

## RESEARCH ARTICLE

# Inhibitory effect of Phenethyl Isothiocyanate Against Benzo[a]Pyrene-Induced Rise in CYP1A1 mRNA and Apoprotein Levels as its Chemopreventive Properties

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### Abstract

**Background:** Phenethyl isothiocyanate (PEITC), the most comprehensively studied aromatic isothiocyanate, has been shown to act as an anti-cancer agent mainly through modulation of biotransformation enzymes responsible for metabolizing carcinogens in the human body. Humans are often exposed to carcinogenic factors, some of which through the diet, such as polycyclic aromatic hydrocarbon benzo[a]pyrene via the consumption of over-cooked meats. Inhibition of the enzymes responsible for the bioactivation of this carcinogen, for example CYP1A1, the major enzyme required for polycyclic aromatic hydrocarbons (PAHs) bioactivation, is recognized as a chemoprevention strategy. **Objective:** To evaluate the inhibitory effects of PEITC against benzo[a]pyrene-induced rise in rat liver CYP1A1 mRNA and apoprotein levels. **Materials and Methods:** Precision cut rat liver slices were treated with benzo[a]pyrene at 1 and 5  $\mu$ M in the presence of PEITC (1-25  $\mu$ M) for 24 hours, followed by determination of CYP1A1 mRNA and apoprotein levels using quantitative polymerase chain reaction and immunoblotting. **Results:** Findings revealed that PEITC inhibited benzo[a]pyrene-induced rise in rat liver CYP1A1 mRNA in a dose-dependent manner as well as the apoprotein levels of CYP1A. **Conclusions:** It was demonstrated that PEITC can directly inhibit the bioactivation of benzo[a]pyrene, indicating chemopreventive potential.

**Keywords:** Polycyclic aromatic hydrocarbons - benzo[a]pyrene - phenethyl isothiocyanate - chemoprevention.

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### Introduction

Human are exposed to chemical carcinogens almost on daily basis. Major sources of these compounds are, for example, polycyclic aromatic hydrocarbon (PAH) derived from tobacco and cooked-food (Hecht, 2003), and other food-derived carcinogens such as heterocyclic amines (HCAs) and nitrosamines (Boyce et al., 2014). In most cases, bioactivation of chemical carcinogens is prerequisite for the formation of electrophiles which form DNA adducts leading to carcinogenesis. Inhibition of the enzymes responsible for the bioactivation of carcinogens such as CYP1A1 and 1A2, the major enzymes required for PAH and HCA bioactivation, respectively (Kobayashi et al., 2009; Wohak et al., 2014) is recognized as a chemoprevention strategy (Liu et al., 2013). Accordingly, the ability of glucosinolates and isothiocyanates (ITCs) to prevent the formation of genotoxic metabolites through modulation of carcinogen-metabolizing enzymes has recently attracted extensive interest worldwide. It has been reported that drinking water supplemented with glucosinolate-rich vegetables, such as Brussels sprouts and

red cabbage, reduced IQ-mediated preneoplastic lesions in F344 rats which was accompanied by induction of hepatic UDPGT and CYP1A2 activities (Kassie et al., 2003). Sulforaphane and its synthetic analogues down-regulated CYP1A2 and directly inhibited CYP1A1-induced activity by benzo[a]pyrene in human cell line (Skupinska et al., 2009).

It has been proposed that PEITC, isolated from watercress, is a potent anti-carcinogen in chemically-induced models of cancer (Kuroiwa et al., 2006). The chemoprevention properties of this isothiocyanate is attributed via a series of mechanisms involving a reduction in the appearance of the toxic metabolites of carcinogens by defeating cytochrome P450-stimulated bioactivation and/or promoting detoxifying enzymes (Konsue and Ioannides, 2008; 2010a, 2010b; Abdull Razis et al., 2011; Devi and Thangam, 2012; Abdull Razis and Mohd Noor, 2013a, 2013b; Abdull Razis et al., 2014). Indeed of its positive react to phase II detoxifying enzymes, its response to phase I cytochrome P450 1A1 is remain unclear. Earlier studies revealed that PEITC is able to modulate CYP1A1 mRNA in rat and human liver (Abdull Razis et al., 2011).

