SPECIES-SPECIFIC THIRD GENERATION (G3) LUCIFERASE CELL BIOASSAYS SHOW DRAMATICALLY INCREASED SENSITIVITY AND MAGNITUDE OF RESPONSE TO TCDD AND OTHER AH RECEPTOR AGONISTS

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Abstract
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related "dioxin-like" chemicals are widespread and persistent environmental contaminants that can cause a variety of species- and tissue-specific toxic and biological effects. Most of these effects are mediated by the Ah receptor (AhR) and involve binding of liganded AhR complex to its specific DNA recognition site and activation of gene transcription. Although the chemically activated luciferase expression (CALUX) bioassay has been used extensively for the directly detection of dioxin-like chemicals from a variety of environmental and biologic materials, the response of the CALUX bioassay at very low dioxin-like chemical levels is inadequate to screening of extracts with low levels of contamination. We have developed novel AhR-responsive luciferase reporter plasmids with increased numbers of DREs and stable transfection of a plasmid containing 20 DREs into a variety of cell lines has produced a series of species-specific cell bioassay systems with a dramatically increased luciferase induction response compared to existing CALUX-type cell lines. The minimal detection limit for TCDD in stably transfected mouse hepatoma (H1L7.5 c3) cells was 100- to 250-fold lower than that of the currently used H1L6.1c2 cells. Overall, these new cell lines have application for the detection of very low levels of dioxin-like chemicals.

Introduction
TCDD and structurally related "dioxin-like" chemicals have been environmental contaminants for nearly five decades, which include polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and biphenyls (PCBs). These compounds are lipophilic and found in environmental, biological, food and other matrices. They are resistant to biological and chemical degradation and known to bioaccumulate and biomagnify in the food chain[1]. Exposure to and bioaccumulation of dioxin and dioxin-like chemicals has been observed to produce a variety of species- and tissue-specific effects, such as tumor promotion, lethality, birth defects, hepatotoxicity, immunotoxicity, dermal toxicity, alterations in endocrine homeostasis, and induction of numerous enzymes[1]. Traditional instrumental analysis methods, such as capillary gas chromatography and mass spectrometry (GC/MS) are established for detection and quantitation of these chemicals and they provide very accurate measurement of many of the congeners of concern. However, these procedures are somewhat costly, time-consuming and impractical for rapid and large-scale sampling and screening analysis. Consequently, various rapid and relatively inexpensive assay systems based on the mechanism by which dioxin-like chemicals express their toxic effects have been developed. A major bioassay system that has gained widespread use over the past 10 years is that of the chemically activated luciferase expression (CALUX) cell bioassay[2]. CALUX is a reporter-gene-based cell bioassay that uses genetically modified cells that respond to chemicals that activate the AhR. The recombinant cells used in the CALUX bioassay contain a stably transfected AhR-responsive firefly luciferase reporter gene that responds to chemicals that can bind to and activate the AhR, leading to the induction of luciferase gene expression. Induction of luciferase in the recombinant CALUX cell line occurs in a time-, dose-, and AhR-dependent and chemical-specific manner, and the amount of induced luciferase activity is directly proportional to the amount and potency of the inducing chemical (i.e. AhR agonist) to which the cells have been exposed[2,3]. Although these CALUX bioassay systems have been used extensively for detection of dioxin-like chemicals, the sensitivity and responsiveness of the reporter gene response in these cells is inadequate to allow them to be used for screening of small amounts of biological (i.e. blood) and/or other matrices (i.e. food) that contains relatively low levels of these compounds. Accordingly, the development of improved (i.e. more responsive and sensitive) CALUX cell bioassays is necessary to meet this application need. Here we describe the development and characterization of cell lines stably transfected with these new AhR-responsive CALUX luciferase reporter plasmids.

Materials and Methods

Construction of luciferase expression vectors. The development and testing of the third generation AhR-responsive plasmid pGudLuc7.X with ″X″ representing the number of DRDs (each with 4 DREs) contained within the plasmid construct was described previously[4].

Stable transfection. To produce the stable clonal cell lines, mouse, rat and human helpatoma (Hepa1c1c7, H4IIe and HepG2, respectively) and guinea pig intestinal adenocarcinoma (GPC16) cells were cotransfected with the desired DRE-luciferase plasmid and pSV2neo using lipofectamine. Following 24 hr of growth in nonselective medium, the transfected cells were split 1 to 10 and replated into selective medium containing the antibiotic G418. The optimal concentration of G418 needed for killing of nontransfected cells was determined previously. After about 2-4 weeks of growth in selective medium, individual cell colonies were identified and cloned. TCDD-inducible luciferase activity of all clones was measured and the clone(s) with the greatest ratio of inducible to constitutive luciferase activity was selected for further analysis.

