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Review article

Advancements in techniques for analyzing cell permeability

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ABSTRACT

Cell permeability is a critical factor in drug development, as it directly determines the absorption and bioavailability of therapeutic compounds. This article thoroughly reviews the techniques used to analyze cell permeability, from traditional methods to innovative approaches. Initially, it delves into the characteristics, cultivation, and applications of the Caco-2 cell line, highlighting its ability to simulate the human intestinal epithelium and noting its limitations, such as the extended cultivation time required and the absence of a mucosal layer. Strategies to enhance the performance of Caco-2-based models are explored, including the use of electrospun nanofiber scaffolds and accelerated differentiation media. Additionally, alternative methods such as the Parallel Artificial Membrane Permeability Assay (PAMPA), the Madin-Darby Canine Kidney (MDCK) cell line, and the everted rat intestinal sac model are examined, evaluating their advantages and disadvantages compared to Caco-2 cells. The use of co-cultures of Caco-2 and mucin-producing HT29-MTX cells is also discussed as a more accurate replication of the human intestinal environment. The article further examines less-explored methods, such as porcine cell-based models and physiological mathematical models, offering new perspectives on the study of cell permeability. Finally, emerging three-dimensional models, including induced pluripotent stem cells, organ-on-a-chip systems, and cell spheroids, are presented, promising greater physiological relevance and improved predictability in permeability studies. This article provides a guide for researchers in selecting suitable methods for their studies, considering factors such as reproducibility, physiological relevance, cost, and cultivation time.

1. Introduction

Understanding cell permeability is pivotal in the development of effective oral medications, as it directly influences the absorption,

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bioavailability, and ultimately the therapeutic success of a drug. Oral administration remains the most favored route due to its convenience, patient compliance, and cost-effectiveness in large-scale production [1]. However, the journey of an active pharmaceutical ingredient (API) from ingestion to systemic circulation is fraught with complex physiological barriers and transport mechanisms that can significantly impact its bioavailability [2]. Bioavailability, defined as the fraction of an administered dose that reaches the systemic circulation in an unchanged form, is affected by numerous factors [3]. These include genetic variations, gastrointestinal pH, presence of food, enzymatic activity, mucus layers, and the expression of transporters and efflux proteins in the intestinal epithelium [4,5]. A thorough understanding and accurate prediction of drug absorption are essential for determining appropriate dosing, maximizing therapeutic efficacy, and minimizing adverse effects [6].

Drug absorption across the intestinal epithelium occurs through various mechanisms: passive transcellular diffusion, facilitated diffusion, paracellular transport, and active transport mediated by specific carrier proteins [7]. While passive diffusion is influenced by the physicochemical properties of the drug, such as lipophilicity and molecular size, active transport involves energy-dependent processes that can enhance absorption or contribute to drug efflux back into the intestinal lumen, as seen with P-glycoprotein [6, 8–10]. The intricate interplay of these mechanisms highlights the necessity for reliable *in vitro* models that accurately simulate human intestinal absorption.

Traditionally, *in vivo* animal studies have been employed to investigate drug absorption. However, ethical considerations, high costs, and interspecies variability limit their applicability [11]. The scientific community has increasingly embraced the principles of the 3Rs (Replacement, Reduction, and Refinement) to minimize animal use in research, spurring the development of alternative *in vitro* models that offer ethical, economic, and scientific advantages [12].

The Caco-2 cell line, derived from human colorectal adenocarcinoma cells, has long been a cornerstone in modeling the human intestinal epithelium due to its ability to differentiate into enterocyte-like cells expressing tight junctions and relevant transporters. Despite its widespread use, limitations such as lengthy culture times, absence of mucus production, and variable expression of transport proteins necessitate the exploration of improved or alternative models [13]. Recent advancements have led to innovative strategies aimed at overcoming these limitations. One such approach involves co-culturing Caco-2 cells with mucus-producing HT29-MTX cells to better replicate the intestinal environment. This co-culture model introduces a mucosal layer, enhancing the physiological relevance of the *in vitro* system [14]. Additionally, techniques like using nanofiber scaffolds or differentiation-inducing media have been employed to accelerate cell differentiation and monolayer formation, reducing culture times and improving assay throughput [15]. Alternative cell lines, such as Madin-Darby Canine Kidney (MDCK) cells, offer advantages in terms of rapid growth and the ability to express transport proteins. However, species differences and expression profiles must be carefully considered when interpreting results [13]. Non-cell-based assays like the Parallel Artificial Membrane Permeability Assay (PAMPA) provide high-throughput and cost-effective means to evaluate passive permeability but lack the capacity to model active transport and efflux mechanisms [16,17].

Emerging technologies are now paving the way for more physiologically relevant models. Induced pluripotent stem cells (iPSCs) offer the remarkable ability to differentiate into any cell type, providing a versatile platform for modeling human tissues, including the intestinal epithelium [18]. Organoids derived from iPSCs or adult stem cells can self-organize into three-dimensional structures that closely mimic the architecture and function of real organs. These organoids exhibit enhanced physiological relevance, capturing the complexity of cell-cell interactions and tissue-specific functions [9]. Organ-on-a-chip systems represent another cutting-edge advancement, integrating microfluidics and living cells to recreate the dynamic microenvironment of human organs [19,20]. These microphysiological systems can simulate mechanical forces, fluid flow, and biochemical gradients, offering unparalleled insight into organ-level functions and inter-organ interactions [21]. For instance, gut-on-a-chip models have been developed to mimic the peristaltic motions and microbial interactions of the human intestine, providing a more accurate platform for studying drug absorption and metabolism [22]. Cellular spheroids, three-dimensional aggregates of cells, also contribute to this new era of modeling by providing a more realistic representation of tissue architecture compared to traditional two-dimensional cultures [23,24]. Spheroids can replicate the gradient of nutrients, oxygen, and signaling molecules found in tissues, which is crucial for studying drug penetration and efficacy, especially in cancer research [25,26].

These innovative models promise to enhance our understanding of cellular permeability and the prediction of therapeutic compound absorption. By addressing the limitations of traditional models through more accurate simulations of human physiology, they facilitate the effective translation of laboratory findings into clinical applications. This article provides insight into the available techniques for studying cellular permeability across various models. By offering an integrative view of current methodologies, we aim to guide researchers in selecting and optimizing models that best suit their needs, advancing the understanding of permeability mechanisms and the development of new therapeutics.

2. Cell permeability analysis techniques

2.1. Caco-2

2.1.1. Basic characteristics

The Caco-2 cell line originates from human colon epithelial carcinoma cells that have, through a specific culture method, the ability to differentiate into a monolayer of juxtaposed cells with brush borders that have very similar properties to the enterocytes that form the small intestine [27], such as: membrane peptidases, important transporters on the membrane surface, such as insulin transporters, peptide transporters, vitamin D transporters, small intestine disaccharidases (lactase, dipeptidylpeptidase IV, sucrase-isomaltase, and aminopeptidase N), and efflux proteins [28]. These factors reinforce the idea that this cell model is an ideal substitute for simulating

the physiological environment, providing an excellent *in vitro* model for the analysis of cell permeability. The following Table 1 directly explains a compilation of the main characteristics of this cell model.

2.1.2. Caco-2 cell culture

Under standard culture conditions, Caco-2 cells reach cell confluence after a period of 7 days [54], the duration of the cell differentiation period may vary according to the conformation adopted for the test. In studies carried out by Hidalgo et al., 1989, a conformation was used where the inserts adopted for carrying out the experiments were coated with collagen, leading to a period of approximately 15–16 days to achieve differentiation, obtaining a morphology similar to that of the columnar epithelium of the intestine.

In more recent studies proposed by Ref. [29], It is described that the cell culture, after being seeded on the filter support, must be cared for for a period ranging from 21 to 29 days, and that on the 21st day of culture, the cells have differentiated and present many transport proteins and brush border hydrolases expressed. Due to these characteristics, this period ends up being adopted in many tests as the time necessary for the differentiation of the monolayer [55–60].

In studies done by Vachon et al. and Ding et al. [30,31], It was classified that the cellular morphology goes through three distinct stages, as shown in Fig. 1, the first stage being called homogeneously undifferentiated, characterized by the development of the brush border in the sub-confluence. The second stage, known as heterogeneously polarized, occurs in 5–20 days post-confluence, being marked by the increase in length and density of the microvilli, a reduction in the surface area occupied by the cells and the development of tight junctions between the cells, these being fully developed 21 days after confluence, this being an adequate period to start the permeability tests, as previously mentioned. The third stage, known as homogeneously polarized and differentiated, occurs 30 days after confluence, being marked by a typical morphology of enterocytes, with a minimal cell surface, with a completely formed brush border, with high, regular and fully formed microvilli.

2.1.3. Establishment of the culture on the transwell plate model

The process of differentiated cell monolayer formation commonly occurs on a Transwell filter support, which can be made of different types of materials, such as polycarbonate, polyester, or polyethylene terephthalate [27]. The membrane surface area and pore size of these filters vary according to the needs of the experiment to be performed. Transwell plates with 24 wells, with pores of approximately 0.4 μm, are widely used to analyze transport mechanisms, and plates with larger pore sizes and membrane surface area are used for other types of analysis, such as analyzing how compounds are metabolized, since larger pores facilitate the diffusion process and the supply of nutrients to the cells. This is of utmost importance for evaluating an experiment, since the compounds need to reach the cells in adequate quantities, to simulate conditions close to those of the human body [27,31].

2.1.4. Ways to assess monolayer integrity

One of the most important points to be taken into consideration during the permeability experiment, using the Caco-2 cell line, is the integrity of the cell membrane and its differentiation capacity [31]. As discussed previously, this monolayer takes approximately 21 days to become suitable for use, but this period may vary according to the polarity of the cells, that is, varying according to the communication between the cells results in a phenotype of coordinated polarity evident at the subcellular level [61]. Among some of the ways in which cell polarity can be assessed, the traditional method, and one of the most common, is based on measuring TEER [62]. By using a Millicell voltmeter, the integrity and permeability of the cell membrane can be assessed quickly and effectively, and is considered suitable for use when it presents a TEER value greater than or equal to 300 Ω [29,62].

In addition to the TEER assessment, other forms of analysis can be used, such as the use of scanning electron microscopy, used for the formation of apical microvilli, the epithelial junctions of the cells that make up the cell monolayer [31,63]. Second, another point that can serve as a predictor for the permeability of the monolayer formed is the transmembrane flow of exposed markers, such as: inulin, polyethylene glycols and radiolabeled mannitol, the latter being the most used, due to its small size, its high difficulty in being metabolized and its low capacity to permeate the membrane, being a good criterion for evaluating the integrity of the monolayer [64,

Table 1
Main characteristics of Caco-2 cells.

Parameter	Description	References
Origin	Human colorectal adenocarcinoma	[29]
Differentiation	After confluence, the culture takes a period of 21 days to reach differentiation.	[29–31]
Cell morphology	Polarized cells that have a brush border on the apical surface. In addition, they have tight junctions between the cells.	[32,33]
Membrane integrity	TEER (>300Ω)	[29,34]
Digestive enzymes	The digestive enzymes expressed by Caco-2 cells are: membrane peptidases and small intestine disaccharidases (lactase, aminopeptidase N, sucrase-isomaltase, and dipeptidylpeptidase IV).	[32, 35–37]
Active transport	Amino acids, sugars, vitamins, hormones.	[38–41]
Membrane ion transport	Na+/K + ATPase, H+/K + ATPase, Na+/H+ exchange, Na+/K+/Cl- cotransport, apical Cl- channels	[27, 42–45]
Non-ionic transporters	Permeability glycoproteins, (P-gp, multidrug resistance protein), multidrug resistance-associated protein (MRP), lung cancer-associated resistance protein (LRP).	[27, 46–48]
Receptors	Vitamin B12, D3 and EGFR (epidermal growth factor receptor).	[49,50]
Cytokine production	IL-6, 8, TNFα, TGF-β1, thymic stromal TSLP, IL-15	[51–53]

*High transepithelial electrical resistance: TEER, lymphopoietin: TSLP.

