A New Biotin Derivative−DOTA Conjugate as a Candidate for Pretargeted Diagnosis and Therapy of Tumors

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The synthesis of a new biotin derivative, the (CO) reduced *N*-aminohexyl biotinamido derivative, designed to be serum biotinidase resistant, and its conjugation to the chelator DOTA through an amide bond at one of the four carboxymethyl chains are described. The ^{90}Y-labeled conjugate was able to bind avidin at different Av/conjugate molar ratios with good results. The preclinical results indicate that this new biotin−DOTA conjugate is a good candidate for pretargeted diagnosis and therapy of tumors.

Introduction

Among the radiometal-labeled compounds with tumor-targeting properties, biotin derivatives carrying macrocyclic chelators, in particular the tetraazamacrocycle DOTA, disclosed new horizons in the radioimmunotherapy of cancer in conjunction with the administration of Av or Sav proteins.[1,2] It has been extensively reported, either in preclinical[3] and clinical[4] studies, that multistep Av/biotin methods result in improved tumor-to-nontumor ratios compared with the more conventional "one-step" approach with radiolabeled monoclonal antibodies (MoAbs).[5] Low toxicity and therapeutic efficacy of ^{90}Y-biotin using a pretargeting system with anti-tenascin MoAbs have been shown in a group of 48 patients with high grade glioma.[1] Pretargeting protocols, based on the Av/biotin system, are currently used for applications in clinical oncology.[6,7] In particular, the flexibility of the three-step approach opened new possibilities to convey onto the tumor a variety of biotinylated cytotoxic effectors.[8,9] However, DOTA−biotin adducts, formed via an amide bond between the sidearm of biotin and the spacer carrying the chelating moiety, are per se easily hydrolyzable by the serum biotinidase, an enzyme which is also able to break biotinyl peptides.[10−12] Different ways have been designed in the past to prevent the recognition at the site of the enzymatic attack (i.e., substitution of the carbon adjacent to the amide NH, alkylation of the amide group, incorporation of D-amino acids at the breaking point of the molecule, etc.)[12−15] in order to look for a good compromise between serum stability and high affinity of the compounds toward Av or Sav. Several synthetic steps were necessary for all these modifications, which, in some cases, resulted in a diminished binding constant with the Av or Sav pockets. This fact prompted us to design and synthesize in a few steps a biotin derivative conjugated to DOTA and devoid of the amide target site of the biotinidase. Reported herein is the synthesis of this new biotin derivative together with in vitro research studies concerning binding to avidin and stability of the molecule labeled with ^{90}Y.

Results and Discussion

Synthesis of Reduced Biotinamidohexylamine−DOTA Conjugate (r-BHD). The first synthetic step (Scheme 1) was the preparation of the Boc protected amide **1** starting from the commercially available mono-protected hexamethylenediamine, in the presence of the system HATU/DIPEA, an effective activating agent used in peptide synthesis. Deprotection of the biotinylamido derivative **1** and reduction by BH_3·THF in heterogeneous phase afforded the amine **3**. In compound **3** the length of the parent biotin side chain, that is crucial for the association with the Av or Sav counterpart,[15] is retained. Moreover, a large conformational freedom of the spacer carrying the DOTA chelator has been ensured. The alkylamino spacer itself corresponds to the length of a lysine residue: previous biological researches demonstrated that this is a suitable distance for binding the biotin moiety to the Av pocket and for the activity of the labeled part.[16] Next, on the basis of preceding coordination studies,[17] stating that also *N*,*N*′,*N*″-triacetic acid cyclen derivatives may well accommodate metal ions as Ca, Y, La, In, and Gd as DOTA does, the tetraazamacrocyclic ligand has been directly bonded to the NH_2 group of derivative **3** through one of the four *N*-carboxymethyl groups, giving rise to compound **4**. Because of the very high polarity of DOTA, the activation of the carboxy group of the tetraazamacrocycle was performed in aqueous solution, by using the water-soluble system EDC/sulfo-NHS.[18] In the past, many efforts have been carried out in order to find the best pH conditions for the stability of the activating reagents and of the related sulfo-NHS esters (ref 19 and references therein). The pH value is a relevant factor also for obtaining the highest percentage of mono DOTA-

