Quantitative projection of human brain penetration of the H₃ antagonist PF-03654746 by integrating rat-derived brain partitioning and PET receptor occupancy

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Abstract

1. Unbound brain drug concentration (C₃pu), a valid surrogate of interstitial fluid drug concentration (C₃ISF), cannot be directly determined in humans, which limits accurately defining the human C₃pu/C₃pu of investigational molecules.
2. For the H₃R antagonist (1R,3R)-N-ethyl-3-fluoro-3-[3-fluoro-4-(pyrrolidin-1-lmethyl)phenyl]-cyclobutane-1-carboxamide (PF-03654746), we interrogated C₃pu/C₃pu in humans and nonhuman primate (NHP).
3. In rat, PF-03654746 achieved net blood–brain barrier (BBB) equilibrium (C₃pu/C₃pu of 2.11).
4. In NHP and humans, the PET receptor occupancy-based C₃pu IC₅₀ of PF-03654746 was 0.99 nM and 0.31 nM, respectively, which were 2.1- and 7.4-fold lower than its in vitro human H₃ Ki (2.3 nM).
5. In an attempt to understand this higher-than-expected potency in humans and NHP, rat-derived C₃pu/C₃pu of PF-03654746 was integrated with C₃pu IC₅₀ to identify unbound (neuro) potency of PF-03654746, nIC₅₀.
6. The nIC₅₀ of PF-03654746 was 2.1 nM in NHP and 0.66 nM in human which better correlated (1.1- and 3.49-fold lower) with in vitro human H₃ Ki (2.3 nM).
7. This correlation of the nIC₅₀ and in vitro hH₃ Ki suggested the translation of net BBB equilibrium of PF-03654746 from rat to NHP and humans, and confirmed the use of C₃pu as a reliable surrogate of C₃pu.
8. Thus, nIC₅₀ quantitatively informed the human C₃pu/C₃pu of PF-03654746.

Introduction

Quantitatively defining exposure–response relationships is a key to increasing the confidence in clinical observations during human trials evaluating novel molecules (Morgan et al., 2012). Based on this and, the free drug hypothesis (Tillement et al., 1988), which states that only unbound drug undergoes a pharmacologic interaction, a critical initial goal of Phase 1 studies is to determine dose-unbound plasma compound concentration (C₃pu)–time profile. Assuming equilibrium between C₃pu and unbound compound concentration at its site of action (either intra- or extracellular), C₃pu could be applied to designing dosing regimens in subsequent clinical studies. However, for the central nervous system (CNS) targets, research (Doran et al., 2012; Hammarlund-Udénæs et al., 2008; Liu et al., 2006, 2009) clearly shows that due to the nature of the blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier (BCSFB), C₃pu does not necessarily reflect CNS active-site exposures. Alternatively, interstitial fluid (ISF) compound concentration (C₃ISF) best determines the desired quantitative pharmacological relationships directly for transmembrane proteins and indirectly for intracellular and intra-membrane targets. Thus, for CNS agents it is essential to know C₃ISF at any time point after any dose to optimally design clinical trials to ensure that the underlying pharmacology is truly tested.

Although micro-dialysis may readily determine C₃ISF in rodent models (Ooie et al., 1997), it has very limited feasibility in higher-order species, particularly in humans. Therefore, high-confidence surrogates of C₃ISF must be identified for clinical application and insight. The most
common clinical proxy for $C_{\text{ISF}}$ is cerebrospinal (CSF) compound concentration ($C_{\text{CSF}}$), but its correlation to $C_{\text{ISF}}$ can be tenuous (de Lange & Danhof, 2002; Shen et al., 2004) and its collection via lumbar puncture can be highly unaccommodating for trial subjects. More recently, however, an increasing body of preclinical evidence, encompassing numerous small molecules with a spectrum of physicochemical properties and BBB transporter liabilities, suggests that unbound brain compound concentration ($C_{\text{b,u}}$) is a valid (and efficiently generated) surrogate of $C_{\text{ISF}}$ in rats (Doran et al., 2012; Hammarlund-Udenaes et al., 2008; Kalvass et al., 2007; Liu et al., 2006). Furthermore, it has been indicated that forecasting large animal (i.e. dog and nonhuman primate (NHP)) $C_{\text{b,u}}$ from a measured $C_{\text{p,u}}$ and a rat acute dose-determined $C_{\text{b,u}}$-to-$C_{\text{p,u}}$ ratio ($C_{\text{b,u}},C_{\text{p,u}}$) is of high confidence for non-efflux transporter substrates that show equilibrium at the BBB in rats (Doran et al., 2012). Therefore, establishing such a molecule’s $C_{\text{b,u}},C_{\text{p,u}}$ in rats should enable the accurate projection of human $C_{\text{b,u}}$ and $C_{\text{ISF}}$ from $C_{\text{p,u}}$ determined at any time point in a clinical trial. But in humans, unavailability of measured $C_{b}$ poses a limitation in bridging $C_{\text{p,u}}$ to $C_{\text{ISF}}$, unless there is a reliable pre-clinical and clinical methodology that will quantitate the relationship between $C_{\text{p,u}}$ and $C_{\text{b,u}}$. While the scope of obtaining a human $C_{b}$ via non-invasive techniques is extremely limited, the availability of PET-derived receptor occupancy (RO) gives an opportunity to identify a clinical method that affords a quantitative measure of $C_{\text{b,u}}$. As PET-derived RO methodology involves a centrally penetrant and pharmacologically selective tracer (Hargreaves & Rabiner, 2014), the collection and measurement of unbound plasma compound concentrations ($C_{\text{p,u}}$) during tracer displacement studies can be confidently used as a surrogate of unbound drug concentration at the target. In addition, a quantitative assessment of measured $C_{\text{p}}$ and PET-RO is commonly used to estimate the potency of a novel chemical entity. This potency estimate underpins the design of dose and dosing regimen of such new molecules in proof-of-concept (POC) studies. Therefore, deviations of potency estimates from human in vitro systems may confound the dose predictions of NCEs in POC stages.

