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Short communication

In vitro biosynthesis, isolation, and identification of predominant metabolites of 2-(4-(2-hydroxyethoxy)-3,5-dimethylphenyl)-5,7-dimethoxyquinazolin-4(3*H*)-one (RVX-208)

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ABSTRACT

The structures of the two predominant metabolites (**M4** and **M5**) of RVX-208, observed both in *in vitro* human and animal liver microsomal incubations, as well as in plasma from animal *in vivo* studies, were determined. A panel of biocatalytic systems was tested to identify biocatalysts suitable for milligram scale production of metabolite **M4** from RVX-208. Rabbit liver S9 fraction was selected as the most suitable system, primarily based on pragmatic metrics such as catalyst cost and estimated yield of **M4** (~55%). Glucuronidation of RVX-208 catalyzed by rabbit liver S9 fraction was optimized to produce **M4** in amounts sufficient for structural characterization. Structural studies using LC/MS/MS analysis and ^{1}H NMR spectroscopy showed the formation of a glycosidic bond between the primary hydroxyl group of RVX-208 and glucuronic acid. NMR results suggested that the glycosidic bond has the β-anomeric configuration. A synthetic sample of **M4** confirmed the proposed structure. Metabolite **M5**, hypothesized to be the carboxylate of RVX-208, was prepared using human liver microsomes, purified by HPLC, and characterized by LC/MS/MS and ^{1}H NMR. The structure was confirmed by comparison to a synthetic sample. Both samples confirmed **M5** as a product of oxidation of primary hydroxyl group of RVX-208 to carboxylic acid.

1. Introduction

2-(4-(2-Hydroxyethoxy)-3,5-dimethylphenyl)-5,7-dimethoxyquinazolin-4(3*H*)-one (RVX-208) (Fig. 1) is a novel BET bromodomain inhibitor. It is an orally active small molecule drug candidate for the treatment of cardiovascular diseases. It is proposed to work by increasing the levels of apolipoprotein A1 (Apo-A1), resulting in an increase in high-density lipoprotein cholesterol (HDL-c), thereby potentially reducing the risk for cardiovascular disease [1,2]. RVX-208 is currently in phase 2 clinical trials for the treatment of atherosclerosis. Identification of the main metabolites is an essential component of understanding the elimination of the parent compound from the body, as well as understanding the safety profile for the drug candidate.

In preliminary *in vitro* metabolite profiling and identification studies using liver fractions from various mammalian species, it was determined that the metabolic pathways of RVX-208 involved the formation of two significant metabolites, a glucuronidated metabolite **M4** and an oxidized metabolite **M5**. Metabolites **M4** and **M5** were also identified as predominant metabolites in follow-up *in vivo* animal studies (unpublished results). Therefore, it was important to establish the exact structures of these metabolites, which required synthesis of authentic **M4** and **M5** suitable for NMR structural characterization. In the absence of prior knowledge of metabolite structures, one of the most effective strategies for the production of metabolites is the use of the actual liver metabolic enzyme systems, typically found in hepatocytes, microsomes, or S9 fractions [3–5]. We applied this biosynthetic strategy to synthesize and characterize the two predominant metabolites (**M4** and **M5**) of RVX-208 to support the ongoing development program.

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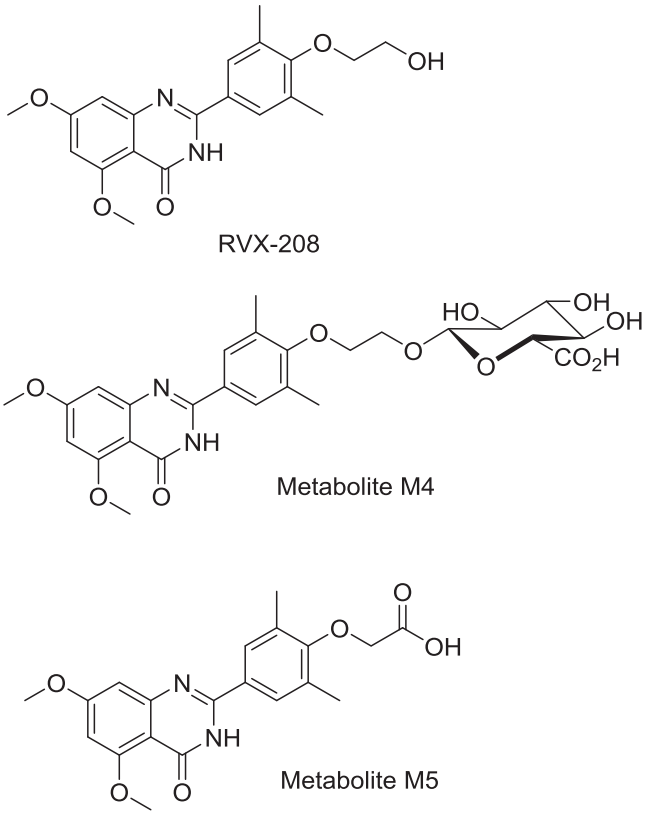


Fig. 1. Structures of RVX-208 and its metabolites **M4** and **M5**.

2. Results and discussion

2.1. Synthesis and structural characterization of glucuronidated metabolite M4

*2.1.1. Screening of biocatalytic systems for production of metabolite **M4***

Initially, a broad spectrum of mammalian liver microsomes (human, mouse, rat, dog, rabbit, monkey, minipig, calf), S9 fractions (from human, mouse, rat, dog, rabbit, monkey, minipig), and twelve human recombinant glucuronyl transferases were screened to identify a system that would be practical for the biosynthesis of **M4**. The initial selection criteria was the estimated yield after 24 h of incubation. Estimated yields of **M4** in reactions catalyzed by mammalian liver microsomes and S9 fractions are shown in Tables 1 and 2, respectively. Metabolite yields were estimated from integrated areas of corresponding peaks in HPLC chromatograms with UV detection at 230 nm. In reactions with UGT enzymes, metabolite **M4** was formed only in trace amounts (estimated yield <1%). Based on results of the screen, microsomes and S9 fraction from rabbit liver and microsomes from calf liver all had an estimated yield above 30% and were selected for further optimization.

