Supporting Information

Discovery of VU2957 (Valiglurax): An $mGlu_4$ Positive Allosteric Modulator Evaluated as a Preclinical Candidate for the Treatment of Parkinson's Disease

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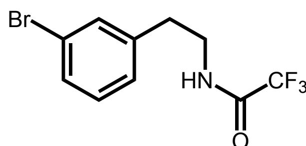
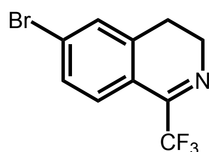
General. All NMR spectra were recorded on a 400 MHz AMX Bruker NMR spectrometer. ^{1}H and ^{13}C chemical shifts are reported in δ values in ppm downfield with the deuterated solvent as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, b = broad, m = multiplet), integration, coupling constant (Hz). Low resolution mass spectra were obtained on an Agilent 6120 or 6150 with ESI source. Method A: MS parameters were as follows: fragmentor: 70, capillary voltage: 3000 V, nebulizer pressure: 30 psig, drying gas flow: 13 L/min, drying gas temperature: 350 ºC. Samples were introduced via an Agilent 1290 UHPLC comprised of a G4220A binary pump, G4226A ALS, G1316C TCC, and G4212A DAD with ULD flow cell. UV absorption was generally observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Waters Acquity BEH C18, 1.0 x 50 mm, 1.7 um. Gradient conditions: 5% to 95% CH_3CN in H_2O (0.1% TFA) over 1.4 min, hold at 95% CH_3CN for 0.1 min, 0.5 mL/min, 55 ºC. Method B: MS parameters were as follows: fragmentor: 100, capillaryvoltage: 3000 V, nebulizer pressure: 40 psig, drying gas flow: 11 L/min, drying gas temperature: 350 ºC. Samples were introduced via an Agilent 1200 HPLC comprised of a degasser, G1312A binary pump, G1367B HP-ALS, G1316A TCC, G1315D DAD, and a Varian 380 ELSD (if applicable). UV absorption was generally observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Thermo Accucore C18, 2.1 x 30 mm, 2.6 um. Gradient conditions: 7% to 95% CH_3CN in H_2O (0.1% TFA) over 1.6 min, hold at 95% CH_3CN for 0.35 min, 1.5 mL/min, 45 ºC. High resolution mass spectra were obtained on an Agilent 6540 UHD Q-TOF with ESI source. MS parameters were as follows: fragmentor: 150, capillary voltage: 3500 V, nebulizer pressure: 60 psig, drying gas flow: 13 L/min, drying gas temperature: 275 ºC. Samples were introduced via an Agilent 1200 UHPLC comprised of a G4220A binary pump, G4226A ALS, G1316C TCC, and G4212A DAD with ULD flow cell. UV absorption was observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Agilent Zorbax Extend C18, 1.8 µm, 2.1 x 50 mm. Gradient conditions: 5% to 95% CH_3CN in H_2O (0.1% formic acid) over 1 min, hold at 95% CH_3CN for 0.1 min, 0.5 mL/min, 40 ºC. For compounds that were purified on a Gilson preparative reversed-phase HPLC, the system comprised of a 333 aqueous pump with solvent-selection valve, 334 organic pump, GX-271 or GX-281

liquid hander, two column switching valves, and a 155 UV detector. UV wavelength for fraction collection was user defined, with absorbance at 254 nm always monitored. Method: Phenomenex Axia-packed Luna C18, 30 x 50 mm, 5 µm column. Mobile phase: CH_3CN in H_2O (0.1% TFA). Gradient conditions: 0.75 min equilibration, followed by user defined gradient (starting organic percentage, ending organic percentage, duration), hold at 95% CH_3CN in H_2O (0.1% TFA) for 1 min, 50 mL/min, 23 ºC. Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification.

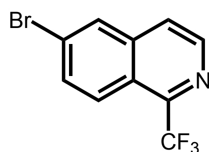


***N*-(3-bromophenethyl)-2,2,2-trifluoroacetamide (9):** A vial was charged with 2-(3-bromophenyl)ethanamine (600 mg, 3 mmol), methylene chloride (20 mL), and trifluoroacetic anhydride (0.45 mL, 3 mmol). Triethylamine (0.84 mL, 6 mmol) was carefully added dropwise. The reaction was stirred for 1 h then concentrated. The residue was purified on silica gel (20% EtOAc/hexanes) to provide the title compound (773 mg, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (ddd, J = 7.9, 1.5, 1.5 Hz, 1H), 7.35 (dd, J = 1.6, 1.6 Hz, 1H), 7.21 (dd, J = 7.7, 7.7 Hz, 1H), 7.12 (d, J = 7.6 Hz, 1H), 6.34 (bs, 1H), 3.61 (ddd, J = 6.8, 6.8, 6.6 Hz, 2H), 2.87 (dd, J = 7.1, 7.1 Hz, 2H); LCMS: RT = 1.007 min; M+H = 296.2; HRMS: Calculated for $C_{10}H_{10}BrF_3NO$, 295.9892; found, 295.9893.



6-bromo-1-(trifluoromethyl)-3,4-dihydroisoquinoline (10): A microwave vial was charged with *N*-(3-bromophenethyl)-2,2,2-trifluoroacetamide (2.6 g, 8.8 mmol), methylene chloride (10 mL), and 2-chloropyridine (1 mL, 10.5 mmol). The vial was cooled to −78 °C and put under an inert nitrogen atmosphere. Triflic anhydride (1M in methylene chloride, 9.6 mmol) was added dropwise. The reaction mixture was allowed to reach rt, then heated under microwave irradiation for 5 min at 160 °C. The solvent was removed under reduced pressure and the residue was purified on silica gel (40% EtOAc/hexanes) to provide the title compound (1.4 g, 56% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 (dd, J = 8.4, 1.8 Hz, 1H), 7.48-7.44 (m, 1H), 7.42 (d, J = 1.6 Hz, 1H), 3.91 (m, 2H), 2.78 (dd, J = 7.9, 7.4 Hz, 2H); LCMS: RT = 0.990 min; M+H = 278.0; HRMS: Calculated for $C_{10}H_8BrF_3N$, 277.9789; found, 277.9787

