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Full Paper

Investigation into the Use of a Diaminodihydroxyaryl Derivative of Ethylenediaminetetraacetic Acid (DAHA-EDTA) for Cu-64 PET Imaging and Radioimmunotherapy

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A diaminodihydroxyaryl derivative of ethylenediaminetetraacetic acid (DAHA-EDTA) was synthesised in two steps and evaluated for Cu-64 radiolabelling of the B72.3 antibody. The ligand complexes Cu-64 rapidly in a pH range 4 to 7. The Cu-64 complex of the parent species *N*,*N*′-bis(carboxymethyl)-*N*,*N*′-bis(2-hydroxyacetanilido)-1,2-diaminoethane (DHA-EDTA) shows good stability in serum at 37°C for up to 72 h. Conjugation of the Cu-64-DAHA-EDTA to the B72.3 antibody was achieved using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as the activating agent. The reaction conditions were optimized for protein concentration and molar ratio of Cu-64-DAHA-EDTA and EDC to antibody. The specific activity of the final [Cu-64-DAHA-EDTA]-B72.3 product was 49 MBq mg^{-1} at the end of synthesis. The biodistribution of [Cu-64-DAHA-EDTA]-B72.3 in LS174t tumour-bearing nude mice was monitored over a 24 h period. Maximum tumour uptake (25.8 ± 7.5 % ID g^{-1}) was achieved at 16 h and maintained at 24 h (21.6 ± 1.8 % ID g^{-1}). Rapid clearance of the [Cu-64-DAHA-EDTA]-B72.3 from the blood resulted in good tumour-to-blood ratios (≈ 3.3) within a shorter period (6 h) than previously reported with B72.3 whole antibody and the LS174t tumour bearing nude mouse model.

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Introduction

Personalised medicine involves the tailoring of therapy for patients to ensure they get the best treatment with minimal side effects.[1] Interest in the use of nuclear molecular imaging techniques such as positron emission tomography (PET) for personalised medicine continues to grow. The high sensitivity and specificity of PET agents allows one to track the radiolabelled biomarker in vivo, and therefore determine the extent of disease and identify dose limiting organs which can cause unwanted side effects. Research into the production of new PET imaging agents and novel methods for radiolabelling biomarkers is important to the success of PET in personalised medicine.

When a PET emitting radioisotope is substituted with a β- or an α-emitting radioisotope, the resultant surrogate agent (radiotherapeutic agent) can then be used to treat the disease. Copper (Cu-64) and copper-67 (Cu-67) are known as a theranostic pair of radioisotopes.[2,3] Cu-64 has a positron emission ($\beta^+ = 0.65$ MeV) that is ideal for PET imaging. Cu-67 has a β emission that is suitable for radiotherapy.

The positron energy for Cu-64 is similar to that of F-18 ($\beta^+ = 0.64$ MeV; $t_{1/2} = 1.8$ h) so one can expect the resolution of Cu-64 PET agents to be comparable to F-18 agents. The longer half-life ($t_{1/2} = 12.7$ h) of Cu-64 is more appropriate for labelling larger molecules such as peptides and proteins and it also permits the transport of the Cu-64 agents across continents.[2c,d,4] High specific activity Cu-64 can be produced on a proton accelerator via various nuclear reactions. For low energy protons, enriched Ni-64 is the target of choice, while different isotopically enriched Zn targets are used when bombarding with moderate to high energy protons.

The Cu-67 half-life is 61.9 h and its β emissions ($\beta^-_{avg} = 121$ (57 %), 154 (22 %), 189 (20 %) keV) have been used in the radiotherapy of systemic disease.[2,3,5,6] Its gamma emissions are ideal for single photon emission tomography (SPECT). Cu-67 has

been produced using high-energy accelerators (> 100 MeV protons) or reactors intermittently over the years. Until now the worldwide supply of Cu-67 has been hampered by low specific activity, poor radiochemical and chemical product purity, and an unreliable supply. However, new cross-section data and the commissioning of several 70 MeV proton cyclotrons around the world, as well as advances in engineering of linear accelerators in recent times, has provided impetus to assess different production routes.[4,5] In particular the photonuclear reaction of $^{68}Zn(\gamma,p)^{67}Cu$ using thick enriched Zn-68 targets has shown significant promise for large scale production of high purity Cu-67.[5,6]

Both of these copper isotopes have been complexed by a wide range of polyaza, polyazacarboxylate, and hexaazacage ligands.[2d,7] The bi-functional chelator (BFC) forms of these ligands are used to radiolabel biomarkers. BFCs possess two functional parts: a chemically reactive group for covalent attachment to the biomarker and a metal binding moiety for complexing the radiometal ion.

The monoclonal antibody B72.3 has been widely investigated for imaging and therapy of cancer. It recognises the tumour-associated protein, (TAG)-72 antigen, that is expressed in several epithelial derived cancers such as colorectal and ovarian.[8] B72.3 antibody was the first radiolabelled (In-111) antibody (OncoScint) to be approved for imaging in ovarian cancer in humans. The B72.3 antibody has been radiolabelled with a range of radionuclides; directly with I-125 and I-131 and indirectly with Tc-99m, In-111, Y-88, Pb-203, Cu-64, and Cu-67 using bi-functional chelators.[2d,7–10] Open chain polyazacarboxylate derivatives of ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA), polyaza and polyazacarboxylatemacrocycles, and hexaaza cage ligands have all been deployed to radiolabel the B72.3 antibody with radiometals. Evaluation of the targeting and stability of the resultant radiolabelled B72.3 immunoconjugate is commonly conducted in LS174t tumour-bearing nude mice. The LS174t is a colorectal cancer cell line that expresses Tag-72 antigen.

Many of the BFCs used are produced by a lengthy synthetic route. It was of interest to us to see if a simpler BFC ligand system could used to radiolabel proteins. In this study a new ligand, a diaminodihydroxyaryl derivative of EDTA (*N*,*N*′-bis (carboxymethyl)-*N*,*N*′-bis(2-hydroxy-5-aminoacetanilido)-1,2-diaminoethane, DAHA-EDTA) was synthesised and evaluated for the radiolabelling of the B72.3 antibody with Cu-64. Conditions for complexing Cu-64 and radiolabelling of the B72.3 antibody are presented. The targetting and clearance properties of the resultant radioimmunoconjugate, [Cu-64-DAHA-EDTA]-B72.3 were investigated in LS174t tumour-bearing nude mice.

Results

The DAHA-EDTA, its precursor *N*,*N*′-bis(carboxymethyl)-*N*,*N*′-bis(2-hydroxy-5-nitroacetanilido)-1,2-diaminoethane (DNHA-EDTA), and parent *N*,*N*′-bis(carboxymethyl)-*N*,*N*′-bis(2-hydroxyacetanilido)-1,2-diaminoethane (DHA-EDTA) derivative were synthesised according to literature methods.[11,12] Ethylenediaminetetraacetic dianhydride was refluxed with a slight excess of the appropriate aminophenol derivative in dry acetonitrile under nitrogen. DAHA-EDTA was synthesised by reducing the nitro groups of DNHA-EDTA at room temperature under nitrogen using an excess of $NaBH_4$ on Pd/C. The purity of each ligand isolated was confirmed by ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry, and FT-IR spectroscopy.

