Sulforaphane-cysteine induces apoptosis by sustained activation of ERK1/2 and caspase 3 in human glioblastoma U373MG and U87MG cells

SAI WU1, YAN ZHOU1, GAOXIANG YANG1, HUA TIAN1, YANG GENG1, YABIN HU1, KAI LIN1 and WEI WU1,2

1Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, 2Institute of Brain Tumor, Beijing Institute for Brain Disorders, Capital Medical University, Beijing, P.R. China

Received September 12, 2016; Accepted February 22, 2017

DOI: 10.3892/or.2017.5562

Abstract. We previously demonstrated that sulforaphane (SFN) inhibited invasion via sustained activation of ERK1/2 in human glioblastoma cells. However, sulforaphane-cysteine (SFN-Cys), an analog of SFN, enriched in plasma with longer half-life, had more potentiality to induce apoptosis. Here we investigated the molecular mechanisms of SFN-Cys-induced apoptosis in human glioblastoma U373MG and U87MG cells. Cell viability assay showed that SFN-Cys inhibited cell viability in a dose-dependent manner. Cell morphology observation also showed SFN-Cys increased the phenotype of cell death in a dose-dependent manner. Furthermore, flow cytometry assay showed that SFN-Cys induced apoptosis significantly in a dose-dependent manner in both cell lines. Furthermore, western blot analysis demonstrated that SFN-Cys induced activation of ERK1/2 in a sustained manner and the activation contributed to upregulation of Bax/Bcl-2 ratio and cleaved caspase 3, and these results can be reversed by the ERK1/2 blocker PD98059. Our results showed that SFN-Cys might be a more promising therapeutic agent versus SFN to resist glioblastoma cells, especially in Taxol-resistant cancer cells.

Introduction

Brain glioblastoma is a commonly occurred tumor with aggressive features. The therapeutic way to treat this disease is to use a surgical resection followed by a combination of radiotherapy and/or temozolomide (TMZ) chemotherapy (1). However, glioblastoma cells often generate resistance to TMZ treatment and the average survival time for patients after general treatment is ~15 months (2,3). Therefore, it is essential to find an effective agent to treat glioblastoma, especially drug-resistant glioblastoma.

Numerous studies have suggested that sulforaphane (SFN) obtained from cruciferous vegetables induced apoptosis in a variety of tumors (4-7). In vivo, SFN is metabolized to produce sulforaphane-glutathione (SFN-GSH), sulforaphane-cysteine-glycine (SFN-CG), sulforaphane-cysteine (SFN-Cys), and sulforaphane-N-acetylcysteine (SFN-NAC) via mercapturic acid pathway (5,8,9). Further studies showed that SFN induced cell apoptosis, inhibited cell proliferation, invasion and angiogenesis (10-14). In human glioblastoma cells, it has been reported that SFN induced apoptosis (15) and our previous study showed that SFN-Cys induced cell apoptosis, inhibited cell proliferation, invasion and angiogenesis (10-14). In human glioblastoma cells, it has been reported that SFN induced apoptosis (15) and our previous study showed that SFN inhibited cell invasion via ERK1/2 signaling pathway (12). Compared with SFN, SFN-Cys inhibits histone deacetylase (HDAC) more efficiently and has longer half-life and retention time in vivo (8,16). HDAC is highly related to cell growth. Hence we assume that SFN-Cys inhibits cell growth and induces apoptosis with higher efficiency, it is important to investigate the underlying mechanisms. These results will provide new insight into the SFN analog anticancer effect, so that we might develop new anticancer agents.

