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Differential suppression of the aryl hydrocarbon receptor nuclear translocator-dependent function by an aryl hydrocarbon receptor PAS-A-derived inhibitory molecule

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Abstract

The aryl hydrocarbon receptor (AhR) heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt) for transcriptional regulation. We generated three N-terminal deletion constructs of the human AhR of 12–24 KDa in size – namely D1, D2, and D3 – to suppress the Arnt function. We observed that all three deletions interact with the human Arnt with similar affinities. D2, which contains part of the AhR PAS-A domain and interacts with the PAS-A domain of Arnt, inhibits the formation of the AhR gel shift complex. D2 suppresses the 3-methylcholanthrene-induced, dioxin response element (DRE)-driven luciferase activity in Hep3B cells and exogenous Arnt reverses this D2 suppression. D2 suppresses the induction of CYP1A1 at both the message and protein levels in Hep3B cells; however, the CYP1B1 induction is not affected. D2 suppresses the recruitment of Arnt to the *cyp1a1* promoter but not to the *cyp1b1* promoter, partly because the AhR/Arnt heterodimer binds better to the *cyp1b1* DRE than to the *cyp1a1* DRE. Interestingly, D2 has no effect on the cobalt chloride-induced, hypoxia inducible factor-1 (HIF-1)-dependent expression of *vegf*, *aldolase c*, and *ldh-a* messages. Our data reveal that the flanking sequences of the DRE contribute to the binding affinity of the AhR/Arnt heterodimer to its endogenous enhancers and the function of AhR and HIF-1 can be differentially suppressed by the D2 inhibitory molecule.

Keywords

AhR; Arnt; DRE binding; HIF-1; AhR deletion constructs

1. Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated signaling molecule which upregulates transcription of cytochrome P450 genes – *cyp1a1*, *1a2*, and *1b1* [1, 2]. Other than this classical response to xenobiotic exposure, this receptor also involves in diverse

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endogenous events such as cell proliferation [3], T cell differentiation [4, 5], hematopoietic stem cell expansion/differentiation [6], lung response to polycyclic aromatic hydrocarbons [7, 8], and glucose tolerance [9] via mechanisms which, for most part, are not clearly defined. For its canonical signaling pathway, the unliganded AhR exists as a cytoplasmic complex which consists of a molecule of AhR, XAP2 (aka ARA9 and AIP), and p23 as well as a dimer of Hsp90 [10–14]. Upon ligand binding, AhR reveals its nuclear translocation signal, leading to its entry into the nucleus via interaction with the importin nuclear core protein [14, 15]. It is likely that the liganded AhR complex translocates into the nucleus; binding of Arnt to AhR causes dissociation of the complex [16]. The AhR/Arnt heterodimer in turn binds to the DRE of the *cyp1a1* promoter, for example, and recruits coactivators which remodel the local chromatin structure, allowing activation of the *cyp1a1* gene transcription to occur [17, 18].

Induction of the CYP1A1 enzyme via AhR is the prototype of the receptor-mediated CYP enzyme induction. CYP1A1 and 1B1 are cytochrome P450 enzymes responsible for xenobiotic metabolism in the human lung [19, 20]. These enzymes convert BaP to epoxides and eventually lead to the formation of diol epoxide – the potent DNA modifier which contributes to the development of lung cancer [21]. Interestingly, the extent of CYP1A1 and 1B1 induction varies noticeably: upon 10 pM TCDD treatment, the message levels of *cyp1a1* can be upregulated to nearly 100-fold in bovine primary cultured hepatocytes whereas the *cyp1b1* message levels can be unchanged [22]. YH439, a non-aromatic thiazole-containing AhR ligand [23], appears to recruit AhR to the *cyp1b1* promoter more effectively than to the *cyp1a1* promoter in Hepa-1c1c7 cells [24]. In addition, human breast cells express higher amounts of CYP1B1 in tumor cell lines when compared with the non-tumor cell lines; the expression of CYP1B1 is responsible for the generation of the carcinogenic 4-hydroxyestradiol [25]. Many researchers have subsequently observed selective CYP induction in breast cancer cell lines. For example, CYP1A1 can be preferentially induced by quercetin over CYP1B1 in MCF10F cells [26]. Tranilast and mexilentine have been shown to selectively induce CYP1B1 but not CYP1A1 protein expression in MDA-MB-468 human breast cancer cell line [27]. It was observed that dioxins recruit AhR more effectively to the *cyp1b1* promoter than to the *cyp1a1* promoter in T47D human breast cancer cell line [28]; however, the precise mechanism is largely unknown.

CA553 (aa 1–295) is a deletion construct of the human AhR [29] and does not contain part of the PAS region which binds ligand and Hsp90 [30]. We have used CA553 to block the Arnt function: CA553 is effective in interfering with the HIF-1 α and Arnt interaction, leading to suppression of the HIF-1 function and xenograft growth [31]. CA553 should retain most of the physical contacts between AhR and Arnt; thus it is not surprising that CA553 blocks all other Arnt-dependent functions which have been tested thus far in our laboratory – these functions include the signaling of AhR [29], ER α [29] and NF κ B (unpublished data). In addition, we examined whether proteins smaller than CA553 would suppress Arnt function. We generated two smaller Arnt-interacting proteins from the human liver cDNA library, namely Ainp1 and Ainp2, which are 59 and 58 amino acids in length, respectively [32]. Ainp1 binds to the HLH region of Arnt [33] whereas Ainp2 binds to the Arnt PAS-A domain (unpublished data). Interestingly, Ainp1 blocks all Arnt functions similarly as CA553 ([34], unpublished data); however, Ainp2 suppresses the ER-dependent activation of gene transcription, promotes the AhR-dependent gene activation, and does not affect the HIF-1 function ([32, 35] and unpublished data). Collectively, our data suggest that a small Arnt-interacting protein may selectively block an Arnt-mediated signaling. In this study, we tested deletions of CA553 to address whether selective blockade of an Arnt function is feasible. We provide evidence that a 12 KDa derivative pertaining to the PAS-A domain of AhR, which we called D2, preferentially suppresses the transcription of the *cyp1a1* gene over the *cyp1b1* gene by suppressing the assembly of the AhR/Arnt/DRE

complex at the *cyp1a1* promoter. Additionally, this D2 molecule preferentially suppresses the signaling of AhR over HIF-1 α .

