Quantitative time-lapse imaging (QTLI)-based analysis of drug-drug interaction mediated by hepatobiliary transporter, multidrug resistance associated protein 2, in sandwich-cultured rat hepatocytes

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**Running Title:** Evaluation of drug-MRP2 interaction by QTLI

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**Abbreviations:** MRP2/Mrp2, multidrug resistance-associated protein 2; SCRH(s), sandwich-cultured rat hepatocyte(s); SCH(s), sandwich-cultured hepatocyte(s); EHBR(s), Eisai hyperbilirubinemic rat(s); SDR(s), SD rat(s); OATPs, organic anion transporter polypeptide(s); OCT, organic cation transporter(s); NTCP, sodium taurocholate cotransporting polypeptide; MK-571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethyl)sulfonyl]methylsulfonyl]propionic acid.
Abstract

There is increasing interest in developing efficient screening platforms to predict drug-induced liver injury. We, therefore, explored a microscope-based analysis to quantitatively evaluate interaction of drugs with multidrug resistance-associated protein 2 (MRP2) essential for hepatic excretion of drugs in sandwich-cultured rat hepatocytes (SCRHs), utilizing 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF) diacetate, which is intracellularly hydrolyzed to fluorescent substrate CDF. Drug-MRP2 interactions were evaluated by measuring the fluorescence change in bile canaliculi in SCRHs in the presence or absence of MRP2 inhibitors, using quantitative time-lapse imaging (QTLI) analysis. Fluorescence was negligible in SCHs from rMrp2-deficient Eisai hyperbilirubinemic rat (EHBR), suggesting that Mrp2 is primarily responsible for CDF accumulation. According to QTLI, rifampicin, cyclosporin A and MK-571 attenuated CDF accumulation in a concentration-dependent manner, with IC50 values (IC50,QTLI) of 3.02, 1.63 and 2.87 μM, respectively. The ratios of IC50 values obtained from the biliary excretion index (BEI) over the IC50,QTLI were 1.34, 1.94 and 1.94, but ratios over IC50s in CDF uptake by rMrp2-expressing membrane vesicles varied more, 6.69, 3.07 and 2.43 for rifampicin, cyclosporin A and MK-571, respectively. When the IC50,QTLI of rifampicin was corrected for the hepatocyte/medium distribution ratio, the relative ratio of IC50,ves/IC50,QTLI was reduced to 2.25 from 6.69 (20.2/3.02) and was close to the ratio for MK-571 (2.43 , 6.96/2.87), which is thought to cross the plasma membrane by passive diffusion. Our results indicate that QTLI is a suitable method to evaluate drug-MRP2 interaction at the bile canalicular membrane, when the hepatocyte/medium distribution ratio in SCRHs is taken into account.
Introduction

The liver is a vital organ, serving to maintain blood sugar and amino acid levels, synthesize proteins, produce biochemicals such as bile salts essential for digestion, and detoxify xenobiotics and drugs. Not only bile salts, but also many conjugated drugs are commonly excreted into bile via bile canalicular membrane transporters. Hence, interaction of conjugated drugs with these transporters may compromise bile salts homeostasis and the detoxification systems of the liver, resulting in severe drug-induced liver injury (Fattinger et al., 2001; Funk et al., 2001). Since unexpected drug-induced hepatotoxicity is one of the major reasons for withdrawal of candidate drugs from the market, there is an urgent need to establish evaluation systems to screen interaction of drugs with bile canalicular membrane transporters in liver, in order to predict \textit{in vivo} drug-induced hepatotoxicity. Furthermore, drug-drug interaction at transporters is of great interest in the clinical development of new drugs (Huang and Woodcock, 2010). However, it is cumbersome to quantitatively evaluate drug-transporter interaction in cytoplasm.

Currently available experimental models to evaluate biliary secretory processes of drugs include liver canalicular membrane vesicles (Tamai and Tsuji, 1987), isolated and cultured hepatocytes (Kukongviriyapan and Stacey, 1990), hepatocyte couplets (Graf et al., 1984), isolated perfused liver (Chandra et al., 2005), and transporter-deficient animal models (Tsuda-Tsukimoto et al., 2006). Use of fresh hepatocytes in culture to assess hepatic transport has gained much support in recent years. Hepatocytes cultured in a sandwich configuration were shown to become repolarized, and to provide a good experimental model to assess hepatic
functions associated with intact bile canaliculi (LeCluyse et al., 1994). Furthermore, biliary excretion of xenobiotics in long-term sandwich-cultured rat hepatocytes (SCRHs) correlates with \textit{in vivo} biliary excretion (Liu et al., 1999). Another advantage of sandwich-cultured hepatocytes (SCHs) is that they can be used to evaluate the biliary excretion index (BEI), which is a measure of the excretion of the candidate compound relative to hepatic uptake (Liu et al., 1999). Despite these advantages of SCRHs, the method is unsuitable for simultaneous screening of candidate compounds because of the cumbersome processes required for bioanalysis of substrate drugs. Although interest in predicting possible interaction of drugs with bile canalicular efflux transporters is growing, currently available cell-free methods, such as membrane vesicles, are generally not suitable to predict the affinity of drugs for transporters from the plasma concentration, because the hepatic cell entry process is not taken into account. From this point of view, SCHs have the advantage of including both the basolateral uptake and bile canalicular efflux transporters. Therefore, we considered that a new microscopy-based method to quantitatively analyze drug-transporter interactions using a fluorescent probe specific to each bile canaliular transporter might be effective as the basis for an efficient and high-capacity screening system.

