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ORIGINAL ARTICLE

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Impact of steroid hormone levels on estradiol-mediated regulation of cytochrome P450 2B6 compared to 1B1 in breast cancer cells

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Abstract

Pharmacogenetic variants of the steroid hormone-metabolizing enzyme cytochrome P450 2B6 (CYP2B6) were reported to be associated with breast cancer (BC) risk and prognosis. *CYP2B6* expression is inducible by estradiol (E2) but induction was demonstrated only under steroid hormone-deprived medium conditions. Physiological conditions, however, even under endocrinological BC treatment, do not correspond to complete steroid hormone depletion. The aim of this study was to investigate the E2-mediated *CYP2B6* and *CYP1B1* regulation under various steroid hormone conditions, including physiological concentrations, in human oestrogen receptor positive (T47D, MCF-7) and negative (MDA-MB-231) BC cell lines. We confirm that steroid-deprived pre-cultivation led to *CYP2B6* upregulation in T47D, but not in MCF-7. However, when pre-cultivated with steroid-containing medium *CYP2B6* was downregulated in T47D and MCF-7, while the addition of physiological E2 concentrations to steroid-deprived medium resulted in a downregulation in T47D. In contrast, *CYP1B1* was never downregulated in any culture condition. Thus, we show that E2-mediated *CYP2B6* regulation in BC cells depends on steroid hormone exposure in a cell line-specific manner. Our data indicates the importance of being careful with conclusions drawn from *CYP2B6* induction findings in vitro, as we demonstrate potential influences of hormonal changes on *CYP2B6* expression, which could impact steroid hormone homeostasis and, consequently, BC risk.

KEYWORDS
breast cancer, CYP2B6, estradiol, regulation, steroid hormones

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1 | INTRODUCTION AND BACKGROUND

Cytochrome P450 2B6 (CYP2B6) is a human drug metabolizing enzyme that accounts for about 2%–5% of total hepatic CYP content[1] and the metabolism of ~10% of clinically applied drugs.[2] Its role in drug metabolism is increasingly relevant, and to date, a wide range of substrates, including the central nervous system acting bupropion, ketamine and propofol, the antiretroviral efavirenz and the antineoplastic cyclophosphamide, have been identified.[2] Beyond its primary hepatic site of action CYP2B6 is also expressed in extrahepatic tissues,[3] where it is involved in drug and steroid hormone metabolism.[2,4,5] Appropriately, oestrogen receptor α (ER-α, gene: *ESR1*) positive breast tumours exhibit higher *CYP2B6* expression levels compared to ER-α negative tumours,[6] and *CYP2B6* has been shown to be inducible by estradiol (E2) via ER-α in breast cancer (BC) cell lines.[7] In vivo studies have demonstrated that pharmacogenetic variants, resulting in lower *CYP2B6* expression and activity, are associated with increased BC risk[8] and poor prognosis in premenopausal BC patients.[9] Since CYP2B6 is involved in the metabolism of E2 to 2-hydroxy-E2,[5] its upregulation may increase the 2-hydroxylation of E2, which is associated with lower BC risk.[10] Consequently, the generation of 4-hydroxy-E2, which readily leads to DNA-toxic quinone metabolite formation and seems to be involved in carcinogenesis,[11,12] may decrease. So far, cell culture approaches to investigate the *CYP2B6* regulation by E2 in BC cells were carried out using E2 deficient medium.[7] However, under physiological conditions, even in menopausal and post-menopausal women, steroid hormones and oestrogenic activity will not ever be fully depleted in plasma or breast tissue.[13,14] Even under circumstances like endocrine therapy in BC, low E2-activity still remains, with certain variability (such as incomplete tamoxifen drug action in conditions of CYP2D6 poor metabolism),[15,16] leading plasma conditions to be more comparable to standard cell culture medium than to the deprived amounts used in previous *CYP2B6* regulation experiments.

The aim of this study was to examine if low steroid hormone levels, as they occur in physiological conditions in vivo or in cell culture medium in vitro, affect the observed E2-mediated induction of *CYP2B6*, and to examine differences between ER-α positive and negative BC cell lines. Additionally, the *CYP2B6* regulation shall be compared in its interplay with CYP1B1, a main E2-metabolizer forming the carcinogenic 4-hydroxy-E2,[17] which was also shown to be inducible via ER-α.[18] Since BC occurs under variable steroid hormone levels and low CYP2B6 activity is associated with BC, understanding the regulation of its expression under different steroid hormone conditions may help to shed further light on the connection between CYP2B6 activity and BC.

Plain English Summary

Pharmacogenetic variants of CYP2B6 are linked to breast cancer risk and prognosis. *CYP2B6* is inducible by oestrogen which was shown under hormone-deprived conditions, not under physiological conditions. This study investigated oestrogen-mediated *CYP2B6* regulation in oestrogen receptor-positive (T47D, MCF-7) and negative (MDA-MB-231) breast cancer cell lines under various hormone conditions. With hormone-deprived pre-cultivation oestrogen upregulated *CYP2B6* in T47D but not in MCF-7. Under hormone presence, *CYP2B6* was downregulated in both cell lines, while with physiological oestrogen concentrations in steroid-deprived medium *CYP2B6* was downregulated in T47D. Thus, oestrogen-mediated *CYP2B6* regulation depends on hormone exposure and cell line, possibly impacting breast cancer risk.

