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MCF-7aro/ERE, a novel cell line for rapid screening of aromatase inhibitors, ERα ligands and ERRα ligands

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ABSTRACT

We have previously generated a breast cancer cell line, MCF-7aro, which over-expresses aromatase and is also ER positive. Recently, this MCF-7aro cell line was stably transfected with a promoter reporter plasmid, pGL3-Luc, containing three repeats of estrogen responsive element (ERE). Experiments using MCF-7aro/ERE have demonstrated that it is a novel, non-radioactive screening system for aromatase inhibitors (AIs), ERα ligands and ERRα ligands. The screening is carried out in a 96-well plate format. To evaluate this system, the cells were cultured overnight in charcoal–dextran stripped FBS medium supplemented with 0.1 nM testosterone or 17β-estradiol, and various concentrations of antiestrogens or AIs. We found that the luciferase activity was induced when the cells were cultured either in the presence of testosterone or 17β-estradiol. Furthermore, a 50% decrease in luciferase activity could be achieved when the cells were cultured in the presence of testosterone together with letrozole, anastrozole, tamoxifen or fulvestrant (concentrations being 75 nM, 290 nM, 100 nM, and 5 nM, respectively), compared to the testosterone-only cultured cells. Using this assay system, we confirmed that 3(2′-chlorophenyl)-7-methoxy-4-phenylcoumarin is an agonist of ER. Furthermore, genestein has been shown to be a ligand of ERRα because its binding could be blocked by an ERRα inverse agonist, XCT790. These results indicate that MCF-7aro/ERE is a novel cell line for rapid screening of AIs, ERα ligands and ERRα ligands.

1. Introduction

Aromatase is the key enzyme in catalyzing the conversion of androgen to estrogen. Estrogen plays a crucial role in breast cancer development and progression. In postmenopausal women, while the blood estrogen levels are very low, an abnormally elevated expression of aromatase is frequently found in breast cancer tissues [1–4]. A high level of aromatase activity sustains the endogenous conversion of estrogen in breast cancer cells, which maintains the survival and progression of tumor growth. To suppress the growth and progression of hormone-dependent cancers, antiestrogens and aromatase inhibitors (AIs) are used as first line treatments or as an adjuvant hormonal therapy immediately after surgery.

Tamoxifen (Nolvadex) is a well-known antiestrogen. It binds to the estrogen receptor (ER) and blocks the effect of estrogen. Beside its antagonistic effect on breast tissue, tamoxifen shows an agonistic effect predominantly in the bone and uterus. Therefore, prolonged use of tamoxifen increases the risk for developing endometrial cancer [5]. Another antiestrogen, fulvestrant (“Faslodex”: ICI 182, 780 or

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Abbreviations: AI, aromatase inhibitor; Cou, 3(2′-chlorphenyl)-7-methoxy-4-phenylcoumarin; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen responsive element; ERR, estrogen-related receptor; HTS, high-throughput screening; ICI, ICI 182, 780 or Faslodex; T, testosterone.

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ICI) was developed as a true antagonist, and showed no differential agonistic effects in different organs/tissues. ICI also has an ER-deregulatory property in that it binds to the ER, thereby causing it to degrade [6].

AIs inhibit the production of estrogen by suppressing aromatase activity. The FDA has approved three third-generation AIs for use as first-line agents against estrogen-dependent cancers [7]. The third-generation AIs include both non-steroidal (letrozole and anastrozole), and steroidal (exemestane) agents. They have recently been proven to be more effective than tamoxifen as a first-line therapy, effective for second-line therapy (e.g. against tamoxifen-resistant disease), and useful for extended adjuvant therapy after tamoxifen [8]. Among these three third-generation AIs, exemestane has been found to be a mechanism-based inhibitor and an aromatase destabilizer [9]. Although these antiestrogens and AIs are powerful drugs to treat hormone-dependent breast cancer, there is a need to develop additional antiestrogens and AIs for breast cancer patients who become resistant to the currently available antiestrogens/AIs.

