SPECIES-SPECIFIC RELATIVE AHR1 BINDING AFFINITIES OF 2,3,4,7,8-
PENTACHLORODIBENZOFURAN EXPLAIN AVIAN SPECIES DIFFERENCES IN ITS
RELATIVE POTENCY

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Running title: Binding affinity of DLCs to avian AHR1
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ABSTRACT

Results of recent studies showed that 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD) are equipotent in domestic chicken (\textit{Gallus gallus domesticus}) while PeCDF is more potent than TCDD in ring-necked pheasant (\textit{Phasianus colchicus}) and Japanese quail (\textit{Coturnix japonica}). To elucidate the mechanism(s) underlying these differences in relative potency of PeCDF among avian species, we tested the hypothesis that this is due to species-specific differential binding affinity of PeCDF to the aryl hydrocarbon receptor 1 (AHR1). Here, we modified a cell-based binding assay that allowed us to measure the binding affinity of dioxin-like compounds (DLCs) to avian AHR1 expressed in COS-7 (fibroblast-like cells). The results of the binding assay show that PeCDF and TCDD bind with equal affinity to chicken AHR1, but PeCDF binds with greater affinity than TCDD to pheasant (3-fold) and Japanese quail (5-fold) AHR1. The current report introduces a COS-7 whole-cell binding assay and provides a mechanistic explanation for differential relative potencies of PeCDF among species of birds.

Keywords: Aryl hydrocarbon receptor, cell-based binding assay, dioxin, COS-7 cells, bird, PeCDF, TCDD

1. Introduction

To aid environmental and human health risk assessments of complex mixtures of dioxins and dioxin-like compounds (DLCs), the World Health Organization (WHO)
established toxic equivalency factors (TEFs) based on the potency of several
polychlorinated dibenzo-\textit{p}-dioxin, polychlorinated dibenzofuran, and polychlorinated
biphenyl (PCB) congeners relative to that of TCDD. TEFs were assigned by an
international panel of scientific experts that considered all available data on the toxic and
biochemical potencies of DLCs published in peer-reviewed scientific journals (Van den
Berg et al., 1998). Separate sets of TEFs were established for mammals, fish, and birds.
These class-specific TEFs are used to calculate toxic equivalent (TEQ) concentrations of
mixtures of DLCs. The TEQ approach assumes that the TEF assigned to each DLC is
the same for all species within a vertebrate class. For example, the WHO-TEF for
2,3,4,7,8-pentachlorodibenzofuran (PeCDF) is 1.0 in birds, indicating that PeCDF and
TCDD are equipotent in birds.

Relative potency (ReP) values used to derive TEFs for birds were obtained from a
small number of \textit{in vivo} and \textit{in vitro} studies, and generally by use of data for only one
avian species, the domestic chicken (\textit{Gallus gallus domesticus}). However, both early
(Kennedy et al., 1996) and more recent studies indicate that the ReP values of some
DLCs vary among avian species (Farmahin et al., 2012; Farmahin et al., 2013a;
Farmahin et al., 2013b; Herve et al., 2010a; Herve et al., 2010b; Manning et al., 2012;
Manning et al., 2013; Zhang et al., 2013). For example, PeCDF and TCDD are
approximately equipotent activators of the aryl hydrocarbon receptor 1 (AHR1) in primary
cultures of domestic chicken hepatocytes (Herve et al., 2010a) and in COS-7 cells
transfected with chicken AHR1 (Farmahin et al., 2012; Farmahin et al., 2013b). In
contrast, PeCDF is a more potent AHR1 activator than TCDD in primary cultures of ring-
necked pheasant (\textit{Phasianus colchicus}) and Japanese quail (\textit{Coturnix japonica})
hepatocytes and in COS-7 cells transfected with pheasant or quail AHR1 (Farmahin et al., 2012; Farmahin et al., 2013b; Herve et al., 2010a). These in vitro findings are in general agreement with those from egg injection studies (Cohen-Barnhouse et al., 2011). Thus, RePs determined in chicken might not be representative of all avian species. In the present study we tested the hypothesis that the differential potency of PeCDF and TCDD among chicken, ring-necked pheasant, and Japanese quail is due to differences in their binding affinities to species-specific AHR1. These experiments required modification of a cell-based binding assay (Dold and Greenlee, 1990) such that it could be used with COS-7 cells transfected with avian AHR1. The modified method measures binding affinities of DLCs to AHR1 expressed in cells. COS-7 cells were used because they express very low levels of endogenous AHR (Ema et al., 1994; Jensen and Hahn, 2001). In addition, we compared the results of the cell-based assay to those obtained with a hydroxyapatite (HAP) binding assay. The results demonstrate important advantages of the cell-based assay and provide new information regarding differences in binding affinity of DLCs to AHR1 among avian species. These data enhance our understanding of the mechanism(s) underlying species differences in AHR activation following exposure to DLCs.

