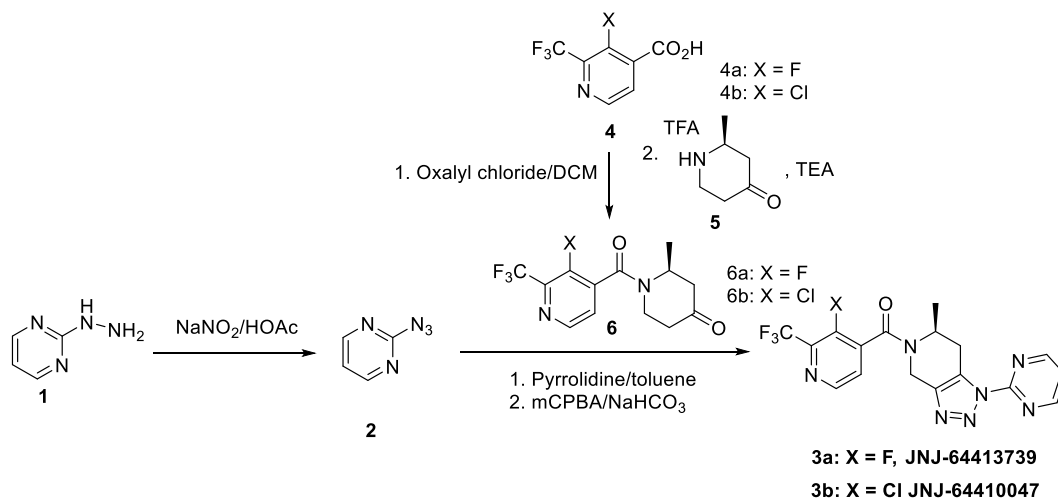


## Synthesis of standard JNJ-64413739 and labeling precursor JNJ-64410047



Compounds 1, 4, and 5 were purchased from Anichem LLC (NJ, USA); ACES Pharma (NJ, USA) and Milestone Pharmtech USA Inc. (NJ, USA); all other reagents and solvents were purchased from commercial sources including Sigma-Aldrich (MO, USA), Thermo Fisher scientific (NH, USA), and VWR International (PA, USA).

**Synthesis:** Compounds 2, 3a/b and 6a/b were prepared according to a published procedure (18).

(S)-(3-fluoro-2-(trifluoromethyl)pyridin-4-yl)(6-methyl-1-(pyrimidin-2-yl)-6,7-dihydro-1H-[1,2,3]triazolo[4,5-c]pyridin-5(4H)-yl)methanone (**3a**, JNJ-64413739)

**3a** was prepared from compound **2** and **6a**. Yield 31% (using compound **6a** as the limiting reagent). MS (ESI): mass calcd. for  $\text{C}_{17}\text{H}_{13}\text{F}_4\text{N}_7\text{O}$ , 407.11;  $m/z$  found, 408.2  $[\text{M}+\text{H}]^+$ .  $^1\text{H}$  NMR (500 MHz, Chloroform- $d$ )  $\delta$  8.95-8.91 (m, 2H), 8.68-8.64 (m, 1H), 7.60-7.56 (m, 1H), 7.49-7.44 (m, 1H), 5.82, 5.65-5.62 (m, 1H), 4.69, 4.19-4.16 (m, 1H), 4.58-4.40 (m, 1H), 3.58-3.37 (m, 2H), 1.42-1.28 (m, 3H).

(S)-(3-chloro-2-(trifluoromethyl)pyridin-4-yl)(6-methyl-1-(pyrimidin-2-yl)-6,7-dihydro-1H-[1,2,3]triazolo[4,5-c]pyridin-5(4H)-yl)methanone (**3b**, JNJ-64410047)

3b was prepared from compound 2 and 6b. Yield 40% (using compound **6b** as the limiting agent). MS (ESI): mass calcd. for  $C_{17}H_{13}ClF_3N_7O$ , 423.08; m/z found, 424.1  $[M+H]^+$ .  $^1H$  NMR (500 MHz, Chloroform-*d*)  $\delta$  8.84-8.79 (m, 2H), 8.65-8.57 (m, 1H), 7.37-7.31 (m, 2H), 5.79-5.72, 5.55 (m, 1H), 4.57, 3.97-3.93 (m, 1H), 4.38-4.30 (m, 1H), 3.44-3.16 (m, 2H), 1.32-1.29, 1.18-1.16 (m, 3H).

