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"CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY BUCHAREST DOCTORAL SCHOOL PHARMACY

The evaluation of various permeation enhancers for facilitating the intestinal absorption of salmon calcitonin and pramlintide

SUMMARY OF THE DOCTORAL THESIS

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Introduction

Over the past half-century, active peptides and proteins have gained significant popularity as pharmacological agents [1]. Advances in bioengineering have led to the development of an increasing number of therapeutic peptides and proteins for the treatment of chronic diseases [2]. This trend is also supported by recent analyses on the development, approval and use of active macromolecules in clinical settings. According to the recent research conducted by Nova One Advisor, the global biologics market was valued at approximately 511 billion USD in 2023 and is projected to reach approx. 1.37 trillion USD by 2033, having a compounded annual growth rate (CAGR) of 10.4% during the period 2024–2033 [3]. Additionally, due to their biological origin, therapeutic peptides and proteins exhibit lower toxicity compared to synthetic molecules, particularly when administered chronically and in appropriate dosages [4]. The primary disadvantage of these pharmacological agents is the necessity of parenteral administration, frequently perceived negatively by patients, especially by patients suffering from chronic diseases such as type 2 Diabetes Mellitus [6]. Although the efficacy and safety of biological drugs are rigorously evaluated during extensive research and development programs, clinical trial outcomes often do not fully translate to the clinical practice settings [7]. A key factor contributing to the limited reproducibility of clinical trial results is poor patient adherence to treatment regimens [7]. While various factors lead to decreased adherence, one of the most important is the complexity of chronic treatments [7]. Therefore, developing technologies aimed at simplifying drug administration, such as sustained-release formulations, oral pharmaceutical formulations and fixed-dose combinations that co-formulate two or more active substances has become essential. Patients with chronic conditions generally prefer oral medications and often opt out of injectable therapies in favor of orals, even when oral alternatives have inferior therapeutic efficacy [6]. However, patient preference for oral formulations poses significant challenges for long-term treatment administration, as macromolecular peptides typically exhibit very low oral bioavailability. Hence, considerable research efforts are being dedicated to developing technologies that enable the oral, nasal and even transdermal delivery of such molecules.

Permeation enhancers (PEs) or absorption enhancers represent a heterogeneous class of excipients that enable macromolecules to penetrate the gastric or intestinal membranes, by temporarily modifying the epithelial barrier [8]. Through this temporary alteration, permeation enhancers facilitate the transport of macromolecules via paracellular or transcellular pathways [8]. Given their diverse mechanisms of action, numerous research projects have extensively investigated their effects [9].

Calcitonin and amylin are hormones with similar peptide structures that bind to the same group of receptors [10]. Salmon calcitonin (sCT) is a peptide with a molecular weight of 3432 Da, composed of 32 amino acids [11]. It is used for the treatment of postmenopausal osteoporosis, hypercalcemia and Paget's disease and also provides relief from bone pain due to it's analgesic effects [12]. Salmon calcitonin is preferred over human calcitonin due to its higher affinity for the calcitonin receptors and approximately 100-fold greater potency [12]. However, a significant drawback of salmon calcitonin therapy is its parenteral route of administration, which may cause substantial patient discomfort or even pain [11], potentially resulting in reduced treatment adherence and compliance. Considering the clinical relevance and molecular properties of salmon calcitonin, substantial research efforts have been conducted to develop an oral formulation of salmon calcitonin, by using permeation enhancers. However, only two such projects have advanced to late-stage clinical research (Phase 3 clinical trials) and none have been approved for clinical use so far [11].

Amylin, another hormone with peptide structure (composed of 37 amino acids), is secreted by the pancreatic beta cells alongside insulin and plays a crucial role in regulating postprandial blood glucose levels [13]. Pramlintide (Pram), a synthetic analog of amylin, has a molecular weight of approximately 3949.9 Da and several structural modifications designed to reduce molecular aggregation [13]. It exhibits similar effects to amylin and was approved in 2005 for the treatment of type 1 and type 2 Diabetes Mellitus under the trade name Symlin® [13]. Similar to salmon calcitonin, pramlintide requires injectable administration, potentially limiting long-term treatment acceptability, considering patient preferences for oral dosage forms [14].

Given the numerous structural similarities between salmon calcitonin and pramlintide, and considering the extensive research efforts dedicated to developing an oral form of salmon calcitonin, the primary objective of this research project is to directly evaluate *in vitro*, using

colorectal adenocarcinoma cell lines (Caco-2 lines), the permeation enhancers previously demonstrating the most pronounced effects on enhancing salmon calcitonin transport (based on research outcomes reported in the literature) and to further investigate their effects on pramlintide.

The research project outlined in this doctoral thesis was structured into four main stages:

Chapter I. General Considerations

1. Advances in the Development of Oral Formulations for Active Peptides [11]

This chapter explains the primary reasons why macromolecular peptides exhibit very low bioavailability when administered orally. These reasons stem from specific anatomical and physiological barriers of the digestive system, as well as the inherent molecular characteristics of therapeutic peptides. Additionally, this chapter provides an overview of the research projects within the pharmaceutical technology field, highlighting both a successful example (oral semaglutide) and the efforts to develop an oral formulation of salmon calcitonin. A significant portion of the information presented in this chapter has been published in the article titled *"Advances In The Development Of Oral Formulations For Calcitonin And Semaglutide"* [11].

