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## Induction of medulloblastoma cell apoptosis by sulforaphane, a dietary anticarcinogen from *Brassica* vegetables

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### Abstract

There is increasing evidence that a variety of natural substances derived from the diet may act as potent chemopreventive agents. In this work, we show that DAOY cells, a widely used model of metastatic medulloblastoma (MBL), are highly sensitive to sulforaphane, a naturally occurring isothiocyanate from *Brassica* vegetables. Sulforaphane induced DAOY cell death by apoptosis, as determined by DNA fragmentation and chromatin condensation. DAOY apoptosis correlates with the induction of caspase-3 and -9 activities, resulting in the cleavage of PARP and vimentin. Both the cytotoxic effect and apoptotic characteristics induced by sulforaphane were reversed by zVAD-fmk, a broad spectrum caspase inhibitor, demonstrating the important role of caspases in its cytotoxic effect. These results identify sulforaphane as a novel inducer of MBL cell apoptosis, supporting the potential clinical usefulness of diet-derived substances as chemopreventive agents.

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**Keywords:** Sulforaphane; Medulloblastoma; Brassica vegetables; Apoptosis

### 1. Introduction

A large number of epidemiological studies have reported strikingly lower cancer rates among individuals who consume large quantities of fruit and vegetables [1,2]. This has led to considerable interest in the identification and characterization of naturally occurring chemopreventive phytochemicals found in the diet capable of inhibiting, retarding or reversing carcinogenesis [3]. Among these, vegetables of the Cruciferae family have received widespread endorsement as beneficial nutrients that can prevent

**Abbreviations:** DAPI, 4',6-diamino-2-phenylindole dihydrochloride; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; rfu, relative fluorescence unit; PEITC, phenylethyl isothiocyanate; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; TNF $\alpha$ , tumor necrosis factor-alpha; zVAD-fmk, z-Val-Ala-Asp(OMe)-fluoromethylketone.

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malignant disease [4]. Consumption of cruciferous vegetables, particularly those of the *Brassica* genus (broccoli, cabbage, cauliflower, radish, mustard, turnip, rutabaga, etc.) has been suggested to reduce the risk of various cancers and to have chemopreventive activity in laboratory animals [5,6]. Numerous studies have shown that the cancer preventive effects of *Brassica* vegetables are related to their unique content in a large variety of thioglycoside conjugates of isothiocyanates, named glucosinolates [7]. Physical damage to cruciferous vegetables by chewing or crushing results in the myrosinase-catalyzed hydrolysis of the glucosinolate content of the vegetables, leading to the production of biologically active isothiocyanates [7]. It is widely accepted that isothiocyanates inhibit tumorigenesis by their inhibition of cytochrome P-450 enzymes involved in the activation of carcinogens as well as by their activation of the phase II detoxification enzymes [8–10]. However, there is also evidence that these compounds directly inhibit the growth of cancer cells in vitro through induction of apoptosis [11]. In this respect, the most extensively studied isothiocyanate, phenylethyl isothiocyanate (PEITC) induces apoptosis in HeLa [12] and leukemia [13] cell lines, a process possibly involving activation of c-Jun N-terminal kinase (JNK) [13–15]. Induction of tumor cell apoptosis by other isothiocyanates remains however much less understood.

Sulforaphane represents a major isothiocyanate from cruciferous vegetables, being particularly abundant in broccoli in which it is stored in its glucoraphanin form [7]. Sulforaphane was initially identified as the principal inducer of Phase II enzymes in broccoli [16] and subsequently shown to possess anticarcinogenic activities [17,18]. Recent data also suggest that this molecule may have a direct inhibitory action on cancer cells. Sulforaphane induces cell cycle arrest of prostate [19], leukemic [20] and colon carcinoma cells [21], leading to apoptotic cell death [19–22]. Whether sulforaphane may similarly act as a potent chemopreventive agent for other types of tumors remain however unclear.

In this study, we tested the proapoptotic effects of sulforaphane and of various dietary-derived substances on a variety of tumor cell lines. Our results show that DAOY cells, a widely used model of metastatic medulloblastoma (MBL), are highly

sensitive to sulforaphane-mediated apoptosis, thereby suggesting that this compound may have chemopreventive properties against pediatric brain tumors.

