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Implementation of macrocycle conjugated antibodies for tumour-targetting

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Abstract - Monoclonal antibodies may be selectively functionalised with macrocyclic ligands, permitting the transport of an imaging or cytotoxic metal radionuclide specifically to a tumour. Antibodies conjugated to [13] and [14]-membered tetraazamacrocycles allow ^{64}Cu or ^{67}Cu labelled antibody to be used for imaging or therapy.

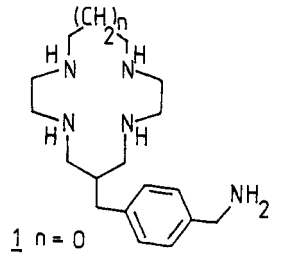
INTRODUCTION

An important feature of modern medical technology has been the development of monoclonal antibodies which when injected bind firmly to tumour associated compounds (antigens) (ref. 1). Although such antibodies themselves are rarely cytotoxic, they can function as specific carriers for the transport of imaging or cytotoxic agents to tumours for use in clinical diagnosis or therapy. A radionuclide-antibody conjugate can be used for both imaging (e.g. a γ or β^+ emitter) or therapy (e.g. a β^- or α-emitter) but in both cases it is essential that the radionuclide does not dissociate from the antibody conjugate, in vivo. Conventionally the metal radionuclide has been complexed by an acyclic chelate (e.g. EDTA or DTPA) which is covalently linked to the antibody. None of these chelates is adequate because the metal tends to dissociate in vivo. In particular the pathway for metal loss involves acid (or metal)-catalysed dissociation so that in the liver and other tissues of locally low-pH metal loss will occur readily. These problems may be obviated by using judiciously chosen macrocyclic ligands which tend to form more kinetically inert complexes with metals.

For radioimmunoscintigraphy, the most promising radionuclides are ^{99m}Tc ($t_{\frac{1}{2}}$ 6.02h, γ), ^{111}In ($t_{\frac{1}{2}}$ 2.83d, γ) for γ-scintigraphy and ^{64}Cu ($t_{\frac{1}{2}}$ 12.8h, β^+) for position emission tomography. Both copper and technetium (as TcO_2^+) are well-known to form thermodynamically stable and kinetically inert complexes with 1,4,8,11-tetraazacyclotetradecane (cyclam) (ref. 2). Our initial efforts therefore were centred on attaching functionalised tetra-aza macrocycles selectively to the antibody. The resultant antibody-macrocycle conjugates have been radiolabelled in aqueous solution and their stability examined in biodistribution studies in animals. The antibody used in this work was B72.3 which binds selectively to tumour-associated glycoprotein found in human breast and colorectal cancers (refs. 3 and 4).

SYNTHESIS AND LINKAGE

A set of four tetra-aza macrocycles was chosen for the initial work using carrier-free ^{64}Cu. Each bears on exocyclic primary amino group for attachment to the antibody (ref. 5). Both 1 and 2 were conveniently prepared by reaction of the corresponding linear tetraamine with diethyl p-cyanobenzylmalonate followed by reduction with borane-tetrahydrofuran. The related cycles 3 and 4 bearing a phenolic substituent which may act as a fifth 'axial' donor (ref. 6),



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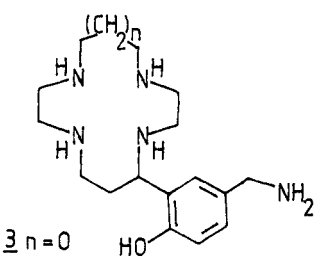
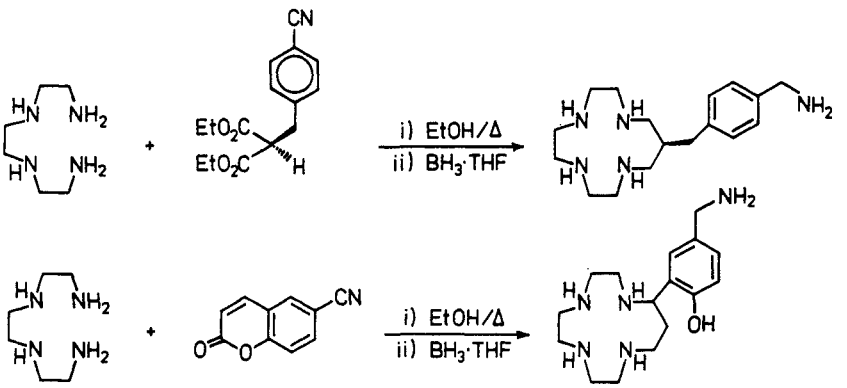
2 n = 1



