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Development of a ^{68}Ga-Labeled Bombesin Analog for Gastrin-Releasing Peptide Receptor-Expressing Prostate Tumor Imaging

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Abstract

Our previous study demonstrated the therapeutic efficacy of ^{177}Lu-DOTA-gluBBN for the treatment of GRPR-expressing prostate tumors. As a matched pair for ^{177}Lu, the development of labeling and molecular imaging technology using ^{68}Ga is needed to introduce the "theranostic" approach. Therefore, the present study described the ^{68}Ga-labeled bombesin analog, ^{68}Ga-DOTA-gluBBN for the imaging of GRPR-expressing prostate tumors. Methods: ^{68}Ga was concentrated and labeled with DOTA-gluBBN using the NaCl method, and the labeling yield was evaluated by iTLC-SG. Human prostate PC-3 tumor cells were used to induce a subcutaneously xenografted-tumor model and a peritoneal metastasized tumor model. PET-CT imaging studies were performed in both animal models. Results: Eluted ^{68}Ga solution containing 5~10% of impurities was purified (>99%) and labeled with DOTA-gluBBN with a high incorporation yield (>98%). The total preparation time from the elution and quality control steps required below twenty minutes. ^{68}Ga-DOTA-gluBBN was clearly visualized in xenografted PC-3 tumors at 1 hr p.i., and the tumor-to-muscle ratio was 1.7-fold higher than that observed using ^{18}F-FDG. In a peritoneal metastasized tumor model, PC-3 tumors were widespread in peritoneum. The metastases could not be specifically visualized by PET imaging, but the tumor uptake was confirmed by ex-vivo autoradiography.

Conclusion: Favorable preclinical results demonstrating specific and effective imaging of GRPR-expressing prostate tumor recommend the further evaluation of ^{68}Ga-DOTA-gluBBN in a clinical study to introduce a theranostic approach for prostate carcinoma patients.

Keywords: PET; Gallium-68 (^{68}Ga); Gastrin releasing peptide receptor; Bombesin; Prostate cancer

Abbreviations: DDW: Deionized Distilled Water; S.C: Subcutaneously; I.P: Intraperitoneal; p.i: Post Injection; FDG: Fluorodeoxyglucose

Introduction

Molecular imaging has led to substantial advances in the diagnosis of cancer, which makes it possible to directly and non-invasively monitor the pathological processes of cancer in real-time. Because the development of a suitable molecular imaging probe is the most important for the molecular imaging, numerous molecules have been discovered in recent years, making targeted molecular imaging possible [1].

Prostate cancer is the most common non-cutaneous malignancy among American men, and it is the second leading cause of cancer death in men in the United States [2]. Because prostate carcinoma cells do not use glucose notably more than normal cells, the most widely used ^{18}F-FDG does not play a prominent role in the diagnosis and staging of prostate cancer; an overall sensitivity of only 57% on a per-patient basis on staging or restaging for 244 prostate cancer patients was reported [3,4]. Although ^{18}F-choline is considered to be the standard diagnostic imaging tool for the clinical assessment of recurrent prostate cancer in Europe, it is not specific for cancer cells. Thus, advances in the imaging of prostate cancer cells may facilitate earlier and more accurate diagnosis and treatment [5].

Bombesin is a neuropeptide ligand that binds to Gastrin-Releasing Peptide Receptors (GRPRs) with high affinity. GRPRs have been shown to be overexpressed in many human tumors, including prostate cancer, breast cancer, small cell lung cancer, ovarian cancers, endometrial cancers, and gastrointestinal stromal tumors [6]. In particular, the overexpression of the GRPR, also called bombesin receptor subtype 2, in prostate cancer cells provides a potential target for the specific diagnosis and therapy of prostate cancer [5].

^{68}Ga is produced from a cost-effective generator, and its half-life is 67.6 min, permitting the production and application of resultant agents. Because ^{68}Ga provides sufficient levels of radioactivity for high-quality images, the examination time and radiation dose to the patient can be minimized. In addition, most therapeutic radionuclides such as ^{177}Lu, are also metals and might allow for theranostic development [7]. According to these advantages of ^{68}Ga, several clinical studies regarding GRPR imaging have been conducted using ^{68}Ga-BZH3, ^{68}Ga-DOTA-BOM and ^{68}Ga-BAY86-7548 [8-10].

