Research Article

Drug Discovery and Regulatory Considerations for Improving *In Silico* and *In Vitro* Predictions that Use Caco-2 as a Surrogate for Human Intestinal Permeability Measurements

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ABSTRACT. There is a growing need for highly accurate *in silico* and *in vitro* predictive models to facilitate drug discovery and development. Results from *in vitro* permeation studies across the Caco-2 cell monolayer are commonly used for drug permeability screening in industry and are also accepted as a surrogate for human intestinal permeability measurements by the US FDA to support new drug applications. Countless studies carried out in this cell line with published permeability measurements have enabled the development of many *in silico* prediction models. We identify several common cases that illustrate how using Caco-2 permeability measurements in these *in silico* and *in vitro* predictive models will not correlate with human intestinal permeability and will further lead to inaccuracies in these models. We provide guidelines and recommendations for improving these models to more accurately predict clinically relevant information, thereby enhancing the drug discovery, development, and regulatory approval processes.

KEY WORDS: Biopharmaceutics Classification System (BCS); biowaiver; Caco-2 cells; Food and Drug Administration (FDA); human intestinal permeability; *in silico* modeling; *in vitro* screening.

INTRODUCTION

In the drug discovery process, the ability of a new molecular entity (NME) to cross biological membranes, particularly the human intestinal mucosa, is crucial in establishing it as a viable drug candidate for clinical development and eventual regulatory approval. The prevalence of oral formulations on the market indicates a strong industry-wide preference for drugs that can permeate the human intestine and be absorbed, further highlighting that poor intestinal permeability is viewed as an unfavorable biopharmaceutical property (1). Since the inception of the Biopharmaceutics Classification System (BCS), which originally demonstrated a relationship between the human jejunal permeability rate of a drug and its extent of absorption (2), the US FDA has supported waivers of *in vivo* bioavailability and bioequivalence studies to facilitate the drug approval process of BCS Class 1 drugs that are highly permeable, highly soluble, and rapidly dissolvable (3). Because human intestinal permeability studies are both costly and difficult (4), studies in the human colon carcinoma cell line, Caco-2, are the most commonly used biological tool for screening the intestinal permeability of NMEs during drug discovery and development

(5,6). As such, measurements in the Caco-2 cell line are accepted by the FDA as a surrogate for human intestinal permeability measurements as part of their BCS guidance (3).

Caco-2 was originally proposed as an *in vitro* model system for studying human intestinal permeability based on its shared characteristics with the small intestinal epithelium of cell morphology, polarity, and enterocytic differentiation (7). Its suitability for intestinal permeability screening was initially confirmed when strong correlations between *in vitro* Caco-2 and *in vivo* human intestinal permeability measurements were observed for some *passively* absorbed drugs (5,8,9). Since then, Caco-2 has been used to screen the intestinal permeabilities of an increasingly diverse population of compounds. Yee (10) had reported that Caco-2 can accurately predict the human intestinal permeability or absorption of compounds regardless of transcellular, paracellular, and carrier-mediated transport mechanisms. However, there are Caco-2 permeability results that disagree with measured human intestinal permeability rates or measured extents of human intestinal absorption (11,12).

Most expert reviews address drug transport processes across Caco-2 monolayers, factors influencing their permeability measurements, and some of the limitations associated with using Caco-2 for human intestinal or absorption predictions (5,13–16). Since Caco-2 has been widely used for screening the intestinal permeabilities of passively and actively absorbed compounds, these expert reviews recommend limiting the application of Caco-2 permeability screening to passive compounds. However, merely using this approach may be unsuccessful based on a recent report

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showing that permeability measures for the majority of drugs on the market include both passive diffusion and active transport (17).

Because discrepancies have only been observed for cases where some highly absorbed drugs have been predicted to be poorly permeable by Caco-2, a consensus of FDA and academic scientists prefer that permeability determinations be based on a pharmacokinetic approach in humans for justifying biowaivers in drug applications rather than the *in vitro* approach, when discordant results between the *in vitro* and *in vivo* methods arise (18). Although the FDA states that human *in vivo* information will “trump” or hold precedence over discordant Caco-2 permeability results in regulatory decisions (18), the lack of available human *in vivo* information during drug discovery to identify when an inaccurate *in vitro-in vivo* permeability prediction occurs with Caco-2 can hinder the drug discovery process in one of two ways.

First, the existence of these variants, questions the reliability of using the Caco-2 cell line as a primary source of screening the intestinal permeabilities of NMEs for lead selection, optimization, and candidate prioritization. Second, Caco-2 permeability data generated during drug discovery are used to develop *in silico* predictive models based on correlations with calculated molecular descriptors that describe permeability requirements. The inclusion of discrepant Caco-2 permeability measurements during *in silico* model development can produce misleading information when using the models for drug design. For these reasons, it is important to identify the types of compounds for which Caco-2 may be deficient in accurately predicting their human intestinal permeabilities, thereby ensuring that the selection of potential drug candidates is not tarnished by inaccurate information during the drug discovery process.

Hence, we sought to identify the cases where using Caco-2 permeability measurements for *in vitro* screening and *in silico* model development purposes can lead to discordant human intestinal permeability predictions. Based on our findings, we propose recommendations on how to use Caco-2 as a primary *in vitro* permeability screening tool and for developing *in silico* predictive models, as well as for improving the current regulatory guidance on how Caco-2 can be used for human intestinal permeability screening during drug discovery and development.

Identifying Compounds for Which Caco-2 May Poorly Predict Human Intestinal Permeability Measurements

Substrates of Highly Expressed Human Small Intestinal Transporters: Peptide, Amino Acid, and Nucleoside Transporters

In vitro Caco-2 permeability measurements have been found to correlate well with *in vivo* human intestinal permeability measurements for passively absorbed drugs, but much less so for compounds that are in part transported by carrier-mediated mechanisms (9,19). In those cases, permeability measurements were found to be much higher in humans than Caco-2 for carrier-mediated compounds, compared to passively absorbed compounds. These workers (9,19) also observed that discrepancies generally correlated with the expression differences of transporters between the

two membranes for which the carrier-mediated compounds are substrates. Hence, differences in the expression of a transporter between Caco-2 and the human intestine may explain differences in the permeability of a substrate between the two membranes, resulting in an incorrect *in vitro-in vivo* permeability prediction.

To determine which transporter substrates' permeabilities may be impacted by transporter expression differences between Caco-2 and the human intestine, we examined the relative expression of transporters in the human small intestine *versus* transporters in the Caco-2 cell line, and the ability of Caco-2 to make an accurate *in vitro-in vivo* permeability prediction for known transporter substrates.

