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RESEARCH ARTICLE

Biological evaluation of avidin-based tumor pretargeting with DOTA-Triazole-Biotin constructed via versatile Cu(I) catalyzed click chemistry

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Abstract

Background: The biotin-avidin interaction remains a gold standard for the two-step pretargeting approach to image tumor sites. We aim to develop two-step pretargeting systems utilizing ^{99m}Tc labeled biotin functionalized macrocyclic chelating agents synthesized using the highly efficient Cu(I) catalyzed azide–alkyne cycloaddition for potential radioimaging applications.

Methods: A facile synthesis of DOTA-Triazole-Biotin, radiocomplexation with ^{99m}Tc and the pretargeting protocol is described. The synthesis features Cu(I) catalyzed click conjugation between biotinylated azide and propynyl functionalized DO3A. ^{99m}Tc radiolabeling was performed to detect the accumulation of avidin as the pretargeting agent. Cytotoxicity was determined using the trypan blue exclusion assay, macrocolony, and MTT assay. Cell uptake studies were performed using radiolabeled DOTA-Triazole-Biotin and compared with avidin treated cells for 2 h. Tumor imaging was performed in U-87MG cell line implanted tumor bearing nude mice and uptake of the radiotracer was estimated.

Results: All compounds have been successfully characterized by NMR and MS spectroscopy. More than 96% radiolabeling efficiency was obtained and the radioconjugate exhibited sufficient stability under physiological conditions.

Conclusion: To summarize, a new candidate for avidin based two-step pretargeting of tumors has been synthesized and evaluated for potential imaging and diagnostic applications. The chelate possesses high stability under physiological conditions, exhibits effective interaction with its avidin target, and low nonspecific retention *in vivo*.

Keywords: Tumor/nontumor ratio, dipolar cycloaddition, tetrazacyclododecane, receptor binding, tumor imaging

Introduction

Among the radiometal-labeled compounds with tumor-targeting properties, biotin derivatives carrying macrocyclic chelators, have disclosed new horizons in the diagnosis and radioimmunotherapy (RIT) of cancer in conjunction with the administration of avidin or streptavidin proteins (Sabatino et al., 2003). Avidin-biotin pretargeting has achieved promising clinical results in imaging and treating various tumors. Lectins (proteins that bind specific sugar molecules on glycoproteins and glycolipids) are expressed at various levels on the surface of tumor cells. Conjugation of cytotoxic agents to glycoproteins recognized by lectins could be useful in the treatment of tumors. Avidin (a highly glycosylated, positively charged protein found in egg white) contains terminal *N*-acetylglucosamine and mannose residues that bind to lectins (Yao et al., 1998). In this study, we tested the ability of avidin, labeled through conjugation to radioactive biotinylated chelate to target intraperitoneal tumors. The high specificity and strong affinity

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($K_d = 10^{-15}$ M) of avidin for biotin plays a significant role in the two-step pretargeting protocol based on administration of the avidin antibody conjugate followed by the radioactive biotin chelate (Gonza´lez & Murphy, 2003; Sakahara & Saga, 1999). The interaction of biotin with avidin has been shown to be stable *in vivo* and is essentially irreversible (Krivickas et al., 2007). Multistep avidin/biotin methods result in enhanced tumor-to-nontumor ratios by placing the radiolabel on a small molecule designed to clear from the circulation and whole body rapidly compared with the conventional "one step" approach with radiolabeled monoclonal antibodies (Hainsworth et al., 2005). Another advantage of biotin-avidin for pretargeting is the tetrameric architecture (four fold valency) of this protein for biotin, providing the potential of modest signal amplification at the tumor site (Chen, 2008). They based their initial pretargeting studies on a DTPA-biocytin analogue and subsequently described the preparation of a DOTA-biotin analogue. Mather and coworkers developed DOTA-Lysine-biotin conjugate as an effector molecule for pretargeted radionuclide therapy.

To simplify the synthesis of biotinylated chelates, the versatile class of Cu(I) catalyzed click chemistry was employed. A novel alkyne-functionalized diethylenetriaminetetraacetic acid (DTTA) chelate has been developed and coupled to a per-azidocyclodextrin core via click chemistry (Bryson et al., 2008). The click chemistry-based synthesis of bistriazole-based polyaminocarboxylic acid derivatives has been elaborated in literature (Camp et al., 2008). Click conjugation refers to a Cu(I) catalysed Huisgen 1,3-dipolar cycloaddition of azides and alkynes, providing 1,4-disubstituted 1,2,3-triazole ring in high yield with minimal work-up and purification of reaction products (Chittepu et al., 2008). It offers in situ generation of Cu(I) salts needed for the catalysis from the stable Cu(II) sulfate/sodium ascorbate redox system as Cu(I) catalysis dramatically improves regioselectivity to afford the 1,4-regioisomer exclusively and increases the reaction rate up to 10^7 times (Bock, 2006).

Another advantage of using click chemistry is that it tolerates most of the organic functional groups, proceeds in a variety of solvents, and performs well over a broad pH and temperature range (Lutz & Zarafshani, 2008). The 100% atom economy and simple product isolation make this reaction useful in various applications ranging from bioorthogonal bioconjugation, polyaminocarboxylic acids synthesis to the construction of peptide bond surrogates (Vundyala et al., 2008). There are important clues that suggest that the triazole group displays structural similarity with the amide bond. The lone pair of the 3-nitrogen mimics the one of the carbonyl oxygen of the amide bond; the polarized C(5)-H bond can act as a hydrogen bonding donor, just like the amide N–H bond, and the electrophilic and polarized 4-carbon is electronically similar to the carbonyl carbon. Since the overall dipolar moment of the triazole system is larger than that of the amide bond, its hydrogen bonding donor and acceptor properties are more marked than those of an amide bond (Knox et al., 2000).

