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Novel 2-amino-isoflavones exhibit aryl hydrocarbon receptor agonist or antagonist activity in a species/cell-specific context

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ABSTRACT

The aryl hydrocarbon receptor (AhR) mediates the induction of a variety of xenobiotic metabolism genes. Activation of the AhR occurs through binding to a group of structurally diverse compounds, most notably dioxins, which are exogenous ligands. Isoflavones are part of a family which include some well characterised endogenous AhR ligands. This paper analysed a novel family of these compounds, based on the structure of 2-amino-isoflavone. Initially two luciferase-based cell models, mouse H1L6.1c2 and human HG2L6.1c3, were used to identify whether the compounds had AhR agonistic and/or antagonistic properties. This analysis showed that some of the compounds were weak agonists in mouse and antagonists in human. Further analysis of two of the compounds, Chr-13 and Chr-19, was conducted using quantitative real-time PCR in rat H4IIE and human MCF-7 cells. The results indicated that Chr-13 was an agonist in rat but an antagonist in human cells. Chr-19 was shown to be an agonist in rat but more interestingly, a partial agonist in human. Luciferase induction results not only revealed that subtle differences in the structure of the compound could produce species-specific differences in response but also dictated the ability of the compound to be an AhR agonist or antagonist. Substituted 2-amino-isoflavones represent a novel group of AhR ligands that must differentially interact with the AhR ligand binding domain to produce their species-specific agonist or antagonist activity and future ligand binding analysis and docking studies with these compounds may provide insights into the differential mechanisms of action of structurally similar compounds.

1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor which, upon activation, induces the transcription of several xenobiotic metabolism genes (Hankinson, 1995; Whitlock, 1999). The receptor binds to a wide range of dissimilar compounds (Denison and Nagy, 2003), the most characterised of which is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The un-activated receptor is located in the cytosol, where the binding site is maintained in the correct configuration by a chaperone protein complex (Bell and Poland, 2000). Once bound to a ligand, such as TCDD, the AhR translocates to the nucleus where it dissociates from the chaperone proteins and binds to a structurally similar protein called the aryl hydrocarbon receptor nuclear translocator (Arnt). This new complex binds to the DNA at specific sites termed dioxin response elements (DRE) where they initiate the transcription of a wide variety of genes including cytochrome P450-1A1 (CYP1A1) and many others responsible for metabolising xenobiotics. For a full review of AhR activation and related responses, see Denison et al. (2011).

While most of the AhR ligands which have been characterised, such as dioxins, are exogenous to the body, endogenous ligands exist and significant agonist activity has been observed in human serum (Connor et al., 2008; Schecter et al., 1999). A few endogenous

Abbreviations: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Chr, 2-amino-isoflavone; qRT-PCR, quantitative real-time polymerase chain reaction.

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or natural AhR ligands have been identified and investigated and dramatic species differences in ligand activity and potency have been reported (Denison et al., 2011; Denison and Nagy, 2003; Nguyen and Bradfield, 2008; Stejskalova et al., 2011; Van der Heiden et al., 2007). Much of the data regarding the potency of AhR ligands comes from a combination of in vivo and in vitro studies in a variety of animal cells and models (Haws et al., 2006). Therefore understanding the mechanisms behind the species-specific differences in the potency of these AhR ligands is important. One of the species related observations is that, in general, most AhR ligands tend to be more potent in rodent cell lines than in human (Budinsky et al., 2010; Xu et al., 2000) and while this difference in potency is most likely due to sequence differences between the ligand binding domain of the rodent and human AhR, it may also be affected by other factors such as ligand pharmacokinetics, metabolism and AhR concentration (Denison et al., 2002).

A well characterised family of natural AhR ligands are the isoflavones which are organic compounds found in various species of the legume family, such as soy beans. The most well known of these compounds are biochanin A, shown to be a relatively strong AhR agonist, and genistein and daidzein, which have been shown to be weak agonists or weak antagonists in mouse Hepa1 and yeast cells and in mice, in vivo (Amakura et al., 2003; Choi and Kim, 2008; Jung et al., 2007; Medjakovic and Jungbauer, 2008; Shertzer et al., 1999; Zhang et al., 2003). Here we report the result of studies examining the species-specific ability of a group of novel substituted 2-amino-isoflavone (Chr) compounds to exert agonistic or antagonistic effects on the mouse, rat and human AhR signal transduction pathway.

2. Materials and methods

2.1. Synthesis of novel 2-amino-isoflavones

The detailed synthesis of all the commercially unavailable isoflavones has been reported elsewhere (Balboni et al., 2012). Compounds Chr-1 (2-amino-3-phenylchromen-4-one) and Chr-13 (2-amino-3-(4′-chlorophenyl)-7-methoxychromen-4-one) were obtained from ChemBridge (San Diego, USA) and Life Chemicals (Braunschweig, Germany), respectively. Chr-19 (6-chloro-3-(4′-methoxy)phenylcoumarin) was synthesised as reported by Quezada et al. (2010). The structures of all Chr compounds used in these studies are presented in Table 1.

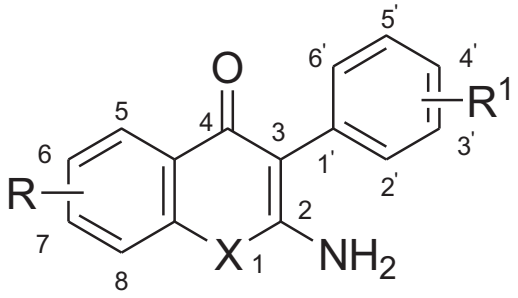
2.2. Materials

TCDD (purity 99%) was purchased from Cerilliant Cambridge Isotope Laboratories (MA, USA). It was dissolved in DMSO then diluted in cell culture medium. All the compounds were prepared as 10 mM solutions in DMSO for the luciferase study then diluted with cell culture medium. Chr-13 and 19 were prepared as 100 mM and 30 mM solutions, respectively, in DMSO for the qRT-PCR study then diluted in cell culture medium. In all cases the final concentration of DMSO was <0.2%. The Chr-13 and 19 compounds were checked for contamination prior to mRNA measurement using gas chromatography/mass spectroscopy (GC/MS) (Fernandes et al., 2004) and no potent TCDD-like AhR agonists, such as polychlorinated dibenzodioxins (PCDD), dibenzofurans (PCDF) or biphenyls (PCB), were found.