In the present study, we focused on MRP2, whose functional expression in bile canaliculi of both liver tissue and cultured hepatocytes is well established. MRP2 plays an essential role in regulation of serum bilirubin level by eliminating its conjugate from the liver, because a genetic defect of MRP2 in humans leads to familial conjugated hyperbilirubinemia, known as Dubin-Johnson syndrome (Kartenbeck et al., 1996; Paulusma et al.,
In addition, MRP2 mediates efflux transport into bile of many structurally diverse xenobiotics and anticancer drugs in either intact or conjugated forms (Kato et al., 2008). We have shown that MRP2 plays an important role in hepatobiliary secretion of drugs by using SCRHS (Fukuda et al., 2008; Fukuda et al., 2010). Therefore, interaction of drugs with MRP2 may cause drug-induced hepatobiliary adverse effects.

Several fluorescent substrates of MRP2, including 5(and 6)-carboxy-2’,7’-dichlorofluorescein (CDF), have been reported. In hepatocytes, the esterified derivative, 5(and 6)-carboxy-2’,7’-dichlorofluorescein diacetate (CDFDA), diffuses across basolateral membranes and is intracellularly hydrolyzed to CDF, which is excreted into bile via Mrp2 (Zamek-Gliszczynski et al., 2003). In the present study, a quantitative method to evaluate interaction of drugs with rat Mrp2-mediated CDF transport into the bile canaliculi formed in SCRHS was sought by utilizing a time-lapse imaging technique. Based on our proposed quantitative time-lapse imaging (QTLI)-based analysis, accumulated fluorescence in bile canaliculi was kinetically analyzed in the presence or the absence of Mrp2 inhibitor. The new method was found to permit reliable evaluation of drug-transporter interaction, as validated by comparison with the results with those of established kinetic assays, such as biliary efflux index (BEI) measurement in SCRHS and the use of Mrp2-expressing plasma membrane vesicles.
Methods

Preparation of SCRHs

Hepatocytes were isolated from male Wistar, SD and Eisai hyperbilirubinemic rats (EHBRs) at the age of 7 to 8 weeks by means of the collagenase perfusion method. Animals were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). All animal experiments were carried out in accordance with the Declaration of Helsinki and the Guidelines of Kanazawa University for the Care and Use of Laboratory Animals. Rat hepatocytes were isolated and sandwich-cultured as described previously (Fukuda et al., 2008). Isolated hepatocytes were plated on collagen-coated tissue culture plates at a density of 0.89 – 1.06 × 10^5 cells/cm². Twenty-four hours later, Matrigel® (BD Biosciences, Franklin Lake, NJ) was overlaid on the cells, and culture was continued for 3 more days under an atmosphere of 5% CO₂ in air at 37°C. At this point, the preparation, which is designated as sandwich-cultured rat hepatocytes (SCRHs), was used for experiments.

Quantification of CDF accumulated in bile canicular spaces in SCRHs

In general, SCRHs were incubated with transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM d-glucose, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, and 25 mM HEPES, adjusted to pH 7.4) containing CDFDA (10 μM). Time-lapse imaging was performed to observe the fluorescence of CDF accumulated in the bile canaliculi for up to 10 min under an automatic fluorescence microscope (BZ-9000, Keyence, Osaka, Japan) under an atmosphere of 5% CO₂ in air at 37°C. The fluorescence was visualized by the microscope utilizing the BZ filter (excitation, 480 ± 30 nm; emission, 510 < nm) at ×20 magnification; during QTLI, a
fluorescence image was taken every 1 min with an exposure time of 22.2 ms by the attached 12-bit CCD camera system. Regions of interest (ROIs), where bile canaliculi were present, were identified by phase-contrast microscopy and defined by free-hand drawing in a section with an area of 150 × 150 µm² within the entire visual field, by means of the BZ-II analyzing software (Keyence). After subtraction of background fluorescence, accumulated fluorescence intensity (per pixel) obtained from 8 ROIs in each section was added together, and the mean value from three individual sections was used to quantitate accumulated CDF in bile canaliculi in each image taken. The general method is illustrated in Supplemental Fig.1. This quantitative time-lapse imaging-based analysis is designated as QTLI in the present study. Interaction of drugs with rMrp2-mediated CDF transport was evaluated by quantifying accumulated fluorescence in bile canaliculi in SCRHs in the presence or the absence of various concentrations of rifampicin, cyclosporin A and 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid (MK-571), which is a competitive inhibitor of both MRP1 and MRP2.

