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RESEARCH PAPER

Effect of Rhamnolipids on Permeability Across Caco-2 Cell Monolayers

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ABSTRACT

Purpose This report describes the effect of rhamnolipids (RLs), an amphiphilic biosurfactant produced by the bacterium Pseudomonas aeruginosa, on the integrity and permeability across Caco-2 cell monolayers.

Methods We measured the trans-epithelial electrical resistance (TEER) and permeability of [^{14}C]mannitol across Caco-2 cell monolayers upon incubation with 0.01–5.0% v/v RLs as a function of incubation time (30, 60, 90, and 120 min). We also studied the recovery of RL-treated Caco-2 cell monolayers upon incubation with Kaempferol, which is a natural flavonoid that promotes the assembly of the tight junctions.

Results TEER of Caco-2 cell monolayers incubated with 0.01–5.0% v/v RLs solution dropped to 80–28% of that of untreated cells. Decline in TEER was associated with an increase in [^{14}C]mannitol permeability as a function of RLs concentration and incubation time with Caco-2 cells. Incubation of RLs-treated Caco-2 cell monolayers with normal culture medium for 48 h did not restore barrier integrity. Whereas, incubation of a RLs-treated Caco-2 cells with culture medium containing Kaempferol for 24 h restored barrier function indicated by the higher TEER and lower [^{14}C]mannitol permeability values.

Conclusions These results show the ability of RLs to modulate the integrity and permeability of Caco-2 cell monolayers in a concentration- and time-dependent fashion, which suggest their potential to function as a non-toxic permeation enhancer.

KEY WORDS Caco-2 cell monolayers · oral drug delivery · paracellular permeability · rhamnolipids · transpethelial electrical resistance

INTRODUCTION

Oral delivery is the most desirable route for administration of macromolecular drugs, however their poor permeation across the small intestinal epithelium has resulted in low oral bioavailability (1). This is due primarily to the restrictive tight junctions (TJ) present between adjacent epithelial cells, which limit the paracellular transport of drugs across the intestinal epithelium. Several classes of absorption enhancers, including fatty acids (2–6), steroids (6–8), phosphonates (9–11), acyl carnitine, alkanoyl-cholines (11,12), as well as mucoadhesive polymers (*e.g.* chitosan) and polyamidoamine (PAMAM) dendrimers (13–18) have been used to increase transport across epithelial barriers (2,6,8,11,19–22). These agents produce their effect by different mechanisms including: i) modulating the structure and localization of tight junction (TJ) complexes and ii) solubilizing membrane components and increasing intracellular calcium concentrations leading to contraction of the actin-myosin filaments and dilation of the TJs (23). While these agents are able to successfully modulate TJs permeability and increase oral bioavailability of drugs both *in vitro* and *in vivo*, their activity is commonly coupled with cytotoxicity due to irreversibly

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compromising the integrity of the intestinal epithelium, which has hindered their clinical use (19,22,24).

