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Graphical Abstract

Summary

Hepatic drug transporters are now recognized as major actors of hepatobiliary elimination of drugs. Characterization of their regulatory pathways is therefore an important issue. In this context, the present study was designed to analyze the potential regulation of human hepatic transporter expression by protein kinase C (PKC) activation. Treatment by the reference PKC activator phorbol 12-myristate 13-acetate (PMA) for 48 h was shown to decrease mRNA expression of various sinusoidal transporters, including OATP1B1, OATP2B1, NTCP, OCT1 and MRP3, but to increase that of OATP1B3, whereas mRNA expression of canalicular transporters was transiently enhanced (MDR1), decreased (BSEP and MRP2) or unchanged (BCRP) in human hepatoma HepaRG cells. The profile of hepatic transporter mRNA expression changes in PMA-treated HepaRG cells was correlated to that found in PMA-exposed primary human hepatocytes and was similarly observed in response to the PKC-activating marketed drug ingenol mebutate. It was associated with concomitant repression of OATP1B1 and OATP2B1 protein expression and reduction of OATP, OCT1, NTCP and MRP2 activity. The use of chemical PKC inhibitors further suggested a contribution of novel PKCs isoforms to PMA-mediated regulations of transporter mRNA expression. PMA was finally shown to cause epithelial-mesenchymal transition (EMT) in HepaRG cells and exposure to various additional EMT inducers, *i.e.*, hepatocyte growth factor, tumor growth factor-β1 or the HNF4α inhibitor BI6015, led to transporter expression alterations highly correlated to those triggered by PMA. Taken together, these data highlight PKC-dependent regulation of human hepatic drug transporter expression, which may be closely linked to EMT triggered by PKC activation.

1. Introduction

Drug transporters, belonging to the solute carrier (SLC) or to the ATP-binding cassette (ABC) transporter families, constitute key-actors of the various steps of drug hepatic elimination [1]. Some of them, located at the sinusoidal pole of hepatocytes, are thus implicated in uptake of drugs into hepatocytes from blood, *i.e.*, the so-called phase 0 of the hepatic drug detoxification system. Others, expressed at the canalicular pole of hepatocytes, are involved in secretion of drugs or drug metabolites into the bile, *i.e.*, the so-called phase 3a of liver detoxification [2]. Additionally, some sinusoidal transporters can mediate back transport of drug metabolites into the blood for a secondary renal elimination [3], *i.e.*, the so-called phase 3b of the hepatic drug processing.

Due to their major role in elimination of drugs, hepatic transporters have been linked to various drug-drug interactions [4] and liver side-effects of drugs [5]. This has recently led drug regulatory agencies to edict recommendations for the characterization of putative interactions of new molecular entities with some of the major hepatic drug transporters [6]. In this context, accurate characterization of molecular and cellular pathways controlling expression and activity level of hepatic drug transporters is likely an important issue. As for other drug detoxifying proteins, various factors, including physiological factors like inflammatory cytokines, hormones, growth factors or endogenous metabolites as well as xenobiotics like drugs and environmental pollutants, have been shown to impair expression and/or activity of drug transporters [7-11]. As for modulation of drug metabolizing enzyme expression, drug-sensing receptors such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are involved in drug transporter regulations [12]. In addition, various non-genomic signaling ways are likely to contribute to transporter regulation [13], notably through modulating the location of transporter to plasma membrane or affecting transporter activity. In this context, the serine/threonine protein kinases C (PKCs) appear to

constitute key actors regulating transporter localization and activity in hepatocytes. Indeed, treatment by the PKC-activating phorbol ester 12-myristate 13-acetate (PMA) leads to retrieval of the sodium-taurocholate cotransporting polypeptide (NTCP/*SLC10A1*) and multidrug resistance-associated protein (MRP) 2 (*ABCC2*) from hepatocyte sinusoidal and canalicular membranes, respectively [14, 15]. It also decreases activity of the sinusoidal organic anion transporting polypeptide (OATP) 1B3 (*SLCO1B3*) through post-translational regulation [16] and induces cholestasis in rat liver, through notably retrieval of the bile salt export pump (BSEP/*ABCB11*) from the canalicular membrane [17].

Because the activation of the PKC-α isoform has been associated with increased expression of the canalicular P-glycoprotein, encoded by multidrug resistance gene (MDR) 1 (*ABCB1*) in rat liver [18], PKCs may regulate, not only transporter localization and activity, but also transporter expression levels in the liver. The exact nature of hepatic drug transporters whose expression level may be changed by PKC activation remains however largely unknown. The present study was therefore designed to gain insights about these points, using primary human hepatocytes and highly-differentiated human hepatoma HepaRG cells, that are fully suitable for investigating hepatic detoxification pathway regulations, including those related to transporters [19, 20]. Our data demonstrate that activation of PKCs by PMA markedly impairs functional expression of various human hepatic drug transporters, which may reflect epithelial-mesenchymal transition (EMT) triggered by the phorbol ester.

2. Materials and methods

2.1. Chemicals and reagents

PMA, verapamil, probenecid, ingenol mebutate, 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) and all-trans retinoic acid (atRA) were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France) whereas carboxy-2,7-dichlorofluorescein

(CF) diacetate was purchased from Invitrogen/Life Technologies (Villebon sur Yvette, France). The PKC inhibitors GF 109203X (also known as bisindolylmaleimide I or Gö 6850), Gö 6983 and Gö 6976 were provided by Calbiochem (La Jolla, CA). Recombinant human hepatocyte growth factor (HGF) and transforming growth factor (TGF) β1 and BI6015 were from R&D Systems (Minneapolis, MN). [^{3}H(G)] taurocholic acid (sp. act. 1.19 Ci/mmol), [6,7-^{3}H(N)] estrone-3-sulfate (E3S) (sp. act. 57.3 Ci/mmol) and [1-^{14}C] tetra-ethylammonium (TEA) (sp. act. 2.4 mCi/mmol) were purchased by Perkin-Elmer (Courtaboeuf, France). Monoclonal antibodies against P-glycoprotein (clone C219), MRP2 (clone M$_2$III-6), MRP3 (*ABCC*3) (clone M$_3$II-9) and breast cancer resistance protein (BCRP/*ABCG2*) (clone BXP-21) were from Alexis Biochemicals (Lausen, Switzerland), whereas monoclonal antibody against phospho-myristoylated alanine-rich C kinase substrate (MARCKS) (Ser159-163) was provided by Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies raised against OATP1B1 (*SLCO1B1*) or OATP2B1 (*SLCO2B1*) have been previously described [21]. All other compounds and reagents were commercial products of the highest purity available.

2.2. Cell culture

Human hepatoma HepaRG cells were cultured as previously described [20]. Briefly, cells were grown in Williams' E medium supplemented with 10% (vol/vol) fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 2 mM glutamine, and 5.10^{-5} M hydrocortisone hemisuccinate for two weeks. Cells were next cultured for additional two weeks in the same medium supplemented with 2% (vol/vol) dimethylsulfoxide (DMSO) in order to get a full differentiation of the cells [22].

Primary human hepatocytes were obtained from adult donors undergoing hepatic resection for secondary tumors, via the Biological Resource Center (University Hospital, Rennes, France), which has obtained the authorization N°DC-2008-630 from the French

Ministry of Health to collect hepatic resections from the digestive surgery department and then to isolate and deliver the hepatocytes used in this study. Hepatocytes, initially prepared by enzymatic dissociation of histologically-normal liver fragments [23], were seeded on plastic dishes at a density of 2.10^5 cells/cm^2 in Williams' E medium (Invitrogen, Cergy-Pontoise, France), supplemented with 10% (vol/vol) fetal calf serum (Perbio Sciences, Brébieres, France), 5 µg/mL bovine insulin (Sigma-Aldrich), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine (Invitrogen). After 24 h, this seeding medium was discarded, and primary hepatocytes were routinely cultured in the fetal calf serum-containing Williams' E medium defined above and supplemented with 5.10^{-5} M hydrocortisone hemisuccinate (Upjohn, Paris La Défense, France) and 2% (vol/vol) DMSO, as reported previously [24]. All experimental procedures complied with French laws and regulations and were approved by the National Ethics Committee.

2.3. Viability assay

Cellular viability was assessed using a colorimetric MTT assay as previously described [25].

2.4. Light microscopy analysis

Light microscopy analysis of monolayer cultures of human hepatocytes was performed using an Axiovert microscope (Carl Zeiss, Le Pecq, France), as previously described [20].

