Mechanisms Underlying Benign and Reversible Unconjugated Hyperbilirubinemia Observed with Faldaprevir Administration in Hepatitis C Virus Patients

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ABSTRACT

Faldaprevir, an investigational agent for hepatitis C virus treatment, is well tolerated but associated with rapidly reversible, dose-dependent, clinically benign, unconjugated hyperbilirubinemia. Multidisciplinary preclinical and clinical studies were used to characterize mechanisms underlying this hyperbilirubinemia. In vitro, faldaprevir inhibited key processes involved in bilirubin clearance: UDP glucuronosyltransferase (UGT) 1A1 (UGT1A1) (IC50 0.45 μM), which conjugates bilirubin, and hepatic uptake and efflux transporters, organic anion–transporting polypeptide (OATP) 1B1 (IC50 0.57 μM), OATP1B3 (IC50 0.18 μM), and multidrug–resistance–associated protein (MRP) 2 (IC50 6.2 μM), which transport bilirubin and its conjugates. In rat and human hepatocytes, uptake and biliary excretion of [3H]bilirubin and its glucuronides decreased on coincubation with faldaprevir. In monkeys, faldaprevir (20 mg/kg per day) caused reversible unconjugated hyperbilirubinemia, without hemolysis or hepatotoxicity. In clinical studies, faldaprevir-mediated hyperbilirubinemia was predominantly unconjugated, and levels of unconjugated bilirubin correlated with the UGT1A1*28 genotype. The reversible and dose-dependent nature of the clinical hyperbilirubinemia was consistent with competitive inhibition of bilirubin clearance by faldaprevir, and was not associated with liver toxicity or other adverse events. Overall, the reversible, unconjugated hyperbilirubinemia associated with faldaprevir may predominantly result from inhibition of bilirubin conjugation by UGT1A1, with inhibition of hepatic uptake of bilirubin also potentially playing a role. Since OATP1B1/1B3 are known to be involved in hepatic uptake of circulating bilirubin glucuronides, inhibition of OATP1B1/1B3 and MRP2 may underlie isolated increases in conjugated bilirubin. As such, faldaprevir-mediated hyperbilirubinemia is not associated with any liver injury or toxicity, and is considered to result from decreased bilirubin elimination due to a drug-bilirubin interaction.

Introduction

Bilirubin is a natural degradation product of heme. With the exception of newborn infants, who are at risk for acute bilirubin encephalopathy and kernicterus, bilirubin is clinically benign in older children and adults (Chowdhury et al., 2001). Bilirubin is tightly bound to albumin and is rapidly cleared from the body, predominantly by the liver via glucuronidation and biliary elimination. Uptake of unconjugated bilirubin into the liver may occur in part via organic anion–transporting polypeptide (OATP) 1B1 and OATP1B3, encoded by SLCO1B1 and SLCO1B3 genes, respectively (van de Steeg et al., 2012), although other mechanisms have also been postulated. Within the liver, bilirubin is exclusively glucuronidated by UDP glucuronosyltransferase (UGT) 1A1 (UGT1A1) to mono- and diglucuronide conjugate forms (Rotger et al., 2005) and then effluxed into the bile by multidrug resistance–associated protein (MRP) 2, encoded by the ABCBC2 gene (Hashimoto et al., 2002). OATP1B1 and OATP1B3 have also recently been reported to have a role in the hepatic uptake and clearance of circulating bilirubin conjugates (van de Steeg et al., 2012). This was evident in Oatp knockout mice, in which a substantially high increase in circulating levels of bilirubin mono- and diglucuronides was demonstrated, compared with only a mild 1.8-fold increase in circulating levels of unconjugated bilirubin (van de Steeg et al., 2012).

Unconjugated, or indirect, hyperbilirubinemia (indirect bilirubin >85% of total bilirubin) can be caused by increased bilirubin production (e.g., hemolysis), inherited defects in...
bilineal processing (e.g., reduced UGT1A1 activity in Gilbert’s syndrome), or drug-mediated reduction in hepatic uptake and/or conjugation of bilirubin (i.e., drug-bilirubin interaction) (Lind and Hyde, 2003). Conjugated, or direct, hyperbilirubinemia (direct bilirubin increases to >50% of total bilirubin) can similarly be caused by inherited defects or drug-mediated reduction in clearance of conjugated bilirubin (e.g., MRP2 polymorphism of Dubin-Johnson syndrome, absence of functional OATP1B1 and OATP1B3 inRotor syndrome, or parenchymal liver disease and biliary obstruction) (Lind and Hyde, 2003).

Iatrogenic elevations in bilirubin can arise during treatment with a wide range of drugs (Ah et al., 2008), including antiviral therapy (Korenblat and Berk, 2005). The mechanisms underlying bilirubin increases are multiple, and the exact mechanisms may be different or somewhat overlapping for various drugs (Korenblat and Berk, 2005). For example, atazanavir; indinavir, rifampicin, and rifampicin are all associated with unconjugated hyperbilirubinemia (Vavricka et al., 2002; Rotger et al., 2005). However, atazanavir and indinavir inhibit UGT1A1 (Zhang et al., 2005) and possibly OATP1B1 (Campbell et al., 2004; Annaert et al., 2010), whereas rifampicin and rifampicin appear to exert their effects predominantly via OATP inhibition, in particular OATP1B1 and OATP1B3 (Vavricka et al., 2002). It is clinically important to determine whether drug-induced hyperbilirubinemia is a consequence of hepatotoxicity, which typically presents as elevation of conjugated or mixed bilirubin and, more importantly, accompanying increases in serum aminotransferases and alkaline phosphatase (Korenblat and Berk, 2005). In contrast, predominantly unconjugated hyperbilirubinemia can often indicate either an extrahepatic effect of the drug, such as hemolysis, or its interference with a specific aspect of bilirubin disposition (Korenblat and Berk, 2005).

