Molecular mechanisms of pruritus in cholestasis
Kremer, Andreas

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Characterization and treatment of persistent hepatocellular secretory failure


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ABSTRACT

Objective: Hepatocellular secretory failure induced by drugs, toxins or transient biliary obstruction may sometimes persist for months after removal of the initiating factor and may then be fatal without liver transplantation. We characterized patients with severe persistent hepatocellular secretory failure (PHSF) and treated them with the pregnane X receptor (PXR) agonist, rifampicin. We also studied the effect of rifampicin on PXR-dependent expression of genes involved in biotransformation and secretion in vitro.

Design: Thirteen patients (age 18-81 years, 6 male) with hepatocellular secretory failure that persisted after removal of the inducing factor (drugs/toxin: 9) or biliary obstruction (4) were identified over 6 years. Six of these patients were screened for ATP8B1 or ABCB11 mutations. All were treated with rifampicin (300 mg daily) for one to ten weeks. Expression of genes involved in biotransformation and secretion was determined by rtPCR in human hepatocytes and intestinal cells incubated with rifampicin (10μmol/L).

Results: Serum bilirubin of patients with PHSF ranged from 264 to 755μmol/L. Normal γGT was found in 10/13 patients of whom 3/6 tested positive for ATP8B1/ABCB11 mutations. Serum bilirubin declined to <33μmol/L after one to ten weeks of rifampicin treatment. In vitro, rifampicin PXR-dependently upregulated biotransformaton phase 1 (CYP3A4), phase 2 (UGT1A1) and phase 3 (MRP2) enzymes/carriers as well as the basolateral bile salt exporter OSTβ.

Conclusion: PHSF may develop in carriers of transporter gene mutations. In severe cases, rifampicin may represent an effective therapeutic option of PHSF. PXR-dependent induction of CYP3A4, UGT1A1, MRP2 and OSTβ could contribute to the anticholestatic effect of rifampicin in PHSF.
INTRODUCTION

Persistent hepatocellular secretory failure (PHSF) after exposure to drugs, toxins, or short-term mechanical biliary obstruction with deep jaundice and progression after removal of the underlying cause is a rare, but often detrimental event. The molecular mechanisms leading to PHSF under these conditions are not understood. Hepatocellular secretion recovers in the majority of patients within days to weeks after stopping the causative drugs or toxins, or removal of the mechanical obstruction.

Medical interventions for PHSF are lacking: Ursodeoxycholic acid (UDCA) has anticholestatic properties in various cholestatic disorders and represents the only approved drug for the treatment of primary biliary cirrhosis (1;2). However, UDCA is ineffective in PHSF when serum bilirubin exceeds 170-255 $\mu$mol/L (10-15 mg/dL) (1;3).

The ligand-activated nuclear receptor, pregnane X receptor (PXR; NR1I2), modulates expression of genes involved in detoxification and elimination of bile salts and other endo- and xenobiotics (4;5). We postulate that treatment with a strong agonist of this transcriptional activator could possibly reverse PHSF.

We defined PHSF as: (i) serum bilirubin $> 255$ $\mu$mol/L ($> 15$ mg/dL), (ii) persistence or increasing elevated bilirubin serum levels ($> 1$ week) after removal of the underlying trigger, (iii) exclusion of obstructive cholestasis by imaging techniques and (iv) no evidence of chronic liver disease before the initiating event (i.e., drug or toxin exposure or transient biliary obstruction by stones or tumor). As therapeutic intervention we chose the pregnane X receptor agonist, rifampicin, which is recommended by European and American guidelines as a second line treatment of severe cholestasis-associated pruritus (1;2).

Here we describe thirteen consecutive patients who fulfilled the diagnosis of PHSF. We searched for a possible genetic background of PHSF in those patients from whom DNA was available. We also investigated the effect of PXR activation by rifampicin on genes involved in biotransformation and secretion (biotransformation phase 1-3) of potential toxins in primary human hepatocytes and human HepG2 hepatoma cells, HepG2 cells
overexpressing PXR (HepG2\textsuperscript{PXR}), HepG2 cells after PXR knock down and the human colon adenocarcinoma HT-29 cell line, for a better molecular understanding of the effects of rifampicin treatment in patients with PHSF.

