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H₆phospa-Trastuzumab: Bifunctional Methylenephosphonate-based Chelator with ⁸⁹Zr, ¹¹¹In and ¹⁷⁷Lu

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Abstract

The acyclic chelator H₆phospa and the bifunctional derivative *p*-SCN-Bn-H₆phospa have been synthesized using nosyl protection chemistry and evaluated with ⁸⁹Zr, ¹¹¹In, and ¹⁷⁷Lu. The *p*-SCN-Bn-H₆phospa derivative was successfully conjugated to trastuzumab with isotopic dilution assays indicating 3.3 ± 0.1 chelates per antibody and *in vitro* cellular binding assays indicating an immunoreactivity value of 97.9 ± 2.6%. Radiolabeling of the H₆phospa-trastuzumab immunoconjugate was achieved with ¹¹¹In in 70–90% yields at room temperature in 30 minutes, while ¹⁷⁷Lu under the same conditions produced more inconsistent yields of 40–80%. Stability experiments in human serum revealed the ¹¹¹In-phospa-trastuzumab complex to be 52.0 ± 5.3% intact after 5 days at 37 °C, while the ¹⁷⁷Lu-phospa-trastuzumab to be only 2.0 ± 0.3% intact. Small animal SPECT/CT imaging using mice bearing subcutaneous SKOV-3 ovarian cancer xenografts was performed, and it was found that ¹¹¹In-phospa-trastuzumab successfully identified and delineated small (~2 mm in diameter) tumors from surrounding tissues, despite visible uptake in the kidneys and bone due to moderate chelate instability. As predicted from stability assays in serum, the ¹⁷⁷Lu-phospa-trastuzumab conjugate served as a negative control and displayed no tumor uptake, with high uptake in bones indicating rapid and complete radiometal dissociation and suggesting a potential application of H₆phospa in transient lanthanide chelation for bone-delivery. Radiolabeling with ⁸⁹Zr was attempted, but even with elevated temperatures of 37 °C, the maximum observed radiometal incorporation over 18 hours was 12%. It can be concluded from this work that H₆phospa is not superior to the previously studied H₄octapa for use with ¹¹¹In and ¹⁷⁷Lu, but improvements in ⁸⁹Zr radiolabeling were observed over H₄octapa, suggesting H₆phospa to be an excellent starting point for elaboration of ⁸⁹Zr-based radiopharmaceutical

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development. To our knowledge, H₆phospa is the best desferrioxamine alternative for ⁸⁹Zr radiolabeling to be studied to date.

Introduction

Recent years have witnessed a surge in interest in the development and application of ⁸⁹Zr-based radiopharmaceuticals for positron emission tomography (PET) imaging.^{1–12} A large part of this attention can be attributed to the intermediate half-life ($t_{1/2} = \sim 3.3$ days) of ⁸⁹Zr, a property that makes the isotope nearly ideal for use with biological vectors that have long circulation times, such as antibodies and nanoparticles.^{1–6, 13} Very few isotopes combine the nuclear properties of an intermediate half-life (2–7 days) with a suitable positron emission for PET imaging, making ⁸⁹Zr uniquely situated amongst its radiometal peers.⁴ To date, the only chelator proven competent enough for use with ⁸⁹Zr is the acyclic hydroxamate-based desferrioxamine (DFO), which can quantitatively radiolabel with ⁸⁹Zr in less than one hour at room temperature; most chelators, it is important to note, cannot adequately complex ⁸⁹Zr under any conditions in aqueous media.^{1, 2, 6, 13} DTPA is currently the best alternative chelator to DFO for ⁸⁹Zr radiolabeling, but can only achieve radiolabeling yields of < 0.1% after 1 hour at room temperature.¹⁴ Despite the excellent radiolabeling properties and sufficient *in vitro* and *in vivo* stability of DFO, over prolonged periods of time, some ⁸⁹Zr can be observed to decomplex, leach out of the DFO chelate, and ultimately accumulate in the skeletal system.^{1, 2, 6, 13} Because of this mild shortcoming of DFO, the goal of discovering a new chelator that can quickly and completely radiolabel ⁸⁹Zr under mild conditions, while concomitantly improving on the thermodynamic stability and kinetic inertness of DFO would be of great interest and utility towards the translation of ⁸⁹Zr from the bench to the clinic. Due to the propensity of Zr(IV) to quickly precipitate, aggregate, and form polynuclear oxo/hydroxo species at typical radiolabeling pHs (2–8), an acyclic chelator with very rapid radiolabeling kinetics is required.² Additionally, due to the ideal pairing of ⁸⁹Zr with heat-sensitive antibodies, the room-temperature radiolabeling properties that most acyclic chelators provide are likewise crucial.

Extending the focus to more common radiometals, ¹¹¹In and ¹⁷⁷Lu are two widely used radiometals that, unlike ⁸⁹Zr, have been employed for decades in both the laboratory and clinic.^{15, 16} These two isotopes are most effectively used as an imaging/therapy pair, with ¹¹¹In typically used for single photon emission computed tomography (SPECT) imaging ($t_{1/2} \sim 2.8$ days) and pre-therapy dosimetry calculations, and ¹⁷⁷Lu typically used for therapy ($t_{1/2} \sim 6.6$ days). Although chelators such as DOTA, CHX-A''-DTPA, and most recently H₄octapa have been found to be effective for use with ¹¹¹In and ¹⁷⁷Lu, new and highly stable acyclic chelators with rapid room temperature radiolabeling kinetics and a variety of physical properties (e.g. charge, denticity, donor atoms) are always of interest.^{17–22}

A number of recent works have illustrated that the use of methylenephosphonate groups in chelators can provide improved radiolabeling properties with a variety of radiometals, most notably accelerated reaction kinetics that allow for faster and lower temperature radiolabel incorporation.^{23–30} In particular, the replacement of carboxylic acid groups with

methylenephosphonates has yielded both improved reaction kinetics, and enhanced chelate stability. Most notable is the example of CB-TE2A, in which the replacement of one (CB-TE1A1P) or both (CB-TE2P) carboxylic acid arms with methylenephosphonate groups resulted in improved radiolabeling kinetics, with *in vitro* and *in vivo* stability being retained or enhanced compared to the CB-TE2A parent chelator.^{23–28} The use of methylenephosphonate groups with our existing “pa” family of acyclic chelators (Chart 1), such as H₂dedpa, H₄octapa, H₂azapa, and H₅decapa is enticing, as it could potentially provide improved radiochemical properties.^{17, 31–33}

The idea of improving radiolabeling kinetics by replacing carboxylic acid chelating arms with methylenephosphonate groups led us to the synthesis of H₆phospa (Chart 1). H₆phospa is a methylenephosphonate derivative of the previously published acyclic chelator H₄octapa, with 8 potential donor arms in an N₄O₄ set (octadentate).¹⁷ Previous attempts to radiolabel the H₄octapa-trastuzumab immunoconjugate with ⁸⁹Zr in a variety of buffers (including HEPES, ammonium acetate, phosphate buffered saline) resulted in no radiometal incorporation as evaluated by radio-iTLC (unpublished data). It was hypothesized that replacement of the acetic acid arms of H₄octapa with methylenephosphonate arms might improve radiochemical yields and reaction kinetics. Fortuitously, the methylenephosphonate derivative of H₄octapa had been previously published by Rodriguez-Blas and co-workers, and so the synthesis of the octadentate chelator H₆L² (which we have now called H₆phospa) was performed using a modified literature procedure in 1 step from the Me₂dedpa precursor,^{34, 35} and the new and unpublished bifunctional derivative *p*-SCN-Bn-H₆phospa was synthesized in 6 steps. Subsequently, *p*-SCN-Bn-H₆phospa was conjugated to the antibody trastuzumab, and radiolabeling experiments were performed with ⁸⁹Zr, ¹¹¹In, and ¹⁷⁷Lu, in order to assess the potential of H₆phospa-based conjugates for application with these three radiometals for molecular imaging agents (SPECT/PET) and therapeutics.

Results and Discussion

Synthesis and Characterization

An important modification of our literature procedure was used to synthesize the acyclic chelator H₆phospa.^{17, 34–36} The precursor Me₂dedpa (**1**) was synthesized according to our previous work in 3 steps utilizing unique 2-nitrobenzenesulfonamide (nosyl) protection chemistry in a cumulative yield of 70–75% (Scheme 1).³⁶ Purification of the Me₂dedpa precursor was performed using neutral alumina, due to the high affinity of this molecule to silica. To afford H₆phospa, a one-pot procedure was used. Me₂dedpa and phosphorous acid were first refluxed together in HCl (6 M) for a short period of time (~1 h), followed by the slow addition of paraformaldehyde to form a transient imine, which could then react with phosphorous acid to yield the methylenephosphonate product. If the paraformaldehyde reagent were added first or added too quickly, the *N*-methylated product would form as a major product, which would greatly reduce yields and complicate purification. The product H₆phospa (**2**) formed an insoluble HCl salt after reducing the crude reaction mixture to dryness *in vacuo*, resuspending in HCl (6 M), and filtering.