Extensive interest in the rational design and synthesis of organic polydentate ligands based on 1, 4, 7, 10-tetraazacyclododecane tetraacetic acid (DOTA) stems mainly from their complexes with radioactive metals (^{64}Cu, ^{99m}Tc) to be used in potential diagnostic/therapeutic applications. These metal complexes find widespread utility as a consequence of their robust steric and electronic properties and well-defined coordination chemistry. The set of coordinating donor atoms (four nitrogens and four oxygens) wraps around a lanthanide (III) ion in a very efficient way to yield complexes endowed with a very high thermodynamic and kinetic stability. On average over 80% of the radiopharmaceuticals used in clinical applications are labeled with ^{99m}Tc ($T_{½} = 6$ h), the short-lived, metastable nuclide having nuclear properties that are almost optimal with regard to a low radiation exposure of the patient.

In an effort to obtain a precursor that can localize and image cancer cells *in vivo* and to pretarget drugs to tumors, ^{99m}Tc labeled DOTA-Triazole-Biotin has been synthesized by conjugating DO3A-functionalized alkyne with biotinylated azide using click chemistry and evaluated for targeted applications.

Materials and methods

2-Bromoethylamine hydrobromide, Sodium azide, Biotin, N-hydroxysuccinimide, Dicyclohexylcarbodimide, Copper sulfate, Sodium ascorbate, Propargyl bromide, and *tert*-butylbromoacetate were purchased from Sigma-Aldrich and Merck. All solvents used were of analytical grades. For reactions to be performed under dry conditions, solvents were dried by usual reported laboratory procedures. 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7-tris (*t*-butyl acetate) was synthesized in our laboratory using the method of Li and Wong (2002). TLC was run on the silica gel-coated aluminum sheets (Silica gel 60 F_{254}, Merck, Germany) and visualized in UV light 254 nm. Radiocomplexation and radiochemical purity was checked by instant thin layer chromatography. ^{99m}Tc was procured from Regional Center for Radiopharmaceuticals (Northern Region), Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, India.

Cell culture

Monolayer cultures of U-87MG (obtained from NIMHANS, Bangalore), were maintained at 37°C in a humidified CO_2 incubator (5% CO_2, 95% air) in DMEM (Sigma, USA) supplemented with 10% fetal calf serum (Biological industries, Israel), 50 U/ml penicillin, 50 µg/ml streptomycin sulfate and 2 µg/ml nystatin. Cells were routinely subcultured twice a week using 0.05% Trypsin (Sigma, USA) in 0.02% EDTA.

Instrumental analysis

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II 400 MHz system (Ultra shield). Mass spectra (ESI-MS in positive and negative ion mode) were performed on in-house Agilent 6310 system ion trap. The receptor binding data were analyzed using iterative curve-fitting program EQUILIBRATE software from graph pad. Radioimaging was performed using HAWKEYE gamma camera and single well type capintec γ- scintillation counter respectively.

Animal models

Animal protocols have been approved by Institutional Ethics Committee. Mice were housed under conditions of controlled temperature 22°C and normal diet. Athymic mice were inoculated subcutaneously with 0.1 ml cell suspension (1×10^6 U-87 MG cells) in the right thigh under sterile conditions.

Synthesis

The ligand [4,7-bis-carboxymethyl-10-(1-{2-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoylamino]-ethyl}-1H-[1,2,3]triazol-4-ylmethyl)-1,4,7,10 tetraaza-cyclododec-1-yl]-acetic acid was synthesized as follows (Scheme 1).

Experimental

Synthesis of 2-Azido-ethylamine (2)

To a solution of 2-bromoethylaminehydrobromide (1 g, 4.87 mmol) in water (10 ml) was added sodium azide (951 mg, 14.6 mmol) and the reaction temperature was raised to 80°C. The reaction was run for 18 h and reaction progress was monitored by TLC. The reaction mixture was cooled to room temperature and extracted with diethyl ether (3 × 50 ml). The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure to obtain colorless oil (85%). The final product was well characterized by ^{1}H, ^{13}C NMR and MS. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 2.88(t, 2H); 3.39(t, 2H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ ppm: 41.32; 54.67; MS(ESI$^+$) m/z: 87[M+H]$^+$

Synthesis of Biotin-N-hydroxysuccinimide ester (3)

To a solution of biotin (1 g, 4.08 mmol) in dimethylformamide (5 ml) was added dicyclohexylcarbodimide (1 g, 4.89 mmol) at 0°C. N-hydroxysuccinimide (562 mg, 4.89 mmol) was added to the reaction mixture and reaction was stirred for 24 h. The reaction mixture was filtered and filtrate was evaporated under reduced pressure to obtain the crude product which was purified by etheration under cold conditions (60%). The final product was well characterized by ^{1}H, ^{13}C NMR, and MS.

