Chapter 13

Cell-Based Assays for Identification of Aryl Hydrocarbon Receptor (AhR) Activators

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Abstract

The Ah receptor (AhR) is a ligand-dependent transcription factor that mediates a wide range of biological and toxicological effects from exposure to structurally diverse synthetic and naturally occurring chemicals. The role of the AhR and its signaling pathway in endogenous physiological functions and its involvement in immune cell development and human diseases has made it a target for development of therapeutic agents. The ability of the AhR to stimulate gene expression in a ligand-specific manner in recombinant mammalian cell lines containing a stably transfected AhR-responsive firefly luciferase or enhanced green fluorescent protein (EGFP) reporter gene permits high throughput chemical screening for AhR activators. The induction of luciferase activity or EGFP fluorescence in these readily available recombinant cell lines occurs in a time-, dose- and AhR-dependent and chemical-specific manner where the magnitude of reporter gene induction is directly proportional to the concentration and potency of the inducing chemical. The AhR agonist activity of positive test chemicals can be confirmed by demonstrating their ability to stimulate expression of CYP1A1, an endogenous AhR-responsive gene, using quantitative real-time PCR. The detailed protocols described here provide step-by-step instructions for detection and characterization of activators of AhR-dependent gene expression that can readily be applied to other appropriate cell lines.

Key words Ah receptor, CALUX, CAFLUX, Luciferase, Green fluorescent protein, Quantitative real time PCR

1 Introduction

The Ah receptor (AhR) is a ligand-dependent transcription factor that not only mediates the induction/repression of expression of a large battery of genes, but it plays a key regulatory role in the production of a broad spectrum of species- and tissue-specific toxic and biological effects of selected AhR ligands [1–6]. Because of their high affinity for the AhR, their metabolic stability and toxicity at extremely low concentrations, halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin)

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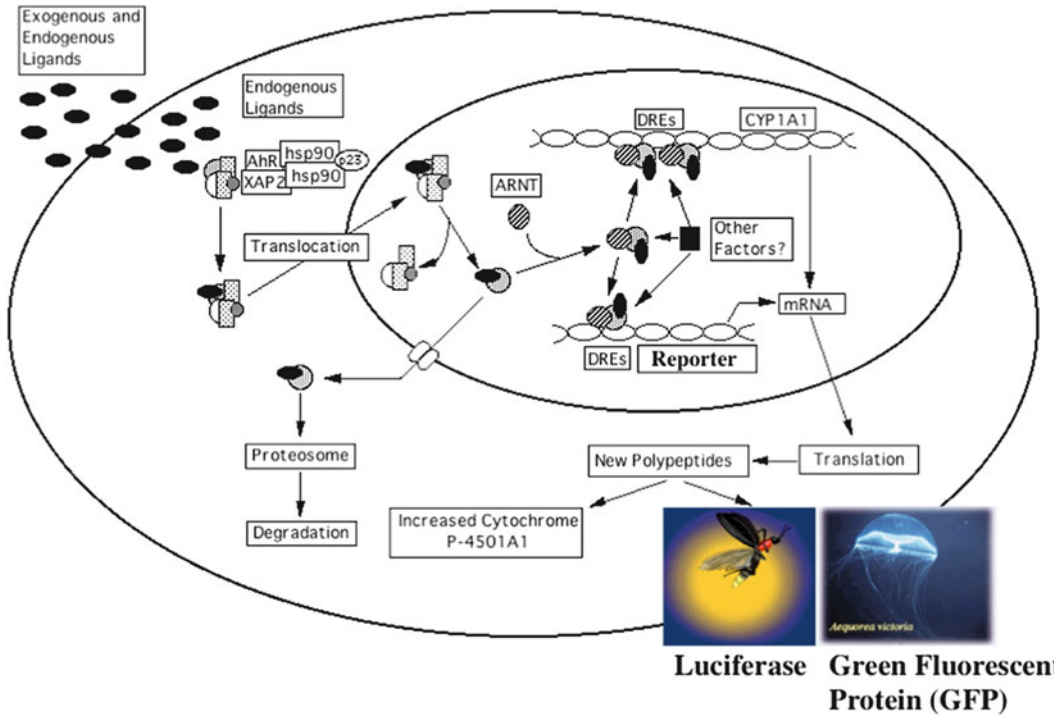

