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Synthesis and evaluation of novel multimeric neurotensin(8–13) analogs

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Abstract—Neurotensin(8–13) is a hexapeptide with subnanomolar affinity to the neurotensin receptor 1 which is expressed with high incidence in several human tumor entities. Thus, radiolabeled neurotensin(8–13) might be used for tumor targeting. However, its application is limited by insufficient metabolic stability. The present study aims at improving metabolic stability by the synthesis of multimeric neurotensin(8–13) derivatives rather than commonly employed chemical modifications of the peptide itself. Thus, different dimeric and tetrameric peptides carrying C- or N-terminal attached neurotensin(8–13) moieties have been synthesized and their binding affinity toward the neurotensin receptor has been determined. The results demonstrate that branched compounds containing neurotensin(8–13) attached via its C-terminus only show low receptor affinities, whilst derivatives with neurotensin(8–13) attached via the N-terminus show IC_{50} values in the nanomolar range. Moreover, within the multimeric neurotensin(8–13) derivatives with neurotensin(8–13) attached via the N-terminus an increasing number of branching units lead to higher binding affinities toward the neurotensin receptor.

1. Introduction

Neurotensin (NT) is a 13 amino acid containing neuropeptide expressed in both the central nervous system and the periphery such as the gastrointestinal tract and the cardiovascular system.[1] NT was first isolated from calf hypothalamus.[2] The physiological and biochemical actions of NT are mediated through binding to NT receptors (NTRs). Up to now, three subtypes of neurotensin receptors have been cloned. Two of them belong to the family of G protein-coupled receptors.[3] All three receptors recognize the same C-terminal 8–13 hexapeptide sequence Arg(8)-Arg(9)-Pro(10)-Try(11)-Ile(12)-Leu(13).[4]

Besides their numerous central and peripheral functions it was reported that NTRs are overexpressed in various human tumors. For example, overexpression of NTR1 was found in primary human tumors such as meningiomas, Ewing's sarcomas, and, most important, in more than 75% of ductal pancreatic carcinomas.[5] Thus, in the last decade many efforts dealt with radiolabeled NT analogs to target NTR1-bearing tumors.[6–8] However, successful in vivo application of radiolabeled NT analogs has been hampered due to the fast peptide degradation by plasma and tissue peptidases.

The rapid proteolytic degradation process might be prevented or reduced by direct molecular modifications within the peptide sequence. For NT several approaches have been envisaged. On one hand, there have been efforts to synthesize stable NT mimetics.[9] On the other hand, the preparation of stable peptide-based NT derivatives has been aimed for. Thus, a cyclic peptide analog, the introduction of reduced peptide bonds and unusual amino acids were investigated.[7,10–15] However, although promising results were obtained in animal studies, none of the attempts to design NT-based peptide radioligands by using stabilized NT analogs for the imaging of NTR1-expressing tumors in vivo gave satisfactory results in humans.[16] Therefore, synthesis of stable radiolabeled NT derivatives with good receptor binding properties and sufficient tumor uptake is still a substantial challenge.

Thus, alternative possibilities for the stabilization of peptides have become an important issue. One possibility might be the formation of these peptides in the form of multiple antigenic peptides (MAPs). MAPs consist of

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a peptide core enabling branching of certain numbers of linear peptides. Very frequently a lysine core is used because lysine has two amino groups. These amino groups double by every branching generation.[17] Furthermore, cores consisting of lysine (β-alanine) have also been described.[18] The functionalization of these branched peptides can be achieved by the attachment of bioactive molecules to the last generation of the core matrix. The resulting dendrimers exhibit two special properties: a globular structure and polyvalency.[19] These multimeric peptides often have a higher in vivo efficacy compared to the monomeric peptides. Thus, MAPs are applied as diagnostics, inhibitors, and as vaccines against parasites, bacteria, viruses, etc.[18]

These interesting properties led to investigations concerning the biological stability of MAPs.[20] A tetrameric NT(8–13) derivative was synthesized and its binding affinity and in vitro stability was compared to that of unmodified NT(8–13). At this, the tetrameric NT(8–13) derivative showed better binding properties to the NTR than monomeric NT(8–13). The stability against proteases was investigated by incubations in human serum. An increased stability of the MAPs could be observed since after an incubation period of 24 h intact MAP could still be found, whereas the monomeric form had been decomposed completely under similar conditions.[20] The unnatural branched structure of the dendritic peptides along with a significantly increased molecular weight seems to have a beneficial effect on the stability.

