Clin Cancer Res* 2004;10:2190–2202. [PubMed: 15041740]
9. Zhang Y, Talalay P, Cho CG, et al. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci USA* 1992;89:2399–2403. [PubMed: 1549603]
10. Matusheski NV, Jeffery EH. Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. *J Agric Food Chem* 2001;49:5743–5749. [PubMed: 11743757]
11. Myzak MC, Ho E, Dashwood RH. Dietary agents as histone deacetylase inhibitors. *Mol Carcinog* 2006;45:443–446. [PubMed: 16652377]
12. Fimognari C, Hrelia P. Sulforaphane as a promising molecule for fighting cancer. *Mutat Res* 2007;635:90–104. [PubMed: 17134937]

13. Karmakar S, Banik NL, Patel SJ, et al. Curcumin activated both receptor-mediated and mitochondria-mediated proteolytic pathways for apoptosis in human glioblastoma T98G cells. *Neurosci Lett* 2006;407:53–58. [PubMed: 16949208]
14. Jiang H, Zhang L, Kuo J, et al. Resveratrol-induced apoptotic death in human U251 glioma cells. *Mol Cancer Ther* 2005;4:554–561. [PubMed: 15827328]
15. Jiang H, Movsesyan V, Fink DW Jr, et al. Expression of human p140trk receptors in p140trk-deficient, PC12/endothelial cells results in nerve growth factor-induced signal transduction and DNA synthesis. *J Cell Biochem* 1997;66:229–244. [PubMed: 9213224]
16. Everitt AV, Hilmer SN, Brand-Miller JC, et al. Dietary approaches that delay age-related diseases. *Clin Interv Aging* 2006;1:11–31. [PubMed: 18047254]
17. Willett WC. Diet, nutrition, and avoidable cancer. *Environ Health Perspect* 1995;103(Suppl 8):165–170. [PubMed: 8741778]
18. Aggarwal BB, Shishodia S. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochem Pharmacol* 2006;71:1397–1421. [PubMed: 16563357]
19. Shimizu M, Shirakami Y, Sakai H, et al. EGCG inhibits activation of the insulin-like growth factor (IGF)/IGF-1 receptor axis in human hepatocellular carcinoma cells. *Cancer Lett* 2007;262:10–18.
20. Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 2000;5:415–418. [PubMed: 11256882]
21. Aziz MH, Kumar R, Ahmad N. Cancer chemoprevention by resveratrol: in vitro and in vivo studies and the underlying mechanisms (review). *Int J Oncol* 2003;23:17–28. [PubMed: 12792772]
22. Morris GZ, Williams RL, Elliott MS, et al. Resveratrol induces apoptosis in LNCaP cells and requires hydroxyl groups to decrease viability in LNCaP and DU 145 cells. *Prostate* 2002;52:319–329. [PubMed: 12210493]
23. Lin HY, Shih A, Davis FB, et al. Resveratrol induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line. *J Urol* 2002;168:748–755. [PubMed: 12131363]
24. Joe AK, Liu H, Suzui M, et al. Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clin Cancer Res* 2002;8:893–903. [PubMed: 11895924]
25. Park JW, Choi YJ, Suh SI, et al. Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity. *Carcinogenesis* 2001;22:1633–1639. [PubMed: 11577002]
26. Mouria M, Gukovskaya AS, Jung Y, et al. Food-derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome C release and apoptosis. *Int J Cancer* 2002;98:761–769. [PubMed: 11920648]
27. Gao X, Xu YX, Divine G, et al. Disparate in vitro and in vivo antileukemic effects of resveratrol, a natural polyphenolic compound found in grapes. *J Nutr* 2002;132:2076–2081. [PubMed: 12097696]
28. Wolter F, Akoglu B, Clausnitzer A, et al. Downregulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. *J Nutr* 2001;131:2197–2203. [PubMed: 11481417]
29. Zhang W, Fei Z, Zhen HN, et al. Resveratrol inhibits cell growth and induces apoptosis of rat C6 glioma cells. *J Neurooncol* 2006;81:231–240. [PubMed: 17031560]
30. Bertl E, Bartsch H, Gerhauser C. Inhibition of angiogenesis and endothelial cell functions are novel sulforaphane-mediated mechanisms in chemoprevention. *Mol Cancer Ther* 2006;5:575–585. [PubMed: 16546971]
31. Weisburger JH. Carcinogenicity and mutagenicity testing, then and now. *Mutat Res* 1999;437:105–112. [PubMed: 10556615]
32. Choi WY, Choi BT, Lee WH, Choi YH. Sulforaphane generates reactive oxygen species leading to mitochondrial perturbation for apoptosis in human leukemia U937 cells. *Biomed Pharmacother*. 2008
33. Park SY, Kim GY, Bae SJ, et al. Induction of apoptosis by isothiocyanate sulforaphane in human cervical carcinoma HeLa and hepatocarcinoma HepG2 cells through activation of caspase-3. *Oncol Rep* 2007;18:181–187. [PubMed: 17549366]
34. Pledgie-Tracy A, Sobolewski MD, Davidson NE. Sulforaphane induces cell type-specific apoptosis in human breast cancer cell lines. *Mol Cancer Ther* 2007;6:1013–1021. [PubMed: 17339367]

