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Research Article

Enhancing the Furosemide Permeability by Papain Minitablets Through a Triple Co-culture *In Vitro* Intestinal Cell Model

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Abstract. The administration of medicines by the oral route is the most used approach for being very convenient. Although it is the most popular, this route also has absorption, and consequently, bioavailability limitations. In this sense, several pharmacotechnical strategies have been used to improve drug absorption, one of which is the use of permeation promoters. Papain is a very versatile plant enzyme that can be used as a permeation promoter of various active compounds. This study aimed to evaluate the safety of papain and the formulation of native papain minitablets to promote *in vitro* permeation of furosemide through an innovative biomimetic triple co-culture model of Caco-2, HT29-MTX, and Raji cells. Regarding permeation, furosemide and metaprolol concentrations are determined with HPLC; those are used to calculate P_{app}. Monolayer integrity was evaluated using TEER and Lucifer Yellow. In the presence of papain, TEER decreased two-fold and the P_{app} of furosemide increased six-fold. The results suggest that native papain minitablets can be used as therapeutic adjuvants to enhance the permeation of drugs significantly improving bioavailability.

KEY WORDS: papain; *in vitro* safety; mucus; oral permeation enhancer; TEER.

Highlights

Triple co-culture cells mimic intestinal epithelium properties with mucus production.

Papain minitablets increase furosemide permeation by six-fold.

Papain promotes safe and effective oral permeation.

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Abbreviations: *ANOVA*, Analysis of Variance; *ATCC*, American Type Culture Collection; *BAPA*, Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride; *BSA*, Bovine serum albumin; *DAPI*, 4′,6-diamidine-2′-phenylindole dihydrochloride; *DMEM*, Dulbecco's modified Eagle's medium; *EC*, Enzyme classification; *ECACC*, European Collection of Cell Cultures; *FBS*, Fetal bovine serum; *FURO*, Furosemide; *GIT*, Gastrointestinal tract; *HBSS*, Hank's balanced salt solution; *Hepes*, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; *LY*, Lucifer yellow; *METO*, Metoprolol; *MTT*, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); P_{app}, Permeability coefficient; *P-gp*, P-glycoprotein; *PPN*, Papain; *RPMI*, Roswell Park Memorial Institute; *SEM*, Scanning electron microscope; *TEER*, Transepithelial electrical resistance; *TX-100*, Triton™ X-100.

INTRODUCTION

Oral drug administration is widely used route for being convenient and patient-friendly. In this regard, the oral route is less painful than the parenteral route and a good compliance route for patients with chronic diseases (1). However, oral absorption depends on drug release and on its permeability through the intestinal membrane. In addition, drug solubility, dissolution, low permeability in the intestinal lumen besides pH variation, presence of enzymes beyond the mucus layer, and first-pass mechanisms can hinder drug absorption and impair therapeutic efficacy (2).

It has been noticed a development of pharmacologically active compound release systems to overcome the challenges of the complex gastrointestinal environment. Of these, oral permeation enhancers can be considered an appropriate pharmacotechnical approach to increase solubility and modulate drug bioavailability, especially, the enhancers that promote a non-permanent change in the intestinal biological barrier with low cell membrane damage and low toxicity (3). Considering these factors, papain (EC 3.4.22.2) has been demonstrated to be a proteolytic plant enzyme that can increase permeation with selective local action on the paracellular permeability of the intestinal epithelium (4) with intense mucolytic activity (5). However, papain has low stability in pharmaceutical formulations. In order to overcome this limitation, solid dosage forms are interesting

options for oral administration of papain due to the absence of water in the formulation (6). In a study conducted by Sharma et al. (7), microsphere formulations of papain for enteral release were developed by emulsion solvent evaporation using hydroxypropyl methylcellulose phthalate, Eudragit L 100, and Eudragit S 100 to prevent gastric inactivation of papain in simulated gastrointestinal pH conditions. These microsphere formulations can act as carriers for oral enzyme delivery, and stability studies have indicated that they have adequate stability at room temperature. In a study by Zafar et al. (8), papain was used to improve the penetration of amoxicillin into the gastric mucosa. Amoxicillin-loaded nanocarriers coated with papain were synthesized by ionic gelation method, and the capacity of this system to enhance mucopermeation was evaluated. The results showed that these nanocarriers were effective in combating *Helicobacter pylori* infection because of the enhanced mucoadhesive and mucopermeation properties of this delivery system.

Modified-release solid oral dosage forms can be developed as monolithic or multiparticulate systems. Multiparticulate pharmaceutical systems include pellets, beads, minitablets, and microcapsules. Minitablets have attracted attention as they have numerous pharmaceutical advantages over other dosage forms (9). According to Tissen et al. (10), minitablets can be produced by direct compression, which is particularly useful for enzymes and other biological substances, because this type of formulation method does not involve wetting the formulation for granulation and subsequent drying. Compared with that of the monolithic system, the smaller size of minitablets ensures better distribution during passage through the gastrointestinal tract and, consequently, a shorter gastric retention time (11). Additionally, as minitablets have low porosity and a smooth surface, they can be easily coated with polymeric films, thus protecting the core from environmental factors such as light, humidity, and air, as well as from the inhospitable environment of the gastrointestinal tract, further ensuring the stability of the active ingredient (12).