The applicability of MAP structures for nuclear medical purposes has already been demonstrated.[21–23] At this, RGD peptides were synthesized in the form of multivalent ligand systems and were labeled with copper-64. It was observed that the dimeric and tetrameric RGD derivatives exhibited a clearly higher binding affinity toward the corresponding receptor than the monomeric peptides. Moreover, the tetrameric peptide showed high tumor uptake and prolonged tumor retention in vivo.[22] Consequently, multivalent peptides seem to represent a promising approach to obtain stable, peptide-based radiotracers.

Therefore, in the present study a series of NT(8–13) derivatives with a multivalent, branched structure were synthesized. It was examined whether it is advantageous to attach the N-termini or the C-termini of the NT(8–13) moieties to the core matrix. In the latter case, a lysine core as described above was used.[17] To attach the N-termini of the NT(8–13) moieties to the core matrix, units consisting of glutamic acid were synthesized. The NT(8–13) moieties were bound to the α-carboxylic group of the C-terminal glutamyl residue and to the γ-carboxylic groups of all glutamyl residues. Furthermore, the influence of a spacer between core matrix and NT(8–13) moieties was examined. The evaluation of the synthesized compounds was carried out on the basis of their in vitro binding affinity for the NTR.

2. Results

Different types of NT(8–13) monomers and multimers were synthesized. The synthesized compounds consist of a core unit and a specific number of unmodified NT(8–13) moieties. Spacers were optionally placed between the core and the NT(8–13) residues. A core unit containing lysine was used to obtain peptides where NT(8–13) is attached via the C-terminus. A glutamic acid core unit was used to couple the N-terminus of NT(8–13) to give dendritic peptides with free C-termini of the NT(8–13) residues.

First, peptides containing a lysine core unit were synthesized. The compounds were obtained using the solid-phase peptide synthesis. Syntheses were performed employing the Fmoc protection strategy. Amino acids were coupled stepwise using HBTU and HOBt as coupling reagents and DIPEA as the base. After cleavage from the resin, HPLC purification was carried out. The obtained peptides showed purities greater than 95%. Dimers were synthesized by introducing Fmoc-Lys(Fmoc)-OH for branching. By insertion of two additional lysine residues, one at each amino group of the previously attached lysine, a tetramer can be prepared. The general synthesis strategy is outlined in Figure 1.

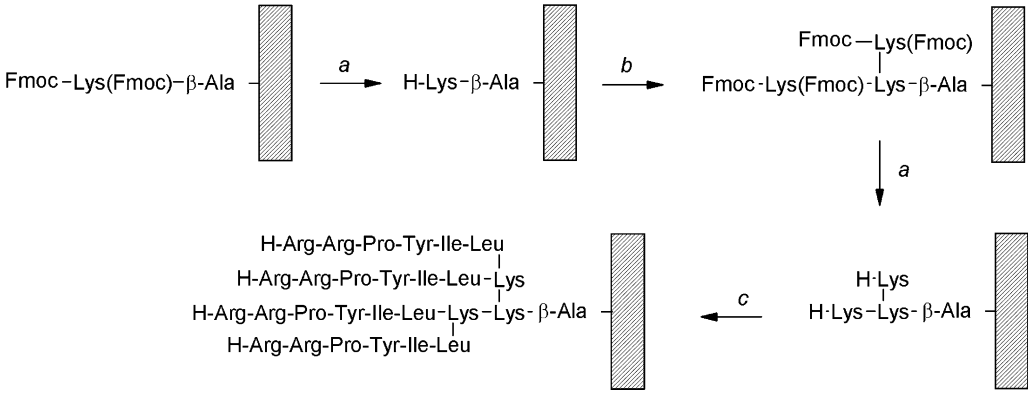

Figure 1. Synthesis of a NT(8–13) tetramer. NT(8–13) residues are attached via the C-terminus. (a) 20% piperidine in DMF, (b) Fmoc-Lys(Fmoc)-OH, HBTU, HOBt, DIPEA, DMF (c) indicates 6 synthesis cycles: 1—Fmoc amino acids for the NT(8–13) sequence, HBTU, HOBt, DIPEA, DMF; 2—20% piperidine in DMF.

