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Improved Synthesis of the Bifunctional Chelating Agent 1,4,7,10-Tetraaza-*N*-(1-carboxy-3-(4-nitrophenyl)propyl)-*N′*,*N″*,*N‴*-*tris*(acetic acid)cyclododecane (PA-DOTA)

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Abstract—A concise synthesis of the bifunctional chelating agent 1,4,7,10-tetraaza-*N*-(1-carboxy-3-(4-nitrophenyl)propyl)-*N′*,*N″*,*N‴*-*tris*(acetic acid)cyclododecane (PA-DOTA) is reported. Difficulties involving the production of partially alkylated products and their removal have been addressed and obviated. After the pure nitro form of PA-DOTA was obtained, conversion to the isothiocyanato form PA-DOTA (**1**, conjugation to HuCC49 and HuCC49ΔCH2 monoclonal antibodies was achieved. Subsequent radiolabeling with ^{177}Lu was performed, demonstrating a useful bifunctional chelating agent suitable for clinical radioimmunotherapy applications.

Introduction

There is continued interest in the synthesis of bifunctional chelating agents (BCAs), their conjugation to proteins and peptides, and subsequent use for sequestering radioactive metal ions for use in radioimmunotherapy and radioimmunoimaging.[1,2] One strict requirement these reagents must meet to be useful for such applications, is that the metal–chelate complex must remain intact in vivo.[3] Many BCAs have been based on acyclic polyaminocarboxylate reagents, such as EDTA or DTPA.[1,4,5] In certain cases, particularly in applications with ^{90}Y and radioisotopes of lanthanide ions, bifunctional EDTA and some DTPA type chelates are not suitably stable due to metal ion release and subsequent accumulation of this isotope in normal tissue.[6,7] BCAs based on 1,4,7,10-tetraazacyclododecane *N*,*N′*,*N″*,*N‴*-tetraacetic acid (DOTA) are attractive since DOTA complexes of Y(III) and lanthanide(III) ions, in general, have exceptional kinetic inertness to metal ion release.[8–10] Studies have been reported using the radioisotopes ^{177}Lu,[11–13] ^{153}Sm,[11] and ^{90}Y,[14–17] with numerous bifunctional derivatives based upon the DOTA template.

The site of the reactive group for protein conjugation of the BCA has been of some interest. Two fundamental possibilities exist; either a chelate with C-functionalization[18] on the macrocycle backbone or N-functionalization through one of the coordinating pendent arms.[16,17,19] One such ligand of the latter category that has been reported is PA-DOTA (Fig. 1).[20] The synthesis presented in the patent literature involves numerous chromatographic steps throughout the synthesis in order to remove a material that is referred to as an isomer.[20] For example, the material recovered after column chromatography in the synthesis of the tetra-ester form of PA-DOTA was reported to be a 2:1 mixture of either 'conformational or geometric isomers' based upon interpretation of ^{1}H and ^{13}C NMR spectra. This 'isomer' was then carried through the remainder of the synthesis. After hydrogenation and saponification, this anomalous isomer was finally separated by column chromatography and identified to actually be 1,4,7,10-tetraaza-*N*-(1-carboxy-3-(4-aminophenyl)propyl)-*N′*,*N″*,-*bis*(acetic acid)cyclododecane (PA-DO3A-NH_2) (Fig. 2). Presence of the heptadentate PA-DO3A in samples of the final product, PA-DOTA, would contribute to a significant overall decreased kinetic inertness to metal ion release

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of the sample.[21,22] Thus, purity of the BCA is of absolute critical importance if the reagent is to be used successfully for in vivo applications. Any doubts as to the quality of the reagent would compromise its continued use in the clinic.

Our laboratories have developed an interest in both the synthesis of PA-DOTA and its subsequent employment as a chelating agent for ^{177}Lu for clinical applications. There are currently no commercial sources of this bifunctional DOTA derivative,[23] due to the prior source having discontinued active research in this area over three years ago. The patent literature provides the only guidance for the synthesis of PA-DOTA. In order for ongoing and future evaluation and experiments to proceed, there exists a need for a concise, straightforward and reproducible synthesis of PA-DOTA, amenable for production of cGMP immunoconjugates.

The humanized antibodies chosen for conjugation in this study were HuCC49 and HuCC49ΔCH2,[24,25] which were developed from the murine monoclonal antibody CC49. These antibodies are reactive with tumor associated glycoprotein (TAG-72), which is expressed on the majority of colorectal, gastric, ovarian, and breast cancers. Radiolabeled CC49 has shown excellent tumor targeting properties and is currently employed in several Phase I and II clinical trials.[12,26,27] Additionally, this antibody has been previously conjugated with PA-DOTA, radiolabeled with ^{177}Lu[12,26–28] and therefore serves as an excellent choice to determine the validity of the PA-DOTA prepared in the report. Unfortunately, a direct comparison of the product described herein to the previously available PA-DOTA is impossible due to its aforementioned discontinuance.

Herein, we report an improved and detailed synthesis of PA-DOTA, wherein the purification concerning potential contamination by PA-DO3A is addressed. The bifunctional ligand obtained is suitable for clinical studies as demonstrated by conjugation to HuCC49 and HuCC49ΔCH2, radiolabeling with ^{177}Lu and tumor localization in an animal model of human colorectal cancer.

