

IN VITRO ANTIPROLIFERATIVE EFFECTS OF SULFORAPHANE ON HUMAN COLON CANCER CELL LINE SW620

Hana Andělová, Emil Rudolf, Miroslav Červinka

Charles University in Prague, Faculty of Medicine in Hradec Králové, Czech Republic: Department of Medical Biology and Genetics

Summary: The isothiocyanate sulforaphane (SF) has been reported to possess chemopreventive efficiency towards various malignancies including colon cancer. Here, we investigated the antiproliferative and pro-apoptotic effects of SF on colon cancer cell line SW620. We found that SF at concentrations of 10–50 μM inhibits cell viability and proliferation of SW620 cells in a time- and dose-dependent manner, with IC_{50} being 26 μM (24 h), 24.4 μM (48 h) and 18 μM (72 h). Also, in the same cells SF caused DNA damage and chromatin condensation after 24 h and 48 h as revealed by phospho-H2A.X western blot analysis and DAPI staining of nuclei. These changes were accompanied by the elevated activity of caspase 3, although after 20 μM SF concentration only. Together, these results indicate that SF suppresses growth of human metastatic colonocytes and induces apoptotic cell death.

Key words: SW620 cells; Caspase 3, Sulforaphane; Apoptosis; Colon cancer; Proliferation; In vitro

Introduction

Colorectal cancer is among the most frequent types of cancers in the world and the second most leading cause of cancer-related death in industrialized countries. In the Czech Republic, this malignancy is the second most frequent neoplastic disease following the lung cancer in males and breast cancer in females. It has been reported that the colorectal cancer incidence is inversely related to the consumption of fruits and vegetables. *In vitro* and *in vivo* experimental studies have identified phytochemicals (i.e. fibers, carotenoids, vitamins and glucosinolates) as potential dietary chemopreventive agents in the development of cancer of the large intestine. In this respect, vegetables of the *Brassicaceae* family, in particular those of the *Brassica* genus (broccoli, cabbage, cauliflower, etc.) have received much attention.

Sulforaphane (SF) [$\text{CH}_3\text{S}(\text{O})(\text{CH}_2)_4\text{N}=\text{C}=\text{S}$] is a naturally occurring isothiocyanate found as its glucosinolate precursor in cruciferous vegetables like broccoli. Its chemopreventive effects are well documented both *in vitro* and *in vivo*. They include (a) inhibition of phase I cytochrome P450 enzymes, (b) induction of phase II metabolism enzymes, (c) antioxidant functions through increased tissue reduced glutathione (GSH) levels, (d) apoptosis-inducing properties, (e) induction of cell-cycle arrest, (f) anti-inflammatory properties and (g) inhibition of angiogenesis. In addition to these individual mechanisms, their multiple mutual interactions are possible to increase chemopreventive potential of this chemical.

In the present study, we aimed to evaluate the antiproliferative and pro-apoptotic effects of SF on metastatic human colon cancer cell line SW620. We present the evidence that SF decreases cell viability and induces apoptosis in the tested colon cancer cells *in vitro*.

Material and methods

Chemicals

Sulforaphane (SF) (Alexis biochemicals, Axxora Corporation, San Diego, USA)

Cell line treatment

Human colon cancer cell line SW620 (ATCC No. CCL-227TM, San Diego, USA) was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sevapharma, Prague, Czech Republic) supplemented with 10 % foetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, USA). Cells were maintained in 37°C in humidified atmosphere with 5 % CO_2 in an incubator. Passaging took place twice a week upon reaching 90 % cell confluency using 0.05 % EDTA/trypsin.

Neutral Red assay

Cells were plated in six parallels in 96-well plates (Nunc, Roskilde, Denmark), left 24 h in an incubator, and then treated with SF (0–100 μM). After 24, 48 and 72 h cells were incubated with neutral red dye dissolved in D-MEM (3 h/37 °C, 100 $\mu\text{g}/\text{ml}$ final concentration, Sigma-Aldrich, Saint Louis, USA). Cells were then washed with phosphate buf-

ferred saline (PBS), fixed in CaCl_2 (1 g/ml in 0.5 % formaldehyde for 15 min), lysed in 1 % acetic acid in 50 % ethanol for 15 min followed by gentle shaking for 15 min so that complete dissolution was achieved. The absorbance was measured in multiplate reader TECAN SpectraFluor Plus (TECAN Austria GmbH, Gröding, Austria) at 550 nm. Results are expressed as percentage of control values.

