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Incorporation of metabolic enzymes to improve predictivity of reporter gene assay results for estrogenic and anti-androgenic activity

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ABSTRACT

Identification and monitoring of so-called endocrine-disrupting compounds has received ample attention; both the OECD and the United States Environmental Protection Agency (US EPA) have designed tiered testing approaches, involving *in vitro* bioassays to prioritize and partly replace traditional animal experiments. Since the estrogen (ER) and androgen (AR) receptor are frequent targets of endocrine disrupting chemicals, bioassays detecting interaction with these receptors have a high potential to be of use in risk assessment of endocrine active compounds. However, in many bioassays *in vivo* hepatic metabolism is not accounted for, which hampers extrapolation to the *in vivo* situation. In the present study, we have developed a metabolic module using rat liver S9 as an add-on to human cell-based reporter gene assays. The method was applied to reporter gene assays for detection of (anti-) estrogens and (anti-) androgens, but can be extended to cell-based reporter gene assays covering a variety of endpoints related to endocrine disruption.

1. Introduction

Over the last two decades, evidence has accumulated that exposure to a group of chemicals with a potential to alter the normal functioning of the endocrine system has been associated with adverse health effects in both wildlife and humans. These endocrine disrupting compounds (EDCs) are defined as exogenous substances or mixtures that alter function(s) of the endocrine system, and consequently cause adverse health effects in an intact organism, its progeny, or subpopulations [1].

Exposure to EDCs has been associated with a wide range of adverse events, including disruption of hormone function, early puberty, effects on reproduction and fertility, diabetes, obesity, and the induction of several hormone-related cancers (breast, testis, prostate) [2–5].

Man-made EDCs have been found in many different areas; they can enter the environment during production, use or disposal. Several EDCs are persistent and tend to bioaccumulate; examples include certain pesticides, polychlorinated biphenyls, and brominated flame retardants [4,6]. Other compounds are reaching significant levels because of their high production volumes, such as certain phthalates, bisphenol A and alkylphenols.

Although the potency of most EDCs is orders of magnitude lower than that of natural hormones, and the exposure concentrations often relatively low, this does not mean that they pose no health hazard. Because it has been shown that the activity of many EDCs is additive, effects of low concentrations of environmental EDCs can add up to pass a threshold of adversity [3,7–9].

Endocrine active substances can act via various modes of action [5], such as activation or inactivation of nuclear receptor-mediated transcription, induction or inhibition of receptor expression, or metabolism of endogenous hormones e.g. through modulation of steroidogenic enzyme expression and function. The Organisation for Economic Co-operation and Development (OECD) has designed a conceptual framework covering several of these steps in a draft testing battery consisting of different information levels [10]. The first level involves gathering of existing data and non-test information, the second level involves data obtained from *in vitro* assays, and higher levels involve *in vivo* experimentation. Likewise, the US EPA has designated a tiered testing system in their Endocrine Disruptor Screening Program (EDSP) [11]. In both testing schedules interaction with the estrogen, androgen, and thyroid hormone sys-

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tem are key elements. In particular, the estrogen receptor (ER) and androgen receptor (AR) have been found to be frequent targets of endocrine disrupting chemicals.

This paper focuses on these two major EDC targets, using CALUX reporter gene assays [12]. The ERα CALUX assay has been validated successfully and is included in an OECD guideline for stably transfected transactivation assays to detect estrogen agonists and antagonists (TG455) [13,14]. More recently a related OECD guideline has been drafted to assess chemical interactions with the androgen receptor, and the AR CALUX assay is currently undergoing validation for The European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) to be incorporated in this guideline [15]. Currently, in the context of the US-EPA Toxicity Forecaster (ToxCast) program a battery of tests has been proposed to predict both of these endpoints [16]. Since application of the ToxCast battery of tests would lead to a significant higher demand on resources than running a single assay for each endpoint, we compared the published results from the EPA battery with experimental results of the CALUX assays.

Several review papers have been published indicating that metabolism is particularly important when testing EDCs, since several hormonally active chemicals are known to be subject to metabolism [5,17,18]. Because compounds can be converted into either more active or less active metabolites, neglecting to determine metabolism can lead to both false negatives and false positives.

In the current study we show that the CALUX system has very low basal metabolic competence. Therefore we have developed a metabolic module using rat liver S9 as an add-on to the CALUX assays; a proof-of-principle of this method was published recently [19]. In genotoxicity testing, mimicking *in vivo* hepatic metabolism using S9 fractions is common practice [20–22]. This approach has also been explored for EDCs [23–25].

The current paper describes the first step towards a validated method to incorporate metabolism in CALUX reporter gene assays for detection of (anti-)estrogens and (anti-)androgens [26]. This provides important data on the role of metabolism in EDC activity, which supports better options for *in vitro/in vivo* extrapolation. For efficiency, the methods were made compatible with automated high-throughput screening.