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Moreover, PEITC has increased CYP1A1 protein and decrease enzymatic activity of CYP1A1 indicating that this aromatic isothiocyanate elicits chemopreventive activity through a mechanism based-inhibition (Konsue and Ioannides, 2010a). Thus, the inhibition of benzo[a]pyrene-induced rise in CYP1A1 expression level is considered to be a helpful biomarker of chemopreventive potential of PEITC. In addition, an established association between modulation of cytochrome P450 1A1 and cancer has been noted (Williams et al., 2000). Therefore, the overall objective of the present study was to evaluate the inhibitory effect of PEITC against benzo[a]pyrene-induced rise in rat liver CYP1A1 mRNA and apoprotein levels.

## Materials and Methods

### Materials

Phenethyl isothiocyanate (PEITC), benzo[a]pyrene, Goat anti-mouse polyclonal antibodies (Sigma Co. Ltd., Poole, Dorset, UK), anti-CYP1A1 monoclonal antibodies (AMS Biotechnology, Abingdon, UK), rat genomic DNA (Novagen, Wisconsin, USA), RNase-free DNase, DNase stop solution, DNase buffer (Promega, Wisconsin, USA), Nucleospin® RNA II (Macherey-nigel GmbH & Co, Düren, Germany), RPMI 1640, Earle's balanced salt solution (EBSS), Minimum Essential Medium Alpha (MEM- $\alpha$ ), gentamicin, fetal calf serum, Superscript II reverse transcriptase, random hexamers, RNase OUT, RNase-free water, and dNTP mix (Invitrogen, Paisley, Scotland), Absolute™ QPCR Mix (Abgene, Epsom, Surrey, UK), were purchased.

### Rat liver samples

The study was conducted according to the Animals (Scientific Procedures) Act 1986. Male sex Wistar albino rats (200-250 g) were bought from B&K Universal Ltd (Charles River, Margate, Kent, UK). These animals were caged at a relative humidity of 30-40 %,  $22 \pm 2$  °C, with an alternate 12-h light: dark cycle with light starting from 07.00 h.

### Precision-cut rat liver slices preparation and incubation

Liver slices (200-300  $\mu$ m) were set from 8 mm cylindrical cores using Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL, USA) as was earlier developed by Hashemi et al. (1999). A 12-well plate was employed to culture the slices with one slice was resided in each well followed by incubation in 1.5 ml media containing test compounds at 37 °C under 95 % air/5% CO<sub>2</sub>. For replication, three different slice pools were prepared, each containing 3 slices, were deployed for each concentration of test compounds.

### Extraction of RNA and quantitative real-time polymerase chain reaction

After 24-hour incubation with benzo[a]pyrene (1 and 5  $\mu$ M) alone or in the existence of PEITC (1-25  $\mu$ M), three slices were deployed for extracting total RNA employing NucleoSpin® RNA II followed by quantification of RNA using Nanodrop spectrophotometer at 260 nm. For reverse

transcription, RNA was primed with random hexamers using a Superscript II reverse transcriptase. cDNA was then amplified employing Absolute™ QPCR Mix comprising 100 nM fluorogenic probe and 400 nM forward and reverse primers. The reactions were performed using ABI7000 SDS Instrument (Applied Biosystems, Warrington, UK) and quantitated utilizing ABI software against a standard curve. CYP1A1 mRNA expression levels were normalized with respect to 18S rRNA and expressed in percentage. The oligonucleotide sequences of the forward and reverse primers, and also probes are as below:

#### CYP1A1

5' primer: GCCTTCACATCAGCCACAGA

3' primer: TTGTGACTCTAACCACCCAGAATC

Probe: TGGCCGTCACCACATTCTGCCTT

#### 18S rRNA

5' primer: CGGTACCACATCCAAGGAA

3' primer: GCTGGAATTACCGCGGCT

Probe: TGCTGGACACAGACTTGCCCTC

### Immunoblot analysis on CYP1A1 apoprotein levels

Rat liver slices were prepared as afore-mentioned and incubated in media containing a range of PEITC concentrations (1-40  $\mu$ M) in the existence and nonexistence of benzo[a]pyrene. Two concentrations of benzo[a]pyrene (0.1 and 10  $\mu$ M) were applied in rat liver slices. Following 24-hour incubation, slices were eradicated from the medium, S9 was prepared and stored at -80 °C. Microsomal fractions were made by multi-step centrifugation when required. Protein concentration was determined according to Bradford (1976) followed by western blot analysis to ascertain whether decrease in CYP1A1 mRNA levels engages, at least in part, a drop in enzyme availability. Microsomal proteins were loaded onto SDS-PAGE 10% (w/v) and subsequently transferred electrophoretically to PVDF membrane. Immunoblot analysis was conducted via exposure to the primary antibodies followed by the compatible HRP-conjugated secondary antibody. The immunoblots were then quantified using densitometry employing GeneTool software (Syngene Corporation, Cambridge, UK), with the control band labelled as 100%.

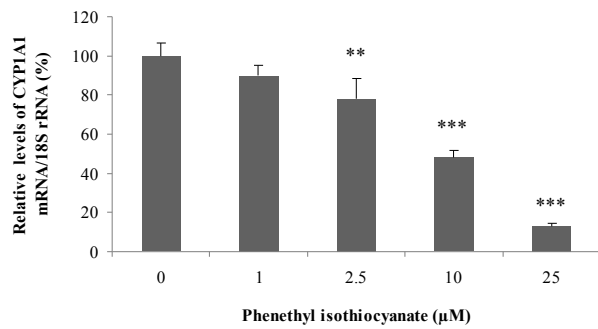
### Statistical analysis

All results are shown in mean  $\pm$  standard deviation. Three different slice pools, each consisting 3 rat slices were used with triplicate analyses for the determination of mRNA levels. Statistical evaluation was performed employing one-way ANOVA followed by Dunnett's post-hoc analysis.

## Results

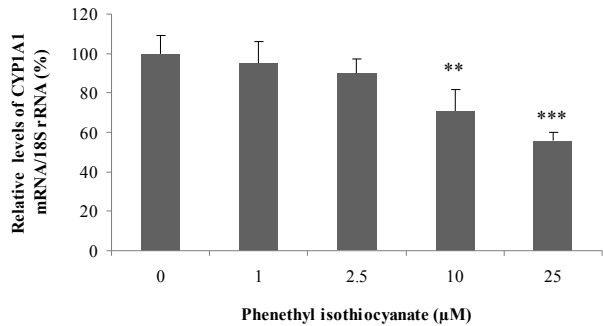
### CYP1A1 mRNA studies

A 24-hour incubation of precision-cut rat liver slices with benzo[a]pyrene (1 and 5  $\mu$ M) alone resulting in rises of CYP1A1 mRNA levels (as expressed in 100%); this effect was inhibited in a concentration-dependent manner by PEITC (Figure 1 and Figure 2). It is worthwhile



**Figure 1. CYP1A1 mRNA Levels in Rat Liver Slices Exposed to PEITC in Combination with Benzo[a] Pyrene.**

Precision-cut rat liver slices were incubated with benzo[a]pyrene (1 μM) in the presence of PEITC (0-25 μM) for 24 h. Total RNA was extracted from tissue slices and the mRNA levels of CYP1A1 were quantified by quantitative RT-PCR methodology (Taqman); mRNA levels were normalized with respect to 18S rRNA and expressed in percentage. Values are presented as mean±SD of three replicates, each containing 3 slices. \*\*, p<0.01; \*\*\*, p<0.001 as compared to 0 μM of PEITC



