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OPTIMISATION OF PULMONARY DRUG DELIVERY OF THE MODEL PEPTIDE DRUG, SALMON CALCITONIN

by

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DECLARATION

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SUMMARY

The use of the pulmonary route is a viable alternative administration route to injection, in particular for drugs with poor systemic bioavailability upon oral delivery such as therapeutic peptides and proteins. However, information about the expression and activity of metabolic enzymes (e.g., peptidases) on disposition of biopharmaceuticals after inhalation is critically missing. This information could help to better understand, if protective measures such as PEGylation, chemical modification or peptidase inhibitors are meaningful for pulmonary delivery of biotechnology medicines. It was the aim of this work to investigate the role of peptidases in pulmonary drug delivery of a model peptide drug, salmon calcitonin (sCT), and to find a suitable formulation for improved pulmonary drug delivery of this peptide. In a first step, mRNA expression levels of selected catabolising peptidases were determined in human lung epithelial cells. The proteolytic enzymes (i.e., carboxypeptidases: CPA1, CPA2, CPB, CPM; gamma-glutamyltransferases: GGT1, GGT2; angiotensin-converting enzymes: ACE, ACE2; aminopeptidases: APA, APB, APN, APP1, APP2, APP3; endopeptidases: 24.11 (neprilysin), 24.15 (thimet oligopeptidase), 24.18 (meprin A); enteropeptidase; trypsin 1, trypsin 2; neutrophilic elastase; dipeptidyl peptidase 4; gamma-glutamylhydrolase) were investigated by semi-quantitative RT-PCR in human bronchial (hBEpC, Calu-3, 16HBE14o-) and alveolar (A549) epithelial cells, respectively. Generally, mRNA encoding peptidases was widely expressed, but obvious differences were observed in the expression pattern between the investigated cell types. Gastrointestinal Caco-2 epithelial cells were used as comparison and expressed the highest number of proteolytic enzymes. Although mRNA expression does not necessarily signify enzyme functionality, the results provide the first comprehensive overview in this field. The investigations continued by studying the fate of the model peptide drug, sCT at the respiratory epithelial barrier with particular emphasis on enzymatic degradation by trypsin, α-chymotrypsin and neutrophil elastase. HPLC studies revealed rapid degradation of sCT by neutrophil elastase, and confirmed the peptide's breakdown by trypsin and chymotrypsin. Abundance of all three peptidases in cell homogenates and supernatants

was observed by Western blotting. However, no or only low activity of the enzymes in both cell supernatants and homogenates were detected by UV measurements with specific substrates. This was likely caused by respective enzyme inhibitors such as alpha-1 anti-trypsin. When co-incubated at 37℃ with cell monolayers or in supernatants *in vitro*, sCT concentration remained unchanged over the period of 2 h, independent of the cell type used. After incubation with homogenates for 2 h, sCT was markedly degraded. It was concluded that peptidases responsible for sCT degradation in the lungs were found to be functionally expressed in human respiratory epithelial cells *in vitro*. Despite the low activity levels measured, it appeared reasonable to protect sCT from enzymatic degradation to further improve its stability and hence, bioavailability when delivered as an aerosol for systemic action. In order to combine the shielding from degrading enzymes with improved membrane permeability, it was the aim of the last project on my work to produce a conjugate of sCT with a polyethylene glycol (PEG) – lipid residue (i.e., DSPE-PEG_{2000}). Size exclusion chromatography (SEC) and diffusion light scattering (DLS) of the synthesis products revealed particles of 12-13 nm diameter. Surprisingly, no covalent conjugation could be confirmed by matrix assisted laser desorption / ionisation mass spectrometry (MALDI-TOF-MS). Equimolar amounts of sCT and DSPE-PEG, however, spontaneously assemble in aqueous solvents, before conjugation can occur. Next, the spontaneously formed complexes – likely micelles - were further characterised for their suitability to improve pulmonary delivery of the peptide drug. DLS, cryo-transmission electron miscroscopy (cryo-TEM) and ^{31}P-nuclear magnetic resonance (NMR) spectroscopy confirmed the presence of small micellar structures. Stability of the micellar complexes against trypsin, α-chymotrypsin and neutrophil elastase was improved compared to the stability of plain sCT *in vitro*. *In vivo* studies using an experimental model of intratracheal aerosolisation into rats showed an enhanced pharmacokinetic performance of the micellar formulation. The herein described PEG-lipid micelles are hence promising carrier systems to enhance the pulmonary delivery of sCT.

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Chapter 1

Introduction

1.1. PULMONARY DRUG DELIVERY

For several years now, delivery of therapeutic agents via the pulmonary route has received significant attention (Patton and Platz 1992, Gonda 2000, Patton and Byron 2007). Forbes and Ehrhardt state three main reasons for the interest in pulmonary drug delivery (i) incessant interest in the treatment of lung diseases by inhalation of pharmaceuticals (ii) the recognition that pharmacokinetic profiles can be improved by absorption from the lung, and (iii) the increased development of peptide and protein drugs which proved unsuitable for oral delivery (Forbes and Ehrhardt 2005). Respiratory diseases, such as asthma, chronic obstructive pulmonary disease (COPD) and pulmonary infections have been treated by inhalation of drugs for over 50 years (Patton *et al.* 2004a). The use of the pulmonary route for systemic administration of drugs is less well established. However, it is becoming an increasingly popular alternative pathway, in particular, for drugs with poor systemic bioavailability upon oral administration e.g., biopharmaceuticals. (Patton *et al.* 1999, Hussain *et al.* 2004, Sakagami 2006, Patton and Byron 2007) According to Patton (1996, 1997), the reasons for this are the favourable drug absorption features of the lung such as its large absorptive surface area and the extensive vascularisation of the respiratory mucosa. Considerable research focuses on inhaled peptides and proteins as an alternative route to injection. The inhalation of pharmaceutical compounds such as therapeutic peptides, proteins, oligonucleotides, and vaccines which are known to suffer from enzymatic degradation and low membrane permeability has been reported. (Patton 1996, LiCalsi *et al.* 1999, Hussain *et al.* 2004, Patton and Byron 2007, Tronde *et al.* 2008) Peptides and proteins that are currently under investigation for systemic treatment by inhalation are shown in Table 1.1 (reviewed in Siekmeier and Scheuch 2008).

Table 1.1. Various proteins under investigation for systemic treatment by inhalation (according to Siekmeier and Scheuch 2008).

Insulin (Exubera®, Afrezza®)	Blood glucose regulating hormone
PTH	Parathyroid hormone, serum calcium regulating
Calcitonin	Opposing the effects of PTH
EPO	Erythropoietin, hematopoietin
IFN-α and -γ	Interferons
Detirelix	Luteinizing hormone-releasing hormone antagonist
GHRH	Growth hormone releasing hormone
Leuprolide	Gonadotropin-releasing hormone agonist
G-CSF	Granulocyte colony-stimulating factor

For reasons that are not fully understood, the lungs exhibit higher bioavailability for macromolecules than any other non-invasive route of delivery (Patton 199, Patton and Byron 2007). However, small natural peptides were observed to be extensively degraded. Peptides with higher molecular weight are less prone to hydrolysis and show higher bioavailabilities. (Adjei and Carrigan 1992, Patton *et al.* 1998, Patton and Byron 2007) Even so, the insulin dose required for inhalation must be approximately 11-times higher than for subcutaneous injection, in order to achieve the same therapeutic effect. A significant proportion of inhaled insulin fails to reach the lower airways, resulting from deposition in the inhaler and upper bronchial tract together with removal by mucociliary transport and/or degradation. (Patton *et al.* 1999, Patton *et al.* 2004b) Pulmonary surfactant represents an additional absorption barrier as it affects the stability and solubility of deposited substances (Patton 1996, Siekmeier and Scheuch 2008) (Figure 1.1).

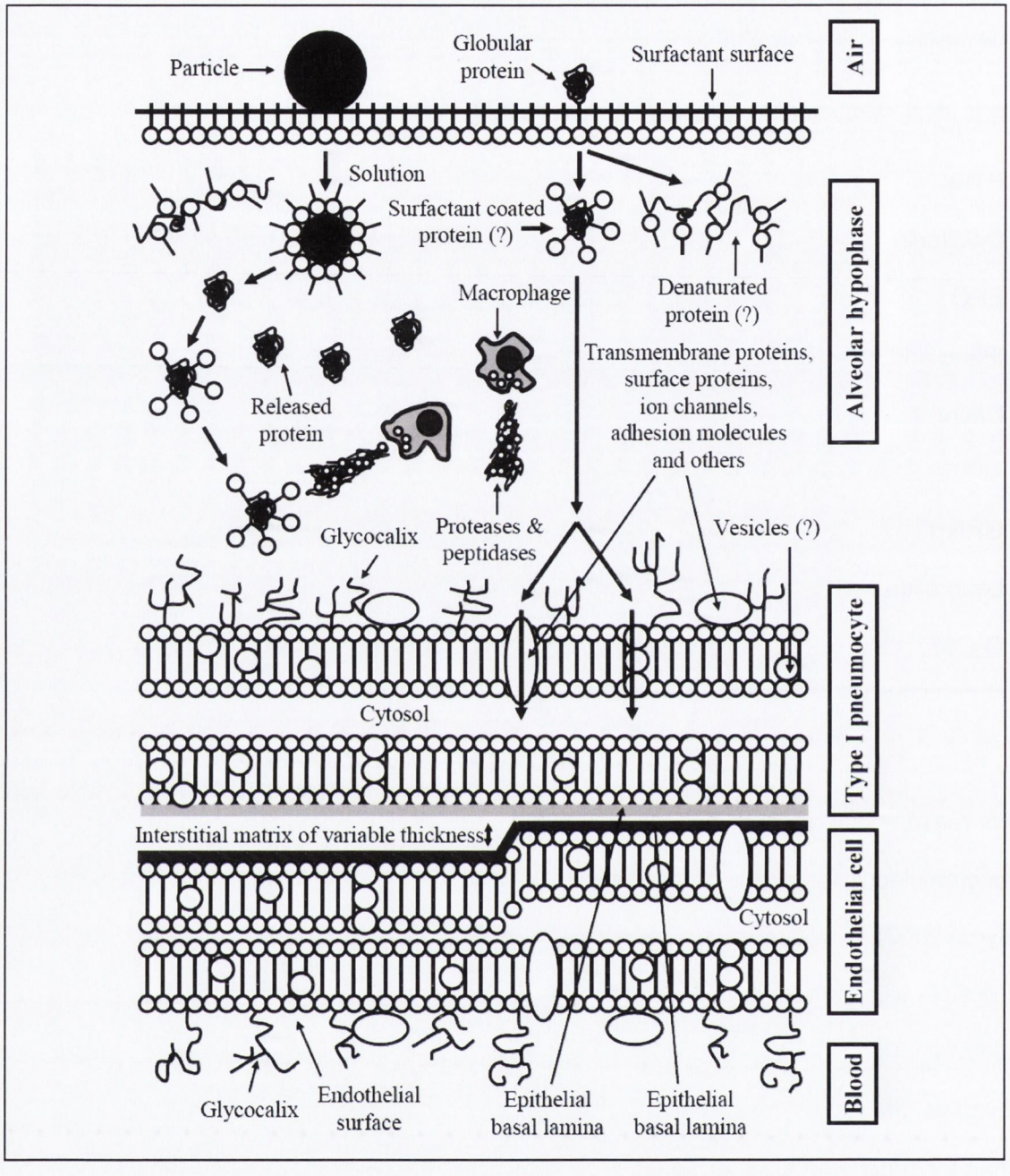

Figure 1.1. Barriers for absorption of peptides and proteins after peripheral/alveolar deposition (copied from Siekmeier and Scheuch 2008, p. 62).

1.2. PEPTIDASES

1.2.1. Introduction

Peptidases are defined as enzymes that hydrolyse peptide bonds between adjacent amino acids (Bergmann and Ross 1936, Woodley 1994). Although all peptide bonds are – in strict terms - chemically identical, the adjacent amino acids create a chemical environment that influences the activity of a peptidase towards a certain peptide bond (Kenny 1977, Woodley 1994). A plethora of enzymes function in the catabolic biotransformation of modern biopharmaceuticals (reviewed in Woodley 1994 and Baumann 2006). Specific cleavage sites of several drug metabolising peptidases are shown in Table 1.2. Peptidases can be divided into endopeptidases, which cleave peptide bonds in the interior of a peptide chain, and exopeptidases, which cleave amino acids from the C-terminal (carboxypeptidases) or from the N-terminal end (aminopeptidases) (Bergmann and Ross 1936, Woodley 1994).

1.2.2. Endopeptidases

Trypsins, chymotrypsins and elastases belong to the serine endopeptidases, which are so named due to a serine residue at the active side (Hartley 1970, Woodley 1994). Trypsin, a mixture of digestive peptidases, was among the first enzymes to be isolated and characterised (Halfon *et al.* 2004). Once activated, trypsin rapidly autocatalyses the production of itself and is involved in the activation of trypsinogen, chymotrypsinogen and zymogen forms of other pancreatic peptidases (Barrett and McDonald 1980, Woodley 2004). It was originally found in the pancreas but trypsin expression has meanwhile been detected throughout the body (Koshikawa *et al.* 1998). Preferable cleavage sites are amide substrates following P1 Arg or Lys residues (Craik *et al.* 1985, Halfon *et al.* 2004). Chymotrypsin, which was found to be the second major proteolytic enzyme in pancreatic juice (see Northrop *et al.* 1939, Graf *et al.* 2004), is - like trypsin and elastase - expressed in its inactive form and is activated by a complex process (reviewed in Woodley 1994). While pancreatic elastase is another important protease present in intestinal fluid

(Woodley 1994), neutrophil elastase is an enzyme associated with lung disease (Tetley 1993). There is evidence to suggest that the regulation of neutrophil elastase is crucial for lung defence and that an imbalance might cause emphysema (Tetley 1993, Reid *et al.* 1999). Due to its ability to degrade the majority of extracellular matrix and key plasma proteins, neutrophil elastase is regarded as one of the most destructive enzymes (Havemann and Gramse 1984).

Besides the secreted serine peptidases, there are other endopeptidases that are membrane-bound (Woodley 1994). For example, neutral endopeptidase-24.11 (neprilysin) is a metallopeptidase localised in the membrane. It preferentially hydrolyses N-terminal peptide-bonds of hydrophobic amino acids and potentially inactivates a multitude of small natural peptides such as substance P, neurokinin A, bradykinin and endothelin. (reviewed in van der Velden *et al.* 1999) Endopeptidase-24.18 (meprin A) exists in a secreted form and membrane-bound forms. Substrates include several physiological peptides (Stephenson and Kenny 1988, Bertenshaw *et al.* 2001), although endopeptidase-24.11 is generally superior towards these substrates. Endopeptidase-24.18, on the otherhand, is capable of cleaving more complex peptides and proteins than endopeptidase-24.11. (Stephenson and Kenny 1988, Choudry and Kenny 1991, Woodley 1994) Enteropeptidase (enterokinase) plays an important role in normal mammalian digestion. It can produce active trypsin by splitting a hexapeptide from the N-terminal side of trypsinogen. In addition, it can hydrolyse peptides and proteins with sites similar to the cleavage sequence of trypsinogen (Kunitz 1939, Sadler 2004). Endopeptidase-24.15 (thimet oligopeptidase I, THOP1) is a cytosolic endopeptidase that hydrolyses a limited number of sites on a bioactive peptide. However, the recognised cleavage sequences exhibit signficant variation. The enzyme is involved in the metabolism of various peptides in the central nervous system and in the periphery such as neurotensin, bradykinin, somatostatin, opioids, and angiotensin I. (Ray *et al.* 2004)

Table 1.2. Substrate specificity of investigated enzymes (modified from Woodley 1994 and Bernkop-Schnürch 1998).

Enzyme	Site of cleavage	Specificity
Trypsin	H_2N□—□—■↓—□—□COOH	Arg, Lys
Chymotrypsin	H_2N□—□—■↓—□—□COOH	Phe, Tyr
Neutrophil elastase	H_2N□—□—■↓—□—□COOH	Ala, Thr, Ile, Val, Ser
CPA1 and CPA2	H_2N□—□—□—□—↓■COOH	Tyr, Phe, Ile, Thr, Glu, His, Ala
CPB1	H_2N□—□—□—□—↓■COOH	Lys, Arg
CPM	H_2N□—□—□—□—↓■COOH	Lys, Arg
GGH	H_2N□—□—□—□—↓■COOH	Glu or other γ-linked amino acid
APA	H_2N■↓—□—□—□—□COOH	Asp, Glu
APB	H_2N■↓—□—□—□—□COOH	Basic amino acids (Lys, Arg, His)
APN	H_2N■↓—□—□—□—□COOH	Many, but especially Ala, Leu
APP	H_2N■↓—□—□—□—□COOH	Pro
GGT1 and GGT2	H_2N■↓—□—□—□—□COOH	γ-Glutamic acid
DPP IV	H_2N□—■↓—□—□—□COOH	Pro, Ala
ACE	H_2N□—□—□—↓■—■COOH	His-Leu
ACE2	H_2N□—□—□—□—↓■COOH	Hydrophobic residues
Endopeptidase-24.11	H_2N□—□—↓■—□—□COOH	Hydrophobic residues
Endopeptidase-24.15	H_2N□—↓■↓—□—□COOH	Many, but only short peptides
Endopeptidase-24.18	H_2N□—↓■↓—□—□COOH	Aromatic amino acids
Enteropeptidase	H_2N□—□—■↓—□—□COOH	$(Asp)_4$-Lys

1.2.3. Exopeptidases

1.2.3.1. Carboxypeptidases. Carboxypeptidases (CP) cleave amino acids from the C-terminus of a peptide. CPA and CPB belong to the secreted enzymes and are the second group enzymes in the small intestine besides the serine endopeptidases (Woodley 1994). CPA1, also known as pancreatic CPA, derives its name from the fact that it exhibits a preference for peptide substrates in which the C-terminal terminal residue has an aromatic group or branched side chain (Auld 2004). An additional member of the carboxypeptidase gene family with similar substrate specificity was named CPA2 (Gardell *et al.* 1988). CPB1 (also named tissue or pancreatic carboxypeptidase B) was initially called basic carboxypeptidase due to its specificity to cleave C-terminal lysine and arginine residues from peptides and proteins (Folk and Gladner 1958, Gladner and Folk 1958, McKay *et al.* 1979). CPM was discovered and characterised as a membrane-bound member of the mammalian metallocarboxypeptidase family. The letter "M" was suggested by Skidgel *et al.* (1989) to point out that CPM is localised on the membrane and to distinguish it from other known mammalian carboxypeptidases. Several peptide hormones that interact with their respective plasma membrane receptors are influenced in their activity by the membrane-bound CPM. (Skidgel *et al.* 1989) Gamma-glutamyl CP (γ-glutamyl hydrolase, GGH) is involved in the metabolism and absorption of folates and anti-folates (Reisenaur *et al.* 1977, for review see Schneider and Ryan 2006). Angiotensin I converting enzyme 1 (ACE) was isolated in 1956 as "hypertension-converting enzyme" by Skeggs *et al.* (1956). As a peptidyl dipeptidase, ACE cleaves C-terminal dipeptides from various substrates with free C-terminus or a C-terminal dipeptide-amide (reviewed in Corvol *et al.* 2004). ACE is involved in the regulation of cardiovascular homeostasis; it cleaves the C-terminal dipeptide from angiotensin I and releases angiotensin II which functions as potent vasopressor (Skeggs *et al.* 1956, Dorer *et al.* 1974). Angiotensin I converting enzyme 2 (ACE2) was the first identified homologue of ACE and has therefore also been referred to as angiotensin-converting enzyme homolog (ACEH) (Tipnis *et al.* 2000). Although ACE and ACE2 show 40% identity in amino acid sequence, ACE2 functions with a CPA-like

action removing only one amino acid from the C-terminus, and not a dipeptide. ACE and ACE2 can both hydrolyse angiotensin I (Ang I), but due to their distinct activities, different products are generated. ACE2 catalyses the conversion of Ang I to Ang 1-9. It was observed that ACE2 is neither affected by inhibitors of ACE, nor by CPA inhibitors. (Donoghue *et al.* 2000, Tipnis *et al.* 2000, Turner and Hooper 2004)

1.2.3.2. Aminopeptidases. The second group of exopeptidases are the aminopeptidases (AP), which are predominantly localised at the membrane (Woodley 1994). APA (glutamyl aminopeptidase) is a membrane-bound enzyme which preferably hydrolyses peptide substrates with an N-terminal glutamyl or aspartyl residue, such as angiotensin II (Nagatsu et al. 1965, Glenner *et al.* 1962, Nagatsu *et al.* 1970). APB activity was originally described as an exopeptidase with the ability to cleave N-terminal basic amino acids from peptide substrates (Hopsu *et al.* 1964). APB has been associated with mechanisms involved in inflammatory processes (Hopsu and Mäkinen 1966) and tumour growth (Saiki *et al.* 1989). APN is a multifunctional enzyme that removes neutral N-terminal amino acids from its substrates, however with little specificity for the amino acid removed. The enzyme exists in a soluble and a membrane form and was found to be widely expressed in many human organs, tissues and cell types. (Gillis *et al.* 1998, van der Velden *et al.* 1998, for review see Luan and Xu 2007) APP was named after its ability to specifically remove the N-terminal amino acid from peptides with a proline residue in the second position (McDonald and Barrett 1986, Harbeck and Mentlein 1991). Three different mammalian APP isoforms have been suggested. They have similar structures and belong to the same subfamily of metallopeptidases but are coded for by different genes (Erşahin *et al.* 2005; http://www.merops.sanger.ac.uk, 17.02.2011). APP1 is a soluble cytosolic form (Holtzman *et al.* 1987, Vanhoof *et al.* 1997, Cottrell *et al.*2000) with broad substrate specificity (Turner and Cottrell 2004). Its physiological role has not yet been characterised, but according to Erşahin *et al.* (2005), APP1 is presumably involved in later stages of protein degradation. In comparison to APP1, APP2 is a membrane-bound enzyme that is

localised on the cell-surface (Kenny *et al.* 1977, Orawski *et al.* 1987), and it seems to participate in the degradation of dietary as well as filtered peptides (Erşahin *et al.* 2005). APP3 is a hypothetical isoform, and its identification has been based on sequence homology (Erşahin *et al.* 2005). Gamma-glutamyl transpeptidase 1 (GGT1, γ-glutamyltransferase) removes the N-terminal γ-glutamyl moiety from a substrate and catalyses its transfer to a dipeptide acceptor (Ball *et al.* 1956, Revel and Ball 1959, Hiratake *et al.* 2004). Its physiological role is to hydrolyse extracellular glutathione so that the resulting amino acids are reabsorbed and available for the intracellular synthesis of glutathione (Hanigan and Ricketts 1993). GGT2 is a second distinct gene which was first described by Pawlak and co-workers (Pawlak *et al.* 1989, Heisterkamp *et al.* 2008). Dipeptidyl peptidase IV (DPP4) is a serine protease which – as the name suggests – release N-terminal dipeptides from peptide substrates, preferably Xaa-Pro (Kenny *et al.* 1976). Physiological substrates are extracellular (neuro)peptides such as substance P, bradykinin, certain chemokines and possibly cytokines like interleukin (IL)-1β and IL-2 (Nausch *et al.* 1990, Hoffmann *et al.* 1993).

The role of these peptidases in the gastrointestinal tract has been thoroughly evaluated (for review see Woodley 1994). Their activity and spatial distribution in the human lung, however, appears less well investigated. The study of lung metabolism would strongly benefit from having reliable experimental models that represent the *in vivo* situation of the human lung. Due to differences in enzyme expression and activity across animal species, data received from animal experiments cannot simply be transferred that which occurs in humans. In recent years, different cellular models of human origin have been developed as experimental models to facilitate investigations of the fate of xenobiotics in the lung. (Ehrhardt *et al.* 2008, Castell *et al.*2005)

1.3. CALCITONIN

1.3.1. Physiology and pharmacology of calcitonin

Calcitonin is a 32-amino-acid peptide hormone that was discovered by Copp and Cameron (1961) as a substance capable of lowering blood calcium. Calcitonin regulates calcium homoeostasis in collaboration with parathyroid hormone and 1,25-dihydroxycalciferol. In mammals, calcitonin is secreted by the C-cells of the thyroid, but its physiologic role is not yet fully understood. (reviewed in Chesnut *et al.* 2008) Its role in calcium regulation, however, has primarily been ascribed to the protection of the skeleton against excessive resorption (Zaidi *et al.* 2002) at times of "calcium stress" such as during pregnancy, growth and lactation. It was reported that the calcitonin serum levels are increased in these periods. (Whitehead *et al.* 1981, Zaidi *et al.* 2002, Woodrow *et al.* 2006, Chesnut *et al.* 2008)

Calcitonin acts directly at the bone, where it inhibits osteoclast activity (Holtrop *et al.* 1974). Osteoclasts are "giant cells adjacent to bone" (Gonzales and Karnovsky 1961, p. 299) consisting of a ruffled border area facing the bone and a surrounding clear area. The bone surface was observed to be disrupted under the ruffled area and it was concluded that bone resorption is mainly caused by the ruffled area of osteoclasts. (Gonzales and Karnovsky 1961, Holtrop *et al.* 1974) It was reported that parathyroid hormone (PTH) increased cross sections with ruffled borders and stimulated bone resorption (Tatevossian 1973, Holtrop *et al.* 1974), while the frequency of ruffled borders was significantly decreased after treatment with calcitonin. It was therefore concluded that osteoclasts can change between active and resting phases. (Holtrop *et al.* 1974)

On the surface of each osteoclast are approximately one million of the 7-transmembrane, G-protein-linked calcitonin receptors (Nicholson *et al.* 1986, Martin *et al.* 1995, Strader *et al.* 1995). Binding of calcitonin stimulates multiple signal transduction pathways resulting in cell calcium efflux and protein kinase C activation. Besides the location of the calcitonin

receptor on osteoclasts, it is expressed by brain, testis, kidney, skeletal muscles, spermatozoa, human primary breast cancer cells and breast cancer cell lines. Its biological effects on these cells, however, are unknown. (Cafforio *et al.* 2009)

1.3.2. Salmon calcitonin

Due to its more flexible structure, salmon calcitonin (sCT) (Figures 1.2 and 1.3) has a much higher intrinsic potency than calcitonin from other species and is therefore the most widely used in the treatment of hypercalcaemia associated with malignant diseases and for the treatment of bone disorders such as Paget's disease or osteoporosis. Another therapeutic benefit is its analgesic effect on both bone pain and nonbone pain. (Nicholson *et al.* 1986, Azria *et al.* 1995, Zaidi *et al.* 2002) Besides its well-established effect on subchondral bone, sCT may have direct effects on chondrocytes and therefore positively affect joint diseases (Sondergaard *et al.* 2010).

In 1968, the isolation of sCT from ultimobranchial glands of salmon, the sequencing of its aminoacid sequence and the first synthesis of the peptide were performed within four months by scientific collaboration involving different research groups (according to Azria *et al.* 1995). Salmon calcitonin is currently on the market in an injectable form (Calcimar®, Miacalcin®) and as nasal spray (Fortical®, Miacalcin®) for the treatment of the aforementioned bone diseases. However, due to inconvenience and low acceptance of those two application forms, it is believed that the full market potential of sCT products has not yet been reached (Guggi *et al.* 2003). Of note, it exhibits poor stability following oral delivery, with at least three peptidases responsible for the enzymatic cleavage of sCT, i.e., neutrophil elastase, trypsin and α-chymotrypsin (Guggi and Bernkop-Schnürch 2003). Thus, inhalation may represent an attractive alternative route for systemic application of sCT.

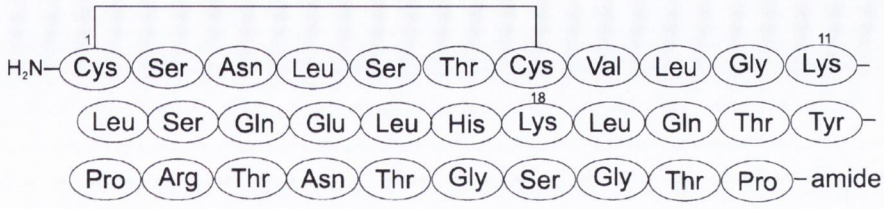

Figure 1.2. Primary structure of the 32 amino acid peptide salmon calcitonin. There is a N-terminal disulfide bridge between the cysteines in positions 1 and 7, and a proline amide residue at the C-terminus. (modified from Lee *et al.* 1999)

Figure 1.3. Salmon calcitonin illustrated in its single α-helical structure including the N-terminal ring (compare structure shown by Andreotti *et al.* 2006). The carbon-backbone and hydrogen are represented in grey, nitrogen in blue oxygen in red, and sulphur yellow. The figure clearly illustrates the amphiphilic properties of sCT which have previously been described by Kaiser and Kézdy (1984) and by Moe and Kaiser (1985). The more hydrophobic residues of the molecule (i.e., residues mainly of carbon and hydrogen, illustrated in grey) face to one side of the helix, while the hydrophilic amino acids (i.e. residues containing nitrogen and oxygen) orient to the opposite side. *The picture was created by Felix Gut, Philipps-Universität Marburg, Germany.*

1.4. PROTEIN MODIFICATION

1.4.1. Introduction

Various approaches to protein modification have been employed in order to improve pharmaceutical and pharmacokinetic properties following injection. In addition, efforts are also being made to identify modifications that facilitate alternative routes of drug delivery such as the oral or pulmonary route. The most common modifications of therapeutic proteins are mutagenesis (creation of protein analogues), chemical modification and design of specific drug delivery systems. However, a reduction in the protein's biological efficacy most frequently accompanies protein modification (Bailon *et al.* 2001, for review see Frokjaer and Otzen 2005). Modified protein drugs currently on the market are listed in Table 1.3.

Table 1.3. Modified proteins approved for marketing (modified from Frokjaer and Otzen 2005).

Product (company)	Drug (route of application)	Modification
Proleukin (Chiron)	Aldesleukin (i.v.)	Analogue
Humalog (Eli Lilly)	Insulin lispro (s.c.)	Analogue
NovoRapid (Novo Nordisk)	Insulin aspart (s.c.)	Analogue
Lantus (Sanofi-Aventis)	Insulin glargine (s.c.)	Analogue
Neulasta (Amgen)	Pegfilgrastim (s.c.)	Mono-PEGylated
Pegasys (Roche)	PEGinterferon α-2a (s.c.)	Mono-PEGylated
PegIntron (Essex Pharma)	PEGinterferon α-2b (s.c.)	Mono-PEGylated
Oncaspar (Medac)	Pegasparaginase (i.m., i.v.)	Mono-PEGylated
Somavert (Pfizer)	Pegvisomant (s.c.)	Multi (4-6)-PEGylated
Levemir (Novo Nordisk)	Insulin detemir (s.c.)	Mono-acylated

1.4.2. Mutagenesis

Protein analogues are most commonly designed by recombinant-DNA technology, whereby a genetically altered protein is synthesised in a special non-pathogenic laboratory strain of bacteria (e.g., *Escherichia coli*) or yeast (e.g., *Saccharomyces cerevisae*). By mutation of amino-acids in the insulin molecule the self-association characteristics and thus time-action profiles can be modified, giving rise to long-lasting or fast-acting insulin analogues (Figure 1.4) (reviewed in Frokjaer and Otzen 2005, Dingermann *et al.* 2010).

Insulin lispro (Humalog®, Lilly) and insulin aspart (NovoRapid®, Novo Nordisk) are fast-acting insulins due to a larger amount of insulin dimers and monomers. (reviewed in Frokjaer and Otzen 2005; http://pi.lilly.com/us/humalog-pen-ppi.pdf, 21.01.2011). Insulin glargine (Lantus®, Sanofi-Aventis) is less soluble at physiological pH due to a shift in the isoelectric point. Insulin glargine therefore precipitates in the tissue after subcutaneous injection resulting in delayed absorption and a relatively flat time-action profile for at least 24 hours. (Heinemann *et al.* 2000)

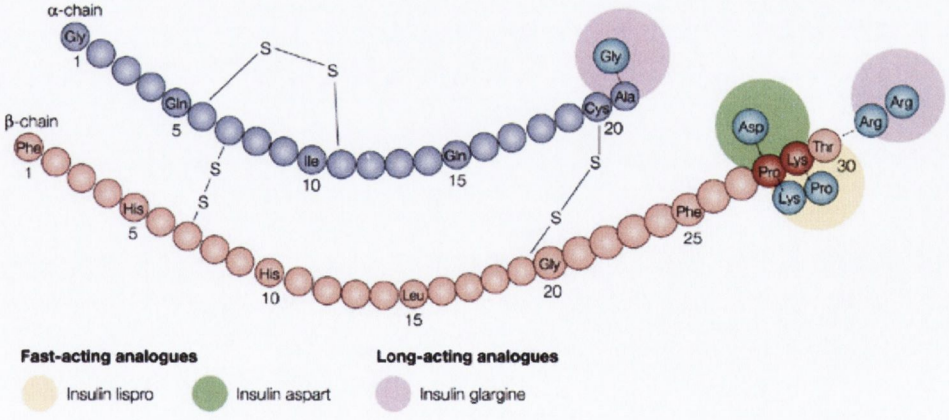

Figure 1.4. Changes in amino acid sequences of the insulin analogues insulin lispro (Humalog®, Lilly), insulin aspart (NovoRapid®, Novo Nordisk) and insulin glargine (Lantus®, Sanofi-Aventis) (modified from Owens 2002).

1.4.3. Chemical modifications

Chemical modifications are manifold but typically include different conjugations with fatty acids or polyethylene glycol (PEG). Conjugation changes the physicochemical parameters of the drug molecule and can therefore alter absorption and the time-action-profile. Generally, due to an increase in molecular weight, the conjugations are expected to show a prolonged circulation time in the blood. (for reviews see Roberts *et al.* 2002, Harris and Chess 2003 and Veronese and Pasut 2005) Although, there is no PEGylated or lipidised sCT derivative currently on the market, chemical modifications of sCT have been performed by several groups.

