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Developing novel hCT derived cell-penetrating peptides with improved metabolic stability

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Abstract

Many promising therapeutics are currently awaiting their clinical application. Due to their low capability of cell membrane crossing, these compounds do not reach their site of action. One way to overcome this problem might be the fusion of these agents to cell-penetrating peptides (CPP), which are able to shuttle various cargoes across cellular membranes. One disadvantage in using CPP in drug delivery is their low metabolic stability. The aim of our work was to increase the proteolytic resistance of the CPP hCT(9–32), a truncated C-terminal fragment of human calcitonin. Thus, we synthesised six modified N-terminally carboxyfluorescein labelled hCT(9–32) derivatives by replacing positions 12 and/or 16 of hCT(9–32) with either *N*-methylphenylalanine or D-phenylalanine, respectively. By using confocal laser scanning microscopy we showed that the modifications did neither affect the peptide internalisation efficiency in HeLa nor HEK 293T cells. The metabolic stability of the peptides was investigated in human blood plasma and HEK 293T cell culture supernatant. To analyse the degradation patterns, we used RP-HPLC and MALDI-TOF mass spectrometry. However, we found for all of the new derivatives high metabolic stabilities. In blood plasma, the half-lives for five of the six peptides increased compared to unmodified hCT(9–32). The degradation patterns showed a distinct stabilisation in the N-terminal part of the modified peptides, in the C-terminal part, we found some cleavage to a minor extent. Furthermore, we studied the conformation of the peptides by CD spectroscopy and demonstrated that they possess no cell toxicity. Since our metabolically more stable compounds are still able to pass the cell membrane they provide powerful tools as drug delivery vectors.

Keywords: Drug delivery; Cell-penetrating peptide; Human calcitonin; Metabolic degradation; Fragmentation pattern

1. Introduction

During the last decade the interest in efficient drug delivery systems increased. Hindered by the lipid bilayer of the cell membrane the access of most therapeutic substances to their target site is restricted. Therefore, a lot of work is done in finding suitable vectors for improving the uptake of such therapeutics. One goal is the fusion of therapeutic agents (by covalent coupling or complexation) with cell-penetrating peptides (CPP). They have the ability to pass the cell membrane and to transport various hydrophilic substances like peptides, proteins and oligonucleotides into the cytoplasma [1,2].

Recently, it was found that human calcitonin (hCT) and its truncated sequence hCT(9–32) possess cell-penetrating properties since they are able to internalise into excised bovine nasal epithelium [3,4]. In further studies, the successful delivery of certain substances by hCT derived peptides was demonstrated like the antiproliferative drug daunorubicin [5], plasmid DNA [6] or the enhanced green fluorescent protein (EGFP) [7].

One disadvantage in using CPP is their low metabolic resistance. Once in the bloodstream or in- or outside (human) cells, they are rapidly degraded. On the one hand this

Abbreviations: CD, circular dichroism; CF, carboxyfluorescein; CLSM, confocal laser scanning microscopy; CPP, cell-penetrating peptide; DIC, diisopropylcarbodiimide; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; hCT, human calcitonin; HOBt, 1-hydroxybenzotriazole; MALDI-TOF, matrix assisted laser desorption ionisation-time of flight; MS, mass spectrometry; RP-HPLC, reversed phase - high performance liquid chromatography; SPPS, solid phase peptide synthesis; TBTU, *O*-(benzotriazol-1-yl)1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TFE, trifluoroethanole; XTT, 2,3-bis(methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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metabolic instability is a limiting factor because it cannot ensure the successful transport of the cargo to its target. On the other hand, the metabolic cleavage of the CPP is one prerequisite for the release of the cargo after internalisation. Furthermore, the metabolic stability influences the clearance of CPP and their cell toxicity. Therefore, keeping in mind an efficient future application in pharmacological fields, the evaluation of its metabolism is for each carrier peptide an important factor.

Previously, the metabolic cleavage of hCT(9–32) after incubation with epithelial models was investigated. Tréhin et al. found that an initial degradation of the peptide occurred mainly in the N-terminal part [8]. Considering that the cargo is coupled N-terminally to the carrier peptide, its effective transport inside the cells could be limited due to degradation by peptidases secreted by the cell layers or located in the cell membrane. Therefore, the purpose of this study was to develop hCT(9–32) analogues with improved metabolic stability in the N-terminal part. Here we demonstrate that our modified peptides were still able to translocate across cell membranes and that they had better metabolic stabilities than hCT(9–32) itself.

