Lack of Contribution of Multidrug Resistance-associated Protein and Organic Anion-transporting Polypeptide to Pharmacokinetics of Regorafenib, a Novel Multi-Kinase Inhibitor, in Rats

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Abstract. *We investigated whether hepatic multidrug resistance-associated protein 2 (ABCC2) is involved in the hepatobiliary excretion of regorafenib, a novel multi-kinase inhibitor, using Sprague-Dawley (SD) rats and Eisai hyperbilirubinemic rats (EHBR) lacking the efflux transporter ABCC2. The involvement of organic anion-transporting polypeptide 1 (OATP1; OATP in humans) and OATP2 in the hepatic uptake of regorafenib and their protein levels in the liver were also investigated in the two rat groups. When regorafenib (5 mg/kg) was administered intravenously, the plasma concentrations of regorafenib were higher in EHBR than those in SD rats. However, the slope of the plasma concentration-time curves was the same for the two groups. Although the apparent biliary clearance of regorafenib in EHBR was lower than that of SD rats, no significant difference in the biliary excretion rate was observed between them, suggesting that regorafenib is not a substrate for ABCC2 and is not excreted into bile by ABCC2. It was also found that the contribution of biliary excretion to the systemic elimination of regorafenib is small. The protein-binding profiles of regorafenib were found to be linear in both rat groups. The binding potency, which was very high in both rat groups (>99.5%), was significantly higher in EHBR than that in SD rats. No significant differences in the plasma concentrations of unbound regorafenib were observed between the two rat groups, suggesting that the differences observed in the pharmacokinetic behaviors of regorafenib between the two rat groups were due to differences in protein-binding. When the protein levels of hepatic OATP1 and OATP2 were measured by immunoblot analysis, the expression of both transporters in EHBR was less than 40% of that in SD rats. The present results suggest that regorafenib is not a substrate for OATP1 and OATP2. These findings suggest the possibility that ABCC2-mediated hepatobiliary excretion and OATP1/OATP2-mediated hepatic uptake do not play important roles in the disposition of regorafenib.*

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A novel multi-kinase inhibitor, regorafenib (Figure 1), inhibits vascular endothelial growth factor receptor (VEGFR) 1, VEGFR2, and VEGFR3, that play key roles in angiogenesis, and fibroblast growth factor receptor (FGFR) 1, platelet-derived growth factor receptor-β (PDGFR-β), tyrosine kinase with immunoglobulin and epidermal growth factor homology domain 2 (TIE2) and the mutant oncogenic kinase KIT, RET, B-RAF (1, 2). Regorafenib is the first oral multi-kinase inhibitor with anti-tumor effect against colonic cancer (3-6), and is therefore used for the treatment of advanced and recurrent colorectal cancer, and of exacerbated gastrointestinal stromal tumor after cancer chemotherapy.

According to "Interview Form 2014" of Bayer HealthCare Pharmaceuticals, Inc., regorafenib is a basic drug and has a high hydrophobicity. For example, the log of the solubility ratio of regorafenib in octanol/water ($\log P_{O/W}$), which represents hydrophobicity, at 5.2 is much higher than that for other tyrosine kinase inhibitors. It is suggested that

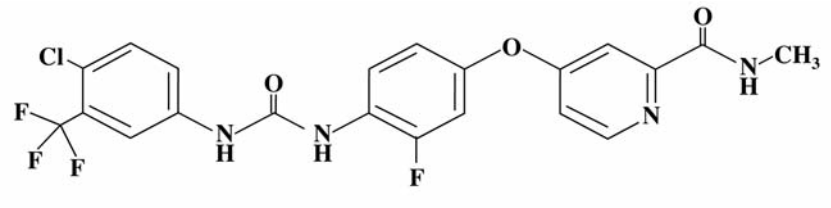

Figure 1. *Chemical structure of regorafenib.*

regorafenib is predominantly metabolized by hepatic cytochrome *P450* (CYP) 3A4 and partly by UDP-glucuronosyltransferase 1A9, and unchanged regorafenib and its metabolites are excreted into the bile, whereas their urinary excretion is the minor excretion route. The glucuronide conjugated metabolite excreted into the bile is de-conjugated by β-glucuronidase, and undergoes enterohepatic circulation as regorafenib does. In addition, regorafenib possesses a very high plasma protein-binding potency (99.5%) among various tyrosine kinase inhibitors. Thus, it is likely that regorafenib has complicated pharmacokinetic characteristics.

