# ASPECTS OF RECEPTOR SCINTIGRAPHY WITH NEUROPEPTIDES

RECEPTOR SCINTIGRAFIE MET NEUROPEPTIDEN

Ik kan over eenzelfde onderwerp niet altijd hetzelfde denken. Ik kan mijn eigen werk niet beoordelen als ik er nog mee bezig ben. Ik moet er net als de schilders eerst een eindje vanaf gaan staan. Hoe ver, denk je?

*Pensées*, Blaise Pascal, 1623-1662.

# ASPECTS OF RECEPTOR SCINTIGRAPHY WITH NEUROPEPTIDES

RECEPTOR SCINTIGRAFIE MET NEUROPEPTIDEN

PROEFSCHRIFT

ter verkrijging van de graad van Doctor

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Eure Kinder sind nicht Eure Kinder.
Sie sind die Söhne und Töchter
der sehnsucht nach sich selbst
Sie kommen durch Euch
aber nicht von Euch.
Und obgleich sie bei Euch sind,
gehören sie Euch nicht.
Ihr dürft Ihnen Eure Lieben schenken
aber nicht Eure Gedanken.
Ihr dürft Ihren Körper ein Heim geben
aber nicht Ihren Seelen.
Den Ihre Seelen wohnen in Hause von Morgen
das Ihr nicht besuchen könnt,
nicht einmal in Euren Träumen.
Ihr dürft Euch bemühen
wie Sie zu sein,
aber sucht nicht,
Sie Euch gleich zu machen.

**Kahlil Gibran**

Aan mijn ouders
Aan Lieneke

*GELEERDEN-PROZA*

*Iedereen weet dat academici niet kunnen schrijven. Eén blik in een dissertatie, één hapje van een congresbundel, en je proeft het: de woorden knarsen als gruis tussen de kiezen, de zinnen drijven als ordeloos wrakhout voorbij, en het betoog blijft duister als een maanloze nacht boven Patagonië.*

BB in de NRC

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## *LIST OF ABBREVIATIONS*

| | |
|---|---|
| ACTH | Adrenocorticotrophin |
| APUD | Amine precursor uptake and decarboxylation |
| ATPase | Adenosine triphosphatase |
| BSA | Bovine serum albumin |
| Bq | Becquerel |
| BH-SP | Bolton-Hunter-Substance P |
| BW | Body weight |
| CT | Computed tomography |
| CP96,345 | Selective non-peptide $NK_1$-receptor antagonist |
| CPM | Counts per min |
| DNA | Deoxyribonucleic acid |
| DOTA | polyazamacrocycle |
| DTPA | Diethylenetriaminepentaacetic acid |
| EDTA | Ethylenediaminetetraaceticacid |
| EGF | Epidermal growth factor |
| GH | Growth hormone |
| GI | Gastrointestinal |
| GRF | Growth hormone-releasing factor |
| GRIF | Growth hormone releasing-inhibiting factor |
| Gy | Gray |
| HPLC | High-performance liquid chromatography |
| HSA | Human serum albumin |
| $IC_{50}$ | Concentration, needed to achieve 50 % inhibition of binding |
| IGF-I | Insulin-like growth factor type I |
| ITLC | Instant thin layer chromatography |
| $K_a$ | Affinity constant |
| $K_d$ | Dissociation constant |
| kD | Kilodalton |
| LHRH | Luteine-hormone-releasing hormone |
| LLI | Lower large intestine |
| MBq | Megabequerel |

| | |
|---|---|
| MEN | Multiple endocrine neoplasia |
| MEM | Minimal essential medium |
| mIBG | Metaiodobenzylguanidine |
| MoAb | Monoclonalantibodies |
| MRI | Magnetic resonance imaging |
| NK | Neurokinin |
| PET | Positron emission tomography |
| PRL | Prolactin |
| PTH | Parathyroid hormone |
| rGH | Rat growth hormone |
| SMS | Sandoz mini somatostatin |
| SP | Substance P |
| SPECT | Single photon emission computed tomography |
| SPR | Substance P receptor |
| SS-R | Somatostatin receptor |
| Sv | Sievert |
| $SS_{14}$ | Somatostatin-14 |
| $SS_{28}$ | Somatostatin-28 |
| t½ | Half-life |
| TRH | Thyrotropin-releasing hormone |
| ULI | Upper large intestine |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 Somatostatin, somatostatin analogues and somatostatin receptors

Somatostatin ($SS_{14}$) is a cyclic disulfide-containing peptide hormone of 14 amino acids (see figure 1). It is present in the hypothalamus, the cerebral cortex, the brain stem, the gastrointestinal tract, and the pancreas. Somatostatin was isolated and characterized by investigators working in the laboratory of Guillemin at the Salk Institute in La Jolla (California) in 1973. Somatostatin receptors have been identified in the gastrointestinal tract, the central nervous system and on many cells of neuroendocrine origin, including the somatotroph cells of the anterior pituitary gland, the thyroid C cells and the D cells of the islets of Langerhans (1,2). However, also non-neuroendocrine cells, such as lymphocytes (3), possess these receptors. In the central nervous system somatostatin acts as a neurotransmitter, while its hormonal activities include the inhibition of the release of growth hormone, insulin, glucagon and gastrin (see ref. 4 for a review).

The general inhibitory effect of somatostatin on hormone secretion of various glands led to the concept of possible beneficial effects of somatostatin in the treatment of diseases based on gland hyperfunction or overproduction of hormones by endocrine-active tumours. However, the tetradecapeptide $SS_{14}$ itself turned out to be unsuitable for routine treatment. This is because after intravenous administration $SS_{14}$ has a very short half-life of $\approx$ 3 minutes in man due to rapid enzymatic degradation and also because of its diversity of action, such as lowering insulin levels. In recent years, successful efforts have been undertaken to synthesize somatostatin analogues that are more resistant to enzymatic degradation by modifying the molecule in various ways with preservation of the biological activity of the original molecule. Introduction of D-amino acids and shortening of the molecule to the bioactive core sequence, resulted in the 8 amino acids-containing somatostatin analogue octreotide (Sandostatin®, see figure 1). Nowadays octreotide is widely used with success in the treatment of the symptoms of neuroendocrine-active tumours, such as growth hormone-producing pituitary adenomas and gastroenteropancreatic tumours (5-7). The biology and clinical application of somatostatin and somatostatin analogues have been described extensively (6-10).

Somatostatin receptor scintigraphy has been performed at the University Hospital Dijkzigt in Rotterdam since 1987. At first with [$^{123}$I-Tyr$^3$]octreotide and since 1989 with [$^{111}$In-DTPA-D-Phe$^1$]octreotide as described by Bakker (11). The visualization of somatostatin receptor-positive tumours in vivo after the administration of [$^{111}$In-DTPA-D-Phe$^1$]octreotide (Octreoscan®) in a large series of patients has been reviewed (12).

Somatostatin receptors are structurally related integral membrane glycopro-

teins. Recently, five different human somatostatin receptor types have been cloned. All subtypes bind $SS_{14}$ and $SS_{28}$ (a polypeptide of 28 amino acids with $SS_{14}$ making up the C-terminus) with high affinity, while the affinity of numerous somatostatin analogues for the five different subtypes differ considerably (13-16). Octreotide binds with high affinity to the hSSTR2 (human somatostatin receptor type 2) subtype, while this analogue has a relatively low affinity for hSSTR3 and hSSTR5 and shows no binding to hSSTR1 and hSSTR4 (13-17). Octreotide scintigraphy is, therefore, based on the visualization of (an) octreotide-binding somatostatin receptor(s) (octreotide receptor(s)), most probably the hSSTR2 and hSSTR5.

**Table 1.** *$IC_{50}$ of octreotide for the five human somatostatin receptor subtypes (17)*

| | SSTR1 | SSTR2 | SSTR3 | SSTR4 | SSTR5 |
|---|---|---|---|---|---|
| $IC_{50}$ for octreotide | $>1$ $\mu$M | 0.4 nM | 35 nM | $>1$ $\mu$M | 7.0 nM |

Recently, several reports have been published on the in vitro binding to somatostatin receptors of another somatostatin analogue, the octapeptide RC-160, which structure is very similar to that of octreotide (figure 1) (18-20). It has been reported that RC-160 has a higher affinity than octreotide for the somatostatin receptor in human breast, ovarian, exocrine pancreatic, prostatic and colonic cancer (18-20). For instance, the possible binding of RC-160 to a somatostatin receptor subtype on human exocrine pancreatic adenocarcinoma, which does not bind octreotide (21), offers a potential advantage for RC-160 over octreotide.

Nearly one-third of human malignant gliomas have an intact blood-brain barrier to contrast agents, and virtually all malignant gliomas and probably most brain metastases have areas of tumour with an intact blood-brain barrier (23-27).

Unlabelled RC-160 may, in contrast to octreotide (22), also pass the intact blood-brain barrier (28). In addition, radiolabelled RC-160 could also represent a benefit in visualizing somatostatin receptor-positive brain tumours with an intact blood-brain barrier. The identification of several somatostatin receptor subtypes enables the development of subtype specific somatostatin analogues, which can be used in the diagnosis and selective treatment of somatostatin receptor-positive lesions (29). Therefore, the eventual application of this new somatostatin analogue will open possibilities, even as a new somatostatin receptor subtype-specific radiopharmaceutical.

**Somatostatin**

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

**Octreotide**

D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

**[$^{123}$I-Tyr$^{3}$]octreotide**

D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr(ol)
|
$^{123}$I

**[$^{111}$In-DTPA-D-Phe$^{1}$]octreotide**

$^{111}$In-DTPA-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

**$^{123}$I-RC-160**

D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-$NH_2$
|
$^{123}$I

**[$^{111}$In-DTPA-D-Phe$^{1}$]RC-160**

$^{111}$In-DTPA-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-$NH_2$

**Figure 1.** *Sequence of somatostatin and some analogues*

**1.2 Attempts to increase the uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in somatostatin receptor-positive tissues by varying mass, specific activity, and by priming with somatostatin analogues.**

In general, in in vitro experiments involving saturable processes (i.e. radioimmunoassays and receptor binding studies) the signal to noise (read the target to background) ratio is often improved by lowering the mass of the radiotracer and/or by increasing its specific radioactivity. In previous studies (30), we reported the visualization of somatostatin receptor-positive tumours in rats by gamma camera scintigraphy using [$^{111}$In-DTPA-D-Phe$^1$]octreotide (see figure 1). In those studies the administered mass of peptide and the radioactive dose were such that >90 procent of the ligand was unlabelled. Theoretically, the presence of unlabelled [DTPA-D-Phe$^1$]octreotide may have a negative effect on the % dose uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in somatostatin receptor-positive tissues due to competition with the same receptor. After the first labelling of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in 1987, the composition of the commercially available $^{111}InCl_3$ has improved. The concentration of the non-radioactive nuclides, competing with $In^{3+}$ for the DTPA-group, has decreased. Therefore the specific radioactivity could be increased 5-fold up to 185 MBq $^{111}$In per $\mu$g [DTPA-D-Phe$^1$]octreotide. We hypothesized that receptor scintigraphy would also show an optimal target to background ratio at the lowest possible mass of peptide with the highest specific radioactivity. Consequently, this would result in a more sensitive imaging technique. In the study presented in Chapter 3, this hypothesis was investigated in rats.

Furthermore, several reports (31,32) suggested a positive effect of the prior administration of unlabeled peptide on the percentage dose uptake of its radioactive counterpart. Therefore, we investigated the effect of intravenous administration of 2 or 10 $\mu$g unlabeled octreotide or of 2 or 10 $\mu$g unlabeled [DTPA-D-Phe$^1$]-octreotide at various time points relative to the injection of [$^{111}$In-DTPA-D-Phe$^1$]-octreotide on the uptake of radioactivity in somatostatin receptor-positive and -negative tissues.

**1.3 Internalization of radiolabelled [$^{125}$I-Tyr$^3$]octreotide and [$^{111}$In-DTPA-D-Phe$^1$]octreotide**

The fate of labelled somatostatin analogues after binding to their receptors is largely unknown. However, this knowledge is essential for a proper choice of radionuclides to label somatostatin analogues for radiotherapy. As will be discussed in detail in section **1.6 Radiotherapeutical considerations** various radionuclides suitable for radiotherapy, e.g. conversion, Auger, and Coster-Kronig electron emitters, are only effective in a short distance of only a few nm up to $\mu$m from their target, e.g. DNA. Radiotherapy achieves its principal effects on both tumours

and healthy normal tissues by the destruction of proliferating cells. This is thought to be the result from damage to cellular DNA caused by ionizing irradiation, especially the production of double strand breaks (33).

Equivocal data have been described with respect to internalization of $SS_{14}$. Receptor-mediated endocytosis of $SS_{14}$ has been demonstrated in rat anterior pituitary cells and in rat islet cells (34-41), whereas other investigators found that [$^{125}$I-Tyr$^{1}$]$SS_{14}$ and [$^{125}$I-Tyr$^{11}$]$SS_{14}$ are not rapidly internalized by $GH_4C_1$ rat pituitary cells and RINm5F insulinoma cells, respectively, probably due to degradation of these radioligands at the cell surface (42,43). Data with respect to metabolism and internalization of somatostatin may have been influenced by the susceptibility of the somatostatin ligands to degradation. Investigations with more stable radioligands, like [$^{125}$I-Tyr$^{3}$]octreotide (44), provide a better insight in the extent of internalization of radiotherapeuticals by somatostatin receptor-positive (tumour) cells.

In the study presented in Chapter 4, it is therefore investigated whether the stable somatostatin analogue [$^{125}$I-Tyr$^{3}$]octreotide is internalized by somatostatin receptor-positive AtT20/D16V mouse pituitary tumour cells and by human GH-secreting pituitary tumour cells. As presented in Chapter 3, we recently found that accumulation of [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide in somatostatin receptor-positive organs showed a tissue-specific bell-shaped function of the injected mass of the radiopharmaceutical. In order to investigate the cellular mechanisms underlying these observations, we also studied the effect of different concentrations unlabelled octreotide on binding and internalization of [$^{125}$I-Tyr$^{3}$]octreotide by the two cell culture systems.

Although still under investigations, some considerations on the internalization of [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide are discussed. We found that during the first 30 minutes after the injection of [$^{123}$I-Tyr$^{3}$]octreotide or [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide in rats specific binding takes place in somatostatin receptor-positive tissues and tumours (ref. 45 and Chapter 6). However, the residence time of both radionuclides in somatostatin receptor-positive tissues and tumours was completely different. Twenty-four hours after the injection of the radiopharmaceutical release of radioactivity from somatostatin receptor-positive tissues and tumours is slower with [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide than with [$^{123}$I-Tyr$^{3}$]octreotide. These results indicate the difference in fate for the two radiopharmaceuticals. The differences likely reflect the different biochemical pathways for iodotyrosine-labelled versus $^{111}$In-DTPA-labelled peptides. Several pathways for iodotyrosine-labelled peptides have been described. LaBadie et al. found that $^{125}$I-labelled asialofetuin (which still has intact affinity for the glycoprotein receptor) was rapidly degraded to mono- and diiodotyrosine in the lysosome which were subsequently deiodinated by a cytoplasmic enzyme (46). Of course this metabolic pathway does not apply to [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide.

As discussed in Chapter 3, in vivo displacement of somatostatin receptor-positive tissue-associated radioactivity is observed when unlabelled ligand is injected 10 min after the radioligand, but not after 20 min, suggesting significant internalization of radiopharmaceutical between 10 and 20 min after injection. This might be the first in vivo and in vitro indication that [$^{111}$In-DTPA-D-Phe$^1$]octreotide is indeed internalized. $^{111}$In-DTPA-labelled receptor targeted proteins are transported to lysomes and the ultimal product $N_\epsilon$-$^{111}$In-DTPA-lysine is trapped in the lysosome (47,48). Receptor-mediated internalization of [$^{111}$In-DTPA-D-Phe$^1$]octreotide will most probably result in degradation to $^{111}$In-DTPA-D-Phe, and this metabolite is also not capable passing the lysosomal and/or other cell membrane(s) (47).

Receptor-mediated endocytosis systems have been described (49,50) where cell surface receptors capture their ligands from the extracellular milieu. The receptor-ligand complex is internalized via invagination of the plasma membrane. The resulting intracellular vesicles, termed endosomes, rapidly acidify, which causes the ligand to dissociate from the receptor, see also figure 2. The ligand is delivered to the lysosome (47) and the receptor recycles back to plasma membrane. The whole process takes approximately 15 min (49), and a single receptor can deliver numerous ligand molecules to the lysosomes. This metabolic pathway of receptor-mediated endocytosis may also hold for the ligand [$^{111}$In-DTPA-D-Phe$^1$]octreotide and its octreotide receptor.

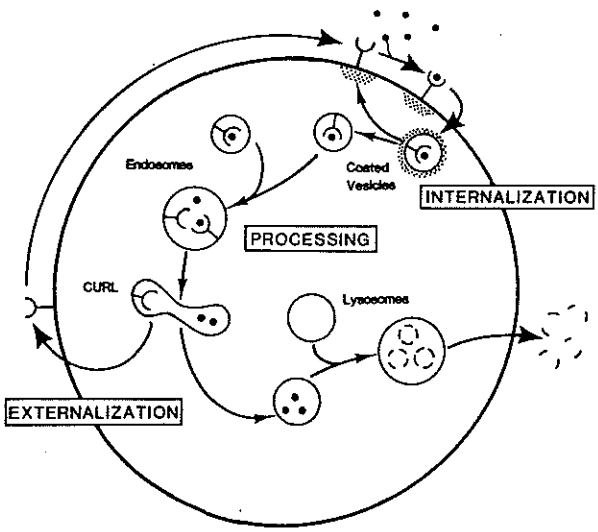


**Figure 2.** *A "basic" model for receptor-mediated endocytosis and receptor recycling (50)*

## 1.4 Blood-brain barrier

Receptors for circulating peptides of gastroenteropancreatic or neural origin (the neuropeptides) are largely located on the plasma membrane of target tissues (51). Owing to the aqueous pores (fenestrae) in capillary walls (52), small peptides (e.g. molecular weight 5 kD or less) readily gain access to the interstitial space and to the receptors on the plasma membrane of the target cells. Unlike the capillaries in any other organ, the microvessels in brain of all vertebrates are characterized by high resistance, epithelial-like tight junctions that essentially cement brain endothelial cells together (53). The barrier formed by the tight junctions of endothelial cells, the blood-brain barrier, prevents all water-soluble substances from entering the brain interstitium from blood (54). It is clear that this blood-brain barrier is an important limiting factor in the application of neuropeptides or neurotransmitters as radiopharmaceutical in brain scintigraphy (23-27,55-59). Although unlabelled and radioiodinated RC-160 may, in contrast to octreotide, pass the blood-brain barrier (21,22), the DTPA-group in the radiopharmaceutical [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160 (Chapter 6) increases its polarity and diminishes its blood-brain barrier permeability. Up to date no DTPA-containing radiopharmaceutical has been reported to pass the blood-brain barrier in amounts suitable for gamma camera scintigraphy.

## 1.5 Substance P

Several radiolabelled peptides are potentially applicable for receptor scintigraphy, as long as they will meet at least the following characteristics:

a. high-affinity and specificity for a characteristic and unique receptor,
b. intact biological activity after radiolabelling, and
c. stability in vivo.

The receptor itself should have an increased expression at the cell surface of tumours or specific pathological processes. In that order, the newly synthesiszed [DTPA-Arg$^{1}$]substance P was investigated.

In 1931, von Euler and Gaddum extracted a potent hypotensive and contractile agent (on smooth muscle) from equine brain and gut (60). This compound was named substance P, where the "P" stands for the "powder" obtained after their extraction procedure. The exact chemical structure of substance P, shown in figure 3, remained unknown for over 40 years. The chemistry, tissue distribution, and pharmacological actions of substance P have been studied extensively (for reviews see refs. 61-63). The term tachykinin was introduced to describe members of the peptide family because of their relatively rapid initiation of smooth muscle contraction, compared with the slower-acting bradykinins. The tachykinin ligands comprise a family of low molecular weight peptide neurotransmitters that share a common C-terminal amino acid sequence, -Phe-X-

Gly-Leu-Met-$NH_2$, in which X represents either Phe, Ile, Tyr or Val (64). The mammalian tachykinins are synthesized primarily by neurons and they include substance P, neurokinin A, neurokinin B, and two N-terminally extended forms of neurokinin A - neuropeptide K and neuropeptide γ. Neurokinin A and neurokinin B are also referred to as substance K and neuromedin K, respectively.

Currently accepted tachykinin receptor nomenclature defines three homologous receptor types: the substance P-preferring $NK_1$ receptor, the neurokinin A-preferring $NK_2$ receptor and the neurokinin B-preferring $NK_3$ receptor. The $NK_1$ receptor is present in both brain and peripheral tissues, and substance P has the highest affinity ($K_d$ 0.5-1 nM) for this receptor. The $NK_1$ receptor has recently been cloned, and the deduced amino acid sequence, 407 residues, contains seven putative membrane-spanning domains and shows a sequence similar to other members of the G-protein coupled receptor family (65). The tachykinin receptors have a wide tissue distribution, and interaction with their ligands is associated with diverse responses such as sensory neurotransmission, immunological responses, both contraction and endothelium-dependent relaxation of vascular smooth muscle, nociception, histamine release, plasma extravasation, inflammation, sexual behaviour and, potentially, nerve regeneration and wound healing. For extensive reviews on the history, nomenclature, synthesis and degradation of tachykinins. the reader is referred to refs.:64 and 65.

**Substance P**

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$

**[$^{111}$In-DTPA-Arg$^1$]substance P**

$^{111}$In-DTPA-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$

**Figure 3**. *Sequence of substance P and [$^{111}$In-DTPA-Arg$^1$]substance P*

The involvement of substance P in the carcinoid syndrome (66) and vascular (migraine) headache (67) has been suggested. In rats with adjuvant arthritis a high concentration of substance P was found in the synovial fluid and a high density of high-affinity substance P receptors in synovial tissue (68). These findings were confirmed in patients with rheumatoid arthritis (69). In addition, very high levels of substance P receptor are expressed in the germinal center of

lymph nodes (70), and on a human astrocytoma cell line (71). Large numbers of high-affinity substance P receptor are found in surgical specimens obtained from patients with inflammatory bowel disease. The substance P-binding $NK_1$ receptor is expressed by arterioles and venules located in the submucosa, muscularis mucosa, external longitudinal muscle, and serosa (70,72).

Consequently, in analogy with the development of [DTPA-D-Phe$^1$]-octreotide and [DTPA-D-Phe$^1$]RC-160, the newly synthesized [DTPA-Arg$^1$]-substance P was prepared and investigated for its binding characteristics in vitro and in vivo.

**Table 2.** *Radionuclides for therapy: physical properties*

| Nuclide | Half-life | $E_\beta$max (keV) | Maximum range |
|---|---|---|---|
| $^{131}$I | 8 days | 606 | 2.4 mm |
| | | 46-330 (IC) | 32-850 $\mu$m |
| | | 0.9-3.1 (Auger) | 0.05-0.33 $\mu$m |
| $^{90}$Y | 64 hours | 2270 | 12 mm |
| $^{186}$Re | 91 hours | 930-1070 | 3.6-4.3 mm |
| | | 53-135 (IC) | 42-200 $\mu$m |
| | | 0.24-7.1 (Auger) | 0.009-1.3 $\mu$m |
| $^{32}$P | 14 days | 1710 | 8.7 mm |
| $^{161}$Tb | 6.9 days | 135-180 | 0.8 mm |
| | | 3-66 (IC) | 0.4-50 $\mu$m |
| | | 1-8 (Auger) | 0.1-1.5 $\mu$m |
| $^{67}$Ga | 78 hours | 82-93 (IC) | 90-110 $\mu$m |
| | | 0.05-9.5 (Auger) | 0.002-2.1 $\mu$m |
| $^{111}$In | 68 hours | 144-245 (IC) | 200-550 $\mu$m |
| | | 0.5-25 (Auger) | 0.02-10 $\mu$m |
| $^{125}$I | 60 days | 3.7-35.5 (IC) | 0.4-20 $\mu$m |
| | | 0.7-30 (Auger) | 0.02-15 $\mu$m |

### 1.6 Radiotherapeutical considerations

Historically, the first example of targeted radionuclide therapy was the treatment of well differentiated thyroid carcinoma with ionic $^{131}$I. This exploits the almost unique property of thyroid cells to concentrate iodide in ionic form (73). The radiotherapeutical treatment of neuroblastomas or catecholamine-producing

phaechromocytomas with the iodocompound $^{131}$I-mIBG (meta-iodo-benzyl-guanidine), which is preferentially taken up by catecholamine-synthesizing cells of the sympathetic nervous tissues, is well established (74). Successful therapy of lymphomas with $^{131}$I-labelled specific antibodies has been reported (75,76). As soon as the success of peptide receptor scintigraphy for tumour visualization became clear, the next step was to label these peptides with $\alpha$- or ß-particles emitters or with Auger, Coster-Kronig or conversion electrons-emitting radionuclides and to perform radiotherapy with these radiolabelled peptides.

A (mono-energetic) conversion electron arises when the atomic nucleus transfers energy to one of its surrounding electrons, enabling it to leave the atom (77). The resulting vacancy is filled by electrons from higher orbitals, which may result in emission of an Auger electron (78) or an Coster-Kronig electron (79). In addition, Auger and Coster-Kronig electrons may arise when vacancies in the electron orbitals is caused by electron capture, in which an orbital electron is captured by the nucleus. For a detailed review on this subject the reader is referred to Bambyrek et al. (77) and to Howell (80).

The applicabality of peptides labelled with nuclides suitable for radiotherapy has to meet at least the following characterisitics:

a. high purity of the radionuclide since only a limited amount of the (radio)ligand can be administered,
b. stability of the radioligand in vivo,
c. a high affinity of the radioligand for its specific receptor, and
d. a long residence time (biological t½ > physical t½) of the radioactivity in/on the target tissue.

Even when these characteristics are met, it is well-known that the empiric in vivo findings will often differ from the calculated estimated dose, largely due to the current short-comings of microdosimetry. For instance, the inhomogeneity of the receptors throughout the target and the potential internalization of the ligand, and the (sub)cellular localisation of the radionuclide are unknown factors (73). Since the energy of conversion, Auger, and Coster-Kronig electrons is usually less than that of ß-particles (74), their Linear Energy Transfer (LET) and consequently their cell kill probability is larger than that for ß-particles. When a radionuclide with a high LET is located within a few nm of the DNA, its radio-toxicity is very high. Therefore, the importance of a given level of heterogeneity depends on the radionuclide. Heterogeneity will be most serious for short-range $\alpha$-emitters and Auger emitters and least serious for long range ß-emitters (73).

For recent reviews on microdosimetry and the radiobiological effectiveness and quality factor for Auger emitters the reader is referred to refs. 73,81-85. The heterogeneity of radionuclide deposition and the short-comings of the MIRD calculations with nonuniform distribution of radioactivity have also been discussed

(refs. 73 and 70, respectively).

Empirical findings from in vitro cell survival experiments demonstrated that intracellular $^{111}$In resulted in a $10^9$-fold higher radiation dose to the cell nucleus than extracellular localized $^{111}$In (86). For most of the peptide-bound radionuclides, theoretically suitable for radiotherapy, the target distribution, their metabolism and consequently the (sub)cellular distribution are still unknown. For a recent review on this subject the reader is referred to Adelstein (87). Positive in vitro results on the therapeutic application of the Auger and conversion electron emitter $^{67}$Ga have been reported (88,89). Dividing cells differ greatly in their response to external beam irradiation, radiosensitivity, and this is probably also true with respect to radionuclide therapy (33).

The usefulness of [DTPA-D-Phe$^1$]octreotide, labelled with the Auger electron emitter $^{111}$In or with the ß-emitter $^{161}$Tb, which both are potentially suitable for radiotherapy is discussed in the appendix papers 1 and 2, respectively. In appendix paper 3 various options are discussed to prepare radiolabelled peptides for receptor-targeted radiotherapy. The application of the well-known ß-emitter $^{131}$I is compared with that of various other candidate radionuclides for therapy.

Various of the above-mentioned data are derived from references 90-92.

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## CHAPTER 2

## AIM OF THE STUDIES

Octreotide receptor scintigraphy is performed at the University Hospital Dijkzigt in Rotterdam since 1987. Initially with [$^{123}$I-Tyr$^3$]octreotide, later with [$^{111}$In-DTPA-D-Phe$^1$]octreotide. To date 1400 patients with various forms of cancer, both classically known as neuroendocrine and non-neuroendocrine have been investigated. Another group of 150 patients with various (auto)immune diseases have also been investigated.

In general in in vitro experiments high specific activities of the radioligand will lead to an increase in sensitivity of detection of either low abundance or low affinity receptor sites. Therefore, since the quality of the commercially available $^{111}InCl_3$ has improved, the possibility was opened to increase the specific activity of [$^{111}$In-DTPA-D-Phe$^1$]octreotide 5-fold. We hypothesized that receptor scintigraphy is best performed using the lowest possible mass with the highest specific activity. In chapter 3 this hypothesis is challenged.

Five different somatostatin receptor subtypes have been cloned sofar. As will be explained in detail in Chapters 3, 4 and 7, the affinity of the somatostatin analogue octreotide for these five different somatostatin receptors subtypes varies. Several reports suggested a higher affinity for some of these subtypes by another somatostatin analogue than octreotide: RC-160. This peptide and its newly synthesized analogue, [DTPA-D-Phe$^1$]RC-160, were radiolabelled and compared in vivo and in vitro with radiolabelled [Tyr$^3$]octreotide and [DTPA-D-Phe$^1$]octreotide, as presented in Chapter 5, 6 and 7.

In analogy of the developments of the above-mentioned radiopharmaceuticals, the potential application of another neuropeptide, substance P, and its newly synthesized analogue, [DTPA-Arg$^1$]substance P, have been investigated for receptor scintigraphy and are presented in Chapter 8 and 9.

As soon as the success of peptide receptor scintigraphy for the visualization of pathological lesions became clear, the following step was the labelling of these peptides with $\alpha$- or ß-particles emitters or with Auger, Coster-Kronig or conversion electrons-emitting radionuclides. Several aspects using peptides labelled with such radionuclides were investigated, as presented in the appendix papers 1, 2 and 3.

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## CHAPTER 3

# THE UNEXPECTED EFFECTS OF DOSE AND SPECIFIC RADIOACTIVITY ON TISSUE DISTRIBUTION OF [$^{111}$In-DTPA-D-Phe$^1$]OCTREOTIDE IN RATS. ATTEMPTS TO OPTIMIZE THE TARGET TO BACKGROUND RATIO

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## ABSTRACT

In order to increase the target to background ratio in receptor scintigraphy, we hypothesized that receptor scintigraphy is best performed using the lowest possible mass with the highest possible specific radioactivity of the radioligand. Since the non-radiochemical composition of the commercially available $^{111}InCl_3$ has been improved after it was first used to label to [DTPA-D-Phe$^1$]octreotide, it was possible to increase the specific radioactivity 5-fold up to 185 MBq $^{111}$In per $\mu$g [DTPA-D-Phe$^1$]octreotide. In the present study we have challenged this hypothesis and investigated the effect of the injected mass and specific radioactivity of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in rats. Uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide showed a bell-shaped function of the injected mass; the optimal mass differed between the somatostatin receptor-positive tissues studied. In contrast, varying specific radioactivity at a constant mass had no significant effect. Moreover, varying mass and specific radioactivity had no effect on the percentage dose uptake in somatostatin receptor-negative organs. Therefore, depending on the tissue, the target to background ratio can be increased by varying the injected mass.

Several reports suggested a positive effect of the prior administration of unlabelled peptide on the percentage dose uptake of its radioactive counterpart. Therefore, we investigated the effect of intravenous administration of 2 or 10 $\mu$g unlabelled octreotide or of 2 or 10 $\mu$g unlabelled [DTPA-D-Phe$^1$]octreotide from

10 min before to 20 min after the injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide. In some of these settings there was a significant increase in the uptake of $^{111}$In in somatostatin receptor-positive organs, in others there was a significant decrease. Since no significant differences were found in background radioactivity and in the percentage dose uptake of $^{111}$In in somatostatin receptor-negative organs, these data indicate that the target to background ratio can be increased by the administration of non-radiolabelled peptide under selected conditions.

In conclusion, in contrast to our hypothesis that receptor scintigraphy shows an optimal target to background ratio at the lowest possible mass of the radiopharma-ceutical with the highest specific radioactivity, we found that the uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in somatostatin receptor-positive organs shows a tissue-specific bell-shaped function of the injected mass of the radiopharmaceutical. This might also apply to somatostatin receptor-positive tumours, the visualization of which may be enhanced by optimizing the mass of [$^{111}$In-DTPA-D-Phe$^1$]octreotide.

## INTRODUCTION

In previous animal studies we reported the visualization by gamma camera scintigraphy of somatostatin receptor-positive tumours in rats by [$^{111}$In-DTPA-D-Phe$^1$]octreotide (1). In those studies the administered mass of [$^{111}$In-DTPA-D-Phe$^1$]octreotide varied between 0.5 and 1 $\mu$g and the radioactive dose was kept constant at 18.5 MBq $^{111}$In. However, the non-radioactive composition of $^{111}InCl_3$ has improved after the first labelling of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in 1987. Therefore, it was possible to increase the specific radioactivity 5-fold up to 185 MBq $^{111}$In per $\mu$g [DTPA-D-Phe$^1$]octreotide, which allows the administration of smaller masses of peptide with the same radioactive dose. We hypothesized that receptor scintigraphy shows an optimal target to background ratio at the lowest possible mass of peptide with the highest specific radioactivity, and this could well result in a more sensitive imaging technique. In the present study this hypothesis and the effects of mass and specific radioactivity of [$^{111}$In-DTPA-D-Phe$^1$]octreotide on the percentage dose uptake and specific binding in several organs were investigated in rats.

Therapy with unlabelled octreotide may have a negative influence on the target to background ratio in [$^{111}$In-DTPA-D-Phe$^1$]octreotide scintigraphy. Surprisingly, Dörr et al. recently reported improved visualization of carcinoid liver metastases by [$^{111}$In-DTPA-D-Phe$^1$]octreotide scintigraphy during treatment with a subcutaneous dose of 600 $\mu$g per day of octreotide (2,3). However, the mechanism(s) and the effect(s) of pretreatment and/or concomitant therapy with unlabelled ligand on receptor scintigraphy have yet not been studied in detail. Somatostatin receptors are structurally related integral membrane glycoproteins. Recently, five different human somatostatin receptor types have been cloned. All subtypes

bind native somatostatin-14 ($SS_{14}$) and $SS_{28}$ (pro-somatostatin with 28 aminoacids) with high affinity, while their affinity for numerous somatostatin analogues differ considerably (4-7). Octreotide binds with high affinity to the SSTR2 (somatostatin receptor type 2) subtype, while this analogue has a relatively low affinity for SSTR3 and SSTR5 and shows no binding to SSTR subtypes 1 and 4 (4-7). Octreotide scintigraphy is therefore based on the visualization of octreotide-binding somatostatin receptors (octreotide receptors), most probably the SSTR2. In the present study, therefore, we also investigated the tissue distribution of $^{111}$In in octreotide receptor-positive (i.e. pituitary, adrenal and pancreas) and octreotide receptor-negative tissues (i.e. kidneys, spleen, liver and soft tissue (thigh) muscle) (1,8) 24 hours after the injection of 0.5 $\mu$g [DTPA-D-Phe$^1$]octreotide labelled with 3 MBq $^{111}$In, while at various time points relative to the injection of the radiopharmaceutical additionally unlabelled octreotide or [DTPA-D-Phe$^1$]octreotide were administered intravenously.

## MATERIALS AND METHODS

*Radiolabelling and Quality Control of the Radiopharmaceutical*

[DTPA-D-Phe$^1$]octreotide and $^{111}InCl_3$ (DRN 4901, 370 MBq/ml in HCl, pH 1.5 - 1.9) were obtained from Mallinckrodt (Petten, The Netherlands). Thirty minutes after the start of the radiolabelling of [DTPA-D-Phe$^1$]octreotide with $^{111}$In, up to a specific radioactivity of 185 MBq $^{111}$In per $\mu$g [DTPA-D-Phe$^1$]octreotide (molar excess of 5- to 10-fold of peptide over $^{111}$In) the efficiency of labelling was over 98 %. The consecutive quality control were performed as described earlier (9). Although it is not excluded that additional groups of the peptide participate in $^{111}$In complexation, radiolabelled product is referred to as [$^{111}$In-DTPA-D-Phe$^1$]octreotide.

*Tissue Distribution and Specific Binding of [DTPA-D-Phe$^1$]octreotide*

In total 123 male Wistar rats (240-260 g) were used in 3 separate experiments (A, B and C, see below). Rats were anaesthetized with ether, and the radiopharmaceutical and/or additional peptides were injected into the dorsal vein of the penis and/or into a sublingual vein. The volume of injection was kept constant at 0.5 mL per rat.

The radioactivity was measured in a dose calibrator (VDC-202, Veenstra, Joure, The Netherlands). In order to study non-specific binding, the rats were injected subcutaneously with 1 mg octreotide in 1 ml 0.05 M acetic acid in 154 mM NaCl 45 min before with [$^{111}$In-DTPA-D-Phe$^1$]octreotide (10). Specific binding was defined as the difference between the tissue uptake of radioactivity in control rats (total binding) and that in animals treated with excess unlabelled peptide (non-specific binding), expressed as % of the injected radioactivity per gram tissue as described before (10).

The ratio of % dose uptake in tissue over soft tissue (thigh) and tissue over blood were calculated for each individual rat. The rats were sacrificed 24 h after administration of [$^{111}$In-DTPA-D-Phe$^1$]octreotide. Blood was collected, and the octreotide receptor-positive anterior pituitary gland, adrenals and pancreas as well as octreotide receptor-negative tissues such as kidneys, spleen, liver and thigh were isolated. Tissue and blood radioactivity were determined using a LKB-1282-Compu-gammasystem (10).

*Experiment A: Effects of Varying the Dose and Specific Radioactivity of [$^{111}$In-DTPA-D-Phe$^1$]octreotide on Specific Binding*

Experiments were performed with 18 groups of 3 male Wistar rats (240-260 g). Nine groups of 3 rats each were injected with 0.02, 0.1 or 0.5 $\mu$g [DTPA-D-Phe$^1$]octreotide with specific activities of 18.5, 55.5 or 185 MBq $^{111}$In per $\mu$g [DTPA-D-Phe$^1$]octreotide. Consequently, the radioactive dose varied between 0.37 and 92.5 MBq per rat. Non-specific binding in tissue was determined in 9 parallel groups of 3 rats each, which were injected with 1 mg octreotide 45 min before the different doses of [$^{111}$In-DTPA-D-Phe$^1$]octreotide as described above.

*Experiment B: Effects of Varying Mass of [DTPA-D-Phe$^1$]octreotide at a Constant Radioactive Dose of [$^{111}$In-DTPA-D-Phe$^1$]octreotide*

Because in experiment A both the dose and mass had varied at the same time, we also investigated the effect of varying mass at a constant radioactive dose. Experiments were performed with 5 groups of 3 male Wistar rats (240-260 g), which were injected with 0.02, 0.1, 0.5, 5 or 50 $\mu$g [DTPA-D-Phe$^1$]octreotide labelled with 3 MBq $^{111}$In. Consequently, the specific radioactivity varied between 150 and 0.06 MBq $^{111}$In per $\mu$g [DTPA-D-Phe$^1$]octreotide.

*Experiment C: Effects of Intravenous Injection of Octreotide or [DTPA-D-Phe$^1$]octreotide at Various Time Points Relative to Administration of [$^{111}$In-DTPA-D-Phe$^1$]octreotide*

Sixteen groups of 3 male Wistar rats (240-260 g) each were injected with 2 or 10 $\mu$g octreotide or [DTPA-D-Phe$^1$]octreotide at -10, 0, 10 and 20 min relative to the injection of 0.5 $\mu$g [DTPA-D-Phe$^1$]octreotide labelled with 3 MBq $^{111}$In. A group of 6 rats injected with radiopharmaceutical only was used as controls. The values of % dose uptake per gram tissues in treated animals are expressed as % of those in the control animals.

*Statistical Analysis*

One-way analysis of variance (ANOVA), was used for statistical analysis. Means were compared using Bonferroni's *t*-test or Newman-Keuls method (11). A $P$ value of $<0.05$ was considered significant.

## RESULTS

*Experiment A: Effects of Varying the Dose and Specific Radioactivity of [$^{111}$In-DTPA-D-Phe$^1$]octreotide on Specific Binding*

Significant specific tissue binding of [$^{111}$In-DTPA-D-Phe$^1$]octreotide was observed in the octreotide receptor-positive anterior pituitary gland, adrenals, and pancreas, but not in the octreotide receptor-negative liver, spleen, kidneys or soft tissue (1) (data not shown).

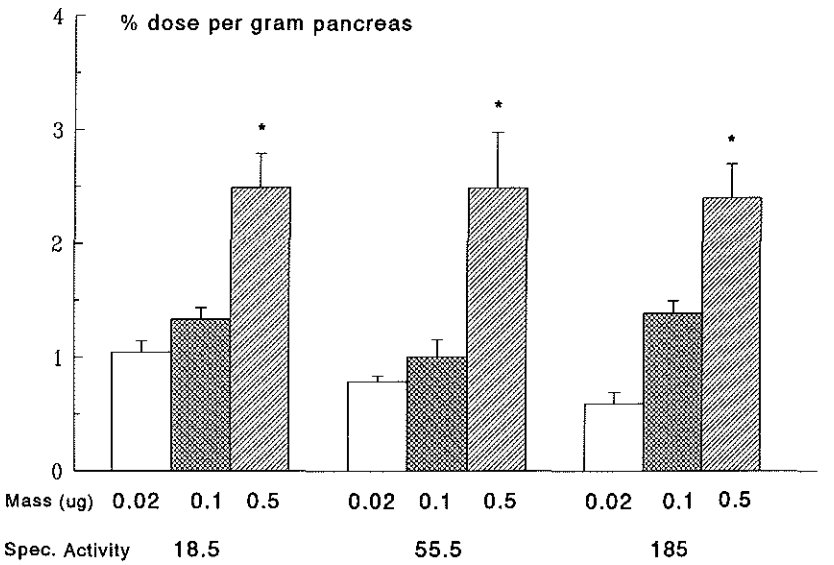


**Figure 1**. *Effects of varying mass of [DTPA-D-Phe$^1$]octreotide (0.02, 0.1 and 0.5 μg) and specific radioactivity (18.5, 55.5 and 185 MBq per μg) on specific binding of [$^{111}$In-DTPA-D-Phe$^1$]octreotide, expressed as % injected dose of radioactivity per g tissue 24 h after injection, (n=3, mean + SD).*
** $P < 0.05$ ; significantly different from 0.02 μg*

Figure 1 shows the effects of mass and specific activity on the specific binding of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in the pancreas. A significantly higher % dose uptake is observed at increasing mass in the range of 0.02, 0.1 and 0.5 μg of [DTPA-D-Phe$^1$]octreotide independent of the specific radioactivity, varying between 18.5 and 185 MBq $^{111}$In per μg [DTPA-D-Phe$^1$]octreotide. Under these conditions the pancreas to soft tissue ratio showed a similar pattern as depicted in Figure 1 (data not shown). Similar patterns in specific binding, although not statistically significant, were found for the adrenals and the anterior pituitary gland (data not shown).

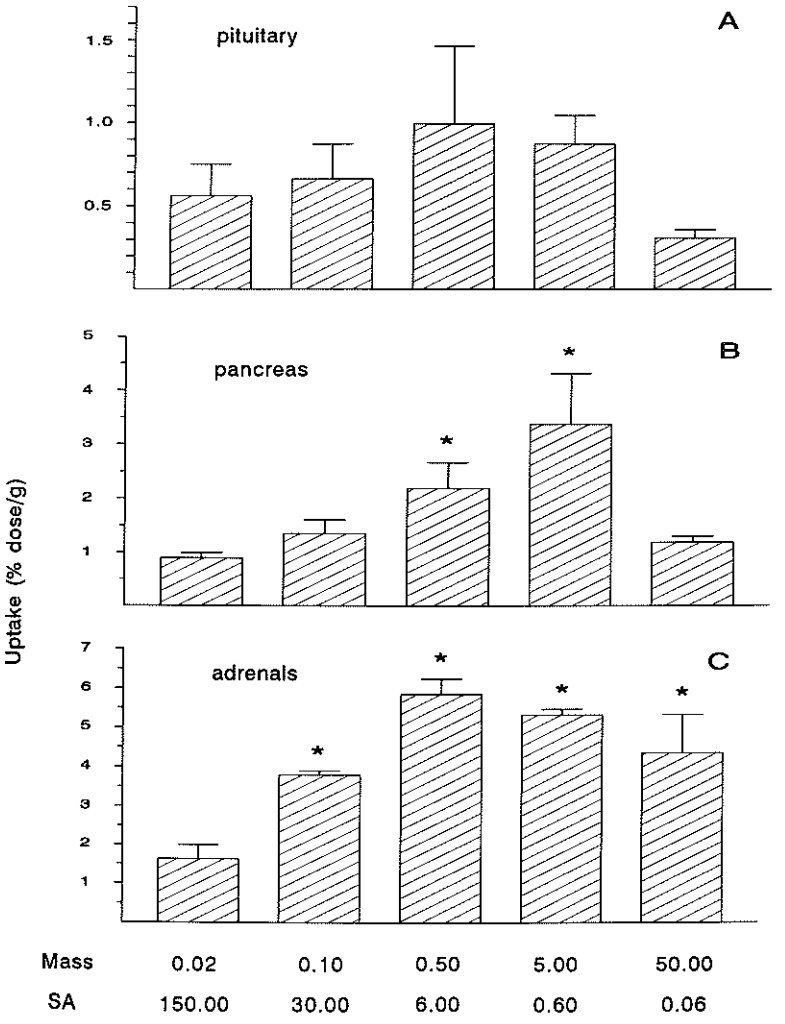


**Figure 2.** *Effects of varying mass of [DTPA-D-Phe$^1$]octreotide labelled with a constant amount (3 MBq) of $^{111}$In on the 24 h uptake of radioactivity in anterior pituitary (A), pancreas (B) and adrenals (C), expressed in % injected dose of radioactivity per gram tissue, (n=3, mean + SD).*
** P <0.05 ; significantly different from 0.02 μg*

*Experiment B: Effects of Varying Mass of [DTPA-D-Phe$^1$]-octreotide at a Constant Radioactive Dose of [$^{111}$In-DTPA-D-Phe$^1$]octreotide*

At a constant radioactive dose of 3 MBq [$^{111}$In-DTPA-D-Phe$^1$]octreotide, there was a biphasic response, i.e. an initial increase followed by a decrease, in a

% dose uptake in the octreotide receptor-positive organs, when the mass of injected [DTPA-D-Phe$^1$]octreotide was increased from 0.02- 50 $\mu$g. The optimum was 0.5 $\mu$g for the anterior pituitary gland (although not significantly different from the other masses), and 5 $\mu$g for the pancreas and 0.5 $\mu$g for the adrenals (Figs. 2 A, B and C). It is remarkable that the % dose uptake in the adrenals is still strongly increased at 50 $\mu$g compared with 0.02 $\mu$g peptide, and in contrast to pituitary and pancreas. The corresponding tissue to soft tissue ratio showed similar profiles (data not shown). No significant differences were found in the ratio of radioactivity in the liver, spleen, blood or kidney versus soft tissue (data not shown).

