Metabolism and Pharmacokinetics of JM6 in Mice: JM6 is Not a Prodrug for Ro-61-8048

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Non standard abbreviations: JM6, 3,4-dimethoxy-N-(4-(3-nitrophenyl)-5-(piperidin-1-ylmethyl)thiazol-2-yl)benzenesulfonamide; KMO, kynurenine 3-monooxygenase; Ro-61-8048, 3,4-dimethoxy-N-(4-(3-nitrophenyl)thiazol-2-yl)benzenesulfonamide; KP, kynurenine pathway; HD, Huntington’s disease; P450, cytochrome P450; IC50, analyte concentration needed to inhibit 50% of the enzyme activity; LC-MS/MS, liquid chromatography tandem mass spectrometry; ESI, electrospray ionization; MRM, multiple reaction monitoring; LLOQ, lower limit of assay quantitation; kel, elimination rate constant; AUC, area under the concentration versus time curve; Ct, last measurable concentration; BSA, bovine serum albumin; MDCKII, Madin-Darby canine kidney; MDR1, multiple drug resistant protein 1; Papp, apparent permeability; SIR, single ion recording; NMR, nuclear magnetic resonance; Clp, plasma clearance; Clint, hepatic intrinsic clearance; Vdss, volume of distribution at steady state; EER, effective efflux ratio.
Abstract

Understanding whether regulation of tryptophan metabolites can ameliorate neurodegeneration is of high interest to investigators. A recent publication describes 3,4-dimethoxy-N-(4-(3-nitrophenyl)-5-(piperidin-1-ylmethyl)thiazol-2-yl)benzenesulfonamide (JM6) as a novel prodrug for the kynurenine 3-monooxygenase (KMO) inhibitor 3,4-dimethoxy-N-(4-(3-nitrophenyl)thiazol-2-yl)benzenesulfonamide (Ro-61-8048) that elicits therapeutic effects in mouse models of Huntington’s and Alzheimer’s diseases (Zwilling et al., 2011). Our evaluation of the metabolism and pharmacokinetics of JM6 and Ro-61-8048 indicate instead that Ro-61-8048 concentrations in mouse plasma after JM6 administration originate from a Ro-61-8048 impurity (<0.1%) in JM6. Following a 0.05mg/kg Ro-61-8048 oral dose alone or co-administered with 10mg/kg JM6 to mice, the Ro-61-8048 AUC\textsubscript{inf} were similar (4300 and 4900 nM×hr, respectively), indicating no detectable contributions of JM6 metabolism to the Ro-61-8048 AUCs. JM6 was stable in incubations under acidic conditions and Ro-61-8048 was not a product of JM6 metabolism \textit{in vitro} (plasma, blood or hepatic models). Species differences in quantitative rate of oxidative metabolism indicate that major circulating JM6 metabolite(s) in mice are unlikely to be major in humans: JM6 is rapidly metabolized via the piperidyl moiety in mouse (forming an iminium ion reactive intermediate) but slowly metabolized in human (\textit{in vitro}) primarily via O-dealkylation at the phenyl ring. Our data indicate that JM6 is not a prodrug for Ro-61-8048 and is not a potent KMO inhibitor.
**Introduction**

Pioneering work by Schwarcz and collaborators has shown that the levels of several metabolites in the kynurenine pathway (KP) are altered in Huntington’s disease (HD) post-mortem human brain samples and in various HD mouse models (Foster et al., 1985; Schwarcz et al., 2010; Schwarcz et al., 1983). Their work further suggested that cytotoxicity induced by quinolinic acid may be involved in the pathophysiology of HD (Sathyasaikumar et al., 2010). Collectively, this body of work prompted us to initiate about five years ago a medicinal chemistry program to develop inhibitors of kynurenine 3-monoxygenase (KMO) as potential therapeutic agents for HD. The therapeutic potential of KMO inhibition in HD was recently further supported by a report that genetic disruption of KMO in a *Drosophila melanogaster* model of HD ameliorated the disease phenotype (Campesan et al., 2011).

The effect of putative KMO inhibitors 3,4-dimethoxy-N-(4-(3-nitrophenyl)-5-(piperidin-1-ylmethyl)thiazol-2-yl)benzenesulfonamide (*JM6*) and 3,4-dimethoxy-N-(4-(3-nitrophenyl)thiazol-2-yl)benzenesulfonamide (*Ro-61-8048*) was recently evaluated in mouse models of Alzheimer’s and Huntington’s disease (Zwilling et al., 2011), where *JM6* prevented spatial memory loss, anxiety deficits, and synaptic loss (in the mouse amyloid precursor protein model), among other signs of disease. In addition, early treatment with *JM6* prolonged survival of R6/2 transgenic HD mice, decreased microglial activation, and prevented synaptic loss. The authors concluded that the effectiveness of *JM6* administration observed in their animal models was driven by the KMO inhibitor *Ro-61-8048*, which they suggested is a metabolic product of *JM6* that they describe as a novel prodrug. A mechanism for *Ro-61-8048* formation from *JM6* was proposed (Zwilling et al., 2011).
While we have never pursued a prodrug strategy, we had characterized JM6 and Ro-61-8048 as putative KMO inhibitors as part of our medicinal chemistry efforts. Our data on JM6 and Ro-61-8048 (unpublished at that time and presented in this report) supported different conclusions and indicated that JM6 is stable when incubated in acidic conditions, is very rapidly and extensively oxidized at the piperidine ring by mouse liver cytochrome P450 enzymes (P450s), but is stable in human liver P450s. In our biochemical assays JM6 is a weak KMO inhibitor (IC$_{50}$ = 19.85 µM for mouse KMO). At the time of evaluation neither compound met our criteria for advancement which included biochemical potency on KMO <100 nM, intrinsic clearance values predicting minimal hepatic metabolism, and similar in vitro qualitative metabolism profile between species, thus we halted their characterization. The discrepancies between our findings and those recently published (Zwilling et al., 2011) prompted us to generate further data to confirm our original findings and explore some of the disagreements.

Data from the studies described in this report demonstrate that JM6 is not a prodrug for Ro-61-8048; the minor impurity of Ro-61-8048 present in the JM6 dose is sufficient to elicit high plasma exposure due to the very slow clearance of Ro-61-8048 in mice.
Materials and Methods

Animal Studies

The intravenous (iv) pharmacokinetics of JM6 and Ro-61-8048 were evaluated in male C57BL/6N mice (wild type strain for the R6/2 mice) at 5 mg/kg as a 1 mg/mL solution in 10:50:40 DMSO:PEG400:H₂O (by volume), filtered prior to dosing.

The pharmacokinetics of JM6 and Ro-61-8048 after oral gavage (po) were evaluated in two separate studies. The first po study comprised three dosing groups where mice received relatively constant levels of JM6 and varying levels of Ro-61-8048 as follows: Group I 10 mg/kg JM6; Group II 9.5/0.5 mg/kg JM6/Ro-61-8048 (w/w), and Group III 9.0/1.0 mg/kg JM6/Ro-61-8048 (w/w). The second po study comprised four dosing groups, each receiving a different dose level of JM6 (0, 10, 25 and 50 mg/kg), co-administered with the same level of Ro-61-8048 (0.05 mg/kg). The JM6 stock used in this second po study contained 0.08% Ro-61-8048 as an impurity, that could not be eliminated (see Results section). To achieve 0.05 mg/kg in the dose with accuracy, an amount of Ro-61-8048 equal to the difference between 0.05 mg/kg and the level of the impurity was added to the dose formulation. Details of the formulation preparation are included in the Supplemental Methods. The oral formulations (10 mL/kg dose volume) were fine suspensions prepared in 10% HydroxyPropyl-β-Cyclodextrin in 50 mM phosphate buffer, pH 7. Analyte concentrations were determined in dose formulation aliquots (100 µL; n=3 per formulation) and were within 15% of target, except for the 5 mg/kg Ro-61-8048 iv dose which quantitated to 4.1 mg/kg and the 10 mg/kg JM6 po dose in Group V which quantitated to 12 mg/kg (pharmacokinetic parameters were adjusted to 10 mg/kg).
Sample Collection and Processing: Terminal blood samples (collected via cardiac puncture into tubes containing K$_2$EDTA) and brain tissues (N=3 per time point and dose group) were obtained at 0.083, 0.25, 0.5, 1, 2, 4, 8 and 24-h post-dose. Blood was centrifuged at 2200 × g for 10 min at 5°C ± 3°C to separate the plasma. Immediately after collection, each brain was rinsed with saline and snap frozen in liquid nitrogen. All samples were stored at -20°C ± 10°C. At the time of bioanalysis each brain was homogenized in acetonitrile:water (3:1, v/v) using a Precellys tissue homogenizer resulting in a brain:solvent ratio of 1:3 (w/v).

Extraction of Test Article From Samples for Bioanalysis: Plasma (25 µL) and brain homogenate (100 µL) from study samples, controls and blanks were dispensed into 96-well plates. Extracting solution (100 µL) consisting of 0.1% formic acid in acetonitrile containing 200 ng/mL diclofenac as the internal standard (IS) was added to all samples except to matrix double blanks and solvent blanks, followed by vortexing and centrifugation (5 min). Supernatants were transferred to a new plate, an aliquot (50 µL) of acetonitrile:MilliQ water (75:25 v/v) was added to the samples, covered and vortexed for 5 min prior to liquid chromatography mass spectrometry (LC-MS/MS) analysis.

Bioanalytical Methods: Analyte concentrations were determined by LC-MS/MS. Reverse phase separation was performed in a Waters Acquity UPLC with a UPLC BEH C18 column (50 x 2.1 mm, 1.7 µm). For quantitation of JM6 and Ro-61-8048 the mobile phase consisted of 0.01% formic acid (v/v) in acetonitrile (B) and 0.01% formic acid (v/v) in milliQ water (A). Elution was initiated with 5% B for 0.2 min, progressed linearly to 95% B over 1 min and maintained for 0.6 min before returning to initial conditions, with a total run time of 2 min. The injection volume was 7 µL. The entire LC eluent was directly introduced to an electrospray ionization (ESI) source operating in the positive ion mode for LC-MS/MS analysis on a Waters
TQD triple quadrupole mass spectrometer with a source temperature of 150°C and a desolvation temperature of 500°C. The mass spectrometer ion optics were set in the multiple reaction monitoring (MRM) mode. The data was processed using QuanLynx software from Waters. Additional details of the bioanalytical methodology are included in the Supplemental Methods.

The assay lower limits of quantitation (LLOQ) were 1.9 nM for JM6 in plasma and brain and 2.4 nM and 5.9 nM for Ro-61-8048 in plasma and brain, respectively.

**Pharmacokinetic Analysis:** Composite non-compartmental pharmacokinetic parameters were calculated from the mean concentrations (n=3) obtained for each time-point using Phoenix WinNonlin, version 5.2.1 (Pharsight Corporation, Cary, NC). For the iv dose the plasma concentration at Time = 0 was back extrapolated from the first two post dose plasma concentrations. For the po dose the concentration at Time = 0 was assumed to be zero. Plasma and tissue concentrations below LLOQ were treated as absent samples.

