Preparation and Characterization of a DOTA−Lysine−Biotin Conjugate as an Effector Molecule for Pretargeted Radionuclide Therapy

James Hainsworth,[†] Peter Harrison,[‡] and Stephen J. Mather*,[†]

Cancer Research UK, Nuclear Medicine Group, St. Bartholomew's Hospital, London, and Bioventix Ltd., 7 Romans Business Park, East Street, Farnham, Surrey GU9 7SX. Received June 28, 2005; Revised Manuscript Received September 5, 2005

Pretargeted radionuclide therapy depends on the establishment of a high concentration of secondary binding sites at a tumor to which low-molecular weight radiolabeled effector molecules can be directed. This study describes the simple synthesis of an effector molecule and its subsequent characterization to determine the extent to which it complied with the ideal requirements of such a compound. (ϵ)-DOTA−(α)-biotinamidolysine (DLB) was synthesized in high yield and purity using conventional SPPS methodology. High radiochemical purities were obtained when labeled with several potentially useful radionuclides. The radiolabeled analogue bound to streptavidin efficiently with a stoichiometry similar to that of native biotin and showed high stability in serum and upon challenge with acid conditions. Biodistribution studies in normal animals showed a rapid rate of clearance from the blood and low retention of radioactivity by normal tissues. This design of effector molecule therefore shows promise for further pretargeted radionuclide therapy studies.

INTRODUCTION

Despite the recent successes in the application of targeted radionuclide therapy for treatment of lymphoma, the relative lack of success of RIT[1] in the treatment of solid tumors has led many researchers to look for alternative strategies, one of the most promising of which is pretargeted radionuclide therapy (for a recent review see ref 1). This involves the preadministration of an antibody conjugate to establish secondary binding sites on the tumor which can then be more efficiently targeted by low molecular weight radiolabeled effector molecules. To prevent these effector molecules from interacting with molecules of antibody conjugate still circulating in the blood, it may be necessary to inject a clearing agent between the first and last reagents in order to reduce the amount of circulating antibody conjugate.

The most widely explored system to date is that based on the biotin−(strept)avidin interaction. Thus the first administration might be that of an antibody−streptavidin conjugate or fusion protein, a suitable clearing agent would be a macromolecular biotin conjugate which remains confined to the vasculature, and the final effector molecule might be a small radiolabeled biotin analogue which is able to diffuse rapidly into the extravascular space surrounding the tumor and bind to the pretargeted antibody−streptavidin conjugate.

It has become clear in recent years that each stage of this targeting regimen needs to be optimized. Not only the reagents themselves but the doses used and the timing between the different stages have all been extensively studied in order to increase the efficacy of the treatment as a whole. This publication is concerned with developments in the design of the last stage in the process, the radiolabeled effector molecule (EM).

The ideal EM should maximize the delivery of a therapeutic radionuclide to the tumor and minimize its uptake by nontarget organs in the body. To achieve this it should have (a) a high affinity for streptavidin, (b) a high stability in vivo, and (c) a favorable pharmacokinetic profile which ensures that it is cleared rapidly from the blood and excreted through the kidneys without any significant retention by organs of excretion and metabolism such as the kidneys and liver. Supplementary benefits include the ability to radiolabel with a range of potentially useful radionuclides and a simple and efficient synthetic route.

The most successful therapies have been achieved using biotin-based EMs which have been labeled with metallic radioisotopes, most commonly yttrium-90. This is achieved by conjugating a chelating agent such as diethylenetraminepentaacetic acid (DTPA) or tetraazacyclododecanetetraacetic acid (DOTA) to biotin. Both bifunctional chelators have the ability to complex a variety of potentially useful metallic radionuclides including those of yttrium, indium, gallium, and lutetium, but DOTA is generally preferred because higher stability metal complexes can be obtained (*2, 3*). In the first DTPA−biotin conjugates to be employed for pretargeting purposes, one of the carboxylic acid groups of DTPA was condensed with the ϵ-amino group of biocytin as shown in Figure 1A (*4*). However, a significant disadvantage of this design is the fact that the amido bond so formed can

* To whom correspondence should be addressed. Phone: +44 207 601 7153. Fax: +44 207 601 7143. E-mail: Stephen.mather@cancer.org.uk.

[†] St. Bartholomew's Hospital.

[‡] Bioventix Ltd.

[1] Abbreviations: DLB, (ϵ)-DOTA−(α)-biotinamidolysine; DOTA, tetraazacyclododecanetetraacetic acid; DTPA, diethylenetraminepentaacetic acid; EM, effector molecule; Fmoc, 9-fluorenylmethoxycarbonyl; HOBT, hydroxybenzotriazole; MTT, 4-methyltrityl; NMP, *N*-methylpyrrolidone; pi, postinjection; RIT, radioimmunotherapy; SPSS, solid-phase peptide synthesis; TFA, trifluoroacetic acid, TIS, triisopropylsilane.