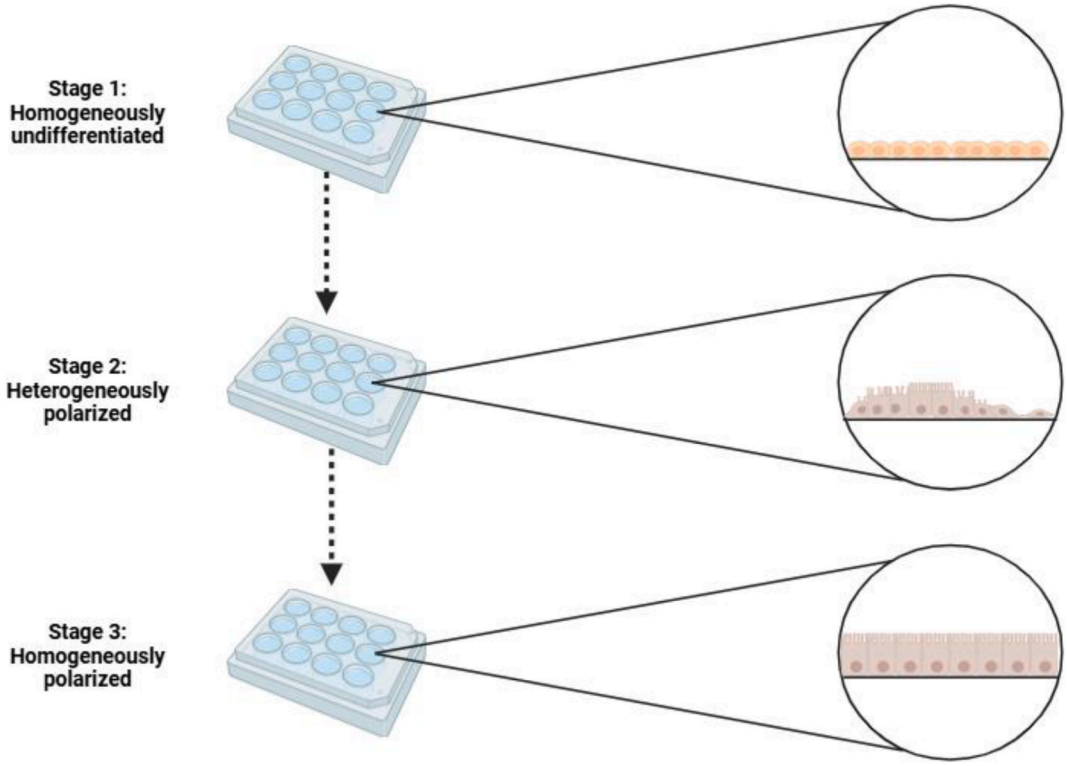

Fig. 1. Graphical representation of the 3 stages that cell morphology goes through (the representation shows the monolayer in a 12-well Transwell System).

65]. Another alternative way of assessing cell polarity is by evaluating the enzymatic activity of brush border marker enzymes, such as invertase, lactase, isomaltase and alkaline phosphatase, enzymes normally expressed by cells of the small intestine and which, when expressed by Caco-2 cells, reflect the degree of differentiation of these cells into cells similar to enterocytes, with this expression being proportional to their differentiation and growth [31,66].

2.1.5. Application of the Caco-2 cell line

The Caco-2 cell line, due to its high capacity to simulate the enterocytes present in the human small intestine, has several applications related to the interaction that the compound being analyzed may have when interacting with the intestinal tissue. Some of these applications are: intestinal metabolism, intestinal absorption [67], intestinal barrier [68], intestinal immunity [69], intestinal adhesion [31,70]. However, the most relevant application in this article is intestinal transport, providing an excellent comparison between the intestinal permeability observed *in vivo* and *in vitro* [31].

The absorption of a drug by the cell in an *in vivo* model is mediated by four basic mechanisms (Fig. 2): passive transcellular transport, a type of transport that accounts for the rapid movement of lipophilic compounds across the cell membrane; passive

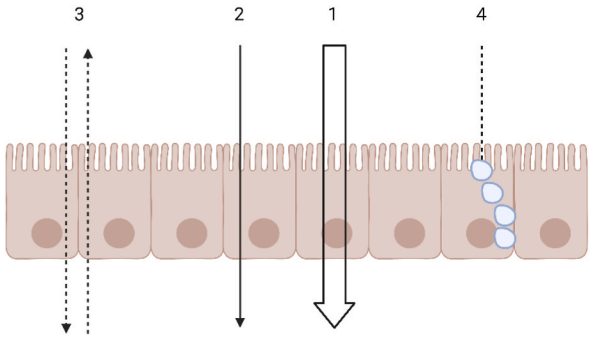

Fig. 2. Schematic drawing of an intestinal epithelium in the process of cellular transport. Passive transcellular pathway is presented as number 1; passive paracellular as number 2; active carrier-mediated transcellular pathways as number 3; and transcytosis routes as number 4.

paracellular transport, a type of transport that accounts for the movement of hydrophilic molecules through the tight junctions of the intestinal epithelium and provides slow absorption of these molecules; transcellular transport pathways mediated by active carriers, a form of compound movement that accounts for the similarity of the drug to the compound it is designed to transport, whether hydrophilic or lipophilic; and transport routes through transcytosis, a low-capacity transport route that is explored for the transport of extremely potent drugs [71].

In a study comparing the absorption of certain drugs in *in vivo* and *in vitro* models, it was observed that the transcellular transport route exhibits excellent permeability in the *in vitro* Caco-2 model, with an absorption discrepancy from the *in vivo* model of only 4 times, making it an excellent avenue for quantitative and qualitative analysis in studies [71,72]. On the other hand, drug absorption through the paracellular route shows a certain deficiency in the *in vitro* model, with an absorption rate approximately 80 to 100 times lower than the *in vivo* model, showing a qualitative nature correlation [54,71]. Regarding the comparison of carrier-mediated and transcellular absorption models, an excellent correlation index in absorption was observed between the study model using Caco-2 and the *in vivo* model, due to the expression of the same transporter proteins, extracellular receptors. However, it is worth noting that these absorption models are highly dependent on the cell lineage selected for Caco-2 cell culture, which can vary in the expression of these factors depending on the lineage [27,71].

2.1.6. Main disadvantages of the method

Even though it is one of the main models used for cell permeability analysis, the Caco-2 cell line has some disadvantages during its use. These include its extremely long cultivation time, a period of approximately 21 days after cell confluence for the cells to differentiate, the difference observed between the intestinal epithelium and the monolayer formed, since the monolayer does not have the diversity of cells that the intestinal epithelium has, which can be reflected in a striking way in the absorption observed in the monolayer [27]. Another difficulty found in the use of the cell line is that this cell model does not take into account the mucus layer that covers the small intestine to measure cell permeability, since this cell line alone is not able to produce this substance. This obstacle is solved through the co-culture of Caco-2 and HT29 cells, a topic discussed further below [27].

2.1.7. Ways to solve the disadvantages related to the use of caco-2 cells and increase the performance of the analysis process

As discussed throughout topic 2, Caco-2 cells are very useful for use in permeability tests, however, the main point that ends up making their use difficult, as previously mentioned, is due to the long cultivation time required for differentiation to be achieved and for them to be evaluated as suitable for use, therefore a reduction in the time required to perform the test would represent an increase in the yield of these tests.

One of the ways that could lead to an increase in income was described by Hu et al. [73], This study, aiming to reduce the time required for cell adhesion and differentiation, replaced the classic transwell model, which has an average culture time of 21 days, with a model based on electrospun nanofiber scaffolds made of polylactic acid, which simulate the natural extracellular matrix, which ultimately improves cell adhesion, proliferation and expression. This modified transwell model provided a reduction in culture time of approximately 5 times, with an integrated monolayer in a period of 4 days, which ended up presenting a TEER value of 422Ω.cm2. In addition to improving the monolayer development period, the change provided by the electrospun nanofiber scaffolds decreased the apparent permeability of phenol red, symbolizing that the model developed into an excellent barrier for small molecules, increased cell differentiation, enzyme and P-gp activity, in addition to improving the *in vitro* predictive potential. These improvements showed that the model made of nanofiber scaffolds can be a good substitute for the conventional transwell model.

Another way to increase assay throughput was described by Uchida et al. [74], In this study, the plate was assembled in a 96-well plate at a concentration of 6.5–10 5 cells/cm 2, and maintained in seeding medium for 48 h and subsequently exposed to a differentiation-inducing medium made of butyric acid for another 48 h. At the end of 4 days after the start of the assay, the cells showed adequate confluence for the cell permeability assay. When comparing the conventional form, a 40 % reduction in P-gp expression was observed, probably due to the short period of culture time, a relevant point for the expression of this protein. This change may benefit experiments in which overexpression of P-gp influences transport. An alternative form was described by Cai et al. [75], this essay showed certain differences when compared with Uchida et al. [74], however, they have the similarity of using a differentiation-inducing medium so that the cultivation time was reduced, in this case, a reduction from 21 days to 7, still presenting a moderate correlation with the *in vivo* and *in vitro* absorption parameters [76].

2.1.8. Application of the caco-2 cell line for the analysis of products of natural origin

Being a highly relevant lineage in clinical trials involving intestinal enterocytes, the Caco-2 cell line is used to analyze a wide range of products, including plant-based compounds that are explored for the treatment of some pathology, such as diabetes.

The review proposed by Schreck et al. [77], The aim of this study is to show the receptors present in intestinal cells, responsible for the absorption of fatty acids, fructose and glucose (also present in Caco-2 cells), and the natural products used to inhibit this process. In this review, more than 70 types of plants were analyzed, and the potential of their products was analyzed with the aim of inhibiting the glucose transporters present in these cells. In addition to the analysis of the inhibition of glucose transporters, this article also analyzed other plant products that aim to inhibit the transport of fructose and intestinal fatty acids, presenting a number of types of plants analyzed that is approximately 7 times smaller than those used for glucose transporters, in the case of fructose, and only the extract of two plants analyzed to inhibit the transport of fatty acids. This ends up showing that the cellular model is well used to analyze the inhibition of glucose and fructose transport, but presents a low exploration index for fatty acids.

2.2. Parallel Artificial Membrane Permeability Assay (PAMPA)

Although the cell lines were well-know for predicted the intestinal absorption, the cell-free systems, like PAMPA, has the vantage point of superior robustness of barriers, in addition to the possibility of adjusting Cell-free systems can be classified into two types, with the first type known as biomimetic barriers, which are formed from (phospho)lipids, and the second type known as non-biomimetic barriers, formed from dialysis membranes [78].

