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Bifunctional ^{64}Cu-labelled macrobicyclic cage amine isothiocyanates for immuno-positron emission tomography

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New macrobicyclic cage amine or "sarcophagine" (sar) bifunctional chelators have been synthesised that form copper complexes of exceptional *in vivo* stability and incorporate isothiocyanate (-NCS) functional groups for conjugation to an antibody. The chelators were synthesised from the methyl-capped complex $[Mg^{II}(CH_3)(NH_2)sar]^{2+}$. Coordination of Mg^{II} within the cavity of the cage amine ligand protects the secondary amine atoms from reacting with the -NCS functional groups. Two different $[Mg^{II}(NCS\text{-}sar)]^{2+}$ derivatives were conjugated to the HER2/neu-targeting antibody trastuzumab and the progress of the reaction monitored by electrospray mass spectrometry. The Mg^{II} ion was removed from the immunoconjugates under mild conditions (0.1 M citrate buffer, pH 6). Labelling of the (CH_3)(*p*-NCS-Ph)sar-trastuzumab conjugate with $^{64}Cu^{II}$, a radioisotope suitable for positron emission tomography (PET), was fast (~5 min) and easily performed at room temperature with high radiochemical purity (>95%). Biodistribution and PET imaging studies *in vivo* showed that ^{64}Cu-labelled (CH_3)(*p*-NCS-Ph)sar-trastuzumab maintained high stability under physiological conditions with high and selective uptake in a HER2-positive cancer cell line. The stability of the copper complex and the 12.7 h half-life of the radioisotope allows clear visualisation of tumours out to 48 h.

Introduction

A radioimmunoconjugate consists of a radionuclide tethered to an antibody or antibody fragment that can be used to deliver radioactivity selectively to target tissue for imaging and treatment of disease.[1] Metal radionuclides are most commonly incorporated into antibodies through a bifunctional chelator. Several monoclonal antibodies and engineered fragments are available that have the potential to be used for targeted imaging and therapy.[2, 3] Positron emission tomography (PET) is a molecular imaging modality with the sensitivity and spatial resolution to detect radiolabelled antibodies *in vivo* for quantitative assessment of physiological events. The positron-emitting isotope ^{64}Cu possesses attractive properties for PET imaging; the relatively low positron emission energy of 653 keV is similar to that of ^{18}F and results in high-quality images, and the half-life of 12.7 hours is suitable for delivery agents with biological residence times of several days. It also provides a potential diagnostic pair for the therapeutic, beta-emitting radionuclide ^{67}Cu.

Antibodies are usually conjugated to a chelator prior to radiolabelling to take full advantage of the half-life of the radioisotope and to minimise radiation exposure to hospital staff. Conjugation and radiolabelling at 37 ºC or below and near neutral pH is critical for maintaining the biological integrity of the antibody. The high molecular weight of monoclonal antibodies has a

profound effect on their biodistribution and metabolism and it can take days to reach optimal target-to-background ratios, therefore it is essential that ^{64}Cu complexes used for radioimmunoconjugates are extremely stable *in vivo*.

Recent research has focussed on developing bifunctional chelators that form thermodynamically stable and kinetically inert coordination complexes with ^{64}Cu for radiolabelling antibodies. The tetraazamacrocycles 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) have been shown to possess sufficient $^{64}Cu^{II}$ complex stability for immuno-PET imaging. However, recent studies on 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA), derivatives of 1,4,8,11-tetraazacyclotetradecane-1,8,-diacetic acid (TE2A), a cross-bridged cyclam based macrocycle with a methane phosphonate pendant group (CB-TE1A1P) and the macrobicyclic hexaamine "sarcophagine" (sar = 3,6,10,13,16,19-hexaaza-bicyclo[6.6.6]icosane) ligands demonstrate milder radiolabelling conditions and higher stability, leading to better quality imaging.[4-11]

Sarcophagine ligands are particularly well-suited for applications that utilise radioactive isotopes of copper as they form extremely stable and kinetically inert Cu^{II} complexes with fast complexation kinetics at room temperature, under dilute conditions, resulting in high specific activity.[12, 13] The bifunctional chelators *N*-[(4-aminophenyl)methyl]-3,6,10,13,16,19-hexaazabicyclo[6.6.6]-icosane-1,8-diamine (SarAr), incorporating an aromatic amine, and 5-(8-amino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]-icosan-1-ylamino-5-oxopentanoic acid (COSar), incorporating an aliphatic carboxylate have both compared favourably in comparisons of radiolabelling efficiency and biodistribution against bifunctional tetraazamacrocycles.[8, 9] SarAr was originally developed to improve the efficiency of the coupling reaction between 1,8-diamino-3,6,10,13,16,19-hexaazabicyclo[6.6.6]-icosane ($(NH_2)_2$sar) and antibodies, however the generation of activated carboxylate esters *in situ* required large excesses of the bifunctional chelator and coupling agent to achieve reasonable incorporation and to reduce competing side-reactions.[13, 14] Bifunctional chelators that incorporate reactive functional groups such as esters, isothiocyanates or maleimides, which react with lysine or cysteine residues of an antibody without the need for additional coupling reagents, are potentially easier to use and therefore more likely to find widespread use. Our earlier approach involved the synthesis of a methyl-capped sar with an aliphatic carboxylate *N*-hydroxysuccinimide ester, which was conjugated to a single-chain antibody for PET imaging of activated platelets.[15] A modified version of SarAr featuring a phenylisothiocyanate functional group (SarAr-NCS) has been used to introduce a cage amine ligand to the B72.3 antibody and to silica particles.[16] Isothiocyanates react with nucleophilic primary and secondary amines.[17, 18] The secondary amines of $(NH_2)_2$sar are nucleophilic and react with alkyl halides and aldehydes in preference to the primary amines.[19-21] When introducing a reactive functional group such as an isothiocyanate, it is important that the bifunctional chelator should react with the desired reactant to the exclusion of all other potential reactants.

