Revealing the importance of carrier-cargo association in delivery of insulin and lipidated insulin

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1. Introduction

Therapeutic peptides and proteins are highly specific [1] and hence promising drug candidates with various pharmacological actions similar to that of endogenous proteins [2]. For replacement therapy, insulin is administered by injection, and oral administration will improve patient convenience. However, large hydrophilic macromolecules, such as insulin, display poor permeation across the intestinal mucosa resulting in low bioavailability [1,3]. To enhance the transepithelial permeation of insulin and other therapeutic peptides, cell-penetrating peptides (CPPs) have demonstrated some potential as delivery enhancing agents [4–6], yet a comprehensive understanding of the mechanism of action comprising effect of intermolecular interplay and membrane interactions needs further exploration. The cationic and amphipathic CPP penetratin was previously shown to deliver macromolecular cargos into cells as well as across biological barriers, such as the intestinal mucosa [6–8]. The penetratin analogues shuffle and penetramax were shown to increase plasma insulin following in situ administration in rats to a
Higher extent when compared to penetratin [9]. Shuffle was designed by rearrangement of the amino acid sequence in penetratin constraining the position of the basic amino acids (Fig. 1a) [10]. Subsequently, penetramax was created by exchanging the terminal arginine and lysine in the sequence of shuffle [9]. Penetratin, shuffle and penetramax all belong to the group of cationic CPPs being rich in arginine and lysine residues [4], which facilitate electrostatic interactions with the negatively charged cell membrane [11]. In addition, the hydrophobic amino acid tryptophan promotes interaction with the lipid bilayer [12]. To further promote hydrophobic interactions with the cell membrane and their delivery potential, lipidation of CPPs has been pursued. Acylation of octaarginines has been utilized for enhancing cellular uptake of small molecules and proteins [13] and stearylation of transportan 10 investigated for improved oligonucleotide transfection [14,15]. We recently demonstrated that lipidated penetratin interacts differently with bilayer membranes when compared to the non-lipidated counterpart [16]. Similarly, lipidation of therapeutic peptides and proteins may be applied to facilitate their transepithelial delivery, as earlier demonstrated for lipidated glucagon-like peptide-2 (GLP-2) and lipidated calcitonin [17,18]. Lipidation of insulin is also applied, but rather to improve albumin binding thereby prolonging the insulin plasma half-life upon injection as is the case for the insulin analogue insulin detemir, the active pharmaceutical ingredient in Levemir® [19]. Insulin detemir was designed by elimination of the C-terminal threonine and addition of a C14-lipid chain to the lysine side chain in the B-chain (Fig. 1b) [19]. Addition of a lipid moiety has previously been demonstrated to enhance intestinal insulin absorption [20,21].

However, the mechanistic understanding of how carrier-mediated permeation of a therapeutic cargo occurs via the paracellular route remains to be elucidated. In the present study, we hypothesize that the ability of selected peptides with demonstrated cell-penetrating properties may function as carrier peptides to interact with a cargo and the cell membrane, which will influence their potential to facilitate transmucosal delivery. Thus, the term carrier defines the property of these excipient peptides to aid delivery of the therapeutic peptide and thus, does not describe uptake through membrane carriers. We anticipate that elucidating this will provide a novel understanding of the mode of action of carrier peptides as transepithelial permeation enhancers. In this context, the impact of cargo lipidation in enhancing hydrophobic interactions with the carrier peptides and cell membranes, and hereby transepithelial permeation, was investigated. Penetratin, shuffle and penetramax were employed as carrier peptides; insulin and lipidated insulin as relevant therapeutic peptide cargos. Interactions between insulin or lipidated insulin with penetratin, shuffle or penetramax were investigated using orthogonal biophysical methods. The carrier peptide-mediated membrane binding, cell uptake and transepithelial permeation of insulin and lipidated insulin were studied in vitro employing the human intestinal Caco-2 cell culture model. Additionally, for insulin pharmacokinetic and pharmacodynamic (PK/PD) studies were performed in rats and complemented with histological analysis of the intestinal tissue.

2. Materials and methods

2.1. Materials

Human recombinant insulin (molecular weight (Mw): 5808 g/mol) and Insuman® Rapid were kindly provided by Sanofi (Frankfurt, Germany). Insulin detemir (14.2 mg/mL, Mw: 5917 g/mol) was isolated from the marketed product Levemir® (Novo Nordisk, Bagsvaerd, Denmark). Penetratin, shuffle and penetramax (Mw: 2247 g/mol, purity: > 95%) were obtained from Synpeptide (Shanghai, China). The 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-(1-glycerol)] (sodium salt) (POPG) were from Avanti Polar Lipids (Alabaster, AL, USA). 2-Morpholineethanesulfonic acid (MES) and 4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (HEPES) were purchased from PanReac AppliChem (Darmstadt, Germany). Hank’s balanced salt solution (HBSS), sodium phosphate and disodium hydrogen phosphate were obtained from Merck (Kenilworth, NJ, USA). Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin, 1-glutamine, non-essential amino acid solution and trypsin-EDTA were purchased from Merck. Fetal bovine serum (FBS) was obtained from GE Healthcare (Cardiff, UK). Fluorescein isothiocyanate (FITC)-labeled dextran (average Mw: 4000 g/mol) was purchased from Merck. (4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Phenazine methosulfate (PMS), bovine serum albumin (BSA) and Triton® X-100 were obtained from Merck. Phosphate-buffered saline was from VWR (Radnor, PA, USA). 16% (w/v) paraformaldehyde and zonula occludens-1 (ZO-1) monoclonal antibody conjugated with Alexa Fluor 488 were purchased from Thermo Fisher Scientific (Walther, MA, USA). Tissue-Tek® Tissue-Clear® Xylene Substitute was obtained from Sakura Finetek (Alphen aan den Rijn, Netherlands). Claudin-3 primary antibody was from Abcam (Cambridge, UK) and biontinylated goat anti-rabbit immunoglobulin secondary antibody was from Vector Laboratories ( Burlingame, CA, USA). Periodic acid solution and Schiff’s reagent were purchased from Merck. Mayer’s acid hema-toxylin was obtained from Region Hovedstadens Apotek (Herlev, Denmark).

![Fig. 1. (a) Amino acid sequences of penetratin and its analogues shuffle and penetramax. Blue: arginine and lysine (R and K, respectively). Orange: tryptophan (W). Underlined: rearranged amino acids. (b) Sequences of insulin with disulfide bonds and its lipidated analogue. Blue: lysine (K). Red: threonine (T). Orange: C14-lipid chain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image_url)
2.2. Insulin and lipidated insulin solutions

Stock solutions of 5 mg/mL insulin in 10 mM HCl in ultrapure water purified by a PURELAB flex (ELGA, High Wycombe, UK) were prepared in NoStick® Hydrophobic Microtubes (Scientific Specialties, Lodi, CA, USA). For preparation of lipidated insulin, LeveMir® samples containing 14.2 mg/mL insulin detemir were dialyzed against 20 mM HCl in ultrapure water in a 1:200 sample-to-dialysis buffer volume ratio using a 3.5K molecular cut-off value (MWCO) Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific). The dialysis was performed twice at room temperature for 2 h and finally overnight at 4 °C with new dialysis buffer. The solution was diluted to 5 mg/mL lipidated insulin in ultrapure water and both stock solutions of insulin and lipidated insulin were filtered through a 0.2 μm LABSOLUTE® syringe filter (Th. Geyer, Renningen, Germany). The absorbance measured at 280 nm using a Nanodrop 2000c (Thermo Fisher Scientific) was used for concentration determination prior storage at −80 °C. The concentration of lipidated insulin was verified before and after the dialysis using reverse phase high performance liquid chromatography (RP-HPLC) as described below for Ultracentrifugation.