Alternatives to these synthetic absorption enhancers include the controlled use of bio-inspired agents shown to modulate TJs permeability under different pathological conditions. In fact, it has been shown that many enteric bacteria and viruses can permeate across the small intestinal epithelium *via* the paracellular route despite their large size (25–27). These enteric pathogens utilize specific virulence factors that are displayed on their surface or secreted into the intestinal lumen to degrade or delocalize TJs proteins (*e.g.* claudins) and allow the diffusion of the pathogen from the small intestine into the systemic blood circulation (25–27). The effect of enteric pathogens on the integrity of the intestinal epithelium motivated the search for biologically-derived molecules that can selectively and reversibly modulate the integrity of the TJs to increase the permeability of therapeutic molecules across the intestinal epithelium. Zonula occludens toxin (ZOT) isolated from *Vibrio cholera*, and its 12 kDa active fragment known as ΔG (28), are the most extensively studied agents in this category of permeability enhancers (26). Both ZOT and ΔG proved to enhance paracellular permeability across rat intestinal epithelium and Caco-2 cells with minimal toxicity (28–31). In this report, we evaluate the effect of natural surfactants known as rhamnolipids (RLs) that are produced by *Pseudomonas aeruginosa* (32) on the permeability across Caco-2 cell monolayers. Rhamnolipids are composed of a hydrophilic mono- or di-rhamnose head attached to two hydrophobic C8 lipid tails (32), which are recognized as the active agent responsible for disruption of the TJs in lung epithelium and invasion of *Pseudomonas aeruginosa* into the systemic circulation (33–35). We hypothesized that RLs can reversibly modulate the integrity of the TJs in Caco-2 cell monolayers in a concentration- and incubation time-dependent fashion resulting in enhanced paracellular permeability (Fig. 1). Therefore, we investigated the change in trans-epithelial electrical resistance (TEER) and transport of [^{14}C]mannitol (paracellular permeability marker) across Caco-2 cell monolayers as a function of RLs concentration and incubation time. These permeability studies were carried out using RLs concentrations that proved to be well tolerated by Caco-2 cells based on the lactate dehydrogenase (LDH) leakage assay. Further, we investigated the effect of Kaempferol, a natural flavonoid that enhances the expression of zonula occluden and promotes the assembly of the TJs (36), on the integrity of Caco-2 cell monolayers after treatment with RLs.

MATERIALS AND METHODS

Materials

Human colorectal adenocarcinoma (Caco-2) cells were purchased from American Type Cell Culture (ATCC, Rockville, VA). [^{14}C]Mannitol (specific activity 51.5 mCi/mmol) was purchased from Moravek Radiochemicals (Brea, CA). Rhamnolipids was purchased as an aqueous 10% w/v solution from Jeneil Biosurfactant Co. (Saukville, WI). Kaempferol and Hanks Balance Salt Solution were purchased from Sigma-Aldrich Inc. (St. Louis, MO). EcoLume™ analytical liquid scintillation fluid was purchased from MP Biomedical (Solon, OH).

Caco-2 Cell Culture

Caco-2 cells were grown at 37°C, 5% CO_2 and 95% relative humidity using Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 0.05% penicillin/streptomycin/amphotericin. Cells were passaged at 80–90% confluency using a 0.25% trypsin/0.20% ethylene diamine teraacetic acid (EDTA) solution. Media was changed approximately every 48 h. Caco-2 cells (passages 26–40) were seeded at 6.5×10^4 cells/cm^2 on polycarbonate 6-well Transwells® (Corning Costar Corporation, Cambridge, MA) (3.0 μm mean pore size) and used for transport experiments 21–28 days after seeding.

Lactate Dehydrogenase (LDH) Leakage Assay

LDH is a cytosolic enzyme that is not normally secreted outside the cell but leaks into the culture medium upon damage to cell membranes. LDH leakage into the apical compartment was used to measure the effect of rhamnolipids on the viability of Caco-2 cell monolayers. Caco-2 cell monolayers cultured in 6-well Transwells® were incubated with rhamnolipids at apical concentrations of 0.05–5.0% v/v for 120 min. Caco-2 cell monolayers were also incubated with blank HBSS or 2% Triton X-100 as negative and positive controls, respectively. LDH leakage into the apical compartments was quantified using LDH assay kit following the manufacturer's specifications (Roche Diagnostics Corporation, Indianapolis, IN). The observed LDH leakage upon incubation of Caco-2 cells with different RLs concentrations was normalized to that observed upon incubation with Triton X-100 (positive control). LDH leakage in response to different RLs concentrations was compared to that observed with Caco-2 cells incubated with blank HBSS (negative control). The observed LDH leakage upon incubation with different RL concentrations was compared to the observed leakage for Caco-2 cell monolayers incubated with regular culture medium (control) using student's *t*-test assuming unequal variance.