^{1}H NMR (DMSO, 400 MHz) δ ppm: 1.22-1.65(m, 6H), 2.48(t, 2H), 2.55(t, 2H), 2.63(s, 4H), 3.12(s, 1H), 4.30-4.40(m, 2H), 6.36 (brs, NH); ^{13}C NMR (DMSO, 400 MHz) δ ppm: 24.91, 28.28, 30.44, 31.21, 33.79, 55.68, 59.61, 61.43, 163.12, 170.72; MS (ESI$^+$) m/z: 341[M+H]$^+$

Synthesis of 5-(2-Oxo-hexahydro-thieno [3, 4-d]imidazol-4-yl)-pentanoic acid (2-azido-ethyl)-amide(4)

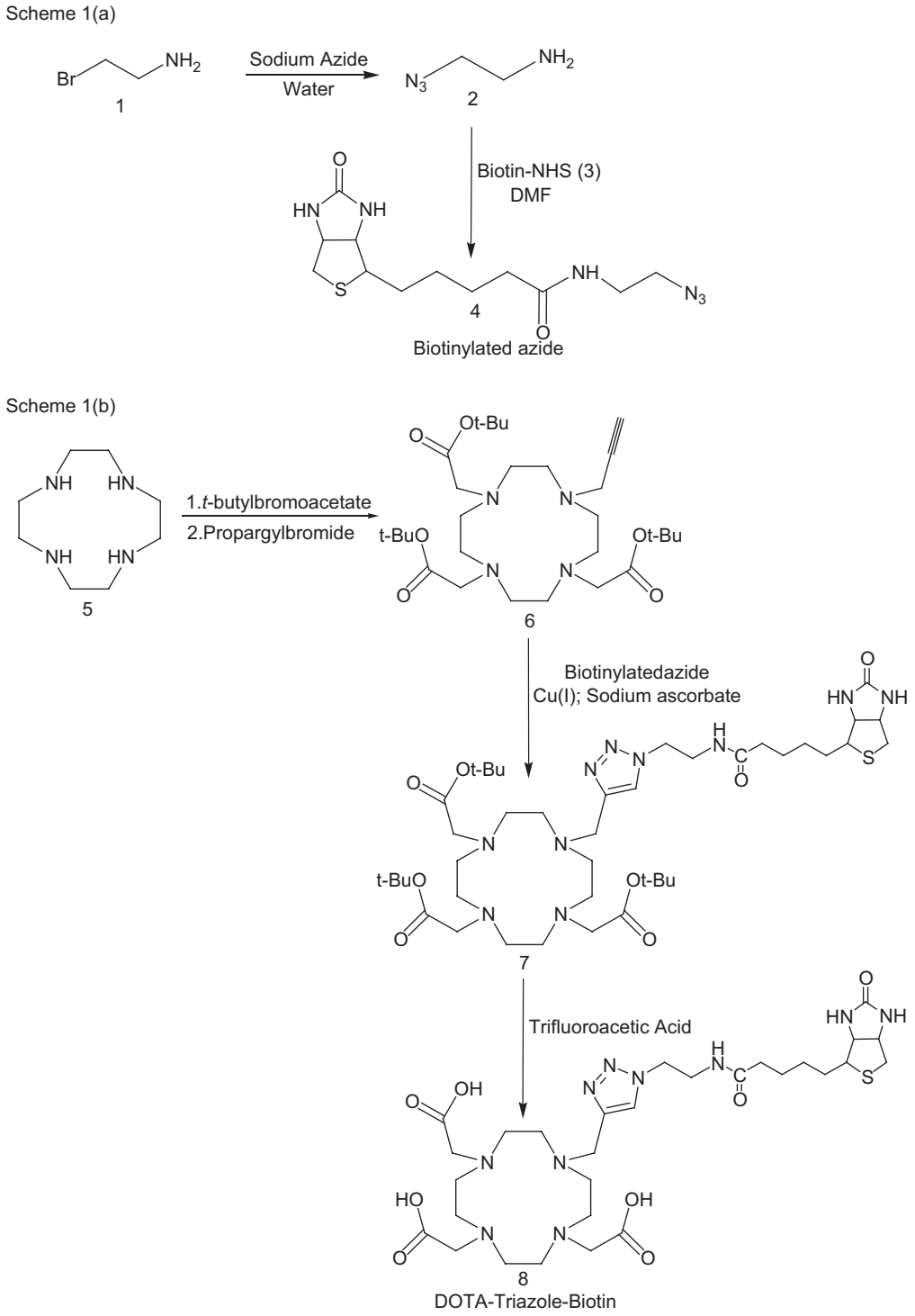
To a solution of the Biotin N-hydroxysuccinimide active ester (500 mg, 1.466 mmol) in dimethylformamide (10 ml) was added triethylamine (740 mg, 7.33 mmol) at room temperature. This was followed by the addition of 2-azidoethylamine (252 mg, 2.932 mmol) to the reaction mixture and the reaction was run for 48 h. The solvent was evaporated under reduced pressure to obtain the crude product. It was purified by column chromatography on a silica gel (eluent: dichloromethane/methanol: 9/1) to afford the desired product as a white solid (70%). The final product was well characterized by ^{1}H, ^{13}C NMR and MS. ^{1}H NMR (DMSO, 400 MHz) δ ppm: 1.21-1.51(m, 6H), 2.19(t, 2H), 2.50(t, 2H), 2.71(s, 4H), 2.83(t, 2H), 3.31(t, 2H); 3.34 (s, 1H), 4.30-4.40(m, 2H), 6.43(brs, NH); ^{13}C NMR (DMSO, 400 MHz) δ ppm: 25.00, 29.71, 31.19, 33.9, 36.21, 55.85, 59.64, 61.51, 163.16, 173.24; MS (ESI$^+$) m/z: 313[M+H]$^+$

