Modulation of the Tight Junctions of the Caco-2 Cell Monolayers by H_2-antagonists

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Purpose. The tight junctions in the intestinal epithelium represent highly specialized intercellular junctions. Ranitidine, an H_2-antagonist, causes a tightening of the tight junctions. Hence, we have investigated the effect of ranitidine and other H_2-antagonists on the function of the intestinal tight junctions.

Methods. Effect of the H_2-antagonists on the tight junctions has been investigated using the transepithelial electrical resistance (TEER) and the transport of mannitol across the Caco-2 cell monolayers.

Results. Four different H_2-antagonists caused an increase in the TEER across the Caco-2 cell monolayers, accompanied by a decrease in the permeability for mannitol. The effect was concentration-dependent and saturable. Ranitidine and famotidine, caused a decrease in their own transport rate across the Caco-2 cells. Ranitidine competitively inhibited the increase in TEER caused by famotidine, whereas compounds which represent molecular fragments of ranitidine had no effect. The relative potency of the four H_2-antagonists in causing an increase in the TEER correlated inversely with the oral bioavailability of these compounds in humans.

Conclusions. We hypothesize that the H_2-antagonists exert their effect on the tight junctions of Caco-2 cells by modulation of interactions among proteins associated with the tight junctional complex.

KEY WORDS: H_2-antagonists; ranitidine; tight junctions; Caco-2; paracellular transport; *in vitro* model.

INTRODUCTION

Oral route is the most convenient and widely used route for the administration of therapeutic agents. In order for an oral drug to be therapeutically effective, it is necessary for the drug to traverse the epithelial lining of the gastrointestinal tract, the major barrier to the absorption of orally dosed drugs. While many lipophilic drugs rapidly partition into the intestinal mucosal membranes and are well absorbed (transcellularly) from the gastrointestinal tract, many hydrophilic drugs are poorly absorbed after oral administration. This is because hydrophilic drugs traverse across the intestinal mucosa via the intercellular spaces (paracellular route); an inefficient process due to (i) small surface area available to enter the intercellular spaces and (ii) presence of the tight junctions which seal the intercellular spaces (1,2).

Using the Caco-2 cell line, whose morphological and biochemical properties closely resemble those of the small intestinal cells (3–6), we have shown that the H_2-antagonist ranitidine traverses the in vitro model of intestinal mucosa predominantly via the paracellular pathway (7). These results are consistent with the observation that the absorption of ranitidine in humans after oral administration is incomplete and that its bioavailability is ~50% (8) despite little or no first pass metabolism. During our studies with ranitidine, we observed that it increases the "tightness" of the tight junctions when present on the apical (AP) side of the Caco-2 cells as evidenced by a significant increase in the transepithelial electrical resistance (TEER). Since, modulation of the tight junctions could potentially influence the absorption of drugs like ranitidine which are predominantly absorbed via the paracellular pathway, we have investigated the effect of ranitidine on the tight junctions of Caco-2 cells. We report here that several H_2-antagonists (see Figure 1 for structures), in addition to ranitidine, cause an increase in the "tightness" of the tight junctions by specific and saturable interactions with some component on the cell surface or of the tight junction complex. We also show that the modulation of the tight junctions by the H_2-antagonists results in decreased permeability of polar molecules, including ranitidine, across Caco-2 cell monolayers.

MATERIALS AND METHODS

Chemicals

[^{14}C]mannitol (radiochemical purity = 99%, specific activity = 55mCi/mmol) was purchased from NEN Research Products, Boston, MA. Ranitidine-HCl, and nizatidine were obtained from Glaxo Research Institute, Research Triangle Park, NC. Famotidine and cimetidine were obtained from Sigma Chemical Co., St. Louis, MO. 5-(Dimethylaminomethyl)furfuryl alcohol was purchased from Aldrich Chemical Co., Milwaukee, WI. The "amine fragment", 2-[(5-N,N-dimethylaminomethyl)-2-furanylmethyl]thioethane amine, was synthesized as described in the literature (9).

