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In house validation of recombinant yeast estrogen and androgen receptor agonist and antagonist screening assays

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ABSTRACT

Besides other modes of action, endocrine disruptors may interact with hormone receptors thereby modifying the physiological function of endogenous hormones. In the present study, we report the results obtained with yeast based assays to detect the (anti-)estrogenic potential (YES) and the (anti-)androgenic potential (YAS) of 105 substances. The results show very high reproducibility and good concordance with literature data of *in vivo* and/or *in vitro* studies: the overall true positive rate, true negative rate and accuracy of the assays were 78%, 95%, and 87% (estrogen agonism), and 70%, 97%, and 90% (estrogen antagonism), 88%, 96%, and 95% (androgen agonism) and 81%, 88%, and 85% (androgen antagonism). Furthermore, the performance of the YES assay has been compared to the HeLa based transcriptional activation assay using 20 compounds. The overall true positive rate, true negative rate, and accuracy obtained for the 20 compounds were 100%, 88%, and 95% (mammalian cell based HeLa assay) and 92%, 86%, and 90% (yeast based YES assay). Taken together, the YES and YAS are robust systems, easy to handle and satisfying the requirements for screening systems that can be applied in programs including the US Environmental Protection Agency's Endocrine Disruptor Screening Program.

1. Introduction

A variety of natural and synthetic chemicals can mimic hormone-like activity through interaction with endogenous hormone receptors, including the estrogen receptor (ER). Estrogenic chemicals identified to date include some organochlorine pesticides, such as *o,p′*-DDT and methoxychlor, and industrial chemicals and byproducts, including some polychlorinated biphenyl congeners, alkyl phenols, and bisphenol A. There is concern that exposure to such compounds might disrupt normal endocrine function, leading to altered reproductive capacity, infertility, endometriosis, and cancers of the breast, uterus, and prostate (Sonnenschein and Soto, 1998). Compounds interfering with physiological androgens include the anabolic steroids R1881 and fluoxymesterone, the herbicide linuron, and the plasticizer benzylbutylphthalate. There is additional concern that some abnormalities in male sexual development may be mediated through androgen receptor (AR) interaction (Foster and McIntyre, 2002; Kelce et al., 1998; Rempel and Schlenk, 2008).

In vitro tests for the detection of endocrine disruption include hormone-responsive mammalian cell proliferation (mitogenesis) and hormone-sensitive transcription of reporter genes (transcriptional activation) in mammalian cell lines and yeasts. *In vivo* estrogen and androgen agonist or antagonist action may occur through either a direct interacting with the steroid receptor, or indirectly, i.e., by non-receptor mediated action, for example, by changing steroid synthesis. In the *in vitro* assays described here estrogen and androgen antagonists are characterized by reduced receptor mediated transcriptional activation. The U.S. EPA has proposed an Endocrine Disruptor Screening Program (EDSP) to evaluate pesticides and other substances for their potential to induce hormone related health effects (EPA 1998). The proposed EDSP consists of a Tier 1 screening battery of *in vitro* and *in vivo* test methods designed to identify substances capable of interacting with the endocrine system, and Tier 2 *in vivo* testing designed to confirm and

Abbreviations: YES, yeast estrogen screening; YAS, yeast androgen screening; A, androgen/-ic; AA, antiandrogen/-ic; AE, antiestrogen/-ic; AR, androgen receptor; E, estrogen/-ic; ER, estrogen receptor; hERα, human estrogen receptor α; hAR, human androgen receptor; TA, transcriptional activation; ERE, estrogen response element; U.S. EPA, United States Environmental Protection Agency; EDSP, Endocrine Disruptor Screening Program; OECD, Organisation for Economic Co-operation and Development; TG, test guideline; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; DINP, diisononylphthalate; R1881, methyltrienolone; 4,4′-DDD, dichlorodiphenyl-dichlorethane; HPTE, dihydroxymethoxychlor; *o,p′*-DDT, 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane; *p,p′*-DDE, 1,1′-(2,2-dichloroethene-1,1-diyl)bis(4-chlorobenzene); DEHP, bis(2-ethylhexyl) phthalate; DMSO, dimethyl sulfoxide; CPRG, chlorophenol red β-D-galactopyranoside; FBS, fetal bovine serum; DCC, dextran-coated-charcoal treated; PC, positive control; VC, vehicle control.