Preparation of environmental samples, chemical treatment, and luciferase analysis. Three environmental sediment samples were extracted and cleaned up using X-CARB to allow isolation of dioixn-like PCDDs, PCDFs and PCBs[5]. Briefly, aliquots of each sample were extracted with toluene and then purified by acid silica column chromatography and a patented carbon column chromatography procedure. Two fractions were subsequently collected from the carbon column: one of which contained chlorinated biphenyls, and the other primarily PCDD/Fs. The PCDD/F-containing fraction was exchanged into dimethylsulfoxide (DMSO) and the luciferase analysis was determined as previously described[4].

Results and Discussion

TCDD-inducible luciferase activity of the mouse cell clone H1L7.5c3. The mouse hepatoma (Hepa1c1c7) cells provide an excellent model cell line because of their high degree of responsiveness to TCDD and related chemicals. This is largely due to the unusually high concentration of AhR complexes in these cells[6]. In preiouvs studies, this cell line was transiently transfected with pGudLuc7.X (X= 1–5 DRDs; 4–20 DREs) and showed response to TCDD with the induction of luciferase activity[4]. This cell line was also stably transfected with these vectors and the induction of luciferase activity by TCDD in the isolated clones was determined. Of all initial cell clones tested, 1 clone H1L7.5c3 exhibited the highest TCDD responsiveness and was selected for further characterization. The dose dependence of luciferase induction in H1L7.5c3 cells was determined by incubation of the cells with increasing concentrations of TCDD and measurement of luciferase activity after 24 hr (Fig. 1). The magnitude of the induction response for the new stable cell line was dramatically greater than that of the currently used CALUX cell line (H1L6.1c2) at all TCDD concentrations, with the maximal luciferase activity for H1L6.1c2 at ~11,000 RLUs and that of H1L7.5c3 at ~700,000 RLUs. Most importantly, although the dioxin-like activity was essentially not detectable in the H1L6.1c2 cells at TCDD concentrations of 10^{-14} to 10^{-12} M, it was easily detected using the H1L7.5c3 cells (Fig. 1). The responsiveness and sensitivity of the new cells was compared to the previous H1L6.1c2 cell bioassay systems and although the estimated EC_{50} of these assays were within an order of magnitude (0.01–0.04 nM), the minimal detection limits (MDLs) for TCDD with H1L7.5c3 cells (0.01 pM) was about two orders of magnitude lower than that of the H1L6.1c2 (3 pM) cells (Fig. 1). Analysis of the time course of luciferase induction revealed that TCDD-inducible luciferase activity in H1L7.5c3 cells was detectable by 1 hr, with the 24 hr time taken as the standard for analysis (data not shown). Interestingly, we observed that the magnitude of TCDD-dependent induction in the H1L7.5c3 cells gradually increased with increasing cell passage number with a maximal luciferase activity of ~150,000 RLUs at 1 nM TCDD in the initial studies[4], increasing to ~700,000 RLUs in the current experiment (Fig. 1). This has been reported in previous studies[7]. The overall magnitude of the induction response in H1L7.5c3 cells has remained relatively consistent, indicating that the recombinant cell lines have been stably established.

Analysis of three environmental sample extracts with the cell clone H1L7.5c3. Sediment samples were collected and cleaned up in order to remove PAHs and other AhR agonists from the desired dioxin-like PCDD, PCDF and PCBs[5]. The TCDD standard was run in parallel with environmental samples as a positive control and

comparative purpose. Similar to the response with TCDD, these cell lines showed the same amplified responses to environmental samples as to TCDD standard solutions. The magnitude of induction of luciferase activity for TCDD in the H1L7.5c3 cells was dramatically greater than in the H1L6.1c2 cells, compare 250,000-400,000 RLUs in the H1L7.5c3 cells to 1,000-4,000 RLUs in H1L6.1c2 cells. For the environmental samples, the luciferase activity in H1L7.5c3 cells was also significantly higher than that in the H1L6.1c2 cells (Fig. 2).