*Experiment C: Effects of Intravenous Injection of Octreotide or [DTPA-D-Phe$^1$]octreotide at Various Time Points Relative to Administration of [$^{111}$In-DTPA-D-Phe$^1$]octreotide*

As shown in Figure 3A, the administration of 2 or 10 $\mu$g octreotide or 2 or 10 $\mu$g [DTPA-D-Phe$^1$]octreotide 10 min prior to the administration of 0.5 $\mu$g [DTPA-D-Phe$^1$]octreotide labelled with 3 MBq $^{111}$In (further referred to as radiopharmaceutical) resulted in significantly lower % dose uptake values in the octreotide receptor-positive anterior pituitary gland. Coinjection of 10 $\mu$g [DTPA-D-Phe$^1$]octreotide or 2 or 10 $\mu$g octreotide with the radiopharmaceutical also significantly lowered the % dose uptake in the pituitary, but coinjection of 2 $\mu$g [DTPA-D-Phe$^1$]octreotide was without effect. Injected 10 min after the radiopharmaceutical, a significant lowering of % dose uptake was observed with 2 or 10 $\mu$g octreotide, and 20 min after the radiopharmaceutical only with 2 $\mu$g octreotide but not with 10 $\mu$g octreotide.

Figure 3B shows significantly higher % dose uptake values in the pancreas after the administration of 2 $\mu$g [DTPA-D-Phe1]octreotide at 0 or 20 min or of 10 $\mu$g [DTPA-D-Phe$^1$]octreotide at -10, 0 or 10 min relative to the injection of the radiopharmaceutical. Figure 3B also shows the significantly lower % dose uptake of radioactivity in the pancreas after the administration of 10 $\mu$g octreotide at -10, 0, and 10 min, but not at 20 min post injection of the radiopharmaceutical.
Figure 3C shows that after the administration of 10 $\mu$g [DTPA-D-Phe$^1$]octreotide at -10, 0 and 20 min relative to the injection of the radiopharmaceutical the adrenal % dose uptake was significantly lowered. However, after the administration of 2 $\mu$g [DTPA-D-Phe$^1$]octreotide 10 and 20 min after injection of the radiopharmaceutical there was a significantly higher % dose uptake in the adrenal.

Figure 3C also presents the significantly lower adrenal % dose uptake after the administration of 2 or 10 $\mu$g octreotide at -10, 0 and 10, but not at 20 min post injection of the radiopharmaceutical.

Since the % dose uptake in all the measured octreotide receptor-negative tissues was unaffected by the intravenous administration of 2 or 10 $\mu$g octreotide or 2 or 10 $\mu$g [DTPA-D-Phe$^1$]octreotide, the calculated ratio of % dose uptake in octreotide receptor-positive tissue *versus* blood or *versus* soft tissue gave similar results as those presented in Figure 3 (data not shown).

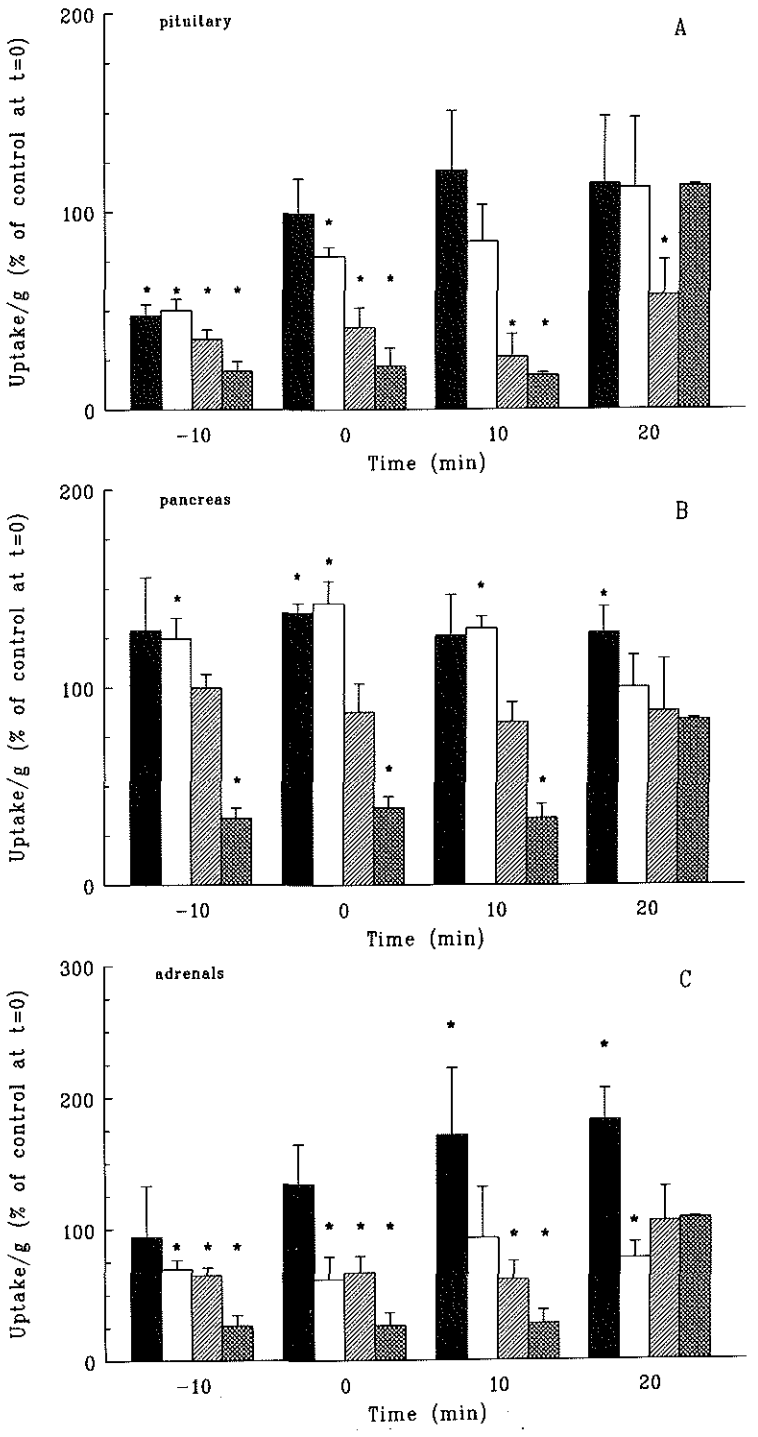
pituitary
A
Uptake/g (% of control at t=0)
200
100
0
-10
0
10
20
Time (min)
pancreas
B
Uptake/g (% of control at t=0)
200
100
0
-10
0
10
20
Time (min)
adrenals
C
Uptake/g (% of control at t=0)
300
200
100
0
-10
0
10
20
Time (min)

**Figure 3.** *Effects of intravenous administration of 2 (black bar) and 10 (open bar) μg [DTPA-D-Phe1]octreotide and 2 (hatched) and 10 (cross-hatched) μg octreotide at indicated time intervals relative to the injection of 0.5 μg (3 MBq) [$^{111}$In-DTPA-D-Phe$^1$]octreotide on the 24 h uptake of radioactivity in anterior pituitary (A), pancreas (B) and adrenals (C). Values are expressed as % dose per g tissue (n=3) relative to that in control rats (100%, n=6). The control values (in % dose per g tissue) were 0.90 ± 0.20 for the pituitary, 2.2 ± 0.3 for the pancreas and 5.0 ± 1.1 for the adrenals. * P <0.05 ; significantly different from control*

## DISCUSSION

In previous animal studies we reported the visualization by gamma camera scintigraphy of octreotide receptor-positive tumours in rats with [$^{111}$In-DTPA-D-Phe$^1$]octreotide (1). In those studies the administered mass of [$^{111}$In-DTPA-D-Phe$^1$]octreotide varied between 0.5 and 1 μg while the radioactive dose was kept constant. However, the non-radioactive composition of $^{111}InCl_3$ has improved after our first preparation of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in 1987. Therefore, it was possible to increase the specific radioactivity 5-fold up to 185 MBq $^{111}$In per μg [DTPA-D-Phe$^1$]octreotide. This increased specific radioactivity would allow the administration of smaller masses of peptide with the same radioactive dose. We hypothesized that receptor scintigraphy shows an optimal target to background ratio at the lowest possible mass of peptide with the highest specific radioactivity, resulting in a more sensitive imaging technique.

To test this hypothesis we investigated whether varying specific radioactivity of [$^{111}$In-DTPA-D-Phe$^1$]octreotide results in changes in the specific and non-specific binding in either octreotide receptor-positive or octreotide receptor-negative tissues, and, consequently, in an altered target to background ratio. We evaluated these parameters by varying the mass and the radioactive dose of [$^{111}$In-DTPA-D-Phe$^1$]octreotide (*Exp. A)*. Unexpectedly, we found that not the lowest possible mass of [DTPA-D-Phe$^1$]octreotide of maximum specific radioactivity was optimal, but that the specific binding to octreotide receptor-positive tissue increased at higher mass of the radiopharmaceutical with an optimum in the low microgram range, depending on the octreotide receptor-positive tissue under study. Since this might also hold for octreotide receptor-positive tumours, an extra parameter has become available to increase the target to background ratio, and hence, the sensitivity to detect such tumours. This was further substantiated by the findings that non-specific binding in the tissues studied did not change with the dose or mass of injected radiopharmaceutical.

Next, we further evaluated the effects of varying mass with a constant radioactive dose of [$^{111}$In-DTPA-D-Phe$^1$]octreotide on the uptake of radioactivity in the octreotide receptor-positive and octreotide receptor-negative tissues (*Exp. B*). In this experiment we found an optimal uptake of radioactivity in the anterior

pituitary gland, the pancreas and the adrenals at 0.5, 5, and 0.5 μg [DTPA-D-Phe$^1$]octreotide, respectively. It remains to be established, however, which peptide mass would be optimal for uptake of $^{111}$In after the administration of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in different octreotide receptor-positive organs and tumours in humans. The reason for these differences in tissue uptake depending on the injected mass of [DTPA-D-Phe$^1$]octreotide has to do with the availability of the radiopharmaceutical to its receptor as well as the processes following the binding of the radiopharmaceutical to its receptor. Relevant factors for receptor accessibilty include the capacity of the radiopharmaceutical to pass biomembranes, the competition by endogenous somatostatin and the rate of blood perfusion of the tissue. For instance the production of somatostatin in the pancreas may contribute to the relatively high optimal dose of 5 μg of [$^{111}$In-DTPA-D-Phe$^1$]octreotide for uptake in the pancreas. This is in contrast to the optimal dose of 0.5 μg for the highly perfused adrenal, which does not produce somatostatin. Other factors include the dissociation constant between the radiopharmaceutical and the receptor, the mode of administration that might influence the concentration and the exposure time of receptor to the radiopharmaceutical, the rate of internalization of the ligand-receptor complex, and the rate of reexpression and/or upregulation of the receptor. All abovementioned parameters illustrate the dynamics and the complexity of the ligand-receptor binding process, in particular in vivo (12).

In a third experiment (*Exp. C),* finally, we evaluated the effects of the intravenous administration of 2 or 10 μg [DTPA-D-Phe$^1$]octreotide or 2 or 10 μg octreotide at various time intervals relative to the injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide on the tissue uptake of $^{111}$In. Also in vitro findings suggest that the optimal ratio between specific and non-specific binding of peptides to the somatostatin receptor-containing cells is not necessarily highest at the lowest ligand concentration. Presky et al. found an increase in the number of somatostatin receptors on $GH_4C_1$ pituitary cells 24 h after the treatment with somatostatin (13). The presented experiments were also performed since we recently observed a rapid, increased internalization of [$^{125}$I-Tyr$^3$]octreotide in normal and tumour pituitary cells by the simultaneous addition of a nanomolar concentration of unlabelled octreotide [Hofland et al. unpublished]. Dörr reported improved visualization of carcinoid liver metastases in patients by [$^{111}$In-DTPA-D-Phe$^1$]octreotide scintigraphy during treatment with a subcutaneous dose of 600 μg of octreotide per day (2,3). These data in patients therefore may well be in accordance with our animal data.

In all the octreotide receptor-positive organs we found a significantly lowered % dose uptake of radioactivity when 10 μg octreotide was administered at -10, 0 and 10 min, but not at 20 min post injection of the radiopharmaceutical. This may be an indication of the limited exposure time of the radiopharmaceutical to its receptor as well as the rate of binding of the radioligand to its receptor and

the subsequent internalization of the peptide-receptor complex. The amount of radioactivity in the octreotide receptor-positive tissues is stable, since no significant differences are found between 4 and 24 h after injection of the radiopharmaceutical (8).

The effects of the administration of octreotide on the inhibition of % uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in the octreotide receptor-positive tissues were more pronounced than the effects of [DTPA-D-Phe$^1$]octreotide administration. This may be due to the difference in affinity between the two somatostatin analogues for the receptor, which is $\approx$ 5-fold lower for [DTPA-D-Phe$^1$]octreotide than for octreotide, as has been demonstrated previously (9). Further possible differences may be due to differences in distribution and metabolism.

In summary, the results of this third experiment indicate that, depending on the type of octreotide receptor-positive tissue, the injection of variable amounts of [DTPA-D-Phe$^1$]octreotide or octreotide at various time points relative to the injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide may be a means to increase the target to background ratio. These mechanisms may be applied to increase the target to background ratio in somatostatin receptor imaging in humans. Preliminairy findings in patients indicate that a specific activity higher than 220 MBq $^{111}$In per 5 $\mu$g [DTPA-D-Phe$^1$]octreotide will lead to decreased quality of scintigraphy, and that uptake in tumours is significantly reduced (14).

## CONCLUSION

In conclusion, in contrast to the hypothesis that the percentage uptake of [DTPA-D-Phe$^1$]octreotide in octreotide receptor-positive tissues is optimal at the lowest possible dose of maximum specific radioactivity, we found in rats that it is a bell-shaped function of the injected mass, being optimal between 0.5 - 5 $\mu$g [$^{111}$In-DTPA-D-Phe$^1$]octreotide. This indicates that the sensitivity of the detection of somatostatin receptor-positive tumour by receptor scintigraphy may be improved by varying the mass of radiopharmaceutical, which has now also been confirmed in patients.

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## CHAPTER 4

# INTERNALIZATION OF A RADIOIODINATED SOMATOSTATIN ANALOGUE, [$^{125}$I- Tyr$^3$]OCTREOTIDE, BY MOUSE AND HUMAN PITUITARY TUMOUR CELLS

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## ABSTRACT

Recently we developed a technique which allows the in vivo visualization in man of somatostatin receptor-positive neuroendocrine tumours after iv injection of [$^{123}$I-Tyr$^3$]octreotide or [$^{111}$In-DTPA-D-Phe$^1$]octreotide. Radiotherapy of such tumours using somatostatin analogues coupled to $\alpha$- or ß-emitting radionuclides has been proposed as an application for radiolabelled somatostatin analogues. In order to develop this concept further it is of importance to know whether the above mentioned radiolabelled somatostatin analogues are internalized by the tumour cells, and whether it might be possible to manipulate the degree of internalization. In the present study we have investigated the internalization of a stable somatostatin analogue, [$^{125}$I-Tyr$^3$]octreotide, by mouse AtT20/D16V pituitary tumour cells and by primary cultures of human GH-secreting pituitary tumour cells.

[$^{125}$I-Tyr$^3$]octreotide showed a time-dependent increasing accumulation in AtT20 cells, with after 4h of incubation, values up to 6-8% of the dose radioligand added. Binding and internalization of [$^{125}$I-Tyr$^3$]octreotide was temperature-dependent. No internalization was observed at 0 °C, at 20 °C only a low amount of radioligand was internalized, while at 37 °C. AtT20 cells showed a high amount of internalization of [$^{125}$I-Tyr$^3$]octreotide. Three out of four human GH-adenoma cell cultures also showed a specific-, time-dependent increasing internalization of [$^{125}$I-Tyr$^3$]octreotide with values of 0.38, 0.54 and 5% of the dose radioligand added.

The simultaneous addition of 1 nM unlabelled octreotide induced a rapid two-fold increase in membrane-binding and internalization of the radioligand in AtT20 cells, while it also significantly increased internalization in two out of three GH-adenoma cell cultures which showed internalization of the radioligand (6- and 0.5- fold, respectively). In one GH-adenoma cell culture 1 nM unlabelled octreotide also induced a significant increase in the amount of membrane-bound radioligand.

In conclusion, a high amount of [$^{125}$I-Tyr$^{3}$]octreotide is internalized, in a specific-, time-, and temperature-dependent manner by mouse AtT20- and human GH-secreting pituitary tumour cells. A nanomolar concentration unlabelled octreotide induces a rapid increase in the amount of [$^{125}$I-Tyr$^{3}$]octreotide internalized by AtT20- and in two out of three human GH-adenoma cell cultures which showed internalization of the radioligand. Because membrane-binding was also increased, this is suggested to be caused by a rapid recruitment of somatostatin receptors at the outer tumour cell membrane.

## INTRODUCTION

Somatostatin receptors (SS-R) are present in all normal target tissues of the peptide, such as the brain, the anterior pituitary gland, and the pancreas. In a variety of human tumours, frequently originating from normal somatostatin (SS)-target tissues, high numbers of SS-R can be detected by classical biochemical binding techniques, as well as by in vitro autoradiography. These tumours include those with amine precursor uptake and decarboxylation (APUD) characteristics (pituitary tumours, endocrine pancreatic tumours, carcinoids, paragangliomas, small cell lung cancers, medullary thyroid carcinomas, pheochromocytomas), as well as meningiomas, well-differentiated brain tumours (astrocytomas), neuroblastomas, lymphomas and some human breast cancers (1). Recently we developed a technique which allows the in vivo visualization in man of the above mentioned SS-R positive tumours after iv injection of [$^{123}$I-Tyr$^{3}$]octreotide (2,3) or [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide (4). Using this technique we showed that certain tumours, especially those with a high number of SS-R's, could still be visualized 48h after injection (1-4). This rather long residence time of radioactivity on human tumours in vivo may suggest that the radioligand is internalized by the tumour cells. Internalization of radioligand is of special importance when radiotherapy of certain SS-R positive human cancers with $\alpha$- or ß-emitting isotopes coupled to SS analogues is considered (5,6).

At present, equivocal data have been described with respect to internalization of SS. Receptor-mediated endocytosis of SS has been demonstrated in rat anterior pituitary cells and in rat islet cells (7-14), whereas other investigators found that [$^{125}$I-Tyr$^{1}$]SS$_{14}$ and [$^{125}$I-Tyr$^{11}$]SS$_{14}$ are not rapidly internalized by $GH_4C_1$ rat pituitary cells and RINm5F insulinoma cells, respectively, probably due to degradation of these radioligands at the cell surface (15,16). Since data with

respect to internalization of SS may have been influenced by the susceptibility to degradation of the SS ligands used in the above-mentioned studies, investigations with respect to internalization of more stable radioligands, like [$^{125}$I-Tyr$^3$]octreotide (17), may provide a better insight in the amount of internalization by SS-R positive (tumour) cells.

In the present study we therefore investigated whether the stable SS-analogue [$^{125}$I-Tyr$^3$]octreotide is internalized by SS-R positive AtT20/D16V mouse pituitary tumour cells and by human GH-secreting pituitary tumour cells. We recently found that accumulation of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in SS-R positive organs showed a tissue-specific bell-shaped function of the injected mass of the radiopharmaceutical (18). In order to investigate the cellular mechanisms underlying these observations we also studied the effect of different concentrations unlabelled octreotide on binding and internalization of [$^{125}$I-Tyr$^3$]octreotide by the two cell culture systems.

## MATERIALS and METHODS

*Cell culture*

AtT20/D16V mouse pituitary tumour cells were obtained from Dr. J. Tooze (European Molecular Biology Organization, Heidelberg, Germany). The cells were cultured in DMEM supplemented with sodium pyruvate (1mM), 10% fetal calf serum (FCS), fungizone (0.5 mg/L), penicillin ($10^5$ U/L), and sodium bicarbonate (2.2 g/L final concentration). The medium was adjusted to pH 7.4 with 1 N NaOH. The cells were passaged once a week using trypsin (0.05%) and EDTA (0.02%). For internalization experiments, the cells were seeded in a density of 0.5 x $10^6$ cells per well in 12-well multiwell plates (Costar, Cambridge, MA) and grown to confluency for 2 days.

Human GH-secreting pituitary tumour tissue from four acromegalic patients was obtained by transsphenoidal operation. A single tumour cell suspension was prepared by enzymatic dispersion of the tissue as described in detail previously (19). The tumour cells were cultured for 4 days at a density of $10^6$ cells per 3 ml in 12-well multiwell plates (Costar) in Minimal Essential Medium (MEM) supplemented with non-essential amino acids, sodium pyruvate (1 mM), 10% FCS, penicillin ($10^5$ U/L), fungizone (0.5 mg/L), L-glutamine (2mM), and sodium bicarbonate (2.2 g/L final concentration). The medium was adjusted to pH 7.4 with 1 N NaOH.

All cells were cultured at 37 °C in a water-jacketed incubator in humidified air with 5% $CO_2$. The media and supplements were obtained from GIBCO (Paisley, UK).

*Radioligands*

The SS analogue [Tyr$^3$]octreotide was iodinated with $^{125}$I by the chloramine-

T method and purified by HPLC, as described previously in detail (20). Specific radioactivity of the radioligand yielded approximately 2000 Ci/mmol.

*Internalization experiments*

On the day of the experiment the AtT20 cells were washed twice with internalization medium. The internalization medium consisted of DMEM supplemented with HEPES (30mM), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin ($10^5$ U/L), fungizone (0.5 mg/L) and 0.2% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis). The medium was adjusted to pH 7.4 with 1 N KOH. The cells were allowed to adjust to the medium for 1 h at 37 °C. Thereafter approximately 200.000 cpm [$^{125}$I-Tyr$^3$]octreotide (approximately 0.1 nM final concentration) was added to the medium and the cells were incubated at 37 °C (or other temperatures when indicated) for a period up to 4 h in quadruplicate without or with excess unlabelled octreotide (1 $\mu$M) in order to determine non-specific membrane binding and internalization. Binding and internalization of [$^{125}$I-Tyr$^3$]octreotide was blocked by excess unlabelled peptide, while unrelated peptides such as GnRH (1$\mu$M) and TRH (1$\mu$M) did not exert any influence.

After the incubation period the cells were washed twice with ice-cold internalization medium. Thereafter, 1 mL sodium acetate (20 mM) in Hanks' Balanced Salt Solution, pH 5.0 (HBSS-Ac), was added to the cells. The cells were then incubated during 10 min. at 37 °C. After 10 min. the supernatant was collected. Thereafter, the cells were washed with HBSS-Ac, and the supernatant was pooled with the supernatant of the previous step. This pooled supernatant fraction, acid-extractable radioactivity, represents membrane-bound radioligand. At the end of the incubation, after the HBSS-Ac treatment, the cells were extracted in 1 N NaOH. The radioactivity in this fraction represents internalized radioligand. This method of separation of membrane bound and internalized radioligand has been described previously by others (21).

Human GH-secreting pituitary tumour cells, which had attached to the bottom of the wells during the 4-day culture (see above), were rinsed twice with internalization medium. 1 mL of this medium was then added to the cells, and incubations were performed as described for the AtT20 cells.

Both cell types studied remained viable in internalization medium during the 4 h incubation period, as determined by trypan blue exclusion.

*SS-R binding studies*

SS-R binding studies were carried out using [$^{125}$I-Tyr$^3$]octreotide as radioligand as described previously (22). Rat brain cortex- or AtT20 cell membrane preparations (corresponding to 15-30 $\mu$g protein) were incubated in a total volume of 100 $\mu$l at room temperature for 60 min with 30,000-50,000 cpm radioligand and increasing concentrations of unlabelled octreotide in HEPES buffer (10 mM

HEPES, 5mM $MgCl_2$, 0.2 g/L bacitracin, pH 7.6) containing 0.2% BSA (Sigma). After the incubation, 1 mL ice-cold HEPES buffer was added to the assay mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 rpm in an Eppendorf microcentrifuge. The remaining pellet was washed twice with ice-cold HEPES buffer, and the final pellet was counted in a gamma-counter. Specific binding was taken to be total binding minus binding in the presence of excess (1 $\mu$M) unlabelled octreotide. Unrelated compounds (TRH, LHRH, EGF), added in a 1000-fold excess were not able to displace [$^{125}$I-Tyr$^3$]octreotide binding.

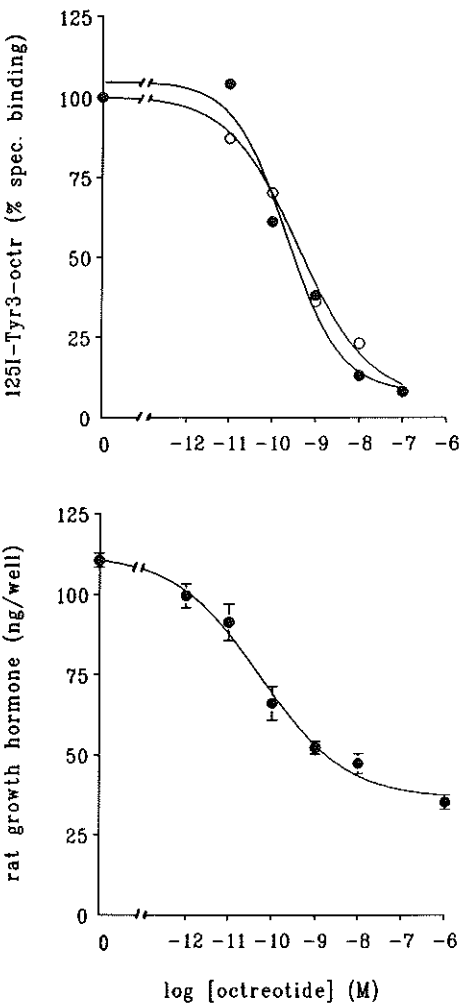


**Figure 1.**

*Upper panel:*
*Displacement of binding of [$^{125}$I-Tyr$^3$]octreotide (upper panel): by unlabelled octreotide to rat brain cortex membranes in internalization medium (O; see Materials and methods section) and in HEPES buffer (●)*

*Lower panel:*
*Dose-dependent inhibition by octreotide of growth hormone release by cultured female rat anterior pituitary cells in internalization medium. Normal female rat anterior pituitary cells were cultured for 7 days in MEM + 10% FCS. On day 4 of culture, the medium was changed. On day 7 of culture 4h incubations with increasing concentrations of octreotide were performed in quadruplicate in internalization medium. GH concentrations in the media were determined as described previously in detail (24)*

*Analysis of data*

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA). When significant overall effects were obtained by ANOVA, multiple comparisons were made by the Newman-Keuls test (23). All data are expressed as the mean ± SE, n=4 wells per time-point or treatment group.

$IC_{50}$ values for displacement of [$^{125}$I-Tyr$^3$]octreotide binding and for inhibition of GH release by cultured rat anterior pituitary cells, were determined by the computerized program GraphPad (ISI Software, Philadelphia, PA, USA).

## RESULTS

*Establishment of the incubation conditions for the study of internalization of [$^{125}$I-Tyr$^3$]octreotide*

In initial experiments we have compared binding of [$^{125}$I-Tyr$^3$]octreotide to rat brain cortex membranes in a regular binding buffer (20 mM HEPES, 5 mM $MgCl_2$, 20 mg/L bacitracin, 0.2% BSA, pH 7.4) with binding of the same radioligand in internalization medium (see Materials and Methods section). We found that the amount of specific binding in internalization medium was approximately 10% of the specific binding measured to membranes diluted in the HEPES-buffer. Displacement of [$^{125}$I-Tyr$^3$]octreotide with increasing concentrations of unlabelled octreotide showed an $IC_{50}$ value of 0.2 nM, which is similar to that found in HEPES-buffer (Figure 1, upper panel). Growth hormone release by normal rat anterior pituitary cells incubated in internalization medium was inhibited in a dose-dependent manner by octreotide. The $IC_{50}$ value of inhibition of GH release was 54 pM (Figure 1, lower panel), which is comparable to that of cells cultured in regular culture medium supplemented with fetal calf serum (25). These data demonstrate that, although the number of SS-binding sites that is measured in internalization medium is lower as compared to that in regular binding buffer, those SS-R which are detected have a dissociation constant in the low nanomolar range and are biologically active.

In order to verify that the method of separation of internalized (acid-resistant) and surface-bound extracellular (acid-releasable) ligand indeed released all binding of radioligand from membrane-receptors, rat brain cortex membranes were incubated with [$^{125}$I-Tyr$^3$]octreotide without or with excess unlabelled octreotide to determine non-specific binding for 1h at 20 °C.

Thereafter, the membranes were washed twice with ice-cold binding buffer and subsequently incubated for 10 min. at 37 °C with HBSS-HAc as described in the materials and methods section. This acid-treatment completely abolished specific binding of [$^{125}$I-Tyr$^3$]octreotide, demonstrating that all membrane-bound radioligand is acid-releasable.

Scatchard analysis of [$^{125}$I-Tyr$^3$]octreotide binding to AtT20 cell membranes revealed a high number of high affinity SS-R (2815 ± 317 fmoles/mg membrane

protein, $K_d$= 0.35 ± 0.05 nM; values are the mean ± SE of four independent determinations).

*Internalization of [$^{125}$I-Tyr$^3$]octreotide by mouse AtT20 anterior pituitary tumour cells*

Figure 2 shows time-dependent specific membrane-binding and internalization of [$^{125}$I-Tyr$^3$]octreotide by AtT20 cells. [$^{125}$I-Tyr$^3$]octreotide showed time-dependent increasing accumulation in these cells. The amount of [$^{125}$I-Tyr$^3$]octreotide internalized was approximately 5% of the dose radioligand added, after 240 min of incubation, and at all time-points studied significantly higher than the amount of radioligand which was membrane-bound. Internalization of [$^{125}$I-Tyr$^3$]octreotide by AtT20 cells was temperature-dependent. At 0 °C, no binding and internalization of [$^{125}$I-Tyr$^3$]octreotide was seen, while the amount of internalized radioligand increased with increasing temperature (20 and 37 °C, respectively; Figure 3).

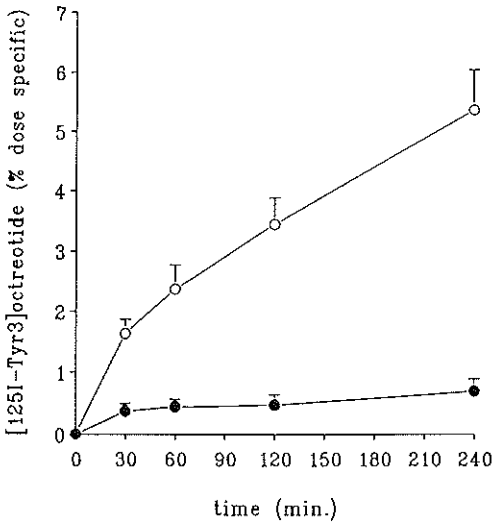


**Figure 2.** *Membrane-binding and internalization of [$^{125}$I-Tyr$^3$]octreotide by AtT20 mouse pituitary tumour cells. (○ : membrane-bound; ● : internalized) Values are expressed as specific binding and internalization and are the mean ± SE of the percentage of dose of radioligand added of three independent experiments*

We also investigated the effect of 1 nM of unlabelled octreotide on binding and internalization of the radioligand. Surprisingly we found an increased internalization of [$^{125}$I-Tyr$^3$]octreotide induced by this low amount of unlabelled octreotide (Figure 4, lower panel). 1$\mu$M unlabelled octreotide completely blocked internalization of the radioligand after 15 min. of incubation ($P<0.01$ vs control cells), while 1 nM octreotide nearly doubled internalization already after 15 min. of incubation ($P<0.01$ vs control cells). At these time-points, however, 1 nM octreotide did not significantly increase the amount of membrane-bound radioligand

(Figure 4, upper panel). Because the increased internalization seems to occur very rapidly, we also studied the effect of 1 nM unlabelled octreotide at several time-points between 0-2.5 minutes of incubation. The results of these experiments are shown in figure 5. Again, 1 nM unlabelled octreotide induced a very rapid (within 1 min. of incubation) two-fold increase in the amount of internalized radioligand (figure 5, lower panel). At the same time however, membrane-bound [$^{125}$I-Tyr$^3$]octreotide was also significantly increased (figure 5, upper panel).

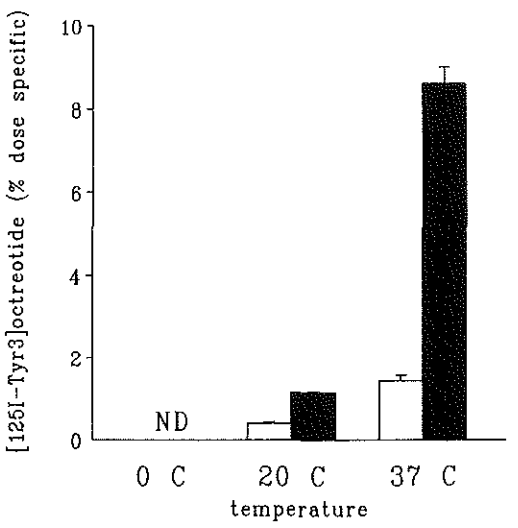


**Figure 3.** *Temperature-dependency of internalization and membrane-binding of [$^{125}$I-Tyr$^3$]octreotide by AtT20 mouse pituitary tumour cells. Values are expressed as specific binding and internalization as the mean ± SE of the percentage of the dose of radioligand added. Open bars: membrane-bound radioligand; filled bars: internalized radioligand; ND means undetectable*

**Table 1.** *Membrane-binding and internalization of [$^{125}$I-Tyr$^3$]octreotide by cultured GH-secreting pituitary adenoma cells from patient 2*

| | [$^{125}$I-Tyr$^3$]octreotide (% dose ± SE) | |
|---|---|---|
| | membrane-bound | internalized |
| control | 1.39 ± 0.07 | 0.79 ± 0.04 |
| octreotide (1μM) | 1.03 ± 0.01 * | 0.25 ± 0.02 * |
| octreotide (1nM) | 1.33 ± 0.10 | 0.64 ± 0.03 ** |

*$10^6$ cells per well were incubated in quadruplicate during 60 min with approximately 0.1 nM [$^{125}$I-Tyr$^3$]octreotide. Thereafter membrane-bound and internalized radioligand was determined as described in the materials and methods section. * $P<0.01$ and ** $P<0.05$ vs control cells*

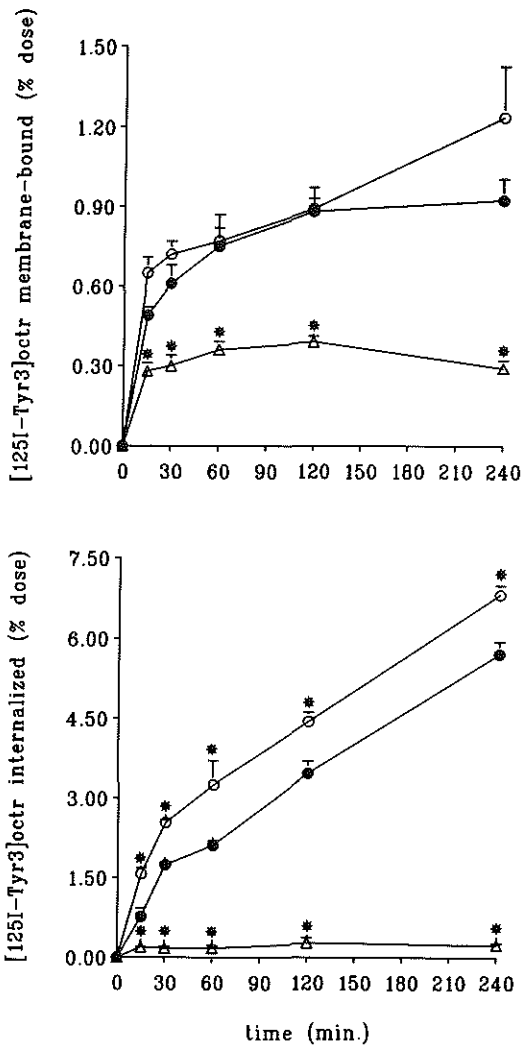


**Figure 4.**
*Effect of 1μM and 1nM unlabelled octreotide on membrane-binding (upper panel) and internalization (lower panel) of [$^{125}$I-Tyr$^3$]octreotide by mouse AtT20 pituitary tumour cells.*
** $P<0.01$ vs control cells.*
*Values are expressed as the mean ± SE of the percentage of dose of radioligand added of three independent experiments.*
*● : control cells;*
*Δ : 1 μM octreotide;*
*○ : 1 nM octreotide*

*Internalization of [$^{125}$I-Tyr$^3$]octreotide by human GH-secreting pituitary adenoma cells*

Similar to the observations in AtT20 cells, human GH-secreting pituitary adenoma cells from patient 1 also showed a time-dependent specific membrane-binding (Figure 6, upper panel) and internalization of [$^{125}$I-Tyr$^3$]octreotide (Figure 6, lower panel). In the presence of 1 nM unlabelled octreotide a rapid increase in both membrane-bound and internalized radioligand was observed. A statistically significant higher amount of radioligand was bound and internalized at all time-points studied. After 60 min. of incubation an approximately six-fold higher amount of radioligand was bound and internalized in the presence of 1 nM

unlabelled octreotide as compared to control cells. The total amount of radioligand that was bound and internalized after 4 h of incubation was also considerably high in the GH-secreting pituitary adenoma cells of this patient (approximately 1.5 and 5%, respectively, of the dose radioligand added).

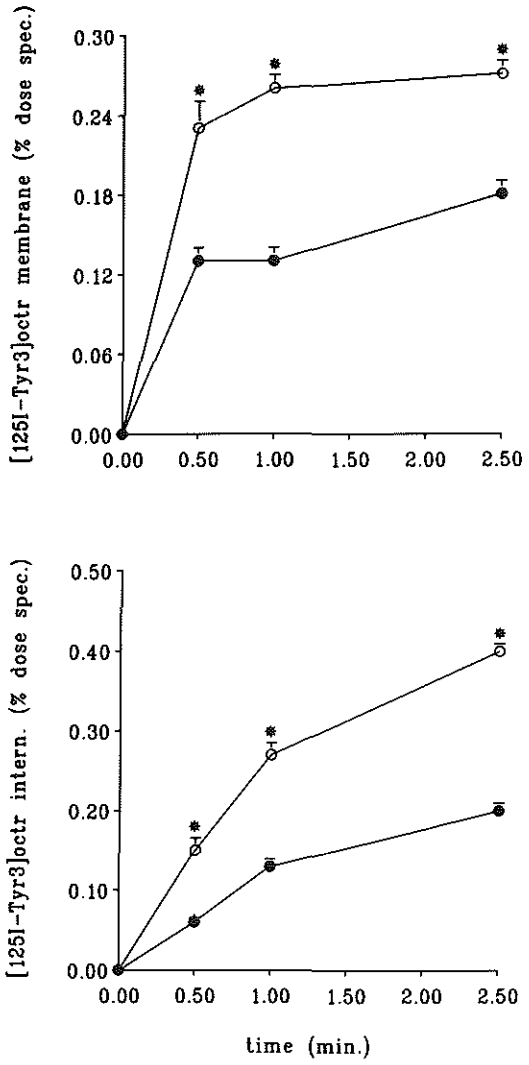


**Figure 5.**
*Rapid increase of membrane-binding (upper panel) and internalization (lower panel) of [$^{125}$I-Tyr$^3$]octreotide induced by 1 nM unlabelled octreotide.*
● *: control;*
○ *: 1 nM unlabelled octreotide.*
* *$P<0.01$ vs control cells.*
*Values are expressed as specific binding and internalization and as the mean ± SE of the percentage of dose of radio ligand added of three independent experiments*

Cultured tumour cells from patient 2, also showed after 60 min. of incubation a relative high amount of [$^{125}$I-Tyr$^3$]octreotide binding and internalization. However, 1 nM of unlabelled octreotide had in these tumour cells no effect on the amount of membrane-bound radioligand, and inhibited the amount of internalized radioligand slightly, but significantly (Table 1). Unfortunately not enough tumour cells had

been obtained from this patient at operation to allow the study of more time-points. Cultured cells of patient no.3 did not bind, nor internalized [$^{125}$I-Tyr$^3$]octreotide between 30 and 240 min. of incubation (data not shown). Cultured cells from patient 4 showed a time-dependent increasing internalization and membrane-binding of [$^{125}$I-Tyr$^3$]octreotide between 30 and 240 min. of incubation (0.38 and 0.09% of the dose radioligand added, respectively, after 240 min. of incubation). The amount of radioligand which was internalized and membrane-bound was lower, however, as compared to the values measured in cells from patients 1 and 2. Using cells from patient 4 we also studied the full dose-response relationship of increasing concentrations of unlabelled octreotide (0.01 to 100 nM) on internalization and membrane binding of the radioligand. Table 2 shows that, after 240 min. of incubation, unlabelled octreotide inhibited in a dose-dependent manner binding of [$^{125}$I-Tyr$^3$]octreotide by cells from this patient, while the amount of radioligand which was internalized was significantly increased in the presence of 1 nM unlabelled octreotide and significantly inhibited by 100 nM octreotide at the same time.

**Table 2.** *Membrane-binding and internalization of [$^{125}$I-Tyr$^3$]octreotide by human GH-secreting pituitary tumor cells from patient 4*

| | [$^{125}$I-Tyr$^3$]octreotide | (% control specific) |
|---|---|---|
| octreotide (nM) | membrane-bound | internalized |
| 0 | 100 ± 8 | 100 ± 4 |
| 0.01 | 96 ± 6 | 103 ± 3 |
| 0.1 | 107 ± 4 | 104 ± 6 |
| 1 | 104 ± 21 | 142 ± 4 * |
| 10 | 61 ± 6 ** | 117 ± 7 |
| 100 | 45 ± 8 * | 35 ± 3 * |

*Values are expressed as mean ± SE of the percentage of specific membrane-binding and internalization by control cells (0 nM octreotide). The amount of radioligand bound to membranes and internalized after 4 h of incubation yielded 0.09 ± 0.01 and 0.38 ± 0.02, respectively, of the dose added.*
** $P<0.01$ and ** $P<0.05$ vs control*

Interestingly, we found that the preoperative subcutaneous administration of 50 μg octreotide induced a significant lowering of serum GH levels in patients 1 and 2, while patients 3 and 4 showed no response of GH levels to octreotide in vivo (Figure 7).

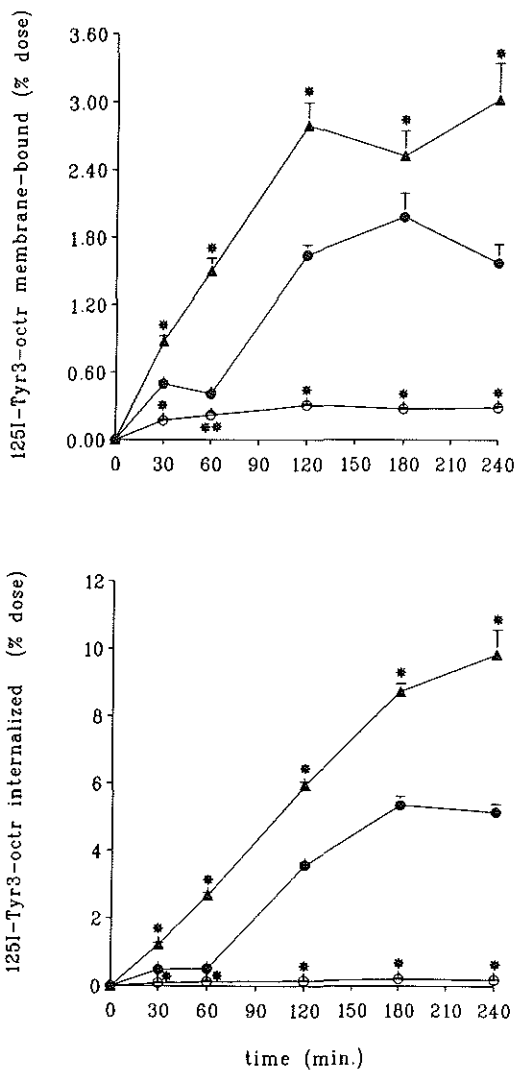


**Figure 6.** *Effect of 1μM and 1nM unlabelled octreotide on membrane-binding (upper panel) and internalization (lower panel) of [$^{125}$I-Tyr$^{3}$]octreotide by human GH-secreting pituitary adenoma cells of patient 1.*
*** $P<0.05$ and * $P<0.01$ vs control cells.*
*Values are expressed as the mean ± SE of the percentage of dose of radioligand added.*
● *: control cells;*
○ *: 1μM octreotide;*
▲ *: 1nM octreotide*

In support of this low sensitivity to octreotide in vivo, we also observed that the cultured cells from patients 1 and 2 were significantly more sensitive to octreotide in vitro, as compared to the cultured tumour cells from patients 3 and 4. GH release by the cultured tumour cells from patients 1, 2, 3, and 4 was inhibited by

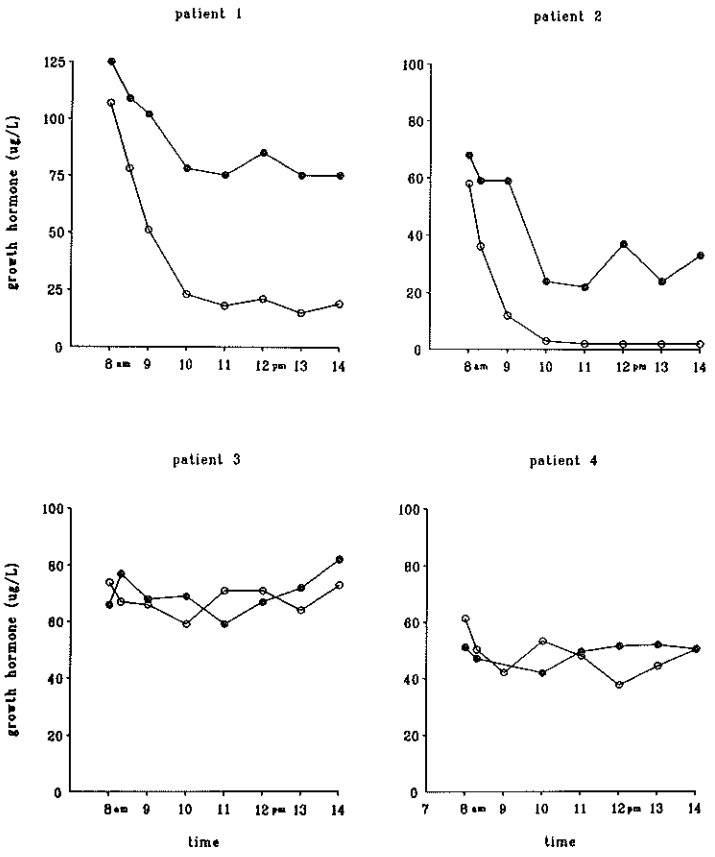


**Figure 7.** *Effect of the administration of 50 μg octreotide subcutaneously on the serum GH levels in four acromegalic patients*
●*: placebo at 8.15 am;* ○*: 50 μg octreotide sc. at 8.15 am*

octreotide (10 nM) by 83 ± 9, 43 ± 2, 27 ± 1, and 16 ± 0 %, respectively. Thus, the cells of patient 1, which showed the highest amount of internalization of [$^{125}$I-Tyr$^3$]octreotide, also showed the highest response of GH release to octreotide in vitro.