Measurement of biliary excretion index (BEI) of CDF in SCRHs

SCRHs were incubated with CDFDA at 37°C for 10 min with (+) or without (-) divalent ions (e.g. Ca²⁺ and Mg²⁺). CDF was extracted as described previously (Fukuda et al., 2008). To achieve a Ca²⁺/Mg²⁺-free condition, both CaCl₂ and MgSO₄ were removed and EGTA at the final concentration of 0.5 mM was added to the general transport buffer described above, then uptake was performed. To quantify CDF accumulation, SCRHs were washed with ice-cold transport buffer at the end of uptake to remove bound CDF, and lysed with
1% Triton X. CDF concentration in the lysate was quantified using a microplate reader (ARVO™ X3, PerkinElmer Japan, Osaka) with a fluorescence filter for excitation (at 490 nm ± 10) and for emission (at 535 nm ± 25). For taurocholate, radioactivity was quantified with a liquid scintillation counter (LSC-5100, Aloka, Tokyo, Japan). BEI (%) was obtained as follows;

\[
\text{BEI} \, (\%) = 100 \times \frac{\text{CDF}_{+\text{Ca}^{2+}} - \text{CDF}_{-\text{Ca}^{2+}}}{\text{CDF}_{+\text{Ca}^{2+}}} \\
\]

where CDF (+Ca\(^{2+}\)) and CDF (-Ca\(^{2+}\)) represent apparent uptake in the presence and absence of divalent cations (Ca\(^{2+}\) and Mg\(^{2+}\)), respectively.

**CDF uptake by transporter-expressing membrane vesicles**

Transporter-mediated CDF uptake was studied by using membrane vesicles prepared from insect Sf9 cells expressing rat and human MRP2, rat Bcrp, and rat Bsep (Genomembrane Inc., Yokohama, Japan). In general, membrane vesicles were incubated with reaction buffer (50 mM MOPS, 70 mM KCl, 7.5 mM MgCl\(_2\), and 200 mM glutathione, adjusted to pH 7.42) at 37°C for 3 min in the presence of 5 mM AMP (control) or ATP. At the end of uptake, the reaction was stopped by adding ice-cold buffer (40 mM MOPS, 70 mM KCl) and membrane vesicles were trapped on the filter (HAWP02500, Millipore, Bradford, MA) by means of a rapid filtration method. To evaluate the direct effect of inhibitor, Mrp2-mediated CDF uptake was measured in the presence or absence of various concentrations of inhibitor in the transport medium. Finally, extracted CDF
was quantified with a microplate reader (ARVO™ X3). ATP-dependent MRP2-mediated transport was expressed as the difference between the intravesicular accumulations of CDF in the presence of AMP and ATP.

**Estimation of intracellular concentration of rifampicin in SCRHs**

Intracellular accumulation of rifampicin by SCRHs for 5 min was measured by HPLC. Rifampicin uptake by SCRHs was performed in the absence of divalent ions as described above. Separation was achieved with an analytical column Cica Mightysil RP-18 GP Aqua (250 mm × 4.6 mm; Kanto Kagaku, Tokyo, Japan) equipped with a guard column (Cica Mightysil RP-18 GP Aqua, 50 mm × 4.6 mm, Kanto Kagaku).

Rifampicin was detected by a UV detector at set at 254 nm (Alliance 2690 separation module/2487 dual absorbance detector, Waters, Milford, MA). The mobile phase consisted of methanol and 10 mM phosphate buffer pH 3.0 (60:40; v/v), and the flow rate was 1 ml/min. The obtained intracellular accumulation of rifampicin was divided by cellular volume (/mg of protein), which was determined by subtracting [14C]inulin uptake (a marker for extracellular fluid) from steady-state [3H]H2O uptake by SCRHs in Ca2+/Mg2+-free buffer.

Intracellular concentration of free rifampicin in SCRHs was estimated by measuring the ratio of bound to unbound form (Cb/Cub) in different concentrations of rat hepatocyte homogenate [10, 20, 30, 50 % (w/v)] by means of an ultrafiltration method utilizing Ultracel® YM-30 (Millipore). The ratio at 100% homogenate was linearly extrapolated from the observed values. Unbound tissue ratio (fT) of rifampicin was obtained as follows:
Data Analysis

Student's t-test was used to assess the significance of difference between in vitro assay results, with $p < 0.05$ as the criterion of significance. To estimate IC$_{50}$ values of inhibitors, the inhibitory effect of inhibitor drugs on substrate drug (indicated as ‘% of Control’) in SCRHs assay and membrane vesicle assay was fitted to the following equations (3), and (4), respectively:

\[
\text{% of Control} = 100 - 100 \times \frac{I_{\text{max}} \times [I]}{(\text{IC}_{50} + [I])}
\]  \hspace{1cm} (3)

where $I_{\text{max}}$, $[I]$ and IC$_{50}$ are maximum inhibitory effect of inhibitor, inhibitor concentration, and the concentration required to inhibit 50% of the fluorescence accumulation measured by QTLI or BEI, respectively,

\[
\text{% of Control} = 100 \times \frac{\text{IC}_{50}}{\text{IC}_{50} + [I]}
\]  \hspace{1cm} (4)

where $[I]$ and IC$_{50}$ are the inhibitor concentration and the concentration required to inhibit 50% of the CDF uptake by membrane vesicles, respectively.
Results

