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Introduction of D-Phenylalanine enhanced the receptor binding affinities of gonadotropin-releasing hormone peptides

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Abstract

The purpose of this study was to examine whether the introduction of D-Phe could improve the GnRH receptor binding affinities of DOTA-conjugated D-Lys⁶-GnRH peptides. Building upon the construct of DOTA-Ahx-(D-Lys⁶-GnRH1) we previously reported, an aromatic amino acid of D-Phe was inserted either between the DOTA and Ahx or between the Ahx and D-Lys⁶ to generate new DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) or DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) peptides. Compared to DOTA-Ahx-(D-Lys⁶-GnRH1) (36.1 nM), the introduction of D-Phe improved the GnRH receptor binding affinities of DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) (16.3 nM) and DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) (7.6 nM). The tumor targeting and pharmacokinetic properties of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was determined in MDA-MB-231 human breast cancer-xenografted nude mice. Compared to ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1), ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) exhibited comparable tumor uptake with faster renal and liver clearance. The MDA-MB-231 human breast cancer-xenografted tumors were clearly visualized by single photon emission computed tomography (SPECT) using ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) as an imaging probe, providing a new insight into the design of new GnRH peptides in the future.

Keywords

Gonadotropin-releasing hormone; receptor binding affinity; breast cancer; SPECT imaging

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Gonadotropin-releasing hormone (GnRH) receptor is an attractive molecular target for developing cancer imaging probes due to its over-expression on breast cancer,^{1,2} prostate cancer,³ ovarian and endometrial cancers.^{4,5} Native GnRH peptide is a hypothalamic decapeptide (pGlu¹-His²-Trp³-Ser⁴-Tyr⁵-Gly⁶-Leu⁷-Arg⁸-Pro⁹-Gly¹⁰-NH₂) with nanomolar binding affinity to the GnRH receptor. Both motifs of pGlu¹-His²-Trp³ and Arg⁸-Pro⁹-Gly¹⁰-NH₂ played important roles in GnRH receptor binding.⁶ Meanwhile, the substitution of Gly⁶ with a D-amino acid enhanced the binding affinity and reduced the metabolic clearance of the peptide.⁷ Thus, the D-Lys⁶-GnRH peptide has been used as a delivery vehicle to target radionuclides^{8,9} or chemotherapy agents^{10–18} to the GnRH receptors for cancer imaging and treatment. Over the past several years, we and others have reported the development and evaluation of radiolabeled GnRH peptides for cancer imaging.^{8,9,19–23} Specifically, the epsilon amino group of D-Lys⁶ in D-Lys⁶-GnRH peptide was selected for conjugation of radionuclides.^{8,9,19–23} Interestingly, the lipophilicity of the moiety attached to the D-Lys⁶ showed a significant impact on both receptor binding affinities and internalization properties of ⁶⁸Ga- and ¹⁸F-labeled D-Lys⁶-GnRH peptides.⁸ The more lipophilic aminohexanoic acid (Ahx) linker was better than the β-Ala linker in retaining high receptor binding affinities and high internalization percentages of the ¹⁸F-labeled D-Lys⁶-GnRH peptides. Direct coupling of the hydrophilic ⁶⁸Ga-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (⁶⁸Ga-DOTA) moiety to D-Lys⁶-GnRH dramatically reduced the receptor binding affinity and internalization percentage of the peptide.⁸

In our previous report,⁹ instead of direct coupling the radiometal chelator DOTA to D-Lys⁶-GnRH, we introduced an Ahx linker between the DOTA and D-Lys⁶-GnRH to enhance the lipophilicity of the moiety attached to D-Lys⁶-GnRH. Interestingly, DOTA-Ahx-(D-Lys⁶-GnRH1) exhibited 36.1 nM GnRH receptor binding affinity. Moreover, the MDA-MB-231 human breast cancer-xenografted tumor lesions were clearly visualized by single photon emission computed tomography (SPECT)/CT using ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) as an imaging probe.⁹ Clearly, the introduction of the hydrocarbon Ahx linker between the DOTA and the side chain of D-Lys⁶ contributed to the success of ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH1) as an imaging probe. Thus, we were interested in whether the receptor binding affinities of the DOTA-conjugated D-Lys⁶-GnRH peptides could be enhanced by improving lipophilicity of the moiety attached to D-Lys⁶-GnRH. In this study, building upon the structure of DOTA-Ahx-(D-Lys⁶-GnRH1), we introduced an aromatic amino acid of D-Phe to improve the lipophilicity of the moiety attached to the D-Lys⁶-GnRH peptide. We hypothesized that the introduction of the aromatic D-Phe between the DOTA and D-Lys⁶-GnRH would enhance the receptor binding affinity of the peptide. To examine our hypothesis, we designed two novel DOTA-conjugated D-Lys⁶-GnRH peptides with the aromatic D-Phe linker. Specifically, the D-Phe was inserted either between the DOTA and Ahx or between the Ahx and the D-Lys⁶-GnRH moiety to examine which position would be better for the conjugation of D-Phe in retaining nanomolar GnRH receptor binding affinity.

Initially, DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) (Figure 1) were synthesized using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and purified by reverse phase-high performance liquid chromatography (RP-HPLC). Both peptides displayed greater than 95% purity after HPLC purification. The peptide identities were confirmed by electrospray ionization mass spectrometry. The calculated molecular weights of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) were 1900.1 Da, whereas the found molecular weights of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) were 1900.2 Da (m/z: [M+3H]³⁺ = 634.4; [M+2H]²⁺ = 950.9) and 1899.9 Da (m/z: [M+3H]³⁺ = 634.3; [M+2H]²⁺ = 950.4), respectively. The GnRH receptor binding affinities of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) were determined using Millipore ChemiScreen™

human GnRH receptor membrane preparations. The IC_{50} values of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) were 7.6 and 16.3 nM, respectively (Figure 2). Interestingly, the introduction of D-Phe displayed a profound impact on the receptor binding affinity of the DOTA-conjugated D-Lys⁶-GnRH peptides. Both DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) exhibited stronger GnRH receptor binding affinities than that of DOTA-Ahx-(D-Lys⁶-GnRH).⁹ The receptor binding affinities of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) were 4.8 and 2.2 times the binding affinity of DOTA-Ahx-(D-Lys⁶-GnRH). It is worthwhile to note that the position of D-Phe also showed a significant impact on the receptor binding affinity of the GnRH peptide. The stronger receptor binding affinity of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) suggested that the position between the Ahx and D-Lys⁶-GnRH moiety was a better position for the conjugation of D-Phe.

DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) displayed stronger GnRH receptor binding affinity than DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH). Thus, we further radiolabeled DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) with ¹¹¹In and evaluated its *in vitro* serum stability. DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was readily labeled with ¹¹¹In in 0.1 M 4-Morpholineethanesulfonic acid (MES) buffer at pH 5.5 with greater than 95% radiolabeling yield. ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was completely separated from its excess non-labeled peptide by RP-HPLC. The retention time of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was 14.9 and 18.2 min, respectively (Figure 3). The radiochemical purity of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was greater than 98%. The specific activity of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was 9.0647×10^8 MBq/g. ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was stable at 37 °C in mouse serum for 4 h. Only intact ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was detected by RP-HPLC after 4 h of incubation in mouse serum (Figure 3). The specific GnRH receptor binding of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) is shown in Figure 4. Incubation of 1 μM of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) blocked 98% of the binding of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) ($p < 0.05$), indicating that the binding of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was GnRH receptor-specific.

To further evaluate the ability of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) to target the GnRH receptors *in vivo*, we determined the biodistribution properties of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) in MDA-MB-231 human breast cancer-xenografted nude mice. The biodistribution results of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) are shown in Table 1. ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) exhibited a rapid tumor uptake. The tumor uptake was 1.02 ± 0.14 and $0.38 \pm 0.07\%$ ID/g at 0.5 and 1 h post-injection. The tumor uptake decreased to 0.20 ± 0.04 and $0.15 \pm 0.03\%$ ID/g at 2 and 4 h post-injection. Meanwhile, ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) displayed high accumulation and prolonged retention in pituitary because GnRH receptors are abundant in pituitary gonadotropes. The pituitary uptake was 38.64 ± 6.75 , 33.68 ± 4.53 and $24.29 \pm 8.31\%$ ID/g at 0.5, 1 and 4 h post-injection. Co-injection of non-labeled DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) peptide significantly blocked the tumor uptake ($0.15 \pm 0.08\%$ ID/g at 1 h post-injection, $p < 0.05$), as well as the pituitary uptake ($0.55 \pm 0.39\%$ ID/g at 1 h post-injection, $p < 0.05$), indicating the tumor and pituitary uptake of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was GnRH receptor-mediated. Overall, ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) exhibited comparable tumor uptake as ¹¹¹In-DOTA-Ahx-(D-Lys⁶-GnRH)⁹ at 0.5, 2 and 4 h post-injection.

¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) displayed a fast blood clearance, leading to the increased tumor-to-blood ratios over time. The tumor-to-blood ratio was 0.75 at 1 h post-injection and 12.89 at 4 h post-injection, respectively. The whole-body clearance of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was rapid, with approximately $86.61 \pm 4.15\%$ of the

injected radioactivity cleared from the urinary system by 1 h post-injection. The renal uptake was 8.14 ± 1.10 , 6.65 ± 1.45 , 7.58 ± 1.10 and $5.33 \pm 0.71\%$ ID/g at 0.5, 1, 2, and 4 h post-injection. Figure 5 shows the radioactive HPLC profile of the urine sample. The urine analysis revealed that most of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was metabolized into two compounds with higher polarities. As compared to ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1),⁹ ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) displayed a faster blood clearance and lower liver and kidney uptake. The blood uptake of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was 53%, 70% and 91% less than that of ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1) at 0.5, 2 and 4 h post-injection. The tumor-to-blood ratios of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) were 2.3 and 9.5 times those of ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1) at 2 and 4 h post-injection. As shown in Figure 6, ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) exhibited 19%, 39% and 63% less liver uptake, and 47%, 15% and 43% less kidney uptake than ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1) at 0.5, 2 and 4 h post-injection.

Finally, we examined the tumor imaging property of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) in MDA-MB-231 human breast cancer-xenografted nude mice. The three-dimensional, coronal and transversal SPECT/CT images are presented in Figure 7. The SPECT images of the tumors accurately matched their anatomical CT images. Flank MDA-MB-231 xenografted tumors were clearly visualized by SPECT/CT using ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) as an imaging probe, highlighting its potential for breast cancer detection. Despite the fact that the MDA-MB-231 human breast cancer-xenografted tumor lesions could be clearly detected using ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) as an imaging probe, ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) didn't display higher tumor uptake which we anticipated. The urine analysis revealed that ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was metabolized into two compounds with higher polarities at 1 h after injection. It was likely that the rapid metabolism of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH), either the degradation of the D-Lys⁶-GnRH peptide or the cleavage of the Ahx-D-Phe linker, contributed to the lower tumor uptake than what we anticipated. Thus, we are interested in investigating whether the tumor uptake of ^{111}In -labeled GnRH peptides could be enhanced by structural modification in future studies. A possible way to improve the tumor uptake is to cyclize the GnRH peptide. The peptide cyclization via metal and lactam bridge has been successful in improving the tumor uptake of radiolabeled alpha-melanocyte stimulating hormone peptides.^{24–27} Accordingly, it would be interesting to develop novel radiolabeled cyclic GnRH peptides for tumor targeting in the future.

The experimental details are presented in References and notes.^{28–35}

In conclusion, the introduction of D-Phe enhanced the receptor binding affinity of the DOTA-conjugated D-Lys⁶-GnRH peptides. DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) exhibited higher receptor binding affinity than DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH). As compared to ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1), ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) exhibited decreased radioactivity accumulation in blood, liver and kidney while retaining the comparable tumor uptake. Successful imaging of MDA-MB-231 human breast cancer-xenografted tumor lesions using ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) as an imaging probe provided a new insight into the design of new GnRH peptides in the future.

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REFERENCES AND NOTES

1. Fekete M, Wittliff JL, Schally AV. J. Clin. Lab. Anal. 1989; 3:137. [PubMed: 2569034]
2. Baumann KH, Kiesel L, Kaufmann M, Bastert G, Runnebaum B. Breast Cancer Res. Treat. 1993; 25:37. [PubMed: 8390871]
3. Halmos G, Arencibia JM, Schally AV, Davis R, Bostwick DG. J. Urol. 2000; 163:623. [PubMed: 10647698]
4. Srkalovic G, Schally AV, Wittliff JL, Day TG Jr, Jenison EL. Int. J. Oncol. 1998; 12:489. [PubMed: 9472084]
5. Gründker C, Emons G. Reprod. Biol. Endocrinol. 2003; 1:65. [PubMed: 14594454]
6. Millar RP. Animal Reproduction Sci. 2005; 88:5.
7. Beckers T, Bernd M, Kutscher B, Kuhne R, Hoffman S, Reissmann T. Biochem. Biophys. Res. Commun. 2001; 289:653. [PubMed: 11726197]
8. Schottelius M, Berger S, Poethko T, Schwaiger M, Wester HJ. Bioconjug. Chem. 2008; 19:1256. [PubMed: 18510351]
9. Guo H, Lu J, Hathaway H, Royce ME, Prossnitz ER, Miao Y. Bioconjug. Chem. 2011; 22:1682. [PubMed: 21749045]
10. Nagy A, Schally AV, Armatis P, Szepeshazi K, Halmos G, Kovacs M, Zarandi M, Groot K, Miyazaki M, Jungwirth A, Horvath J. Proc. Natl. Acad. Sci. U.S.A. 1996; 93:7269. [PubMed: 8692981]
11. Halmos G, Nagy A, Lamharzi N, Schally AV. Cancer Lett. 1999; 136:129. [PubMed: 10355741]
12. Wang X, Krebs LJ, Al-Nuri M, Pudavar HE, Ghosal S, Liebow C, Nagy AA, Schally AV, Prasad PN. Proc. Natl. Acad. Sci. U.S.A. 1999; 96:11081. [PubMed: 10500132]
13. Szepeshazi K, Schally AV, Nagy A, Halmos G, Groot K. Anticancer Drugs. 1997; 8:974. [PubMed: 9436641]
14. Szepeshazi K, Schally AV, Nagy A. Breast Cancer Res. Treat. 1999; 56:267. [PubMed: 10573117]
15. Kahan Z, Nagy A, Schally AV, Halmos G, Arencibia JM, Groot K. Cancer. 1999; 85:2608. [PubMed: 10375109]
16. Bajo AM, Schally AV, Halmos G, Nagy A. Clin. Cancer Res. 2003; 9:3742. [PubMed: 14506166]
17. Kahan Z, Nagy A, Schally AV, Halmos G, Arencibia JM, Groot K. Breast Cancer Res. Treat. 2000; 59:255. [PubMed: 10832595]
18. Chatzistamou I, Schally AV, Nagy A, Armatis P, Szepeshazi K, Halmos G. Clin. Cancer Res. 2000; 6:4158. [PubMed: 11051271]
19. Gali H, Hoffman TJ, Sieckman GL, Owen NK, Katti KV, Volkert WA. Bioconjug. Chem. 2001; 12:354. [PubMed: 11353532]
20. Jalilian AR, Shanehsazzadeh S, Akhlaghi M, Garousi J, Rajabifar S, Tavakoli MB. J. Radioanal. Nucl. Chem. 2008; 278:123.
21. Barda Y, Cohen N, Lev V, Ben-Aroya N, Koch Y, Mishani E, Fridkin M, Gilon C. Nucl. Med. Biol. 2004; 31:921. [PubMed: 15464394]
22. Guo H, Gallazzi F, Sklar LA, Miao Y. Bioorg. Med. Chem. Lett. 2011; 21:5183.
23. Guo H, Hathaway HJ, Royce ME, Prossnitz ER, Miao Y. Bioorg. Med. Chem. Lett. 2013; 23:5484. [PubMed: 24018188]
24. Guo H, Yang J, Gallazzi F, Miao Y. J. Nucl. Med. 2011; 52:608. [PubMed: 21421725]
25. Guo H, Yang J, Gallazzi F, Miao Y. J. Nucl. Med. 2010; 51:418. [PubMed: 20150256]
26. Miao Y, Gallazzi F, Guo H, Quinn TP. Bioconjug. Chem. 2008; 19:539. [PubMed: 18197608]
27. Chen J, Cheng Z, Owen NK, Hoffman TJ, Miao Y, Jurisson SS, Quinn TP. J. Nucl. Med. 2001; 42:1847. [PubMed: 11752084]
28. **Peptide synthesis:** Amino acids and resin were purchased from Advanced ChemTech Inc. (Louisville, KY) and Novabiochem (San Diego, CA). DOTA-tri-*tert*-butyl ester was purchased from Macrocyclics Inc. (Richardson, TX). Two new GnRH peptides were synthesized using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry according to our published procedure⁹ with modifications. Seventy micromoles of resin, 210 μ mol of each Fmoc-protected amino acid and 210

μmol of $(\text{tBu})_3\text{DOTA}$ were used for the synthesis. The intermediate scaffold of pGlu-His(Trt)-Trp(Boc)-Ser(tBu)-Tyr(tBu)-D-Lys(Mtt)-Leu-Arg(Pbf)-Pro-Gly was synthesized on Rink amide resin by an Advanced ChemTech multiple-peptide synthesizer (Louisville, KY). The protecting group of Mtt in the scaffold was removed by 25% hexafluoroisopropanol (HFIP) in the presence of 10% *N,N*-diisopropylethylamine (DIPEA) for further conjugation of the moiety of D-Phe-Ahx-DOTA(tBu)₃ or Ahx-D-Phe-DOTA(tBu)₃. After the conjugation, 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 four times, purified by reverse phase-high performance liquid chromatography (RP-HPLC) and characterized by LC-MS.

29. ***In vitro* GnRH receptor binding assay:** The GnRH receptor binding affinities (IC_{50} values) of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) were determined by *in vitro* competitive binding assay according to the published procedure.⁹ ¹²⁵I-[D-Trp⁶]-LH-RH {pGlu-His-Trp-Ser-Tyr-[¹²⁵I]-D-Trp-Leu-Arg-Pro-Gly-NH₂} was obtained from PerkinElmer Inc. (Boston, MA). The receptor binding assay was replicated in triplicate for each peptide. Briefly, 5 μL of Millipore ChemiScreen™ human GnRH membrane preparations (Billerica, MA) 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^{-12} 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% of polyethylenimine. Each filter was rinsed with 1 mL of ice-cold washing buffer three times. The activities on the filters were measured in a Wallac 1480 automated gamma counter (PerkinElmer, Waltham, MA). The IC_{50} value of each peptide was calculated using Prism software (GraphPad Software, La Jolla, CA).
30. **Peptide radiolabeling with ¹¹¹In:** ¹¹¹InCl₃ was purchased from MDS Nordion, Inc. (Vancouver, Canada) for radiolabeling. DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) showed stronger GnRH receptor binding affinity than DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH). Hence, we further evaluated DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH). ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was prepared in a 0.1 M MES (4-Morpholineethanesulfonic acid) buffer solution at pH 5.5. Briefly, 100 μL of 0.1 M MES buffer solution (pH 5.5), 10 μL of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) solution (1 mg/mL) and 50 μL of ¹¹¹InCl₃ (37–74 MBq in 0.05 M HCl aqueous solution) were added into a reaction vial and incubated at 75 °C for 30 min. After the incubation, the radiolabeled DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was purified to a 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 1 mL/min flow rate. The mobile phase consisted of solvents A (20 mM HCl aqueous solution) and B (100% CH₃CN). The gradient was initiated and kept at 82:18 A/B for 3 mins followed by a linear gradient of 82:18 A/B to 72:28 A/B over 20 mins. Then, the gradient was changed from 72:28 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 at 25 °C for 20 mins to remove the acetonitrile. The pH of the final peptide solution was adjusted to 7.4 with 0.1 N NaOH and sterile normal saline for specific binding and animal studies.
31. ***In vitro* serum stability:** *In vitro* serum stability of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was determined by incubation in mouse serum at 37 °C for 4 h and monitored for degradation by RP-HPLC. Briefly, 100 μL of HPLC-purified ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) solution (~3.7 MBq) was added into 100 μL of mouse serum (Sigma-Aldrich Corp, St. Louis, MO) and incubated at 37 °C for 4 h. After the incubation, 400 μL of a mixture of ethanol and acetonitrile (v:v = 1:1) was added to precipitate the serum. The resulting mixture was centrifuged at 10,000g 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.
32. **Specific human GnRH receptor binding of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH):** The specific human GnRH receptor binding of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was determined using human GnRH receptor membrane preparations obtained from Millipore, Inc (Billerica, MA). Briefly, 50 μL of Millipore ChemiScreen™ human GnRH receptor membrane

preparations were incubated at 25 °C for 3 h with approximately 60,000 cpm of HPLC-purified ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) in 50 μL of binding medium with or without 1 μM of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) peptide blockade ($n=3$). 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 three times and counted in a gamma counter. The experiment was replicated in triplicate.

33. **Biodistribution studies:** All the animal studies were conducted in compliance with Institutional Animal Care and Use Committee approval. MDA-MB-231 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). The biodistribution of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was determined in MDA-MB-231 human breast cancer-xenografted female athymic nude mice (Harlan, Indianapolis, IN). The nude mice were subcutaneously inoculated with 1×10^7 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 21 days post cell inoculation. Each tumor-bearing mouse was injected with 0.037 MBq of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) via the tail vein. Groups of 5 mice were sacrificed at 0.5, 1, 2 and 4 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. The specificity of tumor uptake of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was determined by co-injecting 10 μg of unlabeled DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) at 1 h post-injection.
34. **Tumor imaging and urinary metabolites of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH):** To determine the tumor imaging property of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH), approximately 7.4 MBq of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) was injected into a MDA-MB-231 tumor-bearing nude mouse via the tail vein. The mouse was sacrificed at 1 h post-injection to collect the urine for analyzing the metabolites, as well as for small animal SPECT/CT (Nano-SPECT/CT, Bioscan) imaging. The CT scan 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 the InVivoScope (Bioscan, Washington, DC). The urine sample was directly centrifuged at 16,000g for 5 min before the HPLC analysis. Thereafter, the urine was injected into the HPLC using the gradient described above.
35. **Statistical methods:** Statistical analysis was performed using the Student's t test for unpaired data to determine the significance of differences in GnRH receptor binding, tumor, pituitary, blood, kidney, liver and muscle uptake between ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) with or without 1 μM of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) peptide blockade. Difference at the 95% confidence level ($p < 0.05$) was considered significant.

Linker	Peptide
$X = \text{---C(=O)---(CH}_2\text{)}_6\text{---NH}_2$	DOTA-Ahx-(D-Lys ⁶ -GnRH)
$X = \text{---C(=O)---CH(Ph)---NH---C(=O)---(CH}_2\text{)}_6\text{---NH}_2$	DOTA-Ahx-D-Phe-(D-Lys ⁶ -GnRH)
$X = \text{---C(=O)---(CH}_2\text{)}_6\text{---NH---C(=O)---CH(Ph)---NH}_2$	DOTA-D-Phe-Ahx-(D-Lys ⁶ -GnRH)

Figure 1.

Structures of DOTA-Ahx-(D-Lys⁶-GnRH1), DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH). The structure of DOTA-Ahx-(D-Lys⁶-GnRH1) was cited from our previous report⁹ for comparison.

Figure 2.

The competitive binding curves of the peptides. The IC₅₀ values of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and DOTA-D-Phe-Ahx-(D-Lys⁶-GnRH) were 7.6 and 16.3 nM, respectively.

Figure 3.

UV HPLC profile of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) (A); Radioactive HPLC profiles of ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) (B, T = 0) and its mouse serum stability (C, T = 4 h) after 4 h incubation at 37 °C. The retention times of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) and ¹¹¹In-DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) were 14.9 and 18.2 min, respectively.

Figure 4. Binding of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) on human GnRH receptor membrane preparations with (white) or without (blue) the presence of $1\mu\text{M}$ of DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH). * $p < 0.05$.

Figure 5.

Radioactive HPLC profile of the urine sample of a MDA-MB-231 human breast cancer-xenografted nude mouse at 1 h post-injection of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH). The arrow indicates the retention time of the original ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) prior to the tail vein injection.

Figure 6. Liver (A) and kidney (B) uptake of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) (blue) and ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1) (white). Data of ^{111}In -DOTA-Ahx-(D-Lys⁶-GnRH1) were cited from reference 9 for comparison. * $p < 0.05$.

Figure 7. Three-dimensional (A), coronal (B) and transversal (C) SPECT/CT images of MDA-MB-231 human breast cancer-xenografted tumors at 1 h post-injection of 7.4 MBq of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH). Flank tumor lesions (T) are highlighted with arrows on the images.

Biodistribution of ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) 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).

Table 1

Tissue	0.5 h	1 h	1 h peptide blockade	2 h	4 h
Percent injected dose/gram (%ID/g)					
Tumor	1.02 $\pm$ 0.14	0.38 $\pm$ 0.07	0.15 $\pm$ 0.08 *	0.20 $\pm$ 0.04	0.15 $\pm$ 0.03
Brain	0.05 $\pm$ 0.01	0.01 $\pm$ 0.01	0.02 $\pm$ 0.03	0.01 $\pm$ 0.00	0.01 $\pm$ 0.00
Blood	1.35 $\pm$ 0.37	0.32 $\pm$ 0.21	0.22 $\pm$ 0.07	0.06 $\pm$ 0.02	0.01 $\pm$ 0.01
Heart	0.49 $\pm$ 0.22	0.17 $\pm$ 0.13	0.12 $\pm$ 0.03	0.06 $\pm$ 0.03	0.02 $\pm$ 0.02
Lung	2.22 $\pm$ 0.77	0.57 $\pm$ 0.16	0.36 $\pm$ 0.10	0.21 $\pm$ 0.05	0.10 $\pm$ 0.04
Liver	1.30 $\pm$ 0.39	0.62 $\pm$ 0.10	0.49 $\pm$ 0.16	0.47 $\pm$ 0.11	0.36 $\pm$ 0.04
Spleen	0.37 $\pm$ 0.08	0.21 $\pm$ 0.10	0.08 $\pm$ 0.05	0.10 $\pm$ 0.03	0.07 $\pm$ 0.03
stomach	4.20 $\pm$ 2.68	0.97 $\pm$ 0.45	0.68 $\pm$ 0.12	0.23 $\pm$ 0.12	0.30 $\pm$ 0.28
kidneys	8.14 $\pm$ 1.10	6.65 $\pm$ 1.45	6.89 $\pm$ 0.72	7.58 $\pm$ 1.10	5.33 $\pm$ 0.71
Muscle	0.16 $\pm$ 0.10	0.04 $\pm$ 0.03	0.08 $\pm$ 0.02	0.04 $\pm$ 0.01	0.02 $\pm$ 0.02
Pancreas	0.29 $\pm$ 0.14	0.09 $\pm$ 0.03	0.22 $\pm$ 0.19	0.05 $\pm$ 0.03	0.01 $\pm$ 0.01
Bone	0.32 $\pm$ 0.17	0.07 $\pm$ 0.06	0.13 $\pm$ 0.11	0.04 $\pm$ 0.01	0.07 $\pm$ 0.05
Pituitary	38.64 $\pm$ 6.75	33.68 $\pm$ 4.53	0.55 $\pm$ 0.39 *	38.58 $\pm$ 7.96	24.29 $\pm$ 8.32
Percent injected dose (%ID)					
Intestines	2.81 $\pm$ 1.42	1.56 $\pm$ 0.63	1.76 $\pm$ 1.16	6.01 $\pm$ 6.69	4.59 $\pm$ 2.17
Urine	73.62 $\pm$ 8.02	86.61 $\pm$ 4.15	83.34 $\pm$ 16.08	89.25 $\pm$ 6.98	91.55 $\pm$ 2.57
Uptake ratio of tumor/normal tissue					
Tumor/Blood	0.75	1.18	0.68	3.36	12.89
Tumor/Muscle	6.43	9.92	2.03	5.43	8.49

* $p < 0.05$, significance of difference in tumor and pituitary uptake between ^{111}In -DOTA-Ahx-D-Phe-(D-Lys⁶-GnRH) with or without peptide blockade at 1 h post-injection.