Nosyl protection chemistry was crucial for the synthesis of *p*-SCN-Bn-H₆phospa, as it allowed for deprotection under mild conditions (no strong acid/base or hydrogenation) with

thiophenol and potassium carbonate in tetrahydrofuran at room temperature. The use of benzyl protection chemistry was not feasible for this synthesis, as deprotection would require hydrogenation (Scheme 1, step iv) that would transform the *p*-nitrobenzyl functionality to *p*-aniline. The aniline primary amine would then react (Scheme 1, step v) with paraformaldehyde and phosphorous acid creating unwanted *N*-methyl/methylenephosphonate products, and prevent later isothiocyanate formation. The bifunctional derivative *p*-SCN-Bn-H₆phospa (**10**) was synthesized for the first time in 6 steps with a low cumulative yield of ~4%. Briefly, the isothiocyanate precursor *p*-NO₂-Bn-H₆phospa (**9**) was synthesized through an analogous pathway to H₆phospa, with the *p*-NO₂-Bn-Me₂dedpa precursor made first, followed by a very similar methylenephosphonate formation in HCl (6 M) with paraformaldehyde and phosphorous acid. The *p*-NO₂-Bn-H₆phospa product was purified via semi-preparative reverse-phase (RP) HPLC, hydrogenated to form *p*-NH₂-Bn-H₆phospa, and finally reacted with thiophosgene in chloroform and HCl (3 M) to yield *p*-SCN-Bn-H₆phospa (**10**), which was lyophilized after RP-HPLC purification to yield an off-white powder.

Once synthesized, H₆phospa was reacted with ZrCl₄, In(ClO₄)₃, and LuCl₃ in three attempts to make non-radioactive metal complexes. The In³⁺ complex was identified by low-resolution and high-resolution mass spectrometry, but was too insoluble to perform other characterization techniques, and no crystals were obtained for X-ray solid-state structure determination. The Lu³⁺ complex was also very insoluble, could not be detected by mass spectrometry, and, like the In³⁺ complex, could not be characterized by other techniques (for example, they were not soluble in MeOD, D₂O or DMSO-*d*₆ for NMR spectroscopic analysis). The Zr⁴⁺ complex also could not be detected by mass spectrometry or characterized by other techniques, though it was observed that a fine white precipitate formed shortly after addition of the zirconium salt to an aqueous solution of H₆phospa. Although synthesis of these metal complexes was attempted, none could be confirmed. Radiolabeling experiments (*vide infra*) with ¹¹¹In and ¹⁷⁷Lu have confirmed that coordination of these metal ions does indeed occur, while radiolabeling experiments with ⁸⁹Zr have demonstrated that the H₆phospa complex of this metal forms inefficiently in low yields.

Antibody Modification, ¹¹¹In and ¹⁷⁷Lu Radiolabeling, and *In Vitro* Characterization

Moving beyond radiolabeling efficiency and chemical characterization, the *in vitro* and *in vivo* stability of a chelator will determine its ultimate value for use in radiopharmaceuticals. To this end, a model system based on the HER2/*neu*-targeting antibody trastuzumab and HER2-expressing SKOV-3 ovarian cancer cells was used. After modification of the purified trastuzumab with *p*-SCN-Bn-H₆phospa and subsequent radiolabeling experiments, the resulting radioimmunconjugates were characterized by radio-iTLC, radiometric isotopic dilution assays, cellular binding assays, serum stability challenges, and *in vivo* SPECT/CT imaging using female nude athymic mice bearing subcutaneous SKOV-3 xenografts (Table 1).

The bifunctional chelate derivative *p*-SCN-Bn-H₆phospa was conjugated to purified trastuzumab via incubation under mildly basic conditions (pH 8.5–9.0) with 4 equivalents of

p-SCN-Bn-H₆phospa, and the resulting conjugates were purified via size exclusion chromatography (PD-10, GE Healthcare). Subsequent radiometric isotopic dilution experiments revealed that 3.3 ± 0.1 accessible chelates per antibody had been successfully conjugated (Table 1). H₆phospa-trastuzumab was then radiolabeled with either ¹¹¹In or ¹⁷⁷Lu in NH₄OAc buffer (pH 5.5, 200 mM) for 30 min at room temperature, producing ¹¹¹In-labeled radioimmunoconjugates with acceptable yields (70–90% radiochemical yield) and high specific activities (2.9 ± 0.7 mCi/mg) (Table 1). Radiolabeling experiments with ¹⁷⁷Lu resulted in inconsistent yields (40–80% radiochemical yield) but high specific activities (2.8 ± 0.8 mCi/mg). The radiochemical purity of ¹¹¹In-phospa-trastuzumab was excellent after purification (>99%), but iTLC evaluation of ¹⁷⁷Lu-phospa-trastuzumab showed impurities that eluted at the solvent front, even after purification. This was most likely a result of weakly-bound ¹⁷⁷Lu undergoing ligand exchange between the ¹⁷⁷Lu-phospa-trastuzumab immunoconjugate and EDTA⁴⁻ (50 mM, pH 5) in the aqueous mobile phase (Figure 1).

The ¹¹¹In-phospa-trastuzumab conjugate was produced quickly under mild room temperature conditions, but radiolabeling properties of H₆phospa are admittedly inferior to H₄octapa with both ¹¹¹In and ¹⁷⁷Lu (H₄octapa providing quantitative RCY in < 15 min at RT). However, *in vitro* assays with HER2-expressing SKOV-3 cancer cells designed to calculate the immunoreactive fraction of an antibody at infinite antigen levels revealed an immunoreactivity of $97.9 \pm 2.6\%$ for ¹¹¹In-phospa-trastuzumab (n=9, Table 1). This represents a negligible decrease from the theoretical maximum of 100% assumed for unmodified trastuzumab. Cellular immunoreactivity assays were not performed with ¹⁷⁷Lu-phospa-trastuzumab due to limited stability. The stabilities of the ¹¹¹In/¹⁷⁷Lu-phospa-trastuzumab radioimmunoconjugates were assessed using a human serum transchelation challenge at 37 °C over a period of 5 days (gentle agitation, 300 rpm, Eppendorf Thermomixer) (Table 1). The amount of radiometal that remained bound to the H₆phospa-trastuzumab immunoconjugate was determined using radio-iTLC. ¹⁷⁷Lu-phospa-trastuzumab proved to be very unstable, for the ¹⁷⁷Lu was quickly transchelated by serum proteins (e.g. transferrin), with only 8% of the ¹⁷⁷Lu remaining chelate-bound after 24 hours and only 2% still intact after 5 days (Figure 2). The ¹¹¹In-phospa-trastuzumab construct fared better, with 67% of the ¹¹¹In remaining intact after 24 hours and 53% after 5 days. Included for comparison in Figure 2 are previously obtained human serum stability data of the H₄octapa-based constructs ¹¹¹In/¹⁷⁷Lu-octapa-trastuzumab, both of which quantitatively and rapidly radiolabeled at room temperature in less than 15 minutes, remained > 90% intact throughout an entire 5 day transchelation challenge, and demonstrated excellent *in vivo* stability.³⁶

⁸⁹Zr Radiolabeling

DFO has been used as the chelator of choice for ⁸⁹Zr for over 20 years.² During this time, no challengers have emerged that improve upon it, or, for that matter, display comparable radiochemical properties.² The challenges faced when trying to improve upon DFO are significant, and these difficulties are only amplified by the problematic aqueous chemistry of ⁸⁹Zr.² Although the previously published acyclic chelator H₄octapa has been shown to have great potential for use with the radiometals ¹¹¹In and ¹⁷⁷Lu, unpublished experiments

have revealed its incompetence for radiolabeling with ^{89}Zr . Using a variety of buffers (NH_4OAc , HEPES, PBS, pH 5.5–7.4) and temperatures (RT to 90 °C) no measureable quantity of ^{89}Zr could be incorporated into the H_4octapa -trastuzumab immunoconjugate. Our hypothesis from these observations was that by using methylenephosphonate donor arms in place of the carboxylic acid arms in H_4octapa , we could potentially enhance radiolabeling kinetics and improve radiometal-complex stability with ^{89}Zr . From these observations, we modified the design of H_4octapa by taking advantage of its inherent modularity and replaced its pendant acetic acid chelating arms with methylenephosphonate groups to synthesize H_6phospa .

Radiolabeling experiments with the H_6phospa -trastuzumab construct have proven our original hypothesis correct in a rather anticlimactic way, with the methylenephosphonate groups providing increased ^{89}Zr incorporation up to a maximum yield of 12% (3.3 ± 0.1 chelates per antibody, PBS pH 7.4, 37 °C, 18 hr) (Figure 3). Under the more realistic radiolabeling conditions typically used for ^{89}Zr -DFO - RT, pH 7.0–7.5, 1 hr - radiochemical yields of 7–8% were achieved (Figure S11–12).