Incubation Media

Eagle's minimum essential medium (mod.) 1X (w/Earle's salts and L-glutamine) was obtained from Fisher Scientific, Pittsburgh, PA. Fetal bovine serum (FBS) was purchased from Gibco Laboratories, Grand Island, NY. Nonessential amino acids (NEAA), Hank's balanced salt solution (HBSS), 0.05% trypsin solution (10X), N-hydroxyethylpiperazine-N′-2-ethanesulfonic acids (HEPES), and phosphate buffered saline (PBS) were purchased from Sigma, Chemical Co., St. Louis, MO.

Cell Culture

Caco-2 cells, originated from a human colorectal carcinoma, were obtained from American Tissue Culture Collection, Rockville, MD., and were cultured at 37°C in minimum essential medium, containing 10% FBS and 1% NEAA, in an atmosphere

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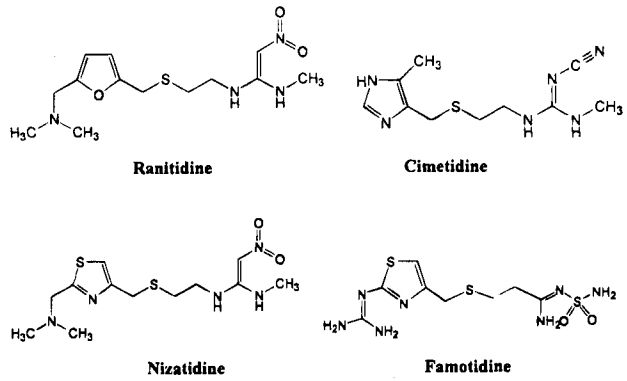

Fig. 1. Structures of H_2-antagonists.

of 5% CO_2 and 90% relative humidity (3,10). Cells were passaged every 3–4 days at a split ratio of 1 to 10 (1.0mL of 0.05% trypsin was used per 75cm^2 flask for trypsinization). Cells were seeded at a density of 80,000 cells/well onto the polycarbonate membranes of 1.0 cm^2 Transwells™ (12mm i.d., 3.0μm pore size, Costar, Cambridge, MA), and allowed to grow to late confluency (20–25 days). Media were changed every 2 days after seeding. The culture medium was replaced with the transport buffer (HBSS containing 25mM glucose and 25mM HEPES buffer pH 7.0), 1h prior to the experiment.

Measurements of TEER

The integrity of the tight junctions was checked by measuring TEER (11) (expressed as $\Omega \times cm^2$) using an EVOM Epithelial Tissue Voltohmmeter (World Precision Instruments, Sarasota, FL) and an Endohm-12 electrode. TEER values were measured before and during the treatment with H_2-antagonists. TEER values were in the range of 320–520 $\Omega \times cm^2$ prior to the experiment. The experiments were initiated by adding 0.4mL of transport buffer containing ranitidine, famotidine, cimetidine, or nizatidine to the AP side of the Transwells™. In the experiment where the effect of ranitidine on the basolateral (BL) membrane was investigated, 1.5mL transport buffer containing ranitidine (at concentration of 10mM, 30mM, 70mM, or 100mM) was added to the BL side of the Transwells™. Cell monolayers were incubated at 37°C and TEER values were measured at desired time points. Three measurements were taken for each concentration.

Transport Studies

Transport experiments were initiated by replacing the apical media with 0.4mL of transport buffer containing drugs. Concentrations of [^{14}C]mannitol, ranitidine, and famotidine were 25μM (55mCi/mmol), 10–200mM, and 5–50mM (the solubility limit of famotidine is 50mM), respectively. Transport rates were monitored by measuring the amount of drugs accumulated in the BL media (1.5mL) for the first 15min. The amount of radiolabeled [^{14}C]mannitol transported was measured by liquid scintillation counting in a Beckman LS-5801 spectrophotometer. The amount of BL ranitidine or famotidine was quantified by HPLC using a BDS Hypersil C18 column (250 × 4.6mm, 5μm) and an isocratic mobile phase (80% 50mM phosphate buffer, pH 6.0 and 20% methanol). Ranitidine and famotidine were monitored by uv at 320nm and 220nm, respectively. All transport experiments were carried out under sink conditions (transport experiments were designed such that less than 10% of the total amount of the compounds was present on the BL side at any given time). The integrity of tight junctions of cell monolayers was checked by measuring the transport of [^{14}C]mannitol and/or measuring TEER prior to the experiment.