2 | MATERIALS AND METHODS

2.1 | Materials

Dimethyl sulfoxide (DMSO) was purchased from Carl Roth (Karlsruhe, Germany). 17β-E2, Dulbeccos's phosphate buffered saline (PBS), Roswell Park Memorial Institute (RPMI) medium with phenol red (order no.: R7388), Dulbeccos modified Eagle-medium (DMEM) (order no.: D6546), trypsin (order no.: T3924) and penicillin–streptomycin solution (P/S) (order no.: P4333) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). RPMI medium without phenol red (order no.: 11835030), charcoal-stripped fetal bovine serum (CS FBS) (order no.: A3382101), TRIzol™ reagent, High-Capacity cDNA Reverse Transcription Kit and PowerUP SYBR Green were purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA). Fetal bovine serum (FBS) (order no.: P40-37500) was purchased from PAN Biotech (Aidenbach, Germany). All primer assays for qPCR were purchased from QIAGEN (Venlo, Netherlands).

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2.2 | Cell culture and cell incubations

T47D, MCF-7 and MDA-MB-231 human BC cell lines were kindly provided by the molecular gynaecology group of the university hospital Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen, purchased from American Type Culture Collection (ATCC). T47D and MCF-7 were cultivated in RPMI medium, supplemented with 10% FBS and 1% P/S. MDA-MB-231 cells were cultivated in DMEM medium, also containing 10% FBS and 1% P/S. All cell lines were maintained at 37°C in a 5% CO_2 environment with a medium change every 2–3 days and subcultured at 80%–90% confluency. For all mRNA expression regulation experiments, all cell lines were plated in phenol red-free RPMI medium, supplemented with either 10% FBS or CS FBS and 1% P/S for 72 h before E2 exposure. To examine the effects of E2-supplementation in a medium containing CS FBS, cells were plated in phenol red-free RPMI medium with 10% CS FBS and 1% P/S supplemented with 0.5 nM E2 for 72 h before cell exposure. A concentration of 0.5 nM E2 was chosen since this is an average amount present in commercially available FBS[19] and represents a physiological concentration.[20] For mRNA expression investigations, 10 nM E2 was applied, as published in other *CYP2B6* and *CYP2B1* induction studies, where this concentration was described as high physiological.[7] Therefore, after cultivation for 72 h in varying medium conditions, T47D, MCF-7 and MDA-MB-231 cells were incubated for 24 h with either 10 nM E2 in DMSO or—as a control—0.1% DMSO only. The pH-indicator phenol red was omitted in all regulation experiments since it shows oestrogenic activity and thus could affect ER-mediated effects.

2.3 | RNA isolation, cDNA synthesis and quantitative real-time PCR (qPCR)

Twenty-four hours after E2 exposure whole RNA was isolated using TRIzol™ Reagent as described by the manufacturer. One microgram of the isolated RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol. The quantification of the cDNA was performed by qPCR using PowerUP SYBR Green and QuantiTect Primer Assays as described in the manufacturer's protocols. Primer assays for the genes *PGK1* (order no.: QT00013776), *RRN18S* (order no.: QT00199367), *CYP1B1* (order no.: QT00209496), *CYP2B6* (order no.: QT00000910), *ESR1* (ER-α) (order no.: QT00044492) and *ESR2* (ER-β) (order no.: QT00060641) were used. For normalization of the cycle threshold (Ct) values, the gene *PGK1* was used in the cell lines T47D and MCF-7, while 18s was utilized in the MDA-MB-231 cells, reflecting their respective suitability as stable reference genes.

2.4 | Statistical analysis

To compare the results of different cell stimulations, significance levels were assessed using the two-tailed non-parametric Mann–Whitney test for unpaired samples. The data are expressed as mean ± SD and were derived from three independent experiments with each two or three replicates. Significance levels were demonstrated by asterisks starting with $p \leq 0.05$ *, followed by ≤ 0.005 **, ≤ 0.001 *** and ≤ 0.0001 ****, respectively, indicating increasing levels of statistical significance. Statistical analyses and graphical representation were performed using GraphPad Prism® 10.

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[21]

3 | RESULTS

To investigate the impact of physiological steroid hormone levels on the induction of *CYP2B6* and *CYP1B1* by E2, we cultivated three BC cell lines for 72 h under three different steroid hormone level conditions. This included the application of CS FBS (steroid hormone deprived), standard FBS (steroid hormone presence) and CS FBS supplemented with 0.5 nM E2 (steroid hormone deprived with low E2 background level). The cells were incubated with 10 nM of E2 for 24 h, RNA was isolated, and qPCR was performed for *CYP2B6*, *CYP1B1*, *ESR1* (ER-α) and *ESR2* (ER-β). To point out the necessity of ER-α for the regulation, we compared the ER-α positive BC cell lines T47D and MCF-7 to the ER-α negative cell line MDA-MB-231. All cell lines showed consistent expression of the reference genes *PGK1* or *18s* after E2 exposure.

