Interaction of Rifampicin and Darunavir/Ritonavir or Darunavir/Cobicistat *In Vitro*

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Running Title: Interaction of Rifampicin with Boosted Darunavir

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ABSTRACT

Treatment of HIV patients co-infected with tuberculosis (TB) is challenging due to drug-drug interactions (DDIs) between antiretrovirals (ARVs) and anti-TB drugs. The aim of this study was to quantify the effects of cobicistat (COBI), or ritonavir (RTV), in modulating DDIs between darunavir (DRV) and rifampicin (RIF) in a human hepatocyte-based *in vitro* model. Human primary hepatocyte cultures were incubated with RIF alone, or in combination with either COBI or RTV for three days, followed by co-incubation with DRV for one hour. Resultant DRV concentrations were quantified by HPLC-UV, and the apparent intrinsic clearance ($CL_{int.app.}$) of DRV was calculated. Both RTV and COBI lowered RIF-induced increases in $CL_{int.app.}$ in a concentration-dependent manner. Linear regression analysis showed that $\log_{10}$ RTV and $\log_{10}$ COBI concentrations were associated with percentage inhibition of RIF-induced elevations in DRV $CL_{int.app.}$ β = -234 (95% CI = -275 to -193; $P < 0.0001$), and β = - 73 (95% CI = -89 to -57; $P < 0.0001$), respectively. RTV was more effective in lowering 10 µM RIF-induced elevations in DRV $CL_{int.app.}$ (IC_{50} = 0.025 µM) than COBI (IC_{50} = 0.223 µM). Incubation of either RTV or COBI in combination with RIF was sufficient to overcome RIF-induced elevations in DRV $CL_{int.app.}$, with RTV more potent than COBI. These data provide the first *in vitro* experimental insight into DDIs between RIF and COBI-boosted or RTV-boosted DRV, and will be useful to inform physiologically-based pharmacokinetic (PBPK) models to aid in optimising dosing regimens for the treatment of HIV-TB co-infected patients.

INTRODUCTION

Approximately 25% of human immunodeficiency virus-1 (HIV)-infected patients worldwide are co-infected with *Mycobacterium tuberculosis* (1, 2), accounting for 390,000 deaths in 2014 (3). Clinical management of HIV-tuberculosis (HIV-TB) patients presents significant challenges, especially in resource-limited settings (2, 4), where virological failure or intolerance to first-line antiretroviral therapy requires the use of HIV protease inhibitors (PIs) (5). PIs largely undergo phase I metabolism by cytochrome p450 3A4 (CYP3A4), and are also substrates of P-glycoprotein (P-gp; ABCB1) (6). Consequently, PIs are commonly administered in combination with pharmacokinetic (PK) "boosters" such as ritonavir (RTV) or cobicistat (COBI), which act by inhibiting CYP3A4-mediated PI metabolism and P-gp-mediated PI efflux, thereby improving the PK profile of PIs by prolonging PI half-life, and increasing PI bioavailability (7-9).

Rifampicin (RIF) is an essential component of short-course anti-TB treatment regimens (2, 10); however, RIF is also a potent inducer of the expression and activity of several metabolic enzymes – including CYP3A4 (11). Co-administering RIF with PIs can result in clinically-significant drug-drug interactions (DDIs), whereby PI bioavailability may be significantly reduced (>75%) (10, 12-14). Consequently, administering standard-doses of RTV-boosted PIs to HIV-TB patients receiving RIF is contraindicated under the current World Health Organisation (WHO) guidelines (15). The search for effective second-line therapeutic options for the treatment of HIV-TB co-infected patients is therefore a research priority (16).

Darunavir (DRV) is chiefly metabolised by CYP3A4 (17), and co-administration of a low-dose of either RTV or COBI together with DRV increases DRV systemic bioavailability

(18, 19). In addition, the high barrier to genetic resistance, as well as the tolerability, safety profile, and potency of DRV - when administered in combination with a low-dose of either RTV (DRV/r), or COBI (DRV/c) - have made these fixed-dose combinations important options for the treatment of HIV-patients (20-22).

Previous studies have demonstrated markedly reduced exposure of RTV-boosted PIs, including atazanavir (ATV) (12), indinavir (IDV) (13), and lopinavir (LPV) (14), as well as an increased risk of hepatotoxicity when RIF is co-administered with these drugs in healthy volunteers. For this reason, studies aimed at investigating DDIs between DRV/r and RIF in HIV-negative subjects have not been undertaken. Similarly, the extent of the DDI between DRV/c and RIF remains unknown. A recent population PK (pop-PK) analysis showed that it was possible to offset the effects of RIF on DRV C_{trough} by increasing the dose of DRV/r administered (23), which raises the possibility that RTV may overcome potential DDIs between DRV and RIF *in vitro* and *in vivo*. The aim of the present study was to quantify - using an *in vitro* model - the extent of DDIs arising from co-incubation of RIF with either RTV or COBI, by specifically measuring the apparent intrinsic clearance ($CL_{int.app.}$) of DRV by primary human hepatocytes.

MATERIALS AND METHODS

Chemicals. DRV (Cat. No.: S1620) and COBI (Cat. No.: S2900) were purchased from Selleckchem (Munich, Germany). RIF (Cat. No.: R3501), RTV (Cat. No.: SML0491), potassium phosphate monobasic (Cat. No.: P0662), methanol (Cat. No.: 34860), and acetonitrile (Cat. No.: 34967) were purchased from Sigma-Aldrich (Poole, UK). Orthophosphoric acid (Cat. No.: 153154D) was purchased from VWR (Lutterworth, UK). HPLC-grade water was produced by an ELGA PureLab system (Veolia Water Technologies, High Wycombe, UK).

