Unravelling the modulation of tight junctions: Molecular mechanisms and permeation enhancement

Brunner, Joël

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THÈSE

présentée aux Facultés de médecine et des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences en sciences de la vie,
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par

Joël BRUNNER

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Thèse N° 158

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Les Facultés de médecine et des sciences, sur le préavis de Monsieur G. BORCHARD, professeur ordinaire et directeur de thèse (Section des sciences pharmaceutiques), Monsieur L. SCAPOZZA, professeur ordinaire (Section des sciences pharmaceutiques), Madame A. MÜLLERTZ, professeure (Department of Pharmacy, University of Copenhagen, Copenhagen, Denmark) et Monsieur C.-M. LEHR, professeur (Department of drug delivery, Helmholtz Institute for Pharmaceutical Research Saarland, Saarland, Germany), autorisent l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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To my family, colleagues and friends…

“He who thinks too much about every step he takes will always stay on one leg.”

Chinese proverb

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English summary

The pharmacokinetic parameters of a therapeutic molecule are crucial. Absorption in the first instance gives the drug the opportunity to enter the body and reach the systemic circulation. Some treatments are poorly or not at all absorbed through the epithelia. They are therefore invasively administered. Facilitating and increasing the absorption of molecules would make it possible to reduce administered doses and also to change the route of administration of certain treatments for non-invasive administration, such as a nasal spray or eye drops.

Tight junctions (TJs) are a set of proteins responsible for holding cells together and regulating the permeability of exogenous nutrients and compounds across the cell layer. Controlled modulation of TJs could increase the absorption of drugs across the mucosa.

The enzyme protein kinase C zeta (PKC ζ) has been shown to be one of the modulators responsible for the expression of TJs proteins such as occludin and Zonula occludens (ZO). This enzyme is also involved in the activation of these proteins to close TJs. Thus, inhibition of PKC ζ activity would prevent closure of TJs and transiently increase the permeability of molecules across the epithelium.

In the structure of PKC ζ, a part called pseudosubstrate (PS) has been identified as the autoinhibitory segment of the enzyme's activity. The creation of a synthetic peptide with the same sequence as this segment could inhibit PKC ζ activity using the enzyme's natural mechanism. Our L-R5 pentapeptide with a myristoylated tail was used, optimised and tested throughout this thesis.

The objectives of this PhD thesis were (1) to attest to the efficacy and non-toxicity of L-R5 in opening TJs, (2) to determine the exact mechanism of inhibition of PKC ζ activity by L-R5, (3) to explore the possibilities of optimisation in the sequence and structure of L-R5 and (4) to produce a L-R5 coupled insulin formulation for nasal administration.

Chapter 1 is a review and overview of the different manners to increase permeability. Several mechanisms have been studied. Some are based on environmental changes in the cell, while others are focused to modulate a specific target in the opening mechanisms of the intercellular junctions. Target-specific permeation enhancers (PEs) have been shown to better control the uptake of drugs and tracer molecules. Moreover, only part of the intracellular function is modulated. However, the transition to clinical phases is often unconvincing for these PEs. More optimisation and research are still needed before a target-specific PE is found on the market.

Chapter 2 demonstrates the effectiveness of L-R5 in increasing permeability. Indeed, the pentapeptide increases the passage of naloxone and fluorescein through a layer of primary

nasal cells. Furthermore, the cytotoxicity of the peptide as well as its haemolytic capacity were measured. Due to some haemolysis observed in the presence of L-R5 due to its myristoyl tail, it was then confirmed that the peptide did not pass through the cell layer, as did naloxone or fluorescein 4 kDa dextran. For this purpose, a UHPLC-MS/MS analytical method was developed and validated. This chapter demonstrated the efficacy of the peptide as well as the safe use in a future *in vivo* application. The putative mechanism of L-R5 to interact with PKC ζ was the first suggestion as an intracellular action. Thus, in order to support the evolution along this thesis, this inaccuracy corrected in the following chapter has been retained as originally thought.

The exact intracellular mechanism of application of L-R5 to cells was still unknown. **Chapter 3** demonstrates that the pentapeptide does not interact with PKC ζ directly, as originally thought. The PS part of the enzyme interacts electrostatically with occludin in order to attach to it and phosphorylate the protein. When L-R5 enters the cell, it competes with this interaction and thus prevents the activation of occludin. TJs therefore do not close, which increases the permeability of the cell layer. In addition, the expression of occludin and ZO-1 is decreased in presence of the peptide. Finally, a proteomic analysis was performed and showed that the impact of L-R5 was much wider than just on TJs. Despite the absence of toxicity, the peptide influences many more intracellular mechanisms than expected. This may be explained by its affinity for all potential PKC ζ target proteins, if not all PKCs. This broad range of action could also provide new opportunities for treatment.

Chapter 4 explores the possibilities of optimising L-R5. For this purpose, a modification of the myristoyl tail was performed, as well as changes in peptidic structures. The modification of the fatty acid was intended to counteract the haemolytic effect of the peptide. Unfortunately, this effect is unavoidable in the presence of fatty acids, and this myristoyl is necessary for the peptide to enter the cell. The D-form of the peptide as well as the peptide with mixed amino acids equally increases the permeability of the 4 kDa dextran fluorescein through the Caco-2 cell layer. In contrast, the replacement of certain amino acids decreased or even cancelled the effect of the peptide on permeability. Finally, a comparison with other PEs in studies or on the market was made. Considering the toxicity, the increase in permeability and the dose administered, L-R5 is comparable, if not better than the other molecules tested.

The final objective of this PhD thesis was to develop a formulation of L-R5 with insulin for nasal application. In **chapter 5**, a formulation of ultra-rapid insulin coupled to L-R5 was tested *in vitro* as well as *in vivo*. An interaction test between a recombinant human insulin and the peptide was also performed by circular dichroism. No influence on the secondary structure of insulin by L-R5 was found. The *in vitro* permeability of insulin through a nasal primary cell layer

could also be increased in the presence of the peptide. On the other hand, the *in vivo* transition was not as successful. The formulation was nasally administered to mice that had developed type 1 diabetes. No significant reduction in blood glucose levels was observed. The development of medical devices for nasal administration in mice would certainly have yielded better results.

In conclusion, it was demonstrated during this PhD thesis that L-R5 is a promising PE. Its intracellular mechanism was demonstrated in order to better optimise the desired effect and the potential structural modifications required. The efficacy of the peptide has been repeatedly demonstrated, as well as its lack of toxicity. Further formulation studies will be required. In addition, proteomics has opened up the ways of action of L-R5 and given new possibilities for applications. Finally, other routes of administration, such as the ocular route, should be considered for the formulation of this peptide.

Résumé en français

Les paramètres pharmacocinétiques d'une molécule thérapeutique sont primordiaux. L'absorption en premier lieu donne la possibilité au médicament d'intégrer l'organisme afin de rejoindre la circulation systémique. Certains traitements sont peu ou pas absorbés à travers les épithélia. Ils sont donc administrés de manière invasive. Faciliter et augmenter l'absorption des molécules permettrait de diminuer les doses administrées et également changer la voie d'administration de certains traitements pour une administration non-invasive, comme un spray nasal ou des gouttes oculaires.

Les jonctions serrées (TJs) sont un ensemble de protéines responsables du maintien entre les cellules ainsi que de la gestion perméabilité de nutriments et composés exogènes à travers la couche cellulaire. Une modulation contrôlée des TJs permettrait d'augmenter l'absorption des médicaments à travers les muqueuses.

Il a été démontré que l'enzyme protéine kinase C zêta (PKC ζ) est un des modulateurs responsables de l'expression des protéines des TJs comme occludine et Zonula occludens (ZO). Cette enzyme est également impliquée dans l'activation de ces protéines pour fermer les TJs. Ainsi, une inhibition de l'activité de PKC ζ empêcherait la fermeture des TJs et augmenterait donc transitoirement la perméabilité des molécules à travers l'*épithélium*.

Dans la structure de PKC ζ, une partie appelée pseudosubstrat (PS) a été identifiée comme le segment auto inhibiteur de l'activité de l'enzyme. La création d'un peptide synthétique possédant la même séquence que ce segment pourrait permettre d'inhiber l'activité de PKC ζ en utilisant le mécanisme naturel de l'enzyme. Notre pentapeptide L-R5 possédant une extrémité myristoylée a été utilisé, optimisé et testé tout au long de ce travail de thèse.

Les objectifs de cette thèse de doctorat étaient (1) d'attester de l'efficacité et de la non toxicité de L-R5 dans l'ouverture des TJs, (2) de déterminer l'exact mécanisme d'inhibition d'activité de PKC ζ par L-R5, (3) d'explorer les possibilités d'optimisation dans la séquence et structure de L-R5 et (4) de réaliser une formulation d'insuline couplée à L-R5 pour une administration nasale.

Le **chapitre 1** est une revue et vue d'ensemble des différents moyens d'augmenter la perméabilité. Plusieurs mécanismes ont été étudiés. Certains se basent sur des changements environnementaux de la cellule, alors que d'autres se spécifient à moduler une cible précise dans le mécanisme d'ouverture des jonctions intercellulaires. Il s'avère que les modulateurs de la perméabilité à cible spécifique permettent un meilleur contrôle de l'absorption des médicaments et molécules traceurs. De plus, seule une partie du fonctionnement intracellulaire est perturbée. Cependant, la transition aux phases cliniques est souvent peu

convaincante pour ces promoteurs d'absorption. Davantage d'optimisations et de recherches sont encore nécessaires avant de trouver un promoteur d'absorption à cible spécifique sur le marché.

Le **chapitre 2** démontre l'efficacité de L-R5 dans l'augmentation de la perméabilité. En effet, le pentapeptide augmente le passage de naloxone et fluorescéine à travers une couche de cellules primaires nasales. De plus, la cytotoxicité du peptide ainsi que sa capacité hémolytique ont été mesurées. En raison d'une certaine hémolyse constatée en présence de L-R5, il a été ensuite confirmé que le peptide ne passait pas à travers la couche cellulaire, à l'instar de la naloxone ou la fluorescéine dextran 4 kDa. Pour cela, une méthode analytique UHPLC-MS/MS a été développée et validée. Ce chapitre aura démontré l'efficacité du peptide ainsi que la garantie d'utilisation dans une future application *in vivo*. Le mécanisme présumé de L-R5 d'interagir avec PKC ζ était la première suggestion d'action intracellulaire. Ainsi, afin d'appuyer l'évolution dans ce travail de thèse, cette inexactitude corrigée dans le chapitre suivant a été conservée telle que pensée originalement.

Le mécanisme intracellulaire exact lors de l'application de L-R5 sur des cellules était toujours inconnu. Le **chapitre 3** démontre que le pentapeptide n'interagit pas avec PKC ζ directement, comme il était pensé initialement. La partie PS de l'enzyme interagit avec occludine des TJs de manière électrostatique afin de s'y attacher et phosphoryler la protéine. Lorsque L-R5 entre dans la cellule, il entre en compétition avec cette interaction et empêche donc l'activation d'occludine. Les TJs ne se ferment donc pas, ce qui augmente la perméabilité de la couche cellulaire. De plus, l'expression d'occludine et de ZO-1 est diminuée en présence du peptide. Enfin, une analyse protéomique a été effectuée et a démontré que l'impact de L-R5 était bien plus large qu'uniquement sur les TJs. Malgré une absence de toxicité constatée, le peptide influe beaucoup plus de mécanismes intracellulaires que prévu. Cela peut être expliqué par son affinité avec toutes les potentielles protéines cibles de PKC ζ, voire de toutes les PKC. Ce large champ d'action pourrait également donner de nouvelles opportunités de traitements.

Le **chapitre 4** explore les possibilités d'optimisation de L-R5. Pour cela, une modification de l'extrémité myristoyl a été effectuée, ainsi que des changements de structures peptidiques. La modification de l'acide gras a été voulue afin de pallier à l'effet hémolytique du peptide. Malheureusement, cet effet est inévitable en présence d'acides gras, et ce myristoyl est nécessaire au peptide pour entrer dans la cellule. La forme dextrogyre du peptide ainsi que le peptide avec des acides aminés mélangés augmentent d'une même importance la perméabilité de la fluorescéine dextran 4 kDa à travers la couche de cellule Caco-2. En revanche, le remplacement de certains acides aminés a diminué voire annuler l'effet du peptide sur la perméabilité. Enfin, une comparaison avec d'autres promoteurs d'absorption en

études ou sur le marché a été faite. En tenant compte de la toxicité, de l'augmentation de la perméabilité et de la dose administrée, L-R5 est comparable, si ce n'est meilleur que les autres molécules testées.

L'objectif final de ce travail de doctorat était une formulation de L-R5 avec de l'insuline pour une application nasale. Dans le **chapitre 5**, une formulation composée d'insuline ultra rapide couplée à L-R5 a été testée *in vitro* ainsi qu'*in vivo*. Un test d'interaction entre une insuline recombinante humaine et le peptide a également été effectué par dichroïsme circulaire. Aucune influence sur la structure secondaire de l'insuline par L-R5 n'a été constatée. La perméabilité *in vitro* de l'insuline à travers une couche de cellules primaire nasale a également pu être augmentée en présence du peptide. La transition *in vivo* n'a pas eu le même succès. La formulation a été administrée par voie nasale à des souris ayant développé le diabète de type 1. Aucune réduction significative de la glycémie n'a été constatée. Le développement de dispositif médicaux pour l'administration nasale chez les souris aurait sûrement donné de meilleurs résultats.

En conclusion, il a été démontré durant ce travail de doctorat que L-R5 est un promoteur d'absorption prometteur. Son mécanisme intracellulaire a été démontré afin de mieux pouvoir optimiser l'effet voulu et les potentielles modifications structurelles nécessaires. L'efficacité du peptide a été maintes fois démontrée, de même que son absence de toxicité. Des études supplémentaires quant à la formulation seront nécessaires. De plus, la protéomique a ouvert le champ d'action de L-R5 et donné de nouvelles possibilités d'applications. Enfin, d'autres voies d'administrations, telles que la voie oculaire, sont à considérer pour la formulation de ce peptide.

Chapter 1: General introduction

Target specific tight junction modulators

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Foreword: The pharmacokinetic parameter “absorption” is fundamental for drugs to obtain good efficacy of action. Increasing the permeability of therapeutic molecules through mucous membranes allows for a reduction in administered doses and possibly allows for some molecules to change from invasive to non-invasive administration. This review explores the different mechanisms that have been developed and tested to increase drug absorption.

This review, entitled “Target specific tight junction modulators”, was published in the journal *Advanced Drug Delivery Reviews* in February 2021 (DOI: 10.1016/j.addr.2021.02.008). The accepted manuscript is presented below.

Author contribution: Part 3 of the manuscript was written by Dr. Sakthikumar Ragupathy. The rest has been written by myself with the support of Prof. Gerrit Borchard.

Declaration of interest:

The authors declare no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Abstract

Intercellular tight junctions represent a formidable barrier against paracellular drug absorption at epithelia (nasal, intestinal) and the endothelium (blood-brain barrier). In order to enhance paracellular transport of drugs and increase their bioavailability and organ deposition, active excipients modulating tight junctions have been applied. First-generation of permeation enhancers (PEs) acted unspecific mechanisms, while recently developed PEs address specific physiological mechanisms. Such target specific tight junction modulators (TJMs) exert the advantage of a defined specific mechanism of action. To date, merely a few of these novel active excipients has entered clinical trials, as their lack in safety and efficiency *in vivo* often impedes their commercialisation. A stronger focus on the development of such active excipients would result in an economic and therapeutic improvement of current and future drugs.

Keywords: epithelial permeability; tight junctions; paracellular pathway; claudin; occludin; Zonula occludens

1. Introduction

Many drugs exist on the market with different routes of administration. One of the main challenges for new drugs is to assure the absorption of these and to deliver them at the right place, at the right moment. Chemical modifications and working with formulations hurdles are daily concerns to optimize drugs bioavailability. Drug candidates of low molecular weight under development are generally of low epithelial permeability and therefore of low bioavailability [1]. Based on the last report by the World Health Organization (WHO), about 35% of oral drugs approved between the years 2000 and 2011 are described as BCS (Biopharmaceutics Classification System) class 3 drugs, characterized by their low permeability and high solubility [2]. Moreover, 85% of drugs developed in Europe and the USA are applied by oral administration [3]. An option currently being explored in order to enhance bioavailability of such drugs is to increase the permeability of the intestinal epithelium. Thereby, parenteral injection, which is associated with safety concerns and does meet with lower patient compliance, may be avoided. The final objective of enhancing oral absorption would be to reach parenteral bioavailability using a safer and more accepted route of administration.

Many different biological barriers impede on oral drug absorption. These barriers form a separation between the body and the environment as well as between distinct fluid compartments. Mucosal epithelia represent the thin layers preventing infections and entry of external organisms and molecules. Mucus is produced by specialized cells and secreted at the apical side as a protective and lubricant liquid [4]. The mucus layer is the first barrier to pass for exogenous compounds. The second are the phospholipid bilayers of the epithelial cells themselves, and the intracellular environment.

Drugs and other molecules can pass through the epithelial cell layer by either the transcellular or paracellular pathways. The transcellular pathway of drug absorption may be characterized by simple passive diffusion of the drug along a concentration gradient. Active (energy-dependent) drug uptake at epithelia involves mechanisms of membrane fusion, transcytosis via vesicle carriers or drug absorption into the cell followed by secretion on the basolateral side into the systemic circulation. Absorption by the paracellular pathway will let the drug pass through the space in between two or three adjacent cells (epithelial, endothelial or blood brain barrier BBB cells), however, diffusion is hampered by several inter-cellular junctions [5] (figure 1). The modulation of both, the trans- and paracellular pathways have been investigated to enhance drug permeability. Transcellular permeation enhancers (PEs) such as fatty acids, bile salts, and others are more frequently applied in clinical studies and are present in marketed drugs than the paracellular PEs mainly due to the proven safety of the former [6]. On the other hand, PEs acting on the paracellular pathway have been shown to enhance the

permeability of even larger molecules [7]. There are for now no paracellular PEs coupled to a drug on the market. This is mainly due to formulation hurdles and *in vitro/in vivo* transition. These struggles coupled to industries more and more focused on developing proteic and/or immunogenic drugs that can reach a size of 150 kDa instead of trying way to absorb them lead to few outcomes for paracellular PEs. On another hand, PEs choice has to be carefully made as the safety or the efficacy would be conceded to the other. However, studies have been more focused on a paracellular PE that would have less toxicity and a local effect.

Desmosomes, mainly composed of desmoplakin, desmogleins and desmosomal cadherins represent the strongest link between epithelial cells [8]. They are absent in endothelium [9]. These junctions have the role of maintaining epithelial structure in case of mechanical stress. They are not only present in epithelia or mucosae, but in all tissues undergoing mechanical movement, such as cardiac or endothelial tissues [10]. Loss of desmosomes has been demonstrated to also provoke cancer progression by losing cell-cell signalling [11].

Two different transmembrane protein families, E-cadherins and catenins, mainly compose adherent junctions (AJs) [12]. E-cadherins are calcium-dependent transmembrane proteins, while catenins are intracellular components. These junctions are responsible for cell-cell adhesion and the maintenance of the cell layer structure. They are also present in endothelium

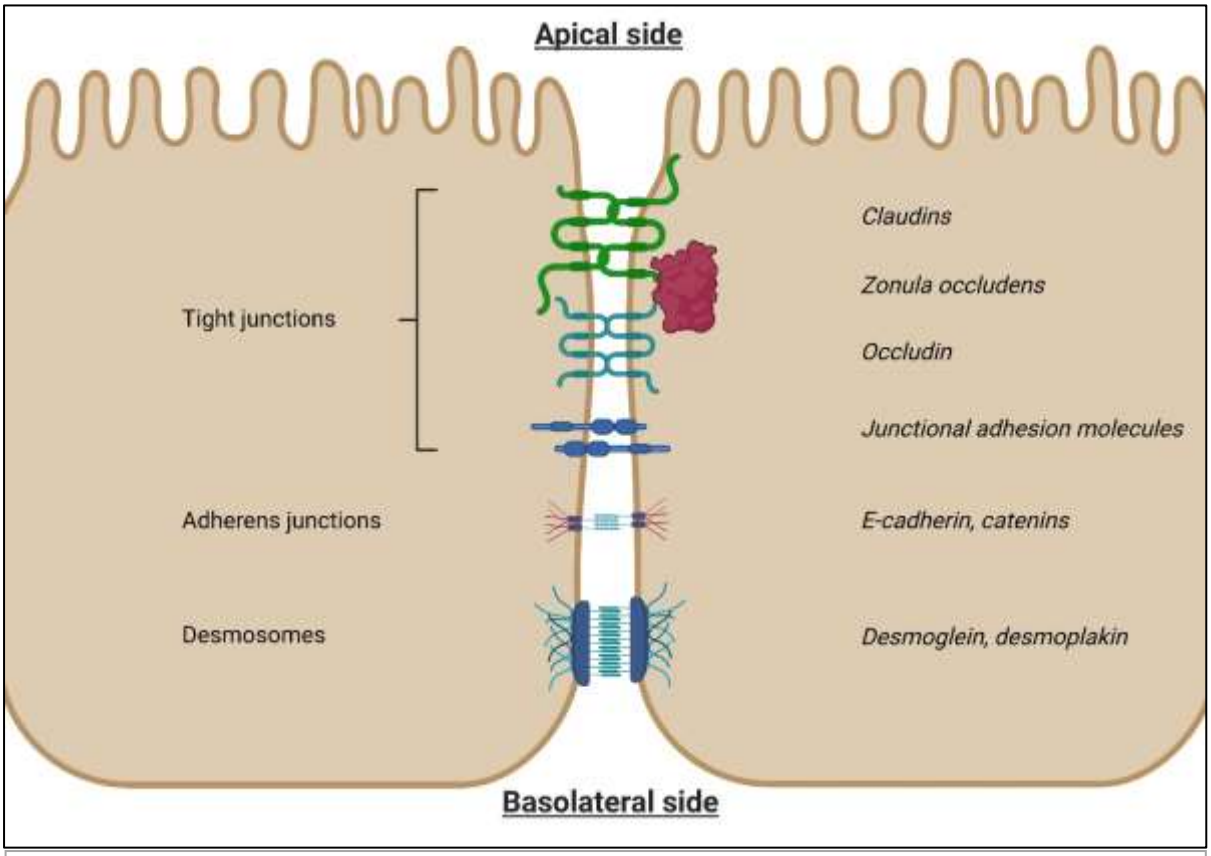


Figure 1: Schematic representation of the different main components of epithelial intercellular junctions.

and BBB but with another type of cadherin: VE-cadherins [13]. AJs are also involved in the intracellular signalling to induce the expression of tight junction (TJ) proteins. In the absence of AJs, cells are not able to form a structured cell layer and TJs cannot be formed. AJs are also responsible for inflammation monitoring by regulating the passage of leukocytes through the cell layer [14]. The association of AJ proteins with the actin cytoskeleton contributes to cell-cell junction movement and organization.

Between the cells near the apical side of the cell layer, a network of proteins forms the so-called tight junctions (TJs) [15] that restrict this space to a pore size between 50-60 Å (intestinal crypt cells) and to a value of < 6 Å in between villus cells [16]. Under normal physiological conditions, only water and solutes such as electrolytes may pass through the paracellular pathway. TJs are therefore regarded as the main barrier against paracellular diffusion and not directly responsible for the cell layer structure [17]. They consist of a system of several proteins, including MARVEL (MAL and related proteins for vesicle trafficking and membrane link) proteins, TAMP (tight junction-associated marvel domain-containing protein), which are involved in barrier regulation (tricellulin, occludin) and MAGUK (membrane-associated guanylate kinase) proteins, which link transmembrane proteins to the cytoskeleton (Zonula occludens) [18]. In addition, TJs also include immunoglobulin JAM (junctional adhesion molecule) [19]. The identification of further TJ proteins is still ongoing [20].

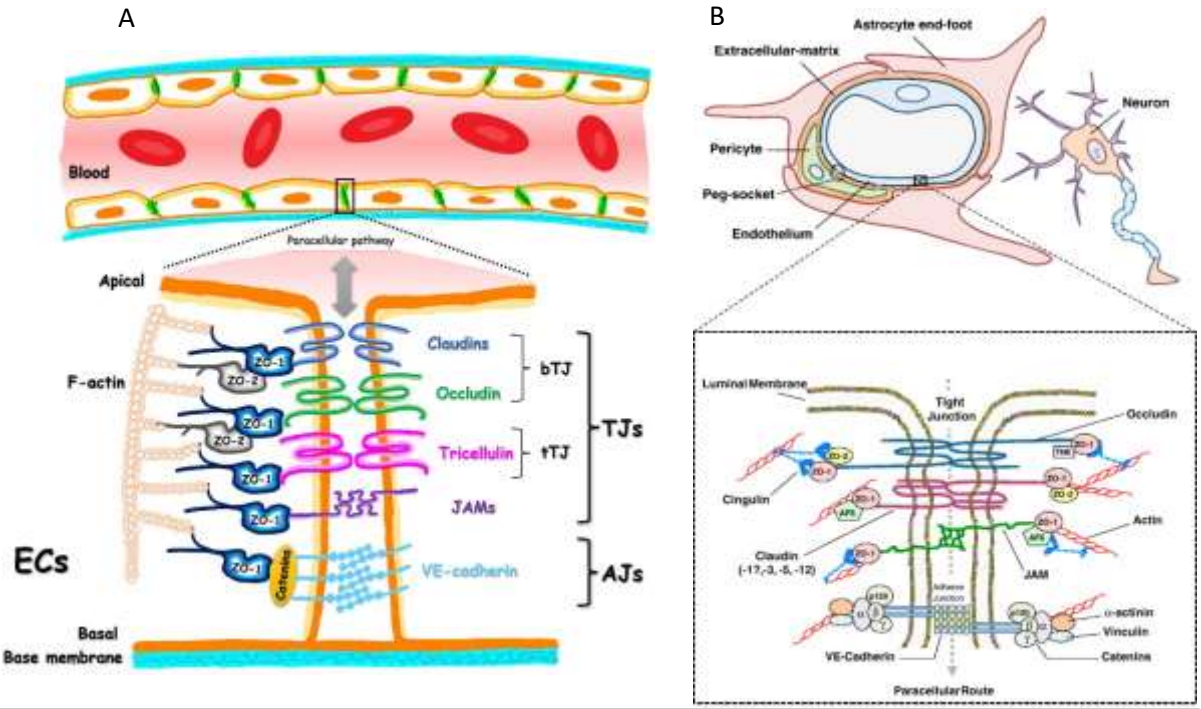


Figure 2: Schematic representation of intercellular junction between A: endothelial cells [21] and B: BBB cells [22].

These semi-permeable junctions can be found in epithelial, endothelial and BBB cells [23]. Some specificities for each cell type can be noted but TJs composition is comparable. Figure 2 shows intercellular junction of endothelial and BBB cells to compare with the figure 1 that represents more epithelial intercellular junctions. The differences between them will be discussed below. The multiple unit structure of TJs includes intracellular and transmembrane components, closing TJs by interacting with each other in a dynamic fashion, however, the complete mechanism remains partially unclear. The TJ closure is regulated by ubiquitination and successive degradation of TJ proteins [24] and mediated by clathrin-mediated endocytosis [25]. In addition, the cell is able to change cell layer permeability by intracellular signalisation via enzymes to modulate TJ protein expression, with claudins being the major proteins involved. The most important proteins involved in TJ formation are discussed in the following.

1.1. Claudins

Claudins are a group of more than 25 types of proteins that are involved in TJ formation [26], with sizes ranging from 21 to 34 kDa (207 to 305 amino acids) [27]. This amount of types may increase as new ones are discovered still today. One of their roles is the capacity to form paracellular pores, with solutes smaller than approximately 4 Å freely passing through such pores [28]. Even if many types of these transmembrane proteins have been sequenced, only a few of them have a known role in the cell and in TJ modulation. Moreover, not all claudins are present in all tissues [29], with some forms being specific to cardiac or intestinal tissues and some types of claudins being co-expressed [30]. For example, only claudins -3, -5 and -12 have been found in the endothelium and the BBB [23] and claudin-5 seems to be absent in epithelium except in the intestine. Claudins almost all have a PDZ domain in their C-terminus linking them to PDZ proteins (e.g., ZO-1, -2 and -3) [31]. For closing TJs, an interaction between claudins of two different cells and a polymerization have to occur.

Claudins expression is regulated by many different known and unknown mechanisms but mainly by catenins. Being involved in TJ formation, studies have shown their presence in cells that do not participate in TJ formation [32]. In fibroblasts, expression of claudins lead to their polymerisation, however, not at the cell membrane, and the belt-like apical cell layer maintained by claudins was not formed. This shows that other components of TJs are necessary for claudin localisation and activity.

1.2. Occludin and tricellulin

The other main component of TJs is occludin, the first transmembrane TJ protein discovered [29]. For this 65 kDa protein merely two different isoforms were identified [33]. These isoforms are suggested to be the result of a mutation during mRNA (messenger RNA) splicing,

however, their role is similar [12]. Occludin interacts with MAGUK such as claudins [18]. Its four transmembrane domains [15] give this protein a strong homology to myelin and the lymphocyte-associated protein (MAL) [29] and permits an assemblage with claudins in TJs [34]. Occludin is mainly expressed in endothelial and epithelial tissues [1,12,17] and is activated or inactivated at the cell membrane. Its expression is regulated by different kinases, mainly by non-receptor tyrosine kinase c-Yes and protein kinase C. Prior its phosphorylation the protein is located in the lateral membrane [29]. Occludin's localisation follows Zonula occludens protein localisation because of their linkage, as it is the case for claudins. However, in contrast to claudins, the two proteins are linked through a PI3K domain instead of the PDZ domain in claudins.

Occludin knock-out cells maintained their restricted permeability and expression of TJs [5], but have shown unexplained phenotypes [29]. These knock-out cells have also shown a stimulated tumour progression [15], whereas an overexpression of occludin was demonstrated to inhibit tumour growth. Modulation of TJ formation has first been suggested as an explanation of this phenomenon. But other studies have shown that occludin is not a required protein for TJ formation but the gene coding for occludin remains important [33]. Occludin expression is a key responsible for TJ formation, however, the expression of occludin alone does not result in TJ, as no spontaneous formation of TJs was detected in non-polarized cells [34]. The importance of occludin expression has also been explored for the treatment against porcine epidemic diarrhea virus (PEDV) infection [35]. Absence of occludin resulted in a higher intestinal infection by the virus and resulted in stronger diarrhea.

A protein similar to occludin has recently been described and named tricellulin [36]. The structure and many pathways of expression and functionality are close to occludin's, being involved in TJ formation between three adjacent cells. Tricellulin is responsible for sealing the three cells together and create a central tube also involved in the passage of macromolecules [37]. Its similarity to occludin is mainly due to the close location of their genes of expression on chromosome 5. An impairment in TJ organization was observed in tricellulin knock-down cells. Lack of tricellulin is further impairing the spreading of *shigella* [38], which appears use tricellulin to diffuse across the cell layer and in between cells.

1.3. Zonula Occludens protein

The third main protein involved in TJ formation is Zonula Occludens (ZO) [29]. Three different ZO multidomain polypeptides (ZO-1, ZO-2 and ZO-3) [39] have been described for this cytoplasmic plaque component [17]. These members of the MAGUK family [40,41] have been suggested to play a role in many TJ and AJ formation pathways. ZO proteins act as scaffolding proteins by binding and regulating the expression of cytoplasmic (cytoskeleton) and

transmembrane components [28]. Gene transcription and cell proliferation, as well as the management of claudin polymerization [26] or cadherin cell-cell adhesion promotion [39] are partly regulated by ZO proteins [42]. Phosphorylation of these proteins remains a necessary step for their activation. This phase is mainly assured by protein kinase C (PKC) and tyrosine kinase [42]. The phosphorylated form has been detected to a higher level in less adherent cells.

Different roles have been assigned to the three ZO isoforms. Besides its importance for the assembly and regulation of TJs, ZO-1 is involved in the regulation of epithelial cell proliferation and density. ZO-1 is entering the nucleus by virtue of its two nuclear localization sequences and binds to the Y-box transcription factor, ZONAB [41]. This is suggested to lead to the nuclear accumulation of cyclin-dependent kinase 4 (CDK4), which is involved in cell cycle G1 phase progression [43]. Like ZO-1, ZO-2 can enter the nucleus and was shown to accumulate there upon heat shock [44]. It interacts with a DNA-binding scaffold attachment factor-B (SAF-B), which is involved in transcriptional regulation. However, in contrast to ZO-1, the depletion of ZO-2 does not appear to affect epithelial permeability in MDCK cells [28]. Finally, ZO-3 does not appear to be indispensable for TJ formation and function, as knock-out cells as well as mouse embryos deficient of the protein did not show a specific phenotype [45].

A depletion of ZO proteins has been shown to result in a delayed formation of TJs and an apical reorganisation of actin and myosin. In an embryonic state, this disruption provokes lethality [39]. Endocytosis of ZO may be responsible for certain types of colitis [40], and the loss of ZO-1 is established in breast cancer cells [41]. ZO-1 expression can be regulated by interferon-γ, and other studies have shown an implication of interleukins in the localization and expression of TJ proteins [46,47]. Inhibition of ZO-2 signaling has been proven to induce bone resorption and osteoclast differentiation [34]. Finally, the involvement of ZO proteins in mechanisms of infection has been studied. The tumour adenovirus E4-ORF1 was shown to bind intracellularly to ZO-2, which has tumor suppressor functions. The resulting disruption of cell layer integrity facilitates tumour progression [48]. Another prominent example is the ZO toxin (Zot) of *Vibrio cholera*. This toxin binds to a receptor at the surface of the cells and successively inhibits ZO proteins [46], enhancing paracellular permeability. This toxin has therefore been studied with respect to its use as a potential permeation enhancer to enhance drug absorption [49].

1.4. Cytoskeleton

The TJ proteins discussed so far are linked to actomyosin filaments and microtubules [29], calcium- and ATP- (adenosine triphosphate) dependent components of the cytoskeleton [1]. Actin is a protein composed by 375 amino acids (42 kDa) existing in two different

conformations: F-actin and G-actin [50]. On another hand, myosin is a family of 18 proteins mainly composed by two heavy chains (MHC) and two light chains (MLC) [51]. Myosin is primarily responsible for junctional tone and assembly [29], whereas actin is mainly involved in cytoskeletal contraction. Cofilin is one of the key proteins to regulate F-actin and G-actin polymerization [52].

The actin cytoskeleton is coupled to TJ proteins such as claudins by weak interaction of the actin binding site at the C-terminal of ZO-1 [53]. This weak interaction appears to be essential for the modulation of TJ permeability, and suggested as a potential therapeutic target, e.g., to enhance drug transport at the blood-brain-barrier.

An endogenous mechanism to regulate epithelial TJ is represented by the phosphorylation of myosin light chain (MLC) through kinases or phosphatases, which induces the contraction of the perijunctional actomyosin ring. The increased phosphorylation of MLC corresponds to an increased permeability and open state of TJs [54]. On the other hand, the contraction of actin circumferential filaments [55] is controlled by calcium and Rho kinase [56,57]. Calcium channels are essential for extracellular calcium to enter the cell, otherwise actin filaments stay relaxed and cell layers lose their barrier function [1]. However, even in the case of loss of this barrier structure, the barrier function is kept [34].

Other extracellular stimuli for the modulation of TJs are pro-inflammatory cytokines and immune cells. As an example, tumor necrosis factor alpha (TNF-α) may induce cytoskeletal contraction at the level of the actomyosin ring through an NF-kB/MLCK mediated pathway, leading to TJ opening [47]. The other example is the cytoskeletal redistribution due to endothelial growth factor also leading to an altered orientation of actomyosin filaments [58].

Cell junctions are maintained by a complex organization and by the regulation of these different components. The intercellular space can be opened or closed to let nutrients and solutes pass through the cell layer by an endogenous mechanism. Absorption of drugs by the paracellular pathway is generally hindered due to their size and charge. By temporarily disrupting and changing the cellular regulation of junction proteins, an increased permeability may be achieved, and drug absorption enhanced. Compounds achieving such modulation of TJs are active excipients called tight junction modulators (TJMs).

2. Classification of tight junction modulators

Increasing the bioavailability of drugs has the goals of increasing therapeutic efficacy, potentially reduce the dose applied, and reduce interindividual variability of resulting pharmacokinetic profiles in the patient [59]. To this end, the chemical structure of the API may be optimized or the synthesis of a prodrug considered. In other cases, for molecules which

cannot respect Lipinski's rule of 5, the formulation itself or the route of administration [60] may be adapted. As an example, a protection of the active substance by an emulsion, enteric coating or parenteral administration can be suggested. In addition more recently, new means of administration have been developed, including the transdermal application of iontophoresis and the use of microneedles have shown promising results [61]. New types of formulations have also been discovered to avoid invasive routes of administration [62]. Another option is to increase the residence time of the drug formulation at the absorptive epithelium to allow for a higher drug uptake. To this end, numerous muco- and bio-adhesive formulations based on polymers (chitosan, polyacrylic acids) have been suggested [6,63].

