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The Proteome of Filter-Grown Caco-2 Cells With a Focus on Proteins Involved in Drug Disposition

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Abstract
Research on the physiology of intestinal cells and drug transport often makes use of Caco-2 cells. In this study, the total protein technique was used to quantify the global proteome of filter-grown Caco-2 cells. The results were compared with proteomes from the human colon and jejunum. There were a total of 8096 proteins found. Thorough examination of proteins that characterize enterocyte development, such as adherence and tight junctions, integrins, and brush-border hydrolases, provided almost exhaustive coverage of the anticipated proteins. Out of the 327 proteins that were found, 112 were solute carriers and 20 were ATP-binding cassette transporters; these proteins were involved in absorption, distribution, metabolism, and excretion. The levels of OATP2B1 were sixteen times more in Caco-2 cells compared to jejunum. At clinically relevant intestine concentrations, OATP2B1 accounted for 60%-70% of the uptake kinetics of pita vastatin, an OATP2B1 substrate, in Caco-2 monolayers. We aimed to understand how this discrepancy affected in vitro-in vivo extrapolations. Together, pita vastatin kinetics and transporter concentrations were used to simulate the role of active transport and membrane penetration in the jejunum. Pita vastatin absorption in vivo is mostly mediated via transmembrane diffusion, as shown by the much decreased transporter contribution (<5%) caused by the lower OATP2B1 expression in the jejunum. The first comprehensive measurement of the Caco-2 proteome produced in a filter has been presented here. To correctly interpret drug transport pathways in the human gut, we also show that transporter expression levels are very important.

Introduction

The human colon carcinoma Caco-2 cell line was originally iso-lated by Fogh et al.[1]Weisbaum et al. introduced the use of filter-grown Caco-2 monolayers for studies of epithelial cell physiologyand reported on its spontaneous differentiation to an enterocyte-like phenotype.[2,3]This paved the way for scientists in the pharmaceutical

field toapplythis cell line for the studyof solutetransportandabsorption.[4-7]Borchardtand Wilsonwerethe first touse Caco-2cellstostudyactivetransportprocesses,includingtransportofbileproteins, vitamins, amino acids, and peptides.6,8-10 Since methods for identifying or knocking off the target transporter were just recently developed, functional studies dominated their groundbreaking work and the subsequent many investigations. Modern mass spectrometry has made it feasible to map the whole Caco-2 cell proteome.11 Gene editing methods like CRISPR-Cas913 may entirely knock out proteins of relevance to better understand their function, and targeted proteomics can quantify collections of proteins, including key drug transporters12. Modeling transport capacity in the intricate cellular environment may be achieved by integrating systems biology methods with protein expression and kinetic characteristics.14

In this study, we used cutting-edge protein quantification tools to examine the worldwide proteome of differentiated Caco-2 cell monolayers. The standard protocol for transport investigations involves growing the cells on filter supports for three weeks. Our next step was to evaluate the worldwide Caco-2 protein expression profile with that of the human jejunum and colon, with a particular emphasis on ADME proteins (transporters, drug-metabolizing enzymes, and others) that are important for drug disposal processes. Lastly, we modeled the jejunal drug permeability via the high affinity and low capacity transporter OATP2B1 (SLCO2B1), which is thought to play a significant role in the intestinal absorption of anionic drug substrates, using the protein concentration data.15-17

Methods

Supply items

The chemicals and cell culture medium were all bought from Sigma-Aldrich or Thermo Fisher Scientific, respectively, located in Waltham, MA and St. Louis, MO. Corning (Corning, NY) supplied the cell culture plates and Transwell permeable filters.

I do research on cell culture.

From passage 95 to 105, Caco-2 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 1% nonessential amino acids. The cells were initially received from the American Type Culture Collection. Following the methods previously mentioned, they were cultivated on Transwell polycarbonate filters with a diameter of 6.5 mm and a pore size of 0.4 mm.18 In a nutshell, the cells were seeded onto the filter at a density of 0.15×10^{1} cells/mL and kept in a medium that included 10% fetal calf serum, 1% nonessential amino acids, 100 U/mL penicillin, and 100 mg/mL streptomycin. The filters were ready for proteomic analysis after being cultured for 21 days.

