The naturally occurring aliphatic isothiocyanates sulforaphane and erucin are weak agonists but potent non-competitive antagonists of the aryl hydrocarbon receptor

Ahmad F Abdull Razis*, Natalya Hanlon, Ewa Soltys, Veronika Krizova, ^aRenato Iori, Kathryn E Plant, Nick Plant and Costas Ioannides

Molecular Toxicology Group, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, UK, and ^a Agricultural Research Council – Industrial Crop Research Centre (CRA-CIN), Via di Corticella, 133, 40128 Bologna, Italy.

* Current address: Department Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.

Corresponding author: Professor C Ioannides
Molecular Toxicology Group
Faculty of Health and Medical Sciences
University of Surrey
Guildford, Surrey
GU2 7XH, UK

Tel. No: +44 1483 689709
Fax No: +44 1483 686401
E-mail: c.ioannides@surrey.ac.uk

Abstract

As the Ah receptor target gene products play a critical role in chemical carcinogenesis, antagonists are considered as potential chemopreventive agents. It is demonstrated in this paper that the isothiocyanates *R,S*-sulforaphane and erucin are non-competitive antagonists of the aryl hydrocarbon (Ah) receptor. Both isothiocyanates were poor agonists for the receptor and elevated CYP1A1 mRNA levels only modestly when incubated with precision-cut rat liver slices. In contrast, the classical Ah receptor agonist benzo[a]pyrene was a potent inducer of CYP1A1 mRNA levels, with this effect being effectively antagonised by the two isothiocyanates. In further studies, it was demonstrated that *R,S*-sulforaphane could both prevent the interaction of and displace already bound benzo[a]pyrene from the Ah receptor, but no concentration dependency was observed with respect to the isothiocyanate. Both erucin and *R,S*-sulforaphane antagonised the benzo[a]pyrene-mediated increase in the CYP1A-mediated *O*-deethylation of ethoxyresorufin in rat precision-cut liver slices. Of the two isomers of *R,S*-sulforaphane, the naturally-occurring *R*-isomer was more effective than the *S*-isomer in antagonising the activation of the Ah receptor by benzo[a]pyrene. Antagonism of the Ah receptor may be a major contributor to the established chemoprevention of aliphatic isothiocyanates

Key words: isothiocyanates; glucosinolates; chemoprevention; sulforaphane; erucin; Ah receptor

Introduction

The aryl hydrocarbon (Ah) receptor is a ubiquitous cytosolic transcription factor that appears to be involved increasingly in many pathophysiological processes such as cancer and immunosuppression, with the underlying mechanisms being currently poorly understood (Abel and Haarmann-Stemmann 2010). Moreover, it regulates enzyme systems such as the CYP1 family of cytochromes P450 and quinone reductase (Kohle and Bock 1989; Safe 2001) which play critical role in the biotransformation of major classes of chemical carcinogens such as polycyclic aromatic hydrocarbons (Ioannides and Lewis 2004). Ligands of this receptor are considered detrimental to the living organism, and include many important classes of chemical carcinogens such as polycyclic aromatic hydrocarbons (Cheung et al. 1993; Pushparajah et al 2008). Briefly, interaction of the agonist with the receptor leads to translocation into the nucleus where the activated receptor interacts with another protein, the aryl hydrocarbon receptor nuclear translocator (Arnt); the heterodimer interacts with its response elements leading to increased transcription of numerous gene products, a number of which are involved in carcinogenesis. Indeed, Ah receptor null mice were refractive to the carcinogenicity of the polycyclic aromatic hydrocarbon benzo[a]pyrene (Shimizu et al 2000). Moreover, experimental studies established that Arnt was required for tumour initiation by benzo[a]pyrene (Shi et al. 2009). It is thus evident that chemicals that function as antagonists of this receptor may be endowed with chemopreventive activity.

R-Sulforaphane [1-isothiocyanato-(4*R*)-(methylsulfinyl)-butane] and erucin [1-isothiocyanato-4-(methylthio)-butane] are two structurally-related isothiocyanates having an aliphatic side chain that are present in substantial amounts in cruciferous vegetables where they

are present as the glucosinolates glucoraphanin and glucoerucin respectively. Consumption of these vegetables has been repeatedly linked to lower cancer incidence in epidemiological studies (Ambrosone et al. 2004; Lam et al. 2009) and it is generally accepted that glucosinolates may be largely responsible for this effect. When the vegetable is disrupted, such as during mastication, the enzyme myrosinase (β -thioglucoside glucohydrolase) comes into contact with the glucosinolate converting it to the isothiocyanate; similar conversion takes place in the human intestine by virtue of microbial myrosinase (Verkerk et al. 2009). Isothiocyanates such as sulforaphane are very well absorbed and achieve high bioavailability in both rat and human (Hanlon et al. 2008a, 2009a). A number of studies in chemically-induced models of cancer have established that sulforaphane and other isothiocyanates are potent anticarcinogens (Kuroiwa et al. 2006; Hecht 2000). The chemopreventive activity of glucosinolates is believed to be mediated by the isothiocyanates through a number of mechanisms including a decrease in the availability of the genotoxic metabolites of chemical carcinogens, impaired cell proliferation and enhanced apoptosis (Zhang 2004). Experimental evidence is presented in the current paper that sulforaphane, as well as erucin, is an antagonist of the Ah receptor and this attribute may be an important contributor to its chemopreventive activity.

