Doctoral Thesis

New stabilised bombesin-analogues as potential tumour imaging agents

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New stabilised bombesin-analogues as potential tumour imaging agents

A dissertation submitted to the
Swiss Federal Institute of Technology Zurich
for the degree of
Doctor of Natural Sciences

presented by

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2005
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Summary

Peptide receptors have been found to represent excellent targets for in vivo cancer diagnosis and therapy. Especially, in a high number of breast and prostate carcinoma over-expression of GRP (Gastrin Releasing Peptide) receptors were found, which led to the development of tumour targeting with bombesin (BBS) analogues with high GRP receptor affinity. BBS analogues labelled with radioisotopes offer a promising way of selective non-invasive imaging and therapy for GRP receptor positive neoplasms.

The aim of this work was the development of $^{99m}$Tc-labelled BBS-analogues to be used for detecting GRP receptor positive tumours by gamma ray scintigraphy and also the chemical characterisation of these peptides. For the pharmacological studies, the analogues were labelled with $\text{fac-}[^{99m}\text{Tc(OH)}_3\text{(CO)}_3]^+$ and for the chemical characterizations we used $\text{fac-}[^{185/187}\text{Re(OH)}_3\text{(CO)}_3]^+$.

With respect to earlier work in the Center of Radiopharmaceutical Science, the bifunctional chelator retro-$\text{N}^2$-carboxymethyl-histidine, short ($\text{N}^2$His)Ac, has shown to be a promising one. The alkylation of the His-chelator ($\text{N}^2$-linked acetate) leads to tridentate binding capacity. Tridentate chelators, which are able to replace all three water molecules of the metallic complex $\text{fac-}[\text{M(OH)}_3\text{(CO)}_3]^+$ are a prerequisite to obtain good in vivo properties. The second function of the bifunctional chelator is coupling it to the amine terminus of peptides. The challenge experiment, where the BBS(8-14), Trp$^8$-Ala$^9$-Val$^{10}$-Gly$^{11}$-$\text{His}^{12}$-Leu$^{13}$-Met$^{14}$-NH$_2$, was labelled and subsequently incubated with equimolar His (free) showed, that the kinetic is fast enough to obtain the thermodynamically more stable complex within the time frame of the usual labelling procedure. This confirmed the postlabelling approach as the suitable method.

Since natural BBS is metabolized very rapidly in vivo, new analogues with different modifications based on the fragment BBS(7-14) have been synthesized. At known and supposed cleavage-sites of the enzymes, natural amino acids have been replaced by non-natural amino acids. Many changes led to the expected improved stability but negatively to a loss of affinity. Two non natural amino acids CyHAla$^{13}$ and Nle$^{14}$ at the place of Leu$^{13}$ and Met$^{14}$, respectively, brought about the best in vivo properties. This change was combined with the introduction of different spacers, namely -$\beta$Ala-$\beta$Ala- and -NH-CH$_2$-CH$_2$-O-CH$_2$-CH$_2$-O-CH$_2$CO-, between the receptor binding amino acid sequence (7-14) of BBS and the metal complex.
The inhibition of $^{125}$I-BBS(1-14) binding (IC$_{50}$) was tested with all unlabelled derivatives for a first selection, and peptides with a value $> 1000$ nM were excluded from further investigations with a few exceptions. These being BBS-II, which is the analogue with the non-modified chain of amino acids and thus the reference for many experiments, and at least one example of each type of substitution. The next step was to investigate the stability in human plasma. Generally, peptides with a half-life of at least 6 hours were followed up by further pharmacological and chemical characterizations. This included metabolic stability in vitro (PC-3 cells), binding properties of the labelled peptides (Lindmo, Scatchard), internalization, externalization as well as their in vivo biodistribution in nude mice bearing human PC-3 xenografts. Some changes in the molecule led to an increase of the stability in plasma. Several new analogues retained the receptor affinity, BBS-IV and BBS-IX showed even higher affinity for GRP receptor in human prostate carcinoma PC-3 cells than the natural bombesin. Receptor-bound analogues were rapidly internalized at 37°C with more than 70% of the activity being internalized after 30 min incubation and remaining almost constant for at least 2 h. For the externalisation experiments we pre-incubated the cells for 45 min with $^{99m}$Tc(CO)$_3$-BBS analogues to allow maximal internalization and then measured the rather rapidly externalized activity. Up to 80% of the activity was released within the first 5 h and consisted of metabolites (peptide fragments and pertechnetate).

In biodistribution studies performed on mice with PC-3 tumour xenografts, the analogues showed a low tumour uptake. In contrast, the uptake in other GRP receptor-positive organs, such as pancreas and colon, was rather high. Furthermore it could be partly inhibited after co-injection with unlabelled BBS, which indicates that this uptake is receptor-specific. The metabolites were excreted via the kidneys. The analysis of the urine with HPLC showed, that no intact BBS analogues were excreted. The spacers had only little influence on the metabolic stability and the receptor affinity of the new BBS-analogues. They brought about a significant increase of the uptake in the GRP receptor rich pancreas and in PC-3 tumour xenografts in nude mice.
Summary

Figure 1. Uptake ratios of the different BBS-analogues based on % of injected dose per gram tissue of the GRP receptor rich Pancreas (Pa) compared to Blood (Bl), Kidney (Ki) and Liver (Li). The improvement is demonstrated by the clear increase from BBS-IV $\rightarrow$ BBS-XXX $\rightarrow$ BBS-38.

Some of the new BBS analogues showed a clear improvement of the target to non-target uptake ratios. These ratios should be $> 1$ for diagnostic application in humans, and thus, one objective of this work was reached. However, the clearance from the target tissues is still rather rapid, even faster than from kidneys or liver. Since in therapy the clearance from tumour must be slower than from all healthy tissues, the BBS-analogues did not fulfil the expectations and thus new analogues need to be developed.
Zusammenfassung


Das Ziel dieser Arbeit war die Entwicklung neuer $^{99m}$Tc-markierter BBS-Derivate, welche Tumore, die GRP-Rezeptor positiv sind, durch Gammastrahlen-Szintigraphie detektieren können, aber auch die chemische Charakterisierung der verschiedenen, synthetisierten Derivate. Die pharmakologischen Studien wurden mit $^{99m}$Tc(OH)$_3$(CO)$_3$$^+$ markierten Peptiden durchgeführt, während man für die chemische Charakterisierung mit $^{185/187}$Re(OH)$_3$(CO)$_3$$^+$ arbeitete.


Modifikationen wurden mit verschiedenen Spacern, welche zwischen der receptorbindenden Aminosäuresequenz (7-14) und dem Metallkomplex eingeführt wurden, kombiniert. Es waren dies die Spacer, -ßAla-ßAla- und -NH-CH₂-CH₂-O-CH₂-CH₂-O-CH₂CO-.


Die Biodistributionsversuche zeigten nur eine geringe Aufnahme der Aktivität in die PC-3 Tumoren der Mäuse. Im Vergleich dazu war die Aufnahme in andere GRP-R positive Gewebe, wie Pankreas und auch Darm eher hoch. Diese Bindungen waren GRP-Rezeptor spezifisch und konnten mit der Co-Injektion von kaltem Peptid (BBS(8-14)) inhibiert werden. Der Urin wurde mittels HPLC analysiert, wobei bestätigt werden konnte, dass ausschließlich Metaboliten ausgeschieden wurden. Mit den Spacern erreichte man eine Verbesserung der Aktivitätsaufnahme in die GRP-R positiven Gewebe, wie Pankreas und PC-3 Tumornokenografte. Auf die metabolische Stabilität und die Rezeptorbinderung haben sie jedoch nur einen geringen Einfluss ausgeübt.
**Zusammenfassung**

Figur 1: Der Vergleich der Aufnahmeverhältnisse von BBS-Analoga in Nacktmäsen mit PC-3 Tumorxenografts, basierend auf den % injizierten Dosen pro Gramm Gewebe. Der GRP-R reiche Pankreas (Pa) wird mit dem Blut (Bl), den Nieren (Ki) und der Leber (Le) verglichen. Die Erhöhung von BBS-IV zu BBS-XXX zu BBS-38 verdeutlicht die erzielten Verbesserungen.

Einige der neuen Bombesinanaloga zeigten eine Verbesserung der Aufnahmerate von Target- zu nicht Targetgewebe. Für die Applikation am Menschen muss diese einen Wert von > 1 aufweisen. Die Bedingung konnte erfüllt werden, jedoch konnte auf die Clearance des Tumors, die klar langsamer verlaufen muss als die aus all den anderen Geweben, keinen wesentlichen Einfluss genommen werden. Dies ist aber eine wichtige Voraussetzung damit ein therapeutischer Einsatz in Betracht gezogen werden kann. Somit entsprechen die Bombesinanaloga noch nicht den gewünschten Erwartungen, was basierend auf den bisherigen Resultaten, die Synthese weiterer Peptide erfordert.
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Aba</td>
<td>Aminobutyric acid</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AVA</td>
<td>5-aminovaleric acid</td>
</tr>
<tr>
<td>bb3</td>
<td>Bombesin receptor subtype 3</td>
</tr>
<tr>
<td>bb4</td>
<td>Bombesin receptor subtype 4</td>
</tr>
<tr>
<td>BBS</td>
<td>Bombesin</td>
</tr>
<tr>
<td>BFCA</td>
<td>Bifunctional chelating agent</td>
</tr>
<tr>
<td>BOC</td>
<td>Tert.-butyloxy-carbonyl</td>
</tr>
<tr>
<td>Bq</td>
<td>Becquerel</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CyHAla</td>
<td>Cyclohexylalanin</td>
</tr>
<tr>
<td>DIC</td>
<td>N,N-diisopropylcarbodiimide</td>
</tr>
<tr>
<td>Dipea</td>
<td>N-ethylisopropylamine</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethan</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s minimum essential medium</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethyleneetriaminepentaacetic acid</td>
</tr>
<tr>
<td>Edta</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Fmoc</td>
<td>N-(9-fluorenyl)methoxycarbonyl</td>
</tr>
<tr>
<td>Fcs</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamin</td>
</tr>
<tr>
<td>GRP</td>
<td>Gastrin releasing peptide</td>
</tr>
<tr>
<td>GRP-R</td>
<td>Gastrin releasing peptide receptor</td>
</tr>
<tr>
<td>H</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HOBt</td>
<td>1-hydroxy-benzotriazole</td>
</tr>
<tr>
<td>KeV</td>
<td>Kilo electron volt</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucin</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>Met</td>
<td>Methionin</td>
</tr>
<tr>
<td>Min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>Ms</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>(Nα hist)Ac</td>
<td>retro Nα-carboxymethyl histidine</td>
</tr>
<tr>
<td>Nle</td>
<td>Norleucin</td>
</tr>
<tr>
<td>NMB</td>
<td>Neuromedin B</td>
</tr>
<tr>
<td>NMB-R</td>
<td>Neuromedin B receptor</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>Pada</td>
<td>2-picolylamine-N,N-diacetic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline solution</td>
</tr>
<tr>
<td>PC-3</td>
<td>Human prostate adenocarcinoma cell line</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>Pro-Ile</td>
<td>Prolin-Isoleucin</td>
</tr>
<tr>
<td>PyBop</td>
<td>Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>RPhplc</td>
<td>Reverse phase-high pressure liquid chromatography</td>
</tr>
<tr>
<td>SPET</td>
<td>Single photon emission tomography</td>
</tr>
<tr>
<td>Tbu</td>
<td>tert-butyl</td>
</tr>
<tr>
<td>Tfa</td>
<td>Trifluoroacetic acid</td>
</tr>
</tbody>
</table>
CHAPTER 1

Target-specific radiopharmaceuticals for cancer
1.1 General consideration

Cancer, of which there are over one hundred forms, will affect one in three western Europeans at some point in their lifetime. Almost every second tissue in the body can seed malignancies, some even several types. Chance of recovery depends on the type of tumour and if it is a primary tumour or whether metastasis has occurred. Today’s standard treatment is surgery, external radiation therapy and chemotherapy, regardless of its limited effectiveness for secondary (metastatic) cancer outside the treated area. In metastasis, cells break away from the cancerous tumour and travel through the bloodstream or lymphatic system and continue to grow elsewhere. In case of small size metastasis imaging and treatment must be systemic, meaning that the tumour agent must be able to reach it via blood stream. This being the primary focus of specific radionuclide therapy. Selective delivery of radiation doses to target tissue would make therapeutic radiopharmaceuticals a suitable addition to other treatment modalities. Radiopharmaceuticals are composed of diagnostic or therapeutic radionuclides, linked to a carrier molecule (radioconjugate). Radiometals conjugation occurs by a bifunctional chelating agent (BFCA). Selection of carrier molecules is based on a biological function, specifically, binding to overexpressed receptors, which are interesting targets for tumour therapy. The reason of this overexpression is unknown, however for our purpose, namely a peptide based tumourtherapy, it is not important whether this over-expression is the primary cause for a normal cell to become cancerous or if a cancer cell inherently develops more receptors. Cancer cells generally show faster rate of growth, higher energy consumption and an increased production of receptor proteins.

1.2 Radionuclides for diagnostic or therapeutic use

The selection for the diagnostic or therapeutic application of a radiopharmaceutical is dependent upon the physical properties of the radionuclide. The ideal properties and a few examples of radionuclides are briefly discussed as well as the reason why we chose technetium and rhenium.

Diagnostic

Among radiopharmaceuticals that are applied diagnostically in nuclear medicine, two major groups can be distinguished: the \( \gamma \)-ray emitting radiopharmaceuticals used in single photon
emission tomography (SPET) and the positron emitting radiopharmaceuticals used in positron emission tomography (PET).

1) **Single photon emission tomography (SPET)**

SPET radiopharmaceuticals are used routinely for diagnostic and imaging purposes. They are characterized by emitting photons with energies in the range of 100-200 keV. The most commonly used SPET- nuclides in oncology are $^{99m}$Tc, $^{123}$I and $^{111}$In. Lower energy $\gamma$-rays are readily absorbed in tissue and therefore less useful for external imaging. Higher energy-emission leads to a higher radiation-dose to patients, but this dose should not be too high (generally, the benefit of a diagnostic outcome is compared with the risks of the radiation burden). The high energetic radiation has to be compensated by reduced activity which leads to reduced signal to noise ratios and to low imaging resolution (more radiation impinges oblique on the detector because it is not absorbed by the collimator). The i.v. injection of the carrier-molecule linked to a $\gamma$-emitter identifies the tumoural lesions as radioactive hot spots in the body. Precise quantification is impossible, but the qualitative evaluation of the data yields reliable results. Lesions as small as 5-10 mm can be detected because of its sensitivity.

2) **Positron emission tomography (PET)**

Other group of diagnostically used radionuclides are $\beta^+$- emitters, such as $^{11}$C and $^{18}$F. After decay, their positron annihilation results in two photons of 511 keV, which will be detected in coincidence. The energy is high enough that there is only little absorption in tissue and allows the measuring of the two photons with same intensity. PET represents the most advanced imaging technique, because it not only allows a three-dimensional image reconstruction but also quantification of activity uptake. It combines the highest degree of sensitivity with a resolution of currently, 5-7 mm. High cost (expensive cyclotron facilities for the production of the nuclides) and short half-lives of these radionuclides limit their application.

**Therapeutic**

Radionuclides used in therapy are characterized by high linear energy transfer (LET) radiation, like $\beta^-$, auger- or $\alpha$-emission. A small amount of $\gamma$-radiation is advantageous because it allows monitoring of the therapeutic agents distribution in patients, however it contributes to the total body radiation dose. Alpha particles are high-energy helium nuclei (5
Chapter 1

-8 MeV) with a tissue penetration about 100 µm. The therapeutic effect can be achieved when the radiopharmaceuticals reach the cell surface (1). Auger electrons with energies up to 30 keV have a tissue penetration about 10 nm and can have a therapeutic effect only if they reach the cell nucleus (2). β⁻ emissions (⁹⁰⁰Y, ¹⁷⁷Lu, ¹⁸⁶/¹⁸⁸Re, ⁶⁷⁷Cu) are negatively charged electrons emitted from the nucleus in a continuum of energies up to a maximum value. Depending on the energy, their ranges are up to 10 mm (Table 1.1). They are effective once they reach the cell environment. β⁻ emitting radionuclides would be advantageous because their radiation extends beyond the bound target cell and can therefore kill surrounding tumour cells, whereas auger electrons have such a short-ranged energy deposition requiring internalization and translocation of the radiation source to the nuclear DNA to kill tumour cells effectively.

Selection of the radionuclides

On the basis of the physical properties, the radionuclide is chosen as needed. Some of them are listed in table 1.1. Not mentioned is ⁶⁴⁴Cu (t½ = 12.7 h), which is a positron-emitter (19%, Eβ⁺max = 656 keV) that has been shown to also have therapeutic potential (39%, Eβ⁻max = 573 keV) when targeted to tumours (3, 4). Copper-64 can be used for different applications, but for neither application it's an optimal candidate. For diagnosis the β⁻ radiations are a burden whereas more of them would be desirable for therapy. It is better and easier to use specific nuclides like technetium or rhenium.

Technetium and Rhenium

Technetium-99m continues to be at the forefront of nuclear medicinal applications due to its availability, ideal nuclear characteristics (t½ = 6.04 h, Eγ = 140 keV (89%)), and well-established labelling chemistry. It is used in more than 85% of the diagnostic scans done each year in hospitals. ⁹⁹⁹Tc is advantageous because of its 6-hour half-life, which is sufficient time for radiopharmaceutical synthesis and application yet short enough to administer higher amounts up to 1 GBq with low radiation burden to patient. Furthermore, ⁹⁹⁹Tc is readily available from commercial ⁹⁹⁹Mo/⁹⁹⁹⁹Tc generators at low cost. The mother nuclide ⁹⁹⁹Mo has a half-life of 66 h and decays to ⁹⁹⁹⁹Tc, which can be eluted with physiological sodium chloride solution (0.15 M), in oxidation state +VII, as sodium pertechnetate Na₃[TcO₄]. Therefore a generator can be used for approximately one week. The development of new radiopharmaceuticals based on ⁹⁹⁹⁹Tc in oxidation state +I has
attracted attention after the discovery of an easy synthetic pathway to obtain the water soluble precursor \([^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+\). Whereas the carbonyls are inert, the labile water molecules can easily be exchanged by a wide variety of ligands. It is an ideal candidate for labelling biomolecules like peptides or proteins using N-heterocycles containing compounds as bifunctional chelating agents (5, 6, 7, 8, 9).

Characterization of \(^{99m}\text{Tc}\) complexes by means of spectroscopy and analytical methods is quite difficult because of the low concentration of technetium in the generator eluate. Thus, chromatography with \(\gamma\)-detection is used. Another possibility for analysis is the comparison with the corresponding, inactive Re-reference complexes (rhenium-185/187) since the coordination behaviour of Tc and Re are found to be quite similar. The reduction potential of rhenium is about 200 mV higher than that of technetium. This is often the reason why often harsher reaction conditions and better reducing agents are needed for the formations of stable complexes with rhenium. Furthermore, rhenium \textit{in vivo}, is more easily oxidized than technetium. In the periodic Table of elements technetium occupies position 43 and rhenium position 75 in the group VIIb. Their atomic radius and volume is quite similar. The Te-CO and Re-CO bond are quite equal and have the same bond length (8). For the need to investigate the pharmacological properties with the foreseen compound for human application, rhenium-185/187 can be used as non-radioactive substitute for the therapeutic \(\beta\)-emitting isotopes rhenium-186 und rhenium-188.