## DISCUSSION

A variety of human neuroendocrine tumours contain receptors for the tetradecapeptide $SS_{14}$ (1). The presence of SS-R on these tumours enabled us to develop a technique which allows in vivo visualization in man of SS-R positive tumours using the radiolabelled SS analogues [$^{123}$I-Tyr$^3$]octreotide and [$^{111}$In-DTPA-D-Phe$^1$]octreotide (2-6). In several tumours (i.e. carcinoids and paragangliomas)

expressing a high amount of SS-R, binding of radioactivity in vivo exceeds estimates of 0.1% of the administered dose per g of tumour tissue. Radiotherapy using SS analogues coupled to $\alpha$- or ß-emitting isotopes has been proposed as an application for radiolabelled SS analogues and has recently been carried out in one patient (5,6,26). In order to develop this concept further it is of importance to know to which extent the above mentioned radiolabelled SS analogues are internalized by SS-R positive tumour cells, and whether it might be possible to manipulate the degree of internalization.

In the present study we demonstrated a specific-, time- and temperature-dependent high amount of internalization of [$^{125}$I-Tyr$^3$]octreotide by mouse AtT20 pituitary tumour cells. In addition, internalization of [$^{125}$I-Tyr$^3$]octreotide by human GH-secreting pituitary tumour cells was found. Several studies have demonstrated receptor-mediated endocytosis of SS in rat anterior pituitary cells (7,9,11,12), in rat islet cells (10,14), and in AtT20 cells (8). Other investigators, however, showed that [$^{125}$I-Tyr$^1$]SS$_{14}$ and [$^{125}$I-Tyr$^{11}$]SS$_{14}$, unlike [$^{125}$I]epidermal growth factor, are not rapidly internalized by GH$_4$C$_1$ rat pituitary cells and RINm5F insulinoma cells, respectively (15,16). These investigators suggested that the radioligands they used were rapidly degraded by membrane proteases. From our present study it is clear that the radioiodinated SS analogue Tyr$^3$-octreotide is internalized in a high amount by AtT20- and human GH-secreting pituitary tumour cells. This is probably related to the high stability of [$^{125}$I-Tyr$^3$]octreotide (17). The parental cyclic octapeptide analogue octreotide (SMS 201-995) was shown to be highly resistant to degradation by pure enzymes and by tissue homogenates (27). Our data are in favor of a further development of the concept of radiotherapy using radiolabelled SS-analogues.

Another interesting observation of our present study is the rapid increased internalization of [$^{125}$I-Tyr$^3$]octreotide when AtT20 cells and human GH-adenoma cells were simultaneously incubated with a low concentration of unlabelled octreotide. We found that 1 nM of unlabelled octreotide caused a very rapid increase in the amount of internalization of [$^{125}$I-Tyr$^3$]octreotide by AtT20 cells (a 2-fold increase within 1 min. of incubation) and by two out of three cultures of human GH-secreting pituitary tumours (up to 6- and 0.5- fold increase respectively) which showed internalization of the radioligand. In the same experiments excess unlabelled octreotide (1$\mu$M) completely blocked internalization of the radioligand. In AtT20 pituitary tumour cells and in one of the human GH-adenoma cell cultures the increased internalization induced by the addition of a low concentration of unlabelled octreotide was accompanied by an increase in the amount of membrane-bound radioligand, which suggests a very rapid increase in the number of SS-R on these cells.

Little is known with respect to the homologues regulation of SS-R expression. In GH$_4$C$_1$ cells Presky and Schonbrunn demonstrated a time-dependent increase in [$^{125}$I-Tyr$^1$]SS$_{14}$ binding by the addition of unlabelled SS$_{14}$, which reached a

maximum value of approximately 200% after 20h (21). In contrast, Srikant and Heisler (28) showed a decrease in the number of SS-R in AtT20 cells after preincubation for 4h with $SS_{14}$ or $SS_{28}$. Finally, in 7315b rat prolactinoma cells chronic exposure (up to 5 weeks) results in a reversible down-regulation of SS-R (29). At present, we have no explanation for the mechanism of the increased binding and subsequent internalization of [$^{125}$I-Tyr$^3$]octreotide by AtT20 tumour cells and by human GH-adenoma cells, induced by a low concentration of unlabelled octreotide. The rapid increase in [$^{125}$I-Tyr$^3$]octreotide binding and internalization seems unlikely to be caused by de novo synthesis of SS-R's, because this increase occurred already within several minutes of incubation with 1 nM unlabelled octreotide. Therefore, the most likely explanation for this phenomenon seems a rapid recruitment of cellular SS-R's to the outside of the cell membrane, although an effect of octreotide on SS-R synthesis cannot be fully excluded. In this respect it is of interest to mention that glucose has been shown to increase the amount of internalized SS in pancreatic islets, probably due in part to the increase in the cell surface SS-R concentration by migration of the secretion vesicles during emiocytosis, which thereby promotes increased transport of SS-R's (30).

Recently, at least five different SS-R subtypes have been cloned. All subtypes bind $SS_{14}$ and $SS_{28}$ with high affinity, while their affinity for numerous SS-analogues differs considerably (31-35). Octreotide binds with high affinity to the human SSTR2 (hSSTR2) subtype, while this analogue has a moderate affinity to hSSTR3 and hSSTR5 subtypes and shows no binding to the hSSTR subtypes 1 and 4 (31-35). More than one hSSTR subtype is expressed in human GH-adenomas. Greenman and Melmed (36) showed in 3 out of 7 and in 9 out of 10 human GH-adenomas expression of hSSTR1 and hSSTR2, respectively. In addition Reubi et al. (37) recently demonstrated in GH-adenomas, by in situ hybridization autoradiography, hSSTR2- (7 out of 7 cases) and hSSTR3- (3 out of 7), but not hSSTR1 expression. In AtT20 cells four SSTR genes (subtypes 1, 2, 4 and 5) are expressed, of which SSTR2 expression predominates (38). At present it is unclear which SSTR subtype is involved in receptor-mediated endocytosis in human GH-adenoma- and AtT20 cells. Studies on internalization of [$^{125}$I-Tyr$^3$]octreotide by cell lines stably expressing SSTR subtypes may help to answer this question.

Our observation of a higher amount of binding and internalization of [$^{125}$I-Tyr$^3$]octreotide when the cells are simultaneously incubated with a low concentration of unlabelled octreotide may explain the results of a recent in vivo study by our group, in which we found that uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in SS-R positive organs (pituitary, pancreas, adrenals) in rats, showed a bell-shaped function of the injected mass (18). Therefore, the highest specific radioactivity may not automatically result in the highest binding and subsequent internalization of the radioligand by the tumour in in vivo SS-R imaging using radiolabelled SS-analogues in patients with SS-R positive tumours. Indeed, preliminary findings in patients indicate that scintigraphy with a specific activity higher than 220 MBq $^{111}$In per 5

μg [DTPA-D-Phe$^{1}$]octreotide will lead to decreased quality, and that uptake in tumours will be significantly reduced (39). Moreover, Dörr et al., recently demonstrated an improved visualization of carcinoid liver metastases in patients by [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide during octreotide treatment (40), which might be due to upregulation of SS-R's by octreotide. Finally it should be mentioned that we have used [$^{125}$I-Tyr$^{3}$]octreotide as a radioligand in our study. It still has to be established, however, whether radiolabelled DTPA-conjugated SS analogues, such as [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide, are internalized to the same extent by SS-R positive tumour cells as non-DTPA conjugated SS-analogues.

In conclusion, a high amount of [$^{125}$I-Tyr$^{3}$]octreotide is internalized, in a specific-, time-, and temperature-dependent manner by mouse AtT20 cells and by human GH-secreting pituitary tumour cells. The amount of internalized [$^{125}$I-Tyr$^{3}$]octreotide by the human GH-adenoma cell cultures correlated well with their in vivo and in vitro responsiveness of GH secretion to octreotide. A nanomolar concentration of unlabelled octreotide induces a very rapid increase in membrane-binding and in the amount of internalization of [$^{125}$I-Tyr$^{3}$]octreotide by AtT20 cells. Two out of three human GH-adenoma cell cultures also responded to this low concentration of unlabelled octreotide with an increase in the amount of internalized radioligand. Thus, a variability exists between human GH-adenomas in the amount of radioligand internalized, and in their response to low concentrations of unlabelled octreotide. It remains to be established therefore, whether our present conclusions also apply to other types of human SS-R positive neuroendocrine tumours.

*ACKNOWLEDGEMENT*

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## CHAPTER 5

# RADIOIODINATED SOMATOSTATIN ANALOGUE RC-160: PREPARATION, BIOLOGIC ACTIVITY, IN VIVO APPLICATION IN RATS, AND COMPARISON WITH [$^{123}$I-Tyr$^3$]OCTREOTIDE

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## ABSTRACT

We have evaluated the potential usefulness of the radioiodinated octapeptide RC-160, a somatostatin analogue, which might serve as a radiopharmaceutical for the in vivo detection of somatostatin receptor-positive tumours. For this purpose $^{123}$I- and $^{125}$I-labelled RC-160 were tested for their biologic activity, and applied in vivo in rats bearing the transplantable rat pancreatic tumour CA20948, which expresses somatostatin receptors. Our group has recently described the in vivo visualization of such tumours in rats and in humans with the radioiodinated somatostatin analogue [Tyr$^3$]octreotide.

Like [$^{123}$I-Tyr$^3$]octreotide, $^{123}$I-RC-160 showed uptake in and specific binding in vivo to somatostatin receptor-positive organs and tumours. However, blood radioactivity (background) was higher, resulting in a lower tumour to blood (background) ratio. We therefore conclude that in this animal model $^{123}$I-RC-160 has no advantage over [$^{123}$I-Tyr$^3$]octreotide as a radiopharmaceutical for the in vivo use as a somatostatin-receptor imager, although, like [$^{123}$I-Tyr$^3$]octreotide, $^{123}$I-RC-160 shows specific binding to different somatostatin receptor-positive organs. Recently differences were reported in affinity between somatostatin and its analogues for somatostatin receptors expressed in different human cancers, like those of the breast, ovary, exocrine pancreas, prostate and colon. Therefore $^{123}$I-RC-160 might be of interest for future use in humans as a radiopharmaceutical for imaging octreotide receptor-negative tumours.

## INTRODUCTION

High numbers of high-affinity somatostatin receptors for both native somatostatin (for structure, see Fig. 1) and the synthetic octapeptide octreotide (Sandostatin®), have been detected on most endocrine tumours, like endocrine pancreatic tumours and carcinoids (1-4). We have recently described the visualization of somatostatin receptor-positive tumours in vivo after the intravenous administration of [$^{123}$I-Tyr$^3$]octreotide (5-9), which results have been confirmed by others (10-12). Radioiodinated [Tyr$^3$]octreotide is frequently used for the in vitro determination of the presence of somatostatin receptors (13). Recently, several reports have been published on the in vitro binding to somatostatin receptors of another somatostatin analogue, the octapeptide RC-160 (14-16). It has been reported that RC-160 has a higher affinity than octreotide for the somatostatin receptor in human breast, ovarian, exocrine pancreatic, prostatic and colonic cancers (14-16). A phase 1 clinical trial with RC-160 in patients with advanced exocrine pancreatic cancer is being performed, and it appears that RC-160 is well tolerated at doses up to 1500 $\mu$g/day (17,18). The possibility of RC-160 binding to a somatostatin receptor subtype on human exocrine pancreatic adenocarcinoma, which does not bind octreotide (19), offers a potential advantage for RC-160 over octreotide as radiolabelled tumour tracer. RC-160 may, in contrast to octreotide (20), also pass the blood-brain barrier (21). This could represent a benefit in visualizing somatostatin receptor-positive brain tumours with an intact blood-brain barrier. We investigated radioiodinated RC-160 (for structure see Fig. 1) for the potential use in scintigraphy in normal rats and in rats bearing the transplantable pancreatic somatostatin receptor-positive tumour CA20948 (13,19,22). A comparison was made with [$^{123}$I-Tyr$^3$]octreotide, and the possible additional value of $^{123}$I-RC-160 as a radiopharmaceutical was evaluated.

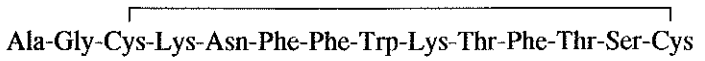
## MATERIALS AND METHODS

*Radiopharmaceuticals*

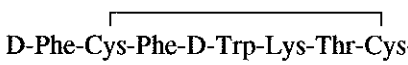
RC-160 was obtained from Peninsula Laboratories (Belmont, Ca, USA). Radioiodination and purification was performed using the technique described by Bakker (6).

[Tyr$^3$]octreotide and RC-160 were labelled with $^{123}$I (specific activity 3.7 TBq $^{123}$I/mg, Medgenix, Belgium) and $^{125}$I (specific activity 0.62 TBq $^{125}$I/mg, Amersham, UK). For the in vivo studies we used the somatostatin analogues labelled with $^{123}$I. The in vitro binding studies were performed with HPLC-purified mono-iodinated somatostatin analogues, since carrier-free radioligands are required for these assays.

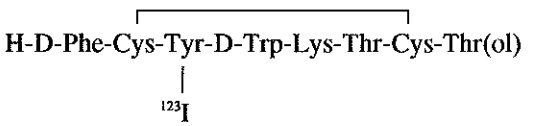
**Somatostatin**




**Octreotide**




**[$^{123}$I-Tyr$^3$]octreotide**




**$^{123}$I-RC-160**

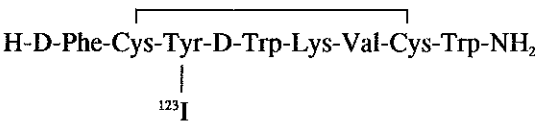



**Figure 1**. *Stuctural formulae of native somatostatin and some analogues. In [Tyr$^3$]octreotide the amino acid Tyr replaces Phe to make radioiodination possible. In RC-160 Tyr is naturally present*

The radioiodination was carried out by adding 2.5 $\mu$g RC-160 in 35 $\mu$l 0.05 M acetic acid and 1.6 $\mu$g chloramine-T in 20 $\mu$l 0.05 M phosphate buffer (pH 7.5) to 200 MBq ($\approx$60 $\mu$l) $^{123}$I or 80 MBq (20 $\mu$l) $^{125}$I in the form of sodiumiodide. The reaction was started by the addition of chloramine-T, representing an only 2.5-fold molar excess over peptide in order to prevent oxidation of the disulfide-bridge of RC-160. The mixture was then vortexed for 1 min. The radioiodination was stopped by adding 1 ml 10 % human serum albumin (Merieux, Lyon, France). After vortexing for 30 s, 20 ml 5 mM ammonium acetate was added. Purification was performed using a SEP-PAK $C_{18}$ reversed-phase extraction cartridge (Waters Associates, Milford, MA), which was washed with 5 ml 70 % ethanol, 5 ml 2-propanol and 5 ml distilled water. After application of the sample, the SEP-PAK cartridge was washed with 5 ml distilled water and radioiodinated RC-160 was eluted with 5 ml 96 % ethanol. The solvent was evaporated at 40 °C under a gentle stream of nitrogen. The residue (approximately 0.5 ml) was diluted with 2.5 ml 154 mM NaCl and 0.05 M acetic acid (pH 3), and the mixture was passed

through a low protein-binding 0.22-$\mu$m Millex-GV filter (Millipore, Milford, MA). The labelling of [Tyr$^3$]octreotide, and the measurements of radioactivity in all fractions were carried out as described before (6). The $^{123}$I-labelled somatostatin analogues for in vivo use were not purified by HPLC and, hence, consisted of mixtures of mono- and di-radioiodinated and non-iodinated peptides (see Results, *Radiolabelling of RC-160*).

All chemicals used were of the highest purity available.

*Biologic activity*

The biologic activity of HPLC-purified mono-$^{125}$I-RC-160 and [$^{125}$I-Tyr$^3$]octreotide was assessed by measuring their potency to inhibit the secretion of rat growth hormone (rGH) from cultured rat pituitary cells as described previously (23).

**Table 1.** *Effect of $^{125}$I-RC-160 and [$^{125}$I-Tyr$^3$]octreotide on secretion of rat growth hormone from cultured rat pituitary cells (n=4)*

| Peptide | Concentration (nM) | Rat growth hormone (ng/ml ± SD) (%) | |
|---|---|---|---|
| Control | | 89.1 ± 9.0 (100) | |
| $^{125}$I-RC-160 | .01 | 91.7 ± 4.6 (103) | |
| | .1 | 95.5 ± 3.0 (107) | |
| | 1 | 77.2 ± 11.1 (87) | * |
| [$^{125}$I-Tyr$^3$]octreotide | .01 | 90.0 ± 4.8 (101) | |
| | .1 | 79.9 ± 11.3 (90) | |
| | 1 | 64.8 ± 4.6 (73) | * |

* *$P<0.05$ vs control*

*Animals and tumours*

Twenty-four male Lewis rats (250-300 gram) were inoculated in both upper hindlegs with the transplantable rat pancreatic tumour CA20948, which has previously been shown to possess somatostatin receptors (19). Sixteen male Lewis rats (250-300 gram) without tumour were used as controls. The rats were anaesthetized with ether. The radiopharmaceuticals were injected in the dorsal vein of the

penis, using siliconized syringes (Sigmacoat, Sigma, St Louis, MO). The dose was 18.5 MBq (0.5 μg) for the $^{123}$I-analogue. The radioactivity of the syringes was measured in a dose calibrator (VDC-202, Veenstra, Joure, The Netherlands) in a standard geometry before and after the injection.

**Table 2.** *Tissue distributions (% of injected dose/g tissue) and specific binding (Δ) of $^{123}$I-RC-160 in tumour-bearing rats after intravenous administration (mean ± SD)*

| Tissue | | 0.5 h p.i. | | 4 h p.i. | | 24 h p.i. | |
|---|---|---|---|---|---|---|---|
| Pancreas | - | 0.91 ± 0.38 | | 0.32 ± 0.08 | | 0.06 ± 0.02 | |
| | + | 0.10 ± 0.01 | | 0.07 ± 0.02 | | 0.021 ± 0.002 | |
| | Δ | 0.81 ± 0.38 | * | 0.25 ± 0.08 | * | 0.040 ± 0.019 | * |
| Adrenals | - | 1.17 ± 0.06 | | 0.54 ± 0.09 | | 0.06 ± 0.03 | |
| | + | 0.20 ± 0.06 | | 0.07 ± 0.01 | | 0.038 ± 0.009 | |
| | Δ | 0.97 ± 0.06 | * | 0.47 ± 0.09 | * | 0.022 ± 0.028 | |
| Pituitary | - | 0.16 ± 0.06 | | 0.08 ± 0.02 | | 0.05 ± 0.05 | |
| | + | 0.021 ± 0.003 | | 0.013 ± 0.002 | | 0.004 ± 0.000 | |
| | Δ | 0.13 ± 0.06 | * | 0.064 ± 0.020 | * | 0.044 ± 0.048 | |
| Brain | - | 0.014 ± 0.002 | | 0.008 ± 0.002 | | 0.0019 ± 0.0009 | |
| Cortex | + | 0.008 ± 0.001 | | 0.003 ± 0.000 | | 0.0013 ± 0.0003 | |
| | Δ | 0.007 ± 0.003 | * | 0.005 ± 0.002 | * | 0.0006 ± 0.0009 | |
| Tumour | - | 0.23 ± 0.07 | | 0.20 ± 0.05 | | 0.037 ± 0.021 | |
| | + | 0.11 ± 0.02 | | 0.08 ± 0.02 | | 0.036 ± 0.003 | |
| | Δ | 0.11 ± 0.07 | | 0.12 ± 0.05 | * | 0.001 ± 0.021 | |
| Kidneys | - | 0.74 ± 0.13 | | 0.43 ± 0.13 | | 0.11 ± 0.03 | |
| | + | 0.53 ± 0.04 | | 0.22 ± 0.05 | | 0.10 ± 0.02 | |
| | Δ | 0.21 ± 0.13 | | 0.21 ± 0.13 | | 0.01 ± 0.03 | |

*Each group contained 4 rats; -: no pretreatment; +: pretreatment with 1 mg unlabelled RC-160 subcutanenously 45 min prior to the injection with $^{123}$I-RC-160; * $P < 0.01$, specific binding significantly different from zero*

In order to study the organ distribution of $^{123}$I-RC-160 and [$^{123}$I-Tyr$^3$]octreotide in the 16 control rats, the rats were allocated to 2 groups for each radiopharmaceutical. Four rats were injected subcutaneously between the scapulae with 1 ml 0.01 M acetic acid containing 154 mM NaCl, and 4 other rats were injected with 1 mg of RC-160 in the same solvent in order to saturate the somatostatin receptors. Forty-five minutes later the rats were injected with $^{123}$I-RC-160. Similarly, the 2 other groups of 4 rats were pretreated with vehicle or 1 mg of octreotide, and were subsequently injected intravenously with [$^{123}$I-Tyr$^3$]octreotide. The rats were sacrificed 4 h after administration of $^{123}$I-RC-160 or [$^{123}$I-Tyr$^3$]octreotide.

The 24 tumour-bearing Lewis rats were divided in 3 groups of 8 rats. In each group 4 rats were injected subcutaneously with 1 mg RC-160 in order to saturate the somatostatin receptor, as mentioned above. The 3 groups of 8 rats were sacrificed at 30 min, 4 h or 24 h after administration of $^{123}$I-RC-160. The radioactivity concentration in various tissues was subsequently measured.

The specific binding was defined as the difference between the individual uptake in the non-saturated tissues and the mean uptake in the saturated tissues, which are expressed as percentages of the injected radioactivity per gram tissue in the respective organs (mean $\pm$ SD) after administration of $^{123}$I-RC-160 or [$^{123}$I-Tyr$^3$]octreotide.

*Data acquisition and statistical analysis*

All results are expressed as the mean $\pm$ SD. Tissue-binding values and effects of rat growth hormone secretion for both radiolabelled petides were evaluated using Student's *t*-test. A $P$ value of $<0.05$ was considered significant. The tissue distribution and metabolism of $^{123}$I-RC-160 and [$^{123}$I-Tyr$^3$]octreotide in vivo were studied by gamma camera (Rota-II, Siemens) scintigraphy and measurement of isolated organs in a LKB-1282-Compugammasystem (6). The radioactivity in blood and urine was analyzed as described previously (6).

## RESULTS

*Radiolabelling of RC-160*

The efficiency of labelling of RC-160 was 40-60 % for $^{125}$I and 70-90 % for $^{123}$I, in agreement with the radioiodination data of [Tyr$^3$]octreotide, as described previously [6]. Purification of the iodination mixture using the SEP-PAK $C_{18}$ reversed-phase cartridge resulted in mainly non-peptide bound radioiodine in the water fraction and more than 99 % peptide-bound radioiodine in the ethanol fraction, revealed by HPLC. In Fig. 2 a typical HPLC elution pattern of the peptides eluted in the ethanol fraction is shown, indicating a radiochemical purity of more than 95 % of mono-radioiodinated RC-160. The simultaneously measured absorbance at 254 nm and radioactivity show a clear separation between radioiodinated RC-160 and non-radioiodinated RC-160.

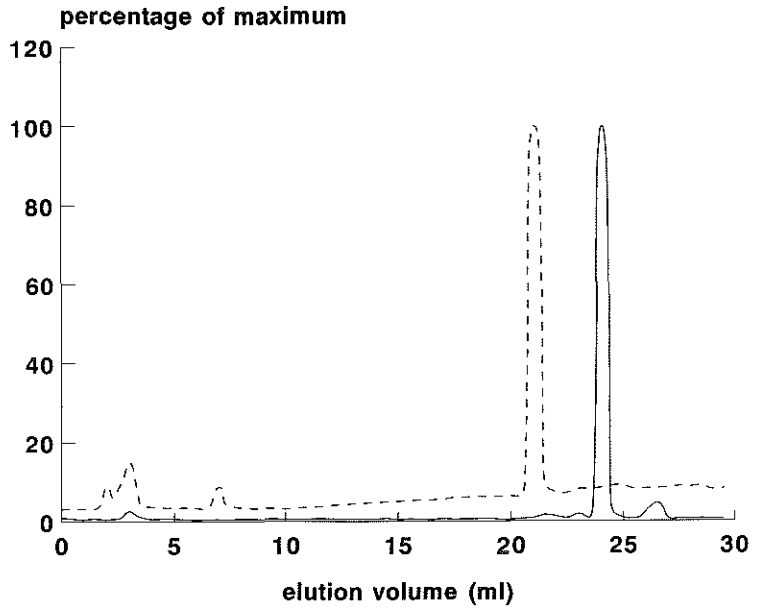



**Figure 2.** *HPLC-elution pattern of the ethanol fraction of SEP-PAK $C_{18}$ purification. Non-radioiodinated RC-160 is measured by UV-absorbance ($\lambda$=254 nm,--), radioiodinated RC-160 and free radioiodide by gamma detection (——). The gamma detected peak at 27 min is the di-iodinated compound*

The acetic acid wash, included in the SEP-PAK purification of [$^{123}$I-Tyr$^3$]octreotide, was omitted from the isolation procedure for $^{123}$I-RC-160, since this wash was found to contain already a substantial fraction ($\approx$ 5-10%) of the radioiodinated RC-160, while only negligible amounts of free radioiodide were detected.

Since the radiolabelling and the SEP-PAK $C_{18}$ separation technique appeared adequate (more than 95 % radiochemical purity of mono-radioiodinated [Tyr$^3$]octreotide and RC-160) HPLC-purification of the radiolabelled somatostatin analogues were not performed. These results were in agreement with the radiolabelling reults of [Tyr$^3$]octreotide (6).

*Receptor binding and specific biologic activity*

In Table 1 the effects are shown of $^{125}$I-RC-160 and [$^{125}$I-Tyr$^3$]octreotide on the secretion of rGH by cultured rat pituitary cells. Both iodinated somatostatin analogues significantly inhibited rGH secretion at 1 nM. Both radioiodinated analogues caused a similar dose-response as the non-radioiodinated counterparts (data not shown).

*Animal studies*

Dynamic scintigraphy of tumour-bearing and control rats after i.v. administration of $^{123}$I-RC-160 and [$^{123}$I-Tyr$^3$]octreotide showed a fast disappearance of the radioactivity from the circulation. With both radioiodinated analogues radioactivity in the blood circulation, as measured above the heart with the gamma camera, decreased in less than 2 min to 50 % of the highest measured radioactivity. This rapid drop of the blood activity can be explained by the phenomenon of distribution of the activity over the whole blood and the interstitial space.

Thirty minutes after injection static images showed a clear uptake of radioactivity in the liver and intestines. The left kidney was seen, as well as excreted activity in the urinary bladder. The right kidney was overprojected by the liver.

Dynamic tumour uptake in situ in the rat during the first 30 min after injection of $^{123}$I-RC-160 was analyzed with the gamma camera. After background correction, using adjacent tissue as reference, no increased uptake in the tumour was found whereas specific binding in the isolated tumour became statistically significant at 4 h (Table 2). As can be seen in Table 2, there is significant specific binding in all the somatostatin receptor-positive tissues analyzed, i.e. pancreas, adrenals, pituitary and brain cortex at 0.5 and 4 h, and in the tumour at 4 h after injection. After 24 h there was still significant specific binding in the pancreas. From 30 min after the administration of $^{123}$I-RC-160, total radioactivity disappeared rapidly from the measured organs and tumours. Statistically significant specific binding was still present 24 h after [$^{123}$I-Tyr$^3$]octreotide injection, in the pancreas, adrenal and pituitary gland (data not shown).

In Table 3 the tissue distributions, 4 h after injection, are compared between pretreated and non-pretreated, non tumour-bearing rats. We found a higher uptake of [$^{123}$I-Tyr$^3$]octreotide in the adrenals, the pancreas and the pituitary gland, than found when using $^{123}$I-RC-160. The effect of pretreatment with unlabelled RC-160 or octreotide is evident in the somatostatin receptor-positive organs for both $^{123}$I-RC-160 and [$^{123}$I-Tyr$^3$]octreotide. Significantly higher specific binding of radioactivity was found after administration of[$^{123}$I-Tyr$^3$]octreotide in the adrenals, the pancreas and the pituitary gland than after administration of $^{123}$I-RC-160. In the rat brain cortex $^{123}$I-RC-160 has a higher uptake and specific binding than [$^{123}$I-Tyr$^3$]octreotide.

Urine samples were obtained 30 min p.i. from tumour-bearing rats (n=4), showing a 13 % $\pm$ 1 % of the total radioactivity in the form of peptide-bound radioiodine. Twenty-four h p.i. the percentage of peptide-bound radioiodine in urine and blood had dropped to 1.3 $\pm$ 0.2 and 1.1 $\pm$ 0.3, respectively, and more than 95 % of the radioactivity in the urine was free radioiodide, which is comparable with the results obtained using [$^{123}$I-Tyr$^3$]octreotide as radioligand (data not shown).

**Table 3.** *Tissue distribution [with (+) and without (-) pretreatment of rats with unlabelled RC-160], and specific binding (Δ) in somatostatin receptor-positive organs (mean ± SD) in non-tumour bearing rats (n=4), 4 h after injection of $^{123}$I-RC-160. The rats in the parallel experiment with [$^{123}$I-Tyr$^3$]octreotide were pretreated with 1 mg octreotide*

| Tissue | | $^{123}$I-RC-160 | | [$^{123}$I-Tyr$^3$]octreotide | |
|---|---|---|---|---|---|
| Pancreas | - | 0.21 ± 0.05 | | 1.03 ± 0.15 | * |
| | + | 0.025 ± 0.006 | | 0.023 ± 0.002 | |
| | Δ | 0.17 ± 0.01 | † | 1.01 ± 0.00 | *† |
| Adrenals | - | 0.19 ± 0.03 | | 0.26 ± 0.02 | * |
| | + | 0.042 ± 0.010 | | 0.027 ± 0.002 | |
| | Δ | 0.15 ± 0.01 | † | 0.23 ± 0.00 | *† |
| Pituitary | - | 0.066 ± 0.007 | | 0.32 ± 0.09 | * |
| | + | 0.0078 ± 0.0020 | | 0.0067 ± 0.001 | |
| | Δ | 0.063 ± 0.002 | † | 0.31 ± 0.00 | *† |
| Brain | - | 0.0052 ±0.0014 | * | 0.0019 ± 0.0002 | |
| cortex | + | 0.0019 ± 0.0003 | | 0.0012 ± 0.0002 | |
| | Δ | 0.0033 ±0.0003 | *† | 0.0007 ± 0.0002 | |

** $P<0.01$ $^{123}$I-RC-160 vs [$^{123}$I-Tyr$^3$]octreotide;*
*† $P<0.01$ specific binding significantly different from zero*

A comparison of the tissue radioactivity concentrations 24 h after injection of $^{123}$I-RC-160 and [$^{123}$I-Tyr$^3$]octreotide is presented in Table 4. [$^{123}$I-Tyr$^3$]octreotide had a higher clearance of radioactivity from somatostatin receptor-negative tissues, such as liver, thymus, blood and lungs and a significantly higher binding in the somatostatin receptor-positive pituitary than $^{123}$I-RC-160. Only in the tumour there was significantly higher binding of radioactivity for $^{123}$I-RC-160 compared to [$^{123}$I-Tyr$^3$]octreotide, but as can be seen from table 2, this is not specific binding.

## DISCUSSION

RC-160 is, like octreotide, a somatostatin analogue with potent hormone secretion-inhibiting characteristics in vivo and in vitro. However, discrepancies with octreotide have been described, especially with regard to binding to a number of human cancers, like those of the breast, ovary, exocrine pancreas, prostate and

colon (14-18,21). Therefore, radioiodinated RC-160 might be an important radiopharmaceutical, having potential advantages over radioiodinated octreotide for the in vivo detection of the last mentioned somatostatin receptor-positive tumours.

In literature no data are available on tissue distribution of the somatostatin analogue RC-160, either in animals or humans. In the present study, therefore, we evaluated the potential use of radioiodinated RC-160 for somatostatin receptor scintigraphy. There was significantly higher uptake and specific binding in somatostatin receptor-positive organs, such as the pancreas, the adrenal and the anterior pituitary gland of $^{123}$I after administration of [$^{123}$I-Tyr$^3$]octreotide than after $^{123}$I-RC-160.

**Table 4.** *Tissue distribution (% of injected dose/g tissue, mean ± SD) in tumour-bearing rats (n=4) 24 h after intravenous injection of 0.5 μg of radioiodinated somatostatin analogue*

| Tissue | $^{123}$I-RC-160 | | [$^{123}$I-Tyr$^3$]octreotide |
|---|---|---|---|
| Spleen | 0.06 ± 0.03 | | 0.028 ± 0.021 |
| Kidneys | 0.12 ± 0.03 | | 0.13 ± 0.01 |
| Liver | 0.17 ± 0.03 | * | 0.027 ± 0.005 |
| Intestines | 0.2 ± 0.0 | * | 0.03 ± 0.01 |
| Thyroid | 320 ± 70 | | 180 ± 50 |
| Thymus | 0.068 ± 0.032 | * | 0.016 ± 0.007 |
| Lungs | 0.12 ± 0.04 | * | 0.033 ± 0.006 |
| Blood | 0.13 ± 0.08 | * | 0.006 ± 0.000 |
| Pancreas | 0.06 ± 0.02 | | 0.11 ± 0.04 |
| Adrenals | 0.06 ± 0.03 | | 0.04 ± 0.01 |
| Pituitary | 0.05 ± 0.05 | * | 0.17 ± 0.02 |
| Brain Cortex | 0.0019 ± 0.0009 | | 0.002 ± 0.001 |
| Tumour | 0.037 ± 0.021 | * | 0.007 ± 0.001 |

** $P<0.01$ $^{123}$I-RC-160 vs [$^{123}$I-Tyr$^3$]octreotide*

In brain cortex of control rats we found at 4 h a low, but statistically significant specific binding of $^{123}$I-RC-160. However, this was caused by a very low amount of tracer (0.0052 % of injected dose per gram) in comparison with other somatostatin receptor-positive tissues, such as the pancreas (0.21 % of injected dose per gram). Since we found a significant difference between the uptake of radioactivity in saturated and non-saturated brain cortex, these data suggests that, in contrast to octreotide, RC-160 and radioiodinated RC-160 are able

to cross the blood-brain barrier, as it has also been reported for cold RC-160 and radioiodinated RC-160 by Banks and Schally (21,24). The presence of the C-terminal amino acid tryptophan in RC-160 (see Fig. 1) enhances the lipophilicity of the molecule, and this might also explain its increased blood-brain barrier permeability and reduced clearance from the tissues and blood.

During the first 30 min after the injection of $^{123}$I-RC-160 no statistically significant uptake was measured in the tumour as measured by gamma camera scintigraphy, and neither after a background correction. This finding is in contrast with the results of the experiments with [$^{123}$I-Tyr$^3$]octreotide as described by Bakker (6). However, 30 min after the injection of $^{123}$I-RC-160, statistically significant specific binding in the isolated tumour was found. Since there is no significant difference between the uptake or specific binding after the injection of [$^{123}$I-Tyr$^3$]octreotide and $^{123}$I-RC-160 in the isolated tumour at 30 min, the relatively low tumour to blood ratio in these experiments is probably the reason for this discrepancy.

## CONCLUSION

$^{123}$I-RC-160 does not seem to have advantages over [$^{123}$I-Tyr$^3$]octreotide as a radiopharmaceutical for somatostatin-receptor scintigraphy, despite the fact that $^{123}$I-RC-160 shows specific high-affinity binding to various somatostatin receptor-positive organs. In contrast to radioiodinated [Tyr$^3$]octreotide and octreotide, which do not pass the blood-brain barrier, our experiments confirm that RC-160 and radioiodinated RC-160 indeed do pass the blood-brain barrier. However, this occurs in low quantities, and consequently the application of radioiodinated RC-160 in nuclear medicine for visualizing somatostatin receptor-positive brain tumours with an intact blood-brain barrier is hampered. In comparison with [$^{123}$I-Tyr$^3$]octreotide the main disadvantage of $^{123}$I-RC-160 is its relatively low tumour to blood (background) ratio, implying poorer in vivo tumour detection.

Apart from the discussed data, it must be emphasized that it has been described by several authors that in comparison to octreotide RC-160 has superior binding characteristics in some human tumours. Therefore, RC-160 and, in spite of its disadvantages, $^{123}$I-RC-160 could open new diagnostic and/or therapeutic applications for patients bearing such tumours. Therefore, in analogy to the development of the $^{111}$In-labelled [DTPA-D-Phe$^1$]octreotide analogue, $^{111}$In-labelled [DTPA-D-Phe$^1$]RC-160 is being prepared and investigated.

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## CHAPTER 6

# A NEW RADIOLABELLED SOMATOSTATIN ANALOGUE [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160: PREPARATION, BIOLOGICAL ACTIVITY, RECEPTOR SCINTIGRAPHY IN RATS AND COMPARISON WITH [$^{111}$In-DTPA-D-Phe$^{1}$]OCTREOTIDE

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## ABSTRACT

We have evaluated the potential usefulness of $^{111}$In-labelled [DTPA-D-Phe$^{1}$]RC-160, derived from the octapeptide somatostatin analogue RC-160, as a radiopharma-ceutical for the in vivo detection of somatostatin receptor-positive tumours. For this purpose $^{111}$In- and $^{115}$In-labelled [DTPA-D-Phe$^{1}$]RC-160 was tested for its biological activity, and applied for somatostatin receptor scintigraphy in vivo to rats bearing the transplantable rat pancreatic tumour CA20948, which expresses somatostatin receptors. We previously described the development of the $^{111}$In-labelled somatostatin analogue [DTPA-D-Phe$^{1}$]octreotide and its use in the in vivo visualization of somatostatin receptor-positive tumours in rats and in humans.

Like [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide, [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160 showed uptake in and specific binding in vivo to somatostatin receptor-positive organs and tumours, and the tumours were clearly visualized by gamma camera scintigraphy. However, as compared to [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide, blood radioactivity (background) was higher, resulting in a lower tumour to blood (background) ratio. Using this animal model we therefore conclude that [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160 has no advantage over [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide as a radiopharmaceutical in the visualization of somatostatin receptors which bind both analogues. However, recent reports suggests the existence of different somatostatin receptor subtypes on some human cancers, which differentially bind RC-160 and not octreotide. These

tumours include cancers of the breast, ovary, exocrine pancreas, prostate and colon. [$^{111}$In-DTPA-D-Phe$^1$]RC-160 might be of interest for future use in such cancer patients as a radiopharmaceutical for imaging somatostatin receptor-positive tumours, which do not bind octreotide.

## INTRODUCTION

High numbers of high-affinity somatostatin receptors for both native somatostatin (for structures, see Fig. 1) and the synthetic octapeptide octreotide (Sandostatin®), have been detected on most neuro-endocrine tumours, like endocrine pancreatic tumours and carcinoids [1-4]. We, and also others have recently described the visualization of somatostatin receptor-positive tumours in vivo after the intravenous administration of [$^{123}$I-Tyr$^3$]octreotide [5-12] and [$^{111}$In-DTPA-D-Phe$^1$]octreotide [13-20]. Several reports have also been published on the in vitro binding to somatostatin receptors of another somatostatin analogue, the octapeptide RC-160 [21-23]. It has been reported that RC-160 has a higher affinity than octreotide for somatostatin receptors in human breast, ovarian, exocrine pancreatic, prostatic and colonic cancers [21-23]. A phase 1 clinical trial with RC-160 in patients with advanced exocrine pancreatic cancer suggests that RC-160 is well tolerated at doses up to 1500 $\mu$g/day [24-25]. The possibility of RC-160 binding to a somatostatin receptor subtype, which does not bind octreotide, for example on human exocrine pancreatic adenocarcinomas [26], offers a potential advantage for RC-160 over octreotide as radiolabelled tumour- tracer. Furthermore, RC-160 and radioiodinated RC-160 may, in contrast to octreotide [27], also pass the blood-brain barrier [28-30]. This could represent a benefit in visualizing somatostatin receptor-positive brain tumours with an intact blood-brain barrier. We recently reported [30] our results with tumour visualization with $^{123}$I-RC-160 in tumour-bearing rats and concluded that, despite the fact that $^{123}$I-RC-160 shows high-affinity binding to various somatostatin receptor-positive tissues and tumours, there was no advantage over [$^{123}$I-Tyr$^3$]octreotide found in binding to the somatostatin receptor-positive tissues and tumours studied sofar.

Therefore, we compared [$^{111}$In-DTPA-D-Phe$^1$]RC-160 with [$^{111}$In-DTPA-D-Phe$^1$]octreotide (for structure see Fig. 1), as radiopharmaceuticals for scintigraphy in normal rats and in rats bearing the transplantable pancreatic somatostatin receptor-positive tumour CA20948 [26,31,32].

## MATERIALS AND METHODS

*Preparation and purification of the radiopharmaceuticals*

RC-160 and [DTPA-D-Phe$^1$]RC-160 were purchased from Sanbio (Uden, The Netherlands). [DTPA-D-Phe$^1$]octreotide and $^{111}$In-$Cl_3$ (DRN 4901) were obtained from Mallinckrodt (Petten, The Netherlands). The radiolabelling of [DTPA-D-Phe$^1$]octreotide and [DTPA-D-Phe$^1$]RC-160 with $^{111}$In and consecutive

quality control were performed as described earlier [33]. Although it is not excluded that additional groups of the peptides participate in $^{111}$In complexation, the labelled products are referred to as [$^{111}$In-DTPA-D-Phe$^1$]RC-160 and [$^{111}$In-DTPA-D-Phe$^1$]octreotide.

**Somatostatin**

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

**Octreotide**

D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

**Tyr$^3$-octreotide**

D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr(ol)

**[$^{111}$In-DTPA-D-Phe$^1$]octreotide**

$^{111}$In-DTPA-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

**RC-160**

D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-$NH_2$

**[$^{111}$In-DTPA-D-Phe$^1$]RC-160**

$^{111}$In-DTPA-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-$NH_2$

**Figure 1.** *Structural formulae of native somatostatin, octreotide, Tyr$^3$-octreotide, [$^{111}$In-DTPA-D-Phe$^1$]octreotide, RC-160 and [$^{111}$In-DTPA-D-Phe$^1$]RC-160*

The radiochemical purity of the radiolabelled somatostatin analogues was greater than 95 %. All chemicals used were of the highest purity available.

*Animals and tumours*

Male Lewis rats (240-260 g) were inoculated in both upper hind legs with the transplantable rat pancreatic tumour CA20948, which has previously been shown to possess somatostatin receptors [26]. Male Lewis rats (240-260 g), not bearing the CA20948 tumour, were used as control rats.

For injection and scintigraphy the rats were anaesthetized with ether.
In order to study specific binding, the rats were injected subcutaneously with 1 mg RC-160 or 1 mg octreotide in 1 ml 0.05 M acetic acid containing 154 mM NaCl or solvent. Forty-five minutes later the rats were injected with either [$^{111}$In-DTPA-D-Phe$^1$]RC-160 or [$^{111}$In-DTPA-D-Phe$^1$]octreotide. Specific binding was defined as the difference between the tissue uptake in tumour-inoculated rats and that in similar animals treated with excess of unlabelled peptide, expressed as percentages of the injected radioactivity per gram tissue (mean $\pm$ SD).

The tracer agents were injected into the dorsal vein of the penis. The dose was 18.5 MBq (0.5 $\mu$g) for both analogues, measured in a dose calibrator (VDC-202, Veenstra, Joure, The Netherlands). The rats were sacrificed 2, or 4, or 24, or 48, or 72 h after administration of the radiolabelled somatostatin analogue. The concentration of radioactivity in various tissues, urine and blood was subsequently measured.

[$^{111}$In-DTPA-D-Phe$^1$]octreotide and [$^{111}$In-DTPA-D-Phe$^1$]RC-160 binds to SEP-PAK $C_{18}$ stationary phase and is, using the separation technique as described by Bakker [6], only eluted with ethanol, while $^{111}$In-DTPA does not retain on SEP-PAK $C_{18}$ columns. Radioactivity in plasma and urine samples which is also eluted with ethanol from the SEP-PAK $C_{18}$ column is termed peptide-bound radioactivity, but is not further characterized.

The radioactivity in blood and urine was analysed as described previously [6].

*Biological activity and radioligand binding studies*

[In-DTPA-D-Phe$^1$]octreotide (labelled with natural indium, i.e. a mixture of 4.23 % non radioactive $^{113}$In and 95.77 % $^{115}$In, $t½ = 6.10^{14}$ years, further on referred to as $^{115}$In) and [$^{115}$In-DTPA-D-Phe$^1$]RC-160 were prepared by mixing the respective [DTPA-D-Phe$^1$]-somatostatin analogues (stock concentration of $10^{-4}$ M) with $10^{-3}$ M $^{115}InCl_3$ (Aldrich) in 0.05 M acetic acid, to a 80-fold molar excess ratio of indium over peptide. The biological activity of [DTPA-D-Phe$^1$]octreotide, [$^{115}$In-DTPA-D-Phe$^1$]octreotide, [DTPA-D-Phe$^1$]RC-160 and [$^{115}$In-DTPA-D-Phe$^1$]RC-160 was assessed by measuring its potency to inhibit the secretion of rat growth hormone (rGH) from cultured rat pituitary cells. The preparation of dispersed

female rat anterior pituitary cells and cell culture conditions have been described earlier [34].

Receptor binding assays were carried out using [$^{125}$I-Tyr$^3$]octreotide as described earlier [35]. Binding curves and $IC_{50}$ for displacement of [$^{125}$I-Tyr$^3$]octreotide binding by unlabelled peptide were calculated from 2 experiments (triplicate determinations) using the computer fitting program of Graphpad (ISI software, Philadelphia, PA, USA).

