Metabolism of LY654322, A Growth Hormone Secretagogue, to An Unusual Diimidazopyridine Metabolite


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Abbreviations:
DQFCOSY, double quantum filtered correlated spectroscopy; GH, growth hormone; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MS^3, 3 stages of tandem mass spectrometry; MS^4, 4 stages of tandem mass spectrometry; MS^n, multiple stages of tandem mass spectrometry; THF, tetrahydrofolate; TOCSY, total correlation spectroscopy
Abstract

LY654322 was rapidly cleared in rats and dogs by renal excretion of parent and metabolism (oxidative and hydrolytic). Among the metabolites identified in the urine of rats and dogs was M25, which was structurally unusual. Indeed, the characterization of M25 and investigation into its disposition relied on the convergence of diverse analytical methodologies. M25 eluted after parent on reverse phase chromatography with an MH+ at m/z 598 (parent + 35 Da). Given its increased lipophilicity and its mass difference compared with parent, it was evident that M25 was not a phase 2 conjugate. Subsequent LC/MS/MS, LC/MS³, LC/MS⁴, and accurate mass experiments identified the structure of M25 as having two replicates of the 1-(4-fluorophenyl)-1-methyl-2-oxo-2-pyrrolidinyl substructure flanking a central aromatic core of composition C₇H₃N₅ that was refractory to fragmentation. Compared with the UV spectrum of parent (λ max = 213 nm), M25 displayed a bathochromic shift (λ max = 311 nm), which substantiated extensive conjugation within the central core. Subsequent NMR analysis of M25 isolated from dog urine coupled with molecular modeling revealed the structure to be consistent with a diimidazopyridine core with two symmetrically substituted 1-(4-fluorophenyl)-1-methyl-2-oxo-2-pyrrolidinyl moieties. Using a structural analogue with a similar chromophore to M25, LC/UV was used to quantitate M25 and determine its urinary disposition. The formation of M25 appears consistent with hydrolysis of LY654322 to an aminimidazole, dimerization of the latter with the loss of NH₃, C-formylation, and subsequent ring closure and aromatization with loss of H₂O.
**Introduction**

Current medical advances have resulted in an increase in human life span and a commensurate increase in the elderly population. Unfortunately, increased longevity has been accompanied by an elevated emergence in conditions of the elderly including frailty – characterized by declining organ function and physical performance. It is thought that the decrease in pituitary growth hormone (GH) with age might be a contributing factor, and that maintenance of GH could be beneficial in the treatment of frailty in the elderly (Lamberts et al., 1997). In order to elicit the biological episodic profile of GH, the development of synthetic peptide GH secretagogues is considered a viable approach to the augmentation of GH secretion (Smith, 2005).

LY654322 (Fig. 1) is a GH secretagogue, and in vivo pharmacokinetic and metabolism studies were conducted as part of late stage discovery studies in rats and dogs. The objectives of these studies were to characterize the pharmacokinetics of parent drug and to delineate major clearance pathways. LY654322 was cleared by renal excretion and hepatic metabolism. Sixteen (16) metabolites were identified altogether, and derived from pathways that involved C-oxidation, N-oxidation and amide hydrolysis. Among these metabolites was M25, which was structurally unique and appeared to be the result of the fusion of two substructures of the parent molecule around an aromatic core. Given its novelty, the characterization of M25 was considered worthwhile. This report describes the identification, urinary disposition and proposed formation of M25.
Methods

Chemicals. Compounds LY654322 (2-methylalanyl-N-[1-[(1R)-1-(4-fluorophenyl)-1-methyl-2-oxo-2-pyrrolidin-1-ylethyl]-1H-imidazol-4-yl]-5-phenyl-D-norvalinamide) and LSN60645 (1,7-dimethyl-1,7-dihydroimidazo[4,5-b:4',5'-e]pyridine) were synthesized at Lilly Research Laboratories.

Animal Studies. All animal experiments were conducted according to protocols approved by the Eli Lilly Animal Care and Use Committee. Male Fischer 344 rats (200 to 275 g) were obtained from Harlan Sprague Dawley, Indianapolis, IN. Female beagle dogs (12.5 to 14.5 kg) were obtained from Marshall Farms, North Rose, NY. LY654322 was formulated in 50 mM phosphate buffer (pH 6) and 10% acacia for intravenous and oral administration, respectively. Animals were dosed following an overnight fast. Food was restored 2 hours post-dose and free access to water occurred throughout.

Sample Collection. Blood samples were collected in heparinized containers on ice, and plasma was harvested by centrifugation. Plasma, urine, and feces samples were stored at -70 °C prior to analysis.