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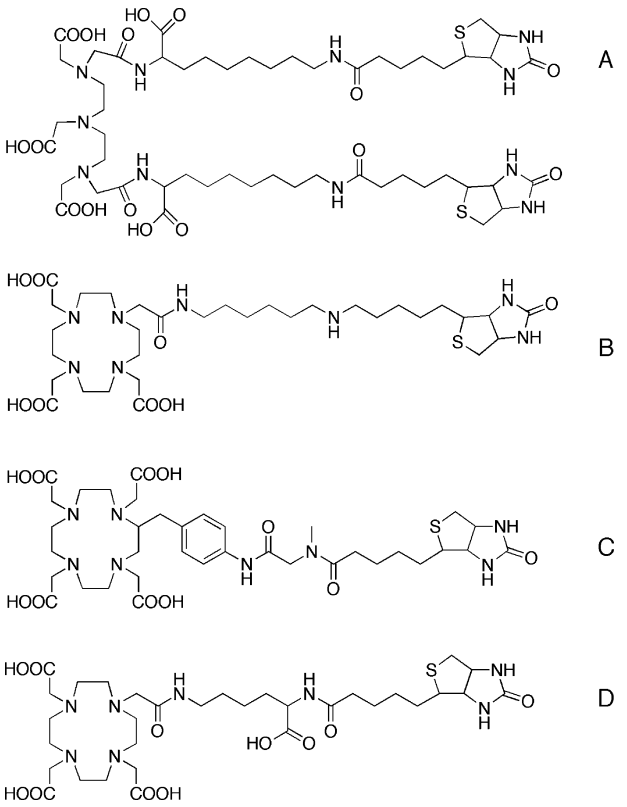


Figure 1. Structure of biotin-based effector molecules. A: DTPA α,ω-bis(biocytinamide). B: DOTA−N-linked-*N*-methylglycyl-biotin. C: Reduced biotinamidohexylamine DOTA. D: (ϵ) DOTA−(α) biotinamidolysine, DOTA

be cleaved by biotinidase, a ubiquitous enzyme present in mammalian serum (*5*) . A number of strategies have been formulated to overcome this problem including the use of reduced or sterically hindered amide bonds to render the linkage stable to biotinidase. Examples are shown in Figures 1B (*6*) and 1C (*7*). A second possible route of instability relates to dissociation of the metal core from the complex in vivo. It is important that the radionuclide remains firmly bound in the complex to ensure that it is not scavenged by other metabolic pathways in the body which would result in an increased radiation dose to normal tissues. The main question in relation to DOTA conjugate design is whether to attach the DOTA chelate to biotin via one of the four pendant carboxylic acid groups or to engineer a point of attachment into one of the ethylene backbones in the molecule. The former approach has the advantage of simpler and easier synthesis but the latter the advantage of potentially greater stability.

For reasons discussed below, we were interested in preparing DOTA conjugates of L-biotin [the mirror image of naturally occurring D-biotin] for use in a novel pretargeting strategy. The synthesis of L-biotin is a complex, six-step procedure, and the material is therefore in limited supply. We therefore wished to develop a simple but efficient route to the preparation of a DOTA−L-biotin conjugate which retained all of the prerequisite properties discussed above. Our preferred design for this molecule is shown in Figure 1D. This molecule has the advantage that it can be prepared using conventional solid-phase peptide synthetic (SPSS) procedures, in high yield. A full characterization of the properties of this EM and the extent to which they complied with our list of requirements was performed.

EXPERIMENTAL PROCEDURES.

Chemicals and Reagents. All reagents were purchased from Sigma-Aldrich unless otherwise indicated**.** Indium chloride ($^{111}InCl_3$) was sourced from Mallinckrodt Medical b.v (Petten, Netherlands), yttrium chloride ($^{90}YCl_3$) from MDS Nordion (Fleurus, Belgium), lutetium chloride ($^{177}LuCl_3$) from Perkin-Elmer Life Sciences, Inc. (Boston, Massachusetts), and cobalt chloride ($^{57}CoCl_2$) from ICN (Basingstoke, Hamps, UK).

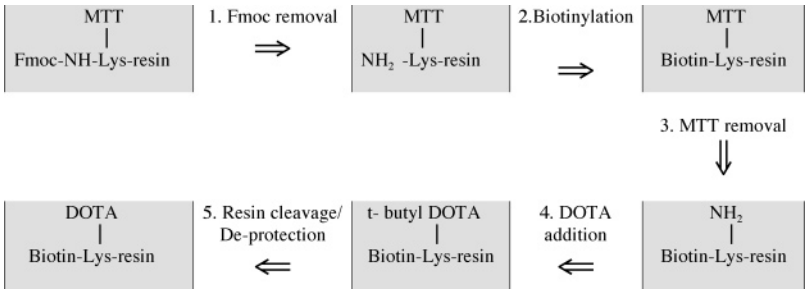
Reverse-Phase Chromatography. HPLC analysis was performed using a Beckman 126 solvent-module pump system with a Beckman 168 diode array detector and a sodium iodide flow through radiochemical detector attached to a Raytest gamma radioactivity monitor. Preparative HPLC was performed using a an Agilent Zorbax 300SB-C8 preparative column (21.2 mm × 25 cm) while for analysis a Galaxy Jupiter 5μ C_{18} column, 300 Å; 4.6 × 250 mm i.d. was used (Phenomenex, Cheshire, England). Data collection was handled by a PC running System Gold Nouveau (Beckman, High Wycombe, England). A mobile phase system comprising water and acetonitrile containing 0.05% TFA was used at a flow-rate of 1 mL per minute.

