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**Expression and transcriptional regulation of ABC transporters and cytochromes P450
in hCMEC/D3 human cerebral microvascular endothelial cells**

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ABSTRACT

We investigated the expression of genes encoding ABC transporters, cytochromes P450 (CYPs) and some transcription factors in the hCMEC/D3 immortalised human cerebral microvascular endothelial cell line, a promising *in vitro* model of the human BBB, and we compared these expressions to a non-brain endothelial cell line (HUVEC) and freshly human brain microvessels. qRT-PCR showed that the *MDR1*, *BCRP*, *MRP1*, *MRP3*, *MRP4* and *MRP5* genes were expressed and that the main CYP gene was *CYP2U1* in hCMEC/D3. The pattern of ABC and CYPs gene expression in hCMEC/D3 differed from HUVEC which did not express *MDR1*. Moreover, expression of P-gp and BCRP was lower in hCMEC/D3 than in human brain microvessels but remain functional as shown by rhodamine 123 efflux assay. The gene encoding the aryl hydrocarbon receptor (AhR), a transcription factor that regulates the expression of some ABC and CYPs was highly expressed in hCMEC/D3 and HUVEC, while the pregnane-X-receptor (PXR) and the constitutive androstane receptor (CAR) were barely detected. We investigated the function of the AhR-mediated regulatory pathway in hCMEC/D3 by treating them with the AhR agonist TCDD. The expressions of two AhR target genes, *CYP1A1* and *CYP1B1*, were increased 26-fold and 28-fold. But the expressions of ABC transporter genes were not significantly altered. We have thus determined the pattern of expression of the genes encoding ABC transporters, CYPs and three transcription factors in hCMEC/D3 and shown that the AhR pathway might afford an original functional transport and metabolic pattern in cerebral endothelial cells that is different from other peripheral endothelial cells.

Keywords: hCMEC/D3 cell line, ABC transporters, CYP, AhR, blood-brain barrier

Running title: ABC transporters and CYP in hCMEC/D3

1. Introduction

The blood-brain barrier (BBB) is a neurovascular unit that is mainly composed of brain microvessel endothelial cells sealed by tight junctions to limit paracellular permeability under physiological conditions. The hCMEC/D3 cell line is an immortalized human cerebral microvascular endothelial cell line that retains most of the morphological characteristics of human blood-brain barrier endothelial cells. The many features of this cell line that have been examined suggest that it is a promising, reliable *in vitro* model of the human BBB for screening potential central nervous system drugs [1-4]. The BBB is not only a morphological barrier; it is also an active physical and metabolic barrier because of the expression of drug efflux transporters and drug-metabolizing enzymes in the endothelial cells. We have therefore investigated the expression of these transporters and enzymes in the hCMEC/D3.

ATP-binding cassette (ABC) efflux transporters, including P-glycoprotein (P-gp, *ABCB1/MDR1*), BCRP (*ABCG2*) and several MRPs (*ABCCs*) are expressed at the luminal membrane of the endothelial cells forming cerebral microvessels, where they actively limit the entry of numerous endogenous compounds and xenobiotics into the brain. P-gp is the best known of the many drug transporters at the BBB [5] and is mainly expressed at the luminal membrane of brain endothelial cells in both humans and animals [6, 7]. BCRP has also been found in primary cultures of human brain endothelial cells [8], and has been immunolocalised at the luminal membrane of human microvessel endothelial cells [9]. MRP1, MRP4, MRP5 and MRP6 have been detected in primary cultures of bovine brain endothelial cells [10], but only MRP1, MRP4 and MRP5 were found in the endothelial cells of human brain microvessels [11]. Although a previous study found P-gp, BCRP, MRP1 and MRP5 in hCMEC/D3 [12], their relative expression have not yet been determined.

The activity of drug metabolizing enzymes, especially phase 1 cytochromes P450 (CYPs), might also indirectly control the cerebral uptake of compounds from the blood [13].

Several CYP isoforms have been detected in the whole human brain (reviewed in [14]) and in isolated rodent brain microvessels [15, 16]. However, no data are available on the expression of CYPs at the human BBB, especially in hCMEC/D3.

The expression of the genes encoding ABC transporters and CYPs is regulated by transcription factors in several peripheral tissues. The pregnane-X-receptor (PXR) induces the expression of P-gp, CYP3A4 and CYP2C9, the constitutive androstane receptor (CAR) that of CYP2B6, while the aryl hydrocarbon receptor (AhR) mainly regulates the expression of CYP1A and CYP1B [17]. Although the presence of PXR, CAR and AhR at the rodent BBB have already been investigated, the findings remain controversial [18-20]. Despite a study showing that hPXR is expressed and functional at the BBB of hPXR transgenic mice, few data are currently available on the expression of these transcription factors at the human BBB. Moreover, expression of transcription factors in hCMEC/D3 has been poorly investigated, with the exception of the study of Zastre et al who showed the up-regulation of P-gp by PXR agonists [21]. Recently, we showed that CAR and PXR genes were only expressed at a very low level in isolated human brain microvessels whereas AhR was expressed at a very high level [22], raising the issue of its putative involvement in the regulation at the human BBB of its known target genes (ABC transporters and CYPs).

This study was performed to determine the expression profiles of the gene encoding ABC transporters, CYPs and the main factors regulating their transcription (PXR, CAR, AhR) in hCMEC/D3. We first compared the hCMEC/D3 profile with that of cultured non-brain human endothelial cells, namely umbilical vein endothelial cells origin (HUVEC), to determine the cerebral specificity of the hCMEC/D3, and with that of freshly isolated human brain microvessels to validate the hCMEC/D3 as a potent useful *in vitro* BBB model. We then focused on the regulation of ABC transporters and CYPs expression by transcription factors in hCMEC/D3 that were also identified in human brain microvessels.

2. Materials and methods

2.1. Reagents

The hCMEC/D3 were grown in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with ascorbic acid, hydrocortisone, basic FGF (Sigma, Saint Quentin Fallavier, France), fetal bovine serum (Eurobio, Les Ulis, France) and Hepes (PAA, Pasching, Austria). HUVEC were cultured in M199 medium, RPMI medium, amphotericine B, and glutamine from Invitrogen (Cergy-Pontoise, France). Type I collagen was obtained from BD Biosciences (Le Pont de Claix, France). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Cerilliant (Round Rock, TX, USA) and rifampicin (RIF), dimethylsulfoxide (DMSO) and Δ^9 -tetrahydrocannabinol (Δ^9 -THC) from Sigma-Aldrich.

Antibodies were obtained from the following sources: monoclonal mouse anti-P-glycoprotein C219 antibodies and monoclonal mouse anti-BCRP BXP-21 from Abcam, Cambridge, UK; rabbit antihuman CYP1B1 peptide serum WB-1B1 from BD Gentest, Woburn, MA; polyclonal rabbit antihuman CYP1A1 antibody was kindly provided by Dr I. de Waziers (Inserm U775S, Paris, France); monoclonal mouse anti- β -actin antibody from Sigma; horseradish peroxidase-conjugated anti-mouse secondary antibodies from Amersham, Buckinghamshire, UK; and alkaline phosphatase-conjugated anti-rabbit secondary antibody from Applied Biosystems, Foster City, CA. Proteins were detected using the ECL system (Amersham Biosciences Europe GmbH, Orsay, France) or the CDP-Star® chemiluminescent substrate (Sigma-Aldrich). Other chemicals and reagents were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) or from Invitrogen (Cergy-Pontoise, France).

2.2. Human brain tissue samples

Human tissues were collected after informed consent had been obtained from each patient, in accordance with the regulations of the ethics committee. Cerebral cortex samples

were obtained in the Department of Neurosurgery at Sainte-Anne Hospital (Paris, France) from patients undergoing surgery for epilepsy or glioma. The neurosurgeons provided samples of brain cortex (grey matter) localised as far as possible from the tumour or the epileptogenic lesion. They were considered to be healthy tissue on the basis of neuroimaging. Information on the patients participating to this study has already been described previously [22]. Samples were stored in RPMI buffer at 4°C for no more than 1 hour.

2.3. Cell culture

Human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr Isabelle Galy-Fauroux (INSERM U765, Paris, France). The human cerebral microvascular endothelial (hCMEC/D3) cells were plated out in T75 flasks coated with type I collagen and grown at 37°C in a humidified atmosphere of 5% CO₂. The cells were used at passages 27 to 32 for all experiments. They were plated out in T75 flasks coated with type I collagen and grown at 37°C under a humidified atmosphere of 5% CO₂. Cells were used at passage 3 of primary culture. When indicated, confluent cells were treated with 25 µM rifampicin (RIF) in DMSO (0.1% v/v) for 24 h, or with 25 nM TCDD in DMSO (0.016% v/v) for 8 h, 24 h, 48 h and 72 h, or with 5 µM Δ9-THC in ethanol (0.01% v/v) for 24 h. Control cells were treated with DMSO 0.1% (v/v), DMSO 0.016% (v/v) or ethanol 0.01% (v/v), as appropriate.

2.4. RNA extraction and reverse transcription

Total RNA was isolated from hCMEC/D3 cells, HUVEC and human brain microvessels using the RNeasy micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, including a DNase I treatment (Rnase-Free Dnase Set, Qiagen SA) to remove genomic DNA. The concentrations and purity of the RNA samples were assessed spectrophotometrically at 260 nm using the Nanodrop® ND-1000 instrument

(NanoDrop Technologies, USA), and the integrity of the RNA was checked by electrophoresis on 0.8 % agarose gels.

Reverse transcription was performed using 1 µg total RNA in a final reaction mixture (20 µL) containing 500 µM of each dNTP, 10 mM DTT, 1.5 µM random hexanucleotides primers (Amersham Biosciences, France), 20 U Rnasin ribonuclease inhibitor (Promega, France) and 100 U superscript II Rnase reverse transcriptase (Invitrogen, France). RT negative controls were prepared by omitting the reverse transcriptase from the mix. All samples were incubated at 25°C for 10 min, then at 42°C for 30 min and at 99°C for 5 min on a thermal cycler (PTC-100 programmable thermal controller, MJ research INC, USA). cDNAs were stored at -80°C.