High-throughput screening (HTS) assays have become one of the major approaches for drug discovery in the modern pharmaceutical industry. So far, the aromatase assay can be carried out in both non-cellular and cell-based formats [10,11]. In both types of assays, aromatase activity is determined by measuring the production of tritiated water from [1β-^{3}H] androstenedione following a 20-min or 1-h incubation,

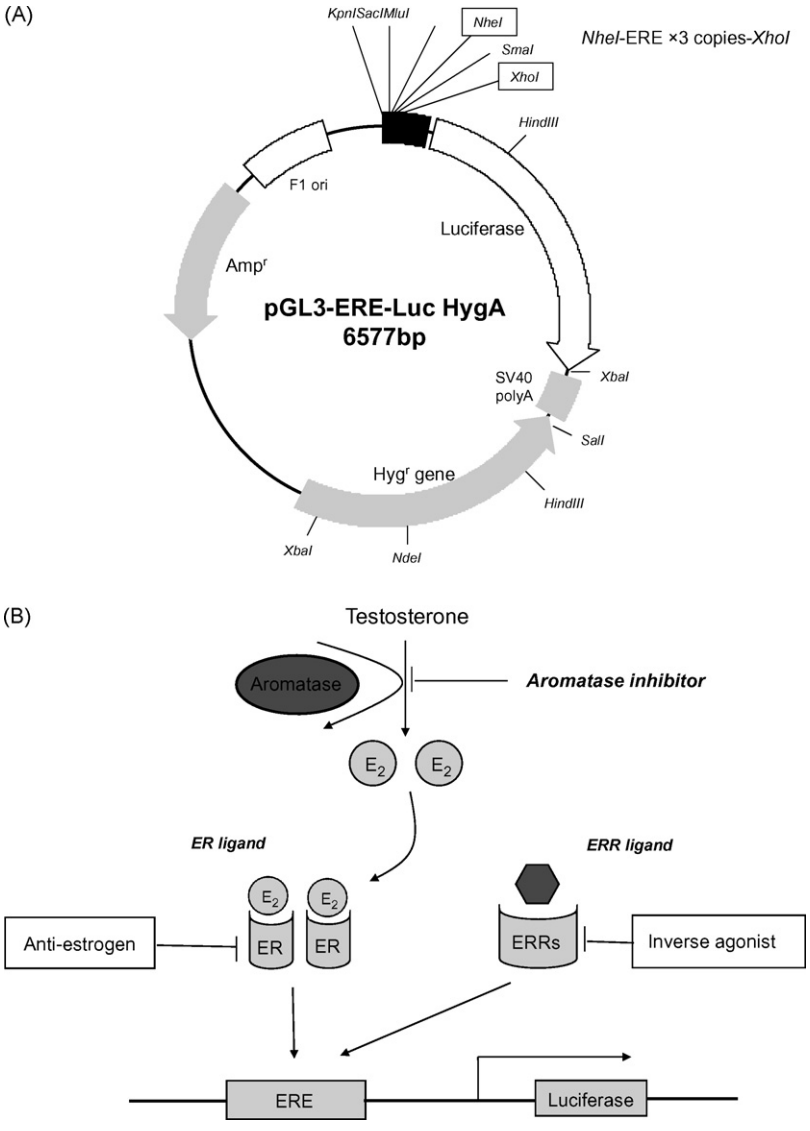

Fig. 1 – (A) Restriction map of pLG3-ERE-Luciferase hygromcyin plasmid. Three repeats of ERE were inserted into NheI and XhoI restriction sites while the hygromycin gene was inserted into XhoI site of the pGL3 promotor plasmid. (B) A schematic diagram to show the working principles of MCF7-aro/ERE cells. T is converted into E_2 by aromatase in MCF7-aro/ERE cells. Then E_2 activates ER. The E_2–ER complex binds to the ERE and induces luciferase expression. Aromatase inhibitor blocks the conversion of T to E_2. Without the formation of the E_2–ER complex, luciferase activity is eliminated. Anti-estrogen binds to ER to prevent ER from binding to E_2, therefore inhibiting luciferase activity. An inverse agonist of ERRs leads to deletion of its luciferase activity.

respectively. Typically, the *in vitro* assay is performed using human placental microsomes that contain a high level of aromatase, and the in-cell assay is performed using MCF-7aro cells or other aromatase-positive cells. These assays require the use of radioactive substrate and are not high throughput methods. Also, human placental microsomes, which are required for this method, may be limited in availability. In consideration of these drawbacks, we have developed a new and reliable in-cell system for aromatase inhibitor screening, which is a non-radioactive, 96-well high-throughput format. In addition, this MCF-7aro/ERE cell line can also be used to screen ligands of ERα and ERRα. Therefore, this is a triple screening system.

This paper describes the construction of pGL3-$(ERE)_3$-Luc expression plasmid which consists of a hygromycin resistant gene, as well as the generation of this MCF-7aro/ERE cell line by transfection. Experiments have been performed to demonstrate that MCF-7aro/ERE is a novel HTS system to screen AI, ER and ERR ligands.

