Effect of sulforaphane on cell growth, G_0/G_1 phase cell progression and apoptosis in human bladder cancer T24 cells

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Abstract. Isothiocyanates (ITCs) from cruciferous vegetables have been shown to be effective in blocking initiation as well as progression of a range of chemically-induced tumors in animal models. In this study, sulforaphane, the most extensively studied ITC, was found to suppress the growth of T24 bladder cancer cells *in vitro* in a dose-dependent manner. Sulforaphane inhibited the proliferation of T24 cells with IC_{50} values 26.9 and 15.9 μM following 24 and 48 h treatments. Sulforaphane (5-20 μM) induced early apoptosis and blocked cell cycle progression at G_0/G_1 phase which was associated with up-regulation of cyclin-dependent kinase inhibitor p27 expression. These results support a role for sulforaphane as an effective agent in the chemoprevention of bladder cancer.

Introduction

To date, more than twenty isothiocyanates (ITCs), both naturally occurring and synthetic, have been shown to suppress the progression of carcinogenesis in both *in vivo* and *in vitro* models. These data are supported by epidemiological studies which have reported an inverse association between dietary intake of ITCs and risk of pancreatic, lung, colon, ovarian and bladder cancer (1-6). Although the epidemiological data are supportive of a role for higher consumption of cruciferous vegetables protecting against bladder cancer (5-7), the data from animal models of bladder cancer are controversial. For example, in two rat models, high levels of ITCs (0.1% of diet) increased incidence of bladder carcinogenesis (8,9). However, at physiological relevant levels, ITCs do exhibit anti-cancer mechanisms such as induction of phase II detoxification enzymes and anti-proliferation that are highly relevant to bladder cancer prevention.

Sulforaphane (SFN) derived from glucoraphenin [4-methyl-sulfinyl-butyl glucosinolate], is abundant in many cruciferous vegetables including broccoli. SFN has been shown to possess bioactivities such as inhibition of phase I enzymes (10), induction of phase II enzymes (11) and to protect against heterocyclic amine-induced DNA adduct formation (12). Other mechanisms such as induction of apoptosis, cell cycle checkpoint (13,14) and inhibition of histone deacetylase (15) and tubulin poly-merization (16) have also been reported.

Ingested ITCs are metabolised principally via the mercapturic acid pathways *in vivo*, forming N-acetylcysteine (NAC) conjugates, which are concentrated and excreted ultimately in urine. Since urine containing NAC conjugates is stored in the bladder, bladder epithelium is a significantly exposed tissue to ITCs (17), and recent data have shown that NAC conjugates have similar anti-proliferation activity to their parent ITC compounds (17). Bladder cancer is a common tumor of the urinary system, and approximately 75% of patients present with superficial cancer, 20% with invasive disease, and the remaining 5% with metastatic disease at first diagnosis. Bladder cancer is three times more common among men than women, and the incidence increases with age (18). If diagnosed early, survival is good, highlighting the importance of a timely and accurate diagnosis (18,19). Most superficial lesions of bladder cancers are removed by transurethral resection (18). However, more than half of the patients experience recurrence in less than 5 years after surgery (17). Consequently, a crucial challenge for patients after removal of primary loci is to prevent recurrence, and the potent chemopreventive role of diet merits further attention. In this study, the effect of SFN on human bladder cancer T24 cell growth and cell cycle block was investigated.

Materials and methods

Reagents. L-SFN ((*R*)-1-isothiocyanato-4-(methylsulfinyl) butane4-methylsulfinylbutyl isothiocyanate, purity ≥98%) was purchased from Sigma (Sigma-Aldrich China Inc., Shanghai, P.R. China). Alkaline phosphatase (AP) system with stabilized

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Abbreviations: SFN, sulforaphane; ITC, isothiocyanates; CKI, cyclin-dependent kinase inhibitor; CDK, cyclin-dependent kinase; PBS, phosphate-buffered saline

Key words: isothiocyanate, sulforaphane, bladder, cell cycle, apoptosis

substrate was purchased from Promega Corp. (Shanghai, P.R. China). RPMI-1640 medium was purchased from Invitrogen Corp., fetal bovine serum (FBS) was purchased from Institutes of Biotechnology, Chinese Academy of Sciences (Shanghai, P.R. China).

