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CALUX® reporter cell lines as sensitive bioassays for monitoring biological activity of various classes of steroid hormones reveal the repressive effect of TCDD on the estrogen receptor

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Introduction

BioDetection Systems B.V. (BDS) is a Dutch company providing biological detection systems for the determination of very low levels of high toxic materials such as dioxins and PCBs, and for the detection of endocrine disrupting materials such as estrogens and anti-estrogens. We recently established a range of highly specific and selective CALUX® bioassays for the detection of steroid hormones (estrogens; ERa-CALUX®, androgens; AR-CALUX®, progestins; PR-CALUX®, and glucocorticoids; GR-CALUX®). Here, we describe the characteristics of these assays, with special reference to interference by dioxins of steroid hormone signaling. While TCDD was shown to be a potent repressor of ER signaling in the ER-CALUX® bioassay containing a dioxin signaling pathway, it was without effect in the novel CALUX® bioassays.

Methods and Materials

DNA constructs

Full length cDNAs for human estrogen receptor a (ERa), human androgen receptor (AR), human progesterone receptor (PR) and human glucocorticoid receptor (GR) were inserted in the expression vector pSG5-neo. The reporter construct 3xEREtataLuc was described previously[1]. The reporter construct 3xHREtataLuc was constructed as followed: 3 tandem repeats of HRE (hormone responsive element specific for AR/PR/GR) oligo's[2] upstream of the minimal adenovirus E_1B TATA promoter sequence were inserted in the multiple cloning site of pGL3-basic.

Stable cell lines

The generation of ER-CALUX® cells (ER-positive human breast tumor cells containing a 3xEREtataLuc reporter construct) was described previously[1]. Stable ERa-CALUX® cells were generated by transfection of ER-negative cells with pSG5-neo-ERa and 3xEREtataLuc plasmids. Other stable CALUX® cell lines (using the same parental human cell line as ERa-CALUX® cells: AR/PR/GR-negative) were made with 3xHREtataLuc reporter plasmid co-transfected with either pSG5-neo-AR, pSG5-neo-PR or pSG5-neo-GR expression plasmid using the calcium phosphate precipitation method. Several clones resulting from each transfection were tested for their response to estradiol (E2) (ERa), dihydrotestosterone (DHT) (AR clones), org2058 (PR clones) or dexamethasone (GR clones) and 1 clone for each CALUX® bioassay was selected for consequent high response. Cells were cultured in DF medium supplemented with 7.5 % foetal calf serum (FCS) and 200µg/ml G418.

CALUX® bioassay

Cells were plated in 96 well plates (6.000-10.000 cells/well) with phenol red free DF medium supplemented with 5% dextran coated charcoal stripped FCS (DCC-FCS) at a volume of 100 µl per well. About 48 h later, medium was removed from the cells and 200 µl of DCC-FCS medium containing the compound(s) of interest (dissolved in DMSO or ethanol, final dilution 1:1000) was added. After 24 h the medium was removed, cells were lysed and measured for luciferase activity as described earlier[1].

Sera
Blood from 15 healthy male and female volunteers was collected in silica coated tubes (Capiject, Terumo Medical Corp.). After centrifugation serum was removed, pooled and stored at –20 °C.
Data analysis
Data are represented as mean values ± SEM from at least three independent experiments with each experimental point performed in triplicate. Dose response curves were fitted using the sigmoidal fit in GraphPad Prism (GraphPad Software, San Diego, CA).

Results and discussion
Performance of CALUX® bioassays
Highly sensitive and responsive CALUX® bioassays were developed (Fig.1). Various androgens tested in the AR-CALUX® bioassay displayed EC_{50} values in the nM range with DHT (0.17 nM) and R1881 (0.16 nM) being the most potent androgens (Fig.1A). EC_{50} values for various progestins tested in the PR-CALUX® bioassay were between 50-125 pM with org2058 (51.3 pM) being the most potent progestin (Fig.1B). The GR-CALUX® bioassay displayed EC_{50} values for various glucocorticoids in

