The JAK1/2 Inhibitor Ruxolitinib Reverses Interleukin-6-Mediated Suppression of Drug-Detoxifying Proteins in Cultured Human Hepatocytes

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Abbreviations:

BSP, bromosulfophthalein; CRP, C-reactive protein; CYP, cytochrome P-450; DDI, drug-drug interaction; DMSO, dimethyl sulfoxide; E3S, estrone-3-sulfate; EC_{50}, half maximal effective concentration; FBS, fetal bovine serum; idDDI, inflammatory disease-related drug-drug interaction; IL, interleukin; JAK, Janus kinase; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; mAb, monoclonal antibody; NTCP, sodium-taurocholate co-transporting polypeptide; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; PBS, phosphate-buffered saline; pSTAT1, phosphorylated STAT1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; STAT, signal transducer and activator of transcription; TEA, tetra-ethylammonium; TP, therapeutic protein**.**

Abstract

The inflammatory cytokine interleukin (IL)-6, which basically activates the JAK/STAT signaling pathway, is well-known to repress expression of hepatic cytochromes P-450 (CYPs) and transporters. Therapeutic proteins, like mAbs targeting IL-6 or its receptor, have been consequently demonstrated to restore full hepatic detoxification capacity, which results in inflammatory disease-related drug-drug interactions (idDDIs). In the present study, we investigated whether ruxolitinib, a small drug acting as a JAK1/2 inhibitor and currently used in the treatment of myeloproliferative neoplasms, may also counteract IL-6 repressing effects towards hepatic detoxifying systems. Ruxolitinib was found to fully inhibit IL-6-mediated repression of CYP (CYP1A2, CYP2B6 and CYP3A4) and transporter (NTCP, OATP1B1 and OCT1) mRNA levels in primary human hepatocytes and differentiated hepatoma HepaRG cells. Such effects were dose-dependent, with ruxolitinib EC_{50} values around 1.0-1.2 µM and thus close to ruxolitinib plasma levels which can be reached in patients. Moreover, they were associated with concomitant restoration of CYP and drug transporter activities in IL-6-exposed HepaRG cells. By contrast, ruxolitinib failed to suppress the repression of drug detoxifying protein mRNA levels caused by IL-1β. The JAK inhibitor and anti-rhumatoid arthritis compound tofacitinib was additionally found to reverse IL-6-mediated suppression of CYP and transporter mRNA expressions. Taken together, our results demonstrated that small drugs acting as JAK inhibitors, like ruxolitinib, counteract IL-6-mediated repression of drug metabolizing enzymes and drug transporters in cultured human hepatocytes. These JAK inhibitors may consequently be hypothesized to restore hepatic detoxification capacity *in vivo* for patients suffering from inflammatory diseases, which may in turn cause idDDIs.

Introduction

Drug-mediated alteration of drug detoxification ways is a major cause of drug-drug interactions (DDIs) (Cascorbi, 2012). Such pharmacokinetics-related DDIs usually involve a small molecule drug, *i.e.*, the perpetrator, which directly inhibits activity of drug metabolizing enzymes or drug transporters or regulate their expression. This will then result in decreased or enhanced clearance of a co-administered drug, *i.e.,* the victim, substrate for these detoxifying proteins. These DDIs therefore implicate small drug perpetrators mostly acting as inhibitors of drug-metabolizing cytochrome P-450 (CYP) or drug transporter activity, through competitive or non-competitive mechanisms, or as inducers of drug metabolizing enzyme expression. Numerous clinical examples of such DDIs have been reported in the literature (Danton et al., 2013). The possibility that a new molecular entity may be the perpetrator or the victim of a putative DDI has to be regulatory investigated by pharmaceutical companies during preclinical drug development (Prueksaritanont et al., 2013).

The increase in the clinical use of therapeutic proteins (TPs) has recently drawn attention to the potential DDIs between TPs and small molecule drugs (Evers et al., 2013). Indeed, inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α are potent repressors of hepatic cytochrome P-450 and drug transporter expression (Morgan et al., 2008; Fardel and Le Vee, 2009), known to commonly lead to reduced clearance of drugs in patients suffering from infectious and/or inflammatory diseases (Morgan, 2009). The use of TPs acting as anti-cytokine drugs, like monoclonal antibodies (mAbs) directed against circulating inflammatory cytokines or cytokine receptors, has consequently been hypothesized to neutralize the repressing effects of inflammatory cytokines towards drug detoxifying protein expression; by this way, it likely restores full hepatic drug detoxification capacity for patients suffering from inflammatory or infectious diseases. This may be the source of DDIs (Harvey and Morgan, 2014), which may in fact be considered as inflammatory disease-related DDIs

(idDDIs). This hypothesis has been validated for TPs targeting the IL-6 pathway in patients with rheumatoid arthritis. Indeed, the mAbs tocilizumab and sarilumab, directed against the IL-6 receptor alpha, as well as the IL-6 neutralizing mAb sirukumab, reduce exposure of co-administered drugs like simvastatin, midazolam or omeprazole (Schmitt et al., 2011; Zhuang et al., 2015; Lee et al., 2017), most likely through reversing IL-6-mediated suppression of CYP expression (Jiang et al., 2016).