Rhenium-186/188 and technetium-99m are congeners, useful for therapeutic and diagnostic application, respectively (9,10). Both have attractive physical properties for application in therapeutic nuclear medicine. \(^{186}\text{Re}\) (\(E_{\beta_{\text{max}}}=1.07\ \text{MeV}\)) has a half-life of about 90 h which is suitable for agents requiring a longer time for tumour localization and normal tissue clearance. \(^{188}\text{Re}\) (\(E_{\beta_{\text{max}}}=2.11\ \text{MeV}\)) has a half-life of approximately 17 h, which makes it a better candidate for larger tumours and smaller biomolecules such as peptides, which are expected to show rapid tumour uptake and faster tissue clearance. \(^{188}\text{Re}\)- and \(^{99m}\text{Tc}\)-labelled radiopharmaceuticals have a similar chemistry (11). Moreover, both radionuclides have long lived mothernuclides (\(^{99}\text{Mo},\ t_{1/2}:\ 66\ \text{h};\ {^{188}}\text{W},\ t_{1/2}:\ 69\ \text{d})\), which allow the production of generators bringing about a widespread clinical application. Re-186 is not suited for a widespread clinical application because it is not cost effective. It is difficult to obtain carrier free in sufficient amounts by the neutron activation in a high flux reactor (normal production, \(^{185}\text{Re}(n,\gamma){^{186}}\text{Re}\)). On the other hand the production with protons (\(^{186}\text{W}(p,n){^{186}}\text{Re}\)) in a cyclotron results in low yield and impurities of various active Re-isotopes.
Table 1.1: Radionuclides suitable for diagnosis or therapy (12)

<table>
<thead>
<tr>
<th>Radiation</th>
<th>Nuclide</th>
<th>Physical half-life</th>
<th>$E_{\text{Max}}$ [MeV]</th>
<th>Maximum range of energy in tissue</th>
<th>$E_\gamma$ [MeV] (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma$</td>
<td>I-123</td>
<td>13.2 h</td>
<td>-</td>
<td>-</td>
<td>0.159 (83)</td>
</tr>
<tr>
<td>$\beta^+$</td>
<td>C-11</td>
<td>20 min</td>
<td>1.0</td>
<td>0.56 mm</td>
<td>0.511</td>
</tr>
<tr>
<td></td>
<td>F-18</td>
<td>110 min</td>
<td>0.6</td>
<td>0.44 mm</td>
<td>0.511</td>
</tr>
<tr>
<td></td>
<td>N-13</td>
<td>10 min</td>
<td>1.2</td>
<td>0.78 mm</td>
<td>0.511</td>
</tr>
<tr>
<td></td>
<td>O-15</td>
<td>2 min</td>
<td>1.7</td>
<td>2.10 mm</td>
<td>0.511</td>
</tr>
<tr>
<td>$\beta^-$</td>
<td>Ag-111</td>
<td>7.5 d</td>
<td>1.05</td>
<td>4.8 mm</td>
<td>0.342 (6)</td>
</tr>
<tr>
<td></td>
<td>Cu-67</td>
<td>2.6 d</td>
<td>0.57</td>
<td>1.8 mm</td>
<td>0.184 (48)</td>
</tr>
<tr>
<td></td>
<td>I-131</td>
<td>8.0 d</td>
<td>0.81</td>
<td>2 mm</td>
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</tr>
<tr>
<td></td>
<td>Lu-177</td>
<td>6.7 d</td>
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<td>1.5 mm</td>
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<tr>
<td></td>
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<td>3.8 d</td>
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<tr>
<td></td>
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<td>17.0 h</td>
<td>2.11</td>
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<tr>
<td></td>
<td>Y-90</td>
<td>2.7 d</td>
<td>2.27</td>
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<tr>
<td>Auger</td>
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<tr>
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<td></td>
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<td>0.03</td>
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<td>0.173 (88), 0.247 (94)</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>At-211</td>
<td>7.2 h</td>
<td>6.8</td>
<td>65 $\mu$m</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bi-212</td>
<td>1.0 h</td>
<td>7.8</td>
<td>70 $\mu$m</td>
<td>0.720 (7)</td>
</tr>
</tbody>
</table>

1.3 Bifunctional chelating agents (BFCA)

The targeting biomolecule has to be modified for labeling with radionuclides. This can be attained by introduction of so called bifunctional chelating agents (BFCA). This makes a stable bond between the radionuclide and the biological part (pharmacophor). The BFCA consists of three parts: a chelating unit for the coordination of the metallic radionuclide, a conjugation group for the covalent attachment to the carrier molecule and a linker. They should form stoichiometrically well-defined complexes that exhibit thermodynamic and kinetic stability with respect to dissociation and stabilization of the current oxidation state of
the radionuclide (13). They must exhibit high labelling efficiencies to achieve good radionuclide labelling yields (>90%) and consequently high specific activity of the radioconjugate. Two possibilities are of special interest: the pre- and postlabelling approach (14). The prelabelling approach involves the formation of a complex of the radionuclide with a bifunctional chelating agent (BFCA) and the subsequent conjugation of the radionuclide-BFCA complex to the carrier-molecule (15). In the postlabelling approach the BFCA is first attached to the carrier molecule to form a BFCA conjugate and then the radionuclide is coupled to the free chelating group of the BFCA (16). Promising BFCAs for the organometallic fac-[M(OH2)3(CO)3]+ (M = 99mTc, Re) are N2O-structures like 2-picolylamine-N,N-diacetic acid (PADA) (15) and Nα-histidinyl acetate (16) (Fig.1). In the special case of BBS with PADA only prelabelling is possible, postlabelling leads to loss of receptor affinity, which can be explained by possible binding of the 99mTc-(CO)3-core to His12.

![structures](image)

**Figure 1.1:** Structures of BFCAs for the fac-[99mTc(OH2)3(CO)3]+ and the corresponding complexes.

The pharmacokinetic profile of a radiopharmaceutical is not only defined by the carrier-molecule itself but also by the properties of the BFCA used. Size, denticity, all over charge and lipophilicity/hydrophilicity of the BFCA has a significant influence to the pharmacokinetics.
1.4 Peptides as carrier molecules

Peptides are molecules consisting of several amino acids linked together with peptide bonds. The size of peptides can vary from molecules with only two amino acids to as many as 50. Peptides exist in natural form and can be quite easily synthesized and modified. They exhibit high affinity to specific receptors, usually in the nano- or subnanomolar range. Another important characteristic is their usual lack of antigenicity. Peptides will usually not cross the blood-brain barrier and they are rapidly excreted from the body. As small and hydrophilic molecules, they show good blood vessel permeability, which allows an easy access to the tumour.

Peptides aimed for use as receptor binding molecules in nuclear medicine have several drawbacks, which need to be overcome. The attachment of a metal complex may interfere with the receptor or cause conformational changes in the binding sequence, which can lead to loss of receptor binding. Peptides are extremely sensitive to peptidases and proteases, but the metabolic stability of the radioconjugate is important for reaching the tumour.

The enzymatic degradation can be blocked or reduced by several ways. The resistance to exopeptidases can be achieved by end-capping (acetylation of the N-terminus, amidating or reducing the C-terminus, N to C cyclization). The resistance to endopeptidases can be achieved by the use of unnatural amino acids, insertion of peptidomimetic sequences or spacers, cyclization of peptides, substitution of peptide bonds and methylation of amide nitrogens.

Neuropeptides for tumour targeting

Neuropeptides represent a group of different families of molecules, which at extremely low concentration are known to act on multiple targets in the human body, namely the brain and the gastrointestinal tract as well as the endocrine system, kidneys, lungs and, the immune-, vascular-, and peripheral nervous system. Neuropeptides control and modulate the function of many key organs and metabolic processes. These peptides not only play prominent roles under normal conditions but also in pathological processes, such as inflammation, cancer and cancer progression. The discovery that certain tumour types overexpress receptors for peptide hormones dates back to the mid-1980s. The high level of expression of somatostatin receptors on various tumour cells has provided the molecular basis for successful use of radiolabelled somatostatin analogues as tumour tracers in nuclear medicine. This led to a wide evaluation of
Chapter 1

tumour receptor expression and development of increasing number of radiolabelled peptide analogues such as somatostatin, bombesin, cholecystokinin, gastrin, substance P, vasoactive intestinal peptide, neurotensin and neuropeptide-Y analogues (17-25). The most commonly used receptor-targeting agents are still few analogues of somatostatin, e.g. $^{111}$In-DTPA]octreotide was approved in June 1994 by the FDA for scintigraphy of patients with neuroendocrine tumours.

It is possible to use peptides as agonists or antagonists. The agonist binds to the receptor and can be internalized but activates the growth of tumour cells. On the other hand, the antagonists should be able to suppress the growth of these cells but is not internalized and remains on the cell surface. Thus far it's not known which one will be more successful for diagnostic or therapeutic applications.

The combination of analogues labelled with different radionuclides and multi-receptor targeting using a combination of peptides are also under investigations (26, 27).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Receptor</th>
<th>Biological effects</th>
<th>Main tumour target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatostatin (Octreotide, Octreotate)</td>
<td>$\text{sst}_2$</td>
<td>Inhibition of hormone and exocrine secretion</td>
<td>Insulinoma, small cell lung cancer</td>
</tr>
<tr>
<td>Vasoactive intestinal peptide</td>
<td>VPAC$_1$</td>
<td>Vasodilation, water and electrolyte secretion in gut</td>
<td>Gastrointestinal cancers (e.g. colorectal)</td>
</tr>
<tr>
<td>Cholecystokinin</td>
<td>CCK$_2$</td>
<td>Gallbladder contractions, exocrine pancreatic excretion</td>
<td>Medullary thyroid cancers</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>NTR$_1$</td>
<td>Vasoconstriction, increase in vascular permeability</td>
<td>Pancreatic cancers</td>
</tr>
<tr>
<td>Substance P</td>
<td>NK$_1$</td>
<td>Hypotension, salivary secretion</td>
<td>Glial tumours</td>
</tr>
<tr>
<td>Bombesin/GRP</td>
<td>GRP</td>
<td>Gut hormone release</td>
<td>prostate and breast cancers</td>
</tr>
</tbody>
</table>
Bombesin-like peptides

Bombesin (BBS) is a tetradecapeptide (pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH2), which was first isolated from amphibians with an N-terminal pyroglutamate residue and C-terminal amidation. Numerous polypeptides with structurally related carboxyl termini were subsequently isolated from amphibians and classified into three subfamilies (bombesin, ranatensin, and phyllolitorin) based upon the sequence of the last three residues in their amidated carboxy-terminal domains. To date, two bombesin-like peptides have been isolated from mammalian tissue: neuromedin B (NMB) in the ranatensin subfamily containing 10 amino acids and gastrin-releasing peptide (GRP) in the bombesin subfamily containing 27 amino acids. They possess structural homology with the C-terminal octapeptide of BBS. BBS, NMB, and GRP exert their effects on target cells by binding to surface G-protein coupled receptors characterized by the typical configuration of seven transmembrane domains. Four receptor subtypes are known: The GRP-preferring receptor (GRP-R), the NMB-preferring receptor (NMB-R), bombesin receptor subtype 3 (bb3), and bombesin receptor subtype 4 (bb4). Although the four subtypes are about 50% identical when their primary amino acid sequences are aligned, the pharmacology of (bb3) is significantly different from the pharmacology of the others. GRP-R, NMB-R, and bb4 all bind bombesin with affinities in the nanomolar range, whereas bb3 binds bombesin with much lower affinity (> 10 μM). The GRP-receptor is frequently expressed on tumours, where prostate and breast cancers are of special interest. The other three subtypes have been poorly examined in human tissues. The bb3 expression is particularly relevant in lung carcinoids and other neuroendocrine lung tumours, whereas gastrointestinal carcinoids preferably express NMB receptors (28, 29).

The physiological role of GRP includes stimulation of enzyme secretion from exocrine glands or stimulation of release of a series of gastrointestinal peptide hormones. It has been established by numerous studies over the past two decades that GRP and its interaction with the GRP receptor promote tumour growth in a number of human cancer cell lines both in culture and nude mice xenografts (30, 31). These findings stimulated the search for potent synthetic bombesin/GRP analogues for the treatment of GRP receptor positive tumours. They may be used as carriers to direct diagnostic or therapeutic radionuclides to GRP-R expressing neoplastic cells with high specificity (21, 32).
1.5 Aim of the project

The neuropeptide bombesin (BBS) and its radiolabelled analogues have great potential for tumour targeting, either for diagnosis (e.g. with $^{99m}$Tc) or therapy (e.g. with $^{188}$Re) of GRP-R positive tumours, especially breast and prostate carcinomas. Since natural bombesin is rapidly metabolised in vivo, this project focused on the development of new, stable bombesin-analogues based on the fragment BBS(7-14). The main goal was the reduction of enzymatic degradation with retention of good binding affinity and improved biodistribution. Thus, unnatural, bulky amino acids, different alkylamides and additional spacers had to be used as addition or substitution of existing parts of BBS(7-14). Accordingly the influence of these changes on the stability of the labelled BBS analogues (in plasma or cell culture) and the receptor affinity were tested first. If the above-mentioned parameters showed reasonable values biodistribution studies are then performed.

A second aim was the development of a radiopharmaceutical applying the “tricarbonyl” method, which was shown to have a great advantage when used with technetium as well as with rhenium, since both metals form robust complexes of almost identical size and structure (octahedral complexes, oxidation state +1). Thus, most experiments could be done with technetium instead of rhenium and had the advantage of a much lower radiation burden (and lower costs). The only disadvantage of the Tc(I)-tricarbonyl method compared to Tc(V)-labelling is lengthy time, which is needed to prepare the labelled compounds.

Further, the advantage of the “tridentate His”, which was already proposed as favourable chelator for binding the Tc-tricarbonyl core, needed to be confirmed.
1.6 References


8. Roger Alberto, Roger Schibli, Daniela Angst, P. August Schubiger, Ulrich Abram, Sonja Abram, Th. A. Kaden. Application of technetium and rhenium carbonyl chemistry to nuclear medicine. Preparation of [NEt4][TcCl3(CO)3] from [NBu4][TcO4] and structure of [NEt4][Tc2(μ-Cl)3(CO)6]; structures of the model complexes [NEt4][Re2(μ-OEt)2(μ-OAc)(CO)6] and [ReBr{-(CH2S(CH2)2Cl}2(CO)3]. *Transition Metal Chemistry* 1997; 22: 597-601.


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CHAPTER 2

New stabilised bombesin-analogues as potential tumour imaging agents
2.1 General Process of peptide evaluation

The development of peptides started with the synthesis of a series of 25 BBS analogues (Table 2.1) and the determination of the ability of the unlabelled peptides to displace the $^{125}$I-BBS from the GRP receptor. The analogues with low affinity (IC$_{50} > 1000$ nM) were excluded from further studies with a few exceptions. These being BBS-II, which is the analogue with the non-modified chain of amino acids and thus the reference for many experiments and, at least one example of each type of substitution. This last point was important to elucidate the influence of the site of change on the metabolic stabilisation.

The remaining BBS analogues were labelled with the $^{99m}$Tc(CO)$_3$ core and the stability in plasma was analyzed as well as the binding to PC-3 cells according to Lindmo. Peptides with promising results were selected and further investigated. Scatchard analysis was performed to determine receptor affinity, internalization into tumour cells was measured and the stability against degradation in PC-3 tumour cells was investigated. A selection of the labelled BBS analogues was injected into nude mice bearing PC-3 tumour xenografts for the evaluation of the biological studies.

Based on the results of these studies further analogues were designed (Table 2.2) and the synthesis was done by Irfan Khan (group of Prof. A.G. Beck Sickinger, Leipzig) or Véronique Maes (Group of Prof. D.A. Tourwé, Brussels). The in vitro characterisation of labelled and unlabelled compounds was generally performed to decide whether an in vivo study with nude mice could be justified.
2.2 Peptide Synthesis

The peptides were obtained by solid phase method using the Fmoc/tBu-strategy. In order to obtain the C-terminal amide, Rink Amide resin was used and Chloro-trityl-chlorid for the alkyl amide peptide. The synthesis of the different peptide analogues was done on an automated robot system (Syro II, MultiSyntech, Bochum, Germany). Fmoc-amino acids were coupled with eight-fold excess in duplicate. The coupling of the ligand (NaHis)Ac was performed in two steps (Scheme 2.1): In a separate vial 6 equivalents bromo acetic acid (the amounts are normalized to the amount of resin used) and 3 equivalents DIC were dissolved in 0.5 ml DCM. After 20 min the obtained anhydride solution was added to a mixture of 1 equivalent resin and 3 equivalents DIPEA. Two hours later the resin was washed with DMF, then 3 equivalents of N-trityl-O-tetritbutyl histidine and 3 equivalents of DIPEA dissolved in 2 ml DMF were added and the mixture was stirred for 24 h. The amide peptides were cleaved from the resin, by addition of TFA and ethandithiol/thioanisol (90:3:7) and allowed to stand for 3 h. After cleavage the peptides were precipitated from cold diethylether, collected by centrifugation, purified by preparative HPLC and lyophilized from the water/tert.-butylalcohol (3:1). Purity and identification of the peptides were analysed by HPLC and MS.

In the case of the alkyl amides we first cleaved the resin without the protecting groups for coupling the amines. Since the linker is acid-labile the peptide can be split off with a very small amount of an acid solution. Therefore we added 25 µl glacial acetic acid, 25 µl trifluorethanol and 200 µl DCM to the resin with the already synthesised peptides. After 1.5 h the solution was pushed through the resin and washed with DCM continuously until the smell of acid disappeared from the resin. Hexane was then used to eliminate the remainder of glacial acetic acid. One equivalent peptide was dissolved in cold DCM (20 mM) and mixed with 5 equivalent amine (isobutylamine or propylamine), 5 equivalent DIPEA and 5 equivalent PyBop. The solution was kept on ice for 5 min and then shaken for 3 h at 20°C. The final procedures were the cleaving of the protecting groups with TFA and ethandithiol/thioanisol (3:7), precipitation, centrifugation, purification and lyophilisation, as described above.
Scheme 2.1: Synthesis of the His-chelator on the amino terminus of the BBS analogues.
Table 2.1: Sequence of BBS-II (unmodified) and new stabilized BBS-analogues obtained by solid phase method using Fmoc-strategy. (CyHAla = Cyclohexyl alanine)

<table>
<thead>
<tr>
<th>BBS-analogues</th>
<th>Chelator</th>
<th>7Aa</th>
<th>8Aa</th>
<th>9Aa</th>
<th>10Aa</th>
<th>11-12Aa</th>
<th>13Aa</th>
<th>14Aa</th>
<th>Amide</th>
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<td>BBS-II</td>
<td>(NαHis)Ac</td>
<td>Gln</td>
<td>Trp</td>
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Table 2.2: The second series of BBS-analogues

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<td>Val</td>
<td>Gly-His</td>
<td>Phe</td>
<td>Nle</td>
<td>NH₂</td>
</tr>
<tr>
<td>BBS-XXX</td>
<td>(NaHis)Ac</td>
<td>Gln Trp</td>
<td>Ala</td>
<td>Val</td>
<td>Gly-His</td>
<td>CyHAla</td>
<td>Nle</td>
<td>NH₂</td>
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<td>BBS-31</td>
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<td>Ala</td>
<td>Val</td>
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<td>Val</td>
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<td>CyHAla</td>
<td>des-Met</td>
<td>NH₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBS-33</td>
<td>(NaHis)Ac</td>
<td>Glu Trp</td>
<td>Ala</td>
<td>Val</td>
<td>Gly-His</td>
<td>CyHAla</td>
<td>des-Met</td>
<td>NH₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BBS-34</td>
<td>(NaHis)Ac</td>
<td>D-Phe</td>
<td>Gln</td>
<td>Trp</td>
<td>Ala</td>
<td>Val</td>
<td>Gly-His</td>
<td>Leu</td>
<td>des-Met</td>
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<tr>
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<td>Gln</td>
<td>Trp</td>
<td>Ala</td>
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<td>CyHAla</td>
<td>des-Met</td>
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<td>Ala</td>
<td>Val</td>
<td>Gly-His</td>
<td>CyHAla</td>
<td>des-Met</td>
<td>Ethylamide</td>
<td></td>
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<tr>
<td>BBS-37</td>
<td>(NaHis)Ac</td>
<td>βAla-βAla</td>
<td>Gln</td>
<td>Trp</td>
<td>Ala</td>
<td>Val</td>
<td>Gly-His</td>
<td>Leu</td>
<td>Met</td>
<td>NH₂</td>
</tr>
<tr>
<td>BBS-38</td>
<td>(NaHis)Ac</td>
<td>βAla-βAla</td>
<td>Gln</td>
<td>Trp</td>
<td>Ala</td>
<td>Val</td>
<td>Gly-His</td>
<td>CyHAla</td>
<td>Nle</td>
<td>NH₂</td>
</tr>
<tr>
<td>BBS-39</td>
<td>(NaHis)Ac</td>
<td>*</td>
<td>Gln</td>
<td>Trp</td>
<td>Ala</td>
<td>Val</td>
<td>Gly-His</td>
<td>CyHAla</td>
<td>Nle</td>
<td>NH₂</td>
</tr>
<tr>
<td>BBS-40</td>
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<td>Ala</td>
<td>Val</td>
<td>Gly-His</td>
<td>3homoleu</td>
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<tr>
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<td>Val</td>
<td>Gly-His</td>
<td>Leu</td>
<td>Nle</td>
<td>NH₂</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(* NH-CH₂-CH₂-O-CH₂-CH₂-O-CH₂CO)

Remark: Roman numbers were used for the first BBS-analogues to prevent misunderstanding when the whole or a fragment of BBS was mentioned (e.g. BBS(1-14) or BBS(7-14)). On the other hand roman numbers > XXX are not easy to read and thus arabic numbering was chosen. The second series of BBS-analogues were gifts either from A.G.Beck-Sickinger or D.Tourvé.

