RESEARCH ARTICLE

Phenethyl isothiocyanate, a naturally occurring phytochemical, is an antagonist of the aryl hydrocarbon receptor

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Scope: The aryl hydrocarbon (Ah) receptor is a ligand-activated transcription factor that is activated by many carcinogens, and its target gene products play a major role in tumour development, so that antagonists of the Ah receptor represent potential chemopreventive agents.
Methods and results: Experimental evidence is presented herein that phenethyl isothiocyanate (PEITC), a phytochemical present in cruciferous vegetables, is such an antagonist. PEITC was a very weak ligand to the Ah receptor, as assessed using the chemical-activated luciferase expression (CALUX) assay, and a poor inducer of CYP1A1 mRNA levels when incubated in precision-cut rat liver slices for 24 h. It antagonised effectively, however, the interaction of benzo[*a*]pyrene to the receptor, being capable of preventing its binding as well as displacing it from the receptor. Moreover, PEITC suppressed in concentration-dependent manner the benzo[*a*]pyrene-mediated rise in rat hepatic CYP1A1 mRNA levels in rat slices. Finally, PEITC antagonised the benzo[*a*]pyrene-mediated increase in the O-deethylation of ethoxyresorufin in both rat and human precision-cut liver slices.
Conclusion: It is concluded that PEITC is an effective antagonist of the Ah receptor in rat and human liver, and this potential may contribute to its established chemopreventive activity.

Keywords:
Ah receptor / Chemoprevention / Cruciferous vegetables / Glucosinolates / Phenethyl isothiocyanate

1 Introduction

Phenethyl isothiocyanate (PEITC), an isothiocyanate with an aromatic side chain, is a phytochemical found in cruciferous vegetables, one of its major sources being watercress, where it is present as a glucosinolate, namely gluconasturtiin [1]. When the vegetables are disrupted, for example during mastication, the enzyme myrosinase (β-thioglucoside glucohydrolase) is released and converts gluconasturtiin into PEITC; microbial myrosinase also contributes to this reaction in the human intestine [2]. Strong epidemiological evidence has been presented of an inverse link between cruciferous vegetable consumption and cancer incidence at a number sites including prostate [3], lung [4], colorectal [5] and breast [6], all being common sites of tumour formation, especially in developed countries. Moreover, in laboratory-based studies, isothiocyanates antagonised the carcinogenicity of chemicals in animal models of the disease [7]. For example, PEITC afforded protection against chemically induced carcinogenesis in the lung, mammary gland, pancreas, colon, forestomach and oesophagus induced by nitrosocompounds, azoxymethane and polycyclic aromatic hydrocarbons [8–13]. One of its principal mechanisms of action is to limit the

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Abbreviations: Ah, aryl hydrocarbon; **ARNT,** aryl hydrocarbon nuclear translocator; **PEITC,** phenethyl isothiocyanate

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availability of the genotoxic metabolites of chemical carcinogens by suppressing their cytochrome P450-mediated bioactivation and/or stimulating their detoxification by up-regulating enzyme systems such as glutathione *S*-transferase, quinone reductase, epoxide hydrolase and glucuronosyl transferase [14–17]. Indeed, exposure to PEITC led to a decrease in DNA-adduct levels in the liver, colon and prostate of rats treated with the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a carcinogen present in cooked food [18].

The aryl hydrocarbon (Ah) receptor was the first ligand-activated transcription factor to be recognised in the regulation of cytochrome P450 enzymes, in this case the CYP1 family of enzymes [19]. A rapidly increasing amount of experimental evidence demonstrated that this receptor plays a pivotal role in the predisposition to or development of numerous pathophysiological conditions, including cancer, such that agonism of this receptor by xenobiotics is considered to lead to a detrimental outcome [20, 21]. Indeed, Ah receptor null mice were refractive to the carcinogenicity of the polycyclic aromatic hydrocarbon benzo[*a*]pyrene [22]. Following interaction of the ligand with the receptor, the complex translocates into the nucleus where it interacts with the aryl hydrocarbon nuclear translocator (ARNT), and the heterodimer binds to dioxin response elements (DREs) leading to a pleiotropic response including up-regulation of CYP1 proteins [23]. Similarly, experimental studies established that ARNT was required for tumour initiation by benzo[*a*]pyrene [24]. The obvious corollary to the finding that the Ah receptor is linked to pathophysiology, is that antagonism of this receptor may reflect potential chemopreventive activity. Indeed, 3′-methoxy-4′-nitroflavone and 6,2′,4′-trimethoxyflavone, both potent antagonists, are considered as potentially beneficial and may possess therapeutic activity [25, 26]. Here, we document for the first time that the naturally occurring phytochemical phenethyl isothiocyanate is a weak agonist but a strong antagonist of the Ah receptor that may contribute to its established chemopreventive activity.