Table 1
Estimated yields (%) of metabolite **M4** in incubations of RVX-208 with liver microsomes.[a]

Source of microsomes	Reaction time (hours)		
	1	**6**	**24**
Male Beagle Dog	0.9	6.4	15.5
Male Cynomolgus Monkey	0.2	1.1	2.9
male Sprague-dawley rat	0.1	0.5	0.7
Male ICR/CD-1 Mouse	0.1	0.3	0.5
Male New Zealand White Rabbit	12.2	22.4	30.3
Pooled Human Mixed Sexes (10 individuals)	0.1	0.6	1.2
Calf	3.7	27.7	35.9
Male Yucatan Minipig	2.5	11.8	14.3

[a] Estimated from the HPLC peak area.

Table 2
Estimated yields (%) of metabolite **M4** in incubations of RVX-208 with S9 Fractions.[a]

Source of S9 Fraction	Reaction time (hours)		
	1	**6**	**24**
Male beagle dog	0.9	1.9	7.3
Male cynomolgus monkey	0.4	1.1	2.2
Male Sprague–Dawley rat	0.3	1.1	2.1
Male ICR/CD-1 mouse	0.4	0.8	0.6
Male New Zealand white rabbit	4.1	16.0	30.9
Pooled human mixed sexes (10 individuals)	0	0.4	0.7
Male Yucatan minipig	1.7	6.5	14.2

[a] Estimated from the HPLC peak area.

*2.1.2. Synthesis, purification and structural characterization of metabolite **M4***

The yield in the glucuronide metabolite synthesis primarily depends on the concentrations of the parent compound and UDPGA (the cofactor for glucuronidation), and the amount of a catalyst added. In the optimization screen, these parameters were varied over a broad range of values at a fixed reaction time of 24 h. The results are shown in Table 3.

In the majority of reaction conditions tested in the optimization screen, the estimated yield of **M4** exceeded 20%, and in several reaction systems it was greater than 50%. Overall, calf microsomes were better catalysts than rabbit microsomes and S9 fraction. However, because rabbit liver S9 fraction offers the most cost effective option compared to other catalysts in Table 3, they were selected as a practical catalyst for synthesis of **M4**. Based on the results of the optimization study, the following reaction conditions were selected for scale-up synthesis of metabolite **M4**: 1 mM RVX-208, 2 mg/mL S9 protein (rabbit), and 10 mM UDPGA.

After 24 h of incubation LC/MS analysis of the reaction mixture showed a 22% yield of **M4**. Following work-up of the reaction and purification by preparative HPLC, a total of 2.6 mg of **M4** was obtained as a white solid with >99% purity as determined by HPLC (see Supporting information).

Table 3
Optimization of reaction conditions for synthesis of metabolite **M4**.

Catalyst	[UDPGA] mM	[Total protein] mg/mL	[RVX-208] mM	Estimated yield, %
Rabbit microsomes	5	4	2	30.0
			0.5	24.1
		1	2	38.4
			0.5	39.5
			0.2	34.6
	25	4	2	48.9
			0.5	54.3
		1	2	39.7
			0.5	49.4
			0.2	49.7
Rabbit S9 fraction	5	4	2	30.3
			0.5	33.0
		1	2	17.5
			0.5	23.2
			0.2	22.3
	25	4	2	35.2
			0.5	41.3
		1	2	16.7
			0.5	25.7
			0.2	21.4
Calf microsomes	5	4	2	50.1
		1	2	40.9
			0.5	56.0

Structural characterization of metabolite **M4** was conducted using LC/MS/MS analysis and ^{1}H NMR spectroscopy. Results of LC/MS/MS analysis suggested that glucuronidation occurred at the hydroxyl group of RVX-208 (Fig. 2). ^{1}H NMR data analysis confirmed the formation of a glycosidic bond between the hydroxyl group of RVX-208 and glucuronic acid (See Supporting information). Analysis of the ^{1}H NMR spectrum in narrow spectral ranges (Fig. 3) allowed determination of the anomeric configuration of the glycosidic bond. Based on the coupling constant for the anomeric proton, which is approximately 8 Hz (panel B in Fig. 3), the glycosidic bond was determined to be in the β-configuration [6]. The proposed structure of metabolite **M4**, derived from LC/MS/MS and NMR data, is shown in Fig. 1.

To verify the structure of metabolite **M4**, it was synthesized chemically from RVX-208 using the synthetic route shown in Scheme 1 (see Experimental section for details). For the synthesis, the initial protection of the amide nitrogen was required to avoid formation of the *N*-glucuronide. Having the 2-*O*-*acetate* glycosyl donor supported the formation of the β-anomer. Both chemically and biosynthetically prepared samples of **M4** exhibit identical LC/MS/MS and ^{1}H NMR data, confirming the structure shown in Fig. 1.

2.2. Synthesis and structural characterization of oxidized metabolite *M5*

For structural identification of metabolite **M5**, conditions of the initial screening protocol used for microsomal incubations were found to be adequate to generate sufficient quantities of **M5**. Using human liver microsomes an analytical amount of **M5** was produced from RVX-208 (See Experimental section for details). After purification by HPLC, the material was subjected to LC/MS/MS characterization. Results of this analysis (Fig. 4) suggested that **M5** is the result of oxidation of the primary alcohol in RVX-208 to a carboxylic acid. To verify the structure of metabolite **M5**, it was synthesized chemically using the synthetic route shown in Scheme 2 (See Experimental for details). The LC/MS/MS and ^{1}H NMR properties of synthetic **M5** are identical to those of the metabolite **M5** prepared using human liver microsomes, thus confirming the structural identity of **M5**.

