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Identification of coregulators involved in estrogen receptor subtype specific binding of the ER antagonists 4-hydroxytamoxifen and fulvestrant

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ABSTRACT

The aim of the present study was to investigate modulation of the interaction of ERα and ERβ with coregulators in the ligand dependent responses induced by the ER antagonistic compounds 4OHT and fulvestrant. Comparison with the MI profiles for the ER agonist E2, will elucidate whether differences in the (ant)agonist dependent interaction of ERα and ERβ with coregulators expressed in MI profiles contribute to the differences in (ant)agonist responses.

To this end, the selected ER antagonistic compounds were first characterized for intrinsic relative potency and efficacy towards ERα and ERβ using ER selective U2OS reporter gene assays, and subsequently tested for ligand dependent modulation of the interaction of ERα and ERβ with coregulators using the MARCoNI assay. Results obtained indicate a preference of 4OHT to antagonize ERβ and find fulvestrant to be less ER specific. MARCoNI assay responses reveal that ERα and ERβ mediated interaction with coregulators expressed in MI profiles are similar for 4OHT and fulvestrant and generally opposite to the MI profile of the ER agonist E2. Hierarchical clustering based on the MI profiles appeared able to clearly discriminate the two compounds with ER antagonistic properties from the ER agonist E2. Taken together the data reveal that modulation of the interaction of ERs with coregulators discriminates ER agonists from antagonists but does not discriminate between the less specific ER antagonist fulvestrant and the preferential ERβ antagonistic compound 4OHT. It is concluded that differences in modulation of the interaction of ERα and ERβ with coregulators contribute to the differences in ligand dependent responses induced by ER agonists and ER antagonists but the importance of the subtle differences in modulation of the interaction of ERs with coregulators between the ER antagonistic compounds 4OHT and fulvestrant for the ultimate biological effect remains to be established.

Abbreviations: 4OHT, 4-hydroxytamoxifen; ATP, adenosine triphosphate; BSA, bovine serum albumin; CCD, charge coupled device; CDTA, 1,2-diaminocyclohexanetetraacetic acid monohydrate; DCC-FCS, dextran-coated charcoal-treated fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; E2, 17β-estradiol; EC_{50}, half maximal effective concentration; EC_{100}, concentration where 100% of the maximal effect is reached; *E. coli*, *Escherichia coli*; EDTA, ethylenedinitrilotetraacetic acid; EEF, Estradiol Equivalence Factor; ER, estrogen receptor; ERE-TATA-luciferase, estrogen responsive element and TATA box binding protein combined with a luciferase gene; FCS, fetal calf serum; GST, glutathione S-transferase; His, polyhistidine; IC_{50}, half maximal inhibitory concentration; LBD, ligand binding domain; MARCoNI, Microarray Assay for Realtime Coregulator – Nuclear Receptor Interaction; MI, modulation index; NEAA, non-essential amino acids; PBS, phosphate buffered saline; R^2, coefficient of determination; RLU, relative light units; SD, standard deviation; SERM, Selective Estrogen Receptor Modulator; TBS, Tris-buffered saline; TR-FRET, time-resolved fluorescence resonance energy transfer; U2OS cells, human osteosarcoma cells; U2OS-ERα cells, stably transfected human osteosarcoma cells expressing 3xERE-TATA-luciferase next to ERα; U2OS-ERβ cells, stably transfected human osteosarcoma cells expressing 3xERE-TATA-luciferase next to ERβ.

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1. Introduction

Estrogens affect cell growth in several tissues [1–3]. These effects are modulated by binding of the estrogenic compounds to estrogen receptor (ER)α and ERβ thereby regulating gene transcription [4–6]. Estrogenic compounds that bind to the ERα and ERβ may be agonists inducing a physiological response similar to that induced by the natural ligand 17β-estradiol (E2) or antagonists, of which the binding results in inhibition of the E2 mediated activation and cellular responses [7,8]. In addition to the nature of the ligand, being an agonist or antagonist, also the intrinsic potency and efficacy (partial or full) of the ligand for the two receptors [7,9], and the ERα/ERβ ratio in the cells or tissue of interest are important for the ultimate biological effect and these factors have been studied in some detail [5–7,10].

ERα and ERβ have been reported to exert counteracting effects on cell proliferation. ERα activation enhances cell proliferation [11], whereas ERβ activation counteracts ERα mediated cell proliferation [7,12–15]. Therefore, ERα antagonism leads to repression of cell growth [16], whereas ERβ antagonism leads to enhanced cell proliferation [17].

It has been suggested that estrogen mediated effects on cell proliferation through the action of ERα and ERβ are involved in the development and progression of cancer of especially estrogen responsive tissues [12,18]. For ER-positive breast tumors as well as for other estrogen-dependent tumors it has been shown that in tumorous tissue compared to normal tissue the ERα/ERβ ratios increase due to a decreased ERβ expression [12]. This role for ER mediated effects on cell proliferation and cancer is also reflected in the use of ER antagonists in hormonal cancer therapy for the treatment of ERα-positive breast tumors [8]. With appropriate endocrine therapy based on ER antagonists, patients with ERα-positive breast cancer have a better prognosis than those with ERα-negative tumors [19].