Ruxolitinib (also known as INCB018424) is a small drug belonging to the emerging class of Janus kinase (JAK) inhibitors and currently clinically used in the treatment of JAK2 *V617F*-positive myeloproliferative neoplasms, including intermediate or high risk myelofibrosis and polycythemia vera (Bose and Verstovsek, 2017). Besides the JAK2 isoform, primarily associated with receptors for the hematopoietic growth factors erythropoietin and thrombopoietin, the JAK1 isoform, which plays a major role in the signaling pathway of inflammatory cytokines, is also potently inhibited by ruxolitinib. With respect to the two other members of the JAK family, tyrosine kinase 2 (TYK2) is additionally targeted by ruxolitinib, whereas JAK3 is less sensitive (Quintas-Cardama et al., 2010). Ruxolitinib is nevertheless considered as belonging to the class of first generation pan-JAK inhibitors (Baker and Isaacs, 2017).

JAK1, JAK2 and TYK2 are thought to play a key role in IL-6 signaling pathway. Indeed, these JAK isoforms are activated/phosphorylated upon IL-6 binding to its receptor and they downstream phosphorylate signal transducer and activator of transcription (STAT) 1 and STAT3, which triggers molecular genomic response to IL-6 (Hunter and Jones, 2015). It is then likely that ruxolitinib may antagonize most of IL-6 effects, which probably contributes to its well-reported anti-inflammatory properties. In this context, it may be hypothesized that ruxolitinib may counteract the suppressing effects of IL-6 towards drug detoxifying proteins, as reported above for TPs targeting the IL-6 pathway. The present study was therefore designed

to investigate this hypothesis, using primary human hepatocytes and highly-differentiated hepatoma HepaRG cells, which are recognized as convenient cell systems to study cytokine effects towards hepatic detoxifying enzymes and transporters (Aitken and Morgan, 2007; Le Vee et al., 2009; Klein et al., 2015; Rubin et al., 2015). Our data demonstrate that ruxolitinib can effectively reverse the repressing effects of IL-6 towards CYPs and drug transporters *in vitro*, suggesting that putative idDDIs with drugs handled by IL-6-targeted detoxifying proteins and co-administrated with ruxolitinib may have to be considered.

Materials and methods

Chemicals and reagents. Ruxolitinib and tofacitinib were provided by Selleckchem (Houston, TX, USA). Verapamil, midazolam and bromosulfophthalein (BSP) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Phenacetine, acetaminophen, bupropion and hydroxy-bupropion were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), whereas 1'-hydroxy-midazolam was provided by Toronto Research Chemicals (Toronto, Canada). [^{3}H(G)] taurocholic acid (specific activity 5 Ci/mmol), [6,7-^{3}H(N)] estrone-3-sulfate (E3S) (specific activity 51.8 Ci/mmol) and [1-^{14}C] tetra-ethylammonium (TEA) (specific activity 3.5 Ci/mmol) were purchased by Perkin-Elmer (Courtaboeuf, France). Recombinant human IL-6 and IL-1β were supplied by R&D Systems (Minneapolis, MN, USA). All other chemicals and reagents were commercial products of the highest purity available. Stock solutions of ruxolitinib were prepared in dimethyl sulfoxide (DMSO), whereas those of IL-6 were done in sterile distilled water containing 0.1 % bovine serum albumin. Control cultures received the same dose of solvents as treated counterparts.

Cell culture. Primary human hepatocytes were obtained from adult donors undergoing hepatic resection for secondary tumours, via the Centre de Ressources Biologiques (CRB) Santé of

Rennes BB-0033-0005 (University Hospital, Rennes, France), which has obtained the authorization N°DC-2008-630 from the French Ministry of Health to collect hepatic resections from the digestive surgery department and then to isolate and deliver the hepatocytes used in this study. Human hepatocytes, initially prepared by enzymatic dissociation of histologically-normal liver fragments (Fardel et al., 1993), were seeded in 24-well or 48-well plastic plates at a density of 2×10^5 cells/cm^2 in Williams' E medium (Invitrogen, Cergy-Pontoise, France), supplemented with 10 % (vol/vol) fetal bovine serum (FBS) (Perbio Sciences, Brébieres, France), 5 µg/mL bovine insulin (Sigma–Aldrich), 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine (Invitrogen). After 24 h, this seeding medium was discarded, and primary hepatocytes were routinely cultured in Williams' E medium defined above and supplemented with 5×10^{-5} M hydrocortisone hemisuccinate (Upjohn, Paris La Défense, France) and 2 % (vol/vol) DMSO, as reported previously (Le Vee et al., 2009). All experimental procedures complied with French laws and regulations and were approved by the National Ethics Committee. Characteristics of hepatocyte donors are indicated in Supplemental Table 1.

Highly-differentiated human hepatoma HepaRG cells were cultured as previously described (Le Vee et al., 2013). Briefly, cells plated in 24-well plates were first grown in Williams' E medium supplemented with 10 % (vol/vol) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine, and 5×10^{-5} M hydrocortisone hemisuccinate for two weeks. Cells were next cultured for additional two weeks in the same medium supplemented with 2 % (vol/vol) DMSO in order to get a full differentiation of the cells (Gripon et al., 2002). HepaRG cells were used for experiments as surrogates for human hepatocytes, notably for CYP activities and drug transport assays, owing to the cost of human hepatocytes and the well-established convenience of using a cell line like HepaRG.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) experiments.