Fig. 1 Mechanisms of Ah receptor (AhR) activation of gene expression and basis for CALUX and CAFLUX cell bioassays. See references [6, 20] for details

and related metabolically stable dioxin-like compounds (DLCs), represent the best studied of all AhR agonists. The toxicity of DLCs appears to be directly related to their metabolic stability and ability to stimulate persistent activation of AhR-dependent gene expression. Consistent with this idea is the recent demonstration that the AhR can bind and be activated by a wide variety of structurally diverse compounds, the majority of which are metabolically labile and do not produce the spectrum of AhR-associated toxic effects [3, 6, 7]. However, all of these compounds stimulate AhR-dependent gene expression by a common mechanistic pathway similar to that of steroid hormones and their receptors [8]. In this mechanism (Fig. 1), the inducing chemical (AhR agonist) diffuses across the plasma membrane of a responsive cell and its specific binding to the cytosolic AhR stimulates nuclear translocation of the AhR protein complex. Once in the nucleus, the AhR is released from its associated proteins following its dimerization with a related nuclear protein called Arnt (AhR nuclear translocator), and formation of the AhR:Arnt heterodimer converts the ligand AhR complex into its high affinity DNA binding form. Binding of the ligand:AhR:Arnt complex to its specific DNA recognition site, the dioxin responsive element (DRE), adjacent to a responsive gene, leads to chromatin disruption and stimulation of transcription of a

wide variety of such responsive genes including CYP1A1 and others encoding phase I and phase II drug/xenobiotic metabolizing enzymes [2, 4, 6].

While early studies were focused on the role of the AhR in mediating the biochemical response to xenobiotics (adaptive induction of metabolic enzymes) and the toxic effects of selected AhR ligands, recent studies have identified key endogenous regulatory roles for the AhR in normal human physiology, tissue and immune cell development and disease [2, 9–14]. These and other related studies identify the AhR as a potential target for the identification and development of therapeutic agents for treatment of several maladies including autoimmune disease, various inflammatory conditions and cancer [13, 15–17]. However, while a wide variety of ligands for the AhR have been identified, few chemicals are suitable for development into useful therapeutic agents. Therefore, the identification of activators of the AhR and AhR signaling pathway is critically needed for such development.

The molecular mechanism of the AhR signaling pathway (ligand binding, DNA binding and nuclear translocation) have been used to develop AhR-based bioassays, the most extensively used bioanalytical approach for detection and characterization of AhR ligands [18–22]. In these systems, incubation of cells in culture for varying lengths of time with test chemicals or extracts containing AhR-agonists leads to the induction of AhR-dependent gene expression that occurs in a time-, chemical-, concentration- and AhR-dependent manner. While numerous AhR-based cell bioassays have been described [18–25], the chemically activated luciferase expression (CALUX) and chemically activated fluorescent expression (CAFLUX) bioassays (Fig. 1), which utilize recombinant cell lines that contain stably transfected AhR-responsive firefly luciferase or enhanced green fluorescent protein (EGFP) reporter genes, respectively, have been extensively used [18, 20, 21, 23]. Treatment of these cells with AhR ligands results in induction of reporter gene activity that is directly proportional to the concentration and potency of the inducing chemical (i.e. its AhR agonist activity) [20, 21, 23]. Although these systems use the same AhR-dependent induction, differences in the characteristics of the respective reporters result in bioassay systems with distinct advantages and disadvantages [20, 21, 24]. While the firefly luciferase reporter gene in the CALUX bioassay system is highly sensitive and responsive, primarily due to enzymatic signal amplification, it has limitations with respect to repeated measurement, relatively high cost for reagents and rapidity for high-throughput screening analysis. By contrast, measurement of EGFP reporter gene activity is more rapid, cost effective, amenable to high throughput and repeated analysis of the same cells and the induction response can be measured in “real time” [20, 24].