In an analysis of 36 transporters, Hilgendorf and colleagues (20) found PEPT1 to be a highly expressed transporter in the human jejunum showing a significantly larger (≥ 10 -fold) difference in transporter expression than any other transporters that they analyzed. The permeabilities of several PEPT1 transporter substrates—cephalexin (21), cephadrine (22), and Gly-Sar (23)—have been shown to correlate with the expression levels of human PEPT1 in Caco-2 cells. Additionally, cephalexin's permeability has been shown to correlate with the expression of rat Pept1 in the rat jejunum. Chong *et al.* (24) were the first to illustrate that Caco-2 is deficient in predicting the high human intestinal absorption of PEPT1 substrates, amoxicillin and cephalexin, where intestinal absorption is directly related to intestinal permeability as defined by the BCS (2) and FDA (3). Hilgendorf *et al.* (20) also found HPT1 to be highly expressed in the small intestine, however, its expression level was comparable between Caco-2 and the human jejunum. Other SLC transporters such as OATPs, OATs, and OCTs were found to be moderately to poorly expressed in the small intestine and their expression differences from Caco-2 were < 10 -fold. Of the efflux transporters they analyzed, BCRP and MRP2 were found to be highly expressed in the small intestine, yet only BCRP had a ≥ 10 -fold difference in expression between Caco-2 and the human jejunum.

Englund and colleagues (25) also found PEPT1 and BCRP to be highly expressed in the small intestine, with variability being ≥ 10 -fold between their human intestinal segments and Caco-2. Seithel and coworkers (26) only found PEPT1 to be highly expressed in the human jejunum with a ≥ 10 -fold difference between the human jejunum and Caco-2. While they measured high expression levels of MRP2 and lower levels of BCRP in the small intestine, the variability in their MRP2 and BCRP expression levels between the small intestine and Caco-2 was < 10 -fold. In a separate analysis of 183 transporters, Sun and colleagues (19) found 87 transporters expressed in the human duodenum, of which at least five transporters that had a high expression in the small intestine and a relative expression of ≥ 10 -fold difference between the human duodenum and Caco-2 cell line. These not only included the peptide transporter, PEPT1, but also members of the family of amino acid, concentrative nucleoside, and equilibrative nucleoside transporters (Table I).

We investigated how transporter expression differences between the Caco-2 and the human intestine affected the *in vitro-in vivo* permeability correlation of Caco-2 and human intestinal permeability data for 30 drugs reported in the literature (4). Unlike Sun *et al.* (19), we conducted the correlations with Caco-2 permeability measurements from

Table I. Highly Expressed Small Intestinal Transporters (19,20,27,28) Where Their Expression Differences Between the Human Small Intestine and the Caco-2 Cell Line Have Been Found to be ≥ 10 -fold

Reference	Transporter name (gene name)	Transporter family/system type	Relative expression differences between the human intestine and Caco-2
Englund <i>et al.</i> (25) ^a	PEPT1 (SLC15A1)	Peptide	>10-fold higher in the ileum than in Caco-2
	BCRP (ABCG2) ^b	Efflux	>10-fold higher in the ileum than in Caco-2
Hilgendorf <i>et al.</i> (20) ^a	PEPT1 (SLC15A1)	Peptide	>10-fold higher in the jejunum than in Caco-2
	BCRP (ABCG2) ^b	Efflux	>10-fold higher in the jejunum than in Caco-2
Seithel <i>et al.</i> (26) ^a	PEPT1 (SLC15A1)	Peptide	10-fold higher in the jejunum than in Caco-2
Sun <i>et al.</i> (19)	PEPT1 (SLC15A1)	Peptide	>15-fold higher level in the duodenum than in Caco-2
	ATB ⁰ (SLC1A5)	Amino acid	>12-fold higher level in Caco-2 than in the duodenum
	GABA/noradrenaline (SLC3A2)	Amino acid	>20-fold higher level in the duodenum than in Caco-2
	CNT2 (SLC28A2)	Nucleoside	>110-fold higher level in the duodenum than in Caco-2
	ENT1 (SLC29A1)	Nucleoside	>25-fold higher level in Caco-2 than in the duodenum

^a These studies did not analyze amino acid and nucleoside transporters.
^b Other sources have reported BCRP to be moderately expressed in the small intestine (26–28) and have <10-fold variability between the small intestine and Caco-2 cell line (26).

datasets taken from the same laboratory so as to avoid interlaboratory variation and selection bias. In Fig. 1, we present the correlation coefficients of human intestinal permeability measurements *versus* those of Caco-2 from eight independent studies where metoprolol was included in the analysis. Of the 30 drugs with available human intestinal permeability data, four (amoxicillin, cephalixin, levodopa, and lisinopril) are known substrates of one or more of the

transporters with a ≥ 10 -fold difference in expression, particularly amino acid or peptide transporters. Including all of the drugs in each dataset, the correlation coefficients (r^2) ranged from 0.409 to 0.917. When the original datasets included any of the amino acid or peptide substrates, the r^2 values ranged from 0.409 to 0.651; when they did not, the r^2 values ranged from 0.835 to 0.917. For the five datasets that included the amino acid or peptide substrates, we found that the

Fig. 1. Effect of inclusion of amino acid and peptide transporter substrates on the *in vitro-in vivo* correlations of Caco-2 and human intestinal permeability measurements for eight studies (29–36), where Caco-2 permeability measurements were available for at least 6 drugs, including metoprolol, of the 30 drugs with available human intestinal permeability measurements. The total number of compounds in each correlation range from 6 to 22, with the average being 11

correlations (Fig. 1) improved when excluding these substrates one by one or completely. When all of the amino acid and peptide substrates were absent from all of the datasets, seven of the eight datasets of Caco-2 permeability measurements correlated well ($r^2 > 0.800$) with human intestinal permeability measurements. The *in vitro-in vivo* permeability correlations are strong even when including substrates of other transporters that are <10-fold difference in expression between the two membranes, suggesting that Caco-2 can predict the human intestinal permeability of substrates of transporters that differ no more than 10-fold between Caco-2 and the human intestine.

