Online Resource 1

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Title: Metabolism and Pharmacokinetic Drug–Drug Interaction Profile of Vericiguat, a Soluble Guanylate Cyclase Stimulator: Results From Preclinical and Phase I Healthy Volunteer Studies

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Methods

Identification of Relevant UGT Isoforms

Isoform-Specific Inhibitor Experiments

Microsomal protein (human liver, intestinal or kidney microsomes, 1.0 mg/mL), 100 mM potassium phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$, and alamethicin (50 µg per mg microsomal protein) were mixed and placed on ice for 15 minutes. Saccharolactone (5 mM) and 1 µM or 10 µM vericiguat (dissolved in 10 mM hydrochloric acid/acetonitrile,1:1) were added and pre-warmed at 37°C for 5 minutes. The reaction was initiated by addition of uridine 5'-diphospho-glucuronic acid (UDPGA; 5 mM) gaining a final volume of 0.5 mL. After 60 minutes at 37°C, reactions were terminated by addition of 250 µL acetonitrile and cooling on ice. Precipitated proteins were removed by centrifugation (14,000 rpm, 3 minutes) and the supernatants were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

In parallel, isoform-specific inhibitors of uridine diphosphate-glucuronosyltransferases (UGTs) were added before initiating the reaction via addition of UDPGA, i.e., atazanavir (inhibitor of UGT1A1, 5 µM), hecogenin (1A4, 10 µM), niflumic acid (1A9, 5 µM), or mefenamic acid (2B7, at higher concentrations, also 1A9, 10 µM).

Correlation Analysis

To further support the isoform identification, vericiguat was incubated with 20 individual human liver microsomal preparations and one sample of pooled human liver microsomes, which had been characterized for their enzymatic activity for two UGT isoform-specific substrates, i.e., 17β-estradiol (UGT1A1) and propofol (1A9). Incubations were performed as described above for microsomal experiments with UGT isoform-specific inhibitors employing vericiguat (1 µM), 17β-estradiol (20 µM), or propofol (100 µM), 0.3–1 mg/mL protein and 15–60 min incubation times. Samples were analyzed for M-1, estradiol 3-glucuronide, or propofol glucuronide, using an HP1290 Infinity liquid chromatograph or an Agilent HP 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled via electrospray ionization interface (TurboIonSpray®) to an atmospheric pressure ionization/tandem mass spectrometer (AB SCIEX API3000 or API3200, Applied Biosystems, MDS Sciex, Ontario, Canada); [^{13}C,$^{2}H_4$]-M-1 (in-house synthesis) or naphthol glucuronide served as the internal standards. Velocity of vericiguat glucuronidation was correlated to respective isoform-specific activity for UGT1A1 and 1A9 employing linear regression analysis.

Investigations Regarding Substrate Properties and Inhibitory Potential Towards Transport Proteins

Online Table 1 Summary of the cell systems, probe substrates, and reference inhibitors used for the respective transport protein investigations

Transporter	Cell system	Probe substrate (concentration)	Reference inhibitor (concentration)
P-gp	LLC-PK1 wild-type and L-MDR1 cells	Digoxin (20 µM) Dipyridamole (2 µM)	Ivermectin (5 µM)
BCRP	MDCKII wild-type and MDCKII-BCRP cells	Topotecan (2 µM) Fluvastatin (2 µM)	Ko143 (1 µM)
OATP1B1	Vector-HEK and hOATP1B1-HEK cells	Pravastatin (5 µM) Estrone-3-sulfate (0.03 µM)	Rifamycin (30 µM) Rifampicin (50 µM)
OATP1B3	Vector-HEK and hOATP1B3-HEK cells	Pravastatin (5 µM) Sulfobromophthalein (0.05 µM)	Rifamycin (30 µM) Rifampicin (5 µM)
OAT1	Vector-HEK and hOAT1-HEK cells	P-aminohippuric acid (10 µM)	Probenecid (100 µM)
OAT3	Vector-HEK and hOAT3-HEK cells	Estrone-3-sulfate (1 µM)	Probenecid (100 µM)
OCT1	Vector-HEK and hOCT1-HEK cells	1-methyl-4-phenylpyridinium iodide (10 µM)	Decynium-22 (50 µM)
OCT2	Vector-HEK and hOCT2 cells	1-methyl-4-phenylpyridinium iodide (10 µM)	Decynium-22 (50 µM)
MATE1	Vector-HEK and hMATE1 cells	Metformin (50 µM)	Cimetidine (100 µM)
MATE2K	Vector-HEK and hMATE2K cells	Metformin (50 µM)	Pyrimethamine (1 µM)
BSEP	Sandwich-cultured human hepatocytes	Taurocholic acid (5 µM)	Cyclosporine A (0.78–50 µM)

BCRP breast cancer resistance protein, *BSEP* bile salt export pump; h, human, *HEK* human embryonic kidney epithelial 293 cells, *Ko143* tert-butyl 3-[(3S,6S,12aS)-9-methoxy-6-(2-methylpropyl)-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydropyrazino[1',2':1,6]pyrido[3,4-b]indol-3yl]propanoate, *LLC-PK1* Lilly Laboratories cell-porcine kidney epithelial cells, *L-MDR1* human MDR1-transfected LLC-PK1 cells, *MATE* multidrug and toxin extrusion protein, *MDCKII* Madin-Darby canine kidney cell line 2, *MDR1* multi-drug resistance protein 1, *OAT* organic anion transporter, *OATP* organic anion transporting polypeptide, *OCT* organic cation transporter, *P-gp* P-glycoprotein

Substrate Properties and Inhibitory Potential of Vericiguat Towards P-gp and BCRP

Studies were performed in a transwell filter system consisting of a small filter inside a well, dividing the well into an apical (above the filter) and a basolateral (under the filter) compartment. The studies were performed at the Genesis Workstation 200 and the Hamilton Microlab Star. Each experiment was performed in triplicate. Before the start of the transport studies, the culture medium was removed and the cells were washed with transport buffer (Hank's balanced salt solution [HBSS] supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], final: 5 mM) to remove residual amounts of medium. Cells were pre-incubated (37°C, 20 or 30 minutes) on both apical and basolateral surfaces with transport buffer. The experiment was initiated by placing the donor solutions on apical (100 µL) or basolateral sides (300 µL) and the receiver solutions on the opposite side. A 37.5 µL sample was taken from the donor compartment (at t = 0 minutes) to confirm the initial concentration of substrate. After 2 hours of incubation at 37°C, samples were taken from both compartments; 37.5 µL was taken from the donor compartment and 150 µL (basal compartment) or 75 µL (apical compartment) was taken from the receiver compartment. In addition, transport buffer was added to the samples to make a total volume of 150 µL. Prior to the analysis, 150 µL of the mixture of ammonium acetate buffer (pH 6.8) and acetonitrile (1:1, v/v) was added to each sample to obtain a total of 300 µL.

