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Establishment of CALUX Bioassay for Dioxin Determination

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ABSTRACT **: Dioxin-like compounds are ubiquitous environmental pollutants that could be accumulated in biological system and toxic to human and wildlife. Given this issues, it is important to develop a reliable dioxin detection methods for a rational risk assessment of dioxin-like compounds. In this study, we tried to set up and validate a sensitive, reliable and rapid bioassay model, CALUX bioassay as a screening tool for routine measurement of dioxin-like compounds in environmental matrices. For the validation of CALUX bioassay, firstly, we performed dose-response assay for 2,3,7,8-TCDD, most potent dioxin-like compound, using two different methods CALUX and EROD assay. Induction of luciferase activity and CYP1A catalyzed EROD activity were dose-dependently induced by 2,3,7,8-TCDD, with initial induction at 0.1 pM and maximal induction at 1 nM. In order to determine whether the CALUX bioassay could predict the effects of dioxin-like compounds, 2,3,7,8-TCDD dose-response from CALUX was compared with that from EROD assay. The correlation coefficient (r^2) was found to be 0.89, indicating a good correlation between two different methods and the possibility of CALUX bioassay as a useful dioxin detecting method.**

Key words : **Dioxin, CALUX bioassay, EROD**

Introduction

There are increasing concerns over the potential adverse effects of Persistent Organic Pollutants (POPs) on human and wildlife. POPs are chemicals that remain intact in the environment for long periods, become widely distributed geographically, and accumulate in the fatty tissue of living organisms. They have toxic properties, resist degradation, bio-accumulate and are transported, through air, water and migratory species, across international boundaries and deposited far from their place of release, where they accumulate in terrestrial and aquatic ecosystems. Therefore, the Stockholm Convention on POPs was adopted in 2001 in response to the urgent need for global action to protect human health and the environment from POPs. 12 chemicals have been listed primarily as POPs (Table 1).

Table 1. POPs in the Stockholm Convention

Chemicals	Pesticides	Industrial Chemicals	By-Products	Annex
Aldrin	+			A
Chlordane	+			A
Dieldrin	+			A
Endrin	+			A
Heptachlor	+			A
Mirex	+			A
Toxaphene	+			A
Hexachloroben-zene	+	+	+	A
PCBs		+	+	A, C
DDT	+			B
Chlorinated dioxins			+	C
Chlorinated furans			+	C

The target chemicals of Stockholm Convention are

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List of Abbreviation: PCB : polychlorinated biphenyls, PCDDs : polychlorinated dibenzo-p-dioxins, PCDFs : polychlorinated dibenzofurans, PAHs : polyhalogenated aromatic hydrocarbons, POPs : Persistent Organic Pollutants, PAHAs : polyhalogenated aromatic hydrocarbons, AhR : aryl hydrocarbon receptor, TEF : Toxicity Equivalency Factor, TEQ : Toxic Equivalency, HRGC/MS : high resolution gas chromatography/mass spectrometry, CALUX : Chemically Activated LUciferase gene eXpression, 2,3,7,8-TCDD : 2,3,7,8-tetrachlorodibenzo-p-dioxin

subdivided into three Annexes; Annex A (Aldrin, Chlordane, Dieldrin, Endrin, Heptachlor, Mirex, Toxaphene, Hexaclorobenzene, and PCBs), Annex B (DDT), and Annex C (Chlorinated dioxins, Chlorinated furans, Hexaclorobenzene and PCBs). The intentionally produced POPs are under the prevention of production and/or usage (chemicals listed in Annex A) and usage restriction (chemicals listed in Annex B). Unintentionally produced POPs, chemicals listed in Annex C, are under the control of continuing minimization and, where feasible, ultimate elimination of quantitative releases.