## 2. Experimental procedures

*Materials and antibodies-* z-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk) was from ICN Biomedical and etoposide was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC), acetyl-Ile-Asp-Thr-Asp-7-amino-4-trifluoromethylcoumarin (Ac-IETD-AFC), and acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (Ac-LEHD-AMC) were purchased from Biosource International (Camarillo, CA). Anti-PARP monoclonal antibody (clone C-2-10) was purchased from Clontech (Palo Alto, CA). Anti-vimentin monoclonal antibody (sc-6260) was purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). Anti- $\beta$ -actin was purchased from Sigma (St-Louis, MO). Anti-Bax monoclonal antibody (clone 6A7) was from BIOSOURCE International (Camarillo, CA) while the anti-Bcl-2 (clone 7) and the anti-paxillin monoclonal antibodies were purchased from Transduction Laboratories (San Diego, CA). Anti-ERK polyclonal antibodies were from Cell Signaling Technology (New England Biolabs, Pickering, ON, Canada). Sulforaphane and other tested natural compounds were purchased from LKT Laboratories (St-Paul, MN) with the exception of PEITC that was from Aldrich.

*Cell culture-* HT-29 and Caki-1 cell lines were cultured in McCoy's 5A medium (Sigma M-4892) containing 10% bovine calf serum (BCS, MediCorp, Montreal, Que.). DAOY and U-87 MG cell line (ATCC #HTB-14) were cultured in minimal essential medium (MEM) supplemented with 1 mM sodium pyruvate and containing 10% heat-inactivated fetal bovine serum (FBS, MediCorp, Montreal, Que.), and antibiotics: 100 units/ml penicillin G, 100  $\mu$ g/ml streptomycin. Bovine aortic endothelial cells (BAEC) obtained from Clonetics (San-Diego, CA) were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% BCS, 100 units/ml penicillin G, 100  $\mu$ g/ml streptomycin, and 1 ng/ml basic fibroblast growth factor (bFGF, Upstate Biotechnology, Lake Placid,

NY). All media and antibiotics were from GibcoBRL (Burlington, ON).

**Fluorimetric caspase-3, caspase-8, and caspase-9 assays-** Cells were grown to about 80% confluence and treated for 18 h with sulforaphane (10  $\mu$ M) or with other inducers of apoptosis such as etoposide (20  $\mu$ M). When required, zVAD-fmk was added 1 h before treatment at a final concentration of 50  $\mu$ M. Cells were collected, washed in cold phosphate-buffered saline (PBS) and lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for 20 min at 4 °C. The lysates were clarified by centrifugation at 16,000g for 20 min. Caspase activities were determined essentially as described previously [23] by incubation with 50  $\mu$ M fluorogenic peptide substrates acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC, caspase-3-specific) or acetyl-Ile-Asp-Thr-Asp-7-amino-4-trifluoromethylcoumarin (Ac-IETD-AFC, caspase-8-specific) or 250  $\mu$ M acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (Ac-LEHD-AMC, caspase-9-specific) in assay buffer [50 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 5 mM DTT, and 1 mM EDTA] on 96-well plates. The release of AFC ( $\lambda_{ex}$  = 400 nm,  $\lambda_{em}$  = 505 nm) or AMC ( $\lambda_{ex}$  = 380 nm,  $\lambda_{em}$  = 460 nm) was monitored for at least 20 min at 37 °C on a SpectraMAX™ Gemini fluorescence plate reader (Molecular Devices). Caspase activities were expressed as relative fluorescence unit (rfu) per second per  $\mu$ g of protein used in the assay [23].

**Cell viability assays-** Cells were grown to about 80% confluence in 35 mm plates and treated for various periods of time. Adherent and nonadherent cells were collected and viability was assessed by mixing aliquots of cell suspensions with an equal volume of 0.4% trypan blue (GibcoBRL). Cells that picked up the dye were considered to be dead.