Coomassie brilliant blue assay

Cells were plated in six parallels in 96-well plates (Nunclon, Roskilde, Denmark). Following exposure to SF (0 μM - 100 μM) for tested time periods cells were fixed in methanol. Next, cells were rinsed with PBS and fixed in a mixture of ethanol and glacial acetic acid (1:50) for 20 min. Following the addition of brilliant blue in ethanol and acetic acid (200 μl /well), cells were incubated for 1 h. Thereafter, desorption solution (0.1 M potassium acetate in 70 % ethanol - 200 μl /well) was added and the plates with cells were left on a shaker for 1 h. The absorbance was recorded at 620 nm with 450 nm of reference wavelength by a multiplate reader TECAN SpectraFluor Plus (TECAN Austria GmbH, Grödig, Austria). Results are expressed as percentage of control values.

Time-lapse videomicroscopy

SW620 cells were seeded into a 25 ml plastic tissue-culture flask (Nunclon, Roskilde, Denmark) and left for 24 h in an incubator with 5 % CO_2 at 37°C. Next day the standard medium was replaced with a medium containing SF (0 μM , 5 μM , 10 μM , 20 μM and 50 μM , respectively). The tissue-culture flask was transferred into a 37° C-heated room where all recordings were performed. Cells were observed continuously over the 72 h period, using an inverted microscope Olympus IX-71 (Olympus Optical CO, Ltd., Tokyo, Japan) equipped with a long-working-distance condenser, and a 20 X phase contrast lens. For time-lapse recording, the microscope was equipped with a Mitsubishi CCD-100E camera (Mitsubishi Corporation, Tokyo, Japan) and connected to a Mitsubishi video recorder HS-S5600 (Mitsubishi Corporation, Tokyo, Japan). The recording was performed in a 480 mode, with a slowing factor of 160 and it continued for 72 h, with a subsequent video analysis. The recorded sequences were converted to digitized format, processed by the software Adobe Premiere 6.0, and analyzed.

Western Blot analysis

Cells were seeded into 75 ml flasks (Nunclon, Roskilde, Denmark) and cultivated for 24 h. Following SF exposure, treated and control cells were harvested at different time intervals, washed with PBS and lysed. Whole cell extracts were prepared in 500 μl of lysis buffer (137 mM NaCl, 10 % glycerol, 1 % n-octyl- β -D-glucopyranoside, 50 mM NaF, 20 mM Tris - pH 8, 1 mM, Na_3VO_4 , Complete TMMini). The lysates were boiled for 5 min/95°C in SDS sample buffer (Tris-HCl pH 6.81, 2-mercaptoethanol, 10 % glycerol,

SDS, 0.1% bromphenol blue) and thereafter they were loaded onto a 12 % SDS/polyacrylamide gel. Each lysate contained equal amount of protein (20 μg) as determined by Bicinchoninic acid assay (Sigma-Aldrich, Saint Louis, USA). After electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane (100 V, 60 min) and incubated at 25°C for 1.5 h with a solution containing 5 % non-fat dry milk, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1 % Tween 20 (TBST). Membranes were incubated with primary antibodies (monoclonal mouse phospho-H2A.X, 1:100, rabbit polyclonal β -actin, 1:100, Cell Signalling Technology, Inc., Danvers, USA) at 4°C overnight followed by five 6 min washes in TBST. Next, the blots were incubated with secondary peroxidase-conjugated antibodies (1:1000, 1 h, 25 °C, Dako A/S, Glostrup, Denmark), washed with TBST and the signal was developed with a chemiluminescence (ECL) detection kit (Boehringer Mannheim-Roche, Basel, Switzerland). Results were photographed by LightBis system (DNR Bio-Imaging Systems, Jerusalem, Israel). Relative quantifications of phosphorylated H2A.X and β -actin expression were measured using GelQuant Ver 2.7 software (DNR Bio-Imaging Systems, Jerusalem, Israel).

