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COMPETITIVE BINDING OF 7-SUBSTITUTED-2,3-DICHLORODIBENZO-*p*-DIOXINS WITH HUMAN PLACENTAL Ah RECEPTOR—A QSAR ANALYSIS

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Abstract—The competitive binding affinities of thirteen 7-substituted-2,3-dichlorodibenzo-*p*-dioxins to the human placental cytosolic aryl hydrocarbon (Ah) receptor were determined using [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin as the radioligand. Multiple parameter linear regression analysis of the competitive binding EC_{50} values for these compounds gave the following equation:

$$pEC_{50}\,(M) = 6.246 + 1.632\,\pi - 1.764\,\sigma_m^0 + 1.282\,HB$$

where π, σ_m^0 and HB are the physiochemical parameters for substituent lipophilicity, meta-directing electronegativity, and hydrogen bonding capacity respectively. The 7-*t*-butyl- and 7-phenyl-2,3-dichlorodibenzo-*p*-dioxins were treated as outliers for the derivation of this equation, and these results suggest that only substituents with van der Waals' volumes $< 40\ cm^3/mol$ were accommodated in the receptor binding site. The equations previously derived from the binding of the 7-substituted-2,3-dichlorodibenzo-*p*-dioxins to the rat, mouse, guinea pig and hamster hepatic cytosolic receptor were different than the correlation obtained using human placental receptor and provide further evidence for the interspecies differences in the molecular and binding properties of the Ah receptor protein.

The aryl hydrocarbon (Ah) receptor is an intracellular receptor protein which has been identified in diverse animal species and mammalian cells in culture [1]. It was first reported by Poland *et al.* [2] that [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin ([^{3}H]TCDD) exibits saturable binding with a high-affinity low-capacity cytosolic protein from C57BL/6J mouse liver. Subsequent research in several laboratories has demonstrated that the interaction of TCDD and related toxic halogenated aryl hydrocarbons with the Ah receptor plays an essential role in the mechanism of action of these chemicals (reviewed in Refs 3–6). However, an endogenous ligand for the Ah receptor has not been identified yet.

The molecular properties of the Ah receptor complexes have been investigated extensively, using [^{3}H]TCDD and other radiolabeled ligands as probes. Initial studies have reported K_D values for the dissociation of Ah receptor complexes in the nanomolar range [2,7–10], but recent studies using ^{125}I-labeled ligands and a kinetic approach have shown that the K_D values are 10^{-11} to 10^{-12} M [11, 12]. The molecular properties of the nuclear and cytosolic Ah receptor complexes are also dependent on the animal species or cellular source of the receptor preparations and on the techniques used to characterize these properties [13–20]. Prokipcak and Okey [20] reported that the apparent molecular masses (M_r) of the cytosolic and nuclear Ah receptor complexes from mouse Hepa-1 cells were 271,000 and 176,000 respectively. Denaturing sodium dodecyl sulfate gel electrophoresis of photoaffinity-labeled cytosolic receptor from several species showed that the molecular weights varied from 95 to 113 kDa [21, 22]. It has also been reported that a 90 kDa heat shock protein is associated with the cytosolic Ah receptor complex [23]; however, the composition and overall molecular structure of the nuclear and cytosolic Ah receptor complexes have not been delineated.

The structure–binding relationships for polychlorinated dibenzo-*p*-dioxin (PCDD), dibenzofuran (PCDF) and biphenyl (PCB) congeners have been investigated using competitive binding assays with [^{3}H]TCDD as the radioligand (reviewed in Refs 4 and 5). The results showed that the most active compounds within each class are substituted in their lateral positions and are approximate isostereomers of TCDD. Safe, Fujita and coworkers have utilized a series of chlorinated dibenzofuran, biphenyl and dibenzo-*p*-dioxins that contained variable substituents at a lateral position to investigate the physiochemical parameters which facilitate the unusually strong Ah receptor–ligand interactions [24–28]. Multiple parameter linear regression analysis of the binding data obtained using 7-substituted-2,3-dichloro- and 2-substituted-3,7,8-trichlorodibenzo-*p*-dioxin analogs and cytosolic receptor preparations from rat, mouse, guinea pig and hamster liver gave different equations for the correlation between binding affinities with the physicochemical characteristics of the

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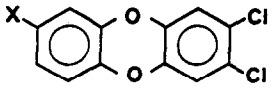

Fig. 1. Structure of 7-substituted-2,3-dichlorodibenzo-*p*-dioxin.

substituents [23, 27]. This study utilized the 7-substituted-2,3-dichlorodibenzo-*p*-dioxin series of congeners (Fig. 1) and [^{3}H]TCDD to investigate the effects of ligand structure on their interactions with a human placental cytosolic Ah receptor preparation [16].