**Staining of apoptotic cells-** DAOY were grown on glass coverslips and were treated with sulforaphane or etoposide for 18 h, as described above. After the treatment, the coverslips were washed twice with PBS, fixed for 60 min in 3.7% paraformaldehyde/PBS at room temperature, washed again twice in PBS and then permeabilized in 0.2% TritonX-100/PBS for 5 min at 4 °C. Permeabilized cells were washed in PBS and the terminal

deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay was performed as described in the In Situ Cell Death Detection Kit (Roche Diagnostic, Laval, Que.). The nuclear morphology of cells was analyzed by staining of DNA with 4',6-diamino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes). For Annexin V staining, DAOY cells grown on coverslips were incubated for 18 h with etoposide or with either 10 or 25  $\mu$ M sulforaphane and apoptotic cells were visualized using the ApoAlert Annexin V kit (Clontech), using the manufacturer protocol.

**Immunoblot analysis-** Cells were harvested and lysed as described for caspase assays and the protein concentration was measured using the BCA protein assay reagent (Pierce, Rockford, IL) and BSA as the standard. Equal amounts of protein samples in sample buffer [62.5 mM Tris-HCl (pH 6.8), 100 mM DTT, 10% glycerol, 2% SDS, and 0.1% bromophenol blue] were heated at 100 °C for 3 min and separated on 0.75 mm-thick, sodium dodecyl sulphate-polyacrylamide gels with a MINI-PROTEAN II apparatus (Bio-Rad, Richmond, CA). Proteins were electroblotted onto 0.45  $\mu$ m-pore diameter PVDF membranes (Roche Diagnostics, Laval, QC) with a semi-dry apparatus (Millipore) in transfer buffer (96 mM glycine, 10 mM Tris, and 20% methanol) for 1 h at 80 mA per gel. Membranes were blocked overnight at 4 °C in Tris-buffered saline [TBS: 20 mM Tris-HCl (pH 7.5), 137 mM NaCl] containing 0.1% (v/v) Tween 20 and 3% BSA. Blots were incubated with primary antibodies in blocking buffer for 2 h at room temperature, followed by a 1 h incubation with a 1:10,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) in the same incubation medium. Immunoreactive bands were revealed with enhanced chemiluminescence Western blotting kit (Renaissance, NEN™ Life Science, Boston, MA) and SuperRX films (Fuji).

### 3. Results

#### 3.1. Sulforaphane induces caspase-3 activity in a medulloblastoma cell line

As a first step towards the identification of diet-derived substances capable of inducing tumor cell

apoptosis, we screened a panel of molecules for their ability to induce cell death of highly invasive tumor cell lines. The inhibitory properties of the molecules were also investigated using an endothelial cell line (BAEC) in order to monitor a potential antiangiogenic effect. Induction of caspase-3 activity was used as a convenient assay to monitor the potential apoptotic action of tested molecules, based on the well-described role of this executioner caspase in apoptosis [24]. Tested compounds include a variety of molecules that have emerged in recent years as potential dietary-derived anticarcinogens such as flavonoids (flavone, epigallocatechin gallate, resveratrol, genistein, quercetin), powerful antioxidants (lipoic acid), indole glucosinolate metabolites (I3C) and isothiocyanates (PEITC, brassinin, sulforaphane).

Cell lines from renal metastatic carcinoma (Caki-1), human glioblastoma (U87), colon carcinoma (HT-29), human MBL (DAOY) as well as BAEC were incubated for 18 h with the compounds (10  $\mu$ M) and caspase-3 activity was monitored in the resulting cell lysates. As shown in Table 1, there are striking differences in the extent of caspase-3 activation triggered by the compounds in the various cell lines. Caki-1 were found to be extremely sensitive to the isothiocyanate PEITC, while this compound elicit moderate effect in HT-29 and BAEC and was totally ineffective on DAOY cells. Perhaps surprisingly, most the other tested compounds had negligible effects on caspase-3 activity associated with all the cell lines with the notable exception of

the isothiocyanate sulforaphane that induced a marked increase (8-fold) in caspase-3 activity of DAOY cells (Table 1) while being inactive on all other cell lines. These results suggest that apoptosis induced by diet-derived substances is highly cell-type specific and that MBL tumor cells may be highly susceptible to treatment with sulforaphane.

