Enzyme Induction and Cytotoxicity of Phenethyl Isothiocyanate and its Glutathione Conjugate towards Breast Cancer Cells

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Abstract

Chemoprevention using isothiocyanates is partly the result of the induction of phase II enzymes for carcinogen detoxification from healthy cells. However, phase II enzyme activity can impair cancer therapeutic agents. The objective of this study was to assess phase II enzyme induction and cytotoxicity of phenethyl isothiocyanate (PEITC) and glutathione conjugate with PEITC (GsPEITC) using MCF-7 and MDA-MB-231 breast cancer cells. Changes of phase II enzymes, glutathione-S-transferase (GST) and NAD(P)H quinone reductase (QR), were measured by colorimetric procedures. Cell viability was determined using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium- bromide) assay. From current results, 24hr exposure to ≤5 μM of PEITC or GsPEITC was not cytotoxic to breast cancer cells. No significant induction of phase II enzyme occurred with a low dose range. By contrast, MCF-7 viability decreased by 50% following 24 hr treatment with 42 ±0.5 μM PEITC or 19. ±0.1 μM GsPEITC. The concentrations for 50% loss of viability (IC50) were 103±0.5 μM PEITC and 32±0.5 μM GsPEITC in tests using MDA-MB-231 cells. Thus, MCF-7 cells were
significantly more sensitive to PEITC or GsPEITC compared to MDA-MB-231 cells. Cytotoxic doses (≥50 µM) of PEITC or GsPEITC increased phase II enzyme activity by a maximum of 500-700-percent. To conclude, cytotoxic doses of PEITC or GsPEITC possess phase II enzyme inducing activity in MCF-7 and MDA-MB-231 breast cancer cells. (225 words)

**Keywords**: isothiocyanates, phase II enzymes, breast cancer cells, glutathione, nutraceuticals

### 1 Introduction

Breast cancer is a leading cause of cancer death in women accounting for 23% of newly diagnosed cancers in 2008 (Globoscan, 2008). Many breast tumours, and the oestrogen-receptor positive (ER+) MCF-7 breast cancer cell-line, show depressed glutathione-S-transferase (GST) activity (Esteller, et al. 1998). However, over-expression of GST may contribute towards resistance for cancer chemotherapeutic agents (Wang, et. al 1999, Perquin, et al. 2001; Oguztuzun, et al. 2011). Genetic polymorphisms affecting GST activity were reported to affect adversely the risk of breast cancer and survival rates (Helzlsouer et al.1998). The GST family has varied roles, ranging from cell detoxification, antioxidant response, and medication of stress-induced apoptosis (Townsend & Tew 2003).

High intakes of cruciferous vegetables, which are rich sources of dietary isothiocyanates, decrease the risk of cancer (Higdon et al. 2007). Chemoprevention by isothiocyanates is attributed partly to the induction of phase II enzymes for carcinogen detoxification in healthy cells and inhibition of phase I enzymes for mutagen activation (Lamy et al. 2011; Singh and Singh, 2012, Dinkova-Kostova 2013). Phenethyl isothiocyanate (PEITC) was selected for the present investigation because it is was recently found to promote cell-cycle arrest and apoptosis in breast cancer cells (Zhang, et al. 2003; Tseng, et al.2004).

Currently, the effect of isothiocyanates on phase II enzymes in breast cancer cells remains uncertain. To our knowledge, no investigations dealing with the phase II enzyme induction by PEITC or the glutathione conjugate with PEITC (GsPEITC) using breast cancer cells have been reported. The relations between phase II enzyme induction activity and cytotoxicity are also uncertain. The aims of this study were, to assess PEITC and GsPEITC cytotoxicity and phase II enzyme inducing activity using MCF-7 (ER+) and MDA-MB-231(ER-) breast cancer cell lines.

### 2 Materials and Methods

Phenethyl isothiocyanate (PEITC; 99%), glutathione, (3-(4,5-Dimethyl thiazol}
-2-yl)-2,5-diphenyltetrazoliumbromide; MTT), menadione (2-methyl-1,4-naphtha-
quinone), 1-chloro-2, 4-dinitrobenzene (CDNB) and other chemicals were from
Sigma-Aldrich Ltd. (Poole, UK). Human breast cancer cell lines were purchased
from American Type Culture Collection (LGC Standards Ltd, Teddington,
Middlesex, UK). Dulbecco’s modified eagle medium (DMEM), foetal bovine
serum (FBS), penicillin-streptomycin and non-essential amino acids, were from
GIBCO® Laboratories (Invitrogen Ltd, UK).

