European Journal of Pharmaceutics and Biopharmaceutics 142 (2019) 353–363

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

A unified in vitro test system for the assessment of tight junction modulators

Daniel Saaber, Stephan Reichl*

Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Mendelssohnstraße 1, 38106 Braunschweig, Germany

ARTICLE INFO

Keywords:
Tight junction modulator
Drug permeation enhancement
MDCK cells
TEER measurement
Drug absorption

ABSTRACT

Tight junction (TJ) modulation is a promising approach for improving drug bioavailability by enhancing the absorption of active pharmaceutical ingredients. However, the application of many different test methods to determine the efficacy of new TJ modulators (TJMs) or to assess different compounds is accompanied by a lack of comparable results, reducing the rational evaluation and commercial marketing of these pharmaceutical excipients. The establishment of unified testing methods can fill this gap and offers the opportunity to compare results from different laboratories. Furthermore, the calculation of a TJ modulation score allows the objective comparison of TJ modulators and facilitates the selection of appropriate candidates.

In this study, eight well-known TJ modulators were tested with a focus on four different in vitro bioassays carried out with MDCK cells. The extent of TJ modulation was determined by transepithelial electric resistance (TEER) measurements and permeability studies with mannitol. To evaluate tolerability, cell viability (MTT) and cytotoxicity (CellTox™ Green) assays were performed, and TEER regeneration was monitored for 24 h after exposure. With the exception of labradimil, seven TJ modulators caused significant TEER reduction of up to 100 %. For five compounds, an enhancement of mannitol permeation was observed. As expected, first-generation enhancers exhibited lower cell compatibility than mechanism-based modulators. Based on the experimental results of this study, for the first time, an evaluation system (tight junction modulator scoring system, TJMSS) is presented that provides a ranking of the tested modulators depending on weighted parameters. Such a system offers the possibility of rational formulation development for drugs requiring improved absorption.

1. Introduction

The modern concepts and methods of drug discovery, including high throughput screening, yield a large number of new promising drug candidates [1], but a multitude of them are related to pharmacokinetic problems such as poor solubility and permeability, limiting their pharmaceutical application [2]. Beyond low permeability after oral administration, many active pharmaceutical ingredients (API) also show poor permeation behavior and drug transport at several other barrier-forming tissues such as the blood brain barrier [3], the cornea [4] or human skin [5]. For this reason, several innovative approaches have been developed in recent decades to overcome these hurdles in drug development and improve drug bioavailability. Such approaches included, for example, prodrug strategies or the inhibition of efflux transporters such as P-glycoprotein, technical innovations such as microneedles or iontophoresis, drug formulation strategies such as the self-microemulsifying drug delivery system (SMEDDS) or nanoparticles and the use of permeation enhancers such as mucoadhesive polymers, bile salts and surfactants [6]. However, all these concepts have the disadvantage of being linked to particular properties of the API or restricted to certain tissues. Out of these considerations, the idea of the modification of drug absorption barriers to increase permeation of various APIs emerged. One possible starting point could be the modulation of tight junctions (TJs), which prevent or decrease paracellular drug transport, in particular of large and hydrophilic molecules [7], thereby forming an important barrier responsible for the low permeability of many chemical entities.

TJs are part of the junctional complex next to gap junctions and desmosomes located in the apical part of the paracellular space, forming a branching network of polymerized protein strands [8]. These strands consist of 4 different integral transmembrane proteins (occludin [9], claudin [10], tricellulin [11] and junctional adhesion molecules A, B and C [12]) that are connected to the actin filaments of the cytoskeleton by zonula occludens proteins [13–15]. This connection enables the specific regulation of TJs to allow the controlled permeation of nutrients or other essential molecules [16]. The key factor for this functionality is the myosin light chain (MLC), a subunit of myosin [17]. MLC activation by phosphorylation leads to the contraction of actin filaments and thereby creates an opening of TJs [18]. This phosphorylation is regulated by the balance between the activities of myosin

* Corresponding author.
E-mail address: S.Reichl@tu-braunschweig.de (S. Reichl).

https://doi.org/10.1016/j.ejpb.2019.07.004
Received 12 February 2019; Received in revised form 28 June 2019; Accepted 2 July 2019
Available online 03 July 2019
0939-6411/

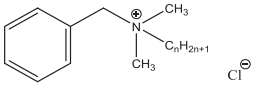
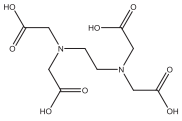
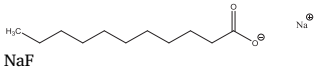
light-chain kinase (MLCK) and myosin light-chain phosphatase (MLCP). Different pathways, triggered by various receptors and kinases, lead to MLCK and MLCP activation or inhibition [19]. Among other things, TJs regulate cell proliferation, differentiation and polarization and contribute to the establishment of the ion gradient [20]. Furthermore, their localization in the paracellular space creates a strong barrier for many xenobiotics. The claudins, with 24 known members belonging to their family, play a decisive role in this so-called fence function [21]. Their expression pattern, particularly their morphology and number of strands, determines the barrier characteristics of different tissues [22]. For example, claudin-5 can be found in especially strong barriers such as the blood brain barrier, cornea or nephron epithelium [23].

Over the years, several TJ modulators have been studied with regard to their drug permeation enhancing properties and can be classified into different groups according to their mechanism of action and tolerability. TJ modulators of the first generation, that do not target specific TJ components, were calcium chelators, e.g., EDTA, and surfactants, e.g., sodium caprate, and were followed at the end of the 1990s by second-generation modulators (targeting specific TJ proteins) such as peptides corresponding to occludin, *Clostridium perfringens* enterotoxin or antisense RNA [24]. Due to the low biocompatibility of these substances along with cytotoxic effects, the development of so-called mechanism-based (MB) TJ modulators seems to be a promising approach [19]. Such compounds, e.g., AT-1002 or PN159, target TJ regulation and are therefore promising to lead to a more gentle and reversible opening of TJs, causing fewer cytotoxic effects in contrast to first- and second-generation modulators. A summary of all TJMs examined in this study is presented in Table 1.

Starting from scratch, what makes a good TJ modulator? The opening of the TJ should be fast, short acting and reversible. A fast and easy method for the evaluation of TJ modulator efficiency can be performed by measuring transepithelial electrical resistance (TEER) as a surrogate parameter for the opening of TJs. In general, two different laboratory methods for TEER measurement are used; for example, single measurements of resistance by chopstick electrodes or cell culture cup chambers and more precise, continuous measurements of impedance under standardized conditions with cellZscope®, ECIS® or xCELLigence® are carried out. The latter are particularly well suited for determining TEER regeneration after the depletion of TJ modulators and thus the reversible opening of TJs. For the evaluation of permeation enhancement, different marker substances such as mannitol, inulin, polyethylene glycol or FITC-dextran can be used [36]. However, TEER measurements can only offer a first indication of whether a substance can enhance API permeation. It must be considered that not every decrease in TEER leads to a higher apparent permeation coefficient (P_{app}) for a drug, and furthermore, with comparable TEER values, P_{app} depends on the paracellular marker used [37], resulting in a general problem with the comparability of results gained from experiments with comparable TEER values and different markers [38–42]. Moreover, P_{app} values obtained for certain tracers can vary greatly among different cell lines [43,44] or tissues. Furthermore, the incubation time of the TJ-modulating compound plays a distinct role and can lead to different results. Beyond efficacy, tolerability is a key feature for distinguishing a good TJ modulator. Miscellaneous cell-based assays are available for the measurement of viability or cytotoxicity [45] as indicators of tolerability, targeting metabolic capacity, membrane integrity or apoptosis markers.

Hence, current research on the efficacy and tolerability of TJ modulators deals with a variety of testing systems and methods and was extensively reviewed by Deli in 2005 [46]. Thus, different cell lines (e.g., Caco-2, HUVEC, MDCK), in vitro models and animal models are used for this application [47]. However, this procedure presents a large disadvantage regarding the comparability and assessment of TJ modulators for several reasons. First, the expression of barrier properties in distinct cell lines or tissues is different, reflected by a large range of TEER values. Second, cell cultures, tissues and animal models are differentially affected by the cell-based assays according to different proliferation and metabolism ratios. Third, the comparison between permeation studies performed using different markers and barriers is limited by their molecular weights, hydrophilicities, barrier properties and associated permeation coefficients. Thus, the selection of suitable permeation enhancers for challenging pharmaceutical applications by

Table 1
Summary of the eight TJ modulators tested in the study.

