LABELLING AND EVALUATION OF NEW STABILISED NEUROTENSIN (8-13) ANALOGUES FOR SPET

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Abstract

Neurotensin(8-13) analogues were biologically stabilised by replacement of the peptide bond between amino acids 8 and 9 by the reduced ψ(CH_2-NH) isostere. DTPA analogues for In-111 labelling and 2-bromo-phenyl-acetyl analogues for radioiodination, showed receptor affinities in the low nanomolar range in combination with a biological half live in human plasma up to 275 minutes. Biodistribution studies in male Wistar rats of metabolically stabilised and non-stabilised ^{111}In-DTPA-NT(8-13) analogues showed a major clearance from the blood through the kidneys. ^{125}I-labelled Neurotensin (8-13) analogues showed accumulation up to 2.2% of the injected dose per g tissue in the liver which might be an important disadvantage when diagnosis of tumours in the gut is aimed. It is strongly suggested that stabilised Neurotensin(8-13) analogues whether labelled with In-111, I-123 and in the near future with Tc-99m, may act as new potential peptidergic radiopharmaceuticals for SPET diagnosis of different NT-receptor positive tumours like non-endocrine pancreas carcinoma, small cell lung carcinoma or colon adeno carcinoma. It is

enticing to speculate that metabolically stabilised Neurotensin(8-13) analogues labelled with an appropriate isotope might be useful in therapy of different human cancers.

1. INTRODUCTION

Neurotensin (NT) is a linear tridecapeptide (p-Glu-Leu-Tyr-Gln-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) which has been first isolated from bovine hypothalamus and bovine small intestine [1-2]. Functional characterisation of this neuropeptide revealed its involvement in intracellular communication in the central nervous system and the gut.

Recent studies revealed the involvement of NT in clonal growth of different human cancers and cancer cell lines. NT and NT receptor (NTR) expression is seen in human non-endocrine pancreas carcinomas, colon adeno carcinomas, meningiomas, astrocytic tumor and small cell lung carcinoma cell lines and may play a role in human prostatic cancer development. Consequently, radiolabelled NT may act as an attractive vector for tumour targeting, a strategy already successfully explored for the somatostatin analogue octreotide, which has become a routine radiopharmaceutical in nuclear medicine. An important advantage of developing radiopharmaceuticals from peptides as NT is there size. As peptides are small molecules, they are rather fast and easily to synthesise and purify. Moreover, the small size of peptides is of the utmost importance for a relatively fast blood clearance by the kidneys, thus leading to low background radioactivity. In this way, radiolabelled peptides are powerful alternatives to monoclonal antibodies showing slow blood clearance, which is an important drawback in scintigraphy of target specific tumours [3].

An important restriction in using natural peptide analogues in developing radiopharmaceuticals is there in vivo stability. NT has an in vivo stability in rat and human of only a few minutes [4-5]. In rat, NT is mainly inactivated by a combination of three metallo-endopeptidases which cleave the peptide on three different peptide bonds [6-7]. A very potent enzyme involved in this inactivation is the metallo-endopeptidase (EP) EP24.15 [8] acting on the amide bond between Arg8 and Arg9.

Different approaches have resulted in developing metabolically stabilised peptides. One of the approaches is to replace the metabolically unstable peptide CO-NH function by a pseudo-peptide isosteric bond unable to be hydrolysed [9-10]. When this peptide function is also involved in receptor interaction, it is important to have access to substitutions that maintain either the amine $\psi(CH_2$-NH) the carbonyl ψ (CO-CH_2) or both ψ(CO-CH_2-NH) functions in order not to lose receptor affinity [11].

Structure-activity studies have shown that the C-terminal hexapeptide NT(8-13) is the minimal fragment required for biological activity [12]. It was shown that ^{3}H-NT(8-13) binds to human brain homogenates to the same receptor sites as ^{3}H-NT but with a 4 times higher affinity: Ki values of 2nM for ^{3}H-NT and 0.5nM for ^{3}H-NT(8-13) were obtained [13]. These receptor sites have been further defined in a low affinity and a high affinity subtype, the latter being responsible for biological activity [14].