1.4.3.1. Lipidisation. Proteins that were chemically attached to fatty acids exhibited, in some cases, increased affinity for serum albumin and therefore a prolonged blood circulation time (Kurtzhals *et al.* 1995 and 1996). Insulin detemir (Levemir®, Novo Nordisk) is an example for a marketed lipidised protein drug. Here, the insulin molecule is chemically attached to myristic acid by acylation (Figure 1.5). Insulin detemir is bound to albumin molecules in the blood and due to its slow release from these complexes it provides a basal insulin level. Glucagon-like peptide-1 (GLP-1), interferon (IFN)-α and desmopressin have also been successfully modified by acylation (Owens 2002, Frokjaer and Otzen 2005).

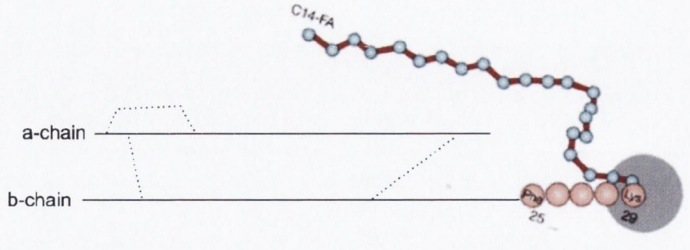

Figure 1.5. Insulin detemir (Levemir®, Novo Nordisk) (modified from Owens 2002).

Two research groups demonstrated that lipidised sCT enhances the transport through biological membranes and therefore increases the area under the curve (AUC) compared to unmodified sCT. In both cases, the disulfide bond in the sCT molecule was reduced and the resulting thiol groups at the cysteine residues conjugated to a palmitic acid derivative (Wang *et al.* 2003, Cheng *et al.* 2007).

Cheng *et al.* (2007) conjugated a ε-maleimido lysine derivative of palmitic acid with sCT via a thio-ether bond and received a non-reversible water-soluble lipid conjugate of sCT (so called "Mal-sCT") (Figure 1.6). Mal-sCT showed improved protective properties against hepatic peptidases but in intestinal fluid its stability was no better than unmodified sCT. After subcoutaneous (s.c.) injection into the rat, Mal-sCT showed increased cellular uptake and assimilable hypocalcaemic activity to sCT (Cheng *et al.* 2007). According to Cheng *et al.* (2007), the more rigid confirmation of Mal-sCT in water may not be able to bind to certain receptor phenotypes which results in a variation in pharmacodynamic response.

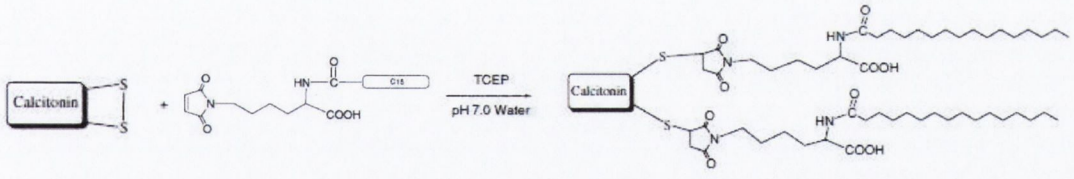

Figure 1.6. "Mal-sCT", a conjugation of a ε-maleimido lysine derivative of palmitic acid with sCT (modified from Cheng *et al.* 2007).

A reversible conjugation was performed by Wang *et al.* (2003) who attached cysteine derivatives of palmitic acid to each of the two thiol groups in the sCT molecule. The reversibly lipidised sCT exhibited enhanced pharmacokinetic and pharmacodynamic properties as well as an increase in bioavailability upon oral administration (Wang *et al.* 2003).

1.4.3.2. PEGylation. To protect protein and peptide drugs from proteolytic degradation, PEGylation is often employed. PEGylation is a process by which a protein, peptide or non-peptide molecule is covalently attached to one or more polyethylene glycol (PEG) chains (Figure 1.7). PEG is a non-toxic, non-immunogenic, non-antigenic, highly water soluble polymer which has been approved by the Food and Drug Administration (FDA) for use as a vehicle or base in foods, cosmetics and pharmaceuticals. (for reviews see Roberts *et al.* 2002, Harris and Chess 2003 and Veronese and Pasut 2005) In solution, each ethylene glycol subunit is associated with two or three water molecules, so that the apparent size of the PEGylated molecules is five to ten times larger than a corresponding soluble protein of similar molecular mass (Kozlowski and Harris 2001, Roberts *et al.* 2002). As a result, the clearance of the PEG-conjugate by the kidneys can be retarded resulting in a prolonged circulation half-life in the body. Further advantages of PEG-conjugates are decreased recognition and degradation by proteolytic enzymes and shielding of antigenic and immunogenic epitopes. PEG is removed from the body intact, in a size-dependant manner by either the kidneys or in faeces. (Katre *et al.* 1987, Monfardini *et al.* 1995, for reviews see Kozlowski and Harris 2001, Roberts *et al.* 2002, Caliceti and Veronese 2003, Harris and Chess 2003, Veronese and Pasut 2005)

Another benefit is the inherent flexibility of PEGylation - pharmacokinetic parameters can be easily altered by the use of a different PEG substituent (Youn *et al.* 2008). Monfardini *et al.* (1995) reported that also the structure of the PEG chain (i.e., branched PEG or linear PEG) changes the characteristics of the PEG-conjugate. For example, peptides conjugated to branched PEG showed greater stability to proteolytic digestion than to conjugates with linear PEG. (Monfardini *et al.* 1995)

Figure 1.7. Molecular formula of polyethylene glycol (PEG).

Amino conjugation is the attachement of PEG via primary amino groups of a peptide or protein. It has been suggested to be "the most common modification and often the first approach in many new PEG-protein projects" (Veronese and Pasut 2005, p. 1452). Due to the wide range of commercially available functionalised PEG moieties (e.g., NHS activated PEG derivatives), conjugations can be easily performed by so called "click chemistry" (see Figures 1.8). However, this method is constrained by the high number of isomers obtained and their complicated purification. A mixture of isomers was approved by the FDA for the first two PEGylated drugs on the market (i.e., Oncaspar® and Adagen®), because reproducibility of the reaction could be demonstrated. The requirements for the approval of new conjugates are stricter and each isomer has to be characterised. (reviewed in Veronese and Pasut 2005)

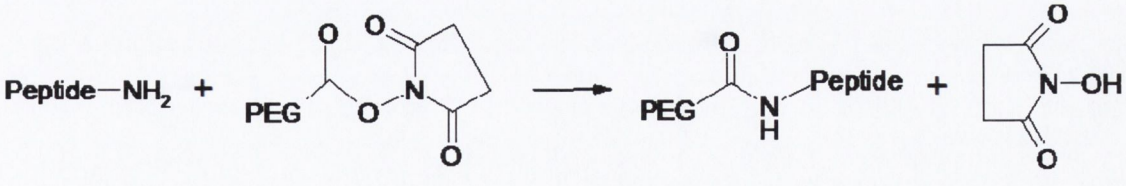

Figure 1.8. An example of a frequently used method of amino conjugation. A primary amino group of a peptide or protein is coupled with a PEG derivative containing a reactive N-hydroxy succinimidyl succinate (NHS) ester group. (modified from Hermanson 2008)

There are currently five PEGylated peptide or protein drugs on the market: Peginterferon alfa-2a (Pegasys®, Roche) for hepatitis B and C treatment, Peginterferon alfa-2b (PegIntron®, Essex Pharma) for the treatment of hepatitis C, Pegvisomant (Somavert®, Pfizer), a human growth hormone (hGH) analog that has been structurally modified to act as a hGH receptor antagonist for the treatment of acromegaly, Pegfilgrastim (Neulasta®, Amgen), a colony stimulating factor to prevent and treat neutropenia and Pegasparaginase (Oncaspar®, Medac) for the treatment of acute lymphatic leukaemia. All the formulations are for subcutaneous (s.c.), intravenous (i.v.) or intramuscular (i.m.) injection. (http://www.fachinfo.de, 16.12.2010) Pegasys®, interferon alfa-2a coupled with 40kDa branched PEG, is a fully characterised mixture of nine different mono-PEGylated isomers. In each isomer the branched PEG chain is attached to a specific lysine residue of the interferon alfa-2a molecule. Although all isomers show antiviral effects, significant differences in the specific activities were observed depending on the position of the PEG chain. (Bailon *et al.* 2001, Foser *et al.* 2003) PegIntron® is a conjugate in which a linear 12 kDa PEG chain is covalently attached to interferone-alfa-2b, primarily to the histidine in position 34. However, the bond is unstable and can easily be hydrolysed upon injection. Thus, it has a shorter half-life and increased clearance compared to Pegasys®. (Foster 2010)

sCT has three possible PEGylation sites: the primary amines of the N-terminus Cys^{1}, as well as Lys^{11} and Lys^{18} (Figure 1.9). PEGylation was reported to significantly improve the stability of sCT in tissue homogenate, most likely due to protection from proteolytic enzymes (Lee *et al.* 1999). Lee *et al.* (1999) produced three different mono-PEGylated sCT derivatives using a linear PEG_{5000} for conjugation.

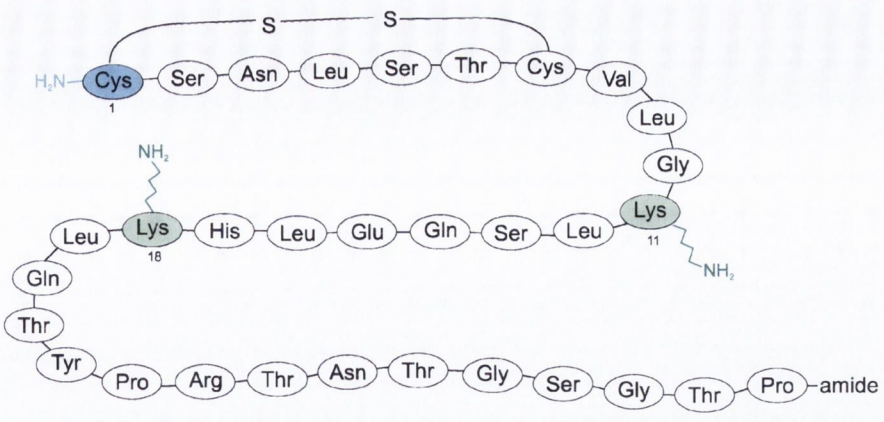

Figure 1.9. Primary structure of salmon calcitonin and the three possible PEGylation sites via primary amino groups (Lee *et al.* 1999).

All three conjugates showed increased resistance to tryptic digestion, but the Lys^{18}-modified sCT derivative was superior to the other two modifications (Lee *et al.* 1999, Youn *et al.* 2006, Youn, Na and Lee 2007). It was further demonstrated that site-specific PEGylation can be performed by the use of protecting groups (Youn *et al.* 2007), or by the use of a certain reaction pH since the N-terminal α-amino group and the ε-amino group of the Lys moiety differ in their reactivity depending on the pH (Na *et al.* 2004). The stability of mono-PEG_{2000}-sCT was tested in homogenates of nasal rat mucosa and showed dramatically reduced sCT degradation and prolonged half-life (Na *et al.* 2004). Shin *et al.* (2004) reported a significantly increased AUC for a similar conjugate compared to unmodified sCT. Youn *et al.* (2008) synthesised three different Lys^{18}-PEGylated sCT derivatives with different sizes of the PEG residues (i.e., 1, 2 and 5 kDa) and investigated their pharmacokinetic properties and suitability for intrapulmonary delivery. Although Lys^{18}-PEG_{5000}-sCT showed best pharmacokinetic parameters and highest resistance towards pulmonary proteolytic enzymes, it was found to have significantly lower hypocalcaemic efficacy than other PEG-sCTs, probably due to its reduced intrinsic bioactivity (~ 30% vs. sCT). In contrast, Lys^{18}-PEG_{2000}-sCT showed the most promising pulmonary potential due to its well preserved bioactivity (> 80% vs. sCT).

1.5. MICELLES

In low concentrations, surface active agents (surfactants) exist in their monomeric form. When a certain concentration is reached, (i.e., the "critical micelle concentration", CMC), surfactant molecules spontaneously aggregate to bigger thermostable micelles (Figure 1.10). This mechanism of self-assembly is due to the amphiphilic nature of the detergent molecules. Their polar head groups interact with water and are faced outwards, while the apolar chains of the detergent molecules are stowed away in the interior of the micelle and thereby shielded from contact with water. Above the CMC, the micellar structures are in equilibrium with their monomeric form. (Sonntag 1977, Helenius *et al.* 1979, Zulauf *et al.* 1989)

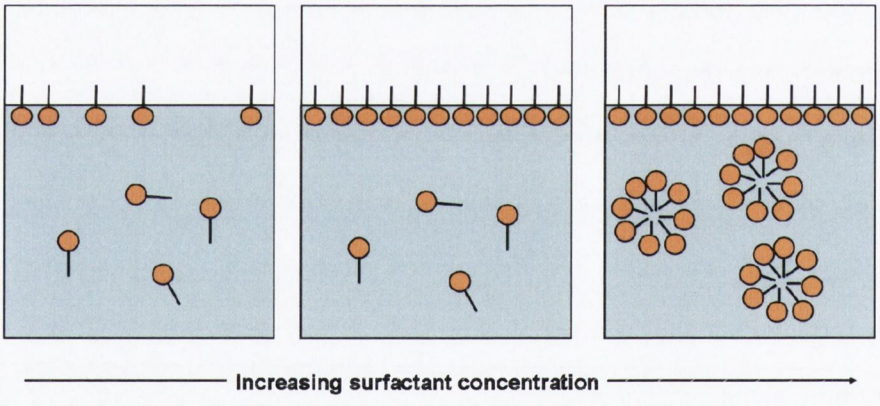

Figure 1.10. Self-assembly mechanism of micelles in dependence of the surfactant concentration. Surfactant molecules preferably arrange in a way that the polar head of the surfactant molecule faces the water, while the apolar tail is exposed to the air at the surface of the solvent or stowed away in the interior of the micelle. By adsorbing at the liquid-air-interface, the surfactant reduces the surface tension of the liquid. Above the CMC, the surface tension remains stable.

Due to a number of favourable properties, polymeric micelles proved suitable as vehicles for drug delivery of poorly water soluble and amphiphilic drugs (Jones and Lereux 1999). Micellar drug carrier systems can improve the solubility of lipophilic drug molecules by solubilising these compounds in the micellar core (Jones and Lereux 1999, Kontoyianni *et al.* 2008). The therapeutic efficacy can be enhanced and side effects can be reduced by attenuating toxicities of pharmaceutical drugs (Croy and Kwon 2006, Zhang *et al.* 2008). And in some cases, sustained release profiles could be achieved (Wang *et al.* 2008, Wei *et al.* 2009). According to Jones and Leroux (1999), the hydrophilic shell of the micelles and their small size may be responsible for prolonged circulation times in the body and accumulation in tumour tissue. Micellar drug carriers containing paclitaxel or other anti-cancer drugs received a lot of attention because they can decrease cytotoxic side effects and prolong the drug retention time in the blood compared to the pure drug after i.v. injection (Zhang *et al.* 1997, Mizumura *et al.* 2001, Han *et al.* 2006, Wang *et al.* 2008, Zhang *et al.* 2008). Micelles as drug carriers for pulmonary application are less well established than micelles for injection and predominantly comprise micelles for gene delivery to the lung (Chao *et al.* 2007, Harada-Shiba *et al.* 2009) and micelles containing drug molecules for the treatment of pulmonary diseases (Gaber *et al.* 2006, Craparo *et al.* 2011, Gilani *et al.* 2011).

1.6. AIMS OF THE STUDY

The aims of this work are three-fold. Firstly, in order to assess the impact of enzymatic degradation of inhaled biopharmaceutical drugs, I sought to investigate enzyme expression of various drug metabolising enzymes at the respiratory epithelial barrier. Specifically, the mRNA expression of 24 peptidases known to be responsible for drug metabolism following oral application was investigated in lung epithelial cells (Calu-3, 16HBE14o-, hBEpC, A549) and the intestinal cell line Caco-2 as comparison.

Secondly, since mRNA expression does not warrant the enzyme's functionality, it was crucial to investigate the protein expression and activity of the metabolising enzymes. In order to study the impact of metabolising enzymes, the fate of a model peptide drug, salmon calcitonin (sCT) at the respiratory epithelial barrier was investigated. In particular, I focused on three peptidases known to be responsible for the degradation of sCT (i.e., trypsin, chymotrypsin and elastase).

Thirdly, I endeavoured to confer protection on sCT from enzymatic degradation. PEGylation enhances the stability of peptide and protein drugs, however it increases both hydrophilicity and size, and might therefore even further reduce their transport across biological membranes. Lipidisation is a chemical modification that can enhance the lipophilicity of sCT, as illustrated by Wang *et al.* (2003) and by Cheng and Lim (2007). Conceivably, by modification of sCT with both PEG and lipid, a drug delivery system that combines the favourable characteristics of increased stability towards degradation, and improved permeability across membranes could be developed. Thus, I sought to modify sCT by conjugation with PEG-lipids, whereby PEG is linked to the free amino groups of sCT.

1.7. REFERENCES

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Chapter 2

Reverse transcription polymerase chain reaction (RT-PCR) analysis of proteolytic enzymes in cultures of human respiratory epithelial cells

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2.1. ABSTRACT

Pancreatic proteolytic digestive enzymes are a major extracellular barrier to the sucessful systemic delivery of biopharmaceuticals via the oral route, wheras in health in the lungs these powerful proteases are less prominent in the extracellular fluids than in the intestinal tract (Siekmeier and Scheuch 2008). Despite this, the absorption of some (but not all) natural peptides and proteins from the lungs may be poor and one has to acknowledge that information on the activity and spatial distribution of proteolytic enzymes in the human lung is scarce. In this thesis, I investigated expression patterns of a series of proteolytic enzymes in several human respiratory cell types on mRNA level in an attempt to better understand the fate of inhaled biopharmaceuticals.

The mRNA expression of proteolytic enzymes (i.e., carboxypeptidases: CPA1, CPA2, CPB, CPM; gamma-glutamyltransferases: GGT1, GGT2; angiotensin-converting enzymes: ACE, ACE2; aminopeptidases: APA, APB, APN, APP1, APP2, APP3; endopeptidases: 24.11 (neprilysin), 24.15 (thimet oligopeptidase), 24.18 (meprin A); enteropeptidase; trypsin 1, trypsin 2; neutrophilic elastase; dipeptidyl peptidase 4; gamma-glutamylhydrolase) was investigated by semi-quantitative RT-PCR in human bronchial (hBEpC, Calu-3, 16HBE14o-) and alveolar (A549) epithelial cells, respectively. Gastrointestinal Caco-2 cells were used as comparison.

Obvious differences were observed in proteinases' expression pattern between the investigated cell types. Although considered to be of bronchial epithelial phenotype, neither Calu-3 nor 16HBE14o- cells matched the mRNA expression pattern of hBEpC in primary culture. Of all investigated cell lines, Caco-2 expresses the highest number of proteases and peptidases.

Although mRNA expression does not necessarily signify enzyme functionality, the results of this chapter provide a first comprehensive overview of peptidase and protease expression and distribution in human lung epithelial cells and are the basis for further investigations.

2.2. INTRODUCTION

The pulmonary route is an increasingly popular means to systemically deliver drugs with poor bioavailability after oral administration; low molecular weight compounds such as dihydroergotamine (Levadex®) and biopharmaceuticals like insulin (Exubera® and Afrezza®) being just two examples (Patton and Byron 2007). The attractiveness of using aerosols for systemic medication is due to the favourable drug absorption features of the lung caused by its large absorptive surface area, extensive vascularisation of the respiratory mucosa and the avoidance of the liver first pass metabolism (Patton 1996). Hence, despite the setback caused by the recent Exubera withdrawal, many therapeutics, among them a plethora of peptides and proteins, are currently being investigated for local and systemic treatment by inhalation (Siekmeier and Scheuch 2008).

Via the lungs, higher bioavailability for macromolecules can be achieved than by any other non-invasive route of delivery (Patton 1996, Dershwitz *et al.* 2000). However, smaller natural peptides usually suffer from high enzymatic degradation, whereas the rate of peptide hydrolysis decreases or is even absent with increasing molecular weight (Adjei and Carrigan 1992, Patton, Nagarajan and Clark 1998, Patton and Byron 2007). Chemical modification to impart peptidase resistance of natural peptides has been widely used to improve their delivery by multiple routes and can greatly improve the bioavailability of peptides delivered to the lungs. Nonetheless, absorption kinetics of biopharmaceuticals are often unpredictable and their bioavailabilities do not correlate very well with molecular weight (Dershwitz *et al.* 2000).

Although literature on the absorption of inhaled biopharmaceuticals by the lungs is available, there is still a lack of detailed information about the processes involved (Patton *et al.* 2010). One of the less well investigated areas in this context is the localisation and activity of proteolytic enzymes in various regions of the lung (e.g., trachea *vs.* bronchus *vs.* bronchiole *vs.* alveolus) and under physiological *vs.* pathological conditions. Studies

on the impact of intestinal and hepatic metabolic activity on drug disposition following oral administration have been frequently conducted (Bernkop-Schnürch 1998), but such information which could help us to better understand if protective measures such as chemical modification (e.g. PEGylation) or co-application peptidase inhibitors are meaningful for pulmonary delivery of biotechnology medicines is still missing.

In Table 2.1 the current state of the art with regards to expression and activity studies of proteolytic enzymes in the (human) lung is summarised. It is obvious that the data is fragmentary and often conflicting and predominantly collected on RNA or protein level, with only a few activity studies. Moreover, investigations of lung peptide metabolism would strongly benefit from having reliable experimental models that represent the *in vivo* situation of the human lung (Castell *et al.* 2005, Sporty *et al.* 2008). The complex architecture of the lung impedes close-up investigations of drug disposition mechanisms *in vivo* or from the isolated organ. Also, due to differences in enzyme expression and activity across animal species, data received from animal experiments cannot simply be transferred that which occurs in humans. (Forbes and Ehrhardt 2005, Castell *et al.* 2005)

In this part of my thesis, I aimed to generate a comprehensive mRNA profile for selected metabolising peptidases expressed in human lung epithelial cells in an attempt to better understand the fate of inhaled biopharmaceuticals.
The terms “peptidases” and “proteinases” often lead to misunderstandings. A “protease” though, is not only active against large proteins, but also against small peptides such as those of therapeutic interest (Woodley 1994). To avoid confusion, in this thesis, any enzyme that cleaves a peptide bond is considered as “peptidase”.

Table 2.1. Specific theoretical cleavage sites of the investigated enzymes (modified from Woodley 1994) and expression in human lung, lung epithelial cells and Caco-2 cultures according to literature.

Enzyme	Site of cleavage	Specificity	Location	Expression in human lung, lung epithelial cells and Caco-2 cultures
Trypsin 1 and 2	H_2N□—□—■↓—□—□COOH	Arg, Lys	secreted	Trypsin 1: - Positive in normal lung tissue with *in situ* hybridisation, negative by Northern blotting (Koshikawa et al. 1998). - Immunohistochemical detection in normal lung tissue (Koshikawa et al. 1998) and A549 cells (Jin et al. 2003). - Negative mRNA signal in A549 by RT-PCR (Jin et al. 2003). Trypsin 2: - Immunohistochemistry demonstrated high expression in bronchial and alveolar epithelium in preterm infants that died of prolonged respiratory distress syndrome compared to weak expression in healthy newborn infants (Cederqvist et al. 2005).
Neutrophil elastase	H_2N□—□—■↓—□—□COOH	Ala, Thr, Ile, Val, Ser	secreted	- Strong expression (by immunohistochemistry) in sputum of cystic fibrosis patients (Gaggar et al. 2007).
CPA1 and 2	H_2N□—□—□—□—↓■COOH	Tyr, Phe, Ile, Thr, Glu, His, Ala	secreted	- No data available
CPB1	H_2N□—□—□—□—↓■COOH	Lys, Arg	secreted	- No data available
CPM	H_2N□—□—□—□—↓■COOH	Lys, Arg	membrane-bound	- High mRNA expression in human lung (Northern blot) (Nagae et al. 1993). - CPM activity in A549 cells (Forbes et al. 1999). - No CPM activity found in Caco-2 cells (Howell et al. 1992).
GGH	H_2N□—□—□—□—↓■COOH	Glu or other γ-linked amino acid	membrane-bound	- Northern blotting and RT-PCR showed weak mRNA expression in human lung (Yin et al. 2003). - Northern blotting showed high expression in A549 cells (Yin et al. 2003).
APA	H_2N■↓—□—□—□—□COOH	Asp, Glu	membrane-bound	- Weak mRNA expression in human lung tissue detected by Northern blotting (Li et al. 1993). - No APA activity found in Caco-2 cells (Howell et al. 1992).

(**Table 2.1.** continued)

Enzyme	Site of cleavage	Specificity	Location	Expression in human lung, lung epithelial cells and Caco-2 cultures
APB	H_2N■↓–□–□–□–□COOH	Basic amino acids (Lys, Arg, His)	membrane-bound	- High mRNA expression in human lung and A549 cells (Northern blot) (Piesse et al. 2002).
APN	H_2N■↓–□–□–□–□COOH	Many, but especially Ala, Leu	membrane-bound, BALF	- Absent from the human pseudo-stratified respiratory epithelium but found in bronchial glands (immunohistochemistry) (Dixon et al. 1994). - APN activity in broncho-alveolar lavage fluid (BALF) by enzyme histochemistry (Van Der Velden et al. 1999). - APN widely distributed in human bronchus: found in connective tissue, secretory epithelium of bronchial glands, perichondrium, nerves and endothelial cells, but absent in bronchial epithelium and smooth muscle (immunohistochemistry and enzymehistochemistry) (van der Velden et al. 1998). - Activity in plasma membrane preparations of A549 cells (Forbes et al. 1999). - Activity in membranes from Caco-2 increasing over the time investigated (Howell et al. 1992).
APP	H_2N■↓–□–□–□–□COOH	Pro	APP1: cytosolic APP2: membrane-bound APP3: putative	- The activity of membrane peptidases (probably referring to APP2) was studied in Caco-2 and APP activity was detected with slight decrease/plateau in activity after day 20 (Howell et al. 1992). APP1: - mRNA expression in human lung (RT-PCR) (Erşahin et al. 2005). APP2: - mRNA expression in human lung (Northern blot and RT-PCR) (Venema et al. 1997). - Low enzyme activity of human lung membranes (microsomes) (Erşahin et al. 2005). APP3: - mRNA of two suggested splicing variants* in human lung tissue (RT-PCR) (Erşahin et al. 2005).

* For this study primers were used that are complementary to sequences in an exon present in both splicing variants. Thus, a single band for the expression of both APP3 variants was received.

(**Table 2.1.** continued)

Enzyme	Site of cleavage	Specificity	Location	Expression in human lung, lung epithelial cells and Caco-2 cultures
GGT1 and 2	H_2N■↓—□—□—□—□COOH	γ-glutamic acid	membrane-bound	GGT1: - Besides the "classic form" of mRNA found in human lung and other organs, an unique, shorter transcript was detected in human lung only (Northern blot) (Wetmore et al. 1993). - Variable GGT staining in immunohistochemical analysis of lung cancers, but absence of the enzyme in normal bronchial surface epithelium (Hanigan et al. 1999). - Activity in A549 cell homogenate (Rahman et al. 1998, Zaman et al. 2006). - Activity in Caco-2 from day 15 on (Howell et al. 1992). GGT2: - no data available
DPP IV	H_2N□—■↓—□—□—□COOH	Pro, Ala	membrane-bound, BALF	- Distribution in human bronchus: expression in submucosal glands and blood vessels. However, no staining of fibroblasts, bronchial epithelium and smooth muscle cells (immunohistochemistry, enzyme histochemistry) (van der Velden et al. 1998). - Activity detected in BALF (enzyme histochemistry), probably secreted in the bronchial lumen by submucosal glands (Van Der Velden et al. 1999, van der Velden et al. 2000). - Increase of activity as well as mRNA expression (Northern blot) with further differentiation of Caco-2 cells (Darmoul et al. 1992).
ACE	H_2N□—□—□—↓■—■COOH	His-Leu	membrane-bound	- Two mRNA species were detected in human tissues with highest expression levels in lung and kidney (Northern blotting) (Tipnis et al. 2000, Donoghue et al. 2000). - Low mRNA expression (RT-PCR) in A549 cells (Wang et al. 1999). - Activity in membranes from Caco-2 with low levels at day 5 and day 8, and a rapid increase in activity after day 8 (Howell et al. 1992).

(Table 2.1. continued)

Enzyme	Site of cleavage	Specificity	Location	Expression in human lung, lung epithelial cells and Caco-2 cultures
ACE2	H_2N□—□—□—□—↓■COOH	Hydrophobic residues	membrane-bound	- No mRNA signal in lung tissue using the Northern blotting technique (Tipnis et al. 2000, Donoghue et al. 2000). - Wide mRNA distribution, including lung parenchyma, lung bronchus, and to a lesser extent pulmonary blood vessels (qRT-PCR) (Harmer et al. 2002). - Protein on the surface of type I and type II alveolar epithelial cells in normal human lungs, as well as in A549 cells. Weak positive staining also in the cytoplasm of bronchial epithelial cells (immunochemistry) (Hamming et al. 2004). - Expression in Calu-3 cells revealed by flow cytometry. Immunofluorescence and confocal microscopy localised ACE2 expression on the surface of Calu-3 cells (Tseng et al. 2005).
Endopeptidase 24.11	H_2N□—□—↓■—□—□COOH	Hydrophobic residues	membrane-bound, BALF	- Very weak activity detected in the human bronchus, where bronchial epithelium was faintly stained (enzyme histochemistry) (van der Velden et al. 1998). - Activity in broncho-alveolar lavage fluid (BALF) by enzyme histochemistry (Van Der Velden et al. 1999). - Low activity in plasma membrane preparations of A549 cells (Forbes et al. 1999). - Activity in Caco-2 cells (Howell et al. 1992).
Endopeptidase 24.15	H_2N□—↓■↓—□—□COOH	Many, but only short peptides	cytosolic	- No data available
Endopeptidase 24.18	H_2N□—↓■↓—□—□COOH	Aromatic amino acids	membrane-bound	- mRNA was detected in Caco-2 cells by RT-PCR. A postconfluental increase of mRNA expression was observed with Northern blotting technique (Lottaz et al. 1999).
Enteropeptidase	H_2N□—□—■↓—□—□COOH	$(Asp)_4$-Lys	membrane-bound	- mRNA was detected in A549 cells, but not in Caco-2 using RT-PCR (Cottrell et al. 2004).

2.3. MATERIALS AND METHODS

2.3.1. Materials

Calu-3 cells (American Type Culture Collection, ATCC HTB-55), A549 (ATCC CL-185) and Caco-2 cells (ATCC HTB-37) were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The 16HBE14o- cell line was a kind gift from Dr. Dieter C. Gruenert (California Pacific Medical Center, San Francisco, CA). Human bronchial epithelial cells (hBEpC) were purchased from Lonza (Verviers, Belgium). All cell culture media and supplements (i.e., bovine pituitary extract (BPE), hydrocortisone, human epidermal growth factor (hEGF), epinephrine, insulin, triiodothyronine, transferrin, gentamicin, amphotericin-B, retinoic acid, foetal bovine serum (FBS), penicillin, streptomycin, non-essential amino acids (NEAA) and sodium pyruvate) were also from Lonza. Transwell Clear filter inserts and 6-well plates were purchased from Corning (Schiphol, The Netherlands). Primers were ordered from Eurofins (Ebersberg, Germany). The RNeasy Mini Kit and the RNase-Free DNase Set were obtained from Qiagen (Crawley, UK). Genomic DNA, Bioscript, $Pd(N)_6$ random hexamers 5'-Phosphate, deoxynucleotide triphosphate (dNTP) mix, diethylpyrocarbonate-treated water, RNAse Inhibitor, BIOTAQ Core kit, HyperLadder IV size marker and agarose were all purchased from Bioline (London, UK). Ethidium bromide was purchased from Sigma-Aldrich (Steinheim, Germany).

2.3.2. Cell culture

hBEpC in primary culture were used at passage numbers 1 to 3 in order to prevent dedifferentiation. The cells were seeded (*performed by Dr Sibylle Endter and Dr Carsten Ehrhardt, Trinity College Dublin, Ireland)* at 50,000 cells/cm^2 on collagen I-coated Transwell Clear filter inserts and cultured under air-interfaced conditions from day 2 onwards in bronchial epithelial basal medium (BEBM) supplemented with BPE, hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferrin, gentamicin, amphotericin-B and retinoic acid. Total RNA was isolated on day 9 of culture.

Calu-3 is a human bronchial epithelial cell line derived from an adenocarcinoma of the lung (Fogh and Trempe 1975). The cells (passage numbers 44 and 56 to 58) were seeded at a density of 75,000 cells/cm^2 in 6-well plates and grown to monolayers in Eagle's Minimum Essential Medium (EMEM, supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1 mM sodium pyruvate and non-essential amino acids). Cells reached confluence by day 8 of culture. Total RNA was isolated after 4, 8 and 12 days to investigate the different stages of gene expression with continuing differentiation of the cells over the time.

The 16HBE14o- cell line was generated by transformation of normal bronchial epithelial cells obtained from a one-year-old male heart-lung transplant patient. Transformation was accomplished with SV40 large T antigen (Cozens *et al.* 1994). Cells (passage numbers 2.65 to 2.66) were grown in 6-well plates at 100,000 cells/cm^2 and cultured in EMEM of similar composition as in the case of Calu-3 cells. Cell monolayer confluence was reached after 3 days, total RNA was isolated after 8 days in culture.

A549 cells are derived from a human pulmonary adenocarcinom (Giard *et al.* 1973). Due to phenotype similarity to type II alveolar epithelial cells, this cell line has been widely utilised in studies of alveolar epithelium function (Sporty *et al.* 2008). Cells (passage number 63) were cultured in 6-well plates with a seeding density of 40,000/cm^2 with Dulbecco's Modified Eagle's Medium : Ham's F-12 (1:1 mix) (DMEM-F12, 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin). A549 cultures reached confluence by day 5 when mRNA was isolated.

Caco-2 cells, derived from colonic adenocarcinoma epithelium, were used as a gastrointestinal reference cell line. The cells (passage numbers 27 to 30) were cultured in 6-well plates in DMEM with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1 mM sodium pyruvate and non-essential amino acids. Total RNA was isolated on day 7 and 14 post seeding.

All cells were cultured at 37℃ and 5% CO_2 atmosphere; all culture media were exchanged every 48 h.

