Buprenorphine, Norbuprenorphine, R-Methadone and S-Methadone Upregulate BCRP/ABCG2 Expression by Activating Aryl Hydrocarbon Receptor in Human Placental Trophoblasts

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Abbreviations: BCRP, Breast cancer resistance protein; ABCG2, the second member of the ATP-binding cassette G family; AhR, aryl hydrocarbon receptor; BUP, buprenorphine; NBUP, norbuprenorphine; P-gp, P-glycoprotein; MET, methadone; R-MET, R-methadone; S-MET, S-methadone; HVT, primary human villous trophoblast; 3-MC, 3-methylcholanthrene; DMSO, dimethyl sulfoxide; HBSS, Hank’s Balanced Salt Solution; XRE, xenobiotic response element; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PXR, pregnane X receptor; CAR, constitutive androstane receptor.
ABSTRACT

Opioid dependence during pregnancy is a rising concern. Maintaining addicted pregnant women on long-acting opioid receptor agonist is the most common strategy to manage pregnancy drug abuse. Methadone (MET) and Buprenorphine (BUP) are widely prescribed for opiate-maintenance therapy. Norbuprenorphine (NBUP) is the primary active metabolite of BUP. These medications can cross the placenta to the fetus, leading to postpartum neonatal abstinence syndrome. Despite their use during pregnancy, little is known about the cellular changes brought about by these drugs in the placenta. In this study, we showed that BUP, NBUP and MET at clinically relevant plasma concentrations significantly induced BCRP mRNA up to 10-fold in human model placental JEG3 and BeWo cells and in primary human villous trophoblasts, and this induction was abrogated by CH223191, an AhR specific antagonist. These drugs increased AhR recruitment onto the AhR-response elements and significantly induced BCRP gene transcription. AhR overexpression further increased BCRP mRNA and protein expression. Knocking down of AhR by shRNA decreased BCRP expression and this decrease was reversed by rescuing AhR expression. Finally, induction of BCRP expression in JEG3 and BeWo cells was accompanied with an increase in its efflux activity. Collectively, we have demonstrated, for the first time, that BUP, NBUP and MET are potent AhR agonists and can induce BCRP in human placental trophoblasts by activating AhR. Given the critical role of BCRP in limiting fetal exposure to drugs and xenobiotics, long-term use of these medications may affect fetal drug exposure by altering BCRP expression in human placenta.
INTRODUCTION

Opioid dependence in pregnant women is on a rise in the US. Public health survey indicates a 15% increase in opiate use during pregnancy between 2009-2013 (Administration, 2014; Volkow, 2016). Opiate use during pregnancy has been associated with physical, mental, and psychological problems for pregnant women and their babies, including preterm delivery and changes in birth weight (Fajemirokun-Odudeyi et al., 2006; Kaltenbach et al., 1998). To lower health risks, several strategies are used to manage pregnancy drug abuse, and current consensus is to maintain opioid-addicted pregnant women on a long-acting opioid receptor agonist to avoid adverse health implications to the mother or fetus/baby (Bart, 2012).

Methadone (MET) maintenance therapy has been the standard of care for opioid addiction (Jones et al., 2005) and is a NIH and WHO accepted standard treatment for opioid-dependent pregnant women (Committee, 2009; National Consensus Development Panel on Effective Medical Treatment of Opiate, 1998). More recently, the FDA approved the use of buprenorphine (BUP) maintenance therapy (Rayburn and Bogenschutz, 2004). BUP is a strong analgesic at low doses and an antagonist at high concentrations (Cowan et al., 1977). It has limited abstinence syndrome and hence a great drug to treat opioid addiction. At present, both MET and BUP are widely used in opiate-maintenance therapy (Strain et al., 1996; Whelan and Remski, 2012). MET is administered as an oral tonic, containing a racemic mixture of R-methadone (R-MET) and S-methadone (S-MET) enantiomers. BUP is administered sublingually and is metabolized to the primary active metabolite norbuprenorphine (NBUP) (Cone et al., 1984).

Placenta, a transient organ, develops during pregnancy to ensure proper exchange of nutrients and waste between the mother and fetus. The placental barrier consists of primarily a
layer of syncytiotrophoblasts that separates the fetal compartment and maternal blood (Cross et al., 1994). Transporter expressed on the apical and basal membranes of the syncytiotrophoblasts play crucial roles in mediating transfer of drugs and xenobiotics across the placental barrier from maternal circulation (Ni and Mao, 2011; Vahakangas and Myllynen, 2009).

Breast cancer resistance protein (BCRP, gene symbol ABCG2) is a major ABC efflux transporter initially discovered in breast cancer cell lines where it confers multidrug resistance (Doyle et al., 1998; Miyake et al., 1999). In normal human tissues, BCRP is highly expressed in the placenta, liver and small intestine (Maliepaard et al., 2001). In the placenta, BCRP is located on the apical membrane of syncytiotrophoblasts that faces the maternal blood (Maliepaard et al., 2001), implicating its role in limiting fetal exposure to drugs and xenobiotics by pumping them from the fetal compartment back to the maternal circulation. In vivo studies using Bcrp1 knockout mice have indeed shown that Bcrp1, the murine homolog of human BCRP, significantly limits fetal exposure to certain BCRP substrates including glyburide, nitrofurantoin and topotecan (Jonker et al., 2000; Zhang et al., 2007; Zhou et al., 2008). Results from placenta perfusion studies support the same conclusion (Kraemer et al., 2006; Staud et al., 2006).

Aryl hydrocarbon receptor (AhR) is a ubiquitously expressed, ligand-activated nuclear receptor which is highly expressed in many adult tissues including human placenta (Dolwick et al., 1993; Manchester et al., 1987). AhR was detectable at high levels in the syncytiotrophoblasts (Jiang et al., 2010). AhR acts as a receptor for a plethora of hydrophobic aromatic compounds including xenobiotics generated from smoking and drugs and can regulate expression of many drug metabolizing enzymes and transporters such as CYP1A1 and BCRP in carcinoma cell lines (Stejskalova et al., 2011a; Tan et al., 2010). While it has been established that AhR is involved in
regulation of BCRP in several cell types, whether BCRP in human placenta can be induced via AhR has not been reported. Among xenobiotic nuclear receptors such as PXR, CAR and AhR, AhR is the only one that is known to be expressed in human placenta (Pavek and Smutny, 2014). AhR can be activated by a range of structurally diverse chemicals/ligands. This activation is associated with nuclear translocation and direct interaction of AhR with xenobiotic response elements (XREs) in the promoter region of target genes to modulate respective gene expression (Denison and Nagy, 2003).

