Journal of Steroid Biochemistry & Molecular Biology 122 (2010) 204–211

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Superinduction of estrogen receptor mediated gene expression in luciferase based reporter gene assays is mediated by a post-transcriptional mechanism

A.M. Sotoca[a,*], T.F.H. Bovee[b], W. Brand[a], N. Velikova[b], S. Boeren[c], A.J. Murk[a], J. Vervoort[c], I.M.C.M. Rietjens[a]

[a] Division of Toxicology, Wageningen University, Tuinlaan 5, 6703 HE Wageningen, Gelderland, The Netherlands
[b] RIKILT, Institute of Food Safety, Wageningen University, 6708 WB Wageningen, The Netherlands
[c] Laboratory of Biochemistry, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

ARTICLE INFO

Article history:
Received 3 March 2010
Received in revised form 21 May 2010
Accepted 18 June 2010

Keywords:
Estrogen reporter gene assay
Genistein
Superinduction
Mechanism of action
Metabolism
Proliferation
Artefact

ABSTRACT

Several estrogenic compounds including the isoflavonoid genistein have been reported to induce a higher maximal response than the natural estrogen 17β-estradiol in *in vitro* luciferase based reporter gene bioassays for testing estrogenicity. The phenomenon has been referred to as superinduction. The mechanism underlying this effect and thus also its biological relevance remain to be elucidated. In the present study several hypotheses for the possible mechanisms underlying this superinduction were investigated using genistein as the model compound. These hypotheses included (i) a non-estrogen receptor (ER)-mediated mechanism, (ii) a role for an ER activating genistein metabolite with higher ER inducing activity than genistein itself, and (iii) a post-transcriptional mechanism that is not biologically relevant but specific for the luciferase based reporter gene assays. The data presented in this study indicate that induction and also superinduction of the reporter gene is ER-mediated, and that superinduction by genistein could be ascribed to stabilization of the firefly luciferase reporter enzyme increasing the bioluminescent signal during the cell-based assay. This indicates that the phenomenon of superinduction may not be biologically relevant but may rather represent a post-transcriptional effect on enzyme stability.

1. Introduction

Isoflavones represent the most important group of phytoestrogens and have structural similarities to endogenous estrogens, suggesting that these compounds might exert their estrogenic effects via the estrogen receptor (ER) [1]. Several *in vitro* assays have been used over the past years to investigate and define the hormonal, including also estrogenic, activity of isoflavones. Indeed, many of these studies have shown that isoflavones exert estrogenic and/or anti-estrogenic activities [2]. An important model isoflavonoid tested in these studies is genistein, which is the main isoflavone present in legumes, particularly soybeans. Diverse biological activities have been associated with genistein including its estrogenicity and chemopreventive and/or antioxidant potential [3,4]. *In vitro*, genistein showed a wide range of cellular activities including inhibition of tyrosine kinase, inhibition of topoisomerase, inhibition of autophosphorylation of the epidermal growth factor receptor, mutagenesis but also antimutagenesis, induction of DNA damaging oxidation but also its prevention, and the promotion of cell death by apoptosis [5]. Some of the estrogenic characteristics of genistein may contribute to protective roles in osteoporosis and menopause symptoms [6], but also to have positive effects against heart diseases, diabetes or even cancer [7]. These proposed beneficial health effects of phytoestrogens in general, and of genistein in particular, have led to a wide range of isoflavonoid based food supplements.

Numerous *in vitro* ER assays have been developed to test the agonism or antagonism of a given substance. Reporter gene assays have become the most popular technique for measuring short-term screening of estrogenic activity. However, a response in an *in vitro* ER agonist or antagonist assay is not sufficient to predict biological or *in vivo* effects.

Genistein and other isoflavones have been shown to be weakly estrogenic and to have relatively low receptor affinity as compared to the natural estrogen receptor ligand 17β-estradiol (E2). However, in some mammalian cell-based assays, using human U2OS bone or Chinese hamster ovarian (CHO) cells, stably transfected to express estrogen receptor (ER) to make them responsive to estrogens, or using MCF7 breast cancer cells containing endogenous ER, the maximal induction of the luciferase reporter gene by genistein has been shown to be substantially higher than the maximal induction by estradiol (E2) [8–12]. In contrast, during cell-based estrogen stimulated proliferation assays, cell proliferation rates induced by genistein are similar to those induced by E2, although occurring at

* Corresponding author. Tel.: +31 317484357; fax: +31 317484931.
E-mail address: ana.sotoca@wur.nl (A.M. Sotoca).

0960-0760/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2010.06.010

higher concentrations [11,13,14]. No "superproliferation" effect by genistein or other isoflavones has been reported so far.

The mechanism underlying the so-called superinduction of estrogen receptor mediated gene expression in luciferase based reporter gene assays and also its biological relevance remain to be elucidated. The aim of the present study was to investigate possible mechanisms underlying this superinduction in reporter gene assays using genistein as the model compound known. Using the ER antagonists ICI 182,780 and RU58668 it was investigated whether the superinduction phenomenon was dependent on ER activation. In a T47D cell proliferation assay it was investigated whether superinduction was also reflected in superproliferation (and thus biologically relevant). Furthermore the possible role for a genistein metabolite with higher induction potency than E2 was investigated. And finally, using RT-PCR, it was quantified whether the increased luciferase activity was a result of increased gene expression and thus mRNA levels or rather a post-transcriptional effect on enzyme stability. Together these experiments provided insight in the mechanism underlying the observed superinduction, pointing at a post-transcriptional effect on enzyme stability and thus an assay artefact rather than at a biologically relevant effect.

2. Materials and methods

2.1. Materials

17β-Estradiol (E2; purity > 98%) and genistein (purity > 98%), acetonitrile and trifluoroacetic acid were purchased from Sigma (Zwijndrecht, The Netherlands). RU58668 was a gift from N.V. Organon (Oss, The Netherlands). Orobol was purchased from APIN Chemicals LTD (Oxon, UK). ICI 182,780 was provided by Zeneca Pharmaceuticals (Cheshire, UK). Dimethyl sulfoxide (DMSO, purity > 99%) was purchased from Acros Organics (Pittsburgh, PA, USA). Fetal calf serum (FCS, Australian origin, 10099), geneticin, G418 and Trizol Reagent were provided by Gibco Invitrogen Life Technologies (Paisley, UK). Phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}), Hank's balanced salt solution (HBSS), nonessential amino acids (100×, 11140-035), growth medium 1:1 mixture of Ham's nutrient mixture F12 and DMEM (31331-028), Alpha-Modified Eagle's Medium (22561-021), and exposure medium phenol-free (21041-025) were supplied by Gibco Invitrogen (Paisley, UK). Dextran-charcoal-treated FCS (DCC-FCS) was heat inactivated (30 min at 56 °C) followed by two 45 min DCC-treatments at 45 °C [15]. Trypsin was obtained from Difco (Detroit, USA; 0.25 g/100 ml in phosphate-buffered saline, PBS). Sodium bicarbonate ($NaHCO_3$ > 99.5%), sodium hydroxide (NaOH) ethylenedinitrotetraacetic acid ($EDTA{\cdot}2H_2O$; Titriplex), magnesium sulfate ($MgSO_4{\cdot}7H_2O$), and 1,4-dithiothreitol (DTT) were obtained from Merck (Darmstadt, Germany). Magnesium carbonate ($(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$) was obtained from Aldrich (St. Louis, MO, USA). *trans*-1,2-Diaminocyclohexane-N,N,N′,N′-tetraacetic acid monohydrate (CDTA) was obtained from Fluka (Buchs, Switzerland). Hygromycin and Diferin were obtained from Duchefa (Haarlem, The Netherlands). ATP was provided by Roche Diagnostics (Mannheim, Germany) and the BCA Protein Assay Kit by Pierce (Bonn, Germany). RNeasy mini kit and SYBR green were provided by Quiagen (Hilden, Germany).