Due to the limited availability of human intestinal permeability rate data, we analyzed the ability of Caco-2 measurements to predict the closest surrogate for such data, namely the human extent of absorption, where we could find much more information available in the literature. Of more than 50 compounds for which *in vitro-in vivo* correlations agreed, we found more than 30 that are known substrates of transporters—such as OAT, OATP, OCT, and P-gp—where expression differences between Caco-2 and the human intestine are <10-fold. This suggests that there may be a likely threshold for transporter expression differences between Caco-2 and the human intestine for Caco-2 measurements to be able to accurately predict the intestinal permeability of transporter substrates. When we examined the lipophilicity of the 66 compounds for which Caco-2 permeability and human extent of absorption measurements were available, we found that the lipophilic compounds (where $\text{Log}P \geq 1$) had high Caco-2 permeability measurements that correctly predicted their high extents of absorption. Hence, it is possible that the lipophilic properties of the compounds (and consequently higher passive permeabilities) may explain the better correlations of OAT, OATP, OCT, and P-gp substrates rather than their <10-fold expression differences between the two membranes. However, we recognize that we could not find studies with Caco-2 permeability measurements for other lipophilic compounds like BCRP substrates and a high-permeability reference standard to evaluate if their human extents of intestinal absorption could be correctly predicted by their physicochemical properties rather than reported ≥ 10 -fold differences in transporter expression between Caco-2 and the human small intestine.

In Table II, we identify 11 hydrophilic drugs where low Caco-2 permeability measurements in the literature do not correctly predict the high extents of absorption of these compounds. These drugs were found to be substrates of members of the amino acid, nucleoside, and peptide transporter families, where the expression differences between Caco-2 and the human intestine are ≥ 10 -fold. We additionally include in Table II three hydrophilic drugs (lisinopril, gabapentin, and lamivudine) that are also substrates of these transporters but have low Caco-2 permeability measurements and low extents of absorption, to demonstrate that Caco-2 cannot discriminate between high and low extents of absorption for these hydrophilic compounds. The passive permeabilities of these compounds are expected to be low, due to the compounds being poorly lipophilic in nature (58). Hence, their ability to exhibit a high intestinal permeability or a high extent of absorption will likely be dependent on the degree of carrier-mediated transport. We examined the literature for Caco-2 permeability measurements between pH 5.0 and 7.5 to see if pH optimization could

possibly improve predictability. Even though pH slightly increased the permeability of some substrates, we could not find any published evidence of changing the permeability from low to high relative to a high-permeability reference standard. Hence, it may not be possible to optimize Caco-2 assays to correctly predict the intestinal permeabilities of these transporter substrates. This may be due to the inherent expression of these transporters in the colonic region, from which Caco-2 is derived, which is much lower than regions of the small intestine (25). Because of the significant difference in expression levels (≥ 10 -fold) for these transporters between Caco-2 and the human small intestine, Caco-2 measurements will be unable to predict which of these compounds have high intestinal absorption.

Differences in the Paracellular Junctions Between Caco-2 and the Human Intestine do not Explain the Inaccurate In Vitro-In Vivo Permeability Predictions by Caco-2 for Hydrophilic Compounds

Hydrophilic compounds are generally believed to use the paracellular rather than transcellular pathway through intestinal membranes because these compounds lack the necessary lipophilic properties to penetrate the cell membrane (8,59). When a hydrophilic compound is observed to have a high intestinal permeability or be highly absorbed, the first hypothesis considered is that this occurs due to paracellular diffusion. Caco-2 has been shown to have a significantly lower number of paracellular pores than the human intestine (60), which may explain why Caco-2 measurements underpredict the intestinal permeabilities of several hydrophilic compounds that are absorbed paracellularly (61). Hence, we were interested in understanding whether the differences between the paracellular pathway in Caco-2 and the human intestine could explain the poor *in vitro-in vivo* permeability predictions of hydrophilic compounds by Caco-2.

In Table III, we list three different cases for the intestinal transport of hydrophilic compounds *via* paracellular passive and transcellular carrier-mediated mechanisms. In Case 1, we identify four drugs where greater than 80% of their intestinal absorption has been estimated to result from paracellular passive diffusion. For these drugs, the intestinal absorption is characterized to be low (less than 90% according to BCS). We could not find a case in the literature when the absorption of a highly absorbed drug (having an extent measure $\geq 90\%$) definitively occurs more than 80% *via* the paracellular pathway. We believe that this does not occur because the paracellular pathway can be saturated (or more colloquially “clogged up”) since it only occupies 0.01–0.1% of the total surface area of the intestine (75,76), and the size-restricting gate function of the paracellular pathway limits permeability (77). Because of this, we further believe that a high extent of intestinal absorption will not be solely a consequence of paracellular diffusion and that an inaccurate *in vitro-in vivo* permeability prediction cannot be explained solely from differences in the paracellular pathways between Caco-2 and the human intestine. Thus, compounds whose intestinal absorption occur $>80\%$ *via* the paracellular pathway will exhibit a low Caco-2 permeability and a low intestinal permeability or extent of absorption.

Table II. Inaccurate Predictions of Human Intestinal Absorption by Caco-2 Explained by Compounds Being Substrates of Highly Expressed Transporters that Differ ≥ 10 -fold in Expression Between The Human Intestine and Caco-2

Compound	cLogP ^a	Transporter substrate	Caco-2 permeability	Extent of absorption
Lisinopril	-1.69	Peptide (37,38)	Low (24,31,37)	Low (24,31)
Amoxicillin	-1.87	Peptide (24,39)	Low (24,30,31)	High (24,30,31)
Cefadroxil	-2.51	Peptide (37,40)	Low (37)	High (41)
Ceftibuten	-1.21	Peptide (42)	Low (43)	High (43)
Cephalexin	-1.84	Peptide (24,37,44)	Low (24,30,31,37,43)	High (4,24,30,31,41,43)
Cephadrine	-1.73	Peptide (37,45)	Low (37)	High (41)
Enalapril	0.67	Peptide (46)	Low (47)	High (4)
Loracarbef	-0.47	Peptide (48)	Low (31)	High (31,41)
Gabapentin	-0.66	Amino acid (49,50)	Low (31)	Low (31)
Levodopa	-2.82	Amino acid (49)	Low (33)	High (4,33)
Ofloxacin	-0.51	Amino acid (51)	Low (52)	High (41)
Pregabalin	-0.92	Amino acid (50)	Low (53)	High (41)
Lamivudine	-1.46	Nucleoside (54)	Low (55)	Low (56)
Zidovudine	0.04	Nucleoside (54)	Low (31,36)	High (31,36)

^a cLogP values taken from Ref. (57).