Stable transfection of pGudLuc7.5 in to cell lines from different species. The effects of increased number of DREs on TCDD detection was further evaluated in cell lines from different species (rat H4IIe, guinea pig GPC16, and human HepG2 cells). These cell lines were stably transfected with pGudLuc7.5, and one clone of each cell line was selected for further analysis. As expected, the overall magnitude of induction response in new cell lines was consistently significantly greater than that of our previously stably-transfected CALUX cell lines (Table 1). These species-specific AhR-responsive recombinant bioassay systems can not only detect TCDD-like AhR agonists, but can be used to examine species differences in TCDD/AhR responsiveness.

The primary attribute of these new generation CALUX cell lines is the dramatically greater magnitude of luciferase activity that is observed at lower inducer concentrations. This would allow more accurate determinations of the induction response at the lower end of the concentration response curve as shown (Fig. 1). While the luciferase activity in the H1L7.5 c3 cells was significantly above control (DMSO) at TCDD concentrations down to 1 x 10^{-14}M (up to 10,000~30,000 RLUs), luciferase activity in the H1L6.1c2 cells was indistinguishable from background until a TCDD concentration of 3 x 10^{-12}M was tested. We also demonstrate the suitability of the H1L7.5c3 cells for the screening of environmental sample extracts for dioxin-like activity. In addition, the dramatically increased level of luciferase activity with the H1L7.5c3 cells suggests that it will be feasible to use these cells in a 384-well microplate format. Conversion to this format would not only reduce the amount of sample extract, cell number and reagents needed for analysis, but also reduce analysis costs. Overall, these new improved cell G3 lines will greatly expand the applications and utilities of the CALUX bioassay for dioxin-like chemicals, particularly for those sample matrices containing low levels of contamination.

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References
1. Giesy J. P., Ludwig J. P. and Tillitt D. E. *Environ Sci Technol* 1994; 28: 128A.
2. Han D., Nagy S. R. and Denison M. S. *Biofactor* 2004; 20: 11.
3. Garrison P.M., Tullis K., Aarts J.M., Brouwer A., Giesy J.P. and Denison M.S. *Fund Appl Toxicol* 1996; 30: 194.
4. He G., Baston D. S., Denison M. S. and Tsutsumi T. *Organoha Comp* 2008; 70: 772.
5. Brown D. J., Orelien J., Gordon J. D., Chu A. C., Chu M. D., Nakamura M., Handa H., Kayama F., Denison M. S. and Clark G. C. *Environ Sci Technol* 2007; 41: 4354.
6. Holmes J. L. and Pollenz R. S. *Mol Pharmacol* 1997; 52: 202.
7. Yang J., Lee H. and Park K. *Chemosph* 2008; 72: 1188.

Fig. 1. Concentration-dependent induction of luciferase gene expression by TCDD in stably-transfected CALUX cell lines. H1L6.1c2 and H1L7.5c3 cells were incubated with the indicated concentrations of TCDD at 37°C for 24 hr, and the luciferase activity was determined as described in Materials and Methods. Values represent the mean ± SD of triplicate determinations and was significantly greater ($p < 0.05$, Student's *t*-test) than control at concentrations equal to or higher than 3×10^{-12} M for H1L6.1c2 cells and 10^{-14} M for H1L7.5c3 cells, respectively.