Our group developed different metabolically stabilised NT(8-13) analogues by introducing $\psi(CH_2NH)$ pseudo-peptide bonds between amino acids 8 and 9. Introducing $\psi(CH_2NH)$ pseudo-peptide bonds at other places in the peptide gave rise to an important drop in receptor affinity. On the other hand, amino acid

replacement of Arg by Lys can lead in higher receptor affinity [15] but also in higher biological effectiveness.

This study investigates the biological consequences of introducing a pseudo-peptide bond, amino acid replacement and attachment of different chelating groups for radiolabelling to different NT(8-13) analogues concerning binding potential, in vitro and biological stability, biological activity and in vivo kinetics.

It is shown that stabilised NT(8-13) analogues show high potency in developing new radiopharmaceuticals for SPET diagnosis of different NTR positive tumours on the one hand and therapy of the same pathologies on the other hand.

2. Experimental

2.1. Peptide synthesis

Both stabilised and non-stabilised NT(8-13) analogues were prepared by solid phase synthesis on a standard Merrifield resin using Boc-main chain protection. Boc-amino acids were coupled by the DCC/HOBt method.

For Iodine labelling, 2-bromo-phenyl acetic acid was coupled to the peptide as described previously [15]. For ^{111}In labelling, DTPA was coupled to the peptide by coupling DTPA-tetra-T-butylester to the peptide with TBTU/ hydroxybenzotriazole.

After cleavage from the resin, both types of peptides were purified by RP-HPLC followed by Mass Spectroscopy and LC-MS analysis.

The synthesis of the metabolically stabilised NT(8-13) analogues was performed as described by Sasaki and Coy [16].. The incorporation of the ψ (CH_2-NH) pseudopeptide bond was performed by reductive amination of a Boc-amino-aldehyde by the solid-phase resin-bond peptide in the prescence of $NaBH_3CN$ as described in detail by Sasaki and Coy [16].

The following NT(8-13) analogues have been synthesised and HPLC purified: DTPA-NT(8-13), DTPA-Lys-ψ (CH_2NH)-Arg-NT(10-13), DTPA-Lys-Arg-NT(10-13), DTPA-Lys-ψ(CH_2NH)-Lys-NT(10-13), DTPA-Lys-Lys-NT(10-13), DTPA-Arg-ψ(CH_2NH)-Lys-NT(10-13), 2-Br-Phe(ac)-NT(8-13), 2-Br-Phe(ac)-Arg-ψ(CH_2NH)-Arg-NT(10-13), 2-Br-Phe(ac)-Lys-Arg-NT(10-13), 2-Br-Phe(ac)-Lys-Lys-NT(10-13) and DOTA-NT(8-13).

2.2. Radiolabeling procedures

2.2.1. DTPA-NT(8-13) analogues: labelling with In-111

The radiosynthesis of non carrier added (n.c.a) ^{111}In-DTPA-NT(8-13) was performed in a kit-formulation. 1.1ml 0.02N HCl and $^{111}InCl_3$ in 0.02N HCl (provided by Mallinckrodt, The Netherlands) were added to 10µg DTPA-NT(8-13), 4.96mg trisodiumcitrate, 0.37mg citric acid, 10mg inositol and 2mg of 2,5-dihydroxybenzoic acid. After 30 minutes at room temperature, the reaction mixture was diluted with 1ml of semi-preparative eluent (H_2O/ACN//TFA, 14/86//0.1, pH=1.9) and injected on the semi-preparative HPLC. The radiolabelled peptide was collected at 25.5min and the collected eluent was diluted with an equal volume of doubly-distilled water. The ^{111}In-DTPA-NT(8-

13) was pre-concentrated on a Baker Bond Octadecyl 100mg mini-column and recovered in 1ml of EtOH/PBS buffer 50/50 pH 7.4 (PBS: 0.14M NaCl, 19mM Na_2HPO_4 and 2.4mM NaH_2PO_4) as described by Terriere et al.[17]. Labelling yields of more than 98% were obtained. The overall yield of the radiosynthesis was about 75% with a radiochemical purity of at least 99.0% and a specific activity of >1700 TBq/mmol.