2.5. Real-time quantitative RT-PCR (qPCR)

The expression of the genes encoding ABC transporters (*MDR1*, *BCRP*, *MRP1-6*, *MRP9*) and transcription factors (*PXR*, *CAR*, *AhR*) was analysed by qPCR on a Light-Cycler® instrument (Roche Diagnostics, Meylan, France) using SYBR Green fluorescence detection. The final reaction mixtures contained 5 µL diluted cDNA, 1 µL LC-FastStart DNA Master SYBR Green kit (Roche Diagnostics, Meylan, France), 0.5 µL of each primer 10 µM, 1.2 µL 10 mM MgCl₂ and 1.8 µL nuclease-free water. Specific primers for each gene were designed using OLIGO 6.42 software (MedProbe, Norway) and were for sequences on separate exons or on exon-exon junctions as far as possible to avoid amplifying genomic DNA. We performed no-template control assays for each primer pair and RT negative controls for each sample; they always produced negligible signals (usually > 40 in Ct value). We checked the size of the specific amplicons of interest and the absence of other PCR products by gel electrophoresis. We also performed melting curve analysis to ensure the specificity of each reaction. Primer sequences are shown in Table 1. All the primers used for qPCR analysis of

ABC transporter genes were checked with positive controls (liver and/or brain cortex). The qPCR of 23 CYPs genes was performed using SYBR Green fluorescence detection on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described [23]. The final reaction mix was composed of 3 μ L diluted cDNA, 5 μ L Absolute QPCR SYBR Green ROX Mix (Abgene, France), 0.3 μ L of each primer 10 μ M and 1.4 μ L nuclease-free water. The primers were kindly supplied by Dr I. de Waziers (University René Descartes, Paris, France). The primers for the 23 CYPs genes are available from Biopredic International (Rennes, France) (<http://www.biopredic.com>).

The genes of interest were those encoding ABC transporters: *MDR1*, *BCRP*, *MRP1*, *MRP2*, *MRP3*, *MRP4*, *MRP5*, *MRP6*, and *MRP9*; the CYPs: *1A1*, *1A2*, *1B1*, *2A6*, *2A7*, *2A13*, *2B6*, *2C8*, *2C9*, *2C18*, *2C19*, *2D6*, *2E1*, *2F1*, *2J2*, *2R1*, *2S1*, *2U1*, *2W1*, *3A4*, *3A5*, *3A7*, *3A43*; transcription factors: *PXR*, *CAR*, and one member of the basic helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) family: *AhR*.

The second derivative method was used, after proportional background adjustment, to determine the cycle at which the log-linear signal was distinguishable from the background, and this cycle number was used as the crossing-threshold value (Ct). Gene expression was evaluated using the Ct value from each sample. A target gene was considered to be quantifiable when the Ct obtained for the less dilute cDNA (1/20) sample was lower than 31. A Ct value of 33 was taken as the detection limit. The $\Delta\Delta$ Ct method was used to determine the relative expression of each target gene relative to those of others of the same family (CYPs and ABC transporters families) in hCMEC/D3, HUVEC and human brain microvessels. The expression of the target gene in each sample was normalised to that of the housekeeping gene encoding TATA box-binding protein (*TBP*): $\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{TBP}}$. The efficacy of each PCR for all CYPs genes was better than 95% [23], as was the efficacy of all other genes tested, making it possible to determine the relative expression of each target gene. Results are

expressed as percentages, with the total expression of all CYPs or ABC transporter genes being set at 100%. The $\Delta\Delta C_t$ method was also used to compare gene expression in hCMEC/D3 and brain isolated microvessels from 3 different human samples, for which the reverse transcription was performed with equal amount of starting mRNA, and to assess the expression of target genes in cells after treatment. This comparison was possible because the expression of the *TBP* gene in hCMEC/D3 and in brain microvessels as well as in hCMEC/D3 treated with vehicle (DMSO or ethanol) and the tested compound (RIF, TCDD or Δ^9 -THC) were not statistically different.

The expression of each target gene in hCMEC/D3 compared to brain microvessels was calculated from the comparative $\Delta\Delta C_t$ parameter using the formula:

$$\Delta\Delta C_t = (Ct_{\text{target gene}} - Ct_{\text{TBP}})_{\text{hCMEC/D3}} - (Ct_{\text{target gene}} - Ct_{\text{TBP}})_{\text{Brain microvessels}}$$

The $2^{-\Delta\Delta C_t}$ values are the difference (-fold) between the amounts of mRNA in the hCMEC/D3 and brain microvessels arbitrarily defined as 1.

The expression of each target gene in vehicle-cells and in treated-cells was calculated as follow: $\Delta\Delta C_t = (Ct_{\text{target gene}} - Ct_{\text{TBP}})_{\text{treated-cells}} - (Ct_{\text{target gene}} - Ct_{\text{TBP}})_{\text{vehicle-cells}}$

The $2^{-\Delta\Delta C_t}$ values are the difference (-fold) between the amounts of mRNA in the treated-cells and vehicle-cells, arbitrarily defined as 1.

2.6. Preparation of microsomal and mitochondria sub-fractions from hCMEC/D3 cells

All steps were carried out at 4°C. Treated hCMEC/D3 were washed 3 times with PBS, scraped free, and collected by centrifugation. These cells were homogenized with a Teflon Potter homogenizer (20 up/down strokes at 800 rpm) in cold buffer H (70 mM sucrose, 210 mM mannitol, 2 mM HEPES and 2 mM EDTA, pH 7.4). The homogenate was then centrifuged at 1500 g for 10 min. The pellet (nuclei and cell debris) was discarded and the supernatant was centrifuged at 12,000 g for 20 min. The resulting supernatant (cytosol and

microsomes) was ultracentrifuged at 100 000 *g* for 1 h. The pellet containing the microsomal fraction was suspended in 50 mM Tris-HCl, 1.15% KCl, 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 10% glycerol, pH 7.4. The 12 000 *g* pellet (containing mitochondria) was suspended in buffer H and centrifuged at 12 000 *g* for 20 min. The supernatant was discarded and the sediment was suspended in cold buffer H. The protein content of the microsomal and mitochondrial fractions was determined with the Bradford reagent (Sigma-Aldrich). They were then stored at -80°C.

2.7. Western blotting

The protein content of each sample was determined with the Bradford reagent (Sigma, Lyon, France) and a bovine serum albumin calibration curve. To compare protein expression in human brain microvessels and in hCMEC/D3, hCMEC/D3 or human microvessel samples were homogenized with a Teflon Potter homogenizer (20 up/down strokes at 800 rpm) in cold buffer H (70 mM sucrose, 210 mM mannitol, 2 mM HEPES and 2 mM EDTA, pH 7.4). The resulting homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant was used as protein samples for further western blotting experiments. Protein samples of human brain microvessels were that from patients n° 9, 10, and 11 of a previous study performed in our laboratory [22].

To test the effect of TCDD on P-gp and BCRP expression in hCMEC/D3, we used the microsomal fractions from vehicle- and TCDD-treated cells that were the same as that used for the EROD assay. All samples were normalised for equal amounts of protein, separated on 8% (P-gp) or 10% (BCRP, CYP1A1, CYP1B1) SDS-PAGE gels and electrotransferred to nitrocellulose membranes (HYBOND, Amersham). Non-specific binding sites were blocked by incubation overnight at 4°C with Tris-base buffer containing 0.1% Tween 20 (TBST) and 5% non-fat milk for P-gp and BCRP, or 0.2% I-Block™ (Applied Biosystem) for CYP1B1.

The membranes were immunoblotted with the C-219 (diluted 1:200), BXP-21 (1:200), antihuman CYP1A1 antibody (1:40 000) and WB-1B1 (1:10 000) for 2 h at room temperature, washed several times in TBST and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:10 000) or alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:20 000) for 45 min at room temperature. The membranes were then exposed to the Amersham ECL system (BCRP, P-gp), or to CDPStar system (CYP1A1, CYP1B1). Signals were revealed with the Bio-Rad ChemiDoc® XRS imaging device (BioRad, Marnes-la-Coquette, France). The blot was then stripped by immersion in 0.1 M acetic acid for 2 h, and reprobed with a monoclonal mouse anti- β -actin antibody (1:10 000). Proteins extracted from HEK-MDR1 cells and from HEK-BCRP cells, both supplied by Dr. RW. Robey (National Cancer Institute, Berkshire, UK), were used as positive controls for human P-gp and BCRP, respectively. Recombinant yeast microsomes expressing human CYP1A1 (provided by Dr I de Waziers, Paris, France) and human CYP1B1 supersomesTM (BD Gentest, Woburn, MA) were used as positive control for CYP1A1 and CYP1B1, respectively.

2.8. P-gp functional assay

The functional activity of P-gp in hCMEC/D3 cells was performed using the Rhodamine 123 (Rh123) efflux assay and flow cytometry analysis. hCMEC/D3 were grown to confluence, trypsinised and counted using a haemocytometer. For each experiment, 3.5×10^6 cells were centrifuged at 300 g for 5 min at 4°C and re-suspended in efflux buffer [RPMI-1640, (Lonza group Ltd, Switzerland)) containing 1 % w/v BSA] at 4°C. From the cell suspension, 2.5×10^5 cells were taken and placed on ice for the duration of the experiment (negative control). The remaining cells were then centrifuged (300 g, 5 min, 4°C), re-suspended at 1×10^6 /ml in efflux buffer containing 10 μ g/ml Rh123 (Sigma-Aldrich, Dorset,

UK) and incubated on ice for 1 h. Rh123-preloaded cells were washed twice in efflux buffer and redistributed into different tubes according to each treatment. For each repetition, 2.5×10^5 cells Rh123-preloaded cells were placed at 4°C in efflux buffer, whilst the remaining cells were incubated at 37°C in efflux buffer with cyclosporin A (40 µM) in vehicle or vehicle only. At 2 h, cells were placed on ice/4°C and then washed twice in ice-cold PBS. Cell suspensions were then read using a FACSCalibur with Cellquest software and the median fluorescence of 10,000 cells per sample recorded.

