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Title page:

MRP2 inhibition by HIV protease inhibitors in rat and human hepatocytes: a quantitative confocal microscopy study.

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Running title page:

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List of non-standard abbreviations

Adverse drug reactions, ADRs; amprenavir, APV; atazanavir, ATZ; biliary excretion index, BEI; 5(6)-carboxy-2',7'-dichlorofluorescein (diacetate), CDF(DA); darunavir, DRV; Dulbecco's modified Eagle's medium, DMEM; Hanks' Balanced Salt Solution, HBSS; indinavir, IDV; lopinavir, LPV; multidrug resistance-associated protein 2, Mrp2/MRP2; nelfinavir, NFV; phosphate buffered saline, PBS; ritonavir, RTV; sandwich-cultured hepatocytes, SCH; sandwich-cultured human hepatocytes, SCHH; sandwich-cultured rat hepatocytes, SCRH; saquinavir, SQV; tipranavir, TPV; William's E medium, WEM

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Abstract

Hepatic drug transporters play a pivotal role in the excretion of drugs from the body, in drug-drug interactions as well as in drug-induced liver toxicity. Hepatocytes cultured in sandwich configuration are an advantageous model to investigate the interactions of drug candidates with apical efflux transporters in a bio-relevant manner. However, the commonly used 'offline' assays (i.e. that rely on measuring intracellular accumulated amounts after cell lysis) are time and resource consuming while data output is often high and variable. In the present study, we used confocal microscopy to investigate the inhibitory effect of all marketed HIV protease inhibitors (10 μM) on the apical efflux transporter multidrug resistance-associated protein 2 (MRP2; ABCC2) by visualizing the biliary accumulation of the fluorescent substrate 5(6)-carboxy-2',7'-dichlorofluorescein (CDF). This method was applied with sandwich-cultured human and rat hepatocytes. Alterations in the biliary excretion index of CDF were calculated based on quantitative analysis of fluorescence intensities in the confocal images. In human hepatocytes, lopinavir followed by tipranavir, saquinavir, atazanavir and darunavir were the most potent inhibitors of MRP2-mediated efflux of CDF. In rat hepatocytes, tipranavir inhibited Mrp2-mediated CDF efflux most potently, followed by lopinavir and nelfinavir. In conclusion, when comparing these findings with previously published data generated in 'offline' transporter inhibition assays, this microscopy-based approach allowed us to investigate the inhibitory effect of drugs on efflux transporters in a very sensitive and non-destructive manner.

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Introduction

Drug-induced hepatotoxicity and adverse drug reactions (ADR) are commonly encountered phenomena during the drug development process. Interactions of drugs with hepatic drug transporters have been implicated to be responsible for a part of these adverse effects (Giacomini *et al.*, 2010). Hepatic drug transporters play an important role in the disposition of many drugs, but also of several endogenous compounds, such as bilirubin and bile acids. It follows that these transporters are important sites of interaction, since competition may emerge for the same transporter binding sites between multiple co-administered drugs on the one hand, and between drugs and endogenous compounds on the other hand. This competition often leads to alterations in the pharmacokinetics and/or toxicity of drugs, but can also pose problems regarding the homeostasis of endogenous compounds (e.g. in case of cholestasis). This implies that, during drug development, it is very important to determine the possible modulatory effects of drug candidates towards drug transporters.

Several model systems are available to explore the transport kinetics of drug candidates by drug transporters. Interaction with uptake transporters is frequently studied using transfected cell lines expressing one specific transporter (Annaert *et al.*, 2010) or using hepatocytes (suspension of in culture) (De Bruyn *et al.*, 2013). However, freshly-isolated or cryopreserved hepatocytes have the advantage of concomitantly expressing the main uptake transporters (for some of them at levels comparable to the *in vivo* situation, which allows to gain insight in the contribution of each transporter in drug uptake (Badée *et al.*, 2015). Considering efflux transporters, available model systems include inside-out membrane vesicles, either prepared from liver tissue (Guyot *et al.*, 2014) or from cells expressing one specific transporter (Köck and Brouwer, 2012), as well as suspended or sandwich-cultured hepatocytes (SCH) (De Bruyn *et al.*, 2013). More specifically, the expression levels of the basolateral efflux transporters MRP3 (*ABCC3)* and MRP4 (*ABCC4*) were shown to be comparable between suspended hepatocytes and the *in vivo* situation, while some apical (canalicular) efflux transporters were shown to undergo partial internalization (Bow *et al.*, 2008;

Lundquist *et al.*, 2014). Importantly, culturing hepatocytes in sandwich configuration for multiple days allows the formation of bile canaliculi and the proper localization of the apical transporters, which makes it a preferred *in vitro* model to study expression, function and regulation of the apical efflux transporters (De Bruyn *et al.*, 2013).