The AUC_{last}, that is the area under the plasma concentration versus time curve to C_t, the last measureable concentration, was calculated using the linear trapezoidal method. When appropriate, the elimination rate constant (k_{el}), was estimated using at least the last three observed concentrations. The portion of the AUC from C_t to infinity (AUC_{last-inf}) was extrapolated from the ratio of C_t/k_{el}. The AUC_{inf} was calculated as AUC_{last} + AUC_{last-inf}. The oral bioavailability (%F) was calculated only for AUCs within the linear dose-response range, by dividing the dose-normalized po AUC_{inf} over the dose-normalized iv AUC_{inf}. 
In Vitro ADME Assays

Metabolic Stability in Plasma, Liver Microsomes and Hepatocytes

Test compound (5 µM, n=2, 37°C) was incubated in pooled plasma or bovine serum albumin (BSA; 45 mg/mL in 0.1 M phosphate buffered saline, pH 7.4). Samples (50 µL) were taken at 0, 10, 30, 120, 240, 360 and 1440 min. Incubations of test compound (1 µM, n=2, 37°C) in pooled liver microsomes (0.25 mg protein/mL in 0.1 M phosphate buffer pH7.4) were initiated with the addition of NADPH (1 mM). Samples (100 µL) were obtained at 0, 5, 10, 20 and 40 min. Test compound (1 µM, n=2, 37°C) was incubated in pooled cryopreserved male hepatocytes (0.25 M cells/mL for mouse and 0.5 M cells for rat and human) in Williams’ MediumE with 4 mM L-glutamine and 2 mM magnesium sulphate; aliquots (100 µL) were taken at 0, 10, 20, 45 and 90 min. Aliquots obtained from the different incubations were added to 150 µL of acetonitrile containing carbamazepine as IS, centrifuged and analyzed by LC-MS/MS.

Stability in Simulated Gastric Fluid

Simulated gastric fluid, pH1.2, was prepared containing 2 g/L sodium chloride, 3.2 g/L of pepsin, 0.7% (v/v) HCl. Gastric buffer, pH 1.2, was also prepared containing 2 g/L sodium chloride and 0.7% (v/v) HCl. Test compound (10 µM) was added to both fluids and samples were mixed at ~25°C on an orbital shaker. Aliquots (50 µL) were taken at 0, 2 and 4 h, added to 150 µL DMSO, mixed and analyzed immediately by LC-UV (λ = 254 nm).

Permeability and Effective Efflux Ratio in Caco-2 and MDCK-MDR1

MDCKII (MDR1 and WT) cell lines were cultured in Transwell plates following the guidelines provided by SOLVO Biotechnology (Budapest, Hungary). The culture period was
3-days and the seeding density $2 \times 10^5$ cells/well. Test compounds (10 µM) were dissolved in Hanks’ Balanced Salt Solution containing 25 mM HEPES (pH 7.4) and added to either the apical or basolateral chambers of the Transwell plate assembly in duplicate. Lucifer Yellow was added to the apical buffer in all wells to assess integrity of the cell layers; wells with Lucifer Yellow permeability above 100 nm/s were rejected. After a 1-h incubation at 37 °C, aliquots were taken from both chambers of each Transwell and added to acetonitrile containing analytical IS (carbamazepine) in a 96 well plate. Analyte concentrations were measured by LC-MS/MS.

The apparent permeability ($P_{app}$) values of test compound were determined for both the apical to basal (A>B) and basal to apical (B>A) permeation and the efflux ratio (B>A: A>B).

For MDCK-cells, the effective efflux ratio was also determined from the ratio observed in MDR1-MDCK cells relative to the ratio observed in wild-type cells.

### Blood-to-Plasma Ratio

Test compound (1 µM, n = 2, 37°C) was incubated in fresh whole blood. Aliquots (100 µL) of whole blood were taken after 30 min of incubation and the remaining sample centrifuged to yield plasma. LC-MS/MS quantitation indicated both analytes distributed preferentially into the plasma compartment (blood-to-plasma ratio ~ 0.6 – 0.8). Bioanalysis in plasma was sufficient to describe systemic pharmacokinetics (data not shown).

### Plasma Protein Binding

Plasma protein binding was determined by equilibrium dialysis following standard procedures. The determinations were done for **Ro-61-8048** since CHDI had determined that **JM6** was not a potent inhibitor of KMO and was not a compound of interest. Triplicate plasma samples fortified with **Ro-61-8048** at 5 and 20 µM (rat and mice) and 10 µM (human) were
added to the donor side, phosphate buffered saline was added to the receptor side of the RED device inserts in Teflon plates and the plates were sealed. Samples were incubated at 37°C and rotated at 200 rpm for 6 hr, and analyzed by LC-MS.

Bioanalysis for In Vitro Samples

Formation of Ro-61-8048 was monitored in all incubations; the LLOQ were 0.01 µM for chemical stability, 0.10 µM for simulated gastric fluid, 0.01 µM for liver microsomes and hepatocytes, and 0.05 µM for whole blood and plasma. The percent of JM6 remaining at each time point, was determined by comparing the instrument response to the time = 0 min (100%). Quantifications (as applicable) were performed against a calibration curve.

Metabolite Identification

In vitro: Incubations (n=4) of JM6 or Ro-61-8048, 1 or 10 µM in 0.5 mg protein/mL or 10 and 25 µM in 1 mg protein/mL or DMSO control were performed in mouse, rat and human liver microsomes with and without NADPH. Aliquots (100 µL) were taken at 0 and 90 min and added to an equal volume of acetonitrile. Incubations in hepatocytes from the same species were performed using the same test compound concentrations in 1M cells, with incubations carried out through 4 hr. All incubations (including DMSO control incubations) were analyzed by LC-MS.

In vivo: Samples from the 50 mg/kg orally dosed animals with JM6 (Groups I and VII) were used. Remaining plasma aliquots were pooled per time point and drug-related material was extracted and proteins precipitated by the addition of solvent using 3 volumes of acetonitrile to 1 volume of plasma. The precipitated mixture was vortexed and centrifuged, the supernatant was then removed for analysis by LC-MS.
**Instrument Conditions and Analysis:** Chromatographic separation of the analytes was achieved with a Waters Acquity UPLC BEH C18 column (2.1 x 50 mm x 1.7 µm), the injection volume was 2 µL and the flow rate was 0.6 mL/min. Mobile Phase B was held isocratic at 2% B for 0.2 min, and increased linearly to 98% in 3.8 min. The column was washed with 98% B for 0.6 min, and equilibrated to starting conditions (2% B), for 0.2 min. Mobile Phases were A (0.01% formic acid in water) and B (0.01% formic acid in acetonitrile). Metabolites were identified using a Waters Xevo-TQ mass spectrometer in tandem to the UPLC, with an electrospray source at following settings: capillary voltage = 3.5 kV, cone voltage = 30 V, extractor voltage = 1.6 V, source temperature = 150°C, desolvation gas temperature = 500°C, desolvation gas flow = 900 L/h, cone gas flow = 50 L/h and collision gas flow = 0.2 mL/min. Incubation extracts were scanned over a mass range of 50 to 1000 amu in both positive and negative ionisation modes. Mass chromatograms were generated for ions observed in the extracts from incubated compounds relative to DMSO controls. In addition, single ion recording (SIR) methods were set up for metabolites thought likely on the basis of the structure of the test compounds. When chromatographic peaks of greater intensity than controls were observed, daughter (fragmentation) spectra were obtained.

**Quantitation of JM6 Metabolites**

To quantify metabolites in liver microsomal incubations for cross-species comparison, JM6 incubations were performed in mouse, rat, or human liver microsomes as described in the metabolite identification studies and reactions were terminated by the addition of an equal volume of methanol. The analysis was performed as described for the metabolite identification studies except that methanol replaced acetonitrile in Mobile Phase B.
Metabolites of JM6 were measured in plasma from Group I mice (10 mg/kg JM6 po). Plasma samples were processed as described above. The mobile phase consisted of 0.01% formic acid (v/v) in methanol (B) and 0.01% formic acid (v/v) in milliQ water (A). Elution was initiated at 2%B for 0.2 min, progressed linearly to 98%B over 3.8 min and maintained for 0.7 min before returning to initial conditions, with a total run time of 5 min. The injection volume was 5 µL.

The mass spectrometer ion optics were set to SIR in the negative ionization mode. The data were processed using the QuanLynx software from Waters. Metabolites M2, M3, M4, M6, M7 and M8 were quantitated using calibration standards prepared from synthetic material dissolved in assay matrix. For the metabolites where synthesized standards were not available (M1, M5, M10, M11, M12 and M13), semi-quantitation was performed by comparison of relative peak intensities against those of a metabolite synthetically available where a similar UV/MRM response factor had been previously determined.

Synthesis and purification of JM6, Ro 61-8048 and metabolites used in these studies

Synthesis of JM6 and Ro 61-8048 According to Zwilling et al., 2011

JM6, using Ro-61-8048 as the precursor, and Ro-61-8048 were synthesized using the synthetic route published by Zwilling et al., 2011 and described in patent WO2008/022281 (Figure 1). JM6 was purified via re-crystallization or semi-preparative HPLC with various columns and mobile phases. Confirmation of the synthetic products was performed by LC-MS and NMR. The impurity profile of JM6 and Ro-61-8048 was determined by a combination of LC-MS and LC-UV. A detailed description of the synthetic steps, product characterization, purification approaches and purity determination can be found in the Supplemental Methods.
Alternate Synthesis of \textbf{JM6}, Without \textbf{Ro 61-8048} as Synthetic Intermediate

\textbf{JM6} was synthesized by scientists at Evotec LTD and AMRI using an alternate path that did not contain \textbf{Ro-61-8048} as a synthetic intermediate following the steps shown in Figure 2. The confirmation of \textbf{JM6} as the final product was performed by LC-MS and NMR, and the impurity profile was determined by a combination of LC-MS and LC-UV methods. A detailed description of the synthetic steps, product characterization, purification approaches and purity determination can be found in the Supplemental Methods.

Synthesis of putative metabolites of \textbf{JM6} 

Putative \textbf{JM6} metabolites were synthesized at Evotec LTD (see Supplemental Methods).

Quantitation of \textbf{Ro 61-8048} in Purified \textbf{JM6} 

Quantification of \textbf{Ro-61-8048} was performed against calibration standard curves by LC-MS/MS analysis using a 1200 RRLC liquid chromatography system with an integrated serial diode array UV detector scanning over a 210 – 400 nm range and a 6410B triple quadrupole mass spectrometer (Agilent Technologies) with an ESI source operating in the positive ionization mode (gas temperature 325 °C, gas flow 11 L/min, nebulizer 55 psi, and capillary 4000 V). Analytes were chromatographed on a Waters Acquity BEH C18 column (30.0 mm x 2.1 mm x 1.7μm) with a Waters Acquity BEH VanGuard pre-column (5.0 mm x 2.1 mm x 1.7μm) using a flow rate of 1.2 mL/min and a column temperature of 60°C. Mobile phases \textbf{A} and \textbf{B} consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Following equilibration at 2% \textbf{B} for 0.5 min the analyte was injected, the gradient was increased linearly from 2% \textbf{B} to 95% \textbf{B} in 4 min, and held constant at 95% \textbf{B} for 0.4 min before returning to 2% \textbf{B} over 0.1 min. \textbf{JM6} and \textbf{Ro-61-8048} were chromatographically resolved. UV detection was
performed over a scan range of 210 – 400 nm. An electrospray ionization source was used with the following parameters: Gas temperature 325 °C, Gas flow 11 L/min, Nebulizer 55 psi, Capillary 4000 V. Additional methodology details are included in the Supplemental Methods.

**Biochemistry**

**Determination of Kynurenine Monooxygenase (KMO) Activity and In Vitro Inhibition**

The KMO enzyme was purified from mouse (C57 BL/6J), rat (Wistar) or human (a section only) livers following published methods (Rover et al., 1997). KMO activity was measured directly by monitoring 3-hydroxy kynurenine (the product of KYN metabolism catalyzed by KMO) by LC-MS/MS. The assay was performed in a 384 well plate with a final volume of 40 µL of 100 mM potassium phosphate pH 7.4, 200 µM NADPH, 0.4 U/mL glucose 6-phosphate dehydrogenase, 3 mM D-glucose 6-phosphate. The reaction mixture consisted of test compound, the mitochondria preparation, NADPH as the cofactor and 100 µM KYN.