2. Materials and methods

2.1. Materials

N^{α}-Fmoc-protected amino acids, 1-hydroxybenzotriazole (HOBt) and 4-(2′,4′-dimethyloxyphenyl-Fmoc-aminomethyl)phenoxy (Rink amide) resin and *O*-(benzotriazol-1-yl)1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were obtained from NovaBiochem (Bad Soden, Germany), diisopropylcarbodiimide (DIC) and XTT (2,3-bis(methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) from Sigma-Aldrich (Taufkirchen, Germany), trifluoroacetic acid (peptide synthesis grade) from Riedel-de-Haen. *O*-(7-azabenzotriazol-1-yl)-1,13,3-tetramethyluronium hexafluorophosphate (HATU), thioanisole, p-thiocresole, piperidine, ethandithiole, trifluoroacetic acid (HPLC grade), menadione (2-methyl-1,4-naphthoquinone), Trypan blue, glycine and 5(6)-carboxyfluoresceine (CF) were purchased from Fluka (Taufkirchen, Germany). *N*,*N*-dimethylformamide, dichloromethane and dimethyl ether were obtained from Biosolve (Valkenswaard, The Netherlands). Acetonitrile (ACN) was from Merck (Darmstadt, Germany). *N*-methylphenylalanine was purchased from Bachem (Weil am Rhein, Germany) and D-phenylalanine from Iris Biotech (Marktredwitz, Germany).

The following side chain protecting groups were chosen: *tert*-butyl (tBu) for Ser, Thr and Tyr; *tert*-butyloxy (tBuO) for Asp and Glu; trityl (Trt) for Asn, Gln and His; *tert*-Butyloxycarbonyl (Boc) for Lys.

For cell culturing the following media and supplements were used: Dulbecco's modified eagle medium (DMEM), RPMI 1640 (with L-glutamine), Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium, fetal calf serum (FCS), L-glutamine, nonessential amino acids and trypsine/EDTA were obtained from Gibco Life Technologies (Karlsruhe, Germany). Glucose was purchased from Serva (Heidelberg, Germany). Cell culture flasks (75 cm^2) and 96-well plates were from TPP (Trasadingen, Switzerland). Glass bottom culture dishes used for the CLSM studies were from MatTek Corporation (Ashland, USA).

2.2. Peptide synthesis

The peptides were synthesised by automated multiple solid-phase peptide synthesis (SPPS) (Syro, MultiSynTech, Bochum, Germany) and hand coupling using the Fmoc-strategy.

The D-phenylalanine modified peptides were obtained by SPPS. In order to get a peptide amide the Rink amide resin was used. The Fmoc-protected amino acids were introduced in 10-fold excess by double coupling procedures (2×36 min) by using in situ activation with DIC and HOBt. The Fmoc removal was carried out with 40% piperidine in DMF, and two times with 20% piperidine for 5 min.

The introduction of *N*-methylphenylalanine occurred manually with activation of TBTU (1 eq) and HOBt (1 eq). The following amino acid was coupled with HATU (1 eq).

The peptides were N-terminally labelled with CF while still bound to the resin with fully protected side chains. Coupling was performed using a 10-fold excess of CF, DIC and HOBt. The overall coupling time was 30 min.

The peptide amides were then cleaved with a mixture of TFA/thioanisole/thiocresole (90/5/5 v/v) within 3 h. The peptides were precipitated from cold diethyl ether, collected by centrifugation and lyophilised from water/*tert*-butyl alcohol (3:1 v/v). Purification of the peptides was achieved by preparative HPLC on RP18 column (Waters, 5 μm. 25×300 mm) by using a linear gradient of 20–60% B in A (A=0.1% TFA in water; B=0.08% TFA in ACN) over 45 min and a flow rate of 15 ml min^{-1}. Identification was performed by MALDI-TOF mass spectrometry (Voyager RP, Perseptive Biosystems) and purity was confirmed by analytical RP-HPLC on a Vydac RP18-column (4.6×250 mm; 5 μm/300 Å) using linear gradients of 10–60% B in A over 30 min and a flow rate of 0.6 ml min^{-1}. For analytical data, see Table 1.

Table 1
Amino acid sequences, molecular masses and half-lives in human blood plasma and HEK 293T cell culture supernatant of hCT(9–32) and the new synthesised derivatives

Peptides	MW [Da][a]		Half-life [h]	
Name and sequence	calc.	exp.	human blood plasma	HEK 293T cell culture supernatant
hCT(9–32)				
LGTYTQDFNKFHTFPQTAIGVGAP-NH_2	2950.4	2952.2	36.2±3.2	650±69
[f^{12}]-hCT(9–32)				
LGT**f**TQDFNKFHTFPQTAIGVGAP-NH_2	2950.4	2952.3	51.2±1.7	1637±129
[f^{16}]-hCT(9–32)				
LGTYTQD**f**NKFHTFPQTAIGVGAP-NH_2	2966.4	2969.2	51.0±4.9	333±35
[$f^{12,16}$]-hCT(9–32)				
LGT**f**TQD**f**NKFHTFPQTAIGVGAP-NH_2	2950.4	2952.5	59.6±7.4	640±47
[*N*-Me-F^{12}]-hCT(9–32)				
LGT-***N*-Me-F**-TQDFNKFHTFPQTAIGVGAP-NH_2	2964.4	2967.5	37.2±4.9	565±31
[*N*-Me-F^{16}]-hCT(9–32)				
LGTYTQD-***N*-Me-F**-NKFHTFPQTAIGVGAP-NH_2	2980.1	2982.3	53.5±8.2	2438±248
[*N*-Me-$F^{12,16}$]-hCT(9–32)				
LGT-***N*-Me-F**-TQD-***N*-Me-F**-NKFHTFPQTAIGVGAP-NH_2	2978.4	2981.6	126.2±15.9	858±247