In some instances, these strategies may not be feasible or sufficiently effective, and the enhancement of the transcellular or paracellular absorption pathway may be considered. The modulation of TJs, e.g., to allow for the absorption of nutrients or the passage of lymphocytes through endothelia during inflammatory events is a naturally occurring process [4,56]. The induced controlled variation of cell layer permeability based on such endogenous mechanism may be suitable to increase drug bioavailability.

To this effect, several different targets such as proteins and enzymes involved in TJ formation, or even the cell membrane itself have been investigated. Secretory transporters (P-glycoprotein or multidrug resistance protein) expressed by intestinal epithelial cells were among the first targets for permeability increase [59], as their inhibition would result in a reduced apical efflux of drugs. However, these transporters have a completely different mechanism and will not be discussed here. Among the different approaches to increase drug absorption, TJ modulators, considered as active excipients in drug formulations, will be discussed in the following.

2.1. Non-specific TJs modulators

Different compounds have been in use as permeation enhancers (PEs), as they were found to generate an increase in drug bioavailability. However, their mechanism of action and specific target in the TJ regulation process remained unknown. These non-specific TJs modulators (TJMs) are included until today in pharmaceutical formulations as well as additives in food [62]. Based on their natural effectiveness, bile salts have been investigated as PEs for pharmaceutical formulations. Their ability to remove the mucus at the luminal side of the intestinal epithelium and to disrupt the organisation of phospholipids of the cell membrane [64], has been demonstrated. Fatty acids have been tested for their ability to disrupt the phospholipid bilayer of the cell membrane [59]. Furthermore, another mechanism of permeability increase has been shown for sodium decanoate through its ability to dilate TJs *in vitro* [65,66]. However, this mechanism of action is difficult to control in an *in vivo* setting.

Early on, disodium ethylenediaminetetraacetate (EDTA) has been suggested for its ability to modulate TJs [67,68]. Through chelation of extracellular Ca^{2+} by EDTA, the homeostasis between intra- and extracellular Ca^{2+} is disturbed, Calcium released form intracellular storage, which finally results in TJ opening. Being rather effective *in vitro*, EDTA is not considered as a suitable PE for oral application, as it is absorbed to a significant extent, which may induce toxicity [52].

The biopolymer chitosan, derived from the ubiquitous chitin, and its derivatives have been the most studied non-specific permeation enhancers [1]. Different mechanisms have been suggested to explain the ability of this polymer to increase permeability. The perturbation of the lipid organisation of the cell membrane and/or the interaction with TJ components have been among the first hypotheses to explain the TJ modulation activity of chitosan [65,69]. In addition, the interaction with actin filaments has been postulated recently [70]. As chitosan is insoluble at physiological pH, the polymer loses its permeation enhancing activity above a pH of 5.5. The overcome this limitation, permanently charged derivatives have been developed that are soluble at a broad range of pH values [71].

The macrogol Solutol® HS15 has been studied to enhance drug permeability at pulmonary, nasal and intestinal epithelia [72–74]. The mechanism of action of this molecule to open TJs

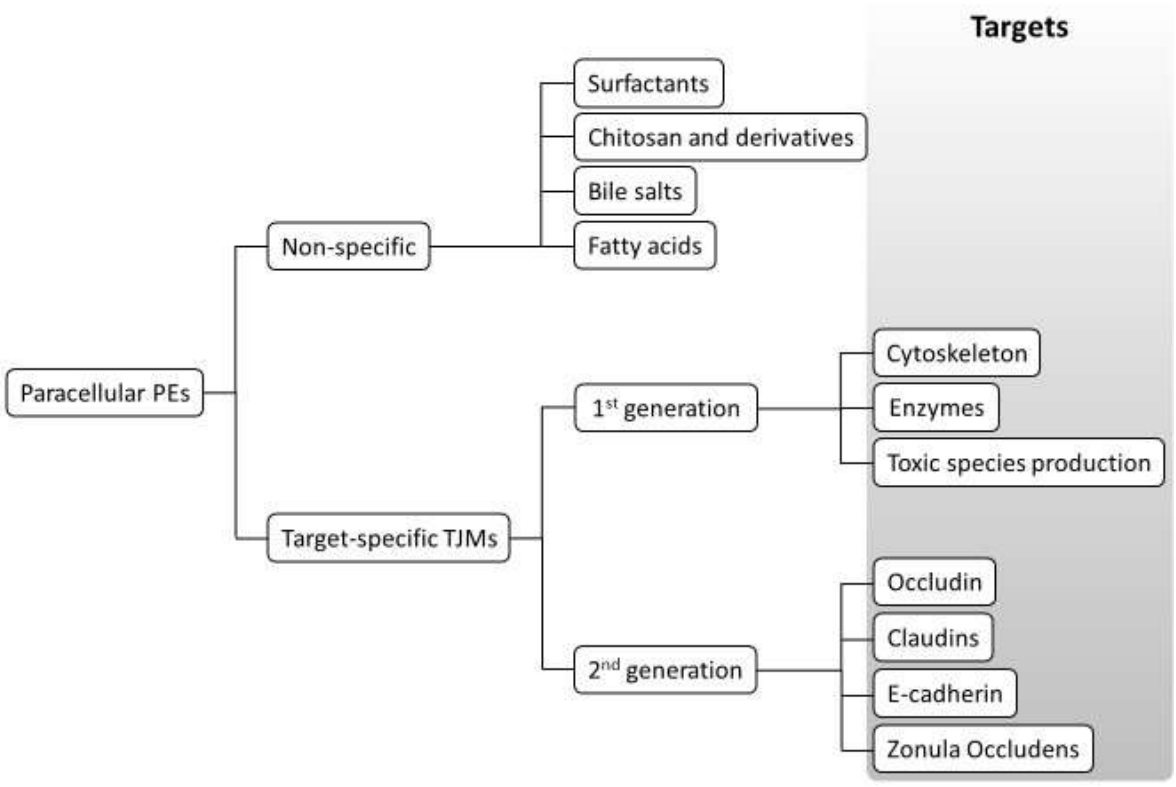


Figure 3: Suggested new classification of TJMs into non-specific and target-specific PEs of first- (targeting non-TJ proteins) and second-generation PEs interacting with TJ proteins.

has been described as an induced redistribution of actin filaments due to membrane perturbation. This rearrangement effectively leads to modulation of cell-cell contacts and increases the intercellular gap. In the studies referred to above, an enhanced permeability of different compounds of increasing molecular weight (mannitol, insulin, fluorescein dextran 4 kDa) was shown. This kind of interaction with actin filaments has been strongly associated to chitosan's mechanism of action, however, in contrast to chitosan, the Solutol® HS15 mechanism of action has more clearly been described. The exact interactions with cellular components, however, still need to be elucidated.

These non-specific TJs modulators lead to an effect on the surface of the cell layer and would be useful in some formulations as they are already present on the market. Even if they are still needed, some disadvantages can be noted. In order to overcome the issue of non-specificity and lack of control of permeation enhancement mechanisms involved, target-specific targets TJ modulators (TJMs) have been developed. More studies and work would be needed on targeted TJMs.

2.2. Target-specific TJs modulators

In 2008, a classification for PEs suggested a categorisation into first- and second-generation TJMs [75], taking into consideration only PEs that increase paracellular transport through TJ modulation. Non-targeted PEs are included in first-generation group and second-generation PEs have a defined target, e.g., a TJ protein (e.g., claudin, occludin). Since 2008, the number and the diversity of PEs has remarkably increased, and a revision introducing more precise differentiations between PEs appears to be timely. We suggest to distinguish between non-specific (e.g., bile salts, surfactants) and targeted PEs, whose effects and mechanisms of action are well known. The group of target-specific TJMs is further divided into first- (targeting non-TJ proteins) and second-generation modulators targeting TJs proteins (figure 3).

2.2.1. Targets of first-generation TJMs

First-generation TJMs modulate TJs through a number of different mechanisms. The main cell constituents involved in TJs that have been targeted by potential PEs are the cytoskeleton (actin filaments), pathways producing toxic species and intracellular enzymes involved in TJ protein regulation. In contrast to non-specific PEs, these potential TJMs have been designed based on their role of interaction with a specific component, yielding higher selectivity and a stronger and controlled effect.

2.2.1.1. Cytoskeleton

As previously described, the cytoskeleton formed by actomyosin filaments is essential for cell-cell interactions. The involvement of the cytoskeleton in TJ modulation is described as being as important as TJ proteins, and modification of its organisation or level of expression would

result in an at least temporary opening of the intercellular gap. Natural bioactive metabolites have also been tested as TJMs *in vitro*. Toxins of the Red Sea sponge *Negombata magnifica*, latrunculin A and B, are used by the organism as a defence mechanism against fish [76]. By binding to cofilin, the F-actin/G-actin equilibrium can be unbalanced.

Latrunculins, as well as cytochalasin D [77] have been shown to act on actin filaments, disrupting the cytoskeleton which results in a change in cell shape, among other effects. Another example of a natural product interacting with the cytoskeleton is bilobalide [78], bioactive metabolite found in *Gingko biloba* leaves. A transient and reversible contraction of actin filaments through stimulation of the adenosine A1 receptor (A1R) mediated phosphorylation of actin-binding proteins and MLC has been observed in endothelial cells of the BBB both *in vitro* and *in vivo*.

As mentioned above, cytochalasin D may act as a PE as well [79,80]. This alkaloid is produced by some fungi as a cell replication modulator by linking to actin filaments [81,82], transiently mediated by MLC [83]. Natural linkage of cofilin to actin is prevented by the association cytochalasin D [84], upon which a modified regulation of actin expression, movement and organisation is induced. Cytochalasin D has been demonstrated to be able to link to G-actin and inhibit usual polymerization. Permeability studies on intestinal ex vivo material have been performed to show the enhanced permeation of mannitol and forskolin coupled to cytochalasin D [85], and the effectiveness of cytochalasin D as a PE has been confirmed [86]. However, a repeated application of cytochalasin D as well as the latrunculins may result in a certain toxicity.

Another experimental PE acting on the cytoskeleton had been identified with capsaicin, an irritant found in plants of the *capsicum genus* [87]. It appears that capsaicin interferes with the permeability of Martin-Darbin Canine Kidney (MDCK) cell monolayers by depolymerization of actin by inducin cofilin activation and decreased the expression of TJ protein occludin. The capsaicin induced reduction in transepithelial electrical resistance (TEER) and increase in paracellular permeability was concentration-dependent and reversible. Looking at these studies on targeting the cytoskeleton, a toxicity is often noted due to this deregulation of intrinsic mechanism. These different ways of defence may lead to promising TJMs but an optimisation is needed.

2.2.1.2. Toxic compounds release

Another strategy of TJ modulation is to interfere with the intracellular metabolism in order to favor the built-up of toxic compounds, finally resulting in the modulation of TJs. One of such targets is the intracellular lipid metabolism. Minimally modified low density lipoproteins (MM-LDL) or oxidized LDL (ox-LDL) have been demonstrated to be atherogenic [88], and induce

reactive oxygen species (ROS) through NAD(P)H oxidase [56] and superoxide dismutase (SOD). The suggested pathway is an attenuation of the expression of desmoglein-2 (DSG-2) and desmocollin-2 (DSC-2), which are involved in the maintenance of cell-cell adhesion. It has been found, however, that only DSG-2 and not DSC-2 is essential to maintain the barrier function in the intestinal epithelium in mice [89]. An active principle of MM-LDL, oxidized-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (Ox-PAPC) [90] can be formed intracellularly. Besides its recently described potential protective activity in septic shock [91], Ox-PAPC induces an increase in intracellular ROS, e.g., in vascular endothelial cells [92], leading to a disruption of the cellular organisation. Finally, the role of Ox-PAPC as a potential PE has been shown by increasing the passage of 10 kDa dextran through the cell layer. The reversibility of this process and its toxicity have not been evaluated, and no further studies have been published on the use OxPAPC as a PE.

Long-chain acylcarnitines composed of 12-18 carbons have been demonstrated to enhance intestinal cell layer permeability *in vitro* [93]. The first suggested mechanism, the triggering of an increase of intracellular Ca^{++}-concentration was rapidly rejected. Recently, a new mechanism has been discovered. In contrast to the first theory, a release of ATP due to administration of carnitine has been proven, which was correlated to the release of ROS [94]. As described, ROS have the capacity to alter cell layer permeability.

2.2.1.3. Sphenopalatine ganglion

The blood-brain barrier (BBB) is a formidable barrier against drug permeation. Its permeability may be modulated by electrical stimulation of the sphenopalatine ganglion (SPG), responsible for the transmission of parasympathetic signals to the cranium [95]. Studies in rats have revealed the two- to six-fold increase of transport across the BBB of marker molecules of molecular weights between 4 and 250 kDa [96]. An increase in BBB permeability by long-time stimulation of the SPG with a low frequency (10 Hz) current has been demonstrated in rats, allowing for the passage of 70 kDa dextran through the BBB [97]. By transmission electron microscopy, the opening of TJs was confirmed, however, the pathway of opening has not been completely understood. The main hypothesis argues the possibility of release of nitrogen species. These toxic molecules have been proven to trigger oxidative stress and alter TJs upon moderate hypoxia followed by reoxination (MHR) [98]. Safety of this technology to increase BBB permeability is to be demonstrated.

2.2.1.4. Phospholipase C

Various enzymes are involved in TJ protein expression, phosphorylation and activation. The enzymatic activity is governed by multiple complex intracellular mechanisms and signals from the extracellular environment. Phospholipase C (PLC) has been investigated as a potential

enzymatic target for permeation enhancement. This enzyme is located at the intracellular part of the cell membrane. Its activation via extracellular receptors has been described to lead to the conversion of phosphatidylinositol (4,5) bisphosphonate (PIP2) into the two second messengers Inositol (1,4,5) triphosphate (IP3) and diacylglycerol (DAG) [99]. These two molecules are respectively responsible for calcium mobilisation and protein kinase activation. After several signalling steps involving actin-myosin filaments and myosin light chain kinase (MLCK) [100], the common final activated cellular component, the cytoskeleton, is contracted and TJs open [101,102].

The medium chain fatty acid (MCFA) of 10 carbons (C_{10}), sodium caprate, has been suggested to stimulate PLC and trigger the activation of the mechanisms described above [103,104]. The first formulation including this PE was suppositories for the rectal application of ampicillin [105]. The effectiveness of sodium caprate for intestinal absorption has been shown, and several formulations have been designed and tested with different active principles for enteric administration [106–108]. The reversibility of the permeation enhancing effect was shown [109,110] as well as the possibility of repeated administration resulting in comparable bioavailability and absence of toxicity [111]. Further experiments to confirm the suggested mechanism of action would be suitable, however, this PE is still one of the most studied *in vitro* and *in vivo* for the application in different formulations [112].

Activation of the bradykinin (BK) B2-receptor has been reported to involve phospholipase C (PLC) in its mechanism of hemodynamic vasodilatory activity [113]. Coupled to involvement of phospholipase A2, nitric oxide and prostaglandins have been shown to also act on vascular and endothelial permeability. Upon bradykinin application and stimulation of tumour vessel B2 receptors, intratumoral blood perfusion was decreased, and permeability microspheres of different sizes (0.05 and 0.1 µm) increased, indicating an increase in vascular pore size [114].

A similar effect was also shown for B2 receptor agonist, nonapeptide RMP-7 (Labradimil), developed to increase BBB permeability in gliomas [115]. RMP-7 was shown to enhance passage of C_{14} carboplatin has been proven after co-administration of bradykinin in adenocarcinoma. An increased permeability of BBB for loperamide and cyclosporine-A has been reported after application of RMP-7. Even if a lower affinity with the receptor has been demonstrated for the nonapeptide compared to bradykinin, its longer plasma half-life has been shown to be sufficient for a stronger effect on the opening of TJs [116]. With no toxicity reported so far [117], according to clinicaltrials.gov RMP-7/carboplatin has completed 2 clinical phases I and 1 phase II studies in patients with brain cancer metastases and pediatric brain tumors. A phase III study for first line treatment of high-grade glioblastoma multiforme was

discontinued not for lack of efficacy or safety, but due to lack of data supporting such clinical trial.

2.2.1.5. MLCK/MLCP balance

As previously described, MLCK is largely involved in TJ modulation through the contraction of the perijunctional actomyosin ring (PAMR) [118]. The contraction and relaxation of PAMR is balanced by the activation of MLCK and MLCP (myosin light chain phosphatase), respectively [119]. MLCP is regulated by Rho kinase and PKC, in contrast to MLCK, which is regulated by PLC. The endocytosis of occludin and the reorganization of TJs is triggered by PAMR phosphorylation [120]. The contraction of PAMR is a naturally occurring process occurring during inflammation and for the absorption of nutrients. At a cellular level, PAMR regulation is responsible for essential cell mechanisms such as proliferation, migration, adhesion, endocytosis and exocytosis [121]. This mechanism of TJ opening is also exploited by bacteria such as enterohaemorragic *Escherichia coli* (EHEC) and *Yersina pseudotuberculosis* [122] to pass the epithelial barrier.

Cytochalasin B (CB) has been identified to alter the cytoskeleton structure through the MLCK/MLCP pathway [123], resulting in the disruption of the polymerisation of actin. *In vitro* studies in Caco-2 cells showed a disruption of TJ protein ZO-1 and enhanced permeability of the epithelial cell layer towards mannitol [124]. Piperazine is another PE that has been suggested to increase permeability of mannitol and 70 kDa molecules across Caco-2 cell layers through its binding to the serotoninergic receptor 5HT4 [125]. Binding results in the release of cyclic adenosine monophosphate (cAMP), which activates MLCK [126]. A further potential PE involving MLCK is the permeant inhibitor of phosphatase (PIP) 640 [127]. This polypeptide has been designed to be able to disturb the equilibrium between MLCK and MLCP [54,128] by inhibiting MLCP. By this intracellular modification, the effect of MLCK is more important and concentration of phosphorylated MLC increased. These different molecules would be more interesting as they have not a vital cellular component as a target. Even if their effect is weaker because of the cascade of reaction, their length of action is more controllable and may result in lower toxicity.

2.2.1.6. Protein kinase C

PKCs are another family of kinases examined as a potential target for PEs. These enzymes are subdivided into 3 groups of 12 isoforms [129]. The conventional (α, βI, βII and γ), novel (δ, ε, η and θ), atypical (ι/λ and ζ) and not attributed (μ, ν) are kinases having different, sometimes opposite roles [130]. These kinases are composed of a catalytic (kinase) domain and a regulatory domain. In some subtypes the domains are connected by a hinge region. It was first thought that the hinge would have to be hydrolysed by caspases to release the kinase

domain [131], however, each isoform appears to be activated by a specific mechanism. PKC α, ε and atypical are responsible for cell proliferation regulation [132]. Diverse targets for PKCs present in the cell, called receptor for activated C kinase (RACK), trigger various intracellular pathways. Proteins involved in TJ formation are also targets of PKCs, with ZO-1 identified to be modulated by PKCs [133].

As previously described, zonula occludens toxin (Zot) is able to transiently open TJs [134]. The underlying mechanism of action of this opening has been first thought to be a direct inhibition of ZO [135]. Later, this toxin has been demonstrated to be able to stimulate actin polymerization by activating protein kinase C-alpha (PKC α) [136]. Zot is involved in causing diarrhea during vibrio cholerae infection. Reducing the length of the peptide form 45 kDa to a 12 kDa fragment called ΔG, its toxic effects at the sufficient concentration to cause TJ opening was removed [46]. Reversible effects on the enhancement of Caco-2 cell layers of several compounds, including mannitol, cyclosporine A, acyclovir, and FITC 10 kDa was shown [49]. The structure of ΔG was then further reduced to a 6 amino acids peptide, only keeping the active part of Zot. This peptide, called AT1002, has primarily been tested for intranasal [137] and topical administration in atopic dermatitis [138]. Permeability enhancement of PEG4000 and cyclosporine A *in vitro* (Caco-2) and after intraduodenal application in rats was confirmed [139]. A version of AT1002 stabilized by C-terminal amidation against hydrolysis at neutral pH values was shown to enhance nasal permeation of mannitol *in vivo* [140].

The effect exerted by AT1002 is reversed by the octameric peptide larazotide [141], possibly through the inhibition of zonulin, a reversible modulator of TJs, has been accepted as the mechanism of action [142]. Larazotide has been examined for its TJ closing activity to treat celiac disease [143], which results in a reduced passage of macrophages through the cell layers, moderation of inflammation and decreased celiac symptoms. A phase II clinical trial involving 80 celiac disease patients revealed that larazotide at low dose could prevent intestinal symptoms upon gluten challenge [144].

Atypical PKC ζ is also a well-studied cytosolic isoenzyme for increasing epithelial permeability. Its activity is not related to the intracellular calcium concentration, but to PIP3 via phosphorylation [130]. A higher activity of the enzyme has also been noted in the presence of DAG [131]. The existence of this enzyme has been first proven in mice brain [145], and successively identified in many tissues and having many different roles, such as the maintenance of cell polarity, mitogen cascade, IL-6 expression or regulation of transcriptional activity of NFκB [146,147]. A role in cancer development has been suggested due to an increased expression in bladder and colorectal tumor cells [148,149]. The possibility to use the level of expression of this enzyme as a diagnostic marker has been suggested.

Atypical PKC ζ has been described as responsible for TJ disruption and redistribution of ZO-1 and occludin [150]. Bronchial barrier function regulation via Toll-like receptor 2 (TLR2) has also been proven to be a main role of PKC ζ in bronchial epithelium *in vitro* [151]. This involvement, in connection with transcription regulation of NFκB, has made this enzyme a pharmacological target for inflammatory diseases of the lungs such as chronic obstructive pulmonary disease (COPD) [147]. The active form of this enzyme is not secreted after hinge cleavage and the release of the pseudosubstrate (PS) part, which acts as an auto-inhibitor on the kinase domain [146]. The hinge domain is partly responsible for the exclusion of the kinase from the nucleus [152]. When the ligand tethers to PKC ζ by dimerization of the PB1 domain, PS also binds to an acidic region of the ligand and frees the substrate region [153]. A short peptide of 13 amino acids has been developed based on PS structure to inhibit PKC ζ [154]. This polypeptide linked to a myristoyl queue has been called ZIP (PKC ζ pseudosubstrate-derived ζ-inhibitory peptide). ZIP has been proven to compete for the link to the acidic region of the ligand and re-engage PS in the substrate region. This would maintain PKC ζ in its active form, however, hindering the kinase form protein phosphorylation and thus from exerting its activity. The disadvantage of this molecule is that in systemic circulation its activity has been shown in too many tissues, particularly in the brain. A local administration has to be performed for this inhibitor. A shorter peptide of five amino acids derived from ZIP called L-R5 has been synthesized to be administered locally to avoid all secondary effects of a systemic distribution [155]. This myristoylated peptide has been demonstrated to open transitory and reversibly TJs in the absence of toxicity.

All of these first-generation PEs have in common the advantage the reversibility of their activity. The fact they act on the cascade of reactions of an indirect target of TJs keeps other pathways of TJ protein expression active. However, the onset of action is slower than for direct inhibitors, which will be described in the following. Moreover, use of toxins fragment or generation of toxic compounds may not deliver the optimal TJMs. Too many risks of cellular toxicity have been noted. On another hand, enzymes modulation seems to assure a better control of TJs opening. Second-generation TJMs will demonstrate a different mechanism of action. Figure 4 resumes the different mechanisms described in the following.

2.2.2. Targets of second-generation TJMs

Second generation TJMs have been originally described as inhibitors or disrupting molecules of TJ proteins such as occludin or claudin [70]. Since then, more TJMs have been developed and new TJ proteins, including PDZ proteins, have been targeted. Even if E-cadherin is not included in the family of TJ proteins, its potential modulation would also increase epithelial permeability, as E-cadherin-mediated cell-cell interactions have been shown to facilitate TJ formation [156].

2.2.2.1. Cadherin

As previously explained, cadherins are not considered as TJ proteins. However, an inhibition or a disruption of this homologous intercellular link between E-cadherin molecules via repetitive extracellular domains (EC 1-5) [157] would also weaken or dislocate TJs. HAV and ADT, EC-1 derived sequences of three amino acids, have been synthesized and interact with EC-1 in the "bulge" and "groove" regions, respectively, and suppress intercellular linkage. HAV has been shown to be crucial for cell adhesion [158]. Different peptides including the HAV sequence have been prepared with varying efficacy and time of action [159]. HAV has been used in combination with antitumoral drugs such as adenanthin, mainly targeting the BBB. A significant decrease of TEER (transepithelial electrical resistance) and an increase of mannitol permeability upon HAV incubation in MDCK cells has been shown. Furthermore, cytotoxicity was detected only after a long term application (24 hours) [160]. Finally, a reversible disruption of E-cadherin organisation was confirmed by immunostaining [161]. This TJM has been confirmed to be a potential PE for the enhancement of permeation of antitumoral drugs through BBB.

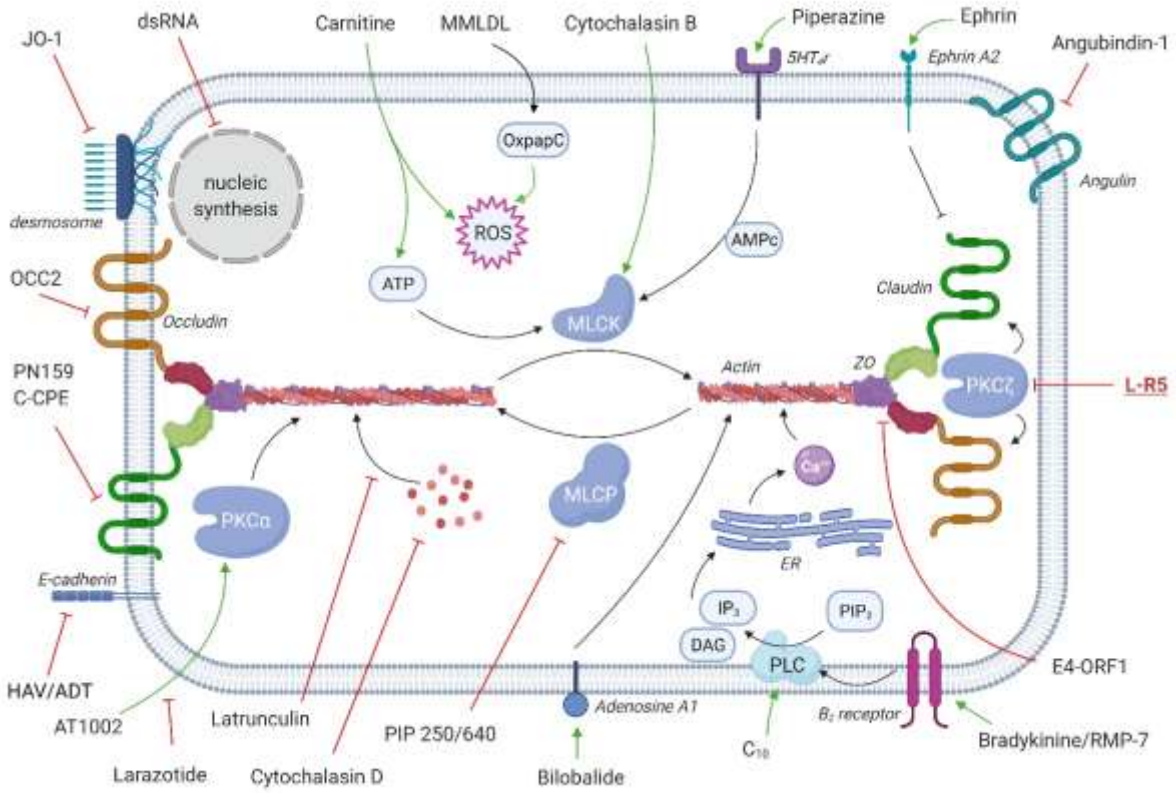


Figure 4: Schematic representation of the different pathways TJ modulation. The green arrows represent a stimulation/activation and the red dashes an inhibition of the target by a TJM.

The other non-TJ protein that has been targeted to increase paracellular permeability is desmoglein 2 (DSG2). As explained above, this protein is involved in cell-cell adhesion. DSG2 has been shown to be expressed in many tissues and overexpression has been detected in many cancers, including more than 60 ovarian cancers [162]. Some adenoviruses have been described to be able to transiently modulate the paracellular space by the cleavage of DSG2 resulting in the reduction in E-cadherin expression [163]. Based on these findings the recombinant adenovirus serotype 3-derived protein JO-1 (junction opener) has been synthesized [164]. Breast cancer has particularly been investigated for a combined therapy of biologics such as trastuzumab (Herceptin) and cetuximab (Erbitux) with JO-1. Mice and even monkeys have been assessed for this TJM leading to encouraging results. Moreover, tumor tissue specificity was revealed in human transgenic mice as DSG2 is more expressed in tumors and JO-1 is more specific for human tissues. Finally, even if no toxicity has been reported, a potential risk of metastasis needs to be considered due to the implication of JO-1 in the epithelial to mesenchymal transition (EMT) pathway. However, this has not been observed in the 20 *in vivo* experiments so far [162].

2.2.2.2. Claudins

Modulating TJs permeability maybe an interesting mechanism as well as a risky one. First of all, the specificity of the claudins targeted has to be established as these proteins are distributed in different tissues and cell types in various expressions [30]. It has been demonstrated that not all claudins can be inhibited by the same inhibitor [165]. Moreover, not all claudins are involved in the same roles in the cells. Some claudins have been described in some inflammation pathways [27] or responsible for osmotic regulation [34]. Claudins have also not obligatorily a role to close barriers [166]. Claudin-3 may have an inversed role on closing barriers. On another hand, some claudins have been noted to be involved in some cancers [167]. These proteins may also be used as cancer markers. Some individual claudins have been linked with some specific cancers, such as claudin-3 in ovarian cancer [168] or claudin-1 and -3 in colorectal cancer [169,170]. On the opposite to these claudins, claudin-6 expression was decreased in gastric cancer [171]. Some claudins have even been demonstrated to be able to exert resistance to cancer therapies [172]. All these discoveries settle that modulating claudins to open TJs may also lead to other consequences such as opening TJs in other tissues, modulate inflammatory pathways or even modify and act on cancers. Specificity and ponderation are important settings to take care for TJs modulators through claudins.

The different claudins have been studied as potential targets for permeability enhancement via several mechanisms. The peptide PN159 (or KLAL/MAP) has been suggested to bind to claudin-4 and -7 resulting in an enhanced paracellular permeability [173]. In addition, PN159

appears to have a concentration-dependent permeabilizing effect on the cell membrane, contributing to overall permeation enhancement. The peptide has been shown to be superior to the use of nanoparticles with respect to its enhancement effects on the permeation of liraglutide through an intestinal cell layer [174].

Compounds involved in endothelial cell development and vascular growth have been studied as they are directly related to cell-cell adhesion. Besides vascular endothelial growth factor (VEGF), the family of ephrins (Eph) and their receptor regulate angiogenesis and have been closely studied as a potential TJMs, as Ephrin-B1 binds to TJ proteins Claudin-1 and Claudin-4 [175,176]. The main tissues studied have been pulmonary epithelia, as ephrin-A1 is expressed in distal lungs and an increased expression has been shown to be related to pulmonary injury [177]. Moreover, ephrin receptors have been demonstrated to be involved in the increase of pulmonary vascular permeability [178]. Different mechanisms have been suggested for ephrin receptors to modulate permeability. First, a destabilization of cell-cell interaction via adherens junctions caused by Eph receptor activation has been shown [179]. Second, Ephrin-A2 has been shown to directly be linked to claudin-4 phosphorylation [180,181]. Both of these mechanisms would trigger a modulation of endothelial permeability. As ephrins are responsible for angiogenesis mainly during foetal growth, the question arises whether these mechanisms still have a use in adults or whether they are remnants of the past. The answer to this question would yield more information on the importance of ephrins as TJMs.

Another toxin from bacteria other than vibrio cholerae was found to be a TJM. The *clostridium perfringens* (CPE) enterotoxin is thought to enable the penetration of the bacteria through the intestinal epithelium [182]. Studies have shown two active parts of the peptide, which have successively been sequenced [183]. The C-terminal part of the toxin (C-CPE) is responsible for opening TJs by interacting with cell junction proteins, and the N-terminal has been demonstrated to be cytotoxic. C-CPE has been isolated assessed as a potential TJM. Diverse derivatives (reduced sequences) have been synthesized and tested on intestinal cell layers, and the interaction with Claudins-3 and -4 was revealed [184]. Binding to the second extracellular loop of these TJ proteins has been established to reversibly increase the paracellular permeability of 4 kDa dextran and FITC-insulin through the cell layer [185,186]. Due to the deletion of the N-terminal part no cytotoxicity was detected. In addition, an interaction with catenins and occludin has also been noted [160]. Further investigations are needed to exactly corroborate modulation of epithelial TJs by C-CPE.

Finally, TJ modulation has been described upon exposure of 16HBE14o- airway epithelial cells to poly-I:C as a model for RNA virus infections, resulting in the down-regulation of the

expression of different claudins in [187]. Apparently, disruption of epithelial TJs occurs via dsRNA induced miR-155 suppression of several claudin proteins, potentially under involvement of the Toll-like receptor 3 (TLR3)/ Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF) signalling pathway.

2.2.2.3. Occludin

Occludin has been a well-studied TJ protein for permeation enhancement. As its mechanism of closing TJs is based on a homologous interaction with another occludin, some synthetic peptides based on the extracellular domain of occludin have been designed [188]. The peptide OCC2, based on the sequence of the second extracellular domain of occludin, has been proven to be effective in the paracellular permeation enhancement of 40 kDa dextran and shown to decrease occludin expression. This TJM has been proven *in vitro* to be specific to occludin and its effect to be reversible. The main field of interest for co-administration with a drug has been the blood-testis barrier (BTB) [189], where the intraperitoneal administration of FSH coupled to OCC2 has shown an increase in the permeability of BTB. Another *in vivo* experiment has been performed by direct injection into rat testes, yielding similar results [190]. A disruption of spermatogenesis was described possibly due to the reversible and concentration-dependent inhibition of Sertoli cell assembly. No further studies on the use of this peptide as a TJM have been published to date.

As previously mentioned, tricellular TJs have been identified. The main proteins identified that compose this junction are tricellulin and angulin 1 to 3 [36]. Tricellulin is present in bicellular TJs and so also close TJs [191]. The iota toxin or angubindin-1 found in *Clostridium difficile* has been shown to interact with angulin-1 [192]. This toxin consists of a domain exerting its toxicity and a second responsible for binding. As it has previously been demonstrated, angulin-1 (also named LSR) is responsible for the recruitment of tricellulin to the tricellular TJs [193]. Different tests have been performed *in vitro*, revealing a decreased expression of angulin-1 and tricellulin at the membrane resulting in increased permeability. Even if these targeted proteins are less abundant than claudins and occludin, an eventual TJM may be effective to increase drug permeation through this interaction.

2.2.2.4. PDZ proteins

Permeability enhancement involving PDZ proteins such as MAGI-1, MUPP1 and ZO-2 as targets has been shown by the human adenovirus type 9, which encodes a carboxyl-terminal PDZ domain-binding motif (PBM) [194]. The oncogenic determinant responsible for the interaction with these proteins is the E4 region e-encoded ORF1 protein (E4-ORF1). PDZ-protein binding induces disruption of cell layer polarity resulting in a higher permeation rate of the virus through the epithelium has been shown. This mechanism appears to be exploited by

a wide variety of different virus families, including influenza virus, severe acute respiratory syndrome coronavirus, and human immunodeficiency virus [195]. The use of PBM motifs as TJMs has been suggested, but to our knowledge not sufficiently explored yet.

Promising results have been noted from these second-generation TJMs. The direct inhibition of TJs protein permits the controlled modulation of only TJs. Moreover, most of these targets are present at the membrane and TJMs may not need to be optimized to enter the cells. However, time of action may be too short or the interaction may be irreversible. These lead to other kinetic problems for the efficacy and the toxicity respectively. A second-generation TJM would be a good candidate to modulate TJs in a formulation but an optimization for the time of action/opening is necessary.

2.2.3. Chemical structures of target specific TJMs

The variety of targets for TJMs leads to also a variety in the chemical structure of these molecules. A representation of these structures has been resumed in table 1. As it can be noted, small molecules such as piperazine or complete proteins such as ephrin can be considered as TJMs. However, some similarities can be found. TJMs targeting membrane receptors are more constructed as small molecules (bilobalide, piperazine). The targets are present out of the cell or at least in the membrane. The interaction can more easily occur. Then, peptidic molecules are more targeting enzymes or TJs proteins (HAV, AT1002, L-R5). The best interaction with a protein may occur with a molecule constituted with the same components (aminoacids). On another hand, big non-peptidic molecules seem to more enter the cell and disturb intrinsic mechanism (latrunculin A, OxPAPC). These similarities may not be rules but designing a new potential TJM would have better effect by following these similitudes.

3. Tight junction modulators in drug delivery

Modulation of TJs is an effective strategy for increasing drug absorption via the paracellular pathway. Under normal conditions, paracellular transport is generally restricted to compounds of molecular radii below 11 Å. TJMs have a unique advantage over transcellular absorption enhancers in terms of effectiveness as they are extremely potent in opening these TJs. Their application can be broad, ranging across various tissue barriers.