It is well-known that the ATP-binding cassette (ABC) drug transporters, multidrug resistance-associated protein 2 (ABCC2), P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) are expressed in a variety of normal organs, including the bile canalicular membrane of hepatocytes, and actively efflux various drugs and endogenous compounds out of the body (7-11). It is suggested that *in vitro*, regorafenib is an inhibitor of ABCB1 and ABCG2, but not a substrate, and that its active metabolites, M2 (*N*-Oxide metabolite) and M5 (*N*-Oxide/*N*-desmethyl metabolite), are substrates of ABCB1 and ABCG2 (12). Although we previously reported that the tyrosine kinase inhibitor, sunitinib, is an *in vivo* substrate of ABCB1 and ABCG2, not a substrate of ABCC2, and is not excreted into bile by ABCC2 (13), there were no data demonstrating whether regorafenib is a substrate of ABCC2 and is transported by ABCC2. It is also known that hepatic ABCC2 plays important roles in the hepatobiliary excretion of glucuronide conjugates of some drugs (14-17). For example, the active metabolite of irinotecan, SN-38, and SN-38 glucuronide are actively excreted into bile by ABCC2 (18, 19). Therefore, although there is no information on whether regorafenib glucuronide is transported by ABCC2, thus we cannot exclude the possibility that ABCC2 is involved in the hepatobiliary excretion of regorafenib and its glucuronide conjugate.

On the other hand, the hepatic organic anion-transporting polypeptide (OATP) family transporters, which are expressed on the basolateral membrane of hepatocytes, are known to play an important role in the hepatic uptake of a variety of drugs (20). Most recently, there are interesting reports suggesting that some tyrosine kinase inhibitors are taken-up into hepatocytes from blood by OATP1B1 *in vitro* (21), and that the tyrosine kinase inhibitors, pazopanib and nilotinib, are the substrate and inhibitor, respectively, of hepatic OATP1B1 (22, 23). We also reported the presence of OATP1B1-mediated hepatic uptake of sunitinib in rats (13). Although there is a suggestion that *in vitro* regorafenib is neither a substrate nor an inhibitor of OATP1B1 and OATP1B3 (12), there is a possibility that regorafenib is taken-up into hepatocytes from blood by uptake transporters, including OATP1B1, *in vivo*.

The aim of the present study was to establish a guideline for the safe use of regorafenib in patients who are scheduled to receive regorafenib therapy. We investigated whether regorafenib and its metabolite, regorafenib glucuronide, are excreted into bile by ABCC2 *in vivo* experiments using Sprague-Dawley (SD) rats and Eisai hyperbilirubinemic mutant rats (EHBR), which have a hereditary deficiency in ABCC2 (24). We also investigated the involvement of OATP1 and OATP2 in the hepatic uptake of regorafenib by analyzing the plasma concentration-time profiles of regorafenib and the protein expression levels of hepatic OATP1 and OATP2 in SD rats and EHBR.

Materials and Methods

Chemicals. Regorafenib was purchased from Active Biochem (Wanchai, Hong Kong, PRC). Sunitinib malate, which was used as an internal standard for the measurement of concentrations of regorafenib in plasma, bile and samples obtained from the *in vitro* protein-binding experiments, was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals were commercially available and were of the highest purity available. All reagents were used without further purification. For animal experiments, regorafenib was dissolved in glycerol formal (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) at a concentration of 1.75 mg/200 μl according to our previous report (25).

Animals and experiments. Male SD rats (age: 9 weeks; body weight: 320 to 345 g) and male EHBR (body weight: 325 to 340 g) of the same age as SD rats were obtained from Japan SLC (Hamamatsu, Japan). The rats were housed under controlled environmental conditions (temperature of 25±1˚C and humidity of 55±5%) with a