Rat Studies. To determine plasma pharmacokinetics, three rats were administered LY654322 by tail vein at 3 mg/kg or oral gavage at 30 mg/kg. Blood was sampled from the jugular vein of rats at 0.05 and 0.25 (intravenous only), and 1, 2, 3, 4, 6, 8, and 10 h post-dose. To determine urinary metabolites, three rats were housed in metabolism cages. Urine was collected at 24 and 48 h.

Dog Studies. To determine plasma pharmacokinetics, three dogs were administered LY654322 by cephalic vein at 1 mg/kg or oral gavage at 3 mg/kg. Blood
was sampled from the jugular vein of dogs at 0.05 and 0.25 (intravenous only), and 1, 2, 3, 4, 6, 8, 10 and 24 h post-dose. To determine urinary metabolites, two dogs were administered LY654322 at 20 mg/kg by oral capsule, housed in metabolism cages and urine collected for 24 h.

**In Vitro Studies.**

*Hepatocytes.* Cryopreserved hepatocytes (In Vitro Technologies, Baltimore, MD) were incubated (1 million cells per incubation) in Hepatocyte Maintenance Medium (Lonza, Walkersville, MD) containing 50 μM LY654322. Conditions were maintained at 37°C in a 5% CO₂ atmosphere. Control incubations without hepatocytes or without substrate were conducted in each study. After 4 h, incubations were sonicated, frozen on dry ice and stored at –70°C until analysis.

*Liver slices.* Rat and dog liver slices (2 slices per incubation) of approximately 8 mm in diameter and 200 to 250 μm in thickness were incubated in Waymouth’s 752/1 medium (Cell and Molecular Technologies, Lavallette, NJ) containing 50 μM LY654322. Conditions were maintained at 37°C in a 95% O₂:5% CO₂ atmosphere. Control incubations without slices or without substrate were conducted in each study. After 24 h, incubations were sonicated, frozen on dry ice and stored at –70°C until analysis.

**Equipment and analytical conditions.**

*Quantitation of LY654322.* LC/MS/MS quantitation of LY654322 in rat and dog plasma was performed on a Sciex API 3000 mass spectrometer (Foster City, CA) by monitoring for MH⁺ with the ion transition m/z 563.4 to 192.4. Plasma samples were extracted by mixing with a 2x volume of acetonitrile, centrifuged and introduced into the mass spectrometer source with a Metachem Monochrom C18 column (2.1 x 50 mm; 5
μm) using a combination of mobile phase A: 0.1% formic acid in water and mobile phase B: 0.1% formic acid in methanol, at a flow rate of 350 μL/min. Mobile phase B was delivered as a gradient from 20% to 75% over 0.8 min and restored to 20% at 0.9 min. The calibration range was 3.9 to 4000 ng/mL. Samples above the limit of quantification were diluted and reanalyzed to yield results within the calibration range.

**Pharmacokinetic analysis.** The pharmacokinetic parameters of LY654322 in plasma were calculated by a noncompartmental method using WinNonlin. The terminal half-life (t<sub>1/2</sub>) was calculated from the first-order elimination rate constant k where t<sub>1/2</sub> = 0.693/k. The area under the plasma concentration time curve, determined by the trapezoidal rule, was extrapolated to infinity using k to determine AUC<sub>0-∞</sub>. Plasma clearance and bioavailability were calculated from AUC<sub>0-∞</sub>, dose and k.

**Identification of metabolites.**

**LC/MS of metabolites.** Urine samples were vortex mixed and centrifuged and transferred to the autosampler for injection. Plasma samples were extracted by mixing with a 2x volume of acetonitrile, centrifuged and concentrated to approximately the original volume. Hepatocyte and liver slice samples were extracted by mixing with an equal volume of acetonitrile, centrifuged and diluted with an equal volume of 0.2% formic acid. Electrospray LC/MS/MS analysis was performed on a Thermo Finnigan LCQ mass spectrometer (San Jose, CA), with a spray voltage of 5.0 kV and a capillary temperature of 225 °C. Product ion spectra were generated at a relative collision energy of 40%. Full scan spectra were acquired in the positive ion mode. There was also in line UV detection with a Finnigan Surveyor PDA. Samples were introduced into the mass spectrometer source via a Supelco Discovery C18 column (2.1 x 150 mm, 5 μm), using a combination...
of mobile phase A: 0.2% formic acid in water and mobile phase B: 10% isopropyl alcohol in acetonitrile, at a flow rate of 200 μL/min. The following profile for mobile phase B was used: initial, 10%; 2 min, 10%; 22 min, 50%; 32 min, 90%; 36 min, 90%; 36.1 min, 10%; 42 min (stop), 10%. Accurate mass measurements were conducted on a Micromass QToF-2/MS using positive ion electrospray, a resolution of 8,500 FWHM, a cone voltage of 40 V, a collision energy of 25 V, and a lock mass of 311.0814 (protonated molecular ion of the sulfadimethoxine standard).

