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Synthesis and Evaluation of Novel Gonadotropin-Releasing Hormone Receptor-Targeting Peptides

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Abstract

The purpose of this study was to develop novel radiolabeled gonadotropin-releasing hormone (GnRH) receptor-targeting peptides for breast cancer imaging. Three novel 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-conjugated GnRH peptides were designed and synthesized. The radiometal chelator DOTA was conjugated to the epsilon or alpha amino group of D-lysine, or the epsilon amino group of L-lysine via an Ahx {aminohexanoic acid} linker to generate DOTA-Ahx-(D-Lys⁶-GnRH1), DOTA-Ahx-(D-Lys⁶-GnRH2) and DOTA-Ahx-(L-Lys⁶-GnRH3), respectively. The conjugation of the DOTA to the epsilon amino group of D-lysine (rather than alpha amino group of D-lysine nor epsilon amino group of L-lysine) maintained the nanomolar GnRH receptor binding affinity. The IC₅₀ values of DOTA-Ahx-(D-Lys⁶-GnRH1), DOTA-Ahx-(D-Lys⁶-GnRH2) and DOTA-Ahx-(L-Lys⁶-GnRH3) were 36.07 nM, 10.6 mM and 4.3 mM, respectively. Since only DOTA-Ahx-(D-Lys⁶-GnRH1) displayed nanomolar receptor binding affinity, the specific GnRH receptor binding of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was determined in human GnRH receptor membrane preparations. Furthermore, the biodistribution and tumor imaging properties of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) were examined in MDA-MB-231 human breast cancer-xenografted nude mice. ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) exhibited specific GnRH receptor binding and rapid tumor uptake (1.76 ± 0.58 %ID/g at 0.5 h post-injection) coupled with fast whole-body clearance through the urinary system. The MDA-MB-231 human breast cancer-xenografted tumor lesions were clearly visualized by single photon emission computed tomography (SPECT)/CT at 1 h post-injection of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1). The profound impact of DOTA position on the binding affinity of the GnRH peptide provided a new insight into the design of novel radiolabeled GnRH peptides. The successful imaging of MDA-MB-231 human breast cancer-xenografted tumor lesions using ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) suggested its potential as a novel imaging probe for human breast cancer imaging.

Keywords

Gonadotropin-releasing hormone receptor; receptor-targeting peptide

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in women in the United States. It was predicted that approximately 209,060 new cases would be diagnosed and 40,230 fatalities would occur in the US in 2010¹. Unfortunately, no curative treatment exists for metastatic breast cancer. Early diagnosis of breast cancer followed by a prompt surgical removal provides patients the best opportunities for cures or prolonged survivals. Mammography is an effective diagnostic tool for primary breast cancer. However, it is less effective for women with breast implants, post-surgical recurrence, or for women under age fifty as the breast tissue tends to be more dense². Meanwhile, the mammography can't detect distant breast cancer metastases. Single photon emission computed tomography (SPECT) and positron emission tomography (PET) techniques^{3,4} are more attractive non-invasive imaging modalities for metastatic breast cancer detection due to their high sensitivities and spatial resolutions. At present, 2-[¹⁸F]fluoro-2-deoxy-D-glucose ([¹⁸F]FDG) is the most commonly used PET imaging agent for the detection of various tumors including breast cancer⁵⁻¹⁵. However, the clinical application of [¹⁸F]FDG is limited¹⁰ by its unsatisfactory sensitivity for small tumors < 1 cm (57%) and for tumors in-situ (25%), and high false-positive results in the circumstance of inflammation. Hence, novel breast cancer-specific imaging probes are urgently needed to improve the detection accuracy for breast cancer.

