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ORIGINAL ARTICLE

Applicability of free drug hypothesis to drugs with good membrane permeability that are not efflux transporter substrates: A microdialysis study in rats

Chun Chen | Hongyu Zhou | Chi Guan | Huanhuan Zhang | Yingying Li | Xue Jiang | Zheng Dong | Yuanyuan Tao | Juan Du | Shuyao Wang | Teng Zhang | Na Du | Junyang Guo | Yaqiong Wu | Zehai Song | Haofei Luan | Yu Wang | Hongwen Du | Shaofeng Zhang | Chen Li | Hang Chang | Tao Wang

Drug Metabolism and Pharmacokinetics (DMPK) Department, Pharmaron, Beijing, China

Correspondence
Tao Wang, DMPK Department, Pharmaron, Taihe Road, No 6, BDA, Beijing, China.
Email: tao.wang@pharmaron.com

Abstract

In clinical pharmacology, the free drug hypothesis has been widely applied in the interpretation of the relationship between pharmacokinetics and pharmacodynamics (PK/PD). The free drug hypothesis assumes that the unbound drug concentration in blood is the same as that in the site of action at steady state. The objective of this study is to demonstrate whether the free drug hypothesis is universally applicable for all drugs. The unbound concentrations of the 18 compounds in blood and in brain interstitial fluids (ISF) at steady state following constant intravenous infusion were simultaneously monitored up to 6 hours via in vivo microdialysis technique. Based on the permeability and efflux ratio (ER), the test compounds can be divided into two classes. Class I includes the compounds with good membrane permeability that are not substrates of efflux transporters (eg, P-gp, BCRP, and MRPs), whereas Class II includes the compounds that are substrates of efflux transporters. The steady-state unbound drug concentrations in blood, brain, and CSF are quantitatively very similar for Class I compounds, whereas the steady-state unbound concentrations in the brain and CSF are significantly lower than those in blood for Class II compounds. These results strongly suggest that the free drug hypothesis is not universal for all drugs but is only applicable for drugs with good permeability that are not substrates of efflux transporters.

KEYWORDS

BBB, efflux transporter, microdialysis, permeability, unbound concentration

Abbreviations: ACD, acid citrate dextrose; aCSF, artificial cerebrospinal fluids; ATP, adenosine triphosphate; BBB, blood-brain barrier; BCRP, breast cancer resistance protein; C_{csf}, concentration in cerebrospinal fluids; CL, clearance; $C_{m,blood/brain}$, unbound concentration in blood/brain measured by microdialysis; CNS, central nervous system; CSF, cerebrospinal fluids; C_{ss}, steady-state concentration; $C_{u,brain}$, unbound concentration in brain measured by equilibrium dialysis; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; EDTA-K_2, ethylenediaminetetraacetic acid dipotassium; ER, efflux ratio; FBS, Fetal bovine serum; $f_{u,brain}$, unbound fraction in brain; HBSS, Hank's balanced salt solution; HPLC/MS/MS, high-performance liquid chromatography combined with tandem mass spectrometry; HP-β-CD, hydroxypropyl-β-cyclodextrin; IACUC, institutional animal care and use committee; ISF, interstitial fluids; $K_{p,uu,brain}$, ratio of unbound brain concentration to unbound blood concentration; MDCK, Madin-Darby Canine Kidney; MDR1, multidrug resistance gene; MRPs, multidrug resistance protein; P_{app}, apparent permeability; PBS, phosphate-buffered saline; PD, pharmacodynamics; P-gp, p-glycoprotein; PK, pharmacokinetics; $T_{1/2}$, half-life; V_{ss}, volume of distribution at steady state.

1 | INTRODUCTION

A basic tenet of clinical pharmacology is that only unbound (free) drug molecules can interact with target receptors that are present on cell membrane or with enzymes that are located inside the cell, and therefore the intensity and duration of drug action are mediated via the time course of unbound drug concentrations at the site of action. With few exceptions, most drug target receptors or enzymes are located outside of the blood circulation in the target tissues. Although unbound drug concentrations in blood can be readily measured, direct assessment of unbound drug concentration at the action site in target tissues is seldom possible due to inaccessibility of the action sites.

For this reason, the unbound drug concentration in blood (plasma) is often used to establish pharmacokinetics-pharmacodynamics (PK-PD) relationship by applying the so-called free drug hypothesis. The hypothesis assumes that the unbound drug concentration in blood is the same as that in the site of action at steady state.[1,2] From literature, the free drug hypothesis appears valid for many drugs. The unbound concentrations of drugs in peripheral tissues and brain are quantitatively similar to those in plasma.[3-7] However, the hypothesis is not applicable for some drugs. For examples, the unbound concentrations of morphine, gabapentin, and atenolol in the brain are significantly lower than those in plasma.[8-11]

Over the years, with the progress of molecular biology, it has become evident that efflux drug transporters (such as P-gp, BCRP, and MRPs) play an important role not only in drug excretion but also in tissue distribution (drug transport), particularly for brain uptake.[12-15] For drugs that are substrates of efflux transporters, at steady state, the unbound drug concentrations in tissues are expected to be lower than unbound drug concentrations in plasma, when efflux transporters are involved in tissue distribution. Based on the involvement of efflux transporters, drugs can generally be categorized into two classes: drugs that are not substrates of efflux transporters (Class I) and drugs that are substrates of efflux transporters (Class II). It is expected that the free drug hypothesis will not be valid for drugs that are substrates of efflux transporters. Conceivably, the free drug hypothesis is also not applicable for drugs that are substrates of influx transporters. Unbound drug concentration in tissues is expected to be higher for influx transporter drugs than that in plasma.

The brain can serve as a suitable target organ to test the free drug hypothesis by comparing unbound drug concentrations in the brain and blood. As part of its protective mechanism, various drug efflux transporters are highly expressed at the BBB.[16,17] Many compounds that enter the brain are efficiently removed by efflux drug transporters. P-glycoprotein (P-gp) is the best-known efflux transporter at the BBB, but others, like breast cancer-resistance protein (BCRP) and multidrug-resistance-associated proteins (MRPs), may also contribute to the removal process. Efflux transporters use the hydrolysis of ATP to transport their substrates up against their concentration gradient.[18,19]

Although the free drug hypothesis has been explicitly used to explain the pharmacokinetics and pharmacodynamics (PK/PD) relationship for several decades, it has not been directly and systemically proven. In most studies that intended to validate the hypothesis, comparison of unbound drug concentrations in the target tissues (such as brain) and in plasma was conducted by indirect measurement of unbound concentrations. In other words, the blood and brain unbound concentrations were indirectly determined by in vitro plasma and brain binding data (unbound fractions), respectively.

For example, Summerfiel et al conducted a study to compare plasma unbound drug concentrations with brain unbound drug concentrations for more than 50 compounds.[20] In this study, the plasma unbound concentrations were calculated from the product of the plasma unbound fraction and plasma total concentration, while the brain free concentrations were calculated from the product of the brain unbound fraction and brain total concentration. Similar approaches have also been adapted by other investigators.[21,22] However, drug binding in brain homogenates may not accurately reflect the binding in intact brain in vivo because tissue homogenates do not take into account the fact that drug binding may differ between interstitial fluid, cells, and subcellular organelles. In a study by Liu et al,[22] unbound drug concentrations of nine compounds measured by microdialysis ($C_{m,brain}$) were compared to the unbound drug concentrations ($C_{u,brain}$) measured by employing unbound fraction in diluted brain homogenates. More than 2-fold differences between the $C_{m,brain}$ and $C_{u,brain}$ were observed in five of the nine test compounds (56%).

The objective of our study is to determine whether the free drug hypothesis is universally applicable to all drugs. Since microdialysis provides a reliable and direct measurement of unbound drug concentrations in the brain and blood in vivo, unbound drug concentrations in brain and blood are measured directly and simultaneously in rats by microdialysis, rather than indirectly calculated from plasma and brain binding.