Name	Formula	Pharmaceutical Use	Mode of action	Molecular Weight	logP
Benzalkonium chloride		Preservative	Cytotoxic interaction with cell membrane and membrane proteins [25,26]	283.9 g/mol	3.45^{+}
EDTA		Chelating agent	Calcium chelator [27]	292.2 g/mol	−0.43*
Sodium caprate		Surfactant	Phospholipase C activation [27,28]	194.2 g/mol	4.09$^{\#}$
Sodium fluoride	NaF	Caries prevention	Myosin light-chain phosphorylation, Cytotoxic effects [29,30]	42.0 g/mol	−0.77$^{\#}$
Sodium nitroprusside	$Na_2[Fe(CN)_5NO]$	Vasodilatator	Myosin light-chain kinase and Protein kinase C activation [31]	261.9 g/mol	0.07$^{\#}$
AT-1002	Phe-Cys-Ile-Gly-Arg-Leu	Permeation enhancer	Protease activated receptor-2 agonist [32]	707.9 g/mol	N/A
Labradimil	Arg-Pro-Hyp-Gly-Thi-Ser-Pro-Tyr(Me)-psi-(CH_2NH)-Arg[x]	Permeation enhancer	Bradykinin B_2 receptor agonist [33]	1098.3 g/mol	−2.5$^{\#}$
PN159	Lys-Leu-Ala-Leu-Lys-Leu-Ala-Leu-Lys-Ala-Leu-Lys-Ala-Ala-Leu-Lys-Leu-Ala	Permeation enhancer	Binding to TJ Proteins [34,35]	1876.5 g/mol	N/A

Listed are their formulas and pharmaceutical use as well as their assumed mode of action as TJM (primary target), molecular weight and octanol/water partition coefficient (logP value). The double-crossed line separates the first-generation enhancers in the first part from the mechanism-based (MB) enhancers in the second part of the table. The logP values were obtained from three different databases (chemspider*, drugbank$^{\#}$ and pubchem^{+}). No logP values were available for the modulators AT-1002 and PN159. Data for benzalkonium chloride, a mixture of alkylbenzyldimethylammonium chlorides, is exemplary for the benzyldimethyloctylammonium chloride. However, neither logP values nor the molecular weight of the TJM allow a conclusion to be drawn as to their modulating properties or the extent of the modulating effect.

[x] Hyp: hydroxyproline, Thi:2-thienyl-alanine, psi: CO-NH is exchanged by CH2NH

formulation scientists is hampered, and the general benefit of TJ modulators independent of APIs and certain tissue characteristics and their universal applicability have been questioned.

This study describes an attempt to solve the problem of the comparability of different TJ modulators and introduces a new method for evaluating TJ modulators. It allows the objective evaluation of different potential TJ modulators and is still adaptable to individual requirements. Based on TEER and permeability data to determine efficiency and regenerative capacity and viability and toxicity assays to evaluate biocompatibility, a score system is proposed that facilitates the uniform assessment of TJMs. The newly developed TJM score condenses data from all experiments into one value to achieve the complete description of a modulator.

2. Materials and methods

2.1. Materials

Phosphate-buffered saline (PBS), Earle's MEM medium with 2.2 g/l $NaHCO_3$ and 2 mM glutamine were purchased from Biochrom (Berlin, Germany). Fetal bovine serum (FBS) and antibiotic solution with 10,000 U/mL penicillin, 10 mg/mL streptomycin sulfate and 25 μg/mL amphotericin B were obtained from Sigma-Aldrich (Darmstadt, Germany).

Except for measurements of multivalent cation-sensitive compounds, all measurements were performed in Krebs-Ringer buffer (KRB), pH 7.4, in 1,000 mL of double distilled water with 116.4 mM NaCl, 5.4 mM KCl, 1.0 mM $NaH_2PO_4{\cdot}H_2O$, 25.0 mM $NaHCO_3$, 15.0 mM HEPES, 5.6 mM D-glucose monohydrate, 0.8 mM $MgSO_4 \times 7{\cdot}H_2O$, 1.8 mM $CaCl_2 \times 2{\cdot}H_2O$. NaCl, $NaHCO_3$ and HEPES were obtained from Carl Roth (Karlsruhe, Germany). KCl and $MgSO_4 \times 7{\cdot}H_2O$ were purchased from Acros Organics (Geel, Belgium). $NaH_2PO_4{\cdot}H_2O$ was purchased from Merck (Darmstadt, Germany). D-glucose monohydrate was obtained from Caelo (Hilden, Germany). For multivalent cationsensitive compounds, calcium- and magnesium-free KRB buffer ($KRB^{-/-}$) was used. OptiPhase SuperMix scintillation liquid was purchased from PerkinElmer (Shelton, Connecticut). Carbon-14-radiolabeled mannitol was obtained from Hartmann Analytic (Braunschweig, Germany). All other chemicals used in this study were of analytical grade. The TJ modulators EDTA, sodium caprate and sodium fluoride were obtained from Sigma-Aldrich. Benzalkonium chloride was purchased from Caelo, and nitroprusside sodium was purchased from Carl Roth. The MB modulators AT-1002 and PN159 were purchased from Caslo (Lyngby, Denmark), and labradimil was received from Bachem (Bubendorf, Switzerland). All modulators were used in concentrations with a logarithmic scale. The choice of mean tested concentration was made according to previously described effective doses for every TJ modulator.

2.2. Madin-Darby Canine Kidney (MDCK) cells I

MDCK type I cells at passage 17 were obtained from the European Collection of Authenticated Cell Cultures (ECACC). This cell line was initially derived from the renal duct of an adult female cocker spaniel. When grown on filters, they form tight monolayers with a polarized morphology. Cells were cultivated in 25 cm^2 tissue culture flasks at 37 °C under a humidified atmosphere with 5 % CO_2. The ECACC-recommended medium Earle's MEM containing 2.2 g/l $NaHCO_3$, 10 % FBS, 2 mM glutamine and 1 % antibiotic/antifungal mixture was used. Growth medium was replaced 3 times per week. Cells were subcultured after reaching confluence. Cell counting was performed using a Z2 Coulter Counter from Beckman Coulter (Krefeld, Germany).

2.3. Transepithelial electrical resistance (TEER) measurement

The cellZscope® from nanoAnalytics (Münster, Germany) was used for continuous evaluation of TEER as a surrogate parameter of tight junction functionality. MDCK cells were grown on 1.12 cm^2, 1.0 μm transparent filter inserts (ThinCerts™, Greiner Bio-One, Frickenhausen, Germany) with a seeding density of 100,000 cells per well. The medium was changed at cultivation days 3, 4 and 5. TEER measurements were performed on day 5 when cells reached a resistance of approximately 3500 $\Omega{\cdot}cm^2$ starting with the replacement of the growth medium by an equivalent volume of KRB, pH 7.4 (500 μL apical, 1500 μL basolateral) and cell incubation for 60 min. After this preincubation, 250 μL of KRB was removed from the insert, and the same volume of double-concentrated sample solution (TJ modulator in KRB) was added to the 250 μL KRB inside the insert to reach the desired concentration. Afterwards, impedance measurements were performed for 180 min with a frequency range of 1 Hz to 100 kHz at 37 °C and 5 % CO_2 inside the incubator.

Subsequently, the cells were carefully washed with fresh KRB and incubated in medium, and TEER was monitored up to 24 h in 30 min time intervals after the start of the experiment to evaluate TEER recovery. The results are given as the relative TEER reduction, defined as the percentage change of the starting value corrected by the baseline value.

2.4. Permeation studies

For permeation studies, MDCK I cells were cultivated on ThinCerts™ filter inserts for 5 days according to impedance measurements as described above. The cellular layer was incubated with 500 μL KRB before permeation. After 60 min, KRB was replaced by 200 μL donor solution containing 1 mM mannitol in KRB spiked with ^{14}C-mannitol to a resulting activity of 0.5 μCi/mL and the TJ modulator at the appropriate concentration. KRB (1200 μL) was used as the acceptor solution in the basolateral compartment. Aliquots (200 μL) were collected from the receiver chamber at fixed time intervals for 210 min and replaced with an equal volume of KRB. Radioactivity was determined using an LS 6500 scintillation counter (Beckmann Coulter, USA) after adding 1.5 mL scintillation liquid cocktail to every sample. The permeation profiles of mannitol were determined by plotting the permeated amount [μg] versus the time [min]. The permeation enhancement factor was calculated as the ratio of the permeated amount of mannitol in the acceptor solution for KRB donor solution with TJM versus KRB donor solution without TJM at the end of the experiment.