Currently, the monolayer Caco-2 cell model, that spontaneously differentiates into polarized cells with clearly defined apical (mucosal) and basolateral (serosal) sites with enterocyte-like properties under conventional cell culture conditions, can be used to assess the safety besides the effect of drug permeability through the mucosal barrier (13). Although this model is considered the gold standard in permeability studies (14), it presents some limitations, including the absence of a mucus layer the absence of M cells (Microfold) (15).

A reliable co-culture model combining Caco-2, HT29-MTX (goblet-like cells), and Raji (B lymphocytes) cells has been established to closely mimic permeability features. Thus, the co-cultivation of these strains can provide improved physiological conditions by making the *in vitro* drug and drug candidate permeability evaluation model more biocompatible with the *in vivo* model by mimicking the intestinal barrier (16–20) especially for the evaluation of drug permeability and toxicological characteristics.

The objective of the present study was to evaluate the cytotoxicity of native papain over Raji and co-culture Caco-2 and HT29-MTX cells and the formulation of native papain minitablets to promote *in vitro* permeation of furosemide using a triple co-culture model of Caco-2, HT29-MTX, and Raji cells.

MATERIALS AND METHODS

Cells and Reagents

Human colon adenocarcinoma cell line (Caco-2) (ATCC® HTB-37™) (passage number 18) and Human Burkitt's lymphoma Raji B cell line (ATCC® CCL-86™) were obtained from ATCC® (Manassas, VA, USA). Human mucus-producing cell line (HT29-MTX-E12) was purchased from ECACC (ECACC 12040401) (Porton Down, Wilts, England) (passage number 50). Alexa Fluor™ 488 Phalloidin, LY, MTT, and PrestoBlue™ were acquired from Invitrogen™ (Carlsbad, CA, USA). Alcian Blue 8GX, BAPA (≥ 98%), BSA, DAPI, FURO (≥ 98%), glutaraldehyde, METO (≥ 98%), PPN pharmaceutical grade (30,000 USP-U/mg), and TX-100 were obtained from Merck KgaA (Darmstadt, HE, Germany). Formaldehyde solution and PrestoBlue™ solution were purchased from Thermo Scientific™ (Waltham, MA, USA). Colloidal silicon dioxide (Aerosil® 200 Pharma) was obtained from Evonik® (Essen, NW, Germany). Magnesium stearate was purchased from Labsynth® (Diadema, SP, Brazil). Microcrystalline cellulose (Microcel® MC-102) was acquired from Blanver (Taboão da Serra, SP, Brazil). Polyvinylpyrrolidone (Kollidon® CL) was obtained from BASF (Ludwigshafen am Rhein, RP, Germany). Pregelatinized starch (Starch 1500®) was purchased from Colorcon® (Harleysville, PA, USA).

Production of Native Papain Minitablets

The raw materials (pregelatinized starch, 43% m/m; microcrystalline cellulose, 43% m/m; pharmaceutical grade native papain, 10% m/m; colloidal silicon dioxide, 1.5% m/m; magnesium stearate, 1.5% m/m; and polyvinylpyrrolidone, 1% m/m) were calibrated in a 425-μm mesh sieve and mixed. The minitablets were produced by direct compression using a rotary press simulator (Mini Express LM 8, Lemaq) fitted with four punches and matrices with a diameter of 2 mm. Compression forces (maximum upper punch forces) between 2 and 4 kN (282–566 MPa) were applied to all minitablets. The rotary press operated at a speed of 42.5 minitablets/min, resulting in tablets with a mass of 9–10 mg.

Characterization of the Minitablets

Weight Variation

Twenty minitablets were individually weighed on an analytical balance (AY 220, Shimadzu Corporation), and the mean, standard deviation, and coefficient of variation were calculated. The limit of variation was 10% (21).

Size

Ten minitablets were selected. Their diameter was measured using a micrometer (436.2 MXRL-25, Starrett), and the mean and standard deviation were calculated.

Hardness

The mechanical resistance of a sample of 10 minitablets was tested using a durometer (298-ATTS, Nova Ética). The minitablets were subjected to radial pressure until fragmentation was noted. The mean, standard deviation, and coefficient of variation were calculated. The acceptance criterion was a pressure of not less than 35 N (22).

In Vitro Dissolution

Dissolution tests were performed using an apparatus 2 (spade). Forty minitablets (each minitablet contains 9.5 mg) were analyzed in 500 mL of dissolution medium (sodium phosphate buffer, pH 6.8) at 37 ± 0.5°C, mimicking an enteric release condition *in vitro*. The rotational speed of spades was set to 100 rpm for 1 h in the dissolution equipment (299-6TS, Nova Ética). Aliquots of this dissolution medium were subsequently collected and used to evaluate the enzymatic activity of papain and for the permeability test.

Evaluation of Papain Proteolytic Activity in Minitablets after Dissolution Testing

The proteolytic activity of papain was quantified using BAPA as a synthetic substrate. Samples were diluted in phosphate buffer pH 7 and incubated in a thermostatic bath at 40°C ± 0.5°C for 45 min. Absorbance was read at 410 nm on the microplate spectrophotometer reader. The method was performed according to Ferraz et al. (23).

