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Effects of triclocarban on the transcription of estrogen, androgen and aryl hydrocarbon receptor responsive genes in human breast cancer cells

Patrick Tarnow *, Tewes Tralau, Danele Hunecke, Andreas Luch

German Federal Institute for Risk Assessment (BfR), Department of Product Safety, Berlin, Germany

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A B S T R A C T

Triclocarban (TCC) is an antimicrobial agent that is used in detergents, soaps and other personal hygiene products. Similarly to triclosan the widespread use of TCC has raised concerns about its endocrine potential. In luciferase-based reporter assays TCC has been shown to enhance estrogenic and androgenic activities following cellular coexposure with estrogen or dihydrotestosterone, respectively. The present study demonstrates that although coexposure with TCC enhances the estrogenic and androgenic readout of luciferase-based reporter cell lines such as HeLa9908 and MDA-kb2, it fails to act as a xenoandrogen on transcriptional level, nor does it induce cell proliferation in the estrogen sensitive E-screen. In addition TCC did not alter the expression of estrogen responsive genes in human mammary carcinoma MCF-7 cells exposed to 17β-estradiol, bisphenol A, butylparaben or genistein.

However, TCC was shown to interfere with the regulon of the aryl hydrocarbon receptor (AhR) as TCC showed a costimulatory effect on transcription of *CYP1A1* and *CYP1B1*, effectively lowering the transcriptional threshold for both genes in the presence of estrogens. It thus seems, that while the induction of the respective luciferase reporter assays by TCC is an unspecific false positive signal caused by luciferase stabilisation, TCC has the potential to interfere with the regulatory crosstalk of the estrogen receptor (ER) and the AhR regulon.

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1. Introduction

Triclocarban (3,4,4′-trichlorocarbanilide, TCC) is an antimicrobial agent commonly added to detergents and personal hygiene products including liquid soaps or soap bars. Apart from its diphenylurea moiety TCC is structurally similar to other widely used antimicrobials such as triclosan (TCS) and hexachlorophene (HCP) (Fig. 1). The use in soaps results in direct human exposure. Liquid soaps contain up to 1.5% of TCC (SCCP, 2005) and for a single shower the absorption of TCC is estimated to be 0.6% (Schebb et al., 2011). Based on an average use of 20 g of soap per shower TCC can therefore be expected to reach concentrations of approximately 1 μM in the blood stream. This was recently confirmed in a study with human volunteers, where the use of TCC containing soap resulted in half-maximal blood concentrations of up to 530 nM (Schebb et al., 2012). Moreover, in the US its ubiquitous use has led to concentrations as high as 6.8 μg/l in environmental water samples (Halden and Paull, 2005). As a halogenated hydrocarbon TCC is hardly biodegradable (Aken et al., 2010; Furukawa and Fujihara, 2008; Solyanikova and Golovleva, 2004) and subsequent levels in sewage sludge easily exceed 50 mg/kg (Heidler et al., 2006). In combination with the frequent use of sewage as fertiliser the poor biodegradability thus further adds to human exposure (Wu et al., 2012).

The high levels of TCC in water and sewage have raised concerns because TCC has been shown to amplify estrogenic and androgenic responses in cell-based reporter assays (Ahn et al., 2008). Androgenic effects were also observed *in vivo*. In castrated rats the coadministration of TCC and testosterone resulted in higher weights of sex accessory organs (Chen et al., 2008). Respective hyperplasias were also found in juvenile animals after they had been treated with TCC (Duleba et al., 2011). Meanwhile the estrogenic effects of TCC *in vivo* are less well investigated. In zebrafish coexposure to 17β-estradiol (E2) and TCC enhanced the transcriptional

Abbreviations: AhR, arylhydrocarbon receptor; AP-1, activator protein; AR, androgen receptor; ATP, adenosine triphosphate; BPA, bisphenol A; BuPa, butylparabene; *CCND1*, cyclinD1, CYP, cytchrome P450; DHT, dihydrotestosterone; E2, 17β-estradiol; EC_{50}, half maximal effective concentration; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERE, estrogen responsive element; *ESR1*, estrogen receptor alpha; *ESR2*, estrogen receptor beta; Gen, genistein; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HSPB8*, heat shock binding protein 8; *NDRG1*, N-myc downstream regulated 1; *PGR*, progesterone receptor; *RPLP0*, ribosomal protein, large, P0; *SARG*, specifically androgen-regulated gene protein; SEM, standard error of the mean; *SORD*, sorbitol dehydrogenase; Sp1, specificity protein; TCC, Triclocarban; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; *TFF1*, trefoil factor 1; *UGT2B15*, glucuronosyltransferase 2B15.

* Corresponding author. Address: German Federal Institute for Risk Assessment (BfR), Max-Dohrn Strasse 8-10, 10589 Berlin, Germany. Tel.: +49 30 18412 3885.

E-mail address: Patrick.Tarnow@bfr.bund.de (P. Tarnow).

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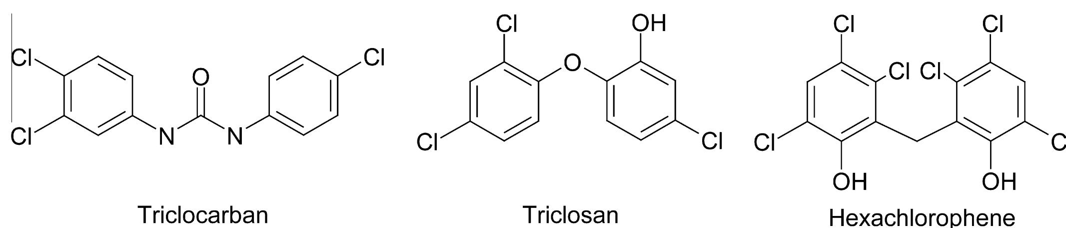

Fig. 1. Chemical structures of the antimicrobials triclocarban (TCC), triclosan (TCS) and hexachlorophene (HCP).

induction of aromatase AroB, while the combination of TCC with the xenoestrogen bisphenol A (BPA) led to reduced expression of *aroB* (Chung et al., 2011).

Estrogens exert their effects mainly via two nuclear receptors, that is estrogen receptor alpha (ERα) and beta (ERβ). Following cognate ligand binding these transcription factors dimerise and bind to specific estrogen response elements (EREs) at the DNA, where subsequent recruitment of co-activators induces target gene expression (Heldring et al., 2007). Alternatively ERs can interact with transcription factors already bound to the DNA, such as AP-1 and Sp1. A third mechanism is the activation of non-genomic pathways, where hormone binding leads to the rapid activation of signalling cascades (Heldring et al., 2007).