Fluorescence microscopy

SW620 cells were grown on coverslips in an incubator with 5 % CO_2 at 37°C for 24 h. After 24 h, the standard medium was exchanged for a medium with tested concentrations of SF and cells were treated for different time intervals. After the treatment, the cells on coverslips were washed with cold PBS, fixed in 3 % paraformaldehyde, labeled with DAPI (1 $\mu\text{g}/\text{ml}$) (Sigma-Aldrich, Saint Louis, USA) for 5 min and mounted into Prolong Gold® medium (Molecular Probes, Inc. Eugene, USA). The cells were examined under a fluorescence microscope Nikon Eclipse E 400 (excitation filter 330–380 nm and emission filter 420 nm) equipped with the digital color matrix camera COOL 1300 (VDS, Vosskühler, Germany). Photographs were taken using the software Nikon Nis Elements (LIM, Prague, Czech Republic) and analyzed. Experiments were done in triplicates.

Activity of caspase 3

SW620 were seeded into 75 ml flasks (Nunclon, Roskilde, Denmark) and left for 24 h in an incubator with 5 % CO_2 at 37 °C. Next day the standard medium was replaced with a medium containing SF (0 μM , 5 μM , 10 μM , 20 μM and 50 μM , respectively). Cells were grown under these conditions for up to 72 h. Caspase 3 Assay Kit, Fluorimetric (Sigma - Aldrich, Saint Louis, USA) was used according to the manufacturer's instructions. Activity of caspase 3 in cell lysates was measured in multiplate reader TECAN SpectraFluor Plus (TECAN Austria GmbH, Gröding, Austria) at 360/465 nm filter combination. The results were expressed as nmol/min/mg of protein.

Statistics

Statistical analysis was carried out with a statistical program GraphPad Prism 4.0 (GraphPad Software, Inc. San Diego, USA) using the one-way Anova test with Dunnett's post test for multiple comparisons. Results were compared with control samples, and means were considered significant if $P < 0.05$.

Results

SF decreases viability and proliferation of SW620 human colon cancer cells

SF at a tested concentration range (0–100 μM) produced time- and dose-dependent inhibition of SW620 cell viability and proliferation as measured by Neutral red up-

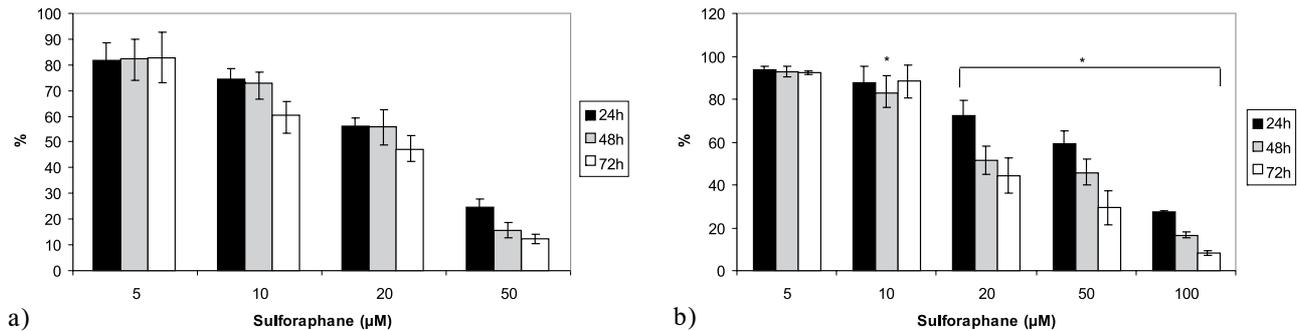


Fig. 1: Effects of sulforaphane (SF) on cell viability and proliferation. SW620 cells were treated with SF concentrations (0–100 μM) for up to 72 h. (a) Neutral red uptake assay, all results are significantly different from control, (b) Coomassie brilliant blue assay. Results are expressed as percentage of control cultures. Values represent the mean \pm SD of three independent experiments. * $P \leq 0.05$, significantly different from control with one way-Anova test and Dunnett's post test for multiple comparisons.