The test compounds were pre-incubated for 5 min at 25°C with the enzyme preparation (0.3 mg protein/ml; specific activity of 1,000±250 nmol/L*min*mg rat and mouse liver enzyme, 700±150 nmol/L*min*mg human liver enzyme). A separate incubation also was performed in the presence of 3 mg/mL of plasma protein, to assess the effects of protein on the binding of the inhibitor to the KMO protein. Controls were included to confirm that KMO activity was not affected in the presence of the plasma protein. Reactions were started by adding KYN (100 µM final concentration), incubated for 40 min (rat and mouse liver lysate) and 60 min (human liver lysate), terminated by addition of 40 µL 10% trichloro acetic acid, vortexed, centrifuged (5 min at 4,000 rpm, 4°C), and submitted to LC-MS/MS.
Enzyme activity kinetic constants were calculated by fitting the data obtained from saturation experiments to Michaelis-Menten equation using the Prism data analysis software (GraphPad Software, Inc.). IC\textsubscript{50} values were determined from 8-point serial 3-fold dilutions starting at a top concentration (3 µM or 30 µM) using the same software. Each IC\textsubscript{50} was determined in triplicates in at least two individual assay runs (n ≥ 2).

**LC–MS/MS analysis:** The LC–MS/MS system used for the KMO assays was composed of a Waters Acquity interfaced with a Waters TQD triple quad mass spectrometer equipped with an electrospray ion source. For LC separation of KYN and 3-hydroxy kynureninea Waters Acquity UPLC BEH C18 column (1.7 µm; 2.1 x 50 mm) connected to a filter guard was used. Flow rate for analysis was 1 mL/min at a column temperature 40°C. The gradient started at 99.9% solvent A (0.1% v/v acetic acid in LC-MS grade water), held for 0.2 min, to 98% solvent B (98% acetonitrile v/v with 0.1% acetic acid v/v) in 0.3 min and held for 0.35 min. The LC was returned to initial conditions within 0.01 min and re-equilibrated for additional 0.4 min. To minimize variation of the injection volumes the 5 µL sample loop was 5-fold overfilled. Sample storage temperature was set to 8°C. MRM transitions specific to each analyte were monitored.
Results

Pharmacokinetics of JM6 and Ro-61-8048 Following a Single Intravenous (iv) Bolus

Following a single iv bolus administration at 5 mg/kg (Table 1; Supplemental Figure 1), the plasma clearance of JM6 was rapid (Clp = 1.9 L/h/kg), and accounted for ~65% of the mouse liver plasma flow of 2.97 L/h/kg (Davies and Morris, 1993). In contrast, the clearance of Ro-61-8048 was 100-fold lower (0.018 L/h/kg, or ~0.6% of the mouse liver plasma flow).

While both compounds had a relatively low volume of distribution at steady state (Vdss), this parameter was ~2.5-fold higher for JM6 when compared to Ro-61-8048 (0.37 vs 0.15 L/kg, respectively). The combined differences in Vdss and Clp yielded a very short terminal half-life (t1/2) for JM6 and a much longer one for Ro-61-8048 (t1/2 = 0.72 and 6.5 h, respectively).

Under non-compartmental pharmacokinetics and constant clearance, the Clp of a compound is estimated as the ratio of dose over AUC. For two compounds administered at the same dose, the ratio of their Clp is inversely proportional to the ratio of their AUCs. In the case of JM6 and Ro-61-8048 which we dosed to mice iv at 5 mg/kg, the AUC ratios (550000 over 5200 nM×h) explained the 105.6-fold difference in clearances. This applies to all doses that yield exposures within the linear range, and provided the plasma clearance mechanisms and rates are the same as those observed in the study used to calculate the compound Clp.

In this same study, following iv administration of JM6 to mice, we detected Ro-61-8048 in plasma with an AUCinf of 2079 nM×h. Assuming that the Ro-61-8048 observed in plasma was the product of JM6 metabolism, we calculated the fraction of the dose of JM6 metabolized to Ro-61-8048 according to Pang and Kwan, 1983:
DMD #46532

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(1) \quad f_{m,iv} = \frac{AUC_{met,DPiv}}{DP_{iv}} \times \frac{Cl_{met}}{kgh L/kg} = \frac{876.2 \mu g/L \times h}{5,000 \mu g/kg} \times 0.018 L/h/kg = 0.0032, \text{ where}
\]

\( met = \text{metabolite of interest (Ro-61-8048)}, f_{m,iv} = \text{fraction of an iv dose of parent drug that is metabolized to met (Ro-61-8048)}, AUC_{met,DPiv} = \text{AUC of the metabolite (Ro-61-8048) after an iv dose of parent (JM6)}, DP_{iv} = \text{iv dose of parent}, Cl_{met} = \text{Clearance of the metabolite of interest.} \)

Thus, assuming that Ro-61-8048 observed in plasma originated from JM6 metabolism, the amount of Ro-61-8048 formed represented a miniscule percentage (0.32%) of a 5 mg/kg iv dose of JM6.

Since we synthesized JM6 using Ro-61-8048 is the precursor (Zwilling et al., 2011) (Figure 1), it was possible that Ro-61-8048 exposures in mice following a JM6 dose originated from Ro-61-8048 contamination in the JM6 dose, thus we explored this possibility. For Ro-61-8048 the ratio of Clp (0.018 L/h/kg) over AUC (876.2 µg/L×h) indicated that a 0.016 mg/kg dose (equivalent to ~0.4% Ro-61-8048 in a 5 mg/kg dose of JM6) was sufficient to achieve the observed AUC in mice plasma. According to our discovery Standard Operating Procedures the JM6 purity for animal dosing was >97% with no individual impurity >0.5%; it was possible that our JM6 stock contained <0.5% Ro-61-8048.

**Effects of Varying Proportions of Ro-61-8048 in JM6 Administered Orally to Mice**

To understand the effect of dosing varying proportions of Ro-61-8048 on exposure, mice were administered a single oral dose of JM6 at 10 mg/kg (Group I), JM6/Ro-61-8048 at 9.5/0.5 mg/kg (Group II), or JM6/Ro-61-8048 at 9.0/1.0 mg/kg (Group III) (Table 2; Supplemental Figure 1). When JM6 was administered alone (10 mg/kg, Group I) maximum JM6 absorption was rapid (Cmax = 0.5 h), and the oral bioavailability was very low (7.2%) probably due to a significant first pass effect rather than poor absorption given the compound’s
high Cl\textsubscript{p} and \textit{in vitro} rate of hepatic metabolism. The \textbf{JM6} AUC\textsubscript{inf} was 1100 nM×h while the \textbf{Ro-61-8048} AUC\textsubscript{inf} was 2-fold higher (2300 nM×h). If the observed \textbf{Ro-61-8048} in plasma was of metabolic origin, according to Pang and Kwan, 1983, it would have corresponded to <0.2% of the \textbf{JM6} dose. Assuming complete absorption of a metabolite formed during first pass, \textbf{Equation 1} can be modified to estimate the fraction of a \textit{po} dose of parent drug that is metabolized to met (f\textsubscript{m,po}); as follows:

\[
(2) \quad f_{m,po} = \frac{AUC_{met,DP_{po}} \times CL_{met}}{DP_{po}} = \frac{955.43 \, \mu g/L \times hr}{10,000 \, \mu g/kg} \times 0.018 \, L/hr/kg = 0.0017
\]

Where AUC\textsubscript{met,DP_{po}} = AUC of the metabolite (\textbf{Ro-61-8048}) after a \textit{po} dose of parent (\textbf{JM6}) and DP\textsubscript{po} = \textit{po} dose of parent.

Alternatively, the oral dose of a compound needed to achieve such AUC can be estimated using the \textit{iv} non-compartmental pharmacokinetic parameters as the product of the clearance times the AUC divided by the oral bioavailability. With an oral bioavailability of 70% for \textbf{Ro-61-8048} (\textbf{Table 2; Group IV}), a 0.023 mg/kg dose (0.018 L/h/kg × 876.2 μg/L×h/0.70) is sufficient to achieve the AUCs observed. A \textbf{Ro-61-8048} dose of 0.023 mg/kg represents ~0.3% in the 10 mg/kg dose of \textbf{JM6} used in our study.

The \textbf{JM6} AUC\textsubscript{inf} were similar for these three dose groups (1100, 1500, and 1100 nM×h for \textbf{Groups I, II} and \textbf{III}, respectively), indicating studies were reproducible and that a 10% difference in \textbf{JM6} dose level is not reflected in the AUC\textsubscript{inf} values due to the compound high Cl\textsubscript{p}.

The \textbf{Ro-61-8048} AUC\textsubscript{inf} for \textbf{Groups II} (0.5 mg/kg) and \textbf{III} (1.0 mg/kg), were ~30% more than dose proportional (53000 and 110000 nM×h, respectively, corresponding to AUC\textsubscript{N} of 110000 and 140000 nM×h, respectively). The slightly more than dose proportional response in \textbf{Ro-61-8048} AUC\textsubscript{inf}, could be the consequence of animal variability or of a change in Cl\textsubscript{p}. This
higher than dose proportional increase in Ro-61-8048 AUC\textsubscript{inf} is not a consequence of JM6 contributions to metabolism, since the JM6 dose decreased from 10 to 9 mg/kg with increasing Ro-61-8048 dose levels. That is, JM6 metabolism contributions to the Ro-61-8048 AUCs should have been lower for Group III.

It is worth noting that the plasma concentrations of Ro-61-8048 between 0-6 h post-dose are similar and decline slowly due to the low Cl\textsubscript{p} and low V\textsubscript{dss} of the compound. Since time-points are not serial, animal variability contributes to the difficulty in defining the t\textsubscript{max}, which for Ro-61-8048 varies between 0.5 – 4 h.

Efforts to Synthesize JM6 Containing 0% Ro-61-8048: Retrospective LC-MS/MS analysis of the JM6 stock used to dose mice confirmed that it contained <0.5% of Ro-61-8048, consistent with our estimate of ~0.3% of the dose determined from the AUC\textsubscript{inf} (see above). To determine whether the presence of Ro-61-8048 in plasma is of metabolic origin, we attempted to obtain a batch of JM6 containing 0% Ro-61-8048 to dose to mice by repurification of the existing supply and by an alternative synthetic route.

Repurification of the Existing JM6 Stock: We further purified our JM6 stock (synthesized as described by Zwilling et al., 2011), via re-crystallization or semi-preparative HPLC with various columns and mobile phases. Regardless of purification process we consistently detected 0.06 – 0.1% Ro-61-8048 in all batches of “purified” JM6.

Alternate JM6 Synthesis Without Ro-61-8048 as Precursor: JM6 was synthesized by two independent CHDI collaborators using a path not involving Ro-61-8048 as precursor (Figure 2 (A)). Yet, Ro-61-8048 was detected at 0.067 and 0.075% in each of these batches. Purity analysis of all intermediate steps indicated that products isolated at the end of Stages 5 and 6 contained low levels of Ro-61-8048.
These data confirm that JM6 undergoes minor degradation to Ro-61-8048. The most probable mechanism of JM6 degradation is proposed in Figure 2 (B).

**Oral Pharmacokinetics of JM6 Co-Administered with Constant Levels of Ro-61-8048**

Since we were unable to obtain JM6 containing 0% Ro-61-8048, to determine whether Ro-61-8048 in plasma originated from metabolism or from the impurity in the dosed material, mice were co-administered a varying levels of JM6 (0, 10, 25 and 50 mg/kg) with constant levels of Ro-61-8048 (0.05 mg/kg).

