https://doi.org/10.31925/farmacia.2025.2.9 **ORIGINAL ARTICLE**

TETRADECYL MALTOSIDE AND DIMETHYL-PALMITOYL-AMMONIO-PROPANE-SULFONATE AS EFFECTIVE PERMEATION ENHANCERS FOR SALMON CALCITONIN AND PRAMLINTIDE: A COMPARATIVE *IN VITRO* STUDY ON CACO-2 CELL LINES

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Abstract

Therapeutic peptides are increasingly used to treat chronic diseases due to their efficacy and safety profiles. However, the benefits observed in clinical trials often don't translate to real-world settings largely due to poor patient adherence caused by inconvenient injectable administration. While oral administration is preferred by chronic patients, it poses significant challenges for peptides including poor gastric absorption, low pH instability and susceptibility to proteolytic degradation. Considering the structural similarities between salmon calcitonin (sCT) and pramlintide (Pram), this *in vitro* study evaluates the efficacy and safety of tetradecyl maltoside (TDM) and dimethyl-palmitoyl-ammonio-propane-sulfonate (PPS) for enhancing the permeability of sCT compared to Pram on Caco-2 cell lines. TDM 0.2 mg/mL significantly increased the 2-hour apparent permeability coefficient (Papp) for sCT by 22% relative to TDM 1 mg/mL, but the effect did not reach statistical significance *versus* PPS 0.1 mg/mL and PPS 0.2 mg/mL. Conversely, PPS 0.1 mg/mL was the most effective permeation enhancer (PE) for Pram, inducing a 517% relative increase in 2-hour Papp *versus* TDM 1 mg/mL (p = 0.00002), 149% *versus* TDM 0.2 mg/mL (p = 0.00018) and 207% *versus* PPS 0.2 mg/mL (p = 0.00008). Thus, PPS 0.1 mg/mL is the most suitable PE for both peptides. At the 2-hour mark, both TDM and PPS significantly lowered transepithelial electrical resistance (TEER) at all concentrations, showing no signs of recovery 6 hours post-exposure. This study highlights the effectiveness of PPS as a viable permeation enhancer for both sCT and Pram, while also emphasizing the efficacy of TDM as a permeation enhancer specifically for sCT.

Rezumat

Datorită profilurilor de eficacitate și siguranță, peptidele terapeutice sunt utilizate din ce în ce mai frecvent în tratamentul bolilor cronice. Însă, adesea beneficiile observate în studiile clinice nu se regăsesc în practica clinică, în mare parte din cauza aderenței scăzute la tratament a pacienților, determinată de administrarea injectabilă. Deși preferată de pacienți, administrarea orală a peptidelor este limitată de absorbția redusă, instabilitatea la pH acid și degradarea proteolitică. Având în vedere similaritățile structurale dintre calcitonina de somon (sCT) și pramlintidă (Pram), acest studiu *in vitro* evaluează eficacitatea și siguranța tetradecil maltozidei (TDM) și a dimetil-palmitoil-amoniu-propansulfonatului (PPS) privind creșterea permeabilității sCT comparativ cu Pram, pe linii celulare Caco-2. TDM 0,2 mg/mL a crescut semnificativ permeabilitatea sCT la 2 ore cu 22% *versus* TDM 1 mg/mL, dar fără semnificație statistică *versus* PPS 0,1 mg/mL și PPS 0,2 mg/mL. În contrast, PPS 0,1 mg/mL a fost cel mai eficace potențiator de permeație (PP) pentru Pram, inducând o creștere relativă a permeabilității la 2 ore cu 517% față de TDM 1 mg/mL (p = 0,00002), cu 149% față de TDM 0,2 mg/mL (p = 0,00018) și cu 207% față de PPS 0,2 mg/mL (p = 0,00008). Astfel, reiese că PPS C1 este PP optim pentru ambele peptide. La 2 ore după expunere, atât TDM cât și PPS (ambele concentrații) au redus semnificativ rezistența electrică transepitelială (RET), fără recuperare la 6 ore după expunere. Acest studiu evidențiază eficacitatea PPS ca PP viabil pentru atât pentru sCT, cât și pentru Pram și întărește totodată eficacitatea TDM ca PP, în special pentru sCT.

Keywords: pramlintide, calcitonin, permeation, enhancers

Introduction

Due to their high potency and selectivity for target receptors, characteristics that provide an attractive efficacy and safety profile, pharmaceutically active peptides and proteins have gained increasing popularity for their use as pharmacological agents [1].

Furthermore, given their biological origins and inherent biodegradability, therapeutically active peptides and proteins generally have a lower toxicity compared to synthetic molecules, especially when administered over extended periods at appropriate doses [2]. Along with the progress made in bioengineering, an increasing number of therapeutic peptides and proteins have

been developed for use in the treatment of chronic diseases [1]. This trend is also observed by the most recent analyses on the development, approval, and use of active macromolecules. Indeed, according to the latest research by Nova One Advisor, the global biologics market size was estimated at USD 511.04 billion in 2023 and is projected to reach around USD 1.374 trillion by 2033, growing at a compound annual growth rate (CAGR) of 10.4% from 2024 to 2033 [3]. The main drawback of these biological medicines is their parenteral route of administration, which many patients, especially those with chronic conditions such as type 2 diabetes, find highly inconvenient [4]. The efficacy and safety of biological medicines are evaluated through large-scale research and development programs consisting of randomized, controlled clinical trials. Such trials are considered the gold standard for researching and assessing the efficacy and safety of medications, because they strictly regulate interventions and minimize external factors, thereby providing clear information about a drug's effects [5]. However, clinical trial findings are often inconsistent with the results observed in real world settings [6]. The primary factor behind the low reproducibility of clinical trial results is poor patient adherence to treatment [6]. Multiple factors underlie poor adherence, including the high complexity of some chronic therapies [6]. As a result, technologies that simplify medication use are needed, such as extended-release formulations, oral peptide delivery, and fixed-dose combinations. Chronic patients strongly prefer oral treatments and may abandon injectables for oral options, even if they are less effective [4]. Many reviews underscore the challenges in formulating oral peptide medications [7, 8]. In our previous work, we examined the obstacles and strategies for developing oral salmon calcitonin (sCT) formulations, as well as the core factors behind the eventual discontinuation of efforts to create an oral form of salmon calcitonin [9]. More recently, we showed that co-formulating sCT with permeation enhancers (PEs) can help overcome its limited intestinal absorption and potentially improve oral bioavailability. Among the PEs evaluated, tetra-decyl maltoside (TDM), a mild surfactant and di-methyl-palmitoyl-ammonio-propane-sulfonate (PPS), a zwitterionic surfactant, were particularly effective in increasing the permeability of sCT on Caco-2 cell lines [10].

