Cannabidiol changes P-gp and BCRP expression in trophoblast cell lines

Objectives. Marijuana is the most commonly used illicit drug during pregnancy. Due to high lipophilicity, cannabinoids can easily penetrate physiological barriers like the human placenta and jeopardize the developing fetus. We evaluated the impact of cannabidiol (CBD), a major non-psychoactive cannabinoid, on P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) expression, and P-gp function in a placental model, BeWo and Jar choriocarcinoma cell lines (using P-gp induced MCF7 cells (MCF7/P-gp) for comparison).

Study design. Following the establishment of the basal expression of these transporters in the membrane fraction of all three cell lines, P-gp and BCRP protein and mRNA levels were determined following chronic (24-72h) exposure to CBD, by Western-blot and qPCR. CBD impact on P-gp efflux function was examined by uptake of specific P-gp fluorescent substrates (calcein-AM, DiOC2(3) and rhodamine123(rh123)). Cyclosporine A (CsA) served as a positive control.

Results. Chronic exposure to CBD resulted in significant changes in the protein and mRNA levels of both transporters. While P-gp was down-regulated, BCRP levels were up-regulated in the choriocarcinoma cell lines. CBD had a remarkably different influence on P-gp and BCRP expression in MCF7/P-gp cells, demonstrating that these are cell type specific effects. P-gp dependent efflux (of calcein, DiOC2(3) and rh123) was inhibited upon short-term exposure to CBD.

Conclusions. Our study shows that CBD might alter P-gp and BCRP expression in the human placenta, and inhibit P-gp efflux function. We conclude that marijuana use during pregnancy may reduce placental protective functions and change its morphological and physiological characteristics.
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Introduction

Until recently it was assumed that prenatal exposure to marijuana is frequently combined with other drugs (e.g. tobacco and alcohol), making cannabis effects difficult to isolate and assess (Kozer and Koren, 2001; Moore et al., 2010). However, the studies of Dekker et al and Hayatbakhsh et al suggested that cannabis use prior to, during the first trimester, or throughout gestation is associated with a higher risk for a low birth weight and neonatal length, as well as preterm labor (Dekker et al., 2012; Hayatbakhsh et al., 2012). Moreover, the presence of the endocannabinoid receptors CB1 and CB2 on placental syncytiotrophoblast (Habayeb et al., 2008a), along with marijuana being the most popular drug of abuse among pregnant population (Brown and Graves, 2013), raised the need to understand the effect of cannabinoids on the placenta.

Cannabidiol (CBD) is one of the most abundant cannabinoids in the marijuana plant (Mechoulam and Shvo, 1963; Mechoulam and Hanus, 2002; Schier et al., 2012). It is a promising candidate for clinical utilization, due to low affinity binding to CB1 and CB2 cannabinoid receptors and no cognitive and psychoactive activity (Zuardi, 2008; Deiana, 2012).

Preliminary data from in vitro models indicate that cannabinoids may interact with human P-gp (ABCB1) and BCRP (ABCG2). Acute and long-term exposures to cannabinoids were shown to alter P-gp expression and function (Holland et al., 2006; Zhu et al., 2006). However, BCRP exposure to cannabinoids demonstrated functional inhibition only (Holland et al., 2007).

P-gp and BCRP both are thought to be protective for the fetus. These ATP-binding cassette (ABC) efflux transporters expressed at the apical membrane of the polarized syncytiotrophoblast layer (Ni and Mao, 2011), and play a significant role in drug transfer across the placental barrier (Lankas et al., 1998; Mao, 2008; Vahakangas and Myllynen, 2009; Myllynen et al., 2010; Eshkoli et al., 2011). P-gp was found to be an anti-apoptotic cellular agent (Smyth et al., 1998; Huls et al., 2009) and BCRP was shown to have a role in placental tissue and syncytial survival by protecting
cells from pro-apoptotic injuries (Evseenko et al., 2007a; Evseenko et al., 2007b; Hardwick et al., 2007; Vahakangas and Myllynen, 2009).

