

p38 MAPK plays a distinct role in sulforaphane-induced up-regulation of ARE-dependent enzymes and down-regulation of COX-2 in human bladder cancer cells

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Abstract. Sulforaphane, a well-characterised dietary isothiocyanate, has been demonstrated to be a potent anti-carcinogenic agent in numerous cancer models, including in bladder cancer cells. In the present study, sulforaphane up-regulated the expression of two Nrf2-dependent enzymes, glutathione transferase (GSTA1-1) and thioredoxin reductase (TR-1), and down-regulated cyclooxygenase 2 (COX-2) in human bladder cancer T24 cells. This action of sulforaphane was associated with the p38 MAPK activity. When a specific p38 MAPK inhibitor, SB202190, was used, both sulforaphane-induced up-regulation of GSTA1-1 and TR-1 and down-regulation of COX-2 were eliminated; in contrast, an activator of p38 MAPK, anisomycin, enhanced the effect of sulforaphane on modulation of GST, TR-1 and COX-2 expression. Moreover, it was established that anisomycin increased nuclear translocation of Nrf2, whereas SB202190 abrogated sulforaphane-induced Nrf2 translocation into the nucleus. In summary, these data suggest that p38 MAPK activation can regulate Nrf2-antioxidant response element (ARE)-driven enzymes and COX-2 expression, thereby facilitating the role of sulforaphane in cancer prevention. This study strongly supports the contention that p38 MAPK is a pivotal and efficient target of sulforaphane in the chemoprevention of bladder cancer.

Introduction

Sulforaphane (SFN), an isothiocyanate (ITC) derived from glucosinolates in cruciferous vegetables, plays a predominant role in cancer prevention in a variety of tissues such as liver, colon and bladder via the induction of antioxidant responsive element (ARE)-dependent enzymes (1,2). A number of

investigations have demonstrated that the major metabolites of SFN, such as N-acetylcysteine conjugates, were as active as the parent compound whilst being almost extensively excreted in the urine. This implicates the bladder as a potential and preferential target organ for SFN (3,4). Moreover, the bladder is sensitive to the induction by dietary inducers of ARE-dependent detoxifying enzymes such as glutathione transferase (GSTA1-1), quinone reductase (QR) (4). In addition, the generation of null or sub-optimal GST-M1 phenotypes through genetic variations or deficiency has been linked with a high risk of bladder cancer in human and rats (5). Thioredoxin reductase-1 (TR-1), another ARE-dependent antioxidant enzyme, protects against potential cell oxidative damage through its binding with substrates such as thioredoxin (Trx), and by reducing hydrogen peroxide and lipid hydroperoxide (6). Interestingly, these enzymes are all associated with SFN-mediated chemoprevention. To date, TR-1 activation by SFN has largely been investigated in non-bladder cancer cell models (7,8). Recently, results from our laboratory have demonstrated that SFN increased TR activity in immortalised human hepatocytes (9). It is accepted that through activating the basic zipper (bZIP) transcription factor, E2-related factor 2 (Nrf2), and binding to ARE in the promoter, SFN can facilitate the induction of ARE-dependent gene expression, thereby protecting against carcinogens and oxidative stress-mediated damage to cells.

Evidence to date suggests that mitogen-activated protein kinase (MAPK) pathways convey the signals stimulated by dietary phytochemicals, such as polyphenols and isothiocyanates (10,11). However, the underlying mechanisms remain unclear since some studies have suggested that MAP kinases positively or negatively modulated the induction of ARE-driven genes (12,13).

Cyclooxygenase-2 (COX-2) is involved in various processes associated with carcinogenesis, metastasis and inflammation, and is considered to be a target of anticancer drugs (14). COX-2 overexpression is often found in human bladder cancer and is closely related with the progression, prognosis and recurrence of bladder cancer (15). Previous studies indicated that COX-2 is a novel target to consider when examining the chemopreventive effect of SFN in bladder cancer. Most importantly, these results showed that the inhibition of COX-2 by SFN is p38 MAPK-dependent (17).