Among the POPs, polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs), so-called "Dioxin-like" compounds, are now in the center of public, regulatory, and scientific attention, especially after several accidents and poisoning incidents proving the toxic effects of dioxin-like compounds have occurred. Dioxin-like compounds are ubiquitous environmental pollutants that could be accumulated in biological system and toxic to human and wildlife. Therefore, it is very important to develop the sensitive, reliable and rapid methods to detect and quantify these chemicals. At present, the determination of dioxin-like compounds has been carried out using the chemical analysis methods or biological analysis methods. Chemical analysis by high resolution gas chromatography/mass spectrometry (HRGC/MS) was the only option and golden standard for detecting dioxin-like compounds until 10 years ago. This method is based on the separation and quantification for dioxin-like compounds from complex mixture. Chemical analysis measures each congener of the selected dioxin-like compounds and multiplies with the TEF values, resulting in TEQ values. It is clear that chemical analysis can measure various dioxin-like compounds quantitatively, selectively and sensitively. However, this chemical analysis has some disadvantages. In the environment, dioxin-like compounds coexist as complex mixture of various congeners and interactions between dioxin-like compounds can modulate their toxic potential. However, chemical analysis methods are not sufficient to identify all the environmental chemicals and to predict combination toxicity and bioavailability in the biological organisms. In addition, chemical analysis methods are usually time-consuming and expensive, due to exhaustive clean up procedure to remove all the interferences and the use of expensive instruments. Thus, recently, considerable attention has been focused on developing of various *in vitro* bioassay methods for a rational risk and hazard assessment of dioxin-like compounds. They allow the analyses of a high number of samples since they are relatively fast, inexpensive, and sensitive. However, these techniques are not yet widely applied for screening or environmental monitoring. The main reasons are probably the lack of validation and the difficulty in interpreting the global biological response of the bioassay. In this study, we evaluated the possibility of a new bioassay method, CALUX bioassay as a pre-screening tool for routine measurement of dioxin-like compounds in the various matrices, such as human blood sample, environmental water sample, and soil sample.

Materials and Methods

Chemicals

2,3,7,8-tetrachlorodibenzo-p-dioxin was generously provided Dr. Chung Y. H. (NIER). DMSO, sodium chloride, sodium acetate, sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), *d*-glucose, ethoxyresorufin, resorufin, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), sodium bicarbonate, glycerol, tris base, phenol, isopropyl alcohol, fluorescarmine, ampicillin-Na salt, lysozyme, sodium dodecyl sulfate (SDS), RNase, and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium pyrubate, penicillin-streptomycin, FBS, trypsin-EDTA, MEM, L-glutamin, and lipofectamine were acquired from Gibco BRL (Rockville, MD, USA). Magnesium chloride, sodium hydroxide, and potassium phosphate monobasic (KH_2PO_4) were purchased from Fisher Chemicals (Pittsburgh, PA, USA). Luciferase assay reagent and reporter lysis buffer were from Promega (Madison, WI, USA). Bacto-tryptone, bacto-yeast extract, and agar were obtained from DIFCO (Detroit, MI, USA) and chloroform was obtained from Mallinckrodt (Paris, KY, USA). Absolute ethanol and acetonitrile was purchased from Hayman (England) and Showa Chemical Co. (Tokyo, Japan), respectively. Agarose and Micro BCA protein assay kit was from FMC (Rockland, ME, USA) and Pierce PERBIO (Rockford, IL, USA), respectively.

Cell lines and Cell Culture Conditions

Mouse liver cell line, Hepa 1c1c7 cells were obtained

from the Korean Cell Line Bank (KCLB, Seoul, South Korea) and were maintained in Minimal Essential Medium (MEM) supplemented with 5% fetal bovine serum and penicillin-streptomycin at 37°C and in 5% CO_2.

CALUX Bioassay

Plasmid; *pmcyp1a1-Luc*

The *pmcyp1a1-Luc*, reporter gene construct was created by cloning mouse *cyp1a1* 5′ flanking region (−1642 ~ +53) into pGL3 vector at Hind III site (Scheme 1). The *pmcyp1a1-Luc* reporter gene construct has the firefly luciferase gene under transcriptional control of mouse *cyp1a1* promoter containing XRE sequence.