3.1 | Regulation of *CYP2B6* and *CYP1B1* by E2 under steroid hormone-starved cultivation

Based on the current research state, *CYP2B6* was only shown to be induced in T47D cells,[7] while *CYP1B1*

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was only induced in MCF-7 cells.[18] Since both cell lines are ER-α positive, we first checked if *CYP2B6* and *CYP1B1* expression is regulated by E2 in the same way, and if we can record the inducibility of both genes in both ER-α positive cell lines. E2 incubation under steroid hormone deprivation resulted in a significant increase of *CYP1B1* mRNA expression in both ER-α positive cell lines (Figures 1B and 2B) and *CYP2B6* mRNA expression in T47D cells (Figure 1A). In MCF-7 cells, *CYP2B6* mRNA expression was not increased (as had been reported before) but was significantly reduced by E2 (Figure 2A). While in T47D cells the mRNA expression of both *ESR1* and *ESR2* was increased (Figure 1C,D), in MCF7 cells *ESR1* mRNA expression was downregulated instead (Figure 2C). E2 incubation did not show a clear regulatory effect on *ESR2* in MCF-7 (Figure 2D). As expected, in the ER-α negative cell line MDA-MB-231, *ESR1* could not be detected and no significant regulation of *CYP1B1*, *CYP2B6* or *ESR2* after treatment with 10 nM E2 was found (Tables 1 and 2).

3.2 | Regulation of *CYP2B6* and *CYP1B1* by E2 under steroid hormone background

The regulation of *CYP2B6* and *CYP1B1* by E2 in BC cells has so far only been shown in steroid hormone-deprived conditions.[7,18] Since this starvation does not represent a physiological state, we wanted to investigate if there are changes in the regulation of these enzymes under physiological conditions. When using standard FBS in medium for 72 h before the 10 nM E2 exposure for 24 h, a significant downregulation of *CYP2B6* mRNA expression in both ER-α positive cell lines T47D and MCF-7 was observed (Figures 3A and 4A). In contrast, the *CYP1B1*

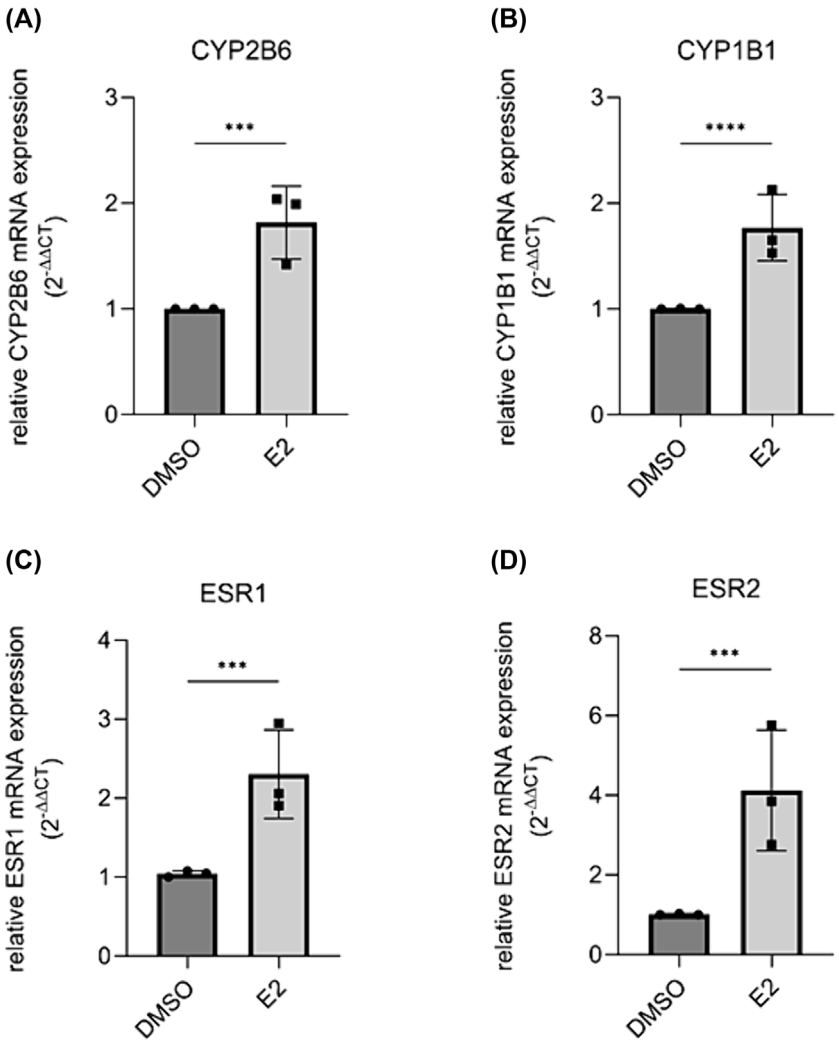


FIGURE 1 Quantitative real-time PCR (qPCR) analysis of cytochrome P450 2B6 (*CYP2B6*), *CYP1B1*, *ESR1* and *ESR2* mRNA expression in T47D cells. After 72 h incubation in Roswell Park Memorial Institute (RPMI) medium without phenol red, with charcoal-stripped fetal bovine serum (FBS), cells were incubated with 10 nM estradiol (E2) for 24 h. mRNA expression of (A) CYP2B6, (B) CYP1B1, (C) ESR1 and (D) ESR2 was analysed via qPCR. Data from three independent experiments with two or three replicates each are shown. Values were normalized to reference gene *PGK1* and compared to dimethyl sulfoxide (DMSO) control. Mean of $2^{-\Delta\Delta CT} \pm$ SD is shown. Statistical significance is based on unpaired Mann–Whitney test.