**Figure 2. CYP1A1 mRNA Levels in Rat Liver Slices Exposed to PEITC in Combination with Benzo[a] pyrene.**

Precision-cut rat liver slices were incubated with benzo[a]pyrene (5 μM) in the presence of PEITC (0-25 μM) for 24 h. Total RNA was extracted from tissue slices and the mRNA levels of CYP1A1 were quantified by quantitative RT-PCR methodology (Taqman); mRNA levels were normalized with respect to 18S rRNA and expressed in percentage. Values are presented as mean±SD of three replicates, each containing 3 slices. \*\*, p<0.01; \*\*\*, p<0.001 as compared to 0 μM PEITC

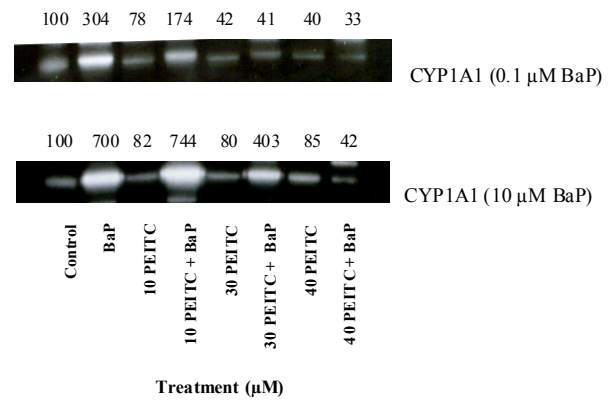
stating that the inhibition of the benzo[a]pyrene-initiated induction of CYP1A1 mRNA levels was seen starting from incubation at 2.5 mM of PEITC.

#### CYP1A apoprotein levels

Immunoblot analysis of CYP1A1 revealed that the inhibition of enzymatic activity was due to a decrease in enzyme availability (Figure 3). At 0.1 μM benzo[a]pyrene, synergistic effect observed at the low 10 μM PEITC concentration, but antagonistic effect was manifested at the higher concentrations of PEITC (Figure 3). At the 10 μM benzo[a]pyrene, synergistic effect was evident at the 10, and to lower extent, 30 μM concentration of PEITC, but a marked antagonistic effect at the 40 μM concentration was seen (Figure 3).

## Discussion

PEITC has the ability to protect against many chemical carcinogens in different organs of animal



**Figure 3. Effect of PEITC on Benzo[a]Pyrene-Mediated CYP1A1 Apoprotein Levels in Rat Liver Slices.**

Liver slices were incubated in culture medium containing 0.1 or 10 μM BaP alone or in combination with PEITC (1-40 μM) for 24 hr. Microsomal proteins were isolated and equal amounts of corresponding protein were loaded on to 10% (w/v) SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. The immunoblot analysis was carried out utilizing mouse anti-rat CYP1A1 primary antibodies followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 30 μg of total protein. Values above blots show % levels of optical density of each band relative to control

models (Wu et al., 2009). The potential basis of protecting mechanisms towards chemical carcinogens involves interference of the metabolic pathways of carcinogens by modulating enzymes, such as CYP1, which are associated with bioactivation (Conney, 2003) and inducing phase II enzymes such as GST and NQO1 (Tan et al., 2010). Following the experimental evidence indicating modulation of carcinogen-metabolizing enzymes by PEITC in human and rat liver slices as well as *in vivo* in rat as described in the published articles (Ioannides and Konsue 2008, Konsue and Ioannides, 2010a, 2010b), the present study was conducted to elucidate the role of PEITC as chemopreventive agent towards distinct carcinogen namely benzo[a]pyrene. This carcinogen was chosen as it was believed to contribute to human cancer risk, as important tobacco-derived and diet-derived carcinogen (Lee and Shim, 2007). Moreover, this compound is known as pro-carcinogen, requiring CYP450-mediated activation, catalyzed by CYP1A1 in the case of benzo[a]pyrene (Uppstad et al., 2010).

