# Novel series of $^{177}$Lu-labeled bombesin derivatives with amino acidic spacers for selective targeting of human PC-3 prostate tumor cells

P. B. PUJATTI [1], J. S. SANTOS [1], R. M. COUTO [1], L. T. U. H. MELERO [1], M. F. SUZUKI [2], C. R. J. SOARES [2], S. R. M. GRALLERT [1], J. MENGATTI [1], E. B. DE ARAÚJO [1]



***Aim.*** **Bombesin (BBN) has demonstrated the ability to bind with high affinity and specificity to GRP receptor, overexpressed on human prostate cancer. A large number of BBN derivatives have been synthesized for this purpose but most of them exhibit high abdominal accumulation, which may represent a problem in their clinical use due to serious side effects to patients. In this study we describe the results of radiolabeling with lutetium-177, stability and *in vivo* studies of novel phenyl-glycine-extended bombesin derivatives. The spacers were inserted to improve bombesin *in vivo* properties and to reduce its target to non-tumor sites.**
***Methods.*** **Preliminary studies were done to establish the ideal conditions for labeling bombesin derivatives. Chromatography systems were applied to determine free lutetium and the stability of the preparations was evaluated either after storing at 2-8 °C or incubation in human serum at 37 °C. *In vivo* experiments included biodistribution, pharmacokinetics and SPECT images and were performed in *Balb-c* and *Nude* mice bearing PC-3 xenografts.**
***Results.*** **The derivatives were labeled with high yield and kept stable at 2-8 °C and are metabolized by human serum enzymes. *In vivo* studies showed fast blood clearance of labeled peptides and rapid excretion, performed mainly by renal pathway. In addition, biodistribution and imaging studies showed low abdominal accumulation and significant and specific tumor uptake of $^{177}$Lu-labeled derivatives.**
***Conclusions.*** **The derivative with longer spacer holds a higher potential as radiopharmaceutical for prostate tumor diagnosis and the derivatives with shorter spacers are potential radiopharmaceuticals for prostate tumor treatment.**

**KEY WORDS:** Bombesin - Lutetium-177 – Prostatic neoplasms - Amino acidic spacer.

*[1]Radiopharmacy Directory, Nuclear and Energy Research Institute (IPEN/CNEN), São Paulo, Brazil*
*[2]Center of Biotechnology, Nuclear and Energy Research Institute (IPEN/CNEN), São Paulo, Brazil*

The improvement in the efficacy and reduction in the toxicity of diagnostic and therapeutic agents remain a goal of clinical practice, especially in oncology.[1] The discovery of specific, high affinity receptors for endogenous peptides expressed on malignant cells prompted the development of several molecules consisting of a peptide carrier linked to an antineoplastic agent. In this field there has been exponential growth in the interest of radiolabeled peptides for diagnosis and therapy.[2] Peptides can be synthesized easily and inexpensively, they have fast clearance and rapid tissue penetration and they are less likely to be immunogenic than antibodies and proteins.[3]

Among the studied peptides, bombesin derivatives are particularly attractive for the development of tumor directed agents. Bombesin (BBN), a 14-amino acid peptide analog of human gastrin releasing pep-

*Fundings.*—CNPq, FAPESP and IPEN/CNEN.

*Acknowledgments.*—We thank Nathanael Gomes for helping with MicroSPECT images, Dr. Áurea Geraldo for HPLC technical support, Prof. Dr. Leoberto Costa Tavares for allowing the computational study in Sybyl 7.2 and Hemocentro of UNICAMP for providing PC-3 cells.

Received on July 9, 2009.
Accepted for publication on October 1, 2010.
Epub ahead of print on November 11, 2010.

Corresponding author: P. Brunelli Pujatti, MD, Radiopharmacy Directory, Nuclear and Energy Research Institute (IPEN/CNEN), Av. Lineu Prestes, 2242, Cidade Universitária, Butantã, CEP 05508-000, São Paulo, Brazil. E-mail: priscillapujatti@yahoo.com.br

tide (GRP) and the related neuromedin B (NMB), was originally isolated from the skin of the frog *Bombina bombina* in 1970.[4] GRP and BBN share a common amino acid sequence in the C-terminal region, which is necessary for their biological activity.[5] Four different bombesin receptor subtypes have been recognized in humans: the neuromedin B receptor (NMB-R), the gastrin-releasing peptide receptor (GRP-R), and the orphan bombesin subtype-3 receptor ($BB_3$). These receptors can be distinguished by their different affinities for the mammalian peptides NMB and GRP, whereas a native ligand for the $BB_3$ has not been identified yet.[6] The fourth BBN receptor ($BB_4$) was isolated from the frog, but has not been found in mammals yet. All bombesin receptors characterized to date are guanine nucleotide binding protein (G-protein)-coupled, with seven transmembrane domains, and active phospholipase C to increase intracellular concentrations of inositol phosphates, diacyl glycerol and calcium.[7]

The interest in using radiolabeled bombesin derivatives as agents for diagnostic imaging and/or systemic radiotherapy of tumors has increased because of the observation that GRP-R are over-expressed in a variety of human tumor cells.[8] GRP-R is not normally expressed by epithelial cells present in colon, lung, and prostate, but is expressed by non-neuroendocrine cells of the pancreas and breast and by most neuroendocrine cells of gastrointestinal tract, lung and prostate. However, GRP receptors are found in high density in a variety of primary and metastatic tumor tissues, such as breast, colon and prostate tumors.[9] In the case of prostate cancer, it has been found a high density of GRP-R not only in the invasive prostatic carcinomas but also in the earliest phase of neoplastic transformation. GRP-R expression by prostate tumor cells seems to be directly and strongly related to the neoplastic condition and its activation regulates tumor cells morphology, differentiation and proliferation.[10] These findings encourage the search of bombesin derivatives which could be radiolabeled and used for imaging and/or delivering a cytotoxic radiation dose to prostate tumor cells.

Some important factors have to be considered in designing radiometal-based bombesin derivatives, such as half-life, mode of decay, cost and availability of the radionuclide.[11] The application of 6.7 day half-life $^{177}$Lu isotope in medicine is spreading in the last few years. It belongs to the group of the rare earth isotopes, finding a continuously wider use both in diagnosis and therapy due to its good radiation properties.[12] The mean range of $^{177}$Lu β particles in tissues is 670 μm, making this radionuclide ideal for treating micro-metastatic disease. Because it also emits γ rays (208 keV, 11% abundance), imaging of $^{177}$Lu-labeled radiotherapeutic agents is possible.[13] In addition, it is relatively easy to conjugate $^{177}$Lu with biologically active compounds. The attachment of the lanthanide to the biomolecule requires a multidentate ligand framework such as DOTA (1,4,7,10-tetraazacyclododecano-1,4,7,10-tetraacetic acid) or DTPA (diethylenetriaminepentaacetic acid), capable of stabilizing the radiolanthanide against *in vivo* transchelation reactions with serum proteins.[5] Clinical studies with $^{177}$Lu-labeled peptides have demonstrated reduced normal tissue damage and the ability to use a single radiolabeled agent for both therapy and imaging.[14]

Several bombesin derivatives have been successfully synthesized and radiolabeled for tumor imaging and therapy and those radiolabeled with lutetium-177 have shown promising results in preclinical studies.[15, 16] Despite the variety of synthesized compounds, one with the optimal characteristics for systemic radiotherapy – including maximal tumor uptake and retention and minimal nontumor tissue uptake and retention – was not reported yet. Most of the studied derivatives exhibit high abdominal accumulation, especially in pancreas and intestine.[16-20] This abdominal accumulation may represent a problem in clinical use of radiolabeled bombesin analogues probably due to serious side effects to patients.

In this context, this work describes the radiolabeling with lutetium-177, stability and preclinical studies of three novel phenyl-glycine-extended bombesin derivatives – $BEFG_1$, $BEFG_3$ and $BEFG_5$ – which have the general structure DOTA-Phe-$(Gly)_n$-BBN(6-14), where n represents one ($BEFG_1$), three ($BEFG_3$) or five ($BEFG_5$) glycine amino acids. The spacers were inserted between the chelator and the bombesin binding sequence in order to improve bombesin *in vivo* properties and to reduce its target to non-tumor sites. We hypothesized that the inserction of the glycines into the spacer would reduce abdominal accumulation of the derivatives and increase renal excretion, as a consequence of the increase in derivatives hydrophilicity.

## Materials and methods

### *Reagents*

The following reagents were supplied from different sources: PC-3 cells (human androgen-independ-

ent prostate adenocarcinoma) from University of Campinas (Brazil); $^{177}LuCl_3$ from IDB (Netherlands); RPMI 1640, trypsin-EDTA and fetal bovine serum (FBS) from Cultilab (Brazil); penicillin-streptomicin from Gibco-Invitrogen (USA); Matrigel high concentration from BD Biosciences (USA); sodium acetate, sodium chloride, sodium citrate, citric acid and trifluoroacetic acid from Sigma-Aldrich Co. (USA), n-octanol, acetonitrile, methanol and ethanol from Merck Chemical Co. (Germany). All used solvents were of analytical grade.