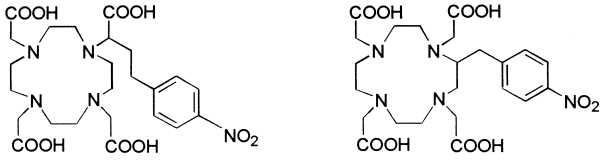

Figure 1. Structure of PA-DOTA-NO_2 (left) and C-functionalized DOTA (right).

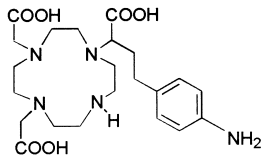

Figure 2. Structure of PA-DO3A-NH_2.

Results and Discussion

Ligand synthesis and characterization

The 1,4,7,10-tetraazacyclododecane (cyclen) framework provides a versatile basis for the synthesis of macrocyclic ligands. The nitrogens of the cyclen ring can be alkylated with various ligating pendent groups, allowing for a convenient method for ligand design. The mono-alkylation of cyclen developed by Kruper and co-workers[29] provides a straightforward route to introducing a functionalized pendent group without selective protection and deprotection of the remaining nitrogens of the cyclen ring.

Thus, the synthesis of PA-DOTA began with the free base cyclen being alkylated in $CHCl_3$ with an alkyl halide of the N-functionization pendent group in a 1.5:1 ratio (Fig. 3). The mono-alkylated derivative (**2**) was predominately formed and isolated in a pure form after column chromatography.[29] This intermediate was then further alkylated with ethyl bromoacetate to introduce the remaining carboxylate functionality of the ligand. After exceedingly careful chromatography, PA-DOTA-NO_2 ester (**3**) was obtained free of any partially alkylated materials and any isomers, conformational or geometric, as determined by the ^1H and ^{13}C NMR spectra. This tetraester was then saponified in concd HCl, followed by hydrogenation to yield PA-DOTA-NH_2 (**5**). Unlike as described in the patent, wherein silica chromatography was performed after each step in the reaction sequence,[20] no further purification was required after alkylation with the ethyl bromoacetate. The PA-DOTA-NH_2 was reacted with thiophosgene in H_2O:$CHCl_3$ to yield PA-DOTA-NCS (**1**), which was then suitable for conjugation to antibodies HuCC49 and HuCC49ΔCH_2. In addition, PA-DOTA-NH_2 ester (**6**) was synthesized as a reference material for comparison to data presented in the patent procedure. Again, the material synthesized here contained only one pair of doublets in the aromatic region of the ^1H NMR spectrum, indicating its purity, unlike the material described in the patent. The route presented here was preferred as it eliminates hydrogenation in an organic solvent, which eliminates a potential fire hazard. Additionally, delaying the formation of the aniline to near the end of the synthesis is preferable since the aniline is somewhat sensitive to decomposition.

In the original patent application,[20] it was reported that after column chromatography the PA-DOTA-NO_2 ester was isolated as a 2:1 mixture of conformational or geometric isomers. This conclusion concerning the number of species present was determined by NMR spectroscopy. This interpretation was presumably prompted by the two pairs of doublets for the aromatic protons reported in the ^1H NMR spectrum. In the synthesis reported here, after column chromatography there is simply one pair of doublets for the aromatic protons. The extra pair of doublets can been observed in the ^1H NMR spectra while monitoring the reaction mixture and in early fractions obtained from column chromatography. These extra doublets are also present before purification of PA-DOTA-NO_2 regardless of (**2**) being

alkylated with either ethyl or methyl bromoacetate and indicated that these extra doublets are not dependent on the choice of alkylating agent. In this preparation, the impurity that gives rise to these extra resonances is present in much smaller amounts, routinely less than 10%, compared to 33% in the patent. In our preparation, this impurity can be removed entirely by exceedingly careful column chromatography to obtain (**3**) without the requirement of any further chromatographic efforts. In subsequent preparations of PA-DOTA in our laboratories, by using a four- to fivefold excess of ethyl bromoacetate and by carefully monitoring the reaction mixture by ^{1}H NMR, column chromatography required to purify the tetra-alkylated product can be reduced to one to two columns.

For a direct comparison to the literature results, preparation of PA-DOTA-NO_2 using methyl bromoacetate, beginning with 4 equiv, was monitored by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of the reaction mixture after 17 h contained two pairs of doublets in the aromatic region and the resonances at 7.38 and 7.46 ppm are in approximately a 2:1 ratio. The volume of the reaction mixture was reduced and more methyl bromoacetate (0.5 additional equiv) was added. After an additional 24 h, the ^{1}H NMR spectrum of the crude reaction mixture appeared to contain essentially only the one doublet at 7.38 ppm. Additionally, two singlets at 3.70 ppm and 3.68 ppm, previously prominent, were now greatly reduced. A sample of PA-DO3A-NO_2 ester was intentionally synthesized for comparison. The resonance at 7.46 ppm in the crude reaction mixture of the PA-DOTA-NO_2 tetramethyl ester was found to be in agreement with the ^{1}H NMR spectrum of the intentionally synthesized PA-DO3A-NO_2 ester. Also, the singlets at 3.70 and 3.68 ppm in the crude reaction mixture are in agreement with those found in the ^{1}H NMR spectrum of the PA-DO3A-NO_2 ester. However, extreme difficulties were encountered in obtaining material that was of acceptable purity in the preparation of the tetramethyl ester. Even after repeated column chromatography and removal of excess methyl bromoacetate and solvents, measurable traces of the PA-DO3A-NO_2 trimethyl ester were apparent in the ^{1}H

