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Development and validation of an analytical method for regorafenib and its metabolites in mouse plasma

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Abstract

An analytical method was developed for measuring the effect of OATP1B2 deficiency on plasma levels of the kinase inhibitor regorafenib and its metabolites regorafenib-N-oxide, N-desmethyl-regorafenib-N-oxide, and regorafenib-N- β -glucuronide (RG) in mice. Compounds were separated by liquid chromatography and monitored by a triple quadrupole mass spectrometer in the selected reaction monitoring mode after positive electrospray ionization. All calibration curves were linear in the selected concentration range ($R^2 = 0.99$). The lower limit of quantification was 5 ng/mL for the four analytes. Within-day precisions, between-day precisions, and accuracies were 2.59–6.82%, 3.97–11.3%, and 94.5–111%, respectively. The identification and structure elucidation of RG, isolated from human urine, was performed by NMR. Compared with wild-type mice given regorafenib (10 mg/kg), deficiency of the drug transporter OATP1B2 *in vivo* had minimal effects on plasma levels of parent drug and the metabolite regorafenib-N-oxide, and N-desmethyl-regorafenib-N-oxide. However, the area under the curve and peak levels of RG were increased by 5.6-fold and 5.1-fold, respectively, in OATP1B2-knockout mice. In conclusion, our analytical method allowed accurate and precise quantitation of regorafenib and its main metabolites in mouse plasma, and is suitable for evaluation of transporter-dependent pharmacokinetic properties of these agents *in vivo*.

1. Introduction

Regorafenib is an orally administered tyrosine kinase inhibitor (TKI) that targets the vascular endothelial growth factor, platelet-derived growth factor, and fibroblast growth factor receptors (1–9), and is used as second-line therapy in intermediate or advanced

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hepatocellular carcinoma patients. Regorafenib has also been approved for the treatment of certain colorectal cancers and advanced gastrointestinal tumors (GIST). In patients with advanced solid tumors, the recommended oral dose of regorafenib is 160 mg (10). Although regorafenib offers a number of important theoretical advantages over conventional cytotoxic agents, the agent is affected by some of the same clinical problems, including a relatively narrow therapeutic window as well as extensive inter-individual pharmacokinetic variability (11). The pharmacokinetic properties of regorafenib have been previously associated with differential expression of certain transporters and enzymes (12, 13). However, the exact mechanisms by which the drug is taken up into hepatocytes and how its metabolites are processed still remain largely unknown.

Previous investigation has suggested that regorafenib undergoes extensive hepatic metabolism through two competing pathways involving CYP3A4 and UGT1A9, which result in the formation of regorafenib-N-oxide (M2), N-desmethyl-regorafenib-N-oxide (M5) and regorafenib-N- β -glucuronide (M7; RG) (14) (Fig.1). Regorafenib, M2 and M5 have been previously detected in plasma of human patients, and regorafenib, M2 and M7 are detectable in urine samples (14, 15) based on liquid chromatography-tandem mass spectrometric (LC-MS/MS) assays (16–18). Details of the *in vivo* glucuronidation of regorafenib, in particular the metabolic disposition of RG and its connection to organic anion transporting polypeptides (19–21), are lacking. In the current work, we describe the development, validation, and application of an assay for regorafenib and its metabolites M2, M5 and RG in mouse plasma by LC-MS/MS.

2. Experimental

2.1. Chemical and reagents

Regorafenib was obtained from LC Laboratories (>99% purity, Woburn, MA) and regorafenib-N-oxide, N-desmethyl-regorafenib-N-oxide, regorafenib- $^{13}\text{CD}_3$ and regorafenib-N-oxide- $^{13}\text{CD}_3$ were obtained from Clearsynth (>99% purity, Mississauga, ON, Canada). Regorafenib-N- β -glucuronide (RG) was obtained from Toronto Research Chemicals Inc. (>99% purity, North York, ON, Canada). Methanol, acetonitrile, formic acid (LC-MS-Grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). RG- $^{13}\text{CD}_3$ was produced as described previously for sorafenib-glucuronide (22). Mouse plasma was obtained from wild-type mice on a DBA/1lacJ background strain.