2. Materials and methods

2.1. Chemicals

Testosterone (T), 17β-estradiol (E_2), tamoxifen, and XCT790 were purchased from Sigma (Sigma chemical, St. Louis, MO), while ICI was purchased from Tocris (Ellisville, MO). Letrozole was provided by Novartis (Basel, Switzerland) and anastrozole was provided by AstraZeneca Pharmaceuticals (Macclesfield, UK). [2,4,6,7-^{3}H] Estradiol (Specific Activity, 95 Ci/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). Genistein and 3(2′-chlorophenyl)-7-methoxy-4-phenylcoumarin (Cou) were purchased from Indofine (Somerville, NJ).

2.2. Construction of plasmids

The luciferase reporter plasmid pGL3-$(ERE)_3$-luciferase, was constructed by inserting a DNA fragment which consisted of three repeats of estrogen responsive element (ERE), 5′-GGTCAGAGTGACC-3′, into a pGL3-promotor vector. This DNA fragment was restricted using NheI and XhoI, and was ligated to linearized pGL3 promotor vector with T4 DNA ligase. After that, the plasmid was cut by SalI and ligated with a hygromycin resistant gene which was generated from a pTRE2hyg vector (Clontech, Mountain View, US).

2.3. Cell culture

A stable aromatase-expressing, ER positive MCF-7 cell line, named MCF-7aro, was previously generated by aromatase cDNA transfection and G-418 selection in this laboratory [11,12]. The pGL3$(ERE)_3$-Luc plasmid, which contains a hygromycin resistant gene, was transfected into MCF-7aro cells using Lipofectin, following the manufacturer's protocol (Invitrogen, Carlsbad, US). The stably transfected cells, MCF-7aro/ERE, were grown in regular MEM medium containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and penicillin/streptomycin with hygromycin and G418 selection at 37 °C in humidified incubators at 5% CO_2.

2.4. Luciferase assay

The levels of ERE-mediated luciferase expression were determined through luciferase activity measurements. Approximately 1×10^4 MCF-7aro/ERE cells (per well) were transferred into 96-well plates containing 200 μl of phenol red-free MEM/well, supplemented with 10% charcoal–dextran-treated FBS. On the following day, the cells were washed once with PBS. Hormones, genistein, antiestrogens and/or AIs dissolved in DMSO were added to the culture medium, and the cells were further incubated for 24 h. Cells were then lysed using 100 μl of reporter lysis buffer (Promega, Madison, WI). Fifty microliters of luciferase substrate (Promega, Madison, WI) was mixed with 40 μl of the cell lysate by a robotic injector and the luciferase assay was carried out using the Victor^2 V plate reader (PerkinElmer, Waltham, MA). The luciferase activity was standardized based on the protein concentration of the cell lysates. The protein concentration was determined by using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA) in the Bradford method.

The degree of inhibition was measured as a percentage of maximum activity induced by T or E_2 for each concentration of inhibitor and was calculated as {[luciferase activity of inhibitor] − background}/{[luciferase activity of T or E_2] − background} × 100%.

2.5. Whole-cell competitive ER binding assay

The procedure was similar to that previously reported by Stoessel and Leclercq [13], with minor modification. Briefly, ER positive MCF-7 cells were suspended in Dulbecco's modified Eagle's medium (phenol red-free) supplemented with 10% charcoal-treated FBS, non-essential amino acids, penicillin (100 U/ml), and streptomycin (100 μg/ml), and were seeded into 24-well plates at a density of 8×10^4 cells/ml per well. Twenty-four hours later, the seeding medium was changed to the same culture medium containing 1 nM of [2,4,6,7-^{3}H] estradiol (E_2) and varying concentrations of either E_2 or the test compounds (1 nM to 1 μM) and incubated at 37 °C for 1 h. The [^{3}H] E_2 containing medium was removed and the cells were

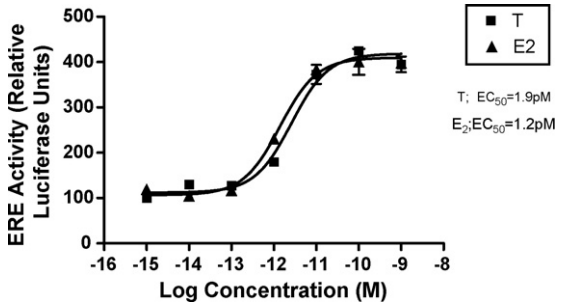

Fig. 2 – Dose–response curves of T or E_2 driven ERE-mediated luciferase activities in MCF-7aro/ERE cells. The T or E_2 stock solutions were added at a 1:1000 dilution (v/v) to MEM medium which was supplemented with 10% of charcoal–dextran-treated FBS. The cells were cultured in this medium for 24 h and the luciferase activity was normalized using protein concentration. The assays were performed in triplicate.