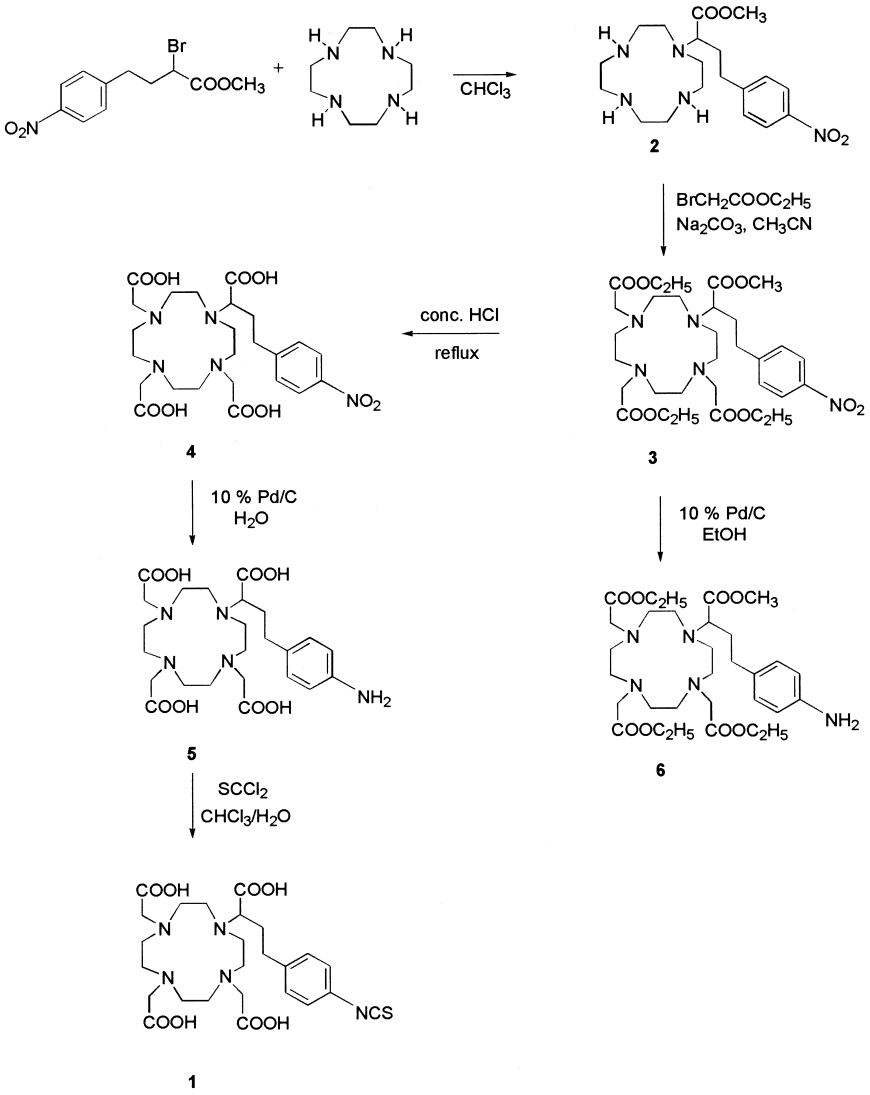

Figure 3. Synthetic scheme of PA-DOTA.

NMR spectrum of PA-DOTA-NO_2 tetramethyl ester. Also, since the methyl ester resonances for the four pendent arms overlapped it was difficult to determine the purity. The PA-DOTA-NO_2 triethyl-methyl ester was found to be preferable for the routine synthesis of this bifunctional ligand for three reasons. First, the tetra- and trace amounts of the tri-alkylated products separated more efficiently during column chromatography. Second, the methyl ester and ethyl ester resonances do not overlap in the ^1H NMR spectrum, making it possible to assess the purity more accurately. Thirdly, chromatography of PA-DOTA-NH_2, in the acid form as outlined in the patent, is eliminated. This obviates elution from silica gel with aqueous components, that may later complicate further modification of the ligand, such as the presence of ammonium salts in the reaction with thiophosgene.

A comparison of the yields of this preparation and that of the patent is not easily addressed. In the synthesis reported herein, to obtain PA-DOTA-NH_2 (**5**) from (**2**), the overall yield was 64%. In the patent, the yield reported after alkylation of (**2**) with methyl bromoacetate was 95%. However, it must be pointed out that this is a crude yield, since the material is reported to be a 2:1 mixture of tetra- and tri-alkylated products. The reduction of this crude material proceeded with a 91% reported crude yield and no yield was reported after column chromatography. Saponification of 1.5 g of the crude PA-DOTA-NH_2 ester, again the 2:1 mixture tetra- and tri-alkyated products, resulted in 0.58 g of the final PA-DOTA-NH_2 as a mixed ammonium and potassium salt after silica gel column chromatography with $CHCl_3$:MeOH:concd NH_4OH (2:2:1) elution. Thus, the calculation of an overall yield for the synthesis of (**5**) via the patent literature is highly problematic. It appears that not only is the PA-DOTA synthesized by the method reported here of a more reproducible purity and is better characterized, but it also is prepared in a higher overall yield.