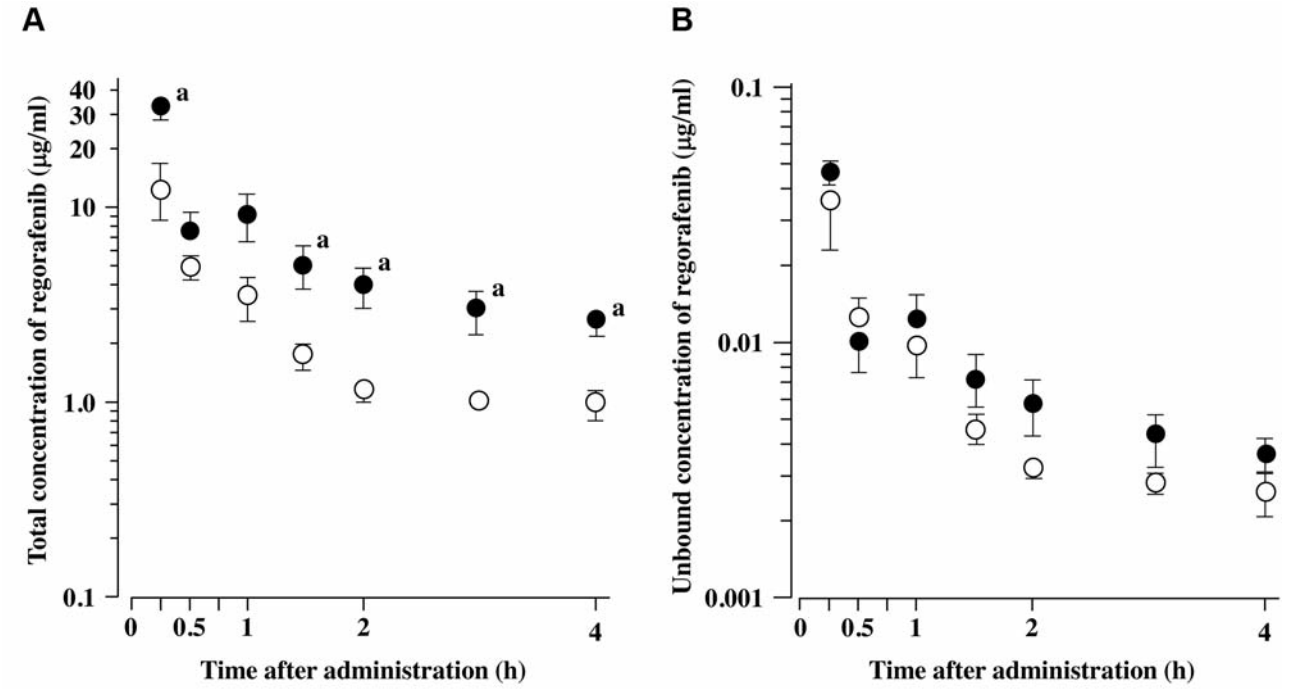

Figure 2. *Semi-logarithmic plot of total (A) and unbound (B) plasma concentration-time data for regorafenib after intravenous injection (5 mg/kg) in Sprague-Dawley (SD) rats and EHBR with cannulated bile ducts. Closed and open circles represent EHBR and SD rats, respectively. [a]Significantly different from SD rats at p<0.05. Each point represents the mean±SE (n=4). When the standard error is small, it is included in the symbol.*

commercial diet and water freely available. All animal experiments were carried out in accordance with the guidelines of Nagoya University for the care and use of laboratory animals (025-020).

For hepatobiliary excretion experiments using SD rats and EHBR, rats under anesthesia by intraperitoneal injection of sodium pentobarbital (30 mg/kg of body weight) were cannulated with polyethylene tubes into the right jugular vein and bile duct. After surgical preparation, the rats received a bolus injection of regorafenib (5 mg/kg). Blood samples (<0.3 ml) were collected at designated time intervals (0.25, 0.5, 1, 1.5, 2, 3 and 4 h after injection of regorafenib) because the hepatobiliary excretion experiments under anesthesia are generally up to approximately 4 h. Plasma samples were obtained from the blood samples by centrifugation at 6,000 ×*g* for 10 min at 4˚C. Bile samples were collected at designated periods (0 to 30, 30 to 60, 60 to 90, 90 to 120, 120 to 180 and 180 to 240 min after injection). The volume of bile samples was measured gravimetrically, with the specific gravity assumed to be 1.0. Plasma and bile samples were stored at −80˚C until analysis. This experiment was carried out under anesthesia with pentobarbital. The body temperature of the animals was maintained at 37˚C throughout the experiments with a heat lamp.

In vitro protein-binding experiment. The plasma protein-binding of regorafenib was determined by the micro partition equilibrium dialysis method using a cellulose membrane (Visking sheet, Sanplatec Corp., Osaka, Japan) with a molecular weight cut-off of 10,000 to 20,000. Blood samples for the protein-binding experiments and livers for immunoblot assay were obtained from SD rats and EHBR (n=4, respectively) by exsanguination from the abdominal aorta under light ether anesthesia. Plasma samples were obtained by centrifugation at 6,000 ×*g* for 10 min. Each liver obtained was stored at −80˚C until immunoblot analysis. Each obtained plasma sample (approximately 3 ml) was mixed to determine the binding profiles of regorafenib. Regorafenib-spiked plasma samples of the desired concentrations (2 to 200 μg/ml) were immediately dialyzed against an equal volume (0.5 ml) of saline at 37˚C for 24 h with slight shaking. The time required to reach equilibrium was determined beforehand. After dialysis, the concentrations of regorafenib on both sides of the membrane were measured.

Assay methods. Concentrations of regorafenib in *in vitro* and *in vivo* each sample were determined by liquid chromatography with tandem mass spectrometric detection (LC-MS/MS; UPLC Accela equipped with LTQ velos; Thermo Fisher Scientific Inc, Waltham, MA, USA). Sample preparation procedures differed between plasma and other samples obtained from *in vitro* protein-binding experiments. Sample mixtures containing 5 μl of plasma or bile, 0.5 ml of 2% phosphate, and 10 μl of the internal standard solution of sunitinib (1 μg/ml methanol) were passed through a solid-phase extraction column, BondElute Plexa PCX (Agilent Technologies, Santa Clara, CA, USA) after pre-conditioning with 500 μl of methanol and 500 μl of distilled water; and the column was then washed and eluted with 1 ml of methanol/acetonitrile (1:1, v/v) solution containing 5% ammonia. The eluent was dried under a stream of nitrogen gas. Finally, the residue was reconstituted in 200 μl of acetonitrile and subjected to LC-MS/MS. On the other hand, 10 μl of the internal standard solution (0.1 μg/ml methanol) was added to 100 μl of saline obtained from *in vitro* protein-binding experiments. Twenty

Table I. *Optimized mass spectrometry parameters for determination of regorafenib and sunitinib.*

Compound	Precursor ion (m/z)	Product ion(m/z)	Polarity	Retention time(min)
Regorafenib	483 [M+H]+	270 (Q-ion)	Positive	3.0
	483 [M+H]+	229 (C-ion)	Positive	
Sunitinib	399 [M+H]+	326 (Q-ion)	Positive	2.5 E/Z
	399 [M+H]+	283 (C-ion)	Positive	

C-ion, Confirmation ion; Q-ion, quantification ion; E/Z, enantiomer.

microliters of the mixture was placed into a new test tube containing 480 μl of acetonitrile. After centrifugation (12,000 ×g for 5 min at 4˚C), the supernatant was injected into LC-MS/MS.