While the CALUX and CAFLUX bioassays have been used extensively for the relatively rapid screening and assessment of the ability of a chemical(s) to stimulate AhR-dependent gene expression, positive results obtained in these assays are often criticized for not directly demonstrating induction of an endogenous AhR-responsive gene (although we have never identified a CALUX-/CALFUX- positive chemical that does not stimulate expression of an endogenous AhR-responsive gene). Accordingly, to confirm this, additional analysis is often needed to demonstrate that a CALUX- or CAFLUX-positive chemical can direct stimulate CYP1A1 gene expression in cells in culture. This is accomplished by measuring CYP1A1 mRNA levels through quantitative real-type PCR (Q-RT-PCR). Together, the combined results from both CALUX/CAFLUX and Q-RT-PCR assays provide strong evidence for the AhR agonist activity of the positive test chemical. The detailed protocols described here provide step-by-step instructions for rapid screening, detection and characterization of activators of AhR-dependent gene expression using CALUX and CAFLUX cell lines and confirmatory analysis using Q-RT-PCR. Additional analysis of positive test chemicals carried out using in vitro AhR ligand binding [26] or DNA binding (gel retardation) (*see* Chap. 12 and [26]) assays can confirm that the test chemical can directly interact with and activate the AhR.

2 Materials

2.1 Equipment

General purpose equipment can be substituted with comparable models from other companies.

1. Laminar flow hood and CO_2 incubator for cell culture.
2. Inverted microscope to examine cells in tissue culture plates.
3. Centrifuge with swinging-bucket rotor for pelleting cells for plating.
4. Benchtop microcentrifuge for preparation of cell lysates for PCR.
5. Orbital shaker platform (such as a Belly Dancer shaker, Denville Scientific, Denville, NJ) for cell lysis.
6. Microplate Luminometer (Anthos Lucy II, Salzburg, Austria or equivalent) with pumps for automatic addition of luciferin reagent to plate wells.
7. Fluostar Microtiter Plate Fluorometer (Phoenix Research Products, Candler, NC or equivalent) with an excitation and emission wavelengths of 485 and 515 nm, respectively.
8. Applied Biosystems 7500 Fast Sequence Detection System (Life Technologies, Grand Island, NY).

2.2 Reagents and Materials

2.2.1 Luciferase/EGFP Reporter Gene Bioassay

1. Trypsin (1×), tissue culture grade, sterile.
2. Phosphate-buffered saline (PBS; 1×), sterile.
3. Alpha-Minimal Essential Media (MEM; Invitrogen, #12000-063) containing 10 % prescreened (**Note 4.1.1**) fetal bovine serum (FBS; Atlanta Biologicals, #S11150).
4. Tissue culture microplates: Sterile 96-well microplates for cell growth and luciferase analysis (white, clear bottom tissue culture microplate for luciferase (Fisher, #07-200-566)) or EGFP analysis (black, clear bottom tissue culture microplate (Fisher, #07-200-565)).
5. Promega Luciferase Assay Lysis Buffer, 5× stock (Fisher, #PR-E1531).
6. Promega Luciferase Assay System (Fisher, #PR-E1501).
7. Microplate white backing tape (PerkinElmer, #6005199).
8. AhR agonist stock solutions (TCDD in DMSO (**Note 4.1.2**) or other non-toxic AhR agonist in DMSO (**Note 4.1.3**)).

2.2.2 Quantitative Real Time PCR Reagents and Materials

1. Trizol reagent (Invitrogen Life Technologies, Grand Island, NY).
2. High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) or High Capacity cDNA Reverse Transcription Kit.
3. TaqMan Fast Universal PCR Master Mix (no AmpErase UNG) (2×).
4. TaqMan Gene Expression assays (20×).