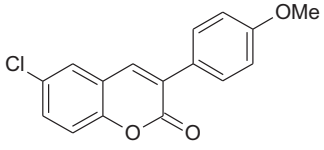
2.3. Cell-lines

The recombinant mouse (Hepa1c1c7) and human hepatoma (HepG2) cell lines (H1L6.1c2 and HG2L6.1c3, respectively) contain a stably transfected plasmid (pGudLuc6.1) which has the firefly luciferase gene (*Photinus pyralis*) under AhR-responsive control of four DREs immediately upstream of the mouse mammary tumour virus (MMTV) viral promoter and luciferase gene (Aarts et al., 1995; Garrison et al., 1996; Han et al., 2004). These cell lines were grown and maintained in α-minimum essential medium (α-MEM; Invitrogen, #12000-063) containing 10% premium foetal bovine serum (Atlanta Biologicals, #S11150). The human breast carcinoma (MCF-7) cells were a kind gift from Dr Tracey Bradshaw (Centre for Biomolecular Science, University of Nottingham, UK) and the rat liver carcinoma (H4IIE-C3) cells (CRL-1548) were purchased from the ATCC. These two cell lines were maintained in minimum essential medium (MEM; Sigma #M2279) containing 10% foetal bovine serum (Sigma #F7524), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma #G1146) and 1% non-essential amino acids (Sigma #M7145). All cell lines were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

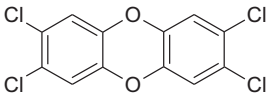
Table 1
Structures of the 2-amino-isoflavones (2-amino-3-phenylchromen-4-one; Chr) compounds and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).



Compound	R	R^1	X
Chr-1	H	H	O
Chr-2	6-Cl	H	O
Chr-3	7-Cl	H	O
Chr-4	H	4′-Cl	O
Chr-5	6-Cl	4′-Cl	O
Chr-6	7-Cl	4′-Cl	O
Chr-7	H	4′-OMe	O
Chr-8	6-Cl	4′-OMe	O
Chr-9	7-Cl	4′-OMe	O
Chr-10	H	3′,4′-$(OMe)_2$	O
Chr-11	6-Cl	3′,4′-$(OMe)_2$	O
Chr-13	7-OMe	4′-Cl	O
Chr-14	7-OMe	3′,4′-$(OMe)_2$	O
Chr-15	7-Cl	H	NH
Chr-16	6-Cl	H	NH
Chr-17	7-OMe	H	O
Chr-18	7-OMe	4′-OMe	O



Chr-19



TCDD

2.4. Measurement of luciferase activity

Cells were plated into 96-well plates and treated with either the indicated concentration of test compounds in 0.1% DMSO, 1 nM TCDD or 0.1% DMSO vehicle for 24 h at 37 °C to measure the agonistic effects of the compounds. The antagonistic properties were tested by incubating cells with 10 μM Chr compound in the presence of 1 nM TCDD (for H1L6.1c2 cells) or 10 nM TCDD (for HG2L6.1c3 cells) for 24 h at 37 °C. After incubation cells were washed twice with phosphate-buffered saline (PBS) followed by addition of cell lysis buffer (Promega) and the plates were then shaken for 20 min at room temperature to allow cell lysis. Luciferase activity in each well was measured using an Orion microplate luminometer (Berthold, Oak Ridge, TN) with automatic injection of Promega stabilised luciferase reagent. Luciferase activity of the DMSO treated samples (background activity) was subtracted from activity obtained with all chemical treatments and the resulting induced luciferase activity for each of the chemicals was normalised to that obtained with a maximally inducing concentration of TCDD (defined as a 100% response). Values significantly different from DMSO background activity (i.e. agonist activity – induction of luciferase activity) or that of TCDD alone (i.e. antagonist activity – inhibition of TCDD-inducible luciferase activity) at $p < 0.05$ were determined by Student's *t*-test.

2.5. Preparation of mRNA

The potency of Chr-13 and 19 was measured by treating rat H4IIE or human MCF-7 cells in a 96-well plate with a cell density of 1.5×10^5 cells/well. Cells were treated with various concentrations of the compounds (Chr: 1 nM–100 μM; TCDD: 100 fM–10 nM) for 4 h to produce a concentration–response curve. A vehicle control of 0.1% DMSO and a 10 nM TCDD control (saturating concentration) were also run alongside each compound. For the antagonistic studies, cells were treated with various concentrations of TCDD (100 fM–10 nM) in the presence of a concentration of antagonist which produced 20% of the maximal response of 10 nM TCDD (or if no agonistic response was obtained, the highest concentration possible). This was determined in the agonistic experiments. Each concentration

Table 2
Sequences of primers and probes for CYP1A1 measurement – forward (f) and reverse (r) primers and probes are indicated. Sequences are shown from 5′ to 3′. FAM: carboxyfluorescein; HEX: hexachlorofluorescein and CY-5: 3′-deoxy-5-(cyanine dye 3)uridine 5′-trisphosphate are the reporter dyes located at the 5′ end of the probe. The quencher dyes, Black Hole-1 or -2 (BH1 or BH2), are at the 3′ end.

Gene	Sequence	GenBank number	Dye
Rat CYP1A1		NM_012540	
Primer (f)	CCACAGCACCATAAGAGATACAAG		
Primer (r)	CCGGAACTAGTTTGGATCAC		
Probe	ATAGTTCCTGGTCATGGTTAACCTGCCAC		FAM-BH1
Rat AhR		NM_013149	
Primer (f)	GCAGCTTATTCTGGGCTACA		
Primer (r)	CATGCCACTTTCTCCAGTCTTA		
Probe	TATCAGTTTATCCACGCCGCTGACATG		HEX-BH1
Rat β-actin		NM_031144	
Primer (f)	CTGACAGGATGCAGAAGGAG		
Primer (r)	GATAGAGCCACCATCCACA		
Probe	CAAGATCATTGCTCCTCCTGAGCG		CY-5-BH2
Human CYP1A1		NM_000499	
Primer (f)	GTTGTGTCTTTGTAAACCAGTG		
Primer (r)	CTCACTTAACACCTTGTCGATA		
Probe	CAACCATGACCAGAAGCTATGGGT		FAM-BH1
Human AhR		NM_001621	
Primer (f)	ATACAGAGTTGGACCGTTTG		
Primer (r)	CTTTCAGTAGGGGAGGATTT		
Probe	TCAGCGTCAGTTACCTGAGAGCCA		HEX-BH1
Human β-actin		NM_001101	
Primer (f)	GACATGGAGAAAATCTGGC		
Primer (r)	AGGTCTCAAACATGATCTGG		
Probe	ACACCTTCTACAATGAGCTGCGTGT		CY-5-BH2

was done in biological triplicates (separate cell samples in individual wells). During the treatment, conditioned medium (second-hand medium from untreated cells) was used to eliminate the effects of weak AhR agonists in the foetal bovine serum such as indirubin (Adachi et al., 2001). The cells were removed using trypsin (Sigma #T4174) and transferred to eppendorf tubes. RNA purification was conducted using Absolutely RNA® Miniprep Kit (Stratagene #400800) as per manufacturer's instructions for small sample sizes. cDNA synthesis was done using High capacity RNA-to-cDNA kit (Applied Biosystems #4387406), samples were incubated for 60 min at 37 °C followed by 5 min at 95 °C using an Eppendorf thermocycler (Germany). Samples with no reverse-transcriptase (No RT) or No RNA were also prepared for control purposes. Samples were stored at −20 °C.