For measurement of regorafenib glucuronide in bile, β-glucuronidase (Helix pomatia, activity >85,000 units/ml; Sigma-Aldrich, St. Louis, MO, USA) was diluted with 0.2% saline solution by 1:3 (v/v). Sample mixtures containing 0.025 ml of bile, 0.02 ml of the diluted β-glucuronidase, 0.125 ml of 100 mM acetate buffer (pH 5.0), and 0.08 ml of distilled water were incubated at 37˚C for 3 h. This reaction solution (0.01 ml) was mixed with 0.49 ml of 0.1% formic acid-acetonitrile solution, and subjected to LC-MS/MS.

Chromatographic separation by LC-MS/MS was performed by an Agilent Poroshell 120 EC-C18 column (2.1×75 mm, 2.7 μm), using a gradient elution mode of water containing 0.1% formic acid (mobile phase A) and of acetonitrile containing 0.1% formic acid (mobile phase B). The initial mobile phase composition was 80% mobile phase A and 20% mobile phase B. The percentage of mobile phase B was maintained for 1 min and then increased to 80% linearly by 1.6 min. After maintaining this percentage for 0.8 min, the mobile phase composition was allowed to return to the initial conditions and allowed to equilibrate for 1.6 min. The injection volume was 3 μl. The total flow rate was 0.5 ml/min. Ionization of regorafenib was performed in the positive electrospray ionization mode. The main parameter settings of the quantitative technique are shown in Table I.

These assays were shown to be linear with respect to the concentrations tested with a correlation coefficient of 0.998. The within-day coefficients of variation for these assays were less than 4.8%. The quantification limit of regorafenib was 7 pg/ml in each sample.

Pharmacokinetic data analysis. Plasma concentration-time data for regorafenib in each rat after a single intravenous administration were analyzed individually using a noncompartmental model. The area under the plasma concentration-time curve (AUC) was calculated by the trapezoidal rule method up to the last measured concentration in plasma. The terminal elimination rate constant (k_{el}) was calculated by determining the slope of the least-squares regression line from the terminal portion of the log concentration-time data. The parameters for unbound regorafenib were estimated in the same manner as that for total regorafenib concentration, where the unbound concentration was calculated using the total plasma concentrations and unbound fraction (f_U) obtained from the protein-binding experiments. However, the pharmacokinetic parameters calculated here represent the apparent values because adequate sampling times at the elimination phase could not be obtained.

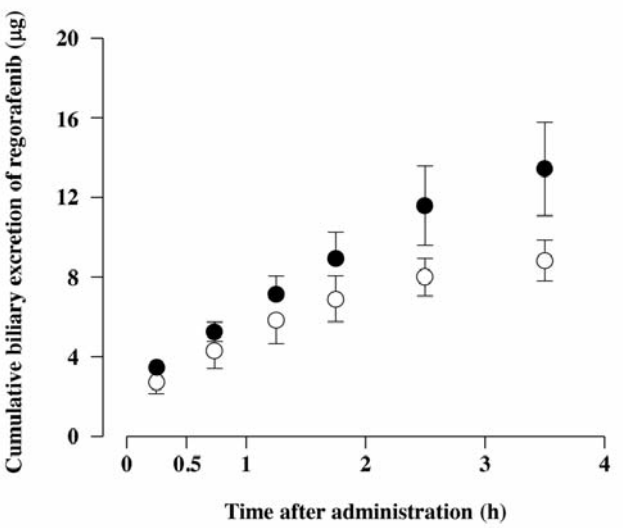

Figure 3. *Cumulative biliary excretion of regorafenib after intravenous injection (5 mg/kg) in Sprague-Dawley (SD) rats and EHBR. Closed and open circles represent EHBR and SD rats, respectively. No significant difference was observed between SD rats and EHBR. Each point represents the mean±SE (n=4). When the standard error is small, it is included in the symbol.*

The biliary clearance (CL_{BILE}) based on the concentration in plasma was calculated by dividing the total amount of regorafenib excreted into the bile between 0.5 and 4 h by the corresponding AUC ($AUC_{0.5-4}$).