2.5. RNA isolation and analysis

Total RNA was isolated from cells using the TRIzol reagent (Invitrogen/Life Technologies). RNA (20 ng) was then subjected to reverse transcription-quantitative polymerase chain reaction (RT-qPCR), using the fluorescent dye SYBR Green methodology

and an ABI Prism 7300 detector (Applied Biosystem, Foster City, CA, USA) [26].The gene primers used for the analysis of transporters, cytochrome P-450 (CYP) 7A1 (also known as cholesterol 7α hydroxylase), PKC isoforms and 18S RNA were exactly as previously described [27, 28]; other primers were plasminogen activator inhibitor-1 (PAI-1) sense, CAGACCAAGAGCCTCTCCAC, PAI-1 antisense, GACTGTTCCTGTGGGGTTGT, E-cadherin sense, TGCTCTTGCTGTTTCTTCGG, E-cadherin antisense, TGCCCCATTCGTTCAAGTAG, vimentin sense, CCAAACTTTTCCCTGAACC, vimentin antisense, GTGATGCTGAGAAGTTTCGTTGA, Snail (also known as Snail1) sense, ACCACTATGCCGCGCTCTT, Snail antisense, GGTCGTAGGGCTGCTGGAA, Slug (also known as Snail2) sense, TGTTGCAGTGAGGGCAAGAA, Slug antisense, GACCCTGGTTGCTTCAAGGA, zinc finger E-box binding homeobox (ZEB) 1 sense, ATCCTGGGGCCTGAAAGCTCAGG, ZEB1 antisense, TGGTGTGCCCTGCCTCTGGT, Twist1 sense, CCAAACTTTTCCTCCCTGAA, and Twist1 antisense, GTGATGCTGAGAAGTTTCGTTGA. Amplification curves of the PCR products were analyzed with the ABI Prism SDS software using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was calculated after normalization to the 18S RNA level, used here as an internal control. Data we routinely expressed as fold factor change comparatively to untreated cells or, alternatively, in arbitrary units relatively to 18S RNA as previously reported [24].

2.6. Western-blot analysis

Protein extracts were prepared from HepaRG cells as previously described [25]. Proteins were then separated on polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. After blocking in Tris-buffered saline containing 4% bovine serum albumin, membranes were incubated overnight at 4°C with primary antibodies directed

against target proteins. Peroxidase-conjugated monoclonal antibodies were thereafter used as secondary antibodies. After washing, immunolabeled proteins were finally visualized by chemiluminescence. Gel loading and transfer were checked by staining membranes with Ponceau red. Intensities of antibody-stained bands and Ponceau red-stained lanes were measured by densitometry using ImageJ 1.40g software (National Institute of Health, Besthesda, MA), allowing to normalize antibody-related staining to Ponceau red-labeling [29].

2.7. Transport assays

Activities of the sinusoidal transporters NTCP, OATPs and organic cation transporter (OCT) 1 (*SLC22A1*) were determined through measuring sodium-dependent-intracellular accumulation of the NTCP substrate taurocholate, probenecid-sensitive uptake of the OATP substrate estrone-3-sulfate, and verapamil-sensitive uptake of the OCT1 substrate TEA, as previously described [20]. In brief, HepaRG cells were incubated with radiolabeled substrates, *i.e.,* for 5 min at 37 °C, in a well-defined transport assay buffer consisting of 5.3 mm KCl, 1.1 mm KH_2PO4, 0.8 mm $MgSO_4$, 1.8 mm $CaCl_2$, 11 mm d-glucose, 10 mm HEPES, and 136 mm*N*-methyl glutamine (sodium-free buffer) or 136 mm NaCl (sodium-containing buffer) and adjusted to pH 7.4. After washing in phosphate-buffered saline, cells were lysed, and accumulation of radiolabeled substrates was determined through scintillation counting. Taurocholate accumulation in the presence of sodium minus accumulation in the absence of sodium, E3S uptake in the absence of probenecid minus uptake in the presence of probenecid, and TEA uptake in the absence of verapamil minus uptake in the presence of verapamil are thought to correspond to NTCP, OATP, and OCT1 activities, respectively [23].

MRP2 activity was determined through measuring the intracellular secretion of the MRP2 substrate CF into bile canaliculi-like structures as previously reported [20]. Briefly,

HepaRG cells were first incubated for 10 min at 37 °C with transport assay buffer containing Ca^{2+} or with the same buffer, except that 1.8 mM $CaCl_2$ was withdrawn and 100 μM EGTA was added, knowing that incubation with this Ca^{2+}-free buffer promotes disruption of tight junctions and opening of bile canaliculi networks. Buffers were then removed and HepaRG cells were further incubated for 10 min at 37 °C in transport assay medium containing 3 μM CF diacetate. After washing with ice-cold phosphate-buffered saline, accumulations of CF into cells + bile canaliculi (Ca^{2+}-containing conditions) and into cells only (Ca^{2+}-free conditions) was determined by spectrofluorimetry using a SpectraMax Gemini SX spectrofluorometer (Molecular Devices, Sunnyvale, CA) (excitation and emission wavelengths were 485 and 535 nm, respectively). Biliary excretion index (BEI) was finally calculated using the following equation:

$$BEI = 100\,x\,\frac{\left[CF\,Accumulation\left(Cells + Bile\,canaliculi\right) - CF\,Accumulation\left(Cells\right)\right]}{CF\,Accumulation\left(Cells + Bile\,canaliculi\right)}.$$

.8. Statistical analysis

Quantitative data were usually expressed as means ± S.E.M.   Data were statistically analyzed using a paired Student's *t* test, analysis of variance (ANOVA) followed by the Dunnett's post-hoc test or the nonparametric Spearman's rank correlation method. The criterion of significance was $p < 0.05$.

3. Results

3.1. Responsiveness of human hepatic HepaRG cells to the PKC-activating agent PMA

PMA, used at a 100 nM concentration previously shown to activate PKCs in various studies [15, 16, 30], was first demonstrated to not alter cellular viability of HepaRG cells up to 48 h of exposure, as assessed by MTT viability assay (Fig. 1A). PMA treatment was next found to induce phosphorylation of MARCKS, a well-established substrate of PKCs [31], in

HepaRG cells (Fig. 1B). PMA was also shown to transiently induce mRNA expression of PAI-1 in HepaRG cells, in agreement with a previous study performed in human hepatoma HepG2 cells [30], and to markedly repress mRNA expression of CYP7A1/cholesterol 7α hydroxylase, a well-known PKC target [32] (Fig. 1C). Taken together, these data indicate that HepaRG cells were fully responsive to the PKC activating agent PMA and were thus suitable for analyzing the putative effects of PMA-mediated PKC activation on human hepatic drug transporter expression.

To more completely characterize the relevance of HepaRG cells with respect to PKC-related studies, we next compared the profile of PKC isoform mRNA expression in HepaRG cells and in primary human hepatocytes. The conventional PKC-α, unlike the other conventional isoforms PKC-β1, PKC-β2 and PKC-γ, and the atypical PKC-ζ and PKC-ι were highly expressed in both HepaRG cells and human hepatocytes (Fig. 1D); mRNA levels of the novel isoforms PKC-δ, PKC-ϵ and PKC-η were also similar in HepaRG cells and hepatocytes, whereas mRNA expression of the novel PKC-θ was barely detectable in the two kinds of hepatic cells (Fig. 1D). PKCs were further ranked according to their mRNA expression level, from the less expressed isoform to the most expressed; Spearman rank correlation method next indicated that profiles of PKC mRNA levels were highly correlated between HepaRG cells and primary human hepatocytes (Fig. 1E).

3.2. Regulation of main human hepatic transporter mRNA levels by PMA

We first analyzed the effects of various exposure times (2 h, 6 h, 24 h and 48 h) to 100 nM PMA on drug transporter mRNA levels in HepaRG cells. As shown in Fig. 2A, PMA decreased expression of the SLC transporters OATP1B1, OATP2B1, NTCP and OCT1, but increased that of OATP1B3. Such effects were time-dependent, with a maximal effect towards transporter mRNA levels occurring after a 24-48 h exposure to the phorbol ester,

excepted for OCT1, for which the decrease was maximal only after a 48 h exposure; a shorter exposure (2 h) failed to alter NTCP, OCT1 and OATP1B3 mRNA levels and only weakly repressed those of OATP1B1 and OATP2B1, whereas an intermediate exposure (6 h) resulted in a moderate and significant repression of OATP1B1, OATP2B1, OCT1 and NTCP mRNAs, without however affecting those of OATP1B3 (Fig. 2A). With respect to ABC transporters, PMA treatment transiently increased MDR1/P-glycoprotein mRNA levels, *i.e.*, MDR1 mRNAs were induced after a 2 h and a 6 h exposure to PMA and thereafter returned to control levels (Fig. 2B); by contrast, it repressed MRP2 and MRP3 mRNA expression, but only after a 48 h exposure, whereas BCRP mRNA expression remained unaffected, whatever the exposure time to PMA. PMA treatment for 24 h or 48 h also markedly reduced BSEP mRNA levels (Fig. 2B).