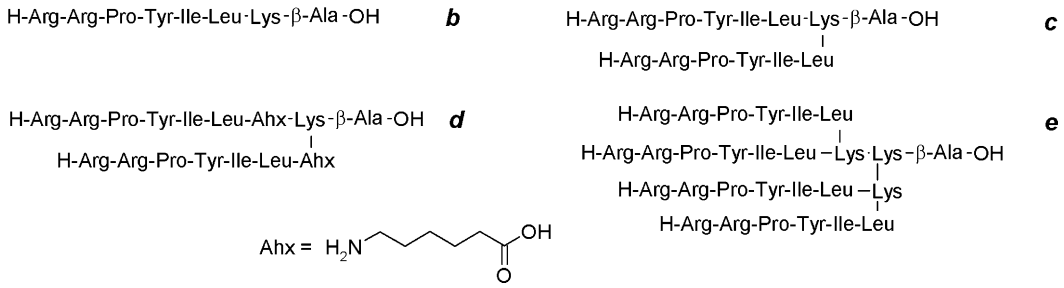

Figure 2. Peptides containing NT(8–13) attached via the C-terminus. Monomeric NT(8–13) derivative **b**, dimeric NT(8–13) derivative **c**, dimeric NT(8–13) derivative **d**, tetrameric NT(8–13) derivative **e**.

Accordingly, the dimer **c** and the tetramer **e** as shown in Figure 2 were synthesized. Another dimer **d** (Fig. 2) bearing a 6-aminohexanoic acid (Ahx) spacer was synthesized. Furthermore, monomer **b** (Fig. 2) was obtained by using Fmoc-Lys(Boc)-OH instead of Fmoc-Lys (Fmoc)-OH to prevent branching. Monomer **b** was prepared to study the influence of a C-terminal substitution of the NT(8–13) moiety and to clarify if there are effects caused by polyvalency.

Receptor binding of NT(8–13) and peptides **b**, **c**, **d**, and **e** was investigated using the human colon adenocarcinoma cell line HT-29. HT-29 cells are reported to express NTR1 on their membranes.[1] They have been used as test system to evaluate a multitude of NT(8–13) derivatives.[7,12,13] The peptides inhibited the binding of [^{3}H]NT, showing a typical sigmoid curve. The determined IC_{50} values are presented in Table 1.

Table 1. Binding properties of branched peptides containing NT(8–13) attached via the C-terminus in HT-29 cells

Peptide	IC_{50} ± SD
NT(8–13)	0.4 nM ± 0.2 nM
Monomer **b**	190 nM ± 3 nM
Dimer without spacer **c**	69 nM ± 19 nM
Dimer with Ahx-spacer **d**	56 nM ± 8 nM
Tetramer **e**	540 nM ± 103 nM

Data are from two experiments in duplicate.

All peptides exhibit IC_{50} values of at least one magnitude higher than unmodified NT(8–13), which is indicative of a drastically reduced binding potential. Dimeric peptides **c** and **d** actually showed lower IC_{50} values than monomeric compound **b**, whereas tetrameric peptide **e** has a substantially higher IC_{50} value compared to the monomeric and the dimeric peptides **b**, **c**, and **d**.

Therefore, another approach using glutamic acid (Glu) as core matrix was explored. Here, the attachment of the NT(8–13) moieties to the core unit was achieved via their N-termini. For the synthesis of dimer **f**, Boc-Glu-OH was employed. It was coupled twice to the resin-bound NT(8–13) residues. Each time 0.25 equiv Boc-Glu-OH was used. The synthesis of peptide **f** is shown in Figure 3.

Furthermore, tetrameric peptide **g** was synthesized using a similar procedure. As the core matrix the Fmoc protected tripeptide Fmoc-Glu-Glu-Glu-OH **a** was used. It was coupled twice to resin-bound NT(8–13). Each time 0.125 equiv Fmoc-Glu-Glu-Glu-OH was used. The general synthesis strategy for tetramer **g** is given in Figure 4.

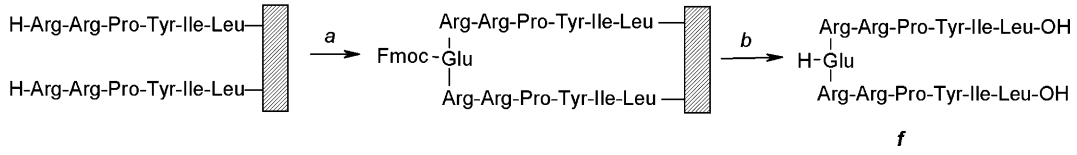

Figure 3. Synthesis of a NT(8–13) dimer **f**. NT(8–13) residues are attached via the N-terminus. (a) Fmoc-Glu-OH, HBTU, HOBt, DIPEA, DMF (b) 1—20% piperidine in DMF; 2—trifluoroacetic acid/dithiothreitol/water/triisopropylsilan 88:5:5:2.