Both functionally and structurally, calcitonin and amylin belong to the same peptide category and act on the same group of receptors, abbreviated as CTR (calcitonin receptors) [11]. Amylin is a peptide hormone (composed of 37 amino acids) co-secreted with insulin by pancreatic beta cells in response to food intake [12]. It complements insulin by helping control postprandial glucose through delaying gastric emptying, inhibiting glucagon secretion, suppressing hunger and stimulating satiety [12]. Pramlintide (Pram) is a synthetic analogue of amylin, with several structural modifications (replacing alanine at position 25 and serine at positions 28 and 29 with proline) to reduce the aggregation and adhesion tendencies seen in amylin. Its molecular mass is approximately 3949.9 Da, placing it among smaller macromolecules [12]. Given its many structural similarities to amylin, Pram has comparable effects, which led to FDA approval in March 2005 for its use in the treatment of both type 1 and type 2 diabetes [12]. Marketed under the brand name Symlin® by Amylin Pharmaceuticals, pramlintide is administered subcutaneously as an injectable solution [12], an approach that may limit its therapeutic potential, especially since patients with diabetes often favour oral medications [4]. Considering the structural similarities between Pram and sCT (32 amino-acids, 3432 Da) [12], this study compares the efficacy and safety of TDM and PPS (with 2 different concentrations, selected based on our previews research findings: 0.2 mg/mL (C1) and 1 mg/mL (C2) for TDM, 0.1 mg/mL (C1) and 0.2 mg/mL (C2) for PPS) [10] in enhancing sCT permeability relative to Pram, *in vitro*, using Caco-2 cell lines. Efficacy was assessed by measuring the apparent permeability coefficient (Papp), while safety was evaluated by measuring the transepithelial electric resistance (TEER). Furthermore, using published data and methodologies [13-15], we developed a mathematical model to estimate how the apparent permeability coefficients of sCT and Pram observed in our *in vitro* study on Caco-2 cell lines, might translate to human jejunal absorption.

Materials and Methods

Materials

Salmon calcitonin (sCT), Pramlintide (Pram) and Tetradecyl maltoside (TDM) were purchased from Sigma-Aldrich Chemie GmbH, Dimethyl-palmitoyl-ammonio-propanesulfonate (PPS) was purchased from Santa Cruz Biotechnology, Inc. The transwell Caco-2 assay system used was the CacoReady 24 Transwell seeded with 21 days differentiated and polarized Caco-2 cells, designed for high-throughput experimentation and purchased from ReadyCell SL. The LC-MS/MS instrumentation used was Waters Acquity PREMIER UPLC and Waters Xevo TQ-Absolute triple quadrupole MS with the Waters Acquity UPLC CSH C18 (2.1 × 30 mm, 1.7 µm) column with pre-column filter. The voltmeter used for TEER measurements was the Millipore Millicell® ERS-2 Volt-Ohm Meter, coupled with the MERSSTX01 electrode, as recommended by ReadyCell. The rest of the materials used were of analytical grade and were obtained from various providers.

Evaluation of PEs efficacy in increasing the permeability of sCT and Pram

The efficacy of PPS and TDM in enhancing the permeability of sCT was evaluated across the Caco-2 cell lines, according to the protocol provided by ReadyCell. Stock solutions of sCT and Pram in DMSO at concentrations 100-fold of the target donor concentration were used for preparation of donor solutions. Donor solutions were prepared in transport buffers by adding 1% DMSO stock solutions to achieve final concentrations in the apical compartments of 0.01 mg/mL for sCT and 0.005 mg/mL for Pram. Permeation enhancers were dissolved directly in the incubation buffer at final concentrations in the apical compartments of 0.1 mg/mL (C1) and 0.2 mg/mL (C2) for PPS, and 0.2 mg/mL (C1) and 1 mg/mL (C2) for TDM. The controls included sCT at 0.01 mg/mL and Pram at 0.05 mg/mL, without permeation enhancers. Additionally, a mix of low- and high-permeability reference compounds (5 µM each) - atenolol, furosemide and ranitidine (low permeability), as well as metoprolol and naproxen (high permeability) - with established apparent permeability coefficients and known human jejunal absorption fractions was used. All controls were subjected to identical incubation and handling conditions. These data were essential for developing a model to estimate how sCT and Pram permeability coefficients translate to human jejunal absorption. 50 µL samples from the apical and basal inserts were collected at the start of the experiment (0 minutes) to achieve a final volume of 250 µL in the apical compartment and 750 µL in the basal compartment. The plate was incubated for 2 hours at 37 °C in 5% CO_2, with orbital shaking at 300 rpm and a 3 mm orbit diameter. At 30 minutes, 60 minutes, 90 minutes and 120 minutes after the start of the incubation, 50 µL samples were collected from the basal compartment and the sample volume was replaced by 50 µL of fresh, warm incubation buffer. All samples were collected in triplicate (n = 3) and the quantitative detections of sCT, Pram and low- and high-permeability reference compounds were conducted *via* liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). The permeation of the investigated compounds was determined by using relative LC-MS/MS peak areas (considering the initial donor solution sample = 100%). The data was used for calculating apparent permeability (Papp), by using the formula below:

$$Papp = \frac{dQ}{dt} \; X \; \frac{1}{A \; X \; C_0},$$

where, dQ/dt represents the amount of product present in the basolateral chamber as a function of time (nmol/s); A represents the area of Transwell = 0.33 cm^2; C_0 represents the initial concentration of compound applied in the apical chamber; Papp values for three low-permeability reference compounds (atenolol, furosemide, and ranitidine) and two high-permeability reference compounds (metoprolol and naproxen) were utilized to develop a predictive model for human absorption [15]. This model was established by correlating each Papp value with its corresponding human effective permeability (hPeff) values reported in the literature [14]. Using the established correlation between Papp and hPeff from these five standard compounds, the human effective permeability for sCT and Pram in the presence of permeation enhancers was extrapolated. Additionally, data from the bio-pharmaceutics classification system (BCS) [14] were employed to correlate the known hPeff values of the reference compounds with their corresponding fractions of dose absorbed. This correlation enabled the extrapolation of hPeff values derived from our Papp measurements, thereby allowing the estimation of the percentage of sCT and Pram doses that could be absorbed in the human jejunum under the influence of PEs.