2 Materials and methods

2.1 Materials

PEITC [*CAS* 2257-09-02], ethoxyresorufin, resorufin, benzo[*a*]pyrene and thiazolyl blue tetrazolium bromide (Sigma, Poole, Dorset, UK), rat genomic DNA (Novagen, Wisconsin, USA), goat polyclonal antibody to lactate dehydrogenase (abcam, Cambridge, UK), NADPH (Melford Laboratories, Ipswich, UK), DNase buffer, DNase stop solution, RNase-free DNase, cell culture lysis reagent, luciferase assay reagent (Promega, Wisconsin, USA), Nucleospin® RNA II, total RNA isolation system (Macherey-nagel GmbH & Co, Düren, Germany), Absolute™ QPCR Mix (Abgene, Epsom, Surrey, UK), Earle's-balanced salt solution (EBSS), foetal calf serum, gentamicin, random hexamers, Superscript II reverse transcriptase and RPMI 1640 with L-glutamine culture medium, minimum essential medium α (MEM-α), dNTP mix, RNase-free water, RNase OUT (Invitrogen, Paisley, Scotland) were all purchased. Goat polyclonal antibody to the Ah receptor (M20:sc-8089), goat polyclonal antibody to Arnt1 (C-19:sc-8076) and donkey anti-goat IgG-HRP were purchased from Santa Cruz Biotechnology (CA, USA).

2.2 Animal and human liver samples

Male Wistar albino rats (200–250 g) were obtained from B&K Universal (Hull, East Yorkshire, UK) or Charles River (Margate, Kent, UK). The animals were housed at $22 \pm 2^{\circ}C$, 30–40% relative humidity, in an alternating 12-h light:dark cycle with light onset at 07.00 h. Liver sections from two human cadaveric livers that could not be used for transplantation purposes were obtained from the UK Human Tissue Bank (The Innovation Centre, Oxford Street, Leicester, UK). Sample A was from a 67-year-old female who died from hypoxic brain injury, and sample B from a 64-year-old male who died as a result of a head injury. The time interval from the moment of donors' death to the beginning of the incubation did not exceed 12 h on both occasions. On receipt, the liver sections were immediately transferred into a sterile container and, after the UW transport solution was carefully decanted, were washed 3–4 times with culture medium.

2.3 Preparation and incubation of rat and human precision-cut liver slices

Rat and human liver slices (200–300 μm) were prepared from 8 mm cylindrical cores using a Krumdieck tissue slicer (Alabama Research and Development, Munsford, AL, USA) as previously described [27]. The multiwell plate procedure, using 12-well culture plates, was used to culture the slices. One slice was placed in each well, in 1.5 mL of culture medium. Slices were incubated under sterile conditions on a reciprocating plate shaker housed in a humidified incubator, at a temperature of 37°C and under an atmosphere of 95% air/5% CO_2. The slices were initially pre-incubated for 30 min in order to slough off any dead cells due to slicing. Three different slice pools, each comprising ten rat or human slices, were used per concentration.

2.4 Assays

Following incubation, slices were removed from culture medium, homogenised and post-mitochondrial supernatants prepared and stored at −80°C. When required, microsomes were isolated by centrifugation ($105\,000 \times g \times 1$ h) and the

dealkylation of ethoxyresorufin [28] and protein content [29] were determined.

2.5 Transcript-level measurement

Precision-cut rat liver slices were incubated with benzo[*a*]-pyrene (2 μM) alone or in the presence of PEITC (1–25 μM) for 4 h. Three slices per sample were used for total RNA extraction, with triplicate analyses being carried out. RNA was extracted using the NucleoSpin® RNA II total RNA isolation system, and was quantified using a Nanodrop spectrophotometer. Total RNA was treated with RNase-free DNase to remove genomic contamination. Reverse transcription was primed with random hexamers and carried out by Superscript II reverse transcriptase according to manufacturer's instructions. To ensure that DNase-treated samples were free from genomic contamination, an RT control was carried out for every RNA sample. cDNA generated from 50 ng was amplified using AbsoluteTM QPCR Mix with 400 nM primers and 100 nM fluorogenic probe in a total reaction volume of 25 μL. Q-PCR reactions were run on the ABI7000 SDS instrument (Applied Biosystems, Warrington, UK) and quantitation was carried out using the ABI proprietary software against a standard curve generated from rat genomic DNA. For the quantitative reverse transcription-polymerase chain reaction, the primers and TAMRA/FAM dual-labelled probes have already been described [30]. Each primer and probe set was designed to amplify sequences within a single exon, so that genomic DNA could be used as a standard.

2.6 AhR ligand-binding assay

Interactions of the PAHs with the Ah receptor were assessed using the chemical-activated luciferase expression (CALUX) assay. In 24-well plates, transfected H1L1.1c2 cells were cultured (7×10^4 cells/mL) in α-MEM supplemented with 10% FBS and penicillin–streptomycin–neomycin antibiotic solution; cells were cultured for 24 h until 50–70% confluent. Cells were then incubated with PEITC and/or benzo[*a*]pyrene, dissolved in DMSO, for 24 h at 37°C and 5% CO_2 in a humid environment, and subsequently washed with PBS; 100 μL of lysis reagent was added into each well and further incubated for 15 min at room temperature. Cell lysates were centrifuged at $13\,000 \times g$ for 2 min, and luciferase activity in the supernatant was determined using the Promega-stabilised luciferase assay reagent according to manufacturer's instructions. Luminescence was read in a Packard Lumicount microplate luminometer with the PlateReader software (Packard Instrument). TCDD (10^{-9} M) served as a positive control, and was used to normalise the binding of other ligands, In studies whose objective was to investigate the ability of PEITC to prevent the binding of benzo[*a*]pyrene to the Ah receptor, cells were incubated initially for 24 h with PEITC (10^{-9} to 10^{-6} M), washed with PBS to remove the free isothiocyanate, and then incubated for a further 24 h in fresh medium containing benzo[*a*]pyrene (10^{-11} to 10^{-5} M). When the objective was to evaluate the potential of PEITC to displace benzo[*a*]pyrene from the Ah receptor, cells were incubated initially with benzo[*a*]pyrene (10^{-11} to 10^{-5} M) for 24-h, were subsequently washed with PBS to remove the unbound aromatic hydrocarbon, and then incubated for a further 2 or 24 h in fresh medium containing PEITC (10^{-9} to 10^{-6} M).