2.2. LC-MS/MS conditions

The LC-MS/MS system consisted of a Vanquish UHPLC system, a TSQ Quantum Ultra triple quadrupole mass spectrometer from Thermo Fisher Scientific, and Thermo Trace Finder General Quan system software (version 3.3). An Accucore Vanquish C18 column (100 $\times$ 2.1 mm, dp = 1.5 μm , Thermo Fisher Scientific) was protected by a corresponding XBridge®BEH C18, 5- μm guard column. The injection volume of sample was 5.0 μL . The temperature of the autosampler rack was 4°C, and the temperature of the column was 40°C. Mobile phase A consisted of water with 0.1% (v/v) formic acid and mobile phase B consisted of acetonitrile: methanol (1:3) with 0.1% (v/v) formic acid. The total run time was 5 min. The optimized gradient started at 0–0.5 min with 10% B; 0.5 – 3.0 min, 95% B; 3.0 –

4.0 min, 95% B; 4.0 - 4.1 min, 10% B; 4.1 - 5.0 min, 10% B. The flow rate was 0.4 mL/min. The MS assay setting with the positive voltage applied to the ESI capillary was 3500 V, and the capillary temperatures was 342°C with a vaporizer temperature of 358°C. Argon was used as a collision gas at a pressure of 1.5 mTorr. Precursor molecular ions and product ions for confirmation and detection of each analyte are shown in Table 1.

2.3. NMR conditions and metabolite isolation

The proton, carbon, HMBC, HSQC, COSY, and fluorine spectra were collected on RG reference materials in D₆-DMSO (Sigma) on a Bruker Avance III HD spectrometer. The fluorine spectrum was on a 400 MHz Ascend magnet, the proton, HMBC, HSQC, and COSY spectra were on a 600 MHz Ultrashield magnet with TXI cryoprobe and the carbon was on a 700 MHz Ascend magnet with TXO cryoprobe. The proton spectra for comparison of a synthetic and urine-purified sample of RG were collected on a 400 MHz spectrometer.

RG was isolated from urine samples obtained from a 63-year old female patient with metastatic colorectal cancer who was treated on a clinical trial (ClinicalTrials.gov Identifier: NCT02800330) with regorafenib given once daily at a dose of 120 mg. The isolation of RG from urine was based on a method that we described previously for sorafenib-glucuronide (22). In brief, urine samples were lyophilized and extracted twice with ethyl acetate, combined, and dried. Samples were reconstituted in methanol and water containing 100 mM MOPS at pH 7. HPLC purification was performed on an Agilent 1200 preparatory HPLC using a Phenomenex Gemini C18 column (250 × 21.2 mm; 5 µm particle size). Mobile phase A was pure water while mobile phase B was pure acetonitrile (Fisher Optima), and a standard gradient from 10-95% B was used in the purification. Fractions containing RG were initially selected on the basis of the UV-Vis spectrum and confirmed by mass spectrometry. RG containing fractions were combined and lyophilized. The lyophilized sample was first reconstituted in D₆-DMSO and a proton spectrum was recorded. The sample was then lyophilized again and reconstituted in 90/10 H₂O/D₂O with 20 mM NaPO₄ at pH 7.0 and a proton spectrum was recorded.

2.4. Working solution, calibration and quality control samples

The working solutions of regorafenib, M2, M5, and RG were prepared by diluting the stock solutions with methanol. The stock solutions of regorafenib, M2, M5, and RG mixed were prepared by dissolving 10 mg with methanol in a 10 mL volumetric flask. The calibration standards ranged in concentrations from 5 – 1,000 ng/mL. The lower limit of quantitation, low, medium, and high quality controls (QC) were prepared at concentrations of 5, 25, 75, 750 ng/mL, respectively. The internal standard (IS) stock solution was prepared at a concentration of 150 ng/mL in acetonitrile: water (3:1, v/v), and stored at –20 °C until use.