Evaluation of PEs safety in increasing the permeability of sCT and Pram

The safety of PPS and TDM was assessed by measuring each PE's impact on transepithelial electrical resistance (TEER) and evaluating the reversibility of this effect. TEER was recorded at 0 minutes, 120 minutes and 360 minutes post-incubation. To isolate the PEs' true effects on the Caco-2 monolayers, the blank resistance (measured in inserts without cells) was subtracted from TEER values at these timepoints. The resulting decrease in TEER for each PE was expressed as a percentage change from baseline.

Statistical analysis

Results are reported as mean ± 1 standard deviation. The statistical significance of all permeability datasets was evaluated using the Tukey Simultaneous test at a 5% significance level ($\alpha = 0.05$). In addition, each permeability dataset obtained for the permeation enhancers was compared with the control dataset (sCT and Pram without PEs) using the Dunnett Multiple Comparison test, also at $\alpha = 0.05$. The same Dunnett Multiple Comparison test was used to assess differences in the TEER datasets compared to the control group at 120 and 360 minutes ($\alpha = 0.05$). Spearman's test was performed for all correlation analyses at a 5% significance level. All statistical calculations were carried out using Minitab Statistical Software, version 18.

Results and Discussion

After 120 minutes, TDM and PPS (both concentrations) produced significant increases in the Papp values of sCT, highlighting a substantial permeation-enhancing effect. However, only TDM C1, PPS C1 and PPS C2 elevated the Papp values of Pram relative to controls, while TDM C2 induced a negligible permeation

enhancing effect on Pram. Notably, the Papp for sCT and Pram controls could not be quantified, as they were below the detection limit of the analytical method used. Both TDM and PPS appear to significantly enhance the permeability of sCT *versus* Pram, as indicated by the higher Papp values for sCT across both enhancers ($p < 0.0001$ for all comparisons), as shown in Figure 1.

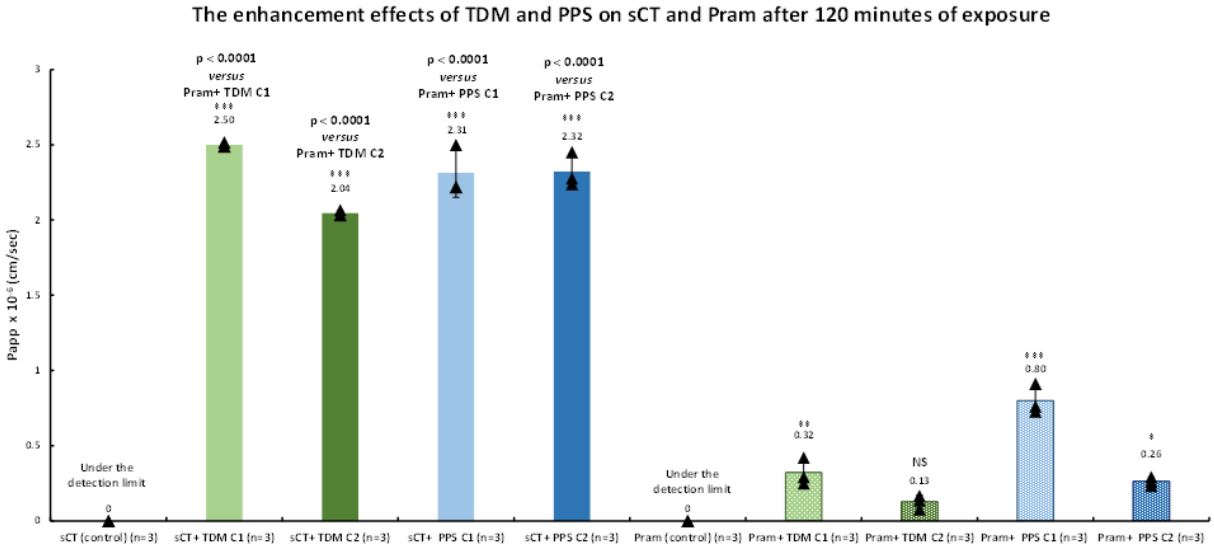

Figure 1.

The enhancement effects of TDM (C1 = 0.2 mg/mL; C2 = 1 mg/mL) and PPS (C1= 0.1 mg/mL; C2 = 0.2 mg/mL) on the permeability of sCT and Pram across the Caco-2 cell lines

Results are expressed as the mean ± SD of three determinations. Each triangle represents one measurement

(NS): $p > 0.05$ compared with control; (*): $p < 0.05$ compared with control; (**): $p < 0.01$; (***): $p < 0.0001$ compared with control

No statistically significant differences were observed in the sCT Papp values between TDM C1, PPS C1 and PPS C2 at the 120-minute mark. However, TDM C1, PPS C1 and PPS C2 exhibited higher permeation increasing effects compared to TDM C2. As shown in Figure 2A, TDM C1 showed a significant 22% relative increase in sCT Papp compared to TDM C2 ($p = 0.02$), PPS C1 demonstrated a significant 13% increase ($p = 0.043$), and PPS C2 achieved a significant 13.6% increase in Papp relative to TDM C2 ($p = 0.036$). The permeation-enhancing effects of both TDM and PPS on sCT became evident early, as sCT accumulation in the basal compartment was already noticeable by the 30-minute mark. As shown in Figure 2B, the divergence in the cumulative amounts of sCT detected in the basolateral compartment reached statistical significance compared to control ($p < 0.0001$) at the first measurement (30 minutes post-exposure) and continued to increase throughout the 120 minutes. The variations of sCT Papp induced by TDM and PPS at 30, 60, 90 and 120 minutes after exposure are illustrated in Figure 2C and Figure 2D, respectively. Notably, TDM C1 maintained a constant Papp for sCT, whereas TDM C2 induced the highest sCT Papp values in the first 30 minutes, followed by a gradual decrease. In contrast, PPS C1 induced the highest sCT Papp at the 120-minute mark, indicating a gradual increase of the permeation enhancing effect, while PPS C2 maintained a steady sCT Papp throughout the incubation period, similar to TDM C1.