The extracellular signal-regulated kinases (ERK1/2) regulate many cellular responses by phosphorylating a number of downstream effectors (12,13,17-20). Transient phosphorylation of ERK1/2 (5-15-min stimulation) contributes to cell growth (18) while sustained phosphorylation of ERK1/2 (>15 min stimulation) causes cell apoptosis (19). Our previous studies demonstrated that SFN inhibited invasion by sustained activation of ERK1/2 to regulate E-cadherin and CD44v6 in human prostate cancer DU145 cells (13) and SFN-Cys suppressed invasion by sustained phosphorylation of ERK1/2 and downregulating galectin-1 in human prostate cancer DU145 and PC3 cells (20). Moreover, SFN inhibited invasion via activating ERK1/2 signaling in human glioblastoma U87MG and U373MG cells. Therefore, we hypothesized that...
SFN-Cys might induce apoptosis by activating ERK1/2 and the apoptosis-related proteins. Both Bax and Bcl-2 are members of the Bcl-2 family regulating the apoptosis of glioblastoma cells (21). Bax contributes to the apoptotic response of glioblastoma cells and the overexpression of Bax increases survival of glioblastoma patients (22). Downregulation of Bcl-2 effectively induced apoptosis in glioblastoma cells (23), while overexpression of Bcl-2 inhibited apoptosis and decreased the effect of radiotherapy or chemotherapy in many other cancers (24). The apoptosis-inducing ability of Bax is suppressed by Bcl-2 binding to its homologous C-terminal domain (25-27). Thus, the increasing Bax/Bcl-2 ratio results in apoptosis in human glioblastoma cells while the decreasing Bax/Bcl-2 ratio represses cell apoptosis and contributes to the resistance of glioblastoma cells to chemotherapeutic agent (28). It is reported that the upregulation of Bax/Bcl-2 resulted in the loss of mitochondrial membrane potential (MMP), inducing cell apoptosis via the intrinsic pathway in glioma cells (29). Since the ratio of Bax/Bcl-2 is regulated by ERK1/2 signaling pathway (30), we hypothesized that SFN-Cys might induce intrinsic apoptosis by upregulating Bax/Bcl-2 ratio mediated via the activated ERK1/2.

Cysteine-aspartic proteases (caspases) constitute a family of proteolytic enzymes and are largely known for their functions in apoptosis (31). The caspases are divided into two groups, the initiator caspases such as caspase 8 and 9 and the effector caspases such as caspase 3 (32,33). Caspase 3 exists as an inactive proenzyme while it is activated after cleaved by initiator caspases (34). Cleaved caspase 3 in turn cleaves multiple cell substrates such as poly(ADP-ribose) polymerase (PARP), leading to cell apoptosis (35). It is reported that SFN activated caspase 3 via ERK1/2 pathway, leading to cell apoptosis in human glioblastoma cells (36). Additionally, caspase 3 is a downstream protein of Bax/Bcl-2, and the upregulation of Bax/Bcl-2 ratio leads to the activation of caspase 3 (28). Taken together, we thought that SFN-Cys might activate ERK1/2, increasing the ratio of Bax/Bcl-2 and upregulating cleaved caspase 3, which led to cell apoptosis in U373MG and U87MG cells. Altogether, our results provide evidence for SFN-Cys inducing apoptosis in glioblastoma, and further explore the underlying mechanisms, facilitating finding more natural products for treating glioblastoma.

Materials and methods

Reagents. D, L-Sulforaphane-L-cysteine (SFN-Cys) and caspase 3 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DMEM/HIGH glucose culture medium was from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Proteineqtech Group, Inc. (Chicago, IL, USA). CCK8 assay kit was from Dojindo Laboratories (Shanghai, China). Annexin V-FITC Apoptosis assay kit was from Genstar (Beijing, China). Mitochondrial membrane potential assay kit with JC-1 was from Beyotime Biotechnology (Shanghai, China). pERK1/2 antibody, ERK1/2 antibody and PD98059 were acquired from Cell Signaling Technology, Inc. (Shanghai, China). The antibodies against Bax and Bcl-2 were from Solarbio (Beijing, China).
for 1 h. After washing, the membrane was scanned by Odyssey Infrared Imaging System (LI-COR Bioscience). The same membrane was stripped and incubated with β-actin antibody for equal loading and normalization.

