Metabolism and Pharmacokinetics of JM6 in Mice: JM6 Is not a Prodrug for Ro-61-8048

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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 (Cell 145:863–874, 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. After a 0.05 mg/kg Ro-61-8048 oral dose alone or coadministered with 10 mg/kg JM6 to mice, the Ro-61-8048 areas under the concentration-time curves (AUCs) from 0 to infinity were similar (4300 and 4900 nM × h, 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 in vitro (plasma, blood, or hepatic models). Species differences in the 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 is slowly metabolized in human (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 Schwarz and collaborators (Schwarz et al., 1983, 2010; Foster et al., 1985) has shown that the levels of several metabolites in the kynurenine pathway are altered in Huntington’s disease (HD) postmortem human brain samples and in various HD mouse models. Their work further suggested that cytotoxicity induced by quinolinic acid may be involved in the pathophysiology of HD (Sathyasaikumar et al., 2010). Taken together, this body of work prompted us to initiate, approximately 5 years ago, a medicinal chemistry program to develop inhibitors of kynurenine 3-monooxygenase (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), in which 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

ABBREVIATIONS: HD, Huntington’s disease; KMO, kynurenine 3-monooxygenase; JM6, 3,4-dimethoxy-N-(4-(3-nitrophenyl)-5-(piperidin-1-ylmethyl)thiazol-2-yl)benzenesulfonamide; Ro-61-8048, 3,4-dimethoxy-N-(4-(3-nitrophenyl)thiazol-2-yl)benzenesulfonamide; P450, cytochrome P450; DMSO, dimethyl sulfoxide; IS, internal standard; LC, liquid chromatography; MS/MS, tandem mass spectrometry; UPLC, ultra-performance liquid chromatography; MRM, multiple reaction monitoring; LLOQ, lower limit of quantitation; AUC, area under the concentration versus time curve; AUCN, dose-normalized AUC; MDCK, Madin-Darby canine kidney; MDR1, multidrug-resistant protein 1; WT, wild type; A, apical; B, basolateral; MS, mass spectrometer; HPLC, high-performance liquid chromatography; KYN, kynurenine.
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).

Although 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 piperidino ring by mouse liver cytochrome P450 enzymes (P450s), and 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 among 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 pharmacokinetics of JM6 and Ro-61-8048 were evaluated in male C57BL/6N mice (wild-type strain for the R6/2 strain) of 24 h postdose. Blood was centrifuged at 2200 g for 15 min. Test compound (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 dicyclofenac 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-Milli-Q water (75:25, v/v) was added, and the samples were covered and vortexed for 5 min before liquid chromatography (LC)-tandem mass spectrometry (MS/MS) analysis.

Bioanalytical Methods. Analyte concentrations were determined by LC-MS/MS. Reverse-phase separation was performed in a Waters Acquity ultraperformance liquid chromatograph with a UPLC BEH C18 column (50 × 2.1 mm, 1.7 μm; Waters, Milford, MA). For quantitation of JM6 and Ro-61-8048, the mobile phase consisted of 0.1% formic acid (v/v) in acetonitrile (B) and 0.01% formic acid (v/v) in Milli-Q water (A). Elution was initiated with 5% B for 0.2 min, progressed linearly to 95% B over 1 min, and was 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 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 were processed using QuanLynx software from Waters. Additional details of the bioanalytical methodology are included in the supplemental data. The assay lower limit of quantitation (LLOQ) were 1.9 nM for JM6 in plasma and brain and 2.4 and 5.9 nM for Ro-61-8048 in plasma and brain, respectively.

Pharmacokinetic Analysis. Composite noncompartmental pharmacokinetic parameters were calculated from the mean concentrations (n = 3) obtained for each time point using Phoenix WinNonlin (version 5.2.1; Pharsight, Mountain View, CA). For the intravenous dose, the plasma concentration at time $t = 0$ was back extrapolated from the first two postdose plasma concentrations. For the oral dose, the concentration at time $t = 0$ was assumed to be 0. Plasma and tissue concentrations below the LLOQ were treated as absent samples.