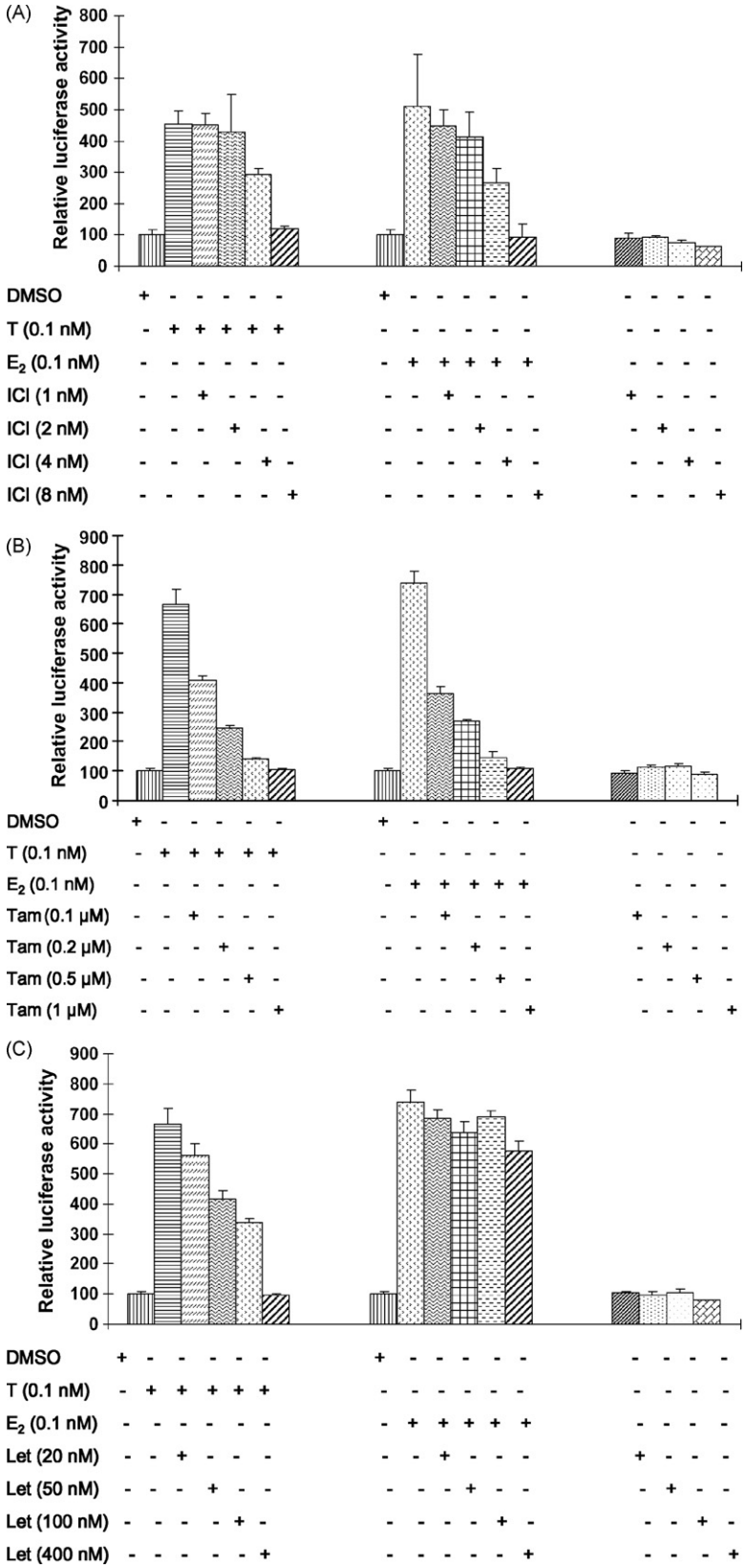

Fig. 3 – The ERE-mediated luciferase activity of MCF-7aro/ERE cells in response to ICI 182, 870 (ICI) (A), tamoxifen (Tam) (B), letrozole (Let) (C) and anastrozole (Ana) (D). The DMSO-treated cells served as a negative control, and the cells cultured with 0.1 nM T or 0.1 nM E_2 which showed maximum ER-triggered transactivation served as the positive control. Inhibition of ERE-mediated luciferase activity was carried out by introducing different concentrations of ICI (A), Tam (B), Let (C) or Ana (D), in the presence of 0.1 nM T or 0.1 nM E_2. In addition, effects of ICI only (A), Tam only (B), Let only (C) or Ana only (D) in the absence of T or E_2, were also examined.