Cell Culture

Caco-2 or HT29-MTX were cultured in 25 or 75 cm^2 culture flasks with DMEM high glucose supplemented with 10% FBS, 1% L-glutamine, and 1% antibiotic solution (10,000 UI/mL penicillin, 10 mg/mL streptomycin, and 1 mg/mL amphotericin B).

Raji cells were cultured in 25 or 75 cm^2 non-treated culture flasks with RPMI 1640 medium supplemented with 10% of FBS, 1% L-glutamine, and 1% antibiotic solution (10,000 UI/mL penicillin, 10 mg/mL streptomycin, and 1 mg/mL amphotericin B).

The cells were incubated at 37°C in the presence of 5% CO_2 until 80% confluence, with replacement of the culture medium every 48 h. All experiments were performed using cultures subjected to at least one subculture passage after thawing, with subsequent passages less than nine.

Cytotoxicity Assays

Raji Cells

Toxicity of PPN on Raji cells was tested using the PrestoBlue™ assay. Briefly, non-adherent Raji cells were seeded in 96-well microplates (15×10^3 cells per well; 45 μL of culture medium per well) followed by eight different PPN concentrations (45 μL per well) with the relative concentration of papain in the well ranging from 16 to 2000 μg/mL. The positive control was a 1% TX-100 solution. The microplates were incubated for 48 h after sample seeding.

Subsequently, cell viability was evaluated using ready-to-use PrestoBlue™ reagent. After 48 h, 10 μL of PrestoBlue™ solution was added to all wells and the microplates were incubated for 2 h. The reading was performed with fluorescence (λ_{ex}: 570 nm; λ_{em}: 610 nm) using a microplate fluorescence reader.

Co-culture of Caco-2 and HT29-MTX Cells

Cell viability assay was evaluated using MTT based on the protocol described by Mosmann (24). To mimic what would occur in the permeation assay, the cytotoxicity assay was performed under the same conditions; *i.e.*, Caco-2 and HT29-MTX cells were seeded in 96-well microplates (20×10^3 cells/well at a physiological ratio of 9:1; 100 μL per well) and incubated overnight. Immediately prior to starting the experiment, the medium was removed from the wells as the cells were exposed to eight different concentrations of PPN (16–2000 μg/mL) and incubated under agitation of 65 rpm for 2 h. The positive control was a 1% TX-100 solution. After 2 h, the supernatant was removed and the cells were carefully washed once with PBS (100 μL/well) and 100 μL of the medium was added to 0.5 mg/mL MTT to form formazan crystals in viable cells. The microplates were incubated for 3 h. Then, the supernatant was removed and 100 μL of ice-cold isopropanol was added to each well to solubilize formazan crystals and the absorbance was read at 570 nm on the microplate spectrophotometer reader.

Permeability Assay

Caco-2 and HT29-MTX cells (2×10^4 cells per well in a physiological proportion of 9:1, respectively) were seeded on the apical chamber of ThinCert™ (0.4 μm porous and 0.33 cm^2 surface area) (Greiner Bio-One, Frickenhausen, BW, Germany) with medium changes every other day. After 14 days of culture, 3×10^3 Raji cells were added to the basolateral compartment and the triple co-culture was maintained for an additional 7 days before the experiments. Then, the cell monolayer was washed with HBSS containing pre-warmed 25 mM HEPES in pH 7.4, and the plate was incubated under stirring (65 rpm) for 15 min. After this, the integrity of the monolayers was evaluated using TEER (Millicell® ERS-2) (Millipore Corporation, Billerica, MA, USA). A resistance reading of 500–600 Ω cm^2 was considered indicative of a confluent monolayer with tight junctions.

The test was performed with the addition of the native papain minitablet solution after the dissolution test concomitant with the addition of the furosemide solution (100 μg/mL) to the apical compartment. Negative and positive controls were 100 μg/mL of furosemide and metoprolol, respectively. The assays were performed in triplicate and unidirectionally from the apical compartment (donor—representing the intestine lumen) to the basolateral one (receptor—representing the systemic circulation) at 37°C, under stirring (65 rpm) for 2 h. The quantification of the model drugs was performed separately using the reverse phase system by HPLC (LC-10A model, Shimadzu Corporation) coupled to a fluorescence detector and a C18 column (150 mm length, 4.6 mm

inner diameter, and 5 μm particle, Eclipse XDB-C18 model, Agilent Technologies) according to Corazza et al. (4).

The permeability coefficient P_{app} (cm/s) was calculated according to the following formula:

$$P_{app} = \frac{V_R}{(A\ x\ C_0)}\ x\ \frac{dQ}{dT}$$

where VR is the volume of the basolateral compartment, A is the surface area of the monolayer, C_0 is the initial concentration in the apical compartment, and dQ/dT is the flux of the drug across the cell layers.

Paracellular permeability was evaluated by the quantification of LY from apical to basolateral compartments. LY concentration was 50 μg/mL. The LY transport was estimated by fluorescence (λ_{ex}: 405 nm; λ_{em}: 535 nm) using a microplate fluorescence reader.