Conjugation, radiolabeling, and biodistribution of ^{177}Lu-PA-DOTA-HuCC49 and ^{177}Lu-PA-DOTA-HuCC49ΔCH2

The conjugation of the PA-DOTA was performed according to routine methods in mildly basic buffer to efficiently react the isothiocyanate group with available amines present on the proteins. A 4:1 ligand to protein molar ratio was chosen as to limit the product to approximately having on the average one chelating agent present on the protein. This level of modification has been noted as being preferable with similar antibodies. Radiolabeling of the conjugates was performed using ^{177}Lu obtained as the chloride salt to obtain nearly identical results for each conjugate; specific activity of 0.6 mCi/mg and greater than 95% radiochemical purity as determined by HPLC.

The tumor localization and biodistribution of ^{177}Lu-PA-DOTA-HuCC49 and ^{177}Lu-PA-DOTA-HuCC49ΔCH2 were determined using groups of 4–5 athymic nude mice bearing LS174T human colon intraperitoneal tumor nodules (~0.4 g) 24 h after injection (Table 1). The median tumor concentrations for ^{177}Lu-PA-DOTA-HuCC49 and ^{177}Lu-PA-DOTA-HuCC49ΔCH2 were 7.9 and 9.4% injected dose per gram (%ID/g), respectively, which did not significantly differ ($p = 0.905$). However, the respective blood concentrations were 0.4 and 0.2% ID/g, which were significantly different ($p = 0.016$). The median tumor:blood ratio of ^{177}Lu-PA-DOTA-HuCC49ΔCH2 (60.2) was significantly higher than with ^{177}Lu-PA-DOTA-HuCC49 (19.4, $p = 0.016$) due to the more rapid elimination from blood. The uptake of ^{177}Lu-PA-DOTA-HuCC49ΔCH2 in kidney (5.8% ID/g) was higher than for ^{177}Lu-PA-DOTA-HuCC49 (2.2% ID/g, $p = 0.016$), while the uptake in liver (9.2 and 6.4% ID/g, respectively) and spleen (4.0 and 1.8% ID/g, respectively) of the two monoclonal antibodies were similar ($p = 0.730$ and $p = 0.191$, respectively). The biodistribution of the ^{177}Lu-PA-DOTA-CC49 conjugate as reported in the patent literature[20] showed greater uptake in the liver (11.54% ID/g), spleen (10.86% ID/g) kidney (7.67% ID/g) and bone (3.65% ID/g) at 24 h as compared to the ^{177}Lu-PA-DOTA-CC49 conjugate reported here (Table 1). This increased liver, spleen, kidney and bone uptake might be indicative of loss of ^{177}Lu from conjugates possibly containing trace amounts of PA-DO3A. No tumor data were given in the patent. There is no comparison available for the ^{177}Lu-PA-DOTA-HuCC49ΔCH2 conjugate, as this immunoprotein has not been previously conjugated with PA-DOTA and labeled with ^{177}Lu.

Table 1. Biodistribution of ^{177}Lu-PA-DOTA-HuCC49 and ^{177}Lu-PA-DOTA-HuCC49ΔCH2 in groups of 4–5 athymic nude mice bearing LS174T human colon cancer xenografts at 24 h after ip injection

Tissue	^{177}Lu-HUCC49 (% ID/g)[a]	^{177}Lu-HUCC49ΔCH2 (% ID/g)[b]
Tumor	7.9(6.6–9.3)	9.4(5.8–14.3)
Blood	0.4(0.3–0.5)	0.2(0.1–0.2)[c]
Kidney	2.2(1.9–2.2)	5.8(4.5–9.3)[c]
Liver	6.4(4.2–7.2)	9.2(5.6–19.0)
Spleen	1.8(1.4–2.9)	4.0(2.5–8.5)
Bone	1.7(1.4–1.8)	2.2(1.1–4.5)

[a] Median with inter-quartile range in parentheses for five mice.
[b] Median with inter-quartile range in parentheses for four mice.
[c] Lu-HuCC49 versus ^{177}Lu-HuCC49ΔCH2 $p < 0.02$.

Conclusion

The major obstacle to the synthesis of cyclen based ligands containing four pendent groups, such as DOTA derivatives, has been the production of the fully alkylated product with minimal trialkylated side-product. By using an excess of ethyl bromoacetate, the tetra-alkylated product can be formed predominately with the partially alkylated products at only 10% or less. However, even when excess alkylating agent has been used for the synthesis of PA-DOTA, producing solely the tetra-alkylated product has been challenging. After repeated and careful chromatography, the ^1H NMR spectrum of the PA-DOTA-NO_2 ester contained only

one set of doublet of doublets in the aromatic region, 7.38 and 8.15 ppm, and not two sets as indicated by the patent application. Once the pure form of PA-DOTA-NO_2 tetra-ester was obtained, the products of saponification and hydrogenation required no further chromatography.