Visualization of Mrp2 activity in SCRHs

When SCRHs were incubated with CDFDA (10 µM), fluorescence per pixel of accumulated CDF in the bile canaliculi was monitored by time-lapse imaging (TLI) every 1 min for up to 5 min, and the quantity of accumulated CDF was expressed in terms of the fluorescence in the ROIs measured by QTLI after subtraction of background fluorescence from cytoplasm or a blank area in the visual field. The fluorescence accumulated in a time-dependent manner for up to 5 min (Fig. 1). In order to evaluate the variability of accumulated fluorescence according to location within the entire visual field, the field was divided to 12 sections of 150×150 µm² (#1 through #12, Fig. 1 inset), and the fluorescence was determined in each section every 1 min for up to 5 min (Fig. 1). The variability of accumulation obtained from 8 ROIs in each section was characterized by the coefficient of variation (CV) at all the time points at which images were taken. The CV was relatively small, ranging from 12.3 to 15.2 %, as summarized (with standard deviations) in Table 1, suggesting that the quantification was reliable. Since variation of fluorescence determined by QTLI was not considered to be significant, we chose any three sections within the image to quantify the fluorescence in the following experiments.

Since CDF is a known substrate of human and rat MRP2, we determined the contribution of rMrp2 to CDF accumulation in bile canaliculi in SCRHs. In the presence of the MRP2-specific inhibitor MK-571, at the concentration of 50 µM, no fluorescence was detectable at the bile canaliculi by means of fluorescence
microscopy (Fig. 2A). CDF accumulation was further evaluated in SCHs from EHBRs that were hereditarily
defective in Mrp2 function (Ito et al., 1997). CDF accumulation in bile canaliculi of SCH from EHBR was
shown to be negligible by fluorescence imaging (Fig. 2B). As shown in Fig. 2C, the quantity of fluorescence
in SDR hepatocytes reached a plateau after 5 min and was determined to be $354.3 \pm 46$ per pixel at 8 min,
whereas the fluorescence was only slightly increased to $50.2 \pm 23.4$ per pixel in EHBR hepatocytes (Fig. 2C).
Fluorescence intensity measured by QTLI in SCHs from SDR and EHBR corresponded well to the
fluorescence images. Since BEI is an established index to evaluate hepatobiliary secretion of drugs, we further
examined the effect of rMrp2 deficiency on the BEI of CDF by measuring CDF uptake by SCRHs in the
presence or absence of divalent ions. As shown in Fig. 2D, the BEI obtained was 47.5% in SDR hepatocytes,
whereas no difference between the uptakes in the presence and absence of divalent ions was observed in
EHBR hepatocytes. This result suggested that accumulation of CDF into bile canaliculi is exclusively
mediated by rMrp2. We also studied CDF uptake for 3 min by plasma membrane vesicles from Sf9 cells
expressing rat bile canalicular membrane transporters, including rat Mrp2, Bsep, and Bcrp, and human MRP2,
in the presence of ATP or AMP. ATP-dependent uptake by rMrp2 was the greatest amongst rat ABC
transporters, and was 16.4-fold higher with ATP than that with AMP. rBcrp- and rBsep-mediated CDF uptakes
were 29.0% and 6.9% of the rMrp2-mediated CDF uptake, respectively. Human MRP2-mediated CDF uptake
was even greater than the rMrp2-mediated CDF uptake (Fig. 3), implying that this methodology is also
applicable to human MRP2 in hepatocytes.
To evaluate interaction of drugs with rMrp2 transport activity, three known substrates and/or inhibitors of MRP2, rifampicin, cyclosporin A and MK-571, were tested for the ability to inhibit CDF accumulation in the bile canaliculi in SCRHs. QTLI showed that the fluorescence intensity obtained at 3 min was decreased in a concentration-dependent manner in the presence of each inhibitor (Figs. 4A, 4B, 4C). Based on the dose-dependent decrease in fluorescence intensity, the apparent IC₅₀ values for rifampicin, cyclosporin A and MK-571 were estimated to be 3.02 µM, 1.63 µM and 2.87 µM, respectively (Table 2, Figs. 4D, 4E, 4F). To validate these IC₅₀ values, we compared them to the IC₅₀ values in the case of BEI. Based on the concentration-dependent effect of these inhibitors on the BEI of CDF, the IC₅₀ values for rifampicin, cyclosporin A and MK-571 were determined to be 4.06 µM, 3.17 µM, and 5.58 µM, respectively (Table 2). The values of relative ratio, expressed as (IC₅₀,BEI/IC₅₀,QTLI), were 1.34, 1.94 and 1.94, as summarized in Table 2. Differences between IC₅₀,BEI and IC₅₀,QTLI were within 2-fold for all three compounds, suggesting that evaluation based on QTLI is compatible with that using the established BEI method.