PAMPA was introduced in 1998 in a 96-well microtiter plate format filled with aqueous buffer solutions (pH 7.4/6.5) with a barrier consisting of a filter (e.g. polyvinylidene fluoride (PVDF)) soaked with phospholipids dissolved in an organic solvent [79], i.e. an artificial membrane, but made from ingredients found in biological membranes [80]. Originally, the first 48 wells (sample) were impregnated with a solution of 1–20 % lecithin in an organic solvent (dodecane, hexadecane, 1,9-decadiene). The remaining 48 wells were moistened with a small volume of a 50 % methanol/buffer solution (v/v). Transport studies are initiated by transferring the stock solution on top of the filter plate into the sample and reference sections, respectively. In general, 0.05 M TRIS, pH 7.4, or 0.05 M phosphate, pH 6.5 buffers were used. The maximum DMSO content of the stock solutions was 5 % [79]. The definition of PAMPA was introduced in 2003, presenting a new method for permeability analysis. This method consists of several parallel wells, each containing a lipophilic permeation barrier, which is confined to a flat area by a fine microfilter, separating two compartments of aqueous nature [81]. In 2004, Bermejo et al. studied the correlation between PAMPA and biological cell models, with good results [82].

This very simple system had a lot of limitations, Due to the potential of this system to retain lipophilic compounds in the membrane, and its inability to simulate paracellular (isn't ideal for small hydrophilic compounds) and active transport, which are essential mechanisms for the absorption process [83], so, a few years after PAMPA introduction, Seo et al. [84] found that varying the membrane lipids composition can affect the permeability of some compounds, and the variations of this model changing (i) the nature of the filter support, (ii) the composition of membrane constituents, (iii) the pH in the donor/acceptor, and (iv) the presence of a "sink" in the acceptor compartment were published to increase the *in vitro–in vivo* correlation [85].

The possibility of change many parameters allows to evaluate different drugs, e.g. the poorly water soluble [80], in addition of different targets like the blood-brain barriers [80], skin [86], buccal [87] and corneal [88], and it allows to develop new methods for analysis of permeability with machine learning algorithms, like the *in silico* quantitative model Artificial Neural Networks (ANN) based on permeability [89]. In addition, PAMPA can be used in different pH, being ideal for pH gradient experiments [90]. The use for natural products was already validated and characterized for the blood-brain barrier with a porcine brain lipid extract providing high phytochemical selectivity [91]. To predict passive intestinal absorption, crude plant extracts were evaluated using a hexadecane membrane. It was observed that the presence of multiple components did not affect the passive permeability of each component individually. However, it was necessary to load a higher amount of the extract [92].

The barrier is directly exposed to the donor environment, that sometimes can be composed by aggressive surfactants, so, this technique is incompatible with some excipients. The integrity of the barrier can be checked by measurement of the TEER, fluorescent or low permeability compounds [78]. Fig. 3 shows a graphical model of the lipid layer belonging to the PAMPA model.

2.3. Madine Darby Canine Kidney (MDCK)

The evaluation of intestinal permeability is one of the most important characteristics in drug discovery and development because this absorption is the main route to analysis the oral bioavailability of drugs [93]. But this absorption is dependent of the intestinal epithelium, which is comprised of absorptive and other functional cells, with physical and biochemicals barriers [94]. MDCK is an epithelial cell line originally derived from the kidney of a normal male cocker spaniel in 1958 and a convenient model for studying transepithelial transport [95]. This assay is based on the transport of compounds by the human P-glycoprotein, also known as multidrug resistance protein 1 (MDR1), so, it cells were submitted to cloning aiming the over-expression of P-gp (MDCK-II-MDR1) [96]. However, a substrate for canine P-gp is not necessarily a substrate for human P-gp and vice versa [97].

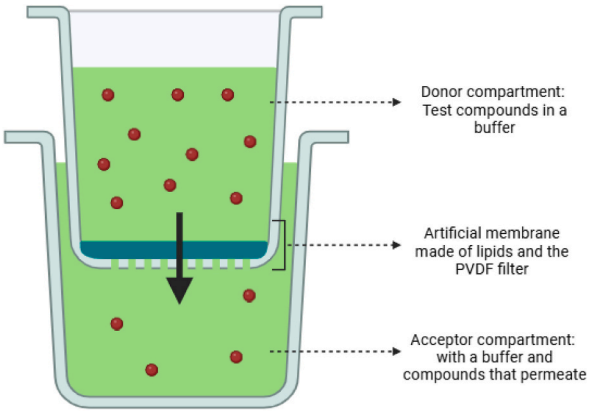

Fig. 3. Graphical representation of PAMPA, showing the donor and receptor compartments, the artificial membrane and the filter made of PVDF.

Like in Caco-2, lipophilic compounds are difficult to analyze because they need high amounts of organic solvents and the cells are not able to tolerate. Moreover, toxic compounds also can affect the cell monolayer and results in non-correct measurements [98]. Due to the fact that MDCK cells have a shorter culture time, leakier tight junctions, and reduced transporter expression compared to Caco-2 cells, they are more suitable for assessing passive permeability, rather than being used to study influx and efflux transporters [99,100]. Thus representing the second major cell model widely used to predict human absorption [101].

There are two different strains of this cell line, being the main difference their junctions, "tigh" with high TEER values for I and "leaky" with low TEER values for II Richardson et al. [102]. These strains were isolated in different times of passage, for that, the older strain (type II) began to express a new protein that may lower the TEER [103]. Besides these differences, Dukes et al. mentions others and explain that the MDCK II is most used unless that the researchers have a specific reason to use the first, reinforcing that the ECACC (European Collection of Cell Cultures) states that MDCK I may display an unstable epithelial phenotype [104].

Among the difficulties of the method is differentiating the paracellular and transcellular transport mechanisms [95] and the canine efflux transporters that may hinder passive permeability measurement due their nonhuman origin and renal origins [105], In addition to the information present in commercially available databases, information is often gathered from humans or rats [104]. The kidney is a complex organ affected by hormones and it's the study of hormonal regulation of kidney functions is made possible through the use of this cell line [106]. Despite these limitations, the method with MDCKII-MDR1 was validated with 50 and 20 different compounds, by Irvine et al. [107] and later by Thiel-Demby [108], and was defined that "A test drug will be classified as highly permeable if the passive permeability equals (±20 %) or exceeds that of labetalol, the reference drug defining the low/high permeability classification boundary".

To validate the integrity of the cells, needs to consider the type of cells that is being used. The culture is maintained in Minimal Essential Medium with 10 % Fetal Bovine Serum (FBS) and 2 mM fresh L-glutamine. Cells are cultured at 37 °C in an environment with 5 % CO_2 and 95 % relative humidity. Cells are expanded when they reach 80–90 % confluence, every 3–4 days, using Trypsin-EDTA solution (Gibco #25300-047). MDCK monolayers are cultured for 3 days before use, very fast when compared to Caco-2 that are cultured for 21–25 days before use [107]. The assay is able to determine whether a drug's permeability change in pH gradients and if it is a P-gp substrate. To validate the method is important to consider the biopharmaceutical classification system of drug permeability [109].

2.4. *Everted rat intestinal sack*

Considering the differences between *in vitro* and *in vivo* tests, the intestinal rat sac model was developed in 1958 to improve the analysis of intestinal permeability using a real tissue *ex vivo* [110]. In 1998 Barthe et al. [111] demonstrated an improved method using the intestinal rat sac, but everted and incubated in warmed tissue culture medium (TC 199) with constant oxygenation for maintenance of structural integrity and cell viability. The experiment is prepared by everting a freshly excised rat proximal small intestine over a glass stirring rod and dividing it into sacs approximately 2.5 cm in length using braided suture silk. After finishing the incubation time, the sacs are cut open and the serosal fluid drained into small tubes. Each tube is weighed before and after the collection of the serosal fluid to accurately calculate the volume of medium collected from inside the sac [112].

To validate the integrity of the rat gut sacs, glucose can be used and quantified in the incubation medium and in the gut sac contents using glucose oxidase – peroxidase (GOD-POD) kit-based assay [113]. The studies also can be conducted using non-everted rat gut sacs with comparable results. In the first case, is possible to reduce the volume of sample utilized, but the higher production of mucus needs to take into account [114]. This assay can be used both the passive and active transport, and allows to use small volumes of the sample [115], but besides was considered quick, inexpensive and useful at that time [116], today we know that, besides simple and efficient, this technique has the disadvantage of ethical commits because the use of animals, in addition the lack of validity of single experiment [117]. Considering that there isn't a standard technique to analysis and there are a lot of other techniques that can't to be correlated, this isn't the first choice. However, can offer many advantages for human investigation because of the similarity between the digestive system of them [118].

2.5. *Caco-2/HT29 Co-culture model*

Cellular models are an efficient and diverse method for studying drug absorption mechanisms at the cellular and molecular levels. In addition, due to their advantages in throughput and reproducibility compared to traditional systems, and also because they mimic *in vivo* physiology with good cost-benefit and without the use of animals, cellular models have proven to be a useful tool in drug discovery and development and have gained increasing interest in the field of scientific research [119].

One of the cell lines studied is the co-culture of Caco-2 and HT29-MTX cells (mucus-secreting goblet cells), which is a human colon adenocarcinoma cell line used to study the biology of human colon cancers and food digestion and bioavailability due to the ability to express characteristics of mature intestinal cells, such as enterocytes or mucus producing cells [120]. Even though this model presents certain difficulties regarding co-culture, this cell type closely mimics intestinal cells, creating a drug absorption model that integrates a mucus barrier into the absorption process [121,122]. The integration of the Caco-2 and HT29-MTX cell lines aims to address the issues encountered when the permeability analysis monolayer is composed solely of Caco-2 cells, such as the absence of a mucus layer covering the intestinal wall [76]. HT29-MTX cells, cloned from the human intestinal cell line HT29 (epithelial cells derived from colorectal adenocarcinoma), spontaneously produce, with the aid of methotrexate, a mucus gel that covers the entire cell surface and, thus, Aligns the results obtained with what is expected under physiological conditions [122,123].

Permeability studies are performed by seeding a proportionally specific mixture of Caco-2 and HT29-MTX cells into Transwell filter

inserts. Subsequently, the co-cultures are maintained under the same conditions used for Caco-2 cell cultures only. After about 20 days in culture, the TEER is measured to estimate cell integrity [121,124].

The two cell lines are easily available and easy to cultivate. The most critical disadvantage of this method is leakage, as Caco-2 and HT29 co-culture models exhibit a significant decrease in TEER with increasing proportions of HT29 cells. In addition, some of the transporters found in normal human intestinal epithelium are not expressed by the two cell lines, and in transport studies the expression of the necessary molecules should be checked. The ratio between Caco-2 and HT29-MTX will be crucial for the formation of homogeneous mucin layer, which is very important for the relevance to the *in vivo* situation [120]. As for the permeability of compounds absorbed by passive transport, it is observed that there is greater permeability in co-culture models (HT29-MTX) compared to the Caco-2 cell monolayer model [122,125].

The co-culture of Caco-2/HT-29MTX is a useful model for the investigation of transport over intestinal epithelium [126] and for bacterial adhesion and invasion [127]. For this, the mucin layer and the permeability of the cell layer is crucial and co-culture will give results that are more similar with the *in vivo* situation than monocultures (Fig. 4) [120]. Despite their wide use, the Caco-2 and HT-29 cell lines are not considered the best *in vitro* model for evaluating colonization capacity and host interaction mechanisms, as they exhibit a mixed phenotype of large and small intestine [119]. In turn, epithelial cells develop an apical and basolateral membrane with distinct structural and functional characteristics. Because cancer cells have a different sugar composition on their cell surface compared to normal cells, it is observed that the results regarding the ability of probiotic bacteria to bind to epithelial cells cultured as monolayers on plastic surfaces do not provide relevant data on adhesion capacity *in vivo* [128,129]. For example, in the monolayer formed by Caco-2 cells, where the cells are cultured on plastic surfaces, it was observed that some bacterial strains, which have good cellular adhesion capacity in healthy intestinal cells, did not show good adhesion in the Caco-2 cell model [130]. This significant difference was also found in the adhesion assay conducted in the unidimensional monolayer and in the functional IPEC-J2 cell model [119,131].