Immunoblot analysis of hepatic OATP1 and OATP2. Each liver obtained for immunoblot analysis of hepatic OATP1 and OATP2 was suspended in 5-fold volumes of 100 mM Tris-HCl buffer (pH 7.4) containing complete protease inhibitors, 1.5 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). The suspension was homogenized with a tight homogenizer (20 strokes up and down) and centrifuged at 1,000×g for 15 min at 4˚C. The supernatant (1 ml) was centrifuged at 100,000×g for 60 min at 4˚C. The pellet was then dissolved in 400 μl of 100 mM Tris-HCl buffer (pH 7.4) containing 0.5% Nonidet P40 (Amresco Inc., Solon, OH, USA). The protein (20 μg) was separated by electrophoresis on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and transferred to a polyvinylidene difluoride membrane (Millipore Company, Bedford, MA, USA). The membrane was blocked in PBS containing 0.1% Tween 20 and 5% nonfat dry milk, and treated with primary antibodies: rat monoclonal antibody against OATP1 and OATP2 (Alexis Biochemicals, San Diego, CA, USA) and mouse monoclonal antibody to β-actin (Santa Crus Biotechnology Inc., Santa Cruz, CA, USA). Immune complexes were visualized using horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (GE Healthcare UK Ltd., Amersham, Buckinghamshire, UK) as the secondary antibody with the enhanced chemiluminescence detection system (ECL, Prime, GE Healthcare). To quantify the relative levels of OATO1, OATP2 and β-actin in each gel, the intensity of the stained bands was measured with the image program (National Institutes of Health, Bethesda, MD, USA).

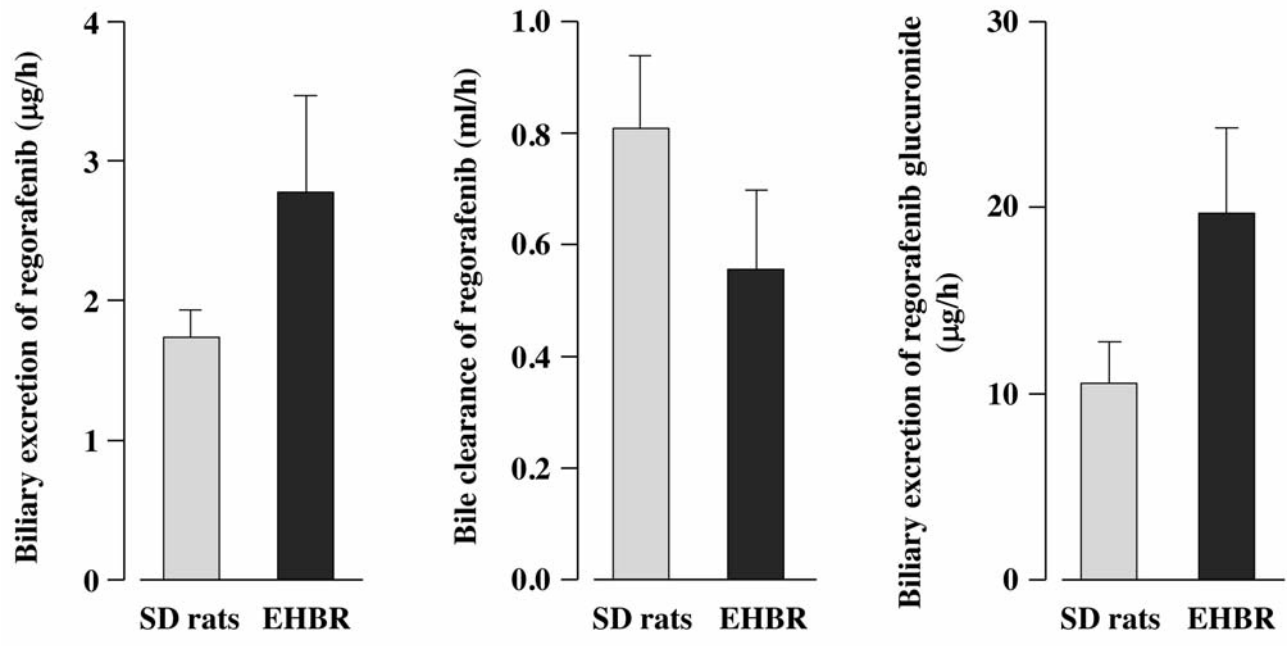

Figure 4. *Biliary excretion rate (left) and bile clearance (center) of regorafenib for 4 h and biliary excretion rate (right) of regorafenib glucuronide in Sprague-Dawley (SD) rats and EHBR between 3 to 4 h. No significant differences were observed between SD rats and EHBR. Each column represents the mean±SE (n=4).*

Statistical analysis. The results are expressed as means±standard error (SE) for the indicated numbers of experiments. Statistical comparisons were assessed by Student's *t*-test. *p*-Values less than 0.05 were considered statistically significant. Correlations between the bound and unbound concentrations were determined by least-squares linear regression analysis. IBM SPSS Statistics Ver. 20 (Stat View software (Armonk, NY, USA) was used for the analysis.

Results

Hepatobiliary excretion of regorafenib in SD rats and EHBR. Mean semilogarithmic plasma concentration-time curves of regorafenib after a single intravenous injection (5 mg/kg) in SD rats and EHBR are illustrated in Figure 2A. As shown in Figure 2A, the total plasma concentration of regorafenib declined biexponentially in both rat groups. The plasma concentrations at 0.25, 1.5, 2, 3, and 4 h after administration in EHBR were significantly higher than those in SD rats. In particular, the plasma concentration at the first sampling point (0.25 h after administration) was approximately 3-fold higher in EHBR (32.1±3.0 μg/ml) than that in SD rats (12.8±4.3 μg/ml). However, the slope of the plasma concentration-time curves in the apparent elimination phase was almost the same in SD rats and EHBR. Namely, no significant difference in the half-life ($t_{1/2}$) was observed between the two rat groups (3.15±0.57 and 2.42±0.30 h, respectively).

