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ORIGINAL ARTICLE

Celecoxib inhibits MDR1 expression through COX-2-dependent mechanism in human hepatocellular carcinoma (HepG2) cell line

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Abstract The role of COX-2 in the regulation of the expression of MDR1, a P-glycoprotein involved in hepatocellular carcinoma cell line, HepG2, was studied in the present investigation. Celecoxib, a selective inhibitor of COX-2, at 25 μM concentration increased the accumulation of doxorubicin in HepG2 cells and enhanced the sensitivity of the cells to doxorubicin by tenfold. The induction of MDR1 expression by PGE_2 and its downregulation by celecoxib or by COX-2 knockdown suggests that the enhanced sensitivity of HepG2 cells to doxorubicin by celecoxib is mediated by the downregulation of MDR1 expression, through COX-2-dependent mechanism. Further studies revealed the involvement of AP-1 in the celecoxib-induced downregulation of MDR1 expression. These experimental studies correlated well with in silico predictions and further suggested the inactivation of the signal transduction pathways involving ERK, JNK and p38. The present study thus demonstrates the usefulness of COX-2 intervention in overcoming the drug resistance in HepG2 cells.

Keywords AP-1 · Celecoxib · COX-2 · HepG2 cells · MDR1 · Simulation

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Introduction

The development of hepatocellular carcinoma (HCC) is a common feature during the natural history of cirrhosis and in advanced chronic liver disease. It has been calculated that 3–5% cases of cirrhosis evolve into HCC. In addition to an early diagnosis, surgery and chemotherapy are the usual methods adopted to reduce the growth of HCC and improve life expectancy of HCC patients. The development of multidrug resistance is the major limitation in chemotherapy of HCC. The most important form of drug resistance is the over-expression of the membrane-associated P-glycoprotein (P-gp), MDR1.

Intrinsic upregulation of MDR1 gene expression has been observed to be associated with primary resistance in untreated HCC of human and rodent origin [5, 16]. It manifests during inflammation-associated processes such as cholestasis [29] as well as in liver regeneration [1]. It was shown that regulation of MDR1 expression was dependent on COX-2 activity [26]. Cyclooxygenase (COX) or prostaglandin H_2 synthase (PGHS) is the enzyme that catalyzes the biosynthesis of prostaglandins (PGs) from the substrate arachidonic acid (AA). Several studies demonstrated elevated levels of COX-2 in different types of human cancer [11, 35, 38]. Celecoxib, a selective COX-2 inhibitor, is being used as an anti-inflammatory agent. Similarly, NSAIDs were shown to enhance the cytotoxic effects of doxorubicin in T98G human malignant glioma cells [28].

Simultaneous over-expression of the COX-2 and MDR1, reported in the regenerative nodules of cirrhotic livers as well as in well-differentiated HCC [20, 23], suggests a possible role for COX-2 in multidrug resistance. Present study is to understand the molecular mechanisms involved in the regulation of MDR1 expression by COX-2.

Materials and methods

Chemicals

PBS, RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (California, USA). Nitrocellulose membrane was from Millipore (Bangalore, India). Phosphatase inhibitor cocktail 1 and 2 and β-actin antibodies were purchased from Sigma-Aldrich (Bangalore, India). Polyclonal antibodies to MDR1 were from Santa Cruz Biotechnology (California, USA). Single-step RT-PCR kit was from AB Gene Technologies (Surrey, UK). Monoclonal COX-2 anti body, PGE_2 estimation kit and PGE_2 were from Cayman Chemical Co., USA. SiRNA for COX-2 was from Santa Cruz Biotechnologies Inc., USA. Celecoxib was a generous gift from Unichem Laboratories, India. Doxorubicin was a generous gift from Dabur Pharma, India. All the other chemicals and reagents were purchased from local companies and are of molecular biology grade.

Cell culture and treatment

Hepatocellular carcinoma (HepG2) cells, expressing MDR1 and COX-2 constitutively, were grown in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine and maintained in a humidified atmosphere with 5% CO_2 at 37°C. The cultured cells were sub-cultured twice each week and the exponentially growing cells were used for all treatments. Celecoxib dissolved in DMSO and doxorubicin dissolved in PBS were used for the treatments. 40 mM stock of celecoxib and 5 mM stock of doxorubicin were employed in this study. At the time of treatment, working solutions were diluted accordingly in RPMI 1640 medium. The drugs were added to the cells, 6 h after the sub-culture. Stock of celecoxib was freshly prepared before every treatment. The final concentration of the vehicle (DMSO) never exceeded 0.1%. HepG2 cells exposed to 0.1% DMSO served as controls.

Effect of celecoxib or doxorubicin on proliferation of HepG2 cells

Cell proliferation was determined by MTT [3-{4,5-dimethylthiazol-2yl}-2,5-diphenyltetrazolium bromide] assay [6]. HepG2 cells (5×10^3 cells/well) were seeded to 96-well culture plate in the presence or absence of celecoxib (1, 10, 25, 50 and 100 µM) or doxorubicin (100 nM, 500 nM, 1 µM, 10 µM, 25 µM and 50 µM) for 12, 24 and 48 h in a final volume of 100 µl. After treatment, the medium was removed and 20 µl of MTT (5 mg/ml of PBS) was added to the fresh medium. After 2 h incubation at 37°C, 100 µl of DMSO was added to each well and plates were agitated for 1 min. Absorbance was read at 570 nm on a multi-well plate reader. Percent inhibition of proliferation was calculated as a fraction of control (control was without celecoxib or doxorubicin).

Synergistic effect of celecoxib and doxorubicin on proliferation of HepG2 cells

HepG2 cells (5×10^3 cells/well) were seeded to 96-well culture plate. Doxorubicin (100 nM, 500 nM, 1 µM, 10 µM, 25 µM and 50 µM) was added in the presence or absence of celecoxib (25 µM) for 24 h in a final volume of 100 µl. After treatment, the medium was removed and 20 µl of MTT (5 mg/ml of PBS) was added to the fresh medium. After 2 h incubation at 37°C, 100 µl of DMSO was added to each well and plates were agitated for 1 min. Absorbance was read at 570 nm on a multi-well plate reader. Percent inhibition of proliferation was calculated as a fraction of control. (Cells treated with 25 µM celecoxib were taken as control for determining the synergistic effects of doxorubicin and celecoxib.)