Synthesis of (4, 7-bis-tert-butoxycarbonylmethyl-10-prop-2-ynyl-1,4,7,10 tetraaza-cyclododec-1-yl)-acetic acid t-butyl ester (6)

To a solution of 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7-tris (*t*-butyl acetate) (500 mg, 0.9727 mmol) in acetonitrile (50 ml) was added potassium carbonate (1.34 g, 9.7276 mmol) under nitrogen. The reaction temperature was raised to 70°C followed by the dropwise addition of propargyl bromide (231 mg, 1.94 mmol). The reaction was stirred at 70°C for 12 h. The reaction mixture was cooled to room temperature, filtered, and the filtrate was evaporated under reduced pressure to obtain the crude product. It was purified by column chromatography on a silica gel (eluent: dichloromethane/methanol: 9/1) to afford the desired product as a brown oil (80%). The final product was characterized by ^{1}H, ^{13}C NMR, and MS. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 1.40(s, 27H), 2.70-2.82(m, 16H), 2.15(s, 1H) 3.31(s, 6H), 3.46(s, 2H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ ppm: 29.32, 43.08, 51.55, 51.94, 56.81, 65.82, 80.87, 172.97; MS (ESI$^+$) m/z: 553[M+H]$^+$

Synthesis of [4-tert-Butoxycarbonylmethyl-7-(2-hydroxy-allyl)-10-(1-{2-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoylamino]-ethyl}-1H-[1,2,3]triazol-4ylmethyl)-1,4,7,10 tetraaza-cyclododec-1-yl]-acetic acid tert-butyl ester (7)

Biotinylated azide (**4**; 200 mg, 0.6410 mmol) and N-functionalized DO3A alkyne (**5**; 354 mg, 0.6410 mmol) were mixed in 1:1 water/*t*-butanol (10 ml). This was followed by the addition of copper(II) sulfate pentahydrate (16 mg, 0.0641 mmol) and sodium ascorbate (127 mg, 0.6410 mmol).The reaction was stirred for 24 h at room temperature. NH_4OH was added to the resulting green solution to trap copper(II). The reaction mixture was extracted with ethyl acetate (3 × 50 ml) and combined organic extracts were dried over sodium sulfate, evaporated under reduced pressure to obtain the crude product. It was purified by column chromatography on a silica gel



Scheme 1. Synthesis of DOTA-Triazole-Biotin via Cu(I) catalyzed click conjugation.

(eluent: dichloromethane/methanol: 9/1) to afford the desired product as a white solid (80%). The final product was well characterized by ^{1}H, ^{13}C NMR and MS. ^{1}H NMR ($CDCl_3$, 400 MHz) δ ppm: 1.22-1.65(m, 6H), 2.19(t, 2H), 2.46-2.67(m, 16H), 3.12(s, 1H), 3.32(s, 6H), 3.56-3.91(m, 6H), 4.30-4.40(m, 2H), 6.36 (brs, NH), 7.38(s, 1H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ ppm: 24.95, 28.09, 29.32, 29.99, 31.88, 33.89, 52.30, 53.89, 55.68, 59.61, 61.43, 163.12, 170.72; MS (ESI^+) m/z: 866$[M+H]^+$

Synthesis of [4,7-bis-carboxymethyl-10-(1-{2-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoylamino]-ethyl}-1H-[1,2,3]triazol-4-ylmethyl)-1,4,7,10tetraaza-cyclododec-1-yl]-acetic acid (8)

To a solution of **6** in anhydrous dichloromethane was added trifluoroacetic acid (5 ml) and the reaction was stirred for 24 h at room temperature. The reaction progress was monitored by TLC. The solvent was evaporated to dryness under reduced pressure to obtain

the crude product. The product was washed well with chloroform (50 ml × 3). Addition of cold diethyl ether led to precipitation of pure product as pale white solid (60%). The final product was well characterized by ^{1}H, ^{13}C NMR, and MS. ^{1}H NMR (D_2O, 400 MHz) δ ppm: 1.89-2.32(m, 8H), 2.64 (s, 2H), 3.03 (s, 1H), 3.25-3.66 (m, 16H), 3.35 (m, 6H), 3.66 (s, 2H), 3.77-4.03(m, 4H), 4.5-4.67(m, 2H), 6.70 (s, 1H); ^{13}C NMR (D_2O, 400 MHz) δ ppm: 22.54-31.45, 33.93, 40.38, 49.39, 50.29, 129.72, 157.23, 166.85, 173.26; MS (ESI^+) m/z: 695.5$[M]^+$ (Figure 1)

Synthesis of radiolabeled DOTA-Triazole-Biotin

DOTA-TB (1 mg) was dissolved in 1 ml double distilled water under nitrogen atmosphere. 100 µl of this solution was taken in glass vial and stannous chloride (50 µl; 1 mg dissolved in 1 ml 10% acetic acid) was added to it followed by the addition of 74 MBq of freshly eluted (<1 h old) ^{99m}Technetium pertechnetate saline solution. The pH of the reaction mixture was adjusted to 7 using 0.1 M sodium carbonate solution. The contents of the vial were mixed well. The vial was allowed to stand for 20 min at room temperature. Standard safety procedures were employed during radiocomplexation of the chelate.

