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Transport mechanisms of hepatic uptake and bile excretion in clinical hepatobiliary scintigraphy with ^{99m}Tc-*N*-pyridoxyl-5-methyltryptophan ☆,☆☆

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ABSTRACT

Introduction: In clinical hepatobiliary scintigraphy, ^{99m}Tc-*N*-pyridoxyl-5-methyltryptophan (^{99m}Tc-PMT) is an effective radiotracer among the ^{99m}Tc-pyridoxylaminates. However, the mechanisms of human hepatic uptake and bile excretion transport of ^{99m}Tc-PMT have not been determined. We thus investigated the transport mechanisms of human hepatic uptake and bile excretion in hepatobiliary scintigraphy with ^{99m}Tc-PMT.

Methods: Four solute carrier (SLC) transporters involved in hepatic uptake were evaluated using human embryonic kidney (HEK) and HeLa cells with high expression of SLC transporters (organic anion transporting polypeptide (OATP)1B1, OATP1B3, OATP2B1, organic anion transporters (OAT)2 and organic cation transporters (OCT)1) after 5 min of ^{99m}Tc-PMT incubation. Metabolic analysis of ^{99m}Tc-PMT was performed using pooled human liver S9. Adenosine triphosphate (ATP)-binding cassette (ABC) transporters for bile excretion were examined using hepatic ABC transporter vesicles human expressing multiple drug resistance 1 (MDR1), multidrug resistance-associated protein 2 (MRP2), breast cancer resistance protein or bile salt export pump. ^{99m}Tc-PMT was incubated for 1, 3 and 5 min with ATP or adenosine monophosphate and these vesicles. SPECT scans were performed in normal and Eisai hyperbilirubinemic (EHBR) model rats, deficient in Mrp2 transporters, without and with verapamil (rat Mdr1 and human MDR1 inhibitor) after intravenous injection of ^{99m}Tc-PMT.

Results: Uptake of ^{99m}Tc-PMT in HEK293/OATP1B1 and HeLa/OATP1B3 was significantly higher than that in HEK293- and HeLa-mock cells. ^{99m}Tc-PMT was not metabolized in the human liver S9. In vesicles with high expression of ABC transporters, uptake of MDR1 or MRP2 was significantly higher at all incubation times. Bile excretion of ^{99m}Tc-PMT was also identified by comparison between normal and EHBR rats with and without verapamil on *in-vivo* imaging.

Conclusions: Human hepatic uptake of ^{99m}Tc-PMT was transferred by OATP1B1 and OATP1B3, and excretion into bile canaliculi via MDR1 and MRP2. ^{99m}Tc-PMT hepatobiliary scintigraphy may be a useful ligand as a noninvasive method of visualizing and quantifying hepatobiliary transporter functionality, which could predict drug pharmacokinetics.

1. Introduction

^{99m}Tc-*N*-pyridoxyl-5-methyltryptophan (^{99m}Tc-PMT) is an effective radiotracer among the ^{99m}Tc-pyridoxylaminates, and is used for the hepatobiliary scintigraphy to diagnose disease of the hepatobiliary function and system in Japan [1,2]. Fig. 1 shows some of the human transporters involved in biliary formation. In human liver, organic anion transporting polypeptide (OATP)1B1, OATP1B3, OATP2B1, organic anion transporters (OAT)2 and organic cation transporters (OCT)1 isoforms are mainly expressed at the sinusoidal membrane as solute carrier (SLC) transporters [3,4]. On the other hand, Adenosine

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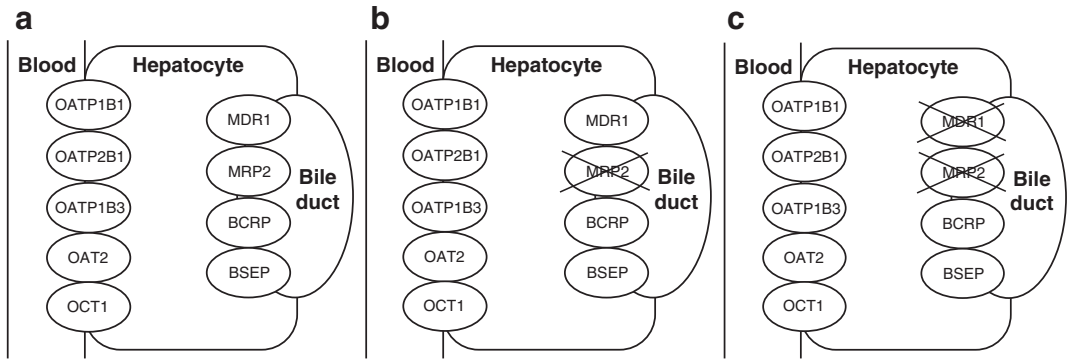