Our previous study demonstrated the targeting and therapeutic efficacy of ^{177}Lu-DOTA-gluBBN for the treatment of GRPR-expressing prostate tumors [11]. To introduce the "theranostic" approach, the development of molecular imaging technology using DOTA-gluBBN is needed, and ^{68}Ga, which is known as the "theranostic twins" of ^{177}Lu, is appropriate for the imaging [12].

Therefore, in this report, we describe the ^{68}Ga radiolabeling of the

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bombesin analog, DOTA-gluBBN, and the preclinical imaging efficacy of ^{68}Ga-DOTA-gluBBN as an imaging modality for GRPR-expressing prostate tumors.

Materials and Methods

Materials

All chemicals were of analytical grade, purchased from Anaspec, and used without further purification (Fremont, CA, US). The glycated bombesin analog, DOTA-gluBBN was synthesized using a previously described method and the molecular structure was shown in Figure 1 [13]. For all experiments, ^{68}Ga produced from a ^{68}Ge/^{68}Ga generator (ITG, Germany) was used. The radioactivity was determined using a Wallac 1470 automated gamma counter (PerkinElmer Life Science, Massachusetts, USA) and an ionizing chamber (Atomlab 200, Bio-dex, New York, USA). The incorporation yield and radiochemical purity (RCP) were determined using a Tracemaster 20 automated TLC-linear analyzer (Berthold, Germany).

Preparation of ^{68}Ga-labeled peptide: ^{68}Ga-DOTA-gluBBN

The ^{68}Ge/^{68}Ga generator was eluted with a total of 4 ml of 0.05 M HCl according to the manufacturer's instructions and concentrated using a NaCl-based ^{68}Ga eluate concentration method as described by Mueller et al. [14]. Briefly, the ^{68}Ga generator eluate was collected by a SCX cation exchange cartridge and eluted from the cartridge with 0.5 ml of a 5 M NaCl solution containing a 12.5 µl of 5.5 M HCl. This eluate was slowly added to 350 µl of ammonium acetate buffer (pH4.5; final 0.25 M) containing ascorbic acid (final 0.1 M) and DOTA-gluBBN in 150 µl of DDW. The mixture was heated by heating block at 100°C for 7 min for the radiolabeling. The iTLC-SG (solvent: 0.5 M sodium citrate buffer, pH4.5) was used to determine the radiochemical purity of the ^{68}Ga-DOTA-gluBBN preparation. To evaluate its radiochemical stability, ^{68}Ga-DOTA-gluBBN with or without adding 9 ml of saline at room temperature was taken at selected times, and analyzed by iTLC-SG.

Preparation of animal models

Cell culture: PC-3 human 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 penicillin, and 100 µg/ml streptomycin (Sigma, Milan, Italy) in an atmosphere of 5% CO2 in air at 37°C for up to approximately 90 % confluence.

Animal models: The protocols used in the animal studies were approved by the Institutional Animal Care and Use Committee at KAERI, and the animals were cared for in accordance with the Guidelines for Animal Experiments.

For the induction of tumor xenografts, PC-3 cells were subcutaneously injected in the right upper flank of male balb/c nude mice at a concentration of 1×10^7 cells/mouse with 100 µl of a 1:1 mixture of culture medium and Matrigel. For the peritoneal metastasized model, 1×10^7 PC-3 cells were intrape ritoneally-injected into male balb/c nude mice with 500 µl of saline.

Positron Emission Tomography-Computed Tomography (PET-CT) imaging

NanoPET/CT scans (Bioscan, USA) were performed using a rodent scanner. ^{68}Ga-DOTA-gluBBN (3.7 MBq/100 µl) was injected into PC-3 tumor mice under isoflurane anesthesia through the tail vein. A ten-minute static scan was acquired at 0, 10, 20, 30, 40, 50 min p.i. for serial images. To compare ^{68}Ga-DOTA-gluBBN with ^{18}F-FDG, 3.7 MBq of each radio-compound was administered into PC-3 tumor mice and a thirty-minute static scan was acquired at 1 hr p.i. In the peritoneal metastasized model, ^{68}Ga-DOTA-gluBBN (3.7 MBq/100 µl) was also injected into the tail vein, and a thirty-minute static scan was acquired at 1 hr p.i. Image analysis was performed using the Bioscan in Vivo Scope software and 0.9.0 AMIDE software.