2.3.3. Primer design

The sequences of the investigated enzymes were obtained from *CHIP Bioinformatics Tools* (http://snpper.chip.org/; last used May 2008). When choosing an exon, the biology of the gene, i.e., the presence of splice variants, truncated versions, etc. were taken into account. All primers were designed against sequences without known single-nucleotide polymorphisms (SNPs) in order to cover all transcript variants. Exons of suitable size (150bp+) and present in all gene transcription variants were validated by using *Primer3* (http://frodo.wi.mit.edu/; last used May 2008) and primers were designed against these sequences (see Table 2.2). Primers were preferably designed within an exon. However, primers for trypsin 2, GGT 1 and GGH there designed including an intron. The selected primer sequences were aligned with *Nucleotide Blast* (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn&MEGABLAST=on&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on; last used May 2008) in order to confirm their selectivity of binding to the specific sequence only. Primers were optimised by PCR with genomic DNA using varying temperatures and $MgCl_2$-concentrations (see Table 2.3).

Table 2.2. Specific primer sequences for RT-PCR analysis. All primers were derived from GenBank, using the displayed accession numbers. Where more than one transcript variant exists, accession number for variant 1 is displayed. Other variants can be accessed through this record.

Peptidase name	Gene	Forward primer 5' to 3'	Reverse Primer 3' to 5'	Product size (bp)	Gen Bank Accession No.
trypsin 1	PRSS1	CTG CCC CCT TTG ATG ATG	TAG CAG TGG CCT GCT GAT AC	151	NM_002769
trypsin 2	PRSS2	GAG CCA GGC TGA GTG TGA A	ACT CCA GGC CTG TTC TTC TG	186 (486)	NM_002770
neutrophilic elastase	ELANE (ELA2)	CCG GTG GCA CAG TTT GTA A	tat tgt gcc aga tgc tgg ag	166	NM_001972
carboxypeptidase A 1	CPA1	TCA AGC CAG TGG AAG CAC TA	gga gga caa gaa ggg tgt ca	217	NM_001868
carboxypeptidase A 2	CPA2	CAG CAA CCC TTG CTC TGA TT	ACC CAT AGG GGA ACA TCA GC	152	NM_001869
carboxypeptidase B 1	CPB1	CTG GGC TTA TGA CCA AGG AA	aag gcc atc agc ttt ctc aa	183	NM_001871
carboxypeptidase M	CPM	ggg agc cac ttg aaa caa aa	cca gtt tat ttt ggg gag ca	250	NM_001874
γ-glutamyl carboxypeptidase	GGH	ttt ccc tgg agg aag tgt tg	tct gga aca ttc tgc tgt gc	245	NM_003878
aminopeptidase A	ENPEP	GTC CGG AGG TGT TTC GAG TA	CGT TCT CCG TGT AGG TGG TT	166	NM_001977
aminopeptidase B	RNPEP	GCC AAG GAG ACT TTT GCA TC	aga ggc cca aga gtc aca ga	245	NM_020216
aminopeptidase N	ANPEP	GTG CTC CAG TGG TTC ACA GA	ggc tca cag gca gag aga ac	188	NM_001150
aminopeptidase P 1	XPNPEP1	GCT TCT TGA CTT GGG TCT GG	TGG TCT CGC TCA CAG CAT AG	152	NM_020383
aminopeptidase P 2	XPNPEP2	caa gag agc aat gcc cct ac	agc cta ccc agg gtt tag ga	161	NM_003399
aminopeptidase P 3 (putative)	XPNPEP3 LOC63929	AGA AGT TTC GGG GTC TTG GT	ctg cca gaa gac acc cat tt	184	NM_022098

(**Table 2.2.** continued)

γ-glutamyl transpeptidase 1	GGT1	cag gct gga cag ttc agt gat	gac agg agg att tgg tgg aa	163 (487)	NM_013430
γ-glutamyl transpeptidase 2	GGT2	GTC CAC CTT CAT CGC TGT G	gac agg agg att tgg tgg aa	229	NM_002058
dipeptidyl peptidase IV	DPP4	gga acc caa gtc caa gca ta	ttg ctg gca gtt tcc ctt ac	204	NM_001935
angiotensin I converting enzyme (isoforms 1 + 2)	ACE	aga ctg gga tgg gaa cac tg	ggg agg gca gga cct tta at	209	NM_000789 NM_152830
angiotensin I converting enzyme 2	ACE2	tcc agg gaa cag gta gag ga	gct cag cac tgc tca aac ac	246	NM_021804
endopeptidase-24.11 (neprilysin)	MME	ctg cct tcc cat gaa tct gt	cag ctg tgg caa caa gaa tg	225	NM_000902
endopeptidase-24.15 (THOP1)	THOP1	gcc tct tgt cat tgt ctg tcc	ctc ctg aca agg gac gtt tc	150	NM_003249
endopeptidase-24.18 (meprin A)	MEP1A	AAA AGG CGA CCC TCA GAA CT	CCC ACT GCA CAC ATG AAA AG	250	NM_005588
enteropeptidase	PRSS7	ACA AGT GTG CCC TGC CTA AT	ccc cct agc att tca ttg ac	225	NM_002772
β-actin	ACTB	AAA CTG GAA CGG TGA AGG TG	AGA GAA GTG GGG TGG CTT TT	171	NM_001101

2.3.4. RNA isolation and reverse transcription polymerase chain reaction

The A549 cell line was investigated by Gaelle Tachon, Trinity College Dublin, Ireland / Université Claude Bernard Lyon, France.

Polymerase chain reaction (PCR) was performed using cells from three to five different isolations. Briefly, total RNA was isolated and purified from cells with an RNeasy Mini Kit according to the manufacturer's instructions. On-column digestion of DNA during RNA purification was performed with an RNase-free DNase Set. Reverse transcription was carried using Bioscript, $Pd(N)_6$ random hexamers 5'-Phosphate, dNTP mix, diethylpyrocarbonate-treated water and RNAse Inhibitor. Complementary DNA samples isolated from different cell lines (corresponding 40 ng RNA) were used for each PCR run with a BIOTAQ Core kit including 0.75 µl, 0.9 µl, 1.1 µl or 1.5 µl (depending on optimum for each primer, see Table 2.3) of 25 mM $MgCl_2$, 1 µl 2.5 mM dNTP mix, 1.25 µl Taq buffer, 0.1 µl Taq polymerase, 0.5 µl 10 pmol/µl of each primer, forward and reverse, and diethylpyrocarbonate-treated water up to a total volume of 12.5 µl. The reaction was incubated at 94℃ for 5 min followed by thermal cyc ling conditions of 35 cycles of 30 s at 94℃, 45 s at 54-62℃ (depending on optimum for eac h primer, see Table 2.3) and 45 s at 72℃. After the last cycle, an additional step of 1 0 min at 72℃ with the Taq polymerase completed any unfinished regions. DNA fragments were separated and visualised by gel electrophoresis, using a 2% agarose gel containing 0.2 µg ethidium bromide per ml gel. A HyperLadder IV size marker was run in parallel. Electrophoresis was carried out at 90 V for 45 min.

2.3.5. Data analysis of mRNA expression of peptidases in the lung

Table 2.4 shows data on the relative number of expressed-sequence clones per million identified in tissue and species-specific databases. Expressed-sequence tags (ESTs) of the various genes were compared using the Gene Expression Omnibus (GEO) repository at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/unigene; 06.09.2010). The gene of interest was entered into

the database and the respective link for *Homo sapiens* was selected. The EST profile shows the distribution of the respective enzyme in various tissues; and for this study, the numbers listed for human lung tissue were selected. Note that the EST profile provides data for human lung, but does not distinguish between different tissues and cell types of the lung.

Table 2.3. Primers were optimised with genomic DNA using varying temperatures and $MgCl_2$-concentrations. The optimum conditions for the primers are summarised in this table.

Primers for respective peptidase	Optimum primer conditions	
	temperature [℃]	volume of 25 mM $MgCl_2$ [µl]
trypsin 1	55.5	0.75
trypsin 2	60.0	0.75
neutrophilic elastase	57.7	0.75
carboxypeptidase A 1	62.0	1.50
carboxypeptidase A 2	60.0	0.75
carboxypeptidase B 1	55.5	0.75
carboxypeptidase M	54.0	0.90
γ-glutamyl carboxypeptidase	57.7	1.10
aminopeptidase A	55.5	0.75
aminopeptidase B	60.0	1.50
aminopeptidase N	60.0	1.50
aminopeptidase P 1	57.7	0.75
aminopeptidase P 2	57.7	0.75
aminopeptidase P 3 (putative)	62.0	0.90
γ-glutamyl transpeptidase 1	62.0	0.90
γ-glutamyl transpeptidase 2	60.0	1.50
dipeptidyl peptidase IV	57.7	0.75
angiotensin I converting enzyme	62.0	0.90
angiotensin I converting enzyme 2	60.0	0.75
endopeptidase-24.11 (neprilysin)	55.5	0.90
endopeptidase-24.15 (THOP1)	55.5	0.75
endopeptidase-24.18 (meprin A)	57.7	0.75
enteropeptidase	60.0	0.90
β-actin	all conditions	all conditions

2.4. RESULTS

2.4.1. Proteolytic enzyme expression

In this part of my thesis, I aimed to generate a comprehensive mRNA profile for selected proteolytic enzymes expressed in human lung epithelial cells. Beta-actin was used as a positive control in all RT-PCR reactions; the negative controls comprising cDNA-negative, RT-negative samples. Semi-quantitative RT-PCR-derived expression profiles of peptidases in different human respiratory epithelial cell models and the human colonic adenocarcinoma cell line, Caco-2, are shown in Table 2.4, using the following symbols to indicate signal intensity compared to the ubiquitously strong expression levels of β-actin: - denoting no detectable expression; + denoting low expression; ++ denoting moderate expression; and +++ denoting high expression. The rating system was based on comparison of expression levels by eye and is therefore somewhat subjective. The idea was, however, to show *if* an enzyme is expressed on mRNA level and if so, to give a *trend* of its expression level rather than absolute numbers. Figure 2.1 illustrates the expression of peptidases in the various cell types; of all investigated enzymes the aminopeptidases showed the most widespread expression profile. Note the levels of β-actin were consistently (+++) throughout all mRNA samples analysed in Table 2.4.

These results show differences in mRNA expression patterns between all investigated cell models. There is an apparent difference between the primary cells (hBEpC) and the immortalised cell lines: neutrophilic elastase and CPB mRNAs could only be detected in hBEpC, but in none of the other cell types, meprin A mRNA was found in hBEpC, but none of the investigated cell lines derived from human lung, whereas CPA1 and APN mRNA were expressed in all the cell types except for hBEpC (Table 2.3).
Although considered to be of bronchial epithelial phenotype, neither Calu-3 nor 16HBE14o- cells matched the mRNA expression pattern of hBEpC in primary culture. The primary cells, Calu-3 and 16HBE14o- showed some similarities, but Calu-3 expresses

more of the studied peptidases' mRNA than 16HBE14o-. Calu-3 also was the only investigated cell type that expresses ACE1 mRNA.

A549 was distinguished from the other cell lines by expressing less peptidase mRNAs than the others: Only 13 of the investigated 23 peptidase mRNAs were detected in A549 cells. From all the other studies cell models, Calu-3 is the one that shows most similarities with the A549 expression pattern.

On the whole, the intestinal cell line Caco-2 indicated the largest spectrum of peptidase mRNA expressed. All selected proteolylic enzymes except neutrophilic elastase, CPB and ACE1 were detected. APP2 mRNA expression was found only in Caco-2 cells, but in none of the investigated cells derived from human lung epithelium.

In Calu-3 and Caco-2 cultures mRNA expression was investigated at different time points. In the case of Calu-3 monolayers, a gradual increase of mRNA expression over time was observed for APN, GGT1, GGT2, DPP IV and ACE2. GGT1 showed a second unspecific band at about 280 bp. Results obtained with mRNA derived from Caco-2 monolayers showed a gradual increase of expression level in the cases of trypsin 1 and 2, THOP1 and enteropeptidase. Slight decreases of expression levels from day 4 to day 8 in Calu-3 can be observed for CPM, neprilysin and THOP. This might be due to analysis of very small amounts, just around detection level or the cells actually express certain mRNA in varying amounts over the time.

2.4.2. GEO profiling

GEO profiling allowed a comparison of my experimental data to cDNA libraries and suggested similar overall expression levels. Neutrophil elastase, aminopeptidase P2, γ-glutamyl transpeptidase 2, ACE2 and carboxypeptidases A1, A2 and B1 were represented with 0 or 2 hits in the GEO database, respectively, and adequately show only moderate, low or no mRNA expression in my experimental results. Aminopeptidase B, which was found strongly or moderately expressed in all cell types investigated, also shows the

greatest number of ESTs in the GEO profile. Carboxypeptidase M mRNA, however, was detected at a lower level in my studies than stated by the GEO figure. The GEO profile correlated best with the expression pattern found in Calu-3 cells.

Table 2.4. RT-PCR expression profile of selected proteolytic enzymes. Transcript expression was examined in a variety of cell culture models including both primary and immortalised cell lines. Relative number of expressed-sequence clones per million of respective enzymes in lung identified in tissue and species-specific databases (Barrett *et al.* 2007) stated as comparison. The number of hits were classified as follows: 0 (-), 1-19 (+), 20-79 (++), 80 and more (+++),* not specified. This table was last updated 06/09/2010.

	hBEpC	16HBE 14o-		Calu-3		A549	Caco-2		GEO profile
	day 9	day 8	day4	day8	day12	day5	day7	day14	lung
trypsin 1	+	-	++	++	++	-	+	++	+
trypsin 2	+++	-	++	++	+++	++	++	+++	*
neutrophilic elastase	+	-	-	-	-	-	-	-	+
carboxypeptidase A 1	-	++	+	+	+	+	+	+	+
carboxypeptidase A 2	++	+	-	-	-	-	+	+	-
carboxypeptidase B 1	++	-	-	-	-	-	-	-	-
carboxypeptidase M	++	+	+	-	++	+	++	++	+++
γ-glutamyl carboxypeptidase	++	+++	++	++	++	+++	+++	+++	++
aminopeptidase A	-	-	-	-	-	+++	++	++	+
aminopeptidase B	++	+++	+++	+++	+++	++	++	++	+++
aminopeptidase N	-	+++	-	+	+++	+	++	++	++
aminopeptidase P 1	+	++	+++	+++	++	++	++	++	++
aminopeptidase P 2	-	-	-	-	-	-	++	+	+
aminopeptidase P 3	-	++	+++	+++	+++	+++	+++	+++	++
γ-glutamyl transpeptidase 1	+	+	+	+	++	++	++	++	++
γ-glutamyl transpeptidase 2	+	-	-	+	++	+	+	+	-
dipeptidyl peptidase IV	++	+++	+	+	+++	++	+++	+++	++
ACE (isoforms 1 + 2)	-	-	+	+	+	-	-	-	++
ACE 2	++	+	+	+	++	-	++	++	-
Endop.-24.11 (neprilysin)	+	++	+	-	+	-	++	++	++
Endop.-24.15 (THOP1)	+++	+	+	-	+	+	+	++	++
Endop.-24.18 (meprin A)	+	-	-	-	-	-	++	++	+
Enteropeptidase	-	-	+	+	+	-	+	++	*
β-actin	+++	+++	+++	+++	+++	+++	+++	+++	+++

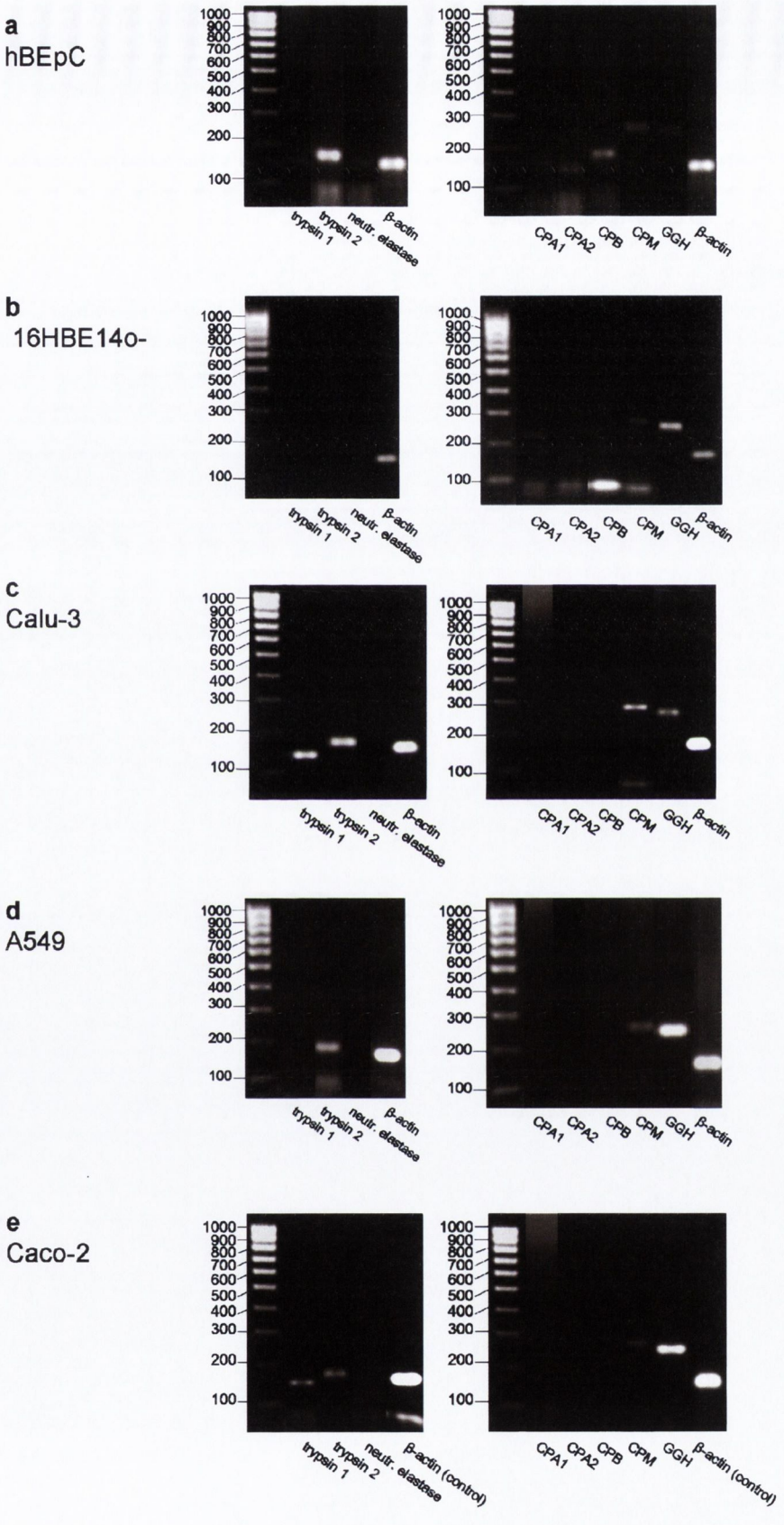
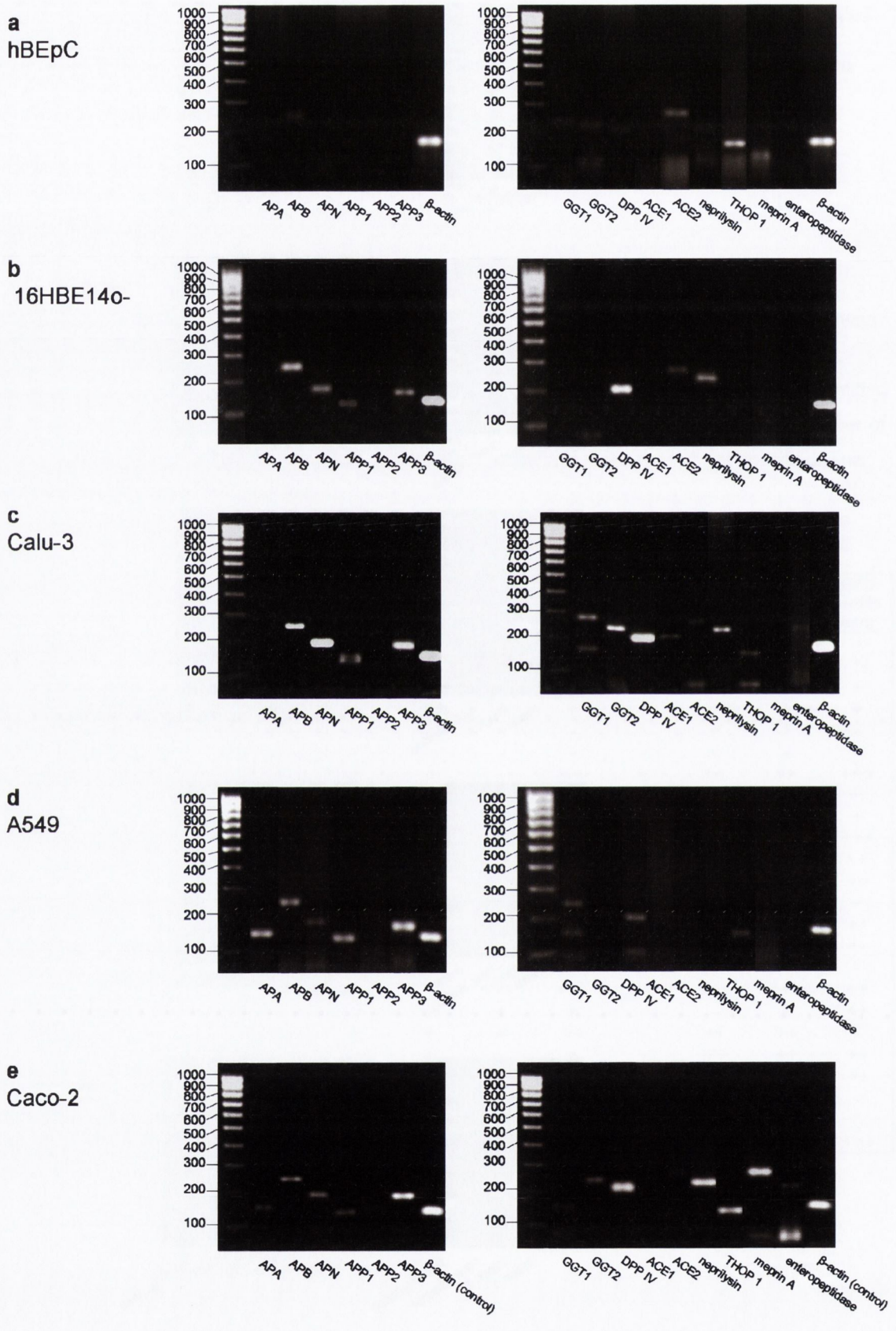


Figure 2.1. Agarose gels showing mRNA expression of the investigated peptidases in hBEpC (a), 16HBE14o- (b), Calu-3 (c), A549 (d) and Caco-2 (e). β-actin was always used as positive control.



(**Figure 2.1.** continued)

2.5 DISCUSSION

To know which proteolytic enzymes are active in the lung is crucial in the context of using the pulmonary route for delivering biopharmaceuticals. Although it has been acknowledged that the lung possesses several defence mechanisms (Patton 1996, Siekmeier and Scheuch 1998) and although some information about enzymes expression and activity has been published (see Table 2.1.), studies that actually focus on peptidases expression and activity in the lung in detail are scarce. In the intestinal tract, the brush border membrane of the epithelial cells is well known to play a role in protein degradation, as it contains at least 15 peptidases that together have a broad specificity and can cleave both proteins and peptides (Woodley 1994). For the small intestine, it is well established that "the lumen and the cell membranes constitute the major enzymatic barriers prior to any absorption". (Woodley 1994, p.67)

As for many peptidases it is not known, if they are present in human lung, and if so, it is not investigated, whether they are expressed in the lung directly or elsewhere. Van der Velden *et al.* (1999) suggested that the presence of peptidases in BALF and in serum is regulated independently of each other and that endopeptidase-24.11, for example, is locally released in the airways. The proteolytic enzymes selected for this study are known to be either secreted into the lumen or to be membrane-bound facing outward from the membrane into the lumen (of the small intestine). Thus, before a protein is absorbed, it is likely to encounter these enzymes on the cell surface. (Woodley 1994) In this chapter, I aimed to investigate whether the same peptidases are expressed in the lung epithelium and if lung epithelium might represent an enzymatic barrier to therapeutic peptides and proteins similar to the intestinal tract. In the case that several isoforms of a respective enzyme exist, they were studied as well, in order to include a broad range of molecules. Aminopeptidase P, for example, has three different forms: besides the membrane-bound APP2 (Kenny *et al.* 1977, Erşahin *et al.* 2005) there is APP1, soluble cytosolic form (Holtzman *et al.* 1987, Vanhoof *et al.* 1997, Cottrell *et al.*2000) with broad substrate specificity (Turner and Cottrell 2004) and a hypothetical isoform APP3 (Erşahin *et al.*

2005). In order to evaluate whether the lung epithelium represents an enzymatic barrier to therapeutic peptides and proteins similar to the intestinal tract, different epithelial cell types derived from the mucosae of lung and intestine were identified for the experiments. All cell types investigated were of human origin. As a surrogate of the bronchial epithelium, two continuously growing cell lines (Calu-3 and 16HBE14o-) as well as primary cells (hBEpC) were studied. This allowed me to compare cells in primary culture with immortalised cells from the same tissue. Both Calu-3 and 16HBE14o- cells are known to form polarised monolayers and functionally express tight junctions, but while the Calu-3 cell line includes ciliated and secretory cells, 16HBE14o- have a non-serous, non-ciliated phenotype (Ehrhardt *et al.* 2008a). It was therefore interesting to investigate, if there are also differences in enzyme expression between two immortalised bronchial epithelial cell lines. The distal lung is regarded as the main area of absorption for inhaled drugs with systemic action, due to its large surface area, the extensive vascularisation and its thin barrier thickness (Patton 1992, Ehrhardt 2008a). So far, no reliable immortalised cell lines possessing the morphology and phenotype of human alveolar epithelium have been reported, hence primary culture of freshly isolated type II pneumocytes is the only available solution. After several days in culture, these ATII cells experience a morphological shift towards ATI-like cells (Ehrhardt *et al.* 2008b). Due to difficulties obtaining human tissue in Ireland, however, I used the alveolar epithelial cell line, A549 in my studies. The inability to express functional cell-cell junction, however, renders A549 cells unsuitable for transport studies (Ehrhardt *et al.* 2008b). This is less relevant for the investigation of peptidases expression though. I am aware that the chosen human pulmonary epithelial cell lines are in so far limited to model for the complete *in vivo* situation as Clara cells and alveolar macrophages are missing from the selection. Moreover, I did not use air-interfaced cultures, which might have an influence on some of the cell properties. The frequently used intestinal epithelial cell line, Caco-2, was studied as comparison, since epithelial peptidases are known to play a role in peptide degradation in the intestinal tract.

The experiments showed that despite obvious differences in expression patterns between the various cell types, the majority of the peptidases' mRNA was indeed expressed in all cell types investigated.

But, which sets of enzymes does a peptide or protein have to encounter when applied via the pulmonary route?

The dissolved molecule has to cross several biological barriers in order to reach the blood stream and to unfold its therapeutic effect systemically. According to Woodley, "the lumen and the cell membranes constitute the major enzymatic barriers prior to any absorption" (Woodley 1994, p.67). Absorption then occurs either via the paracellular pathway (in between adjacent cells), where the drug only faces membrane bound proteases on the apical side of the cell. Tight junctions between the cells limit absorption through this pathway. Only when tight junctions become "leaky", as during inflammatory or allergic conditions (Inagaki *et al.* 1985), macromolecules are allowed to be absorbed through this pathway. Other pathways are the transcellular route (through the epithelial cells), as well as transcytosis and receptor mediated endocytosis, where the drug molecules have to pass intracellularly (Goldberg and Gomez-Orellana 2003). In this thesis, however, the focus was put on peptidases known to be located on the apical side of the brush border membrane in the gastrointestinal tract (GIT), as all protein or peptide drugs have to encounter those enzymes before absorption (Woodley 1994). Other peptidases that might be expressed in the cytosol or in lysosomes of epithelial cells are only discussed when they are an isoform or similar to one of the other investigated enzymes.

Mucus, surfactant and macrophages represent the first barrier to pass. When a particle is inhaled and deposited in the bronchi and bronchioles, a continuously moving layer of mucous carries the particle upwards into the throat where it is swallowed (Patton and Platz 1992).

If the drug reaches the deeper airways and is not removed by mucociliary transport, instead it encounters a set of enzymes that are secreted by lung epithelial cells. Secreted enzymes are the serine endopeptidases, i.e., trypsin and various elastases, and carboxypeptidases A and B (Woodley 1994). While pancreatic elastase is active in the GIT, neutrophil elastase has been regarded as key effector of lung injury (Reid *et al.* 1999) and was therefore investigated in this study.

The mRNA of trypsin 1 and 2, neutrophil elastase and carboxypeptidases A1, A2 and B1 could be detected in various investigated cell types. However, neutrophil elastase and carboxypeptidase B1 were only found to be expressed in the human bronchial primary cells.

Literature provides only little data about secreted peptidases in the lung (see Table 2.1). APN, endopeptidase-24.11 and DPP IV activity was previously detected in BALF, and it was suggested that the presence of these peptidases in serum and BALF "is regulated independently of each other and that the peptidases are locally released into the airways" (Van Der Velden *et al.* 1999, p. 820; van der Velden *et al.* 2000). These peptidases also exist in a membrane-bound form (Woodley 1994), so from my results I can only suggest that the enzymes' mRNA is expressed but it is not possible to confirm the enzymes' localisation. The results of this chapter corroborate that the secreted peptidases under investigation are indeed widely expressed and should be considered qualitatively and quantitatively when peptide or protein drugs are applied via the pulmonary route.

Cell membranes are another barrier to overcome prior to absorption. The membrane-bound peptidases can be divided into endopeptidases that hydrolyse amino acid bonds in the middle of a peptide or protein and exopeptidases which cleave N-terminal amino acids (aminopeptidases) or the C-terminal amino acids from peptides and proteins (carboxypeptidases) (Woodley 1994).

Endopeptidases in the intestinal tract have been thoroughly investigated (reviewed in Woodley 1994). I found mRNA of all four investigated endopeptidases expressed in Caco-

2 cells. Endopeptidase-24.15 is discussed later, so far little is known about where in the lung it is localised (see Table 2.1). Howell *et al.* (1992) detected endopeptidase-24.11 activity in Caco-2 cells and Lottaz *et al.* (1999) showed endopeptidase-24.18 mRNA by RT-PCR. However, Cottrell *et al.* (2004) did not detect enteropeptidase mRNA in Caco-2 using RT-PCR. Information about endopeptidases in the lung is scarce, no information in published on for endopeptidase-24.18 in human lung. The role of endopeptidase-24.11 in chronic inflammation of the airways was investigated by van der Velden and co-workers (van der Velden *et al.* 1998). They detected very weak activity in the human bronchus by enzyme histochemistry. Attribution of this activity to a certain cell type was difficult, nevertheless, the bronchial epithelium was faintly stained (van der Velden *et al.* 1998). Low levels of endopeptidase-24.11 activity in plasma membrane preparations of rat alveolar type I and type II cells, as well as in A549 cells were measured by Forbes *et al.* (1999).

Enteropeptidase initiates the activation of the secreted peptidases mentioned above by cleaving their precursor molecules (Woodley 1994). It has the ability to activate trypsinogen by splitting a hexapeptide from the N-terminal end. In addition, it can hydrolyse peptides and proteins with sites similar to the cleavage sequence of trypsinogen. (Kunitz 1939, Sadler 2004) Active trypsin can then activate other secreted peptidases (Woodley 1994). Interestingly, I found more or less the same expression profiles for trypsin 1 and enteropeptidase which enables trypsin to be activated. Overall, the expression of the various endopeptidases in the investigated human lung cells is absent to moderate with the exception of strong endopeptidase-24.15 expression in hBEpC.

Membrane-bound aminopeptidases (AP) investigated were APA, APB, APN, APP2, γ-glutamyl transpeptidases (GGT) 1 and GGT2, as well as dipeptidyl peptidase (DPP) IV (see Figure 2.1). mRNA of all studied aminopeptidases' was found in Caco-2 cells. Howell *et al.* (Howell *et al.* 1992) investigated the presence of membrane peptidases in Caco-2

monolayers and detected activity of APP, APN, GGT (after 15 days in culture) and DPP IV, but not APA. Darmoul *et al.* (1992) observed an increase of DPP IV activity as well as mRNA expression (Northern blotting) with further differentiation of Caco-2 cells.

APN expression in the lung has received a lot of attention, due to the observation that APN is cell specific surface marker of alveolar type II epithelial cells (Funkhouser *et al.* 1987, Gillis *et al.* 1998). To further support these data, I did not find APN mRNA expression in bronchial primary cells (hBEpC), though it was detected in all investigated immortalised cell types. Forbes *et al.* (1999) reported APN activity in plasma membrane preparations of rat alveolar type I and type II cells, as well as in A549 cells.

GGT1 is most strongly expressed in cells with active secretory or absorptive functions (Hiratake *et al.* 2004) and it is localised to the luminal cell surface (Hanigan *et al.* 1999). Wetmore *et al.* (1993) did not only find the "classic" form of GGT mRNA in human lung, kidney, liver, pancreas, placenta and brain, but also a unique, shorter transcript which was exclusive to the lung. Studies about the exact localisation of GGT in the lung are somewhat conflicting. Dinsdale *et al.* (1992) found GGT in rat lung to be concentrated on the luminal surface of Clara cells and, to a lesser degree, on the surface of type II alveolar pneumocytes. Ingbar *et al.* (1995), however, localised GGT to the apical surface of alveolar epithelial cells, but more densely on type I cells than type II cells, using immuno-electronmicroscopy. Moreover, they found GGT on the apical surface of some ciliated bronchial cells. Rahman *et al.* (1998) and Zaman *et al.* (2006) measured GGT activity in A549 cells and concordantly, moderate GGT mRNA expression in A549 was detected in my experiments.

Literature about GGT2 is scarce and to my knowledge, there is no information about GGT2 expression in lung epithelium. Interestingly, I found an mRNA expression pattern which is quite similar to that of GGT1, although coded for by two different genes.