3 n = 0
4 n = 1

Scheme 1



SYNTHESIS OF [13]-N_4 DERIVATIVES

were synthesised by condensation of 6-cyanocoumarin with the appropriate tetraamine followed by borane reduction (Scheme 1). In 3 and 4 the diprotonated N_4 ring assists the ionisation of the exocyclic phenol by stabilising the resultant phenolate anion and pK_a's of 8.82 and 8.71 (293 K, μ = 0.1) were determined spectrophotometrically for 3 and 4 respectively.

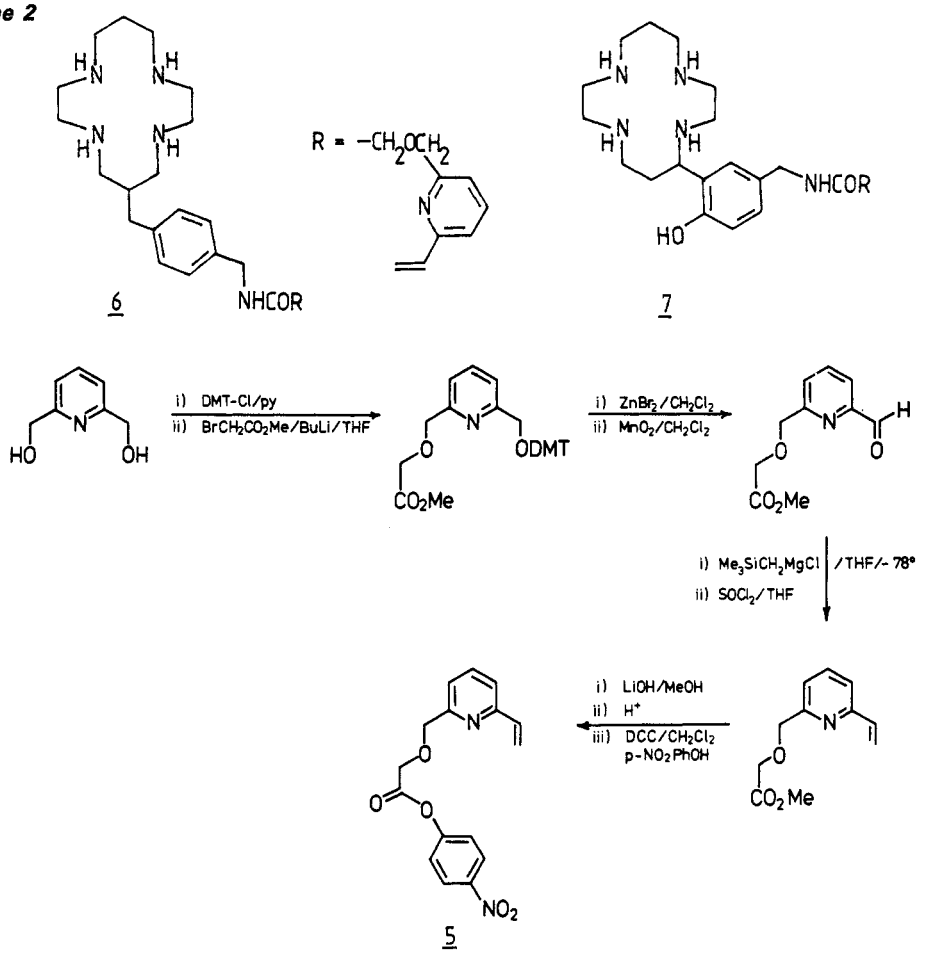
The next problem was to effect a selective linkage of these macrocycles to the antibody. Other approaches which have been advocated for the attachment of C-functionalised EDTA or DTPA ligands involved the use of aryl isothiocyanates or α-bromoamides (refs. 7 and 8). Neither was suitable in this case, nor are they intrinsically chemoselective as they react indiscriminately with antibody primary amine and thiol residues. A bifunctional linker molecule was sought which could be selectively attached to the primary amine group and which bore a second functional group for reaction with a thiol residue on the antibody. The 2- (and 4) vinyl pyridine derivative 5 was synthesised for this purpose (Scheme 2): it reacts selectively with thiol groups in the pH range 5 to 9 while primary and secondary amine groups are unaffected under these conditions (ref. 5). It was therefore possible to form an amide bond by reaction of the cycles 1-4 with the active ester of 5, generating stable and isolable vinylpyridine intermediates 6 and 7. These were susceptible to attack by thiol residues on the antibody to yield stable macrocycle-antibody conjugates.

