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Title: **Valproic acid-induced upregulation of multidrug efflux transporter ABCG2/BCRP via PPAR α -dependent mechanism in human brain endothelial cells**

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Running Title: VPA activates PPAR α to upregulate ABCG2 in hCMEC/D3 cells

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Abbreviations:

ABC, ATP-binding cassette; AED, antiepileptic drug; BBB, blood-brain barrier; BCA, bichinchonic acid; BCRP, breast cancer resistance protein; BECs, brain endothelial cells; BFGF, basic fibroblast growth factor; B2M, beta-2 microglobulin; BPZ, BODIPY FL Prazosin; BSA, bovine serum albumin; CAR, constitutive androstane receptor; CBZ, carbamazepine; CDLC, chemically defined lipid concentrate; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; EBM-2, Endothelial Basal Medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-Phosphate dehydrogenase; HSC70, heat shock cognate protein 70; LEVI, levetiracetam; LTG, lamotrigine; MDT, multidrug transporter; MRP, multidrug resistance-associated

protein; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PDK4, pyruvate dehydrogenase lipoamide kinase isozyme 4; Pgp, P-glycoprotein; PHT, phenytoin; PPAR α , peroxisome proliferator-activated receptor alpha; PPRE, PPAR α response element; PWE, patients with epilepsy; PXR, pregnane X receptor; qPCR, quantitative real-time PCR; SCR, scramble; THBD, thrombomodulin; TPM, topiramate; TR-FRET, Time-resolved fluorescence energy transfer; VC, vehicle control; VPA, valproic acid

ABSTRACT

Despite the progress made in the development of new antiepileptic drugs (AEDs), poor response to them is a rising concern in epilepsy treatment. Of several hypotheses explaining AED treatment failure, the most promising theory is the overexpression of multidrug transporters belonging to ATP-binding cassette (ABC) transporter family at blood-brain barrier. Previous data show that AEDs themselves can induce these transporters, in turn affecting their own brain bioavailability. Presently, this induction and the underlying regulatory mechanism involved at human blood-brain barrier is not well elucidated. Herein, we sought to explore the effect of most prescribed first and second-line AEDs on multidrug transporters in human cerebral microvascular endothelial cells, hCMEC/D3. Our work demonstrated that exposure of these cells to valproic acid (VPA) induced mRNA, protein and functional activity of breast cancer resistance protein (BCRP/ABCG2). On examining the substrate interaction status of AEDs with BCRP, VPA, phenytoin and lamotrigine were found to be potential BCRP substrates. Furthermore, we observed that siRNA-mediated knockdown of peroxisome proliferator-activated receptor alpha (PPAR α) or use of PPAR α antagonist, resulted in attenuation of VPA-induced BCRP expression and transporter activity. VPA was found to increase PPAR α expression and trigger its translocation from cytoplasm to nucleus. Findings from chromatin immunoprecipitation and luciferase assay showed that VPA enhances the binding of PPAR α to its response element in the *ABCG2* promoter, resulting in elevated *ABCG2* transcriptional activity. Taken together, these *in vitro* findings highlight PPAR α as the potential molecular target to prevent VPA-mediated BCRP induction, which may have important implications in VPA pharmacoresistance.

SIGNIFICANCE STATEMENT

Induction of multidrug transporters at blood-brain barrier can largely affect the bioavailability of the substrate antiepileptic drugs in the brains of patients with epilepsy, thus affecting their therapeutic efficacy. The present study reports a mechanistic pathway of breast cancer resistance protein (BCRP/ABCG2) upregulation by valproic acid in human brain endothelial cells via peroxisome proliferator-activated receptor alpha involvement, thereby providing a potential strategy to prevent valproic acid pharmacoresistance in epilepsy.

INTRODUCTION

Antiepileptic drug monotherapy is the initial standard mode used worldwide for epilepsy management (Park et al., 2019). However, one of the biggest concerns till date, is that a major percentage (40-50%) of patients do not respond to first line monotherapy consisting 25-30% refractory cases (Le et al., 2021; Park et al., 2019; Rawat et al., 2018), which is majorly attributed to the inadequate AED concentration reaching the epileptogenic brain (Kwan and Brodie, 2005; Remy and Beck, 2006). One prominent hypothesis explaining this is excessive AED efflux across the blood-brain barrier (BBB) (Löscher et al., 2011). BBB is the most essential component of a healthy central nervous system which allows selective entry of substances into the brain (Haddad-Tovolli et al., 2017). Presence of transport proteins such as members of the ATP-binding cassette (ABC) efflux transporters and solute carrier proteins at the brain endothelial cell membrane controls this transport. Indeed, these transporters have the potential to impede transport of pharmacologically relevant substrate drugs across BBB (Girardin, 2006).

Expression studies performed in the brains of refractory patients with epilepsy (PWE) and in animal model of pharmaco-resistant epilepsy revealed overexpression of multidrug transporters (MDTs) belonging to ABC transporter family such as P-glycoprotein (Pgp/ABCB1), multidrug resistance-associated proteins (MRP1-2/ABCC1-2, MRP4-6/ABCC4-6) and breast cancer resistance protein (BCRP/ABCG2) as the potential phenomenon for epilepsy pharmaco-resistance (Banerjee Dixit et al., 2017; Dombrowski et al., 2001; Volk and Loscher, 2005). Multiple causal factors have been put forward to explain this elevated expression (Leandro et al., 2019). High transporter levels in surgically resected brain tissues of PWE associated with a known structural brain abnormality such as cortical dysplasia or brain tumors, suggest disease etiology as the underlying cause (Leandro et al., 2019). An alternative explanation comes from the data obtained from animal models with

experimentally-induced seizures, showing recurrent seizure activity and the associated neuronal stress as the responsible factor (Bankstahl and Loscher, 2008; van Vliet et al., 2005; van Vliet et al., 2007). However, these studies are few in number and the focus of these experiments has been ABCB1 with very little knowledge on other MDTs. Yet another plethora of studies support the concept that chronic use of AEDs promotes the excessive expression of transporters, possibly indicating a defensive response of body against xenobiotics. This has been widely explored in the literature both *in vitro* and *in vivo*. While multiple AEDs have depicted to exert inductive effect on ABCB1 (Ke et al., 2019; Wen et al., 2008; Yang et al., 2008), this effect on other MDTs is limited and confined to either rat BBB (Lombardo et al., 2008) or other tissue barriers such as intestine, placenta, liver cells, etc. (Grewal et al., 2017; Rubinchik-Stern et al., 2015). Furthermore, there are only two reports available at human BBB discussing the AED-mediated regulation of MDTs and again the transporter investigated is ABCB1. First report by Alms *et al.* in hCMEC/D3 cells demonstrated an upregulatory effect of carbamazepine (CBZ) on ABCB1 activity, albeit the concentration of the CBZ used (100 μ M) exceeded its therapeutic plasma concentration range (Alms et al., 2014). Recently, our group led by Rawat et al. showed downregulation of ABCB1 in hCMEC/D3 cells by valproic acid (VPA) (Rawat et al., 2020). There is no evident result of MDT induction by AEDs and the underlying molecular basis at human BBB, which necessitates further exploration. Besides, induction of the functional expression of MDTs may reduce the bioavailability of AEDs to enter the brain when these drugs are also the substrate of MDTs. Hence, it becomes imperative to systematically investigate the substrate status of AEDs to explain the importance of MDTs in AED efflux.

In the present study, we evaluated the effect of widely prescribed first-line AEDs: phenytoin (PHT), VPA, CBZ and second-line AEDs: lamotrigine (LTG), topiramate (TPM) and levetiracetam (LEVI) on MDTs in hCMEC/D3 cells and determined whether these AEDs

show substrate interactions with MDTs. Further, we explored the molecular basis of AED-mediated transporter regulation. Our data revealed that VPA upregulates expression and activity of BCRP in hCMEC/D3 cells via peroxisome proliferator-activated receptor alpha (PPAR α)-dependent mechanism. In addition, we found VPA, PHT and LTG to show substrate interaction with BCRP. Multiple evidences in mouse models show PPAR α to be an important regulator of ABC transporters suggesting that PPAR α may alter the disposition of substrate toxicants/drugs of these transporters, thus influencing either the drug toxicity or bioavailability (Hoque et al., 2015; Moffit et al., 2006; Wang et al., 2018). Our data substantiates the importance of PPAR α in VPA pharmacoresistance.

MATERIALS AND METHODS

Chemicals and Reagents. Endothelial Basal Medium-2 (EBM-2) was purchased from Lonza (Walkersville, MD). Collagen I (rat tail) and chemically defined lipid concentrate (CDLC) were obtained from Thermo Fisher Scientific (Waltham, MA). Dulbecco's modified Eagle's medium (DMEM), hydrocortisone, ascorbic acid, HEPES, dimethyl sulfoxide (DMSO), PHT, CBZ, VPA, LTG, TPM, LEVI, bovine serum albumin (BSA), Ko143, clofibrate and GW7647 were procured from Sigma-Aldrich (St. Louis, MO, USA). MK886 was from Cayman Chemical Co. (Ann Arbor, MI, USA). Basic fibroblast growth factor (BFGF), fetal bovine serum (FBS), penicillin-streptomycin and BODIPY FL Prazosin (BPZ) were purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture, drug and inhibitor treatment. Immortalized human cerebral microvascular endothelial cell line, hCMEC/D3, was purchased from Cedarlane Laboratories (Burlington, Canada) and was used within passages 25 to 35. They were maintained at 37°C with 5% CO₂ in EBM-2 media supplemented with 1/100 CDLC, 1.4µM hydrocortisone, 5µg/ml ascorbic acid, 10mM HEPES buffer, 1ng/ml BFGF, 2.5% FBS, and 1% penicillin-streptomycin, and cultured on collagen I coated flasks and plates. Human embryonic kidney HEK293 cells were grown in DMEM high glucose (4.5g/l) supplemented with 100 units/ml penicillin, 100µg/ml streptomycin and 10% FBS and were maintained at 37°C in 5% CO₂. PHT, CBZ, VPA, LTG, TPM, MK886, BPZ and KO143 were dissolved in DMSO and LEVI was dissolved in water. The concentration of AEDs used for cell culture treatments were consistent with the recommended serum therapeutic range for individual AEDs in epilepsy patients (Supplemental Table 1). For PPAR α antagonist studies, MK886 was used as a nuclear receptor PPAR α antagonist (Kehrer et al., 2001; Mogilenko et al., 2013). hCMEC/D3 cells were treated with VPA in the presence or absence of MK886 and then BCRP expression

and activity were determined using western analysis and BPZ efflux assay, respectively. MK886 was added to the cells at a final concentration of 8 μ M. DMSO (0.1% v/v) was used as vehicle control (VC) in all experiments unless specified.

Assessment of cell viability. To ensure that the concentrations of the test chemicals did not affect the hCMEC/D3 cell viability, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed. Briefly, cells were plated on collagen-coated 96-well plates for 24h and subsequently treated with varying concentrations of each chemical. Assay was performed after 72h treatment with PHT, CBZ, VPA, LTG, TPM, LEVI and 48h treatment with MK886. 100 μ l MTT (final concentration of 0.5mg/ml; Ameresco, Fountain Parkway, Solon, OH, USA) dissolved in media was added to each well and the plates were incubated at 37°C for 3h. The formed formazan crystals were dissolved in DMSO and absorbance was measured at 570 nm with a reference filter of 630 nm using Infinite 200 PRO multimode plate reader (Tecan, Mannedorf, Zurich, Switzerland). The results were expressed as a percentage ratio of absorbance of treated cells to the absorbance of control cells.

Reverse Transcription and Quantitative Real Time PCR. Total RNA was extracted from treated hCMEC/D3 cells using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. RNA quantity (absorbance 260nm) and purity (absorbance 260nm/absorbance 280nm) were assessed by Infinite 200 PRO NanoQuant (Tecan). 1 μ g of total RNA was reverse transcribed using cDNA synthesis components (Thermo Fisher Scientific). Quantitative real-time PCR (qPCR) was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) and gene-specific primers. Beta-2 Microglobulin (B2M) was used as an internal standard for normalizing gene expression values. Analysis of data was done using the $\Delta\Delta$ Ct method

(Yuan et al., 2006). Results were expressed as fold change of the control group. Gene-specific primer sequences used for qPCR are mentioned in Supplemental Table 2.

Cytoplasmic, nuclear and total protein extraction. Nuclear and cytoplasmic fractions from hCMEC/D3 cells were extracted using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The total cell protein was obtained using radio immune precipitation assay lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail.

Western blot analysis. The protein concentration in the respective lysates was quantified using Bicinchoninic Acid (BCA) protein estimation kit (Pierce Thermo Fisher Scientific Inc., Waltham, MA). Protein sample preparation for PPARA detection was done by heating samples at 95°C for 5 min. For detecting BCRP, samples were deglycosylated using peptide-N-glycosidase F (New England Biolabs, UK) during preparation. Equal amount of protein from each sample were separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. After blocking with 5% BSA at room temperature for 1h, the membranes were incubated at 4°C overnight with primary antibodies to detect BCRP protein (4477, rabbit polyclonal, 1:1000; Cell Signaling Technology, Danvers, MA) (Sridharan et al., 2019), PPAR α (ab24509, rabbit polyclonal, 1:2000; Abcam, Cambridge, MA, US) (Wang et al., 2020), heat shock cognate protein 70 (HSC70, sc-7298, mouse monoclonal, 1:2000; Santa Cruz Biotechnology, Dallas, Texas) (Ahmad et al., 2022), glyceraldehyde-3-Phosphate dehydrogenase (GAPDH, ab8245, mouse monoclonal, 1:10,000; Abcam, Cambridge, MA, US) (Lu et al., 2021), Lamin (sc-376248, mouse monoclonal, 1:1000; Santa Cruz Biotechnology, Dallas, Texas) (Rosain et al., 2022). Subsequently, blots were incubated with respective horseradish peroxidase-conjugated bovine anti-rabbit secondary antibody and goat

anti-mouse antibody and bands were detected using Enhanced Chemiluminescence reagent (Pierce Thermo Fisher Scientific Inc.) on Chemi Doc™ MP Imaging chemiluminescence system (Bio-Rad, Hemel Hempstead, UK). Protein expression was analyzed with the AlphaImager 3400 (Alpha InnoTech corporation, San Leandro, CA) software. HSC70 protein bands were used to normalize the intensity of the target total protein. Bands of Lamin and GAPDH were used to normalize intensities of nuclear and cytoplasmic proteins, respectively.

Functional studies. Functional assay for BCRP was performed using established BCRP substrate, BPZ as described earlier (Hori et al., 2004) with modifications. The assay quantifies a decrease in intracellular BODIPY fluorescence as a result of transporter activity. hCMEC/D3 cells were seeded in a 12-well plate one day prior to drug treatment. Cells were treated with VC or test AED for various time points under study. On the day of assay, cells were washed with 1X phosphate-buffered saline (PBS) to remove drug and incubated with medium containing 250nM BPZ for 30min with or without 1μM Ko143 (BCRP-selective inhibitor) at 37°C in 5% CO₂ (uptake period). Subsequently, cells were washed thrice with ice-cold 1X PBS and incubated in BPZ-free media in presence or absence of 1μM Ko143 for 90 min to allow BPZ efflux to occur (efflux period). Efflux was terminated by washing cells with ice-cold 1X PBS thrice. Cells were lysed by adding 1% triton X-100 followed by incubation at 37°C for 15 min. 20μl of this lysate was aliquoted into 384-well black assay plates and subjected to plate reader for fluorescence detection (excitation: 488 nm; emission: 530nm). The remaining lysate was used for protein estimation by BCA method to normalize the obtained fluorescence with total protein concentration.

ATPase assay. Transport of compound by ABC transporters is coupled with ATP hydrolysis, hence this assay measures the amount of inorganic phosphate generated during

ATP hydrolysis by the substrate drug of the transporter by colorimetric means. The assay was performed using SB BCRP M PREDEASY™ ATPase Kit (Solvo Biotechnology, Budaors, Hungary) according to the manufacturer's instructions. Isolated membranes from MCF-7 cells overexpressing BCRP (5 mg/ml) were incubated with 2% v/v DMSO or water as the VC or the test AED in the presence or absence of sodium orthovanadate. MgATP was added to initiate the reaction. The results were expressed as vanadate-sensitive ATPase activity. In the activation assay, the ability of the test drug to stimulate the baseline ATPase activity is calculated while results of inhibition assay measures test drug-mediated decrease in maximal transporter activity associated with presence of sulfasalazine (known BCRP activator).

Competitive cellular efflux assay. The assay is based on the potential of the test drug to compete with the transporter's known fluorescent substrate for its efflux out of the cell via the transporter. The resulting increase in intracellular fluorescence is estimated. The assay determines the potential substrate interaction of test drug with transporter (Grewal et al., 2017; Kim et al., 2010; Terashi et al., 2000; Wu et al., 2020). In our study, the ability of the AED to compete with the efflux of BPZ through BCRP was measured. Briefly, seeded cells were preincubated for 30 min at 37°C in medium containing BPZ with VC or the AEDs or reference inhibitor, KO143. Cells were washed with 1X PBS and further incubated for 90 min in BPZ-free media continuing with or without Ko143. Termination of assay and fluorescent detection was done as mentioned in the section '**Functional studies**'. Obtained fluorescent intensity was normalized with total protein concentration. The results were represented as fold increase in intracellular BPZ fluorescence over VC.

siRNA down-regulation studies. Gene-specific siRNA and negative control scramble (Catalog no., assay IDs of the siRNA used and the manufacturer has been enlisted in Supplemental Table 3) were transiently transfected into hCMEC/D3 cells using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions.

Briefly, transfection reagent and siRNA were diluted in Opti-MEM® (Gibco, Thermo Fisher Scientific) medium, and then diluted transfection reagent was gently added to the diluted siRNA. After incubation for 20 min at room temperature, the transfection complex was added to the cells. After 6h of transfection, the medium was replaced with fresh Opti-MEM medium and kept further for 18h. Following this, cells were washed with 1X PBS and treated with VPA or VC in EBM-2 complete media and further cultured for indicated time periods to check changes in the gene expression and activity.

Immunofluorescence. hCMEC/D3 cell monolayer was grown onto collagen-coated coverslips in 6-well plates in the EBM-2 complete medium. After treatment with VPA or VC, cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Following this, cells were permeabilized with 0.1% Triton X-100 for 20 min. Non-specific sites were blocked with 1% BSA in PBS for 30 min. Cells were then incubated overnight at 4⁰C with PPAR α primary antibody diluted (1:250) in 1% BSA. After washing with 1X PBS, cells were incubated with the Alexa488-conjugated anti-rabbit secondary antibody (1:1000) for 2h at room temperature. Nuclei were counterstained with 0.5 μ g/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Co.) for 15 min at 37⁰C and mounted on glass slides using ProLong Diamond Antifade Mountant (Invitrogen). Images were acquired using Nikon confocal A1R HD with Ti2-E with 60X Nikon objective (1.4 NA). Fluorescence images were obtained by sequential z stage scanning in two channels (DAPI, Alexa Fluor-488).

To quantify the PPAR α fluorescence signals in the nucleus and cytoplasm, the Image J software was used. Whole cell masks and nuclear masks were generated by applying Huang's thresholding method on PPAR α stained channel and on the DAPI stained channel. Total area and PPAR α fluorescence intensity corresponding to each of the cell was measured. Similarly, the nuclear area and fluorescence intensity corresponding to the nuclear region was measured. The cytoplasmic area and fluorescence signal were obtained by subtracting the

nuclear area and fluorescence from total cellular area and fluorescence, respectively. Nuclear and cytoplasmic fluorescence intensities were then normalized to corresponding areas and the obtained values were divided to determine the nuclear/cytoplasmic (Nuc/Cyt) ratio of PPAR α fluorescence intensity. The average fluorescence intensity for each treatment group per experiment was the mean of all measurements taken from at least 80 cells from 12 random fields.

Chromatin Immunoprecipitation Assay and Quantitative Real-Time PCR.

Treated hCMEC/D3 cells were cross-linked by adding 1% formaldehyde for 10 min at room temperature, followed by quenching using 125 mM glycine solution. Fixed cells were scraped in 1X PBS and centrifuged to obtain the cell pellet. The pellet was resuspended in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, NP-40 (0.5% vol/vol), Triton X-100 (1.0% vol/vol). The samples were kept on ice for 20 min and then centrifuged at 12000 rpm for 1 min at 4⁰C to obtain the nuclear pellet. This pellet was resuspended in 1% SDS lysis buffer and sonication was performed to obtain the sheared chromatin. One-tenth of the sonicated lysates were taken out as the input control. Immunoprecipitation was performed overnight at 4⁰C with 5 μ g anti-PPAR α antibody chromatin immunoprecipitation (ChIP) Grade (ab227074, rabbit polyclonal; Abcam, Cambridge, MA, US) (Murphy et al., 2021). Rabbit IgG was used as a nonspecific control. The resultant chromatin precipitates were captured by Protein A dynabeads (Invitrogen). After washing the whole complex in different buffers as described previously (Gade and Kalvakolanu, 2012), the protein–DNA complex from the antibody was eluted by adding freshly prepared elution buffer (1% SDS, 0.1 M NaHCO₃). Cross-linking was reversed by adding proteinase K to the samples and heating the eluate and input DNA at 65⁰C for 2h. Purified DNA was amplified using qPCR with primers flanking the PPAR α binding sites in the human *ABCG2* promoter regions. The binding sites have previously been verified in the

report by Hoque et al. (Hoque et al., 2012). Primer sequence used were: forward primer 5'-TGGAAGGCTGTGAGTCACTT-3' and reverse primer 5'-AGGACCTTCCTCATTAGGTCAGA-3'. Recruitment was calculated as % of total input.

Plasmid construction. A 345 bp fragment of the human *ABCG2* promoter (-4037bp/-3692bp) containing the PPAR α response elements (PPRE) was PCR amplified using the forward primer 5'-CAACGGTACCCTGGTGCACAGGCATTCA-3' (underlined nucleotides indicate the KpnI site) and the reverse primer 5'-ATAACTCGAGGTTTCAGATTAAAGCCAGC-3' (underlined nucleotides indicate the XhoI site). Amplified fragment was cloned into pCR2.1 TA vector (3929 bp) and then subcloned upstream to the luciferase reporter gene driven by the SV40 promoter into pGL2-promoter vector (5789 bp) linearized with XhoI and KpnI restriction enzymes. The desired final construct (PGL2-prom-ABCG2) was confirmed through sequencing.

Transient Transfection and Dual-Luciferase Assay. For luciferase assay, HEK293 cells (10^5 cells) were cultured in 6 well plates and grown for 24h. Cells were then co-transfected with pRenilla plasmid (20ng/well) and pGL2 reporter plasmid vectors (3 μ g/well) that expressed Renilla and Firefly luciferase activities, respectively. The pGL2 reporter plasmids were pGL2-prom-ABCG2 and pGL2-control. Transfections were performed using polyethylenimine. VPA or VC was added for 24h after transfection and cells were immediately harvested by scrapping. Cells were lysed and the Firefly and Renilla luciferase activity was measured using the Dual-Luciferase Reporter Assay System. Firefly luciferase reporter activity of each data set was normalized to Renilla luciferase activity.

LanthaScreen™ TR-FRET PPAR α competitive binding assay. Interaction of VPA with PPAR α nuclear receptor was studied using a cell-free Lanthascreen TR-TRET ligand-

binding assay (Invitrogen). The assay is based on the principle of competitive displacement of the reference fluorescent ligand (Tracer) from its recombinant terbium-labeled PPAR α LBD. VPA and a selective PPAR α agonist, GW7647 were diluted in DMSO and assay was performed according to the manufacturer's protocol. Results are displayed as percent displacement of the tracer.

Statistical analysis. Data were expressed as mean \pm standard deviation. Statistical significance was assessed by unpaired t-test or one-way ANOVA followed by Dunnett's or Tukey's post hoc test or two-way ANOVA followed by Tukey's post hoc test as indicated against each experimental result. 95% confidence interval accompanying the percent changes was calculated using standard error of the mean between the groups. P-value of less than 0.05 was considered to be statistically significant. The results of the tests have been summarized in Supplemental Table 4.

RESULTS

Effect of first and second-line AEDs on mRNA expression of MDTs in hCMEC/D3 cells. After confirming the absence of any cytotoxicity of AEDs on hCMEC/D3 cells at doses that represent the therapeutic plasma concentration for individual AEDs (Supplemental Fig. 1); we studied the effect of therapeutic doses of AEDs on mRNA expression of ABCC1, ABCC2, ABCC4, ABCC5 and ABCG2. Cells were treated with first-line AEDs (PHT: 40 μ M, 80 μ M; CBZ: 21 μ M, 42 μ M and VPA: 300 μ M, 600 μ M) and second-line AEDs (LTG: 15 μ M, 60 μ M; TPM:15 μ M, 60 μ M and LEVI: 40 μ M, 120 μ M) for 24h. Reverse transcriptase (RT)-qPCR analysis showed a dose-dependent increase in ABCG2 mRNA expression in response to VPA treatment (1.71-fold increase at 300 μ M, 2.43-fold increase at 600 μ M) (Fig. 1). However, no effect of PHT, CBZ, LTG, topiramate (TPM) and levetiracetam (LEVI) was found on expression of transporters under study (Supplemental Fig. 2). We then checked for any time-dependent variations in this induction. It was found that VPA at both the doses induced ABCG2 mRNA at all the time points under study (6h, 12h, 24h and 48h), though the maximum fold change observed was at 24h (Fig. 2).

VPA increases the protein expression and functional activity of BCRP in hCMEC/D3 cells. Similar to the effect on mRNA expression, exposure of hCMEC/D3 cells with VPA (300 μ M, 600 μ M) displayed upregulation of BCRP protein levels starting from 12h to 72h (Fig. 3A). The increase was found to be maximum at 48h (1.74-fold at 300 μ M, 2.09-fold at 600 μ M). A representative blot for 48h protein data is shown in Fig. 3B. To test whether increase in protein expression is also reflected in its functional activity, BPZ efflux assay was performed in the presence of BCRP inhibitor, Ko143. BCRP activity was determined as the ratio between the mean intracellular fluorescence of cells incubated with Ko143 (BCRP inhibited) and cells incubated without Ko143 (non-inhibited) in the presence of VPA, normalized to the corresponding ratio in the absence of VPA. We found that cells

treated with VPA (300 μ M, 600 μ M) for over 48h and 72h resulted in a decrease in BPZ intracellular fluorescence indicating elevated BCRP-specific BPZ efflux (Fig. 3C).

VPA and LTG stimulates the ATPase activity of BCRP. To determine whether VPA or other AEDs (PHT, CBZ, LTG, TPM, LEVI) which are also co-administered with VPA are substrates of BCRP, ATPase assay with the BCRP-overexpressing membrane vesicles was performed for different AEDs over a concentration range and the amount of Pi released from ATP hydrolysis by the test AED was measured by a colorimetric reaction. As seen from the Fig. 4A, C and D, PHT, VPA and LTG stimulated the vanadate-sensitive baseline ATPase activity in the activation assay, indicative of their substrate interaction with BCRP. For CBZ, TPM, and LEVI, no difference from baseline activity was observed (Fig. 4B, E and F). The inhibition assay was done in the presence of test drug and known activator of BCRP-sulfasalazine, which maximally stimulates the ATPase activity. At higher doses of LTG (120 μ M) and CBZ (80 μ M), a reduction in maximally stimulated ATPase activity was observed.

VPA and LTG increases the intracellular accumulation of BPZ. The substrate interaction of PHT, VPA and LTG with BCRP detected in the activation assay was further verified in competitive substrate efflux assay in which BPZ was included as a competing substrate. Fig. 5, A-C shows that co-incubation of PHT (80 μ M), VPA (300 μ M, 600 μ M) and LTG (15 μ M, 60 μ M) with the assay substrate BPZ increased cellular accumulation of BPZ, providing a more definitive evidence of these AEDs as potential substrates of BCRP.

Since the VPA-mediated changes in BCRP expression and activity were observed to be maximum at the higher dose i.e., 600 μ M, all the experiments related to the understanding of mechanistic of this regulation were performed at this dose.

PPAR α silencing/antagonism attenuates VPA-induced BCRP expression and activity. To identify the molecular factors involved in VPA-induced ABCG2 expression, a list of 17 important factors reported to regulate ABCG2 was obtained from the literature (Kukal et al., 2021) (Factors and their primer sequences for qPCR are shown in Supplemental Table 2). Constitutive mRNA expression level for each factor was examined in hCMEC/D3 cells by qPCR (data not shown). Xenobiotic receptors, pregnane X receptor (PXR) and constitutive androstane receptor (CAR), were undetectable in these cells. Thus, siRNA experiments were performed for the remaining factors at mRNA level. Validation data of PPAR α is shown in Fig. 6A, whereas the data for other siRNAs is shown in Supplemental Fig. 3. Of these factors, a 35% [95% CI: 20.66%, 48.33%] decrease in VPA-induced ABCG2 mRNA was observed only when PPAR α was silenced, suggesting the regulation to be PPAR α dependent (Fig. 6B). Further, PPAR α knock-down suppressed VPA-induced BCRP protein and activity by 38.9% [95% CI: 28.68%, 47.92%] and 30.5% [95% CI: 23.76%, 36.82%], respectively (Fig. 6, C-E). For the remaining factors, siRNA knockdown did not affect VPA-induced ABCG2 mRNA (Supplemental Fig. 4).