Radiolabelling DHA-EDTA and DAHA-EDTA with Cu-64

The complexation of Cu-64 with DAHA-EDTA and DHA-EDTA in various buffers (0.1 M sodium acetate, buffered to pH 4, 5, and 6, and 0.1 M sodium phosphate buffer of pH 6 and 7) was investigated. Both ligands complexed (>95 %) the Cu-64 within 10 min at 37°C for all buffers.

As the Cu-64-DAHA-EDTA reaction mixture would be used to conjugate directly to the antibody, pH 6 phosphate buffer was chosen for optimising the specific activity of the Cu-64-DAHA-EDTA complex. Reaction mixtures with ligand-to-Cu molar ratios of 300, 100, 50, 25, 10, 5, 1.5, and 1.2 all gave >95 % complexation within 10 min at 37°C.

Serum Stability of the Cu-64-DHA-EDTA (Parent Species)

The Cu-64-DHA-EDTA complex was incubated in human serum at 37°C. Aliquots were removed from the reaction mixture at 2, 4, 24, 48, and 72 h and separated on a Sephadex G-25 column. Radioactivity elution profiles showed no protein binding of the Cu-64 and more than 95 % of the copper was associated with DHA-EDTA after 72 h.

Radiolabelling of B72.3 Antibody with Cu-64-DAHA-EDTA

Several conditions to conjugate or radiolabel the B72.3 antibody with Cu-64-DAHA-EDTA complex were attempted. These included the use of different buffers, varying the concentrations of protein and the molar ratios of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and Cu-64-DAHA-EDTA to antibody. Varying the buffer conditions (i.e. pH 4 and 5 0.1 M sodium acetate and pH 6, 7, and 8 0.1 M sodium phosphate) showed that the best radiolabelling efficiency (15 %) was achieved using 0.1 M phosphate buffer at pH 6. All other buffer conditions yielded lower radiolabelling efficiencies (≤8 %). Increasing the antibody concentration from 1 to 5 mg mL^{-1} did result in increased labelling efficiency but unfortunately also increased the amount of cross-linked protein. For 3 mg mL^{-1} of antibody, the labelling efficiency was 15 % and the cross-linked protein was 4 %. For reactions with 5 mg mL^{-1} of antibody, the labelling efficiency remained at 15 %, however, the cross-linked protein increased significantly to 22 %. (Note: the molar ratios of EDC and Cu-64-DAHA-EDTA to antibody were set at 500 and 100, respectively). The activating agent, EDC, can cause the antibody to cross-link but it is likely that the Cu-64-DAHA-EDTA complex with its two functional groups (anilino nitrogen) available for covalent attachment to the protein, contributed to the increase in cross-linked antibody. Therefore the optimum antibody concentration for the conjugation reaction was set at 3 mg mL^{-1}.

Additional investigations to optimise the yield of the conjugation reaction involved varying the molar ratio of the Cu-64-DAHA-EDTA complex (5, 10, 20, 50, and 100 molar ratio) and the activating agent, EDC (100, 500, and 1000 molar ratio), to antibody. The optimal molar ratio of EDC to antibody B72.3 was found to be 500. Increasing the EDC concentration to 1000 resulted in increased cross-linked antibody from 4 to 8 %. Table 1 summarises the effect of the molar ratio of Cu-64-DAHA-EDTA to antibody on the radiobelling efficiency of B72.3. The data in Table 1 shows how the radiolabelling efficiency decreases as the molar ratio of Cu-64-DAHA-EDTA to antibody increases, but the number of ligands attached increases. Unfortunately the percentage of cross-linked antibody increases as the molar ratio of Cu-64-DAHA-EDTA to antibody was increased (>20 %).

Table 1. Effect of Cu-64-DAHA-EDTA-to-antibody molar ratio on radiolabelling efficiency

Molar ratio of EDC to antibody: 500 : 1; concentration of antibody: 3 mg mL^{-1}. Reaction mixtures were incubated at 37°C for 1 h

Molar ratio of Cu-64-DAHA-EDTA to antibody	Radiolabelling efficiency [%]	Cu-DAHA-EDTA conjugated to B72.3 antibody
100	15	15
50	23	12
20	28	6
10	34	3
5	40	2

The highest yield and purest product for the conjugation reaction was achieved with an antibody concentration of 3 mg mL^{-1} and a molar ratio of antibody to EDC to Cu-64-DAHA-EDTA of 1 : 500 : 5 in phosphate buffer (pH 6) at 37°C for 1 h.

As the conjugation reaction results in a mixture of products, the crude [Cu-64-DAHA-EDTA]-B72.3 had to be purified further. All reaction mixtures were loaded onto a size-exclusion column (BioSep 3000)-HPLC for further purification. The isolated [Cu-64-DAHA-EDTA]-B72.3 product was free from both Cu-64-DAHA-EDTA and macro-aggregates formed from cross-linked antibody. The radiochemical purity of the [Cu-64-DAHA-EDTA]-B72.3 isolated was 96.7 ± 1.6 % and its specific activity was 49 ± 0.9 MBq mg^{-1} at the end of synthesis and quality control. The relative immunoreactivity of [Cu-64-DAHA-EDTA]-B72.3 was 87 ± 2.6 % of unmodified B72.3. A typical high-performance liquid chromatography (HPLC) profile of crude and purified product is given in the Supplementary Material (see Fig S1, Supplementary Material).

Stability of [Cu-64-DAHA-EDTA]-B72.3

The [Cu-64-DAHA-EDTA]-B72.3 was incubated in pH 6 phosphate buffer at 4°C and 37°C for up to 48 h. Aliquots were removed from the solutions at various time intervals and analysed by size-exclusion HPLC. The data (i.e. UV-vis and radioactivity profiles) showed the purity of the [Cu-64-DAHA-EDTA]-B72.3 was maintained (> 95 %) up to 24 h at both temperatures, however significant breakdown (up to 10 %) was evident after 48 h.

Biodistribution of [Cu-64-DAHA-EDTA]-B72.3 in LS174t Tumour-Bearing Nude Mice

The [Cu-64-DAHA-EDTA]-B72.3 was injected into LS174t tumour-bearing nude mice, which were then sacrificed at 0.5, 1, 2, 3, 4, 5, 6, 16, and 24 h post-injection (p.i.) and their organs harvested. Five animals per time point were used and selected data are summarised in Table 2 and Table 3. A complete set of biodistribution data are provided in Tables S1 and S2 (Supplementary Material).

Table 2 shows the percentage injected dose per gram of organ (%ID g^{-1}) at each time point. Table 3 gives the calculated ratio for tumour to blood, kidney, and liver at each time point. The clearance of [Cu-64-DAHA-EDTA]-B72.3 is bi-phasic with two distinct half lives: a fast α phase of 2.5 ± 0.75 h and a longer β phase of 60 ± 6.5 h.