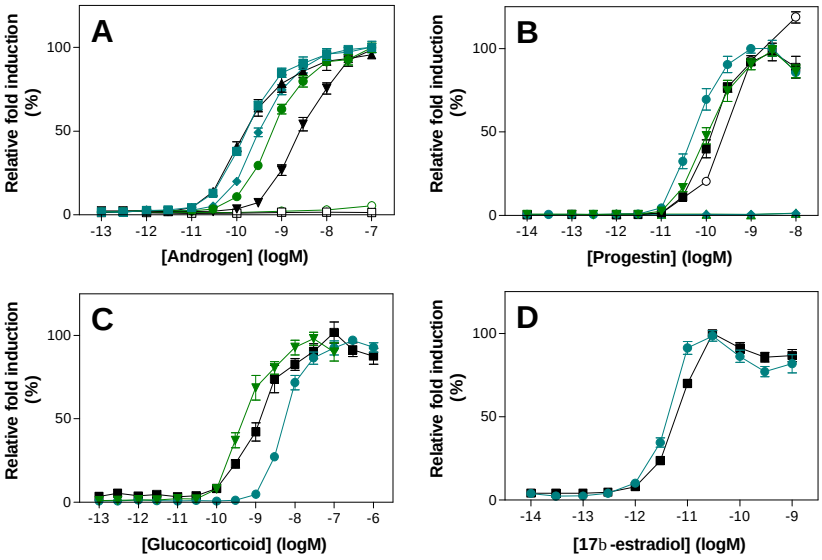

Figure 1: Performance of various CALUX® bioassays. (**A**) Transactivation of AR in the AR-CALUX® cells by the androgens DHT (■), testosterone (●), R1881 (?), nandrolone (◆) or androstenedione (▼) and the progestin org2058 (□) or the glucocorticoid dexamethasone (○). Transcriptional activity is expressed as a percentage of maximal induction by DHT.(B) Transactivation of PR in the PR-CALUX® cells by the progestins MPA (■), org2058 (●) or progesterone (▼), the progestin/androgen R1881 (○) and the glucocorticoid dexamethasone (◆), or the androgen DHT (?). Transcriptional activity is expressed as a percentage of maximal induction by MPA. (C) Transactivation of GR in GR-CALUX® cells by glucocorticoids budesonide (■), hydrocortison (●) or dexamethasone (▼). Transcriptional activity is expressed as a percentage of maximal induction by dexamethasone. (D) Transactivation of ER in the ER-CALUX® cells (■) and in the ERa-CALUX®™ cells (●) by E2. Transcriptional activity is expressed as a percentage of maximal induction by E2.

the 0.5-5 nM range with dexamethasone showing the highest transactivation potential (Fig.1C). Figure 1D shows the sensitivity of two estrogen-specific CALUX® assays. The ER-CALUX® bioassay which contains endogenous estrogen receptors can be used for assessing net mammalian estrogenicity of cocktails of estrogens in *in vivo* or environmental systems, while the ERa specific CALUX® bioassay can be used for measuring relative potency of agonists. Also these bioassays are very sensitive: the EC_{50} for the ER agonist E2 is in the 2-5 pM range for both assays (Fig.1D). The selectivity of the assays towards class-specific hormones was high and no evidence was found for interference by other classes of nuclear hormone receptor ligands in any of the CALUX® bioassays (Fig. 1A, 1B, and data not shown).

Interestingly, the high sensitivity and selectivity of the new assays allowed direct measurements in non-extracted biological samples. As a potential clinical application, human serum was applied directly to the CALUX® bioassays. The presence of androgens in human serum was shown directly by the AR-CALUX® bioassay (Fig.2A: left panel). Surprisingly this serum contains strong anti-androgenic activity, since DHT-mediated AR-transactivation (EC_{50} value of DHT) was completely blocked by the human serum (Fig.2A: right panel). ER-CALUX® cells hardly displayed estrogenic activity in human serum, but instead showed strong antagonistic activity of the serum (Fig.2C: left panel). This is probably due to the high levels of progestin receptors in these cells, since PR is known to show negative cross-talk towards ER. ERa-CALUX® cells do not contain PR and showed the presence of estrogens in human serum. Also no antagonistic activity was observed (Fig.2C: right panel). The presence of progestins in human serum was confirmed in the PR-CALUX® bioassay (Fig.2B: left panel) as was shown indirect by antagonism in the ER-CALUX® bioassay (Fig.2C). No