Briefly, regarding salmon calcitonin the factors contributing to the unsatisfactory outcomes in Phase 3 clinical trials of the two currently developed oral calcitonin formulations (TBRIA® and SMC 021) are detailed, specifically:

- Insufficient gastric absorption, leading to low bioavailability upon oral administration of salmon calcitonin.
- The distribution of calcitonin receptors in other tissues, limiting available concentrations of salmon calcitonin at the target bone tissue.
- The inherent tendency of calcitonin molecules to aggregate and fibrillate, thereby losing potency.

In the case of oral semaglutide, the chapter outlines the factors contributing to the success of this pharmaceutical formulation:

- The use of an innovative co-formulation technology with a permeation enhancer, enabling efficient absorption through the gastric membrane, significantly improving the bioavailability.
- Increased molecular stability, preventing aggregation and degradation of semaglutide in the gastrointestinal environment.
- High selectivity and affinity for GLP-1 receptors, ensuring optimal concentrations and consistent therapeutic effects.
- Extensive clinical evidence demonstrating the efficacy and safety of oral semaglutide in achieving and maintaining glycemic control, cardiovascular safety and weight reduction.

Given the availability of semaglutide in both oral and injectable forms, the chapter concludes with results from an advanced systematic analysis, conducted using the PubMed database. This analysis aimed to identify studies evaluating the impact of oral semaglutide on treatment adherence and persistence among patients with type 2 Diabetes Mellitus. The systematic search results indicated that injectable semaglutide administration is associated with a significantly higher cumulative incidence of treatment discontinuation compared to oral semaglutide. Using oral semaglutide treatment as a reference, the relative risk of discontinuation for injectable semaglutide treatment was 45% higher [15]. Considering the critical importance of patient adherence to treatment, these findings underscore the relevance of the topic addressed in this doctoral thesis and justify ongoing research efforts aimed at developing oral formulations of therapeutic peptides for the treatment of chronic diseases.

Chapter II. Personal Contributions

2. Contributions to the Preliminary Selection of Permeation Enhancers

This chapter presents and analyzes the results of studies identified through a systematic search, aiming to evaluate permeation enhancers for selection in subsequent research stages. To establish the state of the art regarding the use of permeation enhancers for promoting the intestinal absorption of salmon calcitonin, a systematic search was conducted in the MEDLINE

database using the PubMed interface. Both *in vitro* and *in vivo* studies investigating the efficacy and safety of permeation enhancers in increasing the absorbed fraction of salmon calcitonin across various intestinal membrane regions within specific co-formulations were included in this analysis. The identified studies underwent critical appraisal with the primary objective of extracting and synthesizing relevant data on the efficacy and safety profiles of the investigated permeation enhancers. The selection process considered scientific evidence related to the efficacy and safety of permeation enhancers, as well as their feasibility within the planned research context. Personal contributions involved establishing and applying the selection criteria for the permeation enhancers identified in the literature, detailed in the doctoral thesis at pages 32 to 34 and 42 to 46, respectively. Feasibility was assessed based on the associated costs, availability of material resources, complexity of preparation processes and accessibility of required equipment. Consequently, three feasibility categories were defined:

- High feasibility: low costs, straightforward preparation methods, and readily available equipment, creating favorable conditions for continued and expanded research.
- Moderate feasibility: higher costs but still accessible processes and equipment, requiring budget adjustments and prioritization.
- Low feasibility: significant constraints such as prohibitive costs, high technical complexity and lack of specific equipment, substantially limiting research feasibility.

The purpose of this evaluation was to identify and anticipate technical and logistical obstacles that could influence the practical implementation of the proposed methodology, ensuring a balance between scientific rigor and the practical sustainability of subsequent experiments.
Following this process, four permeation enhancers were selected for inclusion in subsequent research stages:

- S-nitroso-N-acetyl-DL-penicillamine (SNAP), a nitric oxide donor
- Sodium taurodeoxycholate (TDC), a bile salt
- Dimethyl-palmitoyl-ammonio-propane sulfonate (PPS), a zwitterionic surfactant
- Tetradecyl maltoside (TDM), a nonionic surfactant

3. Contributions to the *In Vitro* Evaluation of Four Selected Permeation Enhancers: Efficacy and Safety Assessment for Increasing the Transport of Salmon Calcitonin Across Caco-2 Cell Lines [16]

This section outlines personal contributions, highlighted by conducting the first study directly evaluating the effects of SNAP, TDC, PPS, and TDM (selected in the previous stage) on the permeation of salmon calcitonin across Caco-2 cell lines. This research allowed for the ranking of the permeation enhancers, based on their impact on the apparent permeability coefficient values of salmon calcitonin, measured two hours post-exposure (Papp; efficacy parameter) and the transepithelial electrical resistance of the cell lines (TEER; safety parameter). The study is described in detail within the doctoral thesis (pages 49 to 73), and the results have been published in the journal Farmacia [16].