Ex vivo autoradiography

A total of 3.7 MBq of ^{68}Ga-DOTA-gluBBN in 100 µl of saline (n=3) was injected into both PC-3 tumor xenografted mice and the peritoneal metastasized model. For a blocking study, 50 µg of unlabeled peptide was co-injected. At 1 hr p.i., the mice were sacrificed, and tumors in

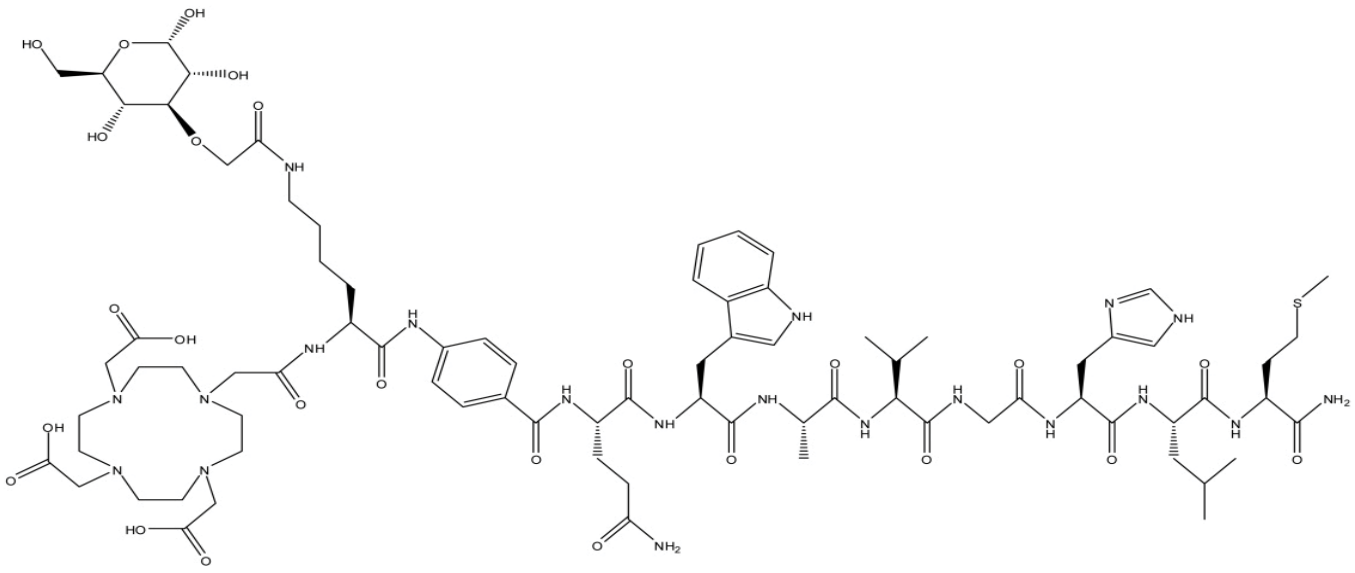

Figure 1: Molecular structure of DOTA-gluBBN [13].

both the right upper flank and peritoneal cavity were collected. The collected tumors were sliced and exposed to film at room temperature for overnight, and analyzed by Cyclone (PerkinElmer, USA).

Statistical analysis

The results are expressed as the mean ± Standard Deviation (S.D.). A nonlinear regression analysis was performed on the *in vitro* data, and statistical analysis of in vivo studies utilized unpaired t test and was analyzed using GraphPad Prism 5.0 software. The level of significance was set at $P<0.05$.

Results

Radiolabeling of DOTA-gluBBN with ^{68}Ga

Previous studies have demonstrated the synthesis of DOTA-Lys (glucose)-4 aminobenzoyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH2 (DOTA-gluBBN). The IC_{50} value of DOTA-gluBBN was 4.67 nM, indicating its specificity and nanomolar affinity to GRPR [13]. In this study, the DOTA-gluBBN was labeled with ^{68}Ga which was purified and concentrated using NaCl-based ^{68}Ga eluate concentration method [14].

