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Valproic Acid induces CYP3A4 and MDR1 genes expression by activation of Constitutive Androstane Receptor and Pregnane X Receptor pathways

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Running Title: Valproic acid induces CYP3A4 and MDR1 via PXR and CAR

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Non-standard abbreviations: CYP, cytochrome P450; kb, kilobase(s); NR, nuclear receptor; RT-PCR, reverse transcriptase polymerase chain reaction; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RXRα, 9-*cis* retinoic acid receptor-α; DMSO, dimethyl sulphoxide; DR3, direct repeat 3; ER6, everted repeat 6; XREM, xenobiotic-responsive enhancer module; PXRE, PXR response element; PBREM, phenobarbital responsive enhancer module; EMSA, electrophoretic mobility shift assay, VPA, valproic acid; Rif, rifampicin

Abstract

In our study, we tested the hypothesis whether valproic acid (VPA) in therapeutic concentrations has potential to affect expression CYP3A4 and MDR1 via constitutive androstane receptor (CAR) and pregnane X receptor (PXR) pathways. Interaction of VPA with CAR and PXR nuclear receptors was studied employing luciferase reporter assays, real time RT–PCR, EMSA, and analysis of CYP3A4 catalytic activity. Using transient transfection reporter assays in HepG2 cells, VPA was recognized to activate CYP3A4 promoter via CAR and PXR pathways. By contrast, significant effect of VPA on MDR1 promoter activation was observed only in CAR co-transfected HepG2 cells. These data well correlated with up-regulation of CYP3A4 and MDR1 mRNAs analyzed by real time RT-PCR in cells transfected with expression vectors encoding CAR or PXR and treated with VPA. Additionally, VPA significantly up-regulated CYP3A4 mRNA in primary hepatocytes and augmented the effect of rifampicin. EMSA experiments showed VPA-mediated augmentation of CAR/RXRα heterodimer binding to DR3 and DR4 responsive elements of CYP3A4 and MDR1 genes, respectively. Finally, analysis of specific CYP3A4 catalytic activity revealed its significant increase in VPA-treated LS174T cells transfected with PXR. In conclusion, we provide novel insight into mechanism, by which VPA affect gene expression of CYP3A4 and MDR1 genes. Our results demonstrate that VPA has potential to up-regulate CYP3A4 and MDR1 through direct activation of CAR and/or PXR pathways. Furthermore, we suggest that VPA synergistically augments the effect of rifampicin in transactivation of CYP3A4 in primary human hepatocytes.

Valproic acid (VPA) is an effective broad-spectrum anticonvulsant used in the treatment of primary generalized tonic-clonic, absence, and partial seizures (Tanaka, 1999). VPA has recently been identified as an inhibitor of histone deacetylase (HDAC) with potential antitumorous activity that has been studied in several clinical trials (Gottlicher et al., 2001; Blaheta et al., 2002; Kramer et al., 2003; Blaheta et al., 2005; Raffoux et al., 2005).

VPA is well known to affect mechanisms that control drug disposition such as activity of hepatic biotransformation enzymes or drug binding to plasma proteins (Rogiers et al., 1995; Wen et al., 2001; Perucca, 2006). Generally, VPA is thought to be an inhibitor rather than inducer of drug metabolizing enzymes (Perucca, 2006). However, this point of view is currently not supported satisfactorily by comprehensive data published in literature. Regarding metabolizing enzymes of cytochrome P450 family (CYPs), Wen and coworkers have demonstrated using *in vitro* methods that VPA in therapeutically relevant concentrations competitively inhibits only human CYP2C9 catalytic activity (Wen et al., 2001). On the other hand, there is clear evidence that VPA has potential to up-regulate expression and activity of several rodent and human genes encoding proteins involved in drug disposition. It was found that prolonged exposure of rats to VPA results in the self-inducing metabolism of the agent (Fisher et al., 1991). Moreover, Rogiers and colleagues have found VPA to be a potent inducer of genes of the rat Cyp2b subfamily; in particular Cyp2b1 and Cyp2b2 (Rogiers et al., 1992; Rogiers et al., 1995). Recently, Eyel and colleagues have found out that VPA does not affect expression of rat Cyp3a2, an orthologue of human CYP3A4; whereas valpromide, the primary amid of VPA that reveals no HDAC inhibitory activity, was shown to induce this gene by a nonspecific mechanism (Eyal et al., 2006). Additionally, these authors have suggested that VPA induces expression and activity of human P-glycoprotein (P-gp, MDR1), a member of the ABC family of drug transporters, in tumor cell lines by mechanism of HDAC inhibition (Eyal et al., 2006). These summarized data indicate that VPA is capable to alter

expression and activity of various CYPs differently. Moreover, VPA has potential to induce MDR1 gene.

We assumed that alteration of expression of these genes could be caused by interaction of VPA with constitutive androstane receptor (CAR; N1I3) and human pregnane X receptor (PXR; SXR; NR1I2). PXR and CAR are ligand-activated nuclear receptors that as heterodimers with retinoid X receptor α (RXRα) up-regulate the transcription of their target genes, such as CYP3A4 and MDR1, by interaction with specific promoter binding motifs (Goodwin et al., 1999). PXR has been shown to be activated by many structurally and chemically diverse ligands. Examples of human PXR activators include xenobiotics such as rifampicin (Bertilsson et al., 1998; Pascussi et al., 2000), the endobiotics lithocholic acid (Staudinger et al., 2001; Xie et al., 2001), progesterone metabolite 5-pregnane-3,20-dione (Jones et al., 2000), and the herbal compound hyperforin (Moore et al., 2000a). In the case of CAR, the experimental substance CITCO, the antimalarial artemisinin drugs, and 5-pregnane-3,20-dione have been recognized as its ligands (Jones et al., 2000; Maglich et al., 2003; Burk et al., 2005b). The barbiturate drug phenobarbital activates CAR indirectly through the increase of CAR translocation from the cytoplasm to the nucleus (Honkakoski et al., 1998; Kawamoto et al., 1999; Goodwin and Moore, 2004). On the other hand, several competitive inverse agonists of CAR have also been discovered such as androstanol, androstenol and clotrimazole (Forman et al., 1998; Moore et al., 2000b).

In this study, we examined whether VPA controls expression of CYP3A4 and MDR1 genes at the transcriptional level via activation of PXR and CAR pathways. This hypothesis was investigated in the human Caucasian hepatocyte carcinoma (HepG2) and human colon carcinoma (LS174T) cells using several reporter luciferase constructs with major promoter regulatory sequences of CYP3A4 and MDR1. In addition, employing real time RT-PCR, we investigated CYP3A4 mRNA expression in primary human hepatocytes treated with VPA.

Additionally, we studied whether VPA augments binding of CAR/RXRα to several response elements of CYP3A4 (DR3, ER6) and MDR1 (DR4) using Electrophoretic Mobility Shift Assay (EMSA).

Our data indicate that VPA is capable to transactivate both CYP3A4 and MDR1 via interaction with CAR pathway; however, VPA-mediated activation of PXR pathway controls only CYP3A4 gene expression. Moreover, we demonstrate that VPA synergizes with rifampicin in transactivation of CYP3A4 in primary human hepatocytes.

Methods

Cell Lines and Chemicals. The human Caucasian hepatocyte carcinoma HepG2 and human colon adenocarcinoma LS174T cell lines were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and were used within 25 passages after delivery and maintained in antibiotic-free DMEM supplemented with 10% fetal calf serum (FCS), 1% sodium pyruvate and 1% nonessential amino acids (Sigma-Aldrich). The latter cell line has been previously shown to have highly inducible expression of CYP3A4 and MDR1 (Geick et al., 2001).

Androstenol (5α-androst-16-en-3α-ol), rifampicin, VPA (2-propylpentanoic acid) and DMSO (dimethyl sulphoxide) were purchased from Sigma-Aldrich. CITCO ((6-(4-chlorophenyl) imidazo [2,1-b][1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime was purchased from BIOMOL (Plymouth Meeting, PA). DMSO or water was used as a solvent of VPA when indicated.