Benzo[a]pyrene is an important environmental carcinogen presented in cigarette smoke, industrial waste by-products (Chen et al., 2002) and food (Hecht, 1999). Average daily intake of benzo[a]pyrene ranges from non-detectable to 389 ng per person per day depending on individual lifestyle (European Food Safety Authority, 2008). The induction of enzymes by benzo[a]pyrene is mainly to enhance its biotransformation, primarily by CYP1 especially CYP1A1 (Uppstad et al., 2010). Such induction is believed to enhance the generation of the genotoxic benzo[a]pyrene 7,8-diol-9,10-epoxide that binds covalently to cellular DNA and initiates carcinogenesis (Nebert et al., 2004). Benzo[a]pyrene is an avid ligand of the Ah receptor and this binding leads to up-regulation of CYP enzyme involved in its metabolism such

as CYP1A1 and CYP1B1 (Nebert et al., 2004; Vondracek et al., 2009). Utilizing precision-cut rat liver slices the aim of this study was to determine whether PEITC can inhibit benzo[a]pyrene-mediated enzyme induction, possibly by interfering with its interaction with the Ah receptor.

The breakdown product of glucosinolates, isothiocyanates have been shown in epidemiology studies to lower cancer risk, and their chemoprevention properties have been proven experimentally (Hecht, 2000). As electrophiles, they are likely to manipulate cellular processes through binding covalently to nucleic acids, proteins, or small molecules and may be indirectly reducing pools of cellular reductants (Nakamura and Miyoshi, 2010). A mechanistic study to determine the effect of isothiocyanates on CYP1A1 activity and expression, and Ah receptor translocation in Mcf7 cells demonstrated that this enzyme was significantly stimulated by benzo[a]pyrene, and isothiocyanates were able to inhibit the rise in activity (Skupinska et al., 2009). A view has been expressed that the inhibition of CYP1A1 enzyme may serve as a useful strategy for cancer chemoprevention (Kleiner et al., 2002).

In the previous studies, exposure of rat liver slices to PEITC led to a modest rise in CYP1A1 mRNA levels at concentration as low as 1  $\mu$ M, and the reduction was observed at the highest concentrations employed (Abdull Razis et al., 2011). Similarly, in primary human hepatocytes both sulforaphane and PEITC raised CYP1A1 mRNA levels (Gross-Steinmeyer et al., 2004). Hepatic CYP1A1 apoprotein levels of rats administered diets containing PEITC was up-regulated at high doses with no concomitant rise in CYP1A1 activity, most probably due to the point that PEITC is a mechanism-based inhibitor (Konsue and Ioannides, 2008; Konsue and Ioannides; 2010a). In line with the ligand-binding studies (Abdull Razis et al., 2011), a benzo[a]pyrene-induced hepatic CYP1A1 mRNA levels was inhibited by PEITC at concentration dependent manner. Similar to CYP1A apoprotein levels, at the high benzo[a]pyrene concentration, synergism was seen at the 10, and to lesser extent, 30  $\mu$ M concentration of PEITC, but a clear inhibition at the 40  $\mu$ M concentration. In agreement, it has been demonstrated that oral administration of sulforaphane to mice resulted in the inhibition of benzo[a]pyrene-induced aryl hydrocarbon receptor activation that consequently decreased cytochrome P450 enzyme activities (Priya et al., 2011).

Induction of CYP1 is known to be regulated through the ligand-activated aryl hydrocarbon receptor (AhR) (Stejskalova et al., 2011). Prior to dissociation from its ligand-binding partners, AhR translocates into the nucleus, subsequently binds to a specific nucleotide sequence of general transcription factors and initiates gene transcription of enzymes including CYP1 (Stejskalova et al., 2011). As a result of suppression of apoprotein levels, the inhibitory effects of PEITC in the current study may involve modulation of the binding of benzo[a]pyrene by PEITC, although the underlying mechanism remains to be established. On the other hand, inhibition mechanism of PEITC on CYP1A may be the result of interactions at the site of the AhR, preventing the binding of benzo[a]

pyrene and/or mechanism-based inhibition leading to loss of activity.

In summary, the present study demonstrated that PEITC inhibits the benzo[a]pyrene-induced rise in hepatic CYP1A1 mRNA and apoprotein levels, and this may add to its established chemopreventive potential.

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