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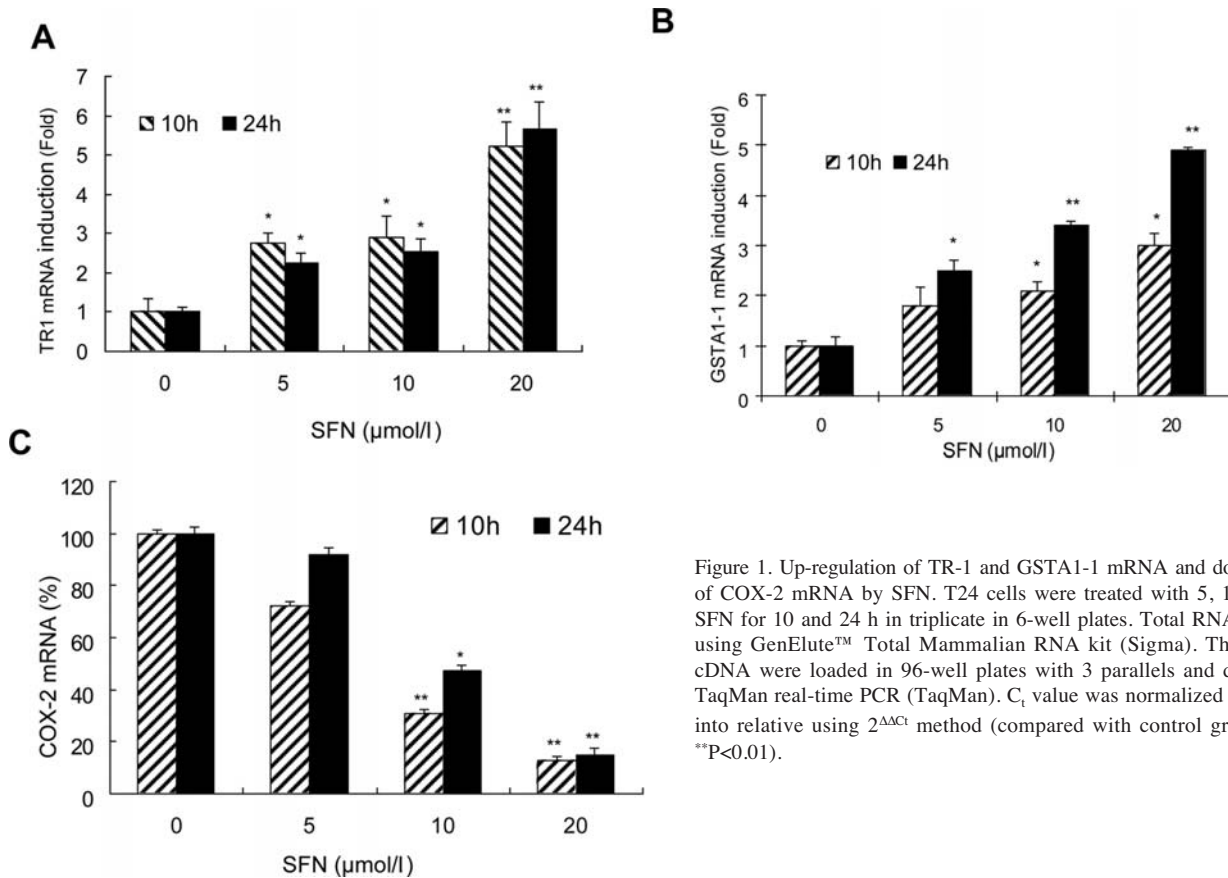