2.3. Dynamic light scattering

The size distribution of complexes between the carrier peptides and insulin or lipidated insulin was investigated by dynamic light scattering (DLS) using a Zetasizer Nano ZSP (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser. Solutions of 15 μM insulin or lipidated insulin alone or in physical mixture with 60 μM penetratin, shuffle or penetramax in MES-HBSS (10 mM MES in HBSS adjusted to pH 6.5 and filtered through a 0.2 μm syringe filter) were measured in disposable cuvettes with a 173° angle of detection. Data was analyzed using Zetasizer Software 7.12 (Malvern Instruments). The hydrodynamic radii were derived by intensity distribution with indication of the relative volume composition. Peaks with a relative volume distribution equal to or less than 5% were excluded.

2.4. Ultracentrifugation

The recovery of the carrier peptides and insulin or lipidated insulin was investigated following ultracentrifugation using an OptimaMAX-XP Ultracentrifuge equipped with a TLA-110 Fixed-Angle Rotor (Beckman Coulter, Brea, CA, USA). Samples containing 15 μM insulin or lipidated insulin alone or in physical mixture with 60 μM penetratin, shuffle or penetramax in MES-HBSS were centrifuged (135,700 g, 25 °C) for 60 min. The supernatant was used for quantification of unassociated insulin, lipidated insulin and carriers by RP-HPLC using a Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with an Aeris PEP-TIDE XB-C18 column (3.6 μm, 100 Å, 100 × 2.1 mm; Torrance, CA, USA) at 40 °C. The mobile phase A consisted of 95% (v/v) ultrapure water containing 0.1% (v/v) TFA mixed with 5% (v/v) acetonitrile and the mobile phase B consisted of 95% (v/v) acetonitrile mixed with 5% (v/v) water containing 0.1% (v/v) TFA. A gradient elution from 20% to 70% mobile phase B over 10 min was used with a flow rate of 0.8 mL/min and an injection volume of 10 μL with duplicate injection. For lipidated insulin, an injection volume of 50 μL was used to obtain sufficient detection. The absorbance was measured using a PDA-detector at 218 nm. The limit of detection (LOD) was determined to be ≤0.9 μM for the carrier peptides and 0.1 μM for both insulin and lipidated insulin.

2.5. Liposome dispersions

Liposomes containing 80:20% (mol/mol) POPC:POPG were prepared by extrusion [22]. Briefly, stock solutions of POPC and POPG in chloroform were mixed in a total lipid concentration of 25 mM and the mixture was dried using a rotatory evaporator for 2 h. The lipid film was washed in absolute ethanol and dried twice for 1 h and finally overnight in order to remove chloroform. The lipid film was rehydrated in MES or phosphate buffer (10 mM MES or phosphate, respectively, in ultrapure water adjusted to pH 6.5 and filtered through a 0.2 μm syringe filter). The lipids were sonicated for 5 min to remove aggregates, vortexed for 1 min every 10 min over 1 h and annealed for 1 h. The liposomes were extruded twice through double layers of 200 nm Nuclepore™ membrane filters (Whatman, Florham Park, NJ, USA) and eight times through a double layer of 100 nm filters (Whatman) to obtain monodisperse liposome dispersions. The liposome size and dispersity were verified by DLS and the lipid content was quantified by a phospholipid kit (MTI Diagnostics, Idstein, Germany) as described by the manufacturer.

2.6. Circular dichroism

The secondary structure of the carrier peptides in mixture with insulin or lipidated insulin in the absence or presence of liposomes was investigated by circular dichroism (CD) using a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK). Samples of 15 μM insulin or lipidated insulin alone or in physical mixture with 60 μM penetratin, shuffle or penetramax with or without liposomes consisting of 1.7 mM 80:20% (mol/mol) POPC:POPG lipids in phosphate buffer were measured in 1 mm quartz cuvette (Hellma Analytics, Müllheim, Germany) for detection in the far UV range at 190–260 nm in 0.5 nm steps. A time-per-point of 1 s and 3 scans were used. Phosphate buffer was used to minimize absorbance below 200 nm. Data was corrected for background, converted to mean residue ellipticity and normalized to baseline. The mean residue ellipticity (MRE) was calculated by Eq. (1).

$$\text{MRE} = (\theta_0) / (1 \times N \times C),$$

where θ is the ellipticity, l is the pathlength, N is the number of residues and C is the concentration. The relative secondary structure content was estimated using an algorithm provided by BeStSel [23].

2.7. Isothermal titration calorimetry

The binding strength between the carrier peptides and liposomes dispersed in MES buffer was investigated by isothermal titration calorimetry (ITC) using a low volume Nano ITC (TA Instruments, New Castle, DE, USA). The liposome dispersion was dialyzed against MES buffer in a 1:200 sample-to-dialysis buffer volume ratio using a 3.5K MWCO Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific). The samples were degassed in order to remove air bubbles. One injection of 0.48 μL and 26 injections of 1.96 μL of liposomes consisting of 34 mM 80:20% (mol/mol) POPC:POPG lipids were titrated into the 60 μM penetratin, shuffle or penetramax diluted in dialysate every 300 s. A stirring rate of 300 rpm and a temperature of 37 °C were used. Titration of liposomes into the dialysate was used to correct for heat of dilution. Data was analyzed using NanoAnalyze software (TA Instruments) applying a one-site fitting model.

2.8. Cell cultivation

Caco-2 cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained in cell culture flasks (Merck) in DMEM supplemented with 90 IU/mL penicillin, 90 μg/mL streptomycin, 2 mM l-glutamine, 0.1 mM non-essential amino acid solution and 10% (v/v) FBS. The cells were grown in 5% CO₂ at 37 °C and detached from the culture flask at approximately 80% confluency with trypsin-EDTA every 7 d. For the in vitro transepithelial permeation study, cell viability assay and immunostaining, 1 × 10³ cells were seeded on each Transwell® filter insert (pore size: 0.4 μm, diameter: 12 mm, area: 1.13 cm², Corning, NY, USA) and grown for 21 d prior experimental use. For the RealTime-Glo™ MT Cell Viability Assay, 6 × 10³ cells were seeded into each well of a white clear bottom 96-well plate (Corning) 24 h prior the experiment. Passages between number 3 and 17 subsequent to thawing were used.
2.9. Transepithelial permeation, membrane binding and cell uptake study