In Case 2, we identify three hydrophilic drugs that are in part (14 to 60%) reported to be paracellularly absorbed and that are also substrates of transporters having a <10 -fold difference in expression between Caco-2 and the human intestine, where the transporters have been reported to be poorly to moderately expressed in the human intestine by Sun *et al.* (19) (Table III). These three drugs exhibit low Caco-2 permeabilities and low extents of absorption we believe due to their intestinal transport being limited by their carrier-mediated and paracellular permeabilities.

In the third case, we identify two hydrophilic drugs (ofloxacin and pregabalin) that are substrates of transporters having a ≥ 10 -fold difference in expression between Caco-2 and the human intestine, and these transporters are also among the most highly expressed transporters characterized by Sun *et al.* (19) in the human intestine (Table III). While these hydrophilic compounds can be in part paracellularly absorbed, it is likely that their affinity for a highly expressed intestinal transporter—such as the amino acid, nucleoside, or peptide transporter—is responsible for their high intestinal absorption as demonstrated in Table II. Pregabalin is an example of a hydrophilic compound

whose high extent of absorption was long believed to result from paracellular diffusion until a highly expressed amino acid transporter was implicated in its intestinal transport (53). Similarly, we believe that the high extent of absorption and low Caco-2 permeability of sotalol likely involves a highly expressed intestinal transporter in contrast to some authors who hypothesize that sotalol's high extent of absorption is due to paracellular diffusion (12,41,74). For these Case 3 drugs, Caco-2 will not be able to predict the high intestinal absorption of these hydrophilic compounds due to the cellular membrane's significant underexpression of relevant transporters compared to the human intestine.

The Need for Larger, Single-Source Caco-2 Permeability Datasets

The existence of variability in Caco-2 permeability measurements between laboratories has been widely recognized (23,78). To help understand the impact of selecting Caco-2 permeability data from multiple sources to develop an *in silico* model, Fig. 2 illustrates the degree of variability in Caco-2 permeability measurements for 20 out of 30 drugs,

Table III. Cases of Intestinal Transport by Paracellular Diffusion and Carrier-mediated Processes for Hydrophilic Compounds and Their Caco-2 Permeabilities and Extents of Absorption

Case	Compound	cLogP ^a	Paracellular contribution	Transporter substrate	Caco-2 permeability	Extent of absorption
1	Acyclovir	-2.42	99% (62)	OAT (63), OCT (63)	Low (36)	Low (64)
	Atenolol	-0.11	80–100% (65)	P-gp (66)	Low (35,36)	Low (4,64)
	Famotidine	-1.17	98% (62)	OAT (67), OCT (67), P-gp (34)	Low (34)	Low (68)
	Ganciclovir	-2.73	99% (62)	OAT (63), OCT (63)	Low (36)	Low (64)
2	Cimetidine	0.19	14–30% (69)	OAT (67), OCT (67), P-gp (70)	Low (36)	Low (4)
	Furosemide	1.90 ^b	55% (69)	OAT (71), P-gp (72)	Low (35)	Low (4)
	Ranitidine	0.67	45–60% (73)	OAT (67), OCT (67), P-gp (70)	Low (36)	Low (4)
3	Ofloxacin	-0.51	8% (62)	Amino acid (51)	Low (52)	High (41)
	Pregabalin	-0.92	ND ^d (53)	Amino acid (50)	Low (53)	High (41)
	Sotalol ^c	0.23 ^b	ND ^d (74)	ND ^d	Low (12)	High (41)

^a cLogP values taken from Ref. (57).

^b Measured LogD_{7.4} values of furosemide and sotalol are -1.54 and -0.79, respectively (57).

^c Hypothesized to belong to this set.

^d ND, not determined.

that is those drugs for which both human intestinal permeability rate measurements are available (4) and multiple sources report their Caco-2 permeability measurements (8,20,29–36,79). Permeability values for each compound were included if metoprolol had also been studied in the Caco-2 evaluation to determine if the observed interlaboratory variability could be mitigated when the measurements were normalized to a reference standard. The ratios of the maximum to minimum Caco-2 permeability measurements found for each compound in the literature are presented in Table IV with and without normalization to metoprolol's permeability.

Permeability values varied greatly for lipophilic compounds and much more so for hydrophilic compounds. The largest interlaboratory variability was observed for substrates with a high affinity for amino acid and peptide transporters, *e.g.*, amoxicillin (180-fold) and levodopa (100-fold), followed by compounds that are paracellularly absorbed, *e.g.*, terbutaline (27-fold) and atenolol (25-fold). With normalization to the reference standard metoprolol, the interlaboratory variability in permeability measurements for hydrophilic compounds worsened for the transporter substrates and for some of the paracellularly absorbed compounds. Even though the variability decreased for some paracellular compounds, the resulting normalized variability was still high. Hence, for hydrophilic compounds, transporter expression and paracellular pathway differences will be a major source of variability across Caco-2 cell lines from different laboratories. Normalization to a reference standard will not successfully reduce the variability in Caco-2 permeability measurements from different laboratories. Lipophilic compounds (such as metoprolol, naproxen, propranolol, and verapamil) that are

Table IV. Variability in Caco-2 Permeability Measurements from Different Investigators

Drug	cLogP ^a	Ratio of maximum to minimum Caco-2 permeability measurement ^b	Ratio of maximum to minimum (with normalization to metoprolol) ^b
<i>Amoxicillin</i>	−1.87	180	508
<i>Cephalexin</i>	−1.84	9.96	77.5
<i>Levodopa</i>	−2.82	100	34.5
<i>Lisinopril</i>	−1.69	5.77	25.4
<i>Atenolol</i>	−0.11	25.4	9.88
<i>Cimetidine</i>	0.19	4.58	4.38
<i>Furosemide</i>	1.90^c	9.29	39.2
<i>Hydrochlorothiazide</i>	−0.37	11.9	32.2
<i>Ranitidine</i>	0.67	7.00	4.98
<i>Terbutaline</i>	0.48	27.4	133
Antipyrine	0.20	7.56	2.74
Carbamazepine	2.38	1.74	1.53
Desipramine	4.47	1.76	1.31
Enalaprilat	0.88	2.98	1.69
Ketoprofen	2.76	5.50	1.57
Metoprolol	1.49	7.78	1.00
Naproxen	2.82	8.47	2.24
Piroxicam	1.89	1.32	1.65
Propranolol	2.75	7.75	3.18
Verapamil	4.47	3.91	2.84

^a cLogP values taken from Ref. (57).

^b The maximum and minimum Caco-2 permeability measurements were determined from Refs. (8,20,29–36,79).

^c Measured LogD_{7.4} values of furosemide is −1.54 (57).