2. Materials and Methods

2.1 Cloning of AHR1 cDNA and preparation of expression constructs

The methods for cloning, sequence analysis, and construction of expression vectors for chicken, ring-necked pheasant, and Japanese quail AHR1 are described
elsewhere in detail (Farmahin et al., 2012). In brief, cDNA amplification kits (Clontech, Foster City, CA, USA) were used to obtain full-length pheasant and Japanese quail AHR1 cDNA (Farmahin et al., 2012) according to protocols similar to those used for chicken AHR1 cloning and full-length cDNA sequencing (Karchner et al., 2006). Full-length cDNAs were ligated into pENTRE/D-TOPO vector (Invitrogen, Burlington, ON, Canada) and subcloned into pcDNA 3.2/V5-DEST vector (Invitrogen).

2.2 Cell culture and transfection

COS-7 (African green monkey kidney fibroblast-like cells), provided by Dr. R. Haché (University of Ottawa, Ottawa, ON, Canada), were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Wisent, St. Bruno, QC, Canada), 1% MEM nonessential amino acids (Invitrogen), and 1% penicillin-streptomycin (Invitrogen; 10,000 unit/mL penicillin, 10,000 μg/mL streptomycin) at 37°C under 5% CO₂. Cells were seeded in 6-well plates at a concentration of 300,000 cells/well in dextran-coated charcoal-treated DMEM supplemented with 10% charcoal stripped FBS and 1% penicillin-streptomycin. Transfection was performed 18 h after plating. Avian AHR1 (chicken, ring-necked pheasant or Japanese quail; 250 ng quantities) and 750 ng salmon sperm DNA (Invitrogen) were transfected into each well. DNA and Fugene 6 transfection reagent (Roche, Laval, QC, Canada) were diluted in OPTI-MEM (Invitrogen). DNA was complexed with 4 μl of Fugene 6 transfection reagent (Roche) and this mixture (100 μl) was added to each well.
2.3 Chemicals

\([^3]H\)TCDD (2,3,7,8-tetrachloro[1,6-\(^3\)H]dibenzo-p-dioxin; specific activity 27.7 Ci/mmol, purified to 99% by high performance liquid chromatography) was purchased from American Radiolabeled Chemicals Inc. (ARC, St. Louis, MO, USA) and provided to us by the Dow Chemical Company. Details concerning the preparation of un-labeled TCDD, PeCDF, and TCDF solutions can be found elsewhere (Herve et al., 2010a). In brief, stock solutions of TCDD, PeCDF, and TCDF were prepared in dimethyl sulfoxide (DMSO) and concentrations were determined by isotope dilution following EPA method 1613 (U.S.EPA, 1994) by high-resolution gas chromatography high-resolution mass spectrometry. Serial dilutions of each chemical were prepared from their respective stocks in DMSO.