Therefore, in the present study, we primarily examined whether AhR in human placental cells can be activated by drugs used to treat drug abuse during pregnancy (BUP, NBUP, R-MET and S-MET) and whether these drugs can induce BCRP expression in human placenta by activating the AhR signaling cascade in human placental cells. Data obtained will have important clinical implications related to the use of tobacco/opiate-maintenance medications during pregnancy.

MATERIALS AND METHODS

Materials and Cell lines: Buprenorphine HCl (RTI Log No: 8982-0899-22, Ref No: SAF 021637), Norbuprenorphine (RTI Log No: 3858-20, Ref No: SAF 021637), R-(-)-Methadone HCl (RTI Log No: 12295-72C, Ref No: SAF 021637) and S-(+)-Methadone HCl (RTI Log No: 12793-31C, Ref No: 021637) were obtained from the central drug repository at NIDA/NIH (Rockville, MD). 3-Methylcholanthrene (3-MC), an established AhR ligand (Abdelrahim et al., 2006) and Ko143, a potent BCRP inhibitor, were from Sigma (St. Louis, MO). CH223191 was purchased from Calbiochem (Billerica, MA). Stocks of these compounds were prepared in
DMSO, aliquoted and stored at -20°C until use; fresh dilutions in appropriate culture medium were prepared and used for every experiment. The final concentration of DMSO was less than 0.1% (v/v) across all treatments. JEG3 (ATCC HTB-36™) and BeWo (ATCC CCL-98) were purchased from ATCC (Manassas, VA). All antibodies used for immunoblotting were from Santa Cruz Biotechnology (Santa Cruz, CA). Human villous trophoblasts (HVTs) (Catalog No: 7120) and Trophoblast Media (Catalog No: 7121) were purchased from ScienCell Research Laboratories (Carlsbad, CA). HVTs were isolated from human placental villi, cryopreserved at passage one and delivered frozen.

**Cell Culture**: JEG3 cells were cultured and maintained in DMEM and BeWo cells were cultured and maintained in F-12K (Invitrogen, Waltham, MA) media, supplemented with 10% FBS (Sigma) and 1 × Anti-Anti (Invitrogen). To eliminate the influence of serum factors and the mild estrogenic effect of phenol red (Berthois et al., 1986), cells were serum starved in phenol red free DMEM or F-12K for 24 h prior to treatment with respective drugs. After 24 h of drug treatment, the cells were harvested and RNA extraction or whole cell lysates were prepared as described below. Human villous trophoblast cells were maintained in Trophoblast Media according to manufacturer’s instruction. The cells were allowed to grow and differentiate for 3 days prior to serum starvation for 24 h followed by drug treatment in serum-devoid Trophoblast Media. After drug treatment for 24 h, HVTs were harvested for RNA extraction or whole cell lysate preparation.

**Total RNA Isolation and Real-time RT-PCR Analysis**: The effects of drug treatment on BCRP mRNA expression were quantified by RT-PCR as follows. Briefly, total RNA was extracted from cells using Trizol reagent (Thermo Fisher Scientific, Waltham, MA) following
manufacturer’s instruction. To eliminate contamination of genomic DNA, all RNA samples were treated with DNase I (Thermo Fisher Scientific) followed by phenol-chloroform precipitation. Complementary DNA was prepared using 2 μg of purified total RNA and SuperScript III reverse transcription kit (Invitrogen) following manufacturers instruction. Real-time PCR reactions were performed using 3 μl of 1:10 diluted single-strand cDNA with specific primers and the SYBR Green 2 × PCR Master Mix (Applied Biosystems, Waltham, MA) on the CFX RT-PCR system (Bio-Rad, Hercules, CA) by initial denaturation at 95°C for 10 min, followed by cycling conditions 95°C for 30 s, 55°C for 30 s and 72°C for 1 min for 30-50 cycles depending on the gene, and finally ending with a hold at 4°C. The specific primer pairs used for human genes were as follows. BCRP/ABCG2: 5’-GCAACATGTACTGGCGAAGA-3’ and 5’-CAGGTAGGCAATTGTGAGGAA-3’; AhR: 5’-CAACCCTTTTCTCTGCCATAA-3’ and 5’-GCCAGGAGGGAACTAGGATT-3’; CYP1A1: 5’-GGACATGACCCCCATCTAT-3’ and 5’-CAGGGCTCTCAAGCACCCTA-3’, 18s ribosomal RNA: 5’-GTGGAGCGATTTGTCTGGTT-3’ and 5’-GAACGCCACTTGTCCCTCT-3’. All primers were obtained from IDT Technologies (Iowa, USA). Quantification of relative mRNA levels was carried out by determining the threshold cycle (C_T) as previously described (Wang et al., 2006). 18s ribosomal RNA (18s rRNA) was used as an internal control. The mRNA levels of target gene were normalized to β-actin as follows: C_T (target gene) – C_T (18s rRNA) = ΔC_T. Then, the relative mRNA levels of target gene after drug treatment were calculated using the ΔΔC_T method: ΔΔC_T (drug treatment) = ΔC_T (drug treatment) – ΔC_T (vehicle). The fold-changes in mRNA levels of target gene upon drug treatment were expressed as 2^-ΔΔC_T. The final concentration of DMSO (vehicle) in all treatments was <0.1% (v/v).
**SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting:** Whole cell lysates were prepared using RIPA buffer (Thermo Fisher Scientific) supplemented with DNase (2 µg/ml) according to manufacturer’s instruction. Following BCA protein quantification (Thermo Fisher Scientific), the protein samples of whole cell lysate (20-30 µg of protein each lane) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting as previously described (Wang et al., 2008; Wang et al., 2006). The following primary antibodies were used: BXP-21 (sc-58222) against BCRP, ACTB (I-19) (sc-1616) against human β-actin, Ah Receptor (B-11) (sc-74571) against AhR, and GAPDH (6C5) (sc-32233) against human glyceraldehyde 3-phosphate dehydrogenase. Human β-actin or GAPDH was detected as an internal control. Relative BCRP protein levels were determined by densitometric analysis of the immunoblots using the ImageJ software (NIH, Bethesda, MD) and normalized to that of β-actin.