[a] All peptides are N-terminally labelled with CF.

2.3. Cell culture

Cells were grown in 75 cm^2 culture flasks to confluency at 37 °C, 5% CO_2 in a humidified atmosphere. HEK 293T cells were grown in Dulbecco's modified eagle medium (high glucose) containing 10% heat-inactivated fetal calf serum and 1% L-glutamine. HeLa cells were cultured in supplemented RPMI 1640 with 10% heat-inactivated fetal calf serum.

Heparinised blood was taken from volunteers of our group. After centrifugation, the supernatant was cooled to −80 °C and samples were used on demand.

2.4. Metabolism studies in human blood plasma and HEK 293T cell culture supernatant

The peptides (44 μM) were dissolved in 1.05 ml human blood plasma and then incubated for 72 h by 37 °C with mechanical shaking (300 rpm). Every 12 h a sample (150 μl) was taken and mixed with 60 μl ACN/ethyl alcohol (1:1 v/v) for plasma protein precipitation. After centrifugation the solvent was filtered two times (pore unit 0.45 μm and 0.22 μm).

The metabolism studies in HEK 293T cell culture supernatant were performed in analogues way. Peptides were dissolved in HEK 293T cell culture supernatant, incubated as described above and after precipitation and centrifugation filtered one time (pore unit 0.22 μm).

Identification of the fragments was performed by analytical RP-HPLC on a Vydac RP18-column (4.6 × 250 mm; 5 μm/300 Å) using linear gradients of 10–60% B in A over 30 min and a flow rate of 0.6 ml min^{-1}. Only the N-terminally labelled fragments were detected by fluorescence measurements.

Since the concentration of the intact peptide decreased linear to the time (data not shown) the half-lives of the peptides in human blood and HEK 293T cell culture supernatant were calculated after linear regression of the data pairs.

Analysis of the peaks was achieved by MALDI-MS studies. Therefore, the peptides (220 μM) were incubated for 72 h (96 h for [*N*-Me-$F^{12,16}$]-hCT(9–32)) in human blood plasma and after incubation the samples were prepared as described above. The samples were recorded by analytical RP-HPLC, the fractions were collected, lyophilised and then analysed by MALDI-TOF-MS.

2.5. Cell viability assay

Cell toxicity of the new peptides was investigated by a XTT based colorimetric cellular viability assay ($n \geq 3$). The tetrazolium salt XTT is cleaved by the succinate-tetrazolium reductase system in the mitochondria of metabolically active cells to a soluble orange formazan dye that is measured by absorbance at 450 nm. Conversion of the tetrazolium compound is therefore a direct indicator of cell viability.

HeLa cells were grown to subconfluency in 96-well plates. Culture medium was removed and 20 and 100 μM solutions of the different CF labelled peptides (CF was also tested for reference) dissolved in OptiMEM were added to the cells. After incubation, the peptide solutions were removed, and the cells were washed three times with OptiMEM. Background was defined by the medium, and untreated cells were used for negative control, as positive control we incubated cells with 70% EtOH for 15 min.

The cells were incubated for 5 h under normal growth conditions and cell viability was subsequently measured by the XTT assay. The XTT solution was prepared in pre-warmed OptiMEM (1 mg XTT/ml), and 1.8 mg menadione was dissolved in 1 ml acetone. Prior to use, menadione solution was added to the XTT solution (10 μl/ml). From this final XTT solution, 50 μl were distributed in each well and incubated at 37 °C and 5% CO_2 for 2 h. Then absorption was measured at 450 nm (Spectrafluor plus, Tecan). To correct for smudges, fingerprints, etc., we measured at a reference wavelength of 650 nm.