Intracellular drug accumulation assays

HepG2 cells (1×10^6 cells/well) were seeded into 6-well culture plates. Cells were incubated with celecoxib at concentrations of 10 and 25 µM for 24 h. To determine intracellular drug accumulation, 50 µM of doxorubicin was added to HepG2 cells and incubated for 2 h. Then, the cells were trypsinized and the final doxorubicin accumulated in HepG2 cells was analyzed using flow cytometer [7]. When cells were incubated with doxorubicin, they take up the drug and kinetics of doxorubicin efflux is dependent on the expression of MDR1. So, if the expression of MDR1 is inhibited, there is more accumulation of doxorubicin, which can be quantified on flow cytometer.

RT-PCR analysis

HepG2 cells were seeded at a density of 5×10^6 in 90 mm culture dishes. Cells were treated with celecoxib (1, 10 and 25 µM) or PGE_2 (6 µg/ml) for 24 h. Cells were harvested and total RNA was extracted using TRIzol reagent from control and treated HepG2 cells. Semi-quantitative RT-PCR was performed with 5 µg total RNA, isolated from HepG2 cells, using one-step RT-PCR kit. Primers used were as follows—human MDR1: forward, 5′-TGA CTA CCA GGC TCG CCA A-3′; reverse, 5′-TAG CGA TCT TCC CAG CAC CTT-3′, which yields 252 bp product [22], actin: forward, 5′-GTT TGA GAC CTT CAA CAC CCC-3′, reverse, 5′-GTG GCC ATC TCC TGC TCG AAG TC-3′, which yields 318 bp product [15]. Reverse transcription was performed at 47°C for 30 min. Subsequent to inactivation

of reverse transcriptase (2 min, 94°C), the samples were subjected to 25 amplification cycles, each consisting of 20 s at 94°C, 1 min at 54°C, and 1 min at 72°C, followed by 5 min at 72°C after the last cycle. The products were analyzed on 1% agarose gel.

COX-2 and MDR1 knockdown analysis

HepG2 cells (1×10^6 cells/well) were seeded into 6-well culture plates. After overnight incubation, cells were transfected with siRNA for COX-2 (Santacruz:sc-44256) and MDR1 (Santacruz:sc-29395) at concentration of 100 nM for 48 h. Total RNA was isolated and RT-PCR analysis (procedure described in "RT-PCR analysis") was performed to estimate the expression of MDR1 in control, COX-2 siRNA transfected, MDR1 siRNA transfected and PGE_2 (6 μg/ml) treated cells.

Preparation of whole-cell extracts and immunoblot analysis

HepG2 cells at a density of 5×10^6 were seeded in 90 mm culture dishes. They were incubated with celecoxib (1, 10 and 25 μM) and PGE_2 (6 μg/ml). Cells harvested were used for preparation of whole-cell extract. The harvested, control and treated, HepG2 cells were washed with PBS and suspended in lysis buffer (20 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin and phosphatase inhibitor cocktail 1 and 2 with 100-fold dilution). After 30 min of shaking at 4°C, the mixtures were centrifuged ($10{,}000 \times g$) for 10 min, and the supernatants were used as the whole-cell extracts. The protein content was determined according to the Bradford method [4]. 100 μg of protein from each treatment was resolved on 10% gels along with protein molecular weight standards, and then transferred onto nitrocellulose membranes. Membranes were stained with 0.5% Ponceau S in 1% acetic acid to check the transfer. The membranes were blocked with 5% (w/v) non-fat dry milk and then incubated with the primary antibodies (COX-2 and MDR1, 1:500 dilution) in 10 ml of antibody-diluted buffer (1× Tris-buffered saline and 0.05% Tween-20 with 1% milk) with gentle shaking at 4°C for 8–12 h and then incubated with peroxidase-conjugated secondary antibodies. Signals were detected using peroxidase substrate, TMB/H_2O_2. Equal protein loading was confirmed by reprobing the nitrocellulose membranes with β-actin antibodies (1:500 dilution).

Electrophoretic mobility shift assay (EMSA)

HepG2 cells at a density of 5×10^6 were seeded in 90 mm culture dishes. Cells were incubated with celecoxib (10 and 25 μM) for 6 h and PGE_2 (6 μg/ml) for 1, 2 and 6 h. Cells were harvested and then used for nuclear protein extraction. The cells were washed with PBS and 200 μl of ice cold lysis buffer (20 mM Tris–HCl, pH 7.5, 10 mM magnesium acetate, 1% NP-40, 1 mM PMSF) was added, and incubated for 5 min on ice with 3–4 vortexings of 10 s each. The nuclei were then harvested by centrifugation at 16,000 rpm for 1 min. The nuclear pellet was resuspended in 40 μl of nuclear protein extraction buffer (420 mM NaCl, 10 mM HEPES, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM DTT and 25% glycerol) and incubated on ice for 30 min with intermittent vortexing of 10 s each. The sample was then centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant collected was used for the mobility shift assay after protein estimation using Bradford assay [4]. Nuclear extracts (8 μg) were incubated with γ-^{32}P-labeled double-stranded oligonucleotide with specific AP-1 binding sequence (5′-CTG AAT CAA CTG CTT CAA-3′) for 30 min at 37°C. DNA–protein complex formed was separated from free oligonucleotides on 6.6% native acrylamide gel. The dried gel was exposed to X-ray film. The specificity of binding was examined by competition with unlabeled oligonucleotide (cold competition).

