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The impact of protein on Caco-2 permeability of low mass balance compounds for absorption projection and efflux substrate identification

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ABSTRACT

This study was to evaluate the mechanistic effect of protein to help better interpret the permeability results for compounds with low mass balance in Caco-2 permeability assay. The absorptive or bi-directional permeability of lipophilic compounds with mass balance were measured across Caco-2 cell monolayers as well as the empty transport devices with or without protein (4% bovine serum albumin, BSA) added to the receiver side. The results from empty transport device study indicated that the filter membrane is a permeability barrier for the low mass balance compounds and protein increases permeability by improving the compound diffusivity through the filter membrane. Caco-2 permeability measured with protein provided better absorption projection. Assuming the amount of compound associated with cells as transported did not correlate with absorption. For efflux substrate identification using Caco-2 bi-directional permeability assay, protein at the receiver side had no significant effect on the conclusion regarding the tested compounds as efflux substrate but increased the permeability measurement from both transport directions. In conclusions, Caco-2 permeability results measured using protein-containing buffer at the receiver side for low mass balance compound seems to provide better correlation with in vivo absorption. The fact that protein at receiver side has minimal effect on efflux substrate identification provides scientific basis for further specific transporter characterization (such as P-gp or BCRP) using specific inhibitors, in which same concentration of inhibitor is used in both sides of the Caco-2 cell system and protein for optimal permeability assessment has to be avoided.

1. Introduction

Caco-2 cell permeability (P_{app}) has been used to predict human intestinal absorption [1–5] and many pharmaceutical companies have been using the Caco-2 cell system as an intestinal permeability screen during lead optimization for the past decade. For passively transported compounds, Caco-2 cell permeability is considered an appropriate method for drug permeability classification based on the Biopharmaceutics Classification System (BCS) [6]. Efflux transporters, such as P-gp, BCRP, MRPs, were demonstrated to be important to drug absorption and tissue distribution [7–10]. Since Caco-2 cell monolayers also express high levels of functional efflux transporters at the apical side [9–12], including P-gp and BCRP, bi-directional permeability assay has been routinely used for efflux substrate identification in drug discovery. Recently, the US FDA Draft Guidance for Drug Interaction Studies (DDI) [13] has recommended the use of bi-directional permeability of Caco-2 cells or MDR-1 transfected cell line for P-glycoprotein (P-gp) substrate and inhibitor determination, which makes the Caco-2 permeability assay important for both drug discovery and development.

With the expanded use of cell monolayer transport systems in drug discovery and development, it has become more important to understand and correctly interpret the Caco-2 permeability data. For compounds with near complete aqueous mass balance (>85%) in the cell transport system, permeability measurements are usually reliable so that using permeability for absorption projection and efflux substrate identification purpose will be straight forward. However, for lipophilic compounds with low mass balance in the Caco-2 system, it can be a challenge.

Because of the difficulty in interpreting permeability measurement for low mass balance compounds, a reliable method is needed for quality permeability measurement when we have better understanding the factors that cause low mass balance and measurement variations. In this report, the permeability and mass balance of proprietary lipophilic compounds and marketed compounds in both empty transport devices and Caco-2 cell plates were studied with

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or without protein (4% BSA to mimic in vivo blood protein concentration) added to the receiver side. Efflux substrate identification using Caco-2 bi-directional permeability assay for marketed and proprietary compounds was also carried out with the same conditions. The characterization was intended to provide more insight into: (1) the optimization of experimental conditions for Caco-2 permeability screening by understanding the non-specific binding in the transport system, and (2) the predictivity of using Caco-2 permeability data measured with protein at receiver side for low mass balance compounds for oral absorption projection and efflux substrates identification.

2. Materials

The Caco-2 cell line was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). Hank's Balanced Salt Solution (HBSS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), L-glutamine, penicillin–streptomycin (PEST), and non-essential amino acids (NEAAs) were obtained from Lonza Walkerwille (Walkerwille, MD). HEPES, D-glucose, trypsin, DMSO and BSA were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA). Transport studies were performed using the HTS Multiwell™ insert system with PET membranes (24-wells, 1.0 μm pore size, 0.33 cm^2 area) and bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ). Scintillation cocktail (Flo Scint III) was purchased from PerkinElmer Life Sciences (Boston, MA). The proprietary cold and radiolabeled test compounds were synthesized within Schering-Plough Research Institute. ^{3}H-digoxin was obtained from PerkinElmer Life Sciences (Boston, MA); ^{3}H-cyclosporin A (CsA) was from Amersham Biosciences UK; ^{3}H-taxol and ^{3}H-prazosin was from Moravek Biochemicals (Brea, CA). All other reagents were obtained from Sigma (St. Louis, MO).

3. Methods

3.1. Cell culture

The cells (passages 32–60) were maintained in a culture medium consisting of DMEM supplemented with 10% FBS, 1% NEAA, 2 mM L-glutamine, and 0.1% PEST. Cells were maintained in an incubator at 37 °C with 5% CO_2 and 90% relative humidity. The cells were subcultured at 70–80% confluency by treatment with 0.25% trypsin containing EDTA.