The recovery of the test compound in the test systems was established at the end of incubation. The amounts of compound in donor and receiver compartments were related to the amount of test compound in the donor compartment at t = 0 hours in each well. A recovery in the range of 80–120% was accepted. Determination of vericiguat, M-1, digoxin, dipyridamole, topotecan, and fluvastatin were conducted via separation by high-pressure LC-MS/MS detection.

OATPs/OCTs/OATs Transporter Uptake Assay

Growth medium was aspirated, and each well was rinsed twice with 0.5 mL HBSS buffer (supplemented with 20 mM HEPES, pH 7.4) and then pre-incubated in 0.5 mL incubation buffer (HBSS buffer, supplemented with 20 mM HEPES, pH 7.4) for 20 minutes at 37°C. The incubation buffer was removed and 200 µL incubation buffer containing the radiolabeled and non-radiolabeled substrates with or without inhibitors was added to each well and incubated at 37°C for designated time intervals. After incubation, the uptake was terminated by aspirating the reaction mixture and washing the cells three times with 0.4 mL ice cold phosphate-buffered saline. Cells were solubilized with 0.6 mL of 1 M NaOH overnight. The entire content of each well (0.6 mL) was transferred to a scintillation vial (Perkin Elmer) and the ^{3}H-respective ^{14}C-content were measured after addition of a 2.5 mL scintillation solvent (Roti®ecoplus; Carl Roth, Karlsruhe, Germany) in a liquid scintillation counter.

Substrate Properties Towards OATPs

Incubation buffer (HBSS supplemented with 20 mM HEPES, pH 7.4) contained [^{14}C]vericiguat or M-1 at a final concentration of 0.5 and 5 µM in the absence and presence of the probe substrates estrone-3-sulfate (10 µM, OATP1B1) or sulfobromophthalein (10 µM, OATP1B3) respective of the probe inhibitor rifampicin (50 µM). After 5 minutes the incubation was terminated. All experiments were conducted on at least two separate days. On each day, all experiments were performed in triplicate.

Substrate Properties Towards OCT1

The incubation buffer contained [^{14}C]vericiguat at a final concentration of 0.5 and 5 µM. After 1 and 5 minutes the incubation was terminated. In addition to the 1 minute incubation time, the inhibitor decynium-22 (50 µM) was added to both concentrations of [^{14}C]vericiguat. Inhibition of hOCT1-mediated 10 µM 1-methyl-4-phenylpyridinium iodide (MPP) uptake by 50 µM decynium-22 was performed as a control experiment. After 1 minute the incubation was terminated. All experiments were conducted on at least two separate days. On each day, all experiments were performed in triplicate.

Inhibitory Potency Towards OCTs

The incubation buffer contained [^{3}H]MPP at a final concentration of 10 µM. To characterize the inhibitory potential of vericiguat and M-1, they were added in two concentrations (5 and 50 µM [for OCT1 investigations] and 0.5 and 5 µM [for OCT2 investigations]). After 1 minute the incubation was terminated. Inhibition of hOCT1/2-mediated MPP uptake by 50 µM decynium-22 was performed as control experiment. All experiments were conducted on at least two separate days. On each day, all experiments were performed in triplicate.

Inhibitory Potency Towards OAT1

The incubation buffer contained [^{3}H]P-aminohippuric acid (PAH) at a final concentration of 10 µM. To characterize the inhibitory potential of vericiguat and M-1, they were added in two concentrations (0.5 and 5 µM). After 5 minutes the incubation was terminated. Inhibition of hOAT1-mediated PAH uptake by 100 µM probenecid was performed as a control experiment in parallel for each test item. All experiments were conducted on at least two separate days. On each day, all experiments were performed in triplicate.

Inhibitory Potency Towards OAT3

The incubation buffer contained [^{3}H]ES at a final concentration of 1 µM. To characterize the inhibitory potential of vericiguat and M-1 they were added in two concentrations (0.5 and 5 µM). After 1 minute the incubation was terminated. Inhibition of hOAT3-mediated estrone-3-sulfate uptake by 100 µM probenecid was performed as a control experiment in parallel for each test item. All experiments were conducted on two separate days. On each day, all experiments were performed in triplicate.

Inhibitory Potency Towards OATPs

Uptake studies were performed at 37°C. Each experiment was performed in triplicate. Before the start of the study, the cell culture medium was removed and replaced with transport buffer. Cells were pre-incubated for 20 minutes at 37°C with buffer followed by pre-incubation for 1 hour with vericiguat or M-1 in buffer. The buffer was removed, and the experiment was initiated by placing 250 µL of the test solution (pravastatin in buffer) onto the cells. After the appropriate incubation time, the media was removed, and the cells were washed three times with 0.9 mL ice cold buffer. The transport buffer was removed and 250 µL of a mixture of ammonium acetate buffer (pH 6.8) and acetonitrile (1:1, v/v) was added to each well and incubated for 20–35 minutes. The supernatant was collected and centrifuged at 16000 g for 3 minutes. 100 µL of the samples were analyzed by LC-MS/MS. Pravastatin was determined in the assay buffer after protein precipitation with the acetonitrile/buffer

containing an internal standard and subsequent filtration followed by separation employing high pressure LC-MS/MS methods.

Inhibitory Potency Towards MATE1/MATE2K

Human embryonic kidney epithelial 293 cells transfected with human *MATE1* and *MATE2K* genes were used to assess the inhibition potential of each test article toward the transporter. The cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 1 mM sodium pyruvate in a humidified incubator. The culture medium was changed three times weekly, and cell growth was observed by light microscopy. When the cells became 80–90% confluent, they were harvested by trypsinization, and the collected cells were seeded onto plates for the uptake studies. The plates were placed in a humidified incubator. Prior to the assay, each cell line was checked with a transporter-specific fluorescent marker compound to confirm the functionality of the transfected transporter.

