Journal of Drug Delivery Science and Technology 55 (2020) 101413

Contents lists available at ScienceDirect

Journal of Drug Delivery Science and Technology

journal homepage: www.elsevier.com/locate/jddst

Papain-cyclodextrin complexes as an intestinal permeation enhancer: Permeability and *in vitro* safety evaluation

Fúlvio G. Corazza[a], Julia V. Ernesto[a], Felipe A.N. Nambu[a], Luma R. de Carvalho[b], Vânia R. Leite-Silva[a], Gustavo H.C. Varca[b], Leandro A. Calixto[a], Daniel P. Vieira[b,*], Newton Andréo-Filho[a], Patricia S. Lopes[a]

[a] Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Departamento de Ciências Farmacêuticas, Universidade Federal de São Paulo (UNIFESP) Campus Diadema, Rua São Nicolau, 210 - Centro - Diadema, São Paulo - SP, CEP: 09913-030, Brazil

[b] Instituto de Pesquisas Energéticas e Nucleares (IPEN-CNEN/SP), Avenida Prof. Lineu Prestes, 2242, Cidade Universitária, São Paulo - SP, CEP 05508-000, Brazil

ARTICLE INFO

Keywords:
Cytotoxicity
Genotoxicity
β-cyclodextrin
2-Hydroxypropyl-β-cyclodextrin
Paracellular transport

ABSTRACT

Oral drug delivery is the main route for drugs administration. However, some drugs have poor permeability across the intestinal barrier. Papain has been widely used in pharmaceutical applications due to its debridement properties and the ability to promote skin permeation of drugs. It is known that papain complexation with cyclodextrins improves its biological stability. In this paper, the ability of the native papain-cyclodextrin complexes is shown be an oral permeation enhancer to furosemide, using a Caco-2 monolayer system to evaluate drug permeability and apparent permeability coefficient. Analysis of the *in vitro* cytotoxicity over CHO–K1, Hep G2 and Caco-2 cell lines and genotoxicity over CHO–K1 and Hep G2 cell lines were also performed. Papain-cyclodextrins complexes did not show any cytotoxicity above 31 μg/mL. No significant genotoxic damage was observed. Papain and cyclodextrin complexes induced almost 2.5-fold increase in furosemide permeation compared to controls, and maintenance of the paracellular integrity of the Caco-2 cells monolayer was confirmed. The papain complexes may be safely applied in pharmaceutical formulations, not only as a therapeutic agent but also as a strategic pharmaceutical adjuvant, promoting permeation of low oral permeability drugs.

1. Introduction

The development of oral drug delivery systems provides huge advantages due to increased patient compliance and greater dose flexibility. However, drug release depends on environmental factors, such as pH, presence of enzymes and gastrointestinal transit. Besides, the therapeutic effect can only occur after drug transport across the intestinal epithelium, so it reaches bloodstream [1]. In order to overcome these bioavailability obstacles, a range of approaches is currently being adopted such as solid dispersions [2], nanostructured systems [3] and peptide permeation enhancers [4].

The peptide permeation enhancers act in a paracellular route, increasing absorption and producing a valuable alternative whereas the interference with dynamic epithelial is rapid and transient [5]. The recent focus on oral drug delivery relying on peptides and proteins encouraged our research on papain as a possible oral permeation enhancer, using furosemide as a low permeability drug model to demonstrate such effects.

Papain (Enzyme Classification 3.4.22.2) is a proteolytic phytoenzyme used in dermatology for the treatment of eschars and wounds due to its broad debriding proteolytic activity [6]. This enzyme acts on dead tissues, helping the removal of necrotic tissue and stimulating wound healing as well as anti-inflammatory processes [7–9]. In addition, the enzyme also holds antimicrobial activity [10], promotes cutaneous [11] and oral permeation [12] without interfering on the integrity of healthy tissues [13].

Towards enhancing the stability and extending its enzymatic activity, among other aspects, papain may be complexed with cyclodextrins [14]. According to Renukuntla et al. [15], Shimpi et al. [16] and Yewale et al. [17], the complexation of protein-derived pharmaceuticals with cyclodextrins is considered a very interesting pharmaco-technical strategy which can contribute to the increase of their stability and guarantee their controlled delivery and enhanced bioavailability.

When evaluating the safety and efficacy of potential permeability enhancers, it should be considered that these bioactive compounds might also have the ability to induce significant damage to the cells.

* Corresponding author.
E-mail addresses: dpvieira@ipen.br, dperezv@usp.br (D.P. Vieira).

https://doi.org/10.1016/j.jddst.2019.101413
Received 9 September 2019; Received in revised form 6 November 2019; Accepted 22 November 2019
Available online 25 November 2019
1773-2247/

This can be tested to determine their impact over cell viability in terms of cytotoxic effects or genotoxic damage [18]. Moreover, it is important to provide experimental evidence on their role as an enhancer for oral permeation of biomolecules. Several lines of evidence correlate the permeability of drugs in Caco-2 cells monolayers with the oral absorption and bioavailability [19–21]. Therefore, this model is considered the gold standard for the assessment of permeability for many molecules [22] and predictive with the in vivo permeability models [23]. However, there is still insufficient information regarding the potential of cyclodextrin-papain complexes in enhancing the oral permeation of drugs with low permeability, and in that sense any advancement is therefore original and crucial for a better understanding of the matter.

Thus, in the present study, we have aimed to evaluate the ability of native papain (PPN) and papain complexed either with β-cyclodextrin (PPN-β-CD) or 2-hydroxypropyl-β-cyclodextrin (PPN-HP-β-CD) to enhance the permeation of furosemide through a Caco-2 cells monolayer, as well as assess the cytotoxicity and genotoxicity profile over CHO–K1, Hep G2, and Caco-2 cell lines.

2. Materials and methods

2.1. Cells and reagents

Chinese hamster ovarian cell line (CHO–K1) (ATCC® CCL-61™), human hepatocellular carcinoma cell line (Hep G2) (ATCC® HB-8065™) and human colon adenocarcinoma cell line (Caco-2) (ATCC® HTB-37™) were used in this research. Alexa Fluor™ 488 Phalloidin, Ethidium Monoazide Bromide (EMA), fluorescent beads, Lucifer Yellow (LY), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT), and SYTOX™ Green were purchased from Invitrogen™ (Carlsbad, CA, EUA). Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPA) (≥98%), Bovine Serum Albumin (BSA), citric acid, 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), colchicine (COLCH), Dimethyl sulfoxide (DMSO), furosemide (≥98%) (FURO), IGEPAL® CA-630, metoprolol (≥98%) (METO), mitomycin C (MTMC), NaCl, PPN pharmaceutical grade (30,000 USP-U/mg), sodium citrate, sucrose, Triton™ X-100 (TX-100) and β-cyclodextrin (β-CD) (1134.98 g/mol) were acquired from Merck KgaA (Darmstadt, HE, Germany). CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Lactate Dehydrogenase (LDH) assay) was provided by Promega (Madison, WI, USA). Formaldehyde solution was acquired from Thermo Scientific™ (Waltham, MA, EUA). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) (1400 g/mol) was obtained from Wacker Chemie AG (Munich, BY, Germany). All the other reagents for cell culture were purchased from Vitrocell® (Campinas, SP, Brazil). The chemical reagents used in this study were of analytical grade.

2.2. Papain-cyclodextrin complexes and their enzymatic activity

Briefly, PPN, β-CD or HP-β-CD (1:10 M ratio) were solubilized in ultrapure water and stirred for 2 h at room temperature, protected from light, using a shaker. The mixture was then frozen at −20 °C for 24 h and freeze-dried (L108 lyophilizer, Liobras) for 7 days at −60 °C and in 36 μm Hg vacuum to obtain the PPN-β-CD and PPN-HP-β-CD complexes.

PPN (native or complexed with cyclodextrins) proteolytic activity was quantified according to Ferraz et al. [24]. Briefly, Samples were properly diluted in cysteine-versene buffer (pH 7) and incubated on a thermostatic bath at 40 °C for 45 min. Absorbance was measured at 410 nm using a microplate spectrophotometer reader.

2.3. Biological assays

2.3.1. Cell culture

CHO–K1 cells were cultured with RPMI 1640 medium 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). Caco-2 or Hep G2 cells were cultured 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). The cells were incubated at 37 °C in a 5% CO_2 atmosphere with controlled humidity. The culture medium was changed every 2–3 days. The cultures were used for testing within 3–10 passages from the initial passage.

2.3.2. Cytotoxicity assays

Cytotoxicity assessment was carried out using the MTT and LDH assay. The extent of mitochondrial impairment was assessed using the MTT assay based on the protocol described by Mosmann [25]. For this assay, CHO–K1, Hep G2 or Caco-2 cells were seeded in 96-well microplates (15×10^3 cells per well; 100 μL of culture medium per well) and incubated overnight for cell adhesion and then exposed to eight different concentrations of PPN, PPN-β-CD and PPN-HP-β-CD (16–2000 μg/mL) for 48 h. 1% TX-100 solution was used as a positive control. After that, 150 μL of culture medium was added to the wells, and the microplates were centrifuged at 1000 rpm and incubated for an additional 3 h to stimulate cell re-attachment of living cells in suspension. After that, cells were carefully washed once with PBS (100 μL/well) and incubated for an additional 3 h with DMEM containing MTT (0.5 mg/mL, 100 μL/well). Subsequently, the supernatant was removed, and 100 μL of ice-cold isopropanol was added in each well to solubilize the formazan formed. The microplate was then shaken on a microplate shaker for 10 min in the dark. Absorbances were measured at 570 nm using a microplate spectrophotometer reader.

The assessment of LDH release was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay. In brief, Caco-2 cells were seeded in 96-well microplates (2.0×10^4 cells/well; 100 μL per well) and incubated for 24 h and then and then exposed to eight different concentrations of PPN, PPN-β-CD, and PPN-HP-β-CD (16–2000 μg/mL) for 8 h. 1% TX-100 solution was used as a positive control. After that, 50 μL of the supernatant of each well was replenished by 50 μL of CytoTox 96® Reagent and then incubated for 30 min. The reaction was interrupted using the Stop Solution. Absorbance was measured at 490 nm using a microplate spectrophotometer reader.

2.3.3. Genotoxicity assays

CHO–K1 (3×10^3 cells/well) or Hep G2 (5×10^3 cells/well) were seeded in 96-well microplates and incubated overnight for cell adhesion and then exposed to three different concentrations of PPN, PPN-β-CD and PPN-HP-β-CD (8, 16 and 31 μg/mL) for 4 h. COLCH (1.1 μg/mL) and MTMC (2.5 μg/mL) were used as aneugenic and clastogenic positive controls, respectively. NaCl 0.9% was used as a negative control for genotoxicity, and 5% DMSO was used as a positive control for cytotoxicity. After that, the cells were carefully washed with PBS followed by the addition of 100 μL of fresh culture medium. The cells were then incubated for 24 h.