Cell line. Human bladder cancer T24 cells, derived from grade III transitional cell cancer of bladder, were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and supplemented with 10% FBS. All cells were grown in 75-cm^2 flasks in a humidified incubator at 37˚C with 5% CO_2. SFN was dissolved in dimethyl sulfoxide (DMSO), the final DMSO concentration in the medium was ≤0.1% (v/v). The vehicle control was RPMI-1640 medium added with equivalent amount of DMSO.

Cell survival assay. A total of 1x10^4 cells per well were seeded in a 96-well plate with 100 μl medium for 4 h. After the cells attached, the original medium was replaced with 200 μl fresh medium containing different concentrations of SFN. After the cells were incubated with SFN for 24 and 48 h, 10 μl 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) was added into each well and the incubation was continued for further 4 h. Then 150 μl medium in each well was aspirated and equal volume of DMSO was added to dissolve the purple crystal dye. The optical absorbance was read on a microplate reader at 570 nm. Results were measured in quadruplicate. The inhibitory rate of cell proliferation was calculated: inhibitory rate (%) = $(A_{control}-A_{treated})/A_{control}$ x 100%.

Measurement of cell growth curve. Growth inhibition of T24 cells by SFN was measured by MTT assay. The cells were seeded from 2x10^6 cells/ml to 2x10^3/ml by a 2-fold serial dilution. Results were measured in quadruplicate. Based on the standard curve, appropriate cell densities for seeding were chosen and exposed to a range of concentrations of SFN (5-20 μM) for 7 days (the medium was changed at 72 h, and the appropriate dose of SFN was added). Results were measured in triplicate. Absorbance in each well was read every 24 h and cell densities were calculated against a standard curve.

Analysis of apoptosis and cell cycle arrest. Early cell apoptosis was measured by a flow cytometry-based Annexin V-FITC Apoptosis Detection Kit, according to manufacturer's instruction (Becton-Dickinson Medical Devices Co. Ltd., Shanghai). Cells undergoing early apoptosis were discriminated from late apoptotic and necrotic cells. Briefly, cells were harvested after the treatment with SFN and washed twice with ice-cold phosphate-buffered saline (PBS). Then the pellets were resuspended in 1 ml binding buffer and cell density was adjusted to 0.5-1.0x10^6 cells/ml. Finally, Annexin V-FITC and propidium iodide (PI) were added into the cell suspensions, stained away from light for 15 min; and apoptotic cells were measured within 1 h using a flow cytometer (BD FACSCanto™).

Cell cycle arrest was detected by flow cytometry analysis. After SFN treatments, cells were harvested using trypsin/EDTA. The pellets were suspended and fixed in 70% ice-cold ethanol at 4˚C for 24 h, then followed by flow cytometric analysis on a flow cytometer after propidium iodide labeling.

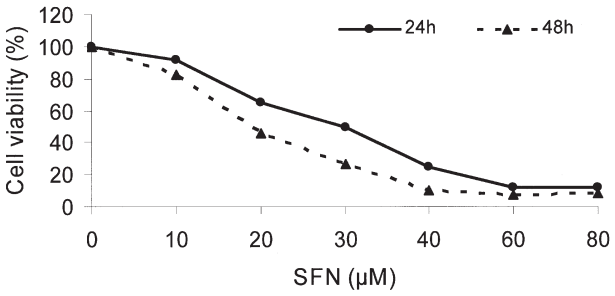

Figure 1. The inhibitory effects of sulforaphane on T24 cells. Cells (10^5/ml) were incubated with sulforaphane at 10-80 μM for 24, 48 h at 37˚C. Each value was calculated as the previous formula for the inhibitory rate. Data are presented as mean ± SD (n=5).

Western blotting. At the end of SFN treatments, cells were harvested, washed with PBS and pelleted by centrifugation. Then cell pellets were suspended in cell lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100) and supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) for about 2 h at 4˚C. At the end of lysis, the lysates were centrifuged at 10,000 x g for 10 min at 4˚C, and the supernatant fractions were used for following Western blot analysis. The denatured sample (40-60 μg protein) was electrophoresed in SDS-PAGE (10-12%) and was transferred to nitrocellulose membranes. The membranes were probed with antibodies, and the bands interested and ß-actin were semi-quantified by scanning the area of the band. The ratio between targeting protein and ß-actin were calculated.