(S)-1-(3-fluoro-2-(trifluoromethyl)isonicotinoyl)-2-methylpiperidin-4-one (**6a**)

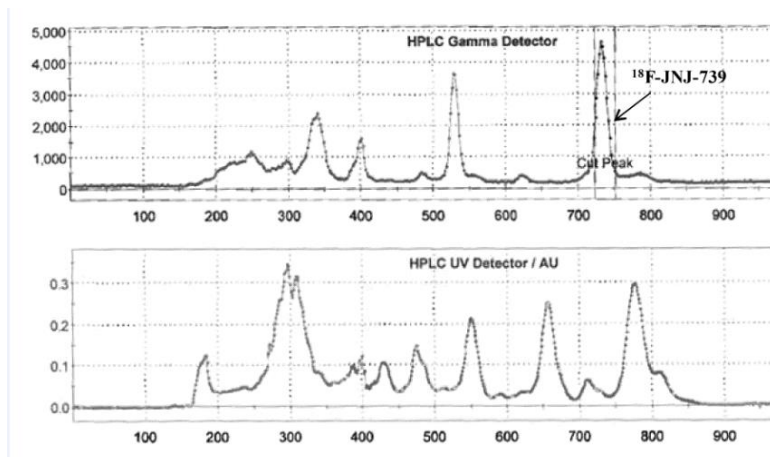
6a was prepared from compound 4a and 5. Yield 71%. MS (ESI): mass calcd. for  $C_{13}H_{12}F_4N_2O_2$ , 304.24 m/z found, 305.1  $[M+H]^+$ .  $^1H$  NMR (500 MHz, Chloroform-*d*)  $\delta$  8.64-8.63 (m, 1H), 7.62-7.59 (m, 1H), 5.36-5.34, 3.36-3.31 (m, 1H), 5.02-4.98, 4.11-4.08 (m, 1H), 3.64 (m, 1H), 2.86-2.31 (m, 4H), 1.59-1.36 (m, 3H).

(S)-1-(3-chloro-2-(trifluoromethyl)isonicotinoyl)-2-methylpiperidin-4-one (**6b**)

6a was prepared from compound **4a** and **5**. Yield 59%. MS (ESI): mass calcd. for  $C_{13}H_{12}ClF_3N_2O_2$ , 320.05; m/z found, 321.1  $[M+H]^+$ .  $^1H$  NMR (500 MHz, Chloroform-*d*)  $\delta$  8.62-8.60 (m, 1H), 7.44-7.19 (m, 1H), 5.33-5.25, 3.61-3.57 (m, 1H), 4.99-4.92, 3.92-3.90 (m, 1H), 3.47-3.22 (m, 1H), 2.79-2.72 (m, 1H), 2.61-2.24 (m, 3H), 1.31-1.26, 1.16-1.15 (m, 3H).

## Radiochemistry

A representative HPLC trace for  $^{18}F$ -JNJ-64413739 is shown below (Supplemental Fig. 1).



**SUPPLEMENTAL FIGURE 1:**  $^{18}\text{F}$ -JNJ-64413739 representative HPLC trace

#### **In Vitro Pharmacology – $K_i$ , $\text{IC}_{50}$ In Human and Rat**

$\text{IC}_{50}$  determination: 1321N1 cells expressing P2X7 receptor orthologs were dissociated 18–24 hours prior to the assay using 0.05% trypsin/EDTA (Invitrogen, Carlsbad, CA), and plated at density of 25,000 cells/well into poly-D-lysine-coated, 96-well, black-walled, clear-bottom plates (Becton-Dickinson, Bedford, MA). On the day of the experiment, cell plates were washed with assay buffer, containing the following: 130 mM NaCl, 2 mM KCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM HEPES, and 5 mM glucose at pH 7.40. After the wash, dye loading was achieved by adding a 2 Calcium-4 (Molecular Devices, Sunnyvale, CA) dye solution to the assay buffer. Cells were stained with the Calcium-4 dye in staining buffer for 30 minutes at room temperature in the dark. Test compounds were prepared at 250x of the final test concentration in neat dimethylsulfoxide. Intermediate 96-well compound plates were pre-pared by transferring 1.2 ml of the compound into 300 ml of assay buffer. A further 3x dilution occurred when transferring 50 ml/well of the compound plate to 100 ml/well in the cell plate. Cells were incubated with test compounds and dye for 30 minutes. Calcium flux was monitored in a Fluorometric Imaging Plate Reader Tetra as the cells

were challenged by adding 50 ml/well of Bz-ATP [29(39)-O-(4-benzoylbenzoyl) adenosine-59-triphosphate tri(triethylammonium)]. The final concentration of Bz-ATP was 250 mM.

