Axon A, May FEB, Gaughan LE, Williams FM, Blain PG, Wright MC.
[Tartrazine and sunset yellow are xenoestrogens in a new screening assay to identify modulators of human oestrogen receptor transcriptional activity.](#)
Toxicology 2012, 298(1-3), 40-51.

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DOI link to article:

<http://dx.doi.org/10.1016/j.tox.2012.04.014>

Date deposited:

26/06/2017

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Tartrazine and Sunset yellow are xenoestrogens in a new screening assay to identify modulators of human estrogen receptor transcriptional activity

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Abstract

Primary biliary cirrhosis (PBC) is a cholestatic liver disease of unknown cause that occurs most frequently in post-menopausal women. Since the female sex hormone estrogen can be cholestatic, we hypothesised that PBC may be triggered in part by chronic exposure to xenoestrogens (which may be more active on a background of low endogenous estrogen levels seen in post-menopausal women). A reporter gene construct employing a synthetic estrogen response element predicted to specifically interact with estrogen receptors (ER) was constructed. Co-transfection of this reporter into an ER null cell line with a variety of nuclear receptor expression constructs indicated that the reporter gene was trans-activated by ER α and ER β , but not by the androgen, thyroid, progesterone, glucocorticoid or vitamin D receptors. Chemicals linked to PBC were then screened for xenoestrogen activity in the human ER α -positive MCF-7 breast cancer cell line. Using this assay, the coal-derived food and cosmetic colourings - sunset yellow and tartrazine - were identified as novel human ER α activators, activating the human ER with an EC_{50%} concentration of 220 and 160nM respectively.

Keywords: food additive; cosmetic additive; estrogen receptors; primary biliary cirrhosis; cholestasis; MCF-7 cells.

1. Introduction

Xenoestrogens are exogenous chemicals that are able to modulate endogenous estrogen activity through for example, structural similarities which result in their interactions with receptors or estrogen metabolising enzymes. A common mechanism of action is for xenoestrogens to show an affinity for estrogen receptors (ER) and subsequently cause endocrine disruption through this interaction (McKenna and O'Malley, 2002). Other chemicals may bind to other receptors which then interact with ERs and modulate their function (Huang *et al.*, 2010).

ERs are members of the nuclear receptor gene superfamily of transcription factors (Tsai and O'Malley, 1994; Hammes and Levin, 2007; see also Nuclear Receptor Signalling Atlas www.nursa.org/10.1621/datasets.02001). Humans possess 2 ER genes, ER α (also termed ESR1 or NR3A1) and ER β (also termed ESR2 or NR3A2). The ER α was first identified and has been the most studied. ER α is a ligand-activated nuclear receptor that functions through binding endogenous estrogens such as 17 β -estradiol (E2) and mediating changes in the expression of genes regulated through ER interaction with specific genomic DNA sequences termed estrogen response elements (ERE) (Tsai and O'Malley, 1994; Hammes and Levin, 2007; see also Nuclear Receptor Signalling Atlas www.nursa.org/10.1621/datasets.02001). ERs also have rapid, non-genomic effects in cells that modulate other signalling pathways such as kinase activities and Ca²⁺ mobilisation (Hammes and Levin, 2007). ER α is expressed in a variety of tissues, most highly in uterine, ovarian, pituitary gland, vas deferens and adipose tissues (Nuclear Receptor Signalling Atlas www.nursa.org/10.1621/datasets.02001) and regulates the expression of greater than 2800 genes in mammary tumour cell lines (Creighton *et al.*, 2006). In normal uterine cells (which express undetectable levels of ER β in mouse), E2 stimulates DNA synthesis, uterine cell proliferation (Leroy *et al.*, 1975; Gerchenson *et al.*, 1984) and directly induces expression of c-jun, jun B and jun D (Cicatiello *et al.*, 1992) as well as ER α (Ing and Tornesi, 1997),

progesterone receptor (Ing and Tornesi, 1997)) and CXCL12 genes (Glance *et al.*, 2009). Therefore, the primary role of ER α is the regulation of sexual reproduction, as exemplified in ER α knock out mice - females are infertile and males have decreased fertility (Lubahn *et al.*, 1993).