(ϵ) DOTA−(α)-Biotinamidolysine (DLB) Synthesis. Classical SPPS methods were used to synthesize the conjugate as described in Scheme 1. Ninhydrin assays were performed at each stage to check completion of the reactions. The starting point was an Fmoc-Lys(MTT)-Wang resin (Novabiochem). Briefly, after removal of the Fmoc protection with 20% piperidine, biotinylation on the α-amino group was performed overnight using a 4-fold molar excess of HOBT activated D- or L-biotin. The ϵ-MTT protection was removed with 1% TFA, 5% TIS in dichloromethane, and DOTA conjugation to the lysine side chain was performed overnight by addition of a 4-fold molar excess of HOBT-activated DOTA-tris(*tert*-butyl) ester (Macrocyclics, TX). Cleavage from the resin and removal of the *tert*-butyl protection was performed with 2.5% TIS, 2.5% water in TFA. The resin was removed by filtration, the DLB precipitated in cold diethyl ether, washed, redissolved in water, and lyophilized. The crude product was purified by reverse-phase HPLC using a C_8 column. The samples were analyzed by reverse-phase HPLC to check their purity, and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Lasermat 2000, Finnigan-Mat, Thermo Electron Corp) was used to verify their molecular mass.

Radiolabeling of DOTA−Biotin Conjugates. The DLB was labeled with indium-111, yttrium-90, and lutetium-177 using the same reaction conditions. Typically, a volume of 10 μL (7 MBq) of the radionuclide was added to an equal volume of 2 M ammonium acetate, pH 5.4, in a 2 mL polypropylene vial. This was followed by 5 μL of a 1 mg/mL solution of DLB in water. The volume was made up to 100 μL with water, and the reaction vial was sealed, heated in a boiling bath for 30 min, and finally quenched with 10 μL of 50 mM EDTA before further analysis. Sodium ascorbate was added to ^{90}Y-labeled solutions at a final concentration of 100 μg/mL to prevent radiolysis.

Assay of DOTA−Lysine Biotin. Because of inaccuracies in weighing small amounts and to compensate for the presence of residual water, the concentration of DLB was confirmed by labeling a sample with $^{57}CoCl_3$ of a known specific activity (*8*). A range of concentrations was labeled with ^{57}Co, quenched with EDTA, and analyzed by ITLC (Pall Life Sciences, Portsmouth, UK) using 10 mM EDTA in 0.1 M ammonium acetate as mobile

Scheme 1. Solid-Phase Synthesis of DLB[a]



[a] Step 1: A volume of 3 mL of 20% piperidine/DMF was mixed with 50 mg of peptidyl resin and incubated for 1 h at room temperature. The reacted sample was centrifuged at 6500 rpm for 1 min, and the supernatant was discarded and replaced with a further 3 mL of 20% piperidine/DMF to ensure complete reaction for another 1 h. The supernatant was discarded, and the pellet evaporated to dryness in a Savant SpeedVac Plus. The resin was washed three times with 1 mL of dimethylformamide and twice with 1 mL of dichloromethane before drying in the SpeedVac. Step 2: The deprotected lysine resin beads were preswollen with 250 μL of NMP, and the excess supernatant was removed. A 4:1 molar excess of biotin (0.12 mmol) to lysine was dissolved in 1 mL of DMSO and 400 μL of NMP. Then 200 μL of 1 M HOBT in NMP was added to the D-biotin solution and left for 3 min. A volume of 200 μL of diisopropylcarbodiimide activator (1 M in NMP) was added to the D-biotin solution and reacted for 30 min. The activated D-biotin solution was then added to the resin and mixed overnight on a 4RT-rocking table (Luckham, Ltd., Burgess Hill, UK). The next day the beads were washed three times with 1.5 mL of NMP. A volume of 500 μL of DCM was used to shrink and dry the beads. Step 3: The resin was washed four times for 3 min with 2 mL of a solution containing 1% TFA, 5% TIS, and 94% DCM. Initially the solution became a fluorescent yellow color, indicating the presence of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) in solution. Washing was continued until the wash solution became colorless again. The beads were then washed in DCM. Step 4: The resin beads were preswollen with 200 μL of NMP. A mass of 115 μg (0.2 mmols) (6× excess) of DOTA-tris(*tert*-butyl ester) (Macrocyclics, Dallas, TX) was dissolved in 700 μL of DMF, and a 200 μL aliquot of 1 M HOBT in NMP was added and mixed for 3 min. A volume of 50 μL of diisopropylcarbodiimide (1 M in NMP) was added and incubated for 30 min at room temperature. The "Activated DOTA" was added to the swollen resin and gently mixed overnight. The resin beads were then washed five times with DMF and twice with DCM. Step 5: A 1 mL aliquot of a cleavage solution containing 2.5% water, 2.5% TIS, and 95% TFA was added to the resin, and the suspension was slowly filtered through a microtube filter over a period of 2 h. The solute was precipitated with 1 mL of cold diethyl ether and centrifuged for 1 min and the supernatant discarded. The pellet was washed with a second 1 mL of ether, the supernatant discarded, and the compound dried in the SpeedVac. The resultant solid was weighed in a microfuge tube on a Sartorius analytical balance (Goettingen, Germany) and then redissolved in water for preparative HPLC purification.

