OPTIMIZATION OF AN *IN VITRO* DNA-BINDING ASSAY FOR IDENTIFICATION OF PARTIAL AGONIST/ANATAGONISTS OF THE AH RECEPTOR

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Introduction

The aryl hydrocarbon receptor (AhR) is a transcription factor mediating toxic and biological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and dioxin-like compounds (DLCs). DLCs are wide-spread environmental contaminants that occur as by-products of chloro-organic synthesis, waste incineration, paper bleaching, metal smelting and other industrial processes. Due to their hydrophobicity, DLCs accumulate in the food chain, and humans can be exposed from fatty foods of animal origin, such as meat and dairy. Exposure to DLCs results in diverse toxic effects including hepatic toxicity, immunotoxicity, cancer promotion, disruption of lipid and glucose metabolism, carcinogenesis, teratogenesis, and mortality in some species[1]. Studies in mice and rats identify the AhR as the mediator of most toxic effects. Guinea pig is one of the most sensitive species to the toxic effects of TCDD. Hepatic cytosolic extract from guinea pig is capable of robust TCDD-dependent activation of DNA binding *in vitro*, and has been extensively used for characterization of putative AhR ligands[2]. A large number of structurally diverse compounds can bind to and activate the AhR[3]. Some compounds, such as alpha-naphthoflavone (ANF) or 3-methoxy-4-nitroflavone (MNF) demonstrate antagonist properties at lower concentrations but act as AhR agonists at higher concentrations[4-9]. The mechanism of this switch remains mostly unknown. It has been previously suggested that agonists and antagonists properties of ANF may differ in a species-specific manner. Specifically, one report found that ANF at 1 μM demonstrated agonist activity with gel retardation assay (GRA) of the guinea pig hepatic cytosol, while rat and mouse hepatic cytosols as well as cell-based assays detected antagonist properties at this concentration[2]. Moreover, mouse AhR and guinea pig AhR reportedly differed in antagonist properties of MNF, which is structurally related to ANF, and these differences were due to a R355I amino acid substitution within the PASB ligand binding domain[10]. Despite wide-spread use of the guinea pig cytosolic GRA for characterization of AhR ligands, limited number of studies have examined pure antagonists[11], and there are no reports of analyzing the activity of partial agonist/antagonists such as ANF in this experimental system. The compounds of this class are increasingly important as proposed therapeutics in various inflammatory conditions, as well as in ameliorating AhR-dependent toxicity. Therefore, there is a pressing need to optimize *in vitro* screening techniques, such as the GRA with guinea pig hepatic cytosol, for optimal detection of antagonist activity of these compounds.

Materials and methods

Preparation of cytosol. Male Hartley guinea pigs (400 g), C57BL mice (20 g) and C3H mice (20 g) were obtained from Charles River Laboratories (Wilmington, MA). All animals were exposed to 12 h light:12 h dark daily and given free access to food and water. Hepatic cytosol was prepared in HEDG (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiotreitol, 10% [v/v] glycerol) buffer as previously described[12]. Cytosol was stored at 80°C until use.
Gel retardation assay was performed as previously described for cytosolic preparations[12] and TNT synthesized AhR and ARNT[13], except 3-(N-morpholino)propanesulfonic acid (MOPS) was used in place of Hepes in MEDG buffer. Guinea pig cytosolic reactions were supplemented with 80 mM KCl where indicated, and TNT reactions were supplemented with 0.16 M KCl. KCl ammounts were adjusted at DNA-binding step acording to the referenced protocols.
Hydroxyapatite ligand binding assay. Cytosol diluted to 8 mg/ml in MEDG 0.1 M KCl was incubated in the presence of 10 nM [^{3}H]TCDD and increasing concentrations of ANF for 1 h, and specifically bound [^{3}H]TCDD was analyzed by the HAP assay[12].
Mutagenesis and cell culture. COS-1 cells were obtained from ATCC and maintained in αMEM (Sigma) supplemented with 10% FBS (Atlanta biologicals) at 37°C and 5% CO_2. Point mutations in the wild type mouse AhR expression plasmid mβAhR/pcDNA3[13] were made using point mutagenesis kit (Stratogene). Transient

transfections using Lipofectamine 2000 (Invitrogen) and lysate analysis using dual luciferase kit (Promega) were performed as previously described[14].

