Intravital multiphoton imaging of rhodamine 123 in the rat liver after intravenous dosing

Xin Liu,1 Camilla A. Thorling,1,2 Lu Jin,1,3 Michael S. Roberts1,2,*

1Therapeutics Research Centre; School of Medicine; University of Queensland; Princess Alexandra Hospital; Woolloongabba, QLD Australia; 2School of Pharmacy and Medical Science; University of South Australia; Adelaide, SA Australia; 3Jiangsu Provincial Key Laboratory of Pharmacology and Safety Evaluation of Material Medica; Department of Pharmacology; Nanjing University of Chinese Medicine; Nanjing, P.R. China

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Abbreviations: AUC, the area under the curve; CyA, cyclosorpin A; IVM, intravital microscopy; MPM, multiphoton microscopy; P-gp, P-glycoprotein; RH123, Rhodamine 123

Intravital imaging with multiphoton microscopy was used to investigate the hepatic disposition of rhodamine 123 (RH123) in the exposed liver of anesthetized rats after intravenous dosing. The role played by the biliary canalicular transporter P-glycoprotein (P-gp) on the disposition of RH123 was explored by administering a P-gp inhibitor, cyclosporine A prior to RH123 administration. The fluorescence intensity of RH123 was defined by multiphoton microscopy using a femtosecond laser excitation wavelength of 900 nm whereas the autofluorescence at an excitation wavelength of 740 nm was used to define the morphology of the liver acini. Intravital imaging showed that RH123 was rapidly taken up from the sinusoids into hepatocytes but slowly eliminated from the cells (half-life 2.89 ± 1.37 h). The presence of cyclosporine A did not affect the uptake of RH123 but markedly increased the fluorescence intensity of RH123 in the liver and was associated with a slower elimination of RH123 from the liver (half-life 11.5 ± 4.24 h). In conclusion, the spatial disposition of RH123 in the rat liver and the effects of a transporter inhibitor on its disposition have been monitored over time using intravital imaging.

Introduction

Intravital microscopy (IVM) is the technique of imaging tissue in living animals using a microscope. IVM allows in vivo visualization of complex dynamic processes over time and space. In its early days, IVM mainly involved bright-field trans-illumination of relatively translucent tissue and was used to examine tissue vasculature and the microcirculation. Today, specificity provided by spectral imaging (specific excitation and emission wavelengths) and/or fluorescent lifetime has enabled IVM to detect and quantify individual solutes and their metabolites and, at the same time, characterize the cellular morphology by autofluorescence or reflectance.1,2 As a consequence, confocal and multiphoton imaging enables focusing at particular depths in tissues and provides information on cellular architecture, metabolic status, and dynamics of exogenously administered solutes at those depths.

Whereas traditional intravital imaging and confocal imaging focus on a specific tissue volume, multiphoton microscopy (MPM) focuses in a specific plane of tissue.1 Confocal and MPM also differ in their methodology for imaging the background cellular structure: confocal uses tissue reflectance whereas MPM uses tissue autofluorescence. The use of a longer excitation wavelength in MPM also leads to a greater imaging depth and less photobleaching. We and others have utilized MPM for non-invasive quantitative imaging of fluorescent molecules in situ and in vivo biological tissues and organs in space (three dimensions), in time, in spectra, in lifetime and in fluorescence anisotropy (total of seven dimensions).1,3 MPM allows dynamic and functional cellular and subcellular imaging in vivo and has been used widely in neuroscience,4 in immunology,5 in cancer research6 and in ophthalmic imaging.7 However, the application of MPM in investigation of xenobiotic hepatic disposition is limited.

We have recently used intravital multiphoton imaging to show that the anionic fluorescent compound fluorescein is rapidly taken up by hepatocyte from sinusoids, metabolized to fluorescein glucuronide and excreted to bile.1,8 In this article, we examine the disposition of a cationic fluorescent dye, rhodamine 123 (RH123). RH 123 is a lipophilic dye commonly used as a mitochondrial-specific stain to measure mitochondrial membrane potential in fluorescence microscopy.9 It has an excitation wavelength of 470 nm and emission wavelength of 580 nm in confocal microscopy. In the liver, its excretion from hepatocytes into the bile is subjected to the activity of the P-glycoprotein (P-gp) transporter protein. As a consequence, RH123 has been used as a marker to measure P-gp transporter function both in vitro and in vivo.10,11 The mechanisms by which RH123 are transported in the liver are illustrated in Figure 1. To date, the hepatic disposition of RH123 has been defined by analyzing outflow perfuse concentration—time profiles after dosing into an isolated perfused rat liver.12 A limitation of using outflow concentrations in...
Results