The transepithelial insulin or lipidated insulin permeation was evaluated using the Caco-2 cell culture model [24]. Caco-2 cell monolayers were washed twice with 0.5 mL and 1.0 mL 37 °C HEPES-HBSS (10 mM HEPES in HBSS adjusted to pH 7.4) on the apical and basolateral side, respectively. The cells were equilibrated to room temperature for 20 min and the monolayer integrity was assessed by measurement of the transepithelial electrical resistance (TEER) in an EndOhm chamber (diameter: 12 mm; World Precision Instruments, Sarasota, FL, USA) equipped with an epithelial voltohmeter (EVOM). The cells were equilibrated to 37 °C for 20 min. The buffer was removed and 370 μL sample containing 15 μM insulin or lipidated insulin and 60 μM penetratin, shuffe or penetramax in MES-HBSS was added to the apical compartment. In addition to insulin and lipidated insulin, the transepithelial permeation of 1 mg/mL FITC-dextran (average Mr: 4,000 g/mol), 0.1 μCi/mL 14C-mannitol and 0.5 μCi/mL 3H-metoprolol was evaluated in the presence of the carrier peptides. Donor samples of 20 μL were collected from the apical compartment at time point 0 min. The filter inserts were transferred to wells pre-filled with 1 mL 37 °C HEPES-HBSS and the cells were incubated on a shaking table (50 rpm, 37 °C) for 3 h. Samples of 100 μL were collected from the basolateral compartment at time points 60, 120 and 180 min for quantification of permeated insulin or lipidated insulin and at time points 15, 30, 45, 60, 90, 120, 150 and 180 min for quantification of permeated dextran, mannitol or metoprolol. Replenishment with 100 μL 37 °C HEPES-HBSS was done to the basolateral side after each sampling. Following the incubation, the cells were washed twice with 37 °C HEPES-HBSS and equilibrated to room temperature for 20 min prior to TEER measurements.

The membrane binding and cell uptake of insulin and lipidated insulin were evaluated using a modified protocol from Trier et al. [17]. Following incubation with insulin or lipidated insulin in physical mixture with penetratin, shuffle or penetramax, the cells were washed thrice with ice-cold HEPES-HBSS, the buffer was removed and the cells were frozen at −80 °C overnight in order to lyse the cells. 250 μL ice-cold HEPES-HBSS was added to the filter and the cells were detached from the filter using a cell scraper twice (Orange Scientific, Braine-l’Alleud, Belgium). The cell suspension was centrifuged (15,700 g, 4 °C) for 20 min using a 5417 R centrifuge equipped with a FA-45-24-11 rotor (Eppendorf, Hamburg, Germany). The supernatant was used for quantification of cell uptake of insulin and lipidated insulin. The protein concentration was determined using a Pierce™ bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA, USA) as described by the manufacturer to normalize the cell lysates to the total protein content obtained from each filter. A volume of 25 μL ice-cold HEPES-HBSS was added to the pellet and the sample was vortexed thoroughly. Then, 75 μL ethanol was added to detach insulin or lipidated insulin from the membrane and the sample was vortexed and centrifuged (15,700 g, 4 °C) for 20 min. The supernatant was transferred to a new tube and the ethanol was evaporated using nitrogen. Subsequently, 200 μL ice-cold HEPES-HBSS was added and the sample was vortexed. The sample was used for quantification of membrane binding of insulin and lipidated insulin.

Insulin and lipidated insulin were quantified by the enzyme-linked immunosorbent assay (ELISA) kit (CrystalChem, Elk Grove Village, IL, USA) as described by the manufacturer. Insulin and lipidated insulin standards and dilutions were prepared from stock solutions. The absorbance was measured at 450 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). FITC-labeled dextran was quantified by fluorescence. The excitation and emission were measured at 495 or 520 nm, respectively, in black clear-bottom 96-well plates using a FLUOstar OPTIMA plate reader (BMG Labtech). 14C-mannitol and 3H-metoprolol were quantified by liquid scintillation. A volume of 2 mL Ultima Gold was added to the samples and the radioactivity was measured using a Packard Tri-Carb 2910 TR liquid scintillation counter (Perkin Elmer).

The apparent permeability coefficient (Papp) was calculated by Eq. (2),

\[ P_{app} = \frac{dQ}{dt} / (A \times C_0), \]

where \( dQ/dt \) is the steady-state flux, \( A \) is the area of the filter insert (1.13 cm²) and \( C_0 \) is the concentration in the donor sample.

2.10. Cell viability assay

Following the in vitro transepithelial permeation study, the cell viability was assessed using the MTS-PMS assay as previously described [25]. Briefly, 320 μL of 240 μg/mL MTS and 2.4 μg/mL PMS in HEPES-HBSS and 1 mL HEPES-HBSS were added to the apical and basolateral side, respectively, of the Transwell® filter. The cells were incubated on a shaking table (50 rpm, 37 °C) for 90 min and 3 × 100 μL samples were transferred from the apical side to a clear 96-well plate. The absorbance was measured at 492 nm using a FLUOstar OPTIMA plate reader (BMG Labtech).

The relative viability was calculated by Eq. (3),

\[ \text{Relative viability} = \frac{(A-C)}{(B-C)} \times 100\%, \]

where \( A \) is the absorbance of samples from cells incubated with insulin or lipidated insulin in presence of penetratin, shuffle or penetramax in MES-HBSS, \( B \) is the absorbance of samples from cells incubated with MES-HBSS and \( C \) is the absorbance of MTS-PMS reagent.

Furthermore, the cell viability was assessed in real time by the RealTime-Glo™ MT Cell Viability Assay kit (Promega) as described by the manufacturer. Briefly, the Caco-2 cells cultured for approximately 24 h were washed twice in 37 °C HEPES-HBSS. Then, 45 μL MT Cell Viability Substrate and NanoLuc® Enzyme in MES-HBSS was added to the cells prior incubation on a shaking table (37 °C) for 5 min. Further, 45 μL test sample was added to reach a final concentration of 7.5–30 μM insulin in physical mixture with 30–120 μM penetratin, shuffle or penetramax in MES-HBSS. The luminescence was measured every 75 s over 5 h at 37 °C using a FLUOstar OPTIMA plate reader (BMG Labtech) with shaking (100 rpm for 2 s) before each measurement.

2.11. Immunostaining

The effect of penetratin, shuffle and penetramax on cell morphology was investigated by confocal laser scanning microscopy (CLSM) using a Zeiss LSM 510 microscope (Zeiss, Oberkochen, Germany). Following incubation with the samples as described for in vitro transepithelial permeation study, the Caco-2 cell monolayers were fixed in 3% (w/v) paraformaldehyde in HEPES-HBSS supplemented with 0.05% (w/v) BSA for 10 min, permeabilized in 0.1% (v/v) Triton® X-100 in PBS for 5 min and blocked in 2% (w/v) BSA in PBS for 30 min. Subsequently, the filter was cut into smaller pieces and ZO-1 was stained with 10 μg/mL ZO-1 monoclonal antibody conjugated with alexa fluor 488 for 2 h. The filter pieces were washed thrice in 2% (w/v) BSA in PBS and mounted on a microscope slide.

2.12. Rat intestinal injection

The intestinal insulin delivery was evaluated in rats. The absorption of insulin was determined after intra-ileal administration to 273–338 g male Sprague Dawley rats (Charles River Laboratories, Sulzfeld, Germany). The rats were acclimated for 7 d at 22 ± 2 °C and 55 ± 10% humidity and then fasted for 18 h prior to the experiment. Surgical procedures were conducted under pentobarbital-induced anesthesia with an initial dose of 65 mg/kg and a maintenance dose of 20 mg/kg/h. A tracheostubus was inserted to ensure free breathing during anesthesia. A silicone catheter was inserted in the proximal ileum for administration of the test samples and a polyurethane catheter was implanted into the carotid artery and portal vein for blood sampling. Prior to
administration of samples, the catheter inserted into the ileum was rinsed with 200 μL saline, corresponding to the dead volume of the catheter. Dosing of the 500 μL sample containing 295 μM (70 IU/kg) insulin and 1180 μM penetratin, shuffle or penetramax in MES-HBSS was done. Subsequently, the catheter was flushed with 200 μL air to ensure administration of the full sample volume. Blood samples of 200 μL were collected from the carotid artery at time points 0, 5, 15, 30, 60, 90, 120, 180 and 240 min and from the portal vein at time points 5, 15, 30 and 120 min. The blood samples were centrifuged (2,900 g) for 10 min in K3-EDTA tubes (Sarstedt, Nümbrecht, Germany) to isolate the plasma. Plasma insulin and glucagon were quantified using an ELISA kits (Merckodia, Uppsala, Sweden) as described by the manufacturer and blood glucose was monitored with a Biosen glucose meter (EKF Diagnostics, Cardiff, UK).