The $AUC_{0.25\text{-}4}$ for regorafenib between 0.25 and 4 h after administration in EHBR was significantly larger than that in SD rats (27.6±5.6 and 10.2±1.82 μg·h/ml, respectively). The cumulative biliary excretion of regorafenib in SD rats and EHBR is illustrated in Figure 3. Although the cumulative biliary excretion of regorafenib was slightly higher in EHBR than SD rats, no significant difference was observed between the two rat groups. The CL_{BILE} and biliary excretion rate (BER) for regorafenib and the BER for the metabolite of regorafenib, regorafenib glucuronide, are illustrated in Figure 4. There were no significant differences in the CL_{BILE} (0.81±0.13 and 0.55±0.14 ml/h, respectively) and BER (1.74±0.19 and 2.78±0.69 μg/h, respectively) of regorafenib between SD rats and EHBR. Regorafenib glucuronide was barely detected in the bile up to 3 h after administration. However, no significant difference in the BER of regorafenib glucuronide (10.6±4.5 and 19.7±9.1 μg/h, respectively) was observed between SD rats and EHBR between 3 to 4 h.

Protein-binding behavior of regorafenib in SD rats and EHBR. The plasma protein-binding profiles of regorafenib in SD rats and EHBR are illustrated in Figure 5. As shown in Figure 5, the relationship between bound and unbound concentrations of regorafenib in SD rats and EHBR was linear, and the f_U of regorafenib in EHBR (0.0014±0.0002) was significantly smaller than that in SD rats (0.0027±0.0003). Regorafenib was

shown to have a very high protein-binding potency, with more than 99.5% binding in both rat groups, but the potency in EHBR (99.86±0.03%) was significantly higher than that in SD rats (99.73±0.03%).

The plasma concentration data for unbound regorafenib were calculated by multiplying by the total plasma concentration-time data for regorafenib by the f_U. When the mean plasma concentrations of unbound regorafenib were plotted against time, the plasma concentrations of unbound regorafenib were still slightly higher in EHBR than those in SD rats (Figure 2B). However, no significant differences in the unbound plasma concentrations were observed between the two rat groups. When the corresponding pharmacokinetic parameters for unbound regorafenib were estimated from the plasma concentration-time curve data for unbound regorafenib in the same manner as for total regorafenib, there were no significant differences in the $AUC_{0.25\text{-}4}$ (27.9±5.0 and 39.8±9.3 ng·h/ml) and CL_{BILE} (294.9±47.3 and 385.1±99.0 ml/h, respectively) for unbound regorafenib between SD rats and EHBR.

Immunoblot analysis of hepatic OATP1 and OATP2 in SD rats and EHBR. The hepatic expressions of the membrane proteins OATP1 and OATP2 in SD rats and EHBR were measured by immunoblot analysis. As shown in Figure 6, the expressions of OATP1 and OATP2 in EHBR were 34.9±7.5% and 21.7±2.1% of those in SD rats, respectively. These results indicate that the ability of OATP1- and OATP2-mediated uptake of endogenous and exogenous substances into hepatocytes from blood is considerably lower in EHBR than SD rats.

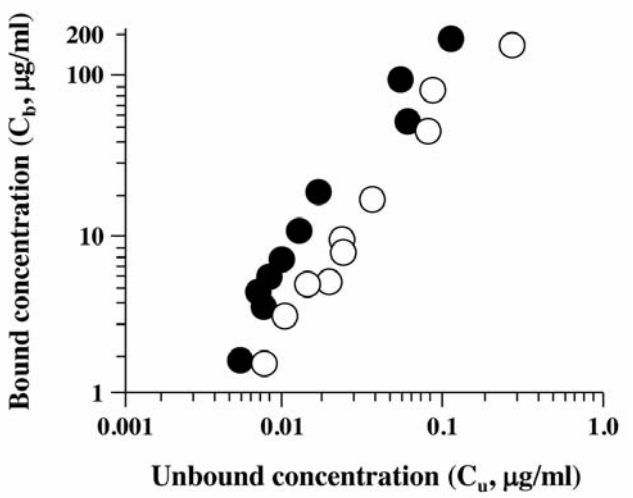

Figure 5. *Protein-binding profiles of regorafenib in plasma of SD rats and EHBR. Each point represents data for regorafenib-spiked plasma samples at designated concentrations. Closed and open circles represent EHBR and SD rats, respectively. No concentration dependency in the protein-binding behavior of regorafenib in plasma of SD rats and EHBR was observed.*

Discussion

Regorafenib, that is used for the oral treatment of patients with metastatic colorectal cancer, causes side-effects such as weakness/fatigue, hand-foot skin reaction, diarrhea, weight loss, infection, and hypertension. To our knowledge, although there is no evidence indicating the relationship between plasma concentrations and side-effects of regorafenib, a high plasma concentration of regorafenib may cause severe damage to the liver. In order to minimize such severe side-effects induced by concomitant drug use, liver dysfunction, over-dosage, and genetic polymorphism of drug-metabolizing enzymes and drug transporters, it may be necessary to understand the pharmacokinetic characteristics of regorafenib. In the present study, we examined whether the efflux transporter ABCC2 and the hepatic uptake transporters OATP1 and OATP2, which correspond to human OATP1B3 and OATP1B1, are involved in the hepatobiliary excretion and hepatic uptake of regorafenib, using SD rats and EHBR.