Radiochemical purity of ^{99m}Tc-DOTA-Triazole-Biotin conjugate

The radiolabeling efficiency was determined using ascending instant thin layer chromatography on ITLC-SG (Paul German, USA) using 100% acetone, ternary mixture of pyridine: acetic acid: water (3:5:1.5) and 0.9% saline as the mobile phase. Each TLC was cut in 0.5 cm fragments and counts of each segment were taken. Hence the percentage of free $Na^{99m}TcO_4^-$, reduced ^{99m}Tc, and complexed ^{99m}Tc could be calculated. ^{99m}Tc DOTA-Triazole-Biotin Conjugate remained at the origin while uncomplexed ^{99m}Tc traveled with the solvent front in acetone.

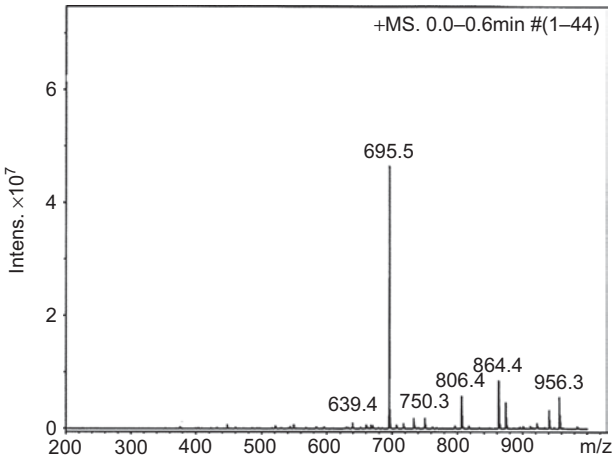


Figure 1. A representative mass spectrum showing the molecular ion peak of DOTA-Triazole-Biotin. MS (ESI^+) *m/z*: 695.5$[M]^+$.

Cytotoxicity studies of DOTA-Triazole-Biotin

Viability assay: Trypan blue exclusion assay

Cell suspension of monolayer culture of U-87MG 1×10^6 cells were prepared and 1:1 dilution of the suspension using a 0.4% trypan blue solution was done after 2 h treatment with the compound. 10 µl of cell suspension was loaded on a hemocytometer. The number of stained cells and total number of cells were counted and percentage viability was calculated.

Macrocolony assay

Monolayer culture of U-87MG cell line was trypsinized and 100 to 1000 cells were plated depending upon the concentrations of the compound in 60-mm petri dishes and incubated at 37°C in 5% CO_2 humidified atmosphere for 8 days. Colonies were fixed in methanol and stained with 1% crystal violet. More than 50 colonies were counted.

MTT assay

Cytotoxicity was determined using the MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide] assay. Exponentially growing cells were plated in a 96 well microtiter plate at a uniform cell density of 4000 cells/well 24 h before treatment. Cells were treated with the varying concentrations of the agent for various time intervals 24 h, 48 h, and 72 h and MTT assays were performed. At the end of treatment, negative control and treated cells were incubated with MTT at a final concentration of 0.05 mg/ml for 2 h at 37°C and the medium was removed. The cells were lysed and the formazan crystals were dissolved using 150 µl of DMSO. Optical density was measured on 150 µl of extracts at 570 nm (reference filter: 630 nm). Mitochondrial activity was expressed as percentage of viability compared to negative control (mean ± SD of triplicate cultures). % of viability = [OD (570–630 nm) test product/OD (570 nm–630 nm) negative control] × 100%. Viability at (0.0001–10 mM) concentration range was plotted against time for DOTA-Triazole-Biotin (Figure 2).

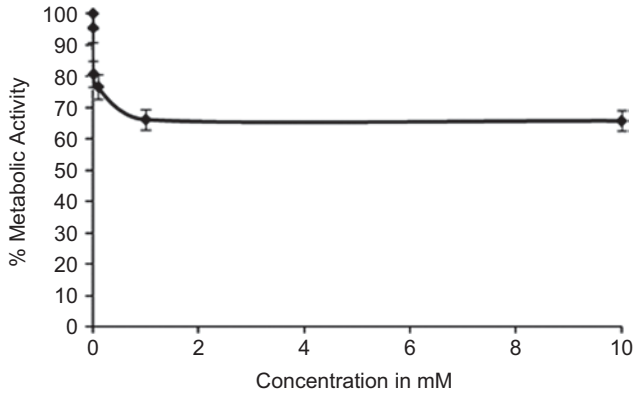


Figure 2. Colorimetric estimation of the mitochondrial activity for cytotoxicity of DOTA-Triazole-Biotin (MTT assay) in U-87MG cell line (0.0001–10 mM concentration range).

Cell uptake assay

Cell uptake studies were performed using radiolabeled DOTA-Triazole-Biotin and compared with avidin-treated cells for 2 h. The specificity of biotin conjugates to bind to cell surface receptors on tumor cells was examined by receptor binding assays on U-87MG cell line grown in normal DMEM (10% serum). Monolayer cultures of the cell lines were washed with HBSS and were then incubated for 2 h in HBSS at 37°C prior to the experiment. Binding experiments were conducted at 37°C. The cell line cultures were then incubated for 40 min with ^{99m}Tc-DOTA-Triazole-Biotin at 37°C in HBSS containing various concentrations (0.001 μM–10 μM) in the absence and presence of the 100 folds excess unlabeled biotin for estimation of total binding and nonspecific binding respectively. Specific binding was obtained by subtracting nonspecific binding from total binding. At the end of each experiment, the cells were washed with cold PBS and 0.9% saline four times. The cell-associated radioactivity was determined by gamma scintillation counting. Scatchard plot analysis was done using EQUILIBRATE software from graph pad (Figure 3).