TEER Measurements

We measured the change in trans-epithelial electrical resistance (TEER) of Caco-2 cell monolayers upon incubation with

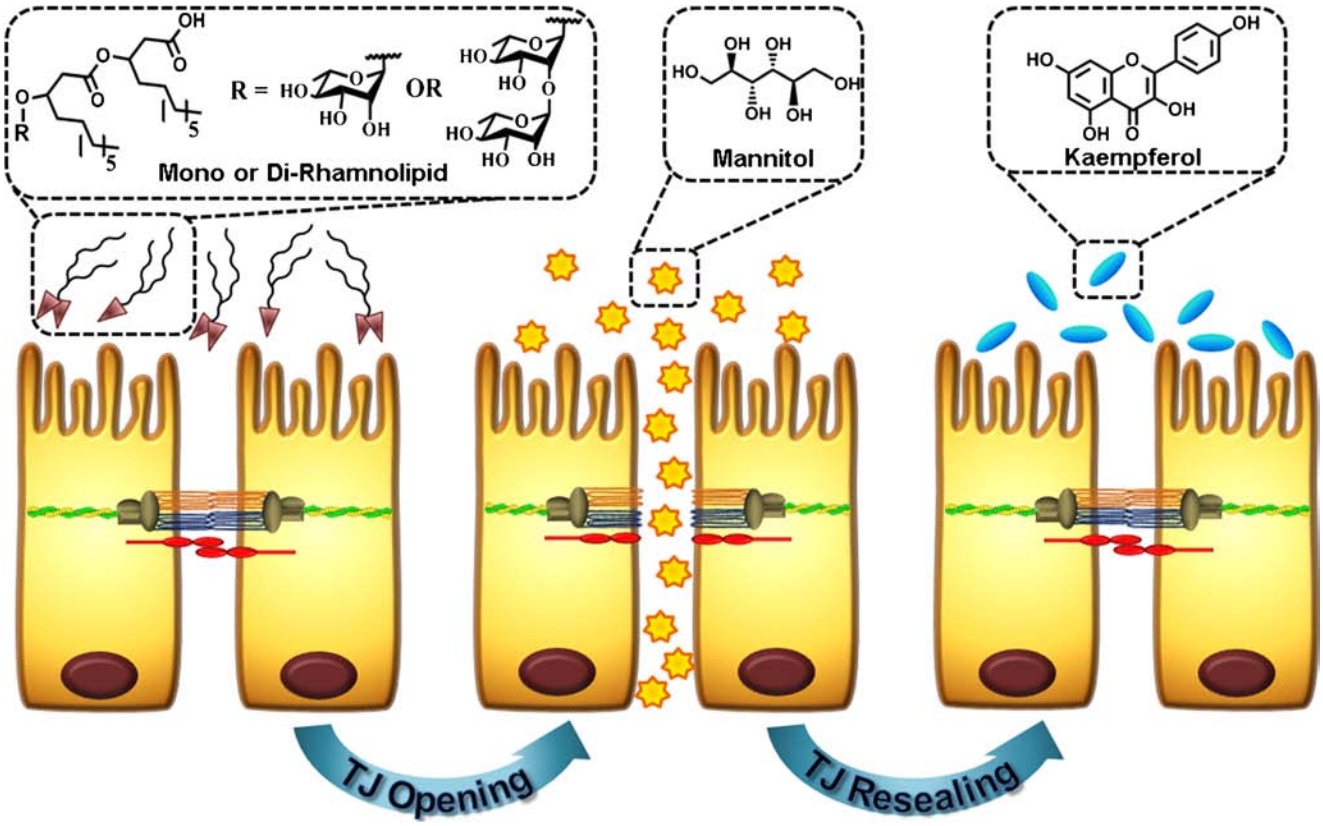

Fig. 1 A schematic drawing showing the ability of rhamnolipids to modulate the tight junctions between adjacent intestinal epithelial cells allowing for enhanced mannitol transport *via* the paracellular route. Incubation to rhamnolipids-treated Caco-2 cells with Kaempferol reseals the tight junctions.

different concentrations (0.01–5.0% v/v) of rhamnolipids. Briefly, a Millicell® ERS meter (Millipore Corporation, Bedford, MA) connected to a pair of chopstick electrodes were inserted in the donor and receiver chambers of treated Caco-2 cells to record the TEER values after 30, 60, 90 and 120 min. of adding the rhamnolipids solution. TEER values of Caco-2 cells incubated with blank HBSS were in the range of 800–1,200Ω.cm^2 and used as a control. All Caco-2 cell monolayers were incubated at 37°C, 5% CO_2 and 95% relative humidity while shaking on an orbital shaker at 50 revolutions per minute (RPM). TEER values were normalized to that recorded directly before adding the rhamnolipids solution.