Primary Hepatocytes. Cryopreserved primary human hepatocytes were purchased from Life Technologies (Cat. No.: HMCPIS; Inchinnan, Scotland). Hepatocytes from a total of four donors were used (**Table 1**).

Stock Solutions. Stock solutions of COBI, DRV, RIF and RTV were freshly prepared in 100% (v/v) methanol at concentrations 6443, 1684.3, 15000 and 6935.4 µM respectively. Prior to use in experiments, all stock solutions were sterile-filtered through a Millex 0.22 µm polyethersulfone membrane (Millipore, Cat. No.: SLGP033RS; Watford, UK), and were either used immediately, or were stored at -20 °C for up to five days prior to use.

Concentrations of drugs used in this study. Primary cryopreserved human hepatocytes were treated with a range of concentrations of test compounds - COBI (0.13—12.76 µM), RIF (0.50—20.00 µM) and RTV (0.01—10.00 µM) - spanning their respective therapeutic plasma concentration ranges in humans, as determined from clinical PK data (24), (25). The concentration of DRV used in experiments (5 µM) was selected from a value within the therapeutic range, and close to the C_{min} of DRV (DRV/r 600/100 C_{min} = 3.58 ± 1.15 µg/ml =

6.03 ± 1.94 µM, (26); DRV/c 800/150 C_{min} = 2.40 ± 1.22 µg/ml = 4.04 ± 2.05 µM, (27)), as obtained from PK data supplied on package inserts (26, 27). Unless otherwise stated, starting drug concentrations quoted within this study refer to the starting total drug concentration present in each case, without adjustment for protein binding. After adjustment for protein binding in Williams' Medium E (WME) incubation medium, the starting unbound concentrations of test compounds used was as follows: COBI (0.068—6.761 µM), DRV (3.800 µM), RIF (0.315—12.600 µM) and RTV (0.001—1.400 µM).

Culture of Primary Human Hepatocytes. Primary cryopreserved human hepatocytes were thawed in Cryopreserved Hepatocyte Recovery Medium (CHRM®, Life Technologies, Cat. No.: CM7000) and were re-suspended in WME plating medium (Life Technologies, Cat. No.: A1217601 supplemented with Hepatocyte Plating Supplement Pack, Life Technologies, Cat. No.: CM3000). Cell viability was determined using a NucleoCounter® NC-100™ (Sartorius Ltd., Epsom, UK). Viable cells were plated on collagen-coated 96-well cell culture plates (Life Technologies, Cat. No.: CM1096) at a density of 6.5 x 10^4 cells per well in 110 µl of WME plating medium. Hepatocytes were incubated in a humidified incubator at 37 °C containing 5% (v/v) CO_2 for five hours prior to removal of the WME plating medium, and overlaying the hepatocyte monolayer with 70 µl per well of Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Life Technologies, Cat. No.: A1413202) diluted in WME incubation medium (Life Technologies, Cat. No.: A1217601, supplemented with Hepatocyte Maintenance Supplement Pack, Life Technologies, Cat. No.: CM4000) to a final concentration of 0.35 mg/ml. Cells were then incubated in a humidified incubator at 37 °C containing 5% (v/v) CO_2 for 24 hours, prior to removal of the WME incubation medium and replacement with 110 µl

of fresh WME incubation medium containing test compounds: COBI (0.128—12.76 µM) together with RIF (0.5—20 µM); or RTV (0.01—10 µM) together with RIF (0.5—20 µM). As a control, hepatocytes were incubated with methanol (0.3% v/v) in WME incubation medium. At 24 hours, and 48 hours post-initial treatment, WME incubation medium containing test compounds was removed, and replaced with fresh WME incubation medium containing test compounds. At 72 hours post-initial treatment all cells were incubated with test compounds together with DRV (5 µM) in WME incubation medium for 60 minutes.

Quantification of Darunavir by HPLC-UV. Following 60 minutes of incubation of hepatocytes with test compounds together with 5 µM DRV, 100 µl of WME incubation medium was removed from each well and was transferred to Corning® Pyrex® 75 x 12 mm borosilicate glass tubes (Appleton-Woods, Cat. No.: KC350) containing 300 µl of 100% acetonitrile. Standards and quality control samples were prepared in WME incubation medium and were treated in the same way. All samples were then vortexed for five seconds, and were dried in a Jouan RC10.22 vacuum centrifuge for six hours at room temperature (18—25°C). After drying, samples were re-constituted in 330 µl of 20% (v/v) acetonitrile in H_2O. One hundred microlitres of the resultant suspension was used to quantify DRV by HPLC-UV.

Chromatographic separation of DRV was achieved using a Waters Atlantis T3 (4.6 x 100 mm, 3 µm) column (Waters, Elstree, UK) equipped with a 10 x 4 mm, 3 µm Fortis C18 Guard (Fortis™ Technologies Ltd., Chester, UK). A Dionex P680 HPLC pump, Dionex ASI-100 automated sample injector and a Dionex UVD170U UV detector (Thermo-Fisher Ltd., Hemel-Hempstead, UK) were used. Mobile phases C (25 mM KH_2PO_4, pH 3.3/orthophosphoric acid) and D (100% acetonitrile) were used in a step-gradient elution as follows: 70% C/30% D from

142 0.0 to 1.5 min, 35% C/65% D from 1.5 to 7.0 min, 20% C/80% D from 7.0 to 9.5 min and 70%
143 C/30% D from 9.5 to 12.5 min. Elution was carried out at room temperature (18—25°C), and
144 the flow rate was maintained at 1.00 ml/min. DRV was quantified at 267 nm and chromatograms
145 were analysed using Chromeleon software (version 6.8; Thermo-Fisher Ltd.). Each experimental
146 condition was assessed in triplicate. The lower limit of detection (LOQ) of DRV was determined
147 to be 0.156 µM. The assay was linear between 0.156 µM and 10 µM (upper LOQ). The mean
148 coefficient of variability (CV) of intra-day precision was 2.6%, whilst the mean CV of intra-day
149 accuracy was 2.0%. The mean CV of inter-day precision was 2.2%, and the mean CV of inter-
150 day accuracy was 1.2%. The mean recovery of DRV from WME was 96.1%.