Conjugation of the isothiocyanato form of PA-DOTA (**1**) to HuCC49 and HuCC49ΔCH2, and subsequent radiolabeling with ^{177}Lu, was successfully performed to demonstrate the binding activity of the conjugates and their ability to target to a tumor in vivo. Thus, a concise reproducible synthesis of the PA-DOTA has provided a reagent suitable for production of clinical grade immunoconjugates for the treatment of malignant disease.

Experimental

Materials and methods

All materials were of reagent grade and used without further purification. The free base form of cyclen was generated by dissolution of the tetrahydrochloride salt (Parish) in H_2O (5–6 g in 20 mL). The pH was raised to 12.5 by addition of solid NaOH and the free base of cyclen was extracted with $CHCl_3$ (6 × 200 mL). The $CHCl_3$ was removed by rotary evaporation and the white solid vacuum dried (recovery ~95%). Chromatography was performed on silica gel 60, 220–440 mesh ASTM (Fluka). Thin-layer chromatography (TLC) was performed on silica gel 60 F-254 plates (EM Reagents). All glassware used in the synthesis of the ligands after hydrolysis was acid-washed and rinsed with distilled deionized water.

^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 instrument at ambient temperature. Chemical shifts are reported downfield relative to TMS ($CDCl_3$) or TSP (D_2O). Chemical ionization mass spectra (CI-MS) were measured using a Finnegan 3000 instrument. Fast atom bombardment mass spectra (FABMS) were measured using a Extrel 400 instrument. High resolution FAB (HRFAB) mass spectra were obtained on a JEOL SX102 mass spectrometer operated at an accelerating voltage of 10 KV. Samples were desorbed from a glycerol matrix using 6 KeV xenon atoms. Mass measurements in HRFAB are performed at 10,000 resolution. Analytical HPLC was performed on a Beckman gradient system equipped with Model 114M pumps controlled by System Gold software and a Model 165 dual wavelength detector set at 254 and 280 nm. An Altex ODS C18 column (4.6 mm × 15 cm) with a 25 min gradient of 100% aqueous 0.05 M triethylammonium acetate to 100% methanol at a flow rate of 1 mL/min was used. Infrared spectra were obtained using a Beckman 4240 spectrophotometer with the samples prepared as Nujol mulls. Size-exclusion HPLC was performed on a Bio-Rad HPLC Pump Model 1330 with a Rainin Instrument Co., Inc. Hydropore SEC 83-S13-C5 column and an isocratic buffer of 10 mM sodium phosphate, 300 mM NaCl and 10% DMSO at a 1 mL/min flow rate.

1,4,7,10-tetraaza-*N*-(1-carbomethoxy-3-(4-nitrophenyl)-propyl)cyclododecane (2). To a solution of cyclen (4.71 g, 27.3 mmol) in amylene stabilized anhydrous $CHCl_3$ (Aldrich) (80 mL) was added over 3 min methyl (*d,l*)-2-bromo-4-(4-nitrophenyl)butanoate (5.49 g, 18.2 mmol).[20] The vial into which the ester had been measured was rinsed with $CHCl_3$ (10 mL) and the solution added to the reaction mixture. The reaction mixture was stirred at room temperature under argon and monitored by TLC (product $R_f = 0.75$, silica gel, 12:4:1 $CHCl_3$:MeOH:concd NH_4OH). In order to accelerate the reaction, the volume was reduced slightly (10–20 mL) by rotary evaporation after 16 h and again after 40 h. After this time, the reaction was complete as determined by TLC. The reaction mixture was purified by column chromatography on silica gel with the above solvent system. Several (2–4) consecutive purifications were required to obtain the product in a pure form. All fractions that contained only the mono-alkylated product, as determined by TLC and ^1H NMR, were combined, reduced to dryness, and vacuum dried. The product was obtained as a thick orange oil (6.20 g, 87%). ^1H NMR (300 MHz, $CDCl_3$) δ 1.7 (br, 3H, NH), 2.01 (m, 1H, C*H*CH_2Ar), 2.14 (m, 1H, C*H*CH_2Ar), 2.52 (m, 4H, CH_2 cyclen), 2.6–3.0 (m, 14H, CH_2 cyclen, CH_2Ar), 3.41 (m, 1H, C*H*($COOCH_3$)), 3.52 (s, 3H, $COOCH_3$), 7.41 (d, 2H, Ar), 8.15 (d, 2H, Ar); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 30.56 (*C*H_2CH_2Ar), 32.56 (CH_2Ar), 45.07, 45.55, 46.95, 48.65 (CH_2 cyclen), 51.14, (*C*H($COOCH_3$)), 62.07 (OCH_3), 123.6, 129.2, 146.3, 149.4 (Ar), 172.7 (C(O)); MS (CI/NH3) *m/e* 394 ($M + H^+$)

