Preliminary Evaluation of Astatine-211-Labeled Bombesin Derivatives for Targeted Alpha Therapy

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There are various diagnostic and therapeutic agents for prostate cancer using bombesin (BBN) derivatives, but astatine-211 (211At)-labeled BBN derivatives have yet to be studied. This study presented a preliminary evaluation of 211At-labeled BBN derivative. Several nonradioactive iodine-introduced BBN derivatives (IB-BBNs) with different linkers were synthesized and their binding affinities measured. Because IB-3 exhibited a comparable affinity to native BBN, [211At]AB-3 was synthesized and the radiochemical yields of [211At]AB-3 was 28.2 ± 2.4%, with a radiochemical purity of >90%. The stability studies and cell internalization/externalization experiments were performed. [211At]AB-3 was taken up by cells and internalized; however, radioactivity effluxed from cells over time. In addition, the biodistribution of [211At]AB-3, with and without excess amounts of BBN, were evaluated in PC-3 tumor-bearing mice. Despite poor stability in murine plasma, [211At]AB-3 accumulated in tumor tissue (4.05 ± 0.73%ID/g) in PC-3 tumor-bearing mice, which was inhibited by excess native BBN (2.56 ± 0.24%ID/g). Accumulated radioactivity in various organs is probably due to free 211At. Peptide degradation in murine plasma and radioactivity efflux from cells are areas of improvement. The development of 211At-labeled BBN derivatives requires modifying the BBN sequence and preventing deamidation.

Key words  astatine-211; targeted alpha therapy; prostate cancer; bombesin

Introduction

Every year, approximately 1.1 million men worldwide are diagnosed with prostate cancer (PC), which is the second-most common cancer among men.1 A significant number of patients with PC develop metastasis.2 Localized PC can be treated with surgery or local radiation therapy; however, patients with metastatic PC generally require systemic therapy, including hormone treatment.3 In addition, many patients with PC develop progressive disease, termed castrate-resistant prostate cancer (CRPC), with increasing prostate-specific antigen levels despite low androgen levels.

Targeted radionuclide therapy (TRT) is a type of radiotherapy targeting cancer cells, in which drugs are conjugated with radionuclides and carriers. TRT using radionuclides that emit α-particles (targeted alpha therapy [TAT]) is an especially attractive treatment strategy for CRPC because some studies have reported excellent results with TAT. For example, [222Ra]RaCl2 (Xofigo) prolongs the overall survival for patients with bone metastasis of CRPC,4 and 222Ac-labeled prostate-specific membrane antigen ligand is effective in patients with metastatic PC.5 Another α-emitting radionuclide, astatine-211 (211At), with a half-life of 7.21 h, is also useful for TAT. TAT has potential advantages for micrometastatic cancer treatment, such as high linear energy transfer and a short range. In the case of 211At, the linear energy transfer is 97 keV/μm and the range is 55–70 μm. These 211At properties enable effective treatment while minimizing damage to surrounding normal tissues.6–9

It has been reported that gastrin-releasing peptide receptors (GRPRs) are overexpressed in various tumors, including PC and breast cancer.9–11 In fact, GRPRs have been used as a target for PC. Bombesin (BBN), a tetradecapeptide isolated from Bombina bombina skin, has high affinity for mammalian GRPRs. BBN can be used as a carrier for PC and many radiopharmaceuticals using BBN derivatives are based on either the complete peptide sequence or its fragments.11–13 However, despite numerous reports of radiopharmaceuticals targeting GRPRs, TRT agents are limited to β-emitting radionuclides, such as 90Y and 177Lu.14 177Lu-AMBA (a GRPR agonist) inhibits tumor growth and increases the survival rate in mice.15 However, BBN derivatives labeled with α-emitting radionuclides, including 211At-labeled BBN derivatives, have not yet been studied.

This study presented a preliminary evaluation of 211At-labeled BBN derivative. We selected bombesin (7–14), which has been used frequently in previous studies,11 as the sequence targeting GRPRs. Because there are no stable isotopes of astatine, we used iodine, which has chemical properties that are similar to astatine, to determine candidates for 211At-labeled BBN sequences. Several nonradioactive iodine-introduced BBN derivatives (IB-BBNs) with different linkers were synthesized and evaluated, and their binding affinity to GRPRs was evaluated. In addition, one BBN derivative was labeled with 211At, and its in vitro and in vivo properties were evaluated.

