Sulforaphane-mediated induction of a phase 2 detoxifying enzyme NAD(P)H:quinone reductase and apoptosis in human lymphoblastoid cells

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The effect of sulforaphane on human lymphoblastoid cells originating from a patient of a high cancer risk was studied. Sulforaphane (SFN) is a naturally occurring substance of chemopreventive activity. In our study, changes in cell growth, induction of apoptosis and phase 2 enzymes as well as glutathione level were examined. Apoptosis was tested by confocal microscopy at three stages: change in mitochondrial membrane potential, caspase activation and phosphatidylserine externalization. We show that SFN increases the activity of the detoxification system: it increases quinone reductase activity at low concentration (0.5–1 μM) and raises glutathione level in a dose-dependent manner. At higher doses (2.5–10 μM) sulforaphane is a cell growth modulator, as it caused cell growth cessation (IC₅₀ = 3.875 μM), and apoptosis inducer. The results obtained suggest that sulforaphane acts as a chemopreventive agent in human lymphoblastoid cells.

Edible brassicas like broccoli, cauliflower, and rape seeds contain substantial quantities of secondary metabolites known as glucosinolates (GLs) as well as the enzyme myrosinase (EC 3.2.3.1). When vegetables are crushed by chopping or chewing,

Abbreviations: D₂R, (l-Asp)₂ rhodamine 110; DTNB, 5,5'-dithio-bis(2-nitrobenzenoic acid); FITC, fluorescein 5(6)-isothiocyanate; ITC, isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; PS, phosphatidylserine; QR, NAD(P)H:(quinone-acceptor) oxidoreductase (EC 1.6.99.2); SFN, sulforaphane.
myrosinase catalyses a rapid hydrolytic reaction, in which the glucosinolates are converted into isothiocyanates (ITC) and other products, depending on the chemical structure of GLs side chains (Fig. 1) (Zhang et al., 1992; Leoni et al., 2000; Fimognari et al., 2002).

Starting from glucorucin (Iori et al., 1999; Fimognari et al., 2002) isolated from broccoli sprouts of *Eruca sativa*, glucoraphanin was obtained through chemoselective oxidation, and controlled myrosinase-catalyzed hydrolysis of this precursor quantitatively afforded sulforaphane (Fig. 2).

![Figure 1. Chemical structure of glucosinolates and their metabolites received after enzymatic hydrolysis by myrosinase.](image1)

![Figure 2. Production of sulforaphane after enzymatic hydrolysis of glucoraphanin.](image2)

Wattenberg (1990) indicates that the putative mechanisms of action of chemopreventive agents involve the suppression of tumor development following elimination of pre-cancerous cells. These mechanisms include the deletion of initiated cells from a tissue by apoptosis so that clonal expansion of the lesion is aborted (Gamet-Payrastre et al., 1998).

Some papers show that ITCs block the proliferation of cancer cells in vitro and induce apoptosis. Gamet-Payrastre et al. (2000) pointed out that SFN induces cell-cycle arrest and apoptosis in HT29 human colon cancer cells (Steinkeller et al., 2001). Our previous study showed that SFN changes cell growth and induces apoptosis in various cell lines, e.g. murine leukemia and human melanoma (Misiewicz et al., 2003).

In this study we evaluated the effect of SFN on cell growth, apoptosis and phase 2 induction. We investigated the path of apoptosis: change in mitochondrial membrane potential, caspase activation and phosphatidylserine externalization. As a marker of phase 2 induction, NAD(P)H: (quinone-acceptor) oxidoreductase (QR) activity and GSH level were measured after SFN exposure.

**MATERIALS AND METHODS**

**Chemicals.** Sulforaphane was synthesized as described previously by Schmidt and Karrer (1948). The purity was 99.8%, as determined by GC. The absorption coefficient in distilled water was determined from absorbance values of eight serial SFN dilutions and at $\lambda = 238$ nm was calculated to be $1066 \text{ M}^{-1} \text{ cm}^{-1}$. MitoLight™ Apoptosis Detection Kit was purchased from Chemicon International, Inc. (Temecula, CA, U.S.A.), (L-Asp)$_2$ rhodamine 110 (D$_2$R) was purchased from Calbiochem and ANNEXIN V Kit from Caltag Laboratories (Burlingame, CA, U.S.A.). All other chemicals were obtained from Sigma.
**Cells.** Human lymphoblastoid cells (G.J. BRCA-1 Ex 5-300), established in permanent cell culture by Epstein-Barr virus immortalization of lymphocytes B originated from a patient with a germline missense mutation in exon 5 of one allele of BRCA-1 gene, were grown in RPMI 1640 medium supplemented with 19% heat inactivated fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin (250 ng/ml), and L-glutamine (2 mM). Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂ and the medium was changed every 48 h.