Effect of Rhamnolipids on Caco-2 Cell Permeability

Permeability of [^{14}C]mannitol (12.3 μM) across Caco-2 cell monolayers was investigated upon incubation with different concentrations (0.01–5.0% v/v) of rhamnolipids. Permeability of [^{14}C]mannitol across Caco-2 cell monolayers in the absence of rhamnolipids was investigated and used as a control. Specifically, Caco-2 cell monolayers were incubated at 37°C, 5% CO_2 and 95% relative humidity while shaking at 100 RPM and samples were collected from the receiver compartment after 30, 60, 90 and 120 min. At the end of the experiment, the donor solutions were collected and assayed to determine the amount of [^{14}C]mannitol remaining in the apical chamber. All samples were mixed with Ecolume scintillation fluid and analyzed using liquid scintillation counting (Beckman Coulter, Indianapolis, IN). Effective permeability coefficient (P_{eff}) of [^{14}C]mannitol in the presence and absence of rhamnolipids was calculated following previously reported method (37). Permeability of [^{14}C]mannitol across the Transwell® filters without cultured Caco-2 cells was determined and labeled filter permeability (P_{filter}). Permeability of [^{14}C]mannitol across Caco-2 cell monolayers (P_m) was estimated by correcting the effective permeability (P_{eff}) for filter permeability (P_{filter}) according to: $P_{eff}^{-1} = P_m^{-1} + P_{filter}^{-1}$ equation. Permeability of [^{14}C]mannitol across RLs-treated Caco-2 cell monolayers was compared to its permeability across untreated cells using student's *t*-test assuming unequal variance.

Effect of Kaempferol on Caco-2 Cell Monolayers

The ability of Kaempferol to restore the integrity of Caco-2 cell monolayers and "seal" the TJs after incubating for 2 h with different concentrations of rhamnolipids was investigated based on the change in monolayer permeability. Briefly, the treatment

solution in the apical chamber was removed at the end of the incubation time (2 h) followed by washing Caco-2 cell monolayers twice with fresh HBSS before adding 1.5 mL of DMEM culture medium to the basolateral chamber. To the apical chamber was added 0.5 mL of DMEM with or without 100 μM Kaempferol. Caco-2 cells were then allowed to recover for 24–48 h under normal culture conditions while changing the culture medium every 24 h. At the end of the recovery period Caco-2 cell monolayers were washed twice with sterile HBSS before investigating TEER and the permeability of [^{14}C]mannitol following the same procedure described earlier. Permeability of [^{14}C]mannitol across RLs-treated Caco-2 cell monolayers was compared to its permeability across untreated cells using student's *t*-test assuming unequal variance.

RESULTS AND DISCUSSION

Cytotoxicity of Rhamnolipids

Rhamnolipids are bacterially-derived surfactants secreted by *Pseudomonas aeruginosa*, which proved to modulate the expression of TJ proteins facilitating bacterial infiltration across lung epithelium and into the basal membrane (34,35). We hypothesized that rhamnolipids can exhibit a similar effect on the intestinal epithelium thus enabling the diffusion of drug molecules and particulate carriers from the gastrointestinal lumen into the systemic circulation. However, it is important that this TJ modulating effect should not be associated with a toxic effect towards intestinal epithelial cells. Using Caco-2 cells as a model for the human intestinal epithelium, we investigated the toxicity of different concentrations of RLs using the leakage of LDH enzyme as an indicator of cytotoxic effects. Incubation of Caco-2 cell monolayers with 0.05–5.0% v/v rhamnolipids caused modest increase (9–13%) in LDH leakage compared to Caco-2 cells incubated with blank HBSS (Fig. 2).