Analysis of Proteins Preparation of Samples

Filters that contained 21-day Caco-2 cultures were subjected to a 5-minute lysis at 100◦C in

a solution that included 0.1 M Tris-HCl, pH 7.8, 2% sodium dodecyl sulfate, and 0.05 M dithiothreitol. Using the multi-enzyme digestion filter-aided sample preparation approach, which included Lys-C and trypsin, samples were processed in 30-kDa ultrafiltration units.To get two fractions per protease, 19 peptides were put onto pipet-tip strong anion exchange columns and eluted at pH 6 and pH 2, respectively.20 The tryptophan fluorescence assay was used to quantify the quantities of proteins and peptides.21Analysis using Liquid Chromatography and Tandem Mass Spectrometry
Following the methods previously detailed, the peptides were separated using a 3-hour acetonitrile gradient in 0.1% formic acid. They were then evaluated on a Q Extractive mass spectrometer (Thermo Fisher Scientific, Germany).22

Analyzing Data

The Macquet software, version 1.2.6.20, was used for the analysis of the mass spectrometric data. The protein sequences in the UniProtKB/Swiss-Prot database, version of May 2013, were searched against peptide MS and MS/MS data in order to identify the proteins. The protein concentrations were determined by applying the total protein method to the raw spectral intensities of the Macquet output.24

Analysis using bioinformatics

Analyzing and Annotating Gene OntologiesUsing the Ontologize software program, version 2.1.26, in conjunction with the human GO annotation database file, the full proteome dataset was annotated using Gene Ontology (GO) keywords.on or around July 22, 2015. The GO Consortium's PANTHER categorization system, version 10.0,27, was used to further categorize the selected proteins by protein class.

Examination of Caco-Related Differentially Expressed ProteinsTwo-sample t-tests, as implemented in Perseus, version 1.5.0.15, were used to identify cells grown on filters or plastic proteins with substantially different amounts in the sample groups. After doing 250 random permutations, a false discovery rate of0.05 was used to determine statistical significance. Proteins that differed significantly were examined using the Ingenuity Pathway Analysis (IPA) program, version 24390178 (Qiagen, Hilden, Germany), which provides data on changed molecular and cellular processes. Evidence derived only from experiments was considered in the study.

Evaluation of the Relationships Between Protein and Messenger RNA Expression via Statistical Means to enable comparison of datasets for distinct sets of transporters, the protein concentrations from this research and the messenger RNA (mRNA) expression levels from three prior studies28–30 were ranked and then adjusted to a scale from 0 to 1.

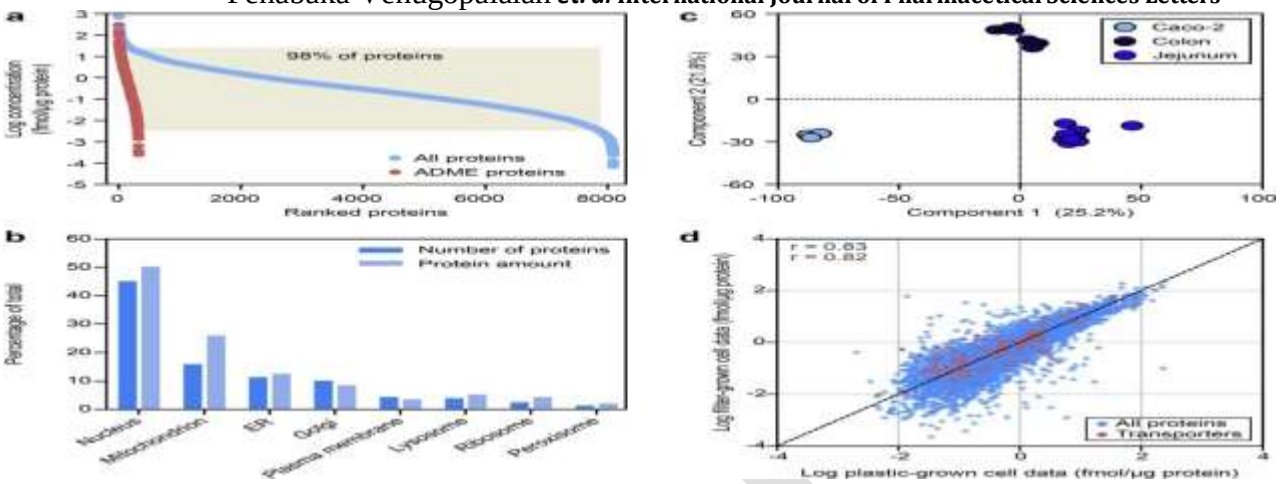
Next, the Spearman rank correlation coefficient (rs) was used to connect the average normalized mRNA expression rankings with the normalized protein concentration ranks.