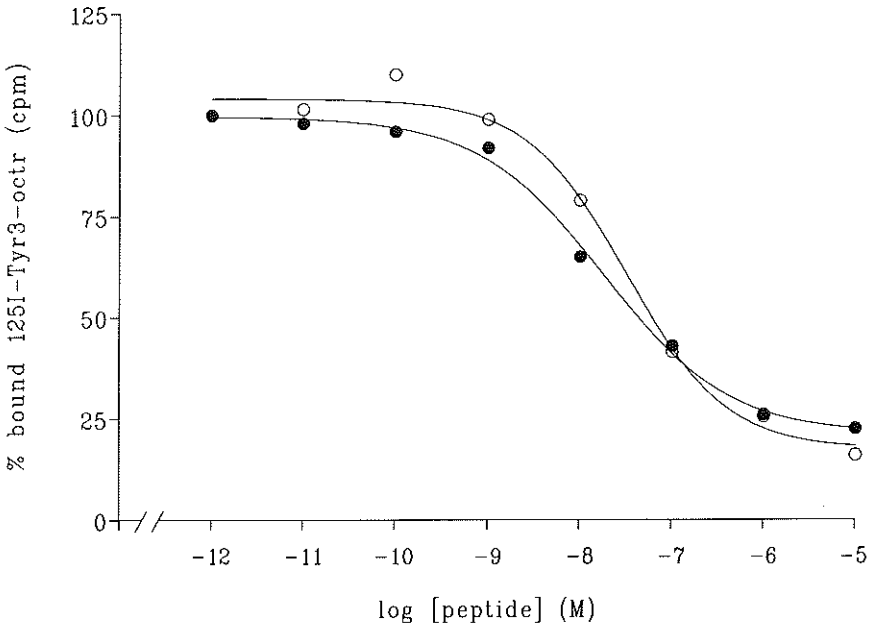



**Figure 2.** *Binding of [$^{125}$I-Tyr$^3$]octreotide to rat brain cortex membranes in the presence of increasing concentrations of [DTPA-D-Phe$^1$]RC-160 (•) and [DTPA-D-Phe$^1$]octreotide (◦), expressed as the percentage of binding in the absence of competing compounds (n=3, maximal SD < 10 %). No significant differences were found at equimolar concentrations*

*Data acquisition and analysis*

All results are expressed as the mean ± SD.

One-way analysis of variance (ANOVA), was used for statistical analysis. For the comparison of means the Newman-Keuls or Bonferroni *t*-test were applied [36]. A *P* value of $<0.05$ was considered significant. The tissue distribution and metabolism of the $^{111}$In-labelled somatostatin analogues in vivo were studied by gamma camera scintigraphy (Rota-II, Siemens)[13] and measurement of $^{111}$In in isolated

organs were performed using a LKB-1282-Compugammasystem [6].

The ratio of tissue-binding values of tissue over soft tissue (thigh) and the radioactivity concentration ratio of tumour over soft tissue were calculated for each individual organ and for the tumours.

## RESULTS

*Quality control, radiolabelling and preparation of the radiopharmaceutical*

Amino acid analysis of RC-160 and [$^{111}$In-DTPA-D-Phe$^1$]RC-160 yielded a peptide content of more than 95 % of the correct amino acid composition. The identity of the peptides was confirmed by fast atom bombardment mass spectrometry, showing molecular weight of 1131 and 1507 dalton for RC-160 and [DTPA-D-Phe$^1$]RC-160, respectively.

Over 95 % efficiency of labelling, tested by ITLC and confirmed by high-performance liquid chromatography (HPLC), was assured when a molar excess of 5- to 10-fold of peptide over $^{111}$In was used, resulting in the formation of [$^{111}$In-DTPA-D-Phe$^1$]RC-160 with a specific activity of 150 MBq $^{111}$In per $\mu$gram [DTPA-D-Phe$^1$]RC-160. The [$^{111}$In-DTPA-D-Phe$^1$]RC-160 was stable for at least 4 h after preparation, and for longer than 24 h if stabilised by either dilution, with for instance saline, or addition of a quencher, such as gentisic acid.

*Receptor binding studies and biological activity*

Fig. 2 shows binding of [$^{125}$I-Tyr$^3$]octreotide to rat brain cortex membranes in the presence of increasing concentrations of unlabelled [DTPA-D-Phe$^1$]octreotide or [DTPA-D-Phe$^1$]RC-160, with $IC_{50}$ values of 20 and 30 nM, respectively.

In Fig. 3 the effects of [$^{115}$In-DTPA-D-Phe$^1$]octreotide, [DTPA-D-Phe$^1$]octreotide, [$^{115}$In-DTPA-D-Phe$^1$]RC-160 and [DTPA-D-Phe$^1$]RC-160 on the secretion of rat growth hormone by cultured rat anterior pituitary cells are shown. There is a dose dependant inhibition of basal rat growth hormone release by these cells, with $IC_{50}$ values of 3, 8, 5 and 15 nM, respectively for the four compounds mentioned above. There were no significant differences between the effects of the two $^{115}$In-labelled and the two non-labelled somatostatin analogues, ($P > 0.05$, Student's *t*-test). Trivalent indium ions, up to a concentration of $8.10^{-5}$ M, did not influence rat growth hormone assay (data not shown).

*Tissue distribution and specific binding*

The radioactivities measured in the isolated tumour and organs, 24 h after injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide and 24, 48 and 72 h after injection of [$^{111}$In-DTPA-D-Phe$^1$]RC-160 are shown in Table 1. Between 24 and 48 hours after injection of [$^{111}$In-DTPA-D-Phe$^1$]RC-160 there is a decrease of radioactivity in most tissues, accompanied by an increase of radioactivity in rat kidney. The uptakes in

the kidney, in the somatostatin receptor-positive tissues and in the tumour were higher for [$^{111}$In-DTPA-D-Phe$^1$]RC-160 than for [$^{111}$In-DTPA-D-Phe$^1$]octreotide. The ratios of tumour over blood and tumour over soft tissue for [$^{111}$In-DTPA-D-Phe$^1$]RC-160 were significantly lower than those found for $^{111}$In-DTPA-D-Phe$^1$]octreotide, mainly because of the very low uptake of radioactivity in soft tissue after injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide (Table 1). Table 1 also shows that even 72 h after injection of [$^{111}$In-DTPA-D-Phe$^1$]RC-160 the remaining radioactivity in blood is still twice the value 24 h after injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide, 0.0061 % *vs* 0.0035 % injected dose per gram. For soft tissue the relative difference is even higher, 0.013 % *vs* 0.002 % injected dose per gram (Table 1).

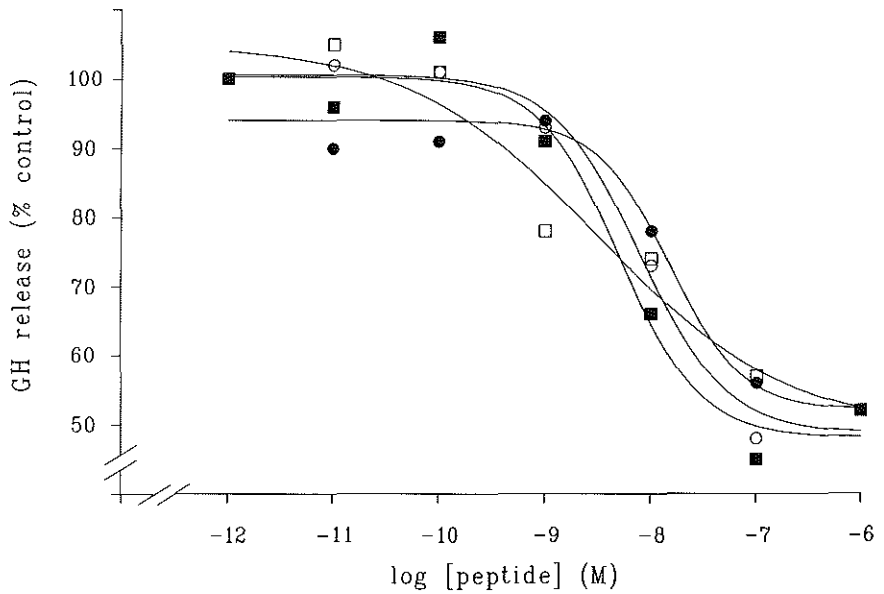



**Figure 3.** *Effects of [$^{115}$In-DTPA-D-Phe$^1$]RC-160 (■), [DTPA-D-Phe$^1$]RC-160 (•), [$^{115}$In-DTPA-D-Phe$^1$]octreotide (□) and [DTPA-D-Phe$^1$]octreotide (○) on the secretion of rat growth hormone from cultured rat pituitary cells (n=4, maximal SD < 15 %). No significant differences were found at equimolar concentrations*

Despite the decrease in radioactivity in most tissues, which are somatostatin receptor-negative tissues, total body radioactivity does not decrease between 24 and 48 h after the injection of [$^{111}$In-DTPA-D-Phe$^1$]RC-160, due to an increase of radioactivity in the kidneys (Table 1). This results in a significantly higher body retention of radioactivity after injection of [$^{111}$In-DTPA-D-Phe$^1$]RC-160 than with [$^{111}$In-DTPA-D-Phe$^1$]octreotide. This higher concentration of radioactivity in the

kidneys after [$^{111}$In-DTPA-D-Phe$^1$]RC-160 is striking, since renal excretion is also the predominant route for clearance of [$^{111}$In-DTPA-D-Phe$^1$]octreotide. For instance even about 50 procent of the injected [$^{111}$In-DTPA-D-Phe$^1$]octreotide is excreted via the kidneys in the first 30 min after injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide, as described earlier by Bakker [13].

**Table 1.** *Tissue distribution (% injected dose per gram tissue, mean ± SD) and tissue ratios in tumour-bearing rats ($n \geq 4$) at indicated time intervals after intravenous administration of 0.5 μg of $^{111}$In-labelled somatostatin analogue*

| Tissue | In-octreotide 24 h | In-RC 24 h | In-RC 48 h | In-RC 72 h |
|---|---|---|---|---|
| Adrenals | 2.2 ± 0.4 | 7.1 ± 2.6 | 0.87 ± 0.12 | 0.73 ± 0.03 |
| Pancreas | 0.92 ± 0.08 | 0.97 ± 0.28 | 0.42 ± 0.14 | 0.46 ± 0.14 |
| Tumours | 0.20 ± 0.02 | 0.69 ± 0.12 | 0.21 ± 0.08 | 0.20 ± 0.08 |
| Pituitary | 0.11 ± 0.02 | 0.35 ± 0.02 | 0.11 ± 0.02 | 0.13 ± 0.01 |
| Brain cortex | 0.0011 ± 0.0001 | 0.011 ± 0.001 | 0.0015 ± 0.0002 | 0.0016± 0.0002 |
| Kidneys | 2.9 ± 0.3 | 5.3 ± 0.8 | 12 ± 1 | 12 ± 2 |
| Liver | 0.031 ± 0.006 | 0.74 ± 0.04 | 0.22 ± 0.03 | 0.26 ± 0.07 |
| Spleen | 0.033 ± 0.018 | 0.93 ± 0.08 | 0.36 ± 0.08 | 0.30 ± 0.04 |
| Intestines | 0.05 ± 0.01 | 0.37 ± 0.09 | 0.12 ± 0.03 | 0.18 ± 0.04 |
| Soft tissue | 0.0022 ± 0.0003 | 0.046 ± 0.003 | 0.011 ± 0.004 | 0.013 ± 0.001 |
| Blood | 0.0035 ± 0.0006 | 0.16 ± 0.02 | 0.0067 ± 0.0006 | 0.0061 ± 0.0012 |
| In toto | 13 ± 2 | 50 ± 4 | 51 ± 5 | 49 ± 2 |
| Tumours vs soft tissue | 97 ± 9 | 12 ± 3 | 25 ± 9 | 13 ± 7 |
| Tumours vs blood | 58 ± 9 | 3.6 ± 0.8 | 32 ± 8 | 30 ± 11 |

In-RC : [$^{111}$In-DTPA-D-Phe$^1$]RC-160
In-octreotide : [$^{111}$In-DTPA-D-Phe$^1$]octreotide

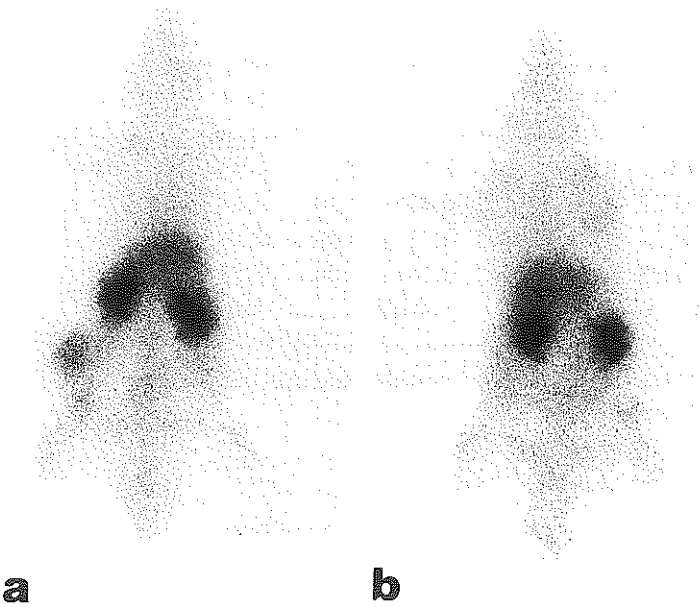
In Table 2 the specific binding at several time intervals after injection of [$^{111}$In-DTPA-D-Phe$^1$]RC-160 and [$^{111}$In-DTPA-D-Phe$^1$]octreotide is given for the somatostatin receptor-positive tissues and tumour. There were no significant differences in binding of either peptide to these sites. Also, no specific binding of either analogue was found in the brain, suggesting that they were unable to pass the blood-brain barrier.

*Metabolism*

SEP-PAK $C_{18}$ columns were used for measuring peptide-bound radioactivity in blood and urine of 4 control rats at 4 h and 24 h after the injection of [$^{111}$In-

DTPA-D-Phe$^1$]RC-160. The percentages peptide-bound radioactivity in blood were 23 $\pm$ 2 at 4 h p.i. and 18 $\pm$ 1 at 24 h p.i.. HPLC-analysis of the ethanol-fractions of the SEP-PAK $C_{18}$ columns of these blood samples confirmed that more than 90 % of this peptide-bound $^{111}$In was intact [$^{111}$In-DTPA-D-Phe$^1$]RC-160 and at 24 h p.i. this figure had dropped below 50 %. The nature of the metabolites of both $^{111}$In-labelled radiopharmaceuticals was not further investigated. The percentage of peptide-bound $^{111}$In in urine-samples was respectively 73 $\pm$ 4 at 4 h , and 75 $\pm$ 2 at 24 h after injection of [$^{111}$In-DTPA-D-Phe$^1$]RC-160. In the urine-samples from two control rats, 24 h after injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide, when most of the radioactivity already has been excreted, more than 90 % of the $^{111}$In was not peptide-bound, as described earlier by Bakker [13].

**Figure 4**. *Static posterior images in rats with unilateral implanted somatostatin receptor-positive CA 20948 tumour, located at the upper part of the right hind leg, 24 h after injection of [$^{111}$In-DTPA-D-Phe$^1$]RC-160. Note the normal uptake of radioactivity in liver and kidneys in both images. There is accumulation of radioactivity in the tumour (a, no preteatment). Notice the absence of accumulation of radioactivity in the tumour in (b). In (b) the rat was pretreated s.c. with 1 mg RC-160 45 min prior to the injection with [$^{111}$In-DTPA-D-Phe$^1$]RC-160 in order to saturate the somatostatin receptors*



*Imaging*

Dynamic images during the first 20 min after injection [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160 or [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide showed a rapid distribution of radioactivity over the whole body. Pretreatment with excess unlabelled RC-160 or octreotide did not affect the blood clearance. Immediately after injection of [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160 or [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide, an increasing amount of radioactivity was measured over the tumours.

From digital static images obtained 24, 48 and 72 h after injection of [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160, whole body retention was found to be approximately 50 procent of the injected dose after 48 h and 72 h, and this retention was predominantly accounted for by radioactivity in the kidneys, see also Table 1. Figure 4 presents static analog images of 2 rats with unilateral somatostatin receptor-positive CA20948 tumours, 24 h after the injection of [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160. Accumulation of radioactivity in the tumour and kidneys was observed. The uptake of radioactivity by the tumour was succesfully inhibited by the pretreatment of unlabelled RC-160 (Fig. 4).

**Table 2.** *Specific binding in somatostatin receptor-positive organs and tumours (percentage injected dose per gram tissue, mean ± SD) in tumour-bearing rats ($n \geq 4$), at indicated time intervals after intravenous administration of 0.5 μg of $^{111}$In-labelled somatostatin analogue*

| Tissue | In-RC 4 h | In-RC 24 h | In-octreotide 4 h | In-octreotide 24 h |
|---|---|---|---|---|
| Adrenals | 5.0 ± 0.3 | 6.1 ± 2.6 | 2.1 ± 0.5 | 1.8 ± 0.4 |
| Pancreas | 0.44 ± 0.22 | 0.56 ± 0.28 | 0.47 ± 0.20 | 0.44 ± 0.15 |
| Tumours | 0.30 ± 0.14 | 0.35 ± 0.12 | 0.21 ± 0.09 | 0.19 ± 0.05 |
| Pituitary | 0.40 ± 0.05 | 0.23 ± 0.02 | 0.16 ± 0.06 | 0.12 ± 0.02 |
| Brain cortex | <0.001 | <0.001 | <0.001 | <0.001 |

*Except for the brain cortex, all organs and tumours had specific binding significantly different from zero, $P < 0.05$*
*In-RC : [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160*
*In-octreotide : [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide*

## DISCUSSION

Like octreotide, RC-160 is a somatostatin analogue with potent hormone secretion-inhibitory and anti-proliferative characteristics in vivo and in vitro. However, discrepancies with octreotide have been described, with regard to

binding to a number of human cancers, such as breast, ovarian, exocrine pancreas, prostate and colon carcinomas [21-23]. Because radiolabelled RC-160 could have advantages over radiolabelled octreotide for the in vivo detection of some of these somatostatin receptor-positive tumours, at first we evaluated radioiodinated RC-160 as a radiopharmaceutical. These studies revealed some major drawbacks of the $^{123}$I-RC-160 as compared to [$^{123}$I-Tyr$^3$]octreotide. The main disadvantage of $^{123}$I-RC-160 was its relatively low tumour to background ratio, implying poorer in vivo tumour detection [30]. In analogy with the development of [$^{111}$In-DTPA-D-Phe$^1$]octreotide we therefore evaluated [$^{111}$In-DTPA-D-Phe$^1$]RC-160. Since for use in animal models no somatostatin receptor-positive tumours are available displaying binding specificity for RC-160 and not for octreotide, we investigated both somatostatin analogues in the CA20948 tumour-model.

The radiolabelling of [DTPA-D-Phe$^1$]RC-160 with (high quality) $^{111}$In is a simple single-step procedure with a high effiency of labelling (> 95%) and does not require special skills nor equipment. We found no significant differences in biological activity between [$^{115}$In-DTPA-D-Phe$^1$]RC-160 and [$^{115}$In-DTPA-D-Phe$^1$]octreotide and the two non-labelled [DTPA-D-Phe$^1$]-somatostatin analogues, as measured by inhibition of rat growth hormone secretion. Recently, however, we found that the non-DTPA conjugated RC-160 was significantly more potent in inhibiting hormone release by normal and tumourous pituitary cells (37). Therefore, a direct comparison between somatostatin analogues with their DTPA-conjugated counterparts should be made with care. The results in the binding studies demonstrated that both non-labelled [DTPA-D-Phe$^1$]-somatostatin analogues are high-affinity and selective ligands for the somatostatin receptor. This was also demonstrated in the in vivo experiments after injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide and [$^{111}$In-DTPA-D-Phe$^1$]RC-160, in which uptake and specific binding in somatostatin receptor-positive tissues and tumours were found. Also, the somatostatin receptor-positive tumours were clearly visualized by gamma camera scintigraphy. However, we did find differences in metabolism and clearance of radioactivity from the blood compartment, soft tissue and other somatostatin receptor-negative tissues. A higher radioactivity concentration and a slower clearance from these tissues were demonstrated for [$^{111}$In-DTPA-D-Phe$^1$]RC-160. The difference in uptake of radioactivity in the liver and excretion in the intestines after injection of [$^{111}$In-DTPA-D-Phe$^1$]RC-160 or [$^{111}$In-DTPA-D-Phe$^1$]octreotide was probably due to a difference in handling of both analogues by the liver [38]. In the perfused rat liver uptake of radioactivity from the medium was 20 % of the dose in the liver during the first hour of perfusion with [$^{111}$In-DTPA-D-Phe$^1$]RC-160, while this was 2 % of the dose for [$^{111}$In-DTPA-D-Phe$^1$]octreotide. Less than 1 % of the dose of both radiolabelled somatostatin analogues were excreted in the bile, where they were in the peptide-bound form.

The presence of the C-terminal amino acid tryptophan in RC-160 (see Fig.

1) enhances the lipophilicity of the molecule, and this may explain the enhanced uptake of [$^{111}$In-DTPA-D-Phe$^1$]RC-160 and/or its metabolites from the liver and its reduced clearance from tissues and blood. The ratios of tumour over blood and tumour over soft tissue for [$^{111}$In-DTPA-D-Phe$^1$]RC-160 are lower than those for [$^{111}$In-DTPA-D-Phe$^1$]octreotide. Although the uptake of radioactivity in the tumour after [$^{111}$In-DTPA-D-Phe$^1$]RC-160 experiments is higher, the clearance of radioactivity from the blood compartment and soft tissue is much slower than for [$^{111}$In-DTPA-D-Phe$^1$]octreotide. This implies a poorer visualization of tumours when the former somatostatin analogue is used. The lack of uptake in brain cortex suggests the inability to penetrate an intact blood-brain barrier for both [DTPA-D-Phe$^1$]-somatostatin analogues. This contrasts with earlier data on RC-160 and radioiodinated RC-160 [28-30], and implies that [$^{111}$In-DTPA-D-Phe$^1$]RC-160 is most probably not suitable for visiualizing human brain tumours with intact blood-brain barrier. However, because mention has been made of somatostatin receptor-positive tumours with other subtypes which do not bind (labelled) octreotide, we want to hypothesize that [$^{111}$In-DTPA-D-Phe$^1$]RC-160 might be applicable in the field of nuclear medicine in the visualization of some human cancers. We currently investigate patients with a negative [$^{111}$In-DTPA-D-Phe$^1$]octreotide gamma camera scintigraphy who have tumours which do not bind octreotide, but belong to the group of tumours which Schally *et al.* suggested to have a high affinity for RC-160 [21-23]. The therapeutic possibilities of [$^{131}$I-Tyr$^3$]octreotide are low [7], since the residence time of $^{131}$I in the tumour is short. This is because radioiodinated [Tyr$^3$]octreotide is rapidly metabolised with release of $^{131}$I in the circulation, as was demonstrated previously in rats [6,30] and patients [7].

Since the residence time in/on the tumourcells of somatostatin analogues with a DTPA-group appears prolonged, it seems worthwhile to investigate if DTPA-peptides labelled with $^{131}$I or other ß-emitting radionuclides would be suitable for radiotherapy. Therefore, [DTPA-D-Phe$^1$, $^{131}$I-Tyr$^3$]octreotide or [DTPA-D-Phe$^1$, $^{131}$I-Tyr$^3$]RC-160 could open new therapeutic applications for patients bearing somatostatin receptor-positive tumours. However, whether the residence time of the radioiodinated somatostatin analogues with a DTPA-group is also prolonged remains to be established, since deiodination of the molecule will than hamper the therapeutic application.

## CONCLUSION

[$^{111}$In-DTPA-D-Phe$^1$]RC-160 does not seem to have advantages over [$^{111}$In-DTPA-D-Phe$^1$]octreotide as a radiopharmaceutical for somatostatin receptor scintigraphy, despite the fact that [$^{111}$In-DTPA-D-Phe$^1$]RC-160 shows specific high-affinity binding to various somatostatin receptor-positive organs. In contrast to radioiodinated [Tyr$^3$]RC-160 and RC-160 which do pass the blood-brain barrier, our experiments show that [$^{111}$In-DTPA-D-Phe$^1$]RC-160 and [$^{111}$In-DTPA-D-

Phe$^1$]octreotide do not pass the blood-brain barrier. In comparison with [$^{111}$In-DTPA-D-Phe$^1$]octreotide the main disadvantage of [$^{111}$In-DTPA-D-Phe$^1$]RC-160 (as we recently also showed for $^{123}$I-RC-160) is its relatively low tumour to blood (background) ratio, implying poorer in vivo tumour detection.

However, previous studies suggest that RC-160 might bind to several human cancer types, which do not bind octreotide. If the existence of different somatostatin receptor subtypes is confirmed, RC-160 and, in spite of its disadvantages, [$^{111}$In-DTPA-D-Phe$^1$]RC-160 may open new diagnostic and/or therapeutic applications for patients bearing such tumours. Further studies in patients need to be performed, with special attention to patients with a negative [$^{111}$In-DTPA-D-Phe$^1$]octreotide gamma camera scintigraphy scan and/or patients with tumours with a higher affinity for RC-160.

Since the DTPA-group appears to prolong the residence time of somatostatin analogues in/on the tumourcells, it seems worthwhile to investigate if DTPA-peptides labelled with $^{131}$I or other ß-emitting radionuclides and a likewise prolonged residence time in/on the tumourcells, would be suitable for radiotherapy.

Consequently, [DTPA-D-Phe$^1$, $^{131}$I-Tyr$^3$]octreotide or [DTPA-D-Phe$^1$, $^{131}$I-Tyr$^3$]RC-160 could then open new therapeutic applications for patients bearing somatostatin receptor-positive tumours.

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## CHAPTER 7

# SOMATOSTATIN RECEPTOR SCINTIGRAPHY USING [$^{111}$In-DTPA-D-Phe$^1$]OCTREOTIDE AND [$^{111}$In-DTPA-D-Phe$^1$]RC-160

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### Somatostatin, somatostatin-analogues and somatostatin receptors

Somatostatin ($SS_{14}$) is a disulfide-containing peptide hormone of 14 amino-acids (for structure, see Fig. 1). It is present in the hypothalamus, the cerebral cortex, the brain stem, the gastrointestinal tract, and the pancreas. In the central nervous system it acts as a neurotransmitter, while its hormonal activities include the inhibition of the release of growth hormone, insulin, glucagon and gastrin (see [1] for a review). Somatostatin receptors have been identified on many cells of neuroendocrine origin, including the somatotroph cells of the anterior pituitary gland, the thyroid C cells and the pancretic islet cells (2,3). However, also non-neuroendocrine, such as lymphocytes (4), may possess these receptors.

The general inhibitory effect of somatostatin on hormone production by various glands led to the concept of possible beneficial effects of somatostatin in the treatment of diseases based on gland hyperfunction or overproduction of hormones by (endocrine-active) tumours. However, the tetradecapeptide somatostatin itself turned out to be unsuitable for routine treatment. After intravenous administration somatostatin has a half-life of $\approx$ 3 min in man, due to enzymatic degradation. In recent years successful efforts have been undertaken to prepare somatostatin analogues that are more resistant to enzymatic degradation by

modifying the molecule in various ways with preservation of the biological activity of the original molecule. Introduction of D-aminoacids and shortening of the molecule to the bioactive core sequence, resulted in the 8 aminoacids-containing somatostatin analogue octreotide (Sandostatin®). Octreotide is currently widely used successfully in the treatment of neuroendocrine-active tumours such as growth hormone-producing pituitary adenomas and gastroenteropancreatic tumours (5,6).

Somatostatin receptors are structurally related integral membrane glycoproteins. Recently, five different human somatostatin receptor types have been cloned. All subtypes bind $SS_{14}$ and $SS_{28}$ (pro-somatostatin with 28 aminoacids) with high affinity, while their affinity for numerous somatostatin analogues differ considerably (7-10). Octreotide binds with high affinity to the hSSTR2 (somatostatin receptor type 2) subtype, while this analogue has a relatively low affinity for hSSTR3 and hSSTR5 and shows no binding to hSSTR subtypes 1 and 4 (7-10). Octreotide scintigraphy is therefore based on the visualization of an octreotide-binding somatostatin receptor (octreotide receptor), most probably the hSSTR2.

Recently, several reports have been published on the in vitro binding to somatostatin receptors of another somatostatin analogue, the octapeptide RC-160, which structure is very similar to that of octreotide (Fig. 1) (11-13). It has been reported that RC-160 has a higher affinity than octreotide for the somatostatin receptor in human breast, ovarian, exocrine pancreatic, prostatic and colonic cancer (11-13). We and others have recently described the visualization of somatostatin receptor-positive tumours in vivo after the administration of [$^{111}$In-DTPA-D-Phe$^1$]octreotide (Octreoscan®; see for a review [14]) or [$^{111}$In-DTPA-D-Phe$^1$]RC-160 (15).

We currently investigate patients with a negative [$^{111}$In-DTPA-D-Phe$^1$]octreotide scintigram who have tumours, which do not bind octreotide but belong to the group of tumours which Schally et al. suggested to have a high affinity for RC-160 (11-13).

## Factors that influence uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in somatostatin receptor-positive tissue, and the target to background ratio

In nuclear medicine the uptake of radioactivity and its residence time in the target, and the target to background ratio are important for visualization. Relevant factors which influence this process include the capacity of the radiopharmaceutical to pass biomembranes, the competition by endogenous ligand and the rate of blood perfusion of the tissue. For instance, the production of somatostatin in the pancreas may be a contributing factor in the relative high optimal dose of 5 $\mu$g of [$^{111}$In-DTPA-D-Phe$^1$]octreotide required for maximum specific binding of the radiopharmaceutical in the rat pancreas (16). This is in contrast to the optimal dose of 0.5 $\mu$g for the highly perfused adrenal, which does not produce somatostatin (16).

Other factors include the dissociation constant between the radiopharmaceutical and the receptor, the rate of internalization and/or degradation of the ligand-receptor complex, and the rate of reexpression and/or upregulation of the receptor. All above-mentioned parameters illustrate the dynamics and the complexity of the ligand-receptor interaction, particular in vivo. A preliminairy study in patients (17) confirmed our data in rats, indicating that uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in somatostatin receptor-positive tissue is a bell-shaped function of the injected mass of the radiopharmaceutical, but further studies are needed to determine which peptide mass is optimal for the uptake of $^{111}$In after the administration of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in different somatostatin receptor-positive organs and tumours in humans. These findings imply that the hypothesis that (somatostatin) receptor scintigraphy shows an optimal target to background ratio at the lowest possible mass of the radiopharmaceutical labelled with the highest specific radioactivity is not correct.

The mode of administration of the radiopharmaceutical is another factor that influences its effective concentration and exposure time to the receptor. In experiments with rats the radiopharmaceutical (3 MBq $^{111}$In labelled with 0.5 $\mu$g [DTPA-D-Phe$^1$]octreotide) was injected over periods of 3, 45 or 180 seconds. The octreotide receptor-positive anterior pituitary, adrenal and pancreas and the octreotide receptor-negative kidneys, liver, spleen, soft tissue (thigh) and blood were isolated after 24 h. The tissue uptake (% injected dose per gram) and the octreotide receptor-positive target to background (blood or soft tissue) ratio were calculated. Only in the adrenal a significant increase in uptake and its target to background ratio was found when the radiopharmaceutical was slowly injected, with a significant increase from 3 to 45 sec, and optimum at 180 seconds. No significant alterations were detected in other tissues.

**Radiotherapy**

The therapeutic possibilities of [$^{131}$I-Tyr$^3$]octreotide are limited (18), since the residence time of $^{131}$I in the tumour is short. This is because radioiodinated [Tyr$^3$]octreotide is rapidly metabolised with release of $^{131}$I in the circulation, as was demonstrated previously in rats (19,20) and in patients (18). Since the residence time in/on the tumour cells of somatostatin analogues with a DTPA-group appears prolonged (14,19), it seems worthwhile to investigate if DTPA-peptides labelled with $^{131}$I or other ß-emitting radionuclides would be suitable for radiotherapy. Therefore, [DTPA-D-Phe$^1$, $^{131}$I-Tyr$^3$]octreotide or [DTPA-D-Phe$^1$, $^{131}$I-Tyr$^3$]RC-160 could open new therapeutic applications for patients bearing somatostatin receptor-positive tumours. However, whether the residence time of the radioiodinated DTPA-somatostatin analogues is also prolonged remains to be established, since deiodination may still hamper the therapeutic application.

**Somatostatin**

Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys

**Octreotide**

D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

**[$^{111}$In-DTPA-D-Phe$^1$]octreotide**

$^{111}$In-DTPA-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

**[$^{111}$In-DTPA-D-Phe$^1$]RC-160**

$^{111}$In-DTPA-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-$NH_2$

**Figure 1.** *Structural formula of somatostatin, octreotide, [$^{111}$In-DTPA-D-Phe$^1$]octreotide and [$^{111}$In-DTPA-D-Phe$^1$]RC-160*

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Submitted

# CHAPTER 8

## SUBSTANCE P RECEPTOR SCINTIGRAPHY: INITIAL STUDIES IN VITRO AND IN RATS

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**ABSTRACT**

We have evaluated the potential usefulness of a new radiolabelled substance P (SP) analogue, [$^{111}$In-DTPA-Arg$^1$]SP, as a radiopharmaceutical for the in vivo detection of SP receptor-positive (SPR+) immunological disorders and tumours. SP, [DTPA-Arg$^1$]SP, and [3-(p-hydroxyphenyl)propionyl-Arg$^1$]SP (Bolton-Hunter-SP,[BH-SP]), inhibited dose-dependently the binding of $^{125}$I-BH-SP to the SPR in rat brain cortex membranes with $IC_{50}$ values of 0.2, 4 and 2 nM, respectively. In an autoradiographic displacement study of the submandibular gland with $^{125}$I-BH-SP as radioligand, an $IC_{50}$ of 2.7 nM was found for [DTPA-Arg$^1$]SP. In vivo metabolism of the radiopharmaceutical in the rat revealed a renal clearance of 50 % of the injected radioactive dose in 30 min, and a rapid enzymatic degradation of the radiopharmaceutical, resulting in an effective t½ of the radiopharmaceutical in blood of ≈ 3 min. Four and 24 h post injection of [$^{111}$In-DTPA-Arg$^1$]SP uptake in and specific binding to SPR+ organs and tumours were found, with a (SPR+) target to background optimum at 24 h. Tissue distribution and ex vivo autoradiographic studies in rats, with and without pretreatment with the selective non-peptide $NK_1$-receptor antagonist CP96,345, showed uptake and specific binding of radioactivity in isolated tumours, submandibular and parotid glands. Visualization of normal SPR+tissues such as the salivary glands by gamma camera scintigraphy, after administration of [$^{111}$In-DTPA-Arg$^1$]SP, was demonstrated in untreated rats. Pathological SPR+ processes were visualized in rats bearing the transplantable pancreatic tumour CA20948, and in rats with adjuvant arthritis, induced by

injecting a homogenate of mycobacteria tuberculosis.

In conclusion: [$^{111}$In-DTPA-Arg$^{1}$]SP can be used successfully to visualize SPR+ processes in vivo by gamma camera scintigraphy.

## INTRODUCTION

Tachykinins are a family of peptides which share a common C-terminal amino acid sequence, -Phe-X-Gly-Leu-Met-$NH_2$, where X represents either Phe, Ile, or Val (1). The mammalian tachykinins include substance P (SP), neurokinin A, neurokinin B, and two N-terminally extended forms of neurokinin A - neuropeptide K and neuropeptide γ. Currently accepted tachykinin receptor nomenclature defines three homologous receptor types: the SP-preferring $NK_1$ receptor, the neurokinin A-preferring $NK_2$ receptor and the neurokinin B-preferring $NK_3$ receptor. The tachykinin receptors have a wide tissue distribution, and interaction with their ligands is associated with diverse responses such as sensory neurotransmission, immunological responses, both contraction and endothelium-dependent relaxation of vascular smooth muscle, nociception, histamine release, plasma extravasation, inflammation, sexual behaviour and, potentially, nerve regeneration and wound healing (for reviews see refs.: 1,2).

**Substance P**
Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$

**[$^{111}$In-DTPA-Arg$^{1}$]substance P**
$^{111}$In-DTPA-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$

**Sendide**
Tyr-D-Phe-Phe-D-His-Leu-Met-$NH_2$

**[$^{111}$In-DTPA-Tyr$^{1}$]Sendide**
$^{111}$In-DTPA-Tyr-D-Phe-Phe-D-His-Leu-Met-$NH_2$

**Figure 1.** *Sequences of synthetic substance P, [$^{111}$In-DTPA-Arg$^{1}$]substance P, sendide and [$^{111}$In-DTPA-Tyr$^{1}$]sendide*

The involvement of SP in the carcinoid syndrome (3) and vascular (migraine) headache (4) has been suggested. In rats with adjuvant arthritis the synovium was found positive for high-affinity substance P receptors (SPR) (5) and was also found in patients with rheumatoid arthritis (6). In addition, very high concentrations of SPR are expressed in the germinal center of lymph nodes (7), and on a human astrocytoma cell line (8). Large numbers of high-affinity SPR are found in

surgical specimen obtained from patients with inflammatory bowel disease. The SPR binding $NK_1$ receptor is expressed by arterioles and venules located in the submucosa, muscularis mucosa, external longitudinal muscle, and serosa (7,9).

In the present study several animal models were used to evaluate the potential usefulness of [$^{111}$In-DTPA-Arg$^1$]SP as radiopharmaceutical for in vivo detection of SPR+ tissues. Firstly, the SPR+ submandibular and parotid gland (10-12) were studied in normal rats. As a second model, rats bearing the SPR+ transplantable rat pancreatic tumour CA20948 (as presented in this paper) were investigated. Thirdly, the possible accumulation of [$^{111}$In-DTPA-Arg$^1$]SP was studied in the joints of rats with adjuvant arthritis, induced by the mycobacteria tuberculosis (13). The specific non-peptide $NK_1$ receptor antagonist CP96,345 (14) was used in these models in order to block specific binding of [$^{111}$In-DTPA-Arg$^1$]SP to SPR.

## MATERIALS AND METHODS

*Preparation and Purification of the Radiopharmaceutical*

[3-(p-hydroxyphenyl)propionyl-Arg$^1$]SP, (Bolton-Hunter-SP, BH-SP), [DTPA-Arg$^1$]SP, the SP antagonist sendide (15) and [DTPA-Tyr$^1$]sendide were purchased from Sanbio (Uden, The Netherlands). For structural formulae, see Fig. 1. Amino acid analysis yielded a peptide content of more than 95 %. The identity of the peptides was confirmed by amino acid analysis and fast atom bombardment mass spectrometry. $^{111}InCl_3$ (DRN 4901, 370 MBq/mL in HCl, pH= 1.5 - 1.9) was obtained from Mallinckrodt (Petten, The Netherlands). The radiolabelling of [DTPA-Arg$^1$]SP with $^{111}$In to a specific activity of 150 MBq $^{111}$In per $\mu$g [DTPA-Arg$^1$]SP, and consecutive quality control by instant thin layer chromatography (ITLC, Silica-gel) and SEP-PAK $C_{18}$ reversed-phase extraction were performed as described earlier (16). High performance liquid chromatography (HPLC) was performed with a Waters 600E multisolvent delivery system, connected to a $\mu$Bondapak $C_{18}$ reversed-phase column (300*3.9 mm, particle size 10 $\mu$m). Elution was carried out at a flow of 1.5 mL/min with a linear gradient of 10 to 50 % acetonitril in 0.1 % trifluoroacetic acid in 30 min, and the latter composition was kept constant for another 5 min.

The radiochemical purity of the radiolabelled SP analogues was greater than 98 %. Although it is not excluded that additional groups of the peptide participate in $^{111}$In complexation, the labelled product is referred to as [$^{111}$In-DTPA-Arg$^1$]SP.

[$^{115}$In-DTPA-Arg$^1$]SP was prepared by mixing [DTPA-Arg$^1$]SP (stock concentration of $10^{-4}$ M) with a 80-fold molar excess of $^{115}InCl_3$ (Aldrich) in 0.05 M acetic acid, as described earlier (17). The binding characteristics of [DTPA-Arg$^1$]SP and [$^{115}$In-DTPA-Arg$^1$]SP to the SPR were examined.

$^{125}$I-BH-SP was purchased from Amersham (UK) and also prepared in our laboratory essentially as described earlier (18). The radioiodination was carried out

by adding 105 μg BH-SP in 150 μl 0.05 M acetic acid and 8 μg chloramine-T in 100 μl 0.05 M phosphate buffer (pH 7.5) to 185 MBq (50 μl) Na$^{125}$I (specific activity 0.62 TBq $^{125}$I/mg, Amersham, UK). The mixture was then vortexed for 1 min. The radioiodination was stopped by adding 1 mL 10 % human serum albumin (Merieux, Lyon, France). After vortexing for 30 s, 2 mL 5 mM ammonium acetate was added. Purification was performed using a SEP-PAK $C_{18}$ reversed-phase extraction cartridge (Waters Associates, Milford, MA), which was washed successively with 5 mL 70 % ethanol, 5 mL 2-propanol and 5 mL distilled water. After application of the sample, the SEP-PAK $C_{18}$ cartridge was washed with 5 mL distilled water and 5 mL 0.5 M acetic acid, and radioiodinated BH-SP was eluted with 1 mL 96 % ethanol. The solvent was evaporated at 25 °C under a gentle stream of nitrogen. HPLC of $^{125}$I-BH-SP was performed on an identical μBondapak $C_{18}$ reversed phase column as mentioned above, and elution was carried out at a flow of 1 mL/min with a linear gradient of 40 to 70 % methanol in 0.05 mM acetate (pH 5.5) in 50 min, and the latter composition was kept constant for another 5 min. The in vitro binding and autoradiography studies were performed with HPLC-purified mono-iodinated $^{125}$I-BH-SP ($^{125}$I-BH-SP).

*Reagents*

The selective non-peptide $NK_1$-receptor antagonist CP96,345 (14) was kindly provided by Pfizer (Capelle aan de ijssel, The Netherlands). Substance P was purchased from Saxon-Biochemicals GMBH (Germany) and luteinizing-hormone-releasing hormone (LHRH), thyrotropin-releasing hormone (TRH) from Hoechst (Germany). Octreotide was from Sandoz (Switzerland) and [DTPA-D-Phe$^1$]octreotide from Mallinckrodt (The Netherlands).

All chemicals used were of the highest purity available.

*Receptor Binding Studies and Autoradiography*

Receptor binding studies were performed with membrane fractions of the submandibular gland, brain stem, brain cortex and the CA20948 tumour. The incubation mixture consisted of 40 kcpm of $^{125}$I-BH-SP, while the remainder of the incubation mixture was identical as described earlier (19). Binding curves and $IC_{50}$ values for displacement of $^{125}$I-BH-SP binding by unlabelled peptide were calculated using the computer fitting program of Graphpad (ISI software, Philadelphia, PA, USA).

Receptor autoradiography was carried out as described earlier (20) with some modifications. In short, the tissue samples were cut on a cryostat (Jung CM3000, Leica, Germany) in 10 μm sections. The sections were mounted onto precleaned gelatin coated microscope slides, and stored at -80 °C. To wash out endogenous SP, sections were preincubated for 15 minutes at roomtemperature with 50 mM Tris-HCl buffer pH 7.4. Thereafter, sections were incubated for 1 h

at 20 °C in 50 mM Tris-HCl buffer pH 7.4 containing 200 mg/L BSA, 40 mg/L bacitracin, and 5 mM $MnCl_2$ to inhibit endogenous proteases, in the presence of iodinated ligand (400 kcpm/mL ≈ 0.1 nM). Non-specific binding was determined on parallel sections by adding unlabelled native SP at a concentration of 1 μM. Incubated sections were washed four times 5 seconds in ice cold 50 mM Tris-HCl buffer pH 7.4 and four times 5 seconds in ice cold distilled water to remove salt, dried quickly, and exposed to Hyperfilm™-$^3$H (Amersham) for 1 week in X-ray cassettes without intensifying screens at -80 °C. Histology was performed on hematoxylin-azophloxine stained sequential cryosections. Specificity of binding was tested by addition of 1μM SP, [DTPA-Arg$^1$]SP, CP96,345, LHRH, TRH and octreotide.

Autoradiographic displacement experiments in the submandibular gland were performed as described earlier (21).

Ex vivo autoradiography was performed on kidney, parotid and submandibular gland and tumour tissue from male Lewis CA20948 tumour-bearing rats. For this study rats were injected intravenously with 3.4 mg CP96,345 per kg body weight (BW) or with vehicle. After 10 minutes the rats were injected with 37 MBq (1 μg) [$^{111}$In-DTPA-Arg$^1$]SP and exsanguinated 24 h later.

*Tissue Distribution and Specific Binding in Control Rats*

Male Wistar rats (220-260 g) were injected under ether anaesthesia into the dorsal vein of the penis and/or into a sublingual vein. In order to find the optimal injected mass of [DTPA-Arg$^1$]SP groups of 3-4 rats were injected with 0.1, 1 or 5 μg [DTPA-Arg$^1$]SP labelled with 3 MBq $^{111}$In. Parallel groups of 3-4 rats were injected similarly 10 min after intravenous administration of 3.4 mg CP96,345 per kg BW in 0.05 M acetic acid in 154 mM NaCl, as described by Bertrand (22).

Specific binding was defined as the difference in tissue uptake of radioactivity between untreated (total binding) and CP96,345-treated (non-specific binding) rats, expressed as percentages of the injected radioactivity per gram tissue, as described before (17). Twenty-four h after administration of [$^{111}$In-DTPA-Arg$^1$]SP the rats were exsanguinated. The following organs and tissues were isolated and subsequently analyzed for radioactivity content: blood, bronchi, lungs, thymus, pancreas, spleen, adrenals, kidneys, aorta, esophagus, jejunum, colon, thigh (soft tissue), liver, exorbital lacrimal gland, parotid and submandibular glands, neck lymph nodes, anterior pituitary gland, brain cortex and striatum. The tissue distribution and metabolism of the $^{111}$In-labelled SP analogues in vivo were also studied by gamma camera scintigraphy (Rota-II, Siemens) (23).

*Imaging in Arthritic and Tumour-bearing Rats*

Adjuvant arthritis was induced in female rats by injection a homogenate of mycobacteria tuberculosis, as described earlier (13). Seventeen days after inoculati-

on the rats had developed an adjuvant reactive arthritis, mostly located in the hind leg joints and at the site of inoculation a granuloma, as confirmed by light microscopy. These rats were injected with 37 MBq (1 $\mu$g) [$^{111}$In-DTPA-Arg$^1$]SP. Since granuloma can be visualized by octreotide scintigraphy (24) rats were injected with 37 MBq (0.5 $\mu$g) [$^{111}$In-DTPA-D-Phe$^1$]octreotide (Octreoscan, Mallinckrodt, Petten, The Netherlands). Imaging was performed 24 h after injection of the radiopharmaceutical.

In a second imaging study two groups of 4 male Lewis rats, bearing the transplantable rat pancreatic tumour CA20948, were also used for imaging. One group was treated intravenously with 3.4 mg CP96,345 per kg BW 10 min prior to the administration of [$^{111}$In-DTPA-Arg$^1$]SP in order to block specific receptor binding of the latter. The other group was similarly treated with vehicle prior to the radiopharmaceutical.