Total RNAs were extracted using the TRI reagent (Sigma-Aldrich). RNAs were then reverse transcribed using the RT kit from Applied Biosystems (Foster City, CA, USA). PCR were next performed using the fluorescent dye SYBR Green methodology and a CFX384 real-time PCR system (Bio-Rad, Hercules, CA, USA), as described already (Le Vee et al., 2009). Gene-specific primers for drug transporters, CYP2B6 and CYP3A4, C-reactive protein (CRP), IL-8 and 18S rRNA were exactly as previously reported (Jigorel et al., 2006; Le Vee et al., 2008). Other primers were CYP1A2 sense, CACTATCAGGACTTTGACAAG, and CYP1A2 antisense, AGGTTGACAATCTTCTCCTG. The specificity of each gene amplification was verified at the end of qPCR reactions through analysis of dissociation curves of the PCR products. Amplification curves were analysed with CFX Manager software (Bio-Rad), using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA tested to the 18S rRNA endogenous reference, using the $2^{(-\Delta\Delta Ct)}$ method. Data were finally commonly expressed comparatively to mRNA expression found in untreated control cells, arbitrarily set at 1 unit for each analysed gene.

Cytochrome P450 activities. CYP3A4, CYP2B6 and CYP1A2 activities were measured through analyzing oxidation of specific substrates, *i.e.*, midazolam, bupropion and phenacetine for CYP3A4, CYP2B6 and CYP1A2, respectively, using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Briefly, hepatocytes were exposed to 50 µM midazolam, 100 µM bupropion or 200 µM phenacetine for 4 h in MEM medium, in the absence of FBS. Production of CYP activities-related metabolites, *i.e.*, 1'-hydroxy-midazolam, hydroxy-bupropion and acetaminophen, was then analyzed in culture supernatants through LC-MS/MS, using an high-performance liquid chromatography Aria system (Agilent, Les Ulis,

France), equipped with a Kromasyl® C18 (4.6 × 150 mm) column (Interchim, Montluçon, France) and coupled to a tandem mass spectrometry TSQ Quantum Ultra (Thermo Fisher Scientific, Villebon sur Yvette, France) fitted with an electrospray ionization source (ESI+). Monitored ion transitions were at 342.0 > 168.0 m/z for 1'-hydroxy-midazolam, 152.0 > 110.0 m/z for acetaminophen and 256.1 > 237.9 m/z for hydroxy-bupropion. Amounts of produced metabolites were finally normalized to total protein cell content, determined by Bradford's method (Bradford, 1976).

Drug transport assays. Activities of the sinusoidal transporters sodium-taurocholate cotransporting polypeptide (NTCP/*SLC10A1*), organic anion transporting polypeptides (OATPs/*SLCOs*) and organic cation transporter 1 (OCT1/*SLC22A1*) were determined through measuring inhibitor-sensitive accumulation of reference substrates, as previously described (Jigorel et al., 2005). In brief, HepaRG cells were incubated for 5 min at 37 °C with radiolabeled substrates, *i.e.*, 30 µM TEA (substrate for OCT1), 4 nM E3S (substrate for OATPs) or 40 nM taurocholate (substrate for NTCP), in the absence or presence of reference transporter inhibitors, *i.e.*, 50 µM verapamil (OCT1 inhibitor) or 100 µM BSP (OATP inhibitor), or in the presence or absence of sodium (for NTCP activity) (Le Vee et al., 2013). The transport buffer used for assays consisted of 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM CaCl2, 11 mM D-glucose, 10 mM HEPES, and 136 mM NaCl, adjusted to pH=7.4; NaCl was replaced by *N*-methylglucamine for transport assays in the absence of sodium. Cells were then washed twice in phosphate-buffered saline (PBS) and subjected to lysis in distilled water. Accumulation of radiolabeled substrates was next determined through scintillation counting of cell lysates and normalized to protein content, determined by Bradford's method. TEA uptake in the absence of verapamil minus uptake in the presence of verapamil, E3S uptake in the absence of BSP minus uptake in the presence of BSP and taurocholate accumulation in the presence of sodium

minus accumulation in the absence of sodium are thought to correspond to OCT1, OATP, and

NTCP activities, respectively (Jigorel et al., 2005).

Immunocytochemistry. HepaRG cells were fixed with a cold solution of acetone for 10 min

on ice. Cells were then washed with PBS and further incubated in PBS containing 4 % bovine

serum albumin for 1 h at room temperature. Cells were next incubated overnight with primary

antibody against phosphorylated-STAT1 (pSTAT1) (Ozyme, Saint-Quentin-en-Yvelines,

France). After two washes with PBS, the primary antibody was detected with anti-rabbit IgG

coupled to Alexa Fluor (Cell Signaling, Leiden, The Netherlands), whereas nuclei were stained

by 4′,6-diamidino-2-phenylindole (DAPI), for 1 h at room temperature. Immunofluorescence

images were finally acquired with a confocal fluorescence microscope LEICA DMI 6000 CS

(Leica Microsystemes SAS, Nanterre, France).

Calculation and statistical analysis. Quantitative data were routinely expressed as means ±

SEM of at least three independent assays, each being performed in duplicate or triplicate. They

were statistically analyzed using analysis of variance (ANOVA) followed by the Newman-

Keuls' post-hoc test. The criterion of significance was $p < 0.05$. Half maximal effective

concentration (EC_{50}) values of ruxolitinib towards cytokine mRNA expression were determined

using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA), through

nonlinear regression based on the four parameter logistic function. Percentages of reduction in

mRNA expression or activity of detoxifying proteins in response to IL-6 or IL-1β treatment

were calculated using the equation (A):

(A)　　% Reduction $_{\text{(mRNA expression or activity)}} = 100 - (\text{Value}_{\text{Cytokine}} \times 100 / \text{Value}_{\text{Control}})$

where Value $_{Cytokine}$ corresponds to mRNA level or CYP or transporter activity in cells exposed to cytokine (IL-6 or IL-1β) and Value $_{Control}$ corresponds to mRNA level or CYP or transporter activity found in control untreated cells.