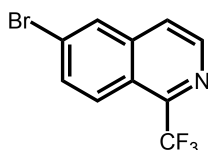
Bischler-Napeiralski Route:



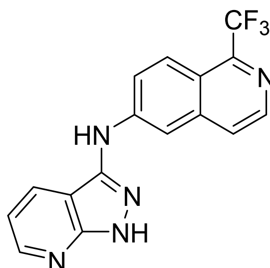
6-bromo-1-(trifluoromethyl)isoquinoline (13): A flask was charged with 6-bromo-1-(trifluoromethyl)-3,4-dihydroisoquinoline (1 g, 3.6 mmol), MnO_2 (3 g, 54 mmol), and *m*-xylene (100 mL). The reaction mixture was heated to 130 °C overnight. After cooling to rt, the reaction mixture was filtered, concentrated,

and the residue was purified on silica gel (10% EtOAc/hexanes) to provide the title compound (437 mg, 44% yield) and carried on crude. LCMS: RT = 1.147 min; M+H = 276.2.

Kuninobu Route:



6-bromo-1-(trifluoromethyl)isoquinoline (13): To a stirred solution of known 6-bromo isoquinoline-2-oxide **12** (100.0 g, 0.44 mol) in dry THF (1.5L, 15 vol) was added molecular sieves (50 g, 3A˚, 50 % w/w) and stirred it for 10 min under argon atmosphere. To it was added trimethyl trifluoromethyl silane (102 mL, 0.66 mol) slowly over a period of 10 min at ambient temperature. Further the reaction mixture was cooled to -20 to-30 ° C, and then KO*t*Bu (50.0 g, 0.44 mol) was added in 3 lots over a period of 30 min. The reaction mass was stirred for 10 min at – 20 to – 30 °C. After completion of reaction (reaction was monitored by TLC and HPLC), it was quenched with water (15 vol) at -20 ° C and product was extracted with MTBE (2 x 10 vol) and it was washed with brine solution (10 vol) then dried over anhydrous sodium sulphate. The organic layer was concentrated under reduced pressure at 45-50 °C to get crude intermediate-5. Intermediate-5 was further purified by using column chromatography (using 60 -120 mesh silica gel) eluted with 5 % ethyl acetate in hexane to get intermediate-5 as an off white solid (46 g, 41 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.70 (d, J = 5.7 Hz, 1H), δ 8.62 (d, J = 5.7 Hz, 1H), δ 8.18 (m, 2H), δ 8.01 (m, 1H), ^{13}C NMR (100 MHz, DMSO-d_6) δ 152.2, 141.6, 132.3, 130.5, 130.0, 129.1, 124.8, 124.6, 126.2, 121.1; HRMS: Calculated for $C_{10}H_5BrF_3N$, 274.9557; found, 274.9561.



***N*-(1*H*-pyrazolo[3,4-*b*]pyridin-3-yl)-1-(trifluoromethyl)isoquinolin-6-amine (VU2957, 7):** To a solution of **13** (100.0 g, 0.36 mol) and commercial 1-(4-methoxybenzyl)-1*H*-pyrazolo[3,4-*b*]pyrdin-3-amine (92.0 g, 0.36 mol) in 1,4-dioxane (1.5 L, 15 vol) was added cesium carbonate (295 g, 0.90 mol) and degassed the reaction mixture with argon for 30 min. Charged $Pd_2(dba)_3$ (18.74 g, 0.018 mol) followed by Xantphos (10.48 g, 0.018 mol) to the reaction mass under argon atmosphere. The reaction mass was heated to 95-100 °C for 1 h. The reaction progress was monitored by HPLC. The reaction mass was cooled to 25-30 °C and filtered through celite bed, filtrate was concentrated at 45-50 °C under vacuum. Cooled the reaction mass to 25-30 °C , diluted with ethyl acetate (20 vol) and washed the organic layer with water (10 vol). The aqueous layer was re-extracted with ethyl acetate (10 vol). Organic layer was combined and washed with 20 % sodium chloride solution. Further the organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure at 40-45 ^{o}C. To the crude reaction mass, methanol (10 vol) was added and stirred for 1 h at ambient temperature. It was filtered and dried under vacuum to get off white solid of **14** as an off white solid (120 g, 73 % yield) and carried on crude into the deprotection step. To a solution of **14** (155.0 g, 0.34 mol) in toluene (1.55 L, 10 vol) was added TFA (1.55 L, 10 vol) in auto clave. The reaction mass was heated to 135 to 140 °C and stirred for 2 h. The reaction progress was monitored by TLC and HPLC. **Note:** TLC shows absence of **14**. After completion of reaction, the reaction mass was cooled to 25-30 ° C, concentrated to minimum volume at 50 – 55 °C under reduced pressure to get thick oil. Further it was basified using cold 10 % aqueous sodium bicarbonate solution (28

vol), stirred for 30 min to get pale yellow precipitate. The precipitate was filtered and washed the residue with water (2 vol). The wet cake was further slurried with water (10 vol) for 30 min at ambient temperature and filtered and dried under vacuum. Further the solid was slurried with ethyl acetate (10 vol) for 1h at ambient temperature and filtered. Residue was washed with ethyl acetate (1 vol) and dried under reduced pressure at 45 to 50 ^{o}C to get pale yellow solid of VU 2957 (100 g, 88 % yield, HPLC purity: 96.14 %).