2.5. Sample preparation

Frozen samples were thawed at room temperature. Plasma samples of 10 µL each were mixed with 20-µL of standard, QC or blank methanol were transferred a 0.6 mL tube, 50 µL of IS was added, and then vortex mixed for 5 min. Tubes were centrifuged at 13,000 rpm for 5 min at 4°C. Supernatants were transferred to autosampler vials, and 5-µL samples were injected for UPLC/MS-MS analysis.

2.6. Method validation

2.6.1. Calibration curve, accuracy and precision—The linearity of regorafenib, M2, M5, and RG curves were evaluated by the peak area ratios of each of the four analytes to the IS relative to the analyte concentrations using a linear regression model. Weighting ($1/\text{area}^2$) was used in all cases. Seven concentration points (5, 10, 50, 100, 250, 500, and 1000 ng/mL) were used in the calibration curves. The coefficient of variation (CV) of the calculated concentration for each standard points was to be less than 15%, and less than 20% for the LLOQ. The accuracy, intra-day precision, and inter-day precision were evaluated on 5 consecutive days, and included LLOQ and QC samples, and calculated as described (23). The back calculated concentration values for QC samples, run in quintuplicate at each concentration level on 5 different days, as well as the between-run and within-run precision values were to be less than 15%.

2.6.2. Selectivity, dilution and recovery—Selectivity was evaluated by analysis of 6 different lots of plasma from untreated mice. The interfering peak areas were to be lower than 10% of the peak area for the LLOQ for each analyte in plasma. A dilution test was also performed to evaluate accuracy at a concentration of 5,000 ng/mL for each analyte, followed by a 1:10 (v/v) dilution in blank mouse plasma. Acceptable accuracy results were to be within $\pm 15\%$. Recovery of regorafenib, M2, M5, and RG was evaluated by comparison of the peak area obtained from blank plasma to that observed in neat solution. QC samples for each analyte were assayed, matrix effects were assessed from a continuous post-column infusion of the analytes in blank mouse plasma extracts, and calculated as described (24).

2.6.3. Freeze-thaw, re-injection, short-term and long-term stability—Freeze-thaw cycles were performed three times using -80°C to room temperature cycles using. QC samples of regorafenib, M2, M5, and RG at low, medium and high concentrations. Re-injection stability was assessed using QC samples for the four analytes left in the autosampler. These QCs were re-analyzed after remaining in the autosampler at 4°C for 24 h. Short-term stability was evaluated for the four analytes at room temperature for 8 h. Long-term stability was evaluated for the four analytes at -80°C temperature for 6 months.

2.7. In vivo pharmacokinetics studies

2.7.1 Animal studies—Male mice knockout for Oatp1b2 [Oatp1b2(−/−)] and age-matched wild-type mice on a DBA/1lacJ background strain were maintained under specific pathogen free conditions in the Ohio State University Laboratory Animal Resources (ULAR), and all mouse experiments were approved by the ULAR Animal Care and Use Committee. Mice were given free access to a standard diet and water in a temperature-controlled with a 12-hour light cycle. Regorafenib was dissolved in a mixture of transcutol: Cremophor EL: sodium chloride (1:1:5). Five mice in each experimental group were given regorafenib orally at a single dose of 10 mg/kg. Blood samples of 30 μL were taken from individual mice at 0.25, 0.5, 1.5, 3, 4.5 and 7.5 h. Samples at 0.25-1.5 h were taken from the submandibular vein using a lancet, samples at 3-4.5 h were derived from the retro-orbital venous plexus using a capillary, and a final samples at 7.5 h was obtained by a cardiac puncture using a syringe and needle. Blood samples were collected in heparin-containing

tubes, and processed by centrifugation for 10 min at 3000×g at 4°C. Plasma supernatants were stored at –80 °C until analysis.