The current manuscript describes a case study validating brain penetration of the potent and selective histamine H$_3$ antagonist (1R,3R)-N-ethyl-3-fluoro-3-[3-fluoro-4-(pyrrolidine-1-methyl)phenyl]cyclobutane-1-carboxamide (PF-03654746, Figure 1; $K_{I_i} = 2.3$ nM) (Wager et al., 2011) in NHP and human. PF-03654746 was studied pre-clinically and in Phase 1 clinical trials for its $C_{\text{p,u}}$-RO relationship using the H$_3$-selective PET ligand $N^\text{[11]}$C-methyl-6-(3-cyclobutyl-2,3,4,5-tetrahydro-1H-benzo[d]azepin-7-yl oxy)-nicotamide ($[^{11}\text{C}]\text{GSK189254}$) (Ashworth et al., 2010). The availability of such a pharmacologically potent, selective and well-validated PET ligand provided a non-invasive opportunity to evaluate PF-03654746 brain penetration in both NHP and humans. Comparison of $C_{\text{p,u}}$-based IC$_{50}$ of PF-03654746 studied by PET RO in NHP and human to in vitro $K_{I_i}$ resulted in additional considerations for the accuracy of the $C_{\text{b,u}},C_{\text{p,u}}$ of PF-03654746 in rat, NHP and human. Towards the accuracy of projecting $C_{\text{b,u}},C_{\text{p,u}}$ in NHP and human using rat-derived $C_{\text{b,u}},C_{\text{p,u}}$, we have proposed an integrated parameter “nIC$_{50}$” which can be compared with in vitro-generated human ($H_3$) $K_{I_i}$. In this paper, we report our findings from these experiments.

### Materials and methods

PF-03654746 was synthesized at Pfizer Worldwide Research and Development (WRD, Groton, CT) as described (Wager et al., 2011). All solvents and buffers were of HPLC grade and procured from Sigma-Aldrich (St. Louis, MO). Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA); pharmacokinetic studies in NHP were performed at WRD (Groton, CT). Male cynomolgus monkeys were fitted with in-dwelling subarachnoid catheters and subcutaneous access ports by Pfizer Veterinary Services (Groton, CT). Rhesus monkeys used in PET imaging studies were housed at the Yale University PET Center (New Haven, CT). All animal care and in vivo procedures were approved by the Pfizer Animal Care and Use Committee and conducted in accordance with the guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). NHP PET studies were approved by the Institutional Animal Care and Use Committee at Yale University and conducted at Yale PET Imaging facility. All clinical studies were conducted in compliance with the ethical principles originating in or derived from the Declaration of Helsinki and in compliance with all International Conference on Harmonization of Good Clinical Practice Guidelines and the International Ethical Guidelines for Biomedical Research Involving Human Subjects. All local regulatory requirements were followed, in the interest of greater protection to the safety of study participants. The radio-ligand used in NHP PET study, $[^{11}\text{C}]\text{GSK189254}$, was produced at the Yale University PET center using a reported procedure (Ashworth et al., 2010).