phase. Under these conditions, DLB-bound ^{57}Co has an $R_f = 0$ while unbound ^{57}Co has an $R_f = 1$. A graph of bound cobalt concentration vs estimated DLB concentration was drawn and the gradient determined by linear correlation from which the true concentration of the DLB was calculated.

Streptavidin Binding of Labeled DOTA−Biotin Conjugates. ^{111}In-labeled DLB was incubated with a series of dilutions of streptavidin (ICN, MP Biomedicals, Irvine CA) ranging from 0.2 pM to 2 μM corresponding to DLB:streptavidin ratios of 10^{-5} to 100:1. The reaction mixtures were incubated for 30 min at 37 °C, and samples were taken and analyzed using Sephadex G-50 spin columns (Amersham Biosciences, Amersham UK) to determine the degree of streptavidin binding.

Serum Stability. To asses the stability of DLB to biotinidase and other serum proteases, 50 μL of ^{111}In-labeled DLB (containing 1 μg of labeled EM) was diluted 10-fold in fresh human serum and incubated with gentle mixing at 37 °C for up to 24 h. Samples were taken at 0, 1, 2, 6, and 24 h, serum proteins were precipitated with an equal volume of acetonitrile, and the supernatant was analyzed by reverse-phase HPLC.

Stability of DOTA−Metal Complexes. The stability of the ^{111}In- and ^{90}Y-DLB complexes were compared with those of the unconjugated DOTA and DTPA radiometal complexes by assessing their resistance to competition by hydrogen ions. The complexes were prepared and diluted with a 10-fold excess of 0.1 M glycine·HCl buffers ranging in pH from 0.5 to 5. After incubation at room temperature for 30 min, the solutions were neutralized by addition of phosphate buffer and analyzed by ITLC using 10% aqueous ammonium hydroxide:methanol (1:1) as mobile phase. Under these conditions, any complexed radioisotope has an $R_f = 1$ while that which has been lost from the complex has an $R_f = 0$.

Biodistribution of ^{111}In-Labeled DLB. The biodistribution of labeled DOTA−biotin conjugate was determined after intravenous administration to normal mice. Twelve normal adult balb/c mice were injected with 35 ng of conjugate labeled with 70 kBq of ^{111}In. Two, six, and twenty-four hours after injection, groups of four mice were killed, and a sample of their blood, a sample of muscle, a femur, both kidneys, the liver, the complete intestine, and the spleen were transferred to preweighed tubes and counted in a suitably calibrated gamma counter together with a series of dilutions of the injected material in order to allow the percentage of the injected dose in each sample to be calculated.

RESULTS

Synthesis of the DOTA−lysine−biotin (DLB) proceeded without difficulty. A practical yield of around 80% was obtained. Although our synthesis was performed manually starting with only 50 mg (0.03 mmol) of loaded resin, no difficulty in scaling the synthesis up to the levels normally performed by automated peptide synthesizers would be expected. The DLB produced was pure as assessed by analytical HPLC (see Figure 2 upper trace) and showed the expected mass of 759 on MS (see Figure 3). Radiolabeling of the effector molecule consistently produced complexes with radiochemical purities greater than 99% as assessed by RP-HPLC (see Figure 2 lower trace). The complexes remained stable in aqueous solution for at least 48 h provided that (in the case of ^{90}Y-labeled DLB) sodium ascorbate was added to prevent radiolysis.

