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Purpose. To characterize the paracellular route of 2/4/A1 monolayers and to compare the permeabilities of incompletely absorbed oral drugs in 2/4/A1 with those in Caco-2 monolayers.

Methods. The cells were cultivated on permeable supports. The 2/4/A1 expression of genes associated with tight junctions was compared with that in the small intestine using RT-PCR. The aqueous pore radii were determined using paracellular marker molecules. The permeabilities of a series of incompletely absorbed drugs (defined as having a fraction absorbed 0 to 80%) after oral administration to humans were studied.

Results. Occludin and claudin 1 and 3 were expressed in 2/4/A1. The pore radius of 2/4/A1 was 9.0 ± 0.2 Å, which is similar to that in the human small intestine, although the pore radius was smaller (3.7 ± 0.1 Å) in Caco-2. The relationship between permeability and fraction absorbed of 13 drugs was stronger in 2/4/A1 than in Caco-2. The relationships were used to predict the intestinal absorption of another seven drugs. The prediction was more accurate in 2/4/A1 (RMSE = 15.6%) than in Caco-2 (RMSE = 21.1%). Further, Spearman’s rank coefficient between FA and permeability was higher in 2/4/A1.

Conclusion. The improved 2/4/A1 cell culture model has a more in vivo-like permeability and predicted the oral absorption of incompletely absorbed drugs better than Caco-2 cells.

KEY WORDS: prediction; drug absorption; Caco-2; paracellular; intestinal epithelia.

INTRODUCTION

The Caco-2 model is used for qualitative and sometimes also quantitative prediction of passive human intestinal permeability to drug-like molecules in drug discovery and development (1–4). However, the quality of the results varies with the compounds studied. Good quantitative to semiquantitative results are obtained when analogous series of drugs that are relatively well absorbed are studied. Such drugs are transported mainly via the transcellular route. However, when incompletely absorbed drugs that have low permeability coefficients (e.g., drugs that are at least partly transported via the paracellular route) are investigated, the results become more scattered and qualitative rather than quantitative.

This is at least partly explained by the fact that Caco-2 cells form less permeable tight junctions than those found in the human small intestinal epithelium, and this results in very low paracellular permeability for the Caco-2 cell monolayers. One difference between the tight junctions in Caco-2 cell monolayers and those in the human small intestine, for example, is a difference in average pore radius (5). Thus, the paracellular permeability of Caco-2 cell monolayers more closely resembles that of the human colon than that of the human small intestine (5,6). In fact, the permeability coefficients of low-permeability drugs and markers can be up to 100 times lower in Caco-2 monolayers than they are in the small intestine in vivo (6). It is clear that Caco-2 cells may not be quantitatively predictive of the intestinal absorption for drugs that are absorbed via the paracellular route in vivo, especially because orally administered drugs are absorbed predominantly from the small intestine. In addition, the low paracellular permeability of Caco-2 cells causes technical and analytic problems that contribute to the scattered results obtained for this group of compounds. A third, less general explanation is transport of poorly permeable compounds by an active transport mechanism that is more abundantly expressed in the intestinal epithelium than in Caco-2 cells (7).

Qualitative results (e.g., classification of compounds into high- and low-permeability drugs) may be sufficient in early screening of libraries of drug-like compounds. However, more quantitative results would be advantageous because such results would result in a better ranking of the individual compounds. Research on cell culture models that better reflect both the transcellular and the paracellular permeability of the human small intestine is therefore of interest. Furthermore, research using cell culture models that more closely resemble the human small intestine will provide both fundamental and applied mechanistic information concerning the role of the paracellular route in drug absorption.

A particularly interesting cell line in this regard is 2/4/A1, a rat intestinal epithelial cell line, which forms polarized monolayers 4–6 days after seeding onto permeable supports (8,9). Initial results showed that the permeability of 2/4/A1 monolayers is comparable to that of the human small intestine (9). This led us to pose the hypothesis that 2/4/A1 cell monolayers are a suitable cell model for studies of slowly and incompletely absorbed drugs that are significantly transported via the paracellular route. Drugs transported via this mechanism include drugs such as atenolol (10,11), H2 antagonists (12,13), furosemide (14,15), and hydrophilic peptides (5,16,17).

We have determined the levels of mRNA that code for various proteins associated with tight junctions in 2/4/A1 cell monolayers in order to characterize the paracellular route in these cells. The 2/4/A1 cell monolayers were cultivated using a new optimized cell culture procedure that enabled us to grow the cells at 39°C, where the cells are in their most differentiated state. The optimized procedure is presented in detail in an accompanying paper (8). We have subsequently determined the average aqueous pore radii of the tight junctions in 2/4/A1 and Caco-2 cell monolayers and compared them. Furthermore, we have established relationships between the absorbed fractions of a set of incompletely absorbed drugs after oral administration to humans (FA) and monolayer permeabilities in 2/4/A1 and Caco-2 cell monolayers. Finally, we have used these relationships to predict the human intestinal absorption of a second set of incompletely

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ABBREVIATION: Å, Angstrom (1 x 10^−10 m)
absorbed drugs. In summary, 2/4/A1 cell monolayers have a paracellular permeability that better reflects the human situation than that of Caco-2 cell monolayers. This in vivo-like paracellular permeability makes 2/4/A1 cell monolayers an interesting alternative to Caco-2 cells and artificial membranes for the prediction of human intestinal passive permeability, especially to incompletely absorbed drugs.