The microplates were then centrifuged (1500 rpm for 10 min), received 8.5 μg/mL of EMA diluted in PBS supplemented with 2% FBS and were exposed to a blue LED light source (440–450 nm, 30 W) during 30 min for the photoactivation of the compound irreversibly linked to the DNA of non-viable cells. After this step, the cells received PBS with 2% FBS following centrifugation to remove the free dye. Cell lysis was performed using a NaCl solution (0.854 mg/mL) containing sodium citrate (1 mg/mL), IGEPAL® (0.3 μL/mL) and the fluorescent dye SYTOX™ Green (0.8 μM). After 60 min at 37 °C, the microplates were centrifuged again and received a second lysis solution based on sucrose (85.6 mg/mL), citric acid (15 mg/mL) and SYTOX™ Green 0.4 μM) supplemented with 5 μL of the beads that were used as an internal standard for the micronuclei calculation. The samples were allowed to rest for 30 min at room temperature prior to automated reading in flow cytometer apparatus (BD Accuri™ C6, BD Biosciences).

The analysis was performed according to Bryce et al. [26]. Briefly,

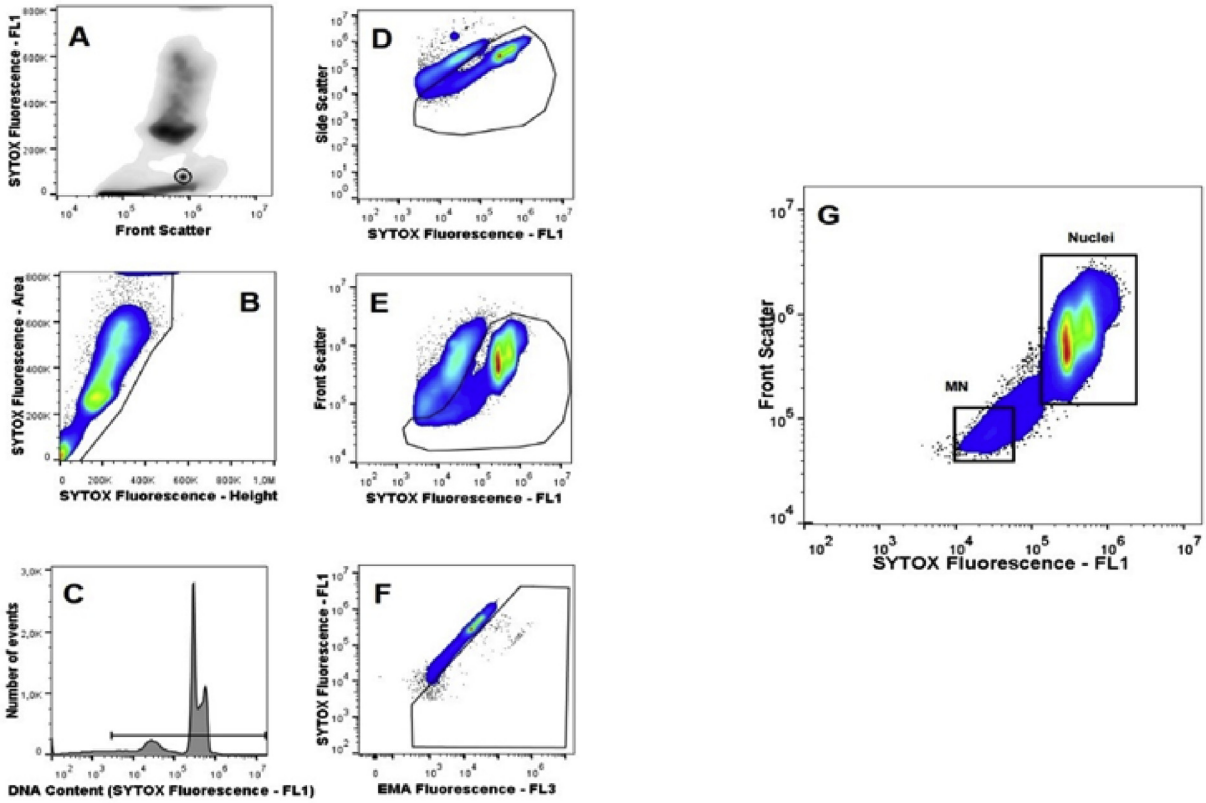

Fig. 1. Gating strategies for the analyses. (A): quantification of the beads (circle) used for the quantitative nuclei analysis; (B): exclusion of nuclei aggregates (doublets); (C): discrimination of events with fluorescence equal to or higher than 1/100 of the lower fluorescence peak (nuclei in G0/G1); (D) and (E): discrimination by complexity and size of the events compared to the fluorescence, respectively; (F): removal of the event associated with non-viable cells (EMA +); (G): final analysis, delimiting the nuclei and micronuclei events.

the events marked with EMA (cell debris and nuclei fragments resulting from apoptotic and necrotic cells) were excluded from the total counting. This step enabled the discrimination of nuclei and micronuclei from viable cells as particles dyed individually with SYTOX™, which were evaluated according to their well-defined size forward scatter (FSC) and fluorescence intensity (FL1). At least 20,000 events in the nuclei regions were considered for the evaluation of each sample (Fig. 1).

2.4. Permeability assays and TEER

The permeability assay was performed according to Hubatsch et al. [27] with some modifications. Briefly, Caco-2 cells were cultured in 24-well ThinCert™ (0.4 μm porous and 0.33 cm^2 surface area) (Greiner Bio-One, Greiner Bio one, Frickenhausen, BW, Germany) for 21 days to reach confluence and differentiation at a seeding density of 2.0×10^4 cells per well and the culture medium was changed every 2–3 days.

The biophysical integrity of the monolayer was examined by measuring the Transepithelial Electrical Resistance (TEER) with an epithelial voltammeter Millipore Millicell® ERS-2 (Millipore Corporation, Billerica, MA, USA). Control measurements from blank inserts were subtracted to calculate the TEER of the cell layer. Resistance values were multiplied by the surface area of the insert and expressed in ohms per centimeter squared. Caco-2 monolayers exhibiting a TEER value of more than 500 Ω cm^2 were used.

Prior to assay, differentiated confluent Caco-2 monolayers were equilibrated in pre-warmed Hank's Balanced Salt Solution (HBSS) buffer pH 7.4 for 15 min. PPN, PPN-β-CD or PPN-HP-β-CD in HBSS were added to the apical compartment, along with 100 μg/mL of furosemide to reach final PPN concentration of 16 μg/mL 100 μg/mL of furosemide and metoprolol were used as negative and positive controls in permeation, respectively. The assays were performed in triplicates 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 permeability coefficient (P_{app}) (cm/s) was calculated according to the following formula:

$$P_{app} = \frac{V_R}{(AxC_0)} x \frac{dQ}{dT}$$

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

The paracellular permeability was assessed by the quantification of LY from the donor to the receptor compartment. The LY concentration used was 50 μg/mL. The LY transport was estimated by fluorescence (λ_{ex}: 405 nm; λ_{em}: 535 nm) using a microplate fluorescence reader.

2.4.1. Furosemide and metoprolol quantification by HPLC

The quantification of the model drugs was performed 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). For furosemide, the mobile phase consisted of monobasic potassium phosphate buffer 20 mM, pH 3.0, and acetonitrile (70:30), while for metoprolol the mobile phase consisted of monobasic potassium phosphate buffer 20 mM, pH 3.0 and methanol (60:40). Both mobile phases were adjusted to 1 mL/per minute flux at room temperature. Furosemide was quantified using λ_{em}: 268 nm and λ_{ex}: 410 nm, and metoprolol with λ_{em}: 230 nm e λ_{ex}: 305 nm. The aliquot injection volume was 20 μL. All solvents were previously filtered

(0.45 μm porous size) prior to the injection in the system.

2.5. Statistical analysis

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

3. Results

3.1. Enzymatic activity

Papain proteolytic activity is the key when it comes to its effects over adherent cell lines, as its cytotoxic action is known to occur via de-attachment effects of cell lines, which impair cell growth. Our experiments revealed that the complexation with cyclodextrin led to no relevant changes in the proteolytic activity of papain traceable by using a specific substrate, whereas the values obtained were around 83% ± 0.1 and 93% ± 0.2 to PPN-β- and PPN-HP-β-CD respectively, after processing with no statistical difference.

Papain proteolytic activity is the key when it comes to its effects over adherent cell lines, as its cytotoxic action is known to occur via detachment effects of cell lines. Regarding the retained enzymatic activity, it was 83% ± 0.1 and 93% ± 0.2 to PPN-β- and PPN-HP-β-CD respectively, relative to the native enzyme. The enzymatic activity of papain was higher preserved in PPN-HP-β-CD after immobilization. This observation could be related to the fact that the overall three-dimensional structure of papain was positively affected after the process due to the complex chemical interaction with cyclodextrins. Such interaction unfolds the catalytic site of enzyme in order to enhance its biological properties [14].

3.2. Cytotoxicity assays

The *in vitro* safety assays are frequently applied in the initial molecular screening and for the evaluation of the activity of those molecules upon cell proliferation and the possible direct toxic effects that eventually cause cell death. Within this context, cytotoxicity assays are considered an important tool for determining or accessing the biological safety of many compounds while performing non-clinical studies [28]. Initially, aiming to understand the toxicity of native papain and papain-β-cyclodextrins complexes, two different approaches were applied for the assessment of cell viability: a) an MTT test to verify the possible toxic effects over the mitochondrial respiratory chain over CHO–K1, Hep G2 and Caco-2 cells (Fig. 2); b) an evaluation of the LDH release after possible damages on the plasma membrane of Caco-2 cells (Fig. 3).

Papain significantly inhibited CHO–K1 cell viability, especially at concentrations above 125 μg/mL ($p < 0.05$) and the calculated IC_{50} values were around 116.6, 81.8, and 98.4 μg/mL for PPN, PPN-β-CD, and PPN-HP-β-CD samples, respectively. Similar results were obtained with Hep G2 cells, with IC_{50} values of 62.6, 71.4, and 68.3 μg/mL for PPN, PPN-β-CD, and PPN-HP-β-CD samples, respectively. Regarding Caco-2 cells, cell viability was inhibited especially at concentrations above 31 μg/mL ($p < 0.05$) if compared to the positive controls, indicating that the mitochondrial biochemical integrity of the cells was preserved in the period of 48 h. It is interesting to note that the compounds with native papain or papain complexed with β-cyclodextrins were not able to cause significant membrane damages during 8 h of incubation ($p < 0.05$), which highlighted the integrity of the cell membrane.

