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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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 (*Gallus gallus domesticus*) while PeCDF is more potent than TCDD in ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*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)

63 established toxic equivalency factors (TEFs) based on the potency of several
64 polychlorinated dibenzo-*p*-dioxin, polychlorinated dibenzofuran, and polychlorinated
65 biphenyl (PCB) congeners relative to that of TCDD. TEFs were assigned by an
66 international panel of scientific experts that considered all available data on the toxic and
67 biochemical potencies of DLCs published in peer-reviewed scientific journals (Van den
68 Berg et al., 1998). Separate sets of TEFs were established for mammals, fish, and birds.
69 These class-specific TEFs are used to calculate toxic equivalent (TEQ) concentrations of
70 mixtures of DLCs. The TEQ approach assumes that the TEF assigned to each DLC is
71 the same for all species within a vertebrate class. For example, the WHO-TEF for
72 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) is 1.0 in birds, indicating that PeCDF and
73 TCDD are equipotent in birds.
74 Relative potency (ReP) values used to derive TEFs for birds were obtained from a
75 small number of *in vivo* and *in vitro* studies, and generally by use of data for only one
76 avian species, the domestic chicken (*Gallus gallus domesticus*). However, both early
77 (Kennedy et al., 1996) and more recent studies indicate that the ReP values of some
78 DLCs vary among avian species (Farmahin et al., 2012; Farmahin et al., 2013a;
79 Farmahin et al., 2013b; Herve et al., 2010a; Herve et al., 2010b; Manning et al., 2012;
80 Manning et al., 2013; Zhang et al., 2013). For example, PeCDF and TCDD are
81 approximately equipotent activators of the aryl hydrocarbon receptor 1 (AHR1) in primary
82 cultures of domestic chicken hepatocytes (Herve et al., 2010a) and in COS-7 cells
83 transfected with chicken AHR1 (Farmahin et al., 2012; Farmahin et al., 2013b). In
84 contrast, PeCDF is a more potent AHR1 activator than TCDD in primary cultures of ring-
85 necked pheasant (*Phasianus colchicus*) and Japanese quail (*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% CO_2. 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 *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}H]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}H]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_2MoO_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}H]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_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_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.

199

200 *2.5.2 Competitive binding analysis*

201 COS-7 cells that were transfected with AHR1 constructs were incubated with
202 graded concentrations of unlabeled TCDD, PeCDF, or TCDF for 15 minutes followed by
203 addition of [^{3}H]TCDD (1 nM nominal concentration) for 105 minutes at 37°C in a 5% CO_2
204 atmosphere. A 10 µl aliquot was taken from each well to determine the [^{3}H]TCDD
205 concentration. The medium was then aspirated, and the cells were washed and lifted
206 using trypsin. The cell suspensions were filtered and washed with acetone, and the
207 radioactivity was measured as described above.

208

209 *2.6 Binding curves*

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

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

$$Y = \frac{B_{max} \times X}{K_d + X}$$

216

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

$$Fractional\,SB = SBA \div SB_{max}$$

where *SBA* is *SB* in the presence of a given concentration of compound A, and SB_{max} is the *SB* of [^{3}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:

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

where *Top* is the fraction of [^{3}H]TCDD specific binding in the absence of a competitor. *Bottom* refers to the fraction of [^{3}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_{max} values for the binding of [^{3}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_ds 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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References

Cohen-Barnhouse, A. M., Zwiernik, M. J., Link, J. E., Fitzgerald, S. D., Kennedy, S. W., Herve, J. C., Giesy, J. P., Wiseman, S., Yang, Y., Jones, P. D., Wan, Y., Collins, B., Newsted, J. L., Kay, D., and Bursian, S. J., 2011. Sensitivity of Japanese quail (*Coturnix japonica*), common pheasant (*Phasianus colchicus*) and white leghorn chicken (*Gallus gallus domesticus*) embryos to in ovo exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,4,7,8- pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF). Toxicol. Sci. 119, 93-103.