The glutathione conjugate with PEITC (GsPEITC) was synthesized as
described previously (Brusewitz, et al.,1977). As a basis for structure
confirmation, the molecular weight of GsPEITC was determined using an Electro
spray ionization mass spectrometer (ESI-MI; Finnigan MAT, San Jose, CA). The
solubility of GsPEITC was determined by suspending (10 mg) using a range of
selected solvents (1 ml) with sonication for 10 minutes. The resulting solutions
were micro-centrifuged (11,000 rpm, 5 min) and non-dissolved GsPEITC pellet
was dried at 60°C to a constant weight. The solubility of GsPEITC was calculated
by weight difference. The chemical stability of GsPEITC was determined by
dissolving with deionized water adjusting to pH 2, 5 and 7, and storing at 37°C.
The concentrations of GsPEITC at different time points were determined by
HPLC.

Cell culture, cytotoxicity and phase II enzyme assay

Cancer cells were cultured using DMEM supplemented with 10% FBS,
1% penicillin-streptomycin and 1% non-essential amino acid. Culture flasks and
96-well microplates were incubated at 37 °C in a 5% CO₂ atmosphere. Cell
viability was determined by the MTT assay (Price, & McMillan, 1990). Cells
previously cultured using 96-microwell plates were treated with 20 μl of MTT,
incubated for 2 hrs at 37°C and treated with 100 μl of lyses buffer (5% sodium
dodecyl sulphate in 50% dimethyl formamide) for 24 hrs to dissolve the blue
formazan crystals formed. Absorbance was measured at 595 nm using a
microplate reader (VERSAmax™, Molecular Devices, Toronto, Canada).

PEITC or GsPEITC (10mM) was dissolved with dimethyl sulfoxide
(DMSO) for toxicity testing, diluted with culture medium, and filter-
sterilized using 0.20-μm cellulose acetate filters. The sterile solution of PEITC or GsPEITC
was further diluted with media before use. Cultured cells were trypsinized,
counted using a NucleoCounter (model NC-3000, ChemoMetec A/S, Denmark),
and seeded (10,000 cells/ well) in 96-microwell plates with 50 μl of culture
medium overnight at 37°C to allow attachment. Cells were treated with 0-1000
μM PEITC or GsPEITC (50 μl) for 24 hrs at 37°C. Growth medium and
isothiocyanate was removed and cells were washed twice with ice-cold PBS (100
μl) prior to the MTT assay (Shoemaker et al. 2004).

Phase II enzyme activities were determined after treatment with
isothiocyanates as described above for cytotoxicity testing. GST was assayed
using a substrate-mixture containing CNDB (1mM), glutathione (1 mM), and
Triton X-100 (0.08%) added to cultured cells in 96-well plates (Habig, et al.1974).
Enzymatic activity was measured after 20 min of incubation at 37 °C using an absorbance microplate reader at 345 nm. The activity of QR activity was evaluated using menadione / MTT as substrate (Prochaska, & Santamaria 1988). Cultured cells were treated with substrate (200 µL/ well) and absorbance readings measured at 610 nm every 4 min for 36 min.

**Statistical analysis**

Experiments were repeated on three occasions and measurements performed in triplicate. Statistical analysis employed the Statistical Package for Social Sciences (SPSS, version 20). Differences between treatments were examined using analysis of variance (ANOVA) with Turkey post hoc analysis; actual probability ($p$) values are cited and $p < 0.05$ is considered significant.