3.3. Genotoxicity assays

In this study, the three lowest non-cytotoxic concentrations (8 μg/mL, 16 μg/mL, and 31 μg/mL) of PPN, as well as PPN-β-CD and PPN-HP-β-CD using CHO–K1 and Hep G2 cell lines were tested according to OECD 487 (2016). The frequency of the micronuclei, the effects of papain and its complexes over CHO–K1 and Hep G2 cell lines were evident after a 4-h incubation period (Fig. 4).

The number of micronuclei formed when CHO–K1 cells (Fig. 4A) were exposed to the positive controls was very significant (COLCH $p < 0.01$ and MTMC $p < 0.0001$), demonstrating that the use of those agents was efficient for the performance of these assays. Furthermore, no significant genotoxic damage was observed in CHO–K1 cells at concentrations of 8, 16 and 31 μg/mL native and complexed papain.

Regarding the Hep G2 cells (Fig. 4B), there was no significant genotoxic damage observed in these cells upon stimuli with any of the concentrations of native and complexed papain. The number of micronuclei formed when the cells were exposed to the positive controls was very significant (COLCH and MTMC $p < 0.0001$), confirming the functionality of the test.

3.4. Permeability assays

HPLC was used to quantify furosemide and metoprolol in the apical and basolateral compartments. Calibration curves were made by linear regression of the peak area *vs.* the concentration. The calibration curves for furosemide and metoprolol were linear for concentrations between 1 and 1000 ng/mL ($R = 0.996$ for both curves). The method was considered specific and met all the analytical specifications for the quantification of both drugs.

The biophysical integrity of the membranes formed by Caco-2 cells cultured in ThinCert™ membranes during the permeability assays was assessed by the determination of TEER during the initial periods and after 72 h of the experiment (Fig. 5).

Based on the lowest non-cytotoxic concentration of samples with native papain or complexed with β-cyclodextrins (16 μg/mL), the permeability assay was performed. P_{app} value of furosemide (alone) is shown in Fig. 6. The P_{app} value of furosemide through Caco-2 cell monolayer was 6.5 ± 0.1, 7.0 ± 0.5, 7.0 ± 1.1 × 10^{-6} cm/s with native papain, papain complexed with β-cyclodextrin and papain complexed with 2-hydroxypropyl-β-cyclodextrin, respectively. The P_{app} of furosemide (negative control) was 2.8 ± 0.0 cm/s. The P_{app} value of metoprolol positive control was 24 ± 1.4 cm/s. It was possible to notice an almost 2.5-fold increase in furosemide permeation with papain and its complexes compared to the control with only furosemide (Fig. 7).

3.5. Fluorescence confocal microscopy

In order to observe certain components of Caco-2 cells, the cells were stained with Alexa Fluor™ 488 Phalloidin (marking actin filaments from the cytoskeleton) and DAPI (marking the nuclei), making it possible to directly observe the monolayer and the network of occlusive junctions formed, so that the adequate morphology could be obtained (Fig. 8).

4. Discussion

There has been a growing interest in new pharmaceutical trend like the oral administration of proteins and peptides [29]. Thus, there is an increasing concern towards evaluating the safety of peptides that are candidates as new drugs or adjuvants compounds for therapeutic applications. Although papain is a proteolytic enzyme commonly used in therapy, the impact of oral enhancer permeation administration of this biomolecule is still unclear, especially due to its trypsin-like mechanism

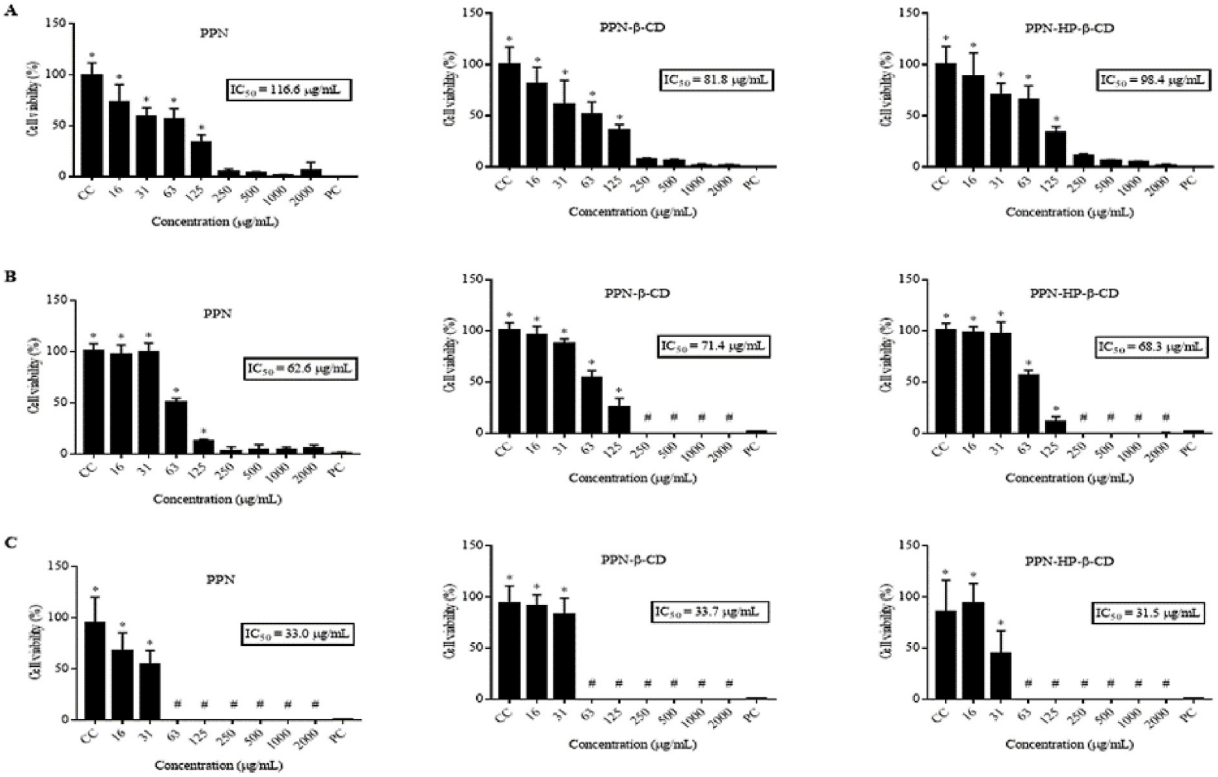

Fig. 2. Cytotoxicity of PPN, PPN-β-CD, and PPN-HP-β-CD on (A) CHO–K1 cells; (B): Hep G2 cells; (C) Caco-2 cells after 48 h of treatment using the MTT assay. CC: cell control; PC: positive control (1% Triton™ X-100); #: absence of cells. The data represent the mean ± standard deviation of two independent tests (n = 12) (*p < 0.05 sample *vs.* positive control followed by One-way ANOVA and Bonferroni test).

of action which leads to cell detachment of adherent cells. Unfortunately, there are not many studies in the reviewed literature regarding the cytotoxicity evaluation of papain complexed with cyclodextrins, evidencing the importance and the pioneering character of this study in providing information about the safety of these compounds.

There was no statistical difference between the proteolytic activity of papain complexed with β-CD or HP-β-CD, which reinforces the fact the multiple inclusion complexes are not taking place in the active site nor in regions capable of blocking the accessibility of the active site. Thus the enzyme interaction with the CDs were proved to be adequate for the preservation of its activity and are in agreement with studies previous conducted by Varca et al. [14].

It was possible to observe a dose-dependent cytotoxic profile, where the papain concentration was directly proportional to the caused cytotoxic effect (Fig. 2). In other words, the papain may alter cell viability in concentrations above the IC_{50} values when the cells are exposed to the samples for a 48 h period. This corroborates the possible increased cell death process caused by cell detachment, which activates apoptotic mechanisms instead of necrosis associated with inflammatory responses [30]. However, the complexes PPN-β-CD or PPN-HP-β-CD were not able to destabilize the membrane to cause a loss of integrity and liberation of LDH to the extracellular milieu (Fig. 3). It is interesting to note that the CHO–K1 rodent cell line was slightly less sensitive to papain if compared to Hep G2 and Caco-2 cells which may indicated the need of performing preliminary *in vitro* tests using human cells instead of other species towards mimicking the human metabolism conditions as the main interest refers to its use for human administration.

In addition to the dose-dependent relationship of the proteolytic of papain over the cells, the exposition time above 24 h was also relevant to the increased cytotoxic effect shared by papain, in accordance with previously reported studies [14,31]. It was reported that papain also holds fibrinolytic activity. Thus, it is suggested that the observed cell death was due to the intense proteolytic activity of papain, which could be effective against the fibrous coatings of tumor cells, corroborating the low viability of Hep G2 and Caco-2 cells exposed to higher concentrations of the native papain and its complexes [32]. Regarding the mechanistic issues, the papain action is known to be related mainly to the cleavage of peptidic bonds between the carboxylic groups of lysine and arginine, and the adjacent residues [33].

The cell adhesion is important for the attachment of cells to one another and also to the extracellular matrix. Therefore, it is considered

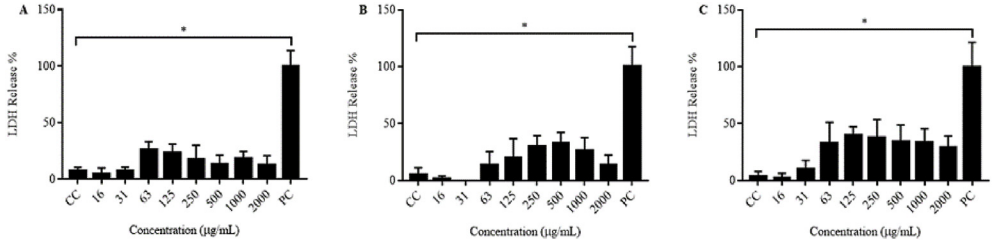

Fig. 3. LDH release of native papain and papain complexed with β-cyclodextrin or 2-hydroxypropyl-β-cyclodextrin using Caco-2 cells and with 8 h of incubation. CC: cells controls; PC: positive control (1% Triton™ X-100); (A): native papain; (B): PPN-β-CD; (C): PPN-HP-β-CD. The data represent the means ± standard deviation of two independent experiments (n = 12) (*p < 0.05 sample *vs.* positive control followed by One-way ANOVA and Bonferroni test).