PGE_2 estimation

HepG2 cells at a density of 5×10^6 were seeded in 90 mm culture dishes. They were incubated with celecoxib (1, 10, 25 μM) for 24 h. At the end of the treatment period, culture medium was collected to determine the amount of PGE_2 secreted by these cells and stored at −80°C. The quantitative analysis of PGE_2 released into the medium was assessed using PGE_2 immunoassay kit as per manufacturer's instructions (Cayman Chemical Company, USA).

In silico studies

Model overview

The model was developed using Teranode's Biological Modeler, which uses MML (Mathematical Modeling Language, developed by the National Simulation Resource at the University of Washington) and allows creation of kinetic equations. The Teranode™ software was used to create and integrate the pathway with ODEs (ordinary differential equations) solved numerically by the Radau method [14]. A scheme of the elements of the model and their connections are shown in Fig. 1.

Model structure and description

Celecoxib-mediated regulation of MDR1 expression has been modeled as follows: (1) COX-2 catalyzes the conversion

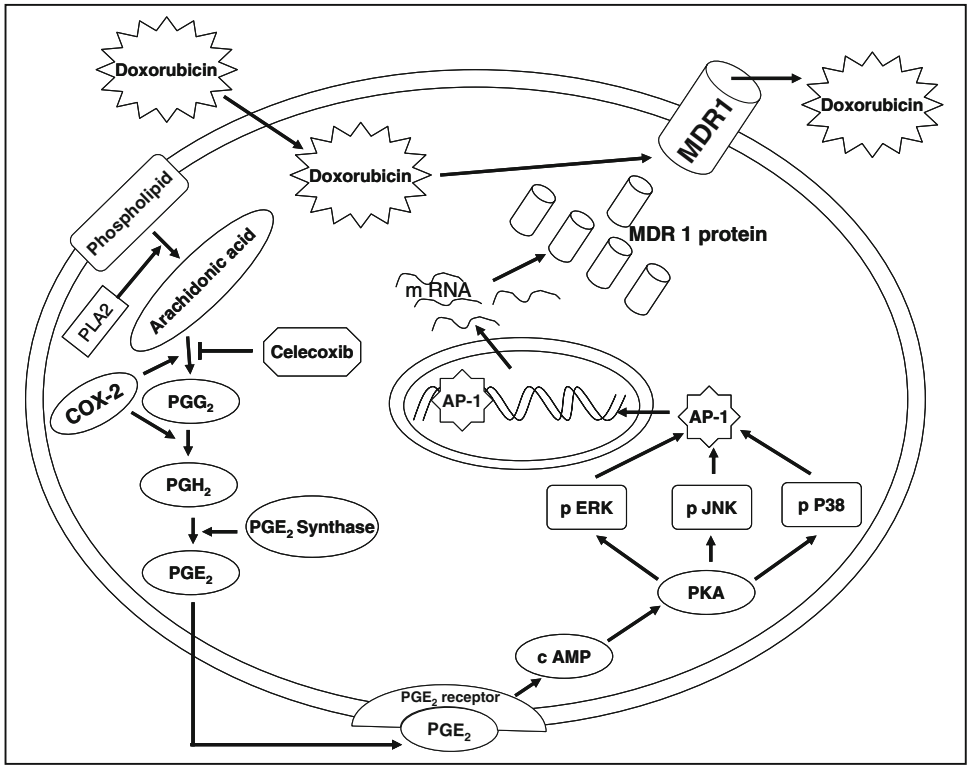

Fig. 1 Schematic representation of the model showing the regulation of MDR1 expression by COX-2 and site of interference by celecoxib

of AA to prostanoids by a two-step process. In the first step, two molecules of O_2 are added to form a short-lived, unstable intermediate prostaglandin G_2 (PGG_2). PGG_2 is then converted to PGH_2 by shedding of single oxygen [37]. (2) PGE_2 synthase catalyzes conversion of COX-derived PGH_2 to PGE_2 through an isomerization reaction [24, 34, 39]. PGE_2 bound prostaglandin (EP2/4) receptor [18, 21] mediates signal transduction by activating PKA pathway [24]. (3) ERK, p38 and JNK in turn are activated by phosphorylation mediated by PKA [9, 13]. (4) The phosphorylated MAPKs subsequently activate the protein c-Jun and c-Fos. Together these two proteins form an active AP-1 transcription complex [8, 27]. AP-1 is the transcription factor regulating the expression of MDR1 [31–33]. MDR1 mRNA is translated to MDR1 protein in the cytoplasm. (5) Doxorubicin is a drug that acts at the level of Topoisomerase II. With the expression of MDR1, there is a rapid extrusion of the drug out of the cell and hence resistance to this chemotherapeutic agent [25]. (6) Celecoxib selectively inhibits COX-2 thereby regulating MDR1 protein levels and increasing the overall doxorubicin retention in the cell. Further details on the model description were included in Supplementary information.

Statistical analysis

Data were reported as the mean ± SE of three independent experiments. Statistical analysis of differences was carried out by one-way analysis of variance (ANOVA).

Table 1 Effect of celecoxib and doxorubicin on the growth of HepG2 cells

Celecoxib	Doxorubicin	IC-50
+	−	35 μM
−	+	5 μM
+	+	500 nM (for doxorubicin)

The percent viable cells were calculated in comparison to control cells. (Control was without doxorubicin or celecoxib for the determination of IC_{50} for celecoxib or doxorubicin. Cells treated with 25 μM celecoxib were taken as control for determining the synergistic effects with both doxorubicin and celecoxib)

A P value less than 0.05 was considered to indicate significance.

Results

Celecoxib and doxorubicin showed synergistic effects on the proliferation of HepG2 cells

HepG2 cells were treated with celecoxib (1–100 μM) and doxorubicin (100 nM to 50 μM) separately for 12, 24 and 48 h and cell proliferation was determined by MTT assay. Under these experimental conditions, a dose-dependent decrease in proliferation of HepG2 cells was observed with an IC_{50} of 35 μM for celecoxib and 5 μM for doxorubicin at 24 h exposure (Table 1).