*Peptides*

The peptides structures were planned by molecular modeling studies and were purchased from piChem (Austria). The computational study was performed using Sybyl 7.2 (version for Linux). All initial structure was built using atoms and structural fragments of the molecular editor. Molecular modelling calculations, graphical representation and structural analyses were carried out using molecular dynamics (MD). Figure 1 shows the structure of the studied phenyl-glycine-extended bombesin derivatives after complexation with lutetium-177.

*Radiolabeling of phenyl-glycine-extended bombesin derivatives with lutetium-177*

Preliminary studies were done to establish the ideal labeling conditions of $^{177}$Lu-labeled $BEFG_1$, $BEFG_3$ and $BEFG_5$. All reagents were prepared with Chelex 100 treated free metal water[21]. Briefly, 20 µg of the peptides were dissolved in 200 µL of sodium acetate buffer (0.4 mol/L, pH 4.5) and 92.5 MBq (2.5 mCi) of $^{177}LuCl_3$ (specific activity 871-920 GBq/mg at radiolabeling) and the reaction mixtures were heated at 90° C for 30 minutes.

*Radiochemical purity determination*

Radiochemical purity was determined by high performance liquid chromatography (HPLC, Shimatzu, Japan) equipped with radioactivity detection (Shell), using RP $C_{18}$ column (Waters, 4.0 x 150 mm, 5 µm, USA), flow rate of 1.5 mL/min with a linear gradient of 10-90% (v/v) 0.1% TFA/acetonitrile and 0.1% TFA/$H_2O$ for 15 minutes. $^{177}LuCl_3$ and the unlabeled peptides (UV detection at 280 nm) were also analysed in the same HPLC system.

Instant thin layer chromatography (ITLC-SG, Gellman Science, USA) was also applied to determine free lutetium, with citrate/citric acid buffer (0.1 mol/L, pH 5.0) as solvent ($R_f$ of labeled peptide was 0.0 and $R_f$ of free lutetium was 0.9-1.0).[21]

In vitro *stability of [$^{177}$Lu]DOTA-Phe-(Gly)$_n$-BBN (6-14)*

To determine the [$^{177}$Lu]$BEFG_1$, [$^{177}$Lu]$BEFG_3$ and [$^{177}$Lu]$BEFG_5$ *in vitro* stability, the preparations were stored at 2-8 °C for 24, 48, 72, 96 and 168 hours. To access $^{177}$Lu-labeled peptides metabolic stability in human serum, 10 mL of human blood was collected, centrifuged twice (10 min, 3000 rpm) to separate the serum and aliquots of 1 mL were spiked with $^{177}$Lu-labeled peptides (24 MBq, 50 µL) and incubated for 1, 4 and 24 hours at 37 °C, followed by ITLC analysis. All experiments were performed in triplicate.

*Experimental partition coefficient determination*

A 25 µL aliquot of radiolabeled peptides was added to a testing tube containing 3 mL of each n-octanol and water, pre-saturated for 24 hours. The tube was vortexed for 1 hour at room temperature and then aliquots (100 µL) of each phase were counted. The partition coefficient was determined by the function: Partition coefficient = $\log_{10}$ (counts in n-octanol phase / counts in water phase). The experiments were performed in quintuplicate.[22]

*Animals*

Animal studies were performed in accordance with International Guiding Principles for Biomedical Research Involving Animals and Guidelines for the Care and Use of Research Animals established by the Animal Studies Committee at Nuclear and Energy Research Institute (IPEN), which approved the protocols (Protocol number 17 – IPEN/CNEN). Male *Balb-c* mice (4 to 8 weeks old, 20-25 g weight) and male *Nude* mice (4 to 8 weeks old, 15-20 g weight) used for *in vivo* experiments were provided by the Animal Facility of IPEN.

In vivo *biodistribution and pharmacokinetic studies in normal* Balb-c *mice*

The radioactive bombesin derivatives (1.85 MBq/100 µL 0.9% NaCl, mass of peptides 0.04 µg) were

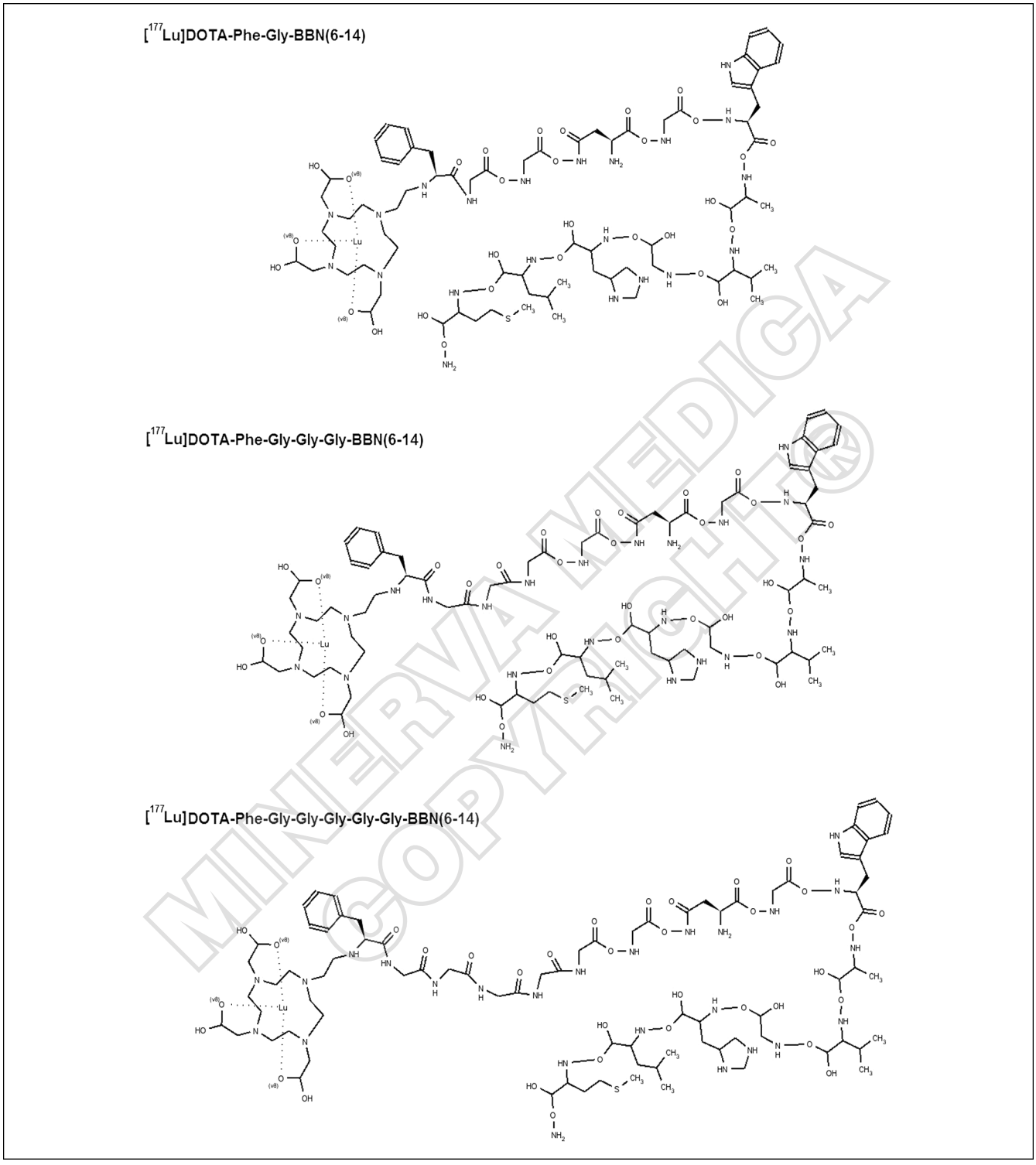




Figure 1.—Molecular structure of $^{177}$Lu-labeled phenyl-glycine-extended bombesin derivatives.

injected intravenously in *Balb-c* mice lateral tail vein. After different time intervals (1, 4 and 24 hours p.i.), the blood was collected and the animals were sacrificed in groups of five. Then, the mice were dissected and vital organs were isolated, weighed and the activities were measured in an automatic gamma counter. The biodistribution of $^{177}$Lu-labeled $BEFG_1$, $BEFG_3$ and $BEFG_5$ were expressed as percentage of injected activity per gram of organ (%IA/g).

Pharmacokinetics studies were performed by measuring [$^{177}$Lu]$BEFG_1$, [$^{177}$Lu]$BEFG_3$ and [$^{177}$Lu]$BEFG_5$ in blood. Blood samples (60 µL) were collected in the orbital plexus vein 1, 5, 30, 60, 120, 240 and 1440 min after intravenous injection in *Balb-c* mice and their activities were measured as described earlier and expressed as percentage of injected activity per millilitre (%IA/mL). The pharmacokinetics parameters were calculated using Biexp [23] software by decomposition of blood clearance curve in two exponential components (fast and slow).

