Research Article

Biphasic modulation of cell proliferation by sulforaphane at physiologically relevant exposure times in a human colon cancer cell line

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Sulforaphane (SFN), a cancer chemopreventive compound derived from broccoli, is able to induce cell cycle arrest and apoptosis in various tumor cell lines. Here we show that cell growth inhibition by SFN follows a biphasic pattern: Transient exposure of 40-16 human colon carcinoma cells for up to 6 h resulted in reversible G_2/M cell cycle arrest and cytostatic growth inhibition even at elevated concentrations, whereas a minimum continuous exposure time of 12 h was necessary for SFN to irreversibly arrest cells in G_2/M phase and subsequently induce apoptosis. IC_{50} values after 12 h of exposure followed by drug-free recovery up to 72 h (6.4–8.1 μM) were indistinguishable from those of chronic exposure for 24 to 72 h (5.4–6.6 μM). Low concentrations of SFN caused a transient decrease in glutathione (GSH) levels followed by GSH induction, which may be related to reversible G_2/M arrest and cytostatic effects. Depletion of GSH does not seem to play a role in SFN-mediated apoptosis induction. Our data clearly contribute to a better understanding of the kinetics of antiproliferative activity of SFN.

Keywords: Broccoli / G_2/M arrest / Glutathione / Reversibility / Sulforaphane

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1 Introduction

Colorectal cancer is the second leading cause of cancer-related deaths in industrialized countries [1]. Strong evidence exists from epidemiological studies that high intake of cruciferous vegetables is associated with a lower risk of developing this disease [2, 3]. Cancer preventive potential of cruciferous vegetables such as broccoli has been attributed to their content of glucosinolates. Through catalytic mediation of myrosinase (β-thioglucosidase), which is released upon physical damage of plant cells (*e.g.*, during cutting or chewing), glucosinolates are hydrolyzed, releasing the corresponding isothiocyanates (ITCs) [4]. ITCs are reactive Michael acceptor-type compounds which rapidly bind to thiol groups, leading to dithiocarbamate formation [5]. Intracellularly, ITCs are conjugated to the tripeptide glutathione (GSH), the most abundant thiol present in the cytoplasm. This generally leads to a transient depletion of GSH, which in turn activates the transcription of γ-glutamylcysteine synthetase (γ-GCS), the key regulatory enzyme in GSH synthesis [6]. Concomitantly, thiol modification of the sensor protein Keap1 activates the Nrf2 pathway, leading to antioxidant responsive element-mediated induction of phase II detoxifying enzymes [7].

The ITC sulforaphane (SFN) was initially identified as the principal inducer of anticarcinogenic protective enzymes from broccoli [8]. Subsequently, chemopreventive efficacy of SFN was demonstrated by inhibition of carcinogen-induced mammary and colon tumorigenesis and of intestinal adenoma formation in $Apc^{Min/+}$ mice [9, 10]. Apart from its modulatory effects on carcinogen metabolism, induction of cell cycle arrest and apoptosis were identified as potential mechanisms contributing to the chemopreventive activities of SFN [4, 11]. In particular, SFN was shown to induce G_2/M cell cycle arrest in a series of cancer cell lines derived from human colon, breast, prostate, and pancreas as well as in human leukemia cell lines [11–13]. On the other hand, non-transformed human T-lymphocytes and cultured human prostate and colon cancer cells were arrested by SFN treat-

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Abbreviations: **BSO,** L-buthionine-*S,R*-sulfoximine; **GSH,** glutathione; **IC_{50},** half-maximal inhibitory concentration; **ITC,** isothiocyanate; **SFN,** sulforaphane

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ment in G_1 phase [11, 14–16]. In many of these cell lines, SFN-mediated cell cycle arrest was accompanied by induction of apoptosis (reviewed in ref. [17–19]).