2.3 Labelling

The peptides were labelled for pharmagological investigations with $^{99m}$Tc(CO)$_3$ [1,2,3]. Approximately 1 ml of a $^{99m}$TcO$_4$ generator eluate in saline (up to about 5 GBq) was added to a mixture of 4.5 mg sodium-boranocarbonate, 2.9 mg borax, 9 mg potassium-sodium tartrate tetrahydrate and 7-8 mg sodiumcarbonate. This solution was heated for 20 min at 100°C. After cooling, the tricarbonyl-solution was neutralized to pH 6.5 with a mixture of 1 M HCl and 0.6 M phosphate buffer. 500 µl of it was mixed with 15 µl of the peptide solution (1mM) and heated for 1 h at 75°C. The labelling was controlled with RP-HPLC.
2.4 Binding assays

We first investigated the affinity of all analogues on prostate carcinomas PC3 cells (the human prostate adenocarcinoma cell line PC-3 was purchased from European Collection of Cell Culture). Cells were maintained in DMEM GLUTAMAX-I supplemented with 1-10% fetal calf serum, 100 IU/ml penicillin G sodium, 100 µg/ml streptomycin sulphate, 0.25 µg/ml amphotericin B. Cell culture was incubated at 37°C in an atmosphere containing 7.5% CO₂. The cells were passaged weekly after detaching them with trypsin/EDTA (0.25%).

*Inhibition studies:* The IC₅₀ value represents the concentration of unlabelled peptide, needed to displace 50% of the binding of the iodinated BBS. PC-3 cells at confluence were placed in 48-well plates (250'000 cells/well). A special binding buffer including protease inhibitors was used, composition as follows: 50 mM HEPES, 125 mM NaCl, 7.5 mM KCl, 5.5 mM MgCl₂, 1 mM EGTA, 5 g/l BSA, 2 mg/l chymostatin, 100 mg/l soybean trypsin inhibitor, 50 mg/l bacitracin. The cells were incubated for 1 h at 37°C with 15'000-25'000 cpm [¹²⁵I-Tyr⁴]BBS(1-14) per well and increasing concentrations of the different unlabeled analogues (0-3'000 nM). The cells were then washed twice with cold PBS 1X and solubilized with 400µl (2x) of 1 N NaOH at 37°C. The bound activities were determined in a γ-counter. IC₅₀ values for the displacement of bound [¹²⁵I-Tyr⁴]BBS by the different analogues were calculated by non-linear regression analysis using GraphPad Prism™. Experiments were performed twice in triplicate. The binding experiments according to Lindmo allowed to judge the quality and yielded the estimated dissociation constant of the labelled peptides. Therefore we used a factor corresponding to the amount of receptors on PC-3 cells, which was determined with earlier synthesised bombesin analogues. We based it on the fact that all PC-3 cells have the same number of receptors. Analogues with a good IC₅₀ value were tested.
**Affinity test according to Lindmo:** PC-3 cells were placed at different concentrations (2 - 0.0625 million cells/well) in 12-well plates. They were incubated for 2 h at 37°C with 1 Mio cpm bombesin analogues (250 μl). Nonspecific binding was determined with 1 μmol/l unlabelled bombesin (8-14). After incubation the supernatant was discarded and the cells washed twice with PBS. To detach the cells the wells were washed 2 times with 500 μl 1N NaOH and the activity measured in a γ-counter. The same method was used to test the affinity of BBS-38 to the cell lines DU-145 and MDA-MB231. We obtained a Kd of 6 ± 1.7 nM for the DU-145 cells, whereas no binding was found with the MDA-MB 231 cells. Experiments were done at least twice in duplicate.

**Saturation studies:** PC-3 cells at confluence were placed in 48-well plates (1 x 10^6 cells/well). Cells were incubated with increasing concentrations (0.001-1 nM) of the ^99m^Tc(CO)3-BBS analogues for 1 h at 37°C in triplicate (final volume, 0.2 ml per well). The concentration of total technetium (^99 + ^99m^Tc), estimated according to Bauer and Pabst [4], was equivalent to 0.002-2 MBq ^99m^Tc activity per well. After washing two times with cold PBS 1X, the cells were solubilized with 400 μl (2x) of 1 N NaOH at 37°C. The bound radioactivity was measured in the γ-counter. Nonspecific binding was determined with 1μM unlabelled BBS(8-14). The protein content was determined in a BIO RAD microplate reader (570 nm) using the Micro BCA Protein Assay Reagent from Pierce. We calculated B_max for BBS II and the cell lines PC-3, ZR75 and BT474. The highest value was obtained for PC-3 with 1660 fmol/mg protein. For the cell line ZR75 we calculated 37.5 fmol/mg protein and for BT474 3 fmol/mg protein. No further tests were done with the BT474 and ZR75 cell lines because of the low values obtained.

### 2.5 Metabolic stability

**Human plasma:** the stability of the ^99m^Tc(CO)3-BBS analogues was tested in plasma obtained from human blood (in heparinized tubes) from healthy volunteers. The peptides were incubated at a final concentration of 3 to 4 MBq/ml (1.5-2 pmol/ml) for various time intervals (up to 24 h) at 37°C. After incubation, proteins were precipitated with acetonitrile/ethanol (1:1) and TFA (0.01%) and centrifuged (10 min., 2 x 10^4 g) at 4°C. The supernatant was filtered and analyzed by RP-HPLC.

**Whole blood (BBS II and BBS III):** the used blood must not be stored for longer than 24 hours at 4 °C. Samples of 1 ml were incubated with 0.5-1.5 MBq for different time periods up to 2h
at 37 °C. After centrifugation (4 min, 4°C, 2x10^4 g) and precipitation the samples were analysed by RP-HPLC.

**Table 2.3: Stability of \([^{99m}\text{Tc}]-\text{BBS II}\) and \([^{99m}\text{Tc}]-\text{BBS III}\) in human plasma and whole human blood. Data are expressed as percentage of intact radioconjugate. (The error of all values were about 4-5%).**

<table>
<thead>
<tr>
<th>Incubation time [min]</th>
<th>Intact BBS-II [%]</th>
<th>Incubation time [min]</th>
<th>Intact BBS-III [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>plasma</td>
<td>blood</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>77</td>
<td>91</td>
<td>5</td>
</tr>
<tr>
<td>20</td>
<td>63</td>
<td>79</td>
<td>10</td>
</tr>
<tr>
<td>40</td>
<td>44</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>60</td>
<td>30</td>
<td>42</td>
<td>60</td>
</tr>
<tr>
<td>120</td>
<td>12</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

We additionally investigated the influence of peptidase inhibitors (Table 2.4) in plasma, blood and in biodistribution studies for BBS-II and BBS-III. Choices of the different peptidase inhibitors were made from information found in literature [5,6,7,8,9].

**Peptidase inhibitors (plasma):** samples of 500 μl plasma were incubated at 37°C for 10-15 min with the following inhibitors: phosphoramidon (100 μM), prolin-isoleucin (50 mM), ACE-inhibitor (100 μM), bestatin (100 μM), 1,10 phenanthroline (1 mM), bacitracin (100 μM) and soyabean trypsin inhibitor (>7000 units/mg; 50 μg/500 μl). The concentration of the inhibitors is in relation to the end-volume of 500 μl. Subsequently we added 25 μl (0.5-1.5 MBq) of the labelled peptides and incubated for 60 min. The final procedures were done as described for human plasma.

**Peptidase inhibitors (blood):** we tested the influence of phosphoramidon (100 μM), prolin-isoleucin (50 mM) and the ACE-inhibitor (100 μM). The procedure is the same as described with plasma.

**Peptidase inhibitor (biodistribution):** the influence of enalapril was tested (15mg/kg). It was injected (p.i.) 45 min before the peptides. (detailed description and results are given under biodistribution)
Table 2.4: Peptidases and their inhibitors

<table>
<thead>
<tr>
<th>Enzym</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutralendopeptidase EC 3.4.24.11</td>
<td>Phosphoramidon</td>
</tr>
<tr>
<td>Aminopeptidase N EC 3.4.11.2</td>
<td>Bestatin</td>
</tr>
<tr>
<td>Metallendopeptidase EC 3.4.24</td>
<td>1,10-Phenanthrolin</td>
</tr>
<tr>
<td>Endopeptidase EC 3.4.24.16</td>
<td>Prolin-Isoleucin</td>
</tr>
<tr>
<td>Angiotensin Converting Enzyme (ACE)</td>
<td>ACE-Inhibitor*</td>
</tr>
<tr>
<td>Prolylendopeptidase u.a.</td>
<td>Bacitracin</td>
</tr>
<tr>
<td>Trypsin u.a.</td>
<td>Soybean Trypsin Inhibitor</td>
</tr>
</tbody>
</table>

* pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro

Figure 2.2. Degradation of \(^{99m}\text{Tc}\)-BBS analogues in human plasma in presence of enzyme inhibitors. control: no inhibitor, PA: Phosphoramidon, 1,10-Ph: 1,10-phenanthroline, P-I: proline isoleucine, ACE I: angiotensin converting enzyme inhibitor

About 70% of BBS II was degraded after 60 min incubation in plasma without the addition of inhibitors. Phosphoramidon (100 µM), bacitracin (100 µM), soyabean trypsin inhibitor (50 µg/500 µl) and bestatin (100 µM) couldn't influence the metabolism. The effect of 1,10-phenanthroline (1 mM) was low and not significant. Pro-Ile (50 mM) increased the stability of the intact peptide to 88% and the ACE-inhibitor (100 µM) to 93% of intact peptide. The combination of both increased the intact peptide part to 100%.

The only inhibitor, which showed an influence on degradation of BBS III, was the ACE-inhibitor (92% intact).
The inhibitors Pro-Ile and ACE-inhibitor showed a similar effect in blood and in plasma. In contrast, phosphoramidon reduced the speed of metabolism in blood, but not in plasma. Thus we can conclude that the neutral endopeptidase EC 3.4.24.11 has a bigger influence in metabolism of BBS-analogues in blood than in plasma.

**Figure 2.2:** Inactivation of bombesin by endopeptidases. (arrows: known sites of cleaving) The bold-marked amino acids represent the binding part of the peptide.

**Human prostate carcinomas PC 3 cells:** the stability in cell culture was evaluated with $2 \times 10^6$ cells. $^{99m}$Tc(CO)$_3$-BBS analogues were added to the cell suspension in PBS (final concentration: 3 to 4 MBq/ml) and incubated at 37°C. At different time points (up to 1 h) cells were lysed by precipitation as described for human plasma and filtered. The filtrates were analyzed by RP-HPLC. All experiments were carried out 2-3 times.
Table 2.5: Binding of the different BBS analogues were tested on human prostate carcinoma PC-3 cells and the half-lives were investigated in human plasma and PC-3 cells. The affinity of the unlabelled analogues is represented by the inhibitory concentration of 50% (IC₅₀) and the affinity of the labelled analogues is represented with the Kᵰ obtained by Lindmo- and Scatchard-experiments. Most BBS-analogues with a IC₅₀ > 1000 nM are not shown. Data are mean ± SD of 2-3 times in triplicate. Metabolic stability of the ⁹⁹mTc(CO)₃-BBS-analogues in human plasma and PC-3 cells at 37°C, analyzed by RP-HPLC. Data are mean ± SD of 2-3 times.

<table>
<thead>
<tr>
<th>BBS-analogues</th>
<th>IC₅₀ (nM)</th>
<th>Kᵰ(Lindmo) (nM)</th>
<th>Kᵰ(Scatchard) (nM)</th>
<th>t₁/₂ (plasma) (h)</th>
<th>t₁/₂ (PC-3 cells) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBS-II</td>
<td>1.9 ± 0.7</td>
<td>0.23 ± 0.29</td>
<td>0.165 ± 0.064</td>
<td>0.5 ± 0.2</td>
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</tr>
<tr>
<td>BBS-III</td>
<td>2.3 ± 1.8</td>
<td>0.93 ± 0.12</td>
<td>0.43 ± 0.124</td>
<td>0.2 ± 0.15</td>
<td>-</td>
</tr>
<tr>
<td>BBS-IV</td>
<td>5.1 ± 1.4</td>
<td>0.44 ± 0.28</td>
<td>0.015 ± 0.07</td>
<td>16 ± 2.8</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>BBS-V</td>
<td>131.9 ± 49.2</td>
<td>0.44 ± 0.33</td>
<td>-</td>
<td>0.4 ± 0.18</td>
<td>-</td>
</tr>
<tr>
<td>BBS-VI</td>
<td>56 ± 11.2</td>
<td>0.34 ± 0.1</td>
<td>-</td>
<td>3 ± 1.4</td>
<td>-</td>
</tr>
<tr>
<td>BBS-VII</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>-</td>
<td>3 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>BBS-VIII</td>
<td>387.4 ± 20.5</td>
<td>-</td>
<td>6 ± 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BBS-IX</td>
<td>14.2 ± 3</td>
<td>0.3 ± 0.3</td>
<td>0.085 ± 0.07</td>
<td>6 ± 1.4</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>BBS-X</td>
<td>266.1 ± 9.5</td>
<td>0.99 ± 1</td>
<td>-</td>
<td>18 ± 2.8</td>
<td>-</td>
</tr>
<tr>
<td>BBS-XI</td>
<td>&gt; 1000</td>
<td>-</td>
<td>-</td>
<td>5 ± 1.41</td>
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<tr>
<td>BBS-XII</td>
<td>619.5 ± 40</td>
<td>11.9 ± 10.3</td>
<td>-</td>
<td>&gt; 24</td>
<td>-</td>
</tr>
<tr>
<td>BBS-XV</td>
<td>352.1 ± 84.9</td>
<td>-</td>
<td>-</td>
<td>&gt; 24</td>
<td>-</td>
</tr>
<tr>
<td>BBS-XVIII</td>
<td>662.1 ± 173</td>
<td>-</td>
<td>20 ± 5.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BBS-XXX</td>
<td>15.7 ± 6</td>
<td>1.6 ± 1.2</td>
<td>0.843 ± 0.836</td>
<td>16 ± 2.8</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>BBS-33</td>
<td>&gt; 1000</td>
<td>-</td>
<td>-</td>
<td>0.2 ± 0.05</td>
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<tr>
<td>BBS-34</td>
<td>21.66 ± 9.7</td>
<td>1.27 ± 0.3</td>
<td>-</td>
<td>6 ± 1.41</td>
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<tr>
<td>BBS-35</td>
<td>79.1 ± 56.8</td>
<td>1.41 ± 0.28</td>
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<td>16.5 ± 2.12</td>
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<tr>
<td>BBS-36</td>
<td>486 ± 428</td>
<td>4.57 ± 0.98</td>
<td>-</td>
<td>6 ± 2.8</td>
<td>-</td>
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<tr>
<td>BBS-37</td>
<td>1.8 ± 0.5</td>
<td>0.34 ± 0.4</td>
<td>-</td>
<td>0.75 ± 0.3</td>
<td>-</td>
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<tr>
<td>BBS-38</td>
<td>5.05 ± 1.7</td>
<td>0.45 ± 0.28</td>
<td>0.18 ± 0.1</td>
<td>10 ± 1.41</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>BBS-39</td>
<td>8.9 ± 5.0</td>
<td>0.22 ± 0.14</td>
<td>0.26 ± 0.01</td>
<td>8 ± 2.82</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>BBS-40</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>-</td>
<td>&gt; 16</td>
<td>-</td>
</tr>
<tr>
<td>BBS-41</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>-</td>
<td>5 ± 1.41</td>
<td>-</td>
</tr>
</tbody>
</table>
2.6 Internalization and Externalization

**Internalization.** PC-3 cells at confluence were placed in 6-well plates (about $1 \times 10^6$ cells/well). Cells were incubated with approximately 4 kBq of $^{99m}$Tc(CO)$_3$-BBS analogues in culture medium for 5, 15, 30, 60 and 120 min at 37°C to allow binding and internalization (total final volume 1 ml per well). Nonspecific internalization was evaluated in the presence of 1µM unlabeled BBS(8-14). After the various incubation times, cells were washed 3 times with PBS 1X to discard unbound peptide. Surface-bound activity was removed twice with a 5 min acid wash (50 mM glycine-HCl, 100 mM NaCl, pH 2.8, 600 µl per well) at room temperature. Cells were solubilized by incubation at 37°C with 600 µl (2x) of 1 N NaOH per well to determine internalized radioligand. Surface bound and internalized activities were measured in a γ-counter. Results were expressed as percentage of total activity in the cells (surface bound and internalized). All experiments were carried out 2-3 times in triplicate.

**Table 2.6:** Internalized activity of $^{99m}$Tc(CO)$_3$BBS-analogues in PC-3 cells at 37°C. Data are given as percentage of acid-resistant activity related to total activity in cells (surface-bound and internalized). Each point represents the mean of 2-3 experiments in triplicate.

<table>
<thead>
<tr>
<th>BBS-analogues</th>
<th>Internalized activity (%) (after 15 min)</th>
<th>Internalized activity (%) (after 120 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBS-II</td>
<td>70 ± 7</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>BBS-IV</td>
<td>77 ± 1.4</td>
<td>82.5 ± 6.5</td>
</tr>
<tr>
<td>BBS-VI</td>
<td>73.5 ± 5</td>
<td>83 ± 4.2</td>
</tr>
<tr>
<td>BBS-IX</td>
<td>79 ± 4.2</td>
<td>82.6 ± 2</td>
</tr>
<tr>
<td>BBS-X</td>
<td>59 ± 4.2</td>
<td>62 ± 8.5</td>
</tr>
<tr>
<td>BBS-XII</td>
<td>65 ± 4.2</td>
<td>65 ± 4.2</td>
</tr>
<tr>
<td>BBS-XXX</td>
<td>74 ± 2.8</td>
<td>83.7 ± 11.8</td>
</tr>
<tr>
<td>BBS-33</td>
<td>45 ± 5.2</td>
<td>45 ± 5.2</td>
</tr>
<tr>
<td>BBS-34</td>
<td>11 ± 2.8</td>
<td>27 ± 2.8</td>
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<tr>
<td>BBS-35</td>
<td>7.5 ± 3.5</td>
<td>30.5 ± 7.7</td>
</tr>
<tr>
<td>BBS-36</td>
<td>13.5 ± 2.1</td>
<td>25 ± 1.4</td>
</tr>
<tr>
<td>BBS-37</td>
<td>66 ± 5.6</td>
<td>79 ± 5.6</td>
</tr>
<tr>
<td>BBS-38</td>
<td>73 ± 1.4</td>
<td>86 ± 5.6</td>
</tr>
<tr>
<td>BBS-39</td>
<td>70.5 ± 3.5</td>
<td>83 ± 1.4</td>
</tr>
</tbody>
</table>
These findings show that cell associated BBS undergoes internalization with remarkable
differences between the different bombesin analogues. Most of the tested radiolabelled BBS
compounds showed to be rapidly internalized. After 15-30 min internalization reached a
maximum and did not change significantly over a period of 120 min. The compounds BBS-
34, BBS-35 and BBS-36 were only slightly internalized, which indicates that these three
analogues are antagonists of the GRP-receptors. The internalization was GRP receptor-
specific since it could be inhibited with 10^{-6} M BBS(8-14).

Table 2.7. Externalized activity of the different $^{99m}$Tc(CO)$_3$-BBS analogues by PC-3 prostate
tumour cells at 37°C. Testing was carried out after 45 min preincubation to allow maximal
binding and internalization. Data represent mean ± SD of 2-3 experiments.