**Stable Overexpression of AhR in JEG3 Cells:** The pcDNA3/AhR expression vector (Fukunaga and Hankinson, 1996) was stably transfected into JEG3 cells to overexpress human AhR as indicated in respective experiments. The corresponding empty vector was used to establish the empty vector control cells with no AhR overexpression.

**Knocking Down and Rescuing AhR:** To knock down AhR in JEG3 cells, a lentiviral shRNA plasmid (pLKO.1) targeting human AhR mRNA and the empty vector purchased from GE Healthcare Dharmacron (Lafayette, CO) were used to prepare lentivirus particles. Lentivirus particles containing sh-AhR or scramble control were produced by co-transfecting HEK293T (Invitrogen) with pMD2Gand psPAX2 virus packaging protein vectors along with the respective pLKO.1 plasmids (scramble or sh-AhR) as previously described (Dever and Opanashuk, 2012) using Lipofectamin 3000 (Invitrogen). Viral particle-containing medium was collected at 42, 72...
and 96 h after transfection, centrifuged twice at 4,000 rpm for 10 min at 4°C and filtered through 0.45 µm low protein binding syringe filter (GE Healthcare, Chicago, IL), aliquoted and stored at -80°C until use. To knock down AhR, JEG3 cells were infected by adding viral particle-containing medium to culture media containing Polybrene (Santa Cruz Biotechnology) at a concentration of 8 µg/ml. Cells with AhR knocked down were obtained by selection in Puromycine for 48 h after infection. Rescue of AhR expression in JEG3 cells with AhR knocked down was performed by transiently transfecting the cells with the pcDNA3/AhR expression vector.

**Luciferase Reporter Plasmids:** The X4-4.2 plasmid containing four consensus AhR binding-xenobiotic response elements (XREs) cloned upstream of the luciferase cassette in the pGL4.2 plasmid was previously used to demonstrate AhR induction by coffee (Ishikawa et al., 2014). The BCRP promoter luciferase plasmids containing the human BCRP promoter regions spanning -1285 to +362 with at least two putative AhR responsive elements (at positions -194/-190 and -59/-55) and spanning -115 to +362 with one putative AhR responsive element (at position -59/-55) cloned into the pGL3 luciferase plasmid was a kind gift of Dr. Douglas Ross (Bailey-Dell et al., 2001). These BCRP promoter luciferase plasmids were previously used to demonstrate induction of luciferase activity by prototype AhR ligands including 3-MC (Tan et al., 2010; Tompkins et al., 2010a).

**Luciferase Reporter Assay:** JEG3 cells stably overexpressing AhR (AHR.OE) or the empty vector (EMT.V) cells were plated in a 24 well plate at a seeding density of 2 × 10⁴ cells/well and grown to confluency, following which they were transiently co-transfected with either X4-4.2 or pGL3-BCRP and pGL3-renilla-luciferase plasmids (Addgene) at a ratio of 100:1, using
Lipofectamine 3000 (Invitrogen) following manufacturers instruction. The cells were cultured in serum containing media for 24 h post transfection. Cells were then serum starved for 24 h and treated with respective compounds at concentrations indicated for 24 h. Following the treatment, the cells were lysed using 1 × PLB (Promega) according to manufacturer’s instructions. Relative luciferase activities were quantified using the Dual-Luciferase assay system (E2920, Promega) and normalized to respective renilla luciferase activities according to manufacturer’s instructions.

**BCRP Activity Assay**: Efflux activities of BCRP before and after drug treatment were measured by quantifying intracellular accumulation of Hoechst33342 as previously described for Jar and BeWo cells (Mason et al., 2014; Xiao et al., 2015) with slight modifications. Briefly, a serum starved confluent monolayer of JEG3 or BeWo cells grown in 96 well plates was treated with a drug or 3-MC at concentrations indicated for 24 h. Cells were then pre-incubated for 1 h with or without 5 µM of Ko143. After the pre-incubation, Hoechst33342 (5 µg/ml) dissolved in fresh media with or without Ko143 (5 µM) was added and incubation was continued for additional 1 h. The reaction was then stopped by adding ice cold Hank’s Balanced Salt Solution (HBSS) buffer and immediately incubating on ice for 5 min, and washed 2 times with ice cold HBSS. The cells were lysed in the lysis buffer (1 mM Tris-HCl, pH 7.4, with 1% Triton X-100). Fluorescence in whole cell lysates was measured with excitation wavelength 355 nm and emission wavelength 460 nm on a BioTek3 microplate reader. The fluorescence was normalized to the amount of protein in cell lysates and used as a measure of intracellular accumulation of Hoechst33342. Induction of BCRP protein expression is expected to decrease intracellular accumulation of Hoechst33342. Inhibition of BCRP by Ko143 is expected to increase intracellular accumulation
of Hoechst33342. The experiments were performed at 37°C in a humidified incubator and plate reader.

**Statistical Analysis:** All data were expressed as means ± SD of at least three independent experiments. Statistical analysis was conducted using two-way ANOVA analysis followed by the Bonferroni correction for multiple comparisons or 2-tailed Student’s *t*-test for paired comparisons. All statistical analysis was performed using the GraphPad Prism 7 software (La Jolla, CA). Differences with *p* value of < 0.05 were considered to be statistically significant.

**RESULTS**

**BUP, NBUP, R-MET and S-MET Induce BCRP mRNA in Trophoblasts.** To determine if the drugs that are used to treat drug abuse during pregnancy can regulate BCRP expression in trophoblasts, we first treated JEG3 cells with BUP, NBUP, R-MET and S-MET at clinically relevant plasma concentration ranges observed in pregnant women (Supplementary Table 1) representing approximately the unbound maximum (*C*\textsubscript{max,u}) plasma concentrations and the *C*\textsubscript{max} plasma concentrations (0.001 µM and 0.01 µM for BUP and NBUP as well as 0.5 µM and 1.5 µM for R-MET and S-MET, respectively). At these concentrations, BCRP mRNA in JEG3 cells was significantly induced approximately 2 – 7-fold by these drugs as compared to the vehicle controls after treatment for 24 h (Fig. 1A and 1B). The fold-induction varied depending on drug used. BUP-mediated induction of BCRP mRNA at 0.01 µM appeared to be smaller than that at 0.001 µM, but the difference was not statistically significant (Fig. 1A). All the drug treatments did not affect cell viability at concentrations used after 24 h treatment (data not shown).
Treatment of JEG3 cells for 12 h did not induce BCRP mRNA (data not shown). Therefore, all subsequent experiments were performed with 24 h treatments at the representative $C_{\text{max}}$ plasma concentrations, as we observed robust induction of BCRP mRNA expression across all the treatment groups at these concentrations.