The rationale was as follows: Since Ro-61-8048 is present in the JM6 stock, increasing the dose level of JM6 administered to mice will result in a proportional increase in the dose level of Ro-61-8048. Consequently, the plasma AUCs for both compounds will also increase in proportion to these increasing dose levels of JM6, precluding us to determine if an increase in Ro-61-8048 were caused by JM6 metabolism or the impurity. Thus, we fixed dose levels of Ro-61-8048 at 0.05 mg/kg, while varying the concentration of JM6. If JM6 is metabolized to Ro-61-8048 then, the Ro-61-8048 AUCs should increase with increasing JM6 dose levels. However, if JM6 is not metabolized to Ro-61-8048 then we should observe similar AUCs for Ro-61-8048 when administered alone or in combination with increasing dose levels of JM6. To achieve that level of accuracy in the dose, an amount of Ro-61-8048 equal to the difference between the intended 0.05 mg/kg dose and the level of the impurity was added to the dose formulations (see Materials and Methods).

When Ro-61-8048 was dosed po at 0.05 mg/kg alone or co-administered with 10 mg/kg of JM6, very similar Ro-61-8048 concentration vs time profiles (Figure 3) and corresponding AUCs were observed (4300 vs 4900 nM×h, for Groups IV and V, respectively, Table 2).
indicating no (or at most negligible) contribution from JM6 degradation/metabolism to the Ro-61-8048 AUC.

The dose-normalized AUCs of JM6 observed following 9 to 10 mg/kg oral doses were consistent across studies and dose combinations (110, 160, 130 and 130 nM×h, for Groups I, II, III and V, respectively, Table 2), indicating good reproducibility between studies. The dose-normalized AUCs of JM6 tended to increase with increasing dose levels, and were 130 nM×h at 10 mg/kg (Group V) and 190 and 190 nM×h at 25 and 50 mg/kg (Groups VI and VII, respectively). The increase in dose normalized AUCs between the 10 mg/kg and the >25 mg/kg doses, is consistent with a change in a clearance route, such as saturation of at least one of the JM6 metabolic paths.

For Groups V, VI, and VII, when the JM6 dose increased from 10 to 25 mg the Ro-61-8048 AUCs increased in a more than dose proportional manner, but remained constant when the JM6 dose doubled (25 and 50 mg/kg). It is improbable that this increase in plasma exposures of Ro-61-8048 resulted from an increased metabolic rate of JM6 since the increase in JM6 were more than dose proportional when the dose increased from 10 to 25 mg/kg, consistent with saturation of the metabolism rate. The possibility that the higher concentrations of Ro-61-8048 originate from increased JM6 (non-enzymatic) degradation at higher dose levels cannot be excluded; if first pass metabolism of JM6 is saturated more JM6 could be absorbed and be available for degradation.

Permeability and Transport of JM6 and Ro-61-8048

Both JM6 and Ro-61-8048 are P-gp substrates, but JM6 has a higher effective efflux ratio (EER = 11 vs 4.2, respectively) indicating greater affinity for this transporter. (Table 3).
Numerically, JM6 had the lowest permeability ($P_{app}$ A-B = 47 vs 170 nm/s, respectively), suggesting that the “low permeability” was a consequence of the P-gp efflux during uptake.

**In Vitro Metabolism and Stability of JM6**

In all *in vitro* incubations with JM6 we monitored the formation of Ro-61-8048. Ro-61-8048 was present in the T=0 as a contaminant (~0.1% of the initial JM6 concentrations) and remained below the Ro-61-8048 LLOQ which was set to quantify 1% turnover of the initial JM6 concentration (see Materials and Methods section) in all incubations.

JM6 was stable ($t_{1/2} > 18$ h) in simulated gastric fluid (pH=1) (**Table 3**), and under the acidic extraction conditions used for bioanalysis by us (0.1% formic acid) or by Zwilling et al., 2011 (6% perchloric acid and diluting 25 µL into an 100 µL extracting solution). JM6 (5 µM) was stable in mouse blood and plasma with $t_{1/2}$ greater than the duration of the incubation of 2.5 h and 24 h, respectively. However, JM6 (1 µM) was very unstable in mouse hepatic microsomes ($Cl_{int} = 220$ µL/min/mg) and hepatocytes ($Cl_{int} = 69$ µL/min/M cells) (**Table 3**).

**Metabolites in Mouse Liver Microsomes and Hepatocytes:** In mouse liver microsomes, most of the JM6 metabolism occurred adjacent to the piperidyl nitrogen. The metabolism pathway that we propose for JM6 (**Figure 4, Supplemental Figure 2**) is consistent with the metabolism path reported for the piperidyl moiety (Baker and Little, 1985; Lin et al., 1996; Testa and Mayer, 2003). Metabolites M2, M3, M4, M6, M7 and M8 were confirmed by comparing their chromatographic retention time and MS/MS spectra to those of synthetic standards. All other metabolites are proposed based on accurate mass and spectral fragmentation. Additional information is included in the Supplemental Results. No additional metabolites were detected in hepatocyte incubations.
Species Differences in In Vitro Metabolism: There were large differences between species in the rate of oxidative metabolism for JM6. The rate was very high in the mouse, high in the rat, but negligible in the human (Clint = 220, 62 and 29 µL/min/mg, respectively). A similar trend was observed in hepatocytes. (Table 3). The primary site of JM6 metabolism also differed between species (Figure 5). In the mouse and rat metabolism occurred primarily at the piperidine ring, while in the human, the minimal metabolism occurred at the phenyl ring.

Metabolites in Mouse Plasma Following po Administration of JM6

The most abundant metabolites in mouse plasma were M3, M4 and M8 (AUC_last = 3600, 7200 and 3700 nM×hr and C_max = 1400, 780 and 1400 nM respectively) (Figure 6). While M4 was not a major metabolite in liver microsomes, we speculate that the high M4 exposures originate from the conversion of M3 to M4 (Figure 4, Path 2) probably catalyzed by aldehyde oxidase which is not present in microsomes. In addition, high exposures do not indicate significant metabolic formation and, since we did not determine the volume of distribution and clearance for each of the metabolites, the relative amount of each metabolite formed in vivo cannot be calculated from these studies. Phase II metabolites (glucuronides, sulphates or amino acid conjugates) were not detected in circulation.

Metabolism and Stability of Ro-61-8048

Species differences in the rate of oxidative metabolism also were observed for R0-61-8048. In liver microsomes the rate of metabolism was negligible in the mouse and rat, but very high in the human (Cl_int = <29, <32 and 220 µL/min/mg, respectively). The same trend was observed in hepatocytes (Cl_int = <14 and 16 µL/min/M cells, respectively) (Table 3). Only two metabolic products, with ml/z = 406 and 392 in positive ion mode, were identified and
corresponded to single and double O-demethylation from the phenyl ring substituents. No additional metabolites were detected in hepatocytes. The proposed metabolism pathway is presented in Figure 7.

**Ro-61-8048** appeared to be the major drug-related component in mouse and rat plasma; four metabolites (including the products m/z = 406 and 392) with were detected in trace amounts by LC-MS comparison. While this is not a quantitative assessment, several changes in instrument conditions to try to improve ionization of these metabolites did not increase the MS signal relative to that of parent.

**KMO Inhibitory Potential of Ro-61-8048, JM6 and Selected Metabolites.**

The IC\textsubscript{50} for mouse, rat and human hepatic KMO inhibition by **Ro-61-8048** were 90, 22 and 170 nM in the absence of plasma protein and $\geq$12,000, 8,000 and 30,000 nM in the presence of 3 mg/mL of plasma protein, respectively (Table 4). The presence of plasma proteins did not affect the KMO activity of the positive controls. The *in vitro* binding of **Ro-61-8048** to plasma proteins in these three species, is high (>99%).

**JM6** and metabolites **M2** and **M8** were weak inhibitors of KMO, with IC\textsubscript{50} $\geq$7 µM for all species tested. We attribute the weak KMO activity to the contribution of **Ro-61-8048** in the **JM6** stock. It was not possible to determine the biological activity of **M2**, **M4**, **M6**, **M7** and **M8** in the absence of **Ro-61-8048** contributions. However, extensive SAR evaluation by CHDI collaborators indicate that any substitution of thiazole adjacent to the sulfur renders compounds inactive against KMO (publication in preparation).
As part of our drug discovery efforts for HD we evaluated JM6 and Ro-61-8048 as putative KMO inhibitors. Our initial assessment of these two compounds indicated that they were not sufficiently potent and that their in vitro metabolic rates in mouse and rat were very different from human. The findings presented in the recent publication by Zwilling et al., 2011 were discrepant with our unpublished data and prompted us to conduct additional studies to explore the disagreements.

Our first conclusion is that JM6 will always contain low levels of Ro-61-8048 under laboratory conditions generally used for storage and animal dosing. Purity analysis of our first batch of JM6 synthesized as described by Zwilling et al., 2011 (using Ro-61-8048 as the precursor) showed a 0.06 – 0.1% Ro-61-8048 impurity. Efforts to purify this batch further did not reduce the level of Ro-61-8048. Synthesis of JM6 by two independent collaborators using an alternate route not involving Ro-61-8048 as a synthetic intermediate did not reduce the level of impurity in the final JM6 batch (0.075% and 0.067% Ro-61-8048 were detected in the JM6 preparation). These findings indicate that JM6 undergoes very low level conversion to Ro-61-8048; JM6 is a zwitterionic compound and analysis of distribution of microspecies in solution predicts that the protonated piperidine will be more abundant between pH 5 and 8, reverting slowly to Ro-61-8048 at neutral conditions (especially if stored in DMSO). We consider that the mechanism proposed for acid-induced release of Ro-61-8048 (Zwilling et al., 2011) is unlikely to occur under physiological conditions. These low levels of Ro-61-8048 could go unnoticed when evaluating compound purity for discovery studies, since they are below the typical 0.5% LLOQ generally used for purity assessment. Undetected low levels together with
the lack of realization that the clearance of Ro-61-8048 is so slow, may have contributed to the erroneous conclusion by Zwilling et al.

Next we conclude that JM6 is stable under acidic conditions and is not metabolized to Ro-61-8048 in vitro. Under the conditions of our studies JM6 was stable in vitro in simulated gastric fluid and under the acidic processing conditions used for bioanalysis. We did not observe formation of Ro-61-8048 following in vitro incubations of JM6 in mouse and rat blood or in mouse, rat or human, plasma, liver microsomes, or hepatocytes (MS/MS LLOQ set up to detect 1% JM6 conversion to Ro-61-8048).