The relative bioavailability (BA) was calculated by Eq. (4),

\[
\text{Relative BA} = \left( \frac{\text{AUC(dose)}}{\text{AUC(dose)ref}} \right) \times 100\%. 
\]  

(4)

where AUC is the area under the curve for plasma insulin following intraintestinal (i.i.) and subcutaneous (s.c.) administration. Similarly, the relative bio potency (BP) was calculated using area over the curve (AOC) for blood glucose. Rats that expired during the study were excluded in data analysis. The study was performed in agreement with license No. /FH/Anz. 1022/1, approved by the Regional Council (Regierungspräsidium) of the State of Hesse, Darmstadt, Germany.

2.13. Histology

The effect of the carrier peptides on rat intestinal tissue was investigated by histoimmunochemistry stainings. Following administration of the samples as described for Rat intestinal injection, the rats were euthanized using pentobarbital 5 or 60 min after dosing. The ileum was isolated 10 cm before and after the silicone cannula and preserved in 100% ethanol and N for blood glucose. Rats that expired during the study were excluded in data analysis. The study was performed in agreement with license No. /FH/Anz. 1022/1, approved by the Regional Council (Regierungspräsidium) of the State of Hesse, Darmstadt, Germany.

3. Results

To explore and understand the impact and mechanism of action that the selected carrier peptides would have on the delivery of a therapeutic peptide cargo, biophysical methods were first employed: Complex formation between insulin or lipidated insulin and the carrier peptides penetratin, shuffle or penetramax was investigated using DLS, ultracentrifugation and CD spectroscopy. Association of the CPPs with liposomes was studied by CD spectroscopy and ITC. Based on these results, the influence of complex formation on carrier peptide-mediated insulin delivery was evaluated in the Caco-2 cell culture model and in rats following intestinal dosing. Complementary to insulin delivery, potential effects of the peptides on the cell monolayer were assessed by TEER measurements, the MTS-PMS assay, a real-time cell viability assay and confocal microscopy in the Caco-2 cell culture model. Moreover, tissue effects subsequent to rat intestinal dosing were studied by histological examination. These complementary studies lead to new information on the use of peptide excipients.

The primary structure of the investigated carrier peptides, insulin and lipidated insulin are presented in Fig. 1.

3.1. Carrier peptide-interactions were affected by insulin lipidation

Molecular interactions between the peptide excipient and the cargo likely will influence the delivery capacity. Here, complex sizes in physical mixtures of penetratin, shuffle or penetramax with insulin or lipidated insulin in a molar ratio of 4:1 were determined from size distributions assessed using DLS (Fig. 2a). Under the chosen experimental conditions, penetratin, shuffle and penetramax alone (without insulin) all appeared to self-assemble into complexes of 308 ± 106 nm, 148 ± 53 nm and 171 ± 67 nm, respectively. An additional signal with a low relative volume distribution as marked by the intensity of the colour was observed for penetramax (34 ± 9 nm, 14%). For insulin alone the signal at 3 ± 1 nm (36%) likely represents hexameric insulin [26]. The hydrodynamic radius of assemblies in samples containing insulin and penetratin was 2 ± 1 nm, whereas larger complexes were observed in samples containing insulin together with shuffle or penetramax (308 ± 105 nm or 413 ± 141 nm, respectively). Lipidation resulted in aggregates, as very large mean sized distributions were observed for lipidated insulin alone (1545 ± 473 nm) as well as in the presence of the carrier peptides (995 ± 404 nm, 1152 ± 430 nm and 859 ± 195 nm for penetratin, shuffle and penetramax, respectively). The DLS measurements were associated with a moderate to high degree of polydispersity with polydispersity indices of 0.2–0.6.

To evaluate the recovery of the carrier peptides and cargo upon physical mixing, they were subjected to ultracentrifugation and the concentration of free carrier peptide and insulin or lipidated insulin was quantified in the supernatant using RP-HPLC (Fig. 2b). Full recovery of the carriers and insulin was observed following centrifugation without mixing, and for the carrier peptides, also when in physical mixture with insulin. The concentration of insulin tended to be lower following centrifugation when in presence of both penetratin, shuffle and penetramax, as 7, 12 and 13% insulin sedimented, respectively, in these mixtures. An average of 94% of lipidated insulin was spun down upon centrifugation of samples only consisting of lipidated insulin. Shuffle
and penetramax appeared to promote further aggregation. Furthermore, in these samples, the concentration of the carriers was lower following centrifugation as compared to when alone (without lipidated insulin).

### 3.2. Secondary structure of insulin-complexes was distinct with penetratin

It is believed that adaptation of an α-helical structure is important for the membrane interaction and subsequent cellular uptake potential of CPPs [27]. Thus, for the application of CPPs as carrier peptides to enhance transepithelial permeation, and not necessarily cellular internalization, it was important to determine the folding propensity of penetratin, shuffle and penetramax, alone or in physical mixture with insulin or lipidated insulin, by CD spectroscopy (Fig. 2c). Liposomes consisting of 34 mM 80:20% (mol/mol) POPC:POPG lipids were included as

Fig. 2. (a) Size distributions determined by DLS and (b) recovery following ultracentrifugation of 15 μM insulin or lipidated (lip.) insulin (grey) in physical mixture with 60 μM penetratin (green), shuffle (red) or penetramax (blue) in MES-HBSS. Mean ± SD (n = 2–3). Significant differences are *: p < 0.05, **: p < 0.01 and ***: p < 0.001 relative to the carrier peptides or insulin alone. The relative volume distribution of the peak is indicated by the intensity of the colour, where the solid dots represent 100%. (c) Secondary structure expressed as CD signal of 15 μM insulin (grey) in physical mixture with 60 μM penetratin (green), shuffle (red) or penetramax (blue) in phosphate buffer investigated by CD. The dashed lines represent the CD signal of a mixture containing both insulin and the carrier peptides subtracted the signal of insulin (black) or the carriers (grey). (d) Representative ITC titrations and (e) hereof derived thermodynamic parameters expressed as dissociation constant (K_d), stoichiometry (n) and enthalpy change (ΔH) for titrations of liposomes consisting of 34 mM 80:20% (mol/mol) POPC:POPG lipids into 60 μM penetratin (green), shuffle (red) or penetramax (blue) in MES buffer. Mean ± SD (n = 3). Significant differences are **: p < 0.01 and ***: p < 0.001 relative to penetratin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Penetratin, shuffle and penetramax were present as random coils in buffer with no evidence of α-helical structure (Fig. S1a, b), whereas some structure (Fig. S1b; 1.5, 1.5 and 1.3%, respectively) was induced by liposomes when using a peptide-to-lipid molar ratio of 1:28. In contrast, insulin and, though to a lesser extent, lipidated insulin displayed α-helical structures (52.0 and 26.1%, respectively) with the characteristic maximum at 193 nm and minima at 208 and 222 nm [28]. Some α-helical structure was observed in samples containing physical mixtures of insulin and carrier peptides; in particular with penetratin (21.4%), but also with shuffle (14.1%) and penetramax (13.1%).