2.9. CYP1A/1B activity assay

We measured the ethoxyresorufin-O-deethylase (EROD) activity in the microsomes and mitochondrial fractions prepared from hCMEC/D3 treated with DMSO (vehicle) or TCDD. The assay medium (pH 7.4) was: 50 mM Tris, 25 mM MgCl₂, EDTA 0.1 mM and 20 µg of protein from the microsomal or mitochondrial fractions. The reaction was started by adding NADPH (62.5 µM final) and ethoxyresorufin (2 µM final). The formation of resorufin at 37°C was monitored for 15 min on a spectrofluorimeter (Tecan, Lyon, France). The excitation and emission wavelengths were 522 nm and 586 nm. The assay was checked using CYP1A2 supersomes (BD Biosciences).

2.10. Statistical analysis

Statistical analyses were done with GraphPad Prism® 4.0 software. The results are expressed as means \pm SD. Student's unpaired *t*-test was performed on Δ Ct values obtained before normalisation to unity to identify significant differences between cells treated with rifampicin, TCDD or Δ 9-THC and vehicle-treated cells. All the tests were two-tailed and statistical significance was set at $p < 0.05$.

1. Results

1.1. Expression of ABC transporter genes in hCMEC/D3, HUVEC and human brain microvessels

The expression of the genes encoding nine ABC transporters was evaluated by qPCR. hCMEC/D3 expressed significant, quantifiable amounts of *MDR1*, *BCRP*, *MRP1*, *MRP3*, *MRP4* and *MRP5* gene transcripts. *MRP2* and *MRP6* mRNAs were detected at a very low level, whereas *MRP9* mRNA was not. The expression profile of the *MDR1*, *BCRP*, *MRP1*, *MRP3*, *MRP4* and *MRP5* genes is shown in figure 1. The expression profile of the ABC transporters genes in human umbilical vein endothelial cells (HUVEC) was also determined and compared to that of hCMEC/D3. *MRP1* was the main ABC transporter gene expressed in HUVEC; lower amounts of the transcripts of *BCRP*, *MRP4* and *MRP5* genes were detected, while *MDR1* and *MRP3* mRNAs were not detectable (Fig. 1).

The *MRP4* and *MRP5* genes were 1.4-fold and 1.3-fold more expressed in hCMEC/D3 than in HUVEC, whereas the *BCRP* and *MRP1* genes were 2.9-fold and 3.7-fold more expressed in HUVEC than in hCMEC/D3. Since *MDR1* and *MRP3* mRNAs in HUVEC were not readily quantified by qPCR due to their very low concentrations, it was difficult to accurately determine the differences in *MDR1* and *MRP3* expression between hCMEC/D3 and HUVEC, but both genes seemed to be about 200-times more expressed in hCMEC/D3 than in HUVEC.

The expression of ABC transporters genes in hCMEC/D3 was also compared to that in freshly human brain microvessels. The figure 3 also shows that *MRP1*, *MRP4* and *MRP5* were more expressed in hCMEC/D3 than in brain microvessels, whereas it was the opposite for the expression of *MDR1* and *BCRP*.

1.2. CYPs genes in hCMEC/D3, HUVEC and human brain microvessels

We also investigated the expression of the genes encoding 23 CYPs from families 1 to 3. The expression profiles of CYP genes in hCMEC/D3 and HUVEC revealed that only 12 CYPs isoforms were detected (Fig. 2). The main isoforms expressed in hCMEC/D3 were *CYP2U1* and *CYP2S1*. The *CYP2R1*, *CYP2B6*, *CYP2E1*, *CYP1A1*, *CYP2D6* and *CYP2C18* genes were weakly expressed (0.2% - 2%), whereas the *CYP1B1* and *CYP2J2* genes were barely detectable. *CYP1A2* and *CYP2C8* were detected but were not quantifiable. None of the other CYP isoforms tested (*CYP2A6*, *CYP2A7*, *CYP2A13*, *CYP2C9*, *CYP2C19*, *CYP2F1*, *CYP2W1*, *CYP3A4*, *CYP3A5*, *CYP3A7* and *CYP3A43*) was detected in hCMEC/D3.

The expression profile of CYPs genes in HUVEC was determined and compared to that of hCMEC/D3 (Fig. 2). *CYP2U1*, *CYP1A1*, *CYP2R1* and *CYP1B1* were the main CYPs genes expressed in HUVEC. All the other CYPs genes were barely detected or not detected. Interestingly, *CYP1A1* and *CYP1B1* were 11-fold and 246-fold more expressed in HUVEC than in hCMEC/D3.

The expression of CYP genes in hCMEC/D3 was also compared to that in freshly human brain microvessels. All the CYP genes detected in hCMEC/D3 were detected in microvessels except *CYP2C18* and were expressed at a similar level in hCMEC/D3 and in brain microvessels, except *CYP2J2* that was up-regulated in hCMEC/D3 (Fig. 3).

1.3. Transcription factors in hCMEC/D3 and HUVEC

Since both ABC transporters and CYPs are regulated by transcription factors like the pregnane-X-receptor (PXR), the constitutive androstane receptor (CAR) and the aryl hydrocarbon receptor (AhR) in several other human tissues than brain, we determined the expression of the genes encoding these transcription factors in hCMEC/D3 and HUVEC by qPCR. *AhR* transcripts were highly expressed in hCMEC/D3 and about twice as abundant in

hCMEC/D3 as in HUVEC, while *PXR* mRNAs were detected but not quantifiable in our experimental conditions in both cell types. *CAR* transcripts were detected in neither hCMEC/D3 nor HUVEC.

1.4. Expression of P-gp and BCRP in hCMEC/D3 and human brain microvessels

Western blotting experiments were performed to compare the expression of P-gp and BCRP in freshly human brain microvessels and in hCMEC/D3. Figure 4 shows western blots of P-gp, BCRP and the reference β -actin in isolated microvessels from 3 patients (see materials and methods) and hCMEC/D3 samples. P-gp was detected at the expected molecular weight (170 kDa) in the isolated microvessels with different intensities in various patients and in hCMEC/D3. BXP-21 antibody was used to detect BCRP protein in isolated human brain microvessels and in hCMEC/D3. A band at the expected molecular weight (70 kDa) was observed for the 3 human samples and in hCMEC/D3. Both BCRP and P-gp were expressed at a clearly lower level in hCMEC/D3 than in isolated microvessels.

1.5. Functional activity of P-gp in hCMEC/D3 cells

In order to assess the functional activity of P-gp in hCMEC/D3 cell line, we measured the efflux of Rh123 from pre-loaded Rh123 hCMEC/D3, a fluorescent dye that is widely known as a substrate for P-gp, at 37°C and with or without cyclosporin A, an inhibitor of P-gp. Flow cytometry data showed that a large amount of Rh123 was able to reach cytoplasm by freely crossing plasma membrane at 4°C, a condition in which active transport is inhibited (Fig. 5). When cells were incubated at 37°C, there was a decrease in the median fluorescence of cells due to P-gp-mediated Rh123 efflux. By contrast, in the presence of cyclosporin A, Rh123 median fluorescence of hCMEC/D3 at 37°C dramatically increased and was close to that observed at 4°C, indicating that Pgp actively extruded Rh123 from hCEMC/D3.

1.6. Effect of Rifampicin (RIF), TCDD and Δ 9-THC on the mRNAs of ABC transporters and CYPs in hCMEC/D3 cells

We investigated the potential function of these transcription factors by treating cells with agonists of PXR and AhR. Cells were incubated with the PXR agonist, RIF (25 μ M) for 24 h (Table 2). The expressions of the *MRP3* and *MRP5* genes were slightly increased, together with a slight increase in the expression of the *CYP2D6* gene. However, an increase in gene expression of less than 1.5-fold probably has little effect on function. The expressions of the other ABC transporters and CYPs genes were not significantly altered.

AhR mRNA were abundant in hCMEC/D3. We therefore investigated the regulation of ABC transporters and CYPs genes by AhR in these cells by treating them for 24 h with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent AhR ligand. TCDD treatment increased the expressions of *CYP1A1* (26.3-fold) and *CYP1B1* (28.4-fold) (Table 2), while the expression of all the other CYPs genes were not altered. The increase in expressions of *CYP1A1* and *CYP1B1* was also observed in the CYPs gene expression profile (Fig. 6). The increases in *CYP1A1* and *CYP1B1* expression occurred after exposure to TCDD for 8 h and remained stable until 72 h (Fig. 7).

hCMEC/D3 were also incubated for 24 h with 5 μ M Δ 9-THC, the psychoactive component of marijuana, whose planar structure resembles that of TCDD, and which may be a AhR ligand [24]. Δ 9-THC did not significantly modulate the expression of any of the ABC transporter genes but slightly increased the expression of the *CYP1A1* gene (Table 2).

1.7. Effect of TCDD on P-gp, BCRP, CYP1A1 and CYP1B1 expression in hCMEC/D3

Although TCDD did not increase the expression of *BCRP* and *MDR1* genes in hCMEC/D3 (Table 2), we studied its effect on P-gp and BCRP proteins expression since AhR

ligands has been reported to increase the amounts of P-gp and BCRP in human liver and caco-2 cells [25-27]. Western blotting analysis of P-gp and BCRP were performed on microsomes from hCMEC/D3 incubated with TCDD or DMSO for 48 h. P-gp and BCRP proteins were detected in cells at their expected molecular weights (170 kDa and 70 kDa, respectively) but their expression were not altered by TCDD (Fig. 8).

CYP1A1 and CYP1B1 proteins were not detected by western blotting in either microsomes or mitochondrial fractions from vehicle-treated or TCDD-treated cells, whereas the positive controls, recombinant yeast expressing human CYP1A1 or CYP1B1 supersomes both produced bands of the expected size (58 kDa and 61 kDa respectively) (data not shown).

1.8. Effect of TCDD on CYP1A1/1B1/1A2 activities

We used the EROD assay to measure the functional activity of TCDD-induced CYP1A1 and CYP1B1 in hCMEC/D3 as it simultaneously assesses CYP1A1/1A2/1B1 activities [28]. This assay was validated by incubating ethoxyresorufin with CYP1A2 supersomes (0.05 to 0.5 pmol of CYP1A2) for 15 min and by measuring the CYP1A2-mediated formation of resorufin in the medium. The lowest CYP1A2 activity (lower limit of quantification) was obtained with 0.05 pmol CYP, corresponding to the activity of 0.5 μ g total protein from CYP1A2 supersomes. This confirmed the high sensitivity of the test. The brain contains CYPs in the endoplasmic reticulum as well as in the inner membrane of mitochondria [29]. We therefore measured the EROD activities of both microsomal and mitochondrial fractions. No EROD activity was detected in either microsomes or mitochondrial fractions when 20 μ g of total proteins from TCDD- or Δ 9-THC treated cells were incubated with ethoxyresorufin for 15 min. The assay also revealed no activity in whole living cultured cells treated with TCDD or Δ 9-THC for 48 h (data not shown).