To evaluate if this induction was cell-type specific and could be replicated in primary human trophoblasts, we next treated BeWo cells and HVTs with the four drugs at $C_{\text{max}}$ concentrations for 24 h. Similar to JEG3 cells, we observed that BCRP mRNA was significantly induced 2 – 5-fold in BeWo cells (Fig. 2D) and 4 – 8-fold in HVTs (Fig. 2F) by these drugs. The results suggest that BUP, NBUP, R-MET and S-MET at clinically relevant concentrations can significantly upregulate BCRP mRNA expression in human placental trophoblasts.

Drug-Induced Increase in BCRP mRNA Expression Is Suppressed by an AhR Antagonist.

We next evaluated the potential involvement of xenobiotic nuclear receptors in mediating drug-induced BCRP gene expression. AhR is the only xenobiotic nuclear receptor highly expressed in human placental syncytiotrophoblasts (Pavek and Smutny, 2014). To evaluate the involvement of AhR in regulating BCRP expression by the drugs in JEG3, BeWo and HVTs, we pretreated the cells with 3 µM of CH223191 which is a potent and highly selective AhR inhibitor (Ishikawa et al., 2014; Zhao et al., 2010) for 12-16 h prior to co-treatment with respective drugs. As expected, all drugs at $C_{\text{max}}$ plasma concentrations significantly induced BCRP mRNA in JEG3 (Fig. 2B), BeWo (Fig. 2D) and HVTs (Fig. 2F) as compared to the vehicle controls. Pretreatment of cells with CH223191 nearly completely abrogated the drug-mediated induction of BCRP mRNA in all the three types of cells, suggesting that BCRP mRNA is likely induced by these drugs via activating AhR. Consistent with these findings, 3-MC which is a known AhR
ligand (Abdelrahim et al., 2006) also induced BCRP mRNA with similar fold-changes (Fig. 2B, 2D and 2F). Furthermore, the four drugs and 3-MC all significantly induced CYP1A1 mRNA and the induction was also completely inhibited by CH223191 (Fig. 2A, 2C and 2E). Since CYP1A1 is a known AhR target gene (Hu et al., 2007; Postlind et al., 1993), the results clearly suggest a role of AhR in the induction of BCRP gene expression by BUP, NBUP, R-MET and S-MET in human placental trophoblasts.

**BUP, NBUP, R-MET and S-MET are AhR ligands.** To further validate whether BUP, NBUP, R-MET and S-MET are AhR ligands, we generated JEG3 cells stably overexpressing AhR. Upon stable transfection of JEG3 cells with a human AhR expression vector, AhR mRNA was significantly increased 5-fold (Fig. 3A). AhR protein expression was also increased in the AhR overexpressing (AHR.OE) cells compared to the empty vector control (EMT.V) and parent (Cnt) cells (Fig. 3B). Fig. 3C shows that treating the EMT.V with 3-MC significantly increased CYP1A1 mRNA ~2-fold compared to the vehicle control possibly due to endogenous AhR. In AHR.OE cells treated with vehicle alone, the levels of CYP1A1 mRNA were ~2 times the levels of CYP1A1 mRNA in the EMT.V cells treated with vehicle alone. Treating the AHR.OE cells with 3-MC further increased CYP1A1 mRNA ~3-fold compared to the levels of CYP1A1 mRNA in the AHR.OE cells treated with vehicle alone (Fig. 3C), consistent with the higher level of AhR expression in the AHR.OE cells.

We next transfected these cells with the AhR luciferase reporter plasmid X4-4.2 that contains 4 consensus AhR XREs (Fig. 3D) and was previously used to demonstrate coffee-mediated AhR activation (Ishikawa et al., 2014). Treating cells with 3-MC clearly increased the luciferase activity (Fig 3E). Again, fold-induction associated with the AhR overexpressing
(AHR.OE) cells treated with 3-MC was much greater than that associated with the empty vector EMT.V cells treated with 3-MC (30-fold versus 3-fold) or with the AHR.OE cells treated with vehicle alone (30-fold versus 10-fold) (Fig. 3E). The results indicate that our model cell system is appropriate for testing AhR ligands. Hence, we treated these cells with respective drugs at $C_{\text{max}}$ plasma concentrations. BUP, NBUP, R-MET and S-MET all significantly increased the luciferase activity 12 – 30-fold in the AHR.OE cells compared to the EMT.V cells treated with vehicle alone or ~10 – 20-fold compared to the AHR.OE cells treated with vehicle alone (Fig. 3F). AhR overexpression alone with no 3-MC or drug treatment also significantly increased the luciferase activity, but at levels generally less than 10-fold (Fig. 3E and Fig. 3F). This is not unexpected as we (Fig. 3C) and earlier studies have shown that just overexpression of AhR can induce expression of its target genes including CYP1B1 compared to the empty vector controls (Wang et al., 2009), suggesting that when AhR is overexpressed, it can enter the nucleus to activate transcription of target gene without ligand-binding. However, in the presence of AhR ligands, expression of target genes is further increased as shown in Fig. 3C. Taken together, these results clearly indicate that BUP, NBUP, R-MET and S-MET are AhR ligands and can activate AhR at clinically relevant concentrations.

BUP, NBUP, R-MET and S-MET Induce the BCRP Promotor Activity via Activating AhR.