Isolation of M25.

Urine from the individual dogs was pooled (approximately 300 mL) and applied directly to two Varian Mega Bond Elut™ C18 SPE columns, which were washed with water (approximately 60 mL each) and eluted with methanol (approximately 50 mL each). The combined eluate was evaporated to dryness and the residue was reconstituted in 2.5 mL of water/acetonitrile (85:15). The sample was centrifuged and the supernatant was fractionated on a Supelco Discovery C18 column (4.6 x 250 mm, 5 μm) using the same solvent system and gradient described above. Fractions were collected in 15-second intervals from 5 to 35 minutes at a flow rate of 4.0 mL/min. Pooling of fractions for M25 was based upon purity and concentration as measured by LC/MS. After pooling, the solvent was evaporated and the dried material was used for NMR analysis.

NMR analysis of M25.

NMR spectra were recorded at 25.0 °C on an Inova 500 MHz NMR spectrometer equipped with a pulsed-field gradient and a Nalorac MDG 500 3-mm probe (Varian Inc., Palo Alto, CA). Compounds were dissolved in approximately 200 μL of CD$_3$OD and transferred to a 3-mm NMR tube. In the case of M25, approximately 40 μL of ND$_4$OD
was added to sharpen the NMR peaks. Chemical shifts were referenced to the residual solvent signal at $\delta$ 3.3 for $^1$H and $\delta$ 49 for $^{13}$C. Two-dimensional NMR experiments including double quantum filtered correlated spectroscopy (DQFCOSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) were performed using Varian standard pulse sequences.

**Molecular modeling of M25.**

Molecular modeling was performed using Maestro and MacroModel software (Schrodinger LLC, 2008). For M25, an extensive conformational search was done using the Mixed Torsional/Low-Mode Sampling procedure (2000 maximum steps), the OPLS2005 forcefield, the GBSA continuum aqueous solvation treatment, and complete energy minimization to convergence. Using the identified M25 global minimum energy structure and an equivalently minimized structure for LSN60645, NMR shielding tensors were calculated using the *ab initio* program Gaussian 03 (Frisch et al., 2004) by Density Functional Theory (B3LYP functional, the 6-31++G(d,p) basis set, and GIAO method) and without geometry optimization. From these, the calculated chemical shift difference for the pyridinyl proton in M25, compared to LSN60645, was estimated by the difference in the calculated isotropic magnetic shielding tensor for the equivalent proton in each molecule.

**Quantitation of M25.**

M25 was quantitated in dog urine by LC/UV using the analogue, LSN60645, as a surrogate analytical standard, since both compounds contained the same diagnostic diimidazopyridine chromophore. LC/UV response at 311 nm was selective for M25 and
LSN60645. LC/UV detection was performed on a Waters 996 photodiode array detector (220 - 500 nm). Chromatography was performed using a Supelco Discovery C18 column (2.1 x 150 mm, 5 μm) with the same mobile phase conditions as described above in LC/MS of Metabolites for the quantitation of M25 in urine, and an isocratic delivery of mobile phase B for the quantitation of LSN60645 in an aqueous solution matrix. The calibration range was 8 to 1000 ng/mL.

LSN60645 and M25 both contained the same chromophore responsible for absorbance at \( \lambda = 311 \) nm, and consequently, their extinction coefficients at this wavelength were assumed to be the same. Since a standard was available for LSN60645, its extinction coefficient was determined by measuring its absorbance at known concentration in chloroform at \( \lambda_{\text{max}} = 311 \) nm on a Spectra Max Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) with 1 cm quartz cuvettes. The extinction coefficient for LSN60645 was calculated to be 12,741 M\(^{-1}\) cm\(^{-1}\).


Results

Pharmacokinetics of LY2456322. The pharmacokinetics of LY654322 were investigated in rats and dogs. Following intravenous administration, mean values for plasma clearance and terminal half-life were 75 mL/min/kg and 2 hours, respectively, in rats and 33 mL/min/kg and 5 hours, respectively, in dogs. Following oral administration, the bioavailability in rats and dogs was 74% and 33%, respectively.

LC/MS/MS of LY654322. The LC/MS/MS spectrum of LY654322 is shown in Fig. 2. MH\textsuperscript{+} at m/z 563 undergoes a facile loss of H\textsubscript{2}O likely through amide – imidic acid tautomerization at either of the two peptide linkages followed by intramolecular condensation to the corresponding imine at m/z 545. Subsequent loss of (CH\textsubscript{3})\textsubscript{2}C=NH from m/z 545 would then afford the ion at m/z 488. Other characteristic fragment ions of parent were m/z 478, m/z 344, m/z 303, m/z 220 and m/z 192. Accurate mass data and proposed elemental compositions of these ions are shown in Table 1.