First, we investigated the hepatobiliary excretion characteristics of regorafenib in bile duct-cannulated SD rats and EHBR after a single intravenous injection. The results obtained showed that the plasma concentrations of regorafenib in EHBR were higher and the $AUC_{2.5\text{-}4}$ in EHBR was significantly larger than those in SD rats, whereas the slope of the plasma concentration-time curves at the elimination phase was almost the same in the two groups. There were also no significant differences in the pharmacokinetic parameters of regorafenib, CL_{BILE} and BER between SD rats and EHBR. It is reported that CYP enzyme activity, including CYP3A2 (which corresponds to CYP3A4 in humans), is significantly lower in EHBR than SD rats (26). Given these findings, it is suggested that CYP3A2-mediated metabolism is partly involved in the changes in the pharmacokinetic behavior of regorafenib observed in this study. Furthermore, since no significant difference in the hepatobiliary excretion of regorafenib was observed between the two rat groups, we can assume that regorafenib is not a substrate for ABCC2, but is excreted into bile by regorafenib-sensitive transporters other than ABCC2. However, it is likely that the contribution of biliary excretion of regorafenib to systemic elimination is small. Furthermore, when the BER of regorafenib and regorafenib glucuronide in SD rats and EHBR were compared, the BER of regorafenib glucuronide in both rat groups was more than 10-fold higher than that of regorafenib in both rat groups. These results suggest that the contribution of the metabolism by either UDP-glucuronosyltransferase or CYP3A2 to the systemic elimination of regorafenib is large. Consequently, these findings suggest the possibility that the observed higher plasma concentrations in EHBR, with no change in $t_{1/2}$, are due to the function of certain regorafenib-sensitive hepatic uptake transporters.

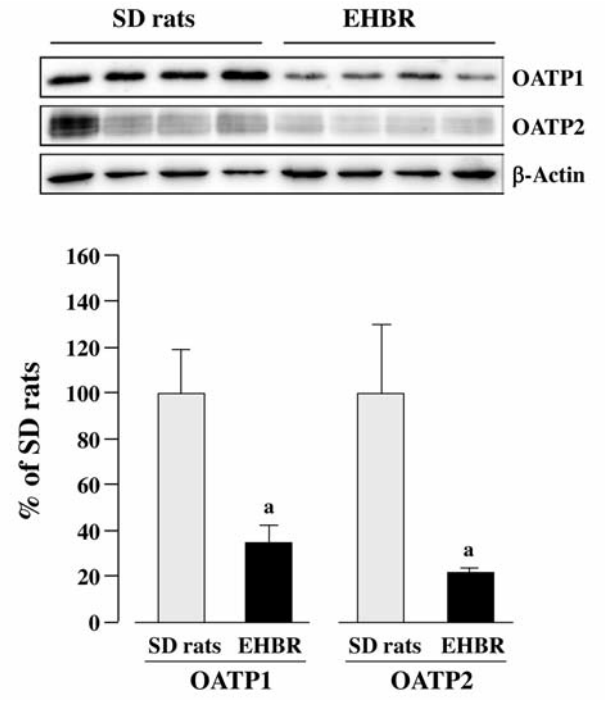

Figure 6. *Expression of OATP1 and OATP2 in Sprague-Dawley (SD) rats and EHBR. Relative levels were derived by taking the values obtained from SD rats as 100.* [a]*Significantly different from SD rats at p<0.05. Each column represents the mean±SE (n=4).*

Plasma protein-binding is known to be a limiting factor in drug disposition, since only the unbound portion of drug is diffused across various cell membranes to be distributed in the body and is subject to metabolism and excretion. EHBR has much higher concentrations of conjugated bilirubin in the plasma (27, 28). Bilirubin is well-known to bind strongly to albumin in plasma. We previously reported that the f_U for micafungin, which has a high degree of protein-binding (>99%), was 1.5-fold higher in EHBR than that in SD rats (29). These findings suggest that the changes in the protein-binding of regorafenib might influence the degree of its hepatic uptake and hepatobiliary excretion.

Secondly, we examined *in vitro* the protein-binding of regorafenib, which has a considerably high protein-binding potency (99.5%) to human plasma proteins, in SD rats and EHBR using an equilibrium dialysis method. Unexpectedly, the protein-binding experiments showed that the degree of the protein-binding of regorafenib in EHBR (99.86%) was significantly higher than that in SD rats (99.73%), which was higher than that in humans, and that the binding profiles of regorafenib were linear in the concentration range studied. It is likely that the protein-binding of regorafenib is not influenced by bilirubin.