2.6. Measurement of CYP1A1 mRNA using qRT-PCR

Measurement of the induction of CYP1A1 mRNA using PCR is a well characterised method of measuring AhR activation with higher sensitivity compared with other methods of measuring AhR activation such as ethoxyresorufin-*O*-deethylase (EROD) (Vanden Heuvel et al., 1994). CYP1A1 and two normalisation genes, β-actin and AhR, were measured using quantitative real-time PCR (qRT-PCR) as a multiplex. This method allows a real-time view of the mRNA levels allowing for a more accurate estimation of mRNA concentration. A complete master mix was prepared containing: 20 μl Taqman® gene expression master mix (Applied Biosystems #4369016), CYP1A1, β-actin and AhR primers (10 μM) and probes (5 μM) and 150 ng cDNA, which was made up to 40 μl with DEPC treated water. The nucleotide sequences for the primers and probes are shown in Table 2. It has been previously shown that these primers and probes can be run in the same reaction without interference (Bazzi et al., 2009; Bell et al., 2007). The Taqman master mix also had an internal dye (ROX) which was used for internal normalisation between wells.

The biological replicate samples were run as two technical replicates (replicate for qRT-PCR measurement only). An aliquot of 20 μl of the complete master mix with cDNA was added to each well of a 96-well qRT-PCR plate. Analysis was conducted using an Applied Biosystems 7500 fast RT-PCR machine with the following protocol: 1 cycle (2 min at 50 °C; 10 min a 95 °C), 40 cycles (20 s at 95 °C; 90 s at 58 °C). A no template control (NTC) was also run alongside the No RT and No RNA. The mRNA levels were measured as C_t values (the cycle at which the fluorescence passes a set threshold and is therefore distinguishable from the background noise) and transferred for analysis using qBasePlus v1.3 (Biogazelle). The qBasePlus software normalised the CYP1A1 mRNA C_t values against the values obtained for β-actin and AhR, then converted them into calibrated normalised relative quantities (CNRQ) (Hellemans et al., 2007; Vandesompele et al., 2002). These values were normalised against 10 nM TCDD-induced CYP1A1 mRNA levels which was defined as 100% or the maximal response. The data was then plotted as concentration vs. normalised CYP1A1 mRNA (% of maximal response) using GraphPad Prism 5 which also calculated the EC_{50}'s and the 95% confidence intervals (95% CI). This allowed comparison of the curves based on their potency at 50% of the maximal effect. It was assumed that all the compounds would reach 100% response therefore the agonist curves were fitted to account for this. The TCDD with antagonist C/R curve was corrected for all the antagonist studies. The EC_{50} was calculated as the halfway point between the ~20% background, due to the antagonist, and the 100% maximal induction. An unpaired *t*-test was used to show whether the EC_{50}s derived from the antagonism curves were statistically different.

3. Results

3.1. Luciferase induction shows that several of the compounds activate AhR-dependent transcription

The two recombinant AhR-responsive luciferase cell culture models, mouse H1L6.1c2 and human HG2L6.1c3 cells, were used to obtain screening data on the AhR agonist activity of the compounds at a single concentration (10 μM). The results from mouse and human are displayed as direct comparisons with the data normalised to the maximal induction response obtained with TCDD (1 nM for H1L6.1c2 cells and 10 nM for HG2L6.1c3 cells) (Fig. 1). The results show that none of the compounds were particularly potent agonists especially in the human cell line and only Chr-3, 15, 16 and 19 induced significant levels of AhR-dependent luciferase reporter gene activity. In the mouse cells, Chr-13, 15, 17 and 19 were the most potent agonists, although only Chr-13 and 15 stimulated AhR-dependent gene expression greater than 30%; relatively low levels of induction were observed by numerous other compounds (i.e. Chr-4, 6, 7, 8, 10, 11, 14, 16, 17 and 18).

To determine the ability of the isoflavones to antagonise mouse and human AhR action, cells were co-incubated with TCDD (1 nM for mouse H1L6.1c2 and 10 nM for human HG2L6.1c3) in the absence or presence of 10 μM of the indicated compound and luciferase activity determined after 24 h of incubation. The data (Fig. 2) shows that while none of the Chr compounds exerted significant antagonistic effects on TCDD dependent activation of AhR signalling in mouse H1L6.1c2 cells, a large number of the compounds (Chr-2, 3, 4, 5, 6, 8, 9, 13, 17, 18 and 19) antagonised TCDD-dependent induction of luciferase in the human HG2L6.1c3 cells. These results demonstrate clear species differences in both the relative potency and functional (agonist and/or antagonist) activity of these compounds. This reduction of AhR-dependent transcription of luciferase, when the isoflavones were incubated in the presence of TCDD, shows that the compounds must have a relatively good affinity for the AhR and that they have very low agonistic

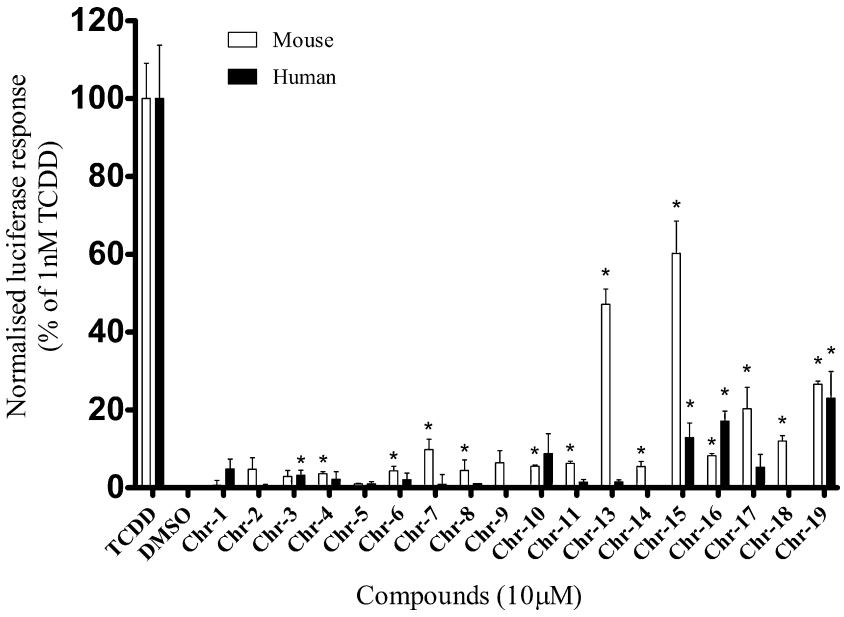


Fig. 1. AhR agonist activity of 2-amino-isoflavone derivatives in recombinant mouse and human hepatoma cell lines – mouse H1L6.1c2 and human HG2L6.1c3 cells were incubated with 10 μM Chr compound, TCDD (1 nM for mouse and 10 nM for human) or 0.1% DMSO control for 24 h. Luciferase activity was measured and normalised against TCDD (maximal response). White bars = mouse, black bars = human. Error bars are S.D., $n = 3$. *Luciferase activity was significantly higher (p-value ≤ 0.05) than that of DMSO control.

efficacy. Table 3 summarises the relative agonist/antagonist activity of the compounds obtained from studies using the recombinant mouse and human luciferase cell lines.