For transport experiments, Caco-2 cells were seeded onto the 24-well HTS Multiwell™ insert filter at a density of 60,000 cells/cm^2. Plates were incubated at 37 °C with 5% CO_2 and 90% relative humidity. The medium was changed every 48 h using a Brandel cell culture automation system (Gaithersburg, MD). The cell monolayers were grown 21–25 days prior to the transport study. The cell batch monolayer integrity and efflux transporter level expression was confirmed with one plate from each batch by testing the ^{14}C-mannitol permeability (<10 nm/s for batch qualification), ^{3}H-digoxin efflux ratio (greater than 6.0), and the transepithelial electrical resistance (TEER) for each monolayer before and after incubation (acceptable range 300–600 Ω cm^2).

3.2. Permeability measurement

Prior to the study, culture medium was removed from both the apical (AP) and basolateral (BL) compartment, and then the cell monolayers were washed twice with warmed (37 °C) HBSS buffer containing 10 mM HEPES, 10 mM D-glucose (TM, pH 7.4). Dosing solution was prepared using TM from a DMSO stock with 1% final DMSO concentration. For AP to BL transport, 0.4 ml dosing solution was added to the AP side, and 1.0 ml of TM with or without 4% BSA was added to the BL side. For BL to AP transport, 1.0 ml dosing solution was added to the BL side and 0.4 ml of TM with or without 4% BSA was added to the AP side. The permeability of each test article was assessed in triplicate unless otherwise stated by incubating the Caco-2 plates for 2 h at 37 °C with 5% CO_2 and 90% relative humidity with shaking using a DPC® Micromix 5 shaker. Donor samples were taken immediately after dosing and at the end of the incubation. Receiver samples were taken at the end of the incubation. For permeability assessments in empty transport devices, the same procedure was followed.

3.3. Compound mass balance study

Caco-2 absorptive permeability and recovery for the low mass balance compounds at 1 and 10 μM concentrations were measured as described in Section 3.2. At the end of the experiment, both AP and BL sides were washed three times with TM buffer. The Caco-2 cell monolayers were retrieved using a cotton swab and put into scintillation vials. Both AP and BL side wells were then washed twice with 1 ml acetonitrile. Washes were pooled and added to 20 ml scintillation vials along with 10 ml of cocktail to each vial. Empty transport devices were treated according to the same procedure.

3.4. Caco-2 sample analysis

Radioactive Caco-2 samples were analyzed using a Packard 2250A Tri-Carb Scintillation Counter. Non-radioactive samples were added to acetonitrile containing an internal standard with a ratio of 1:3 and analyzed by LC/MS/MS. The LC/MS/MS sample analyses was similar to the one described previously [14]. Briefly, two solvent mixtures (A and B) were used for HPLC: A consisted of 0.1% formic acid in 5% aqueous methanol, and B consisted of 0.1% formic acid in 95% aqueous methanol. A Synergy Fusion, 5 μm, 2.0 mm × 30 mm C18 column (Phenomenex, Torrance, CA, USA) with a generic stepwise gradient was used for method development. The MS/MS conditions for monitoring the MRM transitions was optimized and used for subsequent data acquisition and quantification. The mass spectrometer was a Quattro Ultima triple-quadrupole equipped with a four-way MUX® interface (Micromass). Typical mass spectrometer conditions included a source temperature of 150 °C, desolvation gas temperature of 300 °C, desolvation gas flow rate of 1000 l/h, and the electrospray capillary voltage set at 3.5 kV.

3.5. In vivo absorption measurements

All animal study protocols were pre-approved by Schering-Plough Research Institute's Animal Care and Use Committee (ACUC). Absolute absorption in Sprague–Dawley rats was measured by dosing the ^{3}H-labeled compound by both intravenous injection (IV) and oral (PO) administration. Briefly, 100 μCi ^{3}H-labeled compound prepared in 0.4% aqueous hydroxypropyl methylcellulose or 20% hydroxypropyl-β-cyclodextrin was administered to Sprague–Dawley rats intravenously or orally. Plasma samples were taken at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h post-dose. The total radioactivity of the sample at each time point was counted using a Packard 2250A Tri-Carb Scintillation Counter.

3.6. Data analysis

The recovery from the transport device and Caco-2 cells were calculated by dividing the amount of compound recovered from the device or cells by the total dosing amount. The apparent permeability (P_{app}), aqueous recovery, and efflux ratio were calculated

Table 1
Physicochemical properties of the low mass balance compounds.

Compound ID	Molecular weight	*c* log *P*	Polar surface area (PSA)	Number rotatable bonds	Number H_donors	Number H_acceptors
A	422.88	4.8	68.2	6	2	3
B	436.91	5.3	59.4	6	1	3
C	497.00	4.2	79.6	6	2	4
D	588.52	7.2	51.7	7	1	5
E	490.86	3.1	103	7	2	5

Table 2
Permeability and compound disposition of the low mass balance compounds through the filter membrane of the transport device.