3.1. Ocular drug delivery

Drugs administered via topical or systemic routes must overcome several barriers to achieve an effective concentration in the retina and the vitreous. These barriers in ocular drug delivery can be classified as physiological and anatomical. Physiological barriers to ocular drug delivery comprise (i) tear turnover (ii) naso-lachrymal drainage and (iii) blinking. Anatomical

barriers can be further classified as static and dynamic barriers, which reduce drug entry into the anterior segment. Static barriers are the corneal epithelium, stroma, and blood-aqueous barrier (BAB), dynamic barriers include tear drainage, conjunctival blood and lymph flow. TJs are located between the non-pigmented epithelium of the ciliary body. Junctions of the iridial tissues and iris blood vessels limit drug absorption to the anterior chamber of the eye. In the posterior segment the barriers include sclera, Bruch's membrane-choroid (BC), retinal pigment epithelium (RPE) and conjunctiva, which are all static in nature. Both BAB and blood-retinal barrier (BRB) possess TJs between retinal capillary endothelial cells and RPE cells. This limits drug absorption in ocular compartments. Another barrier for drug delivery is the mucin layer present on the surface of cornea and conjunctiva [203–206].

Benzalkonium chloride, an irritant even at low concentration (<0.01%), is used as a preservative in about 74% of the ocular formulations [207]. Its major effect on the corneal epithelium is the decrease of barrier function, inducing dephosphorylation of MLC along with acute ATP release. Accelerated cell desquamation, compromised wound healing and cessation of mitosis have been considered as adverse effects. It also induces variety of cellular damages such as cell membrane damage, adenosine triphosphate depletion, and generation of oxidative stress, activation of AP-1 and NF- KB and cell apoptosis [208].

TJs between corneal epithelial cells offer resistance to the passage of hydrophilic molecules layers. The availability of Ca^{++} ions is crucial for the functionality of these TJs [209]. Polyaminocarboxylic acids such as EDTA, EGTA and ethylenediamine-N, N'-disuccinic acid (EDDS), collectively known as calcium chelators, are capable of sequestering interstitial Ca^{++} ions. These agents are used in topical ocular formulations as stabilizing agents. They also exhibit temporary and reversible permeation enhancement via disruption of TJs and AJs.

3.2. Nasal drug delivery

Low permeability of high molecular weight drugs is regarded as a major limitation in nasal drug delivery [210]. This is mainly due to their hydrophilic nature and large size. The bioavailability of these peptides and proteins ranges between 0.5% to 5% when applied via nasal route [211]. Use of permeation enhancers can help to improve bioavailability of these drugs.

In order for drugs to reach the capillaries below the mucosal layer, several barriers have to be passed. The foremost is the mucus layer, in general lipophilic small molecules can easily pass through the mucus while larger molecular weight drugs like peptides and proteins may find it difficult to pass through. This is due to the fact that hydrophilic molecules are very soluble in mucus and are thus more prone to elimination by mucociliary clearance [212]. For peptides, the intermolecular interactions between the peptide and the glycoprotein chains of the mucus may hinder the drug passage. Drug transport across nasal mucosa can take place through

transcellular or paracellular pathways. Lipophilic drugs usually pass the epithelium by passive diffusion, while specific transporters are expressed for transcellular active transport of certain molecules. As their normal diameters vary between 3.9 and 8.4 Å, TJs limit the passage via the paracellular space [213].

Permeation enhancers for nasal formulations are commonly tested for delivering macromolecules such as calcitonin (Miacalcin) [214]. Of these, surfactant-based permeation enhancers are among the clinically most advanced. Benzalkonium chloride, a cationic surfactant, was used in the nasal formulation for delivering miacalcin (salmon calcitonin). Non-ionic surfactants such as alkyl maltosides and polyethylene glycol stearates are the most advanced nasal permeation enhancers. IntravialTM, a nasal permeation enhancer platform that consists of DDM and tetradecyl maltoside (TDM) has been approved for the use as nasal absorption enhancer of migraine drug sumatriptan (TosymraTM, Dr. Reddy's, Hyderabad, India) [215].

Polyethylene glycol (15) – hydroxystearate (Kolliphor HS15, BASF, Ludwigshafen am Rhein, Germany) is a soluble non-ionic surfactant that is a part of the nasal drug delivery platform CriticalSorbTM (Critical Pharma, Nottingham, UK) [216]. Although it showed promising results in rodents (rats) it failed in large animals or humans. Two target specific tight junction modulators have been evaluated for nasal administration so far. These include (i) *Clostridium perfringens* enterotoxin (CPE) and (ii) Zonula occludens toxin (Zot).

The C-terminal fragment of CPE (C-CPE) modulates the barrier function via claudins [217]. C-CPE was used to increase the nasal absorption of human parathyroid hormone (hPTH) in rats [185]. The fragment is considered a potent absorption enhancer compared to current clinically used enhancers, however, toxicity remains the main problem [218]. Hence, many variants have been synthesized in order to decrease toxicity [219].

Zonula occludens toxin (ZoT) is a protein of *Vibrio cholera* and zonulin is the zot analogue that modulates tight junctions. Hence, efforts were made to design derivatives of ZoT that can be used as absorption enhancers. AT-1002, a hexamer peptide, increases intratracheal absorption of salmon calcitonin in rats by about 5.2-fold [220,221].

3.3. Intestinal drug delivery

C_{10} is the sodium salt of decanoic acid, which is an aliphatic, saturated fatty acid also known as capric acid. FDA approval of C_{10} as a GRAS (generally recognized as safe) ingredient has rendered it an attractive candidate as an absorption promoter. The fact that it has already been registered as an excipient has made C_{10} a key component in a number of formulation technologies including that of gastrointestinal permeation enhancement technology GIPETTM

(Merrion Pharmaceuticals, Ireland). C_{10} is one of the most widely tested absorption promoters since 1982 [103]. Its mechanism of action has been attributed to both paracellular and transcellular effects. *In vitro* studies suggest that C_{10} increases paracellular permeability by modulating tight junctions by relocalization of tight junction proteins occludin and claudin-1, as well as the transmembrane protein ZO-1. It was also shown to contract and redistribute perijunctional actin. The permeation effect is mediated by the activation of phospholipase C, which sequentially activates calmodulin and myosin light chain kinase. The transcellular mode of action may be due to the perturbation of the enterocyte plasma membranes causing leakage due to an increase in membrane fluidity [6,222].

AT1002 has been shown to disrupt TJ protein-protein interactions of ZO-1 with occludin, claudins and myosin 1c [136,223,224] via PAR-2 activation. This modulation is also associated with calmodulin and MLCK activation, resulting in phosphorylation of perijunctional ring myosin and TJ disassembly. Several *in vivo* studies evaluated the potential of AT1002 as a TJ modulator for various trans-mucosal administration routes. AT1002 co-administration significantly increased the intestinal absorption of cyclosporine A in Sprague-Dawley rats [139]. Significant intranasal absorption was observed when AT1002 peptide was used in combination with high molecular weight paracellular markers indicating the potential of AT1002 as an absorption enhancer for nasal delivery of drugs, as well [137]. A combination of AT1002 and ritonavir, when co-administered with bioadhesive polymer carrageenan, also yielded significant nasal absorption [225,226]. AT1002 was also effective as a permeation enhancer in intratracheal (salmon calcitonin) and transdermal application [227]. In combination with the Tat protein, AT1002 significantly improved the permeation of small interfering RNA through epidermis. However, AT1002 suffers from instability under neutral to basic pH conditions [221]. Disulfide dimer formation resulting in total inactivation of AT1002 was prevented by systematic amino acid exchange (cysteine in position 2). A non-dimerizing allyl-glycine derivative of AT1002 was shown to have additionally improved permeability effects. To be effective, AT1002 has to be combined with protease inhibitors for oral administration to protect against gastrointestinal peptide degradation.

Ephrin-A2, a family of receptor tyrosine kinases, disrupts epithelial barrier function by direct phosphorylation of claudin-4. Ephrin-A2 ligand causes vascular permeability in the lungs, resulting in the leakage of albumin into the lungs of rats [177]. High levels of ephrin-A2 mRNA are also expressed in the intestine [228]. Modulation of the ephrin-A2 system may be a possible strategy for increasing pulmonary and intestinal absorption.

Myosin light chain (MLC) acts as a regulatory protein that can undergo reversible phosphorylation to regulate TJs [229]. TJs are closed when MLC is dephosphorylated and

opened when MLC remains phosphorylated [230]. Permeant inhibitors of phosphatase (PIP) 640 and 280 are rationally designed peptides that selectively inhibit the subunits of myosin light chain phosphatase whose function is to prevent de-phosphorylation of myosin light chain kinase by protein-protein interactions. This increases the level of myosin light chain phosphorylation (MLC-p), which leads to an increase in paracellular permeability.

In vitro permeability studies showed that both peptides were able to increase the permeability of fluorescein dextrans of various molecular weights. PIP 640 enhanced permeation of 4 kDA while PIP 250 enhanced the permeation of 4 and 70 kDA dextrans. Insulin delivery using rat intestinal loop instillations resulted in a relative bioavailability of 4% (PIP 640) and 3% (PIP 250), with an event window of 30 to 90 minutes [58]. Further attempts to increase the stability and membrane permeability of both peptides include the introduction of D-isoforms and cationic amino acids [54].

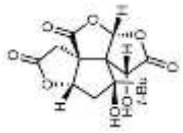
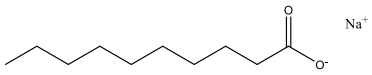
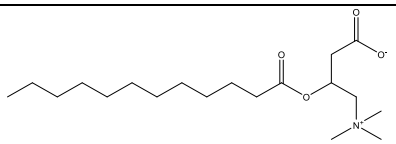
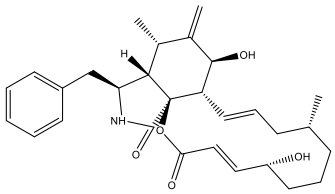
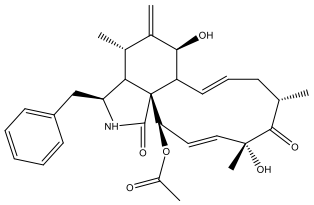
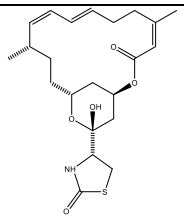
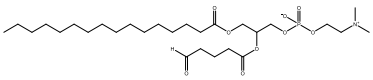
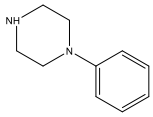
3.4. Delivery across the blood brain barrier

Claudin-5 is crucial for maintaining the barrier integrity at the BBB [231] and hence several modalities were developed around this target to increase drug permeability. Claudin-5 knockout mice died a day after birth indicating the crucial role of this protein [232]. The administration of siRNA against claudin-5 induced BBB permeability of molecules up to 1 kDa without any sign of toxicity [233]. This strategy has also been proven effective in reducing water content in mice with focal cerebral oedema. Interestingly, co-administration of siRNAs against claudin-5 and occludin could increase the permeability of molecules up to 3 to 5 kDa, indicating a synergistic effect [234]. However, the major impediment for translation of siRNA modalities is the lack of robust delivery systems in human [235].

Adeno-associated virus (AAV) carrying the expression system of a doxycycline–inducible short hairpin RNA against claudin-5 was also used to achieve transient BBB modulation in a site and size specific manner [236–238]. Antibodies derived against the extracellular loop of anti-claudin-5 proved to be high affinity, high selective modalities that could bind to claudin-5 and lower barrier integrity of an *in vitro* triple culture model of the BBB without any toxicity. However, no *in vivo* data are available so far.

Another alternative for the specific targeting of TJs proteins are the peptides derived from extracellular domains of junctional proteins. Claudins inhibitory peptides have been derived from two regions: a peptide derived from the C-terminal half of the first extracellular loops including ECH1 or from ECH2 [239,240]. Two D-amino acid peptidomimetics were developed [241,242]. The peptidomimetic C_5C_2 enhanced the permeability of gadolinium chelate at the BBB in mice. However, the disadvantage with these peptides are poor claudins-selectivity due to the nature of heterophilic interaction of claudins.

Table 1: TJMs chemical structures.

ADT/HAV	**Angubindin-1**	**AT1002**
Acetyl-S**HAV**AS-NH_2 Ref: [159]	Aminoacids 421-664 from iota toxin Ib C-terminal Ref: [36]	H-FCIGRL-OH Ref: [140]
Bilobalide	**C_{10}**	**Lauroylcarnitine**
 Ref: [196]	 Ref: [112]	 Ref :[197]
C-CPE	**Cytochalasin B**	**Cytochalasin D**
C-terminal half of CPE Ref:[184]	 Ref: [198]	 Ref: [199]
E4-ORF1	**Ephrin**	**JO-1**
X-(S/T)-X-(V/I/L)-COOH Ref: [194]	Intrinsic protein Ref: [175]	Small recombinant protein from HAdV3 fiber Ref: [200]
Latrunculin A	**Larazotide**	**L-R5**
 Ref: [76]	H-GGVLVQPG-OH Ref: [143]	Myristoyl-ARRWR-OH Ref: [155]
OCC2	**OxPAPC**	**PIP 640**
Aminoacids 184-227 of chick occludin Ref: [188]	 Ref: [201]	RRDYKVEVRR-NH_2 Ref: [127]
Piperazine	**PN159**	
 Ref : [202]	NH_2-KLALKLALKALKAALKLA-amide Ref: [174]	

HAV6 and ADT6, two peptides designed to inhibit cadherin, could strongly modulate BBB permeability. HAV6 is a hexapeptide with a short blood-circulation time. It is known to improve the permeability of albumin only for 10 minutes after its injection into mice [243]. However, HAV6 is not site specific as it also improved the vascular permeability in kidneys, lungs and small intestine and also the epithelial barrier in those tissues [244]. Hence intracarotid artery administration might be a suitable method for these short peptides in order to minimize side effects. Anti-VE cadherin mAbs created extravasation of blood-circulating cells when administered into mice and the mice died within a day.

Truncated fragments of bacterial toxin fragments may be used as PEs upon removal of their toxicity-inducing domain. However, due to their bacterial origin, the risk of immunogenicity remains. *Clostridium perfringens* enterotoxin can open the TJ barrier by binding to several claudins, specifically that of claudin-3 and -4. The C-terminal of *Clostridium perfringens* iota–toxin B was shown to modulate the tricellular TJs by binding to angulin-1 and -3 [36]. Modulation of the BBB in mice and in a zebrafish model was shown to improve drug bioavailability in the brain.

Several efforts have also been made to modulate BBB TJs via targeting non-junctional receptors. The target being mostly kinases. These pharmacological modulators are potent however, they lack size selective loosening of the junctions unlike the TJ protein targeted peptides or antibodies. They also sometimes cause cytoskeletal contractile reactions. An example is RMP-7, an analogue of bradykinin that progressed to clinical phase II for the treatment of glioma [245], however, did not alter the pharmacokinetics of carboplatin at the doses applied during the trial in recurrent malignant glioma patients.

Some of the BBB modulators that target non-junctional receptors include RMP-7, AT-1002, NIBR-0213, Lexiscan A2A, NS1619, Glutamate and Gintonin. These modulators eventually activate one of the kinases downstream and open the paracellular route. These modulators are powerful, cause extensive BBB modulation and their dose should be lowered. On the other hand, the lowering of dose of RMP-7 was one of the reasons why clinical phase II study in patients with glioma of RMP-7 was failed [245]. Hence, it might be challenging to determine the appropriate dose range to open the tight junctions transiently.

Various studies have demonstrated intra-arterial drug delivery methods to be safe and efficacious for a variety of therapeutics [246]. Intra-arterial route may be the ideal route for delivering the target-based BBB tight junction modulators as it enables delivery of high concentration through a targeted vascular territory, while potentially limiting systemic toxicity due to off target effects or due to the co-administered therapeutic agents. Advantages of intra-

arterial route in delivering target based TJMs include (i) local drug delivery (ii) unique pharmacokinetics and (iii) dose advantages.

However, one of the main drawbacks of intra-arterial drug delivery is its extremely complex pharmacokinetics [247,248]. Other factors include inadequate optimization of injection parameters as well as lack of rationalization in drug selection [249,250]. Despite the above-mentioned disadvantages, intra-arterial drug delivery have been widely used in recent years, either off-label or as part of clinical trials especially in the scope of glioblastoma [251,252]. Thus, the trends in recent studies indicate the use of super selective intra-arterial cerebral infusion and less neurotoxic chemotherapies. Most trials continue to use mannitol as the preferred method of hyperosmolar BBB disruption. Usage of target-based BBB TJMs might be a suitable TJs opening agent that can replace unselective mannitol. With optimization and standardization of the techniques of intra-arterial drug delivery along with transient, target selective BBB modulators and improved selection of therapeutic agent, it can offer great benefits to patients [246].

Alternatively, ligands of transporters or receptors can be used to target BBB tight junction mechanisms towards BBB when systemically administered. In this approach, the ligand will be a mere facilitator to deliver the BBB modulator to the BBB. Receptor or transporter ligands can be used to target BBB. On the BBB many receptors are overexpressed, including the transferrin receptor, insulin receptor or LDL receptor–related protein [253,254]. The ligation of these receptors triggers internalization into cells. Thus, the corresponding ligands could be functionalized onto the BBB tight junction modulators to target BBB. Receptor mediated transport is the most widely used and successful strategy to deliver cargo like that of nanoparticles due to the specificity of the interaction between receptors and ligands. However, the targeting ligand – TJ modulator construct must escape the lysosomes post internalization to access the molecular target.

3.5. Tumor drug delivery

The therapeutic outcome of the treatment of solid tumors using a monoclonal antibody (mAB) or cytotoxic drugs depends on tumor mass penetration, target affinity and tissue retention. Studies have shown a positive correlation between up-regulation of tight junction proteins in solid tumors and their resistance to drug therapy, including monoclonal antibodies and chemotherapeutics [255,256]. The epithelial phenotype of solid tumor cells and their capacity to form tight junctions might guard them from immune system attack and chemotherapeutic drugs [257]. Only about 10% of FDA approved mABs are used in therapy of solid tumors, while the others or applied for the treatment of haematological tumors [258]. One of the key reasons for this is that mABs are not able to penetrate into the inner layers of the tumours.

Table 2: Selected properties of TJMs.

TJM	Cell line	Permeation marker	Toxicity	Reversibility	Onset	Ref.
ADT/HAV	MDCK	Mannitol	No	>5 h	30 min	[159]
	Caco-2	-	No	>9 h		[156]
Angubindin-1	EpH4	FITC 40 kDa	-	-	-	[193]
	Caco-2	FITC 40 kDa		42 h	12 h	[36]
	RBT-24	-		48 h	6 h	[269]
AT1002	Caco-2	CsA	No	-	40 min	[139]
	SCBN	FITC 3 kDa	-		1 h	[273]
Bilobalide	hCMEC/HEB	Na-F	No	4 h	1 h	[78]
C_{10}	Caco-2	FD 4 kDa	20 mM	>24 h	20 min	[110]
Carnitine	Caco-2	FD 40 kDa	-	-	5 min	[197]
C-CPE	HNEC	FITC insulin	No	-	1 h	[186]
	MDCK	FD 10 kDa		48 h	4 h	[184]
Cytochalasin B	Caco-2	Mannitol	-	-	10 min	[124]
Cytochalasin D	Caco-2	RVPSL	-	-	2 h	[79]
E4-ORF1	MDCK II	-	-	-	8 h	[194]
Ephrin	bPAEC	FITC 70 kDa	-	-	30 min	[177]
JO-1	T84	PEG 4000	No	25 h	15 min	[163]
Latrunculin	MDCK	Insulin	-	-	15 min	[87]
	Caco-2	FITC-dextran		-	15 min	[84]
Larazotide	Caco-2	FITC 4 kDa	-	2 h	45 min	[274]
L-R5	MucilAir™	FD 4 kDa	No	>3 h	30 min	[155]
OCC2	A6	Dext 40 kDa	No	96h	24 h	[188]
OxPAPC	BAEC	Dext 10 kDa	No	-	1 h	[92]
PIP 640	Caco-2	Dext 70 kDa	No	24 h	15 min	[127]
Piperazine	Caco-2	FD 70 kDa	No	24 h	10 min	[125]
PN159	Caco-2	FD 40 kDa	No	23 h	1 h	[173]

The Her2/neu [259,260] and EGFR [261,262] receptors, which are targets of therapeutic antibodies, are reported to be localized within the basolateral membrane of tumor cells. Target specific tight junction openers (TSTJO) will be promising candidates as antitumor adjuvants, especially when administered by intratumoral administration. A number of trials has been performed and are currently ongoing for intratumoral administration of oncolytic viruses in head and neck cancer [263], and cisplatin in lung cancer [264,265], which may in the future be combined with TJ modulators to further improve efficacy.

JO-1 is a 60 kDa recombinant protein derived from adenovirus serotype 3 (Ad3), which contains the minimal structural domains required for opening intercellular junctions. JO-1 has the ability to activate intracellular signalling that results in transient opening of tight junctions. It has been shown that JO-1 interaction with desmoglein 2 (DSG-2) activates epithelial to mesenchymal transition (EMT) and leads to down regulation of junctional proteins like E-cadherin. The transient opening subsequently increases the intratumoral penetration of therapeutic antibodies like cetuximab and trastuzumab in mice models of lung, colon, gastric, breast and ovarian cancer [34]. JO-1 co-administration also increased efficacy of drugs such as PEGylated liposomal doxorubicin (Doxil®), irinotecan (Camptosar®), and nanoparticle albumin-bound (nab) paclitaxel (Abraxane®). As JO-1 co-administration increased the therapeutic index of chemotherapeutic drugs, the dose of these drugs can be reduced, thereby reducing their side effects [266]. JO-1 was also shown to target predominantly tumor cells compared to normal cells, due to higher expression of DSG-2 in the former. An improved version of JO-1, namely JO-4, has recently been studied for safety and biodistribution when co-administered with Doxil® in transgenic mice and *Macaca fasicularis*. JO-4 was able to render Doxil therapy effective at a dose three times lower than the usual therapeutic dose in a study conducted in a mouse model. The study conducted in *Macaca fasicularis* reported no JO-4 related toxicity [200].

Epithelial junctional opener (JO-1) binds to DSG2 on tumor cells activates pathways involved in epithelial – to – mesenchymal transition (EMT), a process associated with tumor metastasis. This raises the question of whether JO-1 might facilitate metastasis [267]. However, none of the *in vivo* studies conducted in all models used has reported any evidence of increased tumor growth or macroscopic/microscopic signs of metastasis after treatment with JO-1 alone. Moreover, JO-1 injection into Her2/neu-positive HCC1954 tumor bearing mice on the 3rd day did not cause any significant increase in the percentage of circulating Her2/neu- positive cells in the blood [266]. Hence, the hypothesis that the modulation of tumor tight junctions might promote metastasis has to be validated using well controlled experiments. Moreover, the possibility of a target specific tight junction modulator promoting metastasis might depend on several factors such as the molecular target chosen, its expression in the tumor tissue and

whether the treatment module involves a prolonged or multiple activation of the target. Each route of administration exerts its strengths and hurdles, Ocular delivery restrains choices due to sensitivity, intestinal delivery needs a mucosal attachment to release the drug and the TJM at the same place (due to motility), tumor and BBB TJs opening requires cautions and nasal delivery needs more tests and researches to be a better candidate.

4. Translation towards clinical application

4.1. Safety and regulation, bystander absorption

The development of TJMs and their implication in drug delivery is focused on enhancing drug permeability, resulting in a potential decrease in dose in combination with a reduction in side. Moreover, as it has been described before, safer routes of administration than invasive ones can be intended. However, the collection of safety data on a new drug delivery technology such as TJMs is a prerogative for the initiation of clinical trials. There are several aspects that may impede on the safe use of TJMs in drug formulations.

As some TJMs are extracted from bacteria (e.g., iota toxin, AT1002, C-CPE), the assessment of toxicity TJM itself as well as potential residual impurities of the extraction process has to be performed. In addition, the permeation enhancement activity of TJMs must not be regarded as isolated mechanisms, but rather impeding on other regulatory functions of the cell. C-CPE has been shown to bind to Claudin-1, which partially inhibits the toxic and permeation enhancement effect of the toxin. Paradoxically, this binding appears to stabilize C-CPE against enzymatic degradation by proteases, resulting in an enhancement of toxic effects and generating diarrhea in the intestines [268]. TJM activity may activate or enhance the expression of necrosis or apoptosis factors. In addition, the modulation of tricellular TJs by angubindin-1 has been shown to be less toxic than modulation of bicellular TJs [36], and no side effects were detected upon injection of angubindin-1 in mice [269].

The reversibility of TJ modulation is clearly correlated to safety of TJMs [1], and has been examined and confirmed for the majority of TJMs mentioned here. Second generation TJMs have been supposed to exert better reversibility of TJ modulation as they have a more precise target and do not involve intracellular processes [6].

One of the main arguments against the use of TJMs is the potential co-absorption of "bystanders" such as bacteria, viruses and other xenobiotics present in the intestinal lumen. Especially the enhanced of absorption of bacterial cell wall lipopolysaccharide (LPS) and its fragments is of concern, as LPS can cause an inflammatory response through the TLR4 pathway. Diverse parameters have been considered to refute this controversial argument. First of all, bacterial toxin bystanders have a molecular weight between 70 and 900 kDa [222].

While the molecular weight of LPS is > 100 kDa, the maximum molecular weight of a fluorescent dextran (FD) passing through the intestinal epithelium *in vivo* under the enhancement of a TJM was shown to be about 70 kDa [270]. In addition, the intestinal mucosa would also feature the secretion of mucus and fast cell turnover, in addition to peristaltic movement. These properties would not allow for sufficient time for bystanders to pass through the cell layer [6]. These different results and arguments may not be applied to all TJMs, but studies on TJM used in pharmaceutical formulations have indicated safety in terms of co-absorption of bystanders to be less of a problem.

4.2. System for development of TJM

As the improvement of target based TJM has become more and more of interest, new TJMs will be developed based on the understanding of a well-understood mechanism of TJ regulation to increase efficacy and safety, as side effects caused by these molecules have been shown to be reduced compared to non-targeted TJMs [70]. Discovery of tissue-specific TJMs of known mechanism of action would facilitate the development of new drug formulations, which may be hindered by low drug bioavailability. Moreover, new therapeutic options (peptides, proteins) and/or routes of administration will be pursued using these active excipients [271]. The question of sufficient bioavailability will be replaced by the question of drug compatibility with TJMs.

Different systems of classification to guide the decision of selecting the most suitable of TJMs to be used for a certain formulation, route of administration or drug to enhance the permeability, have been established. Different parameters such as toxicity, time of effect onset, kinetics, size of drug to permeate, type of cell/tissue, mechanism of action and reversibility have to be taken into consideration and should include all TJMs. Saaber et al. [61] have created a unified notation factor to compare 8 different TJMs with 4 properties (TEER, permeability of mannitol, viability and cytotoxicity) with respect to their effect on MDCK cells. The scoring system is expressed in a simple equation with possible weighting of each setting. As previously said, there are many details to be taken into consideration and of course not all of them are considered. One of the disadvantages of the proposed system is that it was applied to only one cell line, which additionally may not completely reflect *in vivo* conditions. Therefore, this TJM scoring system may be regarded as an “attempt to solve the problem of the comparability” and not as an absolute comparator. A similar system has been suggested for *in vitro* models of the BBB [272]. Maher et al. [6] reviewed different intestinal permeation enhancers, and resulted in a classification according to the recovery, speed and strength of the effect obtained *in vitro*, in situ and ex vivo. This classification can be applied to determine, which TJM would be suitable for a certain oral formulation and also to compare between a

new intestinal TJM and existing ones. Moreover, it can be used to measure the efficacy of an intestinal PE.

A non-exhaustive list of the different properties of TJMs used to characterize their efficacy and toxicity in different cell lines is shown in table 2. Due to the diversity of conditions under which the TJMs were tested (TJM concentration, cell line, permeation marker, incubation conditions, etc.), a direct comparison between and selection of TJMs for a specific application is not possible.

4.3. Clinical applications

The final steps after adequate TJM discovering are to select a drug to combine with and perform preclinical studies. Even with a potent TJM, new complications may be discovered by combining the API and/or excipients with the PE. Precipitation, chelation or degradation are among these formulation challenges that may be provoked. Stability and compatibility studies have therefore to be performed. Additionally, the importance of the type of formulation can be decisive as not all TJMs can be formulated in solid or liquid form. JO-1 has been proven to be selective for tumors [162,266], and a conjugation to TLR3 agonist poly(I:C) has been designed as an immune-oncologic agent to trigger apoptosis in tumoral cells [275]. Conjugation with polymeric molecules such as PEG has been proven to be as effective as JO-1 alone with the advantage of a better protection and/or delivery of JO-1. On the other hand, the route of administration must also be strongly considered. For example, piperazine has considered for gastro-intestinal application and it has been noted that the pH value was a key parameter for its efficacy as a TJM [276]. RMP-7 has been grafted on liposomes loaded with quercetin [277], which allowed for an increase in quercetin permeability through the BBB to treat Alzheimer disease. This formulation served to protect the PE and at the same time assure the co-localization of API and the active excipients. However, reports have been published for consecutive phase I and II trials, in which no significant advantages were demonstrated [278,279]. JO-4, an affinity enhanced version of JO-1 [162] has also been included in a formulation of PEGylated liposomes, in which the TJM served both the targeting of the liposomes, and to successively enhance the permeation of the API doxorubicin in xenograft model of ovarian cancer [200].

Once the formulation including the TJM is optimally designed, clinical trials may be undertaken. Until now several TJMs shown in figure 4 have been involved in clinical trials. As previously mentioned, larazotide has been tested to enhancement of treating celiac disease [280]. A nasal spray has been tested combining Solutol® HS15 with the parathyroid hormone (PTH) to treat osteoporosis [281]. This molecule has not been considered as a target specific TJM but has been also tested in clinical trials as a PE. However, studies in healthy volunteers

revealed faster clearance from the nasal epithelium accompanied by a lower absorption rate than predicted from preclinical models. PTH has also been combined with carnitine in a tablet by Enteris technologies [222] and tested in phase II in clinical trials.

Table 3: Current clinical status per route of administration.

Route of administration	PE	Clinical status	Drug associated	Reference
Ocular	Benzalkonium chloride	On the market	Dexamethasone	[282]
	EDTA	On the market	Riboflavin	[209]
Nasal / intratracheal	Benzalkonium chloride	On the market	Salmon calcitonin	[214]
	DDM/TDM	On the market	Sumatriptan	[215]
	Solutol®	Fail (not significant)	Insulin	[216]
	C-CPE	Fail (toxicity)	hPTH	[218]
	AT1002	Preclinical	Salmon calcitonin	[220]
Intestinal	AT1002	Preclinical	Cyclosporin A	[139]
	Larazotide	Phase II	-	[280]
	Carnitine	Phase II	PTH	[222]
	EDTA	Phase IIa	Insulin	[283]
	C_{10}	Phase III	Somatostatin	[284]
BBB	RMP-7	Phase II	Carboplatin	[117]
	C-CPE	Preclinical	Taxol	[285]
Intratumoral	JO-4	Preclinical	Doxil®	[266]

The use of MCFA C_{10} in a tablet formulation was realized in GIPET technology [286]. A combination of the widely studied TJM C8 with a somatostatin analogue resulted in promising outcomes in a phase III trial [284], with merely nausea detected as an adverse effect. Non-specific paracellular PEs have also been included in clinical trials. A combination of insulin with bile salts and EDTA (Oramed) [222,283] has been developed to phase IIa clinical trials.

Overall, considering the broad range of TJMs, they are until now rarely involved in clinical trials. The current clinical status of the TJMs is resumed in table 3 per route of administration. This may be due to the fact that the *in vitro* / *in vivo* correlation may not be given, or transition

from animals to humans may not be possible. Lamson *et al*. have reported that one of their problem for intestinal formulation with piperazine as PE was the pH [276]. Targeting may be also a problem depending on the cell type. Endothelial and BBB cells are less accessible than epithelial cells. These last cells can be reached with a local administration, in contrary with BBB or endothelial cells which require an injection or a strong specificity in targeting these cells. Failed clinical trials are rarely published but main hurdles in clinical trials for TJMs are formulations, route of administration and correlation in the different steps (*in vitro*, preclinical tests, clinical trials) and the significant advantage between the original formulation and the potential new one with the TJM. Finally, the development and formulation of a TJM is close to APIs.

5. Conclusions

The benefits of enhancing drug permeation across biological barriers are an enhanced drug bioavailability and potentially reduced side effects. Unspecific permeation enhancers that act by disturbing the cell membrane integrity or homeostasis are being replaced by tight junction modulators that interact with well-defined target molecules and mechanisms. This will in the future allow for a rational assessment of TJM safety and their selection for the enhancement of bioavailability of selected API at biological barriers such as the intestinal and nasal mucosae and the blood-brain barrier, and an increase in efficacy of cancer therapeutics.

6. References

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Chapter 2: Short peptide permeability enhancer

Short peptide sequence enhances epithelial permeability through interaction with atypical protein kinase C zeta

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Foreword: The modulation of tight junctions has recently been a common project of several research groups. This modulation could increase the absorption of drugs. Different cellular mechanisms have been explored. Here, modulation of protein kinase C zeta activity was one of the potential ways to open tight junctions. Our L-R5 peptide was synthesised following the pseudosubstrate sequence of the enzyme in order to inhibit its activity. This chapter demonstrates the possibilities of the peptide as well as the safety of this molecule.

This work, entitled “Short peptide sequence enhances epithelial permeability through interaction with atypical protein kinase C zeta”, was published in the journal *European Journal of Pharmaceutical Sciences* in May 2021 (DOI: 10.1016/j.ejps.2021.105747). The accepted manuscript is presented below, with some further corrections.

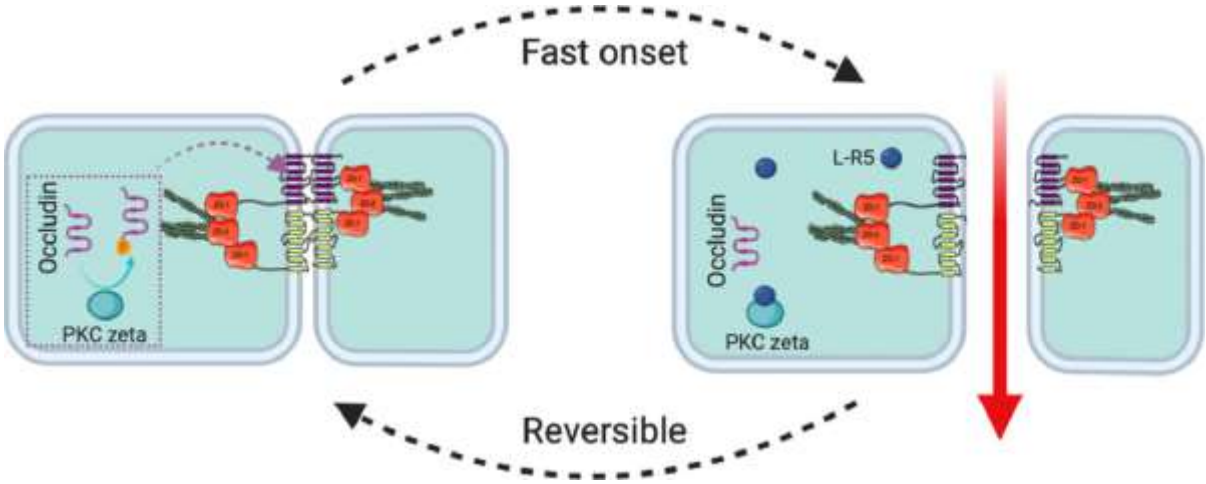
Author contribution: Prof. Gerrit Borchard, Dr. Sakthikumar Ragupathy and I designed and planned the study. Dr. Sakthikumar Ragupathy performed the LDH and CBF experiments as well as peptide synthesis. I was in charge of the cell culture, permeability studies, TEER measurements, haemolysis assays and design and realisation of the analytical methods. Dr. Sakthikumar Ragupathy and I have written the manuscript with the support of Prof. Gerrit Borchard.

Declaration of interest:

The authors declare no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Abstract

We have identified a short peptide sequence (L-R5) acting as partial inhibitor of intracellular atypical protein kinase C zeta, capable of tight junction modulation in terms of reversible and non-toxic drug permeation enhancement. L-R5 is a pentapeptide with a cell-penetrating group at the N-terminus and of the sequence myristoyl-ARRWR. Apically applied *in vitro*, L-R5 transiently increased epithelial permeability within minutes, enhancing apical-to-basolateral (AB) transport of 4 kDa dextran and BCS class III drug naloxone. L-R5 was shown to be stable and bioactive at 37°C over a period of 24 hours. L-R5 was shown to be non-cytotoxic in consecutive exposure studies on primary human nasal epithelial cells by LDH release assay and ciliary beating frequency test. It also proved to be non-cytotoxic in the haemolysis tests. Finally, L-R5 by itself showed very low diffusion across epithelial monolayers, which is of advantage with regard to its expected negligible systemic bioavailability and toxicity. Taken together, these data demonstrate the potential of short peptide partial inhibitor L-R5 to enhance the epithelial paracellular permeability via a reversible mechanism, and in a non-toxic manner.