2.2. Cell lines

The stably transfected human osteosarcoma (U2OS) cell line (ERα-U2OS-Luc) expressing the human ERα in addition to 3xERE(GAGCTTAGGTCACTGTGACCT)-tata-luciferase reporter construct was used as described before [16]. T47D human breast cancer wild type cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The T47D-Luc cell line stably transfected with an estrogen receptor mediated luciferase reporter gene construct was described before [17].

2.3. Cell culture conditions

ERα-U2OS-Luc cells were cultured in a 1:1 mixture of DMEM and F12 (31331-028) buffered with 1260 mg/L $NaHCO_3$, supplemented with 7.5% fetal calf serum (FCS) and 0.5% nonessential amino acids. ERα-U2OS-Luc growth medium was supplemented with geneticin (200 μg/ml) and hygromycin (50 μg/ml) as selection markers. The cells were incubated at 37 °C and 7.5% CO_2 in a humidified atmosphere.

The T47D wild type and T47D-Luc cell lines were cultured in a 1:1 mixture of Ham's nutrient mixture F12 and DMEM (31331-038) supplemented with 5% FCS. The cells were incubated at 37 °C and 5% CO_2 in a humidified atmosphere.

2.4. ERα-U2OS-Luc, T47D-Luc assay

hERα-specific U2OS and T47D luciferase reporter gene assays expressing endogenous ERα were carried out as described previously [11].

2.5. REA bioassay

The yeast estrogen bioassay is based on a yeast cell stably expressing hERα and stably transfected with a yeast enhanced green fluorescent protein (yEGFP) as a reporter gene in response to estrogens. Estrogenic responses using the REA assay were measured as described before [18].

2.6. YES assay

The yeast estrogen screen (YES) was created by expressing human estrogen receptor (hER) and two estrogen response elements (ERE) linked to the β-galactosidase (lacZ) reporter gene. The assay was performed according to methods described before [19].

2.7. Cell proliferation measurements

After 48 h of exposure, proliferation in the ERα-U2OS-Luc and T47D cells was determined by measuring incorporation of 5-bromo-2′-deoxy-uridine (BrdU) into DNA following BrdU Roche's colorimetric protocol. Measurements were performed in a spectrophotometer at 370 nm excitation and 492 nm emission wavelengths.

2.8. HPLC-DAD analysis

Samples from medium in which T47D and ERα-U2OS-Luc cells were exposed for 24 h under the same conditions described above to different concentrations of genistein, however without DCC treated serum in the medium, were collected for HPLC-DAD analysis. The HPLC-DAD system consisted of a Waters (Milford, MA, USA) Alliance 2695 separation module connected to a Waters 2996 photodiode array detector (DAD), equipped with an Alltech (Breda, The Netherlands) Alltima C18 5-μm 150 mm × 4.6 mm reverse phase column with a 7.5 mm × 4.6 mm guard column. Before injection, samples were centrifuged at 16,000 × *g* for 4 min, and 50 μl was injected and eluted at a flow rate of 1 ml/min starting at 0% acetonitrile in nanopure water containing 0.1% trifluoroacetic acid (TFA), increasing to 10% acetonitrile in 5 min, to 15% in the following 16 min, and to 50% in the next 16 min all in nanopure with 0.1% TFA. Thereafter, the percentage acetonitrile was increased to 80% in 1 min. This condition was kept for 1 min as a cleaning step, followed

by a decrease to 0% acetonitrile in 1 min, keeping this condition for 10 min, allowing the column to re-equilibrate at the initial conditions (total run time, 50 min). DAD spectra were detected between 200 and 420 nm, and HPLC chromatograms acquired at 280 nm were used for quantification and presentation.

2.9. LC–MS/MS analysis

Medium of ERα-U2OS-Luc cells exposed to 5 μM genistein under the same conditions as described above but without DCC treated serum in the medium was collected after 24 h of exposure and analyzed by LC–MS/MS. The medium samples were analyzed by injecting 18 μl sample over a 32 mm × 0.10 mm Prontosil 300-3-C18H pre-concentration column (Bischoff, Germany) at a flow of 6 μl/min for 10 min. Compounds were eluted from the pre-concentration column onto a 200 mm × 0.10 mm Prontosil 300-3-C18H analytical column with an acetonitrile gradient at a flow of 0.5 μl/min. The gradient consisted of an increase from 15 to 50% acetonitrile in water containing 1 ml/L formic acid in 16 min. Thereafter the percentage acetonitrile was increased to 80% in 3 min as a column cleaning step. Downstream of the column, an electrospray potential of 1.8 kV was applied directly to the eluent via a solid 0.5 mm platina electrode fitted into a P875 Upchurch microT. Full scan positive mode FTMS spectra were measured between an m/z of 200 and 600 at a resolution of 60,000 on a Thermo electron LTQ-Orbitrap (San Jose, CA, USA). MS/MS scans of the four most abundant singly, doubly or triply charged peaks in the FTMS scan were recorded in data dependent mode in the linear trap (MS/MS threshold = 10,000).

2.10. RT-PCR

RNA was extracted after 6 and 24 h exposure using the TRIzol precipitation method and purified using an RNeasy mini kit protocol for second RNA clean up according to the manufacturer's protocol. Integrity and quantity of the extracted RNA was then assessed by using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., DE, USA). Approximately 1–2 μg of total RNA was collected per sample. Samples were stored at −80 °C until reverse transcription (RT-)PCR using 1 μg of total RNA. First-strand cDNA synthesis was carried out with an oligo(dT)15 primer and Moloney murine leukemia virus reverse transcriptase; during synthesis the recombinant ribonuclease inhibitor RNaseOUT™ was present. The amplification reaction was carried out on a LightCycler (Roche Diagnostics) with gene-specific primers and used the SYBR Green 1 protocol. The following LightCycler protocol was used: 15 min heat start at 95 °C; 45 cycles of denaturation at 95 °C for 30 s, annealing at the optimal annealing temperature for the primer set for 30 s, and extension at 72 °C for 45 s; and a terminal extension at 72 °C for 5 min. Fluorescence detection was carried out at 72 °C. *Luc* mRNA levels were expressed as the *Luc:β-actin* ratio. The oligonucleotide sequences for firefly luciferase were reverse primer: 5′-GCCTCACCTACCTCCTTGCT-3′ and forward primer 5′-CTTCGTGACTTCCCATTTGC-3′; for β-actin the reverse primer was 5′-CCAGAGGCGTACAGGGATAG-3′ and the forward primer was 5′-CACCCCGTGCTGCTGAC-3′.