### Binding of PF-03654746 to plasma proteins and brain homogenate

The unbound fraction of PF-03654746 in plasma of rat, NHP and human, ($f_{u,p}$) and in brain homogenate of rat ($f_{u,b}$) were determined by equilibrium dialysis (Kalvass et al., 2007). (Because initial experiments in rat and human plasma afforded essentially identical unbound fractions of PF-03654746 when tested at concentrations of 100 and 1000 ng/mL, all reported experiments in all matrices occurred at a PF-03654746 concentration of 2 µM.) Aliquots (150 µL, $n = 3$/matrix) were loaded into a 96-well equilibrium dialysis apparatus and dialyzed against an equal volume of 100 mM sodium phosphate buffer (pH 7.4) at 37°C for 6 h in 5% CO$_2$. Subsequently, aliquots of plasma (20 µL), brain (20 µL) and buffer (100 µL) were removed and transferred to 96-well blocks containing acetonitrile (200 µL) and an internal standard. Samples were then centrifuged (3000g for 10 min).
and the resulting supernatants were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Pharmacokinetics studies of PF-03654746 in rat, NHP and human

**Rat**

To each male Sprague-Dawley rat \((n = 4\) rats/time-point/dose\), PF-03654746 was administered at 1 or 3.2 mg/kg, subcutaneously. Animals were euthanized via CO₂ asphyxiation. At each time-point \((0.5, 1, 2\) and \(4\ h\ post-dose\)) whole blood, CSF and brain were harvested. Blood samples were immediately placed on ice before centrifugation \((3000\ g\ for\ 10\ min)\) to yield plasma, which was transferred to \(1.2\ mL\) polypropylene marsh tubes. Cerebrospinal fluid samples were stored on ice immediately after collection. Brain samples were rinsed with water to remove excess blood, blot dried, weighed in a tared vial, and stored on ice until either refrigeration at \(-20°C\) or analysis. During analysis, brain samples were homogenized with three volumes of Millipore water using a Polytron homogenizer. Plasma and brain homogenate samples were extracted using \((2\ ×\ 150\ μL)\) acetonitrile containing an internal standard and vortex-mixed for \(10\ min\). The supernatant was separated by centrifugation \((3000\ g\ for\ 4\ min)\) and collected after each extraction. The extracts were combined and evaporated to dryness using a nitrogen stream at ambient temperature. The residue was reconstituted in \(100\ μL\) of MeOH/H₂O \((50:50,\ v/v)\) and analyzed by LC-MS/MS. Cerebrospinal fluid samples were thawed and an aliquot \((20\ μL)\) was mixed with internal standard \((20\ μL\ of\ a\ 300\ ng/mL\ stock\ in\ methanol)\) and analyzed using LC-MS/MS without extraction.

**Nonhuman primate**

The pharmacokinetics of PF-03654746 in plasma and CSF of male cynomologous monkeys were characterized by administration of 1 mL/kg of a single-bolus dose of PF-03654746 \((n = 5\) cynomologous monkeys/time-point/dose). The doses of 0.167 mg/kg and 0.5 mg/kg of PF-03654746 for IV administration were formulated in saline at concentrations of 0.167 mg/mL and 0.5 mg/mL, respectively. Whole blood \((2\ mL)\) and CSF \((300\ μL)\) were obtained at pre-dose, and at \(0.5, 1, 2, 4, 6, 8, 24,\) and \(30\ h\ post-dose\) of PF-03654746. The collection of CSF was achieved by sterile-needle insertion into the subcutaneous port at the cisterna magna. Whole blood was obtained via a jugular-vein indwelling catheter. Blood samples were collected, processed and analyzed using LC-MS/MS as described previously.

**Human**

Healthy volunteers \((n = 14,\ 18–55\ years\ of\ age)\) were enrolled for collection of plasma and CSF. Subjects received PF-03654746 \((3\ mg,\ QD,\ PO)\), for 12 days. On Day 12, subjects underwent a repeat intra-thecal cannulation procedure \(2\ h\ prior\ to\ receiving\ the\ last\ dose\). Cerebrospinal fluid samples, collected at pre-dose and at \(1, 2, 3, 4, 5\) and \(6\ h\ after\ the\ final\ dose\) of PF-03654746, were flash-frozen in a methanol bath on dry ice and stored at \(-80°C\) until analysis. Blood samples were collected at the same time points as CSF. Serum was harvested by centrifugation \((3000g\ for\ 10\ min)\) of whole blood at \(4°C\). Samples were stored at \(-20°C\) until further processing or analysis.

