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DEVELOPMENT OF A NEW BOMBESIN ANALOG RADIOLABELED WITH LUTETIUM-177: *IN VIVO* EVALUATION OF THE BIOLOGICAL PROPERTIES IN *BALB-C* MICE

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Abstract – In this work we describe the first results of radiolabeling with lutetium-177 (^{177}Lu) and *in vivo* biodistribution and pharmacokinetics studies in normal *Balb-c* mice of a new bombesin analog ($BEFG_2$) – DOTA-Phe-X-BBN(6-14), where X is a spacer of two aminoacids. Bombesin (BBN) is an amphibian analog of human gastrin releasing peptide (GRP). Development of radiolabeled BBN derivatives as agents for diagnostic imaging and systemic radiotherapy has increased considerable because of the observation that GRP receptors (GRPr) are over-expressed in a variety of human tumor cells, such as prostate tumor cells. ^{177}Lu-labeled peptides are attractive due to the excellent radiophysical properties and commercial availability of the radiometal. $BEFG_2$ was successfully labeled with high yield and kept stable for more than 96 hours at 2-8º C and 1 hour in human plasma. Data analysis obtained from the *in vivo* studies showed that the amount of $BEFG_2$ present in plasma decreased rapidly and became almost undetectable at 60 min p.i., indicating rapid peptide excretion, which is performed mainly by renal pathway. In addition, biodistribution and single photon emission tomography showed low abdominal accumulation of ^{177}Lu-DOTA- Phe-X-BBN(6-14), indicating that this analog is a potential candidate for tumors target therapy.

Key words: Bombesin analogs, radiolabeling, lutetium-177, biological properties.

INTRODUCTION

The search for peptide-based radiopharmaceuticals has experienced an important rise in the past decades (2). Success in the area of somatostatin receptor-positive tumor targeting with diagnostic and therapeutic radionuclides has stimulated research toward radionuclide targeting of alternative receptor systems overexpressed in tumors (6). As a consequence, new therapeutical approches are being developed based on these recent advances in understanding the role of other peptides in tumor progression (13).

A high number of regulatory peptides receptors were shown to be overexpressed in various human tumors. They are promising targets for molecular imaging and targeted therapy of cancer, because they are located on the plasma membrane and, upon binding of a ligand, the receptor-ligand is internalized. Among these most relevant peptide receptors, the bombesin receptors are of major interest (16).

Bombesin (BBN), a 14-amino acid peptide originally isolated from European frogs skin, elicit a broad of spectrum of biologic responses, including secretion of adrenal, pituitary, and gastrointestinal hormones; gastric acid secretion; modulation of neuronal firing rate, and regulation of smooth muscle contraction. BBN-like peptides exert their effects on cells by binding to members of a superfamily of G-protein-coupled receptors, characterized by 7 transmembrane domains. There are 4 known subtypes of BBN-related

Abbreviations: BBN, bombesin; **bb3r,** bombesin orphan receptor; **bb4r,** bombesin amphibian receptor; **$BEFG_2$,** DOTA-Phe-X-BBN(6-14), where X is a spacer of two aminoacids; *DOTA*, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; **DTPA,** ***diethylene triamine pentaacetic acid;*** **GRPr,** gastrin releasing peptide receptor; **HPLC,** high performance liquid chromatography; **ITLC-SG,** instant thin layer chromatography; **K_{12},** Intravascular to extravascular space transfer constant; **K_{21},** Extravascular to intravascular space transfer constant; **K_{10},** Intravascular space to excretion system transfer constant; **K_{ss},** Elimination rate constant; **NMBr,** neuromedin B receptor, **p.i.,** post injection; **Rt,** retention time; **$T_{1/2}$,** half-life; **^{131}I,** iodine-131 isotope; **^{177}Lu ,** lutetium-177 isotope; **%IA,** percentage uptake of injected activity per organ; **%IA/g,** percentage uptake of injected activity per gram of organ.

peptide receptors, including GRPr (gastrin-releasing peptide receptor), NMBr (neuromedin B receptor), the orphan receptor bb3r and the amphibian receptor bb4r, although cognate ligands for the last 2 have yet to be described in mammals. GRPr are expressed on a variety human cancers including breast, lung, pancreatic and prostate cancers and their activation regulates tumor cell morphology, differentiation and proliferation as well as upregulating proangiogenic gene expression (5). So, BBN is a promissing peptide for targeting GRPr for molecular imaging and target therapy of those tumors.