*Metabolism*

Male Wistar rats (220-280 g) were sacrificed at 15 min, 1 h, or 24 h after administration of the radiolabelled SP analogue. Blood was collected in EDTA containing tubes and immediately cooled on ice. Since SP is readily metabolized by various enzymes in blood (25,26) samples were immediately centrifugated at 0° C and plasma components were separated by SEP-PAK $C_{18}$ chromatography. Using the separation technique described above (17), [$^{111}$In-DTPA-Arg$^1$]SP binds to SEP-PAK $C_{18}$ stationary phase and is only eluted with ethanol, while $^{111}$In-DTPA is not retained on SEP-PAK $C_{18}$ columns. The radioactivity in plasma and urine samples, which was eluted with ethanol from the SEP-PAK $C_{18}$ column, is termed peptide-bound radioactivity but was not further characterized.

*Data Acquisition and Analysis*

All results are expressed as the mean $\pm$ SD. One-way analysis of variance (ANOVA), was used for statistical analysis. Means were compared using Bonferroni or Newman-Keuls (27). Tissue-binding values were evaluated using Student's *t*-test. A $P$ value of $<0.05$ was considered significant.

## RESULTS

*Radiolabelling and Quality Control of the Radioligand*

The efficiency of labelling of BH-SP with $^{125}$I was 30-35 %. Purification of the iodination mixture using the SEP-PAK $C_{18}$ reversed-phase cartridge resulted in the elution of mainly radioiodide in the water fraction and peptide-bound radioiodine in the ethanol fraction, as revealed by HPLC. Figure 2 shows a typical HPLC analysis of peptide(s) eluted in the SEP-PAK $C_{18}$ ethanol fraction, indicating a radiochemical purity of >95 % of mono-radioiodinated BH-SP. Simultaneous measurement UV-absorbance at 254 nm and radioactivity shows a clear separation

between unlabelled BH-SP, mono- and di-iodinated $^{125}$I-BH-SP with retention volumes of 37, 35.5 and 45 mL, respectively, using the methanol gradient. The retention volume of the commercially available $^{125}$I-BH-SP (specific activity ≈ 2000 Ci per mmol, Amersham, UK) was identical with our mono-iodinated $^{125}$I-BH-SP (data not shown). Since our HPLC-purified mono-iodinated $^{125}$I-BH-SP is carrier-free, its specific activity will also reach this value of ≈ 2000 Ci per mmol for carrier-free mono-iodinated $^{125}$I-labelled peptides.

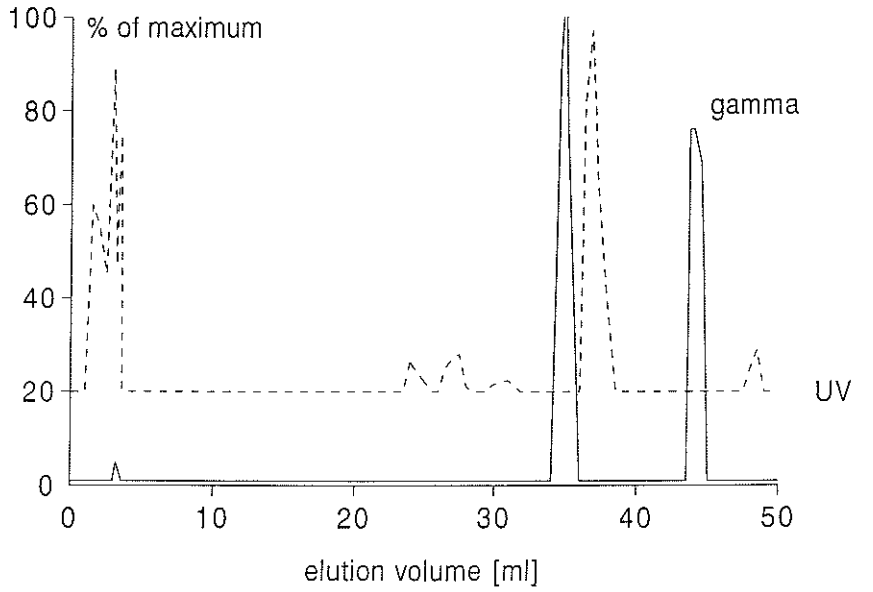


**Figure 2.** *HPLC-elution pattern of the ethanol fraction after SEP-PAK $C_{18}$ purification of the BH-SP radioiodination mixture. Non-radioiodinated BH-SP is measured by UV-absorbance ($\lambda$=254 nm, broken line, with a retention volume of 37 mL), mono- and di-iodinated $^{125}$I-BH-SP are detected by gamma detection (solid line) at 35.5 and 45 mL, respectively*

*Receptor Binding Studies and Autoradiography*

Studies of the binding of $^{125}$I-BH-SP to the SPR in rat parotid gland membranes in the presence of increasing concentrations of SP, [DTPA-Arg$^1$]SP and [$^{115}$In-DTPA-Arg$^1$]SP revealed $IC_{50}$ values of 1, 35 and 31 nM, respectively.

In brain stem membranes $IC_{50}$ values for SP and [DTPA-Arg$^1$]SP were 0.5 and 5 nM, respectively, while for sendide and [DTPA-Tyr$^1$]sendide $IC_{50}$ values were found in the $\mu$molar range. In another study with membrane fractions of the

CA20948 tumour the binding of $^{125}$I-BH-SP was inhibited in the presence of 1 μM of SP, [DTPA-Arg$^1$]SP or CP96,345 to 67 ± 6 %, 66 ± 6 % and 66 ± 0 %, respectively, of the control value in the absence of competitive ligand. In a study with rat brain cortex membranes $IC_{50}$ values of 0.2, 4 and 2 nM were found for SP, [DTPA-Arg$^1$]SP and BH-SP, respectively.

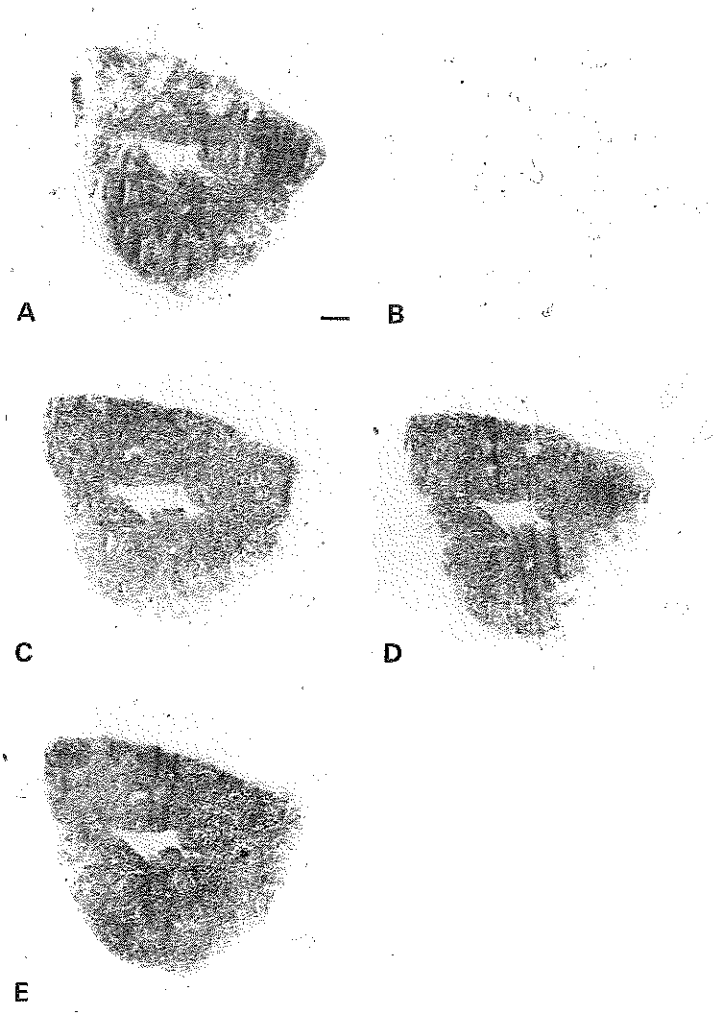


**Figure 3.**
*Autoradiograms showing specific labelling of SPR in the serous part of the rat submandibular gland.*
*(A) shows total binding of $^{125}$I-BH-SP in the absence of unlabelled SP, and (B) non-specific binding in the presence of 1 μM SP.*
*No displacement occurred in the presence 1 μM octreotide (C), LHRH (D) or TRH (E).*
*Bar represents 1 mm*

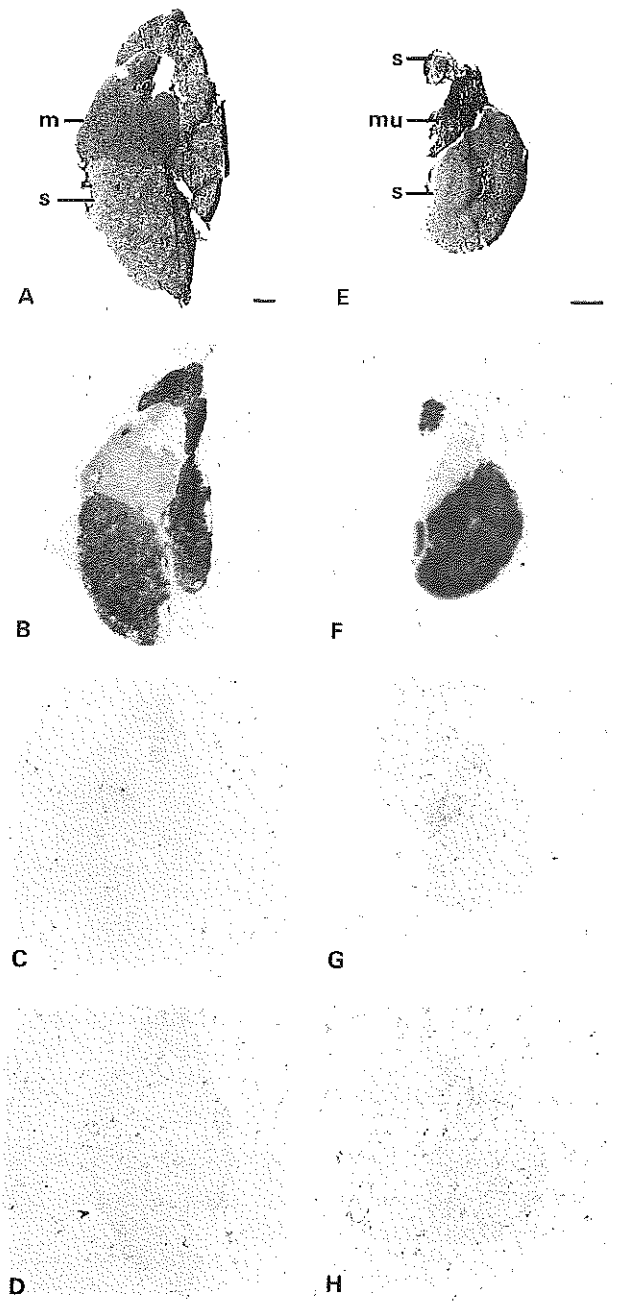


**Figure 4**. *Distribution of SPR in rat submandibular gland (A-D) and in rat parotid gland (E-H). A and E are hematoxylin-azophloxine stained sections. The autoradiograms show total binding of $^{125}$I-BH-SP (B,F) or non-specific binding in the presence of 1 μM SP (C,G) or CP96,-345 (D,H). The serous part of the rat submandibular gland (B) has a high density of high-affinity binding sites to the SPR, whereas the mucous part of the gland has a less high density. The rat parotid gland (F) also has a high density of high-affinity binding sites to the SPR. Some non-specific binding is present on the skeletal muscle tissue, which is located in the section of the parotid gland. M, mucous; S, serous; MU, skeletal muscle. Bar represents 1 mm*

Autoradiography of submandibular gland slices incubated with $^{125}$I-BH-SP demonstrated a high density of SPR in the serous part (Fig. 3), in contrast to the lack of SPR in the mucous part (Fig. 3 and 4). In the presence of 1 $\mu$M SP the binding of $^{125}$I-BH-SP was completely inhibited (Fig. 3B), while in parallel incubations with 1$\mu$M octreotide (Fig. 3C), LHRH or TRH binding of radioactivity was not affected (3D and 3E, respectively). The exorbital lacrimal gland and lymph nodes adjacent to the submandibular gland showed low non-specific binding of $^{125}$I-BH-SP (data not shown). Figure 4 shows the binding of $^{125}$I-BH-SP to SPR in rat submandibular (A-D) and parotid (E-H) gland in the absence (total binding; B,F), or the presence (non-specific binding) of 1 $\mu$M SP (C,G) or 1 $\mu$M CP96,345 (D,H).

Figure 5 shows an autoradiographic displacement study using 10 $\mu$m slices of the submandibular gland and an $IC_{50}$ value of 2.7 nM was found for [DTPA-Arg$^1$]SP.

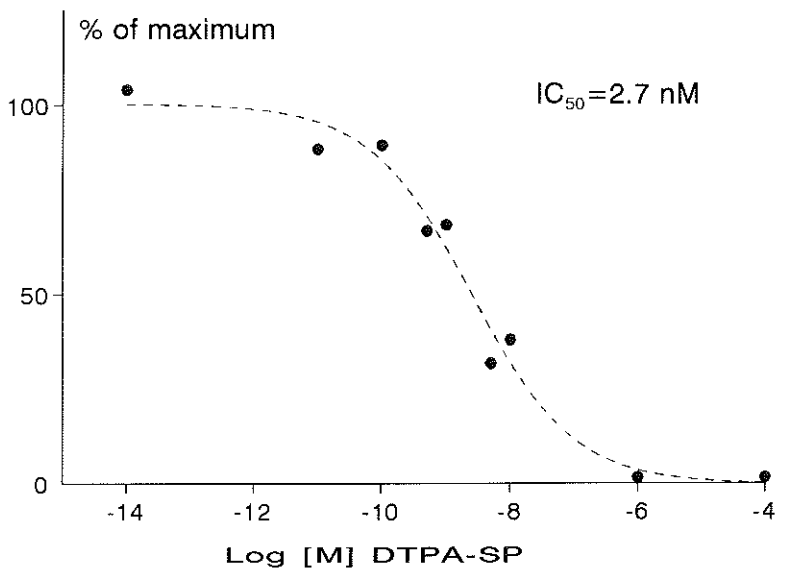


***Figure** 5. Autoradiographic displacement of $^{125}$I-BH-SP on 10 $\mu$m slices of rat submandibular gland. The uptake of radioactivity in the tissue slices was assessed at increasing concentrations of the ligand [DTPA-Arg$^1$]SP*

*Preparation, Radiolabelling, and Quality Control of the Radiopharmaceutical*

An over 98 % efficiency of labelling of the DTPA-conjugated peptides was assured within 15 min, as revealed by ITLC and HPLC, when a 5- to 10-fold molar excess peptide over $^{111}$In was used. Therefore, a specific activity of over 150

MBq $^{111}$In per $\mu$g [DTPA-Arg$^1$]SP or [DTPA-Tyr$^1$]sendide could be achieved. [$^{111}$In-DTPA-Arg$^1$]SP and [$^{111}$In-DTPA-Tyr$^1$]sendide were stable for at least 1 h after preparation, and for longer than 4 h if stabilised by addition of the quencher gentisic acid at a final concentration of 2 mg per mL. The retention volumes for [$^{111}$In-DTPA-Arg$^1$]SP and [DTPA-Arg$^1$]SP with the acetonitrile gradient were 31 and 34 mL, respectively (Fig. 6).

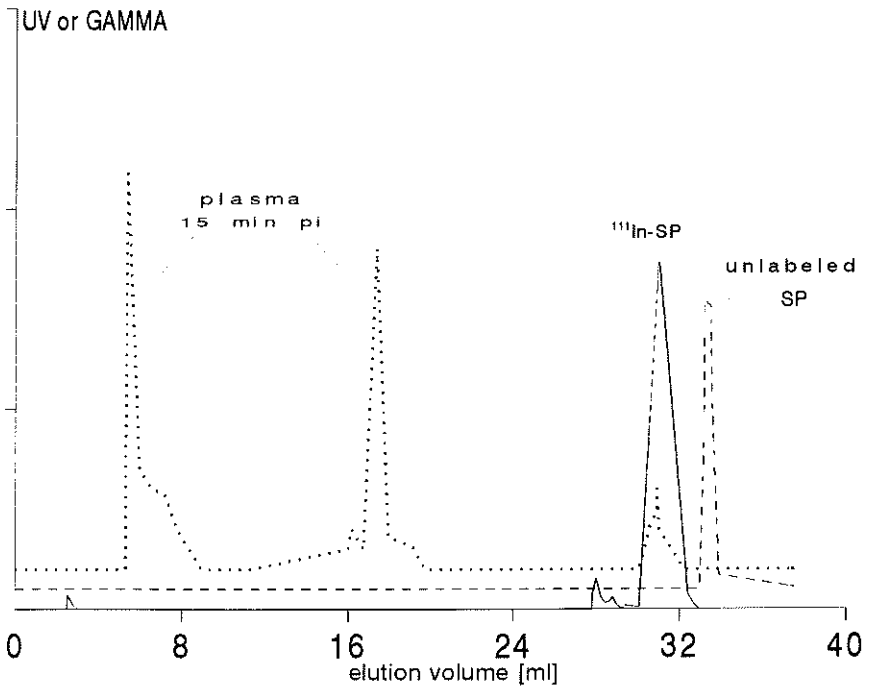


**Figure 6**. *HPLC-elution profile of [$^{111}$In-DTPA-Arg$^1$]SP (——, counts) and [DTPA-Arg$^1$]SP (---, absorbance at 254 nm) with a 10 to 50 % acetonitrile elution gradient. The dotted line represents a HPLC-pattern of the ethanol fraction of SEP-PAK $C_{18}$ extract of serum collected 15 min after injection of the radiopharmaceutical*

*Tissue Distribution and Specific Binding*

The target to background ratio increased between 4 and 24 h, as measured by the uptake of radioactivity in isolated SPR-positive tissues (salivary glands=target) compared with the radioactivity in blood (background). This calculated ratio increased from 23 $\pm$ 11 at 4 h up to 200 $\pm$ 16 at 24 h after the administration of the radiopharmaceutical.

**Table 1.** *Tissue distributions (% injected dose per gram, mean ± SD) in male Wistar rats 24 h after intravenous administration of 3 MBq $^{111}$In labelled with indicated mass of [DTPA-Arg$^1$]substance P*

| Tissue | 0.1 μg (n=3) | 1 μg (n=3) | 5 μg (n=4) |
|---|---|---|---|
| Blood | 0.0062 ± 0.0009 | 0.0055 ± 0.002 | 0.0054 ± 0.0005 |
| Lungs | 0.022 ± 0.004 | 0.020 ± 0.004 | 0.020 ± 0.003 |
| Bronchi | 0.068 ± 0.011 | 0.089 ± 0.020 | 0.069 ± 0.011 |
| Thymus | 0.049 ± 0.004 | 0.088 ± 0.015 | 0.048 ± 0.011 |
| Pancreas | 0.019 ± 0.004 | 0.023 ± 0.005 | 0.022 ± 0.006 |
| Spleen | 0.31 ± 0.07 | 0.32 ± 0.11 | 0.25 ± 0.06 |
| Adrenals | 0.029 ± 0.006 | 0.033 ± 0.001 | 0.055 ± 0.013 |
| Kidneys | 1.1 ± 0.1 | 1.9 ± 0.3 | 2.9 ± 1.1 |
| Aorta | 0.023 ± 0.008 | 0.018 ± 0.008 | 0.044 ± 0.009 |
| Esophagus | 0.049 ± 0.009 | 0.069 ± 0.020 | 0.030 ± 0.005 |
| Jejunum | 1.1 ± 0.11 | 1.4 ± 0.2 | 1.0 ± 0.21 |
| Colon | 0.24 ± 0.03 | 0.22 ± 0.04 | 0.33 ± 0.10 |
| Soft tissue | 0.011 ± 0.002 | 0.019 ± 0.003 | 0.015 ± 0.003 |
| Liver | 0.18 ± 0.03 | 0.24 ± 0.02 | 0.17 ± 0.04 |
| Exorb Lacr | 0.055 ± 0.0004 | 0.095 ± 0.030 | 0.081 ± 0.011 |
| Submandib | 0.65 ± 0.07 | 1.1 ± 0.0 | 1.3 ± 0.2 |
| Parotid | 0.16 ± 0.02 | 0.29 ± 0.05 | 0.32 ± 0.08 |
| Pituitary | 0.020 ± 0.001 | 0.041 ± 0.008 | 0.015 ± 0.004 |
| Brain cortex | < 0.001 | < 0.001 | < 0.001 |
| Striatum | < 0.001 | < 0.001 | < 0.001 |

The radioactivity measured in the isolated tissues, 24 h after injection of 0.1, 1 or 5 μg [DTPA-Arg$^1$]SP labelled with 3 MBq $^{111}$In is shown in Table 1. A high concentration of radioactivity in the kidneys after [$^{111}$In-DTPA-Arg$^1$]SP is evident, since renal excretion is also the predominant route for clearance of [$^{111}$In-DTPA-Arg$^1$]SP. For instance, about 50 % of radioactivity is excreted via the kidneys in the first 30 min after injection of [$^{111}$In-DTPA-Arg$^1$]SP. Most of the tissues mentioned in Table 1 and 2 were selected from the literature because of their SPR content as potential targets, while blood and soft tissue were selected as SPR-negative background. These background values are low, similar to corresponding values after the administration of [$^{111}$In-DTPA-D-Phe$^1$]octreotide (23). The uptake of radioactivity in the SPR+ colon, jejunum, and submandibular and parotid glands is also similar to the values found in somatostatin receptor-positive organs with [$^{111}$In-DTPA-D-Phe$^1$]octreotide scintigraphy (23). However, the uptake of radioactivity in SPR+ adrenals, pituitary, aorta, esophagus and lungs is low. Table 1 shows the total binding and Table 2 the specific binding of varying

amounts of [DTPA-Arg$^1$]SP (0.1, 1 and 5 $\mu$g) labelled with a constant amount of $^{111}$In (3 MBq). In various tissues total and specific binding are optimal with the intermediate mass of the radiopharmaceutical. No significant uptake nor specific binding of [$^{111}$In-DTPA-Arg$^1$]SP was found in the brain cortex and striatum, suggesting that [$^{111}$In-DTPA-Arg$^1$]SP was unable to pass the blood-brain barrier.

In CA20948 tumour-bearing male Lewis rats total uptake amounted to 0.13 $\pm$ 0.01 % injected dose per gram tumour and specific binding to 0.07 $\pm$ 0.01 % dose per g.

**Table 2.** *Specific binding in tissues (% injected dose per gram, mean $\pm$ SD) in male Wistar rats 24 h after intravenous administration of 3 MBq $^{111}$In labeled with indicated mass of [DTPA-Arg$^1$]substance P*

| Tissue | 0.1 $\mu$g | 1 $\mu$g | 5 $\mu$g |
|---|---|---|---|
| Bronchi | 0.044 $\pm$ 0.011 * | 0.047 $\pm$ 0.020 * | 0.048 $\pm$ 0.011 * |
| Thymus | 0.029 $\pm$ 0.004 * | 0.058 $\pm$ 0.015 * | 0.000 $\pm$ 0.011 |
| Spleen | 0.19 $\pm$ 0.07 * | 0.27 $\pm$ 0.04 * | 0.03 $\pm$ 0.06 |
| Esophagus | 0.029 $\pm$ 0.009 * | 0.063 $\pm$ 0.020 * | 0.002 $\pm$ 0.005 |
| Jejunum | 0.86 $\pm$ 0.11 * | 0.60 $\pm$ 0.25 * | 0.0 $\pm$ 0.2 |
| Colon | 0.17 $\pm$ 0.03 * | 0.062 $\pm$ 0.037 | 0.0 $\pm$ 0.1 |
| Submandibul | 0.52 $\pm$ 0.07 * | 0.83 $\pm$ 0.04 * | 0.77 $\pm$ 0.15 * |
| Parotid | 0.15 $\pm$ 0.04 * | 0.21 $\pm$ 0.08 * | 0.16 $\pm$ 0.09 |
| Pituitary | 0.008 $\pm$ 0.004 * | 0.033 $\pm$ 0.008 * | 0.004 $\pm$ 0.004 |
| Exorb Lacri | 0.011 $\pm$ 0.011 | 0.042 $\pm$ 0.030 | 0.011 $\pm$ 0.011 |

* *$P < 0.05$, specific binding significantly different from zero*

*Metabolism*

SEP-PAK $C_{18}$ columns were used for analysis of peptide-bound radioactivity in blood and urine of rats 15 min, 1 h and 24 h after the injection of [$^{111}$In-DTPA-Arg$^1$]SP. The percentage peptide-bound radioactivity in blood was 10 $\pm$ 2, 4 $\pm$ 1 and 0.5 $\pm$ 0.1 respectively. HPLC-analysis of the ethanol-fractions of the SEP-PAK $C_{18}$ extractions of these blood samples showed that less than 25, 15 and 5 %, respectively, of this peptide-bound $^{111}$In was intact [$^{111}$In-DTPA-Arg$^1$]SP. The subsequent t½ of the intact radiopharmaceutical in the first 15 min after the injection was approximately 3 min. This is illustrated for the 15 min serum sample in Fig. 6. The nature of the metabolites of [$^{111}$In-DTPA-Arg$^1$]SP was not further investigated.

The percentage of peptide-bound $^{111}$In in urine-samples collected 1 and 24 h after injection of [$^{111}$In-DTPA-Arg$^1$]SP was less than 10 and 5 %, respectively, of the total radioactivity. More than 90 % of the radioactivity excreted in the urine collected 1 h after injection of the radiopharmaceutical had a molecular weight of

less than 1500 dalton, as shown by PD-10 gel chromatography, i.e. smaller than that of the injected [$^{111}$In-DTPA-Arg$^1$]SP (data not shown).

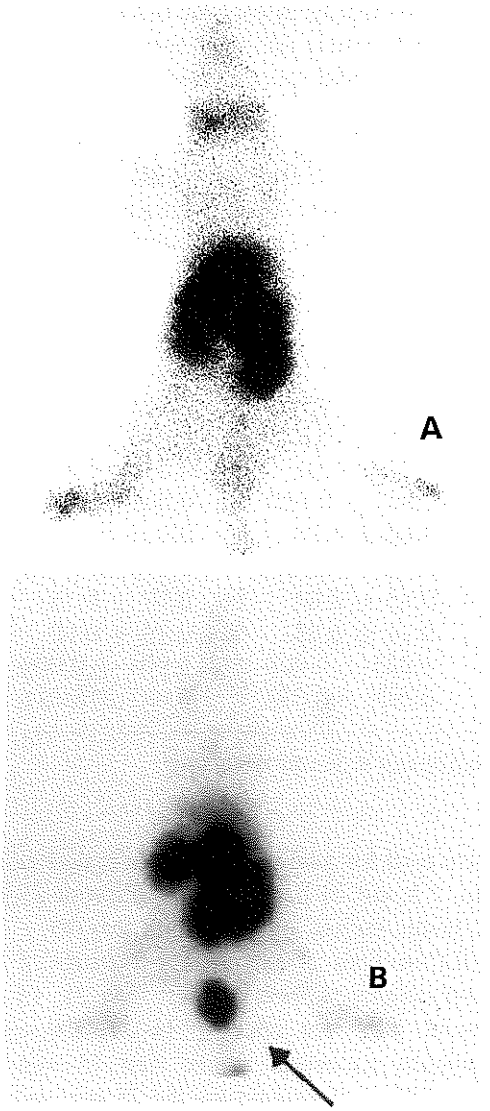


**Figure 7.**
*Static posterior images in rats with adjuvant arthritis, 24 h after injection of 37 MBq (1 μg) [$^{111}$In-DTPA-Arg$^1$]SP (A) or 37 MBq (0.5 μg) [$^{111}$In-DTPA-D-Phe$^1$]octreotide (B). Adjuvant arthritis located in the hind leg joints was induced by injection of a homogenate of mycobacteria tuberculosis.*
*Note the uptake of radioactivity in liver and kidneys in both images. There is accumulation of [$^{111}$In-DTPA-Arg$^1$]SP in the adjuvant arthritis affected hind leg joints (A), as well as salivary glands (A). There is less uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide at these sites (B).*
*The granuloma (arrow) at the site of inoculation is clearly visualized by [$^{111}$In-DTPA-D-Phe$^1$]octreotide (B) but not by [$^{111}$In-DTPA-Arg$^1$]SP (A)*

*Imaging*

Dynamic images during the first 20 min after injection of [$^{111}$In-DTPA-Arg$^1$]SP showed a rapid distribution of radioactivity over the whole body. In the first minutes after the injection of [$^{111}$In-DTPA-Arg$^1$]SP the kidneys were rapidly

visualized and excretion of radioactivity in the urine was demonstrated. The dynamic images were comparable to the those obtained with [$^{111}$In-DTPA-D-Phe$^1$]octreotide (23) and [$^{111}$In-DTPA-D-Phe$^1$]RC-160 (17). From digital static images obtained 24 h after injection of [$^{111}$In-DTPA-Arg$^1$]SP whole body retention was found to be less than 7 procent of the injected dose, and this retention was predominantly accounted for by radioactivity in the kidneys, liver, salivary glands, jejunum and colon (see also Table 1). Figure 7 represents static analog images of 2 rats with adjuvant arthritis and an inflammation granuloma at the site of injection obtained 24 h after the injection of 37 MBq (1$\mu$g) [$^{111}$In-DTPA-Arg$^1$]SP (A) or 37 MBq (0.5 $\mu$g) [$^{111}$In-DTPA-D-Phe$^1$]octreotide (B). The differences in tissue uptake of these radiopharmaceutical are evident. The radiolabelled SP has a high uptake in salivary glands and in the arthritic hind leg joints, but shows less uptake in the granuloma. These findings are in contrast to the rat injected with [$^{111}$In-DTPA-D-Phe$^1$]octreotide (B), which shows no uptake in salivary glands and in the arthritic hind leg joints, but a high uptake at the site of the granuloma.

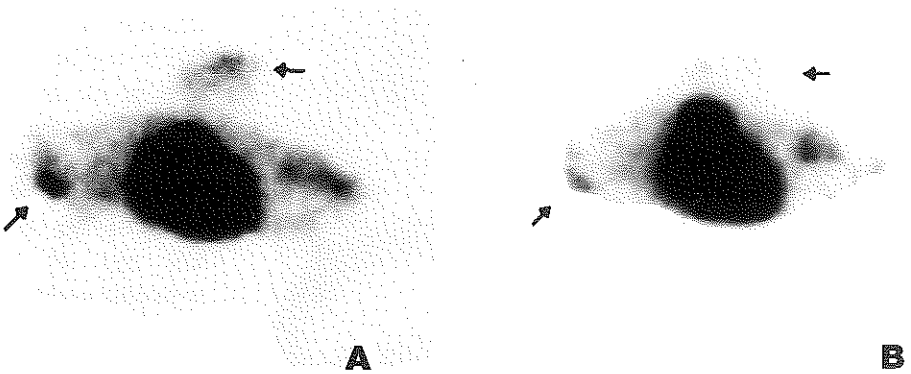


**Figure 8.** *Static lateral images of CA20948 tumour-bearing rats 24 h post injection 37 MBq (1 $\mu$g) [$^{111}$In-DTPA-Arg$^1$]SP. Without (A) or with (B) pretreatment CP96,345. The arrows indicate the salivary gland and the tumour, located at the back of the rat*

Figure 8 presents static lateral analog images of 2 CA20948 tumour-bearing rats 24 h after injection of 37 MBq (1$\mu$g) [$^{111}$In-DTPA-Arg$^1$]SP. Accumulation of radioactivity is observed in the tumour, kidneys and salivary glands (A). Uptake of radioactivity in the tumour, parotid and submandibular gland is reduced by 50 %

after the pretreatment of 3.4 mg per kg BW of the SP antagonist CP96,345 (B), and in agreement with measurement of radioactivity concentrations in the isolated organs. The uptake of radioactivity in the kidneys and liver was not affected by this intervention.

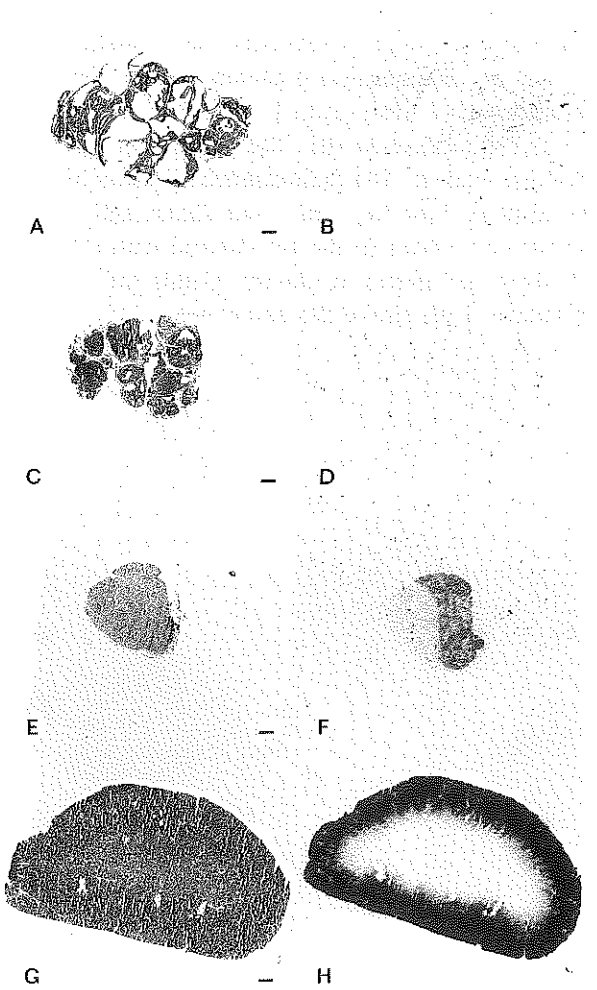


**Figure 9.**
*Ex vivo autoradiography of SPR on a transplantable pancreatic tumour after administration of [$^{111}$In-DTPA-Arg$^1$]SP without (B) or with CP96,-345 (D). Hematoxylin-azophloxine stained sections are shown in A,C,E and G for comparison. Apart from the tumour, accumulation of [$^{111}$In-DTPA-Arg$^1$]SP is seen in the serous part of the submandibular gland (F) and in the cortex of the kidney (H) of the untreated animal*

*Ex vivo autoradiography*

Figure 9 presents ex vivo autoradiography of the transplantable pancreatic tumour CA20948 without (B) or with (D) pretreatment of 3.4 mg CP96,345 per kg BW, submandibular gland (F) and kidney (H), after injection of 37 MBq (1$\mu$g)

[$^{111}$In-DTPA-Arg$^1$]SP. Hematoxylin-azophloxine stained sections (A,C,E,G) are presented for orientation. The autoradiograph of the submandibular gland (F) shows the uptake of radioactivity in the serous acini, and the reduced uptake in the mucous acini, as also demonstrated by morphological staining and in the above-mentioned in vitro autoradiographic study. The parotid gland has mainly serous acini. In the mucous part of the submandibular gland serous and some paneth cells are localised, and these cells seem responsible for the uptake of radioactivity in the mucous part of the gland. The autoradiograph of the kidney (H) demonstrates the uptake of radioactivity in the proximal tubuli and this was not affected by the pretreatment of CP96,345 (data not shown).

## DISCUSSION

$^{125}$I-BH-SP has been shown to be a specific, high affinity radioligand for the $NK_1$ receptor (SPR), and in the present study it was demonstrated that [DTPA-Arg$^1$]SP was a potent competitor for $^{125}$I-BH-SP binding to SPR in isolated membranes from cerebral cortex, brain stem and salivary glands as well as in tissue sections of the submandibular gland. These results suggested that [$^{111}$In-DTPA-Arg$^1$]SP is an attractive pharmaceutical for SPR scintigraphy. Table 1 shows low uptake of [$^{111}$In-DTPA-Arg$^1$]SP in blood and soft tissue (background), while SPR+ organs such as colon, jejunum, submandibular and parotid gland (targets) have higher uptake of radioactivity. Uptake of [$^{111}$In-DTPA-Arg$^1$]SP in the pituitary is low, corresponding to the small number of SPR detected in this tissue (28,29). In contrast to the findings of several other groups (30,31) we could not demonstrate specific uptake in the lungs, although we did find uptake and specific binding in the bronchi. The presence of SPR in smooth muscle, arterioles, and venules has been detected by autoradiography with $^{125}$I-BH-SP as radioligand (7,9), but these represent only a minor part of the total mass of the investigated tissues such as the aorta, bronchi and esophagus; hence the total uptake of radioactivity is low.

The lack of uptake in brain cortex and striatum suggests an inability for [DTPA-Arg$^1$]SP and [$^{111}$In-DTPA-Arg$^1$]SP to penetrate the intact blood-brain barrier, and both analogues are inable to visualize SPR in the central nervous system. These data suggests that only when the blood-brain barrier is affected SPR+ brain tumours, such as astrocytoma and glioma, might be visualized by gamma camera scintigraphy.

The relatively high non-specific binding in comparison to the total uptake of radioactivity in SPR+ tissues (Tables 1,2), is in contrast to octreotide receptor scintigraphy in animals, which is characterized by high specific uptake of radioactivity in the somatostatin receptor-positive tissues. Since homologous blocking of the SPR is not possible for pharmacological reasons, we investigated CP96,345 up to a dose of 3.4 mg i.v. per kg BW, as described by Bertrand (22).

The high level of non-specific tissue uptake of [$^{111}$In-DTPA-Arg$^1$]SP in rats,

as measured in rats pretreated with CP96,345, may be due in part to incomplete blockade of the SPR, since CP96,345 has been shown to have higher affinity for human SPR ($K_i$ 0.5 nM) than for rat SPR ($K_i$ 35 nM) (32-34).

Furthermore, little is known about the pharmacokinetics of CP96,345 in rats. The non-specific binding of $^{125}$I-BH-SP in the presence of CP96,345 in the in vitro autoradiography tissue was relatively low, but the antagonist was added at a high concentration (1 $\mu$M). It is unknown if the concentration required to saturate SPR is reached in vivo after administration of 3.4 mg CP96,345 per kg BW. Therefore, our estimates of the non-specific binding of [$^{111}$In-DTPA-Arg$^1$]SP in tissues using this dose of CP96,345 represent overestimations of the true values. However, also total uptake of [$^{111}$In-DTPA-Arg$^1$]SP in SPR+ tissues remains very low compared with results obtained by octreotide receptor scintigraphy in somatostatin receptor-positive tissues.

We also studied the tissue distribution of [$^{111}$In-DTPA-Arg$^1$]SP after the administration of varying masses of [DTPA-Arg$^1$]SP labelled with a constant amount of $^{111}$In (3MBq) (Tables 1,2). In contrast to general belief that receptor scintigraphy shows an optimal target to background ratio at the lowest possible mass of the radiopharmaceutical with the highest specific radioactivity, we found that the uptake of [$^{111}$In-DTPA-Arg$^1$]SP in SPR+ tissue is usually optimal at intermediate doses.

This might also apply to SPR+ immunological disorders, the visualization of which may be enhanced by optimizing the mass of [$^{111}$In-DTPA-Arg$^1$]SP. This phenomenon is similar to our previously reported findings on the binding of [$^{111}$In-DTPA-D-Phe$^1$]octreotide to somatostatin receptor-positive tissues (35,36). However, the tissue-specific bell-shaped function of [$^{111}$In-DTPA-D-Phe$^1$]octreotide uptake versus the injected mass of [DTPA-D-Phe$^1$]octreotide was explained in part by the homologous upregulation of the somatostatin receptors. It is unknown if SPR is (up-or down)regulated by SP, but it has been reported that SPR is down-regulated by SP-antagonists (37).

In vivo metabolism of [$^{111}$In-DTPA-Arg$^1$]SP in the rat revealed a renal clearance of 50 % of the injected radioactivity in 30 min, and a rapid enzymatic degradation of the radiopharmaceutical, resulting in an effective t½ of the intact radiopharmaceutical in blood of $\approx$ 3 min. The presence of the DTPA-group in [$^{111}$In-DTPA-Arg$^1$]SP does not seem to affect the biological t½ in serum, since similar half-lives have been reported for serum in dogs (2.1 min) (25) and humans (1.6 min) (3). Less than 10 % of the injected dose was found intact in the urine, during the first 30 min after injection of the radiopharmaceutical, which is another indication of the rapid degradation of the radiopharmaceutical. These data are also in contrast to previous reported findings with [$^{111}$In-DTPA-D-Phe$^1$]octreotide (23) and [$^{111}$In-DTPA-D-Phe$^1$]RC-160 (17) scintigraphy in rats, where a larger fraction of the radiopharmaceutical was excreted intact in the urine. Nevertheless, salivary glands, arthritic joints, and the SPR+ tumour were visualized by gamma camera

scintigraphy. These data suggests that the receptor-ligand interactions in vivo must be very rapid, since the concentration of intact radiopharmaceutical in the circulation rapidly diminishes.

Sendide was reported to be a potent and selective $NK_1$ receptor antagonist (15), but in our hands [$^{111}$In-DTPA-Tyr$^1$]sendide did not show selective binding characteristics to the $NK_1$ receptor, neither in the in vitro experiments, nor in vivo.

The intravenously administered [$^{111}$In-DTPA-Tyr$^1$]sendide in rats revealed rapid clearance, but no specific binding to the SPR+ salivary glands or to any part of the gastrointestinal tract was observed.

The binding characteristics of [$^{111}$In-DTPA-Arg$^1$]SP to the human SPR is currently under investigation, since discrepancies between the potencies of several SP analogs in different species have been reported, and the amino acids sequences of rat and human differ by < 10% (for reviews see refs.: 2,38). Different forms of SPR are also reported in rat submaxillary gland (the former nomenclature of submandibular) (39). Before human application, the pharmacological properties of radiolabelled and unlabelled [DTPA-Arg$^1$]SP have to be investigated thoroughly, since SP is known to exert various unwanted cardiovascular effects. The groups of Schaffalatzky (3) and Coiro (40-42), however, have demonstrated that such side effects do not occur when SP is infused at low rates (1.5 pmol.min$^{-1}$.kg$^{-1}$) into humans. However, SP antagonists may be more attractive radiopharmaceuticals for imaging SPR+ processes.

## CONCLUSION

[$^{111}$In-DTPA-Arg$^1$]SP can be used successfully to visualize SPR+ processes in vivo by gamma camera scintigraphy. Further studies in patients with immunological disorders need to be performed, with special attention for SPR+ disorders as proven by autoradiography. We currently investigate patients with inflammatory bowel disease or brain tumours.

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## CHAPTER 9

# THYMUS SCINTIGRAPHY WITH [$^{111}$In-DTPA-Arg$^1$]SUBSTANCE P

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## ABSTRACT

Substance P (SP) is an 11-amino acid neuropeptide and plays an important role in modulating pain transmission through neurokinin 1 and 2 receptors. SP and other tachykinins play a role in the pathogenesis of inflammatory diseases as well. In this study we present results concerning the metabolism of the SP analogue [$^{111}$In-DTPA-Arg$^1$]SP in men and the visualization of the thymus in patients with immune mediated diseases.

*Patients and Methods.* Ten selected patients, with inflammatory bowel disease (5), ophthalmic Graves' disease (1), sarcoidosis (1), Sjögren's syndrome (1), rheumatoid arthritis (1), and systemic lupus erythematosus (1) were investigated. During and after infusion of 200 MBq (2,5 $\mu$g) [$^{111}$In-DTPA-Arg$^1$]SP i.v. and blood pressure, heart rate and oxygen saturation were monitored. Radioactivity was measured in blood, urine and feces during 48 h after injection. Planar and Single Photon Emission Tomography (SPECT) images were obtained 4 and 24 h post injection.

*Results.* After administration of [$^{111}$In-DTPA-Arg$^1$]SP a transient flush was observed in all patients. Degradation of [$^{111}$In-DTPA-Arg$^1$]SP started in the first minutes after administration, resulting in a half-life of 10 min for the total plasma radioactivity and of 4 min for the intact radiopharmaceutical, as identified with HPLC. Urinary excretion amounted in $>95\%$ of the radioactivity within 24 h post injection, and up to 0.05 % was found in the feces up to 60 h. In all patients uptake of radioactivity was found in the parotid glands, areolae of the mammae (in women), liver, kidneys, and urine bladder. In 7 patients a high uptake of [$^{111}$In-DTPA-Arg$^1$]SP was observed in the thymus.

*In conclusion:* [$^{111}$In-DTPA-Arg$^1$]SP is a new radiopharmaceutical and despite its short half-life it can be used to visualize the thymus. This may contribute to the investigation of the role of thymus in immune related diseases. Also, inflammatory

sites in various diseases could be visualized. The exact role of [$^{111}$In-DTPA-Arg$^1$]SP as a radiopharmaceutical in inflammatory diseases remains to be established.

## INTRODUCTION

Substance P (SP) is an 11-amino acid neuropeptide and is known as a powerful member of a family of tachykinins which are characterized by the C-terminal sequence Phe-X-Gly-Leu-Met-$NH_2$ (1). It has been well established that SP plays an important role in modulating pain transmission from peripheral and central primary afferents through neurokinin 1 and 2 receptors.

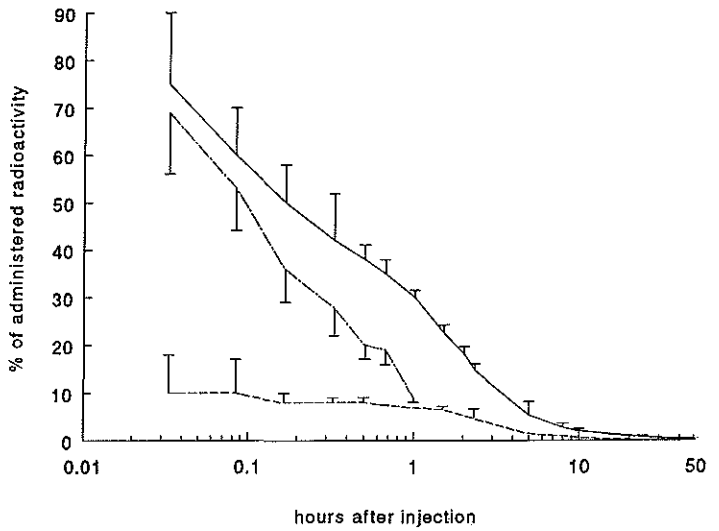


**Figure 1**. *The total plasma (●), peptide bound (▲) and cell bound (○) radioactivity during 48 h after injection*

SP and other tachykinins may play a role in the pathogenesis of inflammatory diseases (2-7). SP was shown to induce degranulation of mast cells in vitro, regulate chemotaxis, release lysosomal enzymes from macrophages, regulate T cell and B cell expansion and production of immunoglobulins (8-11). The thymus seems to be involved in immune mediated diseases. Lymphofollicular hyperplasia of the thymus has been described in autoimmune diseases such as myasthenia gravis, systemic lupus erythematosus, scleroderma, rheumatoid arthritis, Hashimoto's thyreoiditis and Sjögren's syndrome (12-15).
Recently, SP was detected in the peptidergic neurons which innervate the thymus. Further $NK^1$ receptors were demonstrated in the thymus using autoradiography

(16,17). Large numbers of high-affinity substance P receptors (SPR) are found in surgical specimen obtained from patients with inflammatory bowel disease. The SP-binding $NK_1$ receptor is expressed by arterioles and venules located in the submucosa, muscularis mucosa, external longitudinal muscle, and serosa (4).
We developed a scintigraphic technique using an $^{111}In$ labelled SP analogue, ([$^{111}In$-DTPA-Arg$^1$]SP), to visualize affected tissues in immune disorders, e.g. the thymus. In this study results are presented concerning the metabolism of $^{111}In$ labelled [DTPA-Arg$^1$]substance P and the scintigraphic detection of the thymus in immune mediated diseases. This may contribute to the understanding of the role of the thymus in immune disorders.