Figure 1. Up-regulation of TR-1 and GSTA1-1 mRNA and down-regulation of COX-2 mRNA by SFN. T24 cells were treated with 5, 10 and 20 μ M SFN for 10 and 24 h in triplicate in 6-well plates. Total RNA was isolated using GenElute™ Total Mammalian RNA kit (Sigma). The products of cDNA were loaded in 96-well plates with 3 parallels and determined by TaqMan real-time PCR (TaqMan). C_t value was normalized by 18S rRNA into relative using $2^{-\Delta\Delta C_t}$ method (compared with control group, * $P < 0.05$, ** $P < 0.01$).

and grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 μ g/ml penicillin and 100 U/ml streptomycin, 2 mM L-glutamine. Cells were grown in a humidified atmosphere (37°C, 5% CO₂). For Western blot analysis, cells were sub-cultured in 10 cm-dishes at 5x10⁵/ml in duplicate. For RNA isolation, cells were seeded in 6-well plate at 1x10⁵/ml in triplicate.

Total RNA extraction, RT-PCR and real-time PCR. Total RNA was extracted using a GenElute™ Total Mammalian RNA kit (Sigma, UK) according to the manufacturer's instructions. Concentration and purity were determined by absorbance measurements at 260 and 280 nm. Reverse transcriptions (RT) of 1 μ g total RNA, 1 μ l random primer, 0.25 μ l Bioscript Polymerase, 1 μ l dNTP, 4 μ l buffer and 1 μ l RNase inhibitor, were complemented with PCR water to a total volume 20 μ l. The RT reactions were carried out at 70°C for 10 min and 42°C for 1 h.

Target mRNA was quantified by real-time PCR (TaqMan®) using an ABI 7500 Sequence Detection System (Applied Biosystems). Forward/reverse primers and the fluorogenic TaqMan probes were used as described previously (1,8,16). 18S rRNA was used as a housekeeping gene. Real-time PCR was carried out in a 96-well plate (in triplicate) using TaqMan two-step PCR Mastermix Reagent kit. TaqMan threshold cycle number (C_t) was normalized into relative using $2^{-\Delta\Delta C_t}$ method, $\Delta\Delta C_t = (C_{t, Target} - C_{t, 18S})_{treatment} - (C_{t, Target} - C_{t, 18S})_{control}$. Standard curves were constructed using 0.5-100 ng of total RNA in triplicate.

Nuclear fraction extraction and Western blot analysis. T24 cells were harvested following the desired treatment with Trypsin-EDTA (T/E) buffer and the pellets were washed with

In the present study, p38 MAPK has been shown to exert the positive role in the expression of GSTA1-1 and TR-1 induced by SFN through translocating Nrf2 into the nucleus. Combined with the role of p38 MAPK to COX-2 inhibition by SFN, these findings implied that activation of p38 MAPK plays a key role in the anti-carcinogenic effects of SFN. Targeting p38 MAPK could be a promising strategy in the treatment of bladder cancer and these results provide a novel mechanism of SFN in the chemoprevention of bladder cancer.

Materials and methods

Chemicals. SFN (1-isothiocyanato-4-methylsulphanylbutane) was purchased from LKT Laboratory (Alexis Biochemicals, UK). Anti-TR1, anti-Nrf2 (C-20) and anti- β -actin antibodies, and horseradish peroxidase-conjugated (HRP) anti-rabbit and anti-goat antibodies were all purchased from Santa Cruz Biotechnology (USA). Anti-GSTA1-1 antibody was obtained from Calbiochem (UK). Anti-COX-2 antibody was supplied by Upstate Cell Signalling (UK). p38 inhibitor SB202190 was from Tocris (UK). Nuclear Extraction Kit was purchased from Chemicon® International (UK). Protease inhibitor cocktail tablets were obtained from Roche Applied Science (UK). RPMI-1640 medium was purchased from Invitrogen Corporation (UK). Bradford reagent, dimethyl sulfoxide (DMSO), anisomycin and phenylmethylsulphonyl fluoride (PMSF) were all purchased from Sigma (UK).