Plasmids. A chimeric *p3A4-luc* reporter construct containing the basal promoter (–362/+53) with proximal PXR response element (PXRE) and the distal xenobiotic-responsive enhancer module (XREM) (–7836/–7208) of the CYP3A4 gene 5´-flanking region inserted to pGL3-Basic reporter vector was described by Goodwin et al. (Goodwin et al., 1999). Plasmid p-7975(Δ7012–1804) harboring the enhancer of MDR1 gene 5'- flanking region (MDR1E) and the basal promoter was inserted in pGL3-Basic reporter vector as described by Geick et al. (2001), herein it is referred to as *pMDR1-luc. pMDR1E-SV40-luc* reporter plasmid was constructed by insertion of the enhancer region of MDR1 gene from –7881 to –7809 into pGL3-Promoter plasmid (Promega, Madison, WI) in *NheI/BglII* cloning sites upstream of a heterologous promoter SV40. CYP2B6 PBREM reporter gene construct (*pPBREM-SV40-luc*) containing two DR4-type motives (NR1 and NR2) was prepared from *pGL3-Promoter*

plasmid by insertion of the region from -1733 to -1683 of CYP2B6 promoter into *NheI/BglII* cloning sites upstream of a heterologous promoter SV40. Constructed plasmids were sequenced using a Big Dye Terminator Cycle Sequencing Method (Applied Biosystems, MD). The expression plasmid for human PXR receptor, *pSG5-PXR*, was kindly provided by Dr. S. Kliewer (University of Texas, Dallas, TX). The human CAR expression plasmid *pCR3-CAR* was kindly provided by Dr. M. Negishi (NIEHS, Research Triangle Park, NC). The expression plasmid *pSG5-hRXRα* encoding hRXRa cDNA was a generous gift from Dr. C. Carlberg (University of Kuopio, Finland).

Transient Transfection and Luciferase Gene Reporter Assays. All transfection assays were carried out using Lipofectamine®2000 transfection reagent (Invitrogen, Carlsbad, CA) in cells cultivated in the phenol red-free medium containing 10% charcoal-striped FCS, 1% sodium pyruvate and 1% nonessential amino acids according to the manufacture's instruction. HepG2 cells (2×10^5) were seeded into 48-well plates and co-transfected with a luciferase reporter construct (0.4 µg/well) and expression plasmid encoding either CAR or PXR (50 ng/well) 24 h later. Subsequently, cells were maintained in medium supplemented with 10% charcoal/dextran-stripped FBS containing VPA (Sigma-Aldrich) at appropriate concentration for 24 h. Luminescence activity was determined with a luminometer Genios Plus (Tecan, Grödig, Austria) in cell lysate using a commercially available luciferase detection system (Promega, Madison, WI). Additionally, all experiments were in parallel performed using empty *pGL3-Basic* and *pGL3-Promoter* luciferase reporter constructs containing no responsive elements to distinguish non-specific CAR- and PXR- independent effect of VPA on reporter plasmids used. Luminescence of these samples were taken as background that was subtracted in final calculation from luminescence of cell samples transiently transfected with reporter plasmids containing response elements of tested genes.

Resulting data are presented as ratio of luminescence of treated cell samples to control. Luminescence of each sample has been normalized to its protein concentration determined with BCA system (Pierce, Milwaukee, WI).

Real Time RT-PCR Analysis of CYP3A4 mRNA. LS174T cells (1.2×10^5) were seeded into 24-well plates and cultivated for 24 h. Then cells were transfected with CAR or PXR expression plasmids (400ng/well) and appropriate cell samples were exposed to VPA at a concentration of 500 µM for 48 h. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The following primers were used for CYP3A4 (forward primer: 5'- TTCAGCAAGAAGAACAAGGACAA-3'; reverse primer: 5'-GGTTGAAGAAGTCCTCCTAAGC-3'), for MDR1 (forward primer: 5'-TGCTCAGACAGGATGTGAGTTG-3'; reverse primer: 5'-AATTACAGCAAGCCTGGAACC-3' and for housekeeping genes HPRT (Hypoxanthine-Guanine Phosphoribosyl Transferase) (forward primer: 5'-CTGGAAAGAATGTCTTGATTGTGG-3'; reverse primer: 5'-TTTGGATTATACTGCCTGACCAAG -3' and for B2M (β2-microglobulin) (forward primer: 5'-CGTGTGAACCATGTGACTTTGTC-3'; reverse primer: 5'-CATCTTCAAACCTCCATGATGC-3'). cDNA was prepared from 1 µg of total RNA with MMLV transcriptase (Finnzymes, Finland) using oligo(dT)$_{15}$VN primer (Generi-Biotech, Hradec Králové, Czech Republic) and porcine RNase inhibitor (TaKaRa, BIO, Shiga, Japan). Real-Time PCR analysis was performed on an iCycler (Bio-Rad, Hercules, CA). cDNA (40 ng reverse transcribed RNA) was amplified with HotStar *Taq* polymerase (QIAGEN, Valencia, CA) under the following conditions: 3 mM MgCl$_2$, 0.2 mM dNTP, 0.3 µM each primer, 0.025 U/µl polymerase, SybrGreen I in 1:100,000 dilution and fluorescein (10 nM); the temperature profile was 95°C for 14 min; 50 times: 95°C for 15 s, 60 °C for 20 s, 72 °C

for 30 sec; 72 °C for 15 sec; melting curve program 70-95°C. All samples were run in triplicates simultaneously with negative controls. The processing of real-time amplification curves was performed on iCycler software ver. 4.6 (Bio-Rad). Pfaffl´s method was applied for relative quantification of gene expression normalized to endogenous control (housekeeping) gene (Pfaffl, 2001). Results are presented as the means of at least three experiments.

Isolation and CYP3A4 mRNA Analysis in Primary Cell Culture of Human Hepatocytes.

Human tissue samples were obtained according to protocols approved by the Local ethics committee of the Palacky University in Olomouc complying with the current Czech legislation. Hepatocytes were prepared from lobectomy segments resected from adult patients for medical reasons unrelated to our research program. The tissue encompassing the tumor was dissected by a surgeon and sent for anatomopathological studies, while the remaining tissue was used for hepatocytes preparation. No information on the patients was available to us, apart from age, sex, and the reason for surgery. Human liver samples used in this study were obtained from two donors: Donor 1, woman, 69 years old, tumor metastasis; donor 2, woman 46 years old, Caroli syndrome. Hepatocytes were isolated as previously described (Pichard-Garcia et al., 2002). Following isolation, the cells were plated on collagen-coated culture dishes at density 1.4×10^5 cells/cm^2. Culture medium was enriched for plating with 2% FCS (v/v) as described previously (Isom et al., 1985). The medium was replaced with serum-free medium the day after and the cells were allowed to be stabilized for additional 48 – 72 h prior to the treatment with VPA (500 µM) or rifampicin (10 µM) or their combination. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator. Effect of VPA on CYP3A4 mRNA expression was tested in periods of 24 and 48 h. Effect of rifampicin and the combination of VPA and rifampicin were studied in period of 24 h. In the later experiments, DMSO (0.1%) was used as solvent of both VPA and rifampicin. Level of CYP3A4 mRNA

expression was analyzed employing real time RT-PCR according to protocol mentioned above.

Functional Analysis of CYP3A4 Activity. LS174T cells (6×10^5) were seeded into 12-well plates and cultivated for 24 h. Subsequently cells were transfected with CAR or PXR expression plasmids (1.2 µg/well) and then exposed to VPA at a concentration of 600 µM for 48 h. After treatment, cells were washed with PBS and cultivated in serum-free Opti-MEM medium (Gibco) containing testosteron at a final concentration of 150 µM for two h at 37 °C. Then, cells were washed with PBS and lysed with 200 µl SDS (1 %). CYP3A4 activity was detected in total cellular lysate (150 µl) using an established method based on the measuring of a prototypic CYP3A4-mediated testosterone 6β-hydroxylation activity (Guengerich et al., 1986) and modified for the analysis of the metabolite in cell lysate. For the determination of CYP3A4 activity, the metabolite was extracted to dichloromethane. Subsequently, the solvent was evaporated and the sample dissolved in the mobile phase. Concentration of the testosterone metabolite was determined using a Shimadzu Class VP HPLC system (Shimadzu, Tokyo, Japan). Final activity was normalized to sample protein concentration in cell lysate that was measured using BCA assay according to manufacture's instructions (Pierce, Milwaukee, WI).