2.2.2. 2-Bromo-phenylacetic acid NT(8-13) analogues: radioiodination

Radioiodination was performed by using the Cu^+ assisted non-isotopic nucleophilic exchange reaction [18] on the 2-Bromo-phenylacetic acid (2-Br-Phe(ac)) NT(8-13) analogues. To 1mg of the precursor peptide dissolved in 10µl 100%AcOH were added 500µl of stock solution (1mg SnSO4, 25mg 2,5-dihydroxybenzoic acid, 35mg citric acid.H_2O and 500µl glacial acetic acid in 4500µl of water) and 60µl of the copper solution (32.5mg CuSO4.5H2O dissolved in 10ml water). After N_2 flush during 5-10 minutes, the radioiodine solution was added and the reaction mixture was heated at 140°C for 1 hour. Purification was effected by semi-preparative reversed phase HPLC (H_2O/ACN/TFA: 14/86/0.1 v/v mixture, pH 2 on a Vydac 218 TP54 C18 column). Monitoring UV detection at 254nm was used in combination with NaI(Tl) radiomatric detection. An overall labelling yield of 50-72% and a radiochemical purity of at least 99% were obtained.

2.3. In vitro and metabolic stability testing

In vitro biological stability of the ^{111}In-DTPA-NT(8-13), ^{111}In-DTPA-Lys8-ψ(CH_2-NH)-Arg9-NT(10-13) and 2-^{131}I-phenyl acetic acid NT(8-13)

analogues were compared. In vitro stability at pH 7.4 at room temperature was evaluated by analytical HPLC. Metabolical stability testing was performed by using fresh human plasma. Incubation at 37°C was followed by denaturation using a 5% tri-chloro acetic acid / methanol 50/50 (v/v) solution (1/1 plasma). After centrifugation (2000rpm/2min), small aliquots of the supernatants were HPLC analysed.

2.4. Drug competition studies

For receptor competition assays, guinea pig (Pirbright, 300g) bulbus olfactorius membranes were used. After decapitation, the brains were rapidly removed and the bulbus olfactorii were dissected. Tissue samples were homogenised in 15ml of Tris-HCl buffer (50mM, pH 7.4) using a Ultraturrax homogeniser. The homogenate was centrifuged at 16000RPM for 10minutes in a refrigenated Sorvall centrifuge. The pellet was twice rehomogenised and recentrifuged as described above. The final pellet was suspended in Tris-HCl buffer in a dilution of 10mg of original wet tissue/ml. Competition of the binding of 1nM ^{3}H-Neurotensin on 4mg of tissue in a final volume of 500µl was under study.

Analogous binding studies were performed using the human HT29 colon adeno carcinoma cell line. Cells were incubated in a modified Krebs-Ringer Hepes buffer (111mM NaCl, 4mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 20mM Hepes, 0.1% Glucose, 1mM EDTA and 0.1mM Bovine Serum Albumine) at pH 7.4 at a final concentration of $5*10^6$ cells per ml. Competition of the binding of 1nM ^{3}H-Neurotensin was performed on $2*10^6$ cells in a final volume of 500µl.

Incubation at 25°C for 30minutes was followed by rapid filtration under reduced pressure through pre-soaked (2h in pH 7.4 buffer added 1μM of NT) Whatman GF/B glass fiber filters. Filters were rinsed twice with 2ml of Krebs Ringer Hepes buffer with no serum albumin for the HT29 cells and Tris-HCl buffer for the Bulbus Olfactorius homogenates. The filters were placed in plastic scintillation vials containing 2ml of Instagel Gold MV Scintillation fluid. Counting was performed in a Packard Scintillation spectrometer. Inhibition constants (Ki) were calculated by using the following equation (Cheng and Prusoff, 1973):

$$\mathbf{K_i = IC_{50} \times K_d / (K_d + L)}$$

where Kd is the dissociation constant obtained from equilibrium binding experiments and L the concentration of ^{3}H-NT.