Dold, K. M. and Greenlee, W. F., 1990. Filtration assay for quantitation of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) specific binding to whole cells in culture. Anal. Biochem. 184, 67-73.

Ema, M., Matsushita, N., Sogawa, K., Ariyama, T., Inazawa, J., Nemoto, T., Ota, M., Oshimura, M., and Fujiikuriyama, Y., 1994. Human arylhydrocarbon receptor: Functional expression and chromosomal assignment to 7p21. J. Biochem. Tokyo. 116, 845-851.

Fan, M. Q., Bell, A. R., Bell, D. R., Clode, S., Fernandes, A., Foster, P. M., Fry, J. R., Jiang, T., Loizou, G., MacNicoll, A., Miller, B. G., Rose, M., Shaikh-Omar, O., Tran, L., and White, S., 2009. Recombinant expression of aryl hydrocarbon receptor for quantitative ligand-binding analysis. Anal. Biochem. 384, 279-287.

Farmahin, R., Crump, D., Jones, S. P., Mundy, L. J., and Kennedy, S. W., 2013a. Cytochrome P4501A induction in primary cultures of embryonic European starling hepatocytes exposed to TCDD, PeCDF and TCDF. Ecotoxicology 22, 731-739.

Farmahin, R., Manning, G. E., Crump, D., Wu, D., Mundy, L. J., Jones, S. P., Hahn, M. E., Karchner, S. I., Giesy, J. P., Bursian, S. J., Zwiernik, M. J., Fredricks, T. B., and Kennedy, S. W., 2013b. Amino acid sequence of the ligand-binding domain of the aryl hydrocarbon receptor 1 predicts sensitivity of wild birds to effects of dioxin-like compounds. Toxicol Sci. 131, 139-152.

Farmahin, R., Wu, D., Crump, D., Herve, J. C., Jones, S. P., Hahn, M. E., Karchner, S. I., Giesy, J. P., Bursian, S. J., Zwiernik, M. J., and Kennedy, S. W., 2012. Sequence

