Novel Bombesin Analogues Conjugated with DOTA-Ala(SO_3H)-4 aminobenzoic acid and DOTA-Lys(glucose)-4 aminobenzoic acid: Synthesis, Radiolabeling, and Gastrin Releasing Peptide Receptor Binding Affinity

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Abstract - In this study, a novel bombesin (BBN) analogues, DOTA-Ala(SO_3H)-4 aminobenzoyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (DOTA-sBBN) and DOTA-Lys(glucose)-4 aminobenzoyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (DOTA-gluBBN), were synthesized and radiolabeled, and their binding affinities were evaluated. Peptides were prepared by a solid phase synthesis method and their purities were over 98%. DOTA is the chelating agent for ^{177}Lu-labeling, and the DOTA-conjugated peptides were radiolabeled with ^{177}Lu by a high radiolabeling yield (>98%). The Log *P* values of DOTA-sBBN and DOTA-gluBBN were −2.20 and −2.79, respectively. 50.41% of ^{177}Lu-DOTA-sBBN and 72.97% of ^{177}Lu-DOTA-gluBBN were left undegraded by the serum incubation at 37°C for 48 hr. A competitive displacement of ^{125}I-[Tyr^4]-BBN on the PC-3 human prostate carcinoma cells revealed that 50% inhibitory concentration (IC_{50}) were 1.46 nM of DOTA-sBBN and 4.67 nM of DOTA-gluBBN indicating a highly nanomolar binding affinity for GRPR. Therefore, it is concluded that ^{177}Lu-DOTA-sBBN and ^{177}Lu-DOTA-gluBBN can be potential candidates as a targeting modality for the Gastrin-releasing peptide receptor (GRPR)-over-expressing tumors, and further studies to evaluate their biological and pharmacological characteristics are needed.

Key words : ^{177}Lu, Bombesin, Gatrin releasing peptide receptor, Tumor targeting

INTRODUCTION

Bombesin (BBS) is a 14-amino acid neuropeptide that was originally isolated from the skin of the *Bombina* frog and binds with high affinity to gastrin-releasing peptide receptors (GRPR). Among the four identified types of GRPR, the bombesin receptor subtype 2 has been shown to be overexpressed on many human tumors, including prostate, colon, gastric, breast, pancreatic cancer and small cell lung cancer. Thus, radiolabeled BBS analogues show high affinity for this receptor subtype and can be used as radiopharmaceuticals for either the diagnosis or treatment of these cancers (Smith *et al.* 2003b; Okarvi 2004; Smith *et al.* 2005; Mu *et al.* 2010).

It has been shown that the targeting moiety of BBS is the C-terminal amino acid sequences, Trp^8-Ala^9-Val^{10}-Gly^{11}-His^{12}-Leu^{13}-Met^{14}-NH_2, for retaining the receptor binding affinity and preserving the biological activity of BBS-like peptides. Hence, the N-terminal region of the peptide can be used for radiolabeling, and a number of potent BBS analogues have been labeled with a lot of radionuclides such as ^{99m}Tc, ^{111}In, ^{90}Y, ^{64}Cu, ^{177}Lu, ^{68}Ga, or ^{18}F, for targeting

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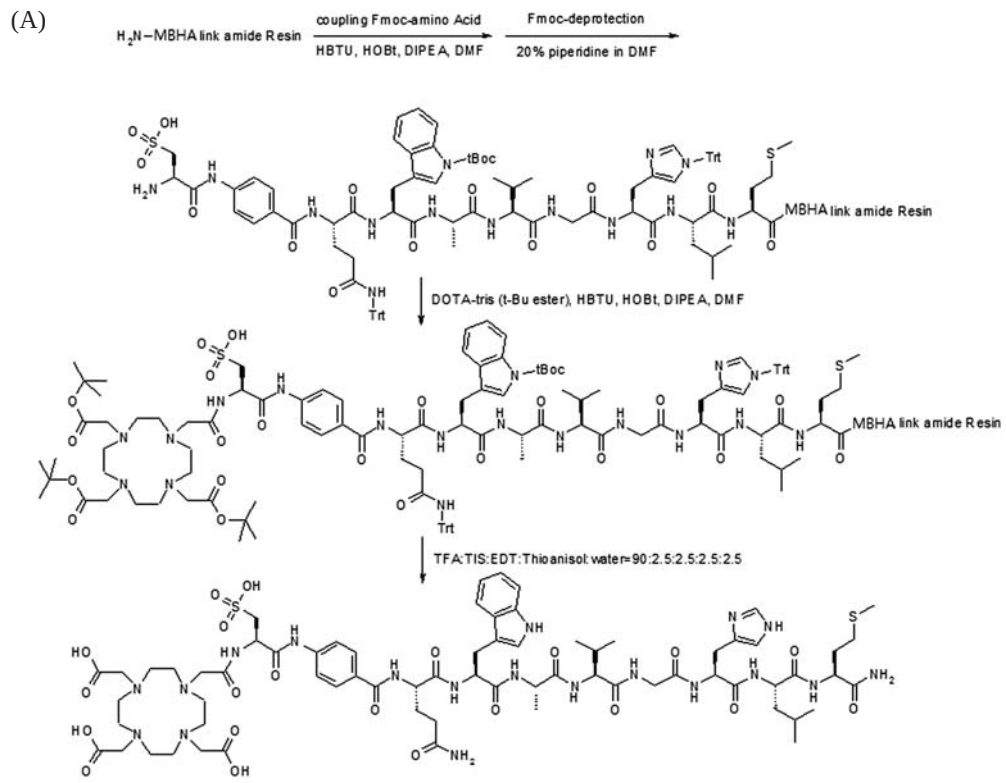