MATERIALS AND METHODS

Cell Culture

Culture media and supplements were purchased from Gibco BRL Life Technologies AB (Taby, Sweden) unless otherwise stated. 2/4/A1 cells [which originate from fetal rat intestine and are conditionally immortalized with a pZipSVtsa58 plasmid containing a temperature-sensitive mutant of the SV40 large T antigen (18)] were expanded as described in the accompanying paper (8). The medium was changed every second day, and the cells were passaged at 80% confluence, approximately every fourth day.

For functional studies, 2/4/A1 cells were seeded at 100 000 cells/cm². The cells were grown in RPMI 1640 supplemented with 6% fetal calf serum (FCS), growth factors, penicillin (100 U/ml), and streptomycin (100 μg/ml) on Transwell polycarbonate filters (0.45-μm pore size, 12 mm in diameter; Costar, Cambridge, MA) coated with EHS extracellular matrix (Promega Corporation, Madison, WI). Cells were used between passage number 23 and 43.

Caco-2 cells were cultivated as described previously (19,20). The 2/4/A1 and Caco-2 cultures were tested negative for Mycoplasma contamination every second month throughout this study.

RT-PCR of Transcripts Coding for Tight Junction Proteins

For the characterization of transcripts coding for tight junction proteins, approximately 7 × 10⁶ filter-grown 2/4/A1 cells were harvested using ice-cold phosphate-buffered saline solution and a cell scraper. Cells were kept on ice during the scraping procedure and subsequently recovered by centrifugation. 2/4/A1 cells and the rat ileal tissue (positive control) were homogenized using a Heidolph DIAX 900 tissue homogenizer equipped with a 6G tool (Heidolph Instruments, Cinnaminson, NJ). RNA was extracted and checked for absence of contaminating genomic DNA, as previously described in the accompanying paper (8). Samples were analyzed after 25, 29, 33, and 37 PCR cycles.

Selection of Drugs

Drugs that are reported in the literature to be predominantly absorbed by passive diffusion from the human intestine were identified as described previously (22). Briefly, drugs whose bioavailability is limited by solubility and pre-systemic metabolism were excluded unless these could be accounted for. In particular, drugs that have a fraction absorbed (FA) of less than or equal to 80% were included in this study. This cutoff value constitutes the border between high- and low-permeability drugs according to Wininwarter et al. (23). However, in order to study the performance of 2/4/A1 monolayers over the entire absorption range, seven drugs with FA 80–100% were also considered initially. In total, 20 drugs were investigated in this part of the study. Eight of these drugs were categorized as sparingly absorbed (FA = 0–20%), five drugs were categorized as intermediately absorbed (FA = 20–80%), and seven drugs were categorized as completely absorbed (FA = 80–100%) across the human intestine.

The sparingly and intermediately absorbed drugs (13 drugs; FA = 0–80%) were subsequently extracted from the data set and used as a training set in order to establish a relationship between permeability and FA. These drugs are listed in Table II. A second set of seven drugs (FA = 0 to 80%) (Table III) was finally selected as an ad hoc test set in order to validate the 2/4/A1 and Caco-2 cell monolayers. The ad hoc test set was chosen on the basis of observed poor performance of Caco-2 in predicting the in vivo permeability or FA for these drugs (Saeho Chong, unpublished results).

The molecular diversity of the data sets was analyzed by principal component analysis (PCA) in SIMCA-P 8.0 using default settings (24). This analysis was performed using the calculated descriptors molecular weight, total surface area and volume (descriptors of molecular size), ClogP, NPSA saturated, NPSA unsaturated, and NPSA (descriptors of hydrophobicity), and PSA and fraction PSA (descriptors of hydrophilicity) for the 13 drugs in the training set and five of the seven drugs in the ad hoc test set.

Drugs

[14C]Acyclovir, [14C]ganciclovir, [14C]PEG (MW 4000), and [14C]phosphonoformic acid were purchased from Moravec Biochemicals (Brea, CA). Acyclovir, atenolol, lactulose, metolazone, phosphonoformic acid, and sulpiride were obtained from Sigma (St. Louis, MO). [14C]Clodronate and unlabeled clodronate (Leiras Co., Turku, Finland) were gifts from Dr. J. Monkkonen (University of Kuopio, Finland). [14C]Mannitol, [3H]rafimose, and [3H]vasopressin were obtained from New England Nuclear (Boston, MA). [3H]Lactulose was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). BMS 189664, BMS 187745, didanosine, BMS 189664, BMS 187745, didanosine.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Expected fragment length</th>
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<tr>
<td>Ocludin</td>
<td>5'-AATGGCATACTCCTCCAACG</td>
<td>5'-AGTCATCCACGCCAACAGGTC</td>
<td>125</td>
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<tr>
<td>Claudin 1</td>
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<td>5'-GAAGGTGTGGCTTGGGATGA</td>
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<tr>
<td>Claudin 3</td>
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<td>5'-GATCTTTGTGGTGGCGACT</td>
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<tr>
<td>Claudin 5</td>
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<td>5'-ACTCCCCACTACGATGTTG</td>
<td>156</td>
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<tr>
<td>Claudin 7</td>
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<td>5'-AGGTATGCAAGCTTGTAT</td>
<td>201</td>
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Table I. Gene-Specific PCR Primers