ANTIPROLIFERATIVE EFFECTS AND METABOLISM OF SULFORAPHANE AND GLUCORAPHANIN FROM BROCCOLI SPROUTS IN HUMAN COLON AND LIVER CANCER CELLS

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ABSTRACT

Broccoli sprouts (BS) provide a good source of glucosinolates, mainly glucoraphanin (GRA), which is hydrolyzed to the biologically active metabolite sulforaphane (4-methylsulfinylbutyl isothiocyanate, SFN). SFN is a naturally occurring cancer chemopreventive agent. The purpose of this study was to obtain the effective dose of a BS extract, the glucosinolate GRA and its metabolite SFN, which showed to be active in inhibiting the growth of three cancer cell lines, Caco-2 and HT-29 human colorectal carcinoma cells; and HEPG2 hepatocellular carcinoma cells by using the colorimetric assay technique MTT. The dose-dependent assay allowed establishing the half maximal inhibitory concentration (IC_{50}) for BS extract (1.67 µM, 1.62 µM and 3.2 µM, in Caco-2, HT-29, HEPG2 cell lines, respectively), and SFN (37.5 µM, 50.9 µM and 69.9 µM was observed in Caco-2, HT-29 and HEPG2 cells, respectively), however, glucoraphanin did not show an antiproliferative effect in the cells under study.
The absorption of sulforaphane metabolites from broccoli sprouts (SFN, SFN-glutathione and SFN-cysteine) by HEPG2 and Caco-2 cells during 3, 6, and 24h was assessed by a selective UHPLC/MS/MS procedure. Concentrations ranging 2 to 90 nmol/L were found within the cells, and concentrations ranging 1 to 60 nmol/L were found released in the medium, depending on the type of analyte under study.
The broccoli sprout extract and its bioactive isothiocyanate sulforaphane effectively inhibit proliferation of cancer cell lines: HT-29 and Caco-2 human colon cells; and HEPG2 human liver cells. Cells are capable of conjugative metabolism, since the SFN mercapturic derivatives could be identified in the incubation medium of HEPG2 and Caco-2 cell lines.
***Keywords**: Brassica, Cytotoxicity, Absorption*

INTRODUCTION

There is consistent epidemiological evidence that a diet rich in vegetables, particularly cruciferous, is inversely related to the risk of suffer certain types of cancer. The chemopreventive benefit of these vegetables is attributed in part to the glucosinolates (GLS), however, the biological activity correspond to their hydrolysis metabolites isothiocyanates (ITC), which are known to stimulate Phase II carcinogen detoxifying enzymes, such as glutathione S-trasferase (GST) and quinone reductase. The most potent stimulator of such enzimes found in broccoli sprouts is sulforaphane (SFN) (1-isothiocyanato-4-(methylsulfinyl)-butane). Also the chemoprevention of SFN by blocking initiation via inhibiting phase I enzymes has been studied. Once cancer is initiated, SFN can act via several mechanisms that modulate cell growth and cell death signals to suppress cancer progression (Clarke, *et al*., 2008). The inactive precursor glucoraphanin (GR) is hydrolyzed to sulforaphane (SFN) by the thiohydrolase myrosinase, found endogenously in broccoli, or by the microflora present in the colon.
The mechanisms by which isothiocyanates might exert their anticarcinogenic effects remain unclear and the evaluation of anticancer activity (antiproliferation) of SFN is very limited. Some recent results suggest that the chemopreventive activities of isothiocyanates may

involve other mechanisms in addition to the activation of detoxifying enzymes activities, as specific mechanisms that could also act at the DNA level or affect signal transduction pathways leading to growth arrest or cell death (Gamet-Payrastre *et al*., 2000).
Caco-2 cells feature many characteristics of intestinal epithelial cells, representing a widely accepted in vitro system for the human metabolism. SFN and other ITC are known to be metabolized in the enterocytes and the liver through the mercapturic acid pathway (Angelino & Jeffery, 2014). An initial reaction between the –N=C=S group of ITC and the cysteine sulfhydryl group of glutathione (GSH) can take place spontaneously and enhanced by glutathione S-transferase (GST). SF-GSH metabolites are found in plasma and urine. The liver is able to carry out enzymatic modifications of the GSH moiety, forming cysteinylglycine-(cys-gly), cysteine-(cys); and the final N-acetyl-cysteine-(NAC-) conjugate is formed in the kidney.
The aim of this work was to examine the effect of lyophilized BS, as a food matrix, the glucosinolate GRA, and its metabolite SFN on the proliferation of three cancer cell lines, Caco-2 and HT-29 human colorectal carcinoma cells; and HEPG2 hepatocellular carcinoma cells using the colorimetric assay method MTT. Different concentrations of BS, GRA and SFN were tested in order to find their IC_{50}. The development of human preventive studies focusing on the components of cruciferous vegetables would be advisable if an inhibitory effect was detected *in vitro*. In this study also the absorption of SFN metabolites of BS extract was studied to identify the possible metabolism in Caco-2 and HEPG2 cells.