**Mitochondrial membrane potential assay.** Mitochondrial membrane potential (MMP) was examined by using the mitochondrial membrane potential kit with JC-1. The cells were plated in 24-well plate and treated with drugs for 24 h. Then, cells were washed with PBS three times and incubated with 250 µl culture medium and 250 µl JC-1 working solution for 20 min in the dark at 37˚C. After that, the cells were washed three times (3 min each time) with ice cold JC-1 washing buffer. Images were captured by a fluorescence microscope at x200 magnification (Axio Imager A2, Zeiss, Jena, Germany). Fluorescent intensity was analyzed by Image-Pro Plus and the level of MMP was calculated as the JC-1 aggregate/monomer ratio.

**Statistical analysis.** Data are expressed as mean ± SD and the differences among the groups were analyzed by one-way ANOVA. P<0.05 was considered to be statistically significant.

**Results**

**SFN-Cys dose-dependently decreases cell viability and changed cell morphology.** CCK-8 assay was utilized to examine the reduction of cell viability after cells were incubated with SFN-Cys in U373MG and U87MG cells. The cells were exposed to 0, 10, 20, 30, 40, 50, 60, 70 and 80 µM SFN-Cys for 24 h. SFN-Cys was found to decrease cell viability in a dose-dependent manner (Fig. 1). Moreover, we found that SFN-Cys decreased cell viability remarkably when cells were exposed to 30 µM SFN-Cys for 24 h (Fig. 1A). Cells treated with SFN-Cys of 0, 15, 30 and 45 µM for 24 h were observed by the light microscope. The cells treated by SFN-Cys were shrunken and exhibited rounded shape compared with the untreated cells. Microscopic images in Fig. 1B showed that the cell morphology exposed to 30 µM SFN-Cys for 24 h began to change.

**SFN-Cys dose-dependently induces cell apoptosis.** Flow cytometry was utilized to prove whether the decrease of cell viability and the changes of morphology (Fig. 1) were caused by cell apoptosis. The cells were treated with 0, 15, 30 and 45 µM SFN-Cys for 24 h before flow cytometry analysis. The cell apoptosis rates are displayed in Fig. 2. We found that SFN-Cys induced cell apoptosis in a dose-dependent manner in both U373MG and U87MG cells. In addition, compared with the untreated control, a significant increase of apoptosis rate was shown in 30 µM SFN-Cys treated cells. Moreover, apoptosis rate in U87MG cells was higher than that in U373MG cells, suggesting the distinct sensitivity to SFN-Cys between U87MG and U373MG cells. Taken together, we chose 30 µM SFN-Cys as an optimal concentration for the following studies.

![Figure 1](image-url)
Figure 2. SFN-Cys dose-dependently induces cell apoptosis in U373MG and U87MG cells. U373MG and U87MG cells were treated with 0, 15, 30 and 45 µM SFN-Cys for 24 h. SFN-Cys induced an increase in both early apoptosis (Annexin V-positive, PI-negative) and late apoptosis (Annexin V/PI-double positive). Data are shown as means ± SD from three separate experiments. *P<0.05 vs. control group, n=3.

Figure 3. SFN-Cys dose-dependently induces ERK1/2 phosphorylation. U373MG and U87MG cells were treated with (A) varying concentrations of SFN-Cys (0, 15, 30 and 45 µM) for 24 h (B) 0 and 30 µM SFN-Cys respectively, PD98059 alone and SFN-Cys with PD98059 combination respectively for 24 h. Data are shown as means ± SD from three separate experiments. *P<0.05 vs. control group. #P<0.05 vs. SFN-Cys only group, n=3.
SFN-Cys dose-dependently activates ERK1/2. U373MG and U87MG cells were treated with increasing doses of SFN-Cys (0, 15, 30 and 45 μM) for 24 h. Western blot analysis showed that SFN-Cys activated ERK1/2 (Thr202/Tyr204) in a dose-dependent way and pERK1/2 was significantly increased at 30 μM of SFN-Cys (Fig. 3A), which was in agreement with our previous studies that SFN and SFN-Cys contributed to the phosphorylation of ERK1/2 in prostate cancer cells (13,20). PD98059, ERK1/2 inhibitor, was utilized to investigate the role of ERK1/2 in SFN-Cys-induced apoptosis. Fig. 3B shows that the phosphorylation of ERK1/2 was significantly diminished in cells treated with both SFN-Cys (30 μM) and PD98059 (25 μM) compared with the cells treated with SFN-Cys alone.