In TLC with sodium citrate, uncomplexed ^{68}Ga can be detected at the origin (possibly colloidal ^{68}Ga) or solvent front due to the formation of ^{68}Ga-citrate [15]. As shown in Figure 2A, the ^{68}Ga solution eluted from the ^{68}Ge/^{68}Ga generator contained 5~10% of impurities, and the volume was 4 ml which was not suitable for experimental purposes. The impurities were successfully removed to below 1%, and the volume was decreased to 0.5 ml by the NaCl method (Figure 2B). The concentrated ^{68}Ga was labeled with DOTA-gluBBN by high radiochemical purity (>98%), and further purification was not required (Figure 2C). A total of 0.22 μg (1.23×10^{-10} mole) of DOTA-gluBBN was routinely used per 1 MBq of the concentrated ^{68}Ga solution to obtain a radiochemical purity of over 98%, and the total preparation time from the elution to the quality control was below twenty-minutes.

Figure 3A shows that the incorporation yield of DOTA-gluBBN with a high amount of radioactivity, 555 MBq ^{68}Ga, was over 98%. In addition, after labeling, the radiochemical purity was maintained for 4 hours, which was a considerable amount of time for imaging using the 68-min short half-life of ^{68}Ga.

PC-3 tumor imaging using ^{68}Ga-DOTA-gluBBN

Nude mice bearing subcutaneous PC-3 tumor xenografts next to the right shoulder were scanned to 60 min post injection and serial PET-CT images were acquired (Figure 4). PC-3 tumors were clearly visualized in all images. In addition, ^{68}Ga-DOTA-gluBBN was rapidly excreted from the blood pool to the urinary bladder through the kidneys, and the highest radioactivity was observed in the urinary bladder at 50 to 60 min post-injection. The pancreas which is a GRPR-positive organ was not visualized because of the location of the cross-section, but it could be seen in Figure 5B.

To compare the imaging efficacy of ^{68}Ga-DOTA-gluBBN with ^{18}F-FDG, 3.7 MBq of each radio-compound was administered and imaged at 1 hr post injection (Figure 5). The biodistribution pattern of two radio-peptide was different, but PC-3 tumors were successfully visualized using both radio-peptides. The tumor-to-muscle ratio of ^{68}Ga-DOTA-gluBBN was 1.7-fold higher than that of ^{18}F-FDG (4.79 for ^{18}F-FDG and 7.96 for ^{68}Ga-DOTA-gluBBN), and

