BIOCHEMICAL PHARMACOLOGY 76 (2008) 1757–1764

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Permeability evaluation of peptidic HCV protease inhibitors in Caco-2 cells-correlation with in vivo absorption predicted in humans

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ARTICLE INFO

Article history:
Received 12 August 2008
Accepted 3 September 2008

Keywords:
HCV protease inhibitor
In vivo and in vitro pharmacokinetics
Human pharmacokinetics prediction

ABSTRACT

The permeability of six peptidic hepatitis C virus (HCV) protease inhibitors, with molecular weights ranging from 500 to 780, was examined in the Caco-2 cell system. All six compounds permeated the cells transcellularly; paracellular permeability, evaluated in the Caco-2 cell system by reducing the calcium concentration in the media to increase the pore size of the tight junctions, most likely contributes only minimally to the oral absorption of the compounds. All six compounds were shown to be efflux substrates displaying concentration-dependent saturation of efflux. The efflux could be blocked by cyclosporine A, a specific P-glycoprotein (P-gp) inhibitor, suggesting that P-gp may be the responsible transporter. Oral absorption in rats was calculated using in vivo oral bioavailability and hepatic extraction ratios. Human oral absorption was projected to be similar to that of rats, as reported previously by comparing rat and human absorption values for 23 marketed drugs. Upon comparison of human oral absorption predicted by Caco-2 permeability and by rat pharmacokinetics, we show a better correlation with Caco-2 permeability obtained at higher compound concentrations, where efflux is saturated, than at lower concentrations. The higher concentrations are likely reflecting the lumen concentrations after in vivo oral dosing. The results presented in this study demonstrate that, when tested at relevant compound concentrations, Caco-2 permeability is useful for predicting the oral absorption of peptidic compounds.

1. Introduction

Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus of approximately 9.6 kb [1]. Upon entering a suitable host cell, the HCV genome serves as a template for cap-independent translation through its 5′ internal ribosome entry site. The resulting 3000-amino acid polypeptide undergoes both co- and post-translational proteolytic maturation by host- and virus-encoded proteases. The virus-encoded protease is responsible for processing the non-structural portion of the polypeptide. Due to the shallow binding site, it is difficult to develop HCV protease inhibitors other than synthetic polypeptides that resemble the natural substrates. The most recent HCV protease inhibitors undergoing clinical evaluations, such as boceprevir [2], BILN-2061 [3], and VX-950 [4], are relatively large peptidic molecules (MW >500), that violate all

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Abbreviations: HCV, hepatitis C virus; AUC, area under the curve; HPLC, high performance liquid chromatography; AP, apical; BL, basolateral; P-gp, P-glycoprotein; BCRP, breast cancer resistant protein.

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doi:10.1016/j.bcp.2008.09.005

Lipinski rules and thus would be predicted to have permeability-limited oral bioavailability.

One of the primary goals during lead optimization of the HCV protease inhibitor discovery program at Schering-Plough was to identify compounds with reasonable membrane permeability. This property often ensures good oral absorption, and sufficient target tissue uptake of the compounds. Physico-chemical properties, such as molecular surface area, polar surface area, hydrogen-bonding potential, and secondary structure have been shown to influence the ability of a molecule to permeate cell membranes [5]. For example, as the polar surface area and the potential for hydrogen-bonding of a molecule increases, the membrane permeability usually decreases. Hence, reduction of the polar surface area by decreasing the molecular weight or by cyclizing part of the molecule may improve the passive diffusion across cell membranes [6]. However, attempts to improve the overall physico-chemical properties of HCV protease inhibitors for improved membrane permeability have not resulted in consistent success.

A second approach for optimizing the membrane permeability relies on in vitro, higher-throughput screening assays. One of the most frequently used in vitro systems for understanding membrane permeability is the Caco-2 cell system. For orally administered drugs, a reasonable relationship has been observed between percent of absorption and Caco-2 cell permeability coefficients [7,8]. It is generally accepted that the permeability coefficient obtained in Caco-2 cells reflects the ability of a compound to permeate through the cell membrane (transcellularly) since the Caco-2 cells have small tight junctions. Caco-2 cells have a high level expression of P-gp and other active efflux transporters, such as BCRP and MRP2, which reduce the apical (AP) to basolateral (BL) permeability by pumping substrates back out of the cells.