2.11. Calculations and statistics

Luciferase induction by genistein in the ERα-U2OS-Luc, T47D-Luc cells, and yEGFP and β-galactosidase induction in yeast cells were compared with the induction of the respective activities by the natural ligand E2. The background induction by the solvent control was set at 0% induction. The maximum induction of luciferase obtained at 12 pM E2 for ERα-U2OS-Luc cells, at 20 pM E2 for the T47D-Luc cells and at 3 nM and 150 pM E2 for the yEGFP and β-galactosidase induction in yeast cells were set as 100%. The data obtained for proliferation as quantified by BrdU incorporation were plotted as induction of proliferation compared to the solvent control (DMSO).

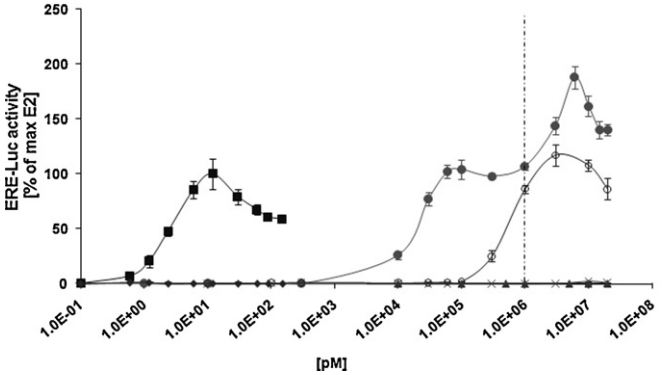

Fig. 1. Induction of ERα-mediated luciferase activity in the ERα-U2OS-Luc cells upon exposure to various (anti)estrogens. (Anti)estrogens tested include E2 (■), E2 + 5 nM ICI 182,780 (♦), genistein (●), genistein + 5 nM ICI 182,780 (○), genistein + 100 nM ICI 182,780 (×), genistein + 180 nM RU58668 (▲). Induction is expressed relative to maximal E2 response set at 100%. Data points represent the mean of triplicate exposure ± standard deviation. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

3. Results

3.1. Activation of ERE-mediated gene expression in the ERα-U2OS-Luc reporter gene assay

Treatment of the human ERα-U2OS-Luc cells with E2 and genistein resulted in dose-dependent expression of luciferase (Fig. 1). Induction of ERα-mediated luciferase expression by E2 occurs at concentrations between 1 and 20 pM. Induction by genistein appears to be biphasic. Exposure to genistein shows a first phase of dose-related luciferase induction at concentrations from 10 to 200 nM and a second phase with a further dose-related increased of luciferase induction at concentrations from 1 to 5 μM. The maximum level of luciferase induction in the first phase of the genistein induction curve is similar to the maximum level of induction by 12 pM E2 and amounts to 104% of the maximum E2 induction at 100 nM genistein. In the second phase, genistein exposure of the cells resulted in a so-called superinduction of luciferase activity amounting to 188% of the maximum level of induction by E2 at 5 μM genistein. When co-exposing cells to E2 with 5 nM of the anti-estrogen ICI 182,780, the luciferase induction by E2 is completely inhibited (Fig. 1). When genistein is co-exposed with 5 nM of the anti-estrogen ICI 182,780, luciferase induction in the high affinity first phase is inhibited and only the low affinity luciferase induction in the second phase remains, as the shape of the curve suggests that only the first part of the genistein dose–response curve was inhibited by ICI 182,780 (Fig. 1). However, the luciferase induced by genistein was totally inhibited by co-exposure with 100 nM ICI 182,780 and 180 nM of another pure ER antagonist, RU58668.

3.2. Activation of ERE-mediated gene expression in the T47D-Luc reporter gene assay

Similar experiments were performed with the human breast cancer cell line T47D-Luc reporter gene assay in order to investigate whether the above described effects of genistein were cell-type specific. The breast cancer derived T47D-Luc cells use the same reporter gene construct that is used in the human ERα-U2OS-Luc cell line. The only difference is that the T47D cells make use of the endogenous ERα, while the ERα-U2OS-Luc cells contain

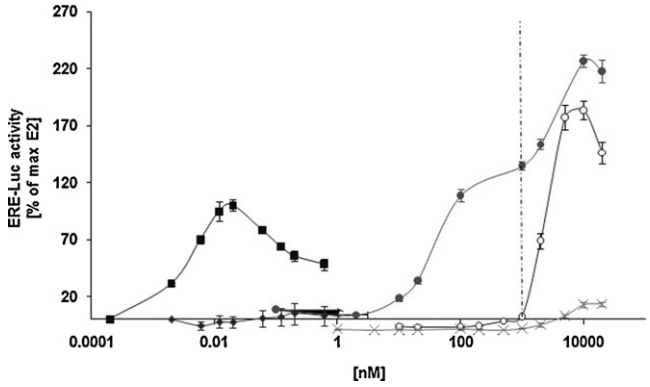

Fig. 2. Induction of ERα-mediated luciferase activity in the ERα-T47D-Luc cells upon exposure to various (anti)estrogens. (Anti)estrogens tested include E2 (■), E2+ 18 nM RU58668 (♦), genistein (●), genistein + 18 nM RU58668 (○) and genistein + 180 nM RU58668 (×). Induction is expressed relative to maximal E2 response set at 100%. Data points represent the mean of triplicate exposure ± standard deviation. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