Analysis of data from this study revealed that TDM (0.2 mg/mL) was the only permeation enhancer showing a statistically significant effect in increasing the apparent permeability coefficient of salmon calcitonin compared to control, SNAP (0.002 mg/mL), and TDC (0.1 mg/mL). Specifically, TDM (0.2 mg/mL) increased the Papp value of salmon calcitonin by 282% compared to control ($p = 0.017$), by 240% compared to SNAP (0.002 mg/mL) ($p = 0.01$) and by 149% compared to TDC (0.1 mg/mL) ($p = 0.036$).

No other statistically significant differences were observed between the permeation enhancers regarding their effects on the Papp values of sCT. Nevertheless, numerically higher Papp values for salmon calcitonin were recorded for solutions containing permeation enhancers compared to control solutions, suggesting a trend of increased permeation associated with all the investigated permeation enhancers, as depicted in Figure 3.1.

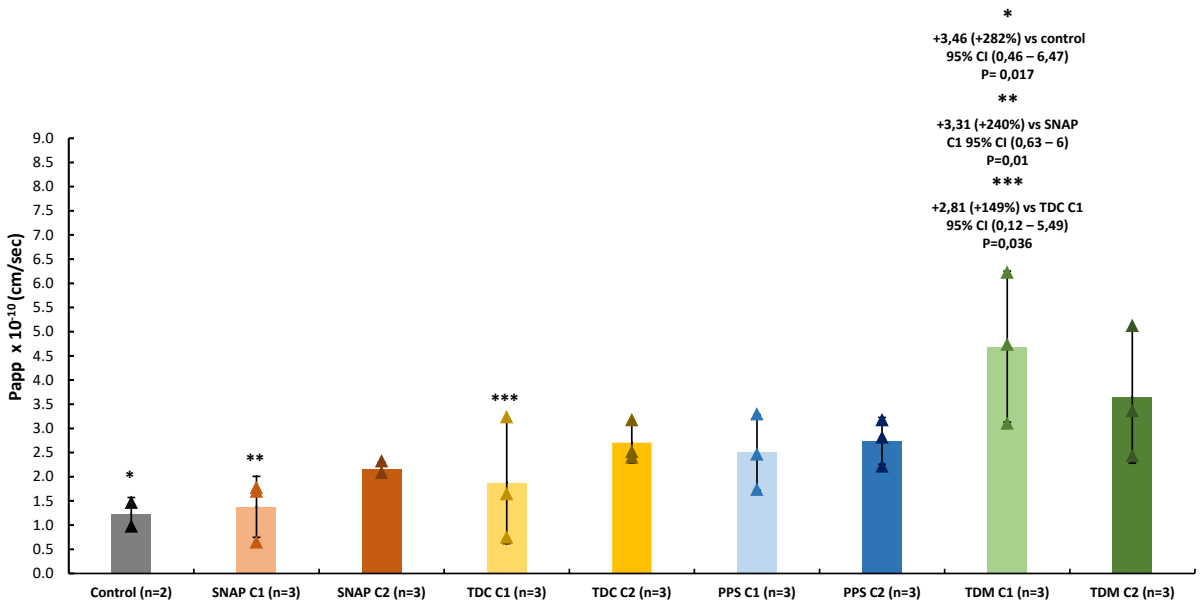

Figure 3.1 The enhancement effects of SNAP (C1 = 0.002 mg/mL; C2 = 0.22 mg/mL), TDC (C1 = 0.1 mg/mL; C2 = 0.25 mg/mL), PPS (C1 = 0.1 mg/mL; C2 = 0.2 mg/mL) and TDM (C1 = 0.2 mg/mL; C2 = 1 mg/mL) on the permeation of sCT across the Caco-2 cell lines

Results are expressed as the mean ± SD of three or two determinations. Each triangle represents one measurement. (*): $p < 0.05$ compared with control; (**): $p < 0.05$ for TDM C1 compared with SNAP C1; (***): $p < 0.05$ for TDM C1 compared with TDC C1.

Compared to control, all investigated permeation enhancers had a statistically significant effect on the TEER values of Caco-2 cell lines, measured two hours post-exposure. No significant differences were observed among the permeation enhancers in terms of their effects on TEER values at two hours post-exposure. The effect of all permeation enhancers was concentration-dependent, with higher concentrations leading to greater numerical reductions in TEER. At 15, 60, and 120 minutes post-exposure, TDM (0.2 mg/mL) exhibited the most pronounced reduction in TEER, whereas SNAP (0.002 mg/mL) demonstrated the weakest effect (Figure 3.2).