2 | MATERIALS AND METHODS

2.1 | Chemicals

In all, 18 compounds with different physiochemical properties were selected for this study. Antipyrine, ganciclovir, ofloxacin, 4-aminoantipyrine, lamotrigine, theophylline, citalopram, digoxin, quinidine, pemetrexed, atenolol, carbamazepine, propranolol, and diltiazem were purchased from MedChemExpress (Monmouth Junction). Acetaminophen was purchased from Sigma-Aldrich. Fexofenadine and sumatriptan were purchased from Tokyo Chemical Industry. Cimetidine was purchased from National Institute for the Control of Pharmaceutical and Biological Products. All the other chemicals were of the HPLC grade or better.

2.2 | Animals

Male Sprague-Dawley rats (8-10 weeks, 250-350 g) were purchased from Sibeifu Biotechnology Co., Ltd. The animals were

acclimatized to the laboratory environment for at least 1 week before the study and were housed in a 12 hour light/12 hour dark cycle environment with free access to food and water. All studies were approved by Pharmaron's Institutional Animal Care and Use Committee (IACUC).

2.3 | Madin-Darby Canine Kidney and Caco-2 cell assays

Bidirectional permeability of test compounds was evaluated using Madin-Darby Canine Kidney (MDCK) II cell line transfected with or without human MDR1 (MDR1-MDCK, Netherlands Cancer Institute), and Caco-2 cell line (American Type Culture Collection). MDCK, MDR1-MDCK, and Caco-2 cells were cultured at 37°C, 5% CO_2, 95% relative humidity with cell culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% Fetal bovine serum (FBS, Invitrogen) and penicillin-streptomycin mixture (Sigma-Aldrich). Cells were incubated for several days until they reached to 80%-90% confluence and then seeded to HTS Transwell-96 Well Permeable inserts (Corning Corporation) at a cell number 5.45×10^5 cells/cm^2 for MDCK and MDR1-MDCK cell, and 2.40×10^5 cells/cm^2 for Caco-2 cell. The plate(s) was incubated for another several days before experiment and medium was replaced daily. At the day of experiment, 1 μmol/L (MDCK and MDR1-MDCK cell) or 5 μmol/L (Caco-2 cell) drug solution prepared in Hank's balanced salt solution (HBSS, Invitrogen) was added in either apical (A) or basolateral (B) compartment, and blank HBSS was added into the other compartment. For MDCK experiment, 10 uM of Cyclosporin A was added into both compartments to inhibit the intrinsic canine P-gp in MDCK cells. After 2 hours of incubation, samples from both sides were collected and stored at −75 ± 15°C until analysis.

The apparent permeability (P_{app}) was calculated as follows:

$$P_{app} = \frac{V_A}{\text{Area} \times \text{time}} \times \frac{C_{acceptor}}{C_{initial,\,donor}} \quad (1)$$

where V_A, Area, and time represent the volume in the acceptor well (0.235 mL for the well on apical side and 0.075 mL for the well on basolateral chamber), surface area of the membrane (0.143 cm^2 for HTS Transwell-96 Well Permeable Supports), and the total transport time in seconds, respectively. $C_{acceptor}$ and $C_{initial,donor}$ represent the concentrations in acceptor wells at the end of incubation and initial concentration in donor chamber, respectively.

The efflux ratio (ER) was determined with the following equation:

$$\text{Effluxratio}\ (\text{ER}) = \frac{P_{app(B-A)}}{P_{app(A-B)}} \quad (2)$$

where $P_{app(B-A)}$ and $P_{app(A-B)}$ indicate the apparent permeability coefficient in basolateral to apical direction (B–A) and apical to basolateral direction (A–B).

2.4 | Rat pharmacokinetic studies

Pharmacokinetic studies of test compounds were conducted in male Sprague-Dawley rats (n = 3). Each of the 18 compounds was administered intravenously to separate groups of animals via tail vein injection. Lamotrigine, acetaminophen, 4-aminoantipyrine, atenolol, quinidine, and fexofenadine were prepared in "10% hydroxypropyl-β-cyclodextrin in water." Digoxin, citalopram, carbamazepine, sumatriptan, and diltiazem were prepared in "DMSO/10% hydroxypropyl-β-cyclodextrin in water (v/v, 5/95)." The rest compounds were prepared in Saline. Blood samples were collected via jugular vein at 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 hours post-dose and placed into EDTA-K_2 coated tubes. Blood was then centrifuged at 4000*g* for 5 minutes to obtain plasma. Samples were stored at −75 ± 15°C before analysis.

2.5 | Rat brain and blood microdialysis studies

Unbound concentrations of the 18 compounds were measured by microdialysis method. Brain and blood microdialysis of each compound was simultaneously conducted in male Sprague-Dawley rats (n = 3-4). CMA 12 guide cannula and a dummy probe (CMA) were implanted on right striatum (coordinates: AP +0.2 mm; ML −3.2 mm; DV −7.0 mm) of animal following Paxinos and Watson's protocol.[23] The cannula and dummy probe were secured to skull with screws and dental cement. Animals were acclimated to laboratory environment for 3-4 days prior to microdialysis study. At 18 hours before dosing, animals were placed into an individual system of freely moving environment, and then blood microdialysis probe (CMA/20, 10 mm, CMA) was implanted into jugular vein while the pre-implanted dummy probe in brain was replaced by brain microdialysis probe (CMA/12, 4 mm, CMA). The two probes were perfused with blank acid citrate dextrose (ACD; 3.5 mmol/L citric acid, 7.5 mmol/L sodium citrate, 13.6 mmmol/L dextrose) and artificial cerebrospinal fluid (aCSF; 147 mmmol/L NaCl, 1.2 mmmol/L $CaCl_2$, 2.7 mmmol/L KCl) solution at a rate of 1 μL/min for 16 hours, respectively. On the day of experiment, each animal received an intravenous bolus loading dose (with the exception of acetaminophen, 4-aminoantipyrine, and ganciclovir) followed by an infusion dose via femoral vein. Dose level and formulation composition of the 18 compounds are listed in Table 1. Brain and blood microdialysis dialysates were collected at 0.5 hours intervals up to 6 hours post-dose. At 6 hours, animals were subsequently euthanized, then cerebrospinal fluids (CSF) and brain samples were collected. All samples were stored at −75 ± 15°C before analysis.

2.6 | Rat microdialysis studies at additional two different brain positions

Unbound brain concentrations of acetaminophen and antipyrine were measured by microdialysis method at additional two different

TABLE 1 Loading doses, infusion rates, formulations, and microdialysis probe recoveries used for the 18 compounds in rat microdialysis study (n = 3; mean ± SD)

Compound name	Loading dose (mg/kg)	IV Infusion dose (mg/kg/h)	Formulation	Probe recovery (blood) (%)	Probe recovery (brain) (%)
Acetaminophen	–	2.50	10%HP-β-CD	42.8 ± 4.9	15.8 ± 5.6
Antipyrine	0.50	0.333	Saline	19.3 ± 1.7	63.7 ± 2.4
4-Aminoantipyrine	–	1.67	Saline	55.3 ± 4.8	18.3 ± 1.3
Lamotrigine	1.00	0.0833	5% DMSO in '10%HP-β-CD'	79.6 ± 6.1	24.9 ± 3.9
Propranolol	5.00	5.00	5% DMSO in '10%HP-β-CD'	59.0 ± 15.0	28.4 ± 9.9
Theophylline	0.70	0.167	Saline	65.9 ± 10.7	12.3 ± 2.0
Carbamazepine	1.00	1.67	5% DMSO in '10%HP-β-CD'	75.4 ± 4.8	32.4 ± 3.4
Citalopram	10.0	3.00	5% DMSO in '10%HP-β-CD'	58.8 ± 8.4	19.1 ± 2.1
Diltiazem	5.00	10.0	5% DMSO in '10%HP-β-CD'	58.4 ± 5.8	25.5 ± 2.7
Ganciclovir	–	3.00	Saline	38.7 ± 28.9	7.70 ± 2.50
Ofloxacin	3.00	3.33	5% DMSO in '10%HP-β-CD'	48.6 ± 9.2	11.2 ± 3.5
Atenolol	6.00	4.17	5% DMSO in '10%HP-β-CD'	31.9 ± 10.0	5.20 ± 3.40
Pemetrexed	2.50	4.17	Saline	22.2 ± 5.9	14.1 ± 2.6
Quinidine	10.0	8.33	5% DMSO in '10%HP-β-CD'	68.9 ± 6.61	22.0 ± 3.0
Cimetidine	8.00	16.7	5% DMSO in '10%HP-β-CD'	43.4 ± 10.0	13.5 ± 0.7
Fexofenadine	10.0	16.7	5% DMSO in '10%HP-β-CD'	55.5 ± 9.6	12.9 ± 3.5
Digoxin	3.00	4.33	5% DMSO in '10%HP-β-CD'	37.7 ± 3.8	31.6 ± 12.9
Sumatriptan	5.00	5.83	5% DMSO in '10%HP-β-CD'	40.9 ± 6.6	6.13 ± 1.56

brain positions of male Sprague-Dawley rats (n = 3). Brain microdialysis probes (CMA/12, 4 mm, CMA) were simultaneously implanted into frontal cortex (AP +3.2 mm, ML +0.8 mm, DV −5.0 mm) and hypothalamus (AP −1.8 mm, ML +0.4 mm, DV −9.2 mm) following the procedure in previous microdialysis study described above. Dose and formulation remained same as they were in the previous microdialysis study. Brain microdialysis dialysates were collected at 0.5 hours intervals up to 6 hours post-dose. All samples were stored at −75 ± 15°C before analysis.