ER β is similarly transcriptionally-activated by E2 (Kuiper *et al.*, 1998). However, expression diverges from ER α , with highest levels in the ovary, lung, epididymis, prostate, colon and specific regions of the brain (Nuclear Receptor Signalling Atlas www.nursa.org/10.1621/datasets.02001). In the normal lung (which expresses low to undetectable levels of ER α - Nuclear Receptor Signalling Atlas www.nursa.org/10.1621/datasets.02001), E2 also induces progesterone receptor expression (Gonzalez-Arenas *et al.*, 2003). However, ER β knockout mice develop relatively normally and have normal fertility (Krege *et al.*, 1998). Ageing ER β knockout males display signs of prostate and bladder hyperplasia (Krege *et al.*, 1998). More recently, effects on lung and brain development have been observed in ER β knockout mice (Patrone *et al.*, 2003; Wang *et al.*, 2001).

Primary biliary cirrhosis (PBC) is a cholestatic liver disease of unknown cause that occurs most commonly in post-menopausal women and is associated with circulating antibodies to mitochondrial proteins within the pyruvate dehydrogenase complex (PDC) (Jones, 2008). The incidence of PBC is increased in geographical areas in close proximity to toxic waste sites (Ala *et al.*, 2006). Population-based space-time clustering suggests a transient environmental agent exposure is causative (McNally *et al.*, 2009). Since estrogens are cholestatic, we therefore hypothesised that xenoestrogens may have a role to play in the development PBC. We have designed and validated a screening assay to identify modulators of human ER transcriptional activity. A range of compounds (xenobiotic lipoic acid analogues, insecticides and food/cosmetic additives), exposure to which have been reported to be associated with an increased risk of developing PBC, have been tested for their

estrogenic potential. We identify, for the first time, several food and cosmetic additives as modulators of the human ER.

2. Materials and methods

2.1. Plasmid Constructs and Materials.

Plasmids directing the expression of a variety of human nuclear receptors – ER α , pCMV5-ER; the androgen receptor (AR), pCDNA3-AR-(1–918); the thyroid hormone receptor (TR), pVP16AD-TR; the progesterone receptor (PR), pM-PR-LBD; the glucocorticoid receptor (GR), pM-GR-LBD and the vitamin D receptor (VDR), pVP16AD-VDR-LBD have been described (Brady *et al.*, 1999; Gaughan *et al.*, 2001). The plasmid directing the expression of ER β , pCDNA3.1-nv5-ER beta was purchased from Addgene, Cambridge, USA). Organophosphates (Greyhound Chemicals, Birkenhead, UK) were a gift from Dr Elaine Mutch (Newcastle University, UK). All other chemicals were purchased from the Sigma Chemical Co. (Poole, Dorset, UK).

2.2. Construction of an estrogen receptor-responsive luciferase reporter construct ((ERE)₃-pGL3). An oligonucleotide containing three synthetic estrogen response elements was synthesised and hybridised with a complementary oligonucleotide (see Figure 1A) to give *SacI* and *XhoI* sticky ends. Each response element consensus site was flanked with an identical nucleotide to its neighbour to promote sensitivity and selectivity for the estrogen receptor (see Supp Table 1). The double stranded fragment was then ligated into pGL3 promoter (Promega, Southampton, UK) and cloned in TOP10 *E. coli* cells (Invitrogen) using standard methods. Recombinant cloned plasmids were screened by restriction digestion screening prior to confirming correct ligation by DNA sequencing.

