CELL BASED ASSAY DIOXIN SCREENING: UTILISATION OF THREE NEW CELLULAR BIOASSAYS FROM DIFFERENT SPECIES AND TISSUES.

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Introduction

The presence of xenobiotics in the food-chain, foodstuffs and various environments are still increasing and one of the worst xenobiotics families is the dioxins and dioxin-like compounds group, well-known for their biostability, high toxicity, food-chain accumulation and interactions with other pollutants such as steroid hormones.

Many, if not all, toxicological effects of dioxins and dioxin-like compounds are driven by the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor. Upon ligand binding, the cytosolic complex receptor is dissociated from his Hsp90 component, translocates to the nucleus, and forms a heterodimer with the Ah receptor translocator (Arnt). This new active complex (AhR-Arnt) binds specifically XRE (Xenobiotic responsive element) sequences which are present in the 5' region of several genes, including genes involved in the phase I and phase II metabolism of xenobiotics[1]. The most study and inducible AhR-dependent gene is the *cyp1a1*, coding for a phase I enzyme metabolising xenobiotics. It has been postulated that abnormal modulation (induction and/or repression) of genes regulated by the AhR lead to the "dioxins" toxic effects [2].

The aim of this work was to set up different cellular assays, which contain the same promoter and reporter gene (firefly luciferase), from various species and tissues. These "home made" cell based assays were compared to commercial assays.

Materials and methods

Plasmid construction. The pTkXRE4-Luc vector contains 4 synthetic XRE sequences (5'GATCCGGGTCCCAGTGCTGTCACGCTAGG 3'), the thymidine kinase promoter (TK) followed by the firefly luciferase gene (as reporter gene). It contains also ampicillin resistance gene (Amp R) and both polyadenylation signals from SV40 virus.

Generation and characterization of stable reporter cell lines. The T47D (ATCC number: HB-133) human breast cancer cell line, HepG2 (human hepatoma, ATCC HB-8065) and H4IIE (rat hepatoma, ATCC number: CRL-1548) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). T47D and HepG2 cells were grown in 75 cm^2 culture flasks in DMEM medium (Dulbecco's modified Eagle's medium) supplemented with 10 % heat-inactivated fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 µg/ml) and amphotericin B, at 37°C. For T47D culture, 1 µg/ml of bovine pancreas insulin was added. H4IIE were grown in 75 cm^2 culture flasks in Minimum Essential Medium (MEM) supplemented with 10 % heat-inactivated fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 µg/ml) and amphotericin B at 37°C.

For stable transfection, our three cell types were seeded at about 70 % confluence in a 60 mm culture dish. The following day, the cells were transfected with 2 µg of pTkXRE4-Luc vector and 0,2 µg pcDNA6 vector (T47D and H4IIE) or pcDNA3 (HepG2) vector for respectively blasticidin or G418 resistance using the Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's instructions.

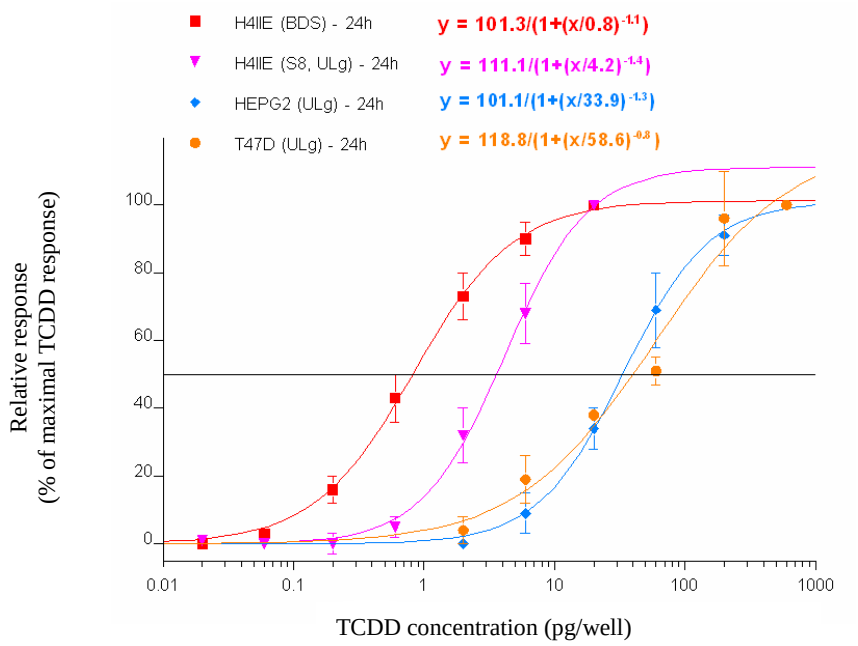
To choose the most inducible clone, TCDD induction was performed by seeding the cells (about 10^5 cells/well in 24-well plates) in fresh medium. After 24h, a 24 hours TCDD treatment was performed, in triplicate, with 30, 60 and 100 pg/ml TCDD concentration respectively for H4IIE, T47D and HepG2. The cells were washed two times in PBS and resuspended in lysis buffer following by a luciferase assay to determine the TCDD induction between treated and not treated cells (control).