Image analysis of RH123. Figure 2 shows a set of images of RH123 fluorescence obtained at different time points from a representative rat administered. Figure 2A and C and Figure 2B and D show RH123 alone and CyA plus RH123, respectively. Images were taken with both 40× (Fig. 2A and B) and 10× objectives (Fig. 2C and D). It is apparent that RH123 appears in the liver sinusoid within 1 min of injection, then its resulting fluorescence intensity further increases in the hepatocyte to about 1 h, and then is eliminated to almost completely disappear from the liver by 6 h. The presence of RH123 in the bile ductules is not evident in the images studied over the 6 h observation period.

The concentration of RH123 in the sinusoids and hepatocytes is expressed as fluorescence intensity per pixel in sinusoids and in hepatocytes. Figure 3 shows time profiles of RH123 in each compartment (Fig. 3A and B) and the ratio of hepatocytes to sinusoids (Fig. 3C) for RH123 alone group and CyA plus RH123 group. A significantly higher AUC 0–6h in hepatocytes was found for CyA plus RH123 group than that for RH123 alone group (969 ± 365 vs 337 ± 267, p < 0.05). In contrast, the AUC 0–6h in the sinusoids was similar in the groups (155 ± 102 vs. 289 ± 199, p > 0.05). A consistently higher hepatocyte to sinusoid fluorescence ratio in the CyA plus RH123 group relative to the RH123 alone group is most evident at later time points. In RH123 alone group, the ratio quickly reached a maximum around 2.4 within 3 min and then declined to and stabilized at around 1.2 after 3 h. In CyA plus RH123 group, this ratio gradually reached maximum of nearly 3 after 1 h and maintained at this level for around 3 h and then started to decrease after 5 h. The elimination of RH123 from hepatocytes was significantly slower (p < 0.05) for CyA plus RH123 group (t 1/2 = 11.5 ± 4.24 h) compared with that for RH123 alone group (t 1/2 = 2.89 ± 1.37 h) as evidenced by the calculated elimination half-life (t 1/2) of RH123 hepatocyte fluorescence intensity.

Plasma levels of RH123. Figure 4 shows the plasma concentration vs. time profiles for RH123 after intravenous administration via the jugular vein. The profiles for RH123 alone and CyA plus RH123 group appear to be identical and each follows a biphasic exponential disposition process. Accordingly, the AUC 0–6h for the RH123 alone group (243 ± 104 ng.h/ml) was not significantly different from that for the CyA plus RH123 group (217 ± 81.7 ng.h/ml) (p > 0.05). The total amount of RH123 eliminated after 6 h was estimated to be 22.4 ± 7.50% and 24.6 ± 4.47% of administered dose for RH123 alone group and CyA plus RH123 group, respectively.

Biliary excretion of RH123. As shown in Figure 5, CyA plus RH123 group showed lower and slower bile excretion of RH123 compared with RH123 alone group. At 6 h, recovery of the administered dose of RH123 in the bile was 4.95 ± 0.89% for RH123 alone group and 1.58 ± 0.75% for CyA plus RH123 group, which was significantly different (p < 0.05).

Discussion

In the present study, we observed the disposition of RH123, a cationic fluorescent dye and P-gp substrate, in rat liver with normal and inhibited P-gp activity (with and without pretreatment of P-gp inhibitor CyA). The dynamics of RH123 fluorescence was individually recorded in two microscopic liver compartments, sinusoid and hepatocyte, respectively. RH123 fluorescence first appeared in the liver within 1 min after bolus injection of RH123 through jugular vein, which indicates a fast uptake of RH123 by the liver. The fluorescence intensity of RH123 in hepatocyte was initially lower than that in sinusoid. Then the intensity in sinusoid rapidly decreased while the intensity in hepatocytes gradually increased, which made the ratio of hepatocyte to sinusoid...
gradually increased. In addition, plasma concentration vs. time profile of RH123 (Fig. 4) showed biphasic exponential decay, which also indicates a fast distribution of RH123 into the tissue. Our results were also consistent with the study in perfused rat liver where an initial rapid decline in the perfusate concentrations of RH123 (indicative of liver uptake) was found to be followed by a very slow decline in its concentration (indicative of release). This may be explained by the selective accumulation of RH123 in mitochondria of hepatocyte that limits its further metabolism and excretion into bile.