2. Material and methods

2.1. Reagents

Human hepatoma Hep3B cells were grown at 37 °C and 5% CO₂ in Advanced MEM (Gibco, Carlsbad, CA) supplemented with 5% HyClone FBS, 2 mM GlutaMAX-I, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin. Cell culture reagents, if not specified, were purchased from Invitrogen (Carlsbad, CA). All chemicals were purchased from Fisher Scientific (Fair Lawn, New Jersey) unless specified otherwise. Guanidine hydrochloride was purchased from USB (Cleveland, Ohio) and arginine was purchased from Sigma (St. Louis, MO). All restriction enzymes were purchased from (New England BioLabs, Ipswich, MA). CoCl₂ was used to chemically mimic hypoxia in Hep3B cells. 3MC, BaP and β NF were used as AhR ligands whereas α NF was used as an AhR antagonist; these chemicals were purchased from Sigma (St. Louis, MO). X-tremeGENE 9 transfection reagent was purchased from Roche Applied Sciences (Indianapolis, IN). The iQ SYBR green supermix was purchased from Bio-Rad (Hercules, CA). His-probe mouse monoclonal IgG H-3 (sc-8039), anti-Arnt rabbit polyclonal IgG H-172 (sc-5580), goat polyclonal IgG N-19 (sc-8077), anti-CYP1A1 mouse monoclonal IgG B4 (sc-25304), anti-CYP1B1 mouse monoclonal IgG G4 (sc-374228), anti-AhR goat polyclonal IgG N-19 (sc-8088), rabbit IgG and mouse IgG were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-Thio mouse monoclonal IgG and anti-c-Myc mouse monoclonal IgG were purchased from Invitrogen (Carlsbad, CA). Anti-AhR rabbit polyclonal IgG SA210 was purchased from Enzo Life Sciences (Farmingdale, NY). IRdye 680 donkey anti-goat IgG, donkey anti-mouse IgG and IRdye 800 donkey anti-rabbit IgG were purchased for LI-COR (Lincoln, NE). Gel shift probes were purchased from Integrated DNA Technologies (San Diego, CA) as follows: IRdye conjugated HRE, OL487 and OL488; IRdye conjugated DRE, OL439 and OL440 (Table I). All other oligonucleotides were purchased from Invitrogen (Carlsbad, CA). The DRE-driven luciferase reporter plasmid pGudLuc1.1 was generous gift from Dr. Mike Denison (University of California at Davis). The pSport and pSport-Arnt plasmids were generous gifts from Dr. Chris Bradfield (University of Wisconsin, Madison). The HRE-driven luciferase reporter plasmid pGL3-Epo was generated by using PCR to amplify the hypoxia enhancer region [29]. The β -galactosidase plasmid pCH110 was purchased from Amersham Pharmacia (Piscataway, NJ). All other plasmids were generated by amplifying the corresponding cDNAs with the specific primers: D1–3 cDNAs were cloned into Xho I and Xba I sites of the pThioHis A plasmid (Invitrogen, Carlsbad, CA) (D1, OL443 and OL444; D2, OL443 and OL445; D3, OL446 and OL444). The pThioHis-CA553 plasmid was generated as previously described [36]. The D2 cDNA was cloned into BamHI and PstI sites of the pQE80L plasmid (Qiagen, Valencia, CA) using OL491 and OL493. The D2 and CA553 cDNAs were cloned into HindIII and XhoI sites of pAAV-MCS plasmid (Stratagene, La Jolla, CA) (D2, OL404 and OL581; CA553, OL272 and OL580). Expression and affinity purification of baculovirus expressed proteins [37] were previously described. Dual-Light luciferase reporter assay was purchased from Applied Biosystems (Foster City, CA). Western analyses were performed using a LI-COR Odyssey imaging system (Lincoln, NE).

2.2. Native purification of AhR deletions D1–3

A single colony of JM109 containing the pThioHis-D1, D2, D3 or CA553 plasmid was inoculated into 100 ml of LB broth with 100 μ g/ml of ampicillin (USB, Cleveland, Ohio), followed by incubation at 37 °C, 250 rpm overnight. The overnight culture was added into 500 ml of fresh LB broth with ampicillin (100 μ g/ml) and 1 mM IPTG (Gold Biotechnology, St. Louis, MO). After incubation for up to 6 h at 250 rpm, bacteria were harvested and

freezed at -80°C , before being resuspended into 22.5 ml of lysis buffer (25 mM HEPES, pH 7.4, 300 mM KCl, and 10% glycerol). Lysozyme (2.5 ml of 7.5 mg lysozyme/ml in lysis buffer) was used to lyse the bacteria. PMSF (1 mM) and leupeptin (2 $\mu\text{g}/\text{ml}$) were added to the bacterial lysate before metal-affinity purification using cobalt agarose beads (Gold Biotechnology, St. Louis, MO). The affinity purified D1–3 and CΔ553 fractions were eluted using lysis buffer containing 0.5 M of imidazole. All fractions were dialyzed into HEDG buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, and 10% glycerol) and stored at -80°C .

2.3. Generation of functional AhR deletion D2 from denatured purification followed by refolding

The induced bacterial pellet was obtained as described under 2.2. Native purification of AhR derivatives D1–3. The pellet was resuspended into 15 ml of lysis buffer (25 mM HEPES, pH 7.4, 6 M guanidine HCl, 300 mM NaCl, and 10% glycerol). The resulting lysate was incubated at room temperature for 30 min and then centrifuged at 16,000g for 30 min before metal-affinity purification. The affinity purified D2 fraction (1.5 ml) was eluted in lysis buffer containing 0.5 M imidazole. Refolding by dialysis was performed as follows: D2 (1.5 ml) was dialyzed using SnakeSkin dialysis tubing (3,500 MWCO, Thermo Scientific, Rockford, IL) in five dialysis buffers (200 ml) in the following order – lysis buffer containing (1) 4 M guanidine HCl; (2) 2 M guanidine HCl; (3) 1 M guanidine HCl and 1 M arginine; (4) 1 M arginine and (5) HEDG buffer. Each dialysis was 2 h except that the last dialysis was performed overnight. The dialyzed samples were centrifuged at 16,000g for 10 min at 4°C to remove any precipitate. The supernatant was considered as the refolded sample and stored -80°C .

2.4. Co-immunoprecipitation assay

For D1–3 and Arnt interaction study, baculovirus expressed human Arnt (5 μl) was incubated with natively purified thioredoxin fusion D1–3 (D1/2, 40 μl ; D3, 20 μl) in HEDG buffer to a 50 μl final volume at 30°C for 10 min. Anti-Arnt H-172 IgG (5 μl) was used to precipitate Arnt (room temperature for 30 min). Dynabeads protein G (Invitrogen, Carlsbad, CA, 5 μl) was added to each sample, followed by incubation for 1 h at 4°C . Beads were washed four times of 5 min each with HEDG buffer containing 0.1% Tween-20. SDS-PAGE electrophoresis sample buffer was then added to each sample, followed by vigorous mixing. The supernatants were subjected for SDS-PAGE and LI-COR Western analysis. For D2 and Arnt C1/2 interaction study, refolded 6HisD2 (30 μl) and thioredoxin fusions of Arnt-C1/C2 (100 μl) in HEDG buffer (400 μl final volume, 25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, and 10% glycerol) were incubated at 30°C for 30 min. After preclearing the sample with dynabeads protein G (2 μl) by rotating at 4°C for 30 min, the supernatant was rotated with either mouse monoclonal His-probe antibody H-3 (10 μl , 2 μg) or mouse IgG (2 μg) for 10 min at room temperature and then 2 h at 4°C . Dynabeads protein G (5 μl) was then added to each sample, followed by rotation at 4°C overnight. After washing beads four times of 5 min each with HEDG buffer containing 0.1% Tween-20, SDS-PAGE electrophoresis sample buffer was added to each sample, followed by vigorous mixing. The supernatants were subjected for SDS-PAGE and LI-COR Western analysis.

2.5. Western blot analysis

The general western protocol using a near-infrared detection method was described previously with slight modification [34]. Wet transfer was performed using the Bio-Rad transfer kit at 300 mA for 120 min at 4°C . Secondary antibody incubation (LI-COR IRDye 680 donkey anti-mouse or anti-goat IgG or IRDye 800 anti-rabbit IgG, 1:10,000) was performed in the blocking buffer for 2 h at room temperature. A LI-COR Odyssey imaging system (Lincoln, NE) was used for analysis.

2.6. Generation of mouse polyclonal antibodies against the AhR deletion D2

The protocol for generating custom polyclonal antibodies using BALB/c mice was published previously [34]. Affinity purified, bacterially expressed 6HisD2 was used as antigen for antibody production.