35. Choi S, Singh SV. Bax and Bak are required for apoptosis induction by sulforaphane, a cruciferous vegetable-derived cancer chemopreventive agent. *Cancer Res* 2005;65:2035–2043. [PubMed: 15753404]
36. Kim BR, Hu R, Keum YS, et al. Effects of glutathione on antioxidant response element-mediated gene expression and apoptosis elicited by sulforaphane. *Cancer Res* 2003;63:7520–7525. [PubMed: 14612554]
37. Sexton E, Van Themsche C, LeBlanc K, et al. Resveratrol interferes with AKT activity and triggers apoptosis in human uterine cancer cells. *Mol Cancer* 2006;5:45. [PubMed: 17044934]
38. Aziz MH, Nihal M, Fu VX, et al. Resveratrol-caused apoptosis of human prostate carcinoma LNCaP cells is mediated via modulation of phosphatidylinositol 3'-kinase/Akt pathway and Bcl-2 family proteins. *Mol Cancer Ther* 2006;5:1335–1341. [PubMed: 16731767]
39. Chaudhuri D, Orsulic S, Ashok BT. Antiproliferative activity of sulforaphane in Akt-overexpressing ovarian cancer cells. *Mol Cancer Ther* 2007;6:334–345. [PubMed: 17237292]
40. Jin CY, Moon DO, Lee JD, et al. Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis through downregulation of ERK and Akt in lung adenocarcinoma A549 cells. *Carcinogenesis* 2007;28:1058–1066. [PubMed: 17183064]

Fig. 1.

Combination treatment with Res and SFN decreases the number of colonies formed in soft agar. U251-GFP cells were plated in 6-well plates with soft agar in the absence or presence of 25 μ M Res, 25 μ M SFN or the combination of Res and SFN for 2 weeks. Cells were then stained with 0.005% crystal violet for 2 h. **a** A representative image of colonies under different treatment conditions. **b** The number of colonies was counted from 20 random fields under the light microscope. ***: $P < 0.001$ vs. Con; **Δ: $P < 0.01$ vs. SFN, $n = 20$

Fig. 2. Combination treatment with Res and SFN prevents cell migration. U251-GFP cells were subjected to in vitro scratch assay. **a**–**e**: images captured by phase contrast microscope. **f**–**j**: images captured by fluorescent microscope. **b** The gap distance was measured and shown by the bar graph. **: $P < 0.01$, ***: $P < 0.001$, vs. Con; ***Δ: $P < 0.001$ vs. Res; $n = 4$

Fig. 3.

Combination treatment with Res and SFN reduces the expression of markers for cell proliferation and cell cycle progression. U251 cells were treated with 25 μ M of Res, 25 μ M of SFN or the combination of Res and SFN for 24 h. Western blot analysis of PCNA (**a**) and cyclin D1 (**b**) was performed. Changes of expression were shown by densitometric analysis.

*, $P < 0.05$, ***: $P < 0.001$, vs. Con; *Δ: $P < 0.5$ vs. Res; $n = 3$

Fig. 4. Combination treatment with Res and SFN induces cell death. U251 cells were treated with 25 μ M of Res, 25 μ M of SFN or the combination of Res and SFN for 24 h. **a** MTS assay, ***: $P < 0.001$ vs. Con; *** Δ : $P < 0.001$ vs. Res; $n = 6$; **b** SRB assay, $P < 0.001$ vs. Con; *** Δ : $P < 0.001$ vs. Res; $n = 5$; **c** LDH release, ***: $P < 0.001$ vs. Con; *** Δ : $P < 0.001$ vs. Res; $n = 6$; **d** U251 cells were treated with combination of Res and SFN at 0, 25, 50 and 100 μ M each for 24 h and BrdU cell proliferation assay was performed, ***: $P < 0.001$ vs. Con, $n = 5$

Fig. 5.

Combination treatment with Res and SFN induces apoptotic cell death. **a** U251 cells were treated with 25 μ M of Res, 25 μ M of SFN or the combination of Res and SFN for 24 h. A representative Western blot analysis of active caspase-3 and β -actin expression was shown. Changes of expression were shown by densitometric analysis. ***: $P < 0.001$, vs. Con; *** Δ : $P < 0.5$ vs. Res; $n = 4$. **b** U251 cells were treated with 25 μ M of Res, 25 μ M of SFN or the combination of Res and SFN for 4 or 24 h. Cytosolic and mitochondrial fractions were isolated using a Mitochondria/Cytosol Fractionation kit (Biovision). Western blot analysis was performed using antibodies against Bax or cytochrome C

Fig. 6.

Combination treatment with Res and SFN reduces the phosphorylation of Akt. **a** U251 cells were treated with 25 μ M of Res, 25 μ M of SFN or the combination of Res and SFN for 24 h. A representative Western blot analysis of phospho-Akt and Akt expression was shown. Densitometric analysis of four Western blots was presented. ***: $P < 0.001$ vs. Con; **Δ: $P < 0.01$ vs. Res; $n = 4$. **b** U251 cells were pretreated with PI3 K inhibitor LY 294002 for 30 min and then with 25 μ M of Res, 25 μ M of SFN or the combination of Res and SFN for 24 h. Western blot analysis was performed using antibodies against phospho-Akt (ser473) and Akt (Cell Signaling). **c** U87 cells were treated with 25 μ M of Res, 25 μ M of SFN or the combination of Res and SFN for 24 h. A representative Western blot analysis of phospho-Akt and Akt expression was shown. Densitometric analysis of four Western blots was presented. **: $P < 0.01$; ***: $P < 0.001$ vs. Con; *Δ: $P < 0.05$ vs. SFN; $n = 3$. **d** HEK293 cells were treated with 25 μ M of Res, 25 μ M of SFN or the combination of Res and SFN for 24 h. Caspase-3 activity assay was performed, $n = 3$