Most significantly, we conclude that JM6 is not a prodrug for Ro-61-8048; given the very slow clearance of Ro-61-8048 a small impurity in the JM6 dose will result in very high exposures. Semi-empirically, this conclusion is supported by the very different iv pharmacokinetic properties and oral bioavailability of these two compounds. When compared to Ro-61-8048, the JM6 Clp in mice is ~100-fold higher and the oral bioavailability is ~7-fold lower (Clp = 1.9 vs 0.018 L/h/kg, and F <10 and ~70%, for JM6 and Ro-61-8048, respectively). These differences in pharmacokinetics are consistent with our in vitro metabolism study results which indicated a very rapid intrinsic clearance for JM6 but a very slow metabolic rate for Ro-61-8048, and suggest the low oral bioavailability and high Clp of JM6 are a consequence of significant oxidative metabolism during absorption and rapid hepatic metabolism during elimination, respectively. In other words, the ~100-fold difference in Clp indicates that similar AUCs will be observed after an intravenous dose of 10 mg/kg JM6 or 0.1 mg/kg Ro-61-8048. However, if these same dose levels are administered orally, metabolism during absorption will cause the JM6 AUC to be ~7-fold lower than the Ro-61-8048 AUC. Combining the effects of Clp and %F, an oral dose of 10 mg/kg of JM6 or 0.014 mg/kg of Ro 61-8048 (<0.2% of the
10 mg/kg JM6 dose) will result in similar AUCs. Experimentally, the conclusion that Ro-61-8048 present in mouse plasma following JM6 administration originates from low levels of Ro-61-8048 in JM6 is supported by the similarity of the Ro-61-8048 concentration vs time profiles and corresponding AUCs observed after a 5 mg/kg oral administration of Ro-61-8048 alone (4300 nM×hr) or co-administered with 10 mg/kg JM6 (4900 nM×h). Extensive JM6 metabolism to Ro-61-8048 would have resulted in much higher Ro-60-8048 upon co-administration with JM6. The Ro-61-8048 C_{max} of 230 nM that we observed following a 10 mg/kg oral administration to mice of JM6 containing ~0.08% Ro-61-8048 was similar to the C_{max} of ~280 nM reported following 7.5 or 25 mg/kg/day JM6 oral administrations to R6/2 or WT mice (Zwilling et al., 2011), suggesting that JM6 preparations used by both groups contained low levels of Ro-61-8048. If metabolism was occurring, it was minimal, since these Ro-61-8048 exposures would have originated from ~0.2% metabolism of the total JM6 dose.

With regards to JM6, following a 10 or 25 mg/kg po dose to mice we observed a C_{max} of 1,100 and 3,400 nM, respectively, lower than the JM6 IC_{50} of 4,000 nM reported by Zwilling et al., 2011 or of 20,000 nM determined by us, and not sufficiently high to inhibit KMO. In our studies, the biochemical potency of Ro-61-8048 on mouse KMO decreased in the presence of plasma proteins (IC_{50} = 90 to \geq 12,00 nM); the Ro-61-8048 binding to plasma proteins is >99%. Considering that the C_{max} previously reported in mice was ~280 nM additional work needs to be conducted, perhaps by dosing Ro-61-8048 directly, to confirm the mechanism of the PD response. While the KMO inhibitory potency of the JM6 metabolites observed in mouse plasma could not be investigated due to the confounding biological effects driven by the presence of Ro-61-8048, extensive SAR evaluation indicates that any substitution of thiazole adjacent to the sulfur renders compounds inactive against KMO (CHDI unpublished data).
Lastly, the different metabolic rates and preferential site of oxidation in vitro between species suggest that the major drug-related components observed in mouse plasma are predicted not to be present in circulation in the human, which will make the translation of the PD effects between species very challenging. In mice and rats in vitro JM6 is rapidly cleaved in vitro at the piperidyl moiety (with formation of an iminium ion), and these metabolites are found in circulation in vivo. In the human, the in vitro metabolism of JM6 proceeds primarily via O-dealkylation at the phenyl ring at a very slow rate.

In summary, our data demonstrates that JM6 is not a prodrug for Ro-61-8048; the minor impurity of Ro-61-8048 present in the JM6 dose is sufficient to elicit high plasma exposure due to the very slow clearance of Ro-61-8048 in mice.
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Authorship Contributions

Participated in research design: Beconi, Yates, Lyons, Matthews
Conducted experiments: Matthews, Clifton, Mead, Winkler, O’Connell,
Contributed new reagents or analytic tools: Prime and Walter
Performed data analysis: Beconi, Yates, Lyons, Matthews, Clifton, Winkler
Wrote or contributed to the writing of the manuscript: Beconi, Yates, Lyons, Matthews, Toledo Sherman, Munoz-Sanjuan, Dominguez
REFERENCES


Footnotes

Competing Interests Statement: CHDI Foundation is a privately-funded not-for-profit biomedical research organization exclusively dedicated to discovering and developing therapeutics that slow the progression of Huntington’s disease. The CHDI Foundation conducts research in a number of different ways; for the purposes of this manuscript, all research was conceptualized, planned, and directed by CHDI scientific staff and conducted at the contract research organizations BioFocus, Saretius, Evotec, and Albany Molecular Research Inc. (AMRI).
Legends for Figures

Figure 1. Synthesis of JM6 (Ro-61-8048 as precursor (Zwilling et al., 2011).

Figure 2. Alternate synthetic route for JM6, not involving Ro-61-8048 as precursor (A) and proposed mechanism of JM6 degradation to Ro-61-8048 (B).

Figure 3. Pharmacokinetics Ro-61-8048 in mice following oral administration alone (Group IV, 0.05 mg/kg) or in combination with JM6 (Group V, 0.05 mg/kg Ro-61-8048 with 10 mg/kg JM6) (N=3 per time point).

Figure 4. Proposed metabolism path for JM6 in mouse liver microsomes.

M2, M3, M4, M6, M7, M8 and JM6 (depicted in blue color) were confirmed by comparing chromatographic retention time and MS/MS spectra with that of synthetic standards, all other structures are proposed based on MS/MS spectra. The iminium ion was not detected, however, it was trapped as M9 or detected as its isomer, M13. M3, M8 and the iminium ion (M9) are the most significant metabolites in mouse liver microsomal incubations. M3, M4, and M8 are the most abundant metabolites in mouse plasma. M11 was present at trace levels. The enzymes responsible for metabolism are proposed; experiments to determine enzyme involvement in metabolism were not conducted. No additional metabolites were detected in hepatocytes.

Ro-61-8048 (<0.1%) was present in the JM6 stock solution (Time = 0) and remained below the assay LLOQ through the incubation.
Figure 5. Metabolite profile of JM6 in mouse, rat and human liver microsomes.

M2, M3, M4, M6, M7, M8, JM6 and Ro-61-8048 were quantified using synthetic standards, the concentrations of the remaining metabolites were estimated by comparison to the closest analogue after determining by comparison of MRM to UV signals, similar ionization intensity. Ro-61-8048 (<0.1%) was present in the JM6 stock solution (Time = 0) and remained below the assay LLOQ through the incubation. JM6 was extensively metabolized in mouse liver microsomes but was very stable in human liver microsomes.

Figure 6. Plasma exposure to drug-related material following a single 10 mg/kg oral administration of JM6 to mice.

Ro-61-8048 in these plasma samples originated from its low level amount in the JM6 dose. (AUC<sub>last</sub>, C<sub>max</sub>, T<sub>max</sub> and T<sub>last</sub> values are presented in Supplemental Table 1)

Figure 7. Ro 61-8048 and primary metabolites in mouse, rat and human liver microsomes

Structures proposed based on MS/MS spectra. Negligible metabolism was observed in mouse liver microsomes, however, oxidative metabolism was extensive in human liver microsomes. No additional metabolites were detected in hepatocytes.
Table 1. Pharmacokinetics of JM6 and Ro-61-8048 following a single intravenous bolus administration to mice

<table>
<thead>
<tr>
<th>Dose Route</th>
<th>Analyte Dosed (Dose Level)</th>
<th>Analyte</th>
<th>AUC$_{\text{last}}$ (nM×h)</th>
<th>AUC$_{\text{inf}}$ (nM×h)</th>
<th>AUCN (nM×h×kg/mg)</th>
<th>Cl$_p$ (L/h/kg)</th>
<th>Vd$_{\text{ss}}$ (L/kg)</th>
<th>MRT (h)</th>
<th>t$_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>JM6 $^a$ (5 mg/kg)</td>
<td>JM6 $^a$</td>
<td>5200</td>
<td>5200</td>
<td>1000</td>
<td>1.9</td>
<td>0.37</td>
<td>0.20</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Ro-61-8048 (5 mg/kg)</td>
<td>Ro-61-8048</td>
<td>1700</td>
<td>2100</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
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</tr>
</tbody>
</table>

$^a$ JM6 in the dose contained <0.4% Ro-61-8048
Table 2. Pharmacokinetics of JM6 and Ro-61-8048 in mice following oral administration alone or in combination

<table>
<thead>
<tr>
<th>Dose Route</th>
<th>Group</th>
<th>Analyte Dosed (Dose Level)</th>
<th>Analyte Measured</th>
<th>AUC\text{last} (nM×h)</th>
<th>AUC\text{inf} (nM×h)</th>
<th>AUC\text{N} (nM×h×kg/mg)</th>
<th>F (%)</th>
<th>C\text{max} (nM)</th>
<th>C\text{maxN} (nM)</th>
<th>T\text{max} (h)</th>
<th>T\text{1/2} (h)</th>
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<tbody>
<tr>
<td>PO</td>
<td>I</td>
<td>JM6 a (10 mg/kg)</td>
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<td>1100</td>
<td>110</td>
<td>7.2</td>
<td>100</td>
<td>110</td>
<td>0.5</td>
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<td></td>
<td></td>
<td>Ro-61-8048</td>
<td></td>
<td>2000</td>
<td>2300</td>
<td>NC</td>
<td>NC</td>
<td>230</td>
<td>NC</td>
<td>4.0 b</td>
<td>6.6</td>
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<td></td>
<td>II</td>
<td>Ro-61-8048 (0.05 mg/kg) with JM6 a (9.5 mg/kg)</td>
<td>JM6</td>
<td>1500</td>
<td>1500</td>
<td>160</td>
<td>9.8</td>
<td>1400</td>
<td>150</td>
<td>0.5</td>
<td>0.9</td>
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<td></td>
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<td>53000</td>
<td>110000</td>
<td>NC</td>
<td>9000</td>
<td>18000</td>
<td>0.5 b</td>
<td>6.9</td>
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<td></td>
<td>III</td>
<td>Ro-61-8048 (1.0 mg/kg) with JM6 (9.0 mg/kg)</td>
<td>JM6</td>
<td>1100</td>
<td>1100</td>
<td>130</td>
<td>7.8</td>
<td>1000</td>
<td>110</td>
<td>0.5</td>
<td>0.7</td>
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<td></td>
<td>Ro-61-8048</td>
<td></td>
<td>110000</td>
<td>110000</td>
<td>140000</td>
<td>NC</td>
<td>17000</td>
<td>21000</td>
<td>0.5 b</td>
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<td>IV</td>
<td>Ro-61-8048 (0.05 mg/kg) alone</td>
<td>Ro-61-8048</td>
<td>4300</td>
<td>4500</td>
<td>90000</td>
<td>70</td>
<td>550</td>
<td>11000</td>
<td>0.5 b</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>Ro-61-8048 b (0.05 mg/kg) with JM6 (10 mg/kg)</td>
<td>JM6</td>
<td>1300</td>
<td>1300</td>
<td>130</td>
<td>13</td>
<td>1900</td>
<td>190</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ro-61-8048</td>
<td></td>
<td>4900</td>
<td>5200</td>
<td>100000</td>
<td>80</td>
<td>530</td>
<td>11000</td>
<td>1.0 b</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>Ro-61-8048 (0.05 mg/kg) with JM6 c (25 mg/kg)</td>
<td>JM6</td>
<td>4800</td>
<td>4800</td>
<td>190</td>
<td>NC</td>
<td>3400</td>
<td>140</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ro-61-8048</td>
<td></td>
<td>8400</td>
<td>8800</td>
<td>180000</td>
<td>NC</td>
<td>800</td>
<td>16000</td>
<td>2.0 b</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>Ro-61-8048 (0.05 mg/kg) with JM6 c (50 mg/kg)</td>
<td>JM6</td>
<td>9600</td>
<td>9600</td>
<td>190</td>
<td>NC</td>
<td>8100</td>
<td>190</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ro-61-8048</td>
<td></td>
<td>7900</td>
<td>NC</td>
<td>160000</td>
<td>NC</td>
<td>19000</td>
<td>160000</td>
<td>4.0 b</td>
<td>6.5</td>
</tr>
</tbody>
</table>

a JM6 in the dose contained <0.4% of Ro-61-8048.b The t\text{max} for Ro-61-8048 is observed between 0.5 and 4 hr and difficult to define precisely in non-serial studies due to the leveled protracted plasma concentration vs time curve driven by the slow Cl\text{p}.
Table 3. *In Vitro* metabolic stability, permeability and transport