### *Cell culture*

Human prostate cancer PC-3 cell line was grown in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin. Cells were kept in humidified air containing 5% $CO_2$ at 37 °C. The cells were grown to 80% confluency, harvested by trypsinization, centrifuged (5 min, 2500 rpm) and then resuspended in PBS.

### In vivo *biodistribution studies in* Nude *mice bearing PC-3 xenografts*

To analyze the affinity of $^{177}$Lu-labeled phenyl-glycine extended bombesin derivatives target to human prostate tumor cells (PC-3) *in vivo*, male *Nude* mice were inoculated subcutaneously with human PC-3 cells (2 x $10^6$) in 0.1 mL phosphate-buffered saline/Matrigel (2:1 v/v) (Lantry *et al.*, 2006). Biodistribution studies were performed as described earlier after 3 weeks with tumors averaging 0.3 g.

### In vivo *blocking studies*

To evaluate the radiopharmaceutical specificity to PC-3 tumor cells, blocking studies were performed with the labeled peptide that exhibited higher tumor uptake. Two groups of three *Nude* mice bearing PC-3 tumor were preinjected with unlabeled peptide (100 µg/100 µL 0.9% NaCl) via the tail vein to act as a receptor blockage.[24] Then, 15 or 45 minutes after preinjection, $^{177}$Lu-labeled peptide (1.85 MBq/100 µL 0.9% NaCl, peptides mass 0.04 µg) was also administered intravenously and the animals were sacrificed 1 hour post injection. The mice were dissected and vital organs were isolated, weighed and the activities were measured in an automatic gamma counter. Data were presented as percentage of injected activity per gram of organ (%IA/g) and compared to biodistribution without administration of cold peptide.



TABLE I.—*Radiochemical yield of the $^{177}$Lu-labeled phenyl-glycine-extended bombesin derivatives after reacting with 92.5 MBq of $^{177}LuCl_3$ at 90° C for 30 minutes (n=6).*

| Bombesin Derivative | Radiochemical Yield (ITLC-SG) (%) | Specific activity (MBq/µg) | Specific activity (GBq/µmol) |
|---|---|---|---|
| [$^{177}$Lu]$BEFG_1$ | 98.4±1.0 | 4.5±0.1 | 7.5±0.1 |
| [$^{177}$Lu]$BEFG_3$ | 99.4±0.2 | 4.6±0.1 | 8.1±0.1 |
| [$^{177}$Lu]$BEFG_5$ | 98.4±0.9 | 4.5±0.1 | 8.5±0.1 |

TABLE II.—*Retention times in HPLC of $^{177}LuCl_3$ and labeled and unlabeled phenyl-glycine-extended bombesin derivatives (n=3).*

| Chemical specie | Molecular weight (g/mol) | Retention time (HPLC) (min) | |
|---|---|---|---|
| $^{177}LuCl_3$ | 283.5 | 1.44±0.4 | |
| | | $^{177}$Lu-labeled peptide | Unlabeled peptide |
| [$^{177}$Lu]$BEFG_1$ | 1645.9 | 7.7±0.3 | 7.3±0.1 |
| [$^{177}$Lu]$BEFG_3$ | 1760.0 | 7.3±0.1 | 6.9±0.3 |
| [$^{177}$Lu]$BEFG_5$ | 1874.1 | 7.2±0.1 | 6.6±0.1 |

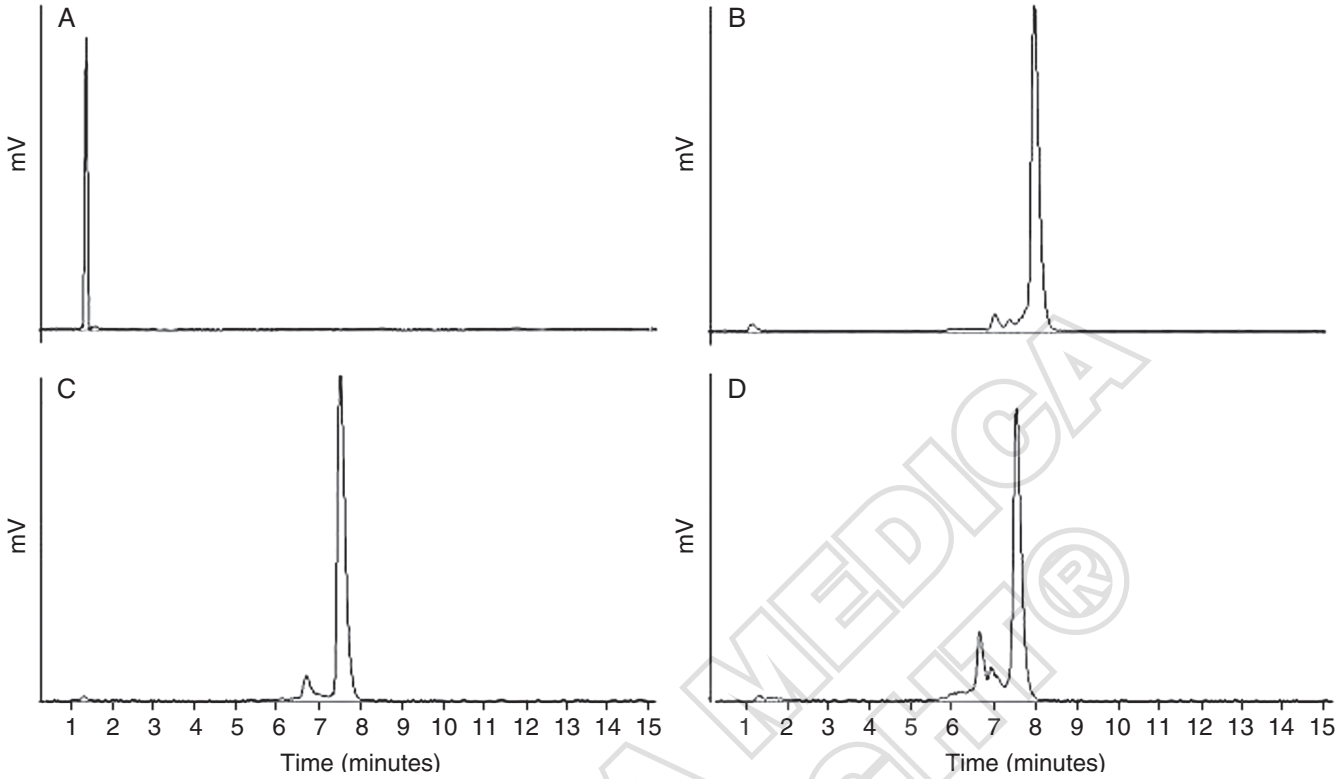


Figure 2.—HPLC profiles using RP $C_{18}$ column of (A) $^{177}LuCl_3$ and (B) [$^{177}$Lu]BEFG$_1$, (C) [$^{177}$Lu]BEFG$_3$ and (D) [$^{177}$Lu]BEFG$_5$.

TABLE III.—*Radiochemical purity (%) determined by ITLC-SG of labeled bombesin derivatives after storing at 4° C for different times (n=3).*

| Incubation time at 2-8 °C (hours) | Bombesin derivative | | |
|---|---|---|---|
| | [$^{177}$Lu]BEFG$_1$ | [$^{177}$Lu]BEFG$_3$ | [$^{177}$Lu]BEFG$_5$ |
| 0 | 99.7±0.1 | 99.5±0.1 | 98.9±0.5 |
| 24 | 98.8±0.1 | 97.5±0.1 | 98.1±0.8 |
| 48 | 98.2±0.4 | 97.2±0.1 | 96.2±1.0 |
| 72 | 96.4±0.1 | 96.6±0.8 | 95.6±0.3 |
| 96 | 97.4±0.5 | 95.8±0.2 | 94.1±1.8 |
| 168 | 93.8±0.1 | 92.6±0.3 | 91.4±1.1 |



*Scintigraphy studies*

Imaging studies were performed in normal male *Balb-c* and *Nude* mice bearing PC-3 tumor at 30 minutes, 1 and 4 hours post intravenous administration of labeled peptides (37 MBq/100 µL 0.9% NaCl). The mice were anesthetized, placed under a gamma camara low-energy high-resolution collimator (LEHR) (Mediso Imaging System, Hungria) and the images were aquired for 180 seconds using a 256x256x16 matrix size and a window set at 208 keV. Imaging studies in *Nude* mice bearing PC-3 tumor were performed with the labeled peptide that exhibit higher tumor uptake in biodistribution studies.