Most estrogenic reporter gene assays use ERE-containing promoters in combination with endogenous or transgenic ERα. Nevertheless, several estrogen responsive genes do not contain classical EREs. Instead these promotors contain ERE half-sites, AP-1- and Sp1-sites or combinations thereof (O'Lone et al., 2004). This suggests the regulation of endogenous genes to be more complex and questions the suitability of assays with readouts that are solely based on ERE-driven gene expression. Therefore this study aimed to compare the results of commonly used reporter gene assays with the effects of TCC on endogenous gene expression in human mammary carcinoma cells. The examined transcripts include androgenic and estrogenic target genes as well as genes of the AhR regulon. Androgenic gene expression was examined in an ER^- background (i.e. MDA-MD-453), while MCF-7 cells were used to test the influence of TCC in combination with E2 and a choice of xenoestrogens typically found in consumer products, cosmetics and foods (Evans et al., 2012).

2. Materials and methods

2.1. Chemicals

Cell culture media were purchased from PAN Biotech (Aidenbach, Germany), charcoal treated FCS was obtained from PAA (Cölbe, Germany) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was a gift from the German dioxin reference lab (BfR, Berlin, Germany). Substrates for the luciferase assays (D-Luciferin, ATP) and reducing agent DTT were obtained from PJK (Kleinblittersdorf, Germany). All other chemicals were purchased from Sigma Aldrich (Munich, Germany). Substances were routinely dissolved in ethanol, with the exception of TCDD and TCC for which dimethylsulfoxide (DMSO) was used.

2.2. Androgen reporter assay

Cell line MDA-kb2 was obtained from the ATCC (ATCC-No. CRL-2713). The MDA-kb2 cell line is a derivative of MDA-MD-453 breast cancer cells. The latter provide a well characterised molecular background for androgenic testing, as they express the androgen receptor (AR) but are negative for ER. Transfection of this cell line with a stable MMTV.luciferase.neo reporter gene construct yielded the MDA-kb2 reporter cell line which is responsive to stimulation of the AR and the glucocorticoid receptor (GR) (Wilson et al., 2002). Upon arrival in the lab cellular transcription of the AR was confirmed by quantitative RT-PCR, as was the absence of transcripts for ER (Fig. S1). Reporter assays were performed as described by Ermler et al. (2010). Briefly, MDA-kb2 cells were maintained in Leibowitz' L-15 medium supplemented with FCS (10% v/v) and grown at 37 °C without the provision of additional CO_2. A week before usage the cells were switched to phenol red free L-15 medium with charcoal treated FCS (5% v/v). Subsequent seeding into 96-well plates was done one day prior to exposure, using a concentration of 10^4 cells per 100 μl and well. Substance exposure was started after 24 h by adding 50 μl of concentrated substance stocks. Dose response curves were measured in triplicate, and controls (1 nM dihydrotestosterone (DHT) and 0.1% ethanol, respectively) were repeated 6-fold.

Measurement of luciferase activity was performed in cellular crude extracts using a Synergy HT plate reader from BioTek (Bad Friedrichshall, Germany). Cells were lysed *in situ* using 50 μl of lysis buffer (0.1 M tris–acetate, 2 mM EDTA, and 1% triton-x, pH 7.8), shaking the plate moderately for 20 min at room temperature. Following cellular lysis 150 μl of luciferase buffer (25 mM glycylglycine, 15 mM $MgCl_2$ and 4 mM EGTA, 1 mM DTT, 1 mM ATP, pH 7.8) and 50 μl of luciferin solution (25 mM glycylglycine, 15 mM $MgCl_2$ and 4 mM EGTA, 0.2 mM luciferin, pH 7.8) were added automatically to each well in order to measure luminescence. All values were corrected for the mean of the negative control and then related to the positive control which was set to 100%.

2.3. Estrogen reporter gene assay

Cell line HeLa9903 was obtained from the JCRB (JCRB-No. 1318). These cells contain stable expression constructs for human ERα and firefly luciferase, respectively. The latter is under transcriptional control of five ERE promoter elements from the vitellogenin gene. The transcription of ERα was confirmed by RT-PCR, as was the absence of AR-transcripts (Fig. S1). The assay was performed according to the OECD test guideline TG455 (OECD, 2009) as follows. Cells were cultivated in phenol red free MEM containing 10% (v/v) of charcoal stripped FCS at 37 °C in an atmosphere with 5% CO_2. For the actual assay cells were seeded into white 96-well polystyrene plates at a concentration of 10^4 cells per 100 μl and well (Costar/Corning, Amsterdam, Netherlands). Test substances were added 3 h after seeding by adding 50 μl of triple concentrated substance stocks to each well. As before dose response curves for treated samples were measured in triplicate, while controls (1 nM E2 or 0.1% ethanol, respectively) were repeated 6-fold. After 24 h of stimulation, cells were washed with PBS and then lysed using 50 μl of lysis buffer and moderate shaking for 20 min at room temperature. Subsequent measurement of luciferase activity was performed analogous to the aforedescribed androgen reporter gene assay. All values were corrected for the mean of the negative controls and then related to the positive controls set as 100%.

2.4. E-screen

Cell line MCF-7 was obtained from the ATCC (ATCC-No. HTB-22) and checked with RT-PCR for transcription of ER, AR, GPR30 and AhR (Fig. S1). Cells were routinely passaged in RPMI 1640 medium containing 10% FCS (v/v), 100 U/ml Penicillin and 100 μg/ml streptomycin and grown at 37 °C in an atmosphere with 5% CO_2. Prior to the actual assays the cells were transferred into hormone-free medium (phenol red free RPMI 1640 with 5% of charcoal stripped FCS). Cells were seeded into 96-well polystyrene plates at a density of 1000 cells per 100 μl and well and were left to settle for 72 h before finally being exposed to the test substance for another 5 days. Dose response curves were measured in triplicate, while controls (1 nM E2 or 0.1% ethanol, respectively) were repeated 6-fold. Following substance exposure the cells were washed twice with PBS before determining cellular protein using bicinchoninic acid (Thermo Scientific, Waltham, MA, USA). After the addition of 25 μl H_2O and bicinchoninic acid solution the reaction was left to proceed for another 30 min at 37 °C before photometrically quantifying peptide triggered Cu-complex formation in a Synergy HT plate reader (λ_{Abs} = 562 nm).