Radiolanthanides are considered to have good nuclear properties for use in radiotherapeutic applications. Attachment of the lanthanide to the biomolecule requires a multidentate ligand framework such as DOTA (1,4,7,10 – tetraazacyclododecane - 1,4,7,10-tetraacetic acid) or DTPA (*Diethylene triamine pentaacetic acid)*, capable of stabilizing the radiolanthanide against *in vivo* transchelation reactions with serum proteins. Lanthanide-labeled BBN-like peptides hold important potential for development of new radiopharmaceuticals for diagnosis and therapy (14). Among the radiolanthanides, the application of lutetium-177 (^{177}Lu) isotope in medicine is spreading in a last few years. This radiolanthanide with physical properties similar to iodine-131 (^{131}I), but with greater chemical stability, has been continuously wider used both in diagnosis and therapy (9). This increasing in ^{177}Lu applicability is due to its good radiation properties. Its half-life of 6.65 days permits to apply more sophisticated procedures both to purify it and to synthesize the radiopharmaceuticals requiring the use of more time and work. Besides, its β^- radiation of 498 keV maximum energy is very suitable for cancer therapy ensuring the interaction range of about 2 mm in a human tissue. The presence of 11% gamma-radiation of 208 keV makes it also suitable for single photon emission tomography (7). 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 (4).

Although a large number of BBN analogs were successfully synthesized and radiolabeled for tumor imaging and therapy and have shown to reduce tumor growth in mice, most of the studied analogs exhibit high abdominal accumulation, especially in pancreas and intestine (2,5,10-12). This abdominal accumulation may represent a problem in clinical use of radiolabeled bombesin analogs probably due to serious side effects to patients. The aim of the present work is to develop a new radiopharmaceutical based on bombesin structure ($BEFG_2$) – DOTA-Phe-X-BBN(6-14) – radiolabeled with lutetium-177 (^{177}Lu) with optimal *in vivo* distribution and pharmacokinetics. X is a spacer of two aminoacids which was inserted between the chelator and the binding sequence in order to improve bombesin *in vivo* properties.

MATERIAL AND METHODS

Reagents

DOTA-Phe-X-BBN(6-14) was provided from piChem and $^{177}LuCl_3$ was obtained from IBD (Netherlands). All other chemicals and reagents required for experiments were of analytical grade and were purchased from Sigma Aldrich Chemical Co.

Preparation of Radiotracer

Preliminary studies were done to establish the ideal labeling conditions for obtaining the highest yield of labeled $BEFG_2$. All reagents were prepared with Chelex 100 treated free metal water. Briefly, $BEFG_2$ (20 μg), 0.4 mol/L sodium acetate buffer (0.2 mL, pH 4.5) and 92.5 MBq (2.5 mCi) of $^{177}LuCl_3$ (in 0.05 M HCl, specific activity 871 – 920 GBq/mg) were heated at 90°C for 30 minutes.

Quality Control

Instant thin layer chromatography (ITLC-SG) was applied to determine free lutetium, with citrate/citric acid buffer pH 5.0 as solvent (Rf of labeled peptide was 0.1-0.3 and Rf of free lutetium was 0.9-1.0) (4). Radiochemical purity was also determined by high performance liquid chromatography (HPLC, Shimatzu) using reversed phase C_{18} columns (Waters, 4.0 x 150 mm, 5 μm) with radioactivity (Shell) detection, flow rate of 1.5 mL/min with a linear gradient of 10-90% (v/v) 0.1% TFA / acetonitrile in 0.1% TFA / H_2O for 15 minutes and the composition was maintained for another 10 minutes.

