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Red clover isoflavones biochanin A and formononetin are potent ligands of the human aryl hydrocarbon receptor

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Abstract

Aryl hydrocarbon receptor (AhR) activation affects the cell cycle and drives cells to apoptosis. Thus, selective AhR modulators (SAhRMs) have previously been implicated in cancer therapy and prevention, particularly for hormone-dependent cancers. In the present study, isoflavones a remedy used to ameliorate menopausal complaints were tested for their potential in transactivating AhR in order to investigate the biological function of red clover isoflavones. The results were compared to the transactivation potentials of other flavonoids and plant-derived indole compounds. We found that the isoflavones biochanin A and formononetin were potent AhR agonists *in vitro*, with EC_{50} values of 2.5×10^{-7} and 1.3×10^{-7} mol/l, respectively. These isoflavones are 10 times more potent compared to the indole compounds indole-3-carbinol and diindolylmethane, publicised as powerful AhR agonists with EC_{50} values of 5.8×10^{-6} and 1.1×10^{-6} mol/l, respectively. Because activated AhR crosstalks with estrogen receptor α, future risk-benefit assessments of isoflavones should take into consideration their AhR transactivating potential.

Keywords: Isoflavones; Biochanin A; Formononetin; AhR; Aryl hydrocarbon receptor; Indole compounds

1. Introduction

Thirty years ago, Poland et al. [1] discovered a protein that binds with high affinity to the toxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This protein was later designated the aryl hydrocarbon receptor (AhR). Subsequent research regarding this receptor focused on the AhR-mediated toxic response that is induced through ligand binding. TCDD and other polyhalogenated hydrocarbons (PAHs) are extremely potent activators of the AhR pathway and induce the expression of AhR-regulated genes that encode for the enzymes of phase I and II metabolic pathways. However, PAHs are not the sole exogenous ligands of the AhR. In fact, the AhR is very promiscuous and binds a wide variety of both agonists and antagonists.

Although there is substantial speculation regarding a potential endogenous ligand [2–8] for AhR, currently there is no clear evidence for any particular moiety. Nevertheless, the existence of an endogenous ligand is supported by a number of studies. Experiments with AhR knockout mice have suggested the AhR may play roles in cardiac physiology and function [9], liver development [10,11], ovary development [12], and immune system regulation [13]. Other studies have established AhR involvement in reproductive physiology [14,15], cell cycle control [16], and apoptosis [17].

Phytoestrogens are plant-derived, non-steroidal chemicals that bind the estrogen receptor. A number of phytochemicals are exogenous AhR ligands, including flavonoids [18–21], the stilben resveratrol [22,23], indole compounds [24], and furocoumarins [25]. Some AhR ligands have also antiestrogenic properties and can be characterized as "endocrine disrupters", because upon binding AhR they may crosstalk with steroid hormone receptors and disturb normal hormone pathways. Interestingly, this crosstalk was recently exploited in the creation of selective AhR modulators (SAhRMs) [26] that can inhibit the development of hormone-dependent cancers and tumours. While endocrine disrupters are mainly anthropogenic chemicals and pollutants, SAhRMs, for the

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most part, are naturally occurring plant compounds or derivates that do not exhibit toxic effects.

The AhR can interact with estrogen receptors (ER) in multiple ways. AhR ligands can also modify estradiol metabolism [27] by increasing 2-, 4-, 6α- and 15α-hydroxylation. In particular, indole compounds alter estrogen metabolism by producing a metabolite ratio of C-2 to C-16 hydroxylated estrogen that is the inverse to that observed in women with breast cancer [28,29]. In a number of *in vitro* studies, AhR ligands were associated with an abolishment of estrogen-induced gene expression [30,31]; presumably, the presence of AhR ligands hindered the ability of the ER to bind to estrogen response elements (EREs) on DNA [32]. Various other examples of AhR–ER interactions are known, and offer a wide range of potential approaches for intervening in ER-dependent diseases.

Foods rich in phytoestrogens or food supplement based on red clover, have beneficial physiological effects. The AhR transactivating potential of many of the so-called phytoestrogens has not been investigated, thus it is not clear whether AhR plays a role in their beneficial effects. Isoflavones are phytoestrogens used for the amelioration of menopausal complaints. Recent clinical studies have demonstrated the beneficial effects of 80 mg per day of red clover isoflavones [33–35]. The spectrum of isoflavones in red clover is different than those found in soy. Red clover is rich in formononetin and biochanin A, while soy has less biochanin A and no formononetin. The isoflavone coumestrol has been shown to have AhR transactivating potential, but only very low amounts are present in standardized red clover isoflavone extracts. Thus, red clover and soy can be expected to produce different biological effects.

