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Patrick Rouimi, Thierry Fricaux, Nicole Gasc, Emilie Amillastre

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P. Rouimi, T. Fricaux, N. Gasc, E. Amillastre and L. Gamet-Payrastre*

UMR1089 Xénobiotiques INRA, 180 Chemin de Tournefeuille, BP3 31931 Toulouse Cedex 9 France.

Phone (33) 561 28 53 83 Fax (33) 561 28 52 44 email : lpayrast@toulouse.inra.fr.

*To whom correspondence should be addressed

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Abstract

Various epidemiological studies have revealed an inverse association between renal cancer and consumption of cruciferous vegetables. Sulforaphane, a natural microconstituant from broccoli, has been shown to exert potent anti-tumoral properties *in vitro* as well as *in vivo* against colon and mammary chemically-induced tumors. Here, we shown in human renal carcinoma cells ACHN cells, that conjugation of sulforaphane to glutathione, a process mainly responsible for its accumulation in various cells, did not change the properties of this isothiocyanate (i.e. cell death, cell cycle arrest in the S-phase and cyclin A and B_1 induced-expression). This result constitutes a good support to possible protective properties against renal cancer described for cruciferous vegetables. Second, our finding seems to be in agreement with the previously described activation of compounds by conjugation to GSH. This may have important implications especially in explaining the effects of rapidly conjugated compounds on tissues and organs distant from the original site of exposure and/or conjugation .

1. Introduction

Epidemiological studies have revealed an inverse correlation between cruciferous vegetables intake and the risk of certain cancer types. Various data suggested that anticancerous effect of cruciferous vegetables observed in animal studies was due to isothiocyanates that occur as thioglucosides conjugates in a variety of edible plants as broccoli and cabbage (1). Many isothiocyanates (ITCs) protect against chemically-induced tumors in a variety of animal organs. Among these, Sulforaphane (SF) is one such ITC that has received a great deal of interest because of potent anti-tumoral properties it displayed in experiments involving carcinogen-treated rats (2). The molecular pathways of SF which mediated these effects haven't been fully elucidated. However, many studies already showed that SF (and other ITCs) was able to induce Phase II drug metabolizing enzymes *in vitro* as well as in animals (3, 4). In addition, evidence is accumulating to indicate that SF inhibits proliferation of cultured cancer cells by inducing cell cycle arrest via the regulation of cell cycle protein levels and/or cyclin-dependent kinase activities (5-8). It has also been shown to induce apoptotic cell death via the caspase or the P53-dependent pathway (9-13). Furthermore some recent findings suggest that SF may be effective as a tumor-suppressing agent and as a chemotherapeutic agent, via histone deacetylase inhibition (14).

In various cellular model (prostate, leukemia, myeloid, breast, colon cancer cells), SF appeared to enter freely (15), to rapidly accumulate by undergoing metabolic conjugation to cellular glutathione (GSH) and then to be exported mainly as a conjugate (GS-SF), possibly via a membrane transporter. Moreover, recent data suggested a common cellular response to various ITCs could exist. Indeed, allyl-ITC, benzyl-ITC and phenetyl-ITC accumulated in leukemia cells were shown to be rapidly exported, mainly as GSH- and cystenylglycine-conjugates, through mechanism apparently involving MRP-1 and Pgp (16).

Anticarcinogenic properties of SF are closely dependent on its cellular accumulation. Zhang et al (15) found that many ITCs rapidly accumulate to very high (up to millimolar levels) levels in cell lines from different species. It appeared that intracellular conjugations of ITCs to GSH was mainly responsible for ITC accumulation, which was abolished by GSH depletion (17). Moreover, cellular GST promoted ITC uptake by enhancing the conjugation reaction. The ultimate point of glutathione conjugate metabolization is conversion to an N-acetyl-cysteine conjugate (SF-NAC), which is excreted in the urine. Zhang (18) showed that SF was almost exclusively accumulated as GSH conjugate in

hepa1c1c7. Hecht et al (19) have demonstrated that SF-NAC possessed chemopreventive activity on experimentally-induced lung cancers in animal studies. Moreover SF-NAC similarly induced growth arrest and apoptosis in human prostate cancer cells (20).