**PATIENTS AND METHODS**

**Human Subjects.** Between 2007 and 2013 thirteen consecutive patients who fulfilled the criteria for persistent hepatocellular secretory failure [(i) serum bilirubin > 255 \(\mu\)mol/L (>15 mg/dL), (ii) persistence or increasing elevated serum bilirubin levels (> 1 week) after removal of the underlying trigger, (iii) exclusion of obstructive cholestasis by imaging techniques and (iv) no evidence of chronic liver disease before the initiating event] were treated with rifampicin (300 mg per day) for one to ten weeks. Patients with a drug-/toxin-induced PHSF (n=9) were at least two weeks off the PHSF-inducing drug/toxin before start of rifampicin treatment. In transient biliary obstruction-induced PHSF (n=4), patients were treated with (multiple) stents, or ongoing biliary obstruction and dilated bile ducts were excluded by ultrasound, MRCP and/or ERCP before start with rifampicin. Other causes of (chronic) liver disease, such as viral, autoimmune, hereditary metabolic or vascular liver diseases, were excluded by biochemical, imaging and histological approaches before start of rifampicin treatment. During and after rifampicin therapy serum liver tests (ALT, AST, \(\gamma\)GT, alkaline phosphatase, bilirubin) were closely monitored to foresee possible hepatotoxicity. The patients were informed about the possible side effects of rifampicin. Since this is an FDA and EMA approved and guideline-recommended drug for use in cholestasis no formal informed consent had to be signed according to the local Medical Ethics Committee’s advice.

**Mutation analysis of \textit{ATP8B1} and \textit{ABCB11}**. All coding exons with flanking intronic sequences of the \textit{ATP8B1} and \textit{ABCB11} genes were sequenced after PCR amplification of DNA from peripheral blood mononuclear cells of 6 patients with normal \(\gamma\)GT after informed consent and were compared to references sequences.
Materials for in vitro experiments. Rifampicin, dimethyl sulfoxide (DMSO) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Steinheim, Germany). Forskolin was purchased from Ascent Science (Cambridge, UK). 6-Ethyl chenodeoxycholate (6-ECDC; INT-747; obeticholic acid) was purchased from Intercept Pharmaceuticals (New York, US). All other chemicals were of the highest purity available.

Primary Human Hepatocyte Isolation. Mature primary human hepatocytes were isolated from liver tissue specimens of three female patients (age between 32 and 40 years) undergoing partial hepatectomy for large liver adenoma. Macroscopic adenoma-free liver tissue was used to isolate primary hepatocytes. Tissue weight ranged from 1 to 4 gram. This procedure was approved by the Medical Ethical Committee of the Academic Medical Center Amsterdam. Before every procedure informed consent was obtained from each patient. Hepatocytes were isolated from small resection samples of human liver by a two-step collagenase treatment, as described by Seglen (6). Cells were cultured in complete Williams E Medium, containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 μmol/L dexamethason-disodiumphosphate, 20 mU/L insulin, 100 U/mL penicillin, 100U/mL streptomycin, 0.25 μg/mL Fungizon, and 1 mmol/L ornithine hydrochloride. Cells were seeded in Primiria 6 wells plates (BD bioscience) at a density of 1×10⁶ cells/well; after 4 hours medium was refreshed. The next day subconfluent cells were incubated for 24 hours in complete Williams E medium containing 10 μmol/L rifampicin or the solvent 0.1% DMSO only (vehicle control).

Cell culture. Human HepG2 hepatoma cells over-expressing PXR and PXR knock-down HepG2 cells were generated using lentiviral transduction as reported previously (7;8). These and control HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Lonza BioWhittaker, Cologne, Germany) supplemented with 10% fetal calf serum, 4 mmol/L L-glutamine and a mixture of antibiotics (5 mg/mL penicillin, 5 mg/mL streptomycin). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. For studying the effect of rifampicin, cells were seeded in 6-well plates at a density of
8×10^5 cells/well until reaching 80% confluence. Subconfluent cells were cultured overnight in serum-free medium containing 0.2% BSA. Following brief washing, cells were incubated for 24 hours in DMEM/0.2% BSA containing 10 μmol/L rifampicin, 10 μmol/L 6-ECDC (INT-747) or rifampicin plus 6-ECDC (10 μmol/L, each). As a solvent control 0.1% DMSO was added to control cells. The human colon adenocarcinoma HT-29 cell line was grown in DMEM supplemented with 10% fetal calf serum, 4 mmol/L L-glutamine and a mixture of antibiotics (5 mg/mL penicillin, 5 mg/mL streptomycin). Cells were incubated at 37°C in a humidified atmosphere containing 10% CO₂. HT-29 cells were differentiated and polarized as described by Cohen (9). Briefly, HT-29 cells were seeded in 6-well plates at a density of 8×10^5 cells/well and were grown up to 20 days and then treated with forskolin for 20 hours. After forskolin was removed the cells were rested for 24 hours in supplemented DMEM. Following brief washing, cells were incubated for 24 hours in DMEM/0.2% BSA containing 10 μmol/L rifampicin or 0.1% DMSO.