The [Cu-64-DAHA-EDTA]-B72.3 appears to be cleared preferentially from the blood via the hepatic system. Table 3 shows that the tumour-to-blood ratio is 2.4, 3.3, 3.2, and 5.6 at 5, 6, 16, and 24 h p.i., respectively. The tumour-to-kidney ratios improve over time (1.5, 1.7, 2.3, and 2.1, respectively). However the tumour-to-liver ratios stay at ~1.0 improving moderately to 1.3 at 24 h p.i.

Analysis of Metabolites

Blood from the mice at each time point was withdrawn into a heparinised syringe, then transferred to microcentrifuge tubes and counted in a gamma counter. Each blood sample was then centrifuged at 2415 *g* for 6 min and the fractions separated, weighed, and then counted in a gamma counter. The recovered plasma from each time point was pooled and a 500 µL aliquot of the combined sample was loaded on to a pre-blocked Sephadex-G25 column (eluent: 0.01 M phosphate buffered saline (PBS) at pH 7.2, flow rate: 1.85 mL min^{-1}). Only two radioactive species were present; they were identified as [Cu-64-DAHA-EDTA]-B72.3 (retention time: 24 min) and Cu-64-DAHA-EDTA (retention time: 48 min). The Cu-64-DAHA-EDTA fractions represented <2 % and 4 % of the total activity in plasma at 6 and 16 h, respectively. At 24 h the percentage of unconjugated Cu-64-DAHA-EDTA increased to 16 % of total activity in plasma.

Dosimetry of Cu-64-DAHA-EDTA-B72.3

MIRDOSE 3 software was used to estimate the dosimetry of the [Cu-64-DAHA-EDTA]-B72.3 in a similar manner to that reported elsewhere.[3,13] Typically, the *Restime* software program (developed by Eric Hetherington, ANSTO) was used to calculate the residence time of radioactivity in the various organs given in Table 2. The residence times were then used to estimate human organ doses with the *MIRDOSE 3* software for a 70 kg reference adult. The analysis assumes identical behaviour and similar pharmacokinetics of [Cu-64-DAHA-EDTA]-B72.3 in mice and humans. Data are presented in Table S3 (Supplementary Material). The total body dose of 0.011 mGy MBq^{-1} is lower than that reported for other radiolabelled immunoconjugates of B72.3.[3,9,10]

Discussion

The study demonstrated that DAHA-EDTA could be prepared in high yield using a simple two-step synthesis. DAHA-EDTA complexation of copper was rapid over the pH range of 4 to 7. A molar ratio of DAHA-EDTA to Cu of 1.2 in phosphate buffer at pH 6.0 gave >95 % labelling efficiency within 10 min at 37°C.

The serum stability of its parent analogue, Cu-64-DHA-EDTA, was found to be comparable to Cu-67-nitrobenzyl-TETA, its macrocyclic counterpart.[14] Surprisingly the Cu-64-DHA-EDTA was found to be more stable (>95 % intact) in serum than Cu-67-DTPA-NH-Bu (<23 %) and Cu-67-1-*p*-nitrobenzyl BEDTA (<14 %) counterparts after 3 days incubation.[14]

Conjugation of Cu-64-DAHA-EDTA to B72.3 antibody is performed in two chemical steps. First the Cu-64-DAHA-EDTA complex is formed in phosphate buffer at pH 6 and the resultant reaction mixture added to the antibody solution (3 mg mL^{-1}) containing an excess of EDC in phosphate buffer at pH 6. The conjugation reaction mixture is incubated at 37°C for 1 h. The final purification step involves size-exclusion chromatography to isolate the [Cu-64-DAHA-EDTA]-B72.3, free from macro-aggregates, unreacted Cu-64-DAHA-EDTA, and by-products. The influence of antibody, EDC, and Cu-64-DAHA-EDTA concentration on the conjugation reaction was investigated. The optimum conjugation reactions conditions were set at

Table 2. Biodistribution of [Cu-64-DAHA-EDTA]-B72.3 in LS174t tumour bearing nude mice

Organ	Biodistribution after various time periods [% ID g^{-1} (standard deviation)][A]					
	1 h	2 h	4 h	6 h	16 h	24 h
Liver	25.2 (3.6)	24.4 (1.5)	25.6 (2.6)	24.4 (3.2)	17.1 (1.2)	16.0 (1.3)
Spleen	6.1 (0.5)	7.3 (1.1)	7.8 (0.7)	7.2 (1.7)	6.4 (1.9)	6.7 (0.7)
Kidney	14.8 (1.3)	14.0 (1.5)	12.3 (1.1)	11.2 (1.1)	9.3 (0.6)	9.8 (0.4)
Muscle	2.1 (1.0)	2.1 (0.5)	1.6 (0.5)	1.0 (0.2)	1.3 (0.4)	1.2 (0.8)
Skin	4.3 (0.6)	5.6 (0.8)	5.1 (1.1)	4.7 (0.9)	3.0 (0.4)	3.3 (0.8)
Bone	3.5 (0.6)	3.6 (0.6)	3.3 (0.4)	2.7 (0.7)	2.8 (1.0)	2.5 (0.2)
Lungs	14.5 (1.1)	14.7 (1.3)	14.5 (1.9)	12.7 (1.8)	10.0 (0.6)	11.0 (0.8)
Heart	8.6 (1.0)	9.1 (0.1)	7.9 (0.3)	6.8 (0.9)	5.2 (0.8)	6.0 (0.5)
Blood	17.7 (1.9)	13.8 (1.3)	9.8 (1.4)	7.7 (0.8)	6.3 (0.8)	3.9 (0.7)
Bladder	5.2 (5.4)	22.6 (17.5)	5.6 (3.1)	4.9 (3.5)	9.6 (12.4)	10.8 (15.6)
Stomach	8.8 (3.1)	9.2 (1.2)	8.7 (1.6)	7.7 (2.2)	4.2 (2.3)	3.5 (1.4)
GIT[B]	9.2 (1.8)	12.1 (1.1)	14.1 (1.5)	13.5 (2.5)	8.5 (1.2)	7.3 (1.2)
Tumour	16.7 (7.9)	27.6 (9.6)	18.9 (2.0)	25.8 (7.5)	20.0 (0.5)	21.6 (1.8)

[A]These values are the average of 5 mice per time point.
[B]Gastrointestinal tract (GIT).

a molar ratio of EDC to Cu-64-DAHA-EDTA to antibody of 500 : 5 : 1 and a 3 mg mL^{-1} concentration of antibody. The purified [Cu-64-DAHA-EDTA]-B72.3 had a specific activity of 49 MBq mg^{-1} at the end of synthesis and quality control. The radiochemical purity was >96 %, and the immunoreactivity of >87 % was considered acceptable for evaluation in animals.

