Involvement of the Transporters P-Glycoprotein and Breast Cancer Resistance Protein in Dermal Distribution of the Multikinase Inhibitor Regorafenib and Its Active Metabolites

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Involvement of the transporters P-gp and BCRP in dermal distribution of the multi-kinase inhibitor regorafenib and its active metabolites

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ABSTRACT

Regorafenib is a multi-kinase inhibitor orally administered to colorectal cancer patients, and is known to often exhibit dermal toxicity. The purpose of the present study was to clarify possible involvement of P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) in the dermal accumulation of regorafenib and its active metabolites M-2 and M-5. Following intravenous administration in *Abcb1a/1b/bcrp*$^{-/-}$ (TKO) and wild-type (WT) mice, delayed plasma clearance of M-2 and M-5, but not regorafenib, was observed in TKO mice compared to WT mice. Elacridar, an inhibitor of both transporters, also caused delayed clearance of M-2 and M-5, suggesting that these transporters are involved in their elimination. Skin-to-plasma concentration ratios of regorafenib, M-2, and M-5 were significantly higher in TKO mice than in WT mice. Elacridar increased skin-to-plasma and epidermis-to-plasma concentration ratios of regorafenib. Basal-to-apical transport of M-2 and M-5 was observed in LLC-PK1-Pgp and MDCKII/BCRP/PDZK1 cells, which was inhibited by elacridar and the BCRP inhibitor Ko143, respectively. The present findings thus indicate that P-gp and BCRP are involved in the accumulation of regorafenib and its active metabolites in the skin, by affecting either their systemic exposure or their plasma distribution in the circulating blood.

Keywords: ABC transporters, Transporters, Skin, P-glycoprotein, Tissue partition

Abbreviations: ABCB1, ATP binding cassette subfamily B member 1; ABCG2, ATP binding cassette subfamily G member 2; BCRP, breast cancer resistance protein; CRC, colorectal cancer; CYP3A4, cytochrome P450 3A4; GIST, gastrointestinal stromal tumor; HFS, hand-foot syndrome; LC-MS/MS, high-performance liquid chromatography/tandem mass spectrometry; LLC-PK1-Pgp, LLC-PK1 cells stably expressing human P-gp; M-2, regorafenib (pyridine)-*N*-oxide; M-5, *N*-desmethyl regorafenib (pyridine)-*N*-oxide; MDCKII/BCRP/PDZK1, MDCKII cells stably expressing both BCRP and PDZK1; P_{app}, apparent permeability coefficient; P-gp, P-glycoprotein

Introduction

Regorafenib is an oral, small-molecule multi-kinase inhibitor that blocks the activity of various protein kinases involved in the regulation of tumor angiogenesis (vascular endothelial growth factor receptor-1, -2, and -3 and TIE2), oncogenesis (KIT, RET, RAF1, and wild-type and V600E mutant BRAF), and the tumor microenvironment (platelet derived growth factor receptor and fibroblast growth factor receptor).[1] Regorafenib showed significant survival benefits in patients with metastatic colorectal cancer (CRC) who had progressed on all available standard therapies or were unable to tolerate standard therapies.[2] In addition, oral regorafenib significantly improved progression-free survival of patients with metastatic gastrointestinal stromal tumors (GIST), compared to placebo.[3] Thus, treatment with regorafenib can benefit these patients, and it has been approved for the treatment of metastatic CRC and GIST.

Despite the significant survival advantages observed in regorafenib treatment, the drug induces characteristic adverse skin reactions such as hand-foot syndrome (HFS), at a high frequency.[4,5] In a meta-analysis of regorafenib trials, HFS was reported to occur at a rate of 61% overall and 20% at grade 3.[5] Such adverse skin reactions sometimes cause dose reduction and/or treatment discontinuation. Therefore, it is clinically important to clarify possible mechanisms for such dermal toxicity.

Regorafenib is metabolized by cytochrome P450 3A4 (CYP3A4) primarily in the liver to form two major active metabolites, regorafenib (pyridine)-*N*-oxide (M-2), and *N*-desmethyl regorafenib (pyridine)-*N*-oxide (M-5),[6,7] which possess pharmacological activities similar to those of regorafenib. Therefore, accumulation of not only regorafenib but also M-2 and M-5 in the skin of hands and/or feet could be important in the induction of HFS. However, the tissue distribution mechanisms of regorafenib and its active metabolites remain unclear.