The AUC$_{last}$, the area under the plasma concentration versus time curve to $t_{last}$, the last measurable 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 $C_{last}$ to infinity (AUC$_{last-infty}$) was extrapolated from the ratio of $C_{last}$/$k_{el}$. The AUC$_{last}$ was calculated as AUC$_{last}$ + AUC$_{last-infty}$. The oral bioavailability (F%) was calculated only for AUCs within the linear dose-response range, by dividing the dose-normalized oral AUC$_{last}$ over the dose-normalized intravenous AUC$_{last}$. The dose-normalized AUC (AUCN) was calculated by dividing the AUC$_{last}$ by the dose level.

In Vitro Absorption, Distribution, Metabolism, and Excretion 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 (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 of protein/mg in 0.1 M phosphate buffer, pH 7.4) 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’ medium E with 4 mM l-glutamine and 2 mM magnesium sulfate; 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 an IS, centrifuged, and analyzed by LC-MS/MS.

Stability in simulated gastric fluid. Simulated gastric fluid, pH 1.2, was prepared containing 2 g/l sodium chloride, 3.2 g/l of pepsin, and 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 of DMSO, mixed, and analyzed immediately by LC-UV ($λ = 254$ nm). Permeability and effective efflux ratio in Caco-2 and MDCK-MDR1 cells. 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 was 2 × 10$^4$ 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 the integrity of the cell layers; wells with Lucifer Yellow permeability greater than 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 compounds 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 MDCK-MDR1 cells relative to the ratio observed in wild-type cells.

**Blood/plasma ratio.** Test compound (1 mM, 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 was centrifuged to yield plasma. LC-MS/MS quantitation indicated that both analytes distributed preferentially into the plasma compartment (blood/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 equilibration dialysis following standard procedures. The determinations were done for Ro-61-8048 because 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 h and analyzed by LC-MS/MS.

**Bioanalysis for in vitro samples.** Formation of Ro-61-8048 was monitored in all incubations; the LLOQ was 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 percentage of JM6 remaining at 0 min (100%). Quantifications (as applicable) were performed against those of a metabolite synthesis, with a total run time of 5 min. The injection volume was 5 µl.

The mass spectrometer ion optics were set to single ion recording in the negative ionization mode. The data were processed using QuanLynx software (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 for which synthesized standards were not available (M1, M5, M10, M11, M12, and M13), semiquantitation was performed by comparison of relative peak intensities against those of the synthetic products formed in mouse, rat, or human liver microsomes as described for 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 p.o. JM6). 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 Milli-Q 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.

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**Synthesis and Purification of JM6, Ro 61-8048, and Metabolites Used in These Studies.** 

*Synthesis of JM6 and Ro 61-8048.* 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 Muchowski et al. (2008) (Fig. 1). JM6 was purified via recrystallization or semipreparative HPLC with various columns and mobile phases. Confirmation of the synthetic products was performed by LC-MS and NMR. The purity 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, purifica-
tion approaches, and purity determination can be found in the supplemental data.

Alternate synthesis of JM6, without Ro-61-8048 as synthetic intermediate. JM6 was synthesized by scientists at Evotec (UK) Ltd. (Abingdon, UK) and AMRI (Albany, NY) using an alternate path that did not contain Ro-61-8048 as a synthetic intermediate following the steps shown in Fig. 2. The confirmation of 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 data.

Synthesis of putative metabolites of JM6. Putative JM6 metabolites were synthesized at Evotec (UK) Ltd. (see supplemental data).