<table>
<thead>
<tr>
<th>BBS-analogues</th>
<th>Externalized activity (%) (after 60 min)</th>
<th>Externalized activity (%) (after 300 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBS II</td>
<td>43 ± 12</td>
<td>92 ± 2.8</td>
</tr>
<tr>
<td>BBS IV</td>
<td>43 ± 11.3</td>
<td>69 ± 8.5</td>
</tr>
<tr>
<td>BBS VI</td>
<td>63 ± 3.5</td>
<td>92 ± 2.8</td>
</tr>
<tr>
<td>BBS IX</td>
<td>52 ± 5</td>
<td>77 ± 9.2</td>
</tr>
<tr>
<td>BBS X</td>
<td>74 ± 9.8</td>
<td>90 ± 4.2</td>
</tr>
<tr>
<td>BBS XII</td>
<td>86 ± 8.5</td>
<td>81 ± 0.7</td>
</tr>
<tr>
<td>BBS XXX</td>
<td>52 ± 2.1</td>
<td>80 ± 5.6</td>
</tr>
<tr>
<td>BBS 33</td>
<td>96 ± 1.4</td>
<td>98 ± 1.4</td>
</tr>
<tr>
<td>BBS 34</td>
<td>87 ± 2.1</td>
<td>96 ± 1.4</td>
</tr>
<tr>
<td>BBS 35</td>
<td>87 ± 1.4</td>
<td>94 ± 2.8</td>
</tr>
<tr>
<td>BBS 36</td>
<td>92 ± 3.5</td>
<td>95 ± 2.8</td>
</tr>
<tr>
<td>BBS 37</td>
<td>24 ± 5.7</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>BBS 38</td>
<td>44 ± 23.5</td>
<td>77 ± 11</td>
</tr>
<tr>
<td>BBS 39</td>
<td>43 ± 1.4</td>
<td>77 ± 1.4</td>
</tr>
</tbody>
</table>

Externalization. PC-3 cells at confluence were placed in 6 cm diameter Petri dishes. Cells
were incubated with about 20 kBq of $^{99m}$Tc(CO)$_3$-BBS analogues in culture medium for 45
min at 37°C to allow maximal internalization into the cells (total final volume 5ml per dish).
The supernatant was discarded and the cells were washed twice with PBS 1X, these were then
incubated again at 37°C with 5ml culture medium for 0, 30, 60, 150 and 300 min. The activity
in the supernatant was measured to determine released activity from the cells. After washing
twice with PBS 1X, surface-bound activity was removed by the acid wash (50 mM glycine-
HCl, 100 mM NaCl, pH 2.8, 0.75 ml per dish) for 5 min, twice, at room temperature. Finally, the amount of remaining internalized activity was recovered by solubilizing cells at 37°C with 1 ml (2x) of 1 N NaOH per dish. Externalized, surface-bound and internalized activities were measured in a γ-counter. Experiments were performed 2-3 times. A rapid time-dependent externalization was found for all the different analogues. HPLC analysis of the supernatant showed that the externalized activity consisted of metabolites.

2.7 Biodistribution studies

Biodistribution studies and tumour uptake of BBS-X and BBS-XII. The biodistributions for BBS-IV, BBS-IX, BBS-XXX, BBS-34 and BBS-39 are described in detail in chapters 3 and 4. All animal experiments were conducted in compliance with the Swiss animal protection laws and with the ethical principles and guidelines for scientific animal trials established by the Swiss Academy of Medical Sciences and the Swiss Academy of Natural Sciences. Studies were performed with 6 to 8 weeks old female CD-1 nu/nu mice (20-25 g), purchased from Charles River Laboratories (Sulzfeld, Germany). For the induction of tumour xenografts, PC-3 cells were subcutaneously injected at a concentration of 5 x 10^6 cells/mouse and allowed to grow for ten days. On the day of the assay the mice received the radioactive conjugate (0.4 to 1 MBq/mouse) intravenously into the tail vein. They were sacrificed by cervical dislocation and dissected. The biodistribution for BBS-X and BBS-XII was evaluated at different post-injection times (1.5h and 5h). Tumours and other tissues (blood, heart, lung, spleen, kidneys, stomach, pancreas, ileum, colon, liver, muscle, bone and brain) were removed and weighed. The amount of radioactivity was determined with a γ-counter. Results were expressed as percentage of injected dose per gram of tissue (% ID/g) referred to total injected activity.
Table 2.8: Biodistribution of the $^{99m}$Tc(CO)$_3$BBS analogues (0.4-1 MBq/mouse) in nude mice with PC-3 xenografts. Results are expressed as percentage of injected dose per gram of tissue (% I.D./g) ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>BBS-X</th>
<th></th>
<th>BBS-XII</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5 h</td>
<td>5 h</td>
<td>1.5 h</td>
<td>5 h</td>
</tr>
<tr>
<td>Blood</td>
<td>0.97 ± 0.22</td>
<td>0.53 ± 0.09</td>
<td>0.49 ± 0.07</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>0.37 ± 0.09</td>
<td>0.18 ± 0.04</td>
<td>0.15 ± 0.03</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Lung</td>
<td>0.69 ± 0.18</td>
<td>0.32 ± 0.05</td>
<td>0.33 ± 0.03</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.29 ± 0.06</td>
<td>0.17 ± 0.03</td>
<td>0.16 ± 0.03</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.09 ± 0.62</td>
<td>1.13 ± 0.07</td>
<td>1.54 ± 0.47</td>
<td>0.35 ± 0.03</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.33 ± 0.07</td>
<td>0.16 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.02 ± 0.11</td>
<td>0.62 ± 0.28</td>
<td>2.71 ± 3.66</td>
<td>0.43 ± 0.13</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.72 ± 0.18</td>
<td>0.28 ± 0.12</td>
<td>0.78 ± 0.36</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Colon</td>
<td>6.24 ± 1.86</td>
<td>3.03 ± 0.15</td>
<td>9.04 ± 7.29</td>
<td>1.79 ± 0.44</td>
</tr>
<tr>
<td>Liver</td>
<td>2.10 ± 0.62</td>
<td>1.09 ± 0.23</td>
<td>2.23 ± 0.82</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>Brain</td>
<td>0.07 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.13 ± 0.03</td>
<td>0.07 ± 0.02</td>
<td>0.12 ± 0.06</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Bone</td>
<td>0.16 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>0.14 ± 0.06</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>Tumour</td>
<td>0.51 ± 0.17</td>
<td>0.27 ± 0.09</td>
<td>0.53 ± 0.17</td>
<td>0.11 ± 0.06</td>
</tr>
</tbody>
</table>

Both analogues exhibited a rapid clearance from blood pool and most of the tissues. The highest uptake was found in colon, liver uptake was the second highest, which may be due to the lipophilicity of the molecules. Brain uptake was very low which indicates that the analogues do not cross the blood-brain barrier and uptake in pancreas and tumour was rather low for BBS-X and BBS-XII.

_Biodistribution studies with additional inhibitor enalapril tested for BBS-II and BBS-III:_
Studies were performed on 8 week old female NMRI mice, purchased from Biological Research Laboratories Ltd. (Füllinsdorf, Switzerland). The biodistribution was investigated with and without injection of the inhibitor enalapril, which was applied i.p. 45 min before injection of the radiolabelled peptides. The biodistribution was evaluated 30 min post-injection times. Tissues (blood, heart, lung, spleen, kidneys, stomach, pancreas, ileum, colon,
liver, muscle, bone and brain) were removed and weighed. The amount of radioactivity was
determined with a $\gamma$-counter. Results were expressed as percentage of injected dose per gram
of tissue (% ID/g) referred to total injected activity.

**Figure 2.3:** biodistribution of $[^{99m}\text{Tc}]$BBS-II after 30 min injection. Uptake of radioactivity
without (n=2) and with enalapril (25mg/kg i.p., n=2). Results were expressed as percentage of
injected dose per gram of tissue (% ID/g) referred to total injected activity.
**Figure 2.4:** biodistribution of $[^{99m}Tc]$BBS-III after 30 min injection. Uptake of radioactivity without (n=2) and with enalapril (25mg/kg i.p., n=2). Results were expressed as percentage of injected dose per gram of tissue (% ID/g) referred to total injected activity.

Highest uptake was found in the intestine and liver. An increased uptake of $[^{99m}Tc]$BBS-III in all tissues occurred with enalapril.

### 2.8 Discussion

Two properties of a peptide based (radio-) pharmaceutical are crucial to bring about a high tumour uptake, first, the peptide should survive sufficiently long in plasma, since it has to be transported from the injection site to the tumour, and second, it should bind with a sufficient high affinity to the specific receptor on the tumour to show a better uptake in the tumour than in healthy tissues. Thus, the BBS chain was modified to obtain a reduced speed of its metabolism and a retained affinity.

The methods to reach this goal were the introduction of non-natural amino acids, either D-amino acids or bulky side chains (such as cyclohexane or tert-butyl at the place of isobutyl). Another idea was the alkylation of the terminal amid. All these changes should reduce the fitting of the BBS analogue in the active site of the enzymes.

After the synthesis of a first series of analogues the screening was started with the determination of the receptor affinity, in that the inhibition of the binding of $^{125}$I labelled BBS was tested (IC$_{50}$ values Table 2.5) with the unlabelled analogues. All the N-alkylated
analogues, both propyl and isobutyl, showed a very weak binding. A less clear effect was found in the case of amino acid substitution, and thus, the receptor affinity of the labelled analogues and their stability in plasma was determined. D-Gln insertion for Gln reduced the affinity of the unlabelled peptide (BBS-V), while the labelled analogue retained the receptor affinity. But there was no better stability in the plasma. The substitution of Ala by D-Ala, Val by Aba and Leu by 3-homoleu brought about a complete loss of affinity. A promising result was obtained the insertion of Tie (BBS-X) and CyhAla (BBS-IV) for Leu\textsuperscript{13}, namely a good receptor affinity of the labelled analogues (BBS-IV slightly better than BBS-X) and considerable increase of the stability in plasma. During the studies we observed a further problem, namely the oxidation of the sulfur of Met, which was assumed to be the reason of some loss of affinity. The handling of the sulphur-free analogues (Nle inserted for Met) was easier. The affinities of the labelled analogues were slightly reduced, when Nle was combined with CyhAla (BBS-XXX), and clearly more reduced with Tie and Nle (BBS-XII). Thus we decided to concentrate on CyhAla for further studies. Also des-Met-NHEt in combination with CyhAla (BBS-36) showed good results. Since the alkylation of the terminal amide is less easy than the insertion of Nle, the latter was selected for the next step in the design of new BBS analogues, namely insertion of spacers between the metal complex and the BBS(7-14) sequence. These spacers showed quite good binding and stability properties and specifically the best results of biodistribution.

The first \textit{in vitro} tests were performed to determine the above-mentioned properties and allowed the selection of the peptides for further studies. This selection led to a non-complete table 2.5, but was done for a practical reason, namely not to waste time with testing of peptides which are useless and for an ethical reason, namely to keep the number of animals low, which have to be killed for the determination of the biodistribution.

Compared to the results obtained in plasma, the improvement of the stability was less effective in human prostate adenocarcinoma PC-3 cells where the analogues were rapidly metabolized (half of the analogues degraded within 15-30 min).

The selection of the site to be stabilised was based on literature data and the detailed investigation of the metabolism by introducing specific enzyme inhibitors. If an enzyme inhibitor was able to improve the stability of a peptide, we concluded, that the known cleavage site of the respective enzyme has to be protected. “Protected” means introduction of non-natural amino acids. These studies showed that the His\textsuperscript{12}-Leu\textsuperscript{13} bond of BBS might easily be metabolized by the neutral endopeptidase 24.11 (EC 3.4.24.11) and the angiotensin converting enzyme (ACE). The investigation with BBS-II and BBS-III and the peptidase
inhibitors in plasma led to new findings, namely, that Pro-Ile (inhibitor of the endopetidase EC 3.4.24.16) and the ACE inhibitor were able to block the enzymatic cleavage of BBS II. BBS III was not protected by Pro-Ile, and degraded even faster than BBS II. According to our interpretation, BBS-III is cleaved by another, so far not identified enzyme. The degradation of BBS by ACE was not known before, but seems to be quite important. To our knowledge, ACE is not very selective with respect to cleaving a peptide bond between specific amino acids, but it seems that, usually, two amino acids of the carboxy terminus are cut off. The His\textsuperscript{12}-Leu\textsuperscript{13} bond is also hydrolyzed by the neutralendopeptidase EC 3.4.24.11 [5], which is inhibited by phosphoramidon. But EC 3.4.24.11 seems not to have such a big influence as ACE, since the ACE-inhibitor increased the stability up to 90%. The reason why phosphoramidon has a smaller influence in plasma than in blood might be due to the endopeptidase EC 3.4.24.11, which exists mainly membrane-bound. It is also found dissolved in blood, but the concentration seems to be too small for having a big influence on metabolism.

If the metabolism of the peptides in the plasma is too fast, the uptake in tumours is diminished, what makes the imaging difficult and the radionuclide therapy impossible. A too fast metabolism in the cells can bring about a too fast externalisation of radioactivity, but if the metabolites are not externalised the metabolism in the cells would not be a problem. For diagnostics metabolism is a minor problem, if it is possible to select an optimal time point for imaging. For therapy it has negative consequences, because of insufficient concentration in tumour and the radio toxicity of the metabolites in the healthy tissues, especially in kidneys and liver. In general it is to say that the biological half-life of radiotherapeutics in the tumour should correspond to 3-4 physical half-lives of the radionuclides.

Almost all investigated BBS analogues seem to be agonists, and thus, they showed a rather rapid and high extent of internalization, which increased with time, reaching a maximum within 15-30 min and remaining then constant up to 2 h. Only the des-Met-NHEt BBS-analogues did hardly internalize, accordingly they are probably antagonists. It is believed that the envisaged therapeutic effect is higher, if the radionuclide is internalized compared to compounds remaining on the cell surface, because of potentially longer retention in the target tissue [10].

When after the internalisation study the plateau of cell related activity was reached, the activity in the supernatant (addition of fresh binding buffer) was cleared and the externalisation of the activity from the cells determined. The stabilised analogues were externalized as rapid as the unmodified analogue [11], and the activity was found mainly in
the form of metabolites. A slower externalization, which would mean longer retention, would be desirable especially for therapeutic purposes.

Biodistribution studies performed in mice bearing PC-3 tumours with the conjugates $^{99m}$Tc(CO)$_3$-BBS-X and XII exhibited a rapid clearance from the blood pool and most non-targeted tissues (Table 2.7). The second highest uptake for both the analogues was liver, most probably because of the lipophilicity of the conjugates. The pancreas, a GRP receptor rich tissue and the tumour showed little accumulation of radioactivity. The low uptake could be due to not sufficient binding properties, although the shelf-life in plasma was very long. The investigation of the biodistribution with BBS-II and BBS-III together with the peptidase inhibitor enalapril showed that the uptake in tissues could be increased. A confirmation for the choice to substitute Leu by CyHAla at position 13.

A third property of radiolabelled peptides is also very important, namely the often observed but unwanted accumulation in the kidneys. Thus the tumour to kidney ratio is an important parameter for peptide evaluation. So far no in vitro test is known, which allows the determination of this characteristic. This point can only be elucidated in vivo. A few peptides show a clear improvement of the properties, and with progress of the work (which is in parallel with the increasing number of the BBS analogues) a lower number of peptides had to be designed and synthesised to obtain higher tumour to kidney ratios.

The results obtained in this work and literature data [12-16] demonstrate that the modifications of the C-terminal structure of BBS is crucial for biological activity, since it influences both metabolism and receptor affinity. While rapid metabolism in plasma prevents tumour uptake, the rapid cellular metabolism is not necessarily a drawback. A slow (or no) externalisation of the metabolites from tumour cells would contribute to a high potential of a BBS analogue to be used as radiopharmaceutical. A long retention of the activity in the tumour is crucial for therapy.
Chapter 2

2.9 References


CHAPTER 3

Chemical and Biological Characterization of New Stabilized Re(CO)$_3$/99mTc(CO)$_3$- Bombesin Analogues

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Biocunjugate Chemistry (submitted)
3.1 Summary

Bombesin (BBS) shows high affinity for gastrin releasing peptide (GRP) receptors which are overexpressed in many human cancers, such as prostate and breast cancer. These receptors are interesting targets for tumor imaging and therapy. Since the natural BBS is very rapidly metabolized in vivo, new analogues have been synthesized based on the fragment BBS(7-14). Here we present three new derivatives (BBS-IV, -IX and -XXX) with different modifications. In BBS-IV, Leu\textsuperscript{13} was replaced by Cyclohexylalanine (CyHAla). BBS-IX has Nle instead of Met at position 14. BBS-XXX includes both modifications, CyHAla and Nle at positions 13 and 14, respectively. In all three, (N\textalpha{His})Ac was coupled to the Gln\textsuperscript{7} as a ligand for labeling with cold Re(CO)\textsubscript{3}\textsuperscript{+} for chemical characterization and \textsuperscript{99m}Tc(CO)\textsubscript{3}\textsuperscript{+} for pharmacological studies. These changes in the molecule led to an increase in plasma stability, especially for BBS-IV and -XXX. Moreover, the new analogues retained the receptor affinity. BBS-IV and -IX showed even higher affinity for GRP receptor in human prostate carcinoma PC-3 cells than the natural sequence whereas the affinity of BBS-XXX was slightly lower. In all cases the affinity was at subnanomolar range. Receptor-bound analogues were rapidly internalized at 37°C. However, the internalized peptides were also rapidly metabolized and the activity was externalized. In biodistribution studies performed in mice with PC-3 tumor xenografts, all three analogues showed a low tumor uptake. In contrast, the uptake in other GRP receptor-positive organs, such as pancreas and colon, was rather high. Furthermore it could be partly inhibited after co-injection with unlabeled BBS which would indicate that this uptake is receptor-specific. Another interesting result is the low kidney uptake. The new analogues are lipophilic and mainly excreted via the hepatobiliar system although liver uptake was not extremely high. Despite the low in vivo tumor uptake observed for these analogues, interesting results were found that encourage to further investigate new \textsuperscript{188}Re(CO)\textsubscript{3}/\textsuperscript{99m}Tc(CO)\textsubscript{3}-labeled bombesin analogues.
3.2 Introduction

Neuropeptide receptors have been shown to be over-expressed in high concentrations in a variety of human tumors and thus they can be used as potential targets for imaging and therapy with radiolabeled neuropeptides (1-5). The successful application of radiolabeled peptide analogues for imaging of neuropeptide receptor-positive tumors (the somatostatin analogues octreotide and octreotate are subject of a huge number of papers) led to the development of peptide analogues based on bombesin (6-9) and neurotensin (10-12) as potential imaging agents. Bombesin (pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂) is a tetradecapeptide, originally isolated from the skin of the amphibian Bombina orientalis (13) with high affinity to the gastrin releasing peptide (GRP) receptor, a G protein-coupled receptor, characterized by the typical configuration of seven transmembrane domains (14). Bombesin (BBS) and its mammalian counterpart GRP produce a wide spectrum of biological responses in the central nervous system as well as in peripheral tissue (15, 16). Apart from the physiological effects, it has been established that GRP/BBS promotes tumor growth in normal and human cancer cell lines. For instance it was shown that BBS can stimulate cell proliferation in the androgen-independent human prostatic carcinoma cell line PC-3 (17, 18). Over-expression of the GRP receptor has been demonstrated in a large number of tumors, however, prostate and breast cancers are of special interest (19-23). Radiolabeled BBS peptides might be of potential interest for GRP receptor scintigraphy for early tumor diagnosis and radiotherapy.

The use of small peptides has advantages because of high affinity and rapid plasma clearance, which may result in a high concentration in the target tissue and good tumor to background ratios. However, peptides are rapidly metabolized in plasma by endogenous peptidases, terminating their biologic effect under physiologic conditions. This problem may be solved since the synthesis and modification of small peptides are rather easy, whereas the design of improved metabolic stability or bioavailability is much more difficult. Three new BBS analogues (BBS-IV, -IX, -XXX, Table 1) in which two natural amino acids have been replaced by non-natural ones to improve metabolic stability have been synthesized and labeled with \( {\text{fac-[}}{^{99m}\text{Tc(OH)₃(CO)₃]}^+ \) (24). Here we present their synthesis and their chemical and pharmacological characterization. This includes specifically NMR with the stable Rhenium congener (Figure 1) of the radioactive Technetium-99m (24), metabolic stability in vitro, binding
properties, internalization, externalization as well as their in vivo biodistribution in nude mice bearing human PC-3 xenografts. The reason of using Re is the molar concentration available, which is below the detection limits of NMR and MS in the case of $^{99m}$Tc (even with a very high amount of radioactivity), and the need to investigate the pharmacological properties with the $^{99m}$Tc labeled compound foreseen for human application.