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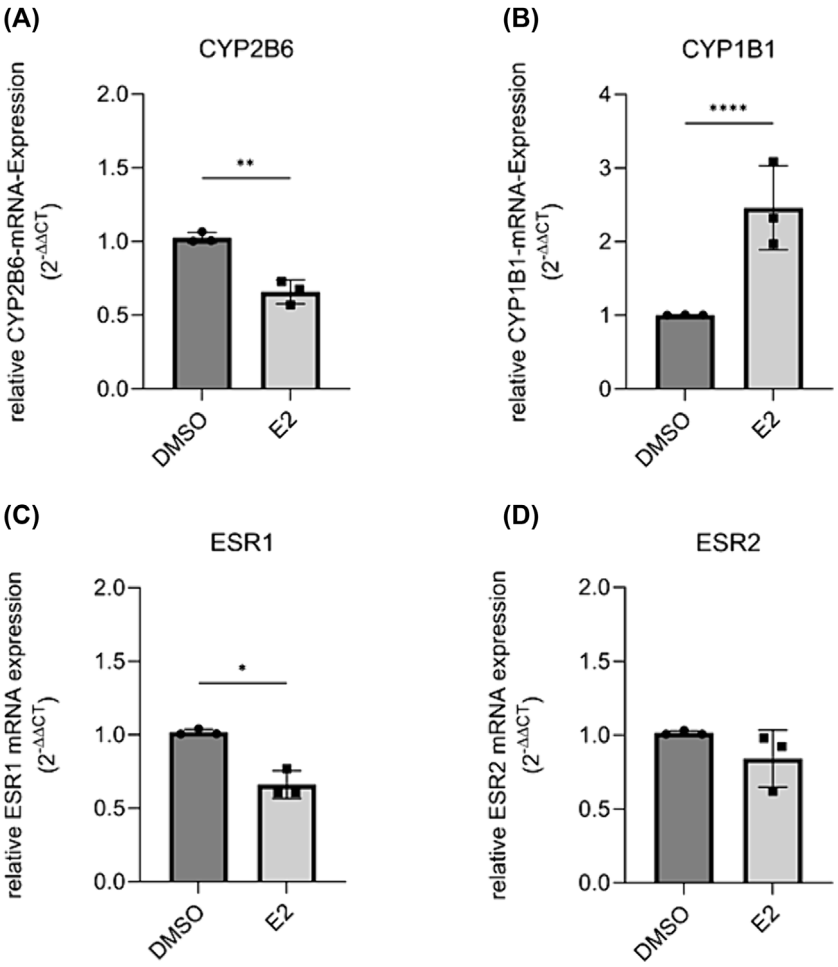


FIGURE 2 Quantitative real-time PCR (qPCR) analysis of cytochrome P450 2B6 (*CYP2B6*), *CYP1B1*, *ESR1* and *ESR2* mRNA expression in MCF-7 cells. After 72 h incubation in Roswell Park Memorial Institute (RPMI) medium without phenol red, with charcoal-stripped fetal bovine serum (FBS), cells were incubated with 10 nM estradiol (E2) for 24 h. mRNA expression of (A) CYP2B6, (B) CYP1B1, (C) ESR1 and (D) ESR2 was analysed via qPCR. Data from three independent experiments with two or three replicates each are shown. Values were normalized to reference gene *PGK1* and compared to dimethyl sulfoxide (DMSO) control. Mean of $2^{-\Delta\Delta CT} \pm$ SD is shown. Statistical significance is based on unpaired Mann–Whitney test.

mRNA induction was unaffected (Figure 3B) compared to the charcoal-stripped conditions (Figure 1B). In both cell lines, the mRNA regulation of *ESR1* correlated with that of *CYP2B6*, while *ESR2* mRNA was increased by E2 again in T47D (Figure 3D). The ER-α negative BC cell line MDA-MB-213 did not show any significant regulation of *CYP1B1*, *CYP2B6* or *ESR2* after E2 treatment (Tables 1 and 2).

3.3 | Regulation of *CYP2B6* and *CYP1B1* by E2 under low E2 level background

Our results so far have shown that the application of E2 to T47D and MCF-7 cells results in an increased expression of *CYP2B6* and *CYP1B1* under the usually applied conditions, where the cells are not exposed to any steroid hormones from standard FBS prior to E2 treatment. Application of the standard FBS including steroid hormones prior to E2 treatment however led to contradicting expression patterns of *CYP2B6* that were concomitant with ER-α expression. To dissect whether this is an effect of the conglomerate of steroid hormones or of the constant presence of E2 as a part of standard FBS, we added 0.5 nM E2, being an average concentration in commercially available FBS,[19] to the steroid-deprived medium and checked for *CYP2B6* downregulation after incubation with 10 nM E2. Indeed, we observed a downregulation of *CYP2B6* mRNA in T47D cells (Figure 3E). In contrast to the use of standard FBS, we additionally recorded a *CYP1B1* downregulation (Figure 3F). In MCF-7 cells, the pre-incubation with 0.5 nM E2 did not result in a significant regulation of neither *CYP2B6* nor *CYP1B1* (Figure 4E,F). The ER-α negative BC cell line MDA-MB-213 did not show any significant regulation of *CYP2B6* or *CYP1B1* after E2 incubation (Table 1). All relative expression values for *CYP2B6* and *CYP1B1* after 24 h of 10 nM E2 incubation under the different conditions are summarized in Table 1 and in Table 2 for both ERs.