Metabolism of LY654322. Plasma and urine samples from rats and dogs dosed with LY654322 were profiled by LC/UV and LC/MS/MS. LY654322 was cleared by urinary excretion of parent and metabolism. Sixteen (16) metabolites were identified between rats and dogs collectively, with considerable overlap occurring between species. Metabolism of parent was related to the following structural regions: (A) aliphatic and N-oxidation, (B) aliphatic and aromatic oxidation and (C) amide hydrolysis (Fig. 1). Among the downstream metabolites of LY654322 was M25 which was identified only in the urine of rats and dogs. M25 was not identified as a metabolite in rat or dog liver.
slices or human hepatocytes, nor was it detected as an impurity in parent drug in incubations where liver slices and hepatocytes were absent. Taken together, these results indicated that M25 was an authentic urinary metabolite of LY654322. M25 was structurally unusual and subject to detailed investigation.

**Characterization of M25.** LC/MS analysis of rat and dog urine identified M25 as eluting after parent on reverse phase chromatography (Fig. 3). Furthermore, its MH$^+$ at m/z 598 exceeded MH$^+$ of parent by 35 Da (Table 2) and was not the product of direct phase 2 conjugation as detailed below. Given its increased lipophilicity and its mass difference compared with parent, it was evident that the metabolic pathway to M25 was not straightforward. Valuable insight into the structure of M25 was gained through a series of MS$^0$ experiments (Fig. 4). MS/MS of M25 afforded ions m/z 220 and m/z 192, which were common with parent and indicated that the 1-(4-fluorophenyl)-1-methyl-2-oxo-2-pyrrolidinyl substructure was retained. The complementary ion to m/z 220 at m/z 379 was fragmented, and the surprising recurrence of ions m/z 220 and m/z 192 suggested that M25 consisted of two replicates of the 1-(4-fluorophenyl)-1-methyl-2-oxo-2-pyrrolidinyl substructure. The complementary ion to m/z 220 at m/z 160 was fragmented, and on this occasion, there was only a weak loss of HCN, indicating that m/z 160 was very stable and likely aromatic. The structure of M25 was further probed with the application of accurate mass MS and MS/MS experiments, which ascribed what was envisioned to be the central core the formula C$_7$H$_5$N$_5$ (neutral molecule), a composition requiring eight double bond equivalents (Table 1). In order to rationalize the existing data, the following structure was proposed: two symmetrically disposed N-[1-(4-fluorophenyl)-1-methyl-2-oxo-2-pyrrolidinyl] substituents to a fused diimidazopyridine.
Since the diimidazopyridine substructure was critical to the characterization of M25, it was used to interrogate the Lilly database for a structural precedent. The hit, LSN60645, a diimidazopyridine analogue, was fortuitous, since it served as a conduit to a series of informative UV and NMR experiments.

The UV spectrum of parent was characterized by a single $\lambda_{\text{max}}$ at 213 nm (Fig. 5A). M25, on the other hand displayed, two $\lambda_{\text{maxima}}$ – one at 208 nm, comparable with parent, and another with a bathochromic shift at 311 nm, consistent with extensive conjugation (Fig. 5B). The UV spectra of M25 matched that of the standard LSN60645, supporting the diimidazopyridine substructure of the central core.

NMR was critical in reconciling the connectivity of the proposed heterocycle core to other substructures of parent in the metabolite. Although the molecular weight of M25 was 35 Da higher than LY654322, the $^1$H-NMR spectrum of the metabolite was strikingly less complex than parent (see Fig. 6 for the aromatic region of LY654322 and M25, and Table 3 for the chemical shift assignments). The aromatic region of the proton NMR spectrum of M25 consisted of only four resonances, two of which ($\delta$ 7.39 br dd; $\delta$ 7.27 br t) were assigned to the 4-flurophenyl substituent analogous to the parent compound (Table 3). Furthermore, the recurrence of the pyrrolidinyl protons and their respective carbons in M25 compared with parent supported the retention of the 1-(4-flurophenyl)-1-methyl-2-oxo-2-pyrrolidinyl substructure in M25 (Table 3). The mutually coupled imidazolyl protons of parent at $\delta$ 7.24 and 7.17 were conspicuously absent in M25 and instead these resonances were replaced by two new resonances at $\delta$ 8.23
(singlet, 2 protons) and δ 6.92 (singlet, 1 proton). The lack of any other resonances, specifically resonances due to the 2-methylalanyl-5-phenyl-D-norvalinyl moiety of the parent, in M25 (Table 3) and the fact that the intensity of the singlet proton at δ 6.92 is approximately one-fourth of the equivalent 4-fluorophenyl protons at δ 7.39 or δ 7.27 revealed that M25 possesses an element of symmetry. Since the UV spectrum of M25 was very similar to LSN60645, a diimidazo pyridinyl derivative, the proton NMR spectrum of M25 was compared with LSN60645 (Fig. 6). The comparison revealed that both spectra showed a singlet resonance with 2 proton intensity in the range δ 8.2 to 8.3, which was assigned to the magnetically equivalent 2 imidazolyl protons of a highly conjugated system. However, the pyridinyl proton (H-19) which resonated at δ 8.06 in LSN60645, a normal chemical shift associated with its chemical environment, appeared with a pronounced up-field shift at δ 6.92 (Δδ = 1.14) in M25.