2.7. Gel shift assay

For AhR/Arnt/DRE gel shift, baculovirus expressed human AhR, Sf9 lysate (7 μ g), thioredoxin or other thioredoxin fusion proteins were incubated in an 11 μ l of final volume of HEDG buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, and 10% glycerol) at 30 °C for 10 min in the presence or absence of β NF/ α NF and baculovirus expressed human Arnt. The protein content of each sample was normalized by BSA. After that, poly-dIdC (1.5 μ g) was added as well as enough KCl to reach 110 mM final concentration. After 10 min incubation at room temperature, IRDye680 conjugated DRE (250 fmol, 2 μ l) was added for another 10 min incubation at room temperature. For HIF-1 α /Arnt/HRE gel shift, baculovirus expressed human Arnt and HIF-1 α and Sf9 lysate (2.5 μ g) were incubated in an 11 μ l final volume of HEDG buffer with thioredoxin or thioredoxin fusion proteins at 30 °C for 30 min. The protein content of each reaction was normalized by BSA. After that, poly-dIdC (Calbiochem, Darmstadt, Germany, 1.5 μ g) and the mutated HRE (75 μ g) were added and enough KCl to reach 110 mM final concentration. After 10 min at room temperature, IRDye 680 conjugated HRE (75 fmol, 1.5 μ l) was added, followed by additional 10 min incubation at room temperature. Orange loading dye (2 μ l) was added to all samples before being resolved on a 5% native polyacrylamide gel (1xTBE) at 4 °C (200 V) for 2 h. After that, the gel was analyzed directly using a LI-COR Odyssey image system.

2.8. Reporter luciferase assay

Hep3B cells were grown in a 24-well plate to about 90% confluence prior to transfection. Before transient transfection, medium was exchanged to Advanced MEM containing 2.5% FBS and 2 mM GlutaMAX-I. In each well, cells were transfected with 100 μ l of Opti-MEM containing 2 μ l of X-tremeGENE 9 and 1 μ g of total plasmid DNA. Afterwards, cells were treated with or without CoCl₂ (100 μ M) in fresh medium for 18 h with or without 3MC (Sigma, St. Louis, MO, 1 μ M) for 6 h at 37 °C before being harvested and analyzed using the Dual-Light luciferase kit. The luciferase activities were normalized by internal β -galactosidase activities.

2.9. RT-qPCR

Hep3B cells (90% confluence) were grown in a 6-well plate. Before transient transfection, medium was exchanged to Advanced MEM containing 2.5% FBS and 2 mM GlutaMAX-I. Opti-MEM (100 μ l) containing X-tremeGENE 9 (8 μ l) and a plasmid (4 μ g) of either pAAV-MCS, pAAV-D2 or pAAV-CΔ553 was added to each well, followed by 30 min incubation at room temperature. After 24 h, medium was changed to Advanced MEM containing 5% FBS. Three days after transfection, cells were treated with fresh medium containing 1 μ M 3MC or DMSO vehicle for 6 h for measuring AhR-dependent target gene induction. For HIF-1 induction, cells were treated with 100 μ M CoCl₂ or water for 16 h. RNA extraction and cDNA synthesis were described previously [32]. RT-qPCR was performed using the Bio-Rad SYBR green supermix and sequence specific primers (Table II) on a Bio-Rad CFX Connect real-time PCR detection system.

PCR conditions (40 cycles) were as follows: 90 °C for 10 sec and 60 °C for 1 min. SYBR Green fluorescence readings were taken at 60 °C when the fluorescence intensity corresponded solely to the PCR product of interest. Normalized fold increase of the

endogenous transcript was determined by the $2^{-\Delta\Delta CT}$ method [38] using 18S as the internal control.

2.10. Microsomes preparation

Hep3B cells (90% confluence) were treated with 10 μ M BaP or DMSO for 6 h on a 10 cm plate. After that, cells were subjected for microsomes preparation as previously described [39].

2.11. Chromatin immunoprecipitation (ChIP) assay

Our ChIP protocol has been previously described [39]. Each sample was from a 10 cm plate of Hep3B cells of 90% confluence. We used anti-Arnt H-172 IgG to determine the Arnt recruitment in all our ChIP assays. Table III lists all ChIP primers used for this study.

2.12. Expression of human AhR in *Pichia pastoris*

The codon optimized full-length human AhR cDNA was purchased from Life Technologies (Grand Island, NY). This cDNA was cloned into SfuI and SacII sites of the pPICZB plasmid (Invitrogen, Carlsbad, CA) to generate pPICZB-hAhR, which expresses the human AhR with a c-myc and a 6-His tags at the C-terminus. The pPICZB-hAhR expression plasmid was linearized with SacI before being electroporated into freshly prepared ySMD1163 *Pichia pastoris* strain. Transformed cells were allowed to recover for 2 h in 1 ml of sorbitol at 30 °C without shaking, followed by addition of 1ml of YPD media. After shaking at 250 rpm for an additional 3 h at 30 °C, cells were plated on a YPD plate in the presence of Zeocin (Invitrogen, Carlsbad, CA, 100 μ g/ml). Transformed colonies were selected and grown on a separate YPD plate for subsequent colony PCR to validate positive clones. For large preparation, single colony from a freshly prepared plate of ySMD1163 carrying the pPICZB-hAhR plasmid was inoculated in BMGY media (50 ml in a 0.5 L flask) and incubated at 29 °C with 250 rpm overnight. The next day when OD₆₀₀ reached 4, cell suspension (26 ml) was centrifuged at 1,500g for 5 min at room temperature. The pellet was washed once with BMMY media (50 ml) and then resuspended in 100 ml of BMMY media containing 0.5% methanol in a 1 L flask to induce the *Pichia pastoris* AhR expression. Induction was allowed at 29 °C with shaking at 250 rpm for 12 h. At harvest, cells were centrifuged at 1,500g for 5 min at 4 °C. After washing once with cold PBS, the pellet was stored at -80 °C. To lyse the *Pichia pastoris* pellet, an equal amount of ZrOB05 beads was added to the pellet in a 5-fold volume of lysis buffer (25 mM HEPES, pH 7.4, 0.3 M KCl, 10% glycerol, 1 mM PMSF, 2 μ g/ml leupeptin). The *Pichia pastoris* cells were lysed in this suspension using Bullet Blender at Speed 8 for 5 min at 4 °C. After centrifuging the lysed cells at 14,000g for 30 min at 4 °C, the supernatant was collected as the loading sample for metal-affinity purification using cobalt agarose beads (Gold Biotechnology, St. Louis, MO). The affinity purified *Pichia pastoris* AhR fractions were eluted using lysis buffer containing 0.5 M of imidazole, followed by dialysis to obtain *Pichia pastoris* AhR in the final buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, 50% glycerol). Expression of *Pichia* AhR was confirmed by western using anti-c-myc and anti-AhR antibodies.

