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An Improved AhR Reporter Gene Assay for Analyzing Dioxins in Soil, Sediment and Fish

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Abstract Our goal was to develop a fast-screening bioassay to determine dioxin levels in the environmental and biological samples from dioxin-contaminated areas. Our original dioxin-responsive-element (DRE)-driven luciferase bioassay (using Huh7-DRE-Luc cells) was modified by reducing the incubation temperature of the cell culture from 37 to 35°C and by adding phorbol-12-myristate-13-acetate, and the modified bioassay was used to examine samples from soil, sediment, and fish. The results of this bioassay were shown to be significantly related to those of the high-resolution gas chromatography/high-resolution mass spectrometry assay of dioxins. The correlative equation was: log (PCDD/Fs I-TEQs) = 1.19 × log (BEQs) − 1.15 with $R^2 = 0.95$ ($p < 0.001$).

Keywords Bioassay · Dioxin · Environment · Contamination

Organochlorine compounds including chlorinated polyaromatic compounds, such polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), are lipophilic, bio-accumulative, and non-biodegradable and thus are recognized as environmental endocrine disruptors. The contamination of dioxin-like compounds in the environment and foodstuffs has raised public concerns due to its potential adverse effects on human health (Wittsiepe et al. 2007). In vitro aryl-hydrocarbon-receptor (AhR) reporter gene assays, e.g., the dioxin-responsive element-driven chemical activated luciferase gene expression (DRE-CALUX) bioassay, are rapid, low-cost, and semi-quantitative methods for large-scale dioxin surveillance (Hoogenboom et al. 2006). In the decade since the development of the DRE-CALUX bioassay, its sensitivity has been improved and its applications have been continuously expanded. Recently, two new in vitro AhR reporter gene assays were developed using the mouse hepatoma cell line Hepa1c1c7, namely the DR-EcoScreen cells/DR-cell assay (Kojima et al. 2011) and the H1L7.5c3 cells/CALUX bioassay (He et al. 2011). The sensitivity of the in vitro AhR reporter gene assay was improved by reducing the incubation temperature from 37 to 33°C and adding dexamethasone (DEX) and phorbol-12-myristate-13-acetate (PMA) to the cell culture medium (Zhao et al. 2010; He et al. 2011). Our previous studies revealed that several non-AhR-ligand chemicals, e.g., As^{3+}, arecoline, and Cd^{2+} could interfere with the in vitro AhR reporter gene assay (Chao et al. 2006, 2007, 2009).

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Therefore, an extensive cleanup procedure is critical for dioxin detection using the in vitro AhR reporter gene assay (Baston and Denison 2011; Chao et al. 2011). Our in vitro AhR reporter gene assay (Huh7-DRE-Luc cells/DRE-driven luciferase assay) combined with a fast cleanup system (a coupled carbon-acid silica column; Cape Technologies, South Portland, ME, USA) was previously reported and data from analyses of dioxins in soil and sediment samples using high-resolution gas chromatograph/high-resolution mass spectrometry (HRGC/HRMS) method were well correlated with those using the AhR reporter gene assay (Chao et al. 2011). The present study aimed to improve the sensitivity of our AhR reporter gene assay by modification of some cell culture conditions.

Materials and Methods

Standard solutions of PCDD/Fs including labeled compound stock, internal standard spiking, cleanup standard spiking, and EPA Method 1613 calibration and verification solutions were obtained from Wellington Laboratories (Ontario, Canada). A liquid standard of seventeen 2, 3, 7, 8-substituted PCDD/F congeners (Cat.# EDF-5416) known as Cerilliant Analytical Reference Standards was from Cambridge Isotope Laboratories, Inc. (Cambridge, MA, USA). CRM DX-1 is a certified dioxins- and furans-containing reference material (reference sediment DX-1 from the National Water Research Institute, Environment Canada, Quebec, Canada). All solvents were pesticide residue grade from Merck (Darmstadt, Germany), Tedia (Fairfield, OH, USA), and Sigma-Aldrich (St. Louis, MO, USA). PMA was purchased from Merck (Darmstadt, Germany). The rapid cleanup system was a coupled acid silica column-activated carbon mini-column (CAPE Technologies, South Portland, ME, USA). Silica gel (100–200 mesh) was purchased from Fisher (Leicestershire, England).

