Published in final edited form as:

Bioconj Chem. 2018 October 17; 29(10): 3483–3494. doi:10.1021/acs.bioconjchem.8b00642.

Synthesis and Evaluation of New Bifunctional Chelators with Phosphonic Acid Arms for Gallium-68 Based PET Imaging in Melanoma

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Abstract

Due to the increasing use of generator-produced radiometal Gallium-68 (⁶⁸Ga) in positron-emission tomography/computed tomography (PET/CT), reliable bifunctional chelators that can efficiently incorporate ⁶⁸Ga³⁺ into biomolecules are highly desirable. In this study, we synthesized two new bifunctional chelators bearing one or two phosphonic acid functional groups, named *p*-SCN-PhPr-NE2A1P and *p*-SCN-PhPr-NE2P1A, with the aim of enabling facile production of ⁶⁸Ga-based radiopharmaceuticals. Both chelators were successfully conjugated to LLP2A-PEG₄, a very late antigen-4 (VLA-4) targeting peptidomimetic ligand, to evaluate their application in ⁶⁸Ga-based PET imaging. NE2P1A-PEG₄-LLP2A exhibited the highest ⁶⁸Ga³⁺ binding ability with molar activity of 37 MBq/nmol under mild temperature and neutral pH. Excellent serum stability of ⁶⁸Ga-NE2P1A-PEG₄-LLP2A was observed, which was consistent with the result obtained from density functional theory calculation. The in vitro cell study showed that ⁶⁸Ga-NE2P1A-PEG₄-LLP2A had significantly longer retention in B16F10 cells comparing to the reported retention of ⁶⁴Cu-NE3TA-PEG₄-LLP2A, although the uptake was relatively lower. In the biodistribution and micro-PET/CT imaging studies, high tumor uptake and low background were observed after ⁶⁸Ga-NE2P1A-PEG₄-LLP2A was injected into mice bearing B16F10 tumor xenografts, making it a highly promising radiotracer for noninvasive imaging of VLA-4 receptors overexpressed in melanoma.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconj-chem.8b00642. DFT Calculation data, Stability results (PDF)

Notes

The authors declare no competing financial interest.

Graphical Abstract

INTRODUCTION

Positron-emission tomography (PET) is a powerful non-invasive medical imaging tool for diagnosis and prognosis of cancer and various other diseases in routine clinical and preclinical settings.^{1–3} Combined with computed tomography (CT) or magnetic imaging (MRI), hybrid PET/CT or PET/MRI provides both anatomical and quantitative physiological information for personalized medicine.¹ Currently, ^{18}F ($t_{1/2} = 109.8$ min), ^{11}C ($t_{1/2} = 20.3$ min), and ^{68}Ga ($t_{1/2} = 67.7$ min) are the three most commonly used radionuclides in clinical PET imaging. Unlike ^{18}F and ^{11}C , which require an expensive cyclotron for production, $^{68}\text{Ga}^{3+}$ is readily available from an in-house $^{68}\text{Ge}/^{68}\text{Ga}$ generator, making it particularly attractive for facilities with limited access to a cyclotron. Given the convenient, economical, and reliable properties of ^{68}Ga production, ^{68}Ga -based radiotracers have shown significant impacts on PET imaging. For example, ^{68}Ga labeled octreotide analogues have proven their usefulness in diagnosis of neuroendocrine tumors, screening of patients, as well as assessing the response of Lutathera radiotherapy in clinics.^{4–8}

To apply a metallic radionuclide in molecular imaging, the radiometal should be radiolabeled with an appropriate bifunctional chelator (BFC) on some biomolecules. The short half-life of ^{68}Ga requires a quick and efficient radiolabeling, preferably under mild conditions without subsequent purification. Thus, there has been an increasing interest in developing efficient BFCs for Ga^{3+} during the last decades (Figure 1). Polyazamacrocyclic chelator 1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetraacetic acid (DOTA) was once considered as a “gold standard” BFC for $^{68}\text{Ga}^{3+}$ for its high kinetic stability. Its bioconjugates, ^{68}Ga -DOTA-Tyr-3-octreotide (^{68}Ga -DOTA-TOC), ^{68}Ga -DOTA-1-Nal³-octreotide (^{68}Ga -DOTA-NOC), and ^{68}Ga -DOTA-Tyr-3-octreotate (^{68}Ga -DOTA-TATE), have been frequently applied in the imaging of somatostatin receptors clinically.^{4–7} However, harsh radiolabeling conditions such as high-temperature or long-term incubation are required for DOTA based tracers to obtain an adequate radiolabeling yield and high molar activity,^{9–11} which may limit their applications in radiolabeling of temperature-, pH- or time-sensitive agents, such as proteins and certain peptides. The current “gold standard” 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and its derivatives, possessing a relative smaller polyazamacrocyclic cage than DOTA, can chelate with ^{68}Ga at room temperature within 10 min under acidic conditions, and the resulting ^{68}Ga -NOTA complexes exhibit great stability.^{10,12–14} Recently, 3-hydroxy-4-pyridinone (HOPO) based chelators have been developed, and the bifunctional derivative CP256 can be labeled with $^{68}\text{Ga}^{3+}$ at room temperature in 5 min at pH 6.5.¹⁵ Chelators with other backbones have also shown

promising labeling properties for ^{68}Ga , including 3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid (PCTA),¹⁶ 1,4,7-triaza-cyclononane-1,4,7-tris[methyl(2-carboxyethyl)phosphinic acid (TRAP),¹⁷ N,N' -bis[2-hydroxy-5-(carboxyethyl)benzyl]-ethyl-enediamine- N,N' -diacetic acid (HBED-CC),^{18,19} 2-(4,7-bis-((1-methyl-1*H*-imidazol-2-yl)methyl) -1,4,7-triazonan-1-yl) - acetic acid (NODIA-Me),²⁰ and 2,2'-(6-((carboxymethyl)-(methyl) amino)-6-methyl-1,4-diazepane-1,4-diyl)diacetic acid (DATA^M).²¹

Recently, we developed a new NOTA-based BFC *p*-SCN-PhPr-NE3TA for effective radiolabeling of ^{64}Cu , which showed better binding selectivity with Cu(II) than Fe(III).²² The chelator was designed to integrate the advantages of both macrocyclic and acyclic frameworks for thermodynamic stability and favorable chelating kinetics for various metals, including Y^{3+} , Bi^{3+} , Lu^{3+} , Cu^{2+} , and Gd^{3+} .^{23–25} We hypothesized that NE3TA and its derivatives may also exhibit promising radiolabeling properties toward ^{68}Ga . It is well-known that the kinetics of complexation and the thermodynamic stability of a complex are affected by the number and the property of substituents attached to the NOTA-based macrocyclic framework. Commonly used functional groups for substituents include carboxylates, phosphonic acids, and phosphinic acids.^{26–29} Prata et al. reported that differences in the number of carboxylate and phosphonic acid groups of NOTA-like chelators could alter the coordination chemistry and the thermodynamic stability between the chelator and ^{68}Ga .²⁹ In their study, the thermodynamic stability constant and stepwise protonation constants of phosphonic acid ligand $\text{Ga}(\text{NOA2P})^{2-}$ (1,4,7-triazacyclononane- N -methylenephosphonic acid- N',N'' -dimethylenecarboxylic acid) were much higher than those of $\text{Ga}(\text{NOTA})$. Therefore, in order to investigate how the phosphonic acid groups affect the stability of the BFCs, besides *p*-SCN-PhPr-NE3TA, we also designed and synthesized two NE3TA derivatives that bear one phosphonic acid group and two phosphonic acid groups in the pendant arm on the macrocyclic 1,4,7-triazacyclononane (TACN) ring, named *p*-SCN-PhPr-NE2A1P and *p*-SCN-PhPr-NE2P1A, respectively. LLP2A-PEG₄ is a peptidomimetic ligand identified from a 1-bead 1-compound library and possesses high specificity to very late antigen-4 (VLA-4, also called integrin $\alpha_4\beta_1$).³⁰ It is a very promising ligand for clinical translation, and has been radiolabeled with different radiometals for PET imaging.^{31–35} In this study, we have all three BFCs conjugated to LLP2A-PEG₄ and the resulting bio-conjugates were radiolabeled with ^{68}Ga under various labeling conditions. ^{68}Ga -NE2P1A-PEG₄-LLP2A, with the favorable radiolabeling conditions, was used for the following *in vivo* biodistribution and PET imaging studies in mice bearing B16F10 melanoma tumors.

RESULTS

Synthesis of BFCs and Bioconjugates.