In order to rationalize the NMR of M25, molecular modeling experiments were performed. A conformational search resulted in a global minimum energy structure (Fig. 7), and other conformations within 2.6 kcal/mole of the global minimum (not shown), all of which positioned the pyridinyl proton perpendicular to the two fluorophenyl rings. Oriented as such, this proton should experience significant shielding due to the aromatic ring current and a pronounced diamagnetic shift. Indeed, calculating the pyridinyl proton chemical shift difference between M25 (in the illustrated conformation) and LSN60645 by ab initio quantum mechanical methods, gave a value Δδ = 0.82, comparable to the up-field shift that was experimentally observed. This supports the contention that in M25 the
ring current from the phenyl groups in close proximity to the pyridinyl proton, should perturb its chemical shift up-field, with respect to the same proton in LSN60645.

Identification of metabolites related to M25 (Table 2).

M2: M2 was identified in the plasma and urine of rats and dogs. The full scan MS of M2 showed MH\(^+\) (C\(_{16}\)H\(_{20}\)FN\(_4\)O) at m/z 303, suggesting an aminoimidazole structure resulting from hydrolysis of the peptide linkage proximal to the imidazole. The LC/MS/MS spectrum of M2 showed several ions consistent with the proposed structure, including m/z 220 (C\(_{13}\)H\(_{15}\)FNO, resulting from loss of 4-aminoimidazole), m/z 204 (C\(_{11}\)H\(_{11}\)FN\(_3\), resulting from loss of N-formylpyrrolidine), m/z 192 (C\(_{12}\)H\(_{15}\)FN, resulting from loss of 4-aminoimidazole and CO), and m/z 84 (C\(_{3}\)H\(_{6}\)N\(_3\), protonated 4-aminoimidazole).

M19: M19 was identified in rat plasma only. The full scan MS of M19 showed MH\(^+\) (C\(_{15}\)H\(_{23}\)N\(_2\)O\(_3\)) at m/z 279, suggestive of the carboxylic acid, complementary to M2, formed by hydrolysis of the peptide linkage proximal to the imidazole. The supporting LC/MS/MS product ion spectrum contained m/z 194 (C\(_{11}\)H\(_{16}\)NO\(_2\), resulting from loss of 3,3-dimethylaziridin-2-one) and m/z 103 (C\(_{4}\)H\(_{11}\)N\(_2\)O, protonated 2-amino-2-methylpropanamide).

M23, M24: M23 and M24 were identified in the urine of dogs, but not rats. The full scan MS of M23 and M24 showed MH\(^+\) (C\(_{22}\)H\(_{28}\)FN\(_4\)O\(_7\)) at m/z 479, suggestive of glucuronide conjugates of M2. M23 and M24 had comparable product ion spectra. Key product ions included: m/z 345, the loss of C\(_{4}\)H\(_{6}\)O\(_5\) from the glucuronide; m/z 303, loss of C\(_{6}\)H\(_{8}\)O\(_6\) from the glucuronide to form the aglycone; m/z 260, loss of the 1-(4-
fluorophenyl)-1-methyl-2-oxo-2-pyrrolidinyl substructure; m/z 242, loss of water from m/z 260; m/z 220, the 1-(4-fluorophenyl)-1-methyl-2-oxo-2-pyrrolidinyl substructure; m/z 192, loss of carbon monoxide from m/z 220. These data define the site of glucuronidation to the aminoimidazole ring.