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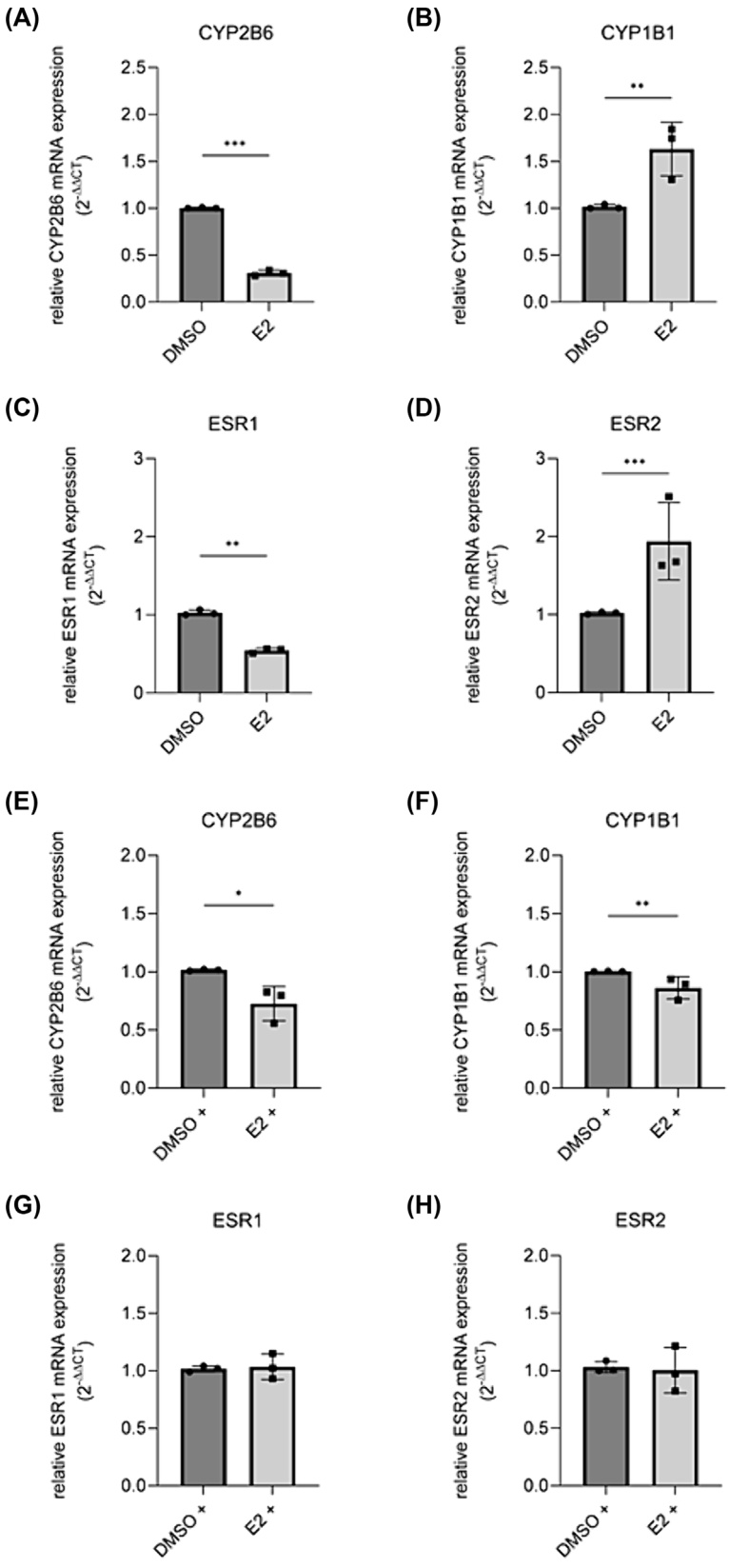


FIGURE 3 Quantitative real-time PCR (qPCR) analysis of cytochrome P450 2B6 (*CYP2B6*), *CYP1B1*, *ESR1* and *ESR2* mRNA expression in T47D cells. Cells were incubated in Roswell Park Memorial Institute (RPMI) medium without phenol red, with (A–D) standard fetal bovine serum (FBS) or (E–H) charcoal-stripped FBS with 0.5 nM estradiol (E2) (+) for 72 h. Afterwards, cells were incubated with 10 nM E2 for 24 h, and mRNA expression was determined for (A, E) CYP2B6, (B, F) CYP1B1, (C, G) ESR1 and (D, H) ESR2 via qPCR. Data from three different experiments with two or three replicates each are shown. Values were normalized to reference gene *PGK1* and compared to dimethyl sulfoxide (DMSO) control. Mean of $2^{-\Delta\Delta CT} \pm$ SD is shown. Statistical significance is based on unpaired Mann–Whitney test.