DISCUSSION

The hCMEC/D3 cell line that we recently derived from a primary culture of cerebral microvessel endothelial cells, has been shown by us and others to display a stable phenotype including most of the properties of the BBB. This model has been validated according multiple criteria [1, 3]. However, very few data are available showing that these cells express the drug efflux transporters that are considered to be phenotypic markers of the human BBB. This study compares the expression of genes encoding several ABC transporters, CYPs and their main transcription factors in hCMEC/D3, human brain microvessels and in non-cerebral endothelial cells, HUVEC, to determine whether hCMEC/D3 have a cerebral endothelium specific profile that differs from that of peripheral endothelial cells. We find that the pattern of ABC transporters and CYPs gene expression in hCMEC/D3 differs from that of HUVEC. Brain microvessel endothelial cells express ABC efflux transporters at their luminal membrane *in vivo* [5]. P-gp, the best known ABC transporter, lies mainly at the luminal membrane of human brain microvessel endothelial cells [7, 30], as well as in primary cultures of human brain microvessel endothelial cells [6, 31, 32]. BCRP, another ABC transporter [33], also lies at the luminal membrane of microvessel endothelial cells, and in primary cultures of brain microvessel endothelial cells [8, 31]. The profile of ABC transporters gene expression in hCMEC/D3 is distinct from that of the non-brain HUVEC. The most remarkable difference is the lack of *MDR1* gene expression in HUVEC. While hCMEC/D3 have been shown to express high amounts of MDR1/P-gp, BCRP and MRP1 mRNAs and proteins [12], their relative abundances were unknown. Our results show that the levels of expression of *MDR1* and *BCRP* in hCMEC/D3 are similar, but lower than in isolated human brain microvessels, a result which was also confirmed at the protein level. However, we confirmed that P-gp was functional in hCMEC/D3, as previously reported using another assay and substrate [12]; it is noteworthy that BCRP was also found functional in hCMEC/D3 [12].

1
2 In contrast to P-gp and BCRP, the expression of MRPs at the human BBB remains
3 controversial. We detected *MRP3* and *MRP4* mRNAs in addition to those of *MRP1* and
4 *MRP5* [12], but the *MRP2* and *MRP6* mRNAs were almost unquantifiable. This is in
5 agreement with a study that detected *MRP1*, *MRP4* and *MRP5* in human brain endothelial
6 cells by immunohistochemistry [34]. The large amount of *MRP1* mRNA in hCMEC/D3 may
7 be due to cell culture since the expression of *MRP1* is known to be up-regulated during cell
8 culture [6]. Similarly, *MRP1* is highly expressed in primary cultures of human brain
9 microvessel endothelial cells [31].

10
11 We have also determined the gene expression profiles of a wide range of CYPs genes
12 in hCMEC/D3, HUVEC and human brain microvessels. CYP activities have been detected in
13 isolated rat brain microvessels [15]. Many studies have shown the presence of several CYPs
14 isoforms in the whole human brain (for review see [14]), but no data are available for human
15 brain microvessel endothelial cells. Our results show that *CYP2U1* is the main gene expressed
16 in hCMEC/D3. *CYP2U1* is a recently described extrahepatic isoform [35] that is expressed in
17 the thymus and brain and is involved in the metabolism of arachidonic acid and other long
18 chain fatty acids [36]. Two other extrahepatic CYP genes, *CYP2S1* and *CYP2R1*, are express
19 in hCMEC/D3. *CYP2S1* has been shown to metabolise naphthalene and *CYP2R1* to be
20 involved in vitamin D metabolism [37]. However, the role of these three isoforms in the
21 metabolism of drugs remains to be demonstrated. The *CYP3A4*, *CYP2C9* and *CYP2D6* genes
22 that are involved in the hepatic metabolism of many marketed drugs are not expressed in
23 hCMEC/D3. We also find that profile of CYPs gene expression in hCMEC/D3 is distinct
24 from that of HUVEC, especially for the *CYP2R1*, *CYP1A1* and *CYP1B1*, which are much
25 more express in HUVEC than in hCMEC/D3, whereas the reverse holds for *CYP2U1* and
26 *CYP2S1*. Thus our findings suggest that the expression profile of CYPs in brain microvessel
27 endothelial cells is quite specific and does not resemble that of peripheral endothelial cells.

Moreover, the gene expression of the main CYP detected in hCMEC/D3 was close to that in isolated human brain microvessels, except for CYP1B1 which was highly expressed in human brain microvessels [22]. Interestingly, we show that many of the major CYP biotransformation enzymes involved in the metabolism of the vast majority of commercial drugs are not highly expressed either in human brain microvessels or in hCMEC/D3, suggesting that the brain endothelium may not play an active role in their metabolism. However, it must be stressed that, brain endothelial cells working in concert with the entire neurovascular unit, our results may not reflect properly the actual drug metabolism capacity of the BBB.

The expression of genes encoding ABC transporters and CYPs in several peripheral tissues is regulated by common mechanisms involving the activation of transcription factors, including two members of the nuclear receptor superfamily: PXR and CAR, and a member of the bHLH/PAS family: AhR [17]. Both PXR and CAR form heterodimers with the retinoid X receptor (RXR) and activate the transcription of some ABC transporters and CYPs genes [38]. Few data are available on the regulation of ABC transporters and CYPs gene transcription in the human brain, especially at the BBB. PXR and CAR have been found in the whole human brain, but not in some regions, including the frontal and temporal lobes and the cerebellum [39]. Fewer studies have examined the expression of PXR and CAR at the human BBB. A recent report indicated that neither *PXR* nor *CAR* were detected in isolated rat brain microvessels [19]. We have detected *PXR* transcripts in hCMEC/D3, but their amounts were very low, and we failed to detect *CAR* mRNA. Treatment of hCMEC/D3 with the PXR agonist, rifampicin, had little effect on the expression of ABC transporters and CYPs genes and induced only a small increase in *MRP3* transcription, as already described in the human hepatoma cell lines HuH7 and HepG2 [40]. *MRP5* expression was also increased after RIF treatment, in accordance with one study reported the up-regulation of *MRP5*, *MDR1* and

$MRP2$ in the brain endothelial cells of epileptic patients treated with anti-epileptic drugs that are ligands of PXR and/or CAR [41]. However, although the expression of $MDR1$ and $MRP2$ genes is induced by RIF via a nuclear receptor mediated pathway [42, 43], hCMEC/D3 incubated with RIF treatment showed no increased expression of those two genes. These results are in contradiction to a report that PXR activation induces the expression of P-gp and Mrp2 in rat brain isolated microvessels [18]. Recently, Zastre *et al* showed that RIF treatment of hCMEC/D3 for 72 h at 10 μ M increased P-gp expression by 1.8-fold [21]; in addition, PXR activation was also shown to induce P-gp activity at the BBB of transgenic mice expressing hPXR [44] and to increase Mrp2 expression at the BBB of rats and hPXR transgenic mice [45]. On the basis of these results, we cannot exclude that we failed to detect P-gp induction in the present study because RIF treatment was limited to 24h. We also report that RIF has no effect on $MRP4$ expression: this is in line with the lack of Mrp4 regulation observed in rat and hPXR transgenic mice brain capillaries after treatment with PXR agonists [46]. We show here that $CYP2D6$ expression is slightly increased by RIF. However, a recent study did not detect any PXR involvement in the regulation of $CYP2D6$ gene expression [47]. Therefore, this slight induction may be mediated by another regulatory pathway. In contrast, $CYP3A4$, $CYP2C9$, $CYP2C8$, $CYP2C19$ and $CYP2B6$ are all induced via PXR [43, 48, 49] but these isoforms remained undetected after treatment with RIF. Our results suggest that there is probably a weak PXR-mediated regulatory pathway in hCMEC/D3.

In contrast to those of PXR and CAR , the transcripts of AhR are abundant in hCMEC/D3 and HUVEC. AhR-target genes have been shown to be induced in HUVEC [50]. Upon ligand binding, AhR forms a heterodimer with the AhR nuclear translocator (ARNT), and so modulates the transcription of target genes like $CYP1$ (for review [51]). Since AhR is functional in mouse brain endothelial cells [20], we investigated the AhR-mediated regulatory pathway in hCMEC/D3 by treating them with TCDD, a potent agonist of AhR. The

expression of *CYP1A1* and *CYP1B1* was strongly induced (26.3- and 28.4-fold) by TCDD treatment. As AhR is known to activate *CYP1A* and *CYP1B* genes [51], this induction may be mediated by the AhR pathway in hCMEC/D3. Interestingly, the induction of CYP1B1 peaked after exposure to TCDD for 8 h and decreased over time. This is frequently observed for compounds that are metabolized by the cells, but is unusual for TCDD for which the induction of its target genes remained most often stable until 72 h. Although we felt that elucidating this result was beyond the scope of the present study, we might hypothesize that TCDD was metabolized by CYP1B1: however, this is unlikely since we failed to detect CYP1A/1B activities in TCDD-treated hCMEC/D3. Alternatively, we hypothesize that a down-regulation of AhR may affect CYP1B1 induction during TCDD incubation. In any case, this decrease in CYP1B1 fold-induction by TCDD over time has also been observed at the protein level with murine cerebral endothelial cells [20]. In this study, CYP1B1 has been detected at the human blood-brain interface of the temporal lobe of patients with glioma and was induced in a human astrocytoma cell line by DMBA, another AhR agonist [52]. However, others have found that CYP1A1 and CYP1B1 proteins are expressed in several mouse blood-brain interfaces and their expression is increased by AhR agonists. These two CYPs have not been detected in brain capillaries [16]. While we find that the expression of *CYP1A1* and *CYP1B1* is induced in hCMEC/D3, the expression and the functional activity of the corresponding proteins were not detected in the microsomes and mitochondria of either control cells or TCDD-treated cells. Several hypotheses may explain such results. First, several normal human tissues have been found to lack CYP1B1 protein despite having the corresponding mRNA [53, 54]. This difference between gene expression and protein content may be due to low protein expression or to post-transcriptional regulation, as in other human cell lines [55]. Interestingly, *CYP1A1* and *CYP1B1* were dramatically less expressed in hCMEC/D3 than in HUVEC and the functional activity of CYP1A/1B measured by EROD