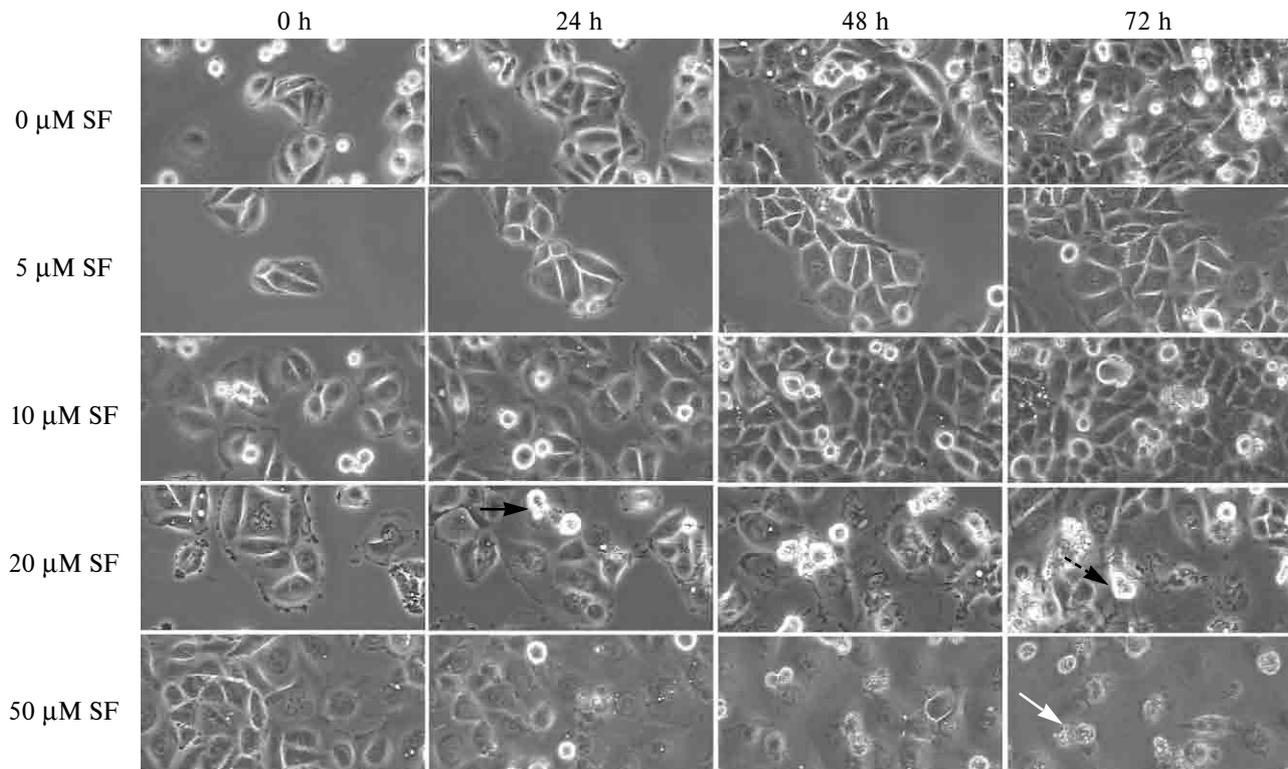


Fig. 2: Time- and concentration-dependent growth inhibition and morphological changes of SW620 cells after the exposure to sulforaphane (SF) at concentrations of 0–50 μM for 72 h. Black arrows indicate apoptotic cells with typical membrane blebbing (full arrow) and late apoptotic cells in terminal shrinkage (dashed arrow). White arrow denotes necrotic cell. Phase contrast (200x). Bar 15 μm .

take assay and Coomassie brilliant blue assay (Fig. 1a,b). Based on Neutral red uptake assay effective cytotoxic concentrations of SF were as follows: $IC_{50} = 26 \mu\text{M}$ at 24 h, $IC_{50} = 24.4 \mu\text{M}$ at 48 h and $IC_{50} = 18 \mu\text{M}$ at 72 h. The results of both assays show a good correlation between SF-dependent loss of cell viability and decreasing cell proliferation during 72 h of treatment. This observation applies for all tested SF concentrations except the lowest (5 μM) and the highest (100 μM). Since cytotoxicity of the highest SF concentration (100 μM) was very marked and occurring early in time, this concentration was omitted from further studies.