The present study introduces a non-destructive technique based on confocal microscopy to quantitatively measure the Mrp2/MRP2 inhibition in rat and human hepatocytes in sandwich culture using CDF. CDF is a well characterized fluorescent substrate of MRP2/Mrp2 (Bow *et al.*, 2008; Heredi-Szabo *et al.*, 2008; Ye *et al.*, 2010; Wissel *et al.*, 2015). More specifically, the inhibitory effect of all marketed HIV protease inhibitors on the Mrp2/MRP2 activity was quantified by determining changes in the biliary excretion index (BEI) of CDF in the presence of these anti-retroviral drugs. The anti-retroviral drugs, which are currently used in the treatment of HIV infection, were chosen as model drugs. Additionally, they often play an important role in many profound drug-drug interactions in the clinic. Moreover, their hepatic disposition constitutes a complex interplay between uptake, metabolism and efflux. These drugs are taken up by the Oatp/OATP family, metabolized by CYP3A enzymes and excreted by the efflux transporters Mdr1/MDR1 and Mrp2/MRP2 (Huisman *et al.*, 2002; Liu and Unadkat, 2013; De Bruyn *et al.*, 2016) .

Finally, the obtained results from the present study were compared with previously published data obtained by a conventional (‘offline’) approach in sandwich-cultured rat and human hepatocytes (SCRH and SCHH) (Ye *et al.*, 2010).

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Materials and methods

Chemicals

Darunavir (DRV) ethanolate, tipranavir (TPV), ritonavir (RTV), atazanavir (ATV) sulfate, and amprenavir (APV) were provided by the National Institutes of Health AIDS Research and Reference Reagent Program (Germantown, MD). Saquinavir (SQV) mesylate, indinavir (IDV) sulfate, nelfinavir (NFV) mesylate, and lopinavir (LPV) were donated by Hetero Drugs Ltd. (Hyderabad, India). William's E medium (WEM), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin-streptomycin (each 10.000 IU/ml), fetal bovine serum, Hanks' Balanced Salt Solution (HBSS) and phosphate buffered saline (PBS) were purchased from Westburg (Leusden, The Netherlands). ITS$^+$ universal culture supplement premix was from BD Sciences (Erembodegem, Belgium). HEPES was purchased from MP Biochemical (Illkirch, France). Collagenase (Type IV), dexamethasone, insulin from human origin, 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate (CDFDA), Percoll® and ECM gel (from Engelbreth-Holm-Swarm murine sarcoma) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Synth-a-Freeze™ was purchased from Life Technologies (Ghent, Belgium).

Methods

Animals

Male Wistar rats in a weight range of 170 – 200 g were purchased from Janvier (Le Genest Saint Isle, France) and were used for isolation of hepatocytes. The animals were housed in the Central Animal Facilities of KU Leuven, according to the Belgian and European laws, guidelines and policies for animal experiments, housing and care. Approval for the experiments was granted by the Institutional Ethical Committee for Animal Experimentation of the KU Leuven. Rats were maintained in a 12 h light–dark cycle with free access to water and standard rat/mouse maintenance food (ssniff Spezialdiäten GmbH, Germany).

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Isolation and cryopreservation of rat hepatocytes

Rat hepatocytes were isolated using a 2-step collagenase perfusion, as previously described (Annaert *et al.*, 2001). Rats were anesthetized using an intraperitoneal injection (120 mg/kg ketamine + 24 mg/kg xylazine i.p.). After isolation, cells were centrifuged (50 × *g*) for 3 min at 4 °C and the pellet was re-suspended in WEM containing 5% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. The hepatocytes were counted three times in a hemocytometer and the viability was determined using the Trypan blue (0.04%) exclusion method. After counting, the hepatocytes were either cultured freshly or cryopreserved for later use. In that case, cells were again centrifuged at 50 g for 3 min at 4°C and the pellet was re-suspended in Synth-a-Freeze™ at a cell density of 10×10^6 cells/mL. Cells were cryopreserved in a programmable controlled rate freezer, Kryo 560–16 (Planer, Sunbury-on-Thames, UK), during a freezing cycle which included a supercooling phase. After reaching − 100 °C, the cryovials were stored in liquid nitrogen until thawing.

Sandwich-cultured rat hepatocytes

Cryopreserved rat hepatocytes were quickly thawed in a 37°C water bath. When all ice was melted, the content was transferred to a mixture of isotonic Percoll® (32%) and thawing medium (DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml insulin and 1 µM dexamethasone) (68%). The cells were centrifuged at 168 g for 20 min at 22°C. Subsequently, the pellet was re-suspended in thawing medium and centrifuged at 50 g for 3 min at 22°C. Next, the cells were re-suspended in seeding medium (WEM containing 10 % FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 µg/ml insulin and 1 µM dexamethasone) at a density of 1.2 million cells/ml. The hepatocytes were then seeded in glass bottom 24-well plates at a density of 26 x 10^4 cells/cm² and placed in an incubator (37°C, 5% CO_2, 95% humidity) (binder CO_2 incubator, binder GmbH). These 24-well plates with glass bottom and a thickness of 160 – 190 µm (MatTek, Ashland, MA, USA) were pre-coated 24

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h before seeding with 50 µg/ml collagen diluted in 0.02 M acetic acid ("*rigid collagen*"). The wells were washed 3 times with PBS before seeding. Unattached cells were aspirated 2 h after seeding by shaking the plate and immediately aspirating the media. Subsequently, cultures were overlaid with ice-cold neutralized collagen solution (~ 1.5 mg/mL, pH 7.4) to obtain a sandwich configuration. After 40 minutes, seeding medium was added to the SCH. The medium was replaced every 24 h with maintenance medium (WEM supplemented with 1% ITS$^+$ universal culture supplement premix, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.1 µM dexamethasone). Cells were used at day 3 of culture time.