Fig. 1. Some of the human transporters involved in biliary formation (a), EHBR model rats (deficient in rat Mrp2 transporters) (b), EHBR model rats with verapamil (competitive rat Mdr1/human MDR1 inhibitor) (c). In the sinusoidal membrane, organic anion transporting polypeptides (OATP)1B1, OATP1B3, OATP2B1, organic anion transporter (OAT)2 and organic cation transporter (OCT)1 isoforms are mainly expressed as several solute carrier (SLC) transporters. In the bile canalicular membrane, multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 2 (MRP2), breast cancer resistance protein (BCRP) and bile salt export pump (BSEP) are expressed as ATP-binding cassette (ABC) transporters.

triphosphate (ATP)-binding cassette (ABC) transporters are mainly located on the bile canalicular membrane in the liver. The types of ABC transporter include multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 2 (MRP2), breast cancer resistance protein (BCRP) and bile salt export pump (BSEP) [3,4].

There are two groups of imaging agents in hepatobiliary scintigraphy [5]. Among ^{99m}Tc-iminodiacetic acid (IDA) analogs, ^{99m}Tc-diisopropyl phenylcarbamoyl IDA (^{99m}Tc-disofenin) [6] has high hepatic extraction via human OATPs [5,7] and rapid bile canalicular excretion via human MRP1 and MRP2 [8]. In addition, ^{99m}Tc-3-bromo-2, 4, 6-trimethyl IDA (^{99m}Tc-mebrofenin) [9] is taken up into the liver by human OATP1B1 [10–12] and OATP1B3 [10,11], and is then excreted by rat Mrp2/human MRP2 [13], but not human OATP2B1 [10], organic cation transporter (OCT)1 [12] or natrium taurocholate cotransporting polypeptide (NTCP) [10–13]. Among the ^{99m}Tc-pyridoxylaminates, ^{99m}Tc-pyridoxylideneglutamate (^{99m}Tc-PG) [14] and ^{99m}Tc-pyridoxylideneisoleusine (^{99m}Tc-PI) [15] have been reported as useful in hepatobiliary scintigraphy. However, the transport mechanisms of hepatic uptake and bile excretion of ^{99m}Tc-PMT have not been determined.

The purpose of this study is to clarify the mechanisms of hepatic uptake of ^{99m}Tc-PMT, a kind of ^{99m}Tc-pyridoxylaminates, using human embryonic kidney 293 (HEK293) and HeLa cells expressing SLC transporters, and the mechanisms of bile excretion using the human hepatic ABC transporter vesicles and *in-vivo* imaging.

2. Material and methods

2.1. Cells and vesicles

For SLC transporters, HEK293 cells expressing human OATP1B1, OATP2B1, OAT2, OCT1 and plasmid vector alone were prepared as described previously [16]. Briefly, HEK293 and HeLa cells were transfected with the respective plasmid DNA, and were then selected with the appropriate antibiotics; cells were designed HEK293/OATP1B1, HEK293/OATP2B1, HEK293/OAT2, HEK293/OCT1 and mock cells, and HeLa/OATP1B3 and mock cells, respectively. All cell lines were grown in Dulbecco's modified eagle medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Carlsbad, CA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO_2.

For ABC transporters, we used vesicles (GenoMembrane Inc., Kanagawa, Japan) with high expression of human MDR1, MRP2, BCRP and BSEP. Experimental kits were also purchased from the GenoMembrane Inc., (Kanagawa, Japan) and were used for experiments with each ABC transporter.