2.6. Pig cell-based methods

In addition to gastrointestinal absorption, renal absorption has also become a very interesting alternative for maintaining the physiological functions of normal cells, also called nucleobases, which are in the process of expansion due to their high therapeutic potential. Pig renal epithelial cells (LLC-PK1) are an example, that consists of an immortalized cell line derived from porcine renal tubular epithelium that expresses organic cation transportes [132]. This cell line has been used in metabolism, transport, toxicity, drug interaction studies and permeability assays. In this type of cell, a Na + -dependent nucleobase transport system, more specifically uracil, was identified, unlike Caco-2 cells, which are independent of this ion. Furthermore, LLC-PK1 cells have the ability to produce plasminogen activator through arecoline, an alkaloid that induces cytotoxicity in many types of cells. Plasminogen activator is a substance that stimulates fibrinolysis and makes LLC-PK1 cells very useful in the investigation of thrombosis cases [133]. Finally, these cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and your similarity with proximal tubular cells *in vivo*, like apical membrane microvilli, high activities of apical membrane enzymes and expression of parathyroid hormone receptors and sodium-dependent glucose transportes, makes LLC-PK1 cells a promising strategy in renal toxicology studies [134,135].

Another well-studied cell type is PSI-1, which are non-fully differentiated mature pig small intestinal epithelial cells [136]. The pig model is closer to humans in terms of genome, organ development, anatomy, physiology and metabolism of the intestinal tract, disease progression and gut-microbe interactions. In addition, PSI-1 cells aren't of tumor origin, so they are a better *in vitro* model for evaluating the epitelial barrier of small intestine and studying mechanisms of pathogen-host interactions, immunological studies and probiotic research than human tumor cell lines [136]. The PSI line is capable of forming a tightly packed epithelial barrier when

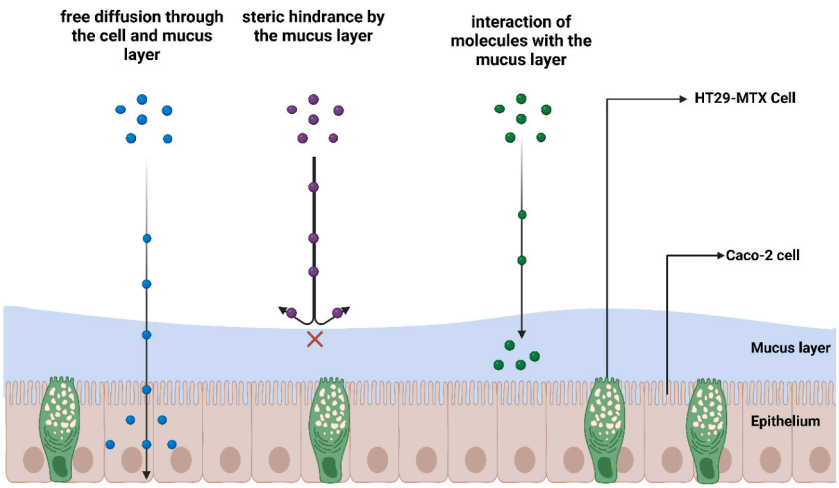

Fig. 4. Graphical representation showing the functioning of the co-culture, the mucus layer and its interaction with the compounds under analysis.

cultured in microporous inserts in the presence or absence of collagen. In addition, the high TEER of PSI clones indicate a highly differentiated epithelial character with high transmembrane transport activity [119,131].

IPEC-J2 line is another type of porcine cells, in this case are porcine intestinal enterocytes isolated from the jejunum of a non-breastfed neonatal piglet. This line is unique because it's derived from the small intestine and is neither transformed nor tumorigenic in nature. These cells mimic human physiology more closely than any other cell line of non-human origin [119]. Therefore, it is an ideal tool to study epithelial transport, interactions with enteric bacteria, effects of probiotic microorganisms and the effect of nutrients and other foods on a variety used parameters, like TEER, permeability and metabolic activity reflecting epithelial functionality [119]. Furthermore, IPEC-J2 cells produce mucins (Muc1 and Muc3), that has been confirmed by RT-PCR and ELISA, respectively [137] and secrete cytokines such as GM-CSF and TNF-α that can establish communication between enterocytes and the immune system [138].

The correct choice of sérum added to the culture medium os this cell is very importante, since the fetal bovine serum causes high resistance and can be beneficial to use when compounds that have a negative effect on monolayer permeability or tight junction structures are investigation. In turn, the porcine serum gives rise to low resistance and normal rates of active transport by increases mucus production [139]. In addition, IPEC-J2 cell monolayers are ready for experimentation after 1 or 2 weeks, faster than compared to the 21-day culture time of Caco-2 cells. A drug transport permeability study showed that IPEC-J2 cells form a more compact monolayer compared to Caco-2 cells. Finally, the maintenance of differentiated characteristics and strong similarities to primary intestinal epithelial cells allow IPEC-J2 cells to be compared to the experimental animal that is used as an *in vivo* model for humans before *in vivo* evaluation [120,140].

3D intestinal epithelial models were constructed in the laboratory from non-carcinogenic lines, which grow on a microporous membrane and allow for cell polarization. On the basolateral side, below the microporous membrane, the epithelial cells are supported by immune system cells, such as dendritic cells and macrophages, which mimic the mucosal lymphoid tissue. In turn, on the apical side of the membrane, the intestinal microbiota is present so that its effects can be studied. Thus, in addition to allowing the cells to polarize with different apical and basolateral parts, the three components mentioned above (epithelia, immune cells and microbiota) are the most important factors in the intestine and that make these models closer to the *in vivo* situation [131].

3. Comparison of different technical of permeability with technical based of Caco-2 cell line

3.1. Comparison of Caco-2 and PAMPA

It is observed that in recent decades, the profile of drugs produced by the pharmaceutical industry has gained an increase in lipophilicity, which has resulted in a need for permeability analysis methods that can measure the permeation capacity of these molecules through passive transcellular transport [141,142]. As observed throughout the article, cell-based analysis techniques are characterized as great protagonists of passive cellular permeability studies, due to the ability to generate reproducible and biorelevant results on a high-throughput basis, however due to certain deficiencies that these methods present other methodologies end up proving necessary for these analyzes, such as cell-free methods, an excellent form of analysis for lipophilic compounds [32,143], the following Table 2 shows the main positive and negative points about cell-based and synthetic membrane models, with some of these points being better worked on in the course of this topic.

As an example of cell-free models, the PAMPA assay stands out as a major player in this scenario, being widely used in studies due to its correlation of results (*in vivo* and *in vitro*) and as a basis for the development of new analysis techniques for cell-free permeability.

A parallel can be drawn between the intestinal permeability analysis model, Caco-2 cells, and PAMPA in order to analyze the feasibility of each method, analyzing parameters of correlation of permeability data obtained, as observed in the studies Kansy et al. [149] which showed a high correlation of permeability data between the Caco-2 and PAMPA models when lipophilic products that use passive transcellular permeation as a transport form were used, both providing a good correlation rate when compared their results with apparent permeability data obtained in *in vivo* assays in rats [150]. In addition, it is observed that for hydrophilic products, the artificial membrane-based model does not appear to be a good form of analysis, due to the inability of a polar, hydrophilic compound

Table 2
Main advantages and disadvantages of cellular models and cell-free models.

Model Type	Advantages	Disadvantages	References
Cellular Model	- Capacity to simulate the environment observed in the small intestine. -Possibility of performing active and passive transport (efficiency depends on cell line used). -Human cell lines are particularly suitable for use.	-Long time required to conduct tests. -Risk of contamination, which complicates the experiment. -High cost to implement. -High variability in results depending on cell differentiation and culture conditions.	[141,144]
Synthetic Model	-Low cost. -Quick use method. -Robust for analyzing compounds with lipophilic characteristics. -Ability to replicate passive transport.	-Absence of enzymes. -Oversimplification of the system. -No membrane transport proteins. -Low compatibility of results based on paracellular transport. -Absence of gastrointestinal tissue morphology reproduction.	[93, 144–148]

to permeate a lipid-based membrane. In addition, PAMPA ends up suffering from the ability to retain lipophilic products in its membrane, potentially acting as a sink for the compounds, depending on the compound's affinity for the membrane [141,149]. Aiming to overcome this deficiency, the artificial membrane model was developed and improved in other models such as Double Sink PAMPA, a method that provides a greater correlation with human data [151].

It is worth noting that for both the cell-free artificial model and the Caco-2 cell line-based model, there are certain factors that can symbolize a barrier in the analysis of permeability results, such as the stirring condition of the water layer, a condition extremely present in living organisms, due to intestinal motility, and which influences the absorption coefficient of the compounds, since the absorption of the compounds when subjected to stirring at 300 rpm was greater than the absorption of the models without stirring and that for values greater than 300 rpm no variation in the absorption coefficient was observed [152].

Another factor to be highlighted is sinking, a parameter that analyzes the adsorption of the analyzed compounds to the bottom of the plate and aims to prevent the backflow of permeated drugs, requiring certain substances such as surfactants. is important to note that parameters such as pH, excipients used in the preparation of the compounds, composition and compatibility of biomimetic media, media that simulate the conditions of the intestinal fluid at different times of the day, should be taken into account during the assay [141,149]. Regarding the preparation time, a clear superiority of the PAMPA model is observed, since the measurements of this model can be made in a total period of approximately 4 h for a cost that ranges from 50 to 100 times less, allowing a high range of analyses in a single day, unlike the benchmark used for permeability analysis, Caco-2, which requires a period of approximately 21 days [153].

In summary, it is found that both methods have a good capacity to provide permeability data, with the artificial membrane model being a faster and cheaper method to perform, being an excellent method of permeability analysis, potentially being a technique that, depending on the lipophilic characteristics of the analyzed drug, financial conditions of the analyst, available time and number of samples to be performed, can replace the technique based on the Caco-2 cell model, due to its equivalent values both with the results provided for comparisons with *in vivo* and *in vitro* models, through comparisons with Caco-2-based models, or act as a tool that strengthens the results obtained by the model in cell analysis models.

3.2. Caco-2/MDCK cell line

As mentioned earlier, among the dosage forms currently used, oral administration is characterized as one of the most widely used and developed within the pharmaceutical industry, requiring techniques that enable the measurement of the permeability capacity that drugs have when in contact with a tissue similar to the epithelial tissue of the human small intestine, in order for this to be carried out in the best way.

Among the techniques used to perform these analyses, *in vitro* cell models gain great prominence within drug development, due to their predictive capacity, lower cost, and shorter analysis time. The cell models used for analysis, the techniques based on cell culture of the Caco-2 and MDCK lines, gain great prominence, being strong candidates for the screening of new drugs, presenting small differences between them that can significantly reflect during the tests [154].

The cell model based on Caco-2 cells, as presented in item 2.1, has been frequently used as an *in vitro* model to assess intestinal permeability, having due to its cell structure being highly similar to that of intestinal enterocytes, permeability results similar to those observed in *in vivo* absorption [155]. However, due to its gradual growth and varied expression of transporters, this model ends up having a low yield in terms of the volume of analyses. The cell permeability assay based on MDCK cells emerges as a viable alternative to the Caco-2 model, considering that they grow and differentiate extremely rapidly compared to Caco-2 cells, taking a period of approximately 3–4 days to be ready for use, symbolizing a considerable reduction in the time required to perform the experiments [156].

Another factor to be considered during the choice of these two cell permeability analysis methods is the presence and role played by P-glycoprotein. This membrane protein, commonly located in the apical region of cells present in the gastrointestinal mucosa, liver, pancreas, brain, and kidney [157,158], has a function associated with multiple drug resistance, acting as a detoxifying secretory system, preventing toxic xenobiotics and toxic metabolites from remaining within cells, performing the transport of these substances to the lumen of the gastrointestinal tract, for example [157,158].