**Effect of Mrp2 inhibitors on CDF Uptake by Mrp2-expressing membrane vesicles**

Previously, interaction of drugs with transport activity of MRP2 has been characterized by using membrane vesicles prepared from cultured and isolated hepatocytes. We, therefore, studied the affinity of rifampicin, cyclosporin A and MK-571 for rMrp2-mediated CDF uptake by Sf9 cell-derived membrane vesicles expressing rMrp2. All three inhibitors reduced ATP-dependent Mrp2-mediated CDF uptake in a concentration-dependent manner (Figs. 5A, 5B, 5C), with IC₅₀ values of 20.2 µM, 5.00 µM and 6.96 µM,
respectively. The values of relative ratio of (IC_{50,ves}/IC_{50,QTLI}) for rifampicin, cyclosporin A and MK-571 were calculated to be 6.69, 3.07 and 2.43, respectively (Table 2). The obtained IC\textsubscript{50} values tended to be higher than those estimated by the QTLI method. This may be due to the difference between the intracellular and extracellular concentrations of inhibitors tested.

**Estimation of intracellular concentration of rifampicin in SCRH**

Amongst the three inhibitors tested, we further examined intracellular concentration of rifampicin in SCRHs, because rifampicin was reported to be taken up into the liver by organic anion transporter polypeptides (OATPs) present at sinusoidal membranes (Tirona et al., 2003). First, the cell volume was evaluated as 2.45 µL/mg based on the uptakes of [\textsuperscript{3}H]H\textsubscript{2}O and [\textsuperscript{14}C]inulin by SCRHs. Next, the unbound fraction of rifampicin in hepatocytes (f\textsubscript{T}) was estimated to be 0.153. Finally, the intracellular concentration of rifampicin was quantified by HPLC analysis after incubation of cells with various concentrations of rifampicin (Table 3). The hepatocyte/medium distribution ratio of rifampicin (termed K\textsubscript{p,SCRH}) was calculated by dividing intracellular concentration by outer medium concentration, and values of 13.7, 14.8, and 19.4 were obtained at the concentrations of 0.5, 1.0 and 5.0 µM in the medium, respectively. Although K\textsubscript{p,SCRH} tended to increase with increasing medium concentration, the concentration of unbound form of rifampicin reached 8.96 µM when the highest K\textsubscript{p,SCRH} was applied. Taking into account the cellular accumulation and intracellular binding, the relative ratio of rifampicin was reduced to 2.25, i.e., similar to the value (IC_{50,ves}/IC_{50,QTLI}) of MK-571, which is considered to cross the plasma membrane by passive diffusion.
Discussion

In the present study, we aimed to develop a microscope-based method for quantitative analysis of hepatobiliary transporter activity, which is important because it affects the pharmacokinetics of xenobiotics, and drug-transporter interactions may cause liver injury. With this aim in mind, we focused on MRP2 function, because an established fluorescent substrate, CDF, is available. Through the entire study, we carefully adjusted exposure time with green filter for CDF so that quantification was not influenced by any color saturation in fluorescence detection. We, therefore, could have shown that our QTLI-based analysis method is a reliable tool to characterize the interaction between compounds of interest and rMrp2 present at the bile canalicular membranes, utilizing CDF as a probe substrate in SCRHs. This method is considered to be superior to previous methods, such as BEI measurement and membrane vesicle assay, in the following respects: 1) tedious bioanalysis for the substrate tested (e.g., by HPLC or radioactivity quantification) after extraction from cell homogenate is unnecessary, 2) alteration of fluorescence can be monitored over time in the same cells in a single well, and 3) bile canalicular accumulation of the substrate can be reliably detected independently of intracellular accumulation. Our successful application of the QTLI method here suggests that the method can be easily adopted for use in efficient screening systems, and ultimately for high-throughput systems.

The first challenge was to validate the quantification based on bile canaliculi within the visual field, because it was impossible to detect fluorescence accumulated in all bile canaliculi in the preparation. The most comprehensive guiding principles, published in the Conference Report of the Washington Conference on...
Analytical Methods Validation for Bioavailability, Bioequivalence and Pharmacokinetic Studies, recommend that accuracy and precision for method validation should not exceed 15 % CV (Shah et al., 1992). In our QTLI method, the precision for the total fluorescence intensity per pixel in 8 ROIs defined based on phase-contrast images of bile canaliculi in any section was about 15 % at most. Therefore, this method could allow us to successfully average fluorescence accumulated in bile canaliculi distributed throughout the visual field, permitting quantification of CDF accumulation by TLI observation. Moreover, our data clearly demonstrate that rMrp2 is exclusively responsible for CDF accumulation in bile canaliculi (Fig. 2). We therefore further evaluated the applicability of this method for assessment of drug-drug interaction on Mrp2 in intact hepatocytes.

In mammalian hepatocytes, influx drug transporter proteins present at sinusoidal membranes of hepatocytes include the sodium taurocholate cotransporting polypeptide (NTCP), organic anion transporters (OATPs and OATs) and cation transporters (OCTs). We have previously shown that several OATP molecules contribute to the hepatic uptake of a wide variety of clinically important drugs (Nakakariya et al., 2008a; Nakakariya et al., 2008b). Thus, OATPs have also been recognized as sites of drug-drug interaction in the liver (Zhang et al., 2006). If we use a fluorescent rMrp2 substrate whose entry is influenced by these transporters, a test inhibitor may disturb the entry of the substrate into hepatocytes, thereby compromising kinetic quantification of the interaction between them. Therefore, in the present study, we used CDFDA, which crosses the plasma membrane predominantly by simple diffusion, because of its high lipophilicity. This feature is favorable for
our proposed QTLI, since CDFDA uptake by hepatocytes was assumed not to be affected by inhibitors tested.