Free thiol groups on the antibody may be generated either by recombitant antibody methods, by the cleavage of the antibody disulphide links to generate F(ab)' fragments or by treatment with 2-iminothiolane. This latter route was followed first and the modified antibody was reacted with the functionalised macrocycles 6 and 7 to yield a stable thioether link between the macrocycle and the antibody. The number of macrocycles linked to each antibody was measured spectrofluorimetrically (rather than by conventional ^{14}C radiolabelling methods) after exhaustive hydrolysis (6M HCl) of the antibody conjugate followed by a standard ortho-phthalaldehyde assay (ref. 9) of the resultant macrocyclic primary amine residues in 1-4. Up to three macrocycles per antibody were measured typically, the detection limit was 5×10^{-11} mol dm^{-3}, and no diminution of immunoreactivity was observed at this level of derivatisation.

LABELLING METHODS AND KINETICS

The introduction of the metal radioisotope into the macrocycle-antibody conjugate needs to be effected both rapidly and selectively in order to optimise the radiolabelling yield and obviate non-specific metal binding to the protein. This is particularly difficult with ^{99m}Tc labelling ($t_{\frac{1}{2}}$ 6.02 h), as reaction of 'reduced technetium' (derived from TcO_4^- in presence of stannous ions) with the macrocycles 1 and 2 in particular is sluggish in the pH range 7 to 9. Binding of technetium by the phenolated macrocycles proceeded more rapidly - for example a 77% incorporation of ^{99m}Tc by 3 [100 μM] was observed (hplc) after 30 minutes at pH 8.95. However, under these conditions, there is appreciable 'non-specific' binding of technetium by the protein. It is necessary therefore to form the technetium(V) dioxo complexes of the vinyl pyridine conjugates 6 and 7 prior to reaction with the antibody. This pre-labelling strategy indeed results in a specifically labelled antibody and in tumour-bearing mice the tumour to blood and tumour to liver ratios were very similar to those obtained with ^{125}I-labelled B 72.3 antibody. The limiting feature of this approach is that two separate steps are involved with a quickly decaying radioisotope, so that unless the linkage and purification steps are fast (< 2 h) the overall radiolabelling yield is low (< 10%).

Scheme 2



The situation is more straightforward with copper. Binding of copper - particularly in the presence of an anionic carboxylate buffer - by the macrocycles 1-4 remains fast even at pH 4 (ref. 10). Under these conditions, non-specific binding of Cu^{2+} to the protein is minimised. Pettit, for example, has shown that for various typical tetrapeptides complexation of Cu^{2+} is strong above pH 5, but at lower pH, free Cu^{2+} does not bind (ref. 11). The rate of incorporation of copper by ligands 1-4 has been studied by stopped-flow spectrophotometry, and the forward rate of association optimised. The more important features of the work are as follows:-

(a) binding of Cu^{2+} by the [13]-N_4 system is fastest (ref. 10).