3. Conclusion

In conclusion, we successfully applied biosynthetic strategy to synthesize the predominant metabolites (**M4** and **M5**) of the clinical drug candidate RVX-208 on a preparative scale. A wide variety of biocatalytic systems were tested for synthetic suitability, the most effective catalysts were identified, and conditions were optimized to achieve practical yields of the target metabolites. The structures of the metabolites were proposed from LC/MS/MS and ^{1}H NMR spectroscopy data, and they were verified by direct chemical synthesis.

4. Experimental procedures

4.1. General procedures and materials

Calf liver microsomes were obtained from CellzDirect (Pittsboro, NC). All other mammalian liver microsomes and mammalian S9 fractions were obtained from In Vitro Technologies, Inc. (Baltimore, MD). Metabolic competency of liver microsomes and S9 fractions was confirmed in a separate control experiment using a standard substrate, testosterone. Components of the NADPH regeneration system and human recombinant UDP-glucuronyl transferases were obtained from BD Gentest (Woburn, MA). RVX-208 was supplied by Resverlogix Corp. (Calgary, Canada). Solvents and reagents for chemical syntheses were used as purchased from commercial suppliers, unless otherwise noted. Synthetic chemical reactions were monitored by thin-layer chromatography (TLC) carried out on

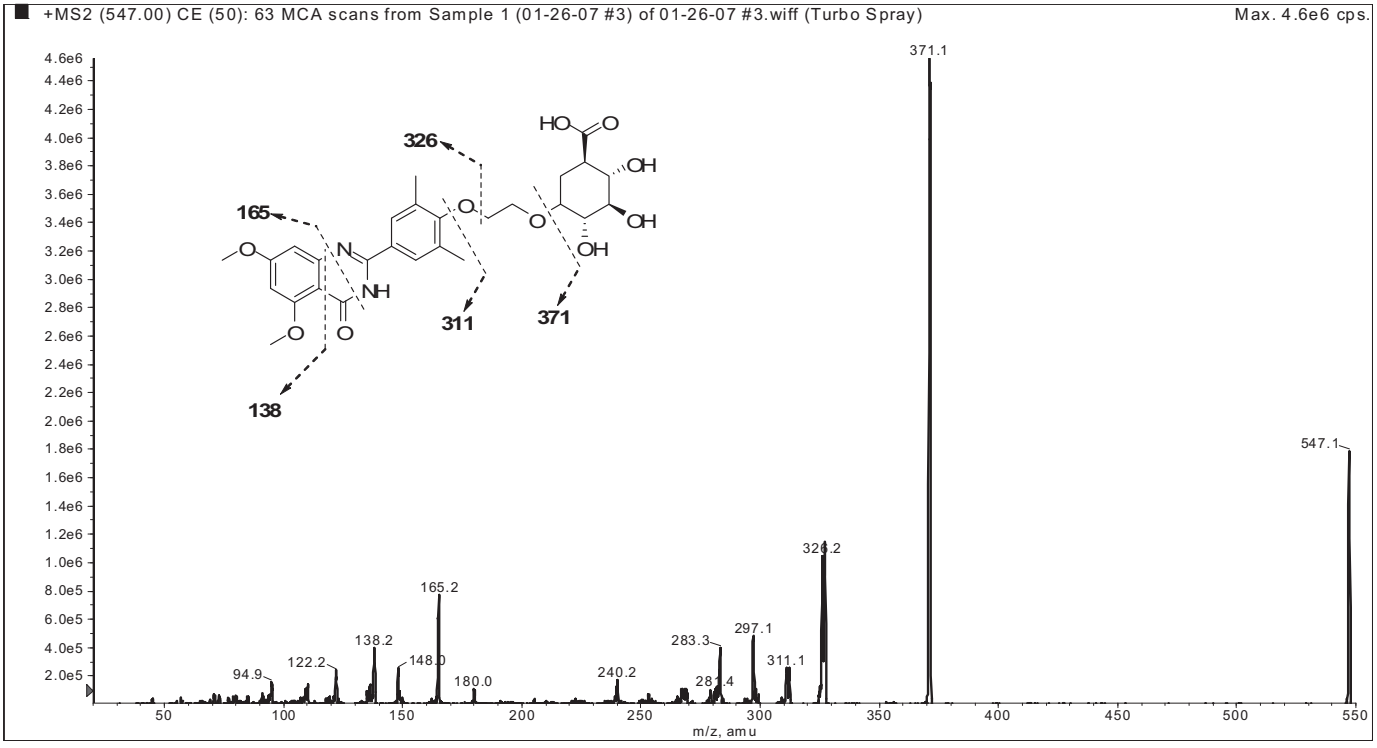


Fig. 2. MS/MS spectrum and fragmentation analysis for metabolite **M4**.