In this study, we tested a selection of phytoestrogens and compared their AhR activation potentials with a variety of known AhR ligands. In addition, we investigated polyphenols that partly exhibit estrogenic activity. AhR activation potencies were measured with a high-throughput bioassay based on yeast as a model organism. These *in vitro* results serve as a guide for further *in vivo* investigations and for clinical studies that focus on the development of plant-based pharmaceuticals and/or herbal remedies.

2. Material and methods

2.1. Chemicals and media

Dimethylsulfoxide (DMSO) was obtained from Sigma–Aldrich (St. Louis, MO). Buffer reagents, *N*-lauroylsarcosine (sodium salt), di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$), potassium chloride (KCl), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), sodium carbonate (Na_2CO_3) and *o*-nitrophenyl-β-galactopyranoside (ONPG), were purchased from Sigma–Aldrich (St. Louis, MO), Fluka (Buch, Switzerland) and Merck (Darmstadt, Germany). *o*-Aminoazotoluene, apigenin, baicalein, 1-benzylimidazole, biochanin A, biotin, biphenyl, butyl paraben, caffeic acid, caffeine, (+)-catechin hydrate, clotrimazole, coumestrol, curcumin, daidzein, diethylstilbestrol, diindolylmethane, diosmin, dioxin, (−)-epicatechin, (±)-equol, 17-β-estradiol, *trans*-ferulic acid, fisetin hydrate, formononetin, 6-formylindolo[3,2-b]carbazole (FICZ), gallic acid, genistein, ICI 182,780, harmaline, indole-3-carbinol, indolo[3,2-b]carbazole (ICZ), ITE (2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid), indomethacine, melatonin, 3-methylcholanthren, β-naphthoflavone, naringenin, nicotine, phloretin, phtalic acid dibutyl ester, piperine, progesteron, quercetin, quinine, *trans*-resveratrol, rhein, rutaecarpine, silibinin, β-sitosterol, tangeretin, theobromin, tryptamin and tryptanthrin were obtained from Sigma–Aldrich (St. Louis, MO), Fluka (Buchs, Switzerland), Biomol International L.P. (Plymouth Meeting, PA), Indofine Chemical Company (New Jersey, USA) and Tocris Cookson (Avonmouth, United Kingdom). The solvents used were HPLC grade.

For the yeast media, yeast nitrogen base was obtained from Difco (Franklin Lakes, NJ), amino acids from Serva Feinbiochemica (Heidelberg, Germany) and dropout medium without tryptophan from Sigma–Aldrich (St. Louis, MO).

2.2. Media preparation for yeast cultivation

Media were sterilised at 121 °C and 10^5 Pa overpressure for 15 min and stored at 4 °C. The Dropout medium without tryptophan (DO-trp), was used for the cultivation of the overnight culture and the yeast stock. GOLD medium with galactose, without tryptophan (GOLD-trp), and adjusted to pH 5.0 was employed for induction. GOLD medium is made of two parts. Part 1 and 2 of the medium were aseptically mixed after autoclaving and a cool down. Part 1 consisted of 0.34 g yeast nitrogen base (without amino acids and ammonium sulphate), 0.67 g ammonium sulphate, 12 mg/l uracil, and amino acids were added as follows: 24 mg/l adenine sulfate, 12 mg/l L-arginine·HCl, 60 mg/l L-aspartic acid, 60 mg/l L-glutamic acid, 12 mg/l L-histidine·HCl, 18 mg/l L-lysine·HCl, 12 mg/l L-methionine, 30 mg/l L-phenylalanine, 225 mg/l L-serine, 120 mg/l L-threonine, 18 mg/l L-tyrosine, 90 mg/l L-valine, and 36 mg/l L-leucine. Part 2 was 1.2% galactose solution in distilled water.

2.3. Yeast AhR screen with β-galactosidase

LacZ-buffer and *Z*-sarcosyl-buffer were adjusted to pH 7.0 and filtered (0.22 μm filter). LacZ-buffer was composed of 60 mM $Na_2HPO_4{\cdot}2H_2O$, 40 mM $NaH_2PO_4{\cdot}2H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, and 1 mM DTT. For the *Z*-sarcosyl-buffer, 0.5% *N*-lauroylsarcosine was dissolved in LacZ-buffer with 2 mM DTT.

o-Nitrophenyl-β-galactopyranoside (ONPG) solution was used for the β-galactosidase assays (4 g ONPG in 1 l of LacZ-buffer). The colour reaction was stopped with 1 M Na_2CO_3.