In contrast, PPS C1 produced a markedly higher permeation-enhancing effect for Pram at 120 minutes, as shown in Figure 3A. Specifically, PPS C1 increased the Pram Papp by 149% relative to TDM C1 ($p = 0.00018$), by 517% relative to TDM C2 ($p = 0.00002$) and by 207% relative to PPS C2 ($p = 0.000083$). Additionally, TDM C1 demonstrated a 146% relative increase in Pram Papp compared with TDM C2 ($p = 0.043$). These findings indicate that the permeation enhancers affect Pram differently than sCT. There were also differences among the PEs in their effects on the cumulative amount of Pram detected in the basal compartment throughout the study, as shown in Figure 3B. Except for TDM C2, all PEs demonstrated permeation-enhancing effects on Pram as early as 30 minutes post-exposure, producing a significant increase in the accumulation of Pram in the basal compartment *versus* the control group ($p < 0.01$). At the 60-, 90- and 120-minute marks, PPS C1 yielded the highest Pram accumulation relative to control, TDM C1, TDM C2 and PPS C2 ($p < 0.0001$). TDM C1 and PPS C2 induced higher accumulations of Pram than TDM C2, with no significant difference between them, while the effect of TDM C2 did not reach statistical significance *versus* control. TDM C1 produced the greatest effect on Papp within the first 30 minutes post-exposure, but declined significantly by the 120-minute mark.

Meanwhile, TDM C2 maintained a low, steady Papp throughout the study, as shown in Figure 3C. The PPS C1 effect on Papp peaked at the 60-minute mark, then decreased significantly at 120 minutes, whereas PPS C2 induced the highest Papp during the first 30 minutes after exposure, as illustrated in Figure 3D.

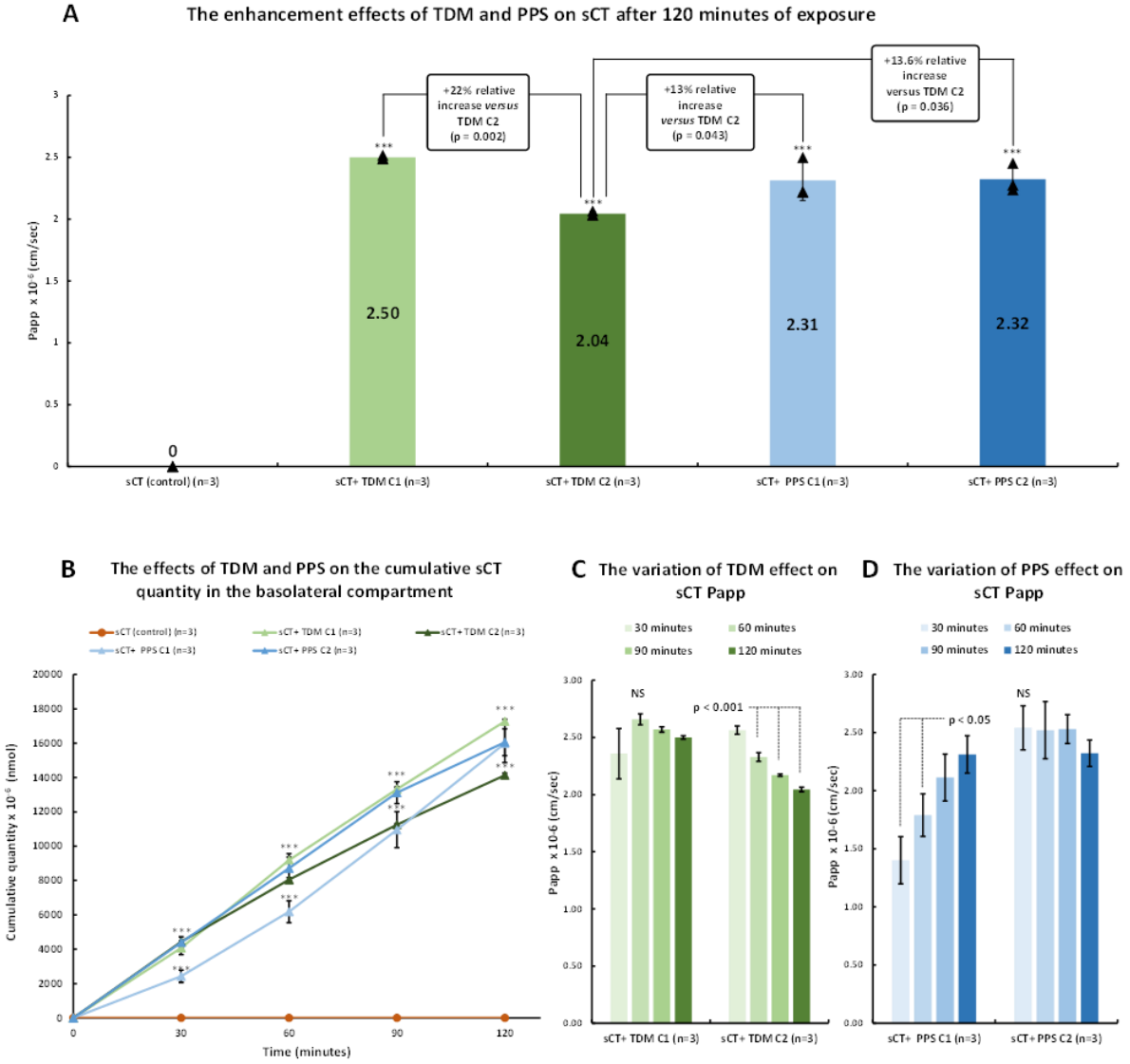

Figure 2.

The enhancement effects of TDM (C1= 0.2 mg/mL; C2= 1 mg/mL) and PPS (C1= 0.1 mg/mL; C2= 0.2 mg/mL) on the permeability of sCT across the Caco-2 cell lines (A) and on the cumulative sCT quantity in the basolateral compartment of the Caco-2 cell lines (B), the variation of the TDM effects on sCT Papp (C) and the variation of the PPS effect on sCT Papp (D)

(A) Results are expressed as the mean ± SD of three determinations; Each triangle represents one measurement; (***): $p < 0.0001$ compared with control

(B) Results are expressed as the mean ± SD of three determinations; (***): $p < 0.0001$ compared with control

(C) Results are expressed as the mean ± SD of three determinations; (NS): $p > 0.05$

(D) Results are expressed as the mean ± SD of three determinations; (NS): $p > 0.05$