### 3. Results and Discussion

Phenethyl isothiocyanate (PEITC) was recently found to be cytotoxic towards breast cancer cells (Zhang, et al. 2003; Tseng, et al. 2004). However, the effect of PEITC on phase II enzyme activity in breast cancer remains uncertain. An increase of phase II enzyme activity is desirable for healthy tissue associated with cancer chemoprevention (Lamy et al. 2011, Singh & Singh, 2012; Dinkova-Kostova 2013). In contrast, for established cancer cells phase II enzymes contribute to increasing resistance towards therapeutic agents (Wang, et. al 1999, Perquin, et al. 2001; Oguztuzun, et al. 2011). The present investigations were initiated to assess the effect of PEITC and GsPEITC on breast cell viability and phase II enzyme induction. GsPEITC is of interest because thiol-conjugates are products of PEITC metabolism (Brusewitz et al. 1977). Moreover, the rate of decomposition of isothiocyanate-thiol conjugates was found to affect their efficacy as phase I enzyme inhibitors (Conaway et al. 2001, Tang et al. 2006).

From preliminary investigations, the synthesis yield for GsPEITC was 62% according to the initial weight of glutathione reacted with PEITC. The molecular weight for GsPEITC was shown by ESI-MS as between $(m/z =) 471.39$ and $(m/z [MH^+] =) 470.95$ in agreement with calculated values. RP-HPLC analysis revealed peak retention times for, GSH, GsPEITC and PEITC of 2.0, 7.4 and 16.3-16.8 min, respectively (data not shown). So GsPEITC eluted before PEITC confirming an increase in hydrophilic character. The solubility characteristics for GsPEITC (Figure 1) showed 93± 3% dissolution using DMSO and 60 ± 7 % or 46 ± 3 % solubility with deionised water (pH 6.5) or water adjusted to pH 2. Conjugation with GSH increased water solubility of PEITC by approximately 60-times (6 mg/ml vs. 0.01 mg/ml). However, GsPEITC was sparingly soluble in the range of organic solvents tested except DMSO (Figure 1).

GsPEITC degrades by dissociation to form free glutathione and PEITC (Conaway, et al., 2001; Tang, et al. 2006). We found the dissociation half-life for
GsPEITC at 37 °C (t½) was 12.6 hrs in water pH 7.0 compared to values of ~1hr to 12.9hr reported previously (cf. Lamy et al. 2011 or review). The t½ for GsPEITC was further increased to 166 hr and 113 hr at pH 5.0 and pH 2.0 indication of low pH stabilization. The marked stability of dry samples of GsPEITC during storage for several months (data not shown) is also noteworthy. In comparison with such results, the t½ value for PEITC was 56.1-64.3 hrs (pH 5.3-pH7.0; 20 °C) decreasing to 1.7 hrs in a medium containing bovine serum albumin due to protein binding (Morse, et al. 1991). Adsorption of PEITC by plastic containers can lead also to losses from solution (Hu & Morris, 2004).

There were no significant changes in breast cancer cell viability with 24 hr exposure to < 5 µM of PEITC or GsPEITC (Figure 2A & 2B). The concentration of PEITC or GsPEITC leading to a 50% reductions of cancer cell viability (IC50) ranged from 19-103 µM (summarized in Table 1). For comparison, the reported IC50 for PEITC treatment of MCF-7 cells was 17.5 µM or 11 µM for with three or 72 hr exposure, respectively. Such data implies that isothiocyanates inhibit cancer cells rapidly within 3 hrs with no major increases after longer exposure (Zhang et al. 2003). By contrast, the IC50 for PEITC treatment of MCF-7 cells was 30.3 µM, 20.8 µM, 15.2 µM, 22.6 µM, or 6.5 µM with one, two, three, six or 48 hr exposure, respectively and so exposure is apparently important (Tseng, et al. 2004).

Past IC50 values for MCF-7 cells are ~2-fold lower compared to values from the present study where investigators dissolved isothiocyanate using protein free media prior to cells treatment (Zhang et al. 2003; Tseng, et al. 2004). In contrast, serial dilution of isothiocyanate solutions using cell culture media (this study) probably led to protein-isothiocyanate interactions (Morse, et al. 1991) which affect bioactivity. The time period for cell pre-culture (24 or 48hrs) prior to isothiocyanate exposure may also be important, since cells in exponential growth phase were more sensitive to drug treatment (Tseng, et al. 2004). Taking such factors into account, the IC50 value for PEITC with respect to breast cancer cells is 20-40 times higher than the reported plasma concentration (1-2 µM) attainable from dietary sources of isothiocyanate (Lamy et al. 2011).