Methods and materials

R,S-sulforaphane, *S*-sulforaphane and erucin (LKT Laboratories, Minnesota, USA), ethoxyresorufin, resorufin, benzo[a]pyrene and thiazolyl blue tetrazolium bromide (Sigma Co. Ltd., Poole, Dorset, UK), rat genomic DNA (Novagen, Wisconsin, USA), goat polyclonal antibody to lactate dehydrogenase (abcam, Cambridge, UK), NADPH (Melford Laboratories Ltd, 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 Alpha (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 (California, USA). *R*-sulforaphane was generated *in situ* from natural glucoraphanin by myrosinase-catalysed hydrolysis as we have described previously (Abdull Razis et al. 2011).

Male Wistar albino rats (200-250 g) were obtained from either B&K Universal Ltd (Hull, East Yorkshire, UK) or Charles River (Margate, Kent, UK). The animals were housed at 22 ± 2 °C, 30-40 % relative humidity, in an alternating 12-h light:dark cycle with light onset at 07.00 h.

Cell toxicity studies

The recombinant mouse hepatoma cell line H1L1.1c2, transfected by Dr. Michael Denison (University of California, U.S.A) (Garrison *et al.*, 1996), was kindly donated by Prof. Aldo Roda (University of Bologna, Italy). 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, were seeded (200 μ l) into 96-well plates and cultured for 24 hours until 50-70 % confluent. These cells were then incubated in culture medium supplemented with *R,S*-sulforaphane or erucin alone (10^{-10} - 10^{-5} M), or in combination of benzo[a]pyrene (10^{-11} to 10^{-5} M) with either *R,S*-sulforaphane or erucin (10^{-6} M) for 24 hours at 37°C and 5 % CO₂ in a humid

environment. An aliquot (10 μ l) of 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, medium was removed and DMSO (100 μ l) was added into the wells, and absorbance was read at 540 nm using an ELISA plate reader. We have already reported a small, but statistically significant, increase in LDH leakage at high concentrations of the sulforaphane isomers (25 μ M); no difference could be discerned between the two isomers (Abdull Razis et al. 2011).

Preparation and incubation of precision-cut rat liver slices

Rat liver slices (200-300 μ m) were prepared from 8 mm cylindrical cores using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL, USA) as previously described (Hashemi et al. 1999). 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% CO₂. 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 10 rat slices, were used per concentration.

Enzyme 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 000g x 1 hour) and the *O*-dealkylation of ethoxyresorufin (EROD) (Burke and Mayer 1974) and protein content (Bradford 1976) were determined.

Transcript level determination

Initially, precision-cut rat live slices were incubated with either erucin or *R,S*-sulforaphane (0-25 μ M) for 24 hours. In subsequent studies, liver slices were incubated with either isothiocyanate (10 μ M) for various periods of time (2-24 h). Finally, precision-cut rat liver slices were incubated with benzo[a]pyrene (2 μ M) alone or in the presence of either erucin or *R,S*-sulforaphane (1-25 μ M) for 4 hours. 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 the 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 50ng was amplified using Absolute™ QPCR Mix with 400 nM primers and 100 nM fluorogenic probe in a total reaction volume of 25 μ l. 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 (Pushparajah et al. 2007). Each primer and probe set was designed to amplify sequences within a single exon, so that genomic DNA could be used as a standard.

Activation of the Ah receptor

Activation of the Ah receptor by isothiocyanates was 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 (minimum essential medium) supplemented with 10% FBS and penicillin-streptomycin-neomycin antibiotic solution; cells were cultured for 24 hours until 50-70 % confluent. Cells were then incubated with the isothiocyanate and/or benzo[a]pyrene, dissolved in DMSO, for 24 hours at 37°C and 5 % CO₂ in a humid environment, and subsequently washed with PBS; 100 μ l of lysis reagent was added into each well and further incubated for 15 minutes at room temperature. Cell lysates were centrifuged at 13000g for 2 minutes, and luciferase activity in the supernatant was determined using the Promega stabilised luciferase assay reagent according to the manufacturer's instructions. Luminescence was read in a Packard Lumicount microplate luminometer with PlateReader software (Packard Instrument Company). TCDD (10^{-9} M) served as a positive control, and was used to normalise the binding of other ligands. The interaction of erucin or *R,S*-sulforaphane (10^{-11} - 10^{-5} M) was initially investigated following incubation for 24 hours. Subsequently, the activation of the Ah receptor by benzo[a]pyrene (10^{-11} - 10^{-5} M) in the absence or presence of *R,S*-sulforaphane or erucin (10^{-9} , 10^{-6} M) was examined under the same conditions. In studies whose objective was to investigate the ability of *R,S*-sulforaphane to prevent the benzo[a]pyrene-mediated activation of the Ah receptor, cells were incubated initially for 24 hours with *R,S*-sulforaphane (10^{-9} , 10^{-6} M), washed with PBS to remove the free isothiocyanate, and then incubated for a further 24 hours in fresh medium containing benzo[a]pyrene (10^{-11} - 10^{-5} M). When the objective was to evaluate the potential of *R,S*-sulforaphane to displace benzo[a]pyrene from the Ah receptor, cells were incubated initially with benzo[a]pyrene (10^{-11} - 10^{-5} M) for 24-hours, were subsequently washed

with PBS to remove the unbound aromatic hydrocarbon, and then incubated for a further 2 hours in fresh medium containing *R,S*-sulforaphane (10^{-9} - 10^{-6} M).

