Elucidating the Mechanism of Cytochrome P450-Mediated Pyrimidine Ring Conversion to Pyrazole Metabolites with the BACE1 Inhibitor GNE-892 in Rats

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Non-standard abbreviations: Aβ, amyloid beta; BACE1, β-site amyloid precursor protein cleaving enzyme 1; CID, collision-induced dissociation; COSY, correlation spectroscopy; HMBC, heteronuclear multiple bond correlation; HPLC, high performance liquid chromatography; HSQC, heteronuclear single quantum correlation; LC-MS/MS, liquid chromatography tandem mass spectrometry; LSC, liquid scintillation counting; NMR, nuclear magnetic resonance; P450, cytochrome P450.
Abstract

An uncommon biotransformation of pyrimidine during the metabolism of GNE-892, a β-secretase 1 inhibitor, was investigated. Three novel metabolites, formed by conversion of pyrimidine to pyrazole, were observed in the 14C-radiolabeled mass balance study in rats. Their structures were characterized by high-resolution mass spectrometry and NMR. Though these metabolites accounted for <5% of the administered dose, their unique nature prompted us to conduct further investigations. The pyrazole-containing metabolites were formed in vitro with rat hepatocytes and liver microsomes, which supported that they were formed during hepatic metabolism. Further, their generation was inhibited by 1-aminobenzotriazole, indicating involvement of P450s. Studies with rat recombinant enzymes identified that CYP2D2 generated the N-hydroxypyrazole metabolite from GNE-892. This biotransformation proceeded through multiple steps from the likely precursor, pyrimidine N-oxide. On the basis of these data, we propose a mechanism in which the pyrimidine is activated via N-oxidation followed by a second oxidative process that opens the pyrimidine ring to form a formamide intermediate. Following hydrolysis of the formamide, a carbon is lost as formic acid together with ring closure to form the pyrazole ring. This article highlights a mechanistic approach for determining the biotransformation of the pyrimidine to a pyrazole for GNE-892.
Introduction

GNE-892 ((R)-2-amino-1,3',3'-trimethyl-7'-(pyrimidin-5-yl)-3',4'-dihydro-2'H-spiro[imidazole-4,1'-naphthalen]-5(1'H)-one) is a novel small molecule that was designed through structure-optimization efforts as a potent inhibitor for BACE1 (β-site amyloid precursor protein cleaving enzyme 1) (IC\textsubscript{50} 48 nM, cellular IC\textsubscript{50} 27 nM) (Hunt et al., 2013). BACE1 is a protease that processes amyloid precursor protein to amyloid beta (Aβ) peptide fragments. The interaction of Aβ peptides with neurons has been implicated in neurodegeneration associated with the progression of Alzheimer’s disease (Hardy and Higgins, 1992; Vassar et al., 1999). BACE1 is, therefore, a promising protein target for the reduction of Aβ levels to interrupt or slow decline in neurocognitive function. The pharmacokinetics and pharmacodynamics of GNE-892 in cynomolgus monkeys provided proof of mechanism for β-secretase inhibition with significantly reduced levels of Aβ in plasma and cerebrospinal fluid following a single oral administration (Liu et al., 2013).

GNE-892 was identified as a potential clinical candidate with promising absorption, distribution, metabolism, and excretion properties. In liver microsomes and hepatocytes, it was moderately stable for rat, and it was stable for monkey and human. The major metabolic pathways were N-demethylation, and hydroxylation at the oxo-imidazole or pyrimidyl-tetrahydro-naphthalene moieties. We recently reported the metabolism in cynomologus monkeys as being mainly oxidative and catalyzed not only by P450s, but also by aldehyde oxidase (manuscript submitted). Interestingly, the \textsuperscript{14}C-radiolabeled metabolism study in monkeys, which was designed as proof-of-concept for microdosing in humans, showed dose dependency in exposure and in the contributions by P450 and aldehyde oxidase to metabolism following single oral dosing.

In the \textsuperscript{14}C-radiolabeled study of GNE-892 in Sprague-Dawley rats, which was conducted to support the toxicological studies, we observed a less common biotransformation pathway involving pyrimidine conversion to pyrazole. The structures for three related metabolites were elucidated using mass spectrometry and confirmed by NMR. Here, we report the results from studies of the formation of these metabolites and mechanistic understanding of this biotransformation pathway.

Materials and Methods

Chemicals and Test Systems. GNE-892 was synthesized at Array BioPharma and Genentech, Inc. \textsuperscript{14}C]GNE-892 was synthesized with a \textsuperscript{14}C label installed at carbon-4 of the pyrimidine moiety (specific activity of 60 mCi/mmol,
radiochemical purity >99.5%) by Quotient Bioresearch (Cardiff, UK) utilizing intermediates from Array BioPharma. All other experimental reagents and chemicals were purchased from vendors at the highest purity that was available. Cryopreserved pooled male Sprague-Dawley rat hepatocytes were from CellzDirect (Research Triangle Park, NC). Rat liver microsomes and recombinant CYP isozymes were from BD Gentest (San Jose, CA).