Metformin, vericiguat, M-1, cimetidine (a known inhibitor of MATE1), and pyrimethamine (a known inhibitor of MATE2K) were prepared in HBSS buffer containing 10 mM HEPES and 15 mM D-glucose (HBSSg) for the inhibitor assessment. All incubations occurred in a humidified incubator. Prior to the assay, the culture medium of the vector control- and transporter-transfected cells was gently aspirated from each well, and the following pre-incubation was performed: the vector control- and the transporter-transfected cells were pre-incubated with HBSSg buffer for 20 minutes. After 20 minutes, the pre-incubation solutions were aspirated, and the inhibitor assessments were initiated. The overall inhibition assay consisted of the following steps: (a) incubation with 500 µL of transporter-specific substrate in the absence and presence of vericiguat and M-1 or known inhibitor for pre-determined periods, (b) at the end of the incubation period, the solution was gently aspirated; the cells were rinsed twice with ice cold HBSSg buffer (1000 µL each time), (c) the cells were lysed with internal standard-containing lysis solution, (d) the lysates were collected for analysis of the probe substrate concentration. The lysis solution was a mixture of acetonitrile and water (3:1, v/v) containing the corresponding internal standard. The concentrations of each test article and metformin were analyzed by LC-MS/MS.

Inhibitory Potency Towards BSEP

The study was performed using hepatocytes from a single human liver plated in a 96-well format. Pre-incubation solutions containing vericiguat, M-1, or a positive control (cyclosporine A [CsA]), were prepared in Plus (+) (buffer containing Ca^{2+}) and Minus (−) (buffer without Ca^{2+}) buffers. Co-incubation solutions containing taurocholic acid (d8-TCA; 5 µM) and vericiguat, M-1, or CsA were prepared in Plus (+) Buffer with 4% bovine serum albumin (BSA). The cell culture medium was removed from the wells and the cells were washed twice with Plus (+) Buffer or Minus (−) Buffer. After the second wash the pre-incubation solution containing test articles or positive control was added. The wells were then incubated for 10 minutes at 37°C. Following the incubation, pre-incubation solutions were removed, and the wells were rinsed once with warm Plus (+) Buffer and Minus (−) Buffer. The wells were aspirated and co-incubation solutions (containing test article or positive control with d8-TCA) were added and incubated for 10 minutes. Following the incubation, the co-incubation solutions were aspirated and discarded. The wells were then washed three times with ice cold Plus (+) Buffer. The plates were frozen at −80°C until

processed for bioanalysis. Treatments using vericiguat or M-1 were compared to the untreated control wells.

Inhibitory Potency Towards UGT Isoforms

Pooled human liver microsomes were purchased from XenoTech (Lenexa, KS, USA). Microsomal protein, 100 mM phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$ and alamethicin (50 µg/mg protein) were mixed and placed on ice for 15 minutes. Saccharolactone (5 mM), the respective probe substrate, and vericiguat (1–50 µM), M-1 (1.6–50 µM), or positive controls were added and pre-warmed at 37°C for 5 minutes. To initiate the reaction, uridine 5'-diphosphoglucuronic acid (4.6–5 mM) was added to gain a final volume of 200 µL or 500 µL. The reference substrates used were estradiol (20 µM; UGT1A1 substrate), 7-ethyl-10-hydroxy camptothecin (5 µM; UGT1A1 substrate), trifluoperazine (60 µM; UGT1A4 substrate), α-naphthol (50 µM; UGT1A6 substrate), propofol (100 µM; UGT1A9 substrate), 1'-hydroxymidazolam (1 µM; UGT2B4 substrate), 3'-azido-3'-deoxythymidine (200 µM; UGT2B7 substrate) or tarenflurbil (20 µM; UGT2B7 substrate).

Vericiguat was dissolved in acetonitrile and M-1 in dimethylsulfoxide. The respective substrates or standard inhibitors (atazanavir [UGT1A1 inhibitor], hecogenin [UGT1A4 inhibitor], niflumic acid [UGT1A9 inhibitor], carvedilol [UGT2B4 inhibitor], and mefenamic acid [UGT2B7 inhibitor]) were dissolved in various solvents resulting in a concentration range of 1.25–4.5% organic solvent in the final incubation mixture. Following incubation for an appropriate time period at 37°C, reactions were terminated by addition of formic acid or acetonitrile containing the respective internal standard. Precipitated proteins were removed by centrifugation and the supernatants were analyzed by LC-MS/MS.

Investigations Regarding CYP Induction Properties of Vericiguat and Metabolite M-1

Cryopreserved hepatocytes were rapidly thawed and purified, followed by plating between two layers of collagen. Hepatocytes were seeded at a density of 100,000 cells/cm^2 on collagen I-coated 96-well plate culture dishes. Incubations were performed in Williams Medium E (phenol red-free) supplemented with 10% (v/v) fetal bovine serum, prednisolone, insulin, glucagon and L-glutamine, penicillin, and streptomycin. Following seeding and attachment of the cells, culture medium was removed, and a collagen gel solution was distributed as a second matrix layer over the cells. At least 3 hours of incubation at 37°C in a culture incubator were allowed for gelation and attachment of the second gel layer. Culture medium was added and changed daily.

Methanolic stock solutions of omeprazole (concentration range in hepatocytes media: 0.023–50 µg/mL), rifampicin (0.014–30 µg/mL), phenobarbital (0.457–1000 µg/mL), pioglitazone (0.091–200 µg/mL), and bosentan (0.023–50 µg/mL) positive controls were prepared prior to use. Stock solutions of vericiguat and M-1 (1 mg/mL and 9 mg/mL, respectively) in 0.02 M hydrochloric acid/acetonitrile or water/acetonitrile were used in the study to prepare the hepatocyte media of vericiguat or M-1.

Hepatocyte cultures were maintained for 24 hours to recover before initiating treatment with vericiguat, or positive controls once daily for three consecutive days and cultured. Cultures were treated with supplemented Williams Medium E (each culture well was treated with 0.04 mL at approximately 37°C and 5.5% CO_2 in humidified air) containing ≤1% v/v acetonitrile (vehicle control), penicillin (negative control), one of eight concentrations of

vericiguat or M-1, omeprazole (a specific CYP1A2 inducer), rifampicin (strong human CYP enzyme inducer), phenobarbital and bosentan (moderate inducers), and pioglitazone (weak inducer) as positive controls. The culture multi-well plates were placed in a humidified culture incubator (37 ± 1°C and 5.5% CO_2 in humidified air).