2.7 | In vivo recovery studies

The recovery of compounds in brain and blood microdialysis probes was determined by an in vivo retrodialysis method. Brain and blood probes were implanted into animals following the procedure in previous microdialysis study.[24] 300 ng/mL of test compounds (250 ng/mL for acetaminophen) in ACD and aCSF were constantly perfused into blood and brain probes for 5 hours, and dialysates were collected at 0.5 hours intervals from 2 to 5 hours Recovery can be calculated by following equation:

$$\text{Recovery}\,(\%) = \frac{(C_{in} - C_{out})}{C_{in}} \times 100\% \quad (3)$$

where C_{in} is the concentration in perfusates while C_{out} is the average concentration of dialysates collected from 2 to 5 hours Samples were stored at −75 ± 15°C before analysis. The recovery data of the microdialysis probes is listed in Table 1.

2.8 | Sample analysis

All samples were analyzed on HPLC/MS/MS systems which consist of Shimadzu LC-30AD pumps (Shimadzu), a rack changer II autosampler (Shimadzu) and either a Shimadzu 8060 (Shimadzu) or an AB Sciex API 4000/5500 (AB Sciex) mass spectrometer. 20 μL of plasma samples was mixed with 200 μL of acetonitrile containing internal standards and centrifuged at 4000*g* for 15 minutes (4°C). The mixture was then thoroughly mixed with 200 μL of acetonitrile containing internal standard and centrifuged at 4000*g* for 15 minutes (4°C). 10 μL of dialysates and CSF samples were mixed with 100 μL of acetonitrile containing internal standard. Supernatants were diluted with appropriate volumes of water before analysis on HPLC/MS/MS.

2.9 | Data analysis

Pharmacokinetic parameters were calculated with WinNolin 8.0 (Pharsight Corporation) by employing a non-compartmental analysis. Graphs and statistical analysis were performed in Prism 7.0 (Graphpad).

ANOVA was used to determine the statistical difference of steady-state unbound concentrations in blood, brain, and CSF. The

statistical difference of the steady-state unbound brain concentrations measured by microdialysis at three different positions was also analyzed by ANOVA. A correlation analysis was performed to evaluate the relationship of the steady-state unbound concentration between brain and blood; and between brain and CSF.

3 | RESULTS

In all, 18 structurally diverse compounds with different physicochemical properties were selected for the rat microdialysis study. Pharmacokinetic studies were conducted to obtain kinetic parameters for calculating loading dose and infusion rate for microdialysis studies for the 18 compounds. Following a single intravenous injection, the kinetic parameters, clearance (CL), volume of distribution at steady state (V_{ss}), and half-life ($T_{1/2}$) in rats were determined as shown in Table 2. Kinetic parameters varied dramatically among the 18 compounds. The plasma CL ranged from 1.48 mL/min/kg for lamotrigine to 97.7 mL/min/kg for diltiazem, while the $T_{1/2}$ varied from 0.823 hours for diltiazem to 15.6 hours for lamotrigine.

For many of the 18 compounds, the pharmacokinetic parameters were in alignment with published data. The mean values of plasma CL were 97.7, 95.9, and 82.8 mL/min/kg, respectively, for diltiazem, citalopram, and propranolol in rats in this study (Table 2), while the reported mean values of rat plasma CL were 90.3, 82.0, and 68 mL/min/kg for the corresponding compounds.[25-27] On the other hand, the mean values of plasma CL were 1.48 and 1.58 mL/min/kg, respectively, for lamotrigine and theophylline in rats in this study (Table 2), while the reported mean values for the corresponding compounds were 0.83 and 2.39 mL/min/kg.[28,29]

Kinetically, approximately five half-lives ($T_{1/2}$) are needed to achieve the target steady-state concentration (C_{ss}) following a constant rate of intravenous infusion. One can use a loading dose to quickly achieve the desired steady-state drug concentration. The loading dose and infusion rate of each drug can be calculated by the following Equations (4) and (5), respectively, using the kinetic parameters and the pre-determined desired C_{ss}.

$$\text{Loading dose} = V_{ss} \times C_{ss} \tag{4}$$

$$\text{Infusion rate} = \text{CL} \times C_{ss} \tag{5}$$

Loading doses and infusion rates for the test compounds are listed in Table 1.

The parental MDCK cells without transfection with efflux transporters were used to measure intrinsic membrane permeability of the 18 compounds, while MDCK cells transfected with human P-gp (MDR1-MDCK) and Caco-2 cell assays were conducted to characterize substrates of efflux transporters. Apical-to-basolateral permeability [$P_{app(A-B)}$], basolateral-to-apical permeability [$P_{app(B-A)}$], and efflux ratios (ER; $P_{app(B-A)}/P_{app(A-B)}$) of the test compounds assessed by MDR1- MDCK monolayers are listed in Table 3. In addition, the $P_{app(B-A)}$ and $P_{app(A-B)}$ and ER values of the test compounds measured by Caco-2 monolayers are presented in Table 4.

The cell membrane permeability ($P_{app(B-A)}$ and $P_{app(A-B)}$) of the 18 compounds measured by the parent MDCK cells is listed in Table 3. Acetaminophen, antipyrine, 4-aminoantipyrine, lamotrigine, propranolol, theophylline, carbamazepine, citalopram, diltiazem, and quinidine showed reasonably good permeability ranging from about 11.0 × 10^{-6} cm/sec (propranolol) to 35.5 × 10^{-6} cm/sec

TABLE 2 Pharmacokinetic parameters of the 18 compounds following an IV single bolus (n = 3; mean ± SD)

Compound name	Dose (mg/kg)	CL (mL/min/kg)	V_{ss} (L/kg)	$T_{1/2}$ (h)
Acetaminophen	5.00	30.5 ± 3.74	2.87 ± 1.44	4.96 ± 2.31
Antipyrine	5.00	4.81 ± 0.77	0.724 ± 0.051	1.99 ± 0.43
4-Aminoantipyrine	5.00	17.7 ± 0.8	0.772 ± 0.018	0.883 ± 0.025
Lamotrigine	5.00	1.48 ± 0.21	1.86 ± 0.18	15.6 ± 3.0
Propranolol	4.38	82.8 ± 12.5	4.64 ± 0.70	0.943 ± 0.062
Theophylline	5.00	1.58 ± 0.52	0.453 ± 0.048	3.42 ± 1.02
Carbamazepine	5.00	18.1 ± 2.9	0.745 ± 0.081	0.993 ± 0.115
Citalopram	2.40	95.9 ± 11.2	6.45 ± 0.64	1.02 ± 0.05
Diltiazem	6.80	97.7 ± 18.8	2.85 ± 0.30	0.823 ± 0.325
Ganciclovir	5.00	21.5 ± 1.4	0.852 ± 0.159	1.35 ± 0.04
Ofloxacin	5.00	32.5 ± 4.9	5.08 ± 2.99	6.32 ± 3.34
Atenolol	5.00	24.5 ± 3.7	2.88 ± 0.44	2.76 ± 0.77
Pemetrexed	10.0	14.0 ± 1.8	0.637 ± 0.272	4.97 ± 1.15
Quinidine	5.00	76.4 ± 3.5	3.80 ± 0.31	1.01 ± 0.03
Cimetidine	10.0	53.7 ± 4.8	3.30 ± 1.93	5.91 ± 4.71
Fexofenadine	5.00	50.9 ± 6.1	1.56 ± 0.38	1.12 ± 0.09
Digoxin	5.00	9.10 ± 1.30	0.376 ± 0.013	1.30 ± 0.26
Sumatriptan	6.70	59.1 ± 5.8	3.11 ± 0.03	0.980 ± 0.011