Ah receptor and Arnt expression

In order to monitor changes in the Ah receptor and Arnt protein expression in H1L1.1c2 cells following exposure to the isothiocyanates or benzo[a]pyrene, 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).

Statistical evaluation

Results are presented as Mean $\pm$ Standard Deviation of 3 pools, each comprising 10 slices. For the determination of transcript level, three slices per sample were used with triplicate analyses being carried out. Statistical evaluation was carried out by one-way ANOVA followed by the Dunnett's post-hoc analysis.

Results

Synthetic *R,S*-sulforaphane, up to a concentration of 10^{-5} M, elicited no toxicity to the H1L1.1c2 cells as exemplified by the MTT assay whereas marginal toxicity was displayed by erucin, but only at the two highest doses (Figure 1A). The toxicity of varying concentrations of benzo[a]pyrene in the presence of each isothiocyanate (10^{-6} M) was also investigated; minimal toxicity was seen in the case of *R,S*-sulforaphane but only at the highest concentration of benzo[a]pyrene (10^{-5} M) whereas no statistically significant change was noted with erucin throughout the concentration range (Figure 1B). We have already reported that under the same conditions benzo[a]pyrene alone did not elicit any toxicity (Abdull Razis et al. 2012a).

Both erucin and *R,S*-sulforaphane were poor agonists to the Ah receptor, as exemplified by the CALUX assay, displaying a <10% activation of the receptor compared with TCDD, with no concentration-dependent effect being evident; in contrast, benzo[a]pyrene was a very good agonist (Figure 2A). Although both isomers of sulforaphane were poor agonists of the Ah receptor, the *R*-isomer was a relatively better activator (Figure 2B). Whereas both *R,S*-sulforaphane and erucin were poor Ah receptor agonists, when incubated with benzo[a]pyrene they antagonised the latter's activation of the receptor, at concentrations of the hydrocarbon $>10^{-10}$ M; interestingly, no concentration-dependent effect for this antagonism could be discerned within the tested range of isothiocyanate concentrations tested (Figures 3A & 3B). Of the two sulforaphane isomers, *R*-sulforaphane was clearly the more effective antagonist, with the effect of benzo[a]pyrene being almost completely suppressed at the highest concentrations of the hydrocarbon (Figures 3C & 3D).

In order to further clarify the antagonistic effects of the isothiocyanates with the Ah receptor, additional studies were conducted using sequential exposure to *R,S*-sulforaphane and

benzo[a]pyrene. Firstly, cells were incubated either in the absence or presence of three concentrations of *R,S*-sulforaphane (10^{-9} , 10^{-7} , or 10^{-6} M) alone for 24 hours, the medium containing free isothiocyanate was removed, followed by a further 24-hour incubation with benzo[a]pyrene (10^{-11} – 10^{-5} M) for 24 hours. Activation of the Ah receptor by the hydrocarbon was markedly suppressed in the cells preexposed to the isothiocyanate, and again the effect was not concentration dependent (Figure 4A). Secondly, in order to establish whether *R,S*-sulforaphane could displace benzo[a]pyrene from the Ah receptor, cells were initially incubated with benzo[a]pyrene (10^{-11} – 10^{-5} M) for 24 hours, followed by the removal of the medium containing and a further 2-hour incubation hour in the absence and presence of *R,S*-sulforaphane (10^{-9} , 10^{-7} , or 10^{-6} M). *R,S*-sulforaphane was effective in antagonising the effect of benzo[a]pyrene on the Ah receptor but the effect was, once again, not concentration-dependent (Figure 4B).

Exposure of the cells to either *R,S*-sulforaphane or erucin resulted in a modest increase in Ah receptor and Arnt expression, but only at the highest concentration employed (10^{-5} M); no statistically-significant change was observed when benzo[a]pyrene (10^{-11} – 10^{-5} M) and either isothiocyanate (10^{-6} M) were incubated together (Figure 5).

Both erucin and *R,S*-sulforaphane elevated CYP1A1 mRNA levels in precision-cut rat liver slices following a 24-hour exposure, in concentration-dependent fashion, maximal rise being achieved at a concentration of 10 μ M with both isothiocyanates (Figure 6A). Incubation of slices with the isothiocyanates (10 μ M) for various time periods revealed that in both cases a significant increase in CYP1A1 mRNA required incubation times longer than 6 hours to be achieved (Figure 6B). Benzo[a]pyrene caused a marked increase in CYP1A1 mRNA levels in rat

slices, and this could be antagonised by *R,S*-sulforaphane and erucin, the former being more effective at the highest concentration examined (Figures 6C).