ER antagonistic compounds for the treatment of breast tumor tissue include tamoxifen [20,21] and fulvestrant [22]. Tamoxifen is a first line breast cancer drug widely used for treatment of ERα-positive breast cancers [23]. It acts by blocking the ER (both ERα and ERβ) [24,25]. Tamoxifen would only properly work in breast cancer cells with relatively high ERα and low ERβ expression, since then tamoxifen will block ERα and thereby reduce ERα induced cell proliferation [17]. 4-Hydroxytamoxifen (4OHT) is the active metabolite of tamoxifen tested in the trans isoform which possesses stronger anti-estrogenic activity than the cis isoform [26]. It has a 100 times higher potency towards both ER isoforms than tamoxifen itself [27]. In competition binding assays with E2, the compound 4OHT has a 1.6 times higher binding affinity for ERβ over ERα [28]. However, tamoxifen and 4OHT are not full ER antagonists, but are Selective Estrogen Receptor Modulators (SERMs) displaying both ER agonistic and antagonistic properties depending on the physiological context [21]. In contrast, fulvestrant is a full ERα and ERβ antagonist and is used as a second line breast cancer drug [29]. Fulvestrant acts by blocking both ERs and reducing cellular levels of ERα [8,30–33]. In tests for its antagonist activity towards E2, it has a 9 times higher antagonistic preference for ERα over ERβ [34].

An important factor that may influence the ultimate gene expression and biological effect induced by estrogenic compounds is the type of coactivators that can bind to the ER-ligand complex. This aspect of interaction of ERs with coregulators has not been studied in great detail. It has been shown that these coregulators influence the ER-mediated activation or repression and transcription of target genes. Coactivators and corepressors have a role in the response to estrogenic and anti-estrogenic compounds, chromatin condensation and mediating transcription [5,10,35,36].

Recently we investigated modulation of the interaction of ERα and ERβ with coregulators in the ligand dependent responses induced by ER agonists [37]. The data obtained revealed that differences in modulation of the interaction of ERα and ERβ with coregulators contribute to the different ligand dependent responses induced by different ER agonists but do not contribute significantly to the differences between ERα and ERβ mediated responses by a given ER agonist. The aim of the present study was to investigate modulation of the interaction of ERs with coregulators upon exposure to the ER antagonistic compounds 4OHT and fulvestrant. To this end, 4OHT and fulvestrant were tested for intrinsic relative potency reflected by IC_{50} and efficacy towards ERα and ERβ using human osteosarcoma U2OS reporter assays and the ligand dependent modulation of the interaction of ERα and ERβ with coregulator induced by the model compounds was investigated using a microarray assay for realtime coregulator – nuclear receptor interaction (MARCoNI) with 154 unique nuclear receptor coregulator peptides of 66 different coregulators.

2. Materials and methods

2.1. Cell culture

The U2OS cell lines, stably expressing ERα or ERβ, in addition to a 3x estrogen responsive element and TATA box binding protein combined with a luciferase gene (3x ERE-TATA-luciferase gene) were kindly provided by the Hubrecht Institute, Utrecht [7].

U2OS-ERα cells were grown in DMEM:F12, a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F12 (Gibco, Paisley, Scotland, 31331-028) supplemented with 10% fetal calf serum (FCS, Invitrogen, Paisley, Scotland, #10099), 0.5% non-essential amino acids (NEAA, Gibco, Paisley, Scotland, 11140-035), 0.2 mg/ml geneticin G418 (PAA Laboratories GmbH, Pasching, Austria, #P02-012) and 0.05 mg/ml hygromycin (Duchefa, Haarlem, the Netherlands, # H0192.0001). U2OS-ERβ cells were grown in 1:1 DMEM:F12 culture medium supplemented with 10% FCS, 0.5% NEAA, and 0.2 mg/ml geneticin G418 [7].

All cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere.

Because phenol red exerts estrogenic activity [38], at least 24 h before exposure to the selected model compounds, cells were washed 3 times with phosphate buffered saline (PBS, Gibco, Paisley, Scotland, #10010-015) and transferred to phenol red free medium (Gibco, Paisley, Scotland, #21041-025) supplemented with 5% hormone-free dextran-coated charcoal-treated fetal calf serum (DCC-FCS, Perbio Science, Waltham, MA, USA, #SH30068.05) and 0.5% NEAA.

2.2. U2OS reporter gene assay

Cells were seeded in 96-well view plates (PerkinElmer, Groningen, the Netherlands, #655180) at a density of 10^5 cells/ml for U2OS-ERα and $7.5 \cdot 10^4$ cells/ml for U2OS-ERβ, 100 μl/well.

Twenty-four hours after seeding, medium was changed to phenol red free medium. Fourty-eight hours after seeding, cells were exposed to the test compounds in triplicate, in phenol red free medium. 17β-estradiol (E2) was chosen as the positive standard estrogenic agonist and used to characterize the antagonist properties of fulvestrant and 4OHT. E2 is known to be both an ERα and ERβ agonist with an approximate 10-fold higher preference for ERα over ERβ as shown in ligand binding experiments with solubilized Sf9 insect cell extracts, and U2OS reporter cell lines [5,39,40]. Progesterone was included as a negative control for ER binding [41,42].

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