Experimental

Generals  211At was produced on CYPRIS MP-30 cy-
clorotn (Sumitomo Heavy Industries, Ltd., Tokyo, Japan) at Fukushima Medical University via $^{209}$Bi($\alpha$, $2n$)$^{211}$At nuclear reaction. $^{211}$At was isolated by dry distillation at 850°C, trapped in polytetrafluoroethylene (PTFE) tube at dry ice/ethanol (EtOH) bath, and elute with chloroform.$^{15}$ $^{[125I]}$-Tyr$^4$-bombesin was purchased from PerkinElmer, Inc. (Waltham, MA, U.S.A.). 9-Fluorenylmethoxy carbonyl (Fmoc)-Met-Rink-Amide MBHA Resin and BBN were purchased from PEPTIDE INSTITUTE, Inc. (Osaka, Japan). 1-(9H-Fluoren-9-yl)-3-oxo-2,7,10-trioxa-4-azadodecan-12-oic acid (Fmoc-AEEA) was purchased from Ark Pharm, Inc. (Arlington Heights, IL, U.S.A.). N-Succinimidyld 3-trimethylstannyl-benzoate ($\text{m}$-MeATE) was purchased from Toronto Research Chemicals (North York, Canada). Other reagents were reagent grade. All chemicals obtained commercially were used without further purification. HPLC analyses were performed on Shimadzu Prominence series coupled to an LC/MS-8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) or Gabi spectrometer (Shimadzu Corporation). LabSolutions Software (Shimadzu Corporation) was applied for controlling the HPLC system and processing data. The WIZARD$^{\text{\textregistered}}$ (PerkinElmer, Inc.) was used.

**Synthesis of BBN Derivatives and Nonradioactive IB-BBN** BBN derivatives shown in Table 1 were manually constructed using Fmoc-based solid-phase methodology using Fmoc-Met-Rink-Amide MBHA Resin and amino acid derivatives protected by Fmoc groups. The peptide chain was constructed in cycles of 1) 10min of deprotection with 20% piperidine in dimethylformamide (DMF), 2) 15min of coupling with 3 equivalents of Fmoc-derivatized amino acid, 1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HBTU), 1-hydroxybenzotriazole hydrate (HOBT), 6 equivalents of N,N-disopropylethylamine (DIEA) and N-methylpyrrolidone (NMP) in DMF. The coupling reaction was then repeated after Kaiser test was positive for the resin after construction of the peptide chain on the resin, the Fmoc protecting group was removed using 20% piperidine in DMF and dry the resin. To cleave peptides from the resin and deprotect, 25 µL of trisopropylsilane, 62.5 µL of water, 62.5 µL of ethanol diethyl ether was added to the filtrate to precipitate crude peptides. The crude peptides were purified by reversed-phase (RP)-HPLC performed with a Cosmosil 5C$_{18}$-AR-II (10×150 mm) at a flow rate of 4 mL/min with 20–30% acetonitrile gradient mobile phase of 0.1% TFA in water for 10min [in the case of 1], with an isocratic mobile phase of 25% acetonitrile in water containing 0.1% TFA [in the case of 2, 4, 5, and 6], or with an isocratic mobile phase of 27% acetonitrile in water containing 0.1% TFA [in the case of 3], respectively. These BBN derivatives were determined by MS and collected. The solvent was removed by lyophilization to provide BBN derivatives as powder.

Nonradioactive IB-BBNs was obtained by coupling with BBN derivatives and 1.75 equivalents of nonradioactive N-succinimidyl 3-iodobenzoate (SIB) in 75% acetonitrile in water containing 0.5% triethylamine as shown in Fig. 1. SIB obtained by previously reported procedures.$^{16}$ The mixture was shaken for 100min and purified by RP-HPLC on Cosmosil 5C$_{18}$-AR-II (10×150 mm) at a flow rate of 4 mL/min with an isocratic mobile phase of 35% acetonitrile in water containing 0.1% TFA [in the case of IB-6], with an isocratic mobile phase of 34% acetonitrile in water containing 0.1% TFA [in the case of IB-3], or with an isocratic mobile phase of 40% acetonitrile in water containing 0.1%

### Table 1. The Sequence and Analytical Data of BBN Derivatives

| Additional sequence | BBN sequence | [M + H]$^+$ | Calc. | Found$^a$ | Yield (%) |
|---------------------|--------------|-------------|-------|-----------|-----------|
|                     |              |             |       |           |           |
|Bombesin             | Pyr Q R L G N Q W A V G H L M-NH$_2$ | — | — | — | — |
|1                    | Pyr Q K L G N Q W A V G H L M-NH$_2$ | — | — | 796.4$^{a}$ | 16.2 |
|2                    | — | Q W A V G H L M-NH$_2$ | 940.5 | 940.5 | 52.7 |
|3                    | (CH$_2$-CH$_2$-O)$_2$ | Q W A V G H L M-NH$_2$ | 1085.5 | 1085.6 | 74.5 |
|4                    | βA βA βA βA | Q W A V G H L M-NH$_2$ | 1153.6 | 1153.6 | 78.7 |
|5                    | βA βA βA S | Q W A V G H L M-NH$_2$ | 1169.6 | 1169.6 | 12.9 |
|6                    | βA βA βA E | Q W A V G H L M-NH$_2$ | 1211.6 | 1211.6 | 26.1 |

$^a$ Pyr: Pyroglutamic acid, βA: β-Alanine, M-NH$_2$: amidated methionine  

$^b$ Mass values were obtained by Electrospray ionization (ESI)-MS.  

$^c$ This calculated value was based on [M + 2H]$^{2+}$.
TFA [in the case of IB-2], respectively. These IB-BBNs were determined by MS and collected. The solvent was removed by evaporator to provide IB-BBNs as clear oil.