During our lead optimization of the HCV protease inhibitor program, about 1000 compounds were evaluated in rat pharmacokinetic studies [9]. Only a small fraction of compounds was selected for further pharmacokinetic evaluation in large animals. In this study, we present six compounds and predictions of their oral absorption in humans based on Caco-2 permeability data and rat pharmacokinetic data.

2. Materials and methods

2.1. Reagents

Test compounds were synthesized within Schering-Plough Research Institute (Kenilworth, NJ). Caco-2 permeability studies were performed using the HTS Multiwell™ insert system (Becton Dickinson Labware; Franklin Lakes, NJ) with PET membrane (24 wells, 1.0-μm pore size, 0.33-cm^2 surface area). All other chemicals were obtained from Sigma Chemical Co (St. Louis, MO).

2.2. Methods

2.2.1. Caco-2 cell culture and permeability

The cells were maintained in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2 mM L-glutamine, and 0.1% penicillin–streptomycin. 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 at a density of 60,000 cells/cm^2 onto an HTS Multiwell™ insert system with PET membranes. Plates were incubated at 37 °C with 5% CO_2 and 90% relative humidity. The medium was changed every 24 h using a Brandel cell culture automation system (Gaithersburg, MD). The cells were grown 21–25 days in the plate prior to the transport studies. The cell membrane integrity was measured by testing the ^{14}C-mannitol permeability for one plate from each batch using a Packard MultiProbe II system (Packard BioScience; Meriden, CT). The ^{14}C-mannitol permeability ranged from 5 to 8 nm/s. In addition, the cell membrane and monolayer integrity were verified by measuring the transepithelial electrical resistance (TEER) across each well before and after transport experiments. TEER readings for each well ranged from 300 to 540 Ω cm^2.

Prior to the study, culture medium was removed from both the AP and BL wells, and the wells were washed twice with warmed Hanks' Balanced Salt Solution (HBSS). The AP wells were replenished with 0.4 mL HBSS buffer containing 10 mM MES, 10 mM D-glucose at pH 6.5, and 10 μM of each compound. The BL wells were replenished with 1.0 mL HBSS buffer containing 10 mM HEPES, 10 mM D-glucose with 4% BSA (pH 7.4). Samples were taken after a 2-h incubation from both the AP (donor) and BL (receiver) wells. The samples were analyzed using LC–MS/MS. Permeability for Caco-2 was calculated by measuring the accumulated amount of a compound transported from donor side to receiver side as a function of time:

$$P_{app} = \frac{dM/dt}{C_0 \times S}$$

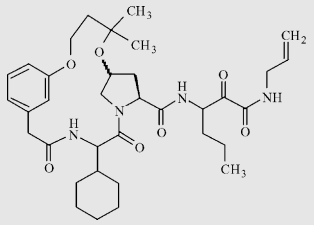
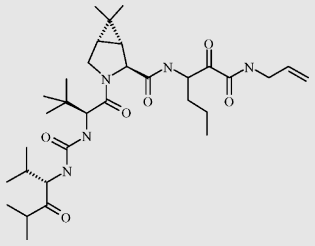
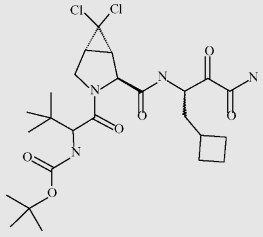
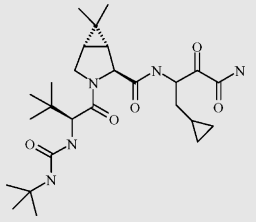
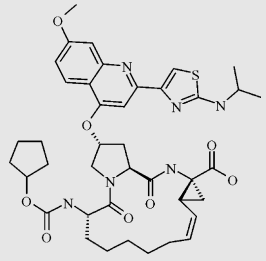
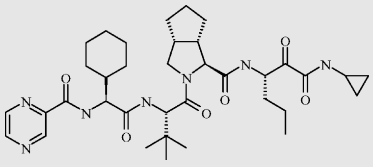
where C_0 was the initial compound concentration, S was the surface area of the membrane on the filter insert, M was the amount of compound transported to the receiver side at measuring time point (t).