Over-expression of gonadotropin-releasing hormone (GnRH) receptors on the breast cancer cells¹⁶⁻²⁰ highlights the potential of GnRH receptor as a molecular target for developing breast cancer-specific imaging probes. Clinical studies showed that 52% of breast cancer specimens removed by surgical resection over-expressed GnRH receptors^{19,20}. Native GnRH peptide is a hypothalamic decapeptide (pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰-NH₂). Both motifs of pGlu¹-His²-Trp³ and Arg⁸-Pro⁹-Gly¹⁰-NH₂ are critical for GnRH receptor binding²¹. The replacement of Gly⁶ with a D-amino acid increases the binding affinity and decreases the metabolic clearance of the peptide²². The D-Lys⁶-GnRH peptide has been used as a delivery vehicle to target the chemotherapy agent (2-pyrrolino-doxorubicin, AN201) to the GnRH receptors for breast cancer treatment²³⁻³¹. The AN201 was coupled to the epsilon amino group of D-Lys⁶ in D-Lys⁶-GnRH to generate a cytotoxic compound named AN207²³. The receptor-targeting AN207 exhibited enhanced remarkable therapeutic efficacy and decreased toxicity compared to the parent chemotherapy agent AN201. A single treatment of AN207 (250 nmol/kg) resulted in 100% cure for the mice bearing MX-1 human mammary carcinomas (GnRH receptor-positive) without apparent toxicity²⁸. These results on AN207 indicated that D-Lys⁶-GnRH could selectively bind the GnRH receptor to target the chemotherapy agent to breast cancer cells.

We hypothesized that the radiolabeled GnRH peptide can specifically bind the GnRH receptors for human breast cancer imaging in this study. We designed and evaluated three novel DOTA-conjugated GnRH peptides to examine our hypothesis, as well as to determine the effect of DOTA position on the binding affinity of the GnRH peptide. Specifically, the metal chelator DOTA was coupled to the epsilon and alpha amino groups of D-Lys⁶ in D-Lys⁶-GnRH via an aminohexanoic acid (Ahx) hydrocarbon linker to yield DOTA-Ahx-(D-Lys⁶-GnRH1) and DOTA-Ahx-(D-Lys⁶-GnRH2), respectively. In a parallel study, the DOTA was coupled to the epsilon amino group of L-Lys⁶ to generate DOTA-Ahx-(L-Lys⁶-GnRH3). The GnRH receptor binding affinities of these three peptides were determined

using Millipore ChemoScreen™ human GnRH receptor membrane preparations. Only DOTA-Ahx-(D-Lys⁶-GnRH1) displayed low nanomolar GnRH receptor binding affinity. Hence, we further evaluated the tumor targeting and imaging properties of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) in MDA-MB-231 human breast cancer-xenografted nude mice.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

Amino acids and resin were purchased from Advanced ChemTech Inc. (Louisville, KY) and Novabiochem (San Diego, CA). DOTA-tri-*t*-butyl ester was purchased from Macrocyclics Inc. (Richardson, TX). ¹¹¹InCl₃ was purchased from MDS Nordion, Inc. (Vancouver, Canada) for radiolabeling. ¹²⁵I-[D-Trp⁶]-LH-RH {pGlu-His-Trp-Ser-[¹²⁵I]Tyr-D-Trp-Leu-Arg-Pro-Gly-NH₂} was obtained from PerkinElmer Inc. (Boston, MA) and ChemoScreen™ Human GnRH receptor membrane preparations were purchased from Millipore, Inc (Billerica, MA) for receptor binding studies. GnRHR antibody (N-20, sc-8682) and the ABC Staining Systems were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA) for immunohistochemistry (IHC) staining. All other chemicals used in this study were purchased from Thermo Fischer Scientific (Waltham, MA) and used without further purification. MDA-MB-231 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA).

Peptide Synthesis

Three GnRH peptides were synthesized using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry. Briefly, the intermediate scaffolds of Dde-HN-D-Lys(Ahx-DOTA(tBu)₃)-Leu-Arg(Pbf)-Pro-Gly, (tBu)₃DOTA-Ahx-D-Lys(Dde)-Leu-Arg(Pbf)-Pro-Gly, and Dde-HN-Lys(Ahx-DOTA(tBu)₃)-Leu-Arg(Pbf)-Pro-Gly were synthesized on Rink amide resin by an Advanced ChemTech multiple-peptide synthesizer (Louisville, KY). Seventy micromoles of resin, 210 μmol of each Fmoc-protected amino acid and 210 μmol of (tBu)₃DOTA were used for the synthesis. The protecting group of Dde in each scaffold was removed by 2% Hydrazine. The moiety of pGlu-His(Trt)-Trp(Boc)-Ser(tBu)-Tyr(tBu) was conjugated to the epsilon amino group of either L-Lys or D-Lys, or the alpha amino group of D-Lys in the intermediate scaffolds. The protecting groups were totally removed by treating with a mixture of trifluoroacetic acid (TFA), thioanisole, phenol, water, ethanedithiol and triisopropylsilane (87.5:2.5:2.5:2.5:2.5:2.5) for 4 h at 25 °C. Each peptide was precipitated and washed with ice-cold ether for four times, purified by reverse phase-high performance liquid chromatography (RP-HPLC) and characterized by LC-MS.