We have already assessed the interaction of CBD with placental BCRP, finding that in both, in vitro and ex vivo systems, CBD inhibited its efflux function (Feinshtein et al., In Press). These results together with other recent findings regarding cannabinoids effect on ABC transporter led us to investigate whether CBD affects placental P-gp on functional and placental P-gp and BCRP on expressional levels (Holland et al., 2006; Zhu et al., 2006; Holland et al., 2007; Holland et al., 2008).

In the present work, the implications of CBD exposure on P-gp and BCRP expression and P-gp function is tested in a human trophoblast-like cell lines BeWo and Jar, as placental model.
Materials and Methods

Materials

BeWo and Jar cells were obtained from Dr. B. Ugele, Ludwig- Maximilians University, Munich, Germany (Feinshtein et al., 2010; Polachek et al., 2010; Feinshtein et al., In Press). MCF7/P-gp cells (a BCRP expressing and P-gp induced cells), were kindly provided by Prof. Esther Priel (Ben Gurion University, Beer Sheva, Israel)(Feinshtein et al., In Press). All materials for cell culture were purchased from Biological Industries (Israel). CBD was a kind gift from Prof. Raphael Mechoulam (The Hebrew University of Jerusalem, Jerusalem, Israel). Calcein-AM, DiOC2(3), rh123 and cyclosporine A (CsA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A list of all antibodies used in the current research is summarized in supplementary table I

Methods

Cell culture and drug treatments: MCF7/P-gp cells were cultured in conditions as previously described (Golan et al., 2009), BeWo and Jar cells were cultured as previously described (Golan et al., 2009; Feinshtein et al., 2010). Briefly, 24h after seeding in 35 x 10 mm cell culture dishes (Corning), growth medium (DMEM for MCF7/P-gp, DMEM/F-12 for BeWo and Jar cells, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C) was replaced with fresh one containing CBD 10 or 15µM (initially dissolved in DMSO) or DMSO (0.08% or 0.12%, respectively, vehicle control). In order to achieve long-term exposure, treatment medium was refreshed every day for 24h, 48h or 72h

Subcellular fractionation: Fractionation procedure was carried out as previously described (Golan et al., 2009), for the isolation of the membrane fraction. The membrane fraction was
diluted in sample buffer 1:3 (10% v/v glycerol, 20% v/v SDS 20%, 5% v/v β-mercaptoethanol, 0.05% w/v bromophenol blue, pH 6.8), boiled for 5 min at 95°C (for BCRP determination) or incubated for 30 min at 37°C (for P-gp determination) and frozen at -80°C until assayed. Aliquots were taken for protein determination using the Lowry assay. Fractions purity was verified using specific markers: Na’/K’ ATPase for membranes and NF/kB p65 for cytosol.

**Immunocytochemistry and Confocal Microscopy**

MCF7/P-gp, BeWo and Jar cells were seeded on cover slips and grown to 70% sub-confluency. Cells were then fixed in PFA for 15min and stained with mouse anti-human CD 243 (MDR-1) antibody, diluted in PBS, containing 3% BSA, for 1h at room temperature, followed by incubation with Alexa Fluor - 488 goat anti-mouse antibody in PBS containing 3% BSA, for 1/2h at room temperature. Cover-slips were washed 3 times and mounted onto glass slides with DAPI (4',6-diamidino-2-phenylindole) -containing fluorescent mounting medium (DAPIFluoromount-G, SouthernBiotech ). Immunofluorescence was detected by an Olympus FV-1000 Spectral confocal laserscanning microscope with excitation at 488nm and emission at .520nm. Image analysis was performed using ImageJ v. 1.40C software.

**FACS analysis of BCRP levels**

Cells were grown to confluency in 6-well culture dishes, trypsinized, counted, and 0.5 *10^6 cells were placed in light resistant vials. Cells were washed three times with cold PBS (and each time centrifuged at 1200g for 5 min), and incubated for 30 min with BSA 3% in PBS. Following additional PBS wash, 50µL of BSA 1% in PBS and antibodies were added (1:10 anti-BCRP FITC conjugated antibody or 1:10 CBL602F FTIC conjugated isotype negative control). Cells were incubated in the dark at 4°C for 1h, washed with ice cold PBS, re-suspended (in 0.5ml PBS) and (analyzed by FACS (BD FACS Vantage).
Immunoblotting

Membrane fractions were thawed on the day of assay. Protein aliquots (60-100µg/lane) were taken for protein separation by SDS-PAGE as previously described (Golan et al., 2009). Semi-quantitative analysis was carried out using a computerized image analysis system (EZQuant-Gel 2.11, EZQuant Biology Software Solutions Ltd., Israel). Equal protein loading was ensured by normalization to Na⁺/K⁺ ATPase (for P-gp) or actin (for BCRP).