Data Analysis

The apparent permeability coefficients (P_{app}, expressed as cm/sec) were calculated from the equation: $P_{app} = (dQ/dt)/C_o*A$ where dQ/dt is the flux (mol/sec), C_o is the initial concentration of drug molecule on the AP side of cell monolayers (mol/mL), and A is the surface area of the porous membrane (cm^o). The means between relevant pairs of data were considered significantly different provided the two-tail p values were less than 0.05 in the student t test.

RESULTS AND DISCUSSION

Effect of H_2-antagonists on Transepithelial Electrical Resistance (TEER) of Caco-2 Cell Monolayers

When ranitidine (200 mM) was applied on to the AP side of the Caco-2 cell monolayers, the TEER value increased by over 2 fold (Figure 2). The effect on the TEER value was maximal at the time of the first measurement, i.e. 5 min after the treatment. Upon longer incubation, the TEER value continued to decrease with time (note that at 60 min TEER values decreased to 60–70% of those at 5 min). This effect must be specific, as mannitol solutions of similar osmolality caused much smaller increase in TEER (data not shown). A small increase in the TEER value was observed when ranitidine (10–70 mM) was applied on to the BL side (Figure 2); however, in comparison with the increase in TEER resulting from the application of ranitidine to the AP side, this increase was substantially lower (compare Figures 2 and 3). Interestingly, the TEER value

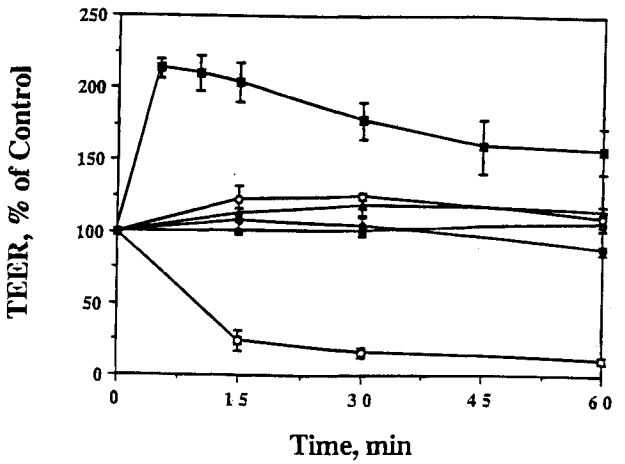

Fig. 2. Effect of ranitidine on TEER when present on the AP (■, 200mM) and BL (△, 0mM; ▲, 10mM; ○, 30mM; ●, 70mM; □, 100mM) side of the Caco-2 cell monolayers. TEER values were measured at different time points after incubating Caco-2 cell monolayers with various concentrations of ranitidine added on to the AP or BL side. Values are the mean of three measurements ± s.d.

decreased to 20% of the control when 100 mM of ranitidine was applied on to the BL side. Mannitol solutions of similar osmolality (~480 mOsm) caused a similar drop in TEER (data not shown). These results indicate that the effect of ranitidine that leads to an increase in TEER by its application to the AP side is likely to be mediated by its interactions with components of the junctional complexes, and that a decrease in TEER by its application at high concentration on the BL side is probably due to an osmotic effect. It is conceivable that ranitidine alters the interactions among proteins (Occludin, ZO-1, ZO-2, 130 kD protein) in the tight junctions (*zonula occludens*) (1,2) or the proteins in the cadherins superfamily present in the desmosomes (*macula adherens*) (12) and/or in the adherans junctions (*zonula adherens*) (13).

At a given time, the TEER value increased as a function of ranitidine concentration on the apical side of the Caco-2 cells (Figure 3). However, the increase in the TEER value appeared to plateau above 200 mM concentration of ranitidine (Figure 3). In addition to ranitidine, three additional H_2-antagonists (cimetidine, famotidine, and nizatidine; see Figure 1 for structures) were evaluated for their ability to modulate TEER of the Caco-2 cell monolayers. As shown in Figure 4, all four H_2-antagonists caused an increase in TEER across Caco-2 cell monolayers. At equimolar concentration, famotidine was the most potent (172% above control) and nizatidine was the least potent (17% above control) H_2-antagonist among the four compounds tested. An immediate recovery in all TEER values was observed after treated cells were washed with fresh buffer.