Under *in vivo* conditions, ITCs like SFN are readily absorbed, metabolized, and excreted *via* the mercapturic acid pathway with peak urinary levels at 1 and 1.5 h after ingestion [4, 11, 20]. The colon is one of the organs with highest exposure to ITCs, both systemically after absorption of free ITC, as well as topologically after ingestion of cooked cruciferous vegetables and subsequent release of ITCs from glucosinolates by the intestinal microflora [4, 11, 21]. In cell culture experiments, SFN and other ITCs accumulate intracellularly in the form of their GSH conjugates and can reach millimolar concentrations. Intracellular accumulation is dependent on sustained uptake; otherwise, ITC–GSH conjugates are rapidly exported from the cells *via* a transporter-mediated process [22]. In this respect, we considered whether the results of *in vitro* studies with incubation times of 24 h or longer would be relevant for the human situation with short transient exposure to ITCs. In particular, using the human colon cancer cell line 40-16, we investigated how (i) the duration of SFN treatment and (ii) drug removal and recovery for up to 72 h would influence cell proliferation and cell cycle progression. In addition, dose-dependent kinetics of GSH depletion and induction were analyzed for a potential correlation with cytotoxic mechanisms.

2 Materials and methods

2.1 Chemicals

Sulforaphane (SFN, CAS no. 4478-93-7) was synthesized as described earlier [23]. All cell culture material was obtained from Invitrogen (Eggenstein, Germany). All material required for flow cytometry was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Fetal bovine serum was provided by PAA Laboratories (Pasching, Austria). McCoy's 5A cell culture medium, sulforhodamine B (SRB), propidium iodide (PI), and RNase A and all other chemicals were obtained from Sigma (Taufkirchen, Germany).

2.2 Inhibition of cell proliferation

The human colon cancer cell line 40-16 derived from HCT116 was provided by B. Vogelstein. We have previously investigated apoptosis-inducing mechanisms of SFN in this cell line [24] and decided to utilize the same cell line for further studies for better comparison of results. McCoy's 5A cell culture medium containing 5% fetal bovine serum was used throughout all experiments. Cells (2.5×10^4 cells/mL) were plated in 96-well plates (200 µL/well). After overnight growth, cells were treated in duplicate with eight serial dilutions of SFN dissolved in DMSO (final DMSO concentration 0.5%) in a concentration range of 0.4–50 µM or with 0.5% DMSO as solvent control for 24, 48, and 72 h. Alternatively, after treatment for either 3, 6, 12, or 24 h, cell culture medium was changed to SFN-free medium, and cells were incubated for up to 24, 48, or 72 h, respectively, to allow recovery. Then the medium was discarded, and cells were fixed using 50 µL of 10% aqueous trichloroacetic acid (TCA) for 30 min at 4°C. Sulforhodamine B staining was performed as described earlier [24]. Calculation of antiproliferative activity was based on the ratio of absorbance readings of treated cells to those of solvent controls (set as 100%) after correction for absorbance of cells present at the time of treatment (day 0 values). Negative values indicate cytotoxic effects. In these cases, day 0 values of the control were used to calculate the percentage of cytotoxicity. Half maximal inhibitory concentration (IC_{50}) values were computed from the results of eight serial two-fold dilutions of SFN tested in duplicate from at least three independent experiments, using TableCurve Windows version 1.0 software (Jandel Scientific, San Rafael, USA) with the equation for a logistic dose–response curve.

2.3 Cell cycle analysis

40-16 cells were plated in 150 mm tissue culture dishes (1.75×10^6 cells/35 mL). After overnight growth, cells were treated with SFN at a concentration of 15 µM or with 0.2% DMSO as a solvent control and incubated for 6, 12, 24, or 48 h. For recovery experiments, cells were treated with SFN for 3, 6, 12, or 24 h before cell culture medium was replaced by SFN-free medium. Incubation with drug-free medium was continued up to 48 h. Attached and floating cells were collected, washed with PBS, pH 7.4, fixed in ice cold 70% ethanol, and stored at −20°C. After washing twice with PBS, cells were incubated with PBS containing propidium iodide (50 µg/mL) and RNase A (100 µg/mL) for 30 min at 37°C. Then they were analyzed by flow cytometry using a FACS Calibur with CELL QUEST PRO software (Becton Dickinson).