2.5. Determination of gene expression

Cell lines MCF-7 or MDA-kb2 were seeded into 12-well plates with hormone-free medium at a concentration of 2×10^5 cells per ml and well. After 48 h of initial incubation the cells were stimulated with test substances for 6 or 24 h, respectively. Following substance treatment cells were washed in PBS and the total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). The extracted RNA (1 μg) was reversely transcribed into cDNA, using a cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA). Relative transcript levels were determined in triplicate by quantitative RT-PCR, using presynthesised Taqman probes or specific primers for a SYBR green master mix (Applied Biosystems, Foster City, CA, USA). Taqman probes and primer sets used were GAPDH (Hs02758991_g1), RPLP0 (Hs999 99902_m1), CYP1A1 (Hs0015 3120_m1), CYP1B1 (Hs00164383_m1), PGR (Hs0155 6707_m1), TFF1 (Hs001 70216_m1), CCND1 (Hs00277039_m1), HSPB8 (Hs00 205056_m1), UGT2B15 (Hs030 08769_g1), ESR1 (Hs01 046812_m1), ESR2 (Hs01100356_m1), AR (Hs00907244_m1) and GPR30 (Hs01922715_s1). The following primers were used in conjunction with SYBR green: SARG-forward (5′-CAG CTA CGA CTT CCT GTC CAC-3)′, SARG-reverse (5′-TGC TGA GTG ATG GTC TCC TCT-3)′, NDRG1-forward (5′-AAC CTG CAC CTG TTC ATC AAT-3′), NDRG1-reverse (5′-GGT CTT TGT TGG GTC CAA TTT-3′), FASN-forward (5′-AAT GTC AAC AAC CTG GTG AG-3′), FASN-reverse (5′-CCC TGT GAT CCT TCT TCA TCA-3′), GAPDH-forward (5′-CTC TGC TCC TCC TGT TCG AC-3′) and GAPDH-reverse (5′-ACG ACC AAA TCC GTT GAC TC-3′). Relative gene expression was calculated using the ΔΔCt method and normalised to expression levels of *GAPDH* or *RPLP0*.

2.6. siRNA mediated gene knockdown

Gene transcription of target genes was knocked down using a commercial siRNA transfection kit (‘HiPerFect’, Qiagen, Hilden, Germany). Briefly, MCF-7 cells were seeded into 12-well plates into hormone-free medium at a density of 1.2×10^5 cells per well. Following a 24 h pre-incubation the transfection was commenced according to the manufacturer’s instructions, using 2 nM of gene-specific or control siRNA, respectively. After 48 h of cellular recovery the efficiency of knockdowns was checked by quantitative RT-PCR.

2.7. Ethoxyresorufin-O-deethylase (EROD) assay

Cytochrome P450 (CYP)-catalysed turnover of 7-ethoxyresorufin by MCF-7 cells was measured in 96-well plates. The cells were seeded into phenol red free RPMI charcoal stripped FCS (5% v/v) at a density of 10^4 cells per well and allowed to rest for 48 h before being subjected to a 24 h test substance exposure. Cells were then washed twice with serum and phenol red free RPMI before 5 μM 7-ethoxyresorufin and 20 μM dicumarol were added to the wells. Subsequent fluorescence readouts were recorded continuously at 37 °C in a Synergy HT microplate reader (λ_{ex} = 530 nm, λ_{em} = 585 nm) and the respetive activity of CYP1 enyzmes was calculated based on a calibration curve with resorufin. Protein concentrations were measured using a BCA protein kit (Thermo Scientific, Waltham, MA, USA). All measurements were performed as 6-fold replicates.

2.8. Thermal shift assay

Protein stability of firefly luciferase (Promega, Madison, WI, USA) was assayed by fluorescent thermal shift (Niesen et al., 2007; Vedadi et al., 2006). Assays were performed in 96-well PCR plates using 3.6 μM of protein, 2 mM ATP and SYPRO orange in 50 mM tris–acetate, pH 7.6. Ligands, such as TCC, were added as indicated. Subsequent thermal shifts ranged from 25 °C to 99 °C at an increment of 1 °C/min and were recorded using the ROX filter set of a HT7500 PCR cycler (Applied Biosystems, Foster City, CA, USA). Data were analysed and the temperature of half-maximal denaturation (T_m) was calculated using the MS Excel spreadsheets as provided by (available at ftp:// ftp.sgc.ox.ac.uk /pub /biophysics) (Niesen et al., 2007; Vedadi et al., 2006).

2.9. Statistical analysis

All experiments were done in triplicate at least. Plotted error bars refer to the standard error of the mean (SEM) and a two-tailed Student’s *t*-test was used to assess significance. Respective *p*-values of $p < 0.05$ are indicated by an asterisk as appropriate.

3. Results

Concerns about an androgenic potential of TCC are mainly fuelled by results obtained from luciferase-based reporter screens (Ahn et al., 2008; Christen et al., 2010). However, the suitability of such systems as sole indicators for potential endocrine activity is disputed (Diel et al., 1999; Baker, 2001; Thorne et al., 2010). To investigate the androgenic potential of TCC this study therefore supplemented a commonly used AR-sensitive cellular luciferase assay with quantitative RT-PCR. The concentration of TCC used in the assays was 1 μM as this corresponds to the maximal levels realistically expected in human blood (SCCP, 2005; Schebb et al., 2012).

3.1. Effects on androgen regulated endogenous target genes of TCC in combination with dihydrotestosterone in MDA-kb2 cells

Initially the reported androgen mediated amplification of luciferase-activity by TCC was reproduced using a MDA-kb2 reporter cell line (Fig. 2). In contrast to the T47D-ARE cell line used previously (Ahn et al., 2008) this cell line originates from human MDA-MB-453 breast cancer cells which were transfected with a MMTV.luciferase.neo reporter construct. The respective reporter is negative for ER but maintains endogenous expression of the AR (Christen et al., 2010). The molecular background thus allows the monitoring of AR-responsive genes, while the luciferase