151 **Measurement of Protein Binding of Drugs in Williams' Medium E Incubation Medium.**
152 The degree of binding of COBI, DRV, RIF or RTV to WME incubation medium was determined
153 using a rapid equilibrium dialysis (RED) base plate (Thermo-Fisher Scientific, Cat. No.: 90004)
154 fitted with RED device inserts (Thermo-Fisher Scientific, Cat. No.: 89810). Five hundred µl of
155 WME incubation medium (Life Technologies, Cat. No.: A1217601, supplemented with
156 Hepatocyte Maintenance Supplement Pack, Life Technologies, Cat. No.: CM4000) alone, or
157 WME incubation medium containing either COBI (5 µM); DRV (5 µM); RIF (5 µM); or RTV (5
158 µM), was placed into separate sample chambers, whilst 750 µl of non-supplemented WME (Life
159 Technologies, Cat. No.: A1217601 alone) was placed in into the corresponding buffer chambers.
160 Each experimental condition was tested in triplicate. Following sealing with Parafilm® 'M'
161 (Sigma-Aldrich), the RED device containing these samples was incubated for five hours at 37 °C
162 with orbital shaking (200 r.p.m.). Following incubation, a 450 µl aliquot was removed from the
163 buffer chamber within each RED device insert and was vortexed for ten seconds with 112 µl

(20% of total final volume) of acetonitrile in a 1.5 ml microcentrifuge tube, prior to transfer to a 300 µl Chromacol fixed insert vial (Thermo-Fisher Scientific), from which 100 µl of the suspension was analysed directly by HPLC-UV, as described below. For WME incubation medium samples, a 450 µl aliquot was removed from each sample chamber within each RED device insert, and was transferred to a 2.0 ml microcentrifuge tube containing 1350 µl of 100% acetonitrile. Samples were then vortexed for five seconds prior to centrifugation at 13,100 x *g* for ten minutes at room temperature. Resultant supernatants were transferred to Corning® Pyrex® 75 x 12 mm borosilicate glass tubes, and were dried in a Jouan RC10.22 vacuum centrifuge at room temperature (18—25°C). After drying, samples were re-constituted in 400 µl of 20% (v/v) acetonitrile in H_2O, and 100 µl of the resultant suspension was used to quantify COBI, DRV, RIF or RTV by HPLC-UV. Chromatographic separation of COBI, RIF and RTV was achieved using a Waters Atlantis T3 (4.6 x 100 mm, 3 µm) column equipped with a 10 x 4 mm, 3 µm Fortis C18 Guard. A Dionex P680 HPLC pump, Dionex ASI-100 automated sample injector and a Dionex UVD170U UV detector were used. Mobile phases A (25 mM KH_2PO_4, pH 3.3/orthophosphoric acid) and B (100% acetonitrile) were used in a step-gradient elution as follows: 70% A/30% B from 0.0 to 2.0 min, 52.5% A/47.5% B from 2.0 to 4.0 min, 35% A/65% B from 4.0 to 6.0 min, 20% A/80% B from 6.0 to 9.0 min, and 70% A/30% B from 9.0 to 12.5 min. Elution was carried out at room temperature (18—25°C), and the flow rate was maintained at 1.00 ml/min. Chromatograms were analysed with COBI and RTV quantified at 220 nm and RIF quantified at 267 nm using Chromeleon software (version 6.8). Each experimental condition was assessed in triplicate. Standards and quality control samples for each drug were prepared and extracted from WME incubation medium to analyse corresponding sample chamber samples, or were prepared in WME medium containing 20% (v/v) acetonitrile for

187 analysis of buffer chamber sample dialysates. The fraction unbound (f_u) of each drug was
188 calculated by dividing the drug concentration quantified in the buffer chamber dialysate with the
189 concentration of drug quantified in sample chamber aliquots. Results are presented as mean f_u ±
190 SD (n = 3).

191 **Calculation of the apparent intrinsic clearance ($CL_{int.app.}$) of Darunavir by Hepatocytes.**
192 The $CL_{int.app.}$ of DRV was calculated based on a previously described method (28). This is
193 summarised in **Equation 1**:

194 **Equation 1:** $CL_{int.app.}$ = (ln2/*in vitro* $t_{1/2}$) x (µl incubation volume/10^6 hepatocytes)

195 Results were expressed as the mean ± SD (µl/min/10^6 hepatocytes) of a total of three
196 donors per condition tested. Three biological replicates were quantified per condition tested,
197 using hepatocytes obtained from three separate donors in each case. All DRV $CL_{int.app.}$ values
198 were calculated using DRV concentrations corrected for DRV protein binding in WME
199 incubation medium.

200

201 **Statistical Analysis.** Statistical analyses were carried out using IBM® SPSS® Statistics (Version
202 22; IBM Corporation, Armonk, NY, USA). All data were assessed for normality using a
203 Shapiro–Wilk test and data were compared using a Mann-Whitney *U* statistical test. Univariate
204 and stepwise-elimination multivariate linear regression analyses (significance threshold = $P <$
205 0.2; $\alpha = 0.05$) were conducted to characterise the influence of co-incubating primary human
206 hepatocytes with various concentrations of RTV or COBI together with RIF on DRV $CL_{int.app.}$.