2.13. Co-precipitation assay

Pichia expressed human AhR (10 μ l), baculovirus expressed human Arnt (2 μ l), salmon sperm DNA (Invitrogen, Carlsbad, CA, 100 μ g), rabbit serum (Sigma, St. Louis, MO, 5 μ l), ThioD2 (6 μ l), and purified *cyp1a1* or *cyp1b1* DNA fragment (1 ng) were incubated in HEDG buffer in a final volume of 50 μ l for 10 min at 30 °C. Thioredoxin was used in place of ThioD2 as the negative control. Formaldehyde was added to 1% final concentration, followed by incubation at room temperature for 10 min to cross link the complex. The reaction was quenched by incubating with 6 μ l of 1.25 M glycine for 10 min at room

temperature. Rabbit anti-Arnt H172 (1 μ g) was then added, followed by incubation for another 30 min. After that, prewashed dynabeads protein G (5 μ l) and 450 μ l of HEDG buffer were added, followed by rotation for 1–2 h at 4 °C to immunoprecipitate the Arnt-containing cross-linked complex. Beads were washed four times for 5 min of each with HEDG buffer containing 5 mM imidazole, 10 mM 2-mercaptoethanol and 0.1% Tween-20 for assaying *cyp1a1* DRE whereas same wash buffer except 0.5% Tween-20 for assaying *cyp1b1* DRE. The washed beads were resuspended with 70 μ l of elution buffer (0.1 M NaHCO₃ and 1% SDS) and then incubated for 7 h at 65 °C to reverse the cross-linking. Samples were treated with Proteinase K (11 μ g, 24 mM Tris-KCl and 6 mM EDTA) for 2 h at 45 °C to degrade proteins. Immunoprecipitated DNA was purified using Promega Wizard SV gel clean-up kit and then used directly for RT-qPCR. Table III shows the primer sets to amplify the 100 bp fragments. PCR conditions (40 cycles) were as follows: 90 °C for 10 sec and 58 °C (annealing and extension) for 1 min. SYBR Green fluorescence readings were taken at 58 °C when the fluorescence intensity corresponded solely to the PCR product of interest. Normalized fold increase of the endogenous transcript was determined by the $2^{-\Delta\Delta CT}$ method [38].

2.14. Statistical analysis

GraphPad Prism 5 software was utilized for statistical analysis. The significant difference was determined by performing one-way (Figs. 6C, 6D, 7, 8A, 8B, and 9E) or two-way (Fig. 5A, 5B, 6A, 6B, 8C, 9B, 9C, and 9D) ANOVA followed by post hoc Bonferroni's multiple comparison test to determine the statistical significance with 95% confidence intervals with * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ and ns, not significant ($p > 0.05$). All error bars in figures represent $\pm$ SD, $n = 3$ with exception of $n = 4$ in a few cases which are mentioned in the figure legends.

3. Results

3.1. AhR deletions D1–3 interact with Arnt

Realizing that the PAS domain is the interaction surface between AhR and Arnt, we generated three deletion constructs of the human AhR PAS domain in an effort to investigate whether binding to a small region of Arnt would preferentially inhibit a specific Arnt function among others. D1 is a 24 KDa human AhR construct (aa 84–295) which covers the whole PAS-A domain of the mouse AhR (Fig. 1). The smaller D2 (12 KDa, aa 84–192) and D3 (12 KDa, aa 191–295) constructs represent the N- and C-terminal half of D1, respectively. These constructs were expressed as a thioredoxin fusion so that they could be used for co-immunoprecipitation studies to determine whether they bind Arnt. Anti-Arnt H-172 IgG co-immunoprecipitated all three constructs (thioredoxin fusions of D1–3) when the baculovirus expressed human Arnt [37] was used as bait (Fig. 2). All three fusions appeared to interact with Arnt similarly (~10% input), but this interaction was rather modest when compared with C Δ 553 (>>10% input), which has been commonly used in our laboratory to bind Arnt in the absence of ligand [36].

3.2. D2 suppresses the formation of the AhR gel shift complex

Next, we examined whether D1–3 would inhibit the formation of the Arnt-containing complex in vitro. In order to have adequate D1–3 to show their effects on the gel shift band in a dose-dependent manner, we generated bacterially expressed thioredoxin fusions of D1–3, with thioredoxin fused at the N-terminus of D1–3. Thioredoxin was used as the negative control for the gel shift experiment. We performed in-gel LI-COR western analysis to normalize the amount of a thioredoxin fusion used in gel shift assays (data not shown). We observed that among D1–3, D2 showed the best dose-dependent suppression of the AhR gel shift complex formation whereas the thioredoxin control did not affect the intensity of the

complex (Fig. 3). However, the extent of suppression by D2 was noticeably weaker than the suppression by CA553, which effectively inhibits AhR function [29]. The identity of the AhR/Arnt/DRE gel shift complex was validated by the observations that (1) anti-Arnt goat IgG, but not goat IgG, abolished the AhR/Arnt/DRE complex and (2) the AhR/Arnt/DRE complex did not form when either Arnt was omitted or an antagonist (α NF) was used in place of an agonist (β NF).

3.3. D2 interacts with the PAS-A domain of Arnt

In an effort to determine the location of Arnt where D2 interacts, we performed co-immunoprecipitation experiment using deletion constructs of the N-terminal Arnt region (Arnt C1, aa 1–160 and Arnt C2, aa 127–356) where interaction between AhR and Arnt occurs. Both refolded 6His fusion of D2 (6HisD2) and native thioredoxin fusion of D2 (ThioD2) interacted with the baculovirus expressed human Arnt, but the refolded 6HisD2 appeared to have a lower background than ThioD2 in the absence of Arnt (Fig. 4, top panels). Since the Arnt deletions (Arnt C1 and C2) for the interaction study were in the form of thioredoxin fusion, we used the refolded 6HisD2 protein (with a different tag) as bait to perform the co-precipitation. We observed that Arnt C2, but not Arnt C1, was co-immunoprecipitated with D2 using anti-His H3 IgG, showing that D2 interacts with the PAS-A domain of the human Arnt (aa 154–362, [40]).

3.4. D2 suppresses the DRE-driven luciferase expression and the 3MC-activated expression of CYP1A1, but not CYP1B1, at the message and protein levels

Since D2 binds to the Arnt PAS-A domain and suppresses the formation of the AhR/Arnt/DRE complex, we examined whether D2 would suppress the AhR-dependent downstream signaling. Results from the reporter luciferase experiment showed that transiently transfected D2 suppressed the 3MC-induced, DRE-driven luciferase expression in a dose-dependent manner (Fig. 5A). This suppression was reversed when exogenous Arnt was present via transient transfection, supporting that this D2 effect is Arnt-mediated (Fig. 5B). The AhR and Arnt protein levels were not altered in the presence of D2 (data not shown), suggesting that the action of D2 likely occurs at the formation of the AhR/Arnt complex. The *cyp1a1* mRNA levels were induced by 25-fold in Hep3B cells after treatment with 1 μ M 3MC for 6 h (Fig. 6A). This induction was suppressed by the transiently transfected D2 to 60%, similar to the control CA553 which is known to suppress the AhR function [29]. The BaP-induced expression of the CYP1A1 protein in Hep3B cells was similarly suppressed by D2 (Fig. 6C), showing that D2 is capable of suppressing the AhR-controlled target gene activity. In contrast, D2 did not alter the induction of CYP1B1 at both the mRNA and protein levels (Fig. 6B and D).

3.5. D2 suppresses the recruitment of Arnt to the *cyp1a1* promoter but not to the *cyp1b1* promoter

Next, we investigated the mechanism for the suppression of the CYP1A1 induction by D2. We performed ChIP assays to address whether D2 would affect the binding of Arnt at the *cyp1a1* promoter. We observed that D2 significantly suppressed the 3MC-mediated recruitment of Arnt to the *cyp1a1* promoter to 50%, comparable to the extent of Arnt recruitment observed in the case of CA553 suppression (Fig. 7). However, D2 did not affect the recruitment of Arnt to the *cyp1b1* promoter, consistent with our finding that D2 has no inhibitory role on the CYP1B1 induction (Fig. 6B and D).