MATERIAL AND METHODS

Standards

Glucoraphanin (GR) and sulforaphane (SFN) were purchased from CRA-CIN (Rome, Italy) and Sigma (St. Louis, MO), respectively. The standards of SFN–gluthatione, SFN–cysteine, and SFN-N-acetylcysteine (SFN–GSH, SFN–Cys, and SFN–NAC, respectively) were from Santa Cruz Biotech (Santa Cruz, CA). All LC–MS grade solvents were obtained from J.T. Baker (Phillipsburg, NJ).

Plant material

Broccoli sprouts (BS) were germinated for 8 days, then were collected, rapidly frozen in liquid N_2 and lyophilized prior analysis. Then, dry samples were hydrolyzed following Cramer and Jeffery (2011) method (Cramer & Jeffery, 2011). All samples tested were filter by 0.22µm. The quantification of GRA and total GLS was carried out in a HPLC-DAD (Baenas, *et al*., 2014; Mellon, *et al.*, 2002).

Citotoxicity assay

Caco2 and HT29 (human colorectal adenocarcinoma cell lines) and HepG2 (human hepatocellular carcinoma cell line) were maintained as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% (v/v) non-essential amino acids, and 1% penicillin/streptomycin (5,000 U/mL) (Lonza, Barcelona, Spain) in the presence of 5% CO_2 at 37°C and humidified atmosphereCells were plated in 96-well plates (1×10^4 cells/well) and grown for 24 h at 37°C in 5% CO_2. Cells were treated with different concentrations of sulforaphane (20, 10, 5, 1, 0.5, 0.1 and 0.05 µM in DMSO 0,1%), glucoraphanin (100, 85, 70, 55, 40, 25 and 10 µM in 0,1% DMSO) and extracted broccoli sprouts (containing 20, 10, 5, 1, 0.5, 0.1 and 0.05 µM of sulforaphane in DMSO 0,1%) dissolved in DMEM. After 24 h of incubation, cell viability was determined using the colorimetric MTT assay (Filipiak *et al*., 2014). The assay was repeated with 3 independent experiment replications. The viability was calculated considering controls containing a solvent control (0.1% DMSO) as 100% viable. DMSO at experimental concentrations was nontoxic to cells.

Metabolism assay

Differentiated Caco-2 and HEPG2 cells (1.5 x 10^5 cells/well) were grown in a 6-well plates and incubated with extracted BS (containing 1 µM SF), during 3, 6 and 24 h. The analysis of SFN and its derivatives (SFN-GSH and SFN- Cys) in cell lines was carried out in a UHPLC/MS/MS method described by Dominguez-Perles, *et al.,* (2014) (Dominguez-Perles *et al.*, 2014).

Statistical analysis

All analysis were conducted by triplicate, processed by Graphpad Prism to determine IC_{50} and SPSS 15.0. to carried out a multifactorial analysis of variance (ANOVA) and Tukey's Test to determine significant differences at *P* values < 0.05.

RESULTS AND DISCUSSION

Analysis of broccoli sprouts

The concentration of GRA was analyzed by HPLC-DAD after the inactivation of the myrosinase activity to prevent the hydrosysis of GRA to SFN (Mellon *et al.*, 2002). Total GLS amount was 377.64 mg·100g fresh weight (F.W.), being GRA the major compound, 160 mg·100g F.W. of the total. In the hydrolyzed extracts of broccoli sprouts, only SFN was detected by UPLC/MS/MS and the concentration was 13 mg·100g F.W. This data is in line with previous reports (Baenas *et al.*, 2014; Dominguez-Perles *et al.*, 2014; Force, *et al.*, 2007).

Cell proliferation

To study the antiproliferative effect of BS and its metabolites on different cell lines, we examined their cytotoxicity on Caco-2, HT-29 and HEPG2 cells. The inhibition of cell growth by BS extract and its metabolites is shown in Figure 1. SFN has been shown to inhibit cell cycle progression, induce apoptotic cell death, and inhibit angiogenesis in a variety of cancer cell types.