Quantitation of Ro 61–8084 in purified JM6. Quantification of 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- to 400- nm range and a 6410B triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) diode array UV detector scanning over a 210- to 400- nm range and a 6410B using a 1200 RRLC liquid chromatography system with an integrated serial quantitation of KYN and 3-hydroxykynureninea Waters Acquity UPLC BEH C18 column (30.0 mm × 2.1 mm × 1.7 μm) with a Waters Acquity BEH VanGuard precolumn (5.0 mm × 2.1 mm × 1.7 μm) using a flow rate of 1.2 ml/min and a column temperature of 60°C. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. After equilibration at 2% B for 0.5 min, the analyte was injected, and the gradient was increased linearly from 2 to 95% B in 4 min and held constant at 95% B for 0.4 min before returning to 2% B over 0.1 min. JM6 and Ro-61-8048 were chromatographically resolved. UV detection was performed over a scan range of 210 to 400 nm. An electrospray ionization source was used with the following parameters: 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 × 2.1 mm × 1.7 μm) with a Waters Acquity BEH VanGuard precolumn (5.0 mm × 2.1 mm × 1.7 μm) using a flow rate of 1.2 ml/min and a column temperature of 60°C. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. After equilibration at 2% B for 0.5 min, the analyte was injected, and the gradient was increased linearly from 2 to 95% B in 4 min and held constant at 95% B for 0.4 min before returning to 2% B over 0.1 min. JM6 and Ro-61-8048 were chromatographically resolved. UV detection was performed over a scan range of 210 to 400 nm. An electrospray ionization source was used with the following parameters: gas temperature, 325°C; gas flow, 11 l/min; nebulizer, 55 psi; and capillary, 4000 V. Additional methodology details are included in the supplemental data.

Biochemical Determination of KMO Activity and In Vitro Inhibition. The KMO enzyme was purified from mouse (C57BL/6J), rat (Wistar), or human (section only) livers following published methods (Röver et al., 1997). KMO activity was measured directly by monitoring 3-hydroxykynurenine (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, and 3 mM tr-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 preincubated for 5 min at 25°C with the enzyme preparation (0.3 mg of protein/ml; specific activity of 1000 ± 250 nmol · min⁻¹ · mg⁻¹ rat and mouse liver enzyme and 700 ± 150 nmol · min⁻¹ · mg⁻¹ human liver enzyme). A separate incubation was also performed in the presence of 3 mg/ml 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 addition of 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 of 10% trichloroacetic acid, vortexed, centrifuged (5 min at 4000 rpm, 4°C), and submitted to LC-MS/MS.

Enzyme activity kinetic constants were calculated by fitting the data obtained from saturation experiments to the Michaelis-Menten equation using Prism data analysis software (GraphPad Software, Inc., San Diego, CA). IC₅₀ values were determined from eight-point serial 3-fold dilutions starting at a top concentration (3 or 30 μM) using the same software. Each IC₅₀ was determined in triplicate 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 quadrupole mass spectrometer equipped with an electrospray ion source. For LC separation of KYN and 3-hydroxykynurenine Waters Acquity UPLC BEH C18 column (1.7 μm; 2.1 × 50 mm) connected to a filter guard was used. The flow rate for analysis was 1 ml/min at a column temperature 40°C. The gradient

Fig. 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). THF, tetrahydrofuran; RT, room temperature.
start at 99.9% solvent A (0.1% v/v acetic acid in LC-MS-grade water), held for 0.2 min, increased 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 reequilibrated for an additional 0.4 min. To minimize variation of the injection volumes, the 5-μl sample loop was 5-fold overfilled. The sample storage temperature was set to 8°C. MRM transitions specific to each analyte were monitored.

Results

Pharmacokinetics of JM6 and Ro-61-8048 After a Single Intravenous Bolus. After a single intravenous bolus administration at 5 mg/kg (Table 1; Supplemental Fig. 1), the plasma clearance of JM6 was rapid (Clp = 1.9 l/h) and accounted for ~65% of the mouse liver plasma flow of 2.97 l·h⁻¹·kg⁻¹ (Davies and Morris, 1993). In contrast, the clearance of Ro-61-8048 was 100-fold lower (0.018 l/h) and accounted for ~0.6% of the mouse liver plasma flow.

Whereas both compounds had a relatively low volume of distribution at steady state (Vdss), this parameter was ~2.5-fold higher for JM6 than for Ro-61-8048 (0.37 versus 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 half-life for Ro-61-8048 (t1/2 = 0.72 and 6.5 h, respectively).

