SUPPLEMENTAL DATA

Comprehensive Evaluation of Liver Microsomal Cytochrome P450 3A (CYP3A) Inhibition: Comparison of Cynomolgus Monkey and Human

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Inhibition Studies with HLM and CLM. *Sample preparation.* Test compounds were dissolved and serially diluted in 100% DMSO (3-fold dilution steps, 10 concentrations) in columns 1 through 10 and 11 through 20 in a 384-well polypropylene microplate (Axygen Scientific, Inc., Union City, CA), using the BenchCel System (Velocity 11 Inc., Menlo Park, CA). Wells in columns 21 - 24 contained 100% DMSO “solvent”; wells in columns 23 - 24 did not receive LM. These wells were used to determine the total (“solvent control”; col. 21 - 22) and background (col. 23 - 24) signals in each assay. Aliquots (5 µL) of these serially-diluted samples were then transferred into 384-well ECHO source plate (LabCyte Inc., Sunnyvale, CA), which was subsequently replicated into 2 copies of the assay-ready plates (Axygen Scientific, Inc., Union City, CA) at 80 nL per well, using the acoustic noncontact liquid dispenser LabCyte ECHO-550 (LabCyte Inc., Sunnyvale, CA). This instrument utilizes acoustic droplet ejection technology, which focuses ultrasonic energy at the meniscus of the fluid sample to eject small droplets of liquid from the surface of the open well. The technology allows an accurate transfer of nanoliter volumes (as 2.5 nL droplets) traveling upwards from the source plate directly into the assay plate.

Assay incubations. To determine the IC₅₀ values for inhibition in the absence of preincubation (0 min preincubation with LM in the presence of NADPH), the test compounds (80 nL, in DMSO) at 10 concentrations were first mixed with 20 µL of the midazolam (2.5 µM final concentration) in the assay buffer (100 mM potassium phosphate, pH 7.4, 2 mM MgCl₂, 1 mM NADPH), then immediately diluted with an equal volume (20 µL) of liver microsomes (0.1 mg/mL) in the same assay buffer, and incubated at 37°C for 5 minutes. To determine the IC₅₀ values for TD inhibition (30-min preincubation with liver microsomes in the presence of NADPH) test compounds were first preincubated for 30 minutes with 20 µL of LM in the assay buffer at 37°C, then diluted with an equal volume of substrate mixture in the same assays buffer, and the reaction was allowed to proceed at 37°C for 5 minutes. At the end of the incubation time, all reactions were terminated by the addition of 40 µL of quench buffer (94% water: 5% acetonitrile: 1% formic acid, v/v) containing [¹³C₃]-1'-hydroxymidazolam (0.1 µM) as internal standard.

The amount of 1'-hydroxymidazolam produced in each well was determined by Aria LX-2 multiplexed LC-MS/MS system (Thermo Scientific, CA, USA). The system consisted of two sets of independent 10ADvp binary pumps (Shimadzu Scientific Instruments, MD, USA) for dual staggered chromatography, a valve interface module (VIM, Thermo Scientific) for stream selection and two modified CTC HTS PAL auto-samplers (Leap Technologies, NC, USA). Sample plates in 384-well plate format were placed on a cooled, open-deck manifold for injection. Aria software (version 1.6.3, Thermo Scientific) controlled all peripheral components. A TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific) was used for mass spectrometric analysis. The MS was equipped with heated electrospray ionization (H-ESI) and used in positive ionization mode. XCalibur 2.2 was used for mass spectrometric data acquisition in the multiple injection chromatogram (MIC) mode.

The LC-MS/MS method used 98:2:0.2 (v:v:v) water/ acetonitrile/ formic acid as mobile phase A, and 98:2:0.2 acetonitrile / water/ formic acid as mobile phase B. The column used was an Ascentis Express C18 column (Sigma-Aldrich, St. Louis, MO, USA) with a dimension of 2cmx2.1mm, 2.7 μ m. The chromatographic gradient started with 5%B at 0.8 mL/min for 5 sec, and ramped in 27 sec to 100%B and maintained for 15 sec at 1.1 mL/min. The gradient was ramped back to 5% B in 1 sec at 0.9 mL/min and equilibrated for 21 sec. For MS/MS conditions, a universal set of source parameters was employed, including a spray voltage at 3.5 kV for positive ionization, a vaporizer temperature at 450°C and a capillary temperature at 275°C. The sheath gas and auxiliary gas settings were 70 and 60 (arbitrary unit), respectively. The selected reaction monitoring (SRM) transition was m/z 342 $\rightarrow$ m/z 203 for 1'-hydroxymidazolam, and m/z 345 $\rightarrow$ m/z 206 for [¹³C₃]-1'-hydroxymidazolam, the tube lens offset was 83 and collision energy was 26 V. The scan widths for both scanning quadrupoles were 1 amu and the scan time was 0.1 sec for each transition.

Midazolam 1'-Hydroxylase Activity with Recombinant Human and Cynomolgus Monkey CYP3A4 and CYP3A5. Selvakumar *et al.* (2014) conducted in-depth kinetic characterization of cynomolgus monkey CYP3A4-catalyzed midazolam 1'-hydroxylation activity. Linearity was maintained at 10 nM protein concentration and 10 min incubation period.

Evaluation of Raloxifene as Inhibitor of Human and Cynomolgus Monkey CYP3A4 and CYP3A5. Inhibition of human CYP3A4, human CYP3A5, cynmolgus monkey CYP3A4, and cynomolgus monkey CYP3A5 was assessed using midazolam as the established substrate (final substrate concentration 2.5 μ M) (Walsky and Obach, 2004). Each assay was performed in a TD format. Specifically, test compounds were preincubated at 37°C with recombinant enzymes (7.5 nM) in the presence of NADPH (1 mM) for 0 and 30 min, followed by 5 min reaction incubation with substrate and NADPH (1 mM). In total, raloxifene was tested 6 times in both 0 and 30 min

preincubation reactions. Assays were performed in 384-well microplates in a total volume of 30 μ L. Automated liquid handling equipment was used in the various steps of the assay process: Bravo (Velocity 11 Inc., Menlo Park, CA), ECHO 550 (LabCyte Inc., Sunnyvale, CA), and Multidrop Combi (Thermo Electron Corporation, Vantaa, Finland). Raloxifene was tested as a single point at each of 10 concentrations ranging from 2.5 nM to 50 μ M. Final dimethyl sulfoxide (DMSO) concentration in the reaction mixture was 0.2%. The reaction was quenched with 30 μ L of water:acetonitrile:formic acid (94:5:1) containing 0.1 μ M internal standard. Microsomal fractions were precipitated by centrifugation at 2500 x g for 10 min prior to LC-MS/MS analysis. The IC₅₀ value for each compound was determined using a four-parameter logistic regression model (see *Data Analysis*).

Figure S1

Michaelis-Menten plot describing midazolam 1'-hydroxylation obtained with HLM and CLM.

Metabolite formation rate is represented on the y-axis, and the x-axis represents midazolam concentration. Error bars represent SD of n = 4 determinations.