

Improved Tumour Targeting with Recombinant Antibody–Macrocyclic Conjugates

Timothy J. Norman,^a David Parker,^{*a} Louise Royle,^{a,c} Alice Harrison,^c Pari Antoniw^b and David J. King^b^a Department of Chemistry, University of Durham, South Road, Durham, UK DH1 3LE^b Celltech Therapeutics Ltd., 216 Bath Road, Slough, UK SL1 4EN^c MRC Radiobiology Unit, Chilton, Didcot, UK OX11 0RD

Linkage of a macrocyclic complexing agent to a spaced tri-maleimide allows formation of a recombinant trivalent antibody by reaction with a Δ -Cys Fab fragment of an engineered human antibody.

Effective tumour therapy in human patients with ^{90}Y -labelled monoclonal antibodies requires a conjugate comprised of an engineered human antibody that clears quickly from the blood and localises selectively in tumour tissue, linked to a bifunctional complexing agent that binds ^{90}Y quickly but does not release the metal ion *in vivo*.^{1,2} The latter problem is resolved using macrocyclic complexing agents based on the tetraazacyclododecane skeleton^{3,4} while the former may be addressed by using antibody fragments which clear more rapidly from the blood after injection.⁵ We report the synthesis of these two ideas in the definition of 12- N_4 azacarboxylate or azaphosphinate conjugates linked to a trivalent recombinant antibody (tri-Fab).⁵

The three step synthesis of the amine functionalised macrocycle, **1**, in a 63% yield from 1,4,7,10-tetraazacyclododecane has been reported previously.⁶ Reaction of the hydrobromide salt of **1** with the *N*-hydroxysuccinimidyl ester of 4-maleimidobutyrate (DMSO, *N*-methyl morpholine, 20 °C) gave the maleimide **2a** (39%) while reaction under identical conditions with an excess of bis(*p*-nitrophenyl) succinate afforded the active ester **2b** (33%), after purification by reverse-phase HPLC. At 310 K, rapid uptake of ^{90}Y by **2a** was observed ($[\mathbf{2a}] = 5 \mu\text{mol dm}^{-3}$, pH 6.5, 0.2 mol dm^{-3} NH_4OAc) and a 91% radiolabelling yield was determined within 10 mins. Comparison of the kinetics of dissociation of **2a** with the C-linked DOTA analogue, **4**,³ (DOTA is 1,4,7,10-tetraazacyclododecanetetra-acetic acid) at low pH indicated that both were remarkably resistant (Table 1) to acid-catalysed dissociation.^{6,7} Such kinetic stability has been shown to correlate well, and predictively, with the *in vivo* stability of the radiolabelled complexes.^{6,8,9} The antibody conjugates of **4** and **2a** were prepared, using murine B72.3 as a model antibody for the purpose of assessing the stability of the ^{90}Y -labelled antibody conjugate in animals. Conjugation of these maleimides to thiol residues on the whole IgG followed by ^{90}Y -labelling of the protein and subsequent HPLC purification followed established methods.^{10,11} After 48 h, the biodistribution of the ^{90}Y in various tissues was examined and the normalised ratio of the ^{90}Y activity in the femur compared to the blood was 0.19 (0.02) for [^{90}Y . **2a**-B72.3] and 0.18 (0.02) for [^{90}Y . **4**-B72.3]. These uniformly low values are indicative of

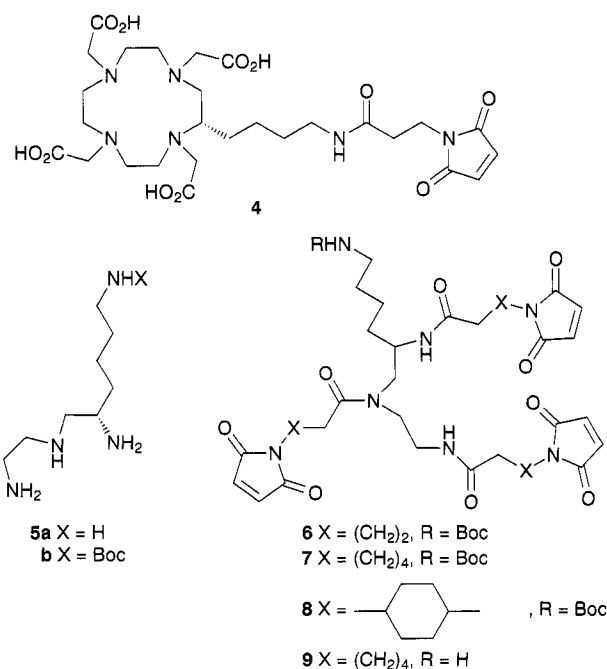
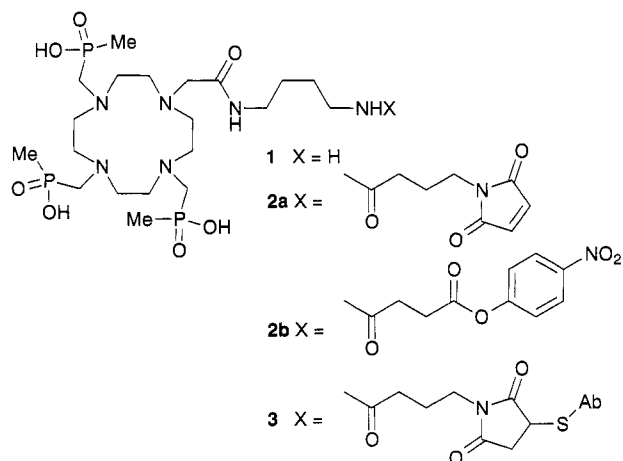
little significant premature ^{90}Y dissociation since free ^{90}Y is known to localise avidly in the bone. Indeed at 48 h the ratio of activity in the femur shaft to blood was 0.06 (0.01) for both conjugates, consistent with the required *in vivo* stability.