In order to test the combination effects, HepG2 cells were treated with varying concentrations of doxorubicin (100 nM, 500 nM, 1 μM, 10 μM, 25 μM and 50 μM) and fixed concentration of celecoxib (25 μM) for 24 h and cell proliferation was determined by MTT assay. In the presence of 25 μM celecoxib, the percent inhibition in the growth of HepG2 cells was much higher at all the concentrations of doxorubicin studied. As a result, the IC_{50} of doxorubicin for HepG2 cells was reduced from 5 μM in the absence of celecoxib to 500 nM in the presence of 25 μM celecoxib (Table 1), i.e. a tenfold reduction.

Celecoxib treatment increased the accumulation of doxorubicin in HepG2 cells

HepG2 cells treated with 25 μM celecoxib (less than its IC_{50} value) for 24 h and then incubated with 50 μM doxorubicin for 2 h showed 56.7% more doxorubicin accumulation compared to the cells without celecoxib treatment (Fig. 2a). In silico predictions and analysis also showed dose-dependent increase in doxorubicin accumulation with celecoxib treatment (Fig. 2b).

Celecoxib regulates MDR1 expression by inhibition of COX-2 enzyme activity

Treatment of HepG2 cells with celecoxib (1, 10 and 25 μM) for 24 h resulted in a dose-dependent reduction of MDR1 expression at mRNA (Fig. 3a) and protein levels (Fig. 3b). Celecoxib inhibited the expression of MDR1 starting from 12 to 48 h time point (data not shown). Addition of PGE_2 (6 μg/ml) to the medium, on the other hand, induced the expression of MDR1 compared to untreated controls. In silico predictions were aligned with experimental trends (bar diagrams in Fig. 3a, b). Experimental and in silico results of celecoxib treatment (1, 10 and 25 μM) for 24 h showed a dose-dependent decrease in levels of PGE_2 (Fig. 3c).

COX-2 knockdown reduced the expression of MDR1

As inhibition of COX-2 activity inhibited the expression of MDR1, the effect of COX-2 depletion on MDR1 expression was tested. Knockdown of COX-2 by siRNA reduced the expression of MDR1 (Fig. 3d). PGE_2 treatment, on the other hand, increased the expression of MDR1 compared to the untreated control cells (Fig. 3d).

Celecoxib-induced inhibition of MDR1 expression is mediated by signal transduction pathway involving MAP kinases and AP-1

Nuclear levels of AP-1, a positive regulator of MDR1 expression, were reduced in a dose-dependent manner in

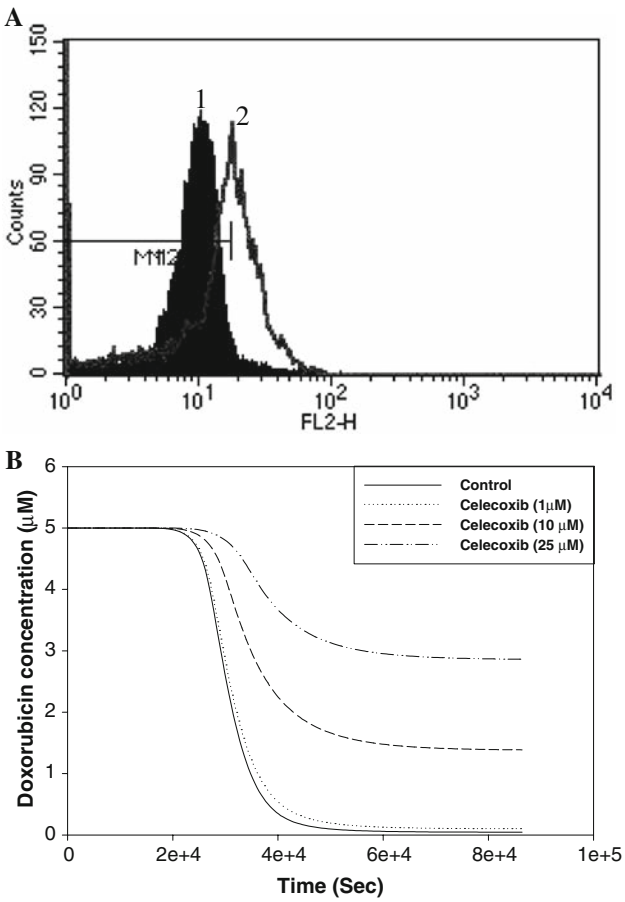

Fig. 2 Measurement of intracellular doxorubicin accumulation by flow cytometer. **a** HepG2 cells were treated with 25 μM of celecoxib for 24 h and then incubated with 50 μM doxorubicin for 2 h. The fluorescence of doxorubicin retained in HepG2 cells was measured with FL2 filter. *Histogram 1* (doxorubicin retained in control cells exposed to 50 μM doxorubicin) overlaid with *histogram 2* (doxorubicin retained in cells treated with 25 μM of celecoxib and exposed to 50 μM doxorubicin). Greater the FL2-Height, greater is the drug retention. **b** *Line plot* showing simulation data of doxorubicin accumulation with celecoxib treatment. Concentration of doxorubicin accumulated in μM on *Y*-axis against time (s) on *X*-axis. This was obtained by running the simulation model with celecoxib concentrations of 0, 1, 10, 25 μM

HepG2 cells treated with celecoxib (10 and 25 μM) for 6 h. Treatment with PGE_2 (6 μg/ml) for 1, 2 and 6 h, on the other hand, showed an increase in the levels of AP-1 in HepG2 cells (Fig. 4). Simulation data of AP-1 translocation correlated well with the experimental data (bar diagrams in Fig. 4). In silico studies showed a dose-dependent decrease in phosphorylated levels of JNK, ERK and p38 with celecoxib treatment (Fig. 5a–c).