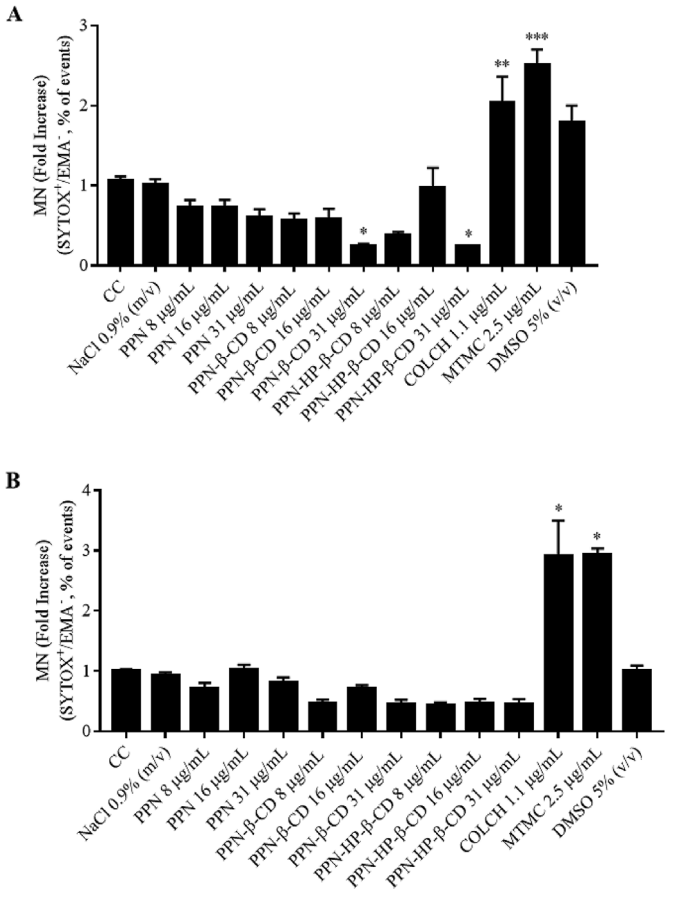

Fig. 4. Relative number of micronuclei (MN) formed after CHO–K1 cells (A) and Hep G2 cells (B) were exposed to PPN, PPN-β-CD, and PPN-HP-β-CD, at the concentrations of 8, 16, and 31 µg/mL for 4 h. The controls were CC: Cell control; NaCl: sodium chloride; COLCH: colchicine; MTMC: mitomycin C; DMSO: dimethyl sulfoxide. The data represent the means ± standard deviation of two independent assays (n = 6) (*p < 0.05; **p < 0.01; ***p < 0.0001 sample *vs*. cells control followed by One-way ANOVA and Bonferroni test).

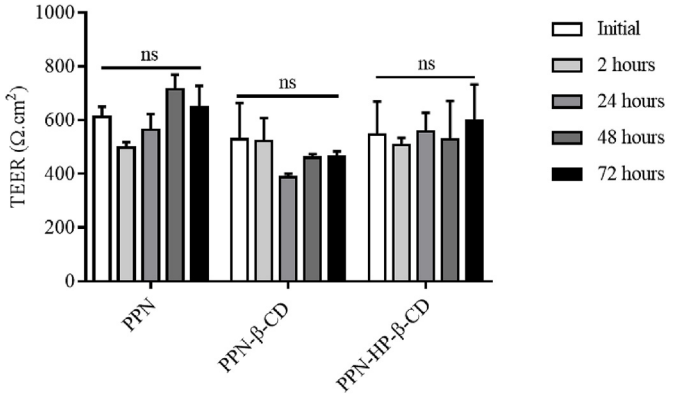

Fig. 5. TEER (Ω.cm^2) in Caco-2 cells monolayer before and after 2 h of exposition with papain samples (16 µg/mL) and furosemide (100 µg/mL). CC: control without treatment; PPN: native papain; PPN-β-CD: papain complexed with β-cyclodextrin; PPN-HP-β-CD: papain complexed with 2-hydroxypropyl-β-cyclodextrin. The data represent the means ± standard deviation (n = 3). ns: not significant.

fundamental for the maintenance of its organizational structure, stability, and for the tissue integrity. In the absence of adhesion to the extracellular matrix, the cells undergo a kind of apoptosis, a process called anoikis. The trigger for this kind of cell death related to the impaired mitochondrial function (intrinsic pathway), or by the activation of certain membrane receptors (extrinsic pathway), which are responsible for the activation of caspases and, in a second moment, endonucleases, as well as for DNA fragmentation that leads to cell death [34]. At higher concentrations, it is suggested that papain may cause those cells to lose connection with the matrix and to become unable to exert their metabolism, resulting in the activation of anoikis, ending on low cell viability in more elevated concentrations.

If on one hand papain might be cytotoxic over adherent cells due to its proteolytic action, on the other hand, the use of cyclodextrins is considered safe for several authors [35–39]. Regarding its safety, cyclodextrins use to form inclusion complexes with biomolecules, such as papain, may promote several benefits, as they may increase the solubility, prolong their stability through the gastrointestinal tract, promote an increased diffusion through the gastrointestinal epithelium and, increase its bioavailability and overall pharmaceutical efficacy [40–42].

In this study, even though the papain complexation with cyclodextrins was not able to diminish the intrinsic toxicity caused by cell detachment, it may improve the stability of the enzyme in pharmaceutical formulations which allows its commercialization since it could extend the shelf life formulations [14,43].

In addition to the *in vitro* cytotoxicity assays, the *in vitro* micronuclei assay using adherent cells provides a trustworthy and important tool for the detection of DNA fragments on the cytoplasm of cells in interphase after the exposition to aneugenic agents (compounds that alter the standard number of chromosomes, possibly preventing the formation of the spindle poles during metaphase of mitosis) or to clastogenic agents (compounds responsible for DNA alkylation that lead to chromosomic disruptions) [44]. The formation of micronuclei is a result of irreversible cell alterations, and the presence of these fragments in the cytoplasm is an important indicator of alterations in the genetic material. Thus, a positive result in this test indicates that the compound induces genetic damages to the cell, contributing to an increased risk of developing cancer [45].

Regarding the assessment of possible genotoxic damages, the investigation of micronuclei is a predictive mean of performing tests for the evaluation of toxicological safety [46]. It is important to mention that the OECD 487 [47] recommends that the cell and micronuclei counting be made by analyzing histological slides. However, there has been an increasing need for automation for this process, especially in the cell counting step and, currently, several research groups perform this assay in a minimalized way, with fewer reagents and materials [48] and with analytic techniques, such as flow cytometry for high-yield screening tests [49].

The choice of the concentrations to be used during the mutagenicity evaluation must consider these preliminary tests of cell cytotoxicity to elect the highest non-toxic concentration to the cells, guaranteeing good cell viability. The OECD 487 [47] recommends the evaluation of at least three concentrations during the in *vitro* micronuclei assay. The concentrations used in this study resulted in at least 70% cell viability in the cytotoxicity assays with the CHO–K1 and Hep G2 cell lines (Fig. 4).

According to OECD 487 (2016), CHO–K1 is one of the cell lines indicated for these assays, and a plethora of studies use this cells to evaluate the genetic toxicity profile of several substances during the non-clinical trials, since these cells are easily cultured and fast-growing, even though they are originated from rodent mammals [50–55]. Several lines of evidence supported that Hep G2 cells are also easily cultured, and besides that they can keep some cellular functions similarly to normal hepatocytes, such as the expression of specific surface receptors, the synthesis, and secretion of plasmatic proteins, and they are able to maintain the activity of certain phase-1 enzymes (oxidative reactions) and phase-2 enzymes (conjugation reactions) from the metabolism related to activation and detox of potentially genotoxic molecules [56–60]. Thus, the fact that Hep G2 cells present similar

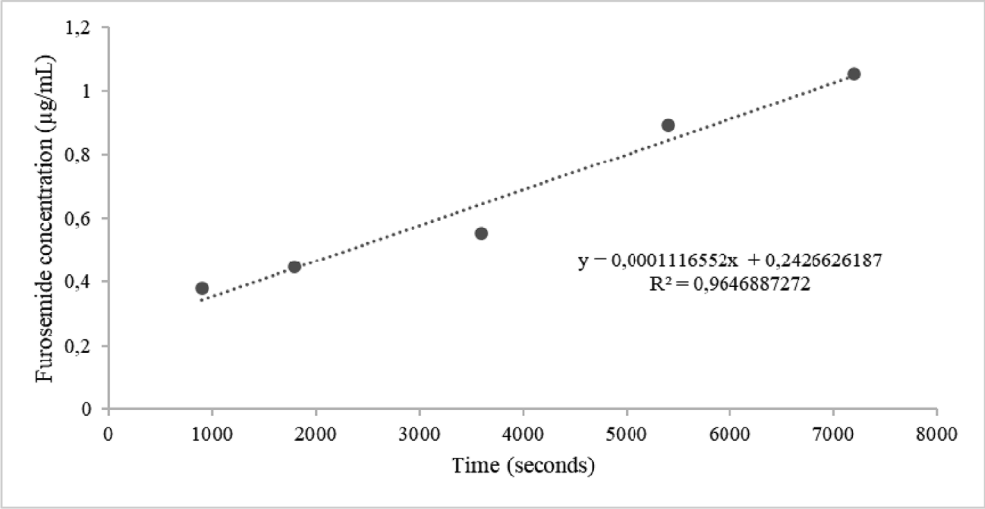

Fig. 6. Permeation profile of furosemide through the Caco-2 cell monolayer in 2 h.

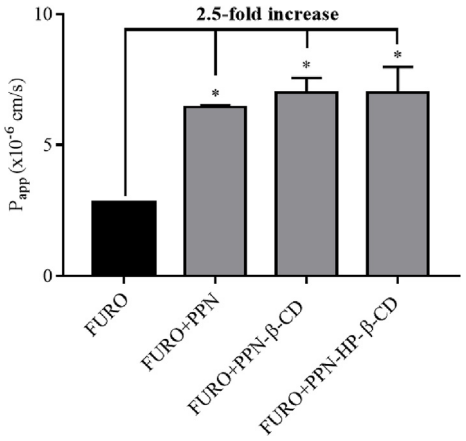

Fig. 7. Permeability coefficient (P_{app}) (cm/s) of 100 µg/mL furosemide, isolated or in the presence of compounds with 16 µg/mL papain, in the Caco-2 cells monolayer after a 2-h exposition. FURO: furosemide; PPN: native papain; PPN-β-CD: papain complexed with β-cyclodextrin; PPN-HP-β-CD: papain complexed with 2-hydroxypropyl-β-cyclodextrin. The data represent the means ± standard deviation (*$p < 0.05$ compared to the negative control (furosemide) followed by One-way ANOVA and Bonferroni test).

characteristics as the human liver can offer a more predictive result regarding the first-passage metabolism of orally-administered drugs, as well as their mutagenic potential after this metabolism [57].