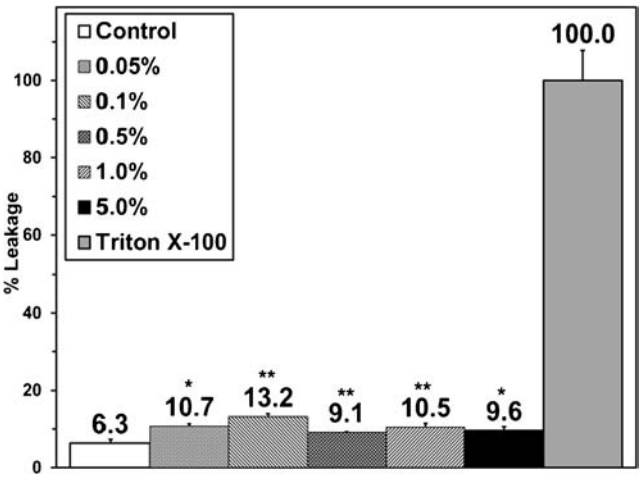

Fig. 2 Change in the LDH leakage upon incubation of Caco-2 cell monolayers with blank HBSS (control) and different concentrations of rhamnolipids (0.05–5.0% v/v) for 120 min. LDH leakage is reported as % of the leakage observed upon incubation of Caco-2 cells with Triton X-100 (positive control). Results are reported as mean ± standard error of the mean ($n = 5$). Statistical difference in % LDH leakage upon incubation with different concentrations of rhamnolipids compared to untreated control group is denoted by * or **, which indicate that $p \leq 0.05$ or $p \leq 0.01$, respectively.

Effect of Rhamnolipids on Integrity of Caco-2 Cell Monolayers

Transmembrane electrical resistance offers a quantitative technique to measure the integrity of the tight junctions that govern solute transport across the paracellular space of endothelial and epithelial monolayers (14,38). We investigated the ability of RLs to modulate the integrity of the TJs in Caco-2 cell monolayers as a function of RLs concentration and incubation time. Results show that trans-epithelial electrical resistance (TEER) of Caco-2 cell monolayers incubated with blank HBSS (control) remained constant for up to 120 min (Fig. 3). Incubation of Caco-2 cells with 0.01% v/v of RLs caused a modest drop in TEER values to 80% of the control group after 120 min of incubation. Increasing the concentration of RLs to 0.05% v/v decreased TEER values to 67% of the control group within 30 min and further declined to 53% after 120 min. Incubation of Caco-2 cells with high RLs concentrations (0.1–5.0% v/v) caused a rapid and sharp decline in TEER values 42–28% of the control group (Fig. 3). These results show that RLs cause a concentration-dependent decline in TEER values of Caco-2 cells indicating the modulation in TJs integrity.

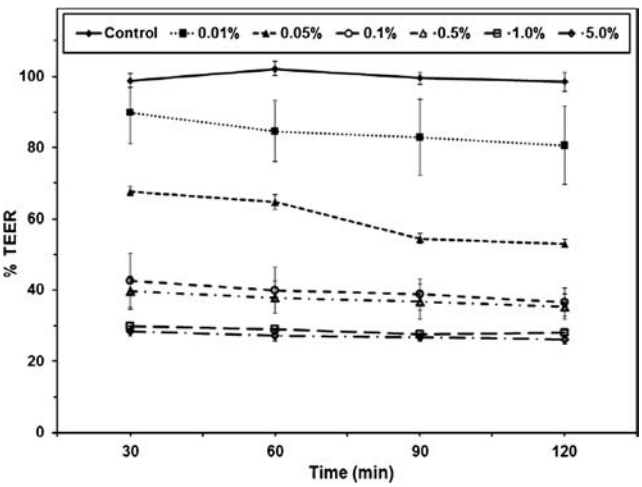

Fig. 3 Effect of blank HBSS (control) and different rhamnolipid concentrations (0.01–5.0% v/v) on trans-epithelial electrical resistance (*TEER*) across Caco-2 cell monolayers as a function of incubation time (30–120 min). TEER values are normalized to initial resistance before the addition of different treatment solutions. Results are reported as mean ± standard error of the mean ($n = 5$).