3 Methods

3.1 Reporter Gene Bioassays

3.1.1 Protocol for Plating Cells into 96-Well Plate

1. General maintenance of cell cultures. Continuous cell lines containing a stably transfected AhR-responsive luciferase reporter gene (mouse H1L6.1c2 (Hepa1c1c7) and human HG2L6.1c3 (HepG2) hepatoma cells [21, 27]) or enhanced green fluorescent protein reporter (EGFP) gene (rat H4G1.1c2 (H4IIe) or mouse H1G1.1c3 (Hepa1c1c7) hepatoma cells [24, 28]) are maintained in alpha-MEM containing 10 % fetal bovine serum. Cells should not exceed 90 % confluence before passaging and cells should be examined prior to trypsinizing and plating.
2. Remove old media from plates by aspiration, rinse cells with PBS, add trypsin solution and incubate for 2–4 min at room temperature or 37 °C (until cells detach from the plates).
3. While the cells are being trypsinized, add a volume of medium/serum to a 50 mL sterile Falcon tube that is at least equal to the volume of trypsin that you will add from the

plates (serum contains trypsin inhibitors). Depending on the number of tissue culture plates, more tubes can be used.

4. Transfer the contents of the culture plate(s) into the Falcon tube(s).
5. Cap and spin cell suspension in a benchtop centrifuge at room temperature for 5 min at 1,100 rpm.
6. In the tissue culture hood, carefully aspirate the media/serum from the centrifuged tubes. Add 10 mL fresh medium/serum to each tube and gently re-suspend the cells (**Note 4.1.4**).
7. An aliquot (10 μL) of re-suspended cells are counted using a hemocytometer or Coulter counter. For the bioassay, the optimal cell density in the 96-well plate format is 750,000 cells/mL, and the counted/re-suspended cells are diluted to this concentration with medium/serum.
8. Add an aliquot of diluted cell suspension (100 μL) to each well of a 96-well plate (clear-bottomed white plate for luciferase analysis or clear-bottomed black plate for EGFP analysis) using a cell trough and multichannel pipette. Cells are typically allowed to attach and grow for 24 h in a tissue culture CO_2 incubator at 37 °C prior to chemical treatment. This typically results in wells containing cells of at least 95 % confluence. Wells should be examined microscopically prior to addition of chemical to ensure that each contains comparable numbers of cells (**Note 4.1.5**).

3.1.2 Protocol for Treating Cells with Test Chemicals

1. In a tissue culture hood, sterilely prepare chemical treatments at the desired concentration at a 1:100 ratio of chemical/sample/control to medium (i.e. 10 μL of test sample is diluted into 990 μL medium) in 7 mL borosilicate glass tubes (autoclaved and baked). Vortex all treatments for several seconds to ensure complete mixing. This volume is sufficient to treat multiple wells, with most analyses carried out in triplicate. In addition to the test samples, each plate contains negative controls (i.e. DMSO and/or solvents used for the test samples), positive controls (i.e. a potent AhR agonist like TCDD (in DMSO) or other AhR agonist) and method blanks (if extracts or mixtures are being examined).
2. Prior to addition of sample treatments, the medium in the 96-well plate(s) can be dumped out with shaking into an appropriate biological waste container containing absorbent material (i.e., bench diaper or paper towels), taking care not to contaminate the cells during this process but to remove as much medium as possible.
3. Carefully fill the appropriate wells of a 96-well microtiter plate with 100 μL of the desired chemical/medium:serum suspension and make sure to note each treatment well.

4. Place the lid(s) back on the treated plate(s) and quickly examine each well microscopically to ensure that cells were not lost during washing and treatment. The plates are placed into a 37 °C incubator for the desired time (4–24 h typically). Alternatively, cells can be incubated with chemicals at 33 °C, which we have found to result in significantly higher luciferase activity and EGFP fluorescence than that obtained at 37 °C ([28]; **Note 4.1.6**).