2.5. Cell viability and toxicity

2.5.1. MTT assay

MDCK cells were cultivated at a density of 10,000 cells/well in a 96-well plate for 24 h. Cells were incubated with 100 μL sample dispersion in KRB per well for 15 min, 30 min, 60 min, 120 min and 180 min. Afterwards, samples were removed, and 100 μL of 0.5 % MTT solution in media was added per well and incubated for 180 min. The MTT solution was replaced by a solubilization solution containing 2.75 g sodium dodecylsulfate, 3.65 g hydrochloric acid, 88.2 g water and 905.4 g isopropyl alcohol. After 12 h of refrigerated storage, the absorbance at 570 nm was determined using a KC4 plate reader from BioTek (Bad Friedrichshall, Germany). After the addition of MTT solution, all further steps performed were protected from light. KRB and 0.1 % Triton-X solutions were used as negative and positive controls, respectively.

2.5.2. Membrane integrity assay

The CellTox™ Green assay from Promega (Mannheim, Germany) was used to determine the cytotoxicity of TJ modulators. The fluorescence-based assay measures changes in membrane integrity that occur as the result of cell death. The cultivation of MDCK cells and sample incubation were performed according to the MTT assay as described above. Subsequently, the CellTox™ Green assay was carried out following the manufacturer's protocol. Fluorescence was measured using a GENios plate reader (Tecan, Männedorf, Switzerland).

2.6. Tight Junction Modulator Scoring System (TJMSS)

Based on the results, a scoring system was proposed to evaluate TJMs. The key parameters and functionality are described below. The TJ modulator score is calculated by spreadsheet analysis, which is provided in the supplementary data. The score can be calculated from TEER measurements at five time points (15, 30, 60, 120 and 180 min), permeation studies and viability/toxicity assays with individual weighting. The scoring is performed using the following Eq. (1):

$$\text{TJ modulator score} = Factor1*-[\text{TEER}] + \frac{1}{25}*Factor2*[permeation] + Factor3 *([viability] + (1 - [toxicity]) + Factor4*(1 + [regeneration]) \quad (1)$$

The four different factors can be chosen as whole numbers ranging from 1 to 5, where one stands for low importance and five stands for high importance. The factors can be individually chosen according to your own requirements. Crucial for different rankings is the weighting ratio between the four parameters. Selecting one or five for all parameters will change the value of the score factor but not the order.

[TEER] is the relative TEER reduction corrected by the relative TEER reduction of control (KRB) at the same time point. [Permeation] is the ratio of permeated mannitol with TJ modulator to permeated mannitol in KRB (control solution) at the chosen time. It is corrected by the empirical factor of $\frac{1}{25}$ to obtain a normalized value with no overestimation of strong permeation enhancement. [Viability] is the metabolic activity determined by MTT assay in relation to the negative control and corrected by the positive control. [Toxicity] is the calculated membrane interference from the CellTox™ Green assay according to manufacturer's protocol. [Regeneration] is the relative TEER reduction corrected by the relative TEER reduction of control (KRB) 24 h after the start of the experiment.

3. Results

In Fig. 1, the relative TEER reduction over 180 min and after a regeneration time of 24 h is presented. All TJ modulators with the exception of labradimil at the concentrations tested were able to cause significant reduction in TEER up to 100 % within the first 15 min of incubation. Most modulators seem to have an all-or-nothing effect, showing no effect or a reduction of approximately 100 %. One millimolar sodium caprate and 10 mM sodium fluoride cause a TEER reduction of approximately 50 %. Among first-generation enhancers exhibiting a reduction of 100 %, only 1 mM EDTA showed TEER regeneration after 24 h. The TEER could also regenerate with the use of 25 mM AT-1002 and 10 μM PN159.

The results of the permeation studies are shown in Fig. 2. In contrast to the significant TEER reduction observed earlier, strong permeation enhancement was detected in this setup only for benzalkonium chloride, EDTA, sodium caprate and PN159 (permeation enhancement factor > 5). PN159 showed a concentration-dependent influence on mannitol permeation, whereas only 1 mM and 10 mM BAC caused permeation enhancement. Interestingly, for 10 mM sodium caprate, a distinctly higher effect was observed than that for 100 mM sodium caprate. This may be because the CMC concentration of 86 mM [48] was exceeded.

Fig. 3 illustrates the results of the MTT assay. First-generation enhancers reduced the metabolic activity of MDCK cells in contrast to most MB modulators. For first-generation enhancers, TEER reduction seems to be linked to the restriction of metabolic activity. In contrast, AT-1002 at TEER-reducing effective concentrations produced a time-dependent decrease in viability; however, the values were at least 50 % or higher. For 10 μM and 100 μM PN159, cell viabilities lower than 80 % were detected, but in contrast to first-generation modulators, 10 μM PN159 was able to reconstitute barrier properties and regenerate TEER to up to 90 % of the levels seen with the control (Fig. 1H).

The results from the CellTox™ Green assay are depicted in Fig. 4. In the case of first-generation enhancers, membrane toxicity was detected only for BAC and sodium caprate. This may be due to their chemical structures and amphiphilic properties. However, for BAC concentrations ranging from 100 μM to 10 mM, contrary to expectation, the determined toxicity levels decrease with higher concentrations, which may be due to fluorescence quenching at higher chloride concentrations [49]. Furthermore, a 180 min incubation with 100 mM sodium fluoride caused a toxicity of approximately 60 %. For the MB modulators AT-1002 and PN159, the MTT results correlate with membrane toxicity measured by the CellTox™ Green assay.

Benzalkonium chloride ranging from 0.005 % (~176 μM) to 0.02 % (~705 μM) is a commonly used preservative in many ophthalmic formulations. It has distinctive cytotoxic potential due to its chemical properties as a cationic surfactant, thereby increasing the ocular bioavailability of numerous topically applied drugs [50–52]. In this study, BAC at concentrations higher than 100 μM (~0.0028 %) caused a fast TEER reduction within the first 15 min. After 24 h of regeneration, cells that were previously incubated with 100 μM BAC showed a remaining TEER reduction of 44 %, whereas incubation with higher BAC concentration did not result in TEER regeneration. For pronounced permeation enhancement, BAC concentrations of 1 mM and higher were necessary and resulted in particularly high permeation enhancement factors up to 20. However, metabolic activity after 15 min incubation was already dramatically decreased to 38 % for 100 μM BAC. For higher concentrations of BAC as well as longer incubation periods, even extremely low metabolic activity values < 5 % were detected.

Ethylenediaminetetraacetic acid acts by depleting extracellular calcium and thereby activates protein kinase C [27]. Concentrations of EDTA above 1 mM caused TEER reduction within 15 min, but this reduction was not as fast as that seen with BAC. After 24 h, the TEER could regenerate completely in the case of 1 mM EDTA, whereas for 10 mM and 100 mM EDTA, the regeneration of barrier integrity was not observed. Permeation enhancement factors up to 8.0 were determined for 10 mM and 100 mM EDTA; however, lower concentrations did not cause improved mannitol permeability. Metabolic activity was above 70 % for all EDTA concentrations after 15 min of incubation but decreased thereafter in both a concentration- and time-dependent manner. Higher variances in these tests were, inter alia, reducible to the partial detachment of cells caused by the calcium-complexing behavior of EDTA. Toxic effects on membrane integrity were not detected for all EDTA concentrations in the CellTox™ Green assay.

A proposed mechanism of action for ***sodium fluoride*** as a TJM is Rho activation and, thereby, myosin light chain phosphorylation [29]. However, the toxic effects of fluoride should also be taken into account [30]. One hundred mM NaF showed fast TEER reduction without regeneration after 24 h, whereas 10 mM NaF exhibited a delayed reduction of 60 % after 30 min incubation time and regeneration up to 100 % after 24 h. Lower concentrations had no effect on the TEER. However, effective NaF concentrations showed no permeation enhancement and reduced metabolic activity.

Nitroprusside sodium effects arise from MLCK and PKC activation [31]. In this experimental setup, 100 mM nitroprusside sodium showed fast TEER reduction up to 100 %, and, for 10 mM nitroprusside sodium, a delayed effect after 90 min with TEER reduction up to 50 % was observed. The regeneration of barrier integrity was only detectable for nitroprusside sodium at a concentration of 10 mM. One hundred millimolar nitroprusside sodium produced 2.5-fold permeation enhancement but reduced the viability of MDCK cells to approximately 40 %.