Finally, CYP1A activity was determined using ethoxyresorufin as the probe substrate. A marked increase in the *O*-deethylation of ethoxyresorufin (EROD) was observed following exposure of the slices to two concentrations of benzo[a]pyrene (1 and 5 μ M); this increase in activity provoked by benzo[a]pyrene was antagonised by both isothiocyanates, but no dose-dependency was apparent (Figure 7); although there may be a threshold concentration of 10 μ M, there is no dose dependency between 10 and 50 μ M. Erucin and *R,S*-sulforaphane failed to elevate this enzyme activity when incubated with the slices alone, under the same conditions.

Discussion

A number of substituted flavones, such as 3'-methoxy-4'-nitroflavone and 6,2',4'-trimethoxyflavone, are potent antagonists of the Ah receptor and are considered to possess potential therapeutic activity (Lu et al 1995; Murray et al. 2010). Here we document for the first time that the naturally-occurring aliphatic isothiocyanates erucin and *R*-sulforaphane, and the synthetic *R,S*-sulforaphane are weak agonists but strong antagonists of the Ah receptor.

Both erucin and *R,S*-sulforaphane are weak agonists of the Ah receptor and in rat slices elevated moderately CYP1A1 mRNA levels, which is consistent with observations made in murine hepatoma Hepa1c1c7 and human HepG2 cells treated with *R,S*-sulforaphane (Anwar-mohamed and El-Kadi 2009). This concords with our previous studies where these isothiocyanates, both *in vivo* and *in vitro*, up-regulated the expression CYP1A2 and CYP1B1 in rat liver, and of CYP1A1 and CYP1B1 in rat lung (Yoxall et al. 2005; Hanlon et al. 2008b, 2008c, 2009b). Clearly, the increase in the expression of these enzymes represents, at least

partly, increased transcription. However, there was no concomitant increase in CYP1 activity as these compounds are effective mechanism-based inhibitors (Yoxall et al. 2005; Hanlon et al. 2008b).

The activation of the Ah receptor by the potent agonist benzo[a]pyrene was impaired by the presence of either of the two isothiocyanates in the incubation. Moreover, pre-treatment of the cells with *R,S*-sulforaphane prior to the addition of benzo[a]pyrene also efficiently prevented the benzo[a]pyrene-mediated activation of the Ah receptor. As initial exposure of the cells to the hydrocarbon, followed by removal of the un-reacted fraction, and subsequent exposure to *R,S*-sulforaphane led to a markedly suppressed activation of the receptor, the isothiocyanate could either displace benzo[a]pyrene from the receptor or impair one of the subsequent steps leading to activation. It is important to emphasise that the antagonistic behaviour of the isothiocyanates towards the agonist benzo[a]pyrene did not display dose-dependency with respect to the isothiocyanate concentration alluding to a non-competitive mode of action that may be a consequence of a conformational change in a functionally important site(s) of the Ah receptor. The current data, however, do not allow us to infer whether the interaction is manifested at the ligand-binding site, subcellular localisation in the nucleus, formation of the heterodimer with Arnt or its subsequent interaction with the DNA response elements. It has been reported that in studies conducted in human mammary tumour Mcf7 cells treated with dibenzo[a,h]anthracene, another polycyclic aromatic hydrocarbon, sulforaphane blocked the translocation of the Ah receptor (Skupinska et al. 2009). It is pertinent to point out that neither isothiocyanate alone nor in combination with benzo[a]pyrene decreased the expression of either the Ah receptor or Arnt proteins, which is supportive of an effect at the post-translational level. In contrast to the isothiocyanates, the flavones that are antagonists of the Ah receptor appear to act competitively

being able to compete with TCDD, an avid ligand for the receptor. Moreover, they appear not to cause a conformational change that would preclude translocation of the receptor into the nucleus or subsequent interaction with Arnt (Murray et al. 2010; Gasiewicz et al. 1996).

In concordance with the receptor-binding studies, both erucin and *R,S*-sulforaphane antagonised the benzo[a]pyrene-mediated rise in CYP1A1 mRNA levels in rat liver slices. Finally, when studies were conducted at the activity level, with CYP1A activity being monitored using the *O*-deethylation of ethoxyresorufin, both erucin and *R,S*-sulforaphane antagonised the benzo[a]pyrene-mediated rise in this activity, but no dose-dependency could be discerned within the concentration range studied. Two concentrations of benzo[a]pyrene were utilised chosen on the basis of preliminary studies where rat liver slices were incubated with benzo[a]pyrene (0-20 μ M) for 24 hours; both the 1 and 5 μ M doses elevated the *O*-deethylation of ethoxyresorufin, with the latter causing maximal induction (results not shown). Consistent with these data, in studies carried out in MCF7 mammary tumour cells sulforaphane prevented the induction of ethoxyresorufin and methoxyresorufin *O*-dealkylase activities but with no concentration-dependency being apparent (Skupinska et al. 2009). It is conceivable that this concentration-independent inhibitory effect of the isothiocyanates on the *O*-deethylation of ethoxyresorufin represents, to some extent, mechanism-based inhibition associated with these compounds (Yoxall et al. 2005; Hanlon et al. 2008b). However, we believe that this is unlikely to be a major mechanism since in studies using isolated rat liver microsomes from animals treated with β -naphthoflavone, a selective CYP1 inducer, mechanism-based inhibition by sulforaphane was observed only at concentrations of at least 50 μ M, whereas in the current studies CYP1 activity induced by benzo[a]pyrene was clearly suppressed at a concentration of only 10 μ M.