The biodistribution of [Cu-64-DAHA-EDTA]-B72.3 in LS174t tumour-bearing mice showed that radiolabelled immunoconjugate cleared from the blood predominantly via the hepatic system (see Tables 2 and 3). The clearance from the blood was bi-phasic, a fast α phase (2.5 ± 0.75 h) and a slower β phase (60 ± 6.5 h). The biological half-life of the [Cu-64-DAHA-EDTA]-B72.3 was significantly faster compared with other radiolabelled whole B72.3 antibody conjugates. For example the Cu-64-SarAr-B72.3 has an α phase of 7.3 ± 0.4 h and a β phase of 100 ± 30 h.[10] The % ID g^{-1} of [Cu-64-DAHA-EDTA]-B72.3 in the blood was 17.7 ± 1.9 % ID g^{-1} at 1 h and decreased dramatically to 8.7 ± 1.6 % ID g^{-1} at 6 h, and to 3.9 ± 0.7 % ID g^{-1} at 24 h p.i. The tumour uptake was also rapid. The % ID g^{-1} tumour uptake increased considerably from 16.7 ± 7.9 % ID g^{-1} at 1.0 h to 18.9 ± 2.0 % ID g^{-1} at 4 h, and to 25.8 ± 7.5 % ID g^{-1} at 6 h p.i. The % ID g^{-1} uptake at the tumour site was maintained up to 24 h post injection (21.6 ± 1.8 % ID g^{-1}). The tumour-to-blood, the tumour-to-liver, and the tumour-to-kidney ratios for [Cu-64-DAHA-EDTA]-B72.3 were 3.3, 1.0, and 2.3, respectively, at 6 h p.i. The tumour/kidney values improved (2.2) at 24 h.

Initially, the rapid blood clearance of [Cu-64-DAHA-EDTA]-B72.3 was thought to be due to instability of the [Cu-64-DAHA-EDTA]-B72.3 in vivo; however, analysis of the blood indicated the Cu-64-DAHA-EDTA was released intact. Approximately 4 % of Cu-64-DAHA-EDTA was released from [Cu-64-DAHA-EDTA]-B72.3 at 16 h p.i. At 24 h this increased to 16 %. Linder and Hazegh-Azam report that free Cu (i.e. Cu-64) in the portal blood and general circulation is generally taken up by serum protein, albumin, and transcuprein.[15] These protein-bound coppers ions are then rapidly deposited in the liver. If the Cu-64/Cu^{2+} has been released from the DAHA-EDTA one would expect to see a continual increase in liver uptake over the 24 h period. In fact the liver-to-tumour ratio remains steady while the total amount of radioactivity in the liver decreases over the 24 h period (see Tables 2 and 3).

Table 3. Ratio of uptake of [Cu-64-DAHA-EDTA]-B72.3 in tumour versus key organs

Tumour to organ	Time					
	1 h	2 h	4 h	6 h	16 h	24 h
Blood	0.9	2.0	1.9	3.3	3.2	5.6
Liver	0.7	1.1	0.7	1.0	0.9	1.3
Kidney	1.1	2.0	1.5	2.3	2.1	2.2

The biodistribution and localisation of other radiolabelled B72.3 antibodies with other BFCs show a broad spectrum of characteristics. Roselli et al. reported that tumour uptake of around 20–30 % ID g^{-1} at 24 h was achieved for the In-111 labelled B72.3[9b] Its tumour-to-blood ratio was reported to be around 2 and its tumour-to-liver ratio was around 0.6. The tumour uptake increased to a maximum of around 40 % ID g^{-1} after 48 and 120 h. The tumour-to-blood ratio for the Cu-64-SarAr-B72.3 agent conducted in our laboratory was 1.4 at 24 h; however, the tumour to liver and kidney ratios were substantially better at 1.9 and 4.0, respectively at 24 h. At 48 h, the Cu-64-SarAr-B72.3 tumour further increased to 38.4 ± 4.8 % ID g^{-1} indicating a more stable compound in the blood than [Cu-64-DAHA-EDTA]-B72.3. The ^{99m}Tc labelled B72.3 reported by Rosenzweig et al. used the diamidedimercaptide BFC for radiolabelling.[9j] Its maximum tumour uptake was only 8 % ID g^{-1} after 24 h. At this time point the tumour-to-blood and tumour-to-liver ratios were found to be 2.6 and 5.4 respectively. Brown et al. also reported the biodistribution of ^{99m}Tc labelled B72.3 and showed the tumour uptake is also low at 7.3 and 10.8 % ID g^{-1} at 6 and 24 h, respectively.[9f] At 6 h, the tumour-to-blood ratio was 0.5 and tumour-to-liver ratio was 1.4, while at 24 h the values were improved; the tumour-to-blood ratio was 1.4 and the tumour-to-liver ratio was 2.5. The difference in the tumour-to-kidney and -liver ratios of these radiolabelled B72.3 agents appears to reflect the natural biological clearance path of the radiometal ion used.

Achieving optimum imaging quality with PET imaging agents requires that the agent localises to the target and clears rapidly from the blood. The [Cu-64-DAHA-EDTA]-B72.3 described in this study, localised to target sites within 6 h and cleared rapidly from the blood, to attain a tumour-to-blood ratio

of ~3.3 by 6 h, which is higher than any other labelled whole B72.3 product (in the same animal model) at this time to our knowledge. If the [Cu-64-DAHA-EDTA]-B72.3 behaves in a similar manner in humans one could envisage imaging on the day of injection. Unfortunately the slow clearance of the [Cu-64-DAHA-EDTA]-B72.3 from the liver suggests this agent would not be as effective as other agents for monitoring tumours in the liver. The use of DAHA may be attractive for other biomarkers that have preferential clearance through the kidneys.

For application in radioimmunotherapy, the [Cu-64-DAHA-EDTA]-B72.3 shows high target-to-non-target ratios for organs such as blood and bone marrow. The total body dose is 0.011 mGy MBq^{-1} (see Table 3), which is lower than that reported for I-131-B72.3, Y-90-DTPA-B72.3, Cu-64-SarAr-B72.3, and Cu-67-SarAr-B72.3 at 0.709, 0.652, 0.027, and 0.137 mGy MBq^{-1}, respectively.[3] This reduced dose to non-target tissues in the body is largely due to the rapid blood clearance of the [Cu-64-DAHA-EDTA]-B72.3. The rate of localisation of radioactivity and the [Cu-64-DAHA-EDTA]-B72.3 stability at the target site over the 6–24 h period, match the half-life (12.7 h) of the Cu-64 well. It is important to remember that calculations of the dosimetry data were made with the results from a biodistribution study in mice. Differences in metabolic rates will affect the biodistribution in humans, altering the final dosimetry of the product. Consequently, the dosimetry results obtained here should be used only as a guide for the expected biodistribution in humans.

Conclusion

The DAHA-EDTA was readily and quantitatively radiolabelled with Cu-64 under mild conditions. The Cu-64-DAHA-EDTA complex conjugation to whole B72.3 antibody was optimised to produce a reasonably high specific activity [Cu-64-DAHA-EDTA]-B72.3 product. Unfortunately, radiolabelling of proteins requires a two-step process and more extensive purification before use. Its biodistribution in tumour bearing nude mice demonstrated unusually fast clearance from the blood and good localisation at the tumour site compared with similar products reported in the literature. The DAHA-EDTA proved to be an effective chelator for radiolabelling whole antibody and is worthy of further investigation for radiolabelling other biomarkers.