*Statistical analysis*

The results are expressed as Mean + SD. Statistical analysis were performed using GraphPad PRISM 5.0 (USA) software using Student´s t-test with two-tailed

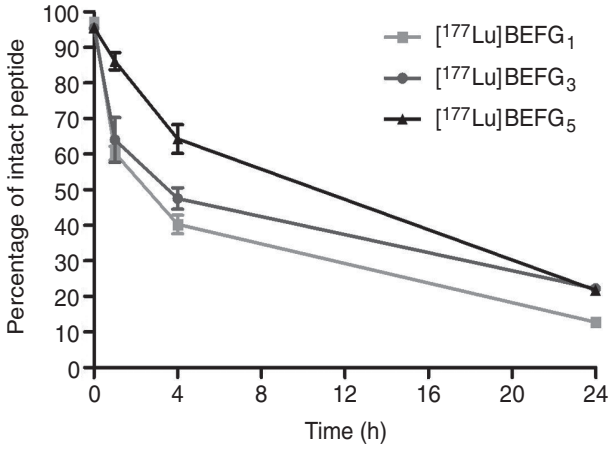


Figure 3.—Representative curve of the time-course degradation of the phenyl-glycine bombesin derivatives radiolabeled with $^{177}$Lu in fresh human serum *in vitro* at 37 °C (n=4).

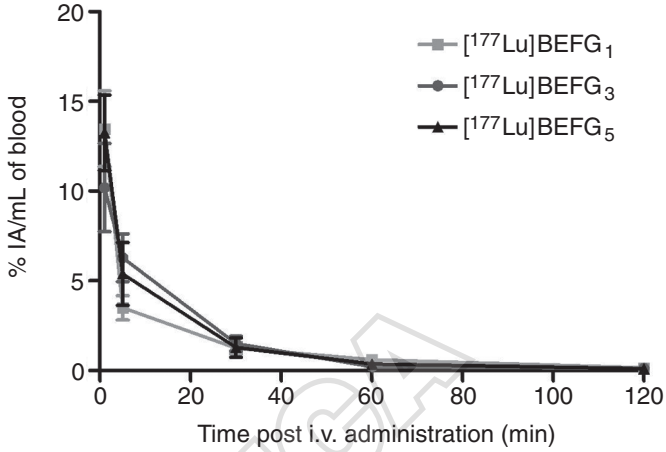


Figure 4.—Blood clearance of $^{177}$Lu-radiolabeled phenyl-glicine-extended bombesin derivatives in male *Balb-c* mice (n=7).

TABLE IV.—*Biodistribution of [$^{177}$Lu]DOTA-Phe-Gly-BBN(6-14) in normal male Balb-c. The results are expressed as % IA/g (n=5).*

| Time p.i./Tissue | 1 hour | 4 hours | 24 hours |
|---|---|---|---|
| Prostate* | 0.01±0.01 | 0.01±0.01 | 0.00 |
| Heart | 0.16±0.06 | 0.03±0.01 | 0.02±0 |
| Lungs | 0.3±0.14 | 0.07±0.01 | 0.09±0.08 |
| Pancreas | 0.16±0.07 | 0.04±0.01 | 0.03±0.01 |
| Spleen | 0.16±0.04 | 0.06±0.01 | 0.07±0.01 |
| Stomach | 0.25±0.1 | 0.12±0.05 | 0.04±0.02 |
| Liver | 0.33±0.08 | 0.12±0.01 | 0.10±0.06 |
| Kidneys | 3.63±1.04 | 2.46±0.35 | 1.31±0.22 |
| Intestines** | 0.32±0.12 | 0.28±0.14 | 0.08±0.03 |
| Skeletal muscle | 0.14±0.04 | 0.05±0.03 | 0.01±0.01 |
| Bone | 0.45±0.14 | 0.17±0.04 | 0.36±0.18 |
| Brain | 0.09±0.03 | 0.01±0.01 | 0.00 |
| Cerebellum | 0.13±0.01 | 0.01±0.01 | 0.01±0.01 |

*Results expressed as % IA/tissue. **Small and large intestine with contents.

TABLE V.—*Biodistribution of [$^{177}$Lu]DOTA-Phe-(Gly)$_3$-BBN(6-14) in normal male Balb-c mice. The results are expressed as % IA/g (n=5).*

| Time p.i. / Tissue | 1 hour | 4 hours | 24 hours |
|---|---|---|---|
| Prostate* | 0.09±0.03 | 0.02±0.01 | 0.00 |
| Heart | 0.08±0.03 | 0.06±0.02 | 0.05±0.03 |
| Lungs | 0.19±0.05 | 0.08±0.03 | 0.05±0.01 |
| Pancreas | 0.08±0.02 | 0.05±0.01 | 0.04±0.01 |
| Spleen | 0.09±0.02 | 0.10±0.02 | 0.11±0.01 |
| Stomach | 0.12±0.09 | 0.22±0.2 | 0.18±0.12 |
| Liver | 0.19±0.04 | 0.20±0.06 | 0.19±0.04 |
| Kidneys | 2.17±0.45 | 3.30±0.44 | 2.08±0.40 |
| Intestines* | 0.17±0.13 | 0.40±0.21 | 0.13±0.03 |
| Skeletal muscle | 0.07±0.03 | 0.04±0.03 | 0.03±0.01 |
| Bone | 0.24±0.03 | 0.70±0.49 | 0.52±0.04 |
| Brain | 0.02±0.01 | 0.00 | 0.00 |
| Cerebellum | 0.04±0.02 | 0.00 | 0.00 |

*Results expressed as % IA/tissue. **Small and large intestine with contents.

TABLE VI.—*Biodistribution of [$^{177}$Lu]DOTA-Phe-(Gly)$_5$-BBN(6-14) in normal male Balb-c mice. The results are expressed as % IA/g (n=5).*

| Time p.i. / Tissue | 1 hour | 4 hours | 24 hours |
|---|---|---|---|
| Prostate* | 0.06±0.05 | 0.01±0.01 | 0.00 |
| Heart | 0.20±0.07 | 0.05±0.01 | 0.02±0.01 |
| Lungs | 0.61±0.3 | 0.10±0.04 | 0.05±0.01 |
| Pancreas | 0.19±0.06 | 0.05±0.01 | 0.03±0.01 |
| Spleen | 0.12±0.04 | 0.07±0.01 | 0.05±0.02 |
| Stomach | 0.25±0.06 | 0.09±0.01 | 0.03±0.01 |
| Liver | 0.24±0.06 | 0.16±0.02 | 0.11±0.04 |
| Kidneys | 2.74±0.67 | 1.93±0.29 | 0.93±0.27 |
| Intestines* | 0.21±0.09 | 0.09±0.06 | 0.03±0.01 |
| Skeletal muscle | 0.11±0.06 | 0.02±0.01 | 0.02±0.01 |
| Bone | 0.32±0.05 | 0.3±0.03 | 0.27±0.07 |
| Brain | 0.01±0.01 | 0.01±0.01 | 0.00 |
| Cerebellum | 0.02±0.01 | 0.01±0.01 | 0.00 |

*Results expressed as % IA/tissue. **Small and large intestine with contents.

TABLE VII.—*Pharmacokinetics parameters determined for phenyl-glycine bombesin derivatives radiolabeled with $^{177}$Lu using Biexp software.*

| Pharmacokinetics parameters | [$^{177}$Lu]BEFG$_1$ | [$^{177}$Lu]BEFG$_3$ | [$^{177}$Lu]BEFG$_5$ |
|---|---|---|---|
| Equation | $C_{(t)} = 694844.69^{-5.11t}+11558.15^{-0.11t}$ | $C_{(t)} = 623853.50^{-5.12t}+5815.18^{-0.14t}$ | $C_{(t)} = 820382.56^{-4.45t}+7539.17^{-0.19t}$ |
| $T_{1/2}$ fast phase (h) | 0.14 | 0.14 | 0.16 |
| $T_{1/2}$ slow phase (h) | 6.15 | 4.90 | 3.70 |
| *$K_{12}$ ($h^{-1}$) | 2.06 | 1.20 | 0.71 |
| **$K_{21}$ ($h^{-1}$) | 0.19 | 0.19 | 0.23 |
| ***$K_{10}$ ($h^{-1}$) | 1.39 | 3.86 | 3.70 |
| Distribution volume (mL) | 93.10 | 42.14 | 28.66 |
| Clearance (mL.$h^{-1}$) | 23.69 | 22.02 | 25.55 |
| ****$K_{ss}$ ($h^{-1}$) | 0.25 | 0.52 | 0.89 |

*Intravascular to extravascular space transfer constant; **Extravascular to intravascular space transfer constant; ***Intravascular space to excretion system transfer constant; ****Elimination rate constant

distribution for paired data and One-way ANOVA analysis of variance for grouped data. Differences at the 95% confidence level ($P<0.05$) were considered significant.