**In Vivo Rat Study and Sample Preparation.** Male bile duct-intact and -cannulated (BDC) Sprague-Dawley rats (n=3 per group) were orally administered a single dose of \([^{14}C]\)GNE-892 at 15 mg/kg (100 μCi/kg) in 1% carboxymethylcellulose, 0.5% Tween, and 5 mM citrate solution. For BDC rats, a solution of taurocholic acid (2.3 mg/mL in 0.9% saline) was infused via the distal (duodenal) cannula at a rate of 0.9 mL/h during the sample collection. Urine and bile were collected on dry ice at approximately 0-8, 8-24, and then at 24-h intervals. Feces were collected on dry ice at intervals of 24 h up to 168 and 96 h post-dose from intact and BDC rats, respectively, and homogenized with 3-5 volumes of 50% (by volume) aqueous isopropyl alcohol. All samples were stored at approximately –20°C until analysis. For metabolite profiling, an equal percentage of urine and bile samples from 0-24 h and feces samples from 0-48 h were pooled to provide a single sample for each matrix that represented greater than 95% of the total radioactivity eliminated by the excretion route. Pooled urine and bile samples were centrifuged and the supernatants were injected directly for metabolite profiling. Pooled fecal homogenates were extracted three times with acetonitrile (ACN) and the extracts were combined, evaporated to dryness by an N-Evaporator (Caliper Life Sciences, Hopkinton, MA), and reconstituted for metabolite profiling.

**Profiling of GNE-892 and Its Metabolites.** LC-MS/MS analysis was conducted on a 20A vp serial (Shimadzu, Columbia, MD) or Accela (Thermo Fisher Scientific, San Jose, CA) HPLC coupled with a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). HPLC separation of in vivo samples was performed by gradient elution from an ACE 3 AR-C18 column (3 μm, 4.6×150 mm; MAC-MOD Analytical, Chadds Ford, PA) with a flow rate of 0.7 mL/min of mobile phases (A) 0.4% formic acid in water adjusted to pH 3.2 with ammonium hydroxide, (B) methanol (MeOH), and (C) ACN. HPLC gradients were initiated with 100% A for 1.5 min with a shallow gradient to 15% B at 3.5 min, 45% B at 68.5 min, and 70% B at 83.5 min. The column was washed with a gradient to 100% C over 4 min, then held for 6.5 min before returning to the initial composition of 100% A within 2 minutes. The system was allowed to equilibrate for 15 min before the next injection. The total sample run time was 111 min. HPLC separation of in vitro samples was performed by gradient elution from a Hypersil Gold column (1.9 μm, 2.1×100 mm, Thermo Scientific) with a flow rate of 0.5 mL/min of mobile phases (A) 0.4% formic acid in water
adjusted to pH 3.2 with ammonium hydroxide, and (B) MeOH. HPLC gradients were initiated with linear gradient from 15 to 35% B over first 10 min, then to 40% B at 11 min, and 100% B at 12 min. The column was washed with 100% B for 2 min before returning to the initial composition of 85% A for 2 min of equilibration before the next injection. The total sample run time was 16 min. The electrospray ion source voltage was set at 5.0 kV and heated capillary temperature at 350°C. A full-scan mass spectrum was acquired at a resolving power of 30,000 (at m/z of 400), and data dependent multi-stage mass spectrometry (MS^n) scans were acquired at a resolving power of 7,500. Accurate mass measurements were performed using external calibration. Hydrogen-deuterium (H/D) exchange experiments were performed by replacing the aqueous and organic components of the mobile phases with deuterium oxide and MeOH-d₄, respectively, and the number of exchanged protons was determined from the observed mass shift.

For plasma and in vitro sample metabolite radioprofiling, fractions of chromatography effluents were collected by time (10-15 sec/fraction) to LumaPlate-96 plates (Perkin Elmer, Waltham, MA), dried by a SpeedVac concentrator (Thermo Scientific) for up to 8 h, and counted for 5 min per well using a TopCount NXT Microplate Scintillation Counter (Perkin Elmer). HPLC radiochromatograms were reconstructed and distribution of individual peaks was determined using Convert and Evaluation (AIM Research Company, Hockessin, DE) or Laura (LabLogic, Brandon, FL) software. Metabolite radioprofiling of excreta samples was performed with online radioflow detection using a β-Ram 5C (LabLogic, Brandon, FL).

**Structural Determination of Pyrazole Metabolite (M16) by NMR.** A purified isolate of M16 (yield ~10 µg) was prepared from pooled fecal homogenates (~95 g) through successive steps of liquid and solid phase extractions and preparative chromatography. Details of the isolation procedures are provided as Supplemental Methods. An aliquot of GNE-892 or M16 was dissolved in 30 µL of ACN-d₃ containing 0.05% (by volume) trimethylsilane as an internal chemical shift reference standard (D, 99.8%, Cambridge Isotope, Andover, MA) and transferred to a 1.7 mm NMR tube, purged with nitrogen and sealed. NMR measurements were performed on an Avance 3, 600 MHz spectrometer equipped with a 1.7-mm TCI Z-gradient CryoProbe (Bruker Daltronics, Billerica, MA). The sample temperature was maintained at 28°C for all data collections, and spectra were acquired using standard Bruker pulse sequences with version 3.1 and patch level 7 TopSpin software (Bruker).