Cell Culture PC-3 (JCRB9110) that has high GRPR expression was obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). PC-3 cells were grown in Ham’s F-12K (Kaighn’s) Medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) with 7% fetal bovine serum (FBS), 1% non-essential amino acids, penicillin (100 units/mL), and streptomycin (100 µg/mL). The cells were cultured at 37°C in humidified air containing 5% CO₂.

In Vitro Competitive Cell Binding Assay: IC₅₀ Determination The binding affinities of native BBN and IB-BBNs for GRPRs were determined by a competitive cell binding assay using [¹¹¹At]Tyr⁴-bombesin as a GRPR-specific radioligand. Experiments were performed as reported previously with slight modification. The IC₅₀ value was calculated by nonlinear regression analysis using GraphPad Prism 7 software (Graph-Pad Software Inc., San Diego, CA, U.S.A.). All experiments were performed triplicate samples. The IC₅₀ values of native BBN, IB-2, IB-3, and IB-4 were averaged from three independent experiments. The IC₅₀ values of other IB-BBNs were resulted from one experiment because the values were worse than that of native BBN.

Preparation of [¹¹¹At]AB-3 The radiosynthesis chart is shown in Fig. 1. N-Succinimidyl 3-[¹¹¹At]astatobenzoate ([¹¹¹At]SAB) was prepared by the following steps. Chloroform of [¹¹¹At]eluate was gently evaporated with nitrogen gas in a vial. m-MeATE (0.1 µmol), 11 µL of N-chlorosuccinimide (1 mg/mL) in methanol (MeOH), and 41 µL of 1% acetic acid in MeOH were added to a reactive vial containing [¹¹¹At]. After leaving to stand at room temperature for 15 min, [¹¹¹At]SAB was purified using RP-HPLC on Cosmosil 5C₁₈-AR-II (4.6 × 150 mm) at a flow rate of 1 mL/min with the gradient mobile phase 40% acetonitrile in water with 0.1% TFA to 60% acetonitrile in water with 0.1% TFA for 20 min. Fractions containing [¹¹¹At]SAB were collected, and the solvent was sub-substituted with acetonitrile by solid-phase extraction using Sep-Pak C₁₈ Plus Light cartridge (Waters, Milford, MA, U.S.A.).

One hundred microliter of [¹¹¹At]SAB in acetonitrile was added to 50 µL of 3 (25–50 µg) in 0.1 M borate buffer (pH 8.5) and shaken at room temperature for 20 min. To stop the reaction, 25 µL of 0.2 M methionine in 0.1 M borate buffer (pH 8.5) was added to reaction mixture and shaken for 5 min. [¹¹¹At]AB-3 was purified by RP-HPLC on Cosmosil 5C₁₈-AR-II (4.6 × 150 mm) at a flow rate of 1 mL/min, with a isocratic mobile phase 35% acetonitrile in water with 0.1% TFA. In order to prevent oxidative degradation, the tubes used to collect fractions were charged with 12.5 µL of 20% sodium ascorbate aqueous solution before collecting fractions, which were collected in 0.25 mL aliquots. Ten microliters of 1% polysorbate solution was added to each fraction containing [¹¹¹At]AB-3, and acetonitrile was removed using an evaporator.

In Vitro and in Vivo Stability Studies To evaluate the stabilities of [¹¹¹At]AB-3, it was diluted 10-fold with phosphate buffered saline (PBS) and the solutions were incubated at 37°C. At 1, 3, 6, and 12 h after the incubation, the solutions were analyzed with RP-HPLC and their radiochemical purities were determined. Furthermore, the stability of [¹¹¹At]AB-3 in murine plasma at 37°C for 2, 15, 30, and 60 min was investigated according to the previously reported method. Nonradioactive IB-3 was also incubated in murine plasma at 37°C for 60 min, and the metabolites of IB-3 were determined by MS.

To determine the radiochemical purity of [¹¹¹At]AB-3 in blood, male BALB/c nu/nu mice (Japan SLC, Inc., Hamamatsu, Japan) were intravenously administered with 100 µL of [¹¹¹At]AB-3 (750 kBq) dissolved in a vehicle. After separation of plasma by centrifugation at 10000 × g for 3 min, an equivalent amount of ice-cold acetonitrile was added to the plasma, and the mixture was centrifuged at 10000 × g for 3 min. The supernatant was filtered through a 0.45 µm filter and analyzed by RP-HPLC to determine the radiochemical purity of each sample.

Cellular Internalization and Externalization Internalization experiments using PC-3 prostate cancer cell lines were performed as described previously with slight modification. Briefly, 2.4 × 10⁶ cells per well in 12-well plates were pre-incubated for 24 h. On the day of the experiment, after cells were rinsed twice with ice-cold Ham’s F-12K (Kaighn’s) medium supplemented by 1% FBS, the cells were supplied with 0.8 mL of fresh medium, and PBS/0.5% BSA buffer (0.1 mL, total series) or 10 µM BBN in PBS/0.5% BSA buffer (0.1 mL, non-specific series). And then [¹¹¹At]AB-3 (10.1 kBq/well) in 0.1 mL PBS/0.5% BSA buffer was added to the well. After incubation for different time intervals (5, 15, 30, 60, and 120 min), the cells were washed twice with ice-cold medium supplemented by 1% FBS and the cells were incubated twice at room temperature in acid wash buffer (50 mM glycine buffer pH 2.8 containing 0.1 M NaCl) for 5 min. The acid wash buffer was collected (membrane-bound radioligand fraction: MRF). Finally, the cells were rinsed with PBS and resolved by adding 0.5 mL × 2 of 1 M NaOH, and then the solution was collected (internalized radioligand fraction: IRF). The radioactivity of MRF and IRF were measured with a γ-counter. The internalized or membrane-bound values were expressed as the percentage of added radioactivity.