## Results

### *Peptides $^{177}$Lu-labeling and quality control*

The analysis of the radiochemical purity by ITLC-SG showed that BEFG$_1$, BEFG$_3$ and BEFG$_5$ were radiolabeled with high yield at the established condition and a specific activity of 7.5, 8.1 and 8.5 GBq/μmol for [$^{177}$Lu]BEFG$_1$, [$^{177}$Lu]BEFG$_3$ and [$^{177}$Lu]BEFG$_5$, respectively, was achieved (Table I). The specific activity of $^{177}$Lu at radiolabeling was 871-920 GBq/mg.

Radiochemical yield obtained by ITLC-SG analysis were confirmed by HPLC analysis, in which free lutetium-177 could be easily separated from the unlabeled and labeled peptides (Table II). In addition, the HPLC radiochromatograms showed no important $^{177}LuCl_3$ (<2%) contamination in the labeling mixtures (Figure 2).

A second peak of shorter RT than the mainly radioactive specie was observed in the HPLC chromatograms of labeled peptides and probably represent the phenyl-glycine-extended bombesin derivatives resulted from oxidation of the methionine residue. This second specie represent less than 10% of total radioactivity present in radiolabeling mixtures.

### In vitro *stability of labeled peptides*

The stability of labeled peptides was evaluated by ITLC-SG after storage at 2-8 °C (Table III). $^{177}$Lu-

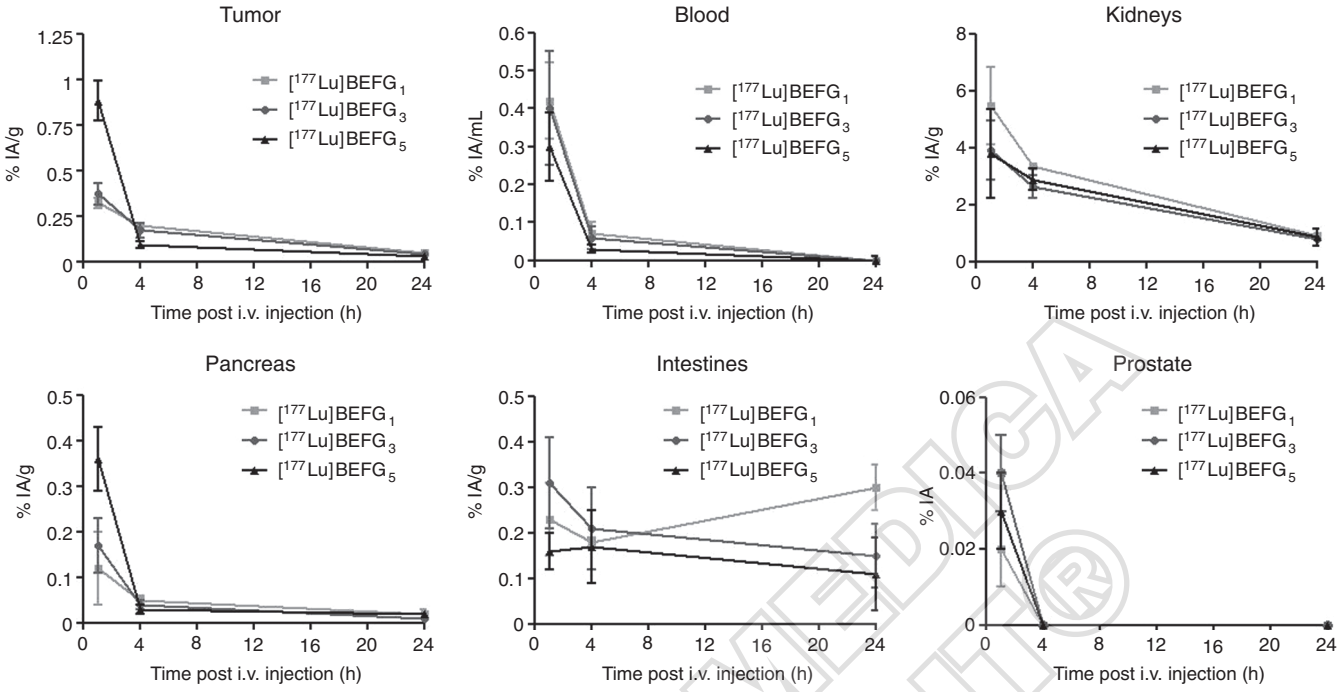


Figure 5.—Biodistribution of [$^{177}$Lu]BEFG$_1$ (squares), [$^{177}$Lu]BEFG$_3$ (circles) and [$^{177}$Lu]BEFG$_5$ (triangles) in PC-3 tumor bearing *Nude* mice at 1, 4 and 24 hours p.i. (n=3).

TABLE VIII.—*Tumor uptake and tumor to tissue ratios of radiolabeled bombesin derivatives in* Nude *mice bearing PC-3 xenografts. The ratios were calculated using the mean of % IA/g data, obtained from biodistribution assay.*

| Bombesin derivative | $^{177}$Lu-BEFG$_1$ | | | $^{177}$Lu-BEFG$_3$ | | | $^{177}$Lu-BEFG$_5$ | | |
|---|---|---|---|---|---|---|---|---|---|
| Time p.i(h) | 1 | 4 | 24 | 1 | 4 | 24 | 1 | 4 | 24 |
| Tumor uptake (% IA/g) | 0.32±0.03 | 0.2±0.01 | 0.05±0.01 | 0.37±0.06 | 0.17±0.04 | 0.04±0.01 | 0.88±0.11 | 0.09±0.02 | 0.03±0.01 |
| Tumor:Blood | 0.75 | 2.74 | 13.9 | 0.93 | 2.85 | 21.30 | 2.99 | 2.69 | 6.91 |
| Tumor:Kidneys | 0.06 | 0.06 | 0.06 | 0.1 | 0.07 | 0.05 | 0.23 | 0.04 | 0.03 |
| Tumor:Intestines | 1.4 | 1.09 | 0.13 | 1.19 | 0.85 | 0.27 | 5.44 | 0.54 | 0.28 |
| Tumor:Pancreas | 2.64 | 4.36 | 2.93 | 2.15 | 4.76 | 3.26 | 2.74 | 2.47 | 1.89 |

phenyl-glycine-extended bombesin derivatives remained stable at 2-8 °C and the radiochemical yield was higher than 90% for more than 168 hours.

After incubation of labeled peptides in fresh human serum, different ITLC-SG chromatograms were obtained according to incubation time, suggesting a metabolic degradation of peptides. Different peaks corresponding to the intact peptides and the probable degradation products were obtained and the radiochemical purity decreased in time (Figure 3). The half-lives in human serum *in vitro* were 3.41 hours, 3.98 hours and 6.11 hours for [$^{177}$Lu]BEFG$_1$, [$^{177}$Lu]BEFG$_3$ and [$^{177}$Lu]BEFG$_5$, respectively.

### *Experimental partition coefficient determination*

Partition coefficient results showed that the radiolabeled peptides exhibit low lipophilicity. The experimental partition coefficient was found as -2.87 + 0.1 for [$^{177}$Lu]BEFG$_1$, -3.04 + 0.2 for [$^{177}$Lu]BEFG$_3$ and -3.09 + 0.1 for [$^{177}$Lu]BEFG$_5$.

### In vivo *biodistribution and pharmacokinetic studies in normal* Balb-c *mice*

Results from biodistribution studies of [$^{177}$Lu]BEFG$_1$, [$^{177}$Lu]BEFG$_3$ and [$^{177}$Lu]BEFG$_5$ performed in

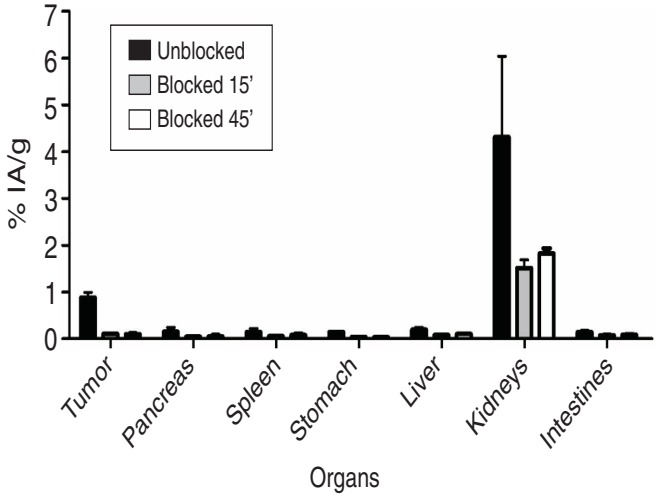


Figure 6.—Biodistribution studies of [$^{177}$Lu]BEFG$_5$ in unblocked and blocked *Nude* mice bearing PC-3 xenografts 1 hour post injection (n=3).

*Balb-c* mice are presented in Tables IV, V and VI, respectively. Appreciable radioactivity could be detected in kidneys up to 24 hours post injection, indicating peptide excretion by renal pathway. Kidneys may be the critical organs for dosimetry. In addition, labeled bombesin derivatives showed low abdominal accumulation, especially in pancreas and intestines.