Ribavirin (RBV) is still an integral part of the treatment of hepatitis C virus (HCV) infection (McHutchison et al., 2009). The main side effect of RBV is hemolytic anemia, which increases the heme load, resulting in usually mild, indirect hyperbilirubinemia that is isolated (i.e., with no other clinical symptoms indicative of liver injury) (Korenblat and Berk, 2005; McHutchison et al., 2009; Perico et al., 2009). Hyperbilirubinemia or anemia has been reported during trials of a number of other HCV protease inhibitors currently approved or in clinical development, such as telaprevir (Zeuzem et al., 2011), asunaprevir (Suzuki et al., 2013), daclatasvir (Suzuki et al., 2013), simprevir (Manns et al., 2011b), and ABT-450 (paritaprevir; AbbVie, Chicago, IL; Poordad et al., 2013). Second-wave direct-acting antivirals, including simprevir, sofosbuvir, and faldaprevir, demonstrate better efficacy over a shorter treatment duration and improved safety profiles, compared with earlier direct-acting antivirals (Asselah and Marcellin, 2014).

Faldaprevir is a highly potent and selective noncovalent NS3/4A protease inhibitor (White et al., 2010) that has demonstrated a strong antiviral response in clinical studies (Manns et al., 2011a). A phase II study in treatment-naïve patients with chronic HCV genotype (G) 1 infection reported significantly improved sustained virologic response rates with faldaprevir in combination with pegylated interferon (PEG-IFN) plus RBV (72–84%) versus Peg-IFN plus RBV alone (56%) (Sulkowski et al., 2013a). Substantial sustained virologic response rates were also observed in patients who were null responders (Sulkowski et al., 2013b) and in treatment-naïve patients (Dieterich et al., 2014). Faldaprevir was generally well tolerated in clinical studies (Sulkowski et al., 2013a,b; Dieterich et al., 2014). At the highest dose (240 mg q.d. or b.i.d.), faldaprevir was associated with jaundice (usually mild) due to cases of isolated, reversible, dose-dependent, predominantly unconjugated hyperbilirubinemia, which was not associated with toxicity or liver injury (Sulkowski et al., 2013a,b). At the lower dose of 120 mg q.d., incidence of mild jaundice was similar to placebo (Sulkowski et al., 2013a).

The clinical and nonclinical studies conducted to further characterize the mechanisms underlying the isolated, unconjugated hyperbilirubinemia observed with faldaprevir are described here. These investigations indicate that the observed elevation in bilirubin levels is due to inhibition of bilirubin clearance by faldaprevir and is not likely to be a toxicity issue. As such, these findings can be considered in clinical decision making regarding the continuation of faldaprevir treatment in the presence of hyperbilirubinemia and jaundice.

### Materials and Methods

#### In Vitro Studies

**Hemolysis.** An in vitro hemolysis assay was performed by DuPont Haskell Laboratory for Health and Environmental Sciences (Newark, DE). Red blood cells collected from fresh whole rhesus monkey blood were washed and diluted with phosphate-buffered saline. Faldaprevir (>200 μg/ml) was mixed with the red blood cell suspension, incubated at 37°C for 30 minutes, and centrifuged (10 minutes, 2000 rpm). Hemoglobin release from lysed red blood cells was measured via the absorbance of supernatant at 540 nm.

**Inhibition of Bilirubin Uptake.** The effect of faldaprevir on [3H]bilirubin uptake was assessed using primary rat hepatocyte monolayers prepared in-house (n = 2) and primary human sandwich-cultured hepatocytes (n = 2; Qualyst Inc., Durham, NC) (Swift et al., 2010). Bilirubin is photolabile, and care was taken to not expose the stock solutions, stocks, and incubations to light. [3H]Bilirubin was purchased from Moravek Biochemicals (Brea, CA), and the proportion of bilirubin isomers was similar to the naturally occurring bilirubin as it was made using tritium exchange. The [3H]bilirubin purchased had a 1α isomer not less than 90%, 1β isomer not more than 4%, and XIII isomer not more than 3%, with overall purity of the isomers together ≥97%. The isomer content and purity were confirmed by high-performance liquid chromatography to match the isomer distribution for bilirubin available from Sigma-Aldrich (St. Louis, MO). [3H]Bilirubin was obtained in 125-μCi, single-use aliquots in amber vials and stored in the dark at −80°C until use. The stock solution in dimethylsulfoxide was made just prior to the experiment. Hepatocytes were incubated 48–96 hours postplating in triplicate with [3H]bilirubin (100 nM) with or without faldaprevir (0.33–100 μM) for 5 minutes in medium containing 0.1% bovine serum albumin. Estrone 3-sulfate (100 μM; Sigma-Aldrich) was used as a positive control for OATP inhibition. Following the incubation, cells were thoroughly washed five times with ice-cold media containing 0.1% bovine serum albumin and 2 mM ascorbic acid to protect bilirubin from photo-oxidation. Hepatocytes were lysed and radioactivity was measured by scintillation counting. In human hepatocytes, active uptake of rosuvastatin, an OATP1B1 and OATP1B3 substrate, and cholecytokinin octapeptide (CCK-8), an OATP1B3 substrate, was monitored to ensure functional expression of relevant uptake transporters. Effect of inhibitors was statistically compared with controls by two-tailed paired t test.