Re-BBS-II was the best soluble analogue. However, the solubility was not good enough to obtain good signal to noise ratios in the NMR spectra and only a few of the expected couplings were seen in the COSY analysis. Thus, a full interpretation of the NMR spectra was not possible despite the possibility to compare it with published data (25). Therefore the reference compounds Na-carbamoylmethyl histidine (L1) and the corresponding rhenium tricarbonyl complex (ReL1) were synthesized and the NMR and MS spectra recorded.

Table 3.1: Sequence of BBS-II (natural) and new stabilized BBS analogues (BBS-IV, -IX and -XXX) obtained by solid phase method using Fmoc-. (CyHAla = Cyclohexyl alanine)

<table>
<thead>
<tr>
<th>Bombesin-analogues</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BBS-II</td>
<td>(NαHis)Ac Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂</td>
</tr>
<tr>
<td>BBS-IV</td>
<td>(NαHis)Ac Gln-Trp-Ala-Val-Gly-His-CyHAla-Met-NH₂</td>
</tr>
<tr>
<td>BBS-IX</td>
<td>(NαHis)Ac Gln-Trp-Ala-Val-Gly-His-Leu-Nle-NH₂</td>
</tr>
<tr>
<td>BBS-XXX</td>
<td>(NαHis)Ac Gln-Trp-Ala-Val-Gly-His-CyHAla-Nle-NH₂</td>
</tr>
</tbody>
</table>

Figure 3.1. Numbering scheme of the Re(CO)$_3$-BBS-II. The arrow points to the bond which is broken in the MS and leads to the main fragments. The carbons C-1 to C-11 of ReL1 and of this peptide are the same.
3.3 Materials and Methods

The Fmoc-protected amino acids (Met, Nle, Leu, CyHAla, His(trt), Gly, Val, Ala, Trp(Boc), Gln(trt) and the Rink amide resin were purchased from NovaBiochem (Läufelingen, Switzerland). Diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland), dimethylsulphoxide (DMSO), ethandithiole, 1-hydroxybenzotriazole (HOBT), N-ethyldiisopropylamine (DIPEA), thioanisole, trifluoroacetic acid (TFA) from Fluka (Buchs, Switzerland). Bombesin (1-14) and N-tertiyl-O-tertbutyl histidine were obtained from Bachem (Bubendorf, Switzerland). The $^{[125}\text{I}-\text{Tyr}]$ bombesin was purchased from Amersham Pharmacia Biotech (Dübendorf, Switzerland). Na$^{[99m}\text{TcO}_4]$ was eluted from a Mallinckrodt $^{99m}\text{Mo}/^{99m}\text{Tc}$ generator (Tyco Healthcare, Petten, The Netherlands) using 0.9% saline. Sodium boranocarbonate was a gift from the provider of the generator and $(\text{N(Et)}_4)_2\text{[Re(CO)}_3\text{Br}_3]$ a gift from Dr. R.Schibli (26).

Cell culture media DMEMGLUTAMAX-I, Trypsin/EDTA, bovine serum albumin and soybean trypsin inhibitor were obtained from Invitrogen (Basel, Switzerland). Foetal calf serum and antibiotic/antimycotic solution were obtained from Amimed (Allschwil, Switzerland). Bacitracin, Hepes and Chymostatin were purchased from SIGMA (Buchs, Switzerland). The working reagent for the protein assay was ordered from SOCOCHIM SA (Lausanne, Switzerland). All chemicals not mentioned above were obtained from either Fluka (Buchs, Switzerland) or Merck (Dietikon, Switzerland).

The peptides were synthesized by an automated Syro robot system from MultiSyn Tech (Bochum, Germany). Correct mass was identified by MALDI-TOF mass spectrometry on a Voyager-DE RP workstation (Applied Biosystems, Darmstadt, Germany). NMR spectra were recorded on a GEMINI 2000, Varian (300 MHz). The HPLC (peptide synthesis) consisted of a RP C-18 column (250 x 4.6 mm, 5 μm, Vydac, Hesperia, USA), flow 0.6 ml/min, gradient 10% to 60% A in B within 30 min, A = acetonitrile/TFA (100:0.008), B = water/TFA (100:0.1). The HPLC analyses of radiolabeled peptides were performed on a Varian ProStar system with a Varian ProStar 230 Solvent Delivery-Pump, a Varian Photodiode Array Detector Model 330, a Varian ProStar Autosampler Model 410 and a radiomatic Flo-one Beta Detector (Packard Canberra), a Macherey-Nagel CC Nucleosil 100-5 C18 reverse phase column (10 μm, 250 x 4.6 mm). HPLC solvents consisted of acetonitril with 0.1% TFA (solvent A), water with 0.1% TFA (solvent B) and methanol (solvent C). The gradient for the labeling control started with a linear gradient 15%A/80%B/5%C to 80%A/20%B/0%C.
(1.2 ml/min) from 0 to 35 min and for the metabolic analyses with 0%A/50%B/50%C to 15%/5%/80% (0.8 ml/min) from 0 to 25 min. The radioactivity was measured with an Auto-γ-counter (Packard Canberra Cobra II) or an ionization chamber (activimeter).

**Synthesis. BBS-II, BBS-IV, BBS-IX, BBS-XXX:** The peptides were obtained by solid phase method using the Fmoc/tBu-strategy. Fmoc-amino acids were coupled with eight-fold excess in duplicate. In order to obtain the C-terminal amide, Rink amide resin was used. The coupling of the ligand was performed in two steps: In a separate vial 6 equivalents (4.7 mg) bromo acetic acid (the amounts are normalized to the amount of resin used) and 3 equivalents DIC were dissolved in 0.5 ml DCM. After 20 min the obtained anhydride solution was added to a mixture of 1 equivalent resin (6.75 μmol) and 3 equivalents DIPEA. Two hours later the resin was washed with DMF, then 3 equivalents Nε-trityl-O-tertbutyl histidine and 3 equivalents DIPEA dissolved in 2 ml DMF were added and the mixture was stirred for 24 h. The peptides were cleaved from the resin, in that TFA and ethandithiol/thioanisol (3:7) was added and allowed to stand for 3 h. After cleavage the peptides were precipitated from cold diethyl ether, collected by centrifugation, purified by preparative HPLC and lyophilized from water/tert-butylalcohol (3:1). The purity and identity of the peptides were analysed by HPLC and MS.

**(NaHis)Ac-amid (L1):** 2.5 g (5.5 mmol) Nε-trityl-O-tertbutyl histidine, 790 mg (5.75 mmol) bromoacetamid and 0.589 ml (5.75 mmol) triethylamine were suspended in 30 ml dichloroethane and gently shaken at room temperature during 48 h. The organic phase was washed twice with water and dried with MgSO₄, yield 2 g (70%). The raw product was purified (100 mg per run) on a semi-preparative HPLC column (optisphere HDO C-18, 250 x 10 mm) using the solvent gradient methanol water acetonitrile, yield 50 mg per run (50%), calculated mass: 510.6 g/mol. MS 511.3 (100%), 512.4 (30%), 513.4 (5%), 269.3 (25%). For de-protection 50 mg of the purified intermediate were dissolved in a mixture of 2 ml dichloromethane and 2 ml TFA and gently shaken at room temperature for about 3 h. The solvents were evaporated and the raw product washed three times with ether, calculated mass: 212.1 g/mol. MS: 213.2 (100%), 158.2 (15%), 116.2 (18%) (several other peaks below 10%).

**ReL1:** 20 mg (0.094 mmol) L1 and 87.7 mg (0.102 mmol) \([N(Et)_4][Re(CO)_3Br_3]\) were dissolved in 10 ml PBS and kept at 75 °C for 24 h. The slightly turbid solution was filtered and aliquots purified on the same column as described above. Fractions eluted
after 15 and 18 min respectively were collected, and evaporated to dryness. The products were dissolved in a mixture of methanol and water for MS and D$_2$O for NMR analysis (see results).

$^{99m}$Tc(CO)$_3$-peptides: About 1ml of a $[^{99m}$TcO$_4$] generator eluate in saline (up to about 5 GBq) was added to a mixture of 4.5 mg sodium-boranocarbonate, 2.9 mg borax, 9 mg potassium-sodium tartrate tetrahydrate and 7-8 mg sodiumcarbonate. The solution was heated for 20 min at 100°C. The tricarbonyl-solution was adjusted to pH 6.5 and then 500 μl mixed with 15 μl of the peptide solution (1mM) and heated for 1 h at 75°C. The labeling was checked with RP-HPLC.

Re(CO)$_3$-BBS-II: A solution of 2.5 mg BBS-II in 2.5 ml water, 1.35 mg [N(Et)$_4$][Re(CO)$_3$Br$_3$] and 60 μl NaOH (0.075 N) was heated for 6 h at 75°C. The suspension was then centrifuged and the supernatant purified with the HPLC. The peaks were analyzed by IonSpec HiResMALDI and NMR.

His-challenging. BBS(8-14) (15 μl, 1mM) (i.e. the analogue without chelator) was labeled with $^{99m}$Tc(CO)$_3$(H$_2$O)$_3^+$ as described above. Then histidine (15 μl, 1mM) was added and the solution kept at 37 and 75°C respectively, for up to 90 min. Samples were taken every 30 min and analyzed by RP-HPLC.

Cell culture. The human prostate adenocarcinoma cell line PC-3 was purchased from European Collection of Cell Culture (Salisbury, England). Cells were maintained in DMEM GLUTAMAX-I supplemented with 1-10% fetal calf serum, 100 IU/ml penicillin G sodium, 100 μg/ml streptomycin sulphate, 0.25 μg/ml amphotericin B. Cell culture was incubated at 37°C in an atmosphere containing 7.5% CO$_2$. The cells were passaged weekly after detaching them with trypsin/EDTA (0.25%).

Metabolic stability in human plasma and in PC-3 cells. The degradation of the radiolabeled conjugates was estimated in vitro in plasma as well as in PC-3 cells. The $^{99m}$Tc(CO)$_3$-BBS analogues were incubated in human plasma from healthy donors at a final concentration of 3 to 4 MBq/ml (1.5-2 pmol/ml) for various time intervals (up to 24 h) at 37°C. After incubation, proteins were precipitated with acetonitrile/ethanol (1:1) and TFA (0.01%) and centrifuged (10 min, 20000g) at 4°C. The supernatant was filtered and analyzed by RP-HPLC.
The stability in cell culture was evaluated with 2 x 10^6 cells. 99mTc(CO)₃-BBS analogues were added to the cell suspension in PBS (final concentration: 3 to 4 MBq/ml) and incubated at 37°C. At different time points (up to 1 h) cells were lysed by precipitation as above and filtered. The filtrates were analyzed by RP-HPLC. All experiments were carried out 2-3 times.

**Binding assays**

**Inhibition studies:** PC-3 cells at confluence were placed in 48-well plates (250'000 cells/well). A special binding buffer including protease inhibitors was used, composition as follows: 50 mM HEPES, 125 mM NaCl, 7.5 mM KCl, 5.5 mM MgCl₂, 1 mM EGTA, 5 g/l BSA, 2 mg/l chymostatin, 100 mg/l soybean trypsin inhibitor, 50 mg/l bacitracin. Cells were incubated for 1 h at 37°C with 15'000-25'000 cpm [¹²⁵I-Tyr⁴]BBS per well and increasing concentrations of the different unlabeled analogues (0-3'000 nM). Cells were then washed twice with cold PBS 1X and solubilized with 400μl (2x) of 1 N NaOH at 37°C. The bound activities were determined in a γ-counter. IC₅₀ values for the displacement of binding of [¹²⁵I-Tyr⁴]BBS by the different analogues were calculated by non-linear regression analysis using GraphPad Prism™. Experiments were performed twice in triplicate.

**Saturation studies:** PC-3 cells at confluence were placed in 48-well plates (1 x 10^6 cells/well). Cells were incubated in triplicate with increasing concentrations (0.001-1 nM) of the ⁹⁹mTc(CO)₃-BBS analogues for 1 h at 37°C (final volume, 0.2 ml per well). The concentration of total technetium (⁹⁹ + ⁹⁹mTc), estimated according to Bauer and Pabst (27), were equivalent to 0.002-2 MBq ⁹⁹mTc activity per well. After 2 washing steps with cold PBS 1X, the cells were solubilized with 400μl (2x) of 1 N NaOH at 37°C. The bound radioactivity was measured in the γ-counter. Nonspecific binding was determined with 1pM unlabeled BBS(8-14). The protein content was determined in a BIO RAD microplate reader (570 nm) using the Micro BCA Protein Assay Reagent from Pierce.

**Internalization.** PC-3 cells at confluence were placed in 6-well plates (about 1 x 10^6 cells/well). Cells were incubated with approximately 4 kBq of ⁹⁹mTc(CO)₃-BBS analogues in culture medium for 5, 15, 30, 60 and 120 min at 37°C to allow binding and internalization (total final volume 1 ml per well). Nonspecific internalization was evaluated in the presence of 1 μM unlabeled BBS(8-14). After the various incubation times, cells were washed 3 times with PBS 1X to discard unbound peptide. Surface-
bound activity was removed by two steps of 5 min acid wash (50 mM glycine-HCl, 100 mM NaCl, pH 2.8, 600μl per well) at room temperature. Cells were solubilized by incubation at 37°C with 600μl (2x) of 1 N NaOH per well to determine internalized radioligand. Surface bound and internalized activities were measured in a γ-counter. Results were expressed as percentage of total activity in the cells (surface bound and internalized). All experiments were carried out 2-3 times in triplicate.

**Externalization.** PC-3 cells at confluence were placed in 6 cm diameter Petri dishes. Cells were incubated with about 20 kBq of 99mTc(CO)3-BBS analogues in culture medium for 45 min at 37°C to allow maximal internalization into the cells (total final volume 5 ml per dish). The supernatant was discarded and the cells washed twice with PBS 1X. Cells were then incubated again at 37°C with 5 ml culture medium for 0, 30, 60, 150 and 300 min. The activity in the supernatant was measured to determine released activity from the cells. After 2 washing steps with PBS 1X surface-bound activity was removed by two steps of 5 min acid wash (50 mM glycine-HCl, 100 mM NaCl, pH 2.8, 0.75 ml per dish) at room temperature. Finally, the amount of remaining internalized activity was recovered by solubilizing cells at 37°C with 1 ml (2x) of 1 N NaOH per dish. Externalized, surface-bound and internalized activities were measured in a γ-counter. Experiments were performed 2-3 times.

**Biodistribution studies.** All animal experiments were conducted in compliance with the Swiss animal protection laws and with the ethical principles and guidelines for scientific animal trials established by the Swiss Academy of Medical Sciences and the Swiss Academy of Natural Sciences. Studies were performed with 6 to 8 weeks old female CD-1 nu/nu mice (20-25 g), purchased from Charles River Laboratories (Sulzfeld, Germany). For the induction of tumor xenografts, PC-3 cells were subcutaneously injected at a concentration of 5 x 10⁶ cells/mouse and allowed to grow for 10-12 days. On the day of the assay mice received the radioactive conjugate (0.4 to 3 MBq/mouse) intravenously into the tail vein. They were sacrificed by cervical dislocation and dissected. For BBS-IV, the biodistribution was evaluated at different post-injection times (1.5, 5 and 24 h). The biodistribution of both BBS-IX and BBS-XXX was tested at 1.5 h post-injection time. In addition, 3 mice per analogue received 300 μg of cold BBS, co-injected with the radiolabeled BBS analogue to determine nonspecific uptake and were killed 1.5 h after injection. Tumors and other tissues (blood, heart, lung, spleen, kidneys, stomach, pancreas, ileum, colon, liver, muscle, bone and
Chapter 3

brain) were removed and weighed. The amount of radioactivity was determined with a γ-counter. Results were expressed as percentage of injected dose per gram of tissue (% ID/g) referred to total injected activity.

3.4 Results

Characterization of the Re compound with NMR and MS. The synthesis of the $^{99m}$Tc as well as natural Re compounds (ReL1 and peptides) yielded two isomers which could be separated by HPLC. The MS results of ReL1 are shown in Table 3.2, Re-BBS-II in Table 3.3, the NMR results of ReL1 is summarized in Table 3.4, while a selected region between 3 and 4 ppm is shown in Figure 3.2 for easier comparison of the data obtained with the peptide fractions. The NMR data of Re-BBS-II are shown in Figure 3.3 with the signals of His and Figure 3.4 with the signals of the α-CH-groups. The region between 4.5 and 7 ppm is not shown, because only the water peak and some background noise is seen, between 0.8 and 1 ppm 11 lines (15 protons), between 1.25 and 1.35 ppm 5 lines (9 protons) and at 1.5 – 1.6 ppm a broad peak composed of many small lines (4 protons) are seen, corresponding to the aliphatic CH$_2$ groups.

Table 3.2: MS data of ReL1 of both HPLC fractions (fr1 and fr2): Molecular mass (M/z) and relative intensity (%), peak with highest intensity = 100

<table>
<thead>
<tr>
<th></th>
<th>fr1:</th>
<th>%</th>
<th>fr 2: M/z</th>
<th>%</th>
<th>Calc mass</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex + Na</td>
<td>504.9</td>
<td>100</td>
<td>505.1</td>
<td>17</td>
<td>505.0</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>502.9</td>
<td>44</td>
<td>503.1</td>
<td>13</td>
<td>503.0</td>
<td>*</td>
</tr>
<tr>
<td>Complex + H</td>
<td>483.7</td>
<td>40</td>
<td>484.2</td>
<td>13</td>
<td>384.0</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>482.7</td>
<td>95</td>
<td>483.1</td>
<td>100</td>
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</tr>
<tr>
<td></td>
<td>481.1</td>
<td>61</td>
<td>481.1</td>
<td>59</td>
<td>481.0</td>
<td>59.7</td>
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<tr>
<td>Re(CO)$_3$-methyl imidazol</td>
<td>353.0</td>
<td>96</td>
<td>353.2</td>
<td>18</td>
<td>353.0</td>
<td>*</td>
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<tr>
<td></td>
<td>351.0</td>
<td>43</td>
<td>351.1</td>
<td>10</td>
<td>351.0</td>
<td>*</td>
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<tr>
<td>Free ligand</td>
<td>214.4</td>
<td>15</td>
<td>213.3</td>
<td>13</td>
<td>213.1</td>
<td></td>
</tr>
<tr>
<td>Re(CO)$_3$-methylamine + H</td>
<td>303.0</td>
<td>40</td>
<td>-</td>
<td>-</td>
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<td>*</td>
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<tr>
<td></td>
<td>301.1</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>301.0</td>
<td>*</td>
</tr>
<tr>
<td>Unidentified fragment</td>
<td>335.1</td>
<td>57</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>333.1</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*) The intensity ratios of the peaks containing Re should be 0.6 and show a difference of two mass units, calculated on the basis of the isotope pattern of natural Re.
Table 3.3: MS data of the Re-BBS-II, the mass of the molecules or fragments are given with intensities in parenthesis. (with the exception of the molecule peak (7%) only peaks with intensities of more than 15% are listed)

<table>
<thead>
<tr>
<th>Calculated fragment</th>
<th>Re-BBS-II, Fraction 1 m/z (%)</th>
<th>Re-BBS-II, Fraction 2 m/z (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecule (sulfoxide) +H</td>
<td>1422 (29), 1423 (20), 1420 (18)</td>
<td></td>
</tr>
<tr>
<td>Molecule + H</td>
<td>1406 (7), 1407 (6), 1404 (4)</td>
<td></td>
</tr>
<tr>
<td>?</td>
<td>850 (21)</td>
<td></td>
</tr>
<tr>
<td>Trp-Ala-Val-Gly-His-Leu-Met(O)-NH₂</td>
<td>828 (57)</td>
<td></td>
</tr>
<tr>
<td>Re-HisAc-Gln</td>
<td>594 (100), 592 (56)</td>
<td>594 (100), 592 (56)</td>
</tr>
<tr>
<td>594 - CO</td>
<td>566 (20)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: NMR data of ReL1 (both HPLC fractions) and L1

<table>
<thead>
<tr>
<th>Position</th>
<th>ReL1, Fraction 1</th>
<th>ReL1, Fraction 2</th>
<th>L1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shift (ppm)</td>
<td>Coupling (Hz)</td>
<td>Shift (ppm)</td>
</tr>
<tr>
<td>8</td>
<td>8.155</td>
<td></td>
<td>8.140</td>
</tr>
<tr>
<td>7</td>
<td>7.108</td>
<td></td>
<td>7.064</td>
</tr>
<tr>
<td>amine</td>
<td>6.500 (broad signal)</td>
<td></td>
<td>6.089 (broad signal)</td>
</tr>
<tr>
<td>4</td>
<td>4.110, 4.121</td>
<td>3.3 Hz (H-5)</td>
<td>3.885 (T)</td>
</tr>
<tr>
<td>5</td>
<td>3.347, 3.359, 3.311, 3.323</td>
<td>3.6, 10.8 (H-4, H-5)</td>
<td>3.371, 3.381 (+shoulders)</td>
</tr>
<tr>
<td>10</td>
<td>3.758, 3.817, 3.841, 3.854</td>
<td>3.9, 12.0 (amine, and H-10)</td>
<td>3.979, 4.009, 4.034, 4.063, 4.166, 4.183, 4.220, 4.236, 5.0, 16.2 (amine and H-10)</td>
</tr>
</tbody>
</table>
Figure 3.2. Selected region of the NMR signals (ppm) of the H bound to C-10 (Ac), C-4 (Hα) and C-5 (Hβ) of ReL1. Fraction 1 contains impurities of the free ligand and fraction 2, while fraction 2 is rather pure (except the non-identified signal between 3.5 and 3.6 ppm).