Bone uptake is commonly assumed as a control of lutetium-177 labeled compounds stability *in vivo*. This tissue actively uptakes free lutetium-177, being a good indicator of radiochemical purity, mainly at the initial time. Bone uptake of $^{177}$Lu-labeled peptides was negligible when compared to pure $^{177}LuCl_3$,[25] indicating the *in vivo* stability of labeled peptides.

The amount of [$^{177}$Lu]BEFG$_1$, [$^{177}$Lu]BEFG$_3$ and [$^{177}$Lu]BEFG$_5$ present in blood decreased rapidly and became almost undetectable at 60 minutes post injection (Figure 4). This rapid clearance was performed mainly by renal pathway, as described earlier in the biodistribution assays. Peptides blood kinetics curves did not differ significantly (P=0.99).

The analysis of blood clearance curves using Biexp software resulted in a biexponential function that represents the distribution from a vascular to an extravascular compartment. Biexp software is a useful tool for comparative analysis of pharmacokinectic parameters. The Table VII summarizes the pharmacokinetics parameters obtained for the studied bombesin derivatives.

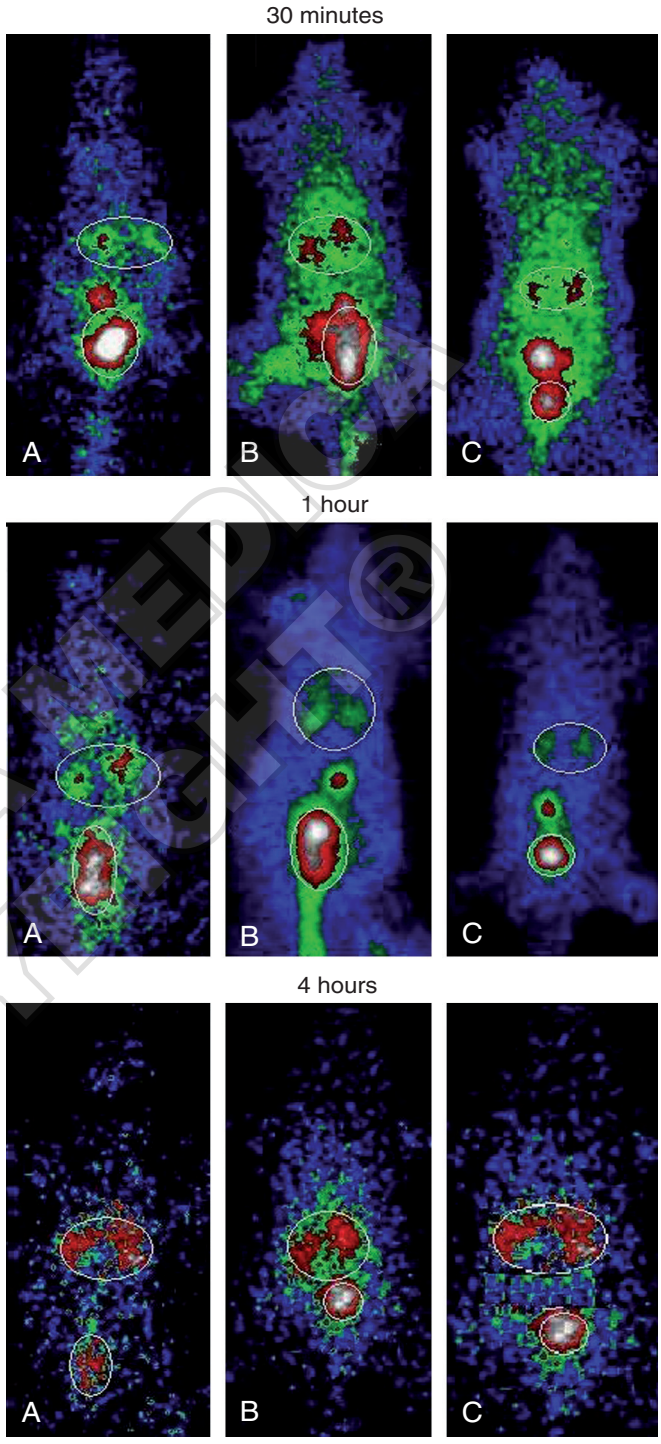


Figure 7.—Scintigraphy images in normal *Balb-c* mice of [$^{177}$Lu] BEFG$_1$ (A), [$^{177}$Lu]BEFG$_3$ (B) and [$^{177}$Lu]BEFG$_5$ (C) 30 minutes, 1 and 4 hours post intravenous administration. The circles indicate kidneys and bladder.

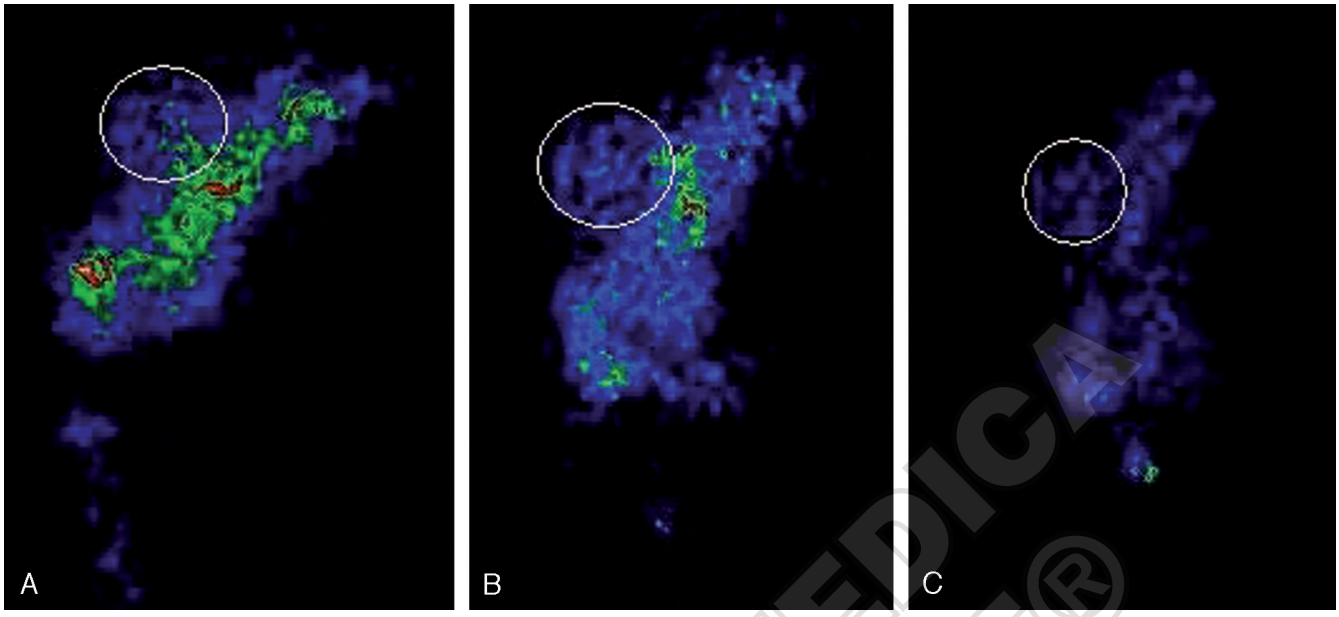


Figure 8.—Scintigraphy images of [$^{177}$Lu]BEFG$_5$ in *Nude* mice bearing PC-3 tumor 30 minutes (A), 1 (B) and 4 hours (C) post intravenous administration. The circles indicate the tumor localization.

In vivo *biodistribution and blocking studies in* Nude *mice bearing PC-3 xenografts*

The biodistribution pattern of the radiolabeled bombesin derivatives in *Nude* mice bearing PC-3 tumor xenografts are summarized in Figure 5. Blood clearances were very rapid, as observed in pharmacokinetics studies. The derivative with larger spacer ([$^{177}$Lu]BEFG$_5$) exhibited significantly higher tumor uptake in the first hour p.i. ($P<0.05$), but not in other times analyzed ($P=0.80$) (Table VIII).

The tumor to tissues ratios for the derivatives over time are shown in Table VIII. Although [$^{177}$Lu]BEFG$_1$ and [$^{177}$Lu]BEFG$_3$ had lower tumor uptake than [$^{177}$Lu]BEFG$_5$, greater tumor to tissues ratios were achieved 4 and 24 hours post injection, indicating higher tumor retention for these two analogues. Exceptions to this were the tumor to kidneys ratios, which were similar for all bombesin derivatives, and the tumor tissues ratios 1 hour p.i, which were higher for [$^{177}$Lu]BEFG$_5$.

Blocking studies performed only with [$^{177}$Lu]BEFG$_5$ reduced tumor and kidneys uptake ($P<0.05$) 1 hour p.i., but not other tissues uptake (Figure 6). The cold BEFG$_5$ blocked approximately 88% of tumor uptake when [$^{177}$Lu]BEFG$_5$ was administered 15 or 45 minutes after pre-injection of unlabeled peptide and the time after pre-injection did not alter the blocking effect.