**RNA isolation and quantification of transcript levels.** Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) and used to generate cDNA with an oligo-dT primer and Superscript III reverse transcriptase (Invitrogen). Realtime PCR was performed at 60°C in a Lightcycler apparatus (Roche) with Lightcycler Faststart DNA Master Plus CYBR Green I (Roche). Transcript levels were normalized to the housekeeping gene 36B4 (acidic ribosomal phosphoprotein P0). Primers used for quantitative PCR experiments are listed in Supplementary Table 1.

**Statistical Analysis**

Statistical differences were evaluated for two groups by Student’s *t*-test and for three or more groups by one-way ANOVA with Bonferroni correction using SPSS (version 18.0). All data are expressed as means ± standard deviations (SD).
RESULTS

Rifampicin normalizes serum bilirubin in patients with persistent hepatocellular secretory failure of different etiology

Between 2007 and 2013, we treated thirteen consecutive patients with rifampicin who presented with PHSF that was progressive after stopping the suspected toxins, drugs, or total parenteral nutrition or after resolving of a mechanical obstruction by stone removal or stenting. The patients diagnosed with PHSF comprised 7 females and 6 males (Table 1). The age at time of onset ranged from 18 to 81 years. Various events contributed to the development of PHSF. One patient developed PHSF after exposure to high amounts of volatile spray-paint (patient A), two patients after antibiotic treatment with flucloxacillin or clavulanic acid (B and C), two (American) patients after abusing anabolic steroids (D and E), three patients after the use of estradiol supplementation (F, G and H) and one patient after start of total parenteral nutrition due to short bowel syndrome (I). Four patients developed PHSF after successful removal of obstruction of the common bile duct due to benign (J, K and L) or malignant obstruction (patient M) (Figure 1 and Table 1).

All patients remained severely jaundiced or even showed increasing serum bilirubin levels despite removal of the initiating cause of cholestasis. Imaging techniques including ultrasound, CT, MRCP and ERCP were used to rule out biliary obstruction in patients. Other causes of (chronic) liver disease were excluded by biochemical and histological markers (Table 1) before treatment with rifampicin (300 mg/day) was initiated. Eleven patients were treated in the Netherlands and two patients were treated in the United States (patients D and E). At the start of rifampicin therapy serum total bilirubin levels ranged from 264 to 751 μmol/L (normal 0-17 μmol/L) (Figure 1), serum transaminases and alkaline phosphatase ranged from 1.5 to 5 times the upper limit of normal (ULN). Ten patients showed normal serum levels of γGT at start of rifampicin treatment. Serum bile salt levels could only be determined in two patients (patient A and K), and were markedly elevated (268 and 225 μmol/L, respectively) at the start of rifampicin treatment.

Duration of rifampicin therapy ranged from one to ten weeks (Table 1). After the start of rifampicin treatment, serum total bilirubin declined rapidly in all patients (Figure 1),
as did serum alkaline phosphatase in the majority of patients (Supplementary figure 1). During and shortly after rifampicin treatment patients were regularly checked for signs and symptoms of adverse events of rifampicin. No side effects were noted. Rifampicin therapy was interrupted in patient L after 3 weeks and was restarted when total serum bilirubin increased again. This led to normalization of serum bilirubin within 8 weeks (patient L Figure 1B).

Taken together, rifampicin dramatically improved hepatobiliary secretion in all thirteen patients with PHSF. No side effects were observed.

**Genetic background of PHSF: Sequencing analysis of ATP8B1 and ABCB11**

Cholestasis with normal serum γGT activity is characteristic for progressive familial intrahepatic cholestasis types 1 and 2 (10). We were able to retrieve genomic DNA from six patients of our PHSF cohort for diagnostic sequencing of both genes. In 3/6 patients mutations were found.

In patient F, sequencing analysis revealed a homozygous mutation in ATP8B1, c.1982T>C leading to an amino acid change Ile661Thr1. This mutation has been described to cause benign recurrent intrahepatic cholestasis type 1 (BRIC1) (11) the symptoms of which had not been observed before and after the episode of PHSF in our patient F.