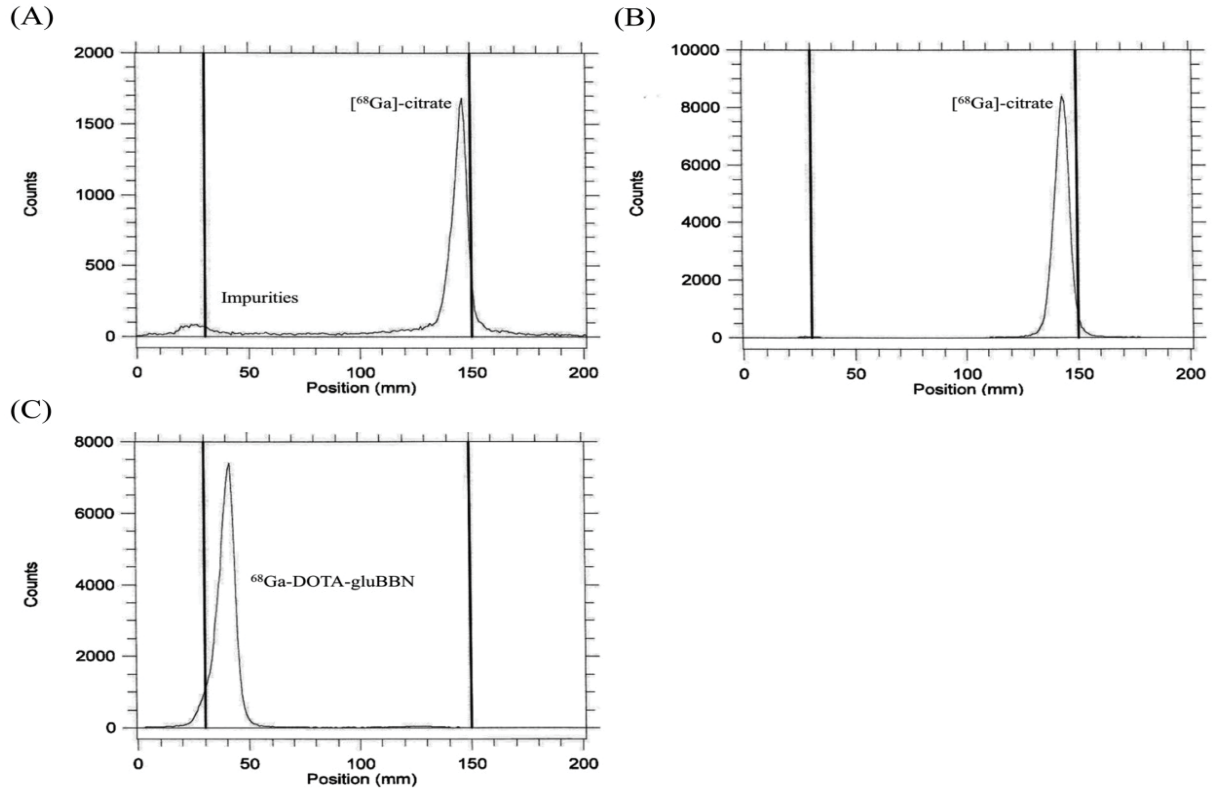

Figure 2: Typical iTLC profiles of eluted ^{68}Ga (A), concentrated ^{68}Ga solution (B), and ^{68}Ga-labeled DOTA-gluBBN. The mobile phase was 0.5 M sodium citrate buffer (pH4.5). Impurities at the origin were removed by the concentration, and ^{68}Ga-labelled DOTA-gluBBN was prepared by high radiochemical purity (>98 %).

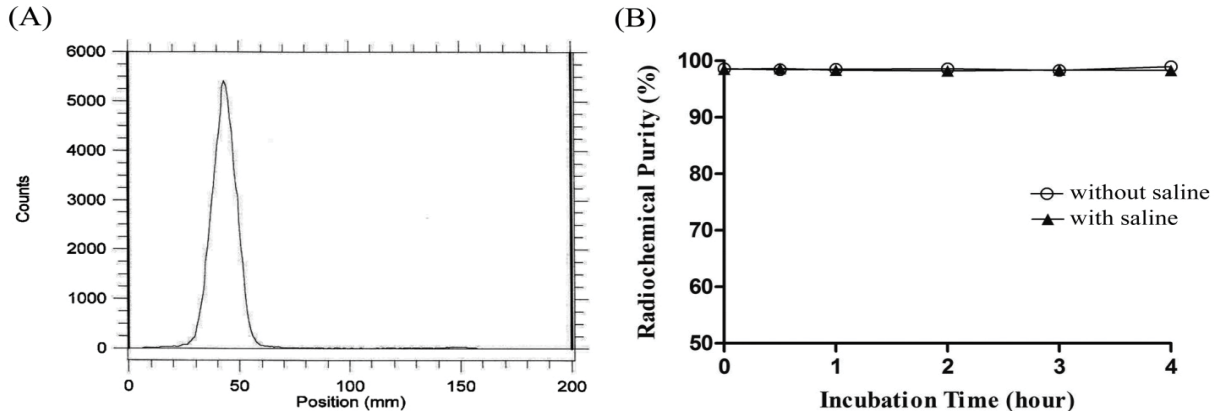

Figure 3: Preparation of 555 MBq of ^{68}Ga-DOTA-gluBBN (A) and the stability at room temperature (B). The ^{68}Ga-DOTA-gluBBN was labeled with high radioactivity over 98%, and the radiochemical purity was maintained for 4 hours.