Under noncompartmental 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 intravenously at 5 mg/kg, the AUC ratios (550,000/5200 nM·h) explained the 105.6-fold difference in clearances. This applied to all doses that yield exposures within the linear range and provided plasma clearance mechanisms and rates that are the same as those observed in the study used to calculate the compound Clp.

In this same study, after intravenous 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) (eq. 1):

\[
\frac{f_{m.i.v.}}{f_{m,o.p.o.}} = \frac{\text{AUC}_{\text{met,DPf,i.v.}}}{\text{DPf,i.v.}} \times \frac{\text{Cl}_{\text{met}} = 876.2 \mu g/l \times h}{5000 \mu g/kg} \times \frac{0.018 l \cdot h^{-1} \cdot kg^{-1}}{0.0032 (1)}
\]

where met is the metabolite of interest (Ro-61-8048), f_{m,i.v.} is the fraction of an intravenous dose of parent drug that is metabolized to met (Ro-61-8048), AUC_{met,DPf,i.v.} is the AUC of the metabolite (Ro-61-8048) after an intravenous dose of parent (JM6), DPf,i.v. is the intravenous dose of the parent drug, and Cl_{met} is the 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 intravenous dose of JM6.

Because we synthesized JM6 using Ro-61-8048 as the precursor (Zwilling et al., 2011) (Fig. 1), it was possible that Ro-61-8048 exposure in mice after 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⁻¹·kg⁻¹) 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 mouse 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 of 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 Fig. 1). When JM6 was administered alone (10 mg/kg, group I), maximal JM6 absorption was rapid (Cmax = 0.5 h) and oral bioavailability was very low (7.2%), probably due to a significant first-pass effect rather than poor absorption, given the high Clp of the compound and in vitro rate of hepatic metabolism. The JM6 AUCinf was 1100 nM·h, whereas the Ro-61-8048 AUCinf was 2-fold higher (2300 nM·h). If the observed Ro-61-8048 in plasma was of metabolic origin, according to Pang and Kwan (1983); it would have corresponded to <0.2% of the JM6 dose. Assuming complete absorption of a metabolite formed during first-pass metabolism, eq. 1 can be modified to estimate the fraction of an oral dose of the parent drug that is metabolized to met (f_{m,o.p.o.}) as follows (eq. 2):

\[
f_{m,o.p.o.} = \frac{\text{AUC}_{\text{met,DPf,o.p.o.}}}{\text{DPf,o.p.o.}} \times \frac{\text{Cl}_{\text{met}} = 955.43 \mu g/l \times h}{10,000 \mu g/kg} \times \frac{0.018 l \cdot h^{-1} \cdot kg^{-1}}{0.0017 (2)}
\]

where AUC_{met,DPf,o.p.o.} is the AUC of the metabolite (Ro-61-8048) after an oral dose of the parent (JM6) and DPf,o.p.o. is an oral dose of the parent.

Alternatively, the oral dose of a compound needed to achieve such an AUC can be estimated using the intravenous noncompartmental pharmacokinetic parameters as the product of the clearance times the oral bioavailability. With an oral bioavailability of 70% for Ro-61-8048 (Table 2; group IV), a 0.023 mg/kg dose (0.018 l·h⁻¹·kg⁻¹ × 876.2 μg/l·h/0.70) is sufficient to achieve the AUCs observed. A Ro-61-8048 dose of 0.023 mg/kg represents ~0.3% in the 10 mg/kg dose of JM6 used in our study.

The JM6 AUCinf values were similar for these three dose groups (1100, 1500, and 1100 nM·h for groups I, II, and III, respectively), indicating that studies were reproducible and that a 10% difference in JM6 dose level is not reflected in the AUCinf values because of the compound’s high Clp.

