

## LABELLING AND EVALUATION OF NEW STABILISED NEUROTENSIN (8-13) ANALOGUES FOR SINGLE PHOTON EMMISSION TOMOGRAPHY (SPET).

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### SUMMARY

Neurotensin(8-13) analogues were biologically stabilised by replacement of the peptide bond between amino acids 8 and 9 by the reduced  $\psi(\text{CH}_2\text{-NH})$  isostere. Diethylenetriaminepentaacetic acid (DTPA) analogues for In-111 labelling and 2-bromo-phenyl-acetyl analogues for radioiodination, showed receptor affinities in the 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 <sup>111</sup>In-DTPA-NT(8-13) analogues showed a major clearance from the blood through the kidneys. <sup>125</sup>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. Neurotensin(8-13) analogues labelled with In-111 or I-123 may act as new potential peptidergic radiopharmaceuticals for SPET diagnosis of neurotensin receptor (NTR) positive tumours.

**Keywords:** Neurotensin, Indium, Radioiodide, *in vitro*, *in vivo*.

## INTRODUCTION

Neurotensin (NT) is a linear tridecapeptide (p-Glu-Leu-Tyr-Gln-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) [1-2] which is among other things involved in clonal growth of different human cancer cell lines and different tumours including non-endocrine pancreas carcinomas, colon adeno carcinomas, meningiomas, astrocytic tumors 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 neurotensin receptor (NTR) positive tumour targeting and therapy. An important advantage of developing radiopharmaceuticals from peptides as NT is their size. The small size of peptides is of the utmost importance for 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 their *in vivo* stability. NT has an *in vivo* stability in rat and humans of only a few minutes [4-5]. In rat, NT is mainly inactivated by a combination of three metallo-endopeptidases which cleave the peptide in three different ways [6-7]. A very potent enzyme involved in this inactivation is the metallo-endopeptidase (EP) EP24.15 acting on the amide bond between Arg8 and Arg9 [8].

Different approaches have resulted in the development of 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  $\psi(\text{CH}_2\text{NH})$  the carbonyl  $\psi(\text{CO-CH}_2)$  or both  $\psi(\text{CO-CH}_2\text{-NH})$  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  $^3\text{H-NT}(8-13)$  binds to human brain homogenates to the same receptor sites as  $^3\text{H-NT}$  but with a 4 times higher affinity:  $K_i$  values of 2nM for  $^3\text{H-NT}$  and 0.5nM

for  $^3\text{H}$ -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  $\psi(\text{CH}_2\text{NH})$  pseudo-peptide bonds between amino acids 8 and 9. Introducing  $\psi(\text{CH}_2\text{NH})$  pseudo-peptide bonds at other places in the peptide gave rise to an important reduction in receptor affinity. On the other hand, amino acid replacement of Arg by Lys can lead to higher receptor affinity [15] as well as higher biological effectiveness.

This paper describes the first *in vitro* and *in vivo* evaluation of different NT(8-13) analogues. The biological consequences of introducing a pseudo-peptide bond, amino acid replacement and attachment of different chelating groups for radiolabelling, binding potential, *in vitro* and biological stability, biological activity and *in vivo* kinetics are under study.

It is shown that the stabilised NT(8-13) analogues show high potential for 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.

## EXPERIMENTAL

### 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  $^{111}\text{In}$  labelling, DTPA was coupled to the peptide by coupling DTPA-tetra-*t*-butylester to the peptide with TBTU/ hydroxy-benzotriazole. After cleavage from the resin, both types of peptides were purified by RP-HPLC and analysed by mass spectroscopy and LC-MS.

The synthesis of the metabolically stabilised NT(8-13) analogues was performed as described by Sasaki and Coy [16]. The incorporation of the  $\psi(\text{CH}_2\text{-NH})$  pseudopeptide bond was performed by reductive amination of a Boc-amino-aldehyde by the solid-phase resin-bound peptide in the presence of  $\text{NaBH}_3\text{CN}$  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- $\psi$  (CH<sub>2</sub>NH)-Arg-NT(10-13), DTPA-Lys-Arg-NT(10-13), DTPA-Lys- $\psi$ (CH<sub>2</sub>NH)-Lys-NT(10-13), DTPA-Lys-Lys-NT(10-13), DTPA-Arg- $\psi$ (CH<sub>2</sub>NH)-Lys-NT(10-13), 2-Br-Phe(ac)-NT(8-13), 2-Br-Phe(ac)-Arg- $\psi$ (CH<sub>2</sub>NH)-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).