Blood kinetics

300 μl of the complex ^{99m}Tc-DOTA-Triazole-Biotin (9.8 MBq activity) was injected intravenously through the dorsal ear vein in normal rabbit. Blood was withdrawn from the other ear vein at different time intervals (5 min to 24 h). Persistence of activity in the circulation was calculated, assuming total blood volume as 7% of the body weight (Figure 4).

Avidin pretargeting scintigraphy

Tumor imaging was performed in U-87 cell line implanted tumor bearing nude mice ($n = 5$) following administration of 100 μl of the labeled conjugate **8** (40 μg, 3.7 MBq activity) pretreated with avidin for 2 h prior to injection. Mice were coinjected with unlabeled biotin to estimate the displacement with respect to radiolabeled biotin-DOTA conjugate. Images were obtained using HAWK-EYE gamma camera, at different time intervals (15 min to 24 h) postinjection (Figure 5). Semiquantative analysis by generation of region of interest (ROI) was done using INTEGRA software to evaluate the uptake of the administered compound at different time intervals.

Biodistribution

U-87 cells were injected subcutaneously in the thigh of the right hind leg of nude mice. When the tumors became conspicuous and approximately of 0.17 g, the mice were used for biodistribution study. An intravenous injection of ^{99m}Tc DOTA-Triazole-Biotin conjugate (100 μl; 3.7 MBq activity) was injected through the tail vein of each mice. Mice were dissected at 1 h, 4 h, and 24 h postinjection; different tissues were isolated, weighed and counted in a gamma counter calibrated for ^{99m}Tc energy. Uptake of the radiotracer in each tissue was calculated and expressed as percentage injected dose per gram of the tissue (%ID/g) (Table 1).

Results

In a two step pretargeting strategy, binding affinity of avidin and biotin have been utilized for tumor targeting. The

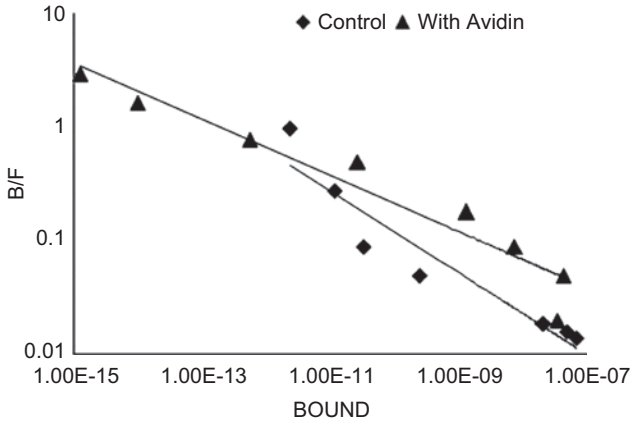


Figure 3. Scatchard Plot of the specific binding data to the ratio of bound to free (B/F) for U-87MG cell line with and without avidin treated cells.

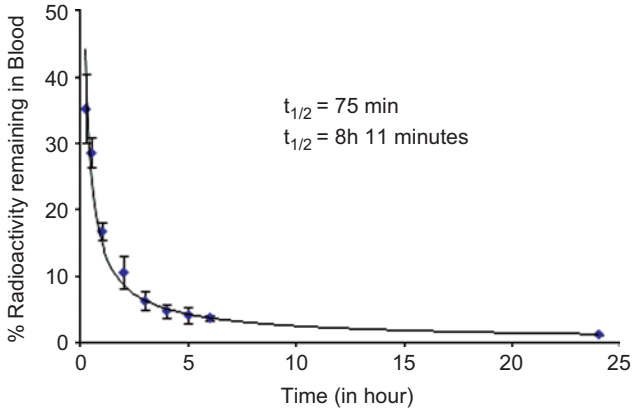


Figure 4. Blood clearance of ^{99m}Tc-DOTA-Triazole-Biotin (9.8 MBq activity) administered through ear vein in normal rabbit.

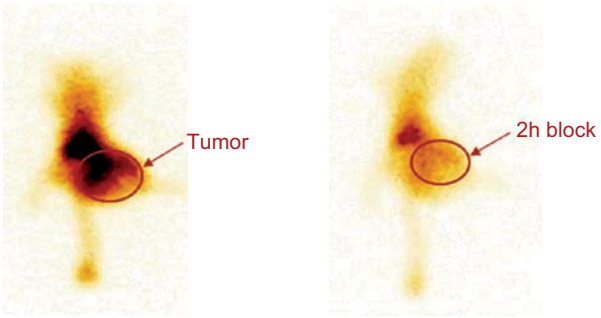


Figure 5. Whole body γ image of female athymic mice with U-87 tumor in right thigh at 1 h following intravenous administration of ^{99m}Tc DOTA-Triazole-Biotin (2.96 MBq activity). Radiotracer uptake can be visualized in tumor with and without coadministration of blocking dose (cold biotin).

Table 1. Biodistribution of ^{99m}Tc-DOTA-Triazole-Biotin in athymic mice following intravenous injection.