To perform an accurate assessment of the properties of the radiolabeled DLB solution, in particular the stoichiometry of its binding to streptavidin, an accurate assessment of its concentration was required. Since only very small amounts of the DLB were required for each assay, very small quantities were weighed out on most

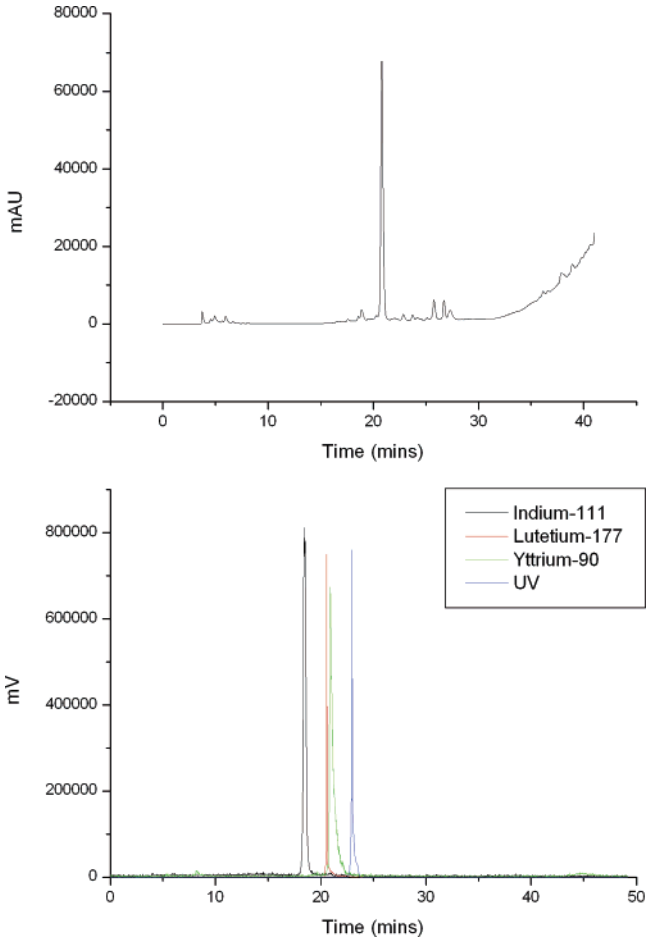


Figure 2. Reverse phase HPLC analysis of DLB. Upper trace UV detection of unlabeled compound. Lower trace radioactive detection of DLB labeled with ^{111}In, ^{90}Y, and ^{177}Lu.

occasions, and, in the knowledge that many peptides are hygroscopic, it was considered likely that this could lead to inaccuracies in the estimated concentrations of the DLB solutions prepared. An accurate assessment was therefore performed by titrating the DLB solution with a known concentration of $^{57}CoCl_3$ solution and plotting the concentration of bound ^{57}Co against the estimated DLB concentration. An example is shown in Figure 4. If the estimated concentration of DLB was correct, then the slope of the line would be 1. In this example the slope is 0.64, indicating that the real concentration of DLB was 1/0.64 or 1.56 times greater than that estimated.

Using a precisely known concentration of ^{111}In-DLB, a titration was performed with a range of streptavidin solutions and the extent of streptavidin binding measured by size-exclusion spin columns. The results show a sigmoidal pattern of binding as seen in Figure 5. With a large molar excess of streptavidin, up to 99% of the ^{111}In-DLB was protein bound. At a DLB:streptavidin ratio of 4:1 (the expected stoichiometric ratio of biotin binding), about 93% of the DLB is protein bound.

A high level of stability of the effector molecule in vivo is required. Two main routes of instability are likely: degradation by serum proteases such as biotinidase and transchelation of the radiometal from the DOTA complex. The first route of degradation was studied by incubating the labeled DLB in fresh human serum for up to 72 h and analyzing the mixture by reverse-phase HPLC. The rate of breakdown was compared with that of ^{111}In-DTPA α,ω-bis(biocytinamide) which is a known biotinidase substrate. No significant breakdown of the ^{111}In-DLB was observed over a 72 h incubation period. This contrasted with a loss of 46% of ^{111}In DTPA α,ω-bis(biocytinamide) in 24 h and a ~100% loss in 72 h. The level of serum protein binding of ^{111}In-DLB after 24 h incubation was found by size-exclusion spin column analysis to be 5%.

Although the high stability of the ^{111}In- and ^{90}Y-DOTA complexes are well documented, it is possible that the removal of one of the pendant carboxylic acid side chains may sufficiently reduce the stability to permit transchelation of the radiolabel in vivo. The stability of ^{111}In- and ^{90}Y-labeled DLB were therefore compared with that of ^{111}In- and ^{90}Y-labeled DOTA as well as ^{111}In- and ^{90}Y-labeled DTPA and ^{111}In- and ^{90}Y-labeled DTPA α,ω-bis(biocytinamide) using a modification of the approach described by Stimmel et al. (*9*). This was performed by diluting the complexes with an excess of glycine·HCl buffers of pH ranging from 0.5 to 5. Under low pH conditions, the high concentration of hydrogen ions would be expected to compete more effectively with the metal, and any loss in stability would result in a more rapid rate of transchelation than that which would be observed at higher pH. The results are shown in Figure 6. It can be seen that at least down to pH 0.5, no difference in stability between ^{111}In- and ^{90}Y-labeled DLB and DOTA complexes can be seen, indicating that the loss of one carboxylate arm has no profound effect upon the rate of loss of metal from the complex. By contrast the DTPA complexes and especially the DTPA α,ω-bis(biocytinamide) complexes (in which two carboxylate groups are used for conjugation) show a much greater rate of metal loss.