Systemic exposure and tissue distribution of therapeutic agents are generally determined by the action of drug-metabolizing enzymes and transporters. In addition to CYP3A4, regorafenib and M-2 are metabolized by UDP-glucuronosyltransferase 1A9 to form inactive glucuronides M-7 and M-8, which are predominantly excreted in urine.[6,7] However, regorafenib and its active metabolites formed by CYP3A4 are mainly excreted in feces.[6,7] Regorafenib is a substrate of the hepatic uptake transporter, organic anion transporting polypeptide 1B1, although detailed information on its role *in vivo* is not available.[8] Regorafenib, M-2, and M-5 have also been reported to be substrates of human ATP-binding cassette transporters, ABCB1 (P-glycoprotein, P-gp), and ABCG2 (breast cancer resistance protein, BCRP).[7,9] Kort et al.[9] proposed that these transporters are primarily involved in accumulation of regorafenib and M-2 in the brain using triple

knockout (*Abcb1a/1b/bcrp*$^{-/-}$, TKO) mice lacking *Abcb1a*, *Abcb1b*, and *bcrp* genes.[9] On the contrary, oral bioavailability of regorafenib was minimally affected by gene knockout of these transporters[9], suggesting their minimal involvement in the gastrointestinal absorption of regorafenib. No information is available on the effect of these transporters on the accumulation of regorafenib or its active metabolites in the skin, despite the impact of dermal toxicity in the clinical application of this drug.

Functional expression of both P-gp and BCRP in the skin has been partially characterized. Gene expression of both transporters was detected in the skin of humans and mice.[10,11] According to immunohistochemical analyses, both transporters are localized to capillary endothelial cells in both species.[12,13] Distribution of intravenously injected substrates to skin is restricted by these transporters.[12] In addition, both P-gp and BCRP are localized to the basal layer of epidermis keratinocytes.[12,13] Interestingly, these transporters have also been proposed to be involved in absorption of their substrates from skin to circulation after topical application, and localization of these transporters in both keratinocytes and endothelial cells may lead to such bidirectional transport.[12,14] However, information on the possible role of these transporters in the skin is still quite limited.

Based on this background, possible involvement of P-gp and BCRP in dermal

accumulation of regorafenib and its active metabolites was examined in the present study. For this purpose, regorafenib, M-2, or M-5 was intravenously administered to both TKO and WT mice to study the role of these transporters, avoiding the effects of gastrointestinal absorption. Sugiyama and coworkers[15] have provided scientific basis for an apparent "synergistic" effect of these two transporters on drug permeation through the blood-brain barrier: loss of these two transporters leads to a far more drastic change in brain distribution compared with loss of a single transporter.[16] In the present study, therefore, we used TKO mice to clearly detect the roles of these transporters in dermal distribution. Effect of elacridar, an inhibitor of P-gp and to a lesser extent, BCRP, was also examined to investigate the role of these transporters in the distribution of regorafenib and its metabolites. *In vitro* transcellular transport studies were also performed to examine possible transport of M-2 and M-5 by these transporters, because such information is lacking and only information on regorafenib transport is available.[9] The *in vitro* and *in vivo* results of the present study may help to elucidate the pharmacokinetic factors associated with regorafenib-induced skin toxicity, benefiting ongoing clinical pharmacology studies in CRC patients treated with regorafenib, including ongoing ones in our research group.

Materials and Methods

Materials

Regorafenib, M-2, M-5, and elacridar were purchased from Toronto Research Chemicals (Ontario, Canada). Ko143 was from Sigma-Aldrich (St. Louis, MO). Imatinib mesylate was from Focus Biomolecules (Plymouth Meeting, PA). Afatinib, bortezomib, cabozantinib, carfilzomib, dabrafenib, dacomitinib, dasatinib, lapatinib, lenvatinib, pazopanib, ponatinib, ruxolitinib, tofacitinib, trametinib, vandetanib and vemurafenib were from AdooQ Bioscience (Irvine, CA). Sorafenib was from LKT Laboratories Inc. (St. Paul, MN). Sunitinib was from Synkinase Pty Ltd (San Diego, CA). Crizotinib was from LC Laboratories Inc. (Woburn, MA). Gefitinib and erlotinib were from Cayman Chemical Company (Ann Arbor, MI). Nilotinib was from Chemscene, LLC (Monmouth Junction, NJ). All other chemicals and reagents were of analytical grade and were obtained from commercial sources.

Animals

Seven- to 9-week-old male TKO and FVB (WT) mice were purchased from Taconic (Hudson, NY) and CLEA Japan (Tokyo, Japan), respectively. ICR mice obtained from Japan SLC (Hamamatsu, Japan) were also used in the inhibition studies.

Mice were housed in a temperature- and light-controlled environment with standard food and tap water provided ad libitum. Animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of Kanazawa University.

Pharmacokinetic Studies in Mice

Mice were fasted overnight with free access to water and anesthetized with an intraperitoneal injection of pentobarbital. Regorafenib, M-2 and M-5 were dissolved in a mixture of saline, tween 80, and dimethyl sulfoxide (98:1:1). Elacridar was dissolved in a mixture of saline, dimethyl sulfoxide, and propylene glycol (1:2:2). Regorafenib (1 mg/3.3 mL/kg body weight), M-2, or M-5 (160 μg/3.3 mL/kg body weight) was administered into the jugular vein. Elacridar (10 mg/2 mL/kg body weight) or vehicle alone was administered into the jugular vein of ICR mice 30 min before the injection of regorafenib, M-2, or M-5. At various intervals after the administration, blood samples were collected through the tail vein. All blood samples were immediately centrifuged to obtain plasma. After the last blood sampling, mice were euthanized, and abdominal skin, brain, liver, kidney, and lung were collected. In some experiments, the skin was divided into epidermis and dermis as described previously.[12] To collect bile samples, the