When the plasma concentrations of unbound regorafenib, as calculated by multiplying the total plasma concentration in each SD rats and EHBR by the f_U (0.0027 and 0.0014, respectively), were plotted against time, no significant differences in the plasma concentration-time profiles and pharmacokinetic parameters for unbound regorafenib observed between SD rats and EHBR. These results suggest that the differences in the pharmacokinetic behaviors of regorafenib between SD rats and EHBR can be explained by the difference in the protein-binding potency between the two groups. In addition, the lower f_U of regorafenib in EHBR may be explained by the hypothesis that the binding site or binding profile for regorafenib on albumin are different from those of bilirubin. However, we cannot exclude the possible involvement of regorafenib-sensitive hepatic uptake transporters in the pharmacokinetic behavior of regorafenib.

It is pharmacokinetically thought that if the protein levels and function of regorafenib-sensitive hepatic uptake transporters in EHBR are impaired, the AUC of regorafenib increases, but the apparent k_{el} and the slope of plasma concentration-time curve are unchanged. Expectedly, the present study showed that the plasma concentrations and $AUC_{0.25\text{-}4}$ of regorafenib were higher in EHBR than SD rats, but the apparent k_{el} was almost the same, suggesting the possibility that the ability of uptake of regorafenib mediated by regorafenib-sensitive hepatic uptake transporters is lower in EHBR. Tyrosine kinase inhibitors, including sorafenib, are known to be substrates of the hepatic uptake transporters OATP1 and OATP2 (22, 23, 30).

There is a report suggesting that EHBR has almost the same function of hepatic OATP2 as SD rats (15). On the other hand, Kuroda and colleagues reported that the hepatic protein expression of both OATP1 and OATP2 in EHBR is one-half of that in SD rats (31). To clarify whether OATP1 and OATP2 are related to the differences in the pharmacokinetic behaviors of regorafenib between SD rats and EHBR, the protein levels of hepatic OATP1 and OATP2 in SD rats and EHBR were measured by immunoblot analysis. The result obtained showed that the expression of hepatic OATP1 and OATP2 in EHBR was considerably lower than that in SD rats, which are supported by results reported by Kuroda and colleagues (31). Surprisingly, despite the expression levels of hepatic OATP1 and OATP2 in EHBR being lower than those in SD rats, no significant difference in the $AUC_{0.25\text{-}4}$ of unbound regorafenib was observed between the two groups. These findings, at least in part, suggest that regorafenib is not a substrate for OATP1 and OATP2, and that regorafenib is not taken-up into hepatocytes by such uptake transporters. In light of biological defense, it is considered that the lower expression of OATP1 and OATP2 on the basolateral membrane of hepatocytes in EHBR is due to prevention of the damage to the liver by accumulation of hepatic toxic substances in the area where

there is no ABCC2 expression on the canalicular membrane of hepatocytes. Given these observations, it is assumed that OATP1 and OATP2 do not play important roles in the pharmacokinetic behavior of regorafenib or that their affinities for regorafenib are low. We can further propose that the majority of systemic elimination of regorafenib in EHBR is dependent on the protein-binding and CYP3A4-mediated metabolism of regorafenib. Moreover, considering that OATP1B1 is termed a genetically polymorphic influx transporter (32), it is a possible that impairment of OATP1B1 function due to genetic polymorphism cannot alter the disposition of regorafenib. However, further studies are needed to clarify the effect of the genetic polymorphism of *OATP1B1* on the clinical pharmacodynamics and pharmacokinetics of multi-kinase inhibitors, including regorafenib.

It is well-recognized that diarrhea induced by anticancer drugs, including irinotecan and 5-fluorouracil, is the most common severe side-effect for patients. Interestingly, Grothey and colleagues reported that in a phase III clinical trial, the incidence of diarrhea was 34% in patients with colorectal cancer who received regorafenib therapy (5). This incidence seems to be higher than that reported with other commonly used anticancer combination therapy regimens against colorectal cancer (33, 34). It is suggested that irinotecan-induced diarrhea is caused by the intestinal luminal accumulation of SN-38, which is produced by de-conjugation of SN-38 glucuronide and excreted into bile, in rats (35, 36). In the present study, we could not confirm the second peak concentration of regorafenib, which shows the presence of enterohepatic recirculation, by the use of bile duct-cannulated rats. Considering that the biliary excretion of regorafenib glucuronide is relatively large, we cannot exclude the possibility that regorafenib-induced diarrhea might be closely associated with enterohepatic re-circulation.

In conclusion, using SD rats and EHBR, the present study demonstrated *in vivo* that regorafenib is not a substrate for ABCC2, OATP1 or OATP2, and that hepatic OATP1 and OATP2 do not play an important role in the disposition of regorafenib. Moreover, although the contribution of hepatobiliary excretion to the systemic elimination of regorafenib is small, regorafenib is likely to be excreted into bile by certain regorafenib-sensitive efflux transporters, including ABCB1 and ABCG2. The findings of this study cannot be extrapolated directly to humans but they may provide useful information for patients with cancer who are scheduled to undergo regorafenib therapy.

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