SFN-Cys increases the ratio of Bax/Bcl-2 and upregulates cleaved caspase 3 by activating ERK1/2. Western blot analysis was utilized to evaluate the expression of the apoptosis regulatory proteins. We have already demonstrated that PD98059 blocked the ERK1/2 signaling pathway activated by SFN-Cys (Fig. 3B). As shown in Fig. 4, the ratio of Bax/Bcl-2 was increased in SFN-Cys-treated cells while the increased ratio was reversed by PD98059 in SFN-Cys with the PD98059 treated cells, indicating that Bax and Bcl-2 were the downstream effectors of ERK1/2. After cells were treated with SFN-Cys for 24 h, the expression of cleaved caspase 3 was significantly increased compared with the untreated cells. PD98059 reversed the expression of cleaved caspase 3 in both PD98059 and SFN-Cys treated cells compared with SFN-Cys only treated cells, suggesting that SFN-Cys induced cell apoptosis through sustained activation of ERK1/2 and subsequently activating caspase 3 in U373MG and U87MG cells. Altogether, these results indicated that SFN-Cys activated ERK1/2, increasing the ratio of Bax/Bcl-2 and upregulating cleaved caspase 3 in U373MG and U87MG cells.

SFN-Cys induces the loss of mitochondrial membrane potential (MMP) by activating ERK1/2. In order to confirm the intrinsic apoptosis pathway which was induced by SFN-Cys, JC-1 mitochondrial membrane potential assay was implemented to analyze mitochondrial membrane potential. Red fluorescence indicated JC-1 aggregated in cells with high MMP, while green fluorescence indicated that JC-1 was presented as a monomer in cells with depolarized mitochondria. In Fig. 5A, the green fluorescence was increased and the red fluorescence was decreased in SFN-Cys only treated cells compared with that in untreated cells. Compared with SFN-Cys only treated cells,
both PD98059 and SFN-Cys treated cells exhibited decreased green fluorescence and the restoration of the red fluorescence. As shown in Fig. 5B, the level of MMP in SFN-Cys only treated cells was significantly decreased compared with the untreated cells. PD98059 reversed the level of MMP in both SFN-Cys and PD98059 treated cells compared with SFN-Cys only treated cells, thus indicating that SFN-Cys induced the loss of MMP which could be reversed by PD98059. Taken together, SFN-Cys induced the loss of MMP by activating ERK1/2.
Discussion

Dietary bioactive components of natural products have been extensively studied for cancer prevention and treatment with low toxicity (37). Of all, sulforaphane (SFN) was demonstrated to be the safest for human cancer therapy (38). Here we demonstrated that sulforaphane-cysteine (SFN-Cys), as a metabolite of SFN in vivo, has potentiality to induce apoptosis. By treating mice with 20 µmoles SFN, SFN was undetectable in brain by 2 h, while SFN-Cys was abundantly detectable in brain by 6 h, indicating that SFN-Cys could penetrate the blood-brain barrier (BBB) to target the tumor with a longer half-life compared with SFN in vivo (8).

The specific hydroxamic acid group of SFN-Cys has greater affinity for Zn\(^{2+}\) of histone deacetylase (HDAC) while SFN has little effect, suggesting that SFN-Cys has the most potentiality to inhibit HDAC (16). Our previous results showed that SFN-Cys inhibited invasion in human prostate cancer cells (20), herein the underlying mechanisms by which SFN-Cys induced cell apoptosis in human glioblastoma cells is demonstrated.