**Effect on Biliary Excretion Index.** The effect of faldaprevir on biliary excretion was assessed using sandwich-cultured rat (SCRH) and human hepatocytes (SCHH) (Qualyst Inc.) (Swift et al., 2010). Cells were preincubated for 10 minutes at 37°C with media containing the divalent cations Ca2+ and Mg2+ (“plus buffer”), which are required for maintenance of the biliary canalculus, or with media devoid of such divalent cations (“minus buffer”). This was followed by incubation with [3H]bilirubin (50 nM) with or without faldaprevir (1, 6, and 12.5 μM) or MK-571 ([5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid)
acid); 50 μM, positive control for uptake/efflux transporters) for 10 minutes. Sodium taurocholate was included as a positive control for biliary excretion. Hepatocytes were washed and lysed, and radioactivity was measured by scintillation counting. Biliary excretion index (BEI) was calculated using the following formula:

\[
\text{BEI} = \left( \frac{\text{Uptake in plus buffer (pmol/well)} - \text{Uptake in minus buffer (pmol/well)}}{\text{Uptake in plus buffer (pmol/well)}} \right) \times 100
\]

The radioactivity assay used does not differentiate between bilirubin and glucuronidated bilirubin, and therefore measures the BEI of the combination of bilirubin and its mono- and diglucuronides. However, with short incubation times, the extent of glucuronidation is expected to be low. The effect of inhibitors was statistically compared with controls by two-tailed paired t test.

**Inhibition of UGT1A1.** The effect of faldaprevir on UGT1A1 activity was determined in human liver microsomes by assessing the rate of glucuronidation of the UGT1A1 substrate estradiol in the presence of faldaprevir. UGT1A1 was activated by preincubation with almethicin for 15 minutes on ice, followed by incubation at 37°C for 5 minutes with faldaprevir (0.07–50 μM) and estradiol (20 μM; Sigma-Aldrich) before initiation of the reaction with uridine diphosphate glucuronic acid (5 mM; Sigma-Aldrich). Reactions were terminated after 5 or 10 minutes, and estradiol 3-glucuronide levels were analyzed by liquid chromatography–mass spectrometry.

**Inhibition of OATP1B1 and OATP1B3.** The effect of faldaprevir on OATP1B1- and OATP1B3-mediated transport of their respective prototypical substrates, [3H]estradiol-17-β-d-glucuronide (0.5 μM; PerkinElmer, Waltham, MA) and [3H]cholecytokinin-8 (1 μM; GE Healthcare, Piscataway, NJ), was evaluated using transiently transfected human embryonic kidney 293 cells. Since individually expressed transporters were evaluated in these experiments, probe substrates were used instead of [3H]bilirubin, due to their ease of use and ease of comparison with other inhibitors. At 16–24 hours postseeding, human embryonic kidney 293 cells were separately transfected with cDNA containing OATP1B1 or OATP1B3 using FuGENE 6 (Promega, Madison, WI). Forty-eight hours post-transfection, transport experiments were conducted at 37°C, during which the OATP1B1- and OATP1B3-expressing cells were incubated with [3H]estradiol-17-β-d-glucuronide (0.5 μM) and [3H]cholecytokinin-8 (1 μM), respectively, in the presence of faldaprevir (0.003–30 μM) for 10 minutes. Following incubations, cells were washed, lysed, and analyzed by scintillation counting.

**Inhibition of MRP2.** The effect of faldaprevir on MRP2 was assessed using MRP2-expressing S99 membrane vesicles (Solvato Biotechnology USA Inc., Boston, MA). Assay conditions were as per the manufacturer’s instructions. Briefly, MRP2 (S99) membrane preparations, the prototypical MRP2 substrate [3H]estradiol-17-β-d-glucuronide (Sigma-Aldrich), and glutathione were preincubated at 37°C for 5 minutes, with or without faldaprevir (0.01–20 μM). The uptake was initiated by addition of adenosine triphosphate. After incubating for 8 minutes, the vesicles were washed and filtered, and the filter plate with vesicles was analyzed for radioactivity by scintillation counting.

**Nonclinical Toxicology Studies**

Liver histology and bilirubin levels were assessed in rhesus monkeys following multiple doses of faldaprevir (80, 175, or 500 mg/kg per day) in a 26-week repeat-dose, study conducted by Covance Laboratories Inc. (Vienna, VA). Laboratory assessments, including bilirubin and alanine aminotransferase (ALT), were measured at multiple time points. Animals were sacrificed at week 26, and histopathologic examination of liver tissue samples was conducted by veterinary pathologists and peer reviewed. All procedures were carried out in compliance with the Animal Welfare Act (2006), the Guide for the Care and Use of Laboratory Animals (revised 2011), and the Office of Laboratory Animal Welfare.