Electrophoretic Mobility Shift Assay (EMSA). Human CAR and RXRα were translated *in vitro* using TNT® Quick Coupled Transcription/Translation Systems (Promega, Delta House, Southapton, USA). The nuclear fraction was isolated from HepG2 cells transfected with hRXRα expression vector using CelLytic™ NuCLEAR™ Extraction Kit (Sigma, Saint Louis, Missouri, USA). The following double-stranded 5'–biotinylated oligonucleotides of specific response elements of both CYP3A4 and MDR1 promoters were used as probes: CYP3A4

DR3 sense, 5'-GAA TGA ACT TGC TGA CCC TCT-3'; CYP3A4 DR3 antisense, 5'- AGA GGG TCA GCA AGT TCA TTC-3'; CYP3A4 ER6 sense, 5'-ATA TGA ACT CAA AGG AGG TCA GTG-3'; CYP3A4 ER6 antisense, 5'-CAC TGA CCT CCT TTG AGT TCA TAT-3'; MDR1 DR4 sense, 5'-CAT TGA ACT AAC TTG ACC TTG C-3' and MDR1 DR4 antisense, 5'-GCA AGG TCA AGT TAG TTC AAT G-3'. The oligonucleotides were synthesized in Generi Biotech, s.r.o. (Hradec Kralove, Czech Republic).

EMSA method was performed according to the protocol published previously with slight modifications (Frank et al., 2003). The binding reactions were performed in a total volume of 20 µl and contained equal amounts (~ 3 µg) of CAR and RXRα, 10x Binding Buffer (100 mM Tris, 500 mM KCl, 10 mM DTT) (Pierce, Milwaukee, WI), Poly (dI·dC) Double Strand (GE Amersham Biosciences, Piscataway, NJ) and 1% NP-40. VPA was tested at final concentrations of 100, 500 and 1000 µM. CITCO, an agonist of human CAR, was used at the concentration of 6.25 µM, androstenol at the concentration of 10 µM. DMSO was used as a solvent of the compounds at maximal final concentration of 0.1%. For supershift experiments, 1 µg of the anti-RXRα rabbit polyclonal IgG antibody was added to the reaction mixture (Santa Cruz Biotechnology, Inc., California, USA). Reactions were preincubated on ice for 10 min before the addition of oligonucleotide probe (20 fmol). Samples were maintained at room temperature for an additional 20 min, and then protein/DNA complexes were resolved on a 5% (w/v) non-denaturing polyacrylamide gel (acrylamide/bisacrylamide 29:1 w/v) in 0.5× TBE buffer (450 mM Tris, 450 mM Boric Acid, 10 mM EDTA). The gels were electrophoresed at 100 V for an h at room temperature. Next, the gels were blotted at 380 mA for 30 min at 4°C and then analyzed by Chemiluminescent Nucleic Acid Detection Module (Pierce, Milwaukee, WI).

EMSA Quantification. EMSA reactions were exposed to X-ray film (Foma Bohemia a.s, Hradec Králové, Czech Republic) and then bands were quantified using computerized densitometry using LabImage densitometry analysis software (Kapelan, Gemany)."

Statistics. One-way ANOVA followed by the Dunnett's Multiple Comparison *post hoc* test or unpaired Student's *t*-test were used for statistical analysis of data using a GraphPad Prism software. Two-way ANOVA with interaction was used to analyze synergistic effect of VPA and rifampicin.

Results

Effect of VPA on CAR- and PXR-mediated Activation of CYP3A4 Promoter in HepG2 Cells Transiently Transfected with *p3A4-luc* Luciferase Reporter Construct. First, we examined whether VPA affects activation of *p3A4-luc* reporter construct through CAR and PXR. We observed statistically significant concentration-dependent increase in CAR- and PXR- mediated transcriptional activation of *p3A4-luc* in HepG2 cells after 24 h exposure to VPA (Fig. 1). VPA at the concentration of 500 μM doubled the activation of *p3A4-luc* in cells co-transfected with CAR expression vector (Fig. 1). In HepG2 cells co-transfected with PXR, we observed even a 4-fold increase in activation of p3A4-luc after treatment with VPA (500 μM) (Fig. 1). In contrast, VPA did not significantly affect transcriptional activation of *p3A4-luc* in HepG2 cells, which were not co-transfected with either CAR or PXR (Fig. 1). We observed that expression of both CAR and PXR in HepG2 cells in the absence of an exogenous ligand resulted in statistically significant activation of *p3A4-luc* reporter, which is in agreement with published data and indicates involvement of endogenous activators of the nuclear receptors in HepG2 cells (Fig. 1) (Goodwin et al., 1999; Goodwin et al., 2002).

Transactivation of CYP3A4 Promoter by VPA in Combination with CITCO or Rifampicin. In these experiments, we examined whether VPA has the ability to affect CAR- and PXR-mediated activation of *p3A4-luc* construct caused by prototypic ligands CITCO (500 nM) in CAR- and Rifampicin (10 μM) in PXR- expressing cells. VPA at tested concentrations in combination with CITCO (500 nM) significantly augmented CAR-mediated activation of *p3A4-luc* compared to CAR expressing cells treated with CITCO ($p<0.01$) (Fig. 2). Similarly, we observed more pronounced effect of VPA in combination with rifampicin (10 μM) on transcriptional activation of *p3A4-luc* via PXR compared to the effect of rifampicin alone ($p< 0.05$) (Fig. 2).

Effect of VPA on CAR- and PXR-mediated Activation of MDR1 Promoter in HepG2 Cells Transiently Transfected with *pMDR1-luc* or Heterologous *pMDR1E-SV40-luc* Luciferase Reporter Constructs. Regarding MDR1, two different luciferase reporter constructs were employed in our study. First, we used *pMDR1-luc* construct containing the distal enhancer region with DR4 responsive motif common for both PXR- and CAR-mediated transactivation of MDR1 gene, and the native basal promoter from -1803 to +281. Using this reporter construct, we observed similar effect of VPA on activation of MDR1 promoter in cells co-transfected with PXR or CAR in comparison with mock-transfected HepG2 cells (Fig. 3A). On the other hand, rifampicin (10 µM) and CITCO (1 µM) significantly activated (~2-fold, $p<0.05$) the *pMDR1-luc* construct in HepG2 cells cotransfected with PXR or CAR expression plasmids under used experimental conditions. This indicates that VPA has probably potential to transactivate this luciferase construct independently on PXR and CAR pathways through a nonspecific mechanism, which is likely related to activation of MDR1 basal promoter (Morrow and Nakagawa, 1994).

In the light of our previous observations demonstrating that heterologous *pMDR1E-SV40-luc* is more responsive to both PXR- and CAR-mediated activation than *pMDR1-luc* reporter, we performed transfection assay with *pMDR1E-SV40-luc* reporter construct containing the MDR1 enhancer upstream of viral SV-40 promoter. Employing the plasmid, we observed that VPA at a concentration of 500 µM activates significantly only CAR-mediated transcription of *pMDR1E-SV40-luc* reporter construct ($p<0.001$) (Fig. 3B). The effect of VPA on activation of pMDR1E-SV40-luc plasmid was comparable with the effect of CITCO (1 µM, 2-fold activation) and with rifampicin (25 µM, 2.3-fold activation, $p<0.05$) in nuclear receptor-transfected cells.

Finally, we used androstenol, an inverse agonist of CAR, to confirm that VPA activates *pMDR1E-SV40-luc* through CAR. Androstenol (10 µM) significantly repressed CAR-

mediated transcriptional activation of *pMDR1E-SV40-luc* in cells exposed to VPA at a concentration of 500 µM ($p<0.05$) (Fig. 3C). In contrast, we observed no effect of androstenol on pGL3-Basic or pGL3-Promoter constructs activities in cells co-transfected with CAR expression vector (data not shown).