Discussion

Over-expression of MDR1, a drug transporter protein, is the primary impediment in cancer chemotherapy. The role

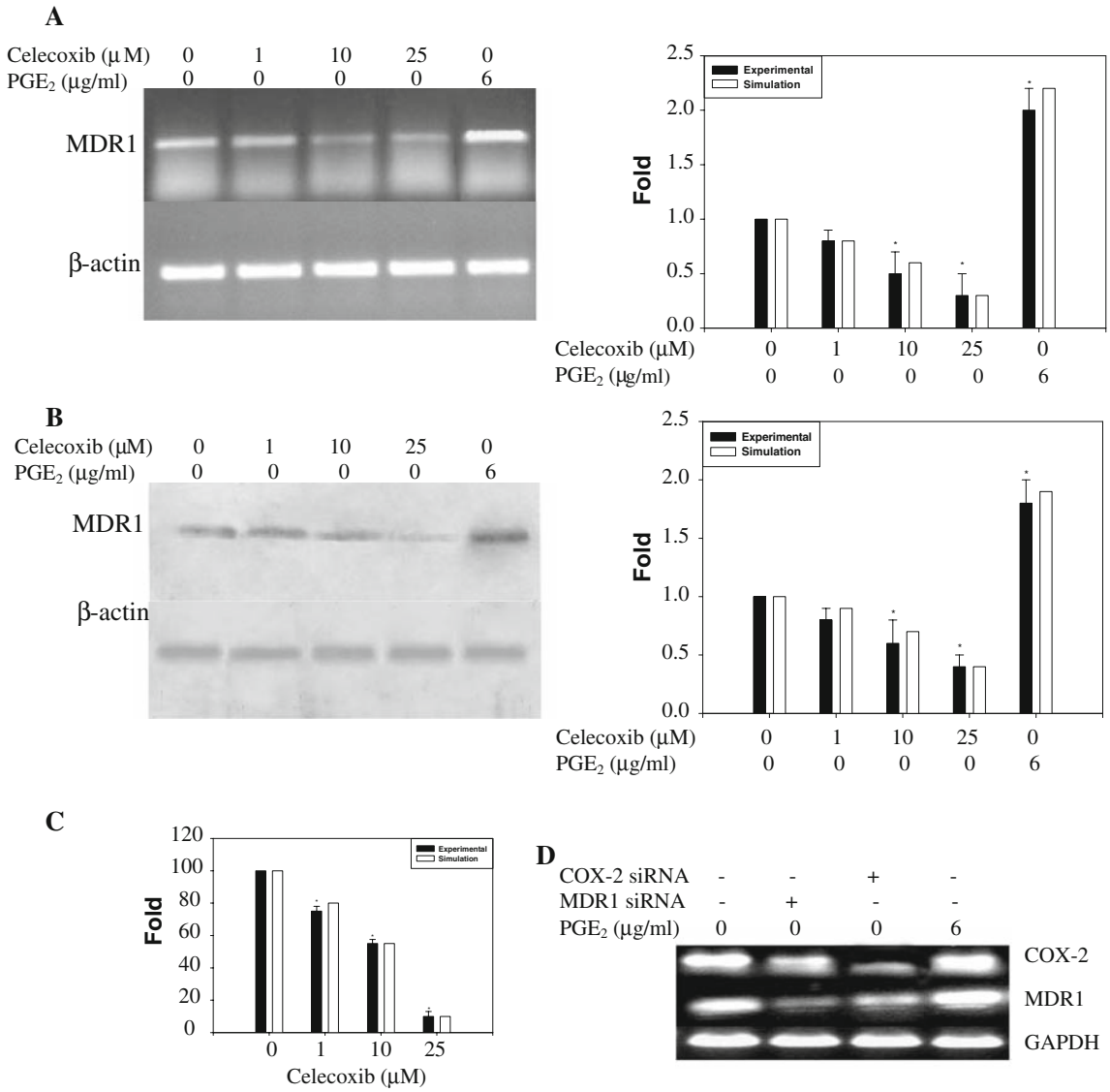

Fig. 3 Effect of celecoxib and COX-2 knockdown on MDR1 expression and PGE_2 release in HepG2 cells. **a** RT-PCR analysis of celecoxib and PGE_2 mediated effect on MDR1 mRNA expression in HepG2 cells. *Bar graphs* denote the fold difference in the expression levels of MDR1 mRNA obtained by experimental and simulation data. **b** Western blot analysis of celecoxib and PGE_2 mediated effect on MDR1 protein expression in HepG2 cells. *Bar graphs* denote the fold difference in the expression levels of MDR1 protein obtained by experimental and simulation data. **c** *Bar graphs* showing the fold difference in the release of PGE_2 in HepG2 cells with celecoxib treatment (1, 10 and 25 μM) obtained by experimental and simulation data. **d** RT-PCR analysis of COX-2 and MDR1 knockdown and PGE_2 mediated effect on MDR1 and COX-2 mRNA expression in HepG2 cells. *Asterisk* denotes statistical significance over control ($P < 0.05$)

of MDR1 in protecting cells from apoptosis induced by chemotherapy has been demonstrated in several cellular systems [17]. Recent studies indicate that the activation of the cyclooxygenase system might be critical event in the development of MDR1-mediated drug resistance [11]. Selective COX-2 inhibitors have been shown to have strong chemopreventive actions against colon cancers in animals and patients with familial adenomatous polyposis [2, 19]. In the present study, treatment of HepG2 cells with celecoxib, a selective COX-2 inhibitor, showed an increased doxorubicin accumulation. Similar increase in the accumulation of doxorubicin with celecoxib treatment was shown in MDA-MB231 cell line, independent of COX-2 and MDR1 but dependent on NF-κB inhibition [36]. Enhanced accumulation of rhodamine 123 was shown in rat glomerular mesangial cells treated with NS-398, a selective COX-2 inhibitor [11]. In the present study also, celecoxib enhanced the accumulation of doxorubicin in HepG2 cells in a dose-dependent manner. As a result of this enhanced accumulation of doxorubicin in the presence of celecoxib, the sensitivity of HepG2 cells to doxorubicin was enhanced by tenfold.