From the luciferase assay results, two compounds were selected for further analysis by qRT-PCR to provide a quantitative measure of their agonism and antagonism of an endogenous gene (CYP1A1). Chr-13 was shown to be a strong agonist (compared to the other Chr compounds) in mouse H1L6.1c2 cells but a strong antagonist in human HG2L6.1c3 cells, indicating a significant species difference. The second compound selected was Chr-19 which was shown to be a weak agonist in both mouse and human cell lines, but more interestingly was also shown to be an antagonist in the human cell line, thus demonstrating that this compound is a partial agonist in human.

3.2. Quantitative real-time PCR (qRT-PCR) measurement of CYP1A1 mRNA

Further analysis was conducted using qRT-PCR in rat H4IIE and human MCF-7 cells. The high degree of similarity in the ligand binding domains of the mouse and rat AhRs (Hahn et al., 1997; Pandini et al., 2009) and similarities in ligand binding specificity and functional activity of mouse and rat AhR were expected to allow us to directly compare the ligand selectivity of human

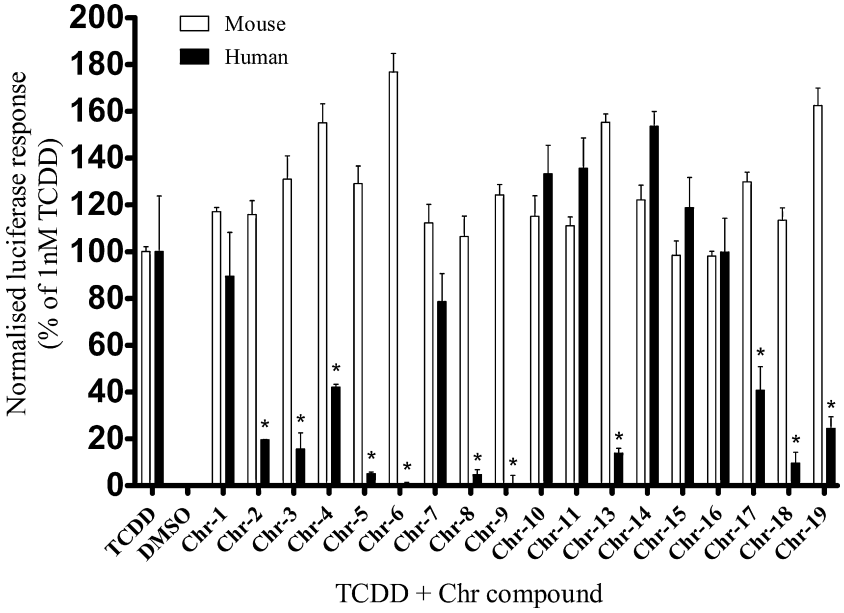


Fig. 2. Antagonistic effects of the compounds when in the presence of TCDD – mouse H1L6.1c2 and human HG2L6.1c3 cells were incubated with 10 μM Chr compound in the presence of TCDD (1 nM for mouse and 10 nM for human cells), TCDD alone or 0.1% DMSO control for 24 h. Luciferase activity was measured and normalised against 1 nM TCDD (maximal response). White bars = mouse, black bars = human. Error bars are S.D., $n = 3$. *Luciferase activity was significantly lower (p-value ≤ 0.05) than that of TCDD alone.

Table 3
Summary of the data obtained from the luciferase induction/inhibition studies. Data obtained by treatment of mouse H1L6.1c2 and human HG2L6.2c3 cells with Chr compounds in the presence (antagonistic) or absence (agonistic) of TCDD. (+) Weak agonist/antagonist, (++) strong agonist/antagonist, (–) no agonistic/antagonistic response detected.

Compound	Mouse		Human	
	Agonist	Antagonist	Agonist	Antagonist
Chr-1	–	–	–	–
Chr-2	–	–	–	+
Chr-3	–	–	+	+
Chr-4	+	–	–	+
Chr-5	–	–	–	++
Chr-6	+	–	–	++
Chr-7	+	–	–	–
Chr-8	+	–	–	++
Chr-9	–	–	–	++
Chr-10	+	–	–	–
Chr-11	+	–	–	–
Chr-13	++	–	–	++
Chr-14	+	–	–	–
Chr-15	++	–	+	–
Chr-16	+	–	+	–
Chr-17	+	–	–	+
Chr-18	+	–	–	++
Chr-19	+	–	+	+

versus rodent AhR signalling pathways. Although some ligand-dependent differences in the relative agonist/antagonist potency have been observed between rat and mouse cells/AhRs, the overall ligand specificity/selectivity of mouse and rat AhRs is very similar (Denison et al., 1999, 2002, 2011). Measurement of CYP1A1 mRNA by qRT-PCR was utilised for these studies as it not only allows detection and a more exacting quantitation of AhR-dependent expression of an endogenous gene (CYP1A1), but it would confirm that the responses examined were not due to selective effects on expression from the integrated luciferase reporter plasmid that might not be seen on other AhR-responsive genes. Human MCF-7 cells were used as they have been shown to be more sensitive at detecting AhR antagonism than HepG2 cells (Zhang et al., 2003). Iwanari et al. (2002) showed that several of the most characterised AhR ligands, such as TCDD and 3-methylchloranthrene (3-MC), showed a comparable pattern of induction of CYP1A1 mRNA in human HepG2 and human MCF-7 cells. The similar pattern of induction observed with a variety of flavonoids in both human HepG2 and human MCF-7 cells indicated that there were minimal inter-tissue differences in response (Zhang et al., 2003). For these studies, Chr-13 and Chr-19 were selected for further analysis as they demonstrated significant differences in their agonist/antagonist activity between human and mouse cells; while Chr-15 was the most potent AhR agonist in these cell lines, it was not examined further because of its lack of antagonistic activity.

Concentration–response (C/R) curves of CYP1A1 mRNA in rat H4IIE and human MCF-7 cells treated with Chr-13, Chr-19 or TCDD is presented in Fig. 3. These studies revealed EC_{50}s of 113 pM (95% CI = 83.0–152 pM) and 661 pM (95% CI = 515–847 pM) for rat and human CYP1A1 induction by TCDD, respectively, showing that the potency of TCDD was 6-fold lower in the human MCF-7 cell line compared with that in the rat H4IIE cells ($p < 0.0001$). Furthermore, it is clear that Chr-13 was significantly less potent than TCDD at activating the AhR. Assuming that Chr-13 can achieve a maximal response, the EC_{50} for rat H4IIE cells treated with Chr-13 was 41.5 μM (95% CI = 35.2–49.0 μM), which is comparable to the data obtained in mouse H1L6.1c2 cells where a 10 μM concentration of Chr-13 gave a 50% response. In human MCF-7 cells, an EC_{50} could not be obtained with the concentrations used. These results indicate that Chr-13 is a relatively weak agonist in rat but is not an agonist in human MCF-7 cells (at the concentrations used), similar