To further examine the regulatory role of PPAR α in VPA-mediated ABCG2 upregulation, we incubated hCMEC/D3 cells with VPA in the presence or absence of PPAR α antagonist, MK886 for 48h (MTT data for MK886 is shown in Supplemental Fig. 5). As shown in figure, addition of MK886 (8 μ M) abrogated the upregulatory effect of VPA on BCRP protein (Fig. 7, A-B) and activity (Fig. 7C) by 50% [95% CI: 30.38%, 65.49%] and 30% [95% CI: 20.1%, 38.9%], respectively.

Treatment of hCMEC/D3 cells with known PPAR α agonist, GW7647 (100nM) and clofibrate (100 μ M) also showed ABCG2 mRNA induction (Supplemental Fig. 6), validating the regulatory effect of PPAR α on ABCG2.

VPA increases PPAR α expression and its nuclear translocation. To get insights into the mechanism of the VPA-mediated ABCG2 overexpression via PPAR α , we first examined the effect of VPA on PPAR α expression. Upon treatment of hCMEC/D3 cells with VPA, *PPAR α* mRNA started to be induced at 3h (1.43-fold) and reached a maximum at 6h (2.04-fold). The increase was continued till 24h (1.76-fold) (Fig. 8A). However, at protein level, the increase was seen till 6h, after which no increase was observed (Fig 8B).

Next, we investigated the possibility whether VPA affects PPAR α translocation in hCMEC/D3 cells. For this, we fractionated the cytoplasmic and nuclear content and checked for the protein expression of PPAR α in each fraction at 0h, 3h and 6h of VPA treatment. GAPDH and Lamin A/C were used as loading marker genes for cytoplasmic and nuclear extracts, respectively. No cross-contamination of nuclear and cytoplasmic fractions was seen. As shown in Fig. 9A, at 0h, PPAR α was found to be expressed in both cytoplasm and nucleus, though nucleus had comparatively lesser levels. However, at 3h and 6h of VPA treatment, an increase in nuclear PPAR α levels could be noted with a simultaneous decrease in cytoplasm, revealing that VPA induces PPAR α nuclear translocation to initiate downstream signaling. Quantitative analysis of the relative nuclear and cytosolic PPAR α protein fold change is shown in Fig. 9B.

Immunofluorescence staining of PPAR α in the fixed hCMEC/D3 cells was also carried out to evaluate the PPAR α translocation. In accordance with the findings of western blot, confocal microscopic imaging and quantitative analysis of PPAR α fluorescence revealed the presence of PPAR α in both cytoplasm and nucleus at 0h. After 3h and 6h of VPA

exposure, a 33.8% [95% CI: 27.35%, 40.22%] and 32.6% [95% CI: 15.4%, 49.98%] increase in Nuc:Cyt ratio of fluorescence was observed (Fig. 9, C-D), respectively, suggesting activation of PPAR α by VPA.

In hCMEC/D3 cells, VPA also increased the mRNA level of PPAR α target genes pyruvate dehydrogenase lipoamide kinase isozyme 4 (*PDK4*) (Janssen et al., 2015) and thrombomodulin (*THBD*) (Shiono et al., 2020) (Fig. 8D). This further corroborated the activating effect of VPA on PPAR α .

VPA enhance the recruitment of PPAR α to *ABCG2* promotor and results in increased promoter activity. The ability of VPA to enhance the binding of PPAR α in the promoter of its target gene *ABCG2* was studied by ChIP assay. Chromatin preparation from cells treated with VC or VPA was immunoprecipitated with anti-PPAR α antibody or negative IgG antibody. *ABCG2* promoter region in the immunoprecipitated complex DNA was amplified using qPCR primers flanking the PPAR α binding region (-3946bp/-3796bp) of *ABCG2* promoter. As shown in Fig. 10A, VPA treatment at 3h and 6h caused enrichment of PPAR α to *ABCG2* promoter region. A representative gel image for recruitment at 3h of treatment is shown in Fig. 10B.

To examine the effect of VPA on *ABCG2* promoter activity driven by PPRE, a 345bp promoter region containing the PPAR α binding site was cloned upstream of the minimal SV40 promoter driving the luciferase reporter gene (PGL2-prom-*ABCG2*). HEK293 cells were then co-transfected with either pGL2-prom-*ABCG2* and pRenilla or control vector and pRenilla followed by incubation with VPA or VC. Firefly luciferase activities were normalized to renilla luciferase activities to consider changes in the transfection variability. Firefly/renilla activity of the VPA-treated cells were then normalized to VC (set as 1) to obtain the relative luciferase activity. VPA was found to increase *ABCG2* luciferase activity

by around 1.42-fold compared to VC, demonstrating *ABCG2* promoter activation by VPA, whereas control vector was not affected by VPA treatment. Fig. 10C depicts the relative luciferase activity of PGL2-control and PGL-prom-*ABCG2*.

VPA displayed weak ligand interaction with PPAR α . To determine whether VPA shows ligand interaction with the PPAR α receptor, LanthaScreen™ TR-FRET PPAR α competitive binding assay was conducted. As depicted in curve between percent displacement and the concentration (Supplemental Fig. 7), while GW7647 showed a strong binding to the receptor with an IC₅₀ value of 0.0224 μ M, VPA started competing with the tracer at a concentration of 556 μ M (within the therapeutic range of VPA). Its IC₅₀ value was achieved at a much higher concentration of 9690 μ M, indicating weak binding of VPA with PPAR α .

DISCUSSION

Despite the availability of 35 AEDs (Jacob et al., 2019), poor response is experienced by a considerable section of PWE. The extensively studied theory behind this is the overexpression of efflux transporters at BBB (Le et al., 2021; Löscher and Potschka, 2002). Earlier studies depict that AEDs have the potential to regulate these MDTs (Alms et al., 2014; Ke et al., 2019). However, AED-mediated regulation of MDTs implicated in drug-resistant epilepsy (other than ABCB1), at the human BBB, is largely unknown. Our results demonstrated an inductive effect of VPA on ABCG2 mRNA, protein and functional activity in hCMEC/D3 cells. Our data goes in-line with a previous report where this induction was demonstrated in placental cells to check the effect of AEDs on placental carriers altering the fetal exposure to certain nutrients (Rubinchik-Stern et al., 2015). A recent report studying the effect of histone deacetylase inhibitors on MDTs in hCMEC/D3 cells showed increase in ABCG2 mRNA at 5mM dose, exceeding the clinical therapeutic plasma concentration range of VPA (You et al., 2019).

Increased expression of BCRP, a well-recognized drug efflux transporter, has been implicated in drug-resistant PWE. Strong BCRP expression was reported in endothelial cells from brain tumors associated with epileptogenic pathology in refractory PWE (Aronica et al., 2005). A retrospective investigation done in epileptogenic tuberous sclerosis specimens (refractory to AEDs), revealed the presence of BCRP in brain endothelial cells (BECs) in addition to the high MDR1 and MRP1 expression (Lazarowski et al., 2006). An upregulation of ABCG2 mRNA was found in hippocampal tissues from patients with mesial temporal lobe epilepsy, demonstrating the potential of ABCG2 as prognostic marker for epilepsy pharmacoresistance (Banerjee Dixit et al., 2017). Recognizing the role of BCRP in MDR phenotype, it is important to know how its expression is regulated by AEDs. Our study reports that VPA elevates BCRP functional expression in hCMEC/D3 cells.

We next assessed the BCRP substrate status of AEDs and found PHT, VPA and LTG as the substrates. CBZ, TPM and LEVI did not show substrate interaction. The observations to some extent corroborated with previous findings. BPZ accumulation assay performed in mouse fibroblasts transduced with human *ABCG2*, in the presence of VPA, demonstrated increase in accumulation of BPZ, indicating its substrate interaction (Cervený et al., 2006). Concentration equilibrium transport assay with MDCK2 cells overexpressing BCRP, identified LTG as the BCRP substrate out of seven AEDs including VPA, CBZ, TPM and LEVI (Romermann et al., 2015). Recently, bidirectional transport assay concluded that BCRP does not contribute in efflux of LEVI (Goncalves et al., 2021). On the contrary, comparison of brain distribution of AEDs between *Mdr1a/1b(-/-)* and *Mdr1a/1b(-/-)/Bcrp(-/-)* mice showed involvement of BCRP in restricting brain access to LEVI (Nakanishi et al., 2013).

The finding that VPA induces BCRP and is also its substrate, led us to investigate the underlying cellular mechanism driving this process at BBB. Using siRNA knockdown and antagonist studies, the role of PPAR α signaling was revealed in this induction. While it is previously demonstrated that PPAR α regulates BCRP expression in mouse intestine and liver (Eldasher et al., 2013; Hirai et al., 2007), hCMEC/D3 cells (Hoque et al., 2012) and rat brain capillaries (More et al., 2017), the involvement of this receptor in VPA-mediated BCRP expression at BBB is a novel finding.

PPAR α is a ligand-activated nuclear receptor, involved majorly in regulation of lipid metabolism. The physiological ligands of PPAR α includes fatty acids and its derivatives (Rakhshandehroo et al., 2010) and is demonstrated to translocate into nucleus upon its activation (Li et al., 2016; Xue et al., 2018). The receptor is shown to get activated by xenobiotics and regulates various genes involved in metabolism and efflux (Chen et al., 2012; Omiecinski et al., 2011). PPAR α activation with clofibrate was reported to upregulate P-gp, Mrp3, Mrp4 and Bcrp in mice liver (Moffit et al., 2006). Mrp3/4 induction along with the

decrease in Mrp2 was observed in mice liver in response to anti-rheumatic drug leflunomide, which involved increase in PPAR α expression. The outcome of this regulation was enhanced hepatic exposure to co-administered drug methotrexate (substrate of Mrp2/3) and liver toxicity (Wang et al., 2018). PPAR α -mediated upregulation of Bcrp was also demonstrated in mouse and rat brain capillaries, indicating PPAR α as a therapeutic target to improve brain drug delivery (Hoque et al., 2015). Thus, these studies substantiate role of PPAR α in influencing the toxicity/bioavailability of drugs which are substrates of such efflux transporters.

We next checked if VPA has effect on PPAR α in hCMEC/D3 cells and found increase in expression and nuclear translocation of PPAR α . This inductive effect has been previously reported in human hepatic cells (Rodrigues et al., 2016; Xu et al., 2019). Moreover, VPA is suggested to be a pan-PPAR activator (Cullingford et al., 2002) and is demonstrated to activate PPAR α LBD in Chinese hamster ovary cells (Lampen et al., 2001).

We speculated that VPA being a fatty acid may behave as ligand for PPAR α receptor to activate it. LanthaScreenTM TR-FRET assay results showed a weak ligand interaction of VPA (at its therapeutic dose) with PPAR α . Since, there might be certain essential proteins involved in enhancing ligand-receptor interaction within the biological system, an effect that cannot be seen in cell-free TR-FRET assay, therefore performing cell-based assays are warranted to confirm the ligand interaction. In addition to ligand-mediated regulation of PPAR α activity, posttranslational modifications of the receptor are also reported to modify its activity, suggesting the possibility that VPA may activate PPAR α via this additional mechanism.

We further showed that exposure of hCMEC/D3 cells to VPA augmented PPAR α binding at PPRE in the *ABCG2* promoter. Luciferase assay in HEK293 cell lines depicted that VPA-mediate *ABCG2* promoter activation via these response elements.

Taken together, the present study demonstrates that VPA upregulates expression and activity of BCRP in human BECs. This involves increase in PPAR α expression, nuclear translocation and its enhanced binding to the *ABCG2* promoter, thus activating the gene's transcriptional activity.

It is worth noting that though VPA activated PPAR α to upregulate BCRP, it did not affect expression of MRP2, despite the reported increase of both these transporters by PPAR α agonists in rat brain capillaries (More et al., 2017). A possible explanation could be the varied species-specific response of human and rat BECs cells towards PPAR α activation (Lawrence et al., 2001). These responses can be attributed to differences in transacting factors in human and rodent cells, affecting PPAR α target gene expression or difference in the human *ABCC2* promoter sequence, rendering the gene unresponsive to PPAR α activation. Further studies are required to determine the mechanism behind these differences.

Till date, conventional AEDs remain the major prescriptions due to their cost-effectiveness and superior efficacy. Among these, VPA is the drug of choice for the first-line treatment because of its effectiveness against broad spectrum of seizure types (Perucca, 2002; Rawat et al., 2020). However, alike other AEDs, VPA also fails to work in one-third of the population (Chen et al., 2019). According to a recent report, resistance to VPA is suggested to be a clinical marker for declaring patients with genetic generalized epilepsy as drug-resistant (Gesche et al., 2017). Considering the significance of VPA in epilepsy treatment, it becomes important to investigate molecular mechanism underlying VPA resistance. Our study highlights PPAR α /BCRP axis as one of the possible mechanisms which could lead to higher VPA efflux from BECs and decrease its intracellular concentration. Future validation of these findings in PPAR α CRISPR knock-out cell line will be of much importance.

Our study has some limitations. First, the AEDs as substrate of BCRP was evaluated through indirect assays. Future experiments should directly quantify the transport of AEDs

via BCRP. One such approach will be to perform high performance liquid chromatography for intracellular drug quantification. Second, this work was performed in hCMEC/D3 cell line, which has relatively low junctional tightness and thus inadequate barrier function compared to primary brain endothelial cells. This limits its use in vectorial drug transport studies for small molecules (Helms et al., 2016). However, despite the limitation of hCMEC/D3 cells to fully recapitulate the human BBB, these cells are reported to retain the expression of most transporters (including ABCG2) and receptors expressed at human BBB and are so far the widely used human brain endothelial cell line to perform gene regulation studies (Weksler et al., 2013). Third, the approach used in this study was *in vitro*. To determine the *in vivo* relevance of the study, data validation utilizing animal models of epilepsy is warranted. PPAR α knockout in such models and evaluating the brain levels of VPA and response to the drug will actually provide the direct evidence of PPAR α as a therapeutic target to overcome VPA pharmacoresistance.

In conclusion, these findings demonstrate that VPA upregulates BCRP in human BECs via PPAR α activation. Moreover, PHT, VPA and LTG displayed substrate interactions with BCRP. Hence, BCRP induction by VPA may likely lead to increased efflux of VPA itself or efflux of PHT/LTG if administered concomitantly. Therefore, targeting of PPAR α may represent a potential therapeutic strategy to address resistance to VPA and possibly other AEDs used in conjunction with VPA.

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FOOTNOTES

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

FIGURE LEGENDS:

Fig. 1 Effect of VPA on mRNA expression of MDTs in hCMEC/D3 cells. Cells were treated with VPA (300 μ M, 600 μ M) for 24h. Total RNA was extracted and subjected to RT-qPCR to determine changes in mRNA levels of ABCC1, ABCC2, ABCC4, ABCC5 and ABCG2. The mRNA levels of these genes were normalized to those of *B2M* and expressed as fold change over VC (0.1% DMSO). The data shown are the means \pm S.D. of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ (One-way ANOVA with Tukey's post hoc test performed on log-converted values).

Fig. 2 Effect of VPA on mRNA expression of ABCG2 in hCMEC/D3 cells at different time points under study. Cells were treated with VPA (300 μ M, 600 μ M) for 6h, 12h, 24h and 48h. Total RNA was extracted and subjected to RT-qPCR to determine changes in mRNA levels of ABCG2. The mRNA level of ABCG2 was normalized to those with *B2M* and expressed as fold change over VC (0.1% DMSO). The data is the mean \pm S.D. of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ (Two-way ANOVA with Tukey's post hoc test performed on log-converted values).

Fig. 3 Effect of VPA on BCRP expression and functional activity in hCMEC/D3 cells. (A) Whole cell lysates from hCMEC/D3 cells treated with VC (0.1% DMSO) or VPA (300 μ M, 600 μ M) for 6h, 12h, 24h, 48h and 72h were subjected to SDS-PAGE and western blot to measure BCRP expression. Bands of HSC were used to normalize the results. Mean densitometric values of the bands obtained from 3 independent experiments was used to calculate fold change over the VC. (B) Representative blot for 48h VPA treatment is shown. (C) hCMEC/D3 cells were treated with VC (0.1% DMSO) or VPA (300 μ M, 600 μ M) for 12h, 24h, 48h and 72h prior to the assay. Intracellular BPZ fluorescence was measured in the presence and absence of the BCRP inhibitor Ko143. Fluorescence intensities were

normalized with total protein content. BCRP activity was determined as the ratio between the mean intracellular fluorescence of cells incubated with KO143 and cells incubated without KO143 in the VPA treated group, normalized to the corresponding ratio in the control (VC) group. Data is the means \pm S.D. of 4 independent experiments. * $p < 0.05$, ** $p < 0.01$ (Two-way ANOVA with Tukey's post hoc test performed on log-converted values).

Fig. 4 Effect of increasing concentrations of AEDs (A) PHT, (B) CBZ, (C) VPA, (D) LTG, (E) TPM and (F) LEVI on vanadate-sensitive BCRP ATPase activity. The ATPase activity is expressed as nmol phosphate generated/mg protein/min. Lower dotted line represents baseline activity (in the presence of 2% v/v DMSO or water) and upper dotted line represents activity of fully activated membrane (in the presence of known BCRP activator sulfasalazine). Activation study measures stimulation of baseline ATPase activity indicating drug as BCRP substrate. Data are presented as means \pm S.D from 3 independent experiments. Statistically significant differences between the baseline and drug-stimulated activity in the activation assay (* $p < 0.05$, ** $p < 0.01$) were determined using one-way ANOVA with Dunnett's post hoc test performed on log-converted values. Inhibition study measures decrease in maximally stimulated BCRP activity indicating interaction of drug with BCRP . Data are presented as means \pm S.D from 3 independent experiments. Statistically significant differences between the maximal membrane activity and drug-treated activity in the inhibition assay (* $p < 0.05$) were determined using one-way ANOVA with Dunnett's post hoc test performed on log-converted values.

Fig. 5 Competitive substrate efflux assay to study substrate relationship of PHT, VPA and LTG with BCRP. hCMEC/D3 cells were co-incubated with either assay substrate BPZ (250nM) and VC (0.1% DMSO) or test AED [(A) PHT, (B) VPA and (C) LTG] or inhibitor of BCRP (1 μ M KO143) and intracellular accumulation of BPZ was measured. Results are reported as the fold increase in intracellular fluorescence in treated cells over control

fluorescence. The data is the mean \pm S.D. of 5 independent experiments. Statistical significance was calculated using one-way ANOVA with Dunnett's post hoc test performed on log-converted values (** $p < 0.01$; BPZ+VC vs. BPZ+PHT/VPA/LTG or BPZ+VC vs. BPZ+KO143).

Fig. 6 Effect of PPAR α knockdown on VPA-induced ABCG2 expression and activity.

Expression of *PPAR α* was silenced by transient transfection of siPPAR α (siRNA specific to human *PPAR α*) in hCMEC/D3 cells. Then, cells were treated with VC (0.1% DMSO) or VPA (600 μ M) for 24h, 48h and 72h to check ABCG2 mRNA, protein and activity, respectively. Scramble (SCR) was used as non-targeting control. (A) RT-qPCR analysis of *PPAR α* expression, mRNA level of *PPAR α* was normalized to those with *B2M*. The data is the mean \pm S.D. of 3 independent experiments. ** $p < 0.01$, VC (SCR vs. siRNA); # $p < 0.01$ VPA (SCR vs. siRNA) (unpaired t-test). (B) RT-qPCR analysis of ABCG2 expression, mRNA level of ABCG2 was normalized to those with *B2M* (C) Representative blot of PPAR α and BCRP is shown; bands of HSC were used to normalize the protein expression (D) Western blot analysis of BCRP expression and (E) Analysis of BCRP functional activity. The data is the mean \pm S.D. of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, N.S., not significant (Two-way ANOVA with Tukey's post hoc test).

Fig. 7 Effect of PPAR α antagonist MK886 on inductive effect of VPA on BCRP expression and activity.

hCMEC/D3 cells were cultured with VC (0.1% DMSO) alone or VPA (600 μ M) alone or VC/VPA in the presence of PPAR α antagonist MK886 for 48h to check effect on ABCG2 protein and activity. (A) Representative blot of BCRP is shown; bands of HSC were used to normalize the protein expression (B) Western blot analysis of BCRP expression and (C) Analysis of BCRP functional activity. The data is the mean \pm S.D.

of 3 independent experiments. $**p < 0.01$, N.S., not significant (Two-way ANOVA with Tukey's post hoc test performed on log-converted values).

Fig. 8 Effect of VPA on expression of PPAR α and its target genes. hCMEC/D3 cells were treated with VC (0.1% DMSO) or VPA (600 μ M) for the indicated time points. (A) RT-qPCR was done to check the effect on PPAR α mRNA expression. *B2M* was used as the reference gene for normalization. (B) Whole cell lysates were subjected to western blot to measure change in PPAR α protein expression. (C) Representative PPAR α blot for 3h is shown. HSC protein bands served as loading control. The data is the mean \pm S.D. of 3 independent experiments. $*p < 0.05$, $**p < 0.01$; VC vs. VPA (Two-way ANOVA with Tukey's post hoc test performed on log-converted values). (D) RT-qPCR was done to check the effect of VPA on mRNA expression of PPAR α target genes *PDK4* and *THBD* at 24h. *B2M* was used as the reference gene for normalization. The data is the mean \pm S.D. of 3 independent experiments. $**p < 0.01$; VC vs. VPA (unpaired t-test performed on log-converted values).

Fig. 9 Effect of VPA treatment on PPAR α translocation to nucleus. (A) hCMEC/D3 cells were incubated with VPA (600 μ M) for 0h, 3h and 6h. Isolated nuclear and cytoplasmic fractions of the cell lysate were subjected to western blot with PPAR α antibody. Lamin A/C and GAPDH was used as nuclear and cytosolic marker respectively. (B) The bar graph illustrates the relative nuclear and cytosolic PPAR α protein expression fold change at 3h and 6h w.r.t expression at 0h. The data is the mean \pm S.D. of 4 independent experiments. $*p < 0.05$, $**p < 0.01$ (One-way ANOVA with Tukey's post hoc test performed on log-converted values). (C) hCMEC/D3 cells were treated with VPA (600 μ M) for 0h, 3h and 6h and subsequently fixed with 4% paraformaldehyde. Fixed cells were incubated with PPAR α primary antibody and Alexa Fluor 488-conjugated secondary antibody. Nuclear staining was performed with DAPI. The intracellular distribution of the fluorescent tag was examined under a confocal microscope. Color in green (Alexa488-conjugated antibody) and blue

(DAPI) represent PPAR α and nucleus, respectively. Arrows indicate cells depicting the translocation. Average PPAR α fluorescence ratios (nuclear to cytoplasm) at 3h and 6h compared to that at 0h were obtained from 4 independent experiments (80 cells per treatment group per experiment). (D) Bar graph shows relative quantification of nuclear: cytoplasmic ratios of PPAR α fluorescence. **p < 0.01 (One-way ANOVA with Tukey's post hoc test performed on log-converted values).

Fig. 10 Effect of VPA on PPAR α recruitment to the *ABCG2* promoter and promoter activation (A) hCMEC/D3 cells were treated with VC (0.1% DMSO) or VPA (600 μ M) for 3h and 6h, and PPAR α recruitment to the region of interest was assessed by ChIP-qPCR. A nonspecific rabbit IgG antibody was included as negative control. Recruitment is presented as percentage of total input. The data is the mean \pm S.D. of 3-4 independent experiments. *p < 0.05, **p < 0.01 (VPA vs VC) (unpaired t-test performed on log-converted values). B. 1% agarose gel image showing qPCR product from 3h treated samples. C. Luciferase reporter gene construct containing the PPRE sequences of the *ABCG2* promoter (pGL2-prom-*ABCG2*) or the pGL2 control vector was cotransfected with renilla-luciferase expression vector into HEK293 cells. Cells were treated with VC (0.1% DMSO) or VPA (600 μ M) for 24h before measurement of luciferase activities. Firefly luciferase activities were normalized to renilla luciferase activities to consider changes in the transfection variability. Firefly/renilla activity of the VPA treated cells were then normalized to VC treatment (set as 1) to obtain the relative luciferase activity fold change. Data is the mean of 3 independent experiments. **p < 0.01 (VPA-treated cells compared with VC treatment set to 1; unpaired t-test performed on log-converted values).

Fig. 1

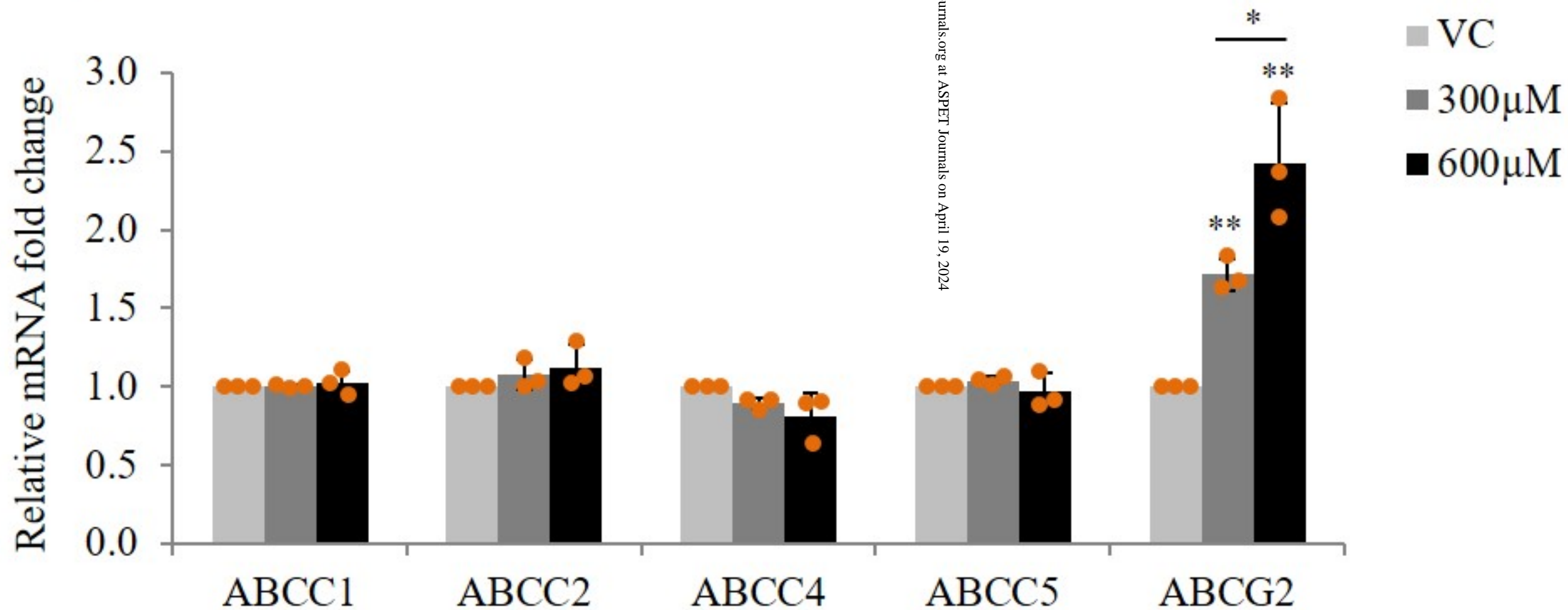


Fig. 2

Relative mRNA fold change
(ABCG2/B2M)