Despite the improved ^{89}Zr radiolabeling yields of H_6phospa -trastuzumab (8–12%) compared to H_4octapa -trastuzumab (~0%), it was not sufficient for H_6phospa to be used as a chelator for ^{89}Zr -based radiopharmaceutical preparations. Although PBS buffer is commonly used for ^{89}Zr radiolabeling, HEPES buffer is a good alternative.^{3, 13, 37–39} ^{89}Zr Radiolabeling experiments with H_6phospa -trastuzumab in HEPES buffer (500 mM, pH 7.1) were attempted, but after 1 hour at room temperature, radiochemical yields of only ~2% were achieved. These results compare poorly to the current “gold standard” chelator desferrioxamine (DFO, Chart 1), which can achieve quantitative radiochemical yields (>95%) at room temperature in less than 1 hour. It is important to note that although H_6phospa has been found to be inferior to DFO, to our knowledge it is the best alternative chelator to be investigated to date. Previously, the best alternative chelator to DFO was DTPA, with a DTPA-antibody conjugate of Zevalin demonstrating ^{89}Zr radiochemical yields of < 0.1% after 1 hour at room temperature.¹⁴

Although the hypothesis that replacing the carboxylic acid pendant arms of H_4octapa with the methylenephosphonate arms of H_6phospa would improve radiolabeling kinetics and radiochemical yields was proven correct, the improvement was modest. However, these results do provide compelling evidence that methylenephosphonate-O donor groups may be superior to traditional carboxylate-O donor groups for ^{89}Zr coordination. The N_4O_4 donor set of the picolinate-based chelators H_4octapa and H_6phospa may be too nitrogen rich, and future chelators should take advantage of the oxophilic nature of Zr(IV) . Taking a lesson from the only suitable ^{89}Zr chelator, DFO (Chart 1), acyclic chelators with O_8 donor sets and binding groups such as hydroxamates, carboxylates, carbonyls, catechols, and hydroxypyridinones are likely most suitable. This valuable insight suggests that H_6phospa is a good starting point for further design modifications towards new ^{89}Zr chelators.

Small Animal SPECT/CT Imaging

Despite the modest radiolabeling performance of H_6phospa -trastuzumab with ^{89}Zr and the non-optimal radiochemical yields and serum stability results with ^{111}In and ^{177}Lu , *in vivo*

imaging was performed to determine the biological behavior of these radio-immunoconjugates. Single photon emission computed tomography (SPECT) was used in conjunction with standard helical X-ray CT for *in vivo* imaging of the ^{111}In and ^{177}Lu radiolabeled H_6phospa -trastuzumab immunoconjugates over a 5-day period. The poor *in vitro* serum stability of ^{177}Lu -phospa-trastuzumab foretold poor *in vivo* stability; however, our laboratory has had a long-standing interest in the delivery of therapeutic doses of lanthanide ions to bone for the treatment of osteoporosis.^{40, 41} Towards this goal, SPECT/CT imaging was performed with the purified ^{177}Lu -phospa-trastuzumab immunoconjugate in order to visualize biodistribution and confirm bone-localization of ^{177}Lu for the potential application of H_6phospa to transiently bind lanthanides for bone-delivery.^{40, 41}

^{111}In -phospa-trastuzumab SPECT/CT images showed significant tumor uptake 24 hours post injection (p.i.), with residual activity remaining in blood circulation, as is typical for antibody constructs. Unlike highly stable ^{111}In and ^{177}Lu radiometal chelates like DOTA (and most recently, H_4octa), some instability and radiometal dissociation can be visualized by moderate uptake in the kidneys and bones/joints (Figure 4). Despite these shortcomings, the SKOV-3 tumors were the most prominent tissue in the SPECT images. As expected from the poor stability results in serum, images of the ^{177}Lu -phospa-trastuzumab construct showed complete decomplexation of the radiometal after 24 hours, with free ^{177}Lu metal ions accumulating almost entirely in the bone (e.g. joints, spine, Figure 5). At 72 hours p.i. the ^{177}Lu -phospa-trastuzumab images looked nearly identical to 24 hours p.i., with no residual activity remaining in blood circulation and no tumor uptake apparent. The ^{177}Lu -phospa-trastuzumab images also serve as negative control experiments, demonstrating the effects of poor chelate stability *in vivo*. Since antibody vectors like trastuzumab have very long biological half-lives, persistent blood uptake is generally a positive sign of stability; not surprisingly, this was not observed for ^{177}Lu -phospa-trastuzumab (Figure 4 vs Figure 5). An interesting aspect of the ^{177}Lu -phospa-trastuzumab SPECT/CT images (Figure 5) is the lack of uptake in the endorecticular system (e.g. liver, spleen) and lungs, with radioactivity almost entirely localizing in the skeletal system. This suggests that there may be a silver lining for H_6phospa with respect to ^{177}Lu , as its failure as a stable chelate for immunoconjugates may be salvaged by its utility in delivering ^{177}Lu to bone.

Conclusions

The acyclic chelator H_6phospa and the bifunctional derivative $p\text{-SCN-Bn-H}_6\text{phospa}$ have been synthesized using unique 2-nitrobenzenesulfonamide (nosyl) protection chemistry, conjugated to trastuzumab (3.3 ± 0.1 chelates per antibody), and evaluated via radiolabeling with ^{89}Zr , ^{111}In , and ^{177}Lu . Radiolabeling of the H_6phospa -trastuzumab immunoconjugate was achieved with ^{111}In in 70–90% yields at room temperature in 30 minutes (2.9 ± 0.7 mCi/mg), and with ^{177}Lu under the same conditions in yields of 40–80% (2.8 ± 0.8 mCi/mg). Human serum stability experiments revealed that the ^{111}In -phospa-trastuzumab immunoconjugate was $52.0 \pm 5.3\%$ intact after 5 days, and ^{177}Lu -phospa-trastuzumab was only $2.0 \pm 0.3\%$ intact. The room temperature radiolabeling conditions afforded by H_6phospa produced the ^{111}In -phospa-trastuzumab conjugate with high immunoreactivity

(97.9 ± 2.6%, as indicated by cellular binding assays). Small animal SPECT/CT imaging was performed using nude athymic mice bearing subcutaneous SKOV-3 xenografts, and it was found that ^{111}In -phospa-trastuzumab successfully identified and delineated small (~2 mm in diameter) tumors from surrounding tissues; however, some uptake was observed in the kidneys and bone due to moderate chelate instability. The *in vivo* behavior of the ^{177}Lu -phospa-trastuzumab immunoconjugate was likewise studied via SPECT/CT, and, as expected from its poor *in vitro* stability in serum, it displayed no tumor uptake and high uptake in the bones and joints, suggesting complete radiometal dissociation. The weak binding of ^{177}Lu by H_6phospa -trastuzumab and the rapid *in vivo* release of ^{177}Lu radiometal ions highlights a potential application of H_6phospa in transient lanthanide chelation for delivery of therapeutic doses to the bone. The radiolabeling efficiency of H_6phospa -trastuzumab was tested with ^{89}Zr , and while H_6phospa offered an improvement over H_4octapa -trastuzumab, the results were modest with 7–8% incorporation of ^{89}Zr after 1 hour at room temperature. DTPA was previously the best alternative chelator to DFO, providing ^{89}Zr -radiolabeling yields of < 0.1% after 1 hour at room temperature. To our knowledge, H_6phospa now provides the second highest ^{89}Zr radiolabeling yields reported to date, with DFO being superior. It can be concluded from this work that H_6phospa is a less suitable chelator for use with ^{111}In and ^{177}Lu when compared to DOTA and H_4octapa , but an improvement for ^{89}Zr radiolabeling. Radiolabeling yields were inferior to the current “gold standard” desferrioxamine (DFO), but valuable insight was gained for the design of new ^{89}Zr chelators, with H_6phospa being an excellent starting point.

Experimental

General Remarks

All solvents and reagents were purchased from commercial suppliers (Sigma Aldrich, St. Louis, MO; TCI America, Portland, OR; Fisher Scientific, Waltham, MA) and were used as received unless otherwise indicated. DMSO used for chelator stock solutions was of molecular biology grade (>99.9%: Sigma, D8418). Methyl-6-bromomethylpicolinate was synthesized according to a literature protocol.¹⁷ Water used was ultra-pure (18.2 MΩ cm⁻¹ at 25 °C, Milli-Q, Millipore, Billerica, MA). The analytical thin-layer chromatography (TLC) plates were aluminum-backed ultrapure silica gel (Siliaplate™, 60 Å pore size, 250 μm plate thickness, Silicycle, Quebec, QC). Flash column silica gel was provided by Silicycle (Siliaflash® Irregular Silica Gels F60, 60 Å pore size, 40–63 mm particle size, Silicycle, Quebec, QC). Automated column chromatography was performed using a Teledyne Isco (Lincoln, NE) CombiFlash® R_f automated system with solid load cartridges packed with flash column silica gel and RediSep R_f Gold® reusable normal-phase silica columns and neutral alumina columns (Teledyne Isco, Lincoln, NE). ^1H and ^{13}C NMR spectra were recorded on Bruker AV300, AV400, or AV600 instruments; all spectra were internally referenced to residual solvent peaks except for ^{13}C NMR spectra in D_2O , which were externally referenced to a sample of $\text{CH}_3\text{OH}/\text{D}_2\text{O}$, and ^{31}P NMR spectra in D_2O , which were externally referenced to 85% phosphoric acid. Low-resolution mass spectrometry was performed using a Waters liquid chromatography-mass spectrometer (LC-MS) consisting of a Waters ZQ quadrupole spectrometer equipped with an ESI electrospray/ chemical ionization ion source and a Waters 2695 HPLC system (Waters,

Milford, MA). High-resolution electrospray-ionization mass spectrometry (EI-MS) was performed on a Waters Micromass LCT time of flight instrument. Microanalyses for C, H, and N were performed on a Carlo Erba EA 1108 elemental analyzer. The HPLC system used for purification of non-radioactive compounds consisted of a semi-preparative reverse phase C18 Phenomenex synergi hydro-RP (80 Å pore size, 250 × 21.2 mm, Phenomenex, Torrance, CA) column connected to a Waters 600 controller, a Waters 2487 dual wavelength absorbance detector, and a Waters delta 600 pump. UV/vis measurements for determining antibody stock solution concentrations were taken on a Thermo Scientific nanodrop 2000 spectrophotometer (Wilmington, DE).

