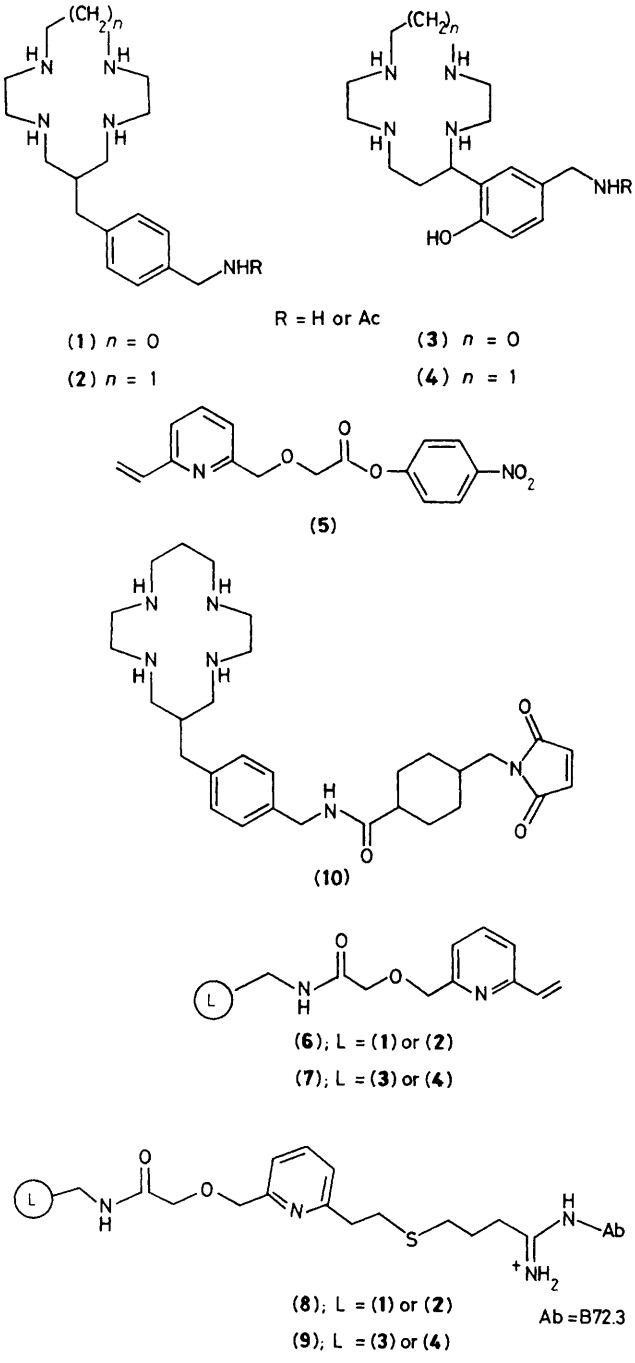
Towards Tumour Targeting with Copper-radiolabelled Macrocycle–Antibody Conjugates

J. Richard Morphy,[a] David Parker,*[a] Ritu Kataky,[a] Alice Harrison,[b] Michael A. W. Eaton,[c] Andrew Millican,[c] Alison Phipps,[c] and Carole Walker[b]

[a] *Department of Chemistry, University of Durham, South Road, Durham DH1 3LE, U.K.*
[b] *M.R.C. Radiobiology Unit, Harwell, Didcot OX11 0RD, U.K.*
[c] *Celltech Ltd., 216 Bath Road, Slough SL1 4EN, U.K.*

Tetra-aza-macrocycles covalently attached to a monoclonal antibody may be efficiently radiolabelled with ^{64}Cu or ^{67}Cu at pH 4, minimising non-specific binding to the protein, giving a kinetically stable conjugate *in vivo*.

The introduction of macrocycle-conjugated monoclonal antibodies is a key feature to the successful application of radiolabelled tumour-localising antibodies for use in tumour imaging and therapy.[1,2] Macrocyclic ligands tend to undergo very slow acid-dependent decomplexation so that a radiolabelled antibody may be stable *in vivo* over a period of days. Two isotopes of copper are of potential use: ^{64}Cu ($t_{\frac{1}{2}}$ = 12.8 h) in positron emission tomography[3] and ^{67}Cu ($t_{\frac{1}{2}}$ = 61.5 h) in radioimmunotherapy.[4] Copper forms well-defined complexes with thirteen- and fourteen-membered tetra-aza-macrocyclic



ligands[5] which are insensitive to decomplexation even at low pH.[6] These ligands have accordingly been covalently attached to the antibody B72.3 (which binds to tumour associated glycoprotein found in human breast and colorectal cancers) and radiolabelling and animal biodistribution data were undertaken.

