Targeting selective activation of M_1 for the treatment of Alzheimer's disease: further chemical optimization and pharmacological characterization of the M_1 positive allosteric modulator ML169

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Supporting Information

Hepatic Microsome Intrinsic Clearance. The metabolism of test compound was investigated in rat hepatic microsomes (BD Biosciences, Billerica, MA) using substrate depletion methodology (% test compound remaining). A potassium phosphate-buffered reaction mixture (0.1 M, pH 7.4) of test compound (1 µM final) and microsomes (0.5 mg/mL) was pre-incubated (5 min) at 37 ℃ prior to the addition of NADPH (1 mM). The incubations, performed in 96-well plates, were continued at 37 ℃ under ambient oxygenation and aliquots (80 µL) were removed at selected time intervals (0, 3, 7, 15, 25 and 45 min). Protein was precipitated by the addition of chilled acetonitrile (160 µL), containing carbamazepine as an internal standard (50 nM), and centrifuged at 3000 RCF (4 ℃) for 10 min. Resulting supernatants were transferred to new 96-well plates in preparation for LC-MS/MS analysis. The in vitro half-life ($t_{1/2}$, min, Eq. 1), intrinsic clearance (CL_{int}, mL/min/kg, Eq. 2) and subsequent predicted hepatic clearance (CL_{hep}, mL/min/kg, Eq. 3) was determined employing the following equations:

1) $t_{1/2}$ = Ln(2) / k ; where k represents the slope from linear regression analysis

(% test compound remaining)

2) *CLint* = (0.693 / $t_{1/2}$) (reaction volume / mg of microsomes) (45 mg microsomes / gram of liver) (20[a] gm of liver / kg body weight); [a]scale-up factors of 20 (human) and 45 (rat)

3) $$CLhep = \frac{Q \cdot CL\,\mathrm{int}}{Q + CL\,\mathrm{int}}$$

Plasma Protein and Brain Homogenate Binding. Plasma protein binding and brain homogenate binding of test compound was determined in rat plasma and rat brain homogenate via equilibrium dialysis employing Single-Use RED Plates with inserts (ThermoFisher Scientific, Rochester, NY). Plasma (220 µL) was added to the 96 well plate containing test article (5 µL) and mixed thoroughly. Subsequently, 200 µL of the plasma-test compound mixture was transferred to the cis chamber (red) of the RED plate, with an accompanying 350 µL of phosphate buffer (25 mM, pH 7.4) in the trans chamber. The RED plate was sealed and incubated 4 h at 37°C with shaking. At completion, 50 µL aliquots from each chamber were diluted 1:1 (50 µL) with either plasma (cis) or buffer (trans) and transferred to a new 96 well plate, at which time ice-cold acetonitrile containing carbamazepine (2x volume) as an internal standard (50 nM) was added to extract the matrices. The plate was centrifuged (3000 RCF, 10 min) and supernatants transferred to a new 96 well plate where they were diluted 1:1 (v/v) in H_2O. The plate was then sealed and stored at –20°C until LC-MS/MS analysis.

Cytochrome P_{450} Inhibition. A four-in-one, 96-well plate assay for determining IC_{50} values against human cytochrome P_{450} 1A2, 2C9, 2D6 and 3A4 was developed based on previous reports (Youdim et al., 2008). Human hepatic microsomes (final concentration of 0.1 mg/mL) and a substrate mixture containing the P_{450} probe substrates phenacetin (10 µM), diclofenac (5 µM), dextromethorphan (5 µM) and midazolam (2 µM) were added to a potassium phosphate buffered solution (0.1 M, pH 7.4) and warmed to 37 °C. The reaction mixture was divided evenly into the 96-well plate and various dilutions of each inhibitor/compound of interest (in duplicate) were then added to this reaction mixture such that the final concentration of each compound ranged from 100 nM to 30 µM. This mixture was allowed to pre-incubate for 15 minutes while shaking at 37 °C. Buffer or NADPH (1 mM) was added and the reaction mixture was incubated for an additional 8 minutes at 37 °C prior to quenching with 2 volumes of ice-cold