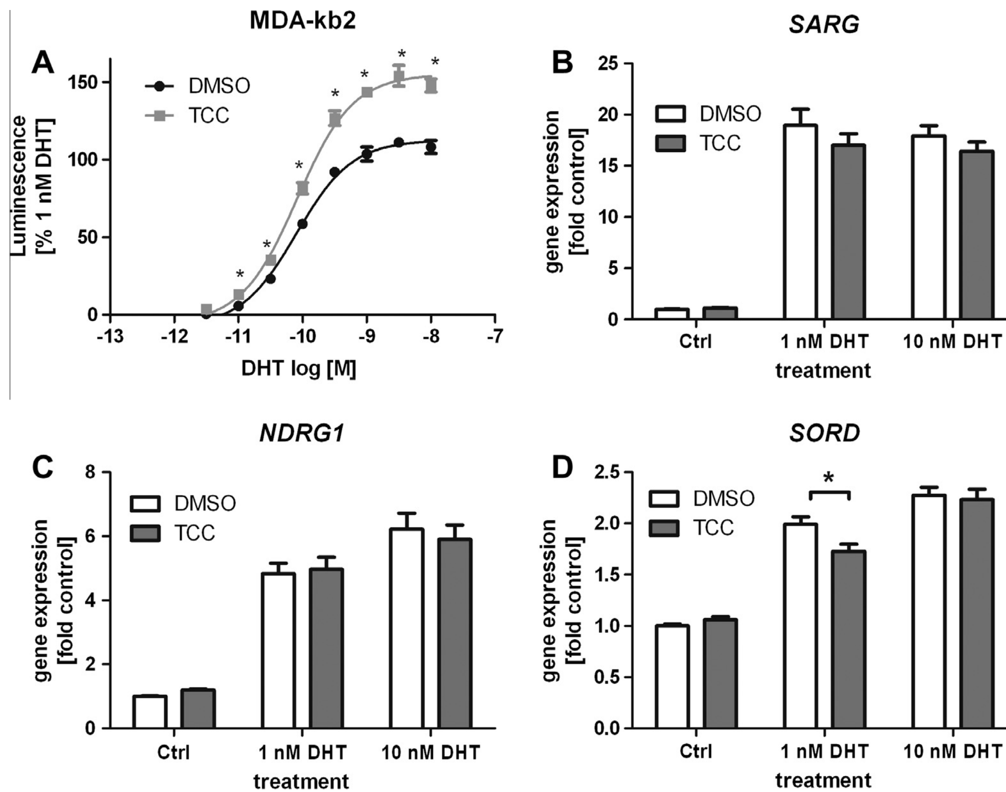

Fig. 2. Exposure to TCC amplifies DHT-triggered luminescence in MDA-kb2 reporter cells (A) but does not influence transcript levels of AR target genes (B–D). Cells were seeded in hormone-free medium for 24 (A) or 48 h (B–D) before being exposed to DHT ±1 μM TCC as indicated. Substance exposure was maintained for 24 h. Reporter gene activity was recorded as substance triggered change in fluorescence and is plotted relative to the activity of the positive control (1 nM DHT) (A). In a parallel experiment transcript levels of *SARG*, *NDRG1* and *SORD* were followed using RT-PCR and SYBR-green. Gene expression levels shown are normalised to endogenous transcription of *GAPDH* (B–D). All data shown represent the mean of three independent experiments (±SEM).

reporter will be responsive to stimulation of the AR as well as the GR. Quantitative RT-PCR confirmed expression of the *AR*, *GPR30* and *AHR* as well as the absence of transcripts for ERα or ERβ (Fig. S1). As anticipated co-exposure to 1 μM TCC and varying concentrations of DHT amplified the luciferase reporter activity by ~40% (Fig. 2A). Meanwhile the corresponding EC_{50} values remained unchanged at 8.0×10^{-11} M and 8.2×10^{-11} M for control and TCC treated cells, respectively. Nevertheless, the suggested

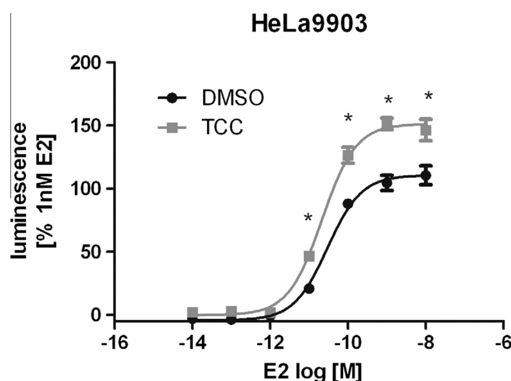

Fig. 3. Effect of TCC in a luciferase dependent estrogen receptor transactivation assay (HeLa9903). Cells containing an ER sensitive firefly luciferase construct were stimulated with increasing concentrations of E2 ±1 μM TCC for 24 h. Any substance triggered changes in luminescence were subsequently recorded using a plate luminometer. All values were corrected for background luminescence and are plotted as percent luminescence of the positive control (1 nM E2). The data shown represent the mean (±SEM) of three independent experiments, each performed in triplicate.

stimulation of the AR is in line with earlier studies, which confirms the functionality of the reporter construct (Christen et al., 2010).

The next aim was to validate the AR-antagonistic function of TCC on a transcriptional level. This was done by RT-PCR, targeting several transcripts known to be regulated by the AR (i.e. *SARG*, *NDRG1* and *SORD*) (Doane et al., 2006). Prior to RNA-extraction the cells were treated for 24 h with 1 or 10 nM DHT ± 1 μM TCC, respectively. Exposure to DHT led to an increased expression of all three transcripts. Yet, in the presence of TCC only *SORD* showed a slight but statistically significant decrease in gene expression ($p = 0.02$) (Fig. 2D). It was thus not possible to confirm the initially observed androgenic effect of TCC on the level of the AR regulon. An unspecific off-target effect of TCC on luciferase should, however, be apparent independent of the receptor investigated. Hence an estrogenic luciferase reporter was used to investigate the effects of TCC in presence of estrogen (E2). The corresponding results were then compared with those of a commonly used proliferation assay, namely the E-screen.

3.2. TCC amplifies the estrogen response in an estrogen dependent reporter gene assay but not in a mitogenic estrogen assay

The effect of TCC on an estrogenic luciferase reporter was studied using HeLa9903 cells, a cell line previously suggested for the detection of xenoestrogens by the OECD and EPA (OECD, 2009). These cells are stably transfected with human ERα and an ERE-driven luciferase reporter gene. The molecular phenotype was verified by quantitative RT-PCR, detecting transcripts of *ESR1*, *GPR30* and *AHR* but not *AR* or *ESR2* (Fig. S1). As described previously (Ahn et al., 2008) cellular co-exposure to E2 and 1 μM TCC resulted in a 50% increase of luciferase signal intensity (Fig. 3). Although

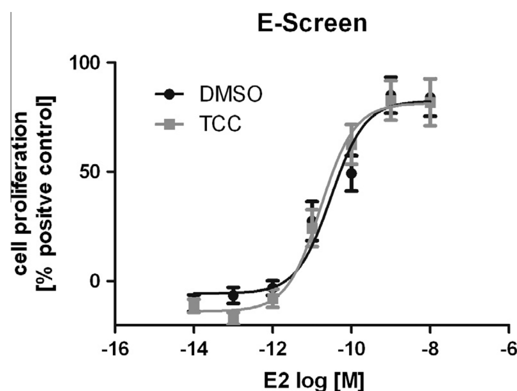

Fig. 4. Effect of TCC on estrogen dependent cell proliferation (E-screen). Following their synchronisation in hormone-free medium MCF-7 breast cancer cells were exposed to different concentrations of E2 ±1 μM TCC. Cell proliferation was quantified as relative protein content per well. The data shown represent the mean (±SEM) of three independent experiments, each performed in triplicate and corrected for the background measured in the negative control.

signal amplification was consistent for all E2-concentrations tested (10^{-11} to 10^{-8} M), the maximal effect was seen at 1 nM E2 and 1 μM TCC (Fig. 3). Higher concentrations of TCC quickly became cytotoxic (Fig. S2).