### Radiolabeling Procedures

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

The radiosynthesis of non-carrier-added (n.c.a) <sup>111</sup>In-DTPA-NT(8-13) was performed in a kit-formulation. 1.1ml 0.02N HCl and <sup>111</sup>InCl<sub>3</sub> in 0.02N HCl (provided by Mallinckrodt, The Netherlands) were added to 10 $\mu$ g 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<sub>2</sub>O/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 <sup>111</sup>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<sub>2</sub>HPO<sub>4</sub> and 2.4mM NaH<sub>2</sub>PO<sub>4</sub>) 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-Bromo-phenylacetic acid NT(8-13) analogues: radioiodination

Radioiodination was performed by using the Cu<sup>+</sup> 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 10 $\mu$ l 100%AcOH were added 500 $\mu$ l of stock solution (1mg SnSO<sub>4</sub>, 25mg 2,5-dihydroxybenzoic acid, 35mg citric acid.H<sub>2</sub>O and 500 $\mu$ l glacial acetic acid in 4500 $\mu$ l of water) and 60 $\mu$ l of the copper solution (32.5mg CuSO<sub>4</sub>.5H<sub>2</sub>O

dissolved in 10ml water). After flushing with N<sub>2</sub> flush for 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<sub>2</sub>O/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) radiometric detection. An overall labelling yield of 50-72% and a radiochemical purity of at least 99% was obtained.

### ***In vitro* and metabolic stability testing**

*In vitro* biological stability of the <sup>111</sup>In-DTPA-NT(8-13), <sup>111</sup>In-DTPA-Lys8-ψ(CH<sub>2</sub>-NH)-Arg9-NT(10-13) and 2-[<sup>131</sup>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. Metabolic stability testing was performed by using fresh human plasma. Incubation at 37°C was followed by denaturation using a 5% trichloroacetic acid / methanol 50/50 (v/v) solution (1/1 plasma). After centrifugation (2000rpm/2min), small aliquots of the supernatants were HPLC analysed.

### **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 refrigerated 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 <sup>3</sup>H-neurotensin on 4mg of tissue in a final volume of 500µl was undertaken.

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<sub>2</sub>, 1.2mM MgSO<sub>4</sub>, 1.2mM KH<sub>2</sub>PO<sub>4</sub>, 20mM Hepes, 0.1% Glucose, 1mM EDTA and 0.1mM Bovine Serum

Albumine) at pH 7.4 at a final concentration of  $5 \times 10^6$  cells per ml. Competition of the binding of  $1 \text{ nM } ^3\text{H-Neurotensin}$  was performed on  $2 \times 10^6$  cells in a final volume of  $500 \mu\text{l}$ .

Incubation at  $25^\circ\text{C}$  for 30 minutes was followed by rapid filtration under reduced pressure through pre-soaked (2h in pH 7.4 buffer added  $1 \mu\text{M}$  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 ( $K_i$ ) were calculated by using the following equation (Cheng and Prusoff, 1973):

$$K_i = \text{IC}_{50} \times K_d / (K_d + L)$$

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

### 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;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.54mM,  $\text{NaHCO}_3$ , 6mM; NaCl, 155mM and Glucose, 2.8mM) kept at  $37^\circ\text{C}$  and gassed with a 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  mixture. Total contraction at the start of the experiment was obtained by using  $3 \times 10^{-6} \text{ M}$  carbachol. After a contact time of 30s, the organ baths were refreshed and the contraction procedure was repeated at intervals of 15min until reproducible result were obtained. Test compounds were used at a concentration of  $3 \times 10^{-8} \text{ M}$ . Relaxations of the test compounds were expressed as percentage inhibition of the total contraction induced by  $3 \times 10^{-8} \text{ M}$  neurotensin.

### ***In vivo* Biodistribution Studies**

Hannover male Wistar rats were injected intravenously in the tail with 20 $\mu$ Ci of n.c.a. radiolabelled peptide. Biodistribution and clearance of <sup>111</sup>In-DTPA-NT(8-13), <sup>111</sup>In-DTPA-Lys $\psi$ (CH<sub>2</sub>-NH)ArgNT(10-13) and <sup>125</sup>I-Phenyl acetyl NT(8-13) was undertaken. Rats were sacrificed by decapitation at 15, 30, 45, 60 and 240 minutes after injection respectively. Blood was collected at the 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 puncture of the bladder. All samples were weighed and directly counted in a single channel gamma counter. Results were expressed as % of the injected dose per gram tissue.

## **RESULTS**

### **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 yields of 98% were obtained. The overall labelling yield as obtained after semi-preparative HPLC purification and recovery was 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 of between 50 and 72%. After HPLC purification an overall labelling yield of 30-50% was obtained with a radiochemical purity of >99% and high specific activity.

### ***In vitro* and *in vivo* evaluation**

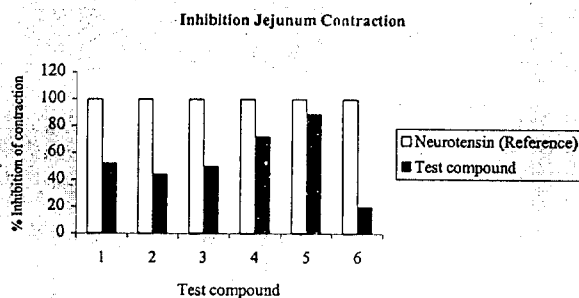
Biological stability data in human plasma of <sup>111</sup>In-DTPA-NT(8-13), <sup>111</sup>In-DTPA-Lys8- $\psi$ (CH<sub>2</sub>-NH)-Arg9-NT(10-13) and 2-[<sup>125</sup>I]-phenyl acetic acid NT(8-13) are listed in Table 1. Introduction of the pseudo-peptide CH<sub>2</sub>-NH isostere between amino acids 8 and 9 gives rise to an important improvement in biological stability from 10 to 275min.

