DEVELOPMENT AND CHARACTERIZATION OF A GREEN FLUORESCENT PROTEIN-BASED RAT CELL BIOASSAY SYSTEM FOR DETECTION OF AH RECEPTOR LIGANDS

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that regulates expression of a battery of genes in a wide range of species and tissues.[1] Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the prototypical and most potent halogenated aromatic hydrocarbon (HAH), and related compounds produces a diverse array of species- and tissue-specific toxic and biological effects.[2] Mechanistically, the inducing chemical enters the responsive cell and binds to the cytosolic AhR. Following ligand binding, the AhR appears to undergo a conformation change that exposes a nuclear localization sequence(s), resulting in translocation of complex into the nucleus. Release of the ligand:AhR from this complex and its subsequent dimerization with a related nuclear protein called Arnt converts the AhR into its high affinity DNA binding form. Binding of the heteromeric ligand:AhR:Arnt complex to its specific DNA recognition site, DRE, upstream of the CYP1A1 and other AhR-responsive genes stimulate transcription of these genes.[1,2]

Proper epidemiological, risk assessment and exposure analysis of TCDD and related HAHs requires accurate measurements of these chemicals both in the species of interest and in various exposure matrices (i.e. biological, environmental, food and feed). While high-resolution instrumental analysis techniques are established for these chemicals, these procedures are very costly, time-consuming and are impractical for large scale sampling studies.[3] Accordingly, numerous bioanalytical methods have been developed for the detection of these chemicals in extracts from a variety of matrices, the majority of which take the advantage of the ability of these chemicals to activate one or more aspects of the AhR-dependent mechanism of action.[4,5] One of the most sensitive bioassay systems developed to date is the so-called CALUX (Chemically Activated Luciferase Expression) assay, which is based on novel recombinant cell lines that contain a stably transfected dioxin (AhR)-responsive firefly luciferase gene. Treatment of these cells with TCDD and related HAHs and polycyclic aromatic hydrocarbons (PAHs), as well as other AhR ligands, results in induction of reporter gene expression in a time-, dose-, AhR-, and chemical-specific manner.[5,6,7] The level of reporter gene expression correlates with the total concentration of the TCDD-like AhR inducers (agonists) present in the sample.[4,5,6,7] Although the firefly luciferase reporter gene contributes to the high degree of sensitivity of the assay, it also has limitations with

respect to our need for a rapid and inexpensive bioassay for high-throughput screening analysis. Accordingly, we previously developed a stably transfected murine cell line containing an AhR-responsive enhanced green fluorescent protein (EGFP) reporter gene.[6] This cell line provided us with a high-throughput cell bioassay system for identification and characterization of AhR agonists and antagonists.[6,8] Here we have extended these studies and describe the development, optimization, and characterization of a stably transfected rat hepatoma (H4II4) cell line that responds to AhR agonists with the induction EGFP. This so-called CAFLUX (Chemically Activated Fluorescent Expression) assay not only has the same sensitivity and chemical specificity as our previously described luciferase-based cell bioassays, but it is easier, more rapid and less expensive, and reporter gene activity can be measured in "real time."

Materials and Methods

Chemicals: TCDD was from Dr Steven Safe (Texas A&M University, USA) and all other HAHs and PAHs were from Accustandards (New Haven, CT).

Construction of EGFP expression vector: The expression vector pGreen1.1 was created by excising the 1846 base-pair (bp) Hind III fragment from the plasmid pGudLuc1.1. This fragment contains the 480-bp dioxin-responsive domain from the mouse CYP1A1 gene inserted upstream of the mouse mammary tumor virus (MMTV) promoter and it confers dioxin responsiveness upon the MMTV promoter and adjacent reporter gene. This fragment was inserted into the Hind III site immediately upstream of the EGFP reporter gene in the plasmid pEGFP-1 (Clontech, Palo Alto, CA).[6]

Cell culture and stable transfections: The rat hepatoma (H4IIE) cell line was maintained in alpha minimum essential medium (MEM) containing 10% fetal bovine serum to 95% confluent (non-selective media). Cells were harvested by trypsinization and resuspended in 500 µl media. Cell suspension was mixed gently with 10 µg of the reporter construct pGreen1.1 DNA by pipetting and transferred to electroporation cuvette. After electroporation cells were placed on ice for 10 minutes before they were split to 10 plates in nonselective media. After 2 days, non-selective media was replaced by selective media (containing 500 mg/l of the antibiotic G418). After growth in selective media for 4 weeks, resistant clones were isolated and screened for the induction of EGFP expression by TCDD (1 nM for 24 h). Clones exhibiting the highest ratio of inducible to constitutive EGFP expression were further characterized.