2.4 Determination of GSH levels

40-16 cells were seeded in 96-well plates (1×10^4 cells/well). SFN (final concentration 0.4–50 µM in 0.5% DMSO) was added 0.5–24 h prior to determination of total GSH levels. Plates were washed with PBS and stored at -80°C until analyzed. GSH was measured as described previously using 5,5′-dithiobis-(2-nitrobenzoic acid) and normalized to protein concentrations determined using bicinchoninic acid [25].

2.5 Statistical analysis

Results are presented as means ± SD of data originating from at least three independent experiments. For statistical

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evaluation, one-way ANOVA followed by Bonferroni's multiple comparison test was applied. Values of $p < 0.01$ were considered as statistically significant.

3 Results

3.1 SFN-induced inhibition of cell proliferation: Influence of exposure and total incubation time

We have shown previously that SFN exhibits cytotoxic effects toward the 40-16 human colon cancer cell line with IC_{50} values of 7.7, 6.6, and 5.7 μM after 24, 48, and 72 h of continuous treatment, respectively [24]. In the present study, we analyzed SFN-induced effects on cell proliferation after shorter exposure times, and determined the reversibility of the observed effects after drug removal. In this respect, we treated 40-16 cells for 3–24 h with SFN in a concentration range of 0.4–50 μM before cell culture medium was replaced by SFN-free medium. Cells were then allowed to recover for 24, 48, and 72 h. Alternatively, SFN-containing medium was not removed. As shown in Fig. 1A, treatment of 40-16 cells with SFN for 3 h followed by recovery up to 72 h resulted in IC_{50} values of 21, 22.3, and 22.6 μM. IC_{50} values were not significantly influenced by the duration of recovery. After 6 h of SFN treatment, IC_{50} values (10.8–13.1 μM) were about 50% lower than those measured after 3 h of incubation, but still twice as high as without recovery (5.4–6.6 μM). In contrast to these short treatment periods, 12 and 24 h incubation with SFN resulted in irreversible cytotoxicity. Specifically, IC_{50} values after 12 h of SFN treatment followed by recovery up to 72 h (6.4–8.1 μM) did not differ significantly from IC_{50} values obtained after 24–72 h exposure without drug removal.

A comparison of profiles of cell growth inhibition after SFN exposure for 3–24 h revealed obvious differences between short (up to 6 h) and longer (12 and 24 h) exposure times. An example of 48 h total incubation is given in Fig. 1B. SFN treatment for 3 and 6 h resulted in cytostatic effects. Even at a concentration of 50 μM, cell numbers (*i. e.*, absorbance values after SRB staining) were still above the initial baseline, indicating inhibition of cell proliferation, but no cytotoxicity. In contrast, after 12 and 24 h exposure, SFN displayed cytotoxic effects at concentrations higher than 12.5 μM, measured by a reduction in cell numbers to values below the initial baseline.

3.2 Effects of SFN on cell cycle progression

SFN has been described to cause G_1 or, more frequently, G_2/M phase cell cycle arrest in various cancer cell lines [11–16]. Therefore, we were interested to know how effects of SFN on cell cycle progression in 40-16 cells would relate to the observed biphasic pattern in cell growth inhibition.

