

Induction of Differentiation in Human Promyelocytic Cells by the Isothiocyanate Sulforaphane

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Abstract. *Background: The consumption of cruciferous vegetables has long been associated with a reduced risk for the occurrence of cancer at various sites. This protective effect is associated with their isothiocyanate content. Sulforaphane (SFN) is by far the isothiocyanate most extensively studied to uncover the mechanisms behind this chemoprotection. In the present study, the ability of SFN to induce cytodifferentiation and apoptosis in a leukemia cell line was investigated. Materials and Methods: Cells were treated with different concentrations of SFN (0-100 μ M). Analysis of cell differentiation was performed by non-specific/specific acid esterase activity. Apoptosis induction was performed by flow cytometry. Results: SFN induced cytodifferentiation toward both granulocytic and macrophagic lineage, mediated by the involvement of phosphatidylinositol 3-kinase/protein kinase C. It also caused a significant increase in the apoptotic cell fraction. Conclusion: These findings suggest that SFN may be a promising antileukemic agent and should encourage further investigation as regards its chemotherapeutic potential.*

Acute myeloid leukemias are malignancies that are raised by the clonal expansion of cells abnormally arrested at some maturation stages of hematopoietic precursors. Although differentiation-inducing therapy by several agents, such as all-*trans* retinoic acid and 1,25-(OH)₂D₃, has favorable outcomes for patients with acute myeloid leukemia (AML), the side-effects, including recurrent disease and hypercalcemia, can be a major obstacle to disease-free survival. The HL-60 cell line, originating from a patient with AML, has been frequently employed as an excellent model for the *in vitro* study of cellular differentiation because it

allows differentiation into either macrophagic or granulocytic lineage according to the inducing stimuli (1, 2).

Sulforaphane (SFN) is a natural molecule showing potential as an anticancer agent and has been extensively studied to uncover the mechanisms behind this activity. There are multiple mechanisms activated in response to SFN, including modulation of metabolic enzymes, induction of apoptotic pathways, suppression of cell-cycle progression, inhibition of angiogenesis and anti-inflammatory activity (3, 4).

In this study, we assessed the inducing effects of SFN on the cellular differentiation of human myelocytic leukemia HL-60 cells. Moreover, we investigated whether apoptosis is involved in the cytodifferentiation-inducing effect of SFN.

Materials and Methods

Chemicals. SFN (CAS registry number 4478-93-7) was purchased from LKT Laboratories (St. Paul, MN, USA). The purity of sulforaphane was 99.37%. The isothiocyanate was dissolved in sterile water and a uniform solution was obtained. The solutions were stocked in the dark at 0-4°C. SFN was used at concentrations of 0.2-100 μ M.

Cells and in vitro culture conditions. The human promyelocytic leukemia cell line HL-60 was obtained from the Istituto Zooprofilattico of Brescia, Italy. Cells were grown in RPMI supplemented with 20% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA), 1% penicillin/streptomycin solution (Sigma), and 1% glutamine solution 200 mM (Sigma). To reduce spontaneous differentiation, cells were never allowed to exceed a density of 1.0×10^6 cells/ml.

Cytotoxicity test. Viability was determined using the trypan blue dye-exclusion test, which distinguishes viable and non-viable cells. HL-60 cells were treated with different concentrations of SFN (0-100 μ M) for 30 h (*i.e.* the exposure time corresponding to the length of the cell cycle). The inhibitory concentration causing 50% cell toxicity following one cell cycle exposure (*i.e.* IC₅₀) was calculated by interpolation from dose-response curves.

Analysis of cell differentiation. Non-specific/specific acid esterase activity suggests differentiation along monocytic/macrophage lineage and monocytic/granulocytic lineage, respectively (5). Assays for α -naphthyl acetate esterase (non-specific acid esterase) and naphthol AS-D chloroacetate esterase (specific acid esterase)

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were performed using cytochemical kits from Sigma (91-A and 91-C) (6). After 72 h of treatment with SFN (0-12.5 μ M), differentiated cells were assessed by microscopic examination (5) of a minimum of 1,000 cells (in duplicate) for each experiment. The results were expressed as percentage of positive cells over total cells. Dimethyl sulfoxide (DMSO) 1% (v/v) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) 100 nM were used as positive control for the monocytic/granulocytic lineage and macrophage lineage, respectively.