In our study we investigated the effect of the NAC- and GS-conjugates of sulforaphane on growth and death in the human renal carcinoma cell line, ACHN. Renal cancer accounts for about 2% of cancers worldwide and increasing incidence has been observed in north America and northern Europe. Currently, chemotherapy and cytokines treatment have produced insufficient response rates with rare complete remission. The role of the diet in etiology of renal cell carcinoma has been investigated in various epidemiological study. From a population-based case-control study in Denmark, it appeared that fats may be associated with renal carcinoma risk (21). Moreover, an inverse association between risk of renal carcinoma and fruits and vegetables intake has been mentioned in a number of studies and remains one of the few consistent dietary findings (22, 23). Mellemgaard et al (24) observed a weak protective effect of cruciferous vegetables, whereas Yuan et al (25) described a significant inverse association between renal cancer and consumption of cruciferous vegetables in population-based case-control study.

Our results show that even after conjugation to GSH, SF inhibited growth of ACHN cell and induced a cell cycle arrest in the S phase correlated with a modulation of cyclin A and B_1 expression.

2. Materials and Methods

Drugs and chemicals- Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Life Technologies (France). Monoclonal antibodies raised against cyclin A and B_1 were obtained from BD Pharmingen (le Pont de Claix, France) and the cdc2 monoclonal antibody from Santa Cruz Technology (Tebu ,France). Monoclonal alpha-tubulin antibody, HRP-labeled secondary antibodies were purchased from Sigma (France). Bovine serum albumine (BSA) was obtained from Roche (Meylan, France). Sulforaphane was obtained from LKT laboratories (St Paul, MN, USA). All other chemicals were purchased from Sigma or from Merck (Darmstadt, Germany) and were of the highest purity available.

Glutathione- or N-acetylcysteine conjugate of sulforaphane was synthesized in our lab according to the method of Kassahun et al. (26) slightly modified. Briefly, GSH or NAC (0.4 mmol) was dissolved in aqueous ethanol (50%, 6 ml) and the pH of the resulting solution was adjusted to 7.8. SF (0.2 mmol) dissolved in ethanol (3ml) was added to the GSH solution and the mixture was stirred at ambient temperature under Ar for 3 hours. After the solvent was evaporated under vacuum, the crude product was solubilized in an H3PO4 pH 2.6 solution and then purified on extraction cartridges (Waters Vac C18 6cc 500 mg). Following solvent evaporation, the final product was dissolved in water and quantified by measuring optical density at 274nm.

Cell culture- ACHN cells were purchased from the European Collection of Cell Culture (ATCC, USA). Routinely, cells were cultured in DMEM containing 25 mM glucose, 43 mM bicarbonate, 100 units/ml penicillin, 100 µg/ml streptomycin at 37°C, supplemented with 10% heat-inactivated FCS and 1% non essential amino-acids (NEAA) under an air/CO_2 (95/5) atmosphere.

In experiments, ACHN cells were seeded at a density of $5x10^4$ cells per ml in 150-mm diameter Primaria dishes in standard medium containing 10% FCS and 1% NEAA. 20 hours after seeding, cell layers were washed with PBS and then incubated in ionic medium (137mM NaCl, 5.36mM KCl, 0.4 mM Na_2HPO_4, 0.8 $MgCl_2$, 1.8mM $CaCl_2$, 20mM Hepes pH 7.4) supplemented with 1% FCS and then treated with SF or GS-SF (15µM). An equivalent amount (0.2%) of solvent (ethanol) was added to control cells. At indicated times, cells were either prepared for flow cytometry analysis or harvested for Western blot.

Estimation of cell death- The number of dead cells was estimated by counting cells floating in the culture medium. Results were expressed as the percentage of dead cell versus total cell number. At the indicated times, floating cells were retrieved from the medium by centrifugation at 2500 RPM for 5 minutes. The pellet was then resuspended, homogenized in 100µl of PBS, diluted in 10 ml of an isotonic solution (Isoton, Beckman Coulter France) and cells were counted using a Coulter Counter (Beckman, France). Attached cells were rinsed in PBS and incubated with 500 µl of trypsin at 37°C for 5 min. Trypsin was inhibited by addition of 1 ml of 5% FCS-containing medium prior to centrifugation of the cell suspension at 2500 RPM for 5 minutes. The obtained pellet was resuspended in 1ml PBS. After homogenization, an aliquot of 100µl of the cell-suspension was diluted in 10 ml isotonic solution for counting.