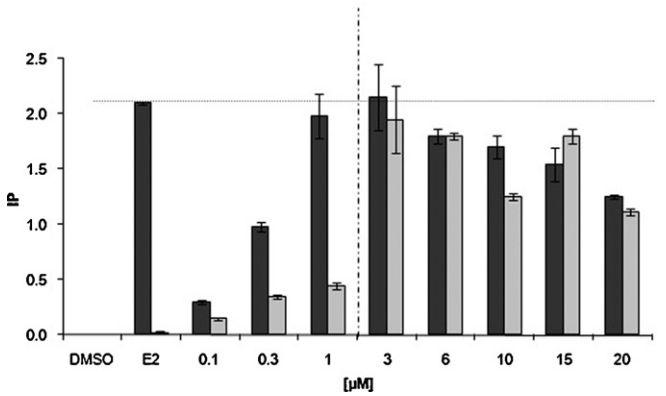

Fig. 3. T47D cell proliferation. Effect of DMSO, estradiol and genistein (black) and DMSO, estradiol and genistein + 10 nM ICI 182,780 (grey) on induction of T47D cell proliferation (IP). The horizontal dotted line indicates the maximal proliferation by E2 at 1 nM. Proliferation was quantified measuring BrdU incorporation after 48 h exposure. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

an extra construct to make them express the hERα. The results shown in Fig. 2 reveal that the responses of E2 and genistein are in the same concentration range and similar to those obtained with the ERα-U2OS-Luc cells. Also the treatment of the T47D-Luc cells with genistein resulted in a biphasic concentration-dependent increase in luciferase activity. Genistein shows a first dose-related luciferase induction at concentrations from 10 to 200 nM (first curve) and a second dose-related increase in luciferase induction at concentrations from 1 to 10 μM (Fig. 2). The maximum level of luciferase activity reached in the first phase at 100 nM genistein amounts to 108% of the maximum induction level reached at 20 pM E2. Genistein-mediated superinduction of luciferase in the second phase amounts to 227% of the maximum level of induction by E2 at 10 μM genistein. When cells were co-exposed to E2 and 18 nM of the anti-estrogen RU58668, the E2 induced luciferase induction was completely inhibited. When cells were co-exposed to genistein and 18 nM of this anti-estrogen, the luciferase induction was only partly inhibited. Again, the shape of the curve shows that low concentrations of the anti-estrogen inhibit mainly the first part of the induction curve. Again, luciferase induction by genistein was completely inhibited by a high concentration of RU58668 (180 nM).

3.3. T47D cell proliferation

In a next series of experiments, it was investigated whether the observed genistein-mediated superinduction of luciferase activity in reporter gene assays would also be reflected in genistein induced ERα-mediated superinduction of cell proliferation, which is a biologically relevant endpoint of estrogenic activity. Since the T47D cell line is known to express endogenous ERα, and to show estrogen induced ERα mediated cell proliferation, cells from the wild type T47D cell line were selected as the model system to investigate the possible occurrence of genistein induced ERα-mediated superinduction of cell proliferation.

As shown in Fig. 3, treatment of T47D wild type cells for 48 h with genistein resulted in a dose-dependent increase in cell proliferation. Proliferation data have been normalized to the proliferation levels obtained at 1 nM E2, the maximum of E2 induced T47D cell proliferation. Fig. 3 shows that the maximal proliferative effect elicited by genistein is similar to that of 1 nM E2 and is reached at a concentration of 1 μM genistein, which is also the genistein concentration inducing the maximum response in the first phase of the luciferase induction in the two reporter cell assays. At concentrations of 3–20 μM genistein, the range where in both reporter gene assays genistein-mediated superinduction was observed, no genistein induced "superproliferation" of the T47D cells was observed.

When cells were co-exposed with the antagonist ICI 182,780 (10 nM), the E2 induced proliferation was completely inhibited, and this concentration was able to reduce genistein induced cell proliferation at genistein concentrations of 0.1–1 μM, but was not able to inhibit the proliferation caused by genistein at 3–20 μM (Fig. 3).

3.4. Genistein metabolite formation

One possible explanation for the observed superinduction by genistein, would be the formation of a metabolite that is also an active ER agonist by itself. Above 1 μM genistein, at which the superinduction occurs, HPLC analysis of the culture medium revealed the formation of a genistein metabolite (M1) (Fig. 4). The medium from ERα-U2OS-Luc cells incubated with 1 and 5 μM genistein was collected upon 24 h exposure. HPLC-DAD chromatograms obtained from medium of genistein-treated cells showed the presence of genistein at a retention time of 36.7 min and another compound (M1) at a retention time of 31.2 min (Fig. 4). Based on the shorter retention time and similarities in the UV spectra, M1 is likely to be a polar metabolite of genistein. The formation of this unknown metabolite (M1) by the ERα-U2OS-Luc cells was significant at concentrations of 0.5 μM genistein and higher.

The samples used for the HPLC-DAD analysis, were subsequently analyzed by LC-FTMS/MS in positive ion mode. The protonated genistein corresponds to the most abundant ion at *m/z* 271. The MS/MS spectrum (with fragments formed at *m/z* 271, 253, 243, 225, 215, 187 and 153) confirmed that the molecular ion $[M+H]^+$ at *m/z* 271 was indeed genistein (data not shown). The M1 metabolite ($[M+H]^+$ ion at *m/z* 287) was subsequently analysed by LC–MS/MS. Fragmentation of the *m/z* 287 ion yielded product ions at *m/z* 269, 259, 241, 231 and 161. The presence of the ion at *m/z* 161 had only been observed earlier with isoflavones as a rearrangement product for a dihydroxylated B-ring fragment [20]. The losses at MH^+ −18 (269), MH^+ −28 (259) and MH^+ 28–28 (231) are typical for hydroxyisoflavones. Taken together, the MS/MS data are consistent with a hydroxylation on the ring B for product M1 identifying the metabolite as 5,7,3′,4′-tetrahydroxyisoflavone, better known as orobol. Subsequently, this metabolite, identified as orobol, was also tested in the ERα-U2OS-Luc reporter gene assay (Fig. 5). Orobol is able to induce reporter gene expression at concentrations above 1 μM reaching a superinduction of 187% at 40 μM.

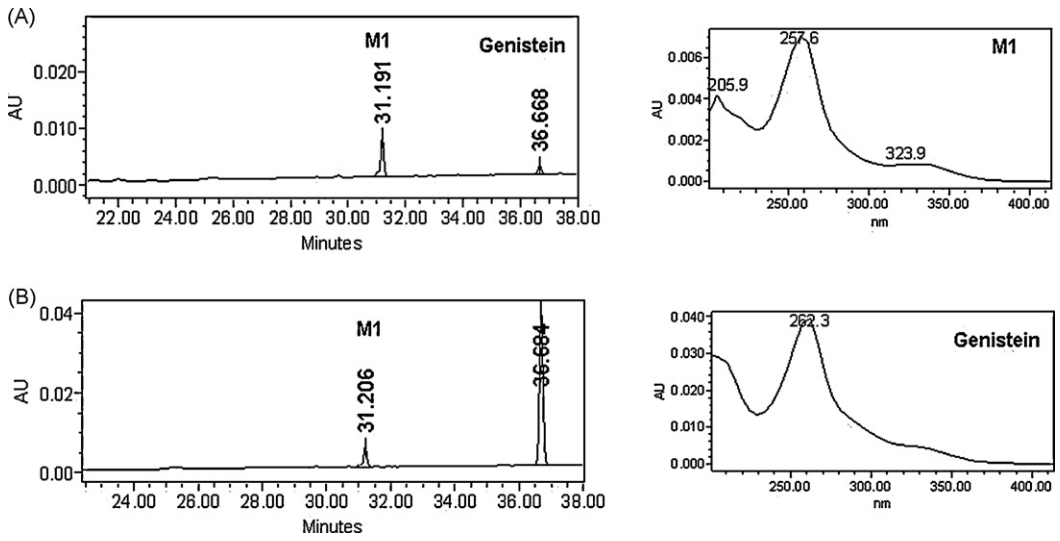

Fig. 4. HPLC analysis. Representative sections of the HPLC chromatograms of ERα-U2OS-Luc medium samples after 24 h of exposure to (A) 1 μM and (B) 5 μM genistein, and the UV spectra belonging to the peaks of M1 (UV_{max} 257.6 nm) and genistein (UV_{max} 262.3 nm).