Effect of VPA on CAR- and PXR-mediated Activation of CYP2B6 PBREM in HepG2 Cells Transiently Transfected with *pPBREM-SV40-luc* Luciferase Reporter Construct.
To elucidate discrepancy between CAR- and PXR- mediated activation of MDR1 promoter by VPA, we analyzed luciferase activity of *pPBREM-SV40-luc* construct in cells transiently transfected with CAR or PXR expression vectors and exposed to VPA (500 µM). *pPBREM-SV40-luc* construct contains two DR4-type responsive elements of the phenobarbital responsive enhancer module (PBREM) of CYP2B6 gene, which has high ability to interact with both CAR and PXR (Goodwin et al., 2001; Faucette et al., 2006). We detected significant up-regulation of *pPBREM-SV40-luc* activity in HepG2 cells co-transfected with both CAR and PXR expression plasmids ($p<0.001$, Fig. 4). Moreover, the activation of the construct was further significantly augmented in cells co-expressing CAR and exposed to VPA (5.8-fold increase, $p<0.001$, Fig. 4). Similarly, VPA increased significantly activation of *pPBREM-SV40-luc* in PXR expressing cells (2-fold, $p<0.05$). Under the same experimental conditions, CITCO (1 µM) increased activation of the plasmid 2.1-fold in the cells expressing CAR and rifampicin (10 µM) 2.4-fold in PXR-transfected cells. Nevertheless, we also detected comparable activation of the luciferase construct by VPA in cells with no exogenous PXR ($p<0.05$) (Fig. 4), which documents that exogenous PXR has minor or no effect in activation of *pPBREM-SV40-luc* by VPA. Thus, we observed a similar pattern in activation of *pPBREM-SV40-luc* and *pMDR1E-SV40-luc* by VPA (Fig. 3B and 4). In both cases, we

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demonstrated that VPA increases CAR-mediated, but not PXR-mediated transactivation of the constructs.

Analysis of VPA-mediated Up-Regulation of CYP3A4 and MDR1 mRNAs in LS174T Cells. To evaluate the ability of VPA to induce CYP3A4 and MDR1 mRNA levels by means of CAR and PXR activation, real time RT-PCR was employed. CYP3A4 and MDR1 mRNAs were quantified in samples of LS174T cells transfected with expression plasmids encoding studied nuclear receptors and exposed to VPA for 48 h. Significant effect of VPA on CYP3A4 mRNA level was observed in cells transfected with both CAR and PXR nuclear receptors yielding a 4.2-fold and 3.8-fold ($p<0.001$ and $p<0.01$) induction, respectively (Fig. 5A). VPA also significantly up-regulated CYP3A4 mRNA in LS174T cells, which were not co-transfected with any nuclear receptor ($p<0.01$) (Fig. 5A). This might be in agreement with high expression of endogenous PXR in LS174T cells, which could at least partly participate in up-regulation of CYP3A4 mRNA by VPA (data in Fig. 1) (Burk et al., 2005b). In case of MDR1, we observed similar profile of VPA-mediated MDR1 mRNA up-regulation. Contrary to transfection assays, we detected statistically significant effect of VPA on MDR1 mRNA expression in PXR transfected LS174T cells (2.0-fold increase, $p<0.05$). However, the MDR1 mRNA level was not statistically significantly different from the level in VPA-treated cells (Fig. 5B). Thus, we suppose that the increase in MDR1 mRNA level after treatment with VPA is caused mostly by mechanism of HDAC inhibition described previously rather than through activation of PXR pathway by VPA (Morrow and Nakagawa, 1994; Jin and Scotto, 1998; Xiao and Huang, 2005). Importantly, we observed statistically significant ($p<0.05$) up-regulation of MDR1 mRNA after co-transfection of LS174T cells with CAR expression vector and treatment with VPA (Fig. 5B). On the other hand, co-transfection of LS174T cells with expression plasmid for either PXR or CAR without exposure to VPA did not result in statistically significant up-regulation of CYP3A4 and MDR1 mRNAs (Fig. 5A and 5B).

Under the same experimental conditions, treatment of LS174T cells with rifampicin (10μM) resulted in 12-fold increase in CYP3A4 mRNA and 7-fold increase in MDR1 mRNA.

Determination of CYP3A4 Catalytic Activity in LS174T Cells Exposed to VPA (600 μM). CYP3A4 induction was subsequently investigated employing functional assay based on determination of specific CYP3A4-mediated testosterone 6β-hydroxylation activity measured in LS174T cell lysate. As shown in Fig. 6, only cells transfected with plasmid encoding PXR yielded statistically significant increase in CYP3A4 catalytic activity after 48 h exposure to VPA at a concentration of 600 µM ($p<0.05$).

VPA induces CYP3A4 mRNA in primary human hepatocytes and synergizes with the effect of rifampicin. To determine whether VPA induces CYP3A4 mRNA and whether it influences the induction by the rifampicin, primary culture of human hepatocytes were treated individually or in combination with VPA (500 μM) and/or rifampicin (10 μM). CYP3A4 mRNA was significantly up-regulated in the hepatocytes exposed to VPA for 48 h taken from both donor 1 (14.1-fold, $p<0.01$) (Fig. 7A) and donor 2 (3.8–fold, $p<0.01$) (Fig.7B). As shown in the Fig 7C, employing unpaired Student's *t*-test, we revealed statistically significant increase of CYP3A4 mRNA compared to control treated with DMSO (0.1%) in cell samples treated with rifampicin (10 µM) for 24 h (donor 1 - 20.1 and donor 2 - 13.6-fold, respectively; p<0.001). In cells cultivated in medium containing VPA (500 µM) and vehiculum (DMSO 0.1%) used for elimination of rifampicin solvent effect, we also detected significant induction of CYP3A4 mRNA relative to control (DMSO 0,1%) ($p<0.01$ and p<0.05, respectively). Furthermore, when VPA (500 µM) and rifampicin (10 µM) were added simultaneously into medium and hepatocytes were cultivated for 24 h, up-regulation of CYP3A4 was synergistic rather than additive (62 and 34 –fold). Employing two-way

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ANOVA, this synergy was found to be statistically significant at the level of $p<0.01$ (Fig. 7C).

Examination of Interactions between CAR/RXRα Heterodimer or CAR Monomer and Responsive Elements of CYP3A4 and MDR1 Promoters in the Presence of VPA Employing a Ligand-dependent EMSA. The interactions between CAR/RXRα heterodimers and nuclear receptor-binding motives of CYP3A4 (DR3, ER6) and MDR1 enhancer (DR4) in the presence of VPA were examined employing EMSA using in vitro-translated CAR and RXRα proteins. CAR forms complex with RXRα, which binds DR3, DR4 and ER6 responsive elements of CYP3A4 and MDR1 even in the absence of a ligand (Goodwin et al., 1999; Geick et al., 2001; Goodwin et al., 2002; Burk et al., 2005a). This complex formation can be enhanced by ligand binding to CAR, which can be revealed employing EMSA (Frank et al., 2004). We detected that VPA at tested concentrations of 500 and 1000 µM increased the formation of CAR/RXRα complex with DR3 responsive element of CYP3A4 promoter (Fig. 8A lane 8; B, lanes 6 and 7). Fig. 8A shows that employing recombinant CAR and RXRα proteins, VPA moderately (by ~ 30%) augmented the CAR/RXRα/DR3 complex in EMSA experiments (Fig. 8A, lane 8). In the next experiments, we used nuclear fraction from HepG2 cells transfected with expression vector encoding RXRα. VPA at the concentration of 500 µM augmented the complex 1.8-fold and at the concentration of 1000 µM VPA increased 4.2-fold binding of CAR/RXRα to DR3 (Fig. 8B, lane 6 and 7). Similarly, as shown in Fig. 8D (lane 6), we detected increased (~ 1.5-fold) binding of CAR/RXRα heterodimer to oligonucleotide containing DR4 motive of MDR1 enhancer in the presence of VPA (1000 µM). Consistently, CITCO, an agonist of human CAR, augmented binding of CAR/RXRα complex to DR3 response elements (Fig. 8A, lane 5; 8B, lane 3) (Maglich et al., 2003). The inverse agonist of CAR androstenol (10 µM) decreased formation of CAR/RXRα complex with response element DR3 in samples treated with CITCO (500 nM) or VPA (500 and 1000 µM) (Fig. 8A,

B, C, D). Interestingly, we observed that VPA at tested concentrations augments interaction of recombinant CAR monomer with DR3 motive of CYP3A4 promoter (Fig. 8C, lane 2). On the other hand, we did not detect any significant and reproducible effect of VPA on interaction of CAR/RXRα heterodimer with ER6 response element of CYP3A4 gene (data not shown). RXRα alone did not reveal any interaction with tested response elements (data not shown). Similarly, we did not detect any complex after incubation of nuclear fraction of HepG2 cells and recombinant RXRα with DR3 oligonucleotide under used conditions (data not shown). These data suggest that VPA could directly interact with CAR/RXRα heterodimer and CAR monomer in binding with DR3 and DR4 response elements of CYP3A4 and MDR1.