In the present study, there were no changes of phase II enzyme activity at low doses of PEITC or GsPEITC. However, cellular GST activity increased by 300% (p < 0.001) and 700% (p = 0.004) after exposure of MCF-7 cells to 50 µM PEITC or GsPEITC, respectively (Figure 3). Also the activity of GST increased by 120 and 300% after the treatment of MDA-MB-231 cells with 50 µM PEITC or GsPEITC, respectively. The QR activity for MCF-7 cells increased by 500% with exposure to 50 µM GsPEITC (p= 0.0119). Further higher degrees of enzyme induction were observed following treatment with 500 µM of isothiocyanate and the data was apparently less reliable owing to the low numbers of viable cells present in the system (Figure 2).

In summary, the findings from this paper suggest that breast cancer cells are insensitive to phase II enzyme induction by low levels of PEITC or GsPEITC but that exposure to toxic doses result in significant rises in GST and QR. There
are no directly comparable investigations of enzyme induction using PEITC with MCF-7 or MDA-DB-123 cells in the literature. Indirect support for our findings, can be seen from previous studies using sulforaphane which also indicate a low sensitivity of cancer cells to phase II enzyme induction. Normal breast epithelial (MCF-10) cells demonstrated a 500% increase in QR activity and a 300% increase of GST activity after treatment with 2 µM sulforaphane (Singletary & MacDonald, 2000). In contrast, treatment of MCF-7 cells with 25 µM sulforaphane produced no increase of GST expression whilst QR activity increased by 20%. When MDA-MB-231 cells were exposed to 25 µM sulforaphane, cell GST activity increased by 5% and QR activity decreased by 21% (Jiang, et al. 2003). Treatment with 0.3 - 3 µM of sulforaphane upregulated 23 genes in normal breast epithelial cells but no changes occured in MCF-7 cells under similar conditions (Telang, et al. 2009a).

The possibility that breast cancer cells exhibit low sensitivity towards phase II enzyme induction needs to be corroborated for a wider group of compounds and, if confirmed, would suggest isothiocyanates pose a low risk of inducing adverse or unwanted nutrient-drug interactions. However, phase II enzyme induction requires a transient accumulation of isothiocyanates within cells (Ye and Zhang 2001). The uptake of isothiocyanates by breast cancer cells is thought to be impaired by their efficient export by multidrug resistance proteins (MRP1 & MRP2) and/or the breast cancer resistance protein (BCRP) but not p-glycoprotein (Hu & Morris, 2004; Ji & Morris, 2005; Telang et al, 2009b). Further research is warranted to consider possible links between phase II enzyme induction, cytotoxicity and the impact of isothiocyanates on cell responses to therapeutic agents.

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References

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Tables 1: Inhibition of breast cancer cells by phenethyl isothiocyanate (PEITC) or glutathione-phenethyl isothiocyanate (GsPEITC)

<table>
<thead>
<tr>
<th>Cell type / Agent</th>
<th>PEITC</th>
<th>GsPEITC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>42.0± 0.51</td>
<td>19±0.10</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>103.2±0.50</td>
<td>32.5±0.53</td>
</tr>
</tbody>
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*IC50 = concentrations leading to 50% decline in cell viability. Rows & columns with different letters are significantly different (p <0.05)

Figure 1: Solubility profile for glutathione-phenethyl isothiocyanate (GsPEITC) conjugate in a selection of organic solvents. Data shows mean ± SE of 3 replicates.
Figure 2: Cytotoxicity of phenethyl isothiocyanate (PEITC) and glutathione-phenethyl isothiocyanate (GsPEITC) conjugate against two breast cancer cell lines; (A) MCF-7 cells, (B) MDA-DB-231 cells. Data shows the mean (%) ± SE for three independent experiments; except where omitted for clarity columns with different letters are significantly different (p<0.05).
Figure 3: Phase II enzyme induction for MCF-7 cells treated with phenethyl isothiocyanate (PEITC) or glutathione-phenethyl isothiocyanate (GsPEITC) conjugate. (A) Glutathione- S-transferase (GST) activity, (B) Quinone reductase (QR) activity. Non-treated cells scaled = 100. Bar shows mean ± SE from three independent experiments; except where omitted for clarity columns with different letters are significantly different (p<0.05).

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