Purification: VU2957 (total quantity: 128 g) (100g from batch No. A11604388, 22 g from batch No. A11604387 and 6.2 g from batch no. A11604333) was stirred with MTBE (10 vol) for 1h at ambient temperature, filtered the solid washed it with MTBE (1.0 vol), dried under reduced pressure for 3 h at 50 - 55 °C to get pale yellow solid of VU2957 in pure form 110.0 g, 86 %, HPLC purity: 99.16 %. ^{1}H NMR (400 MHz, CD_3OD) δ 8.53 (d, J = 2.3 Hz, 1H), 8.50 (dd, J = 4.6, 1.4 Hz, 1H), 8.40 (dd, J = 8.0, 1.5 Hz, 1H), 8.36 (d, J = 5.7 Hz, 1H), 8.20-8.16 (m, 1H), 7.87 (d, J = 5.7 Hz, 1H), 7.79 (dd, J = 9.3, 2.3 Hz, 1H), 7.19 (dd, J = 8.0, 4.6 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 151.3, 149.2, 144.7, 143.2, 140.3, 140.0, 129.6, 124.8, 123.9, 121.8, 121.1, 119.3, 115.7, 115.3, 108.1, 107.6; HRMS: Calculated for $C_{16}H_{10}F_3N_5$, 329.0888; found, 329.0891.

In vitro Pharmacology:

Cell culture. Human $mGlu_4/G_{qi5}$/CHO cells were grown in 90% Dulbecco's Modified Eagle Media (DMEM), 10% dialyzed fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin, 20 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 20 µg/ml proline, 2 mM glutamine, 400 µg/ml G418 sufate (Mediatech, Inc., Herndon, VA) and 5 nM methotrexate (Calbiochem, EMD Chemicals, Gibbstown, NJ). Rat $mGlu_4$/HEK/GIRK cells, as well as HEK/GIRK lines expressing rat $mGlu_2$, $mGlu_3$, $mGlu_7$, $mGlu_8$, and human $mGlu_6$ were cultured in 45% DMEM, 45% F-12, 10% FBS, 20 mM HEPES, 2 mM l-glutamine, 100 units/ml penicillin/streptomycin, 1X nonessential amino acids, 1 mM sodium pyruvate, 700 µg/mL G418, and 0.6 µg/mL puromycin. Rat $mGlu_1$ and $mGlu_5$ HEK cell lines were grown in DMEM, 10% FBS, 20 mM

HEPES, 2 mM l-glutamine, 1X nonessential amino acids, 1 mM sodium pyruvate, 500 μg/mL G418. All cell culture reagents were from Invitrogen (Carlsbad, CA) unless otherwise noted.

Calcium Mobilization Assays. Human $mGlu_4/G_{qi5}$/CHO cells (30,000 cells/20 μl/well) were plated in black-walled, clear-bottomed, poly-d-lysine treated 384 well plates (Greiner Bio-One, Monroe, North Carolina) in DMEM containing 10% dialyzed FBS, 20 mM HEPES, 100 units/ml penicillin/streptomycin, and 1 mM sodium pyruvate (Plating Medium). The cells were grown overnight at 37°C in the presence of 5% CO_2. The next day, the medium was removed and replaced with 20 μL of 2 μM Fluo-4, AM (Invitrogen, Carlsbad, CA) prepared as a 2.3 mM stock in DMSO and mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127 and diluted in Assay Buffer (Hank's balanced salt solution, 20 mM HEPES and 2.5 mM Probenecid (Sigma-Aldrich, St. Louis, MO)) for 45 minutes at 37°C. Dye was removed and replaced with 20 μL of Assay Buffer. For concentration-response curve experiments, compounds were serially diluted 1:3 into 10 point concentration response curves in DMSO, transferred to daughter plates using an Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA), and diluted in Assay Buffer to a 2X final concentration. Ca^{2+} flux was measured using the Functional Drug Screening System 6000 or 7000 (FDSS6000, FDSS7000, Hamamatsu, Japan). After establishment of a fluorescence baseline for 2 seconds (2 images at 1 Hz; excitation, 470 ± 20 nm; emission, 540 ± 30 nm), 20 μl of test compounds were added to the cells, and the response was measured. 142 seconds later, 10 μl (5X) of an EC_{20} concentration of glutamate was added to the cells, and the response of the cells was measured; after an additional 120 seconds, 12 μl (5X) of an EC_{80} concentration of agonist was added and readings taken for an additional 40 seconds. Calcium fluorescence was recorded as fold over basal fluorescence and raw data were normalized to the maximal response induced by glutamate. Potency (EC_{50}) and maximum response values (% Glu Max) for compounds were determined using a four parameter logistical equation using Dotmatics software (Herts, UK) or GraphPad Prism (San Diego, CA); maximal values for data from human $mGlu_4/G_{qi5}$/CHO cell experiments were normalized to the maximal response induced by the $mGlu_4$ control PAM, PHCCC, to

account for day-to-day fluctuations in the maximal response and more effectively drive SAR determinations.

GIRK-Mediated Thallium Flux Assay. Cells were plated into 384 well, black-walled, clear-bottom poly-D-lysine coated plates (Greiner) at a density of 15,000 cells/20 µl/well in DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 100 units/ml penicillin/streptomycin (Assay Media). Plated cells were incubated overnight at 37 ^{o}C in the presence of 5% CO_2. The following day, the medium was removed from the cells and 20 µl/well of 330 nM Fluo Zn^2 (Invitrogen; prepared as a stock in DMSO and mixed in a 1:1 ratio with pluronic acid F-127) in Assay Buffer (Hanks Balanced Salt Solution (Invitrogen) containing 20 mM HEPES pH 7.3) was added to the plated cells. Cells were incubated for one hour at room temperature and the dye was replaced with the 20 µl of Assay Buffer. Glutamate was diluted in Thallium Buffer (125 mM sodium bicarbonate (added fresh the morning of the experiment), 1 mM magnesium sulfate, 1.8 mM calcium sulfate, 5 mM glucose, 12 mM thallium sulfate, 10 mM HEPES, pH 7.3) at 5x the final concentration to be assayed. For concentration-response curve experiments, compounds were serially diluted 1:3 into 10 point concentration response curves in DMSO, transferred to daughter plates using the Echo, and diluted in Assay Buffer to a 2X final concentration. Cell plates and compound plates were loaded onto a Hamamatsu FDSS 6000 or 7000 kinetic imaging plate reader. Baseline readings were taken (10 images at 1 Hz, excitation, 470±20 nm emission, 540±30 nm) and test compounds were added in a 20 µl volume and incubated for 2.5 minutes prior to the addition of 10 µl of Thallium Buffer ± agonist. After the addition of agonist, data were collected for an additional 2 min. The slope of the fluorescence increase beginning five seconds after thallium/agonist addition and ending fifteen seconds after thallium/agonist addition was calculated. Data were analyzed using a four-parameter logistical equation in Dotmatics or GraphPad Prism software.