**General protocol for cell treatment.** Cells were incubated in 6-well plates in 4 ml of medium at a density of 2.5 × 10⁵ cells/ml with SFN used in five concentrations for 48 h before all experiments, unless stated otherwise.

**Cell viability assay.** The culture medium was removed by centrifugation and 1 ml of a 5 mg/ml solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in RPMI-1640 medium without serum and phenol red was added. After 3.5 h of incubation, cells were centrifuged and the MTT solution was removed and propanol was added to each well. When the MTT reduction product, formazan, was solubilized completely in propanol, the absorbance of the solution was measured with a Shimadzu UVmini 1240 spectrophotometer at 570 nm and 690 nm. The final result for each drug concentration was the mean of three experimental data.

Cell viability was also estimated after a short treatment with SFN, and subsequent incubation for 48 h. Cells were grown in 6-well plates at 2.5 × 10⁵ cells/ml with SFN concentration of 1.25, 2.5, and 5 μM. After 10, 30, 60 and 180 min, SFN was removed by changing medium, and the cells were incubated in SFN-free, complete medium for 48 h. After that time the cells viability was determined as above.

**Evaluation of cell culture morphology and cell adhesion.** After SFN treatment, morphology of the cell culture was observed with an Olympus IX 70 inverted microscope, and transmitted light images were taken using an Olympus FV 500 confocal system. The source of light was blue ion argon laser (λ = 488 nm).

**Mitochondrial membrane potential (ΔΨₘ) measurements.** The mitochondrial membrane potential was measured using MitoLight™ Apoptosis Detection Kit (Chemicon International, Inc.). Cells, after 48 h of SFN treatment, were resuspended at 1 × 10⁶ cells/ml in MitoLight™ solution according to the vendor’s protocol. The mitochondrial indicator in the kit is 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine chloride. This dye forms so-called J-aggregates (Smiley et al., 1991). The aggregation leads to a large shift in its absorption and fluorescence spectra to longer wavelengths.

In healthy cells, the dye accumulates forming red fluorescing aggregates (λₑₛ = 585/590 nm). When cells have depolarized mitochondrial, the dye remains in the cytoplasm and fluoresces green (λₑₛ = 527/530 nm).

The fluorescence was measured with a confocal microscope (Olympus Fluo View 500 system equipped with Olympus IX70 microscope). A 40× objective lens (UPlan APO) was used. Argon (488 nm) and He-Ne (543 nm) lasers were employed to excite sequentially the dye monomers and aggregates, respectively. The fluorescence of the monomers was collected through a 505–525 nm bandpass emission filter and the fluorescence of the aggregates was collected by using a 560 dichroic mirror and a 560–610 nm bandpass filter.

**Caspase activity.** The activity of caspases was measured using (L-Asp)₂ rhodamine 110 (D₂R). The nonfluorescent Asp₂-rhodamine 110 conjugate is intracellularly cleaved to the green fluorescent monosubstituted rhodamine 110 and free rhodamine (Hug et al., 1999).

Cells were collected after 48 h of SFN treatment, rinsed with PBS and resuspended in 5 μg/ml D₂R in PBS solution, at 1 × 10⁶ cells/ml.
The fluorescence was measured using the confocal microscope with $\lambda = 488$ nm excitation. The signal was collected using a 510–525 nm bandpass filter.

**Identification of cell death by detection of phosphatidylserine externalization.** Annexin V preferentially binds to phosphatidylserine (PS) (Clarke et al., 2002). Annexin V labelled with FITC (fluorescein 5(6)-isothiocyanate), and PI (propidium iodide) as a vital dye were used to distinguish live, apoptotic and necrotic cells. The lymphoblastoid cells, previously treated with sulforaphane, were stained at $1 \times 10^6$ cells/ml according to the vendor’s protocol and analyzed subsequently with the confocal microscope. A 40× objective was used (UPlan APO), Argon (488 nm) and He-Ne (543 nm) lasers were employed to sequentially excite FITC and PI, respectively.

**Measurement of NAD(P)H:quinone reductase activity.** NAD(P)H: (quinone-acceptor) oxidoreductase (EC 1.6.99.2) (QR) activity was estimated in a direct assay, measuring the NADPH-dependent menadiol-mediated reduction of MTT.