Cell culture. Human bladder cancer T24 cells were obtained from the European Collection of Cell Cultures (ECACC)

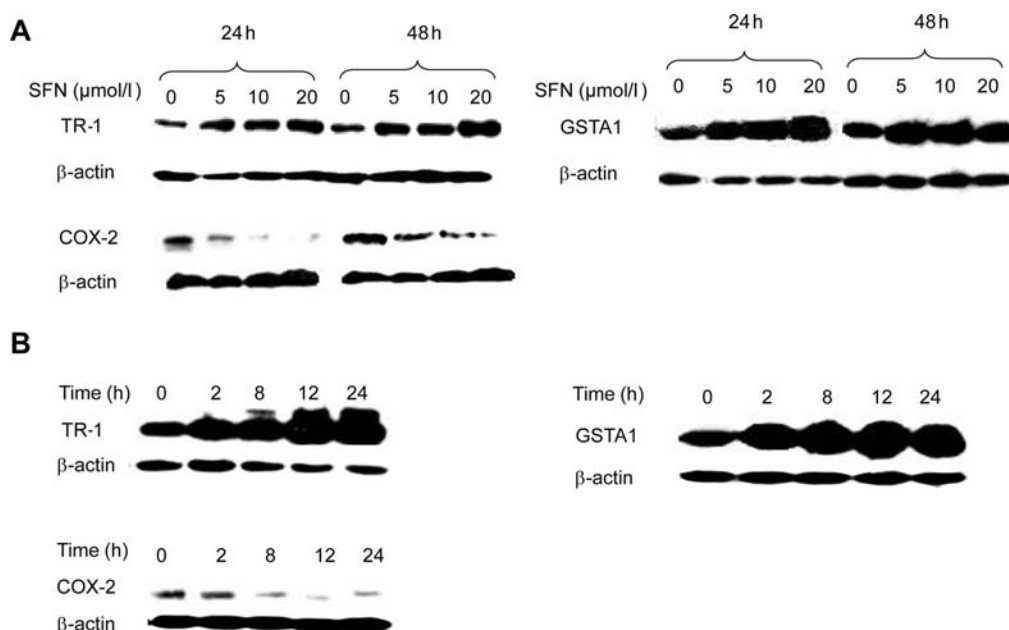


Figure 2. Dose- and time-dependent effect of SFN on TR-1, GSTA1, COX-2 protein expression. Cells grown in 10-cm dishes were treated with 5-20 μ M SFN for 24 and 48 h in duplicate (A). Cells were treated with 20 μ M SFN for 2, 4, 8, 12 and 24 h (B). The pellets were lysated to extract protein for Western blot analysis.

cooled phosphate-buffered saline (PBS). Nuclear- and cytoplasmic extractions were isolated using the Chemicon® Nuclear Extraction Kit. Briefly, the pellet was suspended in cell lysis buffer containing 1% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM PMSF and 1X protease inhibitor cocktail and incubated on ice for 30 min. Thereafter lysates were centrifuged at 10,000 g for 10 min at 4°C and supernatant fractions were used for Western blot analysis.

The protein extractions (10-40 μ g) were run in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved protein bands were transferred onto PVDF membranes (Bio-Rad) using a semi-dry transfer system. The membranes were blocked with 5% fat free dry milk in PBS (pH 7.4) containing 0.1% Tween-20 for 30 min at room temperature, followed by incubation with primary antibodies in PBS overnight at 4°C. The membranes were washed three times and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and then washed again. The targeted protein was visualized with enhanced chemiluminescent (ECL) system (GE Healthcare, UK) according to the manufacturer's instructions. The membranes were then exposed to CL-Xposure film (Perbio Science, UK).

Statistical analysis. All results are expressed as mean \pm SD (from at least three measurements). Data were analyzed by one-way ANOVA, followed by either Dunnett's t-test for separate comparisons with the control group or Sidak's t-test for multiple-group comparisons. Differences were considered to be significant at $P < 0.05$.