To determine whether drug transporters regulated by PMA in HepaRG cells may also be targeted by the phorbol ester in human hepatocytes, primary human hepatocytes were exposed to 100 nM for 24 h before analysis of transporter mRNA expression. Such a PMA treatment did not obviously impair cell viability as demonstrated by light microscopic examination of the human hepatocyte cultures (data not shown). As shown in Fig. 3A, PMA-treated hepatocytes exhibited reduced mRNA expression of OATP1B1, OATP1B3, OATP2B1, NTCP, OCT1 and BSEP, whereas those of MDR1 and MRP3 were induced and those of BCRP and MRP2 remained unchanged. When compared to transporter mRNA regulations occurring in HepaRG cells exposed to PMA for 24 h, some discrepancies were observed for OATP1B3 mRNA levels (increased in HepaRG cells and repressed in human hepatocytes), MRP3 mRNA levels (unchanged in HepaRG cells and induced in human hepatocytes) and MDR1 mRNA levels (unchanged in HepaRG cells and induced in human hepatocytes). However, when transporters were ranked according to their PMA-mediated regulation level, from the most repressed to the most induced, Spearman rank correlation

method indicated that transporter mRNA regulation profiles in response to PMA were significantly correlated between HepaRG cells and primary human hepatocytes (Fig. 3B).

To investigate whether PKC activating agents distinct from PMA may also regulate transporter mRNA expression in HepaRG cells, we finally exposed these highly-differentiated human hepatoma cells to 10 μM ingenol mebutate (also known as PEP005), a small molecule activator of PKCs, used for the topical treatment of actinic keratosis [33] and also exhibiting anticancerous activity [34]. As shown in Fig. 4A, ingenol mebutate repressed mRNA expression of OATP1B1, OATP2B1, NTCP and BSEP, whereas it induced those of OATP1B3 and MRP2. OCT1, MDR1, BCRP and MRP3 mRNA levels remained statistically unchanged in response to ingenol mebutate (Fig. 4A). Analysis of regulation profiles of transporters, ranked from the most repressed to the most induced, indicated that the effects of ingenol mebutate were highly correlated to those of PMA (Fig. 4B).

3.3. Regulation of human hepatic transporter protein expression and activity by PMA

HepaRG cells exposed to PMA for 48 h exhibited significant reduced expression of 75 kDa OATP1B1 and 82 kDa OATP2B1 proteins (Fig. 5A). By contrast, 72 kDa BCRP and 170 kDa P-glycoprotein levels remained unchanged; protein expressions of 190 kDa MRP2 and 169 kDa MRP3 were similarly not obviously altered by exposure to PMA (Fig. 5A).

Treatment by PMA of HepaRG cells for 48 h reduced NTCP activity, *i.e.*, sodium-dependent taurocholate accumulation, as well as OATP activity, *i.e.*, probenecid-inhibitable uptake of E3S, and OCT1 activity, *i.e.*, verapamil-inhibitable accumulation of TEA (Fig. 5B). Exposure to PMA also reduced MRP2-mediated biliary secretion, as assessed by the decrease of BEI for CF (Fig. 5B).

3.4. Involvement of PKCs in PMA-mediated drug transporter regulation

To verify that PMA-triggered regulation of transporters was related to PKC activation, we next analyzed the effects of various chemicals inhibitors of PKCs on PMA-mediated changes in transporter mRNA expression. As shown in Fig. 6, the pan-PKC inhibitors GF 109203X and Gö 6983, used at concentrations that fully block both conventional and novel PKC isoforms [35, 36], suppressed the repressing effects of PMA towards OATP1B1, OATP2B1, NTCP and OCT1 mRNAs in HepaRG cells, without altering basal mRNA expression of these transporters; they also prevented the induction of OATP1B3 mRNAs. This PMA-mediated-OATP1B3 up-regulation was similarly inhibited by Gö 6976, a conventional PKC inhibitor used at a fully active concentration [37] (Fig. 6); by contrast, Gö 6976 failed to suppress the down-regulation of OATP1B1, OATP2B1, NTCP and OCT1 mRNAs in PMA-exposed HepaRG cells (Fig. 6). Gö 6976, unlike Gö 6983 and GF109203X, did not also antagonize the repression of OATP1B1, OATP2B1, NTCP and OCT1 mRNA levels in PMA-treated primary human hepatocytes (data not shown).

3.5. Association of PMA-induced transporter expression changes with EMT

The fact that exposure to PMA led to alterations of mRNA expression of multiple hepatic transporters suggest that such changes may reflect a more global effect of PMA towards hepatic cells, rather than specific effects of PMA towards particular transporters. In this context, the known EMT-inducing effect of PMA [38] has likely to be considered, notably because PMA treatment was found to trigger some morphological changes in HepaRG cells, notably the loss of polygonal epithelial hepatocyte-like morphology and the concomitant appearance of spindle mesenchymal-like morphology (Fig. 7A), which constitute major features of the EMT process [39]. In addition, PMA exposure resulted in decreased mRNA expression of E-cadherin and enhanced mRNA expression of vimentin (Fig. 7B), that

also represent key hall-marks of EMT [40]. Reduced expression of E-cadherin protein concomitantly occurred in response to PMA, as demonstrated by Western-blotting (Fig. 7C). In parallel, PMA enhanced mRNA expression of several transcription factors characterizing EMT [41]: Snail and Slug were thus transiently and early induced after a 2 h exposure to the phorbol ester, whereas ZEB1 and especially Twist1 were later up-regulated (Fig. 7D). Interestingly, the pan-PKC inhibitors GF 109203X and Gö 6983, unlike the conventional PKC-restricted inhibitor Gö 6976, fully suppressed the PMA-triggered repression of E-cadherin and induction of vimentin (data not shown), thus demonstrating that PMA-induced EMT in HepaRG cells was PKC-dependent.

To further establish a link between the EMT process induced by PMA and the profile of transporter expression alteration in HepaRG cells, we investigated whether exposure to other PMA-unrelated inducers of EMT can also result in changes in transporter expression similar to that caused by the phorbol ester. For this purpose, HepaRG cells were treated by growth factors known to trigger EMT, *i.e.*, HGF [42] or TGF-β1 [43], or by the hepatocyte nuclear factor (HNF) 4α antagonist BI6015 [44], because HNF4α suppression results in EMT in hepatocytes [45]. As shown in Fig. 8A, exposure to 20 ng/mL HGF, 10 ng/mL TGF-β1 or 5 µM BI6015 decreased E-cadherin mRNA expression and induced vimentin mRNA expression in HepaRG cells, thus providing evidence that these factors effectively engaged the EMT program in these hepatic cells. HGF, TGF-β1 and BI6015 were next found to impair mRNA expression of various drug transporters (Fig. 8B); notably, OATP1B1, OATP2B1, NTCP and BSEP mRNA expressions were significantly repressed by each of the EMT inducer, whereas OATP1B3 mRNA expression was concomitantly induced (Fig. 8B). Analysis of regulation profiles for transporters, ranked from the most repressed to the most induced, indicated that the effects of HGF, TGF-β1 and BI6015 were statistically correlated to those of PMA (Fig. 8C), thus supporting the conclusion that exposure to each EMT inducer triggers similar

changes in transporter expression in HepaRG cells. In contrast to EMT inducers, atRA, known to also impair expression of drug transporters in hepatocytes in a major way [46], failed to reduce E-cadherin expression and to induce that of vimentin in HepaRG cells (Fig. 8A), which fully supports the fact that atRA does not favor EMT [47]. In addition, changes in profile of transporter mRNA expression induced by exposure to 5 µM atRA for 24 h, including down-regulation of OATP1B1 and OATP2B1 and up-regulation of NTCP and BCRP (Fig. 8B), did not correlate with those caused by PMA (Fig. 8C).

The level of correlation was particularly high between PMA and HGF effects towards transporter expression (ρ=0.96, p<0.0001) (Fig. 8C), suggesting that these two EMT inducers may share common transduction ways for regulating transporters. In this context, PKCs, that are the primary actors involved in PMA effects and that have previously been shown to control some HGF responses [48], may be hypothesized to be also implicated in HGF effects towards transporters in a major way. To investigate this hypothesis, we analyzed the effects of PKC inhibitors on HGF-mediated transporter expression changes. As shown in Fig. 9, GF 109203X, Gö 6983 and Gö 6976 failed to prevent HGF-mediated mRNA repression of OATP1B1, OATP2B1, NTCP and OCT1, thus discarding a role for PKCs in HGF effects towards transporters.