Subtracting the CD signal of insulin alone (Fig. 2c; solid grey line) from the signal of a mixture containing both insulin and penetratin (solid green line) resulted in a curve (dashed black line) similar to that of penetratin alone (solid black line). The same was true vice versa; i.e. subtracting the CD signal of penetratin alone from the signal of the mixture containing both insulin and penetratin resulted in a curve (dashed grey line) similar to that of insulin alone (solid grey line) indicating no structure-modifying intermolecular interactions between carrier and cargo peptide. On the contrary, this phenomenon was not evident for the mixtures containing shuffle or penetramax.

![Graphs showing cell viability and monolayer integrity](image_url)

**Fig. 3.** (a) Cell viability expressed in real time or (b) by end-point values and (c) monolayer integrity of Caco-2 cells incubated with 15 μM insulin or lipidated insulin in physical mixture with 60 μM penetratin (green), shuffle (red) or penetramax (blue) in MES-HBSS relative to cells incubated with buffer assessed by the RealTime-Glo™ MT Cell Viability Assay, the MTS-PMS assay and TEER, respectively. Mean ± SEM (n = 3, N = 2). Significant difference is ***: p < 0.001 relative to cells incubated with buffer. (d) Representative images (n = 1, N = 2) of ZO-1 staining in Caco-2 cell monolayers incubated with 15 μM insulin and 60 μM penetratin, shuffle or penetramax. Observations of changes are marked with white arrows: increased ZO-1 staining at kissing points between the cells. Scale bar = 10 μm. (e) Apparent permeability coefficient (P_app) of 1 mg/mL FITC-labeled dextran (average Mw: 4000 g/mol), 0.1 μCi/mL 14C-radiolabeled mannitol or 0.5 μCi/mL 3H-radiolabeled metoprolol across Caco-2 cell monolayers when applied in physical mixture with 60 μM penetratin, shuffle or penetramax in MES-HBSS. Mean ± SEM (n = 3, N = 3). Significant differences are *: p < 0.05, **: p < 0.01 and ***: p < 0.001 relative to dextran or mannitol and metoprolol, respectively, without the carrier peptides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.3. Liposome-interactions were strongest with penetratin

The differences observed in carrier peptide-cargo complex interactions were further related to the selected peptides’ interactions with membranes. The binding strength between the carrier peptides and liposomes was investigated using ITC (Fig. 2d). The integrated peaks representing heat signals, resulting from injection of liposomes into peptide solutions, displayed sigmoidal curves fitting a one-site binding model. However, extended tailing of the peaks was observed at the inflection region, consequently leading to a less suitable fit when using this model. This phenomenon has previously been reported for penetratin [29]. The thermodynamic parameters obtained from the model describe the binding interactions between the peptides and the membrane mimics (Fig. 2e). The dissociation constants for penetratin, shuffle, and penetramax did not differ significantly. The stoichiometry was, however, significantly higher (p < 0.01) for penetratin with a peptide-to-lipid molar ratio of 1:79 ± 1 as compared to shuffle and penetramax (peptide-to-lipid molar ratios of 1:65 ± 4 and 1:68 ± 2, respectively), whereas the change in enthalpy was significantly larger (p < 0.001) for shuffle when compared to penetratin and penetramax.

3.4. Cell viability was reversibly affected by shuffle and penetramax

To deduce the potential and mechanism of action for carrier peptide-cargo complexes, the cell viability was assessed in real time using the RealTime-GloTM MT Cell Viability Assay (Fig. 3a). 60 μM penetratin alone (without insulin) did not affect the cell viability relative to cells incubated with buffer (Fig. S2b). In contrast, the cell viability decreased in the presence of 60 μM shuffle or penetramax (to 82.9 ± 1.2 and 86.8 ± 2.6%, respectively). For physical mixtures of 15 μM insulin and 60 μM carrier peptide, an initial decrease to 61.9–75.0% of the cell viability was observed followed by a steady recovery over time to 78.8–94.3% relative to cells incubated with buffer (Fig. 3a). The effect of the carrier peptides was concentration-dependent for cells incubated with 120 μM shuffle and penetramax alone (Fig. S2b). A smaller effect on cell viability was observed for 120 μM shuffle and penetramax when in physical mixture with 30 μM insulin (70.3 ± 0.8 and 69.3 ± 2.3%, respectively).

Similar tendencies were found using the traditional end-point MTS-PMS assay (Fig. 3b), with a decreased viability of cells when incubated with shuffle and penetramax alone (51.3 ± 4.3 and 55.5 ± 4.5 %, respectively), as compared to buffer-treated cells.

3.5. Epithelial integrity and junctions were affected by shuffle and penetramax

Eluting to the mechanism of action for enhanced transepithelial permeation effects, the effect of penetratin, shuffle and penetramax on the Caco-2 cell monolayer integrity (Fig. 3c) was first investigated by TEER measurements. Penetratin, both alone and in physical mixture with insulin, did not affect the cell monolayer integrity, whereas shuffle and penetramax reduced the integrity (35.0 ± 4.9 and 56.9 ± 7.2 %, respectively when alone). Interestingly, the effect of penetratin on TEER was smaller when in the presence of insulin (87.6 ± 4.7%). Likewise, the monolayer integrity (Fig. S3b) and cell viability (Fig. S3a) also decreased following incubation with shuffle or penetramax in the presence of the permeation markers dextran, mannitol or metoprolol.

The localization of the tight junction-associated protein ZO-1 was investigated in relation to the cell morphology during incubation with the carrier peptides (Fig. 3d). No major differences were observed in cells incubated with the carriers as compared to cells incubated with insulin only. However, some indications pointed towards that ZO-1 was affected resulting in increased staining intensity at intercellular kissing points, as indicated with arrows. This phenomenon was observed for all samples, yet more profound in cells incubated with shuffle or penetramax both alone and in presence of insulin. The effect was accompanied by restriction of the cells surrounding the kissing point.

3.6. Paracellular permeation was facilitated by shuffle and penetramax

Further relating the effect of the carrier peptides to the permeation of known markers is important to allow for suggestion of their mechanism of action. Thus the permeation across the Caco-2 cell monolayer was assessed for dextran (average Mw: 4000 g/mol), a molecule in a similar size range as insulin (Fig. 3e, Fig. S3c). Shuffle and to a lesser extent penetramax appeared to enhance the transepithelial permeation of dextran with 73-fold (p < 0.001) and 37-fold (p < 0.01), respectively (Fig. 3e). Mannitol and metoprolol were included as paracellular and transcellular markers, respectively. Shuffle and penetramax significantly enhanced the permeation of mannitol (21-fold, p < 0.001 and 15-fold, p < 0.01 increases, respectively) and metoprolol (1.5-fold, p < 0.01 and 1.4-fold, p < 0.05 increases, respectively) across the Caco-2 cell monolayers when compared to mannitol or metoprolol applied alone.