Mucus Staining with Alcian Blue

At day 21 post Caco-2:HT29-MTX seeding, the cells were washed once with PBS and subsequently fixed with cold methacarn (60% methanol, 30% chloroform, 10% acetic acid) in both apical and basolateral compartments for 15 min at 4°C. Next, the cells were washed once with 3% acetic acid. Later, 1% Alcian Blue (pH 2.5) was added to the monolayer and the membranes were observed after the staining (25).

Confocal Fluorescence Microscopy

The confluence of the monolayers was visualized under fluorescence microscopy after 21 days. To cover cells adhering to the ThinCert™ membranes, 100 μL of a diluted solution of Alexa Fluor™ 488 Phalloidin (1:40 in PBS-BSA) was used to label cell membranes. Cell nuclei were counterstained with DAPI solution (1:10,000 in PBS), incubated for 10 min, and washed with PBS. The membranes were cut and mounted on a glass slide for visualization under the fluorescence microscope with appropriate filters (4).

Scanning Electron Microscopy

After 21 days, the cells were washed once with PBS, fixed with glutaraldehyde solution (2.5% in PBS), and incubated for 2 h. Then, they were washed thrice with PBS, dehydrated in an ethanol gradient, and dried using the critical point of CO_2 (Leica Microsystems automatic critical point dryer, EM CPD300). The membranes were then glued on the sample holder (stub) using double-sided adhesive tape containing colloidal carbon, covered with silver and analyzed using SEM (LEO Electron Microscopy Ltd., 435 VP) (26).

Statistical Analysis

The results were expressed as means ± standard deviation. The statistical significance of the differences was determined using one-way ANOVA followed by the Bonferroni post-hoc test. The comparisons were considered statistically significant when the p value was lower than 0.05 ($p < 0.05$). The analyses were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Characterization of the Minitablets

The results of the tests performed to evaluate the physicochemical characteristics of papain minitablets are listed in Table I.

The enzymatic activity of the papain present in the minitablets was calculated using the straight-line equation of the papain calibration curve along the concentration range of 2–100 μg/mL (*i.e.*, $y = 0.0907x - 0.0003$, $R = 0.995$). The papain present in the minitablets had a biological activity of 78.7 ± 3.2%.

Cytotoxicity Tests

To evaluate the toxicity of native papain, eight different concentrations of this enzyme were used in different cytotoxicity assays to verify its possible toxic effect on the mitochondrial respiratory chain of Raji cells and on the co-culture of Caco-2 and HT29-MTX cells. It is interesting to note that papain was not cytotoxic to Raji cells in any of the concentrations evaluated (Fig. 1a). However, for the cell co-culture, concentrations greater than 63 μg/mL of papain caused significantly decreased cell viability (Fig. 1b).

Permeability Assay

The biophysical integrity of the cell monolayer was evaluated by determining the TEER in the initial periods and 2 h after testing. A significant decrease ($p < 0.05$) in the initial TEER value (957 ± 189 Ω.cm^2) compared with the final value (461 ± 50 Ω.cm^2) was observed after the use of the minitablet formulation containing native papain and furosemide (Fig. 2). This indicates that this formulation greatly influenced the opening of the tight junctions between the cells that compose the monolayer.

The P_{app} of furosemide, furosemide in the presence of native papain minitablets, and metoprolol were 0.6 ± 0.2, 3.6 ± 0.4, and 7.1 ± 1.3 × 10^{-6} cm/s, respectively. This indicates that the furosemide permeation increased six-fold in the presence of papain. The native papain promoted a significant permeation of furosemide ($p < 0.05$) (Fig. 3). Furosemide was used both as a negative control and as a model drug for permeability evaluation, whereas metoprolol was used as a positive control. The reversed phase HPLC method was used to quantify furosemide and metoprolol in the samples. The calibration curves for furosemide and metoprolol were linear

Table I. Characterization of Minitablets Produced

Parameter	Results
Weight variation (mg)	9.0 ± 0.7
Size (mm)	2.4 ± 0.1
Hardness (N)	40.8 ± 4.0

Results are the mean ± standard deviation

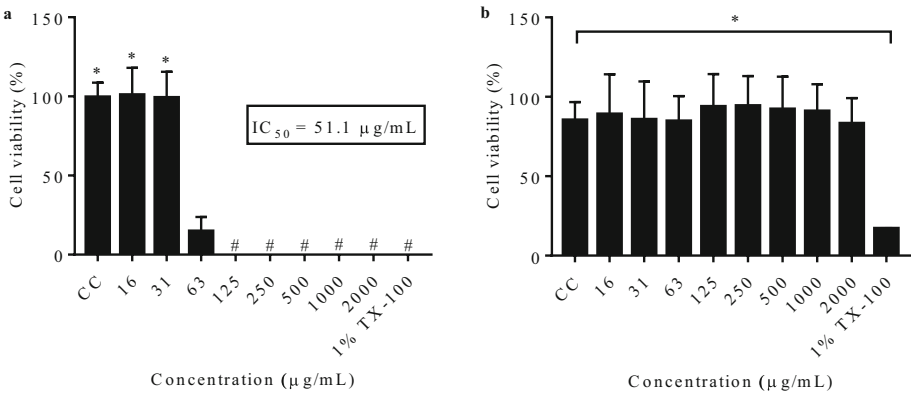

Fig. 1. PPN cytotoxicity in a **a** co-culture of Caco-2 and HT29-MTX cells and **b** Raji cells CC: cell control; PC positive control (1% Triton™ X-100), # absence of viable cells. Results are the mean ± standard deviation of two independent tests ($n = 12$) (*$p < 0.05$; sample *vs.* positive control followed by one-way ANOVA and the Bonferroni test)

over the concentration range of 1–1000 ng/mL (*i.e.*, $y = 11{,}024x + 1455.4$, $R = 0.99$ for furosemide and $y = 7844.4x + 34{,}499$, $R = 0.99$ for metoprolol). The method was considered specific to the quantification of both drugs.