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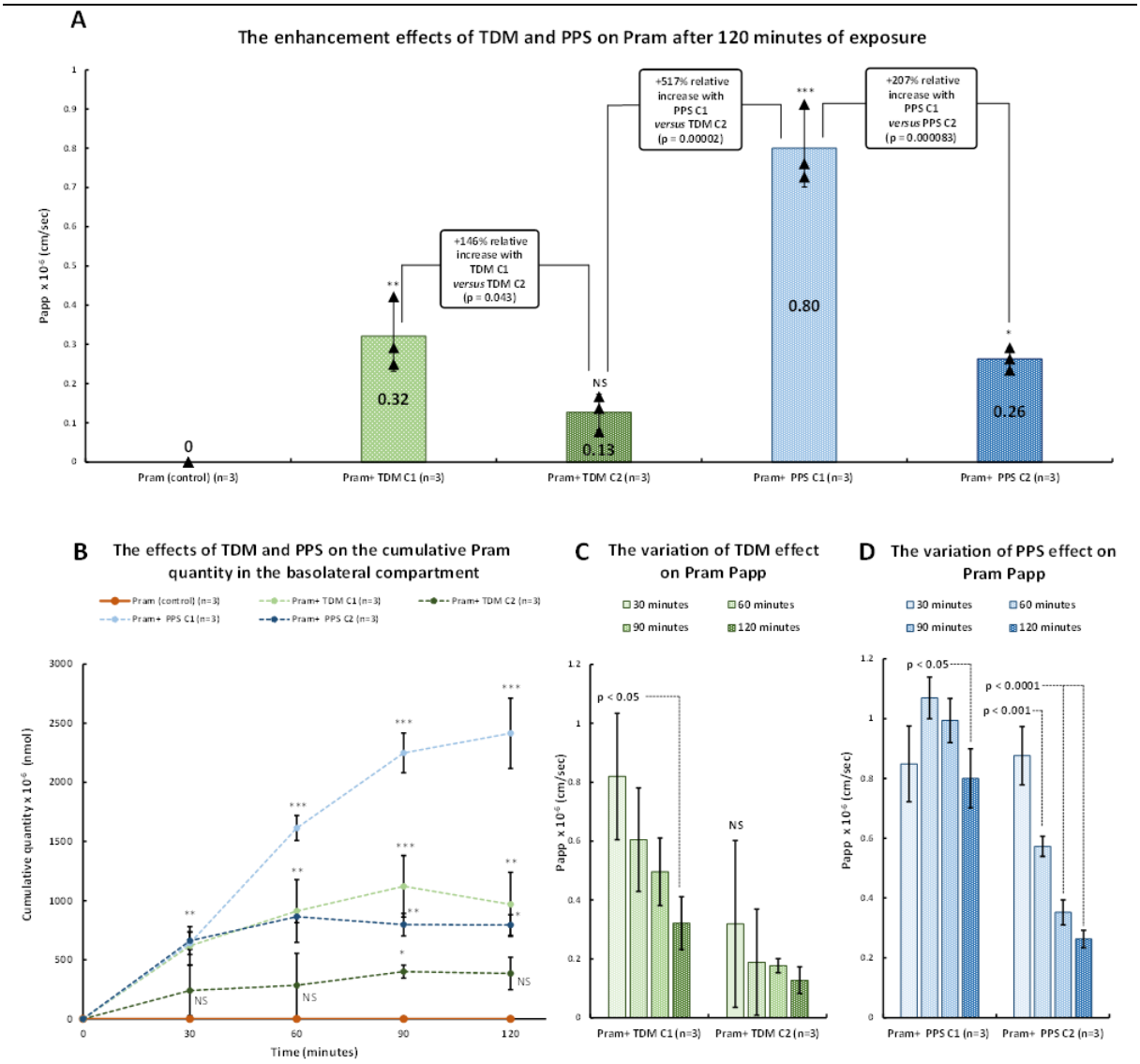

Figure 3.

The enhancement effects of TDM (C1= 0.2 mg/mL; C2= 1 mg/mL) and PPS (C1= 0.1 mg/mL; C2= 0.2 mg/mL) on the permeability of Pram across the Caco-2 cell lines (A) and on the cumulative Pram quantity in the basolateral compartment of the Caco-2 cell lines (B), the variation of the TDM effects on Pram Papp (C) and the variation of the PPS effect on Pram Papp (D)

(A) Results are expressed as the mean ± SD of three determinations; Each triangle represents one measurement; (NS): $p > 0.05$ compared with control; (*): $p < 0.05$ compared with control; (**): $p < 0.01$; (***): $p < 0.0001$ compared with control

(B) Results are expressed as the mean ± SD of three determinations; (NS): $p > 0.05$ compared with control; (*): $p < 0.05$ compared with control; (**): $p < 0.01$; (***): $p < 0.0001$ compared with control

(C) Results are expressed as the mean ± SD of three determinations

(D) Results are expressed as the mean ± SD of three determinations

Relative to the controls, TDM and PPS at all concentrations induced a statistically significant decrease in TEER after 120 minutes of exposure (Figure 4A for sCT and Figure 5A for Pram), with no significant differences observed among the PEs. This finding aligns with the known effects of PEs on TEER, where an increase in Papp corresponds to a decrease in TEER. As shown in Figure 4B for sCT and Figure 5B for Pram, the TEER reduction persisted at 6 hours post-exposure, indicating that the effect was not reversed within this time frame.