Results

Inhibition of IL-6-induced CRP mRNA by ruxolitinib in primary human hepatocytes and HepaRG cells. The responsiveness of primary human hepatocytes and HepaRG cells to IL-6 and ruxolitinib were first determined. To do so, cells were exposed for 24 h to 10 ng/mL IL-6, a concentration previously successfully used to analyze IL-6-mediated regulation of hepatic detoxifying pathways *in vitro* (Le Vee et al., 2009; Klein et al., 2015). As shown in Table 1, this treatment was found to increase mRNA expression of CRP, a reference target of the signaling cascade IL-6/JAK1/STAT3 in human hepatocytes (Zhang et al., 1996). IL-6 concomitantly failed to exert cytotoxic effects, as shown by phase-contrast microscopic examination of the cultures (data not shown), which agrees with previous measurement of cellular viability in IL-6-treated human hepatocytes (Le Vee et al., 2009). In the presence of 5 µM ruxolitinib, IL-6-induced CRP mRNA expression was fully abolished in both primary human hepatocytes and HepaRG cells (Table 1), thus suggesting that ruxolitinib efficiently inhibited the IL-6/JAK/STAT pathway in these cultured hepatic cells. This hypothesis was fully confirmed by the fact that ruxolitinib fully antagonized IL-6-induced phosphorylation of STAT1 and its subsequent nuclear translocation in HepaRG cells (Supplemental Fig. 1).

Attenuation of IL-6-mediated repression of CYP expression by ruxolitinib in primary human hepatocytes and HepaRG cells. In agreement with previous studies (Abdel-Razzak et al., 1993; Aitken and Morgan, 2007) and in comparison to untreated cells, treatment of human hepatocytes by 10 ng/mL IL-6 for 24 h was found to markedly repress mRNA expression of

CYP1A2 (by 95.6 ± 2.9 %), CYP2B6 (by 87.9 ± 3.7 %) and CYP3A4 (by 97.1 ± 2.1 %) in primary human hepatocytes (Fig. 1A). Ruxolitinib used at 5 µM was able to fully reverse these IL-6-mediated suppressions of CYP mRNA expression (Fig. 1A). The JAK inhibitor was similarly efficient to abolish mRNA repression of CYP1A2, CYP2B6 and CYP3A4 caused by longer exposures to IL-6 (48 h and 72 h) (Supplemental Fig. 2). Inhibitory effects of ruxolitinib towards IL-6-related suppression of CYP mRNAs in primary human hepatocytes were next shown to be dose-dependent, with EC_{50} values around 1.1 µM for CYP3A4 and around 1.2 µM for CYP1A2 and CYP2B6 (Fig. 1B); similar ruxolitinib EC_{50} values were found for CYP1A2, CYP3A4 and CYP2B6 mRNA regulation in HepaRG cells (Supplemental Fig. 3). Hepatocyte population from each donor was fully responsive to IL-6, *i.e.*, IL-6 repressed CYP3A4 mRNA levels by more than 97 % for each of the analyzed hepatocyte population, and ruxolitinib EC_{50} values ranged from 1.01 µM to 1.15 µM according to hepatocyte populations (data not shown), thus precluding any major inter-individual difference for the response to the JAK inhibitor.

CYP mRNA and activity levels were next investigated in HepaRG cells exposed to 10 ng/mL IL-6 for 48 h. This treatment time was chosen owing to the known notable half-life of CYP3A4 protein and activity, estimated to 28.9 h in IL-6-exposed human hepatocytes (Ramsden et al., 2015). IL-6 markedly reduced CYP1A2, CYP2B6 and CYP3A4 mRNA expression in HepaRG cells (Fig. 2A). These CYP mRNA repressions were associated with a concomitant significant reduction of CYP1A2, CYP2B6 and CYP3A4 activities (Fig. 2B); CYP1A2-related acetaminophen, CYP2B6-related hydroxyl-bupropion and CYP3A4-related 1'-hydroxy-midazolam formations measured by LC-MS/MS were thus reduced by 67.9 ± 10.9 %, 79.4 ± 8.1 % and 60.8 ± 4.0 %, respectively, in response to exposure to IL-6. Ruxolitinib used at 5 µM was found to counteract IL-6-mediated repression of CYP1A2, CYP26 and CYP3A4 at both mRNA (Fig. 2A) and activity (Fig. 2B) level.

Attenuation of IL-6-mediated repression of drug transporter expression by ruxolitinib in primary human hepatocytes and HepaRG cells. In agreement with previous studies (Le Vee et al., 2009; Yang et al., 2012) and in comparison to untreated cells, treatment of cultured human hepatocytes by 10 ng/mL IL-6 for 24 h was found to markedly repress mRNA expression of the drug transporters NTCP (by 98.0 ± 0.5 %), OATP1B1 (*SLCO1B1*) (by 82.6 ± 5.3 %) and OCT1 (by 75.2 ± 3.9 %) (Fig. 3A). Ruxolitinib used at 5 µM was able to reverse these IL-6-mediated suppressions of transporter mRNA expression (Fig. 3A). The JAK inhibitor was also fully efficient to abolish mRNA repression of NTCP, OATP1B1 and OCT1 caused by longer exposure to IL-6 (up to 72 h) (Supplemental Fig. 4). Inhibitory effects of ruxolitinib towards IL-6-related suppression of transporter mRNAs in primary human hepatocytes were next shown to be dose-dependent, with EC_{50} values around 1.0 µM (for OATP1B1 and OCT1) and 1.1 µM (for NTCP) (Fig. 3B).