Externalization experiments were performed after 60 min of internalization, according to previous reports, with a slight modification. After incubation at 37°C for 60 min, the cells were washed with ice-cold medium, supplemented with 1% FBS, and then re-incubated with serum-free medium for 15, 30, 60, and 120 min. At each time point, the supernatants were collected (released radioactivity). The cells were washed twice with ice-cold PBS and resolved by adding 0.5 mL × 2 of 1 M NaOH, and the solution was collected (remaining radioactivity). The released and remaining radioactivity were measured with a γ-counter. The efflux values were expressed as the ratio of the released radioactivity to the sum of released and remaining radioactivity (uptake activity).

Biodistribution Experiments and Blocking Study The experimental procedures and care of animals were carried out according to the Fukushima Medical University Institute of Animal Care and Use Committee. The animals were housed with free access to food and water at 23°C with a 12-h alternating light/dark schedule. PC-3 tumor-bearing mice were established by subcutaneous injection of approximately 5 × 10⁶ PC-3 cells in PBS(−) into right shoulder of 9-weeks-old male BALB/c nu/nu mice (21–25 g, Japan SLC, Inc.). After 12 d of
inoculation with the cancer cells, mice were intravenously administered 100 µL of $^{211}$At-AB-3 (85 kBq) in vehicle with and without 100 µg of bombesin for the blocking study. The vehicle was saline containing 2% sodium ascorbate and 0.05% polysorbate. At 1 h post-injection, mice were sacrificed, and their tissues were removed, weighed, and the radioactivity were measured. The radioactivity in the tissues was expressed as the percentage of injected dose per gram tissue (%ID/g) and injected dose per tissue (%ID). Blood, plasma, muscle, and bone were assumed to represent 8, 4.3, 48, and 5% of body weight, respectively. An unpaired Student’s $t$-test was used to analyze the in vivo blocking studies. Results were considered statistically significant at $p < 0.05$.

Results and Discussion

**In Vitro Cell-Binding Assay of Nonradioactive IB-BBNs**

To our knowledge, this is the first report on the synthesis of $^{211}$At-labeled BBN derivative. The bombesin (7–14) sequence has often been used in previous studies, so we selected this peptide sequence to develop $^{211}$At-labeled BBN derivatives. Because there are no stable isotopes of astatine, we used iodine as a surrogate to identify $^{211}$At-labeled BBN because of similarity in chemical properties. We synthesized and evaluated several IB-BBNs with various linkers by coupling with SIB and BBN derivatives (Fig. 1). Competitive binding assays using $^{[125]}$I-Tyr4-bombesin were performed to determine an appropriate sequence among the IB-BBNs. The BBN derivatives and IB-BBNs’ amino acid sequences, yield, and analytical data are summarized in Tables 1, 2.

First, we synthesized IB-BBNs with a negative charge (Glu) or a hydroxyl group (Ser) because Garayoa et al. reported that BBN derivatives with a single negative charge lead to significantly higher accumulation in tumor tissue than non-charged BBN derivatives. However, a competitive cell-binding assay showed that IB-BBNs including Ser or Glu, have lower affinity than native BBN (Table 2). In contrast, IB-2, IB-3, and IB-4 had comparable or higher affinity than BBN (Table 2). In preliminary assay, IB-2, IB-3, and IB-4 showed the IC$50$ values, 0.64, 0.22, and 0.34 nM, respectively. Therefore, we decided to evaluate $^{[211]}$At-AB-3 at first, which has a structure like IB-3.

**Preparation of $^{[211]}$At-AB-3** The $^{211}$At-labeled BBN derivatives were synthesized in two steps: $^{[211]}$At-SAB preparation, followed by coupling with 3 (Fig. 1). This process avoided oxidation of a methionine residue. BBN sulfoxide exhibited low receptor binding and biological activity. Tyr$^4$-BBN sulfoxide, the major product when preparing Tyr$^4$-BBN using iodogen, requires sulfoxide reduction and additional HPLC purification. BBN derivative–m-MeATE pre-conjugation means that methionine residues can be oxidized by exposure to an oxidant for labeling, leading to decreased radiochemical yields and/or the need for a reduction process.