As illustrated in Scheme 1, *p*-SCN-PhPr-NE2A1P was synthesized in a 21.9% overall yield. Compound **2** was obtained via a Mannich-like reaction by mixing starting material **1** with paraformaldehyde and diethyl phosphite in tetrahydrofuran (THF). The purified compound **2** reacted with methylsulfonyl chloride in the presence of triethylamine (TEA) in dichloromethane (DCM). However, an unexpected chlorinated compound **3** was obtained

instead of **2**-OMs, as confirmed by $^1\text{H}/^{13}\text{C}$ NMR and MALDI-TOF, which was similar to a previous study.³⁶ The introduction of a phosphonic acid arm to TACN backbone was carried out by the coupling reaction between $\text{NO}_2\text{A}_{\text{tBu}}$ and compound **3** in the presence of *N,N*-diisopropylethylamine (DIEA) in acetonitrile (MeCN) to yield the key intermediate **4**. Chelator *p*- NO_2 -PhPr-NE2A1P (**5**) was obtained after an ester hydrolysis of compound **4**. To prepare the bifunctional version, the nitro group in the intermediate **4** was reduced by 10% Pd/C in methanol under H_2 gas at room temperature to obtain aniline **6**. Another functional group, the carboxylic acid group in compound **7**, was generated by removing the Boc protecting groups in compound **6** using HBr/AcOH at room temperature. The isothiocyanate ($-\text{SCN}$) functional group in compound **8**, which is an amino-activated functional group for further bioconjugation, was then generated with thiophosgene in CHCl_3 . The reaction was carried out at room temperature for 4 h, and the desired compound *p*-SCN-PhPr-NE2A1P (**8**) was obtained as a yellow solid by extraction and lyophilization.

A similar approach was applied to prepare *p*-SCN-PhPr-NE2P1A. As illustrated in Scheme 2, *p*-SCN-PhPr-NE2P1A was synthesized in an overall yield of 10% starting from TACN. In an initial trial, we tried to synthesize compound **11** directly via a Mannich-like reaction by reacting TACN with 2 equiv of both paraformaldehyde and diethyl phosphite in THF. However, significant amounts of byproducts were observed as indicated by TLC, and it was difficult to get pure **11** by either pH-controlled extraction or silica gel chromatography. Therefore, we added additional protection—deprotection steps to obtain compound **11**. Briefly, one of three secondary amino nitrogen atoms in TACN was first protected with a Boc group by reacting with 1-fold of 2-(boc-oxyimino)-2-phenylacetoni-trile (Boc-ON) to afford compound **9**, which was purified by pH controlled extraction in an reasonable yield (60%). Alkylation of **9** with diethyl *p*-toluenesulfonyloxymethyl phosphonate in the presence of K_2CO_3 in MeCN gave the trisubstituted compound **10**. Compound **11** was subsequently obtained by treating compound **10** with trifluoroacetic acid (TFA) in DCM solution. Chelator *p*- NO_2 -PhPr-NE2P1A and its bifunctional version *p*-SCN-PhPr-NE2P1A were then obtained using a similar procedure as *p*- NO_2 -PhPr-NE2A1P and *p*-SCN-PhPr-NE2A1P. Briefly, intermediate **13** was synthesized by coupling compound **12** to compound **11**. Hydrolysis of intermediate **13** gave chelator *p*- NO_2 -PhPr-NE2P1A as a yellow solid. Chelator *p*-SCN-PhPr-NE2P²1A was obtained after reduction of intermediate **13** and subsequent isothiocyanate formation using thiophosgene.

To explore applications of *p*-SCN-PhPr-NE2P1A and *p*-SCN-PhPr-NE2A1P in receptor targeted PET imaging, a peptidomimetic ligand LLP2A-PEG₄ was conjugated to these two BFCs for PET imaging of VLA-4 expression in melanoma xenograft mouse model. By coupling *p*-SCN-PhPr-NE2P1A and *p*-SCN-PhPr-NE2A1P to LLP2A-PEG₄ via a thiourea bond formation, the corresponding products NE2A1P-PEG₄-LLP2A and NE2P1A-PEG₄-LLP2A were conveniently obtained in high yields. In addition, NOTA-PEG₄-LLP2A and NE3TA-PEG₄-LLP2A were also prepared²² to compare their in vivo performances.

Radiolabeling and Stability.

In order to investigate the potential utility of four BFCs for radiolabeling biomolecules with ^{68}Ga , their LLP2A conjugates, NOTA-PEG₄-LLP2A, NE2P1A-PEG₄-LLP2A, NE3TA-

PEG₄-LLP2A, and NE2A1P-PEG₄-LLP2A, were used and their performances at various radiolabeling conditions were compared. LLP2A bioconjugates (100 pmol, ~1 μ M) and ⁶⁸Ga (3.7 MBq) were used for all ⁶⁸Ga radiolabeling experiments with an aimed molar activity of 37 MBq/nmol. The radiolabeling conditions and corresponding results were summarized in Figure 2a. All bioconjugates could achieve nearly identical quantitative radiochemical yields after incubation with ⁶⁸Ga in sodium acetate (NaOAc) buffer (0.1 M, pH 4) at 70 °C within 10 min. However, when the labeling temperature was decreased to 37 °C, NOTA-PEG₄-LLP2A and NE2P1A-PEG₄-LLP2A still showed quantitative radiolabeling with ⁶⁸Ga, while the radiolabeling yields of NE3TA-PEG₄-LLP2A and NE2A1P-PEG₄-LLP2A were only 9% $\pm$ 3% and 30% $\pm$ 4%, respectively. In addition, we also tested the ⁶⁸Ga-labeling at neutral pH condition (0.1 M NaOAc buffer, pH 6.8) for NOTA-PEG₄-LLP2A and NE2P1A-PEG₄-LLP2A at different temperatures. At 70 °C, after being incubated for 10 min, both NOTA-PEG₄-LLP2A and NE2P1A-PEG₄-LLP2A showed quantitative radiolabeling. However, once the incubation temperature dropped to 37 °C, NOTA-PEG₄-LLP2A showed dramatically decreased radiolabeling yield (8% $\pm$ 3%), while NE2P1A-PEG₄-LLP2A could still remain a quantitative radiolabeling yield. Thus, NE2P1A-PEG₄-LLP2A exhibited good ⁶⁸Ga radiolabeling properties under all examined conditions, indicating that *p*-SCN-PhPr-NE2P1A has great potential for preparing ⁶⁸Ga-labeled biomolecules, particularly for those temperature- and acid-sensitive radiotracers.

Serum stability studies were carried out to evaluate the in vitro and in vivo stability of the four ⁶⁸Ga radiolabeled conjugates. Both ⁶⁸Ga-NOTA-PEG₄-LLP2A and ⁶⁸Ga-NE2P1A-PEG₄-LLP2A exhibited high serum stability with less than 5% ⁶⁸Ga disassociation after an 2 h incubation in human serum at 37 °C, while under the same condition, approximately 50% of ⁶⁸Ga disassociated from ⁶⁸Ga-NE2A1P-PEG₄-LLP2A (Figure 2b). To further test the stability of ⁶⁸Ga-NE2P1A-PEG₄-LLP2A, PBS inertness, Zn²⁺ competition, and in vivo metabolism (in B16F10 mice) were performed. ⁶⁸Ga-NE2P1A-PEG₄-LLP2A remained greater than 95% intact after 2 h incubation in both PBS (0.1 M) and ZnCl₂ solution (1 mM). At 30 min post-injection of ⁶⁸Ga-NE2P1A-PEG₄-LLP2A, only 1.1% and 4.8% disassociated ⁶⁸Ga ions were observed in blood and urine respectively (see Figure S1), suggesting substantial in vivo inertness.

DFT Calculation.

The excellent ⁶⁸Ga radiolabeling performance of NE2P1A-PEG₄-LLP2A and the remarkable stability of ⁶⁸Ga-NE2P1A-PEG₄-LLP2A prompted us to further investigate the coordination property of the NE3TA-like chelators with gallium ion. Density functional theory (DFT) calculations were used to generate the optimized geometries and calculate their Gibbs energies. To simplify the calculation process, fully deprotonated phosphonic acid groups were used. All calculations were conducted using commercial software (Gaussian 09 suite, Gaussian software, Wallingford, CT, USA).³⁷ The energies were calculated at B3LYP/6-31G+ +(d,p) level (water as solvent). The optimized structures of these gallium complexes are illustrated in Figure 3, and the relative coordination Gibbs energies are listed in Table S1. All three chelators could bind with gallium and form in-cargo hexa-coordinated complexes with octahedron geometry. In all complexes, the bond distances of Ga-O and Ga-N were approximately 2.0 and 2.1, respectively, which was similar to those of Ga-NOTA

(1.93 and 2.09, respectively)³⁸ and Ga-H₃PrP9 (1.93 and 2.11, respectively)¹⁷ determined from their crystal data. Ga-NE2P1A-PhPr-NCS has the lowest Gibbs energy, confirming that *p*-SCN-PhPr-NE2P1A could form a more stable complex with gallium than *p*-SCN-PhPr-NE3TA and *p*-SCN-PhPr-NE2A1P, which is consistent with the results of the labeling and serum stability studies.