Selectivity Studies

Rat $mGlu_1$ and $mGlu_5$. HEK cells expressing rat $mGlu_1$ or rat $mGlu_5$ were used for selectivity screening and were plated at a density of 15,000 cells/20 µl/well in Assay Media (DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 100 units/mL penicillin/streptomycin). Effects of the test PAM on rat $mGlu_1$ and $mGlu_5$ were assessed using calcium mobilization and by measuring the glutamate concentration-response relationship in the presence and absence of 10 µM PAM. Using a double-addition protocol, compound was added to the cells, followed 2.5 minutes later by a full concentration-response of glutamate. Responses were converted to the maximal response induced by agonist in the presence and absence of compound and plotted using four parameter equation fitting of the concentration-response curve in GraphPad Prism. Compound-induced shifts of the concentration-response relationship were used to assess potential potentiator (left shift of more than 2 fold) or antagonist (right shift of more than 2 fold or depression of the max response by at least 75%) activity of test compound.

Rat $mGlu_2$, $mGlu_3$, $mGlu_4$, $mGlu_7$, $mGlu_8$ and human $mGlu_6$. Compound activity at the rat group II and III mGlus was assessed using thallium flux through G-protein-coupled inwardly-rectifying potassium (GIRK) channels. Cells were plated at a density of 15,000 cells/20 µl/well in Assay Media. Effects of test PAM were assessed by measuring the glutamate concentration-response relationship in the presence and absence of 10 µM PAM. Using a double-addition protocol, PAM was added to the cells, followed 2.5 minutes later by a full concentration-response of glutamate or, in the case of $mGlu_7$, L-AP4. Responses were converted to the maximal response induced by agonist in the presence and absence of compound and plotted using four parameter equation fitting of the concentration-response curve in GraphPad Prism. Compound-induced shifts of the concentration-response relationship were used to assess potential potentiator (left shift of more than 2 fold) or antagonist (right shift of more than 2 fold or depression of the max response by at least 75%) activity of test compound.

Operational modeling and calculation of cooperativity and predicted affinity. Increasing concentrations of VU0652957 were added prior to increasing concentrations of glutamate to human

$mGlu_4/G_{qi5}/CHO$ cells and calcium responses were measured. Shifts of agonist concentration-response curves by allosteric modulators were globally fitted to an operational model of allosterism (Leach, K.; Sexton, P.M.; Christopoulos, A. *Trends Pharmacol. Sci.* **2007**, *28*, 382-389.) below:

$$y = basal + \frac{(E_m - basal)(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n + ([A]K_B + K_AK_B + K_A[B] + \alpha[A][B])^n}$$

where *A* is the molar concentration of the orthosteric agonist; *B* is the molar concentration of the allosteric modulator; K_A is the equilibrium dissociation constant of the orthosteric agonist, and K_B is the equilibrium dissociation constant of allosteric modulator. Affinity modulation is governed by the cooperativity factor α, and efficacy modulation is described by β; these were solved for as a composite parameter, $\alpha\beta$. The parameters τ_A and τ_B relate to the ability of orthosteric agonist and allosteric ligands, respectively, to directly activate the receptor. *Basal,* E_m and *n* represent the basal system response, maximal possible system response, and the transducer function that links occupancy to response, respectively.

Drug Metabolism Methods

In vitro: Protein binding of $mGlu_4$ PAM **7** was determined in plasma via equilibrium dialysis employing Single-Use RED Plates with inserts (ThermoFisher Scientific, Rochester, NY). Briefly 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 article 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 (2 volumes) was added to extract the matrices. The plate was centrifuged (3000 rpm, 10 min) and supernatants transferred to a new 96 well plate. The sealed plate was stored at -20°C until LC/MS/MS analysis.

A cocktail of substrates for cytochrome P450 enzymes (1A2: Phenacetin, 10 μM; 2C9: Diclofenac, 5 μM; 2D6: Dextromethorphan, 5 μM; 3A4: Midazolam, 2 μM) were mixed to assess the ability of compounds to inhibit the major cytochrome P450 enzymes. A reaction mixture of 100 mM Kpi, pH 7.4, 0.1 mg/mL human liver microsomes (HLM) and Substrate Mix was prepared and aliquoted into a 96-deepwell block. Test compound and positive control (in duplicate) were then added such that the final concentration of test compound ranged from 0.1 – 30 μM. The plate was vortexed briefly and then pre-incubated at 37 ºC while shaking for 15 minutes. The reaction was initiated with the addition of NADPH (1 mM final concentration). The incubation continued for 8 min and the reaction quenched by 2x volume of cold acetonitrile containing internal standard (50 nM carbamazepine). The plate was centrifuged for 10 minutes (4000 rcf, 4 ºC) and the resulting supernatant diluted 1:1 with water for LC/MS/MS analysis. A 12 point standard curve of substrate metabolites over the range of 0.98 nM to 2000 nM. The IC_{50} values for each compound were obtained for the individual CYP enzymes by quantitating the inhibition of metabolite formation for each probe substrate. A 0 μM compound condition (or control) was set to 100% enzymatic activity and the effect of increasing test compound concentrations on enzymatic activity could then be calculated from the % of control activity. Curves were fitted using XLfit 5.2.2 (four-parameter logistic model, equation 201) to determine the concentration that produces half-maximal inhibition (IC_{50}).

The *in vitro* biotransformation of compounds was investigated using hepatic S9 subcellular fractions incubated with or without NADPH (1 mM). Reactions were terminated by adding 1 volume of acetonitrile, and proteins were removed by centrifugation. The supernatants were saved for HPLC/UV/MS analysis. Samples were analyzed on a Waters Acquity UPLC system with PDA detector with a flow rate of 0.5 mL/min (A: Water/Acetonitrile 95/5 with 0.1% formic acid; B: Acetonitrile) and a Waters Xevo QTOF mass spectrometer with positive ESI source.