has already been observed in HUVEC whereas we failed to detect any EROD activity in TCDD-treated hCMEC/D3 [50]. Thus, we may postulate that the absence of EROD activity observed in hCMEC/D3 may be due to the very low CYP1A1 and CYP1B1 expression even after induction by TCDD. Second, there is increasing evidence that extrahepatic and especially brain CYPs like CYP1A1 suffer from impaired heme availability, which negatively affects their enzymatic activity especially in microsomes and mitochondria of cell lines and tissue [56, 57]. That may explain the lack of CYP1A/1B catalytic activity observed here. Although CYP1A1 and CYP1B1 proteins were not detected in hCMEC/D3, the activity of AhR observed in these cells suggests that this regulatory pathway may be present at the human BBB and responsible for the regulation of CYP1A1 and CYP1B1. Furthermore, hCMEC/D3 may well be suitable for investigating the capacities of new chemical entities to enhance AhR-mediated CYP1A/1B gene expression. AhR is activated by a wide range of ligands, including polycyclic aromatic hydrocarbons (PAHs) like coplanar polychlorinated biphenyls (PCBs) and benzo[*a*]pyrene, the latter being present in cigarette smoke. These are considered as environment contaminants [58]. These compounds may induce some AhR target genes, including *CYP1A1* and *CYP1B1* at the human BBB. This induction may generate adverse effects, as CYP1B1 activates many procarcinogens and promutagens in potentially neurotoxic metabolites [59]. Although *CYP2S1* gene was shown to be inducible by dioxin via an AhR-mediated regulatory pathway, notably in a human lung cell line [60], no induction of this gene was observed here. This may be due to tissues-specific differences.

In contrast to CYPs, the expression of none of the ABC transporters genes studied was significantly changed by any of the TCDD concentrations tested or by the exposure time. TCDD had no influence on the expression of BCRP mRNA or protein, although two recent studies report the up-regulation of *BCRP* in Caco-2 cells [27] and in primary human hepatocytes [26] treated with AhR ligands (benzo[*a*]pyrene and TCDD respectively).

Similarly, TCDD increased the expression of *MDR1* in primary human hepatocytes [26], although we find no induction of this transporter, in terms of mRNA or protein hCMEC/D3. We find that TCDD does not modify the expression of any of the *MRP* genes, whereas TCDD was shown to induced the expression of Mrp2, Mrp3 and Mrp5 proteins in the mouse liver [61]. These differences may be due to tissue-specific and/or species-specific differences.

The psychoactive component of marijuana, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), is a PAH that is reported to induce expression of the *CYP1A1* gene in murine Hepa-1 cells *via* an AhR-mediated mechanism [24]. Our study of the effect of Δ^9 -THC on the expression of the ABC transporter and CYP genes in hCMEC/D3 showed that *CYP1A1* gene is the only gene slightly induced, suggesting that Δ^9 -THC is not a very potent AhR ligand.

In conclusion, our data provide an extensive characterisation of the ABC transporters and CYPs genes expressed in the human cerebral microvascular endothelial cell line hCMEC/D3, which was previously proposed as a unique model of human BBB. They reveal that (i) hCMEC/D3 cells express the ABC transporters present in freshly isolated human brain microvessels, including functional P-gp and BCRP, although their relative expression appears lower in hCMEC/D3 cells; (ii) expression of the main CYP genes in hCMEC/D3 is close to that in isolated human brain microvessels, except for CYP1B1 which is highly expressed in human brain microvessels; (iii) hCMEC/D3 cells and human brain microvessels express the transcription factor AhR which is involved in up-regulation of CYP1B1 by TCDD in hCMEC/D3 cells. Altogether, these results provide evidence that the hCMEC/D3 cell line may constitute to some extent a valuable model of human brain microvessels for pharmacological, toxicological or metabolic studies; they also strongly suggest that drug metabolism processes at the human BBB are highly dependent upon CYP1B1 and thus significantly differ from those in peripheral organs.

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Abbreviations

ABC: ATP-binding cassette

AhR: aryl hydrocarbon receptor

BBB: blood–brain barrier

BCRP: breast cancer resistance protein

CAR: constitutive androstane receptor

CNS: central nervous system

CYP: cytochromes P450

MDR: multidrug resistance

MRP: multidrug resistance-associated protein

P-gp: P-glycoprotein

PXR: pregnane xenobiotic receptor

Figure legends

Fig. 1. Expression profiles of ABC transporters genes in hCMEC/D3 and HUVEC. Results are expressed as percentages (mean +/-SD) of the total ABC mRNAs in each cell type (n = 4 independent hCMEC/D3 or HUVEC cultures).

Fig. 2. Expression profiles of CYPs genes in hCMEC/D3 and HUVEC. Results are expressed as percentages (mean +/-SD) of the total CYPs mRNAs in each cell type (n = 4 independent hCMEC/D3 or HUVEC cultures).

Fig. 3. Comparative gene expression of ABC transporters and CYPs between human brain microvessels and hCMEC/D3. Results are expressed as the relative gene expression in hCMEC/D3 cells as compared to human brain microvessels normalized to the unity. See materials and methods.

Fig. 4. Expression of P-gp (A) and BCRP (B) in human brain microvessels and hCMEC/D3 by western blotting. Positive controls were proteins extracts from HEK-MDR1 for P-gp and HEK-BCRP cells for BCRP. All samples were mainly soluble and membrane proteins prepared from the supernatant (12,000 g) after cell lysis of hCMEC/D3, HEK-MDR1, HEK-BCRP cells or isolated brain microvessels (see materials and methods). The blots were incubated with the C219 anti-P-gp antibody (A) or the BXP-21 anti-BCRP antibody (B).

Fig. 5. Pgp-mediated drug efflux of Rh123 by hCMEC/D3. Cells in suspension (10^6 cells/ml) were incubated with Rh123 (10 mg/ml) for 1 h at 4°C. Cells were then washed and incubated at 4°C in buffer alone or at 37 °C in buffer alone or containing 40 µM cyclosporin A for 2 h. Results are from one experiment representative of two.

Fig. 6: Expression profiles of CYPs genes in hCMEC/D3 treated with 25 nM TCDD or vehicle for 24 h (results are means \pm SD of 4 independent cell cultures).

Fig. 7: Time-course of the induction of *CYP1A1* and *CYP1B1* genes in hCMEC/D3 treated with 25 nM TCDD and in vehicle-treated cells (results are means \pm SD of 4 independent cell cultures).

Fig. 8: Effect of TCDD on P-gp and BCRP expression in hCMEC/D3 cells. P-gp, BCRP and β -actin proteins detected by western blotting in hCMEC/D3 treated with vehicle or TCDD. Positive controls were proteins extracts from HEK-MDR1 for P-gp and HEK-BCRP cells for BCRP. MW represents approximate molecular weights of these proteins. Each lane was loaded with 20 μ g of total proteins from microsomal preparation (see materials and methods).

Table 1: Sequences of primers used for qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (3'-5')	Length (bp)	GenBank accession*
<i>TBP</i>	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA	132	<u>NM 003194</u>
<i>MDR1</i>	CACCCGACTTACAGATGATG	GTTGCCATTGACTGAAAGAA	81	<u>NM 000927</u>
<i>BCRP</i>	TGACGGTGAGAGAAAACCTTAC	TGCCACTTTATCCAGACCT	122	<u>NM 004827</u>
<i>MRP1</i>	GCCGAAGGAGAGATCATC	AACCCGAAAACAAAACAGG	109	<u>NM 004996</u>
<i>MRP2</i>	CGACCCTTTCAACAACACTACTC	CACCAGCCTCTGTCACTTC	119	<u>NM 000392</u>
<i>MRP3</i>	GTGGGGATCAGACAGAGAT	TATCGGCATCACTGTAAACA	99	<u>NM 020038</u>
<i>MRP4</i>	GCTCAGGTTGCCTATGTGCT	CGGTTACATTTCTCCTCCA	100	<u>NM 005845</u>
<i>MRP5</i>	CAGCCAGTCCTCACATCA	GAAGCCCTCTTGTCTTTTTT	121	<u>NM 005688</u>
<i>MRP6</i>	AGGAGGCCCGAGCTTAGAC	CCTGCCGTATTGGATGCTGT	147	<u>NM 001171</u>
<i>MRP9</i>	ATGCGGTTGTCACTGAAG	GTTGCCTCATCCATAATAAGAAT	115	<u>NM 020298</u>
<i>PXR</i>	CGCTTCCTGAGTCTTTTCA	CGCCTGCCGATGAGTACA	115	<u>NM 003889</u>
<i>CAR</i>	GGGGTTCCAGGTAGAGTTT	GTCGGTCAGGAGAGAAGAG	122	<u>NM 005122</u>
<i>AhR</i>	AGAGGCTCAGGTTATCAGTTT	AGTCCATCGGTTGTTTTTT	132	<u>NM 001621</u>

* gene accession number at www.ncbi.nlm.nih.gov

Table 2: Effects of RIF, TCDD and $\Delta 9$ -THC on the mRNA levels of ABC transporters and CYPs in hCMEC/D3.