Calculation of the half-maximal inhibitory concentration (IC_{50}) of RTV and COBI required to inhibit DRV $CL_{int.app.}$ maximally-induced by 10 µM RIF was completed using DRV $CL_{int.app.}$ data obtained from COBI (donors Lot HU1399, Lot HU1574 and Lot HU1587) and RTV (donors Lot HU1399, Lot HU1587 and Lot HU1621) experiments. Data were firstly normalized by defining the mean maximal elevation in DRV $CL_{int.app.}$ induced by 10 µM RIF alone in each respective dataset as 100%, and plotting remaining values relative to this value. GraphPad Prism® (Version 5; GraphPad Software, Inc. La Jolla, CA, USA) was used to plot the data using the 'log(inhibitor) vs. response' equation and a Least Squares fitting method.

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RESULTS

Assessment of the $CL_{int.app.}$ of Darunavir Following Combination Incubation of Primary Human Cryopreserved Hepatocytes with Ritonavir and Rifampicin. Primary human hepatocytes are commonly used as a tool to predict hepatic metabolic clearance of xenobiotics and DDIs *in vitro* (29, 30). Using this model system, the $CL_{int.app.}$ of DRV was initially calculated under control conditions in which hepatocytes (Lot HU1399, Lot HU1587 and Lot HU1621) were incubated with DRV alone. Experiments aimed at determining the degree of protein binding of DRV within WME incubation medium revealed that the mean f_u of DRV was 0.76 ± 0.07 (n=3; **Table 2**). Following correction for DRV protein binding in WME incubation medium, under control conditions, mean DRV $CL_{int.app.}$ was 10.5 ± 3.8 µl/min/10^6 hepatocytes (n=3). Incubation of human hepatocytes with RIF over 72 hours has been previously shown to induce CYP3A4 enzymatic activity (31, 32). Similarly, in this model system, incubation of hepatocytes with RIF was sufficient to markedly increase $CL_{int.app.}$ of the CYP3A4 substrate DRV at each concentration of RIF tested (0.5—20 µM; **Fig. 1**). The maximal RIF-induced increase (1.9 ± 0.3-fold; n=3) in DRV $CL_{int.app.}$ was observed with 10 µM RIF (**Fig. 1**).

Co-incubation of RIF with RTV reduced 10 µM RIF-induced increases in $CL_{int.app.}$ in a RTV concentration-dependent manner (**Fig. 1**). Notably, RTV (1 µM) was sufficient to overcome the effect of 10 µM RIF on DRV $CL_{int.app.}$, reducing DRV $CL_{int.app.}$ to 0.78 ± 0.25-fold – equivalent to -22% when compared to control levels in which cells were treated with DRV alone (n=3; **Fig. 1**). Increasing RIF concentrations above 10 µM (12.5—20 µM) did not impact the effectiveness of RTV to overcome RIF-elevated DRV $CL_{int.app.}$ (**Fig. 1**). Specifically, 1 µM RTV lowered 12.5 µM RIF-induced and 20 µM RIF-induced DRV $CL_{int.app.}$ by 55% and 47%, to

(8.6 ± 3.2 µl/min/10^6 hepatocytes; *n*=3) and (8.8 ± 3.4 µl/min/10^6 hepatocytes; *n*=3), respectively.

Assessment of the $CL_{int.app.}$ of Darunavir Following Combination Incubation of Primary Human Cryopreserved Hepatocytes with Cobicistat and Rifampicin. In a separate set of experiments, human hepatocytes from three individual donors (Lot HU1399, Lot HU1574 and Lot HU1587) were used to determine the effects of incubating RIF together with COBI upon DRV $CL_{int.app.}$. Under control conditions, where primary human cryopreserved hepatocytes were incubated with DRV alone, DRV $CL_{int.app.}$ was 13.2 ± 1.8 µl/min/10^6 hepatocytes, (*n*=3). Incubation of hepatocytes with RIF (0.5—20 µM) induced a mean increase in DRV $CL_{int.app.}$ of 55.8%. In cells treated with 1 µM RIF, co-incubation with the lowest concentration of COBI tested (0.42 µM) was effective in lowering RIF-induced DRV $CL_{int.app.}$ by 36.9%, yielding a DRV $CL_{int.app.}$ of 12.2 ± 2.8 µl/min/10^6 hepatocytes (*n*=3). Hepatocytes treated with 10 µM RIF exhibited a DRV $CL_{int.app.}$ of 21.6 ± 2.6 µl/min/10^6 hepatocytes (*n*=3). COBI induced a concentration-dependent attenuation of the DRV $CL_{int.app.}$ elicited by 10 µM RIF (**Fig. 2**), with 1.28 µM COBI being sufficient to lower DRV $CL_{int.app}$ to 11.6 ± 2.6 µl/min/10^6 hepatocytes (*n*=3), 13% below DRV control levels. COBI was also effective at reducing $CL_{int.app.}$ elevations induced by higher concentrations of RIF, as co-incubation with 1.28 µM COBI reduced 20 µM RIF-elevated DRV $CL_{int.app.}$ by 46% (12.4 ± 3.9 µl/min/10^6 hepatocytes; *n*=3).