2.2. Uptake experiments with HEK-293 and HeLa cells, and vesicles

^{99m}Tc-PMT was purchased from Japan Medi-Physics Co. (Chiba, Japan). We firstly confirmed expression levels of SLC transporters in the HEK-293 and HeLa cells expressing SLC transporters using ^{3}H-estrone-3-sulfate (^{3}H-E3S) (American Radiolabeled Chemicals Inc., St Louis, MO). One day before the uptake experiments, HEK-293 and HeLa cells expressing a SLC transporter were prepared at 4×10^5 cells/well in 12-well plastic plates ($n = 4$). Cells were pre-incubated for 10 min using modified Hank's balanced salt solution. Each cell was incubated by ^{99m}Tc-PMT (37 kBq) for 5 min and removed from the tissue culture by 0.25% trypsin-EDTA solution (Sigma-Aldrich, St Louis, MO, USA). Then, radioactivity of the cells was measured using a gamma counter. For protein assay of the cells, cellular protein content was measured with a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) using BSA as a standard. Uptake of ^{99m}Tc-PMT in HEK293/OATP1B1, HEK293/OATP2B1, HEK293/OAT2, HEK293/OCT1 and HeLa/OATP1B3 cells was compared with that in mock of HEK293 and HeLa cells.

Bile excretion was measured using ABC transporter vesicles transfected with MDR1, MRP2, BCRP or BSEP ($n = 4$). The vesicles were pre-incubated for 10 min using reaction buffer in the kit. ^{99m}Tc-PMT (37 kBq) was incubated with each vesicle solution for 1, 3 and 5 min with ATP or AMP solution, and radioactivity was measured using a gamma counter. Uptake of ^{99m}Tc-PMT in the ATP solution was compared with that in AMP solution.

2.3. TLC metabolite analysis of ^{99m}Tc-PMT in pooled human liver S9

^{99m}Tc-PMT (370 kBq) was added to each of 3 samples including pooled human liver S9 (BD Biosciences, Woburn, MA) in pure water, 0.167 mM ethylenediaminetetraacetic acid/0.33 M NaK phosphoric acid buffer (pH7.4) with or without a nicotinamide adenine dinucleotide phosphate (NADPH)-generating system. Samples without pooled human liver S9 and NADPH were used as controls. $^{99m}TcO_4^-$ extracted from the ^{99}Mo-^{99m}Tc generator was added to control samples. These samples were stirred and incubated for 5, 10, 30 or 60 min in a water bath at 37 °C and perchloric acid was added to stop the reaction. Metabolite analysis was performed using reverse phase partition thin layer chromatography (TLC), with each sample spotted at 1 μl and 2-butanone/metanol/2 M KCl solution (10/9/1) used as developing solvent. Lengths of the thin layers (8.0 cm) were cut into 1.0 cm sections, and radioactivity was measured using a gamma counter. Relative abundance was calculated using averaged radioactive count for each thin layer divided by a maximum radioactive count in all thin layers. The identity of each metabolite was confirmed based on the Rf values of the TLC spots.

2.4. Animal experiments

Animal experiments were approved by the Chiba University Committee for the Care and Use of Laboratory Animals, and were carried out in accordance with the guidelines for the Care and Use of Laboratory Animals. Three six-week-old male SD normal rats (n = 3) and Eisai hyperbilirubinemic (EHBR) model rats (Fig. 1b), which have Mrp2 deficiency, (n = 3) (280–300 g) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Rats were fasted with no food overnight with water supplied *ad libitum* before experiments. During experiments, rats were anesthetized with 1.2%–1.5% isoflurane (Abbot Laboratories, Green Oaks, IL). A PE-50 catheter was inserted into the femoral vein for ^{99m}Tc-PMT administration. Imaging data were acquired using the dynamic planar protocol with a small animal SPECT/CT scanner FX-3200 (Gamma Medica-Ideas Inc., Northridge, CA) [17]. Rats were placed in the supine position on the scanner bed, and limbs were fixed using surgical tape. The orientation of renal position was determined using a laser beam and CT imaging on the scanner. After setting the radius of rotation to be maximally close to the rats, dynamic scans (30 s × 4 frames, 60 s × 8 frames and 120 s × 10 frames) with a single pinhole collimator were initiated with an intravenous bolus administration of about 74 MBq ^{99m}Tc-PMT using a micro-injection pump CFX1010 (ISIS, Osaka, Japan) for 15–20 s. On the next day, the same EHBR rats were used for experiments using administration of 0.5 mM verapamil, inhibitor of rat Mdr 1 and human MDR1 (Fig. 1c), immediately before a 74 MBq ^{99m}Tc-PMT injection. Voxel sizes were 0.53 × 0.53 × 0.37 mm with a single pinhole collimator. These image data were analyzed using amide's a medical image data examiner, open-source analyzing software tool. Five regions of interest were placed over the liver in ^{99m}Tc-PMT images with reference to CT images. Hepatic uptakes of ^{99m}Tc-PMT were compared among normal rats, EHBR rats and EHBR rats with verapamil.