2.7 Determination of Ah receptor and ARNT expression

In order to monitor changes in the Ah receptor and ARNT protein expression in H1L1.1c2 cells, Western blot analysis was performed. Cell lysates were harvested and the cytosolic fractions isolated. Cytosolic proteins were loaded onto 10% w/v SDS-PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. The immunoblot analysis was carried out by exposure to the goat polyclonal antibody to Ah receptor or goat polyclonal antibody to Arnt1 followed by the appropriate peroxidase-labelled secondary antibody. Lactate dehydrogenase was used as the housekeeping protein for the soluble protein from cell lysate. Immunoblots were performed in triplicate and quantitated by densitometry using the GeneTool software (Syngene Corporation, Cambridge, UK).

2.8 Cell toxicity studies

Cytotoxicity in H1L1.1c2 cells was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). H1L1.1c2 cells, at a density of 7×10^4 cells/mL, that were seeded (200 μL) into 96-well plates and cultured for 24 h until 50–70% confluent. These cells were then incubated in culture medium supplemented with benzo[*a*]pyrene (10^{-11} to 10^{-5} M), PEITC (10^{-10} to 10^{-5} M) and benzo[*a*]pyrene (10^{-11} to 10^{-5} M) with PEITC (10^{-6} M) for 24 h at 37°C and 5% CO_2 in a humid environment. An aliquot (10 μL) of MTT at 5 mg/mL in PBS was added into the wells 2.3 h before completion of the incubation. At the end of incubation, medium was removed and DMSO (100 μL) was added into the wells, and absorbance was read at 540 nm using the ELISA plate reader.

2.9 Statistical evaluation

The results are presented as mean ± standard deviation of three pools, each comprising ten slices. For the determination of transcript level, three slices per sample were used with triplicate analyses being carried out. Statistical evalua-

tion was carried out by one-way ANOVA followed by Dunnett's post-hoc analysis.

3 Results

3.1 Cellular toxicity studies

Neither benzo[*a*]pyrene nor PEITC elicited any toxicity in the H1L1.1c2 cells at the concentrations studied (Fig. 1). Slight toxicity was noted, however, at high concentrations of benzo[*a*]pyrene when incubated with the cells in the presence of PEITC (10^{-6} M).

3.2 Ah receptor studies

PEITC was a weak agonist of the Ah receptor, attaining only an 8% binding compared with TCDD, which was not dose-dependent (Fig. 2A). Benzo[*a*]pyrene, in contrast, was a very good ligand for the receptor, demonstrating dose-dependent agonism, achieving a binding of 80% at a concentration of 10^{-5} M. Benzo[*a*]pyrene-mediated agonism of the Ah receptor was markedly antagonised by PEITC, especially at the lower concentrations of the hydrocarbon $<10^{-6}$ M (Fig. 2B). When the H1L1.1c2 cells were pre-incubated with the isothiocyanate for 24 h, that was subsequently removed, and a further 24-h incubation with benzo[*a*]pyrene carried out, the benzo[*a*]pyrene activation of the Ah receptor was prevented, with no response for benzo[*a*]pyrene concentrations below 10^{-6} M being evident at all concentrations of PEITC tested (Fig. 2C). If the cells were initially exposed to benzo[*a*]pyrene that was subsequently removed and a further incubation conducted for 2 h in the presence of PEITC, the activation of the receptor by etc benzo[*a*]pyrene was impaired, especially at the highest concentrations of the hydrocarbon (Fig. 2D). Once again, this effect was not dependent on the PEITC concentration employed, with all concentrations tested exerting a near-complete abolition of the benzo[*a*]pyrene agonism of the Ah receptor; increasing the incubation time with the isothiocyanate from 2 to 24 h had no further influence (results not shown). Immunoblot analysis demonstrated that exposure of the H1L1.1c2 cells to benzo[*a*]pyrene elevated in dose-dependent manner the protein levels of the Ah receptor and ARNT (Fig. 3). Interestingly, PEITC was also able to elicit a rise in Ah receptor and ARNT protein levels, although this was restricted to the two highest concentrations (Fig. 3). The increase was less pronounced when the cells were incubated in the presence of both compounds (Fig. 3).