To further evaluate if the drugs induced BCRP via activating AhR, we performed luciferase reporter assay with the BCRP promoter luciferase plasmid containing the BCRP promoter region (-1285 to +362) in which there are at least three putative AhR XREs (Tan et al., 2010; Tompkins et al., 2010a) (Fig. 4A). To show whether there is a dose-dependence in activating the BCRP promoter by the drugs, we first treated parent JEG3 cells transfected with the BCRP promoter
luciferase plasmid with drugs at \( C_{\text{max}} \) and \( 10 \times C_{\text{max}} \) concentrations. As shown in Fig. 4C and Fig. 4D, the \( BCRP \) promoter luciferase activity was significantly increased ~2-fold at \( C_{\text{max}} \) concentrations of the four drugs and remained unchanged when drug concentrations were increased to \( 10 \times C_{\text{max}} \). Thus, AhR seemed to be already saturated at \( C_{\text{max}} \) concentrations. Given the nM or low \( \mu \)M concentrations used, these drugs, particularly BUP and NBUP, appear to be highly potent AhR ligands for BCRP induction. We next performed the luciferase reporter assay in AhR overexpressing (AHR.OE) JEG3 cells with 3-MC and the drugs. As expected, 3-MC at 1 \( \mu \)M significantly increased the \( BCRP \) promoter activity ~10-fold in the AHR.OE cells, but only induced the \( BCRP \) promoter activity ~3-fold in the empty vector EMT.V cells (Fig. 4E). On similar lines, treating these cells with drugs at \( C_{\text{max}} \) concentrations led to a significantly higher induction in the \( BCRP \) promoter activity in the AHR.OE cells than that in the EMT.V cells (8 – 10-fold versus ~3-fold) (Fig. 4F). Again, the \( BCRP \) promoter activity was also increased by vehicle alone in the AHR.OE cells, but at levels significantly lower than those induced by 3-MC (Fig. 4E) or the drugs (Fig. 4F). In the EMT.V cells, 3-MC and the drugs slightly increased the \( BCRP \) promoter activity, possibly due to endogenous AhR (Fig. 4E and Fig. 4F). These findings clearly support the conclusion that BUP, NBUP, R-MET and S-MET can induce BCRP gene expression via activating AhR. To further evaluate if the proximal AhR XREs in the \( BCRP \) promoter region are involved in upregulating BCRP gene expression, we transfected parent JEG3 and BeWo cells with either the long (-1285/+362) (Fig. 4A) or the short (-115/+362) (Fig. 4B) \( BCRP \) promoter luciferase plasmid. Treatments with all the drugs significantly induced the \( BCRP \) promoter activities 3 – 5-fold in both types of cells transfected with the long (-1285/+362) \( BCRP \) promoter construct compared to the vehicle controls (Fig. 4G and Fig. 4H). However, all
the treatments did not induce the BCRP promoter activities at all in cells transfected with the short (-115/+362) BCRP promoter construct (Fig. 4G and Fig. 4H). Previous studies have shown that there are one full consensus AhR XRE at position -194/-190 and two putative AhR XREs at positions -391/-387 and -59/-55 (Tan et al., 2010; Tompkins et al., 2010a). The results suggest that the proximal full consensus AhR XRE at position -194/-190 is possibly involved in the induction of the BCRP promoter activity.

**BUP, NBUP, R-MET and S-MET Induce BCRP mRNA and Protein Expression in AhR Overexpressing JEG3 Cells.** We next examined whether the drugs can induce BCRP mRNA and protein expression in AhR overexpressing JEG3 cells at levels higher than those in the empty vector cells. Indeed, drug treatment increased BCRP mRNA 8 – 15-fold in AhR overexpressing cells; however, the induction was generally below 5-fold in the empty vector cells (Fig. 5A). Likewise, 3-MC induced BCRP mRNA ~20-fold in AhR overexpressing cells but only 5 –fold in the empty vector cells (Fig. 5A). These results suggest that overexpression of AhR supports greater induction of BCRP. In parallel, we found that BCRP protein levels were also significantly increased 4 – 6-fold upon drug treatment in AhR overexpressing cells, but only 1.5 – 3-fold in the empty vector cells compared to vehicle controls (Fig. 5B and 5C). Induction of BCRP protein by the drugs in AhR overexpressing cells was significantly greater than that in the empty vector cells (Fig. 5C). In general, the pattern of induction of BCRP mRNA by the drugs is consistent with that of BCRP protein, supporting a transcriptional regulation mechanism of BCRP by the drugs via AhR. 3-MC induced BCRP protein ~3-fold in AhR overexpressing cells and ~2-fold in the empty vector cells compared to vehicle controls (Fig. 5C).
Knocking Down of AhR Diminishes BCRP Induction and Rescuing AhR Expression

**Restores BCRP Induction.** To further confirm that BUP, NBUP, R-MET and S-MET induce BCRP gene expression through activating AhR, we examined the effect of AhR knocking down on BCRP induction by 3-MC and the drugs. As we showed in Fig. 6A, infecting JEG3 cells with viral particles targeting AhR (sh.AHR) reduced AhR protein expression compared to the parent (Cnt) and scramble (sh.SCR) controls. Transfecting the pcDNA3/AhR expression vector (sh.AHR+AHR and sh.SCR+AHR) not only restored but further increased AhR protein expression compared to the controls. 3-MC at 1 μM significantly increased BCRP mRNA ~5-fold in JEG3 cells infected with the scramble viral particles (Fig. 6B, sh.SCR) and knocking down of AhR completely diminished BCRP induction (Fig. 6B, sh.AHR). Overexpression of AhR in the scramble control cells by transiently transfecting the AhR expression vector further increased BCRP mRNA (Fig. 6B, sh.SCR+AHR). Rescuing AhR expression in the AhR knocking down cells by transfecting the AhR expression vector partially re-stored BCRP mRNA expression (Fig. 6B, sh.AHR+AHR). Essentially the same results were obtained for BUP, NBUP, R-MET and S-MET (Fig. 6C and Fig. 6D). For examples, NBUP at 0.01 μM significantly increased BCRP mRNA ~4-fold in the scramble control cells (Fig. 6C, sh.SCR for NBUP) and this induction was completely diminished by knocking down of AhR (Fig. 6C, sh.AHR for NBUP). Overexpression of AhR increased BCRP mRNA in the scramble control cells (Fig. 6C, sh.SCR+AHR for NBUP). Rescuing AhR expression completely re-stored BCRP mRNA expression in the AhR knocking down cells (Fig. 6C, sh.AHR+AHR for NBUP). These results provide further evidence that BUP, NBUP, R-MET and S-MET induce BCRP gene expression by activating AhR.
**Drug Treatment Increases BCRP Efflux Activity.** Hoechst33342 is a model substrate of BCRP and has been widely used for functional evaluation of BCRP (Scharenberg et al., 2002). To test if induction of BCRP mRNA and protein expression is accompanied with increase in BCRP efflux activity, we measured intracellular accumulation of Hoechst33342 in JEG3 (Fig. 7A) and BeWo (Fig. 7B) cells upon drug treatment in the presence or absence of the highly selective BCRP inhibitor Ko143. We found that treatment of cells with either 3-MC at 1 μM or drugs at C_{max} concentrations in the absence of Ko143 led to a significant decrease in intracellular fluorescence after normalization to cellular protein content, indicating an increase in BCRP-mediated Hoechst33342 efflux which is likely due to induction of BCRP protein expression. This decrease in intracellular accumulation compared to the vehicle controls was more prominent in BeWo cells versus JEG3 cells. The presence of Ko143 resulted in a significant increase in intracellular fluorescence compared to that in the absence of Ko143 in both JEG3 and BeWo cells. This increase in intracellular fluorescence is likely caused by inhibiting BCRP efflux activity by Ko143. Collectively, the data suggest that these drugs induce BCRP protein expression, resulting in increased efflux activity of BCRP in human trophoblasts.