2.3. Cell culture. The human breast cancer cell MCF-7 and MDA-MB321 (MDA) lines were originally obtained from the ATCC and Dr Elaine Mutch (Newcastle University, UK)

respectively and were routinely cultured in low glucose Dulbecco's-modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 80 units/ml penicillin and 80 µg/ml streptomycin. Cells were sub-cultured using trypsin. The human biliary epithelial H69 cell line (Grubman *et al.*, 1994) was obtained from Dr Elaine Mutch (Newcastle University) and routinely cultured in low glucose Dulbecco's-modified Eagle medium (DMEM):Ham's F12 medium (2:1) supplemented with 10% (v/v) fetal bovine serum, 80 units/ml penicillin and 80 µg/ml streptomycin, 240ng/ml adenine, 27ng/ml triiodothyronine, 10 µg/ml epinephrine, 1x insulin-transferrin-selenium solution (ITS-solution), and 1µM hydrocortisone.

2.4. Transfection and Reporter Gene Assays. Prior to transfection, MCF-7 and MDA cells lines were cultured for up to four days in culture medium supplemented with charcoal/dextran-stripped serum to reduce the levels of estrogen in the culture medium (May and Westley, 1988). Cells in six well plates were then transfected (per well) with 1.0 µg (ERE)₃-pGL3 and 0.5 µg of a plasmid (RL-TK) encoding the renilla luciferase protein under the control of a thymidine kinase promoter (Promega, Southampton, UK) to control for transfection efficiency with polyethylenimine (MCF-7 cells) or Effectene (MDA cells) transfection reagent according the manufacturer's (Polyplus and Qiagen respectively) instructions. After 24 hours, transfected cells were treated with potential ER agonists/antagonists from a 1000-fold molar concentrated stock in DMSO. Control cells were treated with 0.1% (v/v) DMSO. After 24 hours exposure to treatments, cells were harvested and luciferase and renilla activities determined using a Dual-Luc kit (Promega, Southampton, UK). Luciferase activities were normalised to renilla activities and fold activities then compared to DMSO control treated cells as previously outlined (Haughton *et al.*, 2006).

2.5. RT-PCR. Total RNA was isolated using Trizol (Invitrogen) and RT-PCR performed as previously described (Haughton *et al.*, 2006) using primers given in Table 1. SYBR green (Sigma, Poole, UK) was used to perform quantitative RT-PCR on an Applied Biosystems 7500 fast real-time PCR system.

2.6. Western Blotting. Western blotting was performed as previously described (Haughton *et al.*, 2006). Rabbit polyclonal antibodies to ER α (cat # AB75635) and ER β (cat # AB3576) were purchased from Abcam. An antibody to β -actin was purchased from Chemicon (mouse monoclonal MAB1501). HRP-conjugated anti-IgG antibodies were used for detection using the ECL reagent purchased from GE Healthcare (Amersham, UK).

3. Results

3.1. *Validation of a screening assay for human ER α transcriptional modulators.*

A variety of sequences have been shown to mediate ER-dependent changes in gene transcription and a variety of “consensus” ER binding sites have been proposed. However, there is no absolute consensus on the ER binding sequence and so a synthetic sequence (Figure 1A) based on a consensus sequence derived from a variety of natural human ER response elements (Supp. Table 1) was designed, with the conserved binding site sequences flanked with the same base present at the 5' and 3' end of the conserved binding site, to increase selectivity for the ER.

Although a biliary epithelial cell line might be a preferred cell type to screen xenobiotics for both estrogenic and cholestatic activities, we failed to detect expression of ER genes in the human biliary cell line H-69 (Figure 1B). The human breast cancer MCF-7 cell line was therefore employed. Figure 1B and C indicate that the MCF-7 cell line used in these studies expressed predominantly ER α mRNA and protein after four days culture in medium containing “withdrawn” serum (charcoal-stripped to reduce the constitutive levels of estrogenic compounds present within the culture). ER β mRNA was detected in withdrawn MCF-7 cells, but not protein. The ER α -null MDA cell line also expressed ER β mRNA but not protein. Using 17 β -estradiol (E2) - the major natural estrogen in man - Figure 1D demonstrates that E2 induced the expression of the luciferase reporter gene in (ERE)₃-pGL3 and that the ER antagonist ICI182780 (Wakeling *et al.*, 1991) at 10⁻⁸M inhibited both constitutive and E2-induced reporter gene activity in MCF-7 cells. Other natural estrogens (estrone and estriol), as well as the synthetic estrogen ethinylestradiol, at 10⁻⁸M also induced (ERE)₃-pGL3 reporter gene activity in MCF-7 cells (supplementary Figure 1). Other steroids at this concentration were inhibitory (supplementary Figure 1). In contrast, reporter gene expression was unaffected by E2 and ICI182780 in the ER null MDA cell line (Figure 1D).