3.3 CYP1A1 mRNA studies

When PEITC (1 μM) was incubated with precision-cut rat liver slices, a modest but statistically significant increase in

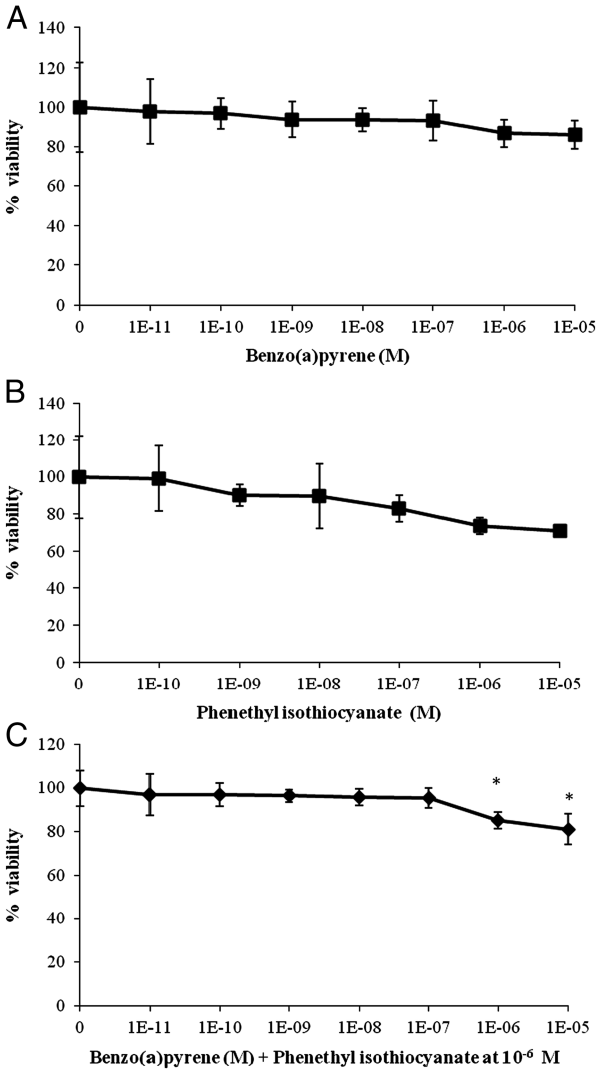

Figure 1. Evaluation of the toxicity of phenethyl isothiocyanate in H1L1.1c2 cells. Cytotoxicity in H1L1.1c2 cells was evaluated using the MTT assay. H1L1.1c2 cells, at a density of 7×10^4 cells/mL, were seeded into 96-well plates and cultured for 24 h. These cells were then incubated in culture medium supplemented with (A) benzo[*a*]pyrene (10^{-11} to 10^{-5} M), (B) phenethyl isothiocyanate (10^{-10} to 10^{-5} M), and (C) benzo[*a*]pyrene (10^{-11} to 10^{-5} M) with phenethyl isothiocyanate (10^{-6} M) for 24 h. An aliquot (10 μL) of 3-(4,5- di methyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 5 mg/mL in PBS, was added into the wells 2.3 h 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 with control.

CYP1A1 mRNA levels was evident following incubation for 8 h or longer (Fig. 4A). A concentration-dependent study revealed that, following incubation for 24 h, a significant increase in CYP1A1 mRNA levels was achieved only at 1 μM, whereas a decrease was evident at the two highest concentrations studied (Fig. 4B). Incubation of rat liver