If the antigen-binding fragments (Fab') of a monoclonal antibody are linked together, giving di-, tri- or tetra-(Fab')s, then faster blood clearance and greater avidity for the tumour-associated antigen ensues.⁵ Given that a maleimide reacts quickly and selectively with a thiol residue, such as that found on a humanised Fab' fragment with a Δ -Cys residue in the hinge region, then a suitable polymaleimide linker should afford a polyvalent recombinant antibody. With this in mind, a trimaleimide was sought that could be linked to a given bifunctional complexing agent, and hence generate the desired triFab conjugate.

Table 1 Dissociation rates of ^{90}Y complexes (at 310 K)^a

Complex ^b	$t_{1/2}/\text{h}$		
	pH1	1.5	2.0
[Y.DOTA] ⁻	13	102	583
[Y.N ₄ P ₄ Me ₄] ⁻	14	34	116
[Y.N ₄ P ₃ CONBN ₂]	145	379	989
[Y. 2a] ⁻	29	91	370
[Y. 4] ⁻	71	254	1370

^a Rates were determined as described in ref. 7 in glycine buffered solutions; ^{90}Y (β^- , $t_{1/2}$ 64 h). ^b N₄P₄Me₄ and N₄P₃CONBN₂ are 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene methylphosphinate) and 10-(*N,N*-dibenzylcarbamoylmethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyltrimethylenetriphosphinate.⁶



The tetraamine **5a**¹² was protected with copper(II) and reacted with BOCN (H₂O, dioxan, Et₃N) followed by treatment with H₂S to yield the mono-BOC amine **5b** (66%). Reaction of **5b** with the appropriate maleimido-acid chloride (CH₂Cl₂, Et₃N) gave the trimaleimides **6**, **7** and **8** in modest yield (16% typically, after HPLC purification). The differing length of the maleimide side-chains (C₃, C₅ vs cyclohexyl) was chosen in order to define whether this perturbed the yield of the derived tri-Fab's. Reaction of each trimaleimide with an excess of freshly prepared Fab(H₂O-DMF; 37 °C; 2 h) was monitored by analytical HPLC (Zorbax GF 250, 0.2 mol dm⁻³ phosphate buffer, pH 7.0, 1 cm³ min⁻¹) and revealed successive formation of mono- di- and tri-Fab species at 9.9, 8.9 and 8.4 mins respectively. The ratio of tri, di- and mono-Fab products depended upon the ratio of reagents used but was independent of the nature of the antibody used (B72.3 vs. h.A-33),[†] and the highest yields of tri-Fab material were obtained with the C₅-spaced maleimide, **7**, giving up to 39% of the tri-Fab' (36% of di-Fab) at a reagent ratio of 5 : 1. At the end of the incubation, remaining thiol groups were capped by addition of an excess of *N*-ethyl maleimide.

Following HPLC purification on a gel filtration column, the integrity of the tri-Fab was established by gel electrophoresis using a standard SDS-Page system under reducing or non-reducing conditions. The immunoreactivity of the product tri-Fab was assessed using a cell-binding assay in which tri-Fab was allowed to compete with fluorescein labelled antibody. Using the h.A-33 Fab', the trivalent recombinant protein based on **8** was found to be 3.4 times more immunoreactive than the parent humanised IgG antibody. This was in line with results obtained with the Lys-Lys-Lys-linked h.A-33 tri-Fab, derived from **10**,⁵ which had an immunoreactivity that was 28% greater than the tri-Fab based on **8**.

Finally reaction of **2b