3.6. Flanking sequences of endogenous DRE alter the binding affinity of AhR to DRE

We generated a 100 bp DRE fragment of the *cyp1a1* promoter, which is analogous to the 100 bp *cyp1b1* promoter fragment we used for ChIP assays (Table III and IV). These two

DRE fragments, which were generated by PCR, have two DRE core sequences in addition to the unique flanking sequences from either *cyp1a1* (DRE5 and DRE6) or *cyp1b1* (DRE2 and DRE3) promoter. We used them to perform an in vitro cross-linking co-precipitation experiment to address whether binding of Arnt to DRE could be affected by the flanking sequences. For this experiment, we used the *Pichia* expressed full-length human AhR, which interacted with the baculovirus expressed human Arnt in the co-immunoprecipitation study (Fig. 8A, top panel). We observed that D2 significantly suppressed the binding of Arnt to the *cyp1a1* DRE fragment to 60% whereas the Arnt binding to the *cyp1b1* DRE fragment was not affected by D2 (Fig. 8A, bottom graphs). To determine whether the endogenous DRE flanking sequences play a role in Arnt binding to the DRE, we performed competitive binding experiment by measuring the Arnt binding to the *cyp1a1* DRE fragment in the presence or absence of an equal amount of the *cyp1b1* DRE fragment, and vice versa. We observed that the *cyp1b1* DRE fragment effectively suppressed 40% of the binding of Arnt to the *cyp1a1* DRE fragment; however, binding of Arnt to the *cyp1b1* DRE fragment was not affected in the presence of the *cyp1a1* DRE fragment (Fig. 8B). Next, we examined whether the flanking sequences contribute to the strong binding of the AhR/Arnt heterodimer to the *cyp1b1* DREs. We generated two mutated fragments of the *cyp1b1* DRE (Table IV) and observed that either changing the -2/+6 nucleotides or five nucleotides flanking the DREs drastically lowered the binding affinity of the AhR/Arnt heterodimer to the *cyp1b1* DREs, confirming that the flanking sequences of the endogenous *cyp1b1* DREs are crucial for AhR/Arnt binding (Fig. 8C).

3.7. D2 does not suppress the HIF-1 α -mediated endogenous target gene expression

We examined whether D2 would suppress the Arnt-dependent HIF-1 signaling. First, we addressed whether D2 would inhibit the formation of the HIF-1 gel shift complex in vitro. Results from the gel shift assay showed that D2 was more effective in abolishing the gel shift complex of AhR than of HIF-1 α (Fig. 9A). Next, we addressed whether D2 would suppress the HIF-1 target gene expression. We observed that although the transiently transfected D2 showed a dose-dependent suppression of the reporter luciferase expression (Fig. 9B), the extent of suppression was less pronounced when compared with the D2 suppression of the DRE-driven luciferase expression (Fig. 5A). This D2 suppression was reversed in the presence of exogenous Arnt, confirming that this D2 effect is Arnt-mediated (Fig. 9C). However, the CoCl₂-dependent induction of three HIF-1 target genes – *veg**f*, *aldolase c* and *ldh-a* – was not altered at the mRNA levels in the presence of D2 (Fig. 9D). Results from the ChIP assay revealed that D2 did not alter the Arnt recruitment to the HRE of the *veg**f* promoter by treating Hep3B cells with 100 μ M CoCl₂ (Fig. 9E). Collectively, although D2 suppressed the HIF1-dependent luciferase activity, this D2 effect was too weak to alter any endogenous HIF1 target gene expression.

4. Discussion

We used three smaller derivatives of the C Δ 553 structure with hopes that binding to a discrete Arnt region would be sufficient in suppressing the Arnt function, and perhaps revealing some selectivity in suppressing different Arnt functions. For example, molecules binding to the (smaller region) PAS-B domain of HIF-1 α or HIF-2 α – such as cyclo-CLLFVY [41], MgcRacGAP [42], a number of small molecules containing aromatic and heterocyclic rings connected with a short linker [43] and acriflavine [44] – are effective inhibitors and hopefuls for cancer treatment. However, selectivity among different Arnt partners remains a key issue of these inhibitors.

In our study, D2, which contains part of the human AhR PAS-A domain and binds to the human Arnt PAS-A domain, is most interesting. All three of our human AhR deletions, D1–

3, bind the full-length human Arnt with similar affinity. This observation was initially surprising since one would assume that D1, which contains the whole PAS-A domain, would have the highest binding affinity when compared with the other two deletions with only partial PAS-A structure. Other researchers showed that the A'α moiety of the mouse AhR PAS-A domain is important for AhR/Arnt interaction: AhR PAS-A mutant lacking A'α appeared to lose its dimerization ability; additionally, the bacterially expressed AhR bHLH-PAS-A mutant lacking A'α did not interact with the Arnt bHLH-PAS-A molecule [45]. In our case, interaction between the full-length Arnt and a small AhR PAS-A derived protein molecule is somewhat different, revealing insights in binding a molecule to the PAS-A domain of the full length Arnt protein. First of all, I must emphasize that CΔ553, which contains the bHLH-PAS-A region (Fig. 1), has by far the highest affinity with the full-length human Arnt when compared with D1–3 (with no bHLH), suggesting that the bHLH domain is essential for strong binding between AhR and Arnt. It appears that in an AhR PAS-A derived protein molecule with no bHLH domain, there is no particular secondary structure within the PAS-A region that has an overriding effect on (weak) interaction with Arnt. Specifically, this observation is referred to D2 and D3: D2 lacks three β-strands (Gβ, Hβ, and Iβ) whereas D3 lacks two β-strands (Aβ and Bβ) and all five α-helices (A'α, Cα, Dα, Eα, and Fα). Indeed it is possible to generate a small Arnt-interacting protein molecule based on the AhR PAS-A region. It is unclear why D1 and D2 appeared to bind similarly to Arnt and yet D2, but not D1 suppressed the formation of the AhR/Arnt/DRE complex, realizing that D2 is part of D1. I would argue that the 3-dimensional structures of Arnt/D1 and Arnt/D2 are quite different that presence of D2 with Arnt is structurally more effective in the interference of the formation of the AhR/Arnt/DRE complex. Nevertheless, this observation revealed that the inherent ability of an inhibitory molecule to bind Arnt per se is not a reliable indicator of a good suppressor of the AhR/Arnt interaction. This D2 inhibitory effect does not necessarily need to occur in the nucleus, since Ainp1, another Arnt-interacting peptide, lowers the nuclear Arnt levels by interacting with Arnt in the cytoplasm [34]. In addition, D2 preferentially suppresses the AhR signaling over the HIF-1 signaling at the chromatin level, revealing a potentially new approach of modulating the Arnt function differentially. Similar differential effects on the interaction of Arnt with different partners has been linked to the Arnt PAS-A domain where D2 interacts [40]. Since D2 contains A'α and three essential hydrophobic residues (L116, A119, and L120) for Arnt interaction [45], it is tempting to speculate that targeting the AhR A'α/Arnt A'α interface is an effective approach to differentially suppress the Arnt-dependent functions.

To unambiguously show that D2 interacts with Arnt, we used two forms of D2 to do so – bacterially expressed thioredoxin fusion and 6His fusion of D2. The 6HisD2 protein is primarily found in the inclusion bodies. However, we are able to purify 6HisD2 using 6 M guanidinium chloride by metal-affinity chromatography, followed by sequential dialysis with the use of arginine to refold the 6HisD2 protein. The refolded 6HisD2 is abundant and functional – this is one of the examples of how we have used this refolding strategy to obtain a number of functional PAS protein constructs in near homogeneity (unpublished data). Similar strategy (without the use of arginine) has been used by others to obtain a purified, functional transactivation domain of AhR for structural studies [46]. In our case, however, arginine appears to be necessary to refold functional 6HisD2 construct.