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extend the Tier 1 results. The proposed Tier 1 screening battery includes *in vitro* ER and AR binding assays and a HeLa cell based transcriptional activation assay. Generally, several reporter gene assays have been described as *in vitro* transcriptional activation (TA) assays to evaluate specific gene expression regulated by specific nuclear receptors such as the estrogen receptors. Following binding of the chemical to a specific receptor and subsequent downstream transcriptional activation reporter gene production is induced. Yeast based reporter assays for steroid-dependent transcriptional activation have long been described by several groups (Beck et al., 2008; Gaido et al., 1997; Mak et al., 1989; Metzger et al., 1988; Purvis et al., 1991; Routledge and Sumpter, 1996; Sanseverino et al., 2009; Sohoni and Sumpter, 1998). In this study, we have used screening systems based on genetically modified yeast cells to detect the (anti-) estrogenic potential (YES) and the (anti-) androgenic potential (YAS) of test substances. Genes, encoding the hERalpha, and the human androgen receptor, respectively, have been integrated into the genome of the yeast strains used. Additionally, the cells contain a plasmid carrying the *lac-Z* gene, which is receptor-dependently expressed and serves as a reporter gene. Recently, OECD TG 455 (OECD 2009) and OPPTS guideline 890.1300 (EPA 2009) utilizing the hERα-HeLa-9903 cell line derived from a human cervical tumor, with two stably inserted constructs encoding the hERα and the firefly luciferase gene as well as an ERE have been adopted.

The aim of the present study was to determine the true positive rate, true negative rate and overall accuracy of the YES and YAS *in vitro* assays to assess estrogen agonistic and antagonistic, androgen agonistic and antagonistic properties. For this purpose a total of 105 test compounds, for which a literature research was performed with respect to their endocrine modulating activity, were assessed for their endocrine activities in the YES/YAS assay. A total of 20 chemicals have been tested in both the YES and HeLa assay to compare their performance in the detection of estrogen agonistic properties.

2. Materials and methods

2.1. Tested chemicals

The 105 chemicals assessed in the in house validation of the YES and YAS assay were of highest available purity (given in parentheses) and purchased from Sigma Aldrich unless noted otherwise. The 105 chemicals tested comprised the following.

2.1.1. Negative compounds (n = 28)

Acetylsalicylic acid (50-78-2; >99%), actinomycin D (50-76-0; 98%), benzalkonium chloride (63449-41-2; ⩾97%), benzoic acid (65-85-0; 99.5%), caffeine (58-08-2; 98.5%), chlormequatchloride (999-81-5; 98.8%), cholesterol (57-88-5; >99%), clofibrate (637-07-0, >98%), colchicine (64-86-8; 95%), di-*n*-hexylphthalate (84-75-3; 98%), dopamine hydrochloride (62-31-7; ⩾99%), dodecyl sulfate sodium salt (SDS, 151-21-3; >99%), Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 67-42-5; 97%), ethanol (64-17-5; 99%), ethyleneglycol (107-21-1; 99.5%), forskolin (66575-29-9; 98%), indomethacin, (53-86-1; 100%), nonylphenol (104-40-5; 99.9%), N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate (16980-89-5; 99%), nonylphenylpolyethylen-glycolacetate (54612-40-7; unknown), ouabain (11018-89-6; ⩾95%), paraoxon (311-45-5; 98%), paraquat (1910-42-5; 99%), sodium azide (26628-22-8; 99.5%), L-thyroxine (51-48-9; ⩾98%), Triton X-100 (9002-93-1; 98%), Tween 20 (9005-64-5; ∼50%), zineb (12122-67-7; 68.4%).

2.1.2. ER agonists (n = 10)

Chlordecone (kepone, 143-50-0; 99.8%), coumestrol (479-13-0; ⩾95%), daidzein (486-66-8; ⩾98%), deoxycholic acid (83-44-3; >99%), diethylstilbestrol (DES, 56-53-1; 99.5%), diethylstilbestrol-dipropionat (130-80-3; ⩾99%), dimethyl formamide (68-12-2; ⩾99%), di-*n*-propylphthalate (131-16-8; 99.7%), estradiol (57-91-0; >98%), estradiol-3-benzoate (50-50-0; 98%).

