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Species Independence in Brain Tissue Binding Using Brain Homogenates

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Abbreviations

ADME = Absorption, Distribution, Metabolism and Excretion

CSF = Cerebrospinal fluid

CNS = Central nervous system

Cyno monkey = Cynomolgus monkey

DPBS = Dulbecco's phosphate buffer solution

F_u = Fraction unbound

IS = Internal Standard

MWCO = Molecular weight cut-off

PD = Pharmacodynamics

PK = Pharmacokinetics

PPB = Plasma protein binding

SD rat = Sprague-Dawley rat

TPSA = Topological polar surface area

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Abstract

Species independence of brain tissue binding was assessed with a large number of structurally diverse compounds using equilibrium dialysis with brain homogenates of seven species and strains (Wistar Han rat, Sprague-Dawley rat, CD-1 mouse, Hartley guinea pig, beagle dog, cynomolgus monkey and human). The results showed that the fraction unbound of the seven species and strains were strongly correlated with correlation coefficients ranging from 0.93 to 0.99. The cross-species/strain correlations were not significantly different from the inter-assay correlation with the same species. The linear correlation between Wistar Han and other species had a slope close to one and an intercept near zero. Based on orthogonal statistical analysis, no correction is needed when extrapolating fraction unbound from Wistar Han rat to the other species or strains. Hence, brain tissue binding of Wistar Han rat can be used to obtain binding of other species and strains in drug discovery.

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Introduction

The free drug hypothesis is a well accepted and widely applied concept in drug discovery and development (Liu et al., 2006; Hammarlund-Udenaes et al., 2008; Lin, 2008; Smith et al., 2010). This hypothesis states that it is the free (unbound) drug at the site of action that exerts pharmacological activity, rather than total drug (bound and unbound) and it is the free drug that is able to distribute from the systemic circulation across membranes to tissues rather than total drug. Provided simple diffusion governs distribution, at equilibrium the free drug concentration in systemic circulation and extravascular compartment will be equal, with total concentrations at a given time being governed by the respective binding constants for the compartments. While these hypotheses are important for understanding the actions of all drugs, they are particularly important considerations for those agents intended to exert their effect on the central nervous system (CNS). The CNS is an extravascular compartment and not directly accessed from systemic circulation due to the blood-brain barrier. In order for a compound to reach a target in the CNS, the free drug in plasma must first cross this barrier. Subsequently, this free drug undergoes binding within the CNS to the brain tissues. Finally, the drug that is free in the CNS is available for pharmacological activity. It therefore follows that trying to understand CNS drug behavior using total drug concentration in the brain or plasma alone can be misleading (Smith et al., 2010). For neuroscience therapeutic targets, accurate understanding of total and free drug concentrations in plasma and brain, particularly brain interstitial fluid, is critical for developing PK/PD relationships, projecting doses and designing clinical studies. Several techniques have been developed to obtain free drug concentration in the brain directly or indirectly (Di et al., 2008; Liu et al., 2008; Di and Kerns, 2011), including *in vivo* microdialysis (direct) (de Lange et al., 1997; Elmquist and Sawchuk, 1997; Hammarlund-Udenaes et al., 1997; Hammarlund-

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Udenaes, 2000; Watson et al., 2006), CSF sampling (indirect) (Shen et al., 2004; Lin, 2008; Friden et al., 2009b), and combination of brain distribution through measuring plasma and brain concentration time courses (Kerns and Di, 2008) and brain tissue binding (quasi-direct) (Kalvass and Maurer, 2002; Mano et al., 2002; Maurer et al., 2005; Summerfield et al., 2006; Liu et al., 2009). This last approach of measuring brain distribution and brain tissue binding, is one of the most common strategies in the pharmaceutical industry to elucidate total and free drug PK relationships in brain and plasma. The approach shows advantages over other methods in that it is widely applicable to compounds with a wide range of physicochemical properties (microdialysis is limited to those compounds for which recovery from apparatus is not prohibitive). Furthermore, it measures the compartment of interest vs. sampling CSF, which is technically not the compartment of interest. Drug concentration in CSF may not be in equilibrium with the biophase where the biological target resides in the brain parenchyma. Drugs can access CSF via both the cerebrovasculature and the choroidal epithelium. Additionally the approach is technically less challenging, more reproducible and higher throughput than the other methods.

For brain tissue binding, both low and high throughput methods using brain homogenates (Kalvass and Maurer, 2002; Mano et al., 2002; Summerfield et al., 2006; Wan et al., 2007) and brain slices (Kakee et al., 1996; Becker and Liu, 2006; Friden et al., 2007; Friden et al., 2009a) have been developed to determine the fraction unbound (f_u) of drugs in brain tissues. Brain slices are considered more physiologically relevant than brain homogenates since the cellular structures (cell membrane, influx and efflux transporters, and intracellular fluids) are preserved in brain slices, whereas these are disrupted in brain homogenates. Nevertheless, data generated

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using the brain homogenate method has good correlation with brain slice data, especially when cytosolic pH partition was corrected for basic compounds (Becker and Liu, 2006; Lin, 2008; Friden et al., 2011). This suggests that nonspecific binding to lipophilic components in the brain is the dominant mechanism for brain tissue binding, and that presence of intact structural elements play a less significant role in determining brain binding. Furthermore, free drug concentration determined from binding studies performed in brain homogenates and brain distribution data has good correlation with direct microdialysis (Friden et al., 2007; Liu et al., 2009) and indirect CSF measurements (Maurer et al., 2005; Liu et al., 2006) of free *in vivo* brain interstitial drug concentration. The advantage of using brain homogenates is that they are readily available from vendors and can be stored frozen and thawed right before experiments, which is much easier than using brain slices. The good predictability and the ease of use makes brain homogenate binding one of the most widely used methods for determining fraction unbound in brain tissues.