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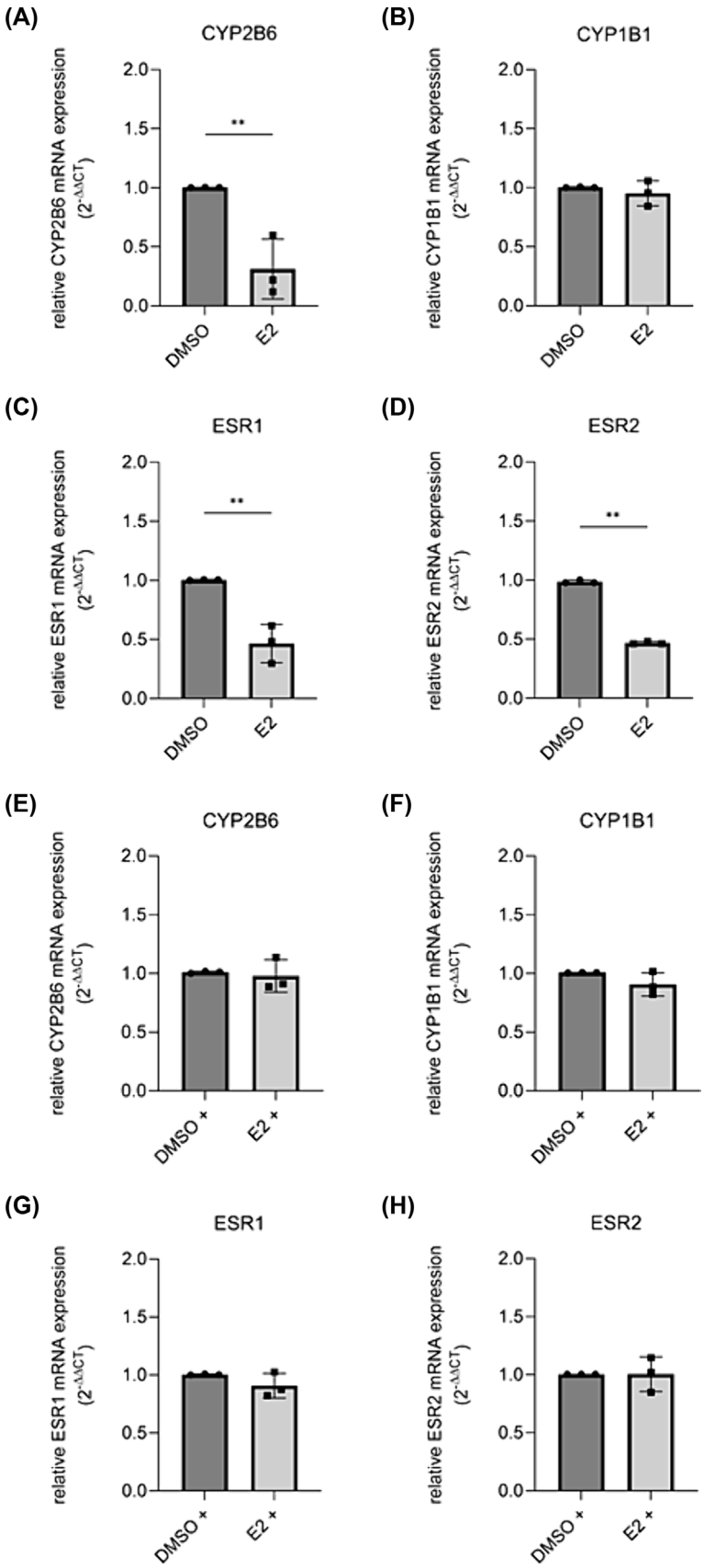


FIGURE 4 Quantitative real-time PCR (qPCR) analysis of cytochrome P450 2B6 (*CYP2B6*), *CYP1B1*, *ESR1* and *ESR2* mRNA expression in MCF-7 cells. Cells were incubated in Roswell Park Memorial Institute (RPMI) medium without phenol red, with (A–D) standard fetal bovine serum (FBS) or (E–H) charcoal-stripped FBS with 0.5 nM estradiol (E2) (+) for 72 h. Afterwards, cells were incubated with 10 nM E2 for 24 h, and mRNA expression was determined for (A, E) CYP2B6, (B, F) CYP1B1, (C, G) ESR1 and (D, H) ESR2 via qPCR. Data from three different experiments with two or three replicates each are shown. Values were normalized to reference gene *PGK1* and compared to dimethyl sulfoxide (DMSO) control. Mean of $2^{-\Delta\Delta CT} \pm$ SD is shown. Statistical significance is based on unpaired Mann–Whitney test.

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TABLE 1 Relative expression values for CYP2B6 and CYP1B1 compared to dimethyl sulfoxide (DMSO) control after 10 nM E2 incubation in different medium conditions in T47D, MCF-7 and MDA-MB-231 cells.

Cell line	Medium condition	*CYP2B6*	*p*-value	*CYP1B1*	*p*-value
T47D	CS FBS	1.85 (±0.39)	**0.0006**	1.73 (±0.27)	**0.0006**
	FBS	0.29 (±0.05)	**0.0079**	1.86 (±0.14)	**0.0079**
	E2 pre-incubation	0.68 (±0.18)	**0.0104**	0.83 (±0.12)	**0.0070**
MCF-7	CS FBS	0.64 (±0.18)	**0.0019**	2.35 (±0.14)	**<0.0001**
	FBS	0.23 (±0.20)	**<0.0001**	0.93 (±0.14)	0.2224
	E2 pre-incubation	0.95 (±0.18)	0.4894	0.87 (±0.09)	0.1135
MDA-MB-231	CS FBS	1.15 (±0.18)	0.2224	1.03 (±0.12)	0.8633
	FBS	0.98 (±0.24)	>0.9999	1.20 (±0.22)	0.1903
	E2 pre-incubation	1.01 (±0.37)	0.8785	0.96 (±0.43)	0.7984

Abbreviations: CS FBS, charcoal-stripped fetal bovine serum; CYP2B6, cytochrome P450 2B6; E2, estradiol; FBS, fetal bovine serum.