Paracellular permeability of the test articles were evaluated by perturbation of Caco-2 cell tight junctions using different Ca^{2+} concentrations (20–1300 μM). The tight junction perturbation was first evaluated using ^{14}C-mannitol as a paracellular permeability probe and ^{3}H-propranolol as a transcellular probe. The compounds were co-dosed with Lucifer yellow (LY), another paracellular transport compound, prepared with HBSS buffer with different Ca^{2+} concentrations.

2.2.2. Rat pharmacokinetic evaluation

All animal work was carried out in compliance with ACUC rules and was approved by a local IACCAC review processes. Dosing solutions were prepared freshly in 20% hydroxylpropyl-β-cyclodextran for intravenous studies or in 0.4% methylcellulose for oral studies. For IV studies male Spraque Dawley rats (~250 g; Charles River, Wisconsin) were surgically prepared with jugular vein and femoral artery catheters at least 2 days prior to dosing. The IV dose was administered via the jugular vein as a bolus dose. Blood samples (0.5 mL) were

Table 1 – Chemical structures, molecular weights, hydrogen bonds, and physico-chemical properties of the HCV compounds

Compound	Chemical structure	MW	# of HB donor and acceptor	clog *P*
S1		639	10	5.49
S2		604	11	6.12
S3		561	10	2.58
S4		506	10	2.79
BILN-2061		775	12	8.39
VX-950		680	11	5.35

collected from the femoral artery. The oral dose was administered by gavage, and blood samples were collected from the femoral vein.

2.2.3. Rat hepatocyte extraction ratio

Stock solutions of the compounds were prepared in methanol and diluted to the desired concentrations in Waymouth's medium. A final test compound concentration of 1 μM was added to pooled cryopreserved human hepatocytes (1 million cells/mL). Samples (1 mL) were incubated in 24-well plates at 37 °C for 2 h in a CO_2 incubator. At 0, 10, 20, 40, 60, and 120 min, a 100-μL aliquot was removed and added to a 96-well plate containing 200 μL acetonitrile with internal standard. All samples were sonicated for 3 min and centrifuged at 2000 × *g* for 15 min, and the supernatants were transferred into a 96-well plate. The plate was stored at 4 °C until analyzed.

2.2.4. Physico-chemical parameters

clog *P* was calculated by Sybyl 7.3 (Tripos Inc; St. Louis, MO), which uses version 4.3 clog *P* algorithm from Biobyte Corp (Claremont, CA).

3. Results

3.1. Physico-chemical properties

Table 1 shows the chemical structures and other parameters of six HCV protease inhibitors. The molecular weights range from 500 (S-4) to 775 (BILN-2061). All six compounds comprise tri- to tetra-peptide backbones and are potent HCV protease inhibitors in a continuous protease activity assay and in a cell-based Replicon assay (data not shown). The total number of hydrogen bond donors and acceptors is between 10 and 12 for the six molecules. The clog *P* values for the compounds range from 2.58 to 8.39, suggesting moderate range (clog *P* = 2–5) of hydrophobicity for membrane permeability with the exception of S2 (clog *P* = 6.12) and BILN-2061 (clog *P* = 8.39).

3.2. Caco-2 permeability and efflux

The AP to BL permeability across Caco-2 cells for five of the six HCV protease inhibitors is limited at low concentration (5 μM), with P_{app} values ranging from 0 to 55 nm/s (Fig. 1). Significantly higher permeability was observed in the BL to AP direction, with P_{app} values range from 60 to 250 nm/s. The efflux ratios (BL to AP/AP to BL) at 5 μM range from 2.4 to 178 of these six HCV protease inhibitors indicate transporter-mediated efflux. At higher compound concentration, a marked increase In the AP to BL permeability was observed, in conjunction with a decrease in the BL to AP permeability (Fig. 1). Cyclosporin A, a known specific inhibitor of P-gp, markedly reduced the efflux ratio to 2 or below for all six compounds.