**Quantification of PF-03654746**

A validated LC-MS/MS method quantified PF-03654746 in serum and CSF. Reconstituted extracts of brain (rat), plasma/serum (rat, NHP and human), or CSF (rat, NHP and human) were analyzed by LC-MS/MS. A 2.0 min gradient was used to achieve separation using a Shimadzu pump (Agilent, Palo Alto, CA). Extracts were injected \((10\ μL)\) onto a Synergi Polar RP C_18\ column \((2.0 \times 50\ mm;\ 4\ μm)\) equilibrated in \(15\ mM\ ammonium\ formate\ (pH\ 3.2)/acetonitrile/MeOH\ (85/5/10)\) at a flow rate of \(0.45\ mL/min\) for \(0.5\ min\) followed by a change in gradient from \(0.5\) to \(2\ min\) to \(5/85\ CH_3CN/MeOH\). The gradient was held from \(2\) to \(3\ min\) and then equilibrated to starting conditions from \(3\ to\ 4\ min.\)**

**Pharmacokinetic calculations**

The pharmacokinetic parameters of maximum plasma drug concentration \(C_{max}\), area under the plasma drug concentration–time curve from time dosed \(t_0\) to the last time-point \(t_{last}\), where the concentration of PF-03654746 was above the lower limit of quantitation \(AUC_{0–t_{last}}\), total plasma clearance \(CL\), steady-state volume of distribution \(V_{ss}\) and half-life \(t_{1/2}\) were determined from individual animals using non-compartmental analysis (WinNonlin, Certara). Human pharmacokinetic parameters were determined using non-compartmental analysis. Concentrations below the limit of quantitation were recorded as \(0\ ng/mL\). Descriptive statistics (geometric mean and coefficient of variation) of the PK parameters \(AUC_{0–t_{last}}, C_{max}\) and \(CL\) were calculated. An analysis of variance model was fit for each log transformed PK parameter.

Inter-compartmental ratios were calculated as follows:

\[
C_{\text{ss},u} = C_x \cdot f_{u,x},
\]

\[
(nIC_{50})_{\text{species}} = \frac{(C_{b,u} : C_{p,u})_{\text{rat}} \cdot (C_{p,u}IC_{50})_{\text{species}}}{(C_{b,u}C_{p,u})_{\text{CSF}}C_{p,u}C_{b,u}} \text{ and } (C_{b,u}C_{p,u})_{\text{CSF}} \text{ are defined previously (Doran et al., 2012).}
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**Positron emission tomography (PET)-derived H3 receptor occupancy of PF-03654746 in NHP and humans**

H₃ RO in rhesus monkeys \((n = 3)\) was measured using PET with the selective H₃ radio-ligand \[^{11}C\]-GSK189254 (Ashworth et al., 2010). A total of 6 PET scans were acquired; two baseline scans and four drug-blocking scans. For each PET scan, a 120 or 150 min dynamic scan was acquired on a High-Resolution Research Tomograph (HRRT) PET scanner.
Individual PET scans. Time-activity curves (TACs) were then generated for quantitation of PF-03654746 in plasma of NHP.

Dynamic images (33 frames over 120 min or 36 frames over 150 min) were reconstructed with a list-mode ordered subset expectation maximization algorithm. For each PET scan, radiotracer concentrations were measured in the cerebellum, thalamus, striatum and occipital cortex; a cerebellar homogenate was used as a standard. For each blocking scan, the difference between the regional baseline TAC values was computed (i.e. $\Delta V_T = baseline V_T - blocked V_T$) and plotted versus the baseline value. If the radio-ligand binds to a single site with uniform affinity and uniform non-specific binding throughout the brain, then this plot of $\Delta V_T$ versus baseline $V_T$ shows a linear relationship, which slope is the degree of RO and which x-intercept is the radiotracer non-displaceable volume $V_{ND}$ (Cunningham et al., 2010). Due to the high affinity of $[\text{11C}]$GSK189254, the PET studies have not been performed with a tracer dose of radio-ligand. Thus, a correction for the usage of a non-tracer (NT) dose of radio-ligand and for carry-over of radio-ligand mass from one PET scan to the next (NTCO correction) was applied as in the human study (Gallezot et al., 2016). For this correction, the in vivo equilibrium dissociation constant $K_E$ of the radio-ligand must be known. This value is unknown in rhesus monkeys, thus the average of values estimated in humans (9.5 pM, Gallezot et al., 2016; 11 pM, Ashworth et al., 2010) and baboons (Papio Anubis) (Salinas et al., 2010) was used. The drug concentration that leads to a 50% blockade of $[\text{11C}]$GSK189254 specific binding ($IC_{50}$) was estimated by fitting the RO versus unbound plasma drug concentration $C_{p,u}$, with the following equation:

$$RO = C_{p,u} / (C_{p,u} + IC_{50}).$$  

In humans, the PET-derived $H_3$ RO of PF-03654746 was measured in healthy volunteers by displacement of the same tracer $[\text{11C}]$GSK189254 (Gallezot et al., 2016).