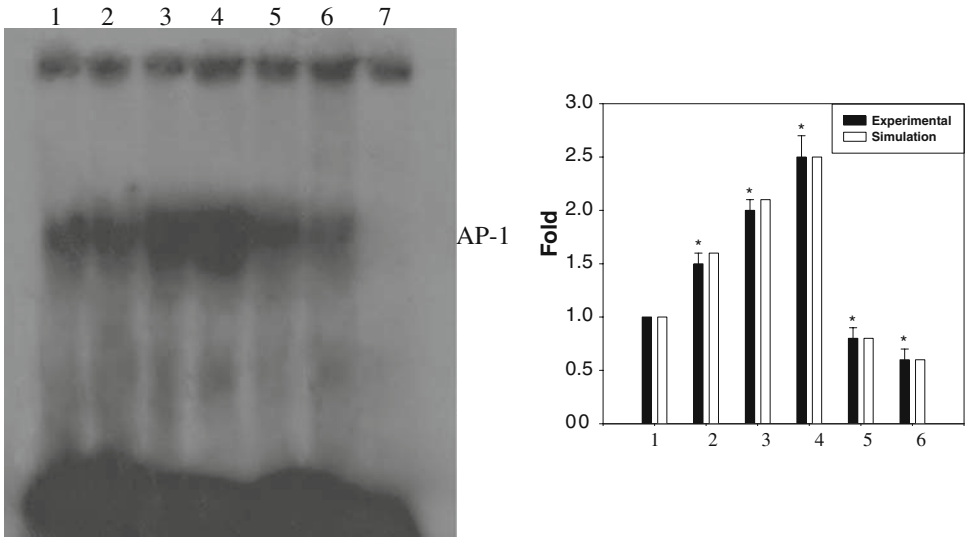

Fig. 4 Effect of PGE_2 and celecoxib on nuclear translocation of AP-1 in HepG2 cells. Electrophoretic mobility shift assay (EMSA) of nuclear extracts using specific AP-1 binding probe in cells treated with celecoxib and PGE_2. *Lane 1* control, *lane 2* PGE_2 (6 μg/ml) 1 h, *lane 3* PGE_2 (6 μg/ml) 2 h, *lane 4* PGE_2 (6 μg/ml) 6 h, *lane 5* celecoxib (10 μM) 6 h, *lane 6* celecoxib (25 μM) 6 h, *lane 7* cold competition. *Bar graphs* denote the fold difference in the AP-1 translocation obtained by experimental and simulation data. *Asterisk* denotes statistical significance over control ($P < 0.05$)

The present study demonstrates the downregulation of MDR1 expression in HepG2 cells, when treated with celecoxib, in COX-2-dependent mechanism. Similar observations were reported in rat glomerular mesangial cells, where in the transfection of COX-2 expression vector resulted in increased expression of MDR1 and its expression was decreased with NS-398 treatment [11]. It was also shown that addition of PGE_2 to the culture medium of rat primary hepatocytes upregulated MDR1b mRNA expression and MDR1-dependent transporter activity [40]. In addition, structurally different cyclooxygenase inhibitors (Indomethacin, Meloxicam, NS-398) were shown to inhibit EGF-induced MDR1 mRNA over-expression, leading to the accumulation rhodamine 123 in rat primary hepatocyte cultures [3]. But the effect of celecoxib on PGE_2 release might be influenced to certain extent on other parameters such as PLA_2 expression [30]. The specific role of COX-2 in MDR1 expression was further supported by COX-2 knockdown experiments. Knockdown of COX-2 by siRNA reduced the expression of MDR1. PGE_2, the enzymatic product of COX-2, binds to prostaglandin receptor [18, 21] and mediates signal transduction by activating PKA pathway [12]. ERK, p38 and JNK in turn are activated by phosphorylation mediated by PKA [9, 13]. These MAP kinases activate c-fos and c-jun, promoting the formation of AP-1 [8, 27]. AP-1 is the key molecule in the regulation of expression of drug transporters [3, 10]. Treatment with celecoxib reduced the nuclear levels of AP-1 in a dose-dependent manner while treatment with PGE_2 showed an increase in the formation of AP-1 in HepG2 cells. The combination of experimental and in silico studies helped in dissecting the signaling pathway involved in the COX-2-dependent mechanism in the regulation of MDR1 protein.

In silico simulation studies utilized the initial levels of components of the pathway, which were calibrated to match the levels obtained via experimental data. The predicted levels of MDR1, COX-2 and AP-1 obtained by in silico simulation studies with celecoxib and PGE_2 treatment corroborated with the experimental results. A gradual increase in doxorubicin retention with increase in celecoxib concentration was observed in both in silico and in experimental studies. Using in silico experiments when K_i values of celecoxib were varied within a range of 300–1×10^{-2} μM, reduction in MDR1 expression (RNA and protein levels) and AP-1 levels was observed with the decrease in K_i values (data not shown). In silico studies further revealed the reduction in the activation of ERK, JNK and p38 by ~1.3-fold with celecoxib (25 μM) treatment when compared to the untreated cells. This decrease in the activation of protein MAP kinases might also be responsible, to a certain extent, for the decrease in MDR1 expression in celecoxib-treated cells.

The experimental and in silico data presented in this study strongly suggest that celecoxib regulates the expression of MDR1 in a COX-2-dependent manner and potentiates the effects of doxorubicin in HepG2 cells. Further, AP-1-mediated signal transduction pathway is involved in the regulation of MDR1 expression by COX-2 in HepG2 cells, whose activity is inhibited by celecoxib treatment. The schematic representation of the model showing the regulation of MDR1 expression by COX-2 and site of interference by celecoxib is shown in Fig. 1. In conclusion, the foregoing studies clearly demonstrate the role of COX-2 in the development of drug resistance and usefulness of COX-2 inhibitor, celecoxib, in overcoming drug resistance in HepG2 cells.