Effect of H_2-Antagonists on the Paracellular Transport

The increase in TEER caused by the H_2-antagonists suggests that these compounds may cause "tightening" of the tight junctions. Therefore, it follows that these H_2-antagonists may inhibit the transport of the paracellularly transported molecules. As shown in Table 1A, ranitidine causes a concentration-dependent decrease in its own permeability (Papp) and that of mannitol across the Caco-2 monolayers. At the highest concentration tested (200 mM), ranitidine causes 3 fold decrease in the permeability of mannitol. Even more noteworthy is the observation that at this concentration, ranitidine causes a 17 fold decrease in its own permeability. As expected, the decrease in the permeability is accompanied by an increase in the TEER value as the concentration of ranitidine is increased in the AP compartment.

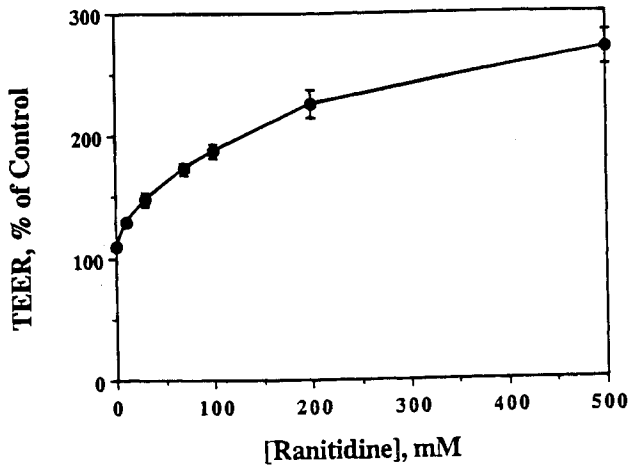

Fig. 3. Relationship between TEER across the Caco-2 cell monolayers and concentration of ranitidine. Cell monolayers were incubated with 10–500mM of ranitidine, added onto the AP side of cell monolayers. TEER values were measured 5 min after the treatment. Percent change of the TEER value was calculated relative to the value at time zero. At least three measurements were taken for each concentration.

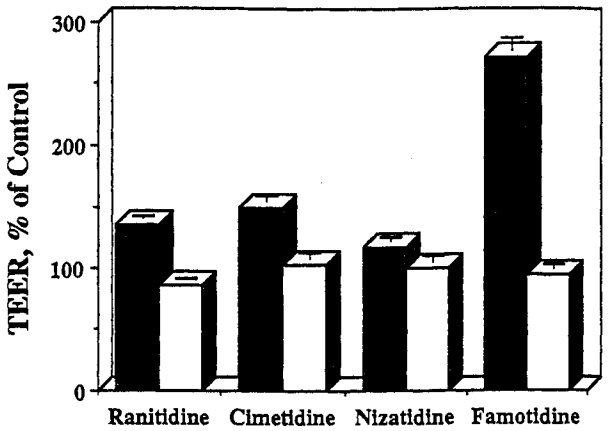

Fig. 4. Comparison of the Effect of H_2-antagonists on TEER of Caco-2 cell monolayers. Cell monolayers were incubated with 50mM of four H_2-antagonists (ranitidine, cimetidine, nizatidine, and famotidine) on the AP side, and the TEER value was measured 15 min after the treatment (solid bar) and 15 min after washing the cells (open bar). Percent change of the TEER value was calculated relative to the TEER value at time zero. At least three measurements were taken for each compound.

Table 1. Permeability of Ranitidine(A), Famotidine(B), and Mannitol in Relation to the Effect of Ranitidine and Famotidine on TEER of Caco-2 Cell Monolayers

(A). Ranitidine[a]

Ranitidine, mM	TEER[b], % of control	Papp[b] × 10^7, cm/sec	
		Ranitidine	Mannitol
0	108 ± 1	—	2.09 ± 0.03
10	121 ± 4	0.51 ± 0.22	2.20 ± 0.39
30	136 ± 5	0.14 ± 0.001	1.14 ± 0.01
70	142 ± 4	0.059 ± 0.01	0.90 ± 0.09
100	150 ± 1	0.05 ± 0.01	0.80 ± 0.05
200	204 ± 13	0.03 ± 0.003	0.70 ± 0.01