Ki determination:  $^3\text{H}$ -A-804598 (N-cyano-N99-[(1S)-1-phenylethyl]-N9-5-quinolinyl-guanidine) was used as the radioligand. P2 membranes were prepared from recombinant cells; 50 mM Tris-HCl (pH 7.4) was added to the cells and homogenized for approximately 30 seconds at high speed. The homogenate was centrifuged at 1500 rpm for 5 minutes followed by careful decanting of the supernatant, which was centrifuged at 32,000g for 30 minutes. Six milliliters of ice-cold assay buffer (50 mM Tris-HCl 1 0.1% bovine serum albumin) was added to the cell pellet. The reaction was incubated for 1 hour at 4°C. The assay was terminated by filtration (GF/B filters presoaked with 0.3% polyethylenimine) and washed with washing buffer (Tris-HCl 50 mM) repeatedly. After drying, the plate Microscint 0 was added to the filters and radioactivity was counted.

### **Liver Microsome Assay**

The liver microsome assay was performed at Cyprotex (Watertown, MA, USA).

Test compounds are incubated over a time course to measure the half life ( $t_{1/2}$ ), from which the following parameters are derived: the in vitro intrinsic clearance ( $Cl_{int}$ ), the hepatic intrinsic clearance ( $Cl_{int, H}$ ), the estimated in vivo hepatic clearance ( $Cl_H$ ), and the extraction ratio (ER) using the simplified well-stirred model. It should be noted that in the simplified well-stirred model the values remain uncorrected for binding to both plasma and microsomal proteins ( $f_u^p$  and  $f_u^{mic}$ ), and the blood to plasma ratio is assumed to be 1.

The test compound is incubated at a defined substrate concentration (typically 1  $\mu\text{M}$ ) in liver microsomes across a time course (typically 0, 5, 10, 20, 40, and 60 minutes). The reaction is terminated

by addition of a suitable organic solvent (typically, acetonitrile, methanol or DMSO). The samples are centrifuged prior to analysis by LC-MS/MS analysis. The relative amount of parent compound remaining in the active incubations vs. the control incubations (t=0 mins) for each compound is measured by peak area comparison.

Typical incubation conditions are: 1  $\mu$ M test compound, 0.5 mg/ml microsomal protein, 1 mM NADPH, 1 mM  $\text{MgCl}_2$ , and 0.1 M phosphate buffer, pH 7.4. Incubation volume is typically 500  $\mu$ L with a final total solvent of 0.01% DMSO and 0.5% acetonitrile.

1. Stock solutions of test compounds (typically 10 mM) are prepared in DMSO and further diluted into a 50:50 solution of acetonitrile:water to a working stock of 0.1 mM.
2. Liver microsomes are thawed and diluted to 0.5 mg/mL in 0.1 M phosphate buffer containing 1 mM  $\text{MgCl}_2$ . The diluted microsomes may be kept on ice and used within 30 min after thawing. NADPH stock solution is prepared and stored on ice.
3. A bulk liver microsomal mixture is prepared by spiking working stock of test compound (e.g. 5  $\mu$ L test compound + 395  $\mu$ L microsomal mixture), followed by thorough mixing.
4. The incubation plate containing the microsomal mixture is situated on a heater shaker unit located on the robot deck and is pre-incubated to 37°C (typically < 5 minutes).
5. The reaction is initiated by the addition of pre-warmed NADPH solution (typically 100  $\mu$ L).
6. Sequential aliquots are removed across a time course (typically 50  $\mu$ L) of the microsomal mixture are removed to a separate plate and quenched with acetonitrile (typically 200  $\mu$ L, which contains a genetic internal standard). Typically, the time points used are 0, 5, 10, 20, 40, and 60 minutes.
7. The plates are centrifuged (10 min at 4000 rpm in a cooled centrifuge) prior to analysis of the supernatant by LC-MS/MS.

8. Typically, generic HPLC–MS/MS conditions are used for sample analysis in which specific SRM transitions are monitored for each compound. These transitions are usually optimized via automated procedures which are specific to the MS instrumentation used.