2.7.2 Pharmacokinetic data analysis—Noncompartmental pharmacokinetic analysis was performed using Phoenix WinNonlin version 7.6 (Certate, USA). The elimination rate constant (K_{el}) was estimated from the slope of the terminal phase of the log plasma concentration–time curve fitted by the method of least-squares and the terminal half-life ($T_{1/2}$) was calculated by $0.693/K_{el}$. The peak plasma concentration (C_{max}) and the time to reach peak plasma concentration (T_{max}) were obtained by visual inspection of the data from the concentration–time curve. The area under the plasma concentration–time curve (AUC) was calculated according to the linear trapezoidal rule. The metabolite to parent ratio (MR) was estimated by $(AUC_{\text{Regorafenib-metabolite}}/AUC_{\text{Regorafenib}}) \times (MW_{\text{Regorafenib}}/MW_{\text{Regorafenib-metabolite}})$.

3. Results and discussion

3.1. UPLC–MS/MS spectrometry and specificity

Data on the mass spectra of regorafenib, M2, M5, and RG as protonated molecular ions [$M + H$]⁺ are provided in Table 1 and Fig. 3 A-D. A positive ion mode with electrospray ionization was used for the UPLC–MS/MS analysis. The run time was 5 min per sample. Under the optimal HPLC conditions, regorafenib, regorafenib-¹³CD₃, M2, M2-¹³CD₃, M5, RG and RG-¹³CD₃ eluted with a retention time of 3.88, 3.77, 3.70, 3.40, 3.88, 3.75, and 3.40 min, respectively. Chromatograms of regorafenib, M2, M5, RG (100 ng/mL each), and the IS (150 ng/mL) in mouse plasma samples are shown in Fig. 4 A-G. Chromatograms of regorafenib, M2, M5, RG (5 ng/mL each), and blank mouse plasma samples are shown in Fig. 5 A-K. Carryover was found to be negligible (less than 10% of LLOQ). The specificity of the method was confirmed by the lack of interference for regorafenib, M2, M5, and RG in blank plasma from 6 different mice.

3.2. Linearity, accuracy, precision, and selectivity

The calibration curves for regorafenib, M2, M5, and RG were linear in the range of 5 to 1,000 ng/mL (R^2 of 0.99). The applied uniform weighting was compared to alternative weighting factors by evaluation of various parameters, including R^2 values being closest to a value of 1.0. The signal to noise ratios for regorafenib, M2, M5, and RG at 5 ng/mL were 202, 95, 120, and 59, respectively. The back-calculated concentrations for the calibration curve points of the four analytes were less than 15% of the nominal concentration, and deviated less than 20% for the LLOQ. The accuracy and precision results are shown in Table 2. The within- and between-run precisions, expressed as coefficients of variation for regorafenib, M2, M5, and RG, were less than 8.32%, 7.42%, 8.1%, 11.34% for the four analytes, respectively, and the observed accuracies were all deviating less than 9.0% of their nominal value. The inter-assay and intra-assay accuracy for the LLOQ, QCs and dilution samples of regorafenib, M2, M5, and RG were between 98.6-105%, 96.2-107%, 97.8-105%, and 94.5-111%, respectively. No interfering peaks were found for each of the four analytes at relevant retention times, and endogenous responses were lower than 2% of the signal at the LLOQ in blank plasma.

3.3. Recovery, matrix effect and dilution

The recovery and matrix effect were evaluated at QC concentrations of 25, 75, and 750 ng/mL. The mean recovery for regorafenib, M2, M5, and RG was found to be >90%, and matrix effects were evaluated in plasma and aqueous solutions (Table 3). No obvious matrix effects were observed with the applied post-column infusion process. The within-run and between-run precisions observed for the diluted QC samples of regorafenib, M2, M5, and RG were less than 8.32, 8.10, 12.0, and 6.69%, respectively, and the corresponding intra-assay and inter-assay accuracies were 90.1, 88.8, 91.3, and 89.1%, respectively.

3.4. Stability

The short-term stability at room temperature for 8 h, the long-term stability at -80°C over a period of 6 months (Table 4), and the stability during 3 freeze-thaw cycles were confirmed for all four analytes, and found to deviate less than 15% of the initial concentrations. This degree of stability allows for multiple samples to be processed conveniently at room temperature. Regorafenib, M2, M5, and RG in a reconstituted solution in the autosampler at 4°C were also found to be stable for up to 24 h, with less than 15% deviation being observed as compared to the initial concentrations.