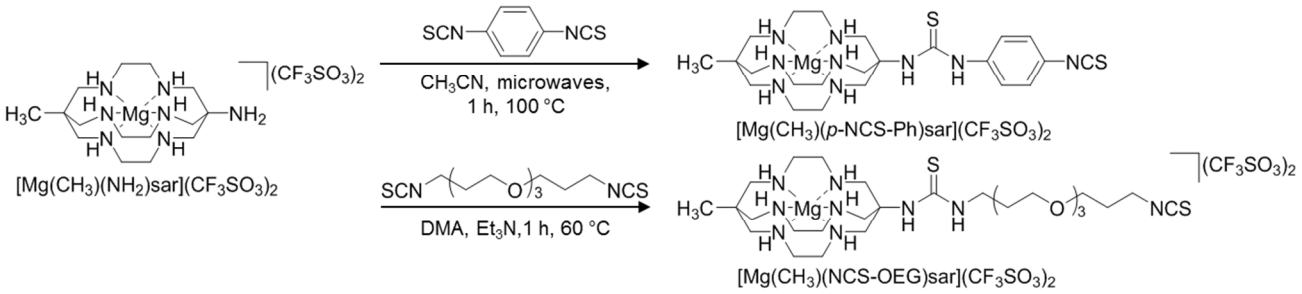
The basis of the present work was an effort to develop the synthesis of stable and versatile sar bifunctional chelators functionalised with an isothiocyanate group utilising the selective protection of the secondary amines by metal ion coordination for facile conjugation to antibodies, while avoiding the need for extreme conditions in order to remove the metal centre prior to radiolabelling with ^{64}Cu. In this work Mg^{II} coordination within the cavity of the cage ligand is used to "protect" the secondary amines and prepare derivatives with isothiocyanate functional groups. The bifunctional ligands have been used to conjugate to the monoclonal antibody trastuzumab, which is used clinically for the treatment of HER2 over-expressing metastatic breast cancer. We show that the magnesium ion can be removed under mild conditions after selectively protecting multiple N-donor sites, while efficient thiourea formation occurs at a nucleophilic ε-amine present on the antibody lysine. The trastuzumab-bifunctional chelator conjugate was radiolabelled with ^{64}Cu and the immuno-PET imaging potential was examined in HER2/neu positive tumour models.

Results

Synthesis

The magnesium complex $[Mg(CH_3)(NH_2)sar](CF_3SO_3)_2$ was prepared by the addition of magnesium trifluoromethanesulfonate to the free base $(CH_3)(NH_2)$sar. Isothiocyanate derivatives were prepared by reacting the symmetrical bis(isothiocyanates) 1,4-phenyl-bis(isothiocyanate) and 1,17-dithioxo-6,9,12-trioxa-2,16-diazaheptadeca-1,16-diene with the primary amine of $[Mg(CH_3)(NH_2)sar](CF_3SO_3)_2$. To obtain a single product containing an isothiocyanate group for conjugation, upwards of 7-fold excesses of the respective bis(isothiocyanates) were used. The complex $[Mg(CH_3)(p\text{-NCS-Ph})sar](CF_3SO_3)_2$ was synthesised in a microwave reactor at 100 ºC for 1 h (Scheme 1). The complex $[Mg(CH_3)(NCS\text{-OEG})sar](CF_3SO_3)_2$ was synthesised under basic conditions at 60 ºC for 1 h (Scheme 1). The reactions were monitored by electrospray ionisation mass spectrometry (ESI-MS) and upon completion diethyl ether was added resulting in precipitation of the product. Centrifugation followed by decanting of the solution successfully removed the remaining bis(isothiocyanate) reactants. The products were characterised by NMR, ESI-MS and elemental analysis.

Scheme 1: Synthesis of [Mg(CH$_3$)(*p*-NCS-Ph)sar](CF$_3$SO$_3$)$_2$ and [Mg(CH$_3$)(NCS-OEG)sar](CF$_3$SO$_3$)$_2$

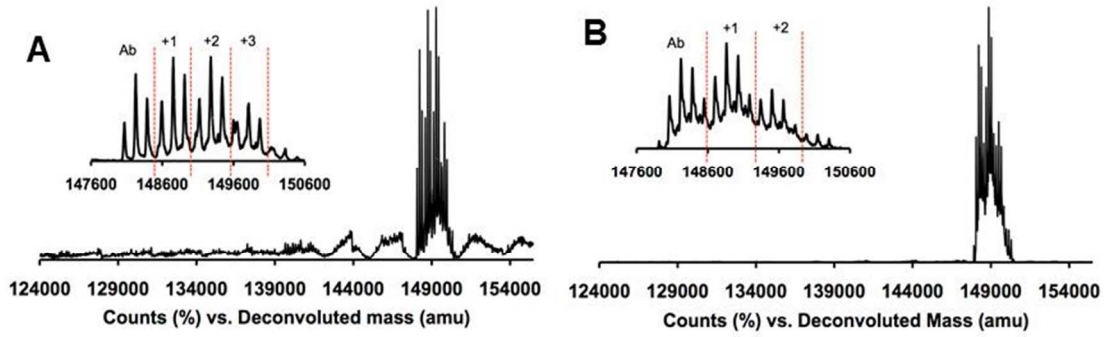


Conjugation to Trastuzumab and Extrusion of Mg^{II}

The isothiocyanate group of both [Mg(CH$_3$)(*p*-NCS-Ph)sar](CF$_3$SO$_3$)$_2$ or [Mg(CH$_3$)(NCS-OEG)sar](CF$_3$SO$_3$)$_2$ was allowed to react with the ε-amines of the lysine residues in trastuzumab to form stable thiourea bonds. A goal was to produce ligand-antibody conjugates with a maximum of five ligands bound to a single antibody as higher ratios can compromise antigen binding and effect biodistribution.[8, 22, 23] Maintaining the chelator to antibody ratio within a narrow range was achieved by careful control of the reaction duration as well as the concentrations and equivalents for each reactant.