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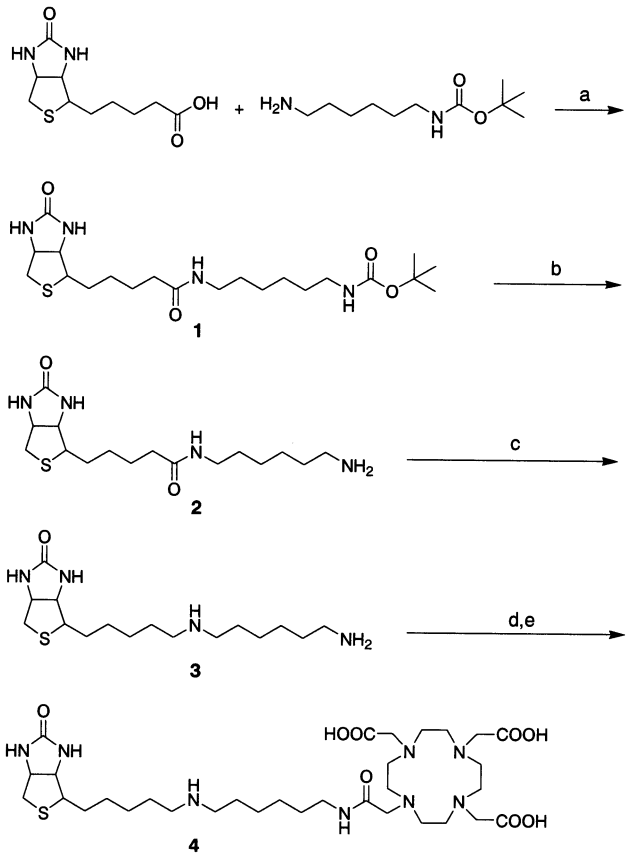
Scheme 1. Synthesis of Compounds **1**−**4**[a]



[a] Reagents and conditions: (a) HATU, NMM, DMF; (b) concd HCl, EtOAc; (c) BH_3, THF; (d) DOTA, sulfo-NHS, EDC, 1 N NaOH, pH 5.5; (e) 1 N NaOH, pH 7.5.

OSSu ester in situ. Because the pK_a value of the fourth carboxy group of DOTA is below 5,[20] acid pH values were mainly considered for the monoactivation. Recently, Shively and co-workers showed that activation at pH 5.5 gave the highest efficiency of conjugation of DOTA-OSSu to cT84.66 antibody.[19] We have investigated the products of EDC/sulfo-NHS-mediated DOTA activation at this pH, by means of negative ions mass spectrometry. At pH 5.5, in our experimental conditions, a relevant amount of parent DOTA [*m*/*z* (abundance): 403 ($M - H^+$, 65%); 425 ($M - H^+ + Na^+$, 42%)], compared to peak of the required monoester [580 ($M - H^+$, 100%)] is still present after 50 min at 4 °C, together with DOTA bis-ester [757 ($M - H^+$, 32%)]. Interestingly, at this time the monoactivated DOTA did not suffer decomposition. However, the highest yield of compound **4** (46%) was obtained when the activation was performed at pH 5.5 and the coupling with the amine **3** at pH 7.5. At this pH the amine is well soluble in water, and the secondary amino group should be largely protonated. Notably, activation and coupling both performed at pH 7.5 afforded compound **4** in 20% yield. The structure of the derivative **4** has been determined by analytical and spectroscopic data as well as by characterizing the amino function. The presence of primary amino groups was excluded by the negative fluorescamine test. Instead, the secondary amino group was detected by the method reported by Feigl[21] (blue-violet spot with sodium nitroprusside and acetaldehyde). The ^{1}H NMR spectrum of the purified compound showed the amide NH at 8.3 ppm while the protonated NH amine

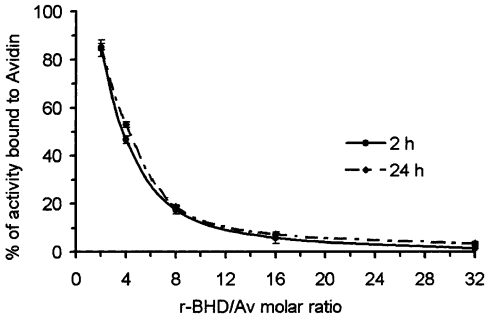


Figure 1. Percentage of activity due to ^{90}Y-labeled r-BHD bound to Av. The aspecific activity retained (in the average of 4%) has been subtracted.