The percentage test compound remaining is calculated as the analytical response of test compound in the active sample divided by the average response of test compound in the control (t=0 min) samples. This result is expressed as a percentage, where the MS signal observed for test compound at t = 0 min is set to 100 % test compound remaining.

The *in vitro* metabolic half-life ( $t_{1/2}$ ) is calculated using the slope of the log-linear regression from the percentage parent compound remaining versus time relationship ( $\kappa$ ),

$$t_{1/2} = -\ln(2)/\kappa.$$

The *in vitro* intrinsic clearance ( $Cl_{int}$ ) (ml/min/mg microsomal protein) is calculated using the following formula:

$$Cl_{int} = \frac{0.693}{t_{1/2}} \times \frac{V_{inc}}{W_{mic\ prot, inc}}$$

Where:  $V_{inc}$  = incubation volume,  $W_{mic\ prot, inc}$  = weight of microsomal protein in the incubation

The hepatic intrinsic clearance ( $Cl_{int, H}$ ) (ml/min/kg) is calculated using the following formula:

$$Cl_{int, H} = Cl_{int} \times MPPGL \times \frac{W_{liver}}{W_{body}}$$

Where MPPGL is the microsomal protein per gram liver i.e.  $W_{body}$  = body weight,  $W_{liver}$  = liver weight

The estimated *in vivo* hepatic clearance ( $Cl_H$ ) (ml/min/kg) may be estimated using the equation for the Well Stirred Model

$$Cl_{H,blood} = \frac{Q_H \times \left( \frac{Cl_{int,H}}{f u_{mic}} \times \frac{f u_p}{B/P} \right)}{Q_H + \left( \frac{Cl_{int,H}}{f u_{mic}} \times \frac{f u_p}{B/P} \right)}$$

Where:  $Q_H$  = hepatic blood flow,  $f u_p$  = the unbound fraction in plasma,  $f u_{mic}$  = the unbound fraction in microsomes, and  $B/P$  = blood to plasma ratio

The extraction ratio (ER) is determined as follows:  $ER = (Cl_H/Q_H)$

#### **MDCK-MDR1 Permeability Assay**

The permeability and efflux of JNJ-64413739 were measured using Madin-Darby Canine Kidney Cells (MDCK) cells transfected with the P-glycoprotein (MDR1). The assay was performed at Cyprotex (Watertown, MA, USA).

MDR1-MDCK cells obtained from the NIH (Rockville, MD, USA) are used between passage numbers 6-30. Cells are seeded onto Millipore Multiscreen Transwell plates at  $3.4 \times 10^5$  cells/cm<sup>2</sup>. They are cultured for 4 days in DMEM and media is changed the day prior to the assay. On day 4 the permeability study is performed. Following culture, the monolayers are prepared by rinsing both basolateral and apical surfaces twice with buffer at pH 7.4 and 37°C. Cells are then incubated with pH 7.4 buffer in both apical and basolateral compartments for 40 min to stabilize physiological parameters.

Buffer at pH 7.4 is then removed from the apical compartment and replaced with test compound dosing solutions. The solutions are prepared by diluting 10 mM test compound in DMSO with buffer to give a final test compound concentration of 10  $\mu$ M (final DMSO concentration adjusted to 1%). The fluorescent integrity marker Lucifer yellow is also included in the dosing solution. The apical compartment inserts are then placed into 'companion' plates containing fresh buffer at pH 7.4. Analytical standards are made from dosing solutions.

For basolateral to apical (B-A) experiments the experiment is initiated by replacing buffer in the inserts then placing them in companion plates containing dosing solutions. Incubations are carried out in an atmosphere of 5% CO<sub>2</sub> with a relative humidity of 95% at 37°C for 60 minutes.

After the incubation period, the companion plate is removed and apical and basolateral samples diluted for analysis by LC-MS/MS. Test compound permeability is assessed in duplicate. On each plate compounds of known permeability characteristics are run as controls.

Test and control compounds are quantified by LC-MS/MS cassette analysis using a 5-point calibration with appropriate dilution of the samples. The starting concentration (C<sub>0</sub>) is determined from the dosing solution and the experimental recovery calculated from C<sub>0</sub> and both apical and basolateral compartment concentrations.