3.1.3 Measurement of Reporter Gene Activity

Luciferase Activity in Lysed Cells

1. Visually examine each well in the 96-well plate under the microscope for cell toxicity (cells rounding up and detaching from the plate) and for cloudiness or a change in color of the media (i.e., precipitation of the test chemicals or culture components or a change in media pH, respectively). If toxicity is observed, the results should be discarded and test chemicals retested at lower concentrations.
2. Dump medium from 96-well plate(s) into appropriate biological waste container as described in 3.1.2.
3. Carefully rinse the wells twice with 100–150 μL PBS and gently dump the liquid into the waste container.
4. Microscopically examine the cells in each well under the microscope to ensure that they were not lost during PBS washing. Firmly tap the inverted plate onto paper towels to remove any remaining PBS.
5. Add 50 μL of room temperature Promega lysis buffer (1×) to each well. For each 96-well plate, prepare the 1× lysis buffer by mixing 1 mL of 5× lysis buffer with 4 mL of MilliQ water and store in glass.
6. Transfer the plate onto an orbital shaker platform (such as a Belly Dancer table) and shake at a moderate speed for at least 20 min to ensure adequate cell lysis.
7. While the cells are lysing, prepare the luminometer and prime the reagent pumps with luciferase substrate. Mix one bottle of room temperature luciferase buffer with one bottle luciferase substrate (buffer and luciferase substrate (luciferin) are from the Promega Luciferase Assay System) and use it to prime the luminometer pumps. Apply white backing tape to each plate containing lysed cells and insert the plate into the luminometer. The luminescence in each well is measured (integrating luminescence over 10 s after a 10 s delay) following automatic injection of Promega stabilized luciferase reagent.
8. Luciferase activity is typically expressed as a percent of the maximum level of induction in a defined number of cells (**Note 4.1.7**) that is produced by a potent AhR agonist such as TCDD (1 nM for rodent cells and 10 nM for human cells) or other AhR agonist (**Note 4.1.8**).

EGFP Fluorescence in Intact Cells

1. EGFP fluorescence levels are measured in intact cells in each well (without the removal of medium), using a microplate fluorometer with excitation and emission wavelengths of 485 and 515 nm, respectively (**Note 4.1.9**).
2. Following fluorescence measurements, the microplate can be returned to the tissue culture incubator and EGFP levels repeatedly measured in the same cells at later times (**Note 4.1.10**).
3. EGFP activity is typically expressed as a percent of the maximum induction fluorescence produced by a potent AhR agonist such as TCDD (1 nM) or other AhR agonist (**Note 4.1.8**).

3.2 Quantitative Real Time PCR Bioassays

*3.2.1 Isolate Total RNA from Cultured Cells (**Note 4.2.1**)*

1. Remove culture medium from plates. Optional: rinse cells with PBS. Tip plates to drain and remove liquid.
2. Add 1 mL Trizol to a 6 cm dish and mix the cells with the Trizol using a scraper (**Note 4.2.2**). If the sample is very viscous and is not sheared well by pipetting up and down through the pipet tip, shear the genomic DNA using a syringe with a 22 gauge needle until the viscosity is reduced (3–4 passes through the needle). Transfer to a microfuge tube and store at −80 °C.
3. Thaw the samples and microfuge them for 10 min at 10,000 × *g*. All centrifugation steps for the rest of protocol are at 4 °C.
4. Transfer the supernatant to a new tube; add 0.2 mL of chloroform and vortex well. Centrifuge for 10 min at 10,000 × *g*.
5. Transfer the top (aqueous) phase to a new tube, taking care to avoid removing any precipitated interphase material. Add 0.5 mL of isopropanol to the aqueous phase, incubate for 10 min at room temperature, and centrifuge for 10 min at 10,000 × *g*.
6. Remove the supernatant with a pipettor or by decanting. Rinse the pellet with 70 % ethanol and re-centrifuging briefly. Remove the supernatant, then re-centrifuge again and remove the remaining liquid with a pipettor. Air-dry the pellet briefly (about 5 min).
7. Dissolve the pellet in 10–50 μL of diethyl pyrocarbonate-treated water. Determine the concentration spectrophotometrically (A^{260}). Store at −80 °C (**Note 4.2.3**).

3.2.2 Prepare the cDNA

1. Mix the following ingredients for one sample: sterile water (4.2 μL), 10× reverse transcriptase buffer (2 μL), 25× dNTP mix (0.8 μL), random primers (2 μL) and reverse transcriptase (1 μL). If you have ten samples, multiply the amounts by 11, enough for each sample plus some extra to cover small pipetting errors.
2. Mix 10 μL aliquots of the above cocktail with 10 μL samples of RNA diluted to 0.2 μg/μL (2 μg total RNA per sample). Incubate for 10 min at 25 °C, then 2 h at 37 °C. Dilute the sample 1:8 with sterile water (140 μL water) and store at −20 °C until needed.