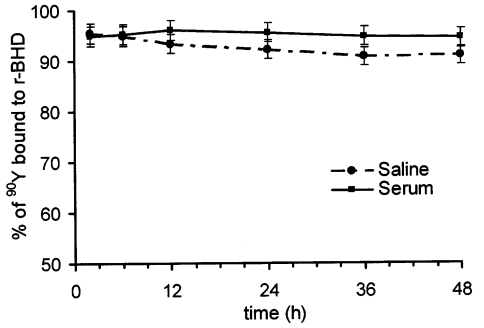


Figure 2. Stability of ^{90}Y-labeled r-BHD in saline and diluted human serum at 37 °C.

group resonated at 8.5 ppm. Interestingly, the synthetic pathway reported in Scheme 1 can be easily scaled up for the preparation of the derivative **4** on large scale.

The in vitro experiments were essential to evaluate the potential role of this new biotin derivative in the clinical settings. Primarily, we attempted to verify whether the conjugation steps and the radiolabeling procedure may alter the natural affinity of biotin for avidin, and then we checked the resistance of the molecule to the action of biotinidase.

Radiolabeling. Labeling yields greater than 97% were routinely achieved.

Studies of Binding to Avidin. The binding studies of the new ^{90}Y-labeled biotin derivative **4**, in the presence of a 1:2 Av/r-BHD molar ratio (which is two times the natural 1:4 molar ratio), showed that about 85% of ^{90}Y-labeled compound **4** was bound to Av, whereas at the natural 1:4 molar ratio only about 50% was bound (Figure 1). The average percentage of activity not specifically retained by the concentrator (about 4%) was subtracted from the counts found in the top part of the concentrator. Data at 2 and 24 h at 37 °C were similar, indicating that the binding occurs rapidly and is almost irreversible despite the linkage with the bulky DOTA molecule.

Stability Studies. Stability studies showed that in both saline and diluted human serum the activity was almost completely associated to the labeled r-BHD molecule up to 48 h (Figure 2). Moreover, the activity dissociated from the ^{90}Y-labeled r-BHD conjugate was not attributable to the cleavage of the side chain by biotinidase. In fact, the amount of cleaved DOTA-^{90}Y, obtained by the counts in the bottom part of the Centricon in the presence of Av excess, was very low (about 3%) and did not increase from 2 to 48 h (in the serum free ^{90}Y was totally bound to serum protein,

personal observation). The release of ^{90}Y in diluted human serum, observed after 48 h of incubation at 37 °C, is also very low and in any case does not represent a major drawback toward the application of this compound in therapy trials. In fact, it has been previously shown that ^{90}Y-biotin presents a very rapid blood clearance with a mean value for $\tau_{blood} = 2.0 \pm 1.1$ h.[1,22] Consequently, we can affirm that biotinidase was not able to break the r-BHD conjugate in a significant extent.

Conclusions. A novel biotin derivative, carrying the DOTA moiety, was synthesized in a few steps by linking the macrocyclic chelator to the diamine that was formed by diborane reduction of the parent *N*-aminohexyl biotinamido derivative. The preclinical evaluations reported in this paper appear very encouraging toward the potential pretargeting applications of this molecule.

Experimental Section

Reagents. DOTA was purchased from Macrocyclics (Richardson, Texas), *N*-Boc-hexamethylenediamine and peptide synthesis grade DMF from Fluka (Germany) and HATU from PerSeptive BioSystems (Framingham, MA). THF was distilled over sodium. TLCs were carried out on SiO_2 (Merck; 60 Å F_{254}) and spots located by UV light (254 and 366 nm), solution of Fluram (fluorescamine; Fluka) in acetone, and/or Cl_2/*o*-tolidine (*o*-tolidine, 75 mg; acetic acid, 3 mL; H_2O, 47 mL; KI, 21 mg). HPLC-grade CH_3CN was purchased from Carlo Erba (Italy). All other chemicals were commercial compounds and were used as received. Melting points were determined on a Büchi 510 apparatus and are uncorrected.