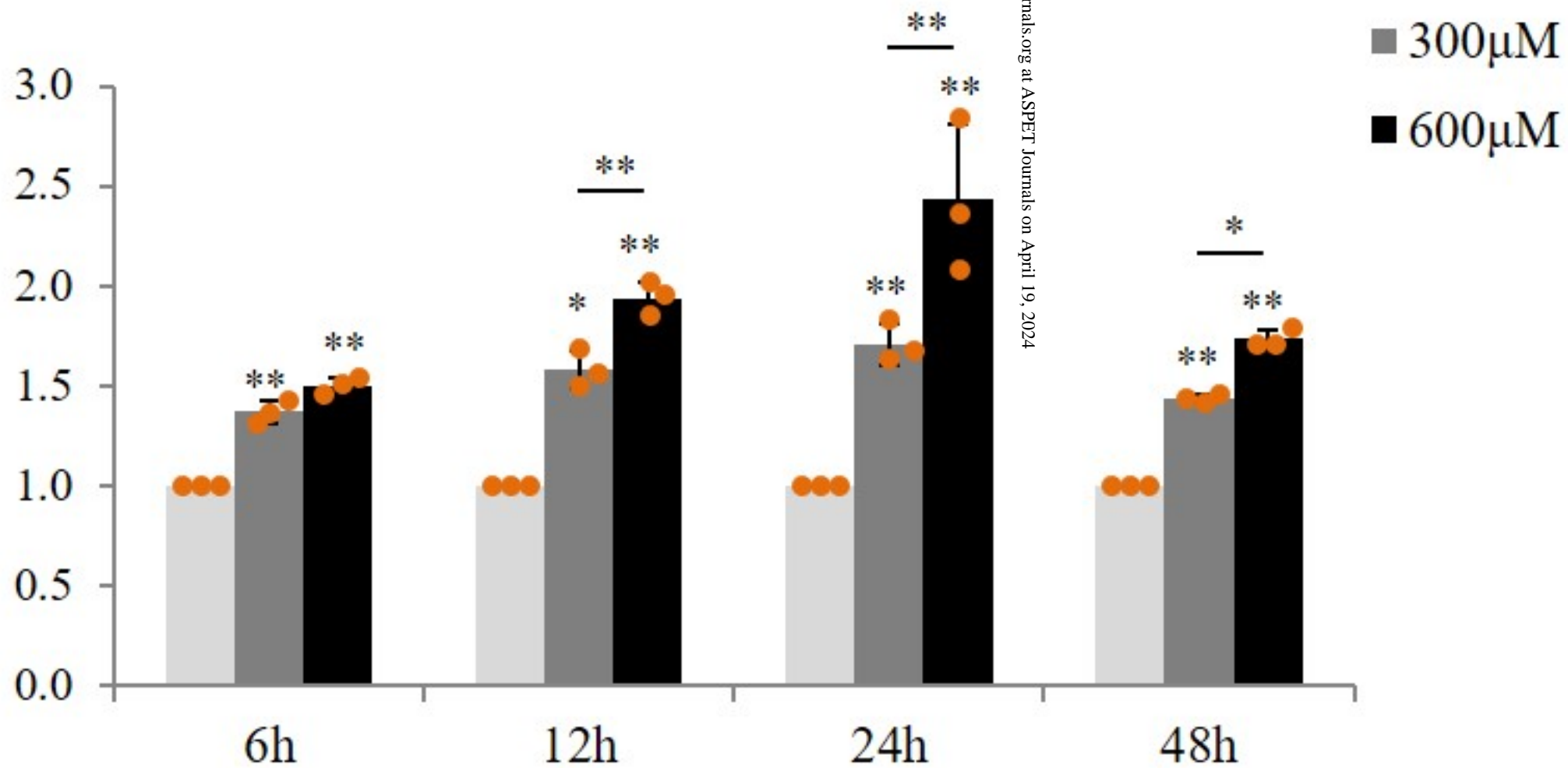
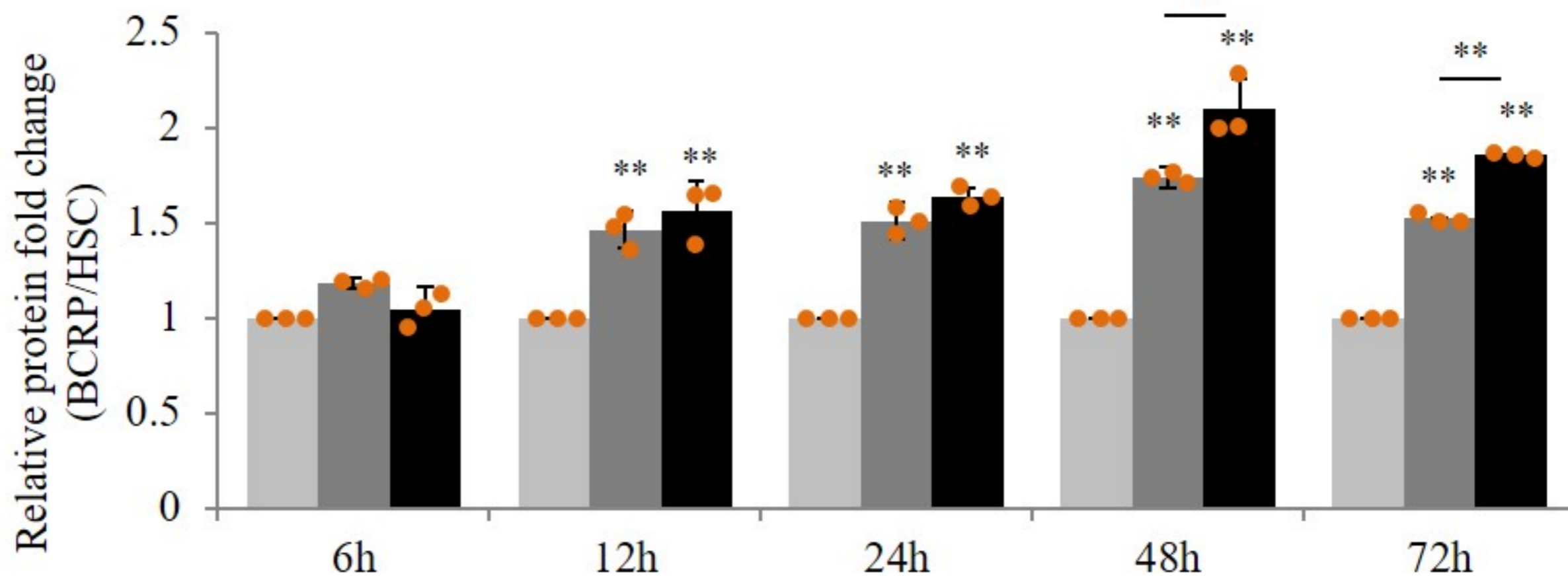


Fig. 3

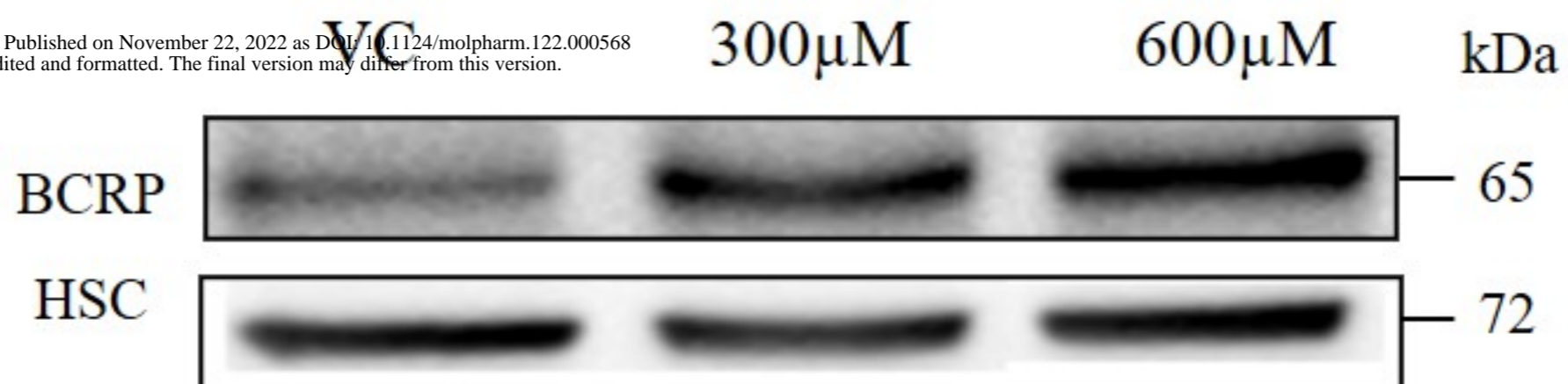
VC 300 μ M 600 μ M

A



B

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C

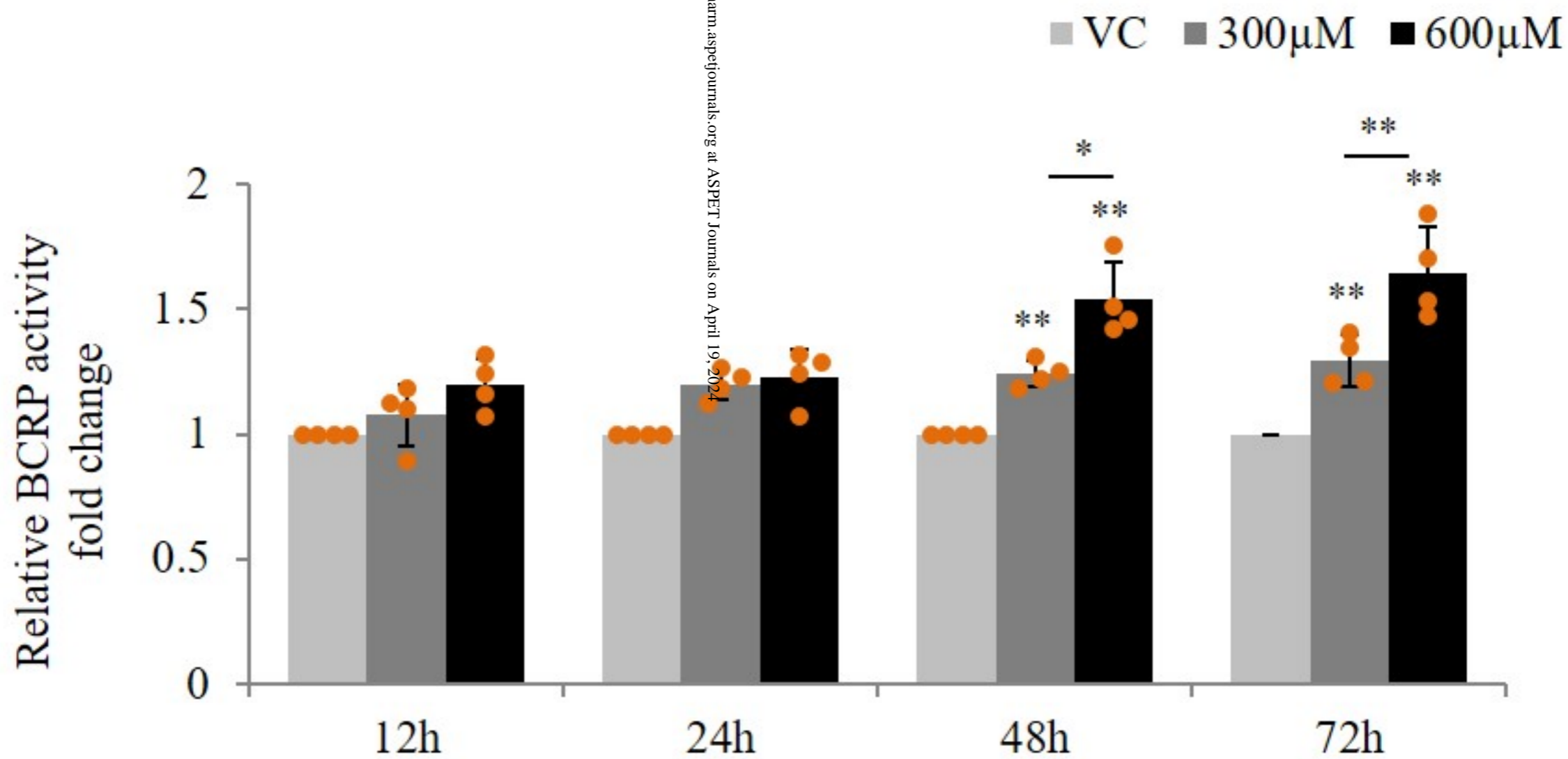


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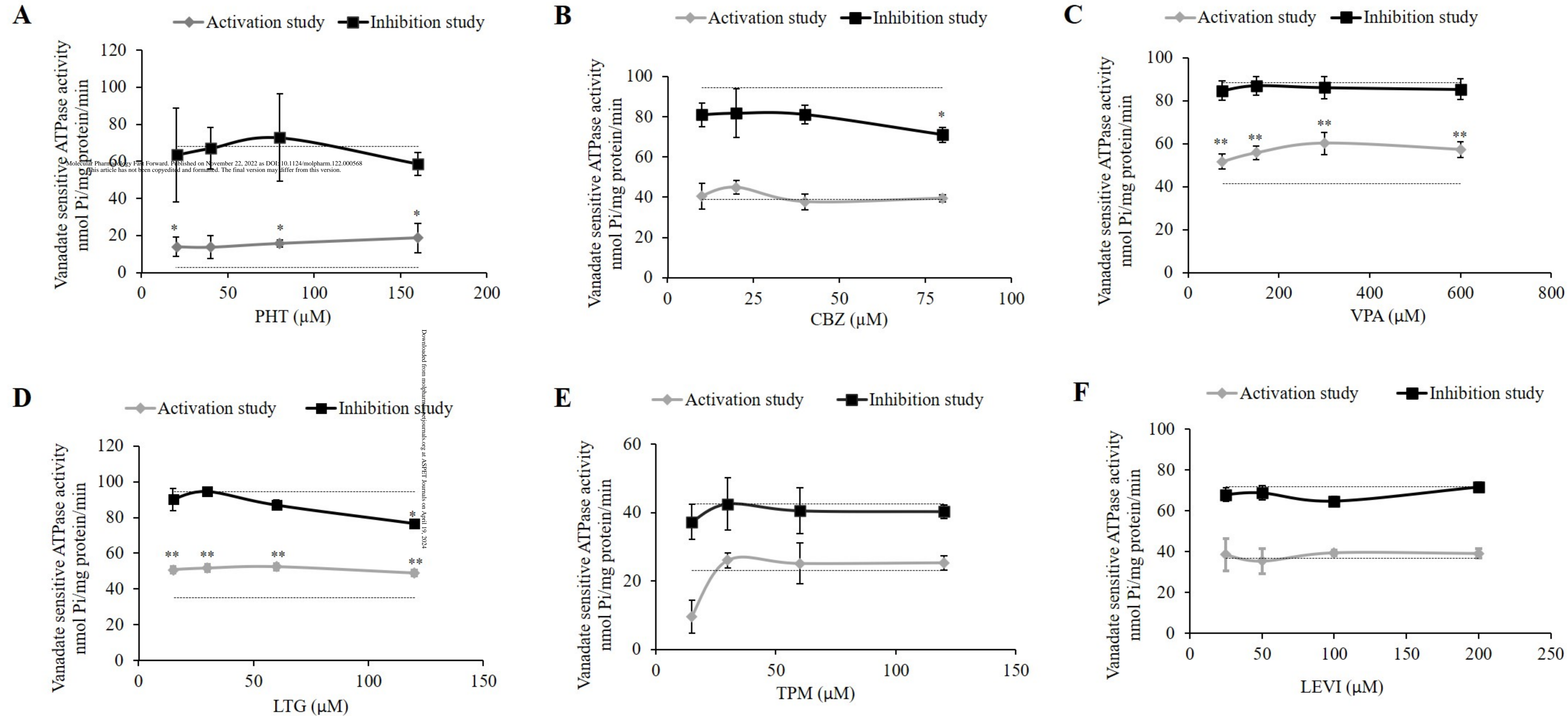
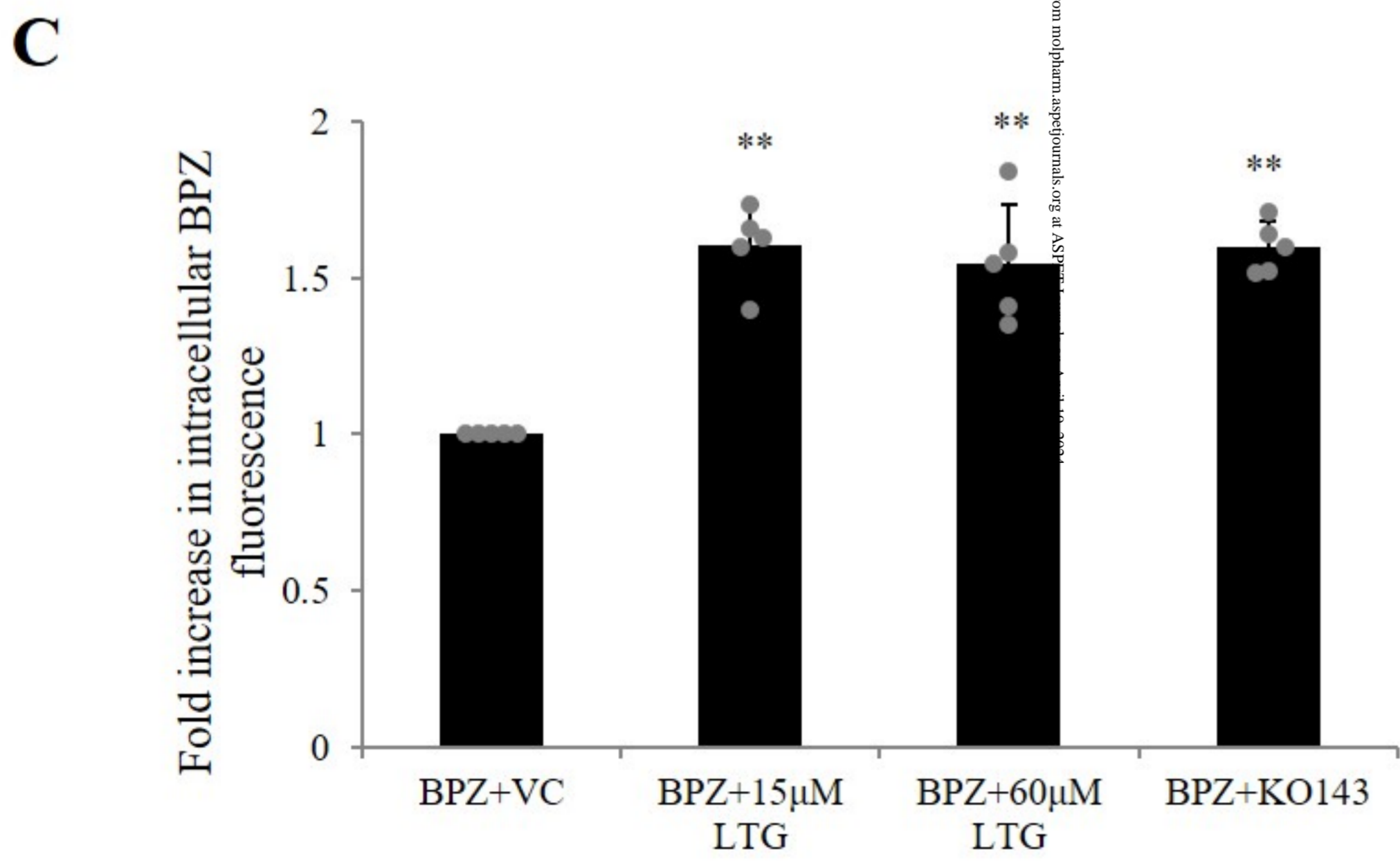
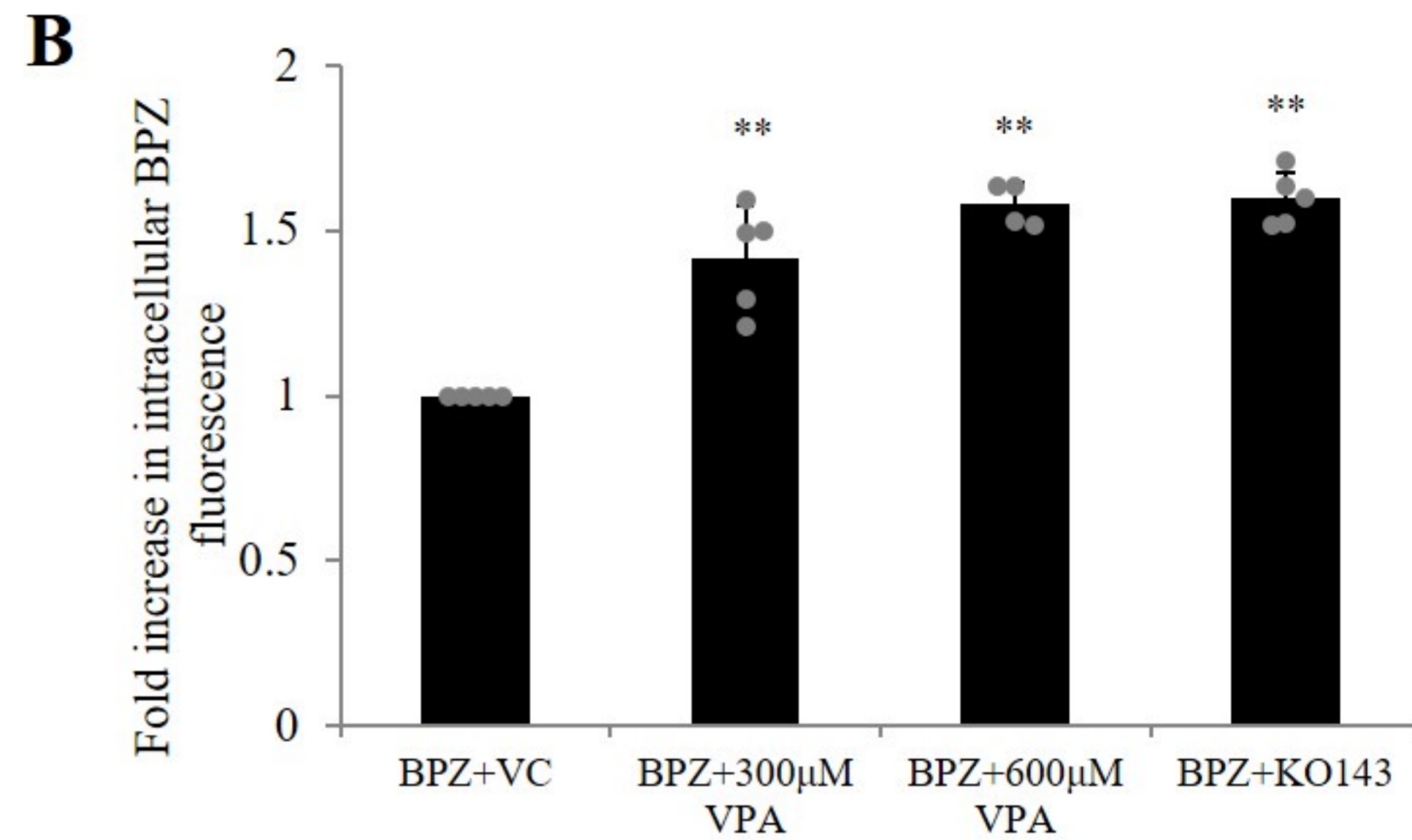
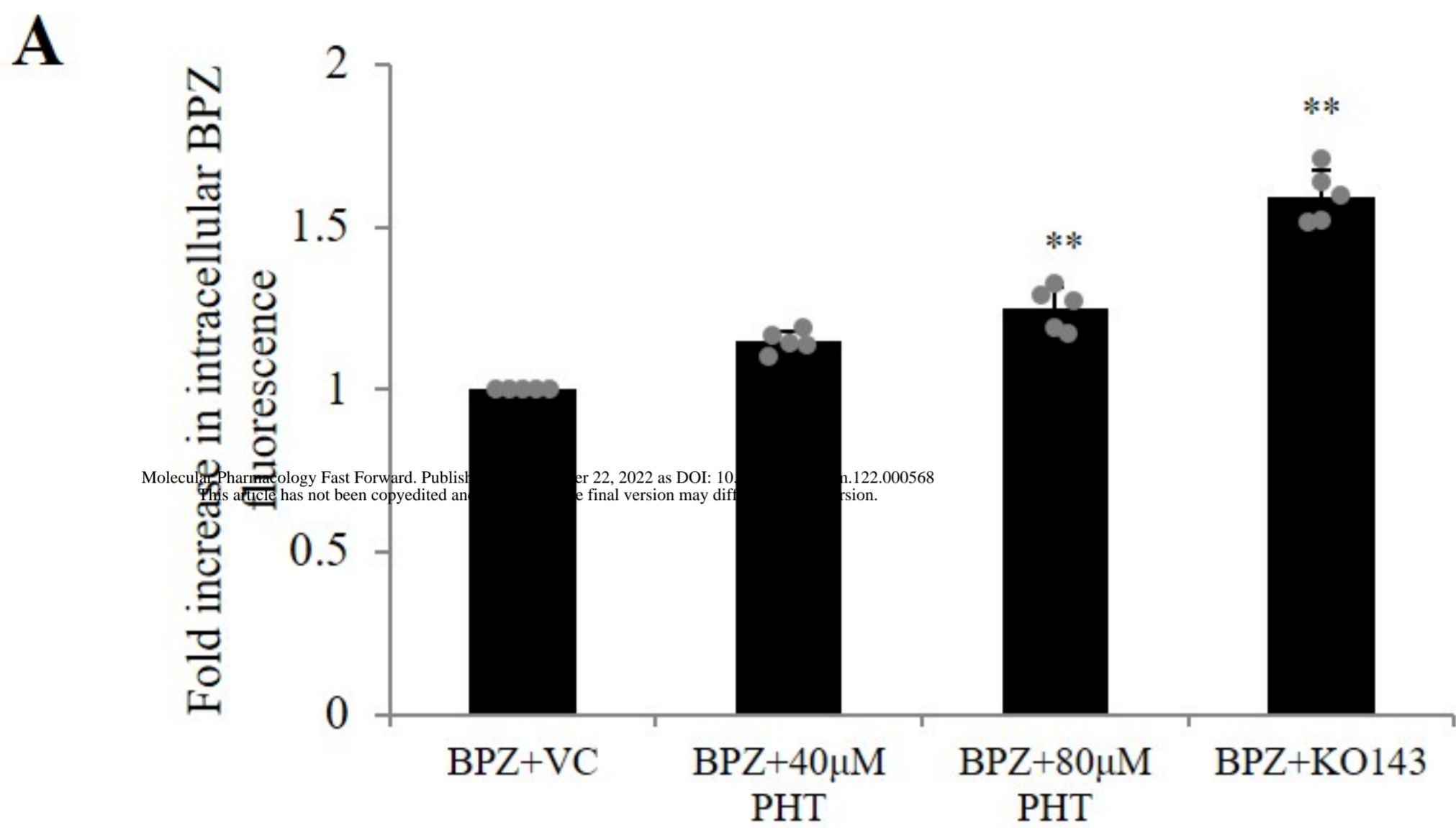


Fig. 5

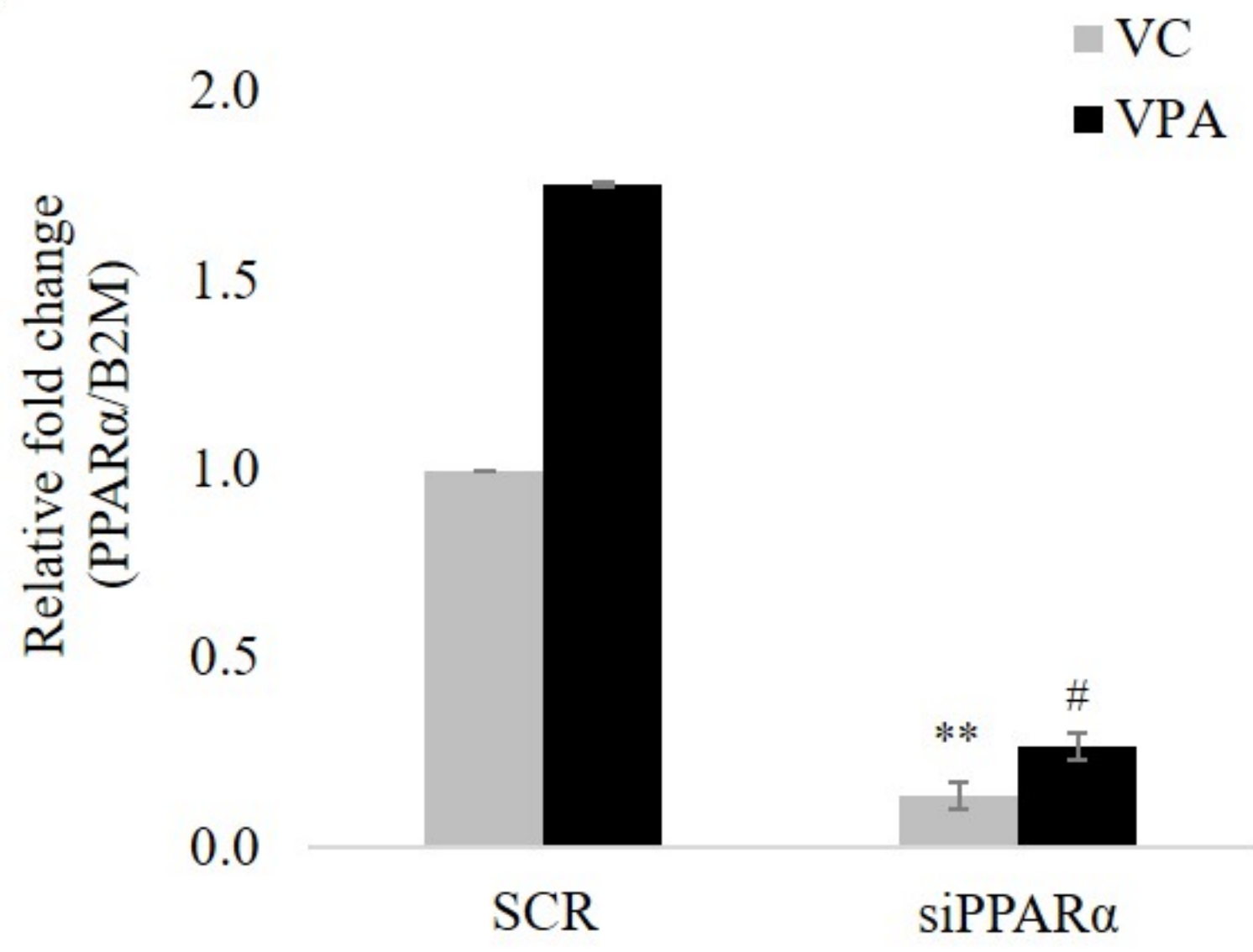


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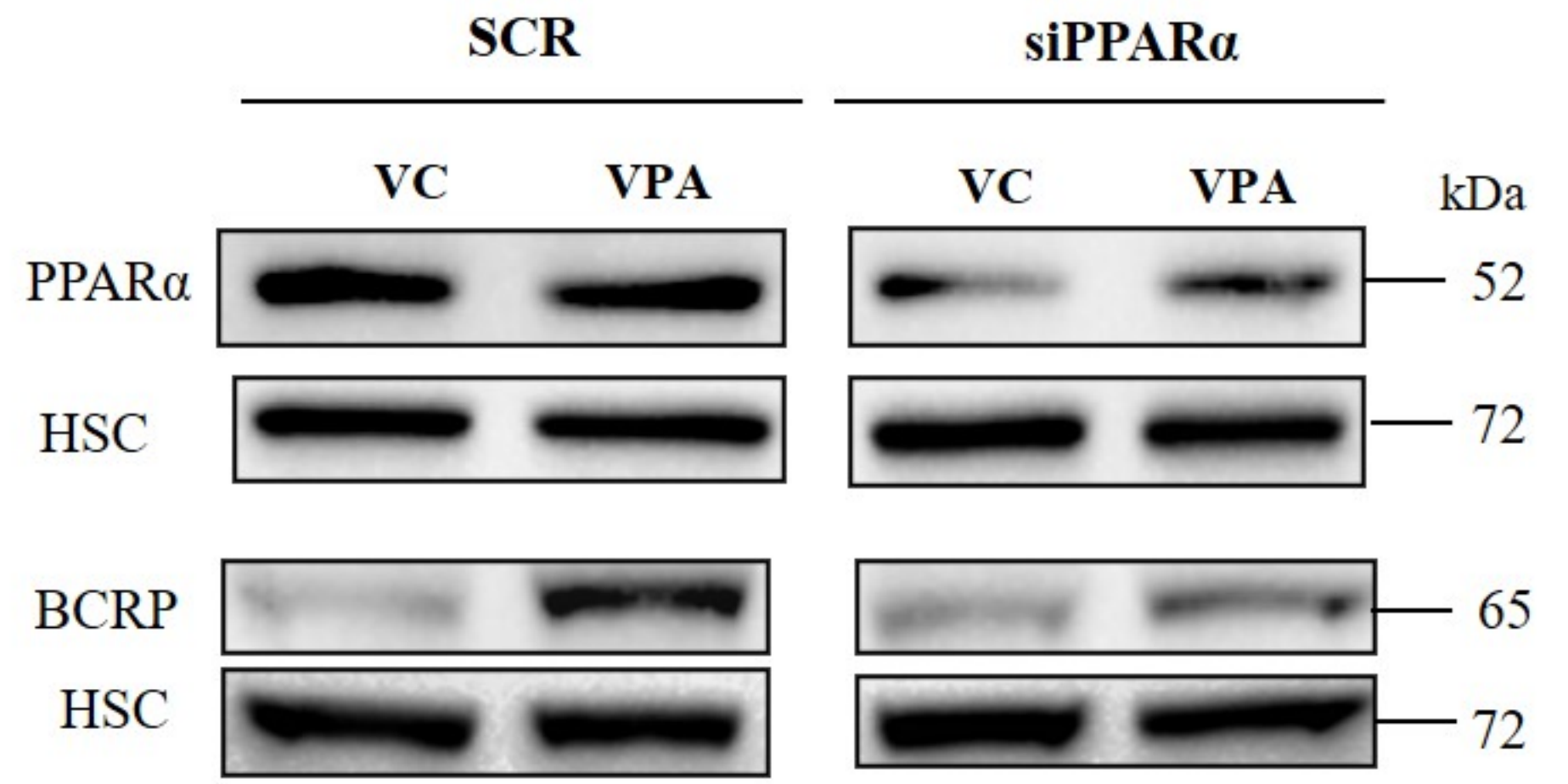
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Fig. 6