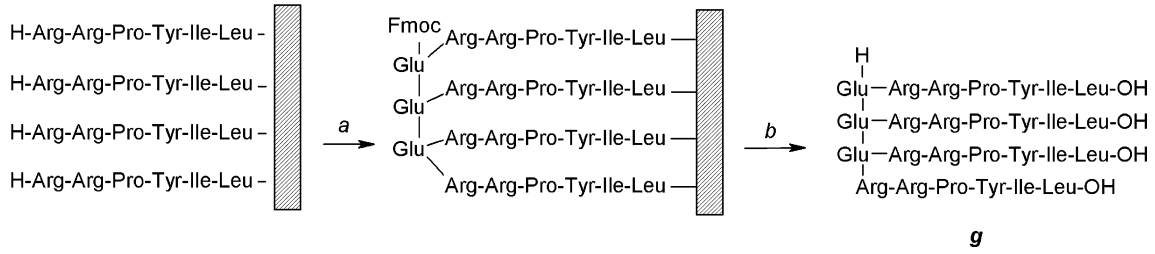

Figure 4. Synthesis of a NT(8–13) tetramer **g**. NT(8–13) residues are attached via the N-terminus. (a) Fmoc-Glu-Glu-Glu-OH, HBTU, HOBt, DIPEA, DMF; (b) 1—20% piperidine in DMF; 2—trifluoroacetic acid/dithiothreitol/water/triisopropylsilan 88:5:5:2.

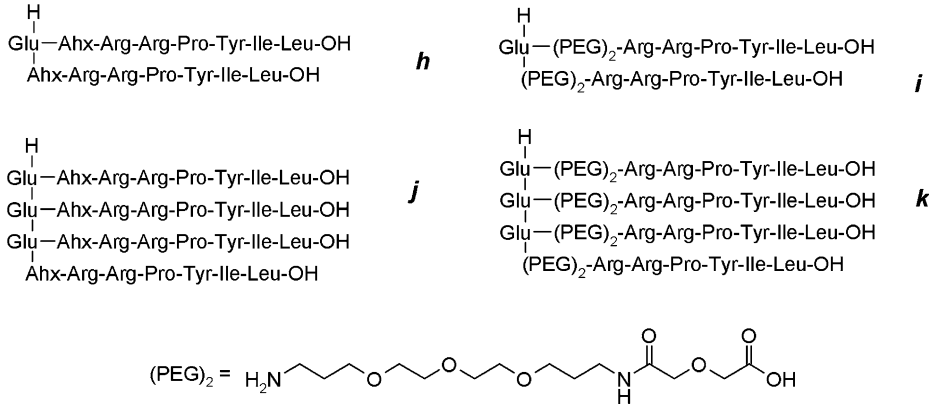

Figure 5. Peptides containing NT(8–13) attached via the N-terminus. Dimeric NT(8–13) derivative **h**, dimeric NT(8–13) derivative **i**, tetrameric NT(8–13) derivative **j**, tetrameric NT(8–13) derivative **k**.

In addition to dimer **f** and tetramer **g**, peptides containing an Ahx or PEG spacer were synthesized. The synthesized dimeric and tetrameric peptides (**h**, **i**, **j**, and **k**) are depicted in Figure 5.

The peptides **f**, **g**, **h**, **i**, **j**, and **k** were also tested with regard to their affinity to the NTR. The resulting IC_{50} values are summarized in Table 2.

The peptides **f**, **g**, **h**, **i**, **j**, and **k** bearing a glutamic acid core unit exhibit significantly lower IC_{50} values and hence better binding properties than the peptides **b**, **c**, **d**, and **e** with a lysine core. Nevertheless, the IC_{50} values of the dimeric peptides **f**, **h**, and **i** are still higher than the IC_{50} value of unmodified NT(8–13) although the C-terminus, which is thought to be essential for sufficient recognition and binding to the NTR, is free. The IC_{50} values of the tetrameric peptides **g**, **j**, and **k** are clearly lower than the IC_{50} values of peptide dimers **f**, **h**, and **i**. The IC_{50} value of the tetramer **g** is reduced to 2.3 nM, which is about 40% of that of dimer **f** (5.2 nM). However, the IC_{50} value of tetramer **g** is also higher than that of unmodified NT(8–13). For the dimeric compounds the insertion of a spacer between NT(8–13) residues and core unit leads to a decline in receptor binding, independent of the type of spacer. In contrast to these findings, at the tetramers the presence of a spacer between branching unit and NT(8–13) moieties does not influence the binding to the receptor or even improves it. Insertion of an Ahx spacer does not significantly change the IC_{50} value as observed for peptide **g** (2.3 nM) and peptide **j** (2.5 nM). However, the use of a PEG spacer leads to a remarkable reduction of the IC_{50} value. Thus, tetrameric peptide **k** containing the PEG spacer showed an IC_{50} value of 0.4 nM which is comparable to that of unmodified NT(8–13) (0.4 nM).