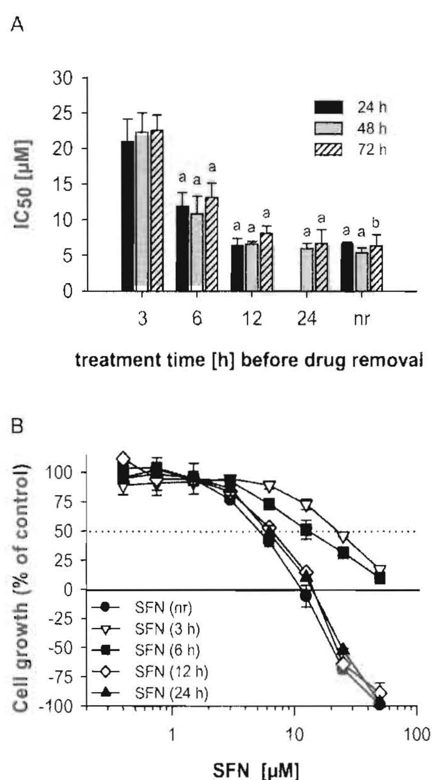

Figure 1. Time-dependent inhibition of cell proliferation by SFN and the effect of drug removal. (A) Inhibition of cell proliferation was determined by sulforhodamine B staining. Cells were treated with eight different concentrations of SFN (0.4–50 μM) or 0.5% DMSO as a solvent control for 3, 6, 12, and 24 h before replacement of SFN-containing cell culture medium by SFN-free medium and further incubation for up to 24 h (black bars), 48 h (gray bars) or 72 h (stippled bars). Alternatively, cells were treated continuously (nr, no removal) for 24 h (black bar), 48 h (gray bar), or 72 h (stippled bar), respectively. IC_{50} values were calculated as described in Section 2. Data are means ± SD of three independent experiments. (a) Significantly different ($p < 0.01$) compared with 3 h SFN treatment within the same total incubation time. (b) Significantly different ($p < 0.01$) compared with 3 and 6 h SFN treatment within the same total incubation time. (B) Profiles of cell growth inhibition after treatment with SFN for 3–24 h as indicated, before cell culture medium was changed to drug-free medium (nr, no removal). Data were taken from one representative cell proliferation experiment with a total incubation time of 48 h. Dose-dependent inhibition of proliferation is shown by values above the baseline, whereas values below the baseline represent cytotoxic effects.

Cells were treated with SFN at a concentration of 15 μM for 6–48 h. Cell cycle distribution was assessed by flow cytometry after DNA staining with propidium iodide. Results are depicted as representative histograms in Fig. 2.

SFN induced a cell cycle arrest in G_2/M phase that was already visible after 6 h (41.4 ± 4.3 *vs.* 29.5 ± 5.2% in the control). The accumulation of cells in G_2/M phase increased with incubation time, with mean percentages of 51.2 ± 3.8

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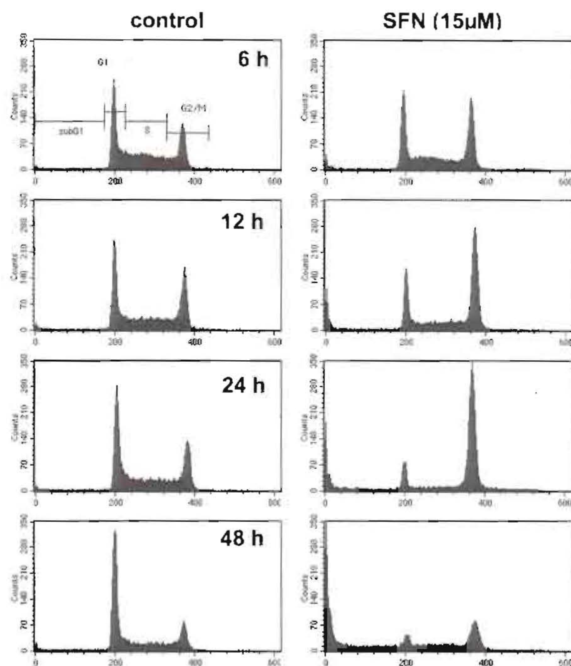

Figure 2. Cell cycle effects of SFN. 40-16 cells were treated with SFN (15 µM) or 0.2% DMSO (untreated control) for 6, 12, 24, or 48 h, respectively. Cell cycle analyses of fixed cells were performed by flow cytometry after staining DNA with propidium iodide. Representative histograms of one out of four independent experiments are depicted.