Log D Measurements.

The octanol/water distribution coefficient (log D value) is considered as one of the important parameters to evaluate the physicochemical properties of radiotracers, which are often correlated with their pharmacokinetic properties. The traditional shake flask method using neutral PBS buffer and octanol was applied to determine the log D values of ⁶⁸Ga radiolabeled conjugates. The results were summarized in Table 1. All tracers exhibited comparable hydrophilic properties with log D values at around -2, and ⁶⁸Ga-NE3TA-PEG₄-LLP2A had the best water solubility among these tracers. Log D values of tracers with phosphorylated chelators were slightly higher than those tracers with fully carboxylated chelators.

Cell Uptake and Cell Efflux.

To determine the specific binding affinity of ⁶⁸Ga-NE2P1A-PEG₄-LLP2A to the VLA-4 receptor, a cell internalization assay was performed using VLA-4 overexpressed B16F10 mouse melanoma cells. The results were compared with those obtained in our previous study using ⁶⁴Cu-NE3TA-PEG₄-LLP2A.²² As shown in Figure 4, ⁶⁸Ga-NE2P1A-PEG₄-LLP2A was rapidly internalized by B16F10 cells within 15 min, and the internalization rate increased over time up to 4 h. In a parallel blocking group that received an additional unlabeled LLP2A-PEG₄ (10 μg), the internalization was significantly blocked at all time-points (*p* < 0.001), indicating ⁶⁸Ga-NE2P1A-PEG₄-LLP2A bound with the VLA-4 receptor highly specifically.

A cellular efflux assay was performed to investigate the cellular retention ability of ⁶⁴Cu-NE3TA-PEG₄-LLP2A and ⁶⁸Ga-NE2P1A-PEG₄-LLP2A in B16F10 cells. The cellular retentions of both tracers decreased rapidly over time within the first hour and then displayed a sustained decrease as more time elapsed.

Biodistribution.

To investigate the tumor uptakes of ⁶⁸Ga-NE2P1A-PEG₄-LLP2A, an ex vivo biodistribution study was conducted using B16F10 xenograft-bearing C57BL/6 mice. As shown in Figure 5, high tumor uptakes were observed, with ± 3.4 , 13.7 ± 2.4 , and $15.2 \pm 2.8\%$ ID/g at 1, 2, and 4 h post injection (pi), respectively. The tumor-to-blood ratios were found to be very high at all examined time-points with the highest at 4 h post injection (18.4 ± 4.7). High tumor/muscle ratios were also observed with 6.7 ± 1.9 , 10.6 ± 2.9 , and 10.3 ± 1.4 at 1, 2, and 4 h post injection, respectively. Undesired high uptake in spleen, bone, and thymus was found, similar to our previous findings.²² A reasonable explanation is that VLA-4 receptors are also abundant in these tissues, especially in young mice. As an indirect proof, the high uptakes in these organs as well as in tumors were significantly reduced after coinjection of excess

amounts of unlabeled LLP2A-PEG₄, indicating the high expression of VLA-4 receptors in these tissues.

PET/CT Imaging.

To visualize the in vivo performance of ⁶⁸Ga-NE2P1A-PEG₄-LLP2A, small animal PET/CT imaging was applied using B16F10 xenograft-bearing C57BL/6 mice model. The static PET/CT images was shown in Figure 6. All tumor xenografts were clearly delineated at 1 and 2 h pi of ⁶⁸Ga-NE2P1A-PEG₄-LLP2A, while the other organs, except for the bladder, showed much lower intensity. In order to verify the specificity of high tumor accumulation, a blocking study was also performed by giving mice the same dose of tracer along with 100 μg LLP2A-PEG₄. As expected, the tumor uptake dropped to the background level.

DISCUSSION

The aim of this study was to develop new NOTA-based BFCs for effective labeling ⁶⁸Ga in the application of PET imaging for cancer patients with overexpressing VLA-4 receptors. In our previous publication, we developed a new Cu(II) specific BFC *p*-SCN-PhPr-NE3TA, which integrated both the macrocyclic and acyclic framework for favorable chelating kinetics for ⁶⁴Cu as well as thermodynamic stability. The resulting ⁶⁴Cu-NE3TA-PEG₄-LLP2A demonstrated promising in vivo kinetic profile and high tumor uptake in mice bearing B16F10 xenografts, which encouraged us to further investigate the potential use of novel TACN derived chelators to complex with other PET radiometals, such as ⁶⁸Ga. In the present study, we developed two novel TACN derived bifunctional chelators bearing one or two phosphonic acid groups, respectively, for ⁶⁸Ga radiolabeling. By replacing the carboxylate group with the more rigid phosphonic acid group, it was believed that it could induce the chelating groups to adopt a better preorganized conformation to facilitate the carbon—metal bond formation during the metal complexation, leading to improved thermal stability. As demonstrated in this study, introduction of the phosphonic acid group did improve the thermodynamic and kinetic stability of the complex between the NE3TA-like chelator and Ga(III). Such an increase in chelator stability upon replacement of carboxylate by phosphonic acid was also observed in the study of Prata et al., as exemplified by Ga(NO₂A₂P)²⁻ and Ga (NOTA).²⁹ Moreover, the peptidomimetic conjugates of *p*-SCN-Bn-NE2P1A, NE2P1A-PEG₄-LLP2A, exhibited good ⁶⁸Ga radiolabeling properties at mild temperatures and neutral pH. The resulting ⁶⁸Ga-NE2P1A-PEG₄-LLP2A also exhibited decent in vitro and in vivo stability. To the best of our knowledge, *p*-SCN-PhPr-NE2P1A is the first NE3TA-based bifunctional chelator for ⁶⁸Ga radiolabeling. In contrast, adding the phosphonic acid group on the pendant arm instead of on macrocyclic nitrogen atoms extends the distance of the pendant phosphonic acid group to the chelation core, which distorted the coordination of the polyhedron, thus affecting the stability of the complex ⁶⁸Ga-NE2A1P-PEG₄-LLP2A.

Cell uptake studies showed that cellular internalization of ⁶⁸Ga-NE2P1A-PEG₄-LLP2A was relatively lower than that of ⁶⁴Cu-NE3TA-PEG₄-LLP2A, which is possibly due to the more negative charges on chelator NE2P1A than those on chelator NE3TA. Despite the relatively lower cell uptake, ⁶⁸Ga-NE2P1A-PEG₄-LLP2A exhibited significantly higher retention in

B16F10 cells as compared to ^{64}Cu -NE3TA-PEG₄-LLP2A at all examined time points (particularly $61.8\% \pm 4.3\%$ vs $12.7\% \pm 1.2\%$ at 2 h, $p < 0.001$), indicating that ^{68}Ga -NE2P1A-PEG₄-LLP2A bound B16F10 cells more tightly and was more difficult to wash out. In summary, ^{68}Ga -NE2P1A-PEG₄-LLP2A exhibited rapid internalization and high retention in B16F10 cells.

The biodistribution results provide quantitative insights to evaluate the in vivo performance of ^{68}Ga -NE2P1A-PEG₄-LLP2A in B16F10 tumor bearing mice. The highest tumor uptake of ^{68}Ga -NE2P1A-PEG₄-LLP2A was achieved at 1 h pi and kept at a high level up to 4 h pi. The tumor-to-muscle ratio of ^{68}Ga -NE2P1A-PEG₄-LLP2A was significantly higher than that of ^{64}Cu -NE3TA-PEG₄-LLP2A at 2 h pi (10.6 ± 2.9 versus 5.8 ± 1.0 , $p < 0.05$), while the differences of tumor uptake values of two tracers had no statistical significance at the same time points. The liver uptake values of ^{68}Ga -NE2P1A-PEG₄-LLP2A were significantly lower than those of ^{64}Cu -NE3TA-PEG₄-LLP2A at 2 h pi ($6.2 \pm 1.2\% \text{ID/g}$ versus $13.1 \pm 1.2\% \text{ID/g}$) and 4 h pi ($4.6 \pm 1.0\% \text{ID/g}$ versus $11.8 \pm 1.0\% \text{ID/g}$). The kidney uptake values of ^{68}Ga -NE2P1A-PEG₄-LLP2A were significantly higher than those of ^{64}Cu -NE3TA-PEG₄-LLP2A at 2 h pi ($11.1 \pm 2.7\% \text{ID/g}$ versus $5.2 \pm 0.4\% \text{ID/g}$) and 4 h pi ($10.1 \pm 0.6\% \text{ID/g}$ versus $4.3 \pm 0.5\% \text{ID/g}$), possibly due to the greater negative charges in chelator NE2P1A than those in the chelator NE3TA. The choice of chelators and radiometals has a significant influence on the in vivo performance of radiotracers, especially on the elimination pathway.