Both SFN-Cys and SFN could significantly decrease cell viability and induce apoptosis at 30 µM as an optimal concentration in U373MG cells and U87MG cells. The IC\(_{50}\) of SFN-Cys was approximately 45 µM (Fig. 1A) while the IC\(_{50}\) of SFN was ~60 µM (12), suggesting that SFN-Cys was a more effective anticarcinogen than SFN. In previous studies, we demonstrated that ERK1/2 was sustainedly activated by SFN-Cys (20 µM) within 48 h in U373MG and U87MG cell lines (data not shown). Sustained activation of ERK1/2 mediated by SFN-Cys is a key trigger to induce apoptosis. The downstream effectors might be involved in apoptosis-related signaling pathways, such as the death receptor pathway and the mitochondria-related pathways. We further demonstrated that SFN-Cys (30 µM) induced cell apoptosis by upregulating Bax/Bcl-2 ratio and causing the loss of mitochondrial membrane potential (MMP) through activating ERK1/2. Apoptosis might also involve multiple transcription factors, including early growth response 1 (EGR-1) and nuclear factor-κB (NF-κB) (39-41). EGR-1 is known to be activated by ERK1/2 and it can bind to the Bax gene promoter to induce the expression of Bax (42,43). Given that SFN activates EGR-1 in glioblastoma (44), SFN-Cys might upregulate Bax by activating EGR-1 mediated by the ERK1/2 signaling pathway. NF-κB can be downregulated by ERK1/2 and the activation of NF-κB contributes to the upregulation of Bcl-2 (45,46). Besides, it was shown that SFN suppressed the activation of NF-κB to induce cell apoptosis, suggesting that SFN-Cys may downregulate Bcl-2 by suppressing the activation of NF-κB mediated by phosphorylating ERK1/2. We also demonstrated that SFN-Cys activated caspase 3 via the ERK1/2 signaling pathway, however, whether SFN-Cys activates caspase 3 by upregulating Bax/Bcl-2 ratio mediated by ERK1/2 is still unclear. The possible cause of the activation of caspase 3 is the release of cytochrome c from the mitochondria regulated by Bax/Bcl-2 (22,47). Bax, Bcl-2, caspase 3 and the disruption of MMP are the hallmarks of intrinsic apoptosis pathway, suggesting that SFN-Cys induced cell apoptosis in human glioblastoma cells via the intrinsic apoptosis pathway. Therefore, the underlying mechanism of caspase 3 activation is likely to be that the SFN-Cys-mediated

![Figure 6. The proposed signaling map for SFN-Cys-induced apoptosis in human glioblastoma cells.](image-url)
increase of Bax/Bcl-2 ratio acts as an apoptotic stimulus resulting in MMP disruption, triggering the release of cytochrome c into the cytosol which then activates caspase 9 and activates caspase 3 subsequently via the ERK1/2 signaling pathway. Alternatively, in addition to cytochrome c, second mitochondria-derived activator of caspases (Smac) can be released into the cytosol as well and bind to the inhibitor of apoptosis (IAP) proteins to deactivate them, resulting in caspase 3 activation and cell death eventually (48). It can be inferred that SFN-Cys may also induce cell apoptosis by triggering the release of Smac and subsequently deactivating the IAP proteins or just regulating the IAP proteins to deactivate them directly. Apart from the intrinsic apoptosis pathway, apoptosis is also sub-classified into two other types of death pathways: the death receptor-mediated (extrinsic) pathway and the endoplasmic-reticulum (ER) stress-mediated apoptosis pathway. The extrinsic pathway involves death receptors, from the tumor necrosis factor (TNF) superfamily, to transmit death signal from the surface to the intracellular signaling pathways, leading to the activation of caspase 8 and the subsequent activation of caspase 3 (49-51). Also, the ER stress apoptosis pathway is triggered by the disturb folding proteins in the ER, leading to the activation of caspase 3 eventually (52). Since these two pathways could both be activated through the ERK1/2 signaling pathway (52,53) and lead to the activation of caspase 3, we thought that SFN-Cys might induce cell apoptosis not only via the intrinsic pathway but via the extrinsic pathway and the ER stress-mediated pathway as well in human glioblastoma cells.