**Clinical Studies**

Clinical findings regarding hyperbilirubinemia were assessed based on serum bilirubin data from one phase I (data on file) and three phase II (Sulkowski et al., 2013a,b; Dieterich et al., 2014) trials of faldaprevir. The clinical trials included in the current investigation were conducted in full compliance with the Guidelines of Good Clinical Practice and the Declaration of Helsinki, and were approved by all institutional review boards, ethical committees, and national authorities. All patients provided written, informed consent.

A randomized, double-blind, placebo-controlled multiple rising dose phase I study in healthy volunteers assessed faldaprevir at 20-, 48-, 120-, and 240-mg doses in a multiple rising-dose, 21- to 28-day trial of healthy volunteers with (n = 9) or without (n = 23) Gilbert’s syndrome (data on file).

SILEN-C1 was a phase Ib, multicenter, randomized, double-blind investigation in 429 treatment-naive patients with HCV G1 infection (Sulkowski et al., 2013a). Patients were randomized to receive 24 weeks of Peg-IFN/RBV plus one of the following: placebo; 120 mg of faldaprevir q.d. with a 3-day Peg-IFN/RBV lead-in (LI), 240 mg of faldaprevir q.d. with LI, or 240 mg of faldaprevir q.d. without LI.

SILEN-C2 was a phase Ib, multicenter, randomized, double-blind investigation in 290 patients with HCV G1 infection and nonresponse to prior Peg-IFN/RBV therapy (Sulkowski et al., 2013b). Patients were randomized to receive 48 weeks of Peg-IFN/RBV plus one of the following: 240 mg of faldaprevir q.d. with Peg-IFN/RBV LI, 240 mg of faldaprevir q.d. without LI, or 240 mg of faldaprevir twice daily with LI.

Both of these phase II studies recorded details of any adverse events occurring, including the dates of onset and resolution, the intensity (mild, moderate, or severe), and whether the adverse effects resulted in treatment discontinuation. Routine laboratory parameters, including bilirubin (total, direct, and indirect), alkaline phosphatase, ALT, and γ-glutamyltransferase, were also evaluated. Following DNA extraction from a separate blood sample, faldaprevir-treated patients from both trials were genotyped for UGT1A1 and OATP1B1 polymorphisms to evaluate whether there was any correlation between alleles at these variants and bilirubin concentrations.

SILEN-C3 was a phase Ib, open-label investigation in 159 treatment-naive patients with HCV G1 infection (Dieterich et al., 2014). Patients were randomized to receive 24 weeks of Peg-IFN/RBV plus 120 mg of faldaprevir daily for 12 or 24 weeks, each with a 3-day Peg-IFN/RBV LI.

**Results**

**In Vitro Studies.** Functional expression of OATP1B1 and OATP1B3 was demonstrated in SCHH by evaluating the uptake of [3H]rosuvastatin and [3H]CCK-8, respectively. Both substrates were actively taken up into hepatocytes with a 5.4-fold uptake of rosuvastatin and 1.6-fold uptake of CCK-8 at 37°C as compared with 4°C, and this uptake was inhibited by rifampycin SV, a known OATP inhibitor (data not shown). Faldaprevir inhibited the uptake of [3H]bilirubin (unconjugated bilirubin) into rat and human hepatocytes in a concentration-dependent manner. Figure 1 depicts the inhibition of bilirubin uptake in one representative hepatocyte preparation for rats and humans. At a clinically relevant concentration of 25 μM, faldaprevir inhibited bilirubin uptake in rat hepatocytes by 43%, and in human hepatocytes by 46%. Estrone 3-sulfate was used as a prototypical OATP inhibitor, and exhibited a 56% inhibition at 25 μM, and 36% decrease in [3H]bilirubin uptake in SCHH.

SCRH and SCHH were used for biliary elimination studies. Sodium taurocholate, which is known to be actively secreted into bile, was used as an indicator of formation of biliary canaliculi. Sodium taurocholate had a high BEI of ~70–80%, and bilirubin and its glucuronides together exhibited a BEI of 10–40% in various preparations of SCHH and SCHH. Faldaprevir reduced the BEI of bilirubin and its glucuronides in a concentration-dependent manner. Faldaprevir at 6 and 12.5 μM resulted in
35 and 56% decreases in bilirubin BEI, respectively, in rat hepatocytes, and 31 and 70% decreases, respectively, in human hepatocytes. MK-571 at 50 μM is both an uptake and efflux transporter inhibitor and was used as a positive control for MRP2 inhibition, and caused a 77 and 59% decrease in bilirubin BEI in SCRH and SCHH, respectively. Figure 2 depicts inhibition of bilirubin BEI by faldaprevir in one representative SCRH and SCHH preparation. Due to limitations of the radiometric detection method used, the biliary excretion studies did not differentiate between excretion of bilirubin and its glucuronides into the biliary canaliculi.