3.7. Transepithelial insulin permeation was enhanced by shuffle and penetramax

To relate the findings on complexation, membrane binding and effects on permeation marker transport, the plasma membrane binding and cellular uptake of insulin and lipidated insulin were evaluated in the Caco-2 cell culture model (Fig. 4a,b). The results were reported as amount obtained from each filter normalized to total protein content. Membrane binding of insulin (Fig. 4a; 0.89 ± 0.07 pmol) was enhanced upon physical mixture with penetratin (1.55 ± 0.13 pmol) and in particular with shuffle (3.66 ± 0.26 pmol) and penetramax (4.29 ± 0.19 pmol). Shuffle (Fig. 4b; 1.43 ± 0.31 pmol, p < 0.001) and penetramax (0.95 ± 0.11 pmol, p < 0.001) significantly enhanced the cellular insulin uptake when compared to insulin alone (0.21 ± 0.04 pmol). Lipidated insulin membrane binding (Fig. 4a; 10.74 ± 1.78 pmol) was enhanced in the presence of penetramin (24.82 ± 2.46 pmol). The carrier peptides did not increase cellular uptake of lipidated insulin (Fig. 4b; 0.33 ± 0.08 pmol).

The transepithelial permeation of insulin or lipidated insulin across monolayers of the Caco-2 cell culture model was evaluated as a result of physical mixture with penetratin, shuffle or penetramax (Fig. 4c). Penetratin did not enhance the transepithelial insulin permeation. Both shuffle and penetramax increased the insulin permeation across the cell monolayer from 0.07 ± 0.01 pmol for insulin alone to 4.48 ± 1.61 and 1.74 ± 0.45 pmol for insulin in physical mixture with shuffle and penetramax, respectively, during 3 h of incubation (Fig. S3d). Based on the flux curves, the apparent permeability coefficients were calculated (Fig. 4c). The apparent permeability coefficient of insulin was increased 64-fold (p < 0.001) and 30-fold (p < 0.01) when in physical mixture with shuffle and penetramax, respectively. Lipidation increased permeation 17-fold when compared to non-lipidated insulin, but this was decreased when in physical mixture with penetratin, shuffle, or penetramax.

3.8. Blood glucose was reduced by insulin when dosed in physical mixture with the carrier peptides

Finally, the relation of findings from both the biophysical investigations and the in vitro assessments was done to in vivo PK/PD readouts. The intestinal insulin delivery was evaluated in rats with blood samples collected from the carotid artery (Fig. 5a,b) and the portal vein (Fig. S4a). Lipidated insulin was not included in the experiment because of the lack of effect of the carrier peptide in increasing its transepithelial permeation in vitro (Fig. 4c). Penetratin (Fig. 5a, Fig. S4d; with the maximum insulin concentration (Cmax) of 2.69 ± 2.56 μg/L) and in particular shuffle (38.31 ± 24.86 μg/L, p < 0.05) and penetramax (28.15 ± 14.47 μg/L) tended to increase the plasma insulin concentration when compared to dosing of insulin without the carrier (0.49 ± 0.56 μg/L). Cmax was reached within 30 min for all test samples (Fig. S4d). The insulin plasma clearance tended to be faster for insulin applied in physical mixture with shuffle and penetramax (Fig. 5a; 60–90
penetratin, respectively (Fig. 5c). Plasma glucagon in blood samples from the carotid artery increased in response to insulin administration 1.14 % or 1.61 ± 0.20 % respectively, when compared to administration of insulin alone. The area under the curve were found for shuffle and penetratin, blood samples collected from the carotid artery (Fig. 5a). Based on the product Insuman® Rapid (lip.) insulin in the Caco-2 cell culture model when in physical mixture with 60–240 min min) as compared to subcutaneous administration of the commercial product Insuman® Rapid. Similar tendencies were observed following blood sampling from the portal vein (Fig. S4a); though higher plasma insulin concentrations were expectedly observed when compared to blood samples collected from the carotid artery (Fig. 5a). Based on the plasma concentration profiles, the area under the curve and the relative bioavailability were calculated (Fig. 5c). A 62- and 64-fold increase of the area under the curve were found for shuffle and penetratin, respectively, when compared to administration of insulin alone. The bioavailability of insulin was 0.02 ± 0.02 % when dosed alone and 1.56 ± 1.14 % or 1.61 ± 1.11 % when in physical mixture with shuffle or penetratin, respectively (Fig. 5c). Plasma glucagon in blood samples from the carotid artery increased in response to insulin administration and peaked shortly after plasma insulin with Tmax of 30–240 min and 60–90 min for insulin administered in physical mixture with shuffle and penetratin, respectively, and 60–240 min for subcutaneous administration of Insuman® Rapid (Fig. S4b). Similar tendencies were observed for plasma glucagon with blood samples collected from the portal vein (Fig. S4c).

Blood glucose levels were reduced by insulin (Fig. 5b, Fig. S4d; with a minimum glucose concentration (Cmin) of 3.66 ± 0.31 mmol/L) when administered in physical mixture with the carrier peptides (2.84 ± 0.44, 1.78 ± 1.06, p < 0.01 and 1.05 ± 0.48 mmol/L, p < 0.001 for penetratin, shuffle and penetratin, respectively). The biopotency of the dosed insulin, calculated based on the pharmacodynamics profiles, was 0.06 ± 0.11 % when alone and 1.36 ± 0.70 % when dosed together with penetratin (Fig. 5c). The blood glucose lowering effect of Insuman® Rapid reached maximum effect later compared to intestinal administration of insulin in physical mixture with the carrier peptides, as the minimum glucose concentration was reached after 120–180 min for the subcutaneous control and after 30–120 min for insulin dosed in physical mixture with the carrier peptides (Fig. S4d).

3.9. Intestinal tissue was affected by shuffle and penetratin

The same combinations of carrier peptide and cargo peptide was evaluated for potential tissue effects resulting from the same procedure of intestinal administration of insulin and the carrier peptides and assessed by histological examination. The tight junction protein claudin-3 (Fig. 5d) was stained to elucidate potential induced effect at the tight junction level. The tissue was further stained with PAS (Fig. 5e) and scored as per visual inspection (Fig. 5f) with a score of 1 corresponding to no visual difference in tissue appearance, and a score of 5 corresponding to extensive cell shedding, surface area reduction and connective tissue retraction. The scores resulting from the individual treatments were averaged. Importantly, the study did not reveal any differences in claudin-3 expression in the intestinal tissue, but some tissue effects (max average score of 2) based on PAS staining were observed for all formulations including the buffer control. These effects were more prominent in rats administered insulin in physical mixture with the carrier peptides, and in particular with shuffle and penetratin.

A reduction of the villi surface area was observed in rats subjected to insulin administered alone (Fig. 5e, grey arrows). Retraction of the connective tissue was observed as a response to all carrier peptides (black arrows), and shedding of the epithelial cells was observed mainly in tissue from rats administered with insulin in physical mixture with shuffle or penetratin (green arrows). Importantly, the general observation was that tissue effects appeared to be most profound in rats euthanized 5 min after dosing as compared to 60 min after dosing (Fig. 5f). It should be noted that the effects were highly variable, as indicated by observations in the samples from only insulin dosing; thus, scores are in all cases average values, e.g. observations of extensive tissue effects with a score of five in one of three rats would be averaged to a score of 2.