Sodium caprate affects TJ function by increasing intracellular calcium levels due to its interaction with the cell membrane, resulting in the contraction of actin filaments [27]. Sodium caprate at concentrations of 10 mM and 100 mM reduced the TEER up to 100 % without regeneration, whereas 1 mM sodium caprate caused TEER reduction of 60 % with complete regeneration compared to the control. However,

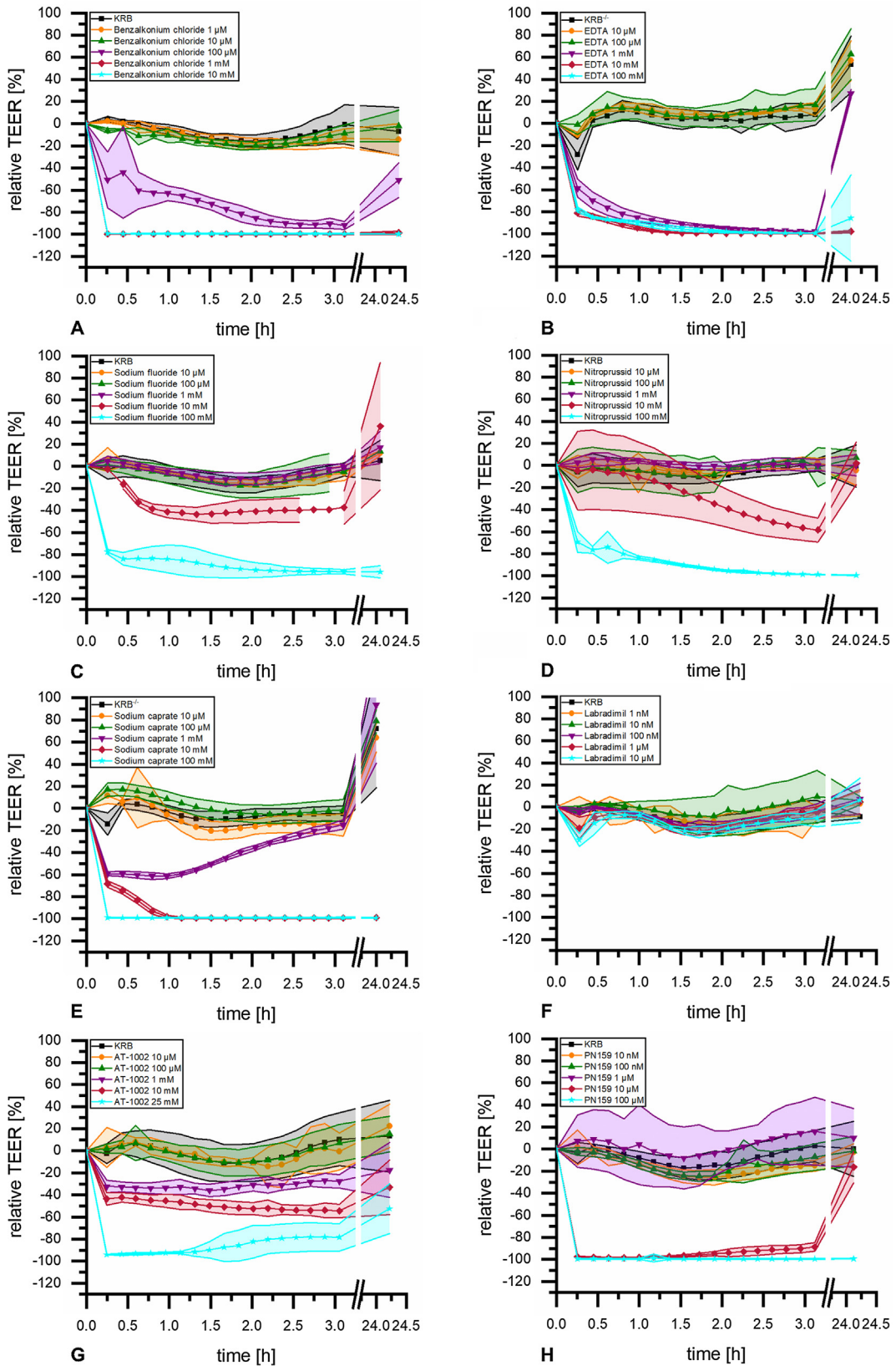


Fig. 1. The effect of eight different tight junction modulators (TJMs) on the transepithelial electrical resistance (TEER) of MDCK monolayers following incubation for 180 min and a recovery period of 21 h is shown. The TEER is expressed with the relative TEER, defined as the first value before adding the TJM, as 0 % and the following values expressed as a percentage change of the starting value. Graphs A–E illustrate the relative TEER development of five different first-generation TJMs, and the relative TEER values of three mechanism-based modulators are depicted in graphs F - H. TEER was continuously measured except for a short break after 180 min for substance depletion and washout. For testing of EDTA and sodium caprate (B, E), polyvalent cation-free KRB ($KRB^{-/-}$) was used, while other experiments were performed with KRB as a reference. The indicated values are the means of two independent experiments with n = 2–3 each ± SD.

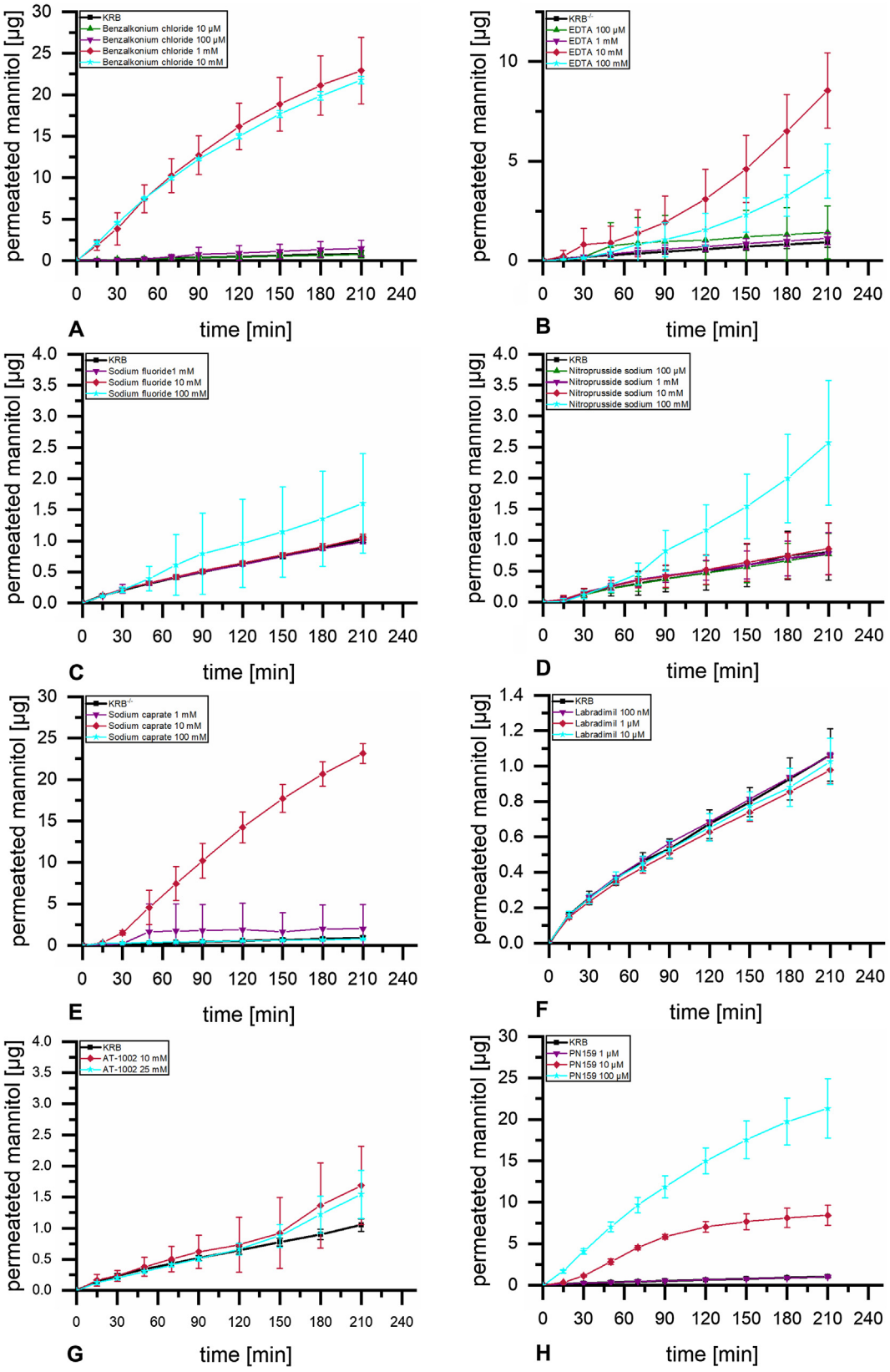


Fig. 2. Permeation profiles for 1 mM mannitol solution in the presence of eight different TJMs using MDCK monolayers as the permeation barrier for an incubation period of 210 min are shown. Figure A–E represent permeation graphs for five first-generation TJMs, whereas permeation graphs for three mechanism-based modulators are depicted in figure F–H. For permeation studies with EDTA and sodium caprate (B, E), polyvalent cation free KRB ($KRB^{-/-}$) was used, while other experiments were performed with KRB as a reference. The indicated values are the means of two independent experiments with n = 2–3 each ± SD.