Species dependence of plasma protein binding is a well known phenomenon. Compounds can bind to specific binding sites of plasma proteins and lead to different fraction unbound in different species per given drug if this specific binding is species-unique. For example, the plasma protein binding (PPB) of Zamifenacin showed marked differences among the various species (f_u : 0.0001 human, 0.0010 dog, 0.0020 rat) (Kratochwil et al., 2004). For this reason, plasma protein binding of drug candidates must be measured in multiple species in order to context total exposures determined in those species at a given dose to what may be predicted to be seen in humans and to develop PK/PD relationships. Similarly, brain tissue binding has been routinely determined in multiple species to account for any potential species dependence.

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However, brain tissue has very different composition than plasma; brain has much higher lipid contents (11% lipid and 7.9% protein) than plasma (0.65% lipid and 18% protein) (Jeffrey and Summerfield, 2007; Di et al., 2008). Similarly, binding to brain tissue is a different phenomenon than binding to plasma tissue. Lipophilicity (Log P) has been shown to be the dominant factor for brain tissue binding of compounds (Wan et al., 2007), suggesting that brain tissue binding might be less sensitive to species than plasma protein binding (non-specific binding to lipids vs. potential specific binding to proteins). Some initial studies with limited species or limited number of test compounds indicated that brain tissue binding is species independent. Wan et al. showed that rat and mouse brain fraction unbound had good correlation ($R^2 = 0.9887$, $N = 25$) (Wan et al., 2007). Summerfield et al. observed that brain fraction unbound among rat, Landrace pig and human correlated well ($R^2 > 0.9$, $N = 21$) (Summerfield et al., 2008). In a review article, Read and Braggio reported that brain fraction unbound was conserved in eight species (dog, cyno monkey, guinea pig, rat, man, marmoset, pig, and mouse) using a limited number of test compounds (7 compounds) (Read and Braggio, 2010). Linear regression was applied in all the studies for data analysis assuming the independent variable X was observed accurately without any random experimental errors. Because all the reported studies either use limited species or limited number of test compounds, the question remains whether the species independence of brain tissue binding is a general phenomenon or if it is only applicable to certain species, certain classes of compounds or to a certain nature of brain binding (e.g., tight vs. mostly free). The goal of this study was to evaluate the degree and nature of potential species differences in brain tissue binding using a large number of test compounds that cover a wide range of physicochemical properties and brain binding characteristics in multiple animal species. From these results, we were able to make conclusions as to if brain binding is species dependent or not,

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for drug-like chemical space in commonly used animal species and strains employed for neuroscience pharmacology, PK, toxicity and efficacy studies. Orthogonal regression was applied to provide more rigorous statistical analysis and this more realistically reflects the fact that both X and Y contains experimental variations. The result of this work will determine if determination of brain binding in a single representative species can replace multispecies determinations. This will be very useful to guide experimental design, increase throughput, reduce cost and to minimize animal usage as it relates to understanding the nature of free and total brain and plasma PK and PK/PD relationships.

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Material and Methods

Materials

Sprague-Dawley rat brain homogenates were purchased from Pel-Freeze Biologicals (Rogers, AR). All other brain homogenates of non-human species (Wistar Han rat, CD-1 mouse, Hartley guinea pig, Beagle dog, Cynomolgus monkey) were ordered as custom products through Bioreclamation Inc. (Hicksville, NY). Human brain tissue (occipital cortex) homogenates was obtained through Tissue Solutions Ltd. (Clydebank, UK) as a custom request. All brain tissues used in the study were mixed genders of male and female with the exception of human, which was male. Test compounds were obtained from Pfizer Global Material Management (Groton, CT) or purchased from Sigma-Aldrich (St. Louis, MO). Pfizer research compound (CP-628374)(Brighty et al., 1999) was used as an internal standard (IS) for LC-MS in both positive and negative ionization modes. Equilibrium dialysis device and cellulose membranes with molecular weight cut off 12-14K were obtained from HTDialysis, LLC. (Gales Ferry, CT). Velocity V11 peelable seals were purchased from BD Falcon (Bedford, MA). Deep 96-well plates of 1.2 and 2.2 mL blocks were from Axygen Scientific Inc. (Union City, CA) and tips of 96 blocks were obtained from Apricot Designs (Monrovia, CA).

Preparation of Brain Homogenates

The homogenates were all prepared by vendors using 1 g brain in 4 mL of Dulbecco's phosphate buffer solution (DPBS) with high speed tissue grinders. The homogenates were further processed using a glass dounce homogenizer (Thermo Scientific, Waltham, MA) to reduce the size of the brain tissues. The homogenates were frozen at -20°C before use.