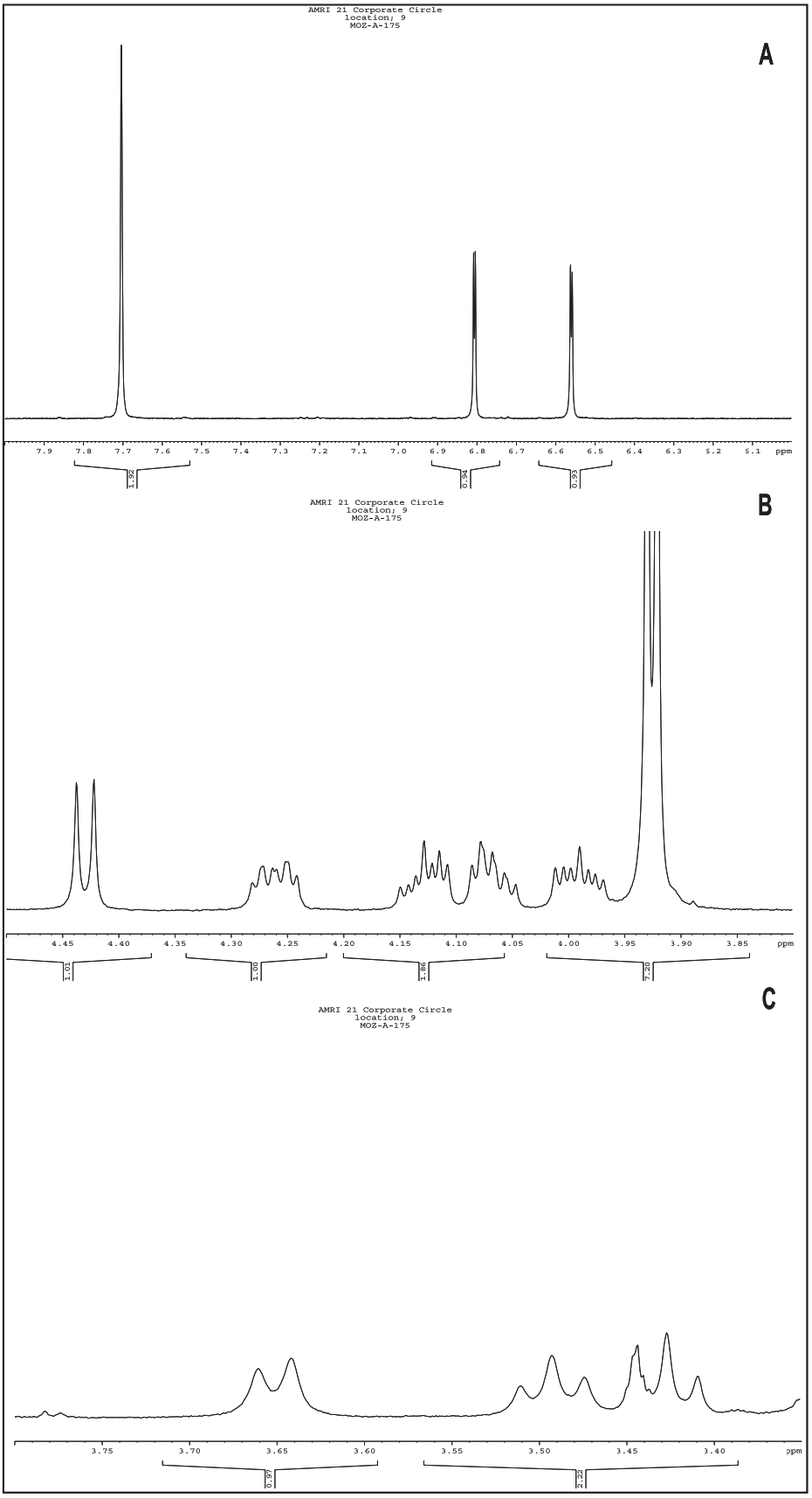
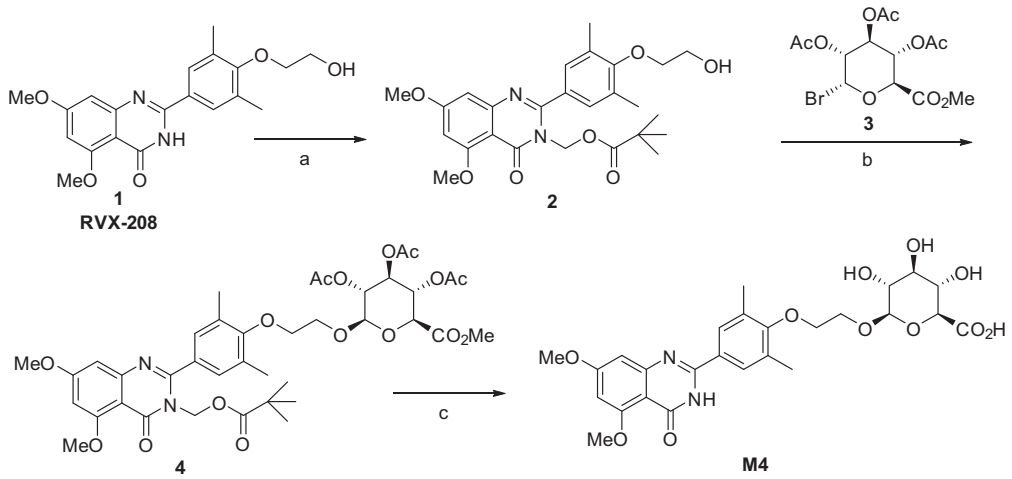


Fig. 3. [1]H NMR spectra of purified metabolite **M4**, showing narrow spectral ranges. Panels: **A**, spectrum in the range from 6.0 to 8.0 ppm; **B**, spectrum in the range from 3.8 to 4.5 ppm; **C**, spectrum in the range from 3.35 to 3.8 ppm.



Scheme 1. Chemical synthesis of metabolite **M4** (see Materials and methods for details). a: NaH, DMF, chloromethylpivalate; b: methylene chloride, AgO; c: NaOMe, MeOH.

Macherey–Nagel (MN) Alugram SIL G/UV_{254} silica gel 60 plates with fluorescent indicator UV_{254} (catalog #818 133) or Sigma–Aldrich silica gel plates (catalog #Z193291-1PAK) using phosphomolybdic acid in absolute ethanol as a developing agent. NMR spectra were recorded on a 400 MHz Varian Mercury spectrometer or a 500 MHz Bruker Avance System, using residual undeuterated solvent as an internal reference. The low resolution mass spectra (MS) were recorded on a Water Micromass ZQ using electrospray ionization-ion trap (ESI) unless otherwise stated. MS/MS analysis was carried out on an API 3200 system (Applied Biosystems). Q1 was set up to select a precursor ion at desired *m/z*, pass the ion through a hexapole collision cell, and the Q3 analyzer then scanned the product ions in the mass range from 0 to 600 amu. Nitrogen was used as the collision gas, and the collision energy was scanned to 100 V.