In Vitro Receptor Binding Assay

The GnRH receptor binding affinities (IC₅₀ values) of DOTA-Ahx-(D-Lys⁶-GnRH1), DOTA-Ahx-(D-Lys⁶-GnRH2) and DOTA-Ahx-(L-Lys⁶-GnRH3) were determined by *in vitro* competitive binding assay according to the published procedure³² with modifications. Briefly, 5 μL of Millipore ChemoScreen™ human GnRH membrane preparations were incubated at 25°C for 3 h with approximately 30,000 counts per minute (cpm) of ¹²⁵I-[D-Trp⁶]-LH-RH in the presence of 10⁻¹¹ to 10⁻⁵ M of each peptide in 95 μL of binding medium {50 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid), 5 mM MgCl₂, 1 mM CaCl₂, pH 7.4, 0.2% bovine serum albumin (BSA)}. After the incubation, 800 μL of ice-cold washing buffer (50 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid), 500 mM NaCl, pH 7.4, 0.1% BSA) was added to each mixture. Each resulting mixture was filtered through a GF/C filter (Whatman, Clifton, NJ) pre-soaked in 1% polyethylenimine. Each filter was rinsed with 1 mL of ice-cold washing buffer for three times. The activities on the filters were measured in a Wallac 1480 automated gamma counter (PerkinElmer,

Waltham, MA). The IC₅₀ value of each peptide was calculated using Prism software (GraphPad Software, La Jolla, CA).

Peptide Radiolabeling with ¹¹¹In

Among these three synthetic GnRH peptides, only DOTA-Ahx-(D-Lys⁶-GnRH1) displayed low nanomolar GnRH receptor binding affinity. Hence, we further evaluated DOTA-Ahx-(D-Lys⁶-GnRH1). ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was prepared in a 0.5 M NH₄OAc buffer at pH 4.5 according to our published procedure³³. Briefly, 50 μL of ¹¹¹InCl₃ (37–74 MBq in 0.05 M HCl aqueous solution), 10 μL of 1 mg/mL DOTA-Ahx-(D-Lys⁶-GnRH1) aqueous solution and 400 μL of 0.5 M NH₄OAc (pH 4.5) were added into a reaction vial and incubated at 75°C for 45 min. After the incubation, 10 μL of 0.5% EDTA aqueous solution was added into the reaction vial to scavenge potential unbound ¹¹¹In³⁺ ions. The radiolabeled DOTA-Ahx-(D-Lys⁶-GnRH1) was purified to single species by Waters RP-HPLC (Milford, MA) on a Grace Vydac C-18 reverse phase analytical column (Deerfield, IL) using the following gradient at a 1 mL/min flow rate. The mobile phase consisted of solvent A (20 mM HCl aqueous solution) and solvent B (100% CH₃CN). The gradient was initiated and kept at 85:15 A/B for 3 mins followed by a linear gradient of 85:15 A/B to 75:25 A/B over 20 mins. Then, the gradient was changed from 75:25 A/B to 10:90 A/B over 3 mins followed by an additional 5 mins at 10:90 A/B. Thereafter, the gradient was changed from 10:90 A/B to 85:15 A/B over 3 mins. The purified peptide sample was purged with N₂ gas for 20 mins to remove the acetonitrile. The pH of final peptide solution was adjusted to 7.4 with 0.1 N NaOH and sterile normal saline for specific binding and animal studies.

In vitro serum stability of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was determined by incubation in mouse serum at 37°C for 2 h and monitored for degradation by RP-HPLC. Briefly, 100 μL of HPLC-purified ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) solution (~3.7 MBq) was added into 100 μL of mouse serum (Sigma-Aldrich Corp, St. Louis, MO) and incubated at 37°C for 2 h. After the incubation, 200 μL of a mixture of ethanol and acetonitrile (V:V = 1:1) was added to precipitate the serum. The resulting mixture was centrifuged at 10,000 g for 5 min to collect the supernatant. The supernatant was purged with N₂ gas for 30 min to remove the ethanol and acetonitrile. The resulting sample was mixed with 500 μL of water and injected into RP-HPLC for analysis using the gradient described above.