**In Vitro Generation of Pyrazole Metabolites.** Hepatocyte incubations were completed with [¹⁴C]GNE-892 (5 µM) in hepatocytes (1×10⁶ cells/mL) suspended in Dulbecco’s modified Eagle’s medium, pH 7.4, for 3 h at 37°C
with gentle shaking. Incubations were terminated with the addition of three volumes of ACN, samples were centrifuged, and the supernatants were concentrated and analyzed by LC-MS/MS and online radiodetection. Separate incubations were conducted in the same manner with pre-incubation for 15 min with 1-aminobenzotriazole (1 mM), a nonselective P450 inactivator.

In vitro screening of rat P450 Supersomes (CYP1A2, 2C6, 2C11, 2C12, 2C13, 2D1, 2D2, 3A1, and 3A2) was performed using a constant amount of P450 (0.04 nmol/mL) supplemented with NADPH (1 mM) in potassium phosphate, pH 7.4 (100 mM), with magnesium chloride (3 mM) and GNE-892 (1 µM) for 60 min at 37°C. For inhibition experiments, selective chemical inhibitors of CYP2D2 (fluoxetine, desipramine, quinine, and quinidine) at 10 µM were pre-incubated for 15 min at 37°C with NADPH (1 mM) and rat liver microsomes (0.5 mg/mL). The reaction was initiated with the addition of GNE-892 (1 µM) and lasted 60 min. At the end of the incubation, the reactions were quenched with an equal volume of cold ACN, and proteins were precipitated. The supernatants were diluted with an equal volume of water for LC-MS/MS analysis. For time-course measurement of metabolite generation by CYP2D2, [14C]GNE-892 was incubated under the same in vitro conditions as non-radiolabeled compound and aliquots were removed at intervals up to 60 min and quenched with three volumes of ACN, then concentrated, and analyzed by HPLC with radiodetection.

Results

Mass Balance and Excretion Profiles. Following the single oral administration of [14C]GNE-892 to Sprague-Dawley rats, 95% of the administered radioactivity was recovered (Table 1). The majority of the radioactivity was recovered in feces (77%), and excretion was rapid and nearly complete after the first 48 h post-dose. For BDC rats, 53% of the administered radioactivity was excreted in bile and 33% in urine, which indicated that at least 86% of the dose had been absorbed.

Metabolite Identification. GNE-892 eliminated predominantly as metabolites that were oxidative in nature and in which the oxo-imidazole, dimethylcyclohexane, or pyrimidine moieties were modified (Figure 1 and Table 2). The structures of metabolites were determined based on high-resolution full scan and MSn mass spectrometry experiments (Figure 2). Since the focus of the described studies was the mechanism for the pyrimidine conversion to pyrazole, the structural elucidation descriptions provided here are only for metabolites where modifications of the pyrimidine moiety were identified.
GNE-892 had a protonated molecular ion ([M+H]+) at \textit{m/z} 336.1820 (C₁₉H₂₂O₅N₅, 0.30 ppm). The product ion at \textit{m/z} 126 corresponded to the intact oxo-imidazole moiety, while product ions at \textit{m/z} 263 and 252 were formed as the result of cleavages of the oxo-imidazole moiety, and corresponded to the pyrimidyl-tetrahydronaphthalene moiety (Figure 2A). The product ions at \textit{m/z} 308, 294, and 276 resulted from the loss of CO, NH₂CN, and its sequential loss of water, respectively. These diagnostic ions were used to identify the site for modification for the identified metabolites.

M5 had a [M+H]+ at \textit{m/z} 352.1773 (C₁₉H₂₂O₂N₅, 1.40 ppm), which was indicative that it had been generated through a mono-oxidation reaction. M5 was identified as an \textit{N}-oxide of GNE-892 on the basis of no increase in the number of exchangeable hydrogens from H/D experiments, the observed fragment ion at \textit{m/z} 335 from the loss of a hydroxyl radical, and its chemical reduction to unchanged drug with the addition of titanium chloride. Product ions at \textit{m/z} 279, 268, and 251 supported that \textit{N}-oxidation had occurred at the pyrimidine (Figure 2B).

M14 had a [M+H]+ at \textit{m/z} 516.2093 (C₂₄H₃₀O₈N₅, 0.83 ppm). Upon fragmentation of the molecular ion, a neutral loss of 176 Da, a characteristic loss for a glucuronic acid conjugate, provided the product ion at \textit{m/z} 340 (no spectra shown). Further fragmentation provided product ion spectra that were comparable to that for M17. Thus, M14 was identified as the glucuronide conjugate of M17.