In addition, CDFDA is not fluorescent itself, but becomes fluorescent after having been hydrolyzed to CDF by intracellular esterases. This feature is also favorable for a probe for QTLI based on determining fluorescence that is susceptible to inhibitors. In the present study, there is a concern that a compound tested may inhibit the hepatic enzyme responsible for the CDF formation resulting in decreased accumulation of fluorescence.

Previously, Vallejo et al reported that bile salt efflux pump (BSEP)-mediated efflux of cholic acid from \textit{Xenopus} oocytes co-expressing BSEP and carboxylesterase 1 (CES1) when they were exposed to methylester of cholic acid was inhibited by neither rifampicin (50 µM) nor cyclosporine A (10 µM). We, therefore, assumed no significant interference occurs in formation of CDF in SCRH in the presence of these drugs.

MK-571 is an established inhibitor selective for both MRP1 and MRP2, and the present study showed that the IC\textsubscript{50,BEI} for MK-571 was comparable to the IC\textsubscript{50,Ves}. Since carboxylesterase does not matter with the affinity obtained from vesicle study using CDF itself, it was considered that MK-571 does not influence the formation of CDF. Thus, inhibition of CDF accumulation by all three inhibitors was thought to be due to a competitive inhibition of Mrp2. In future, it may be necessary to develop a way to load fluoresce substrate of a transporter of interest into hepatocytes by endocytosis/pinocytosis so that it allow us to test compounds without limitation.

CDF was reported to be a substrate of MRP3/\(r\)Mrp3, so it may be extruded to sinusoidal blood by \(r\)Mrp3 present at the basolateral membranes of rat hepatocytes (Zamek-Gliszczynski et al., 2003). Hence, it is
possible that expression of Mrp3 might affect CDF accumulation in bile canaliculi. Although increased hepatic and renal expression of rMrp3 has been reported in EHBR (Kuroda et al., 2004), the CDF uptake in the presence of Ca²⁺ showed no difference between SCHs from SDR and EHBR (Fig. 2D), indicating that the contribution of Mrp3-mediated efflux from cells is likely to be small, and may have little impact on Mrp2-mediated CDF accumulation in bile canaliculi. In addition, an immunohistochemical study confirmed that MRP3 was not detectable in the canalicular membrane domain in hepatocytes (König et al., 1999). Accordingly, rMrp3 may make little contribution to CDF efflux involving bile canaliculi. Furthermore, to evaluate the contribution of other bile canalicular ABC transporters to the CDF accumulation, CDF uptake was measured in membrane vesicles expressing Mrp2, Bcrp or Bsep. CDF uptake mediated by Bsep was significantly less than that by Mrp2, whereas CDF uptake by Bcrp was around 29.0% of that by Mrp2, showing that CDF is a substrate of Bcrp (Fig. 3). In the current study, we used the membrane vesicles prepared from insect Sf9 cells expressing ABC transporters. Since we have shown that rMrp2 transport activity in the same system was well corresponding to its in vivo function (Kato et al., 2008), it was thought that these results reflect physiological function of these transports. Indeed, results obtained from Bcrp-expressing vesicles are consistent to the findings in Bcrp<sup>−/−</sup> mice (Nezasa et al., 2006). Although we were not able to prepare rMdr1-expressing membrane vesicles, there is so far no report indicating that CDF is a substrate of rat and human MDR1. Therefore, QTLI is a promising approach for the evaluation of drug interaction with Mrp2 present at the bile canalicular membrane. Our RT-PCR assays indicated that rBcrp expression is much lower than that of rMrp2, rMdr1a/b or rBsep in SCRHs (data not shown), implying that
the contribution of rBcrp-mediated transport to the net fluorescence of CDF would have had only a marginal
effect on the QTLI in the current study.