Approximately 24 hours after the last treatment, hepatocytes were harvested for messenger RNA (mRNA) isolation. Cell culture medium was removed from each well, washed with 150 µL supplement-free cell culture medium prior to cell lysis, lysed with 100 µL lysis buffer containing proteinase K (50 µg/µL, resulting in a final well concentration of 30 ng/µL). Final cell lysates were stored at −80°C. Isolation of mRNA was done using the Dynabeads mRNA Direct Kit (Life Technologies). Then 150 µL of each cell lysate was mixed with 100 µg magnetic beads, incubated for a few minutes, followed by removal of supernatant. Beads were washed twice with Washing Buffer A and twice with Washing Buffer B with 200 µL and 100 µL per well, respectively. Single-stranded complementary deoxyribonucleic acid (cDNA) was prepared from mRNA with the High Capacity RNA to cDNA Kit (Life Technologies). Next, 20 µL of reverse transcription (RT) Master Mix was added to each well and transcribed for 60 minutes at 37°C using the Gene Amp polymerase chain reaction (PCR) System 9700 thermocycling program (Biometra). The RT Master Mix comprised 10X RT buffer, 25X deoxynucleoside triphosphates, 10X Random hexamers, RNase Inhibitor (20 U/µL), MultiScribe reverse transcriptase (50 U/µL) and RNase-free water. The prepared cDNA samples were stored at −80°C prior to analysis by quantitative RT-PCR.

Incubations of hepatocytes with probe substrates were conducted approximately 24 hours after the last treatment. Spent media was aspirated from the wells and each well was rinsed with pre-warmed Williams Medium E. Medium was aspirated from the wells and reactions were started by addition of 40 µL pre-warmed medium containing a cocktail of probe substrates, phenacetin and testosterone, to each well. The culture multi-well plates were placed in a humidified culture chamber (37°C and 5.5% CO_2 in humidified air) and incubations were carried out for 60 minutes. At 60 minutes the incubation mixture was removed and stored at −18°C until LC-MS/MS analysis. Following phenacetin/testosterone incubations, a same set of wells were rinsed with pre-warmed Williams Medium E and then incubated with pre-warmed medium containing probe substrate S-mephenytoin. Once again, the culture multi-well plates were placed in a humidified culture chamber (37°C and 5.5% CO_2 in humidified air) and incubations were carried out for 4 hours. At 4 hours, the incubation mixture was removed and stored at −18°C until LC-MS/MS analysis. Before analysis, the samples were mixed thoroughly and centrifuged (1500 g for 1 minute at room temperature). Aliquots of supernatant fractions (8 µL from both substrate incubations) were pooled with the respective internal standards, mixed thoroughly, stored for at least 10 minutes at −18°C, centrifuged (3000 g for 10 minutes), and analyzed by LC-MS/MS.

Online Table 2 Summary of bioanalytical determination of analytes in phase I studies

DDI Study	Analyte	Method	LLOQ, µg L^{-1}	ULOQ, µg L^{-1}	Accuracy, %	Precision, %
Mass balance study	Vericiguat	Plasma concentrations were measured using validated LC-MS/MS methods. Concentrations were determined after protein precipitation with acetonitrile containing the internal standards followed by separation employing high-pressure LC-MS/MS. [^{13}C $^{2}H_4$]vericiguat was used as internal standard	0.200	200	99.1–103.13	3.82–5.36
Omeprazole and magnesium/aluminum hydroxide	Vericiguat	Plasma concentrations were measured using validated LC-MS/MS methods. Concentrations were determined in plasma after protein precipitation with acetonitrile/ammonium acetate solution, pH 3.0, (6/1 v/v) including an internal standard ([^{13}C $^{2}H_4$]vericiguat) followed by separation employing high-pressure LC-MS/MS	0.200	200	101–105	1.27–2.26
Ketoconazole	Vericiguat	Plasma concentrations were measured using validated LC-MS/MS methods. Plasma concentrations were determined after protein precipitation with acetonitrile/ammonium acetate solution, pH 3.0, (6/1 v/v) containing an internal standard	0.200	200	94.4–104	1.87–3.83

		([^{13}C ^{2}H$_4$]vericiguat) followed by separation employing high-pressure LC-MS/MS				
Rifampicin	Vericiguat	Plasma concentrations were measured using validated LC-MS/MS methods. Concentrations were determined in plasma after protein precipitation with an acetonitrile buffer; separation was achieved by means of the liquid chromatographic system. [^{13}C ^{2}H$_4$]vericiguat was used as an internal standard	0.200	200	99.5–108	3.24–7.20
Mefenamic acid	Vericiguat	Plasma concentrations were measured using validated LC-MS/MS methods. Concentrations were determined by protein precipitation with acetonitrile. Separation was achieved by means of a liquid chromatographic system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in positive turboionspray ionization mode was applied. [^{13}C$_2$ ^{2}H$_4$]vericiguat was used as an internal standard	0.200	200	98.75–99.9	3.18–5.70
Midazolam	Midazolam	Plasma concentrations were measured using validated LC-MS/MS methods. Concentrations were determined after addition of the internal standard (midazolam-d$_4$ and 1'-hydroxy-midazolam-d$_4$) to a sample aliquot of 0.200 mL, followed by automated liquid-	0.100	100	96.3–99.0	2.08–3.94
	1'-hydroxy-midazolam		0.100	50	88.7–94.8	2.85–3.95

		liquid extraction. Separation was achieved by means of a liquid chromatographic system. For the mass spectrometric detection, a LC-MS/MS in positive turboionspray ionization mode was applied				
Warfarin	R-warfarin	Plasma concentrations were measured using validated LC-MS/MS methods. The internal standard (±) warfarin-d_5 was added to a sample aliquot of 0.100 mL followed by an automated protein precipitation procedure. Separation was achieved by means of a liquid chromatographic system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in negative turboionspray ionization mode was applied	10.0	2000	91.4–111	3.35–21.5
	S-warfarin		10.2	2040	91.5–111	3.06–21.2
	Vericiguat	Plasma concentrations were measured using validated UPLC-MS/MS methods. The internal standard ([^{13}C $^{2}H_4$]vericiguat) was added to a sample aliquot of 0.050 mL, followed by an automated protein precipitation. Separation was achieved by means of an UPLC system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in positive turboionspray ionization mode was applied	1.00	1000	97.7–105	1.13–3.07

