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RESEARCH PAPER

Investigations into the Fate of Inhaled Salmon Calcitonin at the Respiratory Epithelial Barrier

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ABSTRACT

Purpose 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.

Methods Degradation of sCT was assessed by HPLC in cell homogenate, supernatant and intact monolayers of human respiratory epithelial cells (hBEpC, Calu-3, 16HBE14o-, A549) and Caco-2 as comparison at 37°C for 2 h. Breakdown of sCT by trypsin, chymotrypsin and neutrophil elastase was investigated. The presence of enzymes in cell supernatant and homogenate was studied by immunoblot and enzyme activity by model substrate assay. Transport studies across Calu-3 monolayers were performed.

Results sCT concentration remained unchanged over 2 h, when incubated in supernatant or with cell monolayers, independent of cell type studied. When cell homogenates were used, sCT concentrations were reduced to varying extents. sCT was degraded when incubated with enzymes alone. Western blot revealed abundance of all proteinases in cell homogenates and weaker expression in supernatants. Transport studies indicated net-absorptive sCT translocation; presence of bacitracin resulted in increased amount of sCT in receiver compartments.

Conclusions Epithelial proteases play a role in the disposition of sCT after pulmonary delivery.

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INTRODUCTION

Despite the failure of Exubera® (Pfizer's inhalable insulin), oral inhalation remains the most promising non-invasive route for systemic administration of macromolecules (1). The pulmonary route would, however, be even more attractive, if we knew more about the fate of aerosolised medicines after their deposition in the respiratory tract (2). 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 (3). In addition, catabolic enzymes such as the serine endopeptidases, trypsin, chymotrypsin, and various elastases that are either membrane-bound or secreted by respiratory macrophages and epithelial cells can further reduce the bioavailability of the biopharmaceutical (4,5). Whilst the lungs show higher systemic bioavailabilities for macromolecules than any other non-invasive route of delivery (6,7), small natural peptides still suffer from high enzymatic degradation, whereas peptide hydrolysis decreases or is even absent with increasing molecular weight (8).

To better understand processes involved in pulmonary macromolecule disposition on a molecular level, we recently mapped mRNA expression profiles of peptidases and proteinases in respiratory epithelial cells (5). Here, we 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 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% (1). Hence, we and others believe that the full market potential of sCT products has not yet been realised (9). 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) (10–12). Potential cleavage sites of the three enzymes in sCT are illustrated in Fig. 1 (modified from (12)). 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 (13).

In this work, 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. In addition, bi-directional permeation studies across human respiratory epithelial cell monolayers (Calu-3) were carried out to assess if active transport is involved in the process.

MATERIALS AND METHODS

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 (University of California, 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 (N-t-BOC-Ala-p-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 polyviny-