Treatment of HepaRG cells by 10 ng/mL IL-6 for 48 h also markedly suppressed mRNA expression of NTCP, OATP1B1 and OCT1 (Fig. 4A). It concomitantly reduced NTCP, OATP and OCT1 transport activities (Fig. 4B), indicating functional repression of transporters by IL-6, in agreement with previous data (Le Vee et al., 2009). Sodium-dependent uptake of taurocholate, BSP-inhibitable uptake of E3S and verapamil-inhibitable uptake of TEA were thus reduced by 62.4 ± 18.0 %, 46.3 ± 5.8 % and 53.0 ± 18.5 %. Ruxolitinib used at 5 µM was found to reverse IL-6-mediated repression of NTCP, OATP1B1 and OCT1 mRNA levels (Fig. 4A). It concomitantly inhibited IL-6-triggered decrease of NTCP, OATP and OCT1 activity (Fig. 4B).

Lack of ruxolitinib effects towards IL-1β-mediated repression of CYP and transporter mRNA levels in HepaRG cells. To evaluate the specificity of ruxolitinib towards cytokines like IL-6 acting via the JAK/STAT pathway, we next analyzed its potential effects towards IL-

1β-mediated alteration of detoxifying pathways in HepaRG cells. Indeed, IL-1β primarily acts through notably mobilizing myeloid differentiation primary response gene 88 (MYD88)/ IL-1 receptor–activated protein kinases (IRAKs)/ IκB kinases/NF-κB, in a JAK-independent manner (Weber et al., 2010). IL-1β was used at 1 ng/mL, a concentration previously demonstrated to repress many detoxifying proteins in human hepatocytes (Le Vee et al., 2008; Dickmann et al., 2012; Moreau et al., 2017). This IL-1β concentration did not trigger cytotoxicity, as shown by phase-contrast microscopic examination of the cultures (data not shown), which agrees with previous measurement of cellular viability in IL-1β-treated HepaRG cells (Le Vee et al., 2008). IL-1β was found to highly induce mRNA expression of the inflammatory markers CRP and IL-8, as already reported (Le Vee et al., 2008), and to significantly reduce those of various CYPs and transporters (Fig. 5). CYP1A2 and CYP3A4 mRNA levels were thus highly reduced by 99.1 ± 0.3 % and 98.5 ± 0.4 %, respectively, as for those of NTCP, OATP1B1 and OCT1 which were markedly decreased by 91.8 ± 7.6 %, 92.8 ± 2.5 % and 67.7 ± 13.9 %, respectively. Such potent repressions of drug detoxifying protein mRNA levels in response to Il-1β were not significantly impaired by 5 µM ruxolitinib (Fig. 5). Similarly, IL-1β-mediated up-regulation of IL-8 was not counteracted by ruxolitinib, which, by contrast, inhibited that of CRP (Fig. 5), suggesting an implication of the JAK/STAT pathway in CRP up-regulation by IL-1β.

Attenuation of IL-6-mediated repression of CYP and drug transporter mRNA levels by the JAK1/3 inhibitor tofacitinib in HepaRG cells. To determine whether JAK inhibitors structurally distinct from ruxolitinib can also reverse IL-6 mediated suppression of detoxifying protein, we analyzed the effects of tofacitinib (also known as CP-690,550), a marketed JAK1/3 inhibitor currently used for the treatment of rheumatoid arthritis (Vyas et al., 2013). Here, tofacitinib was used at a 3 µM concentration, which may be reached in plasma of patients treated by oral administration of this JAK inhibitor (Dowty et al., 2014; Krishnaswami et al.,

2015). At this concentration, tofacitinib was found to fully reverse IL-6-mediated mRNA induction of the reference target CRP in HepaRG cells (Fig. 6), thus demonstrating that tofacitinib, as ruxolitinib, was efficient to antagonize the JAK/STAT pathway mobilized by IL-6 in such cells. Tofacitinib was next shown to counteract repression of CYP1A2, CYP2B6, CYP3A4, NTCP, OATP1B1 and OCT1 mRNA levels caused by IL-6 in HepaRG cells (Fig. 6).

Discussion

The data reported in the present study indicate that the marketed JAK1/2 inhibitor ruxolitinib can markedly reverse IL-6 mediated repression of hepatic drug detoxifying proteins *in vitro*. Ruxolitinib notably restores mRNA expression of both drug metabolizing enzymes and sinusoidal drug transporters, in primary human hepatocytes and HepaRG cells exposed to IL-6. These ruxolitinib effects are rather specific, as the JAK1/2 inhibitor failed to hinder IL-1β-mediated mRNA suppression of drug detoxifying proteins. Also, tofacitinib, another JAK inhibitor not structurally related to ruxolitinib, counteracted CYP and drug transporter suppression due to IL-6 as well. Furthermore, ruxolitinib concentrations (in the 1 µM range) required to reverse IL-6-mediated repression of detoxifying pathways in primary human hepatocytes were close to those (in the 0.25 µM-1 µM range) previously demonstrated to be needed to efficiently counteract the JAK/STAT pathway in cultured cells (Schonberg et al., 2015). These ruxolitinib effects towards drug detoxifying protein expression consequently most likely implicate its basic inhibitory activity against the JAK/STAT pathway. A role for off-target effects of ruxolitinib, which notably concerns various kinases such as tropomyosin receptor kinase B, activated CDC42 kinase 1, anaplastic lymphoma kinase, RET kinase and Rho-associated protein kinases (Zhou et al., 2014; Rudolph et al., 2016), has thus to be discarded. Importantly, the IL-6 concentration used in the present study (10 ng/mL) has to be