Transportation Trials

Following the procedure outlined earlier, Caco-2 cells (passage 98) were grown on 24-well plates. Prior to equilibrating for 10 minutes at 37◦C, the cells were washed three times with Hank's balanced salt solution, which has a pH of 7.4. The wells were then supplemented with new buffer that included pita vastatin (1-80 mM). Adding ice-cold Hank's balanced salt solution halted transport after 4 minutes. There was a 1 M NaOH lysis reaction. For every concentration of pita vastatin, cellular uptake was measured three times. Data from transportation trials were examined using a Waters Xeon mass spectrometer connected to an ACQUITY UPLC system (Waters, Milford, MA). The next step was to use a water-acetonitrile gradient that included 0.1% formic acid to separate them on a C-18 column (Waters). Measurements were taken using the Target Lynx program developed by Waters. To find the best fit, we used a modified Michaelis-Menten equation with a linear diffusion component, Piffle, and standardized the transport rates against the wells' protein content.31

Analyzing the Regional Permeability of the Jejunum

We used the dissolve package for differential equation solution in R version 3.1.1 (http://www.r-project.org) to simulate the jejunal permeability of pita vastatin using a simplified mechanistic model of intestinal permeability.32 We used the ode function to execute the integration, which chooses an appropriate stiffness integration method automatically. All three parts of the model—the intestines, the cells, and the blood—were based on data from the well-known "Loc-I-Gut" human intestinal perfusion experiment (33), as well as measurements of the small intestine taken from previous studies.34 We hypothesized that compound transport across the basolateral membrane consisted entirely of passive diffusion, whereas compound transport across the apical membrane comprised a mix of transporter-mediated uptake and bidirectional passive Fick's diffusion. The following models were also considered: MDR1/P-gap, BCRP-mediated efflux across the apical membrane, and MRP3-mediated basolateral efflux.