It was observed that the Caco-2 cell line is capable of expressing P-gp on its apical surface, but with some peculiarities, since these cells, in addition to having a long period for P-glycoproteins to be functionally expressed, 17 to 27 days, have variable levels of transporter expression, with this variation depending on several factors, such as the number of passages and treatment of the transwell supports [154,158]. On the other hand, the MDCK cell line, when transfected with the human MDR1 gene, has the ability to highly express P-gp within a conventional culture period of the common MDCK line. During assays performed by Tang et al. [158], the transport mediated by this protein in both cell models was evaluated using [3H]-digoxin as a substrate. In these assays, it was observed that the MDCK-MDR1 cells showed a higher efflux than the Caco-2 cells, with the reasons that may lead to this type of variation in results being the nature of the cell lines, being one canine and the other human, the tumorigenic nature of the lines, one originating from an adenocarcinoma and the other from a renal cancer, these factors being able to induce different lipid patterns in each line, which ends up influencing the functioning of P-glycoprotein, a protein that has its function closely linked to its membrane environment.

Another factor of great relevance to be evaluated during the comparison between the Caco-2 and MDCK cell lines is characterized by the number of passages experienced by the cultured cells and the type of coating of the support used during the experiments, using the presence and absence of type 1 collagen coating as a basis [154]. When assays using collagen-coated supports are performed, it is observed that the Caco-2 cells reach confluence faster and at a higher density than when compared to the model using the uncoated support. In addition, it is found that the permeability values using type 1 collagen-coated supports are higher in the early passages but

tend to decrease and stabilize at higher passage numbers, whereas using uncoated supports, there is a tendency for the cells to develop at a slower rate and present higher permeability values when the cells have gone through many passages [32,154]. When the MDCK cell line is used, it is observed that, unlike the Caco-2 cells, for both types of coatings, the variation between the types of coatings presents a permeability coefficient of variation between higher passage numbers of approximately 7 % [154].

3.3. Everted rat intestinal sack/Caco-2

Intestinal absorption is a critical factor that affects the bioavailability of oral drugs, directly reflecting a drug's potential to permeate the intestinal epithelial barrier [159]. To evaluate this permeability, various experimental models have been developed, among which the everted rat intestinal sac model and Caco-2 cells stand out.

The everted rat intestinal sac model is an *in vitro* method that allows the study of drug transfer and metabolism in the intestine (Fig. 5). First introduced by Wilson et al. [160], this model involves everting a prepared segment of rat small intestine over a glass rod and filling it with a test drug solution. Absorption is assessed based on the difference in the mass of the intestine before and after draining the internal fluid. This method is widely used due to its ability to simulate the intestinal environment and provide valuable information on drug absorption and permeability [161,162].

On the other hand, Caco-2 cells are a human colon adenocarcinoma cell line that, when cultured to confluence, differentiates and forms a polarized monolayer mimicking the characteristics of human intestinal epithelium. This cell model is extensively used to study transepithelial permeability and drug interactions with intestinal cells. It also allows for the measurement of the apparent permeability coefficients of drugs without interference from hepatic metabolism [159,163]. Both models offer significant advantages in studying intestinal permeability. The everted rat intestinal sac provides an environment closer to physiological conditions, including factors like blood flow and the presence of metabolic enzymes [159,164]. However, it requires the use of animals and may present variability between samples. Caco-2 cells, while a simplified model, offer reproducibility and ease of manipulation, making them ideal for screening studies in the early stages of pharmaceutical development [164].

It is important to note that while both models are valuable, they have limitations that must be considered. In the case of the everted intestinal sac, careful monitoring of tissue viability and integrity during the experiment is essential to obtain reliable results. With Caco-2 cells, it is crucial to recognize that, being an *in vitro* model, it does not fully replicate the complexity of the *in vivo* intestine [165, 166].

3.4. Caco-2/co-culture of caco-2-HT29 cells

It is understood that the vast majority of molecules used for therapeutic purposes are administered orally. To ensure systemic availability, The drug needs to penetrate the intestinal epithelium and enter the bloodstream. For this purpose, intestinal barrier cell models provide a timely alternative to identify the uptake of active molecules. Additionally, this model offers the opportunity to evaluate the impacts that excipients have on cellular reactions [167]. Nevertheless, for a selection instrument to be considered

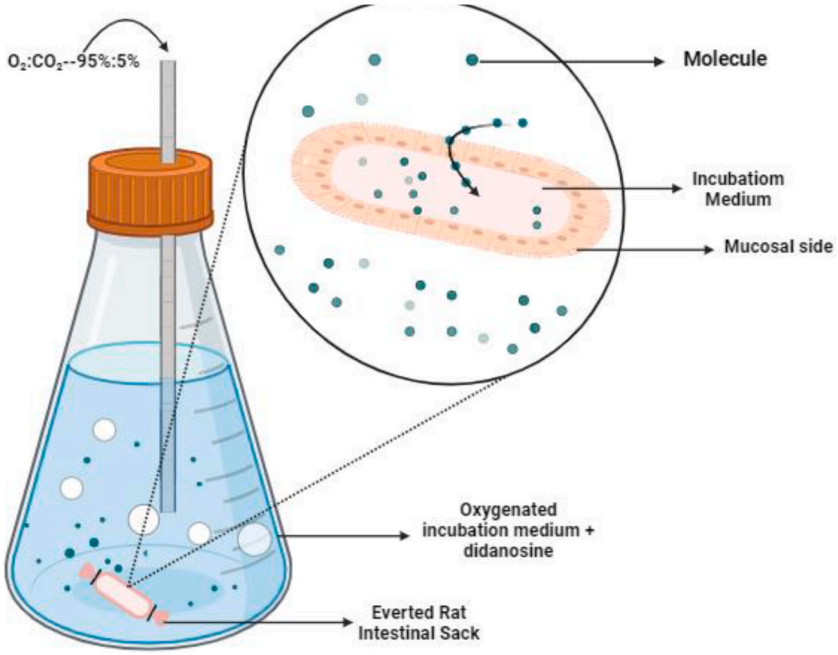

Fig. 5. Graphical representation of the cell permeability analysis model based on the everted rat intestinal sac technique.

suitable, the *in vitro* model must present simple, rapid, and reproducible characteristics, and have the ability to accurately simulate the intestinal epithelium. Over the last 20 years, monolayers of cell lines from human colon adenocarcinoma, the so-called Caco-2 cells, have been widely used to mediate the permeability coefficient of drugs. However, this model ends up presenting excessive limitations, such as the TEER, which is elevated in Caco-2 models when compared to the human intestine, due to the high presence of tight junctions [110].

Several studies highlighted the difficulty of relying solely on the seeding ratio to control permeability in co-culture models. For instance, Hilgendorf et al. [125] demonstrated that Caco-2/HT29-MTX co-culture models exhibited permeability characteristics similar to those of a Caco-2 monolayer, regardless of the initial seeding cell concentration. To replicate the permeability properties of the human intestinal barrier more accurately, they noted that it was necessary to adjust the proportion of goblet cells in the co-culture. This adjustment required the optimization of culture conditions, including modifications to the culture medium composition, culture duration, or the Caco-2/HT29-MTX ratio. Another important limitation they identified was the absence of mucus on the luminal surface of Caco-2 models, which restricted their use in studies investigating the interaction of the absorption barrier with drugs or drug delivery systems designed to improve drug solubility. These limitations, as they explained, were due to the fact that Caco-2 cells represented only one cell type, whereas the intestinal barrier is composed of various cell types, including enterocytes, Paneth cells, goblet cells, stem cells, and endocrine cells. Consequently, they highlighted that the widely used Caco-2 model had significant limitations for absorption studies, emphasizing the need for further improvements. To address these challenges, several mucus-producing goblet cell lines were proposed as alternatives to better mimic the characteristics of the human intestine. Among them, they indicated that the HT29 cell line had proven to be a viable option for advancing drug permeation studies.

Caco-2 and HT29 co-culture models began to be used in combined studies. To closely resemble the characteristics of the intestine, the proportion of HT29 cells in the co-culture needs to be adjusted, which requires optimization of the conditions under which the co-culture will be performed, such as the percentage of Caco-2/HT29 cells, the composition of the culture medium, and the culture time to be adopted. However, studies have emphasized the complexity of using the seeding ratio alone to modulate the permeability obtained through co-culture [167]. Evidence indicates that Caco-2/HT29 co-cultures exhibit similarities to the monolayer formed by Caco-2 cells, with this similarity being independent of the seeding ratio. This fact is justified by the rapid development of Caco-2 cells in culture medium supplemented with 16.5 % fetal bovine serum. Therefore, for the growth of the HT29 cell, the medium to be adopted had to be supplemented with only 10 % fetal bovine serum. It is assumed that the levels of permeability achieved through this co-culture vary and depend on the properties obtained through the drugs. In an analogous case, compounds that are transported by passive paracellular mechanisms permeate the HT29 cell more effectively when compared to Caco-2 cells. In contrast, compounds that demonstrate a lipophilic aspect have similar permeability in both cells [125,168].

Furthermore, some intestinal barrier cell models an ideal *in vitro* model should be practical, quick, consistent, and accurately

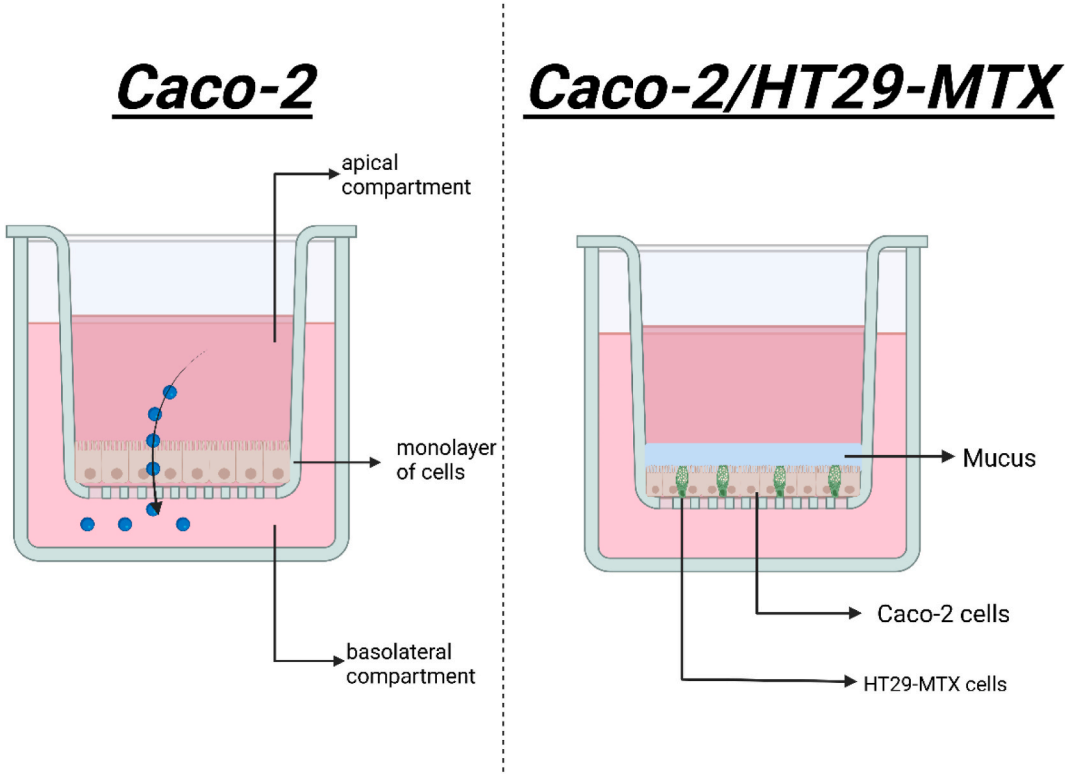

Fig. 6. A comparative with Caco-2 monolayer and co-culture of Caco-2 and HT29-MTX cells.

replicate the intestinal epithelium in order to be effective in screening the absorption of active substances and analyzing the effects of excipients. The monolayers of human colon carcinoma cell lines, the so-called Caco-2, have been widely used over the past twenty years to estimate the apparent permeability coefficient of drugs, Caco-2 cell monolayers present several limitations, including TEER, which is significantly higher in these models, compared to the human intestine due to the overexpression of tight junctions between cells [169]. In Caco-2 models, drug efflux mediated by P-glycoprotein is often estimated to be exaggerated. Additionally, the absence of a mucus layer on the luminal surface of Caco-2 limits the model's relevance for analyzing the interaction of the absorption barrier with formulations or drug delivery systems aimed at increasing the solubility of poorly water-soluble drugs, such as those containing surfactants and lipids [170].