The *C*-functionalised macrocycles, (**1**)—(**4**), were prepared as described earlier,[1] following reaction of diethyl *p*-cyanobenzyl malonate with the appropriate tetra-amine followed by reduction with BH_3·THF (tetrahydrofuran) or *via* condensation of 6-cyanocoumarin with the tetra-amine followed by borane–THF reduction. Reaction of the macrocycles (**1**)—(**4**)

Table 1. Distribution of ^{64}Cu and ^{67}Cu labelled B72.3–macrocycle complex and ^{64}Cu–macrocycle labelled B72.3 in THY 1.2 mice (% I.D. gm^{-1} tissue)

	A(^{64}Cu)[a]	B(^{64}Cu)[b]		C(^{67}Cu)[c]	
Tissue	24h	24h	4h	24h	72h
Blood	18.4	20.0	28.8	19.1	18.1
Kidneys	6.3	5.8	8.2	5.9	6.2
Liver	6.0	8.0	9.3	6.5	5.1
Lungs	8.1	7.8	10.1	7.7	8.6
Spleen	5.1	5.0	5.9	4.1	4.9

[a] **A** = (^{64}Cu + Mac)–B72.3 [Mac = (**2**)]. [b] **B** = ^{64}Cu–(Mac′–B72.3); pH 4.0, 20 °C [Mac′ = (**4**)]. [c] **C** = ^{67}Cu–(Mac′–B72.3); pH 4.0, 37 °C + DTPA wash. (Mac = macrocycle).

(R = H) with the bifunctional linker molecule (**5**)[1] (pH 6.8; 1 : 1, H_2O : dioxan) afforded the desired *exo*-cyclic amides (**6**) and (**7**) which were purified by cation-exchange h.p.l.c. The conjugates (**6**) and (**7**) react selectively with thiol residues on the antibody.[1] These were generated by reactions of B72.3 with 2-iminothiolane (giving typically 3 to 5 thiols per antibody by titration with Ellman's reagent[7]). Incubation of (**6**) and (**7**) with the modified antibody (pH 6, 15 h, 4 °C) gave the desired macrocycle–antibody conjugates (**8**) and (**9**), and excess thiols were capped by reaction with iodoacetamide. Up to two macrocycles per antibody were attached in this manner, with the conjugate exhibiting no loss of immunoreactivity.

At low pH (*e.g.* below 4.5) Cu^{2+} tends not to form stable complexes with proteins. For example at pH 4, species distribution plots indicate that Cu^{2+} does not bind to an array of typical tetrapeptides.[8] Stopped-flow spectrometric studies were undertaken at pH 4, with the aim of maximising the forward-rate of copper association by cycles (**1**) to (**4**). Rates were faster with the [13]-ring cycles[9] and with the non-phenolic systems and were enhanced by the introduction of anionic carboxylate buffers *e.g.* under standard conditions (10^{-3} M ligand, 10^{-4} M Cu^{2+}, pH 3.7, 0.1 M buffer) the rate in succinate ≈ citrate >> acetate. The succinate (succ) system was studied further, and the formation constants for the various Cu–succinate species evaluated (298 K, I = 0.1) by potentiometric titration followed by data analysis using SUPERQUAD.[10] The resultant formation constants [log β: Cu(succ) = 2.59 (±0.01); $Cu(succ)_2^{2-}$ 4.37; $HCu(succ)_2^-$ 9.64] suggested that the anionic copper species are possible reactive intermediates. Accordingly the rate of association was measured as a function of ionic strength, and in the pH range 3.7 to 5.7 the rate varied inversely with ionic strength. Plots of log k_{obs} versus $\sqrt{I}/1 + \sqrt{I}$ (298 K, 0.2 M succinate, 10^{-3} mL, 10^{-4} M Cu^{2+}) were linear with slopes of −2.2 (pH 5.7) and −1.4 (pH 3.7). This is consistent with predominant reaction of the monoprotonated ligand (LH^+) with $Cu(succ)_2^{2-}$ at high pH and $CuH(succ)_2^-$ at lower pH, in contrast to the results from the 'fitted' kinetic analysis for Cu^{2+} association with 'cyclam' (albeit at lower carboxylate concentrations).[9]

The macrocycle–antibody conjugates were radiolabelled with either carrier-free ^{64}Cu or ^{67}Cu, under these optimised conditions (pH 4, 0.2 M succinate, 293 K, 30 min) and radiolabelling yields were compared to a control antibody, B72.3, which bore no macrocycles. After gel filtration the ratio of the activities associated with conjugated and control antibodies was typically >100, and was further enhanced by adding an excess of cyclam or diethylenetriaminepenta-acetic acid (DTPA), after labelling but prior to gel filtration. These labelled antibodies were then injected into nude mice and after a given period of time, the biodistribution of the copper

radiolabel was evaluated (Table 1). For purposes of comparison, the intermediate (**10**) was labelled with ^{64}Cu (pH 6.5, 30 min) and purified by h.p.l.c prior to antibody linkage (60% radiolabelling yield). Data for the 'pre-labelled' and directly labelled antibody–macrocyle conjugates show good agreement: the retention of a high degree of activity in the cardiovascular system (4 h → 70 h) together with low levels of activity in the liver and kidneys† (liver is 30% perfused with blood) strongly suggest that the copper is not being released *in vivo*. Such results augur well for the introduction of copper-radiolabelled antibodies in tumour imaging and therapy.

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† Theoretically the diffusion of an antibody of this size from the blood to extra-cellular tissue should give 50% of the activity in the blood after 24 h. Direct injection of $^{64}CuCl_2$ into mice results in retention of copper in the liver and kidneys.

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