The suggested xenoestrogenic potential of TCC was further examined using the E-screen (Fig. 4). This assay uses the estrogen dependent proliferation of human mammary carcinoma MCF-7 cells as readout. Cellular exposure to E2 triggered a dose dependent increase of MCF-7 cell numbers. Addition of 1 μM TCC, however, failed to have any further proliferative effect. The E2 concentrations producing 50% of the maximal effect (EC_{50}) were comparable between the two assays, ranging from 2.9×10^{-11} M for the luciferase assay to 3.0×10^{-11} M for the E-screen. Nevertheless, while co-stimulation with TCC and E2 resulted in an increase of luciferase triggered luminescence, it failed to have any major effect on E2 dependent cell proliferation or the EC_{50} (2.1×10^{-11} M and 1.6×10^{-11} M).

3.3. Effects of TCC on estrogen regulated endogenous target genes in MCF-7 cells

With hindsight to the previous reports of TCC acting as a xenoestrogen *in vivo* (Chung et al., 2011) potential effects of TCC on the cellular estrogen response were further investigated on a molecular level. This was done using MCF-7 cells. As an established estrogen responsive cell line these cells endogenously express ERα as well as the estradiol-sensitive GPR30 (Fig. S1). In absence of any other reporter constructs they therefore allow a reliable detection of potential transcriptional changes caused by xenoestrogens. Quantitative RT-PCR was therefore used to follow the transcriptional pattern of several estrogen regulated genes in response to co-stimulation with TCC and E2 (10 nM) or various xeno- and phytoestrogens. Bisphenol A (BPA, 10 μM) and butylparaben (10 μM) were chosen as well-characterised xenoestrogens while genistein (10 μM) was used as a phytoestrogen. Analogous to the cellular assays test substance stimulation was maintained for 24 h in presence or absence of 1 μM TCC. In addition cells were also subjected to a 6 h treatment in order to detect any potential short term effects (e.g. as consequence of a short-term exposure, such as a shower with TCC-containing soap). The four transcripts used as molecular readouts for the 6 h treatment (Table 1) were chosen to reflect the various promoter structures of estradiol regulated genes. The promoters of the progesterone receptor (*PGR*) and the trefoil factor 1 (*TFF1* or *pS2*) contain an AP-1 site and an ERE half-site or a combination of several EREs and AP-1 binding sites, respectively (O'Lone et al., 2004; Cavailles et al., 1989). In contrast expression of cyclin D1 (*CCND1*) is regulated by tethered estrogen receptor signalling using Sp1 and AP-1 sites (Liu et al., 2002), whereas the 22 kDa heat shock protein 8 (*HSPB8*) is reported to be partially regulated by non-genomic estrogen signalling (Sun et al., 2007; Madak-Erdogan et al., 2008). Cellular exposure to any of the estrogens resulted in elevated transcript levels for all four genes. Meanwhile treatment with TTC did not have any effect. Neither did exposure to TCC alone alter the transcript levels of any of the ER regulated genes, nor did co-exposure to estrogens and TCC change estrogen-induced levels of gene expression.

The experiment was repeated with a prolonged substance exposure of 24 h (Table 1). Under these conditions expression levels of *CCND1* and *HSPB8* are known to decrease though (data not shown) (Silva et al., 2010). Therefore two other transcripts were chosen as molecular readouts instead, that is the genes for ERα (*ESR1*) and glucuronosyltransferase 2B15 (*UGT2B15)* (Hu and Mackenzie, 2009). The latter also has a prominent role during detoxification of BPA (Völkel et al., 2002; Hanioka et al., 2008). Expression of *ESR1* is reported to be regulated via an ERE half-site, while the transcription of *UGT2B15* is coordinately regulated by E2 via AP-1 sites, imperfect EREs and ERE half-sites (O'Lone et al., 2004; Hu and Mackenzie, 2009; Harrington et al., 2006). As before all transcripts were altered following estrogenic treatment. For *PGR* and *ESR1* treatment with BPA, butylparaben and genistein had an effect similar to E2, unlike *UGT2B15* where the two xenoestrogens had a less pronounced effect. With regard to trefoil factor 1 the prolonged exposure with genistein led to a 10-fold upregulation, a level twice as high as with E2. Again, none of the tested transcripts was influenced either by TCC alone or by co-stimulation with TCC and estrogens. Altogether the experiments therefore did not confirm a potential xenoestrogenic effect of TCC, neither on the molecular level, nor in whole cells (E-screen).

3.4. TCC stabilises firefly luciferase in vitro

Meanwhile the conflicting results for TCC in the various test systems point to an unspecific effect on luciferase. Ligand triggered stabilisation of luciferase has previously been reported to cause false positive readouts (Thorne et al., 2012). We therefore used thermal shift to assay the effects of TCC and ATP on the enzymes heat stability (Fig. 5A). The results showed that TCC indeed directly interacts with firefly luciferase, stabilising the enzyme. The effect is particularly pronounced in the presence of ATP as enzymatic cofactor. Addition of the latter shifted the T_m of luciferase by 3.3 °C. However, with increasing concentrations of TCC this shift increased further to up to 7 °C at 10 μM TCC. No such strong interaction could be seen with structural similar antimicrobials such as TCS and HCP (Fig. 5B). The first did not to stabilise luciferase at all, while the latter only interacted weakly ($\Delta T_m^{5\ \mu M\ HCP} = 2$ °C). Tested in the HeLa9903 estrogen reporter assay both substances were negative (Fig. S2).

3.5. Effects of TCC on target genes of the AhR

Altogether the data indicate that the previously reported effects of TCC as a xenohormone *in vivo* are not related to a direct interaction with the AR or ER. It is well established though that AhR and ERα are connected via a complex regulatory crosstalk mediated by several mechanisms and that interference with this crosstalk can lead to adverse phenotypes (Rataj et al., 2012). On molecular level interactions comprise competition for co-activators as well as AhR mediated protein degradation of ERα by ubiquitinylation (Ohtake et al., 2011). Further on some AhR regulated genes such as *CYP1B1* are also known to be ERE regulated (Tsuchiya et al., 2004).

Table 1
Effect of TCC on target genes of the ER in MCF-7 cells following 6 h or 24 h of co-stimulation with estrogens. Cells were pre-incubated in hormone-free medium for 48 h before being exposed to E2 (10 nM), BPA (10 μM), butylparaben (BuPa, 10 μM) or genistein (Gen, 10 μM) in the presence of ±1 μM TCC, respectively. Relative gene expression of selected ER regulated genes was subsequently quantified with Taqman probes using real-time PCR. Target gene expression was normalised to the transcription level of endogenous *GAPDH* and is reported as fold change compared to the untreated control (mean ± SEM). None of transcriptional differences between DMSO- and TCC-treated samples was found to be statistically significant ($p > 0.05$).