Organs	%ID/g[a] (2 h)	%ID/g[a] (4 h)	%ID/g[a] (24 h)
Blood	1.32 ± 0.22	0.93 ± 0.16	0.24 ± 0.05
Heart	0.23 ± 0.06	0.21 ± 0.05	0.08 ± 0.01
Lungs	1.52 ± 0.13	1.46 ± 0.11	0.47 ± 0.09
Liver	2.7 ± 0.27	2.63 ± 0.28	1.65 ± 0.31
Spleen	0.47 ± 0.12	0.36 ± 0.09	0.22 ± 0.08
Kidneys	7.5 ± 0.68	6.3 ± 0.7	1.53 ± 0.22
Stomach	0.34 ± 0.12	0.3 ± 0.05	0.25 ± 0.04
Intestines	0.51 ± 0.17	0.38 ± 0.08	0.17 ± 0.06
Muscles	0.23 ± 0.04	0.17 ± 0.06	0.11 ± 0.03
Tumor	3.06 ± 0.75	3.65 ± 0.97	1.23 ± 0.24
Tumor/Muscle	13.30 ± 2.08	21.4 ± 1.07	11.18 ± 1.64

[a]Percentage of injected ^{99m}Tc-DOTA-Triazole-Biotin dose per gram following intravenous administration, values shown represent the mean ± standard deviation of data from three animals. [b] Tumor/background ratios based on corresponding % injected dose per gram data.

covalent attachment of bifunctional chelating agents to biotin is a feasible process as biotin has a free carboxylate group and this functional group is not involved in binding of biotin to avidin. The carboxylate group of the biotin was activated by formation of N-hydroxysuccinimide (NHS) ester. For the synthesis of biotin functionalized azide, 2-bromoethylamine was reacted with sodium azide at 80°C followed by its coupling with the biotin-NHS active ester at room temperature. The propynyl derivative was prepared by the alkylation of trisubstitued cyclen with propargyl bromide in presence of potassium carbonate at 70°C. The key step in the synthesis was Cu(I) catalyzed 1, 3-dipolar cycloaddition of the biotinylated azide with the propynyl functionalized DO3A affording 1,4-disubstitued triazole in good yields. The final step involved deprotection of the *t*-butyl groups with trifluoroacetic acid yielding the desired chelate DOTA-Triazole-Biotin. All intermediates and the final compound were successfully characterized by spectroscopic techniques such as ^{1}H, ^{13}C NMR and MS (Figure 1).

Quality control of ^{99m}Tc DOTA-Triazole-Biotin: Ascending thin layer chromatography illustrated that labeled complex remained at the origin while $^{99m}TcO^{-}_{4}$ traveled up with the solvent front in acetone. The yield of the free and complexed ligand could be calculated from above observations. The labeling yield was found to be greater than 98%. Percentage radiolabeling was calculated for different time intervals, namely, 0, 2, 4, 6 and 24 h. The radiolabeled complex exhibited sufficient stability up to 24 h with 97% of the radiolabeling efficiency.

Cytotoxicity studies of DOTA-Triazole-Biotin

The percentage of viable cells for 2 h treatment of the title compound as determined by the trypan blue exclusion assay was found to be 45% at 50 mM. The IC_{50} value for the compound was 40 ± 2.7 mM as calculated from the cell viability assay. U-87 MG showed survival value of 0.88 ± 0.06 at 1 mM for unlabeled compound. Surviving fraction of 0.72 ± 0.05 at 10 mM was observed in the macrocolony assay. Analyzing the MTT assay data at a range of 0.1 μM to 10 mM concentrations, it was observed that at a concentration of 10 mM of compound after 24 h exposure resulted in 35% reduction in the MTT assay which indicated 35% cell death. Whereas at lower concentrations the conjugate did not show any adverse effect on the cell survival as only 5% of the cell death was observed when 1 μM of the DOTA-Triazole-Biotin was incubated with U-87 cell line for 2 h. (Figure 2). Time dependent curve showed regain of the metabolic viability of U87-MG cells at 72 h post treatment.

Cell uptake studies: The ability of DOTA-Triazole-Biotin to bind to U-87 tumor cell lines pretreated with 0.1 mg/ml of avidin was determined by saturation binding assay using ^{99m}Tc-DOTA-Triazole-Biotin as the labeled ligand. Nonspecific binding was obtained by using 100 fold excess of unlabeled biotin. Analysis of the binding curve exhibited saturable binding of the radioconjugate in nM range. Scatchard plot analysis revealed that the labeled compound exhibited high affinity on U-87 cell line with a K_d value of 69 nM. This value was found to be 100 folds more than the K_d (0.37 μM) obtained when the cells were not treated with avidin (Figure 3).

Blood kinetics: Blood kinetics studies in rabbits depicted that there was a rapid clearance of the conjugate **8** from the body as only 16% of injected activity persisted in the circulation at 1 h. After 2 h, the clearance followed a slow pattern and at 24 h approximately 1.05 % activity remained in the blood (Figure 4). The biological half-life was obtained to be $t_{1/2}$ (Fast): 75 min; $t_{1/2}$ (Slow): 8 h and 11 min.

Scintigraphy: Imaging of animals was performed at different time intervals following intravenous administration of the radiolabeled conjugate in five mice. The mice illustrated the beginning of accumulation of activity in tumor at 30 min, which reached to maximum at 1 h (Figure 5) and remained stable for 4 h. Semiquantitative analysis was generated from Region of Interest (ROI) placed over areas counting average counts per pixels with maximum radiotracer uptake at the tumor site and compared to symmetric counterparts with ROI in soft tissues. Target to nontarget (T/NT) ratio at different time intervals was calculated. The ratio of tumor to soft tissue at 1 h was found to be 23 ± 2.3 which then increased to 36 ± 4.2 at 4 h. The radiotracer uptake was decreased at 24 h but the ratio was increased due to the clearance of the labeled compound from the body. The T/NT ratio obtained was 20.8 ± 3.66 at 24 h in tumor bearing nude mice.