To ensure the evaluation of the integrity conditions of the monolayer of Caco-2 and HT29-MTX cells, it is not enough to only measure TEER values. In this case, LY was used as a marker of the paracellular integrity of the monolayer of Caco-2 and HT29-MTX cells during the permeability tests. P_{app} was 3.8×10^{-7} cm/s and can be considered adequate (27).

Mucus Staining with Alcian Blue

Staining with Alcian Blue was performed to prove that HT29-MTX mucus-producing cells were active. It was confirmed that mucus was successfully produced by the HT29-MTX cells (Fig. 4). Alcian Blue is a basic dye characterized by high affinity for acidic mucin glycoproteins. When this dye comes into contact with mucin glycoproteins, it binds with high affinity, providing a dark blue color (28). The mucus comprises mainly water and shrinks to a very fine structure when dehydrated. Owing to this characteristic, mucus is not normally observed in tissue sections fixed in formaldehyde and can be better preserved using methacarn-based Carnoy's fixative (29).

Confocal Fluorescence Microscopy

The morphological evaluation comprised the use of fluorescent markings to visualize certain components of cells using Alexa Fluor™ 488 Phalloidin staining to mark actin filaments present in the cytoskeleton and microvilli, followed by DAPI staining for nucleus marking (Fig. 5).

Scanning Electron Microscopy

The conversion of Caco-2 cells into M cells provided by the presence of Raji cells was confirmed by SEM after 7 days of co-cultivation (Fig. 6). This technique was used to detect any morphological change in the enterocyte in M-cells because a specific marker for the human M-cell has not yet been described.

DISCUSSION

The results of the tests performed to evaluate the physicochemical qualities of the minitablets were within the required parameters. Although the activity of the enzyme present in the formulation was high, it is likely that papain had its activity reduced during the stages of minitablet production, as it is a very sensitive enzyme that presents low stability under certain conditions. As described by Varca et al. (6), decreased enzymatic activity may be owing to oxidation that changes its structure during the production process. Therefore, the inclusion of antioxidants in the formulation may be potentially beneficial for the preservation of papain bioactivity.

The similarity of the Caco-2 cell monolayer model with human enterocytes is fundamental to the evaluation of drug transport. However, in addition to absorbing cells, the *in vivo* intestinal epithelium is also formed of mucus secreting cells that disrupt the homogeneity of this epithelium. Thus, the development of biomimetic models is important for the elucidation of efficient transport mechanisms (30). In this sense, a 9:1 ratio of Caco-2 and HT29-MTX cells,

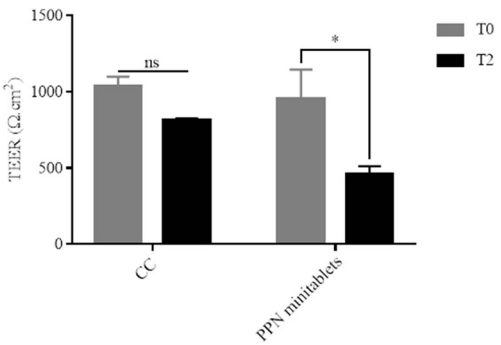

Fig. 2. TEER (Ω cm^2) of the triple co-culture of Caco-2, HT29-MTX, and Raji cells before and after exposure of the cells to the formulation of native papain minitablets and furosemide at a concentration of 100 µg/mL for 2 h. CC cell control, PPN papain, T0 initial time, T2 2 h, ns not significant. The data represent mean ± standard deviation two independent tests ($n = 6$) (*$p < 0.05$; sample *vs.* positive control followed by one-way ANOVA and the Bonferroni test)

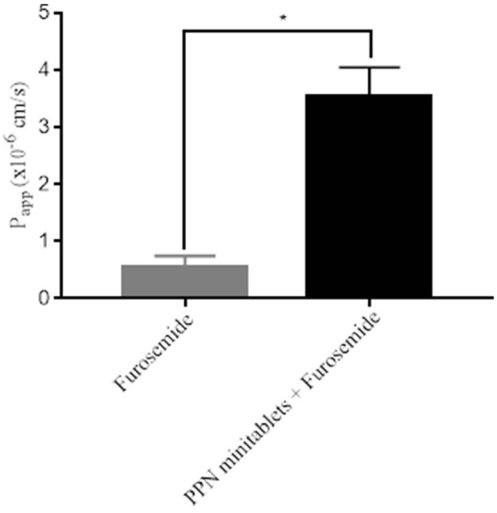

Fig. 3. Permeability coefficient (P_{app}) (cm/s) of furosemide alone at a concentration of 100 μg/mL and in the presence of native papain minitablet formulation through triple co-culture of Caco-2, HT29-MTX, and Raji cells after 2 h of exposure. PPN papain. The data represent mean ± standard deviation of two independent tests ($n = 6$) (*$p < 0.05$; sample *vs.* furosemide followed by one-way *ANOVA* and the Bonferroni test)

respectively, was used in this study, according to the physiological proportion described by Araújo and Sarmento (31).