acetonitrile containing 50 nM of carbamazepine as internal standard. The plates were centrifuged at 4000 RCF (4 ^{o}C) for 10 minutes and the supernatant was removed and diluted with water (1:4, v/v) in preparation for LC-MS/MS analysis. The IC_{50} values for each compound were obtained for the individual P_{450} enzymes by quantitating the inhibition of metabolite formation for each probe; acetaminophen (1A2), 4-hydroxydiclofenac (2C9), dextrorphan tartrate (2D6) and 1-hydroxymidazolam (3A4). Miconazole was included as a positive control broad spectrum P_{450} inhibition. For discrete 2C19 inhibition experiments, a similar assay design was employed with the following exceptions: the probe substrate was S-mephenytoin (40 µM), the NADPH incubation with the reaction mixture went for 30 minutes, the supernatant was reconstituted 1:1 with water for analysis, and the metabolite used for quantitation was 4-hydroxymephenytoin.

***In Vivo* Plasma-Brain Level Studies.** Male Sprague-Dawley rats (n=2) weighing around 250-350 g were purchased from Harlan laboratories (Indianapolis, IN). The animals were acclimated to their surroundings for approximately one week before study initiation and were provided food and water *ad libitum*. For oral plasma-brain level (PBL) studies measuring systemic plasma, hepatic portal vein (HPV) plasma, and brain tissue concentrations, test compound was administered at a 10 mg/kg dose orally (PO) in 10% Tween 80 0.5% Methyl Cellulose in H_2O (v/v), and 1 hour later samples were collected. Whole blood from systemic compartment (cardiac puncture) and HPV was collected into chilled, EDTA-fortified tubes, centrifuged for 10 minutes at 3000 rpm (4 ℃) and stored at -80 ℃ until LC-MS/MS analysis. The brain samples were rinsed in PBS, snap frozen and stored at -80 ℃. Prior to LC-MS/MS analysis, brain samples were thawed to room temperature and subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products) followed by centrifugation at 3500 RCF for 10 minutes. The brain sample supernatants diluted in plasma (4x by volume) and thawed plasma samples were extracted with 10x volume of acetonitrile containing 50 nM carbamazepine (internal standard) in a 96-well plate and then centrifuged at 3500 RCF for 5 minutes. Resulting supernatants were transferred to a new 96-well plate and diluted 1:1 (v/v) with H_2O. A standard curve was prepared using plasma as matrix over a range of 0.5-10000 ng/mL of test compound along with independent quality control (QC) samples at 5, 50, and 500 ng/mL.

Liquid Chromatography/mass Spectrometry Analysis. *In vivo* samples were analyzed via electrospray ionization (ESI) on an AB Sciex Q-TRAP 5500 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-20AD pumps (Columbia, MD) and a

Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 2.1 x 50 mm, 3 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40 ℃. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 30% B after a 0.2 min hold and was linearly increased to 90% B over 0.8 min; held at 90% B for 0.6 min and returned to 30% B in 0.1 min followed by a re-equilibration (1 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500 ℃ and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for test compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciex Analyst 1.4.2 software.

Cytochrome P_{450} inhibition samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 2.1 x 50 mm, 3 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40 ℃. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 10% B after a 0.2 min hold and was linearly increased to 90% B over 1.3 min; returned to 10% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500 ℃ and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciex Analyst 1.4.2 software.

Microsomal stability, plasma protein binding, and brain homogenate binding samples were analyzed on a Thermo Electron TSQ Quantum Ultra triple quad detector via electrospray ionization (ESI) with two Themo Electron Accella pumps (San Jose, CA), and a Leap Technologies CTC PAL autosampler. Analytes were separated by gradient elution on a dual column system with two Waters Acquity BEH C18, 2.1x50 mm, 1.7 µm columns (Milford, MA) heated at 50 ℃. HPLC mobile phase A was 95:5:0.1 Water:Acetonitrile:Formic Acid, while mobile phase B was 95:5:0.1 Acetonitrile:Water:Formic Acid. Pump 1 ran the gradient: 95:5 (A:B) at 800 µL/min hold 0 to 0.5 min, linear ramp to 5:95 (A:B) 0.5 to 1.0min, 5:95 (A:B) hold 1.0 to 1.9 min, return to 95:5 (A:B) at 1.9 min. While pump 1 ran the gradient method, pump 2

equilibrated the other column isocratically with 95:5 (A:B). The total run time was 2.0 minutes per injection. All compounds were optimized using QuickQuan (Thermo Electron) software.