Gene symbol	Ctrl		E2		BPA		BuPa		Gen	
	DMSO	TCC	DMSO	TCC	DMSO	TCC	DMSO	TCC	DMSO	TCC
6 h										
PGR	1.00 ± 0.01	0.97 ± 0.03	24.16 ± 2.33	24.48 ± 2.7	24.11 ± 2.93	28.01 ± 2.87	24.81 ± 1.18	25.97 ± 2.06	29.79 ± 1.92	30.57 ± 3.03
TFF1	1.01 ± 0.05	1.06 ± 0.04	3.41 ± 0.43	3.65 ± 0.35	3.64 ± 0.13	4.13 ± 0.45	3.237 ± 0.30	3.90 ± 0.51	4.96 ± 1.02	5.25 ± 0.58
HSPB8	1.00 ± 0.02	1.00 ± 0.05	10.25 ± 0.67	10.73 ± 1.10	9.43 ± 1.28	10.2 ± 0.51	9.44 ± 0.62	9.69 ± 1.02	12.12 ± 0.52	12.57 ± 0.75
CCND1	1.00 ± 0.01	1.00 ± 0.08	2.41 ± 0.04	2.63 ± 0.09	2.34 ± 0.17	2.46 ± 0.16	2.41 ± 0.03	2.43 ± 0.20	2.59 ± 0.21	2.98 ± 0.22
24 h										
PGR	1.00 ± 0.02	0.89 ± 0.14	23.52 ± 0.77	22.32 ± 0.94	25.31 ± 1.25	24.69 ± 1.86	29.65 ± 2.74	23.10 ± 1.55	29.12 ± 3.47	29.74 ± 4.75
TFF1	1.00 ± 0.02	0.96 ± 0.06	5.51 ± 0.63	5.51 ± 0.40	4.77 ± 0.58	5.01 ± 0.30	4.95 ± 0.41	4.45 ± 0.21	10.83 ± 0.79	9.01 ± 0.78
ESR1	1.00 ± 0.02	1.01 ± 0.03	0.35 ± 0.01	0.35 ± 0.03	0.44 ± 0.04	0.45 ± 0.05	0.48 ± 0.07	0.43 ± 0.07	0.53 ± 0.10	0.52 ± 0.11
UGT2B15	1.00 ± 0.02	1.02 ± 0.08	11.62 ± 0.25	10.72 ± 0.96	5.42 ± 0.61	5.07 ± 0.52	4.61 ± 0.73	3.99 ± 0.50	9.12 ± 2.42	9.08 ± 1.37

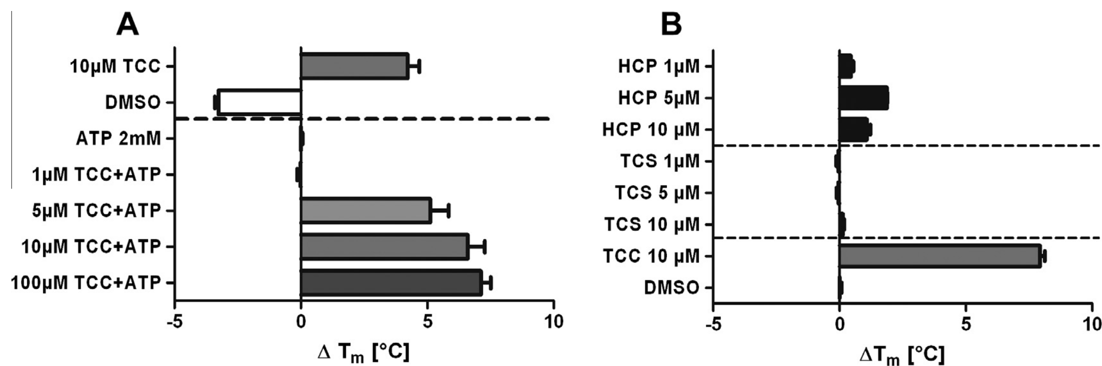

Fig. 5. TCC influences thermal stability of purified luciferase. Thermal enzyme stability (1 °C/min) was assayed using 3.6 μM firefly-luciferase in presence of TCC ± ATP (A) and TCS or HCP with ATP (B) as indicated. Fluorescence of SYPRO-orange was recorded continuously in a thermocycler, covering a temperature interval of 25–99 °C. Different melting curves are plotted as relative changes in T_m and refer to luciferase in presence of 2 mM ATP as stabilising cofactor. The data plotted represent the mean of three independent experiments, each performed in triplicate (±SEM).

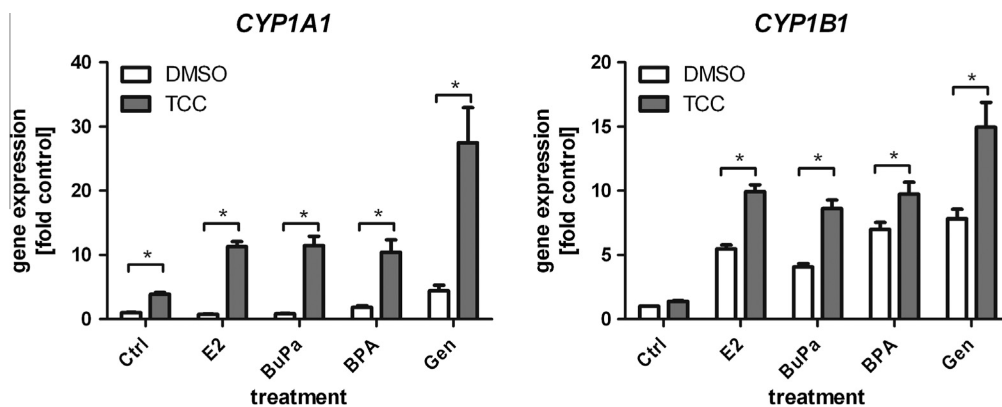

Fig. 6. Effect of TCC on the AhR target genes *CYP1A1* and *CYP1B1* in MCF-7 cells following 24 h of co-stimulation with estrogens. Cells were pre-incubated in hormone-free medium for 48 h before being exposed for another 24 h to E2 (10 nM), BPA (10 μM), BuPa (10 μM) or Gen (10 μM) in the presence of ±1 μM TCC, respectively. Relative gene expression of selected genes was subsequently quantified with Taqman probes using real-time PCR. Target gene expression was normalised to the transcription level of endogenous *GAPDH*. Panels show the results for *CYP1A1* and *CYP1B1*. The data represent the mean of three independent experiments (±SEM).