2.4 HAP binding assays

HAP assays were conducted according to methods described by Gasiewicz and Neal (Gasiewicz and Neal, 1982) and modified by Hahn and colleagues (Karchner et al., 2006) as follows: lysates of AHR1 proteins synthesized by \textit{in vitro} transcription/translation (IVTT) (Farmahin et al., 2012) were diluted in MEEDG buffer [25 mM MOPS, 1 mM EDTA, 5 mM EGTA, 0.02% NaN\(_3\), 10% vol/vol glycerol, 1 mM DTT, protease inhibitor cocktail tablet (PI tablet; Roche ; 1 tablet/ 25 ml buffer); pH 7.5]. DTT and PI tablets were added to the MEEDG buffer on the day of each experiment.
2.4.1 Saturation binding analysis

Diluted IVTT lysates were incubated with \( ^{3}\text{H}\text{]}\text{TCDD} \) at nominal concentrations ranging from 0.05 nM to 10 nM for 2 h and shaken gently at room temperature. A 5 µl aliquot from each incubation tube was used to confirm the concentration of \( ^{3}\text{H}\text{]}\text{TCDD} \).

After 2 h incubation, aliquots (200 µl) of 10% DNA grade HAP (Bio-Rad, Mississauga, ON, Canada) in MEEDMG (MEEDG buffer + 20 mM Na\(_{2}\)MoO\(_{4}\)) were added to glass incubation tubes. The tubes were placed on ice for 15 to 30 min and mixed vigorously every 5 min. The HAP suspension was transferred onto a 25 mm GF/F filter (Whatman, Florham Park, NJ, USA) in a sampling manifold (Millipore, Billerica, MA, USA). After application of a vacuum the filter was washed three times with 800 µl MEEDGT buffer (MEEDG buffer + 0.15% Tween-20). Filters were then transferred to scintillation vials containing 2.5 mL scintillation cocktail (Scintiverse II; Fisher Scientific, Don Mills, ON Canada); radioactivity was measured with a 1450 MicroBeta Trilux scintillation counter (PerkinElmer, Waltham, MA, USA).

2.4.2 Competitive binding analysis

Minor modifications were made to a HAP assay described elsewhere (Jensen et al., 2010; Karchner et al., 2006). In brief, 16.5 µl IVTT lysate diluted with 33.5 µl MEEDG buffer was incubated in glass tubes with unlabeled TCDD, PeCDF, or TCDF at concentrations ranging from 0.01 nM to 300 nM. The tubes were placed in a plate shaker at 220 rpm at room temperature for 15 minutes. \( ^{3}\text{H}\text{]}\text{TCDD} \) (1 nM nominal concentration) was added to the incubation tubes and the tubes were mixed at 220 rpm at room temperature for 105 minutes. The tubes were then transferred to ice and a 5 µl aliquot
was taken from each tube to determine the total concentration of \[^{3}H\]TCDD. The re-
suspended HAP (200 μl) was added to each tube and incubated on ice for 15 to 30 min.
Finally, HAP was washed and radioactivity was measured as described above.

2.5 COS-7 cell binding assays

A cell-based binding assay for measurement of AHR binding in mouse and human
cell lines (Dold and Greenlee, 1990) was modified for use with COS-7 cells expressing
avian AHR1s from transfected plasmids. Cells in 6-well plates that were transfected with
constructs encoding full-length chicken, pheasant, or Japanese quail AHR1 were
incubated for 24 h at 37°C in 5% CO\textsubscript{2} prior to conducting the binding assays.

2.5.1 Saturation binding analysis

Cells that were transfected with Japanese quail AHR1 were exposed to six
concentrations of \[^{3}H\]TCDD (0.1, 0.25, 0.8, 2.5, 8 and 14 nM) for 2 h at 37°C in a 5%
CO\textsubscript{2} atmosphere. A 10 μl aliquot was taken from each well to determine the
concentration of \[^{3}H\]TCDD. After incubation, the medium was aspirated and the cells
were washed with ice-cold PBS and ice-cold 10% fetal calf serum in PBS. The cells were
lifted by incubation with 700 μl trypsin-EDTA (0.05%; Invitrogen) for 5 min at 37°C. DME
medium (700 μl; Invitrogen) was then added to the wells to deactivate the trypsin. The
cell suspension was transferred onto 25 mm GF/F filters (Whatman) that were presoaked
with PBS in a sampling manifold (Millipore). The filters were washed twice with 2.5
ml/filter of acetone that had been pre-cooled to -80°C. The filters were dried by applying
a vacuum for 5 min and radioactivity was measured as described above.
2.5.2 Competitive binding analysis