Specific Human GnRH Receptor Binding of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1)

The specific human GnRH receptor binding of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was determined using human GnRH receptor membrane preparations obtained from Millipore, Inc (Billerica, MA). Briefly, 50 μL of Millipore ChemoScreen™ human GnRH receptor membrane preparations were incubated at 25°C for 3 h with approximately 60,000 cpm of HPLC-purified ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) in 50 μL of binding medium with or without 1 μM of DOTA-Ahx-(D-Lys⁶-GnRH1) peptide blockade. After the incubation, each membrane preparation was mixed with 800 μL of ice-cold washing buffer first, and then filtered through a GF/C filter (Waterman, Clifton, NJ) pre-soaked in 1% polyethylenimine. Each filter was rinsed with 1 mL of ice-cold washing buffer for three times and counted in a gamma counter.

Immunohistochemistry Staining of MDA-MB-231 Human Breast Cancer-xenografted Tumor

The immunohistochemistry staining was performed on MDA-MB-231 human breast cancer-xenografted tumors to demonstrate the GnRH receptor expression. The MDA-MB-231 human breast cancer-xenografted tumors were generated through flank subcutaneous inoculations of MDA-MB-231 cells (1×10⁷ cells/mouse) in female Athymic nude mice. The tumor weights reached approximately 0.3 g at 18 days post cell inoculation. The immunoperoxidase staining of the xenografted MDA-MB-231 tumor slices (4-μm thickness)

were performed according to the protocol of goat ABC staining system (sc-2023) purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Briefly, the tumor slices were treated with 3% H₂O₂ for 15 min followed by a 20 min-treatment with the blocking serum at 25°C. Then, the tumor slices were incubated with primary goat anti-human GnRH antibody (1:40; Santa Cruz Biotechnology, Santa Cruz, CA) for 1.75 h at 25°C. Thereafter, the tumor slices were incubated with biotinylated secondary antibody for 30 min and followed by a 30 min-incubation with AB enzyme reagent. The tumor slices were incubated with the peroxidase substrate for 5 min followed by a dehydration process using ethanol and xylene. After the immunoperoxidase staining, the tumor slices were washed with de-ionized water and counterstained with Gill's formulation #2 hematoxylin. One to two drops of DPX permanent mounting medium were immediately added to the tumor slices after the counterstaining. Then, the tumor slices were covered with glass coverslips and observed by the light microscopy. As controls, the xenografted MDA-MB-231 tumor slices (4-μm thickness) were incubated (without primary goat anti-human GnRH antibody) with secondary biotinylated antibody, AB enzyme reagent and peroxidase substrate, respectively.

Biodistribution and Tumor Imaging

All the animal studies were conducted in compliance with Institutional Animal Care and Use Committee approval. The pharmacokinetics of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was determined in MDA-MB-231 human breast cancer-xenografted female athymic nude mice (Harlan, Indianapolis, IN). The nude mice were subcutaneously inoculated with 1×10⁷ MDA-MB-231 cells on the right flank of each mouse to generate MDA-MB-231 xenografted tumors. The tumor weights reached approximately 0.3 g at 18 days post cell inoculation. Each tumor-bearing mouse was injected with 0.037 MBq of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) via the tail vein. Groups of 5 mice were sacrificed at 0.5, 2, 4 and 24 h post-injection, and tumors and organs of interest were harvested, weighed and counted. Blood values were taken as 6.5% of the whole-body weight.

To determine the tumor imaging properties of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1), approximately 5.6 MBq of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was injected into a MDA-MB-231 human breast cancer-xenografted nude mouse (18 days post the cell inoculation) via the tail vein. The mouse was sacrificed for small animal SPECT/CT (Nano-SPECT/CT®, Bioscan) imaging at 1 h post-injection. The CT imaging was immediately followed by the whole-body SPECT imaging. The SPECT scans of 24 projections were acquired. Reconstructed SPECT and CT data were visualized and co-registered using InVivoScope (Bioscan, Washington DC).

Statistical Methods

Statistical analysis was performed using the Student's t-test for unpaired data to determine the significance of differences between the GnRH receptor binding of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) with or without 1 μM of DOTA-Ahx-(D-Lys⁶-GnRH1) peptide blockade. Difference at the 95% confidence level (p<0.05) was considered significant.