Dosing conc/ID	% Recovery									
Receiver side	P_{app}* (nm/s)		Aqueous**		Filter		Wells		Total	
	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA
1 μM										
A	140 ± 30	283 ± 54	53 ± 2	95 ± 3	5 ± 0.3	3 ± 0.6	21 ± 4	17 ± 2	79	115
B	156 ± 52	267 ± 45	59 ± 2	96 ± 2	5 ± 0.6	3 ± 0.4	30 ± 5	10 ± 2	94	109
C	51 ± 12	190 ± 37	37 ± 0.3	71 ± 1	8 ± 1	7 ± 0.6	43 ± 2	31 ± 3	87	108
D	6 ± 1	13 ± 5	16 ± 0.4	42 ± 2	10 ± 1	10 ± 2	52 ± 6	45 ± 3	78	97
E	45 ± 10	245 ± 57	46 ± 4	78 ± 7	8 ± 2	4 ± 1	23 ± 4	12 ± 2	76	93
10 μM										
A	239 ± 75	337 ± 34	62 ± 2	112 ± 1	2 ± 0.1	1 ± 0.1	10 ± 1	9 ± 1	74	121
B	286 ± 71	345 ± 14	69 ± 5	114 ± 2	2 ± 0.2	1 ± 0.1	13 ± 2	8 ± 1	85	123
C	143 ± 5	333 ± 30	54 ± 2	95 ± 2	4 ± 0.5	2 ± 0.4	36 ± 2	16 ± 1	93	113
D	34 ± 9	130 ± 54	26 ± 4	77 ± 2	5 ± 0.6	3 ± 0.7	54 ± 2	25 ± 1	85	106
E	67 ± 10	288 ± 16	43 ± 3	74 ± 5	8 ± 2	4 ± 1	24 ± 3	12 ± 2	75	90

* Data shown as mean ± SD ($n = 3$).
** Significant aqueous recovery differences were observed with and without addition of protein to the receiver side at both dosing concentrations ($p < 0.05$, *t*-test).

using the following equations:

$$P_{app} = \frac{(dC_R/dt) \times V_R}{S \times C_{D,0\,h}}$$

$$\text{Efflux ratio} = \frac{P\text{app\_BL to AP}}{P\text{app\_AP to BL}}$$

$$\text{Aqueous recovery}\,(\%) = \frac{C_{D,\text{Final}}}{C_{D,0\,h}} \times 100 + \frac{\text{Receiver accumulated amount}}{C_{D,0\,h} \times V_D} \times 100$$

where dC_R/dt is the slope of the cumulative concentration in the receiver compartment versus time; S is the surface area of the cell monolayer; $C_{D,0\,h}$ and $C_{D,\text{Final}}$ are the donor compound concentrations immediately after dosing and at the end of incubation, respectively; and V_D and V_R are the volumes of the donor and receiver compartments, respectively.

The extent of absorption (Fa) was calculated using the following equation with the areas under the total plasma radioactivity versus time curve (AUC) calculated from PO and IV administrations:

$$\text{Fa}\,(\%) = \frac{\text{AUC}_{\text{\_PO(total radioactivity)}}/\text{Dose}_{\text{\_PO}}}{\text{AUC}_{\text{\_IV(total radioactivity)}}/\text{Dose}_{\text{\_IV}}} \times 100$$

3.7. Absorption projection

A correlation of Caco-2 permeability and human absorption was established previously in this lab using the same method as described in the permeability measurement section [15]. The Fa of the compound was then calculated using the following correlation:

$$\text{Predicted\_Fa}\,(\%) = \frac{100}{1 + (P_{app}/15.6)^{-1.04}}$$

4. Results

4.1. Compound permeability and mass balance in the transport device (filter plate)

The permeability and mass balance measured with the empty transport devices of five proprietary lipophilic compounds, whose physicochemical properties are summarized in Table 1, are presented in Table 2 and Fig. 1. The results show high non-specific binding of the compounds to the device wells and filter, especially at lower dosing concentration. The addition of protein at the receiver side and dosing higher concentrations of compound helped to reduce device binding and improved filter permeability and aqueous recovery, which would suggest possible saturation of non-specific binding by the device.

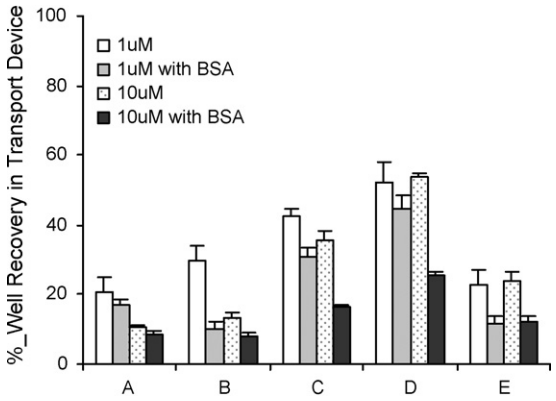


Fig. 1. Compound non-specific binding to the transport device. *: Error bar represents standard deviation ($n = 3$). Significant recovery differences were observed with and without addition of protein to the receiver side at both dosing concentrations ($p < 0.05$, *t*-test).