Purification of ^{177}Lu-$BEFG_2$

Reaction mixtures were purified on Sep-Pak C_{18} reversed phase extraction cartridge (Waters) as described (16). The column was prewashed with 10 mL of ethanol and subsequently activated with 10 mL of distilled water. After application of the sample, the cartridge was washed with 10 mL of distilled water to remove free lutetium-177 followed by 5 mL of methanol to elute the labeled peptide. The solvent was evaporated and the dry residue was dissolved in saline.

Stability of radiolabeled $BEFG_2$

To determine the *in vitro* stability of ^{177}Lu- $BEFG_2$ the preparation was stored at 2-8° C for different times (1 to 7 days) or human serum samples were spiked with ^{177}Lu-$BEFG_2$ (37 MBq/mL) and incubated for 1, 4 and 24 hours, followed by ITLC-SG analysis. All experiments were performed in triplicate.

In vivo studies

Animals

Animal studies were performed in accordance with United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations as well as institutional guidelines. Male *Balb-c* mice (4 to 8 weeks old, 20-25 weight), purchased from IPEN Animal Facility, were used for *in vivo* experiments.

Biodistribution studies

The radioactive analog (1.85 MBq/100μL/mouse) was injected intravenously in mice lateral tail vein. After different time intervals (1, 4 and 24 hours p.i.), the animals were sacrificed in groups of four and the blood was collected. Then, the mice were dissected and vital organs were isolated, weighed and their respective radioactivity was measured in an automatic gamma counter (Packard). The biodistribution of labeled $BEFG_2$ was calculated as percentage uptake of injected activity per organ (%IA) and per gram of organ (%IA/g).

Pharmacokinetics

Kinetics studies were performed by measuring ^{177}Lu-$BEFG_2$ in blood. Blood samples were collected 1, 5, 30, 60, 120, 240 and 1440 minutes after the intravenous injection and their radioactivity were measured as described early. The pharmacokinetics parameters were determined using Biexp software.

Imaging studies

Single photon emission computed tomography (SPECT) images were performed in male *Balb-c* mice 30 minutes, 1, 4 and 24 hours after intravenous injection of ^{177}Lu- $BEFG_2$ (37 MBq/100 μL 0.9% NaCl). The anesthetized mice were 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.

Data analysis

Statistical analysis were performed using Prism 3.0 software. Results were subjected to Student´s *t*-test and expressed as mean ± SD.

RESULTS

Radiolabeling of $BEFG_2$ with Lutetium-177

$BEFG_2$ was radiolabeled with high yield (98.9 ± 1.3%) and a specific activity of 4.6 ± 0.1 MBq/μg was achieved. Sep-Pak purification procedure did not alter the radiochemical purity of the methanol fraction.

The Fig. 1 shows a typical radioactive HPLC profile of ^{177}Lu-labeled $BEFG_2$. The labeled peptide (Rt = 7.4 ± 0.1 minutes) can be clearly separated from free lutetium (Rt = 1.44 ± 0.4 minutes). A second peak of shorter retention time than the mainly radioactive specie was observed in the HPLC profile of labeled peptide, which probably represent the labeled bombesin derivative resulted from oxidation of the methionine residue and represent less than 10% of total radioactivity present in radiolabeling mixture.