Gene	RIF 25 μ M	TCDD 25 nM	$\Delta 9$ -THC 5 μ M
<i>ABC transporters</i>			
<i>MDR1</i>	1.31 \pm 0.25	1.31 \pm 0.17	0.81 \pm 0.12
<i>BCRP</i>	1.03 \pm 0.17	1.90 \pm 1.28	0.95 \pm 0.14
<i>MRP1</i>	1.26 \pm 0.62	1.19 \pm 0.42	0.86 \pm 0.09
<i>MRP3</i>	1.24 \pm 0.12*	1.09 \pm 0.39	0.95 \pm 0.08
<i>MRP4</i>	1.10 \pm 0.38	1.15 \pm 0.16	0.86 \pm 0.08
<i>MRP5</i>	1.36 \pm 0.20**	1.17 \pm 0.18	0.88 \pm 0.07
<i>Cytochromes P450</i>			
<i>CYP1A1</i>	1.81 \pm 1.37	26.31 \pm 10.43***	1.54 \pm 0.31*
<i>CYP1B1</i>	1.27 \pm 0.54	28.37 \pm 17.43**	1.08 \pm 0.16
<i>CYP2B6</i>	1.14 \pm 0.30	0.95 \pm 0.37	0.86 \pm 0.37
<i>CYP2C18</i>	1.04 \pm 0.29	1.38 \pm 0.66	0.88 \pm 0.46
<i>CYP2D6</i>	1.29 \pm 0.22*	0.84 \pm 0.32	0.98 \pm 0.06
<i>CYP2E1</i>	0.98 \pm 0.23	1.21 \pm 0.19	1 \pm 0.23
<i>CYP2J2</i>	0.86 \pm 0.15	0.95 \pm 0.22	0.97 \pm 0.71
<i>CYP2R1</i>	0.88 \pm 0.11	0.97 \pm 0.15	0.92 \pm 0.18
<i>CYP2S1</i>	1.03 \pm 0.08	1.19 \pm 0.02	0.81 \pm 0.1
<i>CYP2U1</i>	0.96 \pm 0.1	0.91 \pm 0.21	0.91 \pm 0.12

Results are expressed in fold-change in gene expression between control (vehicle) cells and treated cells. Cells were exposed for 24 h with each compound. Results are means (\pm -SD) of 4 independent experiments. * p <0.05; ** p <0.01; *** p <0.001.