Figure 3.3. NMR spectra of both fractions of Re-BBS-II: Region of the protons of imidazol (His^{12} and Re-His-complex) and indole (Trp).
Figure 3.4. Selected region of the NMR signals (ppm) of fraction 1 and 2 of the protons H-10 (Ac), H-5 (Re-His complex), the alpha positions of all amino acids and S-methyl (partially oxidized).

**His-challenging.** The labeling of BBS(8-14) with $^{99m}$Tc-tricarbonyl yielded a labeled peptide with a similar retention time as the other BBS analogues with the additional ligand. After addition of free His the transchelation was almost complete (> 90 %) after 30 min at 75 °C, while at 37 °C transchelation was 25 ± 10 % after 1 h and 38 ± 6 % after 90 min. The challenge of labeled BBS-II with even an excess of His did not show any transchelation (8).
Metabolic stability: human plasma and PC-3 cell culture. The degradation of $^{99m}$Tc(CO)$_3$-BBS-IV, -IX and -XXX was determined in human plasma and PC-3 cells (Figure 3.5). In human plasma the BBS analogues were more stable than the natural BBS. Half of the natural BBS was degraded within the first 30 min (8) whereas longer times were required to reach similar percentages of degradation for the BBS analogues (6 h for the compound BBS-IX and 16 h for both BBS-IV and BBS-XXX). On the other hand, a much faster degradation was observed in PC-3 cells. Half-lives of 20 min, 15 min and 15 min were obtained for $^{99m}$Tc(CO)$_3$-BBS-IV, -IX and -XXX, respectively.

![Figure 3.5](image)

**Figure 3.5.** Metabolic stability of the $^{99m}$Tc(CO)$_3$-BBS-analogues in human plasma at 37°C, analyzed by RP-HPLC using a Macherey-Nagel CC Nucleosil 100-5 C18 reverse phase column. Data are expressed as percentage of activity recovered corresponding to intact peptide.

**Binding assays.** The binding affinity to GRP receptors for the different BBS analogues was tested with PC-3 cells.

**Inhibition studies:** the different BBS analogues inhibited the binding of [125I-Tyr$^4$]BBS showing a typical sigmoid curve (Figure 3.6). BBS-II, the analogue with the sequence 7-14 unchanged, showed an IC$_{50}$ of 1.9 ± 0.5 nM, similar to that reported for natural BBS (7, 8). The inhibitory activity of the other BBS analogues was lower (IC$_{50}$ values of 5.0 ± 1.4, 14.1 ± 2.6 and 15.7 ± 6.0 nM for BBS-IV, -IX and -XXX, respectively, Table 3.5).
Figure 3.6. Displacement of $[^{125}\text{I}-\text{Yr}^4]\text{BBS}$ binding from binding sites on PC-3 cells by different BBS analogues (-II, -IV, -IX, -XXX). Results, expressed as percentage of binding, are mean ± SD of 2 experiments in triplicate.

*Saturation studies:* receptor binding of the $^{99m}\text{Tc(CO)}_3$-BBS analogues to intact PC-3 cells was found to be saturable and highly specific. All the analogues showed a high affinity for GRP receptors, at subnanomolar range. The dissociation constant ($K_d$) for BBS-II, the unmodified analogue, was 0.165 nM. The affinity of both $^{99m}\text{Tc(CO)}_3$-BBS-IV and $^{99m}\text{Tc(CO)}_3$-BBS-IX was higher ($K_d$ of 0.015 and 0.085 nM, respectively) whereas $^{99m}\text{Tc(CO)}_3$-BBS-XXX showed a lower affinity ($K_d$ of 0.843 nM). Results are summarized in Table 3.5.

**Table 3.5:** Binding of the different BBS analogues on human prostate carcinoma PC-3 cells. The affinity of the unlabeled analogues is represented with the inhibitory concentration of 50% (IC$_{50}$) and the affinity of the labeled analogues is represented with the $K_d$ obtained in the Scatchard plot of the saturation curves. Data are mean ± SD of 2-3 times in triplicate.

<table>
<thead>
<tr>
<th>BBS analogues</th>
<th>BBS-II</th>
<th>BBS-IV</th>
<th>BBS-IX</th>
<th>BBS-XXX</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC$_{50}$ (nM)</td>
<td>1.9 ± 0.5</td>
<td>5.0 ± 1.4</td>
<td>14.1 ± 2.6</td>
<td>15.7 ± 6.0</td>
</tr>
<tr>
<td>$K_d$ (nM)</td>
<td>0.165 ± 0.064</td>
<td>0.015 ± 0.07</td>
<td>0.085 ± 0.07</td>
<td>0.843 ± 0.836</td>
</tr>
</tbody>
</table>

*Internalization and externalization.* Internalization and externalization experiments were performed in PC-3 cells. The radiolabeled BBS analogues showed to be rapidly internalized in a time-dependent manner. (Figure 3.7). After 30 min, internalization
reached a maximum and did not change significantly over a period of 120 min. Maximal percentages of internalization of 82.5 ± 6.5 %, 82.6 ± 2.0 % and 83.7 ± 11.8 % were obtained for BBS-IV, BBS-IX and BBS-XXX, respectively. Moreover, the internalization was GRP receptor-specific since it could be inhibited with BBS(8-14) I □M. These findings indicate that cell associated BBS undergoes a rapid internalization with no remarkable differences in internalization between the different bombesin compounds.

The externalization of the labeled BBS analogues was measured after 45 min preincubation to allow maximal binding and uptake into the cells. A rapid time-dependent externalization was found for the different analogues. After 30 min 23 % of BBS-IV, 36 % of BBS-IX and 33 % of BBS-XXX corresponded to externalized activity. These values increased to up to 76-77% for all the analogues after 5 h. Results are summarized in Figure 3.7. The externalized activity consisted of metabolites indicating that metabolism in tumor cells was very fast.

**Figure 3.7.** Time-dependent internalization (left graph) and externalization (right graph) of the different 99mTc(CO)3-BBS analogues by PC-3 prostate tumor cells at 37°C. Externalization was tested after 45 min preincubation to allow maximal binding and internalization. Data represent mean ± SD of 2-3 experiments.

**Biodistribution and tumor uptake.** The biodistribution of the different BBS analogues was determined in female nude mice with PC-3 tumor xenografts. All three analogues exhibited a rapid clearance from blood pool and most of the tissues, especially BBS-
XXX. The highest uptake was found in colon for all of them. Liver uptake was the second highest uptake for the three analogues which may be due to the lipophilicity of the molecules. Uptake in pancreas was also high for BBS-IV and BBS-XXX (3.28 ± 1.30 % ID/g and 1.31 ± 0.27 % ID/g, respectively) but not for BBS-IX (0.87 ± 0.38 % ID/g). Tumor uptake was rather low for all the three analogues. Brain uptake was also very low which indicates that the analogues do not cross the blood-brain barrier. Results are shown in Table 3.6. The biodistribution of $^{99m}$Tc(CO)$_3$-BBS-IV was also tested at 5 and 24 h post-injection time (Table 3.6). $^{99m}$Tc(CO)$_3$-BBS-IV uptake decreased rapidly from the GRP-positive tissues, especially in pancreas (uptake 3.28 ± 1.30 % ID/g at 1.5 h and only 0.36 ± 0.14 % ID/g at 5h). Pancreas and colon uptake could be inhibited in more than 50% after co-injection with natural BBS whereas tumor uptake was only slightly reduced. This would prove that the uptake of $^{99m}$Tc(CO)$_3$-BBS-IV in these organs is GRP-specific. On the other hand, a non-specific uptake was found in lung and spleen for this compound.

Table 3.6: Biodistribution of the $^{99m}$Tc(CO)$_3$BBS analogues (0.4-3 MBq/mouse) in nude mice with PC-3 xenografts. Results are expressed as percentage of injected dose per gram of tissue (% I.D./g) ± S.D. (n = 3-7).

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>BBS-IX 1.5 h</th>
<th>BBS-XXX 1.5 h</th>
<th>BBS-IV 5 h</th>
<th>BBS-IV 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.63 ± 0.24</td>
<td>0.18 ± 0.25</td>
<td>0.88 ± 0.74</td>
<td>0.40 ± 0.20</td>
</tr>
<tr>
<td>Heart</td>
<td>0.29 ± 0.23</td>
<td>0.02 ± 0.01</td>
<td>0.37 ± 0.33</td>
<td>0.18 ± 0.12</td>
</tr>
<tr>
<td>Lung</td>
<td>0.54 ± 0.37</td>
<td>0.08 ± 0.03</td>
<td>1.65 ± 1.62</td>
<td>1.16 ± 1.39</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.26 ± 0.10</td>
<td>0.07 ± 0.07</td>
<td>1.07 ± 1.26</td>
<td>0.54 ± 0.64</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.70 ± 0.48</td>
<td>0.44 ± 0.07</td>
<td>2.20 ± 1.57</td>
<td>1.14 ± 0.66</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.87 ± 0.38</td>
<td>1.31 ± 0.27</td>
<td>3.28 ± 1.30</td>
<td>0.36 ± 0.14</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.04 ± 0.41</td>
<td>0.21 ± 0.09</td>
<td>1.04 ± 0.55</td>
<td>0.64 ± 0.33</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.29 ± 0.68</td>
<td>0.64 ± 0.37</td>
<td>1.11 ± 0.31</td>
<td>0.29 ± 0.13</td>
</tr>
<tr>
<td>Colon</td>
<td>6.72 ± 3.16</td>
<td>5.59 ± 1.96</td>
<td>5.25 ± 1.75</td>
<td>2.31 ± 1.04</td>
</tr>
<tr>
<td>Liver</td>
<td>3.82 ± 2.50</td>
<td>1.83 ± 0.75</td>
<td>3.67 ± 2.34</td>
<td>2.13 ± 1.71</td>
</tr>
<tr>
<td>Brain</td>
<td>0.06 ± 0.05</td>
<td>0.01 ± 0.01</td>
<td>0.05 ± 0.04</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.17 ± 0.11</td>
<td>0.03 ± 0.01</td>
<td>0.49 ± 0.90</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>Bone</td>
<td>0.22 ± 0.19</td>
<td>0.03 ± 0.02</td>
<td>0.30 ± 0.24</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>Tumor</td>
<td>0.74 ± 1.08</td>
<td>0.28 ± 0.07</td>
<td>0.63 ± 0.21</td>
<td>0.24 ± 0.09</td>
</tr>
</tbody>
</table>
3.5 Discussion

Earlier studies have shown that His forms very stable complexes with the Tc or Re tricarbonyl moiety if all three donor atoms are available for complex formation (7, 28). However, the formation of two isomers, which was observed by HPLC analysis, might be a disadvantage in the respect of quality or biological activity of a radiopharmaceutical. It was therefore crucial to analyze both compounds. Since the unlabeled peptides showed a single peak, we assumed that the formation of isomers is related to the metal complex and not the receptor binding part of the peptide chain. One confirmation was the equal receptor affinity of the compounds of the separated HPLC fractions, the most important finding with respect to the use as radiopharmaceutical.

The best proof of the formation of two isomers of the metal complex would be the chemical analysis by NMR and MS. This turned out to be difficult for two reasons. Due to the high specific activity of the $^{99m}$Tc it was easy to obtain a high radioactive concentration, but the molar concentrations were always too low for NMR or MS analysis. Such studies could only be done with the Re-compounds. Additionally, the low solubility of the Re-peptides in water did not allow a full interpretation of the spectra. Specifically, the COSY spectra showed only very few of the expected couplings and did not support a reliable interpretation of the NMR spectra of our BBS analogues. Also, the published NMR analysis (25) did not allow the unequivocal assignment of all the NMR signals. Therefore we decided to prepare the reference compound ReL1.

The NMR spectra of the reference compound ReL1 (fraction 1: P1, fraction 2: P2) are in accordance with the R and S conformation of the amine bound to Re (see the structures depicted in Figure 3.8). There are different arguments supporting this assignment. First: the H-10 signals are in accordance with a restricted rotation around the amine-C-10 bond, the coupling with the amine-H and the geminal coupling lead to well separated quartets compound in P2, while the localization above the Re-imidazol bond in P1 is compatible with a signal shift to a higher field. The same shift is observed with the signals of H-C7 and H-C8 of ReL1 compared to L1. Second: one of the H at C-5 interferes slightly with the amine-H in P1 which leads to different chemical shifts, while in P2 C-5 is close to the C-10 (Ac), with less influence on the chemical shift. Third: the different chemical shift of the H-4 is due to the shorter distance to the amide group in P2 compared to P1, additionally, the H-4 is in an equatorial position in both isomers, while the vicinal amine-H is in axial position in P1 and in equatorial position in P2.
Another interpretation is much less probable, but cannot be excluded. The formation of a hydrogen complex containing an additional water molecule was found investigating the Cu histidine complexes (29, 30). However, such a protonated species should lead to a much better separation on HPLC (1+ charged compared to neutral). For the same reason the protonated form should be found in fraction 1. There the acetate-H (H-10) are found at a higher field than in fraction 2, which is in contradiction to expectations, because the protonated amine should lead to a down-field-shift of this signal. The MS gives no indication of an additional water molecule, but this could be lost during activation (evaporation and ionization).

In spite of the incomplete interpretation of the NMR of the BBS analogue, some important conclusions can be drawn. The region around 7 and 8 ppm shows that one His is bound to Re, while the other one is free, which is in agreement with the result of the His-challenging experiment and the chemical shifts of the reference compound ReL1. The area around 3 to 4 ppm shows in part the same signals with Re-BBS-II and ReL1 and of course the additional α-protons of the amino acids. Additionally, similar differences were observed which are in accordance with the above discussed isomers of the metal complex. The region between 0 and 2 ppm shows the methylen- and methyl-H of Leu, Val and Ala. Two peaks which appear in the spectra of both compounds are very sharp singlets (2.83 ppm and 2.02 ppm in the first fraction, 2.89 and 2.07 in the second one). They correspond to the S-CH₃ group of methionine or methionine-sulfoxide (shifted to lower field). The integral of about 1:2 in both cases shows that some oxidation took place, but that oxidation is not the reason of the formation of the two peaks. The additional oxygen was also observed in the MS spectra. Re-injection of the purified products usually showed about 10 to 20 % of the second product in the first fraction, while the second fraction usually was pure. This finding of the oxidation problem together with the good affinity if Met is replaced by Nle, were the reasons to chose Nle for future derivatives.
Figure 3.8. Models of the M(CO)₃ Na-HisAc (R=H or peptide; M=Tc, Re) complexes 1 and 2 obtained in the respective HPLC fraction 1 (left) and fraction 2 (right). H: white, C black (with numbers), N and O grey with atomic symbols. The amine-H (arrow) and the Ac-methylene group (C-10) are found at different positions. The H of the NMR spectra are identified with the number of the respective C atom given here.

Literature data and earlier own studies (31) showed that the His¹²-Lys¹³ bond of BBS may easily be metabolized by the neutral endopeptidase 24.11 (E.C.3.4.24.11) and the angiotensin converting enzyme (ACE). The substitution of Leu by CyHAla at position 13 should have the effect that the peptides do not fit anymore into the cleavage site of enzymes.

The modifications in the 7-14 sequence contributed to increase the plasma stability of the analogues BBS-IV, BBS-IX and BBS-XXX which was clearly better than that of BBS-II (8). However, the improvement in stability was less effective in human prostate adenocarcinoma PC-3 cells where the analogues were rapidly metabolized (half-lives of 20, 15 and 15 min for $^{99m}$Tc(CO)$_3$-BBS-IV, -IX and --XXX, respectively). The rapid cellular metabolism and externalization of the activity (Figures 3.5 and 3.7) would reduce the potential of these BBS analogues to be successfully introduced as radiopharmaceuticals.

The affinity for the GRP receptor was tested in PC-3 cells, where the analogues showed to retain the binding properties of the natural BBS. It was observed for all the derivatives a better affinity for the labeled analogues than for the unlabeled ones (Table 3.5) and we had also observed the same effect for NT analogues with the same ligand (10, 11). All these BBS analogues showed a rather rapid and high extent of
internalization. Internalization increased with time, reaching a maximum within 30 min and remained constant up to 2 h. A similar pattern of internalization was obtained for other BBS analogues already reported (7, 8). It is believed that the envisaged therapeutic effect is higher if the radionuclide is internalized compared to compounds remaining on the cell surface, because of potentially longer retention in the target tissue. However, our new analogues were also rapidly externalized (more than 70 % in the first 5 h, Figure 3.7) as observed for the unmodified analogue (8). These results obtained in binding assays demonstrate that the modifications of the natural sequence affected neither the affinity for the GRP receptor, nor the internalization/externalization of the peptide. This was expected, since the modifications did not dramatically alter the C-terminal structure of the peptide, which is crucial for high affinity binding and biological activity as previously reported (32). The rapid externalization of the internalized activity is likely due to a much more rapid degradation of the analogues in the tumor cells than in plasma. Therefore, new modifications should be considered in order to increase the stability and the retention time in the tumor cells.

Biodistribution studies were performed in PC-3 tumor bearing mice. The conjugates $^{99m}$Tc(CO)$_3$-BBS-IV, -IX and -XXX exhibited a rapid clearance from the blood pool and most non-targeted tissues (Table 6). The second highest uptake for all the analogues was found in the liver. This is most probably due to the lipophilicity of the conjugates. A higher uptake of radioactivity was observed in the pancreas than in the tumor and this is also expectable because mice pancreas is a GRP receptor-rich tissue. The reason for the low uptake in the PC-3 tumors was probably the poor vascularization.

Furthermore, the receptor blocking study confirmed the specificity of the compounds toward GRP receptors. The uptake in GRP receptor-positive tissues (i.e. pancreas and colon) was reduced after co-injection with 300 µg of BBS. In the case of $^{99m}$Tc(CO)$_3$-BBS-IV the uptake in pancreas and colon was reduced in more than 50 % ($3.28 \pm 1.30\%$ ID/g unblocked vs. $1.33 \pm 0.77\%$ ID/g blocked and $5.25 \pm 1.75\%$ ID/g unblocked vs. $2.51 \pm 2.43$ blocked for pancreas and colon, respectively).
3.6 Conclusion

The use of the tricarbonyl-rhenium and -technetium complexes together with His as tridentate ligand is a suitable tool to label biomolecules. The stability of the BBS analogues could be improved but further improvement is needed to obtain higher uptake in GRP receptor-rich tissues compared to liver and kidney.

Acknowledgments

The authors thank Ms. Margaretha Lutz for technical assistance and Ms Regina Reppich for the mass spectrometry. This work has been partly funded by an Oncosuisse-grant, Nr. OCS 01311-02-2003.
3.7 Literature cited


(26) Alberto R., Schibli R. and Schubiger P.A. (1996) Reactions with the technetium and rhenium carbonyl complexes \((\text{N(Et)}_4)_2[\text{MX}_3(\text{CO})_3]\). Synthesis and structure of \([\text{Tc(CN-Bu')_3(CO)}_3(\text{NO}_3)]\) and \((\text{NEt}_4)[\text{Tc}_2(\mu-\text{SCH}_2\text{CH}_2\text{OH})_3(\text{CO})_6]\). *Polyhedron* 15, 1079-1089.