There is also little information concerning the aminopeptidases A, B and P in human lung (see Table 2.1). Compared to the cytosolic form, APP1, the membrane-bound APP2 has more restricted substrate specificity (Simmons 2004, Erşahin *et al.* 2005). Due to its

location on the cell surface of intestinal and renal epithelial brush border membranes, APP2 is assumed to be involved in the catabolism of dietary and filtered peptides (Erşahin *et al.* 2005). In human lung, the presence of APP2 mRNA has previously been reported (Venema *et al.* 1997) but the exact location has so far only been investigated for rat tissue: Chen *et al.* (1991) assumed aminopeptidase P in rats to be located on the luminal surface of the rat pulmonary endothelium.

Immunohistochemical and enzyme-histochemical analysis of the distribution of DPP IV in the human bronchus showed enzyme expression in submucosal glands and blood vessels (van der Velden *et al.* 1998). According to van der Velden *et al.* (1998), no staining of fibroblasts, bronchial epithelium and smooth muscle cells could be observed. However, my results showed DPP IV mRNA expression in all studied cells derived from bronchial epithelium. DPP IV was also found to be present in alveolar cells; my experiments confirmed mRNA expression in A549 cells while DPP IV activity was previously reported in plasma membrane preparations of A549 cells, as well as alveolar type I and type II cells derived from rat (Forbes *et al.* 1999).

Summarising these results, most aminopeptidases showed either strong mRNA expression or no expression at all.

The investigated membrane-bound carboxypeptidases (CP) are CPM, gamma-glutamyl carboxypeptidase (gamma-glutamyl hydrolase, GGH), and angiotensin I converting enzyme (ACE) as well as ACE2. All CP have been studied in human lung by other groups before (see Table 2.1), with ACE2 apparently attracting the highest level of attention.

"CPM was discovered and characterised as the first true membrane-bound member of this class of enzymes" (Li *et al.* 2002, p.189), i.e., the mammalian metallocarboxypeptidases. To elucidate the fact that CPM is membrane-bound and to distinguish it from other known mammalian carboxypeptidases, which are primarily soluble enzymes, Skidgel *et al.* (1989) suggested the letter "M". CPM can "inactivate or modulate the activity of peptide

hormones either before or after their interaction with plasma membrane receptors" because of its localisation on the plasma membrane (Skidgel *et al.* 1989, p. 2236).

ACE was isolated in 1956 as "hypertension-converting enzyme" by Skeggs *et al.* (1956) It cleaves the C-terminal dipeptide from angiotensin I and releases the potent vasopressor angiotensin II (Skeggs *et al.* 1956, Dorer *et al.* 1974), but is also able to cleave C-terminal dipeptides from various other substrates with free C-terminus and to split a C-terminal dipeptide-amide (Corvol *et al.* 2004). Expression of ACE mRNA in human tissues is widespread, amongst others being found in the colon, small intestine and heart, with highest expression levels in lung and kidney (Tipnis *et al.* 2000). There is a somatic isoform found in endothelial, epithelial and neuronal cells and a smaller isoform present in germinal cells. The somatic form is present in the plasma membrane of vascular endothelium, especially in the lung, and mainly catabolises circulating substrates. (Oparil and Haber 1974a and b, Corvol *et al.* 2004)

ACE2 was the first homologue of ACE identified and has therefore also been referred to as angiotensin-converting enzyme homolog (ACEH) (Tipnis *et al.* 2000). Although ACE and ACE2 show 40% identity in amino acid sequence, ACE2 functions as a carboxypeptidase, not as peptidyl dipeptidase. ACE and ACE2 hydrolyse angiotensin I (Ang I), but due to their distinct activities, different products are generated (Donoghue *et al.* 2000, Tipnis *et al.* 2000, Turner and Hooper 2004). In recent years, ACE2 has been reported to play an important role in several lung diseases and it has been identified as severe acute respiratory syndrome (SARS) coronavirus receptor (Kuba *et al.* 2006). Li *et al.* (2008) observed that ACE2 mRNA and activity are dramatically down-regulated in lung fibrosis and they suggested a protective effect of ACE2 against lung fibrogenesis. Considering that ACE2 protein had not been identified in lung before, the most remarkable finding is the surface expression of ACE2 protein on type I and type II alveolar epithelial cells in normal lungs, as well as in the A549 cell line (Hamming *et al.* 2004). Hamming *et al.* (2004) also reported weak positive staining in the cytoplasm of bronchial epithelial cells

In this experimental study, CPM and GGH mRNA was detected in all cell types investigated. In addition to APB, GGH was the peptidase that showed the strongest signals of all peptidases investigated. Interestingly, ACE and ACE2 showed very different expression profiles. While ACE2 was expressed in all cell types but A549, ACE could only be detected in Calu-3 cells.

The final enzymatic barrier to overcome by an absorbed protein or peptide is within the cytoplasma of the epithelial cell (in the case that the drug is actually transcellularly transported).

APP1 is a soluble cytosolic form (Holtzman *et al.* 1987, Vanhoof *et al.* 1997, Cottrell *et al.* 2000) with broad substrate specificity (Turner and Cottrell 2004). No specific physiological role has been ascribed to APP1 yet, but it is presumably involved in later phases of protein catabolsim (Erşahin *et al.* 2005). Erşahin *et al.* (2005) also determined expression levels of the aminopeptidase P isoforms in various human tissues using semi quantitative RT-PCR and found APP1 expression in all tissues investigated including human lung. Endopeptidase-24.15 is expressed in various mammalian tissues with highest expression levels in the brain, pituitary gland, and testis, while much lower activity was found in lung tissue (Chu and Orlowski 1985, Ray *et al.* 2004). However, very little is known about where in the lung endopeptidase-24.15 is localised. Choi *et al.* (1990) found THOP1 expression within the cytoplasm of ciliated epithelial cells of tracheo-bronchial mucosa using immunochemical techniques.

The results of this study show APP1, as well as endopeptidase-24.15 mRNA expression in all cell types investigated, however, with varying expression levels. Although only two among a number of other peptidases were investigated, the results indicate that cytosolic enzymes are widely expressed throughout different respiratory epithelial cell types and might cause degradation of intracellularly processed peptides and proteins.

2.6. CONCLUSIONS

Although expression and activity of individual enzymes have been acknowledged in several studies (see Table 2.1), ,literature providing a comprehensive view on peptidases expression in the human lung is scarce. The results of this chapter of my thesis show a plethora of enzymes being expressed in lung epithelial cells as assessed by RNA analyses. These results also demonstrate that there are significant differences between primary cells and immortalised cells, but also between the different cell lines. Although mRNA expression does not necessarily signify enzyme functionality, the results provide a first overview on peptidase and protease occurrence and distribution in epithelial cells of the lung and the GIT. While it should not be ignored that there are also lysosomal low pH, enzymes in the interstitium, endothelium or other lung cells, as well as alveolar macrophages responsible for degradation of inhaled peptide and protein drugs, this study provides an overview about which peptidases might be present in human lung epithelial cells and are the basis for further, more specific investigations.

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Chapter 3

Investigations into the fate of inhaled salmon calcitonin at the respiratory epithelial barrier

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Pharm Res. (submitted in February 2011)

3.1. ABSTRACT

The fate of inhaled salmon calcitonin (sCT) at the respiratory epithelial barrier was studied with particular emphasis on enzymatic degradation by trypsin, chymotrypsin and neutrophil elastase. Stability of sCT was studied in homogenate, supernatant and on cell monolayers of human respiratory epithelial cells (hBEpC, Calu-3, 16HBE14o-, A549) and Caco-2 as comparison. After incubation at 37℃ for 2 h, samples were serially withdrawn and analysed by HPLC for sCT content. By similar means, degradation of sCT by pure trypsin, chymotrypsin and neutrophil elastase was investigated. The presence of trypsin, chymotrypsin and neutrophil elastase in cell supernatant/homogenate was studied by Western blot and enzymes' activities by UV measurements. The sCT concentration remained unchanged over the period of 2 h, when incubated in supernatant or on cell monolayers, independent of the cell type used. When cell homogenates were studied, sCT concentrations were reduced to varying exents. sCT was also degraded when incubated with pure enzymes. Western blot revealed strong signals for all proteinases in the cell homogenates and weaker, varying expression in supernatants. Epithelial proteases appear to play a role in interactions of sCT with lung epithelium.

3.2. INTRODUCTION

Despite the failure of Exubera® (Pfizer's inhalable insulin), oral inhalation remains a promising non-invasive route for systemic administration of macromolecules (Stevenson 2009). The pulmonary route would, however, be even more attractive, if more detailed mechanisms were known about the fate of aerosolised medicines after their deposition in the respiratory tract (Patton *et al.* 2010). Any drug that is delivered as an aerosol via the lungs has to cross several biological barriers in order to reach the systemic circulation and exert its therapeutic effect. These barriers include, but are not limited to, mucus, ciliary escalator, pulmonary surfactants and macrophages. (Patton and Platz 1992) In addition, catabolic enzymes such as the serine endopeptidases trypsin, chymotrypsin, and various elastases that are secreted into the lumen by epithelial cells can further reduce the bioavailability of the biopharmaceutical (Woodley 1994). Whilst the lungs show higher systemic bioavailabilities for macromolecules than any other non-invasive route of administration (Patton 1996, Patton *et al.* 1998, Dershwitz *et al.* 2000, Patton and Byron 2007), small natural peptides still suffer from high enzymatic degradation, whereas peptide hydrolysis decreases or is even absent with increasing molecular weight (Patton and Byron 2007).

To better understand processes involved in pulmonary macromolecule disposition on a molecular level, I studied the expression profiles of peptidases' mRNA in respiratory epithelial cells (see Chapter 2). In this chapter of my thesis, I chose to study the fate of the 32-amino-acid peptide hormone, salmon calcitonin (sCT), at the blood-air barrier to further advance our understanding of the field. Salmon calcitonin has been approved for the treatment of hypercalcaemia associated with malignant diseases and for the treatment of bone diseases such as osteoporosis and Paget's disease and is currently marketed as injectable solution (Calcimar®, Miacalcin®) as well as nasal spray (Fortical®, Miacalcin®). However, injections are inconvenient for the patient and the nasal formulation shows a very wide relative bioavailability window ranging from 0.3% to 30% (Stevenson 2009).

Bernkop-Schnürch *et al.* (2003) suggested that the full market potential of sCT products has not yet been realised. Limitations such as low bioavailability after nasal application or the drug's poor stability after oral delivery could potentially be avoided by pulmonary administration.

It has been suggested that salmon calcitonin is predominantly degraded by the serine proteases, trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and pancreatic elastase (EC 3.4.21.36) (Dohi *et al.* 1993, Lang *et al.* 1996, Guggi and Bernkop-Schnürch 2003). While pancreatic elastase (chymotrypsin-like elastase) is active in the GIT, neutrophil elastase (3.4.21.37) has been recognised as the pulmonary form of the enzyme, which plays a role in lung injury and lung defence (Reid *et al.* 1999). Potential cleavage sites of the three degrading enzymes in sCT are illustrated in Figure 3.1 (modified from Guggi and Bernkop-Schnürch 2003).

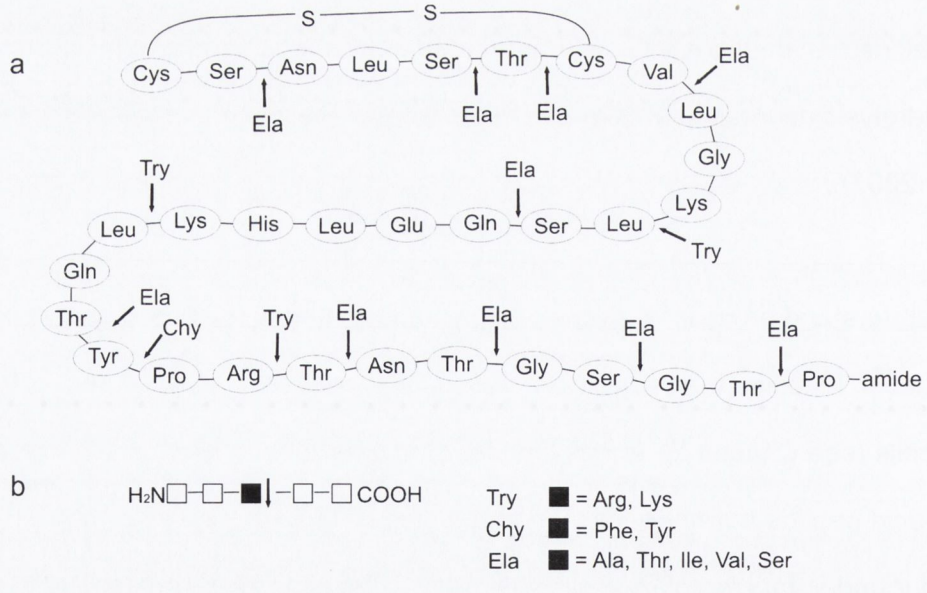

Figure 3.1. Salmon calcitonin (sCT) is a substrate of trypsin (Try), α-chymotrypsin (Chy) and neutrophil elastase (Ela). a) Amino acid sequence of sCT including potential cleavage sites for trypsin, chymotrypsin and neutrophil elastase (modified from Guggi and Bernkop-Schnürch 2003). b) Substrate specificity of Try, Chy and Ela (modified from Woodley 1994).

In this chapter of my thesis, the stability of sCT was tested in pure enzyme solution as well as *in vitro* cell cultures of human lung epithelium to better understand the role these peptidases play in limiting the drug's bioavailability after inhahation.

3.3. MATERIALS AND METHODS

3.3.1. Materials

Calu-3 cells (American Type Culture Collection, ATCC HTB-55), A549 (ATCC CL-185) and Caco-2 cells (ATCC HTB-37) were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). The 16HBE14o- cell line was a kind gift from Dr. Dieter C. Gruenert (California Pacific Medical Center, San Francisco, CA). Freshly isolated human bronchial epithelial cells (hBEpC) were purchased from Lonza (Verviers, Belgium). Cell culture media and media supplements (i.e., sodium pyruvate and non-essential amino acids, bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin, amphotericin-B and retinoic acid) were also purchased from Lonza. All other supplements (i.e., foetal bovine serum (FBS), penicillin and streptomycin) as well as leukocyte-derived neutrophil elastase, N-t-BOC-L-alanine p-nitrophenyl ester (BOC-Ala-NP), N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) and N-acetyl-L-tyrosine ethyl ester monohydrate (ATEE) were purchased from Sigma-Aldrich (Dublin, Ireland). Greiner Bio-One tissue culture plastics were supplied by Cruinn (Dublin, Ireland), except for Corning Transwell Clear filter inserts (1.12 cm^2 surface area, 0.4 µm pore diameter) which were purchased from VWR (Dublin, Ireland).

Trypsin, treated with 1-chloro-3-tosylamido-4-phenyl-2-butanone and α-chymotrypsin, treated with 1-chloro-3-tosylamido-7-amino-2-heptanone were obtained from Worthington (Reading, Berkshire, UK). The BCA protein assay and HPLC grade acetonitrile was purchased from Fisher Scientific (Dublin, Ireland).

Mouse monoclonal anti-chymotrypsin and anti-trypsin antibodies as well as rabbit polyclonal anti-neutrophil elastase antibody were purchased from Abcam (Cambridge, UK). Anti-β-actin mouse monoclonal antibody and proteinase inhibitors, leupeptin and aprotinin, were purchased from Sigma-Aldrich. Cell extraction buffer was obtained from Invitrogen (Karlsruhe, Germany) and the standard protein concentration assay and the immunoblot polyvinylidene fluoride membranes were purchased from Bio-Rad (Hemel

Hempstead, UK). Immobilon Western Chemiluminescent HRP substrate was ordered from Millipore (Carrigtwohill, Ireland). Trifluoracetic acid (TFA) was obtained from Riedel-de Haën (Seelze, Germany). HPLC studies were performed on a LiChroCART®125-4 LiChrospher®100 RP-18 (5 µm) column (Merck, Darmstadt, Germany).

3.3.2. Cell culture

Freshly isolated hBEpC in primary culture were used for up to three passages, in order to prevent phenotypic drift. The cells were seeded (*performed by Dr Sibylle Endter and Dr Carsten Ehrhardt, Trinity College Dublin, Ireland)* at 50,000 cells/cm^2 on collagen I-coated Transwell Clear filter inserts and cultured under air-interface conditions from day 2 onwards in supplemented bronchial epithelial basal medium.

Calu-3 is a human bronchial epithelial cell line derived from an adenocarcinoma of the lung (Fogh and Trempe 1975). The cells (passage numbers 32-43 and 56-58) were seeded at a density of 75,000 cells/cm^2 and grown to monolayers in Eagle's minimum essential medium (EMEM; supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1 mM sodium pyruvate and non-essential amino acids).

The 16HBE14o- cell line was generated by transformation of normal bronchial epithelial cells obtained from a one-year-old male heart-lung transplant patient. Cells (passage numbers 2.62, 2.66, 2.103 and 2.104) were grown at 100,000 cells/cm^2 in EMEM of a similar composition as for the Calu-3 cells.

A549 cells are derived from a human pulmonary adenocarcinoma (Giard *et al.* 1973) and have been widely utilised in studies of alveolar epithelium function (Sporty *et al.* 2008). Cells (passage numbers 72, 79, 80, 98) were cultured with a seeding density of 40,000/cm^2 in Dulbecco's modified Eagle's medium:Ham's F-12 (1:1 mix) (DMEM:F12, 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin).

Caco-2 cells, derived from a colonic adenocarcinoma, were used as gastrointestinal reference. The cells (passage numbers 23, 24, 26, 63, 70) were cultured in DMEM with

10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, 1 mM sodium pyruvate and non-essential amino acids.

All cell types were cultured at 37℃ and 5% CO_2 atmosphere and the medium was changed every other day, until confluence was reached.

3.3.3. Evaluation of the enzymatic digestion of salmon calcitonin

In order to verify that sCT is indeed degraded by neutrophil elastase, HPLC analysis was performed. The breakdown of sCT by trypsin and chymotrypsin was previously reported (Dohi *et al.* 1993, Lang *et al.* 1996) and was also carried out as part of this thesis to affirm these results. Degradation of sCT by all three proteolytic enzymes was investigated after incubation of the peptide with solutions of the pure enzymes for 120 min at 37℃. For elastase studies, 1 mg sCT in 1 ml sodium phosphate solution (50 mM, pH 6.5) was directly added to a 1-unit vial of neutrophil elastase. Samples of 180 µl volume were withdrawn after 0, 10, 30, 60 and 120 min and immediately diluted with a similar volume of 1% TFA, and were then analysed by HPLC. For degradation studies with trypsin, solutions of 1 mg sCT and 10 BAEE units of trypsin per 1 ml potassium phosphate buffer (0.067 M, pH 7.6) were prepared. In the case of chymotrypsin, the solution contained 1 mg sCT and 0.2 ATEE units chymotrypsin per ml of potassium phosphate buffer (0.067 M, pH 7.0). Degradation studies of sCT were carried out for at least four times with each of the respective enzymes, however with varying enzyme concentrations and partly on different HPLC equipment. The enzyme concentrations were adjusted in each experiment in order to find a concentration for each enzyme that shows a consistent degradation within the experiment time of 120 min.

3.3.4. Western blot analysis

These experiments were carried out by Dr Stephen Buckley, Trinity College Dublin, Ireland.

Abundance of each of the three enzymes was investigated in three independent samples of cell supernatant and cell homogenate, respectively. For the latter, cell monolayers were washed with PBS and homogenised in cell extraction buffer supplemented with proteinase inhibitors, leupeptin and aprotinin. Protein sample concentrations were determined using a Bio-Rad standard protein concentration assay according to the manufacturer's instructions. Samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to immunoblot polyvinylidene fluoride membranes. Membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (pH 7.4) for 1 h at room temperature. Incubation with the respective primary antibody was carried out overnight at 4℃, followed by incub ation with HRP-conjugated secondary antibody at room temperature for 1 h. Peroxidase activity was detected with Immobilon Western Chemiluminescent HRP substrate. Images were acquired using a ChemiDoc documentation system (Bio-Rad). To ensure equal loading, protein was normalised to β-actin using an anti-β-actin mouse monoclonal antibody.

3.3.5. Activity of trypsin, chymotrypsin and neutrophil elastase in cell homogenate and in cell supernatant

Enzymatic activities were measured in cell-free supernatant and cell homogenate. Protein concentrations were determined for normalisation using a BCA Protein Assay Kit. Trypsin activity was measured according to a method reported by Schwert and Takenaka (1955). In brief, N-benzoyl-L-arginine ethyl ester (BAEE) is hydrolysed at the ester linkage resulting in an increase of absorbance measured at 253 nm and 25℃ (A_{253}). For trypsin standard solutions, 1 mg trypsin was dissolved in 1 ml 0.001 M HCl and then diluted immediately prior to assay. For the substrate solution, 8.6 mg BAEE were dissolved in 100 ml potassium phosphate buffer (0.067 M, pH 7.6), and A_{253} was then adjusted to 0.575

versus buffer. Three millilitres of substrate solution and 0.2 ml enzyme (standard, supernatant or homogenate) were mixed and pipetted into a 10-mm quartz cuvette and the absorption was determined immediately and then every 10 s over a period of 8 min. The activity was determined after 200 s.

The method of the chymotrypsin assay was also described by Schwert and Takenaka (1955). N-acetyl-L-tyrosine ethyl ester (ATEE) is hydrolysed at the ester linkage resulting in a decrease of absorbance measured at 237 nm and 25℃ (A_{237}). The chymotrypsin standard solution was prepared by dissolving 1 mg chymotrypsin per 1 ml 0.001 M HCl. Immediately prior to assay, this solution was diluted to obtain the standard solutions. Twenty-three and one-half milligrams ATEE was dissolved in 100 ml potassium phosphate buffer (0.067 M, pH 7.0) at 70℃. The sol ution was cooled rapidly and A_{237} was adjusted to 1.2 versus buffer. Two-hundred microlitres of enzyme (standard, supernatant or homogenate) was added to 3.0 ml substrate solution and absorbance at 237 nm was measured every 10 s over a period of 8 min and activity was determined after 200 s.

Neutrophil elastase activity was assayed using a method modified from Cotter and Robinson (1980) and Visser and Blout (1972). For the substrate solution, 3.21 mg BOC-Ala-NP were dissolved in 1 ml acetonitrile and then diluted to 50 ml with sodium phosphate solution (52 mM, pH 6.5 at 37℃). The sub strate solution was equilibrated to 37℃ in a water bath. For the neutrophil elastase s tandard, 0.1 ml buffer and 2.9 ml of substrate solution (resulting in a solution containing 0.20 mM substrate and 50 mM sodium phosphate, also used as the blank value) were directly added to a 1-U vial of neutrophil elastase. The mix was immediately transferred to a 3-ml quartz cuvette and $A_{347.5}$ of test and blank were determined in a spectrophotometer. For measurements, 0.1 ml of cell supernatant, or cell homogenate (preparation as described above) were added to 2.9 ml of substrate solution in a 3-ml quartz cuvette and $A_{347.5}$ of test and blank were determined. The spectrophotometer was thermostatted to 37℃ and $A_{347.5}$ was determined every 10 s over a period of 480 s (standard) or 180 s (supernatant, homogenate) and the activity was determined after 60 s.

3.3.6. Stability studies of sCT in cell supernatant, on cell monolayers and in cell homogenate

The stability of sCT was investigated following incubation in supernatant (i), with cell monolayers (ii) and in cell homogenates (iii). Cells (hBEpC, Calu-3, 16HBE14o-, A549 and Caco-2) were cultured on 6-well plates (for i and ii) or in T-75 tissue culture flasks (for iii) until confluent monolayers were formed. The preparation method is illustrated in Figure 3.2.

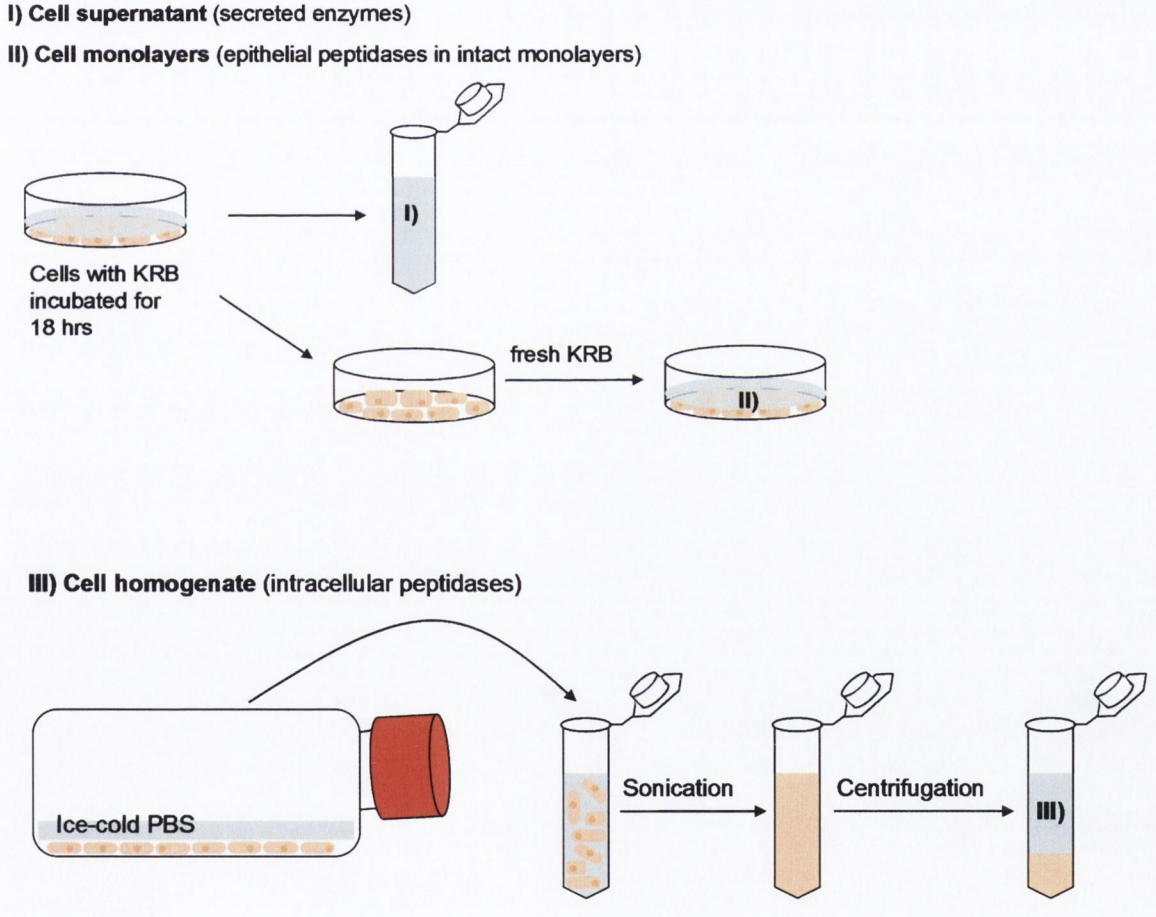

Figure 3.2. Preparation methods for cell supernatant (i), cell monolayers (ii) and cell homogenate (iii) of hBEpC, Calu-3, 16HBE14o-, A549 and Caco-2 cells. sCT was then added to (i), (ii) and (iii), respectively for stability studies.

The cell culture medium of the 6-well plates was aspirated, cell monolayers were washed twice with Krebs-Ringer-Buffer (KRB) and 3 ml freshly prepared KRB were added onto each well. Monolayers with KRB were then incubated for 18 h, after which time the conditioned KRB was collected and centrifuged for 12 min at 3,000 rpm at 4°C. To 1.9 ml of the resulting supernatant (i) 100 µl of a 1% w/v sCT in KRB solution was added. Pre-warmed, fresh KRB (containing sCT) was then added to the cell monolayers (ii) to study interactions of the drug with membrane-bound enzymes. The homogenate was prepared according to the method used by Shah and Khan (2004) with slight modifications. In brief, the cell culture medium was aspirated from the T-75 flasks and the cell monolayers were washed three times with 2 ml ice-cold PBS (pH 7.4). The cells were then scraped off in 1.5 ml ice-cold PBS and homogenised by sonicating the cell suspension for 20 s twice. The samples were centrifuged for 12 min at 3,000 rpm at 4°C. The resultant supernatant was used as cell homogenate (iii). 950 µl cell homogenate were mixed with 50 µl of a 1% w/v sCT in PBS solution. All prepared samples, providing a final sCT concentration of 500 µg/ml (145.7 µM) were incubated at 37°C for 120 min. Samples of 190 µl were withdrawn after 0, 10, 30, 60 and 120 min and immediately diluted with the same volume of 1% TFA stopping solution in order to avoid enzymatic degradation of sCT. Samples were then analysed by HPLC. Three different samples of (i), (ii) and (iii) were prepared of each cell type.

3.3.7. HPLC analytical method

Salmon calcitonin content was analysed by means of reversed phase HPLC. A LC-IOAT VP pump, FCV-10AL VP delivery module, DGU-14A degasser, SIL-10AD VP autosampler, SPD-10A VP UV-VIS-detector and CLASS-VP software (all Shimadzu, Kyoto, Japan) were used. Twenty micro-litres of sample were analysed according to the method described by Shah and Khan (2004) with slight modifications. The mobile phases consisted of 0.1% v/v TFA in water (A) and 0.1% v/v TFA in acetonitrile (B). Briefly, a linear gradient was run at a flow-rate of 1 ml/min: 20-35% B for the first 10 min, 35-37% B

from 10 to 20 min and 37-20% from 20 to 25 min. Detection was achieved at a wavelength of 215 nm. Each sCT concentration was quantified from the corresponding integrated peak area and calculated using the equation of the standard curve (standard solutions from 10 µg/ml to 500 µg/ml).

3.3.8. Statistical data analysis

Outlier detection was performed based on Grubbs' test using GraphPad Software (http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Data of trypsin activity in cell supernatant were analysed by one-way ANOVA and Tukey post test using GraphPad Prism 5.03. Values were considered significant if $P<0.05$.

3.4. RESULTS

3.4.1. Degradation of sCT by trypsin, chymotrypsin and neutrophil elastase

Salmon calcitonin was rapidly degraded in the presence of neutrophil elastase (Figure 3.3a). Moreover, it was confirmed that solutions of trypsin (Figure 3.3b) and α-chymotrypsin (Figure 3.3c) also break down the peptide. The sCT concentration, calculated via the peak area of the chromatogram, decreased with increasing incubation time, whereas the control, i.e., sCT in buffer without any enzymes, was observed to be stable over 120 min. Several peaks of sCT degradation fragments appeared in the chromatograms (Figure 3.3) after 10 min incubation.

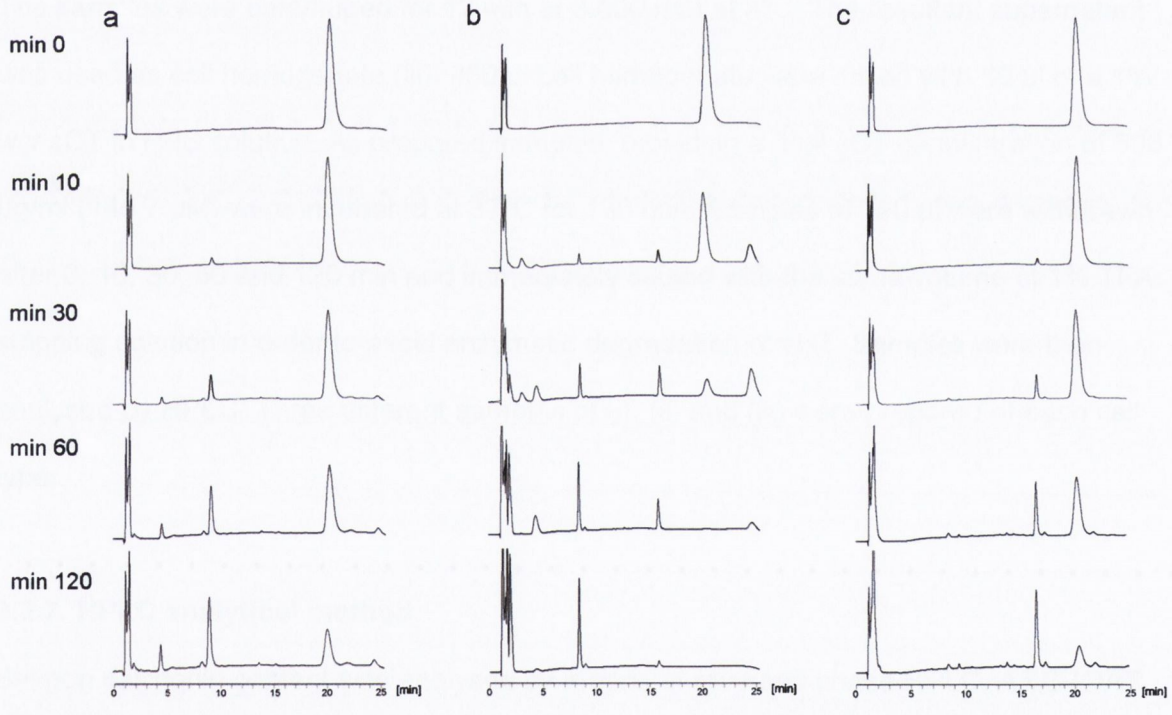

Figure 3.3. Degradation of salmon calcitonin (sCT, 1 mg/ml) in the presence of neutrophil elastase (a), trypsin (b) and α-chymotrypsin (c). The respective enzyme and sCT were incubated at 37°C, samples were withdrawn after 0, 10, 30, 60 and 120 min and analysed by HPLC. Degradation of sCT can be observed in case of all three peptidases.

3.4.2. Protein expression levels of trypsin, chymotrypsin and neutrophil elastase

Western blot analysis was carried out to assess the protein expression profiles for those proteases which were most predominantly involved in the degradation of sCT, namely trypsin, chymotrypsin and neutrophil elastase. As illustrated in Figure 3.4a, trypsin was robustly expressed in the homogenate and supernatant of all pulmonary epithelial cell lines. Similarly, chymotrypsin expression was present in cell homogenates of Calu-3, 16HBE14o- and A549, to comparable degrees, however, there was an absence of chymotrypsin in the supernatant of A549 cells (Figure 3.4b). Interestingly, whilst Calu-3 cells demonstrated strongest expression of neutrophil elastase in the cell homogenate, this was not replicated in the corresponding supernatant (Figure 3.4c). In the case of 16HBE14o- and A549, levels of neutrophil elastase were similar in both their homogenate and supernatant (Figure 3.4c). Caco-2 cells, which were used for comparative purposes, exhibited expression of trypsin and chymotrypsin in both cell homogenates and supernatants (Figures 3.4a and b), however, neutrophil elastase was only very weakly expressed in homogenate and absent in the supernatant (Figure 3.4c).