Co-transfecting (ERE)₃-pGL3 into MDA cells with expression constructs encoding a variety of nuclear receptors demonstrated that only ER α and ER β directed an induction of reporter gene activity in response to E2 (Figure 1E).

The TTF1 gene has been shown to be an ER α -inducible gene in MCF-7 cells (May and Westley, 1988). Primers to a region of this transcript were designed and RT-PCR amplified a single amplicon of the predicted size (Figure 2A). Figure 2B shows that TTF1 mRNA was induced by E2 and that this induction was antagonised by ICI182780 in MCF-7 cells. No induction occurred in MDA cells (Figure 2B).

3.2. Screening a variety of xenobiotics for their ability to modulate human ER α transcriptional function.

Xenobiotic lipoic acid analogues are primarily ER α antagonists. PBC is associated with the presence of anti-mitochondrial antibodies which most commonly immunoreact with the lipoylated E2 component of the pyruvate dehydrogenase complex (PDC-E2) (Jones, 2008). It has been proposed that the mechanism for the initiation of tolerance breakdown in PBC is immune recognition of neo-antigens formed by xenobiotic substitution of lipoic acid in proteins (Leung *et al.*, 2003). Thus, sensitization with proteins substituted with the lipoic acid analogue 6-bromohexanoic acid (6BH) can induce an immune response that cross-reacts with PDC-E2 (Walden *et al.*, 2008).

Figure 3 demonstrates that a number of xenobiotics with the potential to substitute lipoic acid in PDC-E2 modulate ER α transcriptional activity. However, none of these compounds acted as an ER α agonist and many inhibited constitutive reporter gene expression (without causing any overt toxicity, and therefore are unlikely to promote cholestasis via an ER-dependent mechanism).

Insecticides / organophosphates. Pesticides may give rise an autoimmune response although their effects remain poorly defined and little investigated (Holsapple, 2002). However, since some pesticides have been reported to be xenoestrogens (Laws et al., 2000), a range of chlorinated pesticides and organophosphates were examined for their ability to activate the human ER α . Figure 4 demonstrates that only methoxychlor activated the human ER α whereas most compounds had either no effect or inhibited constitutive reporter gene expression (and therefore are unlikely to promote cholestasis via an ER-dependent mechanism).

Food and cosmetic additives. Epidemiological studies have made an association between PBC incidence and cosmetic use (e.g. frequent nail polish use (Gershwin *et al.*, 2005), hair dye use (Prince et al., 2010)). Figure 5 demonstrates that some constituents of cosmetics such as paraben esters, are human ER α agonists (as previously reported (Routledge *et al.*, 1998)), in our assay. In addition the cosmetic and food additives sunset yellow and tartrazine were also shown to be ER α agonists (Figure 5). As previously reported (Kuiper et al., 1998), the phytoestrogen genistein was also shown to be an ER α agonist (Figure 5).

Selected xenoestrogens identified in these studies were then tested for their ability to transcriptionally activate the human ER α . Inhibition of their induction of (ERE)₃-pGL3 reporter by the ER antagonist ICI182780 was also determined, to confirm that the compounds interact with the ER α (and not another trans-activating receptor) in MCF-7 cells. Figure 6A demonstrates that (ERE)₃-pGL3 reporter gene activity induced by E2 as well as by sunset yellow, tartrazine, butylparaben and propylparaben were inhibited by ICI182780. These data were also confirmed at the level of TTF1 mRNA induction (Figure 6B).

Food and cosmetics are potent human ER α modulators of transcriptional function.