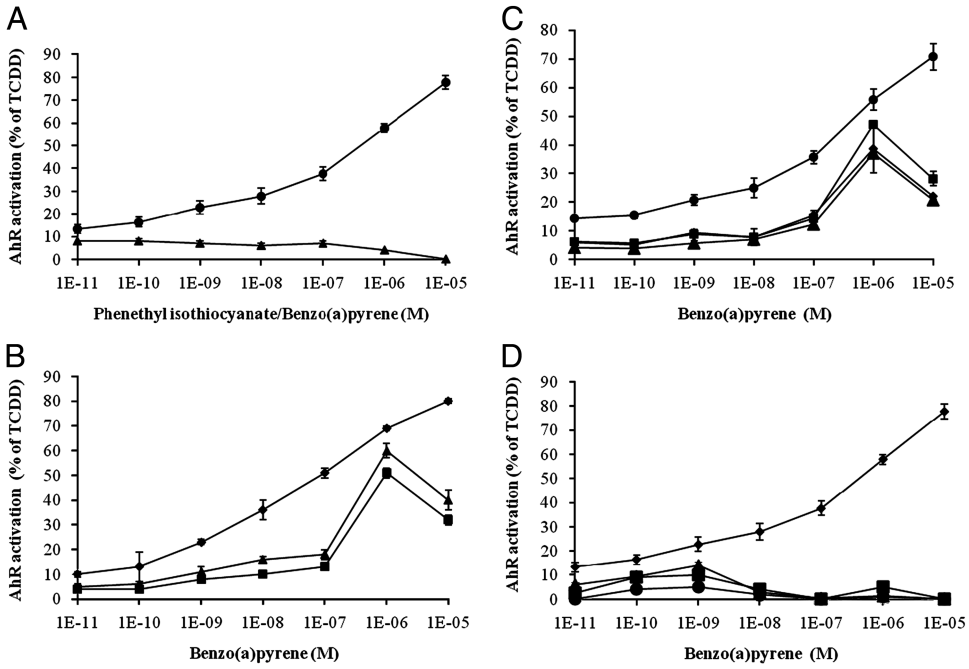

Figure 2. Interactions of benzo[*a*]pyrene and phenethyl isothiocyanate with the Ah receptor. (A) H1L1.1c2 cells (7×10^4 cells/mL) were incubated in culture medium supplemented with benzo[*a*]pyrene (10^{-11} to 10^{-5} M) and phenethyl isothiocyanate (10^{-10} to 10^{-4} M) for 24 h. ▲, Phenethyl isothiocyanate; ●, benzo(*a*)pyrene; (B) H1L1.1c2 cells (7×10^4 cells/mL) were incubated in culture medium supplemented with benzo[*a*]pyrene (10^{-11} to 10^{-5} M) and either phenethyl isothiocyanate (10^{-6} M) or (10^{-9} M) for 24 h. ♦, Benzo(*a*)pyrene, ■, benzo(*a*)pyrene with phenethyl isothiocyanate at 10^{-9} M; ▲, benzo(*a*)pyrene with phenethyl isothiocyanate at 10^{-6} M; (C) H1L1.1c2 cells (7×10^4 cells/mL) were incubated in culture medium supplemented with or without phenethyl isothiocyanate (10^{-9}, 10^{-7}, 10^{-6} M) for 24 h followed by benzo[*a*]pyrene (10^{-11} to 10^{-5} M) for a further 24 h. ●, Benzo(*a*)pyrene; ▲, phenethyl isothiocyanate at 10^{-9} M with benzo(*a*)pyrene; ♦, phenethyl isothiocyanate at 10^{-7} M with benzo(*a*)pyrene; ■, phenethyl isothiocyanate at 10^{-6} M with benzo(*a*)pyrene; (D) H1L1.1c2 cells (7×10^4 cells/mL) were incubated in culture medium supplemented with benzo[*a*]pyrene (10^{-11} to 10^{-5} M) for 24 h followed by phenethyl isothiocyanate (10^{-9}, 10^{-7} and 10^{-6} M) for a further 2 h. ●, Benzo(*a*)pyrene with phenethyl isothiocyanate at 10^{-9} M; ▲, benzo(*a*)pyrene with phenethyl isothiocyanate at 10^{-7} M; ■, benzo(*a*)pyrene with phenethyl isothiocyanate at 10^{-6} M; ♦, benzo(*a*)-pyrene. In all studies, activation of the receptor is expressed as% of TCDD activation (10^{-9} M). Results are presented as mean ± SD of triplicate determinations.

precision-cut liver slices with benzo[*a*]pyrene alone for 4 h caused a huge rise in CYP1A1 mRNA levels; this effect was antagonised by PEITC in a concentration-dependent manner (Fig. 4C). It is worth noting that the antagonism of the benzo[*a*]pyrene-mediated induction of CYP1A1 mRNA levels was observed even with 1 μM PEITC, which was shown (vide supra) to cause a rise in the mRNA levels.

3.4 Ethoxyresorufin O-deethylase

Exposure of rat liver slices to benzo[*a*]pyrene resulted in a marked increase in the CYP1A-mediated O-deethylation of ethoxyresorufin; this rise in activity was antagonised by PEITC in a concentration-dependent manner, with complete antagonism being observed at concentrations > 30 μM (Fig. 5A). In concordance with the rat studies, when precision-cut liver slices from two human donors were incubated with benzo[*a*]pyrene alone, ethoxyresorufin O-deethylase was significantly elevated, but the rise in activity was antagonised by PEITC; once again antagonism was complete at the higher doses of the isothiocyanate used (Fig. 5B and C).

4 Discussion

Extensive studies, largely employing TCDD, have established that ligand binding to the Ah receptor unleashes a plethora of events that are detrimental to the cell and organism [20, 21]. The Ah receptor has been associated with various types of toxicity including developmental toxicity, inflammation, immunotoxicity and tumorigenesis. Consequently, the use of antagonists to block ligand-mediated activation of the Ah receptor may be considered favourable, in particular if these are extensively consumed dietary phytochemicals. Isothiocyanates have been linked epidemiologically to low cancer incidence and their anti-carcinogenic activity has been confirmed in laboratory studies [7]. Being electrophiles, they have the potential to influence cellular processes [31]. A conceivable contributory mechanism of action may be to prevent the binding of