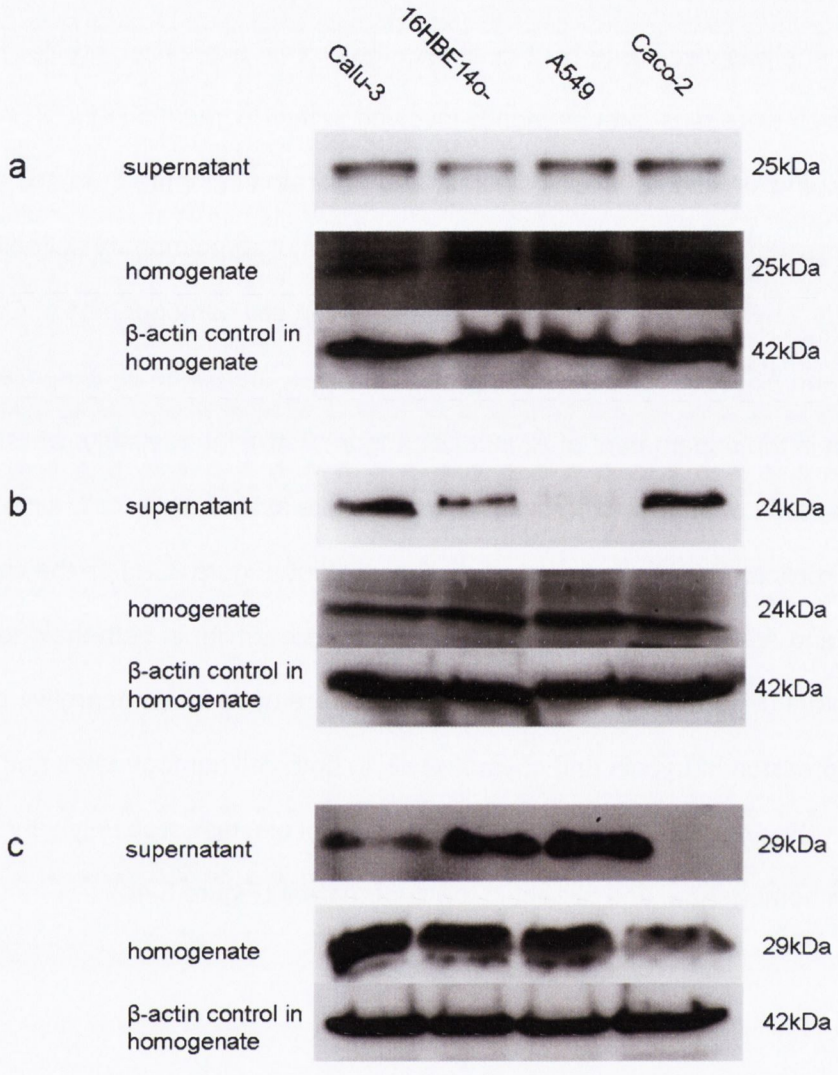

Figure 3.4. Representative Western blots for trypsin (a), chymotrypsin (b) and neutrophil elastase (c) in homogenates and supernatants of Calu-3, 16HBE14o-, A549 and Caco-2 cell monolayers. Samples were investigated after 12 (Calu-3), 7 (16HBE14o-), 5 (A549) and 21 (Caco-2) days in culture.

3.4.3. Activity of trypsin, chymotrypsin and neutrophil elastase in cell homogenates and in cell supernatants

Western blot analysis confirmed the synthesis of proteases involved in the degradation of sCT in various epithelial cell types. However, since a protein's expression does not automatically correspond with its functionality, the activity of trypsin, chymotrypsin and neutrophil elastase was studied in cell supernatant and homogenate (Table 3.1). In cell supernatant, the activity of trypsin was generally low (Table 3.1). It was noted that significantly ($P<0.05$) higher activity levels were obtained in supernatant conditioned by Caco-2 cells compared to that derived from pulmonary monolayers. For chymotrypsin and neutrophil elastase no activity was detected in any of the investigated supernatants (Table 3.1). Determination of the enzyme activity in cell homogenates resulted in unexpected difficulties. For trypsin and chymotrypsin, high fluctuations in absorbance were observed and the activity could therefore not be determined with the necessary reliability. In the case of neutrophil elastase, enzyme activity (expressed in units per mg protein) in cell homogenate was comparable in the three respiratory epithelial cell lines investigated, but was lower in Caco-2 cells. Figure 3.5 shows representative absorption curves obtained in the neutrophil elastase activity assay.

Table 3.1. Activities of trypsin, α-chymotrypsin and neutrophil elastase in cell supernatant and neutrophil elastase activity in cell homogenate of the four investigated cell types. Activities were determined by measuring the change in UV absorbance caused by the reaction with model substrates. Activities are shown in units per mg protein in cell supernatant or homogenate, respectively. Data are represented as means ± SD or average, n = 3-5. (n.d. = not detectable)

	16HBE14o-	Calu-3	A549	Caco-2
trypsin activity in supernatant [BAEE units / mg protein] n=5	29.16 ± 6.87	41.62 ± 10.03	34.93 ± 26.45	147.91 ± 64.65
chymotrypsin activity in supernatant [ATEE units / mg protein] n=3	n.d.	n.d.	n.d.	n.d.
neutrophil elastase activity in supernatant [BOC-Ala-NP units / mg protein] n=3	n.d.	n.d.	n.d.	n.d.
neutrophil elastase activity in homogenate [BOC-Ala-NP units / mg protein] n=3	0.06 ± 0.03	0.05 ± 0.04	0.05 ± 0.01	0.02 ± 0.03

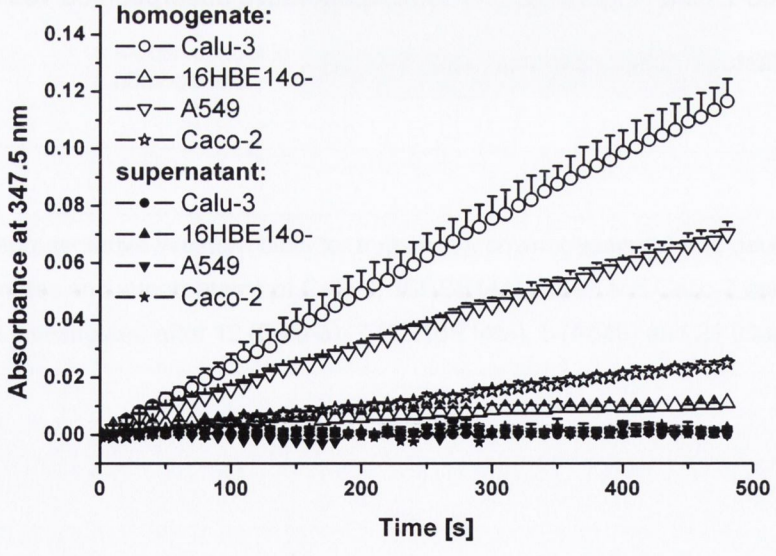

Figure 3.5. Neutrophil elastase activity assay in cell homogenate (hom) and in cell supernatant (sn). An increase in absorbance, caused by the cleavage of the model substrate N-t-BOC-Ala-p-NP, was observed in homogenates of all cell types, investigated. No change in absorbance, however, was observed in any of the investigated supernatants. Data are represented as average ± SD, n = 3.

3.4.4. Stability studies of sCT in cell supernatants, on cell monolayers and in cell homogenate

When incubated in cell supernatants (i) or after incubation with intact cell monolayers (ii), the concentration of sCT remained unchanged over a period of 120 min, independent of the cell type studied (Figures 3.6 and 3.7). After incubation with cell homogenates (iii) however, sCT concentrations were reduced to 82.52±10.53% (hBEpC), 47.19±0.94% (Calu-3), 45.14±20.47% (16HBE14o-), 4.29±2.65% (A549) and 18.50±26.93% (Caco-2) of the original concentration, respectively (Figure 3.8).

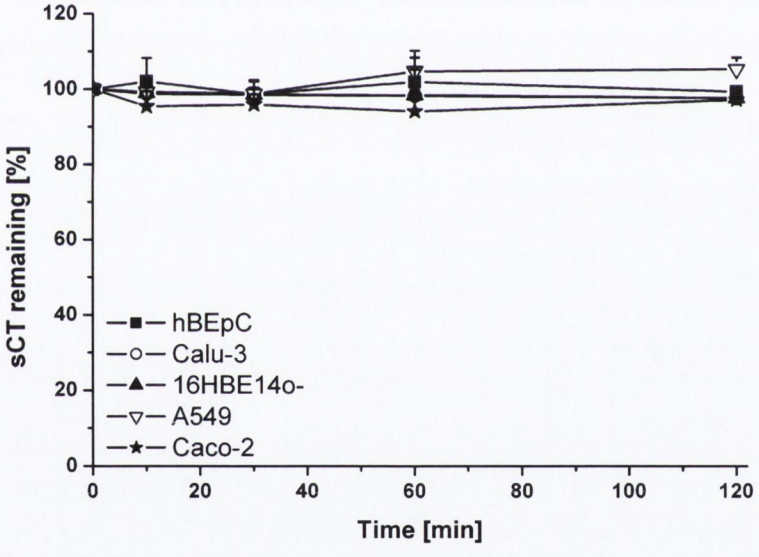

Figure 3.6. Stability of salmon calcitonin (sCT) in cell-free supernatant. The peptide was stable after incubation for 120 min at 37℃. Samples were withdrawn after 0, 10, 30, 60 and 120 min and analysed by HPLC. Peak areas were used to determine the sCT concentrations, here shown as the amount of sCT remaining [%] over the time. Data are represented as means ± SD, n = 3.

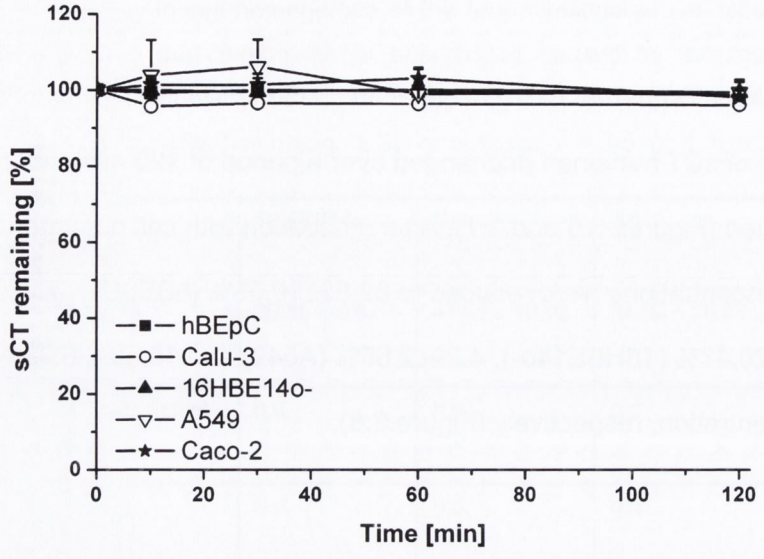

Figure 3.7. Stability of salmon calcitonin (sCT) with cell monolayers. The peptide was stable after incubation for 120 min at 37℃. Samples were withdr awn after 0, 10, 30, 60 and 120 min and analysed by HPLC. Peak areas were used to determine the sCT concentrations, here shown as the amount of sCT remaining [%] over the time. Data are represented as means ± SD, n = 3.

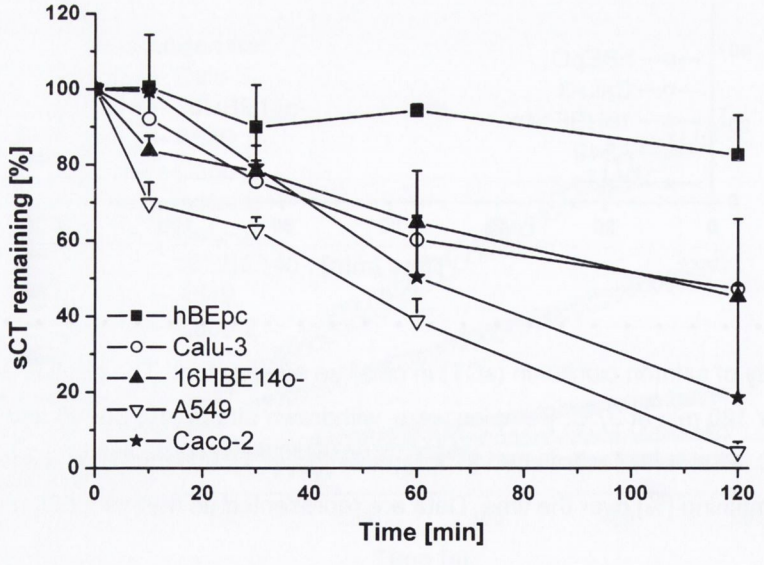

Figure 3.8. Stability of salmon calcitonin (sCT) in cell homogenates. After incubation at 37℃ sCT was rapidly degraded to varying extent, depending on the cell type studied. Samples were withdrawn after 0, 10, 30, 60 and 120 min and analysed by HPLC. Peak areas were used to determine the sCT concentrations, here shown as the amount of sCT remaining [%] over the time. Data are represented as means ± SD, n = 3.

3.5. DISCUSSION

This part of my work addressed potential issues in pulmonary peptide delivery for systemic action, which have been highlighted on a number of occasions (Patton 1996, Patton *et al.* 2010). Currently, there is a dearth of information regarding the fate of inhaled medication, once the drug has left the inhaler device. Enzymes of the peptidases and proteinases families are known to severely hamper non-invasive delivery of biopharmaceuticals (Woodley 1994, Bernkop-Schnürch 1998). The currently available information about spacial expression and activity of peptide degrading enzymes in the lung, however, is far from complete. Indeed, only a few studies address this aspect of pulmonary drug disposition to date (Yamahara *et al.* 1994, Forbes, Wilson and Gumbleton 1999, Baginski *et al.* 2011).

In this chapter, the fate of inhaled salmon calcitonin at the respiratory epithelial barrier was studied, and particular emphasis was placed on evaluating the role that degrading enzymes play in reducing the bioavailability of the peptide drug. Kobayashi *et al.* (1994) already showed that the pulmonary delivery of sCT can indeed be increased when applied together with serine protease inhibitors. However, after the work of Patton (1996) and Kobayashi *et al.* (1994 and 1996), in the 1990's, literature about the stability or degradation of protein and peptide drugs at the lung epithelial barrier has been scarce. The aim of this part of my thesis was, hence, to take a closer look at the fate of an inhaled peptide drug to better understand the impact of peptidases and proteases at the pulmonary epithelial barrier.

Salmon calcitonin was found to be rapidly degraded by neutrophil elastase, an elastase isoform known to be present in human lung (Reid *et al.* 1999, Kawabata *et al.* 2002, Sun and Yang 2004). In addition, I could confirm previous data that trypsin and chymotrypsin are involved in enzymatic breakdown of sCT (Dohi *et al.* 1993, Lang *et al.* 1996, Guggi and Bernkop-Schnürch 2003).

Western blotting revealed abundant expression of all three peptidases in epithelial *in vitro* models of the air-blood barrier, i.e., A549 (alveolar) and Calu-3 and 16HBE14o-

(bronchial) cell monolayers. Surprisingly, neutrophil elastase was found to be expressed in supernatant and homogenate of the studied cell types from human lung, although no mRNA expression was found in the respective cell lines (see Chapter 2). In my opinion, it is unlikely that the PCR experiments delivered false negative results, since the designed primers for neutrophil elastase proved to be working with the primary cells' mRNA and since each experiment was repeated several times. However, different mRNAs within one cell show distinct stabilities. Depending on the lifetime of the respective mRNA, the extent of protein synthesis can be regulated and adapted to the changing requirements. It is therefore possible, that – at the studied timepoints – the protein level was high while mRNA expression was low in order to avoid further protein expression.

Despite the observed protein expression in supernatant and homogenate, only low trypsin activity and virtually no functionally active neutrophil elastase and α-chymotrypsin were discovered in cell supernatants *in vitro*. This might be explained by the presence of physiological enzyme inhibitors such as alpha-1 anti-trypsin or secretory leukocyte antiprotease in the supernatant (Wall and Lunatti 1993, Amelinckx *et al.* 2010). Moreover, Western blot only probes for the presence of an amino acid sequence of the target protein, which does not necessarily warrant the protein's activity. When attempting to measure the enzymes' activity in cell homogenates, I experienced unexpected difficulties in the case of trypsin and chymotrypsin. Substrates cannot only be cleaved by one enzyme, but potentially by any peptidase with preference for the specific amino acid sequence of the substrate. Very likely, due to the enormous number of different enzymes in cell homogenate, many different reactions took place at the same time. The rapid change in composition of the mixture might cause the observed fluctuations in absorbance. Shah and Khan (2004) have previously described this assay in Caco-2 cell homogenate. Reconsidering their report, an apparent error in the calculation can be found, as this group claims to have measured 0.169 µmol trypsin/mg protein. Converting this amount of trypsin results in approximately 4 mg trypsin per mg cell protein. This issue, regretfully, renders the method as such incomprehensible. In general, by lysing cells a

whole bunch of enzymes are released from cell compartments resulting in an enzymatically active mixture that does not represent well an *in vivo* situation. Cell supernatant, by contrast, comprising only secreted enzymes (which are the ones responsible for sCT degradation) comes much closer to the environment in the lung *in vivo*. It was the aim to study homogenate as an enzymatically more active comparison, but activity measurements in supernatant are more valuable. Supernatant of cells is presumably similar to bronchoalveolar lavage fluid (BALF) in regards of peptidases composition. Wall and Lanutti (1993) investigated protease activity in BALF from rat and dog. They reported low or undetectable activity levels of trypsin, chymotrypsin and elastase in both, rat and dog BALF. Although different substrates and conditions were used, their results for trypsin and chymotrypsin are comparable with our findings, i.e., trypsin activity was low in rat and dog BALF and chymotrypsin activity was extremely low in dog BALF and not detectable in rat BALF (Wall and Lanutti 1993). Elastase activies cannot be compared, as Wall and Lunatti determined pancreatic elastase activity, while in this work a substrate for neutrophil elastase was chosen.

When incubated with cell monolayers, supernatants and homogenates *in vitro*, sCT was markedly degraded in the latter case. Particularly, cell homogenates prepared from intestinal Caco-2 and alveolar A549 cell monolayers almost entirely catabolised the peptide within the 2-hour timeframe of the study. In how far studies using cell homogenates have any relevance remains a topic for discussion, although some reports suggested that sCT has notable potential for membrane association and permeation (Lang *et al.* 1998, Gaudiano *et al.* 2005).

An absolute bioavailability of 17% was measured after intratracheal instillation of sCT *in vivo* in rat lungs (Patton, Trinchero and Platz 1994), and relative bioavailabilities of 10-18% for nebulised sCT solution compared to *s.c.* injection in healthy volunteers were reported by Clark *et al.* (2008). Although sCT bioavailability post-inhalation is much higher than can be achieved by any other non-invasive application route, it is desirable to further increase the bioavailability in order to reduce the dose and the risk of potential side effect,

and therefore the cost associated with the therapy. Peptidases and proteinases play an important role in the pulmonary breakdown of sCT. Thus, structural modifications (e.g., by PEGylation) or co-administration of enzyme inhibitors should be investigated to further increase the potency of the aerosol therapy approach. Although this work has some limitations, in that I did not examine other cell types of the lung such as alveolar macrophages, which very likely have a significant impact on peptide drug stability in the lung (Lombry *et al.* 2004), I regard this part of my thesis as an advancing step in expanding the existing knowledge surrounding the fate in inhalable biomacromolecules. Despite their obvious limitation, organotypic *in vitro* models may help to navigate the unchartered terrain of inhalation biopharmaceutics (Sporty *et al.* 2008).

3.6. CONCLUSIONS

The peptidases responsible for sCT degradation in the lungs were found to be functionally expressed in human respiratory epithelial cells *in vitro*. Despite the low activity levels measured in our experiments, it appears reasonable to protect sCT from enzymatic degradation to further improve its stability and hence, bioavailability when delivered as an aerosol for systemic action.

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Chapter 4

Conjugation of sCT with PEG-lipid

4.1. ABSTRACT

Salmon calcitonin (sCT) was demonstrated to be rapidly degraded by peptidases, which were found to be expressed in human lung epithelial cells. It was therefore the aim of this part of my project to shield sCT from proteolytic degradation and to increase its membrane permeability by conjugation with PEG-lipid. DSPE-PEG_{2000}-COOH was activated with EDC and sulfo-NHS to perform an amine-conjugation via the primary amine groups of sCT. The reaction products were analysed by size exclusion chromatography (SEC), dynamic light scattering (DLS) and matrix-assisted laser desorption / ionisation mass spectrometry (MALDI-TOF MS). Two fractions could be separated by SEC, of which one showed particle sizes below 2 nm and the other contained particles of about 13 nm. MALDI-TOF measurements revealed that the 2 nm particles were plain sCT. The 13 nm particles, however, were not a conjugate, but consisted of loosely assembled sCT and DSPE-PEG-COOH. It was further observed that the pure compounds in water – without activation of SEC and sulfo-NHS – spontaneously assembled to complexes of the same diameter. The thin film hydration method – where a PEG-lipid film was prepared and hydrated with aqueous sCT solution - was applied in order to investigate whether sCT and DSPE-PEG-COOH complexes also form under controlled conditions. And it was observed that particles of similar size are formed by all three methods (i.e., mix in water, thin film hydration method and after activation with EDC and sulfo-NHS).

4.2. INTRODUCTION

It was demonstrated in chapter 3 of this thesis that it is reasonable to protect sCT from enzymatic degradation in order to improve its stability and therefore its bioavailability. PEGylation was reported to significantly increase the stability of sCT in tissue homogenate, probably due to a shielding effect from proteolytic enzymes (Lee *et al.* 1999). Lipidisation is a chemical modification that can enhance the cellular uptake of sCT (Cheng *et al.* 2007) and increase the oral bioavailability (Wang *et al.* 2003). Another lipidised sCT analog, "Lipeo-sCT", in which two amphiphilic groups (i.e., hexadodecyl with a triethylene glycol residue), were attached to the peptide (Figure 4.1a), showed prolonged hypocalcaemic activity due to a consecutive release of sCT from lipidised sCT (Cheng and Lim 2009a). Ideally, the favourable characteristics of PEGylation and lipidisation would be combined in one drug molecule. Cheng and Lim had different approaches for the conjugation of sCT with both PEG and lipid. Their aforementioned lipidised sCT (Cheng *et al.* 2007) was additionally conjugated with one or two PEG-chains to the primary amino group of lysins in position 11 and 18 (Figure 4.1b) in order to increase the stability against degrading enzymes. And indeed, mono- ("1PEG-Mal-sCT") and di-PEGylated ("2PEG-Mal-sCT") compounds both showed increased stability in rodent intestinal fluid compared to sCT or lipidised sCT. However, only the mono-PEGylated derivative was active when applied subcutaneously (s.c.) and none of the conjugates showed hypocalcemic activity after oral administration to the rat (Cheng and Lim 2009b). In a second approach Cheng and Lim conjugated a PEG-lipid to sCT via a tri-block linker. Not only was the resulting "Mal-PL-sCT" (Figure 4.1c) more stable towards enzyme degradation than sCT, it also kept some of the hypocalcaemic activity after s.c. injection, though significantly less than unmodified sCT (Cheng and Lim 2010). It was the aim of this part of my thesis to develop a novel PEG-lipid-sCT derivative for pulmonary application that combines increased resistance against enzyme degradation and enhanced cellular uptake. Unlike Cheng and Lim, however, the PEG-lipid was to be linked to sCT via the PEG-chain (Figure 4.1d).

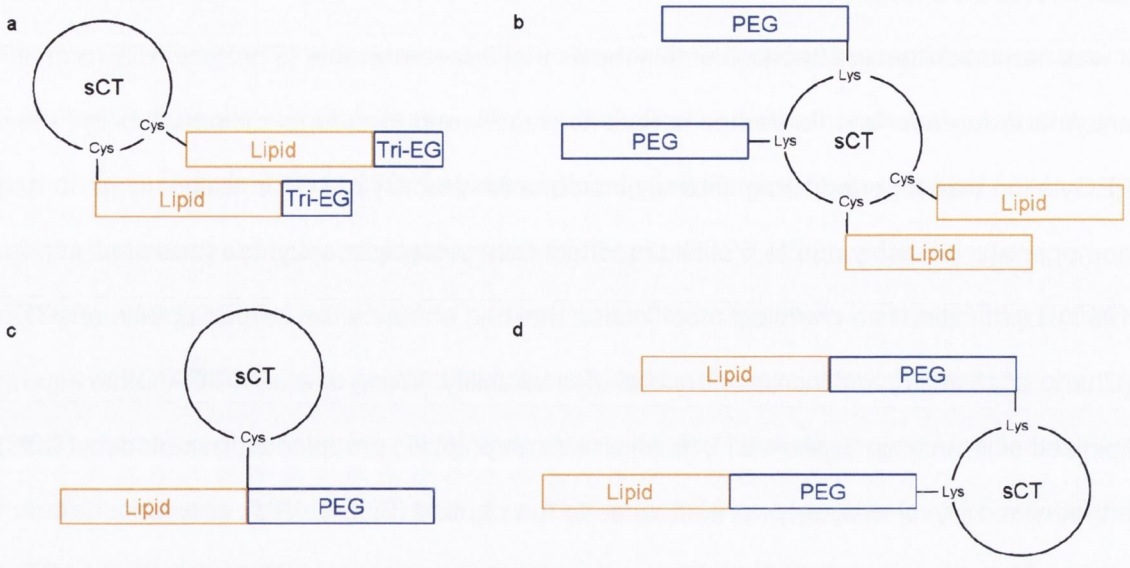


Figure 4.1. Schematic illustration of different sCT derivatives conjugated to both, PEG and lipid: (a) "Lipeo-sCT" in which sCT is linked to two amphiphilic groups consisting of a hexadodecyl chain with a triethylene glycol (Tri-EG) residue (according to Cheng and Lim 2009a), (b) "1PEG-Mal-sCT" or "2PEG-Mal-sCT" with two ε-maleimido lysine derivatives of palmitic acid and one or two PEG chains, respectively, linked to sCT (according to Cheng and Lim 2009b), (c) "Mal-PL-sCT" with a PEG-lipid conjugated to sCT via a tri-block linker (modified from Cheng and Lim 2010) and (d) our approach of coupling sCT to a PEG-lipid via the PEG-chain.

Youn *et al.* (2008) compared Lys^{18}-PEGylated sCT derivatives with three different PEG chain lengths (i.e., 1, 2 and 5 kDa) in regards of their suitability for pulmonary delivery. The PEG_{2000}-conjugate apparently had the most promising properties for pulmonary drug delivery due to its well preserved bioactivity (Youn *et al.* 2008). I, therefore, decided to use a PEG-lipid with a mean PEG residue of 2 kDa (Figure 4.2), i.e., 1,2-distearoyl-sn-glycero-3-phophoethanolamine-N-[carboxy(polyethylene glycol)-2000] ammonium salt (DSPE-PEG-COOH) which can be chemically attached to the primary amino groups of sCT via the activated carboxylic acid group of the PEG-residue (reviewed in Roberts *et al.* 2002; Anabousi *et al.* 2005, Hermanson 2008). Salmon calcitonin has three possible PEGylation sites: the primary amines of cysteine in position 1 (N-terminus) and primary amino residues of lysines in positions 11 and 18 (Lee *et al.* 1999) (Figure 4.3). Ideally, the lipid

chain of the Lipid-PEG-sCT derivative increases the absorption rate and is then cleaved off by esterase once the conjugate has reached the blood stream. The remaining PEG-sCT could then show the favourable characteristics of a PEG_{2000}-conjugate as described by Youn *et al.* (2008).

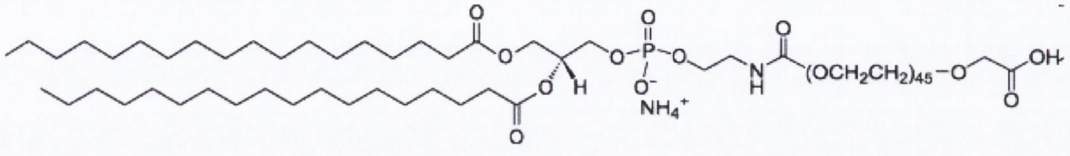


Figure 4.2. Chemical structure of DSPE-PEG-COOH (2,850 Da) (copied from http://www.avantilipids.com/index.php?option=com_content&view=article&id=1088&Itemid=448&catnumber=880120, 30.03.2011). The carboxylic acid group was activated with sulfo-NHS and ECD and then coupled to the primary amino groups of sCT.

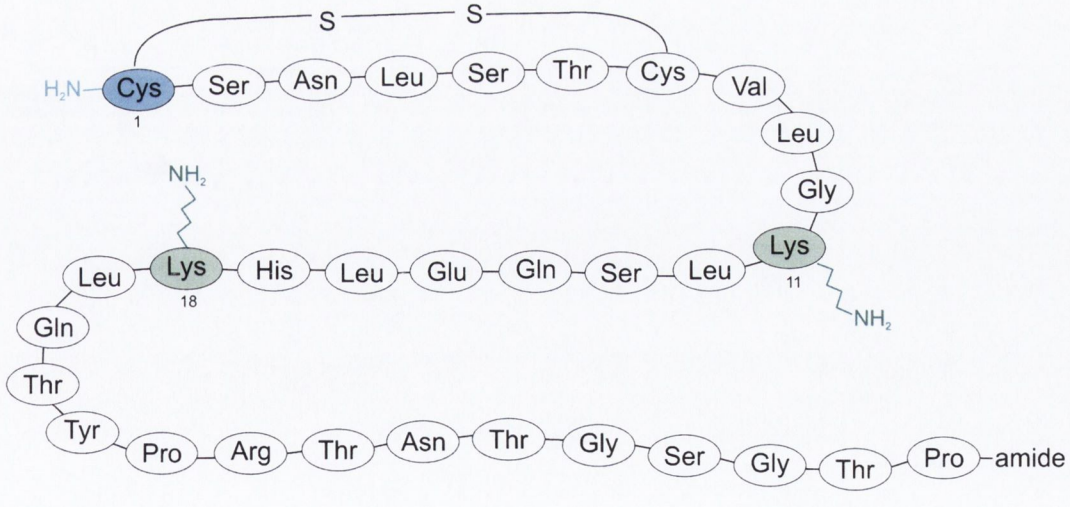


Figure 4.3. Primary structure of salmon calcitonin (MW 3,432 Da) and its three possible PEGylation sites at position 1 (Cys), 11 (Lys) and 18 (Lys) (modified from Lee *et al.* 1999, Guggi and Bernkop-Schnürch 2003).

The carboxylic acid group of DSPE-PEG-COOH (Figure 4.2) is first to be activated with N-hydroxysulfosuccinimide (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimid (EDC) and can then be conjugated to the primary amino groups of sCT, i.e., N-terminus Cys^{1}, as well as Lys^{11} and Lys^{18} (Figure 4.3) (Anabousi *et al.* 2005, Hermanson 2008). According to Hermanson (2008), good results can be achieved in a two-step coupling reaction (Figure 4.4) with change of pH from about 6 to above 7. The first step of the reaction is performed at pH 6, because the hydrolysis rate of esters is much slower and the ester intermediates therefore more stable. To initiate the active ester reaction, which is the second step of the reaction, the pH of the coupling medium has to be raised to above pH 7.0. (Hermanson 2008) Theoretically, there could be three different mono-PEGylated conjugates, three different di-PEGylations and a tri-PEGylated product. Due to sterical hindrance, however, there are some predominant products which can to some extent be influenced by reaction conditions (Na *et al.* 2004, Lee *et al.* 1999).

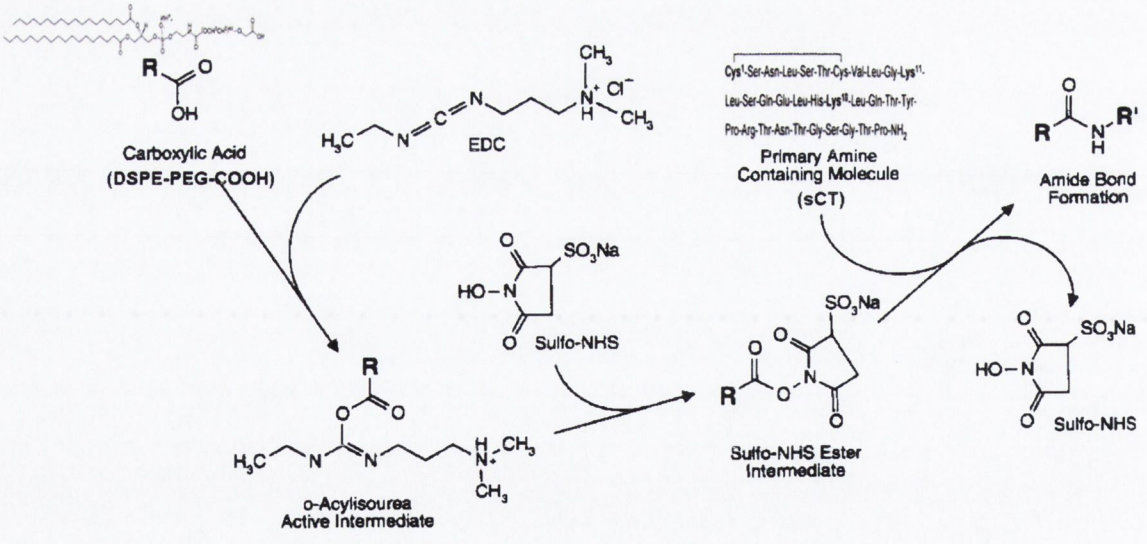


Figure 4.4. Two-step coupling reaction of DSPE-PEG-COOH to sCT. The carboxylic acid group of the PEG-lipid is first to be activated with sulfo-NHS and SEC and then coupled to the primary amino groups on the peptide (modified from Hermanson 2008).