4.1.1. Reactions with mammalian liver microsomes and S9 fractions

A 10 mM stock solution of RVX-208 [7] in DMSO was prepared and used in these experiments. The incubation mixtures, prepared in duplicate, consisted of 0.50 mg microsomal protein, 100 mM potassium phosphate buffer, pH 7.4, 100 μM test compound, and the NADPH regenerating system (1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride), UDPGA (5 mM), and D-saccharic acid lactone (5 mM) in a final volume of 0.5 mL. The reaction mixture minus the microsomal protein or S9 fraction was pre-warmed for 5 min at 37 °C. In experiments with S9 fractions, 0.1 mL of the S9 fraction suspension was added directly to the reaction mixture. In experiments with microsomes, prior to addition to the reaction mixture, ice-cold microsomes were pre-incubated for 20 min with alamethicin (4 μL of alamethicin stock solution in DMSO, 1.5 mg/

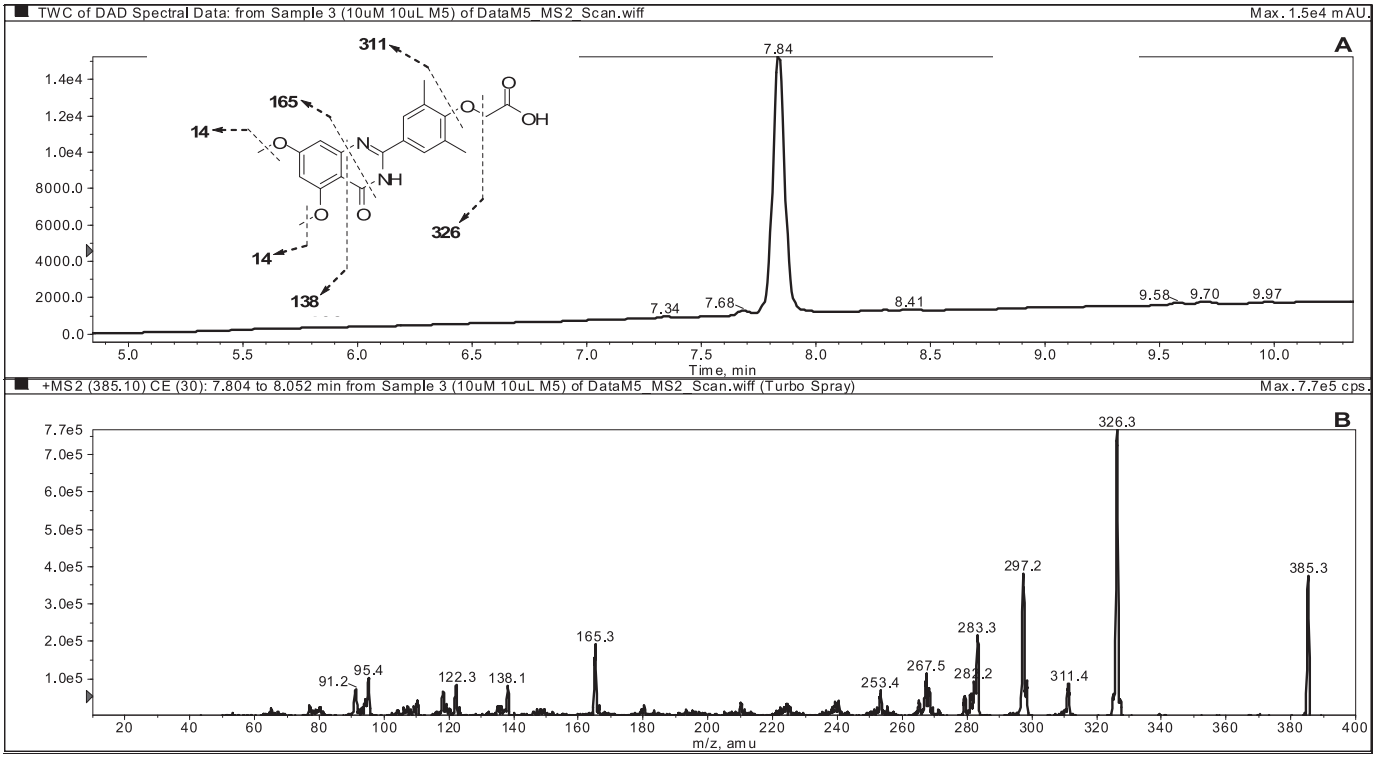
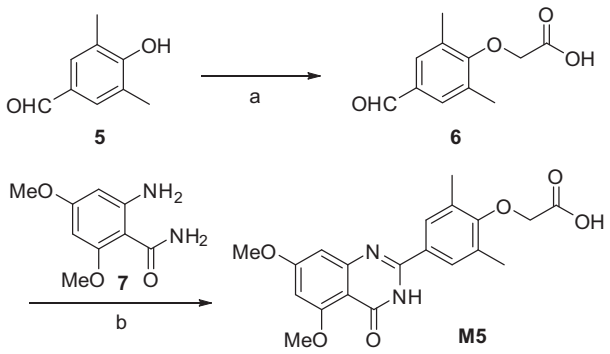


Fig. 4. LC/MS/MS and fragmentation analysis of purified metabolite **M5**. Panels: **A**, UV trace (detection at 230 nm); **B**, MS/MS spectrum.



Scheme 2. Chemical synthesis of metabolite **M5** (see Materials and methods for details). a: Bromoacetic acid, NaOH, water; b: $NaHSO_3$, pTSA, DMAC.

