RESEARCH ARTICLE

Naturally-Occurring Glucosinolates, Glucoraphanin and Glucoerucin, are Antagonists to Aryl Hydrocarbon Receptor as Their Chemopreventive Potency

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Abstract

As a cytosolic transcription factor, the aryl hydrocarbon (Ah) receptor is involved in several patho-physiological events leading to immunosuppression and cancer; hence antagonists of the Ah receptor may possess chemoprevention properties. It is known to modulate carcinogen-metabolising enzymes, for instance the CYP1 family of cytochromes P450 and quinone reductase, both important in the biotransformation of many chemical carcinogens via regulating phase I and phase II enzyme systems. Utilising chemically-activated luciferase expression (CALUX) assay it was revealed that intact glucosinolates, glucoraphanin and glucoerucin, isolated from *Brassica oleracea* L. var. acephala sabellica and *Eruca sativa* ripe seeds, respectively, are such antagonists. Both glucosinolates were poor ligands for the Ah receptor; however, they effectively antagonised activation of the receptor by the avid ligand benzo[a]pyrene. Indeed, intact glucosinolate glucoraphanin was a more potent antagonist to the receptor than glucoerucin. It can be concluded that both glucosinolates effectively act as antagonists for the Ah receptor, and this may contribute to their established chemoprevention potency.

Keywords: Glucoraphanin - glucoerucin - aryl hydrocarbon receptor - CALUX - chemoprevention

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Introduction

The aryl hydrocarbon (Ah) receptor is a cytosolic transcription factor participated progressively in many patho-physiological processes, for example immunosuppression and cancer, with the basic mechanisms being presently investigated. Furthermore, it modulates carcinogen-metabolising enzymes, for instance the CYP1 family of cytochromes P450 and Phase II detoxifying enzymes, including quinone reductase, aldehyde dehydrogenase, glucuronosyltransferase and glutathione S-transferase (Safe, 2001; Abdull Razis and Mohd Noor, 2013) which mainly take place in the biotransformation of major classes of chemical carcinogens (Ioannides and Lewis, 2004). Ligands to this receptor are believed to be harmful to the living organism, and consist of various important groups of chemical carcinogens involving polycyclic aromatic hydrocarbons such as benzo(a) pyrene (Pushparajah et al., 2008; Wohak et al., 2014). The Ah receptor is considered as an orphan receptor with no endogenous ligand being so far identified; the highly toxic, halogenated aromatic hydrocarbon, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is the absolute avid ligand of this receptor identified so far (Hanieh, 2014). In brief, a ligand interacts with the receptor followed by translocation into the nucleus, a location where it act together with another protein, the translocator known as aryl hydrocarbon receptor nuclear translocator (Arnt); the heterodimer interacts with its DNA response elements leading to elevated transcription of several gene products, a number of which are involved in tumourigenesis. In fact, null mice Ah receptor was resistant to the mutagenicity of the polycyclic aromatic hydrocarbon benzo[a]pyrene (Shimizu et al., 2000). In addition, experimental studies demonstrated that Arnt is needed for the initiation of the tumour by the same carcinogen (Shi et al., 2009).

Cassia seed and rosemary that were noted to have health benefit effects were also demonstrated to contain natural Ah receptor agonists (Amakura et al., 2014). Gene expression analyses up-regulated by these agonists showed most of the genes engaged in dioxin-related toxic effects were modulated in the same way. In contrast, antagonists of the Ah receptor-mediated function, for example 3'-methoxy-4'-nitroflavone and 6,2',4'-trimethoxyflavone (TMF), are potentially beneficial and may have therapeutic

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properties (Lu et al., 1995; Murray et al., 2010). It has been shown that in Mcf7 cells, 3'-methoxy-4'-nitroflavone impaired the induction of ethoxyresorufin O-deethylase by TCDD; furthermore, co-treatment of Mcf7 cells with such flavones in the presence of TCDD or [3H]TCDD resulted in dose-dependent suppression of TCDD-induced CYP1A1 mRNA levels and formation of radiolabeled nuclear Ah receptor complex (Lu et al., 1995). Similarly, 6,2',4'-trimethoxyflavone, newly identified as an Ah receptor ligand, was capable of competing with agonists, for example benzo[a]pyrene and TCDD, efficiently deterring AhR-induced transactivation of endogenous targets and a heterologous reporter, such as CYP1A1, independent of cell lineage or species (Murray et al., 2010). Thus, clearly chemicals which function as antagonists of this receptor may function as chemopreventive agents.