Interaction between these six HCV protease inhibitors with peptide transporters, such as Pept1 and Pept2, was evaluated using xenopus oocytes, which express these transporters at high levels (BD Gentest). No significant uptake was observed (data not shown), suggesting that these compounds are not Pepti1 or Pepti2 substrates.

3.3. Paracellular permeability in the Caco-2 cells

Changes in Caco-2 permeability of the compounds were observed at varying Ca^{2+} concentrations. At the highest Ca^{2+} concentration tested (1300 μM), the permeability of mannitol, which permeates primarily through a paracellular mechanism, was low (Fig. 2). At lower Ca^{2+} concentrations, the permeability of mannitol increased. The permeability of propranolol, which permeates through a transcellular mechanism, showed no significant change. The linear correlation in permeability between mannitol and Lucifer yellow (LY) appears to be very good (r^2 = 0.87). The slope of the correlation line is about 1, suggesting both compounds have similar paracellular permeability. As shown in Fig. 3a, the slopes of the correlation lines for S3 and S4 with LY were 0.31 and 0.37, respectively, indicating only partial correlation in their paracellular permeability with LY. Fig. 3b shows no correlation for VX-950 or BILN-2061 with LY, possibly due to the large molecular size, which hinders the paracellular permeability.

3.4. Prediction of human absorption based on the Caco-2 permeability

Caco-2 permeability of 35 marketed compounds with published human oral absorption data was used for comparison with the P_{app} value. A plot of human absorption against the log value of P_{app} is shown in Fig. 4. Best curve fitting showed a sigmoidal correlation between these two parameters with an r^2 value of 0.80, indicating a good correlation.

3.5. Prediction of human absorption based on rat pharmacokinetic data

The in vivo and in vitro pharmacokinetic behavior of the six HCV protease inhibitors is shown in Table 2. The oral bioavailabilities of these compounds range from 10 to 96% in rats. Liver extraction ratios in rats were estimated using rat hepatocytes. CL_{int} was determined then scaled up based on the well-stirred model. The CL_{liver} was used to estimate the extraction ratio (ER) according to the following equation

$$\mathrm{ER} = \frac{\mathrm{CL_{liver}}}{\mathrm{Liver\ blood\ flow}}$$

with a liver blood flow value of 70 mL/min.

The liver extraction ratio was further used to calculate the predicted rat oral absorption of the compounds according to the following hypothetical equation assuming the first-pass effect is primarily due to the liver:

$$\mathrm{Oral\ absorption}(\%) = \frac{\mathrm{Oral\ bioavailability}(\%)}{1 - \mathrm{ER}}$$

As shown previously by Chiou and Barve [10], an excellent correlation was found among oral absorption between rats and humans for 23 marketed drugs. These 23 marketed drugs represent a rather diverse class of structures. Using this relationship, we predict that the oral absorption of these six HCV protease inhibitors will be very similar between rats and humans.