30 μL, were mixed with 0.2 mL of microsomal suspension) and then added to initiate the reaction. After 1 h, 6 h, or 24 h incubation in a shaking water bath at 37 °C, the reactions were stopped by the addition of an equal volume of methanol. After incubation on ice for 15 min, samples were centrifuged (10,000*g*, 15 min, 4 °C) to remove precipitated protein. The supernatants were filtered using a Teflon® syringe filter (4 mm PTFE, 0.45 μm) from Whatman, Inc. (Clifton, NJ) followed by evaporation to dryness in a Centrivap concentrator and Freeze Dry system (both Labconco, Kansas City, MO). Sample residues were resuspended in 80 μL of 50% (v/v) methanol, mixed for 15 s on a vortex mixer, and sonicated for 30 s followed by centrifugation (20,000*g*, 30 s, room temperature). The supernatant was then transferred to HPLC vials for LC/MS analysis.

In experiments to optimize the reaction conditions for the synthesis of metabolite **M4**, stock solutions of RVX-208 in DMSO with concentrations of 0.2 M, 0.05 M, and 0.02 M were prepared and used to obtain final substrate concentrations of 2 mM, 0.5 mM, and 0.2 mM, respectively, after 100-fold dilution. The concentration of microsomal protein in the reaction mixture was 1 mg/mL or 4 mg/mL; the concentration of UDPGA was 5 mM or 25 mM.

4.1.2. Reactions with recombinant UDP-glucuronyl transferases

A 10 mM stock solution of RVX-208 in methanol was prepared and used in these experiments. Incubation mixtures consisted of 100 mM potassium phosphate buffer, pH 7.4, 100 μM test compound, 3.3 mM magnesium chloride, 5 mM UDPGA, and protein (from 0.025 mg/mL to 1 mg/mL, depending on the enzyme) in a final volume of 0.5 mL. The reaction mixture minus the enzyme was pre-warmed for 5 min at 37 °C and the enzyme suspension was added. After 1 h, 6 h, or 24 h incubation in a shaking water bath at 37 °C, the reactions were stopped by the addition of an equal volume of methanol. After incubation on ice for 15 min, samples were centrifuged (10,000*g*, 15 min, 4 °C) to remove precipitated protein. The supernatants were filtered using a Teflon® syringe filter (4 mm PTFE, 0.45 μm) from Whatman, Inc. (Clifton, NJ) followed by evaporation to dryness in a Centrivap concentrator and Freeze Dry system (both Labconco, Kansas City, MO). Sample residues were resuspended in 80 μL of 50% (v/v) methanol, mixed for 15 s on a vortex mixer, and sonicated for 30 s followed by centrifugation (20,000*g*, 30 s, room temperature). The supernatant was then transferred to HPLC vials for LC/MS analysis.

4.1.3. LC/MS analysis for monitoring RVX-208 and its metabolites

LC/MS analysis was performed on a PE SCIEX API 150 system with a PDA detector. Chromatography was done at room temperature using a Waters SunFire C18 column (100 × 2.1 mm, 3.5 μm) with the mobile phase initially composed of 98% of solvent A (0.1% formic acid in water) and 2% of solvent B (0.1% formic acid in acetonitrile). Elution was performed using a linear gradient from 2 to 100% B in 20 min, followed by an isocratic hold for 1 min at a flow rate of 0.35 mL/min. The column was re-equilibrated for 10 min after programming back to the starting solvent mixture over 0.5 min. UV data were acquired at 230 nm with an injection volume of 10 μL of sample solution. The mass spectrometer was operated in positive ion mode with spray voltage set at 5300 V. The ion source temperature was set at 425 °C. The 0.35 mL/min effluent from the HPLC column was directed to an ESI ion source without splitting after UV detection. The mass scan was performed in the range from 200 to 750 amu. The retention time of RVX-208 was 12.3 min.

4.1.4. LC/MS analysis for monitoring testosterone-based reactions

LC/MS analysis was performed on a PE SCIEX API 2000 system with a PDA detector. Chromatography was done at room temperature using a Waters SunFire C18 column (2.1 × 50 mm, 3.5 μm) with the mobile phase initially composed of 10% of solvent A (0.1% formic acid in water) and 90% of solvent B (0.1% formic acid in acetonitrile). Elution was performed with a linear gradient from 10 to 30% B in 12 min, then from 30 to 100% B in 0.5 min, and isocratic hold for 2 min at a flow rate of 0.35 mL/min. The column was re-equilibrated for 8 min after programming back to the starting solvent mixture over 0.5 min. UV data were acquired with total wavelength count (TWC) and the injection volume was 20 μL of sample solution. The mass spectrometer was operated in positive ion mode with spray voltage set at 5400 V. The ion source temperature was set at 450 °C. The 350 μL/min effluent from the HPLC column was directed to an ESI ion source without splitting after UV detection. The retention time of 6β-hydroxytestosterone was 13.8 min.

*4.1.5. Biocatalytic synthesis and purification of metabolite **M4***

All experiments were carried out with the 0.1 M stock solution of RVX-208 in DMSO. Six identical incubations were performed in parallel in 20 mL scintillation vials, each containing 10 mL of the reaction mixture. Each reaction consisted of 20 mg rabbit liver S9 protein, 100 mM potassium phosphate buffer, pH 7.4, 1 mM test compound, the NADPH regenerating system (1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride), and 10 mM UDPGA in a final volume of 10 mL. After 24 h incubation in a shaker at 37 °C, the reaction mixtures were combined, diluted 2-fold with cold methanol, centrifuged, and the supernatant was diluted five-fold with water and passed over a solvated and equilibrated Alltech C18 SPE (4 g) cartridge. The column was washed with 20 mL of acetonitrile and then equilibrated with 20 mL of water. Following sample loading, the absorbent was washed with 40 mL of water. The elution was then performed using acetonitrile (3 × 20 mL), which allowed complete desorption of RVX-208, whereas metabolite **M4** was not eluted. Final elution was performed using 8 mL of 50% (v/v) acetonitrile. The eluate was then injected in 4 mL portions on a Shimadzu LC8A preparative HPLC system consisting of two Shimadzu LC8A pumps, a Shimadzu SPD-10A UV detector, and a SCL-10A system controller. Chromatography was accomplished at room temperature using a Waters Sunfire Prep C18 OBD column (150 × 19 mm, 5 μm) with the mobile phase initially composed of 98% of solvent A (water with 0.1% TFA) and 2% of solvent B (acetonitrile with 0.1% TFA). Elution was performed with a linear gradient from 2 to 100% B in 20 min followed by an isocratic hold at 100% B for 1 min at a flow rate of 20 mL/min. The column was re-equilibrated for 10 min after programming back to the starting solvent mixture over 0.5 min. UV data were acquired at 230 nm. Fractions were collected for the peak eluting at 9.5 min. The pooled sample was concentrated to dryness first by evaporating acetonitrile under reduced pressure and then removing the residual water