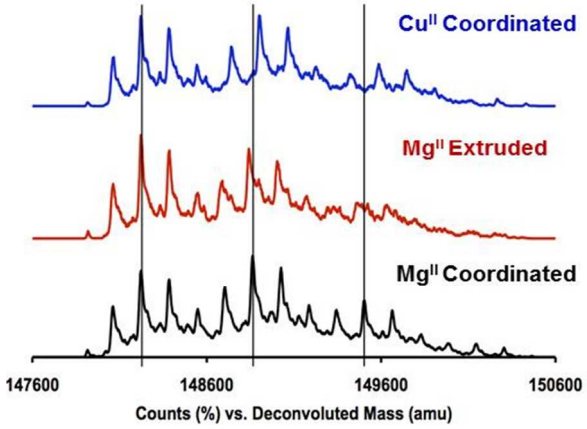
Trastuzumab was diluted in 0.05 M HEPES buffer (pH 8.6) prior to the addition of an aliquot of the bifunctional chelator dissolved in DMSO. All bioconjugation reactions were performed at room temperature. The reactions were monitored by liquid chromatography ESI-MS, a relatively simple and fast method that provides molecular weight data of all species present in the reaction mixture.[24] Signals associated with unreacted trastuzumab were present in all deconvoluted mass spectra of the reaction mixtures. The ESI mass spectrum of trastuzumab displays a signal with a mass-to-charge (m/z) value of 148228 Da and two additional signals of lower intensity (148070 Da and 148387 Da) assigned to the addition or subtraction of one hexose unit, respectively (observed change of ~162 Da per hexose). For the reaction of [Mg(CH$_3$)(*p*-NCS-Ph)sar]$^{2+}$ (529 g/mol) with trastuzumab, five equivalents were used and resulted in the conjugation of up to three complexes per antibody with the m/z distributions centred at 148754, 149281 and 149810 Da (Fig. 1A). The reaction of ten equivalents of [Mg(CH$_3$)(NCS-OEG)sar]$^{2+}$ (641 g/mol) with trastuzumab resulted in the conjugation of up to three complexes per antibody with the m/z distributions centred at 148864 Da, 149504 Da and 150144 Da (Fig.1B). A similar result was achieved with five equivalents of the isothiocyanate when the reaction was left for 12 h. At the end of the specified reaction time the reaction mixtures were diluted with PBS or HEPES buffer and the low molecular weight reactants removed by centrifugal filtration (50 kDa cut-off). The resulting concentrates were washed with buffer and then recovered by inverse filter centrifugation.

Figure 1: Liquid chromatography electrospray ionisation mass spectra of the reaction mixtures of trastuzumab and A) [Mg(CH$_3$)(*p*-NCS-Ph)sar]$^{2+}$-trastuzumab and B) [Mg(CH$_3$)(NCS-OEG)sar]$^{2+}$-trastuzumab



Mg^{II} extrusion from [Mg(CH$_3$)(*p*-NCS-Ph)sar]$^{2+}$ was evaluated in citric acid-citrate buffer at pH 3, 4, 5 and 6. After 5 h incubation the solutions were tested for the presence of the "free" ligand by ESI-MS. Significant loss of Mg^{2+} from the complex was observed at pH 6 and 5, however pH 6 was used, as it is closer to physiological pH. The Mg^{II} was extruded from the cage amine-trastuzumab immunoconjugateby exchanging the buffer to citric acid - citrate buffer (0.1 M; pH 6; 400 µL × 3) using centrifugal filtration. The extrusion of Mg^{II} from the conjugate was monitored by LC-ESI-MS (Fig. 2). Complete extrusion was indicated by a shift in the product signal (red trace) compared to the starting conjugate (black trace) of ~ 24 mass units per chelate. The addition of a solution of Cu(NO$_3$)$_2$ (0.1 mM, pH 7.4) to an aliquot of the product resulted in a shift of ~ 65 mass units per chelate (blue signal). The Mg^{II} free immunoconjugates were concentrated and buffer exchanged into 0.012 M PBS (pH ~ 7.6). For conjugations using approximately eight equivalents of [Mg(CH$_3$)(*p*-NCS-Ph)sar]$^{2+}$ that were allowed to react at pH 8.6 for 2 hours the average ratio of sarcophagine ligands per antibody was estimated to be 1.3±0.3 using a spectrophotometric assay that utilised a water soluble bis(thiosemicarbazone) ligand that forms a 1:1 chromophoric complex with Cu^{II}.

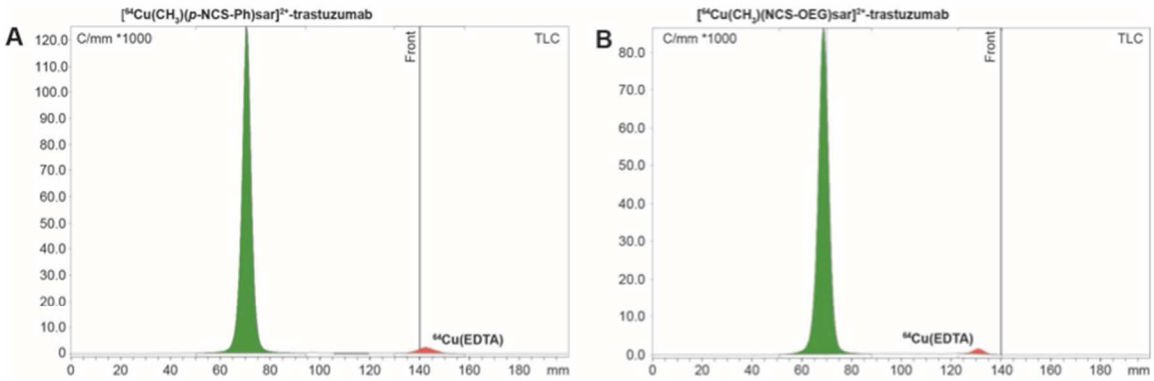
Figure 2: Liquid chromatography electrospray ionisation mass spectra of the reaction mixtures of $[Mg(CH_3)(NCS\text{-}OEG)sar]^{2+}$-trastuzumab (black trace), $(CH_3)(NCS\text{-}OEG)sar$ -trastuzumab (red trace) and $[Cu(CH_3)(NCS\text{-}OEG)sar]^{2+}$-trastuzumab (blue trace). Unreacted traztuzumab was present in all mixtures.