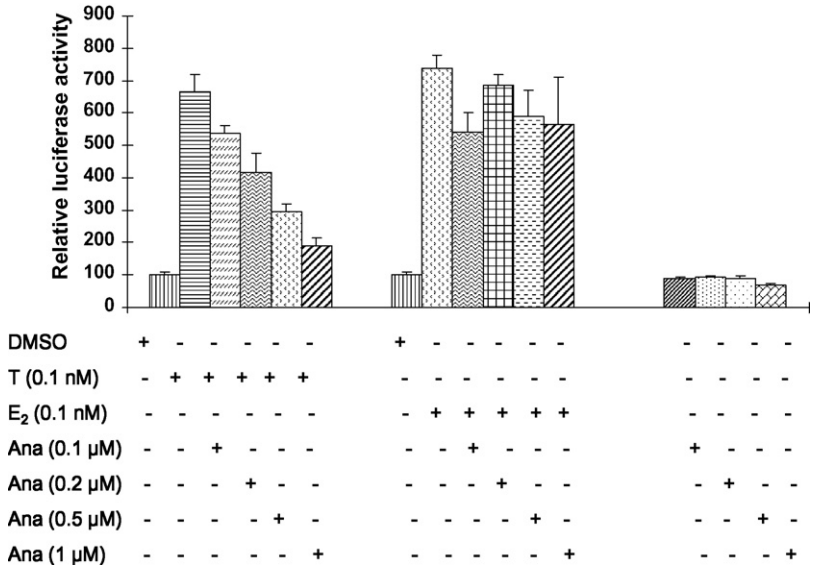

Fig. 3. (Continued).

washed three times with PBS, and 250 μl of 100% ethanol was added to each well for 20 min to solubilize the bound [^{3}H] E_2. The radioactivity in 200 μl of the ethanol extract was determined. The amount of bound [^{3}H] E_2 in the presence or absence of the test compounds was calculated after correcting for non-specific binding as measured by the amount of bound [^{3}H] E_2 in the presence of 1000-fold (1.0 μM) excess of E_2. All compounds were tested with triplicates at each concentration in at least three separate experiments. Binding affinity of Cou is approximately 1/40th of the E_2 binding affinity. Using this method, the EC_{50} values for ICI and tamoxifen are 15 nM and 630 nM, respectively. As a negative control, we did not detect significant amount of [^{3}H] E_2 bound to cells when ER-negative HeLa cells were used.

3. Results

3.1. *Construction of a stable MCF-7aro/ERE cell line*

The MCF-7aro/ERE cell line was generated by transfecting pGL3-$(ERE)_3$-promoter luciferase reporter plasmid into MCF-7aro cells (Fig. 1A). Since MCF-7aro cells were already resistant to G418, MCF-7aro/ERE cells were selected using hygromycin. Several clones were picked, and the clone which could be induced by T or E_2 at maximal luciferase activity was chosen to perform the antiestrogen and AI assays.

3.2. *Inductive response of the ERE-mediated luciferase activity in MCF-7aro/ERE by T or E_2*

To evaluate the utility of MCF-7aro/ERE, we began by assessing the ability of these cells to respond to different concentrations of T and E_2. In these cells, aromatase converts T to E_2, after which E_2 activates ER, triggering luciferase expression through the ERE interaction, as shown in Fig. 1B. The dose dependency of luciferase activity (as an indication of the ERE-mediated luciferase expression) to T or E_2 was assessed. The EC_{50} values for T and E_2 were estimated to be 1.9 pM and 1.2 pM, respectively (Fig. 2). The activity levels of luciferase induced by T were the same as E_2, and they displayed typical sigmoidal dose–response curves. The luciferase activity remained at the basal level when treated at concentrations below 0.1 pM T or E_2, started to increase at 1 pM, and plateaued at 0.1 nM with no further increase of luciferase activity beyond the concentration of 0.1 nM. The ratio of maximal to basal luciferase expression was about 6.

3.3. *Effect of antiestrogens (ICI and tamoxifen) on the ERE-mediated luciferase activity in MCF-7aro/ERE*

Fig. 3A and B shows the inhibitory effects of ICI and tamoxifen on the luciferase activity in the presence of T or E_2. The luciferase activity was at the basal level when MCF7-aro/ERE cells were treated with DMSO, while the luciferase activity reached the maximum at 0.1 nM T or 0.1 nM E_2. The luciferase activity decreased with an increased concentration of antiestrogens (ICI or tamoxifen), both in the presence of 0.1 nM T or 0.1 nM E_2. There was no significant difference between treatment of T + ICI and E_2 + ICI, or T + tamoxifen and E_2 + tamoxifen. At the concentration of 8 nM ICI or 1 μM tamoxifen, the luciferase activity was almost completely inhibited, both in the presence of T or E_2. The calculated EC_{50} of ICI and tamoxifen in the presence of T were 5 nM and 100 nM, respectively, while the EC_{50} of ICI and tamoxifen in the presence of E_2 were 4 nM and 80 nM, respectively (Table 1). As expected, ICI was more effective than tamoxifen in the inhibition of the ERE-mediated luciferase expression. We did not detect any intrinsic ER agonistic activity of ICI or tamoxifen using this assay system.