by lyophilization on a Labconco freeze dryer to give 2.6 mg of **M4** as a white solid.

4.1.6. Biocatalytic synthesis and purification of metabolite *M5*

The reaction of RVX-208 with human liver microsomes was set up as described above in the screening protocol. After 4 h incubation at 37 °C, the reaction mixture was diluted 2-fold with cold methanol, incubated on ice for 15 min, and centrifuged (10,000*g*, 15 min, 4 °C) to remove precipitated protein. The supernatant was filtered using a Teflon® syringe filter (4 mm PTFE, 0.45 μm) from Whatman, Inc. (Clifton, NJ) and injected (20 μL) on an analytical HPLC using the same gradient as described above for LC/MS analysis of RVX-208 and its metabolites. The fractions corresponding to metabolite **M5** (retention time 13.5 min) were collected and submitted to MS/MS analysis.

4.2. Chemical synthesis

4.2.1. 2,2-Dimethylpropionic acid 2-(4-(2-hydroxyethoxy)-3,5-dimethylphenyl)-5,7-dimethoxy-4-oxo-4H-quinazolin-3-ylmethyl ester (*2*)

To a stirred solution of 2-(4-(2-hydroxyethoxy)-3,5-dimeth ylphenyl)-5,7-dimethoxy-3*H*-quinazolin-4-one (**1**; RVX-208) (3.0 g , 8.1 mmol) in DMF (75 mL) under nitrogen atmosphere, sodium hydride (60% in mineral oil, 0.39 g, 9.7 mmol) was added in small portions at room temperature. The reaction mixture was stirred for 1 h, then chloromethylpivalate (1.76 mL, 12.1 mmol) was added, and stirring was continued at room temperature for 24 h. The reaction was quenched with water and the mixture was extracted with ethyl acetate. The crude material was purified by column chromatography (Silica Gel 230–400 mesh; 7/3 methylene chloride/ethyl acetate as eluent; column eluted until compound was isolated as monitored by TLC) to give 2,2-dimethylpropionic acid 2-(4-(2-hydroxyethoxy)-3,5-dimethylphenyl)-5,7-dimethoxy-4-oxo-4*H*-quinazolin-3-ylmethyl ester (**2**) as a white solid. Yield: 2.2 g (56%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20 (s, 2H), 6.95 (d, $J = 2.4$ Hz, 1H), 6.47 (d, $J = 2.4$ Hz, 1H), 6.39 (s, 2H), 3.97 (m, 4H), 3.95 (s, 3H), 3.93 (s, 3H), 2.40 (s, 6H), 2.22 (t, $J = 5.8$ Hz, 1H), 1.21 (s, 9H).

4.2.2. 3,4,5-Triacetoxy-6-(2-(4-(3-(2,2-dimethylpropionyloxymethyl)-5,7-dimethoxy-4-oxo-3,4-dihydroquinazolin-2-yl)-2,6-dimethylphenoxy)ethoxy)-tetrahydropyran-2-carboxylic acid methyl ester (*4*)

To a stirred solution of 2,2-dimethylpropionic acid 2-(4-(2-hydroxyethoxy)-3,5-dimethylphenyl)-5,7-dimethoxy-4-oxo-4*H*-qui nazolin-3-ylmethyl ester (**2**) (3.10 g, 6.40 mmol) in methylene chloride (76 mL), 4 Å molecular sieves (15.4 g) were added under nitrogen. The resulting mixture was stirred for 5 min before commercially available (2*S*,3*S*,4*S*,5*R*,6*R*)-3,4,5-triacetoxy-6-bromotetrahydropyra n-2-carboxylic acid methyl ester (**3**) (2.54 g, 6.40 mmol) was added followed by silver (I) oxide (4.50 g, 19.3 mmol). The reaction mixture was stirred at room temperature in the dark for 3 days. The solid was filtered off and was washed with methylene chloride. The combined filtrates were concentrated and the crude material was purified by column chromatography (Silica Gel 230–400 mesh; 7/3 methylene chloride/ethyl acetate as eluent; column eluted until compound was isolated as monitored by TLC) followed by crystallization from acetone/hexanes (15 mL/25 mL) to give 3,4,5-triacetoxy-6-(2-(4-(3-(2,2-dimethylpropionyloxymethyl)-5,7-dimethoxy-4-oxo-3,4-dihy droquinazolin-2-yl)-2,6-dimethylphenoxy)ethoxy)tetrahydropyran -2-carboxylic acid methyl ester (**4**) as a white solid. Yield: 1.7 g (34%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.18 (s, 2H), 6.95 (d, $J = 2.2$ Hz, 1H), 6.47 (d, $J = 2.2$ Hz, 1H), 6.39 (s, 2H), 5.33–5.24 (m, 2H), 5.14–5.07 (m, 1H), 4.79 (d, $J = 7.6$ Hz, 1H), 4.21–4.12 (m, 1H), 4.04–3.96 (m, 3H), 3.95 (s, 3H), 3.93 (s, 3H), 3.88–3.85 (m, 1H), 3.77 (s, 3H), 2.36 (s, 6H), 2.05 (s, 3H), 2.03 (s, 6H), 1.21 (s, 9H).