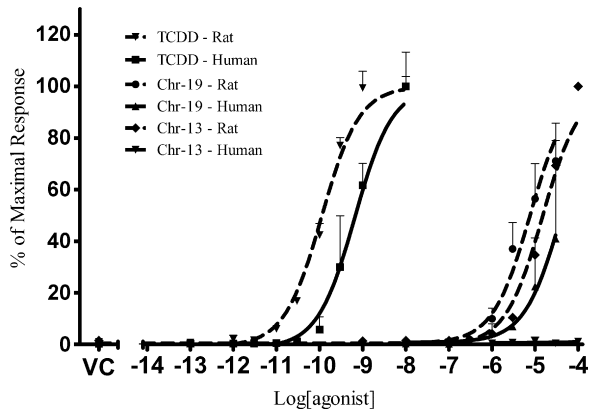


Fig. 3. Concentration-response curve of Chr-13, Chr-19 or TCDD in rat H4IIE and human MCF-7 – cells were incubated with various concentrations of Chr-13, Chr-19 or TCDD for 4 h. qRT-PCR was conducted and the data plotted with CYP1A1 mRNA normalised against β-actin and AhR. The data was further normalised with 10 nM TCDD (rat and human) defined as producing a 100% response. Error bars are S.D., $n = 3$. VC = vehicle control.

to the initial screening results which showed Chr-13 to be agonist in mouse but not human cells. This result is not surprising given the high degree of sequence identity of the mouse and rat AhR ligand binding domain (Pandini et al., 2009).

Investigation of the antagonistic effects of Chr-13 was performed by treating rat or human cells with various concentrations of TCDD but in the presence of a set concentration of Chr-13 which induces ~20% of the maximal agonistic response of 10 nM TCDD. This was estimated to be 10 μM for the rat cells, but no response was detected in human cells, at concentrations up to 100 μM.

The antagonistic effect of Chr-13 in rat H4IIE cells is shown in Fig. 4A. Although the screening data showed that Chr-13 was not antagonistic in the mouse H1L6.1c2 cell line, it was still important to confirm it in the rat H4IIE cell line. The addition of 10 μM Chr-13 to the TCDD C/R curve resulted in a background induction of ~25% of the maximal induction which corresponds well with the data shown in Fig. 3. The EC_{50} for TCDD in the presence of 10 μM Chr-13 was 237 pM (95% CI = 24.9 pM–2.25 nM) which was not significantly different ($p > 0.05$) from the EC_{50} for TCDD alone. The results confirm that Chr-13 has no antagonistic effects under these conditions. Thus, as expected, the rodent AhR signalling pathways behaved similarly. Fig. 4B shows human MCF-7 cells treated with TCDD in the presence and absence of 100 μM Chr-13. Since Chr-13 had no AhR agonistic activity (Fig. 3), there was no elevated background of CYP1A1 mRNA when cells were treated simultaneously with Chr-13 and TCDD. However, the data did show a shift of the TCDD C/R curve to the right, reducing the potency of TCDD by 5-fold compared to TCDD in the absence of Chr-13. The EC_{50} for TCDD in the presence of 100 μM Chr-13 was 3.02 nM (95% CI = 2.55–3.55 nM) which was significantly higher ($p < 0.0001$) than the EC_{50} of TCDD alone. Consequently, these data show that Chr-13 is a weak antagonist in human MCF-7 and an agonist in rat H4IIE cells.

Fig. 3 also shows the C/R curves of Chr-19 and TCDD in rat H4IIE and human MCF-7 cells, as measured by qRT-PCR. The response to TCDD is shown for comparison to Chr-19. Chr-19 was agonistic in both rat H4IIE and human MCF-7 cells, with the compound being approximately 20-fold more potent in rat cells ($p < 0.0001$). Assuming that the compound will attain maximal response, the EC_{50} for Chr-19 in rat H4IIE cells was 7.70 μM (95% CI = 5.22–11.3 μM), and in human MCF-7 cells, the EC_{50} was estimated to be 140 μM (95% CI = 65.4–317 μM). The data shows that Chr-19 is significantly less potent at activating the AhR and inducing CYP1A1 than TCDD.

The antagonistic effects of Chr-19 were examined by treating cells with TCDD in the presence or absence of a set concentration

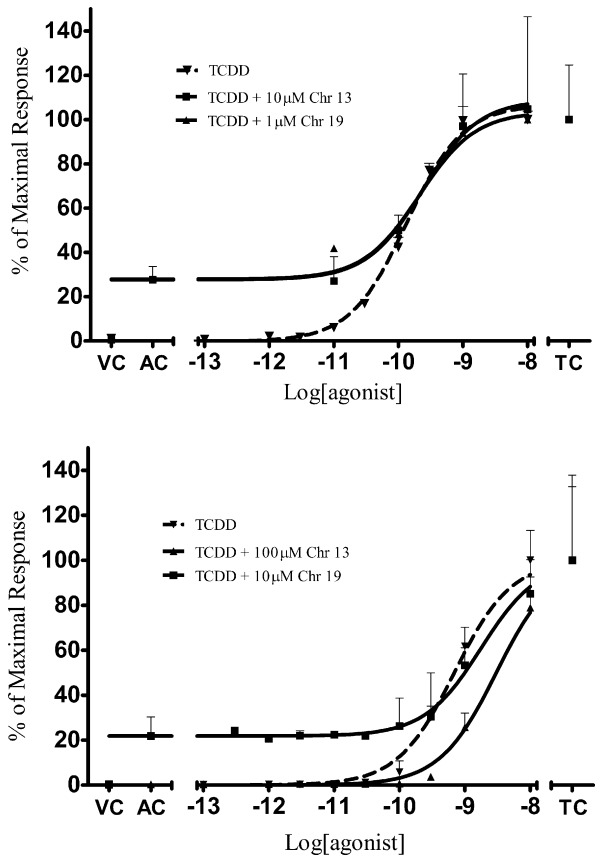


Fig. 4. Concentration–response curve for TCDD in the presence and absence of (A) 10 μM Chr 13 or 1 μM Chr-19 in rat H4IIE, or (B) 100 μM Chr 13 or 10 μM Chr-19 in human MCF-7 – cells were incubated with TCDD (rat: 10 pM–10 nM; human: 100 fM–10 nM) in the presence or absence of Chr-13 or Chr-19 for 4 h. After which, RNA was purified and qRT-PCR was conducted to measure CYP1A1 mRNA. Data was normalised against 10 nM TCDD which has been defined as producing a 100% response. Error bars are S.D., $n = 3$. VC = vehicle control, AC = antagonist only control (Chr-13 or Chr-19 alone) and TC = 10 nM TCDD alone. EC_{50}s are statistically different for human (Chr-13: p-value < 0.0001; hr-19: p-value < 0.004).