considered as supra-physiological. Indeed, in healthy individuals, serum IL-6 concentrations usually range from 1.3 to 10.3 pg/mL and are increased to 2.6 to 123 pg/mL in some patient populations suffering from inflammation-based diseases (Dickmann et al., 2011). Ruxolitinib has therefore to be considered as fully efficient to counteract effects of IL-6, even if the cytokine is used at very high concentrations. By contrast, TPs acting as IL-6 or IL-1β neutralizing mAbs attenuate, but not fully abolish, IL-6 or IL-1β-mediated repression of CYP3A4, through shifting cytokine half maximal inhibitory concentration (IC_{50}) values towards higher values (Dickmann et al., 2011; Moreau et al., 2017).

It is noteworthy that ruxolitinib EC_{50} values (in the 1 µM range) needed to abrogate IL-6 effects towards drug detoxifying proteins in cultured human hepatocytes are much higher than those (in the 5 nM range) required to inhibit JAK1 and JAK2 activities in acellular system assays (Fridman et al., 2010). This may reflect the fact that active free concentrations of ruxolitinib, known to be highly bound to circulating proteins in the human plasma (unbound plasma concentration = 3.3 %) (Shi et al., 2015), are probably low in the FBS-supplemented culture medium used for the *in vitro* treatment of hepatocytes in the present study. Moreover, intracellular hepatic concentrations of unbound ruxolitinib, which remain to be characterized, have likely to be taken into account, because ruxolitinib primarily acts intracellularly, *i.e.*, it blocks JAKs located at the cytoplasmic face of the IL-6 receptor. Finally, metabolic degradation of ruxolitinib by drug metabolizing enzymes, especially CYP3A4 (Shi et al., 2012), may additionally contribute to the relatively high EC_{50} values of ruxolitinib observed in metabolic-competent human hepatocytes.

The marked up-regulation of functional drug detoxifying protein expression by ruxolitinib in IL-6-treated human hepatocyte cultures suggests that the JAK1/2 inhibitor may cause idDDIs, through enhancing detoxification capacities in patients suffering from inflammatory or infectious diseases. This hypothesis is fully supported by the fact that other

drugs targeting the IL-6 pathway, such as the mAbs tocilizumab, sarilumab and sirukumab, have been shown to reduce exposure of co-administered drugs like simvastatin, midazolam or omeprazole (Schmitt et al., 2011; Zhuang et al., 2015; Lee et al., 2017). Moreover, ruxolitinib concentrations required to counteract IL-6-mediated suppression of CYPs and drug transporters *in vitro* are in the 1.0-1.2 µM range, which is close to the range of ruxolitinib maximum plasma concentrations (C_{max}) (0.59-1.29 µM) reached in patients treated with the JAK inhibitor (Ogama et al., 2013). In addition, ruxolitinib *in vitro* effects towards IL-6-related CYP and transporter repression seem to be not transient, *i.e.*, they lasted at least 72 h in cultured human hepatocytes. The clinical use of ruxolitinib may therefore be theoretically efficient to counteract the repressing effect of IL-6 towards drug detoxifying pathways *in vivo*. However, such an effect, and the potential consequences in terms of idDDIs, have not presently been reported in the literature, according to the best of our knowledge. Similarly, tofacitinib, although efficiently counteracting CYP and transporter repression caused by IL-6 in hepatic HepaRG cells, has not been shown to cause clinical idDDIs through enhancing drug metabolism and/or transport. Notably, tofacitinib did not alter the pharmacokinetics of the reference CYP3A4 substrate midazolam in healthy volunteers (Gupta et al., 2012). It similarly failed to impair pharmacokinetics of oral contraceptive steroids in healthy female volunteers (Menon et al., 2016). Nevertheless, it is noteworthy that these pharmacokinetics studies have been performed in healthy subjects, who are unlikely to exhibit inflammation-related altered physiological functions, like IL-6-mediated repression of hepatic detoxifying pathways. By contrast, patients suffering from myeloproliferative neoplasms or rheumatoid arthritis, which correspond to present clinical indications of ruxolitinib and tofacitinib, respectively, commonly exhibit increased plasma levels of IL-6 (Cokic et al., 2015; do Prado et al., 2016; Sollazzo et al., 2016). Pharmacokinetics studies centered on such patients are therefore likely required to verify the hypothesis that marketed JAK inhibitors, including not only ruxolitinib and tofacitinib, but also

baricitinib, very recently approved in European Union for rheumatoid arthritis treatment (Richez et al., 2017), may cause idDDIs through antagonizing the IL-6/JAK/STAT pathway.

It is noteworthy that both ruxolitinib and tofacitinib are primarily metabolized by CYP3A4, which thus appears as a key factor for their detoxification (Shi et al., 2012; Dowty et al., 2014). Inhibition of CYP3A4 activity by ketoconazole has notably been shown to increase total ruxolitinib plasma exposure, whereas induction of CYP3A4 expression by rifampicin decreased it in healthy volunteers (Shi et al., 2012). Through restoring functional expression of hepatic CYP3A4 in patients displaying elevated levels of IL-6, ruxolitinib and tofacitinib may therefore induce their own metabolism. Finally, it is noteworthy that ruxolitinib and tofacitinib reduce plasma levels of IL-6 and other inflammatory cytokines in patients to whom they are administrated (Migita et al., 2014; Tabarroki et al., 2014). Such a down-regulation of inflammatory cytokines may also contribute to putative *in vivo* inhibition of inflammation-related repression of hepatic detoxifying pathway by the JAK inhibitors.