(B). Famotidine

Famotidine[c], mM	TEER[b], % of control	Papp[b] × 10^7, cm/sec		
		Ranitidine	Mannitol	Famotidine
0	129 ± 3	0.45 ± 0.03	1.48 ± 0.16	—
5	115 ± 4	0.34 ± 0.03	1.13 ± 0.09	1.90 ± 0.4
10	145 ± 8	0.25 ± 0.02	0.66 ± 0.05	1.44 ± 0.12
15	179 ± 16	0.15 ± 0.01	0.49 ± 0.08	1.25 ± 0.7
25	191 ± 19	0.09 ± 0.02	0.39 ± 0.04	0.77 ± 0.16
50	235 ± 16	0.08 ± 0.01	0.34 ± 0.02	0.77 ± 0.12

[a] Ranitidine added on to the AP side of the monolayers.
[b] Initial 15min of treatment. Values are the mean of three measurements ± s.d.
[c] Famotidine added on to the AP side of the monolayers.

Similar results were obtained when Caco-2 cells were treated with famotidine (Table 1B).

Specificity of the Effect of H_2-Antagonists on the Tight Junctions of Caco-2 Cells

In order to obtain a better insight into the nature of interactions between H_2-antagonists and the Caco-2 cells that lead to tightening of the tight junctions, we investigated the effect of ranitidine on the modulation of tight junctions by famotidine, the most potent modulator of the tight junctions among the four H_2-antagonists tested. At 25 mM concentration, famotidine caused 2.6 fold increase in the TEER value. In the presence of 25 mM ranitidine, the increase in TEER by 25 mM famotidine was only 1.8 fold (Figure 5). Approximately 30% decrease in the effect of famotidine on TEER of the Caco-2 cells is somewhat of an underestimate of the inhibitory effect of ranitidine on the modulation of the tight junctions by famotidine, as ranitidine (25 mM) itself would contribute to the increase in the TEER value. These results suggest that there is a competition between H_2-antagonists in binding to a specific site at the junctional complex or on the apical membrane of the Caco-2 cells.

The specificity of the interactions of the H_2-antagonists with the Caco-2 cells, leading to the modulation of the tight junctions, was further investigated by comparing the effect of ranitidine on the tight junctions with that of two other compounds representing partial structure of ranitidine. Thus the effect of ranitidine (100 mM) on TEER across the Caco-2 cell monolayer was compared with that of 5-(dimethylaminomethyl)furfuryl alcohol (100 mM) and of the "amine fragment", 2-[(5-N,N-dimethylaminomethyl)-2-furanylmethyl]-thioethane amine (100 mM) (Figure 6). Unlike ranitidine, the "alcohol fragment" did not show any effect on TEER (Figure 6); whereas, the "amine fragment"caused an increase in TEER that was only slightly lower than that caused by ranitidine (Figure 6). These results clearly demonstrate that although the interactions between ranitidine (and other H_2-antagonists) and Caco-2 cells

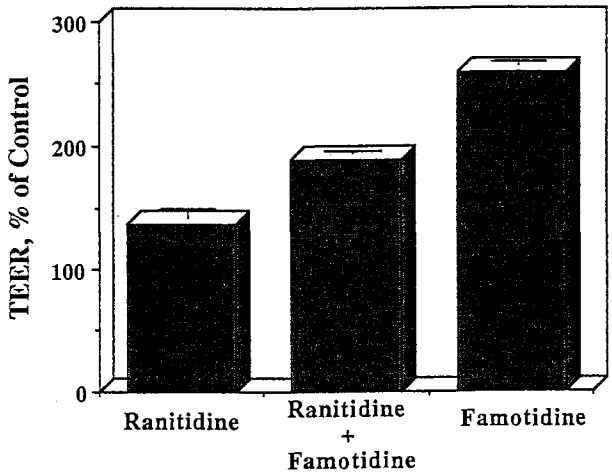

Fig. 5. Inhibition of famotidine-mediated increase in TEER of Caco-2 cell monolayers by ranitidine. Cell monolayers were incubated with 25mM of famotidine, ranitidine, or both for 15 min. Percent change of the TEER value was calculated relative to the TEER value at time zero. Values are the mean of three measurements ± s.d.