(b) rate is dependent on the nature of the buffer used e.g. rate in succinate > citrate >> acetate.

(c) observed rate is dependent upon succinate concentration in pH range 3.6 → 5.7.

(d) the rates increases with decreasing ionic strength and plots of log k versus $\sqrt{I}/1 + \sqrt{I}$ give straight lines with slopes of -2 (pH 5.7) falling to -1.4 (pH 3.7).

(e) the rate increases with temperature quite steeply (E = 63 kJ mol^{-1} for Cu^{2+}/succinate/[13]-N_4).

Under the pseudo-first-order conditions used (L ≥ 10 Cu^{2+}; succinate ~ 0.2 M; L = [13]-N_4), the ionic strength dependence indicates that the species involved in the rate-determining step are of opposite charge. This accords with predominant reation of $[Cu(succinate)_2]^{2-}$ with monoprotonated ligand (pH > 5) and of [Cu(succinate)] with $[LH]^+$ as pH drops. In support of this analysis the formation constants for these species have been measured potentiometrically using 'SUPERQUAD' to compute the refined formation constants (β values for Cu(succ), $Cu(succ)_2^{2-}$ and $HCu(succ)_2^-$ are 2.59, 4.37 and 9.62 resepctively [298 K, I = 0.1] i.e. the dianionic species is > 50% protonated below pH 5.25).

TABLE 1. Comparative animal biodistribution data

Tissue	% Dose gm^{-1}	% Dose
Blood[b]	20 (18.4)	55.6 (55.8)
Kidneys	5.80 (6.33)	2.6 (3.25)
Liver[a]	7.96 (6.01)	13.7 (12.3)
Lungs	7.82 (8.14)	1.41 (1.46)
Spleen	4.97 (5.10)	0.38 (0.38)

[a]liver is 30% perfused with blood.
[b]diffusion of Ab from blood to extra-cellular tissue gives a theoretical value of 50% for blood (24 h).
[c]values in parentheses are for the 'pre-labelled' antibody.

The antibody conjugate of the [14]-N_4 phenolated cycle was labelled (pH 4, 0.2 M succinate, 30 mins) with carrier-free ^{64}Cu (obtained from Harwell) and compared to radiolabelled antibody obtained by binding ^{64}Cu to 7 prior to antibody linkage. Animal biodistribution data are compared in Table 1, and are in good agreement vindicating the use of low pH in order to minimise non-specific copper binding to the antibody. These data suggest that the Cu^{2+} does not dissociate from the antibody conjugate in vivo: if free Cu^{2+} is injected it tends to preferentially accumulate in the liver and kidneys. Such an antibody-macrocycle conjugate is ideally suited therefore for use in ^{64}Cu positron emission tomography studies.

TOWARDS THERAPY

In order to selectively kill a tumour by these methods (radioimmunotherapy) a β^- (or α) radiolablelled antibody conjugate is needed to selectively deliver a sterilising dose of radiation, without affecting healthy tissue. Possible therapeutic radioisotopes are listed in Table 2, including ^{67}Cu - for which the described systems apply. Unfortunately supply of this radioisotope is intermittent, while ^{90}Y is readily available from a ^{90}Sr generator. Several macrocycles have been screened for their ability to both quickly bind yttrium and to form a complex which is stable in vivo. The most promising is the [12]-N_4 tetracarboxylate 8, well known to form kinetically inert lanthamide complexes (ref. 12). The synthesis of a chiral C-functionalised derivative 9 using L-lysine as a precursor has been carried out (Scheme 3). The use of Cu^{2+} to protect the diethylenetriamine sub-unit permits selective acylation of the ϵ-amino group. Such a route is generally applicable to the synthesis of C-functionalised poly-aza macrocyclic ligands. Trials are underway to evaluate the utility of antibody conjugates of ligands such as 9 with the goal of devising a practicable and safe approach to radioimmunotherapy in human patients.