**Urinary Excretion of M25 in Rats and Dogs.** Given the identification of M25 in the urine of both rats and dogs, and the similar UV spectra of M25 and LSN60645 in the region $\lambda = 308$ to 312 nm, UV spectroscopy was exploited as a tool to quantitate M25 in urine and determine the extent of urinary excretion in animals. In dog urine, the chromatographic peak for M25 was well-isolated and the UV range was very selective, allowing M25 to be quantitated and its urinary disposition assessed at 0.3% of the dose in 24 hr. Although M25 was identified in rat urine, it could not be quantitated because of high chromatographic interference.
Discussion

The preclinical pharmacokinetics and metabolism of LY654322 were determined as part of late stage discovery characterization. The pharmacokinetics were characterized in rats and dogs as having high clearance comparable with hepatic blood flow in both species (Davies and Morris, 1993). The compound was extensively metabolized. One of the metabolites identified during the course of these studies was M25, excreted in rat and dog urine, which had LC/MS/MS and UV properties that sparked our interest. The MS4 fragmentation sequence of M25 (m/z 598 → 379 → 160 → 133) coupled with the bathochromic shift in its $\lambda_{\text{max}}$ compared with parent indicated that M25 resulted from substantial molecular modification of LY654322. NMR characterization of M25 and molecular modeling substantiated the dimerization of two imidazo fragments from parent with endogenous C-atom incorporation to afford a diimidazopyridine analogue with a pyridinyl plane of symmetry.

The initial step in the metabolism of LY654322 to M25 is proposed to occur through the hydrolysis of the peptide linker to afford the aminoimidazole M2, which was identified in the plasma and urine of both rats and dogs. Hydrolysis was catalyzed in the liver based on the identification of M2 in rat and dog liver slices and human hepatocytes. M19, the complementary carboxylic acid in the peptide linkage to M2, as well as the downstream M2 glucuronides, M23 and M24, supported the occurrence of M2.

The organ(s), subcellular compartment(s) and conditions which contribute to the formation of M25 from M2 are not known. Although M2 was formed in liver slices and
hepatocytes, M25 was not a metabolite \textit{in vitro}. It is possible that \textit{in vitro} conditions were not optimized for the formation of M25 from M2 or the formation of M25 takes place extra-hepatically.

The subsequent incorporation of the M2 substructure in M25 is envisioned in the metabolic sequence described in Fig. 8. M2 tautomerizes to the imine which undergoes nucleophilic attack by another aminoimidazole molecule to form the intermediate 1, which eliminates NH$_3$ to couple the two aminoimidazole subunits as the $N$-linked condensation product 2. Subsequently, the carbon of the central pyrininium heterocycle is added by formylation through a $C$-centered conjugate nucleophilic attack from one of the imidazo subunits to form 3. The latter is positioned for an entropically-favored intramolecular nucleophilic attack from the $C$-center of the other imidazo subunit to afford ring closure to 4, and ensuing aromatization with the loss of H$_2$O to afford M25. None of the proposed intermediates 1 – 4 were identified as in vivo metabolites in rats or dogs.

The coupling, formylation and cyclization steps in this metabolic scheme are noteworthy. Firstly, the chemical condensation of aminoimidazoles described in the literature (Bredereck et al., 1964a, 1964b), requires high temperatures (>100 °C). The proposed dimerization of M2 is thought to proceed under physiological conditions, although the dimer (intermediate 2) was not detected in liver slices or hepatocytes. It is not known whether this process is enzyme-catalyzed. Secondly, although the $C$-source in its cyclization is conjectural (\textit{vide infra}), experiments were conducted to ensure that single carbon introduction in the formation of M25 was not an artifact of analysis or processing.
In control experiments, formic acid in the mobile phase was replaced with acetic acid or ammonium acetate for LC/MS, and in both instances M25 was present to the same extent. Conversely, urine samples were incubated in 1% formic acid, and no change in M25 was discerned. Taken together, these experiments supported M25 being an authentic metabolite derived from the incorporation of carbon from an endogenous source.
The proposed C-formylation in the formation of M25 is based on precedence in the literature for N- and C-formylations, and invokes $N^{10}$-formyltetrahydrofolate as a carbon source. N-formylation in the metabolism of xenobiotics is an uncommon pathway. It has been described for aromatic (Santti and Hopsu-Havu, 1968; Gothoskar et al., 1979; Tjørnelund et al., 1991) and aliphatic (Mutlib et al., 2002; Obach et al., 2006) amines. Alternatively, N-formylation in the biosynthesis of purines is well-recognized, being mediated by $N^{10}$-formyltetrahydrofolate as the carbon source (Zhang et al., 2008). C-formylation, as a biotransformation, has very little precedence in the literature – one example describes the formation a C-formyl secondary metabolite of phencyclidine mediated through an enamine (Zhao et al., 1991). The reaction was catalyzed by a mitochondrial enzyme with either $N^5$- or $N^{10}$-formyltetrahydrofolate as the carbon source. The C-formylation of intermediate 2 through a conjugate addition (Fig. 8) appears analogous to that described for phencyclidine. In this case, however, the reaction is driven forward by cyclization and aromatization steps to form M25, interestingly analogous to the N-centered formylation and addition steps in purine biosynthesis (Zhang et al., 2008).