^{111}In was cyclotron produced (Advanced Cyclotron Systems, Model TR30) by proton bombardment through the reactions $^{111}\text{Cd}(p,n)^{111}\text{In}$ and was provided by Nordion as $^{111}\text{InCl}_3$ solutions in 0.05 M HCl. ^{177}Lu was procured from Perkin Elmer (Perkin Elmer Life and Analytical Sciences, Wellesley, MA, effective specific activity of 29.27 Ci/mg) as $^{177}\text{LuCl}_3$ in 0.05 M HCl. ^{89}Zr was produced at Memorial Sloan-Kettering Cancer Center on an EBCO TR19/9 variable-beam energy cyclotron (Ebco Industries Inc., British Columbia, Canada) via the $^{89}\text{Y}(p,n)^{89}\text{Zr}$ reaction and purified in accordance with previously reported methods to yield ^{89}Zr with a specific activity of 5.28–13.43 mCi/μg (195–497 MBq/μg).³⁹ Labeling reactions were monitored using silica-gel impregnated glass-microfiber instant thin layer chromatography paper (iTLC-SG, Varian, Lake Forest, CA) and analyzed on a Bioscan AR-2000 radio-TLC plate reader using Winscan Radio-TLC software (Bioscan Inc., Washington, DC). All radiolabeling chemistry was performed with ultrapure water ($>18.2\text{ M}\Omega\text{ cm}^{-1}$ at 25 °C, Milli-Q, Millipore, Billerica, MA) that had been passed through a 10 cm column of Chelex resin (BioRad Laboratories, Hercules, CA). Human blood serum (Sigma, Sera, human, aseptically filled, S7023-100 mL) competition solutions were agitated at 300 rpm and held at 37 °C using an Eppendorf Thermomixer and then analyzed by iTLC using an EDTA mobile phase (50 mM, pH 5). $^{177}\text{Lu}/^{111}\text{In}/^{89}\text{Zr}$ -immunoconjugates were analyzed using iTLC as described above, and purified using PD-10 desalting columns (GE Healthcare, United Kingdom, MW < 5000 Da filter) that were conditioned by elution of 25 mL phosphate-buffered saline (PBS) before use, and Corning 50k MW Amicon® Ultra centrifugation filters. Radioactivity in samples was measured using a Capintec CRC-15R dose calibrator (Capintec, Ramsey, NJ), and a Perkin-Elmer (Waltham, MA) Automated Wizard Gamma Counter was used for creating calibration curves. SPECT/CT imaging was performed using a four-headed NanoSPECT/CT®PLUS camera (Bioscan Inc., Washington DC, USA) with a multi-pinhole focused collimator and a temperature controlled animal bed (Minerve Equipment Veterinaire). The human breast cancer cell line SKOV-3 was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown by serial passage. Animals used were female nude athymic mice.

***N,N'*-[6-(Methoxycarbonyl)pyridin-2-yl]methyl-1,2-diaminoethane (1)**

Compound **1** was prepared according to a previously published method in 3 steps (70–75% cumulative yield).³⁶ ^1H NMR (300 MHz, MeOD, 25 °C) δ : 8.02 (d, J = 7.5 Hz, 2H), 7.94 (t, J = 7.6 Hz, 2H), 7.66 (d, J = 7.5 Hz, 2H), 3.96 (s, 6H), 2.79 (s, 4H). ^{13}C NMR (75 MHz, MeOD, 25 °C) δ : 167.0, 161.4, 148.5, 139.5, 127.6, 124.8, 54.9, 53.4, 50.0, 49.3. HR-ESI-MS calcd. for $[\text{C}_{18}\text{H}_{22}\text{N}_4\text{O}_4+\text{H}]^+$: 359.1719; found: 359.1720, $[\text{M}+\text{H}]^+$, PPM = 0.2.

***N,N'*-(Methylphosphonate)-*N,N'*-[6-(methoxycarbonyl)pyridin-2-yl]methyl]-1,2-diaminoethane (2), H₆phospa**

Compound **2** was prepared according to a modified literature procedure.^{34, 35} To a solution of **1** (74.0 mg, 0.207 mmol) in HCl (5 mL, 6 M) was added phosphorous acid (340 mg, 4.13 mmol), followed by paraformaldehyde in small portions (25.0 mg, 0.827 mmol). The reaction mixture was refluxed overnight, and over a period of 48 hours another 8 equiv of paraformaldehyde was added (50.0 mg, 1.65 mmol). The reaction mixture was concentrated *in vacuo* to form the water-insoluble HCl salt, which was washed with cold HCl (3 M) *ad libitum* to yield **H₆phospa (2)** as a white solid (70%) (Figures S1–S4). ¹H NMR (300 MHz, D₂O, 25 °C) δ: 7.68 (m, 4H), 7.39–7.36 (m, 2H), 4.12 (s, 4H), 3.32 (s, 4H), 2.96 (d, *J* = 11.2 Hz, 4H). ¹³C NMR (75 MHz, D₂O, 25 °C, externally referenced to MeOH in D₂O) δ: 172.1, 153.0, 152.8, 125.8, 123.4, 58.9, 58.8, 52.7, 50.9, 50.4, 50.3. ³¹P[¹H] NMR (121.5 MHz, D₂O, 25 °C, externally referenced to 85% phosphoric acid) 16.0, 2.8. HR-ESI-MS calcd. for [C₁₈H₂₂N₄O₁₀P₂+H]⁺: 517.0889; found: 517.0884, [M+H]⁺, PPM = −1.0. IR (neat, ATR-IR): ν = 1624 cm^{−1} (C=O), 1584 cm^{−1} (C=C py), 1430 cm^{−1} (P=O).

[Lu(phospa)]^{3−} (3). H₆phospa (2)

Metal complex synthesis was attempted, but not confirmed. (14.1 mg, 0.027 mmol) was suspended in 0.1 M HCl (0.5 mL), and LuCl₃·6H₂O (15 mg, 0.033 mmol) was added. The pH was adjusted to 4–5 using 0.1 M NaOH and then the solution was stirred at room temperature. After 1 hour, the reaction mixture was analysed by mass spectrometry, but no product could be identified. After reducing to a white solid *in vacuo*, the product could not be dissolved in MeOD, D₂O, or DMSO-*d*₆, and thus characterization was not performed, and metal complex formation could not be confirmed.

[In(phospa)]^{3−} (4). H₆phospa (2)

Metal complex synthesis was attempted, but not confirmed. (16.1 mg, 0.031 mmol) was suspended in 0.1 M HCl (0.5 mL), and In(ClO₄)₃·6H₂O (20 mg, 0.037 mmol) was added. The pH was adjusted to 4–5 using 0.1 M NaOH, and then the solution was stirred at room temperature. After 1 hour, analysis of the reaction mixture via low-resolution and high-resolution mass spectrometry confirmed the presence of a species consistent with the molecular formula proposed for the indium complex. Similar to the [Lu(phospa)]^{3−} complex, after reducing to dryness *in vacuo*, the white solid could not be dissolved, and therefore further characterization was not obtained, and no crystals suitable for X-ray crystallography were obtained. HR-ESI-MS calcd. for [C₁₈H₂₁InN₄O₁₀P₂ − H][−]: 628.9693; found: 628.9700 [M − H][−], PPM = 1.1.

1-(*p*-Nitrobenzyl)ethylenediamine (5)

Compound **5** was prepared according to a literature preparation,⁴² and was purified with a modified procedure using column chromatography (CombiFlash *R_f* automated column system; 40 g HP silica; A: 95% dichloromethane 5% ammonium hydroxide, B: 95% methanol 5% ammonium hydroxide, 100% A to 30% B gradient) to afford a **7** as brown/amber oil in a cumulative yield of 40% over 3 steps. ¹H NMR (300 MHz, CDCl₃, 25 °C) δ: 7.91 (d, *J* = 8.9 Hz, 2H), 7.18 (d, *J* = 8.5 Hz, 2H), 2.80–2.57 (m, 3H), 2.45–2.31 (m, 2H). ¹³C

NMR (75 MHz, CDCl₃, 25 °C) δ : 147.1, 147.7, 129.4, 122.8, 54.3, 47.5, 41.4. HR-ESI-MS calcd. for [C₉H₁₃N₃O₂+H]⁺: 196.1086; found: 196.1084 [M + H], PPM = -1.0.

