Doctoral Thesis

Kinetics and pathways of intestinal absorption and enzymatic cleavage of therapeutic proteins upon peroral delivery

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Kinetics and Pathways of Intestinal Absorption and Enzymatic Cleavage of Therapeutic Proteins upon Peroral Delivery

A dissertation submitted to the

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Doctor of Natural Sciences

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**Background and Purpose**

There is an increasing use of natural peptides and proteins as therapeutics. For example, eight different protein or peptide drugs have been registered in Switzerland in the last two years\(^1\). In several cases such proteinacious\(^2\) therapeutics are to be applied for long-term treatments. Despite the growing interest in alternative routes of delivery the most popular and desirable method of drug administration remains the oral route. This, however, implies major challenges for the pharmaceutical development and necessitates the invention of efficient and safe delivery systems for peptides and proteins. In addition to the problems which are encountered by all drugs administered perorally, e.g., hepatic first pass metabolism, proteinacious drugs are subjected to the proteolytic activity in the intestine, low intrinsic permeability in the intestinal epithelium and to a certain extent the mucus layer on the intestinal epithelium and the involvement of a secretion pumps, i.e., P-glycoprotein. To overcome these barriers various approaches have been proposed:

- targeting to a particular segment of the gastrointestinal tract in which there is lower enzymatic activity (e.g., the colon)
- using specialised drug carrier systems which shuttle the peptide or protein to its absorption site (e.g., mucoadhesive chitosan-coated liposomes, azo-polymercoated drugs)
- lowering the proteolytic activity (e.g, use of enzyme specific inhibitors)
- improving the resistance to breakdown by structural modification (e.g., cyclisation of peptides)
- inhibition of P-glycoprotein (e.g., verapamil, Tween 80)

To assess the feasibility of peroral delivery of a peptide and/or protein dosing the compound *in vivo* in an animal model or in humans certainly is the method of choice for determination of bioavailability. However, the mechanistic interpretation of the results may be difficult due to the complexity of this model which is mostly due to the overlay of

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\(^1\) IKS 1999, personal communications

\(^2\) Hereafter the term "proteinacious" is used as a synonym for "peptide and protein"
various absorption-controlling factors. Therefore, in order to study the influence of each factor involved in the overall absorption process, the use of *in vitro* and *in situ* methods is essential. Various *in vitro* and *in situ* models are applied, each encompassing different phases of the intestinal absorption process. Thus, the choice of a suitable model has to be in accordance with the specific questions of each investigation. For example, the influence of both luminal and brush border membrane bound enzymes on the stability of a proteinacious compound may be determined by a combination of various *in vitro* models, e.g., incubation studies in the presence of pancreatic extract, purified enzymes, perfusates collected from the intestine, brush border membrane vesicles or incubation studies using excised intestinal mucosa.

Well characterised *in vitro* and *in situ* intestinal models for intestinal transport studies are e.g., the Caco-2 cell culture model, excised intestinal mucosa and single-pass perfusion techniques. The excised mucosa represents a more complex model than the homogeneous Caco-2 cell monolayer with respect to the variety of epithelial cells, the mucus layer, the brush border associated enzymes and membrane associated luminal enzymes. In addition, for the assessment of the effective permeability in different intestinal segments, the excised mucosa model may be the first choice. However, in order to investigate the influence of a specific transport carrier or secretion process such as P-glycoprotein on the membrane permeability the Caco-2 cell model might be more adequate since it is easier to characterise and, thus, specific mechanisms can be defined more easily. In contrast to both, excised mucosa and Caco-2 cell model, the *in situ* single-pass perfusion with concurrent blood sampling from the portal vein allows to investigate solute uptake from the intestine into the blood circulation. Thus, this model is suitable when the assessment of the overall absorption of a compound is the primary focus of interest. In this work, the feasibility of peroral delivery was assessed for IGF-I and, with respect to P-gp, also metkephamid as therapeutically relevant peptide. The influence of the main absorption barriers - enzymatic cleavage, mucus and epithelial transport - on the uptake of proteins was studied by means of
stability and transport studies. This work is divided into 4 chapters as shown below:

- An extended introduction about the assessment of barriers for proteinacious drug uptake, such as enzymatic cleavage, mucus and epithelial transport as related to the feasibility of IGF-I absorption.
- The characterisation of P-glycoprotein-mediated efflux in Caco-2 cell monolayers focusing on the influence of culturing conditions and drug exposure on P-gp expression levels.
- Stability of IGF-I in the gastrointestinal tract of various species in relation to typical substrates of pancreatic proteases.
- Transport of IGF-I across the intestinal epithelium: permeation rates and mechanisms relative to permeability markers.

Each of these aspects will be dealt with in separate chapters.
Abstract

The kinetics and pathways of intestinal absorption and enzymatic cleavage of therapeutic proteins upon peroral delivery are the subject of this work. More specifically, Insulin-like growth factor I (IGF-I) was particularly focussed on. There is a rapidly growing interest in the therapeutic use of natural peptides in medicine, since several endogenous peptides play an important role in the regulatory processes of body functions as enzymes, hormones, neuropeptides or neurotransmitters and cytokines. In various cases these proteinacious drugs are applied for long-term treatments. For these chronic therapies the most convenient method of drug administration is via the oral route. This route, however, presents big challenges to the development of effective delivery systems for peptides and proteins, mainly due to enzymatic degradation in the lumen, the mucus layer on the intestinal epithelium and the low permeability. Chapter I gives an extensive overview of the influence of these three main barriers with special focus on IGF-I.