The images of RH123 in rat liver showed punctuate RH123 fluorescence in hepatocytes with no fluorescence appearing in the nuclei. In contrast, the fluorescence images of an anionic dye fluorescein showed an even distribution in the whole hepatocyte. This difference probably arises from the cationic RH123 being trapped in the hepatocyte mitochondria. Fluorescence of RH123 in bile ductules was not visible in the liver during the 6 h observation period after RH123 injection and is consistent with low concentrations of RH123 in the bile relative to that in the sinusoids or hepatocytes. In contrast, the fluorescence intensity of fluorescein in bile ductules increased gradually and dominates fluorescence intensity in hepatocyte and sinusoid at the later times just prior to elimination. This ability to observe fluorescein but not RH123 in the bile ductules can be accounted for by fast and profound excretion of fluorescein into the bile. Consistent with this difference, only about 5% of administered RH123 is excreted into the bile after 6 h with normal P-gp function (Fig. 5), while more than 20% of the total dose of fluorescein was recovered in the bile after 3 h. Much of the fluorescein excreted in the bile is in the form of a metabolite, fluorescein glucuronide.

It is evident that when P-gp activity is inhibited, the excretion of RH123 into bile is significantly decreased (Fig. 5) and there is a delayed clearance of this compound out of the liver (Fig. 2). In addition, the fluorescence intensity of RH123 in hepatocytes was significantly higher for CyA pretreatment group, compared with that for RH123 alone group as evidenced by the higher AUC of intensity vs. time profile for CyA pretreatment. It should be noted that the absolute intensities varied between animals, consistent with a difficulty in being able to precisely image each liver at the planned imaging depth of 30 μm below the surface and the decrease in fluorescence intensity at deeper depths.

Impairment of P-gp function by CyA pretreatment leads to an inhibition in RH123 biliary excretion and greater hepatocyte RH123 concentrations as shown in Figures 3 and 5. The RH123 fluorescence decline in hepatocytes for CyA pretreatment group (Fig. 2B and D) was slower than in the RH123 alone group (Fig. 2A and C). Interestingly, when P-gp efflux activity was impaired, there was no significant effect on the plasma concentrations of RH123 over time (Fig. 4). These results highlight the danger in potentially misinterpreting pharmacokinetics derived in individual organs. Consistent with our biliary excretion findings (Fig. 5), the AUC of RH123 in perfusate from the isolated perfused livers was significantly increased when the liver was pretreated with CyA. In vivo, we found that the plasma profiles over time (Fig. 4) and hence the total elimination of RH123 at 6 h is similar both with and without CyA pretreatment group.
metabolite, deacetylation and renal excretion. Both the glucuronidated and deacetylated metabolites are excreted into the bile and back into the perfusate in a perfused rat liver preparation. Kunihara et al. suggest that renal and biliary clearance accounts for about 35% and 26% of RH123 in male Sprague-Dawley rats. In principle, CyA should inhibit both biliary and renal P-gp. However, the extent that CyA inhibits RH123 renal excretion via P-gp presently is unclear. An important advantage of intravital imaging in defining hepatic pharmacokinetics is that this organ pharmacokinetics can be defined in vivo so that the overall effects of a drug interacting substance at an individual organ level (such as the liver) can be seen in the context of what effect that substance has on the whole body.

In summary, we used intravital MPM imaging to visualize RH123 disposition in the liver in vivo and used a P-gp inhibitor and whole body pharmacokinetic profiles to better understand RH123 hepatic disposition and its implications for in vivo pharmacokinetics.

### Materials and Methods

**Chemicals.** Rhodamine 123 was purchased from Sigma Aldrich (R8004). Cyclosporine A (Sandimmune®) was manufactured by Novartis and obtained from Princess Alexandra Hospital pharmacy store. Ketamine hydrochloride and ilium xylazil were obtained from Parnell Australia Pty. Ltd. (50266–01) and Troy Laboratories Pty. Ltd. (CD1115), respectively. All other reagents were of analytical grade and obtained from commercial sources.

**Animals.** Male Wistar rats weighting around 300 g were used for all the experiments. Rats were purchased from Animal Resource Centre and were housed in local animal facility for at least one week before the experiments. The temperature was maintained at 20 ± 1°C and humidity of 60% to 75% with artificial light for 12 h (7 a.m. to 7 p.m.). The rats were allowed free access to food and water.