of Chr-19 (i.e. that produces ~20% of maximal induction response). This value was found to be 1 μM and 10 μM for rat and human, respectively. Fig. 4 shows the analysis of antagonistic activity of Chr-19 in (A) rat H4IIE cells and (B) human MCF-7 cells. It can be seen in Fig. 4A that there was no shift of the TCDD curve to the right, which would indicate an antagonist effect. The EC_{50} for TCDD in the presence of 1 μM Chr-19 was 182 pM (95% CI = 31.0 pM–1.07 nM) which was not significantly different ($p > 0.05$) from the EC_{50} obtained from cells treated with TCDD only. Fig. 4B shows human MCF-7 cells treated with TCDD in the presence and absence of Chr-19. An EC_{50} of 1.76 nM (95% CI = 897 pM–3.47 nM) for TCDD with 10 μM Chr-19 was calculated which was statistically significantly higher than that obtained with TCDD alone ($p < 0.05$). This indicates that Chr-19, at 10 μM, reduces the potency of TCDD activation of AhR by 3-fold and is hence a weak antagonist of the AhR. Combined with the data from Fig. 3, which showed Chr-19 was an agonist of human AhR, it can be concluded that this compound is a partial agonist in human MCF-7 cells. Table 4 shows the summary of the results obtained through qRT-PCR.

4. Discussion

The initial data using luciferase-based cell models showed that the majority of these compounds can affect the AhR, some with agonist properties, some with antagonistic properties and some with both (i.e. partial agonists/antagonists). Analysis focused on:

Table 4
Summary of EC_{50}s from potency analysis using qRT-PCR – data obtained in both rat H4IIE and MCF-7 cells measuring the agonistic and antagonistic properties of Chr-13 and Chr-19. *EC_{50}s are significantly different from TCDD alone (p-value ≤ 0.05).

Compound	EC_{50} (95% confidence interval)
Rat	
Chr-13	41.5 μM (35.2–49.0 μM)
Chr-19	7.70 μM (5.22–11.3 μM)
Human	
Chr-13	No agonistic effect
Chr-19	140 μM (65.4–317 μM)
Rat	
TCDD	113 pM (83.0–152 pM)
TCDD + 10 μM Chr-13	237 pM (24.9 pM–2.25 nM)
TCDD + 1 μM Chr-19	182 pM (31 pM–1.07 nM)
Human	
TCDD	661 pM (515–847 pM)
TCDD + 100 μM Chr-13	3.02 nM (2.55–3.55 nM)
TCDD + 10 μM Chr-19	1.76 nM (897 pM–3.47 nM)

(1) their ability to induce luciferase (i.e. to activate the AhR) and (2) to competitively inhibit the ability of TCDD to activate AhR-dependent gene expression. This was done using two cell lines, mouse H1L6.1c2 and human HG2L6.1c3, both containing the stably transfected AhR-responsive firefly luciferase reporter plasmid pGudLuc6.1 (Garrison et al., 1996; Han et al., 2004). These analyses identified two compounds of with divergent species/cell-specific agonist/antagonist response, Chr-13 and 19, that were then further examined for their ability to induce or inhibit expression of an endogenous gene (CYP1A1) using. These latter experiments allowed more accurate quantitation of the relative agonistic/antagonistic activity of the compounds. The ability of the compounds to competitively affect the activity of TCDD allowed measurement of the shift in potency of TCDD and hence measurement of their antagonistic effect. The luciferase screening data for Chr-13 matched well with the results obtained through qRT-PCR, however for Chr-19, although qualitatively the same result was obtained there were some discrepancies. The results obtained in the screening data (Fig. 2) showed that a 10 μM concentration of Chr-19 reduced the response to 10 nM TCDD in human cells by 80% of the maximal response (a 5-fold reduction) whereas when qRT-PCR was used, 10 μM Chr-19 only reduced the response to 10 nM TCDD by 10% (Fig. 4B). One possibility that could explain these divergent results is that there exist inter-tissue differences in the cell lines that modulate the overall AhR-mediated response for this particular compound, although this remains to be determined. Zhang et al. (2003) previously examined AhR activation by a variety of agonists in both HepG2 and MCF-7 cells and while most compounds showed a similar pattern of induction, they also identified several compounds which exhibited differences in potency between the two cells lines and this must relate to cell specific differences as the AhR was identical.

A basic analysis of the structure–activity relationships, based mainly on the screening data, was conducted. Firstly, Chr-2 and 3 were shown to be human AhR antagonists however, if the ether oxygen (position 1; Fig. 5) was substituted with a secondary amine, such as in Chr-15 and 16, the compounds become agonists in human cells instead. Furthermore, the position of the chlorine atom on these molecules (position 6 or 7) had no effect on the agonistic or antagonist activity of the compounds. This is shown by compounds Chr-5, 8 and 16 which have a chlorine atom on position 7, and Chr-6, 9 and 15 which have a chlorine atom on position 6, yet there is no difference in the ability of these compounds to activate or inhibit the AhR (results were the same for all six compounds). The data also suggests that a chlorine atom is required somewhere on the molecule (Chr-1 has no effects), although based on the number of compounds tested, the precise location (position 3, 4 or 4′) does

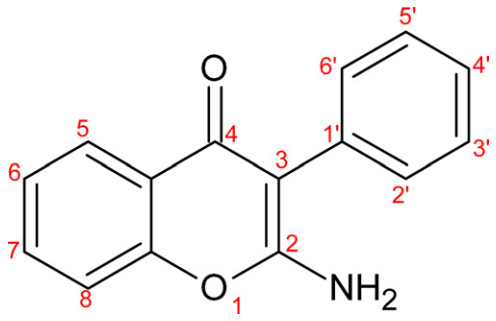


Fig. 5. Position of carbon atoms on the main underlying structure of the Chr compounds.

not seem to affect the compound's properties. The slightly reduced ability of Chr-4 may suggest that there needs to be a chlorine atom on position 6 or 7 in order for it to completely antagonise the AhR at a concentration of 10 μM. The chlorine atoms would provide a high electron density which has been shown to be important for high affinity in similar compounds (Henry et al., 1999).

Chr-13 and 19 are relatively unique in this group of compounds making it difficult to assess what contributes to their species-specific differences in effect. A methoxy group on position 7 is the most likely explanation for why Chr-13 and 17 are agonists in mouse but antagonists in human. Furthermore, as discussed earlier, the chlorine on position 4′ appears to have no effect on AhR binding or activation. The unusual partial agonistic properties of Chr-19 are likely related to the substitution of the amino group on position 2 with a carbonyl group and the removal of the carbonyl group from position 4. If there is a methoxy group on position 3′ it appears that the compound will simply not interact significantly with the AhR, as seen in Chr-10, 11 and 14. Using alpha-naphthoflavone as the backbone structure, Gasiewicz et al. (1996) and Henry et al. (1999) investigated the effect of chemical substituents on AhR activity. They showed that a methoxy group in the 3′ position not only increased affinity for receptor binding (Gasiewicz et al., 1996; Henry et al., 1999), but was very important for antagonist activity (Henry et al., 1999; Lu et al., 1996). The reason for the lack of effect of Chr-1 is unclear as Chr-4 and 7 have antagonistic properties and also have no atom or group on the first benzene ring. Based on the potency of Chr-18 and 19, it would be interesting to test other classes of compounds with similar (AhR binding) structures for instance, chromones and coumarins (such as warfarin).