Results

Effect of SFN on the expression of TR-1, GSTA1-1 and COX-2 in bladder cancer T24 cells. Induction of TR-1 by SFN has

previously been shown in human breast cancer MCF-7 and hepatoma HepG2 cells (7,8). In the present study, TR-1 mRNA was increased after the treatment with 5, 10 and 20 μ M SFN at two time points (10 and 24 h). Five, 10 and 20 μ M SFN induced 2-, 2.9- and 5-fold TR-1 mRNA expression compared with the control respectively (Fig. 1A). SFN induced expression of TR-1 protein in a dose- and time-dependent manner (Fig. 2). Activation of TR-1 by SFN started as early as 2 h after treatment and TR-1 protein increased sharply in time-dependent manner (Fig. 2B). In this study, SFN exhibited a strong induction of TR-1 both in mRNA and protein level in bladder cancer cells, suggesting an antioxidative role of SFN in the chemoprevention of bladder cancer. This is the first time that such an observation has been reported.

GSTA1 induction by SFN has been demonstrated previously at non-transcriptional level in human bladder cancer cells (4). In the present study, SFN (5-20 μ M) induced 2-3-fold and 2.5-5-fold GSTA1 mRNA after 10- or 24-h treatments, respectively (Fig. 1B). Moreover, SFN (5, 10, 20 μ M) up-regulated GSTA1-1 protein expression after 24- or 48-h exposure (Fig. 2A); a similar trend of induction was also observed following a time-course study from 2-24 h (Fig. 2B).

A previous study from our group showed that flavonoids suppressed COX-2 mRNA in human colon adenocarcinoma Caco-2 cells (18). Here, SFN at 5, 10 and 20 μ M decreased COX-2 mRNA expression by 28, 69 and 87%, respectively after 10-h treatment and by 18, 53 and 85%, respectively, after 24-h treatment. Strongest inhibition was found at 10-h treatment (Fig. 1C). For COX-2 protein expression, SFN (5, 10 and 20 μ M) significantly inhibited COX-2 protein after either 24- or 48-h treatment (Fig. 2A), and inhibition of COX-2 protein expression by SFN (20 μ M) was time-dependent (Fig. 2B).