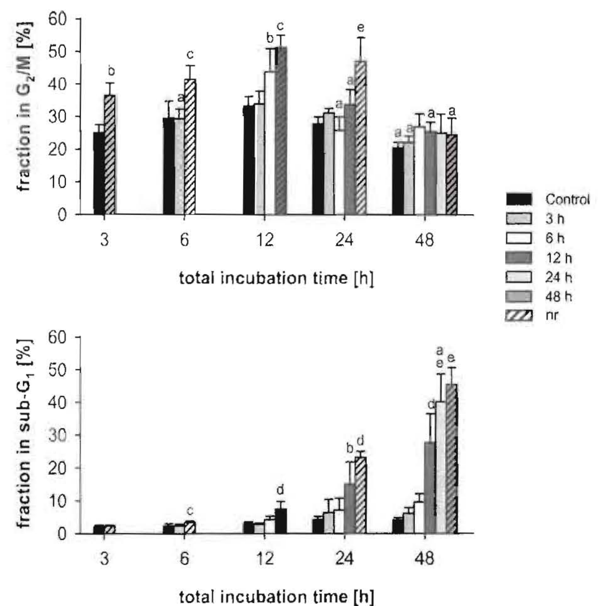

Figure 3. SFN-induced cell cycle effects and the influence of exposure time and drug removal. Data were taken from Table 1. 40-16 cells were treated with SFN (15 µM) or 0.2% DMSO for 3, 6, 12, or 24 h before medium change and drug-free incubation for up to 48 h total incubation time. Alternatively, cells were treated with SFN continuously for 3, 6, 12, 24, or 48 h, respectively (nr, no removal, represented by stippled bars). Cell cycle analyses of fixed cells were performed by flow cytometry after staining DNA with propidium iodide. Data for G_2/M and subG_1 phase are shown as means ± SD of four independent experiments. Letters indicate significant differences as in Table 1.

versus 33.3 ± 2.9% after 12 h and 47.0 ± 7.3 *versus* 27.8 ± 2.1% after 24 h, respectively. After 48 h, the G_2/M arrest was no longer detectable. In contrast, we observed a marked increase in cells in subG_1 phase. This was in good agreement with our earlier investigations on the induction of apoptosis in 40-16 cells by 15 µM SFN [24].

To further explain the results described in Fig. 1, cell cycle analyses were also performed to investigate the influence of the duration of SFN exposure and recovery time on cell cycle distribution. 40-16 cells were exposed to SFN at a concentration of 15 µM for increasing time periods from 3 to 48 h and then harvested or allowed to recover for total incubation times up to 48 h. The results are summarized in Table 1. Most relevant changes in sub-G_1 and G_2/M fractions are depicted in Fig. 3.

SFN treatment for only 3 h already induced a significant increase in the G_2/M fraction of the cells (36.4 ± 4.0%) compared to untreated control cells (25.1 ± 2.4%), concomitant with a reduction of cells in G_0/G_1 phase. After drug removal, cells were able to recover quickly: further incubation for 3 h in drug-free medium (6 h total incubation time) restored the percentage of cells in G_2/M phase to control values.

Continuous exposure to SFN for 6 h provoked a significant G_2/M arrest (41.4 ± 4.3%; compare Figs. 2 and 3), whereas G_0/G_1 and S phases were significantly reduced (for statistical evaluation see Table 1 and footnotes for explanation). The observed G_2/M arrest was still evident after 6 h of recovery in SFN-free medium (12 h total incubation time: 43.8 ± 7.1%). The percentage of cells in S phase as an indicator of cell proliferation was further reduced at this time point (6 h: 26.8 ± 2.0% *vs.* 12 h: 19.5 ± 3.1%). Importantly, another 12 h of recovery (at a total incubation time of 24 h) abolished the G_2/M arrest (25.8 ± 4.1%), although it was clearly evident after 24 h of continuous exposure to SFN (47.0 ± 7.3%; compare Figs. 2 and 3). These results indicated that the G_2/M arrest induced by SFN treatment for 6 h was almost completely reversible.

Continuous SFN exposure for 12 h resulted in the highest accumulation of cells in G_2/M phase (51.2 ± 3.8 *vs.* 33.3 ± 2.9% in the untreated control; compare Figs. 2 and 3). Further incubation in drug-free medium up to 48 h led to a gradual decrease in G_2/M-arrested cells, paralleled by an increase in cells in subG_1 phase, indicative of apoptosis induction (total incubation time 24 h: 14.9 ± 6.9%; 48 h: 27.7 ± 8.8%; compare Fig. 3). Nevertheless, 48 h continuous exposure to SFN was significantly more effective in inducing apoptosis than 12 h treatment plus 36 h recovery time (45.5 ± 5.1 *vs.* 27.7 ± 8.8%). Of notice, we have demon-