Flow cytometry analysis- The effect of SF on cell proliferation was evaluated from cell distribution in the different phases of the cell cycle, using flow cytometry. This was determined by measuring the DNA content in nuclei stained with propidium iodide (DNAcon3 staining kit, Dakocytomation, France). Cells from either control or treated cultures were obtained by trypsinization and added to the pool of floating cells. To the staining solution containing buffer salts, detergent, RNases and chromatin stabilizer reconstituted by the addition of PI solution were added cells washed and resuspended in 100µl PBS . An overnight incubation at 4°C allowed a maximum labeling of the DNA. Cell cycle analysis was performed on a Coulter ELITE flow cytometer through a 630 nm LP filter. Gating on peak versus integrated signals eliminated debris and doublets.$1.5x10^4$ cells were collected per sample. Calculations were performed using MULTICYCLE AV Software (Phoenix Flow System).

Western blot analysis- Whole cell extracts were prepared from each treatment and time point. Briefly, control and SF-treated cells were scraped in the culture medium and centrifuged 2500 RPM during 5 minutes. The pellet was washed twice in PBS before addition of ice-cold lysis buffer (30 mM Tris pH 6.8, containing 5% SDS, 25% glycerol, 2.5% 2-mercaptoethanol, 1mM orthovanadate, 0.05% bromophenol blue and a protease inhibitor cocktail from Sigma). The protein concentration in lysates was determined using the Shaffner and Weissmann method (27). Homogenate protein samples (40µg protein/lane) were fractionated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Ecquevilly, France) by immerged blotting. Membranes were blocked using 5% skimmed dried milk in TBST (20 mM Tris/HCL pH 7.4 containing 0.05% Tween 20) overnight at 4°C. Immunogenicity was detected by incubating the membranes for 60 min at room temperature with the appropriate primary antibody. After washing with saturating buffer, the membranes were incubated with diluted HRP-labeled secondary antibody for 60 minutes. Immunoreactive proteins were then visualized using ECL^{+} (Amersham, Sarclay, France) and quantified after STORM 840 Imager (Molecular Dynamics, Sunnyvale, CA, USA) scanning using the densitometer analysis software. Experiments were performed in triplicate and blots were probed with □-tubulin antibody as an internal control for protein loading.

3. Results

3.1. Effect of GS-SF on ACHN cell growth and death (figure 1)

In human colon carcinoma cells as well as in other cancerous cells, sulforaphane has been shown to inhibit cell growth and to induce apoptosis. Here we investigated the effects of its glutathione conjugate on growth and death using the ACHN cell line and compared them to those of the parent compound (SF). Exposure for 48 hours to 15 µM GS-SF strongly reduced cell growth and resulted in the appearance of floating cells. Inhibition of cell growth by GS-SF was lesser than those obtained in the presence of SF (63% in cells treated with GS-SF versus 87% in cells treated with 15µM SF). Increasing doses (up to 30µM) of SF or GS-SF did not change neither the percent of growth inhibition nor the percent of cells death induced by these compounds. Percent of dead cells (floating cells) was higher in SF than in GS-SF exposure experiments. Up to 30µM SF-NAC (N-acetylcysteine conjugate of Sulforaphane) did not affect cell growth and viability (not shown).

3.2. Evidence for growth arrest (figure 2)

In order to understand cell growth arrest by GS-SF and SF, we next examine the effects of these compounds on the distribution of ACHN cells in the different phases of the cell cycle. Cells, treated for 24, 48 hours and 72 hours with 15µM GS-SF or SF, were stained with propidium iodide and then analyzed for the DNA content using flow cytometry as described in the Material and Methods section. Figure 2 shows that GS-SF and SF displayed similar activities which were not dose-related : i.e. a decrease in the proportion of cells in the G1 phase and a concomitant increase of cell number in the S phase. From these results we can conclude that the glutathione conjugate of SF as well as the parent compound (SF) may interfere with the regulatory pathway of cell cycle progression, which leads to reduced cell growth.