Radiolabelling with ^{64}Cu and Biodistribution in a HER2 Expressing Tumour Model

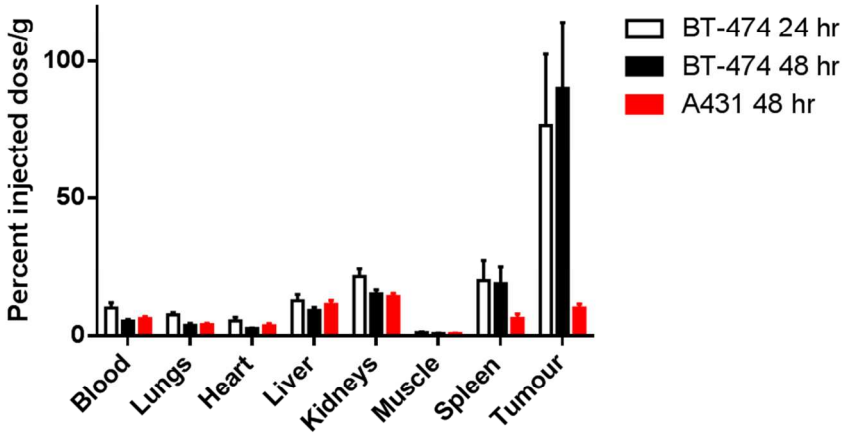
The immunoconjugates, typically 14 μg, were radiolabelled by incubating with $^{64}CuCl_2$ (80 MBq) in 0.01 M PBS buffer (pH 7.4) for 5 min. Radiochemical purity was >95% as measured by radio-TLC (Fig. 3).

Figure 3: Radio-TLC of A) ^{64}Cu-labelled (CH_3)(*p*-NCS-Ph)sar-trastuzumab (green) and ^{64}Cu(EDTA) (red) and B) ^{64}Cu-labelled (CH_3)(NCS-OEG)sar-trastuzumab (green) and ^{64}Cu(EDTA). The origin is located at 70 mm and the solvent front at 140 mm.



The *in vivo* biodistribution of ^{64}Cu-labelled (CH_3)(*p*-NCS-Ph)sar-trastuzumab was investigated in mice bearing BT-474 human breast carcinoma xenografts (high level of HER2 expression) or A431 epidermoid carcinoma (non-HER2 expression) as a negative control (Fig. 4).

Figure 4: *In vivo* biodistribution of ^{64}Cu-labelled (CH_3)(*p*-NCS-Ph)sar-trastuzumab in selected tissues from BT-474 (high level of HER2 expression) tumour-bearing NOD SCID IL2Rγ-null mice at 24 and 48 h post-injection (n = 3). A431 tumour-bearing Balb/c mice were used as a negative control at 48 h post-injection (n = 3).



Effective blood clearance of the tracer was demonstrated in both tumour models. In the BT-474 model, activity remaining in the blood was 7.5 ± 0.8 %ID g^{-1} at 24 h, which reduced to 5.0 ± 0.4 %ID g^{-1} at 48 h. In the A431 model activity in the blood was 6.1 ± 0.4 %ID g^{-1} at 48 h. Uptake in the liver in the BT-474 model was 9.3 ± 1.0 %ID g^{-1} at 24 h, with no significant difference at 48 h (8.6 ± 0.5 %ID g^{-1}) or with the A431 model at 48 h (11.0 ± 0.8 %ID g^{-1}). Uptake of the tracer in the kidneys in the BT-474 model was 15.8 ± 1.0 %ID g^{-1} and 14.1 ± 0.9 %ID g^{-1} at 24 h and 48 h, respectively.

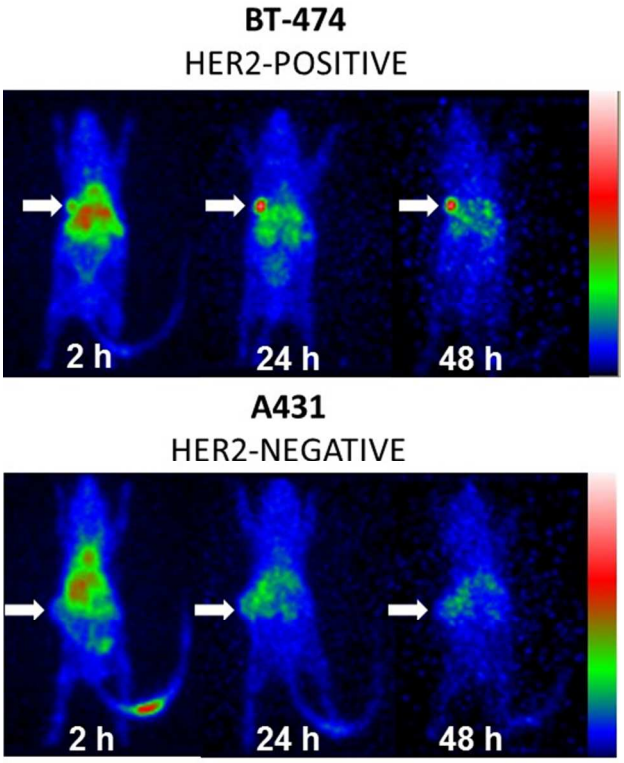
Uptake in the HER2-positive BT-474 tumours was high at 24 h (56.1 ± 9.5 %ID g^{-1}) and was retained at 48 h (82.3 ± 12.1 %ID g^{-1}). Tumour-to-kidneys ratios for the BT-474 tumours at 24 h and 48 h were 3.5 ± 0.5 and 5.9 ± 0.9, respectively and the tumour-to-liver ratios were 5.9 ± 0.8 and 9.5 ± 0.9, respectively ($p < 0.05$).

The tumour uptake of the HER2-negative A431 tumours (9.7 ± 0.8 %ID g^{-1}, $p < 0.01$) was significantly less than the BT-474 tumours at 48 h, demonstrating the receptor-mediated uptake of the tracer. The tumour-to-kidneys and tumour-to-liver ratios in A431 tumours at 48 h were 0.7 ± 0.04 ($p < 0.01$) and 0.9 ± 0.03 ($p < 0.001$), respectively, significantly less than the HER2-positive tumours.