The Ro-61-8048 AUCinf for groups II (0.5 mg/kg) and III (1.0 mg/kg) were ~30% more than dose-proportional (53,000 and 110,000 ng/l·h/mg).
Pharmacokinetics of JM6 and Ro-61-8048 in mice after oral administration alone or in combination

TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Analyte Measured</th>
<th>AUC&lt;sub&gt;last&lt;/sub&gt;</th>
<th>AUC&lt;sub&gt;inf&lt;/sub&gt;</th>
<th>AUCN</th>
<th>F</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;N</th>
<th>T&lt;sub&gt;max&lt;/sub&gt;</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>JM6 (10 mg/kg)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>JM6</td>
<td>1100</td>
<td>1100</td>
<td>110</td>
<td>7.2</td>
<td>100</td>
<td>110</td>
<td>0.5</td>
</tr>
<tr>
<td>II</td>
<td>Ro-61-8048 (0.05 mg/kg) with JM6 (9.5 mg/kg)&lt;sup&gt;a&lt;/sup&gt;</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>
</tr>
<tr>
<td>III</td>
<td>Ro-61-8048 (1.0 mg/kg) with JM6 (9.0 mg/kg)</td>
<td>JM6</td>
<td>49,000</td>
<td>53,000</td>
<td>110,000</td>
<td>N.C.</td>
<td>9000</td>
<td>18,000</td>
<td>0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>IV</td>
<td>Ro-61-8048 (0.05 mg/kg) alone</td>
<td>JM6</td>
<td>110,000</td>
<td>110,000</td>
<td>140,000</td>
<td>N.C.</td>
<td>17,000</td>
<td>21,000</td>
<td>0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V</td>
<td>Ro-61-8048 (0.05 mg/kg) with JM6 (10 mg/kg)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>JM6</td>
<td>4300</td>
<td>4500</td>
<td>90,000</td>
<td>70</td>
<td>550</td>
<td>11,000</td>
<td>0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>VI</td>
<td>Ro-61-8048 (0.05 mg/kg) with JM6 (25 mg/kg)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>JM6</td>
<td>4900</td>
<td>5200</td>
<td>100,000</td>
<td>80</td>
<td>530</td>
<td>11,000</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>VII</td>
<td>Ro-61-8048 (0.05 mg/kg) with JM6 (50 mg/kg)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>JM6</td>
<td>8400</td>
<td>8800</td>
<td>180,000</td>
<td>N.C.</td>
<td>800</td>
<td>16,000</td>
<td>2.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>JM6</td>
<td>9600</td>
<td>9600</td>
<td>190</td>
<td>N.C.</td>
<td>8100</td>
<td>190</td>
<td>0.5</td>
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</tbody>
</table>

N.C., not calculated.
<sup>a</sup> JM6 in the dose contained <0.4% of Ro-61-8048.
<sup>b</sup> The T<sub>max</sub> for Ro-61-8048 is observed between 0.5 and 4 h and is difficult to define precisely in nonserial studies because of the leveled protracted plasma concentration versus time curve driven by the slow Cl<sub>p</sub>.

nM × h, respectively, corresponding to AUCN of 110,000 and 140,000 nM × h, respectively). The slightly more than dose-proportional response in Ro-61-8048 AUC<sub>inf</sub> could be the consequence of animal variability or of a change in Cl<sub>p</sub>. This higher than dose-proportional increase in Ro-61-8048 AUC<sub>inf</sub> is not a consequence of JM6 contributions to metabolism, because 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 and 6 h postdose are similar and decline slowly because of the low Cl<sub>p</sub> and low V<sub>dss</sub> of the compound. Because time points are not serial, animal variability contributes to the difficulty in defining the T<sub>max</sub> which for Ro-61-8048 varies between 0.5 and 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<sub>inf</sub> (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 recrystallization or semipreparative HPLC with various columns and mobile phases. Regardless of the purification process, we consistently detected 0.06 to 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 (Fig. 2A). However, 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 Fig. 2B.