TABLE 3 Permeability and transport data for 18 compounds in MDR1-MDCK and MDCK cell line (n = 4)

	MDCK cell line			MDR1-MDCK cell line		
Compound Name	$P_{app(A-B)}$ (10^{-6} cm/s)	$P_{app(B-A)}$ (10^{-6} cm/s)	Average P_{app} (10^{-6} cm/s)	$P_{app(A-B)}$ (10^{-6} cm/s)	$P_{app(B-A)}$ (10^{-6} cm/s)	Efflux ratio
Acetaminophen	16.7 ± 0.6	14.2 ± 0.9	15.5 ± 1.5	18.5 ± 4.6	14.3 ± 0.9	0.772
Antipyrine	36.8 ± 2.6	25.3 ± 2.2	31.1 ± 6.5	38.2 ± 2.1	25.8 ± 1.9	0.677
4-Aminoantipyrine	43.2 ± 3.7	27.9 ± 1.1	35.5 ± 8.5	39.1 ± 3.4	25.9 ± 1.7	0.662
Lamotrigine	33.1 ± 1.1	21.2 ± 1.0	27.2 ± 6.4	29.4 ± 3.6	20.4 ± 2.5	0.695
Propranolol	13.7 ± 5.3	8.37 ± 3.8	11.0 ± 5.2	18.9 ± 2.6	16.8 ± 0.5	0.889
Theophylline	22.5 ± 2.5	16.5 ± 2.2	19.5 ± 3.8	19.5 ± 1.2	17.4 ± 1.3	0.889
Carbamazepine	34.2 ± 2.6	20.8 ± 2.3	27.5 ± 7.5	34.3 ± 1.2	22.8 ± 0.5	0.665
Citalopram	19.0 ± 3.1	10.9 ± 2.6	15.0 ± 5.1	13.8 ± 3.2	23.7 ± 0.7	1.72
Diltiazem	23.2 ± 2.6	13.6 ± 1.3	18.4 ± 5.5	12.5 ± 3.1	18.7 ± 1.9	1.49
Ganciclovir	0.239 ± 0.372	2.54 ± 2.08	1.39 ± 1.85	0.808 ± 0.162	1.37 ± 0.29	1.69
Ofloxacin	4.87 ± 0.33	4.22 ± 0.12	4.54 ± 0.42	3.79 ± 0.44	4.28 ± 1.02	1.13
Atenolol	0.843 ± 0.670	1.90 ± 0.43	1.37 ± 0.77	2.50 ± 2.33	1.04 ± 1.06	0.416
Pemetrexed	0.452 ± 0.092	0.793 ± 0.148	0.623 ± 0.215	0.709 ± 0.236	0.442 ± 0.252	0.623
Quinidine	24.1 ± 2.7	14.0 ± 0.6	19.1 ± 5.7	8.04 ± 0.12	50.2 ± 0.9	6.24
Cimetidine	0.963 ± 0.260	2.37 ± 0.16	1.67 ± 0.78	1.91 ± 0.93	3.22 ± 0.389	1.68
Fexofenadine	0.809 ± 0.377	1.78 ± 1.15	1.29 ± 0.95	0.300 ± 0.144	0.868 ± 0.247	2.89
Digoxin	2.22 ± 0.34	2.73 ± 0.45	2.48 ± 0.46	0.653 ± 0.098	11.2 ± 2.5	17.1
Sumatriptan	1.07 ± 0.08	2.21 ± 0.37	1.64 ± 0.66	0.683 ± 0.040	1.54 ± 0.59	2.25

(4-aminoantipyrine), while ganciclovir, ofloxacin, atenolol, pemetrexed, cimetidine, fexofenadine, digoxin, and sumatriptan exhibited relatively poor permeability, ranging from 0.623 × 10^{-6} cm/sec (pemetrexed) to 4.54 × 10^{-6} cm/sec (ofloxacin).

According to FDA guidelines, if a net ER of a test compound is ≥2.0 in cells that express P-gp, the compound is classified as a P-gp substrate.30 Therefore, ER of 2.0 is used as a cut-off for classification of efflux transporter substrates. The ER values were less than 2.0 in both MDR1-MGCK and Caco-2 assays for acetaminophen, antipyrine, 4-aminoantipyrine, lamotrigine, propranolol, theophylline, carbamazepine, citalopram, and diltiazem (Tables 3 and 4). These results suggest that these compounds are not substrates of efflux transporters.

In contrast, the ER values were 6.24, 2.89, 17.1, and 2.25, respectively, for quinidine, fexofenadine, digoxin, and sumatriptan when measured by MDR1-MDCK cell assay (Table 3). These results suggest that quinidine, fexofenadine, digoxin, and sumatriptan are P-gp substrates. Consistent with MDR1-MDCK cells, the results of Caco-2 cell assay also suggest that quinidine, fexofenadine, digoxin, and sumatriptan are P-gp substrates. The corresponding ER values were 5.91, 21.6, 48.2, and 2.12 when assessed by Caco-2 cell assay (Table 4). It is of interest to note that the ER values of fexofenadine and digoxin measured by Caco-2 cell assay were much greater than those by MDR1-MDCK cell assay. It is well known that Caco-2 cells express many major efflux transporters, including P-gp, BCRP, and MRPs.[31-33] These results suggest that fexofenadine and digoxin are not only substrates of P-gp but also substrates of other efflux transporters, possibly BCRP and/or MRPs.

Digoxin is a well-known substrate of rat and human P-gps and is often used as a probe in in vitro and in vivo drug-drug interaction involving P-gp.[34,35] In this study, the ER values of digoxin were estimated to be 17.1 and 48.2, respectively, when measured by MDR1-MDCK and Caco-2 assays (Tables 3 and 4). The ER measured in Caco-2 cell assay was markedly greater than in MDR1-MDCK cell assay. These results suggest that digoxin efflux involves not only P-gp but also other efflux transporters, since Caco-2 cells express many efflux transporters. Similar observations were reported by Wang et al[36] The ER value of digoxin was markedly higher in Caco-2 cell assay (ER = 120) than in MDR1-MDCK cell assay (ER = 17).

Similarly, fexofenadine is also known to be a P-gp substrate.[37] In this study, the ER values of fexofenadine were estimated to be 2.89 and 21.6, respectively, for MDR1-MDCK and Caco-2 cell assays (Tables 3 and 4). These results suggest that fexofenadine also involves other efflux transporters in addition to P-gp. The notion that fexofenadine efflux involves multiple efflux transporters is supported by other investigators. A study was conducted to compare the biliary excretion of fexofenadine in wild-type and Mrp2-knockout mice.[38] The authors concluded that more than 50% of biliary excretion of fexofenadine was attributed to Mrp2, with P-gp and Bcrp playing a minor role in mice.

Quinidine is also known to be P-gp substrates.[39] In this study, the ER values of quinidine were estimated to be 6.24 and 9.2, respectively, for MDR1-MDCK and Caco-2 cell assays (Tables 3 and 4). These results suggest that quinidine efflux may only involve P-gp.