Keywords: Tight junction modulators, permeation enhancer, bioavailability, permeability, naloxone

1. Introduction

Modern day drug screening techniques such as high throughput screening have resulted in a majority of drug candidates that encounter physicochemical and pharmacokinetic challenges such as poor solubility and permeability [1]. About 40% of orally administered drugs present on the World Health Organization (WHO) Model List of Essential Medicines belong to Biopharmaceutics Classification System (BCS) class III drugs that suffer from low permeability [2]. Apart from oral administration, several active pharmaceutical ingredients (API) exhibit poor permeation behavior at various other biological barriers such as the blood brain barrier [3], the cornea [4], and the nasal mucosa [5].

To address absorption enhancement across biological barriers, several approaches have been devised in recent decades. The aim of these drug development strategies is to overcome poor absorption resulting in low bioavailability. They include chemical approaches like prodrug strategies, the inhibition of efflux transporters such as P-glycoprotein and formulation approaches such as nanocarrier systems, self-microemulsifying drug delivery system (SMEDDS), microneedles, iontophoresis and the use of absorption enhancers such as cyclodextrins, bile salts and surfactants [6].

However, the techniques to improve drug bioavailability mentioned above have a major disadvantage of being linked to particular properties of the API and/or being restricted to certain tissues. Out of these considerations, the idea of developing tight junction (TJ) modulators as a 'stand-alone' approach has evolved [7]. Reversible and predictable modulation of TJs would increase paracellular drug transport of BCS class III and IV drug candidates and possibly high molecular weight drugs like therapeutic proteins. Predictable TJ modulation offers several advantages including the increase in bioavailability permitting lower doses to be used, which may then result in reduced side effects [8,9].

The major biological barrier limiting the permeation across epithelial monolayers is the apical junctional complex (AJC). The AJC is composed of TJs and adhesion junction structures. TJs are a complex arrangement of proteins between adjacent epithelial cells that regulate passage of ions or molecules through the paracellular space. TJs also determine cell differentiation by giving a clear distinction between the apical and basolateral side of the epithelial cell layer. They are composed of different segments of proteins, namely the transmembrane proteins (claudins, occludin, junctional adhesion molecule (JAM), etc.), and the cytoplasmic scaffolding proteins (ZO-1, cingulin, afadin, MAGI1, etc.). The cytoskeletal proteins of TJs are actin and tubulin [10].

In the past, numerous TJ modulators have been studied and are classified based on their (i) drug permeation enhancing properties, (ii) mechanism of action, and (iii) safety/tolerability.

The first generation of TJ modulators include calcium chelators such as ethylene-diamine tetra acetic acid (EDTA), surfactants such as benzalkonium chloride and sodium salts of fatty acids such as sodium caprate. These modulators are unspecific and were selected empirically using *in vitro* screens [8]. For example, promising candidates like palmitoyl carnitine were later dropped due to their lytic effects on cell membranes that eventually lead to cell lysis [11]. Another example is sodium caprate, which was approved as an absorption-enhancing agent in a rectal ampicillin suppository and causes TJ dilations and enhances paracellular permeability *in vitro* [12]. However, in humans the efficacy is rather associated with non-specific damage to the rectal mucosa rather than paracellular permeability modification [13]. Second generation TJ modulators are target specific and mostly interacting with tight junction proteins. These include peptides corresponding to occludin [14], toxins derived from *Clostridium perfringens* enterotoxin [15,16] and antisense RNA targeting tight junction proteins [17]. These modulators exhibited low biocompatibility along with cytotoxic effects [18].

Progress in the understanding of the molecular architecture and signaling pathways involved in the regulation of TJs has led to the identification of potential targets and safe strategies to reversibly modulate TJs. The 3rd generation tight junction modulators are being developed based on these specific targets with a clear mechanism of action. This approach is entirely different from TJ disruption, induction of morphological changes, membrane interference or other cytotoxic effects that were exhibited by first and second-generation TJ modulators [19]. Thus, development of 'mechanism-based' (MB) TJ modulators is a promising approach that would lead to gentle and reversible opening of tight junctions [9].

MBTJ modulators might have better advantage in terms of translation towards clinics owing to the understanding of mechanism of action and predictability of possible side effects. First generation TJ modulators are the widely studied category of tight junction modulators as some of the have reached clinical trials [20,21]. Several signalling pathways that participate in the regulation of tight junctions have been elucidated. The signalling pathways that regulate TJ function can be broadly grouped as protein kinase C (PKC), Rho-associated protein kinases (Rho/ROCK) and myosin light chain (MLCK) pathways. The crosstalk between the TJ components and these signalling pathways regulates their assembly or disassembly [22]. Phosphorylation of TJ proteins is the most common means by which these kinases involved in the signalling pathways regulate tight junctions [22,23].

A widely acknowledged and elucidated signalling cascade playing an important role in TJ regulation is the MLC II pathway. MLC kinase (MLCK) phosphorylates MLC II to increase TJ permeability while MLC phosphatase (MLCP) dephosphorylates MLC II to restore TJ structure and function [24]. Rho-associated protein kinases (ROCK) can also activate MLC II leading to

an increase in TJ permeability [25]. Comprehensive elucidation of the TJ regulatory signalling cascades have led to studies that performed rational screening to identify MBTJ modulators such as the decapeptide PIP 640 [26]. However, it might be essential to improve the potency of these peptides to increase their translational potential and application in oral dosage formulations [27].

Protein kinase C (PKC) is a family of serine/threonine kinases involved in several cellular functions. PKC isoforms are categorized as conventional (α, β1, β2, γ), novel (δ, ε, η, μ, θ), and atypical (ζ, ι/λ) isoforms. Unlike conventional and novel isoforms, atypical PKC isoforms do not have a C1 domain, which is responsible for membrane localization of other PKC isoforms. Atypical protein kinase C zeta (PKC ζ) is essential for the assembly of tight junction proteins and has been shown to be involved in the maintenance of cell polarity [28,29].

We had previously established the role of PKC ζ in the regulation of epithelial TJs. Toll-like receptor ligation increased barrier function of bronchial epithelial cells via downstream target PKC ζ and an increase in tight junction protein claudin-1 [30]. PKC ζ has been implicated in promoting the junctional assembly of human intestinal epithelial cells (Caco-2) and canine epithelial kidney cells (MDCK). PKC ζ has been shown to phosphorylate occludin and ZO-1 directly, which are essential for epithelial tight junction assembly [31]. We hypothesized that the inhibition of PKC ζ in a controlled manner would lead to a reversible modulation of tight junction permeability. However, irreversible/potent inhibition of PKC ζ might lead to toxic detrimental effects as the enzyme is implicated in vital cellular roles such as cell polarization and proliferation [32]. Zeta inhibitory peptide (ZIP) is a 13-amino acid pseudosubstrate inhibitor of PKC ζ [33]. While ZIP is a reversible and competitive inhibitor [34], regulation of the tight junction permeability by interacting with PKC ζ should occur by partial inhibition in order to maintain PKC zeta's vital cellular functions. We successively decided to optimize the structure of the peptide to design a safe and effective TJ modulator of fast onset of permeation enhancement and being non-toxic for short- and long-term use, which resulted in a myristoylated pentapeptide partial inhibitor of PKC ζ, L-R5 [35]. We here report *in vitro* data on the paracellular permeation enhancement of BCS class III drug, naloxone through the activity of this novel MBTJ modulator.

Today more and more drug candidates are of low solubility and/or permeability [36], classified as BCS III or IV drugs. Moreover, 85% of the drugs developed in Europe and USA are administered orally drugs where the pre-absorption metabolism and the absorption itself represent challenges. One way to avoid these problems is to enhance the permeability of the epithelium and/or to absorb drugs through another epithelium than the intestinal one. The

parenteral route, most suitable in terms of bioavailability is associated with some risks and is not particularly appreciated by patients.

The paracellular passage through the nasal epithelium avoids gastrointestinal metabolism and parenteral safety risks. Additionally, due to high blood perfusion of the nasal epithelium [37,38], drugs absorbed after nasal inhalation reach the systemic circulation fast. Patient compliance is also a real advantage of using the nasal route, however, the problem of absorption through the epithelium is still present. The opioid crisis has become a real problem especially in the USA. In 2010, 2% of the Americans were users of pain relievers and 17.3% of them abused these drugs in the absence of a real indication [39]. Naloxone is used to counter opioid effects by competitive binding to the μ-receptor [40]. Currently, naloxone is principally administered by i.v. injection or by intranasal spray (Narcan®). It has been shown that approximately 4% of the drug administered intranasally passed through the nasal mucosa compared to the i.v. administration [41]. Some studies showed the oral bioavailability of the drug to be even lower [42].

In this study, the efficacy and toxicity of our peptide L-R5 on nasal epithelial cells was investigated. Moreover, the combination of this peptide with naloxone as a potential future treatment was tested.

2. Materials and methods

2.1. Materials

Dichloromethane (DCM), dimethylformamide (DMF), N,N- diisopropylethylamine (DIEA), 2-(1Hbenzotriazol- 1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), hydroxy benzotriazole (HBOT), ethanedithiol (EDT), triisopropylsilane (TIS), trifluoroacetic acid (TFA), Pyridine, Acetonitrile, Ninhydrin-alcohol, Methanol (MeOH), Sodium chloride (NaCl 0.9% + CaCl2 1.25mM + HEPES 10 mM) solution, naloxone were obtained from Sigma-Aldrich, Buchs, Switzerland. Water for injection (WFI) was purchased from Corning, Manassas, VA, USA. Ethanol from purchased from Biosolve Chimie, France. Dulbecco's Minimal Eagle's Medium (DMEM) Hank's balanced salt solution (HBSS) and phosphate buffered solution (PBS) were obtained from Gibco, Zug, Switzerland. Fresh defibrinated sheep blood was obtained from Thermo Fisher Scientific, Zug, Switzerland. Lactate dehydrogenase (LDH) was purchased from Roche, Basel, Switzerland. The tripeptide N-Acetyl-Ala-Arg-Arg (N-Acetyl-ARR, 3 AA) and myristoylated 13-amino acid peptide Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu (Myr-SIYRRGARRWRKL, 13AA, were purchased from Bachem AG, Bubendorf, Switzerland. Primary human nasal epithelial cells MucilAir™ and MucilAir™ cell culture medium were purchased from Epithelix, Plan-les-Ouates, Switzerland. All other

reagents were of analytical grade unless otherwise stated and obtained from commercial sources.

2.2. Cell culture

Primary human nasal epithelial cells MucilAir™ provided in 24-well plates with 6.5 mm inserts; 0.4 µm pore size polycarbonate membrane inserts with a surface are of 0.33 cm^2 were used throughout the study. The inserts were maintained at 37°C and under an atmosphere containing 5% of CO_2. MucilAir™ cell culture medium was utilized and changed every 2-3 days. Based on manufacturers recommendations, a few hours before the experiments the cells were incubated with 100 µl saline solution (NaCl 0.9% + CaCl2 1.25mM + HEPES 10 mM) at the apical compartment for 20 minutes, aspirated to remove the mucus, and then rinsed once with warm phosphate buffered saline (PBS). Before the start of permeability experiments Hank's balanced salt solution (HBSS) 100 µl was added to the apical compartment and 600 µL to the basolateral compartment and allowed to equilibrate for 30 minutes in the incubator.

2.3. Transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was measured after the equilibration of the cells and immediately after the experiments using an EVOM volt-ohmmeter (World Precision Instruments, Stevenage, UK) equipped with chopstick electrodes. The TEER values (Ω cm^2) were calculated by using equation 1:

$$\text{TEER } (\Omega\ cm^2) = (\text{resistance value } (\Omega) - 100\ (\Omega)) \times 0.33\ (cm^2) \quad \text{(Eq. 1)}$$

where 100 Ω is the resistance of the porous membrane the cells were seeded upon, and 0.33 cm^2 is the total surface of the epithelial cell layer. TEER was always measured in warm HBSS.

2.4. L-R5 peptide synthesis

L-R5 peptide (Myr-ARRWR) was prepared by solid phase (SP) synthesis by Fmoc (fluoren-9-ylmethyloxycarbonyl) strategy. The resin was swelled in dichloromethane (DCM) and then drained completely. In order to protect the amino acid, 2-chlorotrityl chloride-based resin was left for swelling in dimethyl formamide (DMF) over 30 minutes. Then, 1.6 g of the first amino acid (Fmoc-L-Arg(Pbf)-OH) from the C-terminus of the peptide together with an Fmoc-protecting group were dissolved in DMF/DCM (Sigma-Aldrich, Switzerland) at a 1:1 ratio. A 10-fold DIEA (N,N-diisopropylethylamine) was added to the Fmoc protected amino acid and left stirring for 30 minutes at room temperature under nitrogen atmosphere. The final product was washed with dimethylformamide (DMF) (3x), dichloromethane (DCM) (3x) and DMF (3x), washed with 5 ml MeOH and then vacuum-dried before the deprotection step.

After each coupling, the resin was deprotected using 20% piperidine in DMF (15 ml/g) for 15 minutes. The resin was then washed with DMF (3x), DCM (3x) and DMF (3x). The completion of the reaction was confirmed using 25% ninhydrin-alcohol solution and 20% phenolic-alcohol solution followed by pyridine addition. The sample was heated at 105 °C for 5 minutes. A deep blue coloration of the resin beads indicated a positive reaction. The coupling was repeated in case of a positive reaction.

Protected amino acid, HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HOBT (hydroxy benzotriazole) and DIEA were dissolved in DMF and then DCM and left to react for 1 hour. The resin was alternately washed three times with DCM and DMF. After coupling of each amino acid, the resin was deprotected followed by ninhydrin assay (positive reaction) and the next amino acid was added to the sequence.

Myr group was added at the N-terminus of the peptide. Then the resin was washed twice with DMF, followed by MeOH, DMF and DCM, and finally vacuum-dried for 10 minutes. The cleavage was carried out using TFA/water/EDT (ethanedithiol)/TIS (triisopropylsilane) (94:2.5:2.5:1 v/v/v/v) during 2 hours. Afterwards, the peptide solution was vacuum-dried and precipitated six times in ether followed by airdrying.

Purification of the crude peptide was performed by HPLC (high-performance liquid chromatography) using eluents A: 0.1% TFA (trifluoroacetic acid) in water and B: 0.1% TFA in 100% ACN and was obtained as a TFA salt after lyophilisation. Standard operating procedure of HPLC was performed using a Venusi MRC-ODS C-18 30 x 250 mm column (at 220 nm) at a 1 ml/minute flow rate with a loading capacity of 3 ml under gradient elution conditions.

2.5. UHPLC-MS/MS method for L-R5 quantification

The HPLC system consisted of a Waters HPLC system equipped with a quaternary pump (Waters 600E Multisolvent Delivery System), online degasser, column heater, autosampler (Waters 717plus) and Waters 996 PDA detector. Data collection and analyses were performed using Millennium 32 Software. The samples for the calibration curve were diluted in a solution of HBSS:ACN (97:3). The range of the calibration curve was established between 0 and 50 µM. Quantification of peptide L-R5 concentration was determined using UHPLC coupled with tandem mass spectrometry. UHPLC-MS/MS analysis was performed using a Waters Acquity UPLC system composed of a binary solvent pump, a sample manager and a Waters XEVO TQ-MS detector. Standards for calibration were prepared in HBSS with 3% ACN.

Gradient separation was performed at 40 °C with a Phenomenex Biozen PS-C18 50 x 2.1 mm column containing 1.6 µm particles. The mobile phase consisted of a gradient increase of ACN

and FA (0.1%) between 5% and 100% in 8 minutes and a corresponding decrease of water and FA (0.1%), followed by 1 minute at 100% of ACN and FA (0.1%), and finally an equilibration of 4 minutes at 5% ACN and FA (0.1%). The flow rate was set at 0.3 ml/minute and the injection volume was 5 µl.

The peptide was detected by electrospray ionization in positive ion mode using multiple reaction monitoring. MassLynx software was used for data integration and analysis. The mass/z of the peptide is 319. The different fragments were detected at 175, 112, 70, 57 and 44.

The limit of detection (LOD) was determined based on Q2 (R1) recommendations from ICH [43] using the limit of the coefficient of variation (15%) by tracing the CV function of concentration. Then the LOQ is 3*LOD. The method was specific for the peptide L-R5 quantification using multiple reaction monitoring at m/z 319; peptide L-R5 eluted at 2.75 ± 0.01 minutes. Intra- and inter-day accuracy and precision were determined using 2.5, 25 and 50 µM standards.

2.6. L-R5 stability test, passage and recovery

The peptide was stored prior to use in NaCl 0.9% at a concentration of 1 g/l. The peptide is completely soluble at this concentration. L-R5's structural stability and activity post incubation at 37°C was tested over a period of 24 hours. A 50-µM peptide solution in HBSS was prepared. This solution was incubated at 37°C in an atmosphere containing 5% CO2 for 0, 3, 6 and 24 hours. Studies were done in triplicate. Peptide stability was evaluated by quantification of the concentration of the intact peptide using the UHPLC-MS-MS method described above and by determining its ability to increase transepithelial permeability in MucilAir™ cell culture.
MucilAir™ cells were cultured as mentioned previously, with fresh warm HBSS in the basal compartment and 100 µL of the different 50 µM L-R5 solutions in HBSS on the apical side. TEER was measured to check the integrity of the epithelial monolayer post experiment, and the passage of the peptide through the cell monolayers (MucilAir™) was quantified by collecting 200 µl of the basolateral medium and quantification of the peptide by UHPLC-MS-MS as described above.

To determine reversibility of permeation enhancement effect, TEER was measured during 24 hours after equilibrium and application of 100 µl of 50 µM and 150 µM L-R5 solution, respectively. The measurements were taken each 5 minutes during 15 minutes and then each 15 minutes during 1 hour and then each hour.

2.7. Toxicity evaluation of L-R5 peptide

A four-week study examined the effect of consecutive and long-term exposure of MucilAir™ cell layers to the L-R5 peptide at one higher (200 µM) and one lower concentration (20 µM). Each week, test substances were administered for four consecutive days and the analysis of ciliary beating frequency (CBF) and lactate dehydrogenase (LDH) release were carried out on the fifth day. In addition, cell layers were examined for morphological changes. The volume of the peptide solution administered was 10 µl/insert and the incubation period was 4 hours after which the test substances were removed, the monolayers washed with saline solution, and the cell layers incubated again in cell culture medium. Saline served as the negative control and isopropanol as positive control. Since the study is a longterm exposure study of 4 weeks and MucilAir™ is an air-liquid interface model, an administration volume 10 µl/insert was used over a surface area of 0.33 cm^2/insert. CBF was measured by a dedicated setup for this purpose. The system consisted of a Mako G030B camera connected to a Zeiss Axiovert 200M microscope with a 5x objective, a PCI card and a specific software package. Cilia beating frequency (CBF), expressed as Hz. were captured at high frequency rate (125 frames per second) at room temperature. CBF was then calculated using Epithelix software (Cilia-X).
Lactate dehydrogenase is a stable cytoplasmic enzyme that is rapidly released into the culture medium upon rupture of the plasma membrane. 100 µl of basolateral medium was collected at each time-point and incubated with the reaction mixture of the Cytotoxicity Detection KitPLUS (LDH) following the manufacturer's instructions (Sigma, Roche 04 744 926 001). The amount of the released LDH was quantified by measuring the absorbance of each sample at 490 nm with a microplate reader. To determine the percentage of cytotoxicity, equation 2 was used (A = absorbance values):

$$\text{Cytotoxicity}(\%)=(A\ (\text{exp value})-A(\text{low control})/A(\text{high control})-A(\text{low control}))*100 \qquad \text{(Eq. 2)}$$

The control value was obtained by apical treatment with 10 % Triton X-100 (24 hours). Triton X-100 causes a massive LDH release and corresponds to 100 % cytotoxicity. The negative controls (nontreated and vehicle) show a low daily basal LDH release of <5 %, which is due to a physiological cell turnover in MucilAir™. Since the study is a long-term exposure study of 4 weeks and MucilAir™ is an air-liquid interface model the LDH release could only be measured at the basolateral side.

2.8. *In vitro* haemolysis assay

Fresh defibrinated sheep blood (Thermo Fisher Scientific, Zug, Switzerland) was used to evaluate the membrane perturbation effect of L-R5. The blood was washed using 5x PBS (Gibco, Zug, Switzerland) and centrifuged at 1500 rpm for one minute. Supernatant was

discarded and the pellet was resuspended in 1 ml of PBS. The above step was repeated 5 times and finally 11 ml of PBS were added. The suspension was stored at 4°C.

A series of L-R5 dilutions between 6.25 and 200 µM was prepared in NaCl 0.9% (Sigma-Aldrich, Buchs, Switzerland). Water for injection (Corning, Manassas, VA, USA) and NaCl 0.9% was used as positive and negative controls, respectively. 50 µl L-R5, L-R5 without the myristoyl, the 13 amino acids peptide and the myristoyl alone with glycine solutions were incubated with 50 µl of the sheep blood suspension (50.3 million cells/ml) in each well of a Corning® 96-well Clear Round Bottom TC-treated microplate for 30 minutes at room temperature under slow shaking. The plate was centrifuged at 3700 rpm for 10 minutes. Supernatant from each well (50 µl) was transferred to a Corning® 96-well Clear Flat Bottom Polystyrene TC-treated microplate containing pre-filled 250 µl of ethanol (Biosolve Chimie, France) in each well and mixed. The absorbance of the plate was read at 412 nm (Biotek Synergy Mx, Sursee, Switzerland) with a maximum of 0.712 and a minimum of 0.072, respectively, for water for injection (WFI) and NaCl 0.9%. The absorbance values obtained for the control group (water for injection) was taken as 100%.

2.9. Permeability studies

The apparent permeability (P_{app}) of FD-4 and naloxone was calculated using equation 3:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0} \quad \text{(Eq. 3)}$$

where C0 is the initial concentration (ng/ml) of the permeant in the donor compartment (apical side), A (cm^2) is the surface area of the cell layers (0.33 cm^2 for 24 well plate inserts) and dQ/dt is the appearance rate of FD-4 or naloxone in the receiver compartment. C0 did not change significantly over the time of study. All experiments were performed in triplicate.

After equilibration and TEER measurement, the peptides were applied to the apical side in 0.25 mM FD-4 solution at a concentration of 50 µM in HBSS. Samples of 100 µl were taken from the basolateral compartment of each well every 30 minutes over a period of 150 minutes, with each volume being replaced by an equal amount of fresh warm buffer to maintain sink conditions. The fluorescence of FD-4 was measured in black 96-well plates using a fluorescence plate reader (BioTek Synergy Mx plate reader, BioTek Instruments GmbH, Lucerne, Switzerland), using excitation and emission wavelengths of 485 and 520 nm, respectively.

The role of the cell-penetrating group (myristoyl) was also evaluated. Fluorescein conjugated dextran of a molecular weight of 4 kDa (FD-4) was used. L-R5 peptides with and without N-

myristoyl group, as well as the myristoyl group alone (attached to glycine) were evaluated at a concentration of 50 µM mixed with the fluorescein (0.25 mM) in HBSS. After equilibration, 100 µl of test solutions were applied at the apical side of the cell layers. Samples of 200 µl were taken from the basolateral side after the first 15 minutes and then every 30 minutes over a period of 180 minutes and replaced with fresh warm HBSS to maintain sink conditions.

61 mM naloxone solutions in HBSS (Gibco) were used to test the permeability of this drug in the presence of L-R5. This concentration was chosen according to the dose used in nasal naloxone sprays (1 and 4 mg/0.1 ml) [44]. L-R5 was dissolved in these solutions at a concentration of 50 µM. A 61 mM solution of Naloxone in HBSS in the absence of L-R5 was used as control. After equilibration and TEER measurement, 100 µl of test solutions were applied at the apical side. Samples were collected from the basolateral side as described above.

The basolateral concentration of naloxone was quantified by HPLC-UV [45] using a Waters HPLC system equipped with a quaternary pump (Waters 600E Multisolvent Delivery System), online degasser, column heater, autosampler (Waters 717plus) and Waters 996 PDA detector. Separation was achieved on a C-18 column (150 mm × 4.6 mm, 5 m). The elution was isocratic with a mobile phase of acetonitrile and 10 mmol/l potassium phosphate buffer adjusted to pH 6.0 with orthophosphoric acid (83:17, v/v). The flow rate was 1.0 ml/min and yielded a backpressure of about 740 psi. The column temperature was maintained at 35 °C, the detection was monitored at a wavelength of 210 nm and injection volume was 20 µl. Data collection and analyses were performed using Millennium 32 Software.

The calibration curve was established using naloxone solutions in HBSS with 12 standards between 0 and 5.5 mM. Repeatability of the method was confirmed by injecting the same sample (naloxone 5.5 mM in HBSS) 6 times. The values for average, standard deviation and relative standard deviation (RSD) for AUC, absorbance and retention time were calculated.

2.10. Statistical analysis

Data are reported as mean ± standard deviation (S.D.). $P<0.05$ was considered as statistically significant. Significance is denoted as *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. The data were analysed using multiple t-test comparisons. The cell viability data were analysed using one-way analysis of variance (ANOVA). $P<0.05$ was considered as statistically significant. Significance is denoted as **$P<0.01$. The data were analysed using Mann-Whitney test.

3. Results

3.1. L-R5 quantification

The method was linear over a concentration range of 0.2-50 µM (R^2 = 0.99). LOD and LOQ of the peptide were determined to be 0.78 and 2.51 µM, respectively (supplementary data). The method was considered accurate and precise as all measured values were within the acceptance limits of validation guidelines of ICH Q2 (R1) [43].

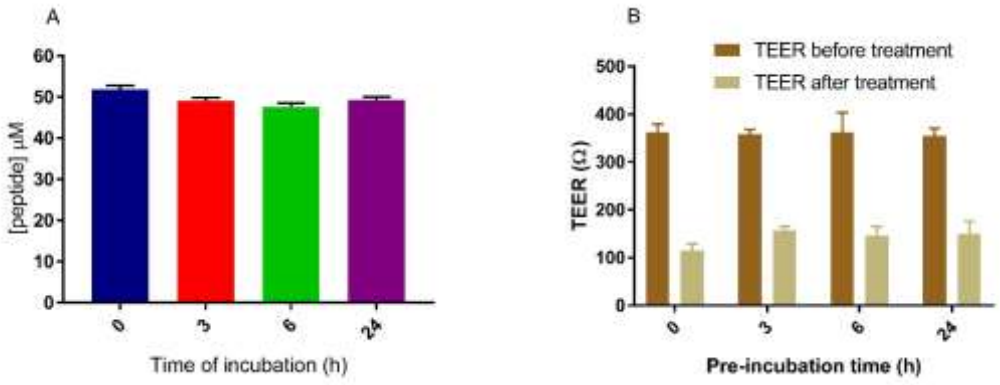


Figure 1: Stability of L-R5 (50 µM) was tested in HBSS at 37°C for time periods of 0, 3, 6 and 24 hours (A) and the corresponding permeability enhancement effect of the peptide was evaluated after different incubation time points using the conditions mentioned above (B). TEER was measured before the application of the L-R5 solutions and 3 hours after. L-R5 remained bioactive in the absence of significant loss of activity during all the incubation times tested on MucilAir™. Values are mean ± S.D. (n=4).

3.2. L-R5 stability test, passage and recovery

We confirmed that the reversibility of the permeation enhancement effect increased with decrease in the length of the peptide. We evaluated the stability of L-R5 at 0, 3, 6, and 24 hours in order to substantiate that the reversibility phenomenon was due to the intrinsic property of the peptide and not due to the lack of stability of the peptide (figure 1A). L-R5 was stable at 37 °C over a time period of 24 hours. Permeability enhancement effects of the peptides incubated at 0, 3, 6 and 24 hours were evaluated using TEER. The peptides exhibited no decrease in permeability enhancement effect over 24 h as shown by the TEER values (figure 1B). All peptides were used at a concentration of 50 µM.

The passage of L-R5 through epithelial monolayers was quantified using a UHPLC-MS-MS method. Peptide concentrations of merely 96.4 ± 93.8 nM in the basolateral compartment (figure 2) were measured, 50-times lower than the concentration of the apically applied peptide solution. This value was well below THE LOQ of 2.51 µM.

We investigated the effect of L-R5 on the TJ barrier function on primary human nasal epithelial cells (MucilAir™) and their recovery over time. L-R5 was tested at concentrations of 50 and 150 µM, respectively. L-R5 exhibited a concentration-dependent effect of decreasing TEER (figure 3). The effect of decrease in epithelial permeability occurs instantaneously within a few minutes. L-R5 showed superior reversibility kinetics without any media change and in a concentration-dependent manner. TEER values completely returned to initial values over a period of about 6 hours for the peptide concentration of 50 µM. A more linear and prolonged permeability enhancement effect was noticed for 13 AA peptide when used at 50 µM. This might be attributed to a longer interaction of the 13 AA peptide with the PKC enzyme. The higher concentration of L-R5 showed a stronger effect but followed the same kinetics.

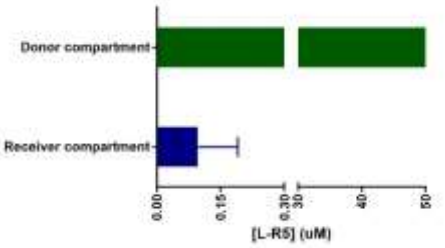


Figure 2**:** Permeation of L-R5 through monolayers of MucilAir™ primary human nasal cells during 3 hours of incubation. Concentration of L-R5 in HBSS in the apical compartment was 50 µM, concentration in the basolateral compartment was quantified by UHPLC-MS-MS, with a LOQ of 2.51 µM. Values are mean ± S.D. (n=3).

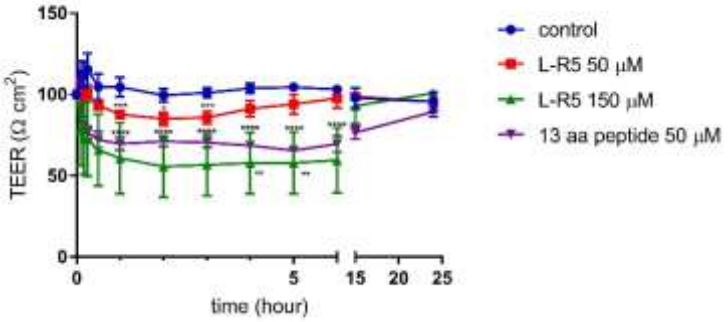


Figure 3: Pentapeptide L-R5 transiently increased the permeability of MucilAir™ primary human nasal epithelial cell monolayers as indicated by the decrease in transepithelial electrical resistance (TEER). TEER values are expressed as percentage of control (the baseline value was > 350 Ω*cm^2). Values represent mean ± SD, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. The data were analysed using multiple t-tests. All experiments were performed at least 3 times during different days with four replicates. Values are mean ± S.D (n = 4).

3.3. Cytotoxicity & ciliary beating frequency (CBF) assay

We investigated the effect of consecutive and long-term exposure of the short peptide partial inhibitor (L-R5) at one higher concentration (200 µM) and one lower concentration (20 µM) on MucilAir™ primary human nasal epithelial cells. CBF test revealed no significant differences between the negative control group (saline) and the L-R5 peptide at both concentrations (figure 4A). The positive control isopropanol increased the CBF (Range 6.6 – 8.75 Hz) as

expected. The LDH test indicated that the peptide at both concentrations showed no cytotoxicity throughout the four-week study (figure 4B).

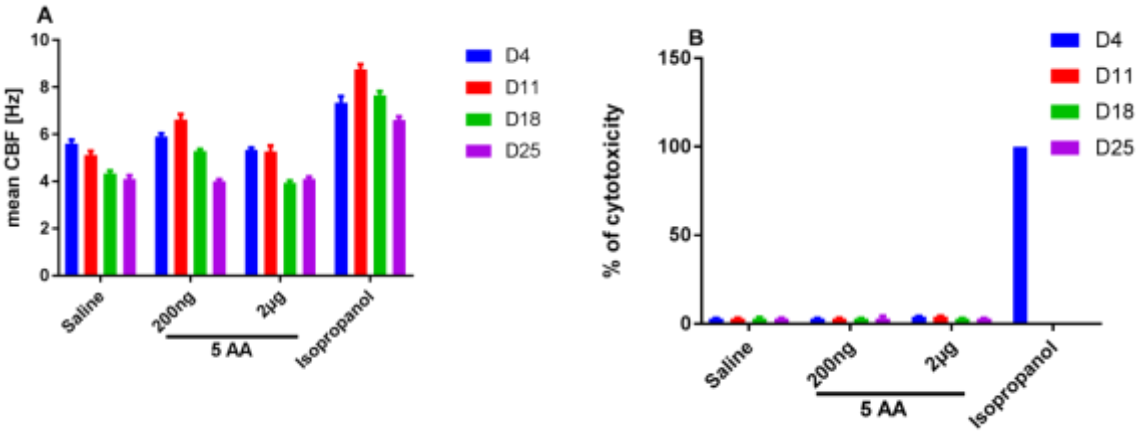


Figure 4: Long-term exposure of MucilAir™ cell monolayers to L-R5 (5 AA) at 20 and 200 µM concentrations by repeated application. Saline was used as negative control and isopropanol as a positive control. (A) Cilia Beating Frequency assay and (B) LDH release assay were measured at measured at days 4, 11, 18 and 25. mean ± S.D. (n=3).

3.4. Haemolysis assay

An *in vitro* haemolysis assay was performed to evaluate if the increase in macromolecular permeability was induced by membrane perturbation. Solutions of L-R5 lacking the myristoyl function of increasing concentrations ranging between 3.125 µM up to 100 µM showed no haemolytic activity (figure 5). On the contrary, myristoyl alone and peptides with myristoyl attachment showed haemolysis. As it will be noted further in the results, the myristoyl alone permeability enhancing properties. The haemolysis due to this fatty acid chain can be suggested to not be responsible for membrane perturbation for other peptides such as L-R5.

3.5. Permeability enhancement of FD-4

We evaluated the ability of L-R5 and zeta inhibitory peptides (ZIP) of various lengths (3 and 13 amino acids) for their ability to increase the paracellular permeability of fluorescein-conjugated dextran used as a model for peptides and proteins. As shown in figure 6, L-R5 and 13 AA PKC zeta inhibitory peptides increased the permeability of FD-4 significantly ($P < 0.001$) compared to the control group. L-R5 increased the permeability of FD-4 across nasal human primary epithelial cells by about 10 times over a time course of 150 minutes, while the increase in permeability caused by the 13 AA peptide was about 25 times. The 3 AA peptide showed no significant difference in permeability compared to the control group.

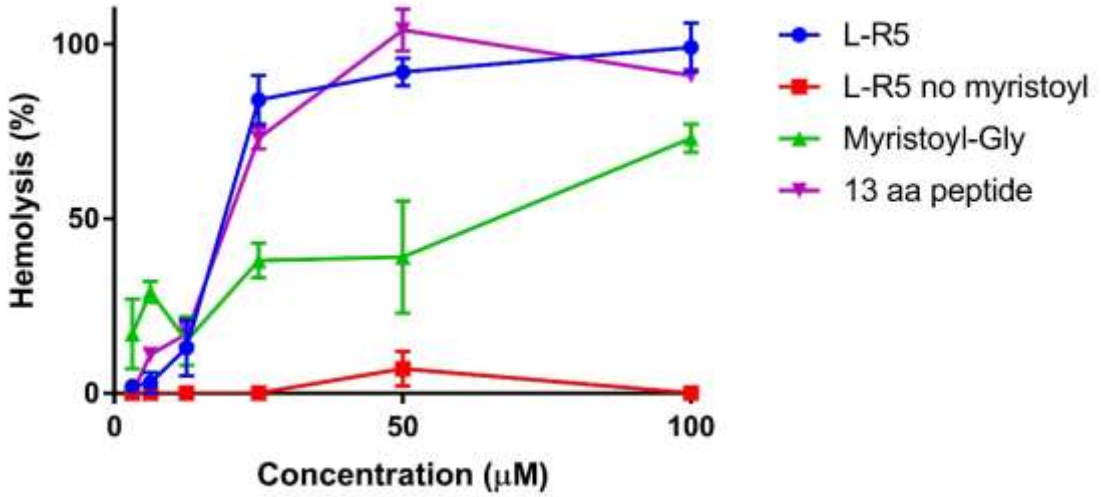


Figure 5: Haemolytic activity measured in fresh defibrinated sheep blood (50.3 × 106 cells/ml), incubated with various concentrations of different sequences of the peptide for 30 minutes at room temperature under slow shaking. NaCl 0.9% and water for injection (WFI) were used as negative and positive control. Values are mean ± S.D.(n=3).