Figure 1

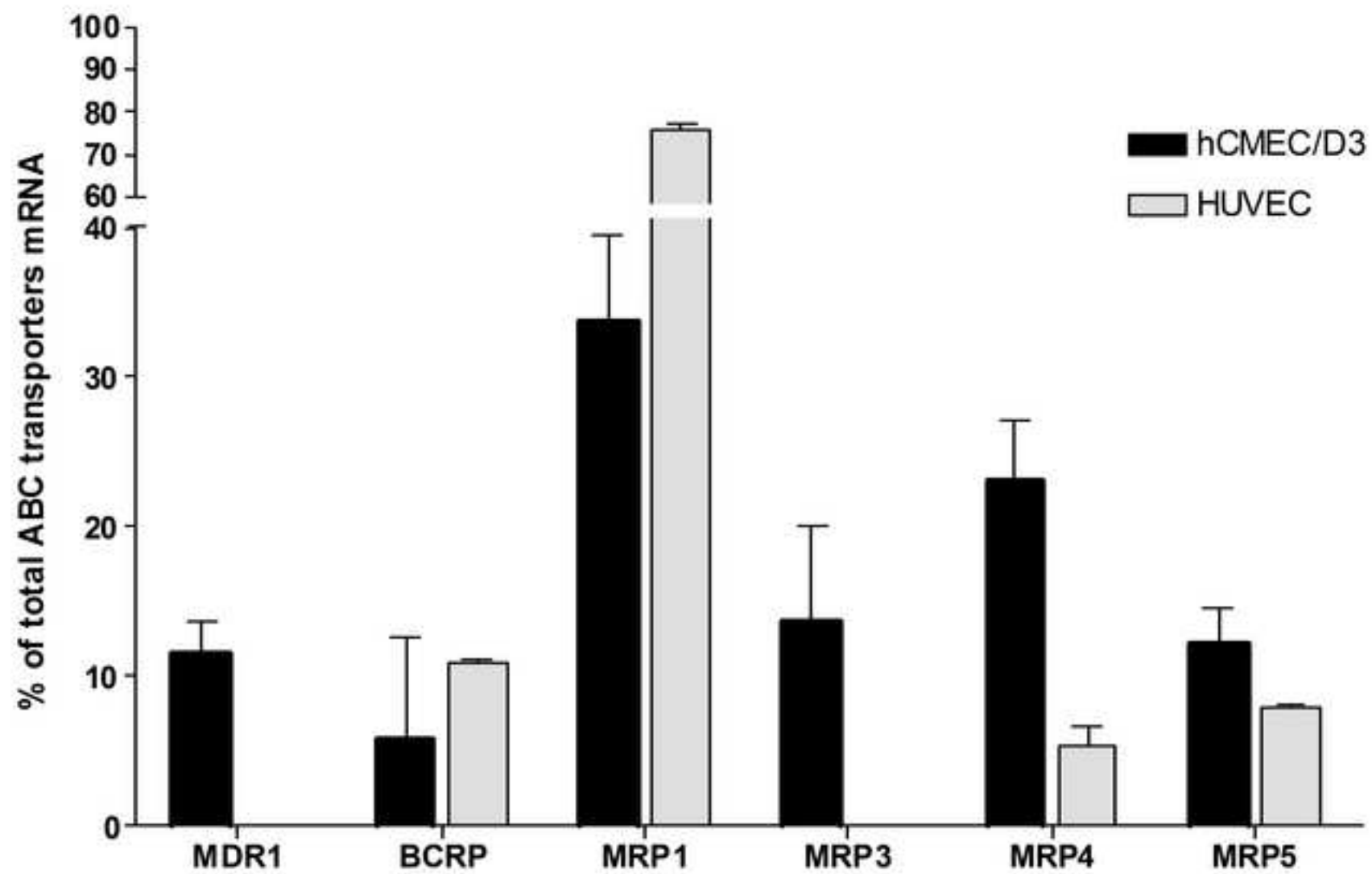


Figure 2

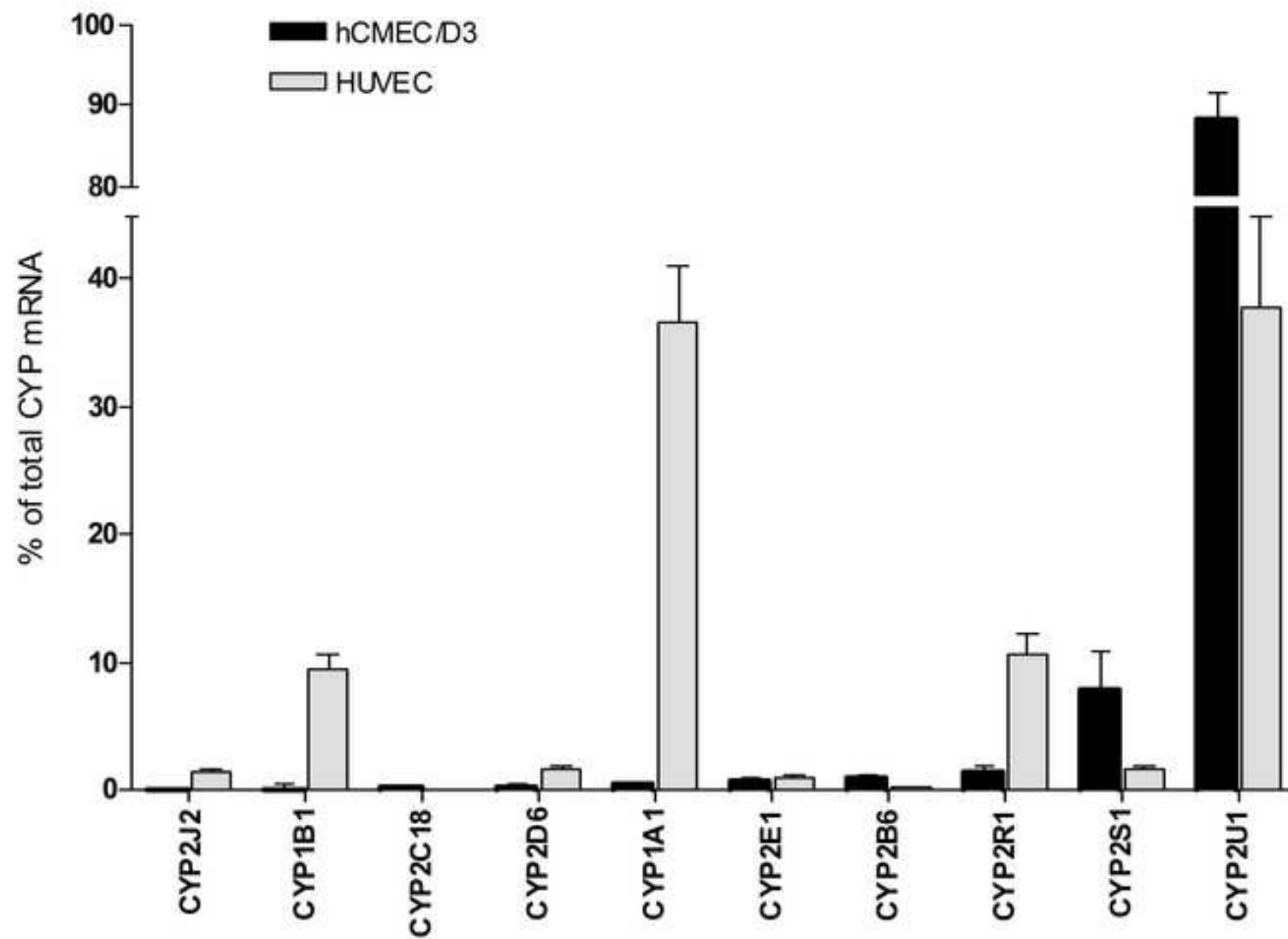


Figure 3

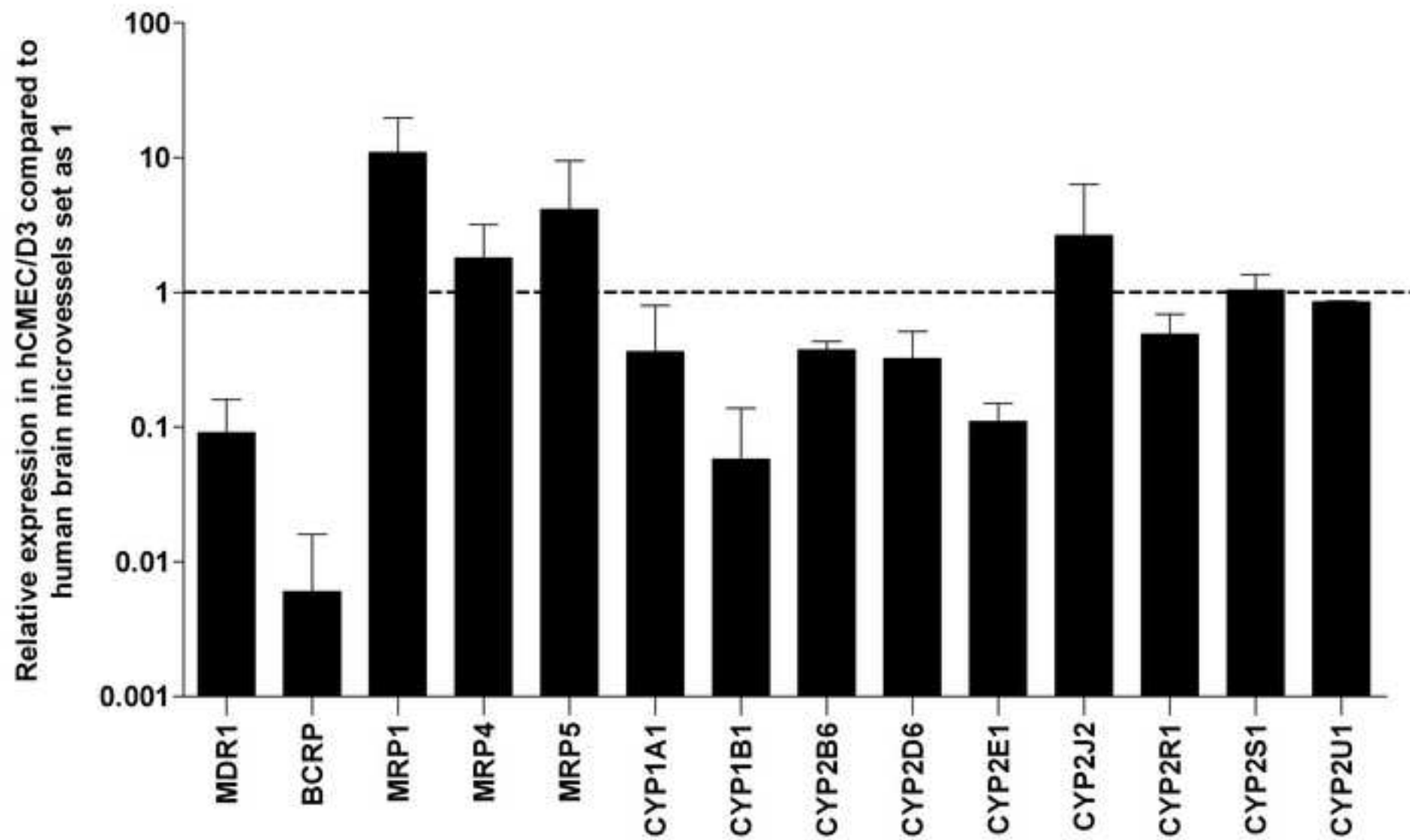
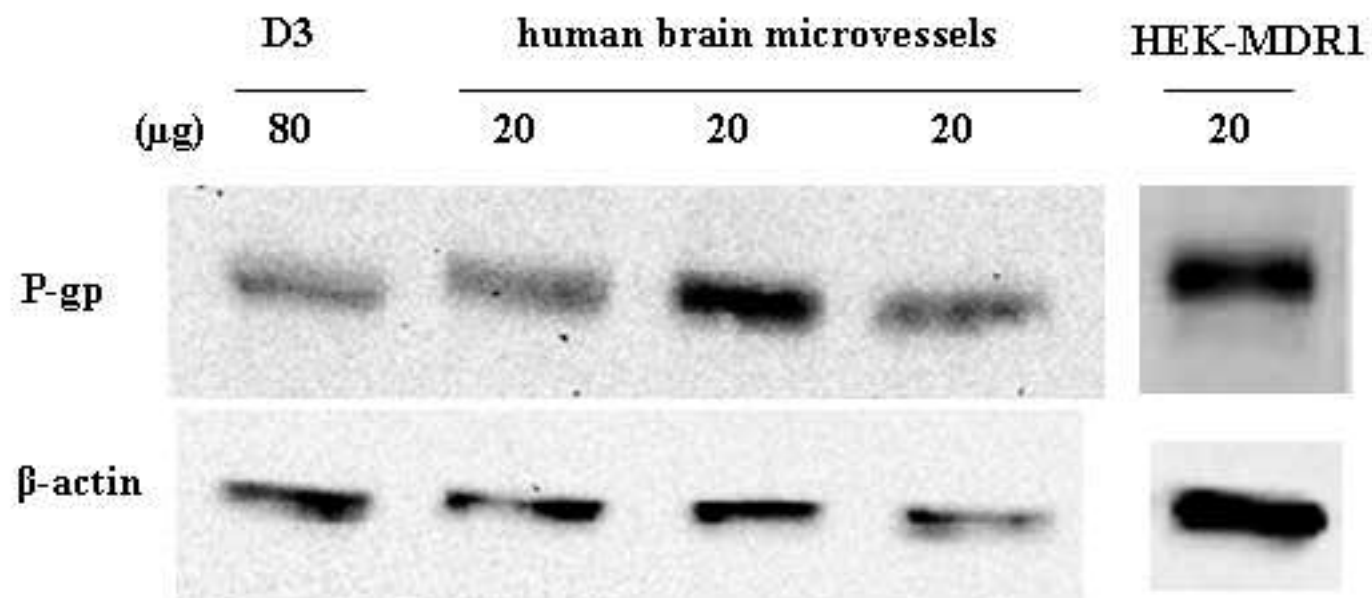