Sandwich-cultured human hepatocytes

Human cryopreserved hepatocytes for sandwich culture (batch #S2203LT) were kindly provided by KaLy-Cell (Plobsheim, France) and were from a Caucasian, 59-year-old woman of who died from a colorectal adenocarcinoma. The hepatocytes were quickly thawed in a 37°C water bath. When all ice was melted, the content was transferred to a mixture of isotonic Percoll® (32%) and thawing medium (68%). The cells were centrifuged at 168 g for 20 min at 22°C. Subsequently, the pellet was re-suspended in thawing medium and centrifuged at 100 g for 3 min at 22°C. Next, the cells were re-suspended in seeding medium at a density of 1.2 million cells/ml. Viability of the thawed hepatocytes was 91 %. The hepatocytes were then seeded in glass bottom 24-well plates, pre-coated with rigid collagen (see SCRH) at a density of 26 x 10^4 cells/cm² and placed in an incubator (37°C, 5% CO_2, 95% humidity). Next, the cells were overlaid with 0.25 mg/mL ECM gel in growing medium after 24 h (day 1). The medium was replaced every 24 h with fresh maintenance medium. Cells were used at culture day 6.

Imaging of CDF efflux

The biliary excretory function of SCRH/SCHH was assessed by quantitative evaluation of the CDF efflux in biliary canalicular network. Cells were washed twice with standard buffer (HBSS

buffered with HEPES (10 mM) at pH 7.4), and then pre-incubated with standard buffer containing a HIV protease inhibitor (10 μM) for 10 minutes. Next, the cells were incubated with CDFDA (1 or 4 μM, as specified in the figure legends) for 10 minutes in the absence or presence of a HIV protease inhibitor (10 μM). All experiments were conducted at 37°C. Then, the culture plate was placed under a Nikon A1R Eclipse Ti confocal microscope with a temperature control unit set at 37°C. Images were acquired using the FITC laser line with excitation wavelength 488 nm. The HV (gain) was set at 100 (default); offset-background was left at 0; laser power was set at 5%; the pinhole was set at 1.2 AU. During picture acquisition, pixel intensity and saturation was monitored and if required, laser power and HV were optimized in order to obtain adequate pixel intensities. Of each condition, three images at a resolution of 1024 x 1024 pixels were taken of random locations within the well.

Calculation of BEI of CDF:

ImageJ software (National Institutes of Health) was used for quantitative analysis of the fluorescent microscopic images. The average pixel intensity of the areas representing the bile canaliculi and cells was determined after manual selection of these regions based upon phase contrast images, taking into account background. The BEI was calculated according to the following formula:

$$BEI(\%) = \frac{(\sum pixel\ intensity)_{bile}}{(\sum pixel\ intensity)_{cells+bile}} \times 100$$

Statistics:

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test. P values less than 0.05 were considered as statistically significant.

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The Spearman rank correlation coefficient was determined between the obtained data in this study and previously published data obtained with the 'offline' assay (Ye *et al.*, 2010). This was done according to the following formula:

$$\rho = 1 - \frac{6 \sum d_i^2}{n(n^2 - 1)}$$

where ρ is the Spearman rank correlation coefficient, d_i is the difference in ranks and n is the sample size.

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Results

The BEI of CDF as a function of incubation time

In a first phase of experiments, we investigated the optimal incubation time of SCRH with CDFDA in order to determine the BEI for CDF. This was done by using CDFDA (4 μM) in absence or presence of the well-known MRP inhibitor MK-571 (10 and 30 μM). Time lapse imaging showed that the BEI for CDF was constant during 10 minutes of incubation for all three conditions; the BEI was about 47% in the control condition, while MK-571 reduced it towards 10% using both tested concentrations (**Figure 1**). Interestingly, although MK-571 at 30 μM clearly reduced the total fluorescence intensity in the image as compared to MK-571 at 10 μM, both concentrations resulted in a similar BEI of CDF. As there was no influence of incubation time on BEI of CDF, we selected 10 minutes as incubation time for the next experiments.

The effect of HIV protease inhibitors on the BEI of CDF in SCRH.

The effect of all HIV protease inhibitors on the BEI of CDF in SCRH at culture day 3 was investigated. **Figure 2** shows the images taken after 10 minutes of incubation with CDFDA (1 μM) in absence and presence of all HIV protease inhibitors (10 μM). These data clearly show a wide range in the inhibitory effect of HIV protease inhibitors on Mrp2-mediated CDF transport. Tipranavir had the most pronounced effect on the BEI of CDF, thereby almost completely blocking the efflux of CDF into the bile canaliculi. This was followed by lopinavir and saquinavir. Indinavir, atazanavir, ritonavir and darunavir did not cause a statistically significant decrease in BEI of CDF. Amprenavir on the other hand increased the BEI of CDF by 24%.

The effect of HIV protease inhibitors on the BEI of CDF in SCHH.

The effect of all HIV protease inhibitors on the BEI of CDF was investigated in SCHH at culture day 6. **Figure 3** shows the images taken after 10 minutes of incubation with CDFDA (1 μM) in absence and presence of all HIV protease inhibitors (10 μM). These data clearly show a

wide range in the inhibitory effect of HIV protease inhibitors on MRP2. Lopinavir had the most pronounced effect on the BEI of CDF, thereby almost completely blocking the efflux of CDF into the bile canaliculi. This was followed by tipranavir, saquinavir, atazanavir and darunavir, respectively. Ritonavir, amprenavir, nelfinavir and indinavir did not cause a statistically significant change in BEI of CDF.