Fig. 1. Solid phase synthesis route and their formula of DOTA-sBBN (A) and DOTA-gluBBN (B), respectively. A sequence of the peptides were DOTA-sBBN : DOTA-Ala(SO_3H)-4 aminobenzoyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 and DOTA-gluBBN : DOTA-Lys (glucose)-4 aminobenzoyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2.

GRP receptor-expressing cancer cells (Hoffman *et al.* 2003; Rogers *et al.* 2003; Zhang *et al.* 2004; Maecke *et al.* 2005; Gourni *et al.* 2006; Zhang *et al.* 2006; Chen *et al.* 2008).

Among the available radionuclides, ^{177}Lu decays by a half-life of 6.71 days with the emission of beta-rays (497 keV), and gamma-rays (113 and 208 keV, 6% and 11%), and it came into the spotlight for the imaging and therapy of tumors. Thus, ^{177}Lu can monitor the *in vivo* localization of the therapeutic radiopharmaceutical injected during treatment by performing a dosimetric evaluation (Lee *et al.* 2009). The well-known chelating agent of radiolabeling peptides with ^{177}Lu is 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). ^{177}Lu exists primarily in an oxidation state of 3+ and is stabilized by hard donor atoms such as nitrogen or oxygen. Furthermore, as the mechanism of ligand coordination is more often ionic than covalent for ^{177}Lu and ^{177}Lu-like elements, multidentate ligand framework of DOTA is often utilized to stabilize the ^{177}Lu against in vivo transligation reactions with serum proteins (Cutler *et al.* 2000; Smith *et al.* 2003a).

BBS has been modified chemically to improve the binding affinity or stability. In particular, the introduction of hydrophilic carbohydrated linker moieties to a ^{99m}Tc-labeled BBS analogue improved the *in vivo* characteristics for the targeting of GRPR. Additionally, the negative-charged ^{18}F-labeled BBS analogue, which has L-cysteic acid [Ala(SO_3H)] moiety in its linker, showed a much higher prostate tumor uptake (Schweinsberg *et al.* 2008; Honer *et al.* 2011).

In this study, we employed Ala(SO_3H)-4 aminobenzoic acid and Lys(glucose)-4 aminobenzoic acid as a linker of BBS_{7-14} with DOTA to prepare radiolabeled candidates for GRPR targeting. A novel DOTA-sBBN and gluBBN were synthesized and radiolabeled, and IC_{50} of the peptides on GRPR-over-expressing human prostate tumor cells were evaluated.

MATERIALS AND METHODS

1. Materials

All chemicals were of analytical grade purchased from a chemical company, and used without further purification.

(B)