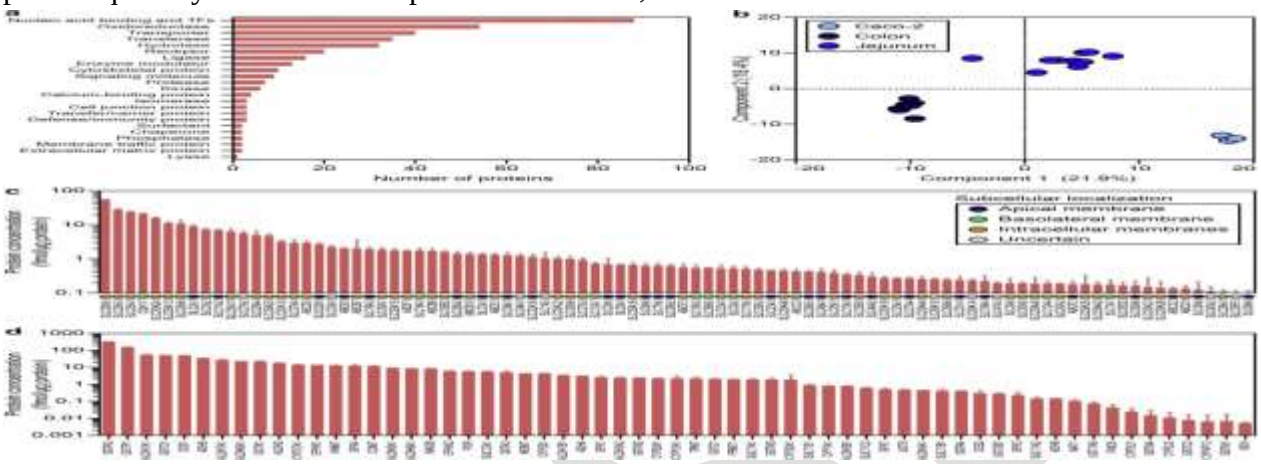
It was presumed that the mixing in the three sections would occur instantly. The usual drug residence period in the human jejunum is around 270 minutes, thus we mimicked the penetration process over that amount of time. By fitting the Caco-2 transport data (see to Transport Experiments), 35 kinetic parameters (Km and Piffle) for the OATP2B1-mediated flow were derived. Using the relative concentration of transporter proteins in the two systems, the maximum transporter activity method was used to scale the Caco-2 data and determine the jejunal OATP2B1 Vmax.36 The quantities of proteins in the jejunum were tripled. This was done to make up for the fact that, according to microscopy of representative tissue slices, jejunal proteomics samples were diluted due to subepithelial tissue. To summarize, human jejunal samples were embedded in paraffin and then cut into 5-mm slices, which were then stained with hematoxylin-eosin. From the subepithelial border of the preparation all the way around each villi, the ratio of epithelial to subepithelial cells was measured in a symmetrical fashion. The biopsies included, on average, one-third epithelial cells. The maximum transporter activity technique was used to scale the Vmax values of the MDR1, BCRP, and MRP3 efflux transporters, according to data from membrane vesicle systems (Valdese et al., unpublished data, 2015). Following adjustment for jejunal transporter concentrations, the corresponding Vmax values used in the models

Final Product

The Entire Proteome of Filter-Grown Caco-2 Cells in Differentiation :Quantitative global proteome analysis was carried out utilizing label-free mass spectrometry to provide a comprehensive understanding of the protein composition of filter-grown Caco-2 cells. From 7.1 × 10—5 to 7.2 × 10^2 fmol/mg total protein, 8096 different proteins were found, with concentrations ranging over seven orders of magnitude. A staggering 98% of these samples had amounts that fell within a 4-logarithmic range (Fig. 1a). At least two peptides were able to identify almost all of the proteins (92.9%), whereas sixty-three percent were able to do so with six or more peptides. The majority of the protein mass, 95%, was made up of the 2400 most abundant proteins (Fig. S1).

The Place of Proteins Within Cells

Fig. 1b shows that the discovered proteins were grouped by key subcellular classes using GO annotations. The proteins derived from the nucleus accounted for 45 percent, or half of the total protein quantity. From the endoplasmic reticulum, 11% and 10%