Unfortunately, there is no report in literature, which introduce reliable ligand-dependent EMSA assay with recombinant PXR at present. Therefore, we could not analyze interaction of VPA with PXR/RXRα complex at present using EMSA method.

Discussion

VPA, an effective broad-spectrum anticonvulsant, has been shown to affect expression of a large number of genes (Bosetti et al., 2004). Some of these recognized alterations in gene expression have been attributed to HDAC inhibitory activity of VPA (Chen et al., 1999; Phiel et al., 2001; Werling et al., 2001; Eyal et al., 2006). In this study, we show for the first time that VPA is capable in clinically relevant range of concentrations < 1000 µM (Centorrino et al., 2003; Allen et al., 2006; Davis et al., 1994; Wen et al., 2001) to up-regulate CYP3A4 and MDR1 genes expression also by a different molecular mechanism via direct activation of CAR pathway. Moreover, we demonstrate that VPA can increase CYP3A4 gene expression and activity through activation of PXR nuclear receptor as well.

Ligand-activated nuclear receptors CAR and PXR up-regulate expression of target genes at the transcriptional level through interaction with specific promoter response elements. Therefore, in order to examine the potential effect of VPA on CAR- and PXR-mediated transcriptional regulation of CYP3A4 and MDR1 genes, we first employed transient transfection experiments with luciferase reporter constructs containing relevant regulatory promoter sequence of tested genes (Goodwin et al., 1999; Goodwin et al., 2001; Goodwin et al., 2002; Burk et al., 2005a). Using transient transfection, we established that the reporter constructs appeared in cells separately from the chromosomal DNA and nucleosome structure, which guaranteed that detected effect of VPA on activation of luciferase constructs is independent on chromatin remodeling caused by VPA-mediated HDAC inhibition.

We demonstrate that VPA induces CAR- and PXR-mediated transactivation of *p3A4-luc* construct containing both DR3 and ER6 responsive elements of CYP3A4 required for CAR- and PXR-mediated regulation of the gene (Goodwin et al., 1999) (Fig. 1). Interestingly, we found out that the combination of VPA with prototypic ligands causes far more pronounced transactivation of the *p3A4-luc* construct (Fig. 2). These data indicate synergistic effect of

VPA and rifampicin on transactivation of CYP3A4 via PXR, which could play an important role during co-treatment with these drugs.

In additional experiments, we examined activation of two different luciferase reporter constructs containing MDR1 enhancer and either native or viral SV40 basal promoter (Burk et al., 2005a). We did not detect any significant CAR- or PXR-dependent effect of VPA on activation of MDR1 gene promoter in HepG2 cells transfected with *pMDR1-luc* reporter construct, although increase in transcriptional activation of this reporter construct was observed in cells exposed to VPA (Fig. 3A). On the other hand, employing *pMDR1E-SV40-luc* reporter construct containing the MDR1 enhancer upstream of SV-40 viral promoter, VPA was identified as an activator of the MDR1 enhancer via CAR pathway (Fig. 3B). Thus, by using of *pMDR1E-SV40-luc* construct we eliminated potential interference of additional transcriptional factors that bind relevant sites in the basal promoter of MDR1 gene such as NF-Y, Sp1, AP-1, NK-κB, C/EBPβ etc. (Scotto, 2003) and analyzed specific CAR-/PXR-mediated activation of the MDR1 enhancer by VPA. Moreover, the reporter plasmid lacks inverted CCAAT motive (Y-box), which was shown to be essential for activation of MDR1 promoter in transient transfection reporter assays by several other HDAC inhibitors (Jin and Scotto, 1998). Despite this fact, significant activation of *pMDR1E-SV40-luc* reporter construct by VPA was observed also in the absence of co-transfected CAR or PXR, which indicates that VPA can also activate MDR1 enhancer of *pMDR1E-SV40-luc* reporter construct (Fig. 3B). We suppose that endogenous CAR or PXR are not involved in the phenomenon as their functional expression in HepG2 cells is very low. However, we cannot exclude potential up-regulation of various transcriptional factors by VPA treatment, which could lead to transactivation of the reporter construct.

Subsequently, interaction of VPA with PXR transactivation pathway has been studied in more detail using another gene reporter construct *pPBREM-SV40-luc* containing two DR4-

type motives of CYP2B6 PBREM region upstream of SV-40 viral promoter, which were

recently demonstrated to have a high affinity to both PXR and CAR (Faucette et al., 2006).

These experiments have revealed significant CAR-mediated, but no PXR-mediated activation

of *pPBREM-SV0-luc* reporter construct by VPA, which is in agreement with the findings

obtained employing of *pMDR1-SV40-luc* reporter construct. Thus, we suggest different effect

of VPA on CAR- and PXR-mediated transcriptional activation of heterologous reporter

constructs *pPBREM-SV40-luc* and *pMDR1-SV40-luc* in comparison with *p3A4-luc* construct

(Fig. 1, 3B, 4). We hypothesize that this discrepancy can be explained considering the report

of Masuyama and coworkers (Masuyama et al., 2005), who demonstrated ligand- and

promoter-specific fashion of PXR-mediated transcription of CYP3A4 and MDR1 genes.

CAR- and PXR-mediated regulation of CYP3A4 and MDR1 genes expression was

subsequently analyzed by real time RT-PCR in samples of LS174T cells (Fig. 5A, B). mRNA

expression of the studied genes was normalized to both HPRT and B2M housekeeping genes

to eliminate false interpretation potentially caused by non-specific VPA-mediated up/down-

regulation of these housekeeping genes. We observed the effect of co-expressed CAR and

PXR on VPA-mediated up-regulation of CYP3A4 mRNA in LS174T cells (Fig. 5A), which

well correlates with the reporter experiments. In case of MDR1, mRNA level was

significantly up-regulated in LS174T cells transfected with CAR expression vector, whereas

VPA-mediated increase of MDR1 mRNA in PXR-expressing cells was not statistically

different from non-transfected cells exposed to VPA (Fig. 5B). Considering data from gene

reporter assay with *pMDR1E-SV40-luc* (Fig. 3A) and *pPBREM-SV40-luc* (Fig. 4), we assume

that observed influence of VPA on MDR1 mRNA expression in LS174T cells transfected

with PXR should be rather attributed to HDAC inhibitory activity of VPA, which was

previously demonstrated to cause MDR1 gene up-regulation (Eyal et al., 2006). Thus, we

hypothesize that the total induction of MDR1 mRNA expression in LS174T cells transfected

with CAR and PXR might be a sum of several VPA effects such as inhibition of HDAC (Eyal et al., 2006) and interaction of VPA with CAR or PXR. On the other hand, we suppose that the effect of VPA on CYP3A4 mRNA up-regulation is mediated by a transcription factor(s), as HDAC inhibition did not result in induction of human CYP3A4 or its rat homologue Cyp3a2 mRNA (Rodrígez-Antona et al., 2003; Eyal et al., 2006). Nevertheless, we hypothesize that HDAC inhibitory activity of VPA synergistically augments the effect of rifampicin in PXR-mediated up-regulation of CYP3A4 mRNA in primary human hepatocytes (Fig. 7C).