gallbladder was cannulated with polyethylene tubing (SP10, Natsume Seisakusho Co., Ltd., Tokyo, Japan), and only bile samples were collected from mice. Cassette dosing of 24 molecularly targeted small molecules was performed by injection of the following three mixtures into the jugular vein: (i) a mixture of crizotinib (297 μg/kg), erlotinib (130 μg/kg), gefitinib (148 μg/kg), imatinib (163 μg/kg), pazopanib (28.9 μg/kg), regorafenib (319 μg/kg), sorafenib (153 μg/kg) and sunitinib (132 μg/kg); (ii) a mixture of dabrafenib (171 μg/kg), dacomitinib (155 μg/kg), dasatinib (161 μg/kg), lenvatinib (141 μg/kg), nilotinib (175 μg/kg), pazopanib (28.9 μg/kg), ponatinib (176 μg/kg), regorafenib (159 μg/kg) trametinib (203 μg/kg), and vandetanib (157 μg/kg); (iii) a mixture of afatinib (160 μg/kg), bortezomib (127 μg/kg), cabozantinib (166 μg/kg), carfilzomib (238 μg/kg), lapatinib (192 μg/kg), pazopanib (28.9 μg/kg), regorafenib (159 μg/kg), ruxolitinib (101 μg/kg), tofacitinib (266 μg/kg) and vemurafenib (162 μg/kg). These mixtures were dissolved in a mixture of saline, tween 80 and dimethyl sulfoxide (88:2:10). At 60 min after the injection, plasma, brain, and abdominal skin were collected. All biological samples were stored at -80 ^{o}C until drug quantification.

Transport studies in cell lines transfected with P-gp or BCRP cDNA

MDCKII cells stably expressing both BCRP and PDZK1

(MDCKII/BCRP/PDZK1) were previously established and grown as described previously.[17] LLC-PK1 cells stably expressing human P-gp (LLC-PK1-Pgp) were supplied from GenoMembrane Co., Ltd. (Yokohama, Japan) and were grown as described previously.[18] Cells were seeded in Transwell polycarbonate inserts (3 μm pore size, 12 mm diameter; Corning, Corning, NY) at a density of 3 x 10^5 cells/well. After 3 days of culture, the cell monolayers were washed twice with transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose,1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES, pH 7.4). After pre-incubation for 30 min with or without inhibitors, the medium was removed, and pre-warmed transport buffer containing regorafenib, M-2, or M5 with or without inhibitors was added to the basal or apical chamber. In the inhibition study, M-2 or M-5 was added to the basal or apical chamber, whereas regorafenib was added to both sides (0, 1, and 10 μM for LLC-PK1-Pgp cells and 0, 0.1, 1, and 10 μM for MDCKII/BCRP/PDZK1 cells). At the designated times, a 100 μL aliquot was sampled from the opposite side and replaced with an equal volume of prewarmed fresh buffer. The medium samples were stored at -80 ^oC until drug quantitation. The efflux ratio was calculated as the ratio of the apparent permeability coefficient (P_{app}) in the basal-to-apical direction to that in the apical-to-basal direction, where P_{app} was calculated as the slope of the regression line in the transport-time profile

of the drug divided by the product of initial drug concentration in the donor chamber and the cell monolayer surface area (1.1 cm^2).

Measurement of drug concentration

Aliquots of plasma and medium samples were mixed with 15 and 6 volumes (v/v), respectively, of methanol with internal standard (sorafenib). Brain, liver, kidney, and lung tissue samples were homogenized in 4 volumes (w/v) of methanol with internal standard, and skin samples were homogenized in 12 volumes (w/v) of the solvent. The mixtures were vortexed, centrifuged (21,500 g, 10 min, 4 °C) twice, and the supernatants subjected to high-performance liquid chromatography/tandem mass spectrometry (LC-MS/MS). Quantification of all drugs was performed using a triple quadrupole mass spectrometer with electrospray ionization (LCMS-8040; Shimadzu, Kyoto, Japan) coupled to a liquid chromatography system (Nexera, Shimadzu). Chromatography was performed by means of gradient elution (flow rate 0.4 mL/min) as follows: 0–0.5 min, 95% A/5% B; 0.5–1.5 min, 95% A/5% B to 35%A/65% B; 1.5–5.5 min, 35% A/65% B to 30%A/70% B; 5.5–6.0 min, 30% A/70% B to 5%A/95% B; 6.0–7.0 min, 5% A/95% B; 7.0–7.1 min, 5% A/95% B to 95% A/5% B (A, water containing 0.1% formic acid and 5 mM ammonium acetate; B, methanol containing 0.1% formic