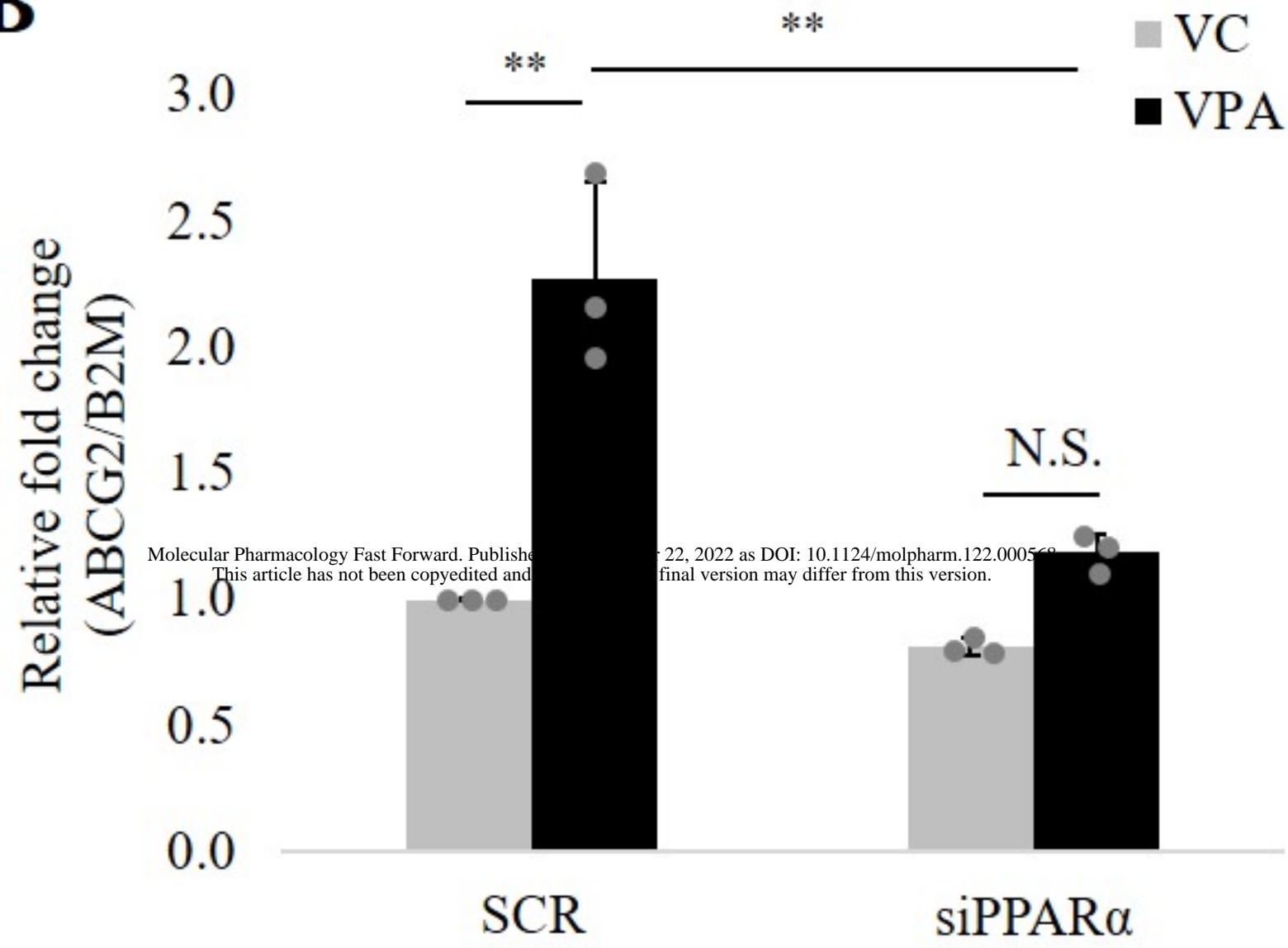
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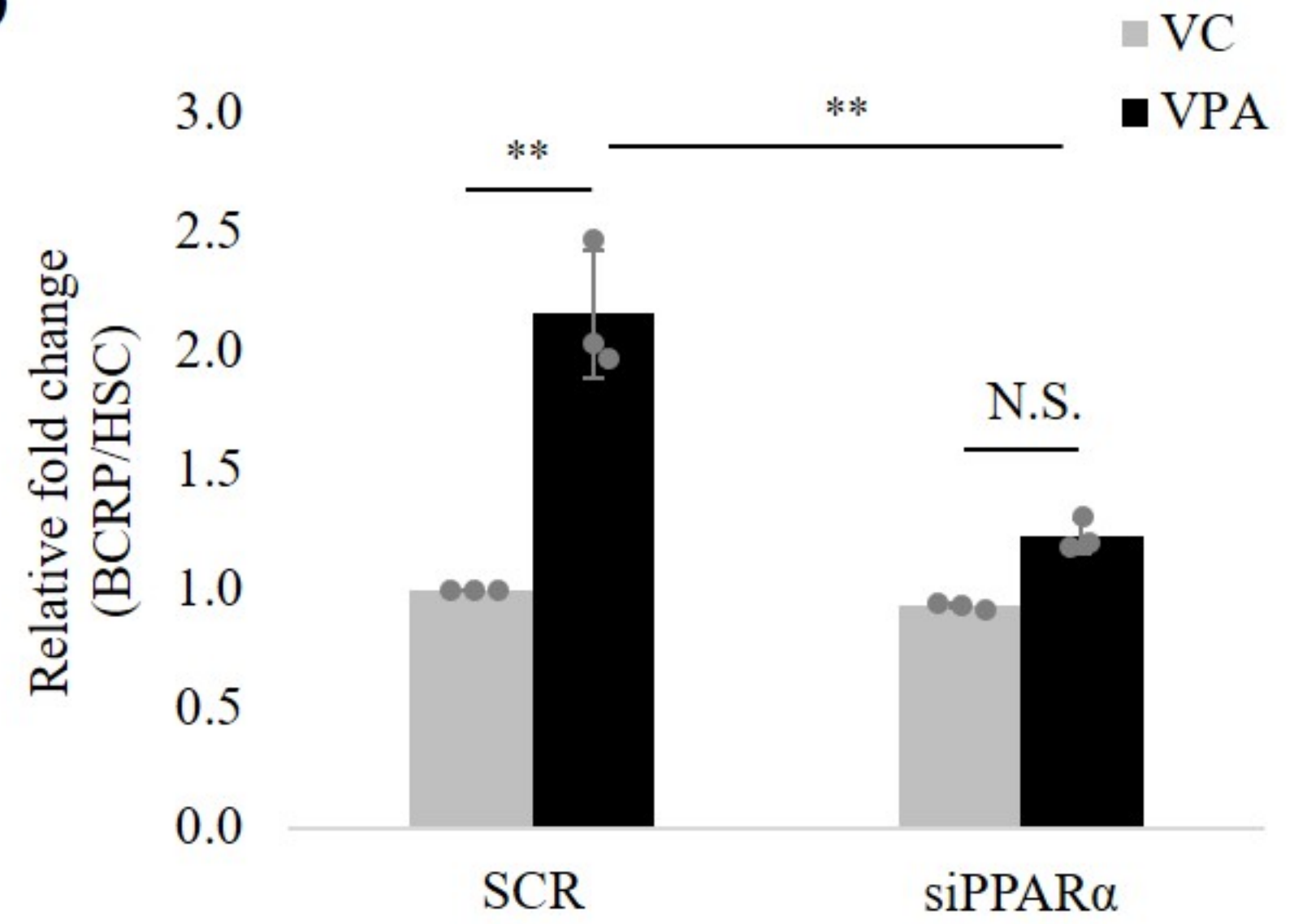
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E

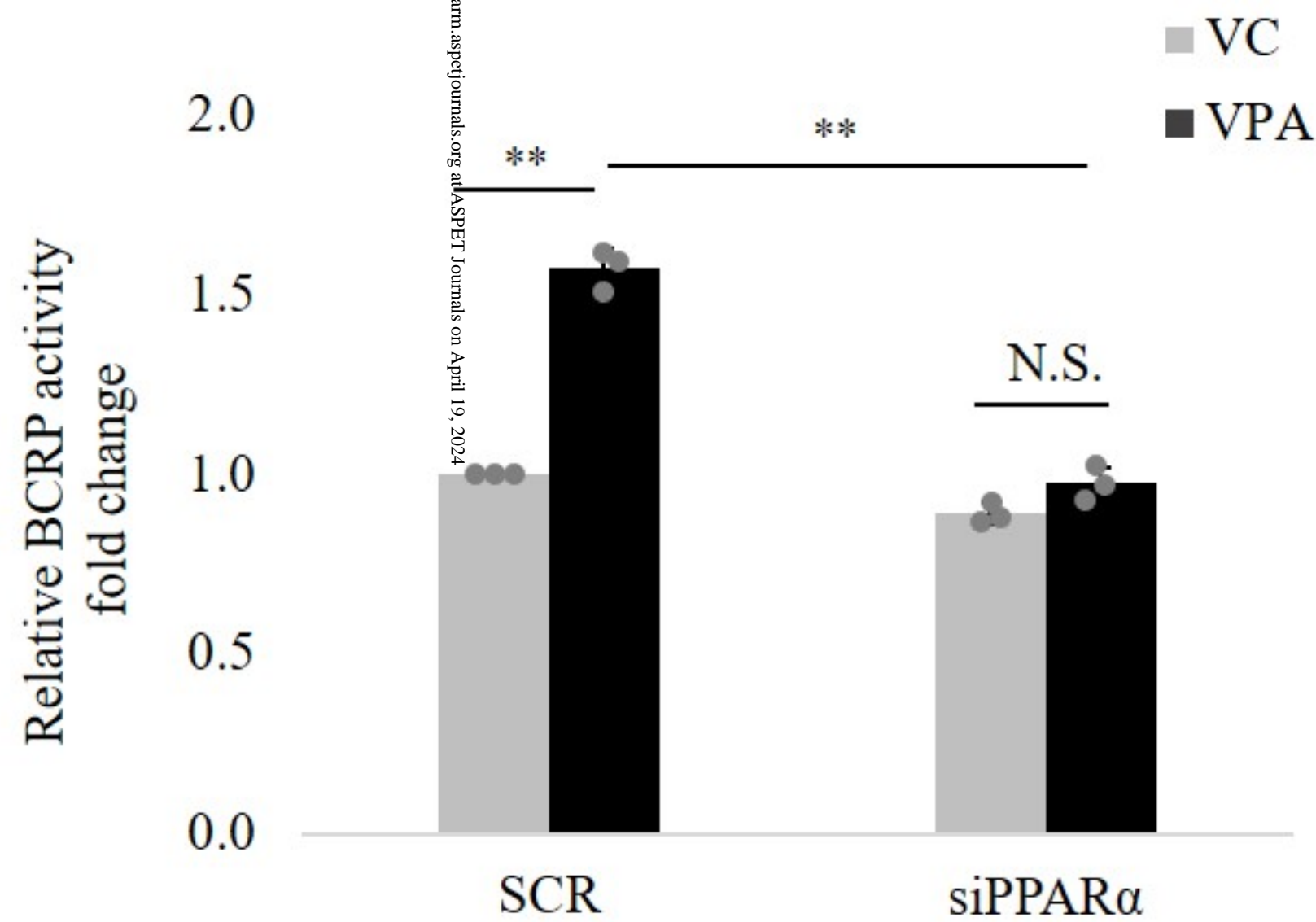
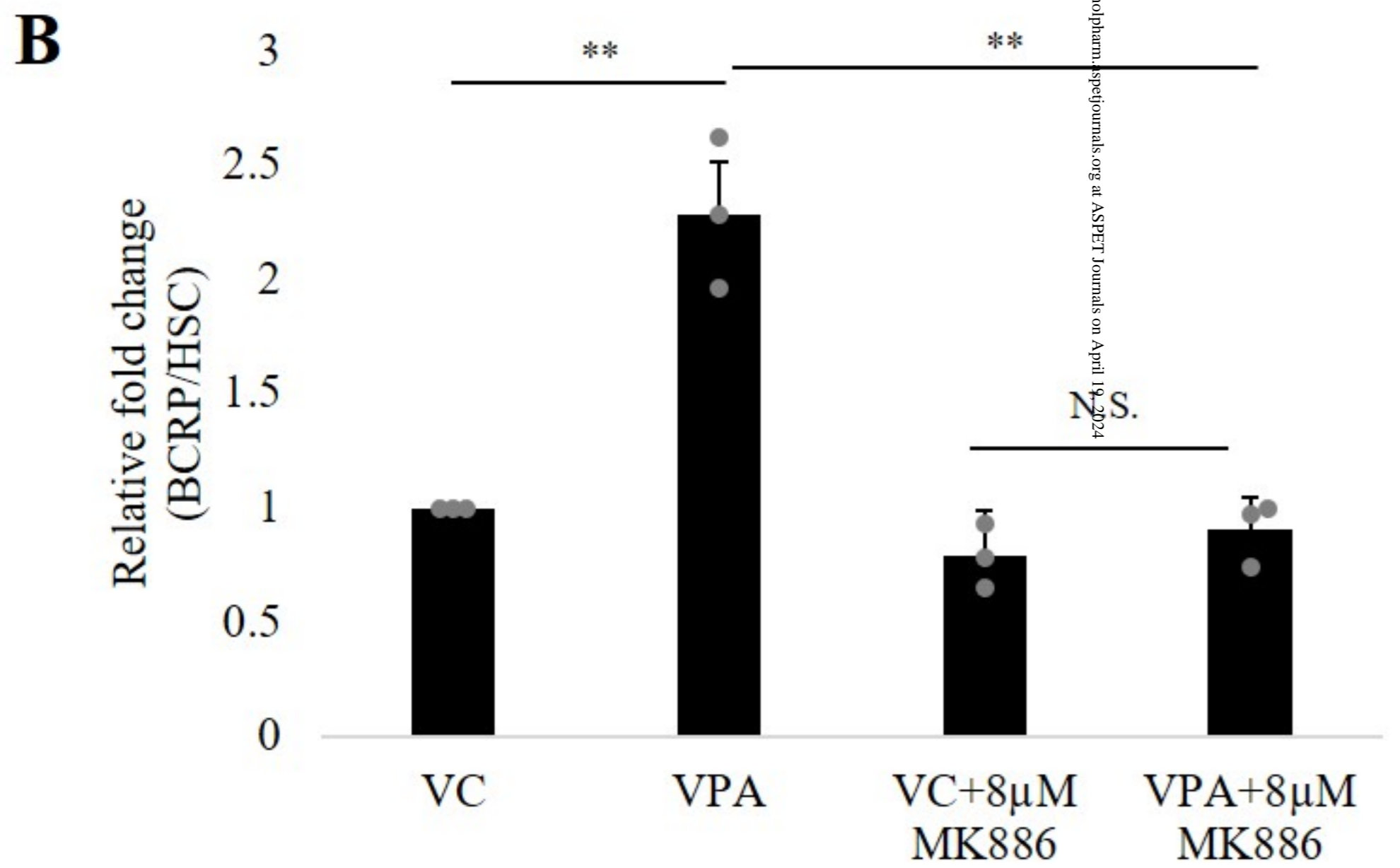
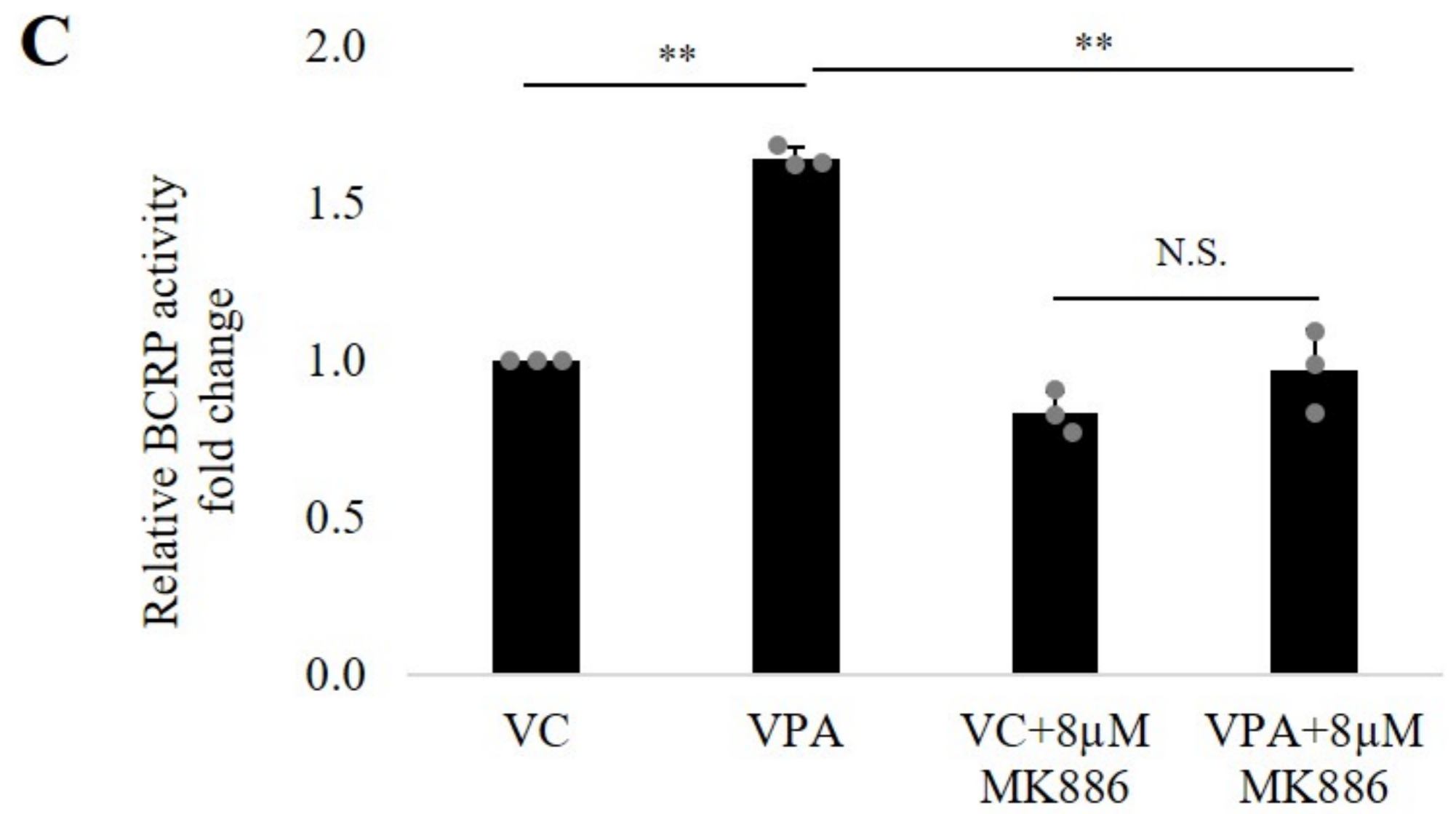
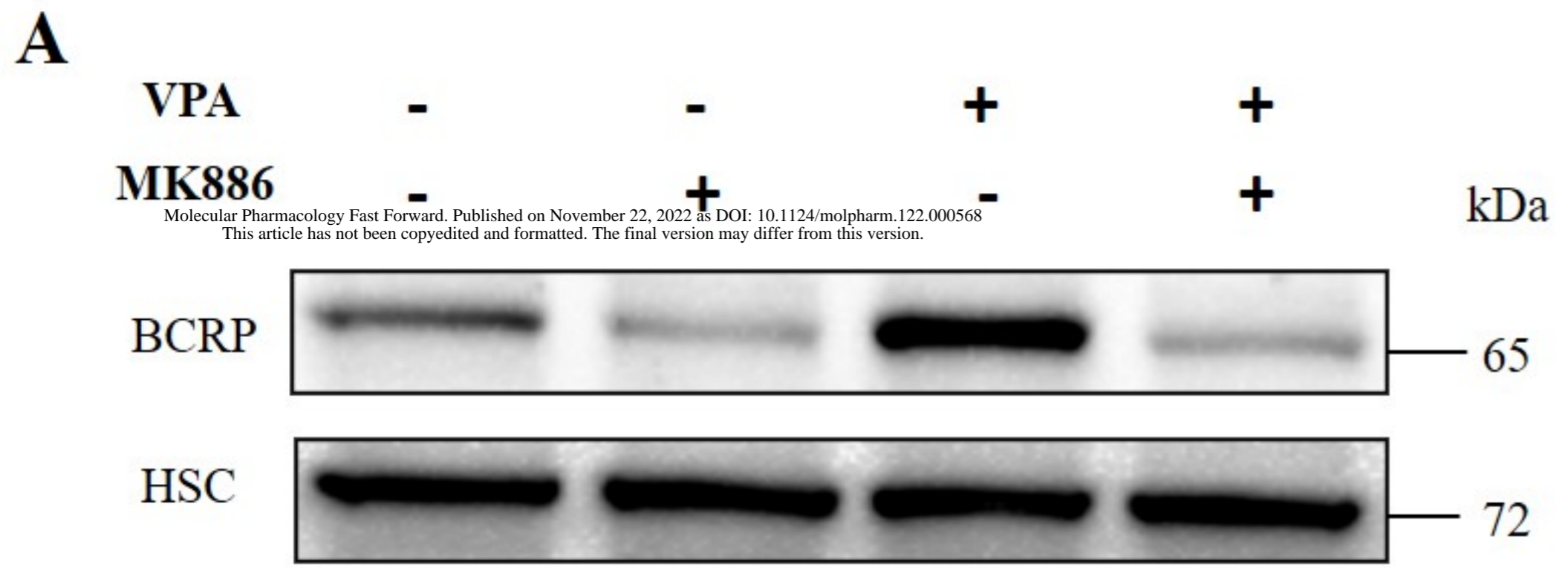
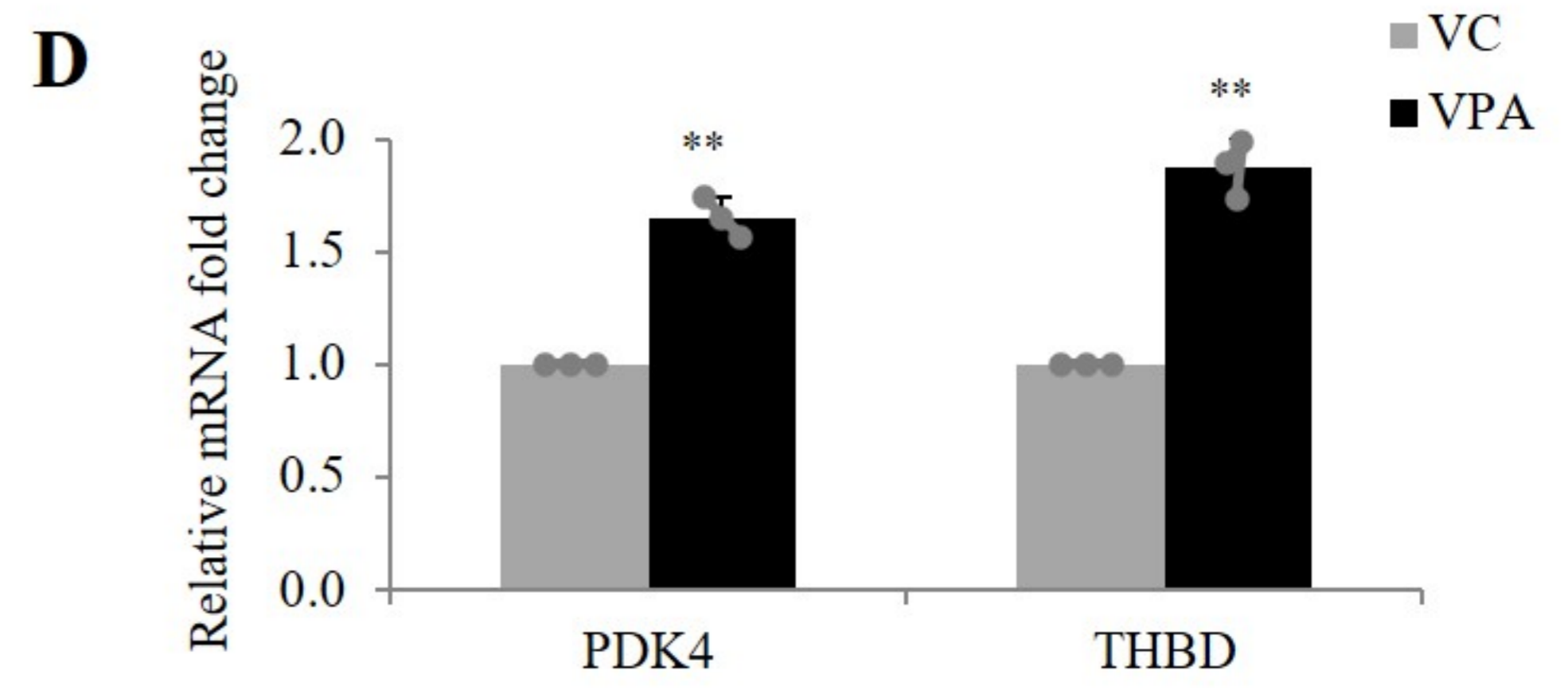
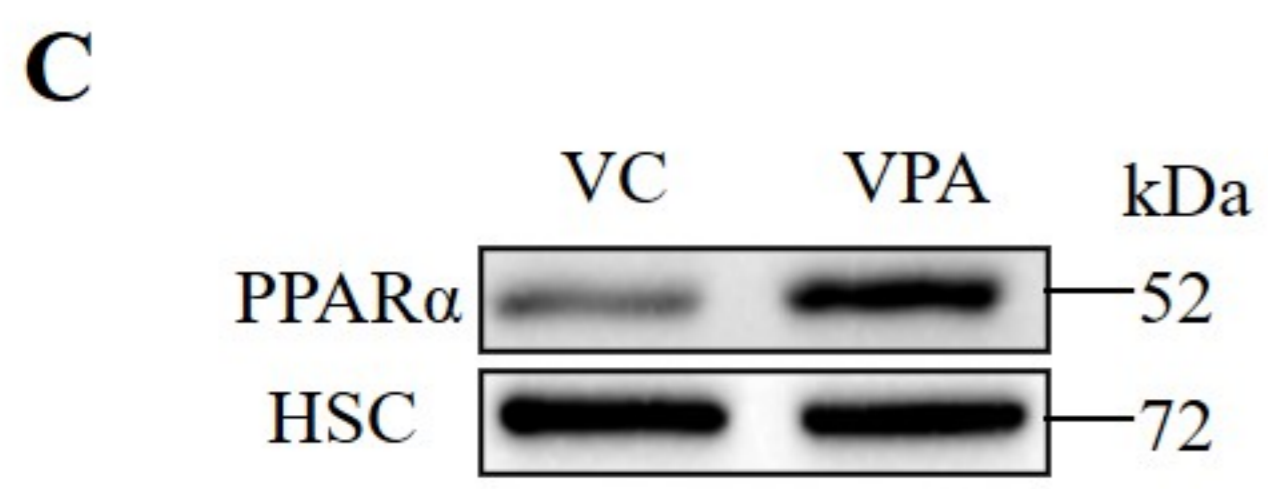
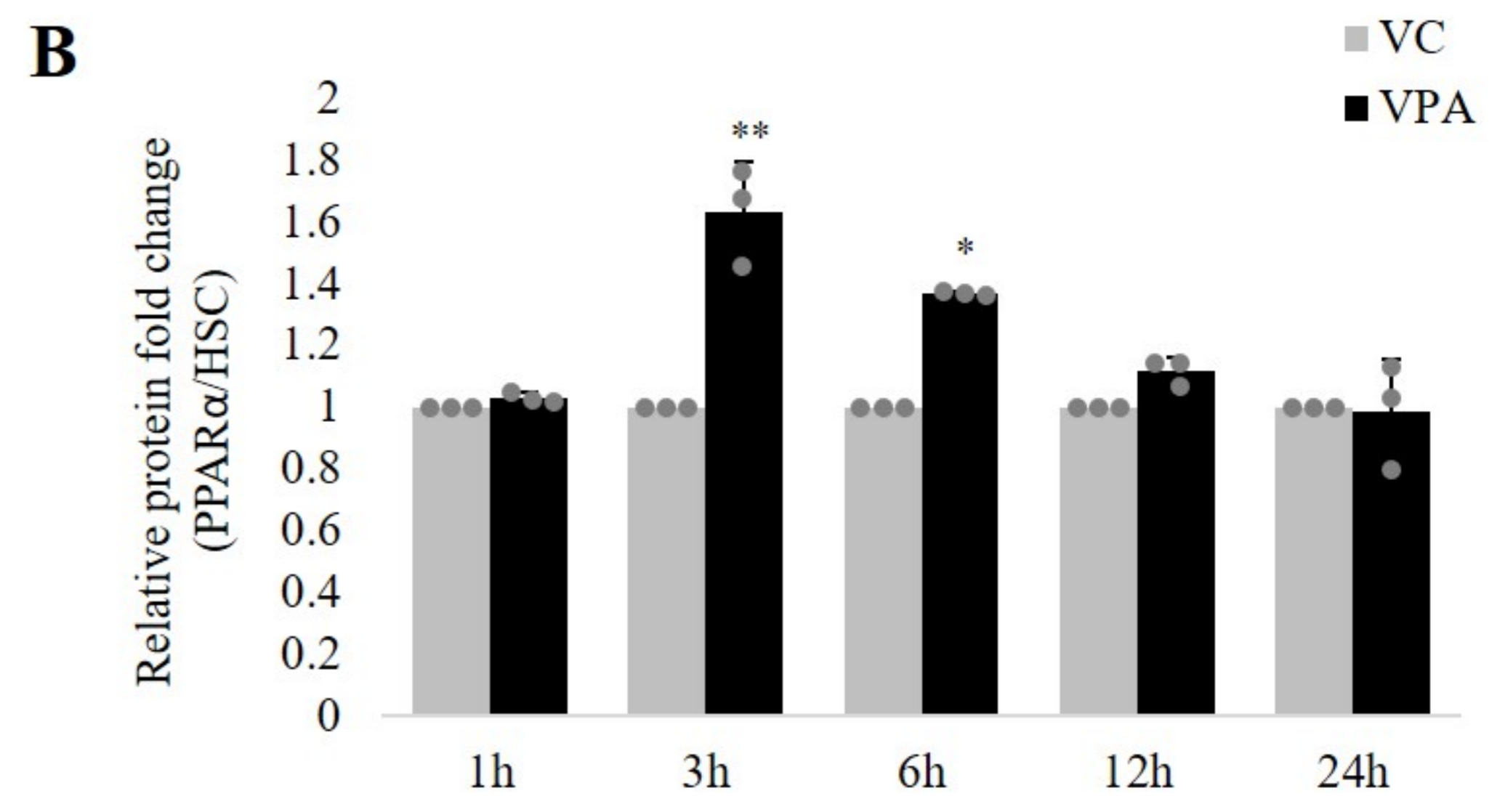
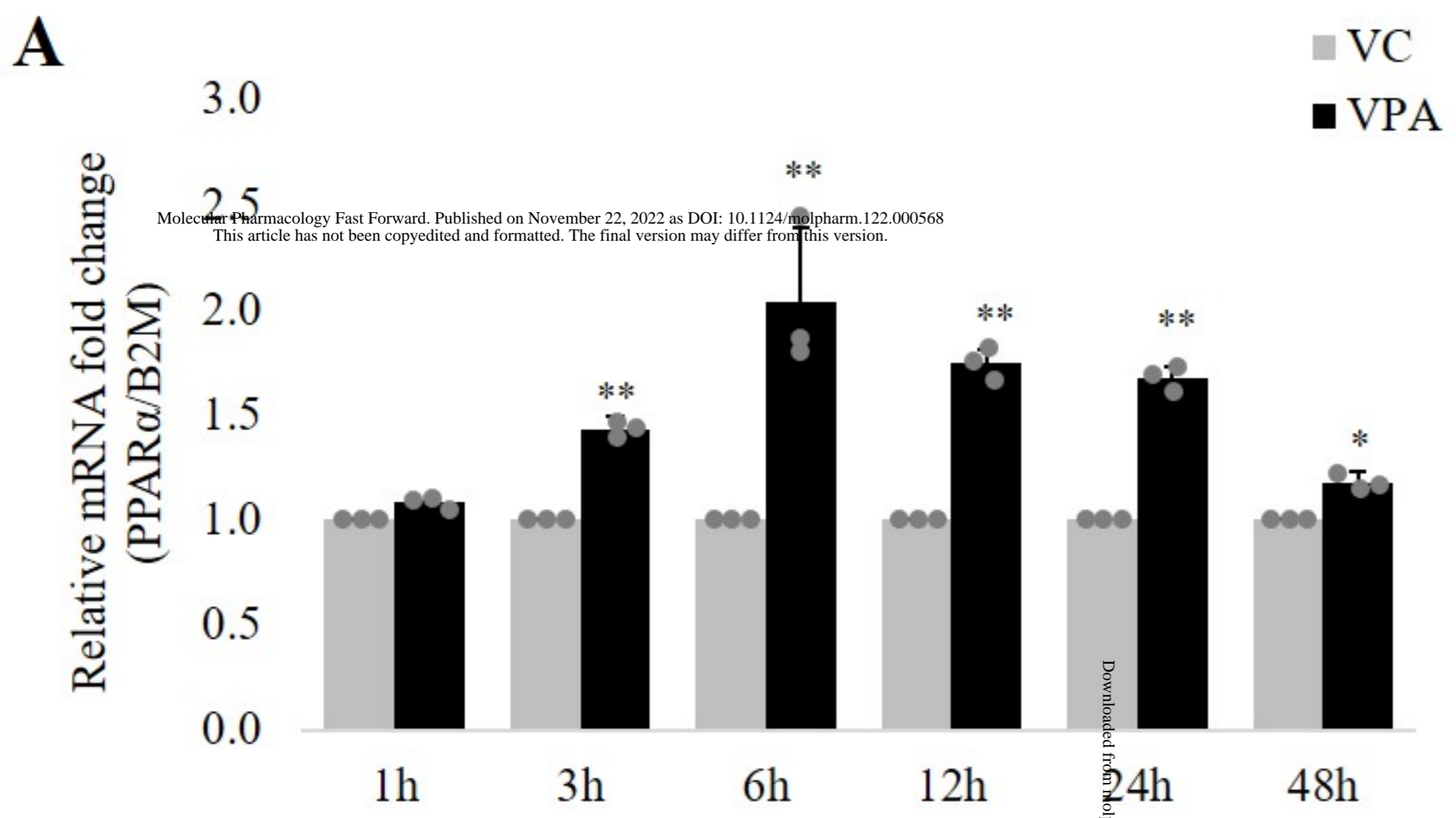