MATERIALS AND METHODS

Chemicals and biochemicals. The synthesis and properties of 7-substituted-2,3-dichlorodibenzo-*p*-dioxins have been described previously [25]. The compounds used in this study were all > 97% pure and includled the 7-methyl, hydroxy, iodo, *t*-butyl, chloro, nitro, phenyl, cyano, trifluoromethyl, bromo, 7,8-$(CH)_4$, hydrogen, and acetyl-2,3-dichlorodibenzo-*p*-dioxins. [^{3}H]TCDD (37 Ci/mmol), unlabeled TCDD and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were synthesized previously in this laboratory as described [8, 24].

Human placental cytosol preparations. Placental cytosol for competition studies was prepared from full-term placentas as previously described [16]. The cytosol used in competition assays was pooled from placental tissue from two individual donors; both were light smokers (less than 20 cigarettes per day). The buffer used was HEDGM [20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)/1.5 mM EDTA/1 mM dithiothreitol/10% glycerol (v/v)/20 mM sodium molybdate, pH 7.6). The final protein concentration in the pooled cytosol was 5 mg/mL.

Receptor assay and competition studies. The concentration of Ah receptor in placental cytosol was determined by sucrose density gradient analysis as previously described [16]. Briefly, cytosol was incubated with 15 nM [^{3}H]TCDD ± competitor for 2 h at 4°. Samples were treated with 0.1 mg dextran-coated charcoal/mg cytosol protein before separation on sucrose gradients. This concentration of charcoal reduces the background radioactivity in the gradient without excessively "stripping" specifically-bound [^{3}H]TCDD from the Ah receptor [16]. The concentration of specific [^{3}H]TCDD binding sites detected in the pooled cytosol with this method was 99 ± 7 fmol/mg cytosolic protein in the absence of competitors. The coefficient of variation in repeated assays on this pool of cytosol was 7% (N = 40).

The ability of the substituted dibenzo-*p*-doxins to compete with 15 nM [^{3}H]TCDD for binding to Ah receptor sites was tested by adding varied concentrations of the competitors to cytosol at the same time that [^{3}H]TCDD was added. Competitors were tested at concentrations ranging from 7.5×10^{-9} M to 1.5×10^{-5} M (0.5 to 1000 times the concentration of [^{3}H]TCDD). Specific binding of [^{3}H]TCDD to the Ah receptor was determined by sucrose density gradient analysis for each sample. Each compound was tested for competition at a minimum of sixteen concentrations. The EC_{50} (concentration required to reduce specific binding of [^{3}H]TCDD by 50%) for each competitor was determined by interpolation from a plot of specific binding versus $\log_{10}$ of the competitor concentration.

Data analysis. Multiple parameter linear regression analysis was determined with the FACOM 382 computer at the Data Processing Center, Kyoto University, Kyoto, Japan, using the known or calculated substituent physical parameters shown in Table 1 as described [24–28].

RESULTS

The pEC_{50} ($-\log EC_{50}$) values for the competitive displacement of [^{3}H]TCDD from human placental Ah receptor preparations by thirteen 7-substituted-2,3-dichlorodibenzo-*p*-dioxins are summarized in Table 1. The most active compound in this series was the naphthyl analog in which the substituent was a fused aromatic ring [i.e. $(CH)_4$] on the lateral positions. This compound was also more active as a competitive ligand than unlabeled TCDF, 3-methylcholanthrene, and dibenzo[*a*,*h*]anthracene which gave EC_{50} values of 60, 15 and 25 nM respectively (unpublished results). Multiple parameter linear regression analysis of the binding data using a series of known or calculated substituent parameters gave Eqn 1 which showed that the relative competitive binding affinities of the 7-substituted-2,3-dichlorodibenzo-*p*-dioxins were dependent on substituent lipophilicity (π), the meta-directing electronegativity values (σ_m^0) and hydrogen bonding (HB) capacity