acid and 5 mM ammonium acetate) on a Cosmosil 2.5C18-MS-II packed column (2.5 μm particle size, 2.0 mm I.D. x 100 mm; Nacalai Tesque, Kyoto, Japan) at 50 °C. Multiple reaction monitoring in positive ion mode was set at 483.00–269.95 for regorafenib, 499.00–201.90 for M-2, 484.90–201.90 for M-5, 487.40–401.00 for afatinib, 368.20–227.10 for bortezomib, 502.10–323.05 for cabozantinib, 721.60–100.05 for carfilzomib, 451.80–260.10 for crizotinib, 520.10–307.10 for dabrafenib, 468.00–317.00 for dacomitinib, 489.30–402.15 for dasatinib, 394.10–278.05 for erlotinib, 447.40–128.10 for gefitinib, 494.20–394.05 for imatinib, 582.30–366.10 for lapatinib, 429.00–372.00 for lenvatinib, 531.00–490.00 for nilotinib, 438.10–357.10 for pazopanib, 533.20–260.05 for ponatinib, 306.90–265.80 for ruxolitinib, 465.00–270.10 for sorafenib, 399.10–283.05 for sunitinib, 313.00–149.00 for tofacitinib, 616.30–254.05 for trametinib, 473.20–453.00 for vandetanib, and 491.20–384.00 for vemurafenib.

Pharmacokinetic analysis

Total clearance (CL_{tot}), distribution volume at steady state (V_{dss}), terminal elimination half-life ($t_{1/2,z}$), and mean residence time (MRT) were estimated in each animal using moment analysis of the plasma concentration profiles obtained at 5, 15, 30,

60 , 120, 360, and 1440 min for regorafenib, and 1, 10, 60, 180 , 360, 480, and 600 min for M-2 and M-5. Tissue-to-plasma concentration (Kp) was obtained by dividing drug concentration in tissue by that in plasma, and the Kp ratio was further calculated by dividing Kp value in TKO mice by that in WT mice.

Statistical Analysis

All values are presented as mean ± S.E.M. The statistical significance of differences was determined using Student's t-test. Differences with a P-value of less than 0.05 were considered to be statistically significant.

Results

Roles of P-gp and BCRP in systemic exposure and dermal distribution of regorafenib and its active metabolites, M-2 and M-5

Regorafenib, M-2, and M-5 were separately injected intravenously in both TKO and WT mice, and their pharmacokinetics examined to clarify the possible role of P-gp and BCRP in their systemic exposure. The plasma concentration profile of intravenous regorafenib in TKO mice showed a slight initial decrease but was almost similar to that in WT mice (Fig. 1A). The plasma concentration of regorafenib in WT mice, but not TKO mice, showed a transient peak approximately 4 h after administration (Fig. 1A). However, the plasma concentration profiles of M-2 and M-5 were significantly higher in TKO mice than in WT mice (Fig. 1B, 1C). Pharmacokinetic parameters were calculated based on the plasma concentration profiles (Table 1). The CL_{tot} values of M-2 and M-5 were higher than that of their parent compound in WT mice (Table 1). The CL_{tot}, $t_{1/2,z}$ and MRT values of M-2 and M-5 were significantly lower in TKO mice than in WT mice (Table 1), indicating higher systemic exposure of the active metabolites but not the parent compound, in TKO mice compared to WT mice.

Concentrations of regorafenib and its active metabolites in each organ were

next examined after intravenous administration of each compound. Skin concentrations of regorafenib, M-2, and M-5 were significantly higher in TKO mice than in WT mice (Fig. 2). A similar trend was observed in both brain and lung (Fig. 2). A difference in M-5 between the two strains was observed in all organs examined (Fig. 2). Tissue distribution was examined by calculating Kp values (Supplementary Fig. 1). The Kp values of regorafenib, M-2, and M-5 in skin and brain of TKO mice were significantly higher than those in WT mice (Supplementary Fig. 1), suggesting the roles of P-gp and/or BCRP in distribution of regorafenib and its active metabolites to these organs. The difference in brain distribution of regorafenib and M-2 between TKO and WT mice was consistent with a previous report by Kort et al.,[9] although their data were obtained after oral administration of only regorafenib.

Effect of elacridar on systemic exposure and dermal accumulation of regorafenib and its active metabolites

To further confirm the roles of P-gp and BCRP in systemic exposure and dermal distribution of regorafenib and its active metabolites, elacridar, an inhibitor of P-gp and BCRP was co-administered with regorafenib, M-2, or M-5 in WT mice, and their plasma and tissue concentrations measured. The plasma concentration profile of

regorafenib with elacridar co-administration showed a slight decrease 1 h post administration, but was similar to that with vehicle co-administration (Fig. 3A). On the other hand, plasma concentrations of M-2 and M-5 were significantly higher in elacridar-treated mice than in control (Fig. 3B, 3C), especially in the terminal phase. The effect of elacridar on M-5 seemed to be more prominent (Fig. 3B, 3C).