Two heterozygous mutations in ABCB11 were disclosed in patients G and H, respectively (Table 2). In patient G, a known PFIC type 2 mutation, c.890A>G leading to a Glu297Gly amino acid change was found (12). In patient H the mutation c.2809 G>A, resulting in a Gly937Arg amino acid change, has not been reported so far in PFIC or BRIC type 2 patients. Recent publications have shown that heterozygous mutations and polymorphisms of ABCB11 may play a role in the development of drug induced liver injury (13-15).
Table 1. Patient characteristics of 13 consecutive patients with severe persistent hepatocellular secretory failure (PHSF) successfully treated with rifampicin

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Underlying cause of cholestasis</th>
<th>Lab tests at the start of rifampicin treatment</th>
<th>Obstructive cholestasis excluded by</th>
<th>Liver histology</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Male</td>
<td>54</td>
<td>Toxic hepatocellular injury: Obese man (BMI 40 kg/m²), developed jaundice and pruritus weeks after paintbrushing 20–25 motorbikes without adequate airway protection (identical episode with identical history 5 years before)</td>
<td>Bilirubin 264 μmol/L, AP 216 μmol/L, γ-GT 107 U/L, AST 162 U/L, ALT 97 U/L, ANA, AMA, LKM neg., HAV, HBV, HCV neg.</td>
<td>Z x US</td>
<td>Extensive steatosis, steatohepatitis and marked intracellular cholestasis</td>
<td>(Pretreatment with cholestyramine, colestevam and naltrexon without effect on pruritus) Rifampicin 300 mg od for 25 days; complete recovery</td>
</tr>
<tr>
<td>B</td>
<td>Female</td>
<td>53</td>
<td>DILI: Fluvoxacin-induced liver and bile duct injury; jaundice and pruritus started 1 week after a 3-week course of fluvoxacin</td>
<td>Bilirubin 278 μmol/L, AP 298 μmol/L, γ-GT 39 U/L, AST 43 U/L, ALT 51 U/L, IgG and IgM normal, ANA, ANCA neg., AMA pos., HAV, HBV, HCV neg.</td>
<td>US MRC</td>
<td>Mild portal inflammation, portal fibrosis and ductopenia, as well as extensive cholestasis compatible with fluvoxacin-induced liver injury, but not PBC</td>
<td>UDCA (450 mg od for 4 weeks without success) Rifampicin, 300 mg od for 4 weeks followed by budesonide 3 mg bid; incomplete biochemical recovery possibly because of ductopenia (fluvoxacin) (UDCA 300 mg tid without effect) Rifampicin 150 mg bid for 10 weeks; complete recovery</td>
</tr>
<tr>
<td>C</td>
<td>Male</td>
<td>76</td>
<td>DILI: Clavulanate-induced liver and bile duct injury after cholecystitis</td>
<td>Bilirubin 487 μmol/L, AP 183 μmol/L, γ-GT 42 U/L, AST 201 U/L, ALT 186 U/L, ANA, AMA, ANCA neg., HAV, HBV, HCV neg.</td>
<td>US CT MRC</td>
<td>Mild cholangitis, extensive cholestasis, compatible with clavulanate-induced hepatopathy</td>
<td>Rifampicin 150 mg bid for 10 weeks; complete recovery</td>
</tr>
<tr>
<td>D</td>
<td>Male</td>
<td>19</td>
<td>DILI: Anabolic steroid-induced liver injury with jaundice and pruritus</td>
<td>Bilirubin 755 μmol/L, AP 250 μmol/L, γ-GT 28 U/L, AST 162 U/L, ALT 65 U/L</td>
<td>US MRC HIDA-</td>
<td>Marked non-inflammatory canalicul cholestasis suggestive of anabolic steroid ingestion</td>
<td>Rifampicin 150 mg od for 3 days, then increased to 300 mg od for 3 weeks; complete recovery</td>
</tr>
<tr>
<td>E</td>
<td>Male</td>
<td>19</td>
<td>DILI: Anabolic steroid-induced liver injury with jaundice and pruritus</td>
<td>Bilirubin 503 μmol/L, AP 577 μmol/L, AST 46 U/L, ALT 36 U/L, γ-GT 25 U/L, HAV, HBV, HCV, EBV neg., ANA, AMA, IgG neg.</td>
<td>US MRC</td>
<td>—</td>
<td>Rifampicin 300 mg od for 3 weeks, then for 1 week 150 mg od; After rapid recovery, rifampicin treatment was stopped.</td>
</tr>
<tr>
<td>F</td>
<td>Female</td>
<td>50</td>
<td>DILI: Oestriadiol-induced liver injury with jaundice and pruritus</td>
<td>Bilirubin 383 μmol/L, AP 556 μmol/L, AST 45 U/L, ALT 33 U/L, γ-GT 64 U/L, HAV, HBV, HCV, EBV neg., ANA neg., ANA slightly pos.</td>
<td>US CT</td>
<td>—</td>
<td>Rifampicin 300 mg od for 4 weeks. After rapid recovery, rifampicin treatment was stopped</td>
</tr>
<tr>
<td>G</td>
<td>Female</td>
<td>18</td>
<td>DILI: Oestriadiol-induced liver injury with jaundice and pruritus</td>
<td>Bilirubin 593 μmol/L, AP 178 μmol/L, AST 87 U/L, ALT 186 U/L, γ-GT 27 U/L, HAV, HBV, HCV, HEV, EBV, CMV, HIV neg., ANA, ANA, ANCA, SMA neg.</td>
<td>US</td>
<td>Marked non-inflammatory canalicul and cytoplasmatic cholestasis, suggestive of DILI</td>
<td>(Pretreatment with cholestyramine and naltrexon without effect on pruritus) Rifampicin 300 mg od for 70 days; complete recovery</td>
</tr>
</tbody>
</table>