In terms of AhR agonism, the most potent of the compounds (Chr-13 and 15) were still 10,000-fold less potent than TCDD at activating the AhR and inducing CYP1A1 mRNA. Their agonistic and antagonistic ability suggests they have similar potency to the mono-ortho-chlorinated polychlorinated biphenyls (PCBs) such as PCB 105 and PCB 118 (Wall, unpublished data). Isoflavones have been shown to have EC_{50} values in the 10 μM range (Amakura et al., 2003), although more potent isoflavones have been identified. Biochanin A, for example, has a similar structure to Chr-7, but with hydroxyl groups on positions 5 and 7, and it was shown to be only 100-fold less potent than TCDD (Medjakovic and Jungbauer, 2008). Daidzein, an isoflavone which is similar in structure to Chr-18 but with hydroxyl groups on positions 7 and 4′ instead of methoxy groups, was shown to be a weak agonist in mouse Hepa1 cells at similar concentrations as Chr-18 (Zhang et al., 2003). Furthermore Zhang et al. (2003) also showed that daidzein had no AhR agonistic activity in human MCF-7 or HepG2 cells, similar to Chr-18. Several compounds with flavone and isoflavone structures were tested in human MCF-7 and mouse Hepa1 cells with most of the compounds having no or very limited AhR activity. Similarly, Zhang et al. (2003) reported species-specific differences by several flavonoid compounds relative to AhR agonist and antagonist activity.

Quercetin and kaempferol (both flavonols) are the most abundant flavonoids found in the diet and based on EROD analysis, Ciolino et al. (1999) reported that quercetin was an AhR agonist and that kaempferol was an antagonist in MCF-7 cells. Several other flavonoid derivatives have also been identified as AhR ligands including galangin, which was shown to act as an AhR antagonist (Ciolino and Yeh, 1999) and chrysin, which was shown to be one of the strongest flavonoid agonists. While chrysin is a partial agonist in human cells it produces no antagonistic effects in rat H4IIE cells demonstrating that many compounds based on the flavonoid structure have species-specific differences (Van der Heiden et al., 2007).

In conclusion, many of the novel 2-amino-isoflavones described here are not only active ligands (agonists/antagonists) of the AhR, but they also produce unusual species differences in response. These analyses have shown that even the slightest substitutions in chemical structure can significantly alter not only the potency of the compound but also its antagonistic potential. Further investigation has quantified a compound which is an agonist of the rat AhR and antagonist of human AhR (Chr-13) and one that is a selective partial agonist of the human AhR (Chr-19). Both of these compounds could be useful tools when investigating the mechanism responsible for ligand-dependent species differences in the activation of the AhR.

Conflict of interest

None.

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References

Aarts, J.M.M.J.G., Denison, M.S., Cox, M.A., Schalk, M.A.C., Garrison, P.M., Tullis, K., de Hann, L.H.J., Brouwer, A., 1995. Species-specific antagonism of Ah receptor action by 2,2′,5,5′-tetrachloro- and 2,2′,3,3′,4,4′-hexachlorobiphenyl. Eur. J. Pharmacol. Environ. Toxicol. 293 (4), 463–474.

Adachi, J., Mori, Y., Matsui, S., Takigami, H., Fujino, J., Kitagawa, H., Miller, C.A., Kato, T., Saeki, K., Matsuda, T., 2001. Indirubin and indigo are potent Aryl hydrocarbon receptor ligands present in human urine. J. Biol. Chem. 276 (34), 31475–31478.

Amakura, Y., Tsutsumi, T., Sasaki, K., Yoshida, T., Maitani, T., 2003. Screening of the inhibitory effect of vegetable constituents on the Aryl hydrocarbon receptor-mediated activity induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Biol. Pharm. Bull. 26 (12), 1754–1760.

Bazzi, R., Bradshaw, T.D., Rowlands, J.C., Stevens, M.F.G., Bell, D.R., 2009. 2-(4-Amino-3-methylphenyl)-5-fluorobenzothiazole is a ligand and shows species-specific partial agonism of the Aryl hydrocarbon receptor. Toxicol. Appl. Pharmacol. 237 (1), 102–110.

Balboni, G., Congiu, C., Onnis, V., Maresca, A., Scozzafava, A., Winum, J.Y., Maietti, A., Supuran, C.T., 2012. Flavones and structurally related 4-chromenones inhibit carbonic anhydrases by a different mechanism of action compared to coumarins. Bioorg. Med. Chem. Lett., In press.

Bell, D.R., Clode, S., Fan, M.Q., Fernandes, A., Foster, P.M.D., Jiang, T., Loizou, G., MacNicoll, A., Miller, B.G., Rose, M., Tran, L., White, S., 2007. Relationships between tissue levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), mRNAs, and toxicity in the developing male Wistar(han) rat. Toxicol. Sci. 99 (2), 591–604.

Bell, D.R., Poland, A., 2000. Binding of Aryl hydrocarbon receptor (AhR) to AhR-interacting protein: the role of hsp90. J. Biol. Chem. 275, 36407–36414.

Budinsky, R.A., LeCluyse, E.L., Ferguson, S.S., Rowlands, J.C., Simon, T., 2010. Human and rat primary hepatocyte CYP1A1 and 1A2 induction with 2,3,7,8-tetrachlorodibenzo-p-dioxin, 2,3,7,8-tetrachlorodibenzofuran, and 2,3,4,7,8-pentachlorodibenzofuran. Toxicol. Sci. 118 (1), 224–235.

Choi, E., Kim, T., 2008. Daidzein modulates induction of hepatic CYP1A1, 1B1, and AhR by 7,12-dimethylbenz[a]anthracene in mice. Arch. Pharmacal Res. 31 (9), 1115–1119.

Ciolino, H.P., Daschner, P.J., Yeh, G.C., 1999. Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. Biochem. J. 340 (3), 715–722.

Ciolino, H.P., Yeh, G.C., 1999. The flavonoid galangin is an inhibitor of CYP1A1 activity and an agonist/antagonist of the aryl hydrocarbon receptor. Br. J. Cancer 79 (9/10), 1340–1346.

Connor, K.T., Harris, M.A., Edwards, M.R., Budinsky, R.A., Clark, G.C., Chu, A.C., Finley, B.L., Rowlands, J.C., 2008. Ah receptor agonist activity in human blood measured with a cell-based bioassay: evidence for naturally occurring AH receptor ligands in vivo. J. Expo. Sci. Environ. Epidemiol. 18 (4), 369–380.

Denison, M.S., Nagy, S.R., 2003. Activation of the Aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu. Rev. Pharmacol. Toxicol. 43, 309–334.