The metabolic stability of $mGlu_4$ PAM **7** was investigated in multi-species hepatic microsomes (BD Biosciences, Billerica, MA) using substrate depletion methodology (% test article remaining). A potassium phosphate-buffered reaction mixture (0.1 M, pH 7.4) of test article (1 μM) and microsomes (0.5 mg/mL) was pre-incubated (5 min) at 37 °C prior to the addition of NADPH (1 mM). The incubations, performed

in 96-well plates, were continued at 37 °C 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 ng/mL), and centrifuged at 3000 rpm (4°C) 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) $t_{1/2} = \mathrm{Ln}(2) / k$; where k represents the slope from linear regression analysis (% test article remaining)

(2) $CL_{int} = (0.693 / t_{1/2})$ (rxn volume / mg of microsomes) (45 mg microsomes / gram of liver) (20[a] gm of liver / kg body weight); [a]species specific scale-up factors

(3) $$CLhep = \frac{Q \cdot CL\mathrm{int}}{Q + CL\mathrm{int}}$$ Q = heptatic blood flow

Liquid Chromatography/Mass Spectrometry Analysis: 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, 3.5 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. 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 0.5 min and returned to 30% 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 °C and mass spectral analyses were performed using multiple reaction monitoring (MRM) 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. For *in vivo* studies, the final PK parameters were calculated by noncompartmental analysis using Phoenix (version 6.2) (Pharsight Inc., Mountain View, CA).

In vivo: All animal studies were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

SD Rat IV/PO PK (conducted at VCNDD)

Male Sprague-Dawley rats (n=2) weighing around 300g were purchased from Harlon laboratories (Indianapolis, IN) and implanted with catheters in the carotid artery and jugular vein. The cannulated animals were acclimated to their surroundings for approximately one week before dosing and provided food and water *ad libitum*. IV cassette PK experiments in rats were carried out according to methods described previously (Bridges et al. *Pharmacol. Res. Perspect.* 2014; reference 49). Briefly, A cassette of compounds (n = 4–5/cassette) were formulated from 10 mM solutions of compounds in DMSO. In order to reduce the absolute volume of DMSO that was administered, the compounds were combined and diluted with ethanol and PEG 400 to achieve a final concentration of 0.4–0.5 mg/mL for each compound (2 mg/mL total) administered in each cassette. The final dosing solutions consisted of approximately 10% ethanol, 40% PEG400, and 50% DMSO (v/v). Each cassette dose was administered IV via the jugular vein to two dual-cannulated (carotid artery and jugular vein) adult male Sprague–Dawley rats, each weighing between 250 and 350 g (Harlan, Indianapolis, IN) for a final dose of 0.2–0.25 mg/kg per compound. Whole blood collections via the carotid artery were performed at 0.033, 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 hours post dose and plasma samples prepared for bioanalysis.

For tissue distribution studies in cassette format, brain dissection and blood collections via the carotid artery were performed at 0.25 hr post dose. Blood samples were collected into chilled, EDTA-fortified tubes, centrifuged for 10 minutes at 3000 rpm (4 °C), and resulting plasma aliquoted into 96-well plates for LC/MS/MS analysis. The brain samples were rinsed in PBS, snap frozen and stored at -80 °C. 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).

Discrete IV PK experiments in rats (n=2 or 3) were carried out analogously at a dose of 1.0 mg/kg in 10% EtOH, 50% PEG 400, 40% saline, while discrete PO PK experiments in rats (n=2 or 3) were carried out using 3-30 mg/kg doses of compounds in a fine microsuspension via oral gavage to fasted animals. Whole blood collections via the carotid artery were performed at 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 hours post dose. Plasma samples were prepared for bioanalysis as described above. Discrete multi-day dosing experiments in male Sprague-Dawley rats (n=3) were carried out by dosing each animal once daily with a 3 mg/kg oral (PO) dose of VU2957 (**7**) and sampling blood over standard PK time points on days 1 and days 4. Plasma samples were prepared for bioanalysis.

CD-1 Mouse IV/PO PK (conducted at Frontage Laboratories)

For intravenous (IV) administration, male CD-1 mice (n=3) were each administered a single 1 mg/kg dose of the test article and then blood samples were collected by submandibular bleeds at 0.033, 0.25, 0.5, 1, 2, 4, 8, and 24 hours post-dosing from each animal. For oral (PO) administration, fasted male CD-1 mice (n=3) were each administered a single 3 mg/kg dose of the test article via oral gavage and then blood samples were collected by submandibular bleeds at 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 hours post-dosing from each animal. Sample preparation and bioanalysis was conducted as described above.

Beagle Dog IV/PO PK (conducted at Frontage Laboratories)

Dogs (male, Beagle, weighing approximately 8-15 kg, n=2) were dosed intravenously (0.5 mg/kg) via cephalic vein and orally (3 mg/kg) by gavage. Blood samples were collected from the jugular vein via needlestick. Blood samples were obtained at 0.083, 0.25, 0.5, 1, 2, 4, 6, 10, and 24 hours (IV) and 0.25, 0.5, 1, 2, 4, 6, 10, and 24 hours (PO) and plasma harvested for analysis. Sample preparation and bioanalysis was conducted as described above.