Time-lapse videomicroscopy records of SW620 cells exposed to different concentrations of SF up to 72 h demonstrated changes in cellular morphology; i.e. cell rounding followed by a loss of adherence with subsequent cell shrinkage and blebbing. While the lowest SF concentration of 5 μM did not cause any morphological alterations in SW620 cells (as demonstrated in Fig. 2, second row), higher concentrations (10, 20, 50 μM) had a significant effect on both cellular morphology as well as cell proliferation rate. Analyses of individual records proved that dynamics and timing of SF-associated inhibitory effects in SW620 cells differed among the higher SF concentrations (Fig. 2, third to fifth row).

SF induces DNA damage in SW620 human colon cancer cells

DNA damage and the resulting signaling in SF exposed SW620 cells was estimated by means of quantification of the amount of phosphorylated histone H2A.X using western blot analysis. In Fig. 3, we demonstrated that all employed SF concentrations induced double strand breaks in DNA of SW620 cells as detected by phosphorylation of histone H2A.X at serine 139. This effect was dose-dependent (5 μM SF - 130 % of control versus 50 μM SF - 290 % of control as measured in relative intensity units) and occurred as early as at 24 h of exposure.

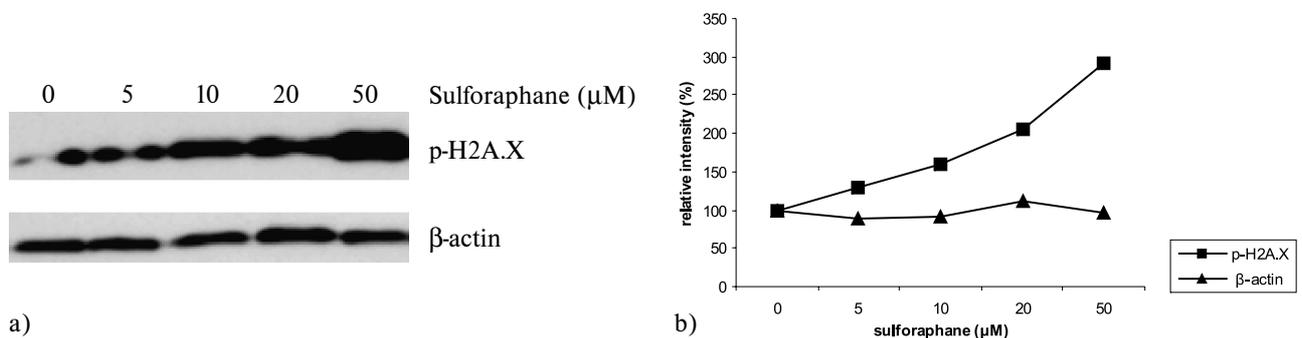


Fig. 3: Levels of phosphorylated H2A.X (p-H2A.X) after 24 h treatment with various sulforaphane (SF) concentrations. SF-treated cells were harvested and p-H2A.X expression was determined by immunoblotting as described under Methods section. (a) Western blot of p-H2A.X and β -actin (control) (b) relative quantification of p-H2A.X and β -actin expression by GelQuant Ver 2.7 software.