When assessed with specific enzymes or transporters, faldaprevir inhibited UGT1A1, OATP1B1, OATP1B3, and MRP2 with IC₅₀ values in the micromolar range (Fig. 3; Table 1). Faldaprevir inhibited UGT1A1 in human liver microsomes with an IC₅₀ value of 0.45 μM. In transfected human embryonic kidney 293 cells, faldaprevir inhibited OATP1B1-mediated uptake of estradiol-17β-D-glucuronide with an IC₅₀ value of 0.57 μM, and inhibited OATP1B3-mediated uptake of cholecystokinin-8 with an IC₅₀ value of 0.18 μM. Uptake of estradiol-17β-D-glucuronide into Sf9 membrane vesicles by MRP2 was inhibited by faldaprevir with an IC₅₀ value of 6.2 μM. Empirical or static mathematical models have been proposed by the US Food and Drug Administration and the European Medicines Agency (European Medicines Agency, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf; US Department of Health and Human Services, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf). These models use the plasma and portal vein concentrations of a drug for predicting its potential to cause in vivo drug-drug interactions based on in vitro inhibition of drug-metabolizing enzymes and transporters. For highly protein-bound drugs, such as faldaprevir, free fraction of 1% is assumed as a conservative estimate while calculating the unbound fractions of the circulating drug, which is then compared with the inhibition parameters to predict potential for in vivo drug-drug interaction. Applying these drug-drug interaction models and using free and total therapeutic concentrations of faldaprevir achieved at doses of 120 and 240 mg, in vivo inhibition of UGT1A1, OATP1B1, OATP1B3, and MRP2 was predicted and could potentially result in a drug-bilirubin interaction (Table 1).
Nonclinical Toxicology Studies. In rhesus monkeys, faldaprevir administration caused a reversible and dose-dependent increase in bilirubin levels (Fig. 4). The faldaprevir-mediated increase in total bilirubin was predominantly due to an increase in unconjugated bilirubin. Increased bilirubin was observed following single administration of faldaprevir at $\geq 20$ mg/kg (data not shown). No gender difference was observed. A 4-week study in rhesus monkeys, using a specific, comprehensive high-performance liquid chromatography assay (details described in the Supplemental Methods), also confirmed that unconjugated bilirubin was the predominant elevated form (Supplemental Fig. 1). No histologic evidence of hepatotoxicity was observed in these monkeys, nor were there any dose-dependent increases in ALT levels (Supplemental Fig. 2).

Additionally, an in vitro assay using fresh rhesus monkey blood confirmed there were no drug-related effects on hemolysis up to the highest concentration examined (115 $\mu$M or 100 $\mu$g/ml), indicating that hyperbilirubinemia did not occur due to increased heme load resulting from hemolysis.

Clinical Studies. In the phase I, multiple rising-dose trial of healthy volunteers ($n = 16$), which was designed to include individuals with Gilbert’s syndrome, incidences of hyperbilirubinemia and peak levels of unconjugated bilirubin during faldaprevir administration correlated with the UGT1A1*28 polymorphism (Fig. 5). Individuals homozygous for the *28 allele ($n = 5$) exhibited bilirubin elevations and had higher peak levels of bilirubin than heterozygous individuals ($n = 4$) or those without the *28 allele ($n = 7$). Among the UGT1A1*28 homozygous individuals, total bilirubin elevations were $\leq 4.6$ times the upper limit of normal. The increase in bilirubin was dose-dependent, occurred on dosing without a significant lag, and was rapidly reversible on cessation of faldaprevir dosing. Furthermore, the hyperbilirubinemia was asymptomatic, reversible, and benign.

In the phase II SILEN-C trials, rapidly reversible hyperbilirubinemia was observed during faldaprevir therapy (Fig. 6, A–C). Hyperbilirubinemia ($> 1.5 \times$ upper limit of normal) was dose-dependent, occurring in 47.8% of patients in the 120-mg q.d. dose group, compared with 83.8% in the 240-mg q.d. dose group (Sulkowski et al., 2013a,b). Elevations in total bilirubin included increases $> 5 \times$ upper limit of normal in up to 13% of patients in the 240-mg q.d. dose group (Sulkowski et al., 2013a,b). Elevations to this extent did not occur in the 120-mg q.d. dose group, although 23% of patients experienced increases in the range of $> 2.5 \times 5 \times$ upper limit of normal (Sulkowski et al., 2013a). The hyperbilirubinemia observed was driven predominantly by increases in the unconjugated form (Fig. 6C), and ratios of direct to total bilirubin were $< 0.5$ (Supplemental Tables 1 and 2). In five patients, ratios of direct to total bilirubin were $>0.5$: no associated ALT increase was observed in four of these patients, whereas in the fifth patient, ALT values did not return to baseline after the end of faldaprevir dosing, even if bilirubin values did, likely due to confounding effects of an inflammatory flare during continued therapy with Peg-IFN plus RBV. The bilirubin elevations were not associated with increases in liver enzymes, such as alkaline phosphatase, ALT, or γ-glutamyltransferase, compared with the control arm (Fig. 6D; Supplemental Tables 1 and 2), or with signs of increased anemia (Sulkowski et al., 2013a,b). No correlation was noted between hyperbilirubinemia and any clinically relevant adverse events (Table 2).