Characterization and optimization of novel cell line for bioassay: One clonal cell line, H4G1.1c2, was selected for further characterization and optimization for microtiter plate (96-well plate format) high throughput analysis of EGFP in intact cells. H4G1.1c2 cells were maintained in selective media. Cells were plated into black clear-bottomed 96-well microplates at 150,000 cells per well and allowed to attach for 24 h. After 24 hours, the media was replaced with non-selective media containing the chemical to be tested. In each plate, wells containing media only, or cells incubated with DMSO and 1nM TCDD were used as blank, negative control and positive control, respectively. After 24 hours of incubation at 33°C, EGFP activity was measured on a Tecan GENios microplate fluorometer with excitation and emission wavelengths of 485 and 515 nm respectively. Dose response relationship analyses for all positive chemicals were carried out and

induced EGFP activity was determined by subtraction of EGFP activity contained in the DMSO negative control.

Results and Discussion

The EGFP reporter gene is a modified form of the green fluorescent protein isolated from *Aequoria victoria* that has been optimized for mammalian expression.[6,9] Rat hepatoma (H4IIE) cells were stably transfected with pGreen1.1, and the induction of EGFP by TCDD in the isolated stably transfected clones was determined. TCDD responsiveness of ~40 initial cell clones was tested, with 18 clones responding to TCDD with a significant induction of EGFP activity. Given the greater degree of TCDD-responsiveness of clone 2, this clonal cell line was further characterized and is hereafter referred to as the H4G1.1c2 cell line.

We have previously reported that induction/expression of EGFP activity is temperature sensitive, with transiently transfected cells grown at 30–33°C exhibiting higher fluorescence than those grown at 37°C.[6] To determine whether the similar temperature dependence is observed with EGFP and to examine the time course of EGFP induction, we examined TCDD-inducible EGFP expression in H4G1.1c2 cells grown at temperatures between 26°C and 37°C. After 24 h of treatment, induction of EGFP activity in cells grown at 33°C was significantly higher than that at all other temperatures (data not shown), which presumably results from low rates of EGFP formation and/or protein folding at temperatures higher or lower than 33°C. The mechanism(s) responsible for this temperature-dependence remains to be investigated.

Based on the need to develop these cells for a high-throughput bioassay, we next examined TCDD-induction of EGFP activity in cells grown in microtiter plates. H4G1.1c2 cells were incubated with DMSO or TCDD (1 nM) for 24 h and the EGFP expression determined (Table 1). TCDD treatment resulted in a 2.4-fold induction of fluorescence, while no change in fluorescence was observed in untransfected hepa1c1c7 cells. These data indicate that the increase in fluorescence is due to the induction of EGFP expression and not to a change in fluorescence of endogenous cellular component(s) in the cells themselves. In addition, these results indicate that approximately 90% of the background fluorescence is derived from components present in the medium. Subtraction of this background fluorescence from all samples revealed that TCDD treatment of H4G1.1c2 cells results in a 15-fold induction of EGFP expression (Table 1).