and in vitro function of chicken, ring-necked pheasant and Japanese quail AHR1 predict in vivo sensitivity to dioxins. Environ. Sci. Technol. 46, 2967-2975.
Gasiewicz, T. A. and Neal, R. A., 1982. The examination and quantitation of tissue cytosolic receptors for 2,3,7,8-tetrachlorodibenzo-p-dioxin using hydroxylapatite. Anal. Biochem. 124, 1-11.
Herve, J. C., Crump, D., Jones, S. P., Mundy, L. J., Giesy, J. P., Zwiernik, M. J., Bursian, S. J., Jones, P. D., Wiseman, S. B., Wan, Y., and Kennedy, S. W., 2010a. Cytochrome P4501A induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. Toxicol. Sci. 113, 380-391.
Herve, J. C., Crump, D. L., McLaren, K. K., Giesy, J. P., Zwiernik, M. J., Bursian, S. J., and Kennedy, S. W., 2010b. 2,3,4,7,8-pentachlorodibenzofuran is a more potent cytochrome P4501A inducer than 2,3,7,8-tetrachlorodibenzo-p-dioxin in herring gull hepatocyte cultures. Environ. Toxicol. Chem. 29, 2088-2095.
Hestermann, E. V., Stegeman J.J., and Hahn, M. E., 2000. Ralative contribution of affinity an intrinsic efficacy to aryl hydrocarbon receptor ligand potency. Toxicol. Appl. Pharmacol. 168, 160-172.
Jensen, B. A., and Hahn, M. E., 2001. cDNA cloning and characterization of a high affinity aryl hydrocarbon receptor in a cetacean, the beluga, Delphinapterus leucas. Toxicol. Sci. 64, 41-56.
Jensen, B. A., Reddy, C. M., Nelson, R. K., and Hahn, M. E., 2010. Developing tools for risk assessment in protected species: Relative potencies inferred from competitive binding of halogenated aromatic hydrocarbons to aryl hydrocarbon receptors from beluga (Delphinapterus leucas) and mouse. Aquat. Toxicol. 100, 238-245.
Karchner, S. I., Franks, D. G., Kennedy, S. W., and Hahn, M. E., 2006. The molecular basis for differential dioxin sensitivity in birds: role of the aryl hydrocarbon receptor. Proc. Natl. Acad. Sci. USA. 103, 6252-6257.
Kennedy, S. W., Lorenzen, A., Jones, S. P., Hahn, M. E., and Stegeman, J. J. 1996. Cytochrome P4501A induction in avian hepatocyte cultures: A promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. Toxicol. Appl. Pharmacol. 141, 214–230.
Manning, G. E., Farmahin, R., Crump, D., Jones, S. P., Klein, J., Konstantinov, A., Potter, D., and Kennedy, S. W., 2012. A luciferase reporter gene assay and aryl hydrocarbon receptor 1 genotype predict the LD(50) of polychlorinated biphenyls in avian species. Toxicol. Appl. Pharmacol. 263, 390-401.
Manning, G. E., Mundy, L. J., Crump, D., Jones, S. P., Chiu, S., Klein, J., Konstantinov, A., Potter, D., and Kennedy, S. W., 2013. Cytochrome P4501A induction in avian hepatocyte cultures exposed to polychlorinated biphenyls: Comparisons with AHR1-mediated reporter gene activity and in ovo toxicity. Toxicol. Appl. Pharmacol. 266, 38-47.
Nakai, J. S. and Bunce, N. J., 1995. Characterization of the Ah receptor from human placental tissue. J. Biochem. Toxicol. 10, 151-159.
Ramadoss, P. and Perdew, G. H., 2004. Use of 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin as a probe to determine the relative ligand affinity of

human versus mouse aryl hydrocarbon receptor in cultured cells. Mol. Pharmacol. 66, 129-136.
U.S.EPA, 1994. Method 1613: tetra- through octa-chlorinated dioxins and furans by isotope dilution HRGC/HRMS. EPA/821/B-94/005.
Van den Berg, M., Birnbaum, L., Bosveld, A. T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X., Liem, A. K., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski, T., 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ. Health Perspect. 106, 775-792.
Zhang, R., Manning, G. E., Farmahin, R., Crump, D., Zhang, X., and Kennedy, S. W., 2013. Relative potencies of aroclor mixtures derived from avian in vitro bioassays: comparisons with calculated toxic equivalents. Environ Sci. Technol. 47, 8852-8861.

Figure Legends

Figure 1. *(A)* Saturation binding of [^{3}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}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}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 [3H]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. *C*ompetitive binding curves of chicken AHR1 for TCDD, PeCDF, and TCDF. *(A) C*ompetitive binding assessed with the HAP binding assay. Chicken AHR1 was expressed by IVTT, incubated with a single concentration of hot ligand ([^{3}H]TCDD) in the presence of various concentrations of TCDD, PeCDF, or TCDD, 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 [^{3}H]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. *C*ompetitive binding curves of *(A)* pheasant and *(B)* quail AHR1 for TCDD and PeCDF. Inhibition of binding of [^{3}H]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.