Analysis of cell differentiation in the presence of kinase inhibitors.

To study the involvement of protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K) in the differentiation of cells exposed to SFN, the effects of the PKC inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) (Sigma) and the PI3-K inhibitor LY-294002 (Sigma) on cytodifferentiation stimulated with SFN were investigated. H-7 and LY-294002 were dissolved in sterile water and methanol, respectively. Cells were incubated for 40 min in the presence of H-7 (20 μ M) or LY-294002 (5 μ M) at 37°C and 5% CO₂ and then treated with SFN 12.5 μ M for 72 h. The assay procedure for non-specific/specific acid esterase activity was followed as described above. Results were expressed as percentage of positive cells over total cells. The percentage of differentiated cells induced by 12.5 μ M SFN was designated as 100%.

Flow cytometry. Micro-flow cytometric analysis was performed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) with the cell assay extension. For cell assays, individual cell fluorescence intensities were measured at two wavelength channels (λ_{ex} 470 nm/ λ_{em} 525 nm, λ_{ex} 635 nm/ λ_{em} 680 nm). The loading of the microfluidic chip was performed according to the manufacturer's recommendations. Briefly, after pre-treatment of the chip with 10 μ l of priming solution (Cell Fluorescence LabChip Kit; Agilent Technologies), a focusing dye solution (LabChip Kit) was applied to the appropriate well of the LabChip for adjustment of the Bioanalyzer optics to each individual chip. Prior to loading of samples, two aliquots (30 μ l each) of Cell Buffer were introduced into the appropriate wells. A total of 6 individual samples with a volume of 10 μ l each was analyzed, on each chip.

Measurement of Annexin V binding by flow cytometry. After treatment with SFN (0-25 μ M) for 24, 48 or 72 h, cells were centrifuged (100g) for 10 min, resuspended at a density of 1×10^6 in 500 μ l of medium containing 10 μ l of Media Binding Reagent (Merck KGaA, Darmstadt, Germany) and 1.25 μ l of Annexin V-biotin (Merck KGaA) and incubated for 15 min. After washing, cells were resuspended in 500 μ l of Binding Buffer (Merck KGaA) and treated with 1 μ l of Cy-5-labelled streptavidin (1 mg/ml; GE Healthcare Biosciences, Uppsala, Sweden) and 0.5 μ l of calcein 500 μ M (Molecular Probes, Eugene, OR, USA). After 30 min, cells were centrifuged and resuspended in Cell Buffer (Agilent Technologies). The chip was prepared for the flow cytometric measurements as described above.

Statistical analysis. All results are expressed as the mean \pm SD. Differences between treatments were evaluated using ANOVA, followed by Dunnett or Bonferroni test, using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered significant.

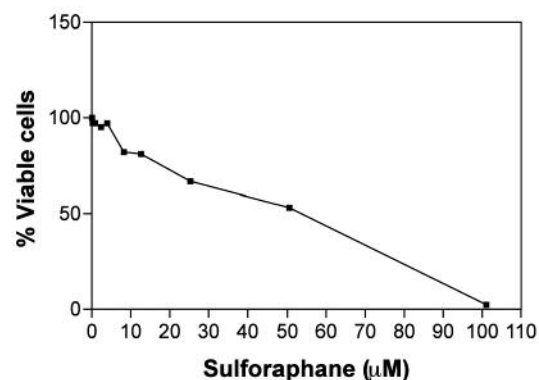


Figure 1. The effect of SFN on HL-60 cell viability.

Results

SFN induced a dose-dependent decrease in cell viability. For example, the proportion of viable cells was 97% at 4 μ M, 81% at 12 μ M, and 67% at 25 μ M (Figure 1). The IC₅₀, as measured by the number of viable cells in cultures after the addition of SFN, was determined as 49.5 μ M.

Since the analysis of cytodifferentiation induction has to be performed at concentrations where the cell viability is higher than 80% (7), the concentrations used for the evaluation of cytodifferentiation by SFN were in the range 1.6-12.5 μ M.