Comparison between the presently obtained transporter inhibition data and previously obtained data with the conventional ('offline') assay.

The Spearman rank correlation coefficient (ρ) between the obtained data in this study using confocal microscopy and previously published data, which was based on a method using cellular accumulation and efflux was determined (Ye *et al.*, 2010). As tipranavir was not included in the study performed by Ye *et al*., this compound was not taken into account in the analysis. ρ values of 0.71 and 0.62 were obtained for the data obtained in rat and human hepatocytes, respectively (Supplemental Table 1).

Discussion

The current study aimed to investigate the use of confocal microscopy to quantitate the inhibitory effect of all clinically relevant HIV protease inhibitors on the activity of Mrp2/MRP2 in SCRH and SCHH. SCH were used as the preferred *in vitro* model since hepatocytes cultured in a sandwich configuration have the ability to form functional biliary networks, and subsequently to maintain the presence of canalicular efflux transporters (LeCluyse *et al.*, 1994; Lecluyse *et al.*, 2000; De Bruyn *et al.*, 2013). Over the past years, SCH have become the gold standard to perform hepatic *in vitro* studies, which allows us to explore the hepatic disposition of compounds, including uptake, metabolism and efflux.

Using CDFDA in cell-based assays has the great advantage that it is non-fluorescent and passively diffuses inside cells where it is rapidly hydrolyzed to fluorescent CDF. In rat hepatocytes, CDF was shown to be transported via Mrp2 into the bile and via Mrp3 into the sinusoidal blood, after which it can undergo cellular re-uptake via Oatp-mediated transport (Zamek-Gliszczynski *et al.*, 2003). Mrp2 was shown to carry out the majority of CDF efflux, but the role of Mrp3 was shown to increase in absence of functional Mrp2 (Ellis *et al.*, 2014). Confocal microscopy was used to visualize and quantify the fluorescence intensity inside the cells and the bile in all conditions tested. In that way, the BEI of CDF (reflecting the ratio of CDF in the bile canaliculi versus CDF in the cells plus bile canaliculi) could be determined. Eventually, this allowed to investigate the effects of HIV protease inhibitors on Mrp2/MRP2-mediated transport in a non-destructive manner.

Nakanishi *et al*. were the first to describe the use of fluorescence microscopy to study Mrp2 inhibition by drugs and/or drug metabolites in rat hepatocytes (Nakanishi *et al.*, 2011, 2012). These investigators looked at a change of fluorescence intensity in the bile canaliculi when potential Mrp2 inhibitors were added. In that way, it is difficult to differentiate between esterase inhibition on one hand and Mrp2 inhibition on the other hand: it is indeed possible that drugs and/or their metabolites affect the esterases converting CDFDA to CDF, which would also lead to a reduction of fluorescence intensity. In the present study, the fluorescence intensity was measured both in the

cells and in the bile canaliculi, taking into account only the effect of drugs on Mrp2/MRP2. Indeed, potential inhibition of the intracellular esterase activity by the drug (metabolite) would not affect the BEI of CDF.

Another advantage of the present approach includes the possibility to specifically explore the interference with Mrp2/MRP2 mediated transport at the canalicular membrane. For instance, it is well-known that typical Mrp2/MRP2 inhibitors are quite often MRP3 inhibitors and that CDF is not only a substrate for Mrp2/MRP2, but also for Mrp3/MRP3 (Zamek-Gliszczynski *et al.*, 2003; Weiss *et al.*, 2007). Based on unpublished in-house data, CDF is a good substrate for rat Mrp3, while it is a poor substrate for human MRP3. Since Mrp3/MRP3 is mainly present at the basolateral side of the hepatocyte membrane (König *et al.*, 1999), it is likely involved in CDF efflux back into the medium (Zamek-Gliszczynski *et al.*, 2003). However, very limited impact is expected from Mrp3/MRP3 (or other Mrp/MRP isoforms located at the basolateral membrane) on the BEI values of CDF in the present study.

Additionally, it is known that CDFDA passively diffuses into the cells, while CDF relies on active uptake mediated by OATP. This means that compounds, which can inhibit the reuptake of CDF, could potentially lead to a decrease of the intracellular amounts of CDF, resulting in a decreased fluorescence intensity in our assay. As canalicular excretion is the driving force of CDF disposition (which can also be seen from the microscopic images), the impact of inhibition of CDF reuptake by OATP in the present assay would be rather low. Moreover, the expression of uptake transporters including the OATP family decreases in function of culture time in SCRH/SCHH (De Bruyn *et al.*, 2013).

To correctly calculate the BEI based on image analysis, saturation of pixel intensity had to be avoided. This was achieved by optimizing laser intensity, gain, and exposure time. An additional advantage of calculating the BEI based on the image analysis is the shorter time required for data generation and processing (as compared to using LC-based determination of cell/medium concentrations for instance).