TABLE 4 Permeability and transport data for 18 compounds in Caco-2 cell line (n = 4)

Compound name	Caco-2 cell line		
	$P_{app(A-B)}$ (10^{-6} cm/s)	$P_{app(B-A)}$ (10^{-6} cm/s)	Efflux ratio
Acetaminophen	19.6 ± 2.1	19.6 ± 1.9	1.00
Antipyrine	36.2 ± 1.2	25.1 ± 0.6	0.693
4-Aminoantipyrine	36.1 ± 2.5	25.8 ± 1.5	0.713
Lamotrigine	27.2 ± 1.9	20.0 ± 0.7	0.737
Propranolol	21.2 ± 3.6	13.8 ± 0.5	0.654
Theophylline	18.4 ± 0.9	27.1 ± 0.4	1.47
Carbamazepine	33.1 ± 2.3	22.0 ± 1.0	0.662
Citalopram	16.0 ± 1.8	17.4 ± 1.4	1.09
Diltiazem	21.7 ± 2.3	20.1 ± 2.3	0.926
Ganciclovir	0.182 ± 0.217	0.474 ± 0.341	2.61
Ofloxacin	4.12 ± 0.49	12.3 ± 0.7	2.99
Atenolol	0.398 ± 0.114	0.595 ± 0.021	1.49
Pemetrexed	0.392 ± 0.207	0.707 ± 0.443	1.81
Quinidine	8.26 ± 3.04	48.8 ± 12.4	5.91
Cimetidine	0.706 ± 0.204	6.49 ± 1.37	9.20
Fexofenadine	0.177 ± 0.055	3.83 ± 1.55	21.6
Digoxin	0.477 ± 0.088	23.0 ± 1.6	48.2
Sumatriptan	0.904 ± 0.283	1.91 ± 0.60	2.12

It is interesting to note that quinidine poorly penetrated the brain even with a good permeability (19.1 × 10^{-6} cm/sec; Table 3). Good permeability of quinidine has also been reported by other investigators. In a study with MDCK-MDR1-NKI cell lines, the permeability was about 60 × 10^{-6} cm/sec for quinidine.[40] In another study, the permeability was about 14 × 10^{-6} cm/sec for quinidine, when Caco-2 cells were used.[41]

It is interesting to note that quinidine has a reasonably good permeability (19.1 × 10^{-6} cm/sec), even though it is a good P-gp substrate (Table 3). There are many good P-gp substrates that show good permeability. For example, indinavir and neflinivir are excellent P-gp substrates with very good permeability. The ER values were 25 and 22, respectively, for indinavir and neflinivir, respectively, while the corresponding values of permeability were 85 × 10^{-6} cm/sec and 197 × 10^{-6} cm/sec.[42]

The ER values of sumatriptan were 2.25 and 2.12, respectively, for MDR1-MDCK and Caco-2 cell assays (Tables 3 and 4). These results suggest that sumatriptan is a P-gp substrate. The notion that sumatriptan is a P-gp substrate is supported by a rat study conducted by Summerfield et al[20] In their rat study, the ER value of sumatriptan obtained from MDR1-MDCK assay was reported to be 2.9, and the steady-state unbound concentrations in the brain was about 20-fold lower than that in plasma.

Although the ER values of ganciclovir, ofloxacin, and cimetidine were less than 2.0 in MDR1-MDCK cell assay, the ER values were greater than 2.0 in Caco-2 assay. The ER was 1.69, 1.13, and 1.68, respectively, for ganciclovir, ofloxacin, and cimetidine in MDR1-MDCK cell assay, while the corresponding ER values were 2.61, 2.99, and 9.2 in Caco-2 assay (Tables 3 and 4). These results suggest that ganciclovir, ofloxacin, and cimetidine are substrates for other efflux transporters (such as BCRP and MRPs), but not P-gp.

In this study, treatment of Caco-2 cells with novobiocin resulted in a marked decrease in the ER of cimetidine, from 9.20 to 3.29 (in supplemental data). Novobiocin is known to be potent BCRP inhibitor.[43] These results suggest that cimetidine efflux may involve efflux transporter BCRP. The notion that ofloxacin is not a P-gp substrate is supported by other investigators. In an in vitro study, neither P-gp nor BCRP affected the efflux transport of ofloxacin. In contrast, it revealed pronounced MRPs involvement in the transfer of ofloxacin.[44] The ER values of ganciclovir were less than 2.0 in MDR1-MDCK assay, and equal to 2.61 in Caco-2 assay (Tables 3 and 4). These results suggest that the compound is a substrate of efflux transporters, but not P-gp. In vitro studies revealed that ganciclovir is a substrate of MRP4.[45,46]

Although the ER values of atenolol and pemetrexed were less than 2.0 in both MDR1-MDCK and Caco-2 cell assays, these two compounds showed poor membrane permeability, suggesting that efflux transporters may be involved in their membrane transport.

Based on the membrane permeability and ER value that were measured by parental MDCK, MDR1-MDCK, and Caco-2 cell lines, the test compounds can be divided into two classes. Class I includes nine compounds (acetaminophen, antipyrine, 4-aminoantipyrine, lamotrigine, propranolol, theophylline, carbamazepine, citalopram, and diltiazem) with good permeability that are not substrates of efflux transporters (eg, P-gp, BCRP, MRPs, etc), while Class II compounds are ganciclovir, ofloxacin, atenolol, pemetrexed, quinidine, cimetidine, fexofenadine, digoxin, and sumatriptan that are substrates of efflux transporters.

The unbound concentrations of the 18 compounds in blood and in brain interstitial fluids (ISF) following constant intravenous infusion were simultaneously monitored via in vivo microdialysis technique. The unbound concentration-time profiles of Class I compounds in the brain and blood are presented in Figure 1, while unbound concentration-time profiles of Class II compounds are shown in Figure 2. As shown in Figures 1 and 2, the unbound drug concentrations of all compounds in blood and brain remained relatively constant from about 1-2 hours up to 6 hours after the commencement of infusion. These results suggest that a steady state was reached rapidly for all Class I and Class II compounds.

As shown in Table 5, the steady-state unbound drug concentrations in blood ($C_{m,blood}$) were quantitatively similar to that in the brain ($C_{m,brain}$) for the Class I compounds. In contrast, the steady-state unbound drug concentrations in the brain were statistically much lower than that in blood for Class II compounds (Table 6). The differences in steady-state unbound drug concentrations between the brain and blood ranged from 3.9-fold for quinidine to 86-fold for pemetrexed, respectively.

For comparison purposes, steady-state unbound drug concentrations in cerebrospinal fluids (C_{csf}) were measured. Rats were