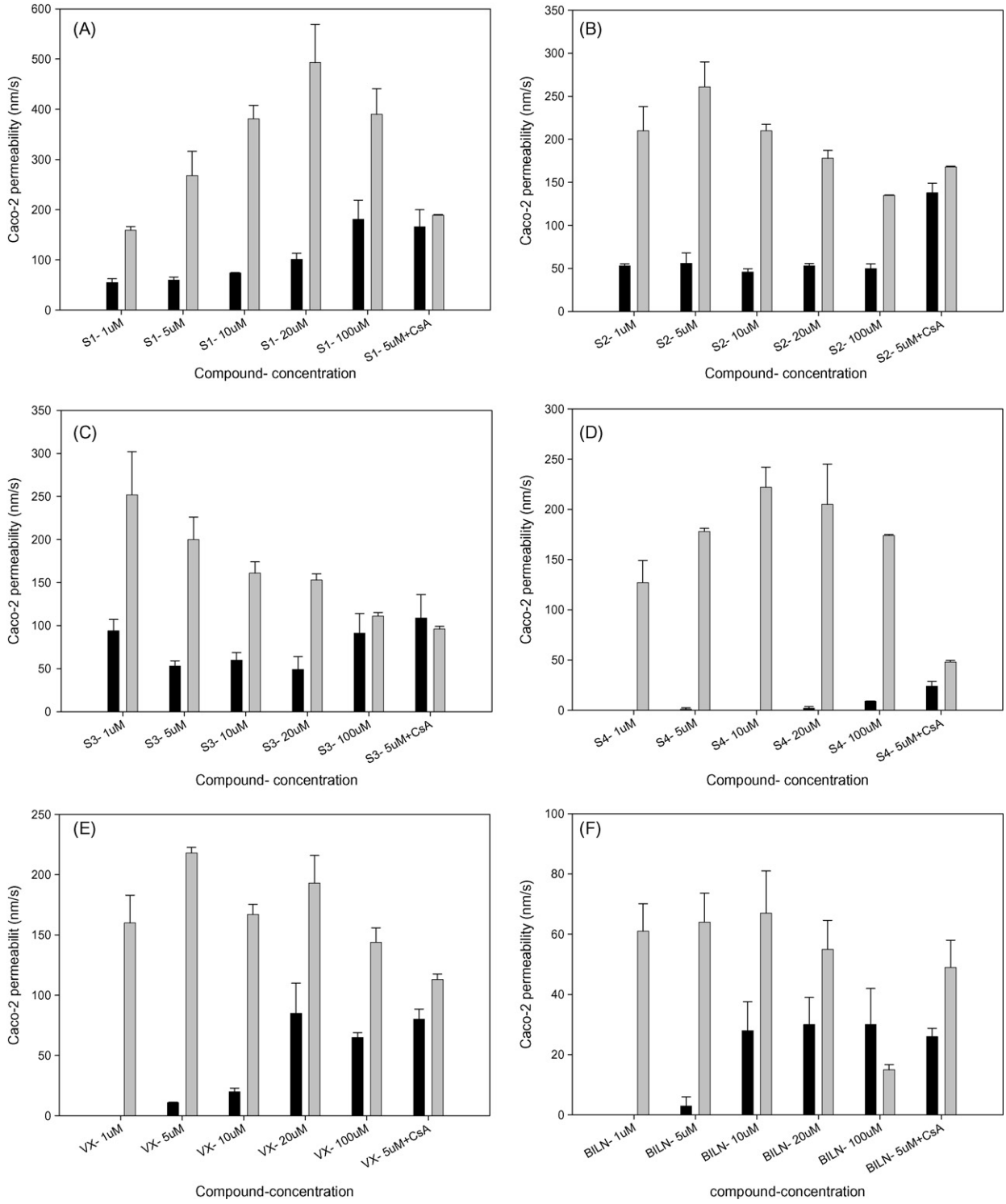


Fig. 1 – AP to BL and BL to AP permeability of six HCV compounds in Caco-2 cells. Filled columns: AP to BL permeability; open columns: BL to AP permeability; A, S1; B, S2; C, S3; D, S4; E, VX-950; F, BILN-2061. The columns represent the mean of duplicate experiments and bars represent standard deviations.

3.6. *Comparison between oral absorption predicted by in vivo and in vitro methods*

Table 2 shows values for oral absorption of the six HCV compounds predicted using Caco-2 permeability data and the plot of oral absorption versus $\log P_{app}$ as a calibration curve (Fig. 4). Two sets of predictions are shown in Table 2: one based on the Caco-2 permeability obtained at a compound concentration of 1 uM and another at a compound concentration of 100 uM. Comparison of the oral

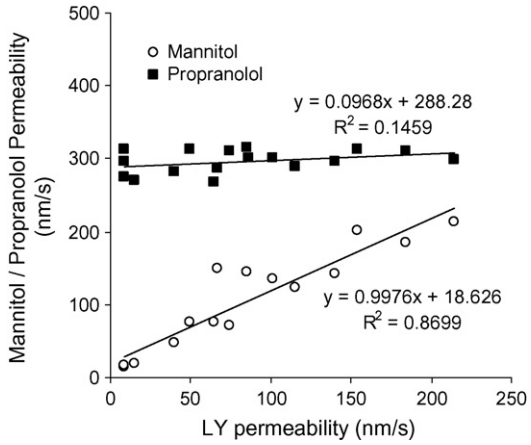


Fig. 2 – Paracelular permeability of mannitol and propranolol in Caco-2 cells. Solid squares represent the Caco-2 permeability of propranolol at various calcium concentrations in the medium. Open circles represent the Caco-2 permeability of mannitol at various calcium concentrations in the medium.