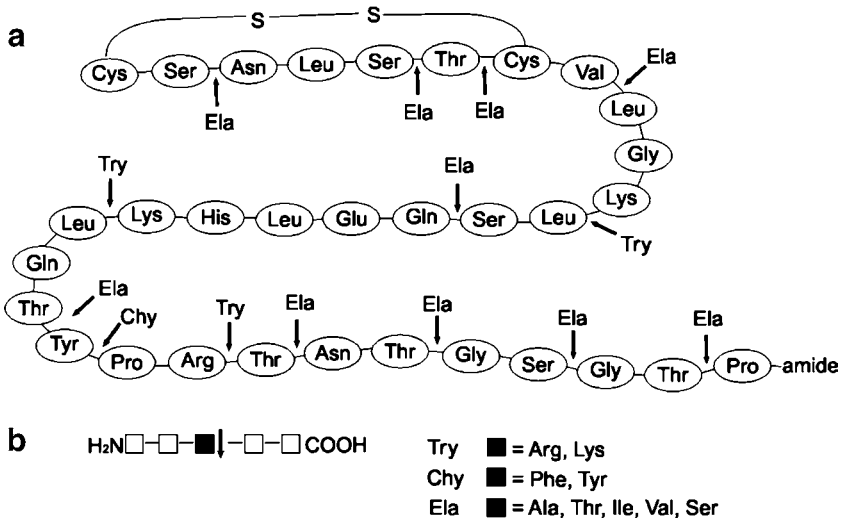

Fig. 1 Salmon calcitonin (sCT) is a substrate of trypsin (Try), α-chymotrypsin (Chy) and neutrophil elastase (Ela). (**a**) Amino acid sequence of sCT including potential cleavage sites for trypsin, chymotrypsin, and neutrophil elastase (modified from (12)). (**b**) Substrate specificity of Try, Chy and Ela (modified from (4)).

lidene fluoride membranes from Bio-Rad (Hemel Hempstead, UK). Immobilon Western Chemiluminescent HRP substrate was ordered from Millipore (Carrigtwohill, Ireland). The sCT ELISA kit was obtained from Bachem (St. Helens, UK) and 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).

Cell Culture

Freshly isolated hBEpC in primary culture was used for up to three passages, in order to prevent phenotypic drift. The cells were seeded 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 (14). 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). For transport studies, Calu-3 monolayers were cultured on permeable Transwell Clear filter inserts and used, when transepithelial electrical resistance (TEER) peaked at 1,143 ± 260 Ω·cm^2, after 12–15 days in culture.

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 (15) and have been widely utilised in studies of alveolar epithelium function (16). 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°C and 5% CO_2 atmosphere, and the medium was changed every other day until confluence was reached.

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 (10,11) and was also carried out by us 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°C. 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, immediately diluted with a similar volume of 1% TFA, and 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).

Western Blot Analysis

Abundance of the three enzymes was investigated in the cell supernatant as well as in cell homogenate. 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°C, followed by incubation 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.

Activity of Trypsin, Chymotrypsin, and Neutrophil Elastase in Cell Homogenate and 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 (17). 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°C (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 (17). 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°C (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 were dissolved in 100 ml potassium phosphate buffer (0.067 M, pH 7.0) at 70°C. The solution was cooled rapidly, and A_{237} was adjusted to 1.2 *versus* buffer. Two-hundred microlitres of enzyme (standard, supernatant, or homogenate) were added to 3.0 ml substrate solution, 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 (18) and Visser and Blout (19). For the substrate solution, 3.21 mg N-t-BOC-Ala-p-NP were dissolved in 1 ml acetonitrile and then diluted to 50 ml with sodium phosphate solution (52 mM, pH 6.5 at 37°C). The substrate solution was equilibrated to 37°C in a water bath. For the neutrophil elastase standard, 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) was 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 thermostated to 37°C, $A_{347.5}$ 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.

Stability Studies of sCT in Cell Supernatant, on Cell Monolayers, and in Cell Homogenate

The stability of sCT was investigated following incubation in supernatant (a), with cell monolayers (b) and in cell homogenates (c). Cells (hBEpC, Calu-3, 16HBE14o-, A549 and Caco-2) were cultured on 6-well plates (for a and b) or in T-75 tissue culture flasks (for c) until confluent monolayers were formed.

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 was 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 (a) 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 (b) to study interactions of the drug with membrane-bound enzymes. The homogenate was prepared according to the method used by Shah and Khan (20) 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 (c). Nine hundred and fifty 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% (*v/v*) TFA solution in order to prevent enzymatic degradation of sCT. Samples were then analysed by HPLC. Three different samples of (a), (b), and (c) were prepared of each cell type.