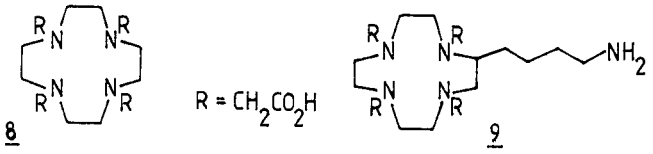


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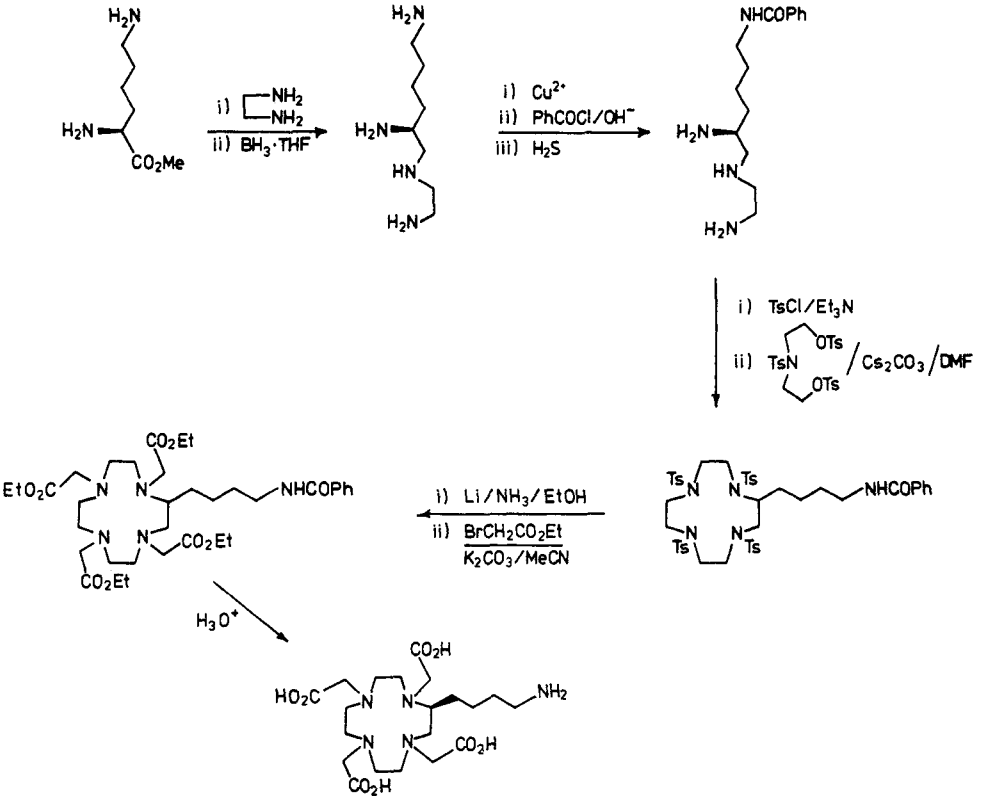
TABLE 2. Therapeutic radioisotopes

Isotope	Half-Life (h)	Dose rate (rad.h^{-1} per μlig^{-1})	Mean Range[b] (mm)	Total Dose[(a)] (rad. : t_∞)
^{67}Cu	62	0.58	0.2	52 (30)
^{90}Y	64	1.96	3.9	180 (180)
^{111}Ag	179	0.82	1.1	212 (198)
^{131}I	193	1.22	0.4	339 (115)
^{161}Tb	166	0.50	0.3	119 (101)
^{188}Re	17	1.91	3.3	47 (44)
^{199}Au	75	0.53	0.1	58 (47)

[a] values in parentheses indicate electron dose.
[b] in healthy tissue.

Scheme 3



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