**DISCUSSION**

Placenta is the first line of protection for the developing fetus. ABC efflux transporters such as P-glycoprotein and BCRP are highly expressed in human placenta and play crucial roles in limiting fetal exposure to drugs and xenobiotics, thus protecting the fetus (Ni and Mao, 2011). Any changes in expression of these transporters in human placenta brought about by chronic use of medications during pregnancy would be expected to affect fetal drug exposure, leading to
detrimental consequences to the fetus. Several reports have detected and quantified BUP, NBUP, R-MET and S-MET in human samples from pregnant women including maternal and umbilical cord plasma as well as fetal meconium (Debelak et al., 2013; Gordon et al., 2010; Jones et al., 2005; Marin and McMillin, 2016), suggesting that these drugs can cross the placental barrier to the fetus and directly interact with the placenta. Therefore, in the present study, we examined the potential of the drugs used to treat drug abuse to alter BCRP expression in human placental trophoblasts and the molecular mechanism behind the changes.

We first demonstrated that these drugs at clinically relevant plasma concentrations observed in pregnant women could significantly induce BCRP mRNA in both the model placental choriocarcinoma cell lines, JEG3 (Fig. 1 and Fig. 2B) and BeWo (Fig. 2D), and the primary HVTs (Fig. 2F). Hence, this drug-induced BCRP expression is not cell-type specific. Interestingly, we noticed that the fold-induction with BeWo cells was generally lower than that with JEG3 cells and HVTs (Fig. 2D versus Fig. 2B and Fig. 2F), which is possibly the consequence of differential basal expression of BCRP in these cells. BCRP expression in BeWo cells is much more abundant than that in JEG3 and primary trophoblasts (Serrano et al., 2007). Hence, fold-induction may be decreased by high baseline expression. Previous studies have shown that differentiation itself can induce or down-regulate transporter expression in trophoblasts (Berveiller et al., 2015); thus, HVT cells were differentiated for 72 h to syncytiotrophoblast prior to drug treatment to avoid the effect of differentiation on BCRP expression. Given the fact that JEG3 and BeWo cells cannot spontaneously differentiate, our results suggest that whether or not the cells are syncytialized is not essential for BCRP induction by these drugs.
Drug metabolizing enzymes and transporters are under control of nuclear receptors such as CAR, PXR and AhR in response to exposure to drugs and xenobiotics. Unlike CAR and PXR, AhR is highly expressed in the first and third trimester placenta, particularly in the syncytiotrophoblast (Jiang et al., 2010; Pavek and Smutny, 2014). Previous studies have shown that human BCRP is a target gene of AhR in various carcinoma cell lines (Tan et al., 2010; Tompkins et al., 2010b). We therefore systematically investigated if BUP, NBUP, R-MET and S-MET can induce BCRP in human placental trophoblasts through AhR. Several lines of evidence support the conclusion that these drugs indeed upregulate BCRP in the human placental cell lines and HVTs by activating AhR. First, we showed that pre-treating JEG3, BeWo and HVT cells with the highly selective AhR antagonist CH223191 nearly completely abrogated induction of BCRP mRNA by the drugs (Fig. 2). Second, 3-MC is a known AhR ligand which has previously been shown to induce CYP1A1 in JEG3 cells (Li et al., 1998). We found that BUP, NBUP, R-MET and S-MET also induced CYP1A1 mRNA in JEG3, BeWo and HVT cells to the similar extent as 3-MC (Fig. 2). Moreover, we confirmed that, just like 3-MC, these drugs at nM or low μM concentrations were able to activate the luciferase reporter activity in JEG3 cells transfected with the X4-4.2 plasmid which contains 4 consensus AhR XREs (Fig. 3), indicating that these drugs are potent AhR ligands. Third, we demonstrated that these drugs were able to directly activate the BCRP promoter likely through activating AhR which binds to at least one proximal full consensus AhR XRE at position -194/-190 in the BCRP promoter region (Fig. 4). Induction of BCRP mRNA by these drugs (Fig. 5C) was accompanied by similar induction of BCRP protein (Fig. 5B and Fig. 5C) as well as increase in BCRP-mediated efflux activity (Fig. 7). Finally, additional evidence came from the effects of altering AhR expression on BCRP
induction. We showed that BCRP induction by the drugs in cells with AhR overexpression was far more robust than that in cells with no AhR overexpression (Fig. 5). Furthermore, knocking down of AhR completely diminished drug-mediated BCRP induction and rescue of AhR expression restored BCRP induction by the drugs (Fig. 6B and Fig. 6C).

A previous study reported no significant induction of BCRP mRNA in primary human trophoblasts treated with prototype AhR ligands including 3-MC (Stejskalova et al., 2011b). The reason of the contradicting results is not known, but may be explained by potentially different differentiation state of primary trophoblasts and different culture conditions used. In vitro differentiation of primary trophoblasts itself can induce or down-regulate transporter expression (Berveiller et al., 2015) and BCRP was shown to be induced by differentiation of primary trophoblasts (Evseenko et al., 2006). Therefore, we differentiated HVT cells for 72 h prior to drug treatment. In addition, compared to the study by Stejskalova et al., (Stejskalova et al., 2011b), the cells used in this study including HVTs were starved in serum-free media prior to drug treatment to eliminate potential effects of serum factors such as hormones on BCRP expression. In that regard, we have reported that 17β-estradiol down-regulates BCRP expression in BeWo cells (Wang et al., 2006).