2.1.3. ER antagonists (n = 3)

Atrazine (1912-24-9; 97.4%), cycloheximide (66-81-9; >94%), ICI 182,780 (129453-61-8; >98%);

2.1.4. AR agonists (n = 3)

4-Androstene-3,17-dione (63-05-8; 98.9%), fluoxymesterone (76-43-7; ⩾99%), methyltrienolone (R1881, 965-93-5; unknown),

2.1.5. AR antagonists (n = 7)

4-*n*-Octylphenol (1806-26-4; 99%), dibutylphtalate (DBP; 84-74-2; 98.7%), diisononylphthalate (DINP, 28553-12-0; 99.5%), di-*n*-amylphthalate (131-18-0; >99%), finasteride (98319-26-7; 99%), ketoconazole (65277-42-1; ⩾99%), vinclozolin (50471-44-8; 99.2%).

2.1.6. ER agonists and ER antagonists (n = 14)

2,4,5-Trichlorophenoxyacetic acid (93-76-5; 97%), 2-ethylhexyl-*p*-hydroxybenoate (5153-25-3; 100%), 4,4′-(1,3-phenylenediisopropylidene)bis-phenol (13595-25-0; 99%), 4,4′-cyclohexylidene-bisphenol (843-55-0; 98%), 4,4′-dihydroxydiphenyl-methane (620-92-8; 98%), 4,4′-sulfonyldiphenol (80-09-1; 98%), 4-*t*-butylpyrocatechol (98-29-3; 97%), clomiphene citrate (50-41-9; unknown), dexamethasone (50-02-2; ⩾98%), genistein (446-72-0; 96%), hydroxytamoxifen (68047-06-3; >98%), *p*-*t*-butylphenol (98-54-4; 99%), resveratrol (501-36-0; 99%), tamoxifen (10540-29-1; >99%);

2.1.7. ER agonists and AR agonists (n = 5)

Dihydrotestosterone (521-18-6; >99%), estrone (53-16-7; 99.3%), 17α-methyltestosterone (58-18-4; ⩾97%), testosterone (58-22-0; 99%), trenbolone (10161-38-8; >95%).

2.1.8. ER agonists and AR antagonists (n = 13)

Dichlorodiphenyl-dichlorethane (4,4′-DDD, 72-54-8; 98.9%), apigenin (520-36-5; ⩾95%), benzophenone (119-61-9; >99%), benzylbutylphthalate (85-68-7; 97.4%), bisphenol A (80-05-7; 98.6%), bisphenol A-dimethacrylate (3253-39-2;>98%), ethinylestradiol (57-63-6; 99%), fenarimol (60168-88-9; 99.8%), kaempferol (520-18-3; 96%), methoxychlor (72-43-5; 99.0 ± 0.5%), 1,1′-(2,2-dichloroethene-1,1-diyl)bis(4-chlorobenzene) (*p*,*p*′-DDE, 72-55-9; >99%), procymidon (32809-16-8; 99.9%), zearalenone (17924-92-4; 99.5%);

2.1.9. AR agonists and AR antagonists (n = 8)

Bis(2-ethylhexyl) phthalate (DEHP, 117-81-7; 99.5%), bicalutamide (90357-06-5; 98.5%), cyproteronacetate (427-51-0; >98%), hydroxyflutamide (52806-53-8; >98%), mifepristone (84371-65-3; ⩾98%), nilutamide (63612-50-0; 100%), progesterone (57-83-0; >99%), spironolactone (52-01-7; >97%).

2.1.10. ER antagonists and AR antagonists (n = 7)

1α,25-Dihydroxyvitamin D3 (calcitriol, 32222-06-3; ⩾99%), 6α-methyl-17αhydroxy-progesterone (medroxyprogesterone, 520-85-4, unknown), corticosterone (50-22-6; 98.5%), flavone (525-82-6; >99%), prochloraz (67747-09-5; 99.5%), vitamin D3 (cholecalciferol, 67-97-0; >99%), xanthohumol (569-83-5, obtained from Saarland University, Germany; unknown).