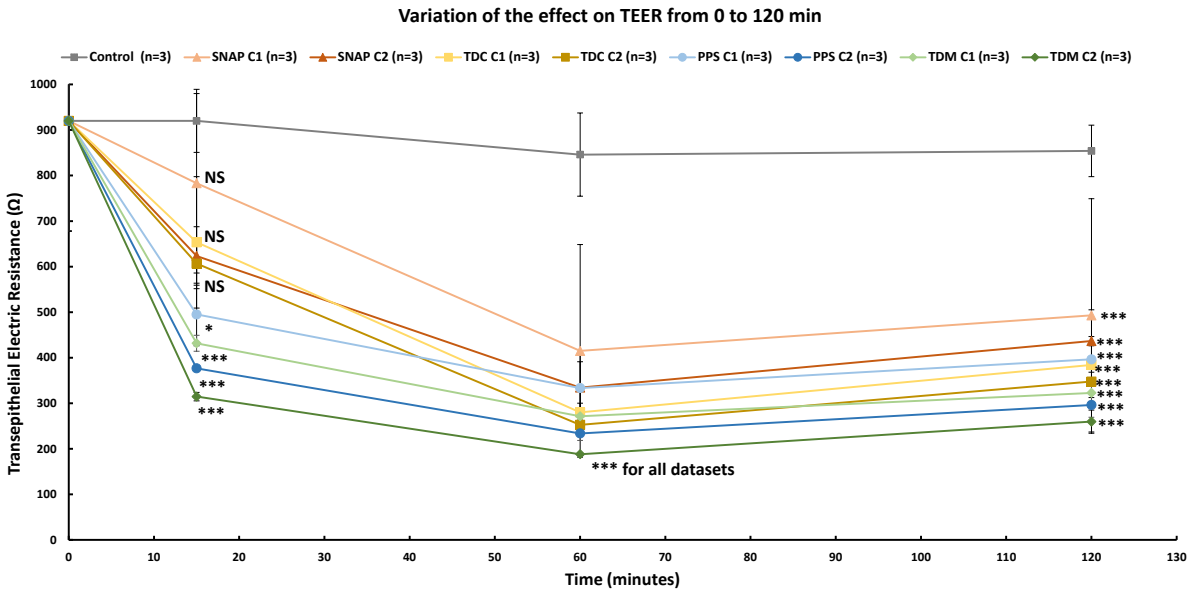

Figure 3.2. The effects of SNAP (C1 = 0.002 mg/mL; C2 = 0.22 mg/mL), TDC (C1 = 0.1 mg/mL; C2 = 0.25 mg/mL), PPS (C1 = 0.1 mg/mL; C2 = 0.2 mg/mL), TDM (C1 = 0.2 mg/mL; C2 = 1 mg/mL) on the TEER of Caco-2 cell lines at 15 minutes, 60 minutes and 120 minutes

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

By correlating these two datasets (Papp and TEER), the study provides a comprehensive understanding of the balance between the efficacy and safety of the analyzed absorption enhancers, thereby contributing to the establishment of selection criteria for the future development of oral pharmaceutical formulations. The study demonstrated that tetradecyl maltoside exhibited significantly greater efficacy in enhancing the transport of salmon calcitonin compared to S-nitroso-N-acetyl-DL-penicillamine and sodium taurodeoxycholate, and similar efficacy to dimethyl-palmitoyl-ammonio-propane sulfonate. An impact on TEER was observed for all evaluated permeation enhancers, consistent with the known class effect of these active excipients. Based on the results of this screening study, tetradecyl maltoside and dimethyl-palmitoyl-ammonio-propane sulfonate were selected for further investigation. As shown in Figure 3.3., TDM C1 exhibited the greatest effect on increasing the Papp value of sCT, followed by TDM C2, PPS C2, TDC C2, PPS C1, SNAP C2, TDC C1, and SNAP C1.

An important aspect that requires further investigation is determining the time interval necessary for TEER to return to baseline, after exposure to each permeation enhancer, as this is essential for a thorough evaluation of the toxicity of these agents.