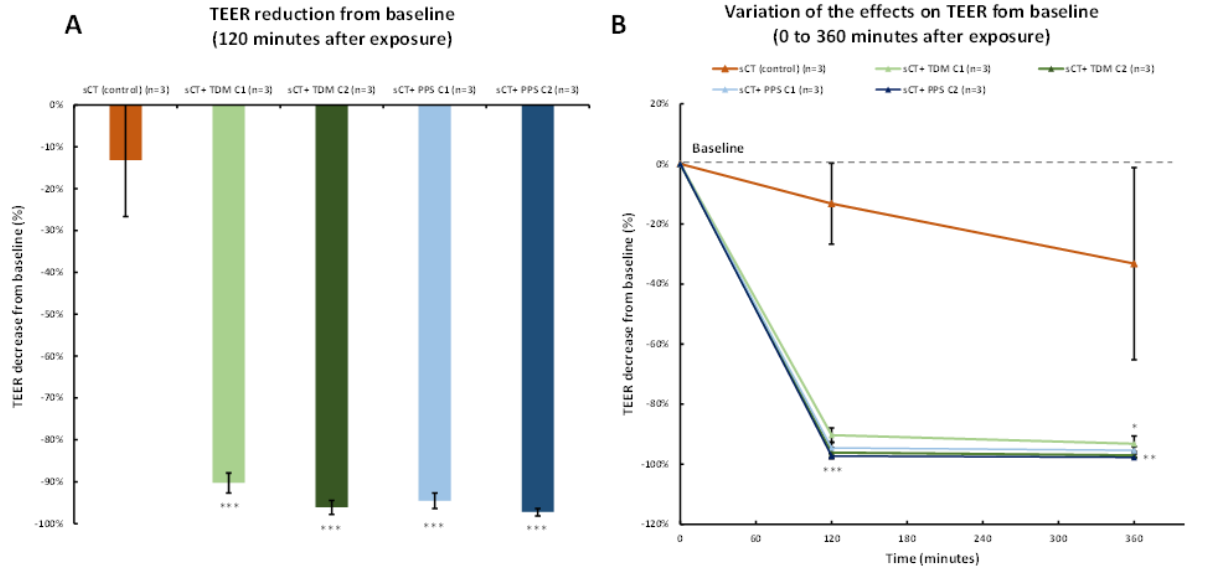

Figure 4.

The effects of sCT in combination with TDM (C1 = 0.2 mg/mL; C2 = 1 mg/mL) and PPS (C1 = 0.1 mg/mL; C2 = 0.2 mg/mL) on TEER, 120 minutes after exposure (A) and the variation of the effects on TEER from baseline to 360 minutes after exposure (B)

(A) Results are expressed as the mean ± SD of three determinations; (***); $p < 0.0001$ compared with control

(B) Results are expressed as the mean ± SD of three determinations (***); $p < 0.0001$ compared with control

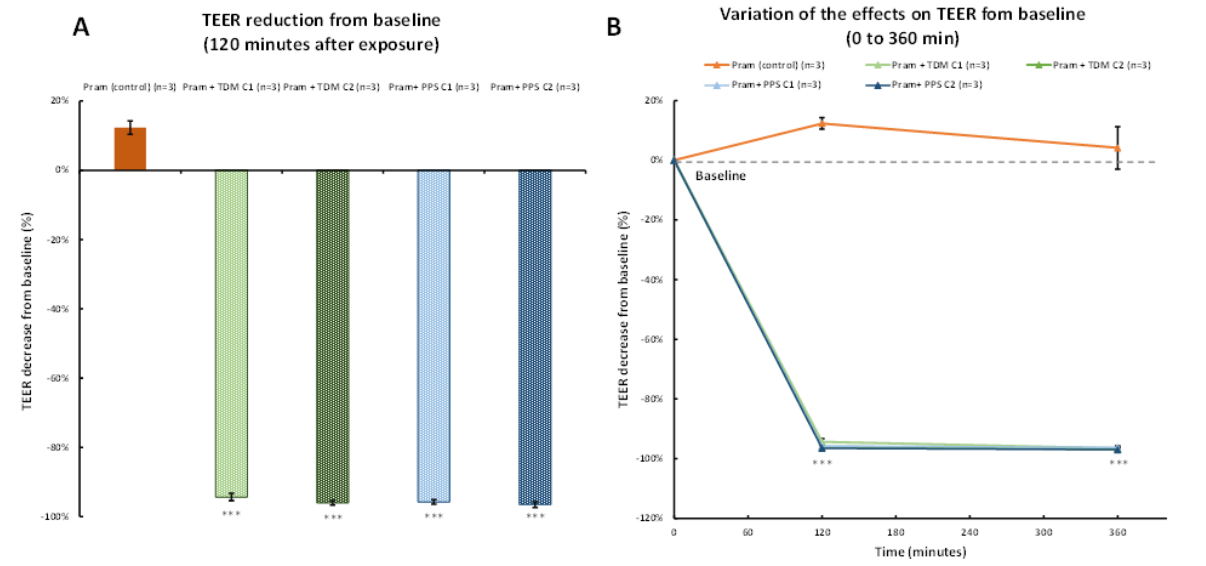

Figure 5.

The effects of Pram in combination with TDM (C1 = 0.2 mg/mL; C2 = 1 mg/mL) and PPS (C1 = 0.1 mg/mL; C2 = 0.2 mg/mL) on TEER, 120 minutes after exposure (A) and the variation of the effects on TEER from baseline to 360 minutes after exposure (B)

(A) Results are expressed as the mean ± SD of three determinations; (***): $p < 0.0001$ compared with control

(B) Results are expressed as the mean ± SD of three determinations; (***): $p < 0.0001$ compared with control

The Papp values for the three low-permeability reference compounds (atenolol, furosemide, and ranitidine) and the two high-permeability reference compounds (metoprolol and naproxen) were used to develop the model for predicting human absorption. This was achieved by correlating each Papp with its known corresponding human permeability (hPeff) values reported in the literature [14]. Table I presents the Papp derived from our Caco-2 assay alongside the corresponding hPeff values for these five standard compounds [14].

Using the correlation between Papp and hPeff observed for the five standard compounds (Spearman rho = 0.93, $p < 0.0001$), the human effective permeability for sCT and Pram induced by the PEs was determined via extrapolation, as shown in the Figure 6.

Table I

Papp values from our Caco-2 assay and the corresponding human effective permeability (hPeff) [14] for 5 standard compounds

Active substance	Mean Papp (x 10^{-6} cm/s) measured in our study	Human Peff (x 10^{-4} cm/s)
Atenolol	0.19 ± 0.03	0.2
Furosemide	0.09 ± 0.02	0.05
Metoprolol	16.3 ± 0.5	1.34
Naproxen	16.3 ± 1.22	8.5
Ranitidine	0.46 ± 0.03	0.27