COS-7 cells that were transfected with AHR1 constructs were incubated with graded concentrations of unlabeled TCDD, PeCDF, or TCDF for 15 minutes followed by addition of [\(^3\)H]TCDD (1 nM nominal concentration) for 105 minutes at 37°C in a 5% CO\(_2\) atmosphere. A 10 μl aliquot was taken from each well to determine the [\(^3\)H]TCDD concentration. The medium was then aspirated, and the cells were washed and lifted using trypsin. The cell suspensions were filtered and washed with acetone, and the radioactivity was measured as described above.

2.6 Binding curves

Specific binding of [\(^3\)H]TCDD is the difference between total and non-specific binding (NSB). NSB was determined by use of (a) unprogrammed lysate for the HAP binding assay (UPL; IVTT lysate that did not have AHR1 expression vector) or (b) a 200-fold excess of unlabeled TCDF for the COS-7 cell binding assay.

The specific binding data were fit to a one-site binding hyperbola curve with the following equation:

\[
Y = \frac{B_{\text{max}} \times X}{K_d + X}
\]

where \(B_{\text{max}}\) is maximum bound receptor, \(X\) is the concentration of free [\(^3\)H]TCDD, and \(K_d\) is the equilibrium dissociation constant. Nonlinear regression analysis was performed with GraphPad (GraphPad Prism 5.0 software, San Diego, CA, USA). To determine the IC\(_{50}\) values, the fractional specific binding (SB) of [\(^3\)H]TCDD was calculated with the following equation:

\[
\text{Fractional SB} = \frac{\text{SB}_A}{\text{SB}_{\text{max}}}
\]
where SBA is SB in the presence of a given concentration of compound A, and $SB_{\text{max}}$ is the SB of $[^{3}\text{H}]$TCDD in the absence of a competitor. The calculated fractional SB data were then analyzed by non-linear regression using a one-site competition equation:

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(X - \log IC_{50})}}$$

where Top is the fraction of $[^{3}\text{H}]$TCDD specific binding in the absence of a competitor. Bottom refers to the fraction of $[^{3}\text{H}]$TCDD binding observed when specific binding sites are occupied with UPL (in the HAP assay) or an unlabeled competitor (in cell-based assay). $X$ is the log of the concentration of the competitor in nM, and $Y$ is the fractional SB at each competitor concentration. Data were fit by unweighted non-linear regression with GraphPad. A one-way ANOVA followed by Tukey’s post-hoc test ($p < 0.05$) were performed to determine statistically significant differences in IC$_{50}$ values for TCDD, PeCDF and TCDF obtained from the HAP binding assay (GraphPad Prism 5.0).

### 2.7 Relative potency

The relative potency (ReP) of PeCDF (or TCDF) compared to TCDD for each AHR1 construct is defined as: IC$_{50}$ of TCDD ÷ IC$_{50}$ of PeCDF (or TCDF).