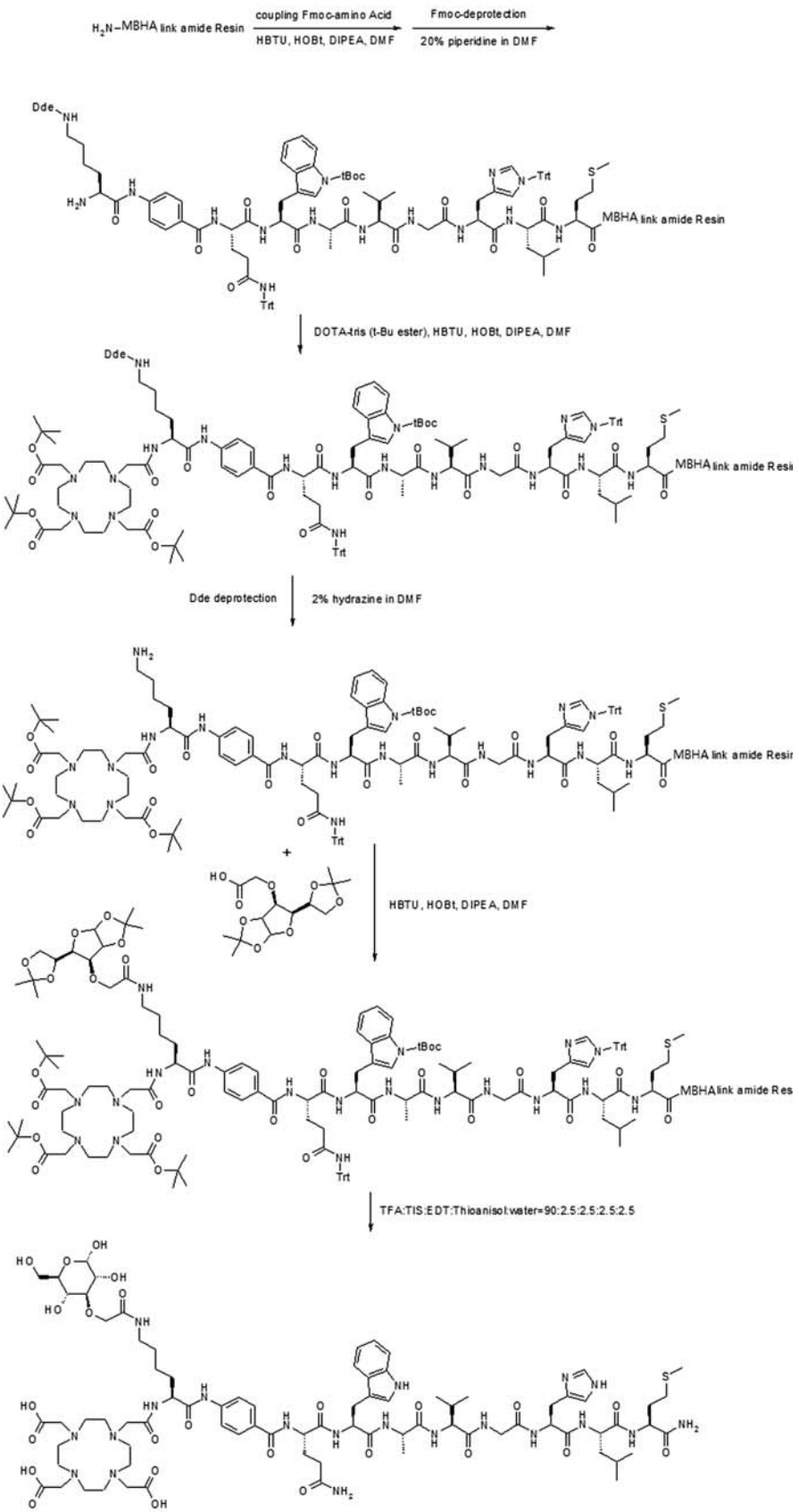

Fig. 1. Continued.