Papp values are expressed as the mean ± SD of 6 determinations

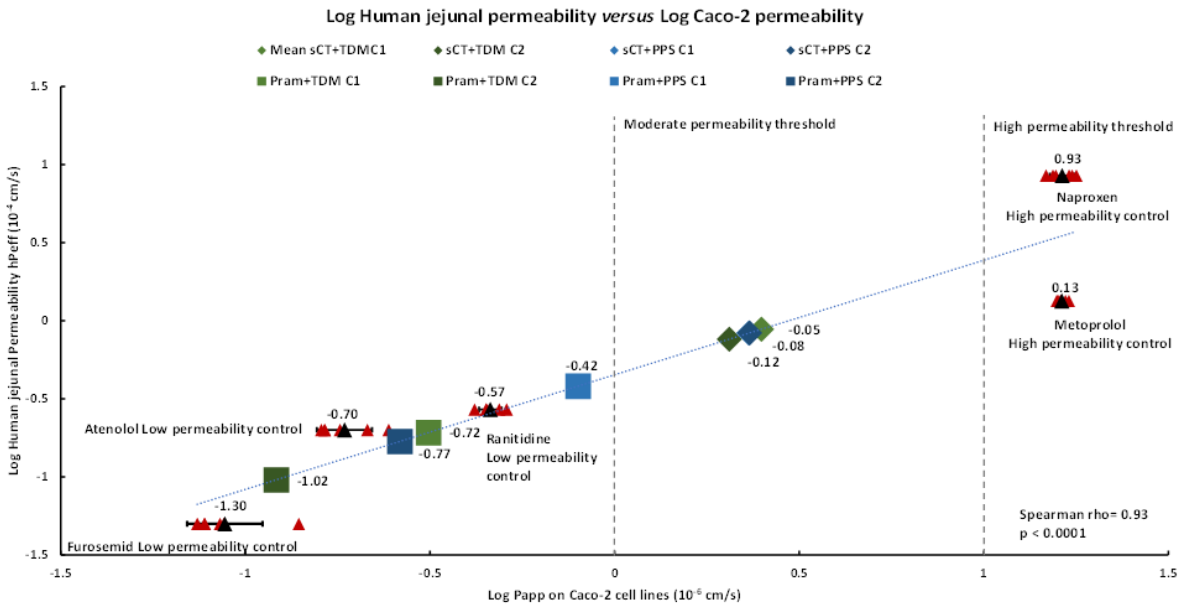

Figure 6.

The relationship between the Papp values from our Caco-2 assay and the corresponding Peff values reported in the literature [14]. For the high- and low-permeability controls, the results are expressed as the mean (black triangle) ± SD of 6 determinations. Each red triangle represents one measurement. For the test compounds, results are expressed as the mean of 3 determinations

By using the data from the biopharmaceutics classification system [14], we established the correlation between the known hPeff values of the tested reference compounds and their corresponding fractions of the doses absorbed (Spearman rho = 0.97, $p < 0.0001$). This correlation enabled us to extrapolate the hPeff values (derived from the Papp values obtained in our study), which in turn allowed us to estimate the percentage of sCT and Pram doses that could be absorbed in the human jejunum under the influence of PEs, as shown in Figure 7.

Table II presents the mean hPeff values obtained for sCT and Pram, calculated based on the Papp values from our study alongside the corresponding estimations of the fractions of sCT and Pram absorbed in the human jejunum.

Table II

hPeff values corresponding estimations of the fractions of sCT and Pram absorbed

Active substance	Mean Human Peff (x 10^{-4} cm/s)	Fraction Dose Absorbed
sCT + TDM C1	0.88 ± 0.003	78% ± 0.05%
sCT + TDM C2	0.76 ± 0.005	76% ± 0.08%
sCT + PPS C1	0.83 ± 0.04	77% ± 0.58%
sCT + PPS C2	0.83 ± 0.02	77% ± 0.41%
Pram + TDM C1	0.19 ± 0.03	60% ± 2.27%
Pram + TDM C2	0.09 ± 0.02	52% ± 3.35%
Pram + PPS C1	0.38 ± 0.03	68% ± 1.01%
Pram + PPS C2	0.16 ± 0.01	59% ± 0.93%

Results are expressed as the mean ± SD of 3 determinations

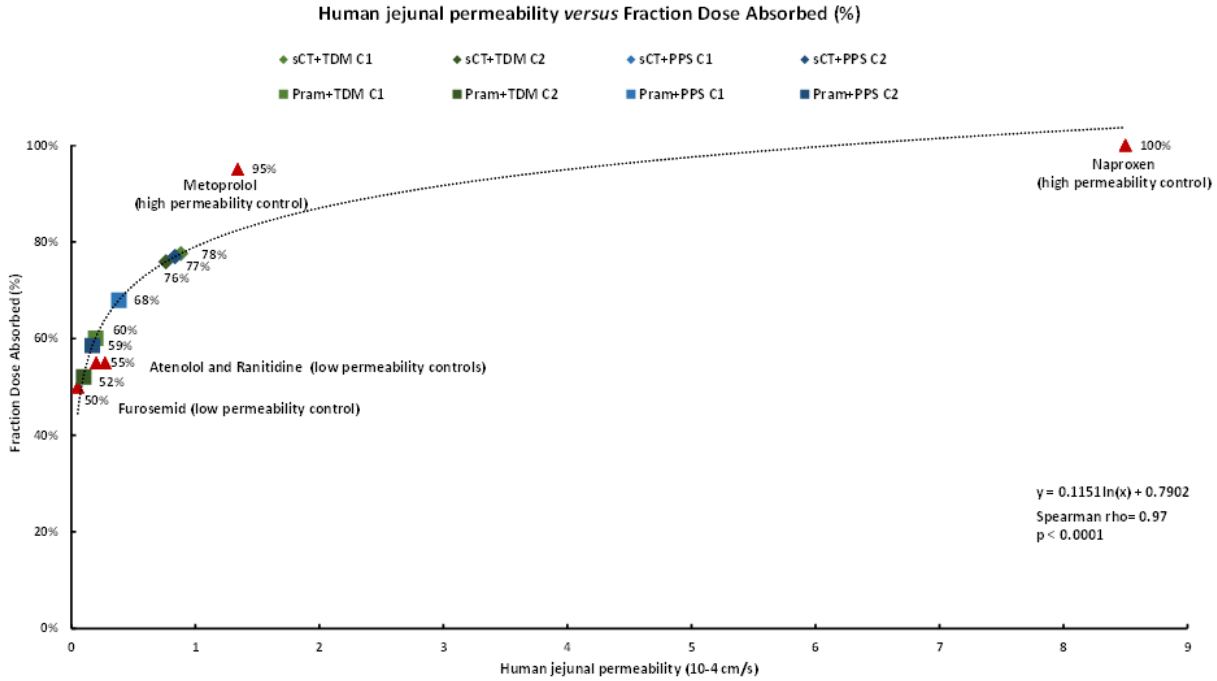

Figure 7.