Statistical analysis. Results are expressed as mean ± SD. Data were analysed by one-way ANOVA, followed by Dunnett's t-test for separate comparisons with the control group or followed by Sidak's t-test for multiple-group comparisons. Differences were considered significant at $p<0.05$.

Results

Effect of SFN on T24 cell growth. Fig. 1 shows that SFN has a significant inhibitory effect on T24 cell growth. The effects of a range of concentrations of SFN (10-80 μM) on cell growth were tested following incubation for 24 and 48 h. The effective dose for suppression of T24 cell growth was observed after 10-40 μM SFN treatments. Inhibitory rates were 8.6±5.1%, 35.1±2.1%, 50.6±1.0% and 75.1±4.3% after 10, 20, 30 and 40 μM SFN, respectively following incubation for 24 h. For 48 h treatment, the inhibitory rates were 17.2±6.7%, 53.8±2.1%, 73.2±0.9% and 89.9±0.7%, respectively over the same range of SFN. The IC_{50} values were 26.9±1.12 μM (24 h) and 15.9±0.76 μM (48 h) respectively.

The effectively lower doses of SFN (10-40 μM) in the inhibition of cell growth were further tested. In the control group, the cells grew rapidly once attached, entering the fast proliferation phase by day 3. Following SFN treatment, no significant cell proliferation was observed in cells treated with 20 μM SFN for 5 days in comparison with initial seeding, after 3 days incubation, the cell numbers had decreased

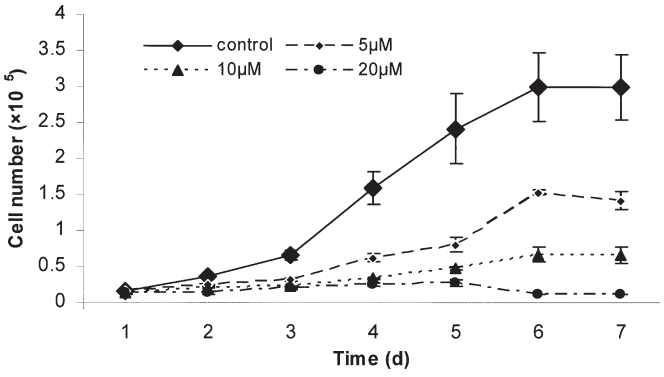

Figure 2. The growth curve of T24 cells with different SFN treatment. Cells were cultured in 96-well plate at 0, 5, 10 and 20 μM SFN medium for 7 days (in triplicates). After the cells in each well were incubated for 72 h, the original medium was aspirated and another medium containing previous concentrations of SFN was added back until the end of experiment. In each day of incubation, cell density in each well was measured by MTT assay.

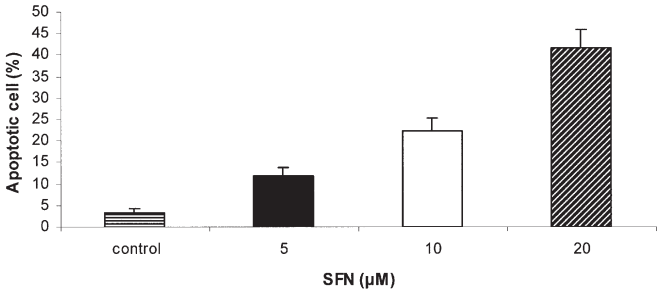

Figure 4. The effects of SFN on early apoptosis in T24 cells. After the incubation with different doses of SFN for 24 h, cells were harvested and washed with PBS. Then the pellets were suspended in ice-cold PBS and apoptotic cells were measured within 1 h. The percentage of apoptotic cells are presented as mean ± SD in comparison with control, p=0.029, p=0.014, p=0.006 in 5, 10 and 20 μM SFN treatments respectively (n=3).

significantly following treatments of cells with 5-20 μM SFN compared to control. The cell number in the control wells was $6x10^4$ and decreased to $3.2x10^4$, $2.2x10^4$, $2.0x10^4$ cells after exposed to 5, 10 or 20 μM SFN. No significant change in cell proliferation was observed in cells treated with 20 μM SFN for 5 days compared to control. Following a 7-day treatment, cell numbers continued to decline to $1.4x10^4$, $0.65x10^4$, $0.11x10^4$ compared to $3x10^5$ in the control, with inhibitory rates of 51.7±5.3%, 77.8±6.2% and 96.3±0.5% respectively (Fig. 2).