The chemoprevention properties of cruciferous vegetables have been ascribed to glucosinolates, a group of sulphur with glycosides, which can be found at significant amounts in these vegetables (Abdull Razis and Mohd Noor, 2013). The putative opinion is that glucosinolates were not directly contributed to the chemoprevention effects, nonetheless their hydrolyzed-products for example the isothiocyanates. It has been presumed that intact glucosinolates, due to their water solubility, could not to reach the blood circulation after oral intake. Nevertheless, it was shown that, glucoraphanin, in any case in dogs and rats, able to absorb intact after oral intake (Bheemreddy and Jeffery, 2007; Cwik et al., 2010). Male F344 rats administered purified glucoraphanin at 150 μmol/kg resulted in 5% of the oral dose remains intact in urine (Bheemreddy and Jeffery, 2007). Glucoraphanin in plasma was detectable in animals administered with glucoraphanin in comparison with control animals; in dog the levels were ranging from 2900 to 15,000 ng/mL after receiving oral dose at 200 mg/kg/day of body weight for 3 days, while in rats the mean concentrations after 13 days dosing at 10, 50, 100, and 500 mg/kg/day were 49.9, 198, 416 and 1630 ng/mL, respectively (Cwik et al., 2010). Moreover, in the rat, glucoraphanin could be condensed to glucoerucin via the reduction of the alkylsulfinyl glucosinolate (Bheemreddy and Jeffery, 2007); glucoerucin differs from glucoraphanin (Figure 1) only by the presence of oxygen on the sulphur atom (Chun et al., 2013). These findings urged us to evaluate whether intact glucosinolates capable of activating the Ah receptor and/or prevent its activation by benzo[a]pyrene. Experimental evidence is presented in these studies that glucosinolates, glucoraphanin and glucoerucin, are antagonists of the Ah receptor and this attribute may be an important contributor to their chemopreventive activity.

Materials and Methods

DMSO, benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Sigma Co. Ltd., Poole, Dorset, UK), luciferase assay reagent and cell culture lysis reagent (Promega, Wisconsin, USA), penicillin–streptomycin–neomycin, foetal calf serum, minimum essential medium α (MEM-α) (Invitrogen, Paisley, Scotland) were commercially available. The recombinant mouse hepatoma cell line H1L1.1c2, transfected by Dr. Michael Denison (University of California, USA), was kindly donated by Prof. Aldo Roda (University of Bologna, Italy).

Glucosinolates isolation

Glucoerucin and glucoraphanin were isolated from Eruca sativa ripe seeds and Brassica oleracea L. var. acephala sabellica, respectively, as previously described (Visentin et al., 1992). Both glucosinolates were extracted using an Ultraturrax homogeniser at average speed for 15 min followed by homogenate centrifugation at 17,700 ×*g* for 30 min. Utilising one-step anion exchange chromatography, glucosinolates were isolated from the extract according to Visentin et al. (1992). The purity of glucosinolate was subsequently improved via gel-filtration conducted using a XK 26/100 column packed with Sephadex G10 chromatography media (Amersham Biosciences), coupled with FPLC System (Pharmacia). All fractions were assessed by HPLC for pure glucosinolates followed by freeze-drying (Wagner et al., 2010). Finally, glucosinolates were characterised employing NMR spectrometry and the purity was checked using HPLC analysis according to the ISO 9167-1 method (EEC Regulation, 1990).

Validation of the Ah receptor procedure

Chemically-activated luciferase gene expression (CALUX) assay, employing recombinant mouse hepatoma H1L1.1c2 cell line transfected with a luciferase reporter gene under the control of dioxin-response enhancers was first validated using TCDD, the highest affinity ligand known for the Ah receptor. In addition, benzo[a]pyrene (B[a]P), a known potent Ah receptor activator, was also investigated.