Fig. 7



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Fig. 8



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Fig. 9

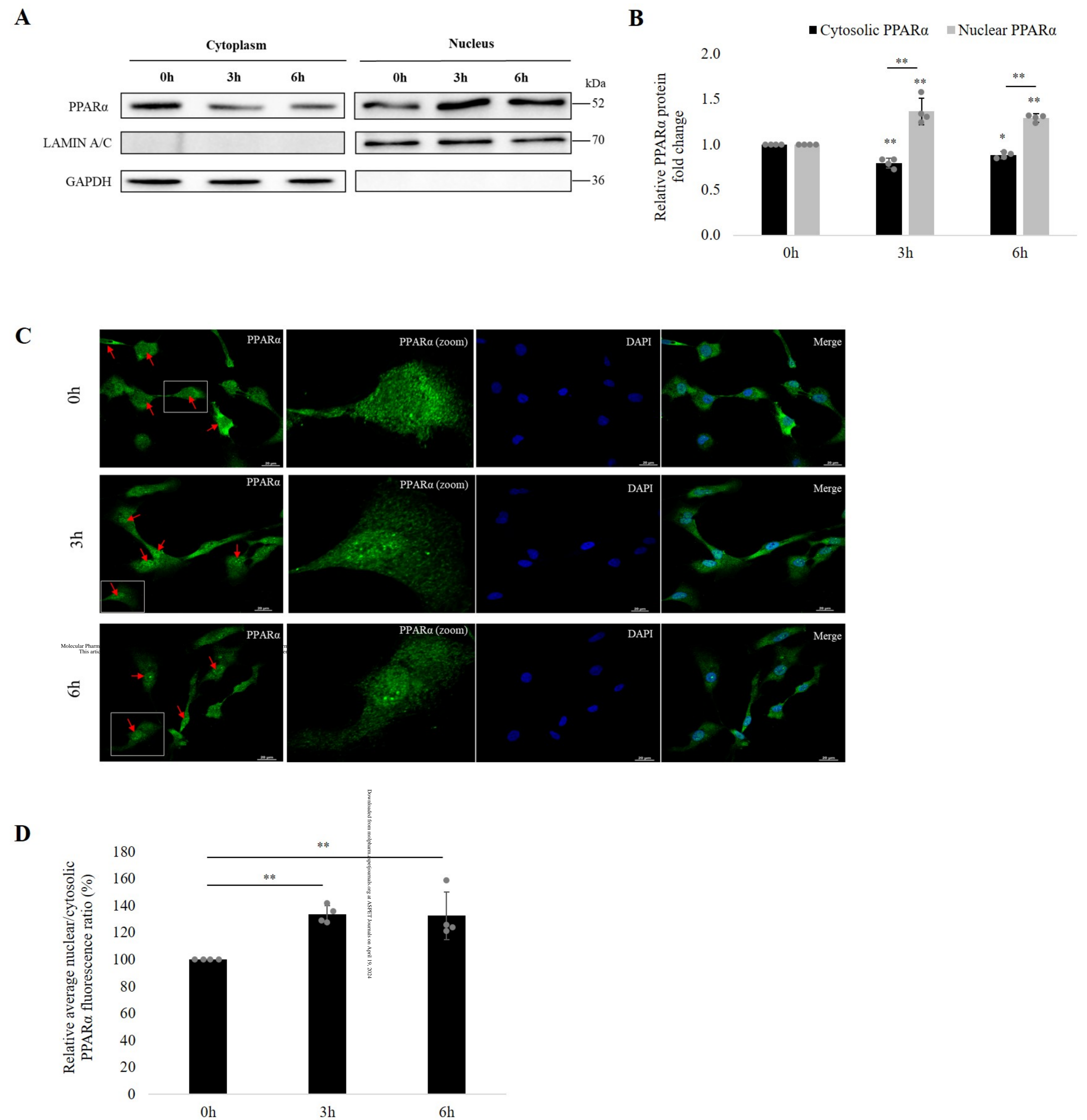
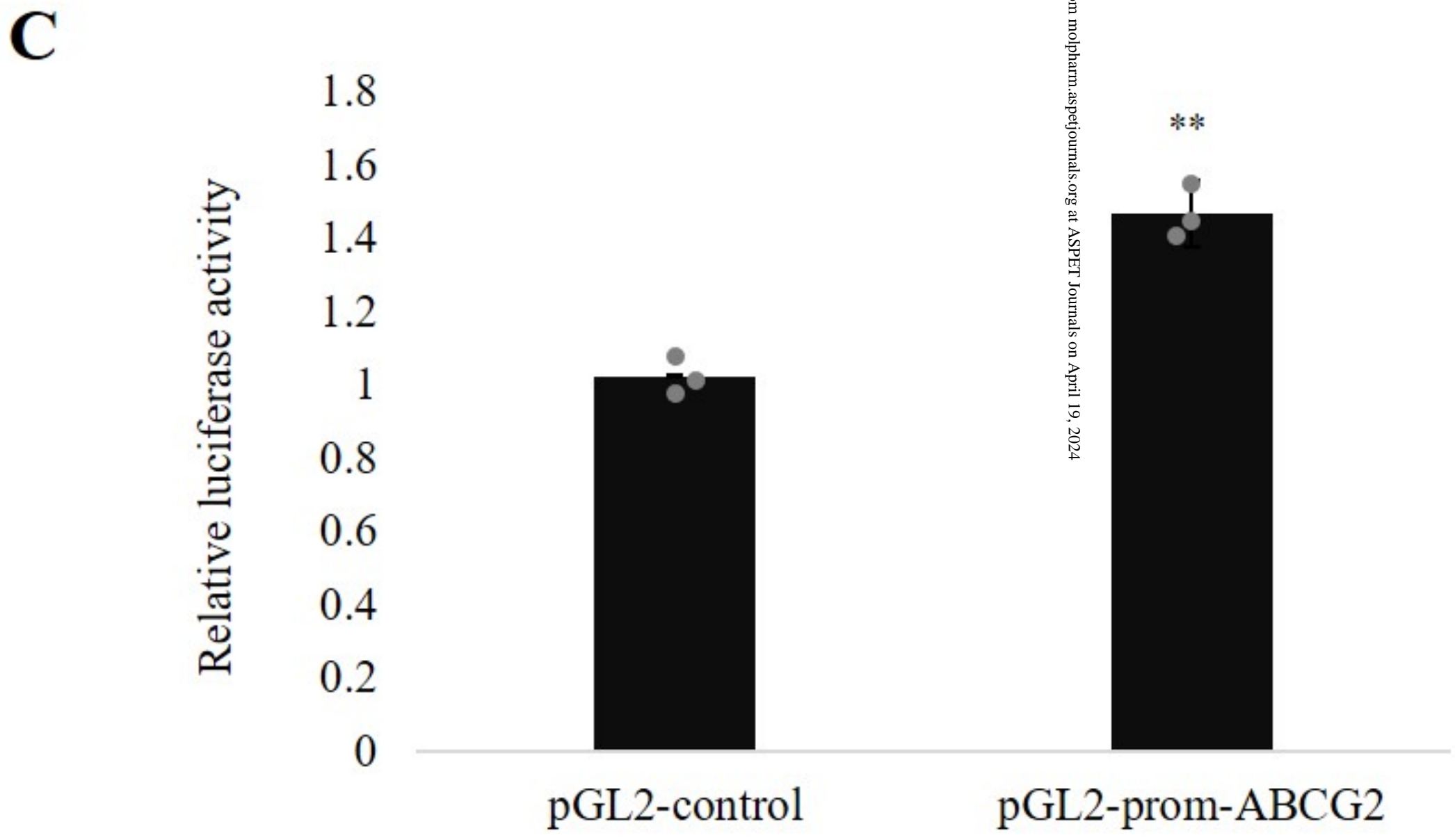
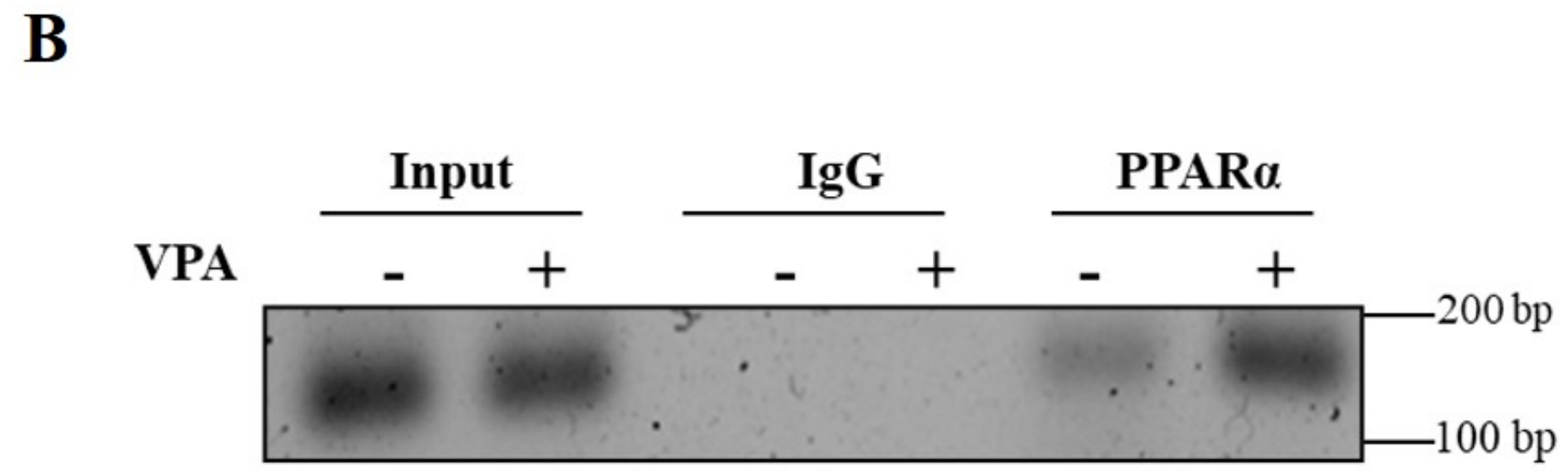
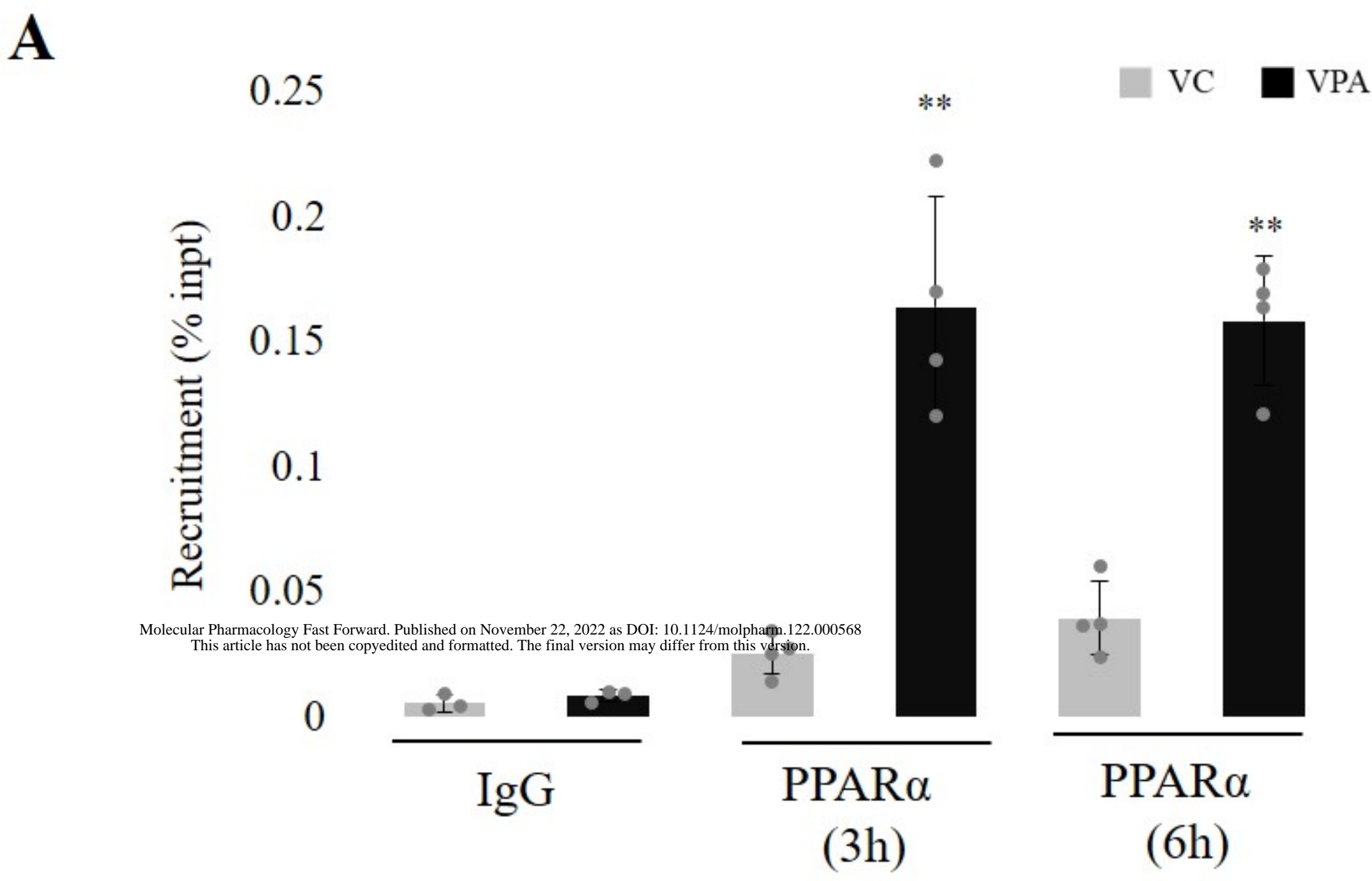


Fig. 10



SUPPLEMENTARY DATA

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Journal Name: Molecular Pharmacology

Valproic acid-induced upregulation of multidrug efflux transporter ABCG2/BCRP via PPAR α -dependent mechanism in human brain endothelial cells

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Supplemental Table 1

Therapeutic plasma concentrations (reference range) of antiepileptic drugs in patients with epilepsy (Johannessen, 2004)

Antiepileptic drug	Therapeutic plasma concentration range (μM)	Doses used in the present study
Phenytoin	40-80	40 μM , 80 μM
Carbamazepine	15-45	21 μM , 42 μM
Valproic acid	300-600	300 μM , 600 μM
Lamotrigine	10-60	15 μM , 60 μM
Topiramate	15-60	15 μM , 60 μM
Levetiracetam	35-120	40 μM , 120 μM

Supplemental Table 2

Sequence of primers used for qPCR

A. Primer sequences for MDTs and B2M		
Gene	Forward Primer	Reverse Primer
<i>ABCC1</i>	5'-TGTGTGGGCAACTGCATCG-3'	5'-GTTGGTTTCCATTTTCAGATGACA-3'
<i>ABCC2</i>	5'-ATATAAGAAGGCATTGACCC-3'	5'-ATCTGTAGAACACTTGACCA-3'
<i>ABCC4</i>	5'-GAGCTGAGAATGACGCACAG-3'	5'-TACGCTGTGTTCAAAGCCAC-3'
<i>ABCC5</i>	5'-GGGAGCTCTCAATGGAAGAC-3'	5'-CAGCTCTTCTTGCCACAGTC-3'
<i>ABCG2</i>	5'- GAAGAGTGGCTTTCTACCTT -3'	5'- GTCCCAGGATGGCGTTGA -3'
<i>B2M</i>	5'-GGCATTCTGAAGCTGACAG-3'	5'-TGGATGACGTGAGTAAACCTG-3'
B. Primer sequences for molecular factor genes		
Gene	Forward Primer	Reverse Primer
<i>PXR</i>	5'-TGCGAGATCACCCGGAAGAC-3'	5'-ATGGGAGAAGGTAGTGTCAAAGG-3'
<i>CAR</i>	5'- GTGCTTAGATGCTGGCATGAGGAA-3'	5'-GGCTGGTGTGATGGATGAACAGATGAG-3'
<i>AhR</i>	5'-ACATCACCTACGCCAGTCGC-3'	5'-TCTATGCCGCTTGGAAGGAT-3'
<i>PPARA</i>	5'-CTATCATTTGCTGTGGAGATCG-3'	5'-AAGATATCGTCCGGGTGGTT-3'
<i>PPARG</i>	5'-AAGGAGAAGCTGTTGGCGGAGA-3'	5'-CAGCCCTGAAAGATGCGGATGG-3'
<i>NRF2</i>	5'-GAGAGCCCAGTCTTCATTGC-3'	5'-TGCTCAATGTCTGTTGCAT-3'
<i>NFKB1</i>	5'-GTGAAGGCCATCCCATGGT-3'	5'-TGTGACCAACTGAACAATAACC-3'
<i>RELA</i>	5'-GCAGAAAGAGGACATTGAGGTG-3'	5'-CTGCATGGAGACACGCACAGGAG-3'
<i>CREB1</i>	5'-ACTGTAACGGTGCCAACTCC-3'	5'-GAATGGTAGTACCCGGCTGA-3'
<i>COX-2</i>	5'- CCTGTGCCTGATGATTGC -3'	5'-CTGATGCGTGAAGTGCTG-3'
<i>TP53</i>	5'-TAACAGTTCCTGCATGGGCGGC-3'	5'-AGGACAGGCACAAACACGCACC-3'
<i>GSK3B</i>	5'-GGTCTATCTTAATCTGGTGCTGG-3'	5'-TGGATATAGGCTAAACTTCGGAAC-3'
<i>JNK1</i>	5'-TGGACTTGAGGAGAGAACC-3'	5'-CATTGACAGACGACGATGATG-3'
<i>cJUN</i>	5'-TTCTATGACGATGCCCTCAACGC-3'	5'-GCTCTGTTTCAGGATCTTGGGGTTAC-3'
<i>MAPK1</i>	5'-CGTGTTCAGATCCAGACCATGAT-3'	5'-TGGACTTGGTGTAGCCCTTGGAA-3'
<i>MAPK3</i>	5'-ACCTGCGACCTTAAGATTTGTGA-3'	5'-AGCCACATACTCCGTCAGGAA-3'

<i>PIK3CA</i>	5'-TGGATGCTCTACAGGGCTTT-3'	5'-GTCTGGGTTCTCCCAATTCA-3'
C. Primer sequences for PPARα target genes		
Gene	Forward Primer	Reverse Primer
<i>THBD</i>	AGCAAGCCCCACTTATTCCC	GGGTGACTCAGGTGAGTTGG
<i>PDK4</i>	GAGGTGGTGTCCCCTGAGAATT	CAAAACCAGCCAAAGGAGCATT

PXR, pregnane X receptor; CAR, constitutive androstane receptor; AhR, aryl hydrocarbon receptor; PPARA, peroxisome proliferator activated receptor alpha; PPARG, peroxisome proliferator activated receptor gamma; NRF2, nuclear factor, erythroid 2 like 2; NFKB1, nuclear factor kappa B subunit 1; RELA; RELA proto-oncogene, NF-KB Subunit; CREB1, cAMP responsive element binding protein 1; COX-2, cyclooxygenase-2; TP53, tumor protein P53; GSK3B, glycogen synthase kinase 3 beta; JNK1, c-Jun N-terminal kinase-1; cJUN, Jun proto-oncogene, AP-1 transcription factor subunit; MAPK1, mitogen-activated protein kinase 1; MAPK3, mitogen-activated protein kinase 3; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; THBD, thrombomodulin; PDK4, pyruvate dehydrogenase lipoamide kinase isozyme 4

Supplemental Table 3

Company source and assay IDs of the siRNAs used in this study.