The biodistribution of ^{111}In-labeled DLB conjugates was studied in normal mice 2, 6, and 24 h after intravenous administration. The results are shown in Table 1. These show a rapid rate of clearance from the blood with very low retention in all tissues except kidney and bone where values ranged from 1 to 2% per gram.

DISCUSSION

Our aim in this exercise was to develop a simple efficient synthesis of a biotin-based effector molecule which provides high, reproducible labeling efficiency, high stability in a biological environment, effective interaction with its streptavidin target, and low nonspecific retention by normal organs and tissues in vivo. The results show that these aims have been largely achieved. The design of our DOTA–lysine–biotin was based on the results of a number of previous pivotal publications. In 1987 Hnatowich and co-workers (*4*) based their initial pretargeting studies on a DTPA–biocytin analogue. Although this firmly established the proof of principle, this effector molecule suffered from a number of shortcomings. The same group subsequently described the preparation of a DOTA–biotin analogue (*10*) and Chinol later showed that DOTA was a superior chelator for this purpose (*11*). The realization that a simple biocytin conjugate was a substrate for biotinidase led to the development of a number of more stable designs (*6, 7*). Wilbur et al. have explored in detail the effects of different substitutions on biotinidase stability (*12, 13*). In particular they showed that substitutions alpha to the biotinamide bond inhibited the activity of the enzyme through steric hindrance (*14*). Wilbur et al. have also studied the effect of different lengths of spacer between the radionuclide and the biotin. Because of their desire to radiolabel with halogens, they developed a series of ether-containing linkers to maintain the hydrophilicity of the molecule. Since DOTA itself confers a high degree of hydrophilicity on the structure,

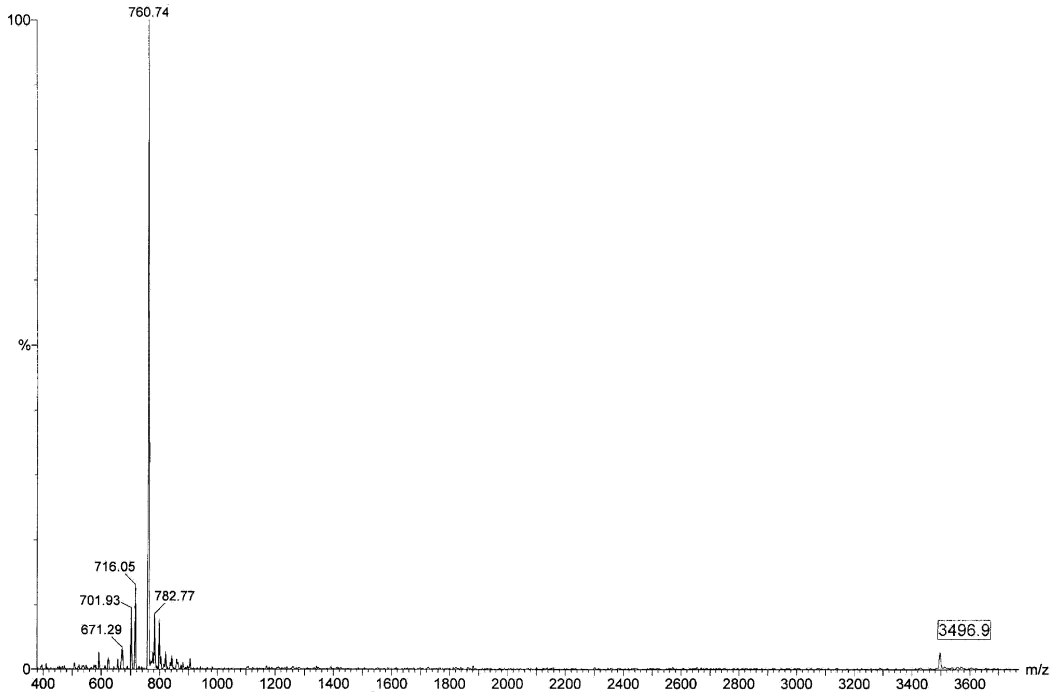


Figure 3. DLB matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometric analysis of DLB. M + H^+ of 760 corresponds to the expected mass of DLB of 759.