The environmental samples of soils ($n = 6$), sediments ($n = 7$), and fish ($n = 3$) were collected from Taiwanese dioxin-contaminated areas in 2010. The samples (10 g each) were extracted with 300 mL of toluene using a Soxhlet extractor. Extracted samples were evaporated to near dryness and then transferred to a coupled carbon-acid silica column for cleanup. The cleanup procedure was previously described (Chao et al. 2011). For the HRGC/HRMS method, all samples were spiked with the different PCDD/F internal standards before extraction and the labeled cleanup standards for PCDD/Fs analysis were added before the cleanup procedure. The extract was analyzed using a HRGC/HRMS system (HP6890/JEOL JMS-700; Hewlett-Packard, Palo Alto, CA, USA; JEOL, Tokyo, Japan) equipped with a DB-5MS 60 m column (J&W Scientific Folsom, CA, USA). The quality assurance (QA)/quality control (QC) procedures met the criteria of the Taiwanese Environmental Protection Administration (EPA). World Health Organization 1998 (WHO_{1998}) toxic equivalency factors (TEFs) and international (I)-TEFs were used in this study (Wittsiepe et al. 2007). For the bioassay, the labeled standards were not added in the extraction and cleanup procedure. After the extraction and cleanup procedure, the final extracts were evaporated to dryness and dissolved in dimethyl sufoxide (DMSO) (100 μL) for the DRE-driven luciferase bioassay (Chao et al. 2011). In brief, Huh7-DRE-Luc cells in DMEM medium were seeded at a density of 1×10^4 cells/well on a 96-well white plate (cat. 36101, Nalge Nunc, Roskilde, Denmark). Following incubation for 24 h at 37°C, the cells were treated with extracts for another 24 h at 35°C. In parallel, other cells were also treated with different concentrations of TCDD (0.1, 0.3, 1, 3, 10, 30, 100, 300, 1,000, 3,000 and 10,000 pM) for 24 h to generate a calibration curve for the assay of TCDD-dependent DRE-driven luciferase activity. If PMA was needed in the extracts or the standards, 10 μL of 8 μM PMA was added to the recombinant hepatoma cells at the 16th h of the incubation period. After treatments, 50 μL of 1× lysis buffer (Promega, Madison, WI, USA) was added per well. Cells were freeze–thawed three times using liquid nitrogen, vortexed (90 rpm at 37°C for 10 min to complete the cell lysis, and assayed using the standard protocol of the Luciferase Assay System (GLOMAX, Promega) to determine luciferase activity. The luciferase activity was expressed as relative light units (RLU)/well. The values of the sigmoid semi-logarithmic TCDD dose–response calibration curve were fitted using the Hill equation (Chao et al. 2011). The luciferase response was converted to a bioanalytical equivalent (BEQ) by comparison of the extract induction responses to a standard curve of TCDD concentrations. BEQs of each sample were independently measured three times. Exploring the correlation between the HRGC/HRMS method and the modified DRE-driven Luciferase bioassay, dioxin I-TEQ levels in soils, sediments, and fish were predicted by BEQ values from this bioassay. The statistical analyses were performed using SPSS 12.0 version.

Results and Discussion

Co-treatment of the recombinant mouse hepatoma cells with TCDD and PMA increased activation of the receptor construct several-fold relative to the cells treated with TCDD alone (Long et al. 1998). This is the first report to examine whether both reducing the incubation temperature (i.e., to 35°C) and adding non-AhR ligand (i.e., PMA) affect TCDD-induced luciferase activation in mammalian recombinant hepatoma cells. Our AhR reporter gene assay cell line, Huh7-DRE-Luc, when treated with TCDD $\leq$ 1,000 pM, showed the highest fold induction of luciferase activity by TCDD at

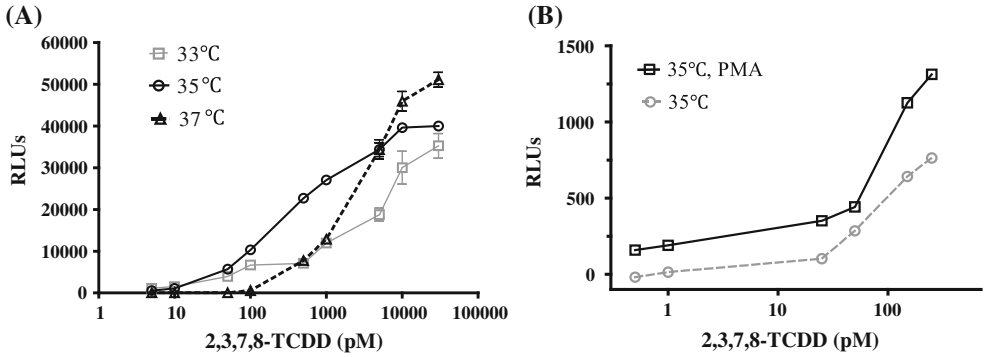

Fig. 1 Effects of temperature and PMA on the Huh7-DRE-Luc cells/DRE-driven luciferase assay. **a** Huh7-DRE-Luc cells were treated with TCDD for 24 h at 33, 35 and 37°C. **b** The cells were also treated with TCDD as indicated for 24 h at 35°C in the presence of 0.8 μM PMA. After treatments, luciferase activity was determined. Data are presented as mean ± SD (n = 3) and are expressed as relative light units (RLUs)