Transepithelial Transport Experiments of sCT

Bi-directional transport experiments were carried out across confluent monolayers of Calu-3 cells. Transwell filter-grown cell monolayers were washed with warm Ringer-HEPES buffer (150 mM NaCl, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2 \cdot 6H_2O$, 6 mM $NaHCO_3$, 5 mM HEPES, 2.8 mM glucose, pH 7.4) and equilibrated in the buffer for approximately 1 h. The buffer in the respective donor side was then replaced with a solution containing sCT at various concentrations (63–475 µg/ml), and monolayers were incubated at 37°C for 60 min. Additional experiments were performed using two concentrations (*i.e.*, 0.1 mM and 1 mM) of bacitracin (a broad-spectrum protease inhibitor (21)) in the transport buffer. Samples were collected from the acceptor compartments, and sCT content was assayed using an ELISA kit. Flux ($\mathcal{J}$ [mol/s]) was determined from steady-state appearance rates of sCT in the receiver fluid. The apparent perme-

ability coefficient, P_{app} [cm/s], was calculated according to Eq. 1:

$$P_{app} = \mathcal{J}/(A \cdot C_i) \quad (1)$$

where C_i [mol/cm^3] is the initial concentration of the drug under investigation in the donor fluid and A is the nominal surface area of cell layers (1.13 cm^2) utilised in this study.

HPLC Analytical Method

Salmon calcitonin content was analysed by means of reversed phase HPLC. An 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) were used. Twenty micro-litres of sample were analysed according to the method described by Shah and Khan (20) 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).

Statistical Data Analysis

Data were analysed by a two-way analysis of variance (ANOVA) with Bonferroni's post-hoc analysis or one-way ANOVA and *t*-test. The significance level used was $P<0.05$.

RESULTS

Degradation of sCT by Trypsin, Chymotrypsin and Neutrophil Elastase

Salmon calcitonin was rapidly degraded in the presence of 1 U of neutrophil elastase (Fig. 2). Moreover, it was confirmed that solutions of trypsin and α-chymotrypsin also break down the peptide (data not shown). The sCT concentration, calculated via the peak area of the chromatogram at 20 min, decreased with increasing incubation time, whereas the control, *i.e.*, sCT in buffer without any enzymes, was observed to be stable over 120 min (data not shown). Several peaks of sCT degradation fragments appeared in the chromatogram (Fig. 2) after 10 min incubation.

