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Differentiating true androgen receptor inhibition from cytotoxicity-mediated reduction of reporter-gene transactivation *in-vitro*

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ABSTRACT

In vitro effect-based reporter assays are applied as biodetection tools designed to address nuclear receptor mediated-modulation. While such assays detect receptor modulating potential, cell viability needs to be addressed, preferably in the same well. Some assays circumvent this by co-transfecting a second constitutively-expressed marker gene or by multiplexing a cytotoxicity assay. Some assays, such as the CALUX®, lack this feature. The cytotoxic effects of unknown substances can confound *in vitro* assays, making the interpretation of results difficult and uncertain, particularly when assessing antagonistic activity. It's necessary to determine whether the cause of the reporter signal decrease is an antagonistic effect or a non-specific cytotoxic effect.

To remedy this, we assessed the suitability of multiplexing a cell viability assay within the CALUX® transcriptional activation test for anti-androgenicity. Tests of both well-characterized anti-androgens and cytotoxic compounds demonstrated the suitability of this approach for discerning between the molecular mechanisms of action without altering the nuclear receptor assay; though some compounds were both cytotoxic and anti-androgenic. The optimized multiplexed assay was then applied to an uncharacterized set of polycyclic aromatic compounds. These results better characterized the mode of action and the classification of effects. Overall, the multiplexed protocol added value to CALUX test performance.

1. Introduction

In vitro testing to detect the specific effects of chemicals is becoming an increasingly important source of information used to assess human health risk (Committee on Toxicity Testing and Assessment of Environmental Agents, 2007; ICCVAM and NICEATM, 2002; OECD, 2005). These assays measure specific cellular functions, including synthetically integrated reporter gene functionalities to enhance the identification and understanding of the toxicological properties of substances. There is currently a particular interest in the use of *in vitro* studies to define the endocrine activity of pure chemicals and detect relevant endocrine active substances in mixtures. In this context, cell-based assays are designed to address specific endocrine mechanisms, such as activation or inhibition of nuclear receptor mediated effects. These tests are then applied as rapid biodetection screening tools to determine inherent endocrine potential, an important source of information used to assess the human health risk of chemicals and mixtures.

The androgen receptor (AR) is an important regulator of male, and to a lesser extent female, sexual health (Gao et al., 2005; Lu et al., 2006). It is a nuclear receptor which when bound to the mammalian androgens testosterone and dihydrotestosterone (DHT) alters gene transcription in favor of more masculine phenotypic characteristics. These effects are generally reversible in adults; however, failure to properly regulate this pathway during periods of fetal development may result in permanent malformations, including feminization of male offspring. Unfortunately, the AR is also capable of binding a wide variety of other ligands. This promiscuity makes the AR a known target for a wide variety of substances with significant potential for dysregulation of androgen signaling.

As a result, testing for disruptions to AR signaling has become an important element within larger *in vitro* batteries to enhance our understanding of the endocrine activity potential of substances. A number of similar transactivation assays are commercially available for measuring alterations in AR-mediated transcription. These cell-based assays typically measure the fluorescent or luminescent signal from a reporter gene under the transcriptional control of an AR-specific

Abbreviations: AR, androgen receptor; EAS, endocrine active substances; DMSO, dimethyl sulfoxide; DHT, Dihydrotestosterone; –, antagonistic activity; PAHs, Polycyclic aromatic hydrocarbons; PANHs or azaarenes, Polycyclic aromatic nitrogen heterocyclics

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promoter region. In the presence of an agonist the fluorescence or luminescence increases, with the further addition of an antagonist, the signal is reduced.

However such assays rely on the underlying health of the cells used, which is largely dependent on basal cellular functions that are also susceptible to toxicological influence from treatment. As these effects on basal cell functions are also likely to affect the specific activities measured in the assay, the cytotoxic effects of unknown substances often confound *in vitro* assay measurements, particularly in situations where the expected measured effect results in a reduction in test signal. This is the case when assessing AR antagonism. Some assays circumvent this conundrum by co-transfecting a second, constitutively expressed reporter gene encoding a different luciferase or a fluorescent protein. But many well-established tests of AR activity, such as the CALUX assay, do not have this feature as part of their methodologies. Thus the cytotoxic effects of unknown substances often confound *in vitro* assay measurements, making the interpretation of results difficult and uncertain.

It should be noted that it is possible to determine whether the cause of the decrease in reporter gene signal is a specific antagonistic effect targeting AR, or a non-specific secondary consequence of changes to cellular viability in such assays. Follow up testing demonstrating the reversibility of the apparent inhibition in the presence of varying concentrations of agonist will confirm a specific antagonistic effect (van der Burg et al., 2010). However, this requires testing several full dose-responses of the putative antagonist, consuming significant amounts of potentially precious sample, increasing the number of assays and resources needed for testing, decreasing the overall assay throughput and considerably delaying the interpretation of results. For these reasons, this approach is suboptimal for mid- to high-throughput screening. In such applications, a direct measure of cytotoxicity, especially one in the same well as that in which the primary test is performed, is preferable to ensure both control of test quality and a proper interpretation of the assay results. Such a multi-parametric strategy is strongly encouraged in current proposals for novel *in vitro* test guidance and regulatory submissions to a variety of regulatory agencies, expert panels, and issues of technical guidance (Committee on Toxicity Testing and Assessment of Environmental Agents, 2007; EPA, 2009; ICCVAM and NICEATM, 2002; OECD, 2005; Parliament, 2008).