2.6. CD spectroscopy

The CD spectra were recorded using a JASCO model J720 spectropolarimeter over 250–180 nm at 20 °C in a N_2 atmosphere. Peptide solutions were in a concentration range of 30–40 μM. Each measurement was repeated 5 times using a thermostable sample cell with a path of 0.02 cm and the following parameters: response time of 0.2 s, scan speed of 20 nm/min, sensitivity of 10 mdeg, step resolution of 0.2 nm, and bandwidth of 2 nm. The CD spectrum of the solvent was subtracted from the CD spectra of the peptide solutions to eliminate the interference from solvent and optical equipment. High-frequency noise was reduced by means of a low-path Fourier transform filter. The ellipticity was expressed as the mean-residue molar ellipticity $[\theta]_R$ in deg cm^2 $dmol^{-1}$.

2.7. Confocal laser scanning microscopy

To investigate the peptide uptake, HeLa and HEK 293T cells were seeded in glass bottom culture dishes, grown to subconfluency and incubated with the CF labelled peptides (50 μM) in OptiMEM for 60 and 90 min, respectively. After incubation, cells were washed two times with ice cold OptiMEM, incubated for 3 min with ice cold Trypan blue (6.5 mM in glycine buffer 1:1 v/v, pH 4.5) for quenching the external fluorescence and washed subsequently two times with ice cold PBS (with 1% glucose).

After the washing and quenching steps we inspected immediately in PBS (with 1% glucose) without fixation using a Leica TCS SP2 AOBS with a PL APO 63×/1.40 oil immersion objective and an Ar (100 mW) 488 nm laser. Untreated cells were used as negative control, cells incubated with 50 μM CF were analysed as reference.

3. Results

3.1. Synthesis

Previous investigations about the metabolic stability of hCT(9–32) revealed that the cleavage sites are located mainly in the N-terminal part before and after positions Tyr^{12} and Phe^{16} (for peptide sequence, see Table 1) [8]. Recently, we reproduced this instability at the N-terminus in degradation experiments using the following two models: human blood plasma and HEK 293T cell culture supernatant. Since we found major cleavage sites between positions Tyr^{12}–Thr^{13} and Phe^{16}–Asn^{17}, we decided to replace the amino acids in position 12 and 16 by either D-phenylalanine or *N*-methylphenylalanine, respectively.

The synthesis occurred both with solid phase peptide synthesis (SPPS) and manual coupling steps by the Fmoc-tBu strategy. All peptides were N-terminally labelled with CF. Finally, all acid labile protecting groups were removed and the peptides were cleaved from the resin by using TFA. After preparative purification by RP-HPLC and analysis by MALDI-TOF-MS, six novel hCT derivatives were obtained. An overview over all new peptides is given in Table 1.

3.2. CD spectroscopy

The conformation of the new peptides was investigated in aqueous solution (pH 7) as well as in 30% TFE solution by using CD spectroscopy. As shown in Fig. 1, all peptides adopt random coil structures in buffer. The observed minima were around 199 nm. Contrarily, the minima of [*N*-Me-F^{16}]-hCT(9–32) and [*N*-Me-$F^{12,16}$]-hCT(9–32) were red-shifted to 190 nm.

With the addition of TFE, hCT(9–32) adopts an α-helical conformation that consists of two negative bands at 205 nm and 225 nm and one positive peak at 194 nm. This is in agreement to previously reported data [9] and resembles that of full length hCT [10]. The D-phenylalanine modified peptides behave very similar to hCT(9–32) and show spectra