### 3. Results and Discussion

Specific binding of TCDD to IVTT-expressed chicken AHR1 was detected by the HAP assay. The $K_d$ and $B_{\text{max}}$ values for the binding of $[^{3}\text{H}]$TCDD with chicken AHR1 were 0.64 ±0.2 nM and 98 ±11 fmol, respectively. Specific binding of TCDD to Japanese quail AHR1 was below the detection limit of the HAP assay (Figure 1, panel A). Failure to
detect weak ligand-receptor interaction by use of the HAP assay was reported elsewhere for human AHR (Nakai and Bunce, 1995) and common tern AHR1 (Karchner et al., 2006). It has been suggested a detergent-washing step in the HAP assay disrupts weak interactions between ligand and AHRs of some species (Karchner et al., 2006). To overcome this limitation of the HAP assay, we modified a cell-based binding assay that was previously developed by Dold and Greenlee (1990). Important modifications included the use of COS-7 cells as the host cells and subsequent transfection of COS-7 cells with avian AHR1. In contrast to results obtained with the HAP assay, specific binding of TCDD to Japanese quail AHR1 expressed in COS-7 cells was detected; the mean $K_d$ value for the binding of $[^3]$H]TCDD to Japanese quail AHR1 was 2.1 nM (Figure 1, panel B). To compare the results obtained from COS-7 cell binding and HAP assays, competitive binding curves of TCDD, PeCDF, and TCDF to chicken AHR1 were obtained and IC$_{50}$ values were determined (Figure 2). The IC$_{50}$ values obtained from the HAP assay were 1.9, 2.1, and 2.1 nM, for TCDD, PeCDF and TCDF, respectively; there were no significant differences in IC$_{50}$ values for the three compounds (ANOVA followed by Tukey’s post-hoc test [$p < 0.05$]). IC$_{50}$ values obtained from the COS-7 cells binding assay were 1.7, 1.1, and 1.6 nM for TCDD, PeCDF, and TCDF, respectively. ReP values calculated from the results of the HAP assay and COS-7 cell binding assay for TCDD, PeCDF, and TCDF were approximately 1.0 (Figure 2 and Table 1).

In cells expressing pheasant and Japanese quail AHR1, the binding affinity of PeCDF was greater than that of TCDD; ReP values were 3 and 5 for pheasant and quail, respectively (Figure 3, panel A and B; Table 1). These results show the same trend observed with hepatocytes and the LRG assay; PeCDF and TCDD induce AHR1-
dependent genes with equal potency in chicken, while PeCDF is more potent than TCDD as an inducer of AHR1-dependent gene expression in pheasant and Japanese quail (Farmahin et al., 2012; Herve et al., 2010a). Although there was generally good agreement between RePs obtained from the binding assay and those measured in the LRG assay (Table 1), the RePs were not always identical. For example, the ReP value obtained from the cell-based binding assay in this study showed that for Japanese quail AHR1 the binding affinity of PeCDF is 5-fold stronger than that of TCDD (ReP=5), while previous data obtained from the LRG assay showed that PeCDF is 20-fold more potent than TCDD in inducing a CYP1A5-mediated reporter gene (ReP=20; Table 1). This is perhaps not too surprising, because the relationship between receptor occupancy and induction of EROD or CYP1A is not always linear (Hestermann et al., 2000).

Transfected cells have been used in previous studies to produce high-levels of AHR expression to conduct binding assays. In those studies, transfected cells were lysed and the cytosolic fraction was extracted to analyze AHR binding to the ligand through charcoal adsorption or HAP assay (Fan et al., 2009) or gel electrophoresis (Ramadoss and Perdew, 2004). In contrast to those studies, here we conducted whole-cell binding assays. The COS-7 whole-cell assay may be particularly useful for species that have low-affinity AHR1 forms (e.g., Japanese quail) because (1) washes with the cold organic solvent inhibit denaturation of proteins, so the ligand-binding complex remains intact during the washes and (2) the ligand-receptor complexes are protected by the cell membrane. The whole-cell assay modified in this study, similar to the HAP assay, is suitable for the analysis of a large number of samples. Therefore, the modified cell-based binding assay can be used as an alternative to the HAP assay. We chose to use COS-7
cells, which express no or very little AHR (Ema et al., 1994), because expression of avian AHR1 in host cells with endogenous AHR would provide heterologous binding sites for DLCs, thus interfering with the binding results.

It would be useful to perform further saturation binding studies to determine the $K_d$s for chicken and pheasant. While the results from such studies would allow comparison of quail AHR1 affinity for DLCs to that of chicken and pheasant AHR1 (i.e., to obtain relative sensitivity (ReS) values), such studies were beyond the scope of this research.

3.1 Conclusion

The results obtained from this study suggest that (1) the COS-7 whole-cell binding assay is useful for species that have low-affinity AHR1 and can be used as an alternative to the HAP binding assay, and (2) the differential potency of PeCDF and TCDD previously reported among chicken, ring-necked pheasant, and Japanese quail AHR1 that has been reported previously from egg injection studies, mRNA expression, and EROD and reporter gene expression studies is due to differences in the relative affinities with which these compounds bind to the AHR1 in each species.