In recent studies we have demonstrated that in both precision-cut rat liver slices and rat hepatoma FAO cells *R*-sulforaphane was more effective than the *S*-isomer in up-regulating phase II detoxification enzymes (Abdull Razis et al. 2011; 2012b). This observation led us to consider the possibility that isomer-dependent differences in their interactions with the Ah receptor may exist. Indeed, *R*-sulforaphane was a better agonist and a far better antagonist of the Ah receptor, demonstrating once again the superior potency of the *R*-isomer.

As phenethyl isothiocyanate is also a potent antagonist of the Ah receptor (unpublished observations), it is reasonable to implicate the isothiocyanate group as the critical structural moiety. Indeed, isothiocyanates have been demonstrated to interact non-specifically with thiol groups of proteins (Thornalley 2003). For example, *R,S*-sulforaphane interacts with cysteine residues of Keap1, leading to activation of the transcription factor Nrf2 resulting in the up-regulation of the antioxidant response element and of phase II detoxification pathways such as quinone reductase (Hu et al. 2011). Moreover, the principal metabolic pathway of isothiocyanates is conjugation with glutathione, the conjugate being further processed and excreted as the mercapturate. This facile interaction with –SH groups is believed to be also responsible for the extensive plasma protein binding of isothiocyanates (Ji et al. 2005). As the Ah receptor has 18 cysteine residues, it is tempting to speculate that isothiocyanates may interact with one or more of these residues, leading to conformational changes that underlie the non-competitive antagonism; however, the available data do not allow for the precise mechanism(s) to be discerned. The importance of cysteine residues has been highlighted in site-directed mutagenesis studies which indicated that cysteine (Cys327) plays an important role in ligand-binding to the Ah receptor (Pandini et al. 2007). However, isothiocyanates can also interact with amino groups to form stable conjugates at physiological conditions. When allyl isothiocyanate

was incubated with bovine serum albumin at pH 7.4, a stable allyl isothiocyanate-lysine conjugate was generated (Nakamura et al. 2009).

In summary the present studies have established that the aliphatic isothiocyanates erucin and *R,S*-sulforaphane are non-competitive antagonists of the Ah receptor, a characteristic that may contribute significantly to their chemopreventive activity. Of the two sulforaphane isomers, the naturally-occurring *R*-sulforaphane was the more potent. Studies that were performed using phenethyl isothiocyanate, having an aromatic substituent, in precision-cut liver slices from human donors suggest that the human Ah receptor, similar to its rat counterpart, can be antagonised by isothiocyanates (unpublished observations). Thus the chemopreventive potential of isothiocyanates, naturally-occurring chemicals that are abundant in human diet, in humans merits further investigation. It is of interest that recently glucoraphanin, the precursor of *R*-sulforaphane, has been engineered into tobacco (Mikkelsen et al. 2010).

Acknowledgements

The authors thank the Malaysian Government for funding this work through a PhD award to one of them (AF Abdull Razis), and Drs M Denison (University of California, Davis, USA) and A Roda (University of Bologna, Italy) for the kind gift of the transfected H1L1. 1c2 cells.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

Abel A, Haarmann-Stemmann T (2010) An introduction to the molecular basis of aryl hydrocarbon receptor biology. *Biol Chem* 391:1235-1248.

Abdull Razis AF, Iori R, Ioannides C (2011) The natural chemopreventive phytochemical R-sulforaphane is a far more potent inducer of the carcinogen-detoxifying enzyme systems in rat liver and lung than the S-isomer. *Intern J Cancer* 128:2775-2782.

Abdull-Razis AF, Konsue N, Dervetzoglou M, Plant KE, Plant K, Ioannides C (2012a) Phenethyl isothiocyanate, a naturally-occurring phytochemical, is an antagonist of the aryl hydrocarbon receptor. *Mol Nutr Food Res* In Press.

Abdull Razis AF., Bagatta M, De Nicola GR. Iori, R, Ioannides C (2012b) Induction of epoxide hydrolase and glucuronosyl transferase by isothiocyanates and intact glucosinolates in precision-cut rat liver slices: importance of side-chain substituent and chirality. *Arch Toxicol* 86:183-194.

Ambrosone CB, McCann SE, Freudenheim JL, Marshall JR., Zhang Y, Shields PG (2004) Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. *J Nutr* 134:1134-1138.

Anwar-Mohamed A, El-Kadi AOS (2009) Sulforaphane induces CYP1A1 mRNA, protein, and catalytic activity levels via an AhR-dependent pathway in murine hepatoma Hepa1c1c7 cells and HepG2 cells. *Cancer Lett* 275:93-101.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* 72:248-254.

Burke MD, Mayer RT (1974) Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Met Disp* 2:583-588.