One major application of this cell bioassay is for identification and characterization of new AhR agonists. Accordingly, we optimized the microplate bioassay. Treatment of these cells with TCDD resulted in induction of reporter gene expression in a time and dose manner (Figure 1). Kinetic analysis revealed that this bioassay had a minimal detection limit (MDL) of 1 pM, an EC50 of 12 pM, and maximal induction at 300 pM, which was identical to the bioassay based on the recombinant mouse cell line, H1G1.1c3, previously developed in our lab.[6]

To confirm the induction of EGFP expression was AhR-dependent and to establish the relative potency of AhR ligands, we examined the ability of selected HAHs and PAHs to induce EGFP expression in H4G1.1c2 cells. The relative rank order potency for HAHs in these cells (Figure 2A) was comparable to our previous results with the luciferase responsive H1L1.1c2 cells and they correlate well with the rank order of potency of these chemicals to bind to and activate AhR.[7] Our

results for the PAHs (Figure 2B) demonstrate the greater induction potency of dibenz(a,h)anthracene (DB(a,h)A) and benzo(k)fluoranthene (BKF) as compared to benz(a)anthracene (BAA) and benzo(a)pyrene (BAP), and are consistent with our previous studies using PAHs.[7] In addition, results from both HAHs and PAHs were identical to the results from the bioassay based on the stably transfected mouse cell line, H1G1.1c3.[6] Overall, these results demonstrated the utility of this cell line as a bioassay system for the detection of chemicals that can activate the AhR signal transduction pathway.

Although the mechanism of AhR activation and minimal detection limit of CAFLUX bioassay are comparable to that of the CALUX bioassay, EGFP has a significant advantage over luciferase as a reporter gene, because it can be repeatedly measured directly in intact living cells in microplates at any selected time point without need for removal of media. This aspect not only results in lower cost (no expensive lysis or reporter gene substrates are needed) and ease and rapidity of measurement (within 1 minute), but it makes the CAFLUX bioassay ideally suited for very high-throughput sample analysis. We have previously developed and validated a recombinant mouse hepatoma cell line containing a dioxin-responsive EGFP reporter gene and have used for analysis of a combinatorial chemical library of ~12500 chemicals.[6, 8] Overall, we have generated and characterized a novel recombinant rat cell line containing the EGFP reporter gene, which will facilitate screening and characterization of AhR agonists rapidly and efficiently. More important both CAFLUX high-throughput bioassay systems developed in our lab will provide an avenue to rapidly screen and identify compounds that may produce species-specific effects.

Acknowledgements

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Table 1. Comparison of the green fluorescent emission from DMSO or TCDD-treated H4G1.1c2 and H4IIE cells.

Treatment	Relative fluorescence[a]	Fold induction[b]	Corrected fluorescence[c]	Fold induction
H4G1.1c2				
DMSO	1394.2 ± 15.5		136 ± 15.5	
TCDD	3327.3 ± 49.0	2.4	2069.1 ± 49	15
H4IIE				
DMSO	1221.8 ± 23.1		-37.6 ± 23.1	
TCDD	1240.8 ± 23.1	1	-17.4 ± 23.1	1
Media alone				
NT[d]	1258.2 ± 10.3		0 ± 10.3	

[a]Values represent the mean ± standard deviation of 3 replicate samples.

[b]Values represent the fold induction of fluorescence over that of the DMSO sample.

[c]Values represent the fluorescence activity after subtraction of fluorescence derived from the "Media-alone" sample.

[d]Not treated (NT).

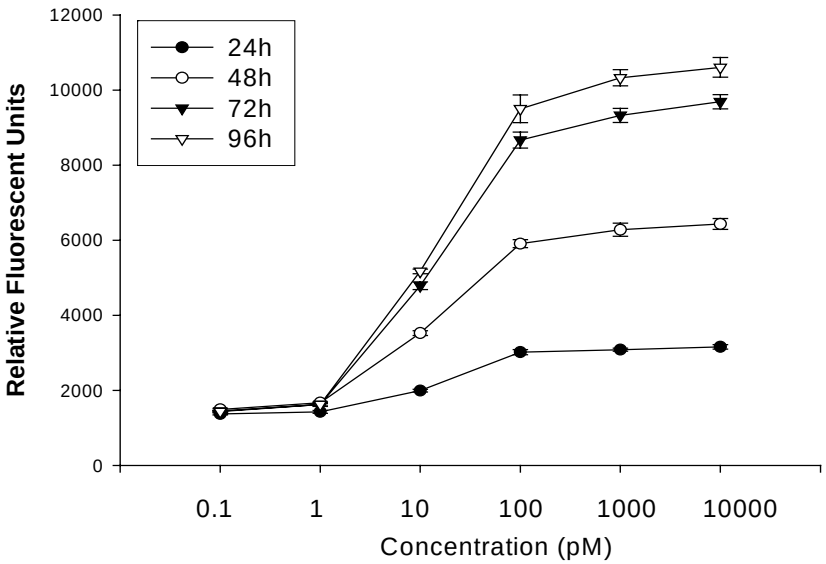

Figure 1. Effect of TCDD dose and incubation time on induction of EGFP gene expression in H4G1.1c2 cells