***N,N*-(2-Nitrobenzenesulfonamide)-1-(*p*-nitrobenzyl)-1,2-diaminoethane (6)**

Compound **6** was prepared according to a literature preparation.³⁶ ¹H NMR (300 MHz, acetone-d₆, 25 °C) δ : 8.18-8.15 (m, 1H), 7.99-7.94 (m, 2H), 7.79-7.57 (m, 5H), 7.33 (d, *J* = 8.5 Hz, 2H), 7.04-6.98 (m, 2H), 4.01 (br s, 1H), 3.41-3.38 (m, 2H), 3.26 (dd, *J* = 3.4, 13.7 Hz, 1H), 2.98 (m, 1H). ¹³C NMR (75 MHz, acetone-d₆, 25 °C) δ : 206.3, 149.2, 148.1, 147.6, 146.7, 135.2, 134.8, 134.3, 133.9, 133.8, 133.6, 131.7, 131.4, 130.9, 126.0, 125.7, 123.9, 57.7, 49.4, 38.2. HR-ESI-MS calcd. for [C₂₁H₁₉N₅O₁₀S₂+Na]⁺: 588.0471; found: 588.0465 [M+Na]⁺, PPM = -1.0.

***N,N*-(2-Nitrobenzenesulfonamide)-*N,N'*-[6-(methoxycarbonyl)pyridin-2-yl]methyl]-1-(*p*-nitrobenzyl)-1,2-diaminoethane (7)**

To a solution of **6** (1.03 g, 1.82 mmol) in dimethylformamide (5 mL, dried over molecular sieves 4 Å) was added methyl-6-bromomethylpicolinate (921 mg, 4.01 mmol) and sodium carbonate (~2 g). The yellow reaction mixture was stirred at 60 °C for 48 h, filtered to remove sodium carbonate, and concentrated *in vacuo*. The crude product was purified by silica chromatography (CombiFlash *R_f* automated column system; 40 g HP silica; A: ethyl acetate, B: petroleum ether, 100% A to 100% B gradient) to yield **7** as white fluffy solid (*R_f* = 0.9 in DCM, 95%). ¹H NMR (300 MHz, CDCl₃, 25 °C) δ : 8.00-7.81 (m, 4H), 7.78-7.70 (m, 2H), 7.65-7.63 (m, 5H), 7.55-7.44 (m, 4H), 7.38-7.36 (m, 1H), 6.98 (d, *J* = 8.5 Hz, 2H), 4.96-4.65 (m, 4H), 4.36 (m, 1H), 3.89 (s, 3H), 3.84 (s, 3H), 3.56-3.50 (m, 1H), 3.39-3.31 (m, 1H), 3.15-3.01 (m, 2H). ¹³C NMR (75 MHz, CDCl₃, 25 °C) δ : 164.9, 164.9, 157.5, 155.9, 147.7, 147.2, 147.0, 147.0, 146.1, 144.6, 137.9, 137.8, 133.7, 133.5, 132.4, 131.8, 131.8, 131.1, 130.0, 129.5, 126.7, 125.6, 124.1, 123.9, 123.9, 122.9, 58.4, 53.4, 52.6, 52.5, 52.2, 49.7, 34.5. HR-ESI-MS calcd. for [C₃₇H₃₃N₇O₁₄S₂+Na]⁺: 886.1425; found: 886.1447, [M+Na]⁺, PPM = 2.5.

***N,N*-[6-(Methoxycarbonyl)pyridin-2-yl]methyl]-*p*-nitrobenzyl)-1,2-diaminoethane (8)**

To a solution of **7** (1.39 g, 1.62 mmol) in tetrahydrofuran (7 mL) was added thiophenol (364 μ L, 3.55 mmol) and potassium carbonate (excess, ~1 g). The reaction mixture was stirred at ambient temperature for 72 hours, while a slow color change from colorless to dark yellow occurred. The reaction mixture was split into two 20 mL falcon tubes, diluted with additional tetrahydrofuran, centrifuged for 5 minutes at 4000 rpm, and then the solvent was decanted. The potassium carbonate in the centrifuge tubes was rinsed with tetrahydrofuran and then centrifuged 5 times, pooled, and concentrated to dryness *in vacuo*. The resulting crude yellow oil was purified by alumina column chromatography (CombiFlash *R_f* automated column system; 24 g neutral alumina; A: dichloromethane, B: methanol, 100% A to 30% B gradient) to yield **8** as clear yellow oil (90%). ¹H NMR (300 MHz, CDCl₃, 25 °C) δ : 8.07-8.04 (m, 2H), 7.96-7.93 (m, 2H), 7.77-7.70 (m, 2H), 7.53-7.46 (m, 2H), 7.34-7.28 (m, 2H), 4.05-3.95 (m, 2H), 3.93-3.92 (s, 6H), 3.91-3.86 (m, 2H), 3.00-2.49 (m, 4H), 2.20 (br s, 2H). ¹³C NMR (75 MHz, CDCl₃, 25 °C) δ : 165.6, 165.5, 160.5, 147.3, 147.2, 147.1,

146.3, 137.3, 130.0, 125.5, 123.4, 123.3, 58.3, 54.9, 52.7, 52.3, 51.7, 39.1. HR-ESI-MS calcd. for $[\text{C}_{25}\text{H}_{27}\text{N}_5\text{O}_6+\text{H}]^+$: 494.2040; found: 494.2039, $[\text{M}+\text{H}]^+$, PPM = -0.2.

***N,N'*-[Methylphosphonate]-*N,N'*-[(6-carboxylato)pyridin-2-yl)methyl]-1-(*p*-nitrobenzyl)-1,2-diaminoethane (9)**

To a solution of **8** (96.0 mg, 0.194 mmol) in HCl (5 mL, 6 M) was added phosphorous acid (320 mg, 3.89 mmol), and the reaction mixture was brought to reflux. Paraformaldehyde (48.0 mg, 1.56 mmol) was added in small portions over 8 hours, and the resulting suspension/solution was refluxed overnight. An additional 8 equiv of paraformaldehyde (48.0 mg, 1.65 mmol) and 20 equiv of phosphorous acid (320 mg, 3.89 mmol) were added over a period of 8 hours and refluxing was continued for an additional night (48 h total). The reaction mixture was concentrated *in vacuo*, and the resulting yellow oil was purified by RP-HPLC on a semi-preparative column (A: 0.1% TFA in deionized water, B: 0.1% TFA in CH_3CN , 5% B to 90% B gradient over 30 minutes) to yield **9** (R_t = 12 minutes) as a white solid (42%) (Figures S5–S7). ^1H NMR (300 MHz, MeOD, 25 °C) δ : 8.12–7.94 (m, 2H), 7.82–7.62 (m, 2H), 7.51–7.34 (m, 3H), 7.24–7.16 (m, 2H), 7.03 (br s, 1H), 4.02–3.46 (m, 3H), 3.15–3.12 (m, 3H), 2.95–1.94 (several m, 7H). ^{13}C NMR (75 MHz, MeOD, 25 °C) δ : 172.7, 172.3, 166.0, 158.2, 152.6, 152.2, 149.1, 145.8, 138.2, 137.7, 130.3, 125.8, 123.7, 123.5, 122.5, 122.3, 58.4, 55.8, 55.6, 54.2, 31.3. ^{31}P [^1H] NMR (121.5 MHz, D_2O , 25 °C, externally referenced to 85% phosphoric acid) 16.4, 15.9. HR-ESI-MS calcd. for $[\text{C}_{25}\text{H}_{29}\text{N}_5\text{O}_{12}\text{P}_2+\text{H}]^+$: 654.1366; found: 654.1359, $[\text{M}+\text{H}]^+$, PPM = -1.1.