Experimental

Materials and Equipment

All materials used were of ACS reagent grade and used without further purification. Ethylenediaminetetraacetic dianhydride, 2-amino-4-nitrophenol, 10 % palladium charcoal, sodium borohydride, sodium azide, and sodium hydroxide were purchased from Sigma–Aldrich. EDTA, acetic anhydride, pyridine, diethyl ether, and acetone were from Ajax Chemical Company, acetonitrile and methanol purchased from Univar, and ethanol from BDH. HPLC grade acetonitrile was purchased from Merck. Sephadex G-25 (20–80 μm) for gel filtration and bovine serum albumin (BSA) were purchased from Aldrich.

All buffers were prepared using Millipore triply de-ionised water. Silica gel impregnated, instant thin-layer chromatography paper (ITLC-SG), and 0.22 μm filters were purchased from Gelman Scientific. Deuterated NMR solvents, D_2O, NaOD and d_6-DMSO, were purchased from Cambridge Isotope Laboratories. Elemental analyses were performed by the Microanalytical Service, The University of Queensland, Australia. ^1H NMR spectra were collected using a JEOL JNM-GX400 spectrometer and ^{13}C NMR spectra were collected using a Bruker Avance DPX 400 Spectrometer. Positive fast atom bombardment (FAB) mass spectra were determined on a JEOL DX-300 mass spectrometer (MS: parent ion (FAB): M/C, LH^+). Melting points are reported uncorrected. Diffuse reflectance FTIR spectra were recorded on a BioRad Digilab FTS-60 in 1 % KBr. A microplate reader was from Dynatech.

High specific activity ^{64}Cu (up to 6.5×10^{14} Bq g^{-1}) was obtained from the National Medical Cyclotron (NMC) as a by-product of the ^{67}Ga production process.

Radioactivity was measured in a CRC-15R Capintec or a Wallac Wizard 1470 gamma counter. Cu-64 in 0.1 N HCl with specific activity that ranged from 2.00 to 7.80×10^8 MBq g^{-1} was supplied by the National Medical Cyclotron, ANSTO.

B72.3 antibody (7 mg mL^{-1}) was purchased from Bioquest, Sydney and concentrated to 54.8 mg mL^{-1} using a Centricon 30 concentrator from Amicon. EDC, BSA, mucin, 5-amino salicylic acid, and Tween 20 were purchased from Sigma. Conjugated rabbit anti-mouse immunoglobulin (RAM-HP) was purchased from DAKO. A 20000 MCO concentrator from Sartorious, 20×20 cm^2 silica gel impregnated glass fibre sheets (Gelman Sciences), and a 0.22 mm filter from Millipore were used. A Waters HPLC was fitted with a 1000 μL sample loop (Rheodyne), a 60×21.2 mm Bio-Sep-Sec S3000 Guard (Phenomenex), and a 600×21.1 mm Bio-Sep-Sec S3000 Column (Phenomenex) was used for purification of the conjugates. The HPLC was fitted with two detectors, UV-vis (set to 280 nm) and a single channel radioactive monitor from EG & Ortec. Data acquisition was conducted using *Value Chrom* software from Bio-Rad Laboratory. A second HPLC (Waters) was used for quality control purposes. This was system fitted with a 50 μL sample loop (Rheodyne), a 75×7.8 mm Bio-Sep-Sec S3000 Guard (Phenomenex), a 300×7.8 mm Bio-Sep-Sec S3000 Column (Phenomenex), and a UV-vis monitor (280 nm). Data acquisition was conducted using *Millennium 245* series software. The outlet was connected to a fraction collector (Pharmacia). Protein concentrations were verified using a Bio-Rad Protein assay kit.

For metabolite studies the Sephadex-G-25 was prepared in the following manner. Sepadex-G-25 resin was swollen in 0.01 M PBS (which contained 0.05 % sodium azide) at pH 7.2 for at least for 3 h or overnight. The swollen Sephadex-G-25 resin was degassed by vacuuming and gentle shaking for 30 min. The resin was loaded into a glass tube; 55 L × 1.2 cm outer diameter fitted with a frits glass base, to a height of 48 cm and the top of the resin was covered with a thin layer of glass wool. The column was then stabilised by passing through 3×125 mL of 0.01 M PBS at pH 7.2 onto the column. Prior to its use the column was blocked by loading 1 mL of 10 % BSA followed by elution with 3×125 mL of 0.01 M PBS at pH 7.2.

Ligand Synthesis and Characterisation

DAHA-EDTA was synthesised in a manner similar to that described previously (see Fig. 1).[11,12]

N,N′-Bis(carboxymethyl)-N,N′-bis(2-hydroxy-5-nitroacetanilido)-1,2-diaminoethane (DNHA-EDTA)

To a solution of 2-amino-4-nitrophenol (1.74 g, 1.59×10^{-2} mol) in dry acetonitrile (100 mL) was added EDTA anhydride (2.0 g, 7.81×10^{-3} mol). The suspension was refluxed under nitrogen overnight. The warm reaction mixture was then filtered and the resultant bright yellow solid was washed with 70 %

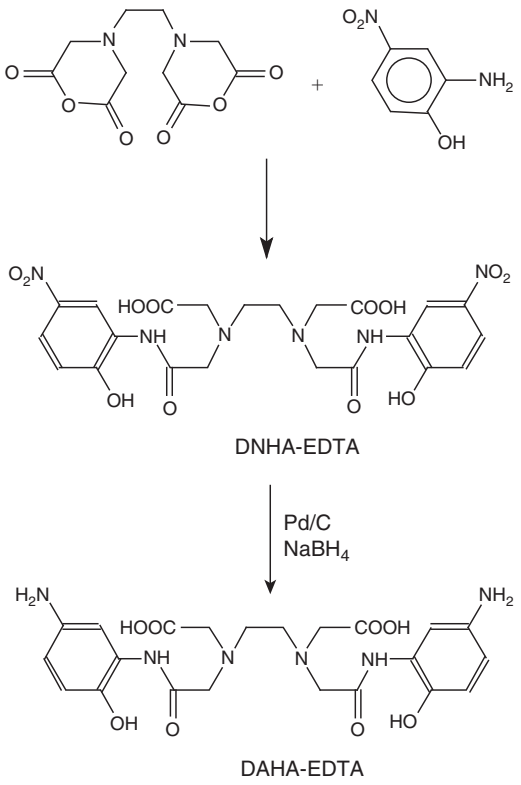

Fig. 1. Schematic for the synthesis of DAHA-EDTA.

ethanol followed by copious amounts of methanol or ethanol then acetone. Yield 3.4 g (81 %). *m/z* (FAB) 564 (MH^+). Mp 222–226°C. λ_{max}/cm^{-1} 1728.4 NHCO, 1693.9 COOH, 3299.3 OH. δ_H (d_6-DMSO) 9.88 (s, 2H, OH), 9.06, 7.89, 6.94 (m, 6H, Ar), 3.56, 3.52 (s, 4H, 4H, CH_2CONH, CH_2COOH), 2.83 (s, 4H, NCH_2CH_2). Anal. Calc. for $C_{22}H_{24}N_6O_{12}\cdot H_2O$: C 45.37, H 4.50, N 14.43. Found: C 45.42, H 4.39, N 14.08 %.