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Equilibrium Dialysis for Brain Binding Study with Brain Homogenates

The dialysis membranes were prepared prior to experiments. The cellulose membranes (MWCO 12-14K) were immersed into de-ionized water for 15 minutes, followed by 15 minutes in 30% EtOH/ de-ionized water, 1 minute in de-ionized water, then at least 15 minutes or overnight in DPBS. The equilibrium dialysis device was assembled according to manufacturer's instructions (http://htdialysis.com/page/1puq4/Operating_Instructions.html). An aliquot of compound dissolved in DMSO stock solution (10 mM) was used to make 100 μ M experimental stock solutions. The experimental stock solutions were diluted 1:100 in the brain homogenate and mixed well with a 96-well pipettor from Soken SigmaPet or Apricot Design PP550 (Monrovia, CA). The final compound concentration for the equilibrium dialysis experiment was 1 μ M with 1% DMSO. A 150 μ L aliquot of brain homogenates spiked with 1 μ M compound was added to one side of the chamber (donor) and 150 μ L of DPBS was added to the other side of the dialysis membrane (receiver). Before incubation, an aliquot of 20 μ L of brain homogenates spiked with 1 μ M of compounds was added into a 96-deep well plate containing 80 μ L of DPBS and 200 μ L of cold acetonitrile (ACN) with mass spectrometry (MS) internal standard (IS, CP-628374(Brighty et al., 1999)). These samples were used for recovery calculation and stability evaluation. The equilibrium dialysis device was covered with Breathe Easy gas permeable membranes obtained from Diversified Biotech (Dedham, MA). Compounds were assessed in triplicate using three equilibrium dialysis devices for each experiment (replicates were between devices rather than within a given device). Equilibrium dialysis devices were placed on a shaking plate at 450 rpm and incubated for six hours in a humidified incubator at 37°C with 5% CO₂. The sampling procedure was designed such that the sample composition was consistent for all the samples to eliminate any potential confounding issues from varying background or

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ionization efficiency during analysis with LC-MS. At the end of the incubation, 20 μL of the brain homogenate samples from the donor wells were taken and added into a 96-deep well plate containing 80 μL of DPBS and 200 μL of cold ACN with IS (1.65 $\mu\text{g}/\text{mL}$). Aliquots of 80 μL dialyzed DPBS were taken from the receiver wells and added to 20 μL of blank brain homogenate and 200 μL of cold ACN with IS in a 96-deep well plate. The plates were sealed and mixed with a vortex mixer (VWR, Radnor, PA) for 3 min, then centrifuged at 3000 rpm and 4°C (Eppendorf, Hauppauge, NY) for 5 minutes. The supernatant was transferred to a new deep well block, sealed and subsequently analyzed using LC-MS/MS as described in the following section.

Instrumentation and Software

An API 3000 triple quadrupole mass spectrometer equipped with Turbo Ion Spray source from Applied Biosystems (Foster City, CA), two Shimadzu LC10-AD pumps (Marlborough, MA) and a Gilson 215 injector (Middleton, WI) were used for sample analysis. Analyst™ 1.4.2 (Applied Biosystems, Foster City, CA), Galileo™ (Thermo Scientific, Waltham, MA) and custom software were applied for data collection, processing and analysis (Janiszewski et al., 2001; Whalen et al., 2006). For each analyte, MRM methods were generated using DiscoveryQuant™ 2.0 software (Applied Biosystems, Foster City, CA) with 3 μM analyte solutions. Samples (25 μL) were injected at 15 sec intervals onto a DB11 1.5 x 5mm column (Optimize Technologies, Oregon City, OR) at a flow rate of 1.2 mL/min with a step gradient: trap and desalt for 0.25 min with 2 mM $\text{NH}_4\text{Ac}/\text{ACN}/\text{MeOH}$ (95/2.5/2.5) then elute from 0.25 to 0.56 minutes with 2 mM $\text{NH}_4\text{Ac}/\text{ACN}/\text{MeOH}$ (10/45/45).

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Calculation of Fraction Unbound

Fraction unbound (f_u) was calculated using equation 1 and 2 as described previously (Kalvass and Maurer, 2002). Briefly, because unbound fractions determined from diluted brain homogenates were higher than undiluted brain tissue. Therefore, corrections were made based on dilution factor (D). Masses in receiver and donor were determined from the area counts in these samples obtained from LC-MS/MS analysis corrected to account for sampling volumes. A dilution factor of five (D=5) was applied in the calculation that reflects the dilution of the brain tissue homogenates.

$$\text{Diluted } f_{u,d} = \frac{\text{Receiver Mass}}{\text{Donor Mass}} \quad \text{Eq (1)}$$

$$\text{Undiluted } f_u = \frac{1/D}{((1/f_{u,d}) - 1) + 1/D} \quad \text{Eq (2)}$$

Where $f_{u,d}$ is the diluted fraction unbound; f_u is the undiluted fraction unbound and D is the dilution factor.

Physicochemical Property Calculations

Physicochemical properties of the test compounds were calculated using various commercial and in house software. Log D and pKa were obtained through ACD/PhysChem Batch (V 9.03, Advanced Chemistry Development, Inc., Toronto, ON, Canada). TPSA was computed according to published equations (Ertl et al., 2000) using in-house software.

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Statistical Analysis

For comparison between species, log transformation was applied to approximate normal distribution to avoid skewed distribution of f_u in its original scale. The mean of the triplicates was calculated after the log transformation, which was equal to the log of the geometric mean. Because the independent variable (X) in simple linear regression is assumed to be observed without random error, orthogonal regression instead of simple linear regression was applied to account for random errors included in all species for both X and Y. The variances of these random errors were assumed to be equal. Orthogonal regression is based on the least squares as in linear regression. However, the difference between observed dependent variable Y and the fitted line is replaced by the distance from data point (X, Y) to the fitted line. Following the orthogonal regression, composite hypothesis of intercept = 0 and slope = 1 was tested. For significance level 0.05, each parameter was tested with level 0.025 using Bonferroni's adjustment. The correlation coefficient between two random variables was calculated, which is a measure of the strength of linear relationship between them. SAS 9.2 (Cary, NC) and customized programs were used for all the statistical analysis (Fuller, 1987).