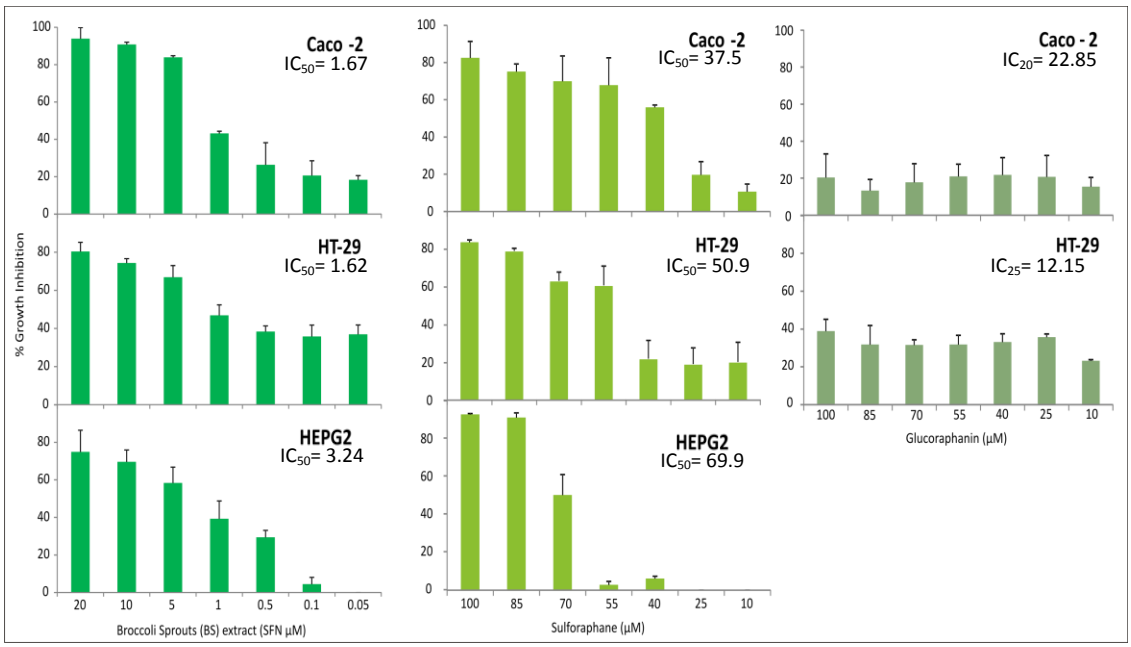

Figure 1. Growth Inhibition (%) of cell lines after application of BS extract, sulforaphane and glucoraphanin solutions. IC_{50} shows the half-maximal inhibitory concentration.

Data obtained was dose and time dependent for BS and SF, except for GRA, which did not achieve the half-maximal inhibitory concentration (IC_{50}) (Figure 1), consistent with observations made in other cancer cell types, including prostate cancer cells (PC3), colon cancer cells (HCT116) (Singh *et al.*, 2004), and Barrett esophageal adenocarcinoma cells (BEAC) (Qazi *et al*., 2010).
The lowest IC_{50} was observed after BS application; the IC_{50} was 1.6 µM and 3.2 µM, in both Caco-2 and HT-29 cells lines, and in HEPG2, respectively. The BS extract showed the highest antiproliferative activity in all cells, even containing lower concentration of SFN. SFN molecule in low concentrations (10 – 0.05 µM) did not reduce the viability of cells (data not shown). The bioactivities of different samples varied with different cell lines. A IC_{50} of 37.5, 50.9 and 69.9 was observed in Caco-2, HT-29 and HEPG2 cells, respectively, after SF application, according to Lubeska *et al*., (2012), who reported a IC50 of 33.4 µM. Gamet-Payrastre, *et al*., (2000) showed that SFN induced a cell cycle arrest in a dose-dependent manner, followed by cell death via an apoptotic process. This cycle arrest was correlated with an increased expression of G_2/M phase cells and the level of cyclins A and B1.

Cell metabolism
The levels of SFN and its derivatives were measured in HEPG2 and Caco-2 cells. After treating the cells with BS extract containing 1µM SFN for 3, 6 and 24h, metabolites were analysed both in whole-cell lysates and in the culture medium (Figure 2). Results after 24h of exposing the HEPG2 cells to the BS extract could not be measured because of the high cytotoxicity of BS in this cell model.