RNA extraction, reverse transcription, Real-Time Polymerase Chain Reaction (qPCR)

Isolation and purification of total RNA from BeWo and Jar cells was carried out using EZ-RNA Kit (Biological Industries, Israel) according to manufacturer’s instructions. 1µg of total RNA was used for reverse transcription using High Capacity cDNA RT kit (Applied Biosystems, Foster City, CA), in 20µl reaction volume. BCRP and P-gp mRNA was measured by qRT-PCR, as indicated in the manufacturer protocol (Applied Biosystems, Foster City, CA), and performed by the Applied Biosystems Real Time PCR system (7500 system), using TaqMan probes and primers for human BCRP, P-gp and actin (Applied Biosystems, Foster City, CA). The cycling conditions for all primers were as follows: hold for 10 min at 95°C, followed by 40 cycles consisting of two steps, 15 s at 95°C (denaturing), and 1 min at 60°C (annealing-extension). The threshold cycle, which correlates inversely with the mRNA levels of target, was measured as the cycle number at which the reporter fluorescent emission increases above a threshold level. P-gp and BCRP mRNA levels were normalized to actin mRNA in the same samples. Results were analyzed by the 2(-ΔΔC(T)) method, demonstrating the relative changes in gene expression from real-time quantitative PCR experiments, using 7500 Software v2.0.4 (Applied Biosystems, Foster City, CA).
P-gp substrate uptake experiments

Cells were pre-incubated for 30 min with CBD 10 or 25 µM (working concentration previously published (Holland et al., 2006; Ligresti et al., 2006; Zhu et al., 2006; Holland et al., 2007; Holland et al., 2008; De Filippis et al., 2011; Arnold et al., 2012; Harvey et al., 2012; Hill et al., 2012; Maor et al., 2012; Solinas et al., 2012; Dudasova et al., 2013; Juknat et al., 2013; Nabissi et al., 2013), and initially dissolved in DMSO) or CsA 20 µM (known P-gp inhibitor used as positive control (Mori et al., 2012)), dissolved in transport buffer (TB) (pH = 7.4) (Feinshtein et al., 2010), while “control cells” were pre-incubated in TB with the correlating concentration of DMSO. Following pre-incubation, P-gp substrates were added (Calcein-AM or DiOC2(3) or rh123) (Minderman et al., 1996; Martin et al., 2003) and cells were further incubated for 30 min.

At the end of incubation plates were treated as previously detailed (Feinshtein et al., 2010), and samples were stored (at −20°C) for further analysis. Intracellular fluorescence of all P-gp substrates was quantified by Infinite M200 microplate reader (Tecan) and normalized to protein amount (determined by Lowry method) (Lowry et al., 1951), or detected by an Olympus FV-1000 Spectral confocal laserscanning microscope and analyzed using ImageJ v. 1.40C software.

Statistical analysis: All statistics and graphs were carried out using GraphPad Prism5 software. Student's t-test or one-way ANOVA followed by appropriate Bonferroni corrections were used.
Results