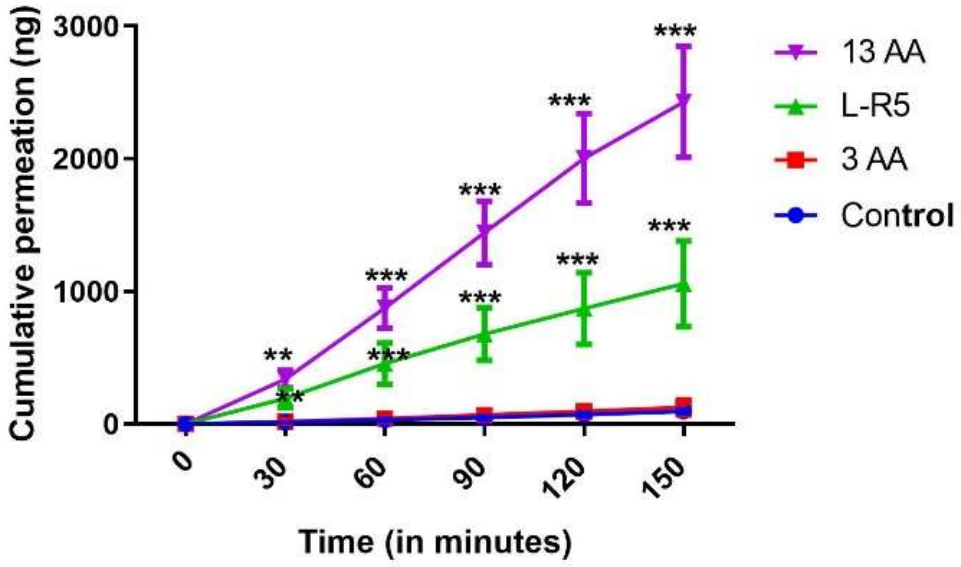


Figure 6: Solutions (50 μM) of three potential PKC ζ inhibitor peptides of different lengths (shorter-N-Acetyl-ARR, 3 AA; Pentapeptide-Myr-ARRWR, L-R5; and longer- Myr-SIYRRGARRWRKL, 13 AA) were compared for their ability to enhance paracellular transport of fluorescein dextran (4 kDa) 0.25 mM across MucilAir™ nasal epithelial monolayers over a period of 150 minutes. Values are mean ± S.D. (n=4). The experiments were repeated at least twice, $^{**}P < 0.01$, $^{***}P < 0.001$. The data were analysed using multiple t-test comparisons.

In addition, the reversibility of permeation enhancement effect or reversibility kinetics was evaluated to ideally design a safe permeation enhancer. Therefore, the apparent permeability (P_{app}) of fluorescein dextran across epithelial cell layers during incubation with L-R5, 13 AA and 3 AA peptides was measured in order to estimate the reversibility of the paracellular permeability effect. The effect of these peptides was reversible as shown by the decrease in P_{app} over time showing that steady state P_{app} values were obtained. L-R5 induced its maximum permeability enhancement at 60 minutes and gradually declined over time as shown by the P_{app} values. The 13 AA peptide induced its maximum permeability enhancement at 90 minutes and gradually declined over time as shown by the P_{app} values. The 3 AA peptide did not show any activity of permeability enhancement.

3.6. Role of the cell membrane penetrating group

We tested the peptide L-R5 without N-myristoyl group for its ability to increase the paracellular transport of macromolecules as well as the effect of the myristoyl fatty acid chain on TJs (figure 7). No increased permeability was noted except for L-R5. It was determined that a cell-permeating group is essential for the partial inhibitor L-R5 to elicit the effect of increasing paracellular permeability. Without the myristoyl group, the peptide showed a permeability pattern not significantly different from the negative control.

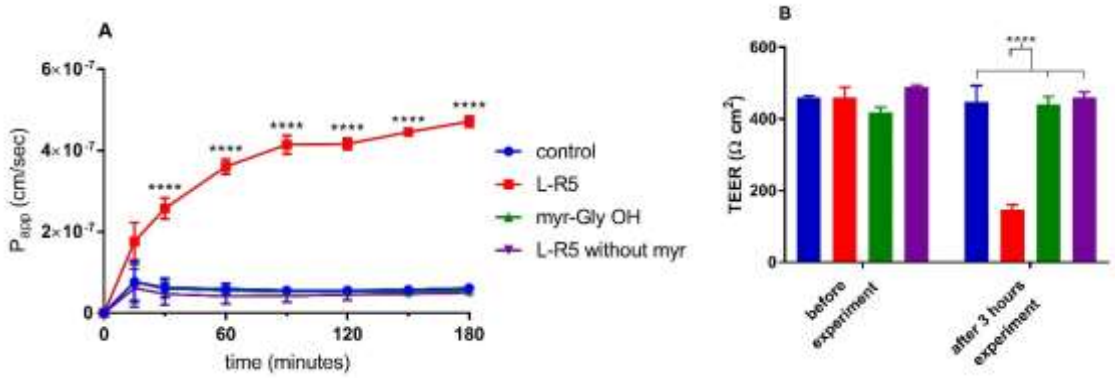


Figure 7: Role of the myristoyl function in L-R5. The permeability of FD-4 (0.25 mM solution) through MucilAir™ epithelial cell layers was measured in the presence of L-R5, L-R5 without myristoyl, or the fatty acid chain linked to glycine (all at a concentration of 50 µM) was investigated over 180 minutes (A). TEER was measured before and after the experiment (B). Values are mean ± S.D. (n=3), ****P < 0.0001. The data were analysed using multiple t-test comparisons.

3.7. Permeability studies of naloxone

R^2 of the AUC based calibration curve for naloxone was 0.9996. Repeatability RSD values were determined as 2.04% (AUC), 0.82% (absorbance) and 1.14% (retention time), respectively. As shown in figure 8, application of solutions of naloxone in combination with L-R5 50 µM very significantly ($P < 0.001$) enhanced drug permeability by 6-fold after 3 hours compared to the control solution (absence of L-R5). In addition, TEER was very significantly lower ($P < 0.0001$) in the presence of the peptide.

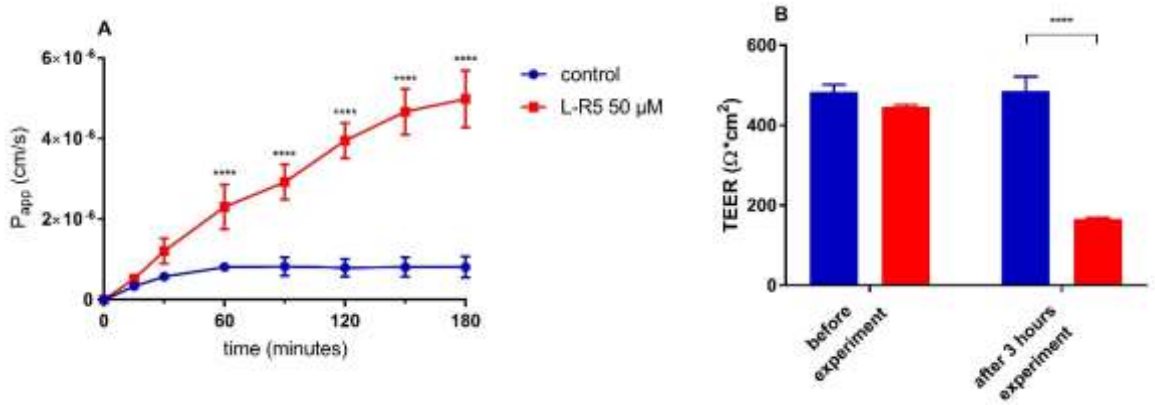


Figure 8: The permeability of naloxone (61 mM) mixed with L-R5 (50 µM) through MucilAir™ epithelial cell layers was investigated over 180 minutes (A). TEER was measured before and after the experiment (B). Values are mean ± S.D. (n=3), ****P< 0.0001. The data were analysed using multiple t-test comparisons.

4. Discussion

We have used a rationally developed cell permeable pentapeptide to stimulate a defined mechanism of action that leads to the transient opening of epithelial TJs. Our approach is based upon our findings from a previous study that demonstrated the barrier function of human bronchial epithelial monolayers were regulated through atypical protein kinase C zeta [30]. In this study, we examined PKC ζ as a potential molecular target to reversibly modulate epithelial TJs as the enzyme plays a vital role in TJ regulation. Our rationale was that the topical application of this cell permeable pentapeptide would result in a local action on TJ function and that this action would be transient due to dilution and elimination from the actual substrates of PKC ζ. The cumulative *in vitro* data presented in this report are consistent with that concept.

L-R5 peptide induced a concentration-dependent increase in permeability, which was completely reversible. The reversibility of the effect is concentration dependent, the higher the concentration the longer the time period to reverse the effect (figure 3). These concentration-

dependent reversibility dynamics must be further investigated in detail for better understanding of the interaction between L-R5 and PKC. Instant decrease in TEER and a concentration-dependent reversibility phenomenon might signify the involvement of PKC ζ in TJ regulation and assembly. PKC ζ is the only PKC isozyme recruited and localized at the cell-cell interface [46]. PKC ζ is continuously active during basal resting conditions and is responsible for the dynamic phosphorylation of occludin at its C-terminal at threonine residues T424, T403, T404, and T438, which was shown to be responsible for the assembly of TJs [47]. Thus, PKC ζ inhibition might cause rapid disassembly of TJs considering its connection with occludin and ZO-1 [31].

The myristoyl group at the N-terminus of L-R5 seems to be an essential component for the peptide to elicit its effect of increasing paracellular permeability. The peptide sequence without the myristoyl group did not show any or negligible effect of increasing permeability (figures 5 and 8). Intracellular delivery of peptides using myristoylation of the API has widely been described [33,48]. The myristoyl chain can be considered as the part allowing the peptide to enter the cell and the 5 amino acids peptide the part that inhibit PKC ζ. This inhibition is partly proven by the fact that ZIP is recognized as a PKC ζ inhibitor and effects noted between 13 AA peptide and L-R5 are very similar. However, L-R5 may inhibit other PKCs as their homology is significant. This has to be investigated by further studies.

It is essential for excipients of nasal formulations to not interfere with the vital functions of the nasal epithelium such as the mucociliary escalator [49]. Mucociliary clearance, which is maintained by the coordinated ciliary activity, is regarded as the key local defense mechanism of the respiratory tract. Ciliary beat frequency (CBF) is the functional parameter of mucociliary clearance and hence it is important to determine the effect of nasally administered formulation excipients such as additives, preservatives and absorption enhancers [50]. We evaluated the effect of long-term and repetitive administrations of L-R5 on the CBF and cellular viability, using human primary MucilAir™ cells that contained a pool of cells collected from 14 different healthy donors. No effect on CBF and cytotoxicity (in terms of LDH release) were detected, potentially indicating the safety of L-R5 in chronic application (figures 4A & 4B). It can be noted that the difference between negative and positive control is not huge. This might be due to lower concentration of isopropanol used. The passage of L-R5 through the epithelial monolayers was found to be negligible at the concentration applied. The amount of L-R5 that permeated through the monolayer was non-quantifiable due to its being lower than the LOQ of 2.51 µM. This appears to allow for several inferences: possibly, most of the L-R5 peptide permeated into the cells by virtue of its myristoyl function, where it remained and exerted its activity [33,48]. Under *in vivo* conditions, we suggest that no or a negligible quantity of the peptide dose applied would reach the systemic circulation, minimizing potential toxic effects

exerted by the peptide. In addition, the observed basolateral L-R5 concentration would have been much higher in case of loss of integrity of the epithelial cell layer. Moreover, since L-R5 is a short peptide it might be more rapidly metabolized or removed by the epithelium adding to the potential safety profile of L-R5.

In our study, naloxone was selected as an example of a BCS class III drug with a clear medical need for absorption enhancement. Based on the results presented here, we suggest that using our L-R5 permeation enhancer technology, the rate of nasal absorption of naloxone would be significantly enhanced. However, a decrease of TEER was noted in figure 9B due to only naloxone's application. Several studies have also noted that naloxone may have the ability to increase the epithelial permeability of solutes and ions [51]. This minor decrease of electrical resistance may be explained by this unexplored phenomenon. Together with the relative ease of nasal application, the combination of L-R5 and naloxone would clearly benefit the patient in an emergency.

Beyond efficacy, tolerability is a key feature for distinguishing a good TJ modulator that has the potential to be introduced into clinical practice. L-R5 appears to have an optimal length as it causes desirable paracellular permeabilization of macromolecules which is fully reversible, a desirable property for avoiding cytotoxicity. The structure of L-R5 of merely 5 amino acids might be attractive in terms of the industrial application due to the relative ease of production and costs of goods (COGs) related to scaleup
and GMP manufacture.

5. Conclusions

We have developed a short peptide partial inhibitor (L-R5) of the intracellular enzyme protein kinase C (PKC). When applied apically *in vitro*, paracellular transport across epithelia is increased within minutes. The effect is perfectly reversible leading to the closure of tight junctions after paracellular drug permeation has occurred. Our study illustrates the following: (i) L-R5 has a fast onset of action and exerts a transient increase of epithelial permeability; (ii) L-R5 increases permeation across epithelial monolayers; (iii) the effects caused by L-R5 are reversible; (iii) *in vitro* data indicate that the long-term and repetitive administration of L-R5 shows no cytotoxicity, no changes in ciliary beat frequency or haemolysis. Studies to further elucidate and potentially optimize the interaction between L-R5 and PKC ζ are currently ongoing.

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Chapter 3: Intracellular mechanism of TJs opening

Impact of L-R5 permeation enhancer on tight junctions opening cellular mechanisms

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Foreword: L-R5 was synthesised to inhibit PKC ζ activity. The structure of the peptide is derived from the pseudosubstrate (PS) sequence of the enzyme. This PS is responsible for maintaining the enzyme in an inactive state. The aim of this chapter is to understand the mechanism of action of L-R5 and its influence on the cellular proteome.

This work, entitled “Impact of L-R5 permeation enhancer on tight junctions opening cellular mechanisms”, was submitted to the journal *Biochemical Pharmacology* in April 2022. The manuscript is presented below.

Author contribution: Prof. Gerrit Borchard and I designed and planned the study for the thermophoresis and Western blot parts. Dr. Alexandre Hainard, Dr. Domitille Schvartz and I planned the proteomic experiment. The thermophoresis experiments were performed by Aurélie Gouiller and myself. I was in charge for the cell culture and the Western blot experiments. The proteomic platform was in charge of the practical part of proteomic experiments. I have analysed the results and written the manuscript with the support of Prof. Gerrit Borchard.

Declaration of interest:

The authors declare no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Abstract

The myristoylated pentapeptide, L-R5, contains an amino acid sequence of the zeta inhibitory peptide (ZIP) portion (pseudosubstrate) of protein kinase C zeta (PKC ζ). As PKC ζ is involved in the modulation of epithelial tight junctions (TJs) through the phosphorylation of TJ proteins, L-R5 was suggested to interact with the enzyme resulting in the enhancement of paracellular permeability. This study shows that L-R5 does not bind to the enzyme but interacts directly with TJ proteins. We show here that the binding of PKC ζ to occludin and its successive phosphorylation is prevented by L-R5, which leads to TJ disruption and enhanced epithelial permeability. Although L-R5 did not show any *in vitro* cytotoxicity, a proteomics study revealed that L-R5 interferes with other regulatory pathways, e.g., apoptosis and immune response. We suggest that structural modification of the peptide may increase the specificity TJ protein-peptide interaction.

Keywords: tight junction, PKC zeta, L-R5, occludin, protein interaction, peptide, phosphorylation

1. Introduction

Tight junctions (TJs) are responsible for the closure of intercellular junctions, thereby modulating the paracellular permeability of epithelial cell layers. TJs are regulated by the interactions of numerous intercellular proteins, with many mechanisms being potentiated by external factors [1]. TJ proteins mainly include the claudins family [2], occludin [3,4] and *zonula occludens* (ZO) group of proteins [5], the expression and activation of which is regulated by many different pathways. It has been shown that phosphorylation of threonine and tyrosine residues of TJ proteins is required to close TJs (figure 1A) [6]. These different phosphorylation reactions do occur by protein kinase C (PKC) [7].

PKCs are a family of serine/threonine kinases [8] that are among the major regulatory enzymes [9] being responsible for the phosphorylation of these residues under certain stimuli [10,11]. PKCs are classified into three different subtypes, called conventional, novel and atypical PKCs [12], based on their respective activation pathway [13]. Atypical PKCs are

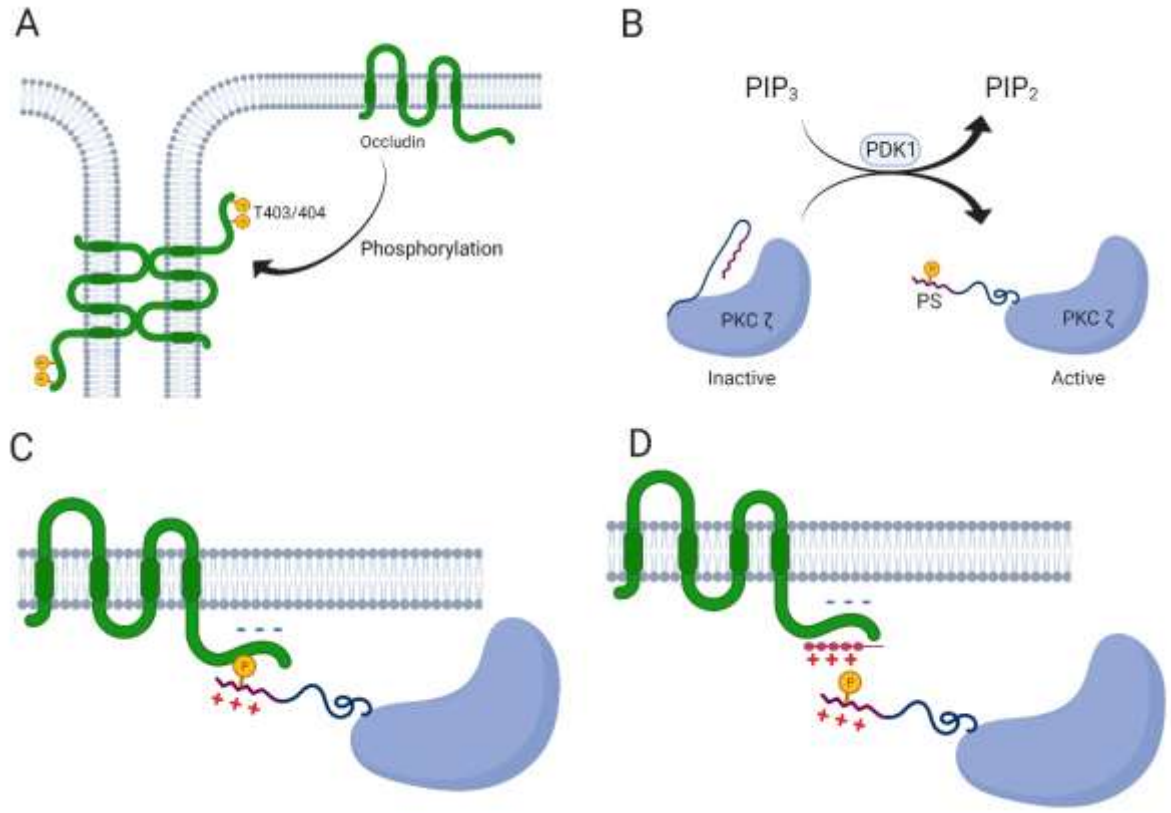

Figure 1A: Pathway of TJ protein occludin activation by phosphorylation of 2 threonines. B: PP2A pathway of PKC ζ activation by removing pseudosubstrate (PS) from the phosphorylation pocket. C: Interaction between PKC ζ PS and occludin for activation of occludin through phosphorylation. D: Mechanism of inhibition of occludin phosphorylation by ZIP.

activated by phosphatidylserine in the PP2A pathway (figure 1B) [14,15], whereas novel PKCs also require diacylglycerol, and the conventional subtype the presence of Ca^{++}. PKCs are also activated by an internal conformational change. A part of the C1 domain, called pseudosubstrate (PS), has been shown to be involved in PKC activation [16]. In addition to the modulation of epithelial permeability, PKCs have been identified to be involved in many different cellular transduction pathways, such as apoptosis, secretion, or cellular proliferation [17]. Clinical trials have been conducted to inhibit PKCs α and β to prevent cancer development [18]. However, none of these inhibitors has been used in a commercial product due to a lack of improved clinical outcome caused by the application of such inhibitors.

PKC ζ has been classified as an atypical PKC [19], which is mainly involved in cell proliferation and survival [12]. Its involvement in cell proliferation has been demonstrated in tumorigenesis [20]. Higher expression of this enzyme has been described in different cancer types and at different stages of tumour development. Examination of colorectal [21], lymphoid and respiratory tumours [22,23] revealed a connection between PKC ζ and tumour development. More specifically, PKC ζ appears to be involved in mitogenic signal transduction [24]. On the other hand, due to its involvement in cell proliferation, PKC ζ has also been shown to be overexpressed in advanced diabetes characterized by pancreatic hyperplasia [25] and to mediate insulin action by phosphorylation of the insulin receptor in adipose tissue [26].

The disruption of PKC ζ activity may trigger serious long-term problems. Activation of NFκB by this kinase is its main role in cell survival [20] and was shown to decrease cell death [18]. Inhibition of PKC ζ is also effective in reducing COPD symptoms [27] and in decreasing epithelial permeability [28]. This TJ modulation involves the enzyme via the Toll-like receptor 2 activation pathway. In addition, PKC ζ reduces epithelial permeability by phosphorylating TJ proteins occludin (threonines 403 and 404) and ZO-1 (serine residues) [29,30]. Inhibition of PKC ζ thus is considered to cause TJ opening and increase in epithelial permeability.

PKCs all contain a pseudosubstrate (PS) part regulating their activity. PS has been shown to keep the enzyme in an inactive state [31] by blocking its catalytic domain [32]. Activation of secondary messengers such as PDK1 releases inhibition of the PS and leaves the enzyme in an active form [33]. Exogenous and artificial peptides of an amino acid sequence resembling (parts of) the PS have been applied as PKC inhibitors [33]. The PKC ζ PS has been sequenced and is located between amino acids 113 and 129 [16]. A myristoylated peptide of a respective amino acid sequence named zeta inhibitory peptide (ZIP) is commercially available. It was initially thought that ZIP would directly inhibit PKC ζ by acting in the same way as PS and keeping the kinase in an inactive state. It has been then noted that ZIP was not specific and also decreased the activity of other PKCs [34]. Furthermore, ZIP still had an inhibitory effect

in the absence of PKC ζ [35]. It has therefore been assumed that ZIP acted through a pathway other than through inhibition of PKC ζ [36]. A recent study showed that PS interacts electrostatically with the targeted proteins to form enzyme-protein complexes [37] coupled to PB1-PB1 (Phox and Bem1) interaction. The addition of ZIP to cellular processes does not directly inhibit PKC ζ but prevents the formation of this complex by competing with the PS of the enzyme (figure 1C+D). Phosphorylation cannot take place, nor can activation of occludin for example. Thus, ZIP does not inhibit the catalytic domain of PKC ζ [38].

In this study, the mechanism of TJ modulation in Caco-2 cells by inhibition of occludin phosphorylation was explored. A reduced myristoylated pentapeptide of ZIP, named L-R5, and several variations were tested previously and were shown to increase drug permeability through epithelial cell layers [39,40]. In this study we examined the mechanism by which L-R5 and its variations, as well as ZIP are are able to increase epithelial cell permeability. Moreover, the influence of L-R5 on the expression of TJ proteins and their extent of phosphorylation were elucidated. Finally, a proteomic analysis on the implication of the peptide on other pathways was performed.

2. Materials and methods

2.1. Peptides and PKC ζ inhibitor

The different peptides L-R5 (myr-ARRWR [40]), D-R5 (amino acids in D form), myristoylated ZIP (13aa), L-R5 of a scrambled sequence (myr-WRARR) referred to here as Sc, and non-myristoylated L-R5 (referred to here as Wo) used in this study were obtained from Bachem AG (Bubendorf, Switzerland). The chemical PKC ζ inhibitor 5-(3-(tert-Butyl)-1-(3-chlorophenyl)-4,5-dihydro-1H-pyrazol-5-yl)-2-fluorophenol [41] (referred to here as In) was a gift by Dr. Engel (University of the Saarland, Germany).

2.2. Microscale thermophoresis (MST)

2.2.1. Protein labelling

The protein construct of PKC ζ active (recombinant enzyme expressed in Sf21 insect cells) was purchased from Eurofins pharma (Dundee, United Kingdom) and the recombinant human C-terminal fragment was purchased from RayBiotech (Peachtree Corners, GA, USA). Fluorescent labelling of both, PKC ζ and occludin was performed following the protocol of coupling the His-tag labelling kit RED-Tris-NTA 2nd generation (Nanotemper Technologies, Munich, Germany) to their respective histidine tail. The fluorescence of the tagged proteins was then measured with the monolith NT.115 (Nanotemper Technologies, Munich, Germany). An excitation LED of 100% was set for the Cap. Scan. The predicted optimal dilution was then calculated to obtain a final fluorescence signal of 100 Raw fluorescence [counts].

2.2.2. Measurement of protein-peptide interactions

Hydrophilic capillaries were used for the different tests because the samples interacted with standard capillaries. The interaction between PKC ζ and occludin was established with serial dilutions of occludin in the assay buffer at concentrations between 0 and 35000 nM and a fixed concentration of 30 nM for PKC ζ. The interaction between PKC ζ and L-R5 was established with serial dilutions of the peptide in assay buffer at concentrations between 0 and 4000 µM and a fixed concentration of 20 nM for PKC ζ. The interaction between occludin and L-R5 was established with serial dilutions of L-R5 in the assay buffer at concentrations between 0 and 25000 µM and a fixed concentration of 50 nM for occludin. The interaction between PKC ζ and occludin in the presence of L-R5 at a sufficient concentration to bind to occludin was established with serial dilutions of occludin in assay buffer at concentrations between 0 and 11300 nM and a fixed concentration of 1.25 mM for L-R5 and 30 nM for PKC ζ. The final volume was 20 µl per dilution. For each experiment, a solution containing only the assay buffer was prepared as a negative control. The different solutions were analysed by MST at 20 %, 40 % and 80 % MST power with a 100 % LED intensity. The laser was switched on for 30 seconds and then switched off for 5 seconds. A repeat measurement was performed after 3 hours of incubation in the capillaries and no significant changes in fluorescence intensity, K_d value and protein adsorption was noted. The results and K_d values were analysed by MO.affinity analysis 3 software (Nanotemper Technologies, Munich, Germany).

2.3. Cell culture

Caco-2 cells (ATCC, Manassas, VA, USA) were cultured in T75 flasks (Merck, Schaffhausen, Switzerland). The cells were incubated at 37°C and a humidified atmosphere containing 5% of CO_2. The medium containing 10 % fetal bovine serum (FBS, Thermofisher, Waltham, MA, USA) was changed every 2-3 days. The Caco-2 cells were passaged every 5 days. The passage numbers were between 33 and 36.

For Western blot experiments, cells were seeded in 12-well plates (Merck, Schaffhausen, Switzerland) at a density of 50'000 cells/cm^2. For the proteomic experiments, the cells were seeded in T10 flasks (Merck, Schaffhausen, Switzerland) at the same initial seeding density. After 7 days and two washes with warm phosphate buffered saline (PBS, Thermofisher, Waltham, MA, USA), the conditions were applied for 1 hour. Peptides were applied at a concentration between 20 and 100 µM. A chemical inhibitor of PKC ζ was also tested. The molecule 4f [41] was used at a concentration of 10 µM in 0.1% DMSO as a positive control. All dilutions were done in 0.9 % saline solution. After incubation, cells for Western blot experiments were lysed with RIPA buffer (Cell signalling, Danvers, MA, USA) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). After centrifugation to

discard cell components, the samples were stored at -20°C. Cells for proteomic analysis were detached with trypsin, washed 3 times with PBS and kept at -80°C until analysis. Samples for proteomic analysis were produced in triplicate for each condition.

2.4. Immunoprecipitation

Specific immunoprecipitation of threonine phosphorylated proteins was performed using the immunoprecipitation kit from Abcam (ab206996, Cambridge, United Kingdom). The lysates used were the same as those described above. The protocol was scrupulously followed with the adaptations described by Wang *et al*. [42]. For 400 µg of proteins, 30 µg of specific threonine phosphorylated antibody (13-9200, Thermofisher, Waltham, MA, USA) was mixed with 30 µl of A/G sepharose beads. Separation of the proteins, antibody and beads was performed by adding the SDS loading buffer (Laemmli buffer, Bio-Rad, Hercules, CA, USA). The immunoprecipitated proteins were used directly in electrophoresis gel.

2.5. Immunoblot analysis

50 µg cell extracts and p-threonine proteins were separated by SDS-polyacrylamide gel electrophoresis (4-15%) (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membrane. Proteins of interest on the membrane were bound to primary antibodies (anti-occludin, anti-ZO-1, anti-F-actin, anti-PKC ζ, Cell signalling technology, Danvers, MA, USA) overnight at 4°C with gentle agitation. These primary antibodies were then recognized by anti-mouse and anti-rabbit antibodies (Li-Cor Biosciences, Lincoln, NE, USA) and detected by Odyssey® imaging system (Li-Cor Biosciences). The signal of each protein was normalised to the signal of the corresponding actin.

2.6. Proteomic

2.6.1. Sample preparation

Cell pellets were resuspended in 100 µl of 0.1 % RapiGest Surfactant (Waters, Milford, MA, USA) in 50 mM ammonium bicarbonate (AB). Samples were heated for 5 min at 100°C. Lysis was performed by sonication (6 x 30 sec.) at 70% amplitude and 0.5 pulse. Samples were kept 30 seconds on ice between each cycle of sonication. Samples were centrifuged for 10 min at 14'000 x g. Protein concentration was measured by Bradford assay and 25 µg of each sample was subjected to protein digestion as follows: the sample volume was adjusted to 100 µl with 0.1 % RapiGest in 50 mM AB. 2 µl of 50 mM dithioerythritol (DTE) were added and the reduction was carried out at 60°C for 1h. Alkylation was performed by adding 2 µl of iodoacetamide 400 mM during 1 hour at room temperature in the dark. Overnight digestion was performed at 37°C with 5 µL of freshly prepared trypsin (Promega AG, Dübendorf, Switzerland) in 50 mM AB at a concentration of 0.2 µg/µl. To remove RapiGest, samples were

acidified with trifluoroacetic acid (TFA), heated at 37°C for 45 min. and centrifuged 10 min. at 17'000 x g. Supernatants were then desalted with a C18 microspin column (Harvard Apparatus, Holliston, MA, USA) according to the manufacturer's instructions, completely dried under speed-vacuum and stored at -20°C.

2.6.2. ESI-LC-MS/MS

Samples were diluted at 1 µg/µl with loading buffer (5% CH_3CN, 0.1% FA). Biognosys iRT peptides were added to each sample and 2 µg of peptides were injected onto the column. LC-ESI-MS/MS was performed on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with an Easy nLC1200 liquid chromatography system (Thermo Scientific, San Jose, CA, USA). Peptides were trapped on an Acclaim pepmap100, C18, 3 µm, 75 µm x 20 mm nano trap-column (Thermofisher) and separated on a 75 µm x 500 mm, C18 ReproSil-Pur (Dr. Maisch GmbH, Ammerbuch, Germany), 1.9 µm, 100 Å, custom-made column. The analytical separation was run for 135 min using a gradient of H_2O/FA 99.9%/0.1% (solvent A) and CH_3CN/H_2O/FA 80.0%/19.9%/0.1% (solvent B). The gradient was run from 8 % B to 28 % B in 110 min, then to 42% B in 25 min, then to 95%B in 5 min with a final stay of 20 min at 95 % B. Flow rate was of 250 nL/min a total run time was of 160 min. Data-Independent Acquisition (DIA) was performed with MS1 full scan at a resolution of 60,000 (FWHM) followed by 30 DIA MS2 scan with fix windows. MS1 was performed in the Orbitrap with an automatic gain control (AGC) target of 1×10^6, a maximum injection time of 50 ms and a scan range from 400 to 1240 m/z. DIA MS2 was performed in the Orbitrap using higher-energy collisional dissociation (HCD) at 30%. Isolation window was set to 28 m/z with an AGC target of 1×10^6 and a maximum injection time of 54 ms.

2.6.3. Data analysis

DIA raw files were loaded into Spectronaut v.15 (Biognosys, Schlieren, Switzerland) and analysed by directDIA using default settings. Briefly, data were searched against the human Reference Proteome database (Uniprot, 2018-06, 21044 entries). Trypsin was selected as the enzyme, with one potential missed cleavage. Variable amino acid modification was oxidized methionine. Fixed amino acid modification was carbamidomethyl cysteine. Both peptide precursor and protein FDR were controlled at 1% (*Q value* < 0.01). Single Hit Proteins were excluded. For quantitation, Top 3 precursor area per peptides were used, "only protein group specific" was selected as proteotypicity filter and normalization was set to "global normalization". The quantitative analysis was performed with MapDIA tool, using the precursor quantities extracted from Spectronaut output. No further normalization was applied. The following parameters were used: min peptides = 2, max peptides = 10, min correl = -1, Min_DE = 0.01, max_DE = 0.99, and experimental_design = replicate design. Proteins were

considered to have significantly changed in abundance with an FDR ≤ 0.05 and an absolute fold change FC≥ |1.5| (log2FC ≥ |0.58|).

3. Results

3.1. L-R5 decreases the affinity between PKC ζ and occludin

Microscale thermophoresis is a method used to quantify the affinity between two molecules (e.g., proteins) by increasing the concentration of the ligand while keeping the concentration of the target fixed. Solutions are heated in capillaries by a laser and the fluorescence of the labelled target protein is measured over time. Three different intensities of the heating laser were applied to better reveal potential complex formation. During the heating of the solution, the target protein diffuses out of the heated region after a certain time, which is dependent on its size and molecular weight. In the case where an interaction between ligand and target occurs, the complex formed shows a larger overall size and molecular weight and will diffuse

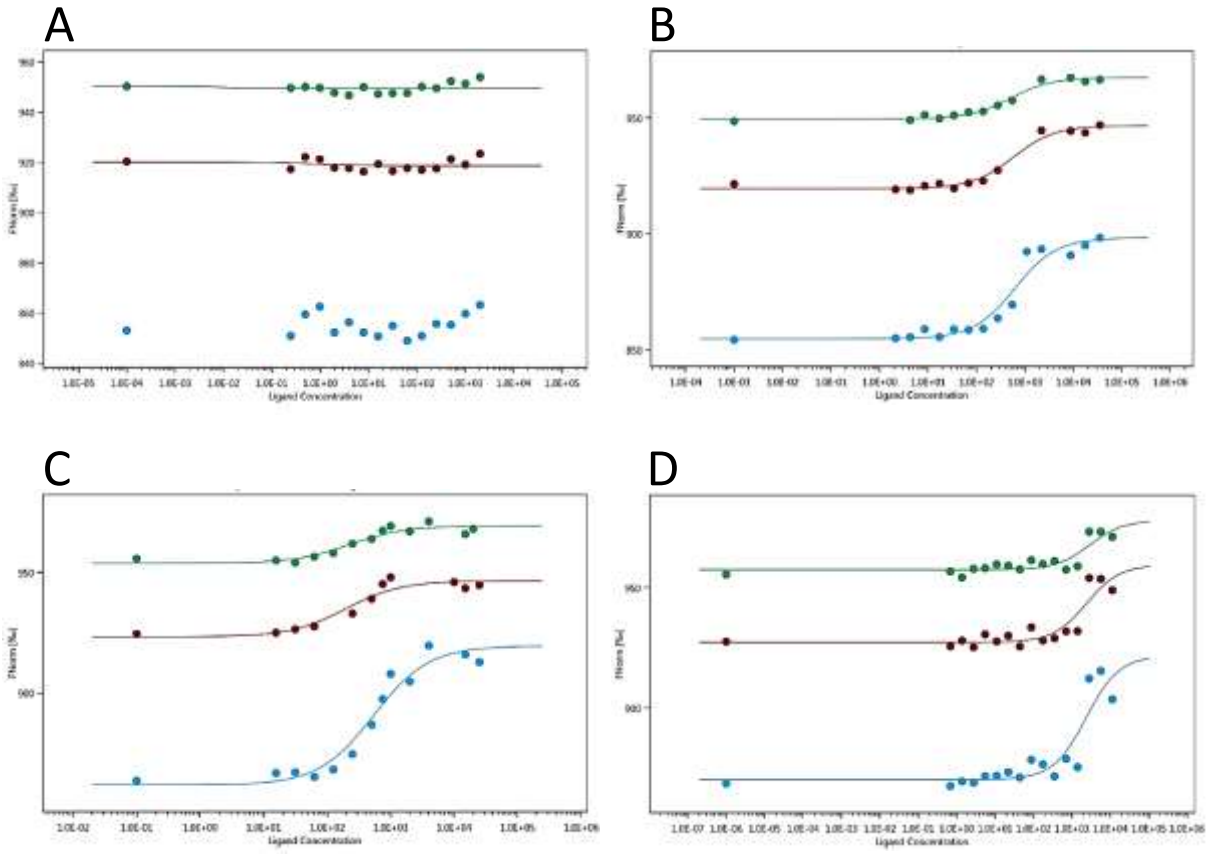

Figure 2: Microscale thermophoresis measurements, green, red and blue lines represent the same samples measured at respectively 20%, 40% and 80% MST intensity. A: interaction of labelled PKC ζ (20 nM) and L-R5 as ligand (0-4 mM); B: interaction of labelled PKC ζ (30 nM) and occludin as ligand (0-35 µM); C: interaction of labelled occludin (50 nM) and L-R5 as ligand (0-25 mM); D: interaction of labelled PKC ζ (30 nM), L-R5 as competitor (1,25 mM) and occludin as ligand (0-11,3 µM). n=2

more slowly when heating is applied. Therefore, the fluorescence during the analysis time will be higher overall and by plotting all results, the dissociation constant K_d can be calculated. This constant was calculated using equation 1 [43]:

$$K_d = \frac{[A]^x \times [B]^y}{[A_x B_y]} \; when \; A_x B_y \Leftrightarrow xA + yB \qquad \text{(Eq. 1)}$$

with A and B, the 2 components (ligand, target) interacting and x and y the 2 stoichiometric factors.