We intentionally examined the DRE regions where other groups have studied for AhR recruitment in our ChIP study. The human *cyp1a1* promoter has been extensively studied for AhR enhancers. Four out of the ten DREs (DRE3–6) within 1,400 nucleotides upstream of the transcription start site appear to be functional by deletion mapping studies [47]. We monitored a 203 bp fragment spanning DRE4–6 for our ChIP study since DRE4–6 are relatively close in proximity whereas DRE3 is a distance away – 400 bp apart from this fragment. In addition, this 203 bp fragment was also measured by at least another group for

ChIP assay [18]. In the case of *cyp1b1*, we utilized a human *cyp1b1* promoter region containing two DREs (DRE2 and DRE3); this region has previously been used for ChIP studies by at least two laboratories [48, 49]. The AhR/Arnt heterodimer binds to DRE3 (–853) of the human *cyp1b1* promoter with relatively high affinity in a gel shift assay when compared among four (DRE2, DRE3, DRE6, and DRE7) out of the eight DREs (–2299 to +25). These four DREs effectively abolish the AhR gel shift complex in a dose-dependent manner [50]. DRE2 (–834) appears to be important for constitutive CYP1B1 expression [51]. Mutations at DRE2 and DRE3 impair the inducibility of CYP1B1 by TCDD [50], suggesting that DRE2 and DRE3 should be monitored for the AhR/Arnt binding. Taken together, we purposely selected the DRE2–3 region of the *cyp1b1* promoter to study the Arnt binding in our ChIP study. However, we cannot rule out the possibility that the untested DREs at the *cyp1a1* and *cyp1b1* promoters also affect the Arnt recruitment by contributing to the tertiary structure that is essential for binding. But we must emphasize that our ChIP results are consistent with the suppressive effect of D2 on the induction of CYP1A1 and CYP1B1 expression.

Interestingly, the differential inhibition of *cyp1a1* and *cyp1b1* expression by D2 was similarly observed when MCF-7 cells were treated with E2 [52]. It appears that modulation of the DNA methylation at the *cyp1a1* promoter is responsible for this E2-mediated suppression of the AhR binding to the DRE. Mechanistically, E2 suppresses the TCDD-mediated induction of the *cyp1a1* message but not the *cyp1b1* message in MCF-7 cells by activating ER α to recruit DNA methyltransferase 3B for gene repression [52]. In addition to the authors' conclusions, it is conceivable that the activated ER α may directly affect the AhR binding, as observed in the case of D2, since ER α physically interacts with AhR [53]. DNA methylation of the *cyp* genes is likely cell specific: although methylation of the *cyp1b1* promoter in MCF-7 cells might not seem important, methylation of the CpG islands close to the transcription start site of the *cyp1b1* promoter in HepG2 cells appears to impair transcription [54]. Additionally, DNA methylation has been proposed to be an epigenetic mechanism to alter *cyp1b1* gene expression in various cell lines [55]. To complicate the matter, it has been reported that E2 induces CYP1B1 by activating ER α to bind to the ERE of the *cyp1b1* promoter in MCF-7 cells [56]; however, other researchers were not able to detect the *cyp1b1* message induction by E2 in the same cell line [57]. Our data certainly reveal an additional mechanism to explain how differential induction of CYP1A1 and CYP1B1 may occur.

It was initially surprising that D2 would suppress the induction of CYP1A1 but not CYP1B1. In vitro DNA selection and amplification strategy had revealed the importance of flanking sequences of the DRE core (GCGTG) in affecting the binding affinity of the AhR/Arnt heterodimer to the DRE [58]. This strategy involves in vitro enrichment for double-stranded DRE sequences that bind favorably to the AhR/Arnt heterodimer. Additional preference at –2 and +6 locations for T and C (TNGCGTGC), respectively, was reported. Of the two *cyp1a1* DREs (DRE5 and DRE6), there is T at the –2 location but an A instead of C at the +6 location whereas both T and C are present in both DREs (DRE2 and DRE3) of the *cyp1b1* promoter. Thus, the AhR/Arnt heterodimer would in theory prefer binding to the DREs of the *cyp1b1* promoter than of the *cyp1a1* promoter.

In an effort to directly address whether the AhR/Arnt heterodimer indeed binds better to the *cyp1b1* DREs than the *cyp1a1* DREs, we performed an in vitro co-precipitation experiment to precipitate the cross-linked complex containing the same length (100 bp) of the DRE fragment from either *cyp1a1* or *cyp1b1*. The 100 bp DRE fragment of the *cyp1a1* promoter was derived from the ChIP PCR fragment (203 bp); this 100 bp fragment preserves the two DRE core sequences (DRE5 and DRE6) – the same number of DREs present in the *cyp1b1* fragment. Our in vitro co-precipitation results proved that AhR prefers binding to the *cyp1b1*

fragment over the *cyp1a1* fragment because of the flanking sequences, even though physical contacts between the fragment and the heterodimer are at the DRE core sequence [59]. Our mutagenesis data certainly supported this theory as well. When we mutated just the -2/+6 locations of DRE5 and DRE6 from T/C to C/A, the binding of the AhR/Arnt heterodimer to this mutated fragment was clearly reduced. This binding was further reduced when we substituted the five DRE flanking nucleotides of *cyp1b1* with the ones from *cyp1a1* (*cyp1b1* DRE2 with *cyp1a1* DRE5; *cyp1b1* DRE3 with *cyp1a1* DRE6); precisely as we would predict if the flanking sequences govern the binding affinity of the AhR/Arnt heterodimer. It has been assumed that differences in the extent of target gene activation by the same transcription factor are often related to the coactivator availability at different promoter regions. Here, we have provided evidence that the ability of a transcription factor to bind to specific enhancer regions, which precedes coactivator recruitment, may be crucial in determining the extent of gene activation.

It is interesting that stability of the endogenous DNA complexes containing Arnt can be distinguished in the presence of D2. We observed that Ainp2, which also binds to the Arnt PAS-A domain, does not inhibit the HIF-1 signaling. However, Ainp2, unlike D2, promotes CYP1A1 induction [32], revealing that the local structure upon binding a molecule to the Arnt PAS-A domain is important in directing the outcome of the binding.

We have used D2, a 12 KDa construct derived from the PAS-A domain of AhR, to selectively block the Arnt function. Although D2 binds to the Arnt PAS-A domain with only modest affinity, it effectively suppresses the formation of the AhR/Arnt/DRE complex at the *cyp1a1* promoter. D2 does not alter the assembly of AhR/Arnt/*cyp1b1* DRE and HIF-1 α /Arnt/*veg*f HRE complexes, which explains why D2 suppresses the CYP1A1 induction and has no effect on the induction of CYP1B1 and HIF-1 target genes. Our data reveal that flanking sequences around the DRE core are important in determining the binding preference of the AhR/Arnt heterodimer to functional DREs.

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Abbreviations

αNF	α -naphthoflavone
AhR	aryl hydrocarbon receptor
Arnt	aryl hydrocarbon receptor nuclear translocator
BaP	benzo[a]pyrene
βNF	β -naphthoflavone
CoCl₂	cobalt chloride
CYP	cytochrome P450
DRE	dioxin response element
HIF-1α	hypoxia inducible factor-1 alpha
HRE	hypoxia response element
3MC	3-methylcholanthrene

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CA553 amino acid sequence which is aa 1–295 of the human AhR (hAhR). This sequence is aligned with the PAS-A domain (aa 110–267) of the mouse AhR (mAhR) [45]. The lined regions represent D2 (aa 81–192) and D3 (aa 191–295) whereas D1 is the combined D2 and D3 sequence (aa 81–295). Asterisks (*) represent identical amino acid sequences between human and mouse AhR. The underlined mAhR sequences represent the secondary structures published by Wu et al. The underlined hAhR sequence (aa 13–86) represents the bHLH domain.

Fig. 2.