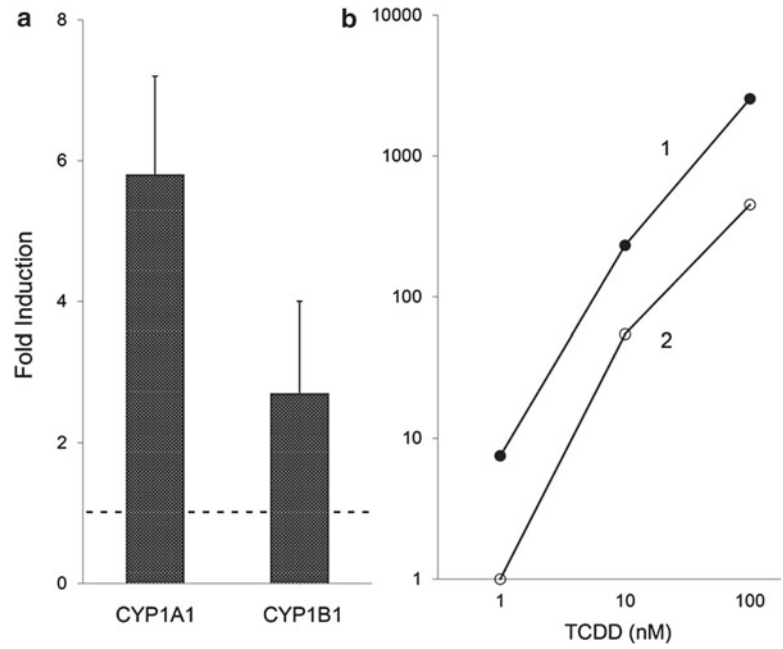

Fig. 2 Illustrated are results for CYP1A1 (#Hs 00153120_m1) and CYP1B1 (#Hs 00164383_m1) using β-actin (#Hs 99999903_m1) as endogenous control. The housekeeping gene GUSB (#Hs 99999908_m1) is also useful in such experiments. (**a**) Human skin organ culture exposed overnight to 10 nM TCDD [29]. Shown are the means ± SDs from three experiments. (**b**) Confluent cultured normal human epidermal cells assayed after overnight TCDD treatment showing a difference in sensitivity in two keratinocyte strains (1 and 2) measured in parallel

3.2.3 Conduct Real Time PCR

1. Set up reactions according to a template sheet plan. Determine how many wells are needed for each probe and then prepare a mixture of Gene Expression Assay probe + master mix. Prepare enough for one extra well to cover pipetting errors. For each well use 0.5 μL of 20× probe + 5 μL of 2× master mix.
2. Pipet 5.5 μL of this mixture into each well according to the template plan, then add 4.5 μL of diluted cDNA from the previous step (**Note 4.2.4**). Seal the plate and wrap it in aluminum foil until it is ready to be run.
3. The plate is inserted in the Applied Biosystems 7500 Fast Sequence Detector and the rates of accumulation of amplicons are measured. The output is then analyzed using the instrument software, which calculates the levels of the target sequence relative to the endogenous control in the various culture conditions being compared (**Note 4.2.5**). Figure 2 shows application to TCDD induction of CYP1A1 and 1B1 in keratinocytes in culture and in the skin [29].

4 Notes

4.1 Luciferase/EGFP Reporter Gene Bioassays

4.1.1. The fetal bovine serum (FBS) used in these and other gene expression-based cell bioassays is a critical factor affecting the level of assay sensitivity and magnitude of AhR-dependent induction of reporter and endogenous gene expression. Thus, prescreening of small amounts of several different lots of FBS from one or more vendors for low background and high signal is strongly encouraged, in order to identify the optimal serum lot. A sufficient amount of the same lot of FBS will not only ensure optimal bioassay characteristics, but also consistent assays over an extended time period.