The interaction of VPA with CAR was investigated in more details using EMSA, in which interaction of VPA with CAR/RXRα heterodimer or CAR monomer (Frank et al., 2003) and responsive elements of both CYP3A4 and MDR1 promoters were examined. In the presence of VPA, we detected augmented binding of CAR/RXRα heterodimer to DR3 responsive element of CYP3A4 and to DR4 responsive element of MDR1 (Fig. 7A, B, C, D). Binding of CAR/RXRα to ER6 element of CYP3A4 was not affected by VPA (data not shown), which might correlate with low affinity of CAR/RXRα complex to bind ER6 motive and with minor role of ER6 in CAR-mediated CYP3A4 gene transactivation (Goodwin et al., 2002). Interestingly, we also observed increased binding of CAR monomer to DR3 motive of CYP3A4 promoter in the presence of VPA (Fig. 7 C, lane 2). Androstenol decreased the binding of CAR/RXRα heterodimer and CAR monomer to DR3 and CAR/RXRα heterodimer to DR4 in samples containing VPA (Fig. 7A, C, D), which provides us additional evidence that VPA transactivates CYP3A4 and MDR1 through CAR. Interestingly, we observed difference in the VPA-activated CAR/RXRα heterodimer binding to DR3 responsive element in EMSA experiments performed employing recombinant CAR and RXRα proteins (Fig. 7A, lane 8) in comparison with the experiments, where recombinant RXRα was replaced with nuclear extract from HepG2 cells transfected with expression vector encoding RXRα (Fig.

7B, lane 7). Binding of CAR/RXRα complex with recombinant *in vitro* translated RXRα to DR3 motive was only slightly augmented in the presence of VPA (~ 30%); however, using nuclear fraction of HepG2 cells transfected with RXRα, we observed more than 4-fold effect of VPA on the CAR/RXRα/DR3 complex formation (Fig. 7B, lane 7). Based on this finding, we hypothesize that nuclear fraction of HepG2 cells could contain another transcription cofactor involved in regulation of CAR/RXRα binding to DR3 in the presence of VPA.

Finally, we analyzed CYP3A4 enzymatic activity in LS174T cells exposed to VPA (600 µM) for 48 h. As shown in Fig. 6, significant increase of CYP3A4 activity was detected only in VPA-treated LS174T cells transfected with PXR expression vector ($p<0.05$). We did not detect any alteration of CYP3A4 enzymatic activity in non-transfected LS174T cells, although LS174T cells express PXR (unpublished data, Burk et al., 2005b). With respect to this fact, we suppose that CYP3A4 catalytic activity can be increased only in cells rich in PXR protein.

In conclusion, we show that VPA mediates transactivation of CYP3A4 and MDR1 genes via direct interaction with CAR nuclear receptor. Furthermore, we present that VPA up-regulates CYP3A4 and its catalytic activity through PXR. With respect to the published data on the effect of VPA in regulation of MDR1 gene expression we suggest that interaction of VPA with CAR signaling pathway together with VPA-mediated HDAC inhibition are the mechanisms involved in up-regulation MDR1 gene. As MDR1 plays an important role in multidrug resistance phenomenon (MDR), we assume that administration of VPA could lead to increase in tumor resistance against many anticancer drugs, which are transported by P-glycoprotein. Finally, our data indicate that VPA in clinically relevant concentrations during treatment of schizoaffective disorders, acute mania and refractory epilepsy, when VPA plasma concentrations range within 500-1000 µM, could cause drug-drug interactions with co-administered drugs, which are metabolized by CYP3A4.

References

Allen MH, Hirschfeld RM, Wozniak PJ, Baker JD and Bowden CL (2006) Linear relationship of valproate serum concentration to response and optimal serum levels for acute mania. *Am J Psychiatry* **163:**272-275.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P and Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci U S A* **95:**12208-12213.

Blaheta RA, Michaelis M, Driever PH and Cinatl J, Jr. (2005) Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. *Med Res Rev* **25:**383-397.

Blaheta RA, Nau H, Michaelis M and Cinatl J, Jr. (2002) Valproate and valproate-analogues: potent tools to fight against cancer. *Curr Med Chem* **9:**1417-1433.

Bosetti F, Bell JM and Manckam P (2004) Microarray analysis of rat brain gene expression after chronic administration of sodium valproate. *Brain Ress Bull* **65:**331-338.

Burk O, Arnold KA, Geick A, Tegude H and Eichelbaum M (2005a) A role for constitutive androstane receptor in the regulation of human intestinal MDR1 expression. *Biol Chem* **386:**503-513.

Burk O, Arnold KA, Nussler AK, Schaeffeler E, Efimova E, Avery BA, Avery MA, Fromm MF and Eichelbaum M (2005b) Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. *Mol Pharmacol* **67:**1954-1965.

Centorrino F, Kelleher JP, Berry JM, Salvatore P, Eakin M, Fogarty KV, Fellman V and Baldessarini RJ (2003) Pilot comparison of extended-release and standard preparations

of divalproex sodium in patients with bipolar and schizoaffective disorders. *Am J Psychiatry* **160:**1348-1350.

Davis R, Peters DH and McTavish D (1994) Valproic acid. A reappraisal of its pharmacological properties and clinical efficacy in epilepsy. *Drugs* **47:**332-372.

Eyal S, Lamb JG, Smith-Yockman M, Yagen B, Fibach E, Altschuler Y, White HS and Bialer M (2006) The antiepileptic and anticancer agent, valproic acid, induces P-glycoprotein in human tumour cell lines and in rat liver. *Br J Pharmacol.*

Faucette SR, Zhang TC, Moore R, Sueyoshi T, Omiecinski CJ, LeCluyse EL, Negishi M and Wang H (2006) Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *J Pharmacol Exp Ther*.

Fisher JE, Nau H and Loscher W (1991) Alterations in the renal excretion of valproate and its metabolites after chronic treatment. *Epilepsia* **32:**146-150.

Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM and Moore DD (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* **395:**612-615.

Frank C, Gonzalez MM, Oinonen C, Dunlop TW and Carlberg C (2003) Characterization of DNA complexes formed by the nuclear receptor constitutive androstane receptor. *J Biol Chem* **278:**43299-43310.

Frank C, Molnar F, Matilainen M, Lempiainen H and Carlberg C (2004) Agonist-dependent and agonist-independent transactivations of the human constitutive androstane receptor are modulated by specific amino acid pairs. *J Biol Chem* **279:**33558-33566.

Geick A, Eichelbaum M and Burk O (2001) Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* **276:**14581-14587.

Goodwin B, Hodgson E, D'Costa DJ, Robertson GR and Liddle C (2002) Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. *Mol Pharmacol* **62:**359-365.

Goodwin B, Hodgson E and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* **56:**1329-1339.

Goodwin B and Moore JT (2004) CAR: detailing new models. *Trends Pharmacol Sci* **25:**437-441.

Goodwin B, Moore LB, Stoltz CA, McKee DD and Kliewer SA (2001) Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol* **60:**427-431.

Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG and Heinzel T (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *Embo J* **20:**6969-6978.

Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T and Waxman DJ (1986) Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J Biol Chem* **261:**5051-5060.

Honkakoski P, Zelko I, Sueyoshi T and Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* **18:**5652-5658.

Chen G, Huang LD, Jiang YM and Manji HK (1999) The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. *J Neurochem* **72:**1327-1330.

Isom HC, Secott T, Georgoff I, Woodworth C and Mummaw J (1985) Maintenance of differentiated rat hepatocytes in primary culture. *Proc Natl Acad Sci U S A* **82:**3252-3256.

Jin S and Scotto KV (1998) Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y. *Mol Cell Biol* **18:**4377-4384.

Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA and Moore JT (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* **14:**27-39.

Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* **19:**6318-6322.

Kramer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters MA, Brill B, Groner B, Bach I, Heinzel T and Gottlicher M (2003) The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. *Embo J* **22:**3411-3420.