CBD impact on BCRP and P-gp protein expression

The changes in P-gp and BCRP protein levels in the membrane fraction of BeWo, Jar and MCF7/P-gp cells were studied. Following determination of proper cell fractionation (Figure 1A), the basal expression of these two transporters in non-treated cells was determined by Western Blot analysis (Figure 1B). Due to unexpected P-gp expression profile in all three cell lines we verified its basal expression by immunocytochemical fluorescent staining (Figure 1C). It can be seen that our BeWo, Jar and MCF7/P-gp cells express detectable levels of P-gp. For P-gp expression, results of Western Blot were confirmed by immunocytochemistry showing that P-gp expression was the highest in MCF7/P-gp and the lowest in Jar cells. For BCRP (ABCG2), results were confirmed by FACS analysis, showing that BCRP expression was much higher in JAR cells compared to BeWo cells (in full accordance with western blot analysis) (Figure 1D). The changes in membrane BCRP and P-gp levels in BeWo cells following long-term exposure to CBD are displayed in Figure 2. Indeed, BCRP levels were significantly increased in a concentration-dependent manner (Figure 2B) that was not time-dependent (Figure 2A). Following long-term exposure to CBD, P-gp protein levels significantly decreased, in a concentration-dependent matter (Figure 2D), with no time-dependent effect (Figure 2C). Similarly, in Jar cells (Figure 3), BCRP protein concentrations was significantly elevated following long term exposure to CBD, in a concentration but not time-dependent effect (Figures 3A, B). Due to very low P-gp initial (baseline) expression in the Jar cell line (Figure 1B, C), the expected down-regulation in P-gp expression following long-term exposure to CBD is demonstrated in experiments of 72h treatment only. P-gp is vaguely visualized in the control group while it is almost undetectable in the CBD treated group (Figure 3C). 48h exposure yielded the same results (P-gp levels were almost undetectable – data not shown), making time and concentration dependent comparisons impractical.
To examine whether CBD effects are cell specific, the same experimental routine was applied to MCF/P-gp cells. In these cells BCRP and P-gp behavior under CBD influence was profoundly different from that seen in choriocarcinoma cell lines. P-gp levels dramatically increased following long-term treatment with CBD, in a concentration- but not time-dependent matter (Figure 4A). At the same time, BCRP expression was not affected by CBD (neither time-, nor concentration-dependent effect was observed) (Figure 4B). Thus, we conclude that CBD has a cell-type specific influence upon long-term cellular exposure. CBD impact on MCF7/P-gp cells was not investigated further.

**CBD impact on BCRP and P-gp mRNA**

To have a glimpse into the mechanism underlying to the changes seen on the protein level in BCRP and P-gp, we further focused to track the changes that occur on mRNA level of these two transporters. In both, BeWo and Jar cells, BCRP mRNA quantification showed elevation following long-term exposure to CBD (Figures 5A, C), and matched the findings on protein level, supporting transcriptional up-regulation. Likewise, P-gp mRNA expression significantly dropped following long-term treatment with CBD, matching the outcomes seen on the protein level (Figures 5B, D), indicating that the changes in P-gp levels following long-term CBD exposure results from transcriptional down-regulation rather than post-transcriptional changes.

**CBD impact on P-gp function**

Only BeWo and MCF7/P-gp cell lines were tested for P-gp inhibition by CBD due to the fact that Jar cells expressed very low P-gp levels. In a preliminary study we observed a non-cell type specific P-gp inhibition by 25µM CBD in both cell lines, as intracellular calcein and rh123 fluorescence was significantly higher (elevation of 101±50% and 70±30%, respectively) in the presence of CBD (Figure 6A, B). We further examined whether lower concentration of CBD
10µM also inhibits P-gp, using two different P-gp specific substrates, calcein-AM and DiOC2(3).

P-gp was inhibited following short-term (1h) exposure to CBD, as significantly more DiOC2(3) and calcein (elevation of 20±8% and 24±9%, respectively) accumulated in the cells (Figure 7A, B).
Discussion

Principal findings of the study: There is a dual effect of CBD on the expression of BCRP and P-gp transporters in trophoblast-like and in MCF7/P-gp cell lines. Under long-term CBD exposure BCRP and P-gp present cell-type specific changes in protein and mRNA levels. Occurring already at the transcriptional level, P-gp protein expression is down-regulated, while that of BCRP is up-regulated. Upon short-term exposure, P-gp efflux function is inhibited by CBD.