N,N′-*Bis(carboxymethyl)*-N,N′-*bis(2-hydroxy-5-aminoacetanilido)-1,2-diaminoethane (DAHA-EDTA)*

Method A: An aqueous solution of sodium borohydride (0.24 g in 2 mL H_2O) was slowly added to an aqueous, nitrogen-purged solution of Pd/C catalyst (50 mg, 1 mL). To this suspension was added DNHA-EDTA (1.0 g) in an ethanol/NaOH solution (10 mL/1 mL, 8 % NaOH). Care was taken to add the latter slowly as the reaction could be vigorous. The suspension was stirred at room temperature for 20 min, or until the solution became clear. Acid (conc. HCl) was added dropwise till excess sodium borohydride was quenched (i.e. effervescence ceased). Pd/C was removed by filtration and the volume of the filtrate reduced under vacuum to dryness. The resultant pale pink powder was dissolved in a minimum of methanol and insoluble salts were removed by filtration. The product was isolated after removal of methanol. Yield: 0.85 g (95 %). *m/z* (FAB) 509 (MH^+). λ_{max}/cm^{-1} 1740.1 NHCO, 1683.3 COOH, 3300 (br) OH. δ_H (d_6-DMSO) 9.33 (s, 2H, O*H*), 8.41–8.28 (m, 6H, Ar), 5.42, 5.35 (s, 4H, 4H, CH_2CONH, CH_2COOH), 3.80 (s, 4H, NCH_2CH_2). Anal. Calc for $C_{22}H_{28}N_6O_8Na_2\cdot NaCl\cdot 5H_2O$: C 34.88, H 5.05, N 11.09. Found: C 34.55, H, 4.86, N 10.57 %.

Method B: Sodium borohydride (0.16 g) was added to an aqueous, nitrogen purged solution of 10 % Pd/C (50 mg, 2 mL H_2O). A concentrated ammonia solution of DNHA-EDTA (10 mL, 28 % NH_3) in ethanol (8 mL) was added to the suspension. The reduction was continued at room temperature for 30 min under N_2 (g) or, until the solution became clear in colour. Concentrated hydrochloric acid was added dropwise to the solution until the evolution of gas ceased. The final pH of the solution was ~6.5. The palladium catalyst was removed by filtration and the filtrate reduced under vacuum. Water was azeotroped using copious amounts of ethanol/acetone. The purple/brown residue was suspended in a minimum volume of ethanol (20 mL) and insoluble salts were removed by filtration. Yield of the isolated product was 0.85 g (95 %). Mp 504°C. λ_{max}/cm^{-1} 1734.0 NHCO, 1684.0 COOH, 2998 (br) OH. δ_H (d_6-DMSO) 7.52 (s, 2H, OH). 6.90–6.81 (m, 4H, Ar), 3.92, 3.76 (s, 4H, 4H, CH_2CONH, CH_2COOH), 3.28 (s, 4H, NCH_2CH_2).

N,N′-*Bis(carboxymethyl)*-N,N′-*bis(2-hydroxyacetanilido)-1,2-diaminoethane (DHA-EDTA)*

The parent species DHA-EDTA of DAHA-EDTA was prepared and used to evaluate the serum stability of the Cu-64 radioabelled complex. To a solution of 2-aminophenol (1.74 g, 1.59×10^{-2} mol) in dry acetonitrile (100 mL) was added EDTA anhydride (2.0 g, 7.81×10^{-3} mol). The suspension was refluxed under nitrogen overnight. The warm reaction mixture was then filtered and the resultant yellow solid was washed with 70 % ethanol followed by copious amounts of methanol or ethanol and then acetone and dried under vacuum. The yield for the yellow powder was 3.52 g (95 %). *m/z* (FAB) 475 (MH^+). Mp 204–205°C. λ_{max}/cm^{-1} 1708.2 NHCO, 1635.8 COOH, 3314.1 OH. δ_H (d_6-DMSO) 8.02 (s, 2H, OH), 6.91–6.72 (m, 8H, Ar), 3.46, 3.38 (s, 4H, 4H, CH_2CONH, CH_2COOH), 2.86 (s, 4H, NCH_2CH_2). δ_C (D_2O/NaOD) 53.97, 59.49, 59.72 (CH_2) 117.89, 120.11, 125.31, 128.2 (CH, Ar), 125.2, 151.46 (Cq),174.57, 180.05 (C=O). Anal Calc. for $C_{22}H_{26}N_4O_8\cdot 3/2H_2O$: C 52.69, H 5.82, N 11.17. Found: C 52.84, H 5.59, N 11.26 %.

Radiolabelling DAHA-EDTA with Cu-64: (Cu-64-DAHA-EDTA)

Preliminary work in our laboratory investigated the optimum buffering conditions for the complexation of Cu-64 with DAHA-EDTA and the parent DHA-EDTA. Buffers ranging from pH 4–7 (0.1 M sodium acetate at pH 4, 5, and 6 and 0.1 M sodium phosphate at pH 6 and 7) were tested. Greater than 95 % of complexation of the Cu-64 was achieved for all buffer conditions investigated.[11]

An aliquot of Cu-64 in 0.1 N HCl solution was first evaporated to dryness and then re-suspended with an aliquot of Milli-water and an equivalent aliquot of 0.1 M phosphate buffer at pH 6. To a 2.8×10^{-8} mol Cu-64/Cu solution (109.9 µL, 394 MBq of Cu-64 in 1 to 1 of H_2O and 0.1 M phosphate at pH 6) was added a 3.4×10^{-8} mol DAHA-EDTA solution (3.8 µL of 20 mg mL^{-1} of DAHA-EDTA in 0.1 M phosphate buffer at pH 6). The mixture was vortexed and then incubated at ~37°C for 10 min. The percentage of complexation or radiolabelling of Cu-64 to DAHA-EDTA was determined by instant thin-layer chromatography (ITLC). The procedure involved depositing a 0.2–1 µL aliquot of the mixture on activated silica impregnated glass fibre strips (1 cm from the bottom). The strips were dried and then developed in Milli-Q water and a trace amount of NH_4OH (typically, 10 mL of Milli-Q water + 150 µL of 32 % of NH_4OH). Once the solvent reached 0.5 cm of the top of the strips, they were dried in air. The ITLC strips were

then cut into 10 portions and each portion was counted using a Wallac Wizard 1470 gamma counter. R_f 1.0 for Cu-64-DAHA-EDTA and 0.0 for Cu-64/Cu^{2+}. The percentage of radioactivity in each fraction was calculated. The radiochemical purity of the Cu-64-DAHA-EDTA was found to be >95 %.

Serum Stability of Cu-64-DAHA-EDTA

The stability of the Cu-64-DAHA-EDTA complex (or release of Cu-64 from the complex) in human serum cannot be determined due to the Cu-64-DAHA-EDTA complex naturally forming salt adducts (via peripheral aniline-nitrogen groups) with the protein. To overcome this problem the Cu-64 complex of the parent species, DHA-EDTA, was formed and assessed as a surrogate for Cu-64-DAHA-EDTA.