Oral Pharmacokinetics of JM6 Coadministered with Constant Levels of Ro-61-8048. Because 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 coadministered 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. Because 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. Therefore, the plasma AUCs for both compounds will also increase in proportion to these increasing dose levels of JM6, precluding our determination whether an increase in Ro-61-8048 was 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,

FIG. 3. Pharmacokinetics of Ro-61-8048 in mice after 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).
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 administered orally at 0.05 mg/kg alone or coadministered with 10 mg/kg JM6, very similar Ro-61-8048 concentration versus time profiles (Fig. 3) and corresponding AUCs were...

<table>
<thead>
<tr>
<th>In Vitro System</th>
<th>Parameter and Units</th>
<th>Ro-61-8048 Mouse</th>
<th>Ro-61-8048 Rat</th>
<th>Ro-61-8048 Human</th>
<th>JM6 Mouse</th>
<th>JM6 Rat</th>
<th>JM6 Human</th>
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<tbody>
<tr>
<td>Metabolic stability</td>
<td></td>
<td></td>
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<td>Liver microsomes</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>
<td>&gt;100/87</td>
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<td></td>
<td>$C_{\text{int}}$, $\mu l \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$</td>
<td>&lt;29</td>
<td>&lt;32</td>
<td>200/230</td>
<td>220</td>
<td>62</td>
<td>&lt;28/29</td>
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<td>Hepatocytes</td>
<td>$t_{1/2}$, min</td>
<td>&gt;200/200</td>
<td>N.D.</td>
<td>88</td>
<td>43</td>
<td>N.D.</td>
<td>170</td>
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<td>$C_{\text{int}}$, $\mu l \cdot \text{min}^{-1} \cdot 1 \text{ million cells}^{-1}$</td>
<td>&lt;14/14</td>
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<td>16</td>
<td>69</td>
<td>N.D.</td>
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<td>$E_{\text{m}}$</td>
<td>&lt;0.5/0.5</td>
<td>N.D.</td>
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<td>0.83</td>
<td>N.D.</td>
<td>0.67</td>
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<td>&gt;24</td>
</tr>
<tr>
<td>Simulated gastric fluid</td>
<td>$t_{1/2}$, h</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>&gt;18</td>
</tr>
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<td>Permeability and transport</td>
<td>$P_{\text{app}}$, A-B/B-A, nm/s</td>
<td>N.D.</td>
<td>N.D.</td>
<td>170/130</td>
<td>N.D.</td>
<td>N.D.</td>
<td>47/5.3</td>
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<td>Efflux ratio</td>
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<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td></td>
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<td>N.D.</td>
<td>N.D.</td>
<td>100/330</td>
<td>N.D.</td>
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<td>7.2/430</td>
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<tr>
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<td>Efflux ratio</td>
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<td>N.D.</td>
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<td>N.D.</td>
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<td>N.D.</td>
<td>N.D.</td>
<td>4.2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>11</td>
</tr>
</tbody>
</table>

N.D., not determined.

Fig. 4. Proposed metabolism path for JM6 in mouse liver microsomes. M2, M3, M4, M6, M7, M8, and JM6 (depicted in blue) were confirmed by comparing chromatographic retention times and MS/MS spectra with those of synthetic standards; all other structures are proposed on the basis of MS/MS spectra. The iminium ion (depicted in green) was not detected; however, it was trapped as M9 or detected as its isomer, M13. M3, M4, and M8 are the most significant metabolites in mouse liver microsomal incubations. M3, M4, and M8 are the most abundant metabolites in mouse plasma. M11 (depicted in red) 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.
very stable in human liver microsomes. JM6 was extensively metabolized in mouse liver microsomes but was observed (4300 versus 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 after 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 among 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 and 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/kg, 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 because 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 (nonenzymatic) degradation at higher dose levels cannot be excluded; if first-pass metabolism of JM6 is saturated, more JM6 could be absorbed and available for degradation.