3.3. Regulation of cell cycle associated protein expression by SF and GS-SF (figure 3).

Cyclins appear to have a major role in cell cycle progression, as their association with cyclin-dependent kinases (CDKs) leads to the subsequent activation of cyclin-CDK complexes. Cells were exposed for 48H either to 15µM GS-SF or SF. Cell layers were then scraped in loading buffer and analyzed for cyclin A and B_1 protein expression as indicated in Materials and Methods section. Western blot studies are presented in figure 3. Our results clearly reveals a nearly identical induction

of cyclin A and B1 expression in SF and in GS-SF-treated cells, which is correlated with the S-phase arrest induced by both compounds.

3.4. SF and GS-SF did not affect GST expression in ACHN cells (figure 4).

SF has been described to modulate GST expression in various cells. These enzymes are though to promote ITC uptake by enhancing conjugation reaction. ACHN cells were incubated with 15 µM GS-SF or SF for 12, 24 or 48 hours. Specific protein expression analysis was performed as described in the Materials and Methods section. Results presented in figure 4 show that none of the tested compounds affects GST isozymes expression in ACHN cells. Thus a modulation of GSTs might not be involved in SF and GS-SF biological effects.

3.5. Mechanism by which SF and GSH-SF induces cell death

In a previous work, we demonstrated occurrence of apoptosis in colon carcinoma HT29 cells exposed to SF (12). Here, flow cytometry analysis of ACHN cell cycle did not reveal any subG1 peak characteristic of an apoptotic cell population, neither under GS-SF nor under SF treatment (figure5A). Moreover, western blot analysis of pro- (bax) and anti-apoptotic (bcl2) protein expression showed no difference between control and SF or GS-SF treated cells. Our results suggest that SF and GSH-SF might not involve bax/bcl2- to induce cell death in ACHN cells. A possible induction of mitotic death by these compounds is discussed.

4. Discussion

In addition to the known risk factors for renal cell carcinoma, i.e. hypertension, obesity and tobacco use, nutritional factor such as meat enriched diet and frequent consumption of poultry may increase the risk for renal cell carcinoma. On the contrary, fruits and vegetables rich diets appear to have protective effect (21, 25). It is particularly noteworthy to study the modulators of cell growth and death in renal cancerous cells, as it is widely regarded that over-expression of anti-apoptotic and growth-promoting regulators contributes to renal carcinoma pathogenesis.

Our results enlighten the main two aspects we wished to investigate in this study. First, sulforaphane, a naturally occurring microconstituant from broccoli, reduces growth in a human renal cancerous cell line. This result constitute a good support to possible protective properties against renal cancer described for cruciferous vegetables (25). Second, our data show that the glutathione-conjugate of SF, which is produced through the major route of ITC metabolisation in rodents and in humans (15), is also an effective inhibitor of cell growth in a renal cancer cell model. Moreover, in the

present work, GS-SF was found, like SF, to modulate expression of cyclins A and B_1, which are two representative S/G2/M regulatory proteins. Our findings seem to be in agreement with the previously described activation of compounds by conjugation to GSH (17, 18, 28, 29). This may have important implications especially in explaining the effects of rapidly conjugated compounds on tissues and organs distant from the original site of exposure and/or conjugation

Biological activity for ITCs (including sulforaphane) metabolites such as the Nacetyl-cysteine conjugates has been recently mentioned. Indeed, SF-NAC has been shown to exert chemoprevention of experimentally induced lung cancers in animals (19). It also significantly reduced the formation of total aberrant crypt foci in the colon in post-initiation studies on the rat model (2). PEITC-NAC alone or in combination with myoinositol was able to inhibit experimental lung tumorigenesis in A/J mice (19). However in our study, SF-NAC did not modify growth and viability in ACHN cells.