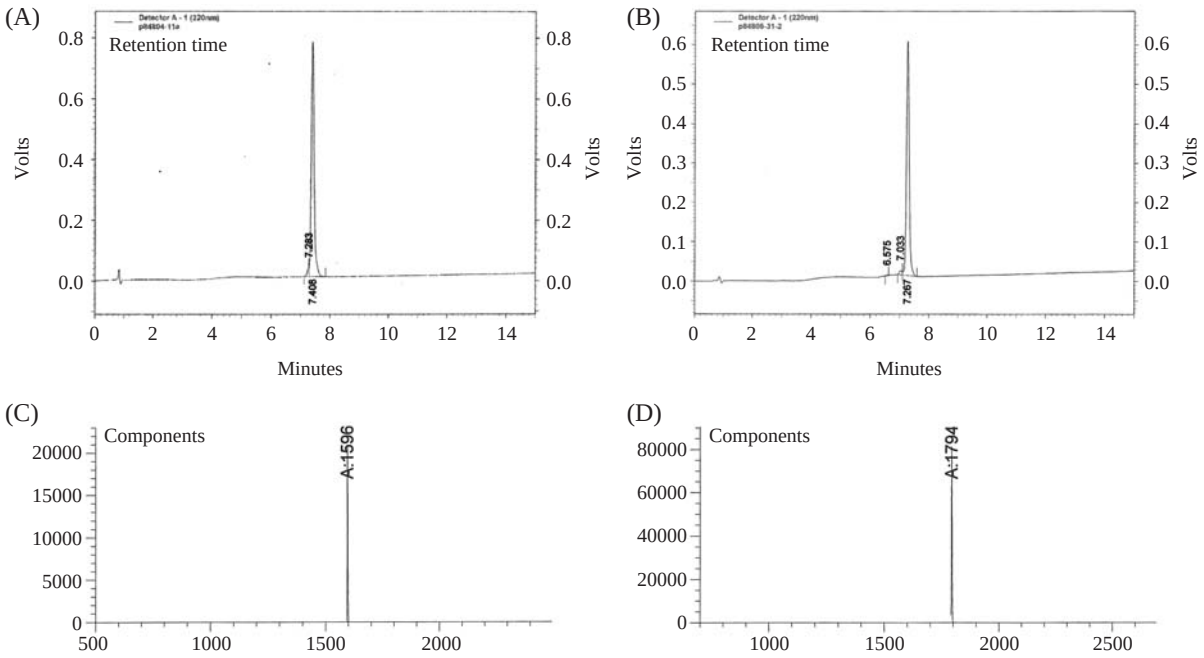

Fig. 2. HPLC analysis of DOTA-sBBN (A) and DOTA-gluBBN (B), and LC/MS profiles of DOTA-sBBN (C) and DOTA-gluBBN (D). The crude products were purified by Shimadzu HPLC equipped with a Capcell pak ^{18}C column and the molecular mass was analyzed on LC-MS. A purity of the peptides was over 98%, and a final MS data of the peptides were equal to the calculated value of the proposed formula.

Automated solid-phase synthesis was accomplished through the use of a Multiple Biomolecular Synthesizer (Peptron, Daejeon, Republic of Korea). Analytical and preparative RP-HPLC was performed on a SHIMAZU prominence HPLC using a Shiseido capcell pak C-18 column. A wavelength of 220 nm was used for UV detection for analytical RP-HPLC. The LC/MS was performed using an HP 1100 series. ^{177}Lu was purchased from Perkin-Elmer (Massachusetts, USA) and the radioactivity was measured using an ionizing chamber (Atomlab 200, Bio-dex, New York, USA). The radiolabeling yield and radiochemical purity (RCP) were determined using a gamma detector-equipped HPLC analyzer (Waters, Milford, USA).

2. Preparation of chelator conjugated peptides

The peptides were prepared through the use of an automated Multiple Biomolecular Synthesizer (Peptron, Daejeon, Republic of Korea). The DOTA-sBBN was synthesized by applying a standard Fmoc (fluorenylmethyloxycarbonyl) strategy as detailed in Fig. 1(A). Briefly, Fmoc-Met-OH conjugated 4-methylbenzhydrylamine (MBHA) resin was used as an anchor polymeric support for a solid phase synthesis. After removing the Fmoc protecting group from resin-bounded Fmoc-Met-OH under a standard cleavage condition (20% Piperidine in *N*,*N*-Dimethylformamide), the linear sequence peptide was prepared by the sequential coupling of Fmoc-Leu-OH, Fmoc-His(Trt)-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Trp(tBoc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-4 aminobenzoic acid, and Fmoc-Ala (SO_3H). For the synthesis of DOTA-gluBBN, Fmoc-Lys (Dde)-OH was coupled instead of Fmoc-Ala(SO_3H) and glucose was attached in 2% hydrazine in DMF after Dde deprotection as shown in Fig. 1(B). DOTA$(OtBu)_3$ was introduced into the peptide by applying HBTU, HOBt, DIPEA and DMF as an activating reagent to ensure efficient coupling. The resulting peptides were cleaved from the polymeric support by treatment with a mixture solvent of 90% TFA containing 2.5% triisopropylsilane (TIS), 2.5% ethanedithiol (EDT), 2.5% thioanisole, and 2.5% deionized water (TFA : TIS : EDT : Thioanisole : H_2O=90 : 2.5 : 2.5 : 2.5 : 2.5). The crude products were purified by Shimadzu HPLC equipped with a Capcell pak ^{18}C column on a binary gradient system at a flow rate of 1.0 ml min^{-1} using an elution solvent of 0.1 % trifluroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile (B) with a gradient elution profile of (B) 0～

10% in 2 min; 10～40% in 10 min; and 40～70% in 2 min. The molecular mass was analyzed on an LC-MS.

3. Radiochemistry of ^{177}Lu-labeled peptides

1) Preparation of ^{177}Lu-labeled peptides

Peptides were dissolved in a 50 mM sodium acetate buffer (pH=5.5) to give a concentration of 10^{-6} mole ml^{-1}. 37 MBq of a ^{177}Lu solution diluted in a 0.05 N HCl was injected into 1×10^{-8} mole of a peptide solution vial to give a final volume of 1 ml, and heated at 90°C for 30 min. The radiolabeling yield and radiochemical purity/stability of the radiolabeled compound were analyzed by a Waters Chromatograph equipped with an X-Terra ^{18}C column. The column was eluted with a binary gradient system with a flow rate of 1.0 ml min^{-1} using an elution solvent of 0.1% TFA in 5% acetonitrile and 0.1% TFA in 95% acetonitrile. The gradient elution profile based on the solution of 0.1% TFA in 95% acetonitrile is as follows: 0%, 5 min; 0～100%, 9 min; 100%, 6 min; 100%, and 2 min with 100% of 0.1% TFA in 5% acetonitrile.