It is reported that caspase 3 executes cell apoptosis, to demonstrate which, Abdi et al administered z-VDV fdmk (a pan-caspase inhibitor) and z-DEVD fdmk (a selective caspase 3 inhibitor) to cells, respectively, and both of the inhibitors resulted in complete inhibition of apoptosis (54). It can be inferred that activated caspase 3 was the executioner to cell apoptosis. Studies also showed that caspase 3 could cleave poly (ADP-ribose) polymerase (PARP) to deprive its abilities of detecting and repairing DNA damage, leading to cell apoptosis ultimately (35,55). The above suggested that the activated caspase 3 which was induced by SFN-Cys could cleave the specific substrates as a proteolytic enzyme, executing cell apoptosis. Thus, caspase 3 might cleave some other substrates in cancer cells as well, such as microtubule associated proteins which play a crucial role in cell apoptosis. In separate studies, we found that caspase 3 cleaved α-tubulin resulting in cell apoptosis (data not shown). The proteolytic activity of caspase 3 is also closely related to the ubiquitin-proteasome system (UPS): caspase 3 cleavage produces substrates such as actomyosin resulting in an increase of proteasome-mediated proteolysis (56). Given that SFN-Cys-activated caspase 3 might cleave α-tubulin to increase proteolysis of UPS, this excessive proteolysis will result in microtubule depolymerization and apoptosis (57). Taxol was used as a microtubule-stabilizing agent to prevent cancers, however, the glioblastoma cells often resist it (58). Luckily, it is reported that SFN could induce cell apoptosis in Taxol-resistant cells (59). These studies suggested that SFN-Cys might be a much more efficient microtubule-targeting agent compared with the traditional agent such as Taxol due to its versatile anticarcinogenic abilities. In addition, SFN can modulate epigenetic events. Previous studies showed that SFN induced cell apoptosis by regulating the level of epigenetic regulators such as polycomb group (PcG) proteins which contribute to chromatin structure modification and gene expression suppression. The SFN-mediated reduction of PcG proteins in a proteasome-dependent way led to an increase of cleaved caspase 3 and cell apoptosis (60). This was consistent with our hypothesis that SFN-Cys-mediated proteolysis of UPS led to cell apoptosis. Thus, we inferred that SFN-Cys might also activate caspase 3 and induce cell apoptosis by modulating the epigenetic regulators mediated via UPS. Moreover, HDAC inhibitor increased the level of caspase 3 mRNA (61). This evidence suggested that the expression of caspase 3 could be epigenetically regulated by HDAC. Recently, HDAC inhibitors are used in epigenetic glioblastoma therapies (62). Given that SFN-Cys was the most potential HDAC inhibitor among SFN and its metabolites, we think that SFN-Cys might induce cell apoptosis through the epigenetic regulation of caspase 3. Therefore, there are multiple ways for SFN-Cys to suppress the tumor progression and further studies needed to be done.

In conclusion, SFN-Cys upregulated Bax/Bcl-2 ratio and activated caspase 3 subsequently via sustained activating ERK1/2 signaling, leading to intrinsic apoptosis in human glioblastoma U373MG and U87MG cells (Fig. 6). The underlying mechanisms that SFN-Cys triggered provided us with more thoughts and information to develop new drugs to treat glioblastoma with high efficacy.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant nos. 81272843 and 81601993).

References

Skała E, Sitarek P, Toma M, Szemraj J, Nieborowska-
Yin XM, Oltvai ZN and Korsmeyer SJ: BH1 and BH2 domains
Wick W, Grimmel C, Wild-Bode C, Platten M, Arpin M
Lee WJ, Hsiao M, Chang JL, Yang SF, Tseng TH, Cheng CW,
Tong WG, Ding Z, Talamonti MS, Bell RH and Adrian TE:
Deschênes-Simard X, Kottakis F, Meloche S and Ferbeyre G:


19. Sitarek P, Skała E, Toma M, Wielanek M, Szemraj J, Nieborowska-