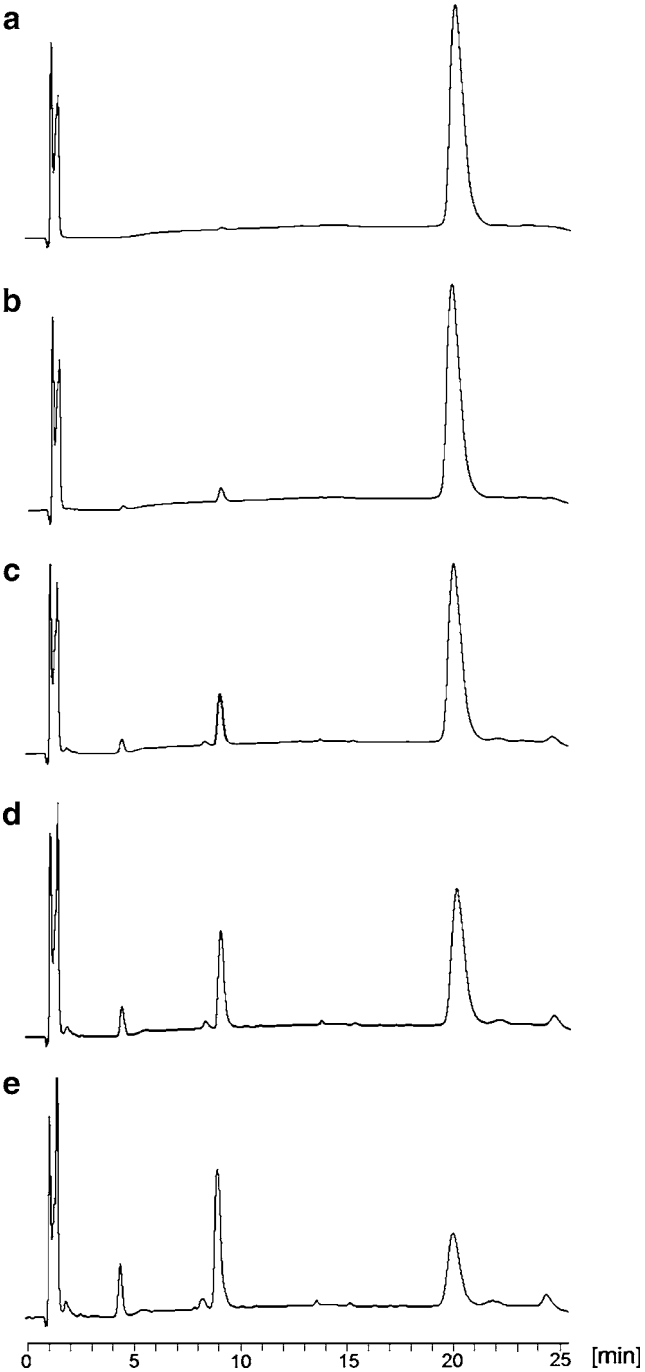

Fig. 2 Degradation of salmon calcitonin (sCT, 1 mg/ml) in the presence of neutrophil elastase (1 U/ml). Neutrophil elastase and sCT were incubated at 37°C; samples were withdrawn after 0 (**a**), 10 (**b**), 30 (**c**), 60 (**d**) and 120 (**e**) min and analysed by HPLC. Significant degradation of sCT can be observed.

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 Fig. 3a, 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 (Fig. 3b). Interestingly, whilst Calu-3 cells demonstrated strongest expression of neutrophil elastase in the cell homogenate, this was not replicated in the corresponding supernatant (Fig. 3c). In the case of 16HBE14o- and A549, levels of neutrophil elastase were similar in both their homogenate and supernatant (Fig. 3c). Caco-2 cells, which were used for comparative purposes, exhibited expression of trypsin and chymotrypsin in both cell homogenates and supernatants (Fig. 3a, b); however, neutrophil elastase was only very weakly expressed in homogenate and absent in the supernatant (Fig. 3c).

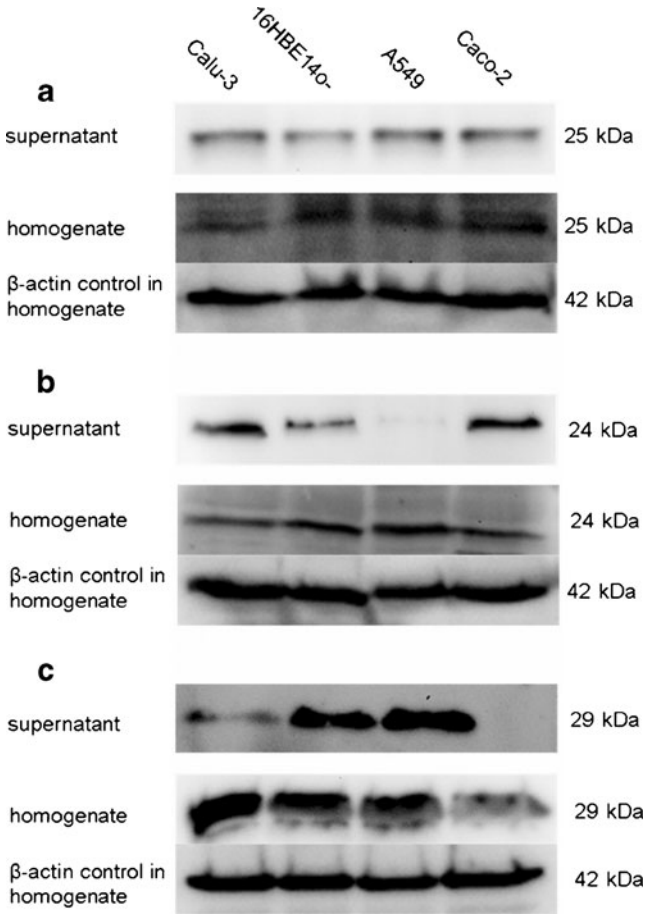

Fig. 3 Representative Western blots for trypsin (**a**), chymotrypsin (**b**), and neutrophil elastase (**c**) in homogenates and supernatants of Calu-3, 16HBE14o-, A549 and Caco-2 cell monolayers. Samples were investigated after 12 (Calu-3), 7 (16HBE14o-), 5 (A549) and 21 (Caco-2) days in culture.