The Cu-64-DHA-EDTA was prepared in a similar manner to that described for Cu-64-DAHA-EDTA above. The Cu-64-DHA-EDTA complex (50 µL) was incubated in 2.5 mL of human serum (filtered at 0.2 µm) and incubated at 37°C. At set intervals (2, 4, 24, 48, and 72 h) 50 µL samples were removed and separated by size exclusion chromatography (Sephadex G-25, Sigma) using a gravity feed column (55 × 0.7 cm) pre-blocked with 1000 µL of 10 % BSA and equilibrated with 0.01 mM PBS at pH 7.2. The UV profile of the eluent was monitored at 280 nm (UV-1 monitor, Pharmacia) for serum proteins and 2 mL fractions of the eluent were collected to assess the amount of radioactivity using a Wallac Wizard 1470 gamma counter. The total activity collected was compared with 50 µL standards (in triplicate) to ensure that there was no activity left on the column. The activity associated with each fraction was measured and the percentage of total radioactivity loaded on the column calculated. The percentage of activity associated with the fractions containing serum proteins and as free complex were calculated to determine the amount of Cu-64 released from the complex.

Radiolabelling of B72.3 Antibody with Cu-64-DAHA-EDTA

Radiolabelling of the B72.3 antibody is based on the formation of an amide linkage between an activated endogenous carboxylic functional group (using EDC) on the antibody and amine substituents of DAHA-EDTA. A schematic of the conjugation reaction is given in Fig. 2.

The conjugation reaction was optimised for the concentration of protein and molar ratios of EDC and Cu-64-DAHA-EDTA. Conditions varied with protein concentration of 1 to 5 mg mL^{-1}, EDC-to-antibody molar ratios of 100–1000, and Cu-64-DAHA-EDTA-to-antibody ratios of 1–100. For example, an aliquot of B72.3 antibody (diluted 1 : 1 with 0.1 M phosphate buffer at pH 6) was added to a 500 mol ratio of 20 mg mL^{-1} of EDC in 0.1 M phosphate buffer at pH 6. To the mixture was added a 5–100 mol ratio of Cu-64-DAHA-EDTA (depending on the specific activity of Cu-64). If the Cu-64 was of specific activity higher than 2×10^8 MBq g^{-1}, Cu-64-DAHA-EDTA was added at a 5–10 molar ratio to antibody. When the specific activity of the Cu-64 was lower than 2×10^8 MBq g^{-1}, the molar ratio of Cu-64-DAHA-EDTA to antibody ranged from 20–100. In each case the reaction mixtures were incubated at 37°C for 1 h.

More specifically a typical conjugation reaction/labelling procedure is as follows: To 2.8×10^{-9} mol (415 µg, 15.2 µL) of B72.3 (27.4 mg mL^{-1}) in 0.1 M phosphate buffer at pH 6 was added 1.4×10^{-6} mol (13.3 µL) of EDC (20 mg mL^{-1} in 0.1 M phosphate buffer at pH 6). To this mixture was added 2.8×10^{-8} mol (114.7 µL) of Cu-64-DAHA-EDTA. The final concentration of antibody was then adjusted to 3 mg mL^{-1} using 0.1 M phosphate buffer at pH 6. The mixture was vortexed and then incubated at 37°C for 1 h. To quench the reaction, a 0.05 to 0.10 molar ratio of EDTA to Cu in 10 mM PBS at pH 7.2 was added. The mixture was then transferred to a 20000 MCO concentrator or ultra-filter, previously wetted with 100 µL of 0.2 M phosphate buffer at pH 8. A further 200 µL of 0.2 M phosphate buffer at pH 8 was added to the filter. The filter was then microcentrifuged for 15 min at 13148 *g*. The protein was washed a further 2–3 times with the same buffer until 85–90 % radiochemical purity of [Cu-64-DAHA-EDTA]-B72.3 was achieved. The [Cu-64-DAHA-EDTA]-B72.3 was then filtered using a 0.22 µm filter.

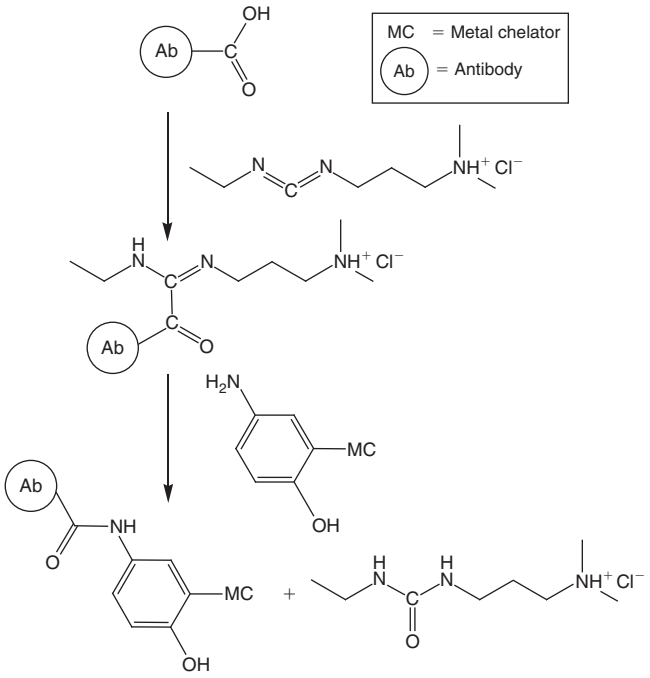

Fig. 2. Schematic of the conjugation of Cu-64-DAHA-EDTA to the antibody.

Final purification of [Cu-64-DAHA-EDTA]-B72.3 was achieved by size exclusion chromatography (using a 600 × 21.1 mm Bio-Sep-Sec S3000 Column) fitted to HPLC. The column was then eluted with 0.01 M PBS at pH 7.2 at a 3 mL min^{-1} flow rate. The retention time of [Cu-64-DAHA-EDTA]-B72.3 was found to be 40.3 min. The fractions containing the radioimmunoconjugate were pooled and concentrated using a 20000 MCO ultra filter. The final product recovered was then assessed for radiochemical purity, protein concentration, and immunoreactivity.

The radiochemical purity of the [Cu-64-DAHA-EDTA]-B72.3 product was determined using size exclusion chromatography (300 × 7.8 mm Bio-Sep-Sec S3000) fitted to HPLC. The column was then eluted with 0.01 M PBS at pH 7.2 at a 1 mL min^{-1} flow rate. Fractions of 0.5 mL were then retrieved. The fractions were then counted using a Wallac Wizard 1470 gamma counter. The percentage radiochemical purity was calculated as a ratio of radioactivity associated with the protein to the total radioactivity loaded on the column.

The specific activity of purified [Cu-64-DAHA-EDTA]-B72.3 was calculated as a ratio of radioactivity associated with radiolabelled antibody to concentration of protein present. The radioactivity was measured using a calibrated Capintec CRC-15R dose calibrator and protein concentration using a micro BioRad Protein

Assay on Immulon-1 96 well plates. The absorbance at 595 nm was measured on a Dynatech MR 7000 ELISA reader. The concentration of the sample was calculated from the standard curve of absorbance versus protein concentration.