| Compound   | [M + H]$^+$ Calc | [M + H]$^+$ Found$^b$ | Yield (%) | IC$50$ (nM)$^d$ |
|------------|------------------|----------------------|----------|----------------|
| Bombesin   | —                | —                    | —        | 0.53 ± 0.01    |
| IB-1       | 911.4$^a$        | 911.7                | 49.5     | 0.85$^d$       |
| IB-2       | 1170.4           | 1170.5               | 62.3     | 0.34 ± 0.29    |
| IB-3       | 1315.5           | 1315.6               | 75.0     | 0.52 ± 0.29    |
| IB-4       | 1383.5           | 1383.7               | 52.6     | 0.33 ± 0.01    |
| IB-5       | 700.3$^b$        | 700.5                | 21.0     | 3.91$^d$       |
| IB-6       | 1441.5           | 1141.6               | 64.8     | 1.67$^d$       |

$^a$ Mass values were obtained by ESI-MS. $^b$ This calculated value was based on [M + 2H]$^2+$. $^c$ Affinities for GRPR were determined with $^{[125]}$I-Tyr$^4$-bombesin in PC-3 prostatic cancer cell line. $^d$ Result of one experiment.
The radiochemical yields following HPLC purification of $^{[211}\text{At}]\text{SAB}$ and $^{[211}\text{At}]\text{AB-3}$ were 79.5±4.4% ($n=3$) and 28.2±2.4% ($n=3$), respectively. The radiochemical purity was >95%, except for preparation for the in vivo stability test (90.2%). Figure 2 shows chromatograms from HPLC following purification. The retention time ($t_R$) of $^{[211}\text{At}]\text{AB-3}$ was 7.9 min, close to that of nonradioactive IB-3 (7.6 min). The delay time between the UV and gamma detectors was approx. 0.5 min. Previous studies have verified the structures of $^{211}$At-labeled compounds by comparing their retention times to corresponding nonradioactive iodine-introduced compounds. 25,26) The similarity of retention times in this study is consistent with previous studies.

**In Vitro and in Vivo Stability Studies** $^{[211}\text{At}]\text{AB-3}$ was highly stable in PBS, even after 12 h (92.1±0.2%), while it gradually degraded within 1 h in murine plasma as shown in Fig. 3(c). Figures 3(a) and 3(b) show the chromatograms of $^{[211}\text{At}]\text{AB-3}$ and nonradioactive IB-3 after 1 h incubation in murine plasma. We identified one degradation product in murine plasma as a truncated peptide, indicating that a peptidase cleaves the amide bond between Trp$^8$ and Ala$^9$. Results showed that the native BBN sequence is unstable in plasma, demonstrating the need for sequence modification to improve its in vivo stability. In cell-binding assay, IB-2 and IB-4 also have GRPR affinity comparable to BBN. However, they have the same BBN sequence as IB-3, so it is easy to predict their instability in murine plasma as $^{[211}\text{At}]\text{AB-3}$. Therefore, their corresponding peptides, $^{[211}\text{At}]\text{AB-2}$ and $^{[211}\text{At}]\text{AB-4}$, were not evaluated further.

In contrast, most of the $^{[211}\text{At}]\text{AB-3}$ existed in an intact form in the blood 2 min after administration into mice (82.9±4.7%), indicating that $^{[211}\text{At}]\text{AB-3}$ can be delivered to the tumor immediately after injection. Unfortunately, we could not determine longer-term stability; because of low radioactivity, we could not analyze the blood sample 5 min after administration.

**Cellular Internalization and Externalization** $^{[211}\text{At}]\text{AB-3}$'s internalization and externalization properties, determined...
in PC-3 cells, are shown in Fig. 4. Results showed that approx. 25% of radioactivity bound to PC-3 cells within 60 min, while approximately 80% of the total bound radioligand was internalized. BBN (1 μM) significantly inhibited radioligand–PC-3 cell binding. This pattern was similar to those reported previously for other BBN derivatives.22,27

As shown in Fig. 4(b), radioactivity was released over time. Radiohalogen-labeled compounds are generally not retained intracellularly after internalization,28 but they may be unsuitable for TAT. Therefore, to improve tumor retention, we might need to consider different 211At-labeling methods, such as using N-succinimidyl 3-[211At]astato-4-guanidinomethylbenzoate ([211At]SAGMB).29,30 This conjugate decomposes into radiometabolites with a positive charge in lysosomes. These radiometabolites have been used successfully for antibody applications. Instead of [211At] SAB, using such reagents for 211At-labeled BBN derivatives might improve radiohalogen retention in tumors.

Unfortunately, the peptide was degraded in mouse plasma because of the native BBN sequence, and the nature of the halogen caused 211At to escape from the cells after internalization. Because of the native BBN sequence, and the nature of the radioactive halogen retention in tumors.