4. Discussion

Previous studies have demonstrated that PKC activation can rapidly modulate drug and bile acid transporter location and activity in hepatocytes, by notably promoting their retrieval from sinusoidal or canalicular membranes [49-51] or modulating their phosphorylation status [16]. The present study demonstrates that *in vitro* PKC activation caused by PMA can also result in marked changes in expression of main hepatic drug transporters. Taken together, these data fully establish PKCs as important regulators of

hepatic transporters and thereby add them to the growing list of signaling proteins and receptors controlling transporter expression, that have likely to deserve attention owing to the recognized role of transporters in pharmacokinetics and drug-drug interactions [1].

Hepatic transporters whose expression is targeted by the PKC activator PMA include major and relevant SLC transporters, such as OATP1B1, OATP2B1 and OCT1, involved in sinusoidal uptake of various marketed drugs such as statins and metformin [2]. These SLC transporters were down-regulated at the mRNA level in both HepaRG cells and primary human hepatocytes exposed to PMA; some of them, *i.e.*, OATP1B1 and OATP2B1, whose membrane location is already known to be rapidly modulated by PKCs [50, 52], were concomitantly decreased at the protein level and also targeted by the PKC activator agent ingenol mebutate. Importantly, OATP and OCT1 activity were reduced in parallel, thus indicating that PMA-mediated repression of transporter expression results into functional consequences. Expression of the bile acid transporters NTCP and BSEP mRNAs were also markedly repressed by PMA in both HepaRG cells and human hepatocytes, thus fully confirming that these bile acid transporters, whose location at the vascular or biliary pole of hepatocytes can otherwise be altered by PMA in a major way [14, 17], correspond to robust targets of PKC-related signaling ways. Interestingly, the apical sodium-dependent bile acid transporter (ASBT/*SLC10A2*), responsible for the reabsorption of the majority of bile acids from the intestinal lumen, and the CYP7A1/cholesterol 7α hydroxylase, that corresponds to the rate-limiting enzyme of the bile acid biosynthetic pathway, are also modulated by PKC activation [32, 53], thus underlining the importance of PKC-related signaling ways for the different steps of bile acid metabolism and transport [49].

Besides BSEP, other canalicular transporters were also regulated by PKC activation. MRP2, whose distribution at the biliary pole is known to be rapidly modulated by PKC activators [15], exhibited thus decreased mRNA expression and activity in HepaRG cells

exposed to PMA for 48 h. MRP3 mRNA expression was similarly reduced after a 48 h exposure to the PKC activator. MRP2 and MRP3 protein levels were however not affected in a major way by PMA. This could be linked to the relative low level of repression of MRP2 and MRP3 mRNA expression in HepaRG cells exposed to PMA for 48 h (by a 1.8- and a 1.4-fold-factor for MRP2 and MRP3, respectively, when compared to untreated cells) or could reflect divergent transcriptional and post-transcriptional effects of PMA towards MRP2 and MRP3 expression. Such differential regulations of mRNA and protein expressions have already been reported for the effects of inflammatory cytokines towards some hepatic transporters [8]. MDR1 mRNA expression was increased by PMA, in both HepaRG cells and human hepatocytes; this up-regulation was however transient in HepaRG cells, *i.e.*, MDR1 mRNAs returned to basal levels after a 24 h exposure to PMA, which may explain why P-glycoprotein content remained unaltered after a 48 h exposure to PMA in HepaRG cells, as demonstrated by Western-blotting.

With respect to the regulation of the sinusoidal ABC transporter MRP3 by PKC activation, a discrepancy was observed between HepaRG cells and human hepatocytes. Indeed, MRP3 mRNA expression was increased in response to a 24 h treatment by PMA in human hepatocytes, whereas it remained unchanged in HepaRG counterparts, and was even decreased in such cells exposed to PMA for 48 h. In the same way, mRNA expression of the sinusoidal SLC transporter OATP1B3 was differently regulated by PMA in HepaRG cells (induction) and human hepatocytes (repression). Such discrepancies between HepaRG cells and human hepatocytes, the reasons for which remain unclear, likely confirm that the former cells cannot be considered as strictly similar to the latter ones with respect to regulation of drug detoxifying pathways, as already demonstrated for transporter regulation by all-trans retinoic acid [46]. Global profiles of transporter regulation by PMA in HepaRG cells and human hepatocytes were however significantly correlated, as well as those of PKC isoform

expression. In the same way, various similarities in detoxifying pathway expressions and regulations between HepaRG cells and human hepatocytes have been reported [54]. The overall and now-well established interest of HepaRG cells as a convenient alternative to the use of human hepatocytes in pharmacological and/or toxicological areas [55] has therefore not to be challenged, even if some differences between these two *in vitro* models have likely to be kept in mind.

With respect to the nature of PKC isoforms implicated in PMA effects towards hepatic drug transporter expression, classical and novel PKC isoforms, that correspond to PKC isoforms classically activated by phorbol esters [56], have likely to be considered, whereas atypical isoforms, that lack the tandem repeat of cystein-rich motifs corresponding to the PKC binding domain of phorbol esters [57], have probably to be discarded. The pan-PKC inhibitors GF 109203X and Gö 6983, unlike the classical PKC inhibitor Gö 6976, were able to prevent the PMA-mediated repression of OATP1B1, OATP2B1, NTCP and OCT1 mRNA expression in both HepaRG cells and human hepatocytes, thus underlining the probable involvement of novel PKC isoforms in these regulations. By contrast, Gö 6976 blocked PMA-mediated induction of OATP1B3 in HepaRG cells, thus suggesting the implication of classical isoforms; in this context, PKCα isoform has likely to be highly suspected because other classical PKC isoforms were very poorly expressed in HepaRG cells (Fig. 1D). Unfortunately, our attempts to identify the exact nature of the PKC isoforms, notably that of novel PKC isoforms, responsible for PMA effects towards transporters were unsuccessful. This was mainly due to the fact that the PKC isoform-specific inhibitor rottlerin, that is thought to electively target PKC-δ [58], was found to exert toxicity towards HepaRG cells (data not shown), thus preventing its use for investigating the involvement of PKC-δ. In addition, siRNA-mediated down-regulations of several PKC isoforms in HepaRG cells, that allowed to reduce PKC isoform mRNA expression by at least approximately 75 % (data not shown),

failed to affect PMA-mediated regulation of transporters; this putatively reflects the fact that basal residual expression of PKCs may be sufficient for PMA response or, alternatively, that PMA response implicates various and redundant PKCs, as already described for some PMA effects [59], thus excluding to prevent it by knocking down a single PKC isoform. Further studies are therefore required to better characterize the nature of PKC isoforms involved in hepatic drug transporter expression regulation.

PMA effects towards drug transporter expression were found to be associated with PMA-mediated EMT, as demonstrated by morphological changes of HepaRG cells, reduced expression of E-cadherin and induction of vimentin and several EMT-related transcription factors. This link between PMA-mediated transporter regulation and *in vitro* EMT was further confirmed by the fact that several inducers of EMT, acting through different pathways, *i.e.,* the receptor tyrosine kinase c-Met for HGF, the SMAD pathway for TGF-β1 and the inhibition of HNF4α for BI6015, led to changes in transporter mRNA expression correlated to those due to PMA in HepaRG cells, but unrelated to PKCs, at least for HGF. By contrast, transporter expression changes due to atRA, which failed to trigger EMT, were not correlated to those caused by PMA. EMT in hepatic cells seems therefore to be associated with a specific profile of transporter mRNA expression alteration, whatever the nature of the EMT inducer. The molecular and cellular bases linking EMT and drug transporter expression remained to be identified, but it may be hypothesized that the de-differentiation program, that usually accompanies EMT [60], may be involved. The repression of specific liver transporters, like OATP1B1, NTCP and BSEP, by PMA and other EMT inducers is consistent with this hypothesis. Interestingly, EMT is a process that is thought to occur in some liver diseases, especially fibrosis and cirrhosis [61]. Alteration in *in vivo* hepatic transporter expression that occurs during fibrosis [62] may therefore be linked to EMT.

The *in vivo* relevance of our findings about PKC activation, EMT and drug transporter regulation remains to be determined. Indeed, if EMT induction by PKC activators such as PMA is well demonstrated *in vitro* for human hepatic cells [63], it is not the case *in vivo*, according to the best of our knowledge. Further studies are therefore required to investigate whether *in vivo* PKC activation in human liver may trigger EMT and alterations of hepatic drug transporter expression levels, which may add to known PKCs-mediated changes in membrane localization of hepatic transporters [15, 49]. The contributions of such hypothetical *in vivo* changes of hepatic transporter expression levels in terms of pharmacokinetics and cholestatic side-effects, that have been described in rat liver exposed to PMA [17], could be next characterized. In this context, it is noteworthy that the PKC activating drug ingenol mebutate probably acts only *in vitro* on hepatic drug transporter expression, owing to the principal local topical use of this compound [33], thus likely discarding any adverse effect of ingenol mebutate towards hepatobiliary secretion of drugs. Potential effects on transporter expression of drugs used as PKC inhibitors, that have enter clinical trials in various diseases such as cancers, diabetes or bipolar disorders [64], may also have to be considered. It is however noteworthy that the pan-PKC inhibitors GF 109203X and Gö 6983 failed to alter basal mRNA expression of drug transporters, thus making unlikely the hypothesis that inhibition of constitutional PKC activity by candidate drugs may change transporter expression in the liver.