The results of the cytotoxicity assays using the monolayer of Caco-2 and HT29-MTX cells through mitochondrial impairment analysis using MTT assay (Fig. 1a) indicated that, in fact, no significant difference in viability was observed in cells treated with concentrations greater than 31 μg/mL in the samples containing papain compared with that in the positive control, thereby showing dose-dependent decreased cellular viability at these concentrations. It is also interesting to note that the exposure time of 2 h was enough for a proteolytic activity of papain on the mucus layer, making it difficult for the papain to access the cells directly and implying a protection by the mucus. This may have happened owing to mucus production secreted by the HT29-MTX cells. According to Köllner et al. (32), papain has mucolytic activity and the mucus produced by HT29-MTX could be considered a substrate for papain, preventing the enzyme from directly reaching the cell junction proteins of this cell monolayer.

Papain is stable and active under a wide range of conditions from pH 4 to 10. However, the enzyme is almost inactive at gastric acid pH. Therefore, the ideal place for papain delivery is the intestine (pH is in the range of 5.0–8.0) (33).

Moreover, papain has a mechanism of action similar to that of trypsin; *i.e.*, it can promote cell detachment through action on transmembrane proteins and junctional adhesion molecules responsible for cell junction and adhesion to the extracellular matrix (33). According to Frisch and Screaton (34), adherent cells are anchorage dependent; *i.e.*, they need adhesion to a contact surface in order to initiate their proliferation. The degradation of these adhesion proteins makes these cells lose contact with the extracellular matrix surface and become incapable of metabolizing, which results in the activation of cellular death mechanisms by loss of adhesion (anoikic) on adhesion cells in higher concentrations; the same behavior was not observed on Raji cells. According to Epstein and Barr (35), the Raji lineage comprises lymphoblastic B cells derived from Burkitt's lymphoma and characterized as suspension cells that do not need to be adhered to a matrix to perform their metabolic activities. Thus, papain did not demonstrate proteolytic activity on these cells, resulting in high cellular viability associated with the various papain concentrations (Fig. 1b). This may corroborate the fact that compounds containing papain are not cytotoxic. However, papain may cause the loss of morphological integrity and the death of the adhesion cells owing to its proteolytic action at higher concentrations (4).

After determining cytotoxicity and the effect of papain samples on cell viability, permeability tests were performed using the triple co-culture model of Caco-2, HT29-MTX, and Raji cells to evaluate if papain promoted the oral permeation of a drug with low permeability. All cell cultures used in these experiments grew on a semipermeable membrane and were cultivated for 21 days to ensure the expression of their specific characteristics so that the ability of compounds containing papain to promote furosemide permeation could be evaluated. According to the Biopharmaceutical Classification System, furosemide is a drug with low permeability (36).

To the best of our knowledge, this is the first study, using this model of cell culture to evaluate the capacity of papain minitablets as an enhancer of drug permeation after the dissolution of the enzyme in a dissolution assay, seeking to mimic *in vitro* the conditions of oral drug administration.

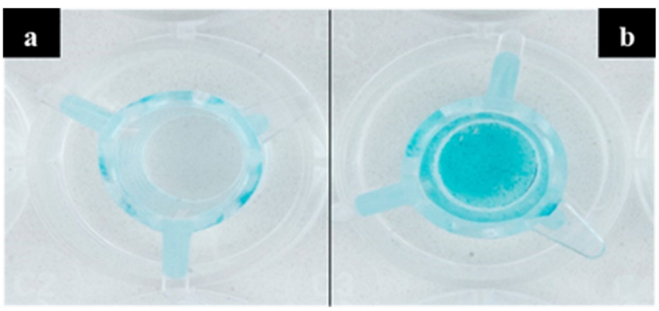

Fig. 4. Mucus staining (blue) produced in HT29-MTX cells in co-culture with Caco-2 and Raji cells fixed with methacarn and stained with Alcian Blue (blue). Polycarbonate membrane of ThinCert™ without cells (white) (**a**). Monolayer of Caco-2 and HT29-MTX mucus-producing cells (**b**)