To our knowledge, food / cosmetic additives such as sunset yellow and tartrazine have not been examined and/or reported to be estrogenic chemicals. Accordingly, we investigated their potency of action in the MCF-7 / (ERE)₃-pGL3 reporter gene assay. Figure 7A illustrates a typical dose – response for E2 and sunset yellow on relative reporter gene expression in MCF-7 cells transfected with (ERE)₃-pGL3. Figure 7B shows the dose-response effect of ICI182780 on E2-induced reporter gene activity. These data were used to calculate EC_{50%} and IC_{50%} values for a range of compounds and Table 2 demonstrates that the food colourings tartrazine and sunset yellow - the most potent xenoestrogens examined in this study - were approximately 50 times less effective than E2 at activating the human ER α in the MCF-7 / (ERE)₃-pGL3 reporter gene assay. Table 2 also demonstrates that the food colourings are more potent activators of the human ER α than the parabens compounds.

4. Discussion

This paper describes the use of a reporter gene system in MCF-7 cells to screen for compounds which have the potential to modulate human ER transcriptional activity. Although, in the context of PBC, it may be preferable to use a biliary epithelial cell line for these studies, the only available human cell line – H-69 – did not express ER (in contrast to biliary epithelial cells *in vivo* (Alvaro *et al.*, 2000; Alvaro *et al.*, 2004). However, the widely employed MCF-7 cell line remains an excellent model system in which to screen for potential estrogenicity in man (Gutendorf and Westendorf, 2001). It is likely that reporter gene expression in the MCF-7 cells employed was predominantly associated with ER α activity since we could not detect ER β protein in these cells (although its mRNA was detectable). The assay system is likely to be relatively specific to ERs since over-expression of a variety of nuclear receptors – in contrast to ER α and ER β – failed to trans-activate the reporter gene

construct in an ER null cell line. However, there are 48 nuclear receptor genes in man (Zhang *et al.*, 2004) and it cannot be excluded that another nuclear receptor expressed in MCF-7 cells might trans-activate the reporter gene.

Interestingly, despite MCF-7 cultures undergoing a four day withdrawal process whereupon the cells are cultured in serum exposed to charcoal (which adsorbs many low molecular weight chemicals such as steroids), reporter gene activity remained further inhibited by the ER antagonist ICI182780 by up to 90%. This suggests that either estrogens remain in the culture despite the withdrawal process or that un-liganded ER remains able to trans-activate the reporter gene construct. If the former is the case, then this assay system has the capacity to respond to lower concentrations of estrogen.

Although the MCF-7 cell provides an assay system for identifying predominantly ER α regulators, the reporter gene construct was also trans-activated by ER β . A cell line expressing ER β – but not ER α – was not identified and so screening for ER β -specific regulators could not be undertaken. Screening a range of compounds identified two food and cosmetic colouring agents – tartrazine and sunset yellow (for structures see supplementary Figure 2) – as human ER activators. To our knowledge, these compounds have not been shown to be xenoestrogen

There are two competing mechanisms proposed for inducing cholestasis in pregnancy or through clinical use of estrogen therapies. The mechanism proposed by Negishi and co-workers is that the expression of many bile acid and drug transporters are transcriptionally repressed by ER α activation (Yamamoto *et al.*, 2006). In contrast, Steiger and co-workers propose that estrogen and/or its hepatic metabolites inhibit the activity of bile acid and drug transporters (Steiger *et al.*, 2000). In either case, xenoestrogens have the capacity to cause cholestasis through the same mechanisms since they are reliant on molecular mimicry of estrogens. A question remains as to the likelihood that tartrazine or sunset yellow could cause an estrogenic effect and cholestatic effect in the liver. Both compounds are water