To date, most known AhR ligands are carcinogens and toxicants such as smoking products. A previous study reported a moderate increase in BCRP protein levels in human placenta from mothers who smoked compared to nonsmokers (Kolwankar et al., 2005). Although the difference in BCRP protein abundance in the placentas between smokers and nonsmokers was not statistically significant possibly due to the small number of samples used (10 each group) and substantial inter-individual variations in BCRP protein expression, the trend is
consistent with our finding that BCRP in human trophoblasts can be induced by AhR ligands. In human placenta, BCRP induction by AhR ligands may represent an adaptive protection mechanism for the placenta and fetus in response to exposure to tobacco smoke, carcinogens or other toxicants which are potent AhR ligands. Many of the AhR ligands and their metabolites could be BCRP substrates. BCRP, upon AhR ligand-mediated induction, facilitates elimination of the toxicants from the placenta to the maternal circulation, thus protecting the fetus. Whether BUP or MET can induce BCRP in human placenta \textit{in vivo} remains to be determined.

BCRP, with its high expression in the small intestine, liver, kidney and many tissue barriers, plays a key role in absorption, distribution and elimination of drugs and xenobiotics (Mao and Unadkat, 2015). Smoking has long been recognized to affect drug disposition. Clinical studies have shown that systemic exposure to irinotecan and erlotinib was significantly lower in smokers than in non-smokers (Hamilton et al., 2006; van der Bol et al., 2007). Although such pharmacokinetic changes were explained by smoking-induced P450 metabolism, induction of BCRP in organs important for drug elimination by smoking products might be an additional contributing factor given the fact that these drugs are excellent BCRP substrates. Nevertheless, caution should be taken to extrapolate the current findings to other tissues. It is worth noting, though, that the opioid agonist oxycodone has been shown to induce BCRP in rat brain (Hassan et al., 2009). Since AhR is known to be expressed in the brain, BCRP in rat brain could possibly be induced by oxycodone through activating AhR. BUP and MET could be chronically used to treat drug abuse among patients who have cancers or are HIV-infected and thus need to take other medications simultaneously. In addition, BUP and MET are strong opioid-agonists used as the fourth line analgesics in cancer patients, and BUP was suggested as a better analgesic than
MET (Lossignol et al., 2013; Nicholson, 2004; Schmidt-Hansen et al., 2015). BCRP confers multidrug resistance in cancers and decreases absorption or facilitates elimination of anti-cancer drugs such as topotecan, irinotecan and flavopiridol which are BCRP substrates (Mao and Unadkat, 2015). Consequently, studies to evaluate the potential of drug-drug interactions via induction of BCRP by BUP or MET would be important for patients who require MET or BUP based therapies.

In summary, this is the first study to demonstrate that BUP, NBUP, R-MET and S-MET are potent AhR ligands and can induce BCRP mRNA and protein expression in human placental trophoblasts at clinically relevant concentrations by activating the AhR signaling cascade. Such findings have important implications in understanding and prediction of clinical drug-drug and drug-toxicant interactions and organ protection.
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AUTHORSHIP CONTRIBUTIONS

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Conducted experiments: Neradugomma NK

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. BUP, NBUP, R-MET and S-MET Induce BCRP mRNA in JEG3 Cells. Confluent monolayers of serum starved JEG3 cells were treated for 24 h with respective drugs at unbound C_{max} (0.01 μM for BUP and NBUP and 0.5 μM for R-MET and S-MET) and C_{max} (0.1 μM for BUP and NBUP and 1.5 μM for R-MET and S-MET) plasma concentrations. Results for BUP and NBUP as well as for R-MET and S-MET are shown in panels A and B, respectively. Shown are means ± SD of 3-6 independent experiments performed in duplicates or triplicates. All data were normalized to the vehicle controls which were set as 1-fold. *indicates significant differences with p value of < 0.05 between the vehicle control and drug treatment groups by two-way ANOVA analysis followed by the Bonferroni correction.

Figure 2. Drug-mediated Fold-Induction of BCRP mRNA Is Suppressed by the AhR Antagonist CH223191. Serum starved JEG3 (A and B), BeWo (C and D) and HVT (E and F) cells were pre-incubated with the AhR inhibitor CH223191 at 3 μM for 12-16 h and then treated with respective drugs at concentrations indicated for 24 h. Cells were harvested and analyzed for expression of CYP1A1 mRNA (A, C and E) and BCRP mRNA (B, D and F). Shown are means ± SD of three independent experiments performed in triplicates. 3-MC was used as a positive control. All data were normalized to the vehicle controls which were set as 1-fold. *indicates significant differences with p value of < 0.05 between with and without CH223191 groups by two-tailed Student’s t-test.
Figure 3. BUP, NBUP, R-MET and S-MET Are AhR Ligands. A shows fold-changes of AhR mRNA levels in JEG3 cells stably transfected with the pcDNA3/AhR expression vector and the corresponding empty vector. B is a representative immunoblot showing AHR protein expression in cells transfected with AhR expression vector (AHROE) compared to parent JEG3 control (Cnt) and the empty vector transfected (EMTV) cells. GAPDH was detected as an internal control. C shows that 3-MC-mediated induction of CYP1A1 mRNA in JEG3 cells with AhR overexpression compared to the empty vector cells. Data in C were normalized to the vehicle controls of EMT.V cells which were set as 1-fold. *indicates significant differences with $p$ value of $< 0.05$ compared to the vehicle-treated EMT.V cells. D schematically illustrates the X4-4.2 plasmid that contains four AhR-binding XREs at a position upstream of the luciferase gene. E denotes that 3-MC significantly induced luciferase activity in AHROE cells transfected with the X4-4.2 plasmid compared to that in the EMT.V cells and vehicle-treated cells. F denotes that BUP, NBUP, R-MET and S-MET significantly induced luciferase activity in AHROE cells transfected with the X4-4.2 plasmid compared to that in the EMT.V cells and vehicle-treated cells. Insert in F shows the data from ligand/drug-treated EMT.V cells compared to vehicle treatment controls. All data shown are means ± SD of three independent experiments performed in triplicates. *indicates significant differences with $p$ value of $< 0.05$ between the respective AHROE and EMT.V cells. #indicates significant difference with $p$ value of $< 0.05$ between the vehicle- and ligand/drug-treated EMT.V cells. $^\$indicates significant differences with $p$ value of $< 0.05$ between the vehicle- and ligand/drug-treated AHROE cells. Statistical analysis was performed by two-way ANOVA analysis followed by the Bonferroni correction.
Figure 4. BUP, NBUP, R-MET and S-MET Active BCRP Transcription. A and B are schematic illustrations of the BCRP promoter luciferase constructs that contain the -1285 to +362 and -115 to +362 regions of the human BCRP promoter, respectively. Treatment with BUP and NBUP (C), R-MET and S-MET (D) significantly induced the luciferase activity in parent JEG3 cells transfected with the -1285/+362 promoter construct. Shown are means ± SD of three independent experiments performed in triplicates. In C and D, data were normalized to the vehicle controls which were set as 1-fold. *indicates significant differences with p value of < 0.05 between the vehicle control (open bars) and drug-treatment groups (filled bars). E shows that 3-MC significantly induced luciferase activity in AhR-overexpressing JEG3 cells (AHR.OE) transfected with the -1285/+362 promoter construct compared to that in the empty vector control JEG3 cells (EMT.V). F denotes that BUP, NBUP, R-MET and S-MET significantly induced luciferase activity in AHR.OE cells transfected with the -1285/+362 promoter construct compared to that in EMT.V cells. Shown are means ± SD of three independent experiments performed in triplicates. *indicates significant differences with p value of < 0.05 between the respective AHR.OE and EMT.V cells. #indicates significant differences with p value of < 0.05 between the vehicle-treated and ligand/drug-treated EMT.V cells. $indicates significant differences with p value of < 0.05 between the vehicle-treated and ligand/drug-treated AHR.OE cells. G and H show luciferase activities in parent JEG3 and BeWo cells, respectively. Cells were transfected with the -1285/ +362 (open bars) or -115/+362 (filled bars) promoter construct. Shown are means ± SD of three independent experiments performed in triplicates. *indicates significant differences with p value of < 0.05 between the cells transfected with -1285/+362 promoter construct and the -115/+362 promoter construct. #indicates significant differences with
\( p \) value of < 0.05 between the vehicle-treated and ligand/drug-treated cells transfected with the -115/+362 promoter construct. $ indicates significant differences with \( p \) value of < 0.05 between the vehicle-treated and ligand/drug-treated cells transfected with the -1285/+362 promoter construct. Statistical analysis for C – H was performed by two-way ANOVA analysis followed by the Bonferroni correction.