**Experimental design.** All animal experiments were approved by animal ethics committee in The University of Queensland. Rats were randomly separated into 2 groups with 4 rats in each group. All rats were anaesthetized by intraperitoneal injecting of ketamine (80 mg/kg) and xylazine (10 mg/kg). The jugular vein and carotid artery was cannulated for drug administration and blood collection, respectively. A midline laparotomy was performed and the bile duct was cannulated for bile collection. The left lobe of the liver was exposed for imaging. Animal was kept anaesthetized during the whole procedure and body temperature was maintained at 37°C by placing them on the heating pad.

One group of rats was injected RH123 alone via jugular vein at the dose of 2 mg/kg, while CyA (2.5 mg/kg) was administered to the other group of rats via portal vein 10 min before the injection of RH123. Rat liver images were acquired immediately and up to 6 h after RH123 injection. Blood samples were collected at 5, 10 and 30 min and 1, 2, 3, 4 and 6 h. Blood samples were centrifuged immediately after collection to obtain plasma samples. Bile was collected in pre-weighted tubes every 10 min until 1 h and every hour until 6 h. All samples were kept at -20°C until analysis.
Intravital imaging. The DermaInspect System (Jen-Lab GmbH) was used for multiphoton imaging of rat livers. An ultra-short (pulse width, 85 femtosecond, repetition rate 80-MHz) pulsed mode-locked tunable Titanium:Sapphire laser (MaiTai, Spectra Physics) was used for excitation. The excitation wavelength was set at 740 nm for liver autofluorescence and at 900 nm for selective detection of RH123. Fluorescence emission in the spectral range of 350 to 650 nm was collected by a bandpass filter (BG39). The laser power was set at 20 mW and the acquisition time for obtaining the images was 7.4 sec per frame. The left lobe of the liver was fixed between an objective (10× or 40×, water immersion, numerical aperture 1.3, Zeiss) and a small adjustable metal plate. The plate was adjusted to suitable position to minimize pressure on the liver and to reduce liver movement during imaging due to respiration. The depth of imaging area was ~30 μm under liver surface. Liver autofluorescence was first obtained at excitation wavelength of 740 nm before the injection of RH123. RH123 fluorescence images were then acquired at the same position with the excitation wavelength of 900 nm immediately after injection and continuously until 5 min. After that, the images were acquired every minute until 10 min, then every 10 min until 1 h, then every 30 min until 6 h.

Plasma and bile sample analysis. Concentration of RH123 in each plasma and bile sample was determined by high performance liquid chromatography using an Angilent C18 column (4.6 × 150 mm, 5 μm). Excitation and emission wavelength was 470 and 580 nm, respectively. The mobile phase consisted of 20 mM KH₂PO₄ buffer and acetonitrile (60:40, v/v) and was delivered at 1 mL/min. The calibration curve was linear within the range of 5–1000 ng/mL for plasma and 0.05–10 μg/mL for bile. Plasma samples were deproteinized by acetonitrile and the supernatant was injected to the instrument. Bile samples were diluted 200 times by water before the injection. The inter- and intra-day accuracy and precision of both methods were within ± 15%.

The area under the plasma concentration vs. time curve (AUC) was calculated following linear trapezoidal rule with extrapolation to infinity (∞) defined by the last concentration divided by the elimination rate constant. The total amount of RH123 excreted at 6 h is given by AUC₀–∞ minus AUC₀–6h. The accumulated excretion of RH123 in the bile up to 6 h was calculated by adding the excretion amounts (concentration × bile weight) for each time interval.

Image analysis. All images were analyzed according to previously reported approach. Briefly, five small areas were randomly selected within hepatocyte and sinusoid compartments in each image, respectively. The fluorescence intensity per pixel in each area was measured by Image J and averaged. The ratio of intensities in hepatocyte to sinusoid fluorescence at each time point in individual animals were calculated and averaged in each group. To compare the trends of intensity changes in hepatocyte between two groups, the half-life of decay of fluorescence intensity in hepatocyte was calculated by fitting the intensity vs. time profile into exponential equation. The AUC for the intensity vs. time curve was also calculated following linear trapezoidal rule for both compartments.

Statistical analysis. All statistics were performed using Prism Graphpad 5. A t-test was used to compare two groups. A p value less than 0.05 was considered to be statistically significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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