In these studies, the rate of treatment discontinuation due to hyperbilirubinemia or jaundice was low. In SILEN-C1, one patient (240-mg q.d. dose) discontinued due to jaundice, whereas none discontinued as a result of hyperbilirubinemia (Sulkowski et al., 2013a). In the SILEN-C2 trial, the rate of discontinuation due to elevated bilirubin and/or jaundice in both the 240-mg q.d./LI and 240-mg q.d. groups was $\leq 1\%$ (Sulkowski et al., 2013b).

A combined genotype analysis in 407 patients receiving the 240-mg q.d. faldaprevir dose in SILEN-C1 and C2 revealed that median indirect, but not direct, bilirubin concentration was dependent on UGT1A1*28 genotype (Supplemental Fig. 3, A and B). In contrast, there was no association found between direct or indirect bilirubin levels and OATP1B1 genotype (Supplemental Fig. 3, C and D). However, the number of subjects was
too few \((n = 2)\) in relevant low-expression genotypes \((^{5/8}_{15})\) to be used to draw statistically meaningful conclusions.

**Discussion**

The investigation described here aimed to characterize the dose-dependent and rapidly reversible elevations in bilirubin observed in HCV G1–infected patients during treatment with faldaprevir (Sulkowski et al., 2013a,b). In vitro studies in primary rat hepatocytes, human hepatocytes, and other enzyme- and transporter-expressing cellular systems; in vivo toxicology studies in rhesus monkeys; and clinical studies in human volunteers and HCV-positive patients were collated to systematically evaluate the molecular mechanisms underlying the predominantly unconjugated hyperbilirubinemia.

Data from in vitro studies in rat and human hepatocytes and other expression systems support the hypothesis that bilirubin elevations are driven by perturbation of bilirubin disposition. Inhibition of \(^{3}\)Hbilirubin uptake by faldaprevir in primary rat and human hepatocytes suggests that, in vivo, faldaprevir may interfere with uptake clearance of unconjugated bilirubin, which may be partially mediated by the OATP transporters. Additional in vitro studies in human liver microsomes and transporter-expressing cell systems demonstrated inhibition of UGT1A1, OATP1B1, OATP1B3, and MRP2 by faldaprevir. The reduction in the \(^{3}\)Hbilirubin BEI by faldaprevir is consistent with these results, which possibly represent a combination of decreased bilirubin glucuronidation via UGT1A1 and efflux of bilirubin glucuronides via MRP2. As shown in Table 1, based on the 2012 European Medicines Agency (http://www.ema.europa.eu).

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**TABLE 1**

Calculation of the drug-drug interaction (DDI) potential for faldaprevir due to inhibition of OATP1B1, OATP1B3, MRP2, and UGT1A1

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<tr>
<th>Transporter</th>
<th>Daily Dose (\text{mg})</th>
<th>(I_{C_{50}}) or (K_{i}^b)</th>
<th>(I_{C_{50}}/K_{i}) (FDA Criterion 1)</th>
<th>(I_{L_{u,inlet}}/IC_{50}) or (I_{L_{u,inlet}}/K_{i}) (EMA, FDA Criterion 2)</th>
<th>(I_{[3H]2}/IC_{50}) (FDA, EMA Criterion 3)</th>
<th>DDI Potential per Regulator Criteria(^d)</th>
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**Fig. 4.** Total and direct bilirubin levels following repeated administration of faldaprevir in rhesus monkeys for 26 weeks.

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\(^d\)EMA, European Medicines Agency; FDA, Food and Drug Administration; \(I_{L_{u,inlet}}\), inlet portal vein concentration estimated per the guidance assuming hepatic blood flow of 1.617 l/min, absorption rate constant of 0.1 min\(^{-1}\), and complete absorption; NA, not applicable.

\(^b\)Total steady-state plasma maximum concentration after oral dosing of faldaprevir in HCV-positive patients.

\(^c\)\(IC_{50}\) value reported for OATP1B1, OATP1B3, and MRP2; mathematically derived \(K_{i}\) value \((IC_{50}/2)\) from experimentally determined \(IC_{50}\) value reported for UGT1A1.

\(^a\)Concentration of faldaprevir in the gastrointestinal tract; \([I]_2 = \text{molar dose in } 250 \text{ ml}; [I]_2 = 552 \mu M\) after an oral dose of faldaprevir at 120 mg q.d.; \([I]_2 = 1104 \mu M\) after an oral dose of faldaprevir at 240 mg q.d.

\(^e\)As per FDA or EMA guidelines for these or comparable enzymes/transporters. FDA criterion 1: in vivo DDI considered possible if \(I_{C_{50}}/K_{i} \geq 0.1\); EMA criterion 1: in vivo DDI considered possible if \(I_{L_{u,inlet}}/K_{i} \geq 0.02\); fraction unbound used is 0.1 as recommended for drugs that are \(>99\%\) protein bound; FDA criterion 2: in vivo DDI considered possible if \(I_{L_{u,inlet}}/IC_{50} \geq 0.25\); EMA criterion 2: in vivo DDI considered possible if \(I_{L_{u,inlet}}/IC_{50} \geq 0.04\) for OATPs and \(>0.25\) for UGT1A1; EMA, FDA criteria 3: in vivo DDI considered possible if \([I]_2/IC_{50} \geq 10\).