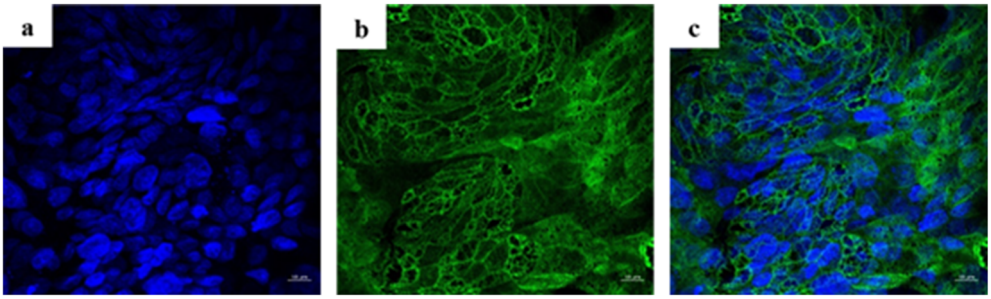

Fig. 5. Evaluation of cell morphology using confocal fluorescence microscopy of a monolayer of Caco-2 and HT29-MTX cells co-cultivated for 21 days. DAPI marking cellular nuclei (**a**); Alexa Fluor™ 488 Phalloidin—marking actin filaments present preferentially on cellular edges and microvilli (**b**); merged (**c**). Scale bar = 10 μm

Furosemide is an example of a low permeability drug widely used in antihypertensive therapy that presents low solubility and permeability (Biopharmaceutical Classification System IV). Furosemide, being considered a weak acid (pKa 3.9), is not capable of crossing the epithelial layer by simple diffusion. Furthermore, furosemide is intensely secreted to the intestinal lumen by P-gp (36–38). These characteristics result in the requirement for high doses of furosemide, which can lead to adverse events. This drug has very limited permeability along the gastrointestinal tract, which contributes to its low bioavailability (39). According to Avdeef and Tam (40), although it presents a low extent of absorption, a small amount of furosemide can be absorbed through paracellular mechanisms, and can thus have therapeutic activity.

Papain contains the Cys-25 residue and histidine (His-159) at its catalytic site (41). The Cys-25 residue has the essential sulfhydryl group, which remains native in the active enzyme, whereas six other cysteine residues form disulfide bridges between them that stabilize the enzyme structure (42). According to Iqbal et al. (43), thiolated polymers can interact with P-gp. In this sense, cysteine residues present in papain may have interacted with P-gp, leading to its inhibition, and consequently, increased furosemide permeation.

The oral bioavailability of many drugs is still impaired owing to low solubility and permeability through the intestinal barrier. Mucus and intestinal epithelium are the main obstacles to the transport of bioactive compounds. The thickness and viscosity of the mucus gel layer form a barrier that can trap the drug and prevent its access to enterocytes (44). Papain showed the capacity to degrade mucus and promote furosemide transport. Similar results regarding decreased mucus viscosity were also reported by Müller et al. (45). Further studies are required to elucidate detailed mechanisms governing the permeation enhancement properties of papain.

The initial TEER values obtained were very close to the values found in the human colon—above 500 Ω cm^2 (46). After the permeability test, the results showed that there was a two-fold decrease in TEER values in systems containing papain compared with the initial measured values (Fig. 2).

The cells that compose the epithelial barrier are linked by intercellular protein junctional complexes, namely, tight junctions, which are located mainly in the apical portion of these cells. These apical junctional complexes allow polarized morphological maintenance of the cellular monolayer and selectively control diffusion along the paracellular way (47).

According to Stremnitzer et al. (48), papain can degrade tight junctions on tight junctional proteins in human primary keratinocytes owing to its proteolytic activity. In addition, Corazza et al. (4) showed that this type of TEER reduction after transport is also explained by the possible reversible papain disruption of tight junctions on intestinal cells and the modulated tight junction permeability thus improved furosemide paracellular transport (Fig. 3).

The manipulation of paracellular permeability has been proposed owing to the transient and reversible opening mechanism of the tight junctions used to increase drug absorption (49). According to Gómez et al. (50),

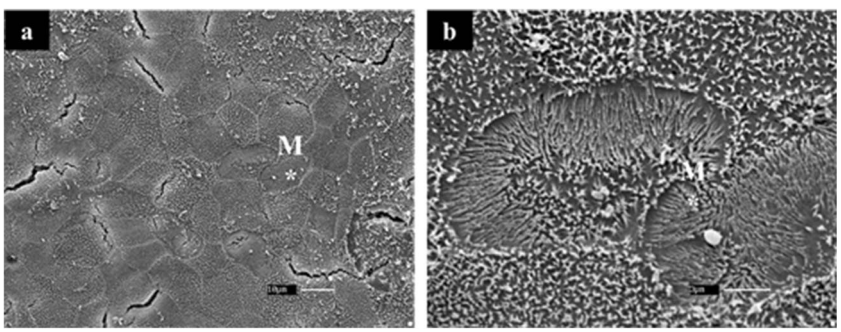

Fig. 6. Identification of M cells using scanning electron microscopy. The cells are identified by the presence of smaller amounts of microvilli on their apical surface. Monolayer of Caco-2 and HT29-MTX cells co-cultivated with Raji cells for 7 days (**a** and **b**). (M) is an M-cell

modifications in the occlusive junctions between adjacent cells decreased the layer of mucus covering the cell surface and change in the fluidity of the cell membrane, and inhibition of glycoprotein-P (P-gp) may contribute to drug permeation. Wang et al. (51) consider that the concomitant administration of paracellular permeation enhancers with low permeability drugs is a strategy to improve oral absorption.