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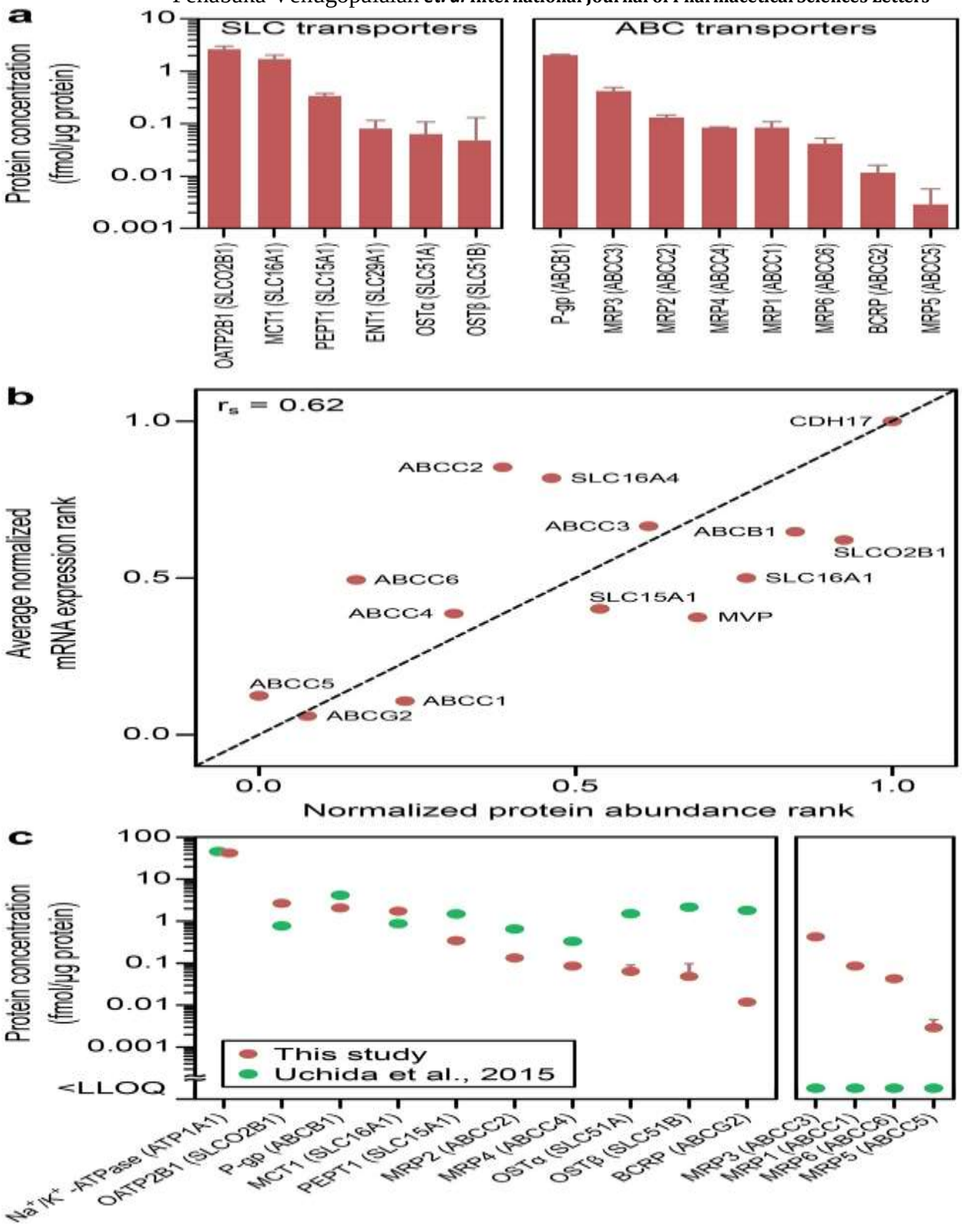

Figure4.Clinicallyrelevanttransportproteinsandcomparisonoftheir expressionlevels with literature data. (a) Protein concentrations of identified transporters listed bythe International Transporter Consortium as being of clinical relevance. (b) Rank-ordercorrelationbetween proteinconcentrationsandmRNA expression levelsfor anumberoftransporters.ThemRNAdataareaveragesfrom3previousstudies.[28-30](c)Comparisonwith a targeted proteomics study of transporters in Caco-2 cells.[12]Ten proteins weredetected above the limit of quantification in the targeted proteomics study (left); 4additional proteins were detected using our global proteomics approach (right).

value of relevance for every biological activity. Proteins increased in filter-grown cells belonged mostly to metabolic pathways (including those involving carbohydrates, amino acids, and other macromolecules) and molecular transport. Conversely, the majority of the proteins that were found to be increased in the plastic-grown cells were associated with activities related to gene expression and cell development (Table 1).