The results suggested that neither native papain nor papain-cyclodextrin complexes may be mutagenic upon contact with human liver cells (Fig. 4). The absence of genotoxic events with papain was also demonstrated by Silva et al. [61], who demonstrated the absence of mutagenicity by papain with concentrations ranging from 5 to 500 µg/mL, using plasmid DNA of prokaryote organisms and the technique of electrophoresis in agarose gel.

Although the use of both cell lines was adequate to predict the absence of genotoxicity from compounds with papain, some authors affirm that the use of human-derived cell lines on the assessment of genetic safety is more trustworthy. Liao et al. [62] compared the use of CHO–K1 and V79 cells (from rodents) to human lymphocytes from peripheral blood for the prediction of micronuclei formation and other genetic alterations when those cells were exposed to different concentrations of mitomycin C and observed that the human cells presented higher selectivity in the cytogenetic assays, especially the *in vitro* micronuclei assay.

Indeed, Fowler et al. [63] highlighted that p53 deficiency in several cell lines from rodents, such as V79 and CHO, makes those cells more susceptible to false-positive results if compared to human cell lines like TK6 and Hep G2. Therefore, the choice of human cells for the *in vitro* micronuclei assay is fundamental for detecting the frequency of genotoxic events with higher precision and reliability.

Diaz et al. [64] considered as positive results those presenting a significant statistic difference ($p < 0.05$) and when compared to controls, those that presented a ≥3-fold increase in the percentage of micronuclei events. The same authors considered as slightly positive those results that presented statistical significance ($p < 0.05$) compared it to the controls, and also a ≥ 2-fold increase, but < 3, in the percentage of micronuclei events. Based on this classification, the positive controls (colchicine and mitomycin C) were considered slightly positive for genotoxicity when compared to cells controls using CHO–K1 cell line, as although they presented a statistically significant difference ($p < 0.05$), the mean micronuclei frequency values were only 2-fold higher than the cell controls. However, regarding Hep G2 cells, the positive controls were considered positive for genotoxicity compared to the cell controls, since the mean micronuclei frequency values presented statistically significant differences to the controls ($p < 0.05$), whose genotoxicity values were at least three times lower

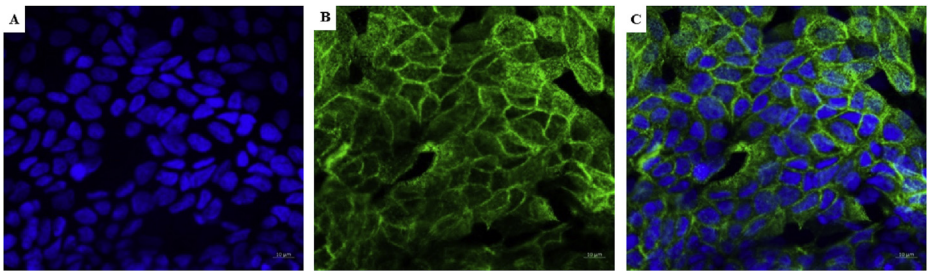

Fig. 8. Fluorescence confocal microscopy of a Caco-2 cells monolayer. (A): DAPI - blue, labeling the nuclei; (B): Alexa Fluor™ 488 Phalloidin - green, labeling the actin filaments present preferentially at the cell borders and microvilli; (C): Merged. Scale bar = 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

than the positive controls, showing that the assay holds a good sensitivity and, therefore, there was a good configuration of positive controls.

OECD 487 [47] outlines that a positive result in the *in vitro* micronuclei assay indicates that the substance induces the loss of chromosome breaks. A negative result indicates that in the applied assay conditions, the substance does not induce chromosomic breaks or loss/gaining of mammal cells in culture. Thus, the results were considered potentially genotoxic for colchicine and mitomycin C (controls of the experiments), and it was possible to affirm that the native papain and papain and cyclodextrin complexes were not able to promote significant cytogenetic alterations and were considered non-genotoxic at the concentrations used in the study.

Regarding the permeability assays, Caco-2 cells that grow attached to permeable membranes maintain some morphological and functional characteristics of the intestinal epithelium. This epithelium is formed by enterocytes, which are strongly attached by protein junction complexes located mainly on the apical portion of those cells and specialized with microvilli on the apical border, metabolic enzymes, and efflux transporters like P-glycoprotein (P-gp), which allow the evaluation of the intestinal permeability under controlled conditions [29]. P-gp is an energy-dependent efflux pump that inhibits the absorption of several pharmaceuticals and is considered one of the main mechanisms of resistance due to the variety of pharmacokinetic parameters it provides [65].

These apical junction complexes are composed of three distinct regions: occlusive junctions, adherent junctions, and desmosomes, which help to maintain the polarized morphology of the cell monolayer. However, according to Cano-Cebrián et al. [66], the occlusive junctions are the main responsible for the selective control of paracellular permeation of nutrients and xenobiotics. The junctions are composed by transmembrane ramified proteins with an extracellular domain, predominantly from the family of claudins and occludins. The extracellular domains form loops that bind to the loops from the domains of the adjacent cells, blocking the intercellular space and creating a seal among neighbor cells. These junctions also connect with the cytoskeleton and stabilize the cell-to-cell adhesion [67]. The occlusive junctions can be opened by intracellular mechanisms (signaling mechanisms involved in the modulation of these junctions) or by extracellular ones (direct interruption of the recognition interactions among the proteins) [68].

Currently, the permeability evaluation of drugs to be orally administered during the initial steps of nonclinical research is mandatory for the development of new formulations, and it is considered an important criterion during the screening and selection of new pharmaceuticals for later clinical investigations [69]. According to Kratz et al. [70], the cell culture is a useful tool to predict the permeability of drugs through the cell monolayer that mimics the barrier condition of the intestinal epithelium. The Caco-2 cells monolayer model for the investigation of permeability and metabolism is widely used as an alternative to the use of animals [71], because it presents an elevated reproducibility and precision and also presents a strong correlation with the absorbed dose in humans [72].

The TEER is a good index of confluence and integrity of monolayers. Initially, the TEER values were above 500 Ω cm^2, indicating that the cells monolayer presented high cohesion due to the significant presence of cell junctions that maintained the integrity of the epithelial barrier during the 21 days of culture. After the application of the samples containing papain, a decrease of TEER values was observed compared to the initial values, although the new values are still considered to be elevated (Fig. 5).

It is interesting to notice that the sample containing papain complexed with β-cyclodextrin reduced TEER after 2 h. It is known that the hydrophobic regions of cyclodextrins can interact with lipids from the plasma membrane, possibly leading to cholesterol depletion and, consequently, destabilizing the cell membrane and favoring the transcellular permeation [37], although the use of cyclodextrins is considered safe for oral administration [73]. Compared to 2-hydroxypropyl-β-cyclodextrin, β-cyclodextrin is more lipophilic. Therefore, it is more likely to form inclusion complexes with specific membrane lipids, such as cholesterol, phosphatidylcholine, and sphingomyelin. This interaction impairs the integrity of the bilayer and leads to a higher fluidity of the membrane. Inversely, the presence of hydroxypropyl groups turns the cyclodextrin more hydrophilic, diminishing its interaction with the aforementioned lipids [74].

Furthermore, decreased TEER values might also be associated with the presence of papain. Though in low concentrations, this enzyme was able to exert its proteolytic action against the proteins from the extracellular matrix and the junction complexes, which are essential for the maintenance of the epithelial barrier integrity [75]. According to Stremnitzer et al. [76], papain in concentrations below 1 μg/mL is able to degrade several proteins related to cell junctions, i.e., claudin-4 and occludins in human keratinocytes *in vitro*.

However, when transport studies were examined, the decreased TEER value recovered and returned to the initial level after 72 h, indicating a good recovery of the cell monolayer integrity. In addition, there was no statistically significant difference between TEER values at the assayed periods. Similar results were found by Guggi and Bernkop-Schnürch [77]. Therefore, in the present study, it is suggested that papain might have promoted a decreased intercellular cohesion, which occurs due to the cell junctions and also modulated the paracellular permeation of furosemide, suggesting that the opening of tight junctions was transient and reversible.

Several regulating agencies recommend the use of Caco-2 cells monolayer for the assessment of intestinal permeability of drugs, as a model for quantifying the permeated fraction and for the classification of permeability according to the Biopharmaceutical Classification System (BCS) [78]. Yee [79] says that a drug is considered poorly absorbed (0–20%) when P_{app} is lower than 1×10^{-6} cm/s, moderately absorbed (20–70%) when P_{app} value is within 1 and 10×10^{-6} cm/s, and well absorbed (70–100%) when P_{app} value is higher than 10×10^{-6} cm/s.

Furosemide is a loop diuretic used for the treatment of edema, congestive heart failure, and hypertension, but also for the treatment of acute and chronic kidney failure [80]. According to BCS, furosemide is classified as class IV, meaning it presents low solubility and low permeability [81]. Granero et al. [82] describe that this low transcellular permeability is due to intrinsic physical-chemical characteristics from the molecule, and to the activation of multiple resistance mechanisms to the drugs. Furosemide, which is considered a weak acid (pKa 3.9), presents an incomplete absorption through the gastrointestinal tract and is not able to cross the epithelial layer just by diffusion. Besides, furosemide is a substrate from P-gp. Therefore, this efflux pump is responsible for the intense secretion of this drug to the intestine lumen, hampering, even more, its absorption.

The permeability coefficient (Papp) of furosemide in the presence of only 2-hydroxypropyl-β-cyclodextrin was approximately 7.01 × 10-6 cm/s. This Papp value was not significantly lower than the furosemide Papp value in the presence of papain-cyclodextrin complexes.

Due to their ability to interact with the epithelial barrier, cyclodextrins can sequester phospholipid components of the bilayer membrane, leading to temporary destabilization of the cell monolayer by decreasing the barrier properties of the lipophilic membrane. In general, this mechanism may facilitate the promotion of furosemide permeation. Besides that, in the presence of cyclodextrins, the physicochemical and biological properties of biomolecules can be very advantageously altered. 2-Hydroxypropyl-β-cyclodextrin has a higher solubility and its self-assembly to soluble active biomolecules, such as papain. This cyclodextrin has a good inclusion complexation and tolerability for biomedical uses. In terms of safety, 2-hydroxypropyl-β-cyclodextrin has less toxicity than β-cyclodextrin [37]. Although studies have shown that cyclodextrins are virtually non-toxic due to their

lack of absorption, it is reported that bacterial β-cyclodextrin degradation and fermentation in the colon may lead to gas production and diarrhea. Moreover, it has been shown that β-cyclodextrin is capable of causing cell lysis in different types of cells, which may lead to hemolysis and renal toxicity. Due to this, they have been considered unsuited for parenteral formulations.