Absence of typical apoptotic protein modulation in cells treated with SF or GS-SF lead us to suggest that a different kind of cell death could take place in our model. Recent findings (30, 31) indicated that SF induced mitotic cell cycle arrest in prostate cancer cells, and the authors suggested a mechanism linked at least to disruption of normal tubulin polymerization. Additional studies are in progress in our lab in order to investigate a possible mitotic catastrophe in GS-SF or SF-treated ACHN cells.

Cellular GSH has been shown to constitute the principal driving force for ITCs accumulation in cells, possibly further enhanced by cellular GSTs (15). However no change in GST expression were observed in SF- or GS-SF-treated cells, suggesting SF effects is less related to the modulation of GST expression than to their enzymatic capacity.

In conclusion, metabolic conjugation of sulforaphane to glutathione, which is the major route of ITC metabolism in rodents and in humans still give rise to growth arrest and death in human renal ACHN cancer cell model.

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Figure legends

Fig. 1. Metabolic conjugation of SF to GSH still give rise to growth arrest and death in ACHN cells. Cells were exposed for 48 hours to either ethanol (controls), or 15µM GS- SF or SF, mixed with the culture medium. The total cell number was obtained by pooling cells attached to the plate to those floating in the culture medium (A). Viable cells were determined using MTT test (B). The number of dead cells was estimated by counting cells floating as described in the Material and Methods section (C). Results are expressed as the percentage of dead cell versus total cell numbers and are the mean ± SD of at least 3 experiments.

Fig. 2. SF and GS-SF induced a cell cycle arrest in the S phase. Attached and floating cells were harvested after treatment for 24, 48 or 72 hours with ethanol (controls), 15µM GS-SF, or 15µM SF. Flow cytometry analysis was performed as described in the Material and Methods section. Values presented are the results ± SD of 4 experiments performed in triplicate.

Fig. 3. ACHN cell cycle arrest induced by SF or its GSH-conjugate is accompanied by an increase in cyclin A and B1 expression. Ethanol (controls), 15µM SF, or 15µM GS-SF was mixed with the culture medium, and cells left at 37° C for 48 hours. Attached and floating cells were collected and harvested and boiled for 5 min in electrophoresis sample loading buffer. Protein samples (40µg) were fractionated by SDS-PAGE and transferred to nitrocellulose membrane as described in the Material and Methods section. Immuno-detection of cyclin A and B was performed on the membranes using specific antibodies. PC corresponds to positive controls. One typical experiment is shown on top of each graph. The results presented are weighted versus alpha-tubulin expression and are the mean of 3 separate experiments.

Fig. 4. SF and GS-SF did not significantly affect GST expression in ACHN cells. After a 48 hours-period of exposure to ethanol (controls), 15µM SF, or 15µM GS-SF mixed with the culture medium, attached and floating cells were harvested and boiled for 5 min in PAGE sample loading buffer. Following SDS-PAGE, proteins (40µg) were transferred to nitrocellulose membrane as described in

the Material and Methods section. Immunodetection of GST classes and subclasses were performed on the membranes using specific antibodies. Typical experiments are presented.

Fig. 5. Cell death induced by SF and GS-SF in ACHN cells might not occur through regulation of Bax and Bcl2 protein expression . After a12, 24 and 48 hours-period of exposure to ethanol (controls), 15µM SF, or 15µM GS-SF mixed with the culture medium, attached and floating cells were harvested in PAGE sample loading buffer and boiled for 5 min. Protein samples (40µg) were fractionated by SDS-PAGE and transferred to nitrocellulose membrane as described in the Material and Methods section. Immunodetection of bax and bcl2 proteins were performed on the membranes using specific antibodies. A typical experiment is presented.

Table I

Neither SF nor GS-SF modify significantly the number of cells at the sub-G1 phase, which is characteristic of an apoptotic process

Treatment	**Percent of total cells in the sub-G1 phase[a]**
Control	2.74 ± 2.19
SF 15 µM	2.19 ± 0.98
GS-SF 15 µM	2.27 ± 1.38

[a] *Values presented are flow cytometry cell-counting into the window of subG1 phase of the cell cycle. Measures after a 48 hours period of exposure to ethanol (controls), 15 µM SF or 15 µM GS-SF as described in the Materials and Methods section. Results are mean ± SD of 4 distinct experiments.*