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Results and Discussion

Selection of Test Compounds and Species

Forty-seven commercially-available compounds covering a wide range of physicochemical properties representing acids, bases, neutrals and zwitterions were selected for brain tissue binding studies with multiple species. The physicochemical properties of the test compounds are summarized Table 1 and the diversity of the physicochemical properties is plotted in Figure 1. The selection of the various physicochemical properties for diversity evaluation was mainly based on properties in Lipinski's rule of five (Lipinski et al., 1997). The compounds show diverse physicochemical properties with Log D ranging from -1.43 to 6.01; MW ranging from 151 to 823; and TPSA ranging from 12 to 220. An addition criteria for compound selection was brain binding characteristics, and the set has been shown to cover a range of fraction unbound spanning 3 log units from 0.0005 to 0.5. The use of the large number of diverse compounds covering various chemical space with a range of properties should allow broader conclusions to be generated that apply across various compounds for brain tissue binding. Species included in the test are those that are commonly used for neuroscience drug discovery and in those studies aimed at understanding CNS drug distribution. The species included were Wistar Han rat, SD rat, CD-1 mouse, Hartley guinea pig, beagle dog, cynomolgus monkey and human.

Comparison of Brain Fraction Unbound Generated in Multiple Species

The brain fraction unbound for the forty-seven compound set was determined in the seven species and strains using equilibrium dialysis with diluted (5X) brain tissue homogenates and the results are shown in Table 2. Concordance of the brain fraction unbound data between species,

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strain and within strain (for Wistar Han, performed to gauge inter-experimental correlation) were analyzed using rigorous statistical orthogonal regression. Orthogonal rather than linear regression was used for this analysis as both X and Y contains random experimental errors. The results showed that brain fraction unbound was highly correlated among the various species with the correlation coefficient ranging from 0.93 to 0.99. No significant species or strain differences in brain fraction unbound were observed. Importantly, the cross-species/strain correlations were not significantly different from the inter-assay correlation that was determined for Wistar Han. Following the orthogonal regression, composite hypothesis of intercept = 0 and slope = 1 was tested and the results are shown in Table 3. For significant difference than intercept = 0 and slope = 1, the p-value has to be less than 0.025. For the statistical tests between Wistar Han rat and other species, the intercepts were all very close to zero (from 0.00788 to 0.166) and the slopes were near 1 (from 0.968 to 1.05), and all the p-values for both the intercepts and the slopes were greater than 0.025, indicating no corrections were needed when extrapolating fraction unbound of other species from Wistar Han rat. The fraction unbound data for all the species are plotted in Figure 2.

The results of the brain binding studies with the 47 diverse drug compounds measured in seven species and strains suggest that there is no significant species and strain dependence in brain fraction unbound, and further, suggests that brain tissue binding is governed predominantly by nonspecific binding. The lack of species differences (or ubiquitous, species-independent nature of brain binding) could potentially be explained by the higher lipid content in brain (Jeffrey and Summerfield, 2007; Di et al., 2008), which is similar across the species tested, leading to higher nonspecific binding than in plasma, or lack of brain proteins in sufficient concentration that

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selectively bind the compounds of interest. In either case it seems reasonable to conclude that nonspecific factors govern brain tissue binding, and that the key elements that determine this non-specific binding (at least in those studies using brain homogenates to determine binding), are highly similar across mammalian species of interest including human.

Conclusions

Brain tissue binding information coupled with other ADME properties is critical for accurate assessment of dose, plasma and brain PK and exposures, and in the development of PK/PD relationships. As such, measuring this parameter remains a critical activity necessary for the study of drugs intended to be used as CNS pharmacological agents. Based on our findings for brain fraction unbound determined in multiple species for a diverse set of drug compounds, we conclude that brain tissue binding is species-independent. A determination of brain fraction unbound in a single species (e.g., Wistar Han rat) can be used as a predictor for brain tissue binding of any preclinical species and strains, as well as human. This finding greatly reduces the cost and resources needed for brain tissue binding measurements performed to help understand CNS behaviors of drugs. Additionally, these findings have great values in helping eliminate brain tissue binding as a possible cause for any observed (or predicted) differences in the behavior of CNS drugs between species.

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Authorship Contributions

Participated in research design: Li Di, John P. Umland, George Chang, Zhen Lin, Dennis O.

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Conducted experiments: John P. Umland, Zhen Lin

Performed data analysis: Li Di, John P. Umland, George Chang, Youping Huang, Zhen Lin

Wrote or contributed to the writing of the manuscript: Li Di, John P. Umland, George Chang,

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Figure Legends

Figure 1. Physicochemical Diversity of Test Compounds

Figure 2. Data of Brain Fraction Unbound of Multiple Species (N = 47). The Y and X axes are fraction unbound of each species and strains in log scale. The 1st column is using Wistar Han Rat 1 as X and the rest of the species as Y. The 1st row is using Wistar Han Rat 1 as Y and the rest of the species as X.