\(^f\)Not applicable, as per FDA or EMA guidelines.
eu/docs/en_GB/document_library/Scientific_guideline/2012/07/ WC500129606.pdf) and draft 2012 US Food and Drug Administration guidance (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ ucm292362.pdf) on drug-drug interactions, therapeutically relevant plasma and predicted portal vein concentrations of faldaprevir are likely to inhibit UGT1A1, OATP1B1, OATP1B3, and MRP2 in vivo. Thus, faldaprevir may interfere with any of these three processes during bilirubin disposition: uptake into the liver via OATP1B1, OATP1B3, and other transport mechanisms; bilirubin glucuronidation via UGT1A1; and efflux of bilirubin glucuronides into the bile via MRP2.

Since the exact contribution of each of these enzymes and transporters in bilirubin clearance is not well established, it is challenging to discern the relative importance of these three processes to faldaprevir-mediated clinical hyperbilirubinemia; however, some inference can be drawn based on whether the increase observed is conjugated versus unconjugated bilirubin. For instance, unconjugated hyperbilirubinemia may be driven by inhibition of uptake and/or glucuronidation, since these processes occur prior to the appearance of bilirubin glucuronides in the circulation. Further support for this hypothesis is provided by literature reports that indinavir, atazanavir, and rifampin cause elevations in unconjugated bilirubin on dosing, potentially due to UGT1A1 or OATP1B1/IB3 inhibition (Vavricka et al., 2002; Campbell et al., 2004; Rotger et al., 2005; Zhang et al., 2005; Annaert et al., 2010; Chang et al., 2013), and that reduced UGT1A1 activity in Gilbert’s syndrome also causes unconjugated hyperbilirubinemia (Strassburg, 2008). A recent study demonstrated that inhibition of OATP1B1/1B3 is associated with clinical cases of drug-induced unconjugated hyperbilirubinemia (Chiu et al., 2014). However, recent reports on Rotor syndrome have clearly shown that a lack of OATP1B1 and OATP1B3 activity actually results in predominantly conjugated hyperbilirubinemia (van de Steeg et al., 2012). All of the transporters involved in bilirubin uptake are not yet known, and the impact of OATP1B1 and OATP1B3 inhibition on unconjugated bilirubin levels depends on the relative contribution of these transporters toward hepatic uptake of bilirubin. Given that both OATP1B1 and OATP1B3 are predicted to be inhibited in vivo, and relative expression of these transporters in the liver is not well established, attempts to differentiate the effect of faldaprevir on OATP1B1 versus OATP1B3 were not considered critical. Although faldaprevir inhibited OATP1B1 and OATP1B3 at therapeutically relevant concentrations, the overall uptake of bilirubin into primary human hepatocytes was only partially inhibited. As such, faldaprevir-mediated inhibition of bilirubin uptake via OATP1B1 and OATP1B3 may play a limited role in the observed unconjugated hyperbilirubinemia, but the exact relative importance in relation to UGT1A1 inhibition is difficult to establish. An increase in conjugated bilirubin may potentially be due to inhibition of the efflux of bilirubin conjugates into the bile via MRP2 and/or their hepatic reuptake from the portal circulation via OATP1B1 and OATP1B3.

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**Mechanisms Underlying Faldaprevir-Mediated Hyperbilirubinemia**

**Fig. 5.** Individual time courses of total bilirubin (micromoles per liter) in healthy volunteers treated for 21–28 days with faldaprevir 240 mg q.d. without the UGT1A1*28 variant (A) and with one or two copies of the UGT1A1*28 variant (B).
Fig. 6. Mean total bilirubin (A), indirect bilirubin (B), bilirubin at the 240-mg dose \((n = 149)\) (C), and ALT (D) levels in treatment-naive HCV G1 patients treated for 24 weeks with faldaprevir plus Peg-IFN/RBV in the phase IIb SILEN-C1 trial. Overall variability in total and direct bilirubin values at steady state was generally between 50 and 60% and remained below 75%. The error bars are not shown to improve visual comparison of central tendencies. The upward error bars for direct bilirubin are shown to indicate that the overall increase, including variability, was limited.
Mechanisms Underlying Faldaprevir-Mediated Hyperbilirubinemia

TABLE 2
Clinical symptoms associated with administration of faldaprevir in chronic HCV G1 patients treated for 24 weeks with Peg-IFN/RBV in combination with placebo or faldaprevir (120 or 240 mg q.d.) in the phase IIb SILEN-C1 Trial

| Drug-metabolizing enzymes and transporters are often involved in clearance of endogenous molecules, such as bilirubin, bilirubin glucuronides, steroids, and creatinine. When such compounds are also used as markers for pathophysiologic processes, comprehensive investigation of both their formation as well as elimination pathways is needed to explain the

Toxicology studies in rhesus monkeys demonstrated a faldaprevir-mediated, dose-dependent increase in unconjugated bilirubin concentrations, which was not associated with microscopic evidence of hepatotoxicity, elevations in liver enzymes, such as ALT, or hemolysis. In both monkeys and humans, plasma bilirubin levels rose immediately following faldaprevir administration, and this was reversible on cessation of therapy. This is consistent with the hypothesis that faldaprevir reversibly inhibits one or more of the processes involved in bilirubin elimination.