Nuclear changes of T24 cells stained by AO/EB. Incubation of T24 cells with 5-20 μM SFN for 24 or 48 h resulted in nuclear changes as observed under an optical microscope. However, significant apoptotic features such as cell shrinkage, condensed chromatin and apoptotic bodies were only observed after treatment with 20 μM SFN for 24 and 48 h. Moreover, the presence of apoptotic cells induced by SFN was further confirmed by fluorescent staining (AO/EB) using a flow cytometric method for early apoptosis detection (Fig. 3).

Measurement of early apoptosis. Some studies have suggested that the effects of cruciferous vegetables may be more important in early carcinogenesis, i.e. initiation, while they may be also important at the tumor promotion or progression stages. To test this hypothesis, the cells were treated with 5, 10 and 20 μM SFN for 24 h and then cells were harvested for the measurements of early apoptosis using a flow cytometry-based AV-FITC Apoptosis Detection Kit. The early apoptotic rates in the treatment groups were 12.0±1.7%,

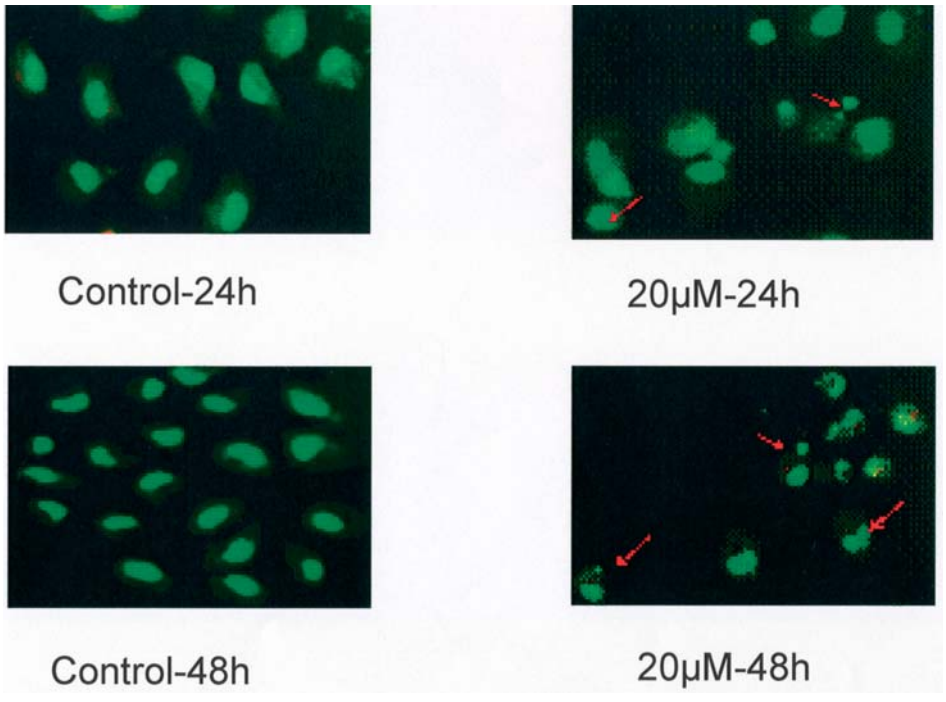

Figure 3. Morphological changes of T24 cell nucleus stained by AO/EB (x200). Cells were harvested after treatment with SFN for 24 and 48 h. Cell pellets were washed with PBS 3 times then suspended and stained with AO/EB in lightness for about 5 min. Then the cell morphological changes of nuclei were observed under a fluorescent microscope.

Table I. The effect of SFN on cell cycle progression of T24 cells (%).