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Table 1. Physicochemical Properties of Test Compounds

#	Compound Name	MW	TPSA	LogD	pKa	Ionic Label
1	Amantadine	151	26.0	-0.69	10.8	Base
2	Amfebutamone	240	29.1	3.27	7.16	Base
3	Aprepitant	534	83.2	4.10	N/A	Neutral
4	Aripiprazole	448	44.8	5.54	6.71	Base
5	Azelastine	382	38.1	1.80	9.16	Base
6	Bromocriptine	655	118	5.07	6.72	Base
7	Bupivacaine	288	32.3	3.15	8.17	Base
8	Buspirone	386	69.6	3.37	6.73	Base
9	Carbamazepine	236	46.3	2.67	N/A	Neutral
10	Chlorpromazine	319	6.48	2.87	9.41	Base
11	Chlorpropamide	277	75.3	0.32	4.64	Acid
12	Citalopram	324	36.3	0.39	9.57	Base
13	Clinafloxacin	366	88.6	-0.61	6.00, 9.09	Zwitterion
14	Clomipramine	315	6.48	3.54	9.46	Base
15	Desipramine	266	15.3	1.43	10.4	Base
16	Diphenhydramine	255	12.5	2.29	8.76	Base
17	Donepezi	379	38.8	3.30	8.80	Base
18	Entacapone	305	128	0.36	5.15	Acid
19	Fluvoxamine	318	56.8	1.15	9.39	Base
20	Ibutilide	385	69.6	1.75	9.95	Base
21	Indomethacin	358	68.5	-0.16	3.96	Acid
22	Lidocaine	234	32.3	1.57	8.53	Base
23	Metoclopramide	300	67.6	0.04	9.65	Base
24	Nicardipine	480	111	4.88	7.30	Base
25	Nimodipine	418	117	3.85	N/A	Neutral
26	Nomifensine	238	29.3	1.46	7.85	Base
27	Nortriptyline	263	12.0	3.17	10.0	Base
28	Paliperidone	426	84.4	0.96	7.86	Base
29	Paroxetine	329	39.7	1.19	10.3	Base
30	Phenytoin	252	58.2	2.49	N/A	Neutral
31	Reboxetine	313	39.7	1.80	8.37	Base
32	Rifampin	823	220	-1.43	4.96, 7.30	Zwitterion
33	Riluzole	234	48.1	2.84	N/A	Neutral
34	Rimonabant	464	50.2	6.01	N/A	Neutral
35	Ritonavir	721	146	5.28	N/A	Neutral
35	Rivastigmine	250	32.8	0.93	8.62	Base
37	Sertraline	306	12.0	2.77	9.47	Base
38	Solifenacin	362	32.8	2.07	9.03	Base

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39	Talinolol	363	82.6	1.35	9.16	Base
40	Telmisartan	515	72.9	4.76	3.83	Acid
41	Terodiline	281	12	1.38	10.5	Base
42	Tolcapone	273	101	1.89	4.78	Acid
43	Trazodone	372	45.8	1.61	6.72	Base
44	Tropisetron	284	45.3	1.04	10	Base
45	UK-240455(Deur et al., 2007)	368	123	-1.27	7.96	Acid
46	Warfarin	308	67.5	0.61	4.5	Acid
47	Zonisamide	212	86.2	-0.1	N/A	Neutral

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Table 2. Comparison of Fraction Unbound (f_u) for Brain Homogenate Binding in Multiple Species

#	Compound Name	Wistar Han 1	Wistar Han 2	SD Rat	CD-1 Mouse	Hartley Guinea Pig	Beagle Dog	Cyno Monkey	Human
1	Amantadine	0.213 ±0.048	0.254 ±0.103	0.147 ±0.010	0.181 ±0.014	0.178 ±0.041	0.218 ±0.060	0.100 ±0.015	0.171 ±0.060
2	Amfebutamone	0.132 ±0.018	0.161 ±0.019	0.171 ±0.021	0.133 ±0.02	0.177 ±0.011	0.213 ±0.082	0.167 ±0.012	0.195 ±0.058
3	Aprepitant	0.001 ±0.000	0.002 ±0.000	0.001 ±0.000	0.002 ±0.000	0.001 ±0.000	0.001 ±0.000	0.002 ±0.000	0.001 ±0.000
4	Aripiprazole	0.001 ±0.000	0.001 ±0.000	0.001 ±0.000	0.001 ±0.001	0.001 ±0.000	0.001 ±0.001	0.001 ±0.000	0.002 ±0.000
5	Azelastine	0.012 ±0.003	0.019 ±0.009	0.009 ±0.003	0.012 ±0.002	0.009 ±0.002	0.011 ±0.004	0.014 ±0.006	0.013 ±0.002
6	Bromocriptine	0.002 ±0.001	0.002 ±0.000	0.001 ±0.000	0.002 ±0.000	0.002 ±0.000	0.002 ±0.000	0.002 ±0.000	0.002 ±0.001
7	Bupivacaine	0.150 ±0.029	0.280 ±0.041	0.098 ±0.018	0.274 ±0.060	0.204 ±0.023	0.211 ±0.011	0.195 ±0.003	0.232 ±0.032
8	Buspirone	0.258 ±0.121	0.269 ±0.050	0.181 ±0.020	0.227 ±0.072	0.209 ±0.038	0.265 ±0.015	0.207 ±0.002	0.263 ±0.030
9	Carbamazepine	0.185 ±0.061	0.171 ±0.034	0.124 ±0.014	0.173 ±0.026	0.128 ±0.021	0.159 ±0.012	0.185 ±0.037	0.171 ±0.021
10	Chlorpromazine	0.006 ±0.000	0.003 ±0.000	0.002 ±0.000	0.003 ±0.001	0.003 ±0.000	0.003 ±0.000	0.004 ±0.001	0.003 ±0.000
11	Chlorpropamide	0.223 ±0.063	0.411 ±0.177	0.668 ±0.000	0.441 ±0.159	0.650 ±0.310	1.827 ±2.256	0.699 ±0.272	0.583 ±0.274
12	Citalopram	0.054 ±0.011	0.050 ±0.005	0.030 ±0.003	0.060 ±0.017	0.038 ±0.013	0.057 ±0.004	0.061 ±0.030	0.047 ±0.011