Cheung Y-L, Gray TJB, Ioannides C (1993) Mutagenicity of chrysene, its methyl and benzo-derivatives and their interactions with cytochromes P-450 and the Ah-receptor; relevance to their carcinogenic potency. *Toxicology* 81:69-86.

Garrison PM, Tullis K, Aarts JM, Brouwer A, Giesy JP, Denison MS (1996) Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. *Fundam App Toxicol* 30, 194-203.

Gasiewicz TA, Kende AS., Rucci G, Whitney B, Willey J (1996) Analysis and structural requirements for Ah receptor antagonist activity: ellipticines, flavones, and related compounds. *Biochem Pharmacol* 52:1787-1803.

Hanlon N, Coldham N, Gielbert A, Kuhnert N, Sauer MJ, King LJ, Ioannides C (2008a) Absolute bioavailability and dose-dependent pharmacokinetic behaviour of dietary doses of the chemopreventive isothiocyanate sulforaphane in the rat. *Br J Nutr* 99:559-564

Hanlon N, Okpara M, Coldham N, Sauer MJ, Ioannides C (2008b) Modulation of rat hepatic and pulmonary cytochromes P450 and phase II enzyme systems by erucin, an isothiocyanate structurally related to sulforaphane. *J Agric Food Chem* 56 :7866-7871.

Hanlon N, Coldham N, Sauer MJ, Ioannides C (2008c) Up-regulation of the CYP1 family in rat and human liver by the aliphatic isothiocyanates erucin and sulforaphane. *Toxicology* 252:92-98.

Hanlon N, Coldham N, Gielbert A, Sauer MJ, Ioannides (2009a). Repeated intake of broccoli does not lead to higher plasma levels of sulforaphane in human volunteers. *Cancer Lett* 284:15-20.

Hanlon N, Coldham N, Sauer MJ, Ioannides C (2009b) Modulation of rat pulmonary carcinogen-metabolising enzyme systems by the isothiocyanates erucin and sulforaphane. *Chem-Biol Inter* 177:115-120.

Hashemi E, Dobrota M, Till C, Ioannides C (1999) Structural and functional integrity of precision-cut liver slices in xenobiotic metabolism: a comparison of the dynamic organ and multiwell plate culture procedures. *Xenobiotica* 29:11-25.

Hecht SS (2000) Inhibition of carcinogenesis by isothiocyanates. *Drug Met Rev* 32:395-411.

Hu C, Eggler AL, Maescar AD, van Breemen RB (2011) Modification of Keap1 cysteine residues by sulforaphane. *Chem Res Toxicol* 24:515-521

Ioannides C, Lewis DFV (2004) Cytochromes P450 in the bioactivation of chemicals. *Curr Topics Medicin Chem* 4:1767-1788.

Ji Y, Kuo Y, Morris ME (2005) Pharmacokinetics of dietary phenethyl isothiocyanates in rats. *Pharma. Res* 22:1658-1666.

Köhle C, Bock KW (1989) Coordinate regulation of human drug-metabolizing enzymes, and conjugate transporters by the Ah receptor, pregnane X receptor and constitutive androstane receptor. *Biochem Pharmacol* 77:686-699.

Kuroiwa Y, Nishikawa A, Kitamura Y, Kanki K, Ishii Y, Umemura T, Hirose M (2006) Protective effects of benzyl isothiocyanate and sulforaphane but not resveratrol against initiation of pancreatic cancers in hamsters. *Cancer Lett* 41:275-280.

Lam TK, Gallicchio L, Lindsley K, Shiels M, Hammond E, Tao X, Chen L, Robinson KA (2009) Caulfield LE, Herman JG, Guallar E, Alberg AJ. Cruciferous vegetable consumption and lung cancer risk: a systematic review. *Cancer Epidemiol Biomarkers Prev* 18:184-195.

Lu YF, Santostefano M, Cunningham BD, Threadgill MD, Safe S (1995) Identification of 3'-methoxy-4'-nitroflavone as a pure aryl hydrocarbon (Ah) receptor antagonist and evidence for more than one form of the nuclear Ah receptor in MCF-7 human breast cancer cells. *Arch Biochem Biophys* 316:470-477.

Mikkelsen MD, Olsen CE, Halkier BA (2010) Production of the cancer-preventive glucoraphanin in tobacco. *Molecul Plant* 3:751-759.

Murray IA, Flaveny CA, DiNatale BC, Chairó CR, Schroeder J., Kusnadi, Perdew GH (2010) Antagonism of aryl hydrocarbon receptor signalling by 6,2'-4'-trimethoxyflavone. *J Pharmacol Exptl Ther* 332:135-144.

Nakamura T, Kawai Y, Kitamoto N, Osawa T, Kato Y (2009) Covalent modification of lysine residues by allyl isothiocyanate in physiological conditions: Plausible transformation of isothiocyanate from thiol to amine. *Chem Res Toxicol* 22:536-542.

Pandini A, Denison MS, Song Y, Soshilov AA, Bonati L (2007) Structural and functional characterization of the aryl hydrocarbon receptor ligand binding domain by homology modeling and mutational analysis. *Biochemistry* 46:696-708.