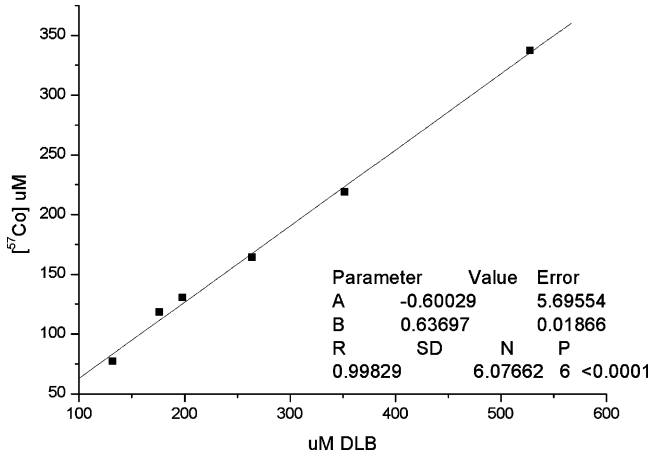


Figure 4. ^{57}Co titration to determine accurate concentration of DLB. A range of concentrations was labeled with ^{57}Co, quenched with EDTA, and analyzed by ITLC. A graph of bound cobalt concentration vs estimated DLB concentration was drawn and the gradient determined by linear correlation from which the true concentration of the DLB was calculated.

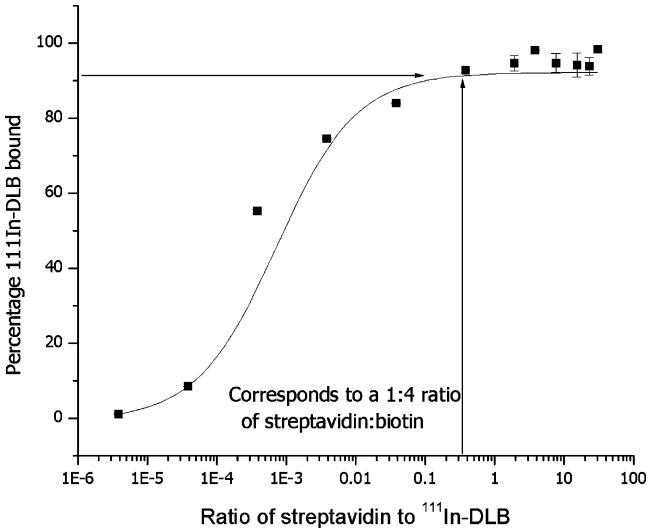


Figure 5. Binding of ^{111}In-DLB to streptavidin. ^{111}In-labeled DLB was incubated with a series of dilutions of streptavidin. After 30 min at 37 °C, samples were taken and analyzed using Sephadex G-50 spin columns to determine the degree of streptavidin binding.

we were not so constrained. For that reason we chose to use lysine as a linker since this would allow us to use standard SPPS chemistry to prepare the effector molecule. By substituting the biotin at the α- rather than the more usual ϵ-amino group of the lysine we ensured that the terminal carboxylate of the peptide provided the necessary steric hindrance of the biotinamide bond (see Figure 1D).

No difficulties were encountered in the synthesis of the effector molecule in acceptable yields. As well as preparing the DOTA−L-lysine−D-biotin described above we also prepared DOTA−L-lysine−L-biotin and achieved similar yields and purities. L-Biotin does not bind to streptavidin, but the rationale for our interest in this compound stems from a desire to combine the advantages of the streptavidin−biotin pretargeting system with those of the bispecific antibody approach (*15*). The streptavidin−biotin system has two significant drawbacks. The first is that streptavidin itself can be highly immunogenic (*16*), and this limits the number of potential administrations that can be made. The second drawback is the likelihood that endogenous biotin present in the blood will compete with the radiolabeled biotin for the limited number of binding sites on the pretargeted streptavidin conjugate (*17*). As effector molecules based on the D-isomer of biotin have many ideal properties (high specific activity, low toxicity, favorable stability, rapid renal excretion), it is expected that compounds based on the L-isomer would retain these advantages. We have therefore raised high affinity antibodies to L-biotin that do not cross-react with D-biotin and would, therefore, not be affected by endogenous biotin in the blood. The work presented in this publication stems from a desire to be able to perform studies directly comparing the two approaches in our laboratory. One of

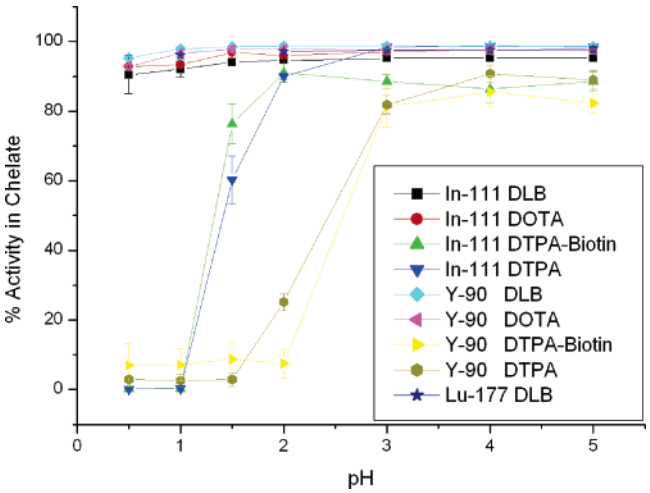


Figure 6. Dissociation of radiometal complexes under challenge at varying pH. The stability of the ^{111}In- and ^{90}Y-DLB complexes were compared with those of the unconjugated DOTA and DTPA radiometal complexes by assessing their resistance to competition by hydrogen ions. The complexes were prepared and diluted with a 10-fold excess of 0.1M glycine·HCl buffers at pH ranging from 0.5 to 5. After 30 min, the solutions were analyzed by ITLC to determine the proportion of intact complex remaining.