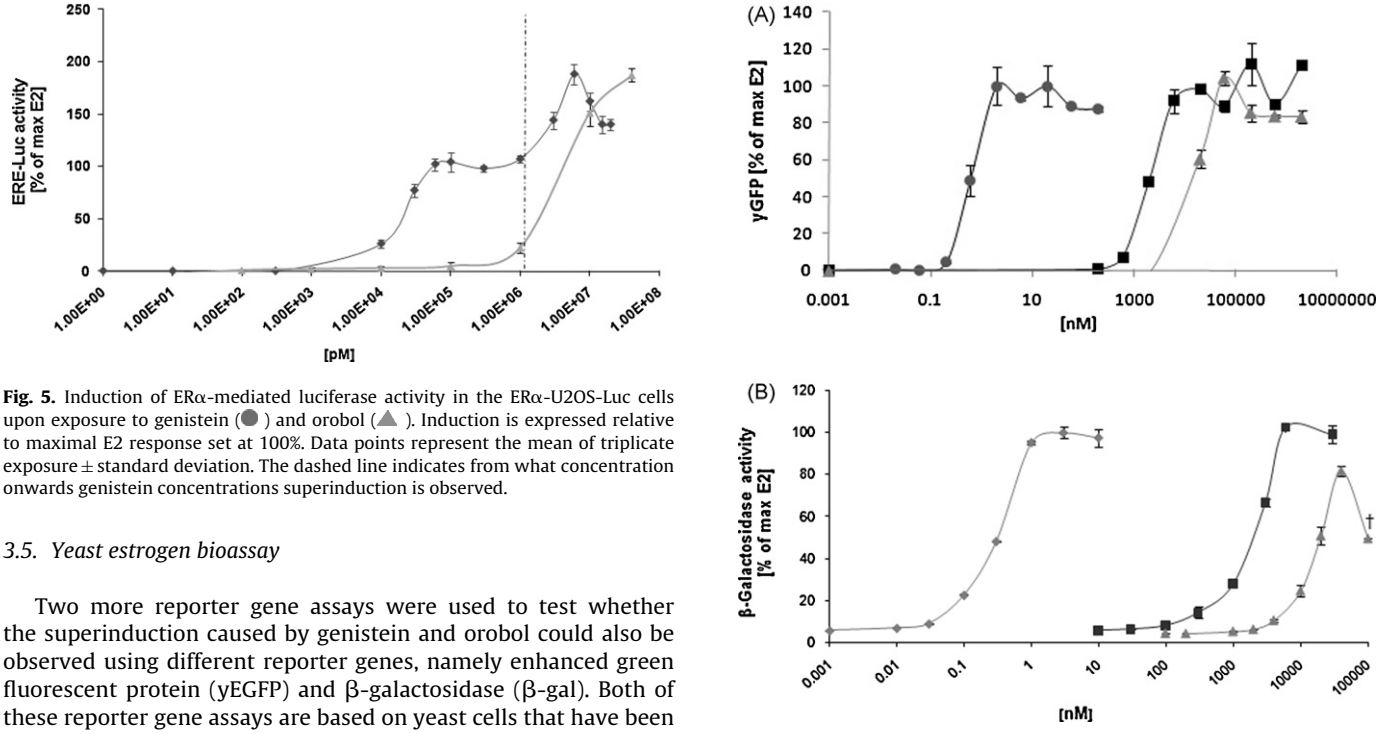

Fig. 5. Induction of ERα-mediated luciferase activity in the ERα-U2OS-Luc cells upon exposure to genistein (●) and orobol (▲). Induction is expressed relative to maximal E2 response set at 100%. Data points represent the mean of triplicate exposure ± standard deviation. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

3.5. Yeast estrogen bioassay

Two more reporter gene assays were used to test whether the superinduction caused by genistein and orobol could also be observed using different reporter genes, namely enhanced green fluorescent protein (yEGFP) and β-galactosidase (β-gal). Both of these reporter gene assays are based on yeast cells that have been previously shown to be metabolically inactive with regard to natural and synthetic estrogens [21,22]. The yeast cells expressing hERα were exposed to genistein and orobol. Fig. 6A and B shows that both genistein and orobol resulted in the same maximal response as E2. Thus, no superinduction was observed in bioassays based on yeast cells and using different reporter genes than firefly luciferase.

Fig. 6. Induction of ERE-mediated reporter gene in yeast estrogen bioassays. (A) Induction of ERE-mediated yEGFP expression in a yeast estrogen bioassay using yEGFP as reporter gene upon exposure to E2 (●), genistein (■) and orobol (▲). Data points represent the mean of triplicate exposure ± standard deviation. (B) Induction of ERE-mediated β-galactosidase expression in a yeast estrogen bioassay upon exposure to E2 (●), genistein (■). Data points represent the mean of triplicate exposure ± standard deviation. (†) Cytotoxicity.

3.6. Genistein and E2 induced luciferase gene expression as detected by RT-PCR

Additional experiments were performed aiming at studying the effect of genistein on luciferase mRNA and/or protein activity and stability. Post-transcriptional activation and stabilization of luciferase mRNA and/or protein might be an alternative explanation for the observed superinduction at high concentrations of genistein (>1 μM) explaining increased luciferase activity without the need for increased transcriptional activity. To actually verify whether or not the increased luciferase activity is a true reflection of increased transcriptional activity, RT-PCR based quantification of luciferase mRNA formation as a function of increasing genistein concentrations was performed and results obtained were compared to the level of mRNA formation induced at optimal levels of E2. To this end, ERα-U2OS-Luc cells were treated with E2 and genistein during 6 and 24 h. RNA from all the samples was extracted, and expression of firefly luciferase gene mRNA was compared with that of the housekeeping gene β-actin by RT-PCR. RT-PCR analysis revealed that the firefly luciferase gene expression was 23-fold