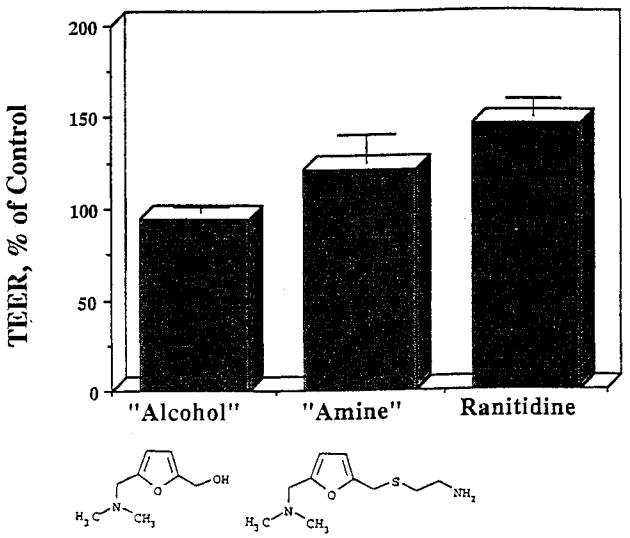

Fig. 6. Effect of molecular fragments of ranitidine on TEER across Caco-2 cell monolayers. TEER values were measured after incubating Caco-2 cell monolayers with 100mM of 5-(dimethylaminomethyl)furfuryl alcohol (structure shown), the "amine fragment" 2-[(5-N,N-dimethylaminomethyl)-2-furanylmethyl]thioethane amine (structure shown), or ranitidine for 15 min. Values are the mean of three measurements ± s.d.

that lead to increase in TEER are specific, the diaminonitrovinyl group may not be critical for the effect.

Relationship between the Potencies of H_2-Antagonists to cause an Increase of TEER and their Oral Bioavailabilities

The observation that ranitidine and the other H_2-antagonists examined in the present study cause "tightening" of the tight junctions raises an important question; i.e. do these compounds affect their own paracellular absorption? In Table 2, we have compared the TEER values of the four H_2-antagonists to their bioavailabilities in humans obtained from literature. It is of interest to note that famotidine, with the highest potency in increasing TEER, has lowest bioavailability; ranitidine and famotidine with intermediate potency have moderate bioavailability; whereas nizatidine, with the lowest potency in increasing TEER, has highest bioavailability (Table 2). These results provide a circumstantial evidence (but not definitive proof) that H_2-antagonists may be affecting their own absorption. Because

Table 2. Relationship between Potencies of H_2-antagonists to cause an Increase in the TEER Values and their Oral Bioavailabilities.

Compound	TEER, % of Control[a]	F, %
Famotidine	272 ± 9	40–49 (ref. 8)
Ranitidine	155 ± 5	51–60 (ref. 15)
Cimetidine	150 ± 4	58–66 (ref. 16)
Nizatidine	117 ± 3	84–112 (ref. 17)

[a] Cell monolayers were treated with 50mM of H_2-antagonists and TEER values were measured at 15min after the treatment. Percent change of the TEER value was calculated relative to the zero-time TEER value and compared to the reported oral bioavailability (%F).

ranitidine, and presumably other three H_2-antagonists examined here, is predominantly transported across intestinal mucosa via the paracelluar pathway (it was reported that ranitidine absorption in the human GI tract is site-specific with the following order: stomach = jejunum > cecum, ref. 7,14), it is reasonable to expect that these H_2-antagonists will affect their own absorption provided that sufficiently high concentrations are attained in the lumen of the GI tract.

In summary, we report here a novel finding that H_2-antagonists have the potential to reduce the epithelial permeability of hydrophilic drugs across the human intestinal mucosa by modulating the properties of tight junctions in the epithelial layer. We have provided a circumstantial evidence that H_2-antagonists could influence their own oral absorption due to their effect on the tight junctions. The effect of H_2-antagonists on the tight junctions is saturable and specific, and is presumably via modulation of interactions among proteins in the tight junctional complex—i.e. the transmembrane protein Occludin and/or the cytoplasmic proteins ZO-1, ZO-2, the 130 kD protein associated with the tight junctions (cf. ref 1,2). The differential effect of ranitidine on TEER, when applied on the AP or the BL side, suggests that the effect of H_2-antagonists on the tight junctions does not require entry of the these agents inside the cells. This is further supported by our previous observations that ranitidine traverses across Caco-2 cells via the paracellular pathway and does not enter the cells significantly (7).

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