Comparison of Cobicistat- and Ritonavir-mediated Reduction of Rifampicin-Induced Darunavir $CL_{int.app.}$. To compare the relative effectiveness of RTV and COBI to attenuate RIF-induced increases in DRV $CL_{int.app.}$, the percentage inhibition of 10 µM RIF-induced elevations in DRV $CL_{int.app.}$ achieved by co-incubation with either COBI (0.13—12.76 µM), or RTV (0.1—10 µM), was determined in comparison to control conditions where cells were treated with 10 µM RIF alone (**Fig. 3**). Following correction for protein binding, the IC_{50} of COBI and RTV - calculated from the percentage-change in DRV $CL_{int.app.}$ under these conditions - was 0.223 µM for COBI and 0.025 µM for RTV (**Fig. 3**). In addition, the maximal inhibition of 10 µM RIF-induced elevations achieved by COBI and RTV were different, with RTV resulting in a 69.5% inhibition of 10 µM RIF-induced increases in DRV $CL_{int.app.}$, whilst COBI-mediated reduction in 10 µM RIF-induced increases in DRV $CL_{int.app.}$ was 56.9% (P=0.05).

Following data normalisation and correction for protein binding, linear regression analysis of the effects of RTV and COBI in combination with RIF at each concentration tested on the percentage change in DRV $CL_{int.app.}$ showed an association between log_{10} RTV unbound concentrations, and log_{10} COBI unbound concentrations and percentage inhibition of RIF-induced DRV $CL_{int.app.}$ of β = -234 (95% CI = -275 to -193; $P < 0.0001$), and β = - 73 (95% CI = -89 to -57; $P < 0.0001$), respectively. Conducting linear regression analysis of the effects of RIF on DRV $CL_{int.app.}$ revealed that RIF exerted a similar effect on DRV $CL_{int.app.}$ in the two independent sets of RTV and COBI experiments, with a positive association observed between RIF unbound concentration and DRV $CL_{int.app.}$ of β = 19 (95% CI = 4 to 34; P=0.017) and β = 16 (95% CI = 4 to 29; P=0.013) in the RTV experiments, and COBI experiments, respectively.

DISCUSSION AND CONCLUSIONS

RIF strongly induces the expression of metabolic enzymes such as CYP3A4 (33-35), and can also induce the activity of drug transporters (36). Collectively, this can result in clinically-relevant DDIs in patients that receive RIF together with other medications (11, 37). These DDIs present challenges for the treatment of HIV-TB patients as several therapeutic options are contraindicated due to known DDIs (10), whilst other potentially viable treatment regimens may either be delayed, or avoided completely, due to hypothetical DDIs that are predicted to occur between anti-TB drugs and ARVs such as PIs. For example, co-administering the standard-dose of any PI with RIF is currently contraindicated under WHO guidelines (15), but the extent of potential DDIs between RIF and PIs has not been determined for all PIs, including DRV. Currently, co-administering dose-adjusted LPV/r, or SQV/r together with RIF is indicated, albeit with the caveat that high levels of toxicity can occur. This raises the possibility that administering other PIs, such as RTV-, or COBI-boosted DRV, together with RIF may also be feasible. The present study addresses this issue by providing the first experimental insight into the effects of co-incubating either RTV, or COBI, together with RIF on DRV $CL_{int.app.}$ in a human hepatocyte-based *in vitro* model of drug metabolism.

Utilisation of human hepatocytes to predict hepatic metabolic clearance of xenobiotics is well-established (29, 30). In this study, incubation of cryopreserved human hepatocytes with RIF increased DRV $CL_{int.app.}$ (**Fig. 1** and **Fig. 2**). This is likely due to induction of CYP3A4 (17, 26), although the effects of RIF on transporters may also be important (30). Uptake transporters such as organic anion transporting polypeptide isoform 1B1 (OATP1B1) (38), and efflux transporters such as P-gp (39), have been shown to play a role in PI elimination, and therefore may also be relevant in the DDIs between RIF and COBI-, or RTV-boosted DRV. Indeed, RIF has been shown to inhibit OATP1B1 (40), and DRV uptake by OATP1B1 and OATP1B3 in

303 transfected CHO cells has also been reported (41). Utilising a pop-PK-model, it has been
304 suggested that OATP3A1 polymorphisms are associated with DRV PK (42), in addition, a recent
305 physiologically-based PK (PBPK) modelling-based study that investigated the PK of DRV/r
306 during pregnancy has also suggested a role for hepatic transporters in DRV disposition (43).

307 Co-incubation of human cryopreserved hepatocytes with COBI and RIF, or RTV and RIF
308 - using concentrations spanning the *in vivo* therapeutic range of these compounds - revealed that
309 both RTV and COBI could reduce RIF-enhanced DRV $CL_{int.app.}$ in a concentration-dependent
310 manner (**Fig. 1** and **Fig. 2**). RTV was more effective than COBI at attenuating the RIF-induced
311 increase in DRV $CL_{int.app.}$, with RTV exhibiting a lower IC_{50} compared to COBI, whilst RTV also
312 achieved greater maximal inhibition of the 10 µM RIF-induced increase in DRV $CL_{int.app.}$
313 compared to COBI (**Fig. 3**). Furthermore, regression analysis revealed a stronger effect of RTV
314 in comparison to COBI for their relative contribution in reducing RIF-induced increases in DRV
315 $CL_{int.app.}$. Due to the more recent approval of COBI, data regarding potential DDIs between
316 COBI and other medications is more limited than that of RTV. The expected differential DDI
317 profiles of COBI and RTV when administered with co-medications have been recently reviewed
318 (44, 45). RTV and COBI both serve as mechanism-based inhibitors of CYP3A4 *in vivo* (46, 47);
319 however, RTV is also known to induce the expression of various metabolic enzymes, including
320 *CYP3A4*, in primary human hepatocytes *in vitro* (34). Very few studies aimed at investigating
321 the relative effects of COBI as an inducer of metabolic enzyme expression have thus far been
322 conducted, although it has been suggested that the induction potential of COBI is less than that
323 of RTV (48), and that COBI is not expected to induce *CYP3A4* expression (27). It was recently
324 suggested that hepatic uptake of RTV occurs chiefly by passive diffusion (49). In addition, RTV