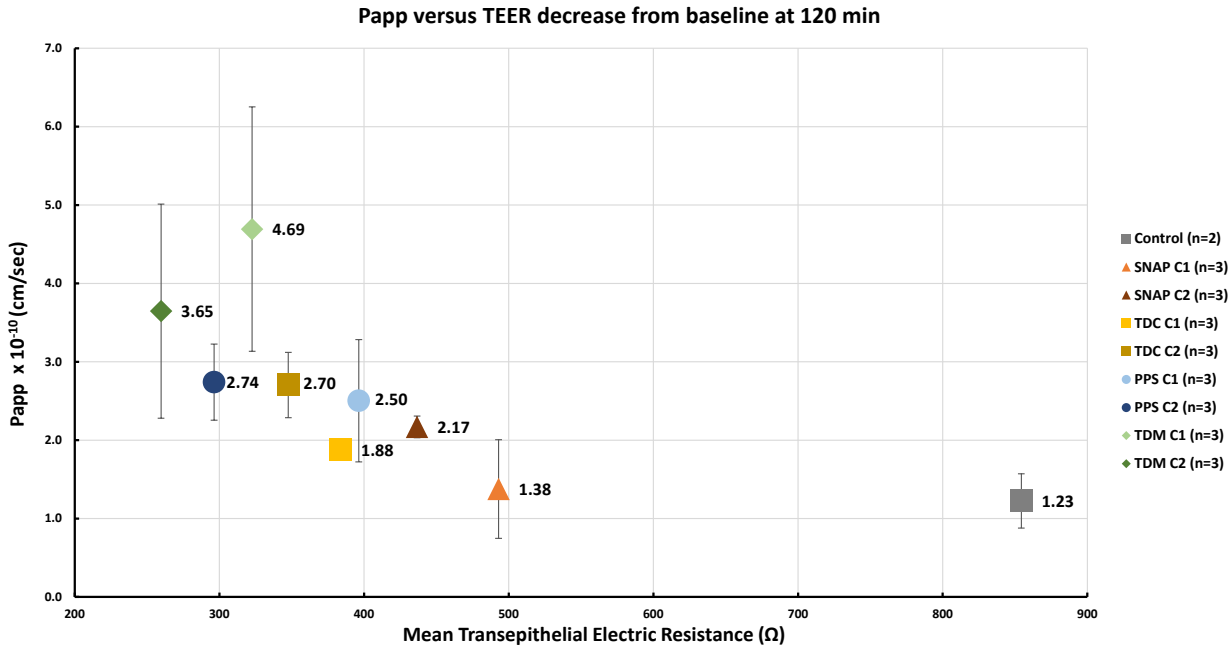

Figure 3.3. The effects of SNAP (C1 = 0.002 mg/mL; C2 = 0.22 mg/mL), TDC (C1 = 0.1 mg/mL; C2 = 0.25 mg/mL), PPS (C1 = 0.1 mg/mL; C2 = 0.2 mg/mL), TDM (C1 = 0.2 mg/mL; C2 = 1 mg/mL) on the permeation of sCT across the Caco-2 cell lines (vertical axis) and on the TEER of Caco-2 cell lines at 120 minutes (horizontal axis)

Results for Papp are expressed as the mean ± SD of three or two measurements and for TEER only as the mean.

Based on the results of this screening study, tetradecyl maltoside and dimethyl-palmitoyl-ammonio-propane sulfonate were selected for further investigation.

4. Contributions to the *In Vitro* Evaluation of the Efficacy and Safety of Tetradecyl Maltoside and Dimethyl Palmitoyl Ammonium Propane Sulfonate in Enhancing the Transport of Salmon Calcitonin and Pramlintide Across Caco-2 Cell Lines and the Utilization of a Mathematical Extrapolation Model for Human Applications [17]

The personal contributions described in this chapter involved conducting the study that evaluated the effects of tetradecyl maltoside and dimethyl-palmitoyl-ammonio-propane sulfonate on salmon calcitonin and pramlintide, utilizing analytical methods with high precision (liquid chromatography coupled with tandem mass spectrometry) and incubation conditions different from those used in the previous study. The research methods and results are described within the doctoral thesis (pages 74 to 102) and the main results have been published in the journal Farmacia [17].

Considering the structural similarities between Pram (37 amino acids, 3949.9 Da) [13] and sCT (32 amino acids, 3432 Da) [11], this study compared the efficacy and safety of TDM and PPS (at two different concentrations, selected based on the results of the previous study: 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) [16] in enhancing the permeation of sCT relative to Pram, *in vitro*, using Caco-2 cell lines. Efficacy was evaluated by measuring the apparent permeability coefficient (Papp), while safety was assessed by measuring the effect of the two enhancers on the transepithelial electrical resistance (TEER) of the cell lines. Additionally, using published data and methodologies [18-20], a mathematical model was developed to estimate how the *in vitro* Papp values might translate to humans.

At the 2-hour mark TDM and PPS (both concentrations), significantly increased the Papp values for sCT, indicating a substantial enhancement of permeation. In contrast, only TDM C1, PPS C1, and PPS C2 resulted in increased Papp values for Pram compared to control, while TDM C2 had a non-significant effect on Papp for Pram. Notably, Papp values for the control solutions (sCT and Pram without permeation enhancers) could not be quantified as they were below the detection limit of the analytical method used. Both TDM and PPS demonstrated a significantly greater increase in the permeation of sCT compared to Pram, as evidenced by the

higher Papp values for sCT in the presence of both enhancers ($p < 0.0001$ for all comparisons), as shown in Figure 4.1.