Some studies using the monolayer of Caco-2 cells as a model to evaluate permeability showed that papain could be considered a good permeation enhancer for compounds that have low permeability through this epithelium. According to Corazza et al. (4), this enzyme can promote the reversible opening of the tight junctions between these cells without destroying the overall intestinal epithelium. In this study, the results showed decreased TEER values and recovery of intestinal epithelium after paracellular diffusion and increased permeation of low permeability compounds.

The mucus layer is an important barrier to drug absorption. Mucus is often secreted by caliciform cells and represents a physical barrier that prevents the diffusion of macromolecules and their permeation through the intestinal epithelium. It largely comprises water and glycoproteins (mucins) and presents hydrogel characteristics that can be fluidized by papain, facilitating drug access to the apical portion of epithelial cells (52). According to Lorkowski (53), mucus may be related to decreased papain activity as a paracellular diffusion enhancer. Thus, mucus may have acted as a substrate for papain, making it difficult for the enzyme to act directly on the tight junctions between cells. In addition, papain may have lowered the viscosity of the mucus, also favoring the transport of furosemide. Proof of the mucolytic activity of papain was reported by Leichner et al. (5), Menzel and Bernkop-Schnürch (54), and Müller et al. (45).

The addition of a third line of immune cells—Raji cells—in this system provides a model compatible with the intestinal Peyer's plate. According to Loo et al. (30), the infiltration of B lymphocytes into the monolayer of Caco-2 and HT29-MTX cells triggers differentiation into a phenotype of M cells from the Caco-2 cells. As M cells specialize in transporting antigens and particles to the underlying immune cells, this is an appropriate model for the study of micro and nanoparticle transport through the *in vitro* M intestinal epithelium (55). Although the presence of M cells demonstrated that the epithelium developed in this study is as biomimetic as possible, the transcytosis capacity of these cells did not increase furosemide permeability.

Mucus was successfully produced by the HT29-MTX cells present in the triple co-culture with Caco-2 and Raji cells, which was confirmed by blue staining visualization (Fig. 4). The mucus was spread all over the cell monolayer even after several washing steps. Therefore, HT29-MTX cells can maintain their intrinsic properties, producing mucus when cultivated with other cell lines. Similar results were reported by other studies (25,56). According to Araújo and Sarmento (31), mucus is only produce by HT29-MTX cells.

Cell monolayer morphology was visualized with confocal fluorescence microscopy using DAPI for nuclear staining and Alexa Fluor™ 488 Phalloidin for actin filament staining in the cytoskeleton and microvilli located on the surface of the cells (Fig. 5). Confluent cell monolayers formed by enterocytes and caliciform cells were visualized. M cells were also observed owing to the absence of microvilli on their apical surface, as reported by Bazes et al. (57) However, as it is difficult to prove the presence of M cells in this system using confocal fluorescence microscopy, this study additionally used scanning electron microscopy.

Caco-2 cells and M cells have distinct surfaces (Fig. 6). Caco-2 cells have a brush border formed by the presence of a large amount of compact and longer microvilli, whereas M cells have disorganized, short, and thicker microvilli along their apical surface and basolateral invaginations that give it the shape of a pocket (58–60). The differentiation of enterocytes in M-cell has been proven by Kernéis et al. (61) through *in vitro* co-cultivation of Caco-2 with lymphocytes from newly isolated murine Peyer plaques. The mechanism of this conversion is not yet known. However, Raji cells may secrete certain cytokines and interact with the monolayer of Caco-2 cells. This contact directly influences the differentiation of as yet undifferentiated Caco-2 cells, leading to morphological and functional changes (62).

In this study, we formulated papain minitablets and performed tests to evaluate their physicochemical and biological characteristics. The parameters of weight variation, size hardness, and dissolution and the evaluation of papain proteolytic activity were considered adequate. In parallel, we established an *in vitro* triple culture model of Caco-2 enterocytes, HT29-MTX goblet-like cells, and Raji B lymphocyte cells, which mimics the *in vivo* intestinal barrier. The integrity and morphology of the cell monolayer were determined by Alcian blue staining of the cell monolayer, scanning electron microscopy, and measurement of the transepithelial electrical resistance values. Additionally, the cytotoxicity and permeability assays showed that owing to its mucolytic activity, papain can facilitate the transport of furosemide through the mucus barrier and increase its permeation through the intestinal epithelium, thus enhancing the bioavailability of the drug.

CONCLUSION

Drug permeability evaluation is fundamental to the prediction of bioavailability. The use of *in vitro* triple co-culture intestinal cells represents the most similar physiological conditions of the organism. In the present study, this model was standardized for the evaluation of samples of native papain minitablets in promoting the permeation of furosemide, a drug known for its low solubility and permeability in biological fluids. In addition to considering these samples as a potent and noncytotoxic single enhancer across the epithelial barrier, the results presented suggest that native papain minitablets contributed to *in vitro* paracellular transport to enhance the overall mechanism of furosemide with potential for therapeutic applications.

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AUTHOR CONTRIBUTIONS

Resources, formal analysis, investigation, and writing-original draft: F.C., J.E., G.V., and D.V.; investigation: F.N. and L.C.; writing–reviewing and funding acquisition: D.V. and V.L.S.; supervision, resources, funding acquisition, and writing–reviewing: D.V, N.A.F, and P.L.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflict of interest.

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