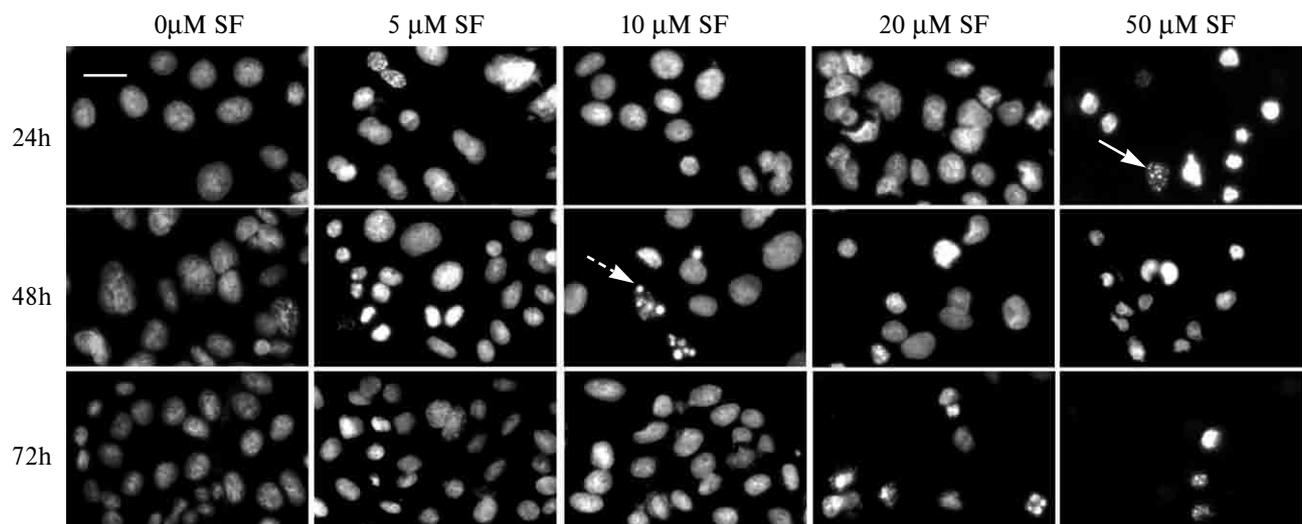


Fig. 4: Apoptotic changes in DAPI stained SW620 nuclei after treatment with sulforaphane (SF) during 72 h. White arrows indicate apoptotic nuclei with early chromatin clumping (full arrow) and late chromatin condensation (dashed arrow). Fluorescent microscopy (200x). Bar 10 μm .

SF activates apoptosis in SW620 human colon cancer cells

Apoptosis in SW620 cells treated with SF was determined by nuclear staining with DAPI. As shown in Fig. 4, apoptotic nuclei with typical chromatin clumped morphology occurred in SF concentrations starting at 10 μM during all followed treatment intervals. In order to confirm apoptosis in the employed model, caspase 3 activity upon the same treatment conditions was carried out. The results indicate that caspase 3 activity was significantly elevated in SF 20 μM treated cells only. All other concentrations did not produce significantly different changes at all treatment intervals (Fig. 5).

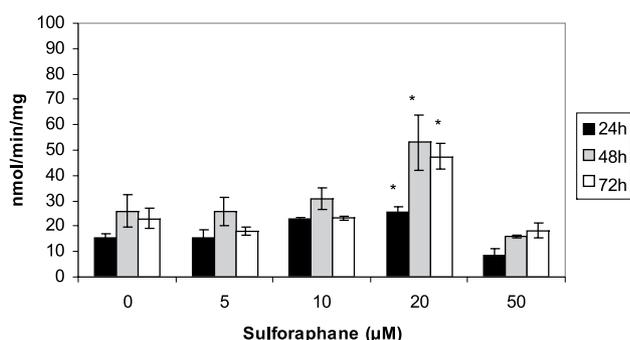


Fig. 5: Effect of sulforaphane (SF) on caspase 3 activity. SW620 cells were treated with SF concentrations (0–50 μM) for up to 72 h. Values represent the mean \pm SD of three independent experiments. * $P < 0.05$, significantly different from control with one way-Anova test and Dunnett's post test for multiple comparisons.

Discussion

Isothiocyanates found in *Brassica* vegetables have been extensively studied as potential chemopreventive agents and their anti-cancer activities have been shown in several cancer models. Sulforaphane, one of the major isothiocyanates derived primarily from broccoli, has been described to inhibit the growth of various cancer cells and to induce apoptosis via numerous mechanisms of which only some are fully understood. Several published studies demonstrated cytotoxic potential of SF towards malignant colonic cells (2, 5); however, experimental evidence concerns only non-invasive and non-metastatic adenocarcinoma models. To determine whether SF is active in advanced stages of colon cancer too, we employed human colon cancer cell line SW620, which is derived from colon cancer metastases in the lymph node.