Table 3
Permeability and compound disposition of the low mass balance compounds in Caco-2 cell plate.

Dosing conc/ID	% Recovery*									
Receiver side	P_{app}* (nm/s)		Aqueous**		Caco-2 cells		Wells		Total	
	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA
1 μM										
A	15 ± 1	24 ± 3	56 ± 2	58 ± 2	28 ± 4	26 ± 3	5 ± 1	5 ± 2	89	89
B	46 ± 3	65 ± 5	62 ± 2	64 ± 1	24 ± 3	26 ± 2	7 ± 1	5 ± 1	93	95
C	23 ± 2	59 ± 9	47 ± 4	51 ± 1	26 ± 2	26 ± 4	10 ± 1	10 ± 1	83	88
D	3 ± 0.2	5 ± 1	16 ± 1	18 ± 2	37 ± 9	52 ± 4	13 ± 1	9 ± 2	68	78
E	3 ± 0.4	18 ± 2	32 ± 2	28 ± 2	41 ± 3	63 ± 10	16 ± 4	6 ± 2	89	96
10 μM										
A	27 ± 1	40 ± 2	62 ± 1	64 ± 1	26 ± 1	23 ± 1	4 ± 1	4 ± 1	91	90
B	53 ± 4	69 ± 3	65 ± 2	71 ± 1	23 ± 4	20 ± 2	4 ± 1	5 ± 1	93	96
C	33 ± 3	80 ± 2	55 ± 3	57 ± 2	23 ± 2	25 ± 3	7 ± 2	5 ± 2	85	86
D	4 ± 0.3	10 ± 2	25 ± 1	30 ± 1	37 ± 6	40 ± 3	17 ± 4	7 ± 4	78	77
E	3 ± 0.3	16 ± 0	47 ± 8	65 ± 14	28 ± 0.4	29 ± 1	7 ± 2	6 ± 1	82	99

* Data shown as mean ± SD ($n = 3$); significantly higher P_{app} with BSA ($p < 0.05$, t-test).
** There were no significant differences in aqueous recovery with and without the addition of protein to the receiver side at either dosing concentration.

4.2. Compound Caco-2 permeability and mass balance

The permeability and mass balance of the compounds in the Caco-2 cell system are summarized in Table 3 and shown in Figs. 2 and 3. The TEER values for the cell monolayers before and after incubation ranged from 300 to 540 Ω cm^2, indicating the monolayer integrities were not affected during experiment. Much less non-specific binding to the transwell was observed in the Caco-2 system than that in empty devices with and without protein (Fig. 2 versus Fig. 1), which suggests non-specific binding to the transport device in Caco-2 cell permeability assays may not be significant. Significant cell association of the lipophilic compounds (Fig. 3 and Table 3) was observed and the addition of protein did not significantly improve the recovery but increased the measured Caco-2 permeability. These observations are consistent with protein increasing diffusivity of molecules through the filter membrane but having limited effect on non-specific binding in the Caco-2 cell system. Higher compound dosing concentration slightly improved aqueous recovery and permeability for the compounds, suggesting that higher dosing concentrations might be used in cell permeability assay when possible. Since the solubility of most lipophilic compounds is limited, buffers with capability of improving solubility but not interfering with the transport properties may be evaluated to increase the dosing concentration, such as the simulated intestinal fluid as used in the study by Fossati et al. [16].

4.3. Correlation of Caco-2 permeability and predicted absorption

Table 4 summarizes the Caco-2 permeability, predicted human absorption (Fa), and rat absorption the tested compounds. As shown previously by Chiou and Barve [17], a unity slope of linear correlation of 64 orally dosed drugs was observed between humans and rats, we predict that the oral absorption of the tested five compounds will be similar between rats and humans. The permeability was calculated using $C_{D,0\,h}$ and $C_{D,Final}$ as the donor concentration, which gave a range of permeability with the consideration that the low mass balance from lipophilic compounds were mostly resulted from decreased donor concentration so that the concentration gradient would change significantly during incubation. Adding $C_{D,Final}$ for calculation may give a reasonable range for the actual compound permeability in Caco-2 cells. The permeability calculated with $C_{D,Final}$ seems to provide better absorption projection with the five tested compounds. For highly lipophilic compound D ($c \log P - 7.2$), which had an extremely low mass balance (<50% at 10 μM with protein), the absorption projection was more uncertain. However, it seems that the absorption using permeability calculated from $C_{D,Final}$ with protein might provide a lower-end absorption estimate for extreme low mass balance compounds.