SFN	G_0/G_1	S	G_2/M	Sub-G_1
0	39.5±2.2	46.1±4.7	13.7±2.8	-
5 μM (24 h)	52.7±1.1[a]	31.6±2.0	14.3±1.1	-
10 μM (24 h)	63.2±1.7[a]	26.3±4.5	11.5±1.8	-
20 μM (24 h)	77.4±4.3[a]	17.3±5.1	5.3±1.1	-
20 μM (48 h)	75.5±4.2[a]	19.2±5.2	6.5±0.5	17.8±2.3

Distribution of the cells in G_0/G_1, S and G_2/M phase were analysed by flow cytometry. Results are expressed as the percentage of total cells. Three independent experiments were performed and mean ± SD are presented. Significant difference from the control; [a]$p<0.01$; -, not detectable.

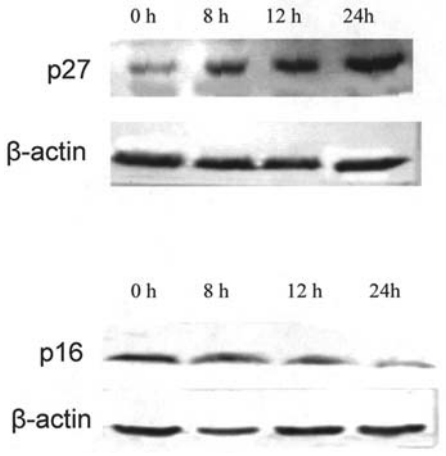

Figure 6. The expression of p27 and p16 protein in T24 cells after the treatment of SFN. The cells were treated with 20 μM sulforaphane for 8, 12 and 24 h. Then the supernatant fractions containing protein lysate were prepared for the determination the expression of p27 and p16 by Western blot analysis.

22.5±2.9% and 41.9±4.1% for 5, 10 and 20 μM SFN treatment, respectively, which is significantly higher than the control 3.3±1.1% (Fig. 4).

Cell cycle arrest and induction of cyclin-dependent kinase inhibitor. Cell proliferation and apoptosis correlated with cell cycle progression. Treatments with 5, 10 and 20 μM SFN for 24 h blocked cells at G_0/G_1 checkpoint, followed by decreasing number of cells in both S and G_2/M phases (Table I). Interestingly, cell apoptosis was induced following 20 μM SFN treatment for 48 h, characterized as sub-G_1 phase ahead of G_0/G_1 phase, while no obvious sub-G_1 cells were observed following 24 h treatments with a range of (5-20 μM) SFN level (Fig. 5). To further understand the mechanism of action, the effect of SFN on the expression of cyclin D and two CKI (p27 and p16) which control the G_0/G_1 were investigated. Following treatment with 20 μM SFN for 8, 12 and 24 h, the expression of p27 was significantly induced compared to control. However, no effect on p16 expression, another CKI, was observed following either 8 or 12 h treatment and level