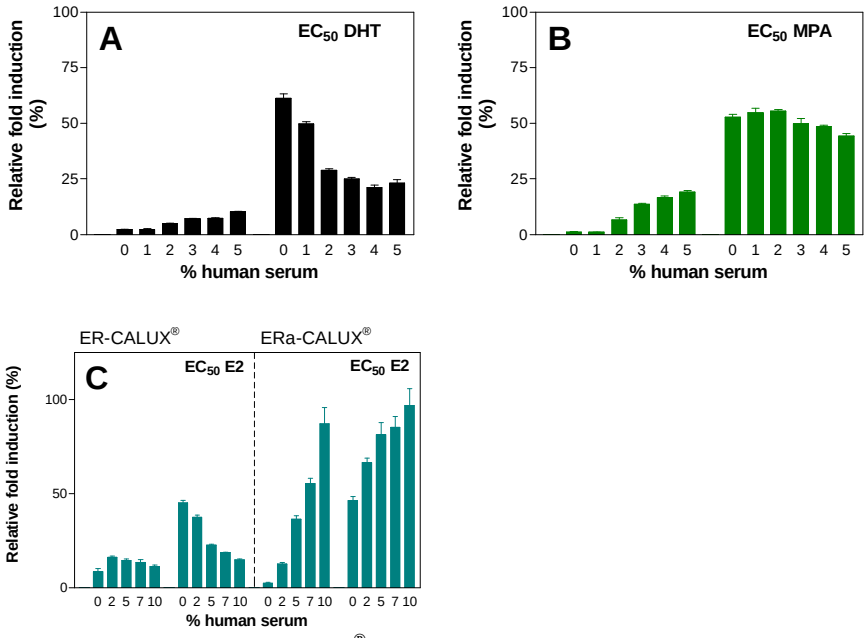

Figure 2: Application of various CALUX® bioassays using human serum. AR-, PR-, ER- and ERa-CALUX® cells were treated with increasing amounts of human serum without or with the EC_{50} value of DHT (A: AR-CALUX®), MPA (B: PR-CALUX®) or E2 (C: ER- and ERa-CALUX®). Transcriptional activity is expressed as a percentage of maximal induction by the reference agonist.

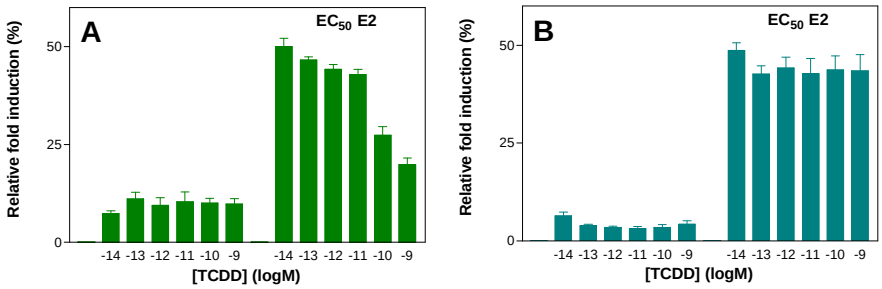

Figure 3: Antagonistic effect of TCDD on ER-signaling in the ER-CALUX® bioassay.
ER-CALUX® (A) and ERa-CALUX® (B) cells were treated with increasing amounts of TCDD without or with the EC_{50} value of E2. Transcriptional activity is expressed as a percentage of maximal induction by E2.

anti-progestagenic activity was observed in human serum (Fig.2B: right panel). The GR-CALUX® bioassay is sensitive enough to demonstrate changing glucocorticoid levels in human patients to be monitored directly. Diurnal endogenous cortisol levels were measured with the GR-CALUX® bioassay and correlated well with cortisol levels measured with a cortisol specific radio immuno assay (data not shown).

TCDD interference of estrogen signaling

The CALUX® bioassays were used to investigate the reported repression of estrogen signaling by TCDD[3]. As shown in Fig.3A, the ER-CALUX® bioassay can be used to show the antagonistic effect on ER: increasing levels of TCDD resulted in decreasing transactivation by E2, confirming earlier results[1]. The ER-CALUX® cells contain active AhR and Arnt (data not shown). The repressive effect is likely caused by a direct interference of estrogen and dioxin signaling pathways at the level of the receptors, since the ER-CALUX® cells express defined synthetic reporter genes that do not allow interference at the promoter level that has been shown to occur with certain promoters containing SP1 binding sites[3]. In ERa- and AR-CALUX® bioassays no agonistic effect of TCDD was observed (Fig.3B and data not shown). This cause of the absence of interference is presently under investigation.

Conclusions

In comparison with other existing reporter gene bioassays, the developed CALUX® bioassays proved to excel in terms of cell line maintenance, fold induction range, minimal detection limit and hormone class specificity. Besides testing the biological activity of different (unknown) hormones or anti-hormones, the CALUX® bioassays can be very useful in screening samples (blood, urine, fat, food, environmental) on (biological active) specific (anti-) hormone content. Furthermore, these bioassays can be used to reveal the molecular mechanisms by which TCDD disrupts endocrine signaling.

References

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