<table>
<thead>
<tr>
<th>In Vitro System</th>
<th>Parameter (units)</th>
<th>Ro-61-8048</th>
<th></th>
<th></th>
<th>JM6</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mouse</td>
<td>Rat</td>
<td>Human</td>
<td>Mouse</td>
<td>Rat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Metabolic Stability</strong></td>
<td>$t_{1/2}$ (min)</td>
<td>$&gt;97$</td>
<td>$&gt;87$</td>
<td>$12 / 14$</td>
<td>$12$</td>
<td>$45$</td>
</tr>
<tr>
<td>Liver Microsomes</td>
<td>$Cl_{int}$ ($\mu$L/min/mg)</td>
<td>$&lt;29$</td>
<td>$&lt;32$</td>
<td>$200 / 230$</td>
<td>$220$</td>
<td>$62$</td>
</tr>
<tr>
<td></td>
<td>$E_h$</td>
<td>$&lt;0.5 / &lt;0.5$</td>
<td>ND</td>
<td>$0.8$</td>
<td>ND</td>
<td>$0.83$</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>$t_{1/2}$ (min)</td>
<td>$&gt;200 / 200$</td>
<td>ND</td>
<td>$88$</td>
<td>$43$</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>$Clint$ ($\mu$L/min/M cells)</td>
<td>$&lt;14 / &lt;14$</td>
<td>ND</td>
<td>$16$</td>
<td>$69$</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>$E_h$</td>
<td>$&lt;0.5 / &lt;0.5$</td>
<td>ND</td>
<td>$0.8$</td>
<td>ND</td>
<td>$0.83$</td>
</tr>
<tr>
<td>Plasma</td>
<td>$t_{1/2}$ (h)</td>
<td>$&gt;24$</td>
<td>$&gt;24$</td>
<td>$&gt;24$</td>
<td>$&gt;24$</td>
<td>$&gt;24$</td>
</tr>
<tr>
<td>Simulated Gastric Fluid</td>
<td>$t_{1/2}$ (h)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Permeability and Transport</strong></td>
<td>$P_{app}$ A-B / B-A (nm/s)</td>
<td>ND</td>
<td>ND</td>
<td>$170 / 130$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MDCK-WT</td>
<td>$E_{efflux}$ Ratio (ER)</td>
<td>ND</td>
<td>ND</td>
<td>$0.8$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MDCK-MDR1</td>
<td>$P_{app}$ A-B / B-A (nm/s)</td>
<td>ND</td>
<td>ND</td>
<td>$100 / 330$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>$E_{efflux}$ Ratio (ER)</td>
<td>ND</td>
<td>ND</td>
<td>$3.2$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MDCK-(MDR1/WT)</td>
<td>$E_{efflux}$ Ratio (EER)</td>
<td>ND</td>
<td>ND</td>
<td>$4.2$</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not determined
Table 4. Determination of the inhibitory potential of Ro 61-8048, JM6, M2 and M8 hepatic lysates of mouse, rat or human KMO

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse KMO</td>
</tr>
<tr>
<td>Ro-61-8048&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90 ± 15</td>
</tr>
<tr>
<td>Ro-61 8048&lt;sup&gt;a&lt;/sup&gt; (3 mg/mL protein)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27,000/12,000</td>
</tr>
<tr>
<td>JM6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20,000 ± 2500</td>
</tr>
<tr>
<td>M2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22,000 ± 0</td>
</tr>
<tr>
<td>M8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27,000 ± 0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± standard deviation of values obtained on three different days with triplicate determinations per day. <sup>b</sup> Individual results obtained on two different days with triplicate determinations per day. The low level activity observed for JM6 could have been from the low level presence of Ro-61-8048 in the JM6, M2 and M8 stocks. All JM6 metabolites synthesized, except for M3, underwent low level degradation to Ro-61-8048; (see Figure 2.(B)) and it was not possible to determine their biological activity in the absence of Ro-61-8048 however, their SAR for activity is not considered favorable.
FIGURE 1

\[
\text{Br} \quad \text{NO}_2 \quad \text{H}_2\text{N} \quad \text{NH}_2 \quad \text{EtOH} \quad 80^\circ\text{C} \quad \text{H}_2\text{N} \quad \text{NH} \quad \text{EtOH} \quad \text{HCHO}
\]

Ro-61-8048

JM6
Supplemental Information

Metabolism and Pharmacokinetics of JM6 in Mice: JM6 is Not a Prodrug for Ro-61-8048

Maria G. Beconi, Dawn Yates, Kathryn A. Lyons, Kim Matthews, Steve Clifton, Tania Mead, Michael Prime, Dirk Winkler, Catherine O’Connell, Daryl Walter, Leticia Toledo Sherman, Ignacio Munoz Sanjuan and Celia Dominguez

Supplemental Methods

Preparation Dose Formulations Containing 0.05 mg/kg Ro 61-8048 in JM6

To achieve 0.05 mg/kg of Ro-61-8048 in the dose with accuracy, an amount of Ro-61-8048 equal to the difference between 0.05 mg/kg and the level of the impurity was added to the dose formulation, as follows.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose of JM6 stock (mg/kg)</th>
<th>Resulting JM6 dose (mg/kg)</th>
<th>Dose of Ro-61-8048 (mg/kg)</th>
<th>Total Ro-61-8048 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>V</td>
<td>10</td>
<td>9.992</td>
<td>0.008</td>
<td>0.042</td>
</tr>
<tr>
<td>VI</td>
<td>25</td>
<td>24.98</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>VII</td>
<td>50</td>
<td>49.96</td>
<td>0.04</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Ro-61-8048 was diluted in dose formulation vehicle and the corresponding volume was added into the JM6 formulation prepared at twice the required concentration, to ensure accuracy of the small amount added.
Additional Details of the JM6 and Ro 61-8048 JM6 Bioanalytical Methodology

Calibration standards for JM6, Ro-61-8048 and JM6 metabolites were prepared in duplicate at each concentration. When synthetic standards were not available, semi-quantitation of the metabolite was performed by comparison of relative peak intensities against those of a metabolite synthetically available where a similar UV/MRM response factor had been previously determined. To help verify no metabolite was missed, 1 h post-dose samples from mice administered a 50 mg/kg po dose of JM6 (plus 0.05 mg/kg Ro-61-8084) were pooled, extracted as described previously and extracts were scanned over a mass range of 340 to 800 amu in both positive and negative ionization mode.

At a minimum, 75% of all the calibration standards and at least two calibration standards per concentration met the accuracy and precision of ±30%. The coefficient of variation (CV%) of the IS signal/area response for the entire run was within ±30%. There was no bias in the accuracy or precision of the calibration standards and no bias or trend in the IS signal/area response for the run to be acceptable. The mean concentrations of the calibration standards at the front end versus those at the back end of calibration curve were within ±15% of each other. All samples were analyzed within 10-days of sample collection.

Synthesis and purification of JM6, Ro 61-8048 and metabolites used in these studies

Synthesis of JM6 and Ro 61-8048 According to Zwilling et al., 2011

JM6 (3,4-dimethoxy-N-(4-(3-nitrophenyl)-5-(piperidin-1-ylmethyl)thiazol-2-yl)benzenesulfonamide), and Ro-61-8048 (3,4-dimethoxy-N-(4-(3-nitrophenyl)thiazol-2-yl)benzenesulfonamide) as described in patent WO2008/022281 (Figure 1). In brief, Ro-61-8048 was prepared by the condensation of 2-bromo-1-(3-nitrophenyl)ethanone with thiourea to produce 4-(3-(nitrophenyl)thiazol-2-amine as the hydrobromide salt, a modification
of the patent procedure which isolated the free base. Subsequent condensation of this salt intermediate with 3,4-dimethoxybenzenesulfonyl chloride in pyridine afforded the final sulfonamide product 3,4-dimethoxy-N-(4-(3-nitrophenyl)thiazol-2-yl)benzenesulfonamide. JM6 was synthesized from Ro-61-8048 via the Mannich reaction with piperidine and formaldehyde.

**Synthesis of the 4-(3-nitrophenyl)-1,3-thiazol-2-amine intermediate (Figure 1, Step 1):**

2-Bromo-1-(3-nitrophenyl)ethanone (10.0 g, 41.0 mmol) and thiourea (3.12 g, 41.0 mmol) were dissolved in EtOH (200 mL) heated to reflux and stirred at this temperature for 2 h. After this period the reaction mixture was cooled to room temperature (RT), the resulting precipitate was collected by filtration and dried under vacuum to give the title compound (10.9 g, 88% yield) as a yellow powder. \( \delta_H (500 \text{ MHz, DMSO}) 8.52 - 8.61 (m, 1H), 8.15 - 8.26 (m, 2H), 7.75 (t, J = 8.04 \text{ Hz, 1H}), 7.49 (s, 1H), 7.95 (br. s, 3H). \) \( m/z \) (ES+) (M+H+) 222.

**Synthesis of 3,4-dimethoxy-N-[4-(3-nitrophenyl)-1,3-thiazol-2-yl]benzene-1-sulfonamide (Ro 61-8048) (Figure 1, Step 2):** 3,4-Dimethoxybenzene sulfonyl chloride (7.44 g, 31.4 mmol) was added portion wise to a stirred solution of 4-(3-nitrophenyl)-1,3-thiazol-2-amine (10.0 g, 33.1 mmol) in dry pyridine (150 mL) and the reaction was stirred at RT under a nitrogen atmosphere for 42 h. After this time, the reaction mixture was concentrated and the resulting residue partitioned between ethyl acetate (150 mL) and ice (100 g). \( \text{NaOH} \) (1 M solution, 100 mL) was carefully added to bring the mixture to pH 9 and the aqueous layer was separated. The organic layer was extracted with \( \text{NaOH} \) (1 M solution, 2 x 50 mL), the aqueous layers were combined, acidified to pH 5 with HCl (6 M solution, 20 mL) and extracted with ethyl acetate (2 x 100 mL). The organic extracts were combined, washed with water (3 x 50 mL) and brine (2 x 50 mL) before being dried (MgSO\(_4\)), filtered and concentrated. The resulting residue was re-crystallised from ethyl acetate to give the title compound (3.12 g, 22% yield) as an off-white
powder. $\delta_H$ (500 MHz, DMSO) 13.30 (br. s., 1H), 8.61 (s, 1H), 8.12 - 8.26 (m, 2H), 7.73 (t, J = 8.04 Hz, 1H), 7.57 (br. s., 1H), 7.46 (dd, J = 0.55, 1.66 Hz, 1H), 7.29 - 7.36 (m, 1H), 7.11 (d, J = 8.51 Hz, 1H), 3.82 (s, 3H), 3.81 (s, 3H). $\text{Tr} = 3.98 \text{ min} \ m/z (\text{ES}^+) (\text{M}+\text{H}^+) 422.$