In summary, LY654322 was metabolized in rats and dogs to M25, which was characterized by LC/MS, UV, NMR and molecular modeling, as an unusual diimidazopyridine. The formation of M25 was rationalized by an initial amide hydrolysis of LY654322 to the aminoimidazole M2, which underwent dimerization, C-formylation, cyclization and aromatization.
Authorship Contributions

Participated in research design: Borel

Conducted experiments: Barbuch, Mattiuz, Jackson, Klimkowski, Rener, Kulanthaivel, Jones

Performed data analysis: Borel, Barbuch, Wheeler, Klimkowski, Rener, Kulanthaivel, Jones

Wrote or contributed to the writing of the manuscript: Borel, Barbuch, Klimkowski, Rener, Kulanthaivel


References


Footnotes

Current affiliation: IsotopicSolutions, LLC, Indianapolis, Indiana (W.J.W.)
Legends for Figures

Fig. 1. Structure of LY654322, showing the principal sites of metabolism. (A) Aliphatic and N-oxidation, (B) aliphatic and aromatic oxidation, and (C) amide hydrolysis.

Fig. 2. LC/MS/MS of LY654322, illustrating fragment ion assignments and proposed structures for the ions at m/z 545 resulting from loss of H2O from parent.

Fig. 3. LC/MS extracted ion chromatogram of dog urine illustrating metabolites related to M25.

Fig. 4. LC/MS, LS/MS3 and LC/MS4 of M25 in dog urine. (A) MS/MS of MH+ at m/z 598, (B) MS/MS of the fragment at m/z 379, (C) MS/MS of the fragment at m/z 160.

Fig. 5. LC UV spectra of (A) LY654322, (B) M25 and LSN60645 superimposed.

Fig. 6. 1H NMR spectra of the aromatic region of (A) LY654322, (B) M25 and (C) LSN60645.

Fig. 7. Molecular model of M25 illustrating a low-energy conformation with the pyridinyl proton perpendicular to the two 4-fluorophenyl rings and predisposed to a diamagnetic shift because of aromatic ring current.
Fig. 8. Proposed metabolism of LY654322 to form M25. Intermediates 1–4 were not identified as metabolites in rats or dogs.
Table 1. Accurate mass data for LY654322 and its metabolite M25.

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<td>398.1992</td>
<td>-0.3</td>
<td>-0.8</td>
</tr>
<tr>
<td></td>
<td>344</td>
<td>C\textsubscript{16}H\textsubscript{26}N\textsubscript{5}O\textsubscript{2}</td>
<td>344.2104</td>
<td>344.2087</td>
<td>1.7</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>179</td>
<td>C\textsubscript{8}H\textsubscript{11}N\textsubscript{4}O</td>
<td>179.0923</td>
<td>179.0933</td>
<td>-1</td>
<td>-5.5</td>
</tr>
<tr>
<td>M25</td>
<td>598</td>
<td>C\textsubscript{33}H\textsubscript{34}N\textsubscript{7}O\textsubscript{2}F\textsubscript{2}</td>
<td>598.2723</td>
<td>598.2742</td>
<td>-1.9</td>
<td>-3.2</td>
</tr>
<tr>
<td></td>
<td>379</td>
<td>C\textsubscript{20}H\textsubscript{20}N\textsubscript{6}OF</td>
<td>379.1683</td>
<td>379.1683</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>220</td>
<td>C\textsubscript{13}H\textsubscript{15}NOF</td>
<td>220.1133</td>
<td>220.1138</td>
<td>-0.5</td>
<td>-2.3</td>
</tr>
<tr>
<td></td>
<td>192</td>
<td>C\textsubscript{12}H\textsubscript{16}NF</td>
<td>192.1187</td>
<td>192.1189</td>
<td>-0.2</td>
<td>-1.0</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>C\textsubscript{7}H\textsubscript{8}N\textsubscript{4}</td>
<td>160.0619</td>
<td>160.0623</td>
<td>-0.4</td>
<td>-2.5</td>
</tr>
</tbody>
</table>
Table 2: LC/MS of LY654322, M25 and its related metabolites.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>MH⁺ (m/z)</th>
<th>Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY654322</td>
<td><img src="image" alt="LY654322 Structure" /></td>
<td>563</td>
<td>rp, dp, ru, du, rs, ds, hh</td>
</tr>
<tr>
<td>M2</td>
<td><img src="image" alt="M2 Structure" /></td>
<td>303</td>
<td>rp, dp, ru, du, rs, ds, hh</td>
</tr>
<tr>
<td>M19</td>
<td><img src="image" alt="M19 Structure" /></td>
<td>279</td>
<td>rp</td>
</tr>
<tr>
<td>M23, M24</td>
<td><img src="image" alt="M23, M24 Structure" /></td>
<td>479</td>
<td>dp, du, ds</td>
</tr>
<tr>
<td>M25</td>
<td><img src="image" alt="M25 Structure" /></td>
<td>598</td>
<td>ru, du</td>
</tr>
</tbody>
</table>

rp = rat plasma, dp = dog plasma, ru = rat urine, du = dog urine, rs = rat liver slice, ds = dog liver slice, hh = human hepatocytes
Table 3. Chemical shift assignments\textsuperscript{a} of LY654322, M25 and LSN60645 in CD\textsubscript{3}OD.