Regarding the papain, it is important to highlight that this phytoenzyme has several beneficial properties, such as stimulating tissue healing, antimicrobial activity and promoting controlled drug permeation, as described in the paper. The complexation of papain with cyclodextrin can promote the stabilization of the enzyme and guarantee the prolongation of its biological activity.

Although presenting an absorption with great variability, a small quantity of furosemide is able to be absorbed through paracellular mechanisms, although this pathway is not considered to be very accessible for the molecules that present charges and high molecular weight [83]. These characteristics corroborate the fact that furosemide has a very limited absorption through the gastrointestinal tract, contributing to its low bioavailability and possibly compromising the therapeutic efficacy.

In the present study, although the biopharmaceutic classification of furosemide suffered no alteration, the P_{app} value of furosemide greatly improved in the presence of papain. Surprisingly, the P_{app} value of this drug associated with the compounds with papain was about 2.5 times higher compared to the P_{app} value from the isolated furosemide (negative control) (Fig. 6). The P_{app} values from furosemide and metoprolol are in accordance with the values found by other authors [84–86].

According to Aungst [87] and Lee et al. [88], the cellular junctions present between cells from the intestine epithelium significantly limit the paracellular permeation of several compounds. Therefore, to modulate the opening of these junctions is an important factor to enhance the bioavailability of low-penetration drugs. Currently, the main intestinal permeation enhancers are small-chain fat acids, acylcarnitines, bile salt derivatives, and EDTA, which exert its action mechanism either by enhancing plasmatic membrane fluidity, consequently enhancing the transcellular permeation, or by altering the modulation of expression and the activity of cell junctions, therefore favoring the paracellular permeation [89].

The concentration of native papain or complexed with β-cyclodextrin used in this study was high enough to dilate the occlusive junctions present in Caco-2 cells and to favor the permeation of furosemide without permanent damage the Caco-2 cell monolayer. Bock et al. [90] evaluated the ability of papain and other proteolytic enzymes, i.e. trypsin, chymotrypsin, and bromelain, in crossing the epithelial barrier and observed that even in low concentrations, papain can lead to a significant decrease in TEER values of the Caco-2 cells by opening the occlusive junctions, facilitating the paracellular transport of compounds with low permeation ability. Guggi and Bernkop-Schnürch [77] also demonstrated that papain is able to enhance the permeation of compounds with low and high molecular weight through the small intestine mucosa. Grabovac et al. [12] developed pills containing heparin with low molecular weight associated with papain and explored its proteolytic activity, aiming to enhance the permeation of biomolecules along the gastrointestinal tract of rats, and observed a significant increase of heparin bioavailability compared to the control group.

LY was used as a marker for the paracellular integrity of Caco-2 cells monolayer. This fluorescent compound presents very low P_{app} values as it permeates only by a paracellular mechanism [91]. The P_{app} value was 4.3×10^{-7} cm/s. According to Wang et al. [92], Papp values lower than 5×10^{-7} cm/s indicate that the monolayer integrity was preserved during the performance of the permeability assays.

The results of the present study are promising for the development of pharmaceutical formulations containing papain complexed with cyclodextrins and for its ability to promote the permeation of other drugs with low oral permeability. The use of cyclodextrins, β-CD and HP- β-CD, did not offer any advantages if compared to the native form of the enzyme in term of safety or enhancement of the permeation effects, but studies have already showed that the complexes present better stability, impacting directly on the desirable stability of the enzyme.

5. Conclusions

In summary, this work proved the *in vitro* safety of papain-cyclodextrin complex and its effectiveness in promoting furosemide permeation, however, the results shown do not include the comparison of β-cyclodextrin alone with furosemide in view of physicochemical aspects and biological aspects of this association.

The results indicated that native papain, as well as β-cyclodextrin-papain and 2-hydroxypropyl-β-cyclodextrin-papain complexes, feature concentration-dependent cytotoxicity, whereas no cytotoxicity was observed at the lowest concentrations investigated over CHO–K1, Hep G2 and Caco-2 cells. The low cell viability values observed were hypothesized to result from papain proteolytic activity which causes cell detachment rather than necrosis originated by direct toxicity or cell death mechanism.

In terms of genotoxicity, native papain and its complexes with cyclodextrins did not induce any effect at any concentration over the above-mentioned cell lines which highlights a possible safety use of the enzyme and its complexes in biomedical applications. No contribution in terms of papain effects over the micronuclei formation was observed for the complexes if compared to the native form of the enzyme.

As for the transport studies, the findings suggested that the enhanced permeation across the intestinal epithelial by papain and papain-β-cyclodextrin complexes might play an essential role in the enhanced intestinal absorption of furosemide.

In conclusion, the results suggest that the complexes containing papain could be applied as pharmacotechnical adjuvants in order to promote a safe permeation of drugs that present low oral permeability.

Author contributions

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

Funding

This work was supported by São Paulo Research Foundation - FAPESP (FAPESP grant #2010/10935-9, #2015/19213-0, #2015/19212-3 and #2016/22916-5).

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors would like to thank Gabriela Nemesio Fazolin from the Center of Chemistry and Environment at Energy and Nuclear Research Institute for the support; to Sheila Schuindt do Carmo and Waldir Caldeira from the Center of Images Acquisition and Microscopy at Institute of Biosciences from the University of São Paulo for the acquisition of fluorescence confocal microscopy images.

References

[1] J.F. Pinto, Site-specific drug delivery systems within the gastro-intestinal tract: from the mouth to the colon, Int. J. Pharm. 395 (2010) 44–52, https://doi.org/10.1016/J.IJPHARM.2010.05.003.

[2] F. Hallouard, L. Mehenni, M. Lahiani-Skiba, Y. Anouar, M. Skiba, Solid dispersions