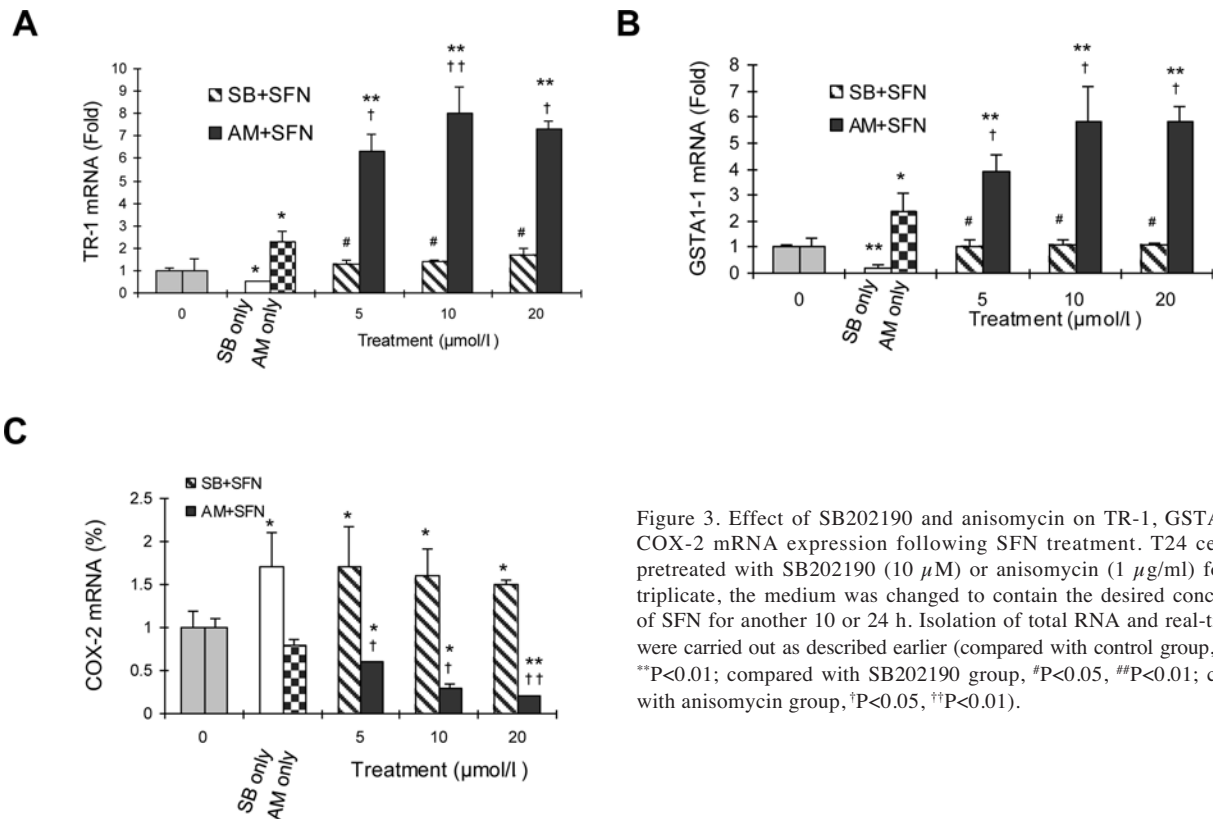


Figure 3. Effect of SB202190 and anisomycin on TR-1, GSTA1-1 and COX-2 mRNA expression following SFN treatment. T24 cells were pretreated with SB202190 (10 μ M) or anisomycin (1 μ g/ml) for 1 h in triplicate, the medium was changed to contain the desired concentration of SFN for another 10 or 24 h. Isolation of total RNA and real-time PCR were carried out as described earlier (compared with control group, * P <0.05, ** P <0.01; compared with SB202190 group, # P <0.05, ## P <0.01; compared with anisomycin group, † P <0.05, †† P <0.01).

Role of SB202190 and anisomycin in SFN-induced TR-1, GSTA1-1 and SFN-inhibited COX-2 expression. SB202190, a p38 inhibitor, has been applied in numerous studies to effectively and specifically inhibit p38 MAPK (17). SB202190 (10 μ M) inhibited mRNA levels of TR-1 and GSTA1-1 by 50 and 80%, respectively. After the cells were pre-treated with SB202190 (for 1 h) and followed with 5, 10 and 20 μ M SFN treatment for further 10 h, TR-1 mRNA induction was attenuated by 1.3-, 1.4- and 1.7-fold, respectively (Fig. 3A). A similar effect was found on TR-1 protein expression, i.e. pre-treatment with SB202190 partly attenuated TR-1 protein induced by SFN (20 μ M), although a weak increase compared to the control values was observed (Fig. 4A). It is of interest that SB202190 exhibits prominent inhibition in GSTA1-1 mRNA. Following SB202190 treatment, SFN failed to induce GSTA1-1 mRNA, rather decreased by 20% of the control (Fig. 3B); similar changes were also observed in Western blot analysis (Fig. 4B). In contrast to the effect on TR-1 and GSTA1-1, SB202190 alone induced COX-2 mRNA 1.4-fold, and reversed the down-regulation of COX-2 by SFN (Fig. 3C).

To further confirm the role of p38 MAPK, anisomycin, an activator of p38 MAPK was applied in the following experiments. The concentration of anisomycin (1 μ g/ml) used was tested for its effective activation of p38 MAPK using Western blot analysis (16). Anisomycin alone induced TR-1 mRNA by 2.3-fold, and displayed further induction in the range of 6-8-fold when co-treated with SFN (5, 10 and 20 μ M) (Fig. 3A), compared to the action of SFN alone (2-5-fold) (Fig. 1A). Likewise anisomycin alone induced GSTA1-1 mRNA by 2.4-fold, and up-regulated ~4-6-fold when co-treated with both anisomycin and SFN (Fig. 3B).