Activity of Trypsin, Chymotrypsin, and Neutrophil Elastase in Cell Homogenates and 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 I). In cell supernatant, the activity of trypsin was generally low (Table I). 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 I). 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. Fig. 4 shows representative absorption curves obtained in the neutrophil elastase activity assay.

Stability Studies of sCT in Cell Supernatants, on Cell Monolayers, and in Cell Homogenate

When incubated in cell supernatants (a) or after incubation with intact cell monolayers (b), the concentration of sCT remained unchanged over a period of 120 min, independent of the cell type studied (Fig. 5, 6). After incubation with cell homogenates (c), 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 (Fig. 7).

Transepithelial Transport of sCT

Fig. 8 shows the apparent permeability (P_{app}) of bi-directional transport studies of sCT across Calu-3 monolayers at different sCT concentration in the donor compartment. A trend towards higher P_{app} values was observed with increasing initial donor concentration; this tendency was, however, not significant. At all concentrations studied, sCT P_{app} values in the absorptive (*i.e.*, apical-to-basolateral) transport direction were greater than their secretive (*i.e.*, basolateral-to-apical) counterparts. Bi-directional transport of sCT was then studied in the presence of 0.1 mM and 1 mM of the enzyme inhibitor bacitracin (Fig. 9). Bacitracin

Table I Activities of Trypsin, A-Chymotrypsin, and Neutrophil Elastase in Cell Supernatant and Neutrophil Elastase Activity in Cell Homogenate of the Four Investigated Cell Types

	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

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)

showed a concentration-dependent effect on sCT P_{app}. At the higher inhibitor concentration absorptive as well as secretive permeability were significantly increased. Intriguingly, the ratio of absorptive over secretive P_{app} decreased from 3.2 (no inhibitor) to 1.3 (0.1 mM bacitracin) and increased again to a value of 3.0 (1.0 mM bacitracin). Transepithelial electrical resistance (TEER) values of the monolayers and flux of the paracellular marker, fluorescein sodium, remained unaffected during all transport studies (data not shown).

DISCUSSION

This work addressed potential issues in pulmonary peptide delivery for systemic action, which have been highlighted on a number of occasions (2,6). Currently, there is a dearth of information regarding the fate of inhaled medication, once the drug has left the inhaler device. Enzymes of the peptidase and proteinase families are known to severely hamper non-invasive delivery of biopharmaceuticals (4,22). Our current understanding 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 (5,23,24).

In this work, 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.* have previously reported that pulmonary delivery of sCT can benefit from co-administration of serine protease inhibitors (21). However, after these initial reports (6,21,25) in the 1990s, little follow-up work on stability of protein and peptide drugs at the lung epithelial barrier has been published.

Salmon calcitonin was found to be rapidly degraded by neutrophil elastase, an elastase isoform known to be present in human lung (13,26,27). In addition, we could confirm previous data that trypsin and chymotrypsin are involved in enzymatic breakdown of sCT (10–12). Western blotting revealed abundant expression of all three peptidases in epithelial *in vitro* models of the air-blood barrier, *i.e.*, A549