When the IC\textsubscript{50} values of MRP2 inhibitors obtained by means of three different methods were compared, there
were greater differences in the IC\textsubscript{50} values between the QTLI (Figs. 4D-F) and plasma membrane vesicles
methods (Fig. 5A-C) than between the QTLI and BEI (Table 2) methods. The relative ratio of IC\textsubscript{50,ves}/IC\textsubscript{50,QTLI}
for the rifampicin was 6.69, which was the greatest among the three methods. The reason for this may be the
difference between the extra- and intracellular concentrations, since IC\textsubscript{50,QTLI} was calculated based on the
concentration in the incubation medium. Rifampicin is known to be a substrate of hepatic-basolateral organic
anion transporting polypeptides, human OATP1B1 and OATP1B3, and rodent Oatp1b2 (Tirona et al., 2003).
Therefore, we measured the intracellular concentration of rifampicin to determine whether or not it is
concentratively accumulated in SCRHs. Indeed, K\textsubscript{p,SCRH} of rifampicin ranged from 13.7 to 19.4, when its
concentration in the medium was 0.5-5.0 µM (Table 3). Thus, intracellular concentrative accumulation of
rifampicin evidently occurs. The IC\textsubscript{50} value based on the intracellular concentration of unbound rifampicin
was estimated to be at most 8.96 µM, when the concentration of rifampicin in the medium was equivalent to
the IC\textsubscript{50,QTLI} obtained by QTLI (3.02 µM). The ratio of IC\textsubscript{50,ves} to the corrected IC\textsubscript{50,QTLI} calculated by
considering the intracellular concentration was decreased from 6.69 to 2.25, i.e., to a level similar to the ratio
for MK-571 (2.43, Table 2), which is considered to cross membranes by passive diffusion. However, there is
still some difference between the IC\textsubscript{50} values. This could be a consequence of hepatic distribution from blood,
which cannot be detected by plasma membrane vesicle methods, and inaccuracy in the estimation of the intracellular free fraction. Another possible explanation is that Mrp2-mediated CDF accumulation in hepatocytes could be inhibited with different affinity by intact and metabolized rifampicin species, whereas only intact rifampicin interacts with rMrp2 in cell-free assay systems, such as membrane vesicle uptake assay. Previous studies have established a significant correlation between biliary excretion of xenobiotics in SCRHs and in vivo biliary excretion (Liu et al., 1999); however, such a correlation has not yet been established for hepatic uptake and intracellular enzyme kinetics. In order to clarify whether or not the observed disassociation in IC₅₀ values between QTLI and membrane vesicles is explained by the ability of QTLI to reflect the biological functions of hepatocytes, further study is needed to establish the relation between in vitro and in vivo behavior of test inhibitors.

In conclusion, we have shown that quantitative TLI-based analysis, designated as QTLI in the present study, is useful to evaluate the interaction of drugs with MRP2 transport activity at the bile canalicular membranes, and the results are consistent with those of the BEI and membrane vesicle methods, if the hepatocytes/medium ratio of the inhibitor is taken into account. There is increasing demand for establishment of highly efficient drug-screening systems that can identify unexpected drug-induced liver injury by candidate drugs. Our new microscope-based screening platform presented here is expected to be suitable for large-scale screening and analysis of interactions of candidate drugs with bile canalicular membrane transporter function in intact hepatocytes.
Authorship Contribution

Participated in research design: Nakanishi, Shibue, Fukuyama, Yoshida, Fukuda, Shirasaka, and Tamai.

Conducted experiments: Nakanishi, Shibue, Fukuyama, and Yoshida.

Contributed new reagents or analytical tools: Nakanishi, Shibue, and Fukuyama

Performed data analysis: Nakanishi, Shibue, Fukuyama, Shirasaka, and Tamai.

Wrote or contributed to the writing of the manuscript: Nakanishi, Shibue, and Tamai.

Other: Acquired funding for the research, Nakanishi, Shirasaka, and Tamai.
References


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Figure Legends

Figure 1

CDF accumulation in bile canaliculi in SCRHs in 12 sections of the visual field. The inset shows a typical fluorescence image captured by the BZ-9000 with 12 individual sections. The time course of fluorescence accumulated in bile canaliculi is shown for up to 5 min. The fluorescence was obtained from 8 ROIs in each section (QTLI method). Each line represents the time-dependent change of fluorescence in one section (#1-#12). Each point represents the summation of the fluorescence obtained from 8 ROIs in each section. Bar = 150 µm

Figure 2

Contribution of rMrp2 to CDF accumulation in bile canaliculi formed in SCRHs. (A) The effect of MK-571 on the CDF accumulation was visualized in SCHs from Wistar rats. SCRHs were incubated with CDFDA (10 µM) for 10 min at 37°C under 5% CO2 in air. Arrowheads represent the CDF accumulation in the bile canaliculi. The pictures are representative of at least three individual experiments. (B) CDF accumulation was measured in SCH from SDR and EHBR for up to 8 min. Each image was taken after incubation with CDFDA (10 µM) for the indicated time at 37°C under 5% CO2 in air. (C) Time course of the fluorescence obtained by QTLI is shown in SCHs from SDR (closed circle) and EHBR (open circle). Each point represents the mean value of fluorescence obtained from three sections ± S.E.M. (D) Accumulation of CDF was measured in SCHs from SDR and EHBR in the presence (closed) or absence (open) of divalent ions. Each column represents the
mean ± S. E. M. (n = 3). *; p<0.05 (Ca²⁺(+) vs. Ca²⁺(-), Student’s t-test)

Figure 3

CDF uptake by membrane vesicles prepared from Sf9 cells expressing rat and human MRP2, rBsep, and rBcrp was measured, and then transporter-mediated CDF uptake was determined by subtracting the uptake in the presence of AMP (4 mM, open column, as control) from that in the presence of ATP (4 mM, closed column). Each column represents the mean ± S.E.M. (n=3). An asterisk (*) indicates a significant difference from the control by Student’s t-test (p<0.05).