2. Materials and methods

2.1. Cell lines and cell culture

The ERα CALUX and AR CALUX cell lines as described by Sonneveld et al., [12] are human U2-OS cells stably transfected with an expression construct for the full-length human ERα- or AR receptor, and a reporter construct consisting of multimerized responsive elements for the cognate receptor coupled to a minimal promoter element (TATA) and a luciferase gene. Cells were maintained as described previously [12]. The Cytotox CALUX, used as a control line for non-specific effects, consists of human U2-OS cells stably transfected with an expression construct constitutively expressing the luciferase gene, and is described in [20]. Wild-type U2-OS cells (HTB-96) and wild-type HepG2 cells (HB-8065; used as control cell line to assess metabolic capacity) were obtained from ATCC.

2.2. Compound selection

The 42 compounds selected for this study were based on several OECD-guidelines. For validation according to the OECD TG455 (Test Guideline for Stably Transfected Transactivation In Vitro Assays to Detect Estrogen Receptor Agonists and Antagonists), 17 positive (agonist) and six negative (no receptor activation) compounds were used. Positives: 17α-estradiol; 17α-ethinylestradiol; 17β-estradiol; 19-nortestosterone; 4-cumylphenol; 4-tert-octylphenol; bisphenol A; butylbenzyl phthalate; coumestrol; diethylstilbestrol; ethyl paraben; genistein; kaempferol; kepone; meso-hexestrol; p,p'-methoxychlor; norethynodrel. Negatives: atrazine; corticosterone; ketoconazole; linuron; reserpine; spironolactone.

In OECD TG457 [27], the VM7Luc4E2 estrogen receptor transactivation test method for identifying estrogen receptor agonists and antagonists, a very similar, largely overlapping list of 27 positive and nine negative compounds is described. Positives: 17-methyltestosterone; 17α-estradiol; 17α-ethinylestradiol; 17ß-estradiol; 4-cumylphenol; 4-nonylphenol; 4-tert-octylphenol; 5α-dihydrotestosterone; apigenin; bisphenol A; butylbenzyl phthalate; chrysin; daidzein; p,p'-DDE; o,p'-DDT; di(2-ethylhexyl) phthalate; dicofol; dibutyl phthalate; diethylstilbestrol; estrone; ethylparaben; fenarimol; genistein; kaempferol; kepone; meso-hexestrol; p,p'-methoxychlor. Negatives: atrazine; corticosterone; flutamide; haloperidol; ketoconazole; linuron; procymidone; reserpine; spironolactone.

Finally, in the OECD Validation Management Group for Non-Animal testing (VMG-NA) paper [17], several preliminary reference compounds are proposed for validation of metabolising systems for ER- and AR- assays: 17β-estradiol; 4-tert-octylphenol; bisphenol A; flutamide; genistein; p,p'-methoxychlor; tamoxifen; testosterone; vinclozolin.

2.3. Chemicals

Aroclor-induced Sprague-Dawley rat liver S9 fraction was obtained from MolTox (11-101). Phenobarbital/β-naphtoflavone (PB-BNF) induced Sprague-Dawley rat liver S9 mix was obtained from MolTox (11-105). NADPH-tetrasodium salt was from Applichem.

17α-estradiol, genistein, glucose-6-phosphate and testosterone were supplied by Wako. 3′-Phosphoadenosine-5′-Phosphosulfate (PAPS) was from Santa Cruz Biotechnology. Apigenin, chrysin, corticosterone, coumestrol, cumylphenol, daidzein, dibutyl phthalate, dicofol, diethylstilbestrol, estradiol, estrone, 17α-ethinylestradiol, ethyl paraben, flutamide, glucose-6-phosphate-dehydrogenase, glutathione (GSH), haloperidol, kaempferol, linuron, meso-hexestrol, 4-nonylphenol, p,p'- Dichlorodiphenyldichloroethylene (p,p'- DDE), p,p'-methoxychlor, norethynodrel, 19-nortestosteron, reserpine, spironolactone, tamoxifen, 4-tert-octylphenol, procymidone, rifampicin, stanozolol, Uridine 5′-diphospho-glucuronic Acid (UDPGA) and vinclozolin were obtained from Sigma. Atrazine, butylbenzyl phthalate, bis-(2-ethylhexyl)phthalate, fenarimol, 17-methyltestosterone and ketoconazole were obtained from Fluka. Bisphenol A was obtained from Aldrich. Kepone was obtained from Supelco. Dihydrotestosterone was obtained from Steraloids. o,p'- Dichlorodiphenyltrichloroethane (o,p'- DDT) was obtained from Chem Service. Tributyltinacetate was obtained from Merck. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) was obtained from Cerilliant.

2.4. CALUX assay procedure

Testing was performed in non-blinded fashion. The automated CALUX assays were carried out as described earlier [28]. In brief, the assay was performed in assay medium, consisting of DMEM without phenol red indicator (Gibco) supplemented with 5% charcoal-stripped fetal calf serum (DCC), 1 × non-essential amino acids (Gibco) and 10 U/ml penicillin and 10 μg/ml streptomycin. A cell suspension in assay medium was made of 1×10^5 cells/ml, and white 384-wells plates were seeded with 30 μl cell suspension/well. After 24 h, exposure medium was prepared. A dilution