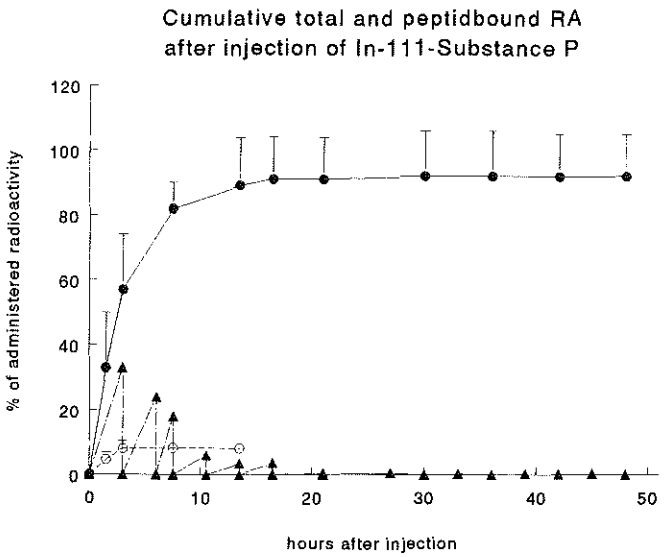


**Figure 2.** *Cumulative urinary excretion pattern of total (●) and peptide-bound (○) radioactivity during 48 h after injection, and (▲) shows the radioactivity in the urinary bladder*

## PATIENTS AND METHODS

*Patients*

Ten selected patients were investigated after informed consent by the patient and approval of the medical ethics committee of our institution. Five with inflammatory bowel disease, one with ophthalmic Graves' disease, one with sarcoidosis, one with Sjögren's syndrome, one with rheumatoid arthritis and one with systemic lupus erythematosus. The diagnosis was established according to the clinical presentation and histopathologic examination of gastrointestinal biopsies in

the patients with inflammatory bowel disease and by liver biopsy in the patient with sarcoidosis. In rheumatoid arthritis, Sjögren's syndrome and systemic lupus erythematosus the diagnosis was made according to the internationally accepted diagnostic criteria. The patient with Graves' disease had lowered serum thyroid stimulating hormone concentration and elevated serum free thyroxine and specific thyroid-stimulating immunoglobulin concentrations. Uptake of radioactivity was investigated in 6 "control patients", 3 with carcinoid tumors, 1 with lung carcinoma and 2 with non-Hodgkin's lymphoma.

*Radiopharmaceuticals*

[DTPA-Arg$^1$]SP was labelled with $^{111}InCl_3$ (370 MBq/mL in HCl, pH= 1.5 - 1.9, Mallinckrodt, Petten, The Netherlands), up to a specific activity of 150 MBq $^{111}$In per $\mu$g [DTPA-Arg$^1$]SP. Quality control by instant thin layer chromatography (ITLC, Silica-gel) and SEP-PAK $C_{18}$ reversed-phase extraction were performed essentiaslly as described earlier (18,19). High performance liquid chromatography (HPLC) was done using a Waters 600E multisolvent delivery system, connected to a $\mu$Bondapak $C_{18}$ reversed-phase column (300 * 3.9 mm, particle size 10 $\mu$m). Elution was carried out at a flow of 1.5 mL/min with a linear gradient of 10 to 50 % acetonitril in 0.1 % trifluoroacetic acid in 30 min, and the latter composition was kept constant for another 5 min.
The radiochemical purity of the radiolabelled SP analogue was greater than 98 %. Although it is not excluded that additional groups of the peptide participate in $^{111}$In complexation, the labelled product is referred to as [$^{111}$In-DTPA-Arg$^1$]SP.

*Imaging*

In our study 2.5 $\mu$g [$^{111}$In-DTPA-Arg$^1$]SP (200 MBq) was infused intravenously in 10 minutes. In all patients blood pressure, heart rate and oxygen saturation of the blood were monitored. Planar and SPECT images were obtained with a large field gamma camera equipped with a medium-energy parallel-hole collimator. SPECT analysis was performed with a Wiener filter on original data. Preset time for images obtained 24 h and 48 h after injection of [$^{111}$In-DTPA-Arg$^1$]SP was 15 min. Planar images were obtained from the head and neck, chest, upper abdomen and lower abdomen. SPECT studies were performed 4 h or 24 h after injection of the radiopharmaceutical.

*Measurement of radioactivity in blood, urine and feces*

Radioactivity in blood, urine and feces was measured with an LKB-1282-Compugamma system or a GeLi-detector equipped with a multi-channel analyser (Series 40, Canberra). Blood was collected in EDTA-containing tubes and immediately cooled on ice. Since SP is readily metabolized by various enzymes in blood, samples were immediately centrifugated at 0° C, and plasma was

fractionated on SEP-PAK $C_{18}$ columns. Using the separation technique described previously (19), [$^{111}$In-DTPA-Arg$^1$]SP binds to SEP-PAK $C_{18}$ stationary phase and is only eluted with ethanol, while $^{111}$In-DTPA is not retained on SEP-PAK $C_{18}$ columns. The radioactivity in plasma and urine samples, which was eluted with ethanol from the SEP-PAK $C_{18}$ column, is termed peptide-bound radioactivity but was not further characterized. Blood samples were collected directly before and 2, 5, 10, 20, and 40 min and 1, 4, 20 and 48 h after infusion. Urine was collected from the time of injection in two 3-h intervals and thereafter in intervals of 6 h until 48 h after injection. If feasible, feces was collected until 72 h after injection. The chemical status of the radionuclide in blood and urine was analysed as a function of time using the SEP-PAK $C_{18}$, HPLC and PD-10 gel filtration techniques as described previously (18,19).

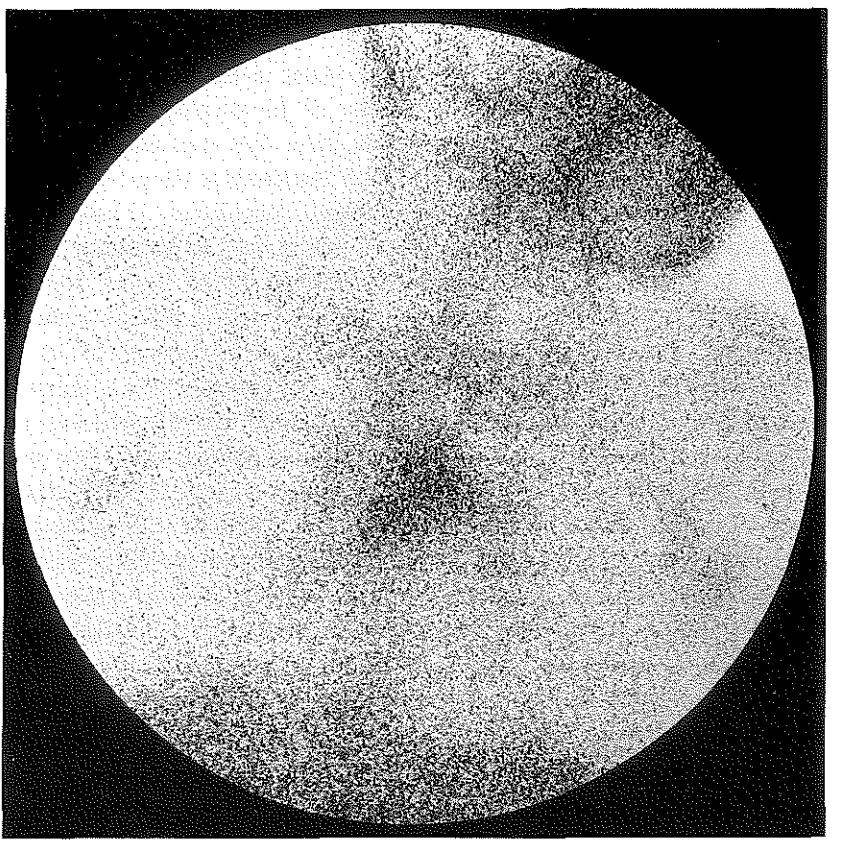

**Figure 3**. *Accumulation of radioactivity in the thymus and salivary glands of a patient with liver sarcoidosis (anterior view of the chest, 24 h post injection)*

## RESULTS

After administration of [$^{111}$In-DTPA-Arg$^{1}$]SP a transient flush was observed in all patients. In one patient with Crohn's disease and anaemia, significant hypotension was observed associated with bradycardia. This patient recovered after 30 seconds in Trendelenburg's position, suggesting the presence of a vasovagal collaps.

In Fig. 1 the time courses of total and peptide-bound radioactivity in plasma are presented until 50 h after administration. The average plasma radioactivity decreased rapidly after injection of [$^{111}$In-DTPA-Arg$^{1}$]SP. Degradation of [$^{111}$In-DTPA-Arg$^{1}$]SP started in the first minutes after administration, resulting in a half-life of 10 min for total plasma radioactivity and of 4 min for the intact radiopharmaceutical, as identified with HPLC.

Renal excretion resulted in the excretion of >90% of the radioactivity in the urine within 24 h post injection as shown in Fig. 2. Up to 0.05 % was found in the feces until 60 h post injection.

In all patients uptake of radioactivity was found in the salivary glands, liver, kidneys, urine bladder and in women the areolae of the mammae were visualized 4 h post injection. The uptake of radioactivity at these sites was supposed to be due to receptor binding (parotid and areolae) or to metabolism (liver, kidneys, urine bladder) of the radionuclide labelled SP analogue. Deposition of radioactivity at other sites was considered due to other causes, which may be pathology related. In 7 patients a high uptake of [$^{111}$In-DTPA-Arg$^{1}$]SP was found in the thymus. The highest uptake of radioactivity in the thymus was observed in the two patients with liver sarcoidosis and Sjögren's syndrome (Fig. 3). CT-scanning of the thorax confirmed an enlarged thymus in both patients. In patients with systemic lupus erythematosus, ophthalmic Graves' disease, rheumatoid arthritis and two patients with inflammatory bowel disease the thymus was visualized as well. No uptake of radioactivity in the thymus was observed in 3 patients with carcinoid tumors, 1 with lung carcinoma and 2 with non-Hodgkin's lymphoma. In addition to the thymus, in the patient with Sjögren's syndrome, the affected parotid glands showed an high uptake of radioactivity which was not observed in other patients. In the patient with unilateral ophthalmic Graves' disease, high uptake of radioactivity was observed in the eye mostly affected (Fig. 4). In 3 out of 5 patients with inflammatory bowel disease accumulation of radioactivity was found at the sites of inflammation (data not shown).

## DISCUSSION

SP is found in the central nervous system as well as in peripheral neuronal circuits. It is involved in the regulation of growth hormone, ACTH and prolactin production (20,21). In the peripheral nervous system SP is found in the primary afferent neurons (A-delta pain fibers and C-fibers) that are involved in the

transmission of noxious stimuli. Bioactive SP has been infused intravenously in humans in doses of 0.5-1.5 pmol.kg$^{-1}$.min$^{-1}$ over 60 min or 0.5-8.0 pmol.kg$^{-1}$.min$^{-1}$ over 20 min without serious side effects. (20,22). A very short half-life of 1.6 min was measured indicating a very high metabolic clearance. In normal plasma SP is degraded by enzymatic hydrolysis involving multiple proteases (23).

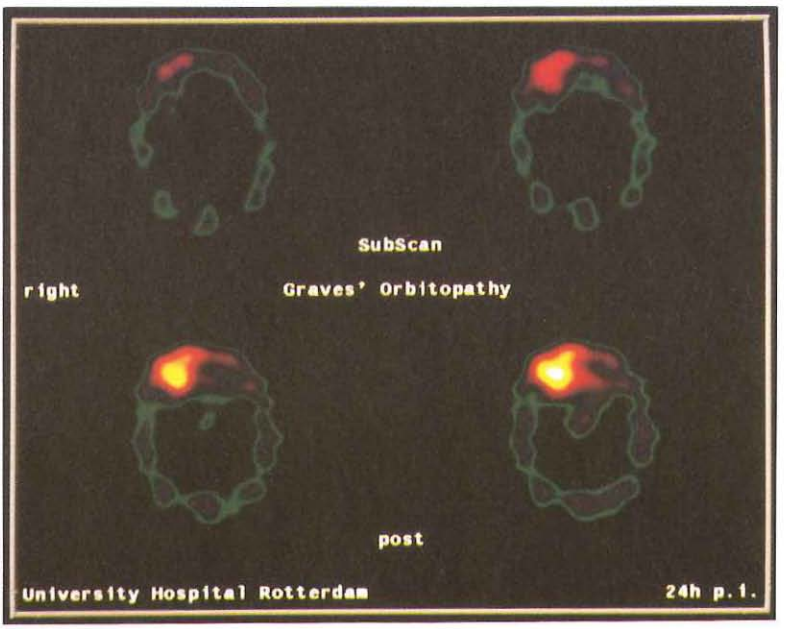


**Figure 4**. *Uptake of radioactivity in a patient with unilateral ophthalmic Graves' disease (SPECT, 24 h post injection)*

In this study the [DTPA-Arg$^1$]SP was injected in patients with immune mediated diseases. In 1 out of 10 patients hypotension was observed with the clinical impression a vasovagal collaps caused the hypotension. After administration of [$^{111}$In-DTPA-Arg$^1$]SP a transient flush was observed in all patients.

A very short half-life of 10 min for plasma radioactivity and of 4 min for the intact radiopharmaceutical was measured. Radioactivity was excreted almost exclusively by the kidneys resulting in a low accumulation of radioactivity in the intestinal tract. Despite, the high degradation rate of this radiopharmaceutical it could visualize affected tissues for instance the thymus and tissues e.g. salivary glands, which are well-known to be SP receptor positive.

In 7 patients with immune mediated diseases the thymus was visualized. In the "control" patients no thymus could be visualized, suggesting a high receptor

expression in immune mediated diseases. SPR have been described recently to be present in the thymus, but their role is still obscure. SP has been identified in nerve fibers of sensory origin in the thymus. The outer cortex of the human thymus contains an one to two cell thick layer that is immunoreactive with antisera against SP. In this region the most immature and recently migrated thymocytes are found emphasizing the role of of neuropeptides in regulating the microenvironment for T cell development (16). The gene encoding both SP and neurokinin-A was shown to be expressed in rat thymus, especially in a subpopulation of cells in the medulla (17).

Also, the thymus is known to play an important role in another immune mediated disease, myasthenia gravis. Microscopy of thymuses revealed in 70 % a follicular hyperplasia of the medulla containing germinal centres resembling a stimulated lymph node (24,25). It may be that primary sensitization against the acetylcholine receptor which plays a crucial role in myasthenia gravis, occurs in the thymus. The reason why SP-receptors are present in the thymus of patients with immune mediated disease, as suggested in our patients, is obscure, but may mean that a similar process of sensitization as is hypothesized in myasthenia gravis, takes place in other auto-immune diseases as well.

Besides the thymus, inflammation sites in two patients with inflammatory bowel disease, the affected orbit in ophthalmic Graves' disease, the salivary glands in Sjögren's syndrome and the joints in rheumatoid arthritis were visualized. Although in patients with inflammatory bowel disease accumulation of radioactivity due to gut stenosis cannot be ruled out, the very low excretion of [$^{111}$In-DTPA-Arg$^1$]SP into the bowel ($<0.05\%$) does not favor this explanation. Therefore, this accumulation is most likely due to the presence of SPR at the sites of inflammation in these patients.

SP is supposed to be involved in inflammatory bowel disease, upregulation of receptor binding sites at the venules and muscle was previously shown by autoradiography (4). In these patients a high-expression of SPR was observed in comparison with control patients (1000-2000 times normal).

SPR were described in inflammatory synovitis in rat and human (6,7). [$^{125}$I]-labelled SP binding to microvascular endothelium paralleled the distribution of SP immunoreactive nerves and had the characteristics of the NK-1 receptor (6).

In this study we present our preliminary results concerning the use of [$^{111}$In-DTPA-Arg$^1$]SP as a new radiopharmaceutical which has a very short half-life in the circulation. The thymus, which is usually involuted after puberty, expressed enough SPR to allow in vivo visualization. The role of the thymus in auto-immune diseases is obscure, only enlargement of thymus has been described in these diseases. Further investigations are necessary to evaluate the role of SPR expression in the thymus in these diseases. Also, inflammatory sites in various diseases could be visualized by SPR scintigraphy. The exact role of [$^{111}$In-DTPA-

$Arg^{1}$]SP as a radiopharmaceutical in autoimmune inflammatory diseases remains to be established.

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## APPENDIX 1

# RADIOTHERAPY WITH A RADIOLABELLED SOMATOSTATIN ANALOGUE, [$^{111}$In-DTPA-D-Phe$^1$]OCTREOTIDE. A case history

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## INTRODUCTION

In 1987, we started octreotide receptor scintigraphy in humans. After a few years of using the radioiodinated [Tyr$^3$]octreotide (1), we switched to [$^{111}$In-DTPA-D-Phe$^1$]octreotide, which has many advantages over the radioiodinated compound (2,3). Patients with various forms of cancer, both classically known as of neuro-endocrine and of non-endocrine nature, have been investigated (4).

The application of these new peptide-receptor agents in scintigraphy, and of many others which will become available in the near future, such as substance P and bombesin or their derivatives, might evoke a combination of optimism and pessimism. After all, the presence of receptors determines the possibility to detect the abnormality or tumour by peptide receptor scintigraphy. Thus, the classical concept of false positives and false negatives really is not applicable to visualization by means of peptide receptor scintigraphy (5). This technique allows primarily an interpretation with regard to receptor presence and not to anatomical abnormalities, as is the case with CT, ultrasound and MRI. Only at a second stage, after showing the localization of an abnormal density of receptors, one might conclude the presence of an anatomical abnormality expressing the peptide receptors, e.g. a primary pancreatic islet cell tumour expressing somatostatin receptors. However, when peptide receptor scintigraphy does not demonstrate (a) lesion(s), suggesting that a tumour or (several of the) metastases do not express (anymore) a certain peptide receptor, this also might be of paramount importance with regard to therapeutic advice to the patient. Dedifferentiation of a tumour is accompanied by loss of a given peptide receptor and this information influences the oncologist's therapeutic decisions. An example is the choice between the use of somatostatin analogues or cytostatic drugs in patients with neuro-endocrine tumours, that are well-differentiated or have become anaplastic, respectively (6,7). With future

(radio)therapy using (radio)labelled peptide(-derivatives), the presence (in the individual patient!) of several undifferentiated metastases, that do not bind a given (radio)ligand as opposed to the well-differentiated metastases, necessitates (an) additional application(s) of a (radio)ligand with appropriate binding characteristics for the undifferentiated tumours, possibly by injection of a "cocktail" of (radiolabelled) ligands. Thus, mapping of the presence, including the localization of the tumours and of the distribution of peptide-receptors on all metastases in the individual patient by peptide receptor scintigraphy may become an attractive, non-invasive, harmless, easy-to-perform tool for an individual therapeutic approach of the cancer patient.

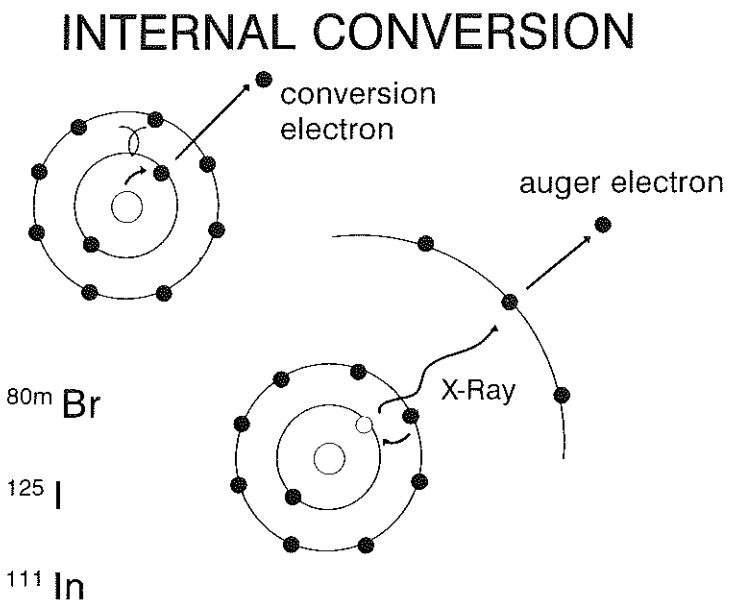


**Figure 1**. *Schematic representation of gamma-ray emission ("internal conversion") leading to the ejection of conversion-electrons (nuclear photons transfer their energy to orbital electrons, which are then ejected from the atom, thereby leaving an electron vacancy, indicated with the small open circle) and Auger-electrons (the latter by X-ray photon interaction with an loosely bound outer electron). The maximum particle range of conversion- and Auger-electrons emitted by $^{111}$In is indicated in Table 1*

In this report we present a patient with an inoperable, metastasized glucagonoma in whom peptide receptor radiotherapy with Auger- and conversion-electrons emitting [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide, affects the growth of the tumour and the

circulating glucagon levels. Because of their more appropriate physical characteristics, future nuclear radiotherapy is directed to the use of $\alpha$- or ß-particles emitting radionuclide labelled peptides.

*Selection of radionuclide for radiotherapy with the radiolabelled somatostatin analogue [DTPA-D-Phe$^1$]octreotide*

For radiotherapeutic applications several radionuclides have been proposed and investigated to be coupled to [DTPA-D-Phe$^1$]octreotide. Radiolabelled [DTPA-D-Phe$^1$]octreotide has an appropriate distribution profile in humans for this purpose. At the moment, theoretically suitable ß-emitting radionuclides are not available in pure form or show a dissociation from the chelated peptide in serum. Therefore, we decided to investigate the antiproliferative effect of the Auger- and conversion-electrons of $^{111}$In-labelled [DTPA-D-Phe$^1$]octreotide. Figure 1 and Tables 1-2 illustrate the origin and the main physical characteristics of these electrons, respectively.

**Table 1**. *Physical characteristics of the radionuclide: $^{111}$In (t½ = 2.83 d)*

| emission-type | E (keV) | R($\mu$m) |
|---|---|---|
| gamma-photons | 171 - 245 | |
| conversion electrons | 144 - 245 | 200 - 550 |
| Auger electrons | 0.5 - 25 | 0.02 - 10 |

**Table 2**. *Energies (E) and yields of conversion- and Auger-electrons of $^{111}$In*

| | E (keV) | Yield (%) |
|---|---|---|
| conversion | 145 - 170 | 10 |
| conversion | 218 - 245 | 6 |
| Auger | 19 - 25 | 16 |
| Auger | 2.6 - 3.6 | 102 |
| Auger | 0.5 | 191 |

## MATERIALS AND METHODS

[DTPA-D-Phe$^1$]octreotide and $^{111}InCl_3$ (DRN 4901, 370 MBq/ml in HCL, pH = 1.5 - 1.9) were obtained from Mallinckrodt Medical BV (Petten, The Netherlands). [DTPA-D-Phe$^1$]octreotide was labelled with $^{111}$In as has been described elsewhere (2). Doses ranged from 1590 MBq to 4810 MBq $^{111}$In, which

were coupled with amounts of [DTPA-D-Phe$^{1}$]octreotide ranging from 10 to 120 μg. The protocol of [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide scintigraphy has been described previously (4). Radiotherapy with [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide was applied after informed consent by the patient and approval by the medical ethics committee of our institution.

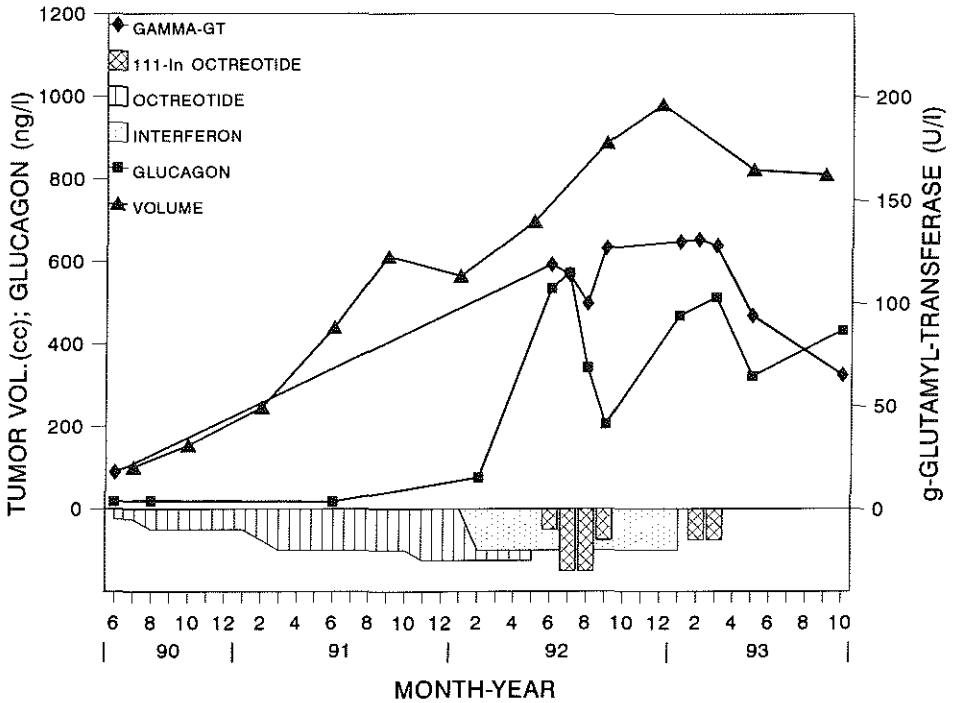


**Figure 2.** *Results of various treatments, including radiotherapy with [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide, in a patient with a metastasized inoperable glucagonoma. From 6/1990 to 5/1992 increasing doses of (not radiolabelled) octreotide (300 to 1500 μg/day) were used and from 2/1992 to 1/1993 Interferon-α (3.10$^{6}$ units three times/week). Because of persistent increase in size of tumour volume during this medical treatment, [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide was administered from 6 to 9/1992 (in 5 doses, total 15022 MBq) and in 2/1993 (2664 MBq) and 3/1993 (2590 MBq). The use of octreotide had been stopped before this radiotherapy. Tumour volume is volume of all abdominal tumours in cc, according to volume measurements of tumours found with whole abdominal CT scanning; glucagon and γ-glutamyl transferase are serum levels*

*Case*

The patient is a 55 years old female, who underwent two abdominal tumour operations in 1988 and 1989, including partial pancreatectomy, splenectomy and dissection of para-aortal lymph nodes. At the second operation liver metastases were not present. Histology showed a neuro-endocrine tumour with positive immuno-histochemistry for glucagon, pancreatic polypeptide, somatostatin, calcitonin, ACTH, gastrin and Carcino embryonic antigen. The patient had been referred to our hospital because of the demonstration of somatostatin receptors on the recurrent metastases according to octreotide scintigraphy. The patient never experienced signs or symptoms of a hormone producing neuro-endocrine tumour. Only elevated serum levels of glucagon were found. Since the excellent clinical condition of the patient at the time of referral, it was decided not to treat her with cytostatics. Treatment with increasing doses of octreotide was started. Eventually therapy with interferon-$\alpha$ was started. Since no antiproliferative effect was observed during this medical treatment, it was decided to investigate the effect of therapeutic doses of [$^{111}$In-DTPA-D-Phe$^1$]octreotide. Neither acute nor longterm side-effects, including on renal, pituitary and bone marrow functions, were observed up to half a year after the last administration of a radiotherapeutic dose of [$^{111}$In-DTPA-D-Phe$^1$]octreotide (data not shown). Figure 2 illustrates the time schedules of treatments applied to this patient and their effects on tumour size and both glucagon and $\gamma$-glutamyl transferase serum levels. Figures 3 - 5 show examples of CT scans related to time, and of [$^{111}$In-DTPA-D-Phe$^1$]octreotide scintigrams. It is evident that both the tumour load, glucagon and $\gamma$-glutamyl transferase levels react benifically to radiotherapy. The decrease in glucagon levels is only transient. In accordance with the decline in $\gamma$-glutamyl transferase levels the CT-scan of the liver shows a clear decrease in the size of liver metastases.

*Dosimetry of [$^{111}$In-DTPA-D-Phe$^1$]octreotide accumulation*

Accumulation of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in the organs with significant uptake, i.e. liver and kidneys, and one selected abdominal tumour (mass = 300 g) were calculated as described before (3). Because of the high radiotherapeutic doses of [$^{111}$In-DTPA-D-Phe$^1$]octreotide that were given, it was possible to scan the patient over a long period. An example of the course of the radioactivity is given in Figure 6. The amount of radioactivity in the liver and the tumour showed a slow decrease (biological half-life > 700 h), whereas the radioactivity in the kidney has a biological half-life of about 270 h. A total of 20 GBq (550 mCi) [$^{111}$In-DTPA-D-Phe$^1$]octreotide was given intravenously in seven administrations (Figure 2). Because of the fact that the patient had a nephrostomy drain on the left side, only 35% of the produced urine entered the bladder. The urine was not collected for measurements of radioactivity. For these reasons the dose to the urinary bladder was estimated using the results described previously (3). The thus

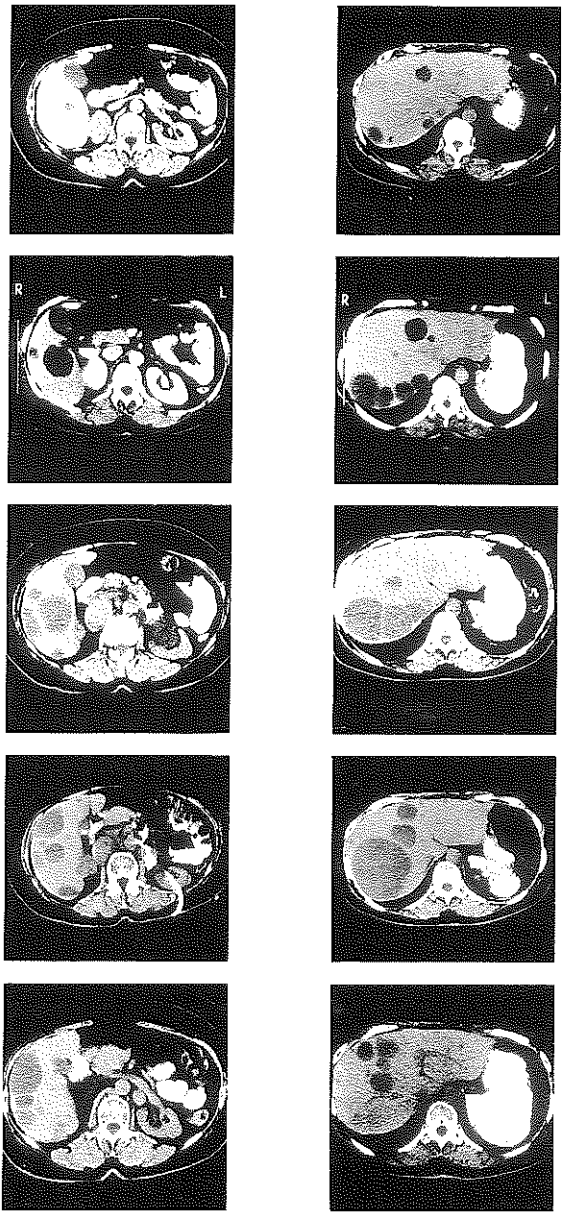


**Figure 3**. *CT scanning of the here reported case with metastasized inoperable glucagonoma, views are from two levels of the liver and related to time : row 1 = 23 july 1990; row 2 = 3 june 1991; row 3 = 4 may 1992; row 4 = 17 december 1992 and row 5 = 30 august 1993*

estimated dose to the urinary bladder wall was 1.3 Gy. Using the MIRDOSE2-program, the calculated doses on the liver and kidneys were 2.4 and 5 Gy, respectively (3). As the tumour had about the same mass as the kidneys (on the basis of the CT scans), and the radiation dose to the tumour is for more than 95% caused by the radioactivity in the tumour, the S-factor for the kidneys was used for the calculation of the dose on the tumour. The estimated apparent radiation dose to the tumour was 13 Gy.

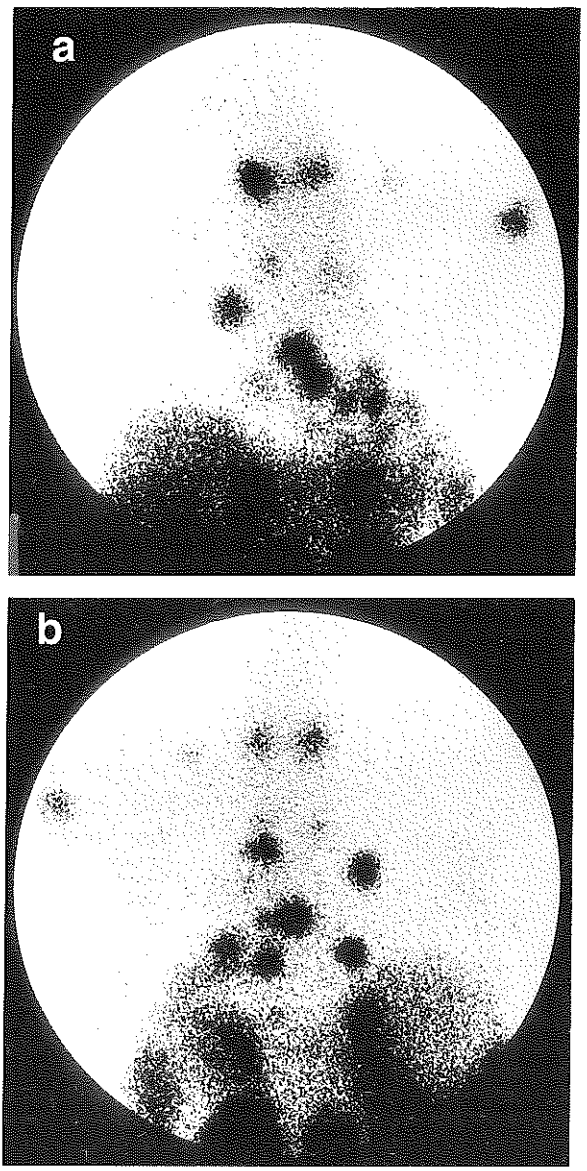


**Figure 4.**
*Planar [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide scintigraphy of the here reported case with metastasized inoperable glucagonoma. Images a and b are anterior and posterior thoracic-abdominal views, respectively. Note the many "hot"-spots at the various sites, representing metastases of the glucagonoma*

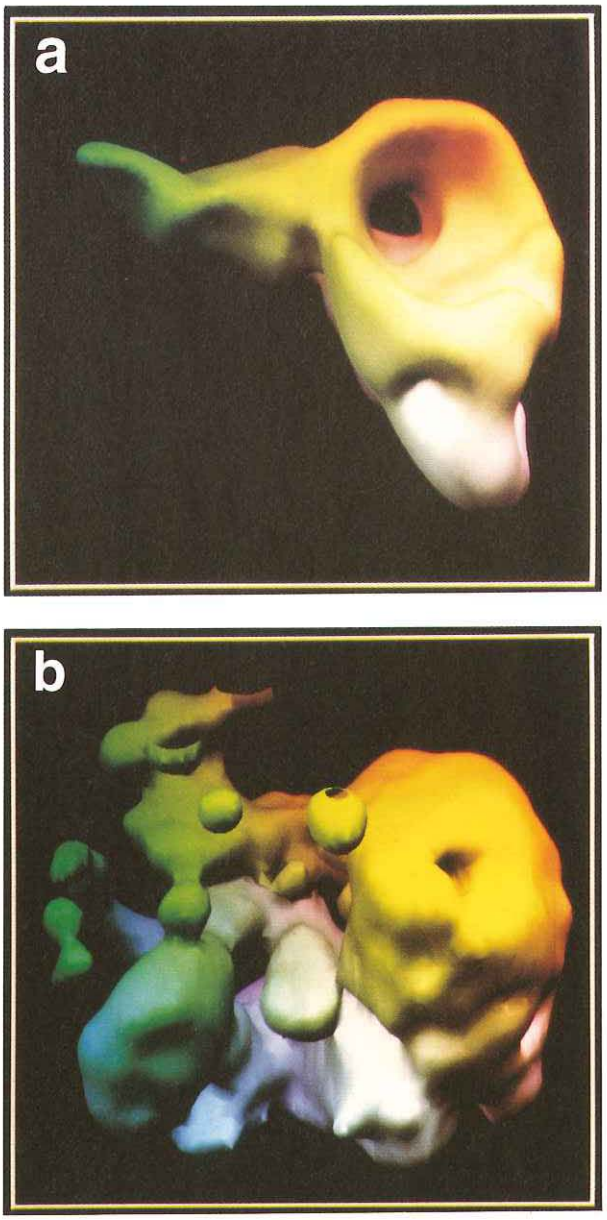


**Figure 5.**
*Three-dimensional representation of the upper abdomen (posterior views) of the here reported case with metastasized inoperable glucagonoma after injection with $^{99m}$Tc-colloid (a) and [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide (b). These scintigrams have been carried out before the second period of radiotherapy with [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide. Note the large "cold" area in the upper part of the right lobe of the liver on the colloid scan and the presence of radioactivity in this area on the [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide scan. This difference is explained by the presence of somatostatin receptor expressing tumour tissue. Furthermore, the [$^{111}$In-DTPA-D-Phe$^{1}$]-octreotide image shows many "hot"-spots at various sites, representing metastases of the glucagonoma*

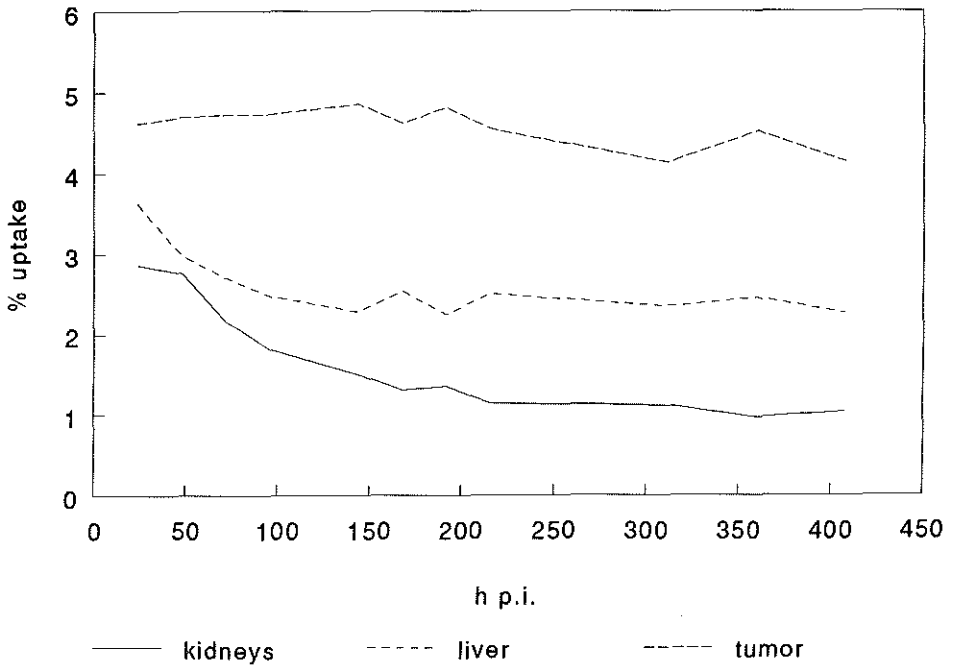


**Figure 6**. *The percentage uptake in the kidneys, liver and abdominal tumour area after a treatment dose of [$^{111}$In-DTPA-D-Phe$^1$]octreotide as function of time after injection*

## DISCUSSION

Scintigraphy with [$^{111}$In-DTPA-D-Phe$^1$]octreotide of the glucagonoma in our patient showed an intense accumulation of radioactivity in the tumour, indicating the expression of a high number of octreotide receptors. Despite the presence of octreotide receptors on the metastases, growth of the tumours continued during octreotide treatment, although a modest antiproliferative effect at an octreotide dose of 1500 $\mu$g/day could not be excluded in retrospect. Also during treatment with the combination of a high dose of octreotide and interferon-$\alpha$ tumour volume continued to increase. Only after a cumulative dose of 20 GBq [$^{111}$In-DTPA-D-Phe$^1$]octreotide, a decrease in total tumour volume in the abdomen of about 20 percent was observed. Furthermore, after both episodes (in 1992 and 1993) of [$^{111}$In-DTPA-D-Phe$^1$]octreotide radiotherapy a (transient) decline in glucagon and $\gamma$-

glutamyl transferase serum levels was observed. Possibly, these changes in serum markers can be regarded as signs of an antiproliferative effect of the high doses of [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide. Such an effect might be comparable to the decline in thyroglobulin levels, which is frequently observed after successful treatment with radioactive iodine for thyroid cancer. It is unlikely that the peptide of [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide itself induced this effect, because of the interval of several weeks between administration of radioligand and the collection of the serum sample for glucagon measurement.

Intense and homogeneous distribution of somatostatin receptors on most glucagonomas is demonstrated by [$^{125}$I-Tyr$^{3}$]octreotide autoradiography (not shown). Of course this is advantageous for peptide receptor therapy with [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide and might partly explain its effect in this case. The apparent radiation dose to the tumour (13 Gy) is calculated according to conventional dosimetry. The application of conventional dosimetry is validated by the fact that the range of several of the ionizing particles emitted is much longer than the average tumour cell diameter. Because of the fact that $^{111}$In is also an Auger-electron emitter (range 0.02 - 10 $\mu$m), the actual radiation dose may be much higher when the $^{111}$In-labelled pharmaceutical is internalized into the cell (Tables 1 and 2). The estimated apparent radiation dose to the tumour (assuming a tumour volume on the basis of the CT scan) with the performed radiotherapy with [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide is relatively low. It is tempting to speculate that the observed response to this radiotherapy (because of the abundance of Auger electrons with a very short particle range) indicates that the [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide is internalized into the tumour cell. In vitro tests will have to confirm this hypothesis. If [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide is not internalized, but only binds to its receptors at the plasma membrane with the observed long residence time, then it may be concluded that the contribution of the conversion electrons (with their longer particle range than the Auger electrons) to the radiotherapeutic effect of [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide is more important.

However, in general, $^{111}$In is not the most appropriate radionuclide for this objective, in asmuch as it lacks the preferable higher energies of $\alpha$- and ß-particles. It is to be expected that the radiotherapeutic use of radionuclides with those higher energies, coupled to small peptides, leads to higher radiation doses and more appropriate particle ranges. Also tumours with an inhomogeneous distribution of peptide receptors may then respond in a favourable way to this kind of treatment. $^{161}$Tb is one of the radionuclides, which is a candidate, because it emits ß-particles and is able to bind to the chelator DTPA without dissociation in blood.

Both peptide receptor scintigraphy and peptide receptor therapy with radiolabelled peptides(-derivatives), like hormones and growth factors, are at the moment in their infancy. Nevertheless, the body distribution of small radiolabelled peptides in humans and the results of scintigraphy with [$^{111}$In-DTPA-D-Phe$^{1}$]octreo-

tide are promising for peptide receptor scintigraphy and peptide receptor therapy . Peptide receptor scintigraphy might not only become a diagnostic localizing tool for most cancer types and their metastases, but it will also form the basis for peptide receptor therapy with radiolabelled peptides. In general, the kidneys are the most critical organs for radiotherapy with radiolabelled peptides. Investigations are being performed to lower the renal accumulation of radioactivity by interrupting the tubular reabsorption of these radioligands by 1. infusion of amino acids, mainly lysine and arginine and 2. transient ATP lowering in renal tubular cells (8,9). Nevertheless, the tolerable radiation dose of the kidneys seems already to be relatively high (10,11). Furthermore, by decreasing the renal accumulation of radioactivity, the cumulative dose of radiolabelled peptides for peptide receptor therapy can be increased, thereby optimizing the ultimate radiation dose to the tumour(s). It is hoped that the latter can also be reached in the future by inducing an up-regulation of the receptor density.

In conclusion, it is to be expected that the arsenal of treatment modalities in oncology in the near future will be extended by peptide receptor therapy using radiolabelled peptides (-derivatives), which are more or less derived from native substances. This contrasts to the larger mouse monoclonal antibodies (10,11), of which the application leads to a higher background radiation and also the forming of human anti-mouse antibodies if administered repeatedly (12).

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## APPENDIX 2

# [$^{161}$Tb-DTPA-D-Phe$^{1}$]OCTREOTIDE: PREPARATION, IN VITRO RECEPTOR BINDING AND BIOLOGICAL ACTIVITY, METABOLISM IN ISOLATED PERFUSED RAT LIVERS AND DISTRIBUTION IN VIVO IN NORMAL AND TUMOUR-BEARING RATS IN COMPARISON WITH [$^{111}$In-DTPA-D-Phe$^{1}$]OCTREOTIDE

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## ABSTRACT

[$^{111}$In-DTPA-D-Phe$^{1}$]octreotide (OctreoScan®) is a radiopharmaceutical that binds to the somatostatin receptor present in certain tissues. It is being used for scintigraphic imaging of somatostatin receptor-positive lesions, such as gastrointestinal pancreatic tumours, neuroblastoma, pheochromocytoma, breast cancer, Hodgkin's lymphoma and small cell lung cancer. New fields of application of radiolabelled somatostatin analogs are e.g. intraoperative scanning using gamma rays emitting radiolabelled somatostatin analogues and the potential use of radiolabelled peptide for radiotherapy. However, for these two purposes, $^{111}$In will not be the radionuclide of first choice. While its characteristics are favourable for scintigraphy (gamma rays of 174 and 247 keV), they are not optimal for intraoperative scanning. This because of the high background radiation due to the relatively hard gamma rays emitted by $^{111}$In. Furthermore, for the second field of investigation, radiotherapy, a beta rays emitting radionuclide is more suitable. The characteristics of $^{161}$Tb are quite ideal for intraoperative scanning: low gamma energy rays (with peaks around 46-48 and 74 keV), and a half-life of nearly seven days. As for the potential use for radiotherapy, $^{161}$Tb emits also hard beta rays. Therefore, the characteristics of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide with respect to specific binding to somatostatin (octreotide) receptors on rat brain cortex membranes, biological activity, metabolism in isolated perfused rat livers and metabolism in vivo in

normal and tumour-bearing rats were determined and compared to those of [$^{111}$In-DTPA-D-Phe$^1$]octreotide.