Co-immunoprecipitation results showing that D1-3 interacts with Arnt. Top, immunoprecipitation (IP) of baculovirus Arnt (Arnt) by anti-Arnt H-172 IgG (αArnt); IgG is rabbit IgG. Anti-Arnt N19 (1:200) was used to detect Arnt. Bottom, anti-Arnt H-172 IgG was used to coimmunoprecipitate thioredoxin fusions of D1-3 (ThioD1, ThioD2 and ThioD3; thioredoxin (Thio) is the negative control) using baculovirus human Arnt as bait. 10% input represents the signal for 10% of ThioD1-3 used to start the experiment. Anti-thio IgG (1:5,000) was used to detect Thio and ThioD1-3. This experiment was repeated once with similar results.

Fig. 3.

Gel shift results showing that D2 inhibits the AhR/Arnt/DRE complex formation. Baculovirus expressed human AhR and Arnt were used in the presence (+) or absence (-) of 10 μ M β NF, 10 μ M α NF and thioredoxin fusions of D1-3 (1X-30X, 1X refers to the normalized amount that has the same intensity of 0.15 μ l of thioredoxin). The protein content was normalized by BSA in all lanes. The upper left arrow represents the AhR/Arnt/DRE complex; the lower left arrow represents the free IRDye labeled DRE; the upper right arrow represents the C Δ 553/Arnt/DRE complex. C Δ 553 is the positive control for comparison. Lanes 1-3 are the same as lanes 6-8 showing the β NF-dependent AhR/Arnt/DRE complex. Lanes 4 (goat IgG, 1 μ l) and 5 (N19, 1 μ l) show that Arnt IgG abolished the gel shift complex. Lanes 9-11 contain 1-30X thioredoxin (cont) as the negative control. Lanes 1-5, 6-11 and 12-23 were performed on separate gels. This experiment was repeated once with similar results.

Fig. 4.

Co-immunoprecipitation results showing that D2 interacts with Arnt C2 but not Arnt C1. Top, both refolded 6HisD2 and thioredoxin fusion of D2 (ThioD2) were co-immunoprecipitated by anti-Arnt H-172 rabbit IgG using baculovirus Arnt as bait. Anti-D2 mouse polyclonal IgG (1:1,000) was used to detect 6HisD2 and ThioD2. Bottom, thioredoxin fusion of Arnt C2, but not thioredoxin fusion of Arnt C1, was co-precipitated by anti-His H3 mouse IgG using refolded 6HisD2 as bait. IP ab represents anti-His H3; IgG represents mouse IgG; 10% input represents 10% of the bait amount used to start the experiment. Anti-Thio IgG (1:5,000) was used to detect Arnt C1 and Arnt C2. This experiment was repeated once with similar results.

A

B

Fig. 5.

Reporter luciferase assay showing that D2 suppresses the 3MC-activated, DRE-driven luciferase activity via an Arnt-dependent mechanism. A, the pAAV-D2 plasmid carrying the D2 cDNA with normalized amount of the parent pAAV EV plasmid (+, 300 ng; ++, 600 ng; +++, 900 ng) was transiently transfected with reporter luciferase and β -gal plasmids into Hep3B cells with (+) or without (-) 1 μ M 3MC. This experiment was repeated three times with similar results. B, the D2, pAAV-D2 plasmid; EV1, pAAV plasmid; Arnt, pSport-Arnt plasmid carrying the full length human Arnt cDNA; EV2, the parent pSport plasmid; +, 200 ng; ++, 600 ng. Various combinations of plasmids were transiently transfected with reporter luciferase and β -gal plasmids into Hep3B cells with (+) or without (-) 1 μ M 3MC. This experiment was repeated three times with similar results.

A

B

C

D

Fig. 6. RT-qPCR and western results showing that D2 suppresses the induction of CYP1A1, but not CYP1B1. 4 μ g of parent pAAV (EV), pAAV-D2 (D2) or pAAV-CΔ553 (CΔ553) plasmid was transiently transfected using X-tremeGENE 9 into Hep3B cells. On day 4 after transfection, cells were treated with 1 μ M 3MC (+) (A and B) or 10 μ M BaP (C and D) or DMSO (–) for 6 h. 18S was used to normalize the *cyp1a1* (A) and *cyp1b1* (B) message levels. The CYP1A1 (C) and CYP1B1 (D) protein levels in microsomes obtained after treatment (EV/DMSO, EV/BaP, D2/BaP, or CΔ553/BaP) were normalized by loading 100 μ g of protein per lane. EV, empty vehicle. CYP1A1 and 1B1 are 53 and 55 KDa, respectively, according to manufacturer's datasheet images. The major lower band in 6C

was intentionally included as loading control. EV/BaP condition (with very small error bar) is arbitrarily set as 1 to normalize data from three separate experiments. Images in C and D are representatives of western images. B4 IgG (1:500) and G4 IgG (1:500) were used to detect CYP1A1 and 1B1, respectively. For 6A, $n = 4$ for plus 3MC and $n = 3$ for minus 3MC; $n = 3$ for all conditions in 6B. The clear column (plus 3MC) in 6A and 6B was arbitrarily set as 1 to normalize the replicate data so that no error bar is shown. For 6C and D, $n = 3$.

Fig. 7.

ChIP results showing that D2 suppresses the Arnt recruitment to the promoter of *cyp1a1* but not *cyp1b1*. Hep3B cells were transfected with nothing (WT and WT-3MC) or 16 μ g of parent pAAV (EV), pAAV-D2 (D2) or pAAV-C Δ 553 (C Δ 553) plasmid. Cells were treated with 1 μ M 3MC or DMSO for 6 h. Anti-Arnt H172 was used to immunoprecipitate the cross-linked complex. 5% input represents 5% of the total lysate amount to start the experiment. This experiment (*cyp1a1* and *cyp1b1*) was repeated three times with similar results. The top images are representatives of the agarose gel images.

A

B

C

Fig. 8.

Co-precipitation results showing that AhR forms a stronger complex at the *cyp1b1* DRE than the *cyp1a1* DRE. A, images (top) showing co-immunoprecipitation of *Pichia* expressed human AhR by anti-Arnt H-172 using baculovirus expressed Arnt as bait. Anti-c-Myc IgG (1:5,000) was used to detect *Pichia* AhR, which contains c-myc tag at the C-terminus. 10% input represents 10% of the total *Pichia* AhR amount used to start the experiment. αArnt, H-172; IgG, rabbit IgG. Bottom graphs show the amount of AhR recruited to the *cyp1a1* DRE fragment (left) or *cyp1b1* DRE fragment normalized by arbitrarily setting second column (AhR and Arnt with no ThioD2 or Thio) as 1. AhR, *Pichia* expressed human AhR; Arnt, baculovirus expressed human Arnt; ThioD2, thioredoxin fusion of D2; Thio, thioredoxin. B, Co-precipitation results with simultaneous presence of equal amount of the *cyp1a1* DRE and *cyp1b1* DRE fragments. Binding of Arnt was quantified by PCR using corresponding primers specific to the *cyp1a1* DRE (left) or *cyp1b1* DRE (right) fragment.

Both fragments are 100 bp and contain two DREs. Middle columns were arbitrarily set to 1 to normalize data. C, Co-precipitation results with equal amount (1ng) of either wild type *cyp1b1* DRE fragment or mutated fragments (*cyp1b1* DRE M1 or *cyp1b1* DRE M2, see Table IV for sequences). The second column was arbitrarily set as 1 to normalize data. A-C were repeated three times with similar results.

A

B

C

D

Fig. 9.