has been shown to induce expression of the efflux transporters *P-gp* (34), and multidrug resistance-associated protein 1 (*MRP1*; *ABCC1*) in primary human hepatocytes *in vitro* (34). DRV is a substrate of P-gp (50) and OATP1A2 and OATP1B1 (38), whilst RTV appears to inhibit P-gp (50), as well as OATP1B1 and OATP1B3 (41), *in vitro,* RTV is also reported to be a substrate of P-gp (51). At the same time, RIF has been described as both a substrate and an inhibitor of OATP1B1 and OATP1B3 *in vitro* (52). In addition, chronic exposure to RIF has been shown to exert an inhibitory effect on P-gp *in vitro* (53), whilst RIF-induced induction of *P-gp/ABCB1* and *OATP1B1* and *ABCC2* expression has also been reported (54). It remains to be seen therefore what the net contribution of transporters such as OATP1B1, OATB1B3 and P-gp may be on plasma levels of DRV *in vivo,* especially when DRV is administered in combination with other compounds such as RIF.

The PK profiles of DRV/r (800/100 mg, *qd*) and DRV/c (800/150 mg, *qd*) in HIV-infected patients are broadly similar (55, 56). However, in a study conducted in healthy volunteers, it has been reported that DRV C_{min} values were 30% lower in individuals treated with DRV/c compared with individuals treated with DRV/r (57). In addition, PK analysis of the PI tipranavir (TPV), when administered in combination with COBI or RTV in healthy volunteers, showed that TPV AUC, C_{max} and C_{tau} levels were significantly lower with COBI compared to RTV (58). Collectively, these studies suggest that the pharmacoenhancment with COBI is not always equal to that of RTV.

Whilst no studies have been conducted investigating the effects of co-administering either DRV/r or DRV/c with RIF on DRV bioavailability, it has recently been shown using a pop-PK modelling approach that administering dose-adjusted DRV/r (1600/200 mg *qd*; 800/100

mg *bid*; or 1200/150 mg *bid*) can potentially overcome the effects of RIF on DRV C_{trough}, albeit with the caveat that RTV-related side-effects may occur and that a higher pill burden would be required (23). These *in silico* findings are in general agreement with the *in vitro* outcomes of the present study. However, extrapolating the *in vivo* significance of *in vitro* data presents multiple challenges (59, 60), and it is difficult to directly infer how the results of the current study may translate *in vivo.* For example, increasing the dose of RTV in combination with a given PI is not always sufficient to overcome the effects of RIF. Indeed, a study of the effects of RIF on the steady-state PK of ATV with RTV in healthy volunteers showed that administering ATV/RTV 300/100 mg, ATV/RTV 300/200 mg, and ATV/RTV 400/200 mg was insufficient to completely overcome the inductive potential of RIF 600 mg (12). In an effort to better understand the absorption, distribution, metabolism and elimination of various compounds, the use of PBPK models has recently gained popularity (61). Various PBPK models have been developed that have proven useful in predicting the effects of administering ARVs in HIV patients with co-morbidities (62). Indeed, a recent study described the development of a PBPK model for predicting clinical DDIs from RIF-based *in vitro* human hepatocyte data (63), and it is therefore hoped that the data presented herein will be of use in the development of PBPK models to predict the effects of co-administering boosted PIs with anti-TB drugs.

In conclusion, the results presented here provide insight into the relative effects of RTV and COBI as pharmacoenhancers of DRV in the presence of RIF in an *in vitro* model of drug metabolism, which can be used in conjunction with PBPK models to rationalise future strategies aimed at optimising treatment regimens for HIV-TB patients. Further work should aim to elucidate the mechanisms that give rise to the differential inhibitory potential of COBI and RTV

demonstrated here, as well as to validate these results *in vivo*. Future studies should also aim to further evaluate the effects of COBI and RTV on gene expression, as well as the effects of these compounds on the expression and activity of various drug transporters *in vitro*. Finally, it would also be of interest to use this model system to evaluate potential DDIs that may occur between RIF and RTV, or COBI, in combination with other PIs, or with other co-medications.

ACKNOWLEDGEMENTS

This work was presented in part at Conference on Retroviruses and Opportunistic Infections (CROI), Boston, Massachusetts, USA, 22—25 February 2016, (Poster Number 459). S. K. has received research grants and/or travel bursaries from ViiV healthcare, Gilead Sciences, Janssen Pharmaceutica NV and Merck. A.O. has received research grants and/or consultancy from AstraZeneca, ViiV Healthcare, Merck and Janssen Pharmaceutica NV, and is a co-inventor on patents relating to drug delivery of anti-infective drugs. M. S. has received research grants from Janssen Pharmaceutica NV and ViiV.

384 **FUNDING INFORMATION**

385 This study was funded by Janssen, Pharmaceutical Companies of Johnson & Johnson. Grant

386 number JXR11327.