Persistent hepatocellular secretory failure
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Underlying cause of cholestasis</th>
<th>Lab tests at the start of rifampicin treatment</th>
<th>Obstructive cholestasis excluded by</th>
<th>Liver histology</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>Female</td>
<td>31</td>
<td>DILI: Oestrogen-induced liver injury with jaundice and pruritus</td>
<td>Bilirubin 565 μmol/L, AP 347 U/L, γ-GT 23 U/L, AST 70 U/L, ALT 55 U/L, HAV, HBV, HCV, EBV neg.</td>
<td>US MRC</td>
<td>–</td>
<td>Rifampicin, 150 mg bid for 2 weeks followed by UDCA 900 mg bid with complete recovery</td>
</tr>
<tr>
<td>I</td>
<td>Female</td>
<td>50</td>
<td>Total parenteral nutrition-induced hepatocellular cholestasis; renal failure with dialysis-associated sclerosing peritonitis and short bowel syndrome</td>
<td>Bilirubin 268 μmol/L, AP 302 U/L, AST 94 U/L, ALT 131 U/L, γ-GT 148 U/L, HAV, HBV, HCV neg.</td>
<td>US</td>
<td>–</td>
<td>Rifampicin 300 mg od; after 4 days of therapy also switch to fat-reduced TPN; rapid recovery</td>
</tr>
<tr>
<td>J</td>
<td>Male</td>
<td>57</td>
<td>Transient biliary obstruction because of choledocholithiasis with colicky RUQ pain and acholic stool</td>
<td>Bilirubin 577 μmol/L, AP 298 U/L, γ-GT 164 U/L, AST 62 U/L, ALT 81 U/L, HAV, HBV, HCV neg.</td>
<td>2× US ERC</td>
<td>Cholestasis and mild nonspecific portal alterations</td>
<td>Rifampicin, 150 mg bid for 2 weeks followed by UDCA 300 mg bid and cholecystectomy; complete recovery</td>
</tr>
<tr>
<td>K</td>
<td>Male</td>
<td>57</td>
<td>Transient biliary obstruction because of choledocholithiasis with colicky RUQ pain and acholic stools</td>
<td>Bilirubin 358 μmol/L, AP 314 U/L, γ-GT 43 U/L, AST 114 U/L, ALT 128 U/L, SMA, AMA, LKM-1, SLA, ANA neg., Anti-HBs pos., HBs-Ag, HBe-Ag neg., HAV, HCV, HIV neg.</td>
<td>4× US ERC</td>
<td>Extensive cholestasis and degenerative alterations</td>
<td>(UDCA 300 mg tid and Colestyrine 4 g od without effect); Rifampicin 300 mg od for 4 weeks followed by cholecystectomy; complete recovery</td>
</tr>
<tr>
<td>L</td>
<td>Female</td>
<td>34</td>
<td>Transient bile duct stenosis after hemi-hepatectomy for colon ca metastasis; biloma after bile leakage (index patient)</td>
<td>Bilirubin 455 μmol/L, AP 183 U/L, γ-GT 33 U/L, AST 186 U/L, ALT 115 U/L.</td>
<td>US CT ERC PTC</td>
<td>–</td>
<td>Rifampicin 150 mg bid for 10 weeks (intermittently stopped for 18 days after 2 weeks of therapy due to a single elevated serum creatinine level (incorrect measurement); complete recovery</td>
</tr>
<tr>
<td>M</td>
<td>Female</td>
<td>81</td>
<td>Transient biliary obstruction because of pancreatic carcinoma</td>
<td>Bilirubin 436 μmol/L, AP 183 U/L, γ-GT 33 U/L, AST 186 U/L, ALT 115 U/L, IgG neg.</td>
<td>US CT 3× ERC (with patent stents)</td>
<td>–</td>
<td>Rifampicin 150 mg bid for 3 weeks. After 4 weeks pylorus-preserving pancreateo-duodenectomy with complete recovery</td>
</tr>
</tbody>
</table>

Normal range of serum lab tests: Total bilirubin (0–17 μmol/L), alkaline phosphatase (AP, 40–120 U/L), AST (0–40 U/L), ALT (0–45 U/L), γ-GT (0–60 U/L).
Persistent hepatocellular secretory failure

Figure 1: Rifampicin improves hepatobiliary secretion in patients with persistent hepatocellular secretory failure.
Total serum bilirubin (μmol/L; after removal of the cause of liver injury) before, during and after treatment with rifampicin in (A) patients with toxin-/ drug-/total parenteral nutrition-induced persistent hepatocellular secretory failure and (B) patients with persistent hepatocellular secretory failure induced after short-term biliary obstruction. Week 0 indicates start of rifampicin treatment.