Activation of the Ah receptor

Utilising chemically-activated luciferase expression (CALUX) assay, PAHs interactions with the Ah receptor were investigated. Transfected H1L1.1c2 cells were cultured at 7 x 10^4 cells/mL) in 24-well plates, in α-MEM supplemented with 10% FBS and penicillin–streptomycin–neomycin; for 24 h up to 50-70% confluent. Cells were subsequently exposed to glucosinolates, glucoerucin and glucoraphanin (10^{-11} - 10^{-5}M), for 24 h at 37ºC and 5% CO_2, and then washed with PBS; 100 mL of lysis reagent was pipetted into each well followed by incubation at room temperature for 15 min. Cell lysates were then centrifuged at 13 000 x*g* for 2 min, and luciferase activity was determined employing Promega-stabilised luciferase assay reagent. Using Packard Lumicount microplate luminometer (Packard Instrument), luminescence was read at 562 nm. The fluoresced light was quantified as relative light units (RLU), corrected for gain and normalised for cell number. Luciferase activity was expressed as percentage of binding of the ligands to the Ah receptor, where TCDD (10^{-9} M) served as a positive control, achieving 100% binding.

Interaction studies between glucosinolates, glucoraphanin and glucoerucin with B[a]P on Ah receptor activation

In studies where the objective was to examine the

ability of glucosinolates, glucoraphanin and glucoerucin to influence the activation of Ah receptor by polycyclic aromatic hydrocarbons, cells were treated with benzo[a] pyrene (10^{-11}-10^{-5} M) in the presence of either glucoraphanin or glucoerucin (10^{-9}, 10^{-6} M), for 24 hours.

Results

Validation of the Ah receptor procedure

Maximum activation was noted at a concentration of 10^{-9} M when the CALUX assay was authenticated

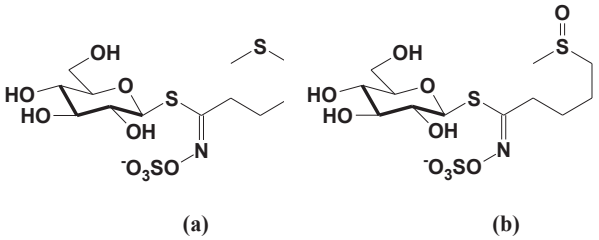

Figure 1. Structure of (A) Glucoerucin and (B) Glucoraphanin

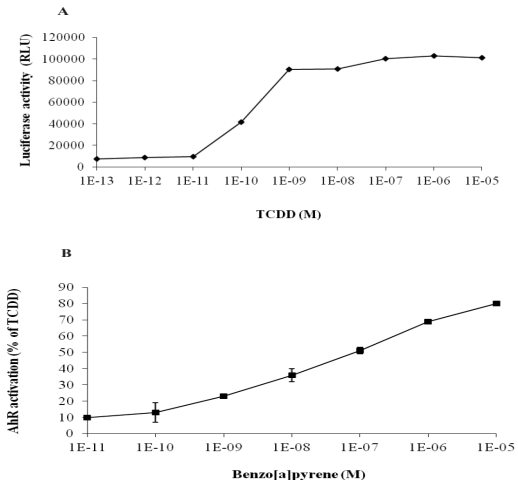

Figure 2. Active of the Ah Receptor by TCDD and Benzo [a] Pyrene. H1L1.1c2 Cells ($7x10^4$ cells/ml) were incubated with TCDD (10^{-13}-10^{-5}M). (A) Benzo [a] Pyrene (10^{-11}-10^{-5}M);(B) for 24 h. The activation ot the receptor is expressed as % of that achieved by TCDD (10^{-9}M). Results are expressed as mean ± SD of triplicate determinations

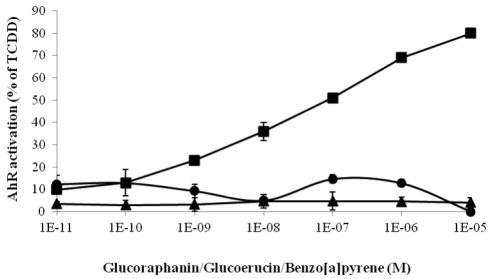

Figure 3. Active of the Ah Receptor by Benzo [a] Pyrene, glucoraphanin. H1L1.1c2 Cells ($7x10^4$ cell/ml) were incubated in culture medium supplemented with benzo [a] pyrene or glucoraphanin or glucoerucin (10^{-11}-10^{-5}M) for 24 h. ■ Benzo(a)pyrene; ▲ glucoraphanin; ● glucoercin. Activation of the receptor is expressed as % of that achieved by TCDD (10^{-9}M). Results are expressed as mean ± SD of triplicate determinations