M16 had a [M+H]+ at \textit{m/z} 324.1812 (C₁₈H₂₂O₂N₅, -1.98 ppm), which indicated one carbon had been lost from GNE-892. This was supported by product ions at \textit{m/z} 296, 264, 251, 240, and 199, which were shifted 12 Da lower than their corresponding ions for GNE-892 (Figure 2C). The product ion at \textit{m/z} 126 accounted for all of the carbons of an unchanged imidazole moiety, which suggested that the loss of carbon likely occurred at the pyrimidine moiety. H/D exchange indicated that M16 had three exchangeable protons, which was one more than the parent. This implied the pyrimidine had been re-cyclized after the loss of carbon and this had introduced an exchangeable hydrogen. To confirm the structure of the five-membered ring moiety, M16 was isolated and purified from fecal samples and characterized by NMR (Figure 3 and Table 3). In the \textsuperscript{1}H NMR, a single signal at 7.84 ppm (2H, br s) was assigned to the two protons in the pyrazole ring. These two protons have similar electronic effects, yielding very similar chemical shifts, in contrast to imidazole, for which the chemical shift of the protons would have been different. Another observation was the disappearance of the signal at 9.09 ppm (1H, broad singlet) from GNE-892, which corresponded to carbon-2 of the pyrimidine. The loss of carbon-2 from pyrimidine was also supported by
observing a 1:1 ratio of the $^{14}$C:$^{12}$C isotopes by mass spectrometry for the pyrazole metabolites when they were generated in vitro from a mix of equal proportions of $^{14}$C and $^{12}$C GNE-892. If carbon-4 or 6 of pyrimidine, which are equivalent carbons, were lost, a change in the $^{14}$C:$^{12}$C isotope ratio (to 2:1) should be observed. Finally, HSQC data were consistent with the predicted $^{13}$C chemical shift for pyrazole carbons 3 and 5 of 132.0 ppm. Hence, M16 was identified as the conversion of pyrimidine to pyrazole metabolite.

M17 had a [M+H]$^+$ at $m/z$ 340.1762 (C$_{18}$H$_{22}$O$_2$N$_5$, -1.86 ppm). Product ions at $m/z$ 312, 280, 267, and 256 were 16 Da higher than the corresponding ions in M16, which supported oxidation had occurred at pyrimidyl-tetrahydronaphthalene (Figure 2D). The loss of a hydroxyl radical provided the ion at $m/z$ 323, and indicated an N-hydroxypyrazole moiety. In product ion spectra for [14C]M17, ion pairs corresponding to $^{12}$C and $^{14}$C were observed at $m/z$ 240/242, and $m/z$ 223/225. The ion abundances were approximately equal within each pair, and were rationalized as equivalent pyrazole cleavages with loses of radiolabel or non-radiolabel carbon. This was consistent with the two pyrazole nitrogens being equal and oxidized with the same probability. In the $^1$H-NMR for M17, that resonances corresponding to the protons of the pyrazole no longer have the same chemical shifts, supporting the formation of N-hydroxylation on the pyrazole nitrogen.

M21 had a [M+H]$^+$ at $m/z$ 370.1881 (C$_{19}$H$_{24}$N$_5$O$_3$, 2.08 ppm), which was consistent with the addition of two oxygen and two hydrogen atoms to GNE-892. The product ion spectra showed a single abundant ion at $m/z$ 325, generated by the loss of NH$_2$C(O)H from the molecular ion (Figure 2E). Further dissociation of $m/z$ 325 generated ions by fragmentation across the intact oxo-imidazole moiety, which were the primary fragmentation pathways observed for the molecular ions of GNE-892 and other metabolites. The favored loss of NH$_2$C(O)H was rationalized to be a facile loss from an opened pyrimidine ring structure, where moieties formed by oxidative ring opening were aldehyde and N-hydroxy moiety.

**Metabolic Profiles.** The mean percentages of GNE-892 and its metabolites that were quantified in urine, feces, and bile, expressed as a percentage of the administered dose, are shown in Table 2. The pyrimidine N-oxide metabolite M5 was detected in urine and feces accounting for 6.1 and 1.1% of the dose, respectively. The pyrazole metabolites M16 and M17 were observed in feces and accounted for 3.4 and 1.2% of the dose, respectively. The glucuronide conjugate of N-hydroxypyrazole metabolite, M14, was detected in bile and accounted for 3.2% of the dose. The pyrimidine-opened metabolite M21 was observed in urine and feces, though it was present at levels that were measureable by mass spectrometry and not by radiodetection.
Metabolite profiles of [14C]GNE-892 in cryopreserved rat hepatocytes demonstrated that parent compound had been fully converted to metabolites following 3 h incubations. The distributions of metabolites as percentages of the total sample radioactivity are summarized in Table 4. At the end of the incubation, the pyrimidine-modified metabolites M5 and M14 had formed and accounted for 23.0 and 10.0% of the sample radioactivity, respectively. The remaining drug was converted to M1, M2, M4, M9, and M13, which were all modified at the dimethylcyclohexane by oxidative processes (Figure 1). These metabolites accounted for 12.9, 4.2, 16.8, 9.8, and 4.0% of the sample radioactivity, respectively, and the remaining radioactivity was distributed over other low-level analytes.