Pushparajah DS, Umachandran M, Plant KE, Plant N, Ioannides C (2007) Evaluation of the precision-cut liver and lung slice systems for the study of induction of CYP1, epoxide hydrolase and glutathione *S*-transferase activities. *Toxicology* 231:68-80.

Pushparajah DS, Umachandran M, Nazir T, Plant KE, Plant N, Lewis DFV, Ioannides C (2008) Up-regulation of CYP1A/B in rat lung and liver, and human precision-cut slices by a series of polycyclic aromatic hydrocarbons; association with the Ah locus and importance of molecular size. *Toxicol In Vitro* 22:128-145.

Safe SS (2001) Molecular biology of the Ah receptor and its role in carcinogenesis. *Toxicol Lett* 120:1-7.

Shi S, Yoon DY, Hodge-Bell KC, Bebenek IG, Whitekus MJ, Zhang R, Cochran AJ, Huerta-Yepez S, Yim S-H, Gonzalez FJ, Jaiswal AA, Hankinson O (2009) The aryl hydrocarbon receptor nuclear translocator (Arnt) is required for tumor initiation by benzo[a]pyrene. *Carcinogenesis* 30:1957-1961.

Shimizu Y, Nakatsuru Y, Ichinose M., Takahashi Y, Kume H, Mimura J, Fuji-Kuriyama Y, Ishikawa T (2000) Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. *Proc Natl Acad Sci USA* 97:779-782.

Skupinska K, Misiewicz-Krzeminska I, Lubelska K, Kasprzycka-Guttman T (2009) The effect of isothiocyanates on CYP1A1 and CYP1A2 activities induced by polycyclic aromatic hydrocarbons in Mcrf7 cells. *Toxicol In-Vitro* 23:763-771.

Thornalley PJ (2003) Isothiocyanates: mechanism of cancer chemopreventive action. *Anti-cancer Drugs* 13:331-338.

Verkerk R, Schreiner M, Krumbein A, Ciska E, Holst B, Rowland I, De Schrijver R, Hansen M, Gerhäuser C, Mithen R, Dekker M (2009) Glucosinolates in *Brassica* vegetables: the influence of the food supply chain on intake, bioavailability and human health. *Mol Nutr Food Res* 53:S219-S265

Yoxall V, Kentish P, Coldham N, Kuhnert N, Sauer MJ, Ioannides C (2005) Modulation of hepatic cytochromes P450 and phase II enzymes by dietary doses of sulforaphane in rats: Implications for its chemopreventive activity. *Int J Cancer* 117:356-362.

Zhang Y (2004) Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action. *Mut Res* 555:73-190.

Figure 1: *R,S*-Sulforaphane and erucin cytotoxicity 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 hours. These cells were then incubated in culture medium supplemented with erucin or sulforaphane (10^{-10} - 10^{-5} M) (A), and benzo[a]pyrene (10^{-11} - 10^{-5} M) with either of the isothiocyanates (10^{-6} M) (B) for 24 hours. An aliquot (10 μ l) 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 At the end of the incubation, medium containing MTT was removed, DMSO (100 μ l) was added into the wells, and absorbance was read at 540 nm. Results are expressed in percentage, as mean $\pm$ SD of triplicate determinations. *, $P < 0.05$ as compared to control. (♦)*R,S*-sulforaphane; (■) Erucin

Figure 2: Activation of the Ah receptor by aliphatic isothiocyanates

(A) H1L1.1c2 cells (7×10^4 cells/ml) were incubated in culture medium supplemented with benzo[a]pyrene (■), *R,S*-sulforaphane (◆) or erucin (▲) (10^{-11} - 10^{-5} M) for 24 hours; (B) H1L1.1c2 cells (7×10^4 cells/ml) were incubated in culture medium supplemented with benzo[a]pyrene(■), *R*-sulforaphane (▲) or *S*-sulforaphane (◆) (10^{-11} - 10^{-5} M) for 24 hours. Activation of the receptor is expressed as % of TCDD activation (10^{-9} M). Results are presented as mean $\pm$ SD of triplicate determinations.

Figure 3: Antagonism of the benzo[a]pyrene activation of the Ah receptor by aliphatic isothiocyanates

(A) H1L1.1c2 cells (7×10^4 cells/ml) were incubated in culture medium supplemented with benzo[a]pyrene (10^{-11} - 10^{-5} M) alone or in combination with *R,S*-sulforaphane (10^{-6} M or 10^{-9} M) for 24 hours. (■) Benzo(a)pyrene alone, (▲) benzo(a)pyrene with *R,S*-sulforaphane (10^{-9} M), (●) benzo(a)pyrene with *R,S*-sulforaphane (10^{-6} M); (B) H1L1.1c2 cells (7×10^4 cells/ml) were incubated in culture medium supplemented with benzo[a]pyrene (10^{-11} - 10^{-5} M) alone or in combination with erucin (10^{-6} M or 10^{-9} M) for 24 hours. (■) Benzo(a)pyrene alone, (▲) benzo(a)pyrene with erucin (10^{-9} M), (●) benzo(a)pyrene with erucin (10^{-6} M); (C) H1L1.1c2 cells (7×10^4 cells/ml) were incubated in culture medium supplemented with benzo[a]pyrene (10^{-11} - 10^{-5} M) alone or in combination with *R*-sulforaphane (10^{-6} M) or (10^{-9} M) for 24 hours. (■) Benzo(a)pyrene alone, (▲) benzo(a)pyrene with *R*-sulforaphane (10^{-9} M), (●) benzo(a)pyrene with *R*-sulforaphane (10^{-6} M); (D) H1L1.1c2 cells (7×10^4 cells/ml) were incubated in culture medium supplemented with benzo[a]pyrene (10^{-11} - 10^{-5} M) alone or in combination with *S*-sulforaphane (10^{-6} M) or (10^{-9} M) for 24 hours. (■) Benzo(a)pyrene alone, (▲) benzo(a)pyrene with *S*-sulforaphane (10^{-9} M), (●) benzo(a)pyrene with *S*-sulforaphane (10^{-6} M). In all studies activation of the receptor is expressed as % of TCDD binding (10^{-9} M). Results are presented as mean $\pm$ SD of triplicate determinations.