2) Serum stability assay

Serum stability was evaluated as described by Nguyen *et al*. with some modification (Leonard and Nguyen 2010). ^{177}Lu-labeled peptides were added to 200 µl of 25% human serum in PBS, and incubated at 37°C for 2 days. 100 µl aliquots of the incubations were taken for the following time periods: 1 and 2 days. The aliquots were mixed with 40 µl of 15% trichloroacetic acid (TCA) and incubated at 4°C for at least 15 min to precipitate the serum proteins. 5 µl of 1 M NaOH was supplemented to the TCA to prevent peptide precipitation. The supernatant was collected for each sample after centrifugation at 13,000 rpm for 10 min and analyzed by HPLC analysis, as described above.

3) Determination of Log *P* value

37 KBq of ^{177}Lu-labeled peptides were dissolved in an equal volume mixture of 1-octanol and a PBS buffer (1 ml : 1 ml). After stirring vigorously for ~20 min, the mixture was centrifuged at a speed of 8,000 rpm for 5 min. 100 µl of samples from both 1-octanol and PBS layers were transferred and the radioactivity was measured using a Wallac 1470 Wizard automated gamma counter (PerkinElmer Life Science). Partition coefficients were measured three different times. The log *P* values were reported as the average of three independent measurements.

4. Biological evaluation of ^{177}Lu-labeled peptides

1) Cell culture

The GRPR-over-expressing PC-3 prostate carcinoma cells were obtained from the American Type Culture Collection (ATCC) and grown in 100 mm culture dishes (Corning, Corning, NY, USA). The cells were cultured in RPMI-1640 (LONZA, Walkersville, MD, USA), supplemented with 10% fetal bovine serum, 100 units ml^{-1} penicillin, and 100 g ml^{-1} streptomycin (Sigma, Milan, Italy) in an atmosphere of 5% CO_2 in air at 37°C for up to approximately a 90% confluence.

2) Competitive binding assay

The IC_{50} values of the peptides were determined using previously described methods with some modifications (Yubin Miao 2008). 1×10^5 PC-3 cells were placed in 12-well plates, and grown for 24 h at 37°C. After replacing the culture media with a FBS free-RPMI-1640, the cells were incubated at 37°C for 1 hr with 20,000 cpm of ^{125}I-[Tyr^4]-BBS (Perkin-Elmer, USA) in the presence of increasing concentrations of the peptide (10^{-6}～10^{-12} M) in a 1 ml binding buffer. The reaction media were collected. Cells were then washed twice with a cold PBS and solubilized with 1 N NaOH for 5 min. The activity was then determined in a gamma-counter. The IC_{50} value for the peptide was calculated through a non-linear regression analysis using the GraphPad Prism5 computer fitting program.

RESULTS AND DISCUSSION

For clinical application, BBN was conjugated to DOTA via a Gly-4 aminobenzoic acid group and labeled with ^{177}Lu. The radiolabeled compound, ^{177}Lu-AMBA was studied for prostate cancer patients in clinical settings (phase I). Lesions in five out of seven patients were successfully seen, and it is concluded that ^{177}Lu-AMBA administration is a safely promising radiopharmaceutical for GRPR over-expressing prostate cancer (Bodei and Nunn 2007).

On the basis of the amino acid sequence of AMBA, we designed and synthesized two novel BBS analogues. Glycation has been reported to provide radiohalogenated somatostatin analogues with excellent physicochemical characte-

ristics. Radiolabeled compounds were more hydrophilic but still exhibited a high receptor binding affinity, improved tumor uptake and tumor-to-nontumor ratios, and reduced hepatobiliary transfer and liver accumulation (Schottelius *et al.* 2002; Wester *et al.* 2002; Wester *et al.* 2003; Schweinsberg *et al.* 2008). In particular, an introduction of hydrophilic glucose into the ^{99m}Tc-labeled BBS analogues increased the receptor binding affinity, and improved the tumor uptake and tumor-to-non-tumor ratios (Schweinsberg *et al.* 2008). Additionally, Ala(SO_3H) as a linker moiety increased GRPR-selective binding affinity (Honer *et al.* 2011). Therefore, we set out to synthesize two BBS analogues which were substituted Gly in the linker moiety of AMBA with Ala(SO_3H) and Lys(glucose). Nonradioactive peptides were synthesized through a solid phase peptide synthesis following the standard Fmoc strategies as shown in Fig. 1.