The in vivo performance of ^{68}Ga -NE2P1A-PEG₄-LLP2A was visualized by the static PET/CT images. The highest radioactivity accumulation was in bladder, indicating that its main elimination pathway was through the urinary system, which was consistent with its Log D profile. Lesion-to-background contrast and liver-to-background contrast observed at 2 h pi were higher than those observed at 1 h pi (Figure 6). The images were similar to those obtained from ^{64}Cu -NE3TA-PEG₄-LLP2A, except a relatively quicker clearance in liver, rendering it a promising tracer for detecting of liver metastasis. The high retention in B16F10 melanoma and the significant decrease in tumor intensity with the coinjection of blocking agents validated the high VLA-4 receptor binding affinity and specificity of ^{68}Ga -NE2P1A-PEG₄-LLP2A.

In conclusion, NE2P1A-PEG₄-LLP2A was radiolabeled with ^{68}Ga in high molar activity at physiologic temperature in neutral pH, and the resulting ^{68}Ga -NE2P1A-PEG₄-LLP2A exhibited high binding affinity and specificity for VLA-4 abundant melanoma, making it a promising tracer for imaging VLA-4 overexpression. Therefore, *p*-SCN-Bn-NE2P1A is a very promising BFC for development of ^{68}Ga -based tracers and may have a significant impact on radiolabeling biomolecules with ^{68}Ga in the future, especially for labeling molecules that cannot tolerate acidic environment and elevated temperature, such as single-chain antibody fragments. Further studies may involve the investigation of the phosphonic acid group's influence on Ga(III) chelation, including fully or partly exchanging the carboxylate groups to phosphonic acid groups on the macrocyclic ring. The synthesis and evaluation of phosphinic acid groups substituted NE3TA-based chelators for Ga(III) chelation are also under investigation in our group.

EXPERIMENTAL PROCEDURES

Reagents and instrumentations.

1,4,7-Triazacyclononane was purchased from Synpartner PharmaTech (Quzhou, China). Aqueous solutions were prepared using ultrapure water (resistivity, 18 M). Compound **1**, **12**, and **NO2A_{tBu}** were synthesized according to our previous report.^{22,36} LLP2A-PEG₄ was synthesized according to the literature.³¹ All other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), unless otherwise specified. The pH was measured using pH test papers (MACHEREY-NAGEL GmbH & Co. KG, Germany). ⁶⁸Ga was eluted from a ⁶⁸Ge/⁶⁸Ga generator (Echert & Ziegler Eurotope GmbH, Germany) by 0.1 N HCl and ⁶⁸Ga was purified by modular-lab (Echert & Ziegler Eurotope GmbH, Germany) using a cation exchange column (Phenomenex, X-C cartridges, Torrance, CA) eluted with 97.5% acetone, 2.25% H₂O, 0.25% HCl (30%, ultrapure) solution (800 μ L). PerkinElmer (Packard) Cobra II Gamma counter and PerkinElmer 2470 WIZARD Automatic Gamma Counter (Waltham, MA) were used to measure γ radiation. ¹H and ¹³C NMR spectra were recorded at temperature 298 K on a Bruker DRX 400 MHz spectrometer equipped with a 5 mm BBO probe (Billerica, MA). ³¹P spectra were recorded at temperature 298 K on a Bruker Avance III 500 MHz spectrometer equipped with a 5 mm BBO probe (Billerica, MA). ESI-MS were measured on a Waters LCT-Premier XE LC-MS station (Milford, MA). High-resolution mass spectra (HRMS) were obtained on Bruker ultrafleXtreme MALDI-TOF/TOF matrix:HCCA (Bruker Corporation). Luna C-18 HPLC columns were from Phenomenex (Torrance, CA, USA). HPLC were performed on a Waters 1525 Binary HPLC pump (Milford, MA) with a Waters 2489 UV/visible detector and a model 106 Bioscan radioactivity detector for purification of peptide conjugates and analysis of their radiolabeled conjugates using two elution buffers (0.1 v% TFA in deionized (DI) water as elution buffer A and 0.1 v% TFA in acetonitrile as elution buffer B). PET/CT data were acquired using an Inveon Preclinical Imaging Station (Siemens Medical Solutions).

Diethyl (((2-Hydroxyethyl)(3-(4-nitrophenyl)propyl)-amino)methyl)phosphonate

(2).—To a solution of **1** (3.5 g, 15.5 mmol) in acetonitrile (MeCN) (50 mL) was added paraformaldehyde (0.7 g, 23.3 mmol) and diethyl phosphite (2.6 g, 18.6 mmol). The resulting mixture was stirred at room temperature overnight. The excess paraformaldehyde was filtered off and the filtrate was evaporated to dryness. The residue was eluted on a silica gel (200–300 mesh) column with 2% MeOH/dichloromethane (DCM) to provide pure **2** (4.8 g, ~82%) as a yellowish oil. ¹H NMR (400 MHz, CDCl₃) δ 8.06 (d, J = 8.6 Hz, 2H, Ar-H), 7.29 (d, J = 8.5 Hz, 2H, Ar-H), 4.07 (p, J = 7.2 Hz, 4H, -OCH₂CH₃), 3.57–3.49 (m, 2H, -CH₂OH), 2.90–2.81 (m, 2H, P-CH₂N-), 2.73–2.60 (m, 6H, -CH₂N- and Ar-CH₂-), 1.86–1.70 (m, 2H, -CH₂CH₂CH₂-), 1.25 (t, J = 7.1 Hz, 6H, -CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 150.02, 146.33, 129.15, 123.59, 62.10 (d, ² J_{C-P} = 7.1 Hz, -CH₂CH₃), 59.69, 58.04 (d, ³ J_{C-P} = 7.9 Hz, -NCH₂-), 56.08 (d, ³ J_{C-P} = 7.5 Hz, -NCH₂-), 50.74, 49.08, 33.12, 28.46, 16.45 (d, ³ J_{C-P} = 5.7 Hz, -CH₃). MALDI-HRMS (matrix: HCCA) calculated for C₁₆H₂₇N₂O₆P, [M + H]⁺ m/z 375.1680, found 375.1680.

Diethyl (((2-Chloroethyl)(3-(4-nitrophenyl)propyl)amino)-methyl)phosphonate

(3).—To a solution of **2** (4.1 g, 11.0 mmol) and triethylamine (TEA) (3.3 g, 33.0 mmol) in

DCM (30 mL) was added dropwise methanesulfonyl chloride (MsCl) (2.5 g, 22.0 mmol) over 10 min at 0 °C. The resulting mixture was allowed to warm to room temperature and stirred overnight. The solution was washed with water (30 mL × 3), dried over Na₂SO₄, filtered, and concentrated to dryness. The residue was purified by a silica gel (200–300 mesh) column eluted with 50% ethyl acetate (EA)/petroleum ether (PE, bp 60–90 °C) to provide pure **3** (3.4 g, ~79%) as a yellowish oil. ¹H NMR (400 MHz, CDCl₃) δ 8.10 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.32 (d, *J* = 8.7 Hz, 2H, Ar-H), 4.19–4.01 (m, 4H, -OCH₂CH₃), 3.51 (t, *J* = 6.8 Hz, 2H, -CH₂Cl), 3.012.87 (m, 4H, P-CH₂N- and -Ar-CH₂-), 2.80–2.64 (m, 4H, -NCH₂-), 1.84–1.70 (m, 2H, -CH₂CH₂CH₂-), 1.29 (t, *J* = 7.1 Hz, 6H, -CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 150.13, 146.37, 129.19, 123.63, 61.97 (d, ²*J*_{C-P} = 7.0 Hz, -CH₂CH₃), 57.10 (d, ³*J*_{C-P} = 6.8 Hz, -NCH₂-), 55.16 (d, ³*J*_{C-P} = 7.3 Hz, -NCH₂-), 50.19 (d, ¹*J*_{C-P} = 160.2 Hz, P-CH₂-N), 41.77, 33.05, 28.88, 16.52 (d, ³*J*_{C-P} = 5.8 Hz, -CH₃). MALDI-HRMS (matrix: HCCA) calculated for C₁₆H₂₆ClN₂O₅P, [M + H]⁺ *m/z* 393.1341, found 393.1341.

Di-tert-butyl 2,2'-(7-(2-(((diethoxyphosphoryl)methyl)(3-(4-nitrophenyl)propyl)amino)ethyl)-1,4,7-triazacyclononane-1,4-diyl)diacetate (4).