Effect of Rhamnolipids on Paracellular Permeability

We investigated the permeability of [^{14}C]mannitol across Caco-2 cell monolayers upon incubation with different concentrations of RLs (0.01–5.0% v/v) to determine if the observed modulation in TJs integrity indicated by the decline in TEER values (Fig. 3) is associated with quantifiable change in paracellular permeability. [^{14}C]mannitol was selected as a permeability marker due to its high aqueous solubility, stability against metabolic enzymes, and exclusive transport *via* the paracellular route (39,40). Results show that apical-to-basolateral permeability of mannitol in absence of RLs (control) remained constant around ~3.5×10^{-6} cm/s at 30, 60, 90, and 120 min (Fig. 4). Incubation of Caco-2 cell monolayers with 0.01% RLs did not affect mannitol permeability at 30 and 60 min but increased to 6.7×10^{-6} cm/s and 5.4×10^{-6} cm/s at 90 and 120 min, respectively. Incubation of Caco-2 cell monolayers with 0.05% RLs caused a time-dependent increase in mannitol permeability, which increased to 6.8×10^{-6}, 7.9×10^{-6}, 13.8×10^{-6}, and 16.2×10^{-6} cm/s after 30, 60, 90, and 120 min of incubation, respectively. Incubation of Caco-2 cell monolayers with higher concentrations of RLs caused a similar time-dependent increase in mannitol permeability that reached 46.4×10^{-6}, 52.3×10^{-6}, 84.4×10^{-6}, and 95.7×10^{-6} cm/s upon incubation for 120 min with 0.1%, 0.5%, 1.0% and 5.0% v/v of RLs, respectively (Fig. 4).

The observed decrease in TEER (Fig. 3) coupled with an increase in mannitol paracellular permeability (Fig. 4) can be a result of the reported alteration in TJs architecture and protein expression in airway epithelium treated with RLs (33–35). For example, Zulianello and co-workers showed that the number of TJs strands between adjacent epithelial cells decreased upon treatment of cultured airway epithelium with RLs (34). Zulianello and co-workers also reported doubling in the number of "loose ends" of the TJs fibril structure in RLs-treated epithelial monolayers compared to untreated ones (34). Other reports showed that treatment of Caco-2 cell monolayers with RLs caused a decline in phosphorylated TJs proteins and reduced the expression of E-cadherin (35). These results show that non-toxic RLs concentrations cause a concentration- and incubation time-dependent increase in paracellular permeability across Caco-2 cell monolayers.

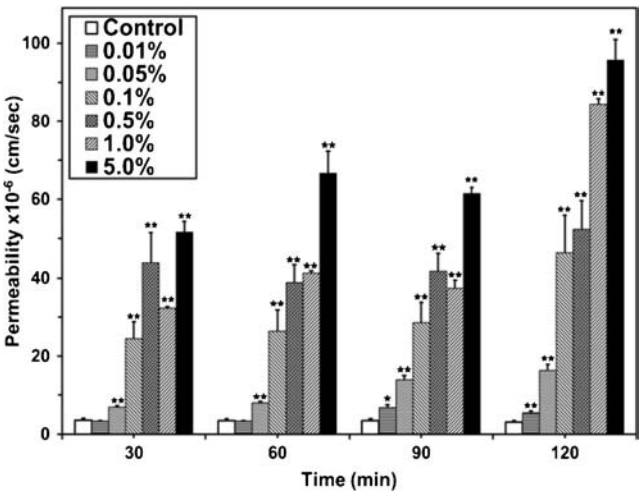

Fig. 4 Permeability of [^{14}C]mannitol across Caco-2 cell monolayers upon incubation with HBSS (control) and different concentrations of rhamnolipids (0.01–5.0% v/v) at different incubation times. Results are reported as the mean + the standard error of the mean ($n = 5$). Statistical difference in [^{14}C]mannitol permeability across treated Caco-2 cell monolayers compared to control group is denoted by * or **, which indicate that $p \leq 0.05$ or $p \leq 0.01$, respectively.