### Results

**Binding of PF-03654746 to plasma proteins and brain homogenate**

In each matrix, PF-03654746 was stable ($\pm 20\%$ of initial concentration) over the incubation period and had insignificant non-specific binding ($<1\%$). The $f_{u,p}$ of PF-03654746 for rat, NHP and human were $70\% \pm 3.5\%$, $70\% \pm 2.4\%$ and $66\% \pm 0.7\%$, respectively; rat $f_{u,b}$ was $70\% \pm 0.7\%$.

**Neuro-pharmacokinetics of PF-03654746 in rat and NHP**

Neuro-pharmacokinetics of PF-03654746 were assessed in rat and NHP. Measured $C_b$ and $C_p$ were converted to unbound matrix concentrations using Equation (1); due to minimal proteins in CSF (Shen et al., 2004), measured $C_{CSF}$ was considered to be fully unbound. For each species, the neuropharmacokinetics of PF-03654746 was assessed by comparing inter-compartmental ratios of PF-03654746 (Table 1).

In rats, PF-03654746 (1 and 3.2 mg/kg, SC) had a linear dose–exposure relationship in brain, plasma and CSF with rapid $t_{max}$ (0.25 h) and elimination ($t_{1/2}$ of 0.5–0.6 h) in all compartments. In rat, mean $C_{b,u}/C_{p,u}$ was 2.1, mean $C_{CSF}/C_{p,u}$ was 0.95, and mean $C_{b,u}/C_{CSF}$ was 2.2, suggesting that PF-03654746 demonstrated equilibrium at both the BBB and BCSFB. This was consistent with an MDCK-MDR1 ER of 1.1, mouse BCRP ER of 1.6 and high permeability ($P_{app,A-n}$: $17 \times 10^{-6} \text{cm/s}$; Pfizer internal data). Although the rat $C_{b,u}/C_{p,u}$ of PF-03654746 is two-fold above unity it does not suggest net active uptake of PF-03654746 at the rat BBB as this deviation from unity could be a combination of assay parameter variability commonly observed in vivo (protein binding, kinetic binding, etc.) as well as in vitro (terminal sampling to obtain rat neuro-PK, plasma PK variability, etc.). Based on the classification system reported by Kalvass et al. (2007), PF-03654746 was classified as net passive diffusion with no indication of active uptake. This inference is also consistent with well-known substrates of net active uptake like diphenhydramine, CE-157119 and oxycodone, where rat $C_{b,u}/C_{p,u}$ is $\geq 3$ (Bostrom et al., 2006; Doran et al., 2012; Shaffer et al., 2014).

In NHP, PF-03654746 had a mean $C_{CSF}/C_{p,u}$ of 0.94. The $t_{1/2}$ of PF-03654746 in NHP plasma and CSF was 0.5 h, suggesting parallel elimination of PF-03654746 from each compartment.

The pharmacokinetic parameters of PF-03654746 in healthy human volunteers following multiple oral doses of PF-03654746 are given in Table 1.

### Table 1. Summary of inter-compartmental ratios of PF-03654746 in rat, NHP and human.

| Species | $C_{b,u}/C_{p,u}$ | $C_{b,u}/C_{CSF}$ | $C_{CSF}/C_{p,u}$ |
|---------|------------------|------------------|------------------|
| Rat     | 2.11             | 2.2              | 0.95             |
| NHP     | ND               | ND               | 0.94             |
| Human   | ND               | ND               | 0.86             |
PF-03654746 at 1.25, 2, 3, 6 and 12 mg, QD, for 14 days is summarized in the supplemental information S1. PF-03654746 demonstrated similar pharmacokinetic characteristics across a 10-fold dose range as observed by a plasma $t_{\text{max}}$ at 3 ± 0.5 h, long $t_{1/2}$ (13.6 ± 2.9 h) and low CL/F (4.0 ± 0.3 mL/min/kg). Subsequently, 3 mg of PF-03654746 was administered to a separate cohort of healthy volunteers for 12 d at which time plasma and CSF were serially collected from $t_0$ (pre-dose) to 6 h post-dose. PF-03654746 demonstrated equilibrium in plasma and CSF as observed by parallel absorption and distribution profiles from 0 to 6 h and $t_{\text{max}}$ at 3 h and 4 h, respectively. Due to a lack of regression points for human CSF, the $t_{1/2}$ of PF-03654746 was not determined. In humans, the AUC$_{0-6\text{h}}$ $C_{\text{CSF}}$: $C_{\text{p,u}}$ of PF-03654746 was 0.86.

**PET-derived RO in NHP**

Figure 2 shows a set of $^{[11]}C$GSK189254 PET images at baseline and blocking conditions (i.e. after PF-03654746 was infused) summed from 60 to 90 min at the level of the striatum, along with the corresponding anatomical MRI images for the same slices. Under the baseline condition, $^{[11]}C$GSK189254 binding was highest in the putamen and caudate, followed by the cerebellum, cortex, thalamus and cerebellar white matter. Regional $^{[11]}C$GSK189254 uptake was significantly reduced under blocking conditions.