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13	Clinafloxacin	0.291 ±0.056	0.148 ±0.043	0.435 ±0.012	0.377 ±0.146	0.167 ±0.090	0.398 ±0.167	0.139 ±0.010	0.157 ±0.100
14	Clomipramine	0.004 ±0.002	0.004 ±0.001	0.003 ±0.000	0.004 ±0.001	0.003 ±0.001	0.004 ±0.000	0.006 ±0.002	0.004 ±0.001
15	Desipramine	0.013 ±0.004	0.013 ±0.005	0.008 ±0.002	0.011 ±0.000	0.007 ±0.002	0.017 ±0.008	0.012 ±0.003	0.011 ±0.000
16	Diphenhydramine	0.046 ±0.031	0.027 ±0.007	0.027 ±0.004	0.054 ±0.022	0.038 ±0.001	0.040 ±0.001	0.047 ±0.010	0.042 ±0.002
17	Donepezil	0.111 ±0.042	0.114 ±0.001	0.070 ±0.008	0.100 ±0.018	0.082 ±0.009	0.134 ±0.016	0.134 ±0.058	0.113 ±0.012
18	Entacapone	0.023 ±0.018	0.021 ±0.004	0.017 ±0.009	0.026 ±0.021	0.019 ±0.004	0.020 ±0.004	0.031 ±0.010	0.025 ±0.003
19	Fluvoxamine	0.013 ±0.002	0.018 ±0.004	0.009 ±0.001	0.034 ±0.025	0.011 ±0.001	0.038 ±0.002	0.044 ±0.009	0.022 ±0.010
20	Ibutilide	0.105 ±0.019	0.104 ±0.029	0.081 ±0.008	0.114 ±0.034	0.075 ±0.008	0.119 ±0.011	0.078 ±0.005	0.091 ±0.010
21	Indomethacin	0.044 ±0.010	0.053 ±0.014	0.028 ±0.007	0.052 ±0.012	0.044 ±0.006	0.035 ±0.004	0.053 ±0.003	0.055 ±0.005
22	Lidocaine	0.292 ±0.096	0.335 ±0.053	0.148 ±0.024	0.334 ±0.100	0.339 ±0.038	0.246 ±0.032	0.239 ±0.020	0.303 ±0.034
23	Metoclopramide	0.345 ±0.091	0.224 ±0.024	0.145 ±0.045	0.230 ±0.014	0.237 ±0.056	0.294 ±0.061	0.413 ±0.199	0.295 ±0.036
24	Nicardipine	0.005 ±0.001	0.006 ±0.001	0.008 ±0.004	0.004 ±0.001	0.004 ±0.000	0.006 ±0.002	0.006 ±0.002	0.006 ±0.001
25	Nimodipine	0.010 ±0.003	0.010 ±0.001	0.009 ±0.000	0.007 ±0.002	0.007 ±0.001	0.007 ±0.000	0.009 ±0.002	0.008 ±0.001
26	Nomifensine	0.086 ±0.019	0.066 ±0.004	0.064 ±0.003	0.078 ±0.006	0.068 ±0.005	0.072 ±0.005	0.068 ±0.004	0.071 ±0.005
27	Nortriptyline	0.007 ±0.002	0.008 ±0.001	0.005 ±0.001	0.008 ±0.001	0.006 ±0.000	0.007 ±0.001	0.009 ±0.002	0.006 ±0.000
28	Paliperidone	0.123	0.092	0.078	0.129	0.101	0.126	0.100	0.116

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		±0.053	±0.011	±0.003	±0.037	±0.006	±0.010	±0.007	±0.007
29	Paroxetine	0.004 ±0.001	0.004 ±0.000	0.006 ±0.005	0.004 ±0.001	0.003 ±0.000	0.004 ±0.000	0.006 ±0.001	0.005 ±0.000
30	Phenytoin	0.128 ±0.040	0.100 ±0.010	0.134 ±0.088	0.088 ±0.012	0.082 ±0.010	0.259 ±0.060	0.114 ±0.015	0.095 ±0.011
31	Reboxetine	0.053 ±0.002	0.042 ±0.008	0.033 ±0.000	0.051 ±0.004	0.043 ±0.005	0.058 ±0.007	0.074 ±0.022	0.046 ±0.004
32	Rifampin	0.133 ±0.060	0.121 ±0.034	0.068 ±0.004	0.121 ±0.041	0.118 ±0.020	0.259 ±0.175	0.162 ±0.012	0.119 ±0.012
33	Riluzole	0.011 ±0.002	0.013 ±0.003	0.009 ±0.001	0.012 ±0.003	0.011 ±0.004	0.008 ±0.002	0.017 ±0.002	0.012 ±0.002
34	Rimonabant	0.0005 ±0.0002	0.0004 ±0.0000	0.0006± 0.0002	0.0004 ±0.0001	0.0004 ±0.0000	0.0005 ±0.0002	0.0006 ±0.0001	0.0005 ±0.0001
35	Ritonavir	0.017 ±0.007	0.017 ±0.012	0.014 ±0.002	0.020 ±0.008	0.018 ±0.002	0.020 ±0.003	0.026 ±0.011	0.021 ±0.005
36	Rivastigmine	0.547 ±0.446	0.338 ±0.002	0.241 ±0.052	0.341 ±0.044	0.389 ±0.035	0.487 ±0.055	0.316 ±0.085	0.345 ±0.108
37	Sertraline	0.001 ±0.000	0.001 ±0.000	0.001 ±0.000	0.001 ±0.000	0.001 ±0.000	0.001 ±0.000	0.001 ±0.000	0.001 ±0.000
38	Solifenacin	0.022 ±0.005	0.019 ±0.003	0.015 ±0.001	0.016 ±0.004	0.014 ±0.001	0.018 ±0.004	0.016 ±0.003	0.017 ±0.001
39	Talinolol	0.141 ±0.037	0.165 ±0.040	0.096 ±0.007	0.117 ±0.026	0.117 ±0.008	0.247 ±0.081	0.176 ±0.074	0.137 ±0.024
40	Telmisartan	0.017 ±0.003	0.013 ±0.002	0.017 ±0.003	0.010 ±0.003	0.008 ±0.002	0.011 ±0.001	0.013 ±0.002	0.014 ±0.005
41	Terodiline	0.030 ±0.006	0.025 ±0.001	0.020 ±0.003	0.049 ±0.007	0.059 ±0.004	0.148 ±0.150	0.071 ±0.012	0.028 ±0.007
42	Tolcapone	0.001 ±0.000	0.002 ±0.000	0.007 ±0.006	0.002 ±0.000	0.002 ±0.000	0.050 ±0.018	0.003 ±0.001	0.003 ±0.000
43	Trazodone	0.085 ±0.017	0.091 ±0.010	0.062 ±0.004	0.086 ±0.019	0.059 ±0.005	0.066 ±0.002	0.082 ±0.022	0.077 ±0.006