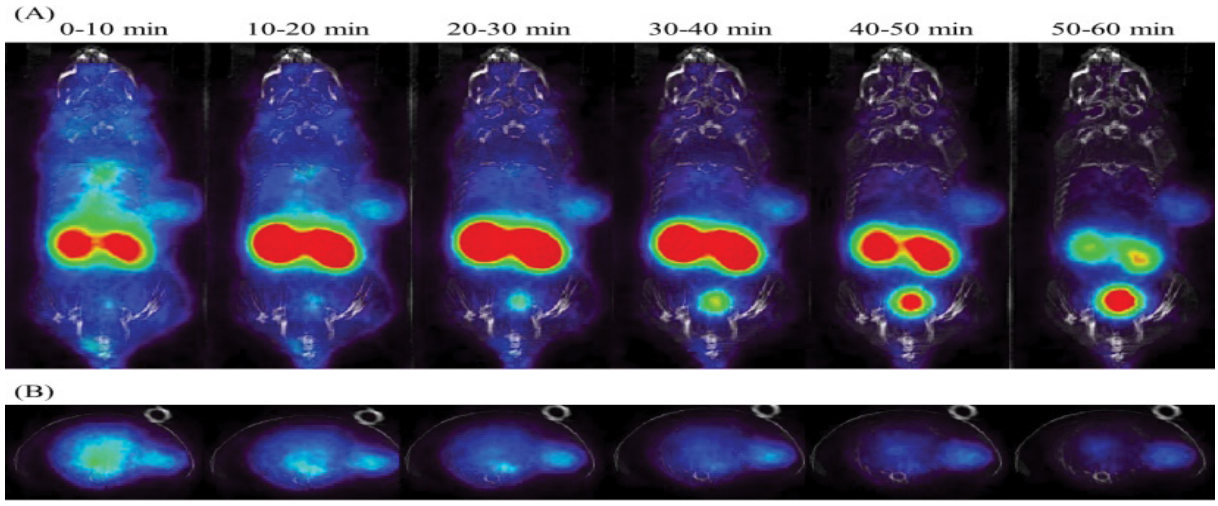

Figure 4: Series of coronal (A) and transverse (B) slices from subcutaneously PC-3 xenografted nude mice. Mice were injected with 3.7 MBq of ^{68}Ga-DOTA-gluBBN and scanned from 0-60min after injection.

^{68}Ga-DOTA-gluBBN was excreted more rapidly from the blood pool than ^{18}F-FDG.

The imaging efficacy of ^{68}Ga-DOTA-gluBBN was evaluated in the PC-3- peritoneal metastasized model. As shown in Figure 6A, intraperitoneal-injected PC-3 prostate cancer cells were diffusely metastasized into the whole peritoneal cavity. Twelve mice were injected, and all mice developed the metastasized PC-3 tumors of various sizes in the peritoneal cavity after 2 months. In Figure 6B, the radioactivity was also distributed diffusely in the whole peritoneal cavity similar to the gross observation; however, the specific metastases could not be visualized. To confirm the uptake of ^{68}Ga-DOTA-gluBBN in S.C.- and I.P.- induced PC-3 tumors, ex vivo autoradiography was performed (Supplement Figure 1). As a result, dark radioactivity was imaged in the subcutaneously xenografted tumor as well as the peritoneal metastasized PC-3 tumor. In addition, the uptake was blocked by co-administration of DOTA-gluBBN, indicating GRPR-specific uptake.

Discussion

Recent developments in cancer research have resulted in various therapeutic technologies. Because of the heterogeneity of patients and tumors, there is an increasing demand for personalized medicine. With such a background, the development of a "theranostic" approach has gained prominence. Patients are selected through a diagnostic study to determine whether a patient will benefit from a therapy. Since the combination of 131I for diagnostic imaging and therapy was established, many theranostic approaches have been performed. In particular, peptide receptor radionuclide therapy using ^{68}Ga/^{177}Lu-DOTA-TOC has been used as an effective and safe treatment option for patients suffering with advanced neuroendocrine tumors [12].

The purpose of this study was to develop ^{68}Ga-labeled DOTA-gluBBN to image GRPR-expressing prostate tumors. With our previous report on ^{177}Lu-labeled DOTA-gluBBN for the treatment of GRPR-expressing prostate tumors [11], these results encourage DOTA-gluBBN to be applied to the theranostic approach.

Several methods to concentrate eluted ^{68}Ga from a ^{68}Ge/^{68}Ga generator have been reported, and recently, Mueller et al. reported a NaCl method using a cationic exchange cartridge. This method utilizes relatively few reagents and comprises a minimal procedure with no subsequent purification steps, making it easy to label chelators with ^{68}Ga [14]. The percentage of impurities in the eluted