In summary, activation of PKCs by PMA was demonstrated to markedly alter functional expression of various human hepatic drug transporters. Such changes, found to be likely linked to the EMT triggered by the phorbol ester, underline the role played by PKCs in drug transporter regulation.

Acknowledgements

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Figure Captions

Fig. 1. PMA-mediated activation of reference PKC targets.

(A) HepaRG cells were either untreated or exposed to 100 nM PMA for 24 h or 48 h. Cellular viability was then determined using the MTT assay. Data are expressed as % of cellular viability found in untreated control cells and are the means $\pm$ SEM of three independent assays. (B) HepaRG cells were either untreated or exposed to 100 nM PMA for 15 min. Phospho-MARKCS content was then analyzed by Western-blotting. Data shown are representative of three independent assays. (C) HepaRG cells were exposed to 100 nM PMA for various lengths of times (from 0 to 48 h). PAI-1 and CYP7A1 mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change comparatively to mRNA levels found in untreated cells and are the means $\pm$ SEM of at least four independent assays. *, $p<0.05$ when compared to untreated cells. (D) PKC isoform mRNA expression in HepaRG cells and primary human hepatocytes was determined by RT-qPCR. Data are expressed in arbitrary units relatively to 18S RNA and are the means $\pm$ SEM of three independent assays. (E) PKCs were ranked according to their mRNA expression level, from the most expressed isoform to the less expressed. Correlation between PKC expression profiles in HepaRG cells and hepatocytes was next analyzed using the Spearman rank correlation method. Spearman's rank coefficient (ρ) and p value are provided on the top of the correlation graph.
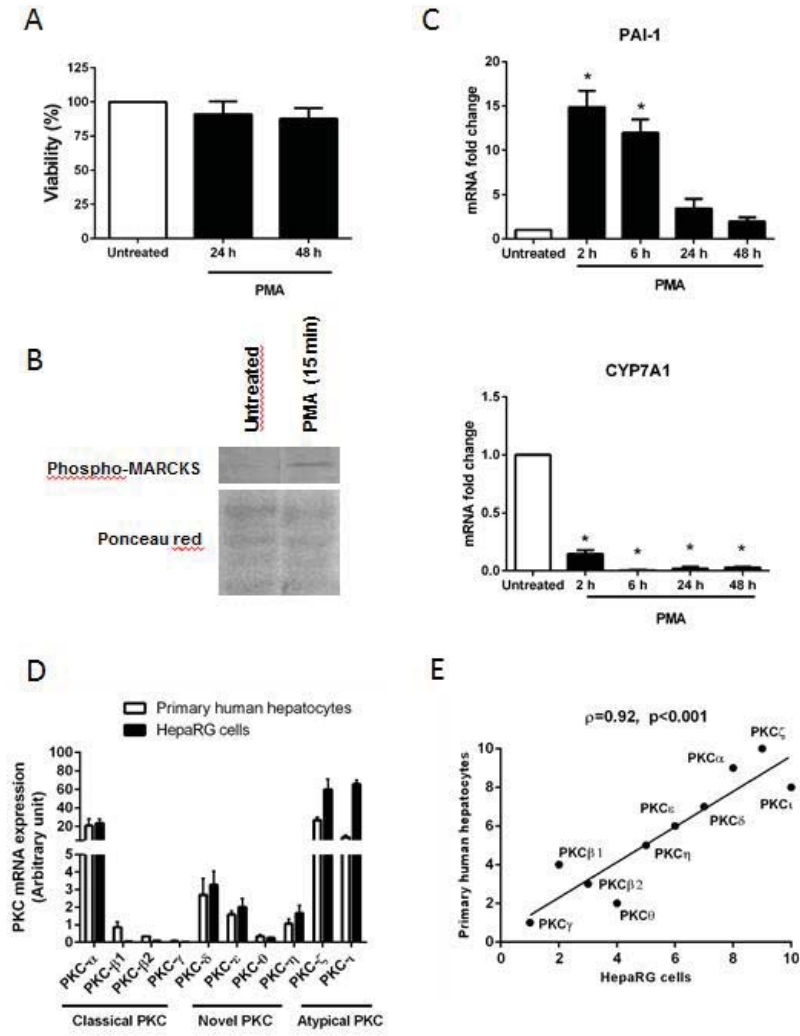
31

Fig. 1

Fig. 2. Effects of PMA on drug transporter mRNA expression in HepaRG cells.

HepaRG cells were exposed to 100 nM PMA for various lengths of times (from 0 to 48 h).

(A) SLC and (B) ABC drug transporter mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change comparatively to mRNA levels found in untreated cells and are the means $\pm$ SEM of at least four independent assays. *, $p<0.05$ when compared to untreated cells.
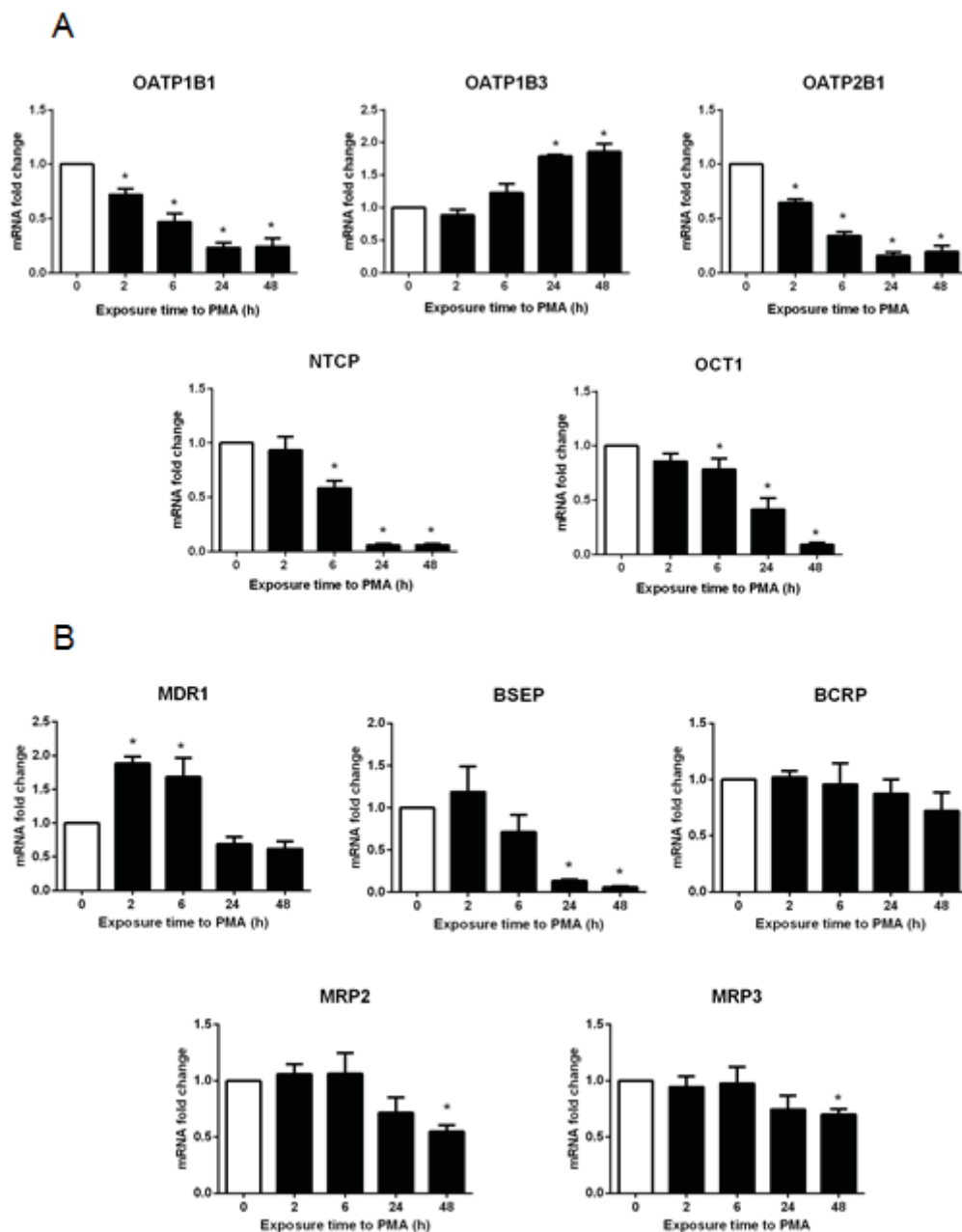
Fig. 2

Fig. 3. Effects of PMA on drug transporter mRNA expression in primary cultured human hepatocytes.