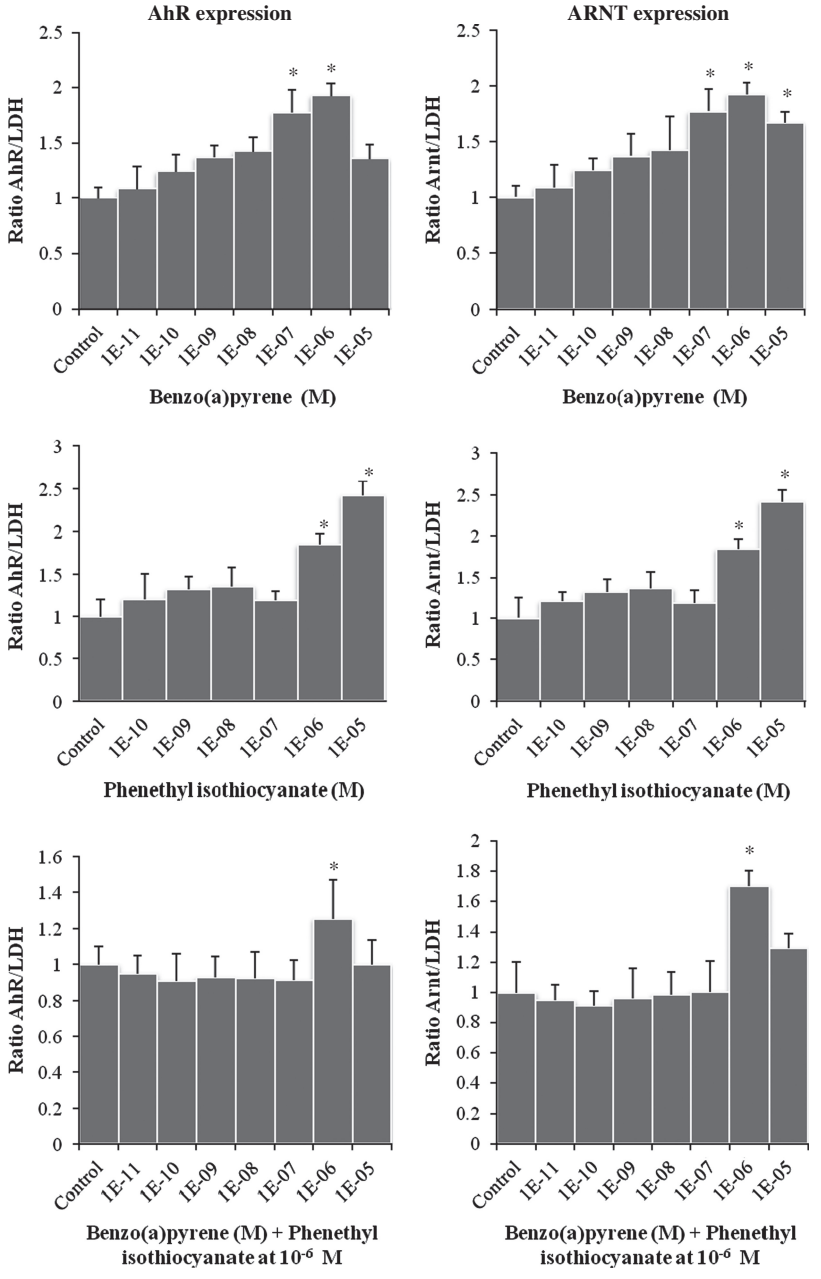

Figure 3. Expression of Ah receptor (AhR) and aryl hydrocarbon receptor nuclear translocator (ARNT) in H1L1.1c2 cells exposed to benzo[*a*]-pyrene and phenethyl isothiocyanate. H1L1.1c2 cells (7×10^4 cells/mL) were incubated with benzo[*a*]pyrene (10^{-11} to 10^{-5} M), phenethyl isothiocyanate (10^{-10} to 10^{-4} M) or a combination of benzo[*a*]pyrene (10^{-11} to 10^{-5} M) with phenethyl isothiocyanate at 10^{-6} M for 24 h. The cell lysates were harvested and the cytosolic fractions isolated. Immunoblot analysis was carried out by exposure to goat polyclonal antibody against AhR or ARNT followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 30 μg of total protein. The blots were stripped and re-probed with anti-LDH antibody to normalise for differences in protein loading. Results are expressed as mean ± SD of triplicate determinations. $^*p < 0.05$ as compared with control.

carcinogenic ligands to this receptor. It is also pertinent to point out that isothiocyanates attain very high intracellular concentrations, measured as the sum of parent compound and glutathione metabolites, and can achieve mM concentrations [32].

PEITC, one of the most extensively studied isothiocyanates, was a poor agonist of the Ah receptor, and when incubated with rat liver slices it only caused a very modest increase in CYP1A1 mRNA levels in comparison with avid ligands such as benzo[*a*]pyrene and TCDD. Significant but relatively modest increases in CYP1A1 mRNA have been reported following exposure of rat and human cells to sulforaphane, an isothiocyanate with an aliphatic side chain [33]; in primary human hepatocytes PEITC and sulforaphane elevated CYP1A1 mRNA levels at concentrations of 10 and 25 μM [34]. When rats were fed diets supplemented with PEITC, up-regulation in hepatic CYP1A1 apoprotein levels was observed at high doses with no parallel increase in CYP1A1 activity, presumably as a result of the fact that PEITC is a mechanism-based inhibitor [14, 35].