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**Figure Legends**

**Figure 1.** (A) Saturation binding of $[^{3}\text{H}]$TCDD to chicken and quail AHR1 assessed with a
HAP binding assay. For both avian species, AHR1 was expressed by IVTT, incubated
with graded concentrations of $[^{3}\text{H}]$TCDD for 2h at room temperature, and analyzed by use
of the HAP assay (refer to Materials and Methods). Specific binding refers to the
difference between total binding and non-specific binding. The average data obtained
from four independent experiments were analyzed to generate one curve fit for chicken.
The specific binding of $[^{3}\text{H}]$TCDD by the quail AHR was undetectable. (B) Saturation
binding assessed with the COS-7 cell binding assay for quail AHR1. COS-7 cells
expressing quail AHR1 were incubated with $[^{3}\text{H}]$TCDD for 2h at 37°C and analyzed.
Specific binding (shown) was calculated as the difference between total binding and non-
specific binding.
**Figure 2.** Competitive binding curves of chicken AHR1 for TCDD, PeCDF, and TCDF.

(A) Competitive binding assessed with the HAP binding assay. Chicken AHR1 was expressed by IVTT, incubated with a single concentration of hot ligand ([3H]TCDD) in the presence of various concentrations of TCDD, PeCDF, or TCDF, incubated for 2h at room temperature, and analyzed according to the filtered HAP assay described in *Materials and Methods*. Each symbol represents the mean value of four replicates; bars indicate standard error.

(B) Competitive binding assessed with the COS-7 cell binding assay for chicken AHR1. Inhibition of binding of [3H]TCDD (single concentration) by various concentrations of TCDD, PeCDF, or TCDF in COS-7 cells expressing chicken AHR1 were determined as described in *Materials and Methods*. Curves were fit to a one-site competition model. Each symbol represents the mean value of two replicates.

**Figure 3.** Competitive binding curves of (A) pheasant and (B) quail AHR1 for TCDD and PeCDF. Inhibition of binding of [3H]TCDD (single concentration) by various concentrations of TCDD or PeCDF in COS-7 cells expressing pheasant or quail AHR1 were determined as described in *Materials and Methods*. Curves were fit to a one-site competition model. Each symbol represents the mean value of at least two replicates.
Figure 1.

A  HAP binding assay

B  COS-7 cell binding assay

specific binding (fmol)

0  2  4  12  14
[free TCDD] (nM)

specific binding (fmol)

0  2  4  10  12
[free TCDD] (nM)

Chicken AHR1
Quail AHR1

Quail AHR1
Figure 2.

A  HAP binding assay

B  COS-7 cell binding assay
Figure 3.

COS-7 cell binding assay

(A) Pheasant AHR1

(B) Quail AHR1
Table 1. Inhibitory concentration 50% (IC\textsubscript{50}) and relative potency (ReP) values determined for chicken, pheasant, and Japanese quail AHR1 using a cell-based assay. Dose-response curves for inhibition of [3H]TCDD binding were generated using data from two to six experiments. Statistical tests could not be performed on the IC\textsubscript{50} values because only one curve fit was generated and only one IC\textsubscript{50} value was derived for each dioxin-like chemical. ReP values were determined for chicken, pheasant, and Japanese quail AHR1 using a HAP binding assay, a cell-based binding assay and a LRG assay. The ReP of PeCDF (or TCDF) compared to TCDD for each AHR1 construct is defined as: IC\textsubscript{50} of TCDD ÷ IC\textsubscript{50} of PeCDF (or TCDF).

<table>
<thead>
<tr>
<th>AHR1 construct</th>
<th>Compound</th>
<th>Cell-based binding</th>
<th>Relative potency</th>
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<tr>
<td></td>
<td></td>
<td>IC\textsubscript{50} (nM)</td>
<td>HAP</td>
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<tr>
<td>Chicken</td>
<td>TCDD</td>
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<tr>
<td></td>
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\textsuperscript{a}Based on in vitro EC\textsubscript{50} values for the luciferase reporter gene assay from Farmahin et al. (2012)