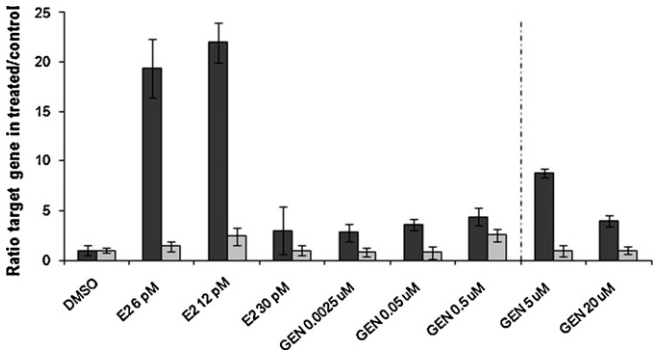

Fig. 7. Relative firefly luciferase mRNA expression levels in ERα-U2OS-Luc cells normalized to the expression of β-actin after 6 h (black column) and 24 h (grey column). The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

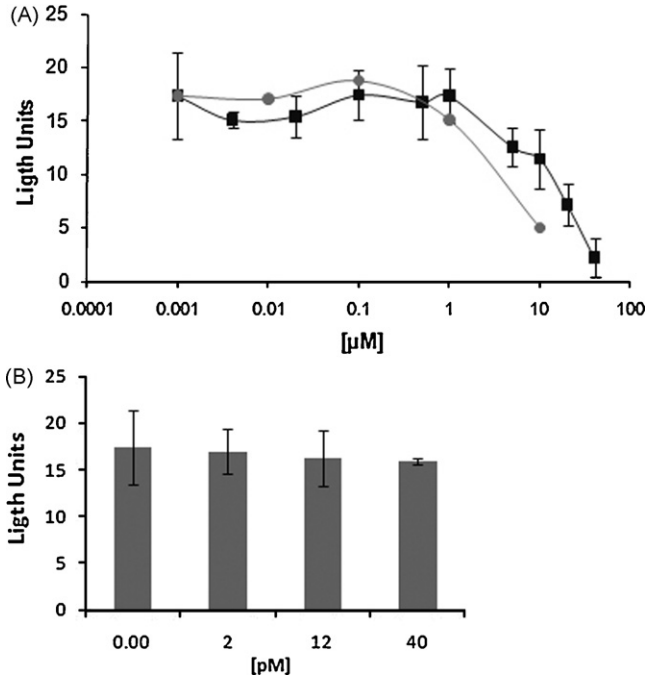

Fig. 8. Genistein and resveratrol inhibit firefly luciferase activity. (A) Firefly luciferase activity after co-incubation with increasing concentrations of genistein (■) or resveratrol (●) or (B) with estradiol. Data points represent the mean of triplicate exposure ± standard deviation. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

up-regulated by 12 pM E2 (Fig. 7). Increasing concentrations of genistein resulted in increased expression of luciferase mRNA, but the maximum level of mRNA induction reached was 9-fold, and never reached the induction factor observed for E2. Moreover, no superinduction of the mRNA level due to genistein treatment at concentration above 1 μM genistein was observed.

Genistein at concentrations above 1 μM increased the luciferase activity. Therefore, in subsequent experiments we used a cell-free biochemical assay to measure the interaction between genistein and the luciferase protein. In order to investigate whether genistein and other compounds causing superinduction may act through interacting with the luciferase enzyme thereby ultimately stabilizing the enzyme, increasing concentrations of genistein, estradiol and resveratrol, another phytoestrogen that induces superinduction, were incubated with a known and constant concentration of pure firefly luciferase protein. The firefly luminescence read-out was dose-dependent with an inhibition of the enzymatic reaction at concentrations above 1 μM for genistein and resveratrol (Fig. 8A), whereas bioluminescence was unaffected by estradiol (Fig. 8B).

4. Discussion

In recent years, several *in vitro* assays have been developed to assess estrogenic activity of chemicals and naturally occurring compounds. From those, several reporter gene assays showed higher transactivation activities, or induction of the reporter gene, for genistein compared to E2. This effect was often called superagonism or superinduction, and, although the mechanism underlying the phenomenon is still unrevealed, it is often suggested that it might be biologically relevant [9]. The present study investigated possible mechanisms behind this superinduction thereby at the same time addressing its biological importance.

The first hypothesis tested the possibility that the induction of the reporter gene was not ER-mediated. Hence, using the ERα-U2OS-Luc cell line, cells were exposed to E2 and genistein in the absence and presence of potent ERα antagonists (ICI 182,780 or RU58668). Using a concentration of the ERα antagonist that fully blocks luciferase induction mediated by E2-ERα (5 nM), the induction of luciferase was only blocked at low genistein concentration while at high concentrations, this was only possible with increased antagonist concentration (100 nM). The same effect was obtained when using the T47D-Luc cell line, another luciferase reporter gene assay based on a cell line that expresses endogenous ERα. Therefore, it is concluded that induction of luciferase is ER-mediated. The different sensitivity of the two phases of luciferase induction by genistein suggests that both processes, although apparently both dependent on ERα mediated luciferase induction, proceed by mechanisms that are at least in part dissimilar and displaying different affinities for genistein.

On the other hand, genistein induces superinduction of the reporter gene in the U2OS-Luc and T47D-Luc cell lines that is not reflected at cell proliferation level. No "superproliferation" was observed in the T47D cell line. This shows that the phenomenon of superinduction is not reflected in this biologically relevant endpoint.

The next hypothesis tested, was whether the superinduction was not due to genistein but to an *in vitro* formed metabolite. This hypothesis originated from the observation that a metabolite was formed at the genistein concentration where superinduction is maximal. The biological activities observed in *in vitro* or *in vivo* studies are often assumed to originate from the parent compounds that are examined, although these may have been subject to biotransformation into one or more structurally different compounds [23,24]. A number of genistein metabolites have been identified [25–27] but most of them have been shown to be less estrogenic than genistein [28,29]. The data presented in this study showed metabolite formation at high genistein concentrations, starting at 0.5 μM and strongly increasing at concentrations up to 5 μM, in the *in vitro* tests using U2OS and T47D cells. Orobol, or 5,7,3′,4′-tetrahydroxyisoflavone, was identified as a main oxidative metabolite, which is in agreement with previously reported data that hydroxylated metabolites of genistein are the most abundant metabolites *in vitro* [30] and that the primary oxidative metabolite of genistein is orobol [20,25].

Therefore, genistein and orobol were tested in the ERα-U2OS-Luc cells, but also in a metabolically inactive yeast estrogen bioassay which uses another reporter gene than luciferase, namely enhanced green fluorescence protein. Additionally, genistein and orobol were measured in another yeast estrogen bioassay using β-galactosidase as a reporter gene. Both compounds were able to show a dose–response curve in the yeast based estrogen bioassays, but neither genistein nor orobol was able to show the superin-

duction as observed in the ERα-U2OS-Luc and T47D-Luc assays. In addition, it was shown that also orobol induced superinduction in the ERα-U2OS-Luc. As orobol does not give rise to higher maximal induction of the reporter genes than genistein, and especially because orobol is less potent than genistein, it can be concluded that orobol is not responsible for the observed superinduction by genistein.