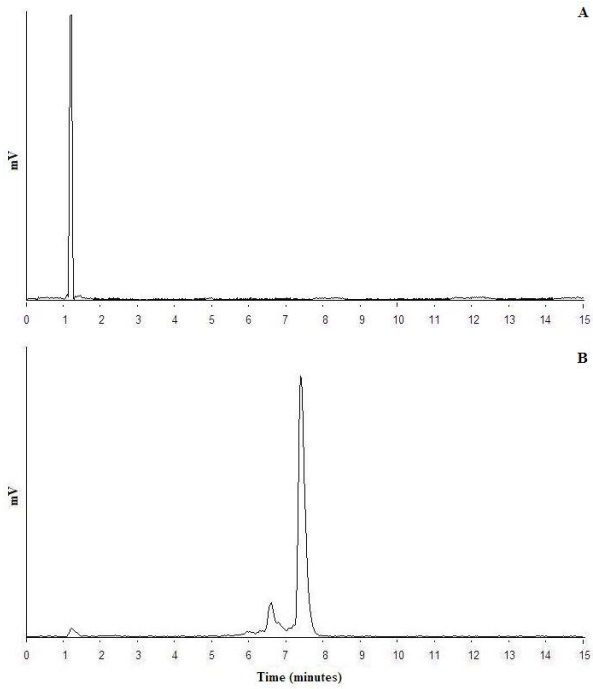

Figure 1. HPLC chromatogram (radioactive) of (A) lutetium-177 and (B) ^{177}Lu-$BEFG_2$.

Stability of ^{177}Lu-$BEFG_2$

The stability of labeled peptide was evaluated by ITLC-SG after both storage at 2-8° C and incubation at 37° C in human plasma. The Table I shows the results obtained from the samples after different times of storage at 2-8° C. ^{177}Lu-$BEFG_2$ remained stable at this temperature and the radiochemical purity of the preparation was higher than 90% for more than 96 hours of storage.

After incubation of the radiolabeled peptide with fresh serum, differences in the ITLC-SG chromatogram were detected in all time intervals analyzed, suggesting a metabolic degradation of ^{177}Lu-$BEFG_2$. These differences were used to calculate the radiochemical purity of the samples and to construct the curve shown in the Fig. 2. *In vitro* ^{177}Lu-$BEFG_2$ half-life in human plasma at 37° C was 3.9 hours.

In vivo studies

Results from biodistribution studies using the ^{177}Lu-labeled peptide performed in *Balb-c* mice are presented in Table II as the percentage of injected activity per organ (% IA) and percentage of injected activity per gram of organ (% IA/g). Appreciable radioactivity could be detected in the kidneys until 24 hours post injection, indicating peptide excretion by renal pathway. Kidneys may be the critical organs for dosimetry. In addition, it could be observed low abdominal accumulation of ^{177}Lu-$BEFG_2$,

especially in pancreas. These renal excretion and low abdominal accumulation could be confirmed by SPECT images acquired 30 minutes and 1and 4 p.i (Fig. 3).

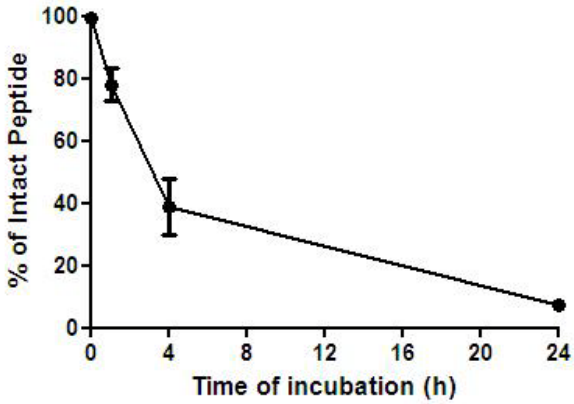

Figure 2. Representative curve of the time-course degradation of ^{177}Lu-BEFG$_2$ in human serum at 37° C. The radiochemical purity decreased to 77.9 ± 5.3, 39.0 ± 9.0 and 7.4 ± 1.5 after 1, 4 and 24 hours of incubation, respectively.

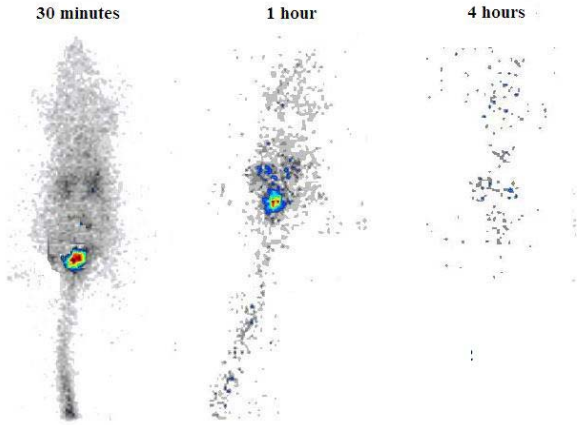

Figure 3. SPECT images of ^{177}Lu-radiolabeled BEFG$_2$ in male *Balb-c* mice. Kidneys and bladder are signalized. The images show fast distribution and renal excretion. Intestine uptake can be observed specially at 1 hour post intravenous injection.