The limitations observed can be attributed to the fact that the Caco-2 model uses only one type of cell, whereas the intestinal barrier is made up of a variety of cells, including enterocytes, goblet cells, Paneth cells, endocrine cells, and stem cells [171]. Co-culture models, which integrate HT29-MTX and HT29-H cell lines with Caco-2, become significant since mucus-producing goblet cells are the second most common type of cell in the intestinal epithelium. Fig. 6 shows a comparison between the monolayer formed only by Caco-2 cells and the co-culture of Caco-2 and HT29-MTX cells:

As noted, the classic permeability analysis techniques have their own peculiarities, presenting both differences and similarities between them, resulting in permeability values that may or may not be similar. Below, a selection of drugs from some therapeutic classes, such as antihypertensives, antibiotics, antiarrhythmics, anti-inflammatories and caffeine for the analysis techniques discussed, will be presented in Table 3.

When analyzing the Papp values presented by the compounds for the different permeability analysis techniques, it was possible to observe that they present a certain similarity between them, showing in most cases, for the same compound, values that do not differ much from the others. This is due to the analysis presented by Ref. [172], by using common transport mechanisms for the techniques presented, such as passive paracellular transport, something common for the techniques shown and which allows for similar results when the compounds use this transport route, however when the compound under analysis presents a predilection for other forms of transport, a striking difference can be observed, such as the case of metoprolol which presents permeability values greater than 20 x 10^{-6} cm/s for the other techniques, while for pampa it presents a value close to zero.

Finally, we conclude this session by stating that the techniques can present similar results and can be interchanged depending on the practitioner's needs and the characteristics presented by the compounds under analysis.

4. New three-dimensional models for drug permeability testing

4.1. Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are a revolutionary type of cell derived from somatic cells that, after a process of genetic reprogramming, acquire the ability to differentiate into any cell type in the body [196], including the three germ layers: ectoderm, mesoderm, and endoderm [197]. Generated from somatic cells using factors such as OCT4, SOX2, KLF4, and c-MYC, iPSCs possess self-renewal and pluripotency properties similar to embryonic stem cells but without the ethical dilemmas associated with the latter [198]. A key advantage of iPSCs is that many types of cells derived from iPSCs are now readily available from commercial sources, which come with a basic level of characterization and quality control that aids in standardization [199]. Additionally, from iPSCs, we can generate human organoids to model permeability under basal conditions and further analyze permeability in response to different stimuli [200]. These characteristics have allowed their application in disease modeling, toxicity testing, drug discovery, and

Table 3
Apparent permeability (Papp) measurements using *in vitro* and *ex vivo* analysis techniques for different compounds. The Papp values (x10^{-6} cm/s) presented correspond to transport in the apical to basolateral direction (A-B).

	Caco-2	PAMPA	MDR-MDCK	Caco-2/ht-29	Everted Rat Intestinal Sack	Class of compounds
Metoprolol	23,7 [172]	~0,2 [173]	67,2 [174]	22,37 ± 0,64 [125]	84 ± 14 [175]	AHT
Furosemide	0,107 ± 0,005 [176]	0,6 [172]	0,7 [174]	1,03 [125]	–	AHT
Caffeine	33.1 ± 2.7 [177]	9.5 ± 0.63 [177]	19,6 ± 0,3 [178]	24.38 ± 0.22 [179]	~126 [180]	
Amoxicillin *	0.69 [181]	1,5 [172]	2.4 ± 0.9 [182]	–	8,52 [183]	antibiotic
Atenolol	1.4 ± 0.4 [155]	0,09 [184]	0,13 ± 0,03 [185]	0.20 ± 0.07 [125]	5,4 ± 2,4 [175]	β-blocker
Isoniazid	36,3 ± 0,3 [186]	5,4 ± 0,07 [187]	–	–	–	antibiotic
Propranolol	32.5 ± 0.3 [155]	12,7 [184]	34,19 ± 1,86 [156]	13.38 ± 0.40 [179]	164 ± 16 [175]	AHT
Salicylic acid	10,1 ± 0,4 [188]	0,013 ± 0,001 [189]	0,8 [174]	–	–	AIF
Digoxin	3.60 ± 0.02 [190]	0.62 [191]	0,26 ± 0,02 [192]	–	31 ± 8,5 [193]	
Verapamil	–	11,5 ± 3,21 [194]	15.2 [174]	41,7 [195]	–	AAR

(*) for amoxicillin only permeability data were found for MDCK and not MDCK MDR, AHT: antihypertensive, AAR: Antiarrhythmic, AIF: anti-inflammatory.

regenerative medicine [201].

One of the most prominent applications of iPSCs is their use in *in vitro* models of the blood-brain barrier (BBB). The BBB is a complex structure that regulates the interaction and molecular exchange between the central nervous system (CNS) and peripheral circulation, maintaining a homeostatic environment essential for brain function [202]. iPSC-derived BBB models allow for the differentiation of these cells into brain microvascular endothelial cells, exhibiting high TEER and expressing tight junction proteins such as claudin-5, which are fundamental features for studying cerebral permeability [203].

Hong et al. [204] examined how the Amyotrophic Lateral Sclerosis-Parkinsonism-Dementia Complex (ALS-PDC) affects cellular permeability and microglial integrity in iPSC-derived brain organoids. Using organoids from ALS-PDC patients, they observed that microglia in the M1 state, associated with a proinflammatory response, affects the permeability of the cellular barrier in the neuronal environment. This inflammatory response not only alters the microglial structure but also compromises barrier function in brain tissue, facilitating a more permeable environment for external agents and increasing susceptibility to the accumulation of toxic proteins such as tau and beta-amyloid, typical of neurodegenerative pathologies. In contrast, restoring the M2 state in microglia through treatment with 10 ng/ml interferon-gamma (IFN-γ) showed a 45 % improvement in permeability and in the microglia's repair function. This translated into a 30 % increase in phagocytosis and a reduction in beta-amyloid accumulation, stabilizing cellular permeability and protecting neurons in the ALS-PDC model. This approach suggests that manipulating permeability and microglial polarization could be a promising strategy for addressing neurodegeneration in ALS-PDC.

On the other hand, Tamò et al. [205] developed an alveolar type II epithelial cell line (AEC II) derived from induced pluripotent stem cells (iPSCs) to study pulmonary pathologies. The iPSCs were differentiated into AEC II using a two-stage protocol, and the resulting cells displayed typical AEC II morphological characteristics, including lamellar body and microvilli formation, as well as the expression of specific proteins like surfactant protein C. To assess the functionality of these cells, TEER was measured in monolayers of LL-iPSC-AEC II as an indicator of epithelial barrier integrity. TEER values increased over time, reaching a peak of 256 $\pm$ 0.544 Ω cm^2 on the third day, followed by a gradual decline. Permeability was evaluated using the paracellular marker Lucifer Yellow, whose apparent permeability coefficient (Papp) progressively decreased as the monolayer formed, recording final permeation values of 3.13 $\pm$ 7.25·10^{-6} cm/s on day 3. Additionally, these cells were shown to respond to inflammatory stimuli; when treated with TNF-α, IL-6 and IL-8 secretion significantly increased, with IL-6 levels reaching 1924.8 $\pm$ 592.7 pg/ml compared to 109.9 $\pm$ 31.2 pg/ml under unstimulated conditions.

Li et al. [206] evaluated the integrity of the BBB in patients with 22q11.2 deletion syndrome, a genetic condition associated with an increased risk of developing schizophrenia. Using human brain microvascular endothelial cells derived from iPSCs from patients, the researchers successfully built a BBB model that allowed measurement of its permeability and TEER. The results showed that the patients' BBB exhibited increased permeability to small and large solutes (NaFl and Dex-70K), with approximately 1.3 and 1.4 times

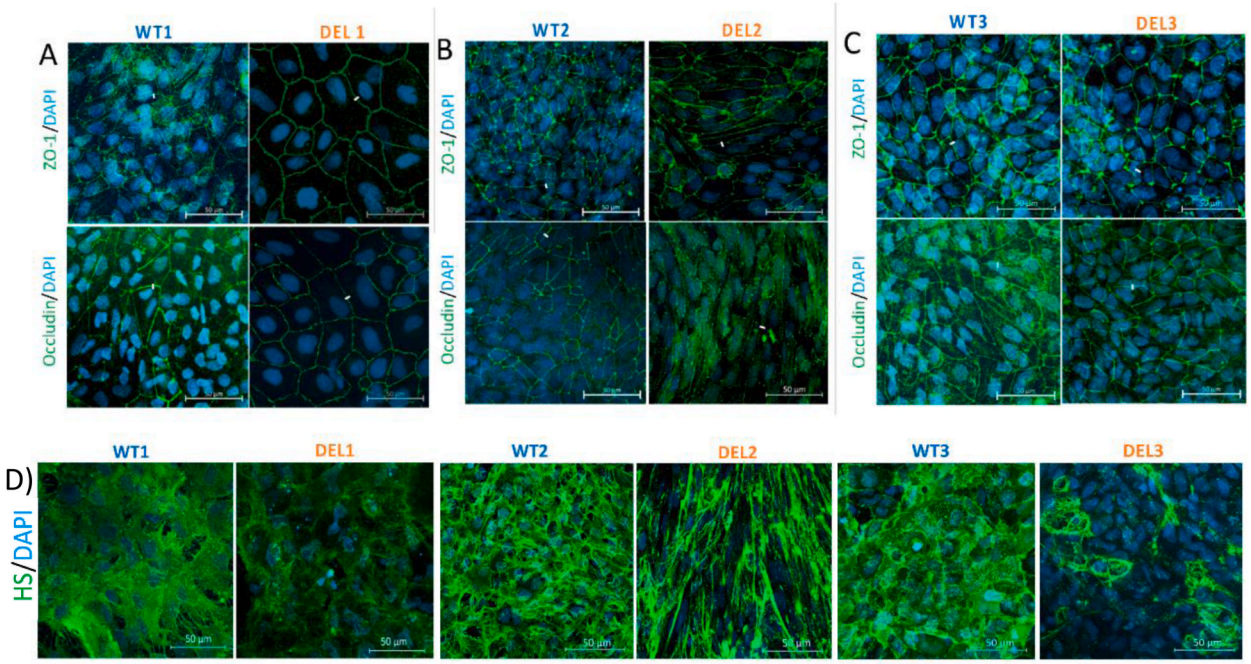

Fig. 7. A-C) Comparison of binding proteins in the iBBB between control (WT) and 22q11.2DS-SCZ (DEL). Confocal images showing tight junction proteins (ZO-1 and occludin) in the iBBB for the control (left panel) and 22q11.2DS-SCZ (middle panel) of three paired iPSCs (right panel). **D)** Comparison of heparan sulfate (HS) intensity in different samples of induced pluripotent stem cells (iPSCs), showing three pairs: each control sample (WT1, WT2, WT3) versus its corresponding 22q11.2 deletion (DEL1, DEL2, DEL3) [206]. Reprint published from open access article by MDPI Copyright 2021.

