RESEARCH ARTICLE

Sulforaphane is Superior to Glucoraphanin in Modulating Carcinogen-Metabolising Enzymes in Hep G2 Cells

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Abstract

Glucoraphanin is the main glucosinolate found in broccoli and other cruciferous vegetables (*Brassicaceae*). The objective of the study was to evaluate whether glucoraphanin and its breakdown product sulforaphane, are potent modulators of various phase I and phase II enzymes involved in carcinogen-metabolising enzyme systems *in vitro*. The glucosinolate glucoraphanin was isolated from cruciferous vegetables and exposed to human hepatoma cell line HepG2 at various concentrations (0-25 μM) for 24 hours. Glucoraphanin at higher concentration (25 μM) decreased dealkylation of methoxyresorufin, a marker for cytochrome P4501 activity; supplementation of the incubation medium with myrosinase (0.018 U), the enzyme that converts glucosinolate to its corresponding isothiocyanate, showed minimal induction in this enzyme activity at concentration 10 μM. Quinone reductase and glutathione *S*-transferase activities were unaffected by this glucosinolate; however, supplementation of the incubation medium with myrosinase elevated quinone reductase activity. It may be inferred that the breakdown product of glucoraphanin, in this case sulforaphane, is superior than its precursor in modulating carcinogen-metabolising enzyme systems *in vitro* and this is likely to impact on the chemopreventive activity linked to cruciferous vegetable consumption.

Keywords: Chemoprevention - glucoraphanin - glucosinolate - isothiocyanate - sulforaphane

Asian Pac J Cancer Prev, **14 (7)**, 4235-4238

Introduction

Sulforaphane [1-isothiocyanato-4-(methylsulphinyl) butane] is a structurally-linked isothiocyanate possessing an aliphatic side chain which is present in large amounts in cruciferous vegetables and present as the glucosinolate, glucoraphanin. Consumption of these vegetables has been repeatedly associated to lower cancer incidence in epidemiological studies, and it is believed that glucosinolates may be liable for this effect (Ambrosone et al., 2004; Lam et al., 2009). Once the vegetable is disrupted via either mastication or chewing, the enzyme so-called myrosinase (β-thioglucoside glucohydrolase) get in touch with the glucosinolate altering it to the isothiocyanate; the same thing happen in the human intestine by microflora myrosinase (Verkerk et al., 2009). Isothiocyanate for instance sulforaphane is well absorbed and reached high bioavailability in rat and human (Hanlon et al., 2008; 2009a). Numerous studies in chemically-induced models of cancer have found that sulforaphane and other isothiocyanates are potent anti-cancer (Hecht 2000; Kuroiwa et al., 2006). In precision cut-rat tissue slices and rat hepatoma FAO cells, sulforaphane displayed its potency in modulating carcinogen detoxifying-enzymes via mechanism-based inhibition (Abdull Razis et al., 2010). The chemoprevention properties of glucosinolates is thought to be initiated by the isothiocyanates over a series of mechanisms including a decrease in the availability of the reactive intermediates of chemical carcinogens, suppression of cell proliferation and induction of apoptosis (Zhang, 2004). Gene expression analysis on apoptosis and inflammation showed significant down-regulation of Bcl-2, COX-2 and IL-1β after treating of HeLa cells with sulforaphane (Sharma et al., 2011). In other study, sulforaphane also induced cytotoxicity and lysosome- and mitochondria-dependent cell death in colon cancer cells with deleted p53, a tumour suppressor protein (Rudolf and Cervinka, 2011). Based on those findings, it was believed that the chemopreventive activity of cruciferous vegetables is completely the result of exposure to degradation products of glucosinolates, such as the aforementioned isothiocyanates, and that the parent glucosinolates make no contribution. Since there is ambiguity whether glucoraphanin or its breakdown-product sulforaphane responsible for the chemopreventive effects, this study was performed *in vitro* employing HepG2 cell line. Our finding is presented in the current paper that sulforaphane is a superior than its precursor

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glucoraphanin in modulating carcinogen-metabolising enzymes and this attribute may be an important contributor to its chemopreventive activity.