1,4,7,10-Tetraaza-*N*-(1-carbomethoxy-3-(4-nitrophenyl)-propyl)-*N′*,*N″*,*N‴*-*tris*(acetic acid, ethyl ester)cyclododecane (3) (PA-DOTA-NO_2 ester). 1,4,7,10-tetraaza-*N*-(1-carbomethoxy-3-(4-nitrophenyl)propyl)cyclododecane (5.92 g, 15.0 mmol), CH_3CN, purged with $Ar_{(g)}$, (170 mL), anhyd Na_2CO_3 (7.12 g) and ethyl bromoacetate (8.93 g, 53.5 mmol) were combined in a 300 mL round bottom flask. The reaction mixture was stirred at room temperature and monitored by TLC (silica gel, 12:4:1, $CHCl_3$:MeOH:concd NH_4OH). After 2 days, the reaction did not appear to be progressing further, but was also incomplete

The reaction mixture volume was reduced to 70 mL by rotary evaporation and allowed to stir for 6 h after concentration. As TLC monitoring indicated that the reaction was still not progressing, additional ethyl bromoacetate (1 mL, 10 mmol) was added to fully alkylate the cyclen ring. After 4 days, the reaction mixture had progressed since the 2 day time point, but was not progressing any further. The reaction mixture was purified by column chromatography. The initial column that was run (silica gel, 2 × 15 in) was poured with 5% MeOH in $CHCl_3$. The reaction mixture was applied and the column eluted with 5% MeOH in $CHCl_3$ (600 mL). The amount of MeOH was increased to 20% (630 mL) and finally the column was eluted with 12:4:1 $CHCl_3$:MeOH:concd NH_4OH (500 mL). Fractions collected within the middle range of the elution with 20% MeOH to the end contained the desired product. These fractions

were combined and reduced to dryness to give the product as a yellow foam (10.76 g). This material contained small (< 10%) amounts of impurities, indicated by an extra doublet at 7.6 ppm in the ^{1}H NMR. This material was re-purified by running consecutive silica gel columns (2–4) with a shallower gradient, 5–10% MeOH in $CHCl_3$ and collection of smaller fractions (10–20 mL). Very late fractions from these columns were deemed pure by their ^{1}H NMR spectra, combined, and rotary evaporated to a foam (total mass 8.96 g, 77%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.3 (m, 9H, CH_3), 1.8–2.2 (m, 14H,-CH_2 cyclen, CH_2CH_2Ar), 2.4–2.6 (m, 4H, CH_2 cyclen), 3.15 (m, 1H, CH(COOCH_3)), 3.4–3.6 (m, 8H, CH_2COOC$_2$H$_5$, CH_2Ar), 3.81 (s, 3H, COOCH_3), 4.1–4.3 (m, 6H, OCH_2), 7.38 (d, 2H, Ar), 8.15 (d, 2H, Ar); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.74 (OCH$_2$CH_3), 25.03 (CH_2CH$_2$Ar), 33.89 (CH_2Ar), 44.64, 47.07, 48.40, 48.33 (CH_2 cyclen), 52.05, 52.47, 54.84 (CH(COOCH_3), CH_2COOC$_2$H$_5$), 59.58, 61.15 (OCH_2, OCH_3), 123.5, 129.5, 146.4, 149.1 (Ar); 173.6, 176.0 (C(O)); MS (CI/NH$_3$) m/e 652 (M + H$^+$). Anal. calcd for $C_{31}H_{49}N_5O_{10}$·H_2O·NaBr: C, 48.19; H, 6.65; N, 9.06. Found: C, 48.17; H, 6.62; N, 9.03.

1,4,7,10-Tetraaza-*N*-(1-carbomethoxy-3-(4-aminophenyl)-propyl)-*N′*,*N″*,*N‴*-*tris*(acetic acid, ethyl ester)cyclododecane (6) (PA-DOTA-NH$_2$ ester). A Schlenk flask was charged with 10% Pd/C (267 mg) and EtOH (6 mL) were under Ar$_{(g)}$, fitted onto an atmospheric hydrogenator, and then saturated with H$_{2(g)}$. A solution of PA-DOTA-NO_2 ester (500 mg, 0.65 mmol) in EtOH (2 mL) was injected into the Schlenk flask. The hydrogenation was allowed to proceed until the uptake of H$_{2(g)}$ had halted. The reaction mixture was filtered through a bed of Celite 577 packed in a medium glass fritted funnel. The filtrate was reduced to dryness by rotary evaporation and vacuum dried to give the product as a pale yellow foam (477 mg, 74%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.27 (m, 9H, -CH_3), 1.7–3.0 (series of mult., 21 H, methyl ester pendent arm, CH_2 cyclen), 3.2–3.5 (m, 8H, CH_2COOC$_2$H$_5$, CH_2Ar), 3.78 (s, 3H, OCH_3), 4.05–4.3 (m, 6H, OCH_2), 6.74 (d, 2H, Ar), 7.08 (d, 2H, Ar); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.92 (OCH$_2$CH_3), 25.09 (CH$_2$CH$_2$Ar), 32.62 (CH_2Ar), 44.40, 47.13, 48.53, 51.56 (CH_2 cyclen), 52.59, 54.96 (CH(COOCH_3), CH_2COOC$_2$H$_5$), 58.30, 61.09 (OCH_2, OCH_3), 115.1, 129.6, 129.9, 145.2 (Ar), 173.5, 173.6, 176.9 (CO); MS (FAB/glycerol) 644 (M + Na$^+$).