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44	Tropisetron	0.062 ±0.021	0.056 ±0.002	0.045 ±0.014	0.059 ±0.016	0.048 ±0.004	0.047 ±0.002	0.064 ±0.015	0.053 ±0.002
45	UK-240455(Deur et al., 2007)	0.266 ±0.069	0.232 ±0.034	0.150 ±0.085	0.545 ±0.192	0.198 ±0.027	0.301 ±0.000	0.230 ±0.086	0.260 ±0.037
46	Warfarin	0.183 ±0.000	0.231 ±0.034	0.199 ±0.07	0.094 ±0.022	0.326 ±0.067	0.384 ±0.127	0.318 ±0.186	0.295 ±0.015
47	Zonisamide	0.327 ±0.147	0.705 ±0.403	0.598 ±0.000	0.299 ±0.081	0.389 ±0.117	0.428 ±0.189	0.386 ±0.068	0.308 ±0.006

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Table 3. Statistical Analysis of Fraction Unbound for Various Species

X	Y	Intercept	Standard Error	P-Value	Slope	Standard Error	P-Value	Correlation Coefficient
Wistar Han 1	Wistar Han 2	0.014	0.042	0.741	0.999	0.025	0.978	0.986
Wistar Han 1	SD Rat	-0.090	0.069	0.201	1.002	0.041	0.970	0.964
Wistar Han 1	CD-1 Mouse	0.056	0.057	0.334	1.028	0.034	0.417	0.976
Wistar Han 1	Hartley Guinea Pig	0.021	0.048	0.656	1.053	0.028	0.064	0.984
Wistar Han 1	Beagle Dog	0.165	0.102	0.113	1.046	0.061	0.454	0.930
Wistar Han 1	Cyno Monkey	0.008	0.055	0.881	0.969	0.033	0.347	0.975
Wistar Han 1	Human	0.010	0.045	0.821	1.002	0.027	0.955	0.984
Wistar Han 2	Wistar Han 1	-0.014	0.042	0.740	1.001	0.025	0.978	0.986
Wistar Han 2	SD Rat	-0.104	0.057	0.084	1.002	0.035	0.950	0.973
Wistar Han 2	CD-1 Mouse	0.042	0.055	0.456	1.028	0.033	0.393	0.977
Wistar Han 2	Hartley Guinea Pig	0.006	0.039	0.873	1.054	0.023	0.026	0.989
Wistar Han 2	Beagle Dog	0.150	0.087	0.094	1.046	0.052	0.386	0.947
Wistar Han 2	Cyno Monkey	-0.005	0.046	0.919	0.970	0.028	0.277	0.982
Wistar Han 2	Human	-0.004	0.033	0.913	1.002	0.018	0.913	0.991
SD Rat	Wistar Han 1	0.090	0.072	0.221	0.999	0.041	0.970	0.964
SD Rat	Wistar Han 2	0.103	0.062	0.101	0.998	0.035	0.950	0.973
SD Rat	CD-1 Mouse	0.149	0.084	0.083	1.027	0.047	0.575	0.954
SD Rat	Hartley Guinea Pig	0.117	0.063	0.072	1.052	0.036	0.150	0.975
SD Rat	Beagle Dog	0.256	0.077	0.002	1.043	0.043	0.328	0.963
SD Rat	Cyno Monkey	0.095	0.068	0.171	0.967	0.038	0.396	0.966
SD Rat	Human	0.100	0.058	0.091	1.000	0.033	1.000	0.976
CD-1 Mouse	Wistar Han 1	-0.054	0.054	0.321	0.973	0.032	0.405	0.976
CD-1 Mouse	Wistar Han 2	-0.040	0.053	0.446	0.972	0.031	0.380	0.977
CD-1 Mouse	SD Rat	-0.145	0.076	0.062	0.974	0.045	0.565	0.954
CD-1 Mouse	Hartley Guinea Pig	-0.035	0.055	0.523	1.025	0.033	0.440	0.978