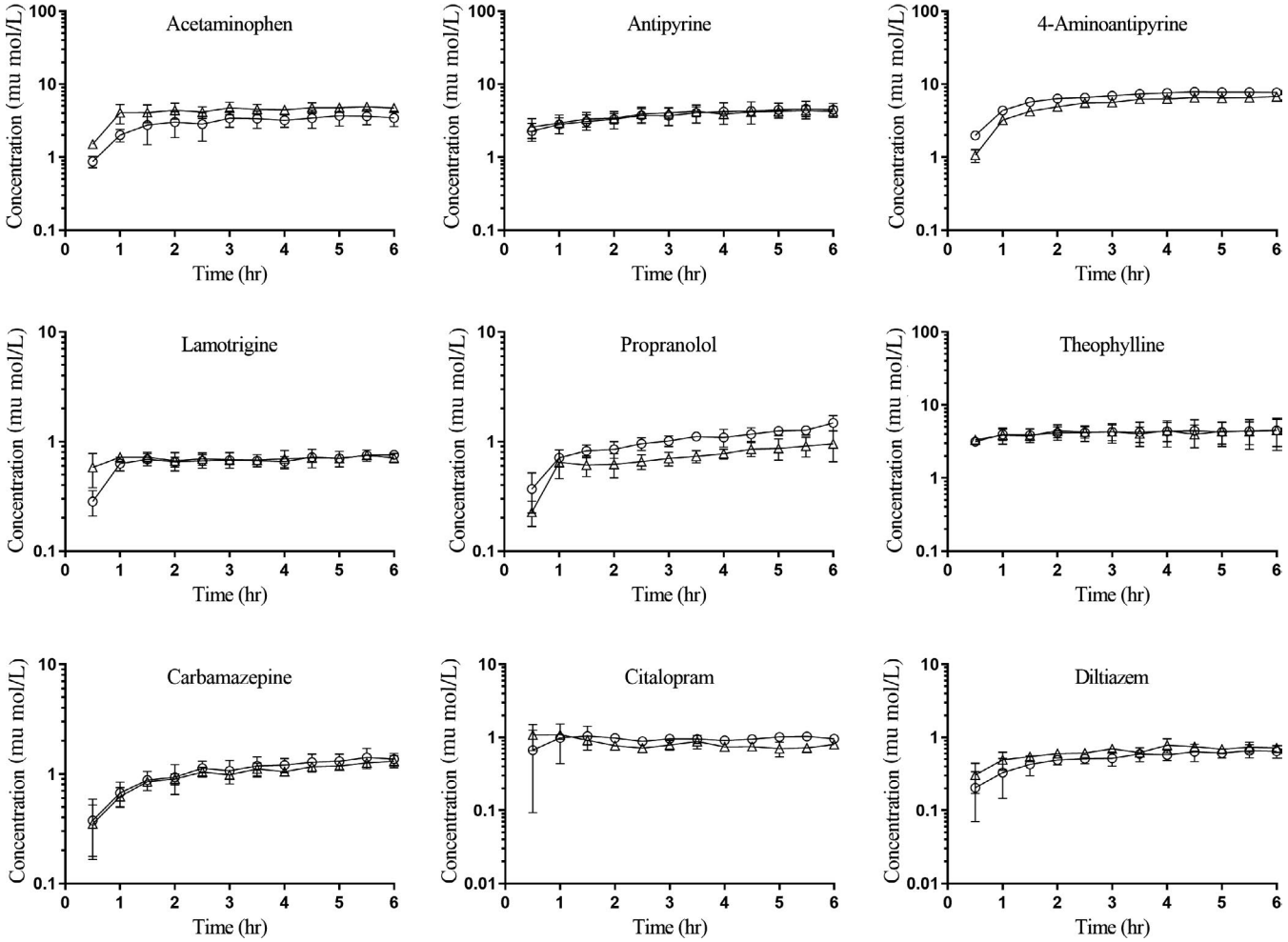

FIGURE 1 Rat unbound drug concentration time profiles of class I compounds in blood (open triangles) and brain (open circles) with good membrane permeability that are not substrates of efflux transporters (mean ± SD, n = 3-4). The unbound drug concentration was simultaneously measured by microdialysis in blood and brain

sacrificed at the end of constant infusion (6 hours), and CSF samples were collected. The unbound drug concentrations in CSF (C_{csf}) were quantitatively similar to the steady-state unbound drug concentrations measured by microdialysis ($C_{m,brain}$) for most compounds of Class I, with the exception of 4-aminoantipyrine, lamotrigine, and carbamazepine (Table 5). The C_{csf} of 4-aminoantipyrine was somewhat lower than $C_{m,brain}$, while the C_{csf} values were slightly higher than the $C_{m,brain}$ for lamotrigine and carbamazepine (Table 5). For Class II compounds, the C_{csf} values were generally lower than $C_{m,brain}$ with the exception of ofloxacin, pemetrexed, and digoxin (Table 6).

As shown in Figure 3, there is a good correlation between the steady-state $C_{m,brain}$ and $C_{m,blood}$ for Class I compounds with a correlation coefficient (R^2) of .937. Similarly, a good correlation between the steady-state $C_{m,brain}$ and C_{csf} of Class I compounds was observed with a correlation coefficient of 0.862. These results suggest that there is a fairly strong relationship between the steady-state $C_{m,brain}$ and $C_{m,blood}$ as well as between the steady-state $C_{m,brain}$ and C_{csf} for compounds with good permeability that are not substrates of efflux transporters.

In contrast, the correlation between the steady-state $C_{m,brain}$ and C_{csf} was poor for Class II compounds. The correlation coefficient was only 0.147 (Figure 3). However, it is a bit surprising to see that there was a reasonably good correlation between the steady-state $C_{m,brain}$ and $C_{m,blood}$ for Class II compounds with a correlation coefficient of 0.803 (Figure 3). It should be noted that the steady-state $C_{m,brain}$ were much lower than the steady-state $C_{m,blood}$ for Class II compounds by a factor of 4-86 fold. Therefore, the "reasonably good" correlation between the steady-state $C_{m,brain}$ and $C_{m,blood}$ for Class II compounds is statistically less meaningful.

A study was conducted to ensure that unbound drug concentrations are evenly distributed within the brain. For this purpose, two compounds, acetaminophen and antipyrine, were selected from Class I compounds. Steady-state unbound drug concentrations of each compound were measured at three different sites of brain: right striatum, left frontal cortex, and left hypothalamus by microdialysis in two separate groups of rats (n = 3). Microdialysis in striatum was conducted in one group of rats, whereas microdialysis at cortex and hypothalamus was conducted in another group of animals. Following constant intravenous infusion, the unbound drug concentrations

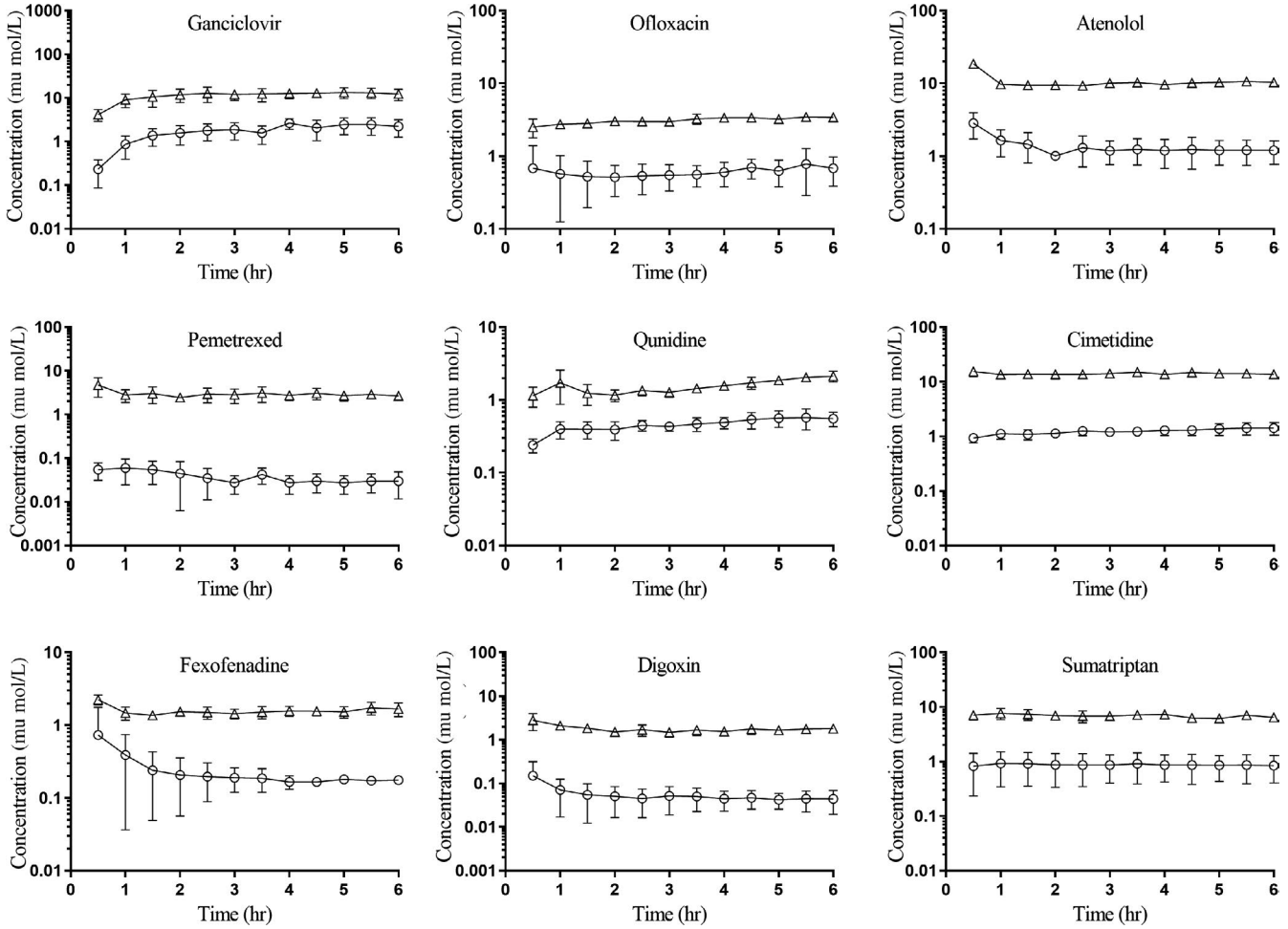

FIGURE 2 Rat unbound drug concentration time profiles of class II compounds in blood (open triangles) and brain (open circles) that are substrates of efflux transporters (mean ± SD, n = 3-4). The unbound drug concentration was simultaneously measured by microdialysis in blood and brain