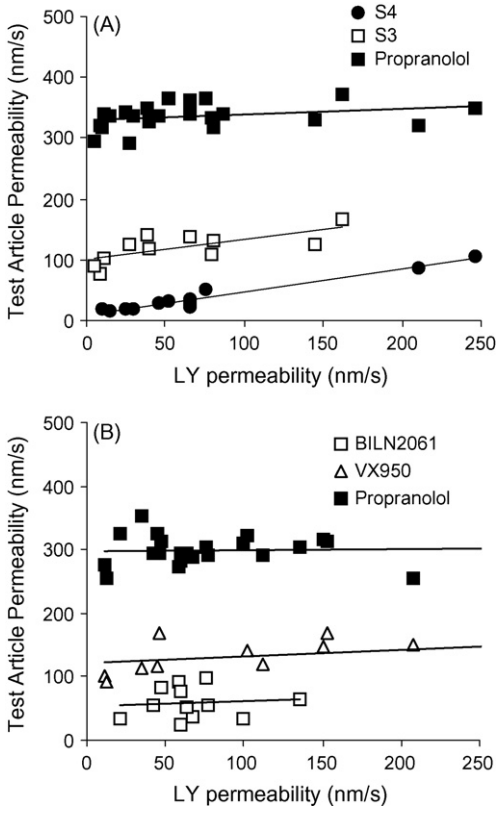


Fig. 3 – Paracelular permeability of HCV compounds in Caco-2 cells. (A) Solid circles, open squares and solid squares represent the Caco-2 permeability of S4, S3 and propranolol, respectively, at various concentrations of calcium in the medium. (B) Open squares, open triangles and solid squares represent the Caco-2 permeability of BILN-2061, VX-950, and propranolol, respectively, at various concentrations of calcium in the medium.

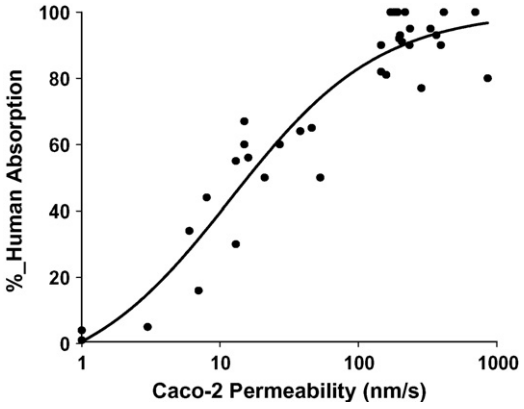


Fig. 4 – Correlation between Caco-2 cell permeability and oral absorption in humans of 35 marketed drugs. A curve-fitting using a sigmoidal equation was performed to represent the relationship between % human absorption vs. the Caco-2 permeability.

absorption predicted using Caco-2 permeability and rat pharmacokinetics showed that the Caco-2 permeability obtained at a compound concentration of 1 μm under-predicted four compounds (S1, S4, VX-950, and BILN-2061) and over-predicted two compounds (S2 and S3). However, using the Caco-2 permeability at high compound concentration (100 uM), three compounds were precisely predicted (S1, S4, and VX-950) and three were over-predicted (S2, S3, and BILN-201).

4. Discussion

Membrane permeability is essential for a drug to enter the systemic circulation after oral administration. Permeability through the gastrointestinal barrier is a complex phenomenon that may involve passive diffusion and active transport via drug transporters. For many peptidic drug candidates, such as the HCV protease inhibitors, membrane permeability presents a major obstacle to achieving oral absorption and delivery to target tissues [11]. Strategies designed to improve the membrane permeability of these types of compounds have emphasized optimizing specific physicochemical properties to facilitate permeability. They include improving lipophilicity, reducing molecular size and charge state, and increasing solubility [12–14]. Despite this emphasis, improvement of physico-chemical attributes for achieving better membrane permeability while maintaining acceptable potency against the target can be very challenging. It is possible that one needs to improve multiple physico-chemical properties in order to achieve a better overall membrane permeability of a given compound. One interesting aspect of these HCV protease inhibitors is the presence of solute-solvent hydrogen bonds. Peptides with well-defined secondary structures, such as β-turns, may exhibit intramolecular hydrogen-bonding, which reduces the hydrogen-bonding potential with the solvent. The free energy required to desolvate such peptides inversely relates to the permeability of the peptides [12].