—To a solution of NO₂AtBu (0.9 g, 2.5 mmol) and *N,N*-diisopropylethylamine (DIEA) (1.0 g, 8.0 mmol) in 50 mL MeCN was added **3** (1.2 g, 2.6 mmol). The resulting mixture was stirred at room temperature for 24 h. After evaporating the solvent, the residue was treated with deionized (DI) water (20 mL) and extracted with DCM. The organic layer was dried and concentrated. The residue was purified by a silica gel (200–300 mesh) column eluted with 210% MeOH in DCM to obtain **4** (0.9 g, ~51%) as a yellowish solid. ¹H NMR (400 MHz, CDCl₃) δ 8.12 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.35 (d, *J* = 8.7 Hz, 2H, Ar-H), 4.08 (p, *J* = 7.2 Hz, 4H, -OCH₂-), 3.68 (brs, 4H, -NCH₂-), 3.56–3.12 (m, 12H, -NCH₂- and -NCH₂CO-), 2.90 (d, *J* = 10.4 Hz, 2H, -NCH₂P-), 2.73 (m, 8H, -NCH₂- and Ar-CH₂-), 1.88–1.78 (m, 2H, -CH₂CH₂CH₂-), 1.47–1.40 (m, 18H, 6 X-CH₃), 1.36–1.23 (m, 6H, -OCH₂H₃). ¹³C NMR (101 MHz, CDCl₃) δ 170.79, 170.72, 149.90, 146.35, 129.26, 123.62, 81.94, 81.57, 62.19, 62.12, 57.89, 56.76, 55.91, 55.81, 53.67, 53.12, 52.82, 51.87, 50.56, 50.43, 49.75, 48.96, 48.81, 44.64, 33.14, 28.11, 16.54, 16.48. MALDI-HRMS (matrix: HCCA) calculated for C₃₄H₆₀N₅O₉P, [M + H]⁺ *m/z* 714.4201, found 714.4217.

2,2'-(7-(2-((3-(4-Nitrophenyl)propyl)(phosphonomethyl)-amino)ethyl)-1,4,7-triazacyclononane-1,4-diyl)diacetic acid (5).

—To a solution of **4** (390 mg, 0.5 mmol) in 2 mL acetic acid at 0 °C was added dropwise 37% HBr in acetic acid (5 mL). The resulting mixture was warmed to room temperature and stirred for 1 d. The solvent was evaporated in vacuo. The residue was taken up in DI water and passed through a 0.45 μm Nylon syringe filter. The aqueous solution was concentrated in vacuo to provide **5** as HBr salts (242 mg, ~80%) as a yellowish solid. ¹H NMR (400 MHz, D₂O) δ 8.02 (d, *J* = 8.7 Hz, 2H, Ar-H), 7.36 (d, *J* = 8.7 Hz, 2H, Ar-H), 3.97 (s, 4H, -CH₂CO₂H), 3.58–3.00 (m, 20H, -CH₂N-), 2.73 (t, *J* = 7.4 Hz, 2H, Ar-CH₂-), 2.12–1.99 (m, 2H, -CH₂CH₂CH₂-). ¹³C NMR (101 MHz, D₂O) δ 176.63, 170.70, 148.72, 146.23, 129.43, 123.86, 56.61, 55.92, 55.86, 51.87, 51.11, 50.91, 50.49, 50.14, 49.54, 49.13, 31.55, 24.19, 20.36. ³¹P NMR (202 MHz, DMSO-*d*₆) δ 13.23. MALDI-HRMS (matrix: HCCA) calculated for C₂₂H₃₆N₅O₉P, [M + H]⁺ *m/z* 546.2323, found 546.2325.

Di-tert-Butyl 2,2'-(7-(2-((3-(4-aminophenyl)propyl)-((diethoxyphosphoryl)methyl)amino)ethyl)-1,4,7-triazacyclononane-1,4-diyl)diacetate (6).—To a solution of **5** (150 mg, 275 μ mol) in MeOH was added 10% Pd/C. The resulting mixture was bubbled with H₂ at room temperature for 3 h. The reaction mixture was filtered and the filtrate was concentrated in vacuo to provide pure **6** (149 mg, ~79%). ¹H NMR (400 MHz, CDCl₃) δ 6.90 (d, *J* = 8.2 Hz, 2H, Ar-H), 6.53 (d, *J* = 8.3 Hz, 2H, Ar-H), 4.10–3.97 (m, 4H, -CH₂CO₂), 3.73–2.49 (m, 26H, -CH₂N-), 2.43 (t, *J* = 7.4 Hz, 2H, Ar-CH₂-), 1.67 (m, 2H, -CH₂CH₂CH₂-), 1.38 (s, 18H, -CH₃), 1.31–1.19 (m, 6H, -CH₂CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 170.71, 144.54, 131.26, 129.09, 115.26, 81.59, 62.20, 62.13, 57.65, 55.54, 53.81, 52.79, 50.72, 50.18, 49.38, 48.57, 32.14, 28.32, 28.13, 16.54, 16.48. MALDI-HRMS (matrix: HCCA) calculated for C₃₄H₆₂N₅O₇P, [M + H]⁺ *m/z* 684.4460, found 684.4471.

2,2'-(7-(2-((3-(4-Aminophenyl)propyl)-(phosphonomethyl)amino)ethyl)-1,4,7-triazacyclononane-1,4-diyl)diacetic acid (7).—To **6** (100 mg, 146 μ mol) at 0 °C in 2 mL acetic acid was added dropwise 37% HBr in acetic acid (5 mL). The reaction mixture was warmed to room temperature and stirred for 1 d. The solvent was evaporated in vacuo. The residue was taken up in DI water and passed through a 0.45 μ m Nylon syringe filter. The aqueous solution was concentrated in vacuo to provide **7** as HBr salts (75 mg, ~90%) as a yellow solid. ¹H NMR (400 MHz, D₂O) δ 7.26 (m, 4H, Ar-H), 3.96 (s, 4H, -CH₂CO₂H), 3.58–2.97 (m, 20H, -CH₂N-), 2.65 (t, *J* = 6.8 Hz, 2H, Ar-CH₂-), 2.00 (s, 2H, -CH₂CH₂CH₂-). ¹³C NMR (101 MHz, D₂O) δ 141.59, 130.11, 127.93, 123.17, 56.68, 55.98, 51.88, 51.12, 50.82, 50.54, 50.46, 50.16, 49.18, 31.08, 24.52. MALDI-HRMS (matrix: HCCA) calculated for C₂₂H₃₈N₅O₇P, [M + H]⁺ *m/z* 516.2582, found 516.2582.

2,2'-(7-(2-((3-(4-Isothiocyanatophenyl)propyl)-(phosphonomethyl)amino)ethyl)-1,4,7-triazacyclononane-1,4-diyl)diacetic acid (8).—To a solution of **7** (16.5 mg, 32 μ mol) in water (0.5 mL) was added a 1 M solution of thiophosgene in CHCl₃ (0.5 mL). The resulting two-phase mixture was stirred intensely for 4 h at room temperature. The aqueous layer was separated and washed with CHCl₃. The aqueous layer was evaporated to dryness to provide **8** (15 mg, ~84%) as a yellowish solid. ¹H NMR (400 MHz, D₂O) δ 7.37–7.15 (m, 4H, Ar-H), 3.90–3.77 (m, 4H, -N-CH₂-CO-), 3.71–2.80 (m, 20H, -N-CH₂-), 2.73–2.59 (m, 2H, Ar-CH₂-), 2.09–1.94 (s, 2H, -CH₂CH₂CH₂-). ³¹P NMR (202 MHz, D₂O) δ 7.29. MALDI-HRMS (matrix: HCCA) calculated for C₂₃H₃₆N₅O₇PS, [M + H]⁺ *m/z* 558.2146, found 558.2152.

tert-Butyl 1,4,7-triazacyclononane-1-carboxylate (9).—To a solution of 1,4,7-triazacyclononane (TACN) (6.2 g, 50.0 mmol) in 50 mL CHCl₃ at 0 °C was added dropwise 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON) (9.8 g, 40.0 mmol) in 100 mL CHCl₃ over 2 h. The resulting mixture was stirred for 72 h at room temperature. The resulting mixture was washed by 10% NaOH (50 mL $\times$ 3) and concentrated. The residue was added 10 mL DI water and adjusted to pH 7 using 1 M HCl and extracted with EA (50 mL $\times$ 3). Then the aqueous layer was adjusted to pH 8 and extracted with EA (50 mL $\times$ 1). The aqueous layer was adjusted to pH 10 and extracted with DCM (50 mL $\times$ 3). The DCM organic layer was dried over Na₂SO₄, filtered, and evaporated to give **9** (5.6 g, ~61%) as a

colorless oil. ^1H NMR (400 MHz, CDCl_3) δ 4.55 (m, 2H, NH), 3.40 (brs, 4H, CO-N- CH_2), 3.16–2.91 (m, 4H, N- CH_2), 2.83 (s, 4H, N- CH_2), 1.42 (s, 9H, - CH_3). ^{13}C NMR (101 MHz, CDCl_3) δ 155.32, 80.6, 53.44, 53.20, 47.84, 47.79, 47.03, 28.45.