TABLE 5 Steady-state unbound drug concentrations in blood, brain, and CSF of class I compounds with good membrane permeability that are not efflux transporter substrates (n = 3-4; mean ± SD)

Compound name	$C_{m,blood}$ (μmol/L)	$C_{m,brain}$ (μmol/L)	C_{csf} (μmol/L)	Efflux transporter substrate
Acetaminophen	4.72 ± 0.46	3.45 ± 0.82	3.94 ± 0.58	No
Antipyrine	4.24 ± 0.74	4.48 ± 0.95	4.98 ± 0.47	No
4-Aminoantipyrine*	6.73 ± 0.32	7.71 ± 0.67	5.82 ± 0.49	No
Lamotrigine*	0.708 ± 0.070	0.757 ± 0.042	1.10 ± 0.09	No
Propranolol	0.954 ± 0.295	1.49 ± 0.24	1.10 ± 0.38	No
Theophylline	4.54 ± 1.82	4.52 ± 2.11	2.71 ± 1.15	No
Carbamazepine*	1.31 ± 0.17	1.37 ± 0.17	1.76 ± 0.16	No
Citalopram	0.804 ± 0.066	0.954 ± 0.091	0.841 ± 0.034	No
Diltiazem	0.722 ± 0.051	0.649 ± 0.127	0.503 ± 0.187	No

*P < .05 (ANOVA), ANOVA test was performed for comparison of $C_{m,blood}$, $C_{m,brain}$, and C_{csf}.

of acetaminophen and antipyrine at the three different sites of the brain remained relatively constant from about 1-2 hours up to 6 hours after the commencement of infusion (data not shown). As shown in Table 7, the steady-state unbound concentrations of acetaminophen and antipyrine were quantitatively similar among the three different sites of the rat brain. An ANOVA test suggests that there was no statistical difference in the steady-state unbound drug concentrations among the three sites. These results strongly suggest that unbound drug molecules are evenly distributed within the brain. Similar observations have been reported by other investigators.[47]

TABLE 6 Steady-state unbound drug concentrations in blood, brain, and CSF of class II compounds that are efflux transporter substrates (n = 3-4; mean ± SD)

Compound name	$C_{m,blood}$ (μmol/L)	$C_{m,brain}$ (μmol/L)	C_{csf} (μmol/L)	Efflux transporter substrate
Ganciclovir*	12.2 ± 3.6	2.23 ± 0.96	0.384 ± 0.056	Yes
Ofloxacin*	3.41 ± 0.25	0.678 ± 0.293	1.06[a]	Yes
Atenolol*	10.4 ± 0.6	1.20 ± 0.43	0.410 ± 0.059	Yes (?)
Pemetrexed*	2.68 ± 0.53	0.0313 ± 0.0193	0.0657 ± 0.0113	Yes (?)
Quinidine*	2.13 ± 0.36	0.552 ± 0.126	0.493 ± 0.191	Yes
Cimetidine*	13.7 ± 0.9	1.42 ± 0.37	0.627 ± 0.094	Yes
Fexofenadine*	1.67 ± 0.36	0.174 ± 0.021	0.0401 ± 0.0064	Yes
Digoxin*	1.81 ± 0.35	0.0443 ± 0.0246	0.119 ± 0.028	Yes
Sumatriptan*	6.49 ± 1.00	0.845 ± 0.439	0.555 ± 0.070	Yes

[a]n = 2, one CSF sample was contaminated with blood and excluded from the mean calculation.
*P < .05 (ANOVA), ANOVA test was performed for comparison of $C_{m,blood}$, $C_{m,brain}$, and C_{csf}.

The authors have shown that the unbound drug concentrations of antipyrine, midazolam, and lamotrigine at the cortex were quantitatively similar to those at the hippocampus in rats.

4 | DISCUSSION

Ideally, rat Mdr1a/1b-MDCK, Mrp-MDCK, and Bcrp-MDCK-transfected cells should be used to characterize the substrate specificity of rat efflux transporters for the 18 compounds used in this rat microdialysis study. Due to unavailability of rat Mdr1a/1b-MDCK, Mrp-MDCK, and Bcrp-MDCK cell lines, human MDR1-MDCK and Caco-2 cells were used in the identification of substrates of efflux transporters. Although species differences in transport activity of efflux transporters have been reported, it is generally accepted that substrate specificity assessed by human efflux transporters closely resembles rat efflux transporters.

In a recent study by Jain et al,[48] the amino acid sequence of P-glycoprotein was highly conserved among mice, rats, and humans (> 90%), suggesting a high structural similarity. Comparison of the binding site interaction profiles of human, rat, and mouse P-gp derived from docking studies with a set of common inhibitors

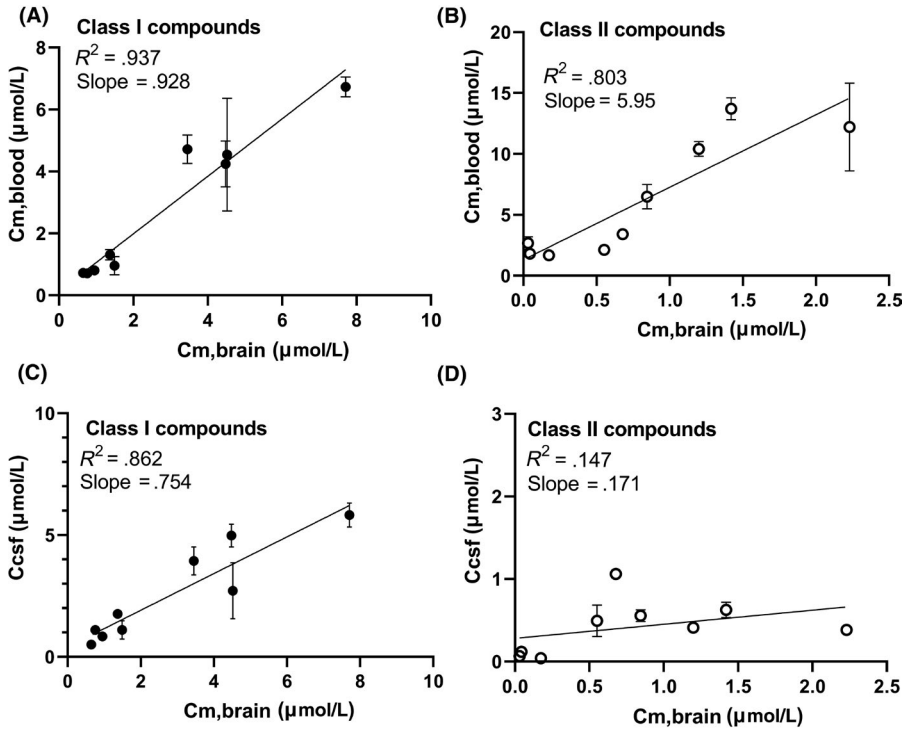

FIGURE 3 The correlation between unbound brain concentration ($C_{m,brain}$) and unbound blood concentration ($C_{m,blood}$, A and B), and unbound CSF concentration (C_{csf}, C and D) at steady state in rats after 6 hours infusion (mean ± SD, n = 3-4)

TABLE 7 Steady-state unbound drug concentrations at three different brain positions with microdialysis method. (n = 3; mean ± SD)

	$C_{m,brain}$ (μmol/L)		
Compound name	**Striatum**	**Frontal cortex**	**Hypothalamus**
Acetaminophen	3.45 ± 0.82	4.79 ± 0.34	4.62 ± 0.82
Antipyrine	4.48 ± 0.95	3.66 ± 1.19	3.81 ± 0.25

Note: There is no statistical difference among the three sites (ANOVA test).

further confirmed that P-gp of rodents and humans share similar binding modes. In another study, the permeability and efflux ratios (ERs) of 31 structurally diverse CNS drugs were compared in human MDR1-MDCK and mouse Mdr1a cell assays. There was a good correlation (R^2 = .92) between the ERs in human MDR1-MDCK and mouse Mdr1a-MDCK cells.[49] Similarly, there was a good correlation (R^2 = .92) of transcellular transport for a series of compounds between rat double transfectant (Oatp4/Mrp2) and human (OATP2/MRP2).[50] Moreover, in vitro study with rat and human colon revealed that digoxin efflux kinetics was virtually identical in rat and human colon. The EC_{50} (the concentration at which half-maximal flux was achieved) of digoxin was 50.6 ± 8.8 and 58.7 ± 15.5 μmol/L, respectively, for rat and human colon, while the corresponding J_{max} (maximal net flux) of digoxin was 2.2 ± 0.3 and 3.1 ± 0.8 nmol/h/cm^2.[51] Inhibitor studies showed that digoxin efflux in intestinal tissues was mainly mediated by P-glycoprotein. Together, these results suggest that functional activities of human efflux transporters are qualitatively and quantitatively similar to those of rat efflux transporters.