Resealing of Caco-2 Cell Monolayers

Successful development of RLs into an adjuvant to enhance oral bioavailability of poorly absorbed drugs requires rapid, efficient, and reversible opening of the TJs to enhance drug's permeability across the intestinal epithelium. Therefore, it is important to assess the ability of Caco-2 cell monolayers to "reseal" the TJs after the removal of RLs. We evaluated the change in TEER values and permeability of [^{14}C]mannitol across RLs-treated Caco-2 cell monolayers after removal of RLs, washing the cells with fresh HBSS, and incubation in culture medium for 48 h. Results show that TEER values of Caco-2 cell monolayers treated with 0.1–5.0% v/v RLs did not recover after 48 h of incubation in regular culture medium (Fig. 5a). Similarly, permeability of [^{14}C]mannitol across RLs-treated Caco-2 cell monolayers remained significantly higher (50×10^{-6}–88×10^{-6} cm/s) than that observed in the control group (Fig. 5c). The inability of Caco-2 cell monolayers to recover and restore barrier function within 48 h of their incubation with RLs is not surprising given that it takes 21 days to establish functioning TJs complexes under normal culture conditions (41).

Kaempferol is a natural flavonoid that has been shown to promote the expression and assembly of TJ proteins (36). Specifically, earlier research showed that incubation of Caco-2 cell monolayers with a 100 μM solution of Kaempferol increases TEER values to 130% of untreated monolayers, which is attributed to increased expression of zonula occludins and caludin TJ proteins and hyper-phosphorylation of occludins (36). Confocal microscopy images of Kaempferol-treated Caco-2 cell monolayers showed increased accumulation of claudin-3 and occludin at the intracellular junctions between adjacent Caco-2 cells (36). These results prompted us to evaluate the ability of Kaempferol to restore TJs integrity in RL-treated Caco-2 cell monolayers *in vitro*. Results show that incubation of RL-treated Caco-2 cell monolayers with a 100 μM Kaempferol solution for 24 h under normal culture conditions restored 60–95% of the TEER values of control (untreated) cells indicating restoration of barrier integrity (Fig. 5b). Similarly, incubating RL-treated