The relationship between the hPeff values and the corresponding fractions of dose absorbed reported in the literature [14]. For the test compounds, results are expressed as the mean of 3 determinations

The model's results show that both TDM and PPS significantly influence the jejunal absorption of sCT, with absorption rates exceeding 70%. Specifically for Pram, the estimated jejunal absorption ranges from 52% with TDM C2 to 68% with PPS C1. Additionally, it is important to highlight that a lower concentration of TDM resulted in a greater increase in the estimated fraction of the dose absorbed for both sCT and Pram compared to a higher TDM concentration. In contrast, the concentration of PPS did not significantly affect the absorption of sCT, whereas it had an inverse effect on the estimated jejunal absorption of Pram. These findings underscore the distinct interactions between the PEs and both sCT and Pram, demonstrating that despite the similarities between the two peptides, the permeation enhancers elicited different permeability increasing effects. This highlights the importance of selecting a PE based on comprehensive testing specific to the peptide in question, rather than relying on data from previous studies involving other peptides. Nevertheless, despite the observed differences, PPS exhibited sufficient efficacy in enhancing the permeability of both sCT and Pram, making it a viable common denominator. The results of this study reinforce our previous findings [10] on the effect of TDM in enhancing the permeability of sCT. Notably, TDM C1 once again demonstrated the highest 2-hour Papp for sCT, achieving statistical significance compared to TDM C2, though not *versus* PPS C1 and PPS C2, despite numerically higher values. These findings add robustness to our results, particularly given the methodological differences between this study and the previous one [10]. Specifically, these differences include the use of distinct Caco-2 cell models (96-well plates with a 0.14 cm² contact surface in the previous study *versus* 24-well plates with a 0.33 cm² contact surface in the current study), the absence of shaking in the earlier study compared to the use of orbital shaking in this study, the employment of different analytical methods (ELISA in the previous study *versus* LC-MS/MS in the current study) and the collection of samples at multiple time points in the current study compared to sampling only at the 2-hour mark in the previous study.

This study has several strengths. First, based on our knowledge, this is the first study to investigate the effects of TDM and PPS on enhancing the permeability of pramlintide. Considering the patients preference for oral treatments [4], the findings of this study could provide insights for future research on other amylin derivatives, which are currently being investigated as injectable treatments for obesity, such as cagrilintide [16], petrelintide [17], KBP-066A [18] and many others [19]. Patients' adherence to treatment is a multifaceted phenomenon, shaped by various socio-demographic and psychological factors [20]. Although, these complexities pose significant challenges, our findings could offer preliminary insights into developing oral formulations of pharmaceutically active peptides, which may potentially contribute to increasing adherence to amylin derivatives, by accounting for patient preferences [4].

Another strength of this study is the use of standardized Caco-2 cell lines, a fundamental model for understanding and predicting the intestinal absorption of pharmaceutical agents. Furthermore, the data were utilized to estimate the dose fractions absorbed in humans, offering necessary insights to inform future research. The use of sCT and Pram as controls provided further clarity on the permeation-enhancing effects of the investigated PEs. Moreover, the evaluation of variability in Papp effects at 30, 60, 90, and 120 minutes offered a deeper perspective on the dynamics of absorption over time.

The study also has a few limitations, including a small sample size and specific challenges associated with the *in vitro* Caco-2 assay. One notable issue is the potential loss of compounds during the assay, such as adhesion to the plate or retention within the cells, which could lead to an underestimation of compound permeability. The lack of TEER recovery at the 6-hour mark further supports the hypothesis that compounds may have been retained within the cells. Another limitation is the absence of TEER measurements beyond the 6-hour exposure period. The lack of TEER recovery observed at the 6-hour mark is concerning, as an irreversible decrease may indicate lasting cellular damage. However, it is plausible that TEER values would have returned to baseline over a longer duration after initial exposure. This hypothesis is supported by findings from another *in vitro* study on Caco-2 cell lines, which showed that short-term exposure to PPS resulted in a sharp decline in TEER values (indicating the opening of intercellular tight junctions) that persisted at the 5-hour mark but returned to baseline levels within 24 hours, highlighting the transient nature of PPS's effects [21]. Additionally, another *in vitro* study on Caco-2 cell lines, evaluating the potency and toxicity of 51 PEs across 11 distinct chemical categories identified PPS as the second most safe and effective PE, providing a viable option for enhancing permeability without causing significant toxicity [22]. This was supported by *in vivo* data on rats, which showed that PPS significantly enhanced sCT absorption without damaging the intestinal epithelium. Regarding TDM, another *in vitro* study on Caco-2 cell lines also observed TDM-induced TEER reductions, suggesting a para-cellular effect [23]. However, this was contested by lactate dehydrogenase (LDH) assay results, which revealed significant cytotoxicity at concentrations that increased Papp values [23]. While Caco-2 studies indicated a possible link between toxicity and permeation enhancement, LDH release from colonic mucosae exposed to the same TDM concentrations was less pronounced [23]. This discrepancy underscores the tendency of Caco-2 models to overestimate damage due to their limited repair capacity compared to *in vivo* systems [23].

Conclusions

In this study, TDM and PPS were shown to significantly enhance the permeability of sCT and Pram on Caco-2 cell lines. Although sCT and Pram share structural and functional similarities, the PEs exhibited distinct effects on each peptide. TDM C1 caused a statistically significant 22% enhancement in the 2-hour Papp for sCT relative to TDM C2. However, the effect of TDM C1 did not reach statistical significance for sCT 2-hour Papp *versus* PPS C1 and PPS C2, despite TDM C1 exhibiting higher numerical 2-hour Papp values. In contrast, PPS C1 proved to be the most effective PE for Pram, inducing a significant 517% relative increase in the 2-hour Papp compared to TDM C1, a 146% increase *versus* TDM C2, and a 207% increase *versus* PPS C2. Consequently, PPS C1 is the most suitable PE for use with both peptides, while TDM C1 is mostly efficacious for sCT. Furthermore, the study identified an inverse relationship between PE concentration and 2-hour Papp enhancement, with lower concentrations of PEs leading to higher 2-hour Papp values for both sCT and Pram. At the 2-hour mark, both TDM and PPS significantly reduced TEER values across all tested concentrations, aligning with the established mechanism of PEs, where increased permeability corresponds to TEER reduction due to the opening of intercellular tight junctions. The lasting decrease in TEER observed 6 hours post-exposure, highlights the importance of conducting extended-duration TEER measurements to thoroughly understand recovery dynamics and assess any potential toxic effects. This study demonstrates the efficacy of PPS as a feasible permeation enhancer for both sCT and Pram and underscores the efficacy of TDM as a permeation enhancer particularly for sCT. These findings represent a step toward achieving oral delivery of peptides with limited gastric absorption, though additional research is needed to further validate these results.

Conflict of interest

The authors declare no conflict of interest.

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