Permeability and Transport of JM6 and Ro-61-8048. Both JM6 and Ro-61-8048 are P-glycoprotein substrates, but JM6 has a higher effective efflux ratio (11 versus 4.2, respectively) indicating greater affinity for this transporter. (Table 3). Numerically, JM6 had the lowest permeability ($P_{app}$ A-B = 47 versus 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) 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 Zwilling et al., 2011 (6% perchloric acid and diluting 25 μl into a 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 and 24 h, respectively. However, JM6 (1 μM) was very unstable in mouse hepatic microsomes (Cl$\text{int} = 220$ μl·min$^{-1}$·mg$^{-1}$) and hepatocytes (Cl$\text{int} = 69$ μl·min$^{-1}$·1 million cells$^{-1}$) (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 (Fig. 4; Supplemental Fig. 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 with those of synthetic standards. All other metabolites are proposed on the basis of accurate mass and spectral fragmentation. Additional information is included in the supplemental data. No additional metabolites were detected in hepatocyte incubations.

Species Differences in In Vitro Metabolism. There were large differences among species in the rate of oxidative metabolism for JM6. The rate was very high in the mouse and high in the rat but negligible in the human (Cl$\text{int} = 220$, 62 and 29 μl·min$^{-1}$·mg$^{-1}$, respectively). A similar trend was observed in hepatocytes. (Table 3). The primary site of JM6 metabolism also differed among species (Fig. 5). In the mouse and rat, metabolism occurred primarily at the piperidine ring, whereas in the human, the minimal metabolism occurred at the phenyl ring.

Metabolites in Mouse Plasma After Oral Administration of JM6. The most abundant metabolites in mouse plasma were M3, M4, and M8 (AUC$_{\text{last}}$, 3600, 7200, and 3700 nM × h and $C_{\text{max}}$, 1400, 780, and 1400 nM, respectively) (Fig. 6). Although M4 was not a major metabolite in liver microsomes, we speculate that the high M4 exposures originate from the conversion of M3 to M4 (Fig. 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 because 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, sulfates, 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 Ro-61-8048. In liver microsomes, the rate of metabolism was negligible in the mouse and rat but very high in the human (Cl<sub>int</sub> = < 29, <32, and 220 μL · min<sup>-1</sup> · mg<sup>-1</sup>, respectively). The same trend was observed in hepatocytes (Cl<sub>int</sub> = <14 and 16 μL · min<sup>-1</sup> · 1 million cells<sup>-1</sup>, respectively) (Table 3). Only two metabolic products, with m/z = 406 and 392 in positive ion mode, were identified and corresponded to the parent.

**KMO Inhibitory Potential of Ro-61-8048, JM6, and Selected Metabolites.** The IC<sub>50</sub> values 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 ≳12,000, 8000, and 30,000 nM in the presence of 3 mg/ml 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%).

**TABLE 4**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mouse KMO</th>
<th>Rat KMO</th>
<th>Human KMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ro-61-8048&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90 ± 15</td>
<td>22 ± 0.15</td>
<td>170 ± 20</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>
<td>8000/8200</td>
<td>&gt;30,000</td>
</tr>
<tr>
<td>JM6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20,000 ± 2500</td>
<td>7100 ± 250</td>
<td>11,000 ± 5800</td>
</tr>
<tr>
<td>M2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22,000 ± 0</td>
<td>11,000 ± 0</td>
<td>39,000 ± 5600</td>
</tr>
<tr>
<td>M8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27,000 ± 0</td>
<td>10,000 ± 0</td>
<td>17,000 ± 750</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.D. of values obtained on 3 different days with triplicate determinations per day.

<sup>b</sup> Individual results obtained on 2 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 (Fig. 2B), and it was not possible to determine their biological activity in the absence of Ro-61-8048. However, their structure-activity relationship for activity is not considered favorable.

<sup>c</sup> Undetected low levels together with the lack of realization that the mechanism proposed for acid-induced release of Ro-61-8048; JM6 is a zwitterionic compound and analysis of distribution of micrometers 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 not likely to occur under physiological conditions. These low levels of Ro-61-8048 could go unnoticed when compound purity is evaluated for discovery studies, because they are below the typical 0.5% LLOQ generally used for purity assessment. However, oxidative metabolism was observed in mouse liver microsomes; however, oxidative metabolism was extensive in human liver microsomes. No additional metabolites were detected in hepatocytes.