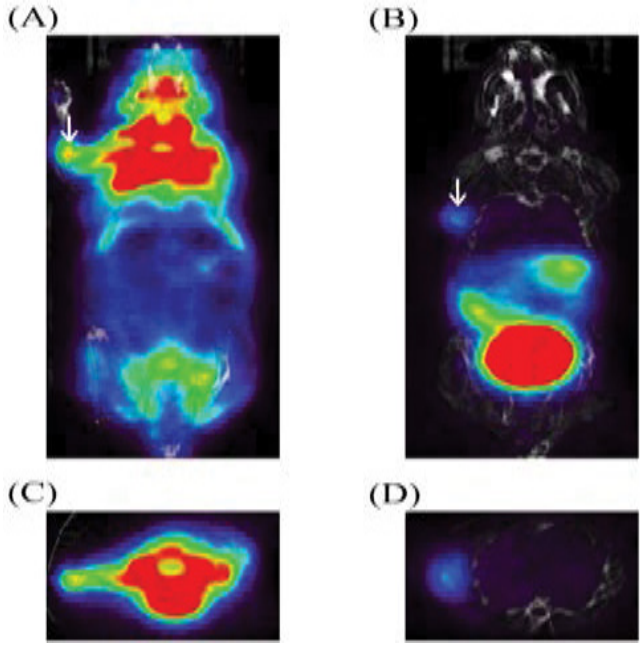

Figure 5: Coronal (A, B) and transverse (C, D) images from subcutaneously PC-3 xenografted nude mice. Images were acquired 1 hr after the injection of 3.7 MBq of 18F-FDG (A, C) and ^{68}Ga-DOTA-gluBBN (B, D), respectively. The tumor-to-muscle ratios were 4.79 for ^{18}F-FDG and 7.96 for ^{68}Ga-DOTA-gluBBN.

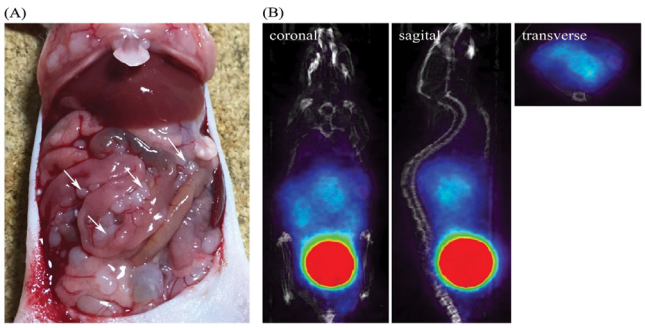

Figure 6: Gross observation of peritoneal metastasized PC-3 prostate tumors (A) and PET images of the mice after the injection of 3.7 MBq of ^{68}Ga-DOTA-gluBBN (B). Diffused metastasis was observed and imaged in the whole peritoneal cavity.

^{68}Ga solution depended on the interval of elution. The longer the interval was, the more impurities were detected, representing 5~10% of the solution. Because the both impurities and ^{68}Ga-DOTA-gluBBN remained at the origin by iTLC-SG with 0.5 M sodium citrate buffer, the elimination of impurities should be completed before radiolabeling. Purified ^{68}Ga was routinely labeled with DOTA-gluBBN (incorporation yield >98%), and the total preparation time was below twenty minutes from the elution of ^{68}Ga solution to the evaluation of radiolabeling yield. Compared with other methods such as HPLC to examine the radiolabeling yield (>10 min), the iTLC-SG method required a shorter time (<10 min) to examine the radiochemical purity.

In addition, the radio of peptide and radioisotope was optimized for labeling. 0.22 μg (1.23×10^{-10} mole) of the DOTA-gluBBN was used for labeling of 1 MBq of ^{68}Ga. Considering that the other DOTA-derivatives such as DOTA-TOC (0.33~0.54 μg per 1 MBq), the optimized labeling condition is useful to avoid the possible side effects caused by high levels of unlabeled peptide [16].

Many studies reported that bombesin derivatives are very radiosensitive in the absence of radiostabilizers. Degradation occurred both during and after radiolabeling, and in particular, the methionine residue of the peptide was found to be readily oxidized to its methionine sulfoxide form [17]. Asti et al. demonstrated that the presence of the oxidized form could lower the RCP of the preparation of ^{68}Ga-labeleled peptide around 80%. On the other hand, ascorbic acid dramatically enhanced the RCP of the radiotracers up to 98%, and presence of free $^{68}Ga^{3+}$ or ^{68}Ga-hydrolyzed products was not detected in any preparation [18]. Hence, ascorbic acid which was the most effective amino acid for radiostability was used in this study. However, the possibility of the presence of an oxidation by-product should be considered in the preparation of the radiotracer because sulfoxide form cannot be detected with TLC.