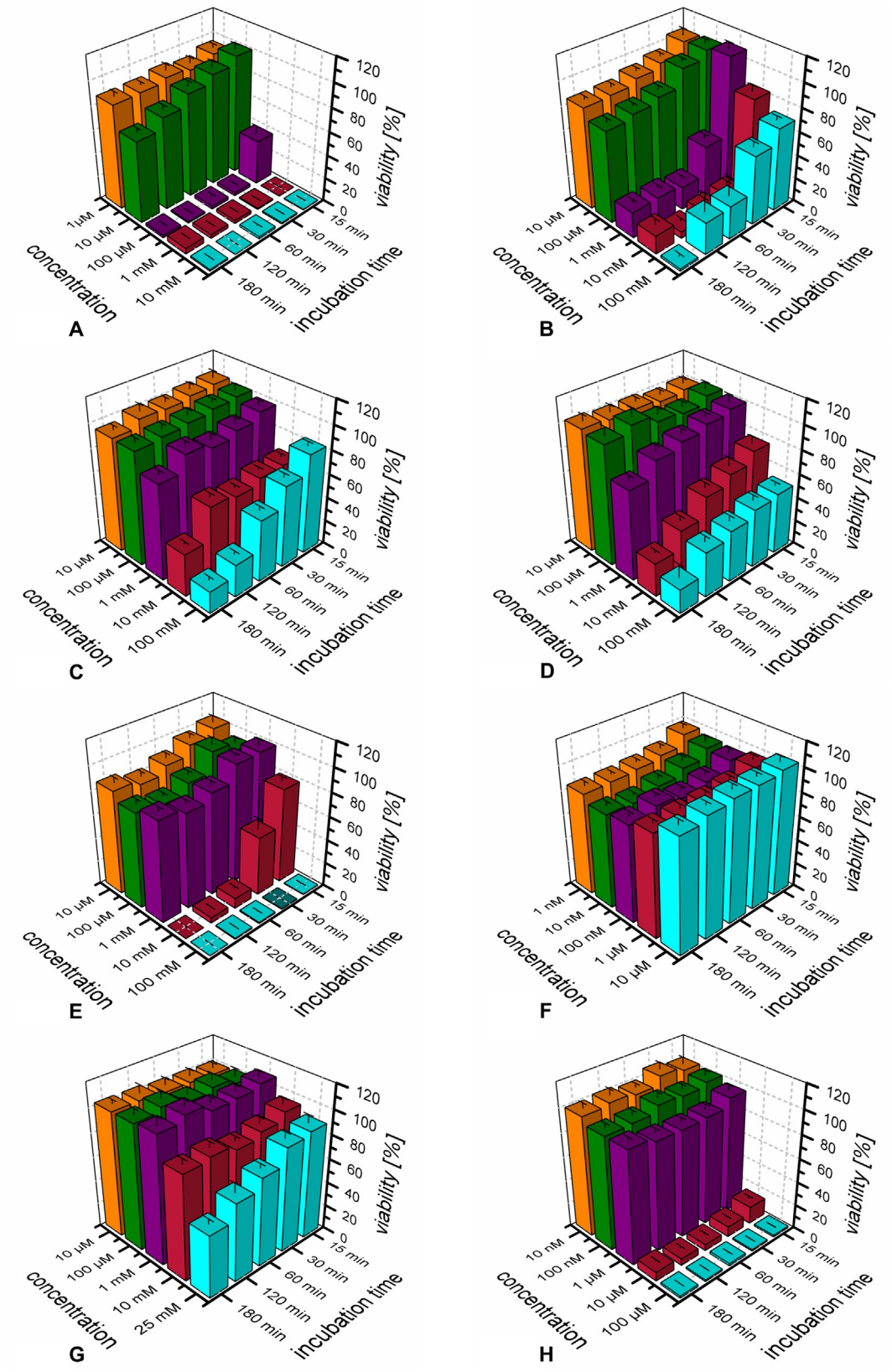


Fig. 3. The metabolic activity of MDCK cells in the presence of eight different TJMs after 15 min, 30 min, 60 min, 120 min and 180 min incubation time determined using the MTT assay and expressed as cell viability is depicted. For EDTA and sodium caprate (B, E), polyvalent cation-free KRB ($KRB^{-/-}$) was used as a negative control, and the other experiments were performed with KRB as a negative control (corresponding to 100 % cell viability). A 0.1 % Triton-X solution was used as a positive control for all experiments (corresponding to 0 % viability). Graphs A – E show MDCK cell viability after incubation with five different first-generation TJMs, whereas viability after treatment with three mechanism-based modulators is depicted in graphs F - H. The indicated values are the means of two independent experiments with n = 5–6 each ± SD.