PET imaging

Small animal PET images of BT-474 tumour bearing NOD SCID IL2Rγ-null mice and A431 tumour bearing Balb/c nude mice at 2 h, 24 h and 48 h post intravenous administration of ^{64}Cu-labelled (CH_3)(*p*-NCS-Ph)sar-trastuzumab are presented in Fig. 5. Tumour uptake was quantified as the maximum standardised uptake value (SUV_{max}) over a volume of interest (VOI) and represents the mean of three tumour bearing animals. The tumour is clearly visible at 2 h post-injection of ^{64}Cu-labelled (CH_3)(*p*-NCS-Ph)sar-trastuzumab in the BT-474 tumour-bearing mice with a maximum standardised uptake value (SUV_{max}) of 2.19 ± 0.08. The SUV_{max} increased significantly to 6.35 ± 0.72 ($p < 0.01$) at 24 h and was 5.99 ± 0.32 ($p < 0.001$) at 48 h. By contrast, uptake in the A431 tumours was significantly less with SUV_{max} of 1.09 ± 0.14 ($p < 0.01$) and 1.84 ± 0.14 ($p < 0.01$) at 2 h and 24 h, respectively, compared to uptake in the BT-474 tumours at the same time points, thus demonstrating the specificity of the tracer.

Figure 5: Small animal PET maximum intensity projection images of HER-2 positive BT-474 tumour bearing NOD SCID IL2Rγ-null mice and non-HER2 expressing A431 tumour bearing Balb/c nude mice at 2 h, 24 h and 48 h post-intravenous administration of ^{64}Cu-labelled (CH_3)(*p*-NCS-Ph)sar-trastuzumab. Arrows indicate tumours. The colour scale for all PET image data shows radiotracer uptake with white and blue corresponding to the highest and lowest activity, respectively.



Discussion

Accurate early screening of monoclonal antibodies in preclinical studies may be assisted by the development of ^{64}Cu-labelled molecular targeting probes. The intermediate half-life of ^{64}Cu enables imaging 48 h post-injection while also limiting the radiation exposure of the patient compared to longer lived radionuclides such as ^{89}Zr, which has also been used to radiolabel trastuzumab.[25] Trastuzumab is a humanised monoclonal antibody selective for human epidermal growth factor receptor 2 (HER2) and has regulatory approval in many countries as a treatment for patients with HER2 over-expressing metastatic breast cancer. The development of PET imaging agents incorporating the antibody trastuzumab has the potential to enable non-invasive detection and accurate evaluation of not only primary but also metastatic HER2-positive breast cancer, thus improving the selection of patients suitable for HER2 targeted therapies and the treatment efficacy.[26] In recent studies of HER2-positive breast cancer patients, both primary tumours and metastases rapidly accumulated ^{64}CuDOTA-trastuzumab enabling visual detection and measurement of uptake after 24 h and out to 48 h.[27, 28] Conjugates incorporating DOTA have demonstrated ^{64}Cu dissociation and subsequent accumulation in the liver and require ^{64}Cu-labelling conditions of ≥40 ºC for 45–60 min.[27-30]

These results suggest that imaging using alternative chelators with improved stability *in vivo* and more favourable radiolabelling conditions could lead to a system that obtains maximum benefit of the 12.7 h half-life of ^{64}Cu. Our aim was therefore to develop a sar bifunctional chelator that can be conjugated to an antibody without compromising the stability of the Cu^{II} chelate or the biological activity of the antibody. In addition, a useful bifunctional chelator must be stable over long periods, resistant to cross-linking but also sufficiently reactive to allow simple and fast conjugation to the antibody. The selective functionalisation of the primary amine groups of cage ligands 1,8-$(NH_2)_2$sar and 1-(CH_3)-8-(NH_2)sar have been achieved by utilising metal coordination to protect the nucleophilic secondary amines.[19, 31-33] The coordination of divalent metal ions within the cavity of the cage amine results in metal complexes where the primary amine is more reactive compared to the analogous Co^{III} complexes, the templating ion required for the synthesis of the ligands. The synthesis of $[Mg(CH_3)(NH_2)sar]^{2+}$ requires neutralisation of the amine donors before complexation of the Mg^{II} ion. Analysis by single crystal X-ray crystal crystallography shows minimal conformational change of the ligand upon complexation of Mg^{II} and the diamagnetic Mg^{II} complexes can be characterised by NMR spectroscopy.[34, 35] Theoretical calculations suggest that the size of the cavity within the parent sarcophagine is sufficient to accommodate Mg^{II}.[36]

Bifunctional chelators incorporating isothiocyanates can be generated by reacting the primary amines of ligands with symmetrical bis(isothiocyanates).[37] The synthesis of $[Mg(CH_3)(NCS\text{-}OEG)sar](CF_3SO_3)_2$ was achieved at ambient pressure and 60 ºC but $[Mg(CH_3)(p\text{-}NCS\text{-}Ph)sar](CF_3SO_3)_2$ synthesis required microwave conditions to complete the reaction.

Coupling of $[Mg(CH_3)(NCS\text{-}OEG)sar](CF_3SO_3)_2$ or $[Mg(CH_3)(p\text{-}NCS\text{-}Ph)sar](CF_3SO_3)_2$ to trastuzumab was very efficient and reproducible giving conjugates with up to three chelators per antibody. The phenyl isothiocyanate of $[Mg(CH_3)(p\text{-}NCS\text{-}Ph)sar](CF_3SO_3)_2$ was more reactive as only a five-fold molar excess was required compared to a ten-fold excess of $[Mg(CH_3)(NCS\text{-}OEG)sar](CF_3SO_3)_2$ to achieve conjugates with similar ligand to antibody ratios.