The integrity of the monolayers throughout the experiment is checked by monitoring Lucifer yellow permeation using fluorimetric analysis. Lucifer yellow permeation is low if monolayers have not been damaged. If a Lucifer yellow Papp value is above QC limits in one individual test compound well, then an n=1 result is reported. If Lucifer yellow Papp values are above QC limits in both replicate wells for a test compound, the compound is re-tested. If on repeat, high Lucifer yellow permeation is observed in both

wells then toxicity or inherent fluorescence of the test compound is assumed. No further experiments are performed in this instance.

The permeability coefficient for each compound ( $P_{app}$ ) is calculated from the following equation:

$$ER = \frac{P_{app}(B-A)}{P_{app}(A-B)}$$

Where  $dQ/dt$  is the rate of permeation of the drug across the cells,  $C_0$  is the donor compartment concentration at time zero and  $A$  is the area of the cell monolayer.  $C_0$  is obtained from analysis of the dosing solution at the start of the experiment.

In addition, an efflux ratio (ER) is calculated from mean A-B and B-A data. This is derived from:

$$ER = \frac{P_{app}(B-A)}{P_{app}(A-B)}$$

### Plasma Protein Binding Assay

The plasma protein binding assay was performed at Cyprotex (Watertown, MA, USA).

Plasma containing test compound at 1  $\mu$ M is incubated at 37°C in wells that are bisected with a semi-permeable membrane. Aliquots of spiked plasma are loaded on one-half of the well and 100 mM phosphate buffer is loaded in equal volume on the other half. After a designated amount of time and after equilibrium is achieved, samples are removed and analyzed from both the buffer and plasma side to obtain free and bound concentrations.

Warfarin tested at 1  $\mu$ M is used to confirm assay conditions. Literature cites warfarin as highly bound by plasma proteins. In-house, warfarin is highly bound in both rat and human, >99% and >98% respectively.

HTDialysis membranes are soaked in DI water for 15-30 minutes prior to assembling the dialysis apparatus. One mL of plasma per compound per concentration of each species is spiked with 10  $\mu$ L of 100  $\mu$ M compound (1  $\mu$ M final concentration) and vortexed lightly for  $\sim$  10 seconds. KPO4 buffer (150-750  $\mu$ L) is added in triplicate to one side of the designated wells and the spiked plasma (150-500  $\mu$ L) is added to the opposite sides. The plate is covered with adhesive sealing film and incubated on a plate agitator (100 RPM) at 37°C for 6 hr (RED) or placed in 37°C incubator overnight (HTDialysis).

For T = 0 samples (1  $\mu$ M reference), a 50- $\mu$ L aliquot (n=3) of spiked plasma is mixed with 50  $\mu$ L of blank buffer and extracted with 200  $\mu$ L of extraction solution (acetonitrile: methanol, 50:50) containing internal standard. Samples are vortexed for 1 minute, kept at room temperature for additional 15 minutes to release compound and to precipitate proteins and centrifuged at  $\sim$ 3200 rpm for 10 minutes. The supernatant is transferred to new tubes and stored sealed.

Post-dialysis, seal is gently removed from the plate and 50  $\mu$ L of incubated plasma is added to 50  $\mu$ L of blank buffer while 50  $\mu$ L of incubated buffer is added to 50  $\mu$ L of blank plasma. All samples are mixed with 200  $\mu$ L of extraction solution (acetonitrile: methanol, 50:50, containing IS) and processed as described above.

For the LC-MS analysis, compounds are tuned and methods developed prior to incubation. Typically this process will be completed using Automaton, an automatic tuning software developed by Applied Biosystems, that selects the optimal mass transition in the MRM (Multiple Reaction Monitoring) mode on a Sciex 4000. A general LC condition is as follows:

Column: Phenomenex Synergi Hydro RP, 4 $\mu$ m, 2.0x50mm or Agilent Zorbax SB-Phenyl, 5 $\mu$ m, 2.1x50mm, flow rate: 0.8 ml/min, solvents: 0.1% formic acid in water or acetonitrile, (buffer A, B, respectively), time: 4 min. Gradient: 0.1 – 1.3 min 2%B 1.3 – 2.4 min 90% B 2.4 – 2.5 min 2% B 2.5 – 4.0 min 2% B, injection amount: 10  $\mu$ L

Samples are immediately analyzed following extracting.