**In Vitro Formation of Pyrazole Metabolites.** Incubations of GNE-892 with nine major recombinant rat P450s demonstrated that CYP2D2 generated M17 to the greatest extent (Supplemental Figure 1). By incubating GNE-892 in rat liver microsomes in the presence of CYP2D2 inhibitors, it was observed that desipramine and quinine were effective at inhibiting the formation of M17, while fluoxetine and quinidine were inhibitory to a lesser extent (16 and 55% of control, respectively) (Supplemental Figure 2). These results were consistent with the reported potencies of these chemicals against rat CYP2D2 (Isobe et al., 2005; Makaji et al., 2010). The residual formation of M17 in inhibition experiments could be attributed to the activity of CYP3A1, which also generated M17 as a recombinant enzyme, albeit to a lesser extent than CYP2D2.

In the incubation of [14C]GNE-892 with recombinant CYP2D2, parent compound was rapidly converted to M5 such that after the first 15 min, unchanged GNE-892 was <5% and M5 was 95% (Figure 4). Through the remaining incubation, M17 was formed and its increase over time was stoichiometrically accounted by the decrease in M5. From 15 to 60 min, M5 decreased from 95 to 88% of the sample radioactivity, whereas M17 increased from 4 to 12%. M21 was also generated in this study, though only at levels detectable with mass spectrometry. No other metabolite was detected by radiodetection during the incubation.

In a two-step incubation, GNE-892 was incubated with recombinant CYP2D2 to generate M5. This reaction was quenched, then individual recombinant rat CYPs were added to the resulting incubate and M17 formation was monitored. Here, we observed that the addition of CYP2D2 was required for converting M5 to M17, and the other tested CYPs did not catalyze this reaction (data not shown).
Discussion

In the characterization of the metabolism and excretion of GNE-892 in rats following a single oral dose, we identified three metabolites that were formed via conversion of the pyrimidine moiety to pyrazole structures. Though examples of metabolic cleavage of pyrimidine exist, it is an uncommon biotransformation pathway (Prakash and Cui, 1997; Sharma et al., 2012). Even more rarely observed has been re-cyclization following pyrimidine scission, which we have found described in only two other cases: (S)-25 and AZD3839 in rats (Lindgren et al., 2013), and vicriviroc in humans (Ghosal et al., 2007). In the cases of (S)-25 and AZD3839, the pyrimidine moiety was converted to imidazole and N-hydroxylated, N-oxidated, and conjugated derivatives in vivo in rats, but were not detected in vitro. In the case of the CCR5 receptor antagonist vicriviroc, the pyrimidine-4,6-dimethyl moiety was converted to a pyrazyl-hydroxylamine. Since, to the best of our knowledge, a mechanistic explanation for this pathway did not exist, and given the report for the metabolism of vicriviroc was precedence for this metabolic pathway in humans, we more closely investigated the pyrimidine conversion by conducting a series of in vitro metabolism studies with GNE-892. From these studies, we identified the enzyme responsible and immediate precursor for the pyrazole, and provide a proposed mechanism for this biotransformation.

The structure of the pyrazole metabolites of GNE-892 were elucidated by mass spectrometry and confirmed by NMR. These data were consistent with contraction of the pyrimidine ring to pyrazole and identified the structures of M16, M17, and M14 as the pyrazole, N-hydroxypyrazole, and glucuronide conjugate of the N-hydroxypyrazole, respectively. The apparent relationship between these metabolites was reinforced by their excretion profiles. M14 was found only in bile; whereas, in bile duct-intact animals, M14 was not present, but M16 and M17 were measured in feces. These results were consistent with M14 being secreted to the intestine in bile and undergoing β-glucuronidase-mediated cleavage to M17 and subsequent reduction to M16 during gastrointestinal transit. We also inferred based on M14 being found only in bile and the pyrazole metabolites not being detected in plasma that liver was likely the major organ for the formation of the pyrazole-containing metabolites.

Given that the pyrimidine conversion to pyrazole requires multiple steps, we sought to understand the enzymes that were involved. Rat hepatocytes were a complete in vitro metabolic system for this biotransformation and generated M14. 1-Aminobenzotriazole was effective at inhibiting this reaction, which indicated that P450s catalyzed at least one step in formation of the pyrazole. Incubation of GNE-892 with individual recombinant rat P450s revealed that CYP2D2 was most likely the rat P450 isoform responsible for the pyrazole formation. This was
also supported by reduced formation of M17 in incubations with rat liver microsomes in the presence of selective chemical inhibitors of CYP2D2. Interestingly, when the oxidative metabolism was studied with microsomes or recombinant P450s, it was M17, the N-hydroxyypyrazole, and not M16, that was generated as the primary pyrazole metabolite.