The commercial cell based assay was the rat hepatoma H4IIE DR-CALUX® cells, purchased from BioDetection System (Amsterdam, The Netherlands). Cells were stably transfected with the upstream region of mouse *cyp1a1* gene (from -1301 to -819) containing four Dioxin Responsive Elements (DREs), the MMTV viral promoter and

the luciferase gene as described by Garrison and co-workers[3].
For stable cell lines characterization and sensitivities comparison, a dose-response curve was established for each recombinant cell line using TCDD as standard. The cells were exposed in triplicate to serial dilutions of TCDD standard solutions during 24h. Dilutions of TCDD standard solutions ranged from 0.3 pM to 10 nM, according to the cell lines.
After incubation, the medium was removed and the cells were lysed. After the addition of luciferin, the luciferase activity was determined using a luminometer (ORION II, Berthold Detection System, Pforzheim, Germany), and reported as Relative Light Units (RLUs). Raw data were then treated as previously described[4].
Guar gum samples were extracted, purified and analyzed as previously described[4].

Results and Discussion

Generation and characterization of stable reporter cell lines. We generated T47D, H4IIE and HepG2 clones using stably transformed with the pTkXRE4-Luc vector and selected the most highly inducible clone S-hum-mam (T47D), S-hum-hep (HepG2) and S-rat-hep (H4IIE). These three stable cell lines responded in a dose dependant manner against TCDD (Figure 1). To compare the sensitivity to dioxins in these three cell based assays, cells were exposed to TCDD in the concentration range 0.3 to 10 nM, according to the cell line. Figure 1 shows the comparison of 4 TCDD calibration curves, obtained with the 4 cell lines studied here. The average EC50 values are 540 pM ± 190 pM (S-hum-mam), 63 pM ± 35 pM (S-rat-hep), 630 pM ± 270 pM (S-hum-hep). These values indicate a better sensitivity of the rat cell lines followed by human cell lines. The commercial DR-CALUX H4IIE cells displayed an EC50 of about 10 pM, showing the best sensitivity.



<u>Figure 1:</u> Dose-response curves obtained with 3 "home made" cell lines (from ULg) compared with the commercial DR-CALUX® H4IIE cells from BDS.

Dioxins and AhR's modulators activities – Tissues and species comparison. Dose-response curves have been established for 7 PCDDs and 10 PCDFs, to which TEF values have been assigned[5], to calculate REP (Relative effective Potency) values in each "home made" cell line. These REP values are compared with those of the commercial cell line and with the WHO-TEF (1998), in table I.
Surprisingly, some dioxins have a REP value higher than the reference dioxin (TCDD) in both human assays, but do not in the rat systems. The dioxins producing a highest REP in both human lines are 1,2,3,7,8-PeCDD, 2,3,4,7,8-PeCDF and 1,2,3,4,7,8-HxCDD. The human tissues seem to be less susceptible to dioxin-like compounds than responsive rodent's cell lines. One explanation would be the lower affinity of AhR for TCDD in human cells in comparison with rodent cells[6].
REP values are in the same range for the two rat hepatoma (from ULg and from BDS) cells. The difference of genetic constructions in these two cell lines does not change their responsiveness to dioxins and dioxins-like compounds.

Table 1: REP values determined in "home made" cell lines (ULG cells) compared to DR-CALUX® H4IIE cells from BDS and to WHO-TEF (1998)[5].

Congener	WHO-TEF (1998)[5]	H4IIE-BDS REP[4]	H4IIE-ULg REP	HepG2-ULg REP	T47D-ULg REP
2,3,7,8-TCDD	1	1	1	1	1
1,2,3,7,8-PeCDD	1	0.5	0.2	1.5	1.4
1,2,3,4,7,8-HxCDD	0.1	0.1	0.1	2.4	1.4
1,2,3,6,7,8-HxCDD	0.1	0.06	0.02	0.08	0.05
1,2,3,7,8,9-HxCDD	0.1	0.06	0.01	0.3	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.03	0.02	0.2	0.1
1,2,3,4,6,7,8,9-OCDD	0.0001	0.0008	0.0003	/	0.00005
2,3,7,8-TCDF	0.1	0.4	0.1	0.4	0.2
1,2,3,7,8-PeCDF	0.05	0.1	0.1	0.2	0.4
2,3,4,7,8-PeCDF	0.5	0.4	0.4	1.6	0.9
1,2,3,4,7,8-HxCDF	0.1	0.08	0.05	0.1	0.7
1,2,3,6,7,8-HxCDF	0.1	0.09	0.04	0.7	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.04	0.1	0.07
1,2,3,7,8,9-HxCDF	0.1	0.1	0.06	0.2	0.07
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.009	0.02	0.008
1,2,3,4,7,8,9-HpCDF	0.01	0.05	0.04	0.2	0.1
1,2,3,4,6,7,8,9-OCDF	0.0001	0.004	0.004	0.01	0.004