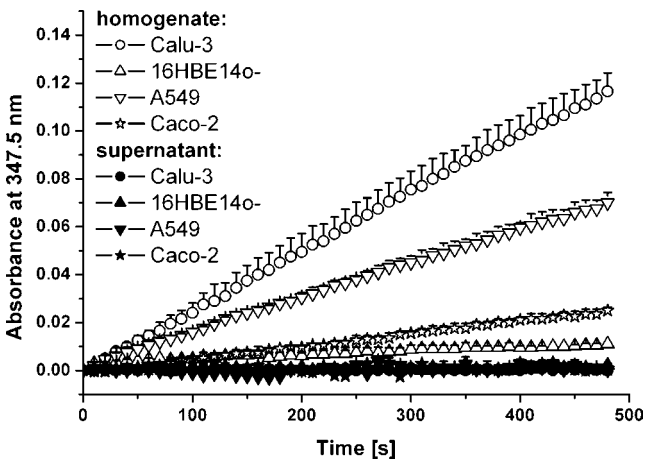

Fig. 4 Neutrophil elastase activity assay in cell homogenate (hom) and in cell supernatant (sn). An increase in absorbance, caused by the cleavage of the model substrate N-t-BOC-Ala-p-NP, was observed in homogenates of all cell types, investigated. No change in absorbance, however, was observed in any of the investigated supernatants. Data are represented as average ± SD, $n=3$.

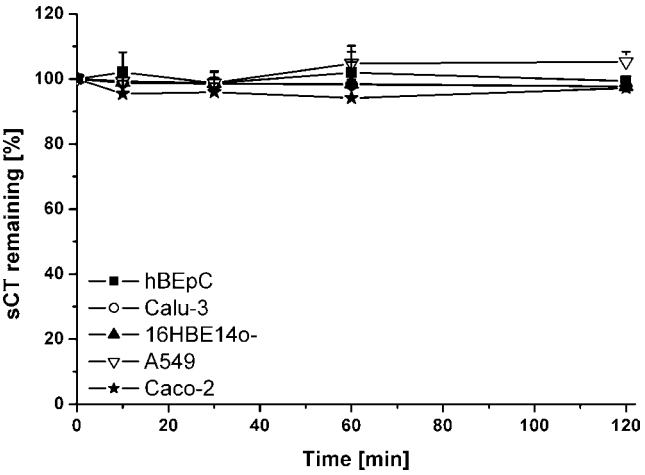

Fig. 5 Stability of salmon calcitonin (sCT) in cell-free supernatant. The peptide was stable after incubation for 120 min at 37°C. Samples were withdrawn after 0, 10, 30, 60 and 120 min and analysed by HPLC. Peak areas were used to determine the sCT concentrations, here shown as the amount of sCT remaining [%] over the time. Data are represented as means ± SD, $n=3$.

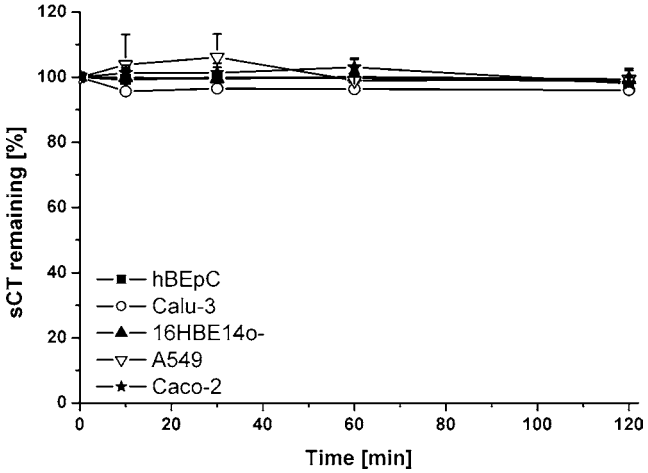

Fig. 6 Stability of salmon calcitonin (sCT) with cell monolayers. The peptide was stable after incubation for 120 min at 37°C. Samples were withdrawn after 0, 10, 30, 60, and 120 min and analysed by HPLC. Peak areas were used to determine the sCT concentrations, here shown as the amount of sCT remaining [%] over the time. Data are represented as means ± SD, $n = 3$.