*Scintigraphy studies*

Scintigraphy images of radiolabeled bombesin derivatives in normal *Balb-c* mice confirmed the results of the biodistribution assays and showed high kidneys uptake until 4 hours p.i., urinary excretion and low abdominal accumulation (Figure 7). The images in *Nude* mice bearing PC-3 tumor xenografts were performed with [$^{177}$Lu]BEFG$_5$ and showed important tumor uptake, especially 30 minutes and 1 hour post intravenous injection (Figure 8). The region of interest (ROI) was calculated as the percentage of tumor region radioactivity compared to whole body radioactivity and was 10.9%, 8.21% and 4.51% 30 minutes, 1 and 4 hours p.i., respectively.

## Discussion

Radiopharmaceuticals for therapeutic applications are designed to deliver a therapeutic dose of radiation to specific disease sites. The ionizing radiation can either damage cellular components in the target tissue directly or indirectly via the free radicals.

However, the destructive potential of therapeutic radioisotopes is not limited to the cellular targets but also to non-tumor tissues.[8] In the case of bombesin analogues, the selectivity is influenced by the length and composition of the spacer group.

In this work, we described the radiolabeling, chemical and biological characterization of a novel series of bombesin derivatives with spacers consisting in a phenylalanine and one, three or five glycine aminoacids. Glycine is the smallest and hydrophilic amino acid. In the proposed bombesin derivatives, a hydrophilic spacer was introduced between bombesin sequence (amino acid 6 to 14) and the chelator group (DOTA), suitable for $^{177}$Lu-labeling procedure. The bombesin derivatives were radiolabeled with high radiochemical yield and a specific activity varying from 7.5 to 8.5 GBq/µmol was obtained.

The specific activity of radiolabeled peptides is an important issue in the development of new radiopharmaceutical, especially if it is for therapeutic applications. Low specific activity can compromise the uptake of the tracer in the tissue of interest *in vivo*, because the cold molecules compete with radioactivity ones for the binding sites, and also can lead to physiological responses due to the presence of the cold peptide in the organism. On the other hand, very high specific activity can cause radiolysis in the solution of the compound, resulting in undesirable impurities.[26] Although the specific activity of radiolabeled bombesin derivatives described in this work is rather low when compared to some analogues described by literature,[16] it is eligible for preclinical evaluation and is comparable to that applied in some studies.[11, 27] However, further studies should be done to optimize the radiolabeling reaction, in order to apply higher radionuclide activity to peptides mass ratios and to produce high specific activity [$^{177}$Lu]BEFG$_1$, [$^{177}$Lu]BEFG$_3$ and [$^{177}$Lu]BEFG$_5$ for clinical investigations. Moreover, high specific peptides can also be achieved applying higher specific activity $^{177}$Lu in radiolabeling reactions.

A second peak was observed in the HPLC chromatograms of [$^{177}$Lu]BEFG$_1$, [$^{177}$Lu]BEFG$_3$ and [$^{177}$Lu]BEFG$_5$, probably related with oxidation of the methionine residue. This specie is probably resulted from radiolabeling conditions including high temperature and acid medium. This methionine sulfoxide residue has already been described for other radiolabeled bombesin derivatives and can be reduced by the addition of antioxidant agents or specific amino acids to the radiolabeling mixtures.[6, 8, 11]

Increasing the number of glycine residues in the spacer has reduced peptides lipophilicity as evidenced by HPLC chromatograms and partition coefficients. Although a little difference among peptides partition coefficients was detected, their slightly reduction with the addition of glycine amino acids in peptides spacers indicate that these amino acids improved the peptides hydrophilicity.

Radiolabeled bombesin derivatives exhibited a high stability at 2-8 °C. Moreover, stability analysis in human serum revealed *in vitro* half-lives higher than the unmodified bombesin, which present a half-life *in vitro* of only 30 minutes.[19] In general, the spacers increased bombesin *in vitro* stability in human serum. The addition of five glycine amino acids in the spacer of bombesin derivatives resulted in slower degradation by human serum enzymes after 4 hours of incubation ($P<0.05$), but not after 24 hours ($P=0.83$). This larger spacer resulted in higher metabolic stability of [$^{177}$Lu]BEFG$_5$, compared to the two other evaluated radiopeptides.

The results of *in vivo* pharmacokinetics studies showed that all $^{177}$Lu-labeled phenyl-glycine extended bombesin derivatives presented fast blood clearance, especially in the first hour p.i.. This fast clearance is important to avoid *in vivo* degradation by human serum enzymes, as observed *in vitro*. Although half-lives of the fast blood clearance components did not differ significantly, some slightly differences on pharmacokinetic parameters could be detected by Biexp software analysis. The addition of glycine amino acids not only increased phenyl-glycine extended bombesin derivatives hydrophilicity, but also improved their *in vivo* kinetics properties: reduction of the second blood component, reduction of $K_{12}$ transfer constant and increasing of the elimination constant ($K_{10}$).

An inconvenient of most studied bombesin analogues is their high *in vivo* uptake by pancreas and intestine due to the high density of GRP receptors in these mice tissues[16,17,18,19,20]. Although GRPr are found in rodent pancreas but rarely in human pancreas, recently investigations using the bombesin analogue $^{177}$Lu-AMBA as targeting vector showed that these receptors are present in human colon and also in smooth muscle and myenteric plexus of the gastrointestinal tract.[9, 28] The dosimetry in these target sites would constitute a problem for the clinical application of radiolabeled bombesin analogues in target therapy.

Kidney retention is an important factor when

considering the development of radiopharmaceuticals for target therapy. Biodistribution studies in *Balb-c* mice demonstrated that [$^{177}$Lu]BEFG$_1$, [$^{177}$Lu]BEFG$_3$ and [$^{177}$Lu]BEFG$_5$ exhibit low kidneys retention, when compared to other bombesin derivatives with moieties containing glycine amino acids.[29, 30] This kidneys excretion and the low liver uptake of radiopeptides confirmed *in vivo* the low lipophilicity represented by partition coefficients. Moreover, *in vivo* studies in *Balb-c* mice showed that the radioconjugates exhibited low abdominal accumulation in pancreas and intestine. Despite pancreatic and intestinal uptake could be related with lower tumor affinity, some studies have shown non linear relation between tumor and pancreatic or intestinal uptake.[19, 31]

Investigations in *Nude* mice bearing human prostate tumor (PC-3) showed that the radiopeptides cleared fast from the blood, but the blood-associated activities were in average higher for *Nude* mice when compared to *Balb-c* mice. As demonstrated in *Balb-c* mice, labeled bombesin derivatives were mainly excreted by renal pathway in *Nude* mice and renal retention was higher in this animal tumor model, but not in intestine and pancreas. Moreover, low prostate uptake of this novel series of bombesin derivatives confirmed previous data that the normal prostate is negative for GRP receptors.[16, 28]

At 1 hour p.i., higher tumor uptake was observed with [$^{177}$Lu]BEFG$_5$ ($P<0.05$). This result is in accordance to literature data [5] and confirmed that there is non linear relation between tumor uptake and pancreatic or intestinal uptake. [$^{177}$Lu]BEFG$_5$ tumor uptake showed to be higher than some bombesin derivatives with important abdominal retention.[19, 31, 32] In addition, *in vivo* tumor uptake of [$^{177}$Lu]BEFG$_5$ allowed PC-3 tumor detection by scintigraphy images, with the advantage of very low abdominal accumulation and background radiation in the images. Finally, results of the *in vivo* competition binding assay, in which a reduction of 88% in tumor uptake 1 hour p.i. was observed with the pre-injection of cold peptide, suggest a specific receptor binding.

No differences in tumor uptake were detected 4 or 24 hours p.i., suggesting that [$^{177}$Lu]DOTA-Phe-(Gly)$_n$-BBN(6-14) derivatives are not internalized or are internalized and rapidly externalized. Further studies are in development in order to elucidate the mechanisms of tumor uptake of these derivatives and to characterize their pharmacological properties as agonists or antagonists of bombesin receptors.

## Conclusions

In this work we studied a novel series of bombesin derivatives for prostate tumor imaging and treatment. The proposed bombesin derivatives were easily radiolabeled with lutetium-177 and presented improved *in vitro* stability and *in vivo* properties. Our results suggested that the derivative [$^{177}$Lu]DOTA-Phe-(Gly)$_5$-BBN(6-14) holds a higher potential as radiopharmaceutical for human prostate tumor diagnosis, because its higher tumor uptake. Also, the derivatives [$^{177}$Lu]DOTA-Phe-(Gly)$_1$-BBN(6-14) and [$^{177}$Lu]DOTA-Phe-(Gly)$_3$-BBN(6-14) are potential radiopharmaceuticals for prostate tumor treatment, because their higher tumor retention. However, dosimetric studies will be developed in order to confirm these hypotheses and modifications in the derivatives structures are under investigation in order to improve their tumor uptake.