***p*-SCN-Bn- H_6phospa , *N,N'*-(methylphosphonate)-*N,N'*-[(6-carboxylato)pyridin-2-yl)methyl]-1-(*p*-benzyl-isothiocyanato)-1,2-diaminoethane (10)**

Compound 9 (52.0 mg, 0.080 mmol) was dissolved in glacial acetic acid (1.5 mL) with hydrochloric acid (1.5 mL, 3 M), palladium on carbon (~10 mg, 20 wt%) was added, and hydrogen gas (balloon) bubbled in via rubber septa and syringe. The reaction mixture was stirred vigorously at RT for 1 hour, then filtered to remove Pd/C and washed *ad libitum* with methanol and hydrochloric acid (3 M). The crude reaction mixture was concentrated *in vacuo*, and without purification, was dissolved in hydrochloric acid (1 mL, 3 M) and then reacted with thiophosgene in chloroform (91 μL , 1.20 mmol) overnight at ambient temperature with vigorous stirring. The reaction mixture was extracted with chloroform (5 $\times$ 1 mL) by vigorous biphasic stirring, followed by decanting and discarding of the organic phase with a pipette in the reaction vial to remove excess thiophosgene. The aqueous phase was diluted to a volume of 4.5 mL with deionized water, and injected directly onto a semi-prep RP-HPLC column for purification (A: 0.1% TFA in deionized water, B: 0.1% TFA in CH_3CN , 5% B to 70% B gradient over 30 minutes). Compound **10** eluted at R_t = 17.5 minutes, was lyophilized overnight, and was isolated as a fluffy off-white solid (14% over 2 steps from **9**) (Figures S8–S10). ^1H NMR (600 MHz, $\text{DMSO}-d_6$, 25 °C) δ : 8.17–7.90 (m, 3H), 7.76–7.63 (m, 3H), 7.46–7.23 (m, 4H), 5.45 (br s, 1H), 4.34–4.32 (m, 2H), 3.94–3.85 (m, 3H), 3.33–3.21 (m, 3H), 3.02–2.94 (m, 3H), 2.61 (s, 1H). ^{13}C NMR (150 MHz, MeOD, 25 °C) δ : 174.5, 169.3, 167.4, 167.2, 160.8, 149.1, 148.6, 140.0, 139.9, 139.0, 137.1, 131.8, 131.7, 131.1, 128.0, 127.0, 126.9, 126.1, 125.3, 125.1, 61.1, 61.1, 38.2, 35.2, 34.7, 34.7. HR-ESI-MS calcd. for $[\text{C}_{26}\text{H}_{29}\text{N}_5\text{O}_{10}\text{P}_2\text{S}+\text{Na}]^+$: 688.1008; found: 688.0998, $[\text{M}+\text{H}]^+$, PPM

= -1.5. IR (neat, ATR-IR): ν = 2075 cm^{-1} (-N=C=S), 1719 cm^{-1} (C=O), 1594 cm^{-1} (C=N py), 1348 cm^{-1} (P=O).

Trastuzumab Bioconjugation

Trastuzumab (purchased commercially as Herceptin, Genentech, San Francisco, CA) was purified using centrifugal filter units with a 50,000 molecular weight cutoff (Amicon® ultra centrifuge filters, Ultracel®-50: regenerated cellulose, Millipore Corp., Billerica, MA) and phosphate buffered saline (PBS, pH 7.4) to remove α - α -trehalose dihydrate, L-histidine, and polysorbate 20 additives. After purification, the antibody was taken up in PBS pH 7.4. Subsequently, 300 μL of antibody solution (150–250 μM) was combined with 100 μL PBS (pH 8.0) and 4 equiv of *p*-SCN-Bn-H₆phospa in 10 μL DMSO, and the pH was adjusted to 9. The reactions were heated at 37 °C for 1 h, followed by centrifugal filtration to purify the resultant antibody conjugate. The final modified antibody stock solutions were stored in PBS pH 7.4 at 4 °C.

¹¹¹In-, ⁸⁹Zr-, and ¹⁷⁷Lu-phospa-trastuzumab Radiolabeling

Aliquots of the H₆phospa-trastuzumab immunoconjugate were transferred to 2 mL microcentrifuge tubes and made up to 1 mL with ammonium acetate buffer (pH 5.5, 200 mM), and then aliquots of ¹⁷⁷Lu or ¹¹¹In were added (~1–3 mCi). The H₆phospa-trastuzumab mixtures were incubated at room temperature for 30–60 minutes at ambient temperature and then analyzed via iTLC with an eluent of 50 mM EDTA (pH 5). 30 μL of EDTA solution (50 mM, pH 5) was then added to the reaction mixtures to quench the reaction, and then the resulting radiolabeled immunoconjugates were purified using size-exclusion chromatography (Sephadex G-25 M, PD-10 column, 30 kDa, GE Healthcare; dead volume = 2.5 mL, eluted with 1 mL fractions of PBS, pH 7.4) and centrifugal column filtration (Amicon® ultra 50k). The radiochemical purity of the final radiolabeled bioconjugate was assayed by radio-iTLC. For ⁸⁹Zr radiolabeling experiments, the same procedure was followed as above, with phosphate buffered saline (PBS, pH 7.4) being used in place of ammonium acetate. ⁸⁹Zr radiolabeling was also attempted in ammonium acetate (200 mM, pH 5.5–6.5) and HEPES buffer (100–500 mM, pH 7–7.2), with PBS ultimately deemed the most suitable buffer. In the iTLC experiments, ¹¹¹In-, ⁸⁹Zr-, and ¹⁷⁷Lu-phospa-trastuzumab remained near the baseline, while ¹⁷⁷Lu³⁺/¹¹¹In³⁺/⁸⁹Zr⁴⁺ ions were coordinated as [¹⁷⁷Lu/¹¹¹In/⁸⁹Zr]-EDTA and eluted with or near the solvent front. iTLC methods were followed from previous work.^{3, 13, 36, 39, 43}

Chelate Number – Radiometric Isotopic Dilution Assay

The number of accessible H₆phospa chelates conjugated to trastuzumab was measured by radiometric isotopic dilution assays following methods similar to those described by Anderson et al. and Holland et al.^{3, 44–46} The experimental procedure was modified to use ¹¹¹In and SKOV-3 ovarian cancer cells.³⁶ All experiments were performed in triplicate. Conjugating 4 equivalents of *p*-SCN-Bn-H₆phospa was found to yield 3.3 ± 0.1 accessible chelates per antibody. Aliquots of [¹¹¹In]InCl₃ (10 μL , <5 kBq [0.1 μCi]) were added to 12 solutions containing 1:2 serial dilutions of non-radioactive In(NO₃)₃ (aq.) (100 μL fractions; 100 – 0.05 nmol, pH 7.5 – 8.5). The mixture was vortexed for 30 s before adding aliquots of

H₆phospa-trastuzumab (20 μ L, 5.0 mg/mL, [100 μ g of mAb, 0.7 nmol], in 0.9% sterile saline). The reactions were incubated at room temperature for 1 h before quenching with EDTA (20 μ L, 50 mM pH 7). The extent of complexation was assessed developing iTLC strips (EDTA, 50 mM) and counting the activity at the baseline and solvent front. The fraction of ¹¹¹In-radiolabeled mAb (Ab) was plotted versus the inverse of the number of nano-moles of In³⁺ and the number of chelates was calculated from the gradient by Equation 1, where c is the number of accessible chelates, $n(\text{mAb})$ is the amount of mAb (nmol), and $n(\text{In}^{3+})$ is the total number of In³⁺ added (nmol).⁴⁷

$$c = A_b \cdot \frac{n(\text{In})}{n(\text{mAb})} \quad (1)$$

***In Vitro* Immunoreactivity Assay**

The immunoreactivity of the ¹¹¹In/¹⁷⁷Lu-phospa-trastuzumab bioconjugates was determined using radioactive cellular-binding assays following procedures derived from Lindmo et al.^{48, 49} To this end, SKOV-3 cells were suspended in microcentrifuge tubes at concentrations of 5.0, 4.0, 3.0, 2.5, 2.0, 1.5, and 1.0 $\times 10^6$ cells/mL in 500 μ L PBS (pH 7.4). Aliquots of ¹¹¹In/¹⁷⁷Lu-phospa-trastuzumab (50 μ L of a stock solution of 10 μ Ci in 10 mL of 1% bovine serum albumin in PBS pH 7.4) were added to each tube ($n = 9$; final volume: 550 μ L), and the samples were incubated on a mixer for 60 min at room temperature. The treated cells were then pelleted via centrifugation (3000 rpm for 5 min), resuspended, and washed twice with cold PBS before removing the supernatant and counting the activity associated with the cell pellet. The activity data were background-corrected and compared with the total number of counts in appropriate control samples. Immunoreactive fractions were determined by linear regression analysis of a plot of (total/bound) activity against (1/[normalized cell concentration]). No weighting was applied to the data, and data were obtained as $n = 9$.

¹¹¹In- and ¹⁷⁷Lu-phospa-trastuzumab Blood Serum Competition Experiments

Frozen human blood serum was thawed for 30 minutes, and 300 μ L aliquots were transferred to 2.0 mL Corning centrifuge vials. A portion of radiolabeled immunoconjugate (~300 μ Ci) was transferred to the blood serum ($n = 3$ for each chelator). Serum competition samples were then incubated at 37 ± 0.1 °C with gentle agitation (300 rpm) and analyzed via iTLC (Bioscan AR-2000) with an EDTA eluent (50 mM, pH 5.0) at time points 0, 24, 48, 72, 96, and 120 hours.

Cell Culture

Human ovarian cancer cell line SKOV-3 was obtained from the American Tissue Culture Collection (ATCC, Bethesda, MD) and maintained in a 1:1 mixture of Dulbecco's Modified Eagle medium: F-12 medium, supplemented with 10% heat-inactivated fetal calf serum (Omega Scientific, Tarzana, Ca), 2.0 mM glutamine, nonessential amino acids, 100 units/mL penicillin, and 100 units/mL streptomycin in a 37 °C environment containing 5% CO₂. Cell lines were harvested and passaged weekly using a formulation of 0.25% trypsin/0.53 mM EDTA in Hank's Buffered Salt Solution without calcium and magnesium.