α: End of first period of treatment with rifampicin in patient L
β: Begin of second period of treatment with rifampicin in patient L
Rifampicin induces expression of genes involved in transport and detoxification in the enterohepatic circulation by PXR-dependent mechanisms.

To understand the effect of the PXR activator rifampicin in PHSF, we investigated its effect on the expression of genes involved in biotransformation and secretion (biotransformation phase 1-3) of potential toxins in liver- and intestine-derived cell lines. In primary human hepatocytes rifampicin stimulated the expression of PXR-sensitive genes: Cytochrome P450 3A4 (CYP3A4; phase 1), responsible for the hydroxylation of bile salts and drugs, and UDP-glucuronosyltransferase 1A1 (UGT1A1; phase 2), responsible for glucuronidation of bilirubin. After 24 hrs both were induced by 10 μM (Figure 2) confirming data previously reported (16;17). Also the expression of the apical ATP-binding cassette (ABC) transporter MRP2 (ABCC2), responsible for the biliary secretion of bilirubin glucuronides and other conjugated organic anions (phase 3), was 1.5-fold induced by this concentration of rifampicin (Figure 2) as described previously (18).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Underlying cause of cholestasis</th>
<th>ATP8B1</th>
<th>ABCB11 (BSEP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>DILI: Estradiol</td>
<td>c.1982T&gt;C</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>DILI: Estradiol</td>
<td>c.1982T&gt;C</td>
<td>p.Glu297Gly (Heterozygote)</td>
</tr>
<tr>
<td>H</td>
<td>DILI: Estradiol</td>
<td>c.890A&gt;G</td>
<td>p.Gly937Arg (Heterozygote)</td>
</tr>
</tbody>
</table>

Table 2: ATP8B1 and ABCB11 (BSEP) mutations in patients with PHSF.

3 Mutation not earlier published.

The organic solute transporter (OSTα-OSTβ) is expressed on the basolateral membrane of epithelial cells mainly of the ileum and colon, liver, kidney and testis were it functions as a transporter of bile acid and steroid conjugates (19;20). Both OSTα and OSTβ are upregulated by farnesoid X receptor (FXR)-dependent mechanisms (21). Notably, OSTβ expression was 67-fold induced in liver tissue of patients with severe obstructive cholestasis, while OSTα expression was induced 3-fold under these conditions (22).
suggests that modulation of OSTβ expression seems to play a critical role in the protection of a cholestatic liver. Here, we found that OSTβ, but not OSTα expression was 2.5-fold induced in primary human hepatocytes exposed to rifampicin (Figure 2).

To prove that rifampicin induces gene expression via the pregnane X receptor (PXR), we subsequently studied its effect in the human HepG2 hepatoma cell line, a HepG2PXR cell line overexpressing PXR, and a HepG2 cell line in which PXR was knocked down. The difference in CYP3A4, UGT1A1, MRP2 and OSTβ expression seen between HepG2 (over)-expressing PXR and HepG2 lacking it indicated that rifampicin indeed induces these genes via PXR-dependent mechanisms (Supplementary Figure 2A-C).

PXR is also expressed in the human intestinal tract. To assess the effect of rifampicin on intestinal cells, we chose the polarized and differentiated HT-29 cell line. Again, rifampicin induced CYP3A4, MRP2 and OST-β expression and tended to induce UGT1A1 expression (p=0.08) (Supplementary Figure 2D).

In contrast to a previous report (23), PXR activation by rifampicin did not lead to induction of the basolateral exporter MRP3 (ABCC3) in primary human hepatocytes and
HepG2 cells, whereas a trend towards induction of MRP3 ($p=0.06$) was observed in HepG2$^{PXR}$ cells (Supplementary Figure 4).