In contrast, anisomycin inhibited COX-2 mRNA by 20%. The additive suppression by SFN and anisomycin was more effective at 5 or 10 μ M SFN treatment, showing 40 and 70% reduction, respectively (Fig. 3C), compared to 8 and 53%, respectively, when only SFN was used (Fig. 1C).

These results were further validated by following the measurement of protein level using Western blotting (Fig. 4). Taken together, these results from both p38 MAPK inhibitor and activator strongly support the contention that p38 exhibits pivotal effects on TR-1, GSTA1 and COX-2 expression.

Effect of SB202190 and anisomycin on the translocation of Nrf2 to the nucleus. SB202190 has previously been found to significantly stimulate translocation of NF- κ B into nucleus binding to the COX-2 promoter, thereby activating COX-2 mRNA expression (16). In the present study, Nrf2, a central regulator in the ARE-related genes expression, was analysed further. When the cells were exposed to anisomycin for 1 h, the expression of phosphorylated Nrf2 (p-Nrf2) in nucleus (around 98 kDa), but not Nrf2 (around 57 kDa) was significantly increased as compared with control (Fig. 5). Most interestingly, SB202190, the specific inhibitor of p38, decreased the nuclear level of p-Nrf2 and had no obvious effect on Nrf2. These findings suggest that SB202190 and anisomycin play a distinct role in the regulation of Nrf2 translocation into nucleus, thereby affecting ARE-dependent gene expression at the transcriptional level.

Discussion

For the first time, it has been demonstrated that p38 MAPK plays an important role in the expression of both ARE-dependent enzymes and COX-2 manipulated by SFN. p38

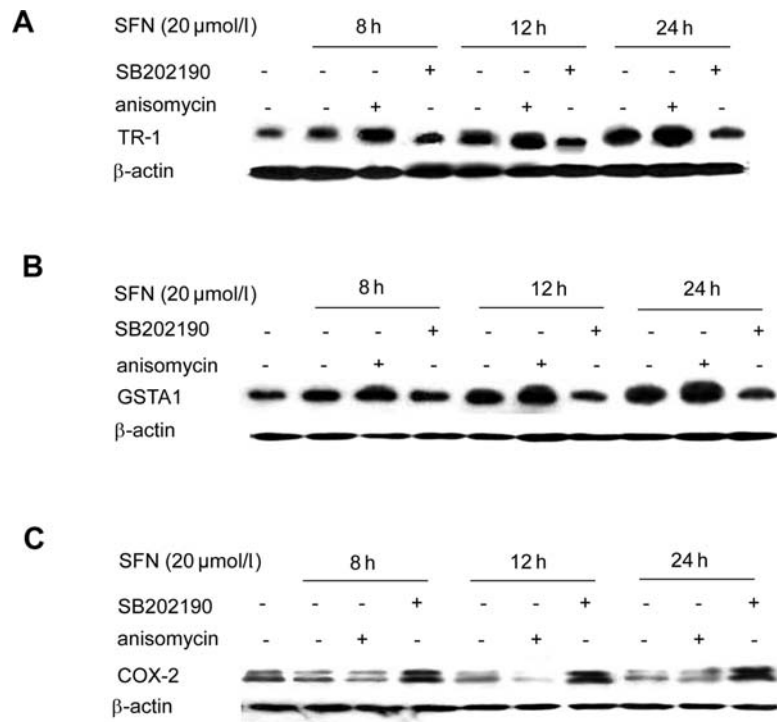


Figure 4. Effect of SB202190 and anisomycin on SFN-mediated expression of TR-1, GSTA1 and COX-2 protein. T24 cells were pre-treated with or without SB202190 (10 μM) and anisomycin (1 μg/ml) for 1 h, then exposed to 20 μM SFN for 8, 12, 24 h in duplicate. The results from Western blot analysis were repeated at least three times.