Table 3 shows the biodistribution of [211At] AB-3 in PC-3 tumor-bearing BALB/c nude mice. Despite peptide degradation concerns, [211At] AB-3 accumulated in tumors 1 h after administration (4.05 ± 0.73%ID/g). In the blocking group, with 100 μg of native BBN, radioactivity accumulation in the PC-3 tumor and pancreas, where GRPR expression is high, decreased significantly compared to the control group. This result indicated that [211At] AB-3 have GRPR-specific uptake in the tumor and pancreas. Additionally, the thyroid uptake was also decreased significantly in the blocking group. We suppose that the blocking agent decreased the deastatination of [211At]AB-3 indirectly and then the radioactivity in thyroid and other tissues in which free 211At accumulates was reduced. BBN derivatives are presumably degraded in the lysosome after binding to the receptor and cellular internalization.31,32 In the case of iodine-labeled compounds with SIB, it is known that radioactive metabolites, which are generated after cell internalization, return to the blood circulation without deiodination.32 However, astatine-labeled compounds seem to be different. It was estimated that oxidative deastatination occurred about 6 × 10⁶ faster than the oxidative deiodination rate in the lysosomes at 37°C by Teze et al.23 Therefore, the astatine-labeled BBN derivatives must be deastatination after cell internalization and then flowed into the blood in this study. If the uptake of [211At]AB-3 into GRPR positive cells is inhibited by the blocking agents, the subsequent deastatination in the cells could be restrained. In contrast, we observed high radioactivity accumulation in the lungs in the blocking group, which has also been reported, but not elucidated, by previous studies.34,35

This study had some limitations. First, [211At]AB-3 accumulation in tumors was relatively high, but other tissues showed higher uptake than tumors. The likely reason is that dehalogenation results in free 211At, which is also supported by previous studies:

- Free 211At accumulates in thyroid, stomach, lungs, and spleen.36–38 Previous study reported that free 211At showed lower accumulation in the thyroid than free iodine and the accumulation of free 211At in thyroid 1h postinjection was approximately 2%ID.36,38 The thyroid accumulation of [211At]AB-3 (0.65 ± 0.09%ID) was not low and must be caused by deastatination. Moreover, [211At]AB-3 also

| Tissue          | %ID/g | %ID    | %ID/g  | %ID    |
|-----------------|-------|--------|--------|--------|
| Blood           | 1.78 ± 0.24 | 2.46 ± 0.34 | 2.01 ± 0.16 | 2.78 ± 0.19 |
| Plasma          | 1.73 ± 0.22 | 1.27 ± 0.18 | 1.80 ± 0.12 | 1.32 ± 0.08 |
| Muscle          | 0.56 ± 0.10 | 5.14 ± 0.90 | 0.65 ± 0.04 | 6.02 ± 0.61 |
| Heart           | 2.19 ± 0.40 | 0.23 ± 0.03 | 2.48 ± 0.23 | 0.25 ± 0.03 |
| Lungs           | 7.28 ± 0.98 | 0.89 ± 0.14 | 138 ± 12.1* | 20.0 ± 1.44* |
| Spleen          | 7.66 ± 1.22 | 0.63 ± 0.12 | 11.6 ± 1.66* | 1.05 ± 0.21* |
| Pancreas        | 4.03 ± 0.38 | 0.64 ± 0.08 | 2.53 ± 0.45* | 0.39 ± 0.05* |
| Testicle        | 2.28 ± 0.38 | 0.19 ± 0.03 | 2.06 ± 0.26 | 0.18 ± 0.02 |
| Stomach         | 12.0 ± 1.47 | 1.77 ± 0.26 | 10.4 ± 1.60 | 1.44 ± 0.23 |
| Small-intestine | 27.0 ± 6.18 | 27.1 ± 5.54 | 22.8 ± 2.43 | 22.1 ± 2.25 |
| Large-intestine | 2.27 ± 0.23 | 0.57 ± 0.10 | 1.75 ± 0.49 | 0.49 ± 0.04 |
| Kidney          | 3.67 ± 0.77 | 1.58 ± 0.33 | 3.82 ± 0.18 | 1.61 ± 0.12 |
| Adrenal gland   | 2.52 ± 0.51 | 0.03 ± 0.01 | 6.67 ± 1.63* | 0.05 ± 0.01* |
| Liver           | 5.27 ± 0.79 | 6.12 ± 0.99 | 11.2 ± 1.56* | 11.7 ± 1.63* |
| Brown fat       | 1.55 ± 0.23 | 0.11 ± 0.02 | 2.28 ± 0.30* | 0.12 ± 0.02 |
| Salivary glands | 18.9 ± 4.20 | 2.45 ± 0.46 | 14.6 ± 1.29* | 1.87 ± 0.32* |
| Thyroid         | 25.1 ± 3.75 | 0.65 ± 0.09 | 15.47 ± 2.32* | 0.30 ± 0.06* |
| Bone            | 1.39 ± 0.22 | 3.21 ± 0.48 | 1.55 ± 0.20 | 3.59 ± 0.48 |
| Brain           | 0.27 ± 0.04 | 0.08 ± 0.01 | 0.29 ± 0.03 | 0.08 ± 0.01 |
| Tumor           | 4.05 ± 0.73 | 0.36 ± 0.12 | 2.56 ± 0.24* | 0.23 ± 0.07 |

Data are expressed as %injected dose per gram tissue (%ID/g) and %injected dose per tissue (%ID). Each value represents the mean ± S.D. for 5 animals. † Data tissues do not contain gastrointestinal contents. Significance was determined using student-t test (*p < 0.05, vs. control group).
showed high accumulation in free $^{211}$At accumulation tissue, such as stomach and salivary glands. When the astatobenzoate structure is used for radiolabeling, deactivation is relatively lower in the case of astatobenzoate-conjugated antibodies. In contrast, deactivation tends to occur with astatobenzoate-conjugated low-molecular-weight compounds, including BBN derivatives. For example, the distribution of astatobenzoic acid and astatobenzoate-conjugated biotin derivatives is similar to that of free $^{211}$At. Therefore, we assumed that the higher radioactivity observed after $[^{211}\text{At}]\text{AB-3}$ injection was due to free $^{211}$At resulting from deactivation. Therefore, preventing deactivation is essential for the therapeutic use of $^{211}$At-labeled BBN derivatives since deactivation reduces the target selectivity of $^{211}$At-labeled compounds.