<table>
<thead>
<tr>
<th>Substructure Position</th>
<th>LY654322</th>
<th>M25</th>
<th>LSN60645</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( ^1H\delta \text{ mult (Hz)} )</td>
<td>( ^{13}C\delta )</td>
<td>( ^1H\delta \text{ mult} )</td>
</tr>
<tr>
<td>1, 1'</td>
<td>7.40 br dd (9, 5)</td>
<td>130.6</td>
<td>7.39 br dd</td>
</tr>
<tr>
<td>2, 2'</td>
<td>7.21 br t (8.5)</td>
<td>117.2</td>
<td>7.27 br t</td>
</tr>
<tr>
<td>3</td>
<td>2.21 s</td>
<td>26.3</td>
<td>2.32 s</td>
</tr>
<tr>
<td>4</td>
<td>3.55 t (7)</td>
<td>49.6</td>
<td>3.53 t</td>
</tr>
<tr>
<td>5</td>
<td>1.78 m</td>
<td>24</td>
<td>1.75 m</td>
</tr>
<tr>
<td>6</td>
<td>1.76, 1.63 m</td>
<td>27.5</td>
<td>1.72, 1.64 m</td>
</tr>
<tr>
<td>7</td>
<td>3.05, 2.67 m</td>
<td>48.7</td>
<td>2.89, 2.74 m</td>
</tr>
<tr>
<td>8</td>
<td>7.24 d (1.5)</td>
<td>134.7</td>
<td>NA</td>
</tr>
<tr>
<td>9</td>
<td>7.17 d (1.5)</td>
<td>109.1</td>
<td>NA</td>
</tr>
</tbody>
</table>

mult = multiplicity, br = broad, m = multiplet, s = singlet, t = triplet, dd = doublet of doublet, obsc = obscured, \( J \) = coupling constant in Hz, NA = not applicable, ND = not detected.

\textsuperscript{a}Assignments were facilitated by the analysis of \( ^1H \), DQFCOSY, TOCSY, HSQC and HMBC spectra.

\textsuperscript{b}Coupling constants are not entered for M25 when identical with those of LY654322.
Table 3 (continued). Chemical shift assignments\textsuperscript{a} of LY654322, M25 and LSN60645 in CD\textsubscript{3}OD.

<table>
<thead>
<tr>
<th>Substructure Position</th>
<th>LY654322 (1H \delta) mult (in Hz)</th>
<th>(13C \delta)</th>
<th>M25 (1H \delta) mult</th>
<th>(13C \delta)</th>
<th>LSN60645 (1H \delta) mult</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,10'</td>
<td>1.30 s</td>
<td>28.4, 28.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>11</td>
<td>4.49 dd (8, 5)</td>
<td>54.4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>1.84, 1.72 m</td>
<td>33.2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>13</td>
<td>1.66 m</td>
<td>28.8</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>14</td>
<td>2.61 m</td>
<td>36.4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>15, 15'</td>
<td>7.13 obsc</td>
<td>129.5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>16, 16'</td>
<td>7.22 obsc</td>
<td>129.4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>17</td>
<td>7.12 obsc</td>
<td>126.9</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>18, 18'</td>
<td>NA</td>
<td>NA</td>
<td>8.23 s</td>
<td>ND</td>
<td>8.30 s</td>
</tr>
<tr>
<td>19</td>
<td>NA</td>
<td>NA</td>
<td>6.92 s</td>
<td>108</td>
<td>8.06 s</td>
</tr>
</tbody>
</table>

\(\text{mult} = \text{multiplicity, br} = \text{broad, m} = \text{multiplet, s} = \text{singlet, t} = \text{triplet, dd} = \text{doublet of doublet, obsc} = \text{obscured, } J = \text{coupling constant in Hz, NA} = \text{not applicable, ND} = \text{not detected.}\)

\textsuperscript{a}\text{Assignments were facilitated by the analysis of } ^1H\text{, DQFCOSY, TOCSY, HSQC and HMBC spectra.}

\textsuperscript{b}\text{Coupling constants are not entered for M25 when identical with those of LY654322.}
Fig 1.
Fig 2.
Fig 3.
Fig 4.
Fig 5.
Fig 6.
Fig 8.