3.4. *Effect of AIs (letrozole and anastrozole) on the ERE-mediated luciferase activity in MCF-7aro/ERE*

Fig. 3C and D shows the inhibitory effects of letrozole and anastrozole on the luciferase activity in MCF-7aro/ERE cells.

Table 1 – Calculated IC_{50} values of ICI 182, 870, tamoxifen, letrozole and anastrozole in the presence of either 0.1 nM T or 0.1 nM E_2

Inhibition of ERE transcriptional activity	Potency EC_{50}(nM)	
	With T	With E_2
ICI 182, 780 (ICI)	5	4
Tamoxifen (Tam)	100	80
Letrozole (Let)	75	N.A.
Anastrozole (Ana)	290	N.A.

N.A., not applicable.

Again, the luciferase activity reached the maximum level when induced by 0.1 nM T or 0.1 nM E_2. The luciferase activity decreased at increased concentrations of letrozole and anastrozole, and in the presence of 0.1 nM T, but there was no reduction of luciferase activity when treated with E_2 + letrozole or E_2 + anastrozole. At the concentration of 400 nM letrozole or 1 μM anastrozole, luciferase activity was completely inhibited in the presence of T, but not in the presence of E_2. The EC_{50} of letrozole and anastrozole in the presence of T were 75 nM and 290 nM, respectively (Table 1), indicating that letrozole was more effective than anastrozole in the inhibition of the ERE-mediated luciferase expression. These results agreed with previously published data [14]. No ER agonistic activity of letrozole or anastrozole was detected using this assay system.

3.5. *Effect of genistein on the ERE-mediated luciferase activity in MCF-7aro/ERE*

Fig. 4 shows that genistein could induce luciferase activity significantly at concentrations of 1 μM and 10 μM. While genistein has been suggested to be an agonist of ER, the luciferase activity could only be slightly suppressed by ICI, but effectively by an ERRα inverse agonist, XCT790 [15,16]. These results confirm our previous findings that genistein is an effective ERRα ligand [17] and demonstrate that MCF-7aro/ERE is also useful for the screening of ERRα ligands.

3.6. *Identification of 3(2′-chlorphenyl)-7-methoxy-4-phenylcoumarin (Cou) as an agonist of ER*

Using a whole-cell competitive ER binding assay, we have found that this coumarin derivative is a ligand of ER (Fig. 5). The binding affinity of this compound is approximately one fortieth that of E_2. As a proof-of-principle experiment, we evaluated the effect of Cou on the ERE-mediated luciferase activity in MCF-7aro/ERE. This compound could induce luciferase activity significantly with an EC_{50} value of 0.24 nM (Fig. 6). The induced luciferase activity could be suppressed by ICI, but not by XCT790 (Fig. 7). These results confirm that Cou is a ligand and an agonist of ER. This compound cannot suppress T-induced luciferase (results not shown), indicating that this compound is not an inhibitor of aromatase.

4. Discussion

The utility of MCF-7aro/ERE as a tool for screening AIs and ERα ligands was carefully evaluated. The dose–response curves induced by T and E_2 overlap (Fig. 2), suggesting that aromatase converts T to E_2 efficiently inside MCF-7aro/ERE cells. The pGL3-$(ERE)_3$-Luc activity was increased more than sixfold with 0.1 nM of T and E_2, compared to the DMSO control. This fold difference was significant enough to allow us to evaluate the potencies of different chemicals. The maximum concentrations of T and E_2 (i.e., 0.1 nM) used in this assay system were 10 times less than that of the traditional protocol [14]. The MCF-7aro/ERE screening system has the advantage over ligand binding assays in that this is a functional assay, i.e., the assay