Gene Symbol	Gene Name	Catalog/Assay ID	Company
<i>AhR</i>	Aryl hydrocarbon receptor	4427038/ s1198	Thermofisher
<i>PPARA</i>	Peroxisome proliferator activated receptor alpha	4427037/ s10881	Thermofisher
<i>PPARG</i>	Peroxisome proliferator activated receptor gamma	4427038/ s10886	Thermofisher
<i>NRF2</i>	Nuclear factor, erythroid 2 like 2	4427037/ s9491	Thermofisher
<i>NFKB1</i>	Nuclear factor kappa B subunit 1	4427037/ s9505	Thermofisher
<i>RELA</i>	RELA proto-oncogene, NF-KB Subunit	4427038/ s11914	Thermofisher
<i>CREB1</i>	cAMP responsive element binding protein 1	4427037/ s3489	Thermofisher
<i>COX-2</i>	Cyclooxygenase-2	4427037/ s11472	Thermofisher
<i>p53/TP53</i>	Tumor protein P53	4427038/ s607	Thermofisher
<i>GSK3B</i>	Glycogen synthase kinase 3 beta	4427038/ s6240	Thermofisher
<i>MAPK1</i>	Mitogen-activated protein kinase 1	4427038/ s11137	Thermofisher
<i>MAPK3</i>	Mitogen-activated protein kinase 3	4427038/ s11140	Thermofisher
<i>PIK3CA</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	4427038/ s10520	Thermofisher
Negative Control No. 1		4390843	Thermofisher
Gene Symbol	Gene Name	Product Number/siRNA ID	Company
<i>JNK1</i>	c-Jun N-terminal kinase-1	NM_002750/ SASI_Hs01_00010441	Sigma-Aldrich
<i>cJUN</i>	Jun proto-oncogene, AP-1 transcription factor subunit	NM_002228/ SASI_Hs02_00333461	Sigma-Aldrich

Supplemental Table 4

Statistical analysis of data

Figure	Comparative groups	Mean difference (95% Confidence Interval of difference)	P value	P value (Statistical test)
Fig. 1 (ABCC1/ B2M)	VC vs 300 μ M	0.00 (-0.05, 0.05)	0.97	0.74 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	-0.01 (-0.06, 0.04)	0.84	
	300 μ M vs 600 μ M	0.01 (-0.04, 0.06)	0.73	
Fig. 1 (ABCC2/ B2M)	VC vs 300 μ M	-0.03 (-0.12, 0.07)	0.64	0.36 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	-0.05 (-0.14, 0.05)	0.33	
	300 μ M vs 600 μ M	-0.02 (-0.11, 0.08)	0.82	
Fig. 1 (ABCC4/ B2M)	VC vs 300 μ M	0.05 (-0.08, 0.18)	0.50	0.15 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	0.09 (-0.03, 0.22)	0.13	
	300 μ M vs 600 μ M	0.05 (-0.08, 0.17)	0.54	
Fig. 1 (ABCC5/ B2M)	VC vs 300 μ M	-0.02 (-0.09, 0.06)	0.79	0.46 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	0.02 (-0.06, 0.09)	0.79	
	300 μ M vs 600 μ M	0.03 (-0.04, 0.11)	0.43	
Fig. 1 (ABCG2/ B2M)	VC vs 300 μ M	-0.23 (-0.34, -0.13)	0.001	<0.001 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	-0.38 (-0.49, -0.28)	<0.001	
	300 μ M vs 600 μ M	-0.15 (-0.25, -0.04)	0.01	
Fig. 2 (ABCG2/ B2M)	VC vs 300 μ M (6h)	-0.14 (-0.21, -0.07)	<0.001	<0.001 (By time points)
	VC vs 600 μ M (6h)	-0.18 (-0.25, -0.11)	<0.001	<0.001 (By treatments)

	300 μ M vs 600 μ M (6h)	0.04 (-0.03, 0.11)	0.65	(Two-way ANOVA with Tukey test)
	VC vs 300 μ M (12h)	-0.20 (-0.27, -0.13)	<0.001	
	VC vs 600 μ M (12h)	-0.29 (-0.36, -0.22)	<0.001	
	300 μ M vs 600 μ M (12h)	0.09 (0.02, 0.16)	0.005	
	VC vs 300 μ M (24h)	-0.23 (-0.30, -0.16)	<0.001	
	VC vs 600 μ M (24h)	-0.38 (-0.45, -0.31)	<0.001	
	300 μ M vs 600 μ M (24h)	0.15 (0.08, 0.22)	<0.001	
	VC vs 300 μ M (48h)	-0.16 (-0.23, -0.09)	<0.001	
	VC vs 600 μ M (48h)	-0.24 (-0.31, -0.17)	<0.001	
	300 μ M vs 600 μ M (48h)	0.08 (0.01, 0.15)	0.015	
Fig. 3A (BCRP/HSC)	VC vs 300 μ M (6h)	-0.20 (-0.42, 0.03)	0.08	<0.001 (By time points) <0.001 (By treatments) (Two-way ANOVA with Tukey test)
	VC vs 600 μ M (6h)	-0.02 (-0.08, 0.05)	0.99	
	300 μ M vs 600 μ M (6h)	-0.06 (-0.12, 0.01)	0.10	
	VC vs 300 μ M (12h)	-0.16 (-0.23, -0.10)	<0.001	
	VC vs 600 μ M (12h)	-0.19 (-0.26, -0.13)	<0.001	
	300 μ M vs 600 μ M (12h)	0.03 (-0.03, 0.09)	0.89	
	VC vs 300 μ M (24h)	-0.18 (-0.24, -0.12)	<0.001	
	VC vs 600 μ M (24h)	-0.21 (-0.28, -0.15)	<0.001	
	300 μ M vs 600 μ M (24h)	0.03 (-0.03, 0.10)	0.80	
	VC vs 300 μ M (48h)	-0.24 (-0.30, -0.18)	<0.001	
	VC vs 600 μ M (48h)	-0.32 (-0.38, -0.26)	<0.001	
	300 μ M vs 600 μ M (48h)	0.08 (0.02, 0.14)	0.004	
	VC vs 300 μ M (72h)	-0.18 (-0.25, -0.12)	<0.001	
	VC vs 600 μ M (72h)	-0.27 (-0.33, -0.21)	<0.001	
	300 μ M vs 600 μ M (72h)	0.09 (0.03, 0.15)	0.001	

Fig. 3C (BCRP activity)	VC vs 300 μ M (12h)	-0.03 (-0.11, 0.05)	0.98	<0.001 (By time points) <0.001 (By treatments) (Two-way ANOVA with Tukey test)
	VC vs 600 μ M (12h)	-0.08 (-0.15, 0.002)	0.06	
	300 μ M vs 600 μ M (12h)	0.05 (-0.03, 0.13)	0.59	
	VC vs 300 μ M (24h)	-0.08 (-0.16, 0.00)	0.048	
	VC vs 600 μ M (24h)	-0.09 (-0.17, -0.01)	0.02	
	300 μ M vs 600 μ M (24h)	0.01 (-0.07, 0.09)	1.00	
	VC vs 300 μ M (48h)	-0.10 (-0.17, -0.02)	0.01	
	VC vs 600 μ M (48h)	-0.18 (-0.26, -0.11)	<0.001	
	300 μ M vs 600 μ M (48h)	0.09 (0.01, 0.17)	0.02	
	VC vs 300 μ M (72h)	-0.11 (-0.19, -0.03)	0.001	
	VC vs 600 μ M (72h)	-0.22 (-0.29, -0.14)	<0.001	
	300 μ M vs 600 μ M (72h)	0.11 (0.03, 0.18)	0.002	
	Fig. 4A (PHT)	Baseline vs 20 μ M	0.02 (0.004, 0.04)	
Baseline vs 40 μ M		0.01 (-0.01, 0.03)	0.44	
Baseline vs 80 μ M		0.02 (0.0003, 0.04)	0.047	
Baseline vs 160 μ M		0.02 (0.006, 0.04)	0.015	
Activated vs 20 μ M		0.01 (-0.04, 0.05)	0.99	0.71 (One-way ANOVA with Dunnett test)
Activated vs 40 μ M		0.02 (-0.03, 0.07)	0.50	
Activated vs 80 μ M		0.01 (-0.04, 0.06)	0.89	
Activated vs 160 μ M		0.006 (-0.04, 0.05)	0.99	
Fig. 4B (CBZ)	Baseline vs 10 μ M	-0.01 (-0.06, 0.04)	0.93	0.31 (One-way ANOVA with Dunnett test)
	Baseline vs 20 μ M	0.02 (-0.04, 0.07)	0.69	
	Baseline vs 40 μ M	-0.02 (-0.07, 0.03)	0.54	
	Baseline vs 80 μ M	-0.008 (-0.06, 0.04)	0.94	
	Activated vs 10 μ M	-0.05 (-0.11, 0.002)	0.06	0.03

	Activated vs 20 μ M	-0.05 (-0.10, 0.006)	0.08	(One-way ANOVA with Dunnett test)
	Activated vs 40 μ M	-0.05 (-0.10, 0.01)	0.11	
	Activated vs 80 μ M	-0.07 (-0.13, -0.02)	0.014	
Fig. 4C (VPA)	Baseline vs. 75 μ M	0.07 (0.03, 0.10)	0.004	0.002 (One-way ANOVA with Dunnett test)
	Baseline vs. 150 μ M	0.06 (0.02, 0.09)	0.008	
	Baseline vs. 300 μ M	0.09 (0.05, 0.13)	0.001	
	Baseline vs. 600 μ M	0.07 (0.03, 0.10)	0.004	
	Activated vs. 75 μ M	-0.03 (-0.08, 0.02)	0.32	0.12 (One-way ANOVA with Dunnett test)
	Activated vs. 150 μ M	-0.02 (-0.07, 0.03)	0.47	
	Activated vs. 300 μ M	-0.03 (-0.08, 0.02)	0.32	
	Activated vs. 600 μ M	0.02 (-0.03, 0.07)	0.70	
Fig. 4D (LTG)	Baseline vs. 15 μ M	0.03 (0.01, 0.05)	0.005	0.001 (One-way ANOVA with Dunnett test)
	Baseline vs. 30 μ M	0.05 (0.03, 0.06)	0.001	
	Baseline vs. 60 μ M	0.05 (0.03, 0.07)	<0.001	
	Baseline vs. 120 μ M	0.04 (0.02, 0.05)	0.002	
	Activated vs. 15 μ M	-0.05 (-0.11, 0.002)	0.06	0.03 (One-way ANOVA with Dunnett test)
	Activated vs. 30 μ M	-0.05 (-0.10, 0.01)	0.08	
	Activated vs. 60 μ M	-0.05 (-0.10, 0.01)	0.10	
	Activated vs. 120 μ M	-0.07 (-0.13, -0.02)	0.014	
Fig. 4E (TPM)	Baseline vs. 30 μ M	-0.007 (-0.05, 0.03)	0.89	0.90 (One-way ANOVA with Dunnett test)
	Baseline vs. 60 μ M	-0.004 (-0.04, 0.04)	0.98	
	Baseline vs. 120 μ M	-0.008 (-0.05, 0.03)	0.86	
	Activated vs. 15 μ M	0.006 (-0.02, 0.03)	0.85	0.32 (One-way ANOVA with Dunnett test)
	Activated vs. 30 μ M	0.01 (-0.02, 0.04)	0.52	

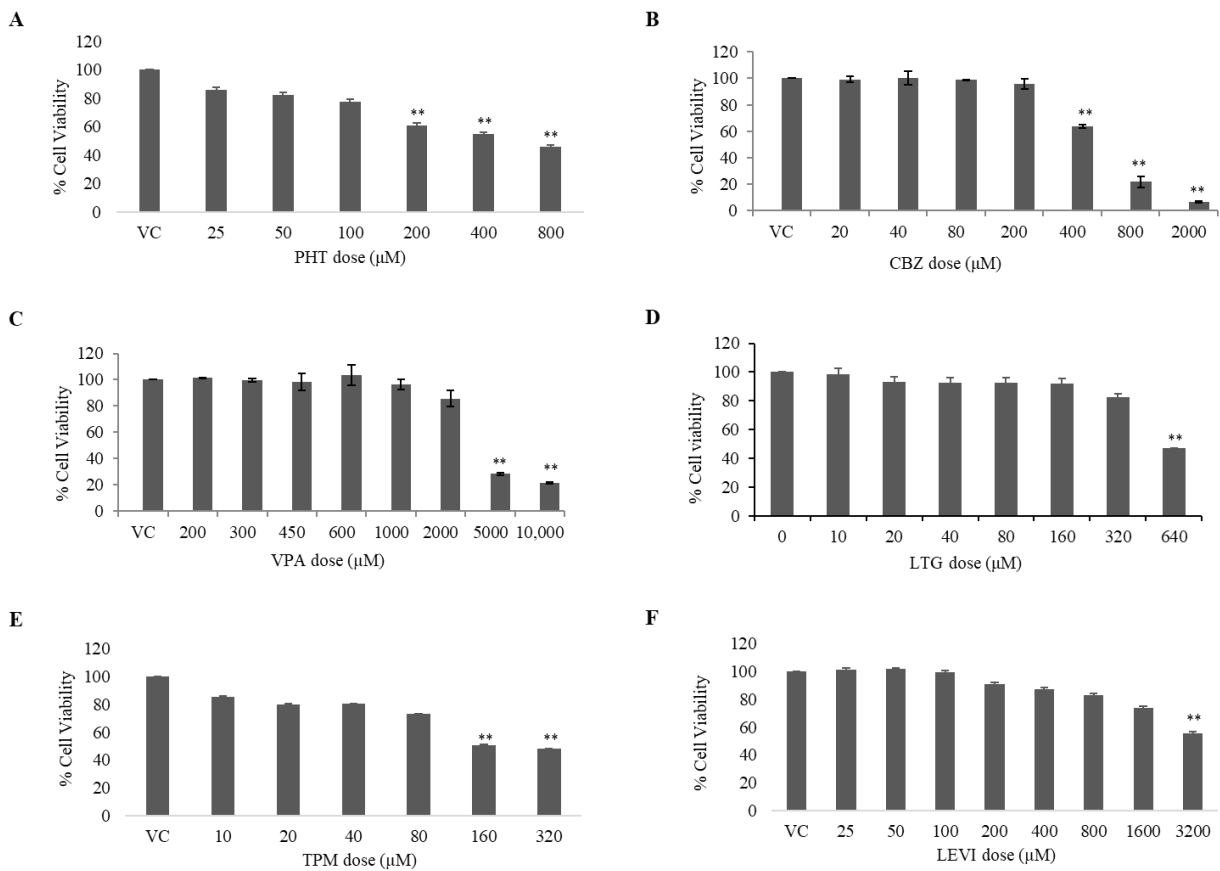
	Activated vs. 60 μ M	0.02 (-0.01, 0.05)	0.17	
	Activated vs. 120 μ M	0.007 (-0.02, 0.03)	0.82	
Fig. 4F (LEVI)	Baseline vs. 25 μ M	0.004 (-0.06, 0.06)	0.10	0.49 (One-way ANOVA with Dunnett test)
	Baseline vs. 50 μ M	-0.002 (-0.06, 0.06)	1.00	
	Baseline vs. 100 μ M	0.02 (-0.04, 0.08)	0.62	
	Baseline vs. 200 μ M	0.02 (-0.04, 0.08)	0.52	
	Activated vs. 25 μ M	0.003 (-0.03, 0.03)	0.98	0.38 (One-way ANOVA with Dunnett test)
	Activated vs. 50 μ M	0.005 (-0.02, 0.03)	0.90	
	Activated vs. 100 μ M	0.01 (-0.02, 0.04)	0.52	
	Activated vs. 200 μ M	0.02 (-0.01, 0.05)	0.25	
Fig. 5A (PHT)	VC vs 40 μ M	0.06 (0.03, 0.09)	<0.001	<0.001 (One-way ANOVA with Dunnett test)
	VC vs 80 μ M	0.10 (0.07, 0.12)	<0.001	
	VC vs KO143	0.20 (0.18, 0.23)	<0.001	
Fig. 5B (VPA)	VC vs 300 μ M	0.15 (0.10, 0.20)	<0.001	<0.001 (One-way ANOVA with Dunnett test)
	VC vs 600 μ M	0.20 (0.15, 0.25)	<0.001	
	VC vs KO143	0.20 (0.15, 0.25)	<0.001	
Fig. 5C (LTG)	VC vs 15 μ M	0.20 (0.15, 0.26)	<0.001	<0.001 (One-way ANOVA with Dunnett test)
	VC vs 60 μ M	0.19 (0.13, 0.24)	<0.001	
	VC vs KO143	0.20 (0.15, 0.26)	<0.001	
Fig. 6B (ABCG2/ B2M)	SCR, VC vs SCR, VPA	1.27 (0.77, 1.77)	<0.001	<0.001 (By siRNA) <0.001 (By drug treatment) (Two-way ANOVA with Tukey test)
	SCR, VC vs siPPAR α , VC	-0.19 (-0.69, 0.32)	0.66	
	SCR, VC vs siPPAR α , VPA	0.19 (-0.32, 0.69)	0.65	

	SCR, VPA vs siPPAR α , VC	1.46 (0.95, 1.96)	<0.001	
	SCR, VPA vs siPPAR α , VPA	-1.08 (-1.59, -0.58)	0.001	
	siPPAR α , VC vs siPPAR α , VPA	0.37 (-0.13, 0.88)	0.16	
Fig. 6D (BCRP/ HSC)	SCR, VC vs SCR, VPA	1.16 (0.79, 1.52)	<0.001	<0.001 (By siRNA) <0.001 (By drug treatment) (Two-way ANOVA with Tukey test)
	SCR, VC vs siPPAR α , VC	-0.06 (-0.43, 0.30)	0.94	
	SCR, VC vs siPPAR α , VPA	0.22 (-0.14, 0.59)	0.28	
	SCR, VPA vs siPPAR α , VC	1.22 (0.85, 1.59)	<0.001	
	SCR, VPA vs siPPAR α , VPA	-0.93 (-1.30, -0.57)	<0.001	
	siPPAR α , VC vs siPPAR α , VPA	0.29 (-0.08, 0.65)	0.13	
Fig. 6E (BCRP activity)	SCR, VC vs SCR, VPA	0.57 (0.47, 0.68)	<0.001	<0.001 (By siRNA) <0.001 (By drug treatment) (Two-way ANOVA with Tukey test)
	SCR, VC vs siPPAR α , VC	-0.11 (-0.21, -0.00)	0.04	
	SCR, VC vs siPPAR α , VPA	-0.03 (-0.13, 0.08)	0.84	
	SCR, VPA vs siPPAR α , VC	0.68 (0.58, 0.78)	<0.001	
	SCR, VPA vs siPPAR α , VPA	-0.60 (-0.70, -0.50)	<0.001	
	siPPAR α , VC vs siPPAR α , VPA	0.08 (-0.02, 0.18)	0.14	
Fig. 7B (BCRP/ HSC)	VC vs VPA	0.36 (0.20, 0.52)	<0.001	0.003 (By -/+MK886) <0.001 (By drug treatment) (Two-way ANOVA with Tukey test)
	VC vs VC+8 μ MMK886	-0.11 (-0.27, 0.05)	0.21	
	VC vs VPA+8 μ MMK886	-0.05 (-0.21, 0.11)	0.77	
	VPA vs VC+8 μ MMK886	0.46 (0.30, 0.62)	<0.001	
	VPA vs VPA+8 μ MMK886	-0.40 (-0.56, -0.24)	<0.001	
	VC+8 μ MMK886 vs VPA+8 μ MMK886	0.06 (-0.10, 0.22)	0.65	
Fig. 7C (BCRP activity)	VC vs VPA	-0.22 (-0.31, -0.12)	<0.001	<0.001 (One-way ANOVA with Tukey test)
	VC vs VC+8 μ MMK886	0.08 (-0.02, 0.17)	0.11	

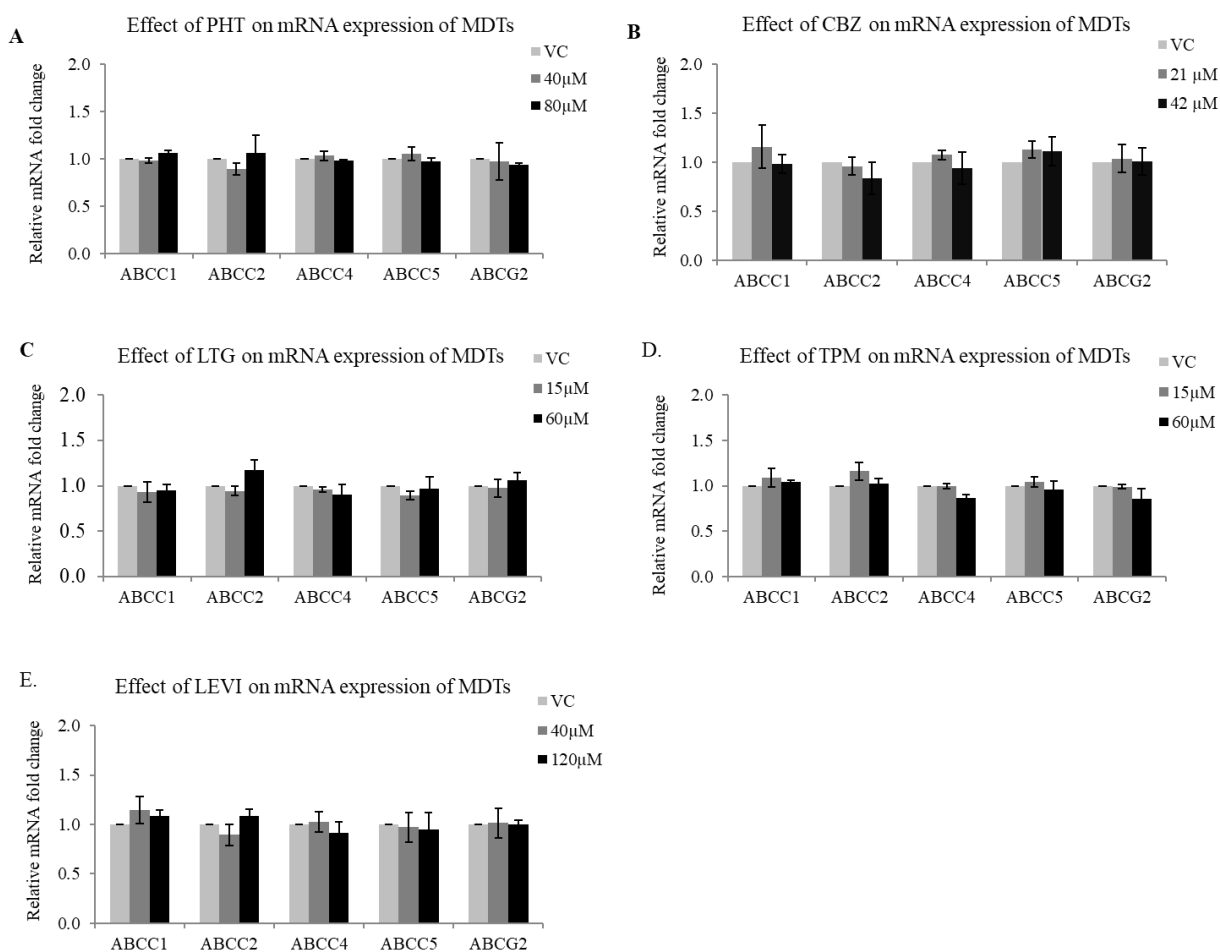
	VC vs VPA+8 μ MMK886	0.02 (-0.08, 0.11)	0.94	
	VPA vs VC+8 μ MMK886	0.29 (0.20, 0.39)	<0.001	
	VPA vs VPA+8 μ MMK886	0.23 (0.14, 0.33)	<0.001	
	VC+8 μ MMK886 vs VPA+8 μ MMK886	-0.06 (-0.15, 0.03)	0.24	
Fig. 8A (PPAR α / B2M)	VC vs 600 μ M (1h)	-0.03 (-0.10, 0.03)	0.80	<0.001 (By time points) <0.001 (By treatments) (Two-way ANOVA with Tukey test)
	VC vs 600 μ M (3h)	-0.16 (-0.22, -0.09)	<0.001	
	VC vs 600 μ M (6h)	-0.30 (-0.37, -0.24)	<0.001	
	VC vs 600 μ M (12h)	-0.24 (-0.31, -0.18)	<0.001	
	VC vs 600 μ M (24h)	-0.23 (-0.29, -0.16)	<0.001	
	VC vs 600 μ M (48h)	-0.07 (-0.14, -0.004)	0.03	
Fig. 8B (PPAR α / HSC)	VC vs 600 μ M (1h)	-0.01 (-0.10, 0.07)	1.00	<0.001 (By time points) <0.001 (By treatments) (Two-way ANOVA with Tukey test)
	VC vs 600 μ M (3h)	-0.21 (-0.29, -0.13)	<0.001	
	VC vs 600 μ M (6h)	-0.14 (-0.22, -0.05)	<0.001	
	VC vs 600 μ M (12h)	-0.05 (-0.13, 0.03)	0.57	
	VC vs 600 μ M (24h)	0.01 (-0.07, 0.09)	1.00	
Fig. 8D	VC vs 600 μ M (24h) – PDK4	-0.22 (-0.25, -0.19)	<0.001	(Unpaired t-test)
	VC vs 600 μ M (24h) – THBD	-0.27 (-0.32, -0.22)	<0.001	
Fig. 9B	0h cytosolic vs 3h cytosolic	0.10 (0.05, 0.15)	<0.001	<0.001 (One-way ANOVA with Tukey test)
	0h cytosolic vs 6h cytosolic	0.06 (0.002, 0.11)	0.04	
	3h cytosolic vs 6h cytosolic	-0.05 (-0.10, 0.01)	0.12	
	0h nuclear vs 3h nuclear	-0.14 (-0.19, -0.08)	<0.001	
	0h nuclear vs 6h nuclear	-0.11 (-0.16, 0.06)	<0.001	
	3h nuclear vs 6h nuclear	0.03 (-0.03, 0.08)	0.67	

	3h cytosolic vs 3h nuclear	-0.24 (-0.29, -0.18)	<0.001	
	6h cytosolic vs 6h nuclear	-0.17 (-0.22, -0.11)	<0.001	
Fig. 9D	0h vs 3h	-0.13 (-0.19, -0.06)	0.001	0.001 (One-way ANOVA with Tukey test)
	0h vs 6h	-0.12 (-0.18, -0.05)	0.002	
	3h vs 6h	0.01 (-0.06, 0.07)	0.95	
Fig. 10A	VC vs VPA (IgG)	-0.20 (-0.67, 0.27)	0.30	(Unpaired t-test)
	VC vs VPA (PPAR α) – 3h	-0.82 (-1.06, -0.58)	<0.001	
	VC vs VPA (PPAR α) – 6h	-0.62 (-0.84, -0.40)	<0.001	
Fig. 10C	VC vs VPA (pGL2-control)	-0.01 (-0.05, 0.02)	0.44	(Unpaired t-test)
	VC vs VPA (pGL2-prom- ABCG2)	-0.17 (-0.20, -0.13)	<0.001	(Unpaired t-test)

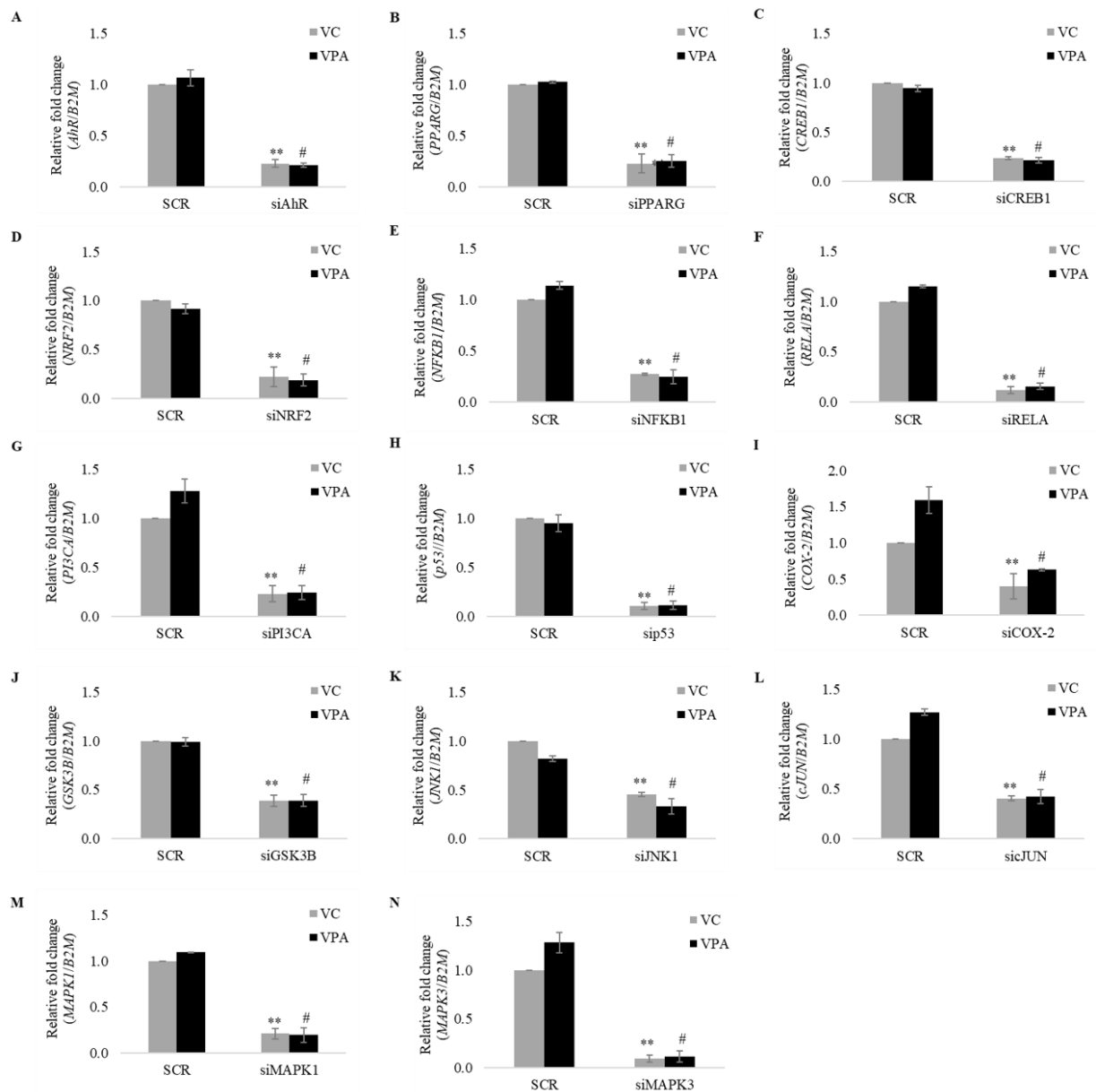
Supplemental Data



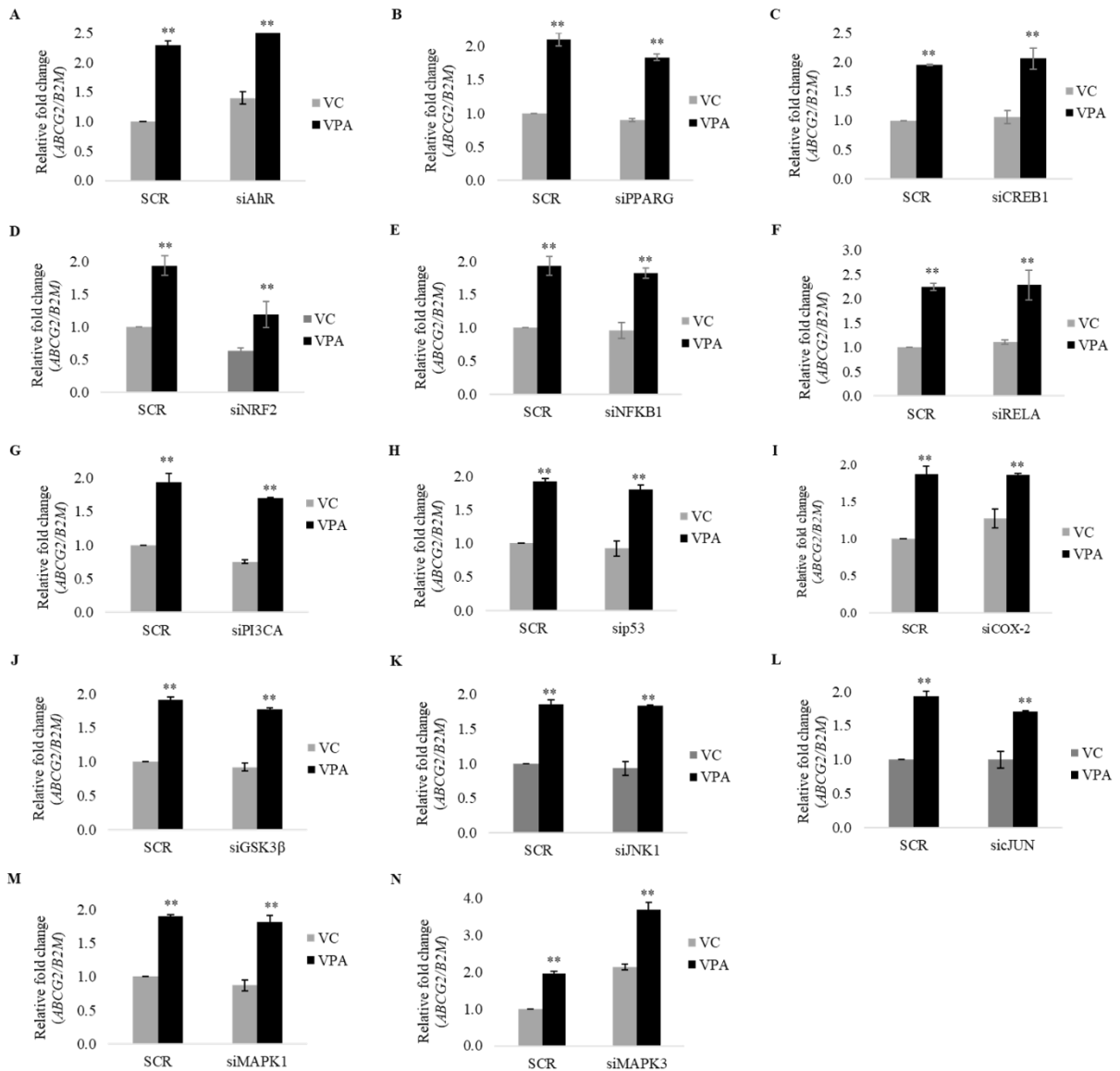
Supplemental Fig. 1. Effect of AEDs on hCMEC/D3 cell viability. Cells were treated with different doses of (A) phenytoin (PHT), (B) carbamazepine (CBZ), (C) valproic acid (VPA), (D) lamotrigine (LTG), (E) topiramate (TPM) and (F) levetiracetam (LEVI) for 72h. After treatment, cells were assessed for % viability using MTT assay. Data is the mean \pm S.D. of 4 independent experiments. ** $p < 0.01$, compared to VC (One-way ANOVA with Dunnett's post hoc test).