Chemistry. ^1H NMR spectra were recorded on a Varian Gemini 200 MHz spectrometer or on a Bruker DRX 500 at CERM (Florence) for DMSO-d_6 solutions. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane. IR spectra were recorded on a Perkin-Elmer model 881 spectrometer for KBr pellets (vbr = very broad, br = broad). The elemental analyses were performed on a Perkin-Elmer 240 C Elemental Analyzer. Semipreparative purification of the final compound was performed on a Phenomenex Jupiter 10 μm C18 300 Å (250 × 10 mm) column using a Beckman System Gold Nouveau apparatus equipped with a diode array detector. Analytical RP-HPLC was used to determine the purity of the fractions, using a Phenomenex Jupiter 5 μm C18 300 Å (250 × 4.6 mm) column. The solvent systems used were: A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). The flow rates were 1 mL/min for analytical HPLC and 4 mL/min for semipreparative HPLC, with the indicated linear gradients. Electrospray ionization mass spectra (ESI-MS) were acquired on a LCQ-Advantage ESI ion trap spectrometer (ThermoFinnigan) for positive and negative ions detection. The samples were diluted 1 to 10 with water and injected directly in the source. Fast atom bombardment mass spectrum (FAB-MS) was registered on a Finnigan-MAT TSQ70 triple stage quadrupole apparatus equipped with an ion tech (Teddington, UK) atom gun with Xenon as bombarding gas.

(3a*S*,4*S*,6a*R*)-*N*-[6-[(1,1-Dimethylethoxy)carbonyl]aminohexyl]hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanamide (1). A solution of *N*-Boc-1,6-diaminohexane hydrochloride (1.03 g, 4.1 mmol) and NMM (0.45 mL, 4.1 mmol) in dry DMF (8 mL) were added to a stirred solution of biotin (1 g, 4.1 mmol) and NMM (0.45 mL) in dry DMF (30 mL). After some minutes a solution of HATU (1.56 g, 4.1 mmol) in DMF (5 mL) was added. The reaction mixture was stirred overnight at room temperature, and after evaporation under reduced pressure the yellow oil was treated with H_2O. The solid precipitate was recrystallized from *n*-propanol to give 1.6 g (3.6 mmol, 88% yield) of compound **1**. The product was pure by TLC (CH_2Cl_2/MeOH = 5:1). Mp 174−176 °C; ^1H NMR (200 MHz) δ 7.74 (t, 1 H, amide NH); ESI-MS: *m*/*e* calcd. (M + H^+) 443.1, found 442.6. Anal. ($C_{21}H_{38}N_4O_4S \cdot 0.3H_2O$) C, H, N.

(3a*S*,4*S*,6a*R*)-*N*-(6-Aminohexyl)hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanamide (2). Concentrated HCl was added to a suspension of compound **1** (1.6 g, 3.6 mmol) in AcOEt (15 mL) (final HCl concentration: ca. 3 M) and the solution stirred for 30 min. The oily product obtained after evaporation was lyophilized and redissolved in H_2O, the pH adjusted to 12 with 2 M NaOH (at 0 °C), and the solution relyophilized. The solid was repeatedly treated with MeOH to separate the inorganic salts and then precipitated by adding ethyl ether, affording compound **2** (1.1 g, 3.2 mmol, 90% yield). The product showed a unique spot on TLC (*n*-propanol/AcOH/H_2O = 1:1:1). Mp 179−182 °C; ^1H NMR, in accordance.

(3a*S*,4*S*,6a*R*)-*N*-(6-Aminohexyl)hexahydro-2-oxo-1*H*-thieno[3,4-*d*]imidazole-4-pentanamine (3). Compound **2** (1.5 g, 4.4 mmol), finely powdered, was suspended in anhydrous THF (15 mL) and slowly added, at 0 °C and under nitrogen, to a solution of 1 M BH_3 in THF (9 mL). The reaction mixture was stirred at 0 °C for 30 min and then refluxed for 10 h, after which time the reduction was monitored by NMR (by quenching a sample with one drop of 3 M HCl). More crops of 1 M BH_3 in THF (1 equiv each) were added, and the suspension was refluxed to complete the reduction. Finally, cold water (10 mL) and 3 M HCl (5 mL) were carefully added, and the mixture was heated to reflux for 3 h. The crude product, collected after evaporation of THF and lyophilization, was purified by reverse-phase column chromatography (Lichroprep RP-8, 40−63 μm; 170 × 20 mm; eluent: $H_2O/CH_3CN/HCl$ = 92:8:0.1, 1 mL/min) affording 1.3 g (3.24 mmol, 74% yield) of pure **3**. The product showed a unique spot on TLC (eluent as for compound **2**). ^1H NMR (200 MHz) δ 8.03 (br, NH_3^+), 8.9 (br, NH_2^+); ESI-MS: *m*/e calcd $[M + H]^+$ 328.23; found 328.2.