### 3.2. Sulforaphane-mediated caspase activity induces medulloblastoma cell apoptosis

The striking specificity of sulforaphane towards DAOY cells led us to investigate in more details its potential proapoptotic effects on this cell line. As shown in Fig. 1A, sulforaphane-induced caspase-3 activity increased over a 24 h period and was detectable at concentrations as low as 2–5  $\mu$ M (Fig. 1B), which is the serum concentration of sulforaphane measured after ingestion of moderate quantities of broccoli sprouts [25], and reached a maximum at approximately 20  $\mu$ M. Interestingly, the induction of caspase-3 activity in DAOY cells by 10  $\mu$ M sulforaphane was much higher than that observed following treatment of these cells with identical concentrations of PEITC and BITC, two isothiocyanates with known proapoptotic properties [11] (Fig. 1C).

To further investigate whether the induction of caspase-3 activity by sulforaphane in the DAOY MBL cell line led to cell death, cells were incubated with 10  $\mu$ M sulforaphane and, at various times, cell

Table 1  
Induction of caspase-3 activity by natural compounds in human tumor cell lines

Compounds	DAOY	Caki-1	HT-29	U-87	BAEC
Flavone	2.3 $\pm$ 0.3	1.1 $\pm$ 0.2	1.1 $\pm$ 0.1	0.9 $\pm$ 0.1	1.1 $\pm$ 0.2
Quercetin	2.5 $\pm$ 0.4	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1	1.0 $\pm$ 0.2	0.9 $\pm$ 0.1
EGCG	1.0 $\pm$ 0.2	1.1 $\pm$ 0.2	1.1 $\pm$ 0.2	0.9 $\pm$ 0.2	1.3 $\pm$ 0.2
Resveratrol	1.0 $\pm$ 0.2	1.1 $\pm$ 0.2	1.2 $\pm$ 0.2	0.8 $\pm$ 0.2	1.2 $\pm$ 0.2
Genistein	0.9 $\pm$ 0.1	0.8 $\pm$ 0.2	1.3 $\pm$ 0.1	0.7 $\pm$ 0.1	1.0 $\pm$ 0.1
Lipoic acid	1.3 $\pm$ 0.2	0.9 $\pm$ 0.1	1.3 $\pm$ 0.1	0.9 $\pm$ 0.2	0.8 $\pm$ 0.1
Indole-3-carbinol	2.0 $\pm$ 0.3	0.9 $\pm$ 0.2	0.9 $\pm$ 0.2	1.0 $\pm$ 0.3	0.9 $\pm$ 0.2
Brassinin	0.8 $\pm$ 0.1	0.9 $\pm$ 0.1	0.8 $\pm$ 0.1	0.8 $\pm$ 0.1	0.7 $\pm$ 0.2
PEITC	0.6 $\pm$ 0.1	10.4 $\pm$ .9	2.4 $\pm$ 0.3	1.9 $\pm$ 0.3	3.7 $\pm$ 0.4
Sulforaphane	8.0 $\pm$ 1.4	1.4 $\pm$ 0.3	0.9 $\pm$ 0.2	1.0 $\pm$ 0.1	0.6 $\pm$ 0.1

Cells were treated with 10  $\mu$ M of the indicated compounds for 18 h and the extent of caspase-3 activity was quantified as described in Section 2. Results are expressed as the *x*-fold induction of caspase-3 activity over untreated cells and are the means  $\pm$  SEM of two distinct experiments.