Digoxin	Digoxin	Plasma concentrations were measured using validated LC-MS/MS methods. The internal standard oleandrin was added to a sample aliquot of 0.200 mL followed by solid phase extraction. Separation was achieved by means of a liquid chromatographic system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in positive turboionspray ionization mode was applied	0.0500	5.00	92.8–95.7	4.06–9.65
	Vericiguat	Plasma concentrations were measured using validated UPLC-MS/MS methods. The internal standard ([^{13}C $^{2}H_4$]vericiguat) was added to a sample aliquot of 0.050 mL, followed by an automated protein precipitation. Separation was achieved by means of an UPLC system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in positive turboionspray ionization mode was applied	1.00	1000	98.8–104	1.48–2.55
Aspirin	Vericiguat	Plasma concentrations were measured using validated HPLC-MS/MS methods. Concentrations were determined after separation by means of a liquid chromatographic system by using a triple quadrupole mass spectrometer in positive ionization	0.200	200	97.2–101.83	2.92–12.3

		mode. [^{13}C $^{2}H_4$]vericiguat was used as internal standard				
Sacubitril/valsartan	Sacubitril	Concentrations were determined after automated protein precipitation with a mixture of acetonitrile and methanol. [$^{13}C_4$]sacubitril and [$^{13}C_4$]LBQ657 were used as internal standards. Separation was achieved by means of a liquid chromatographic system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in positive turboionspray ionization mode was applied	1.00	2500	95.21–101.33	2.81–7.39
	LBQ567		100	10,000	95.73–103.33	3.74–8.91
	Valsartan	Concentrations were determined after automated protein precipitation with methanol. Valsartan-d_9 was used as an internal standard. Separation was achieved by means of a liquid chromatographic system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in positive turboionspray ionization mode was applied	5	5000	101.33–106.80	3.51–5.61
	Vericiguat	Concentrations were determined after automated protein precipitation with a mixture of acetonitrile and ammonium acetate with formic acid in Milli-Q type water. [^{13}C $^{2}H_4$]vericiguat was used as an internal standard.	0.200	200	98.00–110.17	2.54–3.09

		Separation was achieved by means of a liquid chromatographic system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in positive turboionspray ionization mode was applied				
Sildenafil	Sildenafil	Concentrations were determined after addition of the internal standard (sildenafil-d_8) and liquid-liquid extraction with methyl tert-butyl ether. Separation was achieved by means of a liquid chromatographic system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in positive turboIonspray ionization mode was applied	0.200	250	97.60–103.50	2.72–7.81
	Vericiguat	Plasma concentrations were measured using validated HPLC-MS/MS methods. Concentrations were determined after addition of the internal standard [$^{13}C\ ^{2}H_4$]vericiguat and automated protein precipitation with a mixture of acetonitrile and ammonium acetate with formic acid in Milli-Q type water. Separation was achieved by means of a liquid chromatographic system. For the mass spectrometric detection, a triple quadrupole mass spectrometer in positive	1.00	1000	93.88–101.33	1.24–2.58

turboionspray ionization mode was applied

DDI drug–drug interaction, *HPLC* high-performance liquid chromatography, *HPLC-MS/MS* high-performance liquid chromatography-tandem mass spectrometry, *LC-MS/MS* liquid chromatography tandem mass spectrometry, *LLOQ* lower limit of quantification, *ULOQ* upper limit of quantification, *UPLC-MS/MS* ultra-performance liquid chromatography-tandem mass spectrometry.

Incubation of Urine With Human Feces From Phase I Mass Balance Study

Pooled urine (12 mL, 2 mL each from participant) from a collection interval of 0–12 hours was diluted with water (12 mL) and then applied onto solid-phase extraction (Mega BE-C18, 2 g, 12 mL, Agilent Technologies, Waldbronn, Germany; pretreated with acetonitrile followed by water). The cartridge was washed successively with water (10 mL), acetonitrile (2 × 10 mL) and methanol (6 mL), resulting in 4 fractions. After radioactivity analysis, fractions 1 and 3 were discarded. Fractions 2 and 4 were pooled, treated with water (2 mL), and concentrated to about 2.5 mL to yield an almost aqueous solution of the pooled urine samples. This worked up urine sample (500 µL) was added under anerobic conditions to a suspension of fresh human feces (about 0.5 g) in degassed water resulting in a final volume of 5 mL). After incubation for 24 hours at 37°C the incubation mixture was terminated by addition of acetonitrile (2 mL). After centrifugation (5 minutes, 3000 rpm), the supernatant was concentrated and 100 µL were subjected to high performance liquid chromatography with off-line radioactivity detection.

Design of the Phase I DDI Studies

Omeprazole and Magnesium/Aluminum Hydroxide DDI Study

Volunteers were randomized to one of six treatment sequences. All volunteers were planned to receive treatment A (vericiguat 5 mg as a single dose), treatment B (pre-treatment with omeprazole 40 mg daily for 4 days and co-administration with vericiguat 5 mg on the 5th day), and treatment C (co-administration of a 10 mL suspension containing magnesium hydroxide 600 mg/aluminum oxide 900 mg with vericiguat 5 mg [both as a single dose]). The treatment sequences were A-B-C, A-C-B, B-A-C, B-C-A, C-A-B, or C-B-A. A washout period of at least 5 days after administration of vericiguat followed each period.

Ketoconazole DDI Study

The study was conducted in a single-center, randomized, open-label 2-fold crossover design. The study treatment was administered as follows:

Treatment A: a single oral dose of vericiguat 1.25 mg was administered in the morning.

Treatment B: ketoconazole administration (200 mg twice daily) for 2 consecutive days and coadministration with vericiguat 1.25 mg on the third day.

The washout period prior to subsequent study drug administration was at least 12 days.

Rifampicin DDI Study

All volunteers received the following treatments in a fixed sequence:

Treatment A (period 1): a single oral dose of vericiguat 10 mg.

Treatment B (period 2): rifampicin administration (600 mg daily) for 9 days. A single dose of vericiguat 10 mg was administered with rifampicin on the 7th day.

There was a washout phase of 6 ± 1 days between administration of vericiguat in period 1 and the first administration of rifampicin in period 2.

During period 1, plasma samples were obtained at prespecified times on the day of and during the 4 days after administration of vericiguat. During period 2, plasma samples were

taken at prespecified times during the 4th and 7th–9th days of rifampicin administration, and 2 days after the final administration of rifampicin.

Mefenamic Acid DDI Study

The study was conducted in a single-center, randomized, open-label, 2-fold crossover, non-placebo-controlled design.

All volunteers received the following treatments in a randomized order:

Treatment A: a single oral dose of vericiguat 2.5 mg.