Figure 4

A



B

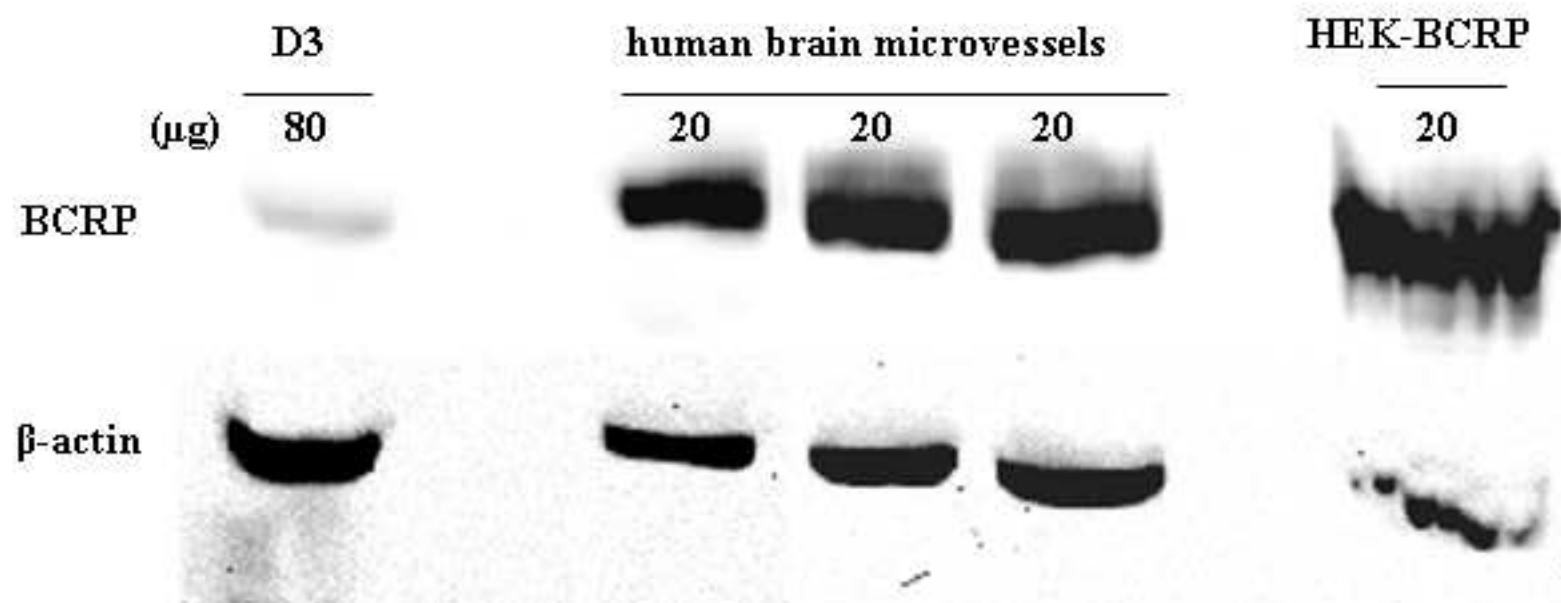


Figure 5

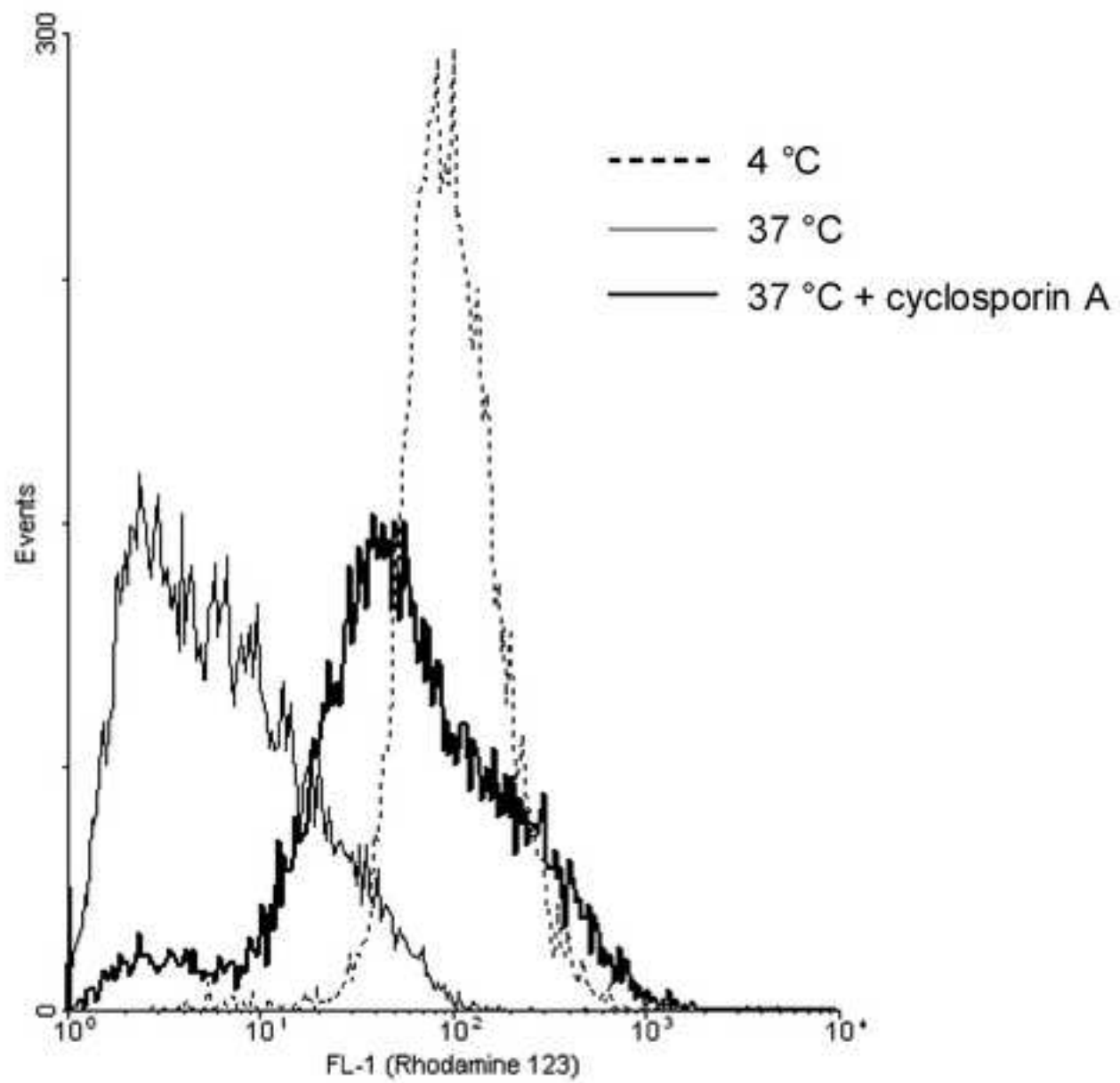


Figure 6

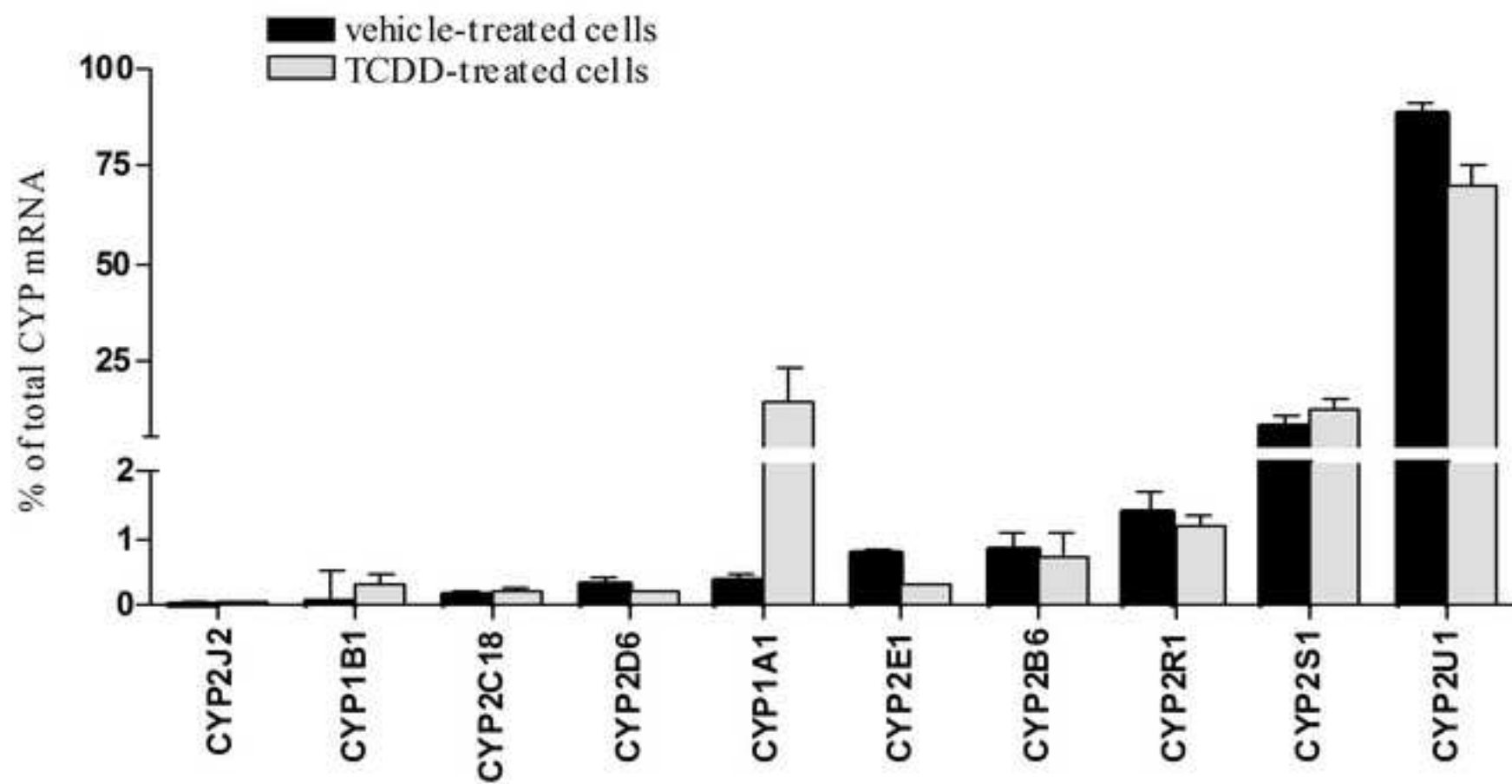


Figure 7

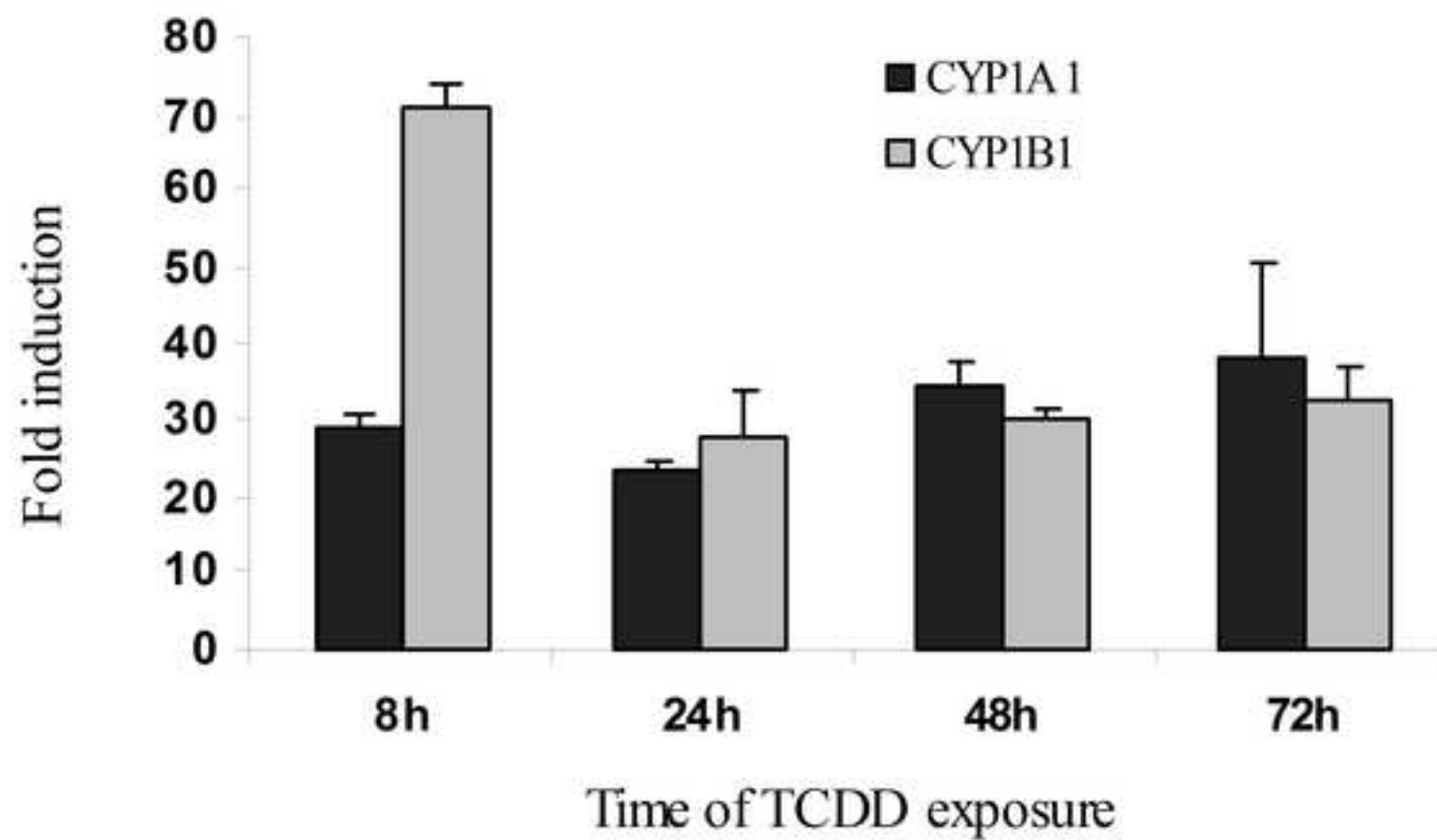


Figure 8