Materials and Methods

Methoxyresorufin, resorufin, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reductase, 2-methyl-1,4-naphthoquinone (menadione), thiazoyl blue tetrazolium bromide (MTT) (Sigma Co. Ltd., Poole, Dorset, UK), β-nicotinamide adenine dinucleotide phoshate reduced (NADPH) (Melford Laboratories Ltd., Ipswich, Suffolk, UK) were all purchased.

Isolation of glucosinolate

Glucoraphanin was isolated and purified from *Brassica oleracea* L. var. acephala sabellica (Cavolo nero di Toscana), according to a procedure developed at CRACIN of Bologna that we have previously described (Visentin et al., 1992; Abdull Razis et al., 2010). Sulforaphane was generated *in situ* by myrosinase-catalysed hydrolysis of natural glucoraphanin (Abdull Razis et al., 2010). Myrosinase (60 units/mg) was isolated from ripe seeds of white mustard (*Sinapis alba* L.), which one unit is defined as the amount of enzyme hydrolysing 1 μmol sinigrin per minute at a pH of 6.5 and 37ºC. Under the same experimental conditions applied, the isothiocyanate is the only enzymic breakdown product of the glucosinolate.

Cells treatment

The HepG2 cell line was kindly donated by Dr. Kate Plant (University of Surrey, UK). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1× non-essential amino acids (NEAA), 100 U/ml penicillin and 100 μg/ml streptomycin, and were grown until 70-80% confluent. At a density of 2×10^5 cells/ml, the cells were seeded (3 ml/well) into six-well plates using the same media, and cultured until reached the confluency at 50-70%. Solutions of the glucosinolate were prepared in Milli Q water and subsequently diluted in serum-free media (0-25 μM) alone or in the presence of myrosinase to generate isothiocyanate. Cells were incubated at 37ºC and 5% CO_2 in a humidified incubator for 24 h.

Enzyme assays

Following incubation, HepG2 cells were washed with ice-cold PBS and harvested in buffer containing 25 mM Tris-HCl, pH 7.4, and 125 mM sucrose (1 ml/well) using a cell scraper and the lysates were resuspended and collected using a 21G needle and a 1 ml syringe. Cells were then sonicated for 20 seconds and centrifuged at 9,000× g for 45 minutes at 4ºC, to prepare the post-mitochondrial supernatant (S9). The supernatant was kept at -80ºC until use. Completely thawed S9 was further centrifuged at 105,000× g for 45 min at 4˚C using a Beckman L7-65 ultracentrifuge with a fixed angle 70 ITI type rotor (Beckman Coulter Ltd, Bedfordshire, UK). The supernatant i.e. cytosolic fraction was removed and kept on ice; the microsomal pellet was resuspended in the same volume of S9 in 0.154 M KCl containing 50 mM Tris-HCl buffer, pH 7.4. The cytosolic and microsomal fractions were immediately used for carrying out enzyme assays. The dealkylation of methoxyresorufin (MROD) (Burke and Mayer, 1974) was determined in the microsomal fraction. The following assays were carried out in the cytosolic fraction: quinone reductase (NQO1) employing MTT [3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] as substrate (Prohaska and Santamaria, 1988), and glutathione *S*-transferase activity (Habig et al., 1974) monitored using CDNB as accepting substrate. Protein concentration was determined in both cellular subfractions using bovine serum albumin as standard (Bradford, 1976).

Glucosinolate toxicity evaluation

Cytotoxicity in HepG2 cells was assessed using MTT. HepG2 cells, at a density of 8×10^3 cells/ml, were seeded into 96-well plates using DMEM supplemented with 10% FBS, 1× non-essential amino acids (NEAA), 100 U/ml penicillin and 100 μg/ml streptomycin and cultured for 24 hours. Solutions of the test compounds and incubation were similar as above, and MTT, at 5 mg/ml in PBS, was added into the wells 2.5 hours before completion of the incubation. At the end of the incubation, DMSO (100 μl) was added into the wells, and absorbance was read at 540 nm using an ELISA plate reader.

Statistical analysis

Enzyme activities are presented as mean±standard deviation. Statistical evaluation was carried out by one-way ANOVA followed by the Dunnett's test.

Results

Effect of glucosinolate on MROD activity

Incubation of HepG2 cells with glucoraphanin at the highest concentration (25 μM) caused a significant decrease in the dealkylation of methoxyresorufin (MROD), a biomarker for CYP1A2 activity; but, in contrast, supplementation of myrosinase to the glucosinolate-containing incubations system up-regulated this enzyme activity at 10 μM (Figure 1).