Influence of two different spacers of Technetium-99m labeled bombesin analogues on the activity uptake in tumors of mice.

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\textit{Nuclear Medicine and Biology (to be submitted)}
Seite Leer / Blank leaf
4.1 Summary

Two spacers have been introduced between the receptor binding amino acid sequence (7-14) of bombesin (BBS) and the His-chelator used for labeling with the 99mTc(CO)3-core. These spacers, namely -βAla-βAla- and 3,6-dioxa-8-amino octanoic acid were combined with BBS analogs containing the natural amino acids Leu13 and Met14 or the non-natural amino acids CyHAla13 and Nle14, which contribute to a better stability of the analogues. The spacers had only little influence on the metabolic stability and the receptor affinity of the new BBS analogs. The dissociation constant obtained for 99mTc(CO)3-BBS-38 ($K_d = 0.18 \pm 0.1 \text{ nM}$) and for 99mTc(CO)3-BBS-39 ($K_d = 0.26 \pm 0.01 \text{ nM}$) were similar to the unmodified analogue ($K_d = 0.18 \pm 0.1 \text{ nM}$). However, the spacers brought about a significant increase of the uptake in the GRP receptor rich pancreas and in PC-3 tumor xenografts in nude mice.
4.2 Introduction

The targeting of overexpressed peptide receptors in tumors by small peptides has become a very strong focus of interest for nuclear medicine [1]. The use of small peptides has advantages because of high affinity and rapid plasma clearance, which may result in a high concentration in the target tissue and good tumor to background ratios. The main drawback of small peptides, namely the rather rapid metabolic degradation, might be overcome by the introduction of non-natural amino acids if the receptor affinity remains unchanged. In the case of Bombesin (BBS) it seems to be important to introduce additionally a spacer between the metal complex used for labeling and the binding sequence of BBS. Such spacers contribute to a higher uptake in GRP-rich tissues.

For labeling with Tc-99m several conjugates with bi-functional chelators were developed [2]. Na-histidinyl acetate [3], PADA-AVA [4], N3S [5], diaminopropionic acid [6], P2S2 [7, 8] and tetraamine [9]. DTPA and DOTA are common for labeling with hard Lewis acid radiometals, like 111In, 67/68Ga, 90Y and the lanthanides [10].

In our laboratory we developed BBS analogues, in which 99mTc or Re (natural) tricarbonyl cores are bound via the ligand system Nα-carbamoylmethyl histidine (NctHisAc) to the N-terminal amine group of BBS(7-14)NH2 (Table 4.1) [3]. Here we present analogues with additional spacer groups. The length of the spacer group and the properties of the radiolabeled complex seem to influence GRP-receptor-binding affinity, uptake of radioactivity in cancer cells and pharmacokinetics of the BBS conjugate. Smith et al. and Hoffman et al. investigated the pharmacokinetics and pancreatic accumulation of different analogues with a series of aliphatic spacer groups, which ranges from 0 to 11 carbon atoms. Constructs in which the aliphatic spacer group ranges from 5 to 8 carbon atoms appear to be the most promising for targeting [5,6,11,12].

BBS analogues in which two natural amino acids (Leu13, Met14) have been replaced by non-natural ones (CyHAla13, Nle14) to improve metabolic stability have already been synthesized and analyzed. The aim of the present work was to combine these findings of stabilization with the advantages of additional spacers.

The complexes M(CO)3((NctHis)Ac) are neutral, while many of complexes mentioned above are either positively or negatively charged. Since we observed solubility problems with our earlier used BBS analogues, we decided to introduce linkers, which are more polar than the aliphatic spacers.
Chapter 4

The study involved pharmacological characterization, like metabolic stability in vitro, binding properties, internalization, externalization and in vivo biodistribution in nude mice bearing human PC-3 or Du-145 xenografts of the new bombesin analogues.

Table 4.1: Sequence of BBS-II (unmodified) and new stabilized BBS analogues (BBS-XXX, BBS-37 -38 and -39) obtained by solid phase method using Fmoc-strategy. (CyHAla = Cyclohexyl alanine)

<table>
<thead>
<tr>
<th>Bombesin-analogues</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBS-II</td>
<td>(NαHis)Ac-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂</td>
</tr>
<tr>
<td>BBS-XXX</td>
<td>NαHis)Ac-Gln-Trp-Ala-Val-Gly-His-CyHAla-Nle-NH₂</td>
</tr>
<tr>
<td>BBS-37</td>
<td>(NαHis)Ac-βAla-βAla-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂</td>
</tr>
<tr>
<td>BBS-38</td>
<td>(NαHis)Ac-βAla-βAla-Gln-Trp-Ala-Val-Gly-His-CyHAla-Nle-NH₂</td>
</tr>
<tr>
<td>BBS-39</td>
<td>(NαHis)Ac-NH-CH₂-CH₂-O-CH₂-CH₂-O-CH₂CO-Gln-Trp-Ala-Val-Gly-His-CyHAla-Nle-NH₂</td>
</tr>
</tbody>
</table>

4.3. Materials and methods

4.3.1 Chemicals

The Fmoc-protected amino acids (Met, Nle, Leu, CyHAla, His(trt), Gly, Val, Ala, βAla, Trp(Boc), Gln(trt), 3,6-dioxo-8-aminocarboxylic acid and the Rink amide resin were purchased from NovaBiochem (Läufelingen, Switzerland). Diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland), D(+)-scucrose, 1,10-phenanthroline, dimethylsulphoxide, ethandithiole, 1-hydroxy-benzotriazole (HOBT), N-ethylidiisopropylamine (DIPEA), thioanisole, trifluoroacetic acid (TFA) from Fluka (Buchs, Switzerland). Bombesin (1-14) and Nτ-trityl-O-tertbutyl histidine, bestatin, prolins-isoleucin (H-Pro-Ile-OH), were obtained from Bachem (Bubendorf, Switzerland). The [125I-Tyr] bombesin was purchased from Amersham Pharmacia Biotech (Dübendorf, Switzerland). Na[^99mTcO₄] was eluted from a Mallinckrodt[^99mTcO₄] generator (Tyco Healthcare, Petten,
The Netherlands) using 0.9% saline. Sodium boranocarbonate was a gift from the provider of the generator and \((N(Et)_4)_2[Re(CO)_3Br_3]\) a gift from Dr. R.Schibli (13). Cell culture media DMEM GLUTAMAX-I, MEM Eagle with 0.1 mM non essential amino acids and 1.0 mM sodium pyruvate, Trypsin/EDTA, bovine serum albumin and soybean trypsin inhibitor were obtained from Invitrogen (Basel, Switzerland). Foetal calf serum and antibiotic/antimycotic solution were obtained from Amimed (Allschwil, Switzerland). Bacitracin, hepes, chymostatin, phosphoramidon, enalapril-maleat and ACE-inhibitor (pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) were purchased from SIGMA (Buchs, Switzerland). The working reagent for the protein assay was ordered from SOCOCHIM SA (Lausanne, Switzerland). All chemicals not mentioned above were obtained from either Fluka (Buchs, Switzerland) or Merck (Dietikon, Switzerland). PBS (phosphate buffered saline) contain a physiological salt concentration, if not otherwise stated.

4.3.2 Equipment

Correct mass was identified by MALDI-TOF mass spectrometry on a Voyager-DE RP workstation (Applied Biosystems, Darmstadt, Germany) and Re labeled analogues by TOF MS ES, LCT Premier, Waters, Switzerland). The HPLC (peptide synthesis) consisted of a RP C-18 column (250 x 4.6 mm, 5µm, Vydac, Hesperia, USA), flow 0.6 ml/min, gradient 10% to 60% A in B within 30 min, A = acetonitrile/TFA (100:0.08), B = water/TFA (100:0.1). The HPLC analyses of radiolabeled peptides were performed on a Varian ProStar system with a Varian ProStar 230 Solvent Delivery-Pump, a Varian Photodiode Array Detector Model 330, a Varian ProStar Autosampler Model 410 and a radiomatic Flo-one Beta Detector (Packard Canberra), a Macherey-Nagel CC Nucleosil 100-5 C18 reverse phase column (10µm, 250 x 4.6 mm). HPLC solvents consisted of acetonitril with 0.1% TFA (solvent A), water with 0.1% TFA (solvent B) and methanol (solvent C). The gradient for the labeling control started with a linear gradient 15%A/80%B/5%C to 80%A/20%B/0%C (1 ml/min) from 0 to 35 min (Bombesin 1) and for the metabolic analyses with 0%A/50%B/50%C to 15%/5%/80% (1 ml/min) from 0 to 25 min (BombesinIIStab1). The radioactivity was measured with an Auto-γ-counter (Packard Canberra Cobra II) or an ionization chamber (activimeter).
4.3.3 Synthesis

BBS-37, BBS-38, BBS-39: Bombesin-analogues were obtained by automated solid phase peptide synthesis with an automated Syro robot system from MultiSyn Tech (Bochum, Germany) using Rink amide resin and the Fmoc/tBu-strategy. Fmoc-amino acids were coupled with eight-fold excess in duplicate. After the synthesis, still on resin, we coupled the chelator (Na-histidinyl acetate) on the analogues. The coupling was performed in two steps: In a separate vial 6 equivalent (4.7 mg) bromo acetic acid (the amounts are normalized to the amount of resin used) and 3 equivalents DIC were dissolved in 0.5 ml DCM. After 20 min the obtained anhydride solution was added to a mixture of 1 equivalent resin (6.75 µmol) and 3 equivalents DIPEA. Two hours later the resin was washed with DMF, then 3 equivalent Nα-trityl-O-tertbutyl histidine and 3 equivalent DIPEA dissolved in 2 ml DMF were added and the mixture was stirred for 24 h. The peptides were cleaved from the resin, in that TFA and ethandithiol/thioanisol (3:7) was added and allowed to stand for 3h. After cleavage the peptides were precipitated from cold diethyllether, collected by centrifugation and lyophilized from water/tert.-butylalcohol (3:1).

4.3.4 Radiolabeling

$^{99m}$Tc(CO)$_3$-peptides: About 1ml of a [$^{99m}$TcO$_4$] generator eluate in saline (up to about 5 GBq) was added to a mixture of 4.5 mg sodium-boranocarbonate, 2.9 mg borax, 9 mg potassium-sodium tartrate tetrahydrate and 7-8 mg sodiumcarbonate. The solution was heated for 20 min at 100°C. After cooling, the tricarbonyl-solution was neutralized top pH 6.5 with a mixture of 1 M HCl and 0.6 M phosphate buffer. An aliquot of 500 µl was mixed with 15 µl of the peptide solution (1mM) and 5µl Tween 4% and heated for 1 h at 75°C.
Re(CO)$_3$-BBS-38 and -BBS-39: A solution of 2.5 mg BBS-analogues in 2.5 ml water, 1.35 mg [N(Et)$_4$][Re(CO)$_3$Br$_3$] and 60 µl NaOH (0.075 N) was heated for 6 h at 75°C. The suspension was then centrifuged and the supernatant purified with the HPLC.

4.3.5 Cell culture

The human prostate adenocarcinoma cell line PC-3 was purchased from European Collection of Cell Culture (Salisbury, England). Cells were maintained in DMEM GLUTAMAX-I supplemented with 1-10% fetal calf serum, 100 IU/ml penicillin G sodium, 100 µg/ml
streptomycin sulphate, 0.25 µg/ml amphotericin B. Cell culture was incubated at 37°C in an atmosphere containing 7.5% CO₂. The cells were passaged weekly after detaching them with trypsin/EDTA (0.25%).

4.3.6 Metabolism

Stability in human plasma. The half-lives of the radiolabeled conjugates were determined in vitro in plasma from healthy donors. The ⁹⁹ᵐTc(CO)₃-BBS analogues at a final concentration of 3 to 4 MBq/ml (1.5-2 pmol/ml) were incubated at 37°C for various time intervals (up to 24 h). After incubation, proteins were precipitated with acetonitrile/ethanol (1:1) and TFA (0.1%) and centrifuged (10 min., 20000g) at 4°C. The pellet was discarded, the supernatant filtered and analyzed by RP-HPLC.

Stability in PC-3 cells. The stability in cell culture was evaluated in vitro in a suspension with 2 x 10⁶ cells in 1 ml PBS without the addition of peptidase inhibitors (control) or with inhibitors. The inhibitors were applied as follows: phosphoramidon 100µM, Pro-Ile 50mM, ACE-Inhibitor 100 µM, 1,10-phenanthroline 1mM and enalapril 100 µM (Table 4.2). The PC-3 cells were incubated for various time intervals (up to 1h) at 37°C with 3 to 4 MBq/ml ⁹⁹ᵐTc(CO)₃-BBS analogues. After incubation cells were lysed and the proteins precipitated by the addition of acetonitrile/ethanol (1:1) and TFA (0.1%) and the obtained turbid solution filtered. The filtrates were analyzed by RP-HPLC. All experiments were carried out 2-3 times.

Stability in kidney and liver homogenates. Liver and kidney, obtained from one mouse were placed in 5 ml sucrose 0.25 M, homogenized (cell smasher RW20) and the content of proteins determined (PIERCE protein assay). We used 10 mg protein of liver or 2.5 mg protein of kidney homogenate and 50 µl (1 MBq) ⁹⁹ᵐTc(CO)₃-BBS analogues. The volume was adjusted with sucrose 0.25 M to 1 ml. After incubation for different times at 37°C, the samples were put on ice and 750 µl of a mixture of acetonitril / ethanol 1:1 + 0.1% trifluoroacetic acid were added to stop the reaction and to precipitate the proteins. The suspensions were centrifuged at 4°C and 20000 rpm for 10 min. An aliquot of the supernatant was filtered by Sep-Pak, which was previously washed with 6 ml H₂O, 6ml EtOH and 2ml H₂O; 50 µl of the filtrate were analyzed by HPLC.

Analysis of urine of mice. The excretion was determined at 1.5 h post-injection (see biodistribution experiments). Immediately after killing, the urine was collected with a syringe, proteins precipitated with acetonitrile/ethanol (1:1) and TFA (0.1%) and centrifuged. The supernatant was filtered and analyzed by RP-HPLC.
Table 4.2: Enzyme inhibitors used for the stability tests and enzymes known to cleave peptides

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoramidon</td>
<td>Neutralendopeptidase EC 3.4.24.11</td>
</tr>
<tr>
<td>Bestatin</td>
<td>Aminopeptidase N EC 3.4.11.2</td>
</tr>
<tr>
<td>1,10-Phenanthrolin</td>
<td>Metalloendopeptidases EC 3.4.24</td>
</tr>
<tr>
<td>Pro-Ile</td>
<td>Endopeptidases EC 3.4.24.16</td>
</tr>
<tr>
<td>ACE-Inhibitor* / Enalapril</td>
<td>ACE</td>
</tr>
</tbody>
</table>

* ACE-Inhibitor: p-Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro

4.3.7 Binding assays

Inhibition studies: PC-3 cells were placed one day before the assay at confluence in 48-well plates (250’000 cells/well). The cells were incubated for 1 h at 37°C in triplicate with 15’000-25’000 cpm \([^{125}\text{I}-\text{Tyr}^4]\)BBS and increasing concentrations of the different unlabeled analogues (0-3’000 nM). A special binding buffer including protease inhibitors was used, composition as follows: 50 mM HEPES, 125 mM NaCl, 7.5 mM KCl, 5.5 mM MgCl₂, 1 mM EGTA, 5 g/l BSA, 2 mg/l chymostatin, 100 mg/l soybean trypsin inhibitor, 50 mg/l bacitracin. The cells were then washed twice with cold PBS. The cell bound activity was solubilized with 400µl (2x) of 1 N NaOH at 37°C and determined in a γ-counter. IC₅₀ values for the displacement of binding of \([^{125}\text{I}-\text{Tyr}^4]\)BBS by the different analogues were calculated by non-linear regression analysis using GraphPad Prism™. Experiments were performed twice.

Saturation studies: One day before the assay PC-3 cells were placed at confluence in 48-well plates (1 x 10⁶ cells/well). The cells were incubated for 1 h at 37°C in triplicate with increasing concentrations (0.001-1 nM) of the \(^{99m}\text{Tc}(\text{CO})_3\)-BBS analogues (final volume, 0.2 ml per well). The concentration of total technetium \((^{99}\text{Tc}+^{99m}\text{Tc})\), estimated according to Bauer and Pabst (14), were equivalent to 0.002-2 MBq \(^{99m}\text{Tc}\) per well. Nonspecific binding was determined with 1µM unlabeled BBS(8-14). After 2 h incubation the cells were washed twice with cold PBS 1X, solubilized (2x) with 400µl of 1 N NaOH at 37°C. The protein content was determined in a BIO RAD microplate reader (570 nm) using the Micro BCA Protein Assay Reagent from Pierce.
4.3.8 Internalization

PC-3 cells were placed one day before the assay at confluence in 6-well plates (about 1 x 10^6 cells/well). The cells were incubated with approximately 4 kBq of ^99mTc(CO)₃-BBS analogues for 5, 15, 30, 60 and 120 min at 37°C to allow binding and internalization (total final volume 1ml per well). Nonspecific internalization was evaluated in the presence of 1μM unlabeled BBS(8-14). After the various incubation times, the cells were washed 3 times with PBS, surface-bound activity was removed by two steps of 5 min acid wash (50 mM glycine-HCl, 100 mM NaCl, pH 2.8, 600μl per well) at room temperature. The cells were solubilized by incubation at 37°C with 600μl (2x) of 1 N NaOH per well to determine internalized radioligand. Surface bound and internalized activities were measured in a γ-counter. Results were expressed as percentage of total activity in the cells (surface bound and internalized). All experiments were carried out 2-3 times in triplicate.

4.3.9 Externalization

PC-3 cells at confluence were placed in 6 cm diameter Petri dishes. The cells were incubated with about 20 kBq of ^99mTc(CO)₃-BBS analogues in culture medium for 45 min at 37°C to allow maximal internalization into the cells (total final volume 5ml per dish). The supernatant was discarded and the cells washed twice with PBS. The cells were then incubated again at 37°C with 5ml culture medium for 0, 30, 60, 150 and 300 min. The activity in the supernatant was measured to determine released activity from the cells. After 2 washing steps with PBS the surface-bound activity was removed by two steps of 5 min acid wash (50 mM glycine-HCl, 100 mM NaCl, pH 2.8, 0.75 ml per dish) at room temperature. Finally, the amount of remaining internalized activity was recovered by solubilizing the cells at 37°C with 1ml (2x) of 1 N NaOH per dish. Externalized, surface-bound and internalized activities were measured in a γ-counter (experiments were performed 2-3 times).

4.3.10 Biodistribution studies

All animal experiments were conducted in compliance with the Swiss animal protection laws and with the ethical principles and guidelines for scientific animal trials established by the Swiss Academy of Medical Sciences and the Swiss Academy of Natural Sciences. Studies were performed with 6 to 8 weeks old female CD-1 nu/nu mice (20-25 g), purchased from...
Charles River Laboratories (Sulzfeld, Germany). For the induction of tumor xenografts, PC-3 cells and DU-145 cells were subcutaneously injected at different concentrations (5-20 x 10^6 cells/mouse) and allowed to grow for 10 days. On the day of the assay the radioactive conjugate (about 3 MBq/mouse) was injected into the tail vein. The mice were sacrificed by cervical dislocation and dissected. The biodistribution of BBS-38 and BBS 39 was evaluated at 0.5, 1.5 and 5 h post-injection (BBS-38 additionally at 24 h). In addition, 3 mice per analogue received 100-300 µg of cold BBS, co-injected with the radiolabeled BBS analogue to determine non-specific uptake and were killed 1.5h (BBS-38), 0.5h and 1.5 h (BBS-39) after injection. Tumors and other tissues (blood, heart, lung, spleen, kidneys, stomach, pancreas, ileum, colon, liver, muscle, bone and brain) were removed and weighed. The amount of radioactivity was determined with a γ-counter. Results were expressed as percentage of injected dose per gram of tissue (% ID/g) referred to total injected activity.