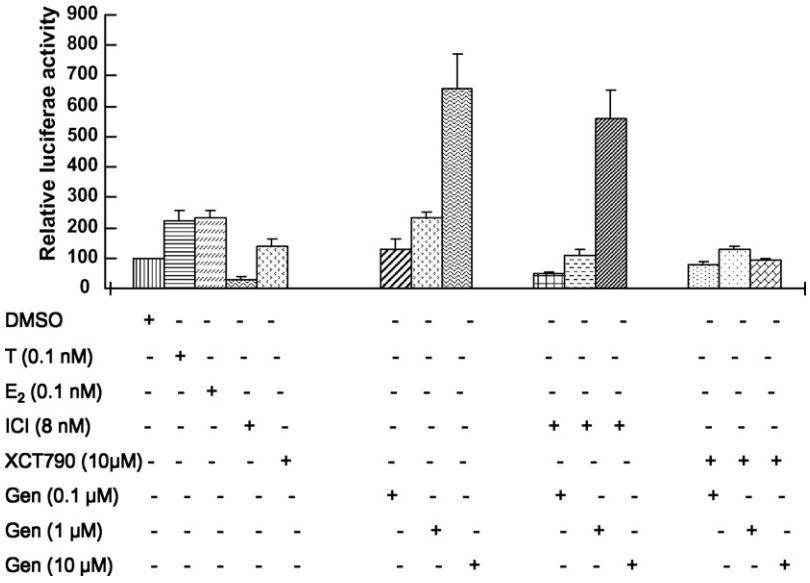

Fig. 4 – The ERE-mediated luciferase activity of MCF-7aro/ERE cells in response to genistein (Gen). The DMSO-treated MCF-7aro/ERE cells served as a negative control, and cells cultured with T or E_2 as the positive control. The luciferase activities induced by Gen, combination of Gen and ICI, or combination of Gen and XCT790 were examined.

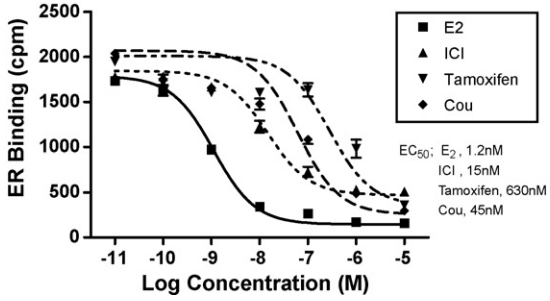

Fig. 5 – Demonstration of 3(2′-chlorphenyl)-7-methoxy-4-phenylcoumarin as a ligand of ER by whole-cell competitive ER binding assay. The experimental conditions are provided in Section 2. The binding of 1 nM [2,4, 6, 7-^{3}H] estradiol was competed with varying concentrations of either E_2 or the test compounds (1 nM to 1 μM). The assays were performed in triplicate.

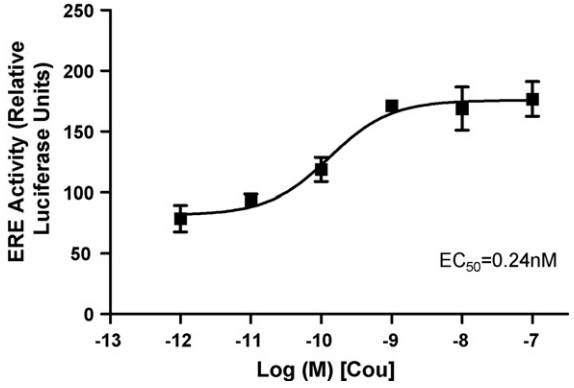

Fig. 6 – Dose–response curves of the ERE-mediated luciferase activity of MCF-7aro/ERE cells driven by 3(2′-chlorphenyl)-7-methoxy-4-phenylcoumarin (Cou). The coumarin stock solutions were added at a 1:1000 dilution (v/v) to MEM medium which was supplemented with 10% of charcoal–dextran-treated FBS. The cells were cultured in this medium for 24 h and the luciferase activity was normalized using protein concentration. The assays were performed in triplicate.

allows us to determine whether the detected chemicals are inhibitors of aromatase, agonists or antagonists of ERα and ERRα. Since it is not a ligand binding assay, K_i or K_d values of the ligands cannot be determined. The screen system is a very sensitive one, as indicated by very low EC_{50} values for T and E_2 (Fig. 2). Due to the fact that we could perform analysis using 0.1 nM T or E_2, the EC_{50} values of tested ligands were typically significantly lower than those determined by ligand binding assays. For example, EC_{50} of Cou determined by the MCF-7aro/ERE screening system (0.24 nM) was significantly lower than that determined by whole-cell competitive ER binding assay (i.e., 45 nM). This feature makes the MCF-7aro/ERE screening system a very sensitive method to screen ligands for ERα and ERRα. However, the high sensitivity of ERα to E_2 requires a total suppression of aromatase activity for aromatase inhibitor screening. Therefore, EC_{50} values of aromatase inhibitors determined by the MCF-7aro/ERE screening system could be slightly higher than those determined by direct enzyme assay.