**Synthesis of 3,4-dimethoxy-N-[4-(3-nitrophenyl)-5-(piperidin-1-ylmethyl)-1,3-thiazol-2-yl]benzene-1-sulfonamide (JM6) (Figure 1, Step 3):** Formaldehyde (37% aq. solution, 5.4 g, 66.6 mmol), followed by piperidine (5.92 mL, 59.9 mmol) were added sequentially to a stirred solution of 3,4-dimethoxy-N-[4-(3-nitrophenyl)-1,3-thiazol-2-yl]benzene-1-sulfonamide (3.30 g, 6.66 mmol) in ethanol (120 mL) and stirred at RT under a nitrogen atmosphere for 3 h. After this time the resulting precipitate was collected by filtration, washed with ethanol (3 x 50 mL), before being dried under vacuum to give the title compound (2.67 g, 75% yield) as a pale yellow powder. $\delta_H$ (500 MHz, DMSO) 12.89 (br. s., 1 H) 8.43 (br. s., 1 H) 8.26 (dd, J=8.20, 0.95 Hz, 1 H) 7.93 (d, J=7.88 Hz, 1 H) 7.75 (t, J=7.96 Hz, 1 H) 7.43 (dd, J=8.35, 1.73 Hz, 1 H) 7.30 (d, J=2.05 Hz, 1 H) 7.08 (d, J=8.35 Hz, 1 H) 3.81 (s, 3 H) 3.80 (s, 3 H) 3.53 (br. s., 2 H) 2.44 (br. s., 4 H) 1.53 (br. s., 4 H) 1.39 (br. s., 2 H). $\text{Tr} = 2.95 \text{ min} \ m/z (\text{ES}^+) (\text{M}+\text{H}^+) 520.$

**Alternate Synthesis of JM6, Without Ro 61-8048 as Precursor**

**Synthesis of 1-(3-Nitro-phenyl)-prop-2-en-1-ol (Figure 2, Step 1):** Vinyl magnesium chloride (1M solution in THF, 79.0 mL, 79.0 mmol) was added drop wise over 10 minutes to a stirred, cold (-70 °C), solution of 3-nitrobenzaldehyde (10.0 g, 66.0 mmol) in dry THF (85 mL) under a nitrogen atmosphere. After warming to 0°C, the reaction was stirred at this temperature for 1 h before being treated with aqueous saturated ammonium chloride (150 mL), followed by water (100 mL), and ethyl acetate (100 mL). The organic layer was separated, and the aqueous extracted with ethyl acetate (2 x 100 mL). The combined organic extracts were dried (Na$_2$SO$_4$), filtered, concentrated and the resulting residue then co-evaporated with dichloromethane (2 x 50
mL) to give the title compound (11.8 g, 99% yield) as a yellow solid. $^1$H NMR (500 MHz, CDCl$_3$) δ ppm 8.27 (s, 1 H) 8.12 - 8.19 (m, 1 H) 7.74 (d, $J$=7.72 Hz, 1 H) 7.54 (t, $J$=7.88 Hz, 1 H) 6.03 (ddd, $J$=17.06, 10.36, 6.46 Hz, 1 H) 5.43 (d, $J$=17.18 Hz, 1 H) 5.27 - 5.35 (m, 2 H) 1.47 - 1.77 (m, 1 H). Tr = 1.63 min (3.5 min method) m/z (ES$^+$) no ionisation.

_Synthesis of 1-(3-Nitro-phenyl)-propenone (Figure 2, Step 2):_ Dess-Martin reagent (30.7 g, 72.4 mmol) was added portion wise over 5 min to a cooled (0 ºC), stirred solution of 1-(3-nitro-phenyl)-prop-2-en-1-ol (10.8 g, 60.3 mmol) in dry dichloromethane (240 mL). The mixture was warmed to RT over 2 h and stirred overnight. Additional Dess-Martin reagent (15.2 g, 35.8 mmol) was added, stirring was continued at RT for 3 h. After this time, the reaction mixture was diluted with dichloromethane (200 mL) and aqueous saturated sodium bicarbonate (300 mL), shaken, filtered, and the organic layer separated. The aqueous layer was extracted with dichloromethane (2 x 300 ml), and the combined organic layers were dried (Na$_2$SO$_4$), filtered and concentrated. The resulting residue was absorbed from dichloromethane (200 mL) onto silica gel (Merck 9385, 130 mL) and the silica purified on a Biotage Isolera machine (750 g column of silica). The column was eluted with ethyl acetate-heptane (1% EtOAC, 99% heptanes to 30% EtOAc / 70% heptanes) to give the title compound (3.33 g, 31% yield) as a white solid. $^1$H NMR (500 MHz, CDCl$_3$) δ ppm 8.78 (s, 1 H) 8.39 - 8.53 (m, 1 H) 8.29 (d, $J$=7.72 Hz, 1 H) 7.72 (t, $J$=7.96 Hz, 1 H) 7.19 (dd, $J$=17.02, 10.56 Hz, 1 H) 6.54 (dd, $J$=17.18, 1.26 Hz, 1 H) 6.08 (dd, $J$=10.56, 1.26 Hz, 1 H). Tr = 1.72 min (3.5 min method) m/z (ES$^+$) no ionisation.

_Synthesis of (3-Nitro-phenyl)-oxiranyl-methanone (Figure 2, Step 3):_ A mixture of hydrogen peroxide (35% solution, 0.99 mL, 11.3 mmol) and 2M sodium hydroxide 1.14 mL, 2.82 mmol) was cooled in ice for 10 min and added drop wise over 5 min to a stirred, cooled (0 ºC), solution of 1-(3-nitro-phenyl)-propenone (1.0 g, 5.65 mmol) in methanol (8.5 mL) and 1,4-
dioxane (5.6 mL). After stirring for 1 h at 0 °C, the reaction was quenched by the addition of acetic acid (1.6 mL) in water (6 mL) and the resulting mixture was concentrated in vacuo to a volume of approximately 6 mL. Water (30 mL) was added, and the mixture extracted with dichloromethane (3 x 30 mL). The combined organic extracts were washed with 50% aqueous saturated sodium sulfite solution (30 mL), separated, and the aqueous phase extracted with dichloromethane (50 mL). The combined organic extracts were dried (Na₂SO₄), filtered and concentrated to give the title compound (0.89 g, 72% yield) as a cream solid. ¹H NMR (500 MHz, CDCl₃) δ ppm 8.89 (t, J=1.73 Hz, 1 H) 8.49 (dd, J=8.20, 1.10 Hz, 1 H) 8.39 (d, J=7.72 Hz, 1 H) 7.71 - 7.79 (m, 1 H) 4.23(dd, J=4.41, 2.36 Hz, 1 H) 3.22 (dd, J=6.15, 4.57 Hz, 1 H) 3.02 (dd, J=6.23, 2.44 Hz, 1 H). Tr = 1.46 min (3.5 min method) m/z (ES⁺) no ionisation.

Synthesis of [2-Amino-4-(3-nitro-phenyl)-thiazol-5-yl]-methanol (Figure 2, Step 4): A mixture of thiourea (1.57 g, 20.58 mmol) and (3-nitro-phenyl)-oxiranyl-methanone (0.79 g, 4.12 mmol) in ethanol (25.0 mL) was stirred overnight at RT under a nitrogen atmosphere. The reaction mixture was then concentrated, absorbed from methanol onto silica gel (15 mL) and the resultant silica was purified on a Biotage machine (100g column of silica gel) eluting with dichloromethane-methanol (0.5% MeOH, 99.5% DCM to 5% MeOH, 95% DCM) to give the title compound (0.29 g, 24% yield) as a yellow solid. ¹H NMR (500 MHz, DMSO-d₆) δ ppm 8.45 - 8.58 (m, 1 H) 8.13 - 8.19 (m, 1 H) 8.01 - 8.08 (m, 1 H) 7.67 - 7.77 (m, 1 H) 7.03 - 7.14 (m, 2 H) 5.48 - 5.59 (m, 1 H) 4.45 - 4.61 (m, 2 H). Tr = 1.08 min (3.5 min method) m/z (ES⁺) 252.

Synthesis of N-[5-Hydroxymethyl-4-(3-nitro-phenyl)-thiazol-2-yl]-3,4-dimethoxy-benzenesulfonamide (Figure 2, Step 5): 3,4-Dimethoxybenzenesulfonyl chloride (0.41 g, 1.73 mmol) was added portion wise over 5 min to a stirred, cooled (0 °C), solution of [2-amino-4-(3-nitro-phenyl)-thiazol-5-yl]-methanol (0.29 g, 1.15 mmol) in pyridine (10 mL) and the reaction
mixture was stirred at RT under nitrogen for 18 hs. After this time, further 3,4-
dimethoxybenzenesulfonyl chloride (0.13 g, 0.57 mmol) was added and stirring was continued
for 3 hs. The reaction mixture was then evaporated and then azeotroped with toluene (2 x 10
mL). The resulting residue was absorbed from methanol onto silica gel (Merck 9385, 10 mL) and
was purified on a Biotage machine (100g cartridge of silica gel) eluting with dichloromethane-
methanol (0.5% MeOH, 99.5% DCM to 4%MeOH, 96% DCM) to give the title compound (0.13
g, 24% yield) as a light brown gum. Tr = 1.59 min (3.5 min method) m/z (ES+) 452.

Synthesis of 7: 3,4-Dimethoxy-N-[4-(3-nitro-phenyl)-5-piperidin-1-ylmethyl-thiazol-2-
yl]-benzenesulfonamide (Figure 2, Step 6): A mixture of N-[5-hydroxymethyl-4-(3-nitro-
phenyl)-thiazol-2-yl]-3,4-dimethoxy-benzenesulfonamide (0.12 g, 0.265 mmol) and thionyl
chloride (2.5 mL) was heated to 60 °C and stirred at this temperature for 2 hs. After this time the
reaction mixture was cooled to RT, concentrated and treated with dry THF (2.5 mL) followed by
piperidine (2 mL) and the resulting mixture was stirred for 30 min at RT. The reaction mixture
was then concentrated, treated with aqueous saturated sodium bicarbonate (20 mL) and extracted
with ethyl acetate (3 x 30 mL). The combined, organic extracts were dried (Na₂SO₄), filtered and
concentrated, the residue was purified on a Biotage machine (25g cartridge of silica gel) and
eluted with dichloromethane-methanol (0.5% MeOH, 99.5% DCM to 4%MeOH, 96% DCM) to
give a yellow solid (51 mg). The yellow solid was stirred with aqueous saturated sodium
bicarbonate (2 mL) for 1 h at RT, the precipitate was filtered off, washed with water (2 x 1 mL)
and dried in a vacuum oven at 40 °C for 15 hs to give the title compound (0.02g, 12% yield) as
an off-white solid. ¹H NMR (500 MHz, DMSO-d6) δ ppm 12.49 - 13.07 (m, 1 H) 8.35 - 8.49 (m,
1 H) 8.26 (d, J=8.51 Hz, 1 H) 7.92 (d, J=7.72 Hz, 1 H) 7.75 (t, J=7.88 Hz, 1 H),7.42 (d, J=8.35
Hz, 1 H) 7.26 - 7.34 (m, 1 H) 7.07 (d, J=8.67 Hz, 1 H) 3.80 (s, 3 H) 3.79 (s, 3 H) 3.38 - 3.63 (m, 2 H) 2.18 - 2.48 (m, 4 H) 1.27 - 1.62 (m, 6 H). Tr = 2.87 min (7 min method) m/z (ES+) 519.