RESULTS

DOTA-Ahx-(D-Lys⁶-GnRH1), DOTA-Ahx-(D-Lys⁶-GnRH2) and DOTA-Ahx-(L-Lys⁶-GnRH3) were successfully synthesized and purified by RP-HPLC. Figure 1 illustrates the synthetic schemes of the GnRH peptides. All three GnRH peptides displayed greater than 90% purity after the HPLC purification. The peptide identities were confirmed by electrospray ionization mass spectrometry. The calculated molecular weights of DOTA-Ahx-(D-Lys⁶-GnRH1), DOTA-Ahx-(D-Lys⁶-GnRH2) and DOTA-Ahx-(L-Lys⁶-GnRH3) were 1752.9, 1752.9 and 1752.9, whereas the found molecular weights of DOTA-Ahx-(D-

Lys⁶-GnRH1), DOTA-Ahx-(D-Lys⁶-GnRH2) and DOTA-Ahx-(L-Lys⁶-GnRH3) were 1752.2, 1752.2 and 1752.4, respectively. The GnRH receptor binding affinities of the peptides are presented in Figure 2. The IC₅₀ values of DOTA-Ahx-(D-Lys⁶-GnRH1), DOTA-Ahx-(D-Lys⁶-GnRH2) and DOTA-Ahx-(L-Lys⁶-GnRH3) were 36.1 nM, 10.6 mM and 4.3 mM, respectively.

We further evaluated DOTA-Ahx-(D-Lys⁶-GnRH1) since only DOTA-Ahx-(D-Lys⁶-GnRH1) exhibited low nanomolar GnRH receptor binding affinity. DOTA-Ahx-(D-Lys⁶-GnRH1) was readily labeled with ¹¹¹In in 0.5 M ammonium acetate solution at pH 4.5 with greater than 95% radiolabeling yield. ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was completely separated from its excess non-labeled peptide by RP-HPLC. The retention times of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) and DOTA-Ahx-(D-Lys⁶-GnRH1) were 12.0 and 8.3 min, respectively. ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was stable in mouse serum at 37 °C for 2 h. Only intact ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was detected by RP-HPLC after 2 h of incubation in mouse serum (Fig. 3). The GnRH receptor binding of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) is shown in Figure 3. Incubation of 1 μM of DOTA-Ahx-(D-Lys⁶-GnRH1) peptide blocked 86% of the binding of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1), indicating that the binding of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was GnRH receptor-specific.

The GnRH receptor expressions in MDA-MB-231 human breast cancer-xenografted tumors slices were confirmed by immunohistochemistry staining. The immunohistochemistry staining results are presented in Figure 4. The GnRH receptor expressions were positively stained in MDA-MB-231 human breast cancer-xenografted tumors. Hence, we used the MDA-MB-231 human breast cancer-xenografted nude mice to determine the tumor targeting and pharmacokinetic properties of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1). The biodistribution results of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) are shown in Table 1. ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) exhibited rapid tumor uptake. The tumor uptake values were 1.76±0.58 and 0.29±0.10 %ID/g at 0.5 and 2 h post-injection. The tumor uptake values decreased to 0.15±0.05 and 0.18±0.07 %ID/g at 4 and 24 h post-injection. Blood clearance of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was fast. The blood uptake was 0.20±0.03 %ID/g at 2 h post-injection. Meanwhile, whole-body clearance of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was rapid, with approximately 95% of the injected radioactivity cleared through the urinary system by 2 h post-injection. The renal uptake values were 15.39±4.59, 8.88±1.26, 9.28±1.18 and 4.43±1.67 %ID/g at 0.5, 2, 4 and 24 h post-injection. Besides the kidneys, the liver was the normal organ with second high uptake after 2 h post-injection. The liver uptake values were 0.77±0.06, 0.97±0.13 and 0.59±0.16 %ID/g at 2, 4 and 24 h post-injection. The tumor imaging properties of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) were examined in MDA-MB-231 human breast cancer-xenografted nude mice. The three-dimensional, coronal and transversal SPECT/CT images are presented in Figure 5. Flank MDA-MB-231 xenografted tumors were clearly visualized by SPECT/CT using ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) as an imaging probe in all three images. The whole-body image (Fig. 5A) showed high tumor to normal organ uptake ratios except for the kidneys and liver.