(alveolar) and Calu-3 and 16HBE14o- (bronchial) cell monolayers. However, only low trypsin activity and 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 anti-protease in the supernatant (28,29). 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 enzyme activity in cell homogenates, we experienced unexpected difficulties in the case of trypsin and chymotrypsin. Often

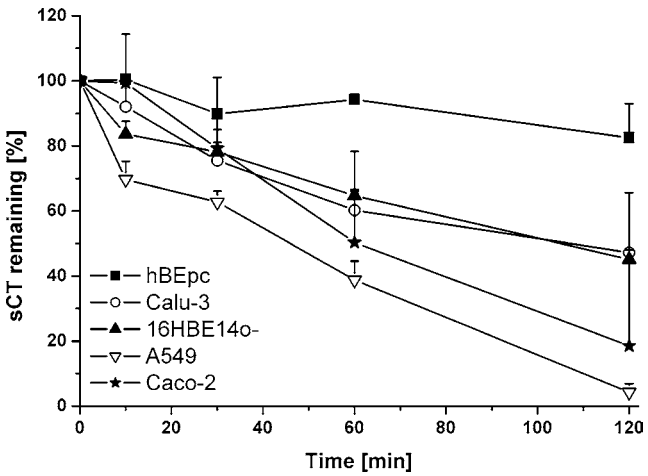

Fig. 7 Stability of salmon calcitonin (sCT) in cell homogenates. After incubation at 37°C, sCT was rapidly degraded to varying extent, depending on the cell type studied. Samples were withdrawn after 0, 10, 30, 60 and 120 min and analysed by HPLC. Peak areas were used to determine the sCT concentrations, here shown as the amount of sCT remaining [%] over the time. Data are represented as means ± SD, $n = 3$.

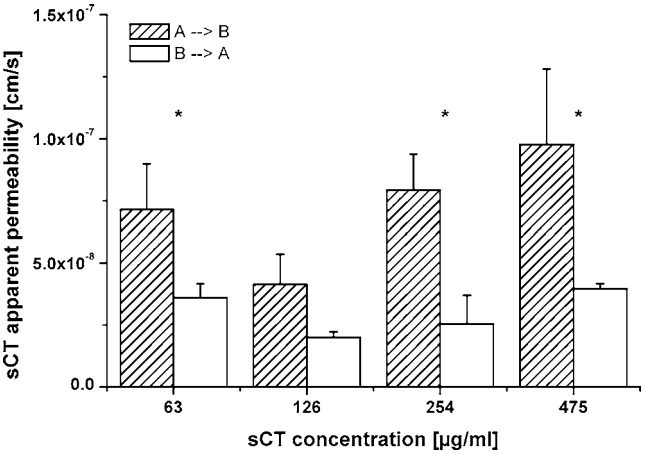

Fig. 8 Bi-directional transepithelial transport of salmon calcitonin (sCT) across Calu-3 cell monolayers at different concentrations. Data are represented as means ± SD, $n = 3$–4. * ($P < 0.05$) within each group.

proteolytic assays are not absolutely specific to one enzyme, as the substrate can be activated by virtually any enzyme that is competent to cleave the amino acid sequence used in the substrate. Due to the high abundance of enzymes in cell homogenate, the number of different reactions taking place simultaneously cannot be controlled, resulting in a rapid change in composition of the test solution and high fluctuation in absorbance.

Moreover, this plethora of enzymes released during the production of cell homogenates is not very representative of the *in vivo* situation, as many of these proteases are limited to relevant cell compartments and are not normally found in extracellular fluids. Although we attempted to study cell homogenate as an enzymatically more active comparison, we believe that activity measurements in cell supernatant are far more informative. Cell supernatant comprising only physiologically secreted enzymes is close to bronchoalveolar