Effect of glucosinolate on Phase II enzymes activities

Neither quinone reductase nor glutathione *S*-transferase

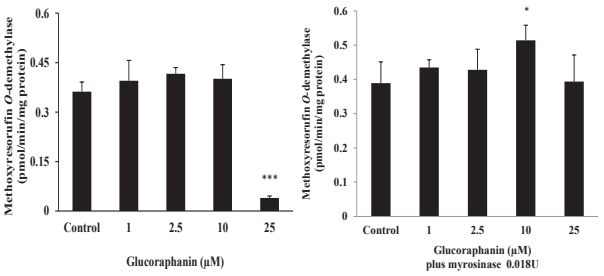

Figure 1. Effect of Glucoraphanin on MROD Activity in Human Hepatoma HepG2 Cells. HepG2 cells were incubated with glucoraphanin (0-25 μM) or glucoraphanin (0-25 μM) plus myrosinase (0.018 U) for 24 h. Activity is presented as mean±SD of three replicates. *p<0.05; **p<0.01; ***p<0.001 when compared to the control (0 μM)

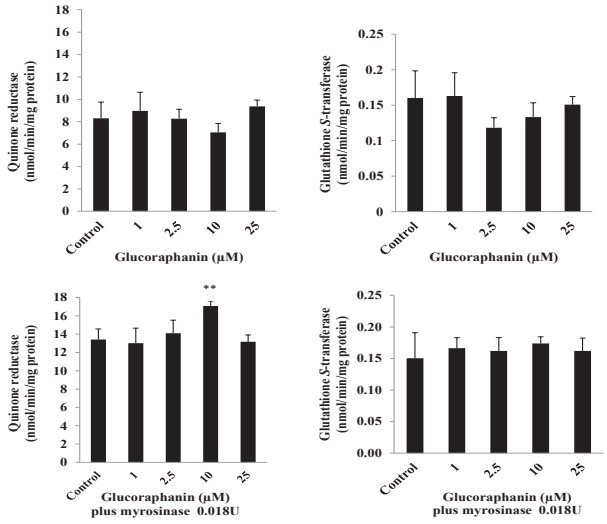

Figure 2. Effect of Glucoraphanin on Quinone Reductase and Glutathione *S*-transferase Activities in Human Hepatoma HepG2 Cells. HepG2 cells were incubated with glucoraphanin (0-25 μM) or glucoraphanin (0-25 μM) plus myrosinase (0.018 U) for 24 h. Activities are presented as mean±SD of three replicates. *p<0.05; **p<0.01; ***p<0.001 when compared to the control (0 μM)

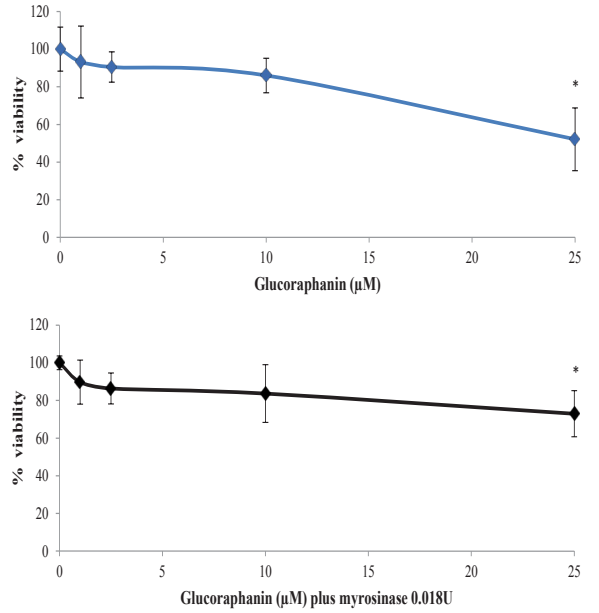

Figure 3. Evaluation of the Toxicity of Glucoraphanin in Human Hepatoma HepG2 Cells. Cytotoxicity in HepG2 cells was evaluated using the MTT assay. HepG2 cells, at a density of 8×10^3 cells/ml, were seeded into 96-well plates and cultured for 24 hours. These cells were then incubated in culture medium supplemented with glucoraphanin (0-25 μM) or glucoraphanin (0-25 μM) plus myrosinase (0.018 U) for 24 hours. An aliquot (10 μl) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 5 mg/ml in PBS, was added into the wells 2.3 hours before completion of the incubation. At the end of incubation, DMSO (100 μl) was added into the wells, and absorbance was read at 540 nm. Results are expressed in percentage, as mean±SD of triplicate determinations.*p<0.05 as compared to control (0 μM)