The concentration of regorafenib and its active metabolites in each organ was next examined after intravenous administration of each compound with or without elacridar. Skin and brain concentrations of regorafenib, M-2, and M-5 were significantly higher in elacridar-treated mice than in control (Fig. 4). In this experiment, the skin was divided into epidermis and dermis. Regorafenib concentrations in both epidermis and dermis were significantly higher with elacridar than without elacridar (Fig. 4D, 4E). The Kp value of regorafenib in skin and brain, but not liver, in elacridar-treated mice was significantly higher than that in control mice (Supplementary Fig. 2), whereas the Kp values of M-2 and M-5 in elacridar-treated mice were not significantly different from those in control mice (Supplementary Fig. 2). Kp values of regorafenib in both epidermis and dermis were also significantly higher in elacridar-treated mice than in control mice (Supplementary Fig. 2D, 2E).

Biliary excretion of M-5

After intravenous injection of M-5 in WT mice, approximately 65% of the dose was recovered in bile during the first 6 h, but only a small percentage was recovered in the bile of TKO mice (Fig. 5).

Interaction of regorafenib and its active metabolites with P-gp and BCRP

In vitro transcellular transport studies were next performed to examine possible interaction of regorafenib and its active metabolites with human P-gp and BCRP. In LLC-PK1-Pgp cells, basal-to-apical transport of regorafenib, M-2, and M-5 was higher than the reverse transport, the efflux ratio of M-5 being highest (Fig. 6A, 6C, 6E). Such directional transport was diminished in the presence of elacridar, the efflux ratios being reduced almost to 1 (Fig. 6A, 6C, 6E). In MDCKII/BCRP/PDZK1 cells, similar directional transport was observed for each compound, and the efflux ratios were reduced in the presence of the BCRP inhibitor Ko143 (Fig. 6B, 6D, 6F). According to package insert information, regorafenib is as a potent inhibitor of BCRP and a modest inhibitor of P-gp *in vitro* (IC_{50} values for BCRP and P-gp are 50 nM and 2 μM, respectively),[6] although no reports of such experimental data can be found. Therefore, the effect of regorafenib on directional transport of M-2 and M-5 was examined in both

cell lines, and it was found that efflux ratios of both M-2 and M-5 were decreased by regorafenib in a concentration-dependent manner (Fig. 6G, 6H). Since regorafenib concentration in the culture medium could decrease due to non-specific adsorption, it was directly measured by LC-MS/MS to account for any such variation. In MDCKII/BCRP/PDZK1 cells particularly, the ratios for M-2 and M-5 were almost completely decreased by regorafenib at ~50 nM (Fig. 6H).

Correlation between distribution of molecularly targeted small molecules in skin and brain due to P-gp and BCRP

The effect of gene knockout and elacridar on tissue concentration and Kp values of regorafenib and its active metabolites was similar in skin and brain (Figs. 2, 4, and Supplementary Figs. 1, 2), prompting us to perform similar studies using additional molecularly-targeted small molecules to verify similarity in their distribution profiles. Three mixtures of therapeutic agents, all of which included both regorafenib and pazopanib, were intravenously injected in both TKO and WT mice, and the Kp ratio, which in principle represents the contribution of transporter-mediated transport over passive transport,[19] was obtained. Among the 24 compounds administered, plasma and skin levels in TKO and WT mice were detectable for 12 compounds, allowing

calculation of their Kp ratios. The skin Kp ratios were significantly correlated with the brain Kp ratios (Fig. 7).

Discussion

Regorafenib exhibits significant survival advantages in a set of cancer patients,[2,3] but treatment frequently induces a characteristic HFS,[4,5] which sometimes results in dose reduction and/or treatment discontinuation. Therefore, understanding of the mechanisms for accumulation of regorafenib and its active metabolites, M-2 and M-5, in the skin could clarify HFS risk factors to improve therapy. The present study has demonstrated, for the first time, that the transporters P-gp and BCRP are involved in accumulation of all of these compounds in the skin, by showing that (i) the disruption of *Abcb1a*, *Abcb1b*, and *Bcrp* genes in mice resulted in significantly higher concentrations of regorafenib, M-2, and M-5 in the skin (Fig. 2); (ii) treatment with elacridar in WT mice also led to an increase in dermal concentration of these compounds (Fig. 4); and (iii) directional transport of all these compounds was observed in both LLC-PK1-Pgp and MDCKII/BCRP/PDZK1 cells, but was diminished in the presence of elacridar and Ko143, respectively. According to the findings, these transporters could be involved in either systemic elimination or dermal distribution of these compounds. In the case of regorafenib, for example, dermal distribution, but not systemic elimination is primarily mediated by these transporters, since the plasma concentration profile after intravenous administration of regorafenib was similar in TKO and WT mice (Fig. 1A), whereas the