Bold italic compounds are known substrates of amino acid and peptide transporters.

Bold compounds are compounds that are paracellularly absorbed.

Fig. 2. The apparent Caco-2 permeability coefficients selected from 11 studies that included metoprolol (8,20,29–36,79) for 20 of the 30 drugs where human intestinal permeability data are available (4). The number of observations (filled circles) per compound is 2 to 11. The lines represent arithmetic mean values

mostly absorbed by passive transcellular processes across the intestinal mucosa (19) also had marked variability in Caco-2 permeability data from different laboratories (Table IV). However, as seen in Table IV, these variances between maximum and minimum permeability measurements are generally lower, and normalization to metoprolol generally decreases the variability.

These findings suggest that the interlaboratory differences are not just a function of Caco-2 cell passage number changing inherent permeability, but instead reflect the marked variability and unpredictability of using Caco-2 permeability measurements from multiple studies for *in silico* correlations. Thus, the selection of particular permeability values from multiple studies could significantly skew correlations using these measurements and produce misleading information. Despite interlaboratory differences, good correlations between Caco-2 and human intestinal permeability measurements can be achieved provided that the data used for the correlations are from the same investigators, as demonstrated earlier with datasets from 7 different investigators. In order to ensure that Caco-2 permeability measurements are a reliable surrogate for human intestinal permeability measurements for *in silico* work, larger datasets of Caco-2 permeability measurements from the same sources are needed for creating accurate *in silico* predictive models.

Table V. Published *In Silico* Predictive Models Using Caco-2 Permeability Measurements

Prediction model	Data taken from multiple sources?	Known substrates of amino acid, peptide, and/or nucleoside transporters included ^a	Suggested paracellular compounds included ^a
Alsenz and Haenel (29)	No	Amoxicillin, Levodopa, Lisinopril, Phenoxymethylpenicillin	Atenolol, Cimetidine, Creatinine, Furosemide, Hydrochlorothiazide, PEG-400, Ranitidine, Terbutaline
Avdeef and Tam (80)	Yes	Amoxicillin, Cephalixin, Enalapril, Levodopa, Lisinopril, Valacyclovir	Atenolol, Cimetidine, Creatinine, Furosemide, Hydrochlorothiazide, multiple PEG compounds, Ranitidine, Terbutaline
Avdeef <i>et al.</i> (81)	Yes	<i>No known substrates</i>	Atenolol, Cimetidine, Nadolol, Terbutaline
Akamatsu <i>et al.</i> (82) ^b	Yes	Amoxicillin, Cefatrizine, Cephalixin, Fosinopril, Loracarbef	Nadolol, Ranitidine
Bergström <i>et al.</i> (83)	No	Amoxicillin, Zidovudine	Acyclovir
Castillo-Garit <i>et al.</i> (84)	Yes	Amoxicillin, Cephalixin, Enalapril, Gly-Pro, Lisinopril, L-Phenylalanine, Penicillin, Zidovudine	Acetubutolol, Acyclovir, Atenolol, Cimetidine, Furosemide, Hydrochlorothiazide, Mannitol, Nadolol, Ranitidine, Scopolamine, Terbutaline
Collett <i>et al.</i> (85)	No	<i>No known substrates</i>	<i>No suggested compounds</i>
Corti <i>et al.</i> (86)	Yes	<i>No known substrates</i>	Acyclovir, Atenolol, Furosemide, Hydrochlorothiazide, Nadolol, Ranitidine
Cruciani <i>et al.</i> (87)	No	Amoxicillin, AZT (Zidovudine), Bretylium, Cephalixin, Gabapentin, Lisinopril, Loracarbef, Zidovudine	Acetubutolol, Atenolol, Hydrochlorothiazide, Sotalol, Terbutaline
Di Fenza <i>et al.</i> (88)	No		Acetubutolol, Acyclovir, Atenolol, Cimetidine, Hydrochlorothiazide, Mannitol, Nadolol, Ranitidine, Terbutaline, Urea
Du-Cuny <i>et al.</i> (89)	Yes	Amoxicillin, Enalapril, Zidovudine	Acetubutolol, Acyclovir, Atenolol, Cimetidine, Furosemide, Hydrochlorothiazide, Mannitol, Nadolol, Ranitidine, Scopolamine, Terbutaline
Ekins <i>et al.</i> (90)	No	<i>No known substrates</i>	<i>No suggested compounds</i>
Ertl <i>et al.</i> (91)	No	<i>No known substrates</i>	Atenolol, Mannitol, Terbutaline
Föger <i>et al.</i> (92)	Yes	<i>No known substrates</i>	<i>No suggested compounds</i>
Fossati <i>et al.</i> (47)	No	Ampicillin, Cefmetazole, Cefoperazone, Cefoxitin, Enalapril, Norfloxacin, Zidovudine	Furosemide, Nadolol, Ranitidine, Terbutaline
Fujiwara <i>et al.</i> (93)	Yes		Acetubutolol, Acyclovir, Atenolol, Cimetidine, Hydrochlorothiazide, Mannitol, Ranitidine, Terbutaline
Goodwin <i>et al.</i> (94)	No	<i>No known substrates</i>	<i>No suggested compounds</i>
Gres <i>et al.</i> (30)	No	Amoxicillin, Cephalixin	Atenolol, Mannitol, PEG-400, Terbutaline
Grice <i>et al.</i> (95)	Yes	Amoxicillin, Bretylium Tosylate, Captopril, Cefadroxil, Cephalixin, Enalapril, Gabapentin, Norfloxacin, Penicillin V, Zidovudine	Acetubutolol, Acyclovir, Cimetidine, Furosemide, Hydrochlorothiazide, Lucifer Yellow, Nadolol, Terbutaline
Guangli and Yiyu (96)	Yes	Amoxicillin, Enalapril, Ceftriaxone, Ganciclovir, Zidovudine	Acetubutolol, Acyclovir, Atenolol, Cimetidine, Furosemide, Hydrochlorothiazide, Mannitol, Nadolol, Ranitidine, Scopolamine, Terbutaline, Urea
Hilgers <i>et al.</i> (97)	No	<i>No known substrates</i>	<i>No suggested compounds</i>
Hou <i>et al.</i> (98)	Yes	Amoxicillin, Enalapril, Ceftriaxone, Ganciclovir, Zidovudine	

Table V. (continued)