Table 2 – Prediction of oral absorption in humans of six HCV compounds based on pharmacokinetics in rats and Caco-2 cell permeability

PK parameters	S1	S2	S3	S4	VX-950	BILN-2061
(%) F_{oral} in rats ($N = 3$)	93 ± 27	16 ± 3	12 ± 5	39 ± 8	26 ± 10	3 ± 2
Rat hepatic extraction ratio	0.54	0.51	0.62	0.25	0.51	0.21
Estimated (%) F_{ab} in human	100 ± 30	32 ± 6	30 ± 12	50 ± 10	52 ± 20	4 ± 3
Estimated (%) F_{ab} in human based on high Caco-2 permeability ($N = 2$)	89 ± 18	79 ± 8	80 ± 20	37 ± 2	64 ± 4	77 ± 30
Estimated (%) F_{ab} in human based on low Caco-2 permeability ($N = 2$)	77 ± 10	77 ± 4	82 ± 12	0	0	0

Values presented are either means or means ± standard deviations.

It has been generally accepted that small hydrophilic solutes permeate the intestinal barrier predominantly through the paracellular route via an aqueous extracellular pathway [15,16]. The main barrier to paracellular diffusion of molecules is the region of the tight junction, which limits the permeation of solutes according to their size and charge. Results from permeability experiments using Ca^{2+}-depleted Caco-2 cells suggest that the HCV protease inhibitors have limited paracellular permeability. These results are consistent with previous findings that molecules with MW >500 are restricted from paracellular permeability [15].

It should be pointed out that the Caco-2 permeability coefficients, may vary significantly from laboratory to laboratory, particularly for compounds that pass through the Caco-2 monolayer via the paracellular route. According to Walter and Kissel [17], permeability coefficients of mannitol in the literature range from 2 to 221 nm/s. Even within the same laboratory, several factors, such as cell source, age of cell culture and culturing conditions, may affect the reproducibility of permeability results. The cell passage number may specifically affect the tight junctions and the expression level of drug transporters, such as P-glycoprotein (P-gp). Nevertheless, it is not difficult to differentiate a highly permeable drug from a poorly permeable drug if the conditions of the assay are carefully maintained. This is particularly true for compounds which are absorbed transcellularly.

Recently, it has been recognized that efflux carried out by transporters, such as P-gp, BCRP, and MRP2, limits the cell permeability of many different chemotypes including peptidic molecules. Since the membrane permeability usually follows first-order kinetics, the efflux may become saturable at higher concentrations of the molecule. Several examples of non-linear pharmacokinetic behavior in humans have been observed due to saturable efflux [18,19]. Based on these examples, the HCV protease inhibitors may have the potential to follow non-linear pharmacokinetics due to P-gp interaction as demonstrated in Caco-2 cells. At low concentrations, the AP to BL permeability of the protease inhibitors in the present study is very limited. At higher concentrations, the AP to BL permeability increased and the efflux ratio decreased, suggesting a saturation of efflux. Based on an estimated fluid volume of 500 mL [20] in the human small intestine, a dose of 700 mg (assuming a 10 mg/kg dose and average 70 kg body weight) would result in a predicted lumenal concentration of 2–2.8 μM. This concentration is much higher than the concentration required for saturating P-gp and exceeds the solubility limit of these compounds.

In summary, this study demonstrated that P-gp efflux in the Caco-2 cell system can confound prediction of oral absorption of a given compound. This is especially true for compounds like the peptidic HCV protease inhibitors, which have low-to-medium intrinsic membrane permeability and borderline physico-chemical properties. If one considers test compound concentrations that saturate efflux and/or are most relevant to the oral dose levels in vivo, it is possible to choose experimental conditions that will provide better prediction of absorption after oral administration in humans.

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