It is concluded that based on the characteristics of $^{161}$Tb (low gamma energy rays with a half-life of nearly seven days, and hard beta rays) combined with the in vitro and in vivo studies performed with [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide, the latter is a promising radiopharmaceutical for both intraoperative scanning and radiotherapy. The latter in particular after inhibition of the renal tubular reabsorption process, to decrease the kidney dose during application. Further studies in patients need to be performed now to see if [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide can indeed open new therapeutic applications for patients bearing octreotide receptor-positive tumours.

## INTRODUCTION

[$^{111}$In-DTPA-D-Phe$^1$]octreotide (OctreoScan®) is a radiopharmaceutical that binds to the somatostatin receptor present in certain tissues. It is being used for scintigraphic imaging of somatostatin receptor-positive lesions, such as gastrointestinal pancreatic tumours, neuroblastoma, pheochromocytoma, breast cancer, Hodgkin's lymphoma and small cell lung cancer (1, review).

Since the availability of this and other radiolabelled somatostatin analogues for in-vivo imaging, the range of diagnostic applications under study is rapidly increasing. One example is the intraoperative scanning using gamma rays emitting radiolabelled somatostatin analogues. This technique is used to guide surgery with respect to the localisation of small somatostatin receptor-positive tumours using a small hand-held probe (2,3). Another field of application that is under examination is the potential use of radiolabelled peptide for radiotherapy.

However, for these two purposes, $^{111}$In will not be the radionuclide of first choice. While its characteristics are favorable for scintigraphy (gamma rays of 174 and 247 keV), they are not optimal for intraoperative scanning, because of the high background radiation due to the relatively hard gamma rays emitted by $^{111}$In. Furthermore, for radiotherapy a beta particles-emitting radionuclide is more suitable.

We have now investigated whether [DTPA-D-Phe$^1$]octreotide labelled with $^{161}$Tb is suitable for above-mentioned applications. The characteristics of $^{161}$Tb are quite ideal for intraoperative scanning: low energy gamma rays (with peaks around 46-48 and 74 keV), and a half-life of nearly seven days. As for the potential use for radiotherapy, $^{161}$Tb emits also hard beta rays. Therefore, the characteristics of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide with respect to specific binding to somatostatin (octreotide) receptors on rat brain cortex membranes, biological activity, metabolism in isolated perfused rat livers and metabolism in vivo in normal and tumour-bearing rats were determined and compared to those of [$^{111}$In-DTPA-D-Phe$^1$]octreotide (4-6).

It appeared from our experiments that, like [$^{111}$In-DTPA-D-Phe$^1$]octreotide, [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide was mostly cleared from the body via the kidneys. We found that in humans a significant amount of [$^{111}$In-DTPA-D-Phe$^1$]octreotide accumulates in the renal parenchyma (about 7% dose, 4 h after injections), because of re-uptake by the tubular cells after glomerular filtration (7). This renal retention reduces both the scintigraphic sensitivity for detection of small tumours in the abdomen and the potential use of the radiolabelled peptide for radiotherapy (8). Therefore, we recently performed studies to determine whether renal uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide could be reduced by inhibiting tubular re-uptake in vivo in the rat (6). In the present study, we tested the effects of increased urinary flow, and administration of lysine (400 mg/kg) or of sodium maleate (400 mg/kg) on tubular reabsorption of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide in vivo in rats.

We also investigated the influence of the labelling efficiency of [DTPA-D-Phe$^1$]octreotide with $^{161}$Tb as well as of rat body weight (age) on organ distribution in rats.

## MATERIALS AND METHODS

*Radiolabelling and Quality Control of the Radiopharmaceutical*

[DTPA-D-Phe$^1$]octreotide and $^{161}TbCl_3$ were obtained from Mallinckrodt Medical B.V. (Petten, The Netherlands). The radiolabelling was performed essentially as described for [$^{111}$In-DTPA-D-Phe$^1$]octreotide (4); except that 20 $\mu$g [DTPA-D-Phe$^1$]octreotide instead of 10 $\mu$g and acetate instead of citrate buffer were used. Only when labelling efficiency was $\geq$ 95%, the radiolabelled product was used for experiments, unless otherwise stated. The $^{161}$Tb was produced at the Free University in Brussels, Belgium, from irradiated $^{160}$Gd.

Highly purified and fatty acid free Bovine Serum Albumin (BSA; Boseral) was a product of Organon Teknika (Oss, The Netherlands). All other reagents were of the highest purity commercially available.

*In Vitro Receptor Binding Studies*

Receptor binding assays were carried out using [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide as described previously for [$^{111}$In-DTPA-D-Phe$^1$]octreotide (4) using rat brain cortex membranes. Binding curves and $IC_{50}$ for displacement of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide by unlabelled [DTPA-D-Phe$^1$]octreotide or [$^{159}$Tb-DTPA-D-Phe$^1$]octreotide were calculated using the computer fitting program of Graphpad (ISI software, Philadelphia, Pa., USA).

*Biological Activity*

The biological activity of [$^{159}$Tb-DTPA-D-Phe$^1$]octreotide was assessed by measuring its potency to inhibit the secretion of rat growth hormone (rGH) from rat pituitary cells as described previously (4).

*Isolated Perfused Rat Liver Studies*

Livers of male Wistar rats (200-250 g body weight) were isolated and perfused in a recirculating system at 37 °C as described previously (9). The magnetically stirred perfusion medium used in all experiments was 150 mL Krebs-Ringer buffer (118 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, and 25 mM $NaHCO_3$) supplemented with 10 mM glucose and 1 % BSA. The pH of the medium was maintained at 7.43 by gassing with carbogen (95 % $CO_2$ and 5 % $O_2$, 400 mL/min). The function of the liver was monitored by its outer appearance, measurement of hydrostatic pressure necessary to maintain a perfusion medium flow of 40 mL/min, bile flow, and pH of the perfusion medium. Livers were preperfused for 30 min. The experiment was started by addition of 370 kBq of tracer to the stirred medium in the central reservoir. Subsequently, 0.5 mL medium samples were taken at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50 and 60 min from a smaller medium reservoir, the height of which determines the hydrostatic pressure. Bile samples were collected during 10 min intervals. The samples were stored at - 20 °C until analysis. The chemical status of the radionuclide in medium and bile samples was analysed as a function of time using ITLC in 1 M acetic acid (pH 5).

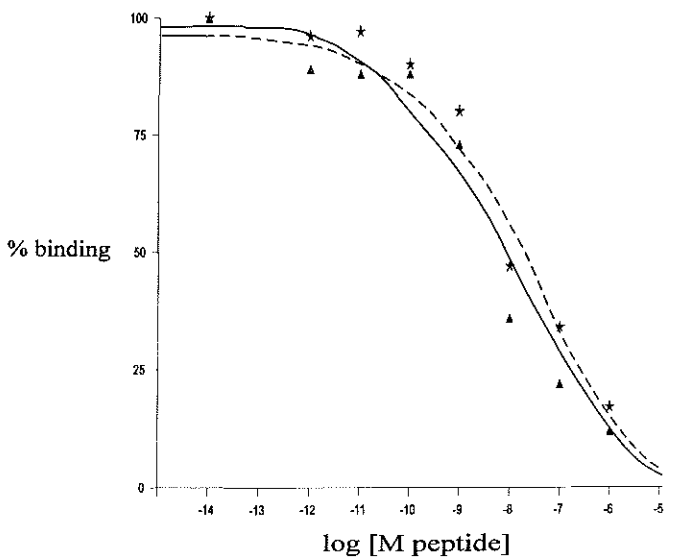


**Figure 1A.** *Binding of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide to rat brain cortex membranes in the presence of increasing concentrations of DTPA-octreotide (▲) or [$^{159}$Tb-DTPA-D-Phe$^1$]octreotide (★), expressed as the percentage of binding in the absence of competing compound*

*Tissue Distribution and Specific Binding of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide in Normal and Tumour-bearing Rats*

Normal male Wistar or Lewis rats or tumour-bearing male Lewis rats (200-250 g, unless otherwise stated) were placed in metabolic cages 24 h prior to the experiment. Tumour rats carried the transplantable rat pancreatic tumour CA20-948, which has previously been shown to possess somatostatin receptors (10), in both upper hind legs. Metabolic cages were used to collect all (radioactive) urine produced during the experiment to follow the urinary clearance of the radioactive compound. The rats were fed with normal rat chow (Hope Farms, Woerden, The Netherlands) or with 35 g/day of rat chow suspended in water (40 gram pellets in 100 mL water). Drinking water was always available ad lib. At t = 0h, rats were injected with 0.2 MBq (0.5 $\mu$g) [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide into the dorsal vein of the penis (injected volume 200 $\mu$L) during ether anaesthesia. The radioactivity was measured in a dose calibrator (VDC-202, Veenstra, Joure, The Netherlands).

In order to determine non-specific binding of the radiopharmaceutical, rats were injected subcutaneously with 0.5 mg octreotide in 1 mL 0.05 M acetic acid in 154 mmol/L NaCl, 40 min before injection of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide. Twenty h after injection of the radiolabelled product, rats were sacrificed and organs were isolated from the rats and subsequently analyzed. The tissue distribution of the $^{161}$Tb-labelled somatostatin analogue was studied by measurement of radioactivity in isolated organs as well as of blood samples, using a LKB-1282-Compu-gammasystem.

*Possible Renal Re-uptake Blockers Tested*

Lysine (400 mg/kg) and sodium maleate (400 mg/kg) were administered intravenously to the rats at physiological pH in volumes of 200 $\mu$L immediately before [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide injection.

*Statistical Analysis*

Results are expressed as mean $\pm$ SD of groups of at least 3 rats. Statistical evaluation was performed using one way analysis of variance followed by comparison among class means and Student's t-test, corrected for multiple pairwise comparisons between means.

## RESULTS

*In Vitro Receptor Binding Studies*

Rat brain cortex membranes were used to study the binding of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide to octreotide receptors in the presence of increasing amounts of [$^{159}$Tb-DTPA-D-Phe$^{1}$]octreotide or unlabelled [DTPA-D-Phe$^{1}$]octreotide (Figure 1A). The binding of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide decreased in the presence of increasing concentrations of both compounds, indicating that the binding process

was saturable and specific. It is also shown that [DTPA-D-Phe$^1$]octreotide competes somewhat better than [$^{159}$Tb-DTPA-D-Phe$^1$]octreotide. The $IC_{50}$ values appeared to be in the nanomolar range, similar to that found for [$^{111}$In-DTPA-D-Phe$^1$]octreotide (4).

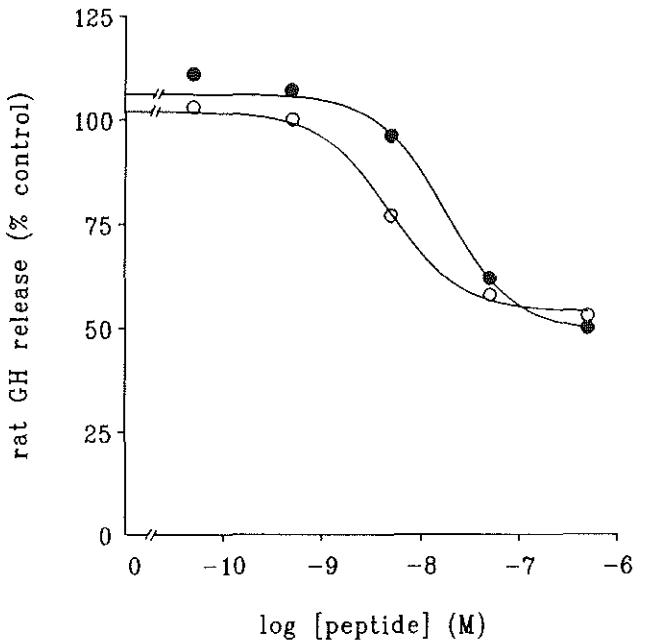


**Figure 1B**. *Effects of [DTPA-D-Phe$^1$]octreotide (o) and of [$^{159}$Tb-DTPA-D-Phe$^1$]-octreotide (●) on secretion of rGH from cultured rat pituitary cells*

*Biological Activity*

Figure 1B shows the effects of [DTPA-D-Phe$^1$]octreotide and of [$^{159}$Tb-DTPA-D-Phe$^1$]octreotide on the secretion of rGH by cultured rat pituitary cells. The radiolabelled somatostatin analogue significantly inhibited rGH secretion in a dose-dependent manner, similar to [$^{111}$In-DTPA-D-Phe$^1$]octreotide (4).

*Isolated Perfused Rat Liver Studies*

In Table 1 is shown that after 1 h perfusion of isolated rat livers with [$^{111}$In-DTPA-D-Phe$^1$]octreotide, 2.2 % of the dose was excreted in the bile, 1.7 % was present in the liver, whereas 95 % was still present in the perfusion medium. The data obtained with [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide show even less liver clearance: after 60 min of perfusion, only 0.09 % was excreted in the bile, and 1.4 % was present in the liver.

**Table 1.** *Peptide-bound radioactivity (PBR) and non-peptide-bound radioactivity (NPBR), expressed as % dose, in perfusion medium and bile after 60 min of perfusion with [$^{111}$In-DTPA-D-Phe$^1$]octreotide (In-oc) or [$^{161}$Tb-DTPA-D-Phe$^1$]-octretide (Tb-oc). TR = total radioactivity in % dose*

| | PBR (medium) | NPBR (medium) | PBR (bile) | NPBR (bile) | TR (liver) |
|---|---|---|---|---|---|
| In-oc | 94.9 ± 0.8 | 0.9 ± 0.1 | 2.2 ± 0.2 | 0.2 ± 0.0 | 1.7 ± 0.2 |
| Tb-oc | 99.6± 1.2* | <0.1* | 0.09 ± 0.0* | <0.1* | 1.4 ± 0.1 |

* *P<0.001 versus [$^{111}$In-DTPA-D-Phe$^1$]octreotide*

**Table 2A.** *Distribution of 0.2 MBq (0.5 μg) [$^{111}$In-DTPA-D-Phe$^1$]octreotide and [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide in several organs of control rats, expressed as % injected dose per g tissue, 20 h after administration*

| Organ | [$^{111}$In-DTPA-D-Phe$^1$]-octreotide | [$^{161}$Tb-DTPA-D-Phe$^1$]-octreotide | |
|---|---|---|---|
| blood | 0.002 ± 0.0003 | 0.0004 ± 0.0001 | * |
| kidneys | 1.52 ± 0.15 | 1.51 ± 0.11 | |
| liver | 0.061 ± 0.012 | 0.13 ± 0.01 | * |
| pancreas | 0.52 ± 0.12 | 0.23 ± 0.03 | * |
| spleen | 0.03 ± 0.005 | 0.02 ± 0.001 | * |
| adrenals | 0.76 ± 0.10 | 0.184 ± 0.02 | * |

* *P<0.001 versus [$^{111}$In-DTPA-D-Phe$^1$]octreotide*

*Tissue Distribution and Specific Binding of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide in Normal and Tumour-bearing Rats in Vivo*

In Table 2 organ distribution of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide and [$^{111}$In-DTPA-D-Phe$^1$]octreotide in normal rats are shown, expressed as % of the injected dose (ID), 20 h after administration. It was found that there were no significant differences in Lewis or Wistar rats with regard to distribution of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide (not shown). [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide is cleared even faster from the blood than [$^{111}$In-DTPA-D-Phe$^1$]octreotide: 20 h after injection only 0.0004 % ID was found in the blood ($P<0.001$ versus [$^{111}$In-DTPA-D-Phe$^1$]octreotide). Kidney uptake of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide and [$^{111}$In-DTPA-D-Phe$^1$]octreotide was similarly high (1.5 % ID). Liver uptake of [$^{161}$Tb-DTPA-D-

Phe$^1$]octreotide was higher than that of [$^{111}$In-DTPA-D-Phe$^1$]octreotide ($P<0.001$), whereas the reverse was true for spleen, pancreas, and adrenals ($P<0.001$), the latter two organs possessing octreotide receptors. In Table 2B is shown that uptake of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide and [$^{111}$In-DTPA-D-Phe$^1$]octreotide in pancreas and adrenals represents mostly specific binding to the octreotide receptors, as uptake in these organs was decreased almost completely ($P<0.001$) by pretreatment of the rats with 0.5 mg unlabelled octreotide.

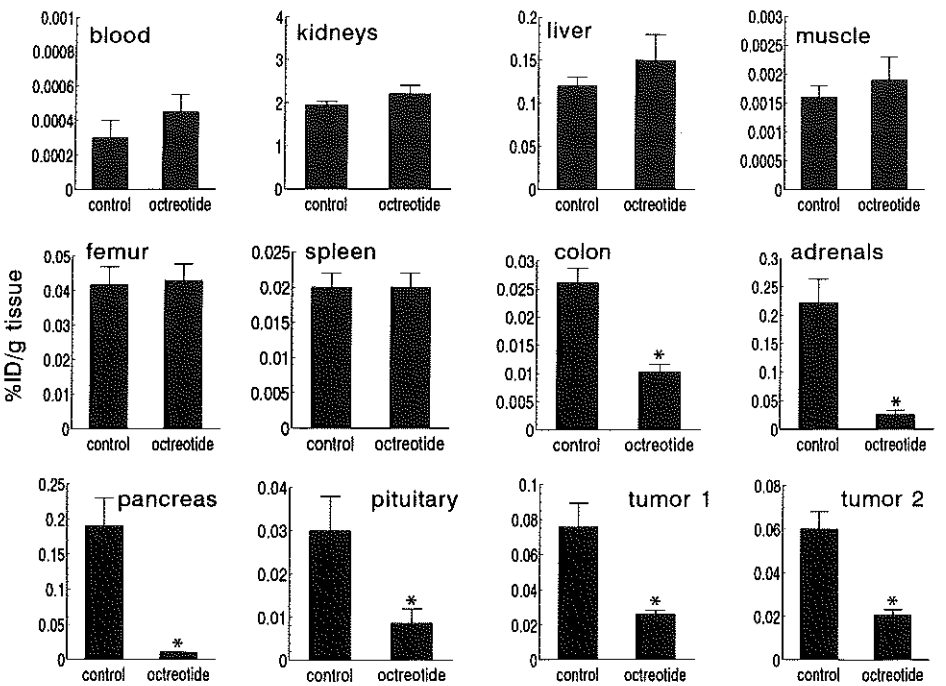


**Figure 2.** *Organ distribution of radioactivity [with (octreotide) or without (control) 40 min pretreatment with 0.5 mg unlabelled octreotide] in tumour-bearing rats, 20 h after administration of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide*

In Figure 2 organ distribution of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide, with or without pretreatment with unlabelled octreotide, is depicted in tumour-bearing rats 20 h after administration of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide. The distribution in the tumour-bearing rats is the same as that seen in normal rats (Table 2A). Uptake in

colon, adrenals, pancreas, pituitary, and tumours appeared to represent mostly specific binding to octreotide receptors, as a major decrease was observed after pretreatment with unlabelled octreotide ($P<0.001$).

**Table 2B**. *Effect of pretreatment (40 min) with 0.5 mg unlabelled octreotide on distribution of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide and [$^{111}$In-DTPA-D-Phe$^1$]octreotide in pancreas and adrenals (organs containing somatostatin receptors) of normal rats, expressed as % of control (= same tracer without pretreatment with unlabelled octreotide, see Table 1A)*

| Organ | [$^{111}$In-DTPA-D-Phe$^1$]-octreotide | | [$^{161}$Tb-DTPA-D-Phe$^1$]-octreotide | |
|---|---|---|---|---|
| pancreas | 1.92 ± 0.20 % | * | 4.34 ± 0.18 % | * |
| adrenals | 1.63 ± 0.22 % | * | 4.52 ± 0.84 % | * |

* *$P<0.001$ versus control*

*Influence of Renal Re-uptake Blockers*

In Figure 3 organ distribution of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide, 20 h after administration, is shown in the presence or absence of lysine (400 mg/kg) or sodium maleate (400 mg/kg), both potent blockers of the renal reabsorption process of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in the proximal tubuli (6). Also for [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide kidney uptake is inhibited significantly by both lysine and sodium maleate, the latter being about twice as potent as the former, showing that also [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide is reabsorbed into the proximal tubular cells of the kidney after glomerular filtration. After administration of lysine, uptake of label in all other organs is not different from the control situation, whereas after sodium maleate administration uptake of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide is significantly increased compared to control ($P<0.001$).

In Figure 4, the influence of urinary flow on tubular re-absorption of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide is depicted. It is shown that rats that eat dry rat chow (points on the left in the Figure) produce less urine (range 5.1-7.5 mL/20h) than rats that eat food suspended in water (points on the right in the Figure; range 12.8-23.1 mL/20 h). However, this increase in urine production on the day of the experiment did not influence the amount of radioactivity in the kidneys. This was also true when the increase in urine production was induced by change in diet already 48 h before the start of the experiment (not shown).

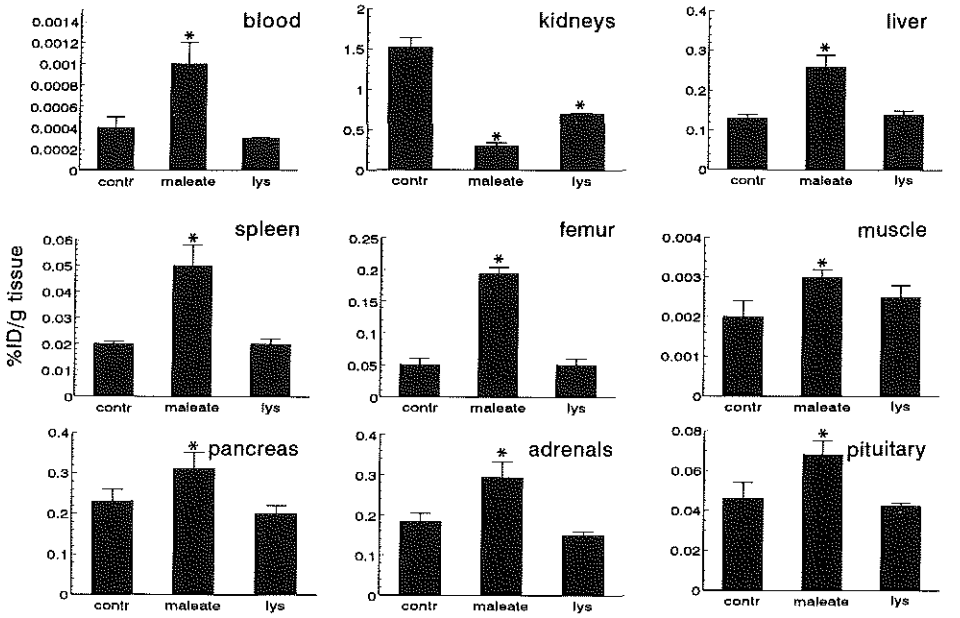


**Figure 3.** *Influence of sodium maleate (400 mg/kg) or lysine (lys; 400 mg/kg) on organ distribution of radioactivity in control rats, 20 h after administration of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide. * $P<0.001$ versus control*

*Influence of Labelling Efficiency on Organ Distribution of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide*

In Figure 5 is shown that a maximal labelling efficiency is very important to reach an optimal organ distribution of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide. 100 % labelling efficiency means that all radioactivity administered to the rats was in the form of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide, whereas 0 % means that it consisted of $^{161}TbCl_3$, in the absence of [DTPA-D-Phe$^1$]octreotide. For organs not possessing octreotide receptors, such as liver, spleen, femur, and muscle the uptake increased significantly with decreasing labelling efficiency ($P<0.001$ versus 100 % labelling). In the kidneys, uptake is about the same for all situations, showing that the Tb not bound to octreotide is taken up with about the same efficiency as [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide. However, for octreotide receptor-possessing organs, such

as pancreas, adrenals and pituitary, it holds that a decrease in labelling efficiency resulted in a significant decrease of uptake of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide in these organs.

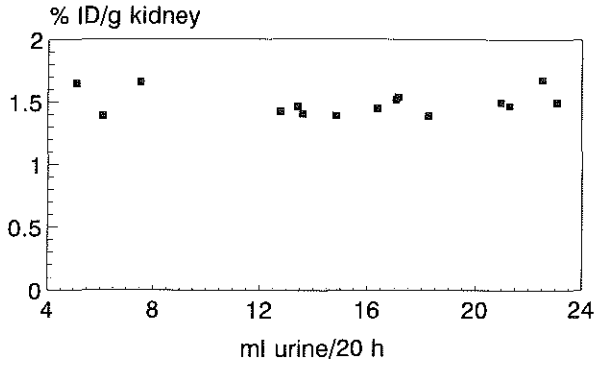


**Figure 4.**
*Influence of urinary flow during the experiment on kidney uptake of radioactivity in control rats, 20 h after administration of [$^{161}$Tb-DTPA-D-Phe$^1$]-octreotide*

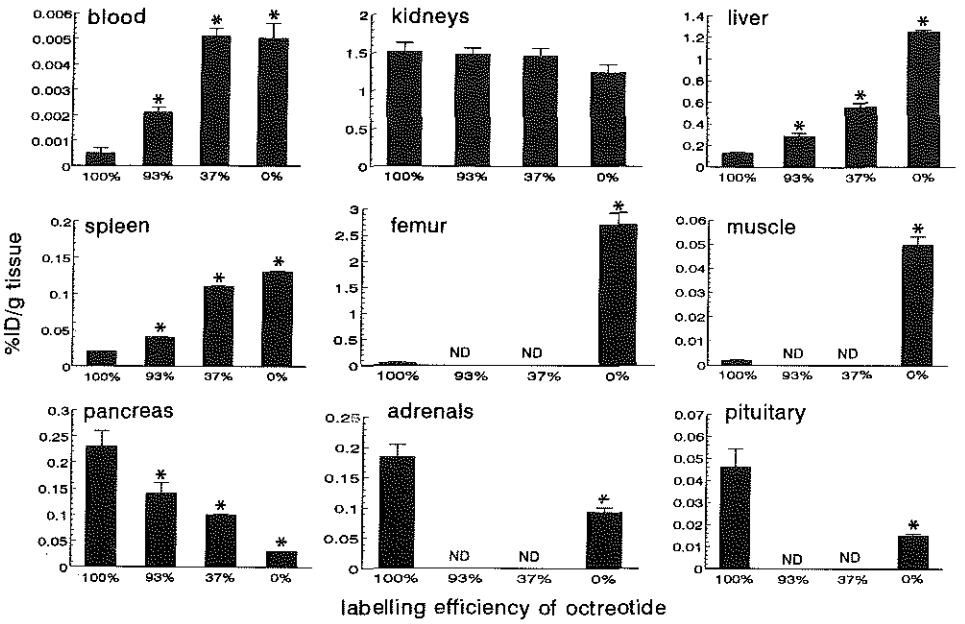


**Figure 5.** *Influence of labelling efficiency of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide on organ distribution in control rats, 20 h after administration of radiopharmaceutical; * $P<0.001$ versus control; ND=not determined*

*Influence of Body Weight on Organ Distribution of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide*

In Figure 6 the influence of body weight on organ distribution of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide is shown, 20 h after administration. Three groups of rats with body weights of 360, 200 and 120 g were tested. It is shown that no significant differences were found in uptake of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide, expressed as % ID/g tissue, between the 3 groups with regard to liver, kidneys and spleen, organs that do not possess octreotide receptors. However, in pancreas and adrenals a significantly lower uptake was found in the 360 g group compared to the control group (= 200 g body weight).

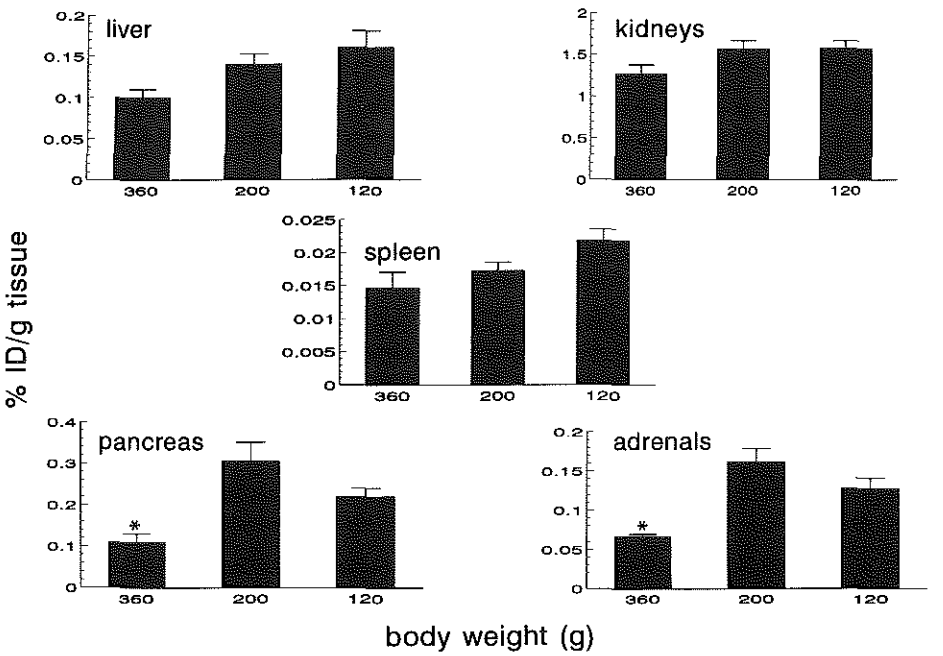


**Figure 6.** *Influence of rat body weight on organ distribution of radioactivity in control rats, 20 h after administration of [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide;*
* *P<0.001 versus control (= 200 g-group)*

## DISCUSSION

The results of the binding studies demonstrate that [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide is a high affinity radioligand for somatostatin receptors, with an affinity comparable to that of [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide (4). This was also demonstrated in the in vivo experiments in control and tumour-bearing rats after injection of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide, in which uptake and specific binding in somatostatin receptor-positive tissues and tumour were found. Somatostatin receptors are structurally related integral membrane glycoproteins. Recently, five different human somatostatin receptor types have been cloned. All subtypes bind native somatostatin-14 ($SS_{14}$) and $SS_{28}$ (pro-somatostatin with 28 aminoacids) with high affinity, while their affinity for numerous somatostatin analogues differ considerably (11-14). Octreotide binds with high affinity to the SSTR2 (somatostatin receptor type 2) subtype, while this analogue has a relatively low affinity for SSTR3 and SSTR5 and shows no binding to SSTR subtypes 1 and 4 (11-14). Octreotide scintigraphy is therefore based on the visualization of octreotide-binding somatostatin receptors (octreotide receptors), most probably the SSTR2.

Rat growth hormone secretion inhibition experiments showed that [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide has a similar potency as [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide (4).

The perfused rat liver is very suitable to investigate several parameters of liver metabolism, such as the disappearance from the perfusion medium, appearance of degradation products and biliary excretion. [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide is taken up even less by the isolated perfused rat liver than [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide as almost no tracer disappears from the perfusion medium. Furthermore, hardly any radioactivity is found in the liver, and excretion into the bile is negligible. In order to find a tumour by intraoperative scanning in vivo, the specific activity expressed in counts per unit of area must exceed the local background radiation. As [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide is not cleared via the liver and thus causes no accumulation of radioactivity in biliary and digestive tract, this radiopharmaceutical would thus be suitable for scanning of tumour receptor accumulation in the upper abdominal region, where many of the small endocrine gastro-entero-pancreatic target tumours are located.

However, the in vivo liver uptake is not in agreement with the liver perfusion studies; in vivo we find a higher liver uptake of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide than of [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide 20 h after administration. At the moment, the reason for this discrepancy between in vivo and in vitro findings is not clear; it is possible that [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide after in vivo administration is less stable than [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide, and that degradation products of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide cause the higher liver uptake as found in vivo, in agreement with our findings that liver uptake of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide increased when the labelling efficiency was suboptimal (Figure 5).

Our findings in Table 2A show that for octreotide receptor-positive organs, such as pancreas and adrenals, uptake of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide is lower than that of [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide. However, as the clearance from the blood of the former compound is faster than that of the latter, tissue/blood ratio is higher in the case of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide than with [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide. In the case of tumours this is a favourable situation for intraoperative scanning.

As for the high uptake in the kidneys: small peptides in the blood plasma are filtered through the glomerular capillaries in the kidneys and subsequently reabsorbed almost completely ($\geq$ 90 %) by the proximal tubular cells by carrier-mediated endocytosis. This is also the case for the radiolabelled octapeptides [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide and [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide. After the subsequent degradation process that takes place in the lysosomes of the tubular cells, their labelled degradation products are "trapped" in the lysozomes (15), causing a high dose of radioactivity in the kidneys. This study in vivo in rats demonstrates that the uptake of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide by the renal tubular cells after glomerular filtration can be reduced by administration of lysine or sodium maleate, in favour of the potential use of the radiolabelled peptide for radiotherapy. As for lysine, the membranes of renal tubular cells contain negatively charged sites, to which positively charged residues of peptides or proteins are thought to bind (16). An inhibition of this binding process may explain the effects by administration of the positively charged amino acid lysine on [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide re-uptake (6,17,18).

The most pronounced effect on tubular re-uptake of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide in this study was exerted by sodium maleate (Figure 3). Sodium maleate has been used to create and study renal tubular dysfunction comparable to the human Fanconi's syndrome (19,20), resulting in aminoaciduria and proteinuria. This compound forms maleyl-CoA by reacting with succinyl-CoA, thereby reducing the cellular CoA supply and inhibiting the citric acid cycle in tubular cells (21). The resulting reduced ATP supply or the reaction of the maleyl-CoA with membrane proteins may inhibit a variety of renal transport systems, including peptide re-absorption (21). The inhibitory effect of sodium maleate, as found in this study, thus indicates that the re-absorption process of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide into the renal tubular cells is energy dependent. The increase of the uptake of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide in all organs by sodium maleate (except for the kidneys), as shown in Figure 3, may be explained by the inhibitory effect of this compound on the renal glomerular filtration rate (22).

Increase in urine production before and during the experiment had no effect on the kidney uptake of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide (Figure 4). The increase in urine production was induced by feeding the rats with chow suspended in water. However, re-uptake of peptides in the renal tubules is not influenced by an

increased urinary flow through the kidneys. The results found here for [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide are in agreement with those for [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide (6).

Figure 5 shows that a maximal labelling efficiency of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide is essential, as with decreasing efficiency the uptake in the octreotide receptor-positive organs decreases, whereas non-specific uptake in the other organs is increased. Even when labelling efficiency was 93 %, the effects on organ distribution were significantly different ($P<0.001$) compared with 100 % labelling efficiency. This decreased specific binding in combination with increased non-specific binding is of course very unfavourable for both intraoperative scanning and radiotherapy.

At last, the effect of rat body weight on [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide organ distribution is shown (Figure 6). As rats gain weight during their whole life, increase in body weight is paralleled by an increase in age and development. Since the same dose of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide was administered to the different groups [with widely different distribution volumes], it is not surprising that uptake in all organs studied is highest in the low-body weight rats, while uptake is lowest is the high-body weight animals. However, for adrenals and pancreas, both octreotide receptor-positive organs, the decrease in uptake in the 360 g group was much more pronounced than in the octreotide receptor-negative organs. This finding may point to developmental changes in expression of the octreotide receptor in these organs, as was found for other somatostatin receptor-positive organs, such as the rat brain and the rat visual system (23,24).

In conclusion: based on the characteristics of $^{161}$Tb (low energy gamma rays with a half-life of nearly seven days, and hard beta rays) combined with the in vitro binding studies, biological activity, and in vivo organ distribution of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide, the latter is a promising radiopharmaceutical for both intraoperative scanning and radiotherapy, the latter in particular after inhibition of the renal tubular reabsorption process, to decrease the kidney dose during application. Further studies in patients need to be performed now to see if [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide can indeed open new therapeutic applications for patients bearing octreotide receptor-positive tumours.

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Submitted

# OPTIONS TO PREPARE RADIOLABELLED PEPTIDES FOR RADIONUCLIDE THERAPY OF TUMOURS EXPRESSING PEPTIDE RECEPTORS

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## ABSTRACT

Radiolabelled peptides are useful for scintigraphy of tumours containing receptors for these compounds. Beta-emitting radionuclides coupled to peptides may be used in radiotherapeutical treatment of such tumours. $^{131}I$ is suggested to be suitable for that purpose. This study deals with (a) the possibilities of radioiodination of peptides using two somatostatin analogues, [Tyr$^3$]octreotide and [DTPA-D-Phe$^1$]RC-160, (b) the required separations after radioiodination, and (c) the influence of high beta fluxes from $^{131}I$ on a peptide during radioiodination and purification steps.

Based on regular therapeutic doses of $^{131}I$ in cancer treatment and previous experience with [$^{111}$In-DTPA-D-Phe$^1$]octreotide, a minimal effective therapeutic dose of 3.7 GBq (38 nmol) $^{131}I$ must be coupled to a maximum of $\approx$ 67 nmol peptide ($\approx$ 100 $\mu$g [DTPA-D-Phe$^1$]octreotide), representing a slight excess of peptide over $^{131}I$.

After radioiodination at low and high molar peptide:iodide ratios the wanted radioiodinated peptide must be isolated from unwanted di-iodinated peptide and unreacted peptide, respectively, as well as from unreacted iodide. Radiolysis by high beta fluxes during labelling and separation steps was simulated by irradiating 30 $\mu$g [DTPA-D-Phe$^1$]octreotide (Fig. 1) by 370 MBq $^{131}I$ in a small volume at about 30 % of the beta flux in the proposed real therapeutic dose. During one h the radiation absorbed dose amounted to 130 Gy. Irradiations were performed from 30 min till 24 h. Afterwards, the composition of the incubation mixture was investigated by HPLC. In the irradiation experiments [DTPA-D-Phe$^1$]octreotide was degraded rapidly (with a half-life of one h) under these conditions. Therefore, during separation steps the peptide will be degraded even faster under the conditions for preparation of a real therapeutic dose (3.7 GBq coupled to 67 nmol peptide).

In conclusion, intact mono-iodinated $^{131}I$-labelled peptides will be very hard to obtain in radiotherapeutic amounts. Direct labelling of the optimal amount of

peptide containing a chelating group such as DTPA with a pure short-lived beta-emitting radiometal (e.g. $^{90}Y$) will be more convenient. Such one-step labelling can be performed in the presence of a suitable radiation quencher, without need of further radiochemical separations.

**[Tyr$^3$]octreotide**

D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

**[$^{111}$In-DTPA-D-Phe$^1$]RC-160**

$^{111}$In-DTPA-D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Trp-$NH_2$

**[$^{111}$In-DTPA-D-Phe$^1$]octreotide**

$^{111}$In-DTPA-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)

**Figure 1**. *Structural formulae of [Tyr$^3$]octreotide, [DTPA-D-Phe$^1$]RC-160, and [DTPA-D-Phe$^1$]octreotide*

## INTRODUCTION

Recently, growing attention has been paid to scintigraphic detection and visualization of various processes containing receptors for peptide hormones by the application of radiolabelled analogues of these hormones. Besides [$^{123}$I-Tyr$^3$]-octretide, nowadays [$^{111}$In-DTPA-D-Phe$^1$]octreotide (known as Octreoscan 111®) is extensively used for scintigraphy of somatostatin receptor-positive tumours (1-3). Furthermore, an increasing number of publications report on application of other radioactive peptides for scintigraphic demonstration of various processes (4-9). It is tempting to presume that such peptides, in case of high tumour accumulation, may also be used for radionuclide therapy, such as $^{131}$I-iodide in the treatment of thyroid cancer and $^{131}$I-MIBG for therapy of neuroblastoma and phaeochromocytoma.

Obviously, $^{131}$I has been proposed for labelling somatostatin analogues for radionuclide therapy of somatostatin receptor-positive tumours (10, 11). Assuming similar tumour accumulations and residence times, radionuclide receptor therapy with $^{131}$I-labelled peptides will require similar doses as being used in $^{131}$I-iodide (12) and $^{131}$I-MIBG therapy (13). Furthermore, usually only a limited amount of a bioactive peptide hormone can be administered without side-effects, which may become a limiting factor in the coupling of a therapeutic amount of radionuclide to such a peptide. Based on our experience with [$^{111}$In-DTPA-D-Phe$^1$]octreotide (14,

15), it was estimated that an effective therapeutic dose of ≈ 3.7 GBq (38 nmol) $^{131}$I had to be coupled to ≈ 100 μg of a ≈ 1.5 kD somatostatin analogue (67 nmol), i.e. nearly a 2-fold excess of peptide over radionuclide. Comparing the molar compound:radionuclide ratios of several regular therapeutic radiopharmaceuticals, it is remarkable that the compound is always present in a large excess over radionuclide (Table 1), even in radioiodinated monoclonal antibodies, taking into account that in each antibody molecule many potential iodine-accepting tyrosine residues are present. Compared with peptide receptor radionuclide therapy of endocrine tumours, other kinds of radionuclide therapy are mostly based on different accumulation mechanisms which are less limited by the mass of the radiopharmaceutical administered. As described before (16), radioiodination of [Tyr$^3$]octreotide using a small excess of peptide over radionuclide resulted in a considerable amount of di-iodinated [Tyr$^3$]octreotide, which is unfavourable as this compound does not bind well to the somatostatin receptor (17). Because of the expected formation of di-iodinated compounds in "therapeutic" reaction mixtures of tyrosine-containing peptides, radiolabelling of two somatostatin analogues, [Tyr$^3$]octreotide and [DTPA-D-Phe$^1$]RC-160 (Fig. 1) at different molar peptide:nuclide ratios was investigated by HPLC. $^{125}$I was used as a model for $^{131}$I, because of its well-characterized specific radioactivity. In contrast to $^{131}$I, the specific radioactivity of $^{125}$I reaches the theoretical value (Table 2) due to its complete different production process, which means that no other iodine isotopes compete with the labelling.

During the necessary HPLC and/or SEP-PAK purification steps after labelling on a therapeutic scale with $^{131}$I, high beta fluxes within small volumes are originated. Therefore, radiolytic effects under such circumstances were simulated by exposing small amounts of peptide in a small volume to $^{131}$I-beta radiation during various time intervals. Afterwards, the reaction mixtures were investigated by HPLC.

## MATERIALS AND METHODS

### *Peptides*

[Tyr$^3$]octreotide, [DTPA-D-Phe$^1$]octreotide and [DTPA-D-Phe$^1$]RC-160 were obtained from Sandoz (Basle, Switzerland), Mallinckrodt Medical (Petten, The Netherlands) and Sanbio (Uden, The Netherlands) respectively.

### *Quality Control of Unlabelled Peptides*

The original peptides were analyzed by reversed phase, high performance liquid chromatography (HPLC) with a Waters 600 E multisolvent delivery system connected to a μBondapak $C_{18}$ reversed-phase column (300 x 3.9 mm, particle size 10 μm) with UV detection (254 nm). Elution was carried out at a flow of 1 mL/min with a linear gradient of 40 % to 80 % (v/v) methanol in 50 mM Na-acetate buffer (pH 5.5) in 20 min and the latter composition was maintained another 5 min.

*Radioiodination of Peptides and Quality Control of Radioiodinated Peptides*

Both peptides were radioiodinated with $^{125}I$ as described before (16). The peptides were radioiodinated under different molar peptide:radionuclide ratios. [Tyr$^3$]octreotide (4.3 $\mu$g to 105 $\mu$g) was labelled with $^{125}I$ (92.5 to 370 MBq) corresponding to molar peptide:radionuclide ratios from 1.7 to 43. [DTPA-D-Phe$^1$]RC-160 (1.3 $\mu$g to 125 $\mu$g) was labelled with $^{125}I$ (37 to 370 MBq) at molar peptide:radionuclide ratios from 1.7 to 31.

After radiolabelling, the peptide components in the reaction mixture were isolated on a SEP-PAK $C_{18}$ reversed-phase extraction cartridge as described before (16), and analyzed with the HLPC-system described above. Eluted radioactivity was monitored on-line using a NaI probe connected to a Canberra single channel analyzer with a recorder. Collected fractions were measured by routine scintillation ounting. If the elution pattern of the radiolabelled peptide consisted of more than one peak, isolated peaks were concentrated and reinjected into the HPLC-system.

**Table 1**. *Molar ligand:radionuclide ratios in therapeutic radiopharmaceuticals*

| treatment of | radionuclide [radioactivity and mass] | compound [mass] | excess of compound over radionuclide |
|---|---|---|---|
| neuroblas-toma | $^{131}I$<br>3700 MBq<br>3.8 x $10^{-8}$ mol | MIBG<br>3.3 mg<br>8 x $10^{-6}$ mol | 210 |
| various malignancies | $^{131}I$<br>3700 MBq<br>3.8 x $10^{-8}$ mol | MoAb<br>20 mg<br>1.3 x $10^{-7}$ mol | 3.4 |
| | | MoAb-fragment<br>20 mg<br>4.0 x $10^{-7}$ mol | 11 |
| | $^{90}Y$<br>3700 MBq<br>2.1 x $10^{-9}$ mol | MoAb<br>20 mg<br>1.3 x $10^{-7}$ mol | 60 |
| | | MoAb-fragment<br>20 mg<br>4.0 x $10^{-7}$ mol | 190 |

| | | | |
|---|---|---|---|
| endocrine tumours | $^{111}$In<br>3700 MBq<br>2.2 x $10^{-9}$ mol | DTPA-octreotide<br>100 $\mu$g<br>6.7 x $10^{-8}$ mol | 30 |
| | $^{131}$I<br>3700 MBq<br>3.8 x $10^{-8}$ mol | peptide<br>100 $\mu$g<br>6.7 x $10^{-8}$ mol | 1.75 |

*Simulation of Radiolysis of Peptides during Purification Steps*

To simulate the consequences of the effect of high beta fluxes emanating from therapeutic amounts of $^{131}$I on microgram amounts of peptide during the mandatory purification steps, a well defined amount (370 MBq) of $^{131}$I was incubated for 30 min to 24 h at room temperature with 30 $\mu$g [DTPA-D-Phe$^1$]octreotide in a small polyethylene cup (reaction volume 317 $\mu$L). In 1 h the radiation absorbed in the reaction volume amounted to 130 Gy. Radiolysis under these circumstances was investigated by HPLC with UV detection at 254 nm.