SFN induced dose-dependent differentiation of HL-60 cells toward the granulocytic lineage (Figure 2A). The highest effect was recorded after treatment with 12.5 μ M SFN, where the fraction of naphthol AS-D chloroacetate esterase-positive cells reached 36%, thus higher than the fraction of naphthol AS-D chloroacetate esterase-positive cells recorded for the positive control (DMSO). SFN also increased the number of α -naphthyl acetate esterase-positive cells, which indicates a differentiation toward the monocytic/macrophagic lineage (Figure 2B), although only at the highest concentration tested.

Previous studies have provided evidence that activation of PKC and PI3-K is necessary for the differentiation of HL-60 cells (8, 9). To determine any relationship between the effect of SFN on cellular differentiation and PKC and/or PI3-K activation, HL-60 cells were treated with specific inhibitors, H-7 and LY-294002, followed by incubation with SFN at the concentration where the highest cytodifferentiation was recorded (12.5 μ M). As illustrated in Figure 2A and B, both H-7 and LY-294002 significantly inhibited HL-60 cell differentiation induced by SFN. In particular, we observed a decrease of 42% for granulocytic differentiation and 68% for macrophagic differentiation. PKC and PI3-K inhibitors were not toxic to the HL-60 cells at the concentrations used in the experiments, as demonstrated by the trypan blue exclusion assay (data not shown).

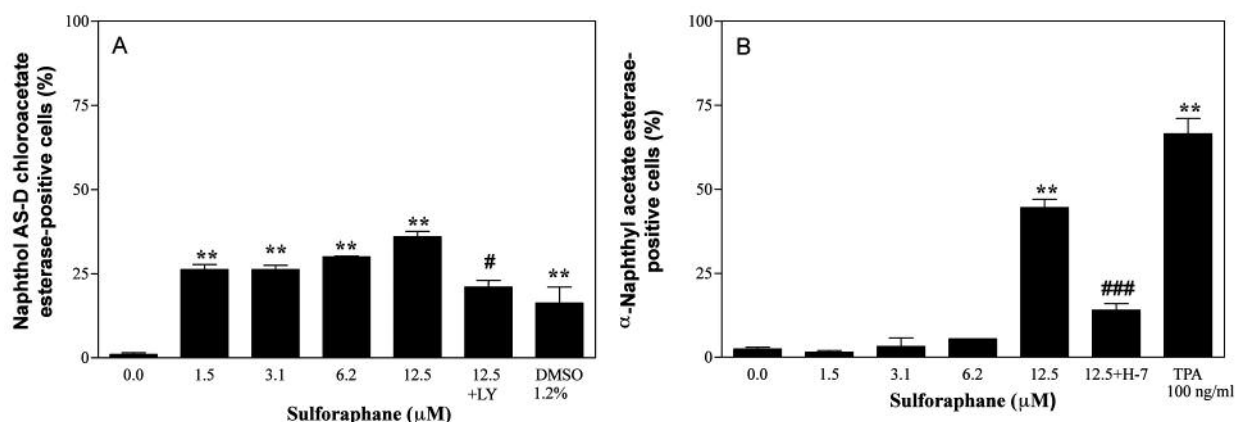


Figure 2. Dose-dependent effects of SFN on HL-60 cell differentiation as evaluated by naphthol AS-D chloroacetate esterase activity (A) and α -naphthyl acetate esterase activity (B), in the presence and absence of H-7 and LY-294002. ** $p < 0.01$ vs. control; # $p < 0.05$ vs. 12.5 μ M; ### $p < 0.001$ vs. 12.5 μ M.

Apoptotic death is a feature of normal terminally differentiated cells (10). We then analyzed the ability of SFN to induce apoptosis. In order to discriminate between apoptosis as a primary effect of SFN and apoptosis as a consequence of terminal differentiation induced by SFN, we analyzed the proapoptotic potential of SFN at three different time-points (24, 48 and 72 h). SFN did not increase the fraction of apoptotic cells after 24 h of treatment (Figure 3). When HL-60 cells were exposed to SFN for 48 or 72 h, we recorded a rise in the fraction of apoptotic cells, which reached approximately four- and three-fold that of the control, respectively (Figure 3).