(A) Primary human hepatocytes were either untreated or exposed to 100 nM PMA for 24 h. Drug transporter mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change in PMA-treated hepatocytes comparatively to mRNA levels found in untreated counterparts and are the means $\pm$ SEM of values from five independent hepatocyte populations. *, $p<0.05$ when compared to untreated cells. (B) Spearman rank correlation analysis of PMA effects towards drug transporter mRNA expressions in primary human hepatocytes versus HepaRG cells. Human hepatocytes and HepaRG cells were exposed to PMA for 24 h; the transporters were ranked from the most repressed to the less repressed. Spearman's rank coefficient (ρ) and p value are provided on the top of the correlation graph.

A

B

$\rho=0.75; p=0.017$
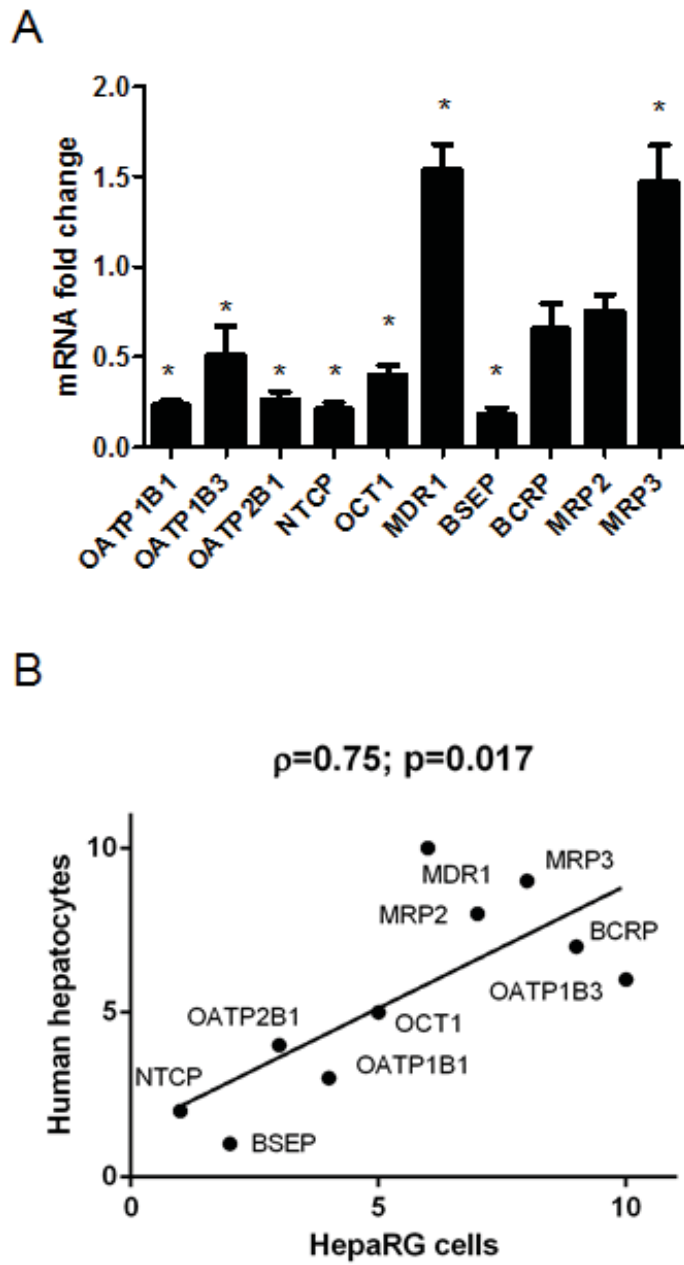
Fig. 3

Fig. 4. Effects of the PKC activator ingenol mebutate on drug transporter mRNA expression in HepaRG cells.

(A) HepaRG cells were either untreated or exposed to 10 μM ingenol mebutate for 24 h. Drug transporter mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change in ingenol mebutate-treated cells comparatively to mRNA levels found in untreated counterparts and are the means $\pm$ SEM of three independent assays. *, $p<0.05$ when compared to untreated cells. (B) Spearman rank correlation analysis of ingenol mebutate versus PMA effects towards drug transporter mRNA expressions in HepaRG cells. HepaRG cells were exposed to ingenol mebutate or PMA for 24 h; the transporters were ranked from the most repressed to the less repressed. Spearman's rank coefficient (ρ) and p value are provided on the top of the correlation graph.
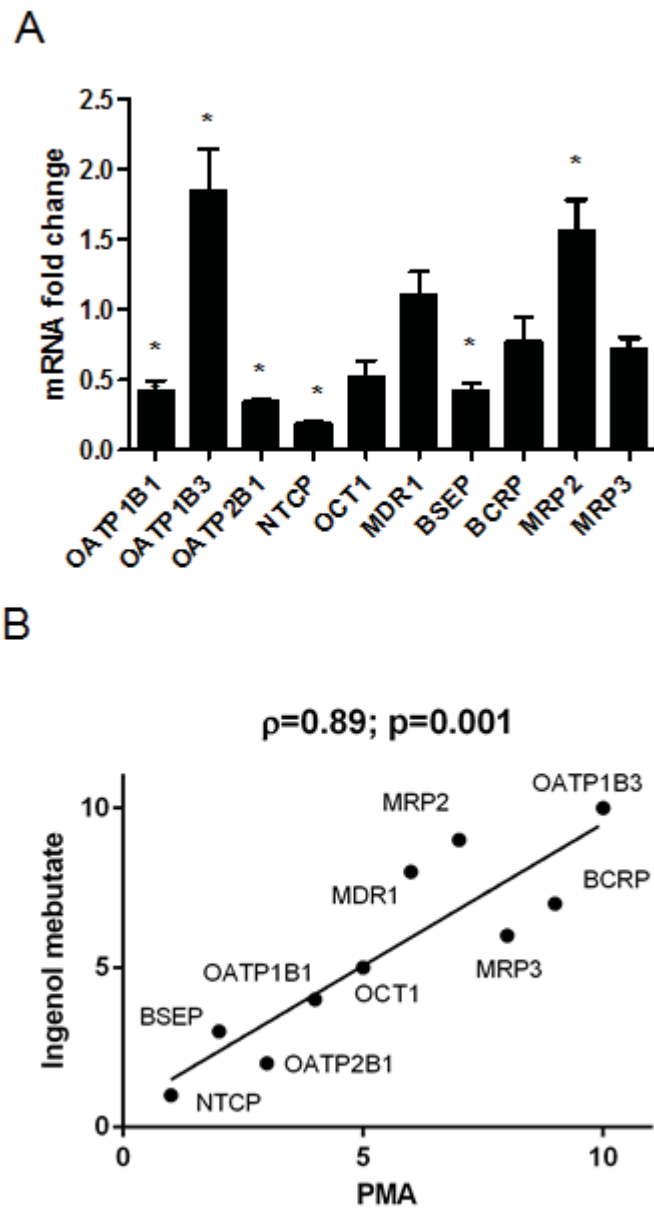
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Fig. 4

Fig. 5. Effects of PMA on (A) drug transporter protein expression and (B) drug transporter activitiy in HepaRG cells.