skin Kp value of regorafenib in TKO mice was significantly higher than that in WT mice (Supplementary Fig. 1A). In contrast, P-gp and BCRP may contribute to both systemic elimination and dermal distribution of M-2 and M-5, because their plasma concentration profiles and Kp ratios after intravenous administration in TKO mice were significantly higher than those in WT mice (Fig. 1B, 1C, Supplementary Fig. 1F, 1K). Possible functional compensation after genetic deletion of P-gp and BCRP should be considered in the pharmacokinetic analyses in TKO mice. In the present study, therefore, the direct effect of an inhibitor elacridar for P-gp and BCRP was also examined, and the inhibitory effect on tissue accumulation was also observed (Fig. 3, 4). Hashimoto et al.[20] examined gene expression of several efflux transporters in both TKO and WT mice, and found that mRNA expression of multidrug resistance associated protein (MRP) 1, 3, 4, and 5 was similar between TKO and WT mice, whereas that of MRP2 was not detected in either of the strains. On the other hand, Oatp1a4 protein expression in brain capillaries of TKO mice was approximately 2-fold lower than that of WT mice.[21] Although regorafenib was reported to be a substrate of human OATP1B1 *in vitro*,[8] influx transporter(s) involved in cellular uptake of M-2 or M-5 have not yet been identified. Influx transporter for regorafenib in mouse skin is also unknown. Further investigation of the uptake mechanism for regorafenib and its metabolites are needed.

The probable involvement of BCRP in accumulation of regorafenib and its metabolites in the skin is likely to prompt further pharmacogenomic studies, especially with regard to possible association between functional polymorphisms in its coding gene *ABCG2* and dermal toxicity. One such known polymorphism is c.421C>A (p.Q141K, rs2231142), which is thought to affect amino acids in the ATP-binding domain of BCRP, leading to impaired BCRP activity *in vitro*.[22] Mechanistic studies suggest that the polymorphism also alters transporter function by lowering its protein expression level through ubiquitin-mediated proteasomal and lysosomal degradation.[22] A compelling body of literature supports the clinical relevance of c.421C>A in human *ABCG2* for multiple substrates, where a significant association of this polymorphism with the pharmacokinetics or pharmacodynamics of several drugs has been identified in genome-wide studies.[23] In fact, a higher systemic exposure to tyrosine kinase inhibitors such as gefitinib, erlotinib, and sunitinib was observed in cancer patients with c.421C>A compared to those without the polymorphism.[24] Similarly, higher systemic exposure and/or higher dermal distribution of regorafenib and its active metabolites may be expected in patients with the c.421C>A polymorphism, leading to dermal toxicity. The frequency of regorafenib-induced HFS of grade 3 or greater in Japanese cancer patients (28%) was higher than that in a non-Japanese, mostly caucasian population

(15%),[25] which may be a reflection of the fact that the frequency of c.421C>A in Asians (30%) was higher than that in Caucasians (10%).[22] Regarding the genetic polymorphisms in *ABCB1* gene, the variants, 1236C>T (p.G412G, rs1128503), 2677G>T/A (p.A893S/T, rs2032582) and 3435C>T (p.I1145I, rs1045642) occur at high allele frequencies and create a common haplotype. Therefore, they have been most widely studied. However, although these polymorphisms of the *ABCB1* gene are sometimes associated with changes in drug disposition, drug response, and toxicity, the findings have been in part conflicting, and the clinical implications remain to be limited.[26] Therefore, these variants in *ABCB1* may not be associated with regorafenib-induced dermal toxicity. On the other hand, Zimmerman et al.[27] have recently reported the existence of a skin transporter for another multi-kinase inhibitor sorafenib, implying possible involvement of multiple transport mechanisms for dermal distribution of regorafenib and its active metabolites. Further studies are warranted to elucidate all the mechanisms of their dermal distribution in humans.

It is also noteworthy that gene deletion of *Abcb1a*, *Abcb1b* and *Abcg2* leads to a slight decrease in plasma concentration of regorafenib (Fig. 1A). A similar decrease in plasma regorafenib concentration was also observed when elacridar was co-administered (Fig. 3A), suggesting that this phenomenon is mediated by either P-gp

or BCRP. Therefore, loss of function of these transporters may also result in a decrease, but not increase, in systemic exposure of the parent compound. Although the exact reasons for such change in systemic exposure of the parent compound are unknown, one of the possible explanations would be the increase in distribution of regorafenib to peripheral organs by the loss of those efflux transporters. This reasoning is supported by the higher volume of distribution at steady state (Vd_{ss}) value in TKO mice compared with WT mice (Table 1). Although distribution to the liver was not affected by the gene deletion (Fig. 2C, Supplementary Fig. 2C), the distribution of regorafenib to the skin of TKO mice was higher than that in WT mice (Fig. 2A, Supplementary Fig. 2A). Therefore, it is likely that distribution to other large organs may also be hindered by these transporters. The other possible explanations would include the absence of enterohepatic recirculation of regorafenib in TKO mice. Plasma concentration of regorafenib was transiently increased for 1-4 h after intravenous administration in WT, but not TKO mice (Fig. 1A, 3A), suggesting enterohepatic recirculation of regorafenib, possibly mediated by P-gp and BCRP. Another possible explanation is that the loss of these transporters may increase regorafenib exposure in the liver, thereby promoting its metabolism to M-2 or other metabolites. This type of transporter-enzyme interplay has already been proposed by Lam et al.[28], indicating that inhibition of efflux transporters

promotes enzymatic metabolism possibly due to the increase in available drug concentration in the liver. In the present study, however, hepatic concentration (Fig. 2C) as well as CL_{tot} (Table 1) of regorafenib in TKO mice were similar to those in WT mice, suggesting that such transporter-enzyme interplay may not be supported by the present findings.