4.3. MATERIALS AND METHODS

4.3.1. Materials

Salmon calcitonin Ph.Eur. (sCT; 3,432 Da) was ordered from Polypeptide Laboratories AB (Limhamn, Sweden), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy (polyethylene glycol)-2000] ammonium salt (DSPE-PEG-COOH; 2,850 Da) was purchased from Avanti Polar Lipids Inc. via Otto Nordwald GmbH (Hamburg, Germany), N-hydroxysulfosuccinimide (sulfo-NHS; 217.14 g/mol) was purchased from Thermo Scientific (Karlsruhe, Germany), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid (EDC; 191.7 g/mol) and Sephadex G-50 and G-100 medium grade and all other chemicals were ordered from Sigma-Aldrich (Steinheim, Germany). Water used was always ultrapure water filtered through 0.2 µm Isopore™ membrane filters (Millipore, Billerica, MA, USA).

4.3.2. Synthesis of sCT-DSPE-PEG-COOH conjugates

The synthesis used was a combination of different methods described by Anabuosi *et al.* (2005), a working colleague at Trinity College Dublin, Dr. Manuela Gaspar, (Sitterberg *et al.* 2010) and general guidelines in the book *Bioconjugate Techniques* (Hermanson 2008). These methods as well as the method used in this thesis are compared in Table 4.1. For the first reaction step, 7.125 mg DSPE-PEG-COOH were dissolved in 1.0 ml phosphate buffer pH 6.6 (0.0025 M). 180 µl sulfo-NHS solution (0.1389 M; 6.032 mg in 200 µl phosphate buffer pH 7.4) and 180 µl EDC solution (0.1389 M; 5.325 mg in 200 µl phosphate buffer pH 7.4) were added and the mixture was left for 15 min at room temperature in order to activate the carboxylic group of the PEG-lipid. The pH was raised to 7.2 by adding one drop 1 M-NaOH. Then, 8.580 mg sCT in a solution of 500 µl phosphate buffer pH 7.4 (0.005 M) were added to the reaction batch. The mix was left at 4℃ for 8 h. The synthesis was performed twice, onc e as described above and a second time without salts (as described in section 4.3.5) for MALDI-TOF MS. The products were then separated by SEC and characterised DLS and MALDI-TOF MS.

Table 4.1. Comparison of different synthesis methods for the conjugation of the activated carboxylic acid group of a protein or PEG-lipid to primary amino groups of a protein.

	Anabousi *et al.* (2005)	**Sitterberg *et al.* (2010)**	**Hermanson (2008)**	**Method used in this thesis**
-COOH	10 µmol lipids (for liposomes) containing 1.78 mg (0.625 µmol) DSPE-PEG-COOH	1 ml liposome suspension with 10 µmol lipids containing 0.17 mg (0.060 µmol) DSPE-PEG-COOH	1-10 mg "-COOH" (here protein)	7.125 mg DSPE-PEG-COOH
Solvent and pH	"appropriate amount" citrate buffer pH 4.0 and 1 ml PBS (pH 7.5) → total volume and pH unknown!	1 ml PBS (pH 7.4)	pH 7.4 OR pH 6.0 where the hydrolysis rate of the active ester intermediate is much slower	1 ml phosphate buffer pH 6.0
EDC concentr. in final solution	180 µl 0.25 M-EDC solution (≦ 0.033 M)	180 µl 0.25 M-EDC solution (0.019 M)	10-fold molar excess to "-COOH" OR 0.05-0.1 M	10-fold molar excess to DSPE-PEG-COOH (0.018 M)
sulfo-NHS concentr. in final solution	180 µl 0.25 M-EDC solution (≦ 0.033 M)	180 µl 0.25 M-EDC solution (0.019 M)	0.005 M	0.018 M
reaction conditions	10 min at RT, then pH adjusted to 7.5	10 min at RT (pH still 7.4)	15 min (pH 6.0), then pH adjusted to > 7.0	10 min at RT, then pH adjusted to 7.2
-NH_2 (protein)	0.78 mg (0.010 µmol) transferrin	1.25 mg (0.017 µmol) transferrin	"-NH_2" in 10-fold molar excess to "-COOH"	8.58 mg sCT
Reaction conditions	8 h at 4℃	8 h at 4℃	2 h at RT	8 h at 4℃
Separation method of products	SEC (Sepharose)	Ultracentrifugation		SEC (Sephadex)
Molar ratio	-COOH 1 EDC 72 sulfo-NHS 72 -NH_2 0.016	-COOH 1 EDC 750 sulfo-NHS 750 -NH_2 0.272	-COOH 1 EDC 10 sulfo-NHS ? -NH_2 10	-COOH 1 EDC 10 sulfo-NHS 10 -NH_2 1

4.3.3. Size exclusion chromatography (SEC)

SEC analysis was used to separate conjugates from free sCT. Sephadex G-50 and G-100 were prepared according to the manufacturer's instructions and were respectively transferred into glass columns of 1 cm diameter. The readily prepared columns were packed to a volume of 20 ml Sephadex. Before use, the columns were thoroughly rinsed with ultrapure water and then equilibrated with the mobile phase (i.e., phosphate buffered saline, PBS, pH 7.4, or ultrapure water). Two hundred microlitres of sample were applied to the column for size-dependent separation. The eluate was collected in fractions of 1 ml or 1.5 ml (depending on the experiment) and the absorbance of each fraction was determined by UV spectroscopy (Ultropsec 3000, Pharmacia Biotech, Freiburg, Germany) at 215 nm.

4.3.4. Physicochemical characterisation

The hydrodynamic diameter was determined by Dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a 10 mW HeNe laser at a wavelength of 633 nm at 25℃. Scatt ered light was detected at a 173° angle with laser attenuation and measurement position adjusted automatically by the Malvern software. Size measurements were performed for three purposes:

i) The SEC fractions of the synthesis products were analysed to determine if the separation was successful (larger compounds such as conjugates are eluted before smaller molecules like sCT). Each collected SEC fraction was measured three times with at least 10 runs.

ii) The size of the separated synthesis products was compared with the size of the two reactants. Pure sCT in PBS (pH 7.4) and pure DSPE-PEG-COOH in PBS (pH 7.4) were measured in regards of their size. In addition, one sample was prepared containing a mix of the two reactants in PBS (pH 7.4) with the same sCT and DSPE-PEG-COOH ratio and concentration (i.e., 7.125 mg DSPE-PEG-COOH and / or 8.58 mg sCT in 860 µl buffer) as

used for the synthesis with EDC and sulfo-NHS. Three size measurements with at least 10 runs were performed for each sample.

iii) Particles formed by the mix of sCT and DSPE-PEG-COOH in water and particles prepared by thin film hydration method were compared in their size. Values given are the mean ± standard deviation of 3 independent samples of which each was measured 3 times with at least 10 runs.

4.3.5. Matrix-assisted laser desorption / ionisation mass spectrometry (MALDI-TOF MS)

Samples were measured by Jan Bamberger and Dr Uwe Linne, Philipps-Universität Marburg, Germany.

For MALDI-TOF MS measurements, the samples had to be salt free. For synthesis, PBS was therefore replaced by phosphate buffer of same pH. The small amounts of phosphate salts and other chemicals left was then diluted / removed by SEC. The samples were eluted with water. MALDI-TOF MS was performed with synthesised conjugates and for comparison with sCT in water, DSPE-PEG-COOH in water and a mix of the two in equimolar amounts in water. The compounds in water were used directly; the synthesised conjugates were first separated by SEC (as described above), then characteristic fractions for both peaks (i.e., fractions 4 + 5 together and fractions 11-13 together) were combined and concentrated by evaporating water under mild conditions at the rotary evaporator (Heidolph Laboratora 4000 efficient). The samples were then embedded into a dihydro benzoic acid (DBH) matrix and measured by MALDI-TOF MS (Bruker Daltonics Autoflex automated high-throughput MALDI-TOF system) with a 337 nm N_2-laser for desorption and ionisation of the molecules.

4.3.6. Preparation of micelles by thin film hydration method

For comparison, DSPE-PEG-COOH sCT micelles were prepared by the thin film hydration method as described elsewhere (Anabousi *et al.* 2005, Bakowsky *et al.* 2008). Briefly, the PEG-lipid was diluted in a mixture of two parts chloroform and one part methanol (v/v) and then dried to a thin film by rotary evaporation (Heidolph Laboratora 4000 efficient, Heidolph Instruments, Schwabach, Germany) under vacuum at 40℃. The equivalent amount of sCT (depending on concentration and ratio to PEG-lipid) was dissolved in water and added onto the lipid film for rehydration. Gentle movement and rotation lead to the formation of micelles. Samples were analysed by DLS as they were 2 h after preparation and also after thorough shaking in the round bottom flask and after sonication for 30 s in an ultrasonic bath.

4.4. RESULTS

4.4.1. SEC

Size exclusion chromatography was performed to separate conjugated sCT-DSPE-PEG from free sCT and other chemicals. Since bigger compounds are eluted before smaller ones, conjugates should be eluted before excessive free DSPE-PEG-COOH and / or free sCT. Only sCT is UV/VIS active, the PEG-lipid does not show absorbance. Figure 4.5 visualises the UV absorbance of the collected fractions forming two distinct peaks. The first peak (ml 6-9) could be caused by a single product or a mixture of different conjugates which were either all of similar size or which were all so big that separation did not occur. The second peak (ml 15-24) is very likely caused by unbound sCT. In Table 4.2, SEC results of the synthesis products are compared and contrasted with an SEC spectrum of the mix of sCT and DSPE-PEG-COOH in water.

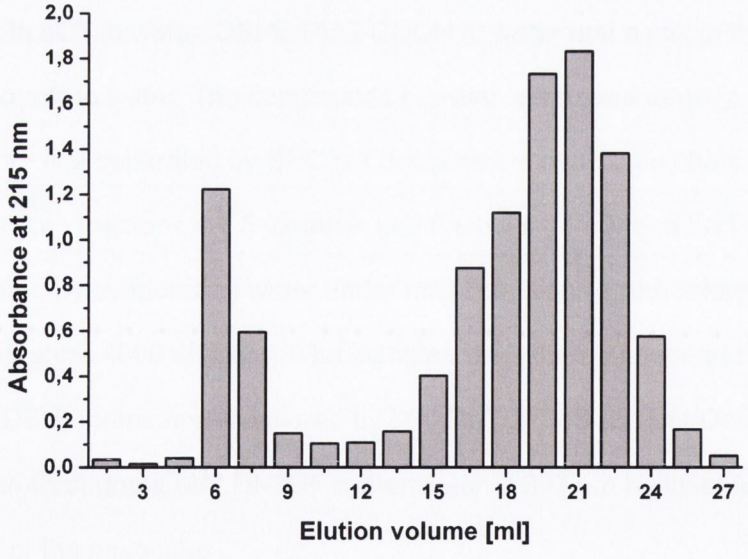


Figure 4.5. Two-step coupling reaction of DSPE-PEG-COOH with sCT. The reaction mixture was separated by SEC (Sephadex G-100, eluent: PBS pH 7.4) and the collected fractions measured with UV / VIS spectroscopy at 215 nm (n=1).

4.4.2. Physicochemical characterisation

Each fraction was measured by DLS to investigate the particle sizes. Fractions 1-6 (i.e., elution volume 0-9 ml) showed a particle diameter of about 13 nm, while fractions 11-17 (i.e., 16.5-25.5 ml) showed particle sizes below 2 nm (here however, the measuring accuracy of the instrument is very low). Fractions in between showed both sizes. The PdI varied strongly between the different fractions: While fraction 4 showed the lowest PdI, 0.26, more diluted samples had a PdI of 1.0. The fact that fractions 1-6 only show one size alludes to a uniform PEGylation rate.

Pure sCT in PBS (pH 7.4) and pure DSPE-PEG-COOH in PBS (pH 7.4) both showed particle diameters below 2 nm. It can therefore be assumed that the second SEC peak indeed represents free sCT and possibly, free PEG-lipid (undetected in UV/VIS). While the sCT peak was high and narrow, DSPE-PEG-COOH showed a broader peak. Surprisingly, the mix of the two reactants in PBS (pH 7.4) showed a single peak with a particle diameter of 13 nm, similar to the product of synthesis with sulfo-NHS and EDC (Table 4.2).

4.4.3. MALDI-TOF MS

The combined fractions 11, 12 and 13 of the SEC spectrum (Figure 4.6a) showed a main peak at 3435.4 (m/z) and a second peak at 3631.1 (m/z) when analysed with MALDI-TOF (Figure 4.6b). Fractions 4 and 5 (Figure 4.6c) did not show a peak at the size of a conjugate, but a distinct peak at 3435.7 (m/z) and a multiple peak around 2813.7 (m/z). Due to different PEG-chain lengths, the multiple peak formation is typical for PEG residues and could also be observed in the MALDI-TOF spectrum of pure DSPE-PEG-COOH in water (Figure 4.7a). Pure sCT in water (Figure 4.7b) revealed a peak at 3436.3 (m/z), similar to that of fractions 11, 12 and 13 (Figure 4.6b). The mix of sCT and DSPE-PEG-COOH (Figure 4.7c) in water showed a MS spectrum similar to that of SEC fractions 4 and 5 (Figure 4.6c). Results are summarised in Table 4.2

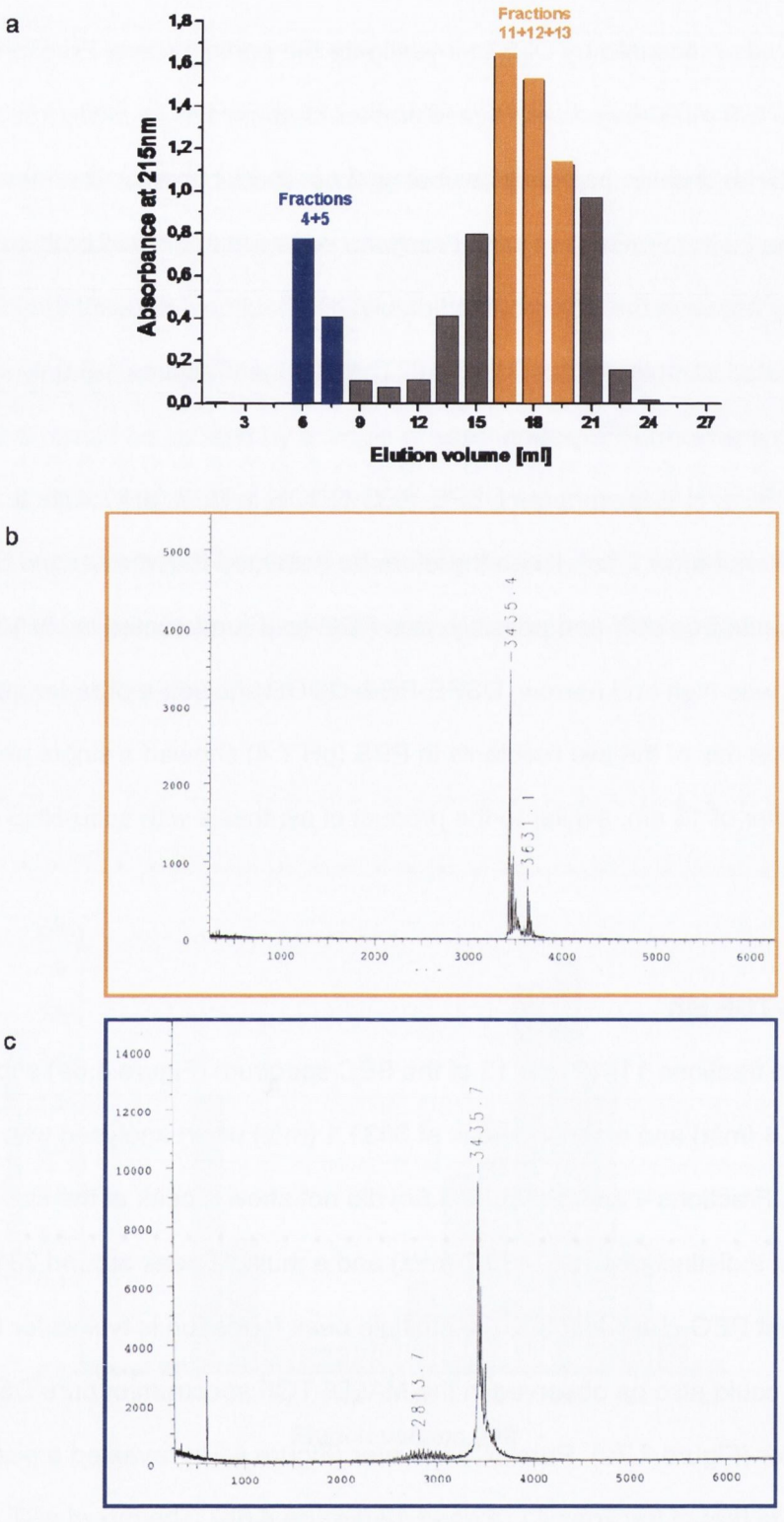


Figure 4.6. MALDI-TOF MS measurements of synthesis products. Molecules are detected according to their mass to charge ratio (m/z). (a) SEC separation of the synthesis products and selection of representative fractions for each of the two peaks to be analysed by MALDI-TOF MS. MS spectra of (b) fractions 11, 12 and 13, representing the second SEC peak and of (c) fractions 4 and 5, representing the first SEC peak with possible conjugates. (n=1)

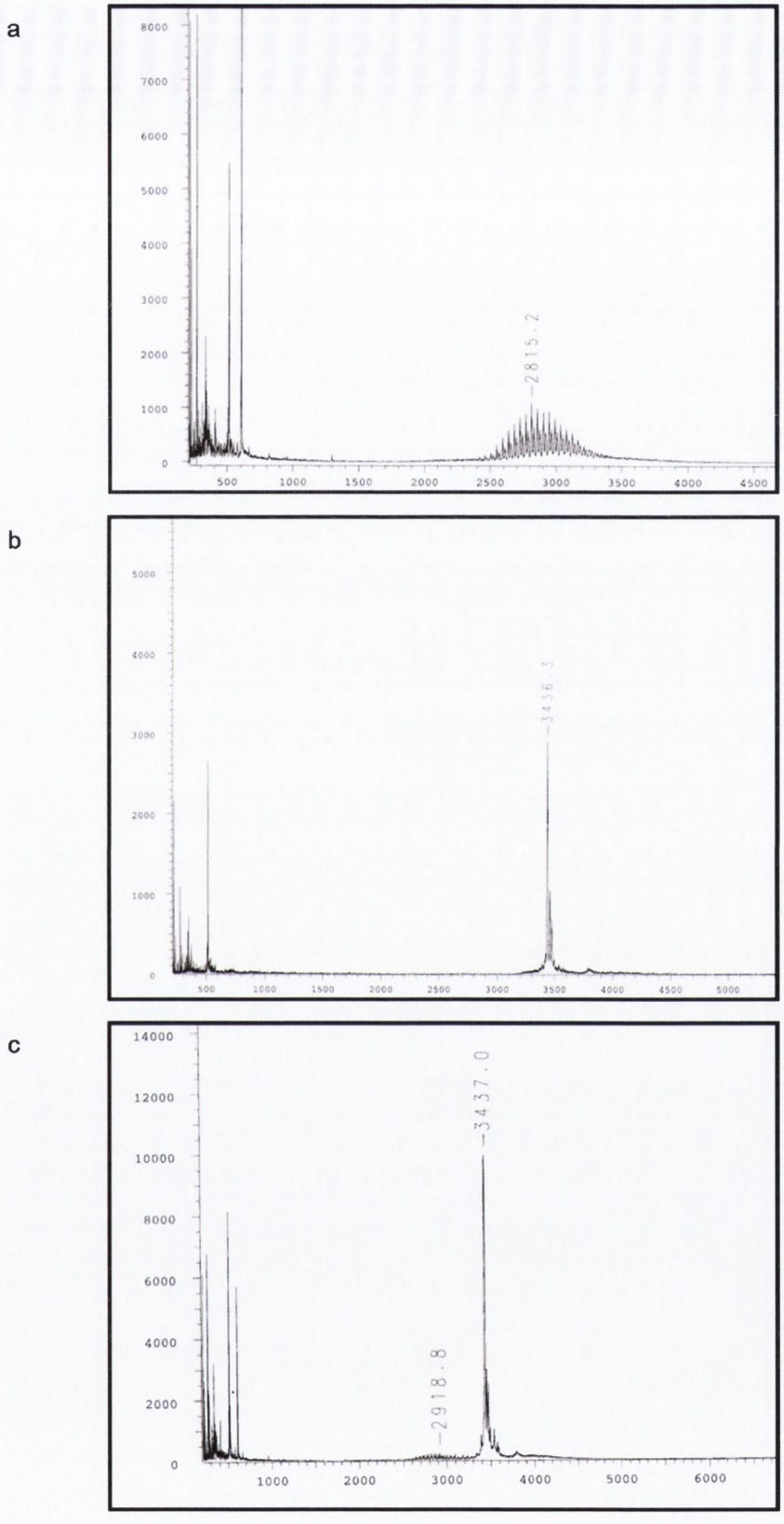
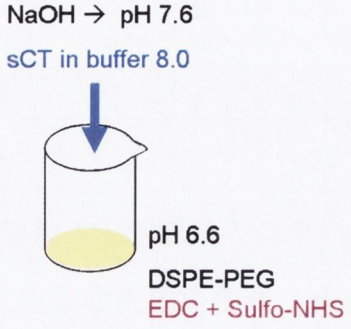
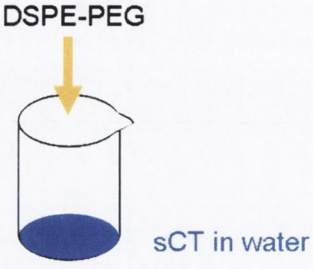
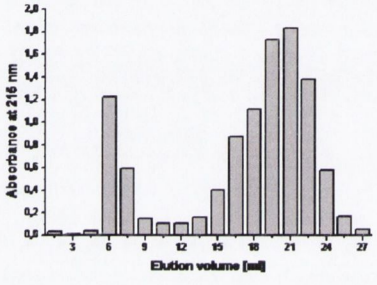
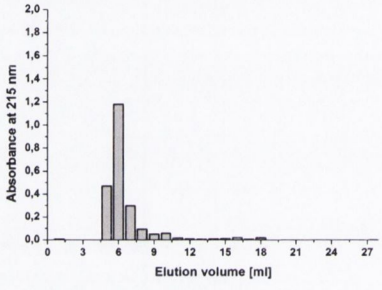


Figure 4.7. MALDI-TOF MS measurements of (a) DSPE-PEG-COOH in water showing the characteristic multiple peaks for PEG residues, (b) sCT in water and (c) the mix of the two in water. (n=1-2)

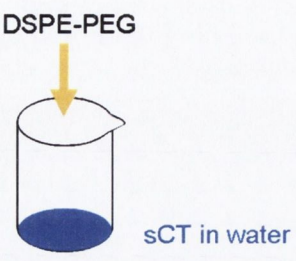
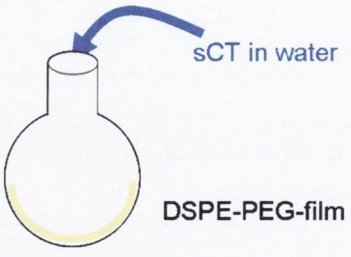
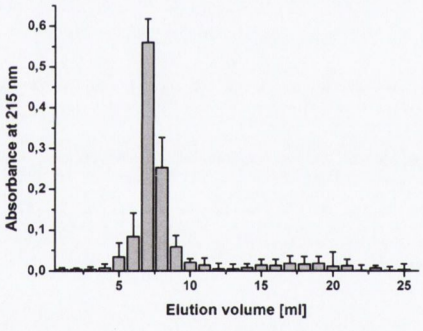
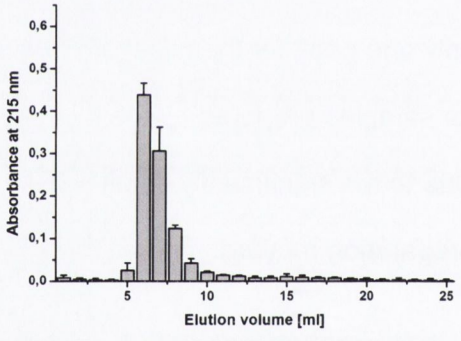
Table 4.2. Summary of SEC, DLS and MALDI-TOF MS results comparing products of the synthesis with EDC and sulfo-NHS with the mix of the reactants in PBS (pH 7.4).

	Synthesis with EDC / sulfo-NHS		**Mix in PBS (pH 7.4)**	
				
SEC (n=1)				
Size *d* [nm] (n=1)	Fractions 1-6: Fractions 11-17:	13 < 2	Mix in PBS: sCT: PEG-lipid:	13 < 2 < 2
MALDI-TOF MS peaks [m/z] (n=1-2)	Fractions 4+5: Fractions 11-13:	3435.7 2813.7 3435.4 3631.1	Mix in PBS: sCT: PEG-lipid:	3437.0 2918.8 3436.3 2815.2

4.4.4. Comparison of the spontaneous assemblies of sCT and DSPE-PEG-COOH in water with particles of the two compounds prepared by thin film hydration method

It was demonstrated that DSPE-PEG-COOH and sCT in equimolar amounts spontaneously assemble to larger particles when mixed in water. The thin film hydration method was used to investigate whether the same product also occurs under controlled conditions, i.e. when lipids are dissolved one after another. The results are summarised in Table 4.3. The particle sizes of the product are 13 nm, independent on the preparation method used. Only after sonication, the particle size was slightly smaller with 12 nm. The PdI, however, was significantly lower for the mix of the compounds in water than for micelles prepared by the thin film hydration method. The PdI of the micelles prepared by the thin film hydration method improved slightly after shaking or sonication, however the protein is vulnerable to degradation. SEC spectra look very similar, in both spectra there is only one peak for the micellar structures and no unbound sCT. The slightly broader peak for samples prepared by the thin film hydration method corresponds with the higher PdI. Due to the significantly smaller ($P<0.05$) PdI, the mere mix in water is the preferable preparation method.

Table 4.3. Particle sizes polydispersity indices (PdI) and SEC spectra of DSPE-PEG-COOH / sCT micelles with regards to the preparation method. Each sample was measured three times with at least ten runs. Values given are mean ± standard deviation of the three measurements (n=3).

	Mix in water	**Thin film hydration method**
		
Size *d* [nm] (n=3)	13 ± 1	13 ± 0 13 ± 1 (after shaking) 12 ± 0 (after sonication)
PdI (n=3)	0.05 ± 0.02	0.53 ± 0.26 0.39 ± 0.11 (after shaking) 0.32 ± 0.02 (after sonication)
SEC (Sephadex G-50) (n=3)		

4.5. DISCUSSION

After the synthesis with EDC and sulfo-NHS, two separate peaks were determined by SEC, suggesting one for conjugates and one for free sCT. However, DLS revealed that the mix of the two compounds in PBS (pH 7.4) or water leads to a peak of similar size than after synthesis with EDC and sulfo-NHS (in both cases about 13 nm diameter). A covalent bonding seemed therefore less likely than a spontaneous aggregation of the two compounds in aqueous solvent. In order to further investigate the bonding type, samples were investigated by MALDI-TOF MS. And here, too, no indication of a covalent bonding was observed. MALDI is such a gentle method that covalent bonds are not destroyed but can be seen in the mass spectrum. In case of a covalent bond between sCT and DSPE-PEG-COOH, a peak at the mass of the conjugate (i.e., 6263.36 = sCT 3431.85 + DSPE-PEG-COOH 2849.51 – water molecule (leaving group) 18.02) should appear in the MS diagram. A loose bond or aggregation such as in micelles would show two separate peaks, one for sCT (3431.85) and another one for the PEG-lipid (2849.51). And exactly those two separate peaks can be seen in the spectra of combined fractions 4 and 5 as well as of the mix of sCT and DSPE-PEG-COOH in water.

The MALDI-TOF spectra also give information about the reaction process of the two-step-synthesis with EDC and sulfo-NHS. In a first step, the activation of carboxylic acid groups with EDC and sulfo-NHS was performed. Due to the multiple peak of the PEG group, it cannot be concluded from the MS diagram if the activation of the carboxylic acid group of DSPE-PEG-COOH with EDC and sulfo-NHS was completed because additional peaks do not stand out in a multiple peak. However, it looks like sCT was itself activated by EDC and sulfo-NHS when added to the mixture in a second step. The MS peak at 3631.1 of fractions 11, 12 and 13 is exactly the size of a sulfo-NHS-ester-derivative of sCT (sCT 3432 + sulfo-NHS 217 – H2O leaving group 18). It seems that part of the sCT is – instead of covalent bonding via amino groups – itself activated on free carboxylic acid groups when added to the DSPE-PEG-COOH / EDC / sulfo-NHS mixture. The activated sCT

does not seem to take part in micelle formation, since it can only be found in the fraction of free sCT and not in the fractions containing micelles.

This might also be a reason why micelle formation is less effective in the presence of EDC and sulfo-NHS. The 1:1 mix in PBS (pH 7.4) showed only one micelle fraction in SEC analysis and no free sCT (Table 4.2). In the presence of EDC and sulfo-NHS, SEC showed a vast peak of free sCT. The activation of carboxylic acid groups and / or the presence of EDC and sulfo-NHS seem to decrease the micelle-forming-properties. Cheng and Lim reported the aggregation of their lipidised sCT and sCT-PEG-lipid conjugates in water into larger particles (Cheng *et al.* 2007, Cheng and Lim 2009 a and b, Cheng and Lim 2010). The results suggest that not only lipidised conjugates, but also a mix of sCT and a PEG-lipid result in the aggregation to larger particles.

4.6. CONCLUSIONS

When sCT and DSPE-PEG-COOH are mixed in an aqueous solvent in a molar ratio of 1:1, spontaneous formation of complexes, likely micelles, with 13 nm diameter occurs. The assembly of sCT and DSPE-PEG-COOH in water happened so quickly that the reaction with ECD and sulfo-NHS was unsuccessful. Particles prepared by the thin film hydration method were of similar size, however, with higher PdI. Hence, for further investigation and characterisation, particles were prepared by the mix of the compounds in water.

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Na, D. H., Y. S. Youn, E. J. Park, J. M. Lee, O. R. Cho, K. R. Lee, S. D. Lee, S. D. Yoo, P. P. DeLuca & K. C. Lee (2004) Stability of PEGylated salmon calcitonin in nasal mucosa. *J Pharm Sci,* 93**,** 256-261.

Roberts, M. J., M. D. Bentley & J. M. Harris (2002) Chemistry for peptide and protein PEGylation. *Adv Drug Deliv Rev,* 54**,** 459-476.

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Chapter 5

Improved pulmonary delivery of salmon calcitonin by assembly into PEG-lipid based micellar complexes

Parts of this chapter have been submitted as:

Baginski, L., O. L. Gobbo, F. Tewes, A. M. Healy, U. Bakowsky & C. Ehrhardt. Improved pulmonary delivery of salmon calcitonin by assembly into PEG-lipid based micellar complexes. *J Control Release. (submitted in March 2011)*

5.1. ABSTRACT

Two micellar formulations of salmon calcitonin (sCT) based on DSPE-PEG_{2000}-COOH or DSPE-PEG_{2000} were investigated for their suitability to improve pulmonary delivery of the peptide drug. Micelles were characterised by DLS, cryo-TEM and ^{31}P-NMR spectroscopy. Their stability against the sCT degrading peptidases, trypsin, α-chymotrypsin and neutrophil elastase was investigated *in vitro*. Studies using an experimental model of intratracheal aerosolisation into rats were performed to test the *in vivo* perfomance of sCT DSPE-PEG micelles. Micelles with a mean hydrodynamic diameter of 12-13 nm spontaneously assembled, when a total concentration of 0.028 mM of DSPE-PEG-COOH and sCT (in a 1:1 molar ratio) or 0.021 mM of DSPE-PEG and sCT (in a 1:1 molar ratio) was exceeded. ^{31}P-NMR and cryo-TEM both confirmed the presence of small micellar structures. Compared to plain sCT, the micellar formulations showed increased stability against enzymatic digestion. The level of protection, however, was higher against chymotrypsin and neutrophil elastase than against trypsin. Data obtained from *in vivo* experiments provided evidence of significantly ($P < 0.05$) higher mean plasma concentrations of sCT, after inhalation of micelles compared to sCT solution, at 60 and 90 min, a significantly ($P < 0.05$) higher AUC_{inf} and a relative bioavailability of 160±55% when compared to plain sCT solution. The herein described PEG-lipid micelles are promising carriers for enhanced pulmonary delivery of sCT.

5.2. INTRODUCTION

Salmon calcitonin (sCT), a 32-amino-acid peptide hormone that has been approved for the treatment of hypercalcaemia associated with malignent diseases and for the treatment of bone disorders such as osteoporosis and Paget's disease. It is currently marketed as injectable solutions (Calcimar®, Miacalcin®) as well as nasal sprays (Fortical®, Miacalcin®). Parenteral administration, however, is not very patient-friendly and the available nasal formulations show a wide window of bioavailability, ranging from 0.3 to 30% relative to injection (Stevenson 2009). Such limitations as well as the drug's poor performance when given orally can potentially be avoided by pulmonary administration (Patton 1996, Siekmeier and Scheuch 2008), as inhalation remains one of the most promising non-invasive route for systemic administration of macromolecules (Patton *et al.* 2010).

The withdrawal of Exubera® (the Pfizer/Nektar inhalable insulin) from the market, resulted in increased scepticism towards pulmonary macromolecule delivery. Afrezza® (MannKind's insulin product), however, has started a new wave of interest in inhalable peptide drugs for systemic action, and it seems to have overcome some of the drawbacks that led to the failure of Exubera® (Neumiller and Campbell 2010). Compared to larger peptides like insulin, smaller compounds often suffer from unusually high enzymatic degradation in the lung (Adjei and Carrigan 1992, Patton *et al.* 1998, Patton and Byron 2007). Nadkarni *et al.* (2011), for example, demonstrated that peptide YY (PYY), an endogenous 36-amino acid peptide and food intake suppressant, showed a remarkable effect on the appetite of rats when administered via the pulmonary route. Nevertheless, the absolute bioavailability remained low, at 12-14%, suggesting a loss of the peptide within the lung by ways other than absorption (Nadkarni *et al.* 2011). Considering the fact, that many catabolising peptidases are synthesised by lung epithelial cells and macrophages (Wall 1995, Hussain *et al.* 2004, Baginski *et al.* 2011), it seems likely that PYY is degraded by proteolytic enzymes to a large extent, before it can reach the systemic circulation.