In the previous report, high amounts of ^{177}Lu-DOTA-gluBBN were rapidly internalized and accumulated in a PC-3 tumor such that the %ID/g of the tumor was 12.42 ± 2.15 at 1 hr p.i. The radio-peptide was quickly cleared from the blood, yielding tumor-to-blood ratios of 39.22 ± 17.36 at 1 hr p.i. and 330.67 ± 131.23 at 24 hr p.i. As a result, the radio-peptide significantly inhibited the PC-3 tumor growth ($P<0.05$) with no treatment-related toxicity in the pancreas and kidneys except for slight glomerulopathy [11].

In accordance with the results, ^{68}Ga-DOTA-gluBBN successfully targeted PC-3 prostate tumors in both the subcutaneous and peritoneal cavities. In the series images, ^{68}Ga-DOTA-gluBBN was already accumulated in the kidneys at 10 min p.i., and the radioactivity was excreted to the urinary bladder at 50 min p.i. The characteristics of fast blood clearance and excretion through the kidneys are advantageous as an imaging agent. Apart from the uptake of radioactivity in the peritoneal area, the PC-3 tumor in the upper body was clearly imaged with low background radioactivity. However, the uptake of radioactivity in the pancreas could hinder efforts to detect tumors in the peritoneal cavity.

Compared with ^{18}F-FDG, ^{68}Ga-DOTA-gluBBN displayed higher tumor-to-muscle ratios in PC-3 tumor xenografted mice. Because there are pros and cons between them, they could be used in a mutually complementing manner. Nevertheless, ^{68}Ga-DOTA-gluBBN has a superior advantage for the treatment of prostate cancer because it can be used for therapy directly in the form of ^{177}Lu-DOTA-gluBBN in the theranostic approach.

Peritoneal carcinomatosis of prostate cancer is a type of rare cancer, although prostate cancer is likely to metastasize to bones. In the literature, peritoneal carcinomatosis is a rare finding in metastatic prostate cancer and is reported in its final stages with multiple metastases [19]. Due to the interest in developing a therapeutic technology for rare cancers recently, we focused on the peritoneal metastases of prostate cancer. Injected PC-3 prostate cancer cells induced diffuse solid tumors in the peritoneal cavity. The precise reasons are not known, but tumors were primarily localized in the pancreas, peritoneal wall, and mesentery. The tumors could not be visualized in PET imaging, but the ex-vivo autoradiography showed the tumor uptake of ^{68}Ga-DOTA-gluBBN. It might be caused by the resolution as well as the uptake of the radiopeptide in other organs including pancreas. However, larger metastases in human might be visualized by the radio-peptide, which can contribute to the development of theranostics for the treatment of rare prostate cancer.

Based on the aforementioned in vivo results, ^{68}Ga-DOTA-gluBBN showed favorable pharmacokinetics, including rapid tumor targeting, rapid blood clearance, and fast renal excretion. These results suggest that ^{68}Ga-DOTA-gluBBN can be a promising diagnostic tool for GRPR-expressing prostate cancer.

If DOTA-gluBBN is applied to the theranostic approach, prostate cancer patients would be diagnosed by PET-CT using ^{68}Ga-DOTA-gluBBN after taking a biopsy for the evaluation of GRPR expression. We previously reported that GRPR is also over-expressed in Korean prostate cancer patients, and there would be many appropriate patients for GRPR-targeted theranostics, including Koream patients [20]. In addition, the diagnosis of prostate cancer can be confirmed, and the tumor burden including metastases can also be assessed using ^{68}Ga-DOTA-gluBBN for functional imaging. These procedures can provide essential information about GRPR density, which can select patients who are suitable for treatment using DOTA-gluBBN [12]. Next, the patients would begin the treatment using ^{177}Lu-DOTA-gluBBN [11], and the absorbed dose to the tumor and the toxicities to normal organs would be evaluated by SPECT-CT, which can make prognosis predictions. After treatment, the prognosis would be determined using ^{68}Ga-DOTA-gluBBN or ^{18}F-FDG. In the future, we can overcome prostate cancer using the theranostic approach.

In conclusion, we reported on ^{68}Ga radiolabeling of DOTA-gluBBN and the imaging efficacy of ^{68}Ga-DOTA-gluBBN in subcutaneously and peritoneal metastasized PC-3 prostate tumors. ^{68}Ga-DOTA-gluBBN exhibited favorable pharmacokinetics and exhibited specific and effective imaging of GRPR-expressing prostate tumors. These data suggest that a clinical study is needed to introduce the theranostic approach for prostate carcinoma patients.

Acknowledgments

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