In the present study, regorafenib at approximately 50 nM was found to almost completely inhibit the transcellular transport of M-2 and M-5 in MDCKII/BCRP/PDZK1 cells (Fig. 6H). Since the medium concentration of regorafenib was directly measured, this concentration range of regorafenib is unlikely to have been affected by loss of regorafenib due to nonspecific adsorption. The maximum plasma regorafenib concentration in cancer patients who received repeated oral doses of regorafenib was reported to be ~7.2 μM,[29] and its unbound concentration was deduced to be 36 nM considering the unbound fraction of regorafenib in human plasma (0.005).[7] We have recently found CRC patients with 11 μM plasma regorafenib in our ongoing clinical study. Taking these considerations into account, inhibition of BCRP by regorafenib could be clinically relevant. The possible interaction of regorafenib and its active metabolites with BCRP (Fig. 6G, 6H) leads to the hypothesis that increase in regorafenib concentration leads to an increase in not only the synthesis of the active

metabolites, but also inhibition of their efflux from skin or other organs. In particular, loss of P-gp and BCRP caused a drastic decrease in CL_{tot} of M-5, compared with that of regorafenib and M-2 (Table 1). *In vitro* transcellular transport studies suggested that M-5 is highly transported by both P-gp and BCRP, as indicated by a higher efflux ratio compared with that of regorafenib and M-2 (Fig. 6). If we also consider that intravenously administered M-5 is largely excreted into the bile (Fig. 5), these transporters could be primarily involved in systemic elimination of M-5, which could be inhibited by a BCRP inhibitor such as regorafenib. Thus, the findings imply possible parent drug-metabolite interactions, which may lead to complex pharmacokinetics of regorafenib and its metabolites, to be clarified by further analyses in humans.

In the present study, elimination phase of regorafenib in circulating plasma was almost unchanged in TKO and elacridar-treated mice, compared with WT mice (Figs 1A, 3A). One of the possible explanations would be small contribution of P-gp and BCRP to overall elimination of regorafenib from systemic circulation. If efflux transporters expressed in the liver other than P-gp and BCRP play major roles in biliary excretion of regorafenib, its systemic elimination in TKO and elacridar-treated mice would be almost unchanged. However, no information is available on whether regorafenib is a substrate of other efflux transporters or not. Regorafenib is also

eliminated by the metabolism. Nevertheless, Kort et al.[9] reported that plasma concentration-time profile of regorafenib in *Cyp3a*$^{-/-}$ mice was almost similar to that in WT mice, whereas that of M-2 in *Cyp3a*$^{-/-}$ mice was much lower than that in WT mice, suggesting that Cyp3a-medaited metabolism may not exhibit so much impact in systemic elimination of regorafenib. Another possible explanation would be the inhibition of P-gp and/or BCRP by regorafenib itself, as demonstrated *in vitro* in the present study (Fig. 6), resulting in minimal difference in biliary excretion of regorafenib between TKO and WT mice at the intravenous dose examined in Fig. 1. Actually, the hepatic concentration of regorafenib was approximately 2,000 ng/g liver (Figs. 2C, 4C), which corresponds to 4 μM if we assume that the specific gravity of the tissue is unity. Since unbound fraction in the liver is not available, if we further assume that the unbound fraction of regorafenib in the mouse liver is equal to that in human plasma (0.005),[6,7] hepatic unbound concentration of regorafenib was estimated to be approximately 20 nM, which was not so much different from the concentration range at which regorafenib inhibits P-gp- and BCRP-mediated transport (Fig. 6G, 6H). This hypothesis may also explain minimal difference in both plasma (Figs. 1A, 3A) and hepatic (Figs. 2C, 4C) regorafenib concentrations between TKO/elacridar-treated and WT mice.

The higher Kp value of regorafenib in the skin of TKO and elacridar-treated mice compared with WT and vehicle-treated mice, respectively (Supplementary Figs. 1, 2), suggested the possible role of P-gp and BCRP as efflux transport systems for this compound in the skin. Since these transporters are expressed on capillary endothelial cells in the skin of mice and humans,[12,13] such efflux transport in the skin may be analogous to that in the blood-brain barrier, in which both P-gp and BCRP play fundamental roles. In fact, the consistent difference in Kp values of regorafenib and M-2 between TKO and WT mice, and elacridar- and vehicle-treated mice, was also observed in the brain (Supplementary Figs. 1, 2). In the present study, further studies were performed using drugs other than regorafenib, and Kp ratios in skin and brain were found to be correlated (Fig. 7), supporting similar roles of these transporters in both organs. However, endothelial cell-cell junctions in the skin may not be as tight as in the brain, leading to the speculation that the paracellular route, in addition to a transcellular one, may act in the dermal distribution of low molecular weight compounds. This may be supported by the present finding that the Kp ratios in skin were generally lower than those in the brain (Fig. 7). Nevertheless, a drastic increase in the concentration of regorafenib metabolites by loss of P-gp and BCRP (Figs. 2, 4) may still indicate the role of these transporters as a determining factor for drug accumulation in the skin. Since