Most likely, a single oxidative step would not have converted the pyrimidine moiety to pyrazole; therefore, we aimed to identify the immediate precursor of the pyrimidine cleavage. Given that carbon-2 is lost from pyrimidine in its conversion to pyrazole, 2-hydroxypyrimidine was first considered as the precursor metabolite. For this purpose, a synthetic standard was tested in rat hepatocytes and liver microsomes; however, no pyrimidine ring-opened or ring-contracted metabolite was detected. Another potential precursor was M5, pyrimidine N-oxide. M5 was an attractive source for the pyrazole metabolites given that it was observed in all samples in which these metabolites were present. Furthermore, it was the most abundant primary oxidative metabolite in rat in vivo and in vitro. Unfortunately, efforts to synthesize M5 were unsuccessful and we were unable to test it directly.

The evidence that M5 was likely the precursor metabolite of the pyrazole was provided by monitoring the formation of metabolites during the incubation of [14C]GNE-892 with recombinant CYP2D2. In these incubations, [14C]GNE-892 was rapidly and nearly quantitatively converted to M5. With continued incubation, M17 formed and mass balance of the radiolabelled material was only explained by accounting for M17 formation by decreases in M5; therefore, we concluded that M5 was the precursor of M17. In surveying these incubations for other drug-related analytes, we observed that M21 was also generated in this study, though it was present at low levels. In addition, when we tested if another P450 isoform could convert M5 to M17 by conducting a two-step incubation, only CYP2D2 was observed to catalyze this reaction.

Based on these findings, we propose the mechanism presented in Figure 5 for pyrimidine biotransformation to pyrazole by P450 enzyme. In the first step, the pyrimidine is oxidized to N-oxide (M5) by P450. Next, to open the pyrimidine ring and re-cyclize as pyrazole, we considered several possibilities for oxidizing M5 at carbon-2 of pyrimidine, which is ultimately lost. Though we did not attempt to study the oxygenating species in the P450-catalyzed reaction, there were observations for GNE-892 that provided us with some insight into this reaction. We first considered an oxo-iron species as an electrophilic oxidant since this is known to act in or, at least, can easily explain most known P450 oxidation reactions (Groves, 2005). Oxidation of M5 by oxo-iron would result in epoxidation or hydroxylation, which is not typically known to lead to ring-opened metabolites. This was also
supported by in vitro testing the 2-hydroxy metabolite and not detecting any ring-opened or pyrazole metabolites. Further, it seemed inconsistent that hydroxylation of the N-oxide pyrimidine of M5, which is less electron rich than the pyrimidine of GNE-892, would occur with an electrophilic oxidant. Since the oxo-iron species did not adequately explain the P450-catalyzed reaction, an alternate oxidant, the peroxo-iron species (Fe-O-O'), was considered (Vaz, 2003). The peroxo-iron species is nucleophilic in nature; therefore, this was consistent with the criterion that a more electron deficient species (N-oxide, M5) is the precursor required for opening the pyrimidine ring. Following formation of the peroxy complex, it seems reasonable that the C-N bond can be cleaved via heterolytic fragmentation and this opens the pyrimidine ring (Figure 5). The resulting formamide intermediate can proceed through two possible pathways. In the first, the formamide intermediate is converted to M21 via a reductive reaction; though the low levels of this metabolite suggested that this is a less favored pathway. The more favored pathway was hydrolysis of the formamide moiety, which proceeds through losing formic acid in a cyclization reaction that forms N-hydroxypyrazole (M17). M17 could then be conjugated with glucuronic acid to form M14 or reduced to M16. As for previous reported examples, it is enticing to suggest that for vicriviroc, the pyrimidine N-oxide and N-hydroxypyrazole metabolites that were observed are the precursor and product of pyrimidine conversion for that drug and that this reaction proceeds according to the same or a similar mechanism in humans as we propose here for GNE-892.

The examples of pyrimidine biotransformation to pyrazole, such as GNE-892, vicriviroc, and (S)-25, demonstrate the potential for carbon atom loss via oxidative biotransformation. This should be considered when selecting the placement of a ¹⁴C radiolabel for metabolism studies (Penner et al., 2012). To date, this biotransformation has been a minor component of total reported metabolism; however, it is conceivable that for other chemicals it occurs to a much greater extent depending on structure, enzymology, or test species. Without a prior knowledge to design experiments that retain volatile components, loss of the radiolabel would result in lower mass balance and a metabolic pathway would not be identified (Roffey et al., 2007). This biotransformation should also be considered in non-radiolabeled studies, especially when human metabolites are being identified for consideration of safety testing. Without a radiolabel to indicate the drug-derived analytes, it is necessary to utilize workflows that search for common and uncommon metabolites. This requires a comprehensive knowledge of possible biotransformations, and underscores the importance of studying the structural requirements, enzymology, and mechanisms of uncommon pathways once they are discovered.
In summary, in the metabolism of GNE-892 in rats, an uncommon P450-mediated conversion of a pyrimidine to a pyrazole was observed. The main enzyme responsible for this biotransformation was CYP2D2 in rat liver. The mechanism involves N-oxidation of pyrimidine, which proceeds through pyrimidine ring opening, loss of formic acid, and a ring closure to form the pyrazole.