HepaRG cells were either untreated or exposed to 100 nM PMA for 48 h. (A) Transporter protein contents were determined by Western-blotting. Left panel, a representative blot is shown for each transporter. Right panel, for each transporter, data were quantified by densitometric analysis, normalized to Ponceau red staining and expressed relative to transporter expression found in untreated cells, arbitrarily set at the value of 100%; they are the means ± SEM of values from three independent assays. *, $p < 0.05$ when compared to untreated cells. (B) Drug transporters activities were determined as described in the Materials and Methods section. Data are the means ± SEM of at least four independent assays. *, $p < 0.05$ when compared to untreated cells.
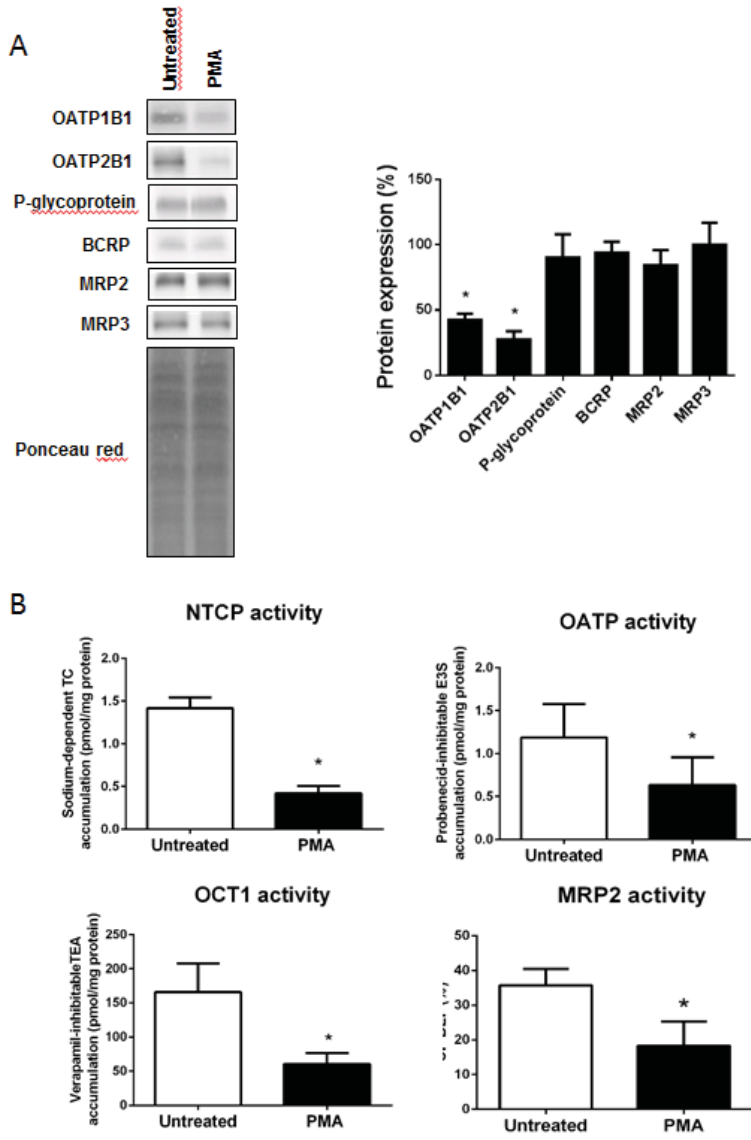
Fig. 5

Fig. 6. Effects of PKC inhibitors on PMA-mediated regulation of transporter mRNA expression in HepaRG cells.

HepaRG cells were either untreated or exposed to 100 nM PMA for 24 h in the absence or presence of 2 μM GF 109203X, 5 μM Gö 6983 or 5 μM Gö 6976. Drug transporter mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change comparatively to mRNA levels found in untreated cells and are the means $\pm$ SEM of four independent assays. *, $p<0.05$ when compared to cells not exposed to PKC inhibitors.
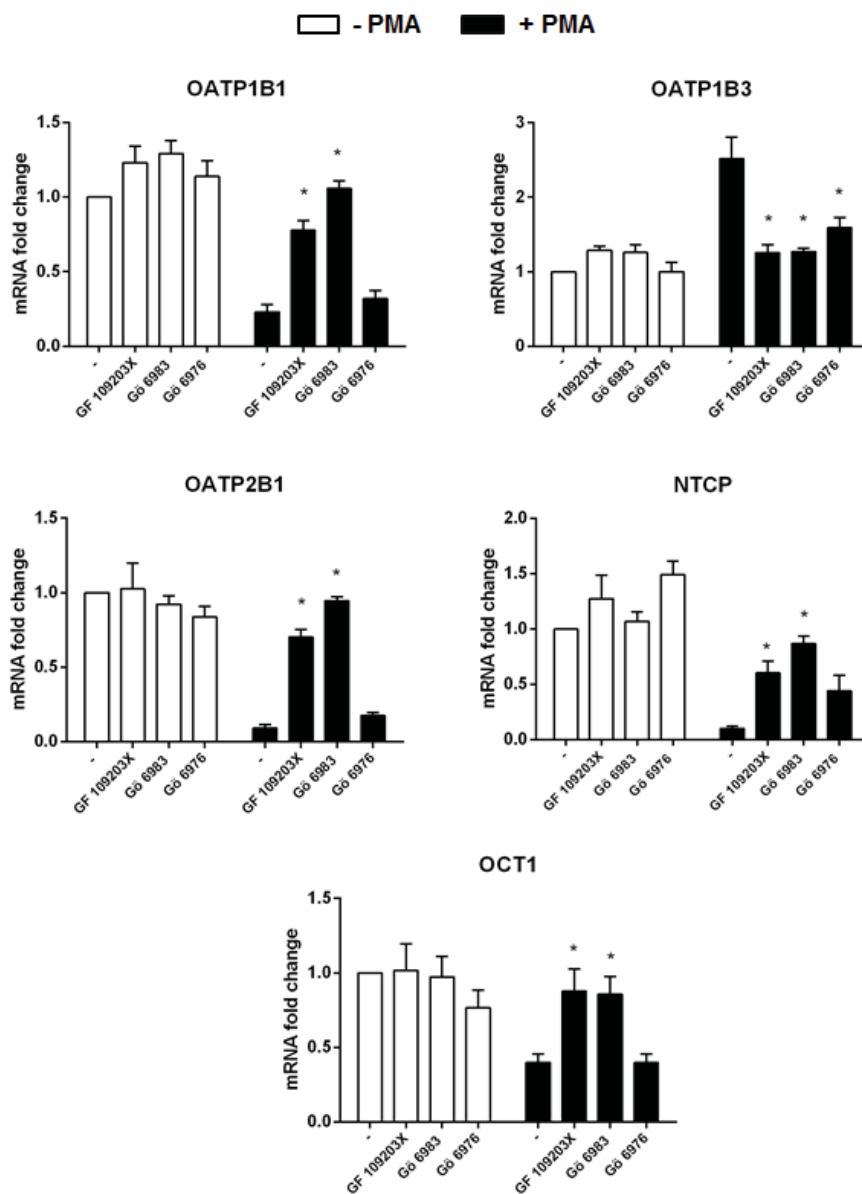
Fig. 6

Fig. 7. PMA-mediated induction of EMT in HepaRG cells.

(A) Light-microscopic pictures of HepaRG cell cultures either untreated or exposed to 100 nM PMA for 24 h. White bar = 25 μm. (B) E-cadherin and vimentin mRNA expressions in HepaRG cells either untreated or exposed to 100 nM PMA for 24 h were determined by RT-qPCR. Data are expressed as fold change comparatively to mRNA levels found in untreated cells and are the means $\pm$ SEM of at least three independent assays. *, $p < 0.05$ when compared to control untreated cells. (C) E-cadherin protein expression in HepaRG cells either untreated or exposed to 100 nM PMA for 24 h or 48 h was analyzed by Western-blotting. Data shown are representative of three independent assays. (D) EMT-related transcription factor mRNA expressions in HepaRG cells exposed to 100 nM PMA for various lengths of times (from 0 to 24 h) were determined by RT-qPCR. Data are expressed as fold change comparatively to mRNA levels found in untreated cells and are the means $\pm$ SEM of three independent assays. *, $p < 0.05$ when compared to control untreated cells.
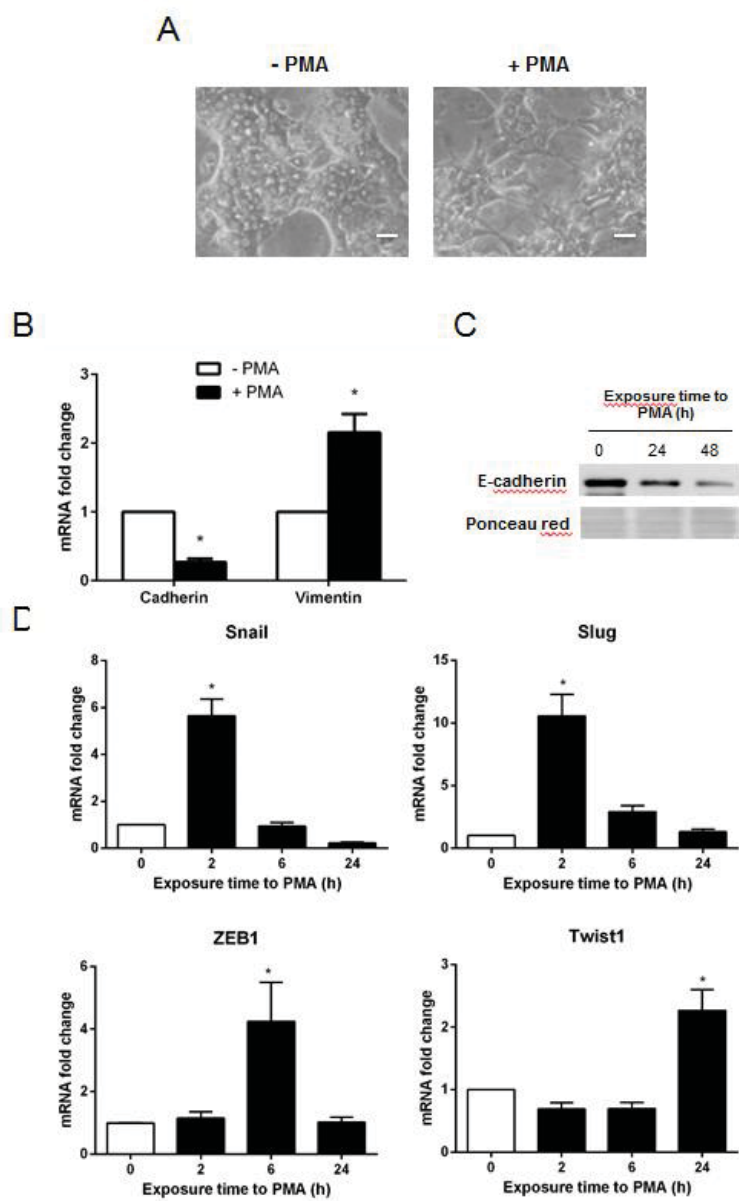
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Fig. 7

42

Fig. 8. Effects of various EMT inducers on drug transporter mRNA expression in HepaRG cells.