In the present study, we evaluated antiproliferative effects of SF on SW620 cells, using concentrations of this chemical up to 100 μM during 72 h. We chose this range of concentrations with respect to available pharmacokinetic evidence. Our data indicate that SF exerted an inhibitory effect on proliferation of SW620 cell line. Despite general

agreement in the literature about antiproliferative effect of SF in colon cancer cells, individual published studies report varying IC_{50} values acquired after treatment with this chemical. The reason for these differences is either different sensitivities of the individual employed proliferation assays (i.e. Sulforhodamine B assay, MTT assay or MTS assay) or the unique genetic profile of used cells. Our own results agree with the former possibility as Neutral red uptake assay yielded $\text{IC}_{50} = 26 \mu\text{M}$ while WST-1 assay produced $\text{IC}_{50} = 60 \mu\text{M}$ (unpublished observation) during 24 h exposure to SF. In addition, it seems that a key role in the sensitivity of malignant colonocytes to SF might be at least partially played by the p53 cell status too. Supporting this notion, there was dramatically increased IC_{50} value in cells with mutated p53 (i.e. Caco-2 cells) in contrary to wild type p53 cells (HCT-116).

Although SF has been known to confer partial protection against DNA damage and, in particular single strand breaks in exposed cells, experimental evidence exists that SF induces oxidative stress which might be ultimately responsible for DNA damage. In order to explore this possibility we determined the levels of phosphorylated H2A.X histone in SF exposed cells during 24 h. It is well documented that histone variant H2A.X is phosphorylated in response to DNA double-strand breaks. We found hyperphosphorylation of H2A.X occurring as early as after 24 h of treatment with all concentrations of SF. Furthermore, the amount of phosphorylated H2A.X in cells increased in a time- and dose-dependent manner. Thus our data clearly show that SF induces double-strand breaks in SW620 cells; whether it is due to its direct DNA interaction or via generation of reactive oxygen species (ROS) remains to be explored. Since there is no evidence that SF directly induces DNA breaks, it is reasonable to assume that SF-dependent oxidative stress is the main DNA-damaging mechanism.

Chromatin condensation is one of the most important nuclear events occurring during apoptosis. A typical pattern of apoptotic nuclei occurred after 24 h of 50 μM SF treatment. Moreover, after 48 h of SF treatment there were apoptotic nuclei in cultures treated with 10 μM and 20 μM SF too. Similar result have been published by Gamet-Payrastra who observed apoptotic nuclei in HT-29 colon cancer cells exposed to 15 μM SF for 48 h.

The established hallmarks of apoptotic cell demise are the activation of executionary proteases (caspases) whose activities lead to the appearance of typical morphological features in dying cells. Caspase 3 is the main executionary protease and the involvement of this enzyme in SF treated cancer cells has been reported in several models, however, there is only one report about the activation of caspase 3 following the treatment with SF in human colon cancer models. We observed a slight elevation in caspase 3 activity in SF treated SW620 cells; still, this observation was limited only to 20 μM SF. The absence of significantly increased caspase 3 activity after treatment with other SF concentrations may have several explanations. Firstly, the elevated ac-

tivity of caspase 3 could be only transient, thus our chosen time intervals did not cover these putative activity peaks. Secondly, SF might induce caspase-independent apoptosis too as has been reported previously. Thirdly, SF may have a special “modulatory” effect to caspase 3 as only a low activation of the same caspase was noted in HT-29 cells after SF treatment. The final proof of the involved caspase 3 in SF induced cell death in SW620 cells, however, might be provided by more direct detection of activated caspase 3 and its targets using for instance immunocytochemical methods. We are planning to address this issue in our future experiments.

In summary, SF in a chosen concentration range of 10–50 μ M inhibits the growth and proliferation of metastatic human colon cancer cells SW620 time- and dose-dependently. These effects may be attributed to SF-associated DNA damage and activation of cell death – apoptosis characterized by transient activation of caspase 3 and chromatin degradation. These results form the basis for further studies into specific molecular targets and mechanisms whereby SF exerts its potential chemopreventive activity in colon cancer cells.

Acknowledgements

This work has been supported by Ministry of Education Research Project MSM 0021620820.

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Submitted June 2007.
Accepted August 2007.

Corresponding author:

Mgr. Hana Andělová, Charles University in Prague, Faculty of Medicine in Hradec Králové, Department of Medical Biology and Genetics, Šimkova 870, 500 38 Hradec Králové, Czech Republic, e-mail: andelova@sujchbo.cz