To our knowledge, this is an improved non-destructive assay which uses confocal imaging to calculate a BEI value and eventually to investigate Mrp2/MRP2 inhibition of drugs/metabolites in cultured hepatocytes using a fluorescent substrate. Most often, assays to screen for efflux transporter inhibitors in cultured cells rely on efflux studies, which use both standard medium (bile canaliculi are intact) and Ca^{2+}/Mg^{2+}-free medium (bile canaliculi are disrupted) to calculate the accumulation of compounds in bile canaliculi, referred to as the conventional "*offline*" method (Ye *et al.*, 2010). Also, transporter-expressed membrane vesicles and transporter-transfected cell lines are regularly used to screen for efflux transporter inhibitors (Förster *et al.*, 2008; Lechner *et al.*, 2010; Morgan *et al.*, 2013). However, in that case, the formation of possible metabolites, which in their turn can lead to possible inhibitory effects, is ignored.

For the series of HIV protease inhibitors, a wide range in the extent of inhibition was observed in both SCRH and SCHH. In SCRH, a slight increase in the BEI of CDF in presence of amprenavir was noticed, for which the specific reason remains uncertain. As mentioned earlier, it would be unlikely that this can be explained by interaction of amprenavir with Mrp3, which was to our knowledge also not demonstrated in literature. There is a possibility that amprenavir increases the BEI of CDF by inhibiting Oatp in rat hepatocytes, which in fact was shown earlier (Annaert *et al.*, 2010). Furthermore, it has been suggested that amprenavir is taken up by an unidentified transporter for which CDF may be a substrate (Liu and Unadkat, 2013). However, it is also very unlikely that amprenavir influences the efflux of CDF mediated by another known canalicular transporter, as it is shown that CDF is not a substrate for Bsep. However, in the same study, it was seen that CDF uptake by Bcrp-expressing membrane vesicles was approximately 29 % of that by Mrp2, meaning that CDF is a weak substrate for Bcrp and thus can function as a partially-selective substrate for Mrp2 (Nakanishi *et al.*, 2012). Another study showed that amprenavir was not an inhibitor, nor a substrate for BCRP (Gupta *et al.*, 2004). The CDF present in the medium may result from bile canaliculi intermittently releasing their contents or may be the result of sinusoidal efflux transport by the hepatocytes via Mrp3. Given the relatively short incubation time (10 min), the latter

mechanism likely predominates in the current experimental conditions. A last hypothesis is that the increased BEI can be attributed to an activation of Mrp2 by amprenavir. However, no evidence was found on this mechanism.

Potent inhibitors for CDF efflux via Mrp2/MRP2 were found to be lopinavir and tipranavir in both rat and human hepatocytes. Nelfinavir was also able to decrease the BEI in rat hepatocytes, but not in human, while darunavir, atazanavir and saquinavir were only able to inhibit CDF efflux in human hepatocytes. According to Ye et al, lopinavir, nelfinavir and indinavir were found to be the most potent Mrp2 inhibitors in rat hepatocytes, while in our study indinavir did not have any effect on the BEI of CDF in both rat and human hepatocytes, which is also the case for ritonavir (Ye *et al.*, 2010). Our obtained *in vitro* data are not entirely in accordance with what was found in literature. For example, it has been shown previously that saquinavir and ritonavir are able to inhibit the Mrp2-mediated transport of methotrexate (Gutmann *et al.*, 1999). However, this discrepancy is likely due to the fact that another probe substrate was used. This substrate-dependency has been shown previously as it is known that MRP2 has more than one non-identical binding site (Gerk *et al.*, 2004) (Zelcer *et al.*, 2003). Ye at al., have applied two Mrp2 substrates, namely CDF and estradiol-17-β-glucuronide, to investigate the possibility of Mrp2 inhibition by HIV protease inhibitors in the same *in vitro* model, which also resulted in substrate-dependent inhibitory effects (Ye *et al.*, 2010). Therefore, a direct comparison between different studies using different probe substrates to investigate the effect of drug on Mpr2/MRP2 mediated transport is not always possible. Moreover, the difference in results between rat and human in the first place can be explained by a species difference in the amino acid sequence of the transporter. It was found that human MRP2 has 78% amino acid sequence identity with rat Mrp2 (Mazur *et al.*, 2012). Moreover, a species difference in Mrp2/MRP2-mediated transport has been shown by several research teams. Additionally, the absolute amount of Mrp2 in rat is about 10-fold higher than the amount of MRP2 in human, as quantified by LC-MS/MS (Li *et al.*, 2009).

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A role for Mrp2/MRP2 in the hepatic disposition of HIV protease inhibitors has been reported in several in vitro studies. Saquinavir, ritonavir and indinavir were identified as MRP2 substrates, while lopinavir was found to be a substrate for both at Mrp2 and human MRP2 (Huisman *et al.*, 2002). However, Bierman *et al*. reported that several HIV PI are poor substrates yet potent blockers for ABC transporters in general (Bierman *et al*., 2010). In our study, especially lopinavir and saquinavir, but not indinavir, decreased the BEI of CDF to some extent in human hepatocytes.