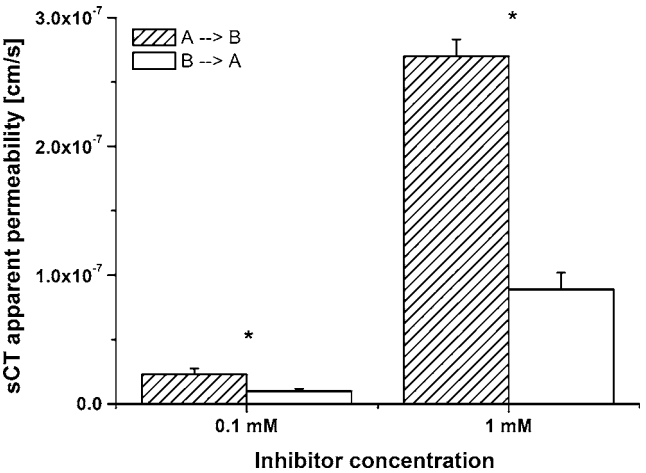

Fig. 9 Bi-directional transepithelial transport of sCT across Calu-3 cell monolayers in the presence of different concentrations (*i.e.*, 0.1 mM and 1 mM) of the peptidases inhibitor, bacitracin.

lavage fluid (BALF). Wall and Lanutti (29) reported low or undetectable activity levels of trypsin, chymotrypsin and elastase in BALF from rat and dog. Albeit studied in other species and using different substrates and experimental conditions, the results obtained are similar to our findings (29). Differences are acknowledged in the case of elastase activies, as Wall and Lunatti determined pancreatic elastase activity, whereas we studied neutrophil elastase.

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 (30,31).

It is noted that the site for systemic absorption of macromolecules is the alveolus rather than the bronchial tract. Reliable *in vitro* models of human alveolar barrier, however, are of limited availability; hence, the Calu-3 cell line was used as an organotypic approximation. Bi-directional transmonolayer permeation studies of sCT have previously been carried out using Caco-2 cells (32–34). The reported permeability coefficients (P_{app}) of 4–15·10^{-8} cm/s (32) and 1.35·10^{-7} cm/s (33) were in the same order of magnitude as observed by us for transport across the bronchial epithelial Calu-3 monolayers. Comparable P_{app} values were also reported for human calcitonin-derived peptide in a similar (*i.e.*, Calu-3 monolayer) set-up by others (35). The noted asymmetry in the bi-directional transport (*i.e.*, the higher absorptive *vs.* secretive flux) was noted and cannot be fully explained at this stage. It was initially believed to be caused by enzymatic degradation which would particularly affect the relatively lower concentration in the receiver compartment. Since the majority of peptidases are associated to the apical aspect of the cell membrane (4) or secreted into the apical fluid, basolateral-to-apical transport should be significantly more affected by this process. When the transport studies were repeated with bacitracin in the transport buffer, the level of asymmetry was indeed decreased, but only at the lower inhibitor concentration. A 10-fold increase in bacitracin concentration resulted in an increase of similar magnitude in P_{app} values in both directions. As TEER and transport of the paracellular marker fluorescein-sodium did not change, the increase in sCT transport was likely caused by enzyme inhibition rather than increased tight junctional permeability. In accordance with this hypothesis, Kobayashi *et al.* (21) found that the *in vivo* absorption of sCT was remarkably enhanced not only by co-administration of absorption enhancers, but also by protease inhibitors (*i.e.*, chymostatin, bacitracin, and potato carboxydase inhibitor).

An absolute bioavailability of 17% was measured after intratracheal instillation of sCT *in vivo* into rat lungs (36), and relative bioavailabilities of 10–18% for nebulised sCT solution compared to *s.c.* injection in healthy volunteers were reported (37). 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 effects, such as dizziness, headaches, distortion of sense of taste, nausea and vomiting, diarrhea and flush (38), and ultimately, the cost associated with the therapy. Peptidases and proteinases play a significant role in the pulmonary breakdown of sCT. In line with this, Youn *et al.* (39) found remarkably improved pharmakokinetics for PEGylated sCT derivatives intended for oral inhalation. Potential shortcomings of the delivery of the peptide can thus be overcome by protein modification. If more knowledge was available on all mechanisms involved in pulmonary inactivation of peptide drugs, structural modifications or co-administrated enzyme inhibitors could be custom tailored and further increase the advantages of aerosol therapy. Although we acknowledge limitations associated with the present work, in that we 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 (40), we regard this report 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 (16).

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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