However, significant species differences in P-glycoprotein transport activity exist between humans and animals.[14,52] In a drug discovery program, human MDR1-MDCK and mouse Mdr1a-MDCK cell assays were established for routine assay. More than 2100 compounds were evaluated for their permeability and efflux ratio. Approximately 25% of the compounds exhibited significant species difference (greater than 2-fold) in P-gp activity between human MDR1 and mouse Mdr1a cell lines.[52] Similarly, significant species differences in transport activity of MRP/Mrp and BCRP/Bcrp were observed across species, when the efflux rates of MRP/Mrp substrate and BCRP/Brcp substrate were evaluated in hepatocytes from rats, dogs, monkeys, and humans.[53]

As shown in Figure 1, the unbound drug concentrations of all Class I compounds in blood and brain remained relatively constant from about 1-2 hours up to 6 hours after the commencement of infusion, suggesting a steady state was reached. The steady-state blood unbound drug concentrations ($C_{m,blood}$) measured by microdialysis were quantitatively very similar to those in the brain ($C_{m,brain}$) measured by microdialysis. There were no statistical differences between the $C_{m,brain}$ and $C_{m,blood}$ for all compounds (Table 5). These results strongly suggest that the free drug hypothesis is applicable to the Class I compounds that have good permeability and are not substrates of efflux transporters. If the free drug hypothesis is applicable to the Class I compounds, the unbound drug concentrations in CSF (C_{csf}) are expected to be quantitatively similar to that in the brain ($C_{m,brain}$). It is unexpected that the C_{csf} of 4-aminoantipyrine, lamotrigine, and carbamazepine was statistically different from $C_{m,brain}$. As shown in Table 5, the C_{csf} of 4-aminoantipyrine was somewhat lower than $C_{m,brain}$ by about 25%, while the C_{csf} values were slightly higher than the $C_{m,brain}$ for lamotrigine (45%) and carbamazepine (25%). The reason for the significant differences between C_{csf} and $C_{m,brain}$ for the three compounds is not clear.

The unbound concentrations of all Class II compounds in blood and brain also remained relatively constant, and a steady state was reached around 1-2 hours after the commencement of infusion (Figure 2). However, the unbound concentrations in the brain were markedly lower than blood for all the compounds. The differences in the steady-state unbound drug concentrations between the brain and blood ranged from 3.9-fold for quinidine to 86-fold for pemetrexed, respectively (Table 6). It is important to point out that steady-state unbound concentrations of drugs in all tissues (organs) should be equal, unless an organ has a clearance process or efflux transport process.[54] It is generally accepted that the brain is not an organ of drug metabolism. Therefore, the marked differences in unbound concentrations between the brain and blood for Class II compounds strongly suggest that these compounds are substrates of efflux transporters.

As shown in Tables 3 and 4, all Class II compounds are substrates of efflux transporters except atenolol and pemetrexed. The ER values of atenolol and pemetrexed were 0.416 and 0.623, respectively, for MDR1-MDCK assay, while the corresponding values were 1.49 and 1.81 for Caco-2 assay. These results suggest that atenolol and pemetrexed are not substrates of efflux transporters (P-gp, BCRP, and MRPs). Kinetically, the steady-state unbound concentrations of a drug in the brain and blood should be equal, unless there is an involvement of carrier-mediated efflux transport of the drug at BBB.[54] If atenolol and pemetrexed are not substrates of efflux transporters, it is expected that the unbound drug concentrations in the brain are quantitatively similar to that in blood. Unexpectedly, the steady-state unbound concentrations of atenolol and pemetrexed in the brain were substantially lower than that in blood (Figure 2 and Table 6). The marked differences in steady-state unbound concentrations between the brain and blood strongly suggest that atenolol and pemetrexed are substrates of some unknown efflux transporters.

In vitro and in vivo studies have revealed that pemetrexed is a good substrate of BCRP and MRPs which are expressed in the BBB acting as efflux transporters.[55] However, conflicting results have been reported by other investigators. Using brain efflux index method, efflux of pemetrexed from brain to blood after intracerebral microinjection was studied in wild-type, Bcrp1$^{-/-}$, and Mrp2$^{-/-}$ mice.[56]

Pemetrexed was eliminated rapidly from the brain to blood circulation even in Bcrp1$^{-/-}$ and Mrp2$^{-/-}$ mice, when the compound was injected into the rat brain. Results from this study indicated that Mrp2 and Bcrp1 did not play an important role in the brain efflux of pemetrexed. Instead, the authors suggested that the involvement of organic anion transporters in the efflux of pemetrexed from the brain, with organic anion transporter 3 (Oat3) being a possibility. The identity of efflux transporters involved in brain efflux of pemetrexed is still unknown.

Atenolol is a hydrophilic compound with low membrane permeability. The steady-state ratio of unbound atenolol concentrations in brain to that in blood (ie, $K_{p,uu,brain}$) was about 0.12 in this study (Table 6). Similar observations were also reported by other investigators. In a rat study using microdialysis, the $K_{p,uu,brain}$ of S-atenolol was reported to be about 0.04 by Chen et al[8] Results from our study and other investigators strongly suggest the possibility of an involvement of transporter-mediated efflux transport from the brain. However, the identity of the efflux transporter(s) is still unknown.

In conclusion, the free drug hypothesis is not universally applicable for all drugs, but only applicable for drugs with good permeability that are not substrates of efflux transporters. If the free drug hypothesis is applicable, the unbound drug concentrations in blood (plasma) at steady state can be used as a reliable surrogate for assessing the unbound drug concentrations at the site of action. It is evident that drugs with poor membrane permeability that are substrates of efflux transporters will not follow the free drug hypothesis. For drugs that are substrates of efflux transporters, the unbound drug concentrations in the brain are expected to be significantly lower than that in blood (plasma). An important lesson learned from this study is that in vitro transport study using transporter-transfected cell lines (such as MDR1-MDCK and/or Caco-2 cells) may sometimes fail to identify substrates of efflux transporters. Supplemental kinetic study in rodents may facilitate accurate identification of efflux transporters. Recently, it has been reported that the unbound concentrations of several drugs in the brain were greater than in plasma, suggesting the involvement of active influx transporters in brain uptake.[20,57] Conceivably, drugs that are substrates of influx transporters are also not expected to follow the free drug hypothesis. The involvement of influx transporters in the brain uptake of drugs is currently an important research topic in our laboratory.

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DISCLOSURE

We, all authors here, declare that there is no relationship, finance, or other kind of conflict of interest.

AUTHOR CONTRIBUTIONS

Participated in research design: Tao Wang, Chun Chen, Hongyu Zhou, Chi Guan, Hang Chang, Chen Li, Hongwen Du, and Shofeng Zhang. Conducted experiments: Chun Chen, Huanhuan Zhang, Teng Zhang, Chi Guan, Yingying Li, Xue Jiang, Yuanyuan Tao, Zheng Dong, Juan Du, Haofei Luan, and Yu Wang. Performed data analysis: Chun Chen, Chi Guan, Hongyu Zhou, Huanghuan Zhang, Shuyao Wang, Na Du, Junyang Guo, Yaqiong Wu, Zehai Song, Hang Chang, Chen Li, Shaofeng Zhang, Hongwen Du, and Tao Wang. Wrote or contributed to the writing of the manuscript: Chun Chen, Chi Guan, Hongyu Zhou, and Tao Wang.

ETHIC STATEMENT

All animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Pharmaron following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

ORCID

Tao Wang https://orcid.org/0000-0001-8047-3809

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