Discussion

In this study, we demonstrated that SFN can induce differentiation in HL-60 promyelocytic leukemia cells. HL-60 cells were found to differentiate into both granulocytes and macrophages. The mechanism by which SFN induces HL-60 cell differentiation remains to be adequately clarified.

Several signal transduction molecules have been found to be involved in HL-60 cell differentiation, such as PKC and PI3-K (8, 9, 11).

SFN is known to activate a number of enzymes involved in stress signaling cascades including protein kinases. SFN targets include stress-activated protein kinases such as JNK, ERK and p38 (3, 12-15). In our study, inhibitors of PI3-K and PKC significantly inhibited the HL-60 cell differentiation induced by SFN. These findings strongly suggest that PI3-K and PKC are involved in the cell differentiation by SFN.

Treatment of HL-60 cultures with SFN caused apoptosis, which may represent an event only partly dependent on cytodifferentiation in our experimental conditions. In fact, after 48 h of treatment with SFN, we had already recorded

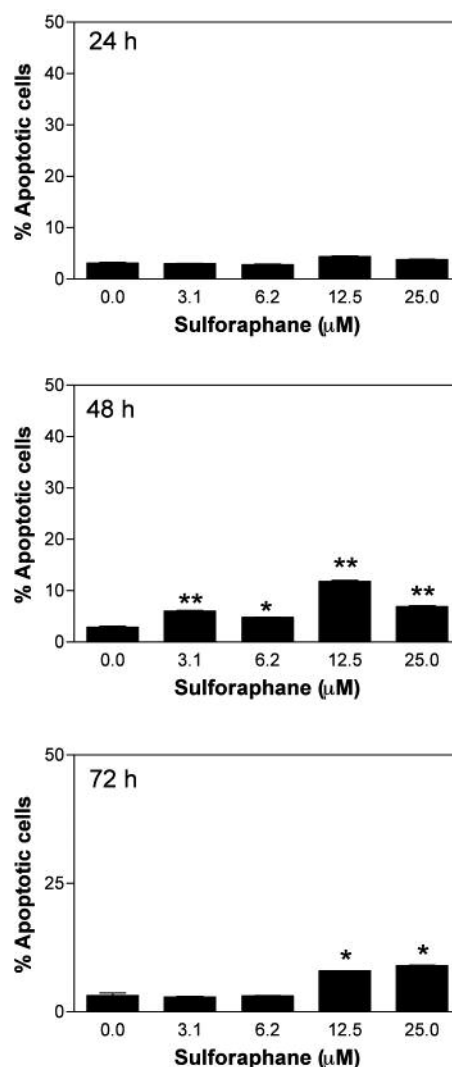


Figure 3. Fraction of apoptotic cells after 24, 48 or 72 h of treatment with SFN. * $p < 0.05$; ** $p < 0.01$ vs. control.

an increase in the apoptotic cell fraction. However, the effects of SFN on HL-60 cells are weak, if compared with its effect on other leukemia cell lines, such as Jurkat cells (16). For example, the dose required to reach IC₅₀ in HL-60 cells was more than three times greater than that recorded for Jurkat cells (49.5 μ M vs. 15.0 μ M respectively), indicating that SFN is probably less cytotoxic in HL-60 cells. Another important difference concerns the proapoptotic effect. The fraction of apoptotic cells induced in Jurkat cells (a seven-fold increase with respect to controls) was more than twice that recorded in HL-60 cells (a three-fold increase with respect to controls).

It is noteworthy that HL-60 cells are *p53* null (17). Thus, the induction of apoptosis in this system indicates that SFN may exert its effects independently of the *p53* gene. However, the analysis of protein levels in Jurkat cells indicated that SFN-induced apoptosis is associated with significant changes in *p53* protein (16). If the apoptotic pathway of SFN is *p53*-dependent, this could represent a possible explanation for the lower sensitivity of HL-60 cells to the cytotoxic and proapoptotic effect of SFN than Jurkat cells.

In conclusion, our results raise the possibility that SFN may be a promising candidate for use in antileukemic chemotherapy and further efforts are warranted to explore its therapeutic potential.

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