Table 2. Binding properties of branched peptides containing NT(8–13) attached via the N-terminus in HT-29 cells

Peptide	IC_{50} ± SD
NT(8–13)	0.4 nM ± 0.2 nM
Dimer without spacer **f**	5.2 nM ± 1.9 nM
Dimer with Ahx-spacer **h**	40 nM ± 17 nM
Dimer with $(PEG)_2$-spacer **i**	21 nM ± 7 nM
Tetramer without spacer **g**	2.3 nM ± 0.8 nM
Tetramer with Ahx-spacer **j**	2.5 nM ± 1.4 nM
Tetramer with $(PEG)_2$-spacer **k**	0.4 nM ± 0.2 nM

Data are from two experiments in duplicate.

3. Discussion

The present study indicates that multivalent NT(8–13) derivatives might be appropriate chemical leads for radiolabeling and subsequent tumor targeting. However, it is important how the attachment of the NT(8–13) moieties to the core unit is realized. The peptides containing NT(8–13) attached via the C-terminus only showed poor receptor binding. The introduction of a spacer between lysine core and NT(8–13) residues did not result in a significant improvement of the receptor binding. Thus, the insufficient binding affinity of the peptides **b**, **c**, **d**, and **e** might result from the blockage of the C-terminus, because during binding of NT(8–13) to the NTR a strong ionic interaction between the C-terminal carboxylic group of NT(8–13) and an arginine residue of the receptor should occur.[24] Moreover, steric bulk also might lead to significantly reduced binding as found for the tetramer **e**. Thus, these NT(8–13) derivatives should not be appropriate structures for the preparation of specific binding radiotracers. These findings are quite contrary to previously published results where analog tetrameric NT derivatives branched via the C-terminus showed better receptor binding than native NT(8–13).[20] However, no clear structure or formula of the used peptides can be found in this publication. Thus, a profound and comparative discussion of potential discrepancies is not possible.

Improved NTR binding properties were obtained for NT(8–13) multimers with NT(8–13) attached via the N-terminus. Here, a polyvalency effect can be found, because the dimeric compound **f** shows an approximately two times higher IC_{50} value compared to tetrameric compound **g**. The introduction of a spacer between core and NT(8–13) moieties resulted in different binding properties for the dimers or tetramers bearing

NT(8–13) attached via the N-terminus, respectively. In contrast to the dimeric peptides, the presence of a spacer does not decline the binding properties of the tetrameric compounds. While the introduction of an Ahx spacer did not influence the IC_{50} value, a PEG spacer led to an improvement of the receptor affinity. The IC_{50} value of the tetramer carrying a PEG spacer **k** was about six times lower than that of the tetramer **g**. This reduction in IC_{50} value might be due to the length or the polarity of the spacer.

In conclusion, we have synthesized different multivalent NT(8–13) derivatives. Attachment of NT(8–13) to the core unit was carried out via the C- or N-termini of the NT(8–13) residues. The peptides containing NT(8–13) attached via the C-terminus exhibited poor binding affinity to the NTR. Therefore, they should not be applicable for tumor targeting. However, the branched derivatives containing NT(8–13) attached via the N-terminus showed binding affinities in the low nanomolar or even subnanomolar range as found with unmodified NT(8–13). Furthermore, duplication of the number of NT(8–13) residues attached to the core results in a bisection of the IC_{50} value, which can be explained by a beneficial polyvalency effect. By introduction of a PEG spacer between core matrix and NT(8–13) moieties a peptide exhibiting comparable receptor affinity than unmodified NT(8–13) was obtained.

Thus, multivalent peptides with NT(8–13) attached via the N-terminus could be appropriate peptide ligands for tumor targeting. The metabolic stability of these compounds still needs to be investigated. For this purpose the peptides should be labeled with the short-lived positron emitter fluorine-18. Fluorine-18 has a half-life of 109.8 min. Radiopharmacological studies of the corresponding ^{18}F-labeled compounds in vivo by means of positron emission tomography (PET) will provide information on biodistribution and catabolism to confirm usefulness of multivalent NT(8–13) derivatives as promising candidates for tumor imaging. Radiolabeling of the peptides can be achieved via acylation with *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB). [^{18}F]SFB was shown to be suitable for radiolabeling of peptides at their primary amino groups.[7,25] Since the here presented NT(8–13) derivatives only exhibit one amino group in each case, adequate [^{18}F]fluorobenzoylated peptides can be prepared. Work on the radiolabeling with fluorine-18 and radiopharmacological investigation is currently in progress.