2.5. Statistical analysis

Statistical significance of differences was determined with Student *t* test or analysis of variance (ANOVA) and Dunnett's test, which were used as multiplex analysis with a population mean value, using JMP software version 9 (SAS Institute Inc., Cary, North Carolina). A *P* value of less than 0.01 or 0.05 was considered statistically significant.

3. Results

Fig. 2 shows the uptake of ^{99m}Tc-PMT in HEK293 and HeLa cells expressing SLC transporters. All HEK293 and HeLa cells expressing SLC transporter showed significantly higher activity using ^{3}H-E3S when compared with HEK293- and HeLa-mock cells ($P < 0.01$). Uptake of ^{99m}Tc-PMT in HEK293/OATP1B1 and HeLa/OATP1B3 was significantly higher than that in HEK293- and HeLa-mock cells (control) ($P < 0.05$ and $P < 0.01$, respectively), while there were no differences in uptake of ^{99m}Tc-PMT in HEK293 cells with OATP2B1, OAT2 and OCT1.

Fig. 3 shows the metabolic analysis of ^{99m}Tc-PMT in pooled human liver S9. Rf values of ^{99m}Tc-PMT and $^{99m}TcO_4^-$ were 0.88 and 0.13, respectively. Values of ^{99m}Tc-PMT in metabolic analysis were 0.88 at 5, 10, 30 and 60 min after metabolic analysis of pooled human liver S9.

Fig. 4 shows the uptake of ^{99m}Tc-PMT with ATP or AMP solution in the vesicles with hepatic ABC transporter (MDR1, MRP2, BCRP, or BSEP) at 1, 3 and 5 min of incubation. Comparing the uptake of ^{99m}Tc-PMT in the ATP and AMP solution, uptake of ATP solution in the vesicles with high expression of MDR1 or MRP2 was significantly higher than that with AMP solution at all incubation times. On the other hand, there were no significant differences in BCRP or BSEP.

Time activity curve of the liver using dynamic imaging with normal and EHBR rats is shown in Fig. 5. In the liver of normal rats, radioactive counts reached a peak at about 2 min and decreased rapidly. On the other hand, in the liver of EHBR rats, peak time and

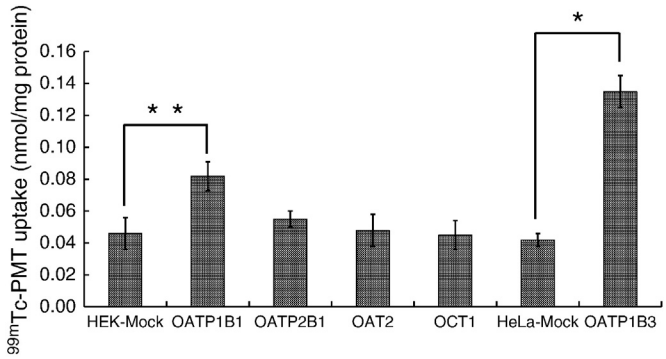

Fig. 2. Uptake of ^{99m}Tc-PMT in human HEK293 and HeLa cells expressing SLC transporter. Uptake in HEK293/OATP1B1 and HeLa/OATP1B3 was significantly higher than that in HEK293- and HeLa-mock cells. ** $P < 0.05$ and * $P < 0.01$ vs. HEK293-mock cells or HeLa-mock cells by the Student *t* test.

washout were delayed when compared with normal rats. In addition, radioactive counts in EHBR rats with verapamil were significantly higher than those in EHBR rats without verapamil. We were able to identify these results in average images of ^{99m}Tc-PMT at 6–10 min in normal rats (Fig. 5b), EHBR rats (Fig. 5c) and EHBR rats with verapamil (Fig. 5d).