increases, respectively, compared to control samples. Additionally, the patients' TEER was 62 % of the value recorded in the controls, indicating significantly reduced barrier integrity. These changes in BBB function were consistent across the three sample pairs evaluated, highlighting the impact of this genetic deletion on barrier structure and function. Furthermore, reduced levels of tight junction

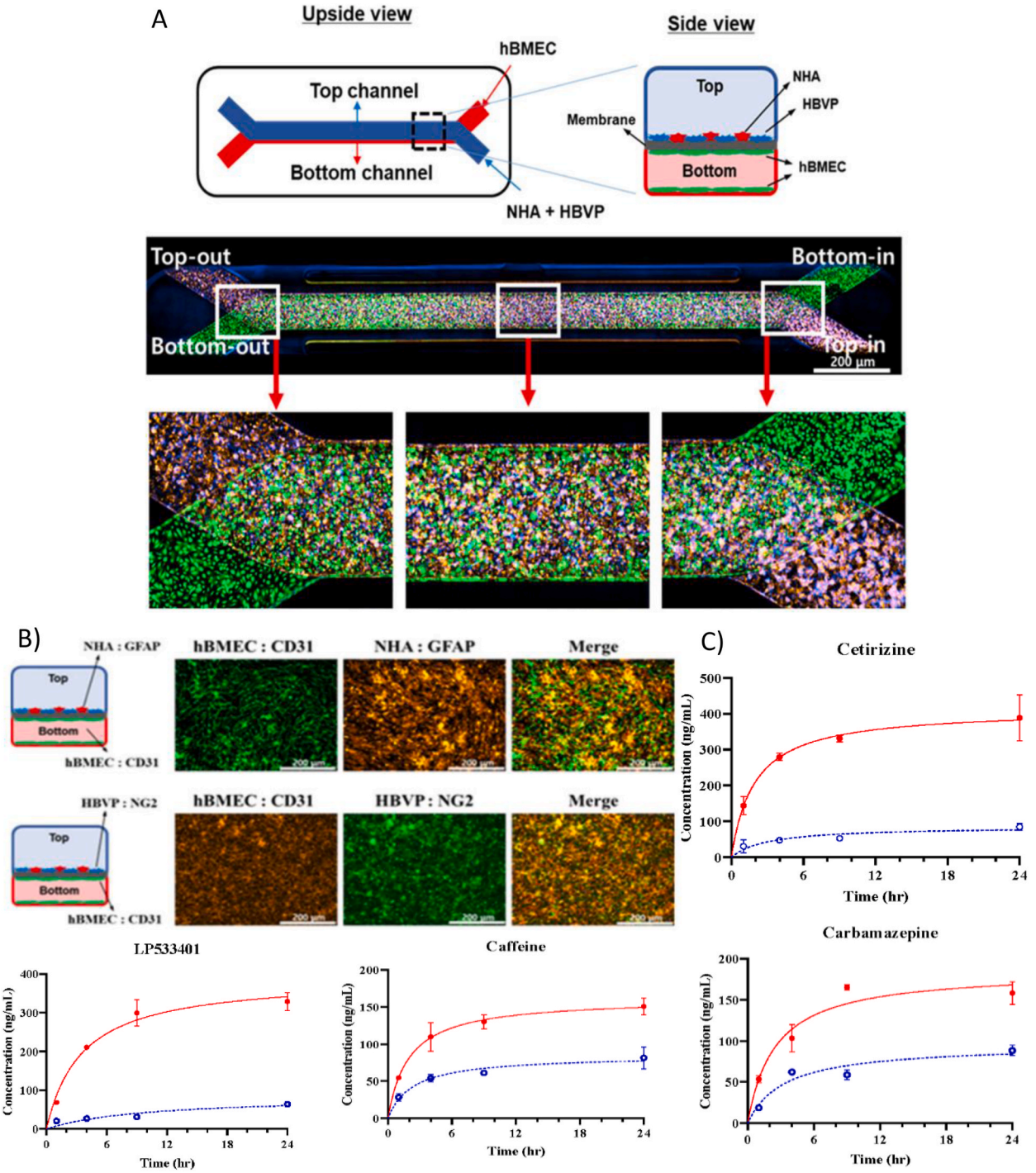

Fig. 8. A) A micro-engineered two-channel chip contained immortalized human brain microvascular endothelial cells (hBMEC, green) on all surfaces of the lower channel, and human astrocytes (NHA, red) and human brain vascular pericytes (HBVP, blue) on the surface of the upper channel. Each cell line was stained with specific markers. **B)** Endothelial cells (stained with CD31), astrocytes (GFAP), and pericytes (NG2). **C)** Observed and estimated drug concentration in the microfluidic system. The blue circles and red dots represent the drug concentration in the blood channel and brain channel, respectively [216]. Reprint published from open access article by MDPI Copyright 2021.

proteins such as ZO-1 and occludin were observed (Fig. 7A), as well as a decrease in the intensity of the endothelial glycocalyx, quantified by the intensity of heparan sulfate, which was only 42 % of the control level (Fig. 7B). These deficits in tight junction proteins and glycocalyx contribute to the BBB integrity loss. Genetic expression analysis identified a significant decrease in the CRKL gene, which is related to VEGF signaling and is crucial for cellular adhesion and barrier stability. These results suggest that CRKL deficiency may be associated with increased BBB permeability in 22q11.2DS.

4.2. Organ-on-a-chip systems

Organ-on-a-chip (OOC) systems represent an emerging technology that replicates human physiological features on microchips, providing advanced platforms for disease modeling, pharmacokinetic and pharmacodynamic studies, and toxicity evaluation in drug development [207]. OOC can simulate the cellular microenvironment and tissue interactions more accurately than conventional 2D models or *in vivo* animal assays, which have limitations in predicting human effects due to interspecies differences [208]. A fundamental component of OOC is microfluidics, enabling precise fluid control at the microscopic scale to recreate physiological flows and mechanical forces, such as shear stress. This is essential for creating dynamic microenvironments, where cells cultured on a chip can interact with a fluid medium simulating physiological condition [209]. This technique facilitates cellular-scale studies and allows for incorporating multiple tissue types on a single chip, such as liver, intestine, and brain, enhancing the biological relevance of toxicity and drug absorption studies [210].

The accuracy in manufacturing these devices often relies on materials like polydimethylsiloxane (PDMS), although this material can adsorb small molecules, affecting responses in toxicity assays. To address this issue, chips with antifouling coatings, such as those based on perfluoropolyether, have been developed to reduce absorption and improve sensitivity in drug evaluation [211]. Moreover, 3D printing technology also plays a crucial role in creating complex structures within these devices, supporting tissue engineering and the study of inter-organ interactions through multi-organ chips [212]. Recent studies highlight the capability of OOC to replicate specific biological barriers, such as the blood-brain barrier and the retinal barrier, providing precise data on permeability and vascular functionality in response to stimuli and pathological conditions such as oxidative stress [213,214].

In this context, Schimek et al. [215] employed an organ-on-a-chip system with a two-organ chip (2OC) to culture and analyze full-thickness human skin equivalents (ftSEs) in a 96-well insert format, optimized for substance permeability testing and future co-culture applications with other tissues, such as liver or kidney. This 2OC system mimics the metabolic interaction between different organs by incorporating two culture compartments connected via microfluidic flow channels, allowing nutrient circulation and maintenance of a physiologically similar environment. The ftSEs cultured in the 2OC exhibited a morphology similar to real human tissue, maintaining cell viability and proliferation in a dynamic environment. This organ-on-a-chip system was designed to perform long-term substance permeability tests and assess skin barrier function, measured through transepithelial resistance and permeation coefficient. With decreasing permeation coefficients over time, the study suggests that the 2OC platform is suitable for modeling complex human tissues and testing the efficacy and toxicity of substances in a multi-organ context, thus reducing the need for animal testing.

Similarly, Yang et al. [216] used a two-channel microfluidic BBB model to evaluate the permeability of ten drugs, addressing a common limitation in the development of neuroactive drugs (Fig. 8A). The BBB-on-a-chip model uses human endothelial cells, astrocytes, and pericytes in channels separated by a polydimethylsiloxane (PDMS) membrane, 7 μm in diameter with 2 % porosity (Fig. 8B). This design allows for physiological-like interactions between cellular components, emulating the effects of flow and shear that enhance the expression of tight junction and transport proteins. To assess permeability, drugs were introduced into the apical (blood) and basolateral (brain) channels, and concentrations were measured at 1-, 4-, 9-, and 24-h using LC-MS/MS. Caffeine and carbamazepine showed high permeability (Papp of 29.99 and 15.77 x 10^{-6} cm/s, respectively), while cetirizine and LP533401 exhibited low permeability, with Papp values of 7.29 and 2.17 x 10^{-6} cm/s (Fig. 8C). The positive correlation between the partition coefficient and Papp validates this model as a predictive tool for BBB permeability, offering advantages over traditional models by avoiding species-specific variations and enabling a more accurate assessment of drug passage across the barrier.

On the other hand, Mosavati et al. [217] developed a placenta-on-a-chip model to study placental barrier permeability, specifically for glucose transport, simulating the maternal-fetal interface. In this device, human umbilical vein endothelial cells (HUVECs) and BeWo trophoblast cells were co-cultured on a porous 400 nm polycarbonate membrane that separates microfluidic channels simulating maternal and fetal blood flows. Experiments revealed that glucose permeability on the cell-free membrane was 1.65 × 10^{-5} cm/s, while in the BeWo/HUVEC co-culture it decreased to 3.7 × 10^{-6} cm/s, reducing glucose transport by 35 % compared to the cell-free model. At an optimal flow of 50 μL/h, glucose concentrations in the inlet channels were adjusted to 7.2 mM (maternal) and 5.6 mM (fetal), reflecting a physiological environment. This device also allows for investigating how factors such as flow rate and membrane porosity affect nutrient transport. For instance, it was observed that at high flows (150 μL/h), diffusion decreases due to increased contribution from convective flow, while greater membrane porosity facilitates glucose transport.

4.3. Cellular spheroids

Multicellular spheroids are a valuable tool in drug permeability and transport research, as they better mimic *in vivo* physiological characteristics compared to 2D models [218]. Various studies have demonstrated that the three-dimensional structure of spheroids allows the formation of nutrient and oxygen gradients, which influence drug permeability in the inner layers of the spheroid [219]. In lung cancer models, doxorubicin resistance in spheroids has been associated with the expression of tight junction proteins like claudin-1, which reduces paracellular permeability and limits drug accumulation in the inner regions of spheroids, where hypoxic cells

less accessible to therapeutic compounds are located [220].