The phase II clinical data in 878 patients with HCV G1 infection are consistent with findings in animal studies, and demonstrate that the unconjugated hyperbilirubinemia arising from faldaprevir administration is benign and not associated with hepatotoxicity. The frequency of hyperbilirubinemia was 47.8 and 83.8% in the 120- and 240-mg q.d. dose groups, respectively; however, discontinuation due to hyperbilirubinemia was limited to 1%. Furthermore, in recently completed phase III studies in HCV-infected patients, hyperbilirubinemia was also predominantly unconjugated, rapidly reversible, and determined not to result from liver injury, based on prospectively defined criteria (Jacobson et al., 2013; Jensen et al., 2013).

Genotyping results from phase II studies corroborate the current understanding that UGT1A1 has an important role in clearance of unconjugated bilirubin (Supplemental Fig. 3). In the SILEN-Č1/C2 studies, the magnitude of the increase in unconjugated bilirubin was inversely associated with UGT1A1 activity (Sulkowski et al., 2013a,b). OATP1B1 genotyping data did not demonstrate any clear trends regarding genotype-dependent differences in bilirubin clearance, which is not surprising since there were very few subjects with low-activity genotypes, and more importantly, OATP1B3 and other as yet unidentified transporters may also facilitate the active uptake of bilirubin into the liver in a compensatory fashion. As such, the genotyping data alone cannot be used to draw conclusions regarding the relative contribution of UGT1A1 inhibition versus hepatic uptake in hyperbilirubinemia associated with faldaprevir, but can be seen as supportive of overall conclusions.

Overall, there was consistency between the nonclinical and clinical data, which, taken together, demonstrate that faldaprevir-mediated hyperbilirubinemia is predominantly driven by rises in unconjugated bilirubin due to inhibition of glucuronidation. Inhibition of hepatic uptake of bilirubin via OATP1B1 and OATP1B3 may also play a role, albeit with a caveat that these transporters may only be partially responsible for hepatic uptake of bilirubin.

Several protease inhibitors in clinical development also lead to elevated bilirubin concentrations. For example, simeprevir has been shown to inhibit OATP1B1 and MRP2, which may explain the mixed conjugated and unconjugated hyperbilirubinemia observed in HCV-infected patients during simeprevir treatment (Kiser et al., 2013). Both asunaprevir and the protease inhibitor ABT-450 have been reported to be associated with bilirubin elevations (Poordad et al., 2013; Suzuki et al., 2013). These observations are consistent with the known inhibitory effect of asunaprevir and ABT-450 on OATP1B1/OATP1B3 (Kiser et al., 2013; Lawitz et al., 2012).

An underlying consideration in discussions of hyperbilirubinemia is the method used to measure bilirubin and its glucuronides. The most common approach to assessment of bilirubin concentrations is the diazo (Jendrassik-Gróf–based) method. The measured concentration of hemoglobin and bilirubin present in the sample is influenced by the process of hemolysis, which can affect the accuracy of the diazo method (Kazmierczak et al., 2002). Furthermore, the method has a degree of inaccuracy in the measurement of the direct:indirect bilirubin ratio at high total bilirubin concentrations. To address the latter point, a high-performance liquid chromatography method was used in the 4-week toxicity study of faldaprevir (250 mg/kg per day) in rhesus monkeys, and results confirmed that elevations in preclinical studies were driven predominantly by indirect, and not direct, bilirubin (Supplemental Fig. 1). Another possible limitation is that reporting of jaundice, including ocular icterus, as an adverse event is a subjective assessment by patients and investigators, as opposed to objective serum bilirubin levels (bilirubin threshold 2–3 mg/dl). Isolated jaundice due to predominantly unconjugated hyperbilirubinemia observed with faldaprevir treatment, without any other relevant finding, should be regarded as a cosmetic issue rather than a clinical toxicity event. The latter would, in contrast, be characterized by predominantly conjugated hyperbilirubinemia in conjunction with increases in liver enzymes, such as ALT and alkaline phosphatase.

Drug-metabolizing enzymes and transporters are often involved in clearance of endogenous molecules, such as bilirubin, bilirubin glucuronides, steroids, and creatinine. When such compounds are also used as markers for pathophysiologic processes, comprehensive investigation of both their formation as well as elimination pathways is needed to explain the
changes observed in their baseline levels in the clinic. Here, we have used a multidisciplinary, integrated approach utilizing clinical and clinical studies to evaluate the hyperbilirubinemia observed during treatment of HCV G1–infected patients with faldaprevir. The rapidly reversible, unconjugated hyperbilirubinemia is not associated with liver injury and appears to occur predominantly via inhibition of glucuronidation by UGT1A1. It is also possible that decreased hepatic uptake of bilirubin via inhibition of OATP1B1 and OATP1B3 partly contributes to hyperbilirubinemia. Inhibition of transporters involved in clearance of conjugated bilirubin (OATP1B1, OATP1B3, and MRP2), or the inherent detection limitations posed by clinical assays for direct bilirubin, may underlie any mild increases observed in conjugated bilirubin levels in the clinical studies. In summary, faldaprevir-associated bilirubin elevations arise due to a drug-bilirubin interaction with UGT1A1 and transporters.

Authorship Contributions

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References

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