Biodistribution: Athymic mice implanted with U-87 cell line exhibited major accumulation in kidneys (7.5%/g) followed by liver (2.7%/g) at 2 h showing that the complex is excreted both by renal as well as hepatobiliary routes (Table 1). The high tumor accumulation of 13.30 ± 0.08 % ID/g at 2 h that remains high 21.4 ± 1.07 up to 4 h post injection was observed. This uptake was significantly blocked by (> 90%) in the animals receiving a high dose of avidin together with the radiolabeled compound **8**. As the background activity gradually

cleared from the body, there was an increase in target-to-nontarget ratios obtained (Table 1).

Discussion

Conventional nuclear medicine imaging with radiolabeled tumor specific agents such as antitumor antibodies can provide high tumor/nontumor ratios but usually with slow signal localization and clearance. This paper describes a method for two-step pretargeting of tumors DOTA-Triazole-Biotin, synthesized through an efficient click reaction of a biotinylated azide and alkyne functionalized DOTA nucleus. In order to increase the amount of radioactivity bound to cancer cells, a new approach in cancer therapy called pretargeting has been employed. The pretargeting protocol involved a two-step procedure comprising prior administration of avidin to establish secondary binding sites on the tumor that could be more efficiently targeted by low molecular weight radiolabeled biotinylated chelate (^{99m}Tc DOTA-Triazole-Biotin). This led to high tumor/nontumor ratios as the radiolabeled biotin chelate was placed on a small molecule avidin designed to clear from the circulation and whole body rapidly eliminating excessive irradiation to nontarget tissues. The extremely high binding affinity of biotin to avidin provided a high tumor targeting efficiency of DOTA-Triazole-Biotin and the tetrameric architecture of avidin (four fold valency for biotin) offered the potential of modest signal amplification at the tumor site.

It is observed that a short distance between avidin and the surface of the biotinylated molecule might result in unsatisfactory binding of the biotinylated chelate to avidin. For sufficient binding capability to avidin, the biotin site and the chelating site of the biotinylated conjugate must be separated by a spacer. Therefore a dynamic strategy involving click chemistry was used to incorporate a highly stable triazole spacer between biotin and the macrocycle as well as to simplify the synthesis of biotin DOTA conjugate. Triazoles are stable to acid and basic hydrolysis and reductive and oxidative conditions, indicative of a high aromatic stabilization. Ever since their debut in 2001, click reactions are finding more and more applications in organic synthesis. This Cu(I) catalyzed dipolar cycloaddition owes its usefulness in part to the ease with which azides and alkynes can be introduced into a molecule and their relative stability under a variety of biological and organic conditions. The synthesis of biotin functionalized azide was a convenient process utilizing readily available starting materials and simple reaction conditions. It was thereupon subjected to Cu(I) catalyzed 1,3-dipolar cycloaddition with its alkyne partner, *namely*, propynyl derivatized DO3A leading to high yield synthesis of DOTA-Triazole-Biotin thereby eliminating the extensive protection and deprotection steps encountered in the routine synthesis of DOTA-biotin conjugates. Click conjugation offered numerous advantages of overall good yields, benign reaction conditions, high functional group tolerance, and easy access to gram-scale preparation of regiospecific 1,4-disubstituted 1,2,3-triazoles.

Radiochemical analysis demonstrated that the chelate formed stable electron donor complexes with ^{99m}Tc with high reproducible labeling efficiency. This compound was examined for its ability to induce cytotoxicity in the cancer cell lines using trypan blue dye exclusion assay to determine the percentage of viable cells. Macrocolony assay was done to test the ability of cells to form colonies after treatment with the compound. MTT assay was done to analyze the metabolic activity posttreatment. Cytotoxicity studies were found to be in good correlation with the different techniques used to investigate the cytotoxicity of the compound. The IC_{50} value of 40 mM was found to be well in range as for imaging 0.102 mmol/kg of the compound is being injected for human scan. The blood clearance studies of the radiolabeled compound **8** showed its high target uptake with the diagnostically useful target-to-nontarget ratio in a short period of time. It exhibited rapid clearance (avidin clearing compound) from blood circulation thereby resulting in the reduction of the background activity. The saturation binding assay revealed that ^{99m}Tc-DOTA-Triazole-Biotin has a high affinity on U-87MG cell line (pretreated with avidin) with a K_d value of 69 nM. The high specificity of **8** results in selective uptake and distribution of the radiolabeled ligand that can be visualized in the high quality images, obtained 1 h after administration in animal models with and without coadministration of blocking dose (biotin), which allows the advantage of comparing *in vitro* receptor binding studies and tumor-bearing animal studies. The biodistribution studies illustrated that ^{99m}Tc-labeled DOTA-Triazole-Biotin persisted in liver up to 4 h and is cleared through renal and hepatobiliary routes. Imaging of tumor-bearing nude mice correlated well with the biodistribution studies. Thus our preclinical evaluations and results demonstrate an encouraging and promising future of this compound in the field of two-step pretargeting.

Conclusion

To summarize, a new candidate for avidin based two-step pretargeting of tumors has been synthesized and evaluated for potential imaging and diagnostic applications. An expedient methodology to derivatize macrocyclic chelating agents with biotin using click chemistry leading to the synthesis of DOTA-Triazole-Biotin has been reported. The chelate possesses high stability in biological environment and exhibits effective interaction with its avidin target and low nonspecific retention *in vivo.*

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