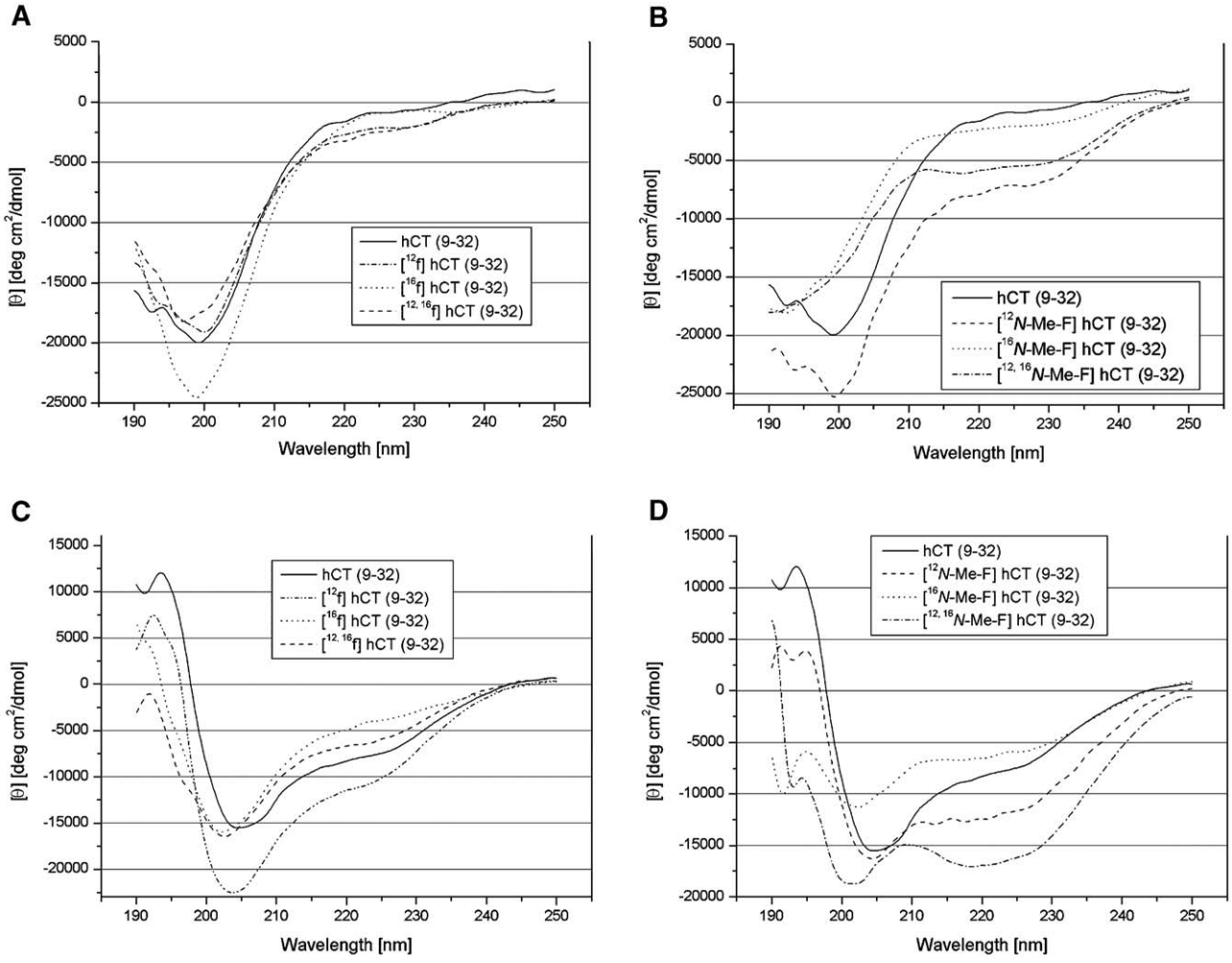

Fig. 1. CD spectra of the CF labelled hCT analogues in aqueous phosphate buffer (pH 7.4) (A, B) and aqueous phosphate buffer (pH 7.4) with 30% TFE (C, D).

indicating α-helical structures with two minima around 203 nm and a maximum around 191 nm. In contrast, the spectra of the *N*-methylphenylalanine modified peptides are more different among each other with minima around 203 nm and 218/225 nm and a maximum around 190/194 nm pointing as well to the formation of α-helices. Interestingly, both [f^{12}]-hCT(9–32) and [*N*-Me-F^{12}]-hCT(9–32) resemble in their structural behaviour hCT(9–32), whereas [f^{16}]-hCT(9–32) and [*N*-Me-F^{16}]-hCT(9–32) adopt the similar conformation to their double modified counterparts [f^{12,16}]-hCT(9–32) and [*N*-Me-F^{12,16}]-hCT(9–32).

3.3. Internalisation studies

We confirmed the effective uptake of the CF labelled peptides by confocal laser scanning microscopy studies using HEK 293T and HeLa cells. Fig. 2 illustrates the entry of the CF labelled compounds in HeLa cells after 60 min incubation time (37 °C; incubation with free CF was for control and with hCT(9–32) as reference). In fact, all variants were still able to cross the cell membrane, thus the structure modification did not affect the translocation into the cytoplasma. In the same way, the internalisation in HEK 293T cells was determined and again, the substances were found to translocate into the cytoplasma (data not shown).

3.4. Cell viability

Cytotoxicity of the compounds was checked by a colorimetric cell viability assay [11]. HeLa cells were incubated for 5 h with the modified peptides at concentrations of 20 μM and 100 μM. Fig. 3 demonstrates that the peptides showed no relevant cytotoxic effects on the cells. This is in agreement to previously reported data [12]. Incubation with CF (20 μM and 100 μM) did also not affect the cell viability. Further, no significant differences between the higher or lower concentrations were observed.

3.5. Metabolic stability of the peptides in human blood plasma and HEK 293T cell culture supernatant

The enzymatic stability of the new hCT derivatives was investigated by using two models as read-out systems: human blood plasma and HEK 293T cell culture supernatant. The feasibility of these two systems for our studies was proved since we could well reproduce the data of Tréhin et al.