**Figure 5. Overexpression of AhR further Enhances BCRP mRNA and Protein Expression.**

A denotes BCRP mRNA expression induced by BUP, NBUP, R-MET and S-MET in AhR-overexpressing JEG3 cells (AHR.OE) compared to the empty vector control (EMT.V) cells. Data were normalized to the vehicle controls in both cell types which were set as 1-fold. Shown are means ± SD of three independent experiments performed in triplicates. * indicates significant differences with \( p \) value of < 0.05 between the EMT.V (open bars) and AHR.OE (filled bars) cells. B shows representative immunoblots illustrating induction of BCRP protein by the drugs in the AHR.OE and EMT.V cells. Human \( \beta \)-actin was used as internal standard. C represents an intensity plot (means ± SD) obtained from three independent immunoblotting experiments. The BCRP protein levels were normalized to \( \beta \)-actin and compared with the BCRP protein levels associated with the EMT.V cells treated with vehicle alone which were set as 100%. * indicates significant differences with \( p \) value of < 0.05 between the respective AHR.OE and EMT.V cells. # indicates significant difference with \( p \) value of < 0.05 between the vehicle-treated and ligand/drug-treated EMT.V cells. $ indicates significant difference with \( p \) value of < 0.05 between the vehicle-treated and ligand/drug-treated AHR.OE cells. Statistical analysis was performed by two-way ANOVA analysis followed by the Bonferroni correction.
Figure 6. Knocking Down of AhR Diminishes BCRP mRNA Induction and Rescuing AhR Expression Restores BCRP mRNA Induction. AhR expression in parent JEG3 cells was knocked down by infecting cells with lentivirus encapsulated shRNA against AhR (sh.AHR). Scrambled shRNA infected cells (sh.SCR) and parent JEG3 cell (Cnt) were used as controls. A denotes a representative immunoblot showing AhR protein levels in parent JEG3 (Cnt), scramble (sh.SCR), scramble cells transfected with the AhR expression vector (sh.SRC+AHR), AhR knocking down (sh.AHR), and AhR knocking down cells transfected with the AhR expression vector (sh.AHR+AHR). GAPDH was used as an internal standard. B shows that 3-MC-induced BCRP mRNA (sh.SCR) was completely diminished by knocking down of AhR (sh.AHR). Rescuing AhR expression by transiently transfecting the cells with the AhR expression vector partially restored BCRP mRNA induction (sh.AHR+AHR). Transfecting the AhR expression vector into JEG3 (sh.SCR) cells further enhanced BCRP mRNA induction (sh.SCR+AHR). C and D illustrate that drug-induced BCRP mRNA (sh.SCR) was completely diminished by knocking down of AhR (sh.AHR). Rescuing AhR expression by transiently transfecting the cells with the AhR expression vector fully restored BCRP mRNA induction (sh.AHR+AHR). Transfecting the AhR expression vector into JEG3 (sh.SCR) cells further enhanced BCRP mRNA induction (sh.SCR+AHR). Shown are means ± SD of three independent experiments performed in triplicates. Data were normalized to vehicle controls which were set as 1-fold. *, and $ indicate significant differences with $p$ value of < 0.05 between the sh.SCR and sh.AHR cells and between the sh.SCR and shSCR+AHR cells, respectively. #indicates significant differences with $p$ value of < 0.05 between the sh.AHR and sh.AHR+AHR cells. Statistical analysis was performed by two-way ANOVA analysis followed by the Bonferroni correction.
Figure 7. Drug treatment increases BCRP efflux activity. JEG3 (A) and BeWo (B) cells were treated with respective drugs at indicated concentrations prior to intracellular Hoechst 33342 accumulation assays in the presence and absence of the BCRP inhibitor Ko143. Shown are means ± SD of four independent experiments performed in triplicates. Data were normalized to the fluorescence associated with the vehicle controls without Ko143 which were set as 100%. $ indicates significant differences with p value of < 0.05 between with and without Ko143 treatment groups. * indicates significant differences with p value of < 0.05 between the vehicle-treated and ligand/drug-treated cells in the absence of Ko143. Statistical analysis was performed by 2-tailed Student’s t-test.