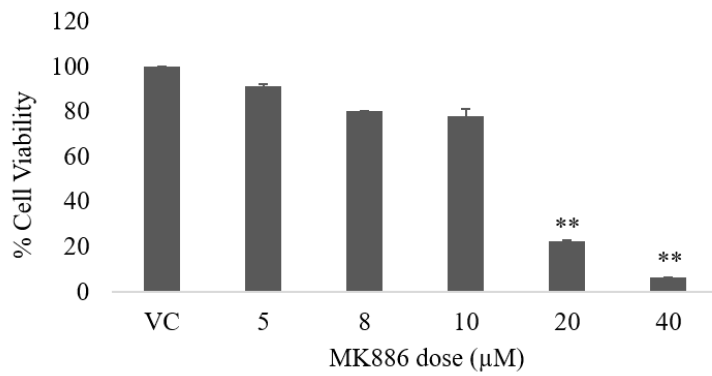
Supplemental Fig. 2. Effect of PHT, CBZ, LTG, TPM and LEVI on mRNA expression of MDTs in hCMEC/D3 cells. RT-qPCR analysis of ABCC1, ABCC2, ABCC4, ABCC5 and ABCG2 mRNA expression in hCMEC/D3 cells treated with (A) PHT (40 μ M, 80 μ M), (B) CBZ (21 μ M, 42 μ M), (C) LTG (15 μ M, 60 μ M), (D) TPM (15 μ M, 60 μ M) and (E) LEVI (40 μ M, 120 μ M) for 24h. The changes in mRNA levels of target genes were normalized with *B2M* and expressed as normalized fold change over VC (0.1% DMSO for PHT, CBZ, LTG and TPM; water for LEVI). The data is the mean \pm S.D. of 3 independent experiments.



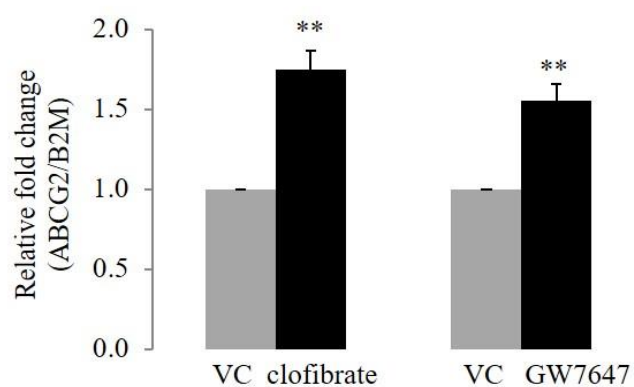
Supplemental Fig. 3. siRNA validation data for 14 molecular factors at mRNA level. hCMEC/D3 cells were transiently transfected with siRNA specific to (A) AhR, (B) PPARG, (C) CREB1, (D) NRF2, (E) NFKB1, (F) RELA, (G) PIK3CA, (H) p53, (I) COX-2, (J) GSK3B, (K) JNK1, (L) cJUN, (M) MAPK1, (N) MAPK3, or the non-targeting control (scramble, SCR). Subsequently, cells were treated with VC (0.1% DMSO) or VPA (600 μ M) for 24h. RT-qPCR analysis was done to check the knockdown of each factor in VC-treated as well as VPA-treated group. The changes in mRNA level of each gene were normalized with *B2M*. The data is the mean \pm S.D. of 3 independent experiments. ** $P < 0.01$, VC (SCR vs. siRNA); # $P < 0.01$ VPA (SCR vs. siRNA) (unpaired t-test).



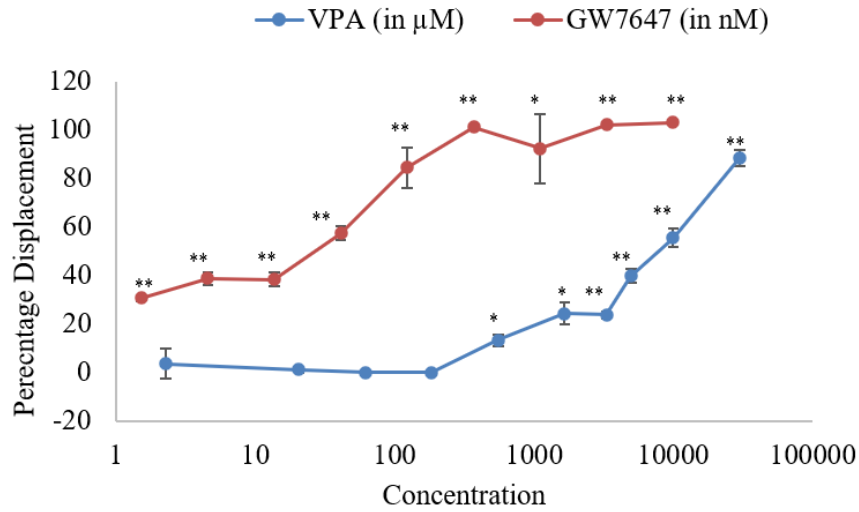
Supplemental Fig. 4. Effect of silencing of molecular factors on VPA-induced ABCG2 mRNA. Expression of the respective factors (A-N) was silenced by transient transfection of gene-specific siRNA in hCMEC/D3 cells. Then, the cells were treated with VC (0.1% DMSO) or VPA (600 μ M) for 24h and RT-qPCR analysis was done to check mRNA expression levels of ABCG2. Scramble (SCR) was used as non-targeting control. The changes in mRNA level of ABCG2 was normalized with *B2M*. The data is the mean \pm S.D. of 3 independent experiments. ** $P < 0.01$, SCR (VC vs. VPA) and siRNA (VC vs. VPA) (Two-way ANOVA with Tukey's post hoc test).



Supplemental Fig. 5. Effect of MK886 on hCMEC/D3 cell viability. Cells were treated with different doses of MK886 for 48h. After treatment, cells were assessed for % viability using MTT assay. Data is the mean \pm S.D. of 4 independent experiments. ** $p < 0.01$, compared to VC (0.1% DMSO) (One-way ANOVA with Dunnett's post hoc test).



Supplemental Fig. 6. Effect of known PPAR α agonist on ABCG2 mRNA in hCMEC/D3 cells. RT-qPCR analysis of ABCG2 mRNA expression in hCMEC/D3 cells treated with 100 μ M clofibrate and 100nM GW7647 for 24h. The changes in the mRNA level were normalized with *B2M* and expressed as normalized fold change over VC (0.1% DMSO). The data is the mean \pm S.D. of 3 independent experiments. ** $p < 0.01$, compared to VC (unpaired t-test).



Supplemental Fig. 7. Binding of VPA to PPAR α LBD in a TR-FRET competitive binding assay. GW7647 was used as a known PPAR α agonist. Data is the mean \pm S.D. of 2 independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to solvent control (1% DMSO, 0% displacement); One-way ANOVA with Dunnett's post hoc test.

References

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SUPPLEMENTARY DATA

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Valproic acid-induced upregulation of multidrug efflux transporter ABCG2/BCRP via PPAR α -dependent mechanism in human brain endothelial cells

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Supplemental Table 1

Therapeutic plasma concentrations (reference range) of antiepileptic drugs in patients with epilepsy (Johannessen, 2004)

Antiepileptic drug	Therapeutic plasma concentration range (μM)	Doses used in the present study
Phenytoin	40-80	40 μM , 80 μM
Carbamazepine	15-45	21 μM , 42 μM
Valproic acid	300-600	300 μM , 600 μM
Lamotrigine	10-60	15 μM , 60 μM
Topiramate	15-60	15 μM , 60 μM
Levetiracetam	35-120	40 μM , 120 μM

Supplemental Table 2

Sequence of primers used for qPCR

A. Primer sequences for MDTs and B2M		
Gene	Forward Primer	Reverse Primer
<i>ABCC1</i>	5'-TGTGTGGGCAACTGCATCG-3'	5'-GTTGGTTTCCATTTTCAGATGACA-3'
<i>ABCC2</i>	5'-ATATAAGAAGGCATTGACCC-3'	5'-ATCTGTAGAACACTTGACCA-3'
<i>ABCC4</i>	5'-GAGCTGAGAATGACGCACAG-3'	5'-TACGCTGTGTTCAAAGCCAC-3'
<i>ABCC5</i>	5'-GGGAGCTCTCAATGGAAGAC-3'	5'-CAGCTCTTCTTGCCACAGTC-3'
<i>ABCG2</i>	5'- GAAGAGTGGCTTTCTACCTT -3'	5'- GTCCCAGGATGGCGTTGA -3'
<i>B2M</i>	5'-GGCATTCTGAAGCTGACAG-3'	5'-TGGATGACGTGAGTAAACCTG-3'
B. Primer sequences for molecular factor genes		
Gene	Forward Primer	Reverse Primer
<i>PXR</i>	5'-TGCGAGATCACCCGGAAGAC-3'	5'-ATGGGAGAAGGTAGTGTCAAAGG-3'
<i>CAR</i>	5'- GTGCTTAGATGCTGGCATGAGGAA-3'	5'-GGCTGGTGATGGATGAACAGATGAG-3'
<i>AhR</i>	5'-ACATCACCTACGCCAGTCGC-3'	5'-TCTATGCCGCTTGGAAGGAT-3'
<i>PPARA</i>	5'-CTATCATTTGCTGTGGAGATCG-3'	5'-AAGATATCGTCCGGGTGGTT-3'
<i>PPARG</i>	5'-AAGGAGAAGCTGTTGGCGGAGA-3'	5'-CAGCCCTGAAAGATGCGGATGG-3'
<i>NRF2</i>	5'-GAGAGCCCAGTCTTCATTGC-3'	5'-TGCTCAATGTCTGTTGCAT-3'
<i>NFKB1</i>	5'-GTGAAGGCCATCCCATGGT-3'	5'-TGTGACCAACTGAACAATAACC-3'
<i>RELA</i>	5'-GCAGAAAGAGGACATTGAGGTG-3'	5'-CTGCATGGAGACACGCACAGGAG-3'
<i>CREB1</i>	5'-ACTGTAACGGTGCCAACTCC-3'	5'-GAATGGTAGTACCCGGCTGA-3'
<i>COX-2</i>	5'- CCTGTGCCTGATGATTGC -3'	5'-CTGATGCGTGAAGTGCTG-3'
<i>TP53</i>	5'-TAACAGTTCCTGCATGGGCGGC-3'	5'-AGGACAGGCACAAACACGCACC-3'
<i>GSK3B</i>	5'-GGTCTATCTTAATCTGGTGCTGG-3'	5'-TGGATATAGGCTAAACTTCGGAAC-3'
<i>JNK1</i>	5'-TGGACTTGAGGAGAGAACC-3'	5'-CATTGACAGACGACGATGATG-3'
<i>cJUN</i>	5'-TTCTATGACGATGCCCTCAACGC-3'	5'-GCTCTGTTTCAGGATCTTGGGGTTAC-3'
<i>MAPK1</i>	5'-CGTGTTCAGATCCAGACCATGAT-3'	5'-TGGACTTGGTGTAGCCCTTGGAA-3'
<i>MAPK3</i>	5'-ACCTGCGACCTTAAGATTTGTGA-3'	5'-AGCCACATACTCCGTCAGGAA-3'

<i>PIK3CA</i>	5'-TGGATGCTCTACAGGGCTTT-3'	5'-GTCTGGGTTCTCCCAATTCA-3'
C. Primer sequences for PPARα target genes		
Gene	Forward Primer	Reverse Primer
<i>THBD</i>	AGCAAGCCCCACTTATTCCC	GGGTGACTCAGGTGAGTTGG
<i>PDK4</i>	GAGGTGGTGTCCCCTGAGAATT	CAAAACCAGCCAAAGGAGCATT

PXR, pregnane X receptor; CAR, constitutive androstane receptor; AhR, aryl hydrocarbon receptor; PPARA, peroxisome proliferator activated receptor alpha; PPARG, peroxisome proliferator activated receptor gamma; NRF2, nuclear factor, erythroid 2 like 2; NFKB1, nuclear factor kappa B subunit 1; RELA; RELA proto-oncogene, NF-KB Subunit; CREB1, cAMP responsive element binding protein 1; COX-2, cyclooxygenase-2; TP53, tumor protein P53; GSK3B, glycogen synthase kinase 3 beta; JNK1, c-Jun N-terminal kinase-1; cJUN, Jun proto-oncogene, AP-1 transcription factor subunit; MAPK1, mitogen-activated protein kinase 1; MAPK3, mitogen-activated protein kinase 3; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; THBD, thrombomodulin; PDK4, pyruvate dehydrogenase lipoamide kinase isozyme 4

Supplemental Table 3

Company source and assay IDs of the siRNAs used in this study.

Gene Symbol	Gene Name	Catalog/Assay ID	Company
<i>AhR</i>	Aryl hydrocarbon receptor	4427038/ s1198	Thermofisher
<i>PPARA</i>	Peroxisome proliferator activated receptor alpha	4427037/ s10881	Thermofisher
<i>PPARG</i>	Peroxisome proliferator activated receptor gamma	4427038/ s10886	Thermofisher
<i>NRF2</i>	Nuclear factor, erythroid 2 like 2	4427037/ s9491	Thermofisher
<i>NFKB1</i>	Nuclear factor kappa B subunit 1	4427037/ s9505	Thermofisher
<i>RELA</i>	RELA proto-oncogene, NF-KB Subunit	4427038/ s11914	Thermofisher
<i>CREB1</i>	cAMP responsive element binding protein 1	4427037/ s3489	Thermofisher
<i>COX-2</i>	Cyclooxygenase-2	4427037/ s11472	Thermofisher
<i>p53/TP53</i>	Tumor protein P53	4427038/ s607	Thermofisher
<i>GSK3B</i>	Glycogen synthase kinase 3 beta	4427038/ s6240	Thermofisher
<i>MAPK1</i>	Mitogen-activated protein kinase 1	4427038/ s11137	Thermofisher
<i>MAPK3</i>	Mitogen-activated protein kinase 3	4427038/ s11140	Thermofisher
<i>PIK3CA</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	4427038/ s10520	Thermofisher
Negative Control No. 1		4390843	Thermofisher
Gene Symbol	Gene Name	Product Number/siRNA ID	Company
<i>JNK1</i>	c-Jun N-terminal kinase-1	NM_002750/ SASI_Hs01_00010441	Sigma-Aldrich
<i>cJUN</i>	Jun proto-oncogene, AP-1 transcription factor subunit	NM_002228/ SASI_Hs02_00333461	Sigma-Aldrich

Supplemental Table 4

Statistical analysis of data

Figure	Comparative groups	Mean difference (95% Confidence Interval of difference)	P value	P value (Statistical test)
Fig. 1 (ABCC1/ B2M)	VC vs 300 μ M	0.00 (-0.05, 0.05)	0.97	0.74 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	-0.01 (-0.06, 0.04)	0.84	
	300 μ M vs 600 μ M	0.01 (-0.04, 0.06)	0.73	
Fig. 1 (ABCC2/ B2M)	VC vs 300 μ M	-0.03 (-0.12, 0.07)	0.64	0.36 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	-0.05 (-0.14, 0.05)	0.33	
	300 μ M vs 600 μ M	-0.02 (-0.11, 0.08)	0.82	
Fig. 1 (ABCC4/ B2M)	VC vs 300 μ M	0.05 (-0.08, 0.18)	0.50	0.15 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	0.09 (-0.03, 0.22)	0.13	
	300 μ M vs 600 μ M	0.05 (-0.08, 0.17)	0.54	
Fig. 1 (ABCC5/ B2M)	VC vs 300 μ M	-0.02 (-0.09, 0.06)	0.79	0.46 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	0.02 (-0.06, 0.09)	0.79	
	300 μ M vs 600 μ M	0.03 (-0.04, 0.11)	0.43	
Fig. 1 (ABCG2/ B2M)	VC vs 300 μ M	-0.23 (-0.34, -0.13)	0.001	<0.001 (One-way ANOVA with Tukey test)
	VC vs 600 μ M	-0.38 (-0.49, -0.28)	<0.001	
	300 μ M vs 600 μ M	-0.15 (-0.25, -0.04)	0.01	
Fig. 2 (ABCG2/ B2M)	VC vs 300 μ M (6h)	-0.14 (-0.21, -0.07)	<0.001	<0.001 (By time points)
	VC vs 600 μ M (6h)	-0.18 (-0.25, -0.11)	<0.001	<0.001 (By treatments)

	300 μ M vs 600 μ M (6h)	0.04 (-0.03, 0.11)	0.65	(Two-way ANOVA with Tukey test)
	VC vs 300 μ M (12h)	-0.20 (-0.27, -0.13)	<0.001	
	VC vs 600 μ M (12h)	-0.29 (-0.36, -0.22)	<0.001	
	300 μ M vs 600 μ M (12h)	0.09 (0.02, 0.16)	0.005	
	VC vs 300 μ M (24h)	-0.23 (-0.30, -0.16)	<0.001	
	VC vs 600 μ M (24h)	-0.38 (-0.45, -0.31)	<0.001	
	300 μ M vs 600 μ M (24h)	0.15 (0.08, 0.22)	<0.001	
	VC vs 300 μ M (48h)	-0.16 (-0.23, -0.09)	<0.001	
	VC vs 600 μ M (48h)	-0.24 (-0.31, -0.17)	<0.001	
	300 μ M vs 600 μ M (48h)	0.08 (0.01, 0.15)	0.015	
Fig. 3A (BCRP/HSC)	VC vs 300 μ M (6h)	-0.20 (-0.42, 0.03)	0.08	<0.001 (By time points) <0.001 (By treatments) (Two-way ANOVA with Tukey test)
	VC vs 600 μ M (6h)	-0.02 (-0.08, 0.05)	0.99	
	300 μ M vs 600 μ M (6h)	-0.06 (-0.12, 0.01)	0.10	
	VC vs 300 μ M (12h)	-0.16 (-0.23, -0.10)	<0.001	
	VC vs 600 μ M (12h)	-0.19 (-0.26, -0.13)	<0.001	
	300 μ M vs 600 μ M (12h)	0.03 (-0.03, 0.09)	0.89	
	VC vs 300 μ M (24h)	-0.18 (-0.24, -0.12)	<0.001	
	VC vs 600 μ M (24h)	-0.21 (-0.28, -0.15)	<0.001	
	300 μ M vs 600 μ M (24h)	0.03 (-0.03, 0.10)	0.80	
	VC vs 300 μ M (48h)	-0.24 (-0.30, -0.18)	<0.001	
	VC vs 600 μ M (48h)	-0.32 (-0.38, -0.26)	<0.001	
	300 μ M vs 600 μ M (48h)	0.08 (0.02, 0.14)	0.004	
	VC vs 300 μ M (72h)	-0.18 (-0.25, -0.12)	<0.001	
	VC vs 600 μ M (72h)	-0.27 (-0.33, -0.21)	<0.001	
	300 μ M vs 600 μ M (72h)	0.09 (0.03, 0.15)	0.001	

Fig. 3C (BCRP activity)	VC vs 300 μ M (12h)	-0.03 (-0.11, 0.05)	0.98	<0.001 (By time points) <0.001 (By treatments) (Two-way ANOVA with Tukey test)
	VC vs 600 μ M (12h)	-0.08 (-0.15, 0.002)	0.06	
	300 μ M vs 600 μ M (12h)	0.05 (-0.03, 0.13)	0.59	
	VC vs 300 μ M (24h)	-0.08 (-0.16, 0.00)	0.048	
	VC vs 600 μ M (24h)	-0.09 (-0.17, -0.01)	0.02	
	300 μ M vs 600 μ M (24h)	0.01 (-0.07, 0.09)	1.00	
	VC vs 300 μ M (48h)	-0.10 (-0.17, -0.02)	0.01	
	VC vs 600 μ M (48h)	-0.18 (-0.26, -0.11)	<0.001	
	300 μ M vs 600 μ M (48h)	0.09 (0.01, 0.17)	0.02	
	VC vs 300 μ M (72h)	-0.11 (-0.19, -0.03)	0.001	
	VC vs 600 μ M (72h)	-0.22 (-0.29, -0.14)	<0.001	
	300 μ M vs 600 μ M (72h)	0.11 (0.03, 0.18)	0.002	
	Fig. 4A (PHT)	Baseline vs 20 μ M	0.02 (0.004, 0.04)	
Baseline vs 40 μ M		0.01 (-0.01, 0.03)	0.44	
Baseline vs 80 μ M		0.02 (0.0003, 0.04)	0.047	
Baseline vs 160 μ M		0.02 (0.006, 0.04)	0.015	
Activated vs 20 μ M		0.01 (-0.04, 0.05)	0.99	0.71 (One-way ANOVA with Dunnett test)
Activated vs 40 μ M		0.02 (-0.03, 0.07)	0.50	
Activated vs 80 μ M		0.01 (-0.04, 0.06)	0.89	
Activated vs 160 μ M		0.006 (-0.04, 0.05)	0.99	
Fig. 4B (CBZ)	Baseline vs 10 μ M	-0.01 (-0.06, 0.04)	0.93	0.31 (One-way ANOVA with Dunnett test)
	Baseline vs 20 μ M	0.02 (-0.04, 0.07)	0.69	
	Baseline vs 40 μ M	-0.02 (-0.07, 0.03)	0.54	
	Baseline vs 80 μ M	-0.008 (-0.06, 0.04)	0.94	
	Activated vs 10 μ M	-0.05 (-0.11, 0.002)	0.06	0.03

	Activated vs 20 μ M	-0.05 (-0.10, 0.006)	0.08	(One-way ANOVA with Dunnett test)
	Activated vs 40 μ M	-0.05 (-0.10, 0.01)	0.11	
	Activated vs 80 μ M	-0.07 (-0.13, -0.02)	0.014	
Fig. 4C (VPA)	Baseline vs. 75 μ M	0.07 (0.03, 0.10)	0.004	0.002 (One-way ANOVA with Dunnett test)
	Baseline vs. 150 μ M	0.06 (0.02, 0.09)	0.008	
	Baseline vs. 300 μ M	0.09 (0.05, 0.13)	0.001	
	Baseline vs. 600 μ M	0.07 (0.03, 0.10)	0.004	
	Activated vs. 75 μ M	-0.03 (-0.08, 0.02)	0.32	0.12 (One-way ANOVA with Dunnett test)
	Activated vs. 150 μ M	-0.02 (-0.07, 0.03)	0.47	
	Activated vs. 300 μ M	-0.03 (-0.08, 0.02)	0.32	
	Activated vs. 600 μ M	0.02 (-0.03, 0.07)	0.70	
Fig. 4D (LTG)	Baseline vs. 15 μ M	0.03 (0.01, 0.05)	0.005	0.001 (One-way ANOVA with Dunnett test)
	Baseline vs. 30 μ M	0.05 (0.03, 0.06)	0.001	
	Baseline vs. 60 μ M	0.05 (0.03, 0.07)	<0.001	
	Baseline vs. 120 μ M	0.04 (0.02, 0.05)	0.002	
	Activated vs. 15 μ M	-0.05 (-0.11, 0.002)	0.06	0.03 (One-way ANOVA with Dunnett test)
	Activated vs. 30 μ M	-0.05 (-0.10, 0.01)	0.08	
	Activated vs. 60 μ M	-0.05 (-0.10, 0.01)	0.10	
	Activated vs. 120 μ M	-0.07 (-0.13, -0.02)	0.014	
Fig. 4E (TPM)	Baseline vs. 30 μ M	-0.007 (-0.05, 0.03)	0.89	0.90 (One-way ANOVA with Dunnett test)
	Baseline vs. 60 μ M	-0.004 (-0.04, 0.04)	0.98	
	Baseline vs. 120 μ M	-0.008 (-0.05, 0.03)	0.86	
	Activated vs. 15 μ M	0.006 (-0.02, 0.03)	0.85	0.32 (One-way ANOVA with Dunnett test)
	Activated vs. 30 μ M	0.01 (-0.02, 0.04)	0.52	