Structural modifications (e.g., by PEGylation) are an effective means to protect the peptide from proteolytic degradation (for reviews see Harris and Chess 2003, Veronese and Pasut 2005). Chemical attachment of PEG residues to polypeptides, however, can be accompanied by a decrease in the drug's bioactivity due to impeded receptor/ligand interactions as was observed for Pegasys®, Roche's PEGylated interferon α-2a (Bailon *et al.* 2001). Different approaches to the conjugation of PEG (Lee *et al.* 1999, Na *et al.* 2004, Shin *et al.* 2004, Youn *et al.* 2006, Youn, Na and Lee 2007) or PEG-lipids (Cheng and Lim 2009 and 2010) to sCT have been reported in the literature. However, only Youn and co-workers focused their work on the pulmonary delivery of the peptide by synthesising three different Lys^{18}-PEGylated sCT derivatives, which strongly differed in regards to their hypocalcaemic efficacy and their ability to shield the peptide from proteolytic enzymes (Youn *et al.* 2008).

The rationale of this chapter was to develop a sCT formulation suitable for pulmonary application that takes advantage of the favourable shielding characteristics of the PEG residues, but at the same time, preserves the bioactivity of the peptide. In micelles, sCT is associated with but not chemically linked to amphiphilic molecules like PEG-lipids. Ideally, the micellar system will provide protection from enzyme degradation prior to absorption, while fully unfolding its therapeutic activity once it has reached the blood stream, resulting in enhanced bioavailability and maintained bioactivity.

5.3. MATERIALS AND METHODS

5.3.1. Materials

Calcitonin (salmon) Ph.Eur. (sCT; 3,432 Da) was purchased from Polypeptide Laboratories (Limhamn, Sweden), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy (polyethylene glycol)-2000] ammonium salt (DSPE-PEG-COOH; 2,850 Da) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ammonium salt (DSPE-PEG; 2,804 Da) were purchased from Avanti Polar Lipids (INstruchemie BV, Delfzijl, Netherlands). Size and count rate were determined in Plastibrand disposable semi-micro-cuvettes (Brand, Wertheim, Germany) and ζ-potential in folded capillary cells (Zetasizer Nano series DTS 1060, Malvern Instruments, Herrenberg, Germany). For size, count rate and ζ-potential measurements, Aqua Ecotainer® (B. Braun, Melsungen, Germany) was used. Quantifoil® S7/2 Cu 400 mesh, holey carbon film grids from Quantifoil® Micro Tool (Jena, Germany) were used. Sephadex G-50, deuterium oxide (D_2O), phosphate buffered saline (PBS) tablets and neutrophil elastase were purchased from Sigma-Aldrich (Steinheim, Germany). Trypsin, treated with 1-chloro-3-tosylamido-4-phenyl-2-butanone and α-chymotrypsin, treated with 1-chloro-3-tosylamido-7-amino-2-heptanone were obtained from Worthington (Reading, United Kingdom). Acetonitrile, HPLC grade far UV, was purchased from Fisher Scientific (Dublin, Ireland), trifluoracetic acid (TFA) from Riedel-de Haën (Seelze, Germany). HPLC studies were performed on a LiChroCART®125-4 LiChrospher®100 RP-18 (5 µm) column (Merck, Darmstadt, Germany). For *in vivo* studies, Vetalar®, Domitor® and Antisedan® from Pfizer (Cork, Ireland) and SoloCath™ catheters from Linton Instrumentation (Diss, United Kingdom) were used. The salmon calcitonin ELISA kit S-1166 was purchased from Bachem (Weil am Rhein, Germany).

5.3.2. Preparation of micelles and physicochemical characterisation

Salmon calcitonin was diluted in ultrapure water and the required amount of DSPE-PEG-COOH or DSPE-PEG, respectively, was added to the solution. Gentle agitation of the mixture assured homogenous distribution and formation of micelles.

SEC analysis was used to separate micelles from free sCT. Sephadex G-50 was prepared according to the manufacturer's instructions and was transferred into a glass column of 1 cm diameter. The readily prepared column was packed to a volume of 20 ml Sephadex. Before use, the column was thoroughly rinsed with ultrapure water and then equilibrated with the mobile phase (i.e., phosphate buffered saline, PBS, pH 7.4, or ultrapure water). Two hundred microlitres of sample were applied to the column for size-dependent separation. The eluate was collected in fractions of 1 ml, 0.5 ml or 90 µl (depending on the experiment) and the absorbance of each fraction was determined by UV spectroscopy (Ultropsec 3000, Pharmacia Biotech, Freiburg, Germany) at 215 nm.

The hydrodynamic diameter and polydispersity index (PdI) of the micelles was determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments) equipped with a 10 mW HeNe laser at a wavelength of 633 nm at 25℃. Scattered light was detected at an angle of 173°, the attenuator wa s manually adjusted to 11 and position to 4.65 mm for all measurements. For data analysis, the viscosity and refractive index of water were used. Values given are the mean ± standard deviation of at least 5 independent samples of which each was measured 3 times with 10 runs. The ζ-potential was measured with the same instrument at 25℃ and a scattering angle of 17° by measuring the electrophoretic mobility by laser Doppler velocimetry (LDV). Samples contained 10^{-3} M sodium chloride and the pH was 7.4. Each sample was measured 3 times with 100 runs. Values given are the mean ± standard deviation.

5.3.3. Determination of the aggregation number of polymer molecules within one micelle

According to Vorobyova and colleagues (2001), the aggregation number p – expressed as the number of polymer molecules within a micelle - can be calculated using Eq. (1)

$$p = \frac{10\pi R_H^3 N_A}{3M[\eta]} \quad (1)$$

where R_H is the hydrodynamic radius, N_A is Avogadro's number, M is the molecular weight of the polymer, and η is the intrinsic viscosity.

5.3.4. Cryo-transmission electron microscopy (Cryo-TEM)

These measurements were carried out by Sabine Barnert and Prof Dr Rolf Schubert, Albert-Ludwigs-Universität Freiburg, Germany.

For cryo-TEM measurements, Quantifoil® S7/2 Cu 400 mesh, holey carbon film grids were prepared according to a standard method described by Fukami and Adachi (1965). A drop of sample (approximately 3 µl) was applied to the grid and the excess was removed with filter paper so that only a thin film remained on the grid covering the perforations. The films were immediately shock-frozen by dipping the grid into liquid ethane (90 K). The samples were then transferred to the microscope (Leo 912 Ω-mega, Leo Elektronenmikroskopie, Oberkochen, Germany) as described elsewhere (Kaiser *et al.* 2003) and examined at 100 K.

5.3.5. Nuclear magnetic resonance (^{31}P-NMR)

Samples were measured by Dr Thomas Kämpchen, Philipps-Universität Marburg, Germany.

^{31}P-NMR measurements were carried out as described by Leal *et al.* (2008). Samples of DSPE-PEG-COOH sCT micelles (equimolar amounts) and DSPE-PEG sCT micelles (equimolar amounts) were prepared and measured in D_2O with 50 ppm spectral width at a resonance frequency of 202.47 MHz (11.7 T) on a JEOL ECA-500 spectrometer (JEOL,

Eching, Germany) using a JEOL broadband observe probe (NM-03520TH5). One thousand scans were accumulated using 4.5 µs pulses. Prior to Fourier transformation, a line broadening of 30 Hz was applied. One to two samples for each formulation were prepared and each sample was measured at room temperature (25℃) and at 37℃.

5.3.6. Determination of the critical micelle concentration (CMC)

The CMC of an equimolar mixture of sCT and the respective PEG-lipid in water was determined by DLS, measuring the intensity of scattered light (in kilo counts per second). For DLS measurements the same instrument with identical setting was used as described above. Samples of the sCT / DSPE-PEG-COOH mixtures in water with total concentrations of 0.003, 0.009, 0.018, 0.024, 0.036, 0.048, 0.072 and 0.14 mM and samples of sCT / DSPE-PEG with total concentrations of 0.006, 0.009, 0.012, 0.018, 0.024, 0.036, 0.048, 0.072 and 0.14 mM were analysed in disposable semi-micro-cuvettes. Values given are the mean ± standard deviation of 5 independent experiments with each experiment comprising 3 measurements of the same sample with 10 runs.

5.3.7. Stability against trypsin, chymotrypsin and neutrophil elastase digestion

The digestion of plain sCT and DSPE-PEG sCT micelles by three sCT degrading enzymes, trypsin, chymotrypsin and neutrophil elastase was investigated according to a method modified from Marschütz and Bernkop-Schnürch (2000). Micelles were prepared in water as described above with PEG-lipid and sCT at equal molarities. A trypsin solution containing 2 BAEE units trypsin per ml PBS (pH 7.4) and a chymotrypsin solution containing 0.2 TAME units chymotrypsin per ml PBS (pH 7.4) were prepared. Five-hundred microlitres of trypsin or chymotrypsin solution were added to the same volume of either plain sCT solution or the micellar complexes. In the case of neutrophil elastase, 500 µl PBS (pH 7.4) and 500 µl of either sCT solution or sCT-containing micelles were directly added to the 1-unit-vials of elastase. All samples containing 1 mg sCT (unmodified or in micelles) and 1 unit trypsin, 0.1 unit chymotrypsin or 1 unit neutrophil elastase,

respectively, per ml solution were incubated at 37°C for 2 h. After 0, 10, 30, 60 and 120 min, samples of 180 µl were withdrawn, immediately diluted with an equal volume of 1% TFA and analysed by HPLC according to the method described below. Following the same method, the stability of DSPE-PEG-COOH sCT micelles was tested against trypsin and chymotrypsin to compare the protective effects of the two micelle formulations. Data are shown as mean ± standard deviation of 3 independent experiments.

5.3.8. High-performance liquid chromatography (HPLC)

The instrument for reverse phase HPLC analyses consisted of a Shimadzu LC-1OAT VP pump, FCV-10AL VP low pressure gradient valve, DGU-14A degasser, SIL-10AD VP autosampler, SPD-10A VP UV-VIS-detector and CLASS-VP software (Kyoto, Japan). Samples were analysed for their sCT content according to a method described by Shah and Khan (2004) with modifications. In brief, the mobile phases consisted of 0.1% v/v TFA-water (A) and 0.1% v/v TFA-acetonitrile (B). A linear gradient was run at a flow-rate of 1 ml/min: 20-35% B for 10 min, 35-37% B from 10 to 20 min and 37-20% from 20 to 25 min. Twenty microlitres of sample were injected and analysed at a wavelength of 215 nm. Each sCT concentration was quantified from the corresponding integrated peak area.

5.3.9. *In vivo* experiments

In vivo experiments were performed by Dr Oliviero Gobbo and Dr Frederic Tewes, Trinity College Dublin, Ireland.

Animals. The experimental protocols employed in this study were approved by the Animal Research Ethics Committee, Trinity College Dublin (TCD) and the Department of Health and Children (Ireland), in accordance with the European Community Directive (86/609). The animals used for experiments were male Wistar rats supplied by the TCD BioResources Unit, with a body weight of 350±50 g. The animals were housed in a thermo-regulated environment with a 12-h light/dark cycle. Food and water were available *ad libitum*.

Surgery. Rats were anaesthetised by an intraperitoneal injection with a mixture of ketamine hydrochloride (Vetalar®) and medetomidine hydrochloride (Domitor®), before a heparinised catheter (SoloCath™) was installed into the left femoral artery for blood sampling. The left femoral vein was cannulated for the administration of sCT solution.

Drug administration. The sCT dose for intratracheal administration was 100 µg/kg in micellar formulation and sCT solution, respectively. The aerosolisation was performed using an AeroProbe™ intracorporeal nebulising catheter controlled by a LabNeb™ unit (Trudell Medical International, London, Canada) according to the manufacturer's protocol. The intravenous bolus administration of sCT (also at 100 µg/kg) was performed by injection of sCT solution or sCT in micelles.

Blood sampling. After drug administration, the sedation was reversed by injection of atipamezole hydrochloride (Antisedan®) and blood sampling was carried out from freely moving animals. Arterial blood samples (200 µl) were serially collected in heparinised tubes 0, 3, 10, 20, 30, 60, 90, 120 and 180 min post sCT administration. Blood samples were centrifuged at 10,000 rcf for 5 min, before plasma was collected and stored at –20°C until further use.

Calcitonin measurement. Samples were assayed using a high-sensitive sCT ELISA kit according to the manufacturer's protocol.

5.3.10. Statistical data analysis

Outlier detection was performed based on Grubbs' test using GraphPad Software (http://www.graphpad.com/quickcalcs/Grubbs1.cfm). Data from stability studies (5.3.7) and *in vivo* experiments (5.3.9) were analysed by F-test and t-test using Excel® software, 2007 (Microsoft, USA). Values were considered statistically significant when $P < 0.05$.

5.3.11. Pharmacokinetic analysis

Pharmacokinetic analysis was carried out by Dr Oliviero Gobbo and Dr Frederic Tewes, Trinity College Dublin, Ireland.

Pharmacokinetic parameters were determined for each individual rat using a two-compartment model. Salmon calcitonin plasma concentration (C_p) versus time profiles were best fitted with Eq. (2) using the least-squares method and the solver function of the Excel® software, 2007 (Microsoft, USA).

$$C_p = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} + C \cdot e^{-ka \cdot t} \quad (2)$$

with $\alpha > \beta$, and C=0 for profiles fitting after *i.v.* administration, and C = –(A+B) for profiles fitting after pulmonary administration. In this equation, the *A, B,* α and β terms were functions of the rate constant *k12, k21, kel* according to Eq. (3) – (5).

$$kel = \frac{\alpha \cdot \beta}{k21} \quad (3)$$

$$k12 = \alpha + \beta - k21 - kel \quad (4)$$

$$k21 = \frac{\alpha \cdot B + A \cdot \beta}{A + B} \quad (5)$$

Area under plasma concentration-versus-time profiles *(AUC)* was calculated by using the linear trapezoidal rule. To determine the AUC infinity (AUC_{inf}), the area remaining after the last measured concentration ($C_{t\to\infty}$) was extrapolated using Eq. (6).

$$AUC_{t\to\infty} = \frac{C_{t\to\infty}}{\beta} \quad (6)$$

The absolute bioavailability (F_{abs}) was calculated by comparison of the sCT AUC_{inf} obtained after intratracheal *(i.t.)* administration to the one obtained by *i.v.* at the same dose using Eq. (7).

$$F_{abs} = \frac{AUC_{\inf\ i.t.}}{AUC_{\inf\ i.v.}} \quad (7)$$

The relative bioavailability (F_{rel}) was calculated by comparing the AUC_{inf} of *i.t.* administered micellar formulation with the AUC_{inf} of the same dose of *i.t.* administered sCT solution using Eq. (8).

$$F_{rel} = \frac{AUC_{\text{inf } micelle}}{AUC_{\text{inf } sCT}} \tag{8}$$

5.4. RESULTS

5.4.1. Physicochemical characterisation of micelles

Micelles with different molar ratios of PEG-lipid and sCT (i.e., 4:1, 2:1, 1:1 and 1:2) were prepared and analysed by DLS and LDV (results are summarised in Table 5.1). Micelles based on DSPE-PEG-COOH and micelles with DSPE-PEG show very similar physicochemical properties. The lowest PEG-lipid:sCT molar ratio at which sCT was completely incorporated in the micelle was 1:1. At this ratio, only a single peak was detected in SEC analyses (Figure 5.1a), whereas higher amounts of sCT resulted in a second peak in the SEC spectrum due to unbound sCT (Figure 5.1b). The 1:1 ratio can hence be considered the saturation ratio of sCT to PEG-lipid, and was chosen for further investigations. Whilst solutions of pure sCT (0.6 mM), DSPE-PEG-COOH (0.6 mM) or DSPE-PEG (0.6 mM) in water both showed particle sizes ≤ 2 nm, spontaneous self-assembly of micelles with a mean hydrodynamic diameter of 12-13 nm was observed, when sCT and a PEG-lipid were added at a 1:1 ratio (each 0.6 mM) in water. Micelles containing DSPE-PEG-COOH showed a PdI of 0.04 ± 0.01 and a ζ-potential of - 2.63 ± 0.32 mV, while for DSPE-PEG sCT systems a PdI of 0.15±0.04 was obtained and a ζ-potential of 1.54±0.20 mV was measured. The slightly more negative ζ-potential of the DSPE-PEG-COOH systems can be explained by the carboxylic acid group.

Table 5.1. Physicochemical characterisation of DSPE-PEG-COOH sCT micelles and DSPE-PEG sCT micelles.

	molar ratio[1]	solvent	size *d* [nm]	zeta potential [mV]	PdI	count rate [kcps]
pure compounds						
sCT	1	water	< 2	n.d.	0.99 ± 0.01	44 ± 3
DSPE-PEG-COOH	1	water	< 2	n.d.	0.20 ± 0.08	120 ± 3
DSPE-PEG	1	water	2 ± 0	- 4.91 ± 6.17	0.13 ± 0.05	147 ± 1
micelles with different molar ratios						
DSPE-PEG-COOH : sCT	1 : 2	water	13 ± 0	- 1.53 ± 0.33	0.06 ± 0.01	1310 ± 3
	1 : 1	water	13 ± 0	- 2.63 ± 0.32	0.04 ± 0.01	1193 ± 5
	1 : 0.5	water	10 ± 0	- 1.71 ± 0.32	0.03 ± 0.01	819 ± 1
	1 : 0.25	water	4 ± 0	- 1.14 ± 0.19	0.18 ± 0.03	289 ± 3
DSPE-PEG : sCT	1 : 2	water	12 ± 0	1.54 ± 0.20	0.08 ± 0.01	989 ± 1
	1 : 1	water	12 ± 0	1.20 ± 0.50	0.06 ± 0.01	999 ± 1
	1 : 0.5	water	12 ± 0	0.70 ± 0.09	0.04 ± 0.01	986 ± 6
	1 : 0.25	water	13 ± 0	-2.00 ± 1.46	0.02 ± 0.01	1004 ± 3
micelles in different solvents						
DSPE-PEG-COOH : sCT	0.5 : 0.5	water	13 ± 0	n.d.	0.08 ± 0.03	525 ± 12
	0.5 : 0.5	saturated NaCl	13 ± 0	n.d.	0.12 ± 0.02	522 ± 6
	0.5 : 0.5	PBS 7.4	12 ± 0	n.d.	0.08 ± 0.02	571 ± 6
DSPE-PEG : sCT	0.5 : 0.5	water	13 ± 0	n.d.	0.17 ± 0.06	573 ± 13
	0.5 : 0.5	saturated NaCl	12 ± 0	n.d.	0.09 ± 0.01	573 ± 10
	0.5 : 0.5	PBS 7.4	12 ± 0	n.d.	0.08 ± 0.01	594 ± 5

n.d. = not determined

[1] One molar part (as stated in the table) is equivalent to a concentration of 0.6 mM, 0.5 part to 0.3 mM, etc.

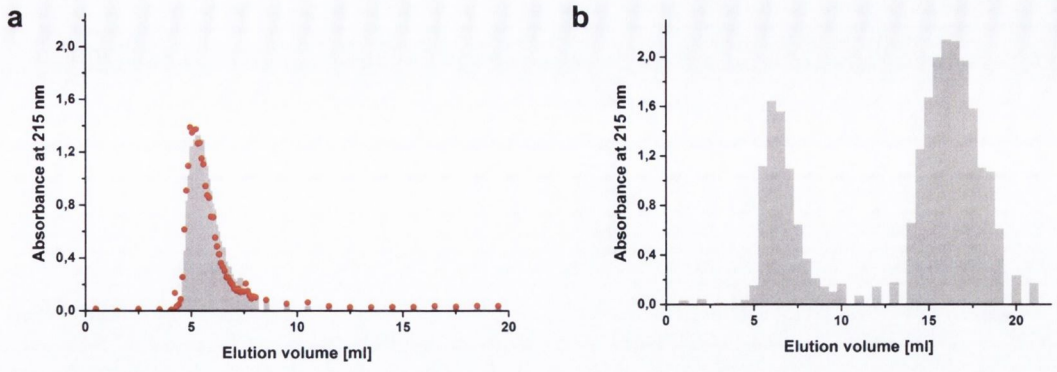

Figure 5.1. SEC spectra of DSPE-PEG-COOH sCT micelles in a 1:1 ratio (a) and 1:2 ratio (b). The 1:1 ratio (a) was measured immediately (grey bars) and at day 5 after preparation (red dots). The spectra both show a single peak and are practically identical. The formulation can therefore be considered as stable for a period of 5 days stored at -4℃. The 1:2 ratio (b) shows an additional peak due to unmodified sCT, while the micelle derived peak at an elution volume of 6 ml is of comparable size to that of the 1:1 ratio. The 1:1 molar ratio can therefore be considered the saturation ratio of sCT to PEG-lipid, (n=1).

5.4.2. Determination of the aggregation number of polymer molecules within one micelle

The method described by Vorobyova *et al.* (2001) was used to determine the number of polymer molecules in a micelle. In this part of my work, micelles are not formed of a single compound, but of a 1:1 ratio of sCT and PEG-lipid. Thus, a molecular weight equal to sCT + DSPE-PEG-COOH (6,282 g/mol) or sCT + DSPE-PEG (6,235 g/mol), respectively, and an intrinsic viscosity of 0.1 dl/g (0.09 for PEG 2000) were used for the estimation of the aggregation number. For a hydrodynamic diameter of 13 nm and 12 nm, respectively – as it was determined by DLS measurements - the aggregation number is approximately 28 in sCT DSPE-PEG-COOH micelles and 22 in DSPE-PEG micelles. One micelle was therefore composed of about 28 or 22 sCT molecules and 28 or 22 PEG-lipid molecules.

5.4.3. Cryo-TEM

Micrographs obtained by cryo-TEM showed multiple spherical structures of a similar size, ranging between 10 - 20 nm, consistent with the data acquired by DLS (Figures 5.2 and 5.3).

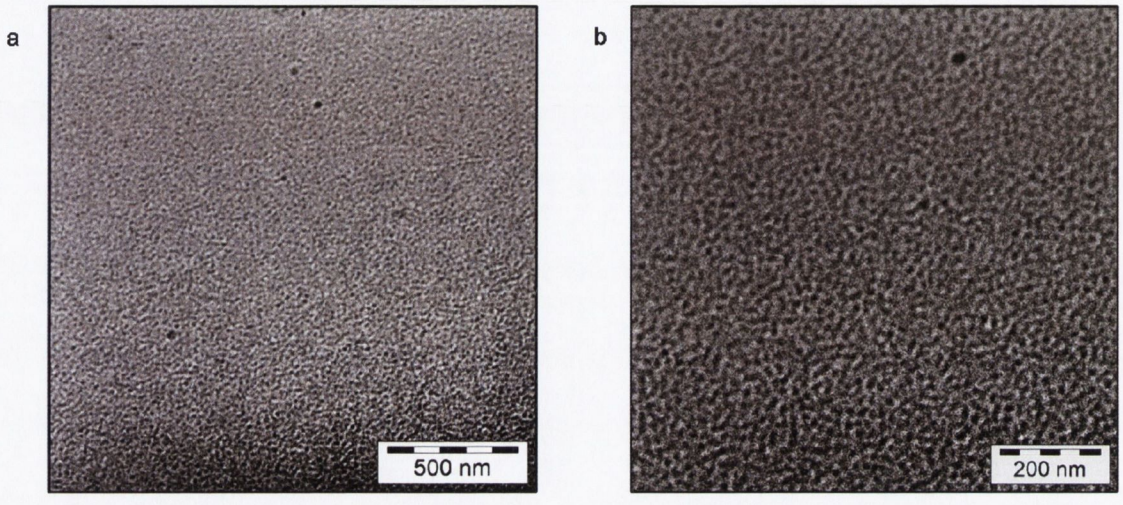

Figure 5.2. Visualisation of DSPE-PEG-COOH sCT micelles by cryo-TEM. Small spherical structures of similar size can be seen at both magnitudes.

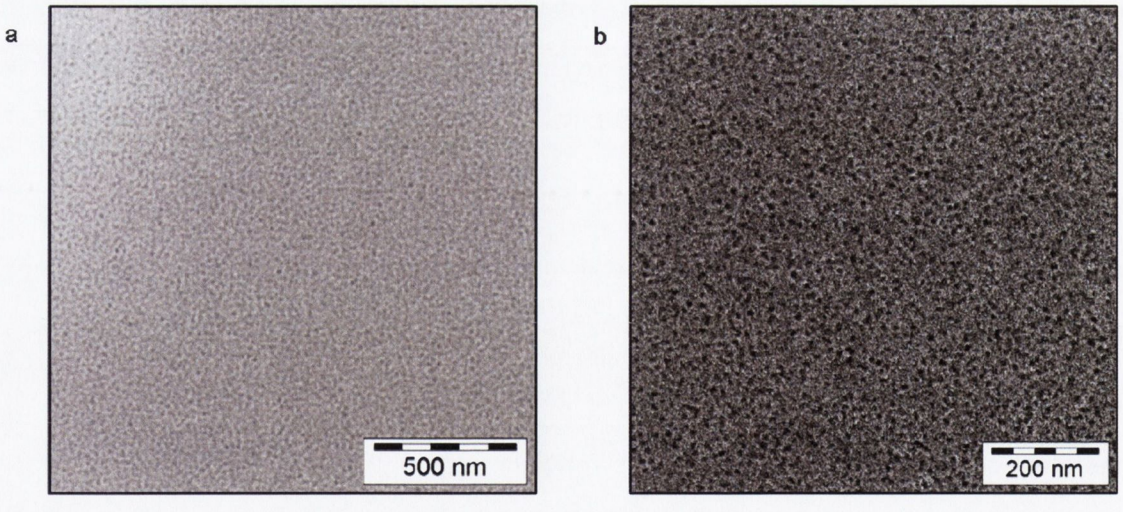

Figure 5.3. Visualisation of DSPE-PEG sCT micelles by cryo-TEM at two different magnifications. The small dark dots indicate monodisperse micelles of spherical shape.

5.4.4. ^{31}P-NMR spectroscopy

Peak formation in ^{31}P-NMR spectra can be used to distinguish between micellar and liposomal formulations (Leal *et al.* 2008). The ^{31}P-NMR signal depends on the molecular order of phospholipids in the particle into which the molecule is embedded. Small micellar particles show symmetric narrow sharp peaklines compared to wider peaks of liposomes with their bigger entity (Cullis and De Kruijff 1978, Leal *et al.* 2008). ^{31}P-NMR spectra of the DSPE-PEG-COOH sCT (Figure 5.4) and DSPE-PEG sCT (Figure 5.5) mixtures all showed symmetric sharp peaks and therefore, confirmed the presence of small micellar structures at room temperature (i.e., 25°C), as well as at body temperature (i.e., 37°C).

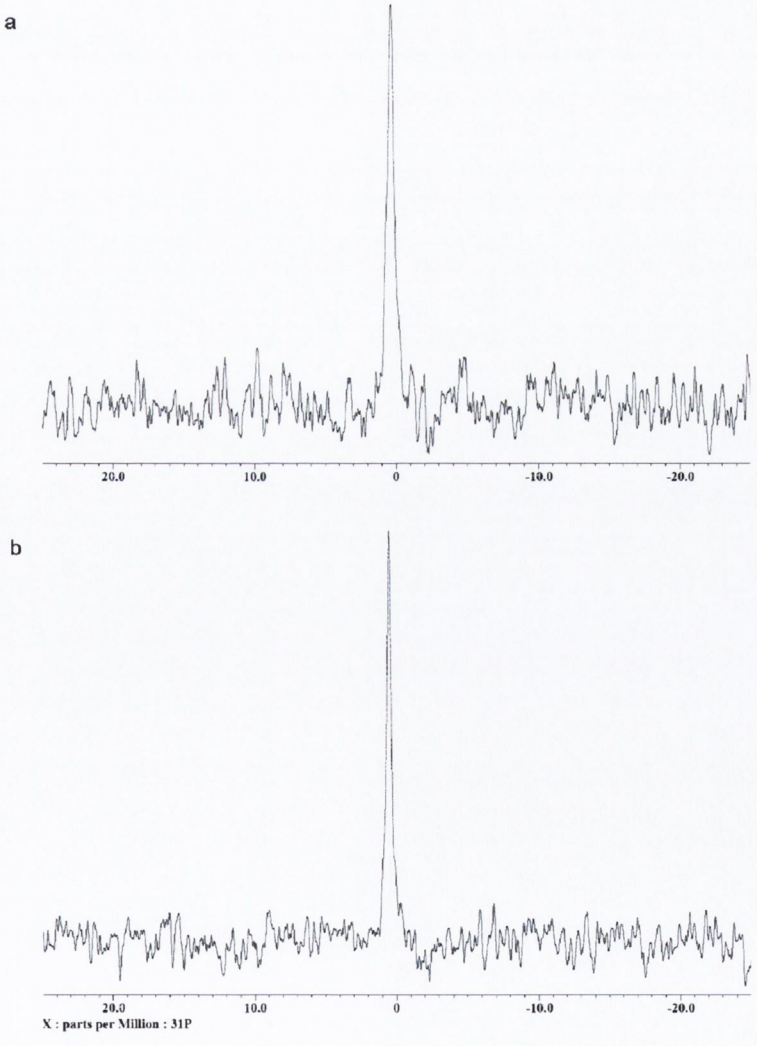

Figure 5.4. ^{31}P-NMR spectra of DSPE-PEG-COOH sCT micelles at 25°C (a) and 37°C (b). Narrow symmetric peaks indicate the presence of small micellar structures at both temperatures (n=2).

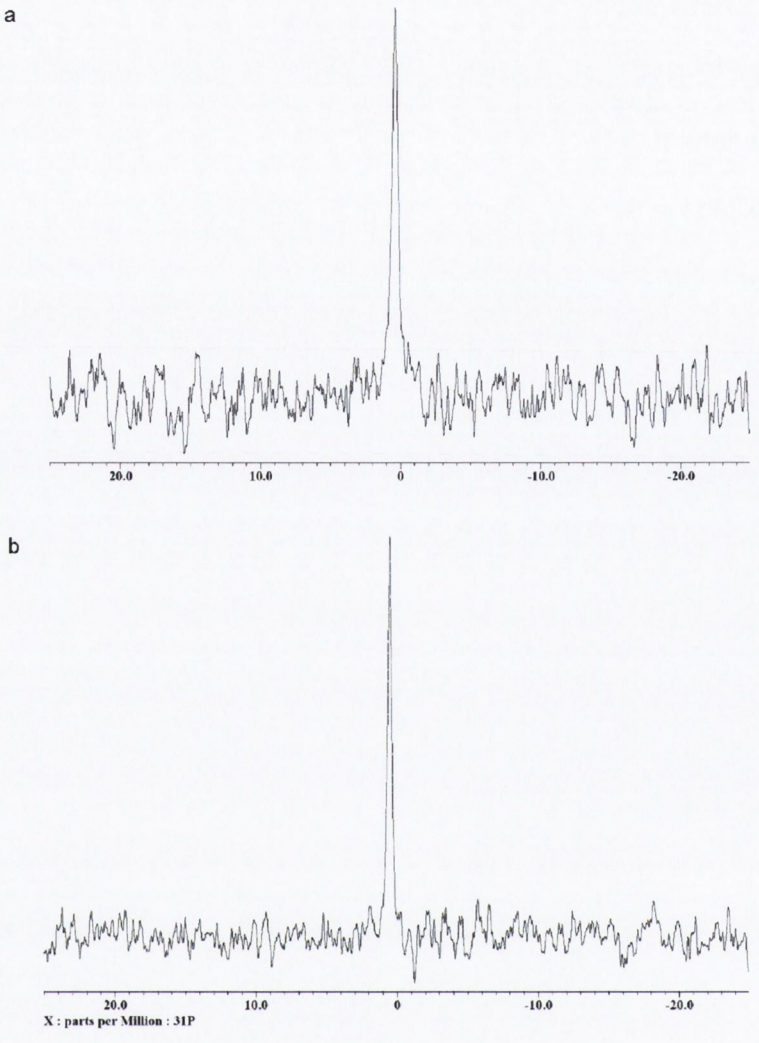

Figure 5.5. ^{31}P-NMR spectra of DSPE-PEG sCT micelles at 25°C (a) and 37°C (b). The narrow symmetric peaks indicate the presence of small micellar structures at both temperatures (n=1).

5.4.5. Determination of the CMC

Plots comparing the intensity of scattered light and the mean hydrodynamic diameter obtained for various concentrations of sCT and DSPE-PEG-COOH or DSPE-PEG, respectively, (at a 1:1 ratio) are illustrated in Figure 5.6. The intersection of the two regression lines of the intensity data (i.e., the CMC) was calculated for each of the two micelle formulations. For DSPE-PEG-COOH sCT micelles the CMC was determined to be 0.028 mM (i.e., 0.014 mM sCT plus 0.014 mM DSPE-PEG-COOH in water) and the CMC of DSPE-PEG sCT was calculated as 0.021 mM (i.e., 0.011 mM sCT plus 0.011 mM DSPE-PEG). At lower concentrations, where surfactant molecules are non-associated monomers (Helenius *et al.* 1979), no reliable size distribution information could be obtained and the autocorrelation functions showed very low intercepts. Once the CMC was reached, however, the scattering intensity showed a linear increase with concentration, and particle sizes of 12-14 nm (depending on the concentration) were measured and autocorrelation functions showed much higher intercepts.