The retention times of the analytical HPLC were found to be 7.41 min and 7.27 min for DOTA-sBBN and DOTA-gluBBN, respectively, and the chemical purities were over 98% (Fig. 2A). The measured ion peaks [M+1 (m/z)] were consistent with the calculated values of the proposed formula. MS data of DOTA-sBBN and DOTA-gluBBN were 1596 and 1794 (calculated=1596.21 and 1793.35). The final peptide sequence of DOTA-sBBNA was DOTA-Ala(SO_3H)-4 aminobenzoyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2, and DOTA-gluBBN was DOTA-Lys(glucose)-4 aminobenzoyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2.

The new conjugates, ^{177}Lu-DOTA-sBBN and ^{177}Lu-DOTA-gluBBN, were routinely prepared at a high yield (>98%) by adding $^{177}LuCl_3$ to an aqueous solution (pH 5.5 ammonium acetate) of the peptides at 90°C for 30 min. The HPLC chromatogram of ^{177}Lu-DOTA-sBBN (Fig. 3A) and ^{177}Lu-DOTA-gluBBN (Fig. 3B) showed a retention time of 11.21 min and 11.15 min, respectively.

Bifunctional chelating agents (BFCA) are used for the preparation of many radiolabeled compounds to introduce radionuclides to the targeting molecules without any distortion of their structure and not reduce their binding affinity and stability. In particular, DOTA is able to strongly chelate many radionuclides such as ^{68}Ga, ^{111}In, ^{149}Pm, ^{212}Pb, ^{90}Y and ^{177}Lu (Ruegg 1990; Depalatis 1995; Pippin 1995; Kukis 1998; Kwekkeboom 1999). Additionally, ^{177}Lu emits medium and lower-energy β-rays (497 keV) and γ-rays (113 and 208 keV, 6% and 11%). Thus ^{177}Lu is considered a suitable radionuclide for performing dosimetry and imaging,

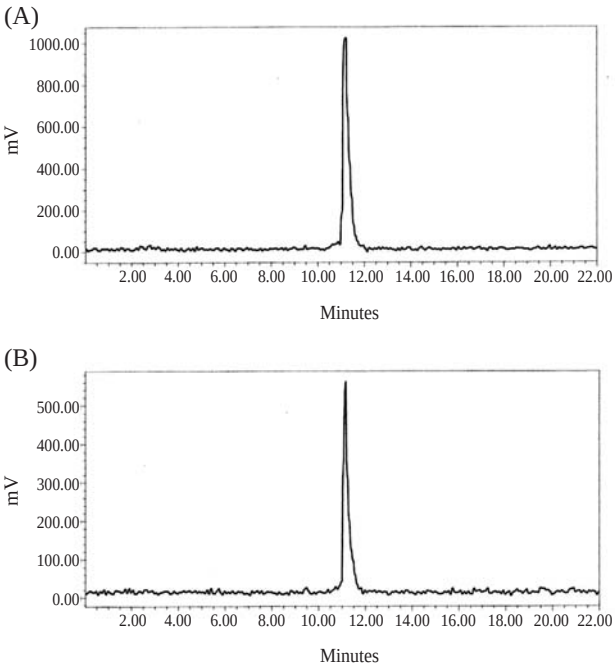

Fig. 3. Typical profiles of ^{177}Lu-DOTA-sBBN (A) and ^{177}Lu-DOTA-gluBBN (B) determined by HPLC analysis using a C-18 column. Radiochemical purity of the peptides was over 98% and further purification was not needed. Retention time: ^{177}Lu-DOTA-sBBN at 11.21 min and ^{177}Lu-DOTA-gluBBN at 11.15 min.

and treating small tumors or metastatic deposits (Miao *et al.* 2005). DOTA-sBBN and DOTA-gluBBN were also easily labeled with ^{177}Lu, and imaging and therapeutic studies might be possible in the next investigations.

To establish the hydrophilicity of the peptides, the octanol/PBS partition coefficients were determined. The Log *P* value for ^{177}Lu-DOTA-sBBN was −2.20. The ^{177}Lu-DOTA-gluBBN was more hydrophilic, with a Log *P* value of −2.79.

Garcia *et al.* reported that the ^{99m}Tc-labeled BBS analogues with Log *P* value between +1 and −1 showed the highest binding affinities with Kd values of <0.5 nM as well as the highest cellular uptake. In addition, a higher hydrophilicity (Log *P*< −1.8) led to a lower affinity and substantial decrease of the internalization (Garcia Garayoa *et al.* 2008). Another report also demonstrated that peptides with a Log *P* value of −0.2∼ −0.5 kept a high affinity for GRPR (Schweinsberg *et al.* 2008). However, the DUP-1 tracer was more hydrophilic (log *P*=−2.41) than the bombesin tracer (log *P*=−0.39). Although the DUP-1 tracer showed a lower binding to tumor cells during the *in vitro* evaluation, the tumor uptake for both tracers was similar,