4. Experimental

4.1. General

Peptide syntheses were performed on an automated multiple peptide synthesizer (Syro I, MultiSyn Tech, Germany). All chemicals were of reagent grade and used without further purification. Fmoc amino acid derivatives used in the peptide synthesis were purchased from Orpegen Pharma, Germany. The reactive side chains of the Fmoc amino acids were protected as follows: Lys, fluorenylmethyloxycarbonyl (Fmoc) or *tert*-butyloxycarbonyl (Boc); Arg, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf); Tyr and Glu, O-*tert*-butyl (O-*t*-Bu). The Fmoc protected spacers 6-aminohexanoic acid and NH_2-$(PEG)_2$-COOH were purchased from Calbiochem-Novabiochem, Germany. Resins and Boc-Glu-OH were purchased from Bachem, Switzerland. *N*,*N*-Diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), *N*,*N*-dimethylformamide (DMF), piperidine and *N*-methyl-2-pyrrolidone (NMP) were purchased from Biosolve Ltd, Netherlands. HBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate), HOBt (*N*-hydroxybenzotriazole), and dithiothreitol were purchased from Calbiochem-Novabiochem, Germany. Triisopropylsilan, dimethylsulfoxide (DMSO), and acetonitrile were purchased from Merck, Germany. [^{3}H]NT ([3,11-tyrosyl-3,5-^{3}H(*N*)]-neurotensin) with a specific activity of 3.7 GBq/μmol was purchased from Perkin-Elmer, USA.

Mass spectra were recorded on a Bruker autoflex II TOF/TOF mass spectrometer. Purification of the peptides was accomplished by semi-preparative C-18 reversed phase chromatography on a HP 1050 system with a Zorbax 300 SB-C18 column (9.4 × 250 mm, 5 μm). CH_3CN in water containing 0.1% TFA as indicated was used as the eluent at a flow rate of 2 mL/min and UV detection at 214 nm. Analytical HPLC was performed using a HP 1100 system equipped with a Zorbax 300 SB-C18 column (9.4 × 250 mm, 5 μm) using gradient elution at a flow rate of 2 mL/min and UV detection at 214 nm. Solvent A: 0.05% of TFA in water; solvent B: 0.04% of TFA in CH_3CN. The following gradient was used: 0 min 10% B, 10 min 20% B, and 15 min 80% B.

4.2. Peptide synthesis

4.2.1. General procedure. The peptides were prepared on alkoxybenzyl alcohol resins with an initial load of 0.6 mmol/g in the case of Fmoc-β-Ala and Fmoc-Glu(O-*t*-Bu), and 0.7 mmol/g in the case of Fmoc-Leu. Syntheses were performed on a 30–60 μmol scale. The Fmoc protecting group was removed with 20% piperidine in DMF for 15 min. The carboxyl group of the incoming amino acid was activated with HBTU and HOBt. Fmoc-amino acid (4 equiv), HBTU (3.6 equiv), HOBt (4 equiv), and DIPEA (7.2 equiv) were dissolved in DMF or NMP, respectively, and added to the resin. The coupling time was 1 h. Coupling was carried out twice for each residue. The peptides were deprotected and cleaved from the solid support with trifluoroacetic acid/dithiothreitol/water/triisopropylsilan 88:5:5:2 for 3 h. The resin was filtered off and the crude peptide was precipitated by adding cold diethyl ether and washed with diethyl ether. The residual ether was removed by evaporation and the peptides were lyophilized and purified.

4.2.2. Fmoc-Glu-Glu-Glu-OH a. The tripeptide **a** was prepared according to the general procedures using the resin preloaded with Fmoc-Glu(O-*t*-Bu). Fmoc cleavage was not accomplished after the coupling of the third amino acid. The product **a** was obtained as a white

powder (28 mg, 90%) and was used without further purification. M_W $C_{30}H_{33}N_3O_{12}$ calculated 627.61, found MALDI-TOF 650.4 $[M+Na]^+$; 666.4 $[M+K]^+$.

4.2.3. Monomeric compound b. The monomeric compound **b** was prepared according to the general procedures using the resin preloaded with Fmoc-β-Ala. As the next amino acid Fmoc-Lys(Boc)-OH was attached. Subsequently, the amino acids for the NT(8–13) sequence were attached stepwise. Compound **b** was obtained as a white powder (14 mg, 20%) after purification by semi-preparative HPLC (20% to 30% CH_3CN within 30 min, t_R = 9.8 min). M_W $C_{47}H_{81}N_{15}O_{10}$ calculated 1016.26, found MALDI-TOF 1017.6 $[M+H]^+$.