4. Discussion

4.1. Biophysical investigation of insulin-carrier peptide interactions

Cargo-CPP interactions have been speculated to be important for the delivery propensity of CPPs when applied for oral therapeutic peptide delivery [30]. New fundamental insight on the potential of penetratin, shuffle and penetratin applied as carrier peptides, in association with insulin or lipidated insulin was reflected in the size distribution of complexes and their recovery was determined by DLS measurements (Fig. 2a) and UC (Fig. 2b), respectively. All the employed carrier peptides appear to self-assemble in aqueous medium (Fig. 2a); consistent with earlier findings for Tat and other CPPs [31,32]. However, full recovery of the carrier peptides was obtained following centrifugation (Fig. 2b) indicating that, either the assemblies are not pelleted or possibly only a small fraction of the carrier peptides in solution self-assemble and obscure the DLS measurement of the monomeric carrier peptide. The size distributions of insulin in physical mixture with penetratin revealed small assemblies of 2 ± 1 nm, similar to that observed in samples with only insulin (Fig. 2a). On the contrary, only minor changes were observed with shuffle or penetratin in absence and presence of insulin, thereby suggesting a different mode of complexation between insulin and penetratin as compared to that with shuffle or penetratin. Kristensen et al. have previously reported a similar difference in the size distributions of penetratin in physical mixture with insulin as compared to shuffle [11].

The nature of this difference in complexation of insulin with penetratin when compared to shuffle and penetratin could not be fully clarified using DLS. Therefore, to further study the interactions between insulin and the carrier peptides, effects of complexation on the secondary structure was investigated by CD spectroscopy (Fig. 2c). The CD signal of a mixture containing both insulin and penetratin was shown to be the sum of the individual signals of the free insulin and penetratin alone, suggesting that insulin does not associate or only loosely associates with penetratin. In contrast, this phenomenon was not evident for mixtures containing shuffle or penetratin, indicating that the CD signals indeed resulted from complexes of insulin and the penetratin.
Figure a: Graph showing plasma insulin levels over time for various treatments.

Figure b: Graph showing blood glucose levels over time for various treatments.

Table c: Summary of AUC and BA for plasma insulin and AOC and BP for blood glucose.

Figure d: Images showing cellular structures treated with different compounds.

Figure e: Images showing the effects of different treatments on tissue structures.

Figure f: Table showing reduction of surface area, retraction of connective tissue, and shedding of epithelial cells over time for different treatments.

(caption on next page)
analogues. The CD spectra thus indicate that complexation induces different folding of shuffle and penetramax with insulin in contrast to that with penetratin, which is consistent with the findings in the DLS measurements (Fig. 2a). These findings correlate well with literature, as the binding strength between surface-immobilized insulin and penetratin has been reported to be lower as compared to shuffle and penetramax when investigated by surface plasmon resonance (9,33). Noteworthy is that in our studies, both insulin and the carrier peptides were freely in solution.

4.2. In vitro & in vivo evaluation of carrier peptide-mediated insulin delivery

Cellular uptake (Fig. 4b) and transepithelial permeation (Fig. 4c) of insulin or lipidated insulin when in physical mixture with the carrier peptides were evaluated in the Caco-2 cell culture model. The difference in complexity, i.e. the complexes formed by mixing insulin with shuffle or penetramax, but not with penetratin (Fig. 2a–c), may partly explain the lack of penetratin-mediated transepithelial insulin permeation (Fig. 4c; shuffle > penetramax > > penetratin). This indicates that association between insulin and the carrier peptides is important for the efficiency of the carriers. Basic and hydrophobic amino acids have earlier been acknowledged as important for the delivery propensity of cell-penetrating peptides as carriers for delivery of a cargo peptide in vivo (9,12). The present study furthermore underlines that the sequential order of the carrier peptide amino acids influences the complexation between the carriers and insulin and thus their ability to mediate transmucosal insulin delivery. This is consistent with the findings of Khafagy et al. & Kamei et al., who screened penetratin analogues for their effect on the insulin plasma concentration profile of following nasal and in situ intestinal administration, thereby revealing the optimized shuffle and penetramax sequences (9,10).

The influence of carrier peptides on insulin absorption was evaluated in rats following intestinal administration (Fig. 5a–c) through PK profiling (insulin and glucagon) and PD profiling (blood glucose). The enhanced insulin bioavailability, when in physical mixture with shuffle or penetramax (Fig. 5c; shuffle = penetramax > penetratin), correlates well with the observed increase in transepithelial insulin permeation in the Caco-2 cell culture model (Fig. 4c; shuffle > penetramax > > penetratin) and is consistent with earlier findings following in situ administration into rat ileal loop segments (9). The lower bioavailability of insulin when in physical mixture with penetratin as compared to shuffle and penetramax, might partly be explained by our findings that penetratin did not complex to a large extent with insulin. Insulin-carrier peptide interactions, as demonstrated for shuffle and penetramax by CD (Fig. 2c), may inhibit the enzymatic degradation of both insulin and the carrier peptides by steric hindrance. Some discrepancies were observed between bioavailability and biopotency (Fig. 5c; penetramax > shuffle > penetratin), with penetramax resulting in a higher biopotency, while a similar insulin bioavailability was observed as compared to that obtained with shuffle. This could likely be a result of the higher Tmax observed for penetramax than for all other formulations (Fig. 5a, S4d), resulting in a prolonged decrease of blood glucose and thus higher AOC and biopotency (Fig. 5b,c).

4.3. Biophysical & in vitro study of lipidated insulin

The size distributions of lipidated insulin displayed large assemblies (Fig. 2a), consistent with an earlier report on the behavior of another lipidated insulin analogue; insulin degludec in absence of phenol (34). Lipidated insulin was spun down under ultracentrifugation when alone but in particular when in physical mixture with the carriers (Fig. 2b), indicating self-assembly; likely due to its hydrophobic conjugate. The lower recovery of the carrier peptides when in physical mixture with lipidated insulin indicates complex formation. Lipidation nevertheless appeared to enhance the transepithelial permeation of the insulin molecule, in accordance with a study by Hashizume et al., where the enhancement where linked to increased lipophilicity and intestinal stability (20). This enhancement was abolished for lipidated insulin in physical mixture with the carrier peptides (Fig. 4c). This could be explained by the self-assembly and/or assembly of lipidated insulin with carrier peptides (Fig. 2a,b) and its extensive cell-membrane binding upon addition to Caco-2 cell monolayers (Fig. 4a). A shorter lipid acid chain (e.g. C12) may be preferred when aiming for improved trans-epithelial translocation as earlier reported for C12-conjugated GLP-2, demonstrating less membrane binding when compared to C16-conjugated GLP-2 (17).

4.4. Biophysical investigation of carrier peptide-liposome interactions

The binding strength between the carrier peptides and liposomes, included as membrane mimics, was investigated by ITC (Fig. 2d, e). The stoichiometry (Fig. 2e; penetratin > penetramax > shuffle) indicates that less penetratin interacts with the liposomes than its analogues. The change in enthalpy (shuffle > penetramax > penetratin) suggests that the shuffle-liposome interaction is associated with higher energy than for penetratin and penetramax. However, the binding constant did not significantly differ among the three carrier peptides. The carrier peptide-liposome interactions reflected in the secondary structure were further investigated by CD (Fig. S1). The carrier peptides did not adapt well-defined α-helical structures in the presence of liposomes (Fig. S1a), as has previously been shown with a lower peptide-to-lipid molar ratio (29). This discrepancy is likely due to a too low amount of anionic POPG lipids in the liposomes relative to the amount of peptide as applied in the present study for induction of α-helices (27,35). Rather, the carrier peptides were estimated to contain a high amount of antiparallel β-sheets in the presence of the membrane mimics (Fig. S1b) in agreement with other studies performed with a similar peptide-to-lipid molar ratio of 1:10 (36).