PET data were fitted well with a 1T compartment model. For each blocking drug dose, Table 2 shows the measured $C_p$ and $C_{\text{p,u}}$ of PF-03654746 and the respective estimates of $H_3$ RO (estimated using the occupancy plot). The $H_3$ RO ranged from 50% to 97%. The $H_3$ RO of PF-03654746 in NHP, increased in a dose-dependent manner with a $C_{\text{p,u}}$-determined IC$_{50}$ of 1.1 nM, as shown in Figure 3. After applying the NTTCO correction, assuming that $^{[11]}C$GSK189254 $K_d$ was 24 pM, the corrected $H_3$ RO ranged from 52% to 98%. The NTTCO corrected NHP $C_{\text{p,u}}$ IC$_{50}$ of PF-03654746 was 0.99 nM (Figure 3). Applying rat-derived $C_{\text{b,u}}$:$C_{\text{p,u}}$ of 2.11 in Equation (2), the NHP IC$_{50}$ of 2.1 nM was essentially equivalent to human in vitro $H_3$ $K_i$ of 2.3 nM. (Note: $K_i$ of PF-03654746 was not determined in in vitro recombinant and/or whole cells system expressing NHP $H_3$ receptors; NHP $K_i$ of PF-03654746 was assumed to be equivalent to human $K_i$ of 2.3 nM based on high sequence homology of $H_3$ receptors between NHP and humans (Yao et al., 2003).)

**PET-derived RO in humans**

The exposure–RO relationship of PF-03654746 in humans has been reported by Gallezot et al. (2016) (*J. Cerebral Blood Flow*) and $C_p$ IC$_{50}$ has been estimated to be 0.144 ng/mL. This resulted in an unbound plasma $C_{\text{p,u}}$ IC$_{50}$ of 0.313 nM, which was 7.4-fold lower than measured in vitro $hH_3$ $K_i$ of 2.3 nM. Using Equation (2) and rat-derived $C_{\text{b,u}}$:$C_{\text{p,u}}$ of 2.11, we calculated human IC$_{50}$ of PF-03654746 as 0.66 nM, which was 3.49-fold lower than the human $K_i$ (2.3 nM) and 3.17-fold lower than NHP PET-RO-derived unbound brain IC$_{50}$ (2.1 nM).

**Discussion**

Efficacious concentrations of an investigational neurotherapeutic, designed for modulation of transmembrane proteins, are determined by $C_{\text{ISF}}$ (Hammarlund-Udenaes et al., 2008). If desired, $C_{\text{ISF}}$ may be measured in rodents (Chaurasia et al., 2007; Hammarlund-Udenaes, 2000; Ooie et al., 1997), and NHP (Rollena et al., 1989), but not so readily in humans (Bouras et al., 2011; Clinckers et al., 2009). For GPCR targets, application of $C_{\text{b,u}}$ as a descriptor of $C_{\text{ISF}}$ has been interrogated in pre-clinical species (Liu et al., 2009). The $C_{\text{b,u}}$: $C_{\text{p,u}}$ of small molecules measured in small and large laboratory animals has been assumed to translate to higher-
order species, including humans. In fact, a recent publication has demonstrated that equilibrium for non-efflux mediated, passively permeable substrates is conserved at the BBB of rat, dog and NHP and that rat-derived $C_{b,u}/C_{p,u}$ can accurately project $C_{b,u}$ in NHP and dog for such compounds (Doran et al., 2012). An unanticipated and large difference in free plasma-based binding potency of PF-03654746 in vivo (NHP/humans) versus in vitro systems resulted in an opportunity to test the accuracy of the translation of $C_{b,u}/C_{p,u}$ from rat to human (Table 3).

In vitro, PF-03654746 demonstrated desirable permeability across physiological membranes and was not a substrate for P-gp or BCRP. In rats, PF-03654746 was rapidly absorbed (plasma $T_{\text{max}}$: 0.5 h post-dose) and demonstrated BBB equilibrium ($C_{b,u}/C_{p,u}$: 2.11). Prior to its clinical progression, it was important to confirm the brain penetration and binding potency of PF-03654746 in a higher-order species (e.g. NHP) using a reliable, potentially non-invasive, and a sensitive methodology. While a terminal neuro-pharmacokinetic study in NHP could accurately define $C_{p,u}$ of PF-03654746 in NHP, availability of a selective and validated H$_3$ radiotracer, $[^{11}\text{C}]$GSK189254, enabled the application of PET-derived RO. This was a potentially non-invasive, pharmacologically specific, and translatable technique to quantitatively evaluate $C_{p,u}$ IC$_{50}$ as a reliable estimate of potency of PF-03654746 in NHP and subsequently in humans. Assuming equilibrium at the BBB, the PET-derived $C_{p,u}$ IC$_{50}$ of PF-03654746 could be readily compared with the in vitro H$_3$ binding $K_i$.