Overall, superinduction by genistein and orobol is especially observed in the luciferase reporter gene assays, and more importantly reporter gene assays using luciferase from firefly as the reporter protein [8–12].

The data obtained can also be used to disregard another hypothesis for the superinduction. We show that the superinduction is not likely to be due to the up-regulation of the ER, as there was no difference between the ERα-U2OS-Luc and T47D-Luc. The T47D-Luc assay uses the endogenous expressed ER which could in theory be up-regulated by normal physiological mechanisms, while the yeast and U2OS cells use a receptor construct that induces a strong and constitutive expression of the human ERα.

Therefore, the next step was to actually verify whether or not the increased firefly luciferase activity was a true reflection of increased transcriptional activity. Therefore, mRNA expression of the firefly luciferase gene was measured in ERα-U2OS-Luc cells after 6 and 24 h exposures to E2 and genistein. After 6 h exposure, the maximum induction of firefly luciferase at the transcript level was significantly higher for estradiol than for genistein-treated cells. At 12 pM E2 luciferase mRNA levels were about 3-fold higher than at 5 μM genistein, a concentration shown before to result in clear superinduction when quantified on the basis of luciferase reporter gene activity. At 24 h, the time of bioluminescent luciferase activity measurements in the ERα-U2OS-Luc reporter gene assay, very low induction levels of luciferase transcription were remaining. These data indicated that the superinduction detected by measuring luciferase activity is not a reflection of real mRNA induction and should more likely be ascribed to a phenomenon that leads to prolonged enzyme activity without the need for increased transcription levels. Therefore, we tested the potential of genistein to act as a stabilizer of the luciferase reporter enzyme.

We evaluated the possible direct interaction of genistein with the reporter enzyme leading to stabilization (decreased degradation) of the enzyme. It has been proposed that certain compounds directly bind to, and stabilize, the firefly luciferase reporter enzyme thereby increasing its half-life [31–33]. The interaction between the bioactive chemicals and the luciferase results in inhibition of the enzyme activity at the same time resulting in stabilization of the enzyme [34,35]. These luciferase-stabilizing compounds are referred to in the literature as luciferase inhibitors [35]. An accumulation of stabilized luciferase reporter enzyme will enhance the observed bioluminescence activity. Superinduction effects have been reported for other estrogenic compounds, e.g. the flavonoid resveratrol, and in all cases, this was specifically reported in firefly luciferase cell-based assays [9,36,37]. Thus, in a cell-free biochemical assay, the possible interaction between genistein, E2 and resveratrol with firefly luciferase was investigated. We observed that resveratrol and genistein but not estradiol can specifically inhibit the bioluminescent enzymatic reaction of the firefly luciferase. Inhibition of the bioluminescence has been suggested to result from direct competitive inhibition of the enzymatic reaction [32]. It can be expected that upon rupture of the cells the inhibitor will dissociate leading to increased activity of the luciferase enzyme due to this stabilizing effect. Thus, genistein at concentrations above 1 μM may interact with luciferase thereby stabilizing the enzyme so that upon lysis of the cells and dissociation of genistein from the enzyme due to dilution, increased activity can be measured as compared to the situation without an added stabilizer/inhibitor. It has been recently shown, that a series of bioactive compounds, that inhibit and thereby stabilize the firefly luciferase enzyme will result in an increased luminescence signal [34].

In conclusion, the data presented in this study strongly indicate that the superinduction caused by genistein can be ascribed to stabilization of the firefly luciferase reporter enzyme increasing the bioluminescent signal during the cell-based assay. This indicates that the phenomenon may not be biologically relevant but may rather represent a post-transcriptional effect on enzyme stability.

Acknowledgement

This research was partially funded by the Graduate School of Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid (VLAG) (Project Number 61.61.100.040).

References

[1] G.G.J.M. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.-A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor {beta}, Endocrinology 139 (10) (1998) 4252–4263.
[2] B. Gutendorf, J. Westendorf, Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens, Toxicology 166 (1–2) (2001) 79–89.
[3] M Murata, K. Midorikawa, M. Koh, K. Umezawa, S. Kawanishi, Genistein and daidzein induce cell proliferation and their metabolites cause oxidative DNA damage in relation to isoflavone-induced cancer of estrogen-sensitive organs, Biochemistry 43 (9) (2004) 2569–2577.
[4] C.E. Rufer, S.E. Kulling, Antioxidant activity of isoflavones and their major metabolites using different in vitro assays, Journal of Agricultural and Food Chemistry 54 (8) (2006) 2926–2931.
[5] S. Rice, S.A. Whitehead, Phytoestrogens and breast cancer—promoters or protectors? Endocrine-related Cancer 13 (4) (2006) 995–1015.
[6] S. Barnes, C. Grubbs, K. Setchell, J. Carlson, Soybeans inhibit mammary tumors in models of breast cancer, Progress in Clinical and Biological Research 347 (1990) 239–253.
[7] P.M. Kris-Etherton, K.D. Hecker, A. Bonanome, S.M. Coval, A.E. Binkoski, K.F. Hilpert, A.E. Griel, T.D. Etherton, Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer, The American Journal of Medicine 113 (9 Suppl. 2) (2002) 71–88.
[8] K. Joung, Y. Kim, Y. Sheen, Assessment of the estrogenicity of isoflavonoids, using MCF-7-ERE-Luc cells, Archives of Pharmacal Research 26 (9) (2003) 756–762.
[9] A Freyberger, G. Schmuck, Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay, Toxicology Letters 155 (1) (2005) 1–13.
[10] E. Sonneveld, J.A.C. Riteco, H.J. Jansen, B. Pieterse, A. Brouwer, W.G. Schoonen, a.B.v.d. Burg, Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities, Toxicological Sciences 89 (1) (2006) 173–187.
[11] A.M. Sotoca, D. Ratman, P. van der Saag, A. Ström, J.A. Gustafsson, J. Vervoort, I.M.C.M. Rietjens, A.J. Murk, Phytoestrogen-mediated inhibition of proliferation of the human T47D breast cancer cells depends on the ER[alpha]/ER[beta] ratio, The Journal of Steroid Biochemistry and Molecular Biology 112 (4–5) (2008) 171–178.
[12] S. Takeuchi, T. Takahashi, Y. Sawada, M. Iida, T. Matsuda, H. Kojima, Comparative study on the nuclear hormone receptor activity of various phytochemicals and their metabolites by reporter gene assays using Chinese hamster ovary cells, Biological & Pharmaceutical Bulletin 32 (2) (2009) 195–202.
[13] M. Maggiolini, D. Bonofiglio, S. Marsico, M.L. Panno, B. Cenni, D. Picard, S. Ando, Estrogen receptor alpha mediates the proliferative but not the cytotoxic dose-dependent effects of two major phytoestrogens on human breast cancer cells, Molecular Pharmacology 60 (3) (2001) 595–602.
[14] K.A. Power, a.L.U. Thompson, Ligand-induced regulation of ERα and ERβ is indicative of human breast cancer cell proliferation, Breast Cancer Research and Treatment 81 (2003) 209–221.
[15] K.B. Horwitz, W.L. McGuire, Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor, The Journal of Biological Chemistry 253 (7) (1978) 2223–2228.
[16] M.E. Quaedackers, C.E. Van Den Brink, S Wissink, R.H.M.M. Schreurs, J.-A. Gustafsson, P.T. Van Der Saag, B. Van Der Burg, 4-Hydroxytamoxifen transrepresses nuclear factor-{{kappa}}B activity in human osteoblastic U2-OS cells through estrogen receptor (ER){{alpha}}, and not through ER{{beta}}, Endocrinology 142 (3) (2001) 1156–1166.
[17] J Legler, C.E. van den Brink, A. Brouwer, A.J. Murk, P.T. van der Saag, A.D. Vethaak, B. van der Burg, Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47D breast cancer cell line, Toxicological Sciences 48 (1) (1999) 55–66.
[18] T.F.H. Bovee, R.J.R. Helsdingen, I.M.C.M. Rietjens, J. Keijer, R.L.A.P. Hoogenboom, Rapid yeast estrogen bioassays stably expressing human estrogen receptors [alpha] and [beta], and green fluorescent protein: a comparison of different