Transfection and Luciferase assay

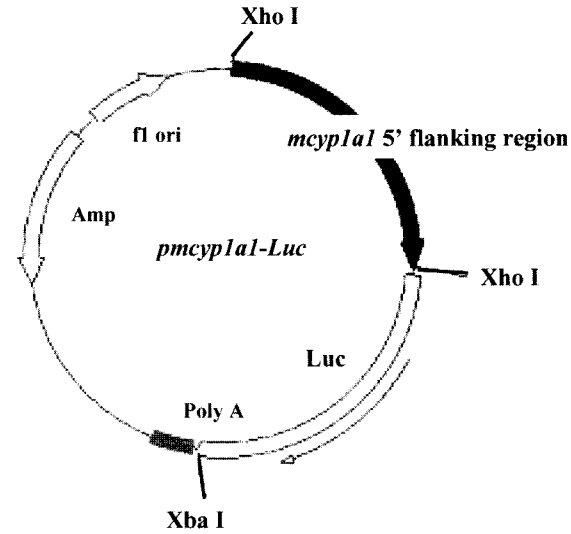
For transient transfection, Hepa 1c1c7 cells were seeded in 48 well plates at a density of 2.5×10^5 cells and transfected with *pmcyp1a1-Luc* using lipofectamine 24 hr after transfection, cells were treated with 2,3,7,8-TCDD standard and serial dilution of samples for 24 hr and lysed for luciferase activity. Luciferase activity was determined by adding luciferin as a substrate and then emitted bioluminescence is detected by a luminometer (Berthold Detection System, Oak Ridge, TN, USA). Luciferase activity was normalized to protein content.

EROD Bioassay

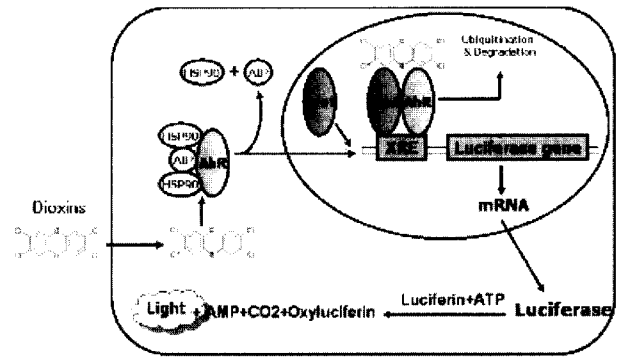
EROD activity of intact cells was measured using a multi-well fluorescence plate reader (FL600, BIO-TEK, Winooski, VT, USA) by Kennedy and Jones's method (Kennedy and Jones, 1994). Hepa 1c1c7 cells were seeded in 48 well plates at a density of 2.5×10^5 cells. After 24 hr, cells were treated with 2,3,7,8-TCDD standard for 24 hr. Sodium phosphate buffer was added to each well of 48 well plates. And then cells were incubated with 7-ethoxyresorufin at 37°C for 10 min. The EROD reaction was initiated with the addition of β-NADPH. The reaction was stopped after incubation for 15 min at 37°C with the addition of fluorescarmine solution (0.3 mg/ml in acetonitrile). After incubation for 15 min at R.T., resorufin fluorescence was measured at 530 nm excitation and 590 nm emission and fluores- camine fluorescence was measured at 360 nm excitation and 460 nm emission.

Statistical Analysis

All numerical data were expressed as the average of the values obtained ± SD. For all experiment significance was determined by conducting a paired Student's *t* test.



Scheme 1. Construct of *pmcyp1a1-Luc*.



Scheme 2. Molecular mechanism of CALUX bioassay.

Results

In this study, we tried to establish and validate a new bioassay method, CALUX bioassay as a pre-screening method to determine dioxin-like compounds in a variety of matrices. CALUX bioassay use genetically modified cells, Hepa 1c1c7 cells transfected with *pmcyp1a1-Luc* construct. The *pmcyp1a1-Luc* construct has the firefly luciferase reporter gene under the transcriptional control of mouse *cyp1a1* promoter containing XRE sequence (Scheme 1). These recombinant cells still contain the complete machinery which is involved in the mode of action of dioxin-like compounds. Thus, created recombinant CALUX cells express luciferase gene upon exposure to dioxin-like compounds. As a consequence, these cells emit luminescence by adding luciferin, substrate for luciferase