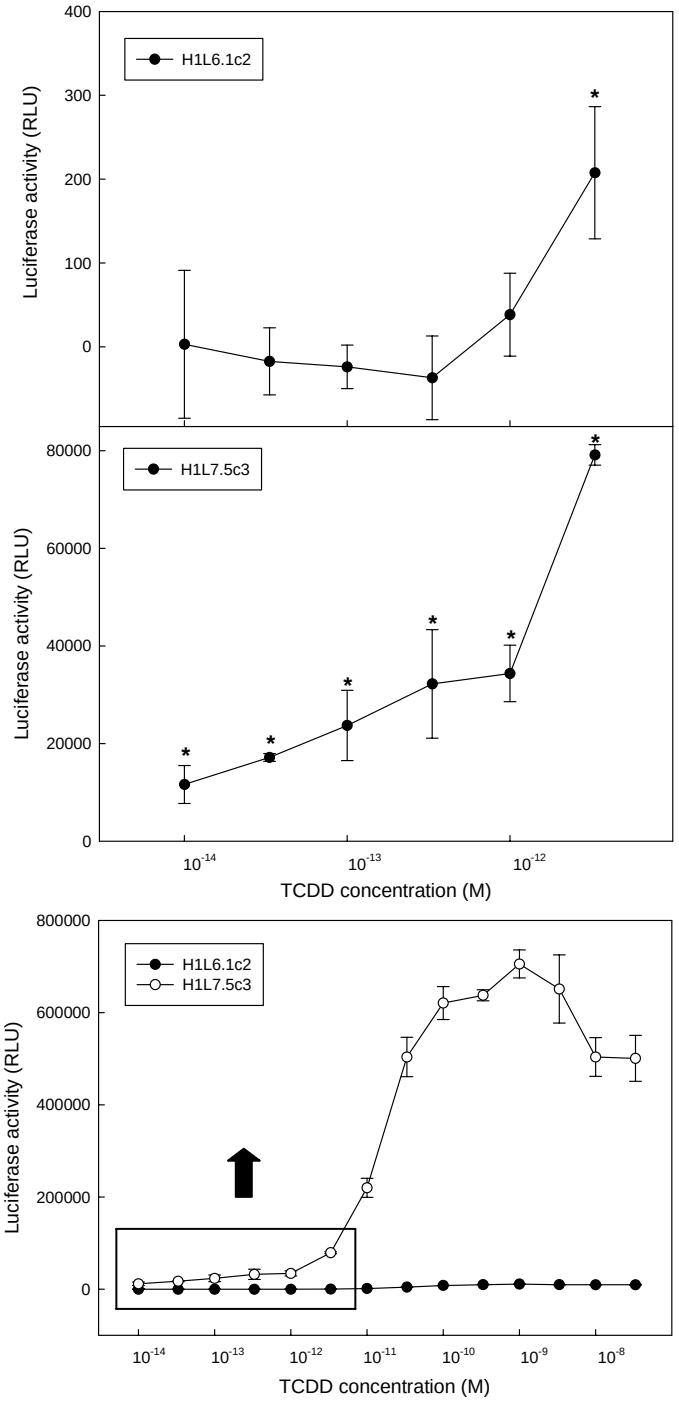

Fig. 2. Induction of luciferase activity by extract of three environmental sediment samples. Mouse hepatoma H1L6.1c2 and H1L7.5c3 cells were incubated with the indicated sample extract for 24 hr, and the luciferase activity was determined as described in Materials and Methods. Values represent the mean ± SD of triplicate determinations.

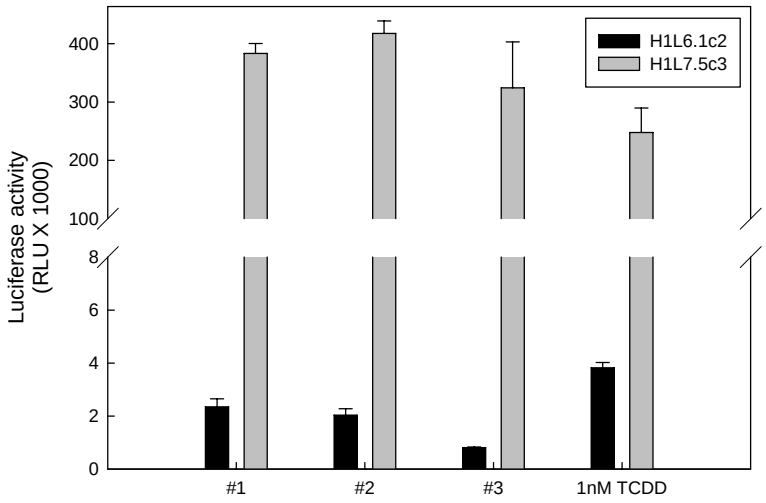

Table 1. TCDD-inducible luciferase activity in several cell lines stably transfected with the expression vectors: pGudLuc1.1, pGudLuc6.1 and pGudLuc7.5.

			Luciferase activity (RLU)		
Cell line	Vector	Clonal cell line	DMSO	TCDD	Fold induction
Mouse					
Hepa1c1c7-wt	pGudLuc6.1	H1L6.1c2	633 ± 77[a]	14114 ± 1807	22
	pGudLuc7.5	H1L7.5c3	7185 ± 610	173294 ± 14689	24
Rat					
H4IIE	pGudLuc1.1	H4L1.1c4	70 ± 8	3058 ± 801	44
	pGudLuc7.5	H4L7.5c1	28007 ± 1873	157063 ± 1141	6
Guinea pig					
GPC16	pGudLuc1.1	G16L1.1c8	410 ± 15	2265 ± 82	6
	pGudLuc7.5	G16L7.5c1	2272 ± 114	40577 ± 4171	18
Human					
HepG2	pGudLuc6.1	HG2L6.1c1	128 ± 17	3550 ± 432	28
	pGudLuc7.5	HG2L7.5c1	4221 ± 750	60982 ± 8509	14

[a] Values are the mean ± SD of triplicate assays.