4. Discussion

In this study, we assessed the human hepatic uptake and bile efflux mechanisms of hepatobiliary scintigraphy with ^{99m}Tc-PMT, a representative ^{99m}Tc-pyridoxylaminate radiotracer. ^{99m}Tc-PMT was mainly incorporated by OATP1B1 and OATP1B3 into the liver, as uptake of ^{99m}Tc-PMT in human HEK293/OATP1B1 and HeLa/OATP1B3 cells was significantly higher than that in mock cells (Fig. 2). The chemical structure of tryptophan in the ^{99m}Tc-PMT has a carboxylic acid group and an anionic charge. In general, OATPs transport anionic system compound with high molecular weight and OATs comparatively transport the compound with low molecular weight [3,4]. We expected that uptake of ^{99m}Tc-PMT involves OATPs or OATs before we performed the experiment and we found that ^{99m}Tc-PMT was only transported by OATPs, as the molecular weight of ^{99m}Tc-PMT is relatively high (>800 Da).

Hepatic uptake of ^{99m}Tc-PMT has the potential to yield metabolic changes. We performed TLC analysis using pooled human liver S9 to confirm the metabolism of ^{99m}Tc-PMT. ^{99m}Tc-PMT was not metabolized in the human liver S9, as the Rf value of ^{99m}Tc-PMT (0.88) was the same as that in control samples with ^{99m}Tc-PMT (0.88) and different from that of $^{99m}TcO_4^-$ (0.13) (Fig. 3). Therefore, we assume that ^{99m}Tc-PMT is not metabolized in clinical hepatobiliary scintigraphy.

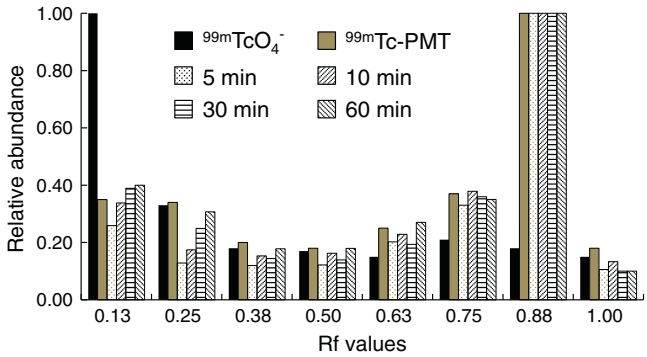

Fig. 3. Metabolic analysis of ^{99m}Tc-PMT in pooled human liver S9. Rf values of ^{99m}Tc-PMT and $^{99m}TcO_4^-$ were 0.88 and 0.13, respectively. Values for ^{99m}Tc-PMT on metabolic analysis were 0.88 at 5, 10, 30 and 60 min in pooled human liver S9.