Eventually, the current data were compared with data obtained from a study which was performed by our research team, applying the conventional 'offline' method (Ye *et al*., 2010). The Pearson correlation coefficient between these two data sets was relatively poor, especially for the data obtained with human hepatocytes. Nevertheless, the three least potent MRP2-inhibiting HIV PI in the study of Ye *et al*. (amprenavir, indinavir, nelfinavir) also turn out to be least potent in the present study. Furthermore, the lack of pronounced MRP2 inhibiting properties of these three HIV PI appears consistent with several other studies on the effects of these HIV PI on MRP1 activity (Jones et al. 2001; van der Sandt et al. 2001; Bierman et al. 2010). At the other end of the spectrum, lopinavir, tipranavir, saquinavir and atazanavir are the most potent MRP2-inhibiting HIV PI in the present study. This 'class of most potent' MRP2 inhibitors is largely in agreement with the data obtained by Ye et al., reporting at least 60% inhibition of MRP2 with the conventional method for lopinavir, saquinavir and atazanavir. For the same HIV PI (and including tipranavir), clear evidence was found in literature that they inhibit MRP1 and/or MRP2 (Agarwal et al. 2007; Janneh et al. 2008; 2009; Bierman et al. 2010). Taken together, there seems to be an acceptable agreement between presently and previously obtained data (including the data obtained by Ye et al.) when considering MRP2 inhibitory potency of HIV protease inhibitors. The relatively large variability (low precision) in the effect of several HIV protease inhibitors on the biliary excretion of CDF in study by Ye et al. remains the most important major difference with the present study. In fact this may explain at least in part the lack of an excellent correlation between both data sets. This variability may be inherent to conventional method since it is based on the assumption that bile

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canaliculi remain intact in the presence of media containing Ca^{2+} and Mg^{2+}. However, the present data show that also in the presence of standard buffer (containing Ca^{2+} and Mg^{2+}), bile canaliculi tend to spontaneously open and close on a regular basis (Supplemental Figure 1). Within the time interval of 70 minutes, some bile canaliculi opened already within 5 minutes while some remained closed throughout the entire experiment. The occurrence of bile canalicular contraction in sandwich culture has been described previously (Reif *et al.*, 2015).

In view of extrapolating these findings on Mrp2/MRP2 inhibition to the *in vivo* situation, the question arises to what extent the hepatocyte/medium concentration ratio of the HIV protease inhibitor *in vitro* corresponds to the liver/plasma concentration ratio of these drugs in vivo. Previously, we determined the Kpuu (intracellular to extracellular unbound concentration ratio) values for ritonavir (Keemink *et al.*, 2015) and atazanavir (Nicolaï *et al.*, 2016) in rat and/or human hepatocytes. These investigations revealed that intracellular exposure to HIV protease inhibitor is the result from a complex interplay between extensive hepatic metabolism and uptake as well as efflux transport. As CYP-mediated metabolic capacity is significantly reduced in cultured primary hepatocytes, differences in intracellular exposure (due to different accumulation kinetics and differences in metabolic clearances) as compared to hepatocytes in vivo can be expected. It follows that future in vitro exploration of Mrp2/MRP2 inhibition should be preferentially conducted at extracellular concentrations yielding intracellular hepatic exposure as observed during *in vivo* studies. PBPK models for HIV protease inhibitors that are currently in development (De Bruyn *et al.*, 2016) should be instrumental in estimating these in vivo intrahepatic exposure values.

Conclusion

Confocal imaging allowed to investigate the inhibitory effect of HIV protease inhibitors on the BEI of CDF in a very sensitive and non-destructive manner. The BEI calculated based on confocal imaging supports a higher throughput and better precision compared to classical methods.

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Authorship Contributions

Conception and design of the work: Nico Holmstock, Jan Snoeys, Pieter Annaert

Conducted experiments: Nico Holmstock

Performed data analysis: Nico Holmstock

Wrote or contributed to the writing of the manuscript: Nico Holmstock, Marlies Oorts, Jan Snoeys, Pieter Annaert

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Footnotes

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Legends for figures:

Figure 1: The effect of MK-571 on the BEI of CDF in sandwich-cultured rat hepatocytes at culture day 3. A: phase contrast and fluorescence images of CDF accumulation in absence (control) and presence of MK-571 (10 and 30 μM). Images taken after 10 minutes of incubation with CDFDA (4 μM). B: the BEI of CDF as a function of incubation time in absence (▲) and presence of MK-571 at 10 μM (●) and 30 μM (■).