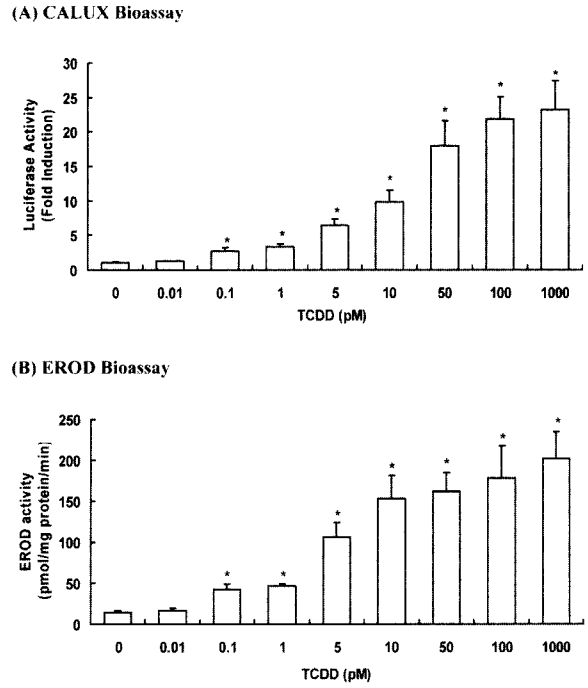


Fig. 1. Dose-response effect of 2,3,7,8-TCDD (A) Hepa 1c1c7 cells transfected with *pmcyp1a1-Luc* were treated with vehicle (0.1% DMSO) or indicated concentrations of 2,3,7,8-TCDD for 24 hr. The luciferase activity was normalized to protein content and expressed as a fold induction compared to that of control. (B) Hepa 1c1c7 cells were treated with vehicle (0.1% DMSO) or indicated concentrations of 2,3,7,8-TCDD for 24 hr. EROD activity was determined as described in materials and methods Data present mean ± S.D. (N = 4) * : Significantly different from control ($p < 0.05$).

enzyme, and emitted luminescence can be easily detected and quantified. The amount of light production is directly related to the amount of dioxin-like compounds in the sample (Scheme 2).

The utility of CALUX bioassay is highly dependent on its analytical sensitivity as well as its rapidity and ease of analysis. Therefore, we carried out dose-response experiment using standard dioxin-like compound, 2,3,7,8-TCDD. Hepa 1c1c7 cells were seeded in 48 well plates at a density of 2.5×10^5 cells and transfected with *pmcyp1a1-Luc*. 24 hr after transfection, cells were treated with vehicle (0.1% DMSO) or various concentrations (0.01 pM~1 nM) of 2,3,7,8-TCDD for 24 hr. As shown in Fig. 1A, induction of luciferase activity was dose-dependent, with initial induction at 0.1 pM and maximal induction at 1 nM. The EC_{50} was calculated to be 14.5 pM. This indicates that the CALUX bioassay model is able to detect a wide range of concentrations between 0.1 pM and 1 nM.

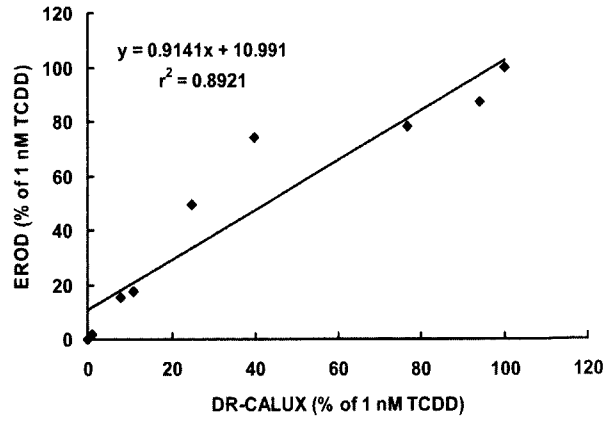


Fig. 2. Comparison of CALUX bioassay and EROD bioassay. Dose-response results from CALUX bioassay (Fig. 1A) and EROD bioassay (Fig. 1B) are expressed as a relative percentage over the 1 nM 2,3,7,8-TCDD treated group. And then each 2,3,7,8-TCDD dose response was compared.

Next, to determine whether the CALUX bioassay could precisely predict the effect of dioxin-like compounds, we compared CALUX bioassay with another most commonly used bioassay method, EROD bioassay. EROD bioassay measures the catalytic activity of CYP1A, which is related with de-ethylation of 7-ethoxyresorufin to resorufin (Li *et al.*, 1999; Safe, 1993; Sanderson *et al.*, 1996). Hepa 1c1c7 cells were treated with various concentrations (0.01 pM~1 nM) of 2,3,7,8-TCDD for 24 hr. CYP1A catalyzed EROD activity was measured in intact cells. As shown in figure 1B, EROD activity was dose-dependently induced by 2,3,7,8-TCDD. The induction gradually increased and reached a plateau at 1 nM. When we compared the results from CALUX bioassay and EROD bioassay, the correlation coefficient (r^2) was found to be 0.89, indicating a good correlation between two different bioassay methods (Fig. 2).