In NHP, the PET-RO $C_{p,u}$-based IC$_{50}$ (0.99 nM) of PF-03654746, determined via blocking of the selective H$_3$ tracer ligand, $[^{11}\text{C}]$GSK189254, enabled the application of PET-derived RO. This was a potentially non-invasive, pharmacologically specific, and translatable technique to quantitatively evaluate $C_{p,u}$ IC$_{50}$ as a reliable estimate of potency of PF-03654746 in NHP and subsequently in humans. Assuming equilibrium at the BBB, the PET-derived $C_{p,u}$ IC$_{50}$ of PF-03654746 could be readily compared with the in vitro H$_3$ binding $K_i$.

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Table 3. Summary of target binding of PF-03654746 in rat, NHP and human.

| Species | In vitro $K_i$ (nM) | $C_{p,u}$ IC$_{50}$ (nM) | $^a$IC$_{50}$ (nM) |
|---------|-------------------|----------------|------------------|
| Rat     | 37                | ND             | ND               |
| NHP     | ND                | 0.99           | 2.1              |
| Human   | 2.3               | 0.31           | 0.66             |

$^a$Calculated using Equation (2) and $(C_{b,u}/C_{p,u})_{rat} = 2.11.$

Figure 3. Displacement of $[^{11}\text{C}]$GSK189254 in NHP at (A) after 2, 7, 0.25 and 0.7 $\mu$g/kg/h of PF-03654746. (B) Relationship of free plasma concentration of PF-03654746 to its RO in NHP.
PET-derived NHP $C_{\text{b,u}}$ IC$_{50}$ and human in vitro $K_i$ was same in magnitude to the observed rat $C_{\text{b,u}}$ of 2.11 (Table 1). This key observation led to extrapolating rat $C_{\text{b,u}}$ to NHP which is well supported via several examples of non-effluxed compounds reported by Doran et al. (2012). We thereby integrated the rat $C_{\text{b,u}}$ of PF-03654746 with its NHP PET-derived $C_{\text{b,u}}$ IC$_{50}$ to define a novel $C_{\text{b,u}}$-based IC$_{50}$ of PF-03654746. This integrated, neuro-pharmacokinetically derived $C_{\text{b,u}}$ IC$_{50}$ is hereafter referred to as “nIC$_{50}$.” Thus, application of Equation (2) to NHP $C_{\text{b,u}}$ IC$_{50}$ of PF-03654746 resulted in NHP nIC$_{50}$ of 2.11 nM. This NHP nIC$_{50}$ of PF-03654746 correlated with greater accuracy to its in vitro human $H_2$, $K_i$ (2.3 nM). This correlation of nIC$_{50}$ with in vitro $H_2$, $K_i$ in rat supported the hypothesis that PF-03654746 was brain penetrant in NHP and was in equilibrium at the NHP BBB with a projected NHP $C_{\text{b,u}}$: of 2.11, similar to that observed in rat.