compounds with both receptor types, The Journal of Steroid Biochemistry and Molecular Biology 91 (3) (2004) 99.
[19] S.F Arnold, M.K. Robinson, A.C. Notides, L.J. Guillette Jr., J.A. McLachlan, A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens, Environmental Health Perspectives 104 (5) (1996) 544–548.
[20] E.S. Roberts-Kirchhoff, J.R. Crowley, P.F. Hollenberg, H. Kim, Metabolism of genistein by rat and human cytochrome P450s, Chemical Research in Toxicology 12 (7) (1999) 610–616.
[21] T.F.H. Bovee, W.G.E.J. Schoonen, A.R.M. Hamers, M. Jorge Bento, A.A.C.M. Peijnenburg, Screening of synthetic and plant-derived compounds for (anti)estrogenic and (anti)androgenic activities, Analytical and Bioanalytical Chemistry 390 (4) (2008) 1111–1119.
[22] T.F.H. Bovee, J.P.M. Lommerse, A.A.C.M. Peijnenburg, E.A. Fernandes, M.W.F. Nielen, A new highly androgen specific yeast biosensor, enabling optimisation of (Q)SAR model approaches, The Journal of Steroid Biochemistry and Molecular Biology 108 (1–2) (2008) 121–131.
[23] M Messina, W. McCaskill-Stevens, J.W. Lampe, Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings, Journal of the National Cancer Institute 98 (18) (2006) 1275–1284.
[24] C.B. Klein, A.A. King, Genistein genotoxicity: critical considerations of in vitro exposure dose, Toxicology and Applied Pharmacology 224 (1) (2007) 1–11.
[25] S.E. Kulling, D.M. Honig, T.J. Simat, M. Metzler, Oxidative in vitro metabolism of the soy phytoestrogens daidzein and genistein, Journal of Agricultural and Food Chemistry 48 (10) (2000) 4963–4972.
[26] S.-M. Heinonen, K. Wähälä, H. Adlercreutz, Metabolism of isoflavones in human subjects, Phytochemistry Reviews 1 (2) (2002) 175–182.
[27] J. Bursztyka, E. Perdu, J. Tulliez, L. Debrauwer, G. Delous, C. Canlet, G. De Sousa, R. Rahmani, E. Benfenati, J.-P. Cravedi, Comparison of genistein metabolism in rats and humans using liver microsomes and hepatocytes, Food and Chemical Toxicology 46 (3) (2008) 939–948.
[28] D. Pugazhendhi, K.A. Watson, S. Mills, N. Botting, G.S. Pope, P.D. Darbre, Effect of sulphation on the oestrogen agonist activity of the phytoestrogens genistein and daidzein in MCF-7 human breast cancer cells, The Journal of Endocrinology 197 (3) (2008) 503–515.
[29] Y. Zhang, T.T. Song, J.E. Cunnick, P.A. Murphy, S. Hendrich, Daidzein and genistein glucuronides in vitro are weakly estrogenic and activate human natural killer cells at nutritionally relevant concentrations, The Journal of Nutrition 129 (2) (1999) 399–405.
[30] D.T. Nguyen, E. Hernandez-Montes, D. Vauzour, A.H. Schönthal, C. Rice-Evans, E. Cadenas, J.P.E. Spencer, The intracellular genistein metabolite 5,7,3′,4′-tetrahydroxyisoflavone mediates G2-M cell cycle arrest in cancer cells via modulation of the p38 signaling pathway, Free Radical Biology and Medicine 41 (8) (2006) 1225–1239.
[31] A. Pfitscher, E. Reiter, A. Jungbauer, Receptor binding and transactivation activities of red clover isoflavones and their metabolites, The Journal of Steroid Biochemistry and Molecular Biology 112 (1–3) (2008) 87–94.
[32] D.S. Auld, N. Thorne, W.F. Maguire, J. Inglese, Mechanism of PTC124 activity in cell-based luciferase assays of nonsense codon suppression, Proceedings of the National Academy of Sciences of the United States of America 106 (9) (2009) 3585–3590.
[33] J.F. Thompson, K.F. Geoghegan, D.B. Lloyd, A.J. Lanzetti, R.A. Magyar, S.M. Anderson, B.R. Branchini, Mutation of a protease-sensitive region in firefly luciferase alters light emission properties, The Journal of Biological Chemistry 272 (30) (1997) 18766–18771.
[34] D.S. Auld, N.T. Southall, A. Jadhav, R.L. Johnson, D.J. Diller, A. Simeonov, C.P. Austin, J. Inglese, Characterization of chemical libraries for luciferase inhibitory activity, Journal of Medicinal Chemistry 51 (8) (2008) 2372–2386.
[35] D.S. Auld, N. Thorne, D.-T. Nguyen, J. Inglese, A specific mechanism for nonspecific activation in reporter-gene assays, ACS Chemical Biology 3 (8) (2008) 463–470.
[36] S.O. Mueller, S. Simon, K. Chae, M. Metzler, K.S. Korach, Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor {alpha} (ER{alpha}) and ER{beta} in human cells, Toxicological Sciences 80 (1) (2004) 14–25.
[37] A. Bakhtiarova, P. Taslimi, S.J. Elliman, P.A. Kosinski, B. Hubbard, M. Kavana, D.M. Kemp, Resveratrol inhibits firefly luciferase, Biochemical and Biophysical Research Communications 351 (2) (2006) 481–484.