soluble and are not likely to be appreciably absorbed via the gut or skin (in the case of cosmetic use). However, gut and skin permeability can be affected by other agents (e.g. alcohol and other agents which are added to cosmetics respectively). There is a wealth of evidence that gut permeability and its consequent effect on inflammation in the liver is a major determinant on the development of alcoholic liver disease (Rao, 2009; Szabo *et al.*, 2010). Given the EC_{50%} values for sunset yellow and tartrazine determined in these studies (220 and 160nM respectively), the question arises as to whether exposure to these dyes could result in a estrogenic effect in man. Taking dietary intake alone (which is not the only source of exposure), their 3 refined exposure analysis indicates that 95th or 97.5th percentile exposure levels are calculated to be potentially up to 7.3 and 5.8 mg/kg body weight/day for tartrazine and sunset yellow respectively (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2009; European Food Safety Authority, 2011). Assuming this maximum dose is taken as a bolus; that only 10% is absorbed and systemically available; that the compounds are completely distributed around the body and that absorption, metabolism and elimination effectively reduce the maximum concentration of the compounds by 50%, serum concentrations of 685nM and 640nM for tartrazine and sunset yellow respectively are calculated. These values are in excess of their EC_{50%} values for the ER α determined in these studies and therefore suggest that in high exposed individuals, intake may be sufficient for these chemicals to have an estrogenic effect. It should also be noted that estrogens have been shown to cause genomic instability – at least in cancer cell lines – via an ER-independent mechanism (Yared *et al.*, 2002; Kabil *et al.*, 2008). Xenoestrogens may also act similarly and this effect may further contribute to cholestatic injury. Since the mechanism appears to be mediated via Src/Raf/Erk signalling mechanism independent of the ER (Kabil *et al.*, 2008), the ER reporter system employed in these experiments would not identify chemicals that might also act via this pathway.

Our data suggest that compounds like tartrazine and sunset yellow – whilst not the cause - may be a component contributor to the development of cholestasis and which in combination with other factors, could pre-dispose to the development of PBC.

Conflict of interest

There are no conflicts of interest.

Acknowledgements

Supported by PhD studentship grant joint-funded by the Medical Toxicology Centre, the British Toxicology Society and the Institute of Cellular Medicine, Newcastle University.

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A

5' CGGGGTCAATTGACCCCGGGTCAATTGACCCCGGGTCAATTGACCC
|||||
3' TCGAGCCCAGTTAAACTGGGGCCAGTTAAACTGGGGCCAGTTAAACTGGGAGCT

B

C

D **E**

Fig. 1. The (ERE)₃-pGL3 reporter gene is transcriptionally regulated by human ER α and ER β . **A**, concatamer of 3 synthetic ERE response elements (shaded) inserted upstream of the minimal promoter sequence in pGL3-promoter luciferase reporter gene construct. **B**, RT-PCR for the expression of the indicated human transcript in a variety of human cell lines transfected with the ERE₃-pGL3 construct. **C**, Western blot for the expression of ER proteins

in cell lines (30 μ g cell protein/lane). **D**, (ERE)₃-pGL3 reporter gene expression after transfection in the indicated cell line - mean and standard deviation normalized ERE₃-pGL3 reporter gene expression from 3 separate transfections in the same experiment, typical of at least 3 separate experiments. *Significantly different ($P > 95\%$) versus the DMSO vehicle treated cells using the Student's T test (two tailed). **E**, (ERE)₃-pGL3 reporter gene expression after transfection of MDA cells with either GFP (as control) or an expression vector encoding the indicated nuclear receptor - mean and standard deviation normalized ERE₃-pGL3 gene expression relative to GFP transfected/DMSO treated cells from 3 separate transfections in the same experiment, typical of at least 2 separate experiments. *Significantly different ($P > 95\%$) versus the GFP-transfected/DMSO vehicle-treated cells using the Student's T test (two tailed).

A **B**

Fig 2. TTF1 mRNA is induced by the ER in MCF-7 cells. **A**, agarose gel electrophoresis of PCR products after quantitative RT-PCR amplification from either DMSO vehicle or E2-treated MCF-7 cells. Each lane represents a PCR product from a separate well of cells from the same experiment, typical of 7 separate experiments. **B**, TTF1 mRNA expression in the indicated cell line after treatment as indicated for 24 hours - mean and standard deviation levels (normalized to 18S rRNA) and expressed as fold the levels in DMSO control-treated cells for each cell line. Results are from 3 separate determinations from the same experiment, typical of at least 3 separate experiments. *Significantly different ($P > 95\%$) versus the DMSO vehicle treated cells using the Student's T test (two tailed).