Subsequent to confidently defining the NHP $C_{\text{b,u}}$: of PF-03654746, we applied the nIC$_{50}$ methodology to accurately define human $C_{\text{b,u}}$: of PF-03654746. In humans, PET-RO determined $C_{\text{p,u}}$ IC$_{50}$ of PF-03654746 was 0.31 nM (Gallezot et al., 2016). This PET-RO-derived $C_{\text{p,u}}$ IC$_{50}$ of PF-03654746 was 7.4-fold lower than the measured human in vitro $K_i$ of 2.3 (±0.09) nM; interestingly, this human $C_{\text{p,u}}$ IC$_{50}$ (0.31 nM) of PF-03654746 correlated better with NHP $C_{\text{p,u}}$ IC$_{50}$ (0.99) and was within 3-fold. However, the 7.4-fold disconnect between PET RO-derived human $C_{\text{p,u}}$ IC$_{50}$ (0.31 nM) and human in vitro $K_i$ (2.3 nM) could not be accounted for, with the observed variance of the in vitro assay. Furthermore, the role of active metabolites contributing to in vivo potency was ruled out based on the observed metabolite profile of non-radiolabeled PF-03654746 in human plasma (Pfizer internal data). Unlike NHP, the 7.4-fold disconnect was dissimilar from the observed rat $C_{\text{b,u}}$: of 2.11; however, building on our conclusions of strong correlations of NHP nIC$_{50}$ with in vitro $H_2$, $K_i$, we applied Equation (2) to human PET-RO $C_{\text{p,u}}$ IC$_{50}$ of PF-03654746. Integration of the human PET-RO-derived $C_{\text{p,u}}$ IC$_{50}$ (0.313 nM) with rat-derived $C_{\text{b,u}}$: (2.11) afforded a human nIC$_{50}$ of 0.66 nM. The human nIC$_{50}$ resulting from application of Equation (2) was in stronger concordance with NHP nIC$_{50}$ (2.1) and was 3.2-fold lower than the in vitro human $K_i$ (2.3 nM). This strong in vitro-to-in vivo correlation (IVIVC) was suggestive of net equilibrium of PF-03654746 at the BBB of rat and human. This validation demonstrated that equilibrium of this non-effluxed substrate was indeed accurately conserved across the BBB of rodents and humans. In fact, the measured $C_{\text{CSF}}$: of PF-03654746 in rat (0.95), NHP (0.94) and human (0.86) implied the conservation of equilibrium at the blood–CSF barrier at the choroid plexus (Saunders, 1986). These data suggested that $C_{\text{p,u}}$ and $C_{\text{CSF}}$ were both excellent surrogates of $C_{\text{b,u}}$. Thus, we report the quantitative evaluation of brain penetration of PF-03654746 using translatable, non-invasive, and a target-specific technique of PET-RO integrated with rodent $C_{\text{b,u}}$:.

Confirmation of a human $C_{\text{b,u}}$: is a key milestone in validating human brain penetration of investigational neuro-active compounds and evaluation of the integrated parameter, nIC$_{50}$, can accurately provide this confirmation in early stages of clinical development. Confidently defining the nIC$_{50}$ parameter during phase I studies, enables confident projection of RO of novel CNS agents by the targeted dose and dosing regimen in POC studies at the target site of action. It should be acknowledged that the accurate projection of $C_{\text{b,u}}$: using the nIC$_{50}$ approach heavily relies on confident measurements of: (a) in vitro target-binding affinity ($K_i$), (b) animal $C_{\text{b,u}}$: of the novel compound (rat in the case of a non-efflux-mediated substrate) and (c) PET-RO-derived $C_{\text{p,u}}$ IC$_{50}$. While high-confidence measurements of in vitro $K_i$ can be obtained, choice of animal $C_{\text{b,u}}$: is of significance in generating the nIC$_{50}$ using Equation (2). As shown previously (Liu et al., 2009; Doran et al., 2012) and with the justification provided by PF-03654746 in the current manuscript, if a compound is a non-efflux substrate and is passively permeable, rat $C_{\text{b,u}}$: can be applied in Equation (2) to quantitate the partitioning of an investigational molecule at the human BBB. In addition, successful application of nIC$_{50}$ approach requires accurate and confident measurement of $C_{\text{p,u}}$ IC$_{50}$. For investigational compounds metabolized to active circulating moieties that may be brain penetrant and may retain significant target potency (van Beijsterveldt et al., 1994), the calculation of nIC$_{50}$ will be confounded by the competition of additional ligand/s at the receptor of interest. Another limitation of this methodology could be envisioned, where $C_{\text{p,u}}$ IC$_{50}$ is measured by applying PET tracers that potentially label multiple enzyme isoforms (Gallezot et al., 2014; Grunder et al., 2008). Similarly, incomplete characterization of the exposure–RO relationship due to factors like solubility-limited PK, limitations on achieving maximal occupancies due to dose-limiting safety and/or receptor desensitization by high intrinsic activity agonists should be considered prior to application of nIC$_{50}$ approach. Nevertheless, in the absence of a measured $C_{\text{b,u}}$ in higher-order species, including humans, the use of an integrated, non-invasive approach to derive nIC$_{50}$ allows for accurate understanding of unbound drug concentration at the pharmacologically relevant target site of action and informs human $C_{\text{b,u}}$:.

Conclusions

This study used PET-derived RO data and experimentally determined primate $C_{\text{CSF}}$: values to strongly suggest that the human and NHP $C_{\text{b,u}}$: of PF-03654746 was essentially similar (i.e. net BBB equilibrium) to that measured in rat. The described studies defined an integrated parameter, nIC$_{50}$, to unambiguously and non-invasively establish the translatability of the neuro-pharmacokinetic properties of PF-03654746, which was not effluxed at the BBB and showed net equilibrium. In future efforts, we suggest testing the accuracy of nIC$_{50}$ approach to compounds that are characterized by net disequilibrium or which may exhibit transporter-mediated disposition at the human BBB.

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Declaration of interest
The authors have no declarations of interest to report.

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