4.1.2. TCDD is one of the most potent AhR agonists and it is the most commonly used agonist in AhR bioassays and is typically dissolved in DMSO. Use of TCDD as the positive control AhR agonist in these assays can be problematic in some laboratories as it is considered a highly toxic carcinogen, even though it is used at extremely low concentrations in these assays, and disposal costs can be high. When working with TCDD take extreme care to avoid contaminating work areas and personnel and use it only in appropriately designated areas with all necessary precautions, including the use of laboratory coats, protective eyewear, disposable benchtop paper, gloves, plastic ware and glassware. Follow all chemical safety guidelines for handling and disposal of these materials. It is particularly important that when handling TCDD or any hazardous chemical dissolved in DMSO or other solvent that you use appropriate solvent resistant nitrile gloves as latex gloves provide little or no barrier to solvent penetration and subsequent chemical exposure. Given the hazards rating associated with TCDD, laboratory use of these chemicals usually requires prior permission of your institutional chemical safety office.

4.1.3. A number of alternative highly potent non-toxic AhR agonists are available, but they have some limitations. Omeprazole is a non-toxic AhR agonist that has been used successfully as positive controls in human AhR cell-based bioassays, but it is a poor AhR agonist in rodent cells [30, 31]. Other non-toxic AhR agonists that have been used as positive controls in rodent AhR bioassays include BNF and indirubin [3, 7, 30–32]. In contrast to TCDD, these non-toxic AhR agonists can be enzymatically degraded within the cells resulting in the reduction in agonist concentration and a decrease in the overall induction of reporter gene activity over time (i.e. induction responses with these non-toxic agonists are transient). However, since use of relatively high

concentrations of these non-toxic ligands in the bioassay can result in maximal induction of AhR-dependent gene expression for extended incubation times, under these conditions they can be used judiciously as positive controls.

4.1.4. During re-suspension, cells must be dispersed so that no clumps of cells remain. This is critical to allow for accurate cell counts for dilution and distribution into microplate wells. Failure to disperse the cells adequately will result in variable numbers of cells in each well and will lead to significant differences in luciferase activity between replicate samples and thus high assay variability with inaccurate potency determinations.

4.1.5. Cells in each well of the microplate must be examined microscopically prior to and after chemical treatment to confirm that each well contains comparable numbers of cells. Not only does this ensure that cells were not lost during the washing and processing steps in the protocol, but it also allows assessment of whether the test chemical produced any cell toxicity. Since luciferase requires lysing of the cells, there can be no post-assay evaluation of cell numbers. Failure to inspect the cells in each well can contribute significantly to variations in the final reporter gene activity.

4.1.6. In most mammalian cell bioassays, cells are passaged and incubated with test chemicals at the standard temperature of 37 °C. However, for recombinant cell bioassays using luciferase or EGFP reporter genes, it has been observed that incubation of treated cells at 33 °C instead of 37 °C results in a dramatic increase in luciferase activity and EGFP fluorescence [28]. This increased activity appears to result from increased activity of the proteins themselves (perhaps due to more optimal folding at the lower temperature) and not from an increase in gene expression. Accordingly, if greater reporter gene activity is desired, the incubation of cells with test chemical should be carried out at 33 °C. However, cells should still be maintained and passaged at 37 °C since this temperature is optimal for cell growth.

4.1.7. The luciferase activity in these assays is based on the sum of the activity present in a defined number of cells within each well of the plate, and activity is not normalized to protein concentration in each well. This is primarily because the detergent present in the Promega lysis buffer interferes with most protein assays (even though some company brochures indicate that their assay is unaffected by detergent). This interference can lead to substantial variation in results and inaccurate determinations of overall luciferase activity.

4.1.8. Reporter gene (luciferase and EGFP) activity is typically expressed as a percent of the maximum induction observed