Both primary human hepatocytes and HepaRG cells were found to be well-responsive to IL-6 and ruxolitinib, thus demonstrating that these cellular models are likely relevant to study the interactions of small drugs with inflammatory cytokines *in vitro*. Cultured human hepatocytes have similarly been used to investigate the effects of TPs towards cytokine-driven regulation of CYPs and transporters (Dickmann et al., 2011; Moreau et al., 2017). Their relevance for the prediction of TP-idDDIs may however be compromised due to the high inter-laboratory variability in CYP suppression by cytokines (Evers et al., 2013). In this context, it is noteworthy that the rather elevated concentration of IL-6 used in the study (10 ng/mL) allows to highly repress CYP3A4 mRNA in analyzed human hepatocyte population from each donor; other drug detoxifying proteins, including CYP1A2, CYP2B6, NTCP, OATP1B1 and OCT1, were also markedly repressed, regardless of the hepatocyte population (data not shown). In addition, ruxolitinib was similarly efficient to counteract IL-6-related repression of CYP3A4

mRNAs, in human hepatocyte population of each donor. Taken together, these data suggest that inter-individual variations for ruxolitinib response may be rather negligible in our experimental conditions. Besides, it should be kept in mind that inflammatory diseases display complex biology, with frequent implication of multiple cytokines, which may interplay themselves. The hepatocyte monoculture has consequently been considered as an oversimplified system, which cannot reproduce the complex biology of *in vivo* inflammatory disease states (Girish et al., 2011; Evers et al., 2013). The analysis of JAK inhibitor effects towards cytokine-regulated detoxifying proteins in a more relevant *in vitro* model, such as the hepatocyte-Kupffer cell coculture system (Nguyen et al., 2015), would be welcome.

In summary, the JAK inhibitor ruxolitinib was shown to potently counteract the repressing effects of IL-6 towards hepatic CYPs and drug transporters. This may be the source of potential idDDIs, through a new mechanism, *i.e.*, a small drug (ruxolitinib) acting as a perpetrator via restoring full hepatic detoxification capacities for patients suffering from inflammation-based diseases. This point has likely to deserve attention, owing to the growing interest for the clinical use of ruxolitinib, tofacitinib, baricitinib and other JAK inhibitors currently in development, for the treatment of various inflammatory and autoimmune diseases (Banerjee et al., 2017).

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Authorship Contributions

Participated in research design: Febvre-James, Fardel

Conducted experiments: Febvre-James, Bruyère

Contributed new reagents or analytic tools: Bruyère, Le Vée

Performed data analysis: Febvre-James, Fardel

Wrote or contributed to the writing of the manuscript: Febvre-James, Bruyère, Fardel

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Legends to figures

Fig. 1. Effects of ruxolitinib on IL-6-mediated mRNA repression of CYPs in primary human hepatocytes.

(A) Primary human hepatocytes were either untreated (control), exposed to 5 µM ruxolitinib or 10 ng/mL IL-6 or co-exposed to 5 µM ruxolitinib and 10 ng/mL IL-6 for 24 h. CYP1A2, CYP2B6 and CYP3A4 mRNA expressions were then determined by RT-qPCR. Data for each transporter are expressed comparatively to mRNA levels found in untreated control cells, arbitrarily set at 1 unit; they are the means ± SEM of values from at least 4 independent human hepatocyte populations. *, $p < 0.05$. (B) Primary human hepatocytes were either untreated or exposed to 10 ng/mL IL-6 in the presence of various concentrations of ruxolitinib (from 0 to 10 µM) for 24 h. CYP1A2, CYP2B6 and CYP3A4 mRNA expressions were then determined by RT-qPCR. Data for each transporter are expressed comparatively to mRNA levels found in untreated control cells, arbitrarily set at 1 unit; they are the means ± SEM of values from at least 4 independent human hepatocyte populations. Ruxolitinib EC_{50} values are indicated at the top of each graph.

Fig. 2. Effects of ruxolitinib on IL-6-mediated repression of CYP mRNA (A) and activity (B) in human hepatoma HepaRG cells.

HepaRG cells were either untreated (control), exposed to 5 µM ruxolitinib or 10 ng/mL IL-6 or co-exposed to 5 µM ruxolitinib and 10 ng/mL IL-6 for 48 h. (A) CYP1A2, CYP2B6 and CYP3A4 mRNA expressions were then determined by RT-qPCR. Data for each CYP are expressed comparatively to mRNA levels found in untreated control cells, arbitrarily set at 1 unit. (B) CYP1A2, CYP2B6 and CYP3A4 activities were determined through measuring the

formation of acetaminophen, hydroxy-bupropion and 1'-hydroxy-midazolam, as described in Materials and Methods. Basal CYP activities in untreated control HepaRG cells were 3.4 ± 1.0 pM acetaminophen/mg protein/min (CYP1A2 activity), 24.1 ± 4.8 pM hydroxy-bupropion/mg protein/min (CYP2B6 activity) and 203.7 ± 25.8 pM 1'-hydroxy-midazolam/mg protein/min (CYP3A4 activity). Data are expressed as fold change when compared to CYP activities found in untreated control cells, *i.e.*, as the ratio CYP activities in treated cells versus that found in untreated cells. (A, B) Data are the means $\pm$ SEM of values from at least 3 independent assays. *, $p < 0.05$.