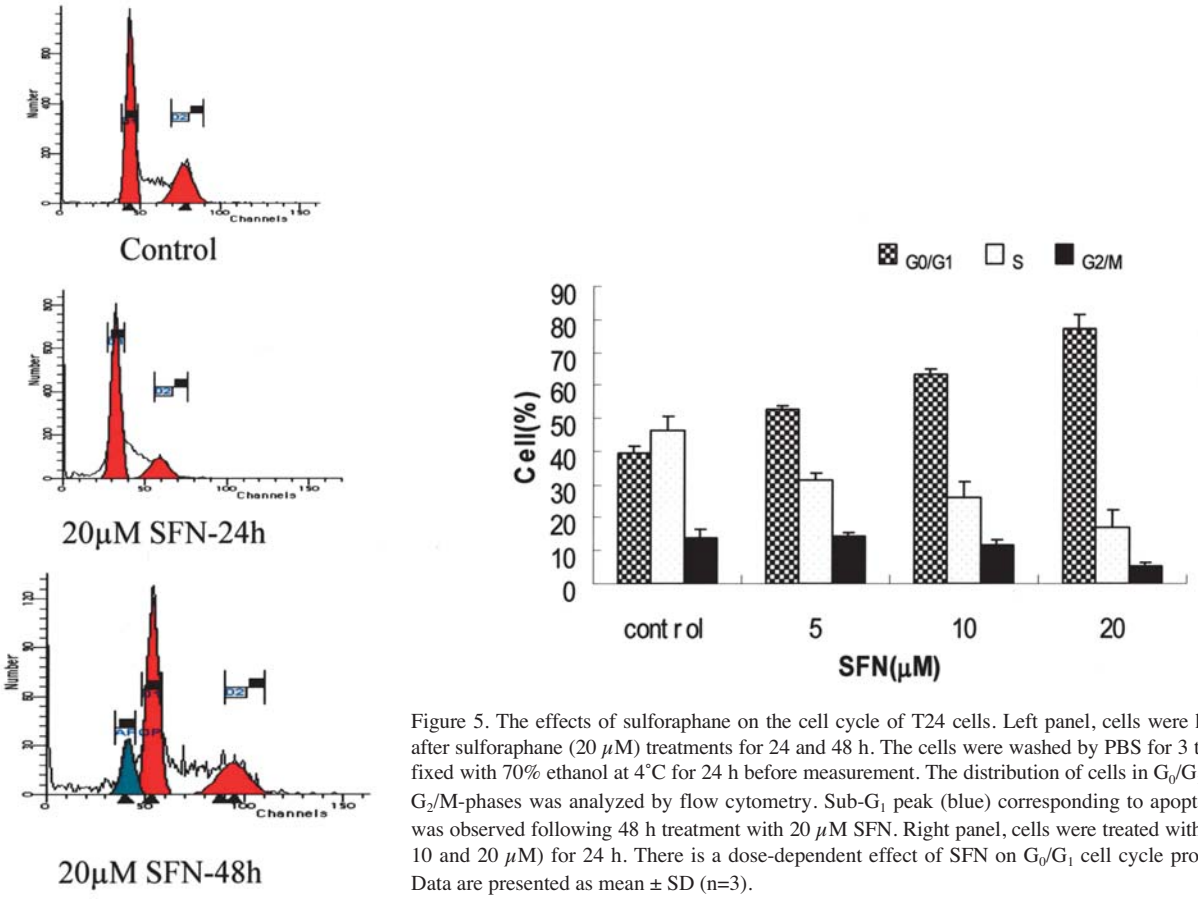

Figure 5. The effects of sulforaphane on the cell cycle of T24 cells. Left panel, cells were harvested after sulforaphane (20 μM) treatments for 24 and 48 h. The cells were washed by PBS for 3 times and fixed with 70% ethanol at 4˚C for 24 h before measurement. The distribution of cells in G_0/G_1-, S- and G_2/M-phases was analyzed by flow cytometry. Sub-G_1 peak (blue) corresponding to apoptotic cells was observed following 48 h treatment with 20 μM SFN. Right panel, cells were treated with SFN (5, 10 and 20 μM) for 24 h. There is a dose-dependent effect of SFN on G_0/G_1 cell cycle progression. Data are presented as mean ± SD (n=3).

of expression decreased at 24 h (Fig. 6). Cyclin D level did not change in this study following treatment with SFN at this time scale (data not shown).

Discussion

Carcinogenesis is a multi-step process in which an accumulation of genetic events within a single cell leads to a progressively dysplastic cellular appearance, deregulated cell growth, and finally, carcinoma (20-22). Based on this hypothesis, cancer chemopreventive strategies must target signal pathways closely related to this process, such as activation and inactivation of some metabolic enzymes of carcinogens, repairing DNA damage, scavenging reactive oxygen species as well as changing the cell signals, altering the expression of genes regulating cell proliferation, apoptosis, and differentiation and decreasing inflammation (23,24). There is growing interest in identifying potential chemopreventive agents in the diet and determining their biological activities, with a view for developing safe and effective chemopreventive agents (25,26).

ITCs derived from cruciferous vegetables have been extensively studied as potential chemopreventive agents and their anti-cancer activities have been shown in several cancer models (27,28), and some of the inconsistent data reported on the relationship between ITCs and bladder cancer is mainly related to the high concentrations used in some of the animal model studies (9,29,30). Since ITCs at relatively lower concentrations (5-20 μM) exhibited inhibitory effects, and these concentrations are achievable through diet, for example, through consumption of 200-400 g Brassica vegetables per day, the potential role of physiologically relevant concentrations in preventing bladder cancer merits further attention (31).