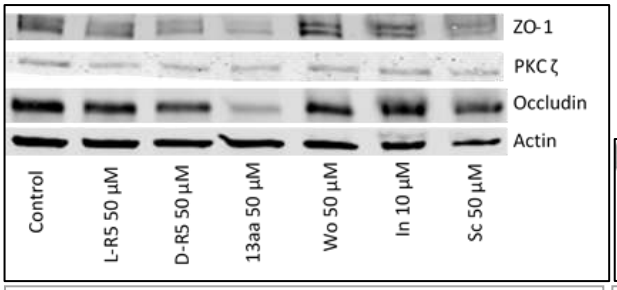

Figure 3: Total protein extracts from Caco-2 cells were immunoblotted for ZO-1, PKC ζ, occludin and actin after different conditions applied for 1 hour: Control (Medium), L-R5 50 µM, D-R5 50 µM, 13aa 50 µM, Wo 50 µM, In 10 µM and Sc 50 µM.

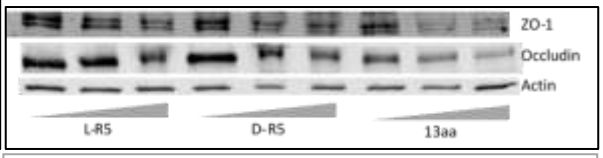

Figure 4: Total protein extracts from Caco-2 cells were immunoblotted for ZO-1, occludin and actin after different gradient conditions for 1 hour: L-R5, D-R5 and 13aa at 20, 50 and 100 µM.

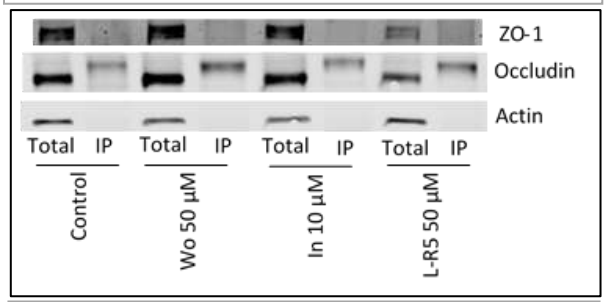

Figure 5: Total protein extracts and immunoprecipitated phospho-threonine protein extracts (IP) were immunoblotted for ZO-1, occludin and actin under different conditions after 1 hour: Control (medium), Wo 50 µM, In 10 µM and L-R5 50 µM.

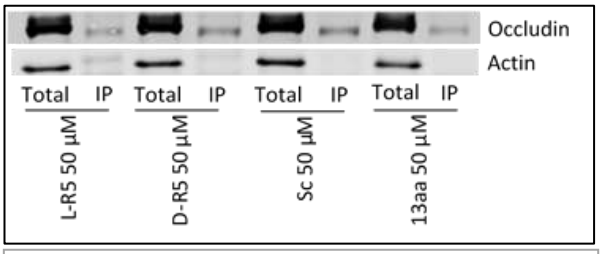

Figure 6: Total protein extracts and immunoprecipitated phospho-threonine protein extracts (IP) were immunoblotted for occludin and actin under different conditions after 1 hour: L-R5 50 µM, D-R5 50 µM, Sc 50 µM and 13aa 50 µM.

First, the different binary interactions were tested between PKC ζ, occludin and L-R5. The myristoylated peptide L-R5 showed no interaction with the enzyme (figure 2A), as no difference of fluorescence over time was noted in the heated region for this solution, even at a concentration of 4 mM for L-R5. On the other hand, occludin interacts with both PKC ζ and

L-R5 (figure 2B and 2C, respectively). An increase in normalized fluorescence over time reveals the formation of a protein-enzyme and protein-peptide complex. The K_d measured for these interactions are 617,33 ± 97,4 nM and 605,67 ± 34,2 µM, respectively. In figure 2D, the interaction between PKC ζ and occludin still occurs but at a higher K_d (2,16 ± 0,59 µM), possibly due to the presence of L-R5.

3.2. TJ protein expression is decreased by L-R5 and analogues

Different peptides and a chemical inhibitor of PKC ζ were applied on Caco-2 cells. The expression of TJ proteins was then quantified by immunoblotting. First, the expression of PKC ζ was not affected by any condition (figure 3). On the other hand, expression of both occludin and ZO-1 were decreased by the myristoylated peptides. The unmyristoylated peptide Wo and the chemical inhibitor In showed no effect on their expression.

To confirm these results, solutions of L-R5, D-R5 and 13aa peptides were applied on the cells at different concentrations to demonstrate the concentration dependence of TJ protein expression (figure 4). As the concentration of the peptides increased, the expression of ZO-1 and occludin decreased. In figure 4, the peptide 13aa is shown to impart a greater reduction of TJ protein expression. The enzyme PKC ζ was not blotted as the peptides were shown to have no effect on its expression.

3.3. Active occludin expression is not affected by ZIP derivatives

It was confirmed that the total amount of ZO-1 and occludin is reduced by the application of ZIP designed peptides (figure 3 and 4). As ZIP is supposed to block the phosphorylation of occludin by PKC ζ, the total amounts of TJ proteins and threonine-phosphorylated TJ proteins were compared (figure 5 and 6). The absence of ZO-1 as well as that of actin can be noted in the immunoprecipitated samples (figure 5). The occludin signal is still only decreased in the presence of L-R5, but not in the presence of both Wo and In. For immunoprecipitated phospho-threonine occludin, the signal between the different conditions is conserved, no significant difference in the expression of p-occludin was found. The same results are shown in figure 6 where the amount of total occludin as well as p-occludin is the same under all conditions.

3.4. The scope of L-R5 is wider than expected

Quantitative proteomic analysis of the different conditions allowed for the identication and quantification of 5115 proteins. By combining data of significantly differently expressed proteins in the control group versus L-R5 treated group (3787 proteins) and In versus L-R5 (3853 proteins), a total of 3573 proteins were found in common between these two comparisons (figure 7). 280 proteins were specifically differentially expressed only in the

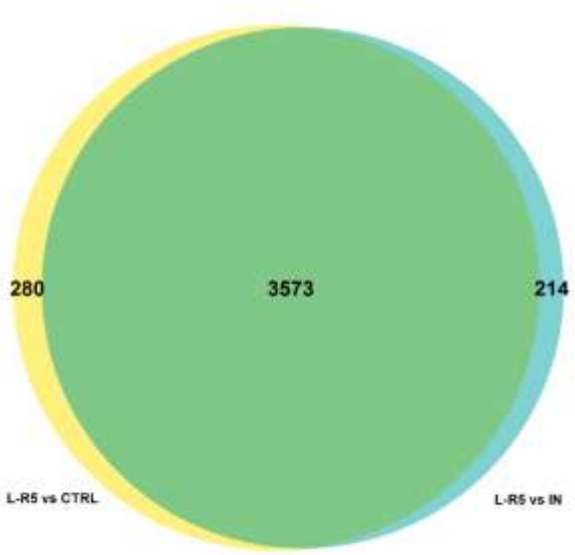

Figure 7: Venn diagram of the significantly regulated proteins in each comparison L-R5 vs CTRL and L-R5 vs In. In total, 4067 proteins were significantly regulated with high confidence (LFDR≤0.05).

control versus L-R5 comparison, and 214 only in the In versus L-R5 comparison. Top 10 pathways influenced in each binary comparison were reported using MetaCore software analysis (figure S4). 6 pathways of 10 were common in control versus L-R5 and In versus L-R5, but none with In versus control. The significance level of affected pathways is represented by their respective -log(*p*-value).

Global relationships among the different conditions were represented by a principal component analysis (PCA) as depicted in figure 8. PCA based on protein level of each replica (represented by a spot), underlines that 77% of the dataset variability is carried by the first principal component and separates the L-R5 group from the two other ones. The second source of variability (PC2) is lower (9.99%) and does not tend to separate sample groups.

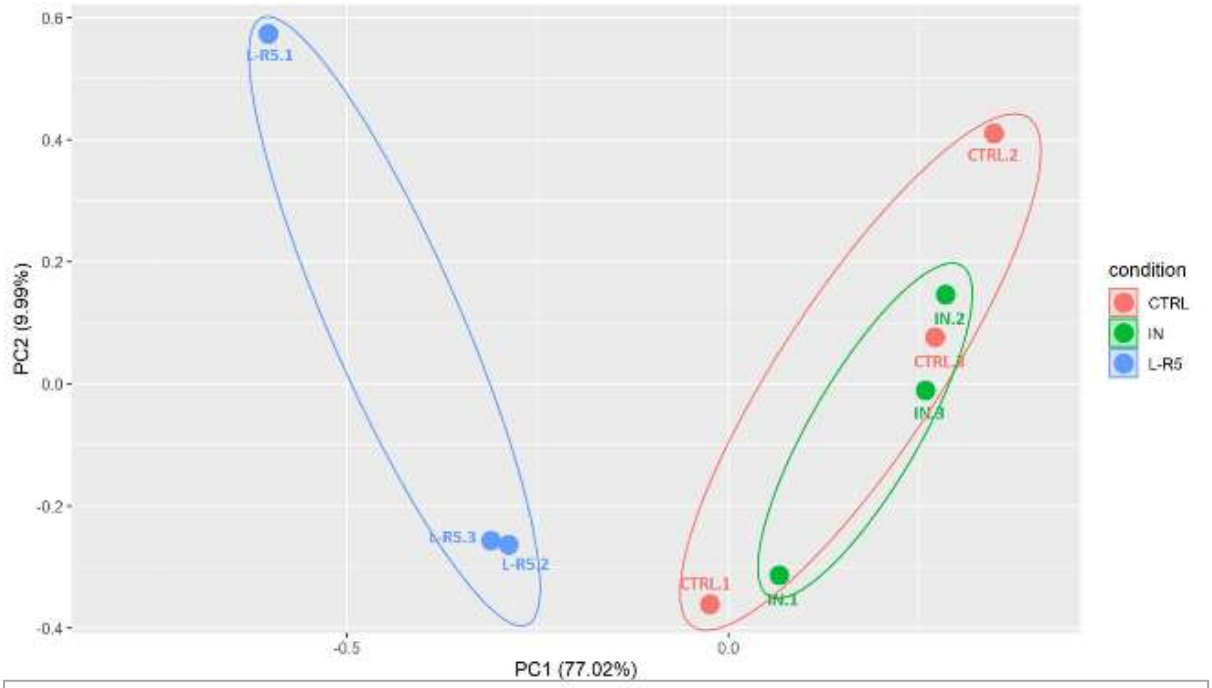

Figure 8: PCA of proteins from Caco-2 cells mediated by 3 conditions: control (red), L-R5 50 µM (blue) and In 10 µM (green). The protein expression profiles were visualized by using the extended data analysis module described in 2.6.3.

PCA demonstrated that the control and In conditions grouped the same proteins but L-R5 grouped completely different expressed proteins. For example, the expression of the protein IRS1 (insulin receptor substrate) was decreased in the comparison L-R5 versus control and L-R5 versus In, but not in the comparison In versus control. In another example, the expression of proteins RICH1 (Rho GTPase-activating protein 17) and CDC 42 (cell division control protein 42 homolog) was increased by L-R5, but not by In alone.

To highlight differentially expressed proteins, a binary comparison between all conditions of downregulated and upregulated proteins was presented in the form of volcano plots (figures S1-S3). The assessment of differential expression of protein levels was done by ANOVA test (cut-off at *p*-value $p \leq 0.05$). 3787 proteins were considered to be significantly differentially expressed between control and L-R5 conditions. Among these, 2384 were upregulated (log 2 (FC) ≥ 0) and 1403 downregulated (log 2 (FC) < 0). 3853 proteins were considered differentially expressed when comparing In and L-R5 conditions. Among these, 2488 were upregulated (log 2 (FC) ≥ 0) and 1365 downregulated (log 2 (FC) < 0). 16 proteins were considered differentially expressed when comparing control and In conditions. Among these, 6 were upregulated (log 2 (FC) ≥ 0), and 10 downregulated (log 2 (FC) < 0).

4. Discussion

Initially, synthetic PS (ZIP for PKC ζ) were used as inhibitors of their respective PKC [33]. However, given the results presented above, the inhibition of PKC ζ does not occur by interaction with the enzyme directly but with the target proteins of this kinase (figure 2A-C). MST analysis provided K_d values, however, it would also be useful to understand whether the interaction between L-R5 and occludin is reversible by determining K_{on} and K_{off} of this interaction [44]. Furthermore, upon binding to occludin, results showed that L-R5 decreased the affinity between PKC ζ and the TJ protein (figure 2D), with K_d values being significantly increased. ZIP is therefore not a direct inhibitor of PKC ζ but acts through competitive binding to the target protein, inhibiting its phosphorylation. The peptide structures may therefore be called "zeta competitor peptides" (ZCPs). Preliminary studies have shown that this interaction is electrostatic in nature due to the positive charges of the arginines of the ZCP [37]. This is confirmed by the same effects seen with the administration of L-R5, D-R5 and Sc (figure 3), with the electrostatic interaction being identical for these three peptides given their equal overall charge. 13aa more strongly decreases TJ protein expression. Its positive charge and its higher complementarity to the target protein provides greater affinity and probably stronger competitive inhibition. However, the peptide without its myristoylated hydrophobic tail can no longer enter the cell [40,45]. Inhibition of PKC ζ by In did not result in a significant decrease in

TJ protein expression. Obviously, the specifical inhibition is not sufficient to have an impact on TJ modulation and decrease expression of TJ proteins.

By competing with the enzyme for binding to occludin, L-R5 prevents phosphorylation of the protein and thus its activation. This prevents successively the closure of TJs [46,47]. ZIP has already been shown to decrease the expression of certain TJ proteins such as occludin and ZO-1 [29]. The ZIP-derived peptide L-R5 has also been shown here to have the same effect (figure 3, 4, S1 and S2). Logic would suggest that the cell increases the production of blocked proteins to compensate for this inhibition. Otherwise, this decrease could be due to i) a tendency of the cell to maintain the same ratio of phosphorylated/non-phosphorylated proteins [48], ii) a recycling of blocked proteins, detected as defective [49,50], or iii) a degradation of these proteins because L-R5 modifies them and makes them unusable or non-viable.

ZO-1 could not be quantified (figure 5) because this protein is phosphorylated at a serine residue [30] and was therefore eliminated during immunoprecipitation. It has previously been shown that inhibition of occludin phosphorylation triggers a decrease in its expression [51]. The results presented above (figure 5 and 6) do not allow the same conclusions to be drawn. Indeed, the expression levels of occludin did not change significantly under the conditions applied in this study. This may be due to an insufficient purification and quantification method, but it may also be likely that phosphorylated occludin (p-occludin) expression does not change. Through the activity of the peptide, the total intracellular concentration of occludin is reduced. In response, the biosynthesis of occludin is activated by the cell. Ideally, the effect of the peptides on all types of occludin should be quantified under each condition, including occludin, threonine-phosphorylated, tyrosine-phosphorylated, serine-phosphorylated and non-phosphorylated occludins. However, Rao speculated that activation of occludin by serine or threonine phosphorylation would not have the same function [52]. Further investigations are still needed to fully unravel the mechanics of occludin phosphorylation inhibition and its consequences.

Previous studies have shown that ZIP is not specifically and competitively inhibiting PKC ζ phosphorylation of TJ proteins [36]. The structural similarities of the PS of the PKC family [23] does explain this non-specificity. Therefore, L-R5 would also be competitive with other PKCs and would interfere with most intracellular mechanisms involving PKCs. The PCA results presented here show that the expression of many proteins is altered by the presence of L-R5 (figure 8), in contrast to In, for which the results are similar to the control condition. Given the absence of cytotoxicity of L-R5 as shown in previous studies [40], this wide range of potential interaction of the peptide was not expected. It may not even translate into safety issues *in*

vivo, however, greater specificity of the peptide for selected TJ proteins could avoid a potential risk.

In this broad field of action, the expression of occludin and ZO-1 proteins is significantly decreased by L-R5 (figures S1 and S2), as previously demonstrated by Western blots. In contrast, In has no impact on their expression (figure S3). Inhibiting PKC ζ alone is not sufficient to decrease their expression, but possibly increase permeability. Concerning L-R5, the volcano plots highlighting the effects of L-R5 (figures S1 and S2) are similar. The proteins affected by the peptide are essentially the same for control and for In (figure 7), and only a few proteins are affected by In condition compared to control. It was mentioned earlier that occludin and ZO-1 are affected by the peptide, but the results also show that claudins 1 and 4 are not. This may be explained by the fact that these proteins are not regulated by PKC ζ [29] and L-R5 is still specific to TJ proteins phosphorylated by PKC ζ, and not all TJ proteins. Furthermore, the expression of occludin and ZO-1 is significantly decreased (figure S1 and S2), which would explain the opening of TJs by L-R5 in addition to the phosphorylation inhibition.

As a proof of concept for L-R5, the expression of PKC ζ-related proteins other than TJ proteins were analysed. In a mechanism of inflammation, the phosphorylation of JAK1 (Janus kinase 1) is mediated by PKC ζ [53]. JAK1 expression is not influenced by L-R5, but its activity is decreased, which is shown by the decreased expression of its target protein, IRS1 [54]. JAK1 phosphorylates this receptor. As for occludin, the activity of the PKC ζ target protein is inhibited by the pentapeptide.

In another intracellular mechanism, PKC ζ is responsible for the phosphorylation of PARD3 (partitioning defective 3 homolog protein) [55]. This protein is then thought to bind to angiomotin [56]. This complex then inhibits the RICH1 protein [57]. The increase in RICH1 expression observed in our study is due to the inhibition of the activity of the angiomotin-PARD3 complex by L-R5. PARD3 is neither phosphorylated nor activated by PKC ζ. These influenced mechanisms confirm the pathway of L-R5. In contrast, no similar results were found in the In versus control condition. The inhibition of PKC ζ by In appears to be too weak or non-existent.

Using the MetaCore software, the 10 most enriched pathways in the different comparisons are listed in figure S4. Pathways enriched in conditions where L-R5 was added (figure S4A and S4B) showed essentially the same results, whether compared to the control or under incubation with In. In contrast, the pathways significantly affected by the In versus control condition are all different from the first 2 conditions (figure S4C).

Only the pathways “chemotaxis lysophosphatidic acid signalling via GPCRs”, “apoptosis and survival NGF/TrkA PI3K-mediated signalling” and “immune response IL-4 signalling pathway” are pathways importantly influenced by L-R5 including PKC ζ. The L-R5 peptide therefore has a very strong influence on the disruption of intracellular mechanisms. This is confirmed by the important -log(*p*-values) of 12-15. The opening of TJs was not observed in these pathways despite the significant influence of L-R5 on this mechanism. It appears that not the regulation of TJs is the mechanism most affected by L-R5, but vesicle formation for intracellular transport (figure S4A). Another type of transport [58] is thus significantly stimulated by the presence of the peptide. In addition, the mechanism of PI3K-mediated apoptosis is also strongly affected by L-R5. As the involvement of PKC ζ in this pathway is proven [59], it is highly conceivable that the peptide interacts with the enzyme’s contribution to this mechanism. One of the target proteins of the enzyme implicated in this pathway is GSK3β [60], whose expression is increased by the presence of the peptide. This results in a significant increase in cell survival [61]. Finally, the “chemotaxis lysophosphatidic acid signalling via GPCR” mechanism is also strongly affected by the peptide. GSK3β linked to PKC ζ is again involved in this pathway [62]. In addition, a decrease in cell proliferation and in formation of adherens junctions, which are complementary junctions to TJs, is observed [63]. Both mechanisms are involved in cancer progression. These results could explain the link between the inhibition of PKCs and the reduction of tumour progression [20].

In is considered as an inhibitor of PKC ζ, but none of the top 10 pathways influenced in the comparison In versus control include PKC ζ. The pathway “development delta- and kappa-type opioid receptors signalling via beta-arrestin” has been referenced as the only one in the list to include PKCs in its mechanism [64]. The effective inhibition of PKC ζ specifically by In is still to be proven *in vitro* or the doses have to be increased. In figure S4C, the common point of these pathways is the involvement of E1A binding protein p300, whose expression is significantly decreased by In. On the other hand, the expression of this protein is increased by the presence of L-R5. As this histone acetyltransferase is not linked to PKC ζ mechanisms [65], it would therefore appear that In interacts with other factors. Moreover, the pathways addressed by In do not imply PKC ζ. The interaction with the kinase is proven [41], but other targets have to be considered as well. However, in view of the wide range of interactions of the peptide, it is legitimate to ask whether the PS derivative modulates TJs by direct interaction, or whether this opening is a result of the sum of all mechanisms affected by the peptide.

The contradictory results between the influence of L-R5 and In can be explained by their different targets. In was synthesised to inhibit PKC ζ, whereas L-R5 blocks the activity of the enzyme by competition with the target proteins. The phosphorylation mechanism cannot be

carried out by PKC ζ or even all PKCs. Therefore, the consequence of L-R5 is not visible on the expression of PKC ζ or their target proteins, but on the proteins secondarily linked with the enzyme. The lack of phosphorylation prevents the activity of the target protein. Occludin for example cannot be active in the presence of L-R5. The subsequent decrease in its expression is due to a negative feedback on the expression mechanism.

5. Conclusions

In this study, the interaction of L-R5, and by extension ZIP and PS of PKC ζ, and PKC ζ with occludin was proven. Furthermore, L-R5 was shown to competes with PKC ζ when binding to occludin, which prevents enzyme-protein interaction. This binding could explain the reason for the opening of TJs in the presence of L-R5. Furthermore, the peptide and its analogues decrease the expression of TJ proteins, in contrast to a specific PKC ζ inhibitor. This decrease may be due to a non-viable modification of the protein. As L-R5 prevents binding between PKC ζ and occludin, the ratio of p-occludin to total occludin should decrease. However, this is not the case. The intracellular regulation of the balance between active and inactive occludin is disturbed by the presence of the peptide and the amount of p-occludin remains the same. The decrease in TJ protein expression was confirmed by proteomics. However, this study showed that L-R5 affects several other mechanisms than just TJ modulation. Finally, competition with PKC ζ is desirable for TJ opening, but this interaction needs more specificity for the TJ proteins involved.

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8. Supplementary data

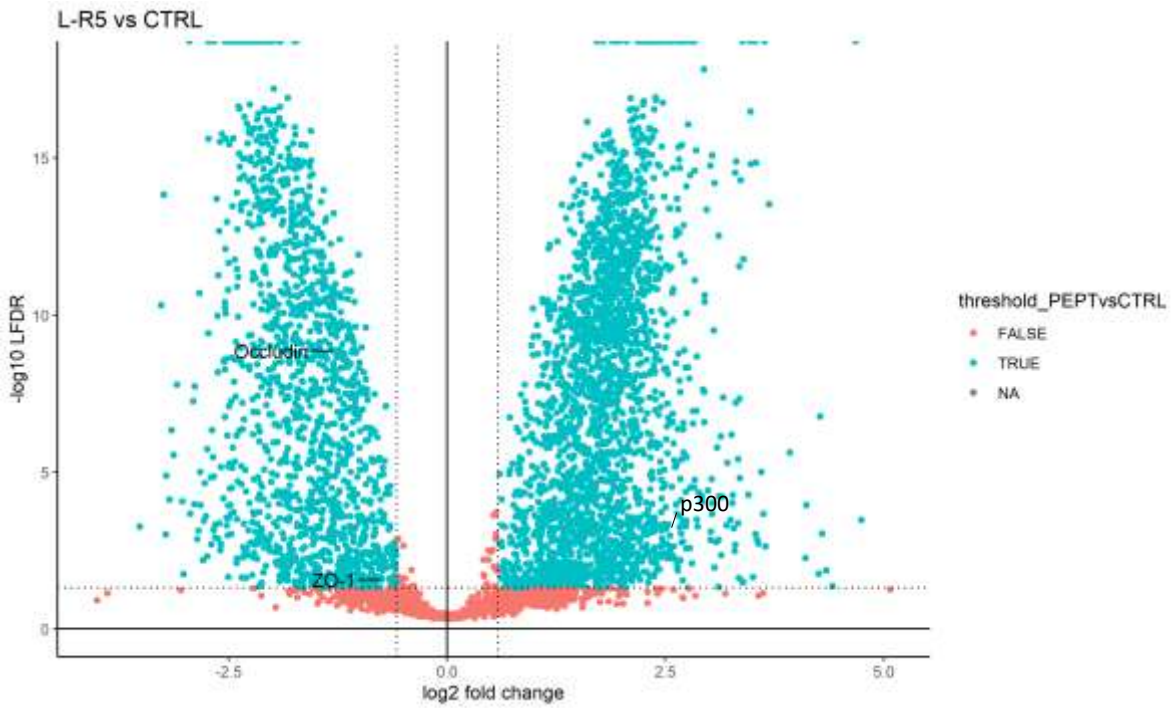

Figure S1: Volcano plot representing log2 fold-change and –log10 LFDR of all the proteins from Caco-2 cells quantified in the comparison L-R5 50 µM vs CTRL. Blue dots represent significantly different proteins, which are above LFDR threshold ≤0.05) and fold-change threshold (log2FC ≥ |0.58|). Proteins p300, occludin and ZO-1 have been highlighted.

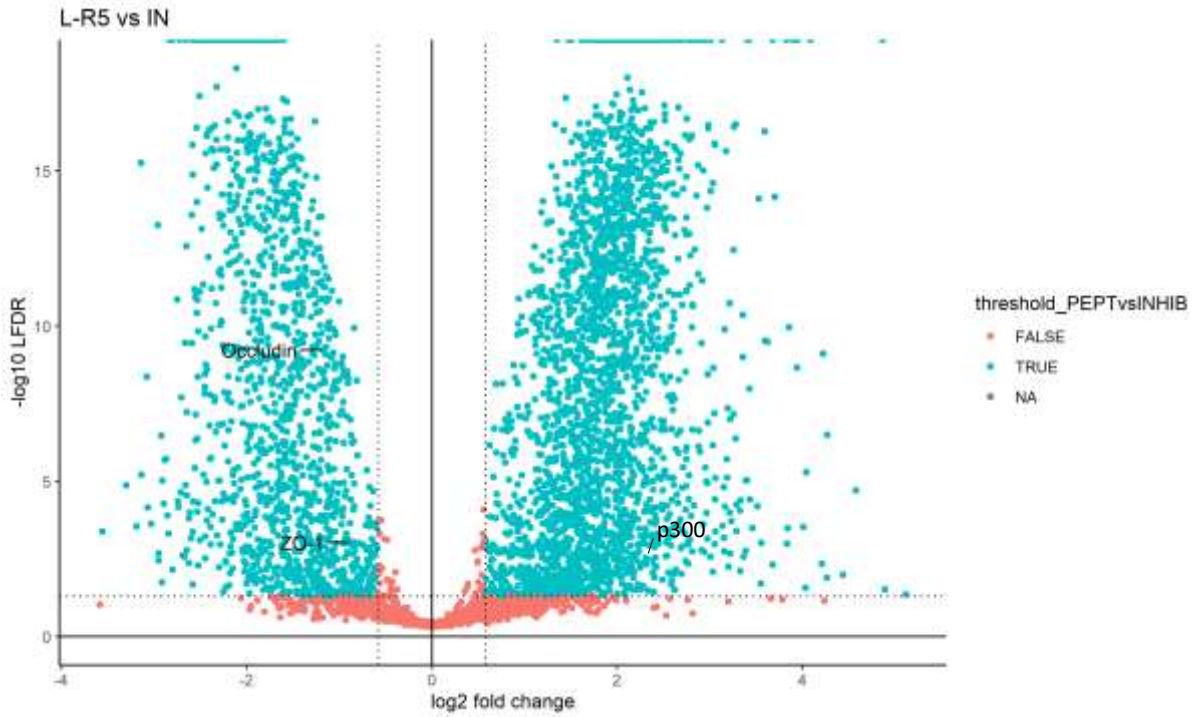

Figure S2: Volcano plot representing log2 fold-change and –log10 LFDR of all the proteins from Caco-2 cells quantified in the comparison L-R5 50 µM vs In 10 µM. Blue dots represent significantly different proteins, which are above LFDR threshold ≤0.05) and fold-change threshold (log2FC ≥ |0.58|). Proteins p300, occludin and ZO-1 have been highlighted.

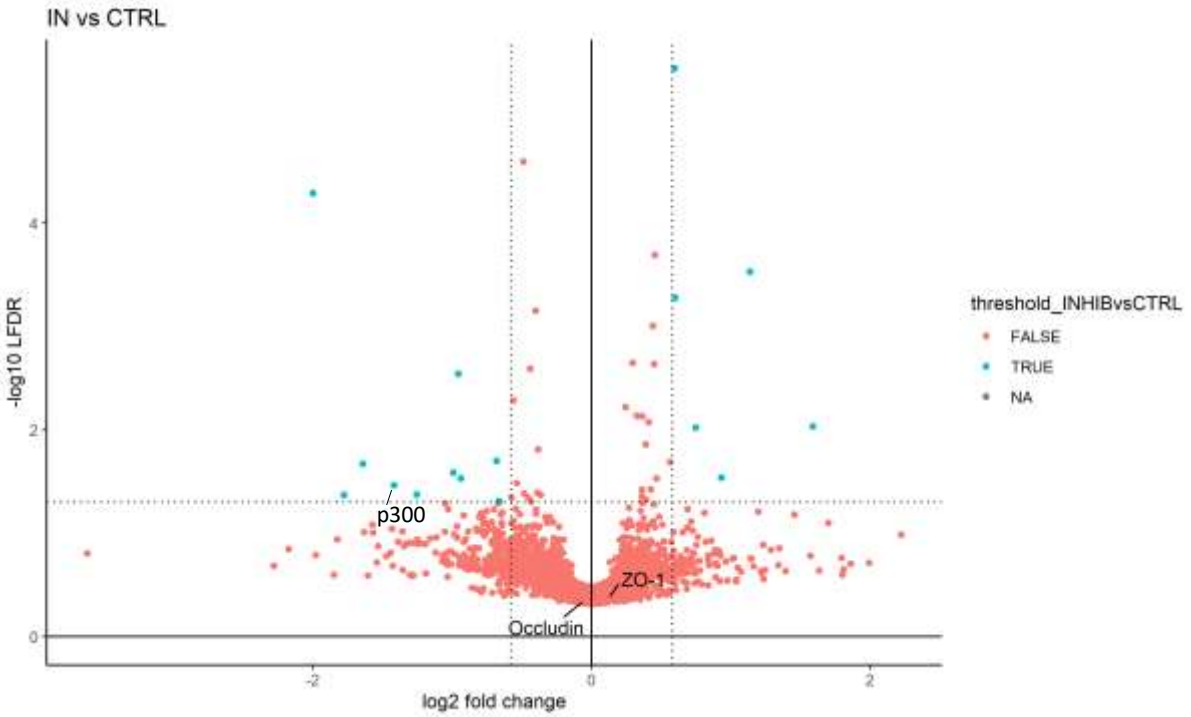

Figure S3: Volcano plot representing log2 fold-change and –log10 LFDR of all the proteins from Caco-2 cells quantified in the comparison In 10 µM vs CTRL. Blue dots represent significantly different proteins, which are above LFDR threshold ≤0.05) and fold-change threshold (log2FC ≥ |0.58|). Proteins p300, occludin and ZO-1 have been highlighted.

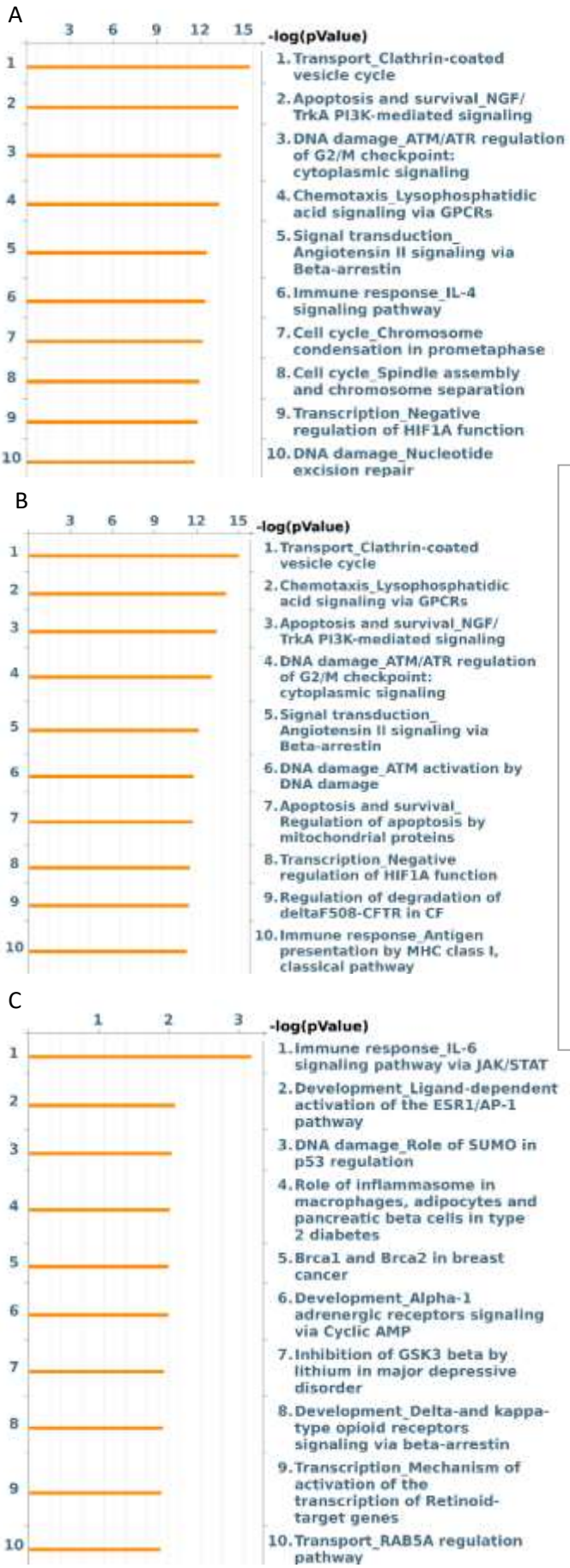

Figure S4: Proteomic pathway analysis (Top 10) as determined by MetaCore analysis. Pathways are listed in order of statistical significance. Orange bars represent the -log(*p*-value) of proteomics analysis. A: Top 10 pathways affected by control versus L-R5 comparison. B: Top 10 pathways affected by In versus L-R5 comparison. C: Top 10 pathways affected by control versus In comparison.

Chapter 4: Peptide structure optimisation

Structure-activity relationship of a peptide permeation enhancer

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Foreword: The structure of the L-R5 peptide was synthesised following the sequence of the PKC ζ pseudosubstrate. The possibility of a more efficient sequence is not excluded. Furthermore, the haemolytic effect of the peptide was due to the presence of a fatty acid. Modulation of this fatty acid necessary for the action of the peptide could decrease or even suppress the induced haemolysis. Finally, a comparison with other permeation enhancers would attest to the effectiveness of L-R5.

This work, entitled “Structure-activity relationship of a peptide permeation enhancer”, was accepted for publication in the journal *Tissue barriers* in April 2022. (DOI: 10.1080/21688370.2022.2060692). The manuscript is presented below.

Author contribution: The studies have been planned, realised and analysed by myself. The manuscript has been written by myself with the support of Prof. Gerrit Borchard.

Declaration of interest:

The authors declare no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Abstract

The pentapeptide L-R5 has previously been shown to transiently increase the permeability of nasal epithelial cell layers *in vitro*, allowing paracellular transport of molecules of up to 4 kDa. Protein kinase C zeta (PKC ζ), a member of a family of serine/threonine kinases was shown to be involved in tight junction modulation induced by L-R5. We show here that the ability of L-R5 to modulate tight junctions is comparable to other permeability enhancers such as bilobalide, latrunculin A or C_{10}. Interaction of the peptide with the target protein occurs via electrostatic interaction, with the presence of positive charges being essential for its functionality. L-R5 is myristoylated to allow quick cell entry and onset of activity. While no epithelial cytotoxicity was detected, the hydrophobic myristoyl rest was shown to cause haemolysis. Taken together, these data show that a structural optimisation of L-R5 may be possible, both from a toxicological and an efficacy point of view.

Keywords: epithelial permeability, Tight junctions, TEER, L-R5, PKC zeta, permeation enhancer

1. Introduction

Drug development is often hampered by the physicochemical properties of the drug candidate. The selection of excipients may lead to an optimisation of key pharmacokinetic parameters such as absorption and distribution [1]. Since the majority of approved drugs are administered orally [2], sufficient intestinal absorption and resulting bioavailability is decisive for drug efficacy and safety. At epithelia, drug absorption can occur by the transcellular or paracellular pathway [3]. It has been estimated that the intercellular (paracellular) space in the intestinal epithelium is about 4 Å [4]. In addition, the passage of molecules through the paracellular space is regulated by networks of proteins forming different types of intercellular junctions. The most important of these connections are tight junctions (TJs) [5], located at the apical side of the epithelial cells.