4.5. In vitro & in vivo evaluation of peptide-induced cell membrane effects

Following incubation in the Caco-2 cell culture model, cell viability was assessed by the MTS-PMS assay (Fig. 3b) and the kinetics hereof using a real time cell viability assay (Fig. 3a). The enhancement of transepithelial insulin permeation (Fig. 4c) appears to be linked to a decrease in viability of the Caco-2 cells. However, this effect was shown to be reversible (Fig. 3a); even in the proliferating, and thus highly sensitive, Caco-2 cells. In addition, the effect of the penetratin analogues on cell viability appears to be more pronounced when administered...
alone as compared to when in physical mixture with insulin; particularly at a peptide concentration of 120 μM (Fig. S2). This is likely due to their complexation with insulin, assuming that complexed carrier peptides affect the cell monolayer to a lesser degree when compared to free peptides (Fig. 2a,c).

Following intestinal administration to rats, the effect on the intestinal tissue was assessed by histological examination (Fig. 5e,f), as well as staining for the tight junction protein claudin-3 (Fig. 5d). Similar to the in vitro studies, the carrier-mediated enhancement of insulin bioavailability (Fig. 5a,c) seems to be linked to tissue effects (Fig. 5f). The tissue effects appeared as shedding of epithelial cells and retraction of connective tissue, which are commonly observed in pathological conditions such as inflammation [37]. The seemingly altered ZO-1 staining at the cell-cell kissing points could relate to redistribution of tight junction proteins, which has previously been linked to intestinal epithelial cell shedding [38]. However, the unchanged villi height, lack of discontinuities at the lateral sides of the epithelium and in the villi crypts, as well as unaltered claudin-3 expression (Fig. 5d) indicate that the effects are in range with effects observed for C10; an absorption enhancer evaluated in clinical trials [39,40]. The effects appear to occur immediately after dosing and to be reversible as less tissue effects were observed 60 min after dosing as compared to effects observed 5 min after dosing, corresponding to the T_{max} for all samples except for that of penetramax (Fig. S4d). Similarly, tissue effects induced by SDS and C10 has been shown to occur immediately with an onset within 15 min and also to be reversible within 1 h [39,41]. Recovery of induced tissue effects could be explained by a decreasing concentration of intact peptide over time due to enzymatic degradation [8]. Although the intestinal epithelium has a high turnover rate, this cannot account for the observed recovery within 60 min, and must rather be explained by the natural dynamics of the intestinal epithelium homeostasis comprising continuous shedding of cells from the villi tips. Some tissue effects were observed in rats following administration of buffer and insulin controls (Fig. 5e,f), indicating some of the tissue effects observed in rats following administration of insulin in physical mixture with the carrier peptides might not be attributed to the carrier peptides, but rather normal conditions in the intestines or effects of experimental procedure.

In general, the risk of adverse effects together with the recognized low oral bioavailability for therapeutic peptides likely will require use of large quantities of both the therapeutic peptide and carrier peptides, as is also the case for implementation of excipients like SNAC to enhance the bioavailability of semaglutide to around 1% [42]. Such obstacles to clinical use of carrier peptides for oral drug delivery also exist and are indeed important to realize. However, due to the technological advancements, synthesis of such functional excipients is not necessarily the major bottleneck for future oral peptide drugs. Design of more stable and potent versions of shuffle and penetramax may be pursued to reduce the dose, although the degradability of the peptide is an important asset that should be investigated further in relation to the observed rapid reversibility in the intestinal epithelium, as any effects could limit future clinical use. However, mechanistic insight is important to facilitate design of carrier peptides with more favorable properties along with head-to-head comparison to other investigated enhancers in oral formulations.

4.6. Mechanistic study of carrier peptide-induced tight junction effects

The monolayer integrity was assessed by TEER measurements (Fig. 3e). Similar to the observed decrease in cell viability, shuffle and penetramax decreased the integrity of the Caco-2 cell monolayer. The decrease in monolayer integrity following incubation with shuffle and penetramax suggests an effect on the tight junctions and thus possibly leading to insulin permeation through the paracellular space. This hypothesis is supported by the observed enhanced transepithelial permeation of the paracellular markers mannitol and dextran, while the increased permeation of the transcellular marker metoprolol was less significant (Fig. 3e). Moreover, induced ZO-1 staining in the cell-cell kissing points was observed (Fig. 3d). A similar effect on ZO-1 in Caco-2 cell monolayers has earlier been demonstrated for the tight junction modulating peptide PN159, but not for the CPPs Pep-1, R8 and β-sheet under the applied experimental conditions [43]. However, due to the simultaneous reduction in cell viability (Fig. 3b), it may also be speculated that the effect on cell viability might cause the reduced integrity or vice versa. However, penetramax in mixtures with model cargos affected the cell viability to a similar extent as shuffle (Fig. 3b, Fig. S3a), whereas the effect of penetramax on monolayer integrity was less pronounced when compared with shuffle (Fig. 3c, Fig. S3b). Together with a lower enhancing effect on transepithelial permeation of model cargos (Fig. 3e, Fig. 4e), it is speculated that the effect of the carrier peptides may not solely be a result of affected cell membrane, but also a more specific effect at the tight junction level leading to increased paracellular permeation. The possible contribution by the paracellular route is contradictory to most current literature, as the most likely mechanism for penetratin is believed to involve a transcellular route [44-46]. One study has previously argued for paracellular transport, due to an effect of carrier peptides on the barrier integrity as evaluated by TEER measurements [47]. However, most studies with these peptides are investigating their cell-penetrating property aiming for intracellular delivery of a therapeutic cargo and would thus not reveal potential enhancement of paracellular transport. Also, transcytosis would also likely not be induced as a result of cellular uptake. In the present study, shuffle and penetramax-mediated cell uptake of insulin was also detected (Fig. 4b); thus, in addition to the paracellular route, a transcellular route may contribute to the net insulin flux across the cell monolayer.

5. Conclusion

The penetratin analogues shuffle and penetramax are demonstrated to be promising candidates for carrier-mediated insulin delivery across biological barriers based on transepithelial insulin permeation in the Caco-2 cell culture model and insulin plasma concentration profiling following intestinal administration to rats. Whereas, lipidation of insulin shows potential for improving the translocation of insulin across the cell monolayer, the carrier peptides did not further enhance the transepithelial permeation of lipidated insulin. Importantly, the effect of the carrier peptides appears to be facilitated by association with the cargo and/or with the cell membrane. Strong association between a carrier and the cell membrane might be linked to a lowering of the cell viability as observed for shuffle and penetramax, but not for penetratin. The effect of the carriers on monolayer integrity, combined with increased transepithelial paracellular permeation of relevant markers, suggest involvement of the paracellular route rather than a transcellular route. Overall, by combining orthogonal in vitro and in vivo methods applied to investigate the mode of action new knowledge is evident and suggest an immediate, but reversible effect on the paracellular dynamics leading to an increased insulin bioavailability.

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