OST$\alpha$-OST$\beta$ serves as an escape cellular efflux path. OST$\alpha$-OST$\beta$ is controlled by the nuclear bile salt receptor FXR. In cholestatic hepatocytes its expression prevents intracellular accumulation of toxic hydrophobic bile salts. To investigate whether PXR and FXR may have cooperative effects on OST$\alpha$-OST$\beta$ expression, we incubated HepG2 cells with rifampicin and the potent FXR agonists, 6-ECDC (INT-747), a well-known inducer of OST$\alpha$-OST$\beta$ mRNA expression. As reported previously, after 24 hr the expression of both OST$\alpha$ and OST$\beta$ was significantly induced by 10 $\mu$M of 6-ECDC (6.2- and 39.0-fold, respectively, Supplementary Figure 3A and 3B). Addition of the PXR agonist, rifampicin had a substantial additive effect on OST $\beta$ expression leading to a 49.8 fold induction in comparison to vehicle control. This additional effect was abolished in HepG2 cells after knock down of PXR (Supplementary Figure 3D).

**DISCUSSION**

Persistent hepatocellular secretory failure (PHSF) is a rare, but life-threatening complication of acute liver injury induced by drugs, toxins, or short-term mechanical obstruction for which no effective treatment is known. In the present study we demonstrate that thirteen consecutive patients who fulfilled the criteria for PHSF were successfully treated with the potent PXR agonist rifampicin. Our findings in human liver and intestinal cells *in vitro* suggest that rifampicin exerts these beneficial effects at least in part by upregulating key detoxification enzymes and export pumps including apical MRP2 and basolateral OST$\beta$ by PXR-dependent mechanisms. Induction of genes involved in detoxification and elimination of bilirubin, bile salts and other endo- and xenobiotics may represent a novel therapeutic strategy in PHSF.

In nine patients, PHSF was observed after toxin- (ill-protected volatile paint-brushing; patient A), drug- (DILI induced by flucloxacillin, clavulanate, anabolic steroid abuse, and estradiol; patients B-H), or total parenteral nutrition-induced liver injury (patient
J) (Figure 1A). Although only 1 in 10,000 to 100,000 patients usually develops DILI due to a specific drug, DILI accounts for 5 to 10% of patients hospitalized for jaundice (24). A recent retrospective study showed that 10% of patients with DILI either died or underwent a liver transplantation (25). Serum bilirubin was significantly increased in deceased patients or those who underwent liver transplantation, and was an independent predictor of mortality (25;26).

In four patients, PHSF developed after previous mechanical obstruction of the biliary tract (choledocholithiasis; transient bile duct stenosis after hemihepatectomy; bile duct stenosis due to pancreatic carcinoma; patients J-M; Figure 1B). Treatment of the initial cause of cholestasis in these patients by removal of the underlying stenosis, and afterwards conformation of a non-obstructive biliary tract by various imaging techniques, did not result in improvement of the jaundice.

Ten of thirteen severely cholestatic patients showed normal serum γGT during the course of PHSF, a clinical characteristic of PFIC and BRIC type 1 and 2. This observation suggested a role for ATP8B1 and/or ABCB11 (BSEP) in the development of PHSF. Therefore, sequence analysis of both genes was performed in six of the PHSF patients who were able and willing to provide blood for analysis and had a normal J-GT. A homozygous mutation was identified in ATP8B1, c.1982T>C leading to an amino acid change Ile661Thr1 in patient F which had been reported in BRIC1 patients (11). BRIC1 is a rare hereditary disease caused by a mutation in ATP8B1 characterized by recurrent episodes of jaundice with pruritus and normal γGT levels (27). Patient F had no clinical or biochemical history of BRIC1, but developed PHSF after the start of a course of estradiol, which has been reported to induce a cholestatic episode in BRIC1 patients (27). In the past rifampicin has been used in the treatment for both low γGT-PFIC and BRIC patients. Although rifampicin relieved pruritus in patients with low γGT-PFIC, it had only a temporary effect on serum bilirubin levels. In contrast, rifampicin was shown to be effective in BRIC, resolving eighteen out of twenty-two cholestatic episodes in seven patients (28). Rifampicin induces hydroxylation and conjugation of bile acids due to upregulation of CYP3A4 and UGT1A1. This results in an increased urinary bile acid excretion. Patient F rapidly
responded to rifampicin with regard to total serum bilirubin and pruritus, and recovered completely from her cholestatic episode,

Heterozygous mutations in \( ABCB11 \) were found in two of six patients analyzed (G, H). Notably, heterozygous mutations of \( ABCB11 \) are known to be associated with drug-induced cholestasis (14).