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References

Identification of human liver cytochrome P450 enzymes involved in biotransformation of vicriviroc, a
CCR5 receptor antagonist. Drug Metab Dispos 35:2186-2195.


Hunt KW, Cook AW, Watts RJ, Clark CT, Vigers G, Smith D, Metcalf AT, Gunawardana IW, Burkard M, Cox AA,
Geck Do MK, Dutcher D, Thomas AA, Rana S, Kallan NC, DeLisle RK, Rizzi JP, Regal K, Sammond D,
amyloid precursor protein cleaving enzyme 1 (BACE1) inhibitors: from hit to lowering of cerebrospinal

Different effects of desipramine on bufuralol 1''-hydroxylation by rat and human CYP2D enzymes. Biol

Biotransformation of two beta-secretase inhibitors including ring opening and contraction of a pyrimidine
ring. Drug Metab Dispos 41:1134-1147.

Mechanistic Pharmacokinetic-Pharmacodynamic Modeling of BACE1 Inhibition in Monkeys:
Development of a Predictive Model for Amyloid Precursor Protein Processing. Drug Metab Dispos
41:1319-1328.

Makaji E, Trambitas CS, Shen P, Holloway AC, and Crankshaw DJ (2010) Effects of cytochrome P450 inhibitors
on the biotransformation of fluorogenic substrates by adult male rat liver microsomes and cDNA-expressed

Penner N, Xu L, and Prakash C (2012) Radiolabeled absorption, distribution, metabolism, and excretion studies in


Footnote

Parts of this work were presented at the 18th North American meeting of the International Society for the Study of Xenobiotics in Dallas, TX on October 14-18, 2012.

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Legend for Figures

Figure 1. Proposed biotransformation pathways of GNE-892 in rats.

Figure 2. The collision-induced dissociation product ion spectra observed for (A) GNE-892 and its metabolites (B) M5, (C) M16, (D) M17, and (E) M21, and interpretation of their fragment ions. Theoretical m/z values are indicated for the proposed fragment ions. For GNE-892, the site of the 14C-label at carbon-4 of the pyrimidine is indicated by the asterisk.

Figure 3. ¹H-¹³C Multiplicity Edited HSQC NMR spectrum of M16 isolated from rat feces. Data were used for assignment of proton and carbon resonances to confirm the pyrazole moiety. CH and CH₃ signals are shown in red and CH₂ signals are shown in blue.

Figure 4. Formation of pyrimidine N-oxide (M5, filled square) and N-hydroxypyrazole (M17, open square) metabolites from [¹⁴C]GNE-892 (filled triangle) in incubation with rat recombinant CYP2D2.

Figure 5. Proposed mechanism for formation of pyrazole-containing metabolites from GNE-892. The numbering for the pyrimidine ring is shown for GNE-892 and the ¹⁴C radiolabel was located at carbon-4 of pyrimidine.
Table 1. Cumulative recovery of administered radioactivity in urine, bile, and feces collected from rats following a single oral dose of GNE-892 at 15 mg/kg (100 µCi/kg)

<table>
<thead>
<tr>
<th>Time period (h)</th>
<th>Cumulative Recovery of Dose (%)</th>
<th>Bile Duct-Intact Rats (mean, n=3)</th>
<th>Bile Duct-Cannulated Rats (mean, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
<td>Total</td>
</tr>
<tr>
<td>0-8</td>
<td>15.3</td>
<td>0.0</td>
<td>15.3</td>
</tr>
<tr>
<td>0-24</td>
<td>17.3</td>
<td>74.2</td>
<td>91.5</td>
</tr>
<tr>
<td>0-48</td>
<td>17.6</td>
<td>77.0</td>
<td>94.6</td>
</tr>
<tr>
<td>0-72</td>
<td>17.7</td>
<td>77.2</td>
<td>94.9</td>
</tr>
<tr>
<td>0-96</td>
<td>17.7</td>
<td>77.3</td>
<td>95.0</td>
</tr>
<tr>
<td>0-120</td>
<td>17.8</td>
<td>77.3</td>
<td>95.1</td>
</tr>
<tr>
<td>0-144</td>
<td>17.8</td>
<td>77.4</td>
<td>95.2</td>
</tr>
<tr>
<td>0-168</td>
<td>17.8</td>
<td>77.4</td>
<td>95.2</td>
</tr>
</tbody>
</table>
Table 2. Identified metabolites and their relative distribution in urine, feces, and bile collected from rats following a single oral dose of GNE-892 at 15 mg/kg