It was predicted that inhibiting the conversion of T to E_2 by AIs as well as reducing ERα availability with antiestrogens

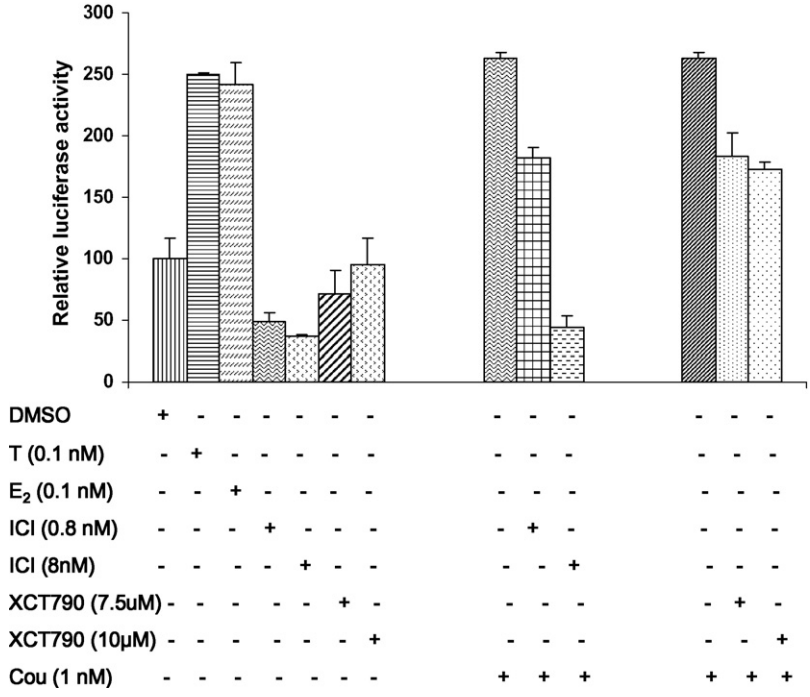

Fig. 7 – The ERE-mediated luciferase activity of MCF-7aro/ERE cells in response to 3(2′-chlorphenyl)-7-methoxy-4-phenylcoumarin (Cou). The DMSO-treated MCF-7aro/ERE cells served as a negative control, and cells cultured with T or E_2 as the positive control. The luciferase activities induced by Cou, combination of Cou and ICI, or combination of Cou and XCT790 were examined.

would result in the suppression of ERE-mediated luciferase activity (Fig. 1B). Through this assay system, as a proof-of-principle study, we confirmed tamoxifen and ICI to be antiestrogens and letrozole and anastrozole to be AIs. Both T- and E_2-induced luciferase activities could be blocked by antiestrogens at a concentration of 8 nM of ICI or 1 μM tamoxifen (Fig. 3A and B). As expected, only T-induced luciferase activity could be blocked by letrozole or anastrozole.

There was an induction of luciferase activity when the MCF-7aro/ERE cells were treated with genistein in the absence of T or E_2. The higher the concentration of genistein, the higher the activity of luciferase could be induced. However, with the combination of genistein and ICI, the luciferase activity was only slightly suppressed. These results suggest that ER is not the major receptor that genistein binds to. Genistein is an isoflavone phytoestrogen which has shown agonistic activity to ER and ERRs [17]. ERRs are able to bind to the ERE. To confirm our previous findings, the genistein-induced luciferase expression was effectively suppressed by XCT790, an inverse agonist of ERRα. Our microarray analysis revealed that there is minimal expression of ERβ in our MCF-7aro cells [18]. This is confirmed by our results indicating that the genistein-induced activity is completely suppressed by XCT790, while genistein has been reported to be a better ligand of ERβ than ERα [19].

To demonstrate the utility of this screening system, an ER ligand (identified by competitive ER binding assay), Cou, was found to be an agonist of ER, probably ERα because this cell line has a minimal expression of ERβ. Using the same system, this compound was found not to be a ligand of ERRα nor an inhibitor of aromatase.

In summary, we have developed a new assay system which is able to screen for ERα ligands and AIs at the same time. The assay is carried out in a 96-well plate, and it facilitates a high-throughput rapid screening. It is sensitive, easy to handle, and reproducible at low cost. Also, the MCF-7aro/EREs can possibly be used to screen ERR agonists and antagonists. This triple screening system will be valuable in searching for chemicals, such as environmental chemicals, that interact with ERα, ERRα and/or aromatase at the same time.

Acknowledgments

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