were affected following exposure of the cells line to the glucosinolate (Figure 2). When myrosinase was added to the glucosinolate-containing incubations, the inductive potential was marked at 10 μM in the case of quinone reductase activity (Figure 2).

HepG2 cells viability following incubation with glucosinolate

Exposure of HepG2 cells line to glucoraphanin alone and/or in the presence of myrosinase to the incubation system, elicited no toxicity unless at the highest concentration (25 μM), as exemplified by MTT assay (Figure 3).

Discussion

Oral administration of glucoraphanin at high doses leads to elevation of cytochrome P450 and glutathione *S*-transferase activities in the liver of rats (Perocco et al., 2006). It is impossible to distinguish whether the increase in these activities is caused by the glucosinolate itself or due to sulforaphane released from the action of intestinal microflora (Getahun and Chung, 1999). In order to answer this query, studies using human hepatoma HepG2 cell lines were conducted to evaluate the capability of glucoraphanin and its breakdown product sulforaphane in modulating cytochrome P450 enzymes of the CYP1 family, the most vital phase I enzymes involved in xenobiotic metabolism (Ioannides and Lewis, 2004), and also phase II detoxifying enzymes such as quinone reductase and glutathione *S*-transferase.

In line with our previous finding (Abdull Razis et al., 2010), MROD and quinone reductase activities were up-regulated when the cells were exposed to glucoraphanin in the presence of myrosinase; demonstrating that the isothiocyanates are the principal contributors to the increased activity rather than the parent glucosinolate. These findings are commensurate with *in vitro* and *in vivo* studies where sulforaphane induced quinone reductase activity both in Hepa1c1c7 mouse hepatoma cell and in male Fisher 344 rats (Hintze et al., 2003). The up-regulation of phase II enzymes by sulforaphane involves the antioxidant response element (ARE), the transcription of which is regulated by Nrf2 (Nuclear factor-erythroid 2 p45-related factor 2) (Juge et al., 2007); thus reflect the potential in preventing the Keap1-mediated degradation of Nrf2. However, glutathione *S*-transferase activity was not affected; discrepancy with Hanlon et al. (2009b) though this may reflect in tissue differences.

In contrast, these findings contradict the work of Scholl et al. (2011) who demonstrated that in the HepG2 cells, glucosinolate glucoraphasatin induced quinone reductase activity of significantly higher than its degradation products that were generated by addition of myrosinase into the cell culture media prior to the addition to the cells; however, in their studies much higher concentrations (50-200 μM) of the glucosinolate were employed. In other study, the methanol extract of radish sprouts containing glucosinolate glucoraphasatin demonstrated high potency in inducing quinone reductase activity in Hepa1c1c7 cells (Lee and Lee, 2006), although it is possible that other compounds in the extract were responsible for this effect. It is important to point out that glucosinolate is an exceptional substrate of myrosinase, so that exposure to

this enzyme at pH 7.4 allows to its complete hydrolysis to produce the isothiocyanate (Papi et al., 2008).

Neither glucoraphanin nor its breakdown product sulforaphane (1-10 μM) caused any toxicity in the HepG2 cells, comparable with the studies where sulforaphane isomers were exposed to HepG2 and FAO cells, respectively (Kassie et al., 2003; Abdull Razis et al., 2011). Similarly, studies employing the MTT assay revealed that sulforaphane (1-10 μM) does not influence cell viability of murine hepatoma Hepa 1c1c7 cells (Anwar-Mohamed and El-Kadi, 2009).

In conclusion, it can be concluded that the breakdown product of glucosinolate, in this case sulforaphane superior than its precursor glucoraphanin in modulating carcinogen-metabolising enzyme systems *in vitro* and this is likely to impact on the chemopreventive activity of cruciferous vegetables.

Acknowledgements

The authors would like to thank the Ministry of Higher Education, Malaysia for providing a research fund.

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