Prediction model	Data taken from multiple sources?	Known substrates of amino acid, peptide, and/or nucleoside transporters included ^a	Suggested paracellular compounds included ^a
Irvine <i>et al.</i> (31)	No	Amoxicillin, AZT (Zidovudine), Bretium Tosylate, Cefazirine Propylene Glycol, Cefuroxime Sodium, Cephalixin Monohydrate, Gabapentin, Lisinopril Dihydrate, Loracarbef Monohydrate, Penicillin V	Acetubutolol, Ayclovir, Atenolol, Cimetidine, Furosemide, Hydrochlorothiazide, Mannitol, Nadolol, Ranitidine, Scopalamine, Terbutaline, Urea
Jung <i>et al.</i> (32)	No	<i>No known substrates</i>	<i>No suggested compounds</i>
Krurup <i>et al.</i> (99)	No	<i>No known substrates</i>	Acetubutolol
Kulkarni <i>et al.</i> (100)	No	Zidovudine	Ayclovir, Atenolol, Mannitol, Nadolol, Terbutaline
Li <i>et al.</i> (34)	No	<i>No known substrates</i>	Ayclovir, Atenolol, Cimetidine, Furosemide, Hydrochlorothiazide, Mannitol, Nadolol, Ranitidine HCl, Sotalol HCl, Terbutaline Hemisulfate
Liang <i>et al.</i> (101)	No	<i>No known substrates</i>	Ranitidine, Terbutaline
Linnankoski <i>et al.</i> (102)	No	Ganciclovir	Acetubutolol, Ayclovir, Cimetidine, Hydrochlorothiazide, Mannitol, Ranitidine, Scopalamine, Urea
Lundquist <i>et al.</i> (103)	No	<i>No known substrates</i>	Atenolol, Creatinine, Mannitol, Nadolol, Terbutaline
Marrero Ponce <i>et al.</i> (104)	No	<i>No known substrates</i>	Mannitol, Terbutaline, Urea
Marrero Ponce <i>et al.</i> (105)	No	<i>No known substrates</i>	Atenolol, Mannitol, Terbutaline
Marrero Ponce <i>et al.</i> (106)	Yes	Amoxicillin, Cephalixin, D-Phe-L-Pro, Gabapentin, Gly-Pro, Lisinopril, L-Phenylalanine, Penicillin G, Zidovudine	Acetubutolol, Ayclovir, Cimetidine, Hydrochlorothiazide, Mannitol, Ranitidine, Scopalamine, Urea
Matsson <i>et al.</i> (107)	No	Glycylsarcosine, Lobucavir, Valacyclovir	Acetubutolol, Atenolol, Cimetidine, Furosemide, Mannitol, Nadolol, Urea
Nordquist <i>et al.</i> (108)	No	AZT (Zidovudine), Benzylpenicillin, L-Alanine, L-Dopa, Gabapentin, Methyldopa, <i>No known substrates</i>	Ayclovir, Atenolol, Cimetidine, Creatinine, Nadolol, Ranitidine, Scopalamine, Terbutaline
Norinder <i>et al.</i> (109)	No	<i>No known substrates</i>	Acetubutolol, Atenolol, Cimetidine, Inulin, Mannitol, Nadolol, Ranitidine, Urea
Norinder and Osterberg (110)	No	<i>No known substrates</i>	Atenolol, Mannitol, PEG, Terbutaline
Osterberg and Norinder (111)	No	<i>No known substrates</i>	Atenolol, Mannitol, Terbutaline
Osterberg and Norinder (112)	Yes	<i>No known substrates</i>	Acetubutolol
Paixão <i>et al.</i> (113)	Yes	Amoxicillin, Ampicillin, Ceftriaxone, Cefuroxime, Cephalixin, Cephadrine, Enalapril, Glycine, Gly-Pro, Levodopa, Lisinopril, Loracarbef, Norfloxacin, Penicillin V, Valacyclovir, Zidovudine	Acetubutolol, Ayclovir, Atenolol, Cimetidine, Famotidine, Furosemide, Hydrochlorothiazide, Mannitol, multiple PEG compounds, Ranitidine, Scopalamine, Sotalol, Terbutaline
Palm <i>et al.</i> (114)	No	<i>No known substrates</i>	Atenolol
Palm <i>et al.</i> (115)	No	<i>No known substrates</i>	Atenolol

Table V. (continued)

Prediction model	Data taken from multiple sources?	Known substrates of amino acid, peptide, and/or nucleoside transporters included ^a	Suggested paracellular compounds included ^a
Parrott and Lavé (116)	No	Ganciclovir, Penicillin V	Acyclovir, Atenolol, Hydrochlorothiazide, Ranitidine, Terbutaline
Pickett <i>et al.</i> (117) ^b	Yes	No known substrates	No suggested compounds
Refsgaard <i>et al.</i> (118) ^b	No	No known substrates	Furosemide, Ranitidine
Ren and Lien (119)	Yes	Zidovudine	Acebutolol, Acyclovir, Atenolol, Cimetidine, Hydrochlorothiazide, Mannitol, Nadolol, Terbutaline
Skolnik <i>et al.</i> (120)	No	Cefuroxime, Cephalixin, Ganciclovir, Lisinopril, Norfloxacin	Acebutolol, Atenolol, Cimetidine, Furosemide, Hydrochlorothiazide, Nadolol, Ranitidine, Terbutaline
Stenberg <i>et al.</i> (121) ^b	No	No known substrates	Atenolol, Cimetidine, Mannitol
Thomas <i>et al.</i> (122)	No	Benazepril, Benzylpenicillin, Breytilium, Enalapril, Gabapentin, Norfloxacin, Ofloxacin	Acebutolol, Acyclovir, Atenolol, Famotidine, Hydrochlorothiazide, Nadolol, Ranitidine, Scopalamine, Terbutaline
Tronde <i>et al.</i> (123)	No	No known substrates	Atenolol, Mannitol, Terbutaline
van de Waterbeemd <i>et al.</i> (124)	No	No known substrates	
Yamashita <i>et al.</i> (125)	Yes	Ganciclovir, Zidovudine	Acebutolol, Acyclovir, Atenolol, Cimetidine, Hydrochlorothiazide, Mannitol, Nadolol, Ranitidine, Scopalamine, Terbutaline
Yazdanian <i>et al.</i> (36)	No	Zidovudine	Acebutolol, Acyclovir, Atenolol, Cimetidine, Hydrochlorothiazide, Mannitol, Nadolol, Ranitidine, Terbutaline, Urea
Zhang <i>et al.</i> (126)	Yes	No known substrates	Acebutolol, Atenolol, Mannitol, Terbutaline
Zhu <i>et al.</i> (127)	Yes	Amoxicillin, Breytilium Tosylate, Captopril, Cefadroxil, Cephalixin, Enalapril, Gabapentin, Norfloxacin, Penicillin V, Zidovudine	Acebutolol, Acyclovir, Cimetidine, Furosemide, Hydrochlorothiazide, Lucifer Yellow, Nadolol, Terbutaline

^a Likely underestimated as these compounds have not been thoroughly characterized.^b Entire dataset not published to examine all of its compounds.