Cannabinoid effects on pregnancy: Cannabis is under extensive use in western society as a recreational drug. The effect of this drug on pregnancy outcome was under constant debate; however, recently cannabis consumption was reported to be associated with adverse pregnancy outcomes, including preterm birth and fetal growth restriction (Dekker et al., 2012; Hayatbakhsh et al., 2012). Although the mechanisms in which phytocannabinoids exert their effects are not well understood, there is accumulating evidence that endocannabinoids (like anandamide) can influence reproduction, including fertilization, implantation, angiogenesis, embryo development and placental growth (Taylor et al., 2007; Habayeb et al., 2008a; Lewis et al., 2012; Solinas et al., 2012; Sun and Dey, 2012). One possible route to convey the effect of endocannabinoids is through the CB1 and CB2 receptors that are express by the human trophoblast and provide a direct target for cannabinoids (Kenney et al., 1999; Habayeb et al., 2008a; Habayeb et al., 2008b). However, not all endocannabinoids exert their effect through the classic cannabinoids receptors. For example CBD, lacks the central effects of cannabis and works through CB1 and CB2 independent model of action (Scuderi et al., 2009). Since our goal was to test the direct cannabinoid effect on trophoblast transporters BCRP and P-gp, and CBD showed the most potent inhibitory effect (among major cannabinoids) on these transporters (Holland et al., 2006; Zhu et al., 2006; Holland et al., 2007) it made CBD the ideal cannabinoid for the present study.
BCRP and P-gp significance in pregnancy: BCRP and P-gp play a key role in transport of drugs and endogenous compounds in the human placenta, affecting the outcome of pregnancy (Robey et al., 2009; Myllynen et al., 2010). They transport a broad variety of structurally diverse compounds, some of which are congruent (Frohlich et al., 2004; Mathias et al., 2005; Zhou, 2008). In addition, BCRP transports a wide range of substrates, including fetal hormonal precursors such as estrone-3-sulfate, naturally occurring carcinogens, porphyries and ceramides (Imai et al., 2003; Krishnamurthy and Schuetz, 2005; Evseenko et al., 2007a; Evseenko et al., 2007b; Mao, 2008; Dietrich et al., 2011). P-gp is expressed in the apical membrane of syncytiotrophoblast and probably is the main placental protective transporter during the first trimester. Its expression (both mRNA and protein) decreases with advancing gestation, being the highest during the first trimester (Gil et al., 2005; Mathias et al., 2005; Sun et al., 2006). BCRP plays an important role as a survival factor in BeWo cells as well as in the human placenta. It is thought to have a protective antiapoptotic role in the trophoblast, regulating their survival under low oxygen conditions (Krishnamurthy and Schuetz, 2006; Yeboah et al., 2006; Evseenko et al., 2007a; Evseenko et al., 2007b). The trophoblast expression of BCRP may change in different pregnancy complications. Indeed, placentas of women with preterm labor and intra-amniotic inflammation had a higher expression of this transporter than those of women with preterm labor without inflammation. In addition, the mRNA expression of BCRP correlated with that of IL-8, which also increased significantly in placentas of women with preterm labor and inflammation, suggesting that the transfer of drugs across the placenta may be altered in cases of preterm labor with inflammation (Mason et al., 2011).

CBD impact on BCRP and P-gp upon long-term exposure: In the current study we have demonstrated for the first time that the exposure of trophoblast-like cell lines BeWo and Jar to CBD is associated with two distinct patterns of effects on the expression of the BCRP and P-gp
transporters. The use of these cell lines, instead of primary trophoblast cultures, results from the
fact that primary trophoblast may rapidly differentiate in culture, continuously changing their
gene and protein expression (Evseenko et al., 2006). Comparison of these parameters in regard to
CBD influences in such dynamic in vitro environment would be almost impossible. Moreover,
BeWo and Jar cell lines, derived from human gestational choriocarcinoma, commonly used as an
in vitro model for trophoblast toxicology studies, and they offer a suitable model to study certain
aspects of human trophoblast physiology, without the aspect of inter-patient variability (Sullivan,
(2004; Khare et al., 2006; Myren et al., 2007)
The first effect that was observed following long-term exposure is that CBD transcriptionally
inhibited P-gp membrane expression (as mRNA levels of P-gp were also dramatically reduced
following long-term exposure to CBD). The long-term inhibitory effect of CBD on P-gp protein
was previously reported in drug-selected human T lymphoblastoid leukaemia cell line
(CEM/VLB(100)) (Holland et al., 2006). The cell specificity of CBD was previously reported
(Khare et al., 2006; Hu et al., 2011), yet our study is the first to detect this phenomenon in
trophoblast-like cell lines. This observation may have clinical implications, in light of the role of
P-gp as one of the key transport mechanisms for numerous drugs in the human placenta,
especially during the venerable period of the first trimester in which all the fetal organs are
formed. Thus, our results might be especially important to women who use cannabis on a regular
basis during the first trimester and are treated with other drugs that are P-gp substrates
The expressional up-regulation of BCRP mRNA and protein in trophoblast-like cell lines
following long-term exposure to CBD is the second novel effect reported in our study. This
observation is similar to that reported in trophoblast of women with preterm labor and
inflammation, suggesting that I) long-term exposure to CBD may elicit an inflammatory response
in trophoblast-like cell lines (Mason et al., 2011); and II) that this phenomenon could be a direct
compensational consequence of P-gp down-regulation, providing a working defense line to the
developing embryo. Interestingly, compensation of such nature was already described in murine
placentas (Hutson et al., 2010). Nonetheless, the signal transduction of CBD action in human
placenta/trophoblast/choriocarcinoma cells has not been clearly reported yet and needs to be
.further elucidated