Denison, M.S., Seidel, S.D., Rogers, W.J., Ziccardi, M., Winter, G.M., Heath-Pagliuso, S., 1999. Natural and synthetic ligands for the Ah receptor. In: Puga, A., Wallace, K.B. (Eds.), Molecular Biology Approaches to Toxicology. Taylor & Francis, Philadelphia, pp. 393–410.

Denison, M.S., Pandini, A., Nagy, S.R., Baldwin, E.P., Bonati, L., 2002. Ligand binding and activation of the Ah receptor. Chem. Biol. Interact. 141 (1–2), 3–24.

Denison, M.S., Soshilov, A.A., He, G., DeGroot, D.E., Zhao, B., 2011. Exactly the same but different: promiscuity and diversity in the molecular mechanisms of action of the Aryl hydrocarbon (dioxin) receptor. Toxicol. Sci. 124 (1), 1–22.

Fernandes, A., White, S., D'Silva, K., Rose, M., 2004. Simultaneous determination of PCDDs, PCDFs, PCBs and PBDEs in food. Talanta 63 (5), 1147–1155.

Garrison, P.M., Tullis, K., Aarts, J.M.M.J.G., Brouwer, A., Giesy, J.P., Denison, M.S., 1996. Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals. Fundam. Appl. Toxicol. 30, 194–203.

Gasiewicz, T.A., Kende, A.S., Rucci, G., Whitney, B., Willey, J.J., 1996. Analysis of structural requirements for Ah receptor antagonist activity. Ellipticines, flavones and related compounds. Biochem. Pharmacol. 52, 1787–1803.

Hahn, M.E., Karchner, S.I., Shapiro, M.A., Perera, S.A., 1997. Molecular evolution of two vertebrate Aryl hydrocarbon (dioxin) receptors (AHR1 and AHR2) and the PAS family. Proc. Natl. Acad. Sci. U. S. A. 94 (25), 13743–13748.

Han, D.-H., Nagy, S.R., Denison, M.S., 2004. Comparison of recombinant cell bioassays for the detection of Ah receptor agonists. Biofactors 20, 11–22.

Hankinson, O., 1995. The aryl hydrocarbon receptor complex. Annu. Rev. Pharmacol. Toxicol. 35, 307–340.

Haws, L.C., Su, S.H., Harris, M., Devito, M.J., Walker, N.J., Farland, W.H., Finley, B., Birnbaum, L.S., 2006. Development of a refined database of mammalian relative potency estimates for dioxin-like compounds. Toxicol. Sci. 89 (1), 4–30.

Hellemans, J., Mortier, G., Paepe, A.D., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8 (2), 1–14.

Henry, E.C., Kende, A.S., Rucci, G., Totleben, M.J., Willey, J.J., Dertinger, S.D., Pollenz, R.S., Jones, J.P., Gasiewicz, T.A., 1999. Flavone antagonists bind competitively with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to the Aryl hydrocarbon receptor but inhibit nuclear uptake and transformation. Mol. Pharmacol. 55, 716–725.

Iwanari, M., Nakajima, M., Kizu, R., Hayakawa, K., Yokoi, T., 2002. Induction of CYP1A1, CYP1A2, and CYP1B1 mRNAs by nitropolycyclic aromatic hydrocarbons in various human tissue-derived cells: chemical-, cytochrome P450 isoform-, and cell-specific differences. Arch. Toxicol. 76, 287–298.

Jung, J., Ishida, K., Nishikawa, J., Nishihara, T., 2007. Inhibition of estrogen action by 2-phenylchromone as AhR agonist in MCF-7 cells. Life Sci. 81 (19–20), 1446–1451.

Lu, Y.-F., Santostefano, M., Cunningham, B.D.M., Threadgill, M.D., Safe, S., 1996. Substituted flavones as aryl hydrocarbon (Ah) receptor agonists and antagonists. Biochem. Pharmacol. 51, 1077–1087.

Medjakovic, S., Jungbauer, A., 2008. Red clover isoflavones biochanin A and formononetin are potent ligands of the human Aryl hydrocarbon receptor. J. Steroid Biochem. Mol. Biol. 108 (1–2), 171–177.

Nguyen, L.P., Bradfield, C.A., 2008. The search for endogenous activators of the Aryl hydrocarbon receptor. Chem. Res. Toxicol. 21, 102–106.

Pandini, A., Soshilov, A.A., Song, Y., Zhao, J., Bonati, L., Denison, M.S., 2009. Detection of the TCDD binding fingerprint within the Ah receptor ligand binding domain by structurally driven mutagenesis and functional analysis. Biochemistry 48, 5972–5983.

Quezada, E., Delogu, G., Picciau, C., Santana, L., Podda, G., Borges, F., Garcia-Morales, V., Vina, D., Orallo, F., 2010. Synthesis and vasorelaxant and platelet antiaggregatory activities of a new series of 6-halo-3-phenylcoumarins. Molecules 15, 270–279.

Schecter, A.J., Sheu, S.U., Birnbaum, L.S., DeVito, M.J., Denison, M.S., Papke, O., 1999. A comparison and discussion of two different methods of measuring dioxin-like compounds: gas chromatography–mass spectrometry and the CALUX bioassay—implications for health studies. Organohalogen Compd. 40, 247–250.

Shertzer, H.G., Puga, A., Chang, C.-Y., Smith, P., Nebert, D.W., Setchell, K.D.R., Dalton, T.P., 1999. Inhibition of CYP1A1 enzyme activity in mouse hepatoma cell culture by soybean isoflavones. Chem. Biol. Interact. 123 (1), 31–49.

Stejskalova, L., Dvorak, Z., Pavek, P., 2011. Endogenous and exogenous ligands of the Aryl hydrocarbon receptor: current state of art. Curr. Drug Metab. 12 (2), 198–212.

Van der Heiden, E., Bechoux, N., Sergent, T., Schneider, Y.-J., Muller, M., Maghuin-Rogister, G., Scippo, M.-L., 2007. Aryl hydrocarbon receptor-mediated agonist/antagonist/synergic activities of food polyphenols are species- and tissue-dependent. Organohalogen Compd. 69, 365–368.

Vanden Heuvel, J.P., Clark, G.C., Kohn, M.C., Tritscher, A.M., Greenlee, W.E., Lucier, G.W., Bell, D.A., 1994. Dioxin-responsive genes: examination of dose–response relationships using quantitative reverse transcriptase–polymerase chain reaction. Cancer Res. 54, 62–68.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3 (7), 1–12.

Whitlock Jr., J.P., 1999. Induction of cytochrome P4501A1. Annu. Rev. Pharmacol. Toxicol. 39, 103–125.

Xu, L., Li, A.P., Kaminski, D.L., Ruh, M.F., 2000. 2,3,7,8-Tetrachlorodibenzo-p-dioxin induction of cytochrome P4501A in cultured rat and human hepatocytes. Chem. Biol. Interact. 124 (3), 173–189.

Zhang, S., Qin, C., Safe, S.H., 2003. Flavonoids as Aryl hydrocarbon receptor agonists/antagonists. Effects of structure and cell context. Environ. Health Perspect. 111, 1877–1882.