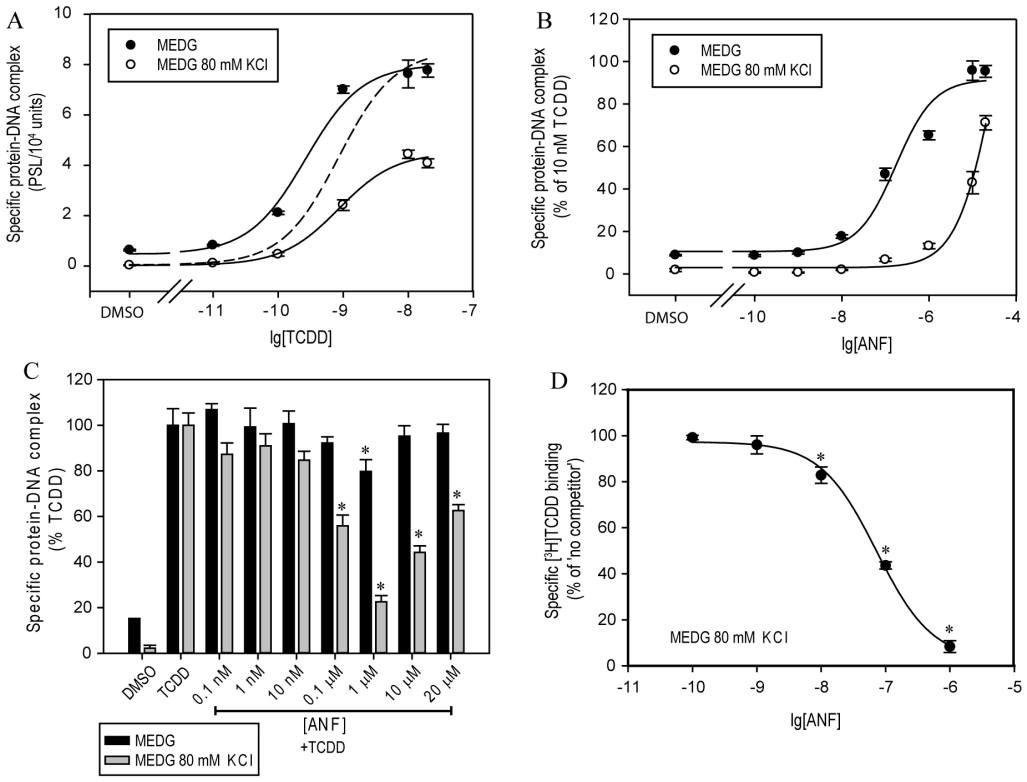

Figure 1. In vitro ANF antagonism with guinea pig cytosol. Guinea pig cytosol was diluted in MEDG -/+ 80 mM KCl and incubated in the presence of increasing TCDD concentrations (A), increasing ANF concentrations (B), 10 nM TCDD and increasing ANF concentrations for 1.5 h and analyzed by gel retardation assay. D. Guinea pig cytosol was incubated in the presence of 2 nM [^{3}H]TCDD and increasing concentrations of ANF for 1 h, and specifically bound [^{3}H]TCDD was analyzed by the HAP assay. (A-D). Results are presented as means ± standard deviations of three independent reactions. *, statistically different from 'TCDD' at P<0.05.

Results and discussion

Preliminary experiments found that ANF was a strong agonist in the guinea pig hepatic cytosol (data not shown). Varying experimental conditions determined that adding KCl into the dilution buffer affected ANF agonist and antagonist properties, with 80 mM optimal for binding analysis. Cytosolic samples were diluted, incubated in the presence of increasing concentrations of TCDD or ANF and analyzed by GRA. Addition of KCl into the dilution buffer decreased the overall intensity of the TCDD-dependent DNA-bound complex and slightly increased the EC_{50} of the TCDD-dependent response (Fig. 1A). Specifically, this value was increased from 0.28±0.05 nM to 0.82±0.03 nM, and the difference was statistically significant. To demonstrate the change in EC_{50} the activation curve in the presence of KCl was normalized to the maximal level of the 'MEDG' reaction (similar values at 10 µM) and plotted as a dash line (Fig. 1A). Addition of KCl had a more dramatic effect on ANF-dependent activation of DNA binding (Fig. 1B), shifting the curve to the right and resulting in a statistically significant increase in EC_{50} from 0.293±0.106 µM to 4.16±1.52 µM. Addition of KCl also resulted in a dramatic change in the antagonist properties of the AhR (Fig. 1C). The reactions were incubated in the presence of 10 nM TCDD (a maximal stimulatory concentration) and increasing concentrations of ANF prior to DNA-binding analysis. When cytosol was diluted with MEDG, little antagonist activity could be detected (there was a small statistical decrease at 1 µM relative to TCDD alone reaction (Fig. 1C)). However, in the presence of KCl, antagonism could be observed with an inverted bell-shaped curve, that is characteristic of partial agonists/antagonists such as ANF (Fig. 1C). The concentration range, at which ANF antagonistic response was observed, closely matched the ANF agonist range in the absence of KCl in dilution buffer (Fig. 1B). A hydroxyapatite ligand binding assay was utilized to examine competitive binding of ANF and [^{3}H]TCDD. The ANF [^{3}H]TCDD competitive displacement curve in the presence of KCl (Fig. 1D) closely matched the ANF antagonist profile. These findings suggest that ANF can bind to the AhR equally well in the presence or absence of KCl in the dilution buffer, and that this