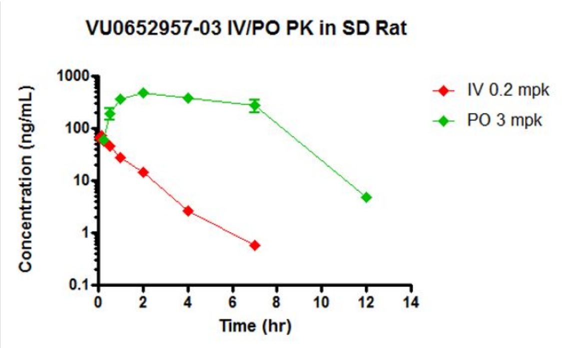
Cyno Monkey IV/PO PK (conducted at Frontage Laboratories)

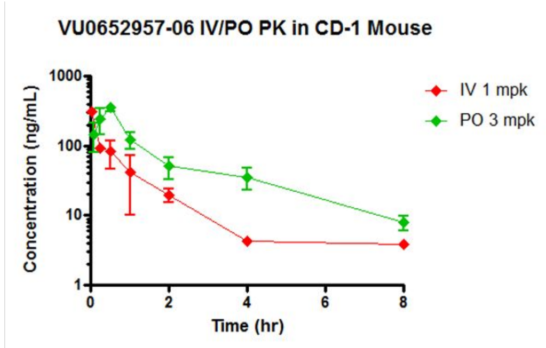
Cynomolgus monkeys (male, n=2) were dosed intravenously (0.2 mg/kg) via implanted catheter and orally (3 mg/kg) by gavage. Blood samples were collected from the femoral vein via needlestick. Blood samples were obtained at 0.083, 0.167, 0.25, 0.5, 1, 2, 4, 8, 10, and 24 hours (IV) and 0.25, 0.5, 1, 2, 4, 8, 12, and 24 hours (PO) and plasma harvested for analysis. Sample preparation and bioanalysis was conducted as described above.

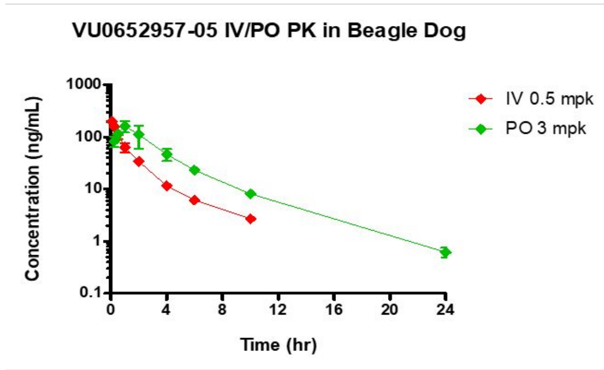
Cyno Monkey Brain Distribution Studies (conducted at Wake Forest University)

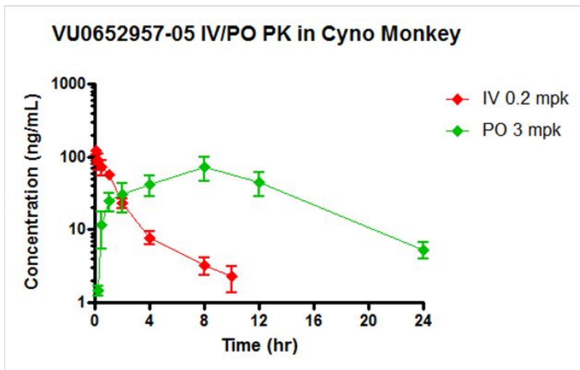
Aged cynomolgus monkeys slated for humane termination (male, n=3) were dosed intravenously (1 mg/kg) via implanted femoral catheter with samples taken at 0.5 hr (plasma and CSF) and 0.67-1 hr (brain). Following euthanasia with ketamine/euthasol, various brain regions were removed and appropriately dissected to yield distinct samples of white matter, striatum, globus pallidus, hippocampus, thalamus, prefrontal cortex, parietal lobe, occipital lobe, and cerebellum. Sample preparation and bioanalysis was conducted as described above. Analysis of independent brain regions yielded similar concentrations of analyte and the data presented represent mean concentrations from all combined regions for each animal and were used in the determinations of brain to plasma partitioning (Kp).

***In Vivo* Behavior (HIC):** As detailed in: Engers, D.W.; Gogliotti, R.D.; Cheung, Y-Y.; Salovich, J.M.; Garcia-Barrantes, P.M.; Daniels, J.S.; Morrison, R.; Jones, C.K.; Blobaum, A.L.; Macor, J.E.; Bronson, J.J.; Conn, P.J.; Lindsley, C.W.; Niswender, C.M.; Hopkins, C.R. *ACS Chem. Neurosci.* **2016**, *7*, 1192-1200.









Supporting Figure 1. Multispecies IV/PO PK of VU2957 (**7**).

GPCR	IC50 (µM)	SD	NM	YMaxObs
Adenosine A2a	> 30.00	0.00	1 of 1	32.34
Adrenergic Alpha 1B	> 30.00	0.00	1 of 1	6.70
Adrenergic Alpha 1D	> 30.00	0.00	1 of 1	22.13
Adrenergic Alpha 2A	> 30.00	0.00	1 of 1	26.04
Adrenergic Alpha 2C	> 30.00	0.00	1 of 1	5.21
Adrenergic Beta 1	> 30.00	0.00	1 of 1	6.48
Adrenergic Beta 2	> 30.0000	0.00	1 of 1	9.23
Cannabinoid CB-1	< 8.991	0.00	1 of 1	53.98
Dopamine D1	> 30.00	0.00	1 of 1	19.05
Dopamine D2	> 30.00	0.00	1 of 1	8.87
Histamine H1	> 30.00	0.00	1 of 1	1.98
Histamine H2	> 30.00	0.00	1 of 1	7.80
Muscarinic M2	> 30.00	0.00	1 of 1	-3.91
Opiate Kappa	> 30.00	0.00	1 of 1	17.43