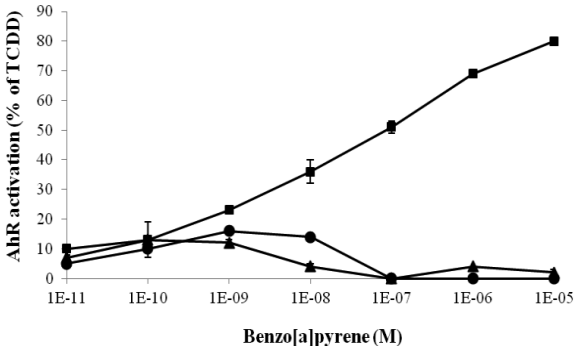

Figure 4. Modulation of the Benzo[a]pyrene Activation of the Ah Receptor by Glucoraphanin. H1L1.1c2 Cells ($7x10^4$ cell/ml) were incubated in culture medium supplemented with benzo [a]pyrene (10^{-11}-10^{-5}M) alone or in combination with glucoraphanin (10^{-6}M or 10^{-9}M) for 24 h. ■ Benzo [a]pyrene alone, ● Benzo[a]pyrene with glucoraphanin (10^{-9}M); ▲ Benzo[a]pyrene with glucoraphanin (10^{-6}M). The activation of the receptor is expressed as % of that achieved by TCDD (10^{-9}M). Results are expressed as mean ± SD of triplicate determinations

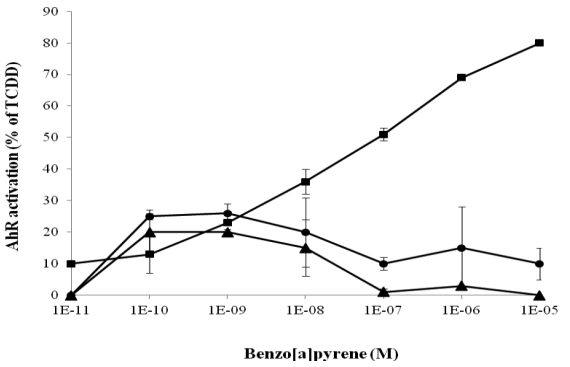

Figure 5. Modulation of the Benzo[a]pyrene Activation of the Ah Receptor by Glucoerucin. H1L1.1c2 Cells ($7x10^4$ cells/ml) were incubated in culture medium supplemented with benzo[a]pyrene (10^{-11}-10^{-5}M) alone or in combination with glucoerucin (10^{-6}M or 10^{-9}M) for 24 h. ■ Benzo[a]pyrene alone, ● Benzo[a]pyrene with glucoerucin (10^{-9}M), ▲ Benzo[a]pyrene with glucoerucin (10^{-6}M). The activation of the receptor is expressed as % of that achieved by TCDD (10^{-9}M). Results are expressed as mean ± SD of triplicate determinations

with TCDD, the highest affinity ligand known for the aryl hydrocarbon (Ah) receptor (Figure 2A) as well as benzo[a]pyrene, with maximum activation occurring at a concentration of 10^{-5} M (Figure 2B).

Activation of the Ah receptor

Glucosinolates were poor ligands in comparison with benzo[a]pyrene, but glucoerucin was found to be a relatively a better ligand than glucoraphanin, achieving 15% and 5% of activation of the receptor, respectively (Figure 3).

Interaction studies between glucosinolates glucoraphanin and glucoerucin with B[a]P on Ah receptor activation

Studies were undertaken to evaluate whether the activation of the Ah receptor by benzo[a]pyrene was modulated in the presence of the glucosinolates glucoraphanin and glucoerucin, the precursors of sulforaphane and erucin respectively, to ascertain whether

these glucosinolates displayed any antagonistic activity. Glucoraphanin at either concentrations of 10^{-6}M or 10^{-9}M, effectively antagonised the benzo[a]pyrene activation to the Ah receptor, with the effect being more marked at the higher concentrations of benzo[a]pyrene (10^{-7}-10^{-5}M) (Figure 4).