The final product was stored at 4 and 37°C for up to 48 h and then analysed by size-exclusion HPLC. The [Cu-64-DAHA-EDTA]-B72.3 radiochemical purity was maintained (>95 %) at 24 h at both temperatures. However, at 48 h breakdown of the radiolablled conjugate was evident with up to 10 % loss of radioactivity from antibody.

Immunoreactivity of [Cu-64-DAHA-EDTA]-B72.3

The immunoreactivity of [Cu-64-DAHA-EDTA]-B72.3 was measured using an enzyme linked immunoabsorbance assay (ELISA) in a similar manner to that described previously.[3,10] Two plates were prepared at the same time. The first one was for standard antibody (unmodified antibody) and the second one was for test antibody (modified antibody, i.e. [Cu-64-DAHA-EDTA]-B72.3). Serial dilutions of mucin from 4 μg mL^{-1} to 2 ng mL^{-1} in 0.05 M PBS at pH 7.2 were added to the first four rows of a 2 Immulon 4, 96 well plate with 0.05 M PBS at pH 7.2 (100 μL). Glycine (i.e. control for non-specific binding) was plated in parallel to the second four rows of the plates. The plates were covered with cling film and incubated overnight at 4°C. Each well was then washed two times with 200 μL of 0.05 % Tween 20 in 0.05 M PBS pH 7.2. The wells were incubated with 200 μL of 0.05 % Tween 20 in 0.05 M PBS at pH 7.2 for at least 2 h at 37°C and then washed three times with 200 μL of 0.05 % Tween in 0.05 M PBS at pH 7.2. Antibodies (standard antibody and test antibody), 100 μL prepared at 4 μg mL^{-1} in 0.05 % Tween 20 in 0.05 M PBS at pH 7.2, were added to each well and then incubated for 1.5 h at 37°C. Each well was then washed three times with 200 μL of 0.05 % Tween 20 in 0.05 M PBS at pH 7.2. A 1/2000 dilution of the second antibody, peroxide-conjugated rabbit anti-mouse immunoglobulins (RAM-HP), in 0.05 % Tween 20 in 0.05 M PBS at pH 7.2, was added to each well. The plates were then incubated for 1 h at 37°C. The excess RAM-HP was removed and the well washed a further three times with 200 μL of 0.05 % Tween 20 in 0.05 M PBS at pH 7.2 and once with 200 μL of 0.05 M PBS at pH 6.8. 5-Amino salicylic acid (5-AS) was prepared by dissolving 100 mg in 100 mL of 10 mM PBS at pH 6.8 and adding 100 μL of 1% freshly prepared H_2O_2. An aliquot (100 μL) of the 5-AS was added to each well and allowed to react at room temperature before reading the absorbance at 410 nm. Specific binding of the antibody was determined by subtracting the absorbance for glycine wells from the total absorbance obtained for each well. A saturation curve, correlating absorbance with antigen concentration, was plotted. A comparison of saturation curve, generated for standard antibody and test antibody was used to assess the relative immunoreactive fractions.

Animal Studies

All animal studies were approved by the Animal Care and Ethic Committee (ACEC) ANSTO under protocol No. 99/143. There were 5 Nu/nu mice (nude mice) for each time point. The studied time points were 1, 2, 4, 6, 16, and 24 h. Five 2–3 week-old mice were implanted with 100 μL of 10×10^6 cell mL^{-1} cell suspension in 0.01 M PBS at pH 7.2. The implant was left to grow for 1 week. The mice were later sacrificed and tumour tissue was harvested, diced to 2×2 mm^2, and kept in chilled sterile media. The diced cells of tumour were then implanted into the hind flanks of mice aged 4 weeks. The tumours were then left to grow for 2 weeks to $\sim 7 \times 7$ mm^2 in size before use for animal studies.

Each mouse was injected with no more than 0.35 MBq or 35 μg of protein of [Cu-64-DAHA-EDTA]-B72.3. After injection with [Cu-64-DAHA-EDTA]-B72.3, at time the point required, the mice were anaesthetised by using CO_2, then sacrificed and dissected. The blood and selected organs were removed, weighed and then counted using a Wallac Wizard 1470 gamma counter. The percentage of injected dose for blood and selected organs as well as the percentage of injected dose per gram of blood of selected organs were calculated. Selected data for % ID g^{-1} are given in Table 2. Table 3 gives the calculated ratio of tumour to blood, liver, and kidney at the various time points.

Determination of Metabolites in Blood Plasma

Blood was withdrawn from each of the sacrificed mice into heparinised syringes and then transferred to a pre-weighed microcentrifuge tube. The blood samples were weighed and then counted using a Wallac Wizard 1470 gamma counter. The counted blood samples were centrifuged for 6 min at 6708 *g* on a microcentrifuge. The supernatant (plasma) from five mice at a certain time point was pooled into a pre-weighed microcentrifuge tube. The fractions were then counted using a Wallac Wizard 1470 gamma counter.

An aliquot of plasma (500 μL) was loaded into a BSA-pre-blocked and pre-equilibrated (0.01 M PBS at pH 7.2) Sephadex G-25 (48 × 1.2 cm) column. The column was then eluted with 0.01 M PBS at pH 7.2 with a 1.85 mL min^{-1} flow rate and the eluent monitored using a UV-1 Pharmacia Monitor at 280 nm. Fractions of 1.85 mL were collected and then counted using a Wallac Wizard 1470 gamma counter.

Dosimetry of [Cu-64-DAHA-EDTA]-B72.3

The dosimetry of [Cu-64-DAHA-EDTA]-B72.3 was determined assuming its biological distribution in mice is similar to humans. Calculations were conducted in a similar manner to that reported previously.[3,16] Briefly, using the biodistribution results obtained for [Cu-64-DAHA-EDTA]-B72.3 in the mice, the radioactivity measured for each organ or tissue was corrected for the natural decay of the radioisotope. The residence time (mCi h) of the radioactivity in each organ was determined by integrating the percentage injected dose curve for that organ (using *Restime* software developed by Eric Hetherington, ANSTO).[3] As no time points were monitored beyond 24 h the residence time of activity in each organ beyond 24 h was considered to be equivalent to the rate of decay of the radionuclide (termed extension activity). Both residence time activity and extension activity were added to determine the total residence time of the product in each organ. As the dose to each organ is a combination of the dose from the tissue of that organ as well as any blood that may be present within it, the total residence time was corrected for blood content in selected organs according to the method of Weber et al..[16] The total residence time was also corrected for activity that was distributed to the tail of the nu/nu mice by a proportional increase within every organ. The *MIRDOSE 3* program was used to determine the dose delivered to each organ in a 70 kg human adult as a consequence of the cumulated activity in those organs. The dose to the whole body was corrected for additional dose resulting from localisation of the product to the tumour mass. Data are summarised in Tables S2 and S3 (Supplementary Material).

Supplementary Material

The typical HPLC profile of crude and purified final [Cu-64-DAHA-EDTA]-B73.2 (Fig S1), complete set of biodistribution data (Table S1), and tumour-to-key organ uptake ratios and the dosimetry data (Tables S2 and S3) are available on the Journal's website.

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