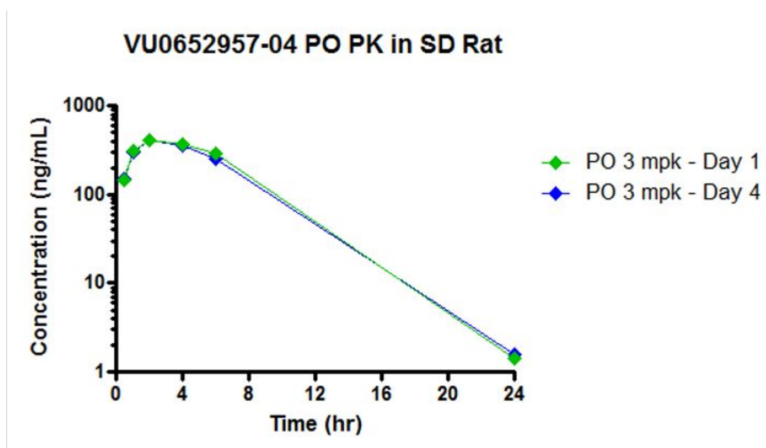
GPCR	IC50 (µM)	SD	NM	YMaxObs
Opiate Mu	> 30.00	0.00	1 of 1	8.96
Serotonin 5HT1B	> 30.00	0.00	1 of 1	13.74
Serotonin 5HT2A (EC50				
Serotonin 5HT2B (EC50				
Serotonin 5HT4	> 30.00	0.00	1 of 1	11.00
Transporters	IC50 (µM)	SD	NM	YMax
Dopamine (DAT)				
Norepinephrine (NET)				
Serotonin (SERT/5HTT)				
NHRs	IC50 (µM)	SD	NM	YMaxObs
Androgen	> 150.00	0.00	1 of 1	19.64
Estrogen ERα	> 150.0	0	1 of 1	38.38
Glucocorticoid	> 150.00	0.00	1 of 1	22.16
Progesterone	> 150.0	0.00	1 of 1	12.96

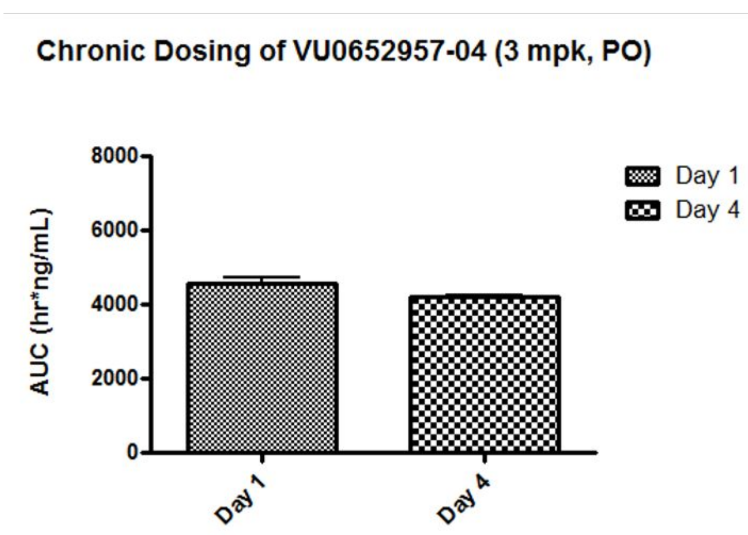
Ion Channels	IC50 (µM)	SD	NM	YMaxObs
Calcium L-type	> 25.00	0.00	1 of 1	
GABA-A (α1β2γ2) Antagonist	> 30.00	0.00	1 of 1	
GABA-A (α1β2γ2) Potentiator (EC50)	> 30.00	0.00	1 of 1	
GABA-A Alpha 5 channel	> 30.00	0.00	1 of 1	
NMDA 1.2A (EC50)				
NMDA 1.2B (EC50)				
NMDA Glutamate				
Cardiac Na+	> 30.00	0.00	1 of 1	
Nicotinic Ach alpha1	> 30.00	0.00	1 of 1	
Nicotinic Ach alpha7	> 30.00	0.00	1 of 1	
Nicotinic Acetylcholine α4β2 (EC50)	> 30.00	0.00	1 of 1	
T-Type Calcium (EC50)	> 25.00	0.00	1 of 1	-1.88
Enzymes	IC50 (µM)	SD	NM	YMaxObs
Acetylcholinesterase	> 30.00	0.00	1 of 1	6.37
MAO-A	> 30.00	0.00	1 of 1	7.53
MAO-B	> 30.00	0.00	1 of 1	8.59
PDE3				
PDE4				

**$5HT_{2B}$: >30 µM; DAT: >30 µM; NET: >30 µM; SERT/5HTT: > 30 µM;
NMDA 1.2A: >30 µM; NMDA 1.2B: >30 µM; NMDA Glutamate: >30 µM;
PDE3: >50 µM; PDE4: > 50 µM**

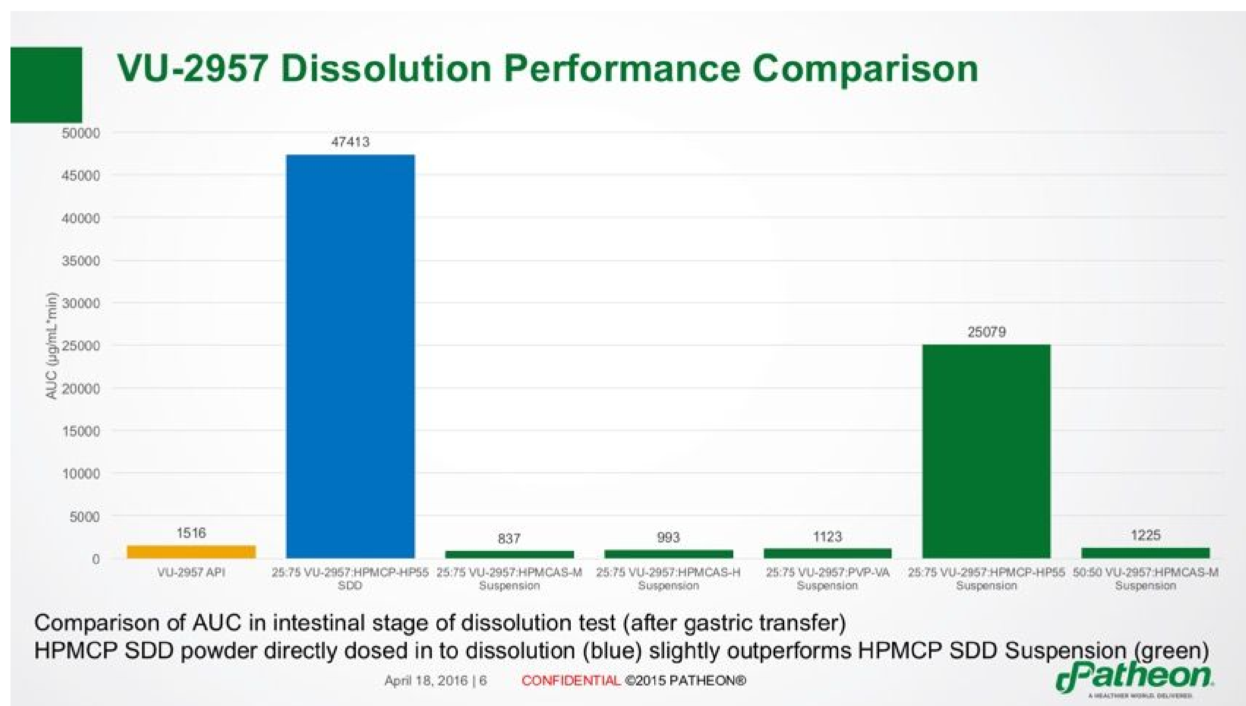
Activities in Eurofins Lead Profiler Panel (Ephys, % inhibition at 10 µM using IonWorks Quattro: Nav1.5=16%, Kv4.3/KChIP2=16.3%, Kv1.5=4.0%, KCNQ1/minK=4.0%, hERG=2.1%, Cav1.2=6.7%, Kir2.1=2.1, HCN4=1.7%