3.5. Metabolite RG identification

All atoms in the synthetic RG sample were assigned with the exception of the oxygens, nitrogens, one of the amide proton, one of the hydroxyl protons, and the carboxylate proton, which were not observed in the NMR spectra. The assignments were made using proton, carbon, fluorine, COSY, multiplicity edited HSQC, and HMBC spectra. The position of the glucuronide was confirmed through an HMBC correlation from the proton on carbon 34 of the glucuronide and carbon 18 of the neighboring aromatic ring (Fig. 2).

During the HPLC method development it became apparent that the behavior of the RG was highly dependent on pH. Indeed, the retention time of the synthetic RG sample was initially very different from the retention time of the RG present in urine samples of a patient. However, once both solutions were buffered to pH 7.0, retention times were congruent. The proton spectrum of the RG from urine in $\text{D}_6\text{-DMSO}$ was different from that seen in the synthetic RG in $\text{D}_6\text{-DMSO}$, likely due to differences in the pH. Therefore, the proton spectra for comparison of the synthetic RG with the RG from urine were both collected in buffer at pH 7.0. Under those conditions, the spectra overlaid properly, confirming that the isolated metabolite from urine has a glucuronic acid moiety attached to regorafenib at the same position as the synthetic, commercially-available version of the compound.

3.6. Pharmacokinetic studies

Previously reported studies have suggested that the hepatocellular uptake of certain TKIs may be regulated by the organic anion transporting polypeptides OATP1B1 and OATP1B3 (19–20), which are highly expressed in the human liver. However, a systematic evaluation of the interaction of regorafenib with OATP1B-type transporters *in vivo*, and how such process affects drug disposition profiles has remained unstudied. To test the interaction of regorafenib and its metabolites with the murine orthologue transporter OATP1B2, we pharmacokinetically characterized regorafenib and its three metabolites in mice with or

without a deletion of OATP1B2. We found that the AUC, $C_{\max}$ and $T_{\max}$ for regorafenib, M2, and M5 were minimally different between male wild-type mice and male OATP1B2(–/–) mice after a single oral administration of regorafenib at a dose of 10 mg/kg (Fig. 6A–C; Table 5), which is in line with previously reported findings (13). However, the $C_{\max}$ and AUC of RG were significantly increased in OATP1B2(–/–) mice at each of the evaluated time points, resulting in 5.6-fold and 5.1-fold increases, respectively, in AUC and $C_{\max}$, compared to wild-type mice (Fig. 6D; Table 5). In addition, we found that the metabolic ratio of RG to regorafenib was increased by 5.3-fold in OATP1B2(–/–) mice (Table 5). The dependence of the metabolic disposition of RG on OATP1B2 function observed here is similar to that previously reported for the glucuronic acid metabolite of sorafenib (SG) (22, 25), whereby OATP1B2 deficiency is associated with altered levels of SG in the systemic circulation.

4. Conclusion

We developed and validated a UPLC-MS/MS method for the quantitative determination of regorafenib and its main metabolites in mouse plasma. Compared to previously reported analytical methods for regorafenib (18, 26–28), the current analytical method requires smaller volumes of plasma while retaining high sensitivity, and thus can be applied to pharmacokinetic studies in mice undergoing repeated sampling. In addition, the method allows for quantitative analysis of RG, an important metabolite of regorafenib that is dependent on OATP1B2 function for subsequent distribution and elimination pathways. Our current preclinical studies are focused on the contribution of RG to enterohepatic recirculation of regorafenib, and on the role OATP1B2 plays in this process.

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Figure 1.

Major metabolic pathways of regorafenib and chemical structures of regorafenib, (B). regorafenib-N-oxide (M2), (C). N-desmethyl-regorafenib-N-oxide (M5), and (D). regorafenib-N-β-glucuronide (M7; RG)

Figure 2.
 ^1H NMR spectra of regorafenib-N- β -glucuronide isolated and purified from patient urine and a synthetic reference standard

Figure 3. MS/MS chromatograms and proposed fragmentation pathways for (A). regorafenib, (B). regorafenib-N-oxide, (C). N-desmethyl-regorafenib-N-oxide, and (D). regorafenib-N-β-glucuronide

Figure 4.