The extrusion of Mg^{II} from cage amine ligands can be achieved under relatively mild conditions especially when compared to the forcing and reducing conditions required for removal of either Co^{III} or Cu^{II} from cage amine complexes. For example, Mg^{II} was used to protect the secondary amine sites of $(NH_2)_2$sar and $(CH_3)(NH_2)$sar, directing reductive alkylation reactions to the primary amines.[21, 32] This approach was used to alkylate the cage amine complex with long-chain hydrocarbons to prepare metallosurfactants where the Mg^{II} ion was removed by heating in concentrated HCl solution.[32] The use of highly acidic conditions is not suitable for the present work as it would denature the antibody and lead to significant loss of biological activity. The extrusion of divalent metal ions from the cage amine ligand is dependent on both pH and the competing ligand.[34, 38, 39] The conditions developed here rely on the use of citrate as both a pH buffer and a competing ligand. Citric acid is a tricarboxylic acid polydentate metal chelator and polyprotic buffer.[40, 41] Solutions of citric acid and citrate are buffered over the pH range 3.0 to 6.2.[42] The Mg^{II} was successfully extruded from the cage amine ligand with citric acid – citrate buffer at pH 6 and monitored with LC-ESI-MS. The citric acid - citrate buffer affinity for the divalent magnesium ion is highest at pH ~ 6, where the tri-anionic citrate form ($C_6H_5O_7^{3-}$) predominates.[43-45]

The radiolabelling and *in vivo* studies focussed on the immunoconjugate $(CH_3)(p\text{-}NCS\text{-}Ph)sar$-trastuzumab due to the greater reactivity of $[Mg(CH_3)(p\text{-}NCS\text{-}Ph)sar](CF_3SO_3)_2$ and the commercial availability of the starting material, 1,4-phenylene bis(isothiocyanate). The new immunoconjugate was radiolabelled with ^{64}Cu at room temperature in ~5 min in >95% radiochemical purity.

The selectivity of ^{64}Cu-labelled $(CH_3)(p\text{-}NCS\text{-}Ph)sar$-trastuzumab was confirmed with *in vivo* biodistribution and PET imaging of BT-474 (high level of HER2 expression) and A431 (non-HER2 expressing) tumour-bearing mice. The tracer exhibited high tumour uptake and retention in the BT-474 tumours that was significantly higher than uptake in the A431 tumours. Importantly, clearance from non-target organs indicated the stability of the sar complex as dissociated ^{64}Cu is thought to bind to copper binding proteins in the liver and kidneys.[28]

In conclusion, the incorporation of an isothiocyanate functional group to a methyl-capped macrobicyclic cage amine ligand produced promising new bifunctional chelators for PET imaging using ^{64}Cu-labelled radioimmunoconjugates. The Mg^{II} ion "protected" the secondary amines for the synthesis of stable isothiocyanate bifunctional chelators but was easily removed under mild conditions. The new bifunctional chelators exhibited facile conjugation and fast radiolabelling at room temperature and were stable *in vivo*. This is the first report of tumour imaging with a sar chelator conjugated to the monoclonal antibody trastuzumab and demonstrated clear tumour uptake and low background noise in a HER2-positive animal tumour model. The chemistry developed here could readily be adapted to other antibodies and is equally applicable to the beta-emitting ^{67}Cu isotope that has potential to be used for radioimmunotherapy. Thus, the $^{64/67}Cu$-labelled sar-trastuzumab immunoconjugates provide a potential example of the 'theranostic' approach that has proven successful in a range of other cancers.

Experimental

General information

Herceptin (trastuzumab) was purchased from Genentech (San Francisco, CA), 2-[4-(2-hydroxyethyl)piperzin-1-yl] ethanesulfonic acid (HEPES) from Ajax Finechem Pty. Ltd. (Scoresby, VIC) and 1,4-phenylene bis(isothiocyanate) were purchased from Sigma (St Louis, MO). All solvents and reagents were purchased from standard commercial suppliers and were used as received. $^{64}CuCl_2$ solution (0.02 M HCl, no carrier added) was produced at Sir Charles Gairdner Hospital (Nedlands, Western Australia) through the $^{64}Ni(p,n)^{64}Cu$ nuclear reaction. Nuclear magnetic resonance (NMR) spectra were acquired on a Varian FT-NMR 500 spectrometer. ^1H and $^{13}C[^1H]$ chemical shifts were referenced to the residual solvent peak, except when $^{13}C[^1H]$ chemical shifts were referenced to acetone in D_2O (δ = 30.89 and 215.94 ppm)[46]. ESI-MS spectra were recorded on an Agilent 6510 esi-TOF LC/MS mass spectrometer (Agilent, Palo Alto, CA). Microanalyses for C, H, and N were carried out by Chemical & Microanalytical Services (CMAS) Pty. Ltd. Belmont, Vic. Radio-TLC analyses were performed using a Raytest Rita-Star TLC scanner using Merck TLC silica gel 60F254 plates (Merck Millipore, Billerica, MA). Briefly, a 2 μL aliquot was removed and added to 10 μL of phosphate buffer (0.1 M, pH 7.4) containing sodium EDTA (10 mM). The solution was spotted onto an ITLC strip and developed with phosphate buffer (0.1 M, pH 7.4) containing sodium EDTA (10 mM). Microwave synthesis was conducted using Biotage Microwave vials (2-5 mL) in a Biotage Initiator 2.0 microwave synthesiser. 1,17-dithioxo-6,9,12-trioxa-2,16-diazaheptadeca-1,16-diene was synthesised using a literature method.[47]

Synthetic procedures

$[Mg(CH_3)(p\text{-}NCS\text{-}Ph)sar](CF_3SO_3)_2$.

A suspension containing $[Mg(CH_3)(NH_2)sar](CF_3SO_3)_2$ (0.05 g, 0.08 mmol) and 1,4-phenylene bis(isothiocyanate) (0.15 g, 0.79 mmol) in acetonitrile (4 mL) was irradiated in a microwave reactor (100 °C, for 1 h). After cooling the supernatant was decanted from a crystalline solid. The supernatant was evaporated to dryness under a flow of nitrogen to give a colourless residue, which was washed with ether (40 mL × 3), followed by acetonitrile (4 mL and 1.5 mL) using a process of centrifugation and decantation. The remaining residue was dried *in vacuo* (8 mg). A second crop was obtained by evaporating the acetonitrile supernatant to