Treatment B: a single oral dose of mefenamic acid 500 mg was given on the morning of the first day, followed by multiple doses of mefenamic acid 250 mg every 6 hours starting from the afternoon of the first day for 3 days. Vericiguat was administered with mefenamic acid on the 2nd day.

There was a washout phase of 14 days between treatments A and B.

Midazolam DDI Study

The study was conducted in a single-center, randomized, non-blinded, crossover design. Thirty-two healthy male volunteers were randomized to one of two treatment sequences (A-B or B-A). The test drugs were administered as follows:

Treatment A: at day 4: a single oral dose of midazolam 7.5 mg in the fed state.

Treatment B: on days 1 to 3: a daily single oral dose of vericiguat 10 mg at 00h in the fed state. At day 4: a single oral dose of midazolam 7.5 mg together with a single oral dose of vericiguat 10 mg in the fed state.

Each volunteer received the abovementioned treatments. Washout phase between treatments A and B was at least 10 days.

Warfarin DDI Study

The study was conducted in a single-center, randomized, double-blind, placebo-controlled, 2-fold crossover design. Vericiguat 10 mg was administered once daily over 9 days.

The effect of multiple doses of vericiguat on the PK of R- and S-warfarin (warfarin 25 mg), administered on the 6th day of vericiguat treatment, was investigated. To investigate a possible effect of warfarin on vericiguat, a PK profile of vericiguat was assessed on day 5 (without warfarin) and on day 6 (with warfarin).

Digoxin DDI Study

All volunteers were planned to receive three treatments: treatment A (vericiguat 10 mg as a single dose), treatment B (digoxin 0.375 mg once daily for 14 days), or treatment C (co-administration of digoxin 0.375 mg with vericiguat 10 mg; vericiguat was given as a single dose followed by consecutive treatment once daily for 8 days). Healthy volunteers were randomized to one of the following treatment sequences: A-B-C or A-C-B, without a washout period between treatments.

Aspirin DDI Study

The aspirin DDI study consisted of a pilot part and a main part. The pilot part of the study had a non-randomized, non-blinded, and non-controlled design. Volunteers were treated

with a single dose of vericiguat 15 mg. The main part of the study was conducted in a randomized, non-blinded, non-controlled, 3-period, 3-fold, crossover design. Volunteers were planned to receive three treatments: treatment A (vericiguat 15 mg as a single dose), treatment B (aspirin 500 mg for 2 days), and treatment C (pre-administration of 500 mg aspirin for a day, followed by co-administration of 500 mg aspirin with vericiguat 15 mg). Volunteers were randomized to one of the following treatment sequences: A-C-B, B-A-C, or C-B-A. Each treatment was separated by a washout period of at least 14 days.

Sacubitril/Valsartan DDI Study

The study was conducted in a single-center, randomized, single-blinded, placebo-controlled design in two parallel treatment groups of 16 volunteers.

The volunteers received vericiguat 2.5 mg (or placebo) alone and a combination of vericiguat 2.5 mg (or placebo) with a fixed dose combination of sacubitril/valsartan 97/103 mg twice daily for 14 days at steady state after a run-in phase of sacubitril/valsartan alone (starting dose of sacubitril and valsartan 49/51 mg twice daily for 14 days and run-in up-titration to the target dose of sacubitril and valsartan 97/103 mg twice daily for 13 days).

Sildenafil DDI Study

The study was randomized, placebo-controlled, and single-blinded, and was performed at one site. Investigator, site staff and healthy volunteers were blinded with respect to treatment randomization. Bioanalytical staff were not blinded.

Healthy volunteers were to be treated for 16 days (day 0 to 15) and were to be randomized to either treatment A (one vericiguat 10 mg tablet per day) or treatment B (one placebo tablet per day). Healthy volunteers on both treatments were to receive sildenafil 25 mg on day 13, sildenafil 50 mg on day 14, and sildenafil 100 mg on day 15.

Online Table 3 Formation of metabolite M-1 following UGT inhibition

Inhibitor	Inhibitor concentration (µM)	Metabolite M-1 formation (percent of control)		
		Human liver microsomes[a]	Human kidney microsomes[b]	Human intestinal microsomes[c]
Atazanavir (UGT1A1)	5.0	75.0	94.8	73.1
Hecogenin (UGT1A4)	10.0	96.2	101.7	96.6
Niflumic acid (UGT1A9)	5.0	49.8	15.1	90.3
Mefenamic acid (UGT2B7, 1A9)	10.0	85.2	73.0	86.9

Normalized enzymatic activity on incubations with 1 µM vericiguat (SD) were:

[a]28300 (6600) [M-1] peak area/mg protein × min,

[b]70000 (18200) [M-1] peak area/mg protein × min,

[c]23700 (7750) [M-1] peak area/mg protein × min.

SD standard deviation, *UGT* uridine diphosphate-glucuronosyltransferase.

Online Table 4 Contribution of UGT1A1 and 1A9 to vericiguat glucuronidation

Human UGT isoform	Glucuronide of selective substrate	Correlation of coefficient (R^2) Metabolite M-1 formation
UGT1A1	17β-estradiol 3-glucuronide	0.610
UGT1A9	Propofol glucuronide	0.728

UGT uridine diphosphate-glucuronosyltransferase.

Online Table 5 Effects of vericiguat on the PK of other drugs: geometric mean, coefficient of variation, and range