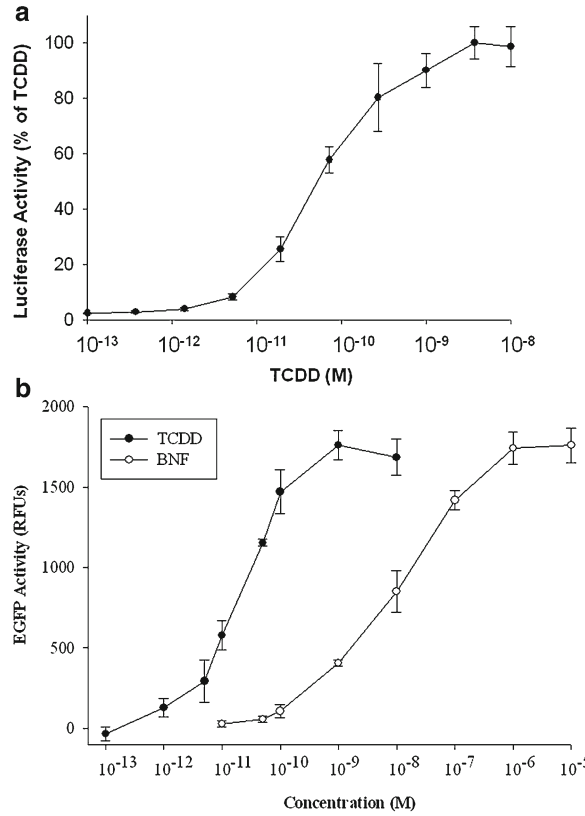

Fig. 3 Concentration-dependent induction of reporter gene activity in CALUX and CAFLUX cell bioassays. (**a**) Induction of luciferase activity by TCDD in mouse hepatoma (H1L6.1c2) CALUX cells. (**b**) Induction of EGFP fluorescence by TCDD and beta-naphthoflavone (BNF) in rat hepatoma (H4G1.1c3) CAFLUX cells

using a potent AhR agonist like TCDD or other AhR agonist, with the luciferase activity (relative light units (RLUs)) or EGFP fluorescence (relative fluorescent units (RFUs)) values representing the mean ± SD of at least triplicate determinations. Figure 3 illustrates the concentration-dependent induction of luciferase activity and EGFP fluorescence by TCDD in the H1L6.1c2 and H1G1.1c3 cell lines, respectively.

4.1.9. In order to allow normalization of EGFP results between experiments, the instrument fluorescence gain setting should be adjusted in each experiment so that the level of EGFP induction by 1 nM TCDD (or a maximal inducing concentration of another AhR agonist) produces a relative fluorescence of 9,000 relative fluorescence units (RFUs).

4.1.10. One major advantage of the EGFP bioassay over the luciferase bioassay is that EGFP fluorescence is measured in intact cells without having to remove the medium, and cells

can be returned to the incubator after they have been read. Luciferase activity typically requires cell lysis. Accordingly, this allows repeated measurements of EGFP fluorescence from the same cells over time and provides an extremely easy avenue in which to examine the time course of induction by test chemicals.

4.1.11. The H1L6.1c2 luciferase cell bioassay protocol described here can readily be applied to other cell lines containing stably-transfected AhR-responsive luciferase reporter genes [19, 20, 25, 33, 34].

4.2 Quantitative Real Time PCR Bioassay

4.2.1. The experimentalist should become familiar with the valuable information included in "Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR" available from Applied Biosystems.

4.2.2. A fume hood is used when working with Trizol, phenol and chloroform. These solutions are discarded as hazardous waste.

4.2.3. In earlier protocols, contaminating genomic DNA is removed typically by pretreatment with DNase (e.g., DNA-Free Kit, Ambion, Austin, TX). This step guarded against forming products during real time PCR from contaminating genomic DNA. Design of primers to span introns so as to yield very large cDNA amplicons with genomic DNA has made this step moot. Even if the amplicon is within a single exon, removing genomic DNA may not be necessary in some cases where the mRNA being measured is abundant.

4.2.4. In real time PCR, a threshold level is set above the baseline but low enough to be within the exponential portion of an amplification curve. The cycle number at which the fluorescence signal arising from accumulation of an amplicon reaches the threshold is called the C_T. Dilutions of cDNA are most convenient when the number of PCR cycles to reach the C_T is in the range of 20–30. The difference between the C_T for CYP1A1 and the GUSB endogenous control gene is calculated for two of the conditions being compared. From the difference between these differences, the relative amount of CYP1A1 can be calculated as the antilog (base 2), called the $\Delta\Delta C_T$ method.

4.2.5. This protocol describes measurement of CYP mRNA levels in cultured cells relative to one or more endogenous housekeeping genes. In keratinocytes, two genes that change little in transcription with many treatment conditions are used for the present purpose for normalization, but this property must be verified for previously untried treatment conditions.

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