4.2.4. Dimeric compound c. The dimeric compound **c** was prepared according to the general procedures using the resin preloaded with Fmoc-β-Ala. As the next amino acid Fmoc-Lys(Fmoc)-OH was attached. Subsequently, the amino acids for the NT(8–13) sequence were attached stepwise. Compound **c** was obtained as a white powder (4 mg, 10%) after purification by semi-preparative HPLC (20–30% CH_3CN within 30 min, t_R = 21.3 min). M_W $C_{85}H_{143}N_{27}O_{17}$ calculated 1815.26, found MALDI-TOF 1816.1 $[M+H]^+$; 908.5 $[(M+2H)/2]^{2+}$.

4.2.5. Dimeric compound d. The dimeric compound **d** was prepared according to the general procedures using the resin preloaded with Fmoc-β-Ala. As the next amino acid Fmoc-Lys(Fmoc)-OH was attached. Subsequently, Fmoc-Ahx-OH and the amino acids for the NT(8–13) sequence were attached stepwise. Compound **c** was obtained as a white powder (11 mg, 9%) after purification by semi-preparative HPLC (20% to 30% CH_3CN within 30 min, t_R = 26.6 min). M_W $C_{97}H_{165}N_{29}O_{19}$ calculated 2041.58, found MALDI-TOF 2042.3 $[M+H]^+$; 1021.6 $[(M+2H)/2]^{2+}$.

4.2.6. Tetrameric compound e. The tetrameric compound **e** was prepared according to the general procedures using the resin preloaded with Fmoc-β-Ala. Fmoc-Lys(Fmoc)-OH was attached twice. Subsequently, the amino acids for the NT(8–13) sequence were attached stepwise. Compound **c** was obtained as a white powder (5.6 mg, 5%) after purification by semi-preparative HPLC (22–30% CH_3CN within 30 min, t_R = 24.6 min). M_W $C_{173}H_{291}N_{55}O_{33}$ calculated 3669.60, found MALDI-TOF 3670.8 $[M+H]^+$; 1835.5 $[(M+2H)/2]^{2+}$.

4.2.7. Dimeric compound f. The dimeric compound **f** was synthesized according to the general procedures using the resin preloaded with Fmoc-Leu. First, the amino acids for the NT(8–13) sequence were attached stepwise. After deprotection of the Arg(8) residue of the NT(8–13) moiety, Boc-Glu-OH (0.25 equiv), DIPEA (1 equiv), HBTU (0.25 equiv), and HOBt (0.25 equiv) were added. The coupling reaction was allowed to proceed for 2 h. This coupling step was repeated once. Deprotection and cleavage was performed according to the general procedures. The dimeric compound **f** was obtained as a white powder (20 mg, 15%) after purification by semi-preparative HPLC (20–80% CH_3CN within 30 min, t_R = 14.3 min). M_W $C_{81}H_{133}N_{25}O_{18}$ calculated 1745.12, found MALDI-TOF 1745.9 $[M+H]^+$, 873.3 $[(M+2H)/2]^{2+}$.

4.2.8. Dimeric compound h. The dimeric compound **h** was synthesized according to the general procedures using the resin preloaded with Fmoc-Leu. First, the amino acids for the NT(8–13) sequence were attached stepwise, then Fmoc-Ahx-OH was added. After deprotection of the Ahx residue, coupling with Boc-Glu-OH was performed as described above. The dimeric compound **h** was obtained as a white powder (8 mg, 12%) after purification by semi-preparative HPLC (20–80% CH_3CN within 30 min, t_R = 13.9 min). M_W $C_{93}H_{155}N_{27}O_{20}$ calculated 1971.44, found MALDI-TOF 1972.6 $[M+H]^+$.

4.2.9. Dimeric compound i. The dimeric compound **i** was synthesized according to the general procedures using the resin preloaded with Fmoc-Leu. First, the amino acids for the NT(8–13) sequence were attached stepwise, then Fmoc-$(PEG)_2$-OH was added. After deprotection of the $(PEG)_2$ residue, coupling with Boc-Glu-OH was performed as described above. The dimeric compound **i** was obtained as a white powder (10 mg, 13%) after purification by semi-preparative HPLC (25–50% CH_3CN within 30 min, t_R = 14.4 min). M_W $C_{109}H_{185}N_{29}O_{30}$ calculated 2381.87, found MALDI-TOF 2382.9 $[M+H]^+$.