tert-Butyl 4,7-bis((diethoxyphosphoryl)methyl)-1,4,7-triazacyclononane-1-carboxylate (10).—To a solution of **9** (3.4 g, mmol) in 100 mL MeCN was added K_2CO_3 (4.6 g, 32.9 mmol) and diethyl *p*-toluenesulfonyloxymethyl phosphonate (10.6 g, 32.9 mmol). The reaction mixture was heated to 70 °C for 8 h. The resulting solution was filtered and concentrated. The residue was purified using silica column (200–300 mesh) eluted with 10% MeOH in DCM to obtain **10** (4.2 g, ~52%) as a colorless oil. ^1H NMR (400 MHz, CDCl_3) δ 4.16–3.97 (m, 8H, - CH_2CH_3), 3.39–3.25 (m, 4H, $\text{CH}_2\text{-P}$), 3.10–2.93 (m, 8H, - CH_3), 2.77 (s, 4H), 1.46–1.32 (m, 9H, - CH_3), 1.32–1.18 (m, 12H, - CH_2CH_3). ^{13}C NMR (101 MHz, CDCl_3) δ 155.31, 79.36, 61.67 (m, 4C, - $\text{OCH}_2\text{-}$), 56.61 (d, $^3J_{\text{C-P}} = 7.6$ Hz, -N- $\text{CH}_2\text{-}$), 55.87 (d, $^3J_{\text{C-P}} = 7.2$ Hz, -N- $\text{CH}_2\text{-}$), 54.88 (d, $^3J_{\text{C-P}} = 4.9$ Hz, -N- $\text{CH}_2\text{-}$), 54.55 (d, $^3J_{\text{C-P}} = 7.0$ Hz, -N- $\text{CH}_2\text{-P}$), 49.26, 48.56, 28.48, 16.47 (m, 4C, - CH_3). MALDI-HRMS (matrix: HCCA) calculated for $\text{C}_{21}\text{H}_{45}\text{N}_3\text{O}_8\text{P}_2$, $[\text{M} + \text{Na}]^+$ m/z 552.2574, found 552.2572.

Tetraethyl ((1,4,7-triazacyclononane-1,4-diyl)bis(methylene))bis(phosphonate) (11).—Compound **10** (1 g) in an ice bath was treated with 10 mL TFA/DCM 1:1, gradually allowed to warm to ambient temperature, and stirred for 12 h. The result mixture was evaporated and coevaporated with 20 mL CHCl_3 3 times to obtain **11** (~100%) as a yellow oil as its TFA salts. ^1H NMR (400 MHz, CDCl_3) δ 4.12–4.01 (m, 8H, - CH_2CH_3), 3.99–3.77 (m, 3H, NH and TFA-H), 3.06–2.96 (m, 4H, $\text{CH}_2\text{-P}$), 2.96–2.67 (m, 12H, N- CH_2), 1.31–1.22 (m, 12H, - CH_2CH_3). ^{13}C NMR (101 MHz, CDCl_3) δ 61.70 (d, $^2J_{\text{C-P}} = 7.0$ Hz, - $\text{OCH}_2\text{-}$), 53.92 (d, $^3J_{\text{C-P}} = 2.8$ Hz, -N- CH_2), 53.65 (d, $^3J_{\text{C-P}} = 6.1$ Hz, -N- CH_2), 51.90 (d, $^1J_{\text{C-P}} = 153.6$ Hz, -N- $\text{CH}_2\text{-P}$), 45.90, 16.51 (d, $^3J_{\text{C-P}} = 5.7$ Hz, - CH_3). MALDI-HRMS (matrix: HCCA) calculated for $\text{C}_{16}\text{H}_{37}\text{N}_3\text{O}_6\text{P}_2$, $[\text{M} + \text{Na}]^+$ m/z 452.2050, found 452.2048.

tert-Butyl 2-((2-(4,7-bis((diethoxyphosphoryl)methyl)-1,4,7-triazonan-1-yl)ethyl)(3-(4-nitrophenyl)propyl)amino)-acetate (13).—To a solution of **12** (1.0 g, 2.3 mmol) and DIEA (0.7 g, 5.6 mmol) in 50 mL MeCN was added **11** (0.7 g, 1.8 mmol). The reaction mixture was stirred at room temperature for 24 h. After evaporating the solvent, the residue was treated with DI water 20 mL and extracted with EA. The organic layer was dried and concentrated. The residue was purified using silica column (200–300 mesh) eluted with 2–10% MeOH in DCM to obtain **13** (0.6 g, ~44%) as a yellow solid. ^1H NMR (400 MHz, CDCl_3) δ 8.11 (d, $J = 8.6$ Hz, 2H, $\text{NO}_2\text{-Ar-H}$), 7.34 (t, $J = 8.9$ Hz, 2H, Ar-H), 4.18–4.02 (m, 8H, 4 X- OCH_2), 3.23 (s, 2H, - $\text{NCH}_2\text{CO-}$), 3.01–2.66 (m, 22H, N- $\text{CH}_2\text{-}$ and - $\text{NCH}_2\text{-P}$), 2.64–2.57 (m, 2H, Ar- CH_2), 1.83–1.69 (m, 2H, - $\text{CH}_2\text{CH}_2\text{CH}_2\text{-}$), 1.42 (s, 9H, - CH_3), 1.30 (m, 12H, 4 X- CH_2CH_3). ^{13}C NMR (101 MHz, CDCl_3) δ 170.94, 149.97, 146.28, 129.38, 123.59, 81.74, 62.19 (d, $^2J_{\text{C-P}} = 6.9$ Hz, - OCH_2), 55.69, 54.13, 53.95, 53.17, 52.56, 51.03, 50.73, 50.01, 33.21, 28.37, 28.09, 16.55 (d, $^3J_{\text{C-P}} = 5.6$ Hz, - OCH_2CH_3). MALDI-HRMS (matrix: HCCA) calculated for $\text{C}_{33}\text{H}_{61}\text{N}_5\text{O}_{10}\text{P}_2$, $[\text{M} + \text{H}]^+$ m/z 750.3966, found 750.3957.

2-((2-(4,7-Bis(phosphonomethyl)-1,4,7-triazonan-1-yl)-ethyl)(3-(4-nitrophenyl)propyl)amino)acetic acid (14).—To **13** (600 mg, 0.8 mmol) at 0 °C in 2 mL acetic acid was added dropwise 37% HBr in acetic acid (5 mL). The resulting mixture was warmed to room temperature and stirred for 1 d. The solvent was evaporated in vacuo. The residue was taken up in DI water and passed through a 0.45 μ m Nylon syringe filter. The aqueous solution was concentrated in vacuo to provide **14** as HBr salts (400 mg, ~86%) as a yellow solid. ¹H NMR (400 MHz, D₂O) δ 7.98 (d, J = 8.3 Hz, 2H, NO₂-Ar-H), 7.31 (d, J = 8.5 Hz, 2H, Ar-H), 4.06–3.92 (m, 2H, COCH₂N), 3.52–2.90 (m, 22H, N-CH), 2.69 (t, J = 7.3 Hz, 2H, Ar-CH₂-), 2.10–1.93 (m, 2H, -CH₂-). ¹³C NMR (101 MHz, D₂O) δ 168.82, 148.64, 146.22, 129.48, 123.85, 54.70, 54.02, 52.62, 51.83, 51.53, 51.12, 50.07, 48.59, 31.52, 24.13. ³¹P NMR (202 MHz, DMSO-*d*₆) δ 15.09. MALDI-HRMS (matrix: HCCA) calculated for C₂₁H₃₇N₅O₁₀P₂, [M + H]⁺ m/z 582.2088, found 582.2097.

tert-Butyl N-(3-(4-aminophenyl)propyl)-N-(2-(4,7-bis-((diethoxyphosphoryl)methyl)-1,4,7-triazonan-1-yl)ethyl)-glycinate (15).—To a solution of **13** (150 mg, 275 μ mol) in MeOH was added 10% Pd/C (20 mg). The resulting mixture was bubbled with H₂ at room temperature for 3 h. The reaction mixture was filtered and the filtrate was concentrated in vacuo to provide pure **15** (173 mg, ~92%). ¹H NMR (400 MHz, CDCl₃) δ 6.95 (d, J = 7.8 Hz, 2H, NO₂-Ar-H), 6.59 (d, J = 8.1 Hz, 2H, Ar-H), 4.09 (p, J = 7.1 Hz, 8H, 4 X-OCH₂), 3.18 (s, 2H, -NCH₂CO-), 3.11–2.51 (m, 22H, N-CH₂- and -NCH₂-P), 2.46 (t, J = 7.5 Hz, 2H, Ar-CH₂), 1.78–1.59 (m, 2H, -CH₂CH₂CH₂-), 1.41 (s, 9H, -CH₃), 1.34–1.22 (m, 12H, 4 X-CH₂CH₃). ¹³C NMR (101 MHz, CDCl₃) δ 170.91, 144.26, 132.05, 129.08, 115.20, 80.78, 61.70 (d, ²*J*_{C-P} = 6.9 Hz), 56.55, 56.04, 55.30, 54.29, 54.10, 52.73, 52.31, 32.54, 31.54, 29.47, 28.15, 22.60, 16.53 (d, ³*J*_{C-P} = 5.8 Hz), 14.08. ³¹P NMR (202 MHz, D₂O) δ 11.53. MALDI-HRMS (matrix: HCCA) calculated for C₃₃H₆₃N₅O₈P₂, [M + H]⁺ m/z 720.4225, found 720.4213.