## References


1. Panigone S, Nunn AD. Lutetium-177-labeled gastrin releasing peptide receptor binding analogues: a novel approach to radionuclide therapy. Q J Nucl Med Mol Imaging 2006;50:310-21.
2. Engel JB, Schally AV, Dietl J, Rieger L, Hönig A. Targeted therapy of breast and gynecological cancers with cytotoxic analogues of peptide hormones. Mol Pharm 2007;4:652-8.
3. Weiner RE, Thakur ML. Radiolabeled peptides in diagnosis and therapy. Semin Nucl Med 2001;31:296-311.
4. Anastasi A, Erspamer V, Bucci M. Isolation and structure of bombesin and alytesin, two analogous active peptides from the skin of the European amphibians Bombina and Alytes. Experientia 1971;26:166-7.
5. Varvarigou AD, Bouziotis P, Zikos Ch, Scopinaro F, de Vicentis G. Gastrin-releasing peptide (GRP) analogues for cancer imaging. Cancer Biother Radiopharm 2004;19:219-29.
6. Maina T, Nock BA, Zhang H, Nikolopoulou A, Waser W, Reubi JC *et al.* Species differences of bombesin analog interactions with GRP-R define the choice of animal models in the development of GRP-R-targeting drugs. J Nucl Med 2005;46:823-30.
7. Patel O, Shulkes A, Baldwin GS. Gastrin-releasing peptide and cancer. Biochim Biophys Acta 2006;1766:23-41.
8. Chen J, Linder KE, Cagnolini A, Metcalfe E, Raju N, Tweedle MF *et al.* Synthesis, stabilization and formulation of [$^{177}$Lu]Lu-AMBA, a systemic radiotherapeutic agent for Gastrin Releasing Peptide receptor positive tumors. Appl Radiat Isot 2008;66:497-505.
9. Thomas R, Chen J, Roudier MM, Vessella RL, Lantry LE, Nunn AD. *In vitro* binding evaluation of $^{177}$Lu-AMBA, a novel $^{177}$Lu-labeled GRP-R agonist for systemic radiotherapy in human tissues. Clin Exp Metastasis 2009;26:105-19.
10. Markwalder R, Reubi JC. Gastrin-releasing peptide receptors in the human prostate: relation to neoplastic transformation. Cancer Res 1999;59:1152-9.
11. Zhang H, Schuhmacher J, Waser B, Wild D, Eisenhut M, Reubi JC *et al.* DOTA-PESIN, a DOTA-conjugated bombesin derivative designed for the imaging and targeted radionuclide treatment of bombesin receptor-positive tumours. Eur J Nucl Med Mol Imaging 2007;34:1198-208.
12. Mikolajczak R, Parus JL, Pawlak D, Zakrzewka E, Michalak W,

Sasinowska I. Reactor produced $^{177}$Lu of specific activity and purity suitable for medical applications. J Radioanal Nucl Chem 2003;257:53-7.
13. Vértes A, Nagy S, Zoltan K. Handbook of Nuclear Chemistry. v.4. Kluwer Academic Publishers: Netherlands, 2003.
14. Kwekkeboom DJ, Bakker WH, Kooij PM, Konijnenberg MW, Srinivasan A, Erion JL *et al.* [$^{177}$Lu-DOTA$^{0}$,Tyr$^{3}$]octreotate: comparison with [$^{111}$In-DTPA$^{0}$]octreotide in patients. Eur J Nucl Med 2001;28:1319-25.
15. Zhang H, Chen J, Waldherr C, Hinni K, Waser B, Reubi JC *et al.* Synthesis and evaluation of bombesin derivatives on the basis of pan-bombesin peptides labeled with indium-111, lutetium-177, and yttrium-90 for targeting bombesin receptor-expressing tumors. Cancer Res 2004;64:6707-15.
16. Lantry LE, Cappelletti E, Maddalena ME, Fox JS, Feng W, Chen J *et al.* $^{177}$Lu-AMBA: Synthesis and characterization of a selective 177Lu-labeled GRP-R agonist for systemic radiotherapy of prostate cancer. J Nucl Med 2006; 47:1144-52.
17. Rogers BE, Bigott HM, McCarthy DW, Manna DD, Kim J, Sharp TL *et al.* MicroPET imaging of a gastrin-releasing peptide receptor-positive tumor in a mouse model of human prostate cancer using a $^{64}$Cu-labeled bombesin analogue. Bioconjug Chem 2003;14:756-63.
18. Smith CJ, Sieckman GL, Owen NK, Hayes DL, Mazuru DG, Kannan R *et al.* Radiochemical investigations of gastrin-releasing peptide receptor-specific [$^{99m}$Tc(X)(CO)$_3$-Dpr-Ser-Ser-Ser-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH$_2$] in PC-3, tumor-bearing, rodent models: syntesis, radiolabeling and *in vitro/in vivo* studies where Dpr = 2,3-Diaminopropionic acid and X = $H_2O$ or $P(CH_2OH)_3$. Cancer Res 2003;63:4082-8.
19. Garayoa EG, Schweinsberg C, Maes V, Rüegg D, Blanc A, Bläuenstein P *et al.* New [$^{99m}$Tc]bombesin analogues with improved biodistribution for targeting gastrin releasing-peptide receptor positive tumors. Q J Nucl Med Mol Imaging 2007;51:42-50.
20. Prasanphanic AF, Nanda PK, Rold TL, Ma L, Lewis MR, Garrison JC, *et al.* [$^{64}$Cu-NOTA-8-Aoc-BBN(7-14)NH$_2$] targeting vector for positron-emission tomography imaging of gastrin-releasing peptide receptor-expressing tissues. PNAS 2007;104:12462-67.
21. de Araújo EB, Caldeira Filho JS, Nagamati LT, Muramoto E, Colturato MT, Couto RM *et al.* A comparative study of $^{131}$I and $^{177}$Lu labeled somatostatin analogues for therapy of neuroendocrine tumors. Appl Radiat Isot 2009;67:227-33.
22. Durkan K, Lambrecht FY, Unak P. Radiolabeling of bombesin-like peptide with $^{99m}$Tc: $^{99m}$Tc-litorin and biodistribution in rats. Bioconjug Chem 2007;18:1516-20.
23. Murphy R, Tonantzitla I. *Biexp*. Puebla: México; 1991.
24. Biddlecombe GB, Rogers BE, de Visser M, Parry JJ, de Jong M, Erion JL, *et al.* Molecular imaging of gastrin-releasing peptide receptor-positive tumors in mice using $^{64}$Cu- and $^{86}$Y-DOTA-(Pro$^{1}$,Try$^{4}$)-Bombesin(1-14). Bioconjug Chem 2007;18:724-30.
25. Haley TJ, Komesu N, Efrus M, Koste L, Uphan HC. Pharmacology and toxicology of lutetium chloride. J Pharm Sci 1964;53:1186-8.
26. Saha, GB. Fundamentals of Nuclear Pharmacy. 5$^{th}$ ed. Springer: United States of America; 2003.
27. Hu F, Cutler CS, Hoffman T, Sieckman G, Volkert WA, Jurisson SS. Pm-149 DOTA bombesin analogs for potential radiotherapy *in vivo* comparison with Sm-153 and Lu-177 labeled DO3A-amide-βAla-BBN(7-14)NH$_2$. Nucl Med Biol 2002;29:423-30.
28. Waser B, Eltschinger V, Linder K, Nunn A, Reubi JC. Selective in vitro targeting of GRP and NMB receptors in human tumours with the new bombesin tracer $^{177}$Lu-AMBA. Eur J Nucl Med Mol Imaging 2007;34:95-100.
29. Parry JJ, Kelly TS, Andrews R, Rogers BE. In vitro and iv vivo evaluation of $^{64}$Cu-labeled DOTA-Linker-Bombesin(7-14) analogues containing different amino acid linker moieties. Bioconjug Chem 2007;18:1110-7.
30. Garrison JC, Rold TL, Sieckman GL, Naz F, Sublett SV, Figueroa SD *et al.* Evaluation of the pharmacokinetic effects of various linking group using the $^{111}$In-DOTA-X-BBN(7-14)NH$_2$ structural paradigm in a prostate cancer model. Bioconjug Chem 2008;19:1803-12.
31. Lane SR, Veerendra B, Rold TL, Sieckman GL, Hoffman TJ, Jurisson SS *et al.* $^{99m}$Tc(CO)$_3$-DTMA bombesin conjugates having high affinity for the GRP receptor. Nucl Med Biol 2008;35:263-72.
32. La Bella R, Garcia-Garayoa E, Langer M, Bläuenstein P, Beck-Sickinger AG, Schubiger PA. In vitro and in vivo evaluation of a $^{99m}$Tc(I)-labeled bombesin analogue for imaging of gastrin releasing peptide receptor-positive tumor. Nucl Med Biol 2002;29:553-60.