dryness under a flow of nitrogen to give a colourless solid. The solid was washed with water (1 mL × 3) using a process of centrifugation and decantation and dried *in vacuo* (22 mg). The two crops were confirmed by NMR spectroscopy to be the same compound (total yield 30 mg 45 %). (Found: C, 36.21; H, 4.95; N, 15.16; calc. for $C_{25}H_{39}F_6MgN_9O_6S_4$: C, 36.26; H, 4.75; N, 15.22). ^{1}H NMR (500 MHz; DMSO-d_6): δ (ppm) 9.60, s, 1H, SCN-C_6H_4-*H*N-C=S; 7.51-7.56, m, AA'BB', 2H, Ar; 7.47-7.51, m, 1H, cage-*H*N-C=S; 7.34-7.41; m, AA'BB', 2H, Ar; 3.83-3.98, m, 3H, NH; 3.43-3.64, m, 6H, cage CH_2; 3.09-3.24, m, 3H, NH; 3.00-2.81, m, 9H, CH_2; 2.35-2.53, m, CH_2; 0.59, s, 3H, CH_3. ^{13}C NMR (125.7 MHz; DMSO-d_6): δ (ppm) 179.4; 138.9; 132.8, NCS; 126.1; 125.2; 123.8; 120.7, q, J_{CF} = 314 Hz, $CF_3SO_3^-$; 57.0, cage CH_2; 55.6, quat. C of amine cage; 54.8, cage CH_2; 48.3, $N(CH_2)_2N$; 35.0, quat. C of methyl cap; 24.2, CH_3. ESI$^+$ (m/z) found 264.63; calc. for $[C_{23}H_{39}MgN_9S_2]^{2+}$ 264.63.

[Mg(CH$_3$)(NCS-OEG)sar](CF$_3$SO$_3$)$_2$. To a stirring solution of 1,17-dithioxo-6,9,12-trioxa-2,16-diazaheptadeca-1,16-diene (0.16 g, 0.53 mmol) and triethylamine (2 drops) in N,N-dimethylacetamide (0.5 mL) at 60 °C was added [Mg(CH$_3$)(NH$_2$)sar](CF$_3$SO$_3$)$_2$ (0.05 g, 0.08 mmol) portion wise over 1 h. The resulting mixture was stirred at 60 °C for 1 h. After cooling, diethyl ether was added and the resulting colourless precipitate was collected by filtration, washed with diethyl ether and dried under vacuum, (29 mg, 39%. (Recrystallised from CD_3CN, found: C, 36.91; H, 5.79; N, 13.27; calc. for $C_{29}H_{55}F_6MgN_9O_9S_4 \cdot 0.5H_2O$: C, 36.69; H, 5.95; N, 13.28). ^{1}H NMR (500 MHz; CD_3CN): δ (ppm) 6.65-6.78, m, 1H, HNC=S; 5.97-6.16, m, 1H, HNC=S; 3.35-3.80, m, 17H, CH_2; 2.90-3.28, m, 18H, CH_2; 2.38-2.60, m, 9H, CH_2; 1.86-1.93, m, 2H, $OCH_2CH_2CH_2N$; 1.69-1.78, m, 2H, $OCH_2CH_2CH_2N$; 0.64, s, 3H, CH_3. ^{13}C NMR (125.7 MHz, CD_3CN): δ (ppm) 182.7, $(NH)_2CS$; 129.5, NCS; 121.9, q, J_{CF} = 314 Hz, $CF_3SO_3^-$; 71.1; 71.0; 76.6; 69.6; 68.0, OEG CH_2; 58.5, $H_3CC(CH_2)_3$; 56.7, $NC(CH_2)_3$; 56.4, $NC(CH_2)_3$; 49.9, 49.8, $HN(CH_2)_2NH$; 43.1, CH_2NCS; 42.6, $CH_2NHC=S$; 36.3, CCH_3; 30.5, CH_2CH_2NCS; 29.4, $CH_2CH_2NHC=S$; 24.3, CH_3. ESI$^+$ (m/z) found 790.32; 754.35; calc. for $[M+CF_3SO_3]^+$ 790.32; $[M+CF_3CO_2]^+$ 754.35.

Trastuzumab Antibody Modification/Thiourea Bioconjugation

The initial concentration of trastuzumab was determined by UV/vis spectroscopy. Briefly, aliquots of trastuzumab (25 µL, 20 µL and 5 µL) were added to MOPS buffer (50 mM; pH 7.1; 3000 µL), and the concentration was determined from the absorbance (ε_{280} = 1.42 mg^{-1} mL cm^{-1}) to be 25 mg mL^{-1} after volume corrections.[48]

To a solution of trastuzumab (4 – 5 mg mL^{-1}, 98.5 µL) in 0.05 M 2-[4-(2-hydroxyethyl)piperzin-1-yl] ethanesulfonic acid buffer (HEPES, pH 8.6) was added a solution of 10 equiv. of [Mg(CH$_3$)(NCS-OEG)sar](CF$_3$SO$_3$)$_2$ (1.7 µL) or 5 equiv. of [Mg(CH$_3$)(*p*-NCS-Ph)sar](CF$_3$SO$_3$)$_2$ (1.3 µL) in DMSO. The reaction was left to stand at room temperature and was monitored by LC-ESI-MS. After 2 h for [Mg(CH$_3$)(*p*-NCS-Ph)sar](CF$_3$SO$_3$)$_2$ or 12 h for [Mg(CH$_3$)(NCS-OEG)sar](CF$_3$SO$_3$)$_2$ the reaction mixture was diluted with phosphate buffered saline (PBS, 300-400 µL, pH 7.6) and the resultant antibody conjugate was purified using centrifugal filter units with a 50 kDa molecular weight cutoff (Amicon ultra, 500 µL, Merck Millipore, Billerica, MA). To the recovered antibody conjugate was added 0.1 M citric acid-citrate buffer (400 µL × 3, pH 6), followed by centrifugation (7000 rpm, 15 min), and the mixture was left to stand at room temperature for 1 h after the final centrifugation, diluted in 0.1 M citric acid-citrate buffer (400 µL, pH 6). This was followed by centrifugation (7000 rpm, 15 min) and centrifugal filtration using 0.1 M citric acid-citrate buffer (400 µL x 3, pH 6). The final modified antibody was purified by centrifugal filtration using PBS (400 µL x 3, pH 7.4) and stored at 4 ºC. The extrusion of Mg^{II} from the conjugate was analysed by LC-ESI-MS.