treatment with regorafenib in patients induces adverse skin reactions such as HFS at a high frequency, these transporters may also be risk factors for the dermal toxicity. This hypothesis needs to be further evaluated in our ongoing clinical studies. In addition, these transporters are also localized on the basal layer of epidermis keratinocytes,[12,13] which include immature, but proliferative epithelial cells. BCRP is also expressed in both epidermal Langerhans cells and dermal interstitial dendritic cells, and is possibly involved in differentiation of myeloid dendritic cell progenitors to Langerhans cells in the skin.[30] Thus, it is possible that the interaction of regorafenib and its active metabolites with BCRP in non-endothelial cells may also be associated with dermal events provoked by regorafenib.

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Figure Legends

Fig. 1. Systemic elimination of regorafenib, M-2, and M-5 after intravenous administration

After intravenous administration of regorafenib (1 mg/kg body weight, A), M-2 (160 μg/kg body weight, B), or M-5 (160 μg/kg body weight, C), the plasma concentration of each compound was measured in WT (closed circles) and TKO (open circles) mice. Each value represents the mean ± S.E.M. (n = 4-9, 3-7, and 4-8 in panels A, B, and C, respectively).

*, Significant difference compared to WT mice ($p<0.05$).

Fig. 2. Tissue concentration of regorafenib, M-2, and M-5 after intravenous administration

After intravenous administration of regorafenib (1 mg/kg body weight), M-2 (160 μg/kg body weight), or M-5 (160 μg/kg body weight), the concentrations of regorafenib (A-E), M-2 (F-J), and M-5 (K-O) were measured at 6 h, 10 h and 10 h, respectively in WT (closed bars), and TKO (open bars) mice. Each value represents the mean ± S.E.M. (n = 4-5, 4, and 4 in panels A-E, F-J, and K-O, respectively).

*, Significant difference compared to WT mice ($p<0.05$).

Fig. 3. Effect of elacridar on systemic elimination of regorafenib, M-2, and M-5 after intravenous administration

At 30 min after intravenous administration of elacridar (10 mg/kg body weight, closed circles) or vehicle alone (open circles), regorafenib (1 mg/kg body weight, A), M-2 (160 μg/kg body weight, B) or M-5 (160 μg/kg body weight, C) was also intravenously administered, and plasma concentration of each compound was measured in ICR mice. Each value represents the mean ± S.E.M. (n = 5).

*, Significant difference compared to vehicle-treated mice ($p<0.05$).

Fig. 4. Effect of elacridar on tissue concentration of regorafenib, M-2, and M-5 after intravenous administration

At 30 min after intravenous administration of elacridar (10 mg/kg body weight, closed bars) or vehicle alone (open bars), regorafenib (1 mg/kg body weight, panels A-E), M-2 (160 μg/kg body weight, panels F-H) or M-5 (160 μg/kg body weight, panels I-K) was also intravenously administered, and tissue concentration of each compound was measured at 6 h after the injection in ICR mice. Each value represents the mean ± S.E.M. (n = 5).

*, Significant difference compared to vehicle-treated mice ($p<0.05$).

Fig. 5. Biliary excretion profile of M-5 after its intravenous administration

Cumulative biliary excretion of M-5 was determined in WT (closed circles) and TKO (open circles) mice. Each value represents the mean ± S.E.M. (n = 4-5).

Fig. 6. Transcellular transport of regorafenib, M-2, and M-5, and inhibition of directional transport of M-2 and M-5 by regorafenib in LLC-PK1-Pgp and MDCKII/BCRP/PDZK1 cells.

In panels A-F, 1 μM of regorafenib (A, B), M-2 (C, D), or M-5 (E, F) was applied to either the basal (circles) or the apical (diamonds) side in the presence (closed symbols) or absence (grey symbols) of elacridar (5 μM) or Ko143 (1 μM) in LLC-PK1-Pgp (A, C, E) or MDCKII/BCRP/PDZK1 (B, D, F) cells, respectively, and transcellular transport to the opposite side was measured. In panels G and H, similar studies of M-2 and M-5 (1 μM each) were performed with regorafenib present on both sides in LLC-PK1-Pgp (G) or MDCKII/BCRP/PDZK1 (H) cells. The abscissa represents mean regorafenib concentration in culture medium at the end of experiments, measured by LC-MS/MS.

Fig. 7. Correlation between Kp ratios of various molecularly-targeted small molecules in skin and brain

Three mixtures of 24 drugs were intravenously administered in TKO and WT mice, and plasma and tissue concentrations were measured at 60 min after the injection (see Methods for details). Data represent mean values of each drug (n = 3-5).