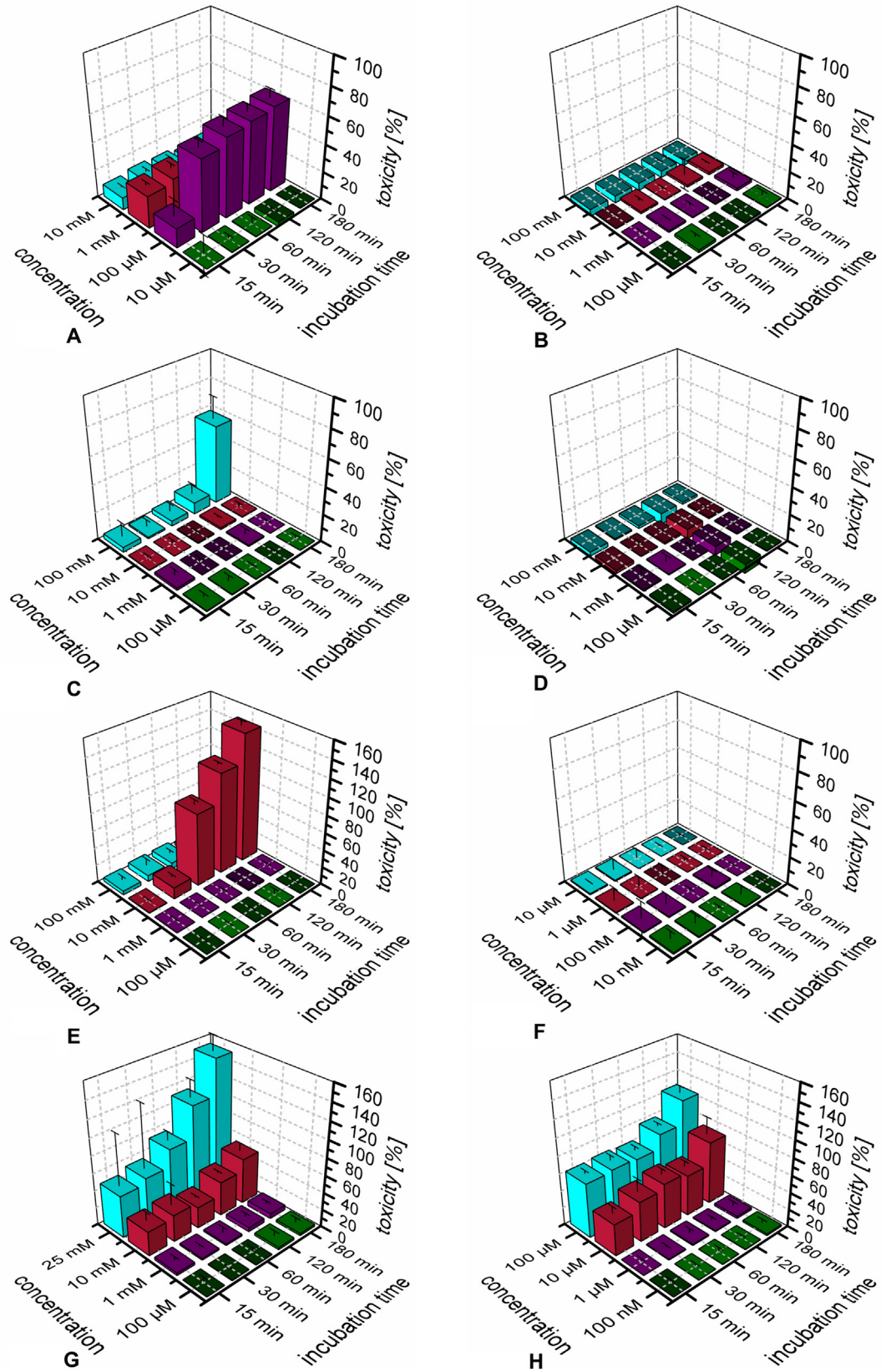


Fig. 4. Cytotoxic effects caused by eight different TJMs after 15 min, 30 min, 60 min, 120 min and 180 min incubation detected as changes of cell membrane integrity via CellTox™ Green assay are presented. Bars represent the relative cytotoxicity. For EDTA and sodium caprate (B, E), polyvalent cation-free KRB ($KRB^{-/-}$) was used as a negative control, and the other experiments were performed with KRB as a negative control (corresponding to 0% toxicity). As a positive control (corresponding to 100 % toxicity) for all experiments, lysis solution was used according to the manufacturer's protocol. Graphs A–E show the cytotoxicity of five different first-generation TJMs, while the cytotoxicity of three mechanism-based modulators is depicted in graphs F–H. The indicated values are the means of two independent experiments with n = 2–3 each ± SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

only for 10 mM sodium caprate was permeation enhancement detectable to a comparable extent as that found for 10 mM BAC. A distinct influence on cell viability was observed for 10 mM and 100 mM sodium caprate. In contrast to 100 mM sodium caprate, the cytotoxic effect of 10 mM sodium caprate was time-dependent.

Labradimil has been reported to increase the permeability of human brain microvascular endothelial cells, e.g., for 70 kDa dextran and inulin [33] and was tested in multiple clinical trials [19]. However, in this experimental setup, labradimil caused only a slight decrease in the TEER. Additionally, no effects of labradimil on permeation studies and the MTT and CellTox™ Green assays were detected.

The MB modulator ***AT-1002,*** known as a PAR2 receptor agonist [32], decreased TEER values at concentrations from 1 mM to 25 mM without resulting in the significant permeation enhancement of mannitol. Twenty-five millimolar AT-1002 showed a time-dependent reduction of metabolic activity, whereas for concentrations below 10 mM, viability was found to be above 80 %. The CellTox™ Green assay indicated time- and concentration-dependent effects on membrane integrity for 10 mM and 25 mM AT-1002.

For ***PN159***, a direct interaction with TJ proteins is hypothesized [34]. PN159 at concentrations of 10 μM and 100 μM showed strong TEER reduction as well as permeation enhancement comparable to BAC, but only for 10 μM was TEER recovery seen. However, both concentrations also ereduced metabolic activity and exhibited intensive effects in toxicity testing.

4. Discussion

Since increasing numbers of drug candidates currently exhibit low bioavailability [53] due to poor solubility and low permeability, a promising approach for improving drug absorption could be the enhancement of paracellular flux across epithelial barriers by tight junction modulation. In particular, advanced scientific understanding of tight junction regulation provides a chance for the targeted development of novel mechanism-based TJ modulators to increase permeability with fewer side effects than modulators from prior generations [54]. Based on the idea of developing TJ modulators for pharmaceutical application, the question of testing routines and parameters to describe their suitability came into focus. However, the current scientific literature reports no consistent approach for the specification of TJ modulators, resulting in a large number of cell lines with different barrier characteristics, a variety of cell viability and toxicity assays and many different permeation markers that are used for this purpose [46]. This has the great disadvantage that many of these studies are not comparable with one another. In fact, numerous TJ modulators have been described in recent decades, but only sodium caprate has been placed on the market and is used in commercial pharmaceutical products [55]. A crucial obstacle for companies could be the lack of valid preclinical data and test systems for selecting an appropriate compound for their purposes. Depending on the application, different characteristics of a TJ modulator are required, which makes comprehensive characterization indispensable. However, timely research regarding selected individual methods and special cell lines or tissues hampers, with respect to the low comparability of results, the comprehensive characterization of TJ modulators. Thus, formulation scientists are faced with the question of what modulator to choose for their purposes. The successful choice of TJ modulator depends on the application site and the dosage form, and one or the other property appears to be of greater importance on a case-by-case basis.

This study aimed to solve these problems by providing a unified test system for the assessment of TJ modulators. For this purpose, performance of the assays is to be fast and easy but nevertheless produce all relevant information for the examination of TJ modulators and provide high comparability for all enhancers. Second, a scoring system should be established on the basis of these results. The assignment of a specific score should simplify the choice of the most appropriate modulator for the respective application. As a consequence, the choice of an appropriate modulator, e.g., a modulator with a rapid response or low toxicity, is simplified and could improve the application of TJ modulators as universal permeation enhancers.

To implement such a test and scoring system, five first-generation modulators (EDTA, benzalkonium chloride, sodium caprate, sodium fluoride and nitroprusside sodium) as well as three MB modulators (labradimil, AT-1002 and PN159) were examined regarding their efficacy and toxicity. The eight tested compounds are known permeation enhancers with different mechanisms of action and differ in their tolerability, effectiveness, response time, TEER reduction and permeation enhancement. For this study, the MDCK I cell line, a well-established cellular model for testing epithelial permeability, was used. MDCK I cells have the advantage of showing high, stable TEER values of 3,500 Ω·cm^2 after five days of cultivation, which makes them very well suited for impedance measurements and permeation assays. Impedance was continuously measured with the cellZscope®, providing standardized and stable conditions. In contrast to simple resistance measurements, impedance measurements are not accompanied by temperature fluctuations or variation in electrode distance that result in different TEER values [56]. Permeation assays were performed with mannitol as a hydrophilic marker, which offers various advantages in comparison to fluorescence-based assays. Mannitol is nonionic and causes fewer interactions with charged TJ modulators. Furthermore, the quantitative analysis is not influenced by quenching effects from several cations and anions [49]. Cell viability was tested by the well-established MTT assay, and the cytotoxic effects of TJ modulators on cell membrane integrity were determined using the commercially available CellTox™ Green assay. Thus, the whole system is kept as simple as possible to provide the fast but still comprehensive and conclusive characterization of TJ modulators. The methods are unified and can be easily transferred to other laboratories to achieve not only intralaboratory but also interlaboratory comparability of different TJ modulators.

Comparison of the results obtained with this test system to those of previous studies that used a different experimental setup shows similar characteristics for some tested substances, but now more objective classification is allowed. For benzalkonium chloride, fast TEER reduction and permeation enhancement due to its high cytotoxicity were observed, as already reported for in vitro [26,50] and in vivo [57] studies. Another investigation revealed smaller cytotoxic effects of EDTA in comparison to those of benzalkonium chloride [58], as also detected in this study. Tomita et al. described the higher permeation enhancement of EDTA but higher TEER reduction of sodium caprate when the two are directly compared [27] using concentrations of 0.25 % (equivalent to 10 mM in this study). We found similar TEER reduction but higher permeation enhancement of sodium caprate. In contrast to our results, Ghadiri et al. recently reported no influence of EDTA on TEER as well as lower cytotoxicity of EDTA and sodium caprate [59]. However, this may be due not only to the use of Calu-3 cells but also to the use of Hank's balanced salt solution, which is not specified as calcium-free and could explain the differing results. AT-1002 has previously been shown to enhance the permeation of PEG 4000, inulin [39] and mannitol [60] in rats, but its effects on cell viability and toxicity were not tested. For labradimil, permeation enhancement was shown for inulin but not for dextran in human brain microvascular monolayers [33]. We were able to prove the TEER reduction and permeation enhancement of MDCK cells incubated with 10 μM and 100 μM PN159. Cui et al. reported cell viability > 80 % when treating the EpiAirway™ model with 25 μM, 50 μM and 100 μM PN159 [35], which is in contrast to the results obtained with our test system. Generally, the results of the present study confirm findings from previous investigations but also indicate the need for standardization of test systems for the comparability of TJ modulators.