binding results in antagonism. However, ANF exhibited primarily agonist activity in the absence of KCl and primarily antagonist activity in the presence of KCl. The ANF antagonist activity closely correlated with its ability to displace [^{3}H]TCDD (compare Fig. 1C to Fig 1D) indicating that ANF antagonism may be simply due to ANF binding within the ligand binding pocket without triggering AhR transformation. This conclusion is consistent with previous studies[8, 9]. The KCl-dependent agonist-to-antagonist switch indicated that the guinea pig AhR is capable of ANF antagonism *in vitro*, but that it was likely masked by ANF agonist activity in the absence of KCl. Addition of KCl inhibited ANF agonism to a larger extent than TCDD-dependent agonism, and the net effect was de-masking of ANF antagonist activity. This KCl-dependent change in the properties of partial agonist-antagonist was also observed for other compounds such as MNF, but pure AhR antagonists CH-223191 and 6,2',4'-trimethoxyflavone[15, 16] demonstrated antagonist response with either dilution buffer (data not shown). These findings suggest that modification of the existing guinea pig GRA protocol with the addition of KCl into dilution buffer may be useful for detection and characterization of partial agonist/antagonists of the AhR. One previous study proposed that mouse and guinea pig AhRs may differ in antagonist properties of MNF and that the specific mutation R355I was responsible for these changes[10]. Since MNF is similar in structure to ANF, one might expect a similar mutation-specific effect on ANF activity with the AhR. To test this possibility, the R355A and R355I mutations of the mouse AhR were generated and tested in transient transfections (in COS-1 cells) with the guinea pig AhR and mouse AhR, as controls (Fig. 2A, B). Following incubation with TCDD (10 nM) and/or ANF (1 µM) for 20 h, the luciferase activity of a DRE-reporter was normalized to Renilla luciferase activity of an internal control in cell lysates. This experiment revealed that although absolute levels of activity differed (Fig. 2A), there were no differences among relative ANF agonist and antagonist activity among the wild type and mutant AhRs (Fig. 2B). This suggests a lack of effect of these mutations on ANF properties, and more generally, a lack of interspecies differences in ANF properties between mouse and guinea pig AhRs. The differences with the previous study[10] may be due to a different compound used (MNF) and/or different experimental system. The ANF agonist and antagonist properties were further examined with GRA protocol using the C57BL and C3H mouse hepatic cytosol, as well as the *in vitro* synthesized AhR and ARNT (using TNT system). As expected, C57BL demonstrated dramatically lower TCDD-dependent protein-DNA complex formation relative to C3H and TNT-expressed proteins (Fig. 3A). ANF possessed strong agonist activity with TNT-expressed proteins (reaching 100% of TCDD levels) and weaker agonist activity with C57BL and C3H AhRs (Fig. 3B). However, despite the presence of KCl in the reaction, TNT-expressed AhR demonstrated a lack of ANF antagonist activity, while either mouse cytosolic system demonstrated strong ANF antagonism even without any added KCl (Fig. 3C). These findings indicate expression system-specific effects on ANF agonist and antagonist activities, since the C57BL and TNT AhR would have identical primary sequence, despite distinct ANF antagonist properties. The differences in ANF

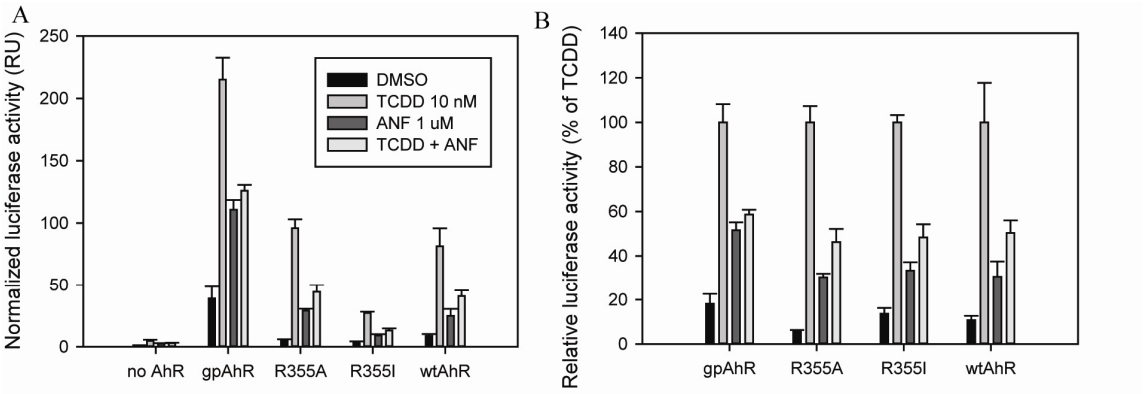

Figure 2. Antagonism with point mutations of mouse AhR. Indicated guinea pig AhR, wild type or mutated mouse AhR plasmid was co-transfected with DRE-responsive firefly luciferase reporter and internal control (Renilla luciferase) into COS-1 cells. Cells were treated for 24 with indicated concentrations of TCDD and/or ANF, or solvent control (DMSO), and luciferase activity of cell lysates was analyzed.
A. Absolute activity normalized to internal control. B. Relative activity normalized to TCDD.