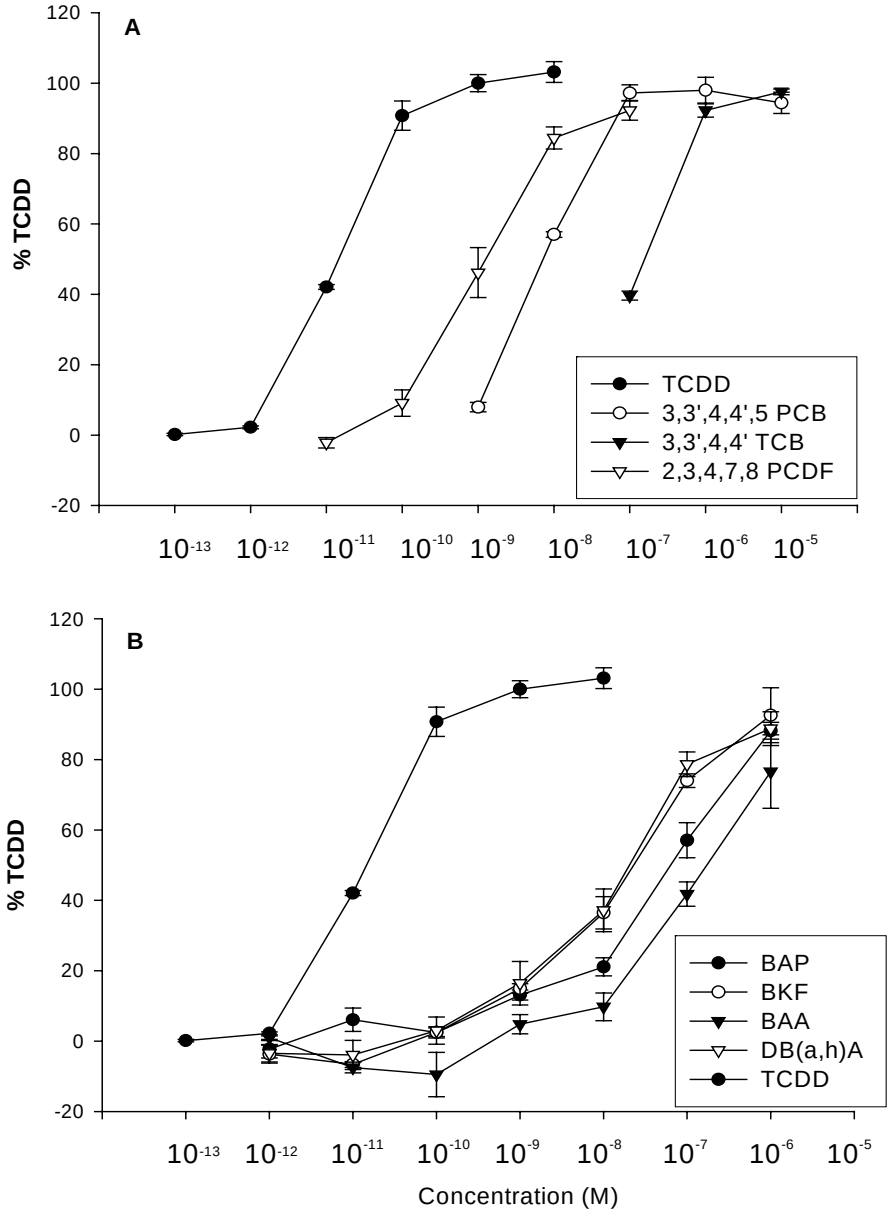

Figure 2. Dose response relationship for EGFP induction by selected HAHs (A) and PAHs (B).