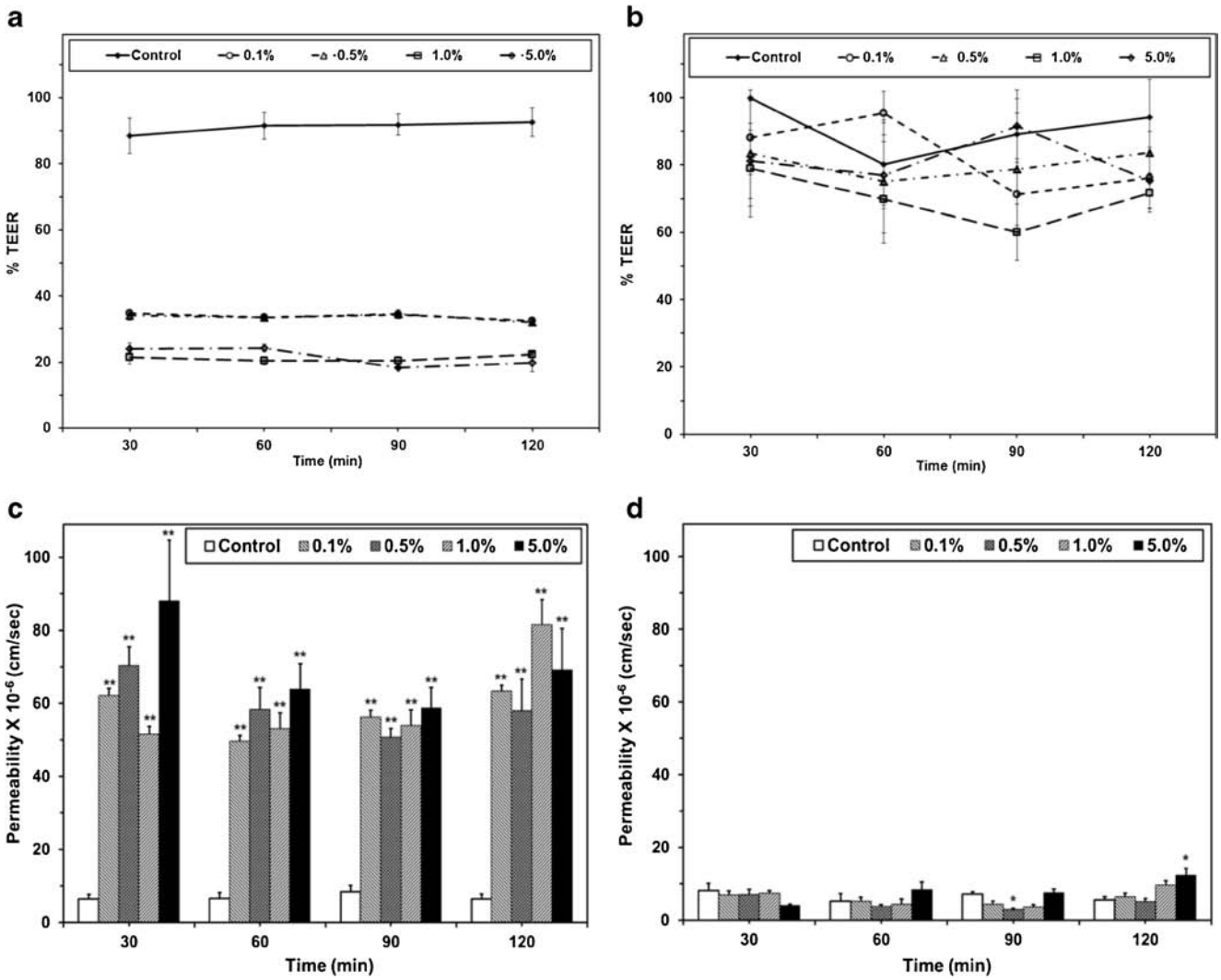

Fig. 5 Recovery of TEER (**a**, **b**) and [^{14}C]mannitol permeability (**c**, **d**) across Caco-2 cell monolayers after incubating with different concentrations of rhamnolipids (0.1–5.0% v/v) for 120 min, followed by incubation with blank DMEM culture medium for 48 h (**a**, **c**) or 100 μM Kaempferol containing DMEM for 24 h (**b**, **d**). Results are reported as the mean + the standard error of the mean ($n = 5$). Statistical difference in TEER values and [^{14}C]mannitol permeability compared to control group is denoted by * or **, which indicate that $p \leq 0.05$ or $p \leq 0.01$, respectively.

Caco-2 cell monolayers with 100 μM Kaempferol solution for 24 h reduced [^{14}C]mannitol permeability to match that of the control (untreated) group at all incubation time points (Fig. 5d). These results collectively indicate the ability of Kaemperol to restore barrier integrity for RLs-treated Caco-2 cell monolayers.

It is important to note that while Kaempferol was necessary to restore TJs integrity in RL-treated Caco-2 cell monolayers *in vitro*, it is likely that the range of repair mechanisms present *in vivo* may restore the integrity of the intestinal epithelium without the need for an external aiding agent like Kaempferol. It is well established that TJs assembly and maintenance are regulated by a number of physiological and pathological stimuli (42,43). For example, the presence of probiotic bacteria has been shown to regulate the assembly of TJs proteins to reduce the permeability and preserve barrier function in many disease states (42).

CONCLUSIONS

Our results show the ability of rhamnolipids to modulate the integrity and increase the paracellular permeability across Caco-2 cell monolayers in a concentration- and incubation time-dependent fashion. Incubation of RLs-treated Caco-2 cells with Kaempferol reseals the tight junctions and restores barrier properties. Rhamnolipids are a promising new class of permeation enhancers that can potentially be used to increase the oral bioavailability of therapeutic drug molecules with minimal non-specific toxicity towards intestinal epithelial cells.

ACKNOWLEDGMENTS AND DISCLOSURES

Charity J. Wallace and Scott H. Medina contributed equally to this research.

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