CBD impact on P-gp upon acute exposure: In agreement with previously published data, we
found that CBD holds inhibiting properties over P-gp efflux function (Holland et al., 2006; Nieri
et al., 2006; Zhu et al., 2006). Moreover, our results, show inhibition of P-gp efflux function in
cell lines that naturally express this transporter. It can be seen that CBD 10µM (Figure. 7) yielded
inhibition effects lower than CBD 25µM (Figure 6). Similarly to results recently presented by us
(Feinshtein et al., In Press), this observation could imply that CBD inhibits P-gp in a
concentration dependent fashion. However, due to different quantification methods used in
.present study, this should be further elucidated

Of note, many of the drugs that are prescribed and considered safe to use during pregnancy are in
fact P-gp or BCRP substrates, like Loratadine and H₂ blockers (i.e. Ranitidine and Cimetidine)
(Collett et al., 1999; Chen et al., 2003; Li et al., 2008; Schwarz et al., 2008; Dahan and Amidon,
2009; Gill et al., 2009; Matok et al., 2010). Our finding may have clinical implications,
suggesting that the use of cannabis during gestation may alter drug transport through the
trophoblast and lead to the absence of a functional placental barrier during the first trimester,
leaving the developing embryo unprotected at this vulnerable period of pregnancy. Moreover, the
trophoblast of pregnant women exposed to marijuana patients may exhibit some resistance to
.apoptotic and inflammatory processes due to the effect of CBD on BCRP expression

Conclusions: Following cannabis consumption, all the drugs that are P-gp substrates can
potentially penetrate the human placental barrier at higher rates when combined with CBD, and
therefore their safety under these conditions is to be questioned. Additionally, changes in
placental BCRP expression profile might lead to altered transplacental transport of BCRP
substrates, such as medications, naturally occurring carcinogens, hormonal precursors and apoptotic molecules, and influence pregnancy outcomes.

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**Figure legends**

**Figure 1:** (A) Cell fractionation: to verify proper fractionation of whole cell lysate, membrane fractions and cytosolic fractions of BeWo, Jar, MCF/P-gp cells were subjected to Western blot analysis. Na⁺/K⁺-ATPase served as membrane marker, and NF-κB (p65 subunit) served as cytosolic marker. (B) P-gp and BCRP basal expression in MCF7/P-gp, Jar and BeWo cells was determined by Western blotting. (C) Immunocytochemistry: fluorescent staining of BeWo, Jar and MCF7/P-gp cells with anti-P-gp antibody and DAPI. NC – negative control. (D) BCRP (ABCG2) expression in choriocarcinoma cell lines BeWo and Jar as demonstrated by FACS analysis.