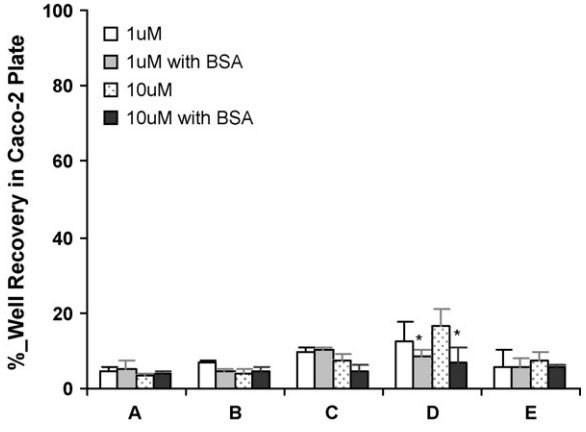


Fig. 2. Compound non-specific binding to Caco-2 cell System. *: Error bar represents standard deviation ($n = 3$). No significant recovery differences were observed with and without addition of protein to the receiver side at both dosing concentrations with the exception of the most lipophilic compound D ($c \log P = 7.2$) ($p < 0.05$, t-test).

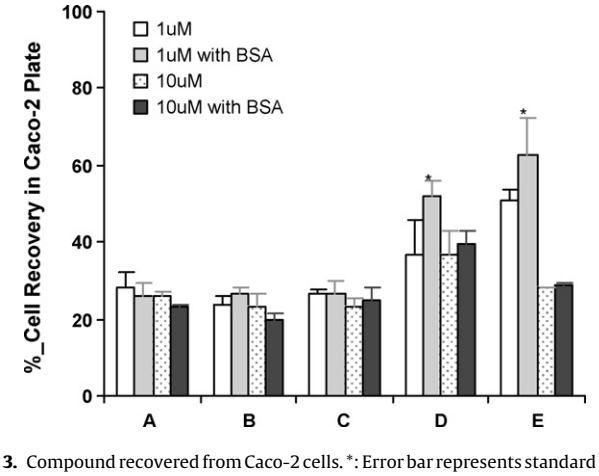


Fig. 3. Compound recovered from Caco-2 cells. *: Error bar represents standard deviation ($n = 3$). There is no significant differences for cell association with or without BSA added to the receiver side at the same dosing concentration with the exception of the most lipophilic compound D and largest PSA compound E at 1 μM ($p < 0.05$, t-test).

Table 4
In vitro and in vivo absorption correlation of the low mass balance compounds.

ID	P_{app} with cells[a] (nm/s)	P_{app} range[b] (nm/s)	AUC PO[c] (μM equiv. h)	AUC IV (μM equiv. h)	Predicted Fa (%)	Fa (%) in rats
A	430 ± 12	40–65	24.5 (13%)	24.8 (34%)	72–82	99
B	409 ± 31	69–106	56.2 (14%)	57.5 (5%)	82–88	98
C	503 ± 51	80–151	12.1 (16%)	15 (14%)	85–92	81
D	678 ± 58	10–15	3.7 (19%)	5.4 (16%)	37–48	69
E	500 ± 47	16–26	2.2 (30%)	5 (7%)	48–63	44

[a] Permeability calculated by counting the amount associated with cells with protein at the receiver side as amount transported after 2-h incubation time dosed at 10 μM.
[b] The low-end permeability was calculated using $C_{D,0\,h}$ and the high-end was calculated using $C_{D,Final}$ as dosing concentration.
[c] Dose normalized to 10 mg/kg for both IV and PO ($n = 3$, number in the parenthesis was the coefficient of variation (CV%) among the three dosing animals for each administration route).

Since there has been a question about how to take the amount associated with the cells into account for absorption projection, we tried to count the amount as transported with a 2-h transit time for absorption. When the amount associated with the cell monolayer was included as transported through the cell monolayer, the resulting permeability values are all high, which do not help to project in vivo absorptions.

4.4. Efflux substrate identification with or without protein in Caco-2 bi-directional permeability assay

The bi-directional permeability of seven marketed compounds measured across transport devices with and without Caco-2 cell monolayers are summarized in Tables 5 and 6, respectively, for system validation purpose. All of the test articles except CsA were freely transported through the transport device with almost complete recovery (>85%), good filter permeability (>200 nm/s) (Table 4) and no bi-directional permeability differences were observed (efflux ratio = 1). The protein significantly improved the filter membrane permeability of CsA through the transport device from both transport directions. For efflux substrate identification using Caco-2 cells (Table 6), all of the four known efflux substrates, namely CsA, digoxin, prazosin, and taxol, had efflux ratio greater than 2.0 with or without protein. To further investigate the protein effect on efflux substrate identification, seven proprietary compounds, which included five low mass balance lipophilic compounds, were evaluated. Tables 7 and 8 summarize the physicochemical properties of the compounds and the Caco-2 bi-directional permeability results, respectively.