Following treatment with estrogens and TCC we therefore also measured the expression of two classical target genes of the AhR, *CYP1A1* and *CYP1B1* (Fig. 6). Used as single substance TCC induced a slight increase in *CYP1A1* expression which was comparable to the treatment with genistein. None of the other estrogens had a comparable effect when used alone. However, in combination with TCC they acted as strong inducers, increasing transcription of *CYP1A1* by up to 20-fold. Moreover, while all estrogens acted as inducers for *CYP1B1*, co-exposure to TCC stimulated transcription even further. On the other hand treatment with TCC alone only had a marginal effect on *CYP1B1* gene expression. The results indicate TCC to be a co-stimulator of the AhR. This is further supported by the fact that siRNA mediated reduction of *AHR* transcript levels to 25% strongly reduced the co-stimulatory effects of TCC and E2 on CYP induction (Fig. 7A). Meanwhile knockdown of *ESR1* produced a similar result. The reduction of ERα by 85% basically

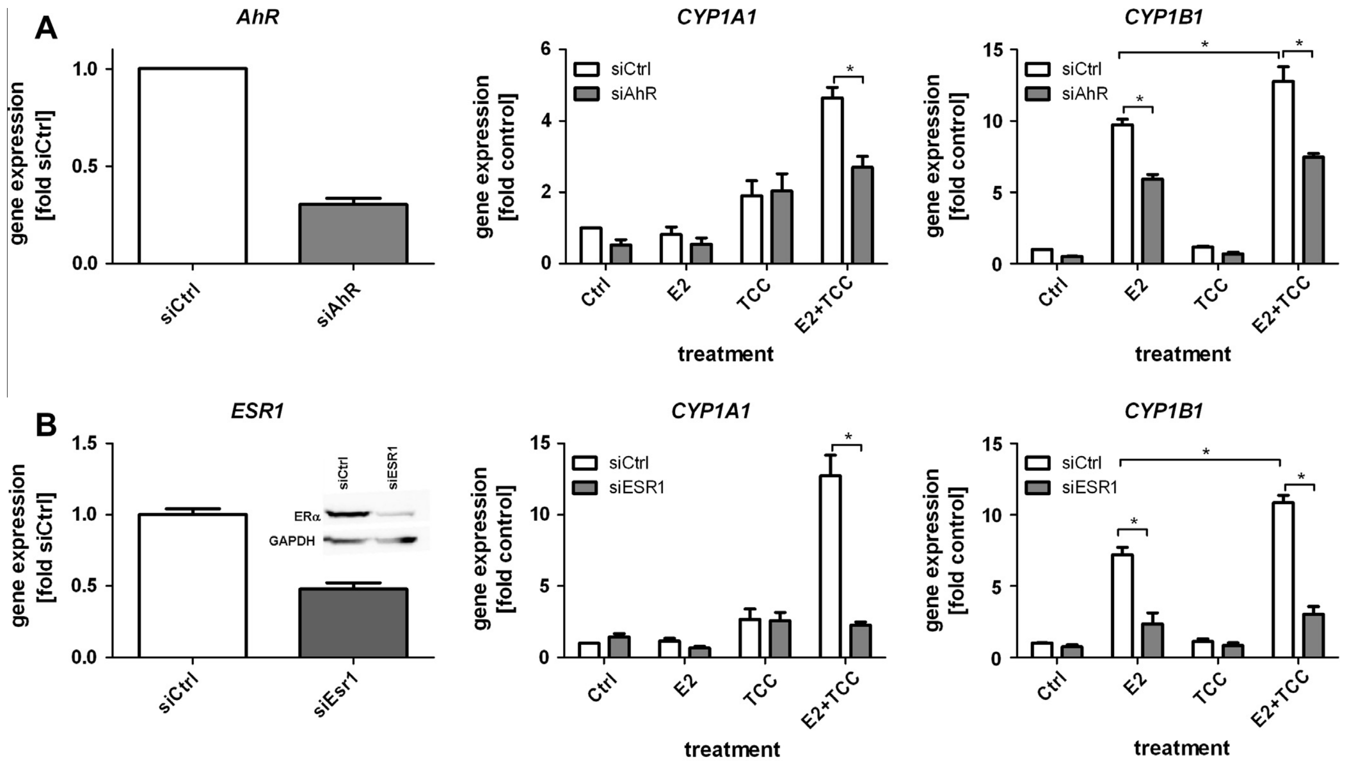

Fig. 7. Effect of AhR and ERα knockdown on transcript levels of *CYP1A1* and 1B1 in MCF-7 cells. Following transfection with gene-specific siRNA the successful knockdown of *AHR* (A) or *ESR1* (B) was confirmed using RT-PCR and by Western Blot (ERα only, inset). Cells were stimulated with 10 nM E2 ±1 μM TCC for 24 h. Transcript levels of *CYP1A1* or *CYP1B1* were quantified using Taqman probes and real-time PCR. Gene expression levels were normalised to the transcription of endogenous *RPLP0*. The data represent the mean of three independent experiments (±SEM).

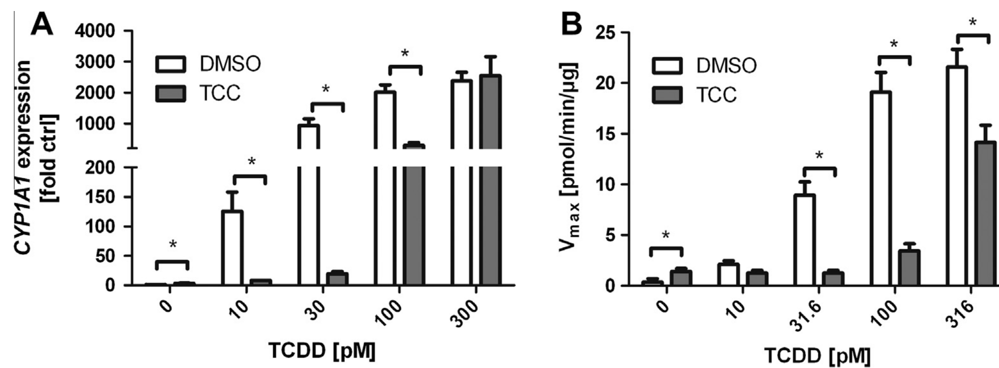

Fig. 8. Antagonistic effect of TCC on TCDD-induced gene expression (A) and enzymatic activity (B) of *CYP1A1* in MCF-7 cells. Cells were pre-incubated in hormone-free medium for 48 h before being exposed for another 24 h to various concentrations of TCDD ±1 μM TCC, respectively. Transcript levels of *CYP1A1* were quantified using Taqman probes and real-time PCR. Gene expression levels were normalised to the transcription of endogenous *GAPDH* (A). Enzymatic activity was followed in a parallel experiment, quantifying *CYP1A1*-catalysed resorufin-formation fluorometrically (B). Data plotted represent the mean of three independent experiments (±SEM).