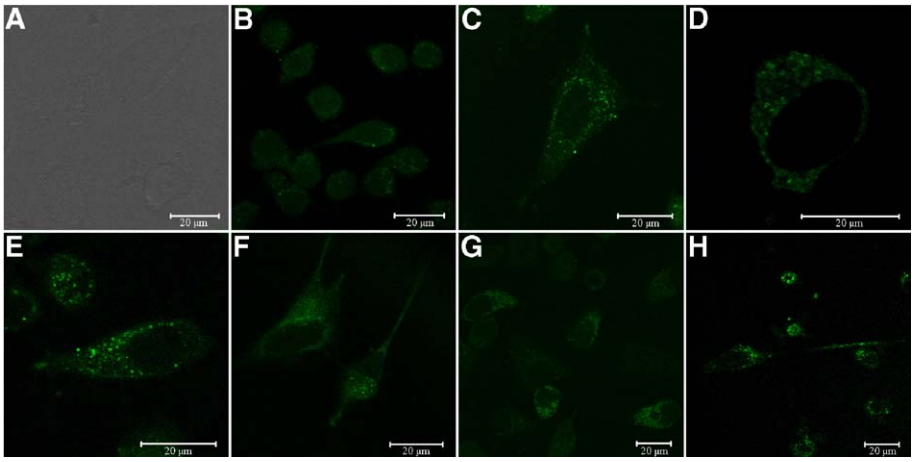

Fig. 2. Cellular uptake of the stabilised peptides in HeLa cells. Cells were incubated with the peptides and CF, respectively, at a final concentration of 50 µM at 37 °C for 60 min (C–F, and H) and 90 min (A, B, G). Washing with Trypan blue quenched remaining external CF fluorescence. Subsequently, the cells were inspected without fixation. (A) CF only, (B) CF-hCT(9–32), (C) CF-[f^{12}]-hCT(9–32), (D) CF-[f^{16}]-hCT(9–32), (E) CF-[f^{12,16}]-hCT(9–32), (F) CF-[*N*-Me-F^{12}]-hCT(9–32), (G) CF-[*N*-Me-F^{16}]-hCT(9–32), and (H) CF-[*N*-Me-F^{12,16}]-hCT(9–32). Each scale bar is 20 µm.

concerning the degradation of hCT(9–32). The CF labelled compounds were incubated in these two media and at certain time points samples were taken and analysed by RP-HPLC. The half-lives were evaluated as described in the Experimental section. In Table 1 are shown the calculated values. Generally, we found a higher metabolic stability of all new compounds compared to unmodified hCT(9–32). The highest half-life in human blood plasma of about 126 h was calculated for the *N*-methylphenylalanine modified peptide [*N*-Me-F^{12,16}]-hCT(9–32), whereas the other peptides showed about one half lower values ranging between 51 and 59 h. In contrast, incubating the peptides with HEK 293T cell culture supernatant showed only minor degradation. Nevertheless, we observed the same trend as in human blood plasma, all peptides (except [f^{16}]-hCT(9–32)) were more stable than hCT(9–32). In the case of the *N*-methylphenylalanine modified substances the most stable compound was again the one with the exchange at both positions 12 and 16 and in the case of the D-phenylalanine modified peptides it was [f^{12}]-hCT(9–32).

3.6. Degradation pattern

Additional degradation experiments should help to clarify the fragmentation pattern. For analysis of the metabolites the modified peptides were incubated with human blood plasma for 72 h (96 h for [*N*-Me-F^{12,16}]-hCT(9–32)) and fractionated by RP-HPLC. Since the peptides were labelled at the N-terminus, only the fragments possessing the CF label could be detected. Fig. 4A shows typical HPLC chromatograms of hCT(9–32) (upper panel) and [*N*-Me-F^{12,16}]-hCT(9–32) (lower panel). The collected HPLC peak fractions were further analysed by MALDI-TOF mass spectrometry (for example, see