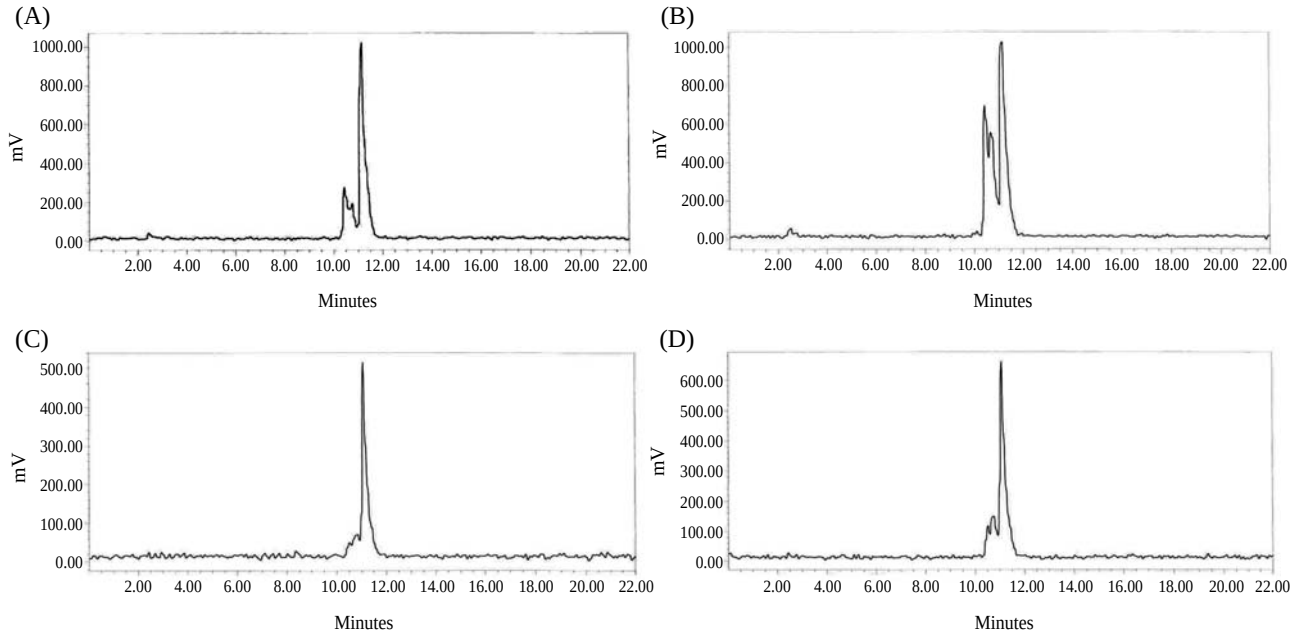

Fig. 4. Serum stability of ^{177}Lu-DOTA-sBBN at 24 hr (A) and 48 hr (B), and ^{177}Lu-DOTA-gluBBN at 24 hr (C) and 48 hr (D). 72.82% at 24 hr and 50.41% at 48 hr of ^{177}Lu-DOTA-sBBN were remained, and 80.21% at 24 hr and 72.97% at 48 hr. % of ^{177}Lu-DOTA-gluBBN were remained.

and the higher hydrophilicity resulted in a greater kidney uptake of DUP-1 (Faintuch *et al.* 2012). Additionally, the IC_{50} value of ^{18}F-NOTA-8Aoc-BBN_{7-14}-NH_2 was very sub-nanomolar, 0.28 nM, though its Log *P* value was −1.47. %ID/g of the PC-3 tumor was 2.15±0.55 at 1 hr p.i, and it was clearly visualized as a PC-3 tumor (Dijkgraaf *et al.* 2012). Although Log *P* value of ^{99m}Tc-labeled BBN_{7-14}-NH_2 was more hydrophilic, −2.39, it was also visualized as a GRPR-over-expressing tumor (Jiyun *et al.* 2008). Therefore, the binding affinity cannot be predicted by its Log *P* value, but it can be expected that the *in vivo* characteristics of more hydrophilic peptide can be better (Schweinsberg *et al.* 2008; Honer *et al.* 2011).

As shown in Fig. 4, ^{177}Lu-DOTA-gluBBN was more stable in human serum than ^{177}Lu-DOTA-sBBN. 37.18% of ^{177}Lu-DOTA-sBBN was degraded for 24 hr after incubation, and 22.41% was more degraded for 24 hr. In contrast, 19.79 % of ^{177}Lu-DOTA-gluBBN was degraded for 24 hr, and 7.24% was more degraded for 24 hr. Because the difference between two peptides is the linker moiety, it seems that the structure using Lys(glucose) as a linker is more stable than that of Ala(SO_3H), and ^{177}Lu-DOTA-gluBBN can be promising considering that glucose might increase the *in vivo* characteristics.