The bone uptake is commonly assumed as a control of lutetium-labeled compounds stability in *in vivo* assays. This tissue actively uptakes free lutetium-177, being a good indicator of radiochemical purity, mainly at the initial time. Bone uptake of ^{177}Lu-BEFG$_2$ was negligible when compared to pure ^{177}LuCl$_3$ (3), confirming no contamination of free lutetium in the preparation.

To determine the pharmacokinetics parameters in mice we performed kinetics studies by measuring ^{177}Lu-BEFG$_2$ in blood. The results of blood analysis are expressed in Fig. 4 and the calculated pharmacokinetics parameters are resumed in Table III. The amount of ^{177}Lu-BEFG$_2$ present in plasma decreased rapidly and became almost undetectable at 60 minutes post injection. This rapid clearance is performed mainly by renal pathway, as described early in the biodistribution assay.

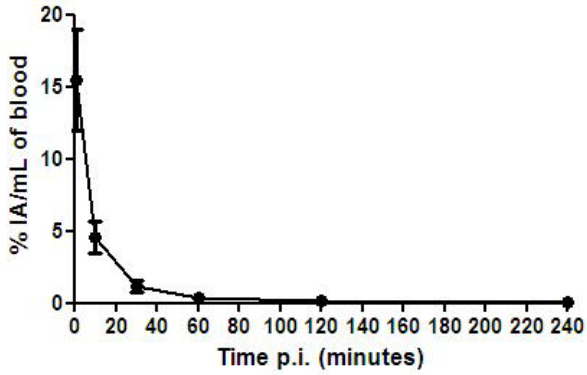

Figure 4. Blood clearance kinetics of labeled BEFG$_2$. Data analysis suggest a two-compartmental model.

DISCUSSION

It is well known that bombesin C-terminal group is required for its central high-affinity binding and biological activity (8). Many efforts in developing derivatives modified at BBN N-terminal group have been done to label bombesin with radiolanthanides and to diminish BBN analogs side effects due to the physiological responses. Several studies with agonists and antagonists that target the relevant receptors for human (GRPr, NMBr and bb3r) have been reported and rapid and high degree of endocytosis of the receptor-bound radiolabeled analogs indicate their agonistic nature as described for agonists, but not for antagonists (1).

We reported in this work the first results of a new bombesin analog radiolabeled with lutetium – ^{177}Lu-DOTA-Phe-X-BBN(6-14) (^{177}Lu-BEFG$_2$) – where X is a spacer of two aminoacids. This spacer was inserted to improve bombesin stability and to increase its hydrophilicity. The *in vitro* half-life of ^{177}Lu- BEFG$_2$ (3.9 hours) was much higher than its *in vivo* half-life in blood (7.8 minutes), indicating that this peptide can target tissues before its degradation by the metabolism in plasma. ^{177}Lu- BEFG$_2$ *in vitro* half-life was also higher than the unmodified peptide (30 minutes) (2). In addition, the hydrophilic spacer led to a great biological pattern with low liver uptake and rapid clearance mainly performed by renal pathway.

Table 1. *In vitro* stability of radiolabeled $BEFG_2$ after storing a 2-8° C for different times.

	Time of incubation at 2-8° C					
Radiochemical	**Immediately**	**24 hours**	**48 hours**	**72 hours**	**96 hours**	**168 hours**
Purity	99.4 ± 0.1	98.3 ± 0.2	97.8 ± 0.6	96.3 ± 0.1	96.9 ± 0.1	94.3 ± 0.1

Table 2. Biodistribution (0.185 MBq) of ^{177}Lu-$BEFG_2$ in normal *Balb-c* mice (n=5).