Table 1. Biodistribution of ^{111}In-DLB in Normal balb/c Mice

	% of injected dose/g tissue (SD) at time point (h)		
tissue	2	6	24
BLOOD	0.09 (0.02)	0.08 (0.02)	0.02 (0)
MUSCLE	0.21 (0.13)	0.41 (0.31)	0.11 (0.07)
BONE	1.31 (0.52)	1.39 (0.91)	0.1 (0.03)
KIDNEY	1.76 (0.27)	1.93 (0.38)	1.19 (0.26)
LIVER	0.29 (0.19)	0.22 (0.11)	0.13 (0.03)
GUT	0.61 (0.51)	0.61 (0.14)	0.27 (0.17)
SPLEEN	0.22 (0.17)	0.24 (0.09)	0.3 (0.32)

the difficulties in exploiting L-biotin as an effector molecule is its difficult synthesis. We obtained the material from an expensive commercial custom synthesis (CMS Chemical, Abingdon, UK) which synthesized the compound from L-mannose according to the method of Ohrui (*18*). Although our DLB synthesis was performed manually on a small scale in order to limit the amount of the expensive L-biotin required, no difficulty in scaling the synthesis up to the levels normally performed by automated peptide synthesizers would be expected. It would also be possible to prepare the corresponding D-lysine analogues (*19*) if desired.

High levels of purity of the DLB and its radiolabeled counterparts were achieved. The molecule was able to bind efficiently to streptavidin in solution with a stoichiometry similar to that of native biotin indicating that the lysine spacer did not significantly inhibit this interaction.

In vitro measures of stability were encouraging. HPLC analysis of the radiolabeled effector molecule after incubation in serum for up to 3 days indicated no loss in purity over this time, indicating that the α-amino-linked molecule was not cleaved by serum proteases such as biotinidase. This is in contrast to the radiolabeled DTPA–ϵ-amino-linked control biotin analogue which was completely degraded over the same time period. However, it is possible that a proportion of this loss can be attributed to transchelation from the DTPA to transferrin present in the serum.

The stability of the radiometal–DOTA complexes were studied by comparing their rate of acid-assisted dissociation in a modification of the method described by Stimmel et al. (*9*). While Stimmel studied the dissociation of various chelates at pH 2 over a period of several days, we chose to study the effect of a range of pH (0.5–5) at a single time of 30 min. The results show that while the yttrium–DTPA complexes start to show dissociation at pH 3, the yttrium–DOTA complexes show no significant dissociation even at pH 0.5. As expected the indium–DTPA complexes are rather more stable than their yttrium counterparts while the indium–DOTA complexes are as stable as the yttrium analogues under the conditions studied. Although it has been shown that DOTA lacking one of the *N*-acetate side chains has a lower thermodynamic stability constant than DOTA itself (*20*), the most important issue in radiopharmaceutical design is that of kinetic stability since the great majority of the yttrium-90 will either clear from the body or decay within a few days. These results suggest that, from a practical point of view, the convenience of using the SPSS-friendly commercially available reagent DOTA-tris(*tert*-butyl) ester is not outweighed by a large decrease in the kinetic stability of the complex and taken together with the serum stability study indicate that this effector molecule would have a suitable stability profile for pretargeting studies.

The final stage of the characterization of the DOTA–lysine–biotin was to study its biodistribution in normal animals. The dose of compound chosen for this study (1.4 μg/kg) was within the range of those chosen for clinical pretargeting studies – 0.7 to 30 μg/kg (*21–23*). The ideal effector molecule would show no significant retention by any normal tissues or organs. With the exception of the bone and kidney, all tissues showed a retention of 0.5% or less. Bone showed an initially quite high value of around 1% but declined to only 0.1% by 24 h while kidney uptake varied between 1 and 2%. With the exception of the high initial bone uptake, these results are similar to those published from similar studies of alternative effector molecules. Goodwin et al. (*24*) showed very similar results with a DOTA–C-linked-isothiocyanatobenzylbiotin analogue in most tissues but only around 0.1% bone uptake at 2 h pi, while Sharkey et al. (*25*) showed similar results but a rapidly clearing kidney retention with DOTA–N-linked (D)-ϵ-lysine–biotin. The biodistribution of a DOTA–N-linked-*N*-methylglycyl-biotin (*26*) is presented in graphical form only but appears to follow a similar pattern of biodistribution.

In conclusion we consider that the design and simple synthetic method described above is suitable for the preparation of radiolabeled effector molecules for pretargeted radionuclide therapy.

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