TJs comprise a network of different proteins, with the most important being occludin [6], zonula occludens proteins (ZO-1, -2) [7] and the claudin family [8]. The modulation of these junctions is a dynamic process triggered by intracellular and/or extracellular stimuli, such as inflammatory signals [9,10]. In addition to managing cell tissue permeability, TJs also play a role in cell differentiation and proliferation, as well as in the establishment of cell polarity [11,12]. In addition, TJs also close the intercellular space between the apical and basolateral sides, and form a transmembrane exchange zone (TJ fence function) [13]. Their regulation is mainly regulated by protein kinases C (PKCs) [14,15]. The controlled modification of these junctions has been one of the pathways used to open the intercellular space, thereby increasing paracellular permeability.

In order to increase oral bioavailability of drugs, different types of permeation enhancers (PEs) have been developed [16] that act through different mechanisms of TJ modulation, but also of other types of intercellular connections such as adherens junctions (AJs). Modulation of these junctions in a selective manner would allow to transiently increase transepithelial permeability. Several PEs have been introduced into the market, such as SNAC and C_{10} [17]. Some of these PEs have a well-described mechanism of action, such as bilobalide, which interacts with the adenosine A1 receptor [18], or latrunculin A, which interferes with the cytoskeleton structure [19]. An issue to be addressed is the potential toxicity of these PEs, as has been described for example for sodium dodecyl sulphate (SDS) [20]. PEs addressing the regulation of TJ proteins such as occludin and ZO-1 [21,22] may directly lead to an increase in epithelial permeability through a well-defined mechanism. Accordingly, PKCs, which regulate the balance of certain TJ proteins are prime targets for such a PE.

PKCs are a family of serine/threonine kinases [23]. These enzymes have been identified to be involved in many intracellular mechanisms, such as apoptosis [24], proliferation [25] or

inflammation [26], and are grouped into conventional, novel and atypical PKC sub-families [27]. PKCs may have opposing roles [16]. An imbalance in their expression was found in certain diseases, such as certain cancer types, where an increase in the expression of certain PKCs is present [28], and in diabetes [29]. Management of this expression imbalance through therapeutic intervention has already been studied, however, until now in the absence of real clinical success [30].

PKC ζ belongs to the atypical sub-family and has been identified to be overexpressed in some cancer types [31]. In addition, this kinase has also been found to be responsible for the activation of the TJ proteins occludin [32] and ZO-1 [33] through phosphorylation of threonine and serine residues, respectively. Phosphorylation takes place through the interaction between PKC ζ and the target proteins mediated by the pseudosubstrate (PS) part of the enzyme through electrostatic interaction [34] (figure 1). The PS is a segment that is part of the enzyme between the amino acids 113 and 129 [35] and has initially a self-inhibitory function on the enzyme keeping it in an inactive state [36]. When PKC ζ is activated, the role of the PS is to interact with the target protein to allow its phosphorylation [34]. Inhibition of PKC ζ may prevent the activation of TJ proteins and the closure of these junctions, increasing paracellular permeability.

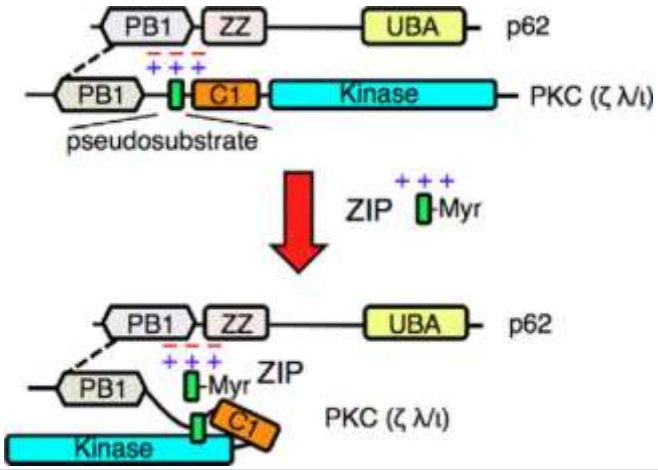

Figure 1: Mechanism of electrostatic interaction between PS of PKC ζ and the target protein (here p62). In a normal situation PS interacts with a part of the target protein (here PB1). The zeta inhibitory peptide (ZIP) competes with the PS and prevents interaction between the enzyme and the protein. Phosphorylation and activation of the target protein does not occur [34].

A myristoylated peptide with the PS sequence of PKC ζ (zeta inhibitory peptide ZIP) has been commercialised as an inhibitor of the enzyme [37]. Inhibition of the kinase by ZIP was originally thought to be achieved by taking the place of the PS to keep PKC ζ in an inactive state.

However, it was recently shown [34] that this peptide does not interact with the enzyme. A ZIP-derived peptide (L-R5) tested in our laboratory showed that the inhibition of PKC ζ activity was not achieved by interacting with the enzyme, but with the target protein [38]. The interaction between PS and occludin for example was competitively blocked by the presence of L-R5, inhibiting phosphorylation and thus activation of TJ proteins.

The increase in permeability by L-R5 has already been demonstrated [39], as has its lack of epithelial cytotoxicity *in vitro*. This study further characterises L-R5, compares the peptide to other PEs, assesses the toxicity of several fatty acids potentially replacing the myristoyl rest towards an improved efficacy of the peptide.

2. Materials and methods

2.1. Materials

Phosphate buffered saline (PBS) solution, sodium pyruvate, Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin (penstrep), non-essential amino acids (NEAA), foetal bovine serum (FBS), Hank's balanced saline solution (HBSS), trypsin EDTA and saline solution (NaCl 0.9%) were obtained from Gibco (Zug, Switzerland). Latrunculin A, bilobalide, sodium caprate (C_{10}) (Table 1), collagen solution PureCol, myristoyl-Gly (myr), methyl myristoleate (myro), methyl octanoate (octa) and methyl palmitate (palm) were obtained from Sigma-Aldrich (Buchs, Switzerland). Sodium fluorescein, fluorescein dextran 4 kDa, fluorescein dextran 150 kDa and fresh defibrinated sheep blood were obtained from Thermo Fisher Scientific (Zug, Switzerland). Water for injection (WFI), T75 flasks, 96-well plates as well as 24-well plates with 6.5 mm inserts of 0.4 µm pore size and a surface area of 0.33 cm^2 were obtained from Corning (Root, Switzerland). Sodium dodecylsulfate (SDS) was purchased from Merck (Schaffhausen, Switzerland). Ethanol 99.8% was purchased from Biosolve (Dieuze, France). Caco-2 cells were purchased from ATCC (HTB-37, Manassas, USA). WST-1 reagent was purchased from Roche (Basel, Switzerland). All peptides were obtained from Bachem AG (Bubendorf, Switzerland), their respective structures are detailed in Table 2.

2.2. Cell culture

Mycoplasma-free Caco-2 cells were cultured in T75 flasks in a humidified atmosphere at 37°C and 5% CO_2. These cells were used at passage numbers 35-39. Cells were cultured in DMEM supplemented with 10% FBS, 1% PenStrep, 1% NEAA and 1% sodium pyruvate. The medium was changed every 2 to 3 days and the cells were passaged using trypsin every 5 days at a split ratio of 1:3. For toxicity studies, cells were seeded at a density of $4.5*10^4$ cells/cm^2 in 96-well plates. 100 µl of medium was added and cells were incubated for 2 days. 24-well plate

inserts were coated for 3 hours with a 0.003% collagen solution in PBS before seeding cells at a density of $6*10^4$ cells/cm^2. 600 µl and 100 µl of medium were changed every 2 to 3 days on the basolateral and apical side, respectively, for 21 days. Before the start of permeability experiments, cells were equilibrated in warm HBSS with the same volumes for 30 minutes in the incubator under the conditions described above.

Table 1: Permeation enhancers used and their suggested mechanism of action.

Permeation enhancer	**Mechanism of action**	**Concentration reported**	**Reference**
Latrunculin A	Prevents actin repolymerization, disrupts actin cytoskeleton	0.2 µM	[40]
Bilobalide	A1R-mediated phosphorylation of actin-binding proteins	5 µM	[41]
Sodium caprate	Reversible removal of tricellulin from tricellular tight junction	8.5 mM	[20]
Sodium dodecylsulfate	Integration into cell membrane increasing cell membrane fluidity causing loss of integrity and increase in permeability	2 mM	[42]

Table 2: Designation and structures of peptides used.

Peptide designation	**Peptide structure**
L-R5	L-myr-ARRWR
D-R5	D-myr-ARRWR
Scrambled	L-myr-WRARR
L-A5	L-myr-AARWR
L-W5	L-myr-ARRAR
ZIP	L-myr-SIYRRGARRWRKL

myr: myristoyl; ZIP: zeta inhibitory peptide

2.3. Assessment of cytotoxicity

Impact of the peptides and fatty acids (FA) on cell proliferation as a measure of toxicity was tested by WST-1 assay. The peptide solutions in NaCl 0.9% had concentrations between 25 and 100 µM, and FA solutions between 50 µM and 5 mM. SDS 0.1 % in water for injection and culture medium were used as positive control and negative controls, respectively. The prepared cell plate was taken out of the incubator and 100 µl of the different solutions were applied, mixed with culture medium, and incubated for 24 hours under conditions as described above. Finally, the supernatant was removed and a mix (100 µl) of WST-1 reagent and cell culture medium at a ratio of 1:1 was added. Absorbance was measured at 450 nm and 690 nm after 2 hours with a plate reader (Biotek Synergy Mx, Sursee, Switzerland). The signal measured by the second wavelength was considered as the baseline. To determine the percentage of cellular viability, equation 1 was used:

$$\text{Cytotoxicity}\ (\%) = \frac{\text{Abs}_{\text{exp value}} - \text{Abs}_{\text{neg control}}}{\text{Abs}_{\text{pos control}} - \text{Abs}_{\text{neg control}}} \times 100 \qquad \text{(Eq. 1)}$$

2.4. *In vitro* haemolysis assay

As previously described [39], fresh defibrinated sheep blood was used to evaluate the influence of peptides and fatty acids on the integrity of the cellular membranes. The blood was washed using PBS and centrifuged at 1500 rpm for 1 minute. Supernatant was discarded and the pellet was resuspended in 1 ml of PBS. These steps were repeated 5 times. Finally, 11 ml of PBS were added, and the suspension was stored at 4°C. Solutions of peptides and fatty acids (myr, myro, octa, palm) at concentrations between 1 mM and 5 µM were prepared in HBSS. WFI was used as a positive control and HBSS was used as a negative control. 50 µl of each condition were incubated with 50 µl of washed sheep blood suspension (47.2 million cells/ml) in each well of a round bottom 96-well plate for 30 minutes under slow agitation at room temperature. The plate was centrifuged at 3700 rpm for 10 minutes. 50 µl of supernatant of each well was then transferred and mixed with 250 µl of ethanol in a flat bottom 96 well plate. The absorbance was then read at 412 nm (Biotek Synergy Mx, Sursee, Switzerland) with a maximum of 0.724 and a minimum of 0.095, respectively for WFI and HBSS. The absorbance values obtained for the positive control were defined as 100% haemolysis and the negative control as 0%.

2.5. Transepithelial electrical resistance

Transepithelial electrical resistance (TEER) was measured as previously described [39] right after the equilibration of the cells and immediately after the permeability experiments using an EVOM volt-ohmmeter (World Precision Instruments, Stevenage, UK) equipped with chopstick electrodes. TEER values were calculated by using equation 2:

$$\text{TEER}\ (\Omega\ \text{cm}^2) = \left(\text{resistance value}\ (\Omega) - 100\ (\Omega)\right) \times 0.33\ (\text{cm}^2) \quad \text{(Eq. 2)}$$

Where 100 (Ω) is the resistance of the porous membrane coated with the collagen layer the cells were seeded upon, and 0.33 cm^2 is the total surface of the epithelial cell layer and of the insert. TEER was always measured in warm HBSS.

2.6. Permeability studies

As previously described [39], the apparent permeability (P_{app}) of fluorescein dextran 4 kDa (FD-4) and 150 kDa (FD-150) was calculated using the equation 3:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0} \quad \text{(Eq. 3)}$$

Where C_0 is the initial concentration (10^6 ng/ml) of the permeant in the donor compartment (apical side), A is the surface area of the cell layers (0.33 cm^2 for inserts in 24 well plates) and dQ/dt is the appearance rate of FD-4 or FD-150 in the receiver compartment (basolateral side). All experiments were performed in triplicate.

After equilibration and TEER measurement, the peptide or FA solutions containing 0.25 mM FD-4 or 6.6 µM of FD-150 in HBSS were applied to the apical side. The concentration of the other PEs was determined based on the literature referring their permeability enhancing effect. Instead of peptides or FA, bilobalide [41], latrunculin A [40], SDS [42] and C_{10} [20] were applied at a concentration of 5 µM, 0.2 µM, 2 mM, 8.5 mM, respectively. Samples of 100 µl were taken from the basolateral compartment of each well each 15 minutes during the first half hour, and then each 30 minutes for a final period of 3 hours, with each volume being replaced by an equal volume of warm HBSS to maintain sink conditions. The fluorescence of FD-4 and FD-150 was then measured in black 96 well plates at excitation and emission wavelengths of 485 and 520 nm respectively, using a plate reader (Biotek Synergy Mx, Sursee, Switzerland). A calibration curve was established to determine the concentration of the fluorescent compound over time at the basolateral side.

2.7. Statistical analysis

Data are reported as mean ± standard deviation (S.D.). Statistical significance was considered at a *p*-value < 0.05. Significance is denoted as *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. The data were analysed using two-way analysis multiple comparison of variance (ANOVA) with GraphPad Prism. The data were analysed using multiple t-test comparisons.

3. Results

3.1. L-R5 compared to other PEs

The L-R5 peptide had already shown its ability to increase the permeability of molecules through an epithelial cell layer [39]. This increase in permeability and decrease in TEER was compared to other PEs with different mechanisms of action (Table 1). L-R5 was compared to bilobalide and latrunculin A (figure 2A and 2B), and subsequently to C_{10} and SDS (figure 2C and 2D). The passage of FD-4 is further increased by L-R5 compared to latrunculin A and bilobalide, which showed similar increases in permeability. The decrease in TEER, however, is comparable between the 3 PEs. By contrast, both SDS and C_{10} drastically decreased TEER at concentrations tested. Epithelial permeability caused by C_{10} showed a strong increase only after 150 minutes, which corroborates data previously reported in literature [17]. On the other hand, the effect of surface-active agent SDS is considered to be due to its toxicity [43].

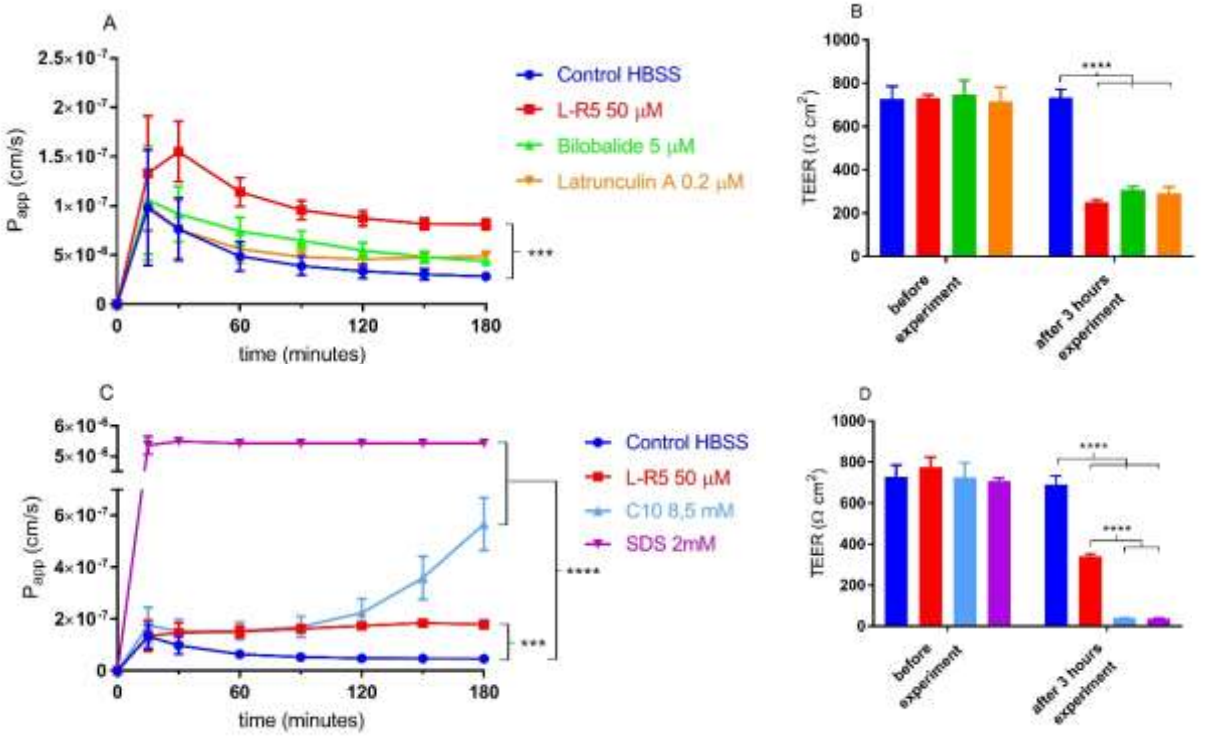

Figure 2: Comparison of permeability enhancement between L-R5 and other PEs. The permeability of FD-4 (0.25 mM solution) through Caco-2 epithelial cell layers was measured in the presence of L-R5 50 µM, Bilobalide 5 µM and Latrunculin A 0.2 µM over 180 minutes (A). TEER was measured before and after the experiment (B). The permeability of FD-4 (0.25 mM solution) through Caco-2 epithelial cell layers was measured in the presence of L-R5 50 µM, C_{10} 8.5 mM and SDS 2 mM over 180 minutes (C). TEER was measured before and after the experiment (D). Values are mean ± S.D. (n=3), ***P < 0.001, ****P < 0.0001. The data were analysed using multiple t-test comparisons.

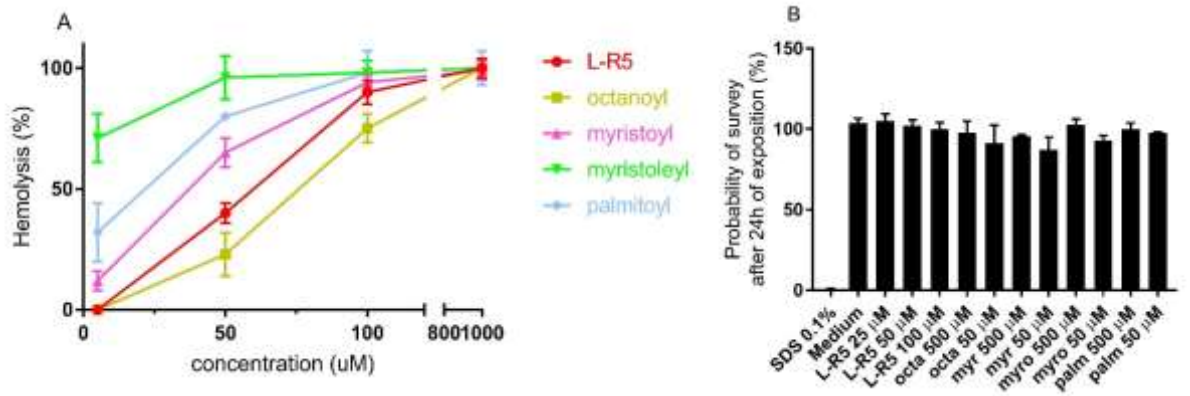

Figure 3: Toxicity comparison between L-R5 and FAs. The haemolytic activity measured in fresh defibrinated sheep blood (47.2 × 10^6 cells/ml), incubated with various concentrations (5-1000 µM) of L-R5 and FAs (octanoyl, myristoyl, myristoleyl, palmitoyl) for 30 minutes at room temperature under slow shaking. HBSS and water for injection (WFI) were used as negative (0%) and positive (100 %) controls, respectively (A). Cytotoxicity was measured by WST-1 test. Caco-2 cells were incubated for 24 hours with various concentrations of L-R5 (25-100 µM) and FAs (50 and 500 µM). SDS 0.1% and cell culture medium were used as positive (0%) and negative control (100%), respectively (B). Values are mean ± S.D. (n=3).

3.2. Influence of fatty acids on permeability and toxicity

The impact of length or state of saturation of the fatty acid moiety on *in vitro* cytotoxicity and permeability was examined. A haemolysis test was performed on defibrinated blood cells (figure 3A) with fatty acids of different chain lengths (C8:0 octanoyl, C13:0 myristoyl and C16:0 palmitoyl) as well as an unsaturated myristoleyl (C13:1) moiety and L-R5, which is myristoylated. The extent of haemolysis was detected to increase with the chain length of the fatty acid. Furthermore, the unsaturated fatty acid showed a higher haemolytic index than the other fatty acids, despite being shorter than palmitoyl. In addition to the haemolysis test, a WST-1 cell toxicity test was performed (figure 3B). Solutions of the same fatty acids (50 and 500 µM) and the L-R5 peptide (25-100 µM) were incubated with Caco-2 cells for 24 hours. In contrast to the haemolysis test, no significant toxicity was revealed by this test.

The permeability and opening of TJs was also tested for these FAs in comparison to the L-R5 peptide (figure 4A and 4B). The fatty acids as well as the peptide were incubated with Caco-2 cell layers for 3 hours after application to the apical side and the permeability of FD-4 was

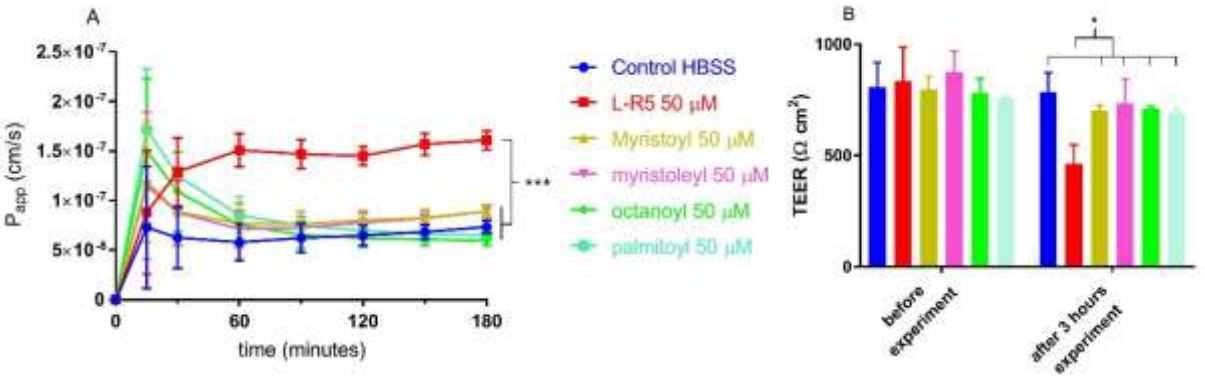

Figure 4: Comparison of permeability enhancement between L-R5 and other PEs. The permeability of FD-4 (0.25 mM solution) through Caco-2 epithelial cell layers in the presence of L-R5, myristoyl, myristoleyl, octanoyl and palmitoyl (all at a concentration of 50 µM) was investigated over 180 minutes (A). TEER was measured before and after the experiment (B). Values are mean ± S.D. (n=3), **P* < 0.05, ****P* < 0.001. The data were analysed using multiple t-test comparisons.

quantified. No significant increase in permeability was observed compared to HBSS control, with the exception of L-R5, which increased significantly the passage of FD-4 through the Caco-2 cell layers. The inability of fatty acids to increase permeability either by TJ modulation or toxic effects was further documented by the maintenance of the initial TEER value during incubation. However, the initial increase in permeability profile in the presence of FAs is different from that in the presence of L-R5.

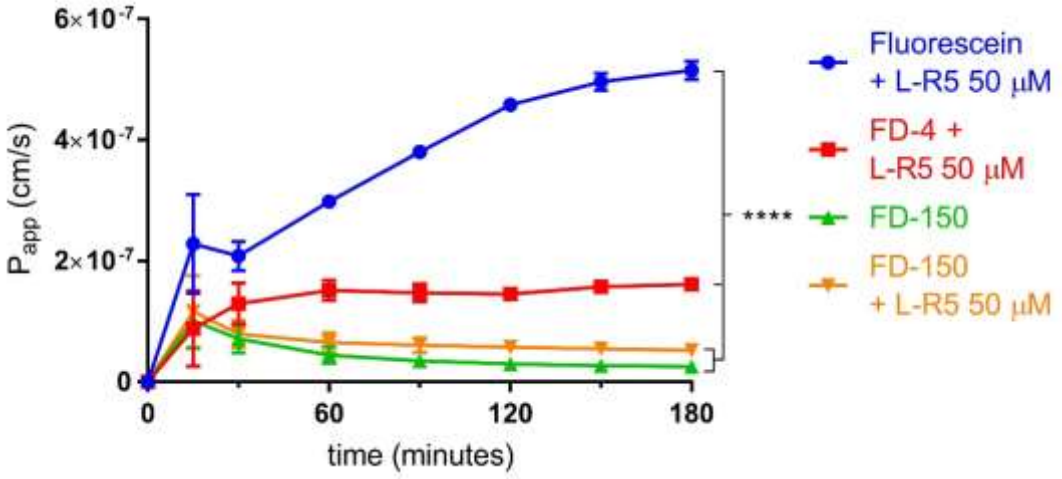

Figure 5: Size-dependent increase in permeability by L-R5. The permeability of sodium fluorescein, FD-4 and FD-150 (all 0.25 mM solution) through Caco-2 epithelial cell layers was measured in the presence of L-R5 (50 µM) over 180 minutes. Values are mean ± S.D. (n=3), *****P* < 0.0001. The data were analysed using multiple t-test comparisons.

3.3. TJs opening range with L-R5

The increase in permeability for FD-4 caused by L-R5 has been demonstrated previously [39]. In order to detect the extent of permeability increase for compounds of increasing molecular weights, the passage through Caco-2 cell layers of sodium fluorescein, FD-4 and FD-150 in the presence of L-R5 was measured (figure 5). As expected, fluorescein sodium showed a higher permeability compared to FD-4. In contrast, FD-150 showed a permeability in the presence of the peptide, which was not significantly different from the control group. A molecular weight of 150 kDa therefore appears to be the limit of opening of TJs caused by L-R5 activity.

3.4. Structural modifications of L-R5 and their impact on permeability enhancement

As electrostatic and possibly hydrophobic interaction between L-R5 and the target protein may play a role in the modulation of TJs, several structural modifications were applied to the peptide. For the peptide L-W5, the hydrophobic amino acid tryptophan was replaced by the neutral amino acid alanine, and in the case of peptide LA-5, one of the positively charged arginines was replaced by alanine. The passage of FD-4 through cell layers of Caco-2 cells in the presence of these peptides was measured in addition to TEER measurements (figure 6). The removal of a positive charge (LA-5) appears to negate any effect on TJ modulation, with FD-4 P_{app} data showing no significant difference to the control group. By contrast, the peptide

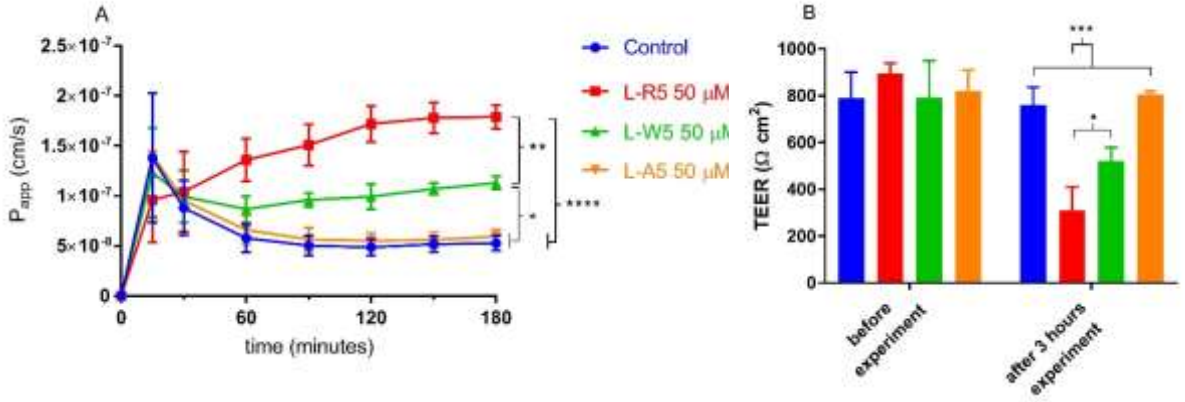

Figure 6: Permeability enhancement comparison between L-R5 and structural modifications of L-R5. The permeability of FD-4 (0.25 mM solution) through Caco-2 epithelial cell layers in the presence of L-R5, L-R5 with the tryptophan replaced by an alanine and L-R5 with the first arginine replaced by an alanine (all at a concentration of 50 µM) was investigated over 180 minutes (A). TEER was measured before and after the experiment (B). Values are mean ± S.D. (n=3). *P < 0.05, ***P < 0.001. The data were analysed using multiple t-test comparisons.

without tryptophan (LW-5) still has an influence on the increase in FD-4 permeability. However, the effect is significantly lower than with L-R5.

The same peptide as L-R5 using D-amino acids (D-R5), as well as L-R5 with a scrambled sequence of amino acids (myr-WRARR) and ZIP were compared with respect to their effect on permeability and TEER in Caco-2 cell monolayers (figure 7). All 5-amino acid peptides, including the scrambled peptide, were shown to influence the permeability of FD-4 and the TEER of the cell layer in the same way, with FD-4 permeability increased and the TEER significantly decreased compared to the control group. Furthermore, the ZIP peptide increased the passage of FD-4 and decreased TEER to a higher extent than the other peptides.

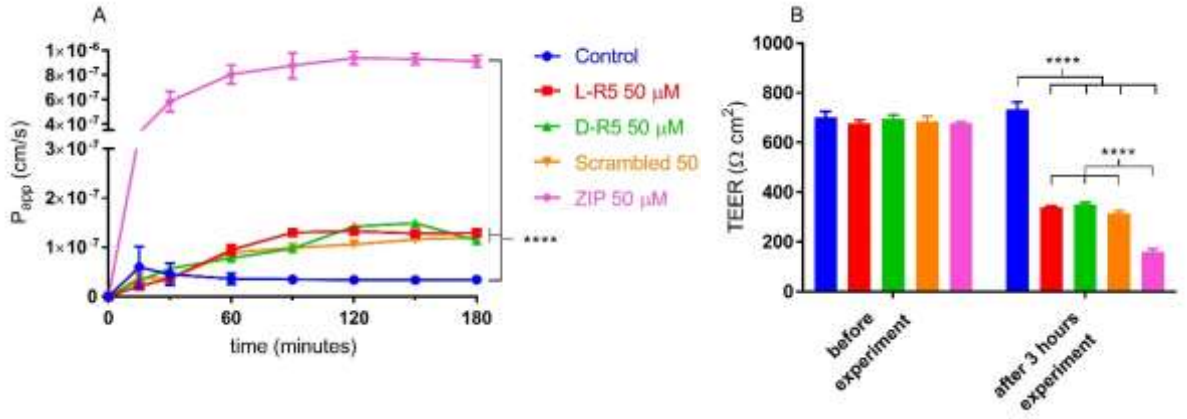

Figure 7: Permeability enhancement comparison between L-R5, its D form (D-R5), its scrambled form and ZIP. The permeability of FD-4 (0.25 mM solution) through Caco-2 epithelial cell layers was measured in the presence of L-R5, D-R5, scrambled L-R5 and ZIP (all at a concentration of 50 µM) over 180 minutes (A). TEER was measured before and after the experiment (B). Values are mean ± S.D. (n=3). ****P < 0.0001. The data were analysed using multiple t-test comparisons.

4. Discussion

The aim of this study was to investigate more deeply the ability of L-R5 to transiently open TJs. This opening seems to be able to increase the absorption of molecules with a molecular weight < 150 kDa. Haemolytic and cytotoxic tests were performed. In addition, a comparison with other PEs was carried out in order to attest the effect of L-R5. Finally, structural modifications of the peptide were applied in order to further elucidate the mechanism of action of the peptide.

L-R5 is more successful in increasing the permeability of FD-4 compared to the PEs bilobalide and latrunculin A (figure 2A). The mechanisms of action of these two molecules are similar, as they both influence the cytoskeleton of the cell. By interacting with the adenosine A1 receptor, bilobalide causes contraction of the actin filament, which transiently opens the intercellular junction [41]. Latrunculin A enters the cell and disrupts the cytoskeletal filaments, distorting the cell and allowing larger molecules to pass through the cellular junctions [40]. The opening induced by L-R5 appears to lead to a higher permeability with a faster onset, however, it should be noted that the applied concentrations of the other two molecules necessary to open TJs are much lower. An equivalent concentration may give the same or even better results. The problem is that since latrunculin A is derived from a sponge toxin [44], an increase in concentration may lead to safety issues. By contrast, bilobalide showed no cytotoxicity at a concentration of 625 µM and may be more effective at higher concentrations if no increase in toxicity is observed [41]. The fast increase in the 15 first minutes of each condition may be due to a hormetic effect [45], even if this difference is not significant. More specifically, the modification of the ambient environment will inevitably force the cell to an adaptation. A greater disruption of the cell membrane by FAs is also a potential reason for this increase. This stress will lead to a short increase of the paracellular permeability. Another explanation of this short greater enhancement can be explained by a potential increase in the permeability of FD-4 by FAs forming micelles.

L-R5 activity was also compared to C_{10} and SDS (figure 2C). SDS is supposed to increase transcellular permeability [46]. However, the concentration used here was reported to be toxic at 2 mM as reported in literature [43]. The increase in permeability is therefore probably due to cell death induced by this surfactant. TEER results for C_{10} would suggest that it was also toxic at the concentration of 8.5 mM (figure 2D). This molecule is thought to be responsible for a redistribution of TJ proteins [47]. The permeability profile shows that the increase in FD-4 passage is gradual. C_{10} is therefore more effective than L-R5, but at a much higher concentration. Although no toxicity has been demonstrated for C_{10} [48,49], administration of a reduced quantity may be beneficial for cellular health. On another hand, a lower submicellar concentration of C_{10} has been shown to be more toxic due to medium chain fatty acid (MCFA) synthesis [50]. The critical micellar concentration (CMC) may be pivotal for C_{10} toxicity. For now, C_{10} has been widely tested, above CMC at 8.5 mM as a permeation enhancer without a proven toxicity.

Although L-R5 has previously been shown not to cross epithelial cell layers, potentially avoiding systemic circulation [39], the haemolytic effect of the peptide was tested (figure 3A). Haemolysis occurs when the erythrocyte's cell membrane is disrupted. The haemolysis induced by L-R5 appears to be directly related to the fatty acid to which it is attached. The

fatty acid moiety is necessary for its uptake into the cell. The larger the fatty acid, the greater the haemolytic effect. In addition, the unsaturated fatty acid induces haemolysis to a greater extent, which can be explained by a strong deformation of the cell membrane [51]. In view of these results, a reduction in the length of the fatty acid bound to L-R5 would be desirable. However, this would result in a reduction in the penetrability of the peptide into the cell, and thus a reduction in the permeation effect, thus a need to increase the dose administered. Increasing the peptide concentration does not appear to be toxic to the cell (figure 3B). Furthermore, none of the fatty acids showed cytotoxicity in epithelial Caco-2 cell layers, even at high concentrations of 500 µM. The haemolytic effect induced by the myristoyl tail would not have any consequence on *in vivo* application as we previously demonstrated that the peptide does not pass through the epithelial cell layer [39].

It may be considered that the fatty acid is responsible for the increase in permeability, as it disrupts the membrane. However, as shown in figure 4A, the passage of FD-4 in the presence of fatty acids through the Caco-2 cell layer remains similar to the control condition. Furthermore, TEER is not reduced by the fatty acids either (figure 4B). All these results therefore confirm that L-R5 penetrates the cell by disrupting the cell membrane without being toxic and increases the permeability of FD-4 by a mechanism other than cell membrane disruption.

The permeability of FD-150 is only slightly increased by L-R5 (figure 5). Very few non-specific paracellular PEs [16] were shown to increase the paracellular permeation of such large molecules, such as chitosan particles or liposomes [52,53]. 150 kDa therefore appears to be the limiting size for increased permeability induced by L-R5. However, an increase in peptide concentration may be sufficient to be more effective. 150 kDa is the average size of antibodies and therefore the largest treatments that can be prescribed [54]. These treatments are therefore administered in an injectable manner as absorption through an epithelium is impossible without a PE or carrier. Being able to formulate this type of treatment in a non-invasive dosage form would help to increase safety and reduce treatment costs. More *in vitro* and *in vivo* research is needed to assess the suitability of L-R5 with a therapeutic drug.