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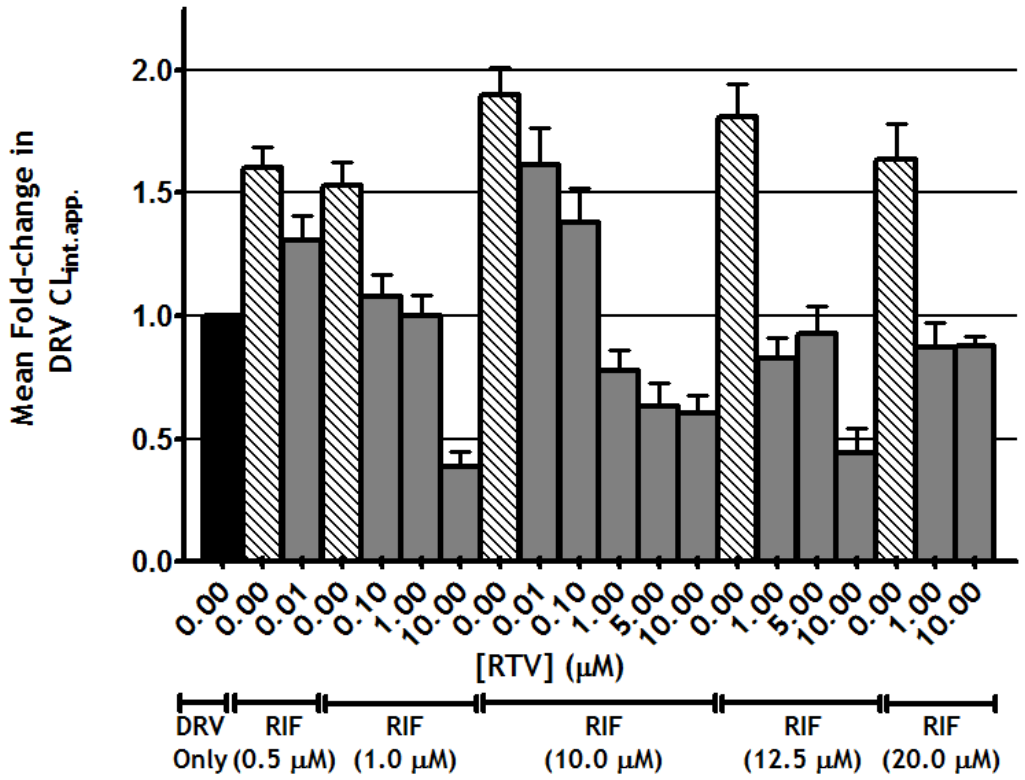
563 **FIGURES AND FIGURE LEGENDS**

564 **FIGURE 1**



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566

567 **Figure 1: Effects of rifampicin alone, or in combination with ritonavir, on mean DRV**

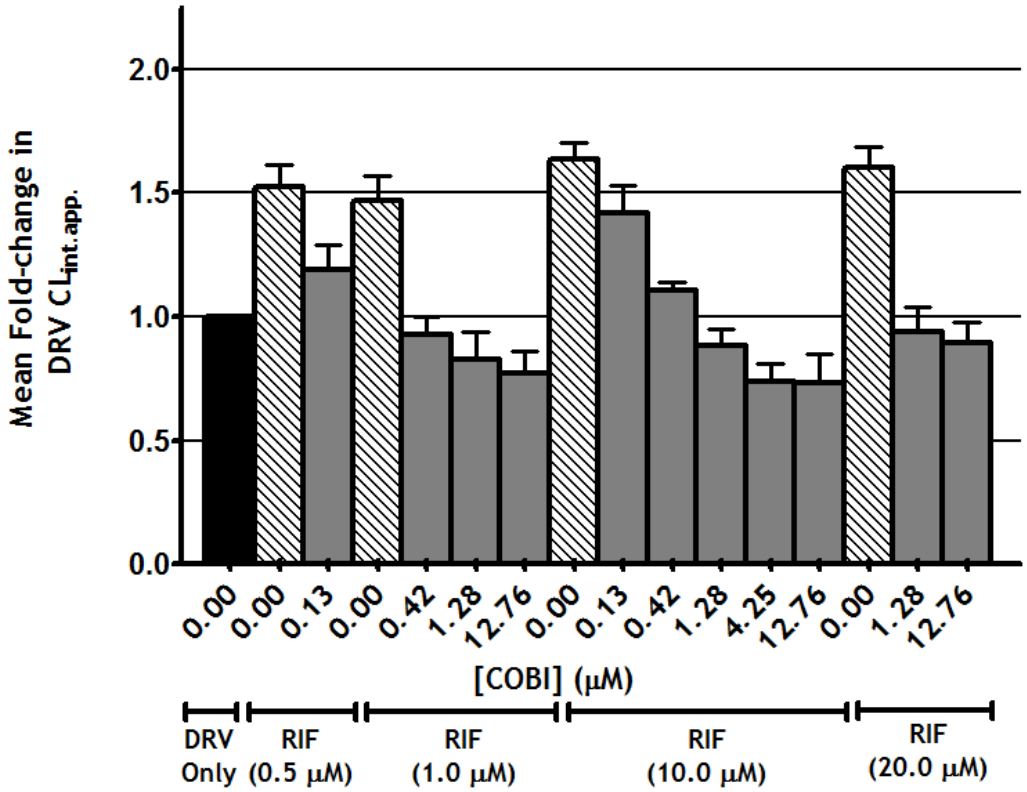
568 **$CL_{int.app.}$ in primary human hepatocytes *in vitro*.** Cryopreserved primary human hepatocytes

569 were incubated with rifampicin alone (RIF; 0.5—20 μM), hatched bars; or with RIF (0.5—20

570 μM) together with ritonavir (RTV; 0.01–10 μM), grey bars, for 72 hours. All cells were then

incubated with RIF (0.5—20 µM), or RIF (0.5—20 µM) together with RTV (0.01–10 µM) as described above, together with darunavir (DRV; 5 µM), for 60 minutes. Control cells were treated with DRV (5 µM) alone for 60 minutes (black bar). The results shown represent the mean DRV $CL_{int.app.}$ from three biological replicates measured in hepatocytes from three independent donors (Lot HU1399, HU1587 and HU1621). Error bars: SD.