Synthesis of putative metabolites of JM6

Synthesis of N-[5-(Hydroxymethyl)-4-(3-nitrophenyl)-1,3-thiazol-2-yl]-3,4-dimethoxybenzene-1-sulfonamide (M2): A 37% solution of formaldehyde in water (1 mL, 13.35 mmol) was added in one portion to a stirred solution of 3,4-dimethoxy-N-[4-(3-nitrophenyl)-1,3-thiazol-2-yl]benzene-1-sulfonamide (0.16 g, 0.38 mmol) in DMSO (0.6 mL) at RT under a nitrogen atmosphere. The resulting precipitate was dissolved by the addition of NaOH (21 mg, 0.53 mmol) in water (0.6 mL). The solution was then heated directly at 50ºC in a pressure tube for 16 h. After this time, the reaction was cooled to RT and EtOAc (10 mL) was added. The organic layer was washed sequentially with water (10 mL), brine (5 mL) and 10% citric acid (10 mL). The organic layer was separated and the aqueous further extracted with EtOAc (2 x 5 mL). The combined organic layers were washed with brine (5 mL), dried over MgSO4, filtered and then concentrated. The resulting residue was purified by prep HPLC to give the crude title compound (0.18 g, 98% yield) as a yellow solid. δH (500 MHz, MeOD) 8.42 (t, J=2.05 Hz, 1 H) 8.28 - 8.34 (m, 1 H) 7.91 (dt, J=7.84, 1.36 Hz, 1 H) 7.73 (t, J=7.96 Hz, 1 H) 7.55 (dd, J=8.51, 2.05 Hz, 1 H) 7.44 (d, J=2.05 Hz, 1 H) 7.06 (d, J=8.51 Hz, 1 H) 4.57 (s, 2 H) 3.88 (s, 3 H) 3.87 (s, 3 H). Tr = 3.40 min m/z (ES+) (M+H+) 452.

Synthesis of N-[5-(aminomethyl)-4-(3-nitrophenyl)-1,3-thiazol-2-yl]-3,4-dimethoxybenzene-1-sulfonamide (M8): Formaldehyde (37% aqueous solution, 0.12 g, 0.49 mmol), followed by diallylamine (0.06 mL, 0.49 mmol) were added sequentially to a stirred solution of 3,4-dimethoxy-N-[4-(3-nitrophenyl)-1,3-thiazol-2-yl]benzene-1-sulfonamide (0.21 g, 0.49 mmol) in ethanol (10 mL) and stirred at RT under a nitrogen atmosphere for 3 h. After this
time the resulting mixture was concentrated to give N-(5-\{[bis(prop-2-en-1-yl)amino]methyl\}-4-(3-nitrophenyl)-1,3-thiazol-2-yl)-3,4-dimethoxybenzene-1-sulfonamide (2.67 g, 75% yield) as an orange gum which was taken forwards directly without further purification. \( \text{Tr} = 1.76 \ \text{min} \) \( m/z \) (ES\(^+\)) (M+H\(^+\)) 531. N,N-Dimethyl barbituric acid (0.18 g, 0.2 mmol) and Pd(PPh\(_3\))\(_4\) (0.13 g, 0.11 mmol) were added portion wise over 5 min to a solution of N-(5-\{[bis(prop-2-en-1-yl)amino]methyl\}-4-(3-nitrophenyl)-1,3-thiazol-2-yl)-3,4-dimethoxybenzene-1-sulfonamide (0.26 g, 0.46 mmol) in DCM (20 mL) and the mixture was heated to reflux under a nitrogen atmosphere for 16 h. After this time, the reaction mixture was cooled to RT and concentrated. The resulting residue was dissolved in DMSO (2 mL) and purified by Prep HPLC to give the title compound (0.003 g, 2% yield) as a yellow powder. \( \delta_H \) (500 MHz, MeOD) 8.34 (t, J = 1.73 Hz, 1H), 8.23 (dd, J = 1.42, 8.20 Hz, 1H), 7.86 (d, J = 7.88 Hz, 1H), 7.65 (t, J = 7.96 Hz, 1H), 7.53 (dd, J = 2.05, 8.35 Hz, 1H), 7.46 (d, J = 1.89 Hz, 1H), 6.97 (d, J = 8.51 Hz, 1H), 4.17 (s, 2H), 3.84 (s, 3H), 3.83 (s, 3H). \( \text{Tr} = 2.64 \ \text{min} \) \( m/z \) (ES\(^+\)) (M+H\(^+\)) 451.

**JM6 Purification and Purity Determination**

JM6 was purified by sequential triturations in boiling EtOH, followed by EtOH at RT and by further triturations in acetone at RT. After each trituration stage the compound was isolated by filtration, dried under vacuum and submitted to NMR and LC-MS analysis.

NMR data was collected using a Bruker 500MHz, in deuterated DMSO. The spectrum was consistent with that reported in the patent for JM6 (WO2008/022281). LC-MS analysis was conducted using a Shimadzu LCMS-EV2010 system with integrated serial HPLC-UV-MS instruments. Source parameters were as follows: CDL temperature 230°C, Heater block temperature 250 °C, Nebulizer flow 1.5 L/min, Q-Array DC 10 V, Q-Array RF 130 V. The chromatography was conducted on an Atlantis dC18 column (Waters Corporation).
(2.1mmx100mm; 3µm) using a flow rate of 0.6 mL/min and a column temperature of 40°C. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The column was equilibrated with 5% B for 0.5-min. After analyte injection, the gradient increased linearly from 5% B to 100% B in 5 min, and was held constant at 100% B for 0.4 min. UV detection was performed at 215 nm; mass detection was performed over the range 150-750Da. The molecular ion corresponding to JM6 \((m/z = 519)\), the primary reaction product eluted at 2.95 min and represented 98.6% of the UV trace. In the original stock of JM6 used for the first set of studies, impurities were present at 3.40 min (1.1% UV area; \(m/z = 477\)) and 4.37 min (0.3% UV area; \(m/z = 699\)), in the positive ionization mode.

**Quantitation of Ro 61-8084 in Purified JM6 (Additional Details)**

Three independent control stock solutions were prepared: (1) JM6 stock solution, where JM6 (0.20 mg) of was dissolved in 1 mL of acetonitrile:water (1:1, v:v) to give a 0.2 mg/mL solution. (2) JM6 “spiked” with Ro-61-8048, where JM6 (0.20 mg) was dissolved in 1 mL for a 0.02 mg/mL solution of Ro-61-8048 in acetonitrile:water (1:1, v:v), and (3) Ro-61-8048, where Ro-61-8048 (0.20 mg) was dissolved in 10 mL acetonitrile:water (1:1, v:v), followed by serial dilution to achieve a 100 ng/mL solution.

Ro-61-8048 calibration standards were prepared by dissolving 1.00 mg of Ro-61-8048 in 10 mL acetonitrile:water (1:1, v:v; 0.1 mg/mL), followed by serial dilutions in acetonitrile:water (1:1, v:v), to achieve concentrations of 1250, 625, 250, 125, 62.5, 37.5 (LOQ), 25, 18.75, 12.5 ng/mL. The calibration curve was considered acceptable when individual concentrations were within ±15% of the expected value (±20% for the LLOQ). Data were processed using the MassHunter Quantitative Analysis software.
The spiked sample was analyzed using full scan mass detection switching between positive and negative ion modes coupled with diode array UV detection. The scan range was 100 – 1000 Da for 500 ms, the fragmentor voltage was 135 V. Calibrants and other samples were analyzed using MRM and diode array UV detection. The most intense MRM transition (m/z 422.1→137.2, fragmentor = 60 V, CE= 12 V) was used to quantify **Ro-61-8048**, transitions m/z 422.1→77.2 and m/z 422.1→153.2 were used as secondary qualifiers.
Supplemental Results

Metabolites in Mouse Liver Microsomes and Hepatocytes

Metabolism was initiated by hydroxylation $\alpha$ to the piperidyl nitrogen via Path 1, where hydroxylation occurred at the ring (M5, $m/z$ 533.61, 2-hydroxypiperidine), or Path 2, with hydroxylation at the adjacent methyl (M1, $m/z$ 533.61, N-(5-hydroxy(piperidin-1-yl)).

M5 is proposed to be in equilibrium with an iminium ion reactive intermediate which is further oxidized to the lactone (M6, $m/z$ 531.60), followed by the ring-opened acid (M7, $m/z$ 550.61), and subsequent N-dealkylation (M8, $m/z$ 449.50).

M1 primarily underwent non-enzymatic cleavage to form an aldehyde (M3, $m/z$ 448.61, N-(5formyl)), and further reduction likely by aldehyde reductase to yield N-5(hydroxymethyl) (M2, $m/z$ 450.61). It is also possible for M1 to undergo N-dealkylation releasing the piperidine to yield N-5(hydroxymethyl) (M2, $m/z$ 450.61), and be further oxidized to the aldehyde (M3, $m/z$ 448.61, N-(5formyl)), not depicted in Figure 4. M1 is also proposed to be in equilibrium with the iminium ion reactive intermediate. Subsequent oxidation to the acid (M4, $m/z$ 464.46) was detected at very low levels only when incubated at higher (10 µM) JM6 concentrations. M3 oxidation to form M4 is most likely catalyzed residual non-CYP enzyme activity.

O-demethylation of JM6 (M10 and M11) at the di-methoxy benzenesulfonamide moiety was negligible in vitro. Demethylation of JM6 metabolites was not detected.

The presence of an iminium ion reactive intermediate was inferred from the formation of a cyanide adduct M9 in trapping experiments and the presence of a possible iminium isomer, dehydro-piperidine (enamine M13 $m/z$ 515) when methanol was used as extraction solvent. When mouse liver microsomal incubations were enriched with potassium cyanide, a new metabolite, M9 was detected. This metabolite showed a neutral loss of -27 Da, characteristic of a
cyanide adduct, when the multiple reaction monitoring (MRM) transition of 544 to 517 amu was monitored by LC-MS/MS. (Supplemental Figure 2 (A) and 1 (B)). Comparison of the M9 to the JM6 spectra (Supplemental Figure 2 C) and 5 (D)), suggested that cyanide conjugation occurred in the piperidine ring, the fragment ion of m/z 434 in both spectra indicates the remaining part of the molecule is intact.
Supplemental Figures

Supplemental Figure 1. Concentration vs time profiles of JM6 and Ro-61-8048 supporting the pharmacokinetic summaries presented in Table 2 of the manuscript.

PO Doses - Group I = 10 mg/kg JM6; Group II = 9.5 mg/kg JM6/0.5 mg/kg Ro-61-8048; Group III = 9.0 mg/kg JM6/1 mg/kg Ro 61-8048; Group IV = 0.05 mg/kg Ro-61-8048, Group V = 10 mg/kg JM6/0.05 mg/kg Ro-61-8048; Group VI = 25 mg/kg JM6/0.05 mg/kg Ro 61-8048; Group VII = 50 mg/kg JM6/0.05 mg/kg Ro-61-8048. The graph in panel B is presented in the main manuscript as Figure 3, its “Y”-axis was brought to the same scale as the other graphs on this figure.
Supplemental Figure 2. Characterization of the iminium ion reactive intermediate by cyanide trapping.

Total ion chromatogram (TIC) of multiple reaction monitoring (MRM), 544 to 517 amu (loss of 27 Da), following JM6 incubations in mouse liver microsomes in the absence (A) and presence (B) of 10 mM potassium cyanide. MS/MS spectra of protonated JM6 (C; m/z 519) and protonated cyanide adduct M9 (D, m/z 544) with proposed fragmentation.
Supplemental Table 1 Plasma exposure to drug-related material following a single 10 mg/kg oral administration of JM6 to mice.

<table>
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<th>Metabolite</th>
<th>$\text{AUC}_{0-\text{last}}$ (nM x h)</th>
<th>$T_{\text{last}}$</th>
<th>Observed $C_{\text{max}}$ (nM)</th>
<th>Observed $T_{\text{max}}$ (h)</th>
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<td>JM6</td>
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