Discussion

There has been considerable scientific, regulatory, and public concern over the POPs with regard to their release and potential adverse effects on environment and human health. They have toxic properties, resist degradation, bio-accumulate and are transported, through air, water and migratory species, across international boundaries and deposited far from their place of release, where they accumulate in terrestrial and aquatic ecosystems. Therefore, the Stockholm Convention on

POPs was adopted in response to the urgent need for global action to protect human health and the environment from POPs in 2001. Among them, dioxin-like compounds are now in the center of attention, because some of these compounds exhibit a wide spectrum of toxic effects; carcinogenicity, teratogenicity, hepatotoxicity, and immunotoxicity (Safe, 1986; Poland *et al.*, 1982; Gao *et al.*, 1998, Schecter; 1994).

In that context, a rapid, inexpensive, and reliable method to detect and quantify dioxin-like compounds is mandatory. For an integrated risk assessment of dioxin-like compound, it is important to know both their concentration and toxicity. In the past, these procedures often rely on accurate chemical analytical techniques, such as HRGC/MS, HPLC, or other methods. However, chemical analysis methods have some limitations, such as high analysis costs, lack of rapidity, and the need for large sample volumes. In addition, congener-specific toxicity data are not included although the application of TEF has been used to estimate the relative biological and toxicological potency of a given mixture of dioxin-like compounds. Moreover, a number of studies demonstrated non-additive interactions between dioxin-like compounds (Bannister *et al.*, 1987; Haake *et al.*, 1987; Biegel *et al.*, 1989; Davis and Safe, 1989, 1990; Harper *et al.*, 1995). The non-additive (antagonistic) interactions reported to date have been observed at relatively high concentrations of both agonist and antagonist and the importance of these interactions in human and other animal at lower exposure levels is unknown. However, in environment, dioxin-like compounds invariably exist as complex mixtures of them and these non-additive interactions represent important limitations of the TEF approach for risk assessment of dioxin-like compounds.

As an alternative to the chemical analysis, a number of bioassay methods have been developed. Recently, various *in vitro* bioassay based on AhR-dependent mechanism have been developed for the identification and characterization of AhR activating compounds, because it is well known that toxicological and biological effects caused by dioxin-like compounds are commonly mediated through AhR (Nebert *et al.*, 1993; Lucier *et al.*, 1993). Among them, we adopted Chemically Activated LUciferase gene eXpression (CALUX) *in vitro* bioassay, CALUX bioassay. At present, they are not yet widely applied for screening or monitoring of dioxin-like compounds in various environmental matrices because validation data, such as cross-validation data between different bioassays and inter-laboratory cross-validation data with same technology are deficient.

In this study, we demonstrated that CALUX bioassay was sensitive and reliable dioxin detection tool. In the dose-response experiment using prototype AhR activator, 2,3,7,8-TCDD, it induced dose-dependent stimulation of luciferase activity in a wide range of concentrations. 0.1 pM TCDD began to increase the luciferase activity and 1 nM TCDD showed maximal luciferase induction. EC_{50} was calculated to be 14.5 pM. In addition, CALUX bioassay was highly correlated with EROD bioassay ($r^2 = 0.890$). This high correlation between the EROD bioassay and CALUX bioassay was also reported by other groups (Behnisch *et al.*, 2002; Murk *et al.*, 1996; Sanderson *et al.*, 1996; Zhang *et al.*, 2002).

In conclusion, the CALUX bioassay can be recommended as an "early warning system" for routine measurement of dioxin-like compounds in various environmental matrices because it has a great deal of advantages; that is available with smaller sample volume, smaller finance, and shorter testing time. Moreover, CALUX bioassay can provide congener-specific toxicity data although cell based bioassay does not consider uptake, tissue distribution, and metabolism of dioxin-like compounds. Therefore, this bioassay method can help to give a first indication as to whether the unknown compound will bind to the AhR and whether it may have the potential to cause dioxin-like effects when considering the addition of more compounds to dioxin-like compounds list. Although it is currently unknown what specific chemicals in these samples are responsible for the observed induction response, this bioassay system is a highly effective method for screening large numbers of potentially exposed samples. Once positive samples are identified, chemical analysis such as GC/MS and/or HPLC can be used in order to identify the specific compounds or congeners responsible for the induction response and to assess specific exposure of samples. This combination of chemical analysis and biological analysis will provide further information as to potential exposure to AhR ligands and activators in the inexpensive and relatively rapid manner.

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