As shown in Table 8, protein at receiver side significantly improves Caco-2 permeability measurements from both transport directions for the compounds with or without mass balance issues except for the least lipophilic compound #1 ($c\log P = 1.7$). All of the compounds were sufficiently identified as efflux substrates (efflux ratio ≥2.0) with or without protein, which also correlates well with the low brain/plasma ratio (<1) in rats (Table 7), with the exception of compound #4 which became highly permeable when protein was added. Efflux ratios also decreased with protein for most of the tested compounds, which is consistent with the more significant increase in $P_{app\_AP\,to\,BL}$ as compared to that of $P_{app\_BL\,to\,AP}$. The observed trend of better BL to AP recovery than AP to BL recovery, which is also a general observation from our discovery bi-directional permeability screening for efflux substrate with high $P_{app\_BL\,to\,AP}$, could due to the active efflux transport function at the apical side resulting less cell association for lipophilic efflux substrates.

Table 5
Bi-directional permeability of standard compounds through the filter membrane of the transport device.

Compound ID	Filter P_{app} (nm/s)[a]				% Aqueous recovery[a]				Efflux ratio	
	AP to BL		BL to AP		AP to BL		BL to AP			
Receiver side	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA
CsA	28 ± 2	172 ± 11	31 ± 0	194 ± 28	16 ± 1	80 ± 6	29 ± 1	86 ± 3	1.1	1.1
Prazosin	725 ± 28	492 ± 19	640 ± 46	473 ± 10	101 ± 2	120 ± 6	87 ± 3	91 ± 3	0.9	1.0
Taxol	524 ± 22	479 ± 13	494 ± 21	519 ± 16	86 ± 3	120 ± 3	75 ± 2	95 ± 10	0.9	1.1
Digoxin	780 ± 28	511 ± 19	626 ± 2	484 ± 12	107 ± 16	111 ± 5	94 ± 0	102 ± 2	0.8	0.9
Mannitol	876 ± 17	533 ± 55	800 ± 42	575 ± 22	106 ± 5	110 ± 3	97 ± 2	86 ± 2	0.9	1.1
Urea	871 ± 13	594 ± 18	854 ± 11	623 ± 11	100 ± 0	106 ± 1	98 ± 2	90 ± 0	1.0	1.0
Propranolol	708 ± 23	440 ± 11	718 ± 49	493 ± 36	93 ± 0	104 ± 6	94 ± 9	90 ± 10	1.0	1.1

[a] Data shown as mean ± SD ($n = 3$).

Table 6
Bi-directional permeability of standard compounds in Caco-2 plates.

Compound ID	Caco-2 P_{app}* (nm/s)				%_Aqueous recovery*				Efflux ratio	
	AP to BL		BL to AP		AP to BL		BL to AP			
Receiver side	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA
CsA	15 ± 1[a]	23 ± 0[a]	57 ± 1[a]	69 ± 1[a]	58 ± 5.2	61 ± 5	34 ± 2	48 ± 5	3.7	3.0
Prazosin	122 ± 3	129 ± 3	370 ± 15	372 ± 15	92 ± 1.6	91 ± 0	100 ± 2	100 ± 3	3.1	2.9
Taxol	39 ± 1	43 ± 1	225 ± 0	233 ± 3	92 ± 1.3	94 ± 2	89 ± 1	90 ± 2	5.8	5.3
Digoxin	16 ± 1	16 ± 0	150 ± 3	164 ± 10	98 ± 1.9	104 ± 2	97 ± 1	101 ± 2	9.7	10.5
Mannitol	5 ± 0	5 ± 0	4 ± 0	3 ± 0	98 ± 0.8	100 ± 1	101 ± 3	98 ± 1	0.8	0.6
Urea	47 ± 0	47 ± 0	49 ± 1	47 ± 0	98 ± 1.0	97 ± 3	96 ± 1	98 ± 0	1.1	1.0
Propranolol	395 ± 30	341 ± 3	229 ± 12	182 ± 22	89 ± 6.6	85 ± 3	93 ± 1	99 ± 3	0.6	0.5

[a] P_{app} significantly increased with BSA ($p < 0.05$, t-test).

* Data shown as mean ± SD ($n = 3$).

Table 7
Test compounds used in Caco-2 bi-directional permeability assay.