Furthermore, in blood-brain barrier models, spheroids allow for the evaluation of therapeutic agents' penetration ability through a functional barrier with specific permeability characteristics, where factors like the presence of tight junctions and efflux pump activity, such as P-glycoprotein regulate the transport of molecules into the central nervous system [221]. A study on using spheroids to model the blood-brain barrier highlights how culturing endothelial cells, pericytes, and astrocytes in spheroids can simulate the cellular

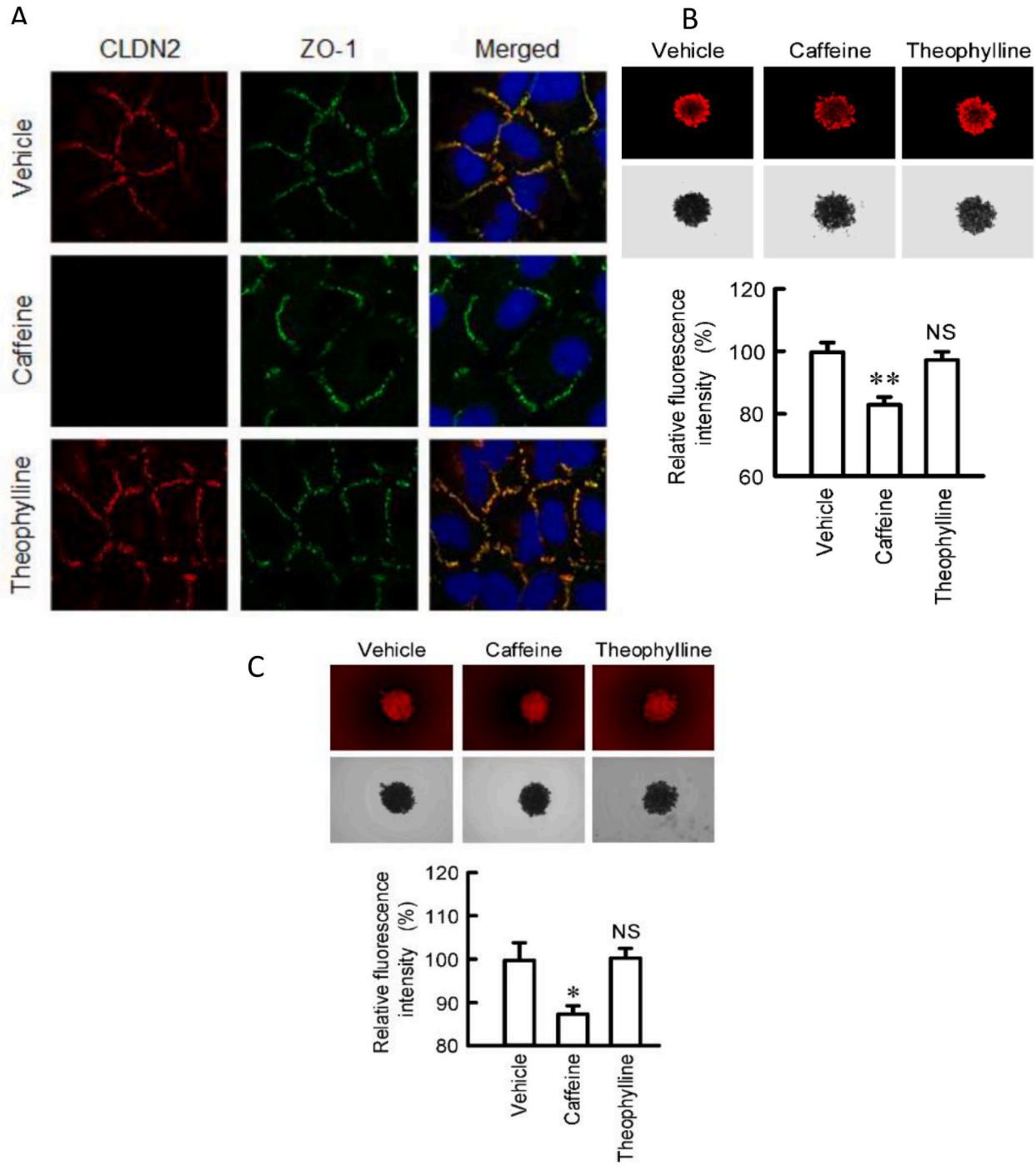

Fig. 9. A) Effect of caffeine and theophylline on the cellular localization of CLDN2 and ZO-1. The cellular localization of CLDN2 and ZO-1 is indicated by red and green fluorescence signals, respectively. **B)** Cells incubated with the ROS marker (CellRox Deep Red) showed significantly lower fluorescence intensity in caffeine-treated cells. **C)** Cells incubated with the hypoxia marker (LOX-1) showed reduced fluorescence intensity, indicating lower hypoxia in caffeine-treated cells [227]. Reprint published from open access article by MDPI Copyright 2021.

organization of this barrier, providing a robust model for evaluating compounds that may cross it [219].

In another field, liver spheroids have been used to study drug metabolism and permeability, employing multiscale mathematical models that allow for analyzing transport and metabolism dynamics within the spheroid. This offers deeper insights into how cellular permeability and spatial distribution affect drug distribution in liver tissues, contributing to a more accurate prediction of hepatotoxicity for new drugs [222]. Additionally, intestinal spheroids have been developed to study drug absorption and transport at the intestinal level, as they replicate extracellular matrix interactions and show faster polarization and differentiation than conventional 2D models, making them ideal for evaluating drug absorption more representatively of *in vivo* physiological conditions [223].

The use of multicellular spheroids in permeability studies has also enabled innovations in techniques such as electropermeabilization, used in tumor spheroids to enhance cytotoxic drug delivery to inner layers. It has been observed that the use of transient electrical pulses can effectively permeabilize cells in the spheroid core, allowing for uniform drug distribution of agents like bleomycin and cisplatin throughout the multicellular structure [224]. Similarly, spheroid models that emulate intestinal architecture, such as structured multicellular spheroids, allow for advanced differentiation and replicate characteristics of the human intestinal mucosa, offering an ideal platform for studying permeability and immune behavior in response to intestinal pathogens, underscoring these models' versatility in pharmacological and infection applications [225]. Finally, recent studies on the development of microfluidic arrangements for spheroids of different sizes have demonstrated that spheroid size variability significantly affects cellular response and compound penetration [226].

Eguchi et al. [227] investigated how caffeine affects Claudin-2 (CLDN2) protein expression and its impact on permeability and toxicity in human A549 lung adenocarcinoma cell spheroids. Experiments showed that caffeine, at a concentration of 200 μM, reduces CLDN2 levels by approximately 80 %, promoting its degradation in lysosomes. Theobromine also showed similar effects in reducing CLDN2, while theophylline had no impact. Additionally, caffeine decreased CLDN2 expression in the cell contact region, where it colocalizes with the tight junction protein zonula occludens-1 (ZO-1) (Fig. 9A). This change significantly increased paracellular permeability, raising the flux of doxorubicin (DXR) by 40 % and lucifer yellow by 30 %. TEER also increased by 50 %, indicating a CLDN2-mediated alteration in tight junction structure and function. Besides these permeability effects, caffeine intensified DXR accumulation in A549 cell spheroids, increasing the toxicity of DXR and cisplatin in these lung cancer cells, while theophylline showed no effects in this regard. Additionally, caffeine reduced Nrf2-mediated stress signaling, lowering oxidative stress levels and decreasing the expression of antioxidant genes such as HO-1, NQO-1, and GCLM. Finally, caffeine may have a protective effect against oxidative stress (Fig. 9B) while facilitating the action of chemotherapeutic drugs by reducing hypoxia in the tumor microenvironment (Fig. 9C).

Similarly, Boccellino et al. [228] explored the impact of curcumin on prostate cancer (PC) cells and spheroids derived from these cells. Two PC cell lines with different metastatic potentials (DU145 and PC-3) were evaluated, comparing the effects of curcumin with chemotherapeutic drugs such as cisplatin, paclitaxel, and docetaxel. Curcumin demonstrated a dose-dependent reduction in cell viability, similar to the chemotherapeutic agents, and decreased EGFR-mediated signaling and ERK activation, both implicated in prostate cancer progression. Regarding permeability and spheroid modeling, three-dimensional spheroids were formed from DU145 and PC-3 cells in an extracellular matrix, providing a more realistic environment to study treatment effects in a tumor-like setting. In DU145 spheroids, curcumin treatment at a concentration of 6.9 μg/mL reduced spheroid size by 40 %, while combining it with cisplatin (25 ng/mL) reduced the size by approximately 60 % after 12 days of treatment. For PC-3 spheroids, treatments achieved around a 50 % reduction in spheroid size compared to the control, which typically grew six-fold under untreated conditions. Cell viability in both cell lines decreased by up to 80 % in the presence of chemotherapeutic agents combined with curcumin, compared to a 50 % reduction with curcumin alone. These findings indicate that curcumin not only reduces spheroid size and viability but also enhances the effect of chemotherapeutic drugs, promoting apoptosis and affecting the structural integrity and permeability of three-dimensional spheroids in a more realistic tumor model.

Bong et al. [229] evaluated intestinal drug permeability using a 3D Caco-2 cell spheroid system, optimizing key variables such as cell number (5000–10,000 per spheroid), initial compound concentration, and incubation time. This approach facilitated a more accurate simulation of human intestinal absorption compared to the traditional two-dimensional method (PAMPA), achieving an 85 % correlation with human absorption in 22 commercial compounds, versus 70 % in the PAMPA method. Caco-2 cell spheroids mimic the multicellular structure and function of intestinal villi, improving predictability in pharmacokinetics studies and drug discovery. The spheroid architecture allows for a more realistic evaluation of transepithelial transport and paracellular absorption, capturing permeability variations for both hydrophilic and lipophilic compounds. Furthermore, their multilayer morphology facilitates the analysis of progressive drug diffusion and retention, increasing sensitivity in distinguishing compounds with varying absorption levels. This method is positioned as a more precise tool in estimating compound efficacy and transport, surpassing PAMPA assay in predictive accuracy.

5. Conclusion

In this article, we have explored in detail the various techniques used to assess cell permeability, from classic Caco-2 cell-based models to innovative three-dimensional technologies. Caco-2 cells have been fundamental in understanding passive and active transport in human intestinal absorption. However, they face limitations, such as the extended time required for their cultivation and the lack of a mucosal layer that accurately reflects the intestinal environment. The introduction of co-cultures with HT29-MTX cells has helped to overcome some of these limitations by adding a mucosal layer, bringing the results closer to conditions observed in living organisms. On the other hand, methods such as PAMPA and the use of MDCK cells offer advantages in terms of speed and cost, though they present challenges in accurately replicating active and paracellular transport mechanisms. Advances in three-dimensional models, including the use of induced pluripotent stem cells, spheroids, and organ-on-a-chip systems, represent a major leap in our

ability to simulate the complexity of human tissue. These models provide a more realistic environment for studying cellular interactions and drug responses, improving the accuracy of permeability studies. Ultimately, the choice of the most suitable technique for evaluating cell permeability should be based on the specific characteristics of the compound under study, the absorption mechanisms involved, and the available resources. Combining traditional models with innovative approaches offers a robust strategy to advance the development of new drugs, enhancing precision in predicting their absorption and therapeutic efficacy. It is essential to continue optimizing and standardizing these models to maximize their potential. Furthermore, exploring new technologies and methodologies will allow for a more comprehensive understanding of cell permeability and how drugs interact at the cellular level. This integrative approach will not only benefit the development of more effective and safer drugs but also contribute to a better understanding of human physiology and the cellular basis of various diseases.

CRediT authorship contribution statement

Vanderson de Jesus da Silva: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Christian Shleider Carnero Canales:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Giulia Polinário:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Vinicius Oliveira Chesna:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Rafaela Melo Pogianeli:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Edson Reinaldo Júnior:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Mauro Evaristo Venceslau Junior:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Maria Fernanda Ortolani Pollini:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Andréia Bagliotti Meneguin:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition. **Fernando Rogério Pavan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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