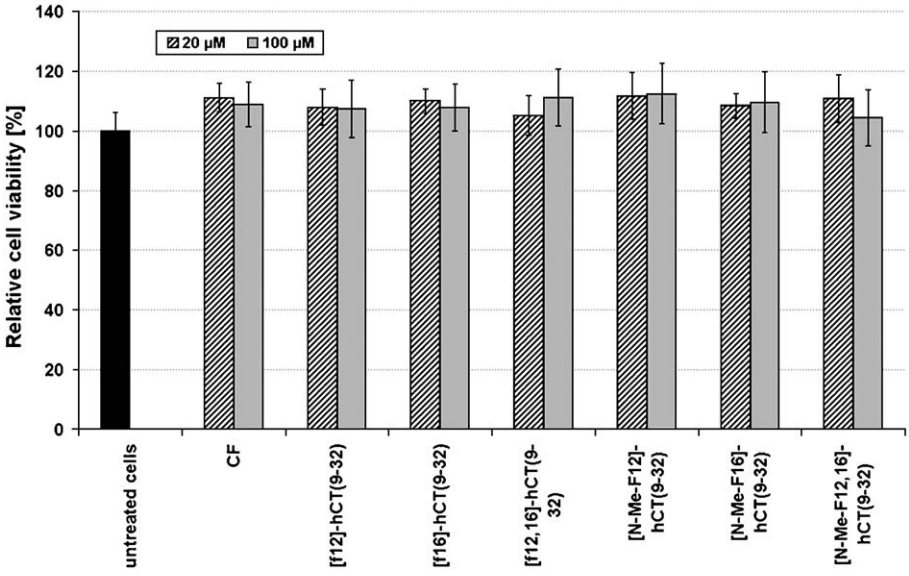

Fig. 3. Relative cell viability of HeLa cells incubated with different concentrations of the stabilised peptides at 37 °C for 5 h. The cell viability of untreated cells (black column) was set 100%. Striped columns represent a peptide concentration of 20 µM and grey columns 100 µM.

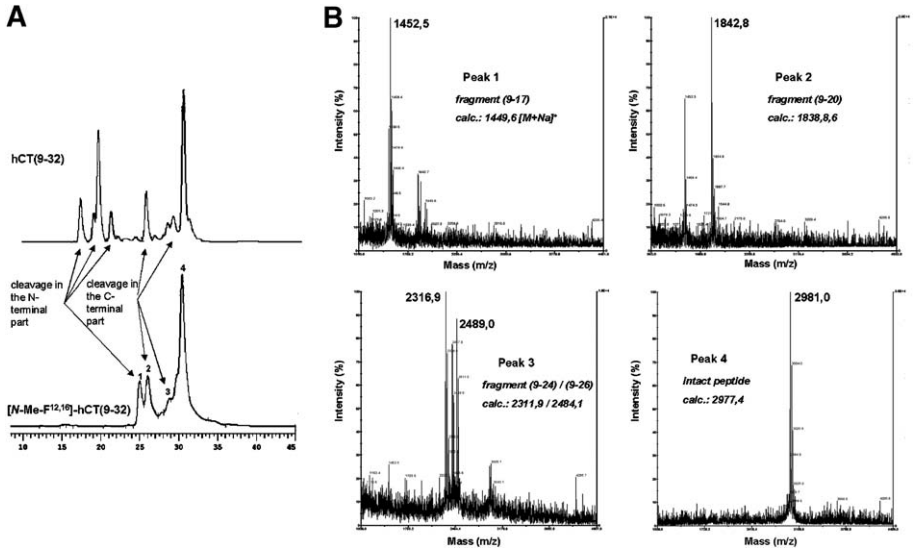

Fig. 4. (A) RP-HPLC chromatographs of CF labelled hCT(9–32) (up) and CF labelled [*N*-Me-F^{12,16}]-hCT(9–32) (bottom) after metabolic degradation in human blood plasma. Most of the N-terminal cleavage sites in hCT(9–32) were stabilised in the modified peptide. (B) MALDI-TOF mass spectra of the metabolites of CF-labelled [*N*-Me-F^{12,16}]-hCT(9–32) after degradation in human blood plasma. The peaks represent $[M+H]^+$.

Fig. 4B). The observed *m*/*z* values of the MALDI spectra corresponded in most cases to $[M+H]^+$. The suggested cleavage sites of the modified peptides are depicted in Fig. 5. As can be seen, the fragment hCT(9-17) is found for all peptides after degradation.

For the compound [f^{12,16}]-hCT(9–32), this cleavage site was also detected but the amount of the hCT(9-17) fragment was not as high as for the other peptides. Interestingly, both the D-Phe modification at position 12 and at position 16 stabilise the peptide at position 12. But [f^{12}]-hCT(9–32) differs in its degradation pattern since it was also degraded before and after Phe^{16}.

The *N*-methylphenylalanine modified peptides showed similar cleavage patterns, they all were cleaved off after position Asn^{17} and His^{20}. [*N*-Me-F^{12}]-hCT(9–32) was additionally cleaved off between positions Thr^{13}–Gln^{14}. This degradation was prevented by the amino acid replacement at position 16 with *N*-methylphenylalanine.

All peptides were degraded in the C-terminal part between Gln^{24}–Thr^{25} and Ala^{26}–Ile^{27}. Nevertheless, as illustrated in Fig. 5, the degradation in the C-terminal part occurred only to a minor extent.

Fig. 5. Scheme of suggested metabolic cleavage sites of CF labelled modified hCT analogues after incubation with human blood plasma. Different colours of the arrows indicate the amount of detected metabolites as analysed by the peak intensity of the RP-HPLC chromatographs; black > grey > light grey. X = *N*-methylphenylalanine.