With the prime objective of making future research on TJ modulators on the one hand comparable and on the other hand adjustable to the appropriate requirements and needs, we introduce a new *tight*

junction modulator scoring system (*TJMSS*, see also Section 2.6). It is designed to be as simple and flexible as possible without requiring additional experiments. Hence, it results, in the style of FMEA analysis, in a scoring system that includes the parameters of TEER reduction, permeation enhancement, viability, toxicity, and TEER regeneration with individual weighting factors from 1 to 5, finally combining all data describing a TJ modulator into one value (TJ modulator score). Final evaluation by spreadsheet application to calculate distinct scoring values is a fast and easy way to compare modulators with regard to certain applications from one set of data without further research (the corresponding Excel file has been made available in the supplementary data).

In the following, the selection of a suitable TJM for three drug application routes using the TJMSS is briefly presented. Although the TJMSS always uses the same data set, the results (TJ modulator score) are strongly dependent on the user's selected settings (i.e., incubation time and weighting of the TEER reduction, permeation enhancement, viability and TEER regeneration parameters with factors from 1 to 5). The first case considers ophthalmic products with challenging dosage forms. Due to the multilayered structure and expression of TJs, the cornea forms a strong absorption barrier for many ocular drugs. Moreover, lacrimation and eyelid blinking decrease the precorneal residence time of APIs, resulting in a very low bioavailability. Therefore, to increase paracellular drug absorption, ophthalmic formulations need a TJM with a short response time, and to avoid corneal damage, good tolerability is required. The effectiveness and tolerability of a TJM is of particular interest during the first 15 min after application because eye drops are quickly diluted and washed out by the tear fluid. Thus, in this case, we searched for a modulator with a short incubation time (choosing “15 min”) and weighted permeation enhancement and viability with a factor of “3″. Based on these assumptions, the TJMSS proposes labradimil, EDTA and sodium caprate as suitable enhancers. In the second case, the blood brain barrier (BBB) represents another physiological barrier that can hardly be passed by many potential APIs, e.g., for the therapy of neurological diseases. Considering the BBB, a longer TJM response time is assumed due to its systemic circulation time, and as a consequence, the TJM should exhibit good tolerability for a longer period of time. Accordingly, the incubation time was set to “60 min”, the weighting factors for TEER reduction and permeation enhancement were set to “3”, and the weighting factor for viability was set to “5”. For these settings, TJMSS proposes sodium caprate, labradimil and sodium fluoride as the most suitable enhancers. In the third case, e.g., oral administration, TEER regeneration may potentially be of great importance to reduce the unintended enhancement of other compounds because of the opened paracellular space to a minimum. Therefore, with an incubation time of “30 min” and weighting factors for permeation enhancement, TEER reduction and regeneration set to “3”, benzalkonium chloride, PN159 and sodium caprate seem to be the most suitable enhancers. Remarkably, for all of the chosen applications, sodium caprate, the only commercially used TJ modulator, is among the three suitable enhancers. Nevertheless, the result of scoring and ranking of the TJMSS always depends on the selected parameters and factors.

5. Conclusions

The experimental setup of this study provided data for the adequate characterization of TJ modulators, showing their specific strengths and weaknesses. The TJMSS established on the basis of these data allows us to evaluate TJ modulators for the first time. Thus, this is the first study proposing a unified procedure that tests TJ modulators and makes results about different compounds or from different laboratories comparable, with the aim of giving a promising class of permeation enhancers better access to the market. Furthermore, a guideline is offered for the structured testing of TJ modulators by the unification of present approaches that establishes a general applicable strategy for the evaluation of TJMs. Previous problems of comparability are reduced by the unified test system, and the novel TJMSS facilitates the interpretation of the results obtained with this test system, particularly in relation to the specific application of the TJM.

However, even though the TJMSS enables the uniform evaluation of TJ modulators, the effects of these modulators on the target tissue still have to be tested. In addition, possible interactions between TJM and API or the drug delivery system and their impact on modulating effects are currently not considered by the TJMSS. Therefore, TJMSS could be extended in the future so that only TJMs with desired physicochemical properties are included in the ranking. Furthermore, to validate the newly introduced TJMSS, further studies should be conducted to investigate whether this in vitro procedure can be correlated with in vivo studies. This could make a valuable contribution to preclinical formulation development. Furthermore, this study could form the essential basis to create a TJ modulator database to establish the widespread use of this promising class of permeation enhancers in the market.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.07.004.

References

[1] Sergei Dikler, Scott A. Busby, W. Adam, G. Hill (Eds.), MALDI-HTS beyond 100,000 samples per day in Drug Discovery, 2016.
[2] L.Z. Benet, F. Broccatelli, T.I. Oprea, BDDCS applied to over 900 drugs, The AAPS J. 13 (2011) 519–547.
[3] W.M. Pardridge, The blood-brain barrier: bottleneck in brain drug development, NeuroRx J. Am. Soc. Exp. NeuroTherapeutics 2 (2005) 3–14.
[4] M.L. Sears, Pharmacology of the Eye. Ocular Pharmacokinetics, Springer Berlin Heidelberg, Berlin, Heidelberg, 1984.
[5] K. Basler, S. Bergmann, M. Heisig, A. Naegel, M. Zorn-Kruppa, J.M. Brandner, The role of tight junctions in skin barrier function and dermal absorption, J. Control. Release Off. J. Control. Release Soc. 242 (2016) 105–118.
[6] V.K. Pawar, J.G. Meher, Y. Singh, M. Chaurasia, B. Surendar Reddy, M.K. Chourasia, Targeting of gastrointestinal tract for amended delivery of protein/peptide therapeutics: strategies and industrial perspectives, J. Control. Release Off. J. Control. Release Soc. 196 (2014) 168–183.
[7] J.M. Anderson, C.M. van Itallie, Physiology and function of the tight junction, Cold Spring Harbor Perspect. Biol. 1 (2009) a002584.
[8] J.P. Chalcroft, S. Bullivant, An interpretation of liver cell membrane and junction structure based on observation of freeze-fracture replicas of both sides of the fracture, J. Cell Biol. 47 (1970) 49–60.
[9] M. Furuse, T. Hirase, M. Itoh, A. Nagafuchi, S. Yonemura, S. Tsukita, Occludin: a novel integral membrane protein localizing at tight junctions, J. Cell Biol. 123 (1993) 1777–1788.
[10] M. Furuse, K. Fujita, T. Hiiragi, K. Fujimoto, S. Tsukita, Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin, J. Cell Biol. 141 (1998) 1539–1550.
[11] J. Ikenouchi, M. Furuse, K. Furuse, H. Sasaki, S. Tsukita, S. Tsukita, Tricellulin constitutes a novel barrier at tricellular contacts of epithelial cells, J. Cell Biol. 171 (2005) 939–945.
[12] I. Martìn-Padura, S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, D. Simmons, E. Dejana, Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration, J. Cell Biol. 142 (1998) 117–127.
[13] A.S. Fanning, B.J. Jameson, L.A. Jesaitis, J.M. Anderson, The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton, J. Biol. Chem. 273 (1998) 29745–29753.
[14] M. Itoh, M. Furuse, K. Morita, K. Kubota, M. Saitou, S. Tsukita, Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins, J. Cell Biol. 147 (1999) 1351–1363.
[15] G. Bazzoni, O.M. Martinez-Estrada, F. Orsenigo, M. Cordenonsi, S. Citi, E. Dejana, Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin, J. Biol. Chem. 275 (2000) 20520–20526.
[16] J.L. Madara, R. Moore, S. Carlson, Alteration of intestinal tight junction structure and permeability by cytoskeletal contraction, Am. J. Physiol. 253 (1987) 61.
[17] H.F. Lodish, Molecular Cell Biology, 3. print ed., 6. ed., Freeman, New York, NY, 2008.
[18] E.D. Le Shen, E.D. Black, W.I. Witkowski, V. Lencer, E.E. Guerriero, J.R. Schneeberger, Turner, Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure, J. Cell Sci. 119 (2006) 2095–2106.
[19] D. Saaber, S. Wollenhaupt, K. Baumann, S. Reichl, Recent progress in tight junction modulation for improving bioavailability, Expert Opin. Drug Discov. 9 (2014) 367–381.