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CD-1 Mouse	Beagle Dog	0.106	0.087	0.228	1.016	0.052	0.756	0.946
CD-1 Mouse	Cyno Monkey	-0.044	0.054	0.411	0.943	0.032	0.077	0.975
CD-1 Mouse	Human	-0.044	0.053	0.410	0.975	0.031	0.422	0.977
Hartley Guinea Pig	Wistar Han 1	-0.020	0.045	0.653	0.949	0.025	0.052	0.984
Hartley Guinea Pig	Wistar Han 2	-0.006	0.037	0.872	0.949	0.021	0.020	0.989
Hartley Guinea Pig	SD Rat	-0.111	0.057	0.057	0.950	0.032	0.130	0.975
Hartley Guinea Pig	CD-1 Mouse	0.035	0.055	0.530	0.975	0.031	0.429	0.978
Hartley Guinea Pig	Beagle Dog	0.140	0.075	0.068	0.990	0.043	0.822	0.960
Hartley Guinea Pig	Cyno Monkey	-0.010	0.0343	0.769	0.921	0.019	0.0002	0.990
Hartley Guinea Pig	Human	-0.009	0.0267	0.730	0.951	0.015	0.002	0.994
Beagle Dog	Wistar Han 1	-0.158	0.090	0.085	0.956	0.056	0.434	0.930
Beagle Dog	Wistar Han 2	-0.143	0.077	0.071	0.956	0.048	0.364	0.947
Beagle Dog	SD Rat	-0.246	0.065	0.0004	0.959	0.040	0.308	0.963
Beagle Dog	CD-1 Mouse	-0.104	0.081	0.203	0.984	0.050	0.752	0.946
Beagle Dog	Hartley Guinea Pig	-0.141	0.070	0.050	1.010	0.043	0.823	0.960
Cyno Monkey	Wistar Han 1	-0.009	0.057	0.880	1.032	0.035	0.362	0.975
Cyno Monkey	Wistar Han 2	0.005	0.048	0.919	1.031	0.029	0.291	0.982
Cyno Monkey	SD Rat	-0.098	0.067	0.150	1.034	0.041	0.412	0.966
Cyno Monkey	CD-1 Mouse	0.047	0.058	0.423	1.061	0.036	0.095	0.975
Cyno Monkey	Hartley Guinea Pig	0.011	0.037	0.770	1.086	0.023	0.001	0.990
Cyno Monkey	Beagle Dog	0.155	0.073	0.041	1.078	0.045	0.089	0.962
Cyno Monkey	Human	0.001	0.033	0.981	1.033	0.020	0.108	0.991
Human	Wistar Han 1	-0.010	0.045	0.820	0.999	0.027	0.955	0.984
Human	Wistar Han 2	0.004	0.033	0.914	0.998	0.020	0.913	0.991
Human	SD Rat	-0.100	0.055	0.076	1.000	0.033	1.000	0.976
Human	CD-1 Mouse	0.045	0.056	0.421	1.026	0.033	0.434	0.977

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Human	Hartley Guinea Pig	0.010	0.028	0.731	1.051	0.017	0.004	0.994
Human	Beagle Dog	0.152	0.073	0.044	1.043	0.044	0.331	0.962
Human	Cyno Monkey	-0.001	0.032	0.981	0.968	0.019	0.097	0.991

Figure 1

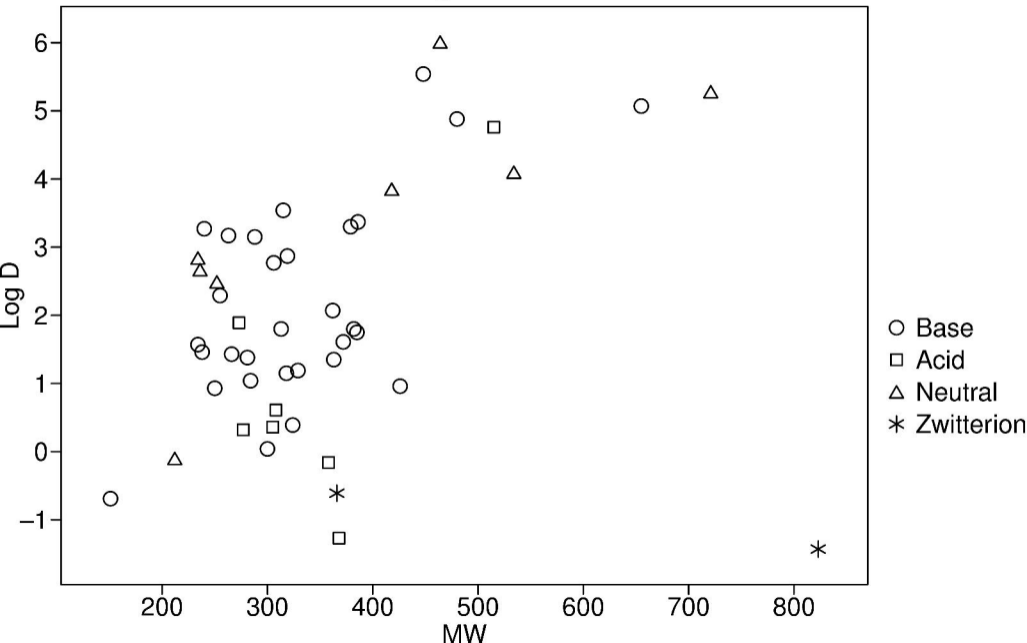


Figure 2