4.2.3. 6-(2-(4-(5,7-Dimethoxy-4-oxo-3,4-dihydroquinazolin-2-yl)-2,6-dimethylphenoxy)-ethoxy)-3,4,5-trihydroxytetrahydropyran-2-carboxylic acid (*M4*)

A 0.05 M solution of sodium methoxide in methanol (129 mL, 6.40 mmol) was added to 3,4,5-triacetoxy-6-(2-(4-(3-(2,2-dimethylpropionyloxymethyl)-5,7-dimethoxy-4-oxo-3,4-dihydro quinazolin-2-yl)-2,6-dimethylphenoxy)ethoxy)tetrahydropyran-2-carboxylic acid methyl ester (**4**) (0.90 g, 1.1 mmol) at 0 °C under nitrogen. The reaction progress was monitored by TLC and mass spectroscopy. After disappearance of the starting material, water (0.5 mL) was added to hydrolyze the methyl ester over 2–3 h at room temperature. The reaction mixture was treated with amberlite H^+ until pH became neutral. The amberlite was filtered off and washed with methanol. The crude material was purified by Biotage reverse phase column chromatography (KP-C18-HS, 35–70 μm, 90 A; 25/75 to 40/60 gradient of MeOH/H_2O as an eluent; column eluted until compound was isolated as monitored by TLC) to give 6-(2-(4-(5,7-dimethoxy-4-oxo-3,4-dihydroquinazolin-2-yl)-2,6-dim ethylphenoxy)ethoxy)-3,4,5-trihydroxytetrahydropyran-2-carbox ylic acid (**M4**) as a white solid. Yield: 186 mg (34%). Mp 208.6–210.8 °C; UV (λ, log ε): 211 (1.70), 258 (1.68) nm; Purity by HPLC: 97.7%; ^{1}H NMR (400 MHz, CD_3OD): δ 7.70 (s, 2H), 6.79 (d, $J = 2.2$ Hz, 1H), 6.55 (d, $J = 2.2$ Hz, 1H), 4.42 (d, $J = 7.7$ Hz, 1H), 4.32–4.25 (m, 1H), 4.16–4.09 (m, 1H), 4.08–4.03 (m, 1H), 4.02–3.95 (m, 1H), 3.92 (s, 3H), 3.91 (s, 3H), 3.59 (d, $J = 9.0$ Hz, 1H), 3.50–3.40 (m, 1H), 3.28 (d, $J = 9.0$ Hz, 1H), 2.40 (s, 6H). Exchangeable protons (CO_2H, OH and NH) are not detected. MS (ES^+) *m/z*: 547.24 (M+1). Analysis calculated for $C_{26}H_{30}N_2O_{11}\cdot 4H_2O$ (618.52), %: C 50.48; H 6.19; N 4.53. Found, %: C 50.11; H 5.55, N 4.80. The material (M4) was hydroscopic and prolonged drying did not reduce water content. Water content by Karl Fisher varied between 5.5 and 10.7%.

4.2.4. 4-Formyl-2,6-dimethylphenoxyacetic acid (*6*)

A solution of sodium hydroxide (2.5 g, 63 mmol) in water (65 mL) was added to a mixture of bromoacetic acid (5.3 g, 38 mmol) and 3,5-dimethyl-4-hydroxybenzaldehyde (**5**) (1.9 g, 13 mmol) in water (30 mL). The reaction mixture was stirred at 100 °C for 24 h, then the solution was acidified (pH ~2) with conc. HCl. The resulting brown solid was isolated, washed with water, dried, and purified by column chromatography (Silica Gel 230–400 mesh; 0–10% gradient of methanol in methylene chloride as an eluent; column eluted until compound was isolated as monitored by TLC) to give 4-formyl-2,6-dimethylphenoxyacetic acid (**6**) as a light brown solid. Yield 0.40 g (15%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.90 (s, 1H), 7.59 (s, 2H), 4.54 (s, 2H), 2.39 (s, 6H).

4.2.5. 2-(4-(5,7-Dimethoxy-4-oxo-3,4-dihydroquinazolin-2-yl)-2,6-dimethylphenoxy)-acetic acid (*M5*)

To a solution of 2-amino-4,6-dimethoxybenzamide (**7**) (0.15 g, 0.76 mmol) in DMAC (5 mL) were added 4-formyl-2,6-dimethylphenoxyacetic acid (**6**) (0.16 g, 0.76 mmol), sodium hydrogen sulfite (Assay > 58.5%, 0.150 g, 0.84 mmol) and *p*-toluenesulfonic acid monohydrate (15 mg, 0.076 mmol). The reaction mixture was stirred at 150 °C for 3 h, cooled to room temperature, and water (40 mL) was added. The yellow precipitate was filtered, washed with water and small amount of methanol, then triturated with 10% methanol in diethyl ether to give 0.084 g of compound which was further purified by preparative HPLC to give 47 mg (16%) of **M5** as a white solid. Selected data for **M5**: Mp 281–282 °C; UV (λ, log ε): 209 (2.58), 261 (2.80) nm; Purity by HPLC: 96.2%; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.96 (br s, 1H), 11.85 (s, 1H), 7.90 (s, 2H), 6.74 (d, $J = 2.2$ Hz, 1H), 6.52 (d, $J = 2.2$ Hz, 1H), 4.46 (s, 2H), 3.89 (s, 3H), 3.84

(s, 3H), 2.31 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.8, 164.9, 161.7, 160.4, 158.6, 153.8, 153.1, 131.3 (2 carbons), 129.0 (2 carbons), 128.3, 105.4, 101.9, 98.3, 69.5, 56.6, 56.3, 16.8 (2 carbons).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.03.062.

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