A number of cell viability tests have been well-described in the literature targeting many mechanisms of toxicity. Some assays are designed to count cells, either directly through nuclear staining in high content analysis or indirectly using nucleic acid levels (cyanine dye staining). Others highlight the numbers of dead cells *e.g.* cell impermeable dye staining. Still others measure only viable cells using measures of basal cellular activities: proliferation (^{3}H thymidine incorporation), proteolysis (glycylphenylalanyl-aminofluoroumarin cleavage), esterase cleavage (calcein and fluorescein derivatives), mitochondrial potential (rhodamine dyes), the presence of reducing agents (tetrazolium or resazurin dye reduction), or ATP concentration (a variety of luciferin-based assays). The latter category may quantify viable cell numbers only indirectly, but these assays possess certain advantages; most especially that they require the use of a plate-reader rather than microscopy, flow cytometry, or high-content analysis instruments and are consequently higher-throughput.

Thus, the solution to the difficulty in assessing effects of interest like AR antagonism is often the multiplexing of assay protocols measuring basal cellular activities into those established for primary targets. However, these assays must be sufficiently sensitive if possible using the same detection method (*e.g.* fluorescence or luminescence), such that changes in cell viability that are observable in the primary test are also detectable in the secondary cell viability endpoint. Data from treatments at cytotoxic concentrations can then be excluded from evaluation as exceeding the upper testing limit in a manner similar to the Maximum Tolerated Dose is applied to *in vivo* studies (EPA, 2009; ICCVAM and NICEATM, 2002), quantified as the ratio between specific and cytotoxic effects (cytotoxicity index or Z-scoring) or simply interpreted as qualitatively different by taking this cytotoxic context into account (Committee on Toxicity Testing and Assessment of Environmental Agents, 2007).

Unfortunately, many currently accepted *in vitro* tests for anti-androgenicity, including the CALUX® transactivation assay, do not include an in-well cytotoxicity measure as part of their protocol. Among the assays for basal cellular functions described above, protocols quantifying ATP concentration by luminescence generally have a low limit of quantification and are thought to be sufficiently sensitive for inclusion in luminescent reporter gene assay procedures. We evaluated the suitability of multiplexing one such commercially available test, the RealTime-Glo™ MT cell viability assay from Promega, onto the CALUX® anti-androgenicity assay already established in our lab.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO), flutamide (CAS No. 13311-84-7), 5α-Dihydrotestosterone (DHT, CAS No. 521-18-6), menadione (CAS No. 58-27-15, purity > 98%), cadmium chloride ($CdCl_2$) (CAS No. 10108-64-2), etoposide (CAS No. 33419-42-0), methoxychlor (CAS No. 72-43-5), vinclozolin (CAS No. 50471-44-8), acridine (CAS No. 260-94-6, purity ≥ 97%), benzo[*a*]pyrene (CAS No. 50-32-8, purity ≥ 96%), anthracene (CAS No. 120-12-7, purity 97%), benz[*a*]-anthracene (CAS No. 56-55-3, purity 99%), benz[*a*]acridine (CAS No. 225-11-6, purity 99.5%), benz[*c*]acridine (CAS No. 225-51-4, purity 99.8%), dibenz[*a,h*] anthracene (CAS No. 53-70-3, purity 97%), dibenz[*a,j*]acridine (CAS No. 224-42-0, purity 99%), dibenz[*a,h*]acridine (CAS No. 226-36-8, purity 99%) and dibenz[*c,h*]acridine (CAS No. 224-53-3, purity 99%), were purchased from Sigma-Aldrich (Buchs, Switzerland).

Various concentrations of each test substance were prepared to stock concentrations in DMSO. A vehicle control of DMSO was always included within each concentration-range. Similarly, 300 nM DHT or 1500 nM stanozolol stock solutions were also prepared in DMSO. Immediately prior to treatment, either the 300 nM DHT or 1500 nM stanozolol stock was diluted 1000-fold in assay medium; this 0.3 nM DHT or 1.5 nM stanozolol agonist-supplemented assay medium was used to dilute the antagonist stock solutions in DMSO 1:200 in preparation for treatment.

The CALUX® AR cell line, consisting of human U2OS osteosarcoma cells co-transfected with constructs of a human androgen receptor (AR) and a luciferase reporter gene under the direct transcriptional control of repeated copies of the respective hormonal response element (Sonneveld et al., 2005), were licensed from BioDetection System (BDS; Amsterdam, Netherlands). CALUX cells were cultured in 75 cm^2 cell culture flasks (VWR, Dietikon, Switzerland) at 37 °C, 5% CO_2 and 100% humidity. The cells were routinely grown in cell culture media containing DMEM/F12 supplemented with non-essential amino acids (1%), fetal bovine serum (7.5%) (Life Technologies, Zug, Switzerland), 10 units/ml penicillin and 10 μg/ml streptomycin (Sigma-Aldrich, Buchs, Switzerland) and G418 (0.2 mg/ml) (Roche, Mannheim, Germany), as a selection antibiotic. Maintenance cultures were sub-cultured using trypsin (Sigma-Aldrich, Buchs, Switzerland) twice per week and re-suspended 1:3 to 1:8 in growth medium.

2.2. Chemically Activated LUciferase gene eXpression (CALUX) reporter gene assay

All cells destined for experimentation (passages 14–33) were re-suspended in assay medium instead of growth medium at subculture and seeded into 96-well tissue culture treated polystyrene plates (VWR, Dietikon, Switzerland) at a density of 10,000 cells per well. The assay medium was identical to DMEM/F12 growth medium; except that the hormonally-active phenol red pH indicator, G418, and fetal bovine