Representative chromatograms of mice plasma spiked with a concentration of 100 ng/mL in (A) regorafenib, (B) regorafenib-N-oxide (C). N-desmethyl-regorafenib-N-oxide, (D) regorafenib-N-β-glucuronide, as well as 150 ng/mL-concentrations of (E) regorafenib-¹³CD₃, (F) regorafenib-N-oxide-¹³CD₃, (G) and regorafenib-N-β-glucuronide-¹³CD₃

Figure 5. Representative chromatograms of mouse plasma spiked with a 5 ng/mL concentration of (A) regorafenib, (B) regorafenib-N-oxide, (C) N-desmethyl-regorafenib-N-oxide, or (D) regorafenib-N- β -glucuronide, as well as representative chromatograms of blank mouse plasma (H-K).

Figure 6.

Mean plasma concentration-time profiles of (A) regorafenib, (B) regorafenib-N-oxide, (C) N-desmethyl-regorafenib-N-oxide, and (D) regorafenib-N-β-glucuronide, following an oral administration of regorafenib (10 mg/kg) to wild-type mice and OATP1B2-knockout mice (mean ± SD, n=5)

Precursor molecular ion/product ion for quantification, precursor molecular ion/product ion for confirmation and detection parameters collision energy (CE)/RF Lens (V) for each analyte

Table 1

Analyte	Polarity	Precursor (m/z)	Product (m/z)	Confirmation (m/z)	Collision Energy (V)	RF Lens (V)
Regorafenib	Positive	483.14	270.000	288.058	31.640	111
Regorafenib-N-oxide	Positive	499.12	303.986	229.000	24.461	120
N-desmethyl-regorafenib-N-oxide	Positive	485.12	202.000	290.000	31.590	113
Regorafenib-N-β-glucuronide	Positive	659.15	483.111	269.986	15.916	86
Regorafenib-13CD3	Positive	487.16	274.058	N/A	32.247	109
Regorafenib-N-oxide-13CD3	Positive	503.17	308.058	N/A	24.815	121
Regorafenib-N-β-glucuronide-13CD3	Positive	663.11	486.986	N/A	20.466	94

Table 2
Assay performance data for the quantitation of regorafenib and its metabolites in mouse plasma

	Concentration (ng/mL)	Regorafenib			Regorafenib-N- β -glucuronide		
		Intra-Assay (%)	Inter-Assay (%)	Accuracy (%)	Intra-Assay (%)	Inter-Assay (%)	Accuracy (%)
LLOQ	5	5.12	8.42	99.3	5.63	7.42	101
QC 1	25	3.66	7.05	105	4.75	5.17	104
QC 2	75	2.59	4.01	105	3.41	4.83	107
QC 3	750	5.11	6.78	98.6	4.33	5.69	96.2
Dilution (1:10)	500	5.66	8.32	90.1	4.75	6.69	89.1

	Concentration (ng/mL)	Regorafenib-N-oxide			N-desmethyl-regorafenib-N-oxide		
		Intra-Assay (%)	Inter-Assay (%)	Accuracy (%)	Intra-Assay (%)	Inter-Assay (%)	Accuracy (%)
LLOQ	5	4.36	4.33	97.8	6.82	11.3	94.5
QC 1	25	4.24	3.97	102	6.54	7.35	109
QC 2	75	3.72	4.59	105	4.03	4.81	111
QC 3	750	3.71	6.64	98.7	6.08	8.63	96.8
Dilution (1:10)	500	5.85	8.1	88.8	7.66	12.	91.3