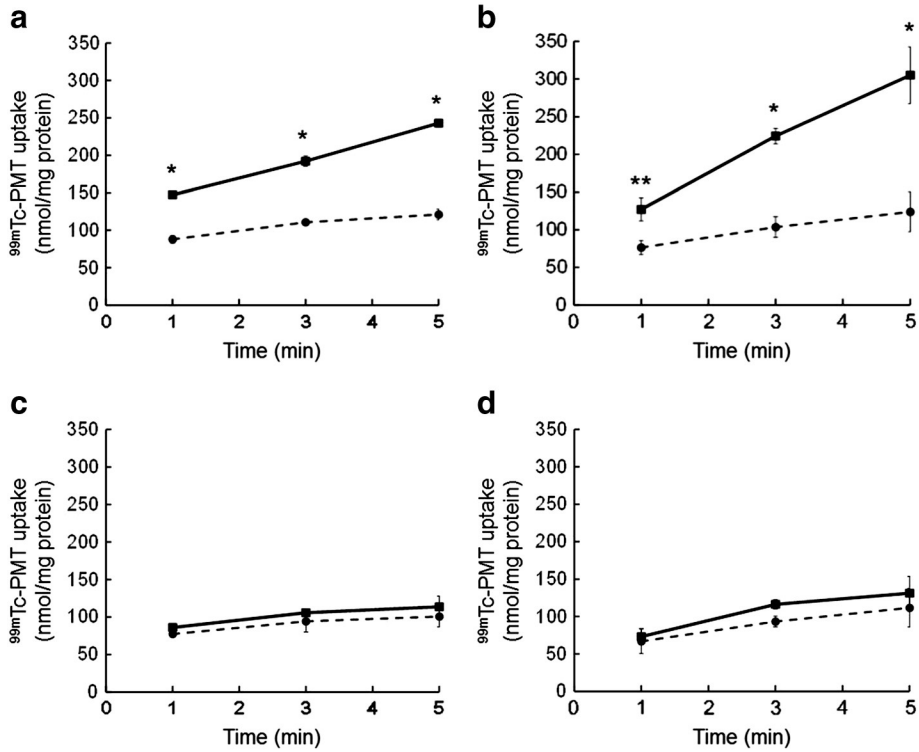

Fig. 4. Uptake of ^{99m}Tc-PMT with ATP (■)or AMP (●) solution in human hepatic ABC transporter vesicles expressing MDR1 (a), MRP2 (b), BCRP (c) or BSEP (d). In the case of vesicles with high expression of MDR1 and MRP2, uptake of ^{99m}Tc-PMT with ATP solution increased significantly as compared to that with AMP solution at 1, 3 and 5 min. ** $P < 0.05$ and * $P < 0.01$ vs. AMP solution by the Student t test.

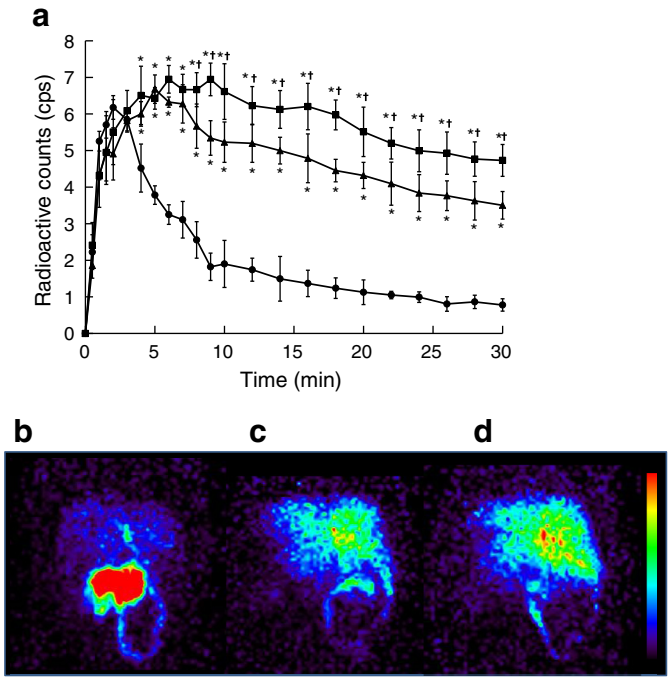

Fig. 5. Time activity curve of hepatic radioactivity in dynamic imaging with normal rats (●), EHBR rats without verapamil (▲) and EBHR rats with verapamil (■). In the liver (a), peak time and washout in EHBR rats were delayed when compared with the normal rats. Radioactive counts in EHBR rats with verapamil were significantly higher than those in EHBR rats without verapamil. This trend was visible in the average images for ^{99m}Tc-PMT from 6 to 10 min in normal rats (b), in EHBR rats (c) and in EHBR rats with verapamil (d). * $P < 0.01$ vs. normal rats. † $P < 0.05$ vs. EHBR rats by Dunnett's test after ANOVA.

^{99m}Tc-PMT is secreted into the gallbladder with bile in humans. Using vesicles with high expression of ABC transporter, we found that ^{99m}Tc-PMT was excreted via MDR1 and MRP2 because ^{99m}Tc-PMT was incorporated into the vesicles with high expression of human MDR1 and MRP2 using ATP dependent transport (Fig. 4).