abolished all co-stimulatory effects of E2 and TCC on *CYP1* gene transcription (Fig. 7B). It therefore appears that AhR as well as ERα are essential for the co-stimulatory effect of TCC on CYP1 expression. A direct interference of TCC with the AhR has also been suggested by Ahn et al. who identified TCC to be a weak AhR antagonist in cells treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Ahn et al., 2008). Treatment of TCDD-exposed MCF-7 cells with 1 μM TCC indeed inhibits endogenous expression of *CYP1A1* (Fig. 8A). The inhibitory effect is maintained throughout a concentration range of 10–100 pM TCDD, above which TCC seems to be outcompeted. An EROD assay further confirmed these results, showing that TCC also inhibited CYP1A1 mediated resorufin formation (Fig. 8B). This inhibition of a classical AhR cascade is in contrast to the co-stimulation of estrogenic CYP-induction seen before and demonstrates a differentiated effect of TCC on the AhR signalling cascade.

4. Discussion

This study investigated the endocrine effects of TCC using different *in vitro* assays. Despite its widespread use and its disputed role as an endocrine disruptor there are only few studies that looked into the molecular effects of TCC exposure. Most of the published data about the estrogenic or androgenic effects of TCC come from studies that used luciferase-based reporter assays. These

cellular assays are ideal for high-throughput screening due to their ease of handling and their automated readout. Hence they have become a tool of choice for the screening and investigation of potential endocrine disruptors and environmental pollutants. An androgenic action of TCC has been suggested repeatedly based on various androgenic transactivation assays (i.e. T47D-ARE cells, MDA-kb2 cells, or transiently transfected LnCaP or C4-2B cells) (Duleba et al., 2011; Chen et al., 2008; Blake et al., 2010; Ahn et al., 2008; Christen et al., 2010). The MDA-kb2 luciferase assay used in this study indeed confirmed TCC to enhance the DHT mediated luciferase signal. Yet, TCC failed to increase transcription of several androgen responsive genes when tested in the same molecular background. This suggests an interaction of TCC with luciferase instead. The latter is confirmed further by the results of the estrogenic reporter assays.

The estrogenic effect of TCC was previously shown in BG1-ERE cells (Ahn et al., 2008). Derived from ovarian cancer cells, this cell line has been stably transfected with an ERE-driven firefly luciferase reporter construct. Receptor ERα is expressed endogenously in these cells. In contrast the HeLa9903-reporter recommended by the OECD and EPA (OECD, 2009) supplies the ERE-driven luciferase construct as well as the ERα transgenetically. Nonetheless, the previously reported estrogen amplifying effect of TCC was also seen with the HeLa9903 cells. In addition, the exposure triggered increase of luminescence and the dose response curves for TCC were comparable to those published by Ahn et al. (2008). However, TCC did not show any further xenoestrogenic activity in a subsequent proliferation assay (Soto et al., 1995). Moreover, the expression of known estrogen responsive genes remained unaffected as well. The only notable exception was *CYP1B1*, a known target gene of the ER as well as the AhR (Tsuchiya et al., 2004; Shen et al., 1994).

Altogether the results suggest that the effects seen with TCC in luciferase-based transactivation assays are due to interference with firefly luciferase, rather than being triggered by ERα or the AR. Similar false positives have been reported in previous high-throughput screens (Thorne et al., 2010). A recent screen of the NIH Molecular Libraries Small Molecule Repository identified 12% of the 360,864 molecules to be inhibitors of firefly luciferase (Thorne et al., 2012). In some cases inhibition paradoxically resulted in an increase of the luminescence signal, probably because of enzyme stabilisation (Sotoca et al., 2010). Such a mode of action is also supported by the PubChem Bioassay Database (http://pubchem.ncbi.nlm.nih.gov) which quotes a preliminary EC_{50} of 8.9 μM TCC for the inhibition of luciferase. Thermal shift assays indeed confirmed a strong stabilising interaction of TCC with luciferase at ligand concentrations above 5 μM. The effective concentration for TCC is likely to be even lower in cellular assays as these have more physiological buffer conditions.

In absence of a direct receptor interaction the androgenic and estrogenic effects seen with TCC *in vivo* are thus likely to be the result of a mechanism different from classical AR- or ER-signalling (Chen et al., 2008; Duleba et al., 2011; Chung et al., 2011). A prime target for endocrine crosstalk is the AhR, which is known to influence the cell's response to estrogens as well as androgens (Morrow et al., 2004; Wormke et al., 2003; Ohtake et al., 2007). Our results indeed show an interference of TCC with the AhR regulon. In presence of the model substrate TCDD it acts as an antagonist for the AhR, effectively inhibiting TCDD-triggered induction of CYP1A1. In addition, exposure to TCC was sufficient to increase transcription of *CYP1A1*, while co-exposure together with estrogens led to strong induction of *CYP1A1* and *CYP1B1*. As classical phase I enzymes CYP1B1 and CYP1A1 are regulated by AhR, the latter exclusively so (Nebert et al., 2004). Monooxygenase CYP1B1 on the other hand is known to be also co-regulated by estrogens (Tsuchiya et al., 2004). However, the fact that TCC failed to show estrogenic effects but clearly acted co-stimulatory on CYP1B1 expression points to an AhR-mediated response. The observation of TCC as a moderate agonist of the AhR is further supported by Yueh et al. who report induction of *CYP1B1* at near cytotoxic concentrations (5–25 μM TCC) (Yueh et al., 2012; Ahn et al., 2008). At these high concentrations *CYP1B1* gene induction did not require co-stimulation with estrogens. The effect depended nevertheless on the presence of functional ERα, which is consistent with the results of the ERα knockdown in this study. It thus seems, that while the induction of the respective luciferase reporter is an unspecific false positive effect caused by luciferase stabilisation, TCC has the potential to interfere with the regulatory crosstalk of the estrogen receptor and the AhR regulon.

Reporter gene assays are a simple and fast tool to screen for hormonal activity. However, they should be used with their limitations in mind and results should be verified with independent assays in order to reduce false positives and false negatives alike (Bovee and Pikkemaat, 2009). For substances that can directly interact with luciferase, such as TCC, the respective reporter assays are an unsuitable tool to investigate any potential endocrine properties. As shown in this study TCC has the potential to lower the transcriptional threshold of classical AhR target genes such as *CYP1A1* and *CYP1B1*. Endocrine effects observed *in vivo* might thus not be directly mediated by interaction with the AR or ER but result from an interference with the AhR regulon. Hence future molecular hazard assessments should focus on the possible co-exposure to TCC and xenoestrogens.

Conflict of interest

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2013.03.003.

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