SKOV-3 Xenograft Mouse Models

All experiments were performed under an Institutional Animal Care and Use Committee-approved protocol, and the experiments followed institutional guidelines for the proper and humane use of animals in research. Six- to eight-week-old athymic nu/nu female mice (NCRNU-M) were obtained from Taconic Farms Incorporated (Hudson, NY). Animals were housed in ventilated cages, were given food and water *ad libitum*, and were allowed to acclimatize for approximately 1 week prior to treatment. After several days, SKOV-3 tumors were induced on the right shoulder by a subcutaneous injection of 1.0×10^6 cells in a 100 μ L cell suspension of a 1:1 mixture of fresh media/BD Matrigel (BD Biosciences, Bedford, Ma).

^{111}In - and ^{177}Lu -phospha-trastuzumab SPECT/CT Imaging Studies

Mice with SKOV-3 ovarian cancer xenografts (right shoulder, ~2 mm diameter) were imaged with ^{111}In ($n = 2$) and ^{177}Lu ($n = 2$) labeled immunoconjugates, using a four-headed NanoSPECT/CT[®]PLUS camera (Bioscan Inc., Washington DC, USA) with a multi-pinhole focused collimator and a temperature controlled animal bed unit (Minerve equipment veterinaire). Nine pinhole apertures with a diameter of 2.5 mm were used on each head, with a field of view (FOV) of 24 mm. Settings of the ^{111}In energy peaks were 245 and 171 keV, and the ^{177}Lu peaks were 208, 112, and 56 keV. A CT at 45 kVp was acquired (180 projections, pitch of 1). Based on the helical CT topogram, SPECT images were obtained over a range of 85 mm. For H₆phospha-trastuzumab mice were administered doses of ~800–810 μ Ci ^{111}In -labeled (specific activity ~3 μ Ci/ μ g, ~250 μ g of immunoconjugate per mouse) and ~575 μ Ci of ^{177}Lu -labeled (~3 μ Ci/ μ g, ~190 μ g) agents each in 200 μ L of sterile saline (0.9% NaCl), via intravenous tail vein injection. Approximately 5 minutes prior to SPECT/CT image acquisition, mice were anesthetized via inhalation of 2% isoflurane/oxygen gas mixture (Baxter Healthcare, Deerfield, IL), placed on the scanner bed, and anesthesia was maintained during imaging using a reduced 1.5% isoflurane/oxygen mixture. Animals were imaged at 24, 72, and 120 h p.i. for ^{111}In , and 24 / 72 h p.i. for ^{177}Lu to determine the general $^{111}\text{In}/^{177}\text{Lu}$ -phospha-trastuzumab biodistribution and tumor uptake. ^{177}Lu -immunoconjugate mice were imaged for 1.5–2 hours each, and ^{111}In -immunoconjugate mice were imaged for 45–60 min each. Image collection was performed using NuLine software (V1.02 build 009), and images were processed and reconstructed using InVivoScope (2.00 patch3, 64 bit) and HiSPECT (v 1.4.3049) software.

Animal Protocol

All animal experiments were performed according to a protocol approved by Memorial Sloan-Kettering Cancer Center's Institutional Animal Care and Use Committee (#08-07-013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and references

1. Zeglis BM, Lewis JS. Dalton Trans. 2011; 40:6168–6195. [PubMed: 21442098]
2. Deri MA, Zeglis BM, Francesconi LC, Lewis JS. Nucl. Med. Biol. 2013; 40:3–14. [PubMed: 22998840]
3. Holland JP, Divilov V, Bander NH, Smith-Jones PM, Larson SM, Lewis JS. J. Nucl. Med. 2010; 51:1293–1300. [PubMed: 20660376]
4. Holland JP, Williamson MJ, Lewis JS. Mol. Imaging. 2010; 9:1–20. [PubMed: 20128994]
5. Ikotun OF, Lapi SE. Future Medicinal Chemistry. 2011; 3:599–621. [PubMed: 21526898]
6. Chang AJ, DeSilva R, Jain S, Lears K, Rogers B, Lapi S. Pharmaceuticals. 2012; 5:79–93. [PubMed: 24288044]
7. Perk L, Visser O, Stigter-van Walsum M, Vosjan M, Visser G, Zijlstra J, Huijgens P, van Dongen G. Eur. J. Nucl. Med. Mol. Imaging. 2006; 33:1337–1345. [PubMed: 16832633]
8. Börjesson PKE, Jauw YWS, Boellaard R, de Bree R, Comans EFI, Roos JC, Castelijns JA, Vosjan MJWD, Kummer JA, Leemans CR, Lammertsma AA, van Dongen GAMS. Clin. Cancer Res. 2006; 12:2133–2140. [PubMed: 16609026]
9. Perk LR, Visser GWM, Vosjan MJWD, Stigter-van Walsum M, Tijink BM, Leemans CR, van Dongen GAMS. J. Nucl. Med. 2005; 46:1898–1906. [PubMed: 16269605]
10. Verel I, Visser GWM, Boellaard R, Stigter-van Walsum M, Snow GB, van Dongen GAMS. J. Nucl. Med. 2003; 44:1271–1281. [PubMed: 12902418]
11. Wooten A, Madrid E, Schweitzer G, Lawrence L, Mebrahtu E, Lewis B, Lapi S. Applied Sciences. 2013; 3:593–613.
12. Walther M, Gebhardt P, Grosse-Gehling P, Würbach L, Irmeler I, Preusche S, Khalid M, Opfermann T, Kamradt T, Steinbach J, Saluz H-P. Appl. Radiat. Isot. 2011; 69:852–857. [PubMed: 21397511]
13. Zeglis BM, Mohindra P, Weissmann GI, Divilov V, Hilderbrand SA, Weissleder R, Lewis JS. Bioconjugate Chem. 2011; 22:2048–2059.
14. Perk LR, Visser OJ, Stigter-van Walsum M, Vosjan MJWD, Visser GWM, Zijlstra JM, Huijgens PC, Dongen GAMS. Eur. J. Nucl. Med. Mol. Imaging. 2006; 33:1337–1345. [PubMed: 16832633]
15. Wadas TJ, Wong EH, Weisman GR, Anderson CJ. Chem. Rev. 2010; 110:2858–2902. [PubMed: 20415480]
16. Ramogida CF, Orvig C. Chem. Commun. 2013; 49:4720–4739.
17. Price EW, Cawthray JF, Bailey GA, Ferreira CL, Boros E, Adam MJ, Orvig C. J. Am. Chem. Soc. 2012; 134:8670–8683. [PubMed: 22540281]
18. Camera L, Kinuya S, Garmestani K, Wu C, Brechbiel MW, Pai LH, McMurphy TJ, Gansow OA, Pastan I, Paik CH, Carrasquillo JA. J. Nucl. Med. 1994; 35:882–889. [PubMed: 8176477]
19. Förster GJ, Engelbach MJ, Brockmann JJ, Reber HJ, Buchholz HG, Mäcke HR, Rösch FR, Herzog HR, Bartenstein PR. Eur. J. Nucl. Med. 2001; 28:1743–1750. [PubMed: 11734910]
20. Wu C, Kobayashi H, Sun B, Yoo TM, Paik CH, Gansow OA, Carrasquillo JA, Pastan I, Brechbiel MW. Bioorg. Med. Chem. 1997; 5:1925–1934. [PubMed: 9370037]
21. Brechbiel MW, Gansow OA, Atcher RW, Schlom J, Esteban J, Simpson D, Colcher D. Inorg. Chem. 1986; 25:2772–2781.