Some structural modifications were applied to L-R5. It was shown that the presence of positive charges, conferred by arginine, is essential for the activity of the peptide (figure 6A and 6B). In addition, the presence of a hydrophobic amino acid provides complementarity in the interaction, as the replacement of tryptophan by alanine significantly reduces the effect of the peptide. Further investigation on the correlation between peptide structure and its activity may lead to the development of more specific PKC ζ competitors. As an example, phosphorylation of TJ protein occludin by PKC ζ occurs at the C-terminal domain of the protein (between

amino acids 403-438) [55]. Specificity of the inhibition of this phosphorylation step may be achieved by adapting the sequence of small peptide inhibitors, possibly by increasing the number of positive charges. However, this would have to be weighed against a potential increase in toxicity caused by the higher cationic charge density of the compound.

The other peptides tested were D-R5, L-R5 with mixed amino acids (scrambled) and ZIP (figure 7). D-R5 and scrambled peptides showed the same effect on FD-4 permeability as L-R5. The effect of D-R5 was expected, as it has the same sequence as L-R5. The use of D-R5 would be useful if *in vivo* as a more metabolically stable form [56], and a longer effect would potentially be visible due to its slower degradation. The effect of ZIP is stronger than for L-R5, which can be explained by its greater complementarity with the sequence of the target protein. ZIP has the same sequence as the PS and therefore the sequence that interacts with the protein *in vivo*. The advantage of using L-R5 instead of ZIP is found in the reduction of the time of action [39], resulting in shorter disturbance of TJ integrity. The use of a peptide with a scrambled sequence compared to L-R5 resulted in a comparable activity, as was expected seen the nature of peptide-protein interaction.

The use of L-R5 as a PE can add value to a formulation for drug candidates of low bioavailability. Its effect is comparable to other PEs and no cytotoxicity has been revealed. Furthermore, the opportunities for combination with drugs are wide given the possibility of increasing the paracellular permeation of molecules with a molecular weight of up to 150 kDa.

5. Conclusion

Modulation of TJs by interfering with PKC ζ phosphorylation of TJ proteins has been shown to be suitable to increase epithelial permeability. The application of L-R5 peptide to intestinal cells *in vitro* is non-toxic and effective in transiently increasing the permeation of molecules with a suggested upper molecular weight limit of 150 kDa. Furthermore, the efficacy of permeation enhancement of the peptide is comparable to other PEs. Optimisation of L-R5 in terms of target protein affinity and adaptation of activity kinetics appears to be possible by modification of the nature of the amino acids included. Finally, increase in activity through these modifications must be weighed against safety aspects.

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Chapter 5: Insulin formulation for nasal administration

Permeation enhancement of insulin upon nasal administration

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Foreword: Diabetes is one of the most prevalent diseases in the world, and type 1 diabetics require daily or even multiple daily injections of insulin. In our lab, we have demonstrated L-R5 has the ability to transiently open TJs. This opening increases the absorption of drugs across the epithelium. The combination of insulin with L-R5 could allow diabetics to treat themselves with insulin by non-invasive administration, such as a nasal spray.

Author contribution: Prof. Gerrit Borchard, Dr. Sakthikumar Ragupathy and I designed and planned the study for the formulation part. Dr. Cecilia Jiménez-Sánchez, Florian Visentin, Dr. Sakthikumar Ragupathy and I planned the *in vivo* experiments with the support of Prof. Pierre Maechler. The *in vivo* studies were performed by Florian Visentin with the assistance of Dr. Cecilia Jiménez-Sánchez and myself. I was in charge for the cell culture, permeability studies, TEER measurements and circular dichroism experiments. I have written the manuscript with the support of Prof. Gerrit Borchard and Prof. Pierre Maechler.

Declaration of interest:

The authors declare no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

Abstract

Diabetes is one of the most common diseases in the world and also one of the most studied. The main treatment for type 1 diabetes is subcutaneous injections of insulin due to its poor absorption through the mucosa. Combining insulin with an absorption enhancer may allow non-invasive administration by enhancing its absorption. The peptide L-R5 has previously been shown to increase the *in vitro* permeability of molecules across the nasal epithelium. The combination of this myristoylated pentapeptide with insulin resulted in a significant increase of the passage of the protein through the epithelial cell layer. Furthermore, no significant interaction with the secondary structure of insulin was observed. Finally, nasal administration of a formulation composed of rapid insulin and L-R5 did not produce significant results in reducing blood glucose levels in diabetic mice. Optimisation of the formulation, as well as the use of a device adapted to nasal administration in mice, is still necessary.

Keywords: Insulin, peptide, tight junctions, L-R5, diabetes

1. Introduction

Diabetes is one of the most recurrent diseases in the world [1]. The prevalence of diabetes in the world was 2.8% in 2000 and is constantly increasing [2]. By 2030, 4.4% of the population could be affected by this disease. The main causes are an increase in hepatic glucose production, a decrease in glucose utilisation by target tissues (adipose tissue, muscles, liver) and/or a decrease in insulin secretion by pancreatic β-cells [3]. Diabetes treatment is currently limited to slowing the progression of the disease.

Diabetes is characterised by blood sugar levels above 7 mM [4]. Several types of diabetes have been identified. The main ones are MODY (maturity onset diabetes of the young), type 1 diabetes and type 2 diabetes [5]. MODY are already detectable at birth [6], and are caused by a genetic variation resulting in reduced insulin secretion upon glucose stimulation. Type 1 diabetes is an autoimmune disease [7] caused by a stress yet unexplained leading to immunogenic recognition and suppression of pancreatic β-cells. Absence of insulin production is prevalent in type I diabetes patients. Type 2 diabetes is mainly defined by an unhealthy diet and lifestyle [8]. The hyperglycaemia noted in this type of diabetes is due to insulin resistance in the target tissues storing sugars in the form of glycogen (liver and muscle) or triglycerides (adipose tissue). Type 2 diabetes appears progressively as a result of too high insulin demand and β-cell weakness [9].

There are many different treatments for the different types of diabetes. Type 1 diabetes is mainly treated with daily subcutaneous injections of insulin. Insulin analogues with different rapidity of action have been synthesised to suit different diabetes profiles and patient preferences [10]. Rapid-acting (or even ultrarapid-acting) insulins are used at each meal to compensate for the excess glucose provided by food [11]. Slow insulins are used to manage blood glucose levels throughout the day with a single injection [12]. These two types of insulin are often combined to treat type 1 diabetes. In addition, different treatments were identified to alleviate insulin resistance and interacting with the mechanism of action of insulin. Some of these treatments increase cellular energy production and glucose consumption [13], while others increase insulin sensitivity of target tissues [14]. Despite these treatments, the best way to treat diabetes is to improve lifestyle by having a healthy diet and regular physical activity.

The parenteral route is used generally for insulin administration because of its poor absorption upon application by other routes [15]. Nasal [16] and enteral [17] formulations have been tested in order to avoid invasive administration of the therapeutic protein to patients. Indeed, poor patient compliance and a high risk of infection are present with the invasive route [18,19]. Nasal formulations have been extensively studied due to the high vascularisation of this epithelium [20]. The nasal formulation of insulin allowed for better absorption of the protein in

the central nervous system, indicating a nose-to-brain transport mechanism [21]. However, insulin levels in the blood were not increased via this route, probably due to merely low levels of insulin pass through the endothelium of blood vessels, insufficient to treat diabetes. Thus, usually the systemic bioavailability of insulin upon nasal administration is poor [22]. Moreover, the clearance is around 15-20 minutes [23] and several metabolic enzymes degrade the peptide before its absorption can occur. This indicates that either insulin passes through the epithelia, blood vessels and the blood-brain barrier very rapidly [24], or is transported into the central nervous system through the neurons of the olfactory bulb. This discovery may be important for the treatment of Alzheimer's disease as insulin may play an important role of the treatment of this disease [25].

The systemic bioavailability of biomolecules can be improved by association with permeation enhancers [26]. Modulation of tight junctions (TJs) has already been studied to increase insulin absorption [27]. C-*Clostridium perfringens* enterotoxin (C-CPE) was shown to increase insulin uptake *in vitro* [28]. Modulation of TJs was also previously tested using the peptide L-R5 [29]. The D-form of the peptide showed the same effect on TJ opening [30] and would be more suitable for *in vivo* experiments as it would be metabolized more slowly [31]. A combination of the peptide with an insulin formulation could allow for increased permeation of the therapeutic protein through the nasal epithelium and achieve sufficient blood insulin concentration to treat diabetes and reduce blood glucose levels.

This study demonstrates the efficacy of the formulation of a rapid insulin with the L-R5 peptide. The increase in insulin absorption *in vitro* is significant. However, further studies are needed for the development of an optimal formulation.

2. Materials and methods

2.1. Materials

Sodium chloride (NaCl 0.9% + $CaCl_2$ 1.25mM + HEPES 10 mM) solution, human recombinant insulin, chlorohydric acid, sodium hydroxide and FITC-insulin were obtained from Sigma-Aldrich (Buchs, Switzerland). Water for injection (WFI) was purchased from Corning (Manassas, VA, USA). Hank's balanced salt solution (HBSS) and phosphate buffered solution (PBS) were obtained from Gibco (Zug, Switzerland). The pentapeptide D-R5 (myristoyl-ARRWR-OH) was obtained from Bachem AG (Bubendorf, Switzerland). Primary human nasal epithelial cells MucilAir™ and MucilAir™ cell culture medium were purchased from Epithelix (Plan-les-Ouates, Switzerland). The Accu-check glucometer was obtained from Roche diagnostics (Rotkreuz, Switzerland). All other reagents such as Apidra® insulin injectable solution were of analytical grade unless otherwise stated and obtained from commercial sources.

2.2. Cell culture

As previously described [29] primary human nasal epithelial cells MucilAir™ provided in 24-well plates with 6.5 mm inserts equipped with 0.4 µm pore size polycarbonate membrane inserts with a surface are of 0.33 cm^2 were used throughout the study. The inserts were maintained at 37°C and under an atmosphere containing 5% CO_2. MucilAir™ cell culture medium was utilized and changed every 2-3 days. Based on the manufacturer's recommendations, a few hours before the experiments the cells were incubated with 100 µl saline solution (NaCl 0.9% + $CaCl_2$ 1.25mM + HEPES 10 mM) at the apical compartment for 20 minutes, aspirated to remove the mucus, and then rinsed once with warm phosphate buffered saline (PBS). Before the start of permeability experiments 100 µl Hank's balanced salt solution (HBSS) was added to the apical compartment and 600 µL to the basolateral compartment and allowed to equilibrate for 30 minutes in the incubator.

2.3. Circular dichroism (CD) spectroscopy measurements

The secondary structure of the human recombinant insulin was analysed by CD spectroscopy. These measurements were performed using a JASCO J-815 spectropolarimeter (Jasco, Hiroshima, Japan) with a 180-700 nm photomultiplier (exel-308). CD spectra were recorded from 260 to 190 nm. Solutions were stored in 0.1 cm cuvettes (Quartzglas Suprasil®, Thermofisher scientific, Waltham, MA, United States) during the measurement. The temperature was controlled at 22°C. Each spectrum is the results of 3 accumulations recorded in degrees. The data interval was 0.5 nm as well as the data pitch, the bandwidth was 2.00 nm, the scanning speed was 20 nm/min and the response 1 second. The solutions were prepared in purified water. Insulin solutions were prepared at a concentration of 100 µM. The pH was adjusted at 2, 4.5, 7 and 12 with HCl and NaOH 1 M. The peptide was diluted at 50 µM.

The CD signal values obtained at 210 nm were used to calculate the amount in α-helix (%) of human recombinant insulin [32]. The mean residue ellipticity (MRE) was calculated using equation 1:

$$MRE = \frac{Ellipticity\ (mdeg)}{C_p \times N \times l} \quad \text{(Eq.1)}$$

with C_p the concentration of insulin (µM), N the number of amino acids (51 for this human recombinant insulin) and l the length of the cuvette (1 mm). Then the ratio of α-helix was calculated using equation 2 [32]:

$$\alpha - helix\ (\%) = -\frac{MRE_{210nm} - 4000}{33000 - 4000} \times 100 \quad \text{(Eq.2)}$$

2.4. *In vitro* permeability studies

As previously described [29], the apparent permeability (P_{app}) of FITC-insulin was calculated using equation 3:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0} \quad \text{(Eq. 3)}$$

where C_0 is the initial concentration (ng/ml) of the permeant in the donor compartment (apical side), A (cm^2) is the surface area of the cell layers (0.33 cm^2 for 24 well plate inserts) and dQ/dt is the appearance rate of FITC-insulin in the receiver compartment. C_0 did not change significantly over the time of study. All experiments were performed in triplicate.

The peptide D-R5 was applied to the apical side in 0.15 mM FITC-insulin solution at a concentration of 50 µM in HBSS. Samples of 100 µl were taken from the basolateral compartment of each well every 30 minutes over a period of 180 minutes, with each volume being replaced by an equal amount of fresh warm buffer to maintain sink conditions. The fluorescence of FITC-insulin was measured in black 96-well plates using a fluorescence plate reader (BioTek Synergy Mx plate reader, BioTek Instruments GmbH, Lucerne, Switzerland), using excitation and emission wavelengths of 485 and 520 nm, respectively.

2.5. Generation of β-cell-specific *Phb2* knockout mice

The ß-cell specific prohibitin-2 knockout mouse (β-$Phb2^{-/-}$) is a model of monogenic type 2 diabetes with progressive decline of ß-cell function and survival [33]. As previously described [34] all animal experiments were conducted at the University of Geneva Medical Centre with the approval of the animal care and experimentation authorities of the Canton of Geneva (GE/4/20). Mice were maintained on a 12-h dark/12-h light cycle with water and food *ad libitum* and genotyped using primers [33]. Since the phenotype of β-$Phb2^{-/-}$ mice is similar between males and females [33], only females were used for experiments; the age of the mice was between 4 and 5 weeks. All β-$Phb2^{-/-}$ and $Phb2^{fl/fl}$ mice were generated as previously described [33] and maintained on a mixed genetic background to avoid inbred strain specific phenotypes. BKS.Cg-Dock7m$^{+/+}$Lepr^{db}/J mice (*db/db* and heterozygous db/+ mice as controls) were purchased from Charles River Laboratories Italia (Calco, Italy).

2.6. *In vivo* glycemia studies

Mice were fasted 6 hours before the beginning of the experiments. The glycemia was measured before the beginning of the experiments by collecting blood from the tail vein. The two formulations were both composed of Apidra® insulin at a concentration of 1 U/kg (for mice weighing 25 g) diluted in saline solution. One of the formulations was also composed of D-R5

at a concentration of 100 µM. 20 µl of the formulations was applied in mice nostrils (10 µl in each nostril). The glycemia was then measured each 15 minutes during 1 hour and then each 30 minutes for 1 hour and a final measurement after a total of 3 hours.

2.7. Statistical analysis

Data are reported as mean ± standard deviation (S.D.). P<0.05 was considered as statistically significant. Significance is denoted as **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. The data were analysed using multiple t-test comparisons. The data were analysed using one-way analysis of variance (ANOVA).

3. Results

3.1. L-R5 influence on insulin secondary structure

Circular dichroism (CD) was used to determine the secondary structure of the human recombinant insulin and its behaviour under different pH conditions. Figure 1 shows the CD spectra of human recombinant insulin at different pH values. A common spectrum is revealed with a major minimum and a minor minimum at 210 and 222 nm, respectively. Moreover, a strong peak is seen at 195 nm (figure 1A-1C), which disappeared at pH=12.

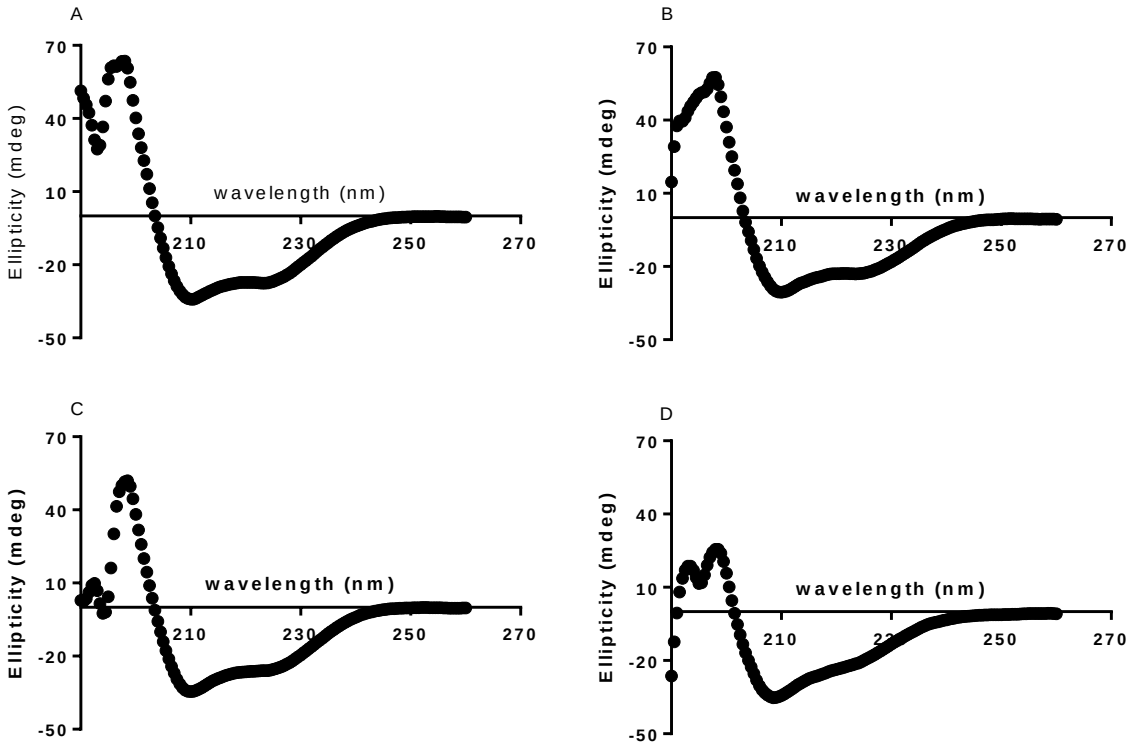

Figure 1: Circular dichroism (CD) spectra of human recombinant insulin 100 µM in water at pH=2 (A), pH=4.5 (B), pH=7 (C) and pH=12 (D) in the far UV range.

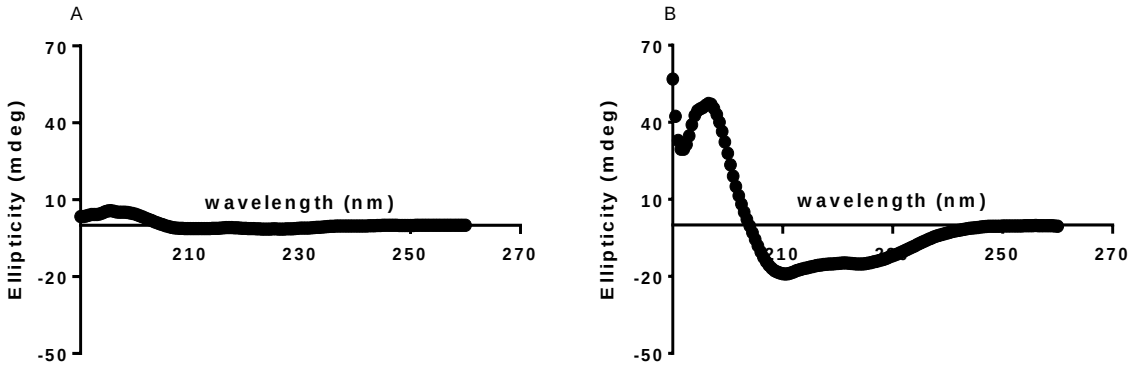

Figure 2: Circular dichroism (CD) spectra of L-R5 50 µM in purified water at pH=4.5 (A) and human recombinant insulin 100 µM with L-R5 50 µM in water at pH=4.5 (B) in the far UV range.

CD measurements were performed on the peptide L-R5 alone and on the mix of L-R5 and human recombinant insulin at pH=4.5 (figure 2). as expected, almost no ellipticity was detected for the L-R5 solution (figure 2A). On the other hand, a similar elliptic profile was shown for the mix (figure 2B) as well as for insulin alone. However, the intensity of the peaks at 210 and 222 nm is comparatively reduced.

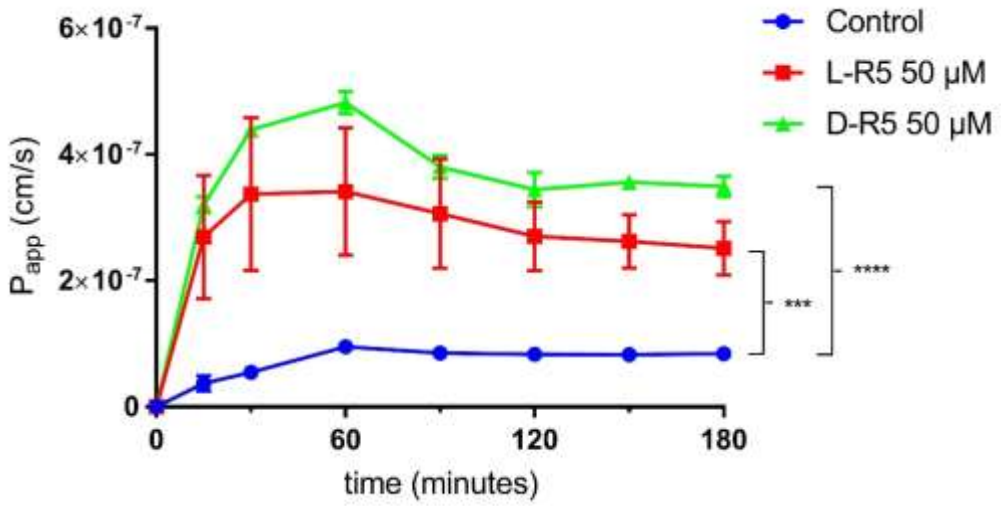

Figure 3: The permeability of FITC-insulin (0.15 mM solution) through MucilAir™ epithelial cell layers alone or in the presence of L-R5 and D-R5 (both at a concentration of 50 µM) was investigated over 180 minutes. Values are mean ± S.D. (n=3), ***P < 0.001, ****P < 0.0001. The data were analysed using multiple t-test comparisons.

The ratio of α-helix of insulin was calculated for each condition (table 1). The composition in α-helix is not affected by the pH except for a slight change at pH=4.5. On the other hand, the ratio of α-helix was decreased upon the addition of L-R5.

3.2. Insulin permeability *in vitro*

Table 1: Mean residue ellipticity (MRE) and α-helix ratio at 210 nm of insulin at different pH values.

pH of the solution	MRE (deg cm^2 $dmol^{-1}$)	α-helix (%)
2	-6703.5	36.9
4.5	-5981.1	34.4
7	-6777.9	37.1
12	-6736.8	37.0
4.5 + L-R5 50 µM	-3715.9	26.6

The ability of L-R5 and D-R5 to enhance FITC-insulin permeability through MucilAir™ epithelial cell layers was measured. As shown in figure 3, a significantly increased passage of FITC-insulin was noted in the presence of the two peptides compared to the control condition. A more important permeation enhancement was observed in the presence of D-R5 than for L-R5.

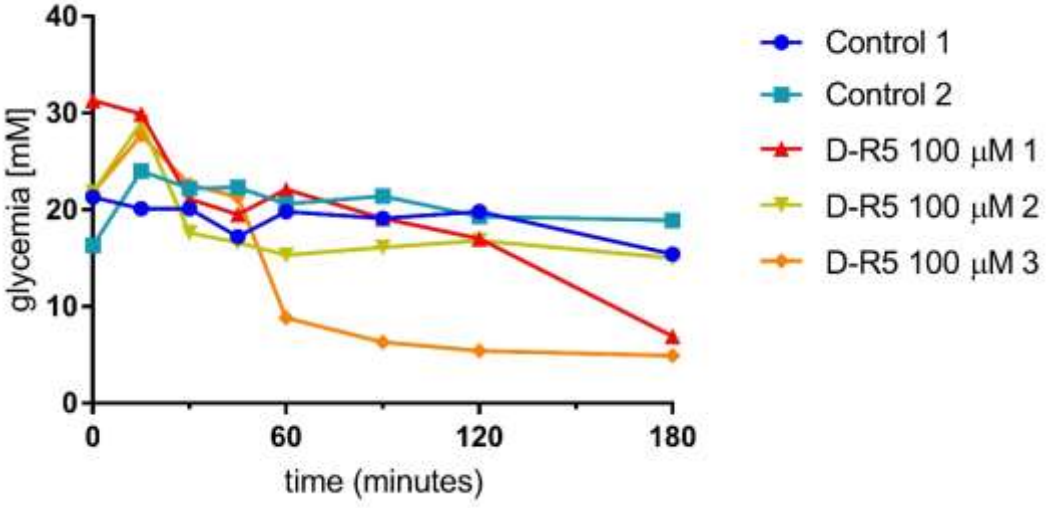

Figure 4: A solution of Apidra® insulin (1U/kg) was nasally administered to 5 diabetic mice. D-R5 100 µM was added in the formulation for 3 of the mice. Glycemia was then measured at certain time points over 180 minutes.

3.3. *In vivo* administration of insulin in combination with D-R5

A formulation of Apidra® insulin and of the insulin mixed with D-R5 100 µM was intranasally administered to mice (1U/kg), respectively, and glycemia measured over time (figure 4). A decrease in glycemia was apparent in the presence of the peptide. Glycemia of control mice was not affected by the administration of insulin alone. The glycemia of one of the three mice receiving D-R5 in combination with insulin was only slightly decreased compared to the two other mice receiving the same formulation. Finally, the glycemia profile is different for the two most efficient measurements (mouse 1 and 3 receiving the complete formulation). Glycemia in these animals did not decrease at the same speed.

4. Discussion

In vitro tests were conducted to determine the plausibility of a nasal formulation combining insulin with a tight junction opening peptide (L-R5 or D-R5). Potential modification in the secondary structure of human recombinant insulin was examined at different pH values. It was previously described that insulin is mainly composed of an α-helix [35], which is essential for its pharmacological activity. It has previously been shown [36] that the solution pH influences the overall charge of insulin and therefore its solubility. A pH close to neutrality causes aggregation of the protein. The CD spectra show no difference in the secondary structure of the recombinant human insulin tested at different pH values (figure 1). The α-helix ratio remains the same at acidic, neutral and basic pH. The pH=4.5 was tested as a limiting pH. Indeed, the pH of a nasal formulation should be between 4.5-6.5 to avoid irreversible damage to the nasal mucosa [37]. In order to avoid aggregation in the nasal formulation of peptide and insulin, pH=4.5 was chosen instead of a more neutral pH.

To examine the influence of L-R5 on the secondary structure of recombinant human insulin, the CD spectrum of L-R5 alone was established at pH=4.5 (figure 2A). Due to the short sequence of the pentapeptide, no secondary structure was observed, and no noisy changes in the CD spectrum of insulin were caused by the intrinsic spectrum of L-R5.

The global shape of the CD spectrum of recombinant human insulin (figure 1B) does not change in the presence of L-R5 (figure 2B). The overall structure is therefore preserved. In contrast, the intensity of the elliptical low points is reduced. In addition, the α-helix content is also reduced (table 1). A modification or interaction with α-helices can be supposed in the presence of L-R5. Given the affinity of L-R5 for other proteins (PKC ζ target proteins) [38], an interaction is possible with insulin, especially since insulin is a charged molecule. It is sufficient that negative charges are present in the α-helix to obtain an interaction [39]. Despite this

probable interference, the spectral profile of insulin remains very similar to the one of the protein alone in pH=4.5 solution.

The association of L-R5 and D-R5 with insulin was then performed by quantifying the permeability of FITC-insulin (figure 3). Both peptides significantly increased the passage of the protein through MucilAir™ cell layers. A 3- to 5-fold increase in P_{app} was observed in the presence of either peptide. Opening of TJs by these peptides and enhancement of permeability of a fluorescent marker had already been shown [29]. However, the increase in the permeability of a therapeutic protein is only now assured. Furthermore, the D-R5 peptide seems to be more effective possibly due to a decreased intracellular metabolism of the D-form peptide [31]. In order to ensure the pharmacodynamics of the peptide, the use of D-R5 will therefore be more appropriate for *in vivo* experiments.

Apidra® (insulin glulisine) is effective in the treatment of diabetes [40,41]. However, its administration is invasive. Nasal administration of this insulin has been tested, and its mucosal biocompatibility has been proven [42]. However, glycemia levels were not affected upon nasal application. The combination of insulin with an absorption promoter was examined [43], and conclusive and promising results in terms of lowering blood glucose levels were observed. In the current study, preliminary results were promising and indicated the potential for an effective combination of insulin and D-R5 (figure 4). Unfortunately, further experiments did not show repeatable results, with the difficulty of nasal administration in conscious mice probably being a decisive factor for the variability of the data. Furthermore, it cannot be excluded that an interaction between the peptide and insulin is present. More stability studies are needed to obtain a functional formulation. Finally, it is also possible that the dose of D-R5 peptide is not sufficient *in vivo* to open TJs sufficiently wide and quickly.

5. Conclusion

Nasal delivery of injectable drugs is one of the most promising alternatives to parenteral application. Diabetes is a recurrent disease worldwide that requires patients to apply daily injections of insulin. Nasal absorption of insulin is poor and requires an effective absorption promoter to achieve sufficient blood concentrations. L-R5 has been shown to be effective in opening TJs resulting in permeation enhancement, with both L- and D-forms of L-R5 increasing insulin permeability *in vitro*. Preliminary conclusive results were observed by *in vivo* nasal application of this formulation. However, the repeatability of the results was not achieved. Future studies will be conducted to obtain an effective formulation.

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General conclusions and perspectives

A wide variety of tests have been performed to characterise the L-R5 pentapeptide. The effectiveness of the peptide in transiently and reversibly increasing the permeability of TJs has been repeatedly demonstrated. Furthermore, by developing a UHPLC-MS/MS method for quantifying L-R5, the localisation of the peptide in the apical and intracellular space could be demonstrated. Despite a haemolytic effect due to the hydrocarbon chain, the insufficient passage of the peptide through the nasal cell layer ensures systemic safety. Acute cellular toxicity has been ruled out, but in the case of a repeated administration, further investigations of long-term toxicity are required once a stable and effective formulation is established. The combination of L-R5 with naloxone provided a first pilot formulation to study the peptide's activity in the presence of another chemical molecule. Formulating the pentapeptide with other therapeutic molecules are further tests to be conducted.

L-R5 was originally synthesised based on the sequence of the PKC ζ PS. The peptide was therefore intended to interact with the enzyme to keep it in an inactive form. However, extensive studies showed that the activity of the enzyme was actually inhibited by L-R5 interacting with target proteins of PKC ζ. As an example, L-R5 competes with the enzyme by binding electrostatically with occludin. This competition is one of the mechanisms causing the opening of TJs. In addition, a decrease in expression of TJ proteins occludin and ZO-1 was observed in the presence of L-R5. TJs are thus affected by different mechanisms. A better understanding of the whole intracellular influence of the peptide is needed, especially since the impact of L-R5 is not restricted to TJs proteins. Many other intracellular mechanisms are affected by the activity of the pentapeptide. This information therefore requires a thorough introspection of the field of action of L-R5. New possibilities for the use of the peptide may emerge. Based on the large number of targets of the peptide, other pathways could be of therapeutic interest. Structural optimisation of the peptide to increase affinity and selectivity for a specific target would avoid or reduce its influence on other mechanisms. Further proteomic analysis would provide useful information. Coupling with metabolomics studies would surely reveal these crucial data.

The comparison of L-R5 with other PEs in studies and on the market attested to the potential competition of the peptide. In addition, structural optimisation of L-R5 was explored in order to possibly achieve a higher effect, better affinity and lower haemolytic toxicity. The attempt to avoid haemolysis due to the myristoyl extremity by modifying it failed. Any fatty acid causes red cells disruption and this lipophilic part is necessary for the peptide to enter the cell and exert its activity. Once its action is complete, the metabolism of the peptide is not known. It is assumed that L-R5 is recycled by the cell like any other L-peptide. Further investigations of

the metabolism and elimination of L-R5 would ensure its safe use. Furthermore, the opening of TJs by L-R5 seems to be limited to 150 kDa molecules. Further testing of the correlation between the administered peptide dose and extent of TJ opening would be desirable. A formulation with nanoparticles or micelles may then be considered if the opening is sufficient.

Some amino acids were found to be essential for the action of the peptide, such as tryptophan or the positive charges of arginines. A complete modification of the molecule could be envisaged. For this, the lipophilicity as well as the intrinsic charges of the molecule must be preserved or at least replaced by an equivalent. The creation of a non-peptide chemical molecule that simulates the same interactions as L-R5 would bring significant advantages, such as slower metabolism, better affinity and easier optimisation.

The combination of L-R5 with insulin was formulated. First of all, only a minimal interaction was found with the secondary structure of the protein. These premises are not sufficient to ensure the absence of interference. Physical interaction tests, as well as activity tests of the two compounds, need to be carried out in order to guarantee the effectiveness of the formulation. L-R5 has nevertheless demonstrated its ability to increase the permeability of labelled insulin. Although the activity of the therapeutic protein was not certainly conserved, the enhancement of its passage to the basolateral side of the cell layer is promising. Combination with larger therapeutic molecules would be a further step in the future to explore the different possibilities of formulations with L-R5. Finally, the nasal formulation of insulin glulisine associated with the pentapeptide was tested in mice. This pilot project did not give significant or repeatable results. However, with an optimisation of the formulation and delivery device, repeatable values could be achieved. The results would therefore be more reliable. Other combinations with L-R5 are in consideration, including a formulation with an antibody that would be administered nasally, or an ocular formulation. Once again, stability and efficacy tests will have to be carried out.

This PhD thesis did not establish an effective nasal formulation *in vivo*. However, the technical, mechanistic and structural characteristics of L-R5 have been duly investigated and now make it possible to foresee a nasal or ocular formulation containing the peptide. All these results have provided a better overview of the routes to formulation and efficacy.

Glossary

ACN.....Acetonitrile

AJ…..……..……Adherens junction

ATP……Adenosine triphosphate

BBB……Blood-brain barrier

BCS..……..……Biopharmaceutics classification system

β-PHB2……..…….β-cell specific prohibitin 2

BTB……..……Blood-testis barrier

cAMP……..……Cyclic adenosine monophosphate

CBF……..……Ciliary beating frequency

CD……Circular dichroism

CDC 42…….. cell division control protein 42 homolog

CMC……..…..Critical micellar concentration

COPD……..……Chronic obstructive pulmonary disease

CPE……..……..……*Clostridium perfringens* enterotoxin

CPP……..……Cell-penetrating peptide

DAG……...Diacylglycerol

DCM……..……Dichloromethane

DMF…….……Dimethylformamide

DSG……..……..Desmoglein

EDTA……Ethylenediaminetetraacetate

EHEC……..…Enterohemorragic *Escherichia coli*

EMT……..……Epithelial to mesenchymal transition

FA……..……Fatty acid

FC……..Fold change

FD……Fluorescein dextran

FDR……..False discovery rate

FITC……..…..Fluorescein isothiocyanate

ICH……..….International council for harmonisation

IP……..Immunoprecipitation

IP3……Inositol triphosphate

IRS1……..Insulin receptor substrate 1

JAM……Junctional adhesion molecule

JO-1……Junction opener

K_d……Dissociation constant

LDH……..Lactate deshhydrogenase

LOD……Limit of detection

LOQ……Limit of quantification

MAGUK……Membrane-associated guanylate kinase

MARVEL……MAL and related proteins for vesicle trafficking and membrane link

MB……...Mechanism-based

MCFA……Medium chain fatty acid

MLC……Myosin light chain

MLCK……Myosin light chain kinase

MLCP……Myosin light chain phosphatase

MMLDL……Minimally modified LDL

MODY……Maturity onset diabetes of the young

MRE……Mean residue ellipticity

mRNA……...Messenger RNA

MST……..Microscale thermophoresis

PAMR……Perijunctional actomyosin ring

P_{app}……..Apparent permeability

PARD3……Partitioning defective 3 homolog

PB1……..Phox and Bem1

PCA……Principal component analysis

PDK1……..Pyruvate dehydrogenase lipoamide kinase isozyme 1

PE……Permeation enhancer

PEDV……Porcine epidemic diarrhea virus

PIP2……Phosphatidylinositol bisphosphonate

PI3K……..Phosphoinositide 3-kinase

PKC……Protein kinase C

PLC……..……Phospholipase C

PP2A……Protein phosphatase 2

PS……Pseudosubstrate

PTD……..……Protein transduction domain

RACK……Receptor for activated C kinase

RICH1……Rho GTPase-activating protein 17

ROCK……..….Rho-associated protein kinase

ROS……..……Reactive oxygen species

SD……..…….Standard deviation

SDS……....Sodium dodecylsulfate

SMEDDS……Self-microemulsifying drug delivery system

SPG……..……Sphenopalatine ganglion

TEER……..……Transepithelial electrical resistance

TFA……Trifluoroacetic acid

TJ……..……Tight junction

TJM……..……..……Tight junction modulator

TLR……..……..……Toll-like receptor

UHPLC……..…….Ultra high performance liquid chromatography

VEGF……Vascular endothelial growth factor

WFI……..Water for injection

WHO……..……World health organisation

ZCP……..Zeta competitory peptide

ZIP……..…PKC zeta pseudosubstrate-derived zeta-inhibitory peptide

ZO……..……..……Zonula occludens

Zot……..……..……Zonula occludens toxin