2.5. Biological activity Studies

Biological activity of the non-radiolabelled NT(8-13) analogues was evaluated. Assays were based on the biological potency obtained from the peptides to relax pre-contracted isolated longitudinal smooth muscle strips of the guinea pig jejunum (isotonic contraction and relaxation). Pirbright Guinea pigs of female sex (250g) were killed by decapitation. The jejunum was removed and rinsed. Segmental strips of 2cm length were dissected. These strips were suspended in an organ bath of 20ml and connected to an isotonic transducer under a preload of 1g (Displacement Transducer Control Unit, Janssen Scientific Instrument Division). The organ bath was filled with De Jalon solution (KCl, 5.6mM; $CaCl_2{\bullet}2H_2O$, 0.54mM, $NaHCO_3$, 6mM; NaCl, 155mM and Glucose, 2.8mM) kept at 37°C and gassed with a 95% O_2 and 5% CO_2 mixture. Total

contraction at the start of the experiment was obtained by using 3×10^{-6}M carbachol.. After a contact time of 30s, the organ baths were refreshed and the contraction procedure was repeated at intervals of 15min until reproducable result were obtained. Test compounds were used at a concentration of 3×10^{-8}M. Relaxations of the test compounds were expressed as percentage inhibition of the total contraction induced by 3×10^{-8} M Neurotensin.

2.6. In vivo Biodistribution Studies

Hannover male Wistar rats were injected intravenously in the tail with 20µCi of n.c.a. radiolabelled peptide. Biodistribution and clearance of ^{111}In-DTPA-NT(8-13), ^{111}In-DTPA-Lysψ(CH_2-NH)ArgNT(10-13) and ^{125}I-Phenyl acetyl NT(8-13) was under study. Rats were sacrified by decapitation at 15, 30, 45, 60 and 240 minutes after injection respectively. Blood was collected at time of decapitation. Organs including heart, lungs, kidneys, spleen, jejunum, ileum, colon ascendens and liver were quickly removed, washed and dried. Urine was collected by punction of the bladder. All samples were weighed and counted in a single channel gamma counter. Results were expressed as % of the injected dose per gram tissue.

3. RESULTS

3.1. Radiolabelling procedures

For labelling with In-111, DTPA was substituted as chelating group on the 8 position of NT(8-13). Under the above described conditions, radiolabelling

Table I. Biological Stability in Human Plasma.

Analogue	t ½ (*)
NT	1.5
^{111}In-DTPA-NT(8-13)	10
2-^{131}I-Phenylacetyl-NT(8-13)	12
^{111}In-DTPA-NT-Lys8-(CH_2NH)-Arg9-(8-13)	275

*Biological halflive in human plasma in minutes.

yields of 98% is obtained. The overall labelling yield as obtained after semi-prep HPLC purification and recovery is 75% in combination with a radiochemical purity of at least 99% and a specific activity of >1700 TBq/mmol.

Radioiodination using the copper assisted nucleophilic non isotopic displacement reaction on the brominated precursor molecules in reducing and acidic conditions yielded various labelling yields between 50 and 72%. After HPLC purification an overall labelling yield of 30-50% is obtained with a radiochemical purity of >99% and high specific activity.

3.2. In vitro and in vivo evaluation

Biological stability in human plasma of ^{111}In-DTPA-NT(8-13), ^{111}In-DTPA-Lys8-ψ(CH_2-NH)-Arg9-NT(10-13) and 2-^{131}I-phenyl acetic acid NT(8-13) is listed in Table I. Introduction of the pseudo-peptide CH_2-NH isostere

between amino acids 8 and 9 gives rise to an important improvement in biological stability from 10 to 275min.

Table II shows the Ki values obtained from drug competition studies using ^{3}H-NT both for binding on Bulbus Olfactorius (B.O.) and HT29 cells. The dissociation constant Kd for ^{3}H-NT was calculated from equilibrium saturation experiments; 0.7nM and 3.5nM for the B.O. and HT29 model respectively. As obtained from Scatchard plots, total binding capacity (Bmax) values are 25 and

Table II. Inhibition of the binding of ^{3}H-Neurotensin on Bulbus Olfactorius (B.O.) homogenates and HT29 cells.