We treated our patients with rifampicin (300 mg/day), a potent activator and high affinity ligand of the pregnane X receptor (PXR). PXR acts as a broad-specificity sensor of xeno- and endobiotics and regulates expression of various target genes predominantly in liver and intestine involved in uptake, detoxification and excretion of endo- and xenobiotics (biotransformation phase 0-3) and bile salt synthesis (29;30). Here, we confirmed the effect of rifampicin \textit{in vitro} on key detoxification enzymes and exporters including CYP3A4, UGT1A1, and MRP2 in different human liver and intestinal cell lines.

The organic solute transporter \( \alpha - \beta \) (OST\( \alpha \)-OST\( \beta \)) is responsible for secretion of bile salts and conjugated steroids across the basolateral membrane of hepatocytes into the portal circulation (31). OST\( \beta \) is critically required for transport activity of the OST\( \alpha \)-OST\( \beta \) heterodimer (32). The nuclear receptor FXR is considered to be the main regulator of OST\( \alpha \) and OST\( \beta \) expression (21). Here, we demonstrate that rifampicin induces OST\( \beta \) in both human liver and intestinal cell lines in a PXR-dependent manner. Notably, rifampicin had an additive effect on OST\( \beta \) expression during potent FXR activation. In human liver, a 7-fold higher expression of OST\( \alpha \) than OST\( \beta \) has been described. Upregulation of OST\( \beta \) will affect the transport activity of the heterodimer and help in the removal of cholestatic toxins from the hepatocyte. Rifampicin also reduces the expression of cholesterol 7\( \alpha \)-hydroxylase (CYP7A1) thereby reducing the de novo synthesis of bile salts (33).

Prolonged treatment with rifampicin for four to twelve weeks is associated with hepatotoxicity particularly under cholestatic conditions in up to 12% (34) whereas treatment periods of less than two weeks appear safe in patients with chronic cholestasis (35). No rifampicin-related hepatotoxicity was observed in our cohort.
In conclusion, we show that short-term treatment with the PXR agonist rifampicin appears to be safe and effective in patients with PHSF. PHSF is a new disease entity defined by persistence of cholestasis for > 1 week after stopping the causative drug or toxin or removal of the extrahepatic biliary blockade. In some of these patients we found genetic defects of hepatocellular transport. We are aware that these are uncontrolled observations but the effect of rifampicin in these patients seems robust enough to warrant a large placebo-controlled study with cross-over design.

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SUPPLEMENTARY DATA

Supplementary Figure 1: Serum Alkaline Phosphatase in patients with persistent hepatocellular secretory failure. Serum alkaline phosphatase (U/L; after removal of the cause of liver injury) before, during and after treatment with rifampicin in patients with persistent hepatocellular secretory failure. Week 0 indicates start of rifampicin treatment. Patient B developed histology-proven ductopenia with an increase of alkaline phosphatase over months and recovery after combined treatment with UDCA and budesonide. For patient L see legend figure 1.
Supplementary Figure 2: Rifampicin induces CYP3A4, UGT1A1, MRP2 and OST-β in human HepG2 hepatoma cells and human intestinal HT-29 cells in a PXR-dependent manner. (A) Human HepG2 hepatoma cells, (B) human HepG2 cells overexpressing PXR, (C) human HepG2 cells after successful knockdown of PXR with short-hairpin RNA lentiviral transduction and (D) human colonic HT-29 cells were incubated with rifampicin (10 μM, dark grey bars) or DMSO (0.1%, v/v) for 24 hrs (n=3-6). Total RNA was isolated to generate cDNA and CYP3A4, UGT1A1, MRP2, MRP3, OST-α and OST-β mRNA levels were determined using qPCR using 36B4 as a reference gene. Data are presented as mean ± SD; * p<0.05, ** p<0.01.
Supplementary Figure 3: PXR and FXR co-activation induces OST-β expression in human HepG2 hepatoma cells. (A) OST-α and OST-β mRNA levels were determined in human HepG2 hepatoma cells after incubation with rifampicin (10μM), 6-ECDC (10μM) or co-incubation of rifampicin and 6-ECDC for 24 hrs. (C) OST-α and OST-β mRNA levels were determined in human HepG2 hepatoma with knockdown of PXR after incubation with rifampicin (10μM), 6-ECDC (10μM) or co-incubation of rifampicin and 6-ECDC for 24 hrs. (n=3) Data are presented as mean ± SD; * p<0.05, ** p<0.01.
Supplementary Figure 4: MRP3 expression is marginally affected by rifampicin in human liver cells. Expression of the basolateral organic anion transporter MRP3 (ABCC3) is not altered in (A) primary human hepatocytes, (B) the human hepatoma cell line HepG2 and (D) the differentiated intestinal HT-29 cell line, but tends to increase (p=0.06) in (C) HepG2-PXR cells after PXR activation with rifampicin (10 μmol/L, 24 hrs). Mean ± SD, n=3.