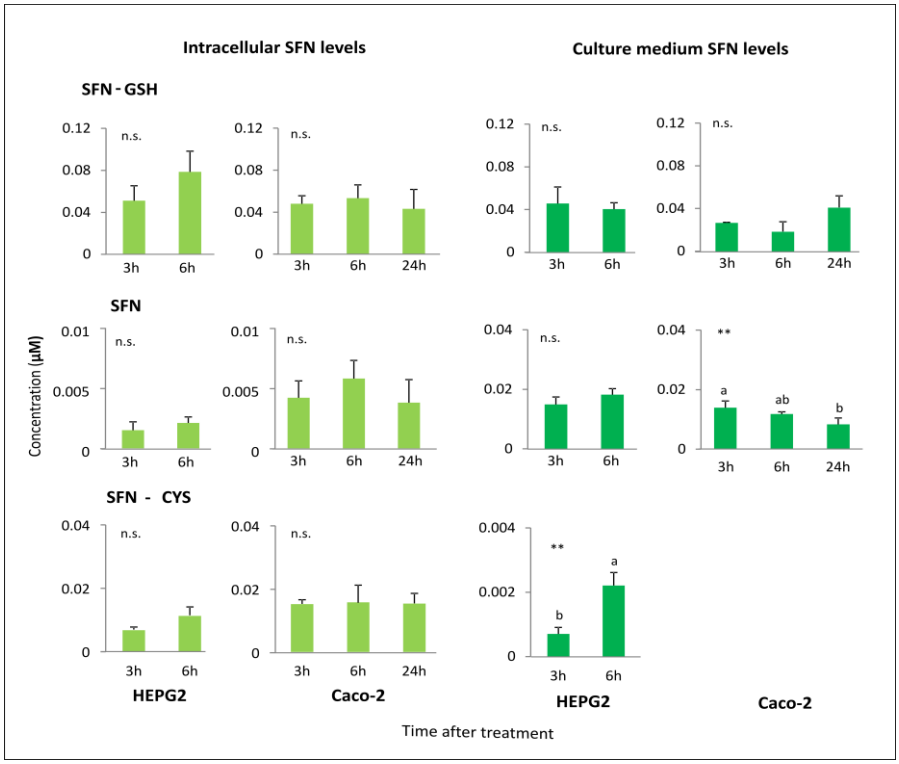

*Figure 2. Concentration (µM) of sulforaphane (SFN) and its metabolites sulforaphane-glutathion (SFN-GSH) and sulforaphane-cysteine (SFN-CYS) in the interior of HEPG2 and Caco-2 cells and their release in the culture medium after the application of broccoli sprouts extract containing 1µM of sulforaphane. Lower case letters show statistically significant differences at **p<0.01 and n.s. not significant p>0.05.*

SFN in cells, both intracellular and in the medium was principally accumulated as glutathione (GSH) conjugates. This process not only requires the action of the Phase II enzyme glutathione S-transferase (GST), it also depletes the cell of GSH, resulting in a rapid increase

in GSH production within the cell, therefore assisting carcinogen metabolism on two fronts. Phase II enzyme induction is considered the most likely contributing factor to the anticarcinogenicity of SFN, although the inhibitory effect on cell proliferation have been confirmed in other in vitro experiments (Frydoonfar, 2004). If the cells were treated with a GSH-depleting agent, the SFN could not be accumulated in the cells, and the subsequent induction of phase II enzymes was blocked (Kim, *et al*., 2003).
Results showed also the presence of SFN and SFN-CYS, after the application of lyophilized BS extract to the HEPG2 and Caco-2 cells, in both media and cells (Figure 2). Concentration of SFN-CYS was similar in both cells lines After 6h, a higher content of SFN and its metabolites were found in both cell line lysates compared to 3h and 24h of incubation (Figure 2).
Cells are capable of conjugative metabolism, since, as we have shown in our experiments, SFN mercapturic derivatives could be identified in the incubation medium, as well as in the cell lysate. SFN effect as modulator of absorption and metabolism in enzymatic systems has been proved before (Lubelska *et al*., 2012). Hence, these cell lines are a good model for the examination of metabolism regulation, even there are significant differences with human enterocytes (Petri *et al.*, 2003).

CONCLUSIONS

The results of this study indicate that broccoli sprouts and sulforaphane inhibit proliferation of cancer cell lines providing support to the role of *Brassica* foods in reducing the risk of certain cancers (Higdon, *et al*., 2007), nonetheless, it is necessary to study the potential synergy of SFN combined with other food components, as in this work, broccoli sprouts extract, with lower sulforaphane concentration,had a greater antiproliferative effect than SFN its self, which may lose their bioactivity. On the other hand, concentrations of SFN-metabolites found in inside Caco-2 and HEPG2 cells showed absorption of SFN after the application of broccoli sprouts extract and its conjugation as mercapturic derivatives, assisting carcinogen metabolism in the cell. These results should encourage further *in vivo* assays to understand their metabolism and bioavailability, and preventive efficacy as therapeutic agents within the confines of a clinical trial for any form of cancer. The consumption of broccoli sprouts or their use as ingredient in food industry would enrich the composition in health-promoting bioactives of new foods.

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