Figure 4

Effect of rifampicin (A), cyclosporin A (B), and MK-571 (C) on CDF accumulation in bile canaliculi was visualized in SCRHs from Wistar rats. SCRHs were incubated with CDFDA (10 µM) for 3 min at 37°C under 5% CO₂ in air. Dose-dependent inhibition of rifampicin (D), cyclosporin A (E) and MK-571 (F) on the accumulated CDF fluorescence analyzed by QTLI was fitted to equation (3) as described in Materials and Methods. SCRHs were incubated with or without the respective inhibitor for 3 min. Each point represents the mean ± S.E.M. (n=15-21). An asterisk (*) indicates a significant difference from the control by Student’s t-test (p<0.05).
Figure 5

Effect of rifampicin (A), cyclosporin A (B), and MK-571 (C) on the CDF uptake by membrane vesicles from Sf9 cells expressing rMrp2. CDF uptake by Mrp2-expressing membrane vesicles in the presence of various concentrations of Mrp2 inhibitors was measured. The Mrp2-mediated uptake was determined by subtracting the uptake in the presence of AMP from that in the presence of ATP. Dose-dependent inhibition of rifampicin, cyclosporin and MK-571 on CDF uptake was fitted to equation (4) as described in Materials and Methods.

Data represent the mean ± S.E.M. (n=3). An asterisk (*) indicates a significant difference from the control by Student’s t-test (p<0.05).
### Table 1

**Variation of fluorescence intensity determined by QTLI from each section**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Fluorescence intensity determined by QTLI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>1</td>
<td>50.7</td>
<td>7.7</td>
</tr>
<tr>
<td>2</td>
<td>135.2</td>
<td>16.6</td>
</tr>
<tr>
<td>3</td>
<td>250.4</td>
<td>31.9</td>
</tr>
<tr>
<td>4</td>
<td>384.9</td>
<td>54.4</td>
</tr>
<tr>
<td>5</td>
<td>487.9</td>
<td>70.3</td>
</tr>
</tbody>
</table>

The CDF accumulation was visualized in SCH from Wistar rats. SCRHs were incubated with CDFDA (10 µM) for 5 min at 37°C under 5% CO₂ in air, and fluorescence images were taken every 1 min. Row fluorescence values in the all 12 sections as shown in Figure 1 inset were analyzed by QTLI as described in the Materials and Methods. Each value shows the mean value of fluorescence (per pixel) from 12 sections with the S.D. and C.V.
Table 2

IC\textsubscript{50} values of rifampicin, cyclosporin A, and MK-571 for rMrp2-mediated CDF transport obtained by three different methods

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC\textsubscript{50} values (µM)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QTLI</td>
<td>BEI</td>
<td>Vesicle</td>
</tr>
<tr>
<td></td>
<td>IC\textsubscript{50,QTLI}</td>
<td>IC\textsubscript{50,BEI}</td>
<td>Relative ratio</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>3.02 ± 2.13</td>
<td>4.06 ± 0.06</td>
<td>1.34</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>1.63 ± 0.74</td>
<td>3.17 ± 0.29</td>
<td>1.94</td>
</tr>
<tr>
<td>MK-571</td>
<td>2.87 ± 0.09</td>
<td>5.58 ± 0.31</td>
<td>1.94</td>
</tr>
</tbody>
</table>

Table summarizes the all IC\textsubscript{50} values obtained by the three methods. Relative ratio was calculated by dividing IC\textsubscript{50,ves} by IC\textsubscript{50,QTLI}. Each value represents the mean ± SEM.
Table 3

Hepatocytes/medium ratio ($K_{p,SCRH}$) and estimated unbound concentration of rifampicin in SCRHs

<table>
<thead>
<tr>
<th>Rifampicin concentration (µM)</th>
<th>Medium</th>
<th>Hepatocyte</th>
<th>$K_{p,SCRH}$</th>
<th>Estimated unbound rifampicin (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>6.8 ± 0.4</td>
<td></td>
<td>13.7</td>
<td>6.33</td>
</tr>
<tr>
<td>1.0</td>
<td>14.8 ± 2.4</td>
<td></td>
<td>14.8</td>
<td>6.83</td>
</tr>
<tr>
<td>5.0</td>
<td>97.1 ± 7.3</td>
<td></td>
<td>19.4</td>
<td>8.96</td>
</tr>
</tbody>
</table>

$K_{p,SCRH}$ was obtained by dividing rifampicin concentration by the concentration in the medium. Finally, estimated unbound rifampicin concentration was calculated by multiplying $K_{p,SCRH}$ by the unbound fraction of rifampicin in hepatocytes ($f_T$: 0.153).
Figure 1

The figure shows a graph plotting fluorescence intensity (in pixels) against time (in minutes). The graph includes a grid with labels from 1 to 12, indicating different experimental conditions or samples. The fluorescence intensity increases with time, suggesting a steady increase in the measured signal over the five-minute period.
Figure 2

A

+MK-571 (50 µM) Control

Phase Contrast Fluorescence

B

EHBR SDR

1 min 3 min 5 min 8 min

Control +MK-571 (50 µM)
Figure 3

CDF Uptake (µL/mg protein/5min)

rBsep  rBcrp  rMrp2  hMRP2

*
Figure 4

A  Rifampicin

B  Cyclosporin A

C  MK-571

D  Rifampicin

E  Cyclosporin A

F  MK-571

% of Control

Concentration (µM)

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Figure 5

A  Rifampicin  B  Cyclosporin A  C  MK-571

% of Control  Concentration (µM)

0  0.1  1  10  100  120

0  0.1  1  10  100  120

0  0.1  1  10  100  120

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