Membrane Proteins at the Brush Border

The presence of an apical brush boundary membrane, which houses several enzymes that hydrolyze peptide bonds, is a characteristic of differentiated intestinal epithelial cells.38,39 Valin (VIL1) is one of the 100 most highly expressed proteins (59 fmol/mg protein) and provides structural support for microvilli at the apical brush boundary. Figure 2a also shows the presence of many enzymes that are known to be border-localized. Two of the most popular markers for enterocytic differentiation, sucrase-isomaltose and intracellular alkaline phosphatase, were found among them. A completely differentiated Caco-2 phenotype was indicated by their high levels, which were 9.6 and 4.7 fmol/mg protein, respectively.2

The Proteins That Connect Adherents

Both endothelial and epithelial tissues have adherence junctions, which are significant intercellular protein complexes. The structure is connected to the actin cytoskeleton by means of cadherin adhesion receptors, which in turn are connected to a set of related cytoplasmic proteins, vinculin, and catenins.40 Specifically, E-cadherin and P-cadherin, also known as CDH-1 and CDH-3, were located here. Among the two, E-cadherin was more numerous by more than 100-fold (Fig. 2b), indicating its prevalence in epithelial tissue. There were detectable levels of vinculin and all three catenin's (p120 catenin, are-catenin, and b-catenindCTNND1, CTNNA1, and CTNNB1). Additionally, three members of the pectin family, afamin, EPLIN, and PLEKHA7 (MLLT4, LIMA1, and PLEKHA7) were shown to be proteins involved with adherence junctions. As a result, Caco-2 cells had several essential adherence junction components.

Closed-Loop Proteins

The intestinal epithelial cells have tight connections that allow some ions and tiny polar solutes to pass through, creating a semipermeable paracellular barrier.41 Using hydrophilic integrity indicators like mannitol and polyethylene glycol, as well as transepithelial electrical resistance,

Caco-2 cells create a paracellular barrier that is more robust than the small intestine in vivo and in vitro.42,43 dollars Paracellular barrier functions in the intestinal epithelium are carried out by the 27-member claudin family (CLDN) of junction proteins.44,45 In tight junctions, the claudins and other extracellular proteins like occluding (OCLN) make up the extracellular compartment. Intracellular scaffolding proteins like ZO-1 (TJP1) link the claudins to the cytoskeleton, which includes actin and microtubules. Proinflammatory cytokines, bacterial toxins, and pharmaceutical excipients are only a few examples of the local environmental factors that might affect the intracellular proteins with phosphorylation sites. Because of its potential to improve biopharmaceutical and medication absorption in the intestines, tight junction control has attracted a lot of attention from the pharmaceutical industry.46The distribution of the claudins differs between cell types, and they are either barrier- or channel-forming. Fig. 2c shows that Caco-2 cells expressed the barrier-forming clay-dins claudin-1, -3, and -4. Claudin-2, -7, and -15 are claudins that have paracellular channel activities and were identified in Caco-2 cells. It is worth noting that claudins 1 and 3, which create barriers, were much more common.