properties between the TNT-expressed AhR and C57BL cytosolic AhR could be utilized to further elucidate the ANF-dependent mechanisms. However, the TNT experimental system would not be useful in identification and characterization of antagonist properties of ANF and similar compounds *in vitro*. In contrast, either guinea pig cytosol in the presence of KCl or C3H mouse cytosol without added KCl demonstrated high level of DNA binding and antagonist properties of ANF, indicating potential applicability of these methods for *in vitro* screening protocols in detection and characterization of partial AhR agonist/antagonists.

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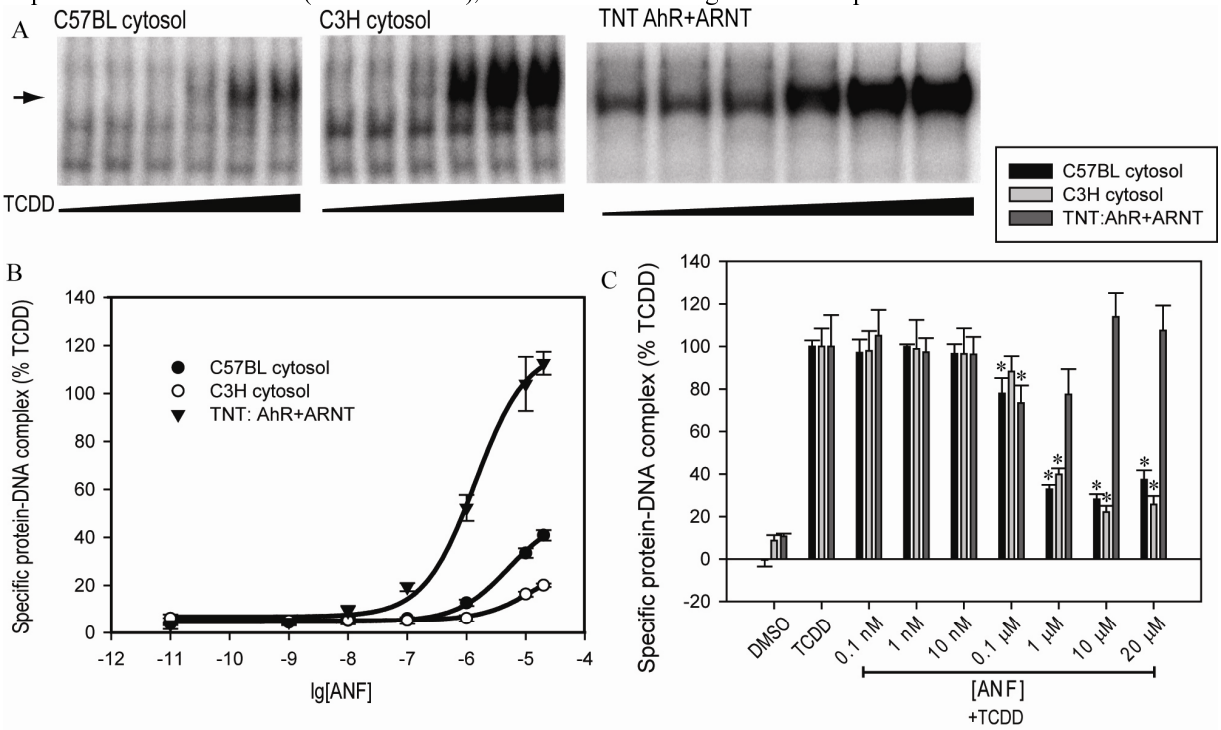

Figure 3. Variability in ANF antagonist properties among mouse AhR experimental systems *in vitro*. Mouse C57BL or C3H hepatic cytosol was diluted to 8 mg/ml in MEDG; mouse in vitro synthesized AhR and ARNT were diluted at 1:1:8 in MEDG 0.2 M KCl. Reactions were incubated in the presence of increasing TCDD concentrations (A), increasing ANF concentrations (B), and increasing ANF concentrations and/or TCDD (10 nM) (C) for 2.5-3 h at room temperature and analyzed by gel retardation assay. The values are means +/- standard deviations of three independent reactions; *, statistically different from 'TCDD' at $P<0.05$ as determined by the Student's t-test.

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