443 **Figure 1.**

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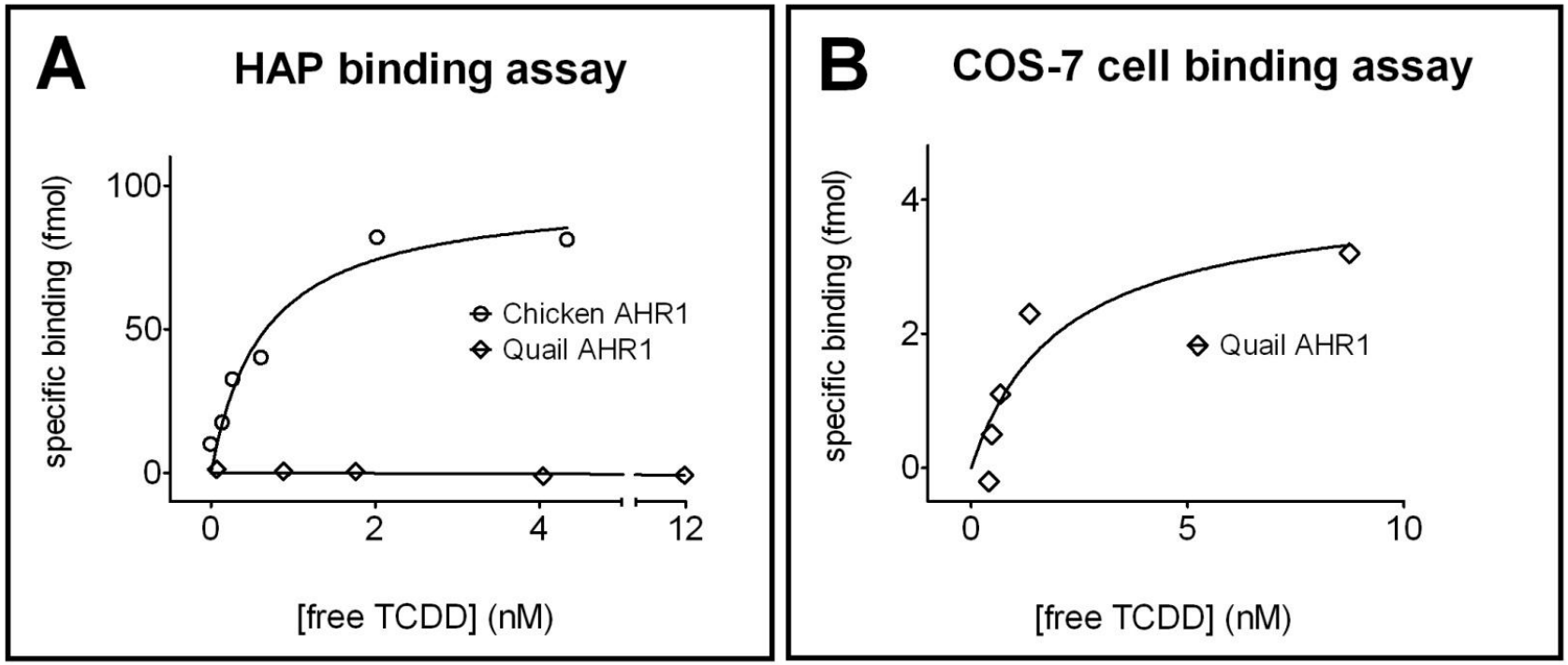