Second, the ease of peptide degradation, shown in stability studies, also inhibits ideal tumor accumulation. All BBN derivatives reported in this study contain native peptide sequences and have common problems; therefore, conversion to a peptidase-resistant sequence is essential. Third, radioactivity efflux from cells after internalization also reduces radioactivity accumulation in tumors, decreasing therapeutic efficacy. Unfortunately, the drug design of $^{211}$At-labeled BBN derivative used in this study was inappropriate. Developing $^{211}$At-labeled BBN derivatives will need the BBN sequence to be modified, in addition to preventing deactivation and radioactivity efflux from cells.

Finally, the antitumor effects of $[^{211}\text{At}]\text{AB-3}$ were not investigated in this study due to the possibility of serious side effects. As the results of biodistribution studies, $[^{211}\text{At}]\text{AB-3}$ was specifically accumulated in tumor, suggesting the possibility of exhibiting antitumor effects. On the other hand, higher accumulations than tumor were observed in many other tissues, especially in the small intestine. Studies about toxicity of $^{211}$At have shown that the major cause of death after administration of free $^{211}$At is gastrointestinal epithelial sloughing and focal transmural necrosis. Meanwhile, if the accumulation of $[^{211}\text{At}]\text{AB-3}$ in the small intestine was mainly located in the contents, the toxicity to the wall for the alpha-emitter could be minimal, as shown in the cases of $[^{223}\text{Ra}]\text{RaCl}_{2}$. However, it was not clear because the radioactivity in the small intestine between the wall and the contents was not discriminated in this study.

**Conclusion**

We developed novel $^{211}$At-labeled peptide with an affinity like BBN for GRPRs. In tumor-bearing mice, GRPR-specific $[^{211}\text{At}]\text{AB-3}$ accumulation in tumors was not enough because of the low stability of murine plasma and radioactivity efflux from cells, as shown in *in vitro* experiments. In addition, there was nonspecific radioactivity accumulation, which was likely derived from deactivation. Taken together, $^{211}$At-labeled BBN derivative development requires modifying the BBN sequence and $^{211}$At-labeling methodology in order to increase tumor uptake.

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**Conflict of Interest**

The authors declare no conflict of interest.