Analogue	Ki (nM) B.O. (1)	Ki (nM) HT29 (2)
Neurotensin	**1.5**	**1.0**
DTPA-NT(8-13)	**19.0**	**62.0**
^{115}In-DTPA-NT(8-13)	**n.t.**	**3.9**
DTPA-NT-Lys8-(CH_2NH)-Arg9-(8-13)	**13.0**	**n.t.**
DTPA-NT-Lys8-(CH_2NH)-Lys9-(8-13)	**7.4**	**n.t.**
DOTA-NT(8-13)	**n.t.**	**17.0**
2-Br-Phenylacetyl-NT(8-13)	**5.0**	**4.2**
2-Br-Phe(ac)-NT-Arg8-(CH_2NH)-Lys9-(8-13)	**4.2**	**n.t.**

(1) Ligand : 1 nM ^{3}H-Neurotensin , S.A. = 92 Ci/mmol. 4mg of fresh tissue homogenate was used in a final volume of 500µl. Kd = 0.7nM.

(2) Ligand : 1 nM ^{3}H-Neurotensin , S.A. = 92 Ci/mmol. 2×10^6 cells were used in a final volume of 500µl. Kd = 3.5nM.

Results of duplicate experiments. (n.t.) : not tested

Table III. Biodistribution in Wistar rats.

^{111}In-DTPA-Lys8ψ(CH_2-NH)Arg-NT(10-13)

Organ	%ID/g			
	15'	30'	60'	240'
Blood	0.4	0.3	0.2	0.0
Kidney	1.8	1.8	1.0	0.3
Liver	0.1	0.1	0.1	0.0
Heart	0.1	0.1	0.1	0.0
Spleen	0.1	0.1	0.1	0.1
Lung	0.4	0.3	0.2	0.1
Duodenum	0.2	0.1	0.1	0.0
Jejenum	0.2	0.2	0.1	0.0
Ileum	0.1	0.2	0.1	0.0
Colon	0.2	0.1	0.1	0.1

2-^{125}I-Phenylacetyl-NT(8-13)

Organ	%ID/g			
	15'	30'	60'	240'
Blood	0.3	0.2	0.2	0.0
Kidney	3.5	5.7	2.3	0.3
Liver	0.7	2.2	1.1	0.1
Heart	0.1	0.1	0.1	0.0
Spleen	0.1	0.2	0.1	0.0
Lung	0.3	0.4	0.2	0.0
Duodenum	0.2	0.8	0.8	0.1
Jejenum	0.2	0.7	0.6	0.2
Ileum	0.1	0.8	0.8	0.2
Colon	0.1	0.1	0.1	0.0

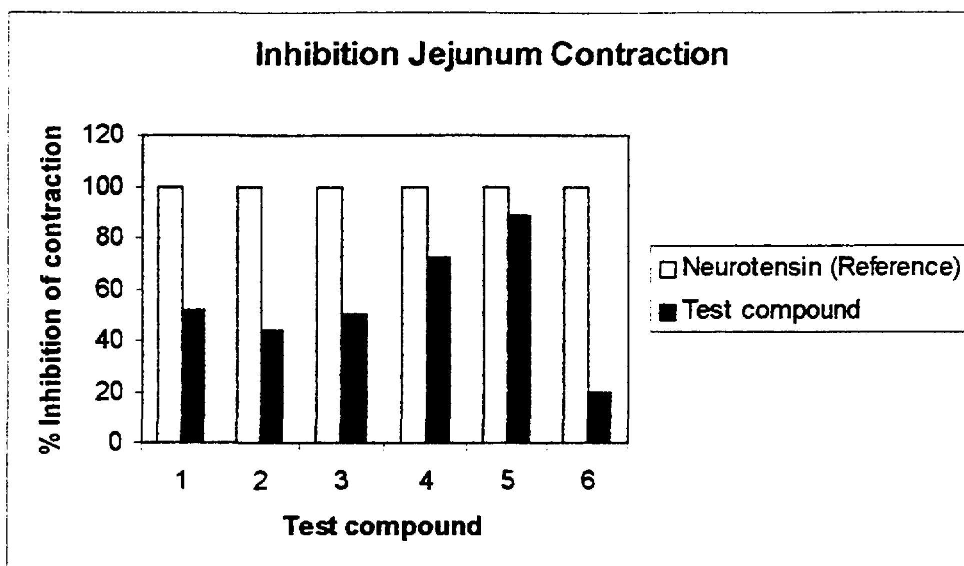

FIG. 1: Inhibition of the contraction of different NT(8-13) analogues as percentage of the contraction induced by parent NT.