Low absorption of peptides may result from their high hydrophilicity and molecular weight, respectively. Secretion processes may contribute to the limited absorption of various drugs. An important example for an efflux-pump is P-glycoprotein (P-gp). P-gp is known to secrete various peptide drugs. Caco-2 cells are a relevant in vitro model to study the influence of such a secretion process on the permeability of drugs. Therefore, the influence of cell culture conditions and previous drug exposure on P-gp expression levels in Caco-2 cells was determined (Chapter II). In this study, the expression of P-gp is demonstrated (i) visually by confocal laser scanning microscopy (CLSM), (ii) functionally by transport studies with substrates of the efflux pump and (iii) quantitatively by flow cytometry (FCM) analysis using specific monoclonal antibodies (anti P-gp MRK 16 as an external antibody and C219 P-GlycoCheck as an internal antibody). Trypsinisation of the cells post confluency led to a decrease of P-gp expression levels, while prior trypsinisation led to an increase after long term cultivation. Culturing the cells on polycarbonate filters did not elicit a significant change of P-gp
expression over time in culture, whereas in plastic flasks (polystyrene) a decrease was detected. Using CLSM a strong fluorescence on the apical side of Caco-2 cell monolayers was observed, as a result of incubation with MRK 16 as primary and IgG Cy5 as secondary antibody. Previous drug exposure of the cells showed that verapamil, celiprolol and vinblastine induced the P-gp expression, while metkephamid (MKA) decreased the P-gp expression level as compared to the control. Permeation studies consolidated the immunohistochemical results that P-gp is expressed in the Caco-2 cells, albeit at variable concentrations which requires careful control when this model is to be used in quantitative structure/secretion studies (Chapter II). For talinolol and MKA a higher transport from basolateral to apical than in the reverse direction was found. Incubation of the cell monolayer with MRK 16 reduced the secretory process to the apical side, but did not influence the $^3$H-mannitol flux.

IGF-I is a 7648 Da protein consisting of 70 amino acids and is considered among others as a long-term treatment for type II diabetes.

In order to investigate the peroral route of administration as an alternative to the parenteral injection treatment, the metabolism of IGF-I in the gut was investigated in the presence of crude porcine pancreatic enzymes (CPPE) from pig and flushings of the small and large intestine from pig, rat and dog. Moreover, incubation studies with purified pancreatic enzymes which are present in the intestine, such as aminopeptidase M, carboxypeptidase A, $\alpha$-chymotrypsin and trypsin, were performed in order to determine the most active enzymes responsible for the metabolism of IGF-I (Chapter III).

IGF-I was degraded in the jejunum, ileum and colon. Significant intra- as well as inter-species differences of IGF-I stability were observed. IGF-I was less stable in the ileum as compared to jejunum and colon of all species examined. It could be shown that IGF-I was mainly degraded by chymotrypsin ($t_{1/2} = 2.7$ min) and trypsin ($t_{1/2} = 34.6$), whereas in the presence of aminopeptidase M and carboxypeptidase A it was stable within 90 min. Instability could be improved by adding serineprotease inhibitors such as aprotinin, soybean trypsin inhibitor and N$\alpha$-p-tosyl-L-lysine
chloromethyl ketone (TLCK) or casein. While casein strongly increased
the stability of IGF-I in all segments of the three species, the stability of N-
acetyl-L-tyrosin ethyl ester and N-benzoyl-L-tyrosin ethyl ester, both
typical substrates for chymotrypsin, was only partially increased.
Interaction studies with casein and IGF-I elucidated that under the
conditions used ~ 70 % of IGF-I was bound to casein leading to the
conclusion that the specific protection of IGF-I occurred due to interaction
with casein.

It could be shown that IGF-I was mainly degraded by
chymotrypsin- and trypsin-like enzymes and that the stability in the
intestine could be clearly increased by adding a mixture of chymotrypsin
and trypsin inhibitors or casein. We assessed the stability of IGF-I in
relation to substrates of the pancreatic enzymes. A degradation rate of 35
nmol mL⁻¹ min⁻¹ in human jejunal fluid was predicted. Additionally, the
stability of IGF-I showed a significant inter-species difference.

The influence of the permeability barrier in the intestine for IGF-I
bioavailability was investigated. The transport of IGF-I across intestinal
epithelium was characterised with respect to its permeation mechanism and
also its magnitude in relation to permeability markers. Two systems were
applied in order to study IGF-I absorption: permeation studies across rat,
human and porcine excised mucosa in Ussing chambers and single-pass
perfusion studies in rats (Chapter IV).

Across porcine, human and rat mucosa of the colon and jejunum,
only a low permeability of IGF-I was observed. Addition of casein
increased IGF-I permeability significantly, yet permeabilities were still
lower than PEG 4000 permeability, which is known as a non-absorbable
drug. There was no significantly different permeation across rat, porcine
and human mucosa. Through a mixture of chymotrypsin- and trypsin-
inhibitors IGF-I permeability increased clearly, but in contrast to casein
this mixture damaged the integrity of the mucosa indicated by the
increased flux of PEG 4000. Saturable permeation kinetics was observed
for IGF-I across porcine mucosa. IGF-I plasma concentrations were not
significantly increased after single-pass perfusion compared to endogenous
plasma concentrations, moreover, casein did not seem to have an
enhancing effect when this system was applied, which was in accordance with the permeation studies.

This study thus elucidated the permeation kinetics of IGF-I in relation to permeability markers. Moreover, the influence of various enhancers on the permeability of IGF-I was demonstrated. Also, the influence of target site in the gut on the IGF-I absorption was shown.

Generally, peroral delivery of IGF-I is clearly limited by its enzymatic stability and its low absorption. However, we could show several approaches to increase the stability and permeability of IGF-I.