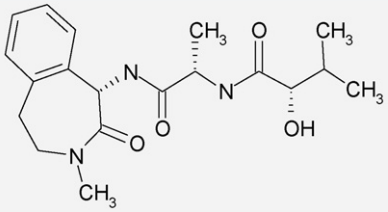
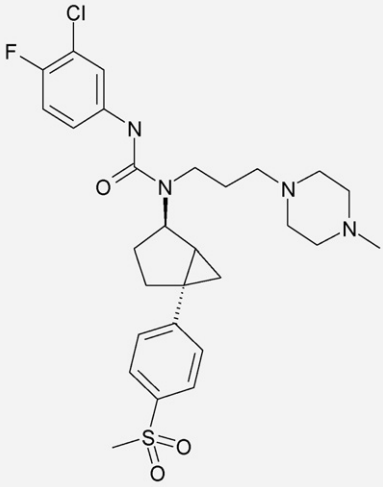
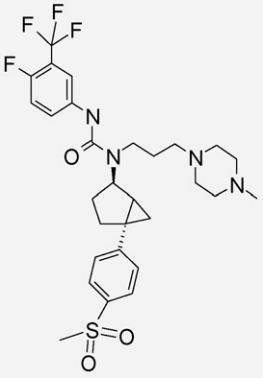
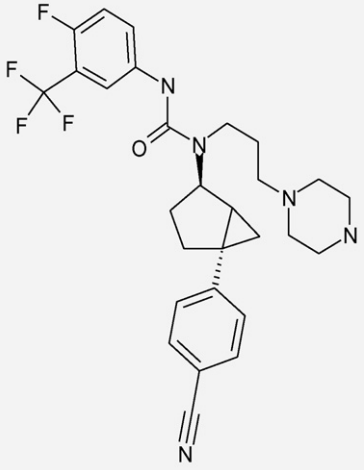
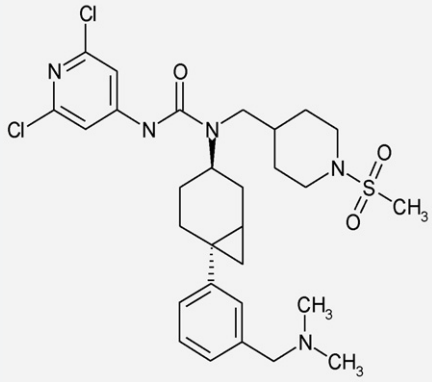
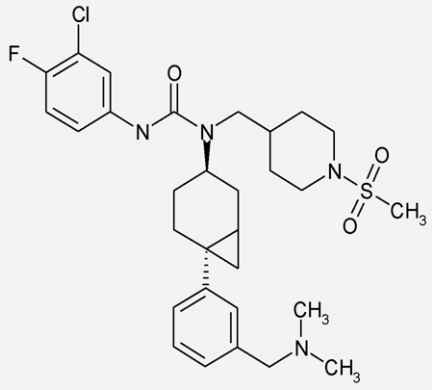
Compound ID	Mol. wt.	*c* log *P*	PSA	Brain/plasma ratio in rats[a]	Structure
# 1 (LY-450,139)	361	1.66	99	0.1	
#2	563	2.77	73	0.1	
#3	597	3.16	73	<1	
#4	574	4.01	131	NA[b]	NA
#5	530	4.74	71	0.7	

Table 7(*Continued*)

Compound ID	Mol. wt.	*c* log *P*	PSA	Brain/plasma ratio in rats[a]	Structure
#6	609	4.88	86	<1	
#7	591	5.07	73	0.1	

[a] The concentration ratio of brain and plasma levels (Br/Pl) was measured with a cassette rapid rat screening (CARRS) method [18]. Briefly, two rats were dosed at 10 mg/kg orally with the test compound. Plasma samples and brain tissue was collected at 6 h post-dosing and analyzed for concentration.
[b] Not available.

5. Discussion

Caco-2 permeability with low mass balance has always been an issue when we try to interpret the results for absorption projection and efflux substrate identification. With expanded applications of Caco-2 permeability assay in drug discovery and development, more understanding of the measured permeability would help to better interpret the results. A simple incubation method that could efficiently improve permeability measurement for better absorption and/or efflux substrate projection for low mass balance lipophilic compounds is in need in drug discovery as well as development. Ingels and Augustijins [19] had proposed different options to address the low mass balance issue due to adsorption to the transport device and/or non-specific binding to the cells, which included the use of BSA and/or surfactant in transport buffer. Multiple studies have also observed improved Caco-2 permeability of lipophilic compounds when BSA was added to the receiver side [20–22], but how the protein enhances permeability has not been fully examined. Recently, Lakeram investigated the effect of different experimental conditions on Caco-2 permeability measurement for a series of p-hydroxybenzoate ester compounds with a range of lipophilicities (log *P* 1.96–5.69) [23]. The authors demonstrated that with the optimized conditions for three parameters, including BSA, surfactant and different stirring rate, the permeability measure-

Table 8
Caco-2 bi-directional permeability of test compounds.