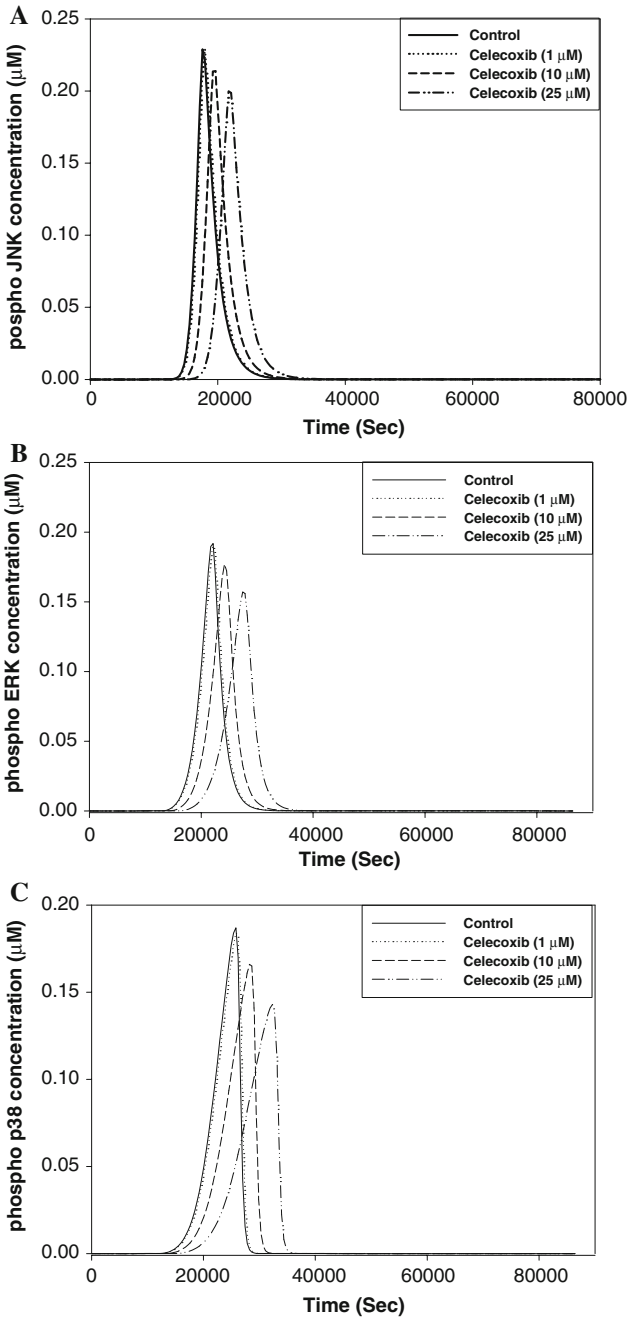

Fig. 5 In silico simulation data on the effect of celecoxib on phosphorylation of JNK, ERK and p38 in HepG2 cells. *Line plot* showing simulation data of phospho JNK (**a**), phospho ERK (**b**) and phospho p38 (**c**) concentration in μM on *Y*-axis against time (s) on *X*-axis with celecoxib treatment. This was obtained by running the simulation model with celecoxib concentrations of 0, 1, 10, 25 μM. At 25 μM of celecoxib concentration, the levels of phosphorylated forms of JNK, ERK, p38 were decreased by ∼1.3-fold

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References

1. Arino PA, Gottesman MM, Pastan I (1990) Regulation of multidrug resistance gene in regenerating rat liver. Cell Growth Differ 1:57–62
2. Bertagnolli MM, Eagle CJ, Zauber AG, Redston M, Solomon SD, Kim K, Tang J, Rosenstein RB, Wittes J, Corle D, Hess TM, Woloj GM, Boisserie F, Anderson WF, Viner JL, Bagheri D, Burn J, Chung DC, Dewar T, Foley TR, Hoffman N, Macrae F, Pruitt RE, Saltzman JR, Salzberg B, Sylwestrowicz T, Gordon GB, Hawk ET (2006) Celecoxib for the prevention of sporadic colorectal adenomas. N Engl J Med 355:873–884
3. Bhushan A, Abramson R, Chiu JF, Tritton TR (1992) Expression of c-fos in human and murine multidrug-resistant cells. Mol Pharmacol 42:69–74
4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254
5. Bradley G, Sharma R, Rajalakshmi S, Ling V (1992) P-glycoprotein expression during tumor progression in the rat liver. Cancer Res 52:5154–5161
6. Campling BG, Pym J, Galbraith PR, Cole SP (1988) Use of the MTT assay for rapid determination of chemosensitivity of human leukemic blast cells. Leuk Res 12:823–831
7. Chan JY, Chu AC, Fung KP (2000) Inhibition of P-glycoprotein expression and reversal of drug resistance of human hepatoma HepG2 cells by multidrug resistance gene (mdr1) antisense RNA. Life Sci 67:2117–2124
8. Chen Y, Hughes-Fulford M (2000) Prostaglandin E2 and the protein kinase A pathway mediate arachidonic acid induction of c-fos in human prostate cancer cells. Br J Cancer 82:2000–2006
9. Dalle S, Longuet C, Costes S, Broca C, Faruque O, Fontes G (2004) Glucagon promotes cAMP-response element-binding protein phosphorylation via activation of ERK1/2 in MIN6 cell line and isolated islets of Langerhans. J Biol Chem 279:20345–20355
10. Daschner PJ, Ciolino HP, Plouzek CA, Yeh GC (1999) Increased AP-1 activity in drug resistant human breast cancer MCF-7 cells. Breast Cancer Res Treat 53:229–240
11. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN (1994) Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 107:1183–1188
12. Fujino H, Salvi S, Regan JW (2005) Differential regulation of phosphorylation of the cAMP response element-binding protein after activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. Mol Pharmacol 68:251–259
13. Gallagher ED, Xu S, Moomaw C, Slaughter CA, Cobb MH (2002) Binding of JNK/SAPK to MEKK1 is regulated by phosphorylation. J Biol Chem 277:45785–45792
14. Hairer E, Wanner G (1999) Stiff differential solved by Radau methods. J Comput Appl Math 111:93–111
15. Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI, Rigas B (1996) Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. Biochem Pharmacol 52:237–245