Maglich JM, Parks DJ, Moore LB, Collins JL, Goodwin B, Billin AN, Stoltz CA, Kliewer SA, Lambert MH, Willson TM and Moore JT (2003) Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes. *J Biol Chem* **278:**17277-17283.

Masuyama H, Suwaki N, Tateishi Y, Nakatsukasa H, Segawa T and Hiramatsu Y (2005) The pregnane X receptor regulates gene expression in a ligand- and promoter-selective fashion. *Mol Endocrinol* **19:**1170-1180.

Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL and Kliewer SA (2000a) St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc Natl Acad Sci U S A* **97:**7500-7502.

Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL and Kliewer SA (2000b) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* **275:**15122-15127.

Morrow CS and Nakagawa M (1994) Reversible transcriptional activation of mdr1 by sodium butyrate treatment of human colon cancer cells. *J Biol Chem* **269:**10739-10746.

Pascussi JM, Drocourt L, Fabre JM, Maurel P and Vilarem MJ (2000) Dexamethasone induces pregnane X receptor and retinoid X receptor-alpha expression in human hepatocytes: synergistic increase of CYP3A4 induction by pregnane X receptor activators. *Mol Pharmacol* **58:**361-372.

Perucca E (2006) Clinically relevant drug interactions with antiepileptic drugs. *Br J Clin Pharmacol* **61:**246-255.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29:**e45.

Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA and Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem* **276:**36734-36741.

Pichard-Garcia L, Gerbal-Chaloin S, Ferrini JB, Fabre JM and Maurel P (2002) Use of long-term cultures of human hepatocytes to study cytochrome P450 gene expression. *Methods Enzymol* **357:**311-321.

Raffoux E, Chaibi P, Dombret H and Degos L (2005) Valproic acid and all-trans retinoic acid for the treatment of elderly patients with acute myeloid leukemia. *Haematologica* **90:**986-988.

Rodrígez-Antona C, Bort R, Jover R, Tindberg N, Ingelman-Sundberg M, Gómez-Lechón M and Castell JV (2003) Transcriptional regulation of human CYP3A4 basal expression by CCAAT enhancer-binding protein .... *Mol Pharmacol* **63:**1180-1189.

Rogiers V, Akrawi M, Vercruysse A, Phillips IR and Shephard EA (1995) Effects of the anticonvulsant, valproate, on the expression of components of the cytochrome-P-450-mediated monooxygenase system and glutathione S-transferases. *Eur J Biochem* **231:**337-343.

Rogiers V, Callaerts A, Vercruysse A, Akrawi M, Shephard E and Phillips I (1992) Effects of valproate on xenobiotic biotransformation in rat liver. In vivo and in vitro experiments. *Pharm Weekbl Sci* **14:**127-131.

Scotto KV (2003) Transcriptional regulation of ABC drug transporters. *Oncogene* **22:**7496-7511.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* **98:**3369-3374.

Tanaka E (1999) Clinically significant pharmacokinetic drug interactions between antiepileptic drugs. *J Clin Pharm Ther* **24:**87-92.

Wen X, Wang JS, Kivisto KT, Neuvonen PJ and Backman JT (2001) In vitro evaluation of valproic acid as an inhibitor of human cytochrome P450 isoforms: preferential inhibition of cytochrome P450 2C9 (CYP2C9). *Br J Clin Pharmacol* **52:**547-553.

Werling U, Siehler S, Litfin M, Nau H and Gottlicher M (2001) Induction of differentiation in F9 cells and activation of peroxisome proliferator-activated receptor delta by valproic acid and its teratogenic derivatives. *Mol Pharmacol* **59:**1269-1276.

Xiao JJ and Huang Y (2005) Chemoresistance to depsipeptide FK228 [(E)-(1S,4S,10S,21R)-7-[(Z)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo[8,7,6]-tricos-16-ene-3,6,9,22-pentanone] is mediated by reversible MDR1 induction in human cancer cell lines. *J Pharmacol Exp Ther* **314:**467-475.

Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ and Evans RM (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci U S A* **98:**3375-3380.

Footnotes

Legends for figures

Fig. 1. Effect of VPA on CAR- and PXR-mediated Transactivation of CYP3A4 Promoter. HepG2 cells were transiently transfected with *p3A4-luc* reporter construct containing the basal promoter (–362/+53) with proximal PXR response element and the distal xenobiotic-responsive enhancer module (XREM) (–7836/–7208) of CYP3A4 (0.4 µg/well) and either *pCR3-CAR* or *pSG5-PXR* expression vector (50 ng) using Lipofectamine®2000 transfection reagent according to manufacture's instructions. Transfected HepG2 cells were maintained in medium containing VPA at indicated concentrations for 24 h. Luciferase activities are normalized to protein concentration and expressed as fold activation of non-treated cells transfected with *p3A4-luc*. All means ±S.D. were calculated from quadruplicates of a representative experiment and analyzed using ANOVA followed by Dunnett's test.

[#] $p<0.05$, [###] $p<0.001$ – statistically different from non-treated cells transfected *with p3A4-luc;*
* $p<0.05$, ** $p<0.01$ - statistically different from VPA non-treated cells co-transfected with either *pCR3-CAR* or *pSG5-PXR.*

Fig. 2. Transactivation of CYP3A4 Promoter by VPA in Combination with CITCO or Rifampicin. HepG2 cells were transiently transfected with *p3A4-luc* reporter construct (0.4 µg/well) and *pCR3-CAR* or *pSG5-PXR* expression plasmid (50 ng) using Lipofectamine®2000 transfection reagent according to manufacture's instructions and subsequently exposed to VPA in combination with CITCO (500 nM) or Rifampicin (Rif; 10 µM), prototype ligands of CAR and PXR, respectively, for 24 h. All means ±S.D. were calculated from quadruplicates of a representative experiment and analyzed using ANOVA followed by Dunnett's test. Data are presented as fold activation of non-treated cells transfected only with *p3A4-luc.*

* $p<0.05$, ** $p<0.001$ - statistically different from cells co-transfected with either *pCR3-CAR* or *pSG5-PXR* and treated with either CITCO or rifampicin (Rif).

Fig. 3. Effect of VPA on CAR- and PXR-mediated Activation of Reporter Constructs of MDR1 Gene. HepG2 cells were transiently transfected with *pCR3-CAR* or *pSG5-PXR* expression plasmid (50 ng) and appropriate reporter construct (0.4 µg/well) using Lipofectamine®2000 transfection reagent according to manufacture's instructions. Transfected HepG2 cells were maintained in medium containing tested compounds at indicated concentrations for 24 h. (**A.**) Effect of VPA on CAR- and PXR-mediated transactivation of *pMDR1-luc* construct containing the enhancer region of MDR1 gene (-7975/-7013) and the basal promoter from -1803 to +231. (**B.**) VPA-mediated transcriptional activation of *pMDR1E-SV40-luc* construct containing the enhancer region of MDR1 gene and viral promoter SV40 in cells co-expressing exogenous CAR or PXR. (**C.**) Influence of androstenol (Andol), an inverse agonist of CAR, on CAR-mediated transcriptional activation of *pMDR1E-SV40-luc* in HepG2 cells treated with VPA. HepG2 cells were transiently transfected with *pCR3-CAR* (40 ng) and *pMDR1E-SV40-luc* construct (0.4 µg/well) using Lipofectamine®2000. Control and VPA-treated cells were exposed to DMSO (0.1 %) to eliminate influence of the androstenol solvent. Data are presented as fold activation of non-treated cells transfected only with appropriate reporter construct *pMDR1E-luc* or *pMDR1E-SV40-luc*. All means ±S.D. were calculated from quadruplicates of a representative experiment.

$p<0.001$ – statistically different from non-treated cells transfected only with reporter construct; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ – statistically different from VPA non-treated cells co-transfected with either *pCR3-CAR* or *pSG5-PXR* expression vectors; ## $p<0.01$ - statistically different from cells transfected with reporter plasmid and exposed to VPA.