The reaction mixture was prepared for each set of assays as previously described (Prochaska & Santamaria, 1988).

Cells were collected, centrifuged at 150 × $g$ and lysed by 20 min incubation at room temperature with lysing solution (2 mM EDTA, 0.9% digitonin, pH 7.8). After the incubation, 200 µl of cell lysate was transferred to a disposable cuvette and 800 µl of reaction mixture was added. After 10 min the absorbance of MTT was measured at 610 nm. The absorbance in a cuvette with reaction mixture only was subtracted from the measured absorbance values. QR activity was expressed in mU/mg protein. One unit of enzyme activity is the amount of enzyme catalyzing conversion of 1 µM of the substrate to product in one minute at temperature 25°C. The absorption coefficient of reduced MTT was set at the level of 11300 M$^{-1}$ cm$^{-1}$ at $\lambda = 610$ nm according to Prochaska and Santamaria (1988).

The protein content was assessed by the Bradford assay. All experiments were made in triplicate.

**Total glutathione quantification.** Total glutathione level was assessed using a protocol that involves oxidation of GSH by 5,5′-dithio-bis(2-nitrobenzenoic acid) (DTNB) (Hedley & Chow, 1994). In the process GSSG and TNB (5-thio-2-nitrobenzoic acid) are formed (Tietze, 1969).

A standard curve was prepared using chemically reduced GSH as a standard.

Cells were collected, rinsed with PBS, and lysed on ice for 1 h with lysis buffer containing 0.6% sulfosalicylic acid. Samples were centrifuged at 14000 r.p.m. for 20 min at 4°C. Supernatant was transferred to new tubes, and stored at −20°C.

Measurements were taken in disposable cuvets at 412 nm.

All reagents were made as stock solutions in buffer A (125 mM sodium phosphate buffer, 6.3 mM Na$_2$EDTA, pH 7.5). Stock solutions were: 6 mM DTNB, 0.3 mM NADPH, glutathione reductase 50 U/ml.

One hundred microliters of the sample, 100 µl of DTNB (0.6 mM) and 700 µl of NADPH (0.21 mM) were mixed in a cuvette, filled to of 1 ml with buffer A and incubated for 10 min at 37°C. The reaction was initiated by addition of 10 µl of glutathione reductase and the initial reading at 412 nm was taken. The formation of TNB was monitored for 30 min. The amount of glutathione was determined from a calibration curve, in which the rate of change in A$_{412}$ is plotted against GSH equivalents. The result was expressed as the amount of GSH per mg of total protein.

**RESULTS**

SFN was tested at five concentrations: 0.5–10 µM. The lowest concentration used in-
creased cell viability, while from 1 μM to 10 μM a linear decrease in the cell viability was observed (Fig. 3).

IC₅₀ for SFN in lymphoblastoid cells was calculated at 3.9 μM.

The minimum time of exposition to SFN required to decrease the cell viability was also tested. As shown in Fig. 4, the change after 10 min was significant, especially for 5 μM, while treatment with SFN for 30 min had no significant effect on cell growth. When the cells were grown in the presence of SFN for 60 and 180 min, a dose- and time-dependent decrease in viability was observed. As a control at each time point, cells without SFN added were used.

Treatment with SFN affected also cell culture morphology. As shown in Fig. 5, at a low concentration (0.01 μM) the test compound stimulates cells to form clusters. In the culture treated with 5 μM SFN the cells separate, but there are still some clusters remain. At 10 μM SFN almost no clusters left.

The SFN treated cells expressed morphological features of apoptotic cells, like chromatin condensation, formation of apoptotic bodies and cell shrinkage. The occurrence of apoptosis was confirmed by confocal studies.

Figure 3. The viability of lymphoblastoid cells versus sulforaphane concentration.

Cells were cultured in the presence of 0.5, 1, 2.5, 5 and 10 μM SFN and cell viability was measured by MTT assay after 48 h. The viability is expressed as the mean percentage of control ± S.D. (standard deviation) of three independent experiments.

Figure 4. The viability of lymphoblastoid cells versus sulforaphane concentration after short treatment.

Cells were incubated with 1.25, 2.5 and 5 μM SFN for 10, 30, 60 and 180 min and subsequently cultured for 48 h in complete medium. The viability, measured by MTT test, is expressed as percentage of control. Experiment was in duplicate.

Figure 5. Change in cell culture morphology after treatment with 0 (A), 0.01 (B) and 5 μM (C) SFN.