Analogue	t 1/2 (*)
NT	1.5
<sup>111</sup> In-DTPA-NT(8-13)	10
2- <sup>131</sup> I-Phenylacetyl-NT(8-13)	12
<sup>111</sup> In-DTPA-NT-Lys8-(CH <sub>2</sub> NH)-Arg9-(8-13)	275

\*Biological half-life in human plasma in minutes.

**Table 1.** Biological Stability in Human Plasma.

Table 2 shows the  $K_i$  values obtained from drug competition studies using <sup>3</sup>H-NT both for binding on Bulbus Olfactorius (B.O.) and HT29 cells. The dissociation constant  $K_d$  for <sup>3</sup>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 ( $B_{max}$ ) values are 25 and 37 fmoles/mg tissue binding law affinity and high affinity sites for the B.O. model and 42 fmol/ 10<sup>6</sup> cells binding only high affinity sites for the HT29 model. All analogues show  $K_i$  values in the low nanomolar range. DTPA-NT(8-13) analogues show 5 to 10 fold lower affinities by comparison to the 2-bromophenyl 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.



**Figure 1**



Biological effects were between 20 and 89% of the effect induced by parent NT for 2-Br-Phe(ac)-NT(8-13) and 2-Br-Phe(ac)-Arg-ψ(CH<sub>2</sub>NH)-Lys respectively. The use of partial agonists in developing peptidergic radiopharmaceuticals should be preferable to 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 diagnostic and/or therapeutic qualities of the tracer.

Analogue	Ki (nM)	Ki (nM)
	B.O. <sup>(1)</sup>	HT29 <sup>(2)</sup>
Neurotensin	1.5	1.0
Ac-NT(8-13)	9.0	0.1
DTPA-NT(8-13)	19.0	20.0
<sup>115</sup> In-DTPA-NT(8-13)	n.t.	3.9
DTPA-NT-Lys8-(CH <sub>2</sub> NH)-Arg9-(8-13)	13.0	n.t.
DTPA-NT-Lys8-(CH <sub>2</sub> NH)-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 <sub>2</sub> NH)-Lys9-(8-13)	4.2	n.t.

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

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

**Table 2.** Inhibition of the binding of <sup>3</sup>H-Neurotensin on Bulbus Olfactorius (B.O.) homogenates and HT29 cells.

Biodistribution data in Wistar rats with 20µCi of <sup>111</sup>In-DTPA-NT-Lys8ψ(CH<sub>2</sub>NH)Arg-NT(10-13) and 2-<sup>125</sup>I-Phenylacetyl-NT(8-13) are reproduced in Table 3. 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 being studied.

<sup>111</sup>In-DTPA-Lys8ψ(CH<sub>2</sub>-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
Jejunum	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

<sup>111</sup>In-DTPA-Lys8ψ(CH<sub>2</sub>-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
Jejunum	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-<sup>125</sup>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
Jejunum	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

Table III. Biodistribution in Wistar rats.

All test compounds were used at  $3 \times 10^{-8}$  M. Mean values of duplicate experiments are shown. The following test numbers were screened (values  $\pm$  SD): 1. DTPA-NT(8-13) ( $55 \pm 14\%$ ), 2. 2-Br-Phe(ac)-Lys $\psi$ (CH<sub>2</sub>-NH)Arg-NT(8-13) ( $44 \pm 19\%$ ), 3. DTPA- Lys $\psi$ (CH<sub>2</sub>-NH)Arg-NT(8-13) ( $50 \pm 14\%$ ), 4. DTPA- Lys $\psi$ (CH<sub>2</sub>-NH)Lys-NT(8-13) ( $72 \pm 11\%$ ), 5. 2-Br-Phe(ac)-Arg $\psi$ (CH<sub>2</sub>-NH)Lys-NT(8-13) ( $89 \pm 6\%$ ), 6. 2-Br-Phe(ac)-NT(8-13) ( $20 \pm 1\%$ ).

## 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 applications. The use of DTPA-NT(8-13) analogues with a reduced peptide bond of the  $\psi$ (CH<sub>2</sub>-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 analogues show an important clearance by the liver. Analogous results were seen in biodistribution studies using neutral charged radiolabelled somatostatin analogues [19], suggesting the need for 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.

## ACKNOWLEDGMENTS

The research was partly supported by 'Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch Onderzoek in de Industrie' (I.W.T.) and 'Fonds voor Wetenschappelijk Onderzoek' (F.W.O.). The authors wish to thank Janssen Research Foundation, Belgium and Resolution Pharmaceuticals, Ontario-Canada, and Prof M. Mareel of the division of Experimental Oncology of the University of Gent, Belgium for their scientific support.

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