4.2.10. Tetrameric compound g. The tetrameric compound **g** was synthesized according to the general procedures using the resin preloaded with Fmoc-Leu. First, the amino acids for the NT(8–13) sequence were attached stepwise. After deprotection of the Arg(8) residue of the NT(8–13) moiety, Fmoc-Glu-Glu-Glu-OH (0.125 equiv), DIPEA (1 equiv), HBTU (0.125 equiv), and HOBt (0.125 equiv) were added. The coupling reaction was allowed to proceed for 2 h. This coupling step was repeated once. Deprotection and cleavage was performed according to the general procedures. The tetrameric compound **g** was obtained as a white powder (12 mg, 10%) after purification by semi-preparative HPLC (20–70% CH_3CN within 30 min, t_R = 18.5 min). M_W $C_{167}H_{271}N_{51}O_{38}$ calculated 3601.34, found MALDI-TOF 3602.1 $[M+H]^+$, 1801.4 $[(M+2H)/2]^{2+}$.

4.2.11. Tetrameric compound j. The tetrameric compound **j** was synthesized according to the general procedures using the resin preloaded with Fmoc-Leu. First, the amino acids for the NT(8–13) sequence were attached stepwise, then Fmoc-Ahx-OH was added. After deprotection of the Ahx residue, coupling with Fmoc-Glu-Glu-Glu-OH was performed as described above. The tetrameric compound **j** was obtained as a white powder (5 mg, 8%) after purification by semi-preparative HPLC (20–70% CH_3CN within 30 min, t_R = 19.4 min). M_W $C_{191}H_{315}N_{55}O_{42}$ calculated 4053.98, found MALDI-TOF 4055.1 $[M+H]^+$, 2028.1 $[(M+2H)/2]^{2+}$.

4.2.12. Tetrameric compound k. The tetrameric compound **k** was synthesized according to the general procedures using the resin preloaded with Fmoc-Leu. First, the amino acids for the NT(8–13) sequence were attached stepwise, then Fmoc-$(PEG)_2$-OH was added. After deprotection of the $(PEG)_2$ residue, coupling with Fmoc-Glu-Glu-Glu-OH was performed as described above. The tetrameric compound **k** was obtained as a white powder (4 mg, 8%) after purification by semi-preparative HPLC (20–70% CH_3CN within 30 min, t_R = 18.6 min). M_W $C_{223}H_{375}N_{59}O_{62}$ calculated 4874.83, found MALDI-TOF 4876.1 $[M+H]^+$, 2438.6 $[(M+2H)/2]^{2+}$.

4.3. Cell culture

The cells HT-29 were obtained from ECACC-Oxford, England (ECACC Ref. No: 91072201). They were routinely cultured in tissue culture T-25/75 flasks (Cellstar®, Greiner bio-one) with McCoy's 5a medium containing Glutamax-I (Gibco-BRL), 10% fetal bovine serum (Sigma), and penicillin/streptomycin (10,000 U/mL, Gibco). For the experiments cells were detached by trypsin/EDTA (0.02–0.05%).

4.4. In vitro binding studies

HT-29 cells were plated on tissue culture T-25/75 flasks. Cells were grown in the culture media in humidified 5% CO_2/95% air at 37 °C. In the T-25/75 flasks the cells reached confluence after 3–5 days. The experiments were conducted at confluence with cell densities between 4×10^6 and 7×10^6 cells/ml (equivalent to about 300–700 μg protein) of passage 5–20.

The experiments were initiated by preparation of a dilution series of the peptides. A 1 mM stock solution of the peptides dissolved in DMSO was prepared. Final concentrations of 10 μM, 6 μM, 1 μM, 600 nM, 200 nM, 100 nM, 60 nM, 10 nM, 6 nM, 2 nM, and 1 nM were obtained by dilution with Tris buffer (50 mM Tris–HCl, 1 mM EDTA, 0.1% BSA, and 0.5 mM *o*-phenanthroline, pH 7.4) containing 10% DMSO. The cells in the flasks were washed three times with Tris buffer. They were subsequently removed from the flask and resuspended in 8 ml of Tris buffer. The cell suspension was then homogenized in a glass homogenizer. 200 μl of the cell homogenate was mixed with 100 μl of the peptide solution and 500 μl of Tris buffer. 200 μl of [^{3}H]NT was added and the mixture was allowed to incubate for 30 min at room temperature. Non-specific binding was assessed in the presence of 100 mM NT(8–13).

Separation of bound and free ligand was performed by rapid filtration through Whatman GF/B glass fiber filters using a Brandel cell harvester (Gaithersburg, MD, USA). Filters were washed with three volumes of Tris buffer and were placed into vials with 4 ml scintillation cocktail (ULTIMA Gold®, Packard Instruments, USA). The [^{3}H]NT remaining on the filters was quantified by liquid scintillation spectrometry (TriCarb®, Packard Instruments, USA).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.05.024.

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