(A, B) HepaRG cells were either untreated or exposed to 20 ng/mL HGF, 5 µM BI6015 or 5 µM atRA for 24 h or to 10 ng/mL TGF-β1 for 48 h. (A) E-cadherin and vimentin mRNA and (B) drug transporter mRNA expressions were determined by RT-qPCR. Data are expressed as fold change comparatively to mRNA levels found in untreated cells and are the means $\pm$ SEM of at least three independent assays. *, $p<0.05$ when compared to control untreated cells. (C) Spearman rank correlation analyses of HGF, TGF-β1, BI6015 or atRA versus PMA effects towards drug transporter mRNA expressions in HepaRG cells. HepaRG cells were exposed to PMA, HGF or BI6015 for 24 h or to TGF-β1 for 48 h; the transporters were ranked from the most repressed to the less repressed. Spearman's rank coefficient (ρ) and p value are provided on the top of the correlation graphs.
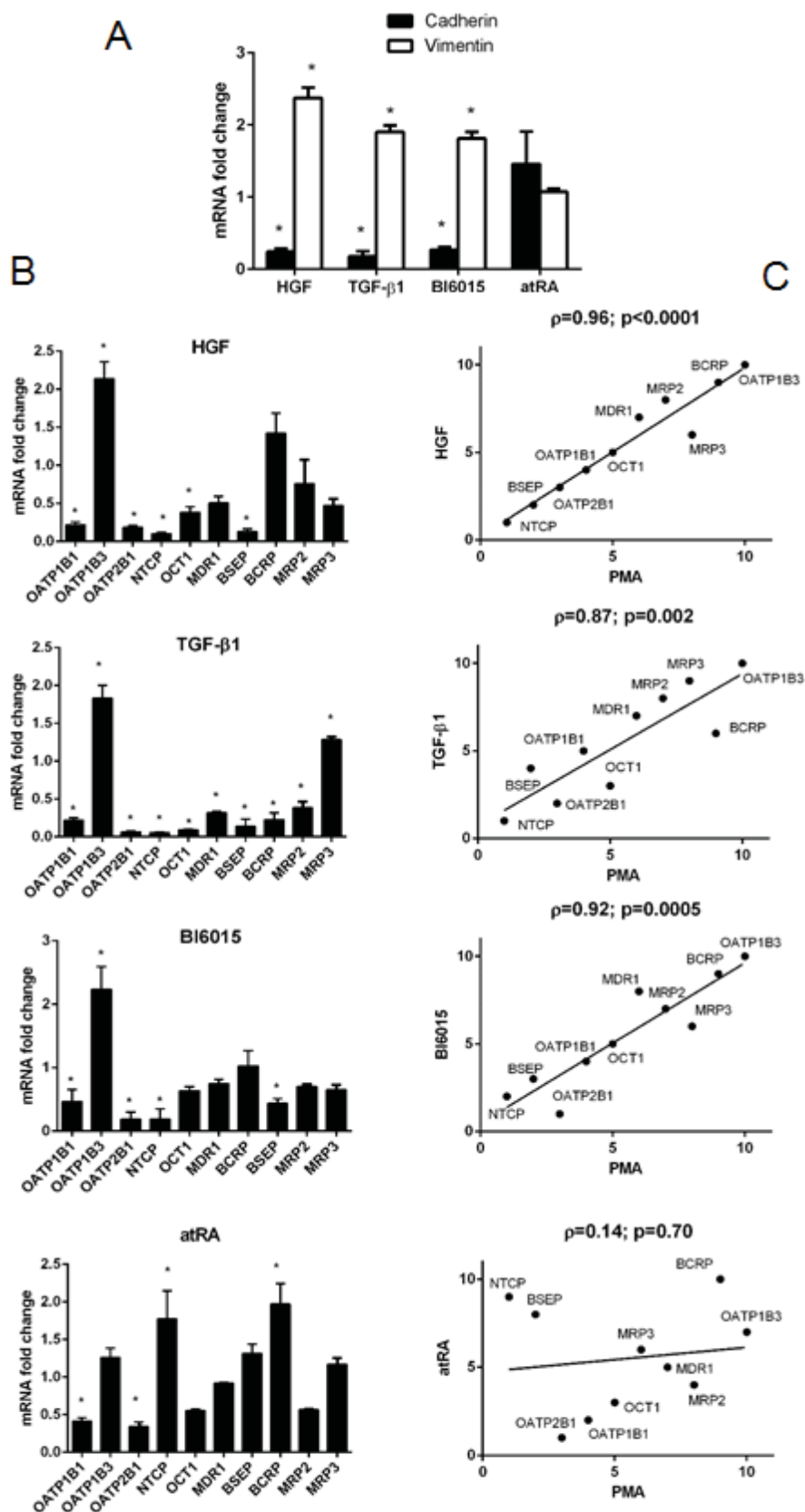
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Fig. 8

Fig. 9. Effects of PKC inhibitors on HGF-mediated regulation of transporter mRNA expression in HepaRG cells.

HepaRG cells were either untreated or exposed to 20 ng/mL HGF for 24 h, in the absence or presence of 2 μM GF 109203X, 5 μM Gö 6983 or 5 μM Gö 6976. Drug transporter mRNA expressions were next determined by RT-qPCR. Data are expressed as fold change comparatively to mRNA levels found in untreated cells and are the means $\pm$ SEM of three independent assays. *, $p<0.05$ when compared to cells not exposed to PKC inhibitors.
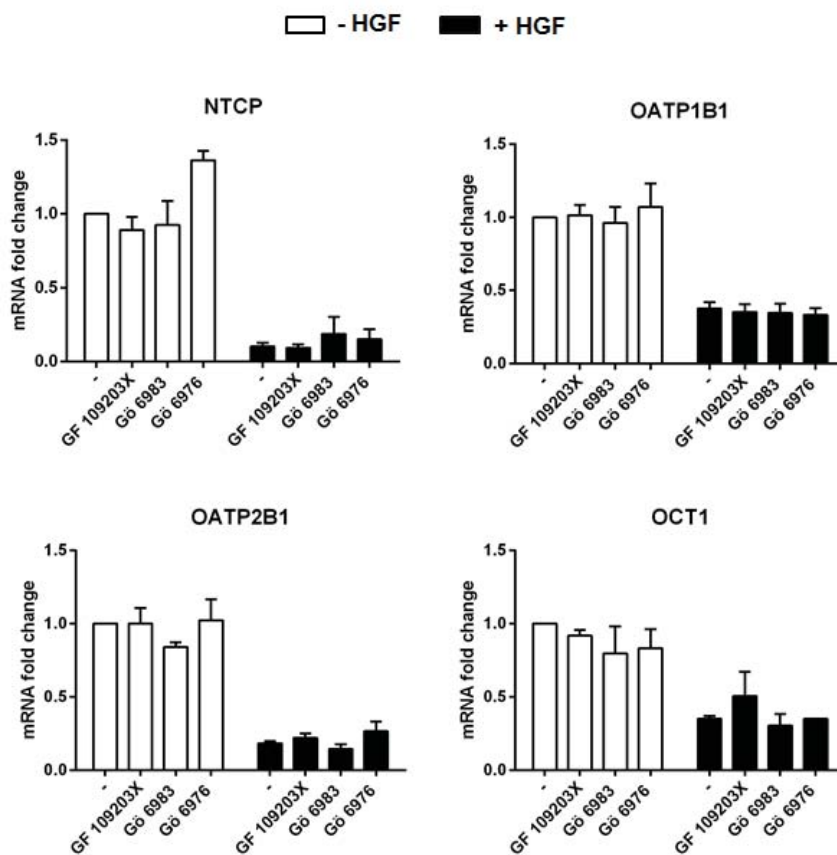

Fig. 9