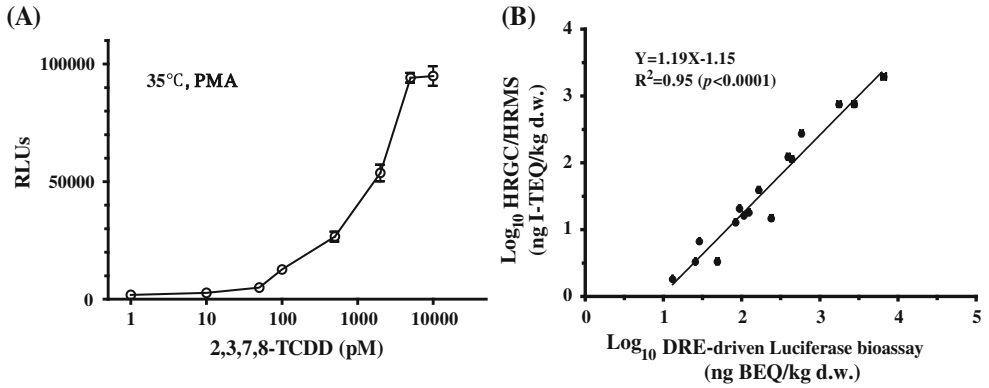

Fig. 2 **a** Dose–response induction of luciferase activity by TCDD. Huh7-DRE-Luc cells were treated with TCDD for 24 h at 35°C in the presence of 0.8 μM PMA. After treatments, luciferase activity was determined. Data are presented as mean ± SD (n = 3) and are expressed as relative light units (RLUs). **b** A linear regression was determined between the HRGC/HRMS assay values and the bioassay values ($R^2 = 0.95$, $p < 0.001$, n = 16). Thirteen environmental samples (6 from soil and 7 from sediment) and 3 biological samples (from fish) were included. The mathematic equation was log (PCDD/Fs I-TEQs) = 1.19 × log (BEQs) − 1.15.

incubation temperature 35°C (Fig. 1a); two previous studies reported a similar finding (Zhao et al. 2010; He et al. 2011). In the report by Zhao et al., luciferase activity of a recombinant Hepa1c1c7 cell, i.e., H1L6.1c2, treated with sediment extracts at 33°C was significantly increased (5–15-fold) above the luciferase activity of the same cells with the same treatment at 37°C (Zhao et al. 2010). Using Hepa1c1c7 cells stably transfected with a new CALUX plasmid with an increased DRE number, namely H1L7.5c3 or third generation (G3) CALUX cells, the same research team showed that TCDD treatment at 33°C caused a 4- to 10-fold increase in luciferase activity above that caused by TCDD treatment at 37°C (He et al. 2011). Figure 1b also shows that addition of PMA significantly enhanced the TCDD-induced luciferase activation by 1.8–3.2-folds, consistent with previous findings (Zhao et al. 2010). Results in Fig. 1 clearly show that incubation of Huh7-DRE-Luc cells with PMA at 35°C markedly improved the sensitivity of the cells used in TCDD detection.

Huh7-DRE-Luc cells were treated with TCDD at 35°C in the presence of 0.8 μM PMA and a dose–response induction of luciferase activity by TCDD was demonstrated in Fig. 2a, showing a semi-logarithmic dose–response calibration curve with a sigmoid appearance ($R^2 > 0.995$, $p < 0.001$). Treatments with 0.8 μM (800 nM) PMA at 35°C could enhance the TCDD-induced luciferase activation when TCDD was higher than 3 pM. Moreover, Huh7-DRE-Luc cells were treated with a liquid standard (EDF-5416) or a certified reference material (DX-1) at 35°C in the presence of PMA. As shown in Table 1, the resultant BEQ levels by EDF-5416 (10X dilution) or DX-1 (1X, 10X, and 100X dilution) were 4.22–5.71 times higher than WHO_{1998}-TEQ levels. As compared with the previous assay using Huh7-DRE-Luc cells treated at 37°C (Chao et al. 2011), stability and sensitivity of the present AhR reporter gene assay using the cells treated at 35°C in the presence of PMA were obviously improved (data not shown).

Table 1 Huh7-DRE-Luc cells treated with a liquid standard (EDF-5416) or a certified reference material (DX-1) at 35°C in the presence of PMA

Certified standards		DRE-driven luciferase bioassay					
Standard	Concentration	N[a]	Mean	Range	SD	RSD	Ratio[b]
EDF-5416							
10X[c]	7.50[d]	9	42.9	37.4–46.4	4.29	10.0	5.71
(ng WHO_{1998}-TEQ/mL)			(ng BEQ/mL)			(%)	
CRM DX-1 [g]							
1X	466	6	1970	1,894–2,039	121	6.15	4.22
10X	46.6	12	221	113–279	28.7	13.0	4.75
100X	4.66	9	23.6	15.9–28.6	2.74	11.6	5.06
(ng WHO_{1998}-TEQ/kg d.w.[e])			(ng BEQ/kg d.w.)			(%)	

[a] N is the number of measurements

[b] Ratio = (the BEQ value of the bioassay)/(the certified TEQ value of the standard)

[c] 10X means that the standard was diluted to 1/10

[d] The original concentration was 75.0 ng WHO_{1998}-TEQ/mL

[e] ng WHO_{1998}-TEQ/kg dry weight (d.w.)