N-(3-(4-Aminophenyl)propyl)-N-(2-(4,7-bis-(phosphonomethyl)-1,4,7-triazonan-1-yl)ethyl)glycine (16).—To **15** (80 mg, 110 μ mol) at 0 °C in 2 mL acetic acid was added dropwise 37% HBr in acetic acid (5 mL). The reaction mixture was warmed to room temperature and stirred for 1 d. The solvent was evaporated in vacuo. The residue was taken up in DI water and passed through a 0.45 μ m Nylon syringe filter. The aqueous solution was concentrated in vacuo to provide **16** as HBr salts (50 mg, ~91%) as a yellow solid. ¹H NMR (400 MHz, D₂O) δ 7.32–7.20 (m, 4H, Ar-H), 3.98 (s, 2H, NCH₂CO-), 3.51–2.94 (m, 22H, -N-CH₂-), 2.64 (t, J = 7.3 Hz, 2H, Ar-CH₂-), 2.10–1.89 (m, 2H, -CH₂CH₂CH₂-). ¹³C NMR (101 MHz, D₂O) δ 168.86, 141.48, 130.09, 127.92, 123.15, 54.70, 54.06, 52.65, 51.82, 51.78, 51.55, 51.49, 50.95, 50.03, 48.56, 31.04, 24.38. MALDI-HRMS (matrix: HCCA) calculated for C₂₁H₃₉N₅O₈P₂, [M + H]⁺ m/z 552.2347, found 552.2347.

N-(2-(4,7-Bis(phosphonomethyl)-1,4,7-triazonan-1-yl)-ethyl)-N-(3-(4-isothiocyanatophenyl)propyl)glycine (17).—To a solution of **16** (14 mg, 25 μ mol) in water (0.5 mL) was added a 1 M solution of thiophosgene in CHCl₃ (0.5 mL). The resulting two-phase mixture was stirred intensely for 4 h at room temperature. The aqueous layer was separated and washed with CHCl₃. The aqueous layer was evaporated to dryness to provide

17 (12.5 mg, ~83%) as a yellowish solid. ^1H NMR (400 MHz, D_2O) δ 7.23–7.14 (m, 4H, Ar-H), 3.87 (s, 2H, COCH_2N), 3.46–2.86 (m, 22H, N-CH), 2.67–2.57 (m, 2H, Ar- CH_2 -), 2.06–1.89 (m, 2H, $-\text{CH}_2$ -). MALDI-HRMS (matrix: HCCA) calculated for $\text{C}_{22}\text{H}_{37}\text{N}_5\text{O}_8\text{P}_2\text{S}$, $[\text{M} + \text{H}]^+ m/z$ 594.1911, found 594.1919.

Synthesis of NE2A1P-PEG₄-LLP2A.

To a solution of LLP2A-PEG₄ and DIEA (3 equiv) in 100 μL DMF was added *p*-SCN-Bn-NE2A1P (1.2 equiv). The mixture was reacted at room temperature for 4 h. The solvent was lyophilized, and the residue was purified using HPLC. HPLC method: Luna C-18 (150 $\times$ 4.6 mm), 18% acetonitrile with 0.1% TFA for 3 min then increase to 35% acetonitrile with 0.1% TFA over 30 min with flow at 1.5 mL/min and UV absorbance at 280 nm; retention time, 19.7 min. ESI-MS: observed, m/z $[\text{M}+2\text{H}]^{2+} = 865.397$, calculated, $[\text{M}+2\text{H}]^{2+} = 865.417$.

Synthesis of NE2P1A-PEG₄-LLP2A.

To a solution of LLP2A-PEG₄ and DIEA (3 equiv) in 100 μL DMF was added *p*-SCN-Bn-NE2P1A (1.2 equiv). The mixture was reacted at room temperature for 4 h. The solvent was lyophilized, and the residue was purified using HPLC. HPLC method: Luna C-18 (150 $\times$ 4.6 mm), 18% acetonitrile with 0.1% TFA for 3 min then increase to 35% acetonitrile with 0.1% TFA over 30 min with flow at 1.5 mL/min and UV absorbance at 280 nm; retention time, 22.6 min. ESI-MS: observed, m/z $[\text{M}+2\text{H}]^{2+} = 883.409$, calculated, $[\text{M}+2\text{H}]^{2+} = 883.406$.

Radiolabeling.

100 pmol of bioconjugates in 100 μL 0.1 M NaOAc buffers (pH 4.0 and pH 6.8) were added 3.7 MBq ^{68}Ga . The molar activity was aimed at 37 MBq/nmol. The mixture was vortexed for 10 s, and incubated in a thermomixer at 37 $^\circ\text{C}$ and/or 70 $^\circ\text{C}$ for 10 min. The radiolabeling yield was determined by radio-HPLC. HPLC method: Luna C-18 (150 $\times$ 4.6 mm), 5% acetonitrile with 0.1% TFA for 5 min, then increased to 100% acetonitrile with 0.1% TFA over 2 min and the ratio held for 8 min with flow at 1.5 mL/min. Free ^{68}Ga comes out at 2.7 min and the radiolabeled conjugates come out at 11.1 min.

Stability.

Serum stability: ^{68}Ga labeled bioconjugates (~3.7 MBq) were mixed with 0.5 mL human serum, and the resulting mixtures were incubated at 37 $^\circ\text{C}$ for 2 h. After incubation, plasma protein was precipitated with 0.5 mL acetonitrile and centrifuged at 14 000 rpm for 5 min. The supernatants were collected and more than 90% activity were recovered determined by a dose calibrator. The radio-purity was determined by radio-HPLC. More than 95% intact radiolabeled conjugates were observed.

Kinetic inertness: ^{68}Ga -NE2P1A-PEG₄-LLP2A (~3.7 MBq, 10 μL) was added to solutions of 0.1 M PBS, and 1 mM ZnCl_2 (each 0.5 mL). The resulting mixtures were incubated at 37 $^\circ\text{C}$ for 2 h. The kinetic inertness was determined by radio-HPLC. HPLC method: starting with 5% acetonitrile with 0.1% TFA, and then increased to 90% acetonitrile with 0.1% TFA over 5 min, and the ratio was kept for 10 min with flow at 1.0 mL/min. Free ^{68}Ga comes out at 4 min and the radiolabeled conjugate come out at 10 min.

In vivo metabolism: Two C57BL/6 mice were injected intravenously with ^{68}Ga -NE2P1A-PEG₄-LLP2A (~20 MBq/mouse). The mice were sacrificed after 30 min. The blood sample were collected and combined. The blood sample was centrifuged at 14 000 rpm for 5 min to remove red blood cells. The supernatant was further treated with acetonitrile and centrifuged at 14 000 rpm for 5 min to remove plasma protein. The supernatant was used for radio-HPLC analysis. Urine sample were also collected, combined, treated with acetonitrile, and centrifuged at 14 000 rpm for 5 min. The supernatant was used for radio-HPLC analysis.

DFT Calculation.

All calculations were performed using Gaussian09 suite of programs. Density functional theory method was used, employing uB3LYP hybrid functional.³⁹ Geometry optimization was done using a combined basis set 6-31+G(d,p). Frequency calculation was done at the same level of theory as geometry optimization, to confirm the stationary points to be minima. Single point energy calculations were done using B3LYP method at a larger basis set 6-311+ +G(d,p). Solvent effect was accounted for using self-consistent reaction field (SCRF) method, using SMD model and UAKS radii.^{40,41} Water was used as the solvent. (See the Supporting Information for detailed calculation data.)

Log D Measurements.