DISCUSSION

There is considerable interest to develop receptor-targeting peptide radiopharmaceuticals for cancer imaging including breast cancer. For instance, radiolabeled bombesin (BBN) peptides³⁴⁻³⁷ have been utilized to target the gastrin-releasing peptide (GRP) receptors for breast cancer imaging. Both ^{99m}Tc-Cyc-Aca-BBN(2-14)NH₂³⁴ and ^{99m}Tc-RP527³⁵ have been successfully used to visualize breast cancer in human, confirming the feasibility of using receptor-targeting radiolabeled peptides for breast cancer detection. In this study, the GnRH receptor is an attractive molecular target due to its over-expression on human breast

cancer cells as well as tissue samples^{16–20}. Wild-type GnRH peptide (pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰-NH₂) can bend around the flexible Gly⁶ for GnRH receptor binding. The substitution of Gly⁶ with a D-amino acid enhances the binding affinity and reduces the metabolic clearance of the peptide²². It is known that both motifs of pGlu¹-His²-Trp³ and Arg⁸-Pro⁹-Gly¹⁰-NH₂ play crucial roles in GnRH receptor binding²¹. Several radiolabeled GnRH peptides have been reported over the past decade^{38–40}. Initially, a bifunctional chelating agent (BFCA) of P₂S₂-COOH {6,8-bis-[3-(bis(hydroxymethyl)phosphanyl)propylsulfanyl]octanoic acid} was conjugated to the epsilon amino group of D-Lys⁶ in D-Lys⁶-GnRH peptide for ^{99m}Tc/¹⁸⁸Re radiolabeling³⁸. The P₂S₂-D-Lys⁶-GnRH peptide was readily labeled with ^{99m}Tc/¹⁸⁸Re with greater than 88% radiolabeling yields, demonstrating the feasibility of using the P₂S₂-COOH as a BFCA for GnRH peptide radiolabeling with ^{99m}Tc/¹⁸⁸Re. However, neither receptor binding affinities nor biodistribution properties were reported for ^{99m}Tc/¹⁸⁸Re-P₂S₂-D-Lys⁶-GnRH³⁸. Thereafter, a backbone metal cyclization strategy was employed to cyclize the N-terminus and C-terminus of the GnRH peptides to develop ^{99m}Tc-labeled GnRH peptides³⁹. Unfortunately, the backbone metal cyclization dramatically decreased the GnRH receptor binding affinities of the peptides, confirming that both N-terminus and C-terminus need to be reserved for strong GnRH receptor binding.

In 2008, ¹⁸F- and ⁶⁸Ga-labeled GnRH peptides were reported for GnRH receptor-targeting⁴⁰. The motif of *p*-fluorobenzoyloxime acetyl (FBOA) was conjugated to the epsilon amino group of D-Lys⁶ in D-Lys⁶-GnRH via the β-Alanine (β-Ala) or Ahx linker for ¹⁸F radiolabeling, whereas the DOTA was directly coupled to the epsilon amino group of D-Lys⁶ in D-Lys⁶-GnRH for ⁶⁸Ga radiolabeling. The results on receptor binding affinity and internalization property indicated that the lipophilicity of the moiety attached to the D-Lys⁶ showed a significant impact on both receptor binding affinity and internalization property. The Ahx linker was more lipophilic and better than the β-Ala linker in terms of maintaining high receptor binding affinity and high internalization percentage of the GnRH peptide. Direct coupling of the hydrophilic ⁶⁸Ga-DOTA moiety to D-Lys⁶-GnRH dramatically reduced the receptor binding affinity and internalization percentage of the peptide³⁶. No biodistribution result was reported for ¹⁸F- and ⁶⁸Ga-labeled GnRH peptides.

In this study, instead of direct coupling the DOTA to the D-Lys⁶, we conjugated the DOTA to the D-Lys⁶ via the Ahx linker to enhance the lipophilicity of the moiety attached to D-Lys⁶-GnRH while remaining nanomolar GnRH receptor binding affinity of the peptide. Since both alpha and epsilon amino groups of D-Lys⁶ can be used for DOTA conjugation, we separately conjugated the DOTA to each amino group of D-Lys⁶ to determine which amino group was better for DOTA conjugation in terms of GnRH receptor binding affinity. The IC₅₀ values of DOTA-Ahx-(D-Lys⁶-GnRH1) and DOTA-Ahx-(D-Lys⁶-GnRH2) revealed that the epsilon amino group of D-Lys⁶ was better than the alpha amino group in maintaining nanomolar GnRH receptor binding affinity. The conjugation of the DOTA to the alpha amino group of D-Lys⁶ dramatically decreased the GnRH receptor binding affinity by 293-fold. Furthermore, we conjugated the DOTA to the epsilon amino group of L-Lys⁶ via the Ahx linker to examine the potential impact of D- and L-configuration of the Lys⁶ on the GnRH receptor binding affinity. The IC₅₀ values of DOTA-Ahx-(D-Lys⁶-GnRH1) and DOTA-Ahx-(L-Lys⁶-GnRH3) suggested that the D-configuration of the Lys⁶ played a key role in maintaining the GnRH receptor binding affinity as well. The replacement of D-Lys⁶ with L-Lys⁶ sacrificed the GnRH receptor binding affinity by 119-fold. The dramatic differences in GnRH receptor binding affinities among these three novel GnRH peptides demonstrated the profound impact of the DOTA position on the binding affinity of the GnRH peptide.