$$pEC_{50}\,(M) = 6.246 + 1.632\,\pi - 1.764\,\sigma_m^0 + 1.282\,HB \quad (1)$$

$$(\pm 0.878)\ (\pm 1.099)\ (\pm 1.800)\ (\pm 1.436)$$

where N = 11, SD = 0.468, r = 0.838

(N = the number of binding values used for determining the equation, s is the standard deviation, and r is the correlation coefficient). The calculated pEC_{50} values for the 7-substituted-2,3-dichlorodibenzo-*p*-dioxins using this equation are also summarized in Table 1. Both the bulky *t*-butyl and phenyl substituents with van der Waals' volumes of 41.8 and 45.8 cm^3/mol, respectively, were treated as outliers for this calculation. Comparable studies using hepatic cytosols from other animal species also showed that the bulky *t*-butyl and phenyl substituents were outliers, and this was consistent with a maximum molecular volume or size requirement for the lateral substituents [24–27].

DISCUSSION

Based on the structure–binding relationships observed for several classes of halogenated aryl hydrocarbons, Poland and Knutson [4] suggested that the most avid ligands would fit into a 3×10 Å rectangle with halogen atoms substituted in the four corners. These dimensions do not account for the relatively higher Ah receptor binding affinities of other ligands which include polynuclear aromatic

Table 1. Competitive binding EC_{50} values for the 7-substituted-2,3-dichlorodibenzo-*p*-dioxins to human placental cytosolic receptor and a summary of substituent physicochemical parameters π, HB and σ_m^0

Substituent	EC_{50} (nM) (Observed)	pEC_{50} (M) (Observed)	pEC_{50} (M) (Calculated)	π	σ_m^0	HB
Me	45	7.35	7.14	0.48	−0.06	0
OH	350	6.46	6.79	−0.43	0.02	1
I	50	7.30	7.63	1.22	0.34	0
t-Bu*	450	6.35	9.34	1.80	−0.09	0
Cl	450	6.35	6.95	0.83	0.37	0
No_2	1000	6.00	6.46	0.11	0.71	1
Ph*	20	7.70	9.21	1.86	0.04	0
CN	230	6.64	6.14	−0.18	0.62	1
CF_3	15	7.82	7.23	1.10	0.46	0
Br	70	7.16	7.19	0.98	0.37	0
$(CH)_4$	5	8.30	8.19	1.28	0.08	0
H	500	6.30	6.25	0	0	0
CO_2Me	35	7.46	7.17	0.16	0.350	1

* These two compounds are treated as outliers for the derivation of Eqn 1.

hydrocarbons, β-naphthoflavone and heteropolynuclear aromatic hydrocarbons [9, 29–32]. Gillner *et al.* [30] suggested that a binding site with dimensions of 6.8 × 13.7 Å was more consistent with the data obtained for the different classes of chemicals which exhibit affinity for the Ah receptor.

Several approaches have been utilized to investigate the nature of the unusually strong molecular interactions between TCDD and related compounds and the Ah receptor binding site. For example, Murray *et al.* [33] have calculated electrostatic potential maps for TCDD and related congeners with different potencies and suggested that negative lateral regions play an essential role in the interaction of TCDD with the Ah receptor. It has also been proposed that the molecular polarizability of individual halogenated aromatic hydrocarbons is the critical physiochemical parameter which governs receptor–ligand interactions [34, 35].