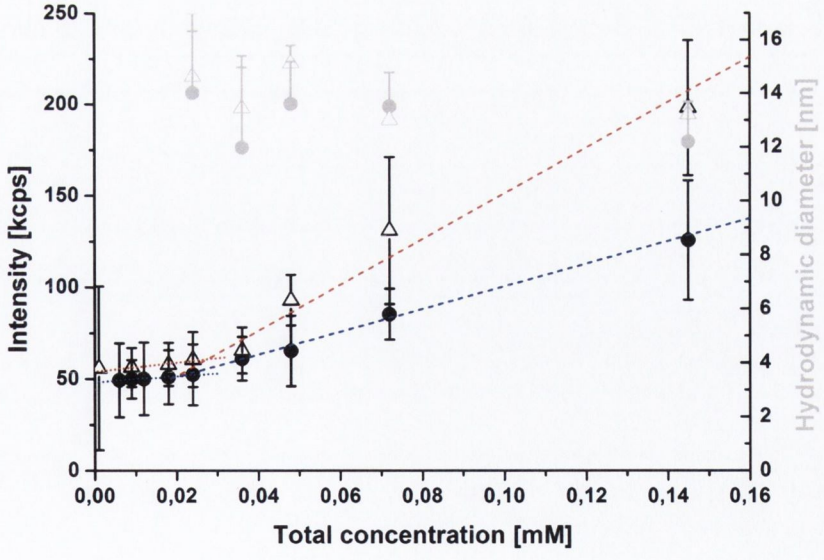

Figure 5.6. The intensity of scattered light (black) and the mean hydrodynamic diameter (grey) obtained for different concentrations of equimolar amounts of sCT and DSPE-PEG-COOH (Δ) or DSPE-PEG (•), respectively. The intersection of the respective two regression lines of the intensity data (i.e., the CMC) was calculated to be 0.028 mM for DSPE-PEG-COOH (red lines) and 0.021 for DSPE-PEG (blue lines). Values are given as means ± SD; n = 5.

5.4.6. Stability against trypsin, chymotrypsin and neutrophil elastase digestion

A protective effect of the DSPE-PEG sCT micelles against enzymatic degradation was observed, when compared to plain sCT. The degradation profiles of the 3 different enzymes are illustrated in Figure 5.7. When incubated with trypsin, degradation of sCT micelles and plain sCT showed a small, albeit significant ($P < 0.05$) advantage for the micellar formulations after 120 min, with the sCT content of micelles being reduced to 39.02±0.88% of the original concentration, compared to 36.60±1.01% in the case of plain sCT. Chymotrypsin reduced sCT to 56.29±0.96% in the micelles after 120 min, which was significantly ($P < 0.05$) higher than the 45.89±3.19% remaining for plain sCT. Neutrophil elastase dramatically reduced micellar sCT to 27.18±8.06% of the original concentration, whereas 8.80±11.48% of plain sCT was left after 120 min. This difference, however, was not significant ($P > 0.05$). Figure 5.8 summarises the stability data of incubation with the 3 different enzymes after 120 min. The stability of sCT in DSPE-PEG-COOH micelles was tested against trypsin and chymotrypsin and compared to the micellar formulation with DSPE-PEG (Figure 5.9). Looking at the graphs, the stability of the two micellar formulations against trypsin and chymotrypsin was comparable. After 120 minutes, the sCT content of the DSPE-PEG-COOH formulation was reduced to 37.33±2.24% when incubated with trypsin and to 58.74±0.42% after incubation with chymotrypsin. In the presence of chymotrypsin, sCT in DSPE-PEG-COOH micelles was slightly but significantly ($P < 0.05$) more stable than sCT in DSPE-PEG micelles after 120 min.

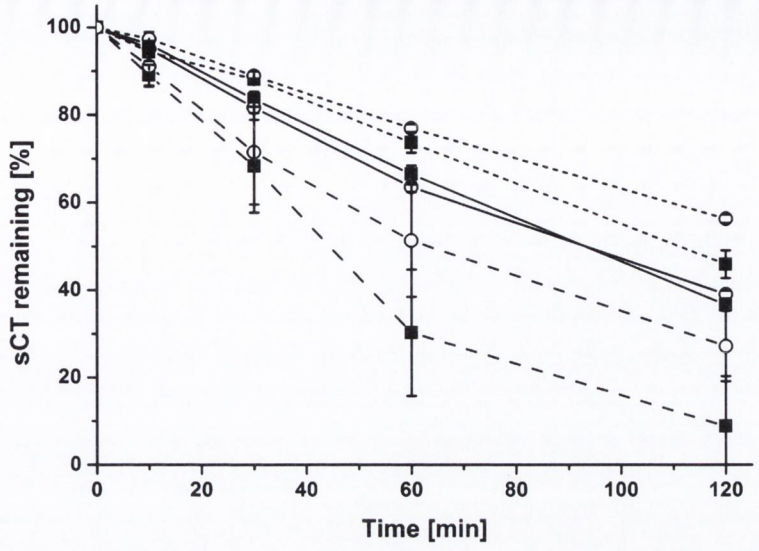

Figure 5.7. Stability of plain sCT (■) compared to sCT assembled into DSPE-PEG micelles (○) in the presence of 1 unit trypsin (solid line), 0.1 unit α-chymotrypsin (dotted line) or 1 unit neutrophil elastase (dashed line), respectively. Samples were withdrawn after 0, 10, 30, 60 and 120 min of incubation at 37℃ and analysed by HPLC. Peak areas were used to determine the sCT concentrations, here shown as the amount of sCT remaining [%] over time. Data are represented as mean ± SD; n = 3.

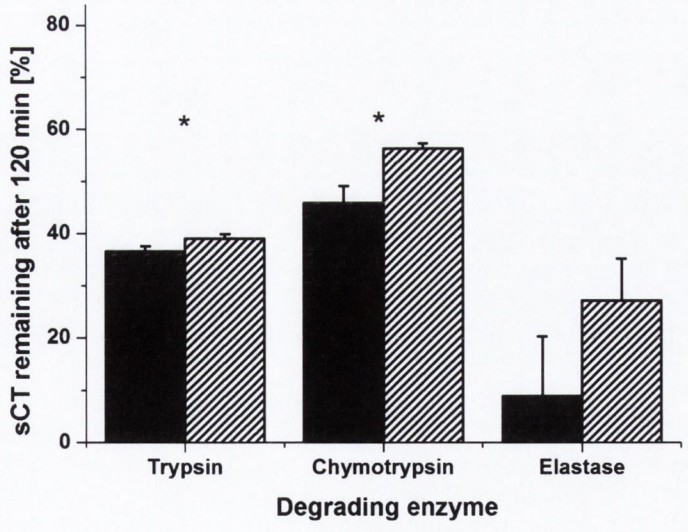

Figure 5.8. Protection of free sCT (black bars) and DSPE-PEG sCT micellar complexes (shaded bars) against enzymatic digestion. The peptide was incubated in the presence of trypsin, α-chymotrypsin or neutrophil elastase for 120 min at 37℃, before samples were analysed by HPLC. The amount of sCT remaining [%] of the original concentration is represented as means ± SD; n = 3; * $P < 0.05$.

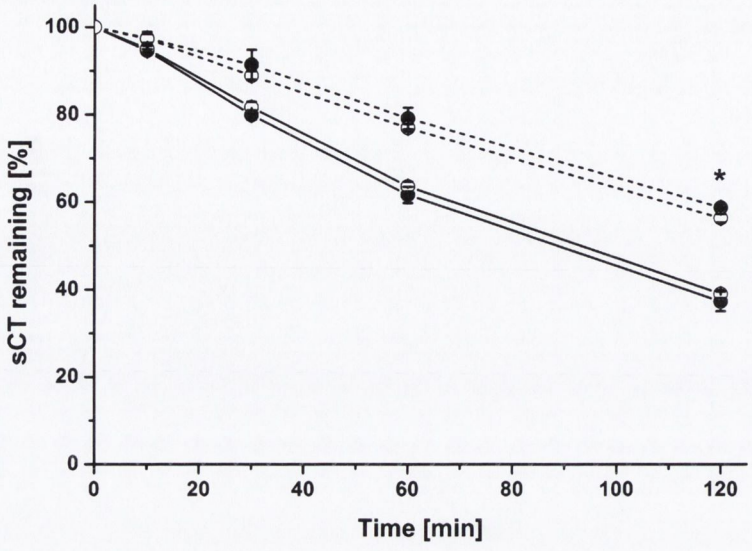

Figure 5.9. Stability of sCT DSPE-PEG-COOH micelles (●) compared to sCT assembled into DSPE-PEG micelles (○) in the presence of 1 unit trypsin (solid line) and 0.1 unit α-chymotrypsin (dotted line), respectively. Samples were withdrawn after 0, 10, 30, 60 and 120 min of incubation at 37℃ and analysed by HPLC. Peak areas were used to determine the sCT concentrations, here shown as the amount of sCT remaining [%] over time. Data are represented as mean ± SD; n = 3, * $P < 0.05$.

5.4.7. *In vivo* experiments

The individual plasma concentration *vs.* time profiles of intratracheally administered sCT DSPE-PEG micelles and sCT solution are shown in Figures 5.10 and 5.11. When compared to free sCT in solution, PEG-lipid micelles resulted in higher mean plasma concentrations at all time points, which were significantly different ($P < 0.05$) at 60 and 90 min (Figure 5.12). The maximum plasma concentration (c_{max}) and the time at which it was reached (t_{max}) were both slightly increased for the micellar formulation compared to *i.t.* administered sCT solution. Table 5.2 summarises the pharmacokinetic parameters, AUC_{inf}, F_{abs}, F_{rel}, c_{max} and t_{max}. The AUC_{inf} for sCT micellar complexes was 23±8 min·ng/ml/µg·kg, and therefore significantly higher than the AUC_{inf} for free sCT which was determined as 14±6 min·ng/ml/µg·kg. Relative and absolute bioavailabilities were hence, both significantly ($P < 0.05$) increased by more than 60% (Table 5.2).

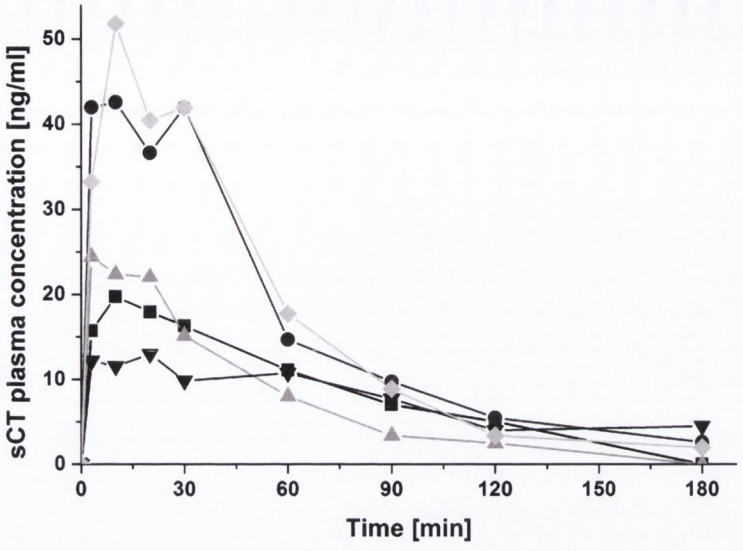

Figure 5.10. Plasma concentrations of intratracheally aerosolised micellar complexes of sCT (100 µg/kg). The graphs show the individual plasma concentrations (ng/ml) of five investigated rats.

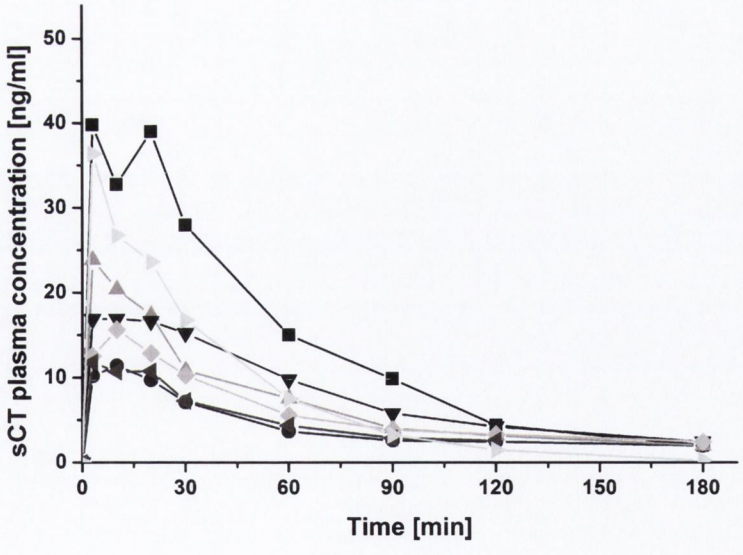

Figure 5.11. Concentration time profiles of intratracheally applied sCT solution (100 µg/kg). The graphs show the sCT plasma concentration (in ng/ml) at the given time points of the seven investigated rats.

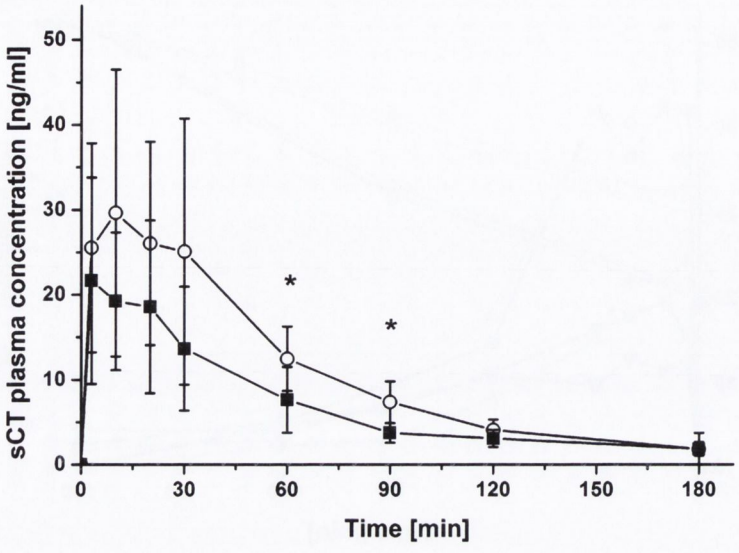

Figure 5.12. Plasma concentrations of intratracheally aerosolised sCT solution (■) (n = 7) and DSPE-PEG micelles (○) (n = 5), both given at a concentration of 100 µg/kg. Data is represented as mean ± SD. * $P < 0.05$.

In addition to the pulmonary application, micelles were also injected *i.v.* into rats. The mean sCT plasma concentrations compared to plasma concentrations of *i.v.* injected sCT solution of the same concentration are illustrated in Figure 13. Although sCT plasma concentrations were slightly higher after injection of the micellar formulation, there is no significant difference in the mean AUC_{inf} so that the micellar formulation cannot be regarded as superior to sCT solution. Pharmacokinetic data are summarised in Table 5.2.

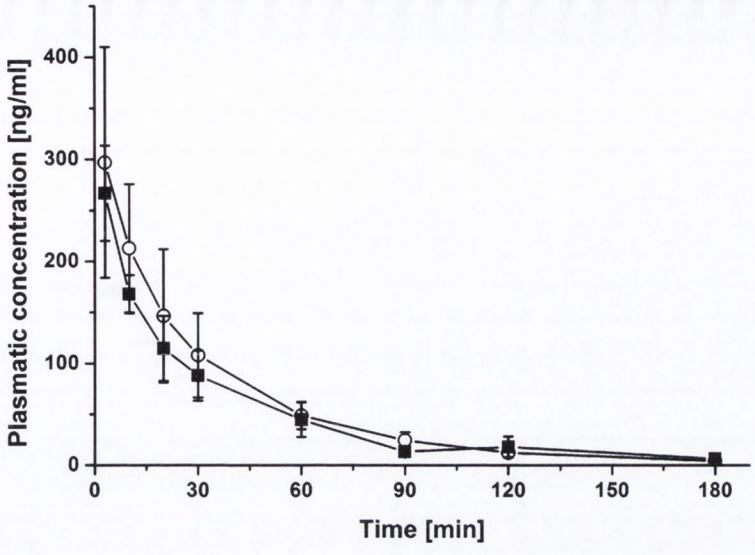

Figure 5.13. Plasma concentrations of *i.v.* injected sCT solution (■) (n = 7) and DSPE-PEG micelles (○) (n = 5), both given at a concentration of 100 µg/kg. Data is represented as mean ± SD. * $P < 0.05$.

Table 5.2. Pharmacokinetic parameters of intratracheally aerosolised and injected sCT (100 µg /kg) and PEG-lipid sCT micelles (n=5-7).

	sCT solution (*i.v.*)	**DSPE-PEG micelles (*i.v.*)**	**sCT solution (*i.t.*)**	**DSPE-PEG micelles (*i.t.*)**
AUC_{inf} [min*ng/ml/µg*kg]	125±35	113±28	14±6	23±8
F_{abs} [%]	100±28	90±22	11±5	18±6
F_{rel} [%]			100±39	160±55
c_{max} [ng/ml]	489±48	524±224	22±12	30±17
t_{max} [min]			5±4	7±4

5.5. DISCUSSION

This work set out to determine if micellar complexes could result in an improvement in the pulmonary delivery of salmon calcitonin. Micelles prepared from sCT and DSPE-PEG_{2000} or DSPE-PEG_{2000}-COOH, respectively were characterised by DLS, LDV and ^{31}P-NMR. In addition, cryo-TEM measurements visualised small spherical micelles (Johnsson and Edwards 2003). The two investigated micellar formulations (both composed of a 1:1 ratio) showed comparable physicochemical properties. *In vitro*, the micellar PEG-lipid sCT formulations proved to be more stable against degradation by trypsin, chymotrypsin and neutrophil elastase than plain sCT. *In vivo* experiments showed that plasma concentrations were significantly higher post micellar administration than after aerosolisation of sCT solution after 60 and 90 min and the bioavailability (absolute and relative) of micellar formulations was significantly increased ($P < 0.05$) compared to the sCT solution. As the effect observed *in vivo* was more pronounced than the increase in stability *in vitro*, other effects than the shielding from catabolising peptidases may contribute to the increase in bioavailabitity of the micellear formulation compared to plain sCT. Due to different particle sizes, the particle size distribution after inhalation of the micellar formulation may be more favourable for drug absorption than in particle distribution of nebulised sCT solution. Spreading of micelles might occur post deposition, so that the sCT molecule cannot only be shielded from degradation by the surrounding PEG-lipid molecules, but the tenside could also act as a permeation enhancer. In the same context, toxicity and thus barrier impairment have to be considered.

After intratracheal instillation of sCT *in vivo*, an absolute bioavailability of 17% was measured by Patton *et al.* (1994) in rat lungs, and Clark *et al.* (2008) reported relative bioavailabilities of 10-18% for nebulised sCT solution compared to *s.c.* injection in healthy volunteers. It is, however, desirable to further increase the efficiency of peptide delivery, in order to reduce local side effects, and also the costs associated with the therapy. In chapter 3 of this thesis I showed that peptidases and proteinases play a significant role in

the pulmonary breakdown of sCT and that therefore formulations that protect from proteolytic degradation are a key element of improving macromolecule delivery via the lungs. Several groups have demonstrated that direct conjugation of sCT with PEG alone or PEG and lipids can enhance the peptide's stability against digestion by proteases (Lee *et al.* 1999, Na *et al.* 2004, Shin *et al.* 2004, Youn *et al.* 2006, Youn *et al.* 2007, Youn *et al.* 2008, Cheng and Lim 2009 and 2010). Direct PEGylation, however, generally comes at the cost of significantly reduced bioactivity (Bailon *et al.* 2001, Harris and Chess 2003, Youn *et al.* 2008). My aim was, therefore, to develop a more transient delivery approach based on PEG-lipid-peptide self assembly.

The amphiphilic properties of the α-helical salmon calcitonin have previously been reported and it was suggested that the hydrophobic residues of the molecule face to one side of the helix, while the hydrophilic amino acids orient to the other (Kaiser and Kézdy 1984, Moe and Kaiser 1985, Andreotti *et al.* 2006). It therefore is likely, that DSPE-PEG associates with sCT in such a way that the PEG-groups loosely interact with the hydrophilic components of the sCT molecules and together form the outer part of the micelle, whereas the lipid-chain and lipophilic residues of sCT are stowed in the interior of the micelle. Moreover, it was reported several years ago that proteins are able to bind equimolar amounts of amphiphilic tensides, and that this binding was caused by hydrophobic interactions (Reynolds and Tanford 1970). Reynolds and Tanford (1970) also emphasised that the protein was not incorporated in the interior of a micelle nor interacted with the surface of tenside micelles, but that the tenside monomers interacted with the macromolecule and together formed micelle-like structures. A hypothetical structure of DSPE-PEG sCT micelles is illustrated in Figure 5.14.

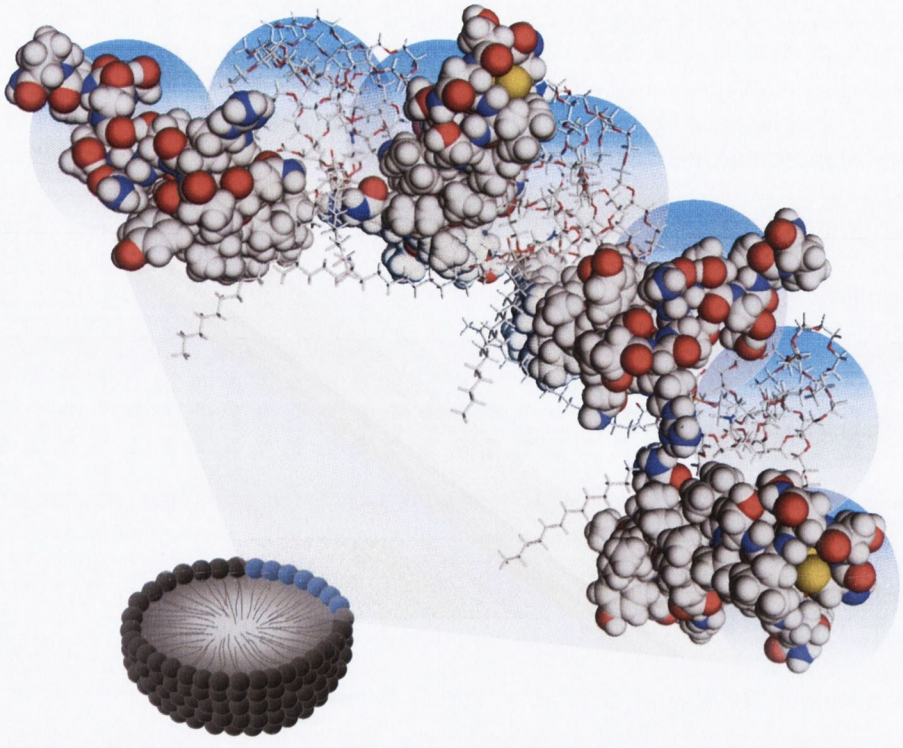

Figure 5.14. Hypothetical structure of DSPE-PEG sCT micelles. To better distinguish the different molecules, DSPE-PEG is displayed as stick model, whereas sCT is visualised as space-filling model. It should be noted that not only DSPE-PEG is an amphiphilic molecule, but also sCT (Kaiser and Kézdy 1984, Moe and Kaiser 1985, Andreotti *et al.* 2006). DSPE-PEG and sCT molecules are hypothetically arranged alternating with the lipophilic aspect of sCT and the lipid chains of DSPE-PEG facing the interior of the micelle, and the hydrophilic component of both molecules forming the outer shell oriented towards the aqueous phase. The presented model was designed to illustrate the possible interactions between sCT and the PEG-lipid within the micellar structure and does not necessarily correspond with the exact location of sCT within the micelle. Scale and aggregation number in the model were freely chosen and did not take into account the calculated aggregation number. *The DSPE-PEG stick models and sCT space models were created by Felix Gut, Philipps-Universität Marburg, Germany.*

To my knowledge, this is the first report on micellar systems comprising a peptide drug and a PEG-lipid being tested for pulmonary application with the aim of achieving a systemic action. Cheng and Lim observed the assembly of PEG-lipid sCT conjugates in water into larger particles. Conjugates with a fatty acid molecule and one or two separate PEG_{5000}-chains, associated to particles with radii of 34±17 nm and 68±21 nm, respectively (Cheng and Lim 2009). Conjugates of sCT and a PEG_{5000}-lipid molecule resulted in complexes with a radius of 30±9 nm (Cheng and Lim 2010). However, the data of this part of my thesis suggest that not only conjugates, but also mixtures of the non-conjugated compounds (i.e., PEG-lipid and sCT) result in association into micelles, and that these systems are suitable carriers for peptide drugs. Similar observations have recently been published by Castelletto *et al.* (2007), Kastantin *et al.* (2010) and Lim *et al.* (2011). Castelletto *et al.* (2007) and Kastantin *et al.* (2010) thoroughly investigated the interaction of bovine serum albumin (BSA) with DSPE-PEG_{2000} micelles and found that BSA possesses binding sites for the adsorption of PEG-lipid monomers leading to the formation PEG-lipid protein complexes. Lim *et al.* (2011) observed that DSPE-PEG_{2000} self-associated spontaneously with the 30-amino acid peptide, glucagon-like peptide 1 (GLP-1). After subcutaneous injection, the micellar GLP-1 showed promising anti-inflammatory properties in the treatment of acute lung injury. With a diameter of 14±3 nm the GLP-1 micelles were of comparable size to the sCT micelles of this study. Lim *et al.* prepared DSPE-PEG-micelles first and subsequently added the peptide and found a lipid:peptide saturation molar ratio of 13:1 (Lim *et al.* 2011), whereas my data indicate a lipid:peptide saturation molar ratio of 1:1. The differences in preparation might explain the observation of Lim and co-workers that some GLP-1 molecules spontaneously associate to the DSPE-PEG micelles (Lim *et al.* 2008 and 2011), whereas my results support the hypothesis of Reynolds and Tanford (Reynolds and Tanford 1970) that the peptide interacts with the tenside monomers to form micellar structures, but that the sCT is not incorporated into micelles formed solely of tenside.

5.6. CONCLUSIONS

This work was a first step in introducing a new formulation for pulmonary sCT delivery. Micelles of a 1:1 ratio of DSPE-PEG and sCT showed increased stability against catabolic enzymes, and an enhanced pharmacokinetic performance.

5.7. REFERENCES

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Chapter 6

Conclusions

Although much remains to be explored in the field of pulmonary drug delivery of peptide and protein drugs, the presented work provides novel insights into the presence of peptidases expression in the human lung and their influence on the degradation of biopharmaceuticals, herein, demonstrated using the model peptide drug, salmon calcitonin (sCT).

Currently, a lot of research efforts are focused on the modification of biopharmaceuticals in order to increase their bioavailability; also after pulmonary delivery. However, even more effective peptide modifications can be achieved, if more information was available on the fate of drugs once they have left the inhaler device. For sure, only a small fraction of the inhaled drug actually reaches the deeper lungs and can potentially be absorbed. Technologically advanced inhaler devices have been a major step forward to reassure that a greater amount of the drug reaches the deep lung and to increase patient compliance. But what use is the best inhaler device when the bulk of therapeutic protein is gone, before it reaches the blood stream and can unfold its pharmacological effect?

In the case of many peptidases it is not known, whether they are functionally expressed in the human lung, and if so, it is not well understood, what their spatial distribution is and where exactly - in the case of secreted enzymes - they unfold their action. My data showed a plethora of proteolytic enzymes being expressed in lung epithelial cells as assessed by mRNA analyses. The study is somewhat limited, however, in that mRNA expression levels were evaluated semi-quantitatively by the eye only. The aim was, nevertheless, to show if peptidases are expressed at all, and to give a trend about their expression level. In this respect, this study provides a first overview about which drug metabolising peptidases might be present in human lung epithelial cells and are the basis for further, more specific investigations. It is noteworthy that there were obvious differences in peptidases' expression between cell lines and primary cells, but also

between the different cell lines. If cell lines are used for metabolism studies, it should be thoroughly considered which cell line is suitable for such experiments.

In further investigations, I could show that peptidases that are mainly responsible for the catabolism of sCT are abundantly expressed on protein level. Intruigingly, neutrophil elastase was not found to be expressed on mRNA level, but on protein level in cell lines derived from human lung. However, mRNA expression may be reduced when a certain amount of protein is reached. To further investigate this matter, mRNA and protein expression of neutrophil elastase could be investigated and compared on several days in culture. Trypsin, which was confirmed to catabolise sCT, was not only found to be expressed on mRNA level, but also protein expression and activity in supernatant were detected. Very likely, many other peptidases for which I have shown mRNA expression are also involved in the degradation of therapeutic proteins at the epithelial barrier.

PEG-modifications of peptides and proteins provide an effective means to shield the molecules from proteolytic degradation and thus, prolong their circulation time. However, PEGylation is usually accompanied with a reduction – sometimes even the complete loss – of bioactivity. The micellar formulation with sCT was an attempt to use the shielding effect of PEG, but preserve the bioactivity, as the PEG-lipid was not covalently bound to the peptide. In the case of osteoporosis treatment, to actually prove increased bioactivity and a positive effect on the bone structure, the bone density would have to be measured. This, however, is a complicated and costly procedure which could not be realised in our laboratory. Increased sCT plasma levels and resulting reduced calcium plasma levels are often used as markers for bioactivity since the regulation of plasma calcium levels by sCT has been ascribed to the protection of the skeleton against excessive resorption (compare Chapter 1). Salmon calcitonin plasma levels after the application of different formulations, does not necessarily correspond with the respective bioactivities. However, increased sCT plasma levels are a prerequisite for the therapeutic effect of sCT on the bone. The

comparison of sCT plasma levels after application of different formulations therefore allows to draw conclusions about which formulation is more effective than another.

The fact that a protein and a PEG-lipid form such stable assemblies was surprising and interesting. For this part of my work, many experiments were performed, before I could finally put all pieces together and conclude that the self-assembled structures are micelle-like formations. Additional experiments such as cryo-TEM and P-NMR were performed to confirm this hypothesis. With their improved stability against peptidases and the enhanced pharmacokinetic properties, the PEG-lipid based micelles are a formulation approach that merits further investigations and modifications. Other chain lengths of the PEG and / or the lipid residues, branched PEG-chains or a mixture of different PEG-lipids might further improve the effectiveness of the formulation. It would also be interesting to investigate the mechanisms that are responsible for the increase in bioavailability and to find an answer to related questions e.g., do the micelles spread on the surfactant covered epithelium? Do the surrounding PEG-lipid molecules protect a sCT molecule from proteolytic degradation? What happens to the PEG-lipid in the lung? Is it absorbed or degraded or does it accumulate? Does the PEG-lipid also act as permeation enhancer? And if so, is the enhanced permeation caused because of barrier impairment?

There are still many unanswered questions in the field of pulmonary drug delivery, but with this work I aimed to answer some of them. With the knowledge of expression patterns of the relevant drug catabolising enzymes, more specific and effective peptide formulations can be developed. Besides the shielding from enzymes, the co-application of specific enzyme inhibitors or permeability enhancers can individually be applied. A potent formulation with a technologically advanced and patient friendly delivery device may be the key to successful pulmonary delivery of peptide drugs.

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II. LIST OF ABBREVIATIONS

ACE	Angiotensin I converting enzyme 1
ACE2	Angiotensin I converting enzyme 2
Ala	Alanine
Ang	Angiotensin
ANOVA	Analysis of variance
AP	Aminopeptidase
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATEE	N-acetyl-L-tyrosine ethyl ester monohydrate
AUC	Area under the curve
BAEE	N-benzoyl-L-arginine ethyl ester hydrochloride
BALF	Broncho-alveolar lavage fluid
BCA assay	Bicinchoninic acid assay
BEBM	Bronchial epithelial basal medium
BOC-Ala-NP	BOC-L-alanine p-nitrophenyl ester
Bp	Base pair
BPE	Bovine pituitary extract
BSA	Bovine serum albumine
cDNA	Complementary deoxynucleotide triphosphate
CMC	Critical micelle concentration
COPD	Chronic obstructive pulmonary disease
CP	Carboxypeptidase
Cryo-TEM	Cryo-transmission electron microscopy
Cys	Cysteine

DBH	Dihydro benzoic acid
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPP4	Dipeptidyl peptidase IV
DSPE-PEG	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ammonium salt
DSPE-PEG-COOH	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy (polyethylene glycol)-2000] ammonium salt
e.g.	*exempli gratia*, for example
ed., eds	Editor, editors
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's Minimum Essential Medium
EPO	Erythropoietin
Eq.	Equation
ESTs	Expressed-sequence tags
et al.	*et alii*, and others
etc.	*et cetera*, and other things, and so forth
FBS	Foetal bovine serum
FDA	Food and Drug Administration
G-CSF	Granulocyte colony-stimulating factor
GEO	Gene Expression Omnibus
GGH	Gamma-glutamyl hydrolase

GGT	Gamma-glutamyl transpeptidase
GHRH	Growth hormone release hormone
GIT	Gastrointestinal tract
Gln	Glutamine
GLP-1	Glucagon-like peptide-1
Glu	Glutamic acid
Gly	Glycine
hBEpC	Human bronchial epithelial primary cells
hEGF	Human epidermal growth factor
hGH	Human growth hormone
His	Histidine
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish peroxidase
i.e.	*id est*, in other words
i.m.	Intramuscular
i.v.	Intravenous
IFN	Interferon
IL	Interleukin
Ile	Isoleucine
KRB	Krebs Ringer Buffer
LDV	Laser Doppler velocimetry
Leu	Leucine
Lys	Lysine
MALDI-TOF MS	Matrix assisted laser desorption / ionisation – time of flight – mass spectrometry
mPEG	Methoxy-polyethylene glycol
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry

NCBI	National Center for Biotechnology Information
NEAA	Non-essential amino acids
NHS	N-hydroxy succinimidyl succinate
NMR	Nuclear magnetic resonance
p.	Page
PBS	Phosphate buffered saline
PdI	Polydispersity index
$Pd(N)_6$	Random hexamers 5'-phosphate
PEG	Polyethylene glycol
PEGylation	Conjugation with PEG
Ph.Eur.	*Pharmacopoea Europaea,* European Pharmacopeia
Phe	Phenylalanine
^{31}P-NMR	Phosphorous-31 NMR
Pro	Proline
PTH	parathyroid hormone
PYY	Peptide YY
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
s.c.	Subcutaneous
SARS	Severe acute respiratory syndrome
sCT	Salmon calcitonin
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography

Ser	Serine
SNP	Single-nucleotide polymorphism
sulfo-NHS	N-Hydroxysulfosuccinimide
TCD	Trinity College Dublin
TEER	Transepithelial electrical resistance
TFA	Trifluoracetic acid
THOP1	Thimet oligopeptidase I, endopeptidase-24.15
Thr	Threonine
Tri-EG	Triethylene glycol
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine
vs.	*versus*