Fig. 4. Effect of VPA on CAR- and PXR-mediated Activation of PBREM Region of CYP2B6 Promoter. HepG2 cells were transiently transfected with *pCR3-CAR* or *pSG5-PXR* expression plasmid (50 ng) and *pPBREM-SV40-luc* reporter construct containing two DR4-type motives of CYP2B6 PBREM (0.4 µg/well) using Lipofectamine®2000 transfection reagent according to manufacture's instructions. Transfected HepG2 cells were maintained in medium containing VPA (500 µM) for 24 h.

$p<0.001$ – statistically different from non-treated cells transfected only with reporter construct; * $p<0.05$, *** $p<0.001$ – statistically different from VPA non-treated cells co-transfected with either *pCR3-CAR* or *pSG5-PXR* expression vectors.

Fig. 5. Analysis of VPA-mediated up-regulation of CYP3A4 and MDR1 mRNAs. LS174T cells were transfected with *pCR3-CAR* or *pSG5-PXR* expression plasmids (400ng/well) using Lipofectamine®2000 transfection reagent according to manufacture's instructions and exposed to VPA (500 µM) for 48 h. mRNA expressions of tested genes were determined employing real time RT-PCR and normalized to HPRT housekeeping gene. Effect of VPA (500 µM) on CYP3A4 (**A.**) and MDR1 (**B.**) mRNA expression is presented as a fold increase to control non-treated cells. Data are the means ±S.D. of three individual cell samples; * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

Fig. 6. Determination of CYP3A4 Catalytic Activity. LS174T cells were transfected with expression plasmids encoding CAR or PXR. Subsequently, cells were treated with VPA (600 µM) and specific CYP3A4-mediated testosterone 6β-hydroxylation activity was detected after 48 h. Concentration of the 6β-testosterone metabolite was determined using HPLC system in cell lysate. Final activity was normalized to sample protein concentration. Bars indicate the means ± S.D. calculated from three samples; * $p<0.05$.

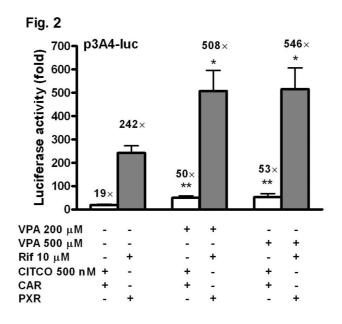**Fig. 7. VPA synergizes with rifampicin in induction of the CYP3A4 mRNA in primary human hepatocytes.** Primary human hepatocyte cultures were cultivated together with VPA and/or rifampicin (Rif) either individually or in combination, and then assayed using real time RT-PCR. CYP3A4 mRNA expression is normalized to HPRT housekeeping gene and presented as a fold increase to control non-treated cells. Values represent the means ± standard deviation (S.D.) of three independent wells. **(A.** and **B.)** 48-h exposure of primary hepatocytes to VPA (500 µM) up-regulates CYP3A4 mRNA (p <0.01). This effect of VPA was analyzed employing unpaired Student's t-test. **(C.)** Rifampicin, VPA or a combination of these two drugs significantly up-regulate CYP3A4 mRNA to control (0.1% DMSO-treated cells) with p values of * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ (unpaired Student's t-test) after 24 h of treatment. When given in combination, the induction is in both cases statistically synergistic rather than additive, at [##]$p<0.01$ (analyzed by two-way ANOVA with interaction).

Fig. 8. The ability of CAR/RXRα heterodimer to bind CAR response elements of CYP3A4 (DR3, ER6) and MDR1 (DR4) in the presence of VPA was investigated using EMSA as described in *Methods*. (**A.**) The complex formation of CYP3A4 DR3 with CAR/RXRα was examined with *in vitro* translated wild-type CAR and RXRα proteins in the presence of solvent (water or DMSO 0.1% where indicated), the CAR agonist CITCO (6.25 µM; lane 5 and 7), the inverse agonist of CAR androstenol (10 µM; lane 6,7 and 9), VPA (1000 µM; lane 3, 8 and 9) or CITCO-androstenol (Andol) and VPA-androstenol combinations (lane 7 and 9). (**B.**) Combined gel shift/supershift experiments were performed using *in vitro* translated wild-type CAR protein and the nuclear fraction of HepG2 cells transfected with RXRα expression vector. The concentration-dependent effect of VPA (100, 500, 1000 µM; lane 5-7) on CAR/RXRα complex was studied. The band supershift was

performed with anti-RXRα antibody (1 µg; lane 8). (**C.**) Effect of VPA on CAR monomer interaction with CYP3A4 DR3. The experiments were performed using *in vitro* translated CAR without RXRα and with VPA (1000 µM) and androstenol (10 µM) as an inverse agonist of CAR. (**D.**) The capability of VPA (1000 µM; lane 6) to affect CAR/RXRα heterodimer interaction with MDR1 DR4 response element was tested using *in vitro* translated wild-type CAR and RXRα proteins. CITCO (6.25 µM) and androstenol (10 µM) were used as a known ligand and inverse agonist of CAR, respectively. Representative gels are shown. Band signal intensity was quantitated by densitometry and data are expressed as -fold increase (fold) relative to their vehicle controls (DMSO or water).

Fig. 1

VPA 200 μM	-	-	-		+	+	+		-	-	-
VPA 500 μM	-	-	-		-	-	-		+	+	+
CAR	-	+	-		-	+	-		-	+	-
PXR	-	-	+		-	-	+		-	-	+

Fig. 2

Fig. 3

A.

B.

C.

Fig. 4

VPA 500 μM	-	-	-	+	+	+
CAR	-	+	-	-	+	-
PXR	-	-	+	-	-	+

Fig. 5

A.

CYP3A4 mRNA (fold)

VPA 500 μM	-	+	-	+	-	+
CAR	-	-	+	+	-	-
PXR	-	-	-	-	+	+

B.

MDR1 mRNA (fold)

VPA 500 μM	-	+	-	+	-	+
CAR	-	-	+	+	-	-
PXR	-	-	-	-	+	+

Fig. 6

Fig. 7

Fig. 8

A. CYP3A4 DR3

lane	1	2	3	4	5	6	7	8	9
H₂O	+	+	+	-	-	-	-	-	-
DMSO 0.1%	-	-	-	+	+	+	+	+	+
CITCO 6.25 μM	-	-	-	-	+	-	+	-	+
Andol 10 μM	-	-	-	-	-	+	+	-	+
VPA 1000 μM	-	-	+	-	-	-	-	+	+
CAR	-	+	+	+	+	+	+	+	+
fold		1.0	1.1	1.1	1.2	1.0	0.8	1.3	1.1

CAR/RXRα complex ▬

free probe ▬

B. CYP3A4 DR3

lane	1	2	3	4	5	6	7	8
H₂O	+	-	-	-	+	-	+	+
DMSO 0.1%	-	+	+	+	-	-	-	-
CITCO 6.25 μM	-	-	+	+	-	-	-	-
Andol 10 μM	-	-	-	+	-	-	-	-
VPA 100 μM	-	-	-	-	+	-	-	-
VPA 500 μM	-	-	-	-	-	+	-	-
VPA 1000 μM	-	-	-	-	-	-	+	-
IgG RXRα 1μg	-	-	-	-	-	-	-	+
fold	1.0	1.1	1.3	1.2	1.0	.8	4.2	

band shift ▬

CAR/ RXRα - NF complex ▬

C. CYP3A4 DR3

lane	1	2	3	4
DMSO 0.1%	+	+	+	+
Andol 10 μM	-	-	+	+
VPA 1000 μM	-	+	+	-
fold	1.0	1.4	0.9	0.3

CAR monomer ▬

D. MDR1 DR4

lane	1	2	3	4	5	6	7
DMSO 0.1%	-	+	+	+	+	+	+
CITCO 6.25 μM	-	-	+	-	+	-	-
Andol 10 μM	-	-	-	+	+	-	-
VPA 1000 μM	-	-	-	-	-	+	+
CAR	-	+	+	+	+	+	+
fold		1.0	0.9	0.6	0.6	1.5	1.0

CAR/RXRα complex ▬