In Silico Work Considerations

In silico predictive models of intestinal permeability have been proposed to replace a large part of conventional Caco-2 screening in drug discovery (13). For these models to be reliable and accurate, their predictions will be greatly dependent on the quality of Caco-2 permeability measurements that are used as a substitute for human intestinal permeability measurements in their development and validation.

Table V presents some common practices of selecting Caco-2 permeability measurements for datasets used to generate *in silico* models. We observed that these datasets used similar absolute Caco-2 permeability measurements to determine the high versus low-permeability classification of the compounds. However, we show in Table IV that the absolute Caco-2 permeability measurements for compounds (e.g., the high-permeability reference standard, metoprolol) vary for different studies. Hence, rather than using a standardized permeability cut-off for determining Caco-2 permeability, it is most accurate to determine the permeability of a compound relative to a permeability reference standard as recommended by the FDA (3). Out of 55 published *in silico* models, more than 34% have Caco-2 permeability measurements in their datasets from more than one source. We show in Table IV the variability associated with selecting compounds from more than one source. We found that the variability of Caco-2 permeability measurements for lipophilic compounds could only be minimized with normalization to a reference standard. We show that the variability in Caco-2 permeability measurements for hydrophilic compounds is high regardless of normalization to a reference standard. Hence, it is most appropriate to select Caco-2 permeability measurements for developing *in silico* models from the same source. We were only able to achieve good *in vitro*–*in vivo* permeability correlations when datasets of Caco-2 permeability measurements originated from the same laboratory. Selecting particular permeability measurements from different laboratories for *in silico* datasets not only introduces bias or variability in the correlations, but can also result in generating models that produce misleading information for drug design.

As discussed earlier, paracellular pathway and transporter expression differences between Caco-2 and the human intestine can affect the *in vitro* predictions of human intestinal permeabilities for hydrophilic drugs. At least 87% of the *in silico* models in Table V use datasets containing Caco-2 permeability measurements for hydrophilic drugs, defined here as compounds having $\text{Log}P$ (or $\text{Log}D_{7.4}$) values <1 (120). We observed that estimations of the human intestinal permeability or absorption of these compounds were generally underpredicted. Complete exclusion of all hydrophilic compounds from training and validation datasets produces models that are restricted to a confined chemical space. Hence, for the 87% of *in silico* models that also include implied paracellularly absorbed compounds, rather than eliminating these compounds from datasets, we suggest that correction factors should be in place to adjust for the pore radius of tight junctions in Caco-2 monolayers to improve their paracellular permeability prediction (128).

While it is generally accepted that substrates of carrier-mediated transporters have higher *in vivo* permeabilities than those measured in Caco-2, we recognize that there are many examples of compounds where a low permeability in Caco-2 is reflective of a low permeability *in vivo*. However, we show

that these compounds are likely substrates of transporters that have <10 -fold variability in expression between Caco-2 and the human small intestine. For compounds that are particularly substrates of highly expressed intestinal transporters that have a ≥ 10 -fold variability in expression between Caco-2 and the human small intestine such as the amino acid, nucleoside, and peptide transporters, we show not only that their human intestinal permeability predictions are incorrect but that when Caco-2 permeability measurements are included in *in vitro*–*in vivo* correlations, poorer correlations are obtained. Similarly, it is very possible that 50% of the published models that include Caco-2 permeability measurements for these substrates as part of their training and/or validation datasets during *in silico* model development can give skewed, misleading correlations and therefore these compounds should be excluded from correlations. In fact, we found that the models could not predict the high intestinal permeability or absorption of these substrates. The only scenario where including these substrates may not impact an *in silico* correlation is perhaps when predicting passive intestinal permeability. Consequently, these *in silico* models will not accurately predict the high intestinal permeability of hydrophilic compounds in drug discovery, which we believe will only be seen through their affinity for carrier-mediated transporters that are significantly underexpressed in the Caco-2 cell line.

In Vitro Screening Considerations

It is very important to accurately determine whether a drug candidate has desirable biopharmaceutical properties, such as high intestinal permeability, during drug discovery. With a standardized, validated permeability method, Caco-2 can predict the high intestinal permeability of lipophilic compounds relative to a permeability standard such as metoprolol. We found that this same case does not apply to some hydrophilic compounds. Because hydrophilic drugs represent about 27% of oral drugs presently on the market, and approximately the same percentage of NMEs being investigated by the industry (129), it is crucial to recognize the limitations Caco-2 may have as the primary screening source of NMEs for lead selection, lead optimization, and candidate prioritization.

It was discussed earlier that the intestinal permeability of hydrophilic compounds will likely be dependent on either passive paracellular diffusion and/or carrier-mediated transport mechanisms. Strategies for improving the oral absorption of hydrophilic compounds have either attempted to enhance drug absorption *via* the paracellular pathway (75,130,131), or to create prodrug forms of the NME that target highly expressed intestinal transporters, such as the amino acid (132,133), nucleoside (134), and peptide transporters (135,136). We did not find any evidence to support that manipulating the paracellular pathway increased the intestinal permeability or absorption of a hydrophilic compound from low to high (i.e., changing a drug from BCS Class 3 to Class 1). Hence, we believe that the latter strategy of targeting a highly expressed intestinal transporter may be more successful in improving the intestinal permeability or BCS absorption of a hydrophilic NME.

For compounds that are paracellularly absorbed, Caco-2 will underpredict their human intestinal permeabilities due to the lesser number of paracellular pores in Caco-2 compared

to the human intestine (60). Correcting for their paracellular differences can improve their paracellular permeability prediction (128). However, Caco-2 is not suitable for estimating the contribution of the paracellular pathway in the intestinal transport of NMEs because it lacks the sensitivity to detect changes in the paracellular pathway due to the presence of a lesser number of pores. As such, other systems have been proposed as better methodologies than Caco-2 for evaluating paracellular intestinal permeability (60,61,137).