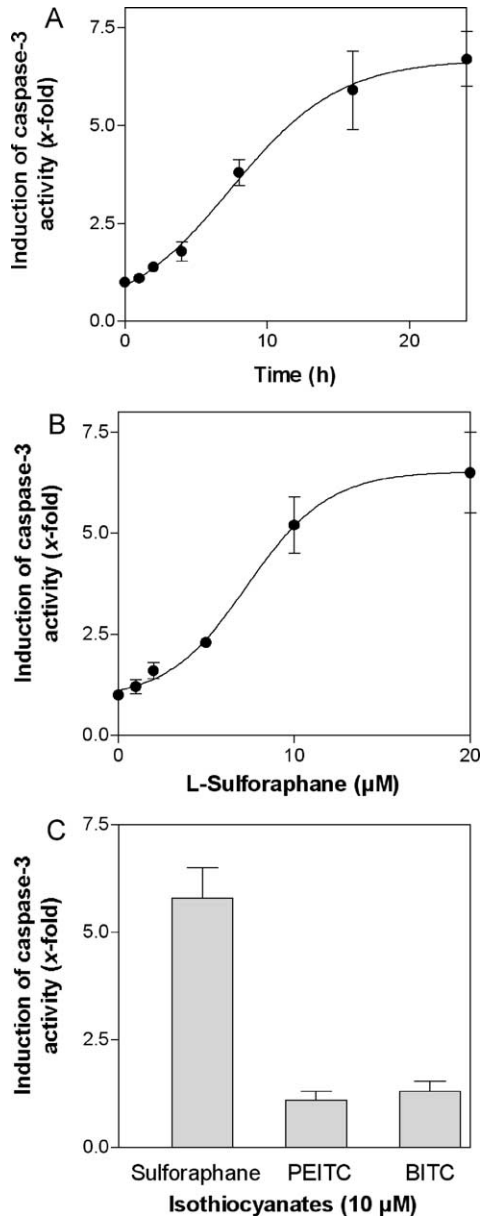


Fig. 1. Dose- and time-dependence of sulforaphane-mediated increase of caspase-3 activity in DAOY cells. Subconfluent DAOY cells were either left untreated or treated with  $10 \mu\text{M}$  sulforaphane for various periods of time (A) or for 18 h in the presence of increasing concentrations of the compound (B). Extracts from control and treated cells were used to determine DEVDase (caspase-3) activity, as described in the experimental procedures section. C. DAOY were treated for 18 h with  $10 \mu\text{M}$  of sulforaphane, PEITC or BITC and the extent of caspase-3 activation was monitored. Results represent the means  $\pm$  SEM of two experiments performed in duplicate.

viability was measured by exclusion of trypan blue. Fig. 2 shows that sulforaphane induced 60% cell death of DAOY after about 24 h of treatment, the extent of cell death being comparable to that induced by etoposide. The viability of other cell lines, such as U-87 (glioblastoma), was however, not altered by sulforaphane, further demonstrating the preferential action of this compound on DAOY (results not shown). Importantly, sulforaphane-induced cell death was greatly reduced by the presence of zVAD-fmk, a broad-spectrum inhibitor of caspases, indicating that the observed cell death is caspase-dependent (Fig. 2).

To further determine if sulforaphane-induced cell death was caused by programmed cell death rather than by necrosis, we next examined DNA condensation and fragmentation, two fundamental characteristics of apoptotic cells. DNA fragmentation in the nuclei of apoptotic cells was monitored using the TUNEL assay, in which DNA fragmentation is visualized after incorporation of fluorescein-dUTP, while DAPI staining was used to show chromatin condensation. As shown in Fig. 3A, DAOY cells treated for 18 h with  $10 \mu\text{M}$  sulforaphane were markedly stained by the TUNEL assay, whereas

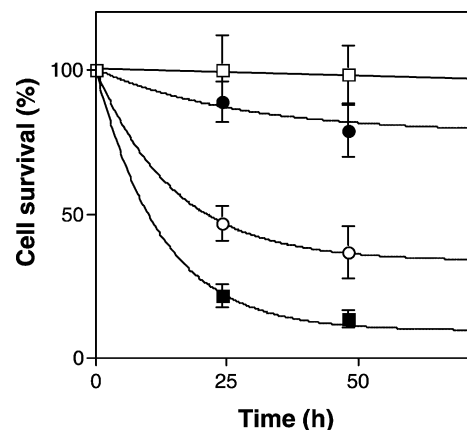


Fig. 2. Sulforaphane induces DAOY cell death. Subconfluent cells were either left untreated ( $\square$ ) or treated with  $10 \mu\text{M}$  sulforaphane ( $\circ$ ) or with  $20 \mu\text{M}$  etoposide ( $\blacksquare$ ) for 0, 24, 48, and 72 h. DAOY were also treated with sulforaphane in the presence of  $50 \mu\text{M}$  zVAD-fmk ( $\bullet$ ). Adherent and nonadherent cells were collected and viability was assessed by Trypan blue exclusion. Cell death is expressed as the percentage of cells incorporating the dye relative to the total amount of cells. Results represent the means  $\pm$  SEM of two experiments performed in duplicate.