Cells after 48 h of treatment were observed in 6-well plates under inverted microscope with argon-ion laser as light source. Objective 10× UPlanFL.
Cells were tested for the markers of apoptosis: change in $\Delta \psi_{\text{mt}}$, caspase activation and phosphatidylserine externalization. As shown in Fig. 6, control cells had polarized mitochondrial membrane, and the dye accumulated forming red fluorescing aggregates.

Figure 6. Images of cells stained with MitoLight$^\text{TM}$ Apoptosis Detection Kit (right).

Isoplots (left) of red and green fluorescence intensity over the area of a single cell pointed with an arrow. Cells were incubated with 0 (A), 1 (B), 2.5 (C) and 5 $\mu$M (D) SFN for 48 h and examined by confocal microscopy to detect changes in the mitochondrial membrane potential $\Delta \psi_{\text{mt}}$: aggregates (red fluorescence) and monomers (green fluorescence) of the mitochondrial potential-sensitive MitoLight$^\text{TM}$ dye.
gates. After treatment with 1 μM SFN, a partial depolarization of mitochondrial membrane occurs and the appearance of free dye in the cytoplasm, fluorescing green, is observed. Simultaneously a decrease in the intensity of red fluorescence occurs, which indicates a change in $\psi_{\text{mt}}$. The mitochondrial membrane becomes completely depolarized when cells are treated with 2.5–10 μM SFN.

The second marker of apoptosis tested was the activation of aspartate-specific cysteine proteases (caspases). The cells were loaded with non-fluorescent Asp$_2$-rhodamine 110 conjugate, which is intracellularly cleaved to the green fluorescent monosubstituted rhodamine 110 and free rhodamine by active caspases. Images were taken with 40× objective (UPlanAPO).

Cells were stained with FITC-conjugated annexin V and with PI to discriminate between live, apoptotic and necrotic cells. In the control, only few cells were annexin V$^+$/PI$^+$, while in cells treated with 5 μM SFN, most were annexin V$^+$/PI$^-$, or annexin V$^+$/PI$^-$ (not shown). In cell culture treated with 1 μM SFN we did not detected a significant increase in the number of apoptotic cells, the first noticeable rise in early apoptosis was observed in cells incubated with 2.5 μM SFN.

Table 1. Effect of sulforaphane on QR activity and intracellular GSH concentration in human lymphoblastoid cells

<table>
<thead>
<tr>
<th>SFN [μM]</th>
<th>QR activity [mU/mg prot.]</th>
<th>GSH [nM/mg prot.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>36.057 ± 4.0</td>
<td>83.53 ± 7.9</td>
</tr>
<tr>
<td>0.5</td>
<td>86.869 ± 7.2</td>
<td>115.82 ± 13.2</td>
</tr>
<tr>
<td>1.0</td>
<td>73.805 ± 5.9</td>
<td>200.41 ± 17.6</td>
</tr>
<tr>
<td>2.5</td>
<td>135.037 ± 8.7</td>
<td>180.06 ± 11.9</td>
</tr>
<tr>
<td>5.0</td>
<td>152.212 ± 10.0</td>
<td>165.14 ± 12.4</td>
</tr>
<tr>
<td>10.0</td>
<td>186.902 ± 15.1</td>
<td>41.68 ± 25.9</td>
</tr>
</tbody>
</table>
In order to determine QR activity, the NADPH-dependent menadiol-mediated reduction of MTT assay was performed (Table 1). SFN was found to induce rapidly and significantly QR activity. The CD value was calculated (CD, concentration required to double the QR activity) at 0.35 \( \mu M \).

Glutathione as the major cell protector against free radicals and electrophiles plays an important role in defending cells against many xenobiotics. In this experiment, cells treated with SFN were tested for intracellular glutathione concentration, both reduced (GSH) and oxidized (GSSG). It was found that SFN increases GSH level in lymphoblastoid cells (Table 1). The increase was rapid for lower SFN concentrations, which were non-toxic and did not cause apoptosis in these cells. With a decrease in cell viability, the GSH level also decreased below the control level.

**DISCUSSION**

Sulforaphane has been shown to activate phase 2 enzymes (Brooks et al., 2001; Jiang et al., 2003), increase GSH concentration (Zhang & Talalay, 1998; Brooks et al., 2001; Zhang & Callaway, 2002) and to induce apoptosis in many kinds of cells, like Hep1c1c7, MCF-7, HeLa, LNCaP, HT29, T lymphocytes (Yu et al., 1998; Gamet-Payrastre et al., 2000; Bonnesen et al., 2001; Chiao et al., 2002; Fimognari et al., 2002).