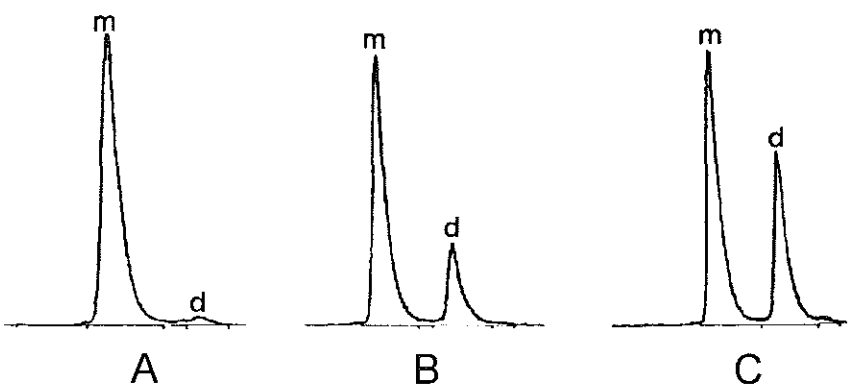


**Figure 2.** *Typical HPLC elution patterns of the reaction mixtures after radioiodination of [Tyr$^3$]octreotide at three different molar peptide:radionuclide ratios: 43 (A), 3.5 (B), and 1.7 (C), measured by on-line gamma-counting.*
*m = mono-iodinated peptide; d = di-iodinated peptide*

## RESULTS

*Peptide Composition before and after Radioiodination*

The HPLC-elution pattern (measured at UV 254 nm) of [Tyr$^3$]octreotide showed one peak (data not shown), while those of [DTPA-D-Phe$^1$]RC-160 and [DTPA-D-Phe$^1$]octreotide consisted of 3 peaks (data not shown), corresponding to data of the manufacturers.

Peptide radiolabelling yields amounted from 31 % to 93 % as measured by SEP-PAK elution. Typical examples of the radioactive peptide composition in the reaction mixtures with different molar peptide:radioiodide ratios are shown in Fig. 2 ([Tyr$^3$]octreotide) and Fig. 3 ([DTPA-D-Phe$^1$]RC-160). Radioiodination of [Tyr$^3$]octreotide yielded 96 %, 75 %, and 60 % mono-iodinated derivative at molar peptide:radionuclide ratios of respectively 41, 3.5, and 1.7. In case of [DTPA-D-Phe$^1$]RC-160 at molar peptide:radionuclide ratios of 17 and 1.7 for the corresponding mono-iodinated compound respectively 82 % and 56 % were found. Thus, at higher molar peptide:radionuclide ratios much more mono-iodinated than di-iodinated peptides are formed. In the case of [DTPA-D-Phe$^1$]RC-160) the HPLC elution profile of the mono-iodinated compound shows three peaks, like the original unlabelled compound (see above). Each of these three peaks yielded after reinjection into the HPLC again the same three original peaks, indicating that this peptide exists in three interconvertable forms (data not shown).

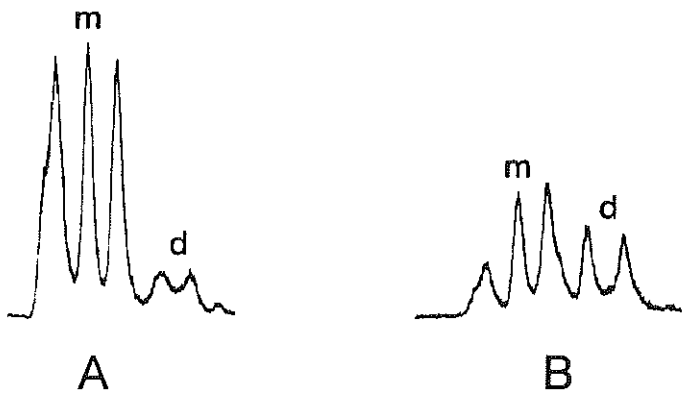


**Figure 3**. *Typical HPLC elution patterns of the reaction mixtures after radioiodination of [DTPA-D-Phe$^1$]RC-160 at two different molar peptide:radionuclide ratios: 17 (A) and 1.7 (B), measured by on-line gamma-counting.*
*m = mono-iodinated peptide; d = di-iodinated peptide*

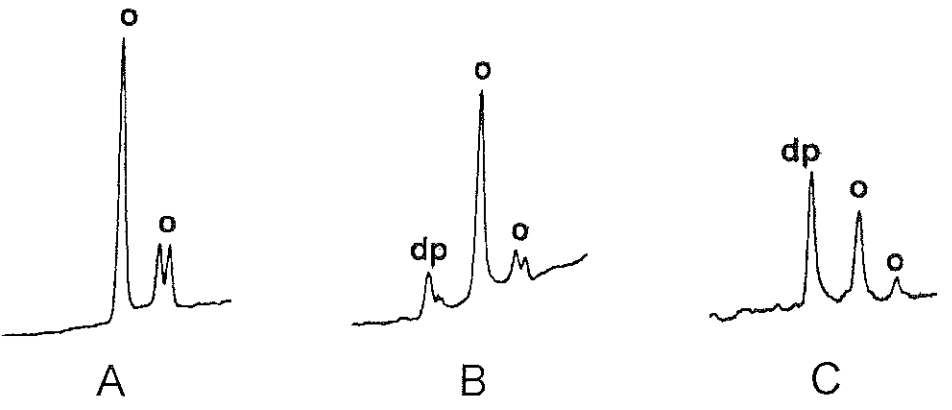


**Figure 4**. *Typical HPLC elution patterns before (A), after 30 min (B), and 4 h (C) irradiation of 30 μg [DTPA-D-Phe$^1$]octreotide by 370 MBq $^{131}I$ iodide, measured by UV-absorption at 254 nm. o = original peptide; dp = degradation products*

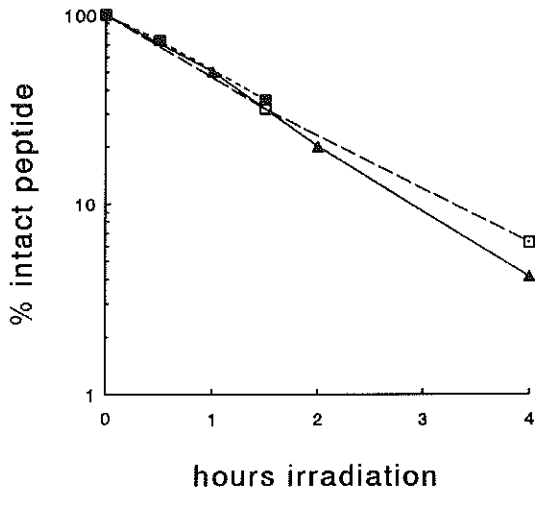


**Figure 5**. *Remaining [DTPA-D-Phe$^1$]octreotide after irradiation by $^{131}I$ as function of time expressed as percentage of the origininal amount, measured by UV-absorption at 254 nm (experiment 1 [▲], 2 [□], and 3 [■])*

*Radiolysis by High Beta-fluxes*

In Fig. 4 the degradation of [DTPA-D-Phe$^1$]octreotide by the beta radiation of $^{131}$I is shown under circumstances (370 MBq $^{131}$I and 30 $\mu$g peptide in 317 $\mu$L reaction volume), simulating those occurring during separation steps. The figure shows the HPLC elution profiles of the original compound and after 30 min and 4 h of irradiation. After irradiation new UV-peaks are formed while the peak of the original peptide deminishes. After 24 h no original peptide nor any degradation-product were detectable anymore (data not shown). The irradiation experiment shows that the original peptide is destroyed under these conditions with a half-life of about 1 h (Fig. 5).

## DISCUSSION

With the growing number of publications on applications of radiolabelled peptides for peptide receptor scintigraphy the question is raised whether such peptides may be used in radionuclide therapy of receptor-positive lesions. As these compounds are accumulated by specific, saturable receptor binding, efficient radionuclide receptor therapy of tumours requires an optimal mass of peptide labelled with a sufficient amount of a suitable radionuclide.

*Optimal Mass of Peptide*

In order to deliver the highest possible radiation dose to a tumour an optimal mass of peptide labelled to high specific activity needs to be administered. The optimal amount of peptide is dependent on various and often unknown parameters, such as saturation as well as possible homologous upregulation of somatostatin receptors at higher peptide doses (and competition with endogenous somatostatin). This is suggested by animal experiments, in which specific accumulation of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in somatostatin receptor-positive tissues was enhanced by injecting an increased amount of peptide (18). Similar observations, although not expressed in absolute accumulation estimates were made in patients who showed enhanced visualization of tumours when they were investigated with [$^{111}$In-DTPA-D-Phe$^1$]octreotide during octreotide treatment (19, 20). Besides, not only saturation, but also unwanted side-effects limit the administration of bioactive peptides. Scintigraphy after administration of [$^{111}$In-DTPA-D-Phe$^1$]octreotide with different amounts of unlabelled [DTPA-D-Phe$^1$]octreotide suggested an optimal range of that 5-120 $\mu$g (3.3 - 80 nmol) peptide (14, 15). A similar mass of radiolabelled somatostatin analogue will therefore be required for radionuclide therapy, assuming that its metabolic properties are comparable with that of [$^{111}$In-DTPA-D-Phe$^1$]octreotide. For a somatostatin analogue of $\approx$ 1.5 kD the presumed optimal therapeutic dose will amount to 67 nmol (100 $\mu$g) peptide, since the optimal target/non target ratio with the highest radionuclide uptake (% dose) is reached with this amount of [$^{111}$In-DTPA-D-Phe$^1$]octreotide during scintigraphy (14).

*Choice of a Suitable Radionuclide*

Thusfar, radionuclide therapy is generally based on the emission of medium- and high-energy beta particles. However, recently attention has also been paid to radionuclides that are commonly used for gamma camera scintigraphy. It is suggested that these "diagnostic" radionuclides may also find therapeutic applications on the basis of emission of their conversion- and Auger-electrons. Because of their low energy the range of these charged particles is very short and, thus, the radiation energy is deposited within the direct vicinity of the desintegrating radionuclide. This category includes among others $^{67}$Ga and $^{111}$In (21-24). The question is whether such a radionuclide, coupled to a peptide, is sufficiently internalized by the cell so that the emitted low-energy particles will reach the nucleus. That this is the case was recently suggested by the successful treatment of a glucaconoma patient with [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide (15). However, of all radionuclides, $^{131}$I has gained the most widespread use as therapeutic agent.

**Table 2.** *Theoretical (=lowest) and practical masses of 3.7 GBq radionuclide*

| Radionuclide | theory [nmol] | practice [nmol] |
|---|---|---|
| $^{131}$I | 6.17 | 38.2 |
| $^{90}$Y | 2.07 | 2.07 |
| $^{186}$Re | 2.91 | 133 |
| $^{32}$P | 11 | 11 |
| $^{114m}$In | 38.3 | ? |
| $^{161}$Tb | 5.29 | ? |
| $^{67}$Ga | 2.53 | 2.53 |
| $^{111}$In | 2.17 | 2.17 |
| $^{125}$I | 46.0 | 46.0 |

*Labelling Peptides with $^{131}$I*

By far the most applied form of radionuclide therapy is the treatment of hyperthyroidism, goitre and well differentiated thyroid carcinoma with $^{131}$I (iodide). In addition, $^{131}$I is successfully used in the form of $^{131}$I-mIBG in therapy of neuroblastomas and phaeochromocytomas based on its accumulation in chromaffin granules of adrenergic tumour tissue of these tumours.

Receptor-mediated radionuclide accumulation is determined by the affinity constant, the number of receptors on the tumour and the local concentration of the radioligand, which depends on the in vivo metabolism of the radiopharmaceutical. Usually, only a limited mass of a bioactive peptide can be administered to the patient.

It can therefore be calculated that radiolabelled peptides must have a much higher radionuclide content than other regular radiotherapeuticals (Table 1). Thus, for receptor-mediated radionuclide therapy the ligand should be labelled to a maximum specific activity without loss of affinity for the receptor. At the highest specific radioactivity commercially available the proposed 3.7 GBq $^{131}$I (38 nmol, see Table 2) iodide may be bound to the presumed optimal mass (100 $\mu$g = 67 nmol peptide). Incorporation of more than one iodine atom into the tyrosine of [Tyr$^3$]octreotide results in the loss of receptor binding (17). Our peptide experiments demonstrate that low excesses of peptide over radionuclide result in relatively large fractions of di-iodinated compounds, while a large excess of peptide over radionuclide causes the formation of mainly the mono-iodinated compounds (Fig. 2 & 3) with, of course, much peptide remaining unlabelled. Preparative HPLC-separations will thus always be required to separate the wanted radiopeptide from (a) the unlabelled peptide, (b) the di-iodinated peptide, and (c) unreacted radioiodide. During the necessary purification on HPLC and/or SEP-PAK columns the radiopeptide is concentrated in small volumes and, hence, is subject to radiolytic damage (Fig. 5). Therefore, in spite of its useful application in other forms of radionuclide therapy, $^{131}$I seems unsuitable for radionuclide peptide receptor therapy. Rather, direct radiolabelling (see later) in the presence of a suitable radiation quencher like gentisic acid (25), and without the need of separation steps will be preferred for preparing radiopeptides for therapy.

*Direct Radiolabelling of Chelator-conjugated Peptides with Radiometals*

A well-known method in the daily practice of nuclear medicine is the simple one-step preparation of radiopharmaceuticals that does not require additional purification steps. Peptides and proteins conjugated with chelators, like the polyaminopolycarboxylic acids EDTA, DTPA and DOTA, are suitable for such a one-step labelling procedure with radiometals. $^{111}$In for diagnostic purposes and $^{90}$Y for therapeutic purposes are frequently used to label proteins conjugated with DTPA groups. Although the binding between this radionuclide and the chelator appeared to be stable in vitro, $^{90}$Y turned out to be released from the DTPA-group in vivo, resulting in unfavourable bone accumulation of $^{90}$Y. This is especially the case when the DTPA group is introduced by reaction of DTPA cyclic anhydride with a lysine residue, as one acetic acid group is used for binding to the protein and, hence, is not available for $^{90}$Y complexation (26). In carbon backbone-linked DTPA-groups such as SCN-Bz-DTPA $^{90}$Y is far more tightly bound under physiological conditions, probably because all five carboxyl groups participate in $^{90}$Y complexation. In addition, peptides may also be derivatized with polyazamacrocycles such as the DOTA group which bind $^{90}$Y with very high affinity (27).

Other therapeutic radiometals suitable for one-step inclusion in the DTPA-group, like $^{161}$Tb, are not yet generally available.

By absence of a convenient suitable method for obtaining $^{131}$I-labelled peptides, at this moment the one-step labelling procedure using chelator-conjugated peptides to bind beta-decaying radiometals, like $^{90}$Y, $^{114m}$In and $^{161}$Tb is the method of choice, although the avalability of ultra-pure radionuclides is still limited. Such radiolabelling has namely to meet the same specific activity requirements as described above (i.e. the highest possible activity of a radionuclide coupled to $\approx$ 67 nmol peptide), which seems possible for many short-lived radionuclides in absence of their isotopes and other metallic contaminants. In Table 2 the theoretical numbers of mols corresponding with 3.7 GBq of some radionuclides are compared with the practical attainable values. This illustrates, that certainly not all short-lived radionuclides will reach the high therapeutic specific activity required for coupling a high therapeutic radionuclide dose to a relatively small amount of peptide. $^{90}$Y is a good candidate in this respect, but few if any peptides are available conjugated with a suitable chelator such as the DOTA group. Our preliminary experience shows that $^{161}$Tb binds well to [DTPA-D-Phe$^1$]octreotide (unpublished results) and may be suitable for radionuclide peptide receptor therapy. At last, the problem of high physiological accumulation of chelator-conjugated radiotherapeuticals in vital organs, such as the kidneys, remains to be solved.

## CONCLUSION

Intact mono-iodinated $^{131}$I-labelled peptides are hard to obtain in radiotherapeutic amounts. For peptide receptor radionuclide therapy direct labelling of a radiometal like $^{90}$Y or $^{161}$Tb to a chelator-conjugated peptide in the presence of a suitable radiation quencher appears far easier attainable, without the need for further separations. However, still many problems on radionuclide purity and synthesis of suitable chelator-conjugated peptides have to be solved.

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## GENERAL DISCUSSION, SUMMARY, AND CONCLUSIONS

Somatostatin receptor scintigraphy has been performed since 1987, initially with [$^{123}$I-Tyr$^3$]octreotide and since 1989 with [$^{111}$In-DTPA-D-Phe$^1$]octreotide. In general, both radiopharmaceuticals have been used at < 10 % of the maximum specific radioactivity. This, however, was thought to be unfavourable, because the established line of thought with regard to saturable processes such as receptor binding, is that sensitivity receptor scintigraphy can be optimized by

a. lowering the mass of the radiotracer or
b. by increasing the specific activity.

This is so because unlabelled ligand will compete with the labelled ligand for the same receptor. Therefore, the injection of less unlabelled peptide will result in an increase of the % dose uptake of the radioligand. Provided that the non-specific binding is unaffected, the sensitivity of this technique will then indeed be increased.

We thus hypothesized that the sensitivity of somatostatin receptor scintigraphy technique could be increased by using the lowest possible mass at the highest possible specific activity. Since the non-radioactive composition of $^{111}InCl_3$ has improved after its first use in the labelling of [DTPA-D-Phe$^1$]octreotide in 1989, the specific activity of [$^{111}$In-DTPA-D-Phe$^1$]octreotide could be increased 5-fold up to 185 MBq per $\mu$g. As described in Chapter 3, the effect of injected mass of [DTPA-D-Phe$^1$]octreotide labelled at various specific activities were investigated in rats. We did not find significant differences in uptake of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in somatostatin receptor-negative organs (background), and, to our surprise, the uptake in somatostatin receptor-positive tissues showed a tissue-specific bell-shaped function of the injected mass. So, in contrast to our hypothesis, the uptake was not optimal at the lowest possible mass, while varying specific activity at a constant injected mass did not affect the % dose uptake.
Since the findings probably extend also to somatostatin receptor-positive tumours, an extra parameter has become available to increase the sensitivity to detect these tumours. Indeed, preliminary findings in patients indicated that a specific activity higher than 220 MBq per 5 $\mu$g [DTPA-D-Phe$^1$]octreotide will lead to a decreased quality of scintigraphy, and that uptake in tumours is significantly reduced.

Furthermore, as presented in Chapter 3, several reports suggested a positive effect of the prior administration of unlabelled octreotide on the percentage dose uptake and internalization of its radioactive counterpart. We evaluated the effects of the intravenous administration of 2 or 10 $\mu$g of unlabelled octreotide or [DTPA-

D-Phe$^1$]octreotide at various time points relative to the administration of the radiopharmaceutical. In all the somatostatin receptor-positive tissues, we found a significant change in % dose uptake of radioactivity, depending on the mass, the ligand and the tissue under study. In summary, these findings indicate that the injection of variable amounts of ligand at various time points relative to the injection of [$^{111}$In-DTPA-D-Phe$^1$]octreotide may also be a means to increase the target to background ratio in somatostatin receptor scintigraphy.

In Chapter 4, the binding and internalization of [$^{125}$I-Tyr$^3$]octreotide by mouse AtT20/D16V pituitary tumour cells and by human growth hormone-producing pituitary tumour cells have been investigated in order to get insight into the metabolism of the radioligand. Internalization of the radioligand is of special importance when radiotherapy of certain somatostatin receptor-positive human tumours with $\alpha$- or ß-emitting radiolabelled somatostatin analogues is considered. We found extensive internalization of [$^{125}$I-Tyr$^3$]octreotide which was dependent on time, and temperature and ligand concentration. Addition of 1 nM unlabelled octreotide induced a rapid increase in membrane binding and internalization of the radioligand. However, in patients investigated with [$^{123}$I-Tyr$^3$]octreotide, a short residence time of radioactivity was found in the somatostatin receptor-positive target tissues. Therefore, [$^{131}$I-Tyr$^3$]octreotide will most probably not be suitable as a radiotherapeutical (see also Appendix 3).

The presence of the DTPA-group in [$^{111}$In-DTPA-D-Phe$^1$]octreotide has major effects on its tissue residence time. With this radiopharmaceutical, we found in patients a biological half-life of $>700$ hours in liver and in tumours, and of $\approx$ 270 hours in the kidneys (see also Appendix 1). These observations indicate tissue- and structure-specific differences in metabolism for somatostatin analogues.

In another experiment, described in Chapter 3, in vivo displacement of somatostatin receptor-positive tissue-associated radioactivity is observed if unlabelled octreotide is injected 10 min after [$^{111}$In-DTPA-D-Phe$^1$]octreotide, but not after 20 min. In conjunction with the long tissue residence time, these findings represent strong evidence for significant internalization of [$^{111}$In-DTPA-D-Phe$^1$]octreotide in vivo.

Recent reports have characterized the ligand specificity of the 5 different somatostatin receptors sofar identified. However, little is still known about the tissue (tumor)-specific expression of these receptors, and, hence, the tissue (tumor)-specific uptake of somatostatin analogues.

In Chapters 5, 6 and 7 the preparation, biological activity, in vivo application in rats and receptor scintigraphy of [$^{123}$I- or $^{125}$I- Tyr$^3$]RC-160, and [$^{111}$In-DTPA-D-Phe$^1$]RC-160 are presented and compared with [$^{123}$I- or $^{125}$I-Tyr$^3$]octreotide, and [$^{111}$In-DTPA-D-Phe$^1$]octreotide, respectively. The radiolabelled RC-160 analogues

all showed uptake and specific high-affinity binding to various somatostatin receptor-positive organs. In contrast to RC-160 and [$^{123}$I-Tyr$^{3}$]RC-160, which do pass the blood-brain barrier, it was shown that [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160 does not pass the blood-brain barrier. Compared to [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide, the main disadvantage of[$^{123}$I-Tyr$^{3}$]RC-160 and [$^{111}$In-DTPA-D-Phe$^{1}$]RC-160 is their relatively low somatostatin receptor-positive tissue to blood (background) ratio, implying poorer sensitivity. However, labelled RC-160 may be potentially useful for the scintigraphy of tumours expressing somatostatin receptor isoforms capable of binding RC-160 but not octreotide. Perhaps, SSTR5 is an example of such a RC-160-preferring somatostatin receptor.

Several other radiolabelled peptides may have potential use as a radiopharmaceutical in receptor scintigraphy. The 11 amino acids-containing neurotransmitter substance P, which is among others involved in several immunological processes, is such a candidate. As described in Chapter 8 and 9, substance P was conjugated with a DTPA-group and labelled with $^{111}$In. [$^{111}$In-DTPA-Arg$^{1}$]substance P was subsequently tested in several in vitro tests to establish its intact binding characteristics, and investigated as a radiopharmaceutical for the detection of immunological disorders in rats and in patients. In vivo metabolism of the radiopharmaceutical in the rat revealed a renal clearance of 50 % of the injected radioactive dose in 30 min half-life as well as rapid enzymatic degradation resulting in an effective half-life of the intact radiopharmaceutical in blood of only $\approx$ 3 min. Despite this very short half-life the visualization of normal substance P receptor-positive tissues, such as the salivary glands, was demonstrated in control rats by gamma camera scintigraphy. Pathological substance P receptor-positive processes were also visualized in rats bearing the transplantable pancreatic tumour CA20948, and in rats with mycobacteria tuberculosis-induced arthritic joints. In analogy with the [DTPA-D-Phe$^{1}$]octreotide experiments, as described in Chapter 3, we also found a tissue-specific bell-shaped uptake of radioactivity in substance P receptor-positive tissues as a function of the injected mass of [DTPA-Arg$^{1}$]substance P in rats. However, the clinical relevance of these findings is limited because of the side effects of substance P administration to humans.

In Chapter 9, we describe the investigation of ten selected patients with inflammatory bowel disease (5), ophthalmic Graves' disease (1), sarcoidosis (1), Sjögren's syndrome (1), systemic lupus erythematosus (1), and reumatoid arthritis (1) after the injection of 200 MBq (2.5 $\mu$g) [$^{111}$In-DTPA-Arg$^{1}$]substance P. The pharmacological characteristics of the latter were evident; even during a 10 min intravenous infusion of 2.5 $\mu$g [$^{111}$In-DTPA-Arg$^{1}$]substance P a transient flush was observed, as was also reported for substance P in healthy volunteers. Degradation of [$^{111}$In-DTPA-Arg$^{1}$]substance P started after the first min after administration,

resulting in a half-life of 10 min for plasma radioactivity and of 4 min for the intact radiopharmaceutical, as identified by HPLC. Urinary excretion amounted to in >95 % of the injected dose of radioactivity in 24 h, while as low as 0.05 % was found in the feces collected up to 60 h after the injection. In all patients uptake was found in the parotid glands, aurolae of the mammae (in women), liver, kidneys, and urine bladder. In seven patients a high uptake of [$^{111}$In-DTPA-Arg$^1$]substance P was found in the thymus. Based on these findings, we conclude that [$^{111}$In-DTPA-Arg$^1$]substance P is a new radiopharmaceutical and, despite its short half-life, can be used to visualize the thymus. This may contribute to the investigation of the role of thymus in immune disorders. However, after the pharmacological characteristics of [$^{111}$In-DTPA-Arg$^1$]substance P in patients became evident, the search for radiolabelled substance P antagonists suitable for gamma camera scintigraphy was continued, but sofar, without results. Several D-amino acids-containing and DTPA-conjugated substance P analogues were also investigated in vivo, but they showed much less specific binding to substance P receptor-positive tissues in vivo than [$^{111}$In-DTPA-Arg$^1$]substance P. The search for more stable substance P agonists may not be a useful strategy, since the pharmacological effects of substance P, as a consequence, will also be prolonged. Therefore, the intravenously administered mass of such a substance P analogue will have to be minimal, which may be too low for optimal tissue (tumour) uptake.

Seven of the 10 peptide bonds of substance P are hydrolyzed by various peptidases, such as angiotensin converting enzyme (ACE) or neutral endopeptidase (NEP). Inhibitors of ACE or NEP may be an interesting addition to the radiopharmaceutical to prolong its half-life, but they may also potentiate the unwanted cardiovascular tachykinin responses.

In the Appendix papers 1, 2 and 3 we describe the development and the application of somatostatin analogues labelled with the Auger electron-emitter $^{111}$In, and the ß electron-emitters $^{161}$Tb and $^{131}$I, respectively. In Appendix 1, a case history of the successful radiotherapy with [$^{111}$In-DTPA-D-Phe$^1$]octreotide is presented in a patient with an inoperable metastasized glucagonoma. After a cumulative dose of 20 GBq [$^{111}$In-DTPA-D-Phe$^1$]octreotide a decrease in total abdominal tumour volume of $\approx$ 20 % was observed, combined with a (transient) decline in levels of glucagon and γ-glutamyl transferase. These findings suggest an antiproliferative effect of the radiotherapy with high doses of [$^{111}$In-DTPA-D-Phe$^1$]octreotide. However, in general, $^{111}$In is not the most appropriate radionuclide for radiotherapy, since it lacks the higher energies of α- and ß-particles. Radiotherapy of tumours with an inhomogenous distribution of peptide-receptors will also require these higher energies. For that reason we specially evaluated the hard ß-emitter [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide.

In Appendix 2, the in vitro receptor binding and biological activity of [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide, its metabolism in isolated perfused rat livers and its tissue distribution in normal and tumour-bearing rats were investigated, and compared with [$^{111}$In-DTPA-D-Phe$^{1}$]octreotide. We found, specific high-affinity binding to the somatostatin receptor and biological activity for the $^{159}$Tb- or $^{161}$Tb-labelled [DTPA-D-Phe$^{1}$]octreotide. Combined with the in vivo organ distribution, [$^{161}$Tb-DTPA-D-Phe$^{1}$]octreotide might be a promising radiopharmaceutical, that can be used both for radiotherapy and as a tool for intraoperative scanning.

In Appendix 3 the frequently mentioned potential use of $^{131}$I-labelled somatostatin analogues for radiotherapy is evaluated. This was done with special attention to the radiochemistry and the technical aspects of the production of radiotherapeuticals.

In the presence of 370 MBq $^{131}$I, somatostatin analogues showed extensive radiolytic damage in aqueous solution with a half-life of only 1 h.

Consequently, we conclude that intact mono-iodinated $^{131}$I-labelled peptides will be very hard to obtain, especially if a supposed dose of 3700 MBq $^{131}$I-labelled peptide is required. For peptide-receptor radionuclide therapy direct labelling of a radiometal like $^{90}$Y or $^{161}$Tb to a chelator-conjugated peptide in the presence of a suitable radiation quencher may be far easier to obtain. However, still many interesting problems on radionuclide purity and synthesis will have to be solved.

## SAMENVATTING, DISCUSSIE EN CONCLUSIES
### ook voor de leek

*Inleiding*

Somatostatine is een hormoon; het is onder andere aanwezig in de hypothalamus, de buitenste regionen van de hersenen, de hersenstam, op verschillende plaatsen in het maag-darm kanaal en in het pancreas (de alvleesklier). Bij het functioneren van een hormoon is een receptor voor de herkenning van het hormoon noodzakelijk; zo wordt somatostatine herkend door specifieke somatostatine receptoren. In vakterminologie wordt gesproken over ligand (=somatostatine) en receptor. In het centraal zenuwstelsel (CZS) is somatostatine op deze zelfde wijze via receptoren werkzaam als een neurotransmitter, dat wil zeggen het is betrokken bij de communicatie in het CZS. Somatostatine is ook buiten het CZS via de somatostatine receptoren betrokken bij bijvoorbeeld de remming van de afgifte van andere hormonen, zoals groeihormoon, insuline, glucagon en gastrine. Deze remmende werking heeft geleid tot de behandeling met somatostatine van patiënten met ziekten die veroorzaakt worden door te hard werkende klieren of tumoren die hormonen uitscheiden.

Somatostatine heeft in de bloedbaan een zeer korte werkzame periode van $\pm$ 3 minuten. Dit was een belangrijke reden om een soortgelijk peptide te maken die langer werkzaam was. Verscheidene van deze peptiden (analoga) worden nu over de gehele wereld gebruikt als geneesmiddel bij patiënten met klieren die teveel hormonen uitscheiden of tumoren met somatostatine receptoren. Een van deze somatostatine analoga is octreotide. Een andere werkzame variant van octreotide is [DTPA-D-Phe$^1$]octreotide: dit analogon bezit een groep (chelator) die de mogelijkheid geeft om hieraan op een eenvoudige en snelle wijze radioaktieve stoffen zoals $^{111}$In ("indium-111") te koppelen. Deze radioaktieve verbinding werd in het Academisch Ziekenhuis Rotterdam "Dijkzigt" ontwikkeld en wordt sinds 1989 routinematig toegepast voor de diagnostiek bij patiënten met verdenking van bijvoorbeeld te snel werkende klieren of tumoren die somatostatine receptoren bezitten.

*Wat is er onderzocht*

De in dit proefschrift met radioaktiviteit gelabelde (gemerkte) peptiden kunnen in principe op twee manieren worden gebruikt:

1. voor de **diagnostiek** van onder andere tumoren met behulp van "nucleaire foto's" (scintigrammen) en
2. voor de **behandeling** van deze tumoren (radionuclide therapie).

Na een inleiding wordt in dit proefschrift in hoofdstuk 3 een onderzoek met [$^{111}$In-DTPA-D-Phe$^1$]octreotide beschreven met als doel te onderzoeken of voor het aantonen van afwijkingen en tumoren **alle** somatostatine moleculen die zich kunnen

binden aan een somatostatine receptor, na inspuiting in rat of mens, radioaktief gemerkt dienen te zijn. Op deze manier hebben namelijk somatostatine moleculen die zich binden aan de somatostatine receptoren geen last van ongemerkte somatostatine moleculen die zich ook binden aan dezelfde receptor. En daarbij geldt: hoe meer receptoren gebonden zijn met gemerkte moleculen, des te beter zouden de afbeeldingen moeten zijn. Aangezien de kwaliteit van het $^{111}InCl_3$ ("indium-111-chloride") sinds de eerste labeling van [DTPA-D-Phe$^1$]octreotide sterk is verbeterd, kon deze hypothese worden getoetst. De resultaten waren volkomen onverwacht: in de rat werd een relatie gevonden tussen de toegediende massa van het ligand ([DTPA-D-Phe$^1$]octreotide) en de percentage dosis opname in somatostatine receptor-positief weefsel, maar niet tussen de "puurheid" van het in te spuiten gemerkt hormoon en de percentage opname. Dit aspect werd ook onderzocht bij patiënten, en uit de voorlopige resultaten is gebleken dat ook bij de mens een minimum aan toegediende massa van het ligand essentieel is.

In hoofdstuk 3 wordt ook een onderzoek beschreven waarbij een poging wordt gedaan de opname van het radioaktieve deel van het [$^{111}$In-DTPA-D-Phe$^1$]octreotide te beïnvloeden met als doel de opname van radioaktiviteit in het weefsel selectief te verhogen. Het bleek dat de opname van radioaktiviteit in de verschillende somatostatine receptor-bevattende weefsels inderdaad kan worden beïnvloed door verschillende hoeveelheden van verschillende liganden op verschillende tijden ten opzichte van het gemerkte ligand toe te dienen.

[Tyr$^3$]octreotide is weer een andere variant van het octreotide, die ook aan somatostatine receptoren bindt. Met de synthese van [Tyr$^3$]octreotide werd het mogelijk octreotide te merken met radioaktief jodium. Deze eigenschap opende speciale onderzoekmogelijkheden in het laboratorium. Voorbeelden van radioaktief jodium zijn $^{123}$I ("jodium-123") , $^{125}$I en $^{131}$I. Uit eerdere onderzoeken met [$^{123}$I-Tyr$^3$]octreotide met ratten, maar ook bij patiënten was gebleken dat de verblijftijd van dit radioligand kort is. Om geschikt te zijn voor radionuclide therapie, zou juist een langere verblijftijd gunstig zijn. Daarom mag worden aangenomen dat [Tyr$^3$]octreotide minder geschikt is voor radionuclide therapie.

De aanwezigheid van een chelator (-DTPA) in het andere radioligand, [$^{111}$In-DTPA-D-Phe$^1$]octreotide, bleek belangrijke effecten op de verblijftijd van de radioaktiviteit in de weefsels te hebben. Uit onderzoeken bij patiënten is gebleken dat de biologische halfwaarde tijd van $^{111}$In in de lever en in de tumor meer dan 700 uren bedraagt, en in de nieren ongeveer 270 uren. Hieruit blijkt ook al een verschil in stofwisseling in deze weefsels en ook voor de twee radioliganden. Dit zou octreotide-liganden met een DTPA-groep geschikter maken voor radionuclide therapie.

In een ander experiment, beschreven in hoofdstuk 3, is verdringing van radioaktiviteit uit weefsels gevonden indien 10 minuten na toediening van het

radioligand een hoeveelheid van het niet gemerkte ligand wordt toegediend. Dit treedt niet op indien het ongemerkte ligand 20 minuten later wordt toegediend. Samen met de genoemde langere verblijftijd van de radioaktiviteit in de weefsels suggereren deze waarnemingen dat er opname van radioaktiviteit in de weefsels plaatsvindt, wanneer [$^{111}$In-DTPA-D-Phe$^1$]octreotide gebruikt wordt. Dit is uiteraard van belang wanneer soortgelijke stoffen voor radionuclide therapie gebruikt gaan worden, teneinde de kern van de kankercellen te bestralen.

Voor de eventuele ontwikkeling van radionuclide therapie met [Tyr$^3$]octreotide of [DTPA-D-Phe$^1$]octreotide gemerkt met de daarvoor geschikte radioaktieve stoffen, is verdere verdieping van de kennis vereist over de lokalisatie en de verblijftijd in de cel van het radioaktieve deel van deze verbindingen. Deze onderzoeken zijn beschreven in hoofdstuk 4 en werden uitgevoerd met cellen van de hypofyse ("hersenaanhangsel"). Deze cellen bezitten een hoge dichtheid aan somatostatine receptoren. Het bleek dat een zeer geringe toevoeging (1 nM) van het octreotide aan de hypofysecellen de hoeveelheid van het aantal somatostatine receptoren op de membraan van de hypofyse cellen snel deed toenemen, en dat er tevens een versnelde opname van het radioligand werd waargenomen. Dit fenomeen is wellicht ook de verklaring van de waarnemingen uit hoofdstuk 3 waarbij een minimum aan toegediende massa van het ligand vereist is om een hogere opname van radioaktiviteit in somatostatine receptor bevattend weefsel te realiseren.

In de literatuur zijn enkele studies beschreven betreffende een ander somatostatine analogon, een cyclisch octapeptide RC-160, dat veel overeenkomsten vertoont met octreotide. De receptor bindende eigenschappen van het RC-160 zouden superieur zijn aan octreotide in tumoren van de borst, eierstokken, exocriene alvleesklier, prostaat en de dikke darm. Bovendien zou RC-160, in tegenstelling tot octreotide de barrière tussen bloed en hersenen kunnen passeren. Deze eigenschappen waren aanleiding om de mogelijkheden van RC-160 te onderzoeken. De resultaten hiervan zijn beschreven in de hoofdstukken 5, 6 en 7. RC-160 en het nieuw gesynthetiseerde [DTPA-D-Phe$^1$]RC-160, en de beide radioliganden $^{123}$I-RC-160 en [$^{111}$In-DTPA-D-Phe$^1$]RC-160 vertoonden alle, in vitro, receptor bindende eigenschappen en biologische aktiviteit. Bij proeven met dieren werden de bindende eigenschappen bevestigd. De eigenschappen van RC-160 en [DTPA-D-Phe$^1$]RC-160 waren in ons testsysteem vergelijkbaar met die van octreotide, respectievelijk [DTPA-D-Phe$^1$]octreotide. Bovendien was door de toevoeging van de polaire DTPA-groep aan het [DTPA-D-Phe$^1$]RC-160 de barrière tussen de bloed en hersenen weer een barrière voor dit radioligand. Vooral de langzamere klaring van radioaktiviteit van [$^{111}$In-DTPA-D-Phe$^1$]RC-160 uit de somatostatine receptor-ontbrekende weefsels en het bloed, vergeleken met [$^{111}$In-DTPA-D-Phe$^1$]octreotide, resulteert in een lagere verhouding van de stapeling van radioaktiviteit in de somatostatine receptor bevattende weefsels ten opzichte van

achtergrond (bloed) en dus ook in een minder gunstige afbeelding op het scintigram. Ondanks de minder goede eigenschappen van RC-160 ten opzichte van octreotide, zijn er theoretisch nog steeds mogelijkheden voor diagnostische en radionuclide therapeutische toepassingen voor RC-160 en [DTPA-D-Phe$^1$]RC-160. Dit geldt voor de tumoren die bepaalde vormen van de somatostatine receptor hebben en die octreotide niet en RC-160 mogelijk wel kunnen binden.

Verschillende radioaktief gemerkte peptiden zijn potentieel kandidaat voor receptor scintigrafie. Een voorbeeld hiervoor is substance P. Dit is een neurotransmitter die onder andere betrokken is bij verschillende immunologische processen.

In de hoofdstukken 8 en 9 worden de onderzoeken beschreven met substance P waaraan een DTPA-groep is gekoppeld. Hierdoor is ook bij deze stof een directe, snelle labeling met $^{111}$In mogelijk. De receptor bindende eigenschappen van het [$^{111}$In-DTPA-Arg$^1$]substance P werden onderzocht en bleken intact te zijn gebleven. Substance P wordt in het lichaam door verschillende enzymen snel afgebroken en voor [$^{111}$In-DTPA-Arg$^1$]substance P geldt dit eveneens. De halfwaarde tijd van het intacte [$^{111}$In-DTPA-Arg$^1$]substance P in bloed van rat en mens is 3-4 minuten. De speekselklieren in rat en mens vertonen een hoge dichtheid aan substance P receptoren, en ondanks de korte halfwaarde tijd is het toch mogelijk om deze weefsels zichtbaar te maken op het scintigram. In de rat werden ook andere weefsels met een bewezen verhoogde substance P receptor-expressie onderzocht en ook deze weefsels konden worden afgebeeld. Hoewel de onderzoeken met [$^{111}$In-DTPA-Arg$^1$]substance P bij de mens nog beperkt zijn, werden ook in de mens afwijkingen op de scintigrammen gevonden met een duidelijk (oorzakelijk) verband met het ziektebeeld. Dat de biologische aktiviteit van het (radio)ligand bewaard is gebleven wordt duidelijk waargenomen: iedere patiënt krijgt bij de toediening van $\pm$ 2,5 $\mu$g [$^{111}$In-DTPA-Arg$^1$]substance P een kortdurende flush ("opvlieger"). Ontstekingen bij verschillende ziekten konden eveneens duidelijk afgebeeld worden. Bij een deel van de onderzochte patiënten was de thymus (zwezerik) opvallend zichtbaar. Bij verschillende autoimmuun-ziekten is vergroting van de thymus beschreven; de rol van de thymus bij deze ziektebeelden is echter nog onduidelijk. De plaats van [$^{111}$In-DTPA-Arg$^1$]substance P in de scintigrafie van bijvoorbeeld ontstekingen en de rol van de thymus bij autoimmuun-ziekten zal in de toekomst nog moeten worden bepaald.

In de appendix hoofdstukken 1, 2 en 3 worden de ontwikkelingen en de toepassingen beschreven van somatostatine analoga die gemerkt zijn met de radioaktieve stoffen $^{111}$In, $^{161}$Tb ("terbium-161") en $^{131}$I. De fysische verschillen tussen deze radioaktieve stoffen zijn groot. Ten eerste heeft $^{111}$In een aantal soorten van straling, zoals gamma-straling waar in de nucleaire geneeskunde gebruik van wordt gemaakt voor afbeeldingen op het scintigram. De tweede belangrijke soort

van straling van $^{111}$In vormen de Auger- en conversie-elektronen; deze straling is ongeschikt voor afbeelden in de nucleaire geneeskunde. Deze elektronen zijn wel geschikt voor therapeutische toepassingen, maar alleen als het uiteenvallende radionuclide op de juiste plaats terecht komt. Deze elektronen hebben namelijk een relatief korte dracht ("reikwijdte"), dat wil zeggen dat deze energierijke elektronen vaak niet verder reiken dan een zeer klein deel van een cel of tumor. De juiste plaats voor de therapie is in dit geval in of vlakbij de celkern, want dit deel van de cel is het meest gevoelig voor het uiteindelijke doel: het toebrengen van onherstelbare stralingsschade. Hiermee zijn we terug bij het onderwerp uit hoofdstuk 4, waarbij het belang van de kennis van de lokalisatie en verblijftijd van het radionuclide werd aangegeven.

In het appendix hoofdstuk 1 wordt melding gemaakt van de eerste en bemoedigende therapeutische toepassing van [$^{111}$In-DTPA-D-Phe$^1$]octreotide bij een patiënt met tumoren die niet verwijderd konden worden. De behandeling is in delen uitgevoerd met een totale dosis van 20 GBq [$^{111}$In-DTPA-D-Phe$^1$]octreotide. Er werd een verkleining van de tumor in de buik van 20 % waargenomen, gecombineerd met een tijdelijke daling van stoffen in het bloed die door de tumor werden gemaakt. De voortgang van deze nieuwe therapie wordt op het moment in internationaal verband verder ontwikkeld.

Ondanks dit succes geldt in het algemeen dat $^{111}$In niet het ideale radionuclide is voor therapeutische toepassingen. Het radionuclide $^{161}$Tb heeft niet alleen de reeds eerder genoemde gamma-straling, maar ook beta-straling. Deze hoog-energetische beta-straling heeft een veel grotere dracht dan de Auger- en conversie-elektronen van bijvoorbeeld $^{111}$In. Bovendien is door deze grotere dracht van het $^{161}$Tb de mogelijke niet-gelijke verdeling van de somatostatine receptoren in een tumor niet meer beperkend. De cellen in een tumor die geen expressie van somatostatine receptoren vertonen kunnen nu toch via een "kruisvuur" vanuit de belendende cellen die wel radioaktiviteit hebben opgenomen worden bestraald. De onderzoeken met [DTPA]-gemerkte peptiden met $^{161}$Tb zijn gaande. De receptor-bindende eigenschappen en de biologische aktiviteit van het [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide zijn intact gebleken, en de resultaten van de studies naar de verdeling van de radioaktiviteit in de organen in de rat zijn hoopgevend. Bovendien is het stralingsniveau rondom een met [$^{161}$Tb-DTPA-D-Phe$^1$]octreotide ingespoten patiënt laag. Met speciale richtingsgevoelige stralings-detectie apparatuur zijn er mogelijkheden voor de anatomische plaatsbepaling van kleine tumoren tijdens een operatie.

In het appendix hoofdstuk 3 worden de mogelijkheden van de produktie en de toepasbaarheid van het $^{131}$I-gemerkte [Tyr$^3$]octreotide en voor de therapie met dergelijke radioaktieve peptiden beschreven. Speciale aandacht is besteed aan de radiochemie en aan de technische aspecten van deze produktie. Een aantal

somatostatine analoga bleken in een waterig milieu, in een klein volume en in de aanwezigheid van 370 MBq $^{131}I$, sterk onderhevig te zijn aan radiolyse (stralingsschade), resulterend in een halfwaarde tijd van deze peptiden van 1 uur. Dientengevolge concluderen wij dat therapeutische hoeveelheden van zelfs 3700 MBq $^{131}I$ van het intacte mono-gejodeerde [$Tyr^3$]octreotide of andere analoga moeilijk, zo niet onmogelijk kunnen worden geproduceerd. Radioaktieve peptiden voor de radionuclide therapie van tumoren zijn wellicht eenvoudiger en beter te produceren via een directe labeling van een -chelator groep bevattend peptide met een radiometaal zoals $^{90}Y$ ("Yttrium-90") of $^{161}Tb$. Dit zal bijvoorbeeld ook kunnen gebeuren in de aanwezigheid van een stof die de schade van radiolyse weet te beperken, een zogenaamde "quencher". Ook wat dit onderwerp betreft is nog verder onderzoek vereist.

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## CURRICULUM VITAE

W.A.P. Breeman werd op 11 september 1951 geboren te Rotterdam geboren. Zijn jeugd bracht hij door op Curaçao (Nederlandse Antillen). De middelbare schoolopleiding startte hij aan het Peter Stuyvesant College (Curaçao), en werd vanaf 1965 voortgezet aan de HBS in Oud-Beijerland. Na het vervullen van zijn militaire dienstplicht begon hij de avondstudie aan het van 't Hoff instituut te Rotterdam. Het diploma HTS-chemie werd in 1979 behaald. Gedurende 1969-1976 werd werkervaring als analist opgedaan in verschillende laboratoria. Vanaf 1976 was hij verbonden aan de Medische Faculteit van de Erasmus Universiteit te Rotterdam, tot 1980 op het laboratorium van de afdeling Cardiochemie (Thorax-centrum, hoofd van de afdeling dr J.W. de Jong), en aansluitend tot 1983 op de afdeling Biochemie I (hoofd van de afdeling Prof. dr W.C. Hülsmann). In 1977 behaalde hij het "C"-diploma Deskundigheid Stralenbescherming aan het Interuniversitair Reactor Instituut te Delft. Vanaf 1983 is hij werkzaam binnen de afdeling Nucleaire Geneeskunde van het Academisch Ziekenhuis Rotterdam.

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*Pensées*, Blaise Pascal, 1623-1662.

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