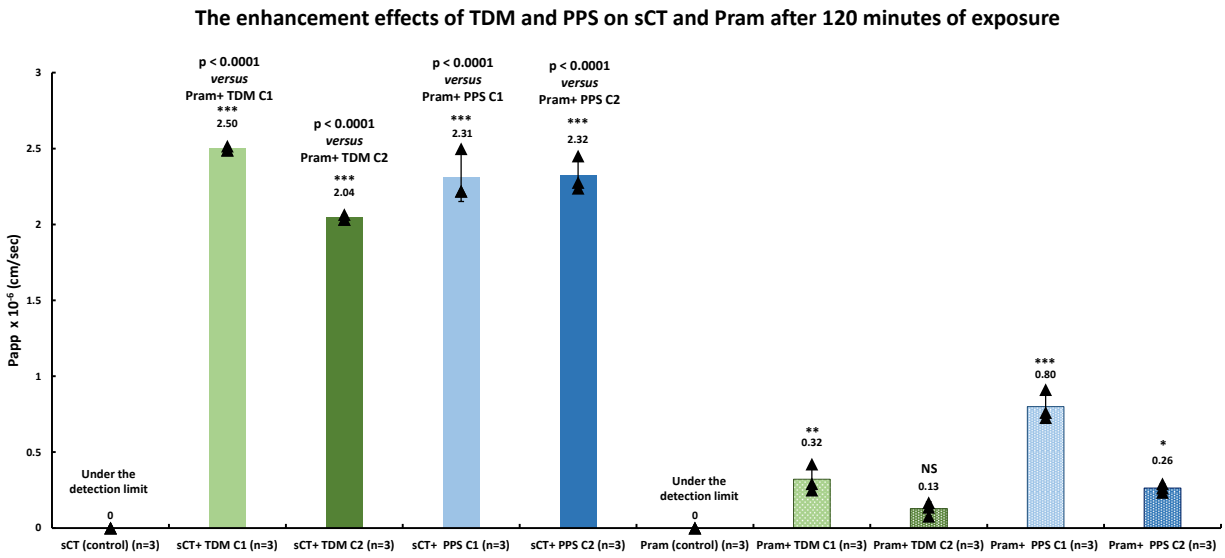

Figure 4.1. The enhancement effects of TDM (C1 = 0.2 mg/mL; C2 = 1 mg/mL) and PPS (C1= 0.1 mg/mL; C2 = 0.2 mg/mL) on the permeation of sCT and Pram across the Caco-2 cell lines

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.

Compared to the values obtained for the control solutions, TDM and PPS at all tested concentrations induced a statistically significant decrease in TEER values at 120 minutes post-exposure (Figure 4.2.A for sCT and Figure 4.3.A for Pram), with no significant differences observed between the studied permeation enhancers. This observation is consistent with the known effects of these permeation enhancers on TEER as part of their mechanism of action (an increase in Papp values is associated with a decrease in TEER values). As illustrated in Figure 4.2.B for sCT and Figure 4.3.B for Pram, TEER values remained low even at 6 hours post-exposure, suggesting that the effect was not reversible within this timeframe.

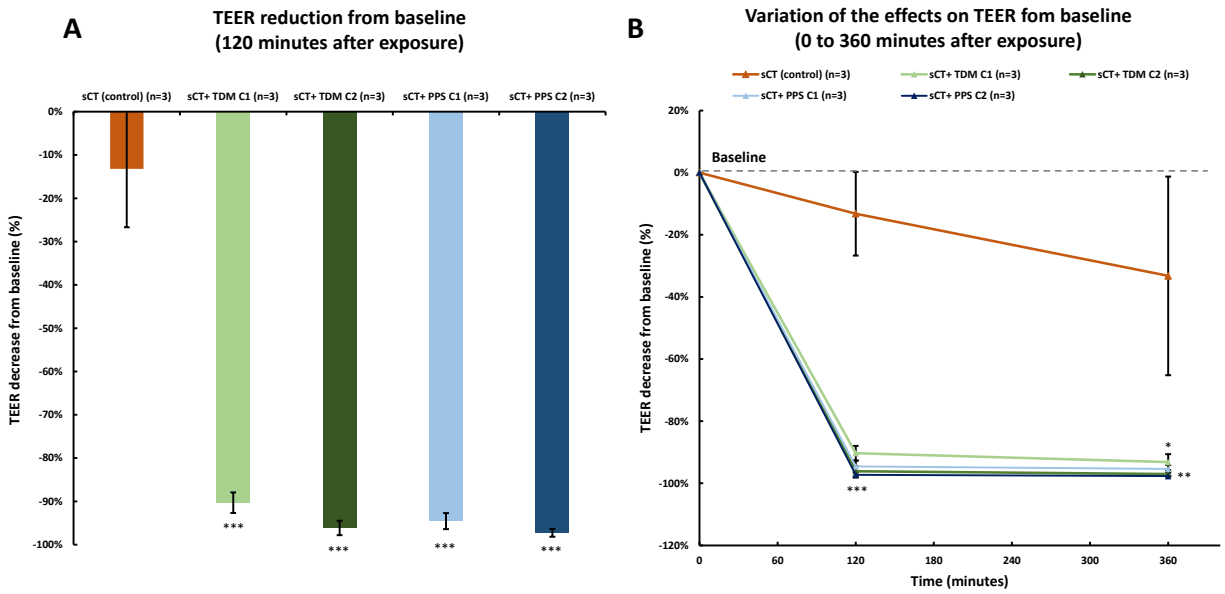

Figure 4.2. The effects of sCT in combination with TDM (C1 = 0.2 mg/mL; C2 = 1 mg/mL) and PPS (C1 = 0.1 mg/mL; C2 = 0.2 mg/mL) on TEER, 120 minutes after exposure (A) and the variation of the effects on TEER from baseline to 360 minutes after exposure (B)

(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.