DDI study	Medication	PK parameter	Geometric mean (range)	CV, %
Midazolam	Midazolam	AUC, μg*h L^{-1}	117 (61.4–219)	34.84
		C_{max}, μg L^{-1}	27.2 (14.7–75.8)	41.93
	Midazolam with vericiguat	AUC, μg*h L^{-1}	96.4 (38.4–175)	34.36
		C_{max}, μg L^{-1}	20.9 (11.9–38.3)	31.26
Warfarin	R-warfarin (warfarin with placebo)	AUC, μg*h L^{-1}	73,000 (46,100–94,800)	20.06
		C_{max}, μg L^{-1}	1130 (875–1520)	13.06
	R-Warfarin (warfarin with vericiguat)	AUC, μg*h L^{-1}	71,900 (45,700–101,000)	21.30
		C_{max}, μg L^{-1}	1130 (939–1590)	13.12
	S-warfarin (warfarin with placebo)	AUC, μg*h L^{-1}	53,600 (33,800–103,000)	29.72
		C_{max}, μg L^{-1}	1110 (891–1530)	13.02
	S-warfarin (warfarin with vericiguat)	AUC, μg*h L^{-1}	52,500 (28,500–117,000)	32.71
		C_{max}, μg L^{-1}	1090 (886–1490)	12.66
Digoxin	Digoxin	$AUC_{\tau,md}$, μg*h L^{-1}	18.6 (11.5–30.3)	21.36
		$C_{trough,}$ μg L^{-1}	0.683 (0.351–1.200)	26.76
	Digoxin with vericiguat	$AUC_{\tau,md}$	19.6 (12.9–27.0)	20.01
		$C_{trough,}$ μg L^{-1}	0.687 (0.351–0.992)	24.47
Sacubitril/valsartan	Sacubitril	$AUC_{0–12,md}$	2110 (1140–3400)	29.73
		$C_{max,md}$	1830 (711–4760)	59.62
	Sacubitril (sacubitril/valsartan with vericiguat)	$AUC_{0–12,md}$	2520 (1340–4740)	43.85
		$C_{max,md}$	2270 (537–6030)	68.71
	LBQ657	$AUC_{0–12,md}$	84,100 (63,200–111,000)	18.75
		$C_{max,md}$	11300 (9280–13,500)	13.29
	LBQ657 (sacubitril/valsartan with vericiguat)	$AUC_{0–12,md}$	73,800 (40,300 –104,000)	24.81
		$C_{max,md}$	10,500 (6790–15,000)	19.61
	Valsartan	$AUC_{0–12,md}$	23,000 (17,800–36,400)	20.25

		$C_{max,md}$	4760 (3020–8000)	27.16
	Valsartan (sacubitril/valsartan with vericiguat)	$AUC_{0–12,md}$	23,900 (12,100–39,100)	41.05
		$C_{max,md}$	4640 (2360–6960)	31.66
Sildenafil	Sildenafil 100 mg with placebo	$AUC_{0–22}$, µg*h L^{-1}	1230 (364–2170)	43.77
		C_{max}, µg L^{-1}	340 (110–600)	40.24
	Sildenafil 100 mg with vericiguat	$AUC_{0–22}$, µg*h L^{-1}	1390 (566–2530)	42.48
		C_{max}, µg L^{-1}	398 (174–725)	46.07

AUC area under the concentration time curve, $AUC_{0–12,md}$ AUC from time 0–12 hours after multiple dose administration, $AUC_{0–22}$ AUC from time 0–22 hours, $AUC_{\tau,md}$ AUC within the dosing interval after multiple dosing, C_{max} maximum concentration within a dosing interval, $C_{max,md}$ C_{max} within a dosing interval after multiple dose administration, *CV* coefficient of variation, *DDI* drug–drug interaction.

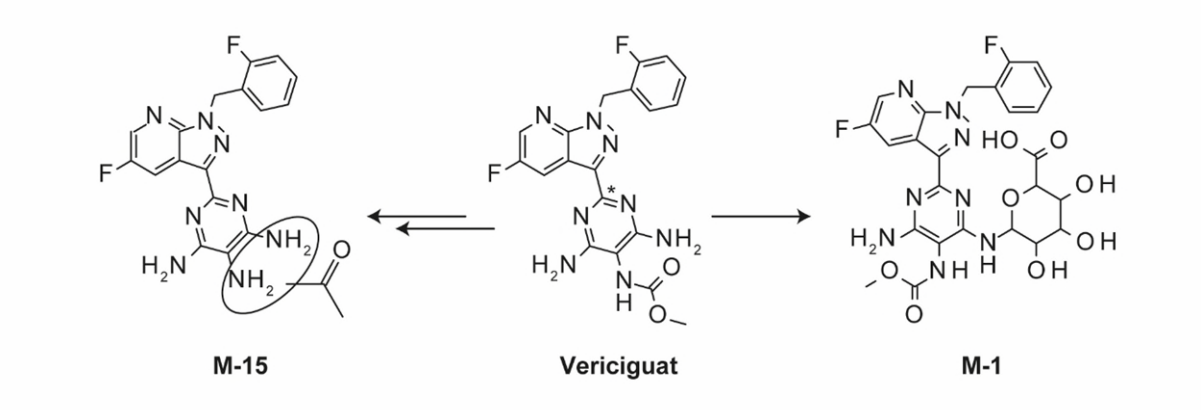
Online Table 6 Effects of co-medications on the PK of vericiguat: geometric mean, coefficient of variation, and range

DDI study	Medication	PK parameter	Geometric mean (range)	CV, %
Omeprazole and magnesium/ aluminum hydroxide	Vericiguat	AUC, µg*h L^{-1}	2490 (1840–3460)	23.24
		C_{max}, µg L^{-1}	142 (54.5–242)	45.75
	Vericiguat with omeprazole	AUC, µg*h L^{-1}	1690 (1150–2230)	21.53
		C_{max}, µg L^{-1}	72.7 (41.8–91.9)	22.73
	Vericiguat with magnesium/aluminum hydroxide	AUC, µg*h L^{-1}	1820 (1220–2440)	20.24
		C_{max}, µg L^{-1}	78.3 (50.8–95.0)	17.31
Ketoconazole	Vericiguat	AUC, µg*h L^{-1}	774 (532–1140)	21.57
		C_{max}, µg L^{-1}	37.2 (26.7–52.1)	18.45
	Vericiguat with ketoconazole	AUC, µg*h L^{-1}	868 (626–1210)	19.69
		C_{max}, µg L^{-1}	41.2 (32.2–68.1)	17.66
Rifampicin	Vericiguat	AUC, µg*h L^{-1}	4930 (2390–7490)	26.68
		C_{max}, µg L^{-1}	245 (127–372)	27.82
	Vericiguat with rifampicin	AUC, µg*h L^{-1}	3520 (2520–5330)	23.07
		C_{max}, µg L^{-1}	224 (143–445)	25.21
Mefenamic acid	Vericiguat	AUC, µg*h L^{-1}	1580 (1130–2250)	18.66
		C_{max}, µg L^{-1}	75.7 (57.5–92.8)	16.95
	Vericiguat with mefenamic acid	AUC, µg*h L^{-1}	1900 (1420–2670)	18.55
		C_{max}, µg L^{-1}	73.6 (64.3–91.2)	11.20
Warfarin	Vericiguat	$AUC_{\tau,md(0-24)}$, µg*h L^{-1}	5180 (3450–9660)	25.54
		$C_{max,md}$, µg L^{-1}	348.3 (260–642)	19.78
	Vericiguat with warfarin	$AUC_{\tau,md}$	5330 (3700–9830)	25.47
		$C_{max,md}$	360.2 (272–618)	21.12
Digoxin	Vericiguat	AUC, µg*h L^{-1}	6120 (3810–12,900)	24.52
		C_{max}, µg L^{-1}	269 (198–374)	20.82