Fig. 3. Screening lipoic acid analogues for their ability to modulate human ER transcriptional activity. (ERE)₃-pGL3 reporter gene expression after transfection in MCF-7 cells and treatment as indicated - mean and standard deviation normalized (ERE)₃-pGL3 reporter gene expression from 3 separate transfections in the same experiment, typical of at least 3 separate experiments. *Significantly different ($P > 95\%$) versus the DMSO vehicle treated cells using one way ANOVA (two tailed).

Fig. 4. Screening insecticides and organophosphates for their ability to modulate human ER transcriptional activity. (ERE)₃-pGL3 reporter gene expression after transfection in MCF-7 cells and treatment as indicated - mean and standard deviation normalized (ERE)₃-pGL3 reporter gene expression from 3 separate transfections in the same experiment, typical of at least 3 separate experiments. *Significantly different (P > 95%) versus the DMSO vehicle treated cells using one way ANOVA (two tailed).

Fig. 5. Screening food and cosmetic additives for their ability to modulate human ER transcriptional activity. (ERE)₃-pGL3 reporter gene expression after transfection in MCF-7 cells and treatment as indicated - mean and standard deviation normalized (ERE)₃-pGL3 reporter gene expression from 3 separate transfections in the same experiment, typical of at least 3 separate experiments. *Significantly different (P > 95%) versus the DMSO vehicle treated cells using one way ANOVA (two tailed).

Fig. 6. Confirmation of human ER activation by xenobiotics. A, (ERE)₃-pGL3 reporter gene expression after transfection in MCF-7 cells and treatment as indicated - mean and standard deviation normalized (ERE)₃-pGL3 reporter gene expression from 3 separate transfections in the same experiment, typical of at least 3 separate experiments. ICI182780 or its vehicle control solvent were added to cells 24 hours prior to addition of potential ER

activator and re-treated at the time of addition of potential ER activator. Significantly different ($P > 95\%$) versus the ^{*}DMSO vehicle treated cells or [#] potential ER activator only-treated cells using one way ANOVA (two tailed). **B**, TFF1 mRNA expression in the indicated cell line after treatment as indicated for 24 hours - ICI182780 or its vehicle control solvent were added to cells 24 hours prior to addition of potential ER activator and re-treated at the time of addition of potential ER activator. Data are the mean and standard deviation levels (normalized to 18S rRNA) and expressed as fold the levels in DMSO control-treated cells for each cell line. Results are from 3 separate determinations from the same experiment, typical of at least 3 separate experiments. ^{*}Significantly different ($P > 95\%$) versus the DMSO vehicle treated or [#] potential ER activator only-treated cells using one way ANOVA (two tailed).

Fig 7. Dose-response regulation of (ERE)₃-pGL3 reporter gene expression. A – B, (ERE)₃-pGL3 reporter gene expression after transfection in MCF-7 cells and treatment with the indicated activator or antagonist - mean and standard deviation normalized (ERE)₃-pGL3 reporter gene expression from 3 separate transfections in the same experiment, typical of at least 3 separate experiments.

Supplementary FIG. 1. Estrogens transactivate the (ERE)₃-pGL3 construct in MCF-7 cells. (ERE)₃-pGL3 reporter gene expression after transfection in MCF-7 cells and treatment as indicated - ICI182780 or its vehicle control solvent were added to cells 24 hours prior to addition of potential ER activator and re-treated at the time of addition of potential ER activator. Data are the mean and standard deviation normalized (ERE)₃-pGL3 reporter gene expression from 3 separate transfections in the same experiment, typical of at least 3 separate experiments. *Significantly different ($P > 95\%$) versus the DMSO vehicle treated cells using the Student's T test (two tailed). \$Significantly different ($P > 95\%$) versus ICI182780 only treated cells using one way ANOVA (two tailed).

17 β – estradiol (E2)

Tartrazine

Sunset yellow

Supplementary FIG. 2. Structures of E2, tartrazine and sunset yellow.