22. Harrison A, Walker CA, Parker D, Jankowski KJ, Cox JPL, Craig AS, Sansom JM, Beeley NRA, Boyce RA, Chaplin L, Eaton MAW, Farnsworth APH, Millar K, Millican AT, Randall AM, Rhind SK, Secher DS, Turner A. *Int. J. Rad. Appl. Instrum. [B]*. 1991; 18:469–476.
23. Ferdani R, Stigers DJ, Fiamengo AL, Wei L, Li BTY, Golen JA, Rheingold AL, Weisman GR, Wong EH, Anderson CJ. *Dalton Trans.* 2012; 41:1938–1950. [PubMed: 22170043]
24. Suzuki K, Satake M, Suwada J, Oshikiri S, Ashino H, Dozono H, Hino A, Kasahara H, Minamizawa T. *Nucl. Med. Biol.* 2011; 38:1011–1018. [PubMed: 21982572]
25. Dumont RA, Deininger F, Haubner R, Maecke HR, Weber WA, Fani M. *J. Nucl. Med.* 2011; 52:1276–1284. [PubMed: 21764795]
26. Fani M, Del Pozzo L, Abiraj K, Mansi R, Tamma ML, Cescato R, Waser B, Weber WA, Reubi JC, Maecke HR. *J. Nucl. Med.* 2011; 52:1110–1118. [PubMed: 21680701]
27. Sprague JE, Peng Y, Fiamengo AL, Woodin KS, Southwick EA, Weisman GR, Wong EH, Golen JA, Rheingold AL, Anderson CJ. *J. Med. Chem.* 2007; 50:2527–2535. [PubMed: 17458949]
28. Boswell CA, Sun X, Niu W, Weisman GR, Wong EH, Rheingold AL, Anderson CJ. *J. Med. Chem.* 2004; 47:1465–1474. [PubMed: 14998334]
29. Notni J, Šimek J, Hermann P, Wester H-J. *Chem. Eur. J.* 2011; 17:14718–14722. [PubMed: 22147338]
30. Notni J, Hermann P, Havlíková J, Kotek J, Kubíček V, Plutnar J, Loktionova N, Riss PJ, Rösch F, Lukeš I. *Chem. Eur. J.* 2010; 16:7174–7185. [PubMed: 20461824]
31. Boros E, Ferreira CL, Yapp DTT, Gill RK, Price EW, Adam MJ, Orvig C. *Nucl. Med. Biol.* 2012; 39:785–794. [PubMed: 22381779]
32. Bailey GA, Price EW, Zeglis BM, Ferreira CL, Boros E, Lacasse MJ, Patrick BO, Lewis JS, Adam MJ, Orvig C. *Inorg. Chem.* 2012; 51:12575–12589. [PubMed: 23106422]
33. Boros E, Ferreira CL, Cawthray JF, Price EW, Patrick BO, Wester DW, Adam MJ, Orvig C. *J. Am. Chem. Soc.* 2010; 132:15726–15733. [PubMed: 20958034]
34. Mato-Iglesias M, Balogh E, Platas-Iglesias C, Toth E, Blas Ad, Blas TR. *Dalton Trans.* 2006:5404–5415. [PubMed: 17102866]
35. Balogh E, Mato-Iglesias M, Platas-Iglesias C, Toth E, Djanashvili K, Peters JA, de Blas A, Rodriguez-Blas T. *Inorg. Chem.* 2006; 45:8719–8728. [PubMed: 17029383]
36. Price EW, Zeglis BM, Cawthray JF, Ramogida CF, Ramos N, Lewis JS, Adam MJ, Orvig C. *J. Am. Chem. Soc.* 2013; 135:12707–12721. [PubMed: 23901833]
37. Vosjan MJWD, Perk LR, Visser GWM, Budde M, Jurek P, Kiefer GE, van Dongen GAMS. *Nat. Protocols.* 2010; 5:739–743.
38. Perk L, Vosjan M, Visser G, Budde M, Jurek P, Kiefer G, van Dongen G. *Eur. J. Nucl. Med. Mol. Imaging.* 2010; 37:250–259. [PubMed: 19763566]
39. Holland JP, Sheh Y, Lewis JS. *Nucl. Med. Biol.* 2009; 36:729–739. [PubMed: 19720285]
40. Mawani Y, Cawthray JF, Chang S, Sachs-Barrable K, Weekes DM, Wasan KM, Orvig C. *Dalton Trans.* 2013; 42:5999–6011. [PubMed: 23299719]
41. Barta CA, Sachs-Barrable K, Jia J, Thompson KH, Wasan KM, Orvig C. *Dalton Trans.* 2007:5019–5030. [PubMed: 17992287]
42. Kumar Mishra A, Panwar P, Chopra M, Kumar Sharma R, Chatal J-F. *New J. Chem.* 2003; 27:1054–1058.
43. Zeglis BM, Sevak KK, Reiner T, Mohindra P, Carlin SD, Zanzonico P, Weissleder R, Lewis JS. *J. Nucl. Med.* 2013; 54:1389–1396. [PubMed: 23708196]
44. Holland JP, Caldas-Lopes E, Divilov V, Longo VA, Taldone T, Zatorska D, Chiosis G, Lewis JS. *PLoS One.* 2010; 5:e8859. [PubMed: 20111600]
45. Anderson CJ, Connett JM, Schwarz SW, Rocque PA, Guo LW, Philpott GW, Zinn KR, Meares CF, Welch MJ. *J. Nucl. Med.* 1992; 33:1685–1691. [PubMed: 1517844]
46. Anderson CJ, Schwarz SW, Connett JM, Cutler PD, Guo LW, Germain CJ, Philpott GW, Zinn KR, Greiner DP, Meares CF, Welch MJ. *J. Nucl. Med.* 1995; 36:850–858. [PubMed: 7738663]
47. Smith-Jones PM, Fridrich R, Kaden TA, Novak-Hofer I, Siebold K, Tschudin D, Maecke HR. *Bioconjugate Chem.* 1991; 2:415–421.

48. Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA Jr. J. Immunol. Methods. 1984; 72:77–89. [PubMed: 6086763]
49. Lindmo T, Bunn PA Jr. Methods Enzymol. 1986; 121:678–691. [PubMed: 3523136]

Figure 1.

A) iTLC radiochromatograph of the unpurified radiolabeling mixture of ¹¹¹In-phospa-trastuzumab (30 minutes, 25 °C, 2.64 mCi/mg); B) iTLC of ¹¹¹In-phospa-trastuzumab after purification by PD-10 column; C) iTLC trace of the unpurified radiolabeling mixture of ¹⁷⁷Lu-phospa-trastuzumab (30 minutes, 25 °C, 2.23 mCi/mg); and D) iTLC trace of ¹⁷⁷Lu-phospa-trastuzumab after purification, showing ¹⁷⁷Lu transchelated from the weakly-bound ¹⁷⁷Lu-phospa-trastuzumab to EDTA⁴⁻ (~200 µg of antibody conjugate used for each reaction).

Figure 2.

The stability of $^{177}\text{Lu}/^{111}\text{In}$ -phospa-trastuzumab in human blood serum over 120 h, agitated at 300 rpm and 37 °C, analyzed via radio-iTLC elution with EDTA mobile phase (50 mM, pH 5), compared to previously obtained values for $^{177}\text{Lu}/^{111}\text{In}$ -octapa-trastuzumab as a reference.³⁶

Figure 3.

Radiolabeling results of H₆phospa-trastuzumab with ⁸⁹Zr in phosphate buffered saline (pH 7.4), showing results at both room temperature and 37 °C with H₆phospa-trastuzumab (3.3 ± 0.1 chelates per antibody), revealing poor radiometal incorporation of < 12% even after 18 hours (~300 µg of antibody conjugate used for each reaction and ~1 mCi of ⁸⁹Zr).

Mouse 1

Mouse 2

Figure 4. SPECT/CT images of ^{111}In -phospha-trastuzumab in female nude athymic mice bearing SKOV-3 tumor xenografts in the right shoulder (diameter ~2 mm), imaged at 24, 72, and 120 hrs p.i., showing significant tumor uptake as well as some uptake in the bone (knee joints and shoulders) and kidneys (outline is visible upon magnification) due to chelate instability (~800–810 μCi injected activity, ~150–200 μg antibody per mouse).

Figure 5.

SPECT/CT images of ^{177}Lu -phospa-trastuzumab in female nude athymic mice bearing SKOV-3 tumor xenografts in the right shoulder (diameter ~2 mm), imaged at 24 and 72 hrs p.i., showing negligible tumor uptake and primarily bone uptake at all time points (~575 μCi injected activity, ~150–200 μg antibody per mouse).

Scheme 1.

Synthesis of **H₆phospa (2)** and **p-SCN-Bn-H₆phospa (10)**, (i) paraformaldehyde (12 eq.), phosphorous acid (20 eq.), HCl (5 mL, 6 M), reflux, 48 h, 70%; (ii) THF, NaHCO₃, 2-nitrobenzenesulfonyl chloride (2.2 equiv), RT, 24 h, 74%; (iii) DMF, Na₂CO₃, methyl-6-bromomethylpicolinate (2.2 equiv), 60 °C, 48 h, 95%; (iv) THF, thiophenol (2.2 equiv), K₂CO₃, RT, 72 h, 90%; (v) paraformaldehyde (16 eq.), phosphorous acid (40 eq.), HCl (5 mL, 6 M), reflux, 48 h, 42%; (vi) 3 mL of (1:1) glacial acetic acid:3 M HCl, Pd/C (20 wt%), H₂ (g) balloon, RT, 1 h, used without further purification (vii) thiophosgene in DCM (15 equiv), HCl (2 mL, 3M), RT, 24 h, 14% over steps vi-vii.

Chart 1.

The current “gold standard” chelators for ¹¹¹In/¹⁷⁷Lu (DOTA, as well as CHX-A''-DTPA and the recently published and promising H₄octapa), DFO, previously published members of the “pa” family, and the new entrant H₆phospa.

Table 1

Chemical and *in vitro* biological characterization data for ^{111}In - and ^{177}Lu -phospa-trastuzumab radioimmunoconjugates.

Immunoconjugate	Isotope	Radiolabeling conditions and yield	Chelates / mAb ^d	Specific activity (mCi/mg)	Immunoreactive fraction (%) ^b	Serum stability 120 h (%) ^c
H ₄ phospa-trastuzumab	^{111}In	30 min, 25 °C, 70–90%	3.3 ± 0.1	2.9 ± 0.7	97.9 ± 2.6	52.0 ± 5.3
	^{177}Lu	30 min, 25 °C, 40–80%	3.3 ± 0.1	2.8 ± 0.8	n/a	2.0 ± 0.3

^a Isotopic dilution assays, n = 3.

^b Determined immediately prior to *in vivo* experimentation, n = 9.

^c Calculated in human serum at 37 °C for 120 h, n = 3.