<table>
<thead>
<tr>
<th>Analyte</th>
<th>([M+H]^+) (m/z)</th>
<th>Retention time(^a) (min)</th>
<th>Biotransformation</th>
<th>Percentage of Dose in Rats</th>
<th>Bile Duct Intact (mean, n=3)</th>
<th>Bile Duct Cannulated (mean, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNE-892</td>
<td>336</td>
<td>73.6</td>
<td>Parent compound</td>
<td>2.8</td>
<td>33.3</td>
<td>7.1</td>
</tr>
<tr>
<td><strong>Pyrimidine-Modified Metabolites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>\n</td>
</tr>
<tr>
<td>M14</td>
<td>516</td>
<td>74.6</td>
<td>(N)-Hydroxypyrazole formation + Glucuronidation</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M16</td>
<td>324</td>
<td>76.4</td>
<td>Pyrazole formation</td>
<td>ND</td>
<td>3.4</td>
<td>ND</td>
</tr>
<tr>
<td>M17</td>
<td>340</td>
<td>77.4</td>
<td>(N)-Hydroxypyrazole formation</td>
<td>D</td>
<td>1.2</td>
<td>D</td>
</tr>
<tr>
<td>M21</td>
<td>370</td>
<td>79.6</td>
<td>Pyrimidine opening</td>
<td>D</td>
<td>D</td>
<td>D</td>
</tr>
<tr>
<td><strong>Other Metabolites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>\n</td>
</tr>
<tr>
<td>M2</td>
<td>352</td>
<td>26.0</td>
<td>Oxidation</td>
<td>1.3</td>
<td>9.6</td>
<td>2.2</td>
</tr>
<tr>
<td>M3</td>
<td>352</td>
<td>40.1</td>
<td>Oxidation</td>
<td>0.6</td>
<td>9.5</td>
<td>0.6</td>
</tr>
<tr>
<td>M4</td>
<td>366</td>
<td>40.4</td>
<td>Dioxidation + Dehydrogenation</td>
<td>0.9</td>
<td>5.4</td>
<td>2.1</td>
</tr>
<tr>
<td>M6</td>
<td>322</td>
<td>66.0</td>
<td>(N)-Demethylation</td>
<td>0.6</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>M7</td>
<td>352</td>
<td>67.0</td>
<td>Oxidation</td>
<td>0.1</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>M9</td>
<td>368</td>
<td>28.3</td>
<td>Dioxidation</td>
<td>0.4</td>
<td>0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>M10</td>
<td>352</td>
<td>35.8</td>
<td>(N)-Demethylation + Dioxidation + Dehydrogenation</td>
<td>0.4</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>M11</td>
<td>338</td>
<td>54.0</td>
<td>(N)-Demethylation + Oxidation</td>
<td>0.3</td>
<td>ND</td>
<td>0.3</td>
</tr>
<tr>
<td>M12</td>
<td>352</td>
<td>55.8</td>
<td>Oxidation</td>
<td>0.2</td>
<td>1.4</td>
<td>0.5</td>
</tr>
<tr>
<td>M13</td>
<td>350</td>
<td>58.0</td>
<td>Oxidation + Dehydrogenation</td>
<td>0.9</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>M15</td>
<td>368</td>
<td>75.6</td>
<td>Dioxidation</td>
<td>0.4</td>
<td>ND</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15.2</td>
</tr>
</tbody>
</table>

\(^a\) Retention times listed were observed for in vivo samples using the 111 minute HPLC method. For in vitro samples analyzed with the 16 min HPLC method, GNE-892, M5, M17, and M21 were observed at 8.1, 4.9, 10.7, and 10.9 min, respectively.

Urine and bile were 0-24 h, feces were 0-48 h.
ND, not detected; D, detected by mass spectrometry
Table 3. $^1$H NMR spectroscopy data of GNE-892 and M16

<table>
<thead>
<tr>
<th>Proton</th>
<th>Chemical Shift in ppm (J in Hz) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1H, 8.90 br s</td>
</tr>
<tr>
<td>5</td>
<td>1H, 8.90 br s</td>
</tr>
<tr>
<td>7</td>
<td>1H, 7.14 br s</td>
</tr>
<tr>
<td>14</td>
<td>1H, 7.51 dd (8.0, 1.7)</td>
</tr>
<tr>
<td>15</td>
<td>1H, 7.28 d (8.0)</td>
</tr>
<tr>
<td>17</td>
<td>1H, 2.76 dd (16.0, 2.4)</td>
</tr>
<tr>
<td></td>
<td>1H, 2.63 dd (16.0, 2.4)</td>
</tr>
<tr>
<td>19</td>
<td>2H, 1.69 d (12.5)$^b$</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>3H, 3.04 s</td>
</tr>
<tr>
<td>23</td>
<td>3H, 0.99 s</td>
</tr>
<tr>
<td>24</td>
<td>3H, 1.13 s</td>
</tr>
<tr>
<td>25</td>
<td>1H, 9.09 br s</td>
</tr>
</tbody>
</table>

s = singlet, br s = broad singlet, d = doublet, dd = double doublet.

$^a$ In acetonitrile-$d_3$.

$^b$ The signal was in the shoulder of the solvent residual peak.
Figure 3
Figure 4

![Graph showing the percentage of sample radioactivity for different analytes over time.](image-url)
Figure 5