D2 preferentially suppresses the signaling of AhR over HIF-1 α . A, Gel shift results showing that ThioD2 (1 4X to 42X, see Fig. 3 legend for 1X definition) is more effective in suppressing the AhR gel shift complex (lanes 5–8) than the HIF-1 gel shift complex (lanes 1–4).). Protein content was normalized by BSA in all lanes. This experiment was repeated once with similar results. B, Reporter luciferase assay results showing that D2 suppresses the CoCl₂-activated, HRE-driven luciferase activity. The pAAV-D2 plasmid carrying the D2 cDNA with normalized amount of the parent pAAV EV plasmid (+, 300 ng; ++, 600 ng; +++ , 900 ng) was transiently transfected with reporter luciferase and β -gal plasmids into Hep3B cells with (+) or without (–) 100 μ M CoCl₂. C, EV1, pAAV plasmid; EV2, pSport plasmid; D2, pAAV-D2 plasmid; Arnt, pSport-Arnt plasmid carrying the full-length human Arnt cDNA. Various combinations of plasmids (++, 400 ng) were transiently transfected with reporter luciferase and β -gal plasmids into Hep3B cells with (+) or without (–) 100 μ M CoCl₂. This experiment was repeated three times with similar results. D, RT-PCR results (*vegfr*, n = 3; *aldolase c*, n = 4; *ldh-a*, n = 4 for plus CoCl₂ and n = 3 for minus CoCl₂)

showing that induction of the HIF-1 target genes message levels is not altered by D2. 4 μ g of parent pAAV (EV), pAAV-D2 (D2) or pAAV-C Δ 553 (C Δ 553) plasmid was transiently transfected using X-tremeGENE 9 into Hep3B cells. On day 4 after transfection, cells were treated with 100 μ M CoCl₂ or DMSO for 16 h. 18S was used for normalization. The clear column (plus CoCl₂) in each panel was arbitrarily set as 1 to normalize the replicate data so that no error bar is shown. E, ChIP results showing that D2 does not suppress the Arnt recruitment to the *veg*f promoter. Hep3B cells were transfected with 16 μ g of parent pAAV (EV), pAAV-D2 (D2) or pAAV-C Δ 553 (C Δ 553) plasmid and then treated with or without 100 μ M CoCl₂ for 16 h. The top images are representatives of the agarose gel images. α -Arnt, anti-Arnt H-172 IgG. 5% input represents 5% of the total lysate amount used to start the experiment. B-D were repeated three times with similar results.

Table I

A list of oligonucleotides used for this study.

OL272	sense	5'-CGAAGCTTCACCATGAACAGCAGCAGC-3'
OL404	sense	5'-CAAGCTTGCATCATGGATGTTGCATTAAAAATCCTCCCCTACTGAAAG-3'
OL439	sense	5'-5IRD680/TCGAGTAGATCACGCAATGGGCCCAGC-3'
OL440	antisense	5'-5IRD680/TCGAGCTGGGCCCATTGCGTGATCTAC-3'
OL443	sense	5'-GCCTCGAGGATGTTGCATTAAAAATCCTCCCCTACTGAAAG-3'
OL444	antisense	5'-CTCTAGATCCTAGAAAGTCTAGTTTGTGTTTGGT-3'
OL445	antisense	5'-CTCTAGATCCTATTCTCAATTCCTTGTCAGACTC-3'
OL446	sense	5'-CAACTCGAGGAAGAAGCCACTGGTCTCCCCAGACAGTAGTC-3'
OL487	sense	5'-/51 RD680/GCCCTACGTGCTGTCTCA-3'
OL488	antisense	5'-/5IRD680/TGAGACAGCACGTAGGGC-3'
OL491	sense	5'-GCGGATCCGATGTTGCATTAAAAATCCTCCCCTACT-3'
OL493	antisense	5'-CCCTGCAGCTATTCTTCAATTCCTTGTCAGACTC-3'
OL499	sense	5'-GCCGAGATCTCGGATGTTGCATTAAAAATCCTCCCCTACTGAAAG-3'
OL501	antisense	5'-CTGGATCCCCTATTCTTCAATTCCTTGTCAGACTC-3'
OL580	antisense	5'-CGCCTCGAGCTAGAAGTCTAGTTTGTGTTTGGT-3'
OL581	antisense	5'-CGCCTCGAGCTATTCTTCAATTCCTTGTCAGACTC-3'

Table II

A list of RT-PCR primers used for this study.

<i>cyp1a1</i>	Forward	OL90 5' GGCCACATCCGGGACATCACAGA 3'
	Reverse	OL91 5' TGGGGATGGTGAAGGGGACGAA3'
<i>cyp1b1</i>	Forward	OL333 5' CACCAAGGCTGAGACAGTGA 3'
	Reverse	OL334 5' GATGACGACTGGGCCTACAT 3'
<i>vegf</i>	Forward	OL94 5' CCTCCGAAACCATGAACTTT 3'
	Reverse	OL95 5' AGAGATCTGGTTCCCGAAAC 3'
<i>aldolase c</i>	Forward	OL88 5' ATAATGGTGTTCCTTCGTCCGA 3'
	Reverse	OL89 5' GTCACCTCGCATGTGGGAGACGT 3'
<i>ldh-a</i>	Forward	OL86 5' GCCCGACGTGCATTCCCGATTTCCTT 3'
	Reverse	OL87 5' GACGGCI ICTCCCTCTTGCTGACG 3'
18S	Forward	OL96 5' CGCCCCCTCGATGCTCTTAG 3'
	Reverse	OL97 5' CGGCGGGTCATGGGAATAAC 3'

Table III

A list of ChIP assay primers used for this study.

<i>cyp1a1</i>	203bp	Forward	OL329 5' TAAGAGCCCCGCCCCGACTCCCT 3'
		Reverse	OL330 5' TAGCTTGCGTGCGCCGGCGACAT 3'
	100bp	Forward	OL665 5' CTCCCCCTCGCGTGACTGC 3'
		Reverse	OL666 5' TGCCCAGGCGTTGCGTGAGA 3'
<i>cyp1b1</i>	100bp	Forward	OL607 5' ATATGACTGGAGCCGACTTTCC 3'
		Reverse	OL608 5' GGCGAACTTTATCGGGTTGA 3'
<i>vegf</i>		Forward	OL482 5' CAGGAACAAGGCCTCTGTCT 3'
		Reverse	OL483 5' TGTCCCTCTGACAATGTGCCATC 3'

Table IV

A list of 100 bp endogenous DRE fragments used for co-precipitation experiment in Fig. 8. The $-2/+6$ flanking locations are underlined. The mutated nucleotides are capitalized and in italic.

<i>cyp1a1</i> 100 bp DRE fragment used in Fig. 8A–B:	
<u>DRE6</u>	<u>DRE5</u>
5'-ctccccctc <u>GCGTG</u> actgcgagc...cctccggctctt <u>CACG</u> Caagcctgggca-3'	
<i>cyp1b1</i> 100 bp DRE fragment used in Fig. 8A–C:	
<u>DRE3</u>	<u>DRE2</u>
5'-...gaagcggcg <u>CACG</u> Caagcccagctcc <u>CACG</u> Caaggggagg...-3'	
<i>cyp1b1</i> 100 bp mutated DRE fragment 1 (<i>cyp1b1</i>DRE M1) used in Fig. 8C:	
<u>DRE3</u>	<u>DRE2</u>
5'-...gaagcggc <u>TCACG</u> Ca <u>G</u> agcccagctcc <u>TCACG</u> Ca <u>G</u> aggggagg...-3'	
<i>cyp1b1</i> 100 bp mutated DRE fragment 2 (<i>cyp1b1</i>DRE M2) used in Fig. 8C:	
<u>DRE3</u>	<u>DRE2</u>
5'-...gaag <u>GCAGTCACGCGAGGG</u> ccag <u>CTTCTACGCAACGC</u> gga...-3'	