16. Huang C, Wu M, Xu G, Li D, Cheng H, Tu ZX, Jiang HQ, Gu JR (1992) Over expression of the mdr1 gene and P-glycoprotein in human hepatocellular carcinoma. J Natl Cancer Inst 84:262–264
17. Johnstone RW, Ruefli AA, Tainton KM, Smyth MJ (2000) A role for P-glycoprotein in regulating cell death. Leuk Lymphoma 38:1–11
18. Kataoka K, Takikawa Y, Lin SD, Suzuki K (2005) Prostaglandin E2 receptor EP4 agonist induces Bcl-xL and independently activates proliferation signals in mouse primary hepatocytes. J Gastroenterol 40:610–616
19. Kawamori T, Rao CV, Seibert K, Reddy BS (1998) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res 58:409–412
20. Koga H, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K, Harada M, Kusaba T, Tanaka M, Kimura R, Nakashima Y, Nakashima O, Kojiro M, Kurohiji T, Sata M (1999) Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor differentiation. Hepatology 29:688–696
21. Kotani M, Tanaka I, Ogawa Y, Usui T, Mori K, Ichikawa A, Narumiya S, Yoshimi T, Nakao K (1995) Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP3 subtype generated by alternative messenger RNA splicing: multiple second messenger systems and tissue-specific distributions. Mol Pharmacol 48:69–79
22. Marroni M, Agrawal ML, Kight K, Hallene KL, Hossain M, Cucullo L, Signorelli K, Namura S, Bingaman W, Janigro D (2003) Relationship between expression of multiple drug resistance proteins and p53 tumor suppressor gene proteins in human brain astrocytes. Neuroscience 121:605–617
23. Nagasue N, Dhar DK, Makino Y, Yoshimura H, Nakamura T (1995) Over expression of P-glycoprotein in adenomatous hyperplasia of human liver with cirrhosis. J Hepatol 22:197–201
24. Ouellet M, Falgueyret JP, Ear PH, Pen A, Mancini JA, Riendeau D (2002) Purification and characterization of recombinant microsomal prostaglandin E synthase-1. Protein Expr Purif 26:489–495
25. Park JG, Lee SK, Hong IG, Kim HS, Lim KH, Choe KJ, Kim WH, Kim YI, Tsuruo T, Gottesman MM (1994) MDR1 gene expression: its effect on drug resistance to doxorubicin in human hepatocellular carcinoma cell lines. J Natl Cancer Inst 86:700–705
26. Patel VA, Dunn MJ, Sorokin A (2002) Regulation of MDR-1 (P-glycoprotein) by cyclooxygenase-2. J Biol Chem 277:38915–38920
27. Roesler WJ, Simard J, Graham JG, McFie PJ (1994) Characterization of the liver-specific component of the cAMP response unit in the phosphoenolpyruvate carboxykinase (GTP) gene promoter. J Biol Chem 269:14276–14283
28. Roller A, Bahr OR, Streffer J, Winter S, Heneka M, Deininger M, Meyermann R, Naumann U, Gulbins E, Weller M (1999) Selective potentiation of drug cytotoxicity by NSAID in human glioma cells: the role of COX-1 and MRP. Biochem Biophys Res Commun 259:600–605
29. Schrenk D, Gant TW, Preisegger KH, Silverman JA, Marino PA, Thorgeirsson SS (1993) Induction of multidrug resistance gene expression during cholestasis in rats and non human primates. Hepatology 17:854–860
30. Shaik MS, Chatterjee A, Jackson T, Singh M (2006) Enhancement of anti-tumor activity of docetaxel by celecoxib in lung tumors. Int J Cancer 118:396–404
31. Silverman JA, Schrenk D (1997) Expression of the multidrug resistance genes in the liver. FASEB J 11:308–313
32. Silverman JA, Thorgeirsson SS (1995) Regulation and function of the multidrug resistance genes in liver. Prog Liver Dis 13:101–123
33. Sukhai M, Piquette-Miller M (2000) Regulation of the multidrug resistance genes by stress signals. J Pharm Pharm Sci 3:268–280
34. Thoren S, Weinander R, Saha S, Jegerschold C, Pettersson PL, Samuelsson B, Hebert H, Hamberg M, Morgenstern R, Jakobsson PJ (2003) Human microsomal prostaglandin E synthase-1: purification, functional characterization, and projection structure determination. J Biol Chem 278:22199–22209
35. Tucker ON, Dannenberg AJ, Yang EK, Zhang F, Teng L, Daly JM, Soslow R, Masferrer JL, Woerner BM, Koki AT, Fahey TJ III (1999) Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. Cancer Res 59:987–990
36. van Wijngaarden J, van Beek E, van Rossum G, van der Bent C, Hoekman K, van der Pluijm G, van der Pol MA, Broxterman HJ, van Hinsbergh VW, Lowik CW (2007) Celecoxib enhances doxorubicin-induced cytotoxicity in MDA-MB231 cells by NF-kappaB-mediated increase of intracellular doxorubicin accumulation. Eur J Cancer 43:433–442
37. Vogel C (2000) Prostaglandin H synthases and their importance in chemical toxicity. Curr Drug Metab 1:391–404
38. Wolff H, Saukkonen K, Anttila S, Karjalainen A, Vainio H, Ristimaki A (1998) Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res 58:4997–5001
39. Xu L, Han C, Lim K, Wu T (2006) Cross-talk between peroxisome proliferator-activated receptor delta and cytosolic phospholipase A(2)alpha/cyclooxygenase-2/prostaglandin E(2) signaling pathways in human hepatocellular carcinoma cells. Cancer Res 66:11859–11868
40. Ziemann C, Schafer D, Rudell G, Kahl GF, Hirsch-Ernst KI (2002) The cyclooxygenase system participates in functional mdr1b overexpression in primary rat hepatocyte cultures. Hepatology 35:579–588