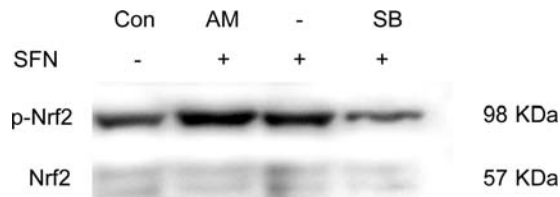


Figure 5. Effect of SB202190 and anisomycin on the translocation of Nrf2 into the nucleus. T24 cells were treated independently and differently as follows: 1640 medium, anisomycin (1 μg/ml) for 1 h, SB202190 (10 μM) for 1 h and SFN (20 μM) for 4 h in duplicate. After 1-h exposure to anisomycin or SB202190, the cells were changed with complete 1640 medium for another 4 h. Nuclear components were then isolated using Nuclear Extraction Kit and subjected to immunoblotting with Nrf2 antibody. The results were repeated at least three times.

activation positively modulates the induction of GSTA1-1 and TR-1 by SFN, on the other hand, enhances the effect of SFN on down-regulation of COX-2. These data strongly suggest that activation of p38 MAPK plays a predominant role in the chemoprevention of SFN; a similar suggestion has also been made for human breast carcinoma MCF-7 cells (19).

In accordance with these findings, positive regulation of p38 MAPK was also reported in other ARE-dependent enzymes, such as HO-1, γ-GCS (20,12). However, only a few studies have reported the converse, that p38 MAPK negatively controls ARE-dependent enzymes. Keum *et al* (13) found that p38 MAPK overexpression suppressed SFN-induced heme oxygenase-1 (HO-1) expression in human hepatoma HepG2 cells. The underlying mechanism remains to be

clarified and the specificities in stimuli and cell types should also be considered.

In non-stimulated cells, Nrf2 is sequestered in the cytoplasm by its inhibitor Keap1. Upon activation, Nrf2 translocates into nucleus, binds to ARE sites in the promoter regions of many detoxification and antioxidant genes, leading to coordinated up-regulation of downstream targets that enhance cellular detoxification and antioxidative potency (21). Targeting Nrf2 activation has been generally accepted as an effective strategy of chemoprevention (22). As demonstrated in this study, Nrf2 activation was mediated by p38 MAPK following exposure to SFN; the accumulation of Nrf2 in nucleus, resulted in GSTA1-1 and TR-1 expressions. A similar signalling pathway, through which HO-1 was regulated by other inducer in breast cancer MCF-7 cells, has also been reported (20).

Accumulating evidence suggests ITCs such as SFN are the most promising and preferential chemopreventive agents in bladder cancer compared to other tissues (4,23). In contrast to the multiple functions of SFN displayed in other cancer models, only a few studies such as activating phase II detoxifying enzymes (24), blocking cell cycle progression (25), inducing apoptosis (26), have been investigated in bladder cancer. Based on the preferential potency of SFN in bladder cancer described here, however, further extensive and intensive investigations are required.

Tissue damage mediated by oxidative stress is an important factor in carcinogenesis (27). TR-1, as a protectant against of oxidative injury, has been investigated in cancer cells of various tissues but not the bladder (7,8). The current study identifies that TR-1 induction by SFN was also involved in the chemoprevention of bladder cancer.

To date, p38 has been targeted by an enormous array of anticancer drugs (28). Activation of p38 exhibits its anti-cancer role in various ways, for example by blocking cell cycle progression (29), inducing apoptosis (30) and suppressing oncogene (31), as well through ARE-driven genes and COX-2 (16). Taken together, these results clearly established that activation of p38 MAPK may be pivotal in the therapy and prevention of bladder cancer with dietary isothiocyanates. Activation of p38 MAPK may contribute to a stronger chemoprotective effect of SFN. Understanding the key signalling pathways of SFN-mediated gene expression may offer a valuable additional strategy for chemoprevention of bladder cancer.

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