**Discussion**

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 the mouse and rat were very different from those in the 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 to 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 micrometers 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 not likely to occur under physiological conditions. These low levels of Ro-61-8048 could go unnoticed when compound purity is evaluated for discovery studies, because 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 after 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 notably, we conclude that JM6 is not a prodrug for Ro-61-8048; given the very slow clearance of Ro-61-8048m a small impurity in the JM6 dose will result in very high exposures. Semiempirically, this conclusion is supported by the very different intravenous pharmacokinetic properties and oral bioavailability of these two compounds. Compared with Ro-61-8048, the JM6 Clp in mice is ~100-fold higher and the oral bioavailability is ~7-fold lower (Clp = 1.9 versus 0.018 l·h⁻¹·kg⁻¹ 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 that 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.

If the effects of Clp and %F are combined, an oral dose of 10 mg/kg JM6 or 0.014 mg/kg Ro-61-8048 (~<0.2% of the 10 mg/kg JM6 dose) will result in similar AUCs. The conclusion that Ro-61-8048 present in mouse plasma after JM6 administration originates from low levels of Ro-61-8048 in JM6 is supported experimentally by the similarity of the Ro-61-8048 concentration versus time profiles and corresponding AUCs observed after a 5 mg/kg oral administration of Ro-61-8048 alone (4300 nM x h) or coadministration with 10 mg/kg JM6 (4900 nM x h). Extensive JM6 metabolism to Ro-61-8048 would have resulted in much higher Ro-61-8048 upon coadministration with JM6. The Ro-61-8048 Cmax of 230 nM that we observed after a 10 mg/kg oral administration to mice of JM6 containing ~0.08% Ro-61-8048 was similar to the Cmax of ~280 nM reported after a 7.5 or 25 mg·kg⁻¹·day⁻¹ JM6 oral administration 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, because these Ro-61-8048 exposures would have originated from ~0.2% metabolism of the total JM6 dose.

With regard to JM6, after a 10 or 25 mg/kg p.o. dose to mice, we observed a Cmax of 1100 and 3400 nM, respectively, lower than the JM6 IC50 of 4000 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 (IC50 = 90 to ≥12,000 nM); the Ro-61-8048 binding to plasma proteins is >99%. Considering that the Cmax previously reported in mice was ~280 nM, additional work needs to be done, perhaps by dosing Ro-61-8048 directly, to confirm the mechanism of the Parkinson’s disease response. Whereas the KMO inhibitory potency of the JM6 metabolites observed in mouse plasma could not be investigated because of the confounding biological effects driven by the presence of Ro-61-8048, extensive structure-activity relationship evaluation indicates that any substitution of thiazole adjacent to the sulfur renders compounds inactive against KMO (CHDI, unpublished data).

Last, the different metabolic rates and preferential site of oxidation in vitro among 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 Parkinson’s disease 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 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.

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We gratefully acknowledge the contribution and dedication of the scientific staff at our contract research organizations BioFocus, Sarepta, Evotec, and Albany Molecular Research Inc. (AMRI) and, specifically, the contributions from Dr. Vadim Mozhave and Dr. Yuri Khmelnitsky (AMRI), who conducted the trapping experiments and proposed the structure of the iminium ion intermediate trapped as the cyanide adduct M9. We also gratefully acknowledge Simon Noble, Director, Scientific Communications at CHDI, for the outstanding job in the technical writing of this manuscript.

Authorship Contributions

Participants in research design: Beconi, Yates, Lyons, and Matthews.

Conducted experiments: Matthews, Clifton, Mead, Winkler, and O’Connell.

Contributed new reagents or analytic tools: Prime and Walter.

Performed data analysis: Beconi, Yates, Lyons, Matthews, Clifton, and Winkler.

Wrote or contributed to the writing of the manuscript: Beconi, Yates, Lyons, Matthews, Toledo-Sherman, Munoz-Sanjuan, and Dominguez.

References


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