Figure 2: Effect of HIV protease inhibitors on BEI of CDF in sandwich-cultured rat hepatocytes at culture day 3. Cells were pre-incubated with HIV protease inhibitors (10 μM) for 10 minutes, prior to incubation with CDFDA (1 μM) for 10 minutes in absence or presence of HIV protease inhibitors. A Control; B amprenavir; C atazanavir; D darunavir; E indinavir; F lopinavir; G nelfinavir; H ritonavir; I saquinavir; J tipranavir. Bars represent the mean ± S.D. (n = 3). Statistical significance between the different conditions was evaluated using one-way ANOVA followed by Dunnett's test. Significantly different from control condition; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 3: Effect of HIV protease inhibitors on BEI of CDF in sandwich-cultured human hepatocytes at culture day 6. Cells were pre-incubated with HIV protease inhibitors (10 μM) for 10 minutes, prior to incubation with CDFDA (1 μM) for 10 minutes in absence or presence of HIV protease inhibitors. A Control; B amprenavir; C atazanavir; D darunavir; E indinavir; F lopinavir; G nelfinavir; H ritonavir; I saquinavir; J tipranavir. Bars represent the mean ± S.D. (n = 3). Statistical significance between the different conditions was evaluated using one-way ANOVA followed by Dunnett's test. Significantly different from control condition; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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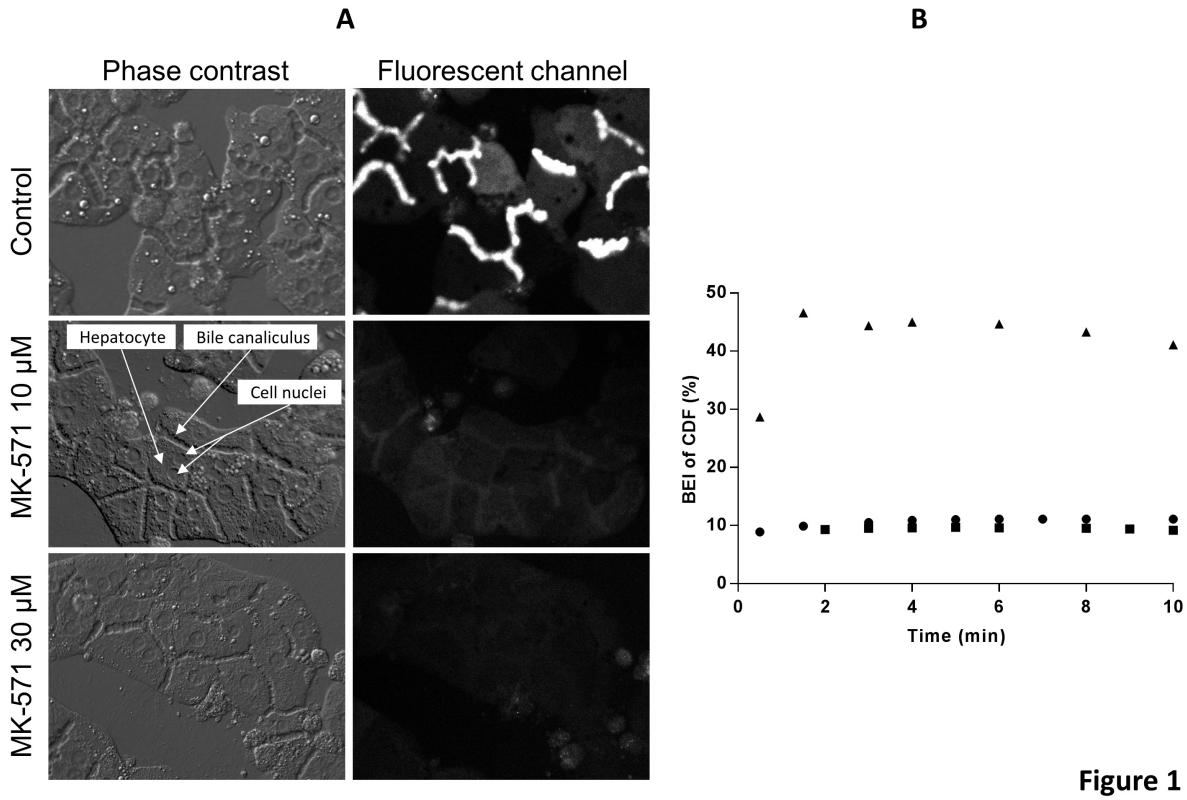

Figure 1

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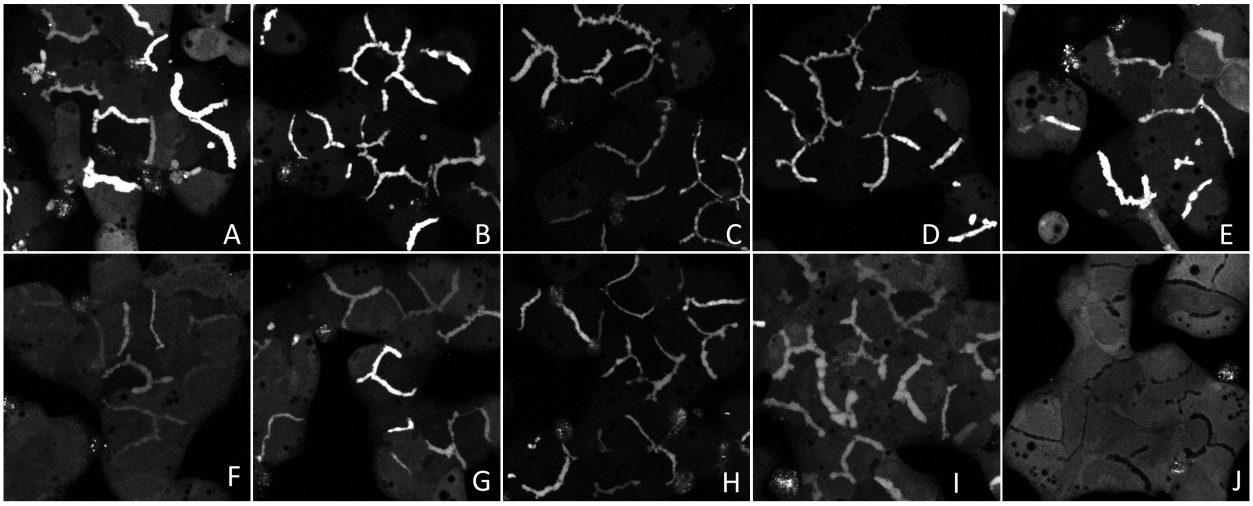