Trastuzumab Conjugate LC-ESI-MS Sample Preparation

Aliquots of the samples for analysis were removed and diluted to a final antibody concentration of 0.1 mg mL^{-1} in 20% acetonitrile, 0.1% trifluoroacetic acid (TFA) and injected onto an Agilent Poroshell 300SB-C18 column (2.1 x 75 mm, 5 µm) connected to a Agilent 6510 esi-TOF LC/MS mass spectrometer (Agilent, Palo Alto, CA). LC-ESI-MS utilised a 0.5 mL min^{-1} flow rate, gradient elution of buffer A = 0.1% aqueous formic acid and buffer B = acetonitrile/0.1% aqueous formic acid (20 to 90% B in A at 9 min).

Procedure for ^{nat}Cu-labelling of (CH$_3$)(NCS-OEG)sar-trastuzumab. A solution of Cu(NO$_3$)$_2$ (0.1 mM, 1 µL) was added to (CH$_3$)(NCS-OEG)sar-trastuzumab in 0.012 M PBS (8 µL pH 7.4). The reaction mixture was incubated at room temperature overnight and monitored by LC-ESI-MS.

Spectroscopic determination of ligand to antibody ratio

A method for the determination of ligand to antibody ratio was developed using a water-soluble bis(thiosemicarbazone) Na[H$_2$L] (see supporting information for the chemical structure of Na[H$_2$L]) that forms a chromophoric 1:1 complex with Cu^{II} (ε_{460} = 1.1(0) × 10^{-4} M^{-1} cm^{-1}).[49, 50] A solution of Na[CuL] was prepared in 50 mM MOPS buffer (930 µL, pH 7.1) by adding an aliquot of 1 mM Cu(NO$_3$)$_2$ (40 µL) into a 1.8 mM solution of Na[H$_2$L] (30 µL) (solution 1). The absorbance of the Na[CuL] solution was measured from 200 to 700 nm. An additional three solutions (solutions 2-4) were prepared containing 1 mM Cu(NO$_3$)$_2$ (40 µL), MOPS buffer (pH 7.1, 924 µL; 921 µL and 918 µL, respectively), and (CH$_3$)(*p*-NCS-Ph)sar-trastuzumab (22 mg mL^{-1}; 6 µL, 9 µL and 12 µL, respectively). To each of these solutions was added an aliquot of 1.8 mM Na[H$_2$L] (30 µL) and the resulting solutions were left overnight before the absorbance was measured from 200 to 700 nm. Using the equation $\Delta A_{460\ nm} = A_{460\ nm\ (solution\ 1)} - A_{460\ nm\ (solution\ 2\text{-}4)}$, the concentration of (CH$_3$)(*p*-NCS-Ph)sar was determined by [(CH$_3$)(*p*-NCS-Ph)sar] = $(\Delta A_{460\ nm})/(1.1 \times 10^{-4}\ M^{-1})$. The ligand to antibody ratio [(CH$_3$)(*p*-NCS-Ph)sar]/[(CH$_3$)(*p*-NCS-Ph)sar-trastuzumab] was measured to be 1.3 ± 0.3.

^{64}Cu labelling of (CH$_3$)(*p*-NCS-Ph)sar-trastuzumab

^{64}Cu (~80 MBq) in 0.01 M PBS (pH 7.4, 160 µL) was added to ~14 µg of (CH$_3$)(*p*-NCS-Ph)sar-trastuzumab in PBS (pH 7.4, 10 µL). The reaction mixture was incubated at room temperature for 5 min and then the radiochemical purity was determined by radio-TLC (> 95%).

***In vivo* biodistribution**

All *in vivo* experiments were performed with institutional animal ethics committee approval and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th Edn. NOD SCID IL2Rγ-null mice were inoculated subcutaneously on the right flank with BT-474 cells in 50% Matrigel. As a negative control Balb/c nude mice were given xenografts of non-HER2-expressing A431 cells in 50% Matrigel. When tumours reached a volume of approximately 100 mm^3, the mice were injected intravenously via a tail vein with ^{64}Cu-labelled (CH_3)(*p*-NCS-Ph)sar-trastuzumab (4-10 MBq). At 24 or 48 h post-injection the mice (n = 3) were euthanised and selected tissues were taken, weighed and counted on a γ counter (187-950-A100 MCA, Biomedex Medical Systems).

Small animal PET imaging

BT-474 and A431 tumour bearing mice as described above were injected intravenously via a tail vein with ^{64}Cu-labelled (CH_3)(*p*-NCS-Ph)sar-trastuzumab (8-10 MBq). At 2, 24 and 48 h post-injection the animals were anaesthetised in 2.5% isoflurane and 50% O_2 in air and placed on the bed of a Philips Mosaic small animal PET scanner and imaged over 10 min. The images were reconstructed using a 3D RAMLA algorithm as described previously.[51] Quantification was performed using software developed in-house (MARVn).[52] All data are presented as mean ± standard error, n = 3. Statistical significance was determined with two-tailed unpaired t-tests using GraphPad Software Online and GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA), with $p < 0.05$ considered statistically significant.

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Electronic Supplementary Information (ESI) available: [^{1}H NMR spectra of $[Mg(CH_3)$(*p*-NCS-Ph)sar$](CF_3SO_3)_2$ and$[Mg(CH_3)$(NCS-OEG)sar$](CF_3SO_3)_2$, the chemical structure of $Na[H_2L]$ and selected UV-vis absorbance spectra for the spectroscopic determination of ligand to antibody ratio]. See DOI: 10.1039/b000000x/

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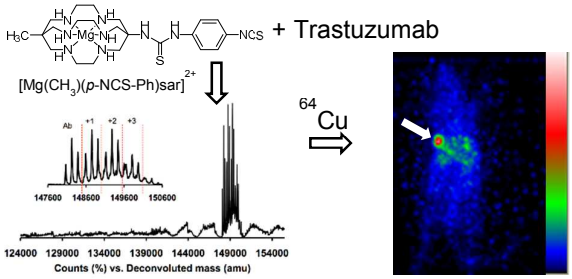
Table of Contents Entry



Sarcophagine ligands with isothiocyanate functional groups were conjugated to trastuzumab and radiolabelled with copper-64 and the conjugate was used to image HER2-positive tumours using positron emission tomography.