10-[2-[6-[5-[(3a*S*,4*S*,6a*R*)-Hexahydro-2-oxo-1*H*-thieno-[3,4-*d*]imidazol-4-yl]-1-pentylamino]-1-hexylamino]-2-oxoethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic Acid (4). A solution of sulfo-NHS (95 mg, 0.44 mmol) in water (1 mL) was added to a solution of DOTA·3H_2O (0.2 g, 0.44 mmol) in water (2 mL), and the mixture was adjusted to pH 5.5 with 1 M NaOH. To the solution, kept at 4 °C, was added, dropwise, EDC (0.084 g, 0.44 mmol) in water (1 mL). The mixture was stirred at room temperature for 20 min, a solution of **3** (0.088 g, 0.22 mmol) in water at pH 7.5 (2 mL) was slowly added, and the pH was monitored with 1 M NaOH. After two h at room temperature, the solution was lyophilized. The crude product was purified by semipreparative RP-HPLC (10 to 15% of B in 20 min) affording compound **4** (0.119 g, 45.6% yield). HPLC (10 to 15% of B in 20 min): t_R = 12.6 min; IR (KBr) ν_{max} 3430 vbr, 3099, 2940, 2860, 1679 br cm^{-1}; ^1H NMR (500 MHz) δ 8.27 (br, amide NH), 8.54 (br, NH_2^+). FAB-MS: *m*/*e* calcd $[M + H]^+$ 715.9, found 715.6; ESI-MS: found 715.4. Anal. ($C_{32}H_{58}N_8O_8S \cdot 4TFA \cdot H_2O$) C, H, N.

Radioactive Materials. $^{90}YCl_3$ in hydrochloric acid (0.04 M) was purchased from AEA Technology (Harwell, UK).

Radiolabeling. Radiolabeling was carried out at a specific activity of 37 MBq ^{90}Y/10 μg of r-BHD (**4**), buffering with 1 M ammonium acetate pH 7.0. After being heated for 25 min at 85 °C, the mixture was diluted to 1 mL with saline. The labeling yield was determined by Instant Thin Layer Chromatography (ITLC SG, Gelman Science, Ann Arbor, MI) in saline, adding a molar excess of Av and EDTA to an aliquot of the radiolabeled mixture. In this system, Av/biotin derivative complex remains at the starting point while free ^{90}Y (bound to EDTA) migrates with the solvent front. The strip was cut in two halves and counted in a β-counter (Tri-Carb 2900TR Liquid Scintillation Analyzer, Packard).

Studies of Binding. Different molar ratios of ^{90}Y-labeled r-BHD toward Av were studied. The same amount of radiolabeled r-BHD (1 μg) was reacted with decreasing amounts of Av (Mw 66 kDa), to obtain 1:2, 1:4, 1:8, 1:16, and 1:32 Av/r-BHD molar ratios. The mixtures were incubated at 37 °C and analyzed at 2 and 24 h using Centricon YM-10 concentrators (Millipore, Bedford, MA, 10kDa M_w cutoff). Aliquots from the top and bottom parts of the Centricon were counted in the β-counter as previously described (see above). To evaluate the

activity aspecifically bound to the Centricon, an ^{90}Y-labeled r-BHD aliquot was centrifuged without adding Av. All runs were performed in duplicate.

Stability Studies. Aliquots from the labeling mixture were diluted with saline or human serum/saline 1:1 solution alternatively. After 2, 6, 12, 24, 36, and 48 h of incubation at 37 °C, two aliquots (10 μL) of both the mixtures were drawn: one was added to Av molar excess and the other was added to saline. The samples were centrifuged in Centricon YM-10 and subsequently washed with 3 × 200 μL saline. The presence of ^{90}Y-labeled r-BHD/Av complex, free ^{90}Y ion, and biotinidase-cleaved ^{90}Y-DOTA, potentially generated during incubation, was determined by the activity counts of the top and bottom parts of the Centricon with and without Av excess (see above).

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Appendix

Abbreviations: Av, avidin; DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo(4,5-*b*)pyridinium 1-hexafluorophosphate 3-oxide, *O*-(7-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; NMM, *N*-methylmorpholine; Sav, streptavidin; sulfo-NHS, *N*-hydroxysulfosuccinimide sodium salt, monosodium 1-hydroxy-2,5-dioxo-3-pyrrolidinesulfonate.

Supporting Information Available: ^{1}H NMR data, more detail of the radiolabeling procedure, and biological studies as well as elemental analyses data are available free of charge via the Internet at http://pubs.acs.org.

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