Supporting Figure 2. Ancillary pharmacology profile of VU2957 (**7**).

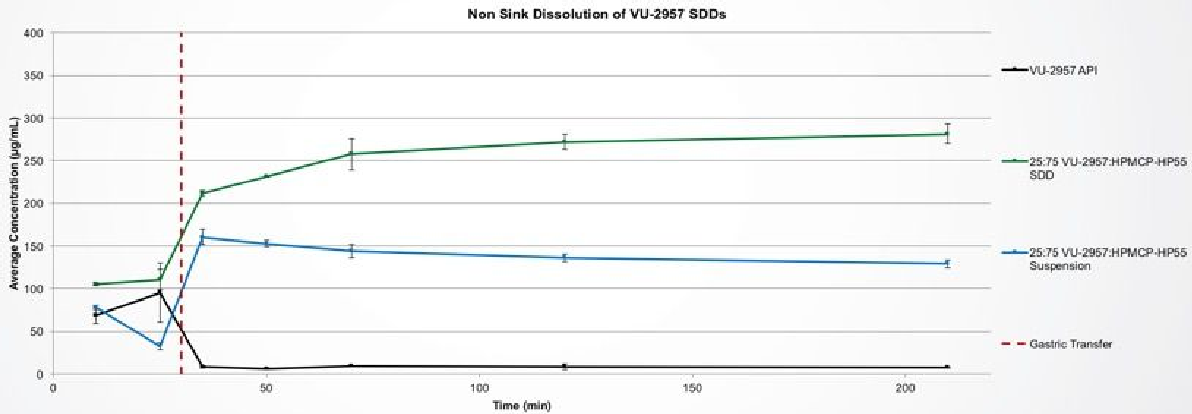




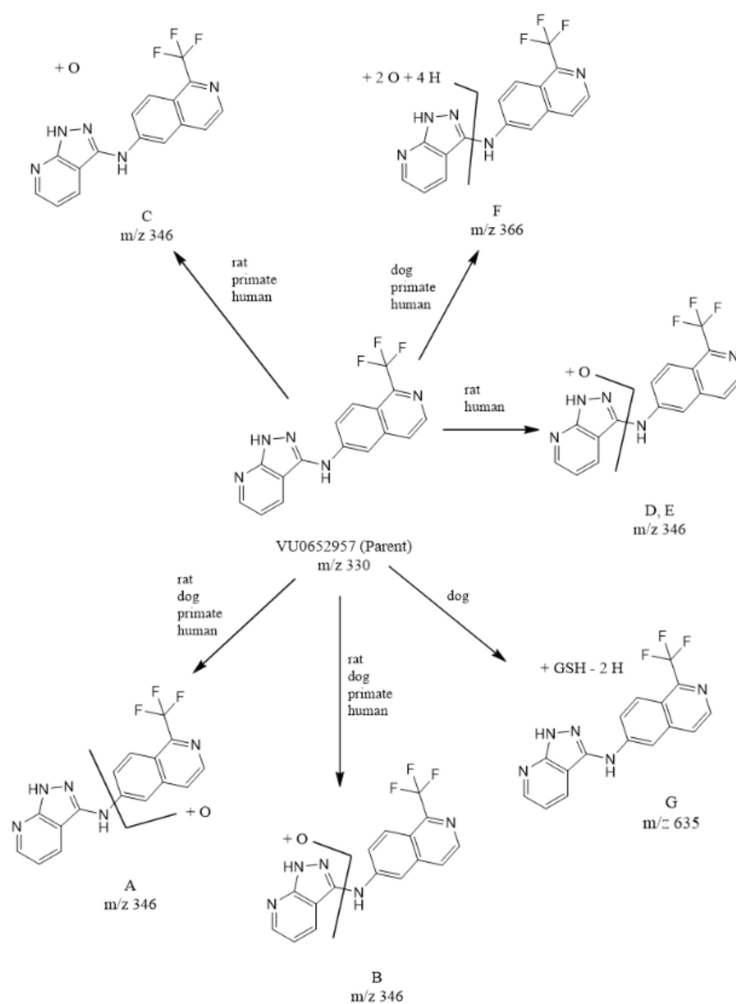
Supporting Figure 3. Chronic oral dosing of VU2957 (**7**) for 4 days at 3 mg/kg PO. No change in exposure confirms lack of auto-induction of metabolism potential of CYP1A2.



Supporting Figure 4. VU2957 dissolution performance as AI and as various spray-dried dispersion (SDD) formulations.



Supporting Figure 5. VU2957 non-sink dissolution performance as AI and as various spray-dried dispersion (SDD) formulations.



Supporting Figure 6. Metabolite identification studies for VU2957 (**7**) across species (rat, dog, primate and human S9 ± NADPH). No NADPH-independent metabolites were observed in any species.