We studied the effect of SFN on lymphoblastoid cells. When studying the chemoprotective activity of a compound, several factors are usually tested: phase 2 enzyme induction, increase in cellular GSH level, and apoptosis induction.

The cell viability assay was performed to evaluate the cytotoxicity of SFN and to find non-toxic concentrations. The concentration of 1 \( \mu M \) SFN and higher caused a decrease in cell viability in a dose dependent manner. IC\(_{50}\) was calculated at the level of 3.9 \( \mu M \). SFN concentration of 0.5 \( \mu M \) increased cell viability. In addition, the cell morphology changed after SFN treatment. In control conditions, the cells were grown in clusters, almost none were separated. SFN at concentration 1 \( \mu M \) caused separation of the cells, and at 5 \( \mu M \) almost all cells were separated.

Next, we examined the mechanisms of the cell viability decrease. The change in mitochondrial potential during apoptosis occurs at the early stage of the process (Palmer et al., 2000). The control cells had polarized mitochondrial membrane, and the dye used as an indicator of \( \Psi_{mt} \), accumulated forming aggregates, that fluoresce red. After treatment with 1 \( \mu M \) SFN, a partial depolarization of mitochondrial membrane occurred and the appearance of free dye in the cytoplasm, fluorescing green, was observed. Simultaneously a decrease in the intensity of red fluorescence occurred, indicating the change in \( \Delta \Psi_{mt} \). The mitochondrial membrane became completely depolarized when cells were treated with 2.5–10 \( \mu M \) SFN. Mitochondrial membrane depolarization is linked to the release of the numerous factors from the mitochondrion, many of them pro-apoptotic, like Apaf-1 (Purging-Koch & McLendon, 2000) and AIF (apoptosis initiating factor) (Goerke et al., 2002). This leads to caspase activation. D_2R is a substrate for a variety of caspase family members and can detect them simultaneously, which upon activation of these enzymes, gives a strong, clear signal. A significant increase in the fluorescence intensity was detected in cells after 5 \( \mu M \) SFN treatment. The further route of cell death was studied by detecting phosphatidylserine externalization, using the annexin V assay (Willingham, 1999). We observed that at 2.5 \( \mu M \) SFN early apoptosis occurred. At higher concentrations apoptosis was more frequent, and more advanced. In the cells treated with 1 \( \mu M \) SFN we did not observe a significant increase in apoptosis, comparing to the control.

Among phase 2 enzymes, glutathione S-transferase and quinone:NAD(P)H oxido-
reductase are studied most frequently. Because of a wide range of GST family enzymes specificity, it is difficult to evaluate total GST activity in cells (Kolm et al., 1995). We have chosen QR as a marker of phase 2 enzymes activity. SFN was tested as a potential activator of QR activity in lymphoblastoid cells, and was found to induce QR activity at very low concentrations, comparing to those needed to induce apoptosis. The CD value was calculated at 0.35 μM.

QR is also essential in diverting quinones from generation of reactive oxygen species, and consequently from depleting glutathione level (Zhao et al., 1997). To confirm this activity of SFN as a QR inducer in the examined cells, the study of intracellular GSH level was performed. Both reduced and oxidized glutathione were measured using the DTNB–GSSG reductase recycling assay. The increase in GSH level was rapid, for the lowest SFN concentration used (0.5 μM). At 1 μM SFN the increase was the most significant.

At 1 μM SFN, the highest induction of phase 2 enzyme QR was observed as well as the first signs of apoptosis.

It was previously shown that in healthy people’s blood, after ingesting 200 μmoles of broccoli sprouts isothiocyanates (largely SFN), the blood plasma concentration of ITC 3 h after digestion reached less than 1 μM (Ye et al., 2002). Our study shows that in this concentration range, SFN causes elevation of phase 2 enzyme and GSH, constituting a very effective mechanism of elimination of potential carcinogens. Apoptosis is induced at higher SFN concentrations (≥ 2.5 μM).

Our study shows that SFN in physiological ranges of concentration causes phase 2 induction and does not induce apoptosis. Programmed cell death appears only after treatment of cells with SFN concentrations higher than those occurring in human blood after ingestion of isothiocyanates.

In conclusion, our results demonstrate for the first time that SFN acts as a chemo-preventive agent in human lymphoblastoid cells.

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