1,4,7,10-Tetraaza-*N*-(1-carbomethoxy-3-(4-nitrophenyl)-propyl)-*N′*,*N″*-*bis*(acetic acid, ethyl ester)cyclododecane (6) (PA-DO3A-NO$_2$ ester). 1,4,7,10-Tetraaza-*N*-(1-carbomethoxy-3-(4-nitrophenyl)propyl)-cyclododecane (380 mg, 0.97 mmol), CH_3CN (10 mL), anhydrous Na_2CO_3 (409 g) and methyl bromoacetate (298 mg, 1.95 mmol) were combined in a 25 mL round bottom flask. The reaction mixture was stirred at room temperature for 17 h after which it was applied to a silica gel column. It was eluted first with 5% MeOH in $CHCl_3$ and then increased to 10% MeOH to obtain the PA-DO3A-NO_2 ester. Solvents were removed by rotary evaporation and the orange foam obtained was vacuum dried overnight (100 mg, 19%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.7 (br, 1H, NH), 2.08 (m, 1H, CH_2CH$_2$Ar), 2.22 (m, 1H, CH_2CH$_2$Ar), 2.7–3.3 (series of mult., 17H), 3.3–3.44 (m, 3H), 3.48 (s, 3H) 3.68 (s, 3H, CH$_2$COOCH_3) 3.70 (s, 3H, CH$_2$COOCH_3), 3.75 (s, 3H, COOCH_3), 7.46 (d, 2H, Ar), 8.18 (d, 2H, Ar); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 30.74, 32.44 (functionalized pendent), 46.89, 47.38, 47.86, 49.56 (CH_2 cyclen), 51.44, 51.57, 57.33, 65.71 (pendent arms and OCH_3), 123.6, 129.2, 146.4, 148.8 (Ar); 170.5, 171.7, 172.3 (C(O)); MS (CI/NH3) m/e 538 (M + H$^+$)

1,4,7,10-Tetraaza-*N*-(1-carboxy-3-(4-nitrophenyl)propyl)-*N′*,*N″*,*N‴*-*tris*(acetic acid)cyclododecane (4) (PA-DOTA-NO$_2$). To a 250 mL round bottom flask was added concentrated HCl (100 mL) (Baker Ultrapure) and PA-DOTA-NO_2 ester, (6.7 g, 8.7 mmol). This was heated at 100–110°C for 6 h. The aqueous HCl was removed by rotary evaporation and the residue taken up in water (2–3 mL). This solution was then lyophilized to give PA-DOTA-NO_2 as a pale yellow solid (6.49 g, 99%). ^{1}H NMR (300 MHz, D_2O, pH 1.0) δ 2.0 (m, 1H, CH(COOH)), 2.19 (m, 1H, CH(COOH)), 2.6–4.0 (series of mult., 25H, cyclen, pendent arms), 7.53 (d, 2H, Ar); 8.20 (d, 2H, Ar); (300 MHz, D_2O, pH = 13) δ 1.75 (m, 1H, CH(COOH)), 2.0–3.4 (series of mult., 26H, cyclen, pendent arms), 7.51 (d, 2H, Ar); 8.19 (d, 2H, Ar); analytical HPLC t_R = 10.7 min; M + H$^+$ calcd for $C_{24}H_{36}N_5O_{10}$ 554.2462, found [HRFAB] m/e = 554.2480, error = + 3.3 ppm. Anal. calcd for $C_{24}H_{35}N_5O_{10}$, 3HCl, H_2O·NaCl: C, 38.52; H, 5.52; N, 9.36. Found: C, 38.42; H, 5.39; N, 9.36.

1,4,7,10-Tetraaza-*N*-(1-carboxy-3-(4-aminophenyl)propyl)-*N′*,*N″*,*N‴*-*tris*(acetic acid)cyclododecane (5) (PA-DOTA-NH$_2$). A Schlenk flask was charged with 10% Pd/C (332 mg) and H_2O (10 mL) and fitted onto an atmospheric hydrogenator. The apparatus was flushed with H$_{2(g)}$ two times to fully saturate the catalyst. A solution of PA-DOTA-NO_2 (1.5 g, 2.0 mmol) in water (10 mL) was injected via syringe into the flask. The hydrogenation was allowed to proceed until the uptake of H$_{2(g)}$ had halted. The reaction mixture was filtered through a bed of Celite 577 packed in a medium glass fritted funnel. The slightly pinkish filtrate was reduced to dryness by rotary evaporation and the light purple residue taken up in water (1–2 mL). This was then lyophilized to give PA-DOTA-NH_2 as a light purple solid (1.18 g, 84%). ^{1}H NMR (300 MHz, D_2O, pH 1) δ 2.0 (m, 1H, CH(COOH)), 2.4 (m, 1H, CH(COOH)), 2.4–4.2 (series of mult., 25H, cyclen, pendent arms), 7.41 (d, 2H, Ar), 7.51 (d, 2H, Ar); ^{1}H NMR (300 MHz, D_2O, pH = 13) δ 1.6 (m, 1H, CH(COOH)), 1.9 (m, 1H, CH(COOH)), 2.1–3.2 (series of mult., 25H, cyclen, pendent arms), 6.81 (d, 2H, Ar), 7.13 (d, 2H, Ar); analytical HPLC t_R = 8.4 min; M + H$^+$ calcd for $C_{24}H_{38}N_5O_8$ 524.2720 found [HRFAB] m/e = 524.2678, error = −8.2 ppm.