576 **FIGURE 2**



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578

579 **Figure 2: Effects of rifampicin alone, or in combination with cobicistat, on mean DRV**

580 **$CL_{int.app.}$ in primary human hepatocytes *in vitro*.** Cryopreserved primary human hepatocytes

581 were incubated with rifampicin (RIF; 0.5—20 μM), hatched bars; or with cobicistat (COBI;

582 0.13–12.76 μM) and RIF (0.5—20 μM), grey bars, for 72 hours. All cells were then incubated

583 with RIF (0.5—20 μM), or RIF (0.5—20 μM) together with cobicistat (COBI; 0.13–12.76 μM)

584 as described above, together with darunavir (DRV; 5 μM) for 60 minutes. Control cells were

585 treated with DRV (5 μM) alone for 60 minutes (black bar). The results shown represent the
586 mean DRV $CL_{int.app.}$ from three biological replicates measured in hepatocytes from three
587 independent donors (Lot HU1399, HU1574 and HU1587). Error bars: SD.

588

589 **FIGURE 3**

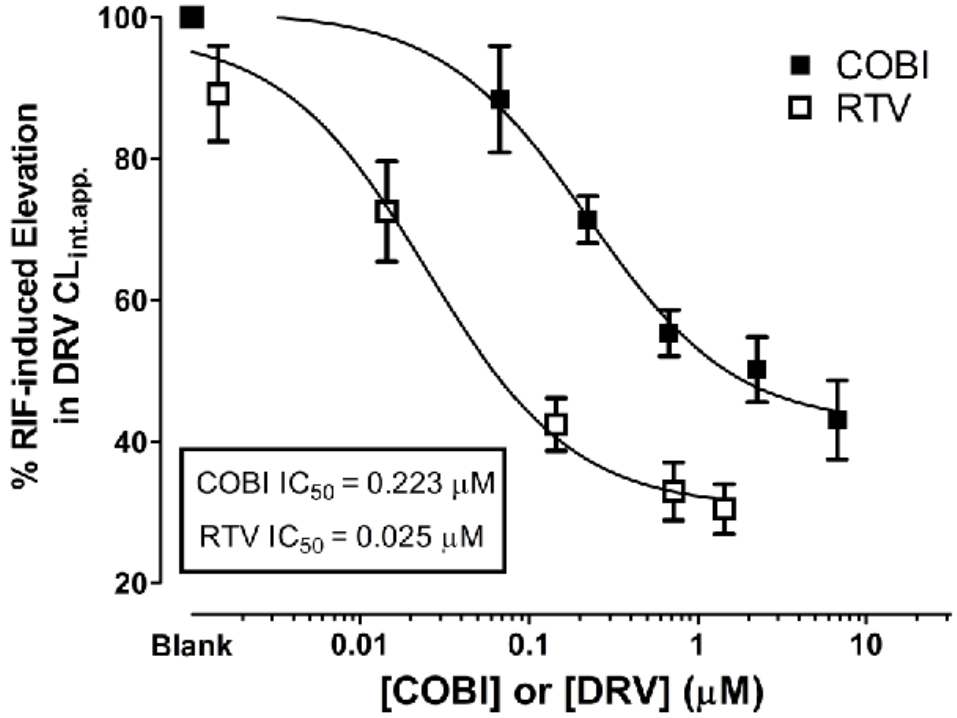

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Figure 3: **Comparative effectiveness of COBI and RTV at lowering RIF-induced DRV $CL_{int.app.}$ in human primary hepatocytes *in vitro***. Graph shows the relative effects of cobicistat (COBI) and ritonavir (RTV) on inhibition of 10 μM rifampicin (RIF)-induced elevations in DRV $CL_{int.app.}$ in cryopreserved primary human hepatocytes. Cells were co-incubated with RTV (starting total concentrations of 0.1—10 μM; donors HU1399, HU1587 and HU1621), or COBI (starting total concentrations of 0.13—12.76 μM; donors HU1399, HU1574 and HU1587) in combination with RIF (10 μM) for 72 hours prior to co-incubation with DRV (5 μM) for one hour. Each condition was tested in triplicate in each donor. The concentrations of RTV and COBI plotted represent the unbound concentrations present in WME incubation medium following correction for protein binding. Untreated control (blank) was assigned a value of 0.001 μM in each case. Error bars: SEM.

609 **TABLES**

610 **TABLE 1**

611 **Table 1:** Donor Information for Cryopreserved Primary Human Hepatocytes Used

Donor	Sex	Race	Age	Medications	Drug Use
HU1399	Female	Caucasian	72	Insulin glargine: 10 units *qd*; Metoprolol: 100 mg *qd*; Lisinopril hydrochlorothiazide: 20/12.5 mg *qd*; Calcium + Vitamin D: 500 mg *qd*; Multivitamin: *qd*; Aspirin: 81 mg *qd*	Historic long-term tobacco use
HU1574	Male	Caucasian	70	Atorvastatin: 80 mg *qd*; Lisinopril: 5 mg *qd.*; Aspirin: 81 mg *qd*; Tamsulosin: 4 mg *qd*	None reported
HU1587	Female	Caucasian	43	Vitamin D oral; Multivitamin oral; Calcium + Vitamin D + Vitamin K	None reported
HU1621	Male	Caucasian	66	Pazopanib: 800 mg *qd*	Rare alcohol use. Historic tobacco use

612

613 **TABLE 2**

614 **Table 2:** Fraction Unbound (f_u) Values in Williams' Medium E (WME) Incubation Medium of

615 each Compound Used in This Study (mean ± SD; n=3)

Compound	Fraction Unbound (f_u) in WME Incubation Medium (mean ± SD)
COBI	0.53 ± 0.04
DRV	0.76 ± 0.07
RIF	0.63 ± 0.05
RTV	0.14 ± 0.01

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