**References**

1. Torre L. A., Bray F., Siegel R. L., Ferlay J., Lortet-Tieulent J., Jemal A., *CA Cancer J. Clin.*, 65, 87–108 (2015).
2. Kelly S. P., Anderson W. F., Rosenberg P. S., Cook M. B., *Eur. J. Nucl. Med. Focus*, 4, 121–127 (2018).
3. Attard G., Parker C., Edes R. A., Schroder F., Tomlins S. A., Tanlock I., Drake C. G., de Bono J. S., *Lancet*, 387, 30–32 (2016).
4. Parker C., Nilsson S., Heinrich D., et al., *N. Engl. J. Med.*, 369, 213–223 (2013).
5. Kratochwil C., Bruchertseifer E., Giesel F. L., Weis M., Verborg F. A., Mortaghy F., Kopka K., Apostolidis C., Haberkorn U., Morgenstern A., *J. Nucl. Med.*, 57, 1941–1944 (2016).
6. Zalutsky M. R., Vaidyanathan G., *Curr. Pharm. Des.*, 6, 1433–1455 (2000).
7. Vaidyanathan G., Zalutsky M. R., *Curr. Radiopharm.*, 1, 177–196 (2008).
8. Guérard F., Gestin J. F., Brechbéli M. W., *Cancer Biother. Radio - pharm.*, 28, 1–20 (2015).
9. Parry J. J., Andrews R., Rogers B. E., *Breast Cancer Res. Treat.*, 101, 175–183 (2007).
10. Markwalder R., Reubi J. C., *Cancer Res.*, 59, 1152–1159 (1999).
11. Sancho V., Di Florio A., Moody T. W., Jensen R. T., *Curr. Drug Deliv.*, 8, 79–134 (2011).
12. Baratto L., Jadvar H., Iagaru A., *Mol. Imaging Biol.*, 20, 501–509 (2018).
13. Ferreira C. A., Fuscaldi L. L., Townsend D. M., Rubello D., Barros A. L. B., *Biomed. Pharmacother.*, 87, 58–72 (2017).
14. Lantry L. E., Cappelletti E., Maddalena M. E., Fox J. S., Feng W., Chen J., Thomas R., Eaton S. M., Bogdan N. J., Arunachalam T., Reubi J. C., Raju N., Metcalfe E. C., Lattuada L., Linder K. E., Swenson R. E., Tweedle M. F., Nunn A. D., *J. Nucl. Med.*, 47, 1144–1152 (2006).
15. Washiyama K., Oda T., Sasaki S., Aoki M., Guerra Gomez F. L., Longobotti M., Nishijima K.-A., Takahashi K., *J. Med. Imaging Radiat. Res.*, 50, S42 (2019).
16. Ogawa K., Takeda T., Yokokawa M., Yu J., Makino A., Kiyono Y., Shiba K., Kinuya S., Odani A., *Chem. Pharm. Bull.*, 66, 651–659 (2018).
17. Maddalena M. E., Fox J., Chen J., Feng W., Cagnolini A., Linder K. E., Tweedle M. F., Nunn A. D., Lantry L. E., *J. Nucl. Med.*, 50, 2017–2024 (2009).
18. Chen X., Park R., Hou Y., Tohme M., Shahinian A. H., Bading J. R., Conti P. S., *J. Nucl. Med.*, 45, 1390–1397 (2004).
19. Aoki M., Odani A., Ogawa K., *Ann. Nucl. Med.*, 33, 317–325 (2019).
20. Maina T., Nock B., Nikolopoulos A., Sotiriou P., Loudos G., Main - tas D., Cordopatis P., Chiotellis E., *Eur. J. Nucl. Med. Mol. Imaging*, 29, 742–753 (2002).
21. Garcia Garavoa E., Schweinsberg C., Maes V., Brans L., Blauenstein P., Tourwe D. A., Schibli R., Schubiger P. A., *Bioconj. Chem.*, 19, 2409–2416 (2008).
22. Yan Y., Chen K., Yang M., Sun X., Liu S., Chen X., *Amino Acids*, 41, 439–447 (2011).
23. Ogawa K., Aoki M., Kadono S., Odani A., *PLOS ONE*, 13, e0195067 (2018).
24. Vigna S. R., Giraud A. S., Reeve J. R. Jr., Walsh J. H., *Peptides*, 9, 923–926 (1988).
25. Ogawa K., Takeda T., Mishiro K., Toyoshiba A., Shiba K., Yoshi - mura T., Shinhara A., Kinuya S., Odani A., *ACS Omega*, 4, 4584–4591 (2019).
26. Ogawa K., Mizuno Y., Washiyama K., Shiba K., Takahashi N., Koizaka I., Watanabe S., Shinhara A., Odani A., *Nucl. Med. Biol.*, 46, 119–125 (2019).
27) La Bella R., Garcia-Garayoa E., Bahler M., Blauenstein P., Schibli R., Conrath P., Tourwe D., Schubiger P. A., *Bioconjug. Chem.*, 13, 599–604 (2002).

28) Cavina L., van der Born D., Klaren P. H. M., Feiters M. C., Boerman O. C., Rutjes F., *Eur. J. Org. Chem.*, 2017, 3387–3414 (2017).

29) Choi J., Vaidyanathan G., Koumarianou E., Kang C. M., Zalutsky M. R., *Nucl. Med. Biol.*, 56, 10–20 (2018).

30) Vaidyanathan G., Affleck D. J., Bigner D. D., Zalutsky M. R., *Nucl. Med. Biol.*, 30, 351–359 (2003).

31) Grady E. F., Slice L. W., Brant W. O., Walsh J. H., Payan D. G., Bunnett N. W., *J. Biol. Chem.*, 270, 4603–4611 (1995).

32) Chitneni S. K., Koumarianou E., Vaidyanathan G., Zalutsky M. R., *Molecules*, 24, 3907 (2019).

33) Teze D., Sergentu D. C., Kalichuk V., Barbet J., Deniaud D., Galland N., Maurice R., Montavon G., *Sci. Rep.*, 7, 2579 (2017).

34) Garcia-Garayoa E., Ruegg D., Blauenstein P., Zwimpfer M., Khan I. U., Maes V., Blance A., Beck-Sickinger A. G., Tourwe D. A., Schubiger P. A., *Nucl. Med. Biol.*, 34, 17–28 (2007).

35) Zhang X., Cai W., Cao F., Schreibmann E., Wu Y., Wu J. C., Xing L., Chen X., *J. Nucl. Med.*, 47, 492–501 (2006).

36) Garg P. K., Harrison C. L., Zalutsky M. R., *Cancer Res.*, 50, 3514–3520 (1990).

37) Lundh C., Lindencrona U., Schmitt A., Nilsson M., Forssell-Aronsson E., *Cancer Biother. Radiopharm.*, 21, 591–600 (2006).

38) Larsen R. H., Slade S., Zalutsky M. R., *Nucl. Med. Biol.*, 25, 351–357 (1998).

39) Wilbur D. S., Hamlin D. K., Chyan M. K., Kegley B. B., Quinn J., Vessella R. L., *Bioconjug. Chem.*, 15, 601–616 (2004).

40) McLendon R. E., Archer G. E., Garg P. K., Bigner D. D., Zalutsky M. R., *Int. J. Radiat. Oncol. Biol. Phys.*, 35, 69–80 (1996).