Comparison of commercial and "home made" H4IIE cells to screen for dioxins in real samples. Two samples of guar gum were extracted and analyzed using both ULg and BDS H4IIE assays (Figure 2).
Even if the H4IIE-luciferase assay developed in ULg is less sensitive than the BDS DR-CALUX® assay, it allows to measure dioxins in both A and B guar gum samples at the same concentration: between 1 and 1.5 pg of TCDD equivalent per well for sample A, and between 5 and 6.5 pg of TCCD equivalent/well, for the extract of sample B diluted 10 times. These concentrations for sample B seem to be relevant, since the 100-fold extract of sample B displays a concentration of 0.5 pg TCDD equivalent /well. These results show that "home made" H4IIE cells could be used to determine the TCDD equivalent concentration in a real sample

In conclusion, on the contrary to rat cell lines, luciferase reporter gene assays developed using human tumour mammary gland and human hepatoma seem to be not sensitive enough to perform dioxin screening at the regulatory levels in food and feed. However, these cell lines could be interesting to study the interaction of pure compounds or sample extracts with the human Ah receptor.

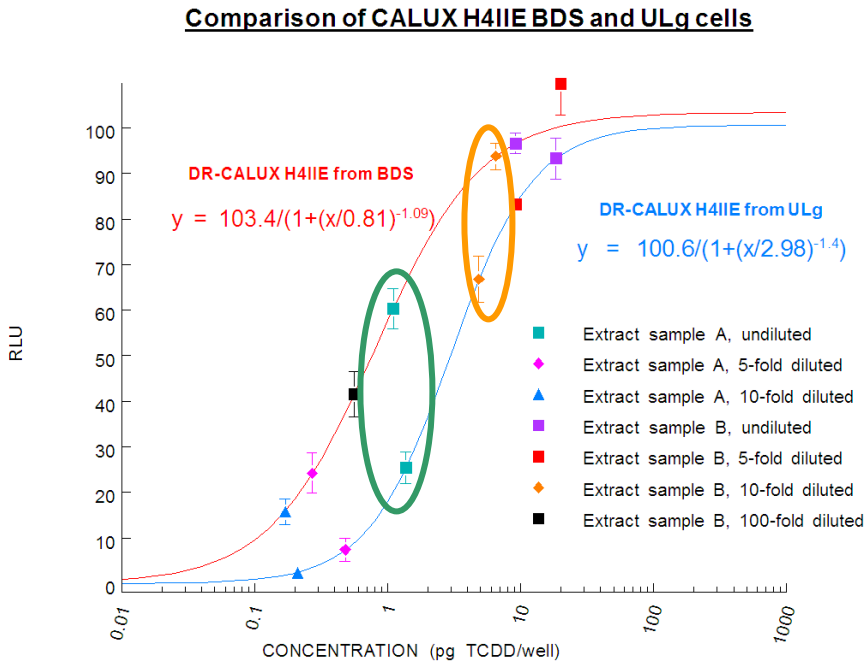


Figure 2: Analysis of two guar gum samples (A and B) with a H4IIE-luciferase cell based assay from BDS (DR-CALUX) and from ULg. Coloured lines represent the TCDD standard curves, while coloured dots correspond to the RLU measured for the sample extracts, as indicated in the legend of the graph. All analyses were performed with 0.4% DMSO in the culture medium. For the undiluted extract, one well contains the dioxins extracted from 0.32 g of sample.

References

1. Schmidt, J.V. and C.A. Bradfield, *Ah receptor signaling pathways.* Annu Rev Cell Dev Biol, 1996. **12**: p. 55-89.
2. Gasiewicz, T.A., et al., *Analysis of structural requirements for ah receptor antagonist activity: Ellipticines, flavones, and related compounds.* Biochemical Pharmacology, 1996. **52**(11): p. 1787-1803.
3. Garrison, P.M., et al., *Species-specific recombinant cell lines as bioassay systems for the detection of 2,3,7,8-tetrachlorodibenzo-p-dioxin-like chemicals.* Fundam Appl Toxicol, 1996. **30**(2): p. 194-203.
4. Scippo, M.-L., et al., *DR-CALUX(R) screening of food samples: evaluation of the quantitative approach to measure dioxin, furans and dioxin-like PCBs.* Talanta, 2004. **63**(5): p. 1193-1202.
5. Van den Berg, M., et al., *Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife.* Environ Health Perspect, 1998. **106**(12): p. 775-92.
6. Ramadoss, P. and G.H. Perdew, *Use of 2-azido-3-[125I]iodo-7,8-dibromodibenzo-p-dioxin as a probe to determine the relative ligand affinity of human versus mouse aryl hydrocarbon receptor in cultured cells.* Mol Pharmacol, 2004. **66**(1): p. 129-36.