**Figure 2:** Long-term exposure of BeWo cells to CBD: changes in membrane BCRP and P-gp expressed levels. Changes in BCRP - CBD concentration-dependent (B) and time-dependent (A). Changes in P-gp - CBD concentration-dependent (D) and time-dependent (C). No statistical significance between 48 and 72h time points. Data is displayed in means ± s.d. of at least three (n=9) independent experiments of each concentration or time point. One representative blot is presented for each experimental group. One-Way ANOVA, followed by Bonferroni’s multiple comparison test, **p<0.01, ***p<0.0001 compared to control or comparison between groups as indicated.

**Figure 3:** Long-term exposure of Jar cells to CBD: changes in membrane BCRP and P-gp expressed levels. Changes in BCRP - CBD concentration-dependent (B) and time-dependent (A). Changes in P-gp (C). Data is displayed in means ± s.d. of at least three (n=12) independent experiments of each concentration or time point. One representative blot is presented for each experimental group. One-Way ANOVA, followed by Bonferroni's multiple comparison test (Student's t test for (C)), *p<0.05, ***p<0.0001 compared to control or comparison between groups as indicated.
Figure 4: Long-term exposure of MCF/P-gp cells to CBD: changes in membrane P-gp (A) and BCRP (B) expressed levels. Protein levels are given as percent of control levels. Data is displayed in means ± s.d. of at least three (n=9) independent experiments of each concentration or time point. One representative blot is presented for each experimental group. One-Way ANOVA, followed by Bonferroni’s multiple comparison test. **p<0.01, ***p<0.0001 compared to control or comparison between groups as indicated.

Figure 5: Long-term exposure of BeWo and Jar cells to CBD: changes in BCRP and P-gp mRNA levels. (A), (C) changes in BCRP mRNA in BeWo and Jar cells (respectively). (B), (D) changes in P-gp mRNA in BeWo and Jar cells (respectively). Values are given as fold of change compared to control. Data is displayed as means ± s.d. of at least three (n=6) independent experiments for each time point. One-Way ANOVA, followed by Bonferroni’s multiple comparison test. *p<0.05, **p<0.01, ***p<0.0001 compared to control.

Figure 6: Non-cell type specific effect of CBD 25µM on P-gp dependent calcein and rh123 intracellular efflux in (A) BeWo and (B) MCF7/P-gp cells. Data presented from at least 2 independent experiments (n=33, n=11, respectively), as means ± s.d. Statistical significance determined by Student's t-test. ***p<0.0001.

Figure 7: CBD 10µM inhibition of P-gp dependent efflux of calcein (A) and DiOC2(3) (B). Data presented from at least 2 independent experiments (n=9, n=6, respectively), as means ± s.d. Statistical significance determined by One-Way ANOVA, followed by Bonferroni’s multiple comparison test for (A) and student's t-test for (B). ***p<0.0001, *p<0.05.
Figure 1

Cell fractionation, P-gp and BCRP basal expression
(A) membranes and cytosols

- BeWo
- Jar
- MCF7/P-gp

membrane associated protein Na+/K+ ATPase ~ 100 KDa

cytosolic protein NF-κB, p65 ~ 65 KDa

(B) BeWo, Jar, MCF7/P-gp

- P-gp
- BCRP
- actin

(C) DAPI, P-gp, Merge, NC - Merge

- BeWo
- Jar
- MCF7/P-gp

(D) ABCG2 Expression In BeWo Cells. Examined By FACS.

- Non Stained BeWo cells
- Non Specific Antibody
- ABCG2 Antibody

ABC2 Expression in JAR Cells. Examined By FACS.

- Non Stained JAR Cells
- Non Specific Antibody
- ABCG2 Antibody
Figure 2

Long-term exposure of BeWo cells to CBD: changes in P-gp and BCRP protein levels.
Figure 3

Long-term exposure of Jar cells to CBD: changes in P-gp and BCRP protein levels.
Figure 4

Long-term exposure of MCF/P-gp cells to CBD: changes in P-gp and BCRP protein levels.

(A) MCF7/P-gp – P-gp

(B) MCF7/P-gp – BCRP
Figure 5

Long-term exposure of BeWo and Jar cells to CBD: changes in BCRP and P-gp mRNA levels.
Figure 6

Non-cell type specific inhibition of P-gp by CBD 25µM.
Figure 7

Inhibition of P-gp by CBD 10µM in BeWo cells.