Time p.i.	**1 hour**		**4 hours**		**24 hours**	
	% AI	**% AI/g**	**% AI**	**% AI/g**	**% AI**	**% AI/g**
Heart	0.02 ± 0.01	0.16 ± 0.03	0.01 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Lungs	0.06 ± 0.01	0.29 ± 0.05	0.01 ± 0.01	0.09 ± 0.01	0.01 ± 0.01	0.04 ± 0.01
Pancreas	0.03 ± 0.01	0.11 ± 0.03	0.01 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Spleen	0.01 ± 0.01	0.09 ± 0.01	0.01 ± 0.01	0.08 ± 0.02	0.01 ± 0.01	0.04 ± 0.01
Stomach	0.07 ± 0.02	0.26 ± 0.07	0.01 ± 0.01	0.04 ± 0.01	0.02 ± 0.01	0.07 ± 0.05
Liver	0.27 ± 0.05	0.21 ± 0.05	0.14 ± 0.02	0.11 ± 0.01	0.08 ± 0.01	0.05 ± 0.01
Kidneys	0.98 ± 0.11	2.16 ± 0.28	0.69 ± 0.16	1.59 ± 0.17	0.34 ± 0.04	0.78 ± 0.16
Intestines*	0.71 ± 0.21	0.32 ± 0.10	0.41 ± 0.20	0.17 ± 0.10	0.51 ± 0.20	0.20 ± 0.11
Skeletal Muscle	1.38 ± 0.71	0.12 ± 0.07	0.43 ± 0.25	0.03 ± 0.01	0.16 ± 0.10	0.01 ± 0.01
Bone	1.16 ± 0.30	0.36 ± 0.11	0.95 ± 0.60	0.30 ± 0.15	0.73 ± 0.13	0.18 ± 0.11
Brain	0.01 ± 0.01	0.03 ± 0.01	0.00	0.00	0.00	0.00
Cerebellum	0.01 ± 0.01	0.06 ± 0.02	0.00	0.00	0.00	0.00

* Intestines with content.

Table 3. Calculated pharmacokinetics parameters for ^{177}Lu-BEFG$_2$ in male *Balb-c* mice. Blood sample data was adjusted by two phases with a fast and a slow decay.

Pharmacokinetics Parameters	**Value for ^{177}Lu-BEFG$_2$**
Equation	$C(t) = 736715.69^{-5.49t} + 13405.34^{-0.284t}$
$T_{1/2}$ fast phase (min)	7.8
$T_{1/2}$ slow phase (min)	329
$^{*}K_{12}$ (h^{-1})	1.28
$^{**}K_{21}$ (h^{-1})	0.37
$^{***}K_{10}$ (h^{-1})	4.11
Distribution volume (mL)	31.76
Clearance ($mL.h^{-1}$)	29.20
$^{****}K_{ss}$ (h^{-1})	0.91

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

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 (2,5,10-12). Although GRPr are found in rodent pancreas but rarely in human pancreas, these receptors are present in high density in human colon (15) and colon dosimetry would constitute a problem for the clinical application of radiolabeled bombesin analogs in target radiotherapy. Biodistribution and SPECT images studies showed ^{177}Lu- BEFG$_2$ uptake by the intestine, but this bind was lower than that decribed for other bombesin agonists. This lower pancreatic and intestinal uptake of ^{177}Lu- BEFG$_2$ could means lower tumor affinity, but some studies have shown non linear relation between tumor uptake and pancreatic or intestinal uptake (2). So, BBN analogs which show low uptake by these organs can also bind to tumor cells that overexpress GRPr.

This study described a very promising peptidic ligand for radiolabeling with diagnostic and/or therapeutic radiometals. ^{177}Lu- BEFG$_2$ was successfully radiolabeled with lutetium-177 and may be an useful tool for targeted diagnosis and radiotherapy of bombesin receptor-positive tumors, like prostate and breast cancer. Further studies are in development to evaluate ^{177}Lu-BEFG$_2$ target to human prostate carcinoma PC-3 cells in *Nude* mice. Modifications at BEFG$_2$ spacer will be also investigated to produce different analogs with improved pharmacokinetics and high specific receptor affinity.

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Other articles in this theme issue include references (17-24).

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