1,4,7,10-Tetraaza-*N*-(1-carboxy-3-(4-isothiocyanatophenyl)propyl)-*N′*,*N″*,*N‴*-*tris*(acetic acid)cyclododecane (1) (PA-DOTA-NCS). A 1 M solution of $SCCl_2$ in $CHCl_3$ (0.39 mL) was added to (**5**) (150 mg, 0.21 mmol) dissolved in H_2O (2 mL) in a 25 mL flask. The mixture

was stirred rapidly for 2 h at room temperature. The aqueous layer was decanted with a pipettor into a round bottom flask and the $CHCl_3$ layer washed with H_2O (2 × 1 mL). The combined aqueous portions were slightly pink in color and were reduced to approximately 1–2 mL by rotary evaporation. The solution was then lyophilized to give PA-DOTA-NCS as a pale-yellow solid (160 mg, 100%). ^1H NMR (300 MHz, D_2O, pH 1.5) δ 1.9 (m, 1H, CH(COOH)), 2.2 (m, 1H, CH(COOH)), 2.6–4.2 (series of mult., 25H, cyclen, pendent arms), 7.32 (quart., 4H, Ar); analytical HPLC $t_R = 16.2$ IR (Nujol) 2000 cm^{-1}; M-H^+ calcd for $C_{25}H_{34}N_5O_8S$ 564.2128 found [HRFAB] $m/e = 564.2144$, error = +2.7 ppm.

Conjugation of PA-DOTA to HuCC49 and HuCC49ΔCH2. A solution of PA-DOTA-NCS (**1**) was prepared (0.5 mg/mL) in 0.1 M H_3BO_4, pH 8.5. HuCC49 (6 mg/mL) and HuCC49ΔCH_2 (4.2 mg/mL) were diluted with borate buffer to 1.5 mg/mL and 1.35 mg/mL, respectively. The PA-DOTA-NCS solution (3.6 μL) was added to each antibody solution (100 μg). The reaction mixtures were incubated at 37°C for 4 h at a final protein concentration of ~10 μM. The solutions were purified using a BioSpin 6 chromatography column (Bio-Rad Laboratories, Hercules, CA) equilibrated with 0.1 M NH_4OAc pH 5.5. The conjugates were collected and stored at 4°C until needed.

Radiolabeling of PA-DOTA-HuCC49 and PA-DOTA-HUCC49ΔCH2 with ^{177}Lu. $^{177}LuCl_3$ (initial specific activity ~4000 Ci/mmol) was produced and purified at the University of Missouri–Columbia Research Reactor. The $^{177}LuCl_3$ (10 μL) was diluted with 0.1 M NH_4OAc, pH 5.5 (90 μL). An aliquot of this ^{177}Lu stock solution (35 μL, 410 μCi) was added to HuCC49 conjugate (30 mL, 45 μg) or to HuCC49ΔCH2 conjugate (30 μL, 50 μg). The mixtures were incubated at 37°C for 30 min and purified using a BioSpin 6 column equilibrated with acetate buffer. Both radiolabeled conjugates had a specific activity of 0.6 mCi/mg and were >95% radiochemically pure as demonstrated by size-exclusion HPLC. The immunoreactivities of ^{177}Lu-PA-DOTA-HuCC49 and ^{177}Lu-PA-DOTA-HuCC49ΔCH2 were ~45 and 35%, respectively, as determined against mucin coated beads by competitive inhibition of radiolabeled antibody with varying concentration of unlabeled antibody. The percent immuoreactivity was calculated by linear extrapolation to the binding at infinite antigen excess.[30]

Animal tumor model. LS174T human colon cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Athymic nude mice obtained from the National Cancer Institute Frederick Research Laboratory (Frederick, MD) were injected ip with 1×10^8 LS174T tumor cells. Two μCi of ^{177}Lu-PA-DOTA-HuCC49 and ^{177}Lu-PA-DOTA-HuCC49ΔCH2, respectively, were injected ip into groups of 4–5 athymic nude mice 8 days after tumor cell inoculation. The mice were sacrificed 24 h after injection of the radiolabeled monoclonal antibodies. Tumor and normal tissues were dissected, weighed, and counted in a well-type gamma counter. The uptake of ^{177}Lu-PA-DOTA-HuCC49 and ^{177}Lu-PA-DOTA-HuCC49ΔCH2 in tumor and normal tissues, and determination of tumor to normal tissue ratios, is summarized by the median and the interquartile range. These are the appropriate measures for central tendency and variability for data such as these when the sample sizes are small. The Wilcoxon Rank-Sum test was used to compare the uptake in tissues and tumor to normal tissue ratios between ^{177}Lu-PA-DOTA-HuCC49 and ^{177}Lu-PA-DOTA-HuCC49ΔCH2.

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