	Vericiguat with digoxin	AUC, μg*h L^{-1}	5990 (3510–9120)	24.80
		C_{max}, μg L^{-1}	268 (180–368)	19.44
Aspirin	Vericiguat	AUC, μg*h L^{-1}	8120 (4100–12,900)	33.74
		C_{max}, μg L^{-1}	402 (142–710)	53.78
	Vericiguat with aspirin	AUC, μg*h L^{-1}	8130 (4420–13,300)	33.62
		C_{max}, μg L^{-1}	414 (161–700)	42.80
Sacubitril/ valsartan	Vericiguat	$AUC_{0–24}$, μg*h L^{-1}	975 (761–1260)	14.79
		C_{max}, μg L^{-1}	82.8 (63.1–112)	18.84
	Vericiguat with sacubitril/valsartan	$AUC_{0–24}$, μg*h L^{-1}	904 (717–1140)	12.29
		C_{max}, μg L^{-1}	75.2 (57.6–99.9)	14.52
Sildenafil 100 mg	Vericiguat	$AUC_{0–24,md}$, μg*h L^{-1}	6430 (3350–10,400)	26.98
		$C_{max,md}$, μg L^{-1}	485 (268–701)	25.22
	Vericiguat with sildenafil 100 mg	$AUC_{0–24,md}$, μg*h L^{-1}	6770 (4640–10,200)	21.79
		$C_{max,md}$, μg L^{-1}	499 (342–698)	19.64

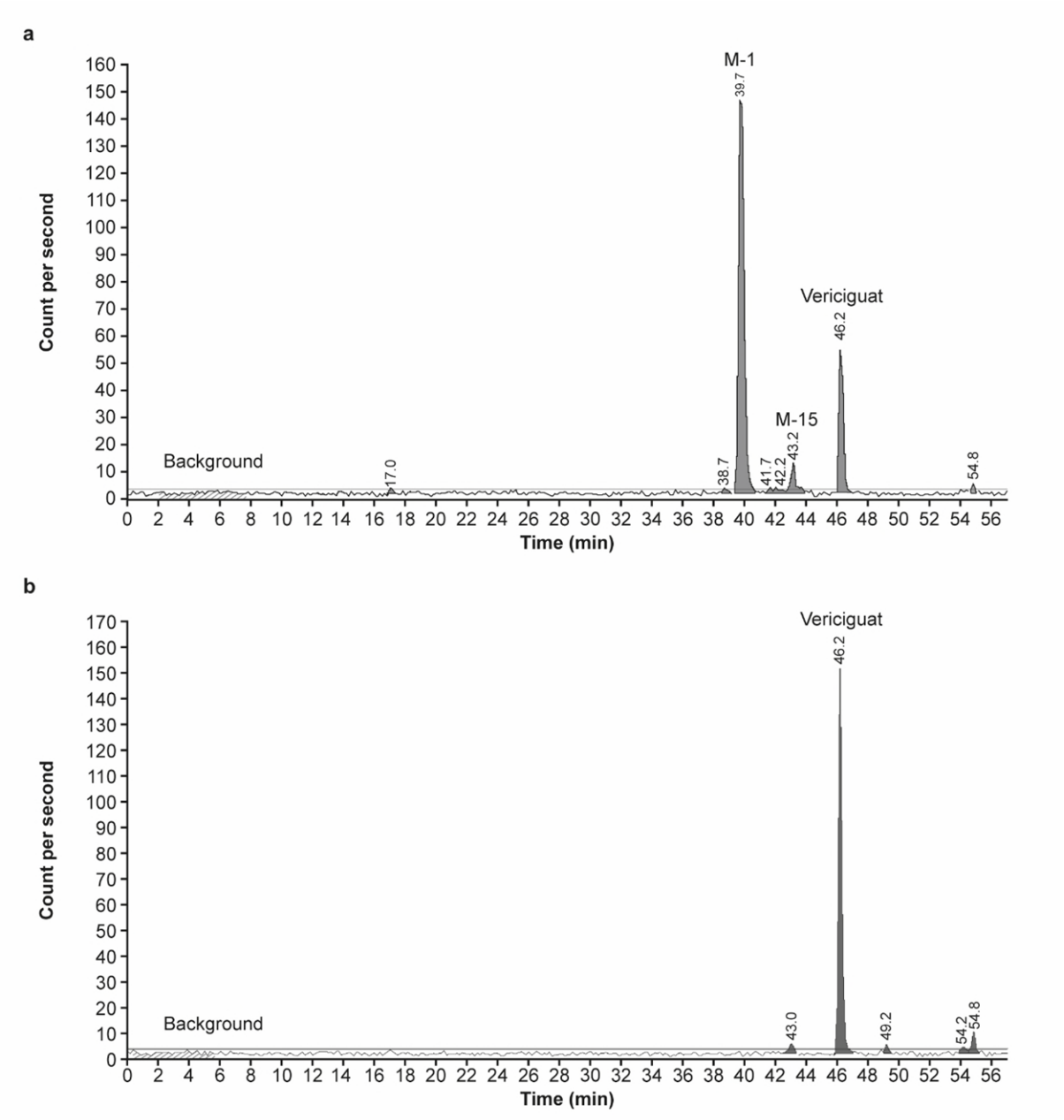
AUC area under the concentration time curve, $AUC_{(0–24)}$ AUC from time 0 to 24 hours, $AUC_{(0–24),md}$ $AUC_{0–24}$ after multiple dose administration, $AUC_{\tau,md}$ AUC within the dosing interval after multiple dosing, $AUC_{\tau,md(0-24)}$ $AUC_{\tau,md}$ for τ from 0 to 24 hours after administration of Vericiguat, C_{max} maximum concentration within a dosing interval, $C_{max,md}$ C_{max} within a dosing interval after multiple dose administration, *CV* coefficient of variation, *DDI* drug–drug interaction.

Online Fig. 1 Biotransformation of vericiguat.



*Position of ^{14}C label.

Online Fig. 2 Detection of vericiguat and its metabolites by HPLC in a) urine after workup and b) after incubation of purified urine with human feces.



HPLC high performance liquid chromatography.