TABLE 2 Relative expression values for ESR1 and ESR2 compared to dimethyl sulfoxide (DMSO) control after 10 nM E2 incubation in different medium conditions in T47D, MCF-7 and MDA-MB-231 cells.

Cell line	Medium condition	*ESR1*	*p*-value	*ESR2*	*p*-value
T47D	CS FBS	2.26 (±0.55)	**0.0006**	3.57 (±1.41)	**0.0006**
	FBS	0.61 (±0.11)	**0.0079**	2.45 (±0.49)	**0.0079**
	E2 pre-incubation	1.00 (±0.14)	0.7984	0.93 (±0.14)	0.3823
MCF-7	CS FBS	0.72 (±0.20)	**0.0008**	0.92 (±0.20)	0.1615
	FBS	0.41 (±0.18)	**<0.0001**	0.54 (±0.14)	**<0.0001**
	E2 pre-incubation	0.90 (±0.16)	0.2224	0.92 (±0.19)	0.6048
MDA-MB-231	CS FBS	-	-	0.86 (±0.30)	0.1903
	FBS	-	-	1.03 (±0.24)	0.7962
	E2 pre-incubation	-	-	0.94 (±0.24)	0.6454

Abbreviations: CS FBS, charcoal-stripped fetal bovine serum; E2, estradiol; FBS, fetal bovine serum.

4 | DISCUSSION

We examined the impact of steroid hormone exposure on the E2-mediated regulation of *CYP2B6* mRNA and compared it to *CYP1B1* in different BC cell lines by mimicking physiological E2 conditions in the culture medium. In these cell lines, upregulation of *CYP2B6* by E2 has been shown under steroid hormone-deprived medium conditions, which is not similar to the physiological conditions in breast tissue. To understand how this regulation works under low levels of steroid hormones, we studied the effects of E2 on the expression of *CYP2B6*, *CYP1B1*, *ESR1* and *ESR2* in three BC cell lines under three different medium conditions.

First, we replicated the induction of *CYP2B6* mRNA expression in T47D cells by incubating for 24 h with 10 nM E2 under steroid hormone-deprived medium conditions, using CS FBS in the culture medium prior to E2 incubation. This was already shown in the same cell line T47D by Lo et al.[7] For CYP1B1, Tsuchiya et al.[18] have demonstrated enzyme induction in MCF-7 cells by E2. In both studies, the necessity of ER-α for the E2-mediated regulation of both CYPs had been demonstrated, which is underlined by our findings of the simultaneous induction of *CYP2B6* and *CYP1B1* probably via ER-α in T47D cells and no changes in expression of both CYPs in ER-α negative MDA-MB-231 cells after E2 incubation.

However, in MCF-7 cells, *ESR1* mRNA was downregulated by E2 incubation, under steroid hormone-deprived culture conditions, which was already shown by others[22] and may explain the *CYP2B6* downregulation. In contrast, *CYP1B1* induction was not affected here and generally did not clearly correlate with *ERS1*

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regulation, in any of the tested conditions. This difference in regulation between *CYP2B6* and *CYP1B1* may be explained by the role of the aryl hydrocarbon receptor (AhR) in *CYP1B1* regulation and its interplay with ER-α.[23,24]

The observed cell line-specific differences between T47D and MCF-7 were also noticed by Lo et al., who identified ER-α bound regions in T47D and compared them to known data on MCF-7 cells.[7] Despite both cell lines representing the same subtype, with over 50% sequence identity only 36% of ER-α binding sites overlapped, demonstrating clear differences in ER-α action of these two BC cell lines.[7]

Since there are no studies considering the E2-mediated regulation of these extrahepatically active E2 metabolizing CYP enzymes under constant exposure to steroid hormones during culture conditions, we investigated this by culturing the cells using medium with standard FBS prior to E2 exposure. We saw that *CYP1B1* induction remained in T47D cells but not in MCF-7, which may also be explainable by cell line-specific differences in ER-α signalling. In contrast, both ER-α positive cell lines T47D and MCF-7 showed a downregulation of *CYP2B6* as well as *ESR1* mRNA expression after E2 incubation, indicating a decrease of CYP2B6 enzyme by E2, possibly via ER-α under physiological conditions. A possible cause for this downregulation by E2 may be an altered activation programme of respective steroid hormone receptors through the additional steroid hormones. In that scope, the progesterone receptor (*PR*), an E2-regulated gene[25] can associate with ER-α in BC cells leading to a unique gene expression pattern, causing opposite effects compared to sole ER-action, for example, anti-proliferative effects.[26] Further, the steroid hormone testosterone also seems to be counteracting E2 effects by inhibiting its proliferative character in breast tissue[27] and repressing E2 signalling.[28]

We, therefore, conclude that the observed contrary regulation of *CYP2B6* expression could be caused by an interplay of ER-α with various steroid hormones such as progesterone or testosterone that are found in conventional FBS for cell culture.