Table 3

Recovery and matrix effect of regorafenib and its metabolites in mouse plasma

Concentration (ng/mL)	Regorafenib		Regorafenib-N- β -glucuronide	
	Recovery (%)	Matrix Effect (%)	Recovery (%)	Matrix effect (%)
25	99.8 $\pm$ 1.69	0.44 $\pm$ 3.53	99.8 $\pm$ 4.30	1.93 $\pm$ 4.62
75	99.3 $\pm$ 1.76	-2.25 $\pm$ 2.51	99.7 $\pm$ 1.20	-1.12 $\pm$ 2.33
750	97.9 $\pm$ 2.11	-5.09 $\pm$ 10.0	97.2 $\pm$ 4.00	-4.25 $\pm$ 7.85
Concentration (ng/mL)	Regorafenib-N-oxide		N-Desmethyl-regorafenib-N-oxide	
	Recovery (%)	Matrix Effect (%)	Recovery (%)	Matrix effect (%)
25	99.8 $\pm$ 2.33	0.54 $\pm$ 3.1	101 $\pm$ 4.30	-7.08 $\pm$ 4.70
75	99.8 $\pm$ 0.32	-0.91 $\pm$ 2.39	106 $\pm$ 2.15	7.28 $\pm$ 4.46
750	95.9 $\pm$ 1.19	-5.92 $\pm$ 8.70	93.1 $\pm$ 12.6	-4.56 $\pm$ 11.2

Table 4
Short-term and long-term stability of regorafenib and its metabolites in mouse plasma

Time	Concentration (ng/mL)	Regorafenib		Regorafenib-N- β -glucuronide	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Short-term (8 hr)	25	2.64	99.1	2.21	102
	75	0.39	104	0.98	107
	750	3.71	100	3.03	98.0
Long-term (6 months)	25	2.64	96.2	2.75	99.5
	75	4.20	98.5	2.26	101
	750	3.70	91.8	2.72	90.9

Time	Concentration (ng/mL)	Regorafenib-N-oxide		N-Desmethyl-regorafenib-N-oxide	
		CV (%)	Accuracy (%)	CV (%)	Accuracy (%)
Short-term (8 hr)	25	2.41	98.2	5.11	106
	75	1.69	103	2.33	112
	750	2.07	101	3.37	100
Long-term (6 months)	25	1.00	98.6	5.63	109
	75	3.07	100	1.58	104
	750	3.25	93.5	2.77	90.4

Table 5

Pharmacokinetic parameters of regorafenib and its metabolites

Wild-type mice	Regorafenib	Regorafenib-N-oxide	N-desmethyl-regorafenib-N-oxide	Regorafenib-N- β -glucuronide
$C_{\max}$ (ng/mL)	3300 $\pm$ 424	360 $\pm$ 129	22.9 $\pm$ 11.5	35.48 $\pm$ 5.97
$T_{\max}$ (h)	2.70 $\pm$ 1.5	5.40 $\pm$ 2.92	7.5	1.60 $\pm$ 0.89
$T_{1/2}$ (h)	14.9 $\pm$ 0.74	N/A	N/A	N/A
AUC_{last} ($\mu\text{g}^* \text{h/mL}$)	18.0 $\pm$ 2.6	1.93 $\pm$ 0.51	0.065 $\pm$ 0.035	0.180 $\pm$ 0.032
MR (%)		10.5	0.36	0.73
OATP1 B2(−/−) mice	Regorafenib	Regorafenib-N-oxide	N-desmethyl-regorafenib-N-oxide	Regorafenib-N- β -glucuronide
$C_{\max}$ (ng/mL)	3644 $\pm$ 172	405 $\pm$ 20.6	27.9 $\pm$ 7.17	179 $\pm$ 61.4 [*]
$T_{\max}$ (h)	1.5	5.80 $\pm$ 1.86	7.5	2.1 $\pm$ 0.82
$T_{1/2}$ (h)	12.5 $\pm$ 1.75	N/A	N/A	N/A
AUC_{last} ($\mu\text{g}^* \text{h/mL}$)	19.3 $\pm$ 2.15	2.38 $\pm$ 0.14	0.099 $\pm$ 0.026	1.01 $\pm$ 0.353 [*]
MR (%)		12	0.51	3.84 [*]

*P<0.05 vs wild-type mice