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Table 1. Summary of reversibility experiments of SFN-induced cell cycle effects (mean ± SD)

Total incubation time (h)	$SubG_1$ (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
Control (no SFN treatment)				
3	2.1 ± 0.5	47.0 ± 3.5	25.9 ± 3.0	25.1 ± 2.4
6	2.3 ± 0.7	36.5 ± 3.7[a)]	31.9 ± 3.9[a)]	29.5 ± 5.2
12	3.1 ± 0.5	37.0 ± 1.9	26.4 ± 0.9[a)]	33.3 ± 2.9
24	4.3 ± 0.9	42.8 ± 2.3	24.4 ± 2.1	27.8 ± 2.1
48	4.1 ± 0.7	56.1 ± 4.5[a)]	19.1 ± 3.3	20.4 ± 1.8[a)]
Treatment with SFN (15 μM) for 3 h				
3	2.4 ± 0.2	36.9 ± 1.6[b)]	24.4 ± 3.2	36.4 ± 4.0[b)]
6	2.4 ± 0.5	42.4 ± 1.7[a)]	25.9 ± 1.6[b)]	29.3 ± 3.0[a)]
12	2.8 ± 0.4	42.8 ± 4.8	19.4 ± 2.1[b)]	33.9 ± 4.0
24	6.3 ± 4.1	37.8 ± 3.0	22.7 ± 3.7	31.1 ± 1.4
48	6.1 ± 1.8	50.7 ± 2.5[a)]	20.2 ± 2.6	22.1 ± 2.0[a)]
Treatment with SFN (15 μM) for 6 h				
6	3.4 ± 0.5[c)]	28.2 ± 6.0[c)]	26.8 ± 2.0[b)]	41.4 ± 4.3[c)]
12	4.2 ± 1.1	30.9 ± 10.1	19.5 ± 3.1[a),b)]	43.8 ± 7.1[b)]
24	7.1 ± 3.6	37.0 ± 10.1	23.4 ± 5.4	25.8 ± 4.1[a)]
48	9.6 ± 2.5	43.4 ± 7.3[b)]	18.6 ± 3.5	26.9 ± 4.0
Treatment with SFN (15 μM) for 12 h				
12	7.4 ± 2.4[d)]	21.6 ± 3.6[c)]	18.7 ± 2.0[b)]	51.2 ± 3.8[c)]
24	14.9 ± 6.9[b)]	35.7 ± 10.6	11.1 ± 1.5[a),d)]	33.6 ± 4.7[a)]
48	27.7 ± 8.8[d)]	23.2 ± 6.3[d)]	16.0 ± 3.1	25.4 ± 2.9[a)]
Treatment with SFN (15 μM) for 24 h				
24	23.2 ± 1.8[d)]	16.1 ± 2.6[e)]	12.2 ± 4.4[d)]	47.0 ± 7.3[e)]
48	40.2 ± 8.5[a, e)]	12.3 ± 1.4[d)]	16.0 ± 1.4	25.0 ± 5.9[a)]
Treatment with SFN (15 μM) for 48 h				
48	45.5 ± 5.1[e)]	14.0 ± 6.8[d)]	14.4 ± 3.5	24.4 ± 5.2

a) Mean significantly different ($p < 0.01$) from preceding total incubation time without SFN treatment (control) or within the same SFN treatment time.
b) Significantly different ($p < 0.01$) from untreated control of the same total incubation time.
c) Significantly different ($p < 0.01$) from untreated control and from 3 h treatment with SFN within the same total incubation time.
d) Significantly different ($p < 0.01$) from untreated control and from 3 and 6 h treatment with SFN within the same total incubation time.
e) Significantly different ($p < 0.01$) from untreated control and from 3, 6, and 12 h treatment with SFN within the same total incubation time.

strated recently by Western blotting *via* the detection of poly (ADP-ribose) polymerase cleavage that the percentage of cells in $subG_1$ phase caused by SFN treatment directly correlated with apoptosis induction in the 40-16 cell line [26].