A further confounder in standard FBS that could interact with ER-α mediated gene regulation is the constant presence of E2 specifically.

To assess this possibility, we cultivated the cells with CS FBS supplemented with an average FBS contained concentration of 0.5 nM E2,[19] before exposure to 10 nM E2 for 24 h. In T47D cells, the pre-incubation with E2-supplementation resulted in a decrease of *CYP2B6* and *CYP1B1* mRNA, demonstrating a clear impact of background E2-levels on the E2-mediated regulation of both enzymes.

E2 induces the expression of *ESR2*, probably via ER-α (Figure 3D). ER-β was shown to negatively impact ER-α expression and downregulate mRNA and protein levels of ER-α inducible genes.[29,30] Although both ERs share many target genes, ER-β actions highly depend on the presence of ER-α and their interplay.[31] ER-α/β heterodimers show lower regulatory activity than ER-α/α homodimers,[32] while in the absence of ER-α, ER-β can partially compensate the role of ER-α in transcriptional regulation.[30] Thus, despite Lo et al. showing an induction of *CYP2B6* by overexpression of ER-α or ER-β,[7] the downregulation of *CYP2B6* and *CYP1B1* we demonstrated by E2 under these conditions may be influenced by an involvement of ER-β-mediated counter-regulation under non-artificial conditions.

In MCF-7 cells no significant regulation was observed by E2 after E2 pre-incubation. Since MCF-7 cells express higher levels of ER-α compared to T47D, while both express similar low levels of ER-β,[33,34] this opposite result between both cell lines may be due to the higher ratio of ER-α over β in MCF-7, causing less impact of ER-β in terms of ER-α-signalling.

In contrast to Lo et al.,[7] we could not detect native CYP2B6 or CYP1B1 in the used BC cell lines by western blot. To strengthen our findings on the regulation of CYP2B6 which are limited by studies on mRNA level, further investigations should contain protein expression measurements. Therefore, quantification of CYP enzyme function and activity by drug metabolism measurements using CYP-specific probe drugs would be the most meaningful method, being considered as an aim for future studies.

CYP2B6 variants with reduced activity or expression have been mentioned to correlate with higher risk or poor prognosis in terms of BC.[8,9] We showed that *CYP2B6* mRNA was downregulated after a sudden and strong increase in E2 levels under the impact of a low-level E2 background. These conditions may represent the hormonal make-up during postmenopausal hormone replacement therapy (PHRT), which is also associated with increased BC risk.[35] Further, our results underline the previously shown strong reduction of CYP2B6 activity under PHRT.[36]

Higher or lower CYP2B6 activity may lead to respective changes in exposure concentrations to either 2-hydroxy-E2 or 4-OH-E2, of which the latter is cancerogenic and may be formed in higher amounts in the absence of CYP2B6 activity. Thus, the differential regulation of *CYP2B6* expression by low *versus* high E2 concentrations may have an impact on local metabolism and degradation of steroid hormone metabolites. Also, endocrine BC therapy inefficiency or resistance could result in an increase of systemic E2 levels that might influence *CYP2B6* regulation in breast (cancer) tissue and therefore

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BC prognosis. While CYP2B6's involvement in E2 metabolism is described,[5] its role in the metabolism of testosterone is more clear.[4,37] In the human body, testosterone can be converted to E2 by aromatases, resulting in higher E2 levels depending on testosterone levels. Consequently, reduced CYP2B6 levels might result in increased testosterone and thus further increased E2 concentrations. Since both steroid hormones are associated with increased BC risk,[38] this may be prognostically relevant.

In contrast to *CYP2B6*, upregulation of *CYP1B1* by E2 seems to be more independent of the availability of steroid hormones, since in T47D cells the E2-mediated induction is visible under both cultivation conditions, using CS or standard FBS. Only pre-incubation of low E2 levels alone reduced its expression in T47D. Since *CYP1B1* is also regulated via AhR[39] and *CYP2B6* is not, this difference may explain the discrepancy in E2-mediated regulation, as AhR was shown to interact with ER-α.[23,24] Because elevated CYP1B1 activity in breast tissue is associated with an increase of BC risk,[40] this less modifiable increase in expression dependent on steroid hormone presence or absence may be a relevant risk factor in different hormonal circumstances.

Taken together, our study demonstrates that *CYP2B6* is rather downregulated than induced by E2 in both ER-α positive cell lines T47D and MCF-7 under a low and physiological steroid hormone background. The previously reported induction of *CYP2B6*, which was shown under steroid hormone deprivation conditions, may probably not be accurate among different hormonal situations. When comparing E2 effects on *CYP2B6*, the induction seen in steroid hormone deprivation conditions, is completely inverted in T47D cells under these conditions. E2-mediated *CYP1B1* induction seems to be less affected by changes in steroid hormone levels.

Translating out in vitro findings to potential consequences in vivo, an E2 level increase from a low to a high physiological level, occurring with PHRT, has the potential to lead to a *CYP2B6* downregulation, possibly affecting steroid hormone metabolism, hormonal balance and therefore BC risk. Further, lower CYP2B6 expression might decrease CYP2B6-dependent testosterone metabolism in breast tissue and thereby increase local E2.

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CONFLICT OF INTEREST STATEMENT

None of the authors declared any conflict of interest with the work that has been published in this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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