In contrast to PEITC and in agreement with our previous studies [30], benzo[*a*]pyrene was a very good agonist for the Ah receptor. In the presence, however, of PEITC the activation of the receptor by benzo[*a*]pyrene was markedly

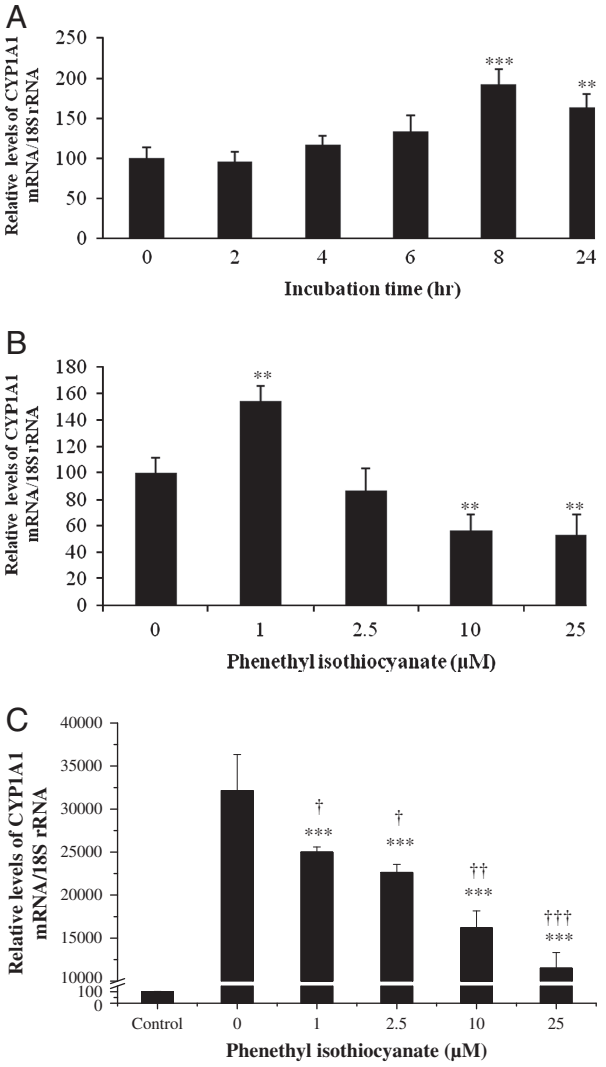

Figure 4. CYP1A1 mRNA levels in rat liver slices exposed to phenethyl isothiocyanate alone or in combination with benzo[*a*]pyrene (A). Precision-cut rat liver slices were incubated in culture medium containing 1 μM phenethyl isothiocyanate for various time periods (0–24 h); (B) precision-cut rat liver slices were incubated with phenethyl isothiocyanate (0–25 μM) for 24 h; (C) precision-cut rat liver slices were incubated with benzo[*a*]-pyrene (2 μM) in the presence of phenethyl isothiocyanate (0–25 μM) for 4 h. In all the three studies, total RNA was extracted from tissue slices and the mRNA levels of CYP1A1 were quantified by quantitative RT-PCR methodology (Taqman); mRNA levels were normalised with respect to 18*S* rRNA. Values are expressed as mean ± SD of three replicates, each containing three slices. In studies (A) and (B) $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ as compared with 0 time/concentration; in study (C) $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ as compared with control; $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$; $^{\dagger\dagger\dagger}p<0.001$ as compared with 0 μM phenethyl isothiocyanate.

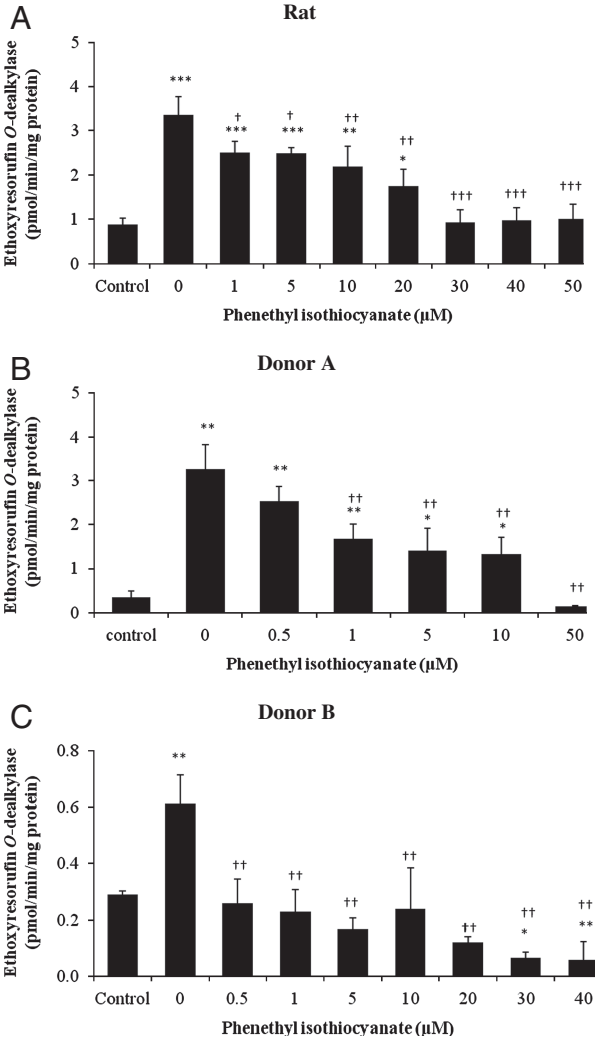

Figure 5. Effect of phenethyl isothiocyanate on the benzo[*a*]-pyrene-mediated up-regulation of ethoxyresorufin O-deethylase activity in rat (A) and human precision-cut liver slices (B). Liver slices were incubated in culture medium containing benzo[*a*]-pyrene (0.1 μM) alone or in combination with phenethyl isothiocyanate (1–50 μM) for 24 h. Values are presented as mean ± SD of three replicates, each containing 10 slices/mL. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ as compared with control; $^{\dagger}p<0.05$; $^{\dagger\dagger}p<0.01$; $^{\dagger\dagger\dagger}p<0.001$ as compared with 0 μM phenethyl isothiocyanate.