DOTA-Ahx-(D-Lys⁶-GnRH1) displayed 36.1 nM GnRH receptor binding affinity, warranting its further evaluation. We chose to radiolabel DOTA-Ahx-(D-Lys⁶-GnRH1) with ¹¹¹In, which is an attractive diagnostic radionuclide and is commercially available. Importantly, the switch from the ⁶⁸Ga-DOTA moiety to ¹¹¹In-DOTA moiety could further reduce one more negative charge of the radiometal-DOTA moiety since ¹¹¹In required one more -COOH group from the DOTA for coordination compared to ⁶⁸Ga. The radiolabeling of DOTA-Ahx-(D-Lys⁶-GnRH1) with ¹¹¹In was rapidly and efficiently (> 95% radiolabeling yield). The metal chelator DOTA formed a stable complex with ¹¹¹In. ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) was stable in serum at 37°C for 2 h (Fig. 3). ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) exhibited GnRH receptor-specific binding, with 86% of the binding being competed off by 1 μM of non-radiolabeled DOTA-Ahx-(D-Lys⁶-GnRH1) peptide blockade. The expressions of the GnRH receptors on MDA-MB-231 human breast cancer-xenografted tumor lesions were confirmed by immunohistochemistry staining (Fig. 4). ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) exhibited rapid tumor uptake (1.76 ± 0.58 %ID/g at 0.5 h post-injection) coupled with fast whole-body clearance in MDA-MB-231 human breast cancer-xenografted nude mice. As presented in Figure 5, the MDA-MB-231 human breast cancer-xenografted tumors were clearly visualized by SPECT/CT using ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) as an imaging probe at 1 h post-injection, highlighting the potential use of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) for human breast cancer imaging. The radioactivity accumulation in kidneys and liver were observed in the SPECT/CT images as well. The kidneys were the normal organs with the highest radioactivity uptakes, which was consistent with the biodistribution results (Table 1). It is worthwhile to note that a positively-charged side chain of Arg⁸ is available in ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1). Therefore, the renal uptake was likely attributed to the electrostatic interaction between the positively-charged ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) and negatively-charged renal tubular cells. A possible way to reduce the renal uptake of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) would be the co-injection of positively-charged lysine, which was successful in decreasing the renal uptakes of ¹¹¹In-labeled alpha-melanocyte stimulating hormone (α-MSH) peptides by 70%⁴¹.

Hydrocarbon linkers with various lengths were successfully coupled between the DOTA and bombesin peptide to optimize the receptor binding affinities of bombesin peptides⁴². The DOTA-conjugated bombesin peptides with the linkers ranging from 5-carbon to 8-carbon exhibited 0.6–1.7 nM receptor binding affinities. Either shorter or longer hydrocarbon linkers dramatically reduce the receptor binding affinity by 100-fold³⁴. Despite the fact that the Ahx linker was utilized to improve the lipophilicity of the metal-DOTA moiety attached to D-Lys⁶-GnRH in this study, more studies would be needed to determine whether the Ahx linker is ideal or not for low nanomolar GnRH receptor binding of the peptide. Hence, it would be interesting to examine the impact of the hydrocarbon linker on GnRH receptor binding affinity of the peptide in the future.

In conclusion, the DOTA position displayed a profound impact on the binding affinity of the GnRH peptide. The coupling of DOTA to the epsilon amino group of D-Lys⁶ maintained nanomolar GnRH receptor binding affinity of the peptide, providing a new insight into the design of novel radiolabeled GnRH peptides. The successful imaging of MDA-MB-231 human breast cancer-xenografted tumor lesions using ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) highlighted its potential as a novel imaging probe for human breast cancer imaging.