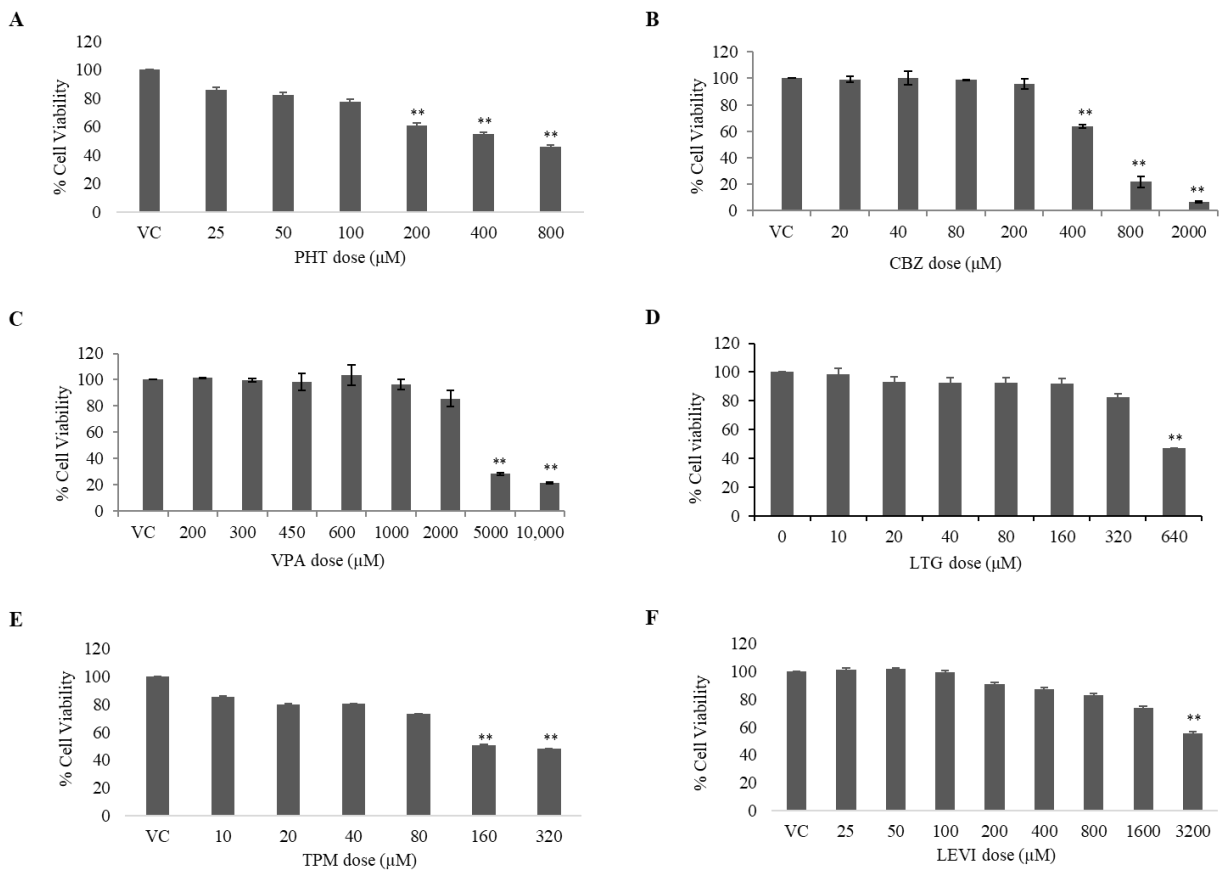
	Activated vs. 60 μ M	0.02 (-0.01, 0.05)	0.17	
	Activated vs. 120 μ M	0.007 (-0.02, 0.03)	0.82	
Fig. 4F (LEVI)	Baseline vs. 25 μ M	0.004 (-0.06, 0.06)	0.10	0.49 (One-way ANOVA with Dunnett test)
	Baseline vs. 50 μ M	-0.002 (-0.06, 0.06)	1.00	
	Baseline vs. 100 μ M	0.02 (-0.04, 0.08)	0.62	
	Baseline vs. 200 μ M	0.02 (-0.04, 0.08)	0.52	
	Activated vs. 25 μ M	0.003 (-0.03, 0.03)	0.98	0.38 (One-way ANOVA with Dunnett test)
	Activated vs. 50 μ M	0.005 (-0.02, 0.03)	0.90	
	Activated vs. 100 μ M	0.01 (-0.02, 0.04)	0.52	
	Activated vs. 200 μ M	0.02 (-0.01, 0.05)	0.25	
Fig. 5A (PHT)	VC vs 40 μ M	0.06 (0.03, 0.09)	<0.001	<0.001 (One-way ANOVA with Dunnett test)
	VC vs 80 μ M	0.10 (0.07, 0.12)	<0.001	
	VC vs KO143	0.20 (0.18, 0.23)	<0.001	
Fig. 5B (VPA)	VC vs 300 μ M	0.15 (0.10, 0.20)	<0.001	<0.001 (One-way ANOVA with Dunnett test)
	VC vs 600 μ M	0.20 (0.15, 0.25)	<0.001	
	VC vs KO143	0.20 (0.15, 0.25)	<0.001	
Fig. 5C (LTG)	VC vs 15 μ M	0.20 (0.15, 0.26)	<0.001	<0.001 (One-way ANOVA with Dunnett test)
	VC vs 60 μ M	0.19 (0.13, 0.24)	<0.001	
	VC vs KO143	0.20 (0.15, 0.26)	<0.001	
Fig. 6B (ABCG2/ B2M)	SCR, VC vs SCR, VPA	1.27 (0.77, 1.77)	<0.001	<0.001 (By siRNA) <0.001 (By drug treatment) (Two-way ANOVA with Tukey test)
	SCR, VC vs siPPAR α , VC	-0.19 (-0.69, 0.32)	0.66	
	SCR, VC vs siPPAR α , VPA	0.19 (-0.32, 0.69)	0.65	

	SCR, VPA vs siPPAR α , VC	1.46 (0.95, 1.96)	<0.001	
	SCR, VPA vs siPPAR α , VPA	-1.08 (-1.59, -0.58)	0.001	
	siPPAR α , VC vs siPPAR α , VPA	0.37 (-0.13, 0.88)	0.16	
Fig. 6D (BCRP/ HSC)	SCR, VC vs SCR, VPA	1.16 (0.79, 1.52)	<0.001	<0.001 (By siRNA) <0.001 (By drug treatment) (Two-way ANOVA with Tukey test)
	SCR, VC vs siPPAR α , VC	-0.06 (-0.43, 0.30)	0.94	
	SCR, VC vs siPPAR α , VPA	0.22 (-0.14, 0.59)	0.28	
	SCR, VPA vs siPPAR α , VC	1.22 (0.85, 1.59)	<0.001	
	SCR, VPA vs siPPAR α , VPA	-0.93 (-1.30, -0.57)	<0.001	
	siPPAR α , VC vs siPPAR α , VPA	0.29 (-0.08, 0.65)	0.13	
Fig. 6E (BCRP activity)	SCR, VC vs SCR, VPA	0.57 (0.47, 0.68)	<0.001	<0.001 (By siRNA) <0.001 (By drug treatment) (Two-way ANOVA with Tukey test)
	SCR, VC vs siPPAR α , VC	-0.11 (-0.21, -0.00)	0.04	
	SCR, VC vs siPPAR α , VPA	-0.03 (-0.13, 0.08)	0.84	
	SCR, VPA vs siPPAR α , VC	0.68 (0.58, 0.78)	<0.001	
	SCR, VPA vs siPPAR α , VPA	-0.60 (-0.70, -0.50)	<0.001	
	siPPAR α , VC vs siPPAR α , VPA	0.08 (-0.02, 0.18)	0.14	
Fig. 7B (BCRP/ HSC)	VC vs VPA	0.36 (0.20, 0.52)	<0.001	0.003 (By -/+MK886) <0.001 (By drug treatment) (Two-way ANOVA with Tukey test)
	VC vs VC+8 μ MMK886	-0.11 (-0.27, 0.05)	0.21	
	VC vs VPA+8 μ MMK886	-0.05 (-0.21, 0.11)	0.77	
	VPA vs VC+8 μ MMK886	0.46 (0.30, 0.62)	<0.001	
	VPA vs VPA+8 μ MMK886	-0.40 (-0.56, -0.24)	<0.001	
	VC+8 μ MMK886 vs VPA+8 μ MMK886	0.06 (-0.10, 0.22)	0.65	
Fig. 7C (BCRP activity)	VC vs VPA	-0.22 (-0.31, -0.12)	<0.001	<0.001 (One-way ANOVA with Tukey test)
	VC vs VC+8 μ MMK886	0.08 (-0.02, 0.17)	0.11	

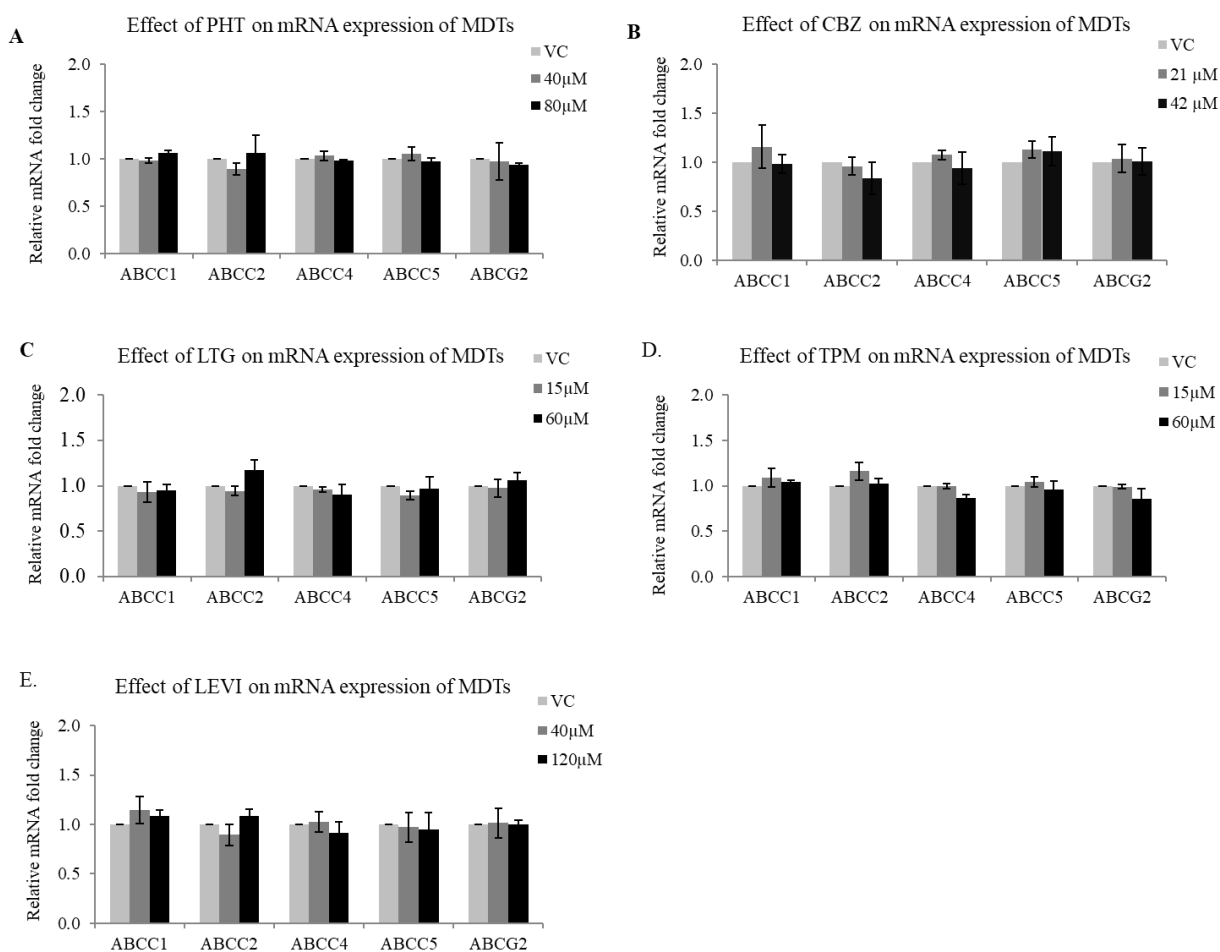
	VC vs VPA+8 μ MMK886	0.02 (-0.08, 0.11)	0.94	
	VPA vs VC+8 μ MMK886	0.29 (0.20, 0.39)	<0.001	
	VPA vs VPA+8 μ MMK886	0.23 (0.14, 0.33)	<0.001	
	VC+8 μ MMK886 vs VPA+8 μ MMK886	-0.06 (-0.15, 0.03)	0.24	
Fig. 8A (PPAR α / B2M)	VC vs 600 μ M (1h)	-0.03 (-0.10, 0.03)	0.80	<0.001 (By time points) <0.001 (By treatments) (Two-way ANOVA with Tukey test)
	VC vs 600 μ M (3h)	-0.16 (-0.22, -0.09)	<0.001	
	VC vs 600 μ M (6h)	-0.30 (-0.37, -0.24)	<0.001	
	VC vs 600 μ M (12h)	-0.24 (-0.31, -0.18)	<0.001	
	VC vs 600 μ M (24h)	-0.23 (-0.29, -0.16)	<0.001	
	VC vs 600 μ M (48h)	-0.07 (-0.14, -0.004)	0.03	
Fig. 8B (PPAR α / HSC)	VC vs 600 μ M (1h)	-0.01 (-0.10, 0.07)	1.00	<0.001 (By time points) <0.001 (By treatments) (Two-way ANOVA with Tukey test)
	VC vs 600 μ M (3h)	-0.21 (-0.29, -0.13)	<0.001	
	VC vs 600 μ M (6h)	-0.14 (-0.22, -0.05)	<0.001	
	VC vs 600 μ M (12h)	-0.05 (-0.13, 0.03)	0.57	
	VC vs 600 μ M (24h)	0.01 (-0.07, 0.09)	1.00	
Fig. 8D	VC vs 600 μ M (24h) – PDK4	-0.22 (-0.25, -0.19)	<0.001	(Unpaired t-test)
	VC vs 600 μ M (24h) – THBD	-0.27 (-0.32, -0.22)	<0.001	
Fig. 9B	0h cytosolic vs 3h cytosolic	0.10 (0.05, 0.15)	<0.001	<0.001 (One-way ANOVA with Tukey test)
	0h cytosolic vs 6h cytosolic	0.06 (0.002, 0.11)	0.04	
	3h cytosolic vs 6h cytosolic	-0.05 (-0.10, 0.01)	0.12	
	0h nuclear vs 3h nuclear	-0.14 (-0.19, -0.08)	<0.001	
	0h nuclear vs 6h nuclear	-0.11 (-0.16, 0.06)	<0.001	
	3h nuclear vs 6h nuclear	0.03 (-0.03, 0.08)	0.67	

	3h cytosolic vs 3h nuclear	-0.24 (-0.29, -0.18)	<0.001	
	6h cytosolic vs 6h nuclear	-0.17 (-0.22, -0.11)	<0.001	
Fig. 9D	0h vs 3h	-0.13 (-0.19, -0.06)	0.001	0.001 (One-way ANOVA with Tukey test)
	0h vs 6h	-0.12 (-0.18, -0.05)	0.002	
	3h vs 6h	0.01 (-0.06, 0.07)	0.95	
Fig. 10A	VC vs VPA (IgG)	-0.20 (-0.67, 0.27)	0.30	(Unpaired t-test)
	VC vs VPA (PPAR α) – 3h	-0.82 (-1.06, -0.58)	<0.001	
	VC vs VPA (PPAR α) – 6h	-0.62 (-0.84, -0.40)	<0.001	
Fig. 10C	VC vs VPA (pGL2-control)	-0.01 (-0.05, 0.02)	0.44	(Unpaired t-test)
	VC vs VPA (pGL2-prom- ABCG2)	-0.17 (-0.20, -0.13)	<0.001	(Unpaired t-test)

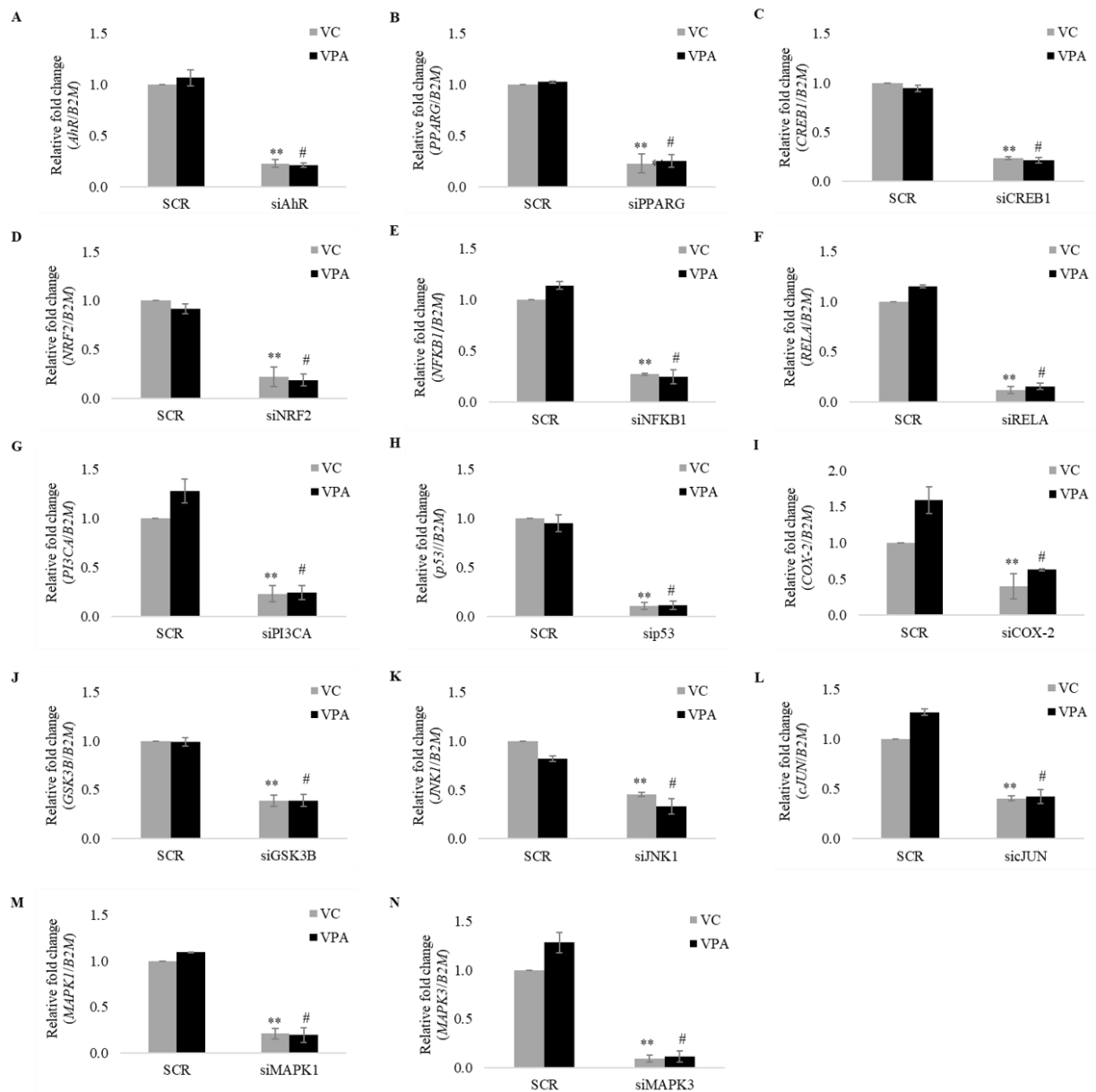
Supplemental Data



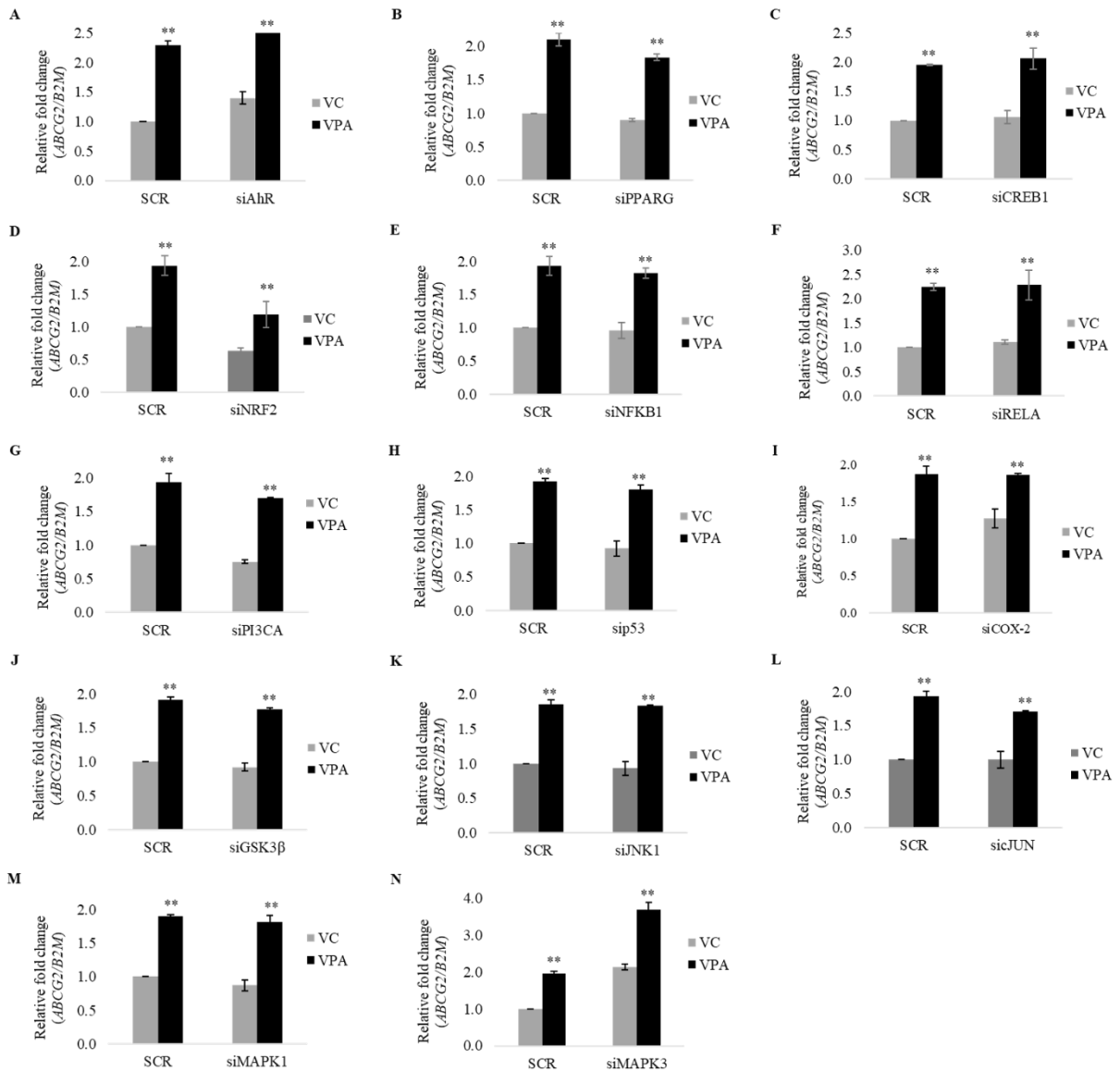
Supplemental Fig. 1. Effect of AEDs on hCMEC/D3 cell viability. Cells were treated with different doses of (A) phenytoin (PHT), (B) carbamazepine (CBZ), (C) valproic acid (VPA), (D) lamotrigine (LTG), (E) topiramate (TPM) and (F) levetiracetam (LEVI) for 72h. After treatment, cells were assessed for % viability using MTT assay. Data is the mean \pm S.D. of 4 independent experiments. ** $p < 0.01$, compared to VC (One-way ANOVA with Dunnett's post hoc test).



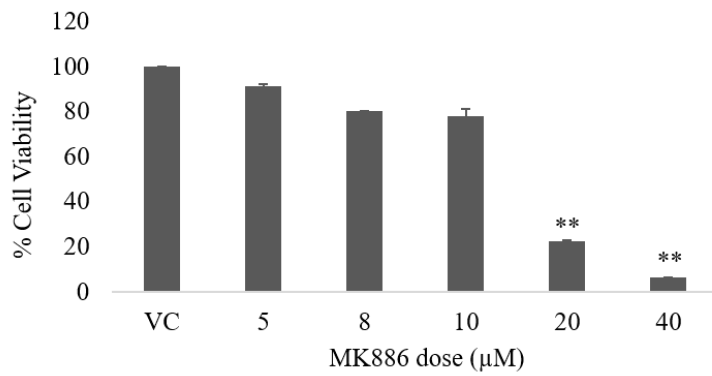
Supplemental Fig. 2. Effect of PHT, CBZ, LTG, TPM and LEVI on mRNA expression of MDTs in hCMEC/D3 cells. RT-qPCR analysis of ABCC1, ABCC2, ABCC4, ABCC5 and ABCG2 mRNA expression in hCMEC/D3 cells treated with (A) PHT (40 μ M, 80 μ M), (B) CBZ (21 μ M, 42 μ M), (C) LTG (15 μ M, 60 μ M), (D) TPM (15 μ M, 60 μ M) and (E) LEVI (40 μ M, 120 μ M) for 24h. The changes in mRNA levels of target genes were normalized with *B2M* and expressed as normalized fold change over VC (0.1% DMSO for PHT, CBZ, LTG and TPM; water for LEVI). The data is the mean \pm S.D. of 3 independent experiments.



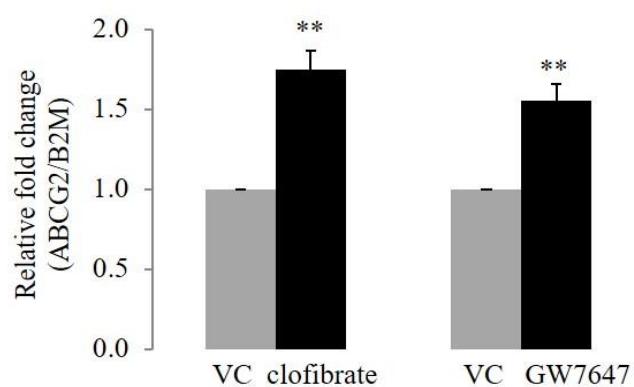
Supplemental Fig. 3. siRNA validation data for 14 molecular factors at mRNA level. hCMEC/D3 cells were transiently transfected with siRNA specific to (A) AhR, (B) PPARG, (C) CREB1, (D) NRF2, (E) NFKB1, (F) RELA, (G) PIK3CA, (H) p53, (I) COX-2, (J) GSK3B, (K) JNK1, (L) cJUN, (M) MAPK1, (N) MAPK3, or the non-targeting control (scramble, SCR). Subsequently, cells were treated with VC (0.1% DMSO) or VPA (600 μ M) for 24h. RT-qPCR analysis was done to check the knockdown of each factor in VC-treated as well as VPA-treated group. The changes in mRNA level of each gene were normalized with *B2M*. The data is the mean \pm S.D. of 3 independent experiments. ** $P < 0.01$, VC (SCR vs. siRNA); # $P < 0.01$ VPA (SCR vs. siRNA) (unpaired t-test).



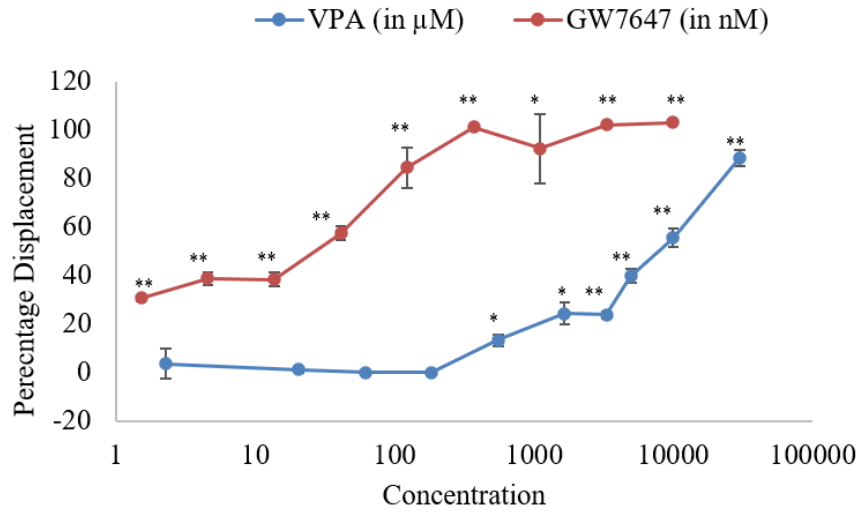
Supplemental Fig. 4. Effect of silencing of molecular factors on VPA-induced ABCG2 mRNA. Expression of the respective factors (A-N) was silenced by transient transfection of gene-specific siRNA in hCMEC/D3 cells. Then, the cells were treated with VC (0.1% DMSO) or VPA (600μM) for 24h and RT-qPCR analysis was done to check mRNA expression levels of ABCG2. Scramble (SCR) was used as non-targeting control. The changes in mRNA level of ABCG2 was normalized with *B2M*. The data is the mean \pm S.D. of 3 independent experiments. ** $P < 0.01$, SCR (VC vs. VPA) and siRNA (VC vs. VPA) (Two-way ANOVA with Tukey's post hoc test).



Supplemental Fig. 5. Effect of MK886 on hCMEC/D3 cell viability. Cells were treated with different doses of MK886 for 48h. After treatment, cells were assessed for % viability using MTT assay. Data is the mean \pm S.D. of 4 independent experiments. ** $p < 0.01$, compared to VC (0.1% DMSO) (One-way ANOVA with Dunnett's post hoc test).



Supplemental Fig. 6. Effect of known PPAR α agonist on ABCG2 mRNA in hCMEC/D3 cells. RT-qPCR analysis of ABCG2 mRNA expression in hCMEC/D3 cells treated with 100 μ M clofibrate and 100nM GW7647 for 24h. The changes in the mRNA level were normalized with *B2M* and expressed as normalized fold change over VC (0.1% DMSO). The data is the mean \pm S.D. of 3 independent experiments. ** $p < 0.01$, compared to VC (unpaired t-test).



Supplemental Fig. 7. Binding of VPA to PPAR α LBD in a TR-FRET competitive binding assay. GW7647 was used as a known PPAR α agonist. Data is the mean \pm S.D. of 2 independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to solvent control (1% DMSO, 0% displacement); One-way ANOVA with Dunnett's post hoc test.

References

Johannessen SI (2004) Therapeutic drug monitoring of antiepileptic drugs, in *Drug Monitoring and Clinical Chemistry* (Hempel G ed) pp 221-253, Elsevier: Amsterdam, The Netherlands.