impaired, but no concentration-dependent effect was noted. As PEITC is such a weak agonist, its effect on the binding of benzo[*a*]pyrene is unlikely to involve a competitive mechanism. In order to evaluate this further, the H1L1.1c2 cells were initially incubated with the isothiocyanate which was washed off before exposure to benzo[*a*]pyrene. Once again the receptor activation by the hydrocarbon was effectively prevented, but as in the previous study no concentration-dependent effect could be discerned. Finally, in order to evaluate whether PEITC could displace benzo[*a*]pyrene already bound to the receptor, the cells were first incubated with the hydrocarbon which was then washed off, before the cells were incubated with the isothiocyanate; receptor activation by benzo[*a*]pyrene was attenuated in the cells

following treatment with the isothiocyanate. Displacement was complete by 2 h of incubation with PEITC with no significant improvement in displacement with a longer incubation (24 h). Clearly, PEITC can prevent the activation of the Ah receptor by benzo[*a*]pyrene, even when the two compounds are not simultaneously in the incubation system, arguing against a competitive mechanism.

In concordance with the ligand-binding studies, PEITC antagonised the benzo[*a*]pyrene-mediated increase in hepatic CYP1A1 mRNA levels. Moreover, a similar antagonism was observed at the activity level, when the O-deethylation of ethoxyresorufin was used as biomarker. PEITC prevented the benzo[*a*]pyrene-mediated increase in activity even at a concentration of 1 μM, the lowest concentration employed in these studies, and a concentration achievable following exposure to dietary levels of the isothiocyanate [36]. In order to ensure that the observations made in rat extend to human, liver precision-cut slices from two donors were incubated with benzo[*a*]pyrene alone or in the presence of PEITC. As in rat, the rise in the O-deethylation of ethoxyresorufin was antagonised by the isothiocyanate. It is likely that in both species, at least partly, the decrease in activity reflects the ability of this isothiocyanate to cause mechanism-based inhibition [35]. However, it is pertinent to point that it is the phenobarbital-inducible CYP2B enzymes that appear to catalyse the metabolism of PEITC to the metabolite(s) responsible for mechanism-based inhibition rather than the benzo[*a*]pyrene inducible CYP1 enzymes in both rat and human, so that mechanism-based inhibition is not likely to be the dominant mechanism [35, 37–39].

The available experimental data do not allow us to define the mechanism(s) through which PEITC acts as an Ah receptor antagonist, whether it impairs the binding of the ligand or influences subsequent transcriptional and/or post-transcriptional events. However, it may be inferred that lower Ah receptor and ARNT levels do not play a role, as PEITC did not decrease their concentration in the H1L1.1c2 cells, and in fact even increased the levels at the higher concentrations. No agonist-induced degradation of these proteins was observed with either benzo[*a*]pyrene or PEITC at any of the concentrations studied. The fact that aliphatic isothiocyanates are similarly endowed with antagonistic activity (unpublished observations) implicates the isothiocyanate group in the mechanism of action. Indeed, in a recent study [40] it was demonstrated that isothiocyanates could suppress CYP1A1/A2 activity induced by polycyclic aromatic hydrocarbons in a human mammary tumour cell line. Isothiocyanates are electrophiles that readily interact with sulfhydryl groups [31] and can thus interact with intracellular proteins such as the Ah receptor. It may be speculated that interaction of PEITC with a critical cysteine may alter the conformation of the receptor rendering it unable to interact with ligands such as benzo[*a*]pyrene or to recruit co-activators. In fact, it has already been reported that sulforaphane and benzyl isothiocyanate can interact with cysteine molecules of proteins [41, 42]. Furthermore, sulforaphane interacts with cysteine residues of Keap1, leading to activation of the transcription factor Nrf2 that results in the up-regulation of the antioxidant response element and of phase II detoxifcation enzymes such as quinone reductase [43]. There are 18 cysteine residues within both the human and rat Ah receptor protein sequences, several of which are located within functionally important regions of the receptor protein. Previous work has demonstrated that a CW216 mutation within the Ah receptor significantly impacts on DNA binding [44]. Such data support the hypothesis that isothiocyanate interaction with cysteine residues within the Ah receptor may influence functionality, although further work is required to evaluate this hypothesis.

In conclusion, we have demonstrated that PEITC is an antagonist of the Ah receptor in rat and human liver, and this may contribute to its established anti-carcinogenic activity. Since isothiocyanates are widespread in extensively consumed cruciferous vegetables, are rapidly absorbed after oral intake achieving good bioavailability, they are among the most promising dietary chemopreventive phytochemicals.

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