Table 2 Analysis of PCDD/F levels in environmental and biological samples with Huh7-DRE-Luc cells/DRE-driven luciferase assay

Samples	N	Mean	Range	SD	RSD (in %)
Soil[a]					
Soil-1	15	2760	1050–4520	204	7.37
Soil-2	18	394	182–789	49.1	12.5
Soil-3	6	166	115–216	17.66	4.27
Soil-4	3	84.0	55.4–144	10.08	12.0
Soil-5	3	107	81.0–124	4.28	4.01
Soil-6	3	49.0	32.3–76.4	4.40	8.98
Sediment[a]					
Sediment-1	18	6570	3270–9940	952	14.5
Sediment-2	12	584	473–753	61.3	10.5
Sediment-3	9	1760	1650–2510	20.3	11.6
Sediment-4	6	124	119–128	10.6	8.58
Sediment-5	6	94.0	81.9–106	6.20	6.59
Sediment-6	3	440	343–555	44.1	10.0
Sediment-7	3	25.6	12.7–52.7	3.05	11.9
Fish					
Whole fish[b]	24	13.1	2.31–19.3	2.10	16.1
Fish meat[c]	24	28.8	20.2–36.6	3.46	12.0
Fish viscera	27	239	96.1–314	40.0	16.8

[a] The unit was ng BEQ/kg d.w

[b] The whole fish of Perth herring (*Nematalosa come*)

[c] The meat of Tilapia (*Oreochromis niloticus niloticus*)

[d] The viscera of Tilapia (*Oreochromis niloticus niloticus*); the unit was ng BEQ/kg w.w

As shown in Table 2, levels of PCDD/Fs range from 49.0 to 2,760 ng BEQ/kg dry weight (d.w.) for 6 soil samples, from 25.6 to 6,570 ng BEQ/kg d.w. for 7 sediment samples, and from 13.1 to 239 ng BEQ/kg wet weight (w.w.) for 3 fish samples. The relative standard deviations (RSD) of the tested samples were between 4.01 and 16.8 %. PCDD/F

measurements with RSD value $\leq$15 % meet the Taiwanese EPA criteria of the in vitro AhR bioassay method (NIEA S901.60B). Notably, fish viscera have a significantly higher BEQ value than whole fish and fish meat. Because levels of I-TEQs and BEQs of 16 samples were non-normally distributed by the Kolmogorov–Smirnov test, the data were logarithmically transformed to approximate normal distribution. The relationship between I-TEQs and BEQs was determined by a linear regression model with the equation: log (PCDD/Fs I-TEQs) = 1.19 × log (BEQs) − 1.15 (R^2 = 0.95) (Fig. 2b).

Several strategies have been used in the in vitro AhR reporter gene assay to improve the sensitivity of dioxin analysis. The strategies include modification of the original reporter plasmid (Van Langenhove et al. 2011), development of a new recombinant reporter plasmid (Novotna et al. 2011), increase in the copy number of DRE in the promoter (He et al. 2011; Kojima et al. 2011), addition of PMA or DEX to the cell culture medium (Zhao et al. 2010), and reduction in incubation temperature from 37 to 33°C (Zhao et al. 2010; He et al. 2011). Among these strategies, PMA or DEX addition to the cell culture medium and reduction in incubation temperature from 37 to 33°C or 35°C are the easier ways to improve detection sensitivity of already established in vitro AhR reporter gene assays, such as our Huh7-DRE-Luc cells/DRE-driven luciferase assay. We have previously demonstrated that the addition of a coupled carbon-acid silica column (CAPE column) to the Huh7-DRE-Luc cells/DRE-driven luciferase assay is a rapid and useful semi-quantitative green technique for large-scale dioxin surveys particularly for the hotspot areas (Chao et al. 2011). Here, we further show that altering the incubation conditions (adding PMA at 35°C) is able to markedly enhance the sensitivity of the Huh7-DRE-Luc cells/DRE-driven luciferase assay of dioxins, suggesting a potential environmentally friendly technique for high-throughput screening of dioxins. This high-throughput screening technique in conjunction with the HRGC/HRMS method may significantly facilitate large-scale survey for dioxin, particularly for environmental dioxin in heavily contaminated areas and dioxin contamination in foodstuffs.

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