Fig. 3. Effects of ruxolitinib on IL-6-mediated mRNA repression of drug transporters in primary human hepatocytes.

(A) Primary human hepatocytes were either untreated (control), exposed to 5 µM ruxolitinib or 10 ng/mL IL-6 or co-exposed to 5 µM ruxolitinib and 10 ng/mL IL-6 for 24 h. CRP, IL-8, NTCP, OATP1B1 and OCT1 mRNA expressions were then determined by RT-qPCR. Data for each transporter are expressed comparatively to mRNA levels found in untreated control cells, arbitrarily set at 1 unit; they are the means $\pm$ SEM of values from at least 4 independent human hepatocyte populations. *, $p < 0.05$. (B) Primary human hepatocytes were either untreated or exposed to 10 ng/mL IL-6 in the presence of various concentrations of ruxolitinib (from 0 to 10 µM) for 24 h. NTCP, OATP1B1 and OCT1 mRNA expressions were then determined by RT-qPCR. Data for each transporter are expressed comparatively to mRNA levels found in untreated control cells, arbitrarily set at 1 unit; they are the means $\pm$ SEM of values from at least 3 independent human hepatocyte populations. Ruxolitinib EC_{50} values are indicated at the top of each graph.

Fig. 4. Effects of ruxolitinib on IL-6-mediated repression of drug transporter mRNA (A) and activity (B) in human hepatoma HepaRG cells.

HepaRG cells were either untreated (control), exposed to 5 µM ruxolitinib or 10 ng/mL IL-6 or co-exposed to 5 µM ruxolitinib and 10 ng/mL IL-6 for 48 h. (A) NTCP, OATP1B1 and OCT1 mRNA expressions were then determined by RT-qPCR. Data for each transporter are expressed comparatively to mRNA levels found in untreated control cells, arbitrarily set at 1 unit. (B) NTCP, OATP1B1 and OCT1 activities were determined through measuring sodium-dependent uptake of taurocholate, BSP-inhibitable uptake of E3S and verapamil-inhibitable uptake of TEA, as described in Materials and Methods. Data are expressed as fold change when compared to transporter activities found in untreated control cells, *i.e.*, as the ratio transporter activity in treated cells versus that found in untreated cells. Absolute levels of transporter-mediated uptake of substrates in untreated control HepaRG cells were 1.59 ± 0.34 pmol/mg protein (taurocholate), 0.35 ± 0.05 pmol/mg protein (E3S) and 148.5 ± 76.9 pmol/mg protein (TEA). (A, B) Data are the means $\pm$ SEM of values from at least 3 independent assays. *, $p < 0.05$.

Fig. 5. Effects of ruxolitinib on IL-1β-mediated repression of CYP and transporter mRNA levels in human hepatoma HepaRG cells.

HepaRG cells were either untreated (control), exposed to 5 µM ruxolitinib or 1 ng/mL IL-1β or co-exposed to 5 µM ruxolitinib and 1 ng/mL IL-1β for 24 h. (A) CRP, IL-8, CYP1A2, CYP3A4, NTCP, OATP1B1 and OCT1 mRNA expressions were then determined by RT-qPCR. Data for each drug detoxifying protein are expressed comparatively to mRNA levels found in untreated control cells, arbitrarily set at 1 unit. Data are the means $\pm$ SEM of values from at least 5 independent assays. *, $p < 0.05$; NS, not statistically significant (p>0.05).

Fig. 6. Effects of tofacitinib on IL-6-mediated repression of CYP and drug transporter mRNA levels in human hepatoma HepaRG cells.

HepaRG cells were either untreated (control), exposed to 3 µM tofacitinib or 10 ng/mL IL-6 or co-exposed to 3 µM tofacitinib and 10 ng/mL IL-6 for 24 h. CRP, CYP and drug transporter mRNA expressions were then determined by RT-qPCR. Data are expressed comparatively to mRNA levels found in untreated control cells, arbitrarily set at 1 unit; they are the means ± SEM of values from 4 independent assays. *, $p < 0.05$.

TABLE 1

Effects of ruxolitinib on IL-6 mediated induction of CRP mRNA levels in primary human hepatocytes and HepaRG cells[a]

Treatment	CRP mRNA levels[b]	
	Primary human hepatocytes	HepaRG cells
None (control)	1.0 ± 0.0	1.0 ± 0.0
Ruxolitinib	0.2 ± 0.0	0.3 ± 0.2
IL-6	6223 ± 2667*	1258 ± 125.7*
Ruxolitinib/IL-6	0.2 ± 0.0§	0.3 ± 0.1§

[a] Cells were either untreated (control), exposed to 5 µM ruxolitinib or 10 ng/mL IL-6 or co-exposed to 5 µM ruxolitinib and 10 ng/mL IL-6 for 24 h. CRP mRNA expression was then determined by RT-qPCR.

[b] CRP mRNA levels are expressed comparatively to mRNA levels found in untreated control cells, arbitrarily set at 1 unit; they are the means $\pm$ SEM of values from 6 independent human hepatocyte populations or 5 HepaRG cell cultures.

*, $p < 0.05$ when compared to control cells; §, $p < 0.05$ when compared to IL-6-treated cells.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6