ID	P_{app} (nm/s)[a]				% Aqueous recovery				Efflux ratio[c]	
	AP to BL		BL to AP		AP to BL		BL to AP			
Receiver side	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA	TM	TM with BSA
#1	12 ± 2	17 ± 0	183 ± 10	191 ± 8	112 ± 11	112 ± 13	113 ± 1	109 ± 0	15	11
#2	31 ± 0	72 ± 9[b]	237 ± 32	360 ± 15[b]	61 ± 1	84 ± 5	101 ± 12	117 ± 4	7.6	5.0
#3	40 ± 4	116 ± 30[b]	216 ± 23	325 ± 8[b]	64 ± 0	84 ± 10	93 ± 8	111 ± 3	5.4	3.5
#4	98 ± 7	284 ± 39[b]	418 ± 1	533 ± 11[b]	92 ± 4	101 ± 5	108 ± 2	113 ± 14	4.3	1.9
#5	6 ± 2	34 ± 14[b]	87 ± 1	205 ± 2[b]	44 ± 4	51 ± 3	84 ± 10	90 ± 2	15	6.0
#6	4 ± 1	8 ± 1[b]	113 ± 15	239 ± 22[b]	48 ± 3	53 ± 3	88 ± 4	84 ± 10	26	29
#7	2 ± 2	23 ± 10[b]	194 ± 26	283 ± 4	62 ± 10	59 ± 7	100 ± 2	97 ± 6	88	12

[a] Data shown as mean ± SD ($n = 3$).
[b] P_{app} with BSA significantly higher than that without BSA ($p < 0.05$, *t*-test).
[c] All test compounds are identified as efflux substrates (efflux ratio ≥2.0) with or without protein added to the receiver side except the highly permeability compound #4 with protein in the receiver side (efflux ratio dropped from 4.3 to 1.9), which could be a poor efflux substrate.

ment for the most lipophilic compound was improved. However, with three experimental conditions that need to be optimized, the approach might not be practical in drug discovery, where large number of compounds with diverse structure series needs to be assayed on a weekly basis.

In this study, a better correlation was observed between the projected absorption with the Caco-2 permeability measured with protein and the absorption in rats. Though BSA has been known for some time to improve permeability of lipophilic compounds in crossing Caco-2 cell monolayers, the mechanism of how BSA increases the permeability has not been fully demonstrated in the literature. It had been widely believed that protein helped to reduce non-specific binding and maintain sink condition. However, the results from current study demonstrated that protein has little effect on non-specific binding in the Caco-2 cell system, which could be the results that the cell insert has been pre-treated with serum during cell culture process so that non-specific binding would have been prevented anyway. We were able to demonstrate with the filter plate permeability assessment that the BSA increase Caco-2 permeability is most likely due to the increased diffusivity through the filter membrane that the cells grown on. This observation is important since it would provide the rational of using excipients (or "sink agent"), including protein, surfactant, etc., at receiver side to reduce filter resistance for lipophilic compounds in Caco-2 assay in order to get a more accurate permeability measurement. Furthermore, since addition of protein has minimal effect on non-specific binding and aqueous recovery, for a more rigorous Caco-2 permeability study, such as for BCS permeability classification purpose, a pre-test for non-specific binding may not be needed if sink agent will be used at the receiver side.

With better understanding of the protein mechanistic effect on Caco-2 absorptive permeability measurement, the same strategy has been applied to Caco-2 bi-directional permeability assay, namely using protein at receiver sides (either basolateral or apical side), for efflux substrate identification and absorption projection purpose. Also, with the development of using Caco-2 assay to identify specific transporters (such as P-gp and BCRP), it would be important to investigate whether the efflux properties can still be adequately assessed without BSA for low mass balance compounds. In this report, the effect of BSA on bi-directional permeability measurement using commercial and proprietary compounds was investigated. The results have shown that BSA improved permeability measurement from both transport directions but there was minimal effect on efflux substrate identification except for highly permeable compound (compound #4 in Table 8). These observations suggest that it would be beneficial to routinely use BSA in Caco-2 bi-directional permeability assay so that the results from one experiment can be used for both absorption projection (including BCS permeability classification) and efflux substrate identification.

For further specific transporter characterization with inhibitors using Caco-2 bi-directional permeability assay as suggested by FDA DDI draft guidance [11], same concentration of inhibitor (such as CsA, Zosuquinar, Elacridar), most of which are also lipophilic, is expected to be added to both donor and receiver sides in order to maintain similar inhibitor concentration at both AP and BL sides. Since the diffusivity through the filter membrane of the low mass balance compounds can be significantly limited without adding BSA so would be the accuracy of the measured permeability. In this study, it has been shown that the efflux substrate identification seems not significantly different with or without protein. It would suggest that similar extent of efflux inhibition by inhibitor for both transport directions could be achieved without optimal permeability assessment for low mass balance compounds (i.e. not using BSA) so that the specific transporter responsible for the efflux transport in Caoc-2 cells could be reasonably characterized.

In summary, the Caco-2 permeability results measured using protein-containing buffer at the receiver side for low mass balance compound seems to provide better correlation with in vivo absorption. For extremely low mass balance compound (less than 50%), Caco-2 permeability with protein may be used to provide lower-end estimates for absorption. Further specific transporter characterization (such as P-gp or BCRP) using specific inhibitors at both sides of the Caco-2 cell transport system, where protein cannot be used, will be expected to reach reasonable conclusion about the specific transporter even though the measured permeability may not be optimal for low mass balance compound.

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