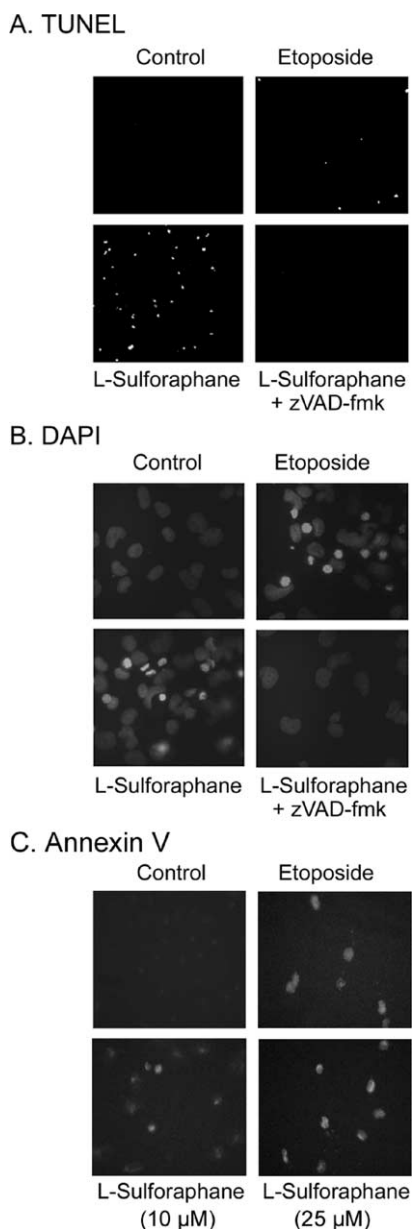


Fig. 3. Sulforaphane treatment induces DNA fragmentation. (A) DAOY grown on coverslips were either left untreated (control) or treated with 10  $\mu$ M sulforaphane in the absence or in the presence of 25  $\mu$ M zVAD-fmk or with 20  $\mu$ M etoposide as a positive control. Fragmented DNA was detected by the TUNEL assay (B) Cells were treated as described in A and DNA condensation was detected by staining with DAPI, as described in Section 2. (C) Cells were treated with etoposide or with either 10  $\mu$ M or 25  $\mu$ M sulforaphane and externalization of phosphatidylserine was monitored by Annexin V staining.

labeling was not observed when sulforaphane-treated cells were cultured in the presence of zVAD-fmk. Nuclei from untreated cells were weakly stained by DAPI, whereas the nuclei from sulforaphane-treated cells contained bright fluorescent spots that are characteristic of condensed chromatin (Fig. 3B). Here again, chromatin condensation was markedly reduced by zVAD-fmk, further emphasizing the essential role of sulforaphane-induced caspase activities in DAOY apoptosis. Induction of apoptosis of DAOY cells by sulforaphane was confirmed by the externalization of phosphatidylserine, as visualized by Annexin V staining of treated cells (Fig. 3C).

### 3.3. Sulforaphane increases intracellular activities of caspase-3 and caspase-9 and the cleavage of PARP and vimentin

Since caspase-3 is an executioner caspase that can be activated through both caspase-9 and caspase-8-dependent pathways [24], we next determined the extent of activation of these initiator caspases following treatment of DAOY with sulforaphane. As shown in Fig. 4, a 18 h incubation with 10  $\mu$ M sulforaphane resulted in a marked increase in caspase-9 activity, as determined using a fluorogenic peptide substrate specific for this caspase. No caspase-9 activities were detected in extracts from cells treated in the presence of 50  $\mu$ M zVAD-fmk, confirming that the protease activity measured was due to caspases and not to other types of proteases. However, under these experimental conditions, we failed to detect any caspase-8 activity towards its specific substrate (Fig. 4). Treatment of the cells with tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) in the presence of cycloheximide, a prototypical activator of caspase-8 activity, resulted in a very weak caspase-8 activity (results not shown), suggesting that DAOY may contain very low levels of this enzyme, as recently suggested for various MBLs [26]. These results suggest that the observed increase in caspase-3 activity observed following treatment of DAOY by sulforaphane is most likely to be a consequence of the activation of the caspase-9 pathway in these cells.