This laboratory has utilized another approach for investigating ligand–receptor binding site interactions. The relative binding affinities of 2-substituted-3,7,8-trichlorodibenzo-*p*-dioxins, 7-substituted-2,3-dichlorodibenzo-*p*-dioxins, 8-substituted-2,3-dichlorodibenzofurans, 8-substituted-2,3,4-trichlorodibenzofurans, and 4′-substituted-2,3,4,5-tetrachlorobiphenyls have been determined using rat hepatic cytosol [24–28]. These compounds all contain a single lateral substituent, and ten or more analogs were synthesized for each of the five series of compounds. Multiple parameter linear regression analysis of the binding data was determined, and the resulting equations correlate binding data for individual compounds with the physicochemical characteristics of the substituents. The results of these studies showed that for over fifty individual compounds, substituent lipophilicity (π) was an important physicochemical property associated with Ah receptor–ligand interactions. Analysis of the competitive binding data (Table 1) for the human placental Ah receptor preparations confirmed that for the 7-substituted-2,3-dichlorodibenzo-*p*-dioxins, substituent π values were also important parameters for ligand–receptor interactions. Two additional terms, namely substituent hydrogen bonding (HB) capacity and meta-directing electronegativity (σ_m^0) values, were also required for development of Eqn 1 for the human placental receptor preparations.

Previous studies using the substituted chlorinated analogs have also shown that several other substituent characteristics were significant factors in correlating binding data with substituent physicochemical parameters. For example, Eqns 2–5 summarize the correlation between receptor binding EC_{50} values for the 7-substituted-2,3-dichlorodibenzo-*p*-dioxins and substituent physicochemical parameters for rat, mouse, guinea pig and hamster liver cytosolic Ah receptor [28].

$$pEC_{50} = 6.10 + 1.24\,\pi \quad \text{(rat)} \tag{2}$$

$$pEC_{50} = 5.28 + 0.95\,\pi + 0.93\,E_s \quad \text{(mouse)} \tag{3}$$

$$pEC_{50} = 6.13 + 0.94\,\pi + 5.79\,\sigma_p \quad \text{(guinea pig)} \tag{4}$$

$$pEC_{50} = 6.38 + 0.70\,\pi \pm 1.23\,\sigma_p \quad \text{(hamster)} \tag{5}$$

The results showed that at least two additional substituent parameters, namely their Taft constants (E_s, a steric property) and electronegativity (σ_p), were also important for the development of the correlations. It was apparent that Eqns 2–5 were significantly different from one another and from the equation derived from the human placental receptor binding data using the same series of substituted ligands. The development of Eqn 1 from the observed binding data (Table 1) required that both the phenyl- and *t*-butyl-substituted analogs be treated as outliers. Inspection of the results in Table 1 shows that, with the exception of these two compounds, there was a good correlation between the observed and calculated pEC_{50} values (i.e. < 0.60 log units) for eleven of the 7-substituted-2,3-dichlorodibenzo-*p*-dioxins. In contrast, the differences between the observed and calculated pEC_{50} values for the phenyl

and *t*-butyl analogs were 1.51 and 2.99 log units respectively. In previous studies with the 7-substituted-2,3-dichlorodibenzo-*p*-dioxins and hepatic cytosols from several species, the bulky *t*-butyl- and phenyl-substituted analogs were also treated as outliers for deriving Eqns 2–5. These results suggest that there are common steric or size requirements (i.e. molecular volumes $< 40\ cm^3/mol$) for the substituents which are independent of the source of the cytosol.

In summary, these studies confirmed that for a series of 7-substituted-2,3-dichlorodibenzo-*p*-dioxins the equation derived by multiple parameter regression analysis of the binding data from human placental receptor preparations was different from the corresponding correlations obtained using rat, guinea pig, mouse and hamster hepatic cytosol. The differences in the properties of human and rodent placental receptor were also apparent from previous studies by Lucier *et al.* [36] who showed that for several samples the Ah receptor was essentially nondetectable in human placenta using rodent assay conditions for the binding studies. These results, and previous studies using 2-substituted-3,7,8-trichlorodibenzo-*p*-dioxin analogs [25, 28] and cytosol from the same species, suggest that there are significant interspecies differences in their respective binding sites and their interaction with ligands. These data complement recent photoaffinity labeling studies which reported that the M_r values for the denatured photolabeled receptor from several species varied from 95 to 113 kD [21, 22]. The biological significance of these interspecies differences in the molecular properties of the Ah receptor has not been determined and is being investigated currently in our laboratories.

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