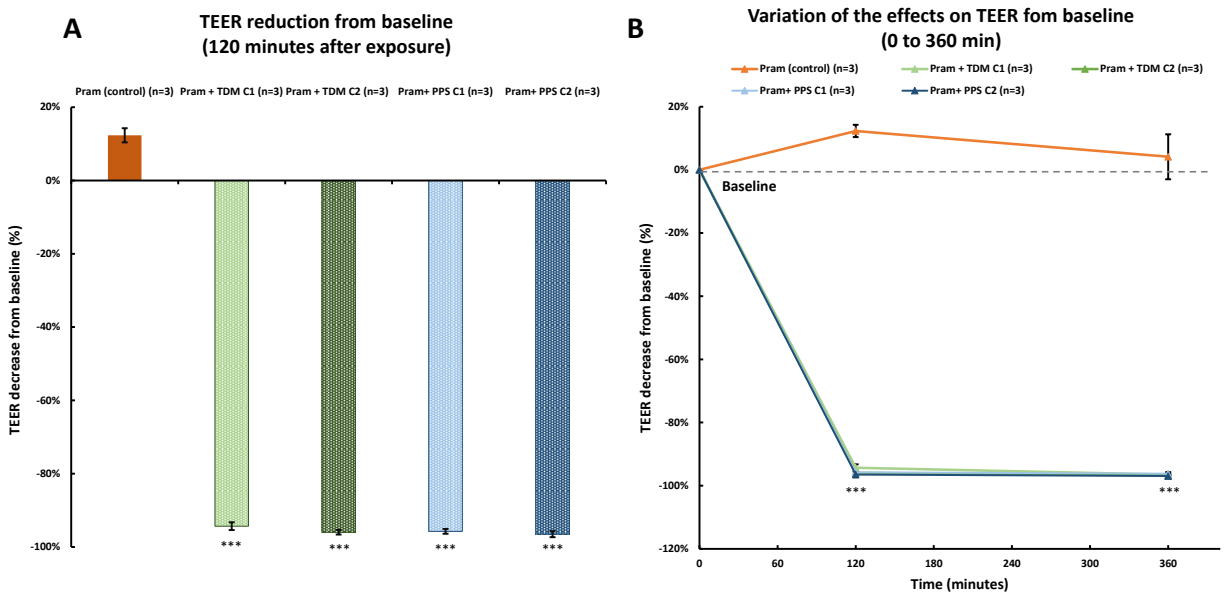

Figure 4.3. The effects of Pram in combination with TDM (C1 = 0.2 mg/mL; C2 = 1 mg/mL) and PPS (C1 = 0.1 mg/mL; C2 = 0.2 mg/mL) on TEER, 120 minutes after exposure (A) and the variation of the effects on TEER from baseline to 360 minutes after exposure (B)

(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 study demonstrated that PPS is an optimal permeation enhancer for both active peptides and reinforced the conclusion of the first study by showing that TDM exhibits high efficacy in enhancing the permeation of Caco-2 cell lines for salmon calcitonin. Based on the predictive model of human absorption, it was observed that both TDM and PPS significantly influence the jejunal absorption of sCT, with absorption rates exceeding 70%. For Pram, the estimated jejunal absorption varied from 52% with TDM C2 to 68% with PPS C1 (Figure 4.4).