4. Discussion

Since an increasing number of therapeutics like oligonucleotides, peptides and proteins are not able to penetrate biological membranes due to their hydrophilic nature and molecular size, their utility for both the "classical pharmacy" and the growing field of gene and protein therapy remains poor. One possibility to increase the bioavailability of such compounds is their fusion to suitable carrier molecules, e.g. cell-penetrating peptides, that are able to shuttle various cargoes across cellular and epithelial barriers. During the last years, a lot of research was done to identify and investigate several cell-penetrating peptides like penetratin [13], HIV-Tat protein derived peptides [14], MPG [15], transportan [16] or hCT derived peptides [17]. However, one disadvantage in using CPP is their low metabolic stability. The peptide–cargo complex should be metabolically stable until the site of action is reached, here the active cargo should be released effectively and after that an efficient clearance of the CPP is important to prevent local or systemic toxicity.

A number of studies demonstrated the potential of hCT-derived peptides as appropriate drug delivery vectors, since they were used to efficiently shuttle several cargoes into various cell types [3–7]. However, Trehin et al. investigated the metabolic stability of several C-terminal hCT fragments when in contact with three epithelial models: Calu-3, MDCK and TR146 [8]. In the presence of all three cell models, hCT(9–32) was observed to be susceptible to higher rates of

metabolic degradation and was cleaved mainly in the N-terminal site, namely around the amino acid positions Tyr^{12} and Phe^{16}.

Our aim was to improve the enzymatic resistance of hCT(9–32) in order to increase its efficiency as drug carrier. To achieve this purpose, we modified hCT(9–32) at positions Tyr^{12} and/or Phe^{16} by replacing D-phenylalanine and *N*-methylphenylalanine, respectively. Both modifications should lead to a metabolic stabilisation of the peptide. In case of an *N*-methylation, this should be due to a decrease in steric accessibility for proteolytic enzymes; using non-physiological D-amino acids, the probable incompatibility with native enzymes is taken for advantage. Indeed, it was shown that a CPP composed of D-amino acids was no longer subject to any metabolic degradation [18]. Due to the higher costs of commercially available D-tyrosine and *N*-methyltyrosine position 12 was replaced with *N*-methylated phenylalanine and D-phenylalanine, too, because of the structural similarity of tyrosine and phenylalanine, disadvantageous effects were not expected after the exchange of tyrosine to the phenylalanine derivative.

We investigated the metabolic stability of our modified hCT(9–32) derivatives in contact with human blood plasma. There exist several examples in the literature describing the use of human blood as protease source to estimate the in vivo peptide stability [18–20]. Besides, the feasibility of this system for our studies was demonstrated since we were able to reproduce the data of Tréhin et al. for hCT(9–32). Furthermore, the degradation of the new peptides was determined in HEK 293T cell culture supernatant because this cell-line is used for most of our research studies.

As was expected, the modifications at position 12 and/or 16 increased the metabolic stability in blood plasma as well as in HEK 293T cell supernatant compared with unmodified hCT(9–32) in nearly any case (see also Table 1). Analysing the metabolites after incubating with blood plasma proved that the modifications stabilise the peptides in the replaced amino acid positions. Through the modifications, there appeared a new fragment by the cleavage between positions Asn^{17}–Lys^{18} that was found in the cleavage pattern of every new peptide. In addition, we detected metabolic cleavage in the C-terminal site. Since this occurred only to a minor extent, it is probably not worth to stabilise this part of the peptides. The enormous stability of the peptides in HEK 293T cell supernatant suggests that they were almost protected from degradation. Whether this finding is due to low protein content has to be elucidated. But still, the trend was the same as in blood plasma and evidences once more the higher metabolic stability of the new peptides.

Our CD spectroscopic measurements revealed for all peptides a random coil conformation in aqueous solution and a transformation of the peptides into an α-helical structure in the presence of TFE. This is in agreement to previously reported data [9]. The tested peptides were still able to internalise into HeLa and HEK 293T cells as proven by CLSM studies. Furthermore, there were no qualitative differences between hCT(9–32) and its new derivatives concerning the cell uptake of the peptides. Obviously, the translocation efficiency of the substances was not abolished by the modifications. Moreover, the pictures displayed a punctuated intracellular distribution of the peptides. This is due to an endocytotic uptake mechanism that was already demonstrated for hCT(9–32) by previous CLSM experiments [7]. More recently, for the branched hCT derivative hCT(9–32)-br a lipid-raft mediated endocytosis was proven [21]. However, quantitative investigations of the cellular uptake are outstanding. It is likely that an improved metabolic stability could entail an increase in uptake efficiency.

In conclusion, we synthesised six new derivatives of the CPP hCT(9–32) that were more metabolically stable compared to hCT(9–32) in the two investigated systems. Further, we could show that the hCT(9–32) analogues were indeed stabilised in their N-terminal part as was suggested by our thesis. Since the capability to cross the cell membrane was not abolished and cell toxicity was not monitored these hCT-derived CPP could provide promising drug delivery vectors of a new generation.

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