for oral administration: an overview of the methods for their preparation, Curr. Pharmaceut. Des. 22 (2016) 4942–4958, https://doi.org/10.2174/1381612822666160726095916.
[3] S.A.A. Rizvi, A.M. Saleh, Applications of nanoparticle systems in drug delivery technology, Saudi Pharm. J. 26 (2018) 64–70, https://doi.org/10.1016/J.JSPS.2017.10.012.
[4] G. Guidotti, L. Brambilla, D. Rossi, Cell-penetrating peptides: from basic research to clinics, Trends Pharmacol. Sci. 38 (2017) 406–424, https://doi.org/10.1016/j.tips.2017.01.003.
[5] M. Sánchez-Navarro, J. Garcia, E. Giralt, M. Teixidó, Using peptides to increase transport across the intestinal barrier, Adv. Drug Deliv. Rev. 106 (2016) 355–366, https://doi.org/10.1016/j.addr.2016.04.031.
[6] L. Shi, R. Ermis, K. Lam, J. Cowart, P. Attar, D. Aust, Study on the Debridement Efficacy of Formulated Enzymatic Wound Debriding Agents by in Vitro Assessment Using Artificial Wound Eschar and by an in Vivo Pig Model: Technical Article, Wound Repair Regen, (2009), https://doi.org/10.1111/j.1524-475X.2009.00545.x.
[7] F. Figueiredo Azevedo, L.P. Santanna, V.C. Bóbbo, E.A. Libert, E.P. Araújo, M. Abdalla Saad, M.H.M. Lima, Evaluating the effect of 3% papain gel application in cutaneous wound healing in mice., wounds a compend, Clin. Res. Pract. 29 (2017) 96–101.
[8] L. Monetta, Uso da papaína nos curativos feitos pela enfermagem, Rev. Bras. Enferm. 40 (1987) 66–73, https://doi.org/10.1590/S0034-71671987000100012.
[9] D. Singh, R. Singh, Papain incorporated chitin dressings for wound debridement sterilized by gamma radiation, Radiat. Phys. Chem. 81 (2012) 1781–1785, https://doi.org/10.1016/J.RADPHYSCHEM.2012.06.010.
[10] Z.S. Silva Jr., Y.-Y. Huang, L.F. de Freitas, C.M. França, S.B. Botta, P.A. Ana, R.A. Mesquita-Ferrari, K.P. Santos Fernandes, A. Deana, C.R. Lima Leal, R.A. Prates, M.R. Hamblin, S.K. Bussadori, Papain gel containing methylene blue for simultaneous caries removal and antimicrobial photoinactivation against Streptococcus mutans biofilms, Sci. Rep. 6 (2016) 33270, https://doi.org/10.1038/srep33270.
[11] P.S. Lopes, C.A.S. de Olive Pinto, A.R. Baby, M.V.R. Velasco, M.E. Taqueda, T.M. Kaneko, Evaluation of in vitro percutaneous enhancement effect of papain and pequi oil on diclofenac sodium permeation through human skin, Rev. Bras. Ciencias Farm. 44 (2008) 225–231, https://doi.org/10.1109/ICSM.2011.6080838.
[12] V. Grabovac, T. Schmitz, F. Föger, A. Bernkop-Schnürch, Papain: an effective permeation enhancer for orally administered low molecular weight heparin, Pharm. Res. 24 (2007) 1001–1006, https://doi.org/10.1007/s11095-006-9226-8.
[13] M.L. Flindt, Allergy to alpha-amylase and papain, Lancet 1 (1979) 1407–1408.
[14] G.H.C. Varca, N. Andréo-Filho, L.F. Fraceto, T.M. Kaneko, H.G. Ferraz, N.M. Esteves, M.G. Issa, M.B. Mathor, P.S. Lopes, Thermal characterization and cytotoxicity of complexes formed by papain and cyclodextrin, J. Biol. Phys. 33 (2007) 463–475, https://doi.org/10.1007/s10867-008-9098-8.
[15] J. Renukuntla, A.D. Vadlapudi, A. Patel, S.H.S. Boddu, A.K. Mitra, Approaches for enhancing oral bioavailability of peptides and proteins, Int. J. Pharm. 447 (2013) 75–93, https://doi.org/10.1016/j.ijpharm.2013.02.030.
[16] S. Shimpi, B. Chauhan, P. Shimpi, Cyclodextrins: application in different routes of drug administration, Acta Pharm. 55 (2005) 139–156.
[17] C. Yewale, S. Patil, A. Kolate, G. Kore, A. Misra, Oral absorption promoters: opportunities, issues, and challenges, Crit. Rev. Ther. Drug Carrier Syst. 32 (2015) 363–387, https://doi.org/10.1615/CritRevTherDrugCarrierSyst.2015011865.
[18] A. Adan, Y. Kiraz, Y. Baran, Cell proliferation and cytotoxicity assays, Curr. Pharmaceut. Biotechnol. 17 (2016) 1213–1221.
[19] P. Artursson, J. Karlsson, Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells, Biochem. Biophys. Res. Commun. 175 (1991) 880–885, https://doi.org/10.1016/0006-291X(91)91647-U.
[20] K.-C. Cheng, C. Li, A.S. Uss, Prediction of oral drug absorption in humans–from cultured cell lines and experimental animals, Expert Opin. Drug Metabol. Toxicol. 4 (2008) 581–590, https://doi.org/10.1517/17425255.4.5.581.
[21] N. Marsousi, F. Doffey-Lazeyras, S. Rudaz, J.A. Desmeules, Y. Daali, Intestinal permeability and P-glycoprotein-mediated efflux transport of ticagrelor in Caco-2 monolayer cells, Fundam. Clin. Pharmacol. 30 (2016) 577–584, https://doi.org/10.1111/fcp.12219.
[22] J.B. Lee, S.H. Son, M.C. Park, T.H. Kim, M.G. Kim, S.D. Yoo, S. Kim, A novel in vitro permeability assay using three-dimensional cell culture system, J. Biotechnol. 205 (2015) 93–100, https://doi.org/10.1016/j.jbiotec.2014.12.019.
[23] H. Lennernäs, Human intestinal permeability, J. Pharm. Sci. 87 (1998) 403–410, https://doi.org/10.1021/js970332a.
[24] C.C. Ferraz, G.H.C. Varca, M.M.D.C. Vila, P.S. Lopes, Validation of in vitro analytical method to measure papain activity in pharmaceutical formulations, Int. J. Pharm. Pharm. Sci. 6 (2014) 658–661.
[25] T. Mosmann, No Title, 65 (1983) 55–63. doi:10.1016/0022-1759(83)90303-4.
[26] S.M. Bryce, J.C. Bemis, S.L. Avlasevich, S.D. Dertinger, In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity, Mutat. Res. Toxicol. Environ. Mutagen. 630 (2007) 78–91, https://doi.org/10.1016/j.mrgentox.2007.03.002.
[27] I. Hubatsch, E.G.E. Ragnarsson, P. Artursson, Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers, Nat. Protoc. 2 (2007) 2111–2119, https://doi.org/10.1038/nprot.2007.303.
[28] X. Liu, D.P. Rodeheaver, J.C. White, A.M. Wright, L.M. Walker, F. Zhang, S. Shannon, A comparison of in vitro cytotoxicity assays in medical device regulatory studies, Regul. Toxicol. Pharmacol. 97 (2018) 24–32, https://doi.org/10.1016/J.YRTPH.2018.06.003.
[29] P. Lundquist, P. Artursson, Oral absorption of peptides and nanoparticles across the human intestine: opportunities, limitations and studies in human tissues, Adv. Drug Deliv. Rev. 106 (2016) 256–276, https://doi.org/10.1016/j.addr.2016.07.007.
[30] T. Decker, M.-L. Lohmann-Matthes, A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity, J. Immunol. Methods 115 (1988) 61–69, https://doi.org/10.1016/0022-1759(88)90310-9.
[31] A. Müller, S. Barat, X. Chen, K.C. BuiUI, P. Bozko, N.P. Malek, R.R. Plentz, Comparative study of antitumor effects of bromelain and papain in human cholangiocarcinoma cell lines, Int. J. Oncol. 48 (2016) 2025–2034, https://doi.org/10.3892/ijo.2016.3411.
[32] K. Sahu, M. Kaurav, R.S. Pandey, Protease loaded permeation enhancer liposomes for treatment of skin fibrosis arisen from second degree burn, Biomed. Pharmacother. 94 (2017) 747–757, https://doi.org/10.1016/j.biopha.2017.07.141.
[33] K.M. Babu, Silk : Processing, Properties and Applications, Woodhead Publishing in association with the Textile Institute, 2013.
[34] P. Paoli, E. Giannoni, P. Chiarugi, Anoikis molecular pathways and its role in cancer progression, Biochim. Biophys. Acta Mol. Cell Res. 1833 (2013) 3481–3498, https://doi.org/10.1016/j.bbamcr.2013.06.026.
[35] A. Asai, T. Okuda, T. Yamauchi, Y. Sugiura, H. Okamoto, Safety evaluation of dry powder formulations by direct dispersion onto air–liquid interface cultured cell layer, Biol. Pharm. Bull. 39 (2016) 368–377, https://doi.org/10.1248/bpb.b15-00791.
[36] S. Gould, R.C. Scott, 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD): a toxicology review, Food Chem. Toxicol. 43 (2005) 1451–1459, https://doi.org/10.1016/j.fct.2005.03.007.
[37] T. Irie, K. Uekama, Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation, J. Pharm. Sci. 86 (1997) 147–162, https://doi.org/10.1021/js960213f.
[38] F. Leroy-Lechat, D. Wouessidjewe, J.-P. Andreux, F. Puisieux, D. Duchêne, Evaluation of the cytotoxicity of cyclodextrins and hydroxypropylated derivatives, Int. J. Pharm. 101 (1994) 97–103, https://doi.org/10.1016/0378-5173(94)90080-9.
[39] K. Toyoda, T. Shoda, C. Uneyama, K. Takada, M. Takahashi, Carcinogenicity study of beta-cyclodextrin in F344 rats, Food Chem. Toxicol. 35 (1997) 331–336.
[40] T. Loftsson, D. Duchene, Cyclodextrins and their pharmaceutical applications, Int. J. Pharm. 329 (2007) 1–11, https://doi.org/10.1016/j.ijpharm.2006.10.044.
[41] K. Uekama, F. Hirayama, T. Irie, Cyclodextrin drug carrier systems, Chem. Rev. 98 (1998) 2045–2076.
[42] J. Zhang, P.X. Ma, Cyclodextrin-based supramolecular systems for drug delivery: recent progress and future perspective, Adv. Drug Deliv. Rev. 65 (2013) 1215–1233, https://doi.org/10.1016/j.addr.2013.05.001.
[43] G. Zulli, P.S. Lopes, M.V.R. Velasco, M.T.S. Alcântara, S.O. Rogero, A.B. Lugao, M.B. Mathor, Influence of gamma radiation onto polymeric matrix with papain, Radiat. Phys. Chem. 79 (2010) 286–288, https://doi.org/10.1016/j.radphyschem.2009.08.025.
[44] M. Kirsch-Volders, T. Sofuni, M. Aardema, S. Albertini, D. Eastmond, M. Fenech, M. Ishidate, S. Kirchner, E. Lorge, T. Morita, H. Norppa, J. Surrallés, A. Vanhauwaert, A. Wakata, Report from the in vitro micronucleus assay working group, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 540 (2003) 153–163, https://doi.org/10.1016/j.mrgentox.2003.07.005.
[45] M. Kirsch-Volders, G. Plas, A. Elhajouji, M. Lukamowicz, L. Gonzalez, K. Vande Loock, I. Decordier, The in vitro MN assay in 2011: origin and fate, biological significance, protocols, high throughput methodologies and toxicological relevance, Arch. Toxicol. 85 (2011) 873–899, https://doi.org/10.1007/s00204-011-0691-4.
[46] J.A. Heddle, M. Fenech, M. Hayashi, J.T. MacGregor, Reflections on the development of micronucleus assays, Mutagenesis 26 (2011) 3–10, https://doi.org/10.1093/mutage/geq085.
[47] Organisation For Economic Co-Operation And Development (OECD/OCDE), Organisation for Economic Co-operation and Development., Guideline for Testing of Chemicals Test No. 487: in Vitro Mammalian Cell Micronucleus Test, (2016), p. 29.
[48] H. Dong, H. Sun, J. Zheng, A microchip for integrated single-cell genotoxicity assay, Talanta 161 (2016) 804–811, https://doi.org/10.1016/j.talanta.2016.09.040.
[49] S.M. Bryce, S.L. Avlasevich, J.C. Bemis, S.D. Dertinger, Miniaturized flow cytometry-based CHO-K1 micronucleus assay discriminates aneugenic and clastogenic modes of action, Environ. Mol. Mutagen. 52 (2011) 280–286, https://doi.org/10.1002/em.20618.
[50] M.J. Aardema, R.D. Snyder, C. Spicer, K. Divi, T. Morita, R.J. Mauthe, D.P. Gibson, S. Soelter, P.T. Curry, V. Thybaud, G. Lorenzon, D. Marzin, E. Lorge, SFTG international collaborative study on in vitro micronucleus test, Mutat. Res. Toxicol. Environ. Mutagen. 607 (2006) 61–87, https://doi.org/10.1016/j.mrgentox.2006.04.002.
[51] M.-Y. Chien, Y.-T. Lin, F.-C. Peng, H.-J. Lee, J.-M. Chang, C.-M. Yang, C.-H. Chen, Gastroprotective potential against indomethacin and safety assessment of the homology of medicine and food formula cuttlebone complex, Food Funct. 6 (2015) 2803–2812, https://doi.org/10.1039/c5fo00638d.
[52] N.M. Esteves-Pedro, A.C.D. Rodas, C.A. Dal Belo, Y. Oshima-Franco, M.G. dos Santos, P.S. Lopes, Implementation of the three Rs in the human hazard assessment of Brazilian medicinal plants: an evaluation of the cytotoxic and genotoxic potentials of Dipteryx alata Vogel, Altern. Lab. Anim. 39 (2011) 189–196.
[53] I.Z. Ocampo, P. de Queiroz Souza Passos, L. Ramirez de Carvalho, C.A. Lira da Cruz, N.M. Esteves-Pedro, F. Medeiros da Silva, O.Z. Higa, L.A.P. Dias, K. Okazaki, D.P. Vieira, In vitro cytotoxic and genotoxic evaluation of peptides used in nuclear medicine (DOTATATE and Ubiquicidin 29-41) in CHO-K1 cells, Cytotechnology 68 (2016) 2301–2310, https://doi.org/10.1007/s10616-016-0024-9.
[54] G.S. Santos, S. Tsutsumi, D.P. Vieira, P. Bartolini, K. Okazaki, Effect of Brazilian propolis (AF-08) on genotoxicity, cytotoxicity and clonogenic death of Chinese