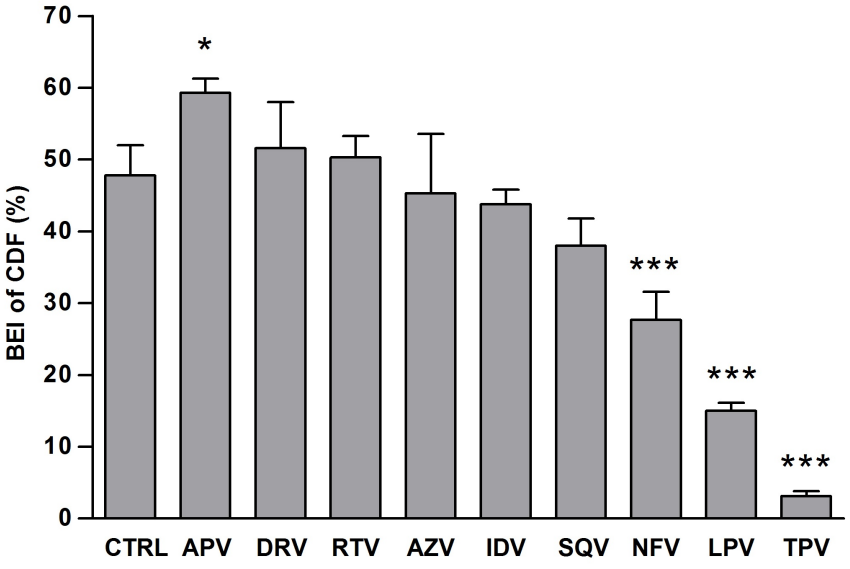

Figure 2

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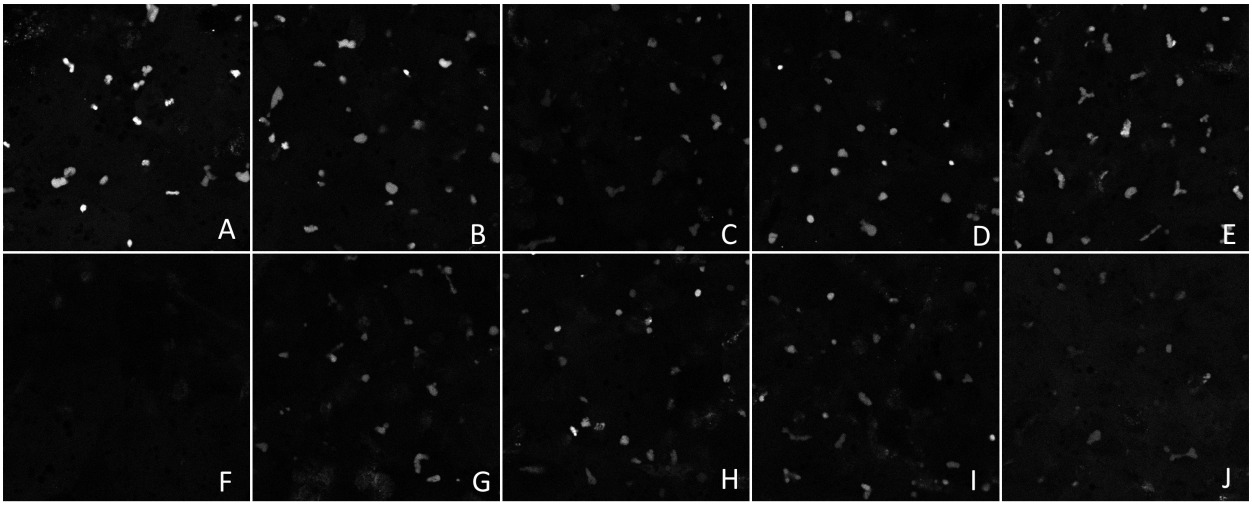

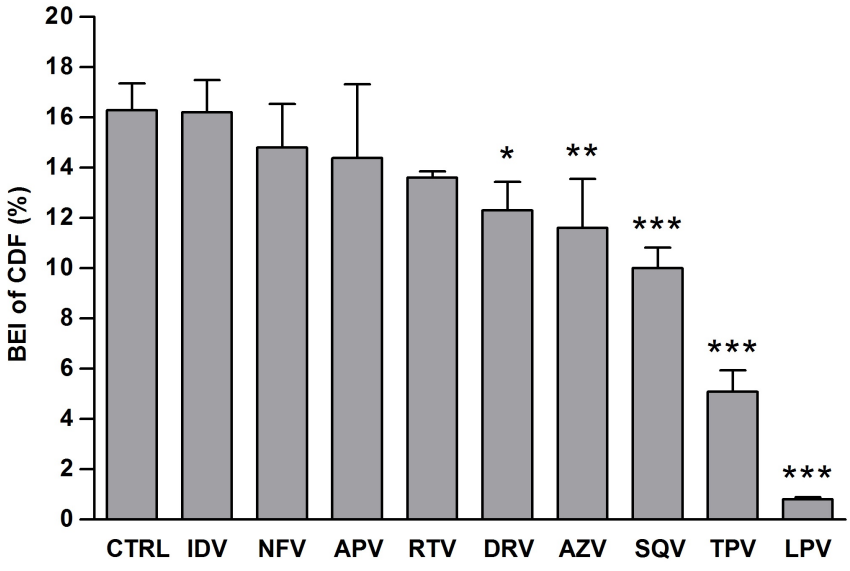

Figure 3