Total compound concentration is determined as  $P'/P_0$ , where  $P'$  and  $P_0$  are the peak ratios in post dialysis and T0 plasma samples, respectively. Free compound concentration is calculated as  $B'/P_0$ , where  $B'$  is the peak ratio in post dialysis buffer samples.

$$\% \text{ unbound} = \left( \frac{\text{free concentration}}{\text{total concentration}} \right) \times 100$$

### Brain Tissue Binding Assay

The brain tissue binding assay was performed at Cyprotex (Watertown, MA, USA).

1 in 10 diluted brain tissue homogenate is prepared by adding 9 mL PBS (pH=7.4) to 1 g of brain tissue. Brain tissue homogenate, containing test compound at 5  $\mu$ M, is incubated at 37°C in the Rapid Equilibrium Dialysis (RED) Device. The RED device consists of a Teflon 48-well base plate which contains disposable inserts. These inserts are bisected by a semi-permeable (MWCO= 8 kD) membrane, creating two chambers. Aliquots (300  $\mu$ l) of spiked 1 in 10 diluted brain tissue homogenate are loaded in to one chamber and phosphate buffered saline (PBS pH=7.4, 500 $\mu$ l) is loaded into the other. The plate is then sealed and placed in a shaking incubator at 37 °C for 5 h. After 5 h, samples are removed and analyzed

from both the buffer and brain tissue homogenate side to obtain free and bound concentrations. These concentrations are then used to calculate the percentage compound bound to brain tissue (%BTB).

Venlafaxine and fluoxetine tested at 5  $\mu$ M are used to confirm assay conditions.

Test compounds are received as neat compounds and diluted to 2 mM in DMSO. The final concentration in brain tissue homogenate is 5  $\mu$ M.

RED inserts are in the RED base plate. Blank 1 in 10 diluted brain tissue homogenate and 2mM stock solutions are supplied to the robot. In addition, RED device and plates are placed on the robot deck (Tecan, Freedom EVO)

The robot automatically spikes brain tissue homogenate at 5  $\mu$ M (final DMSO concentration in the incubation is 1%), mixes and fills RED device with buffer and spiked homogenate. All BTB measurements are performed in triplicate. The RED device is sealed and is allowed to incubate for 5 h at 37 °C on a vortex shaker (500 rpm) in an air incubator.

Calibration curves across an appropriate concentration range and quality control (QC) samples are prepared by the robot. These are used for quantitative analysis of the homogenate and buffer samples.

After incubation, the RED device is automatically sampled with the robot by taking 25  $\mu$ l homogenate and 50  $\mu$ l buffer, which is transferred to a 96 well plate. After addition of matrix (25  $\mu$ l homogenate or 50  $\mu$ l buffer, 50  $\mu$ l DMSO and 300  $\mu$ l acetonitrile) the samples are ready for LC-MS/MS analysis.

Samples are analyzed by LC-MS/MS in Multiple Reaction Monitoring (MRM) mode. One LC-MRM method is used for all tuned compounds, using optimal mass transitions and MS settings found with

tuning. Absolute concentrations of test compound in buffer and homogenate samples is quantified as measured by the calibration curve.

HPLC conditions: column: Waters Xbridge C18 3.5µm 4.6x50mm, Flow rate: 1.2mL/min, column temperature: Room temperature, solvent A: H2O+0.1%FA+0.5% ACN, solvent B: ACN+0.1%FA, run time: 3 min, gradient profile: 0.00 min 5 %B, 1.5 min 95 %B, 1.8 min 95 %B, 1.9 min 5 %B, 3.0 min 5% B

The formula used for determining the apparent unbound fraction ( $f_{u,app}$ ) is as follows:

$$f_{u,app} = \frac{[A]_{buffer}}{[A]_{homogenate}}$$

where  $[A]_{homogenate}$  is the concentration measured in the homogenate and  $[A]_{buffer}$  the concentration measured in the buffer.

Since homogenates are diluted (in this case 10 times) the  $f_{u,app}$  has to be corrected for the dilution factor in order to get the real unbound fraction in brain tissue ( $f_{u,brain}$ ). This is accomplished with the following formula:

$$f_{u,brain} = \frac{f_{u,app}}{D \times f_{u,app} - D \times f_{u,app}}$$

Where D is the dilution factor. Subsequently, the percentage compound bound to brain tissue (%BTB) is determined as follow:

$$\%BTB = (1 - f_{u,brain}) \times 100\%$$