[20] G. Karp, K. Beginnen, Molekulare Zellbiologie, 1., dt. Auflage ed., Springer, Berlin, 2005.
[21] M. Lal-Nag, P.J. Morin, The claudins, Genome Biol. 10 (2009) 235.
[22] P. Claude, D.A. Goodenough, Fracture faces of zonulae occludentes from "tight" and "leaky" epithelia, J. Cell Biol. 58 (1973) 390–400.
[23] D. Günzel, A.S.L. Yu, Claudins and the modulation of tight junction permeability, Physiol. Rev. 93 (2013) 525–569.
[24] M. Kondoh, T. Yoshida, H. Kakutani, K. Yagi, Targeting tight junction proteins-significance for drug development, Drug Discov. Today 13 (2008) 180–186.
[25] R.W. Yee, The effect of drop vehicle on the efficacy and side effects of topical glaucoma therapy: a review, Curr. Opin. Ophthalmol. 18 (2007) 134–139.
[26] A. Narai, S. Arai, M. Shimizu, Rapid decrease in transepithelial electrical resistance of human intestinal Caco-2 cell monolayers by cytotoxic membrane perturbents, Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 11 (1997) 347–354.
[27] M. Tomita, M. Hayashi, S. Awazu, Absorption-enhancing mechanism of EDTA, caprate, and decanoylcarnitine in Caco-2 cells, J. Pharm. Sci. 85 (1996) 608–611.
[28] T. Lindmark, Y. Kimura, P. Artursson, Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in Caco-2 cells, J. Pharmacol. Exp. Ther. 284 (1998) 362–369.
[29] P. Wang, A.D. Verin, A. Birukova, L.I. Gilbert-McClain, K. Jacobs, J.G. Garcia, Mechanisms of sodium fluoride-induced endothelial cell barrier dysfunction: role of MLC phosphorylation, Am. J. Physiol. Lung Cell. Mol. Physiol. 281 (2001) 83.
[30] S. Dogan, H. Gunay, G. Leyhausen, W. Geurtsen, Chemical-biological interactions of NaF with three different cell lines and the caries pathogen Streptococcus sobrinus, Clin. Oral Invest. 6 (2002) 92–97.
[31] F.L. Cardoso, D. Brites, M.A. Brito, Looking at the blood-brain barrier: molecular anatomy and possible investigation approaches, Brain Res. Rev. 64 (2010) 328–363.
[32] S. Gopalakrishnan, N. Pandey, A.P. Tamiz, J. Vere, R. Carrasco, R. Somerville, A. Tripathi, M. Ginski, B.M. Paterson, S.S. Alkan, Mechanism of action of ZOT-derived peptide AT-1002, a tight junction regulator and absorption enhancer, Int. J. Pharm. (Int. J. Pharmaceut.) 365 (2009) 121–130.
[33] J.B. Mackic, M. Stins, S. Jovanovic, K.S. Kim, R.T. Bartus, B.V. Zlokovic, Cereport (RMP-7) increases the permeability of human brain microvascular endothelial cell monolayers, Pharm. Res. 16 (1999) 1360–1365.
[34] P.H. Johnson, S.C. Quay, Advances in nasal drug delivery through tight junction technology, Expert Opin. Drug Deliv. (Expert Opin. Drug Del.) 2 (2005) 281–298.
[35] K. Cui, S.-C. Chen, M.E. Houston, S.C. Quay, Tight junction modulator peptide PN159 for enhanced mucosal delivery of therapeutic compounds, United States Patent 7,244,709 B2, 2005.
[36] K. Matter, M.S. Balda, Functional analysis of tight junctions, Methods (San Diego, Calif.) 30, 2003, pp. 228–234.
[37] P.J. Gaillard, A.G. de Boer, Relationship between permeability status of the blood-brain barrier and in vitro permeability coefficient of a drug, Eur. J. Pharm. Sci. 12 (2000) 95–102.
[38] N. Kirschner, J.M. Brandner, Barriers and more: functions of tight junction proteins in the skin, Ann. N. Y. Acad. Sci. 1257 (2012) 158–166.
[39] K.-H. Song, A. Fasano, N.D. Eddington, Enhanced nasal absorption of hydrophilic markers after dosing with AT1002, a tight junction modulator, Eur. J. Pharm. Biopharm. (Eur. J. Pharm. Biopharm. Off. J. Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V) 69 (2008) 231–237.
[40] T. Inamura, T. Nomura, R.T. Bartus, K.L. Black, Intracarotid infusion of RMP-7, a bradykinin analog: a method for selective drug delivery to brain tumors, J. Neurosurg. 81 (1994) 752–758.
[41] S.M. Krug, M. Amasheh, I. Dittmann, I. Christoffel, M. Fromm, S. Amasheh, Sodium caprate as an enhancer of macromolecule permeation across tricellular tight junctions of intestinal cells, Biomaterials 34 (2013) 275–282.
[42] C.J. Watson, M. Rowland, G. Warhurst, Functional modeling of tight junctions in intestinal cell monolayers using polyethylene glycol oligomers, Am. J. Physiol. Cell Physiol. 281 (2001) 97.
[43] Van Itallie, M. Christina, J. Holmes, A. Bridges, J.L. Gookin, M.R. Coccaro, W. Proctor, O.R. Colegio, J.M. Anderson, The density of small tight junction pores varies among cell types and is increased by expression of claudin-2, J. Cell Sci. 121 (2008) 298–305.
[44] J.D. Irvine, L. Takahashi, K. Lockhart, J. Cheong, J.W. Tolan, H.E. Selick, J.R. Grove, MDCK (Madin-Darby canine kidney) cells: A tool for membrane permeability screening, J. Pharm. Sci. 88 (1999) 28–33.
[45] R.I. Freshney, Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, sixth ed., Wiley-Blackwell, Hoboken, NJ, 2010.
[46] M.A. Deli, C.S. Abrahám, Y. Kataoka, M. Niwa, Permeability studies on in vitro blood-brain barrier models: physiology, pathology, and pharmacology, Cell. Mol. Neurobiol. 25 (2005) 59–127.
[47] M.A. Deli, Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery, Biochim. Biophys. Acta, Biomembr. 1788 (2009) 892–910.
[48] F.E. Stanley, A.M. Warner, E. Schneiderman, A.M. Stalcup, Rapid determination of surfactant critical micelle concentrations using pressure-driven flow with capillary electrophoresis instrumentation, J. Chromatogr. A 1216 (2009) 8431–8434.
[49] Quenching of Fluorescence, in: J.R. Lakowicz (Ed.), Principles of Fluorescence Spectroscopy, Springer US, Boston, MA, 2006, pp. 277–330.
[50] H. Sasaki, T. Nagano, K. Yamamura, K. Nishida, J. Nakamura, Ophthalmic preservatives as absorption promoters for ocular drug delivery, J. Pharm. Pharmacol. 47 (1995) 703–707.
[51] H. Sasaki, K. Yamamura, T. Mukai, K. Nishida, J. Nakamura, M. Nakashima, M. Ichikawa, Modification of ocular permeability of peptide drugs by absorption promoters, Biol. Pharm. Bull. 23 (2000) 1524–1527.
[52] S. Majumdar, K. Hippalgaonkar, M.A. Repka, Effect of chitosan, benzalkonium chloride and ethylenediaminetetraacetic acid on permeation of acyclovir across isolated rabbit cornea, Int. J. Pharm. 348 (2008) 175–178.
[53] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Adv. Drug Deliv. Rev. 46 (2001) 3–26.
[54] M. Kondoh, A. Takahashi, K. Yagi, Spiral progression in the development of absorption enhancers based on the biology of tight junctions, Adv. Drug Deliv. Rev. 64 (2012) 515–522.
[55] M. Kondoh, K. Yagi, Tight junction modulators: promising candidates for drug delivery, Curr. Med. Chem. 14 (2007) 2482–2488.
[56] B. Srinivasan, A.R. Kolli, M.B. Esch, H.E. Abaci, M.L. Shuler, J.J. Hickman, TEER measurement techniques for in vitro barrier model systems, J. Lab. Automat. 20 (2015) 107–126.
[57] M. Kusano, M. Uematsu, T. Kumagami, H. Sasaki, T. Kitaoka, Evaluation of acute corneal barrier change induced by topically applied preservatives using corneal transepithelial electric resistance in vivo, Cornea 29 (2010) 80–85.
[58] S. Burgalassi, P. Chetoni, D. Monti, M.F. Saettone, Cytotoxicity of potential ocular permeation enhancers evaluated on rabbit and human corneal epithelial cell lines, Toxicol. Lett. 122 (2001) 1–8.
[59] M. Ghadiri, P.M. Young, W. Jarolimek, G.E.R. Grau, B.G.G. Oliver, D. Traini, The effect of non-specific tight junction modulators on the transepithelial transport of poorly permeable drugs across airway epithelial cells, J. Drug Target. 25 (2017) 342–349.
[60] K.-H. Song, N. Eddington, The influence of stabilizer and bioadhesive polymer on the permeation-enhancing effect of AT1002 in the nasal delivery of a paracellular marker, Arch. Pharm. Res. 35 (2012) 359–366.