Activation of caspase-3 (and of other executioner caspases) leads to the degradation and inactivation of key cellular proteins such as DNA repair, signaling, and structural proteins [24]. Among these,

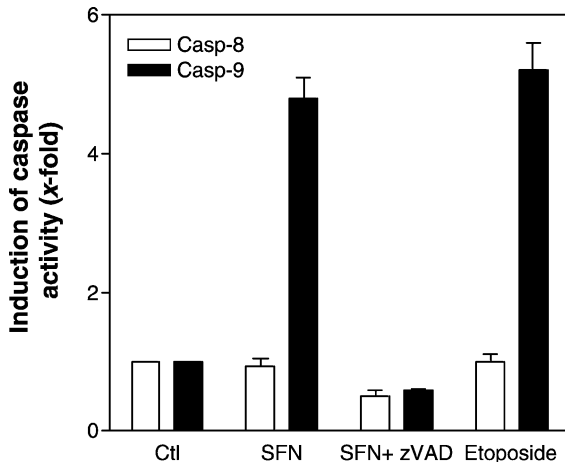


Fig. 4. Sulforaphane treatment induces caspase-9 activity in DAOY. DAOY were either left untreated or treated for 18 h with 10  $\mu$ M sulforaphane or 20  $\mu$ M etoposide in the absence or in the presence of zVAD-fmk. Extracts from control and treated cells were used to determine LEHDase (caspase-9) or IETDase (caspase-8) activities as described in Section 2. Results represent the means  $\pm$  SEM of two experiments performed in duplicate.

the cleavage of poly(ADP-ribose) polymerase (PARP) and of the microfilament-associated vimentin represents a well described response of cells undergoing caspase-mediated apoptosis. As shown in Fig. 5, sulforaphane induced cleavage of PARP characterized by the appearance of a 25-kDa fragment and also induced cleavage of vimentin, characterized by the appearance of a 21-kDa fragment of this protein. These fragments are also observed upon treatment of the cells with etoposide, a known inducer of the cleavage of these proteins. The cleavage of PARP and vimentin is mediated by caspases since addition of zVAD-fmk abolished the formation of the fragments (Fig. 5). Treatment of the cells with sulforaphane had however no significant effect on the cellular levels of  $\beta$ -actin (Fig. 5), nor on those of ERK, paxillin, Bcl-2 and Bax (data not shown). Overall, these results indicate that sulforaphane induces DAOY apoptotic cell death through caspase-mediated pathways.

#### 4. Discussion

MBL is a malignant tumor of the cerebellum that accounts for approx. 20% of primary brain tumors in

children [27]. Current treatment of MBL with radio- and chemotherapy subsequent to surgical resection results in a 5-year survival rate exceeding 80% [28]. However, 30–50% of all patients eventually experience tumor progression or late relapse with high rates of mortality [28]. As such, there is considerable interest in identifying complementary approaches that may increase these low salvage rates.

In this respect, there is currently considerable interest in the chemopreventive properties of natural compounds present in the diet. This interest stems from accumulating evidence that dietary intake of these substances is inversely correlated with the development of cancer and that administration of a number of these compounds to animals can prevent or reverse the development of cancers [3]. In the course of our study, we observed that DAOY, a hyperdiploid human MBL cell line that is extensively used for the study of MBL, was highly sensitive to sulforaphane, the predominant isothiocyanate of broccoli. Sulforaphane induces caspase-3 and -9 activities, but not caspase-8 activity, suggesting that the observed cytotoxicity of sulforaphane on these cells involves the mitochondrial-mediated apoptotic pathway [24]. The increase in caspase-3 activity was correlated with major alterations in DAOY cells that are characteristic of apoptosis including DNA fragmentation, chromatin