Figure 4: Activation of the Ah receptor by benzo[a]pyrene: interactions with *R,S*-sulforaphane

(A) H1L1.1c2 cells (7×10^4 cells/ml) were incubated in culture medium supplemented with *R,S*-sulforaphane (10^{-9} M, 10^{-7} M and 10^{-6} M) or vehicle for 24 hours followed by benzo[a]pyrene (10^{-11} - 10^{-5} M) for a further 24 hours. (◆) Benzo(a)pyrene with *R,S*-sulforaphane (10^{-9} M), (●) benzo(a)pyrene with *R,S*-sulforaphane (10^{-7} M), (▲) benzo(a)pyrene + *R,S*-sulforaphane (10^{-6} M), (■) benzo(a)pyrene alone. (B) H1L1.1c2 cells (7×10^4 cells/ml) were incubated in culture medium supplemented with benzo[a]pyrene (10^{-11} - 10^{-5} M) alone for 24 hours followed by *R,S*-sulforaphane (10^{-9} M, 10^{-7} M, 10^{-6} M) for a further 2 hours. (■) Benzo(a)pyrene alone, (◆) *R,S*-sulforaphane (10^{-9} M) with benzo(a)pyrene, (●) *R,S*-sulforaphane (10^{-7} M) with benzo(a)pyrene, (▲) *R,S*-sulforaphane (10^{-6} M) with benzo(a)pyrene; in all studies activation of the receptor is expressed as % of TCDD activation (10^{-9} M). Results are presented as mean $\pm$ SD of triplicate determinations.

Figure 5: Expression of the aryl hydrocarbon receptor nuclear translocator (Arnt) and the Ah receptor (AhR) in H1L1.1c2 cells exposed to benzo[a]pyrene and aliphatic isothiocyanates.

H1L1.1c2 cells (7×10^4 cells/ml) were incubated with *R,S*-sulforaphane or erucin (10^{-10} - 10^{-5} M) for 24 hours. In a further study, the cells were incubated with *R,S*-sulforaphane or erucin (10^{-6} M) in combination with benzo[a]pyrene (10^{-11} - 10^{-5} M) for 24 hours. The cell lysates were harvested and the cytosolic fractions isolated. Immunoblot analysis was carried out by exposure to goat polyclonal antibody against Arnt (A) or AhR (B) followed by the appropriate peroxidase-labelled secondary antibody. Each lane was loaded with 30 μ g of total protein. The blots were stripped and re-probed with anti-LDH antibody to normalize for differences in protein loading. Results are expressed as mean $\pm$ SD of triplicate determinations. *, $P < 0.05$ as compared to control.

Figure 6: CYP1A1 mRNA levels in rat liver slices exposed to aliphatic isothiocyanates alone or in combination with benzo[a]pyrene.

(A) Precision-cut rat liver slices were incubated with *R,S*-sulforaphane or erucin (0-25 μ M) for 24 hours. (B) Precision-cut rat liver slices were incubated in culture medium containing 10 μ M *R,S*-sulforaphane or erucin for various time periods (0-24 hr); (C) Precision-cut rat liver slices were incubated with benzo[a]pyrene (2 μ M) in the presence of either *R,S*-sulforaphane or erucin (0-25 μ M) for 4 hours. In all 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 normalized with respect to 18S rRNA. Values are expressed as mean $\pm$ SD of three replicates, each containing 3 slices.

In studies (A) and (B) *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared to 0 time/concentration; in study (C) *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared to 0 μ M isothiocyanate; †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$ as compared to control.

Figure 7: Effect of aliphatic isothiocyanates on the benzo[a]pyrene-mediated up-regulation of ethoxyresorufin *O*-deethylase (EROD) activity in rat liver precision-cut slices.

Precision-cut rat liver slices were incubated in culture medium containing benzo[a]pyrene (5 μM) alone or in combination with (A) erucin or (B) *R,S*-sulforaphane (1-50 μM) for 24 hours. At the end of the incubation, microsomes were isolated and EROD activity determined. Control EROD activity was 1 pmol/min/mg protein. Values are presented as mean ± SD of three replicates, each containing 6-10 slices/ml. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$