Similarly, Figure 5 illustrates that benzo[a]pyrene activation of the Ah receptor appeared to be suppressed at the higher concentrations (10^{-7}-10^{-5}M) when H1L1.1c2 cells exposed with benzo[a]pyrene (10^{-11}-10^{-5}M) in the presence of glucoerucin (10^{-9}M or 10^{-6}M) for 24 hours. When comparing the antagonistic effects between glucoraphanin and glucoerucin at 10^{-9}M on the activation of the Ah receptor by benzo[a]pyrene, glucoraphanin was found comparatively a better antagonist than glucoerucin (Figures 4 and 5).

Discussion

Many studies, utilising TCDD, have ascertained that ligand binding to the Ah receptor unleashes a plethora incidents that are harmful to the cell and organism (Hanieh, 2014). This receptor has been linked to different types of toxicity including developmental toxicity, tumourigenesis, immunotoxicity and inflammation. It is also clear that the Ah receptor plays an important role in human cancer through interaction with signaling pathways in a cell-specific manner, suggesting that this receptor may be a helpful device in the early detection and healing of cancer (Tsay et al., 2013). As a result, the role of antagonists to block ligand-mediated activation of the Ah receptor may be beneficial, particularly if these are widely-consumed phytochemicals with proven safety. The hydrolysed product of glucosinolates, isothiocyanates has been noted in epidemiology studies to lower cancer risk, and their anti-cancer properties have been approved in laboratory studies (Barouki et al., 2007). As electrophiles, they are likely to manipulate cellular processes via binding covalently to nucleic acids, proteins, or small molecules and may be indirectly reducing pools of cellular reductants (Hecht, 2000). A mechanistic study to determine the effect of isothiocyanates on CYP1A1 and CYP1A2 activity and expression, and Ah receptor translocation in Mcf7 cells demonstrated that both enzymes were significantly stimulated by benzo[a]pyrene, and isothiocyanates were able to inhibit the rise in activity (Nakamura et al., 2010). A view has been expressed that the inhibition of CYP1A1 and CYP1A2 enzymes may serve as a useful strategy for cancer chemoprevention (Cho and Yoon, 2015). A likely causative mechanism of action of chemopreventive phytochemicals may be to avert the activation of Ah receptor by carcinogenic ligands such as benzo[a]pyrene.

In studies to assess whether the glucosinolate precursors of sulforaphane and erucin displayed any antagonistic effect on the activation of the Ah receptor by benzo[a]pyrene, both glucosinolates revealed a clear antagonistic effect, with glucoraphanin being relatively a better antagonist than glucoerucin. It has already been demonstrated that both glucosinolates induced the O-dealkylations of methoxy- and ethoxyresorufin (Abdull Razis and Mohd Noor, 2013), markers for CYP1 activity that is regulated by the Ah receptor through transcriptional activation (Okino et al., 2009). In other studies, glucosinolates such as sinigrin, glucoiberin, progoitrin and glucosinalbin were capable of inhibiting the level of β-naphthoflavone-induced CYP1A1 expression in Ah receptor-replete cells, and the inhibition effect was found to be dependent on the side chain of the glucosinolate (Whitlock, 1999). Meanwhile, a pronounced induction in CYP1A1 mRNA expression has been noted following exposure of HepG2 cells to another glucosinolate, glucoraphasatin (Wang et al., 1997), which could reflect activation of the Ah receptor by this glucosinolate.

In conclusion, the present studies demonstrate for the first time that glucosinolates, glucoraphanin and glucoerucin, are poor agonists but potent antagonists of the Ah receptor, properties that may attribute significantly to their established chemopreventive potency. As glucosinolates are widely consumed from cruciferous vegetables, are quickly absorbed following oral intake attaining good bioavailability, making them among the most potent dietary chemopreventive phytochemicals, and their function as antagonists of the Ah receptor attribute to their anti-carcinogenic activity.

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