hamster ovary (CHO-K1) cells irradiated with 60 Co gamma-radiation, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 762 (2014) 17–23, https://doi.org/10.1016/j.mrgentox.2013.11.004.
[55] K. Tilmant, H.H.J. Gerets, P. De Ron, C. Cossu-Leguille, P. Vasseur, S. Dhalluin, F.A. Atienzar, The automated micronucleus assay for early assessment of genotoxicity in drug discovery, Mutat. Res. Toxicol. Environ. Mutagen. 751 (2013) 1–11, https://doi.org/10.1016/j.mrgentox.2012.10.011.
[56] P.F. Dehn, C.M. White, D.E. Conners, G. Shipkey, T.A. Cumbo, Characterization of the human hepatocellular carcinoma (hepg2) cell line as an in vitro model for cadmium toxicity studies, In Vitro Cell. Dev. Biol. Anim. 40 (2004) 172, https://doi.org/10.1290/1543-706X(2004)40<172:COTHHC>2.0.CO;2.
[57] M.T. Donato, A. Lahoz, J.V. Castell, M.J. Gómez-Lechón, Cell lines: a tool for in vitro drug metabolism studies, Curr. Drug Metabol. 9 (2008) 1–11.
[58] M.M. Hoshina, L.D. Santos, M.S. Palma, M.A. Marin-Morales, Cytotoxic, genotoxic/antigenotoxic and mutagenic/antimutagenic effects of the venom of the wasp Polybia paulista, Toxicon 72 (2013) 64–70, https://doi.org/10.1016/j.toxicon.2013.06.007.
[59] S.C. Sahu, J. Njoroge, S.M. Bryce, J.J. Yourick, R.L. Sprando, Comparative genotoxicity of nanosilver in human liver HepG2 and colon Caco2 cells evaluated by a flow cytometric in vitro micronucleus assay, J. Appl. Toxicol. 34 (2014) 1226–1234, https://doi.org/10.1002/jat.3065.
[60] M. Uhl, C. Helma, S. Knasmüller, Evaluation of the single cell gel electrophoresis assay with human hepatoma (Hep G2) cells, Mutat. Res. 468 (2000) 213–225.
[61] C.R. da Silva, M.B.N. Oliveira, E.S. Motta, G.S. de Almeida, L.L. Varanda, M. de Pádula, A.C. Leitão, A. Caldeira-de-Araújo, Genotoxic and cytotoxic safety evaluation of papain (*Carica papaya* L.) using in vitro assays, J. Biomed. Biotechnol. 2010 (2010) 1–8, https://doi.org/10.1155/2010/197898.
[62] P.-H. Liao, R.-H. Lin, M.-L. Yang, Y.-C. Li, Y.-H. Kuan, Evaluation of differential representative values between Chinese hamster cells and human lymphocytes in mitomycin C-induced cytogenetic assays and caspase-3 activity, Toxicol. Ind. Health 28 (2012) 174–180, https://doi.org/10.1177/0748233711409974.
[63] P. Fowler, K. Smith, J. Young, L. Jeffrey, D. Kirkland, S. Pfuhler, P. Carmichael, Reduction of misleading ("false") positive results in mammalian cell genotoxicity assays. I. Choice of cell type, Mutat. Res. Toxicol. Environ. Mutagen. 742 (2012) 11–25, https://doi.org/10.1016/j.mrgentox.2011.10.014.
[64] D. Diaz, A. Scott, P. Carmichael, W. Shi, C. Costales, Evaluation of an automated in vitro micronucleus assay in CHO-K1 cells, Mutat. Res. Toxicol. Environ. Mutagen. 630 (2007) 1–13, https://doi.org/10.1016/j.mrgentox.2007.02.006.
[65] L. Benet, C. Cummins, C. Wu, Unmasking the dynamic interplay between efflux transporters and metabolic enzymes, Int. J. Pharm. 277 (2004) 3–9, https://doi.org/10.1016/j.ijpharm.2002.12.002.
[66] M.J. Cano-Cebrián, T. Zornoza, L. Granero, A. Polache, Intestinal absorption enhancement via the paracellular route by fatty acids, chitosans and others: a target for drug delivery, Curr. Drug Deliv. 2 (2005) 9–22.
[67] B.M. Gumbiner, Cell adhesion: the molecular basis of tissue architecture and morphogenesis, Cell 84 (1996) 345–357.
[68] S. Maher, R.J. Mrsny, D.J. Brayden, Intestinal permeation enhancers for oral peptide delivery, Adv. Drug Deliv. Rev. 106 (2016) 277–319, https://doi.org/10.1016/J.ADDR.2016.06.005.
[69] D. Oltra-Noguera, V. Mangas-Sanjuan, A. Centelles-Sangüesa, I. Gonzalez-Garcia, G. Sanchez-Castaño, M. Gonzalez-Alvarez, V.-G. Casabo, V. Merino, I. Gonzalez-Alvarez, M. Bermejo, Variability of permeability estimation from different protocols of subculture and transport experiments in cell monolayers, J. Pharmacol. Toxicol. Methods 71 (2015) 21–32, https://doi.org/10.1016/j.vascn.2014.11.004.
[70] J.M. Kratz, M.R. Teixeira, L.S. Koester, C.M.O. Simões, An HPLC-UV method for the measurement of permeability of marker drugs in the Caco-2 cell assay, Braz. J. Med. Biol. Res. Rev. Bras. Pesqui. Medicas Biol. 44 (2011) 531–537.
[71] G.L. Amidon, H. Lennernäs, V.P. Shah, J.R. Crison, A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability, Pharm. Res. 12 (1995) 413–420.
[72] F. Ingels, S. Deferme, E. Destexhe, M. Oth, G. Van den Mooter, P. Augustijns, Simulated intestinal fluid as transport medium in the Caco-2 cell culture model, Int. J. Pharm. 232 (2002) 183–192.
[73] T. Loftsson, M.D. Moya-Ortega, C. Alvarez-Lorenzo, A. Concheiro, Pharmacokinetics of cyclodextrins and drugs after oral and parenteral administration of drug/cyclodextrin complexes, J. Pharm. Pharmacol. 68 (2016) 544–555, https://doi.org/10.1111/jphp.12427.
[74] R. Challa, A. Ahuja, J. Ali, R.K. Khar, Cyclodextrins in drug delivery: an updated review, AAPS PharmSciTech 6 (2005) E329–E357, https://doi.org/10.1208/pt060243.
[75] V. Dalet-Fumeron, L. Boudjennah, M. Pagano, Binding of the cysteine proteinases papain and cathepsin B-like to coated laminin: use of synthetic peptides from laminin and from the laminin binding region of the β1Integrin subunit to characterize the binding site, Arch. Biochem. Biophys. 358 (1998) 283–290, https://doi.org/10.1006/abbi.1998.0868.
[76] C. Stremnitzer, K. Manzano-Szalai, A. Willensdorfer, P. Starkl, M. Pieper, P. König, M. Mildner, E. Tschachler, U. Reichart, E. Jensen-Jarolim, Papain degrades tight junction proteins of human keratinocytes in vitro and sensitizes C57BL/6 mice via the skin independent of its enzymatic activity or TLR4 activation, J. Investig. Dermatol. 135 (2015) 1790–1800, https://doi.org/10.1038/jid.2015.58.
[77] D. Guggi, A. Bernkop-Schnürch, Improved paracellular uptake by the combination of different types of permeation enhancers, Int. J. Pharm. 288 (2005) 141–150, https://doi.org/10.1016/j.ijpharm.2004.09.023.
[78] H. Pham-The, T. Garrigues, M. Bermejo, I. González-Álvarez, M.C. Monteagudo, M.Á. Cabrera-Pérez, Provisional classification and in silico study of biopharmaceutical system based on caco-2 cell permeability and dose number, Mol. Pharm. 10 (2013) 2445–2461, https://doi.org/10.1021/mp4000585.
[79] S. Yee, In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man–fact or myth, Pharm. Res. 14 (1997) 763–766.
[80] S. Berkó, G. Regdon, E. Ducza, G. Falkay, I. Erős, In vitro and in vivo study in rats of rectal suppositories containing furosemide, Eur. J. Pharm. Biopharm. 53 (2002) 311–315.
[81] M. Lindenberg, S. Kopp, J.B. Dressman, Classification of orally administered drugs on the World Health Organization Model list of Essential Medicines according to the biopharmaceutics classification system, Eur. J. Pharm. Biopharm. 58 (2004) 265–278, https://doi.org/10.1016/j.ejpb.2004.03.001.
[82] G. Granero, M.R. Longhi, M.J. Mora, H.E. Junginger, K.K. Midha, V.P. Shah, S. Stavchansky, J.B. Dressman, D.M. Barends, Biowaiver monographs for immediate release solid oral dosage forms: furosemide, J. Pharm. Sci. 99 (2010) 2544–2556, https://doi.org/10.1002/jps.22030.
[83] A. Avdeef, K.Y. Tam, How well can the caco-2/Madin – Darby canine kidney models predict effective human jejunal permeability? J. Med. Chem. 53 (2010) 3566–3584, https://doi.org/10.1021/jm901846t.
[84] M.B. Fernandes, J.E. Gonçalves, L.C. Tavares, S. Storpirtis, Caco-2 cells permeability evaluation of nifuroxazide derivatives with potential activity against methicillin-resistant Staphylococcus aureus (MRSA), Drug Dev. Ind. Pharm. 41 (2015) 1066–1072, https://doi.org/10.3109/03639045.2014.925919.
[85] C. Li, T. Liu, X. Cui, A.S. Uss, K.-C. Cheng, Development of in vitro pharmacokinetic screens using caco-2, human hepatocyte, and caco-2/human hepatocyte hybrid systems for the prediction of oral bioavailability in humans, J. Biomol. Screen 12 (2007) 1084–1091, https://doi.org/10.1177/1087057107308892.
[86] S. Skolnik, X. Lin, J. Wang, X.-H. Chen, T. He, B. Zhang, Towards prediction of in vivo intestinal absorption using a 96-well caco-2 assay, J. Pharm. Sci. 99 (2010) 3246–3265, https://doi.org/10.1002/jps.22080.
[87] B.J. Aungst, Absorption enhancers: applications and advances, AAPS J. 14 (2012) 10–18, https://doi.org/10.1208/s12248-011-9307-4.
[88] J.H. Lee, A. Sahu, W. Il Choi, J.Y. Lee, G. Tae, ZOT-derived peptide and chitosan functionalized nanocarrier for oral delivery of protein drug, Biomaterials 103 (2016) 160–169, https://doi.org/10.1016/j.biomaterials.2016.06.059.
[89] F. McCartney, J.P. Gleeson, D.J. Brayden, Safety concerns over the use of intestinal permeation enhancers: a mini-review, Tissue Barriers 4 (2016) e1176822, , https://doi.org/10.1080/21688370.2016.1176822.
[90] U. Bock, C. Kolac, G. Borchard, K. Koch, R. Fuchs, P. Streichhan, C.M. Lehr, Transport of proteolytic enzymes across Caco-2 cell monolayers, Pharm. Res. 15 (1998) 1393–1400.
[91] B. Press, D. Di Grandi, Permeability for intestinal absorption: Caco-2 assay and related issues, Curr. Drug Metabol. 9 (2008) 893–900.
[92] X. Wang, M. Meng, L. Gao, T. Liu, Q. Xu, S. Zeng, Permeation of astilbin and taxifolin in Caco-2 cell and their effects on the P-gp, Int. J. Pharm. 378 (2009) 1–8, https://doi.org/10.1016/j.ijpharm.2009.05.022.