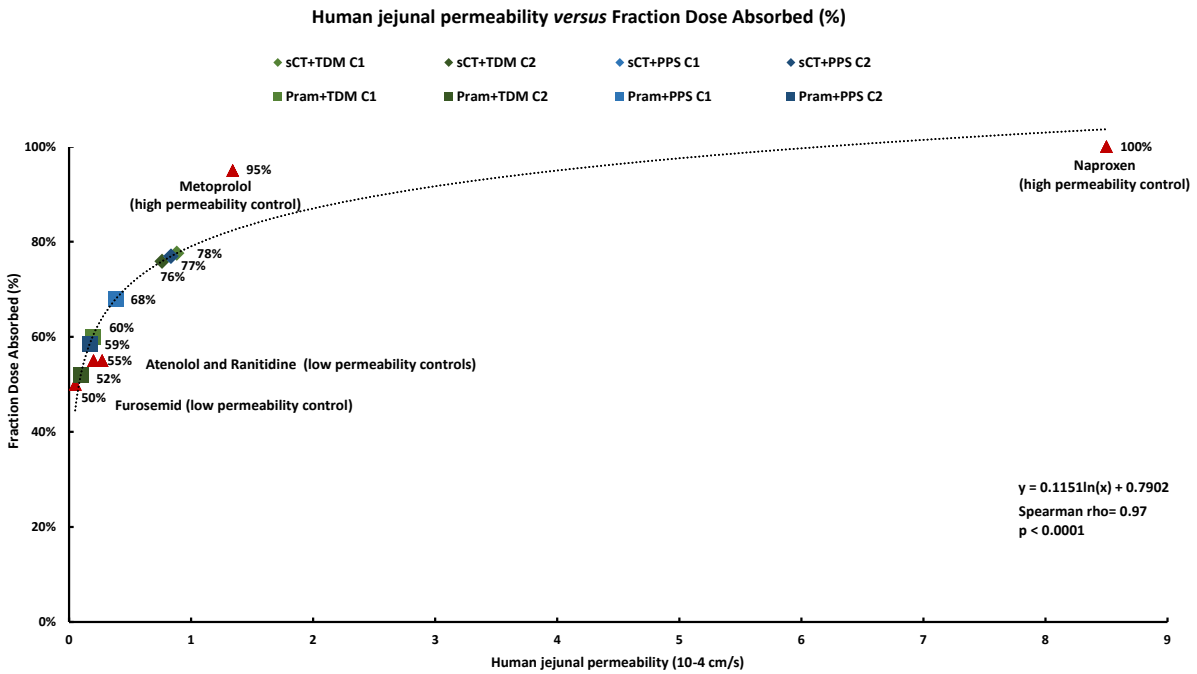

Figure 4.4. The relationship between the hPeff values and the corresponding fractions of dose absorbed reported in the literature

For the test compounds, results are expressed as the mean of 3 determinations.

This *in vitro* study demonstrated that TDM and PPS significantly improve the permeation of sCT and Pram across Caco-2 cell lines. Although sCT and Pram share certain structural and functional similarities, the investigated permeation enhancers exhibited distinct effects on each peptide. The study results indicate that PPS is the most suitable permeation enhancer for co-formulation with both peptides, while TDM is particularly effective in enhancing the permeation of sCT.

5. General Conclusions

The primary objective of this research project was to identify those permeation enhancers with the most pronounced effect on increasing the transport of salmon calcitonin and to conduct a direct comparison of their effects (*in vitro*, using Caco-2 cell lines) on both salmon calcitonin and pramlintide. In the initial phase, four permeation enhancers (SNAP, TDC, TDM, and PPS)

were selected based on efficacy and safety data reported in the literature, as well as on their feasibility within the context of the planned research. These enhancers were subsequently evaluated in an *in vitro* screening study, aimed at ranking them according to their efficacy and safety for increasing the transport of salmon calcitonin from the apical to the basolateral compartments of the Caco-2 cell lines. This represents the first study directly evaluating the effects of SNAP, TDC, PPS, and TDM on the permeation of sCT.

Based on the data provided by this study, only TDM and PPS were selected for the next research phase, to investigate their effects on both salmon calcitonin and pramlintide. Although differences were observed between the effects on sCT and Pram, the study results demonstrated that PPS achieved significant efficacy in enhancing the permeability of Caco-2 cell lines for both peptides, recommending it as a viable common permeation enhancer.

To assess the relevance of the obtained data, a mathematical model based on published methods and data was developed. The model was used to estimate the extent to which the apparent permeability coefficients determined *in vitro* could be extrapolated to humans. The modeling results indicated that TDM and PPS could enable absorption rates exceeding 77% for sCT and over 52% for Pram, thus supporting the potential of these enhancers to facilitate intestinal absorption under human physiological conditions.

Overall, the results of this research project, obtained using standardized Caco-2 cell lines (a fundamental model for understanding and predicting intestinal absorption of active substances), represent a promising beginning towards developing a technology that could allow the oral administration of salmon calcitonin and pramlintide. Furthermore, these findings may provide an important basis for future studies involving other calcitonin or amylin derivatives currently under development for the treatment of diabetes, obesity, and other metabolic diseases.

This research project marks only an initial step toward developing a technology for the oral administration of active peptides. Additional studies are required to validate these results and continue the research efforts. Further evaluation of the effects of these permeation enhancers on a broader range of peptides could accelerate the development of innovative oral therapies, directly impacting patient treatment adherence and long-term therapeutic efficacy.

List of Published Scientific Papers on the Research Topic

1. **Hamza A**, Șaramet G, Actualities In Endocrine Pharmacology: Advances In The Development Of Oral Formulations For Calcitonin And Semaglutide. *Acta Endocrinol (Buchar).*, 2020; 16(3): 383-387. (Chapter 1)
   DOI: https://doi.org/10.4183/aeb.2020.383
   Link: https://acta-endo.ro/Archive/Abstract?doi=2020.383
   Indexed PubMed (PMID: 33363667)
   ISSN: 1843 - 066X (online) and 1841 – 0987 (print)
   Impact Factor 2020: 0.877

2. **Hamza A**, Șaramet G, A comparative study of four permeation enhancers for increasing the transport of salmon calcitonin on Caco-2 cell lines. *Farmacia.* 2024; 72(6): 1342-1352. (Chapter 3)
   DOI: https://doi.org/10.31925/farmacia.2024.6.13
   Link: https://farmaciajournal.com/issue-articles/a-comparative-study-of-four-permeation-enhancers-for-increasing-the-transport-of-salmon-calcitonin-on-caco-2-cell-lines/
   ISSN: 2065-0019 (online) and 0014-8237 (print)
   Impact Factor: 1,6 (2022)

3. **Hamza A**, Prisada RM, Șaramet G. 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. *Farmacia.* 2025;73(2):338–49. (Chapter 4)
   DOI: https://doi.org/10.31925/farmacia.2025.2.9
   Link: https://farmaciajournal.com/issue-articles/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/
   ISSN: 2065-0019 (online) and 0014-8237 (print)
   Impact Factor: 1,6 (2022)

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