4.4 Results

4.4.1 Synthesis and labeling

The retention-times of the Tc-99m labeled peptides are summarized in (Table 3). The free peptides were eluted about 4 min before the labeled BBS-analogues. In general, both pertechnetate and tricarbonyl represented only 0-5% and 0-1%, respectively, of the total activity after labeling. The rest of the activity corresponded to the 99mTc(CO)₃-labeled peptides. The mass spectrometry analysis of the free and Re-BBS-analogues were listed in (Table 4.3).

Table 4.3: Results of the purity and identity test of the BBS analogues: HPLC retention time and mass of free and labeled peptides

<table>
<thead>
<tr>
<th>BBS-analogue</th>
<th>retention of 99mTc labeled peptide (min)</th>
<th>Calculated mass of the free peptide (g/mol)</th>
<th>Measured mass of the free peptide (m/z, z=1)</th>
<th>Measured mass of the Re-peptide (g/mol) (m/z, z=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBS-37</td>
<td>16.4</td>
<td>1277.6</td>
<td>1276.9</td>
<td>-</td>
</tr>
<tr>
<td>BBS-38</td>
<td>16.8</td>
<td>1299.6</td>
<td>1298.7</td>
<td>785.1 (Re-187)</td>
</tr>
<tr>
<td>BBS-39</td>
<td>17.6</td>
<td>1302.5</td>
<td>1301.7</td>
<td>786.6 (Re-187)</td>
</tr>
</tbody>
</table>
4.4.2 Metabolism

In human plasma $^{99m}$Tc(CO)$_3$-BBS-38, and -39 were clearly more stable than $^{99m}$Tc(CO)$_3$-BBS-37, which showed a similar stability as natural bombesin (50% degradation within 30 min) [3] (Fig.4.1).

A much faster metabolism was observed in PC-3 cells than in plasma. After 30 ± 5 min incubation only 50% of the analogue 38 was still intact. The half-life of BBS-39 was 22 ± 5 min. The enzyme-inhibitors 1,10 phenanthroline and Pro-Ile showed an inhibitory effect on both analogues in same degree. Phosphoramidon, enalapril and the ACE-inhibitor only influenced the metabolism of BBS-38, whereas the metabolism of BBS-39 remained unchanged.

The analysis of metabolism in kidney homogenates showed 24% of intact BBS-38 after an incubation of 15 min, while a complete degradation in liver homogenates was detected. The urine collected 1.5 h after injection of $^{99m}$Tc(CO)$_3$-labeled BBS-38 and BBS-39 was analyzed by RP-HPLC. Only metabolites were excreted.

Figure 4.1: Metabolic stability of the $^{99m}$Tc(CO)$_3$-BBS-analogues in human plasma at 37°C, analyzed by RP-HPLC. Data are expressed as percentage of activity recovered corresponding to intact peptide.
4.4.3 Binding assays

Inhibition studies: The different BBS analogues inhibited the binding of $[^{125}\text{I}-\text{Tyr}^4]\text{BBS}$ showing a typical sigmoid curve (Fig. 4.2). The unlabelled BBS-analogue 37 showed the same inhibitory activity (IC$_{50}$: 1.8±0.46 nM) as Bombesin(7-14) (IC$_{50}$: 1.9±0.5 nM). The values of the other BBS analogues were higher (IC$_{50}$ = 5.05±1.7 nM and 8.9±0.5 nM for BBS-38 respectively BBS-39), indicating a lower affinity for GRP receptors (Fig. 2). A further increase was seen with the Re-compounds, namely IC$_{50}$ = 22±6 nM for Re(CO)$_3$-BBS-38 and 20±2 nM for Re(CO)$_3$-BBS-39.

Saturation studies: in saturation studies, specific binding of $^{99m}\text{Tc(CO)}_3$-labeled BBS-38 and BBS-39 to intact PC-3 cells was found to be saturable. Specific binding increased with increasing concentration and finally reached a plateau. Both BBS-38 and-39 showed a high affinity for GRP receptors. The dissociation constant obtained for $^{99m}\text{Tc(CO)}_3$-BBS-38 (K$_d$ = 0.18 ± 0.1 nM) and for $^{99m}\text{Tc(CO)}_3$-BBS-39 (K$_d$ = 0.26 ± 0.01 nM) were similar to that obtained for BBS-II, the unmodified analogue (K$_d$ = 0.18 ± 0.1 nM).

Experiments were performed 2 times in triplicate.

![Figure 4.2: Displacement curves of $[^{125}\text{I}-\text{Tyr}^4]$bombesin by the free BBS analogues and the Re-compound with the stabilized analogues BBS38 and BBS-39.](image-url)
4.4.4 Internalization

After binding the receptor, the analogues were rapidly internalized (Fig 4.3). In all cases BBS-37, BBS-38 and BBS-39, the percentage of internalization increased with time and reached after 2 h a maximum of 80% of the cell related activity. The internalization could be inhibited with 1 µM BBS(8-14). All three analogues showed a low non-specific binding.

![Graph showing internalization of analogues over time](image)

**Figure 4.3:** Time course internalization of $^{99m}$Tc(CO)$_3$-BBS-analogues in PC-3 cells at 37°C. Data are percentage of acid-resistant activity related to total activity in cells (surface bound and internalized). Each point represents mean of 2 experiments in triplicate.

4.4.5 Externalization

The labeled BBS-analogues were allowed to internalize for 45 min, then the extracellular activity was removed. The observed externalizations were rapid as shown in Fig.3. The externalized activity was analyzed by HPLC, it consisted of metabolites and intact peptides were always below the detection limit.

4.4.6 Biodistribution and tumor uptake

The tumor uptake with different numbers of PC-3 cells (5 to 20 Mio), the addition of matrigel and tumor growth of 10 days showed only small differences, the best result was seen with 10 Mio cells/tumor. The matrigel did not show any improvement. In one experiment we
investigated mice with two different tumors, existing of PC-3 or DU-145 cells. The uptake was tested for the analogue 38 and showed a slightly higher uptake in the DU-145 xenografts. The highest uptake was found in pancreas and colon. Both could be inhibited by more than 70% after co-injection with natural BBS for BBS-38. Tumor uptake was rather low for BBS-38 and -39. About 50% for BBS-38 could be inhibited. The pancreas to kidney ratio after 30 min has a value of 10.6 for BBS 38 and 6.5 for BBS-39. The ratios pancreas to liver are 3.6 and 1.5 for the analogue 38 respectively 39.

The results are shown in (Table 4.4). The biodistribution of $^{99m}$Tc(CO)$_3$-BBS-38 was tested at 0.5, 1.5, 5 and 24 h, for $^{99m}$Tc(CO)$_3$-BBS-39 at 0.5, 1.5 and 5 h.

Table 4.4: Biodistribution of the $^{99m}$Tc(CO)$_3$BBS analogues ( 3 MBq/mouse) in nude mice with PC-3 xenografts. Results are expressed as percentage of injected dose per gram of tissue (% I.D./g) ± S.D. (n = 3-7).
4.5 Discussion

The experience with radiolabeled bombesin analogs in studies with mice suggest that in this case a spacer between the binding sequence of the amino acids 8-14 and the metal complex contributes to a high uptake in GRP rich tissues. The reason of this behavior would be easy to understand, if the metal complex due to its size or charge would interfere with the receptor. However, many BBS analogs with or without spacer have been found to have similar affinities, while the in vivo uptake clearly depends on the length of the spacer. Accordingly we also chose spacers to study the in vitro and in vivo properties of $^{99m}$Tc(CO)$_3$-BBS analogues. Since the negative charge of the ligand (NaHisAc) is compensated by the +1 charge of the Tc-carbonyl core, the labeled BBS analogs are neutral and usually not well soluble in water. For this reason we avoided a long aliphatic chain an introduced two spacers with slightly polar bonds, namely βAla-βAla and 8-amino-3,6-dioxa-octanoic acid (an ethyleneglycol derivate).

The affinity of the derivatives for the GRP receptors in PC-3 human prostate adenocarcinoma cells was the same with the natural bombesin and BBS-37, while BBS-38 and BBS-39 were less efficient to inhibit the binding of $[^{125}$I-Tyr$^4$]BBS but still slightly more efficient than BBS-XXX. Fig 2 shows that the complex formation with the Re(CO)$_3$-core brings about a further reduction in the affinity. The $K_d$ values (determined according to Scatchard with Tc-labeled BBS-38 and BBS-39) were in the low nanomolar range, similar for both analogues and only with slight difference compared to BBS-II.

After binding to the receptor at 37°C, the $^{99m}$Tc(CO)$_3$-BBS analogues were rapidly internalized. The activity increased with time, reaching high values after 30 min, without any significant changes up to 2 h. Most of the internalized activity was externalized within the first 5 h in form of metabolites. The externalization of the three BBS analogs was quite similar. We expected in contrary that the different spacers would lead to different rates of externalization, specifically because the influence of these spacers are assumed to be bigger in the case of small metabolites compared to the larger intact BBS analogs. These results demonstrate that the modifications affected neither the affinity, nor the internalization and externalization. The modifications did not alter the C-terminal structure of the peptide, which is crucial for high affinity binding and biological activity as reported [15, 16, 17].

The proteolytic degradation of the three analogs determined in vitro in human plasma showed the expected result, namely a slower degradation of BBS-38 and BBS 39, i.e. the analogs containing non-natural amino acids. Compared to the earlier published analogs without spacer
(BBS-IV and BBS-XXX) the new compounds were less stable, indicating that new degradation sites were introduced. In contrast to these results, the degradation in PC-3 cell culture of the new analogues was slower than the degradation of the analogs without linker. According to earlier experience the degradation in PC-3 cell culture was still faster than in plasma. Thus an unexpected sequence of the stability was found: BBS without spacer in plasma > BBS with spacer in plasma > BBS with spacer in PC-3 cells > BBS without spacer in PC-3 cells. Since bombesin analogues have blocked amino terminals and lack basic amino acid residues, they cannot readily be degraded by aminopeptidases and trypsin like enzymes, respectively (18), thus endopetidases are responsible for the degradation of neuropeptides (19, 20, 21).

Analysis of the stability in PC-3 cell culture and in presence of endopeptidase-inhibitors (Table 4.2) showed a similar result with 1,10-phenanthroline and Pro-Ile. In the case of BBS-38 a similar effect of reduced degradation was found with phosphoramidon, enalapril and the ACE-inhibitor, whereas with BBS-39 no effect was observed. These two peptides only have different spacers, thus we would not expect a different metabolism.

Speculations in this regard would be: Surface bound and internalized peptides are distributed in different parts of the cells, because of the different properties of the spacers (e.g. absorption of a part of the peptide in the cell membrane due to the lipophilicity). Thus, the concentration of the peptides in presence of the enzymes is depending on the spacer, which influences the observed speed of degradation. A second explanation could be: The different properties of the spacers influence the folding of the peptide, which influences the binding to the active center of the peptidase resulting in different kinetics of the hydrolysis of the amide bond. As a third possible reason one could assume that the small difference of the receptor affinity influences the release of the peptides after internalization. This would again be an effect of the concentration of the free peptides inside the cell.

A slow metabolism in plasma is a prerequisite for good tumor uptake of labeled peptides because only the intact peptide which reaches the tumor will be taken up. In the cells not only the metabolism but also externalization is important. Externalized intact peptides will show reuptake but not metabolites. If metabolites are externalized extremely slowly, the metabolism plays a minor role because the activity is still retained. Only fast metabolism and fast externalization leads to a rapid clearance of the activity from the tumor.

The analysis of the stability of the bombesin derivatives in liver and kidney homogenates showed also a rather fast metabolism. This finding is in good agreement with results from
analysis of urine collected 1.5 h after injection of $^{99m}$Tc(CO)$_3$-BBS-38 in mice, where no intact peptide but only metabolites were found.

The biodistribution studies were performed in PC-3 and once in DU-145 tumor bearing mice. The $^{99m}$Tc-labelled conjugates BBS-38 and BBS-39 showed the highest uptake in the pancreas after 0.5 h post-injection but only little uptake in the tumor xenografts. Similar results have been found with many other BBS analogues and it seems therefore to be a problem of the model and not of our compounds. The reason could be rather small and poorly be vascularized tumors. Therefore we tried out other cell lines but they all showed less or even no binding of the BBS analogues in vitro and with the best of them (DU-145) not a significantly better result in vivo. Again with PC-3 cells we investigated the effect of tumor inoculation with 10 Mio and 20 Mio cells per tumor and the effect of matrigel (according to the producer, "The high protein concentration also allows the BD Matrigel™ Matrix Plug to maintain its integrity after subcutaneous injection into mice."). The "matrigel-tumors" showed the lowest uptake while in the case with 20 Mio cells the highest uptake was found, however, the differences were small and not significant (P $\approx$ 0.3).

The activity was very rapidly cleared from blood, tumor and all organs. This behavior is rather favorable in the healthy organs, but in the tumor we would like to see a very slow clearance to get good tumor to healthy tissue ratios. The high liver uptake is most probably because of the lipophilicity of the conjugates, while the colon uptake is due to both a specific binding and clearance from the liver via bile and intestine.

The new compounds confirm the observation of other groups, namely that a spacer increases the uptake of BBS analogues in GRP rich tissues. However, we have not found a significant influence on the stability or affinity. One could expect that the activity could be retained in the cells if the externalization is influenced by the spacer, thus leading to better tumor to organ ratios at late time points. But this was not the case; the clearance was similar from all the organs, or even faster from GRP rich tissues.
4.5 References


17. Alberto R., Schibli R. and Schubiger P.A. Reactions with the technetium and rhenium carbonyl complexes (N(Et)$_4$)$_2$[MX$_3$(CO)$_3$]. Synthesis and structure of [Tc(CN-Bu)$_3$(CO)$_3$](NO$_3$) and (NEt$_4$)[Tc$_2$(μ-SCH$_2$CH$_2$OH)$_3$(CO)$_6$]. Polyhedron, 15 (1996) 1079-1089.


CHAPTER 5

Conclusion
5.1 Conclusion

Peptide receptors have been found to represent excellent targets for in vivo cancer diagnosis and therapy (1-5). One group of tumours of particular interest in this respect are neuroendocrine tumours, such as breast and prostate cancers. A promising candidate for tumour targeting is the tetradecapeptide bombesin (BBS). BBS analogues labelled with radioisotopes offer a promising way of selective non-invasive imaging (e.g. with $^{99m}$Tc) and therapy (e.g. with $^{188}$Re) for GRP receptor-positive neoplasms (6-10). The development of aqueous-based synthesis of the organometallic precursor fac-[M(OH$_2$)$_3$(CO)$_3$]$^+$ ($M = Tc, Re$) by Alberto et al. brought about new possibilities for their application in radiopharmacy (11). The use of the tricarbonyl-rhenium and -technetium complexes together with His as tridentate ligand is a suitable tool to label biomolecules, such as bombesin. The main advantage of this method is the formation of robust Tc and Re complexes of almost identical structure and size, and also equal in vivo properties. The main disadvantage is the time consuming labelling process.

The kinetically controlled reaction can yield both, labelling of the terminal His (chelator) or His$^{12}$ in the chain of BBS. The challenge experiment showed that within reasonable time (about one hour), the thermodynamically favoured tridentate complex is formed. Additionally the NMR data confirmed the presence of a free His and the feasibility of the postlabelling approach.

Two properties of a peptide based (radio-) pharmaceutical are crucial to bring about a high tumour uptake, first, the peptide should survive sufficiently long in plasma, since it has to be transported from the injection site to the tumour, and second, it should bind with a sufficient high affinity to the specific receptor on the tumour to show a better uptake in the tumour than in healthy tissues.

Thus, the BBS chain was modified with the aim of slowing down its metabolic rate while retaining its affinity to the receptor. The results showed, that these two combined prerequisites are not easily fulfilled. The methods to reach this goal were the introduction of non-natural amino acids, either D-amino acids or bulky side chains (such as cyclohexane or tert-butyl at the place of isobutyl). Literature data (12) and own studies showed that the His$^{12}$-Leu$^{13}$ bond of BBS is easily metabolized by the neutral endopeptidase 24.11 (E.C.3.4.24.11) and the angiotensin converting enzyme (ACE). Other ideas like the alkylation of the terminal amid to prevent the peptide from the cleavage of aminopeptidases or the introduction of non-natural...
amino acids at other sites in the peptide chain were not successful. All these changes should reduce the fitting of the BBS analogue in the active site of the enzymes.

During the studies another problem was observed, namely the oxidation of the sulfur of Met, which was assumed to be the reason of some loss of affinity. The handling of the sulphur-free analogues (Nle instead of Met) was easier.

The substitutions of Leu by CyHAla at position 13 and Met by Nle at position 14 showed a clear increase of the stability in plasma with no or very little loss of affinity, we thus decided to concentrate on CyhAla and Nle for further studies.

The experience with radiolabelled bombesin analogues in studies with mice suggest that in this case a spacer between the binding sequence of the amino acids 7-14 and the metal complex contributes to a high uptake in GRP receptor rich tissues (13-15). Accordingly, the influence of two different spacers with long chains and slightly polar bonds, namely βAla-βAla and 8-amino-3,6-dioxa-octanoic acid (an ethylene-glycol derivate) were tested. The in vivo results showed a strong improvement of the uptake ratios of the pancreas or tumour compared to healthy tissues (like liver, kidneys or blood), specifically with the βAla-βAla – linker, this confirmed our expectations.

However, no significant influence on the stability or affinity had been found. One expected the activity to be retained longer in the cells if externalisation is influenced by the spacer, this would lead to better tumour to organ ratios at late time points. This was not the case though; the clearance was similar from all the organs, or even faster from GRP receptor rich tissues.

In general, all compounds tested in mice showed a very rapid clearance of activity from blood, tumour and all other organs. This behaviour is rather favourable in the healthy organs, but in the tumour a slower clearance would be desirable to get good tumour to healthy tissue ratios. The high liver uptake is most probably because of the lipophilicity of the conjugates, while the colon uptake is due to both a specific binding and clearance from the liver via bile and intestine. A high uptake of radioactivity was observed in the pancreas, which is expectable since pancreas of mice is a GRP receptor-rich tissue. Similar results have been found with many other BBS analogues (13, 14) and it seems therefore to be a problem of the model and not of our compounds. Another reason may be the rather small and poorly vascularized tumours, so we also tried out other cell lines. They all showed less or even no binding of the BBS analogues in vitro and with the best cells (DU-145) not a significantly better result in vivo.
A third property of radiolabelled peptides is also very important, namely the often observed but unwanted accumulation in the kidneys. Thus, the tumour to kidney ratio is an important parameter for peptide evaluation. So far no in vitro test is known, which allows the determination of this characteristic. This point can only be elucidated in vivo. Since many BBS analogues were excluded from in vivo tests (it is ethically not acceptable to use and kill animals to test compounds with low receptor affinity), only few peptides can be judged in this respect. While some analogues showed a higher uptake in kidneys than in many other organs, the analogues with the additional linker (BBS-38 and BBS-39) showed nice (low) uptake values.

The results of both in vitro and in vivo tests show that of the many peptides synthesised most failed in at least one of the three aspects (i.e. the required properties are high receptor affinity, high stability in plasma, low kidney uptake). A few peptides show a clear improvement of the properties, especially in comparing target to non-target uptake ratios. For diagnostic application in humans, this ratio for tumour to kidney should be >1, and thus, one objective of this work was reached. Unfortunately, specifically for therapy, the clearance from tumour must be slower than from all healthy tissues and in this respect the aim could not yet be reached. The interesting results, which have been found, encourage the development of further derivatives to be used in nuclear medicine. Specifically, the different properties of the BBS analogues with the spacers indicate that exchanging the spacer, further improvement is possible without affecting the receptor affinity.
5.2 References


Publications and Presentations

Publications


García-Garayoa E., Rüegg D., Ullah Khan I, Bläuenstein P., Zwimpfer M., Blanc A., Beck-Sickinger A., Schubiger P.A. Chemical and Biological Characterization of New Stabilized Re(CO)$_3$-$^{99m}$Tc(CO)$_3$-Bombesin Analogues. *Bioconjugate Chemistry (submitted)*


Presentations

Rüegg D., Bläuenstein P., García-Garayoa E., Schubiger P.A. New stabilised bombesin analogues as potential tumour imaging agents. *Congress of the Swiss Society for Medical Radiology, June, 2003, Lucerne (Switzerland).*

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