The balance between the proliferation and apoptosis in the cell homeostasis is sustained in normal tissue. Therefore, the effect of a compound on cell proliferation is often used as a measure for testing its efficiency in cancer prevention and treatment. Treatment with 10-40 μM SFN for 24, 48 h significantly suppressed cell growth in a dose-dependent manner, in agreement with other findings (32). Studies (33-36) have convincingly shown that following absorption ITCs are rapidly conjugated with intracellular thiols such as GSH, cysteine and NAC, and therefore excreted in urine. The half-life of the accumulated ITC equivalent in human prostate cancer LNCaP cells was only about 1 h (27). So continuous intracellular accumulation and biological efficacy is only possible when there is a continuous presence of ITC to allow frequent cellular uptake of ITC. Following treatment with 5 μM SFN for 3 days, the inhibitory rates increased from 9.0±1.5% (day 1), 34.3±1.5% (day 2) to 51.6±5.2% (day 3). On the 4th and 5th day, the inhibitory effects continued to increase by 61.6±4.4% and 66±5.9%, respectively because of re-treatment with freshly added SFN at day 3. The inhibitory rates were decreased to 52±3.4% and 51.7±5.3% by the 6th and 7th day following exposure to lower levels of SFN in T24 cells. Therefore, these results indicate that frequent treatments with lower SFN may be more effective dose of suppression of bladder cancer cell growth, especially for highly differentiated carcinoma cells such as T24 cells.

It is well known that three crucial mechanisms including protecting DNA by modulating carcinogen metabolizing enzymes, reducing oxidative stress and inhibiting cell proliferation are involved in the suppression of cancer development by ITCs. The fluorescent staining with AO/EB indicated SFN can induce T24 cell nuclear changes with classic features of apoptosis in some instances. Early apoptosis was most significantly induced by lower level of SFN (5 μM) treatment and increased with higher concentration of SFN used. These results suggest that the anti-proliferative effect induced by SFN is partly related to the induction of early apoptosis.

G_1 is a period when many diverse signals intervene to influence cell division and growth. Therefore, G_1 control has become an important target of oncogenes, tumor suppressor genes, as well as for cancer therapy. These results showed that 5-20 μM SFN blocked the T24 cell-cycle at G_0/G_1 phase although there are also studies that showed ITCs blocking cell cycle at the G_2/M checkpoint (13,32). Available evidence suggests that cell accumulation in either G_0/G_1 or G_2/M phases of cell cycle not only depends on ITCs used but also the cell types studied (37). Unlike S phase and M phase, the steps controlling entry and progression through G_1 are largely dependent on cell type (38). To clarify the blocking effect of SFN on G_0/G_1, the related regulating proteins were further explored. To enter S phase all cells must activate CDKs which are protein kinases that require binding to a cyclin subunit to be catalytically competent. G_1 CDKs include Cdk2, Cdk4/6, which generally combine with E-type cyclins (E1, E2), cyclin A and cyclin D (39). Cyclin-CDK complexes are essential for normal cell cycle progression. Except for cyclins, another important modulating protein of CDKs is their inhibitory protein, called cyclin-dependent kinase inhibitor (CKI). CKIs can latch onto cyclin-CDK complexes and disrupt their catalytic centre, delaying or inhibiting the entry to S phase. One of these inhibitors, p27, functions as an integral brake for the cell cycle, while others, such as p15, p16, p21 are mediators of cytostatic signals. Therefore, in orchestrating the G_1/S transition, several components are involved in cancer, such as overexpression of cyclin D, a decrease in levels of p27 (or other CKIs in G_1 phase), ultimately activating CDK and leading to excessive cell proliferation. Our results suggested that G_0/G_1 phase arrest in T24 cells induced by SFN mainly occur as a result of the regulation of p27.

In summary, the inhibitory effects of SFN on the proliferation of T24 cells were observed using relatively low concentration achievable through dietary intake. There was a clear time-dependent effect of SFN on suppression of cell growth. SFN at lower concentrations induced early apoptosis and G_0/G_1 phase arrest in T24 cells. The overexpression of p27 following treatment with SFN indicated that the blocking of G_0/G_1 phase was mainly associated with upregulation of p27. Therefore, these data provided further evidence that SFN is a promising chemopreventive/therapeutic agent against bladder cancer, and the chemopreventive effect is optimally achieved by frequent consumption of isothiocyanates at dietary relevant concentrations.

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