All test compounds were used at 3×10^{-8}M. Mean values of duplicate experiments are shown. The following test numbers were screened (values ± SD): **1**. DTPA-NT(8-13) (55±14%), **2**. 2-Br-Phe(ac)-Lysψ(CH_2-NH)Arg-NT(8-13) (44± 19%), **3**. DTPA- Lysψ(CH_2-NH)Arg-NT(8-13) (50±14%), **4**. DTPA- Lysψ(CH_2-NH)Lys-NT(8-13) (72±11%), **5**. 2-Br-Phe(ac)-Argψ(CH_2-NH)Lys-NT(8-13) (89± 6%), **6**. 2-Br-Phe(ac)-NT(8-13) (20±1%).

37 fmoles/mg tissue binding law affinity and high affinity sites for the B.O. model and 42 fmol/ 10^6 cells binding only high affinity sites for the HT29 model. All analogues show Ki values in the low nanomolar range. DTPA-NT(8-13) analogues show 5 to 10 fold lower affinities in comparison to the 2-bromo-phenyl acetic acid-NT (8-13) analogues.

Relaxation of the guinea pig jejunum induced by different stabilised and non-stabilised NT(8-13) analogues are expressed as percentage of the inhibition induced by native NT. Results are reproduced in Figure 1. No analogue showed total antagonistic effects. Biological effects were between 20 and 89% of the

effect induced by parent NT for the 2-Br-Phe(ac)-NT(8-13) and the 2-Br-Phe(ac)-Arg-ψ(CH_2NH)-Lys respectively. The use of partial agonists in developing peptidergic radiopharmaceuticals should be preferred above the use of full agonists in order to minimize biological effects. On the other hand, a minimal agonistic effect should be required in order to provoke internalisation which might enhance diagnostical and/or therapeutical qualities of the tracer.

Biodistribution in Wistar rats with 20μCi of ^{111}In-DTPA-NT-Lys8ψ(CH_2NH)Arg-NT(10-13) and 2-^{125}I-Phenylacetyl-NT(8-13) are reproduced in Table III. Both types of radiolabelled NT(8-13) analogues show fast blood clearance by the kidneys up to 90% of the injected dose within 4h. The radioiodinated analogue shows an important clearance by the liver up to 2.2 % of the injected dose per gram tissue which might be an important disadvantage when visualisation of tumours in the gut is aimed.

4.0. CONCLUSION

The results of our studies show that both DTPA and 2-bromo-phenyl-acetic acid NT(8-13) analogues show very high and receptor specific binding properties which makes these peptides very interesting in developing new radiolabelled vectors for SPET diagnosis or therapy. On the other hand, the finite biological stability of these peptides is an important disadvantage limiting all properties. The use of DTPA-NT(8-13) analogues with a reduced peptide bond of the ψ(CH_2-NH) type between amino acids 8 and 9 being Arg or Lys, combines high biological stability with binding properties similar to those of their non-stabilised analogues. Using neutral charged radioiodinated phenyl acetic acid NT(8-13)

analogues as radiopharmaceuticals might be limited when visualisation of the gut is aimed as these analogous show an important clearance by the liver as seen in rats. Analogous results where seen in biodistribution studies using neutral charged radiolabelled Somatostatin analogues [19], suggesting the need of a charged peptide in order to avoid clearance by the liver.

NT(8-13) analogues with other chelating groups like DOTA for Y-90 labelling and 2-Methyl-Gly-L-Ser-L-Cys-Gly (Resolution Pharmaceuticals, ON) for labelling with Tc-99m are currently under development.

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