The half life of ^{177}Lu-AMBA was 38.8 hr in human serum and 3.1 hr in murine serum (Lantry *et al.* 2006). Compared with these results, ^{177}Lu-DOTA-gluBBN and ^{177}Lu-DOTA-sBBN were more stable than ^{177}Lu-AMBA in human serum. It might be caused by the kind of linker. As Ala(SO_3H) already showed a high stability, the linkers used in this study might be more stable than natural amino acid, Gly (Honer *et al.* 2011).

Although most bombesin agonist-based peptides showed a low stability, antagonist-based peptides such as ^{18}F-BAY 86-4367 showed a higher stability that was not degraded in human serum for 2 hr. The targeting sequence used in ^{18}F-BAY 86-4367 was [N methyl gly^{11}, statine^{13}, Leu^{14}] BBN_{7-14} (Honer *et al.* 2011). Unnatural amino acid can play a role in increasing the stability of the peptide. Therefore, ^{177}Lu-DOTA-sBBN and ^{177}Lu-DOTA-gluBBN can be more stable by substituting more stable amino acid for natural amino acid in its targeting moiety.

The *in vitro* GRPR binding affinities and specificities of DOTA-sBBN and DOTA-gluBBN were assessed via a competitive displacement assay using ^{125}I-[Tyr^4]-BBN as the radioligand. It was found that ^{177}Lu-DOTA-sBBN and ^{177}Lu-DOTA-gluBBN were able to compete with ^{125}I-[Tyr^4]-BBN bound to PC-3 prostate carcinoma cells (Fig. 5). The IC_{50}

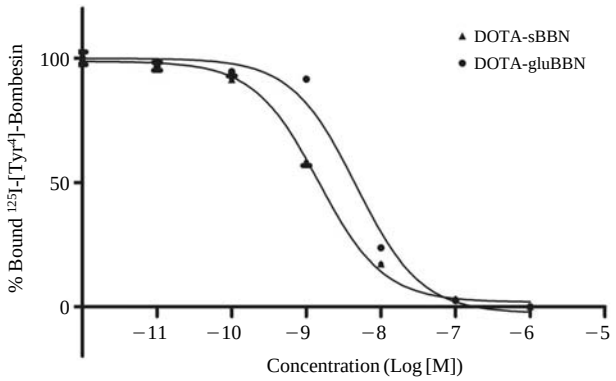

Fig. 5. Competitive binding of ^{125}I-[Tyr^4]-Bombesin on PC-3 cells by a treatment of DOTA-sBBN (▲) and DOTA-gluBBN (●). Results expressed as a percentage of binding are mean ± SD in triplicate. 1×10^5 PC-3 cells were incubated at 37 ˚C for 1 hr with 20,000 cpm of ^{125}I-[Tyr^4]-Bombesin (Perkin-Elmer, USA) in the presence of increasing concentrations of the peptides ($10^{-6} \sim 10^{-12}$ M) in a 1 ml binding buffer. The IC_{50} value was 1.46 nM for DOTA-sBBN and 4.67 nM for DOTA-gluBBN.

values were 1.46 nM and 4.67 nM for DOTA-sBBN and DOTA-gluBBN, respectively. IC_{50} of the AMBA was 4.74 nM similar with two peptides, and it targeted PC-3 tumor successfully. %ID g^{-1} of the PC-3 tumor was 6.35 ± 2.23 at 1 hr p.i (Lantry *et al.* 2006). IC_{50} of ^{177}Lu-DOTA-AR was even 18 nM and it was accumulated in xenografted PC-3 tumor by 10.56 ± 0.70%ID g^{-1} at 1 hr p.i. (Abiraj *et al.* 2011). These results were encouraging to apply two peptides as a targeting modality for a GRPR-over-expressing PC-3 tumor. Because the hydrophilicity is altered by their linker moiety, further studies to evaluate the pharmacokinetics of ^{177}Lu-DOTA-sBBN and ^{177}Lu-DOTA-gluBBN are needed.

^{177}Lu-DOTA-sBBN and ^{177}Lu-DOTA-gluBBN showed high stability and binding affinity on GRPR. Therefore, their pharmacokinetic characteristics and therapeutic efficacy are planned to be evaluated in our next investigation. As *in vitro* targeting abilities of the peptides were shown, promising biological and pharmacological characteristics can be expected in further *in vivo* studies.

CONCLUSION

In conclusion, we developed two novel BBS derivatives, ^{177}Lu-DOTA-gluBBN and ^{177}Lu-DOTA-sBBN. To increase the hydrophilicity, Ala(SO_3H)-4 aminobenzoyl or Lys(glucose)-4 aminobenzoyl were introduced into the linker moiety, which did not affect the binding affinity on GRPR. As the feasiblility to target GRPR is shown, their biological and pharmacological characteristics should be evaluated in the next investigation.

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