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Figure 1.
Synthetic schemes of three novel GnRH peptides.

Figure 2.

The competitive binding curves of the GnRH peptides. The IC_{50} values of DOTA-Ahx-(D-Lys⁶-GnRH1), DOTA-Ahx-(D-Lys⁶-GnRH2) and DOTA-Ahx-(L-Lys⁶-GnRH3) were 36.1 nM, 10.6 mM and 4.3 mM, respectively.

Figure 3.

Radioactive HPLC profiles of ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1) (A, T=0) and its mouse serum stability (B, T=2 h) after 2 h incubation at 37°C. The retention time of ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1) was 12.0 min; Binding of ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1) on human GnRH receptor membrane preparations (C) with (□) or without (■) the presence of 1 μM of DOTA-Ahx-(D-Lys⁶-GnRH1). *P<0.05.

Figure 4. Immunohistochemistry staining of GnRH receptor expressions in MDA-MB-231 human breast cancer-xenografted tumor (A, $\times 400$). The MDA-MB-231 xenografted tumor exhibited strong brown cytoplasmic staining. As a comparison, the MDA-MB-231 xenografted tumor were stained without primary goat anti-human GnRH antibody (B, $\times 400$).

Figure 5. Three-dimensional (A), coronal (B) and transversal (C) SPECT/CT images of MDA-MB-231 human breast cancer-xenografted tumor at 1 h post-injection of 5.6 MBq of ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1). Flank breast cancer lesions (T) were highlighted with arrows on the images.

Table 1

Biodistribution of ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1) in MDA-MB-231 human breast cancer-xenografted nude mice. The data were presented as percent injected dose/gram or as percent injected dose (Mean $\pm$ SD, n=5).

Tissue	0.5 h	2 h	4 h	24 h
Percent injected dose/gram (%ID/g)				
Tumor	1.76 $\pm$ 0.58	0.29 $\pm$ 0.10	0.15 $\pm$ 0.05	0.18 $\pm$ 0.07
Brain	0.34 $\pm$ 0.28	0.03 $\pm$ 0.02	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01
Blood	2.87 $\pm$ 0.71	0.20 $\pm$ 0.03	0.11 $\pm$ 0.02	0.04 $\pm$ 0.02
Heart	1.11 $\pm$ 0.19	0.13 $\pm$ 0.04	0.09 $\pm$ 0.06	0.10 $\pm$ 0.06
Lung	3.17 $\pm$ 0.69	0.28 $\pm$ 0.12	0.18 $\pm$ 0.10	0.11 $\pm$ 0.03
Liver	1.60 $\pm$ 0.29	0.77 $\pm$ 0.06	0.97 $\pm$ 0.13	0.59 $\pm$ 0.16
Spleen	0.80 $\pm$ 0.10	0.23 $\pm$ 0.04	0.39 $\pm$ 0.01	0.31 $\pm$ 0.11
Stomach	0.66 $\pm$ 0.37	0.31 $\pm$ 0.31	0.50 $\pm$ 0.56	0.04 $\pm$ 0.01
Kidneys	15.39 $\pm$ 4.59	8.88 $\pm$ 1.26	9.28 $\pm$ 1.18	4.43 $\pm$ 1.67
Muscle	0.51 $\pm$ 0.34	0.03 $\pm$ 0.03	0.12 $\pm$ 0.03	0.16 $\pm$ 0.02
Pancreas	0.55 $\pm$ 0.04	0.15 $\pm$ 0.02	0.13 $\pm$ 0.08	0.11 $\pm$ 0.08
Bone	0.72 $\pm$ 0.19	0.35 $\pm$ 0.26	0.70 $\pm$ 0.50	0.37 $\pm$ 0.26
Skin	3.07 $\pm$ 0.91	0.26 $\pm$ 0.04	0.24 $\pm$ 0.03	0.22 $\pm$ 0.05
Uptake ratio of tumor/normal tissue				
Tumor/blood	0.61	1.45	1.36	4.50
Tumor/muscle	3.45	9.67	1.25	1.13
Percent injected dose (%ID)				
Intestines	1.43 $\pm$ 0.10	0.42 $\pm$ 0.10	0.63 $\pm$ 0.21	0.16 $\pm$ 0.02
Urine	73.12 $\pm$ 5.36	94.61 $\pm$ 0.60	94.59 $\pm$ 0.27	96.81 $\pm$ 0.72