After 24 h incubation with SFN, G_2/M phase arrest similar to that observed after 12 h was evident. At the same time point, a substantial portion of cells was apoptotic (23.2 ± 1.8% $subG_1$ *vs.* 4.3 ± 0.9% in the untreated control). When cells were incubated with SFN for 24 h and allowed to recover for further 24 h without SFN, there was no significant difference in the percentage of cells in $subG_1$ phase compared to 48 h SFN treatment (40.2 ± 8.5 *vs.* 45.5 ± 5.1%; compare Fig. 3).

Taken together, short treatment times with SFN for 3 and 6 h led to a transient cell cycle arrest in G_2/M phase, which was reversible after drug removal. After exposure for 12 and 24 h, the early G_2/M arrest turned into apoptosis induction with total incubation times of 24 and 48 h, indicated by an increase in the percentage of cells in $subG_1$ phase. Exposure of 40-16 cells to SFN for 12 h was sufficient for an irreversible commitment to cell death, which confirmed the results obtained with the cytotoxicity experiments depicted in Fig. 1.

3.3 Effects of SFN on cellular GSH levels

GSH depletion was suggested to play a role in SFN-induced cell cycle arrest and apoptosis [13, 27]. We therefore tested whether the kinetics of thiol depletion would be related to the growth inhibition profile observed with SFN. 40-16 cells were treated for 30 min to 24 h with SFN at concentrations of 6.25, 12.5, and 25 μM. In addition, we used L-buthionine-*S,R*-sulfoximine (BSO), a specific inhibitor of g-GCS [6], as positive control for GSH depletion.

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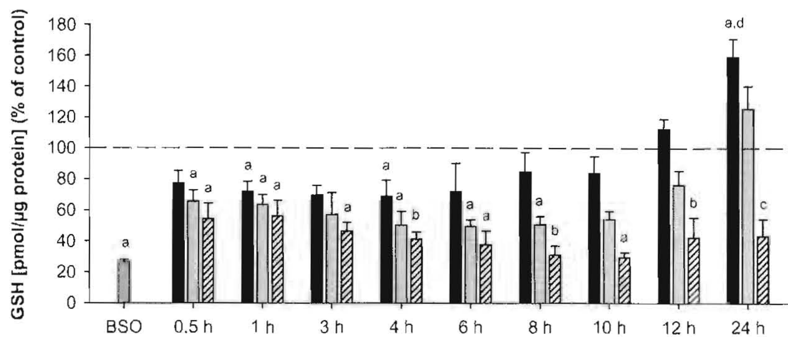

Figure 4. Effects of SFN on total intracellular GSH levels. 40-16 cells were treated with SFN for 0.5–24 h. Results obtained with SFN at 6.25 µM (black bars), 12.5 µM (light gray bars), and 25 µM (stippled bars), and BSO (10 µM, 16 h, dark gray bar) as a positive control are shown as a percentage of the respective solvent control. Means ± SD significantly different ($p < 0.01$) from (a) respective control, (b) control and 6.25 µM, (c) control, 6.25 and 12.5 µM, (d) preceding time point with identical SFN treatment ($n = 3$). GSH levels in the controls were in the range of 55–80 pmol/µg protein.

Treatment with BSO at a concentration of 10 µM for 16 h markedly lowered GSH levels to 26.4 ± 1.5% of the untreated control (Fig. 4). Of note, BSO exerted no cytotoxicity, since protein levels were not changed compared to control cells (data not shown). SFN treatment led to a rapid depletion in GSH levels within 30 min, especially at the two higher concentrations. After treatment with SFN at a concentration of 6.25 µM, GSH levels transiently decreased up to 4 h, followed by gradual elevation to 159.5 ± 11.7% of the untreated control after 24 h, indicative of an induction of GSH synthesis. A similar trend was observed with the intermediate concentration, although the drop in GSH was more pronounced and longer lasting (minimum 49.5 ± 4.1% after 6 h), and GSH resynthesis was slower with lower maximum induction, at least up to 24 h. In contrast, when cells were treated with 25 µM SFN, GSH levels dropped to a minimum of 29.5 ± 3.2% after 10 h and did not recover, suggestive of irreversible cytotoxicity.