Among the IDA group, ^{99m}Tc-mebrofenin has commonly been applied to hepatobiliary scintigraphy worldwide [9]. ^{99m}Tc-mebrofenin is transferred by human OATP1B1 [10–12] and OATP1B3 [10,11] into the liver, and by human MRP2 [13] into the bile. ^{99m}Tc-disofenin [6] has also been proposed for hepatological scintigraphy and has been reported to be taken up by OATP into the liver, [5,7] and excreted by human MRP1 and MRP2 into the bile [8]. From the results of these IDA groups, we expected that human OATP and MRP may be also involved in hepatic uptake and efflux of ^{99m}Tc-pyridoxylaminates, including ^{99m}Tc-PMT. However, these IDA groups were not evaluated other human ABC transporters except MRPs.

Among other imaging agents, ^{99m}Tc-hexakis-2-methoxyisobutylisonitrile (^{99m}Tc-MIBI) has been used clinically for myocardial perfusion imaging, and it is a substrate for human MDR1 [8,13,18], MRP1 [8,19] and MRP2 [8] in hepatocytes and/or cancer cells. Recently, new agents have been developed for hepatobiliary scintigraphy with positron emission tomography (PET) [20,21]. These agents are also transported by human OATPs and MRP2. However, PET lacks versatility because a cyclotron is required to produce radioisotopes. Among the agents for magnetic resonance imaging for measurement of hepatic function, gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA) is transferred via human OATP1B1, OATP1B3 and NTCP, but not OATP2B1, into the liver [22], and is excreted via rat Mrp2 into the bile [23]. Human ABC transporters have not been assessed with Gd-EOB-DTPA. In addition, we are unable to use Gd-EOB-DTPA for patients with Gd allergic reactions, serious kidney damage or hyperbilirubinemia. Therefore, ^{99m}Tc hepatobiliary scintigraphy is the best method to assess SLC and ABC transporters in hepatocytes. Among all imaging agents, metabolic analysis has

scarcely been performed. If these agents are metabolized in the liver, metabolic substances will be involved in the excretion of agents from hepatocytes into the bile on *in-vivo* imaging. On the other hand, as ^{99m}Tc-PMT is not metabolized and unchanged ^{99m}Tc-PMT is excreted into the bile, ^{99m}Tc-PMT is suitable for measuring accurate hepatobiliary transporter activity in clinical hepatobiliary scintigraphy.

As shown in Fig. 4, it was confirmed that ^{99m}Tc-PMT is transferred into bile via rat Mrp2 in a comparison between normal and EHBR rats (Fig. 1b), which is deficient in Mrp2 [24], as washout of ^{99m}Tc-PMT from liver to bile was rapid in normal rats, but was slow in EHBR rats. Furthermore, washout was delayed in EHBR rats with verapamil (rat Mdr1 and human MDR1 inhibitor) (Fig. 1c). The distribution changes of ^{99m}Tc-PMT in EHBR rats without verapamil to normal rats were larger than those in EHBR rats with verapamil to EHBR rats without verapamil because the effect of MRP2 may be larger than that of MDR1 on the excretion into bile canaliculi of ^{99m}Tc-PMT. Therefore, we reconfirmed the ^{99m}Tc-PMT was excreted via MDR1 and MRP2 on *in-vivo* imaging.

Numerous chemotherapeutic drugs for tumor treatment has been taken up into the liver via OATPs, metabolized and excreted via MDR1 and/or MRP2 into the bile [4] and act as both substrates and inhibitors of hepatic transporters such that unexpected and unwanted interactions are frequency observed. Hepatobiliary scintigraphy may be able to measure the hepatobiliary transporter activity on pharmacokinetics of the chemotherapeutic drugs [7] and diagnose hepatic diseases associated with a particular transporter dysfunction. It is important to measure hepatobiliary transporter activity before administration of chemotherapeutic drugs because the drugs cause multiple side effects for patients. Therefore, ^{99m}Tc-PMT imaging will be useful as a noninvasive method to visualize and quantify altered hepatobiliary transporter activity and to predict drug pharmacokinetics.

5. Conclusion

This study showed that the ^{99m}Tc-PMT is mainly accumulated in hepatocytes by OATP1B1 and OATP1B3, and unchanged ^{99m}Tc-PMT is excreted into bile canaliculi via MDR1 and MRP2 in clinical hepatobiliary scintigraphy. ^{99m}Tc-PMT hepatobiliary scintigraphy may be useful as a noninvasive method of visualizing and quantifying hepatobiliary transporter functionality, which could predict drug pharmacokinetics.

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