Several hydrophilic drugs may be highly absorbed because of their carrier-mediated transport. As such, we do not recommend assessing the BCS classification of hydrophilic compounds using the *cLogP* method, where hydrophilic compounds are generally classified as being poorly absorbed due to their low distribution coefficients (138). For example, this method could not differentiate between BCS Class 1 zidovudine and BCS Class 3 lamivudine, thereby classifying both drugs as BCS Class 3 compounds. The better affinity for a nucleoside transporter may be the reason for zidovudine being highly absorbed and lamivudine being moderately absorbed and ineligible to be a BCS Class 1 drug.

It is likely that Caco-2 permeability measurements will incorrectly predict the high intestinal permeabilities of many hydrophilic compounds because of significant transporter expression differences between Caco-2 and the human intestine, such as for the amino acid, nucleoside, and peptide transporters (19). We could not find any published evidence of an optimized Caco-2 permeability method to accurately predict the high intestinal permeability of these hydrophilic substrates. As a result, we recommend using alternate or additional methods to Caco-2 for not only determining the intestinal permeability of hydrophilic compounds, but also for evaluating the substrate specificity of highly expressed intestinal transporters on the intestinal permeability of hydrophilic compounds. While alternate *in vitro* systems (37,45) may be suitable for evaluating their substrate specificity, these cell lines may under- or overexpress these transporters relative to levels in the human intestine, which in turn can under- or overpredict the permeability of these compounds. Hence, the most suitable methodology to characterize overall carrier-mediated transport and the intestinal permeability of these compounds may be using the rat intestine (139).

Regulatory Considerations

With current regulatory standards conveying the importance of compounds that have high intestinal permeability (3), drug discovery and development programs that use Caco-2 as a primary permeability screening tool may be discarding viable drug candidates that are incorrectly predicted to be poorly permeable. For example, we have shown how hydrophilic compounds that are substrates of members of the amino acid, nucleoside, and peptide transporter families can have high intestinal permeabilities and be highly absorbed. However, due to the significant difference in the expression of these transporters between Caco-2 and the human intestine, the high intestinal permeability of these compounds may be incorrectly predicted to be poorly permeable by Caco-2.

The current FDA BCS Guidance for Industry (3) can be viewed as perpetuating the misconception that methods for

evaluating intestinal permeability should be limited to studying passively transported compounds. Not only have we discussed earlier why this is extremely difficult, but Simon and colleagues (140) have shown that four of the nine high-permeability model drugs, and all four low-permeability model drugs, that are recommended for evaluating permeability in the Guidance for Industry, exhibit significant active intestinal transport. Whenever the regulatory agency has found discrepancies between Caco-2 and an *in vivo* method (18), where they use the *in vivo* information to “trump” *in vitro* data, it is likely that the difference in permeability observed is due to a carrier-mediated mechanism that is lacking in the Caco-2 cell line. Thus, it is important for the Guidance for Industry to clarify that Caco-2 will likely exhibit passive permeability, while the other human or animal methods for evaluating intestinal permeability may involve carrier-mediated transport. When intestinal permeability measured across the Caco-2 cell line is high (*i.e.*, the passive permeability of the compound is high), then the total permeability will still be high according to the BCS, even if there is a carrier-mediated component not being characterized in Caco-2. However, when Caco-2 (or passive) permeability is low, hydrophilic compounds that are substrates for transporters (*e.g.*, amino acid, nucleoside, and peptide transporters) can still exhibit a BCS Class 1 high permeability. Hence, we suggest that the FDA in its Guidance for Industry should caution drug discovery programs that are evaluating hydrophilic compounds from using Caco-2 as a primary screening source. Moreover, they should recommend using methods to classify permeability (such as those in humans or rats) that may be more representative of the absorptive processes for these compounds, thereby enabling programs to correctly assess the human intestinal permeability of these compounds.

We believe that not all of the model drugs selected by the FDA are appropriate for establishing the suitability of a permeability method. For example, the list of model drugs includes amoxicillin, a peptide transporter substrate, which, as discussed earlier, can yield discrepancies in permeability between *in vitro* and *in vivo* methods. Therefore, we recommend that amoxicillin be deleted from the list of model drugs and possibly be substituted by another lowly permeable drug that is more passively absorbed.

CONCLUSIONS

Several recent articles have extensively elaborated on the importance, concepts, and problems of drug permeation across biological membranes in drug discovery, development, and regulatory evaluation (17,141,142). Many of these expert reviews have concluding remarks stating that better systems need to be implemented in order to achieve good *in vitro*–*in vivo* correlations that are crucial for progress in the field. While it seems ideal to work towards developing new *in vitro* systems that can better predict the *in vivo* performance of NMEs, it is not practical to continue using current *in vitro* permeability systems in drug discovery, development, and regulatory settings, all the while neglecting the known limitations of these systems.

Industry and regulatory scientists recognize the need for *in silico* and *in vitro* models that can accurately forecast *in vivo* information and serve as a reliable surrogate for animal and human studies, thereby facilitating the discovery and development pipeline, as well as the regulatory approval

processes (17,143). The Caco-2 cell system is an example of such an *in vitro* methodology that can produce discordant human intestinal permeability predictions. With a standardized, validated permeability method, Caco-2 can predict the human intestinal permeability of lipophilic compounds. However, it is not suitable for predicting the human intestinal permeability of hydrophilic compounds, particularly those that are substrates of highly expressed intestinal transporters such as members of the amino acid, nucleoside, and peptide transporter families. To date, rat intestinal tissues have been the most successful at predicting the human intestinal permeability of these compounds.

Apart from cautioning drug discovery programs that are interested in these compounds and urging them to use alternate or additional permeability methods during their permeability screening process, we also caution investigators developing *in silico* predictive models from using such Caco-2 permeability measurements as a surrogate for human intestinal permeability values when developing their models. The inclusion of such data can distort *in silico* correlations, and should be excluded altogether. Because the absolute Caco-2 permeability measurements of compounds vary among different Caco-2 studies, permeability classification should be made relative to a reference standard rather than an absolute permeability cut-off. Significant amounts of variability can be introduced into *in silico* models when their training and validation datasets consist of Caco-2 permeability measurements taken from more than one source. Normalization to the same reference standard in each dataset may minimize the interlaboratory variability for lipophilic compounds. However, this is unsuccessful for hydrophilic compounds. As such, *in silico* modelers should not use Caco-2 permeability measurements in their *in silico* model development from more than one source. Lastly, regulatory agencies should recommend that drug discovery and development programs use alternative permeability methods to Caco-2, such as those involving human or animal intestinal tissues, for assessing the intestinal permeability of these NMEs and supporting their new drug applications.

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