4 Discussion

In the present study, we demonstrated that the time-dependent profile of cell growth inhibition by SFN was biphasic. Short exposure times up to 6 h led to cytostatic inhibition of cell proliferation, which was observed with concentrations up to 50 µM and was sustained up to 72 h even after drug-removal. Further, short exposure times resulted in a transient G_2/M arrest, which was rapidly reversible when exposure was terminated after 3 h followed by drug-free incubation, and longer-lasting, but still reversible when cells were treated for 6 h with 15 µM of SFN. On the other hand, treatment for at least 12 h caused cytotoxic inhibition of cell proliferation and was necessary to efficiently induce cell death. Detailed analyses of molecular mechanisms and a potential causal relation between cell cycle arrest in G_2/M phase and apoptosis induction are currently underway (Pappa *et al.*, in preparation).

Gamet-Payrastre *et al.* [28] investigated reversibility of SFN-induced apoptosis in HT29 human colon cancer cells. A minimum 24 h treatment was required for irreversible induction of cell death. Synchronized cells treated with SFN were unable to re-enter the cell cycle, but cell proliferation was initiated without signs of cytotoxicity after SFN removal. Thus, resting cells in G_0 seemed to be resistant toward SFN. Zhang *et al.* [29] treated various cancer cell lines with SFN, phenethyl-ITC, allyl-ITC, and benzyl-ITC for 72 h or for 3 h followed by 69 h drug-free incubation. Interestingly, while IC_{50} values of SFN were significantly lower after 72 h continuous treatment compared to 3 h plus 69 h recovery time, IC_{50} values of the other three ITCs were similar for both time regimes. Thus, SFNs biphasic growth inhibition profile with delayed initiation of cytotoxicity seems to be unique for this ITC.

Consistent with our data, SFN-induced G_2/M arrest was reported to be irreversible after 16 h in PC-3 human prostate cancer cells [30]. In that study, the authors postulated that G_2/M cell cycle arrest was due to oxidative stress-induced DNA damage. Using the dye 2′,7′-dichlorodihydrofluorescein diacetate sensitive to reactive oxygen species, we could not detect any significant enhancement of oxidative stress after treatment of 40-16 cells with 15 µM SFN for several time points (data not shown).

A recent report demonstrated that significant oxidative stress and resulting DNA damage was correlated with depleted pools of mitochondrial and nuclear GSH, but not with cytoplasmic GSH levels [31]. Consistently, in the present study, reduced GSH levels due to inhibition of GSH synthesis by BSO [32] did not result in toxic effects or cell growth inhibition. Also, time- and dose-dependent depletion and resynthesis of GSH by SFN was not clearly related with cytotoxic potential. SFN treatment for 6 h at a concen-

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tration of 12.5 μM, which we assume to produce comparable effects on cell cycle and apoptosis induction as 15 μM used for the data summarized in Table 1, reduced GSH levels by 50%. This was associated with a transient cell cycle arrest in G_2/M (Table 1) and cytostatic inhibition of cell proliferation (Fig. 1), but was not sufficient to induce cell death. In contrast to short incubation times, continuous exposure to 12.5 μM SFN for 12 and 24 h resulted in GSH resynthesis, while causing sustained G_2/M arrest and commitment to undergo apoptosis. Higher concentrations of SFN resulted in stronger GSH depletion, but were merely cytostatic when exposure was discontinued after 3 and 6 h (Fig. 1B). Taken together, these data indicate that GSH depletion does not play a major role in SFN-induced apoptosis in the 40-16 cell line, but may be related to the observed G_2/M cell cycle arrest and cytostatic cell growth inhibition. Cytoplasmic GSH depletion may also be an indicator for intracellular SFN accumulation, which needs to be sustained for at least 12 h to cause irreversible cytotoxicity.

Further pharmacokinetic investigations need to clarify which organs may be affected by SFN or its metabolites for 12 h or longer after ingestion of cruciferous vegetables. Regarding the colon, the situation is unique due to the fact that this organ is exposed to SFN both systemically and topologically. The length of exposure to SFN and its metabolites then depends on the way in which vegetables are consumed (raw *vs.* cooked) and on intestinal transit time.

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