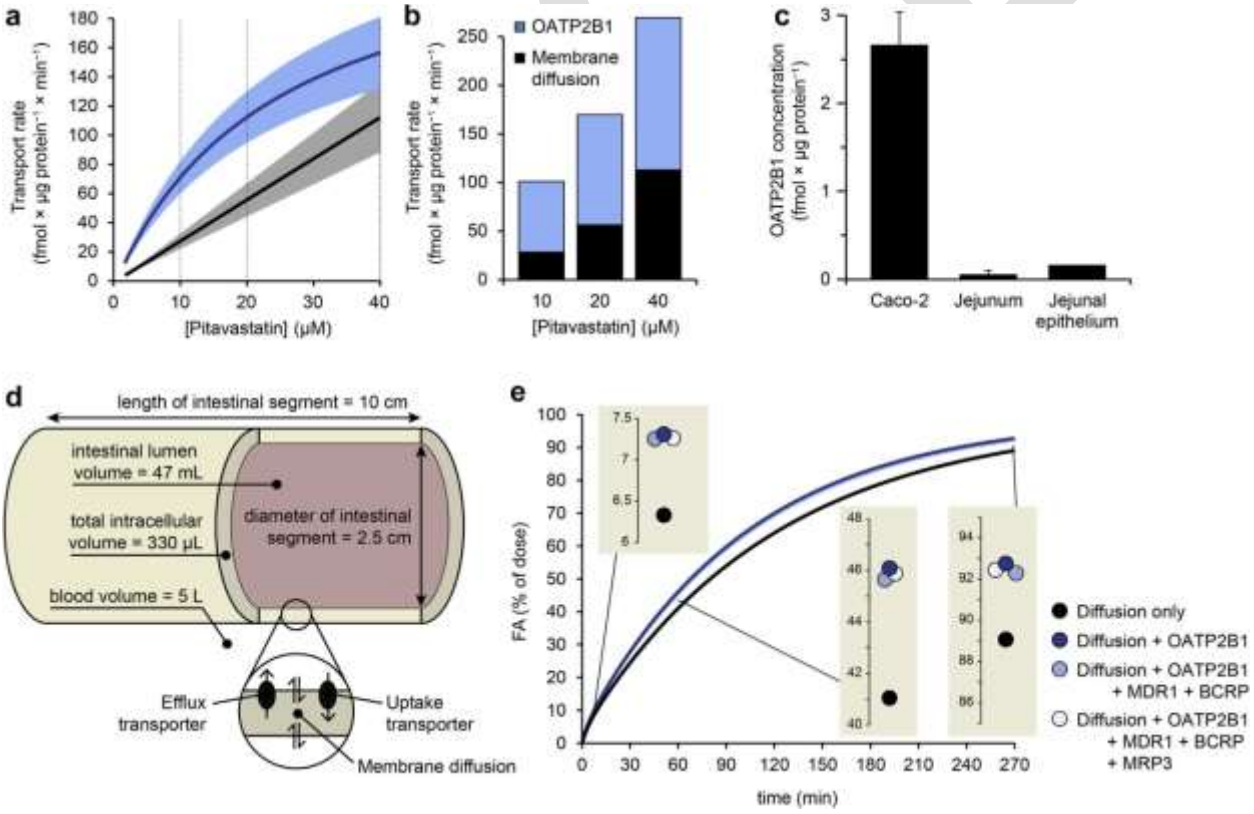

Figure5.Transportofpita vastatininCaco-2cellsandscalingtohumanjejunum.(a)Transportkineticsofpita vastatininCaco-2cells.ThebluelineindicatesthesaturableMichaelis-Menten transport component, and the black line the passive diffusion component. Shaded areas indicate standard errors of the parameter fitting. (b) Contribution

fromOATP2B1andpassivemembranediffusiontothetransportofpita vastatin,atconcentrationsexpectedinthesmallintestineafterstandardclinicaldoses(10,20,and40mM,assuming dissolution of 1, 2, and 4 mg pita vastatin in 250 mL). (c) OATP2B1 protein concentrations in Caco-2 cells and human jejunal tissue. The rightmost bar shows the jejunalprotein concentration corrected for epithelial-to-subepithelial tissue in the analyzed samples. (d) Setup of the intestinal permeability model. The model was constructed to mimic atypical humanintestinalperfusionexperimentina10-cmintestinalsegmentandconsistedof3compartments(intestine,intracellular,andblood). Intercompartmentdrugtransportwasimplementedasbidirectionalpassivediffusionand/ortransporter-mediatedfluxacrosstheapicalandbasolateralmembranes.(e)Simulationofpita vastatinab-sorptionovertime,basedonkineticparametersandjejunaltransporterconcentrations.Blacklineandpointsindicateresultsfromsimulationswithonlypassivetransmembrane diffusionincludedinthemodel.Bluelinesandpointsindicateresultsfrommodelsthatinaddition topassivemembranediffusionalsoincorporatedtransporter-mediatedflux:models including OATP2B1; OATP2B1, MDR1, and BCRP; or OATP2B1, MDR1, BCRP, and MRP3, are shown in increasingly lighter blue color. Insets show the simulated absorption attime ¼ 5, 60, and 270 min.

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Conflict of Interest

The Author declares that there is no conflict of interest.

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