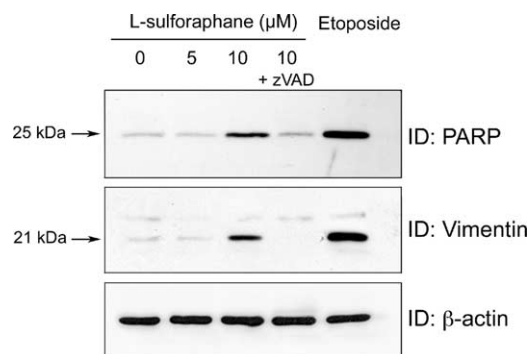


Fig. 5. Western blot analysis of DAOY following treatment with sulforaphane. DAOY cells were either left untreated or treated with increasing concentrations of sulforaphane, in the absence or in the presence of 50  $\mu$ M zVAD-fmk or with 20  $\mu$ M etoposide. After 18 h, cells were harvested and cell extracts were prepared. 20  $\mu$ g of protein were subjected to SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto PVDF. Immunoblot analysis was performed with antibodies directed against PARP, vimentin and  $\beta$ -actin.

condensation and cleavage of key cellular substrates such as PARP and vimentin. Since these events and the overall cytotoxicity of sulforaphane were reversed by co-incubation with the broad spectrum caspase inhibitor zVAD-fmk, it is clear that sulforaphane-mediated DAOY cell death occurs through caspase-dependent apoptosis.

Interestingly, the induction of DAOY apoptosis by sulforaphane appears to be much stronger than that previously reported for a number of other tumor cell lines. For example, a 30 h incubation of prostate cancer cells with as high as 50  $\mu\text{M}$  sulforaphane was reported to induce 50% cell death [19], while a 48 h incubation with 30  $\mu\text{M}$  sulforaphane was necessary to detect significant growth arrest of Jurkat T-leukemia cells [20]. Moreover, a 48 h incubation with 15  $\mu\text{M}$  sulforaphane was required to induce significant apoptosis of HT-29 colon carcinoma cells, as detected by chromatin condensation [22]. Under our experimental conditions (10  $\mu\text{M}$ , 18 h incubation), sulforaphane failed to induce significant caspase-3 activity in all tested cell lines, with the exception of DAOY cells, further supporting the concept that these cells are uniquely sensitive to this compound.

The mechanisms responsible for this apparent selectivity of sulforaphane towards DAOY remains however unclear. DAOY cells closely mimic the in vivo gene expression profiles of human MBLs, expressing 98% of the genes consistently expressed in these tumors [29]. Of these, a number of genes of the PDGFR alpha and of the Ras/MAPK pathways were shown to be closely associated with metastatic MBL, such as DAOY [29]. MBL are also highly sensitive to inhibition of Hedgehog pathway activity [30]. Whether sulforaphane-mediated interference with these pathways is responsible for the preferential induction of DAOY apoptosis by this compound is currently under investigation.

The possible role of diet in the etiology of brain tumors remains largely unknown. Consumption of vegetables, including some members of the *Brassica* genus (cauliflower, cabbage, broccoli) has been inversely correlated with brain cancer mortality [31–33], suggesting that these vegetables contain beneficial components that can reach the brain and prevent the development of cancers. Accordingly, at least one isothiocyanate, PEITC, was shown to be

present in rat brain following administration of the compound by gavage [34], supporting the bioavailability of these compounds to brain cells.

In summary, our results show that MBL cells are highly sensitive to relatively low concentrations of sulforaphane. Human exposure to sulforaphane occurs primarily through consumption of certain cruciferous vegetables, particularly broccoli, in which the sulforaphane glucosinolate, glucorapharin accounts for almost 60% of all the broccoli glucosinates [25,35]. Isothiocyanates from broccoli were found to be rapidly absorbed, reaching a peak concentration of about 2  $\mu\text{M}$  after ingestion of 200  $\mu\text{mol}$  of broccoli isothiocyanates [25], which correspond to a concentration that is in the range of those required to induce cytotoxicity in our studies. Further studies aimed at the determination of the beneficial effects of sulforaphane on MBL growth in vivo should provide interesting informations on the potential clinical usefulness of this compound in the treatment of pediatric brain tumors.

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