447 **Figure 2.**

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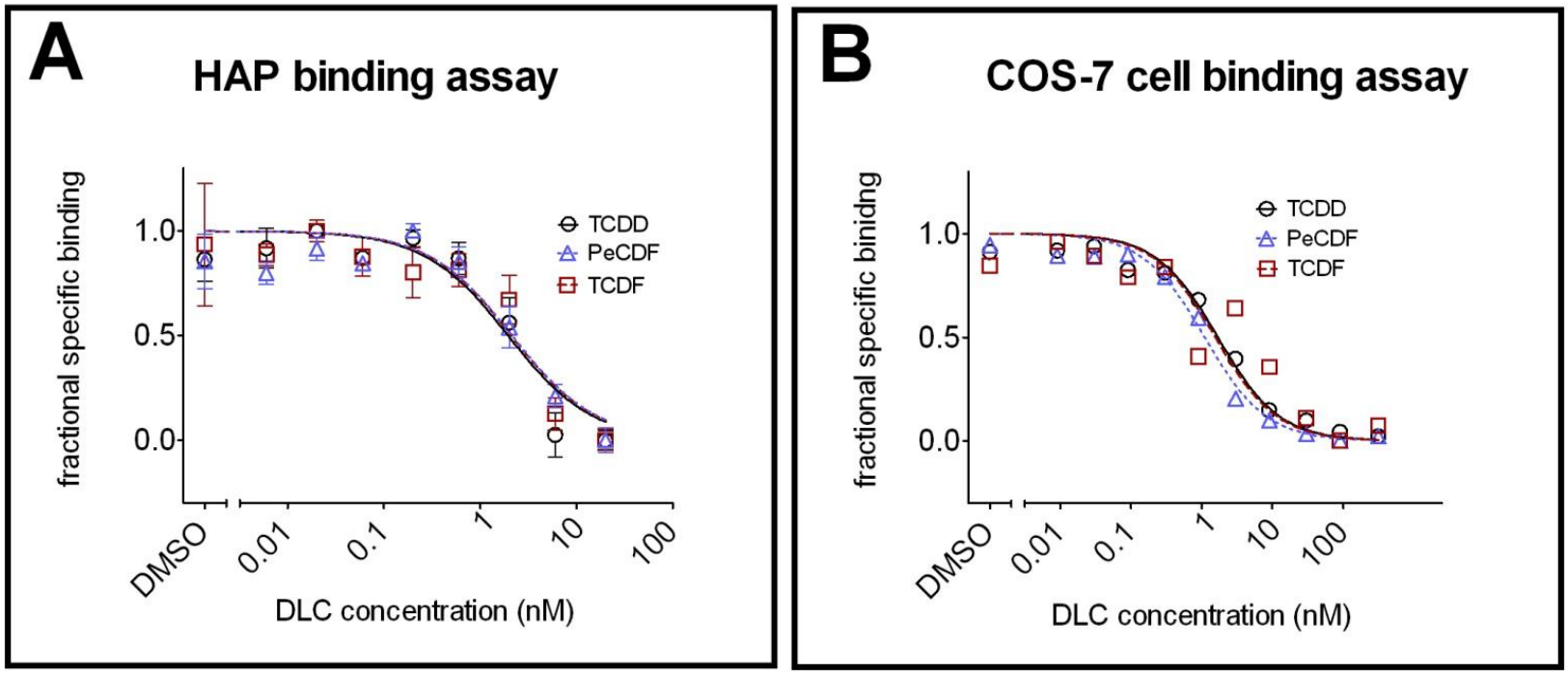

Figure 3.

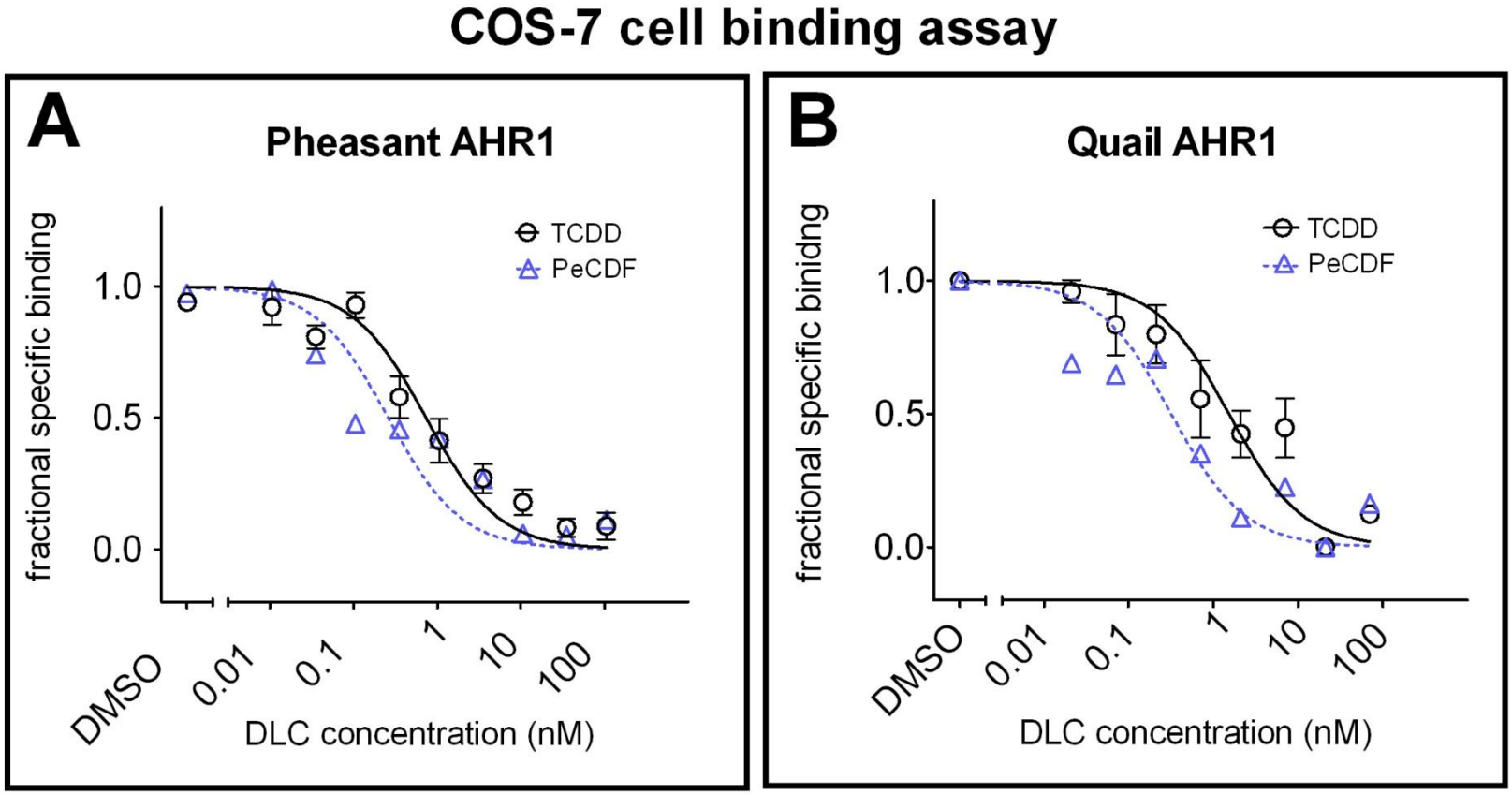
COS-7 cell binding assay
A
Pheasant AHR1
fractional specific binding
TCDD
PeCDF
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
DLC concentration (nM)
B
Quail AHR1
fractional specific binidng
TCDD
PeCDF
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
DLC concentration (nM)

Table 1. Inhibitory concentration 50% (IC_{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_{50} values because only one curve fit was generated and only one IC_{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_{50} of TCDD ÷ IC_{50} of PeCDF (or TCDF).

		Cell-based binding	Relative potency		
AHR1 construct	Compound	IC_{50} (nM)	HAP	Cell-based	LRG[a]
Chicken	TCDD	1.7 (1.1 - 2.7)	1	1	1
	PeCDF	1.1 (0.80 - 1.5)	1	1	1
	TCDF	1.6 (0.78 – 3.1)	1	1	0.4
Pheasant	TCDD	0.74 (0.53 – 1.0)	NA	1	1
	PeCDF	0.26 (0.16 -0.42)	NA	3	4
Quail	TCDD	1.5 (0.87 – 2.6)	NA	1	1
	PeCDF	0.32 (0.13 – 0.78)	NA	5	20

[a]Based on *in vitro* EC_{50} values for the luciferase reporter gene assay from Farmahin et al. (2012)