Radiolabeled bioconjugates (5 μL) were added to 1 mL of octanol and 1 mL of PBS (10 mM, pH 7.4). The mixture was vortexed for 10 min and then centrifuged for 10 min. Both layers were counted in a gamma counter. The log D was calculated based on formula of $\log D = \log([M]_{\text{oct}}/[M]_{\text{aq}})$. Data are presented as mean of triplicate measurements.

Cell Studies.

B16F10 cell line was purchased from the American Tissue Culture Collection (ATCC). All cell handling was aseptically performed in a laminar flow hood. The B16F10 cells were cultured in Dulbecco's Modified Eagle Medium, supplemented with 10% FBS, penicillin (100 unit/mL), streptomycin (100 $\mu\text{g/mL}$), L-glutamine (300 $\mu\text{g/mL}$), and sodium pyruvate (100 mg/mL), glucose (4.5 g/L), and maintained at 37 °C, 5% CO₂.

Internalization Assay.

Cells were seeded in 12-well plates (200 000 cells per well) 24 h prior to the experiment. Before the experiment, cells were washed with 1 mL HBSS twice and 1 mL media (DMEM with 0.1% BSA and 1 mM Mn²⁺) was added to each well. Cells were then incubated with ^{68}Ga -NE2P1A-PEG₄-LLP2A (~10 pmol per well) with and without the addition of excess LLP2A-PEG₄ (10 μg per well) to determine nonspecific internalization. At each time point (15 min, and 1, 2, and 4 h) radioactive media were aspirated, and the plate was washed twice with HBSS (pH 7.2). To collect the surface-bound fraction, each well was treated with 20 mM sodium acetate-HBSS (pH 4.0) and was incubated at 37 °C for 10 min. After removal of the surface-bound fraction, cell pellets were dissolved in 0.5% SDS. All of the fractions were counted for radioactivity on the gamma counter. The radioactivity in each fraction was measured in a well counter (Packard Cobra II gamma counter). The amount internalized was

the amount of activity in the final cell pellet, corrected for activity in the blocked fractions and background activity. The protein content of each cell lysate sample was determined (BCA Protein Assay Kit, Pierce). The measured radioactivity associated with the cells was normalized to the amount of cell protein present (% AD/mg protein).

Cell Efflux Assay.

Cell efflux assays were performed to compare the cell retention ability of ^{68}Ga -NE2P1A-PEG₄-LLP2A in B16F10 cell line. Cells were seeded in a 96 well plate 24 h prior the experiment. Cells were incubated with ^{68}Ga -NE2P1A-PEG₄-LLP2A (~10 pmol per well) in culture media contained 1 mM MnCl₂ for 2 h at 37 °C. Media was then removed and cells were washed twice with HBSS and fresh media without MnCl₂ was added to the cells. At 0, 30, 60, and 120 min following fresh media addition cells were washed twice and lysed with 0.5% SDS and the activity was determined using a gamma counter. 100% internalized dose was defined as total internalized at 2 h post probe addition.

Animal Model.

Four-week-old female C57BL/6 mice purchased from Jackson laboratories (Bar Harbor, Maine) were used in this study. All animal studies were performed under the Guide for the Care and Use of Laboratory Animals under the auspices of Division of Laboratory Animal Resources (DLAR) of the University of Pittsburgh. This study was approved by the IACUC Committee. For xenograft B16F10 tumors, mice were injected subcutaneously at the right shoulder with one million cells in PBS and BD matrigel (1:1).

Small Animal PET/CT Imaging.

Mice bearing B16F10 xenograft (4 mice per group) were injected intravenously with ^{68}Ga -NE2P1A-PEG₄-LLP2A (5.5–7.4 MBq per mouse) with and without 100 μg unlabeled LLP2A-PEG₄. At 1 and 2 h after injection, mice were anesthetized with 2% isoflurane and small-animal PET/CT was performed. Static images were collected for 10 min using a small animal Inveon PET/CT scanner (Siemens Medical Solution), with a tangential and radial full width at half-maximum of 1.5 mm at the center of the field of view and 1.8 mm at the edge of the field of view. PET and CT images were coregistered using Inveon Research Workstation (IRW) software (Siemens Medical Solutions). PET images were reconstructed with the ordered-subsets expectation maximization 3-dimensional/maximum a posteriori probability algorithm, and the analysis of images was done using IRW. Regions of interest were drawn using the CT scan, and the associated PET activities were calculated using the IRW software.

Ex Vivo Biodistribution.

Mice bearing B16F10 xenograft (4 mice per group) were injected intravenously with ^{68}Ga -NE2P1A-PEG₄-LLP2A (5.5–7.4 MBq per mouse) with and without 100 μg unlabeled LLP2A-PEG₄. The tumor-bearing mice were sacrificed at 1 h, 2 h, and 4 h after injection. The blood, heart, intestines, lungs, liver, spleen, kidneys, muscle, bone, thymus, and tumor were harvested, weighed, and counted in a gamma-counter. The percentage injected dose per

gram of tissue (%ID/g) was determined by decay correction for each sample normalized to a standard of known weight, which was representative of the injected dose.

Statistical Analysis.

Significance analysis of the data was performed by unpaired *t* test using the Prism software (GraphPad Software Inc., San Diego, CA). Multiple groups comparisons were performed by one-way ANOVA with the Greenhouse-Geisser correction. A value of $P < 0.05$ was considered significant and $P < 0.01$ was considered highly significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Biomedical Imaging and Bioengineering grant R21-EB020737, the National Natural Science Foundation of China (81801738), the Fundamental Research Fund for the Chinese Central Universities of Huazhong University of Science and Technology (HUST) (2017KFYXJJ235, 2016YXMS140, 2016YXMS147, 2016JCTD109, and 2014TS090), opening foundation of Hubei key laboratory of molecular imaging (02.03.2017–187), research foundation of Wuhan Union Hospital (02.03.2017–12) and Natural Science Foundation of Hubei Province, China (2016CFB379).

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Figure 1.
Structures of gallium chelators and the peptidomimetic ligand LLP2A-PEG₄.

Figure 2.

^{68}Ga labeling results of four bioconjugates after incubation in 0.1 M NaOAc buffer at different labeling conditions for 10 min (a). Serum stability of ^{68}Ga labeled bioconjugates (~37 MBq/nmol) after incubation in human serum at 37 °C for 2 h (b). ($n = 3$).

Figure 3.
DFT optimized structure of gallium and NE3TA-like complexes.

Figure 4. Cell internalization (a) and efflux (b) results of ^{68}Ga -NE2P1A-PEG₄-LLP2A and ^{64}Cu -NE3TA-PEG₄-LLP2A (both 10 pmol per well with molar activity at ~37 MBq/nmol) in B16F10 cells. ** $P < 0.001$, $n = 4$.

Figure 5. Biodistribution of ^{68}Ga -NE2P1A-PEG₄-LLP2A (5.5–7.5 MBq, ~37 MBq/nmol) with or without a blocking agent (100 μg unlabeled LLP2A-PEG₄) in mice bearing B16F10 tumor (a). Tumor-to-nontumor ratios (b). ($n = 4$).

Figure 6. Maximum intensity projection (MIP) PET/CT images of mice bearing B16F10 tumors at 1 and 2 h pi of ^{68}Ga -NE2P1A-PEG₄-LLP2A (~37 MBq/nmol) and blockade at 1 h (coinjected with 100 μg unlabeled LLP2A-PEG₄). Arrows indicate tumors.

^aReaction conditions: (a) Paraformaldehyde (1.5 equiv), Diethyl phosphite (1.2 equiv), THF; (b) Methanesulfonyl chloride (2 equiv), TEA, DCM; (c) Compound 3 (1.06 equiv), DIEA, MeCN; (d) 10% Pd/C, MeOH; (e) 37% HBr/AcOH; (f) CS₂, CHCl₃, H₂O.

Scheme 1.

Synthesis of *p*-SCN-PhPr-NE2A1P^a

Scheme 2.

Synthesis of *p*-SCN-PhPr-NE2P1A. Reaction conditions: (a) Boc-ON (0.8 equiv), CHCl₃; (b) Diethyl *p*-toluenesulfonyloxymethyl phosphonate (2.2 equiv), K₂CO₃, MeCN; (c) 50% TFA/DCM; (d) 11 (0.8 equiv), DIEA, MeCN; (e) 10% Pd/C MeOH; (f) 37% HBr/AcOH; (g) CSCL₂, CHCl₃, H₂O

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Table 1.Log D of ^{68}Ga Radiolabeled Conjugates ($n = 3$)

	^{68}Ga -NOTA-PEG ₄ -LLP2A	^{68}Ga -NE3TA-PEG ₄ -LLP2A	^{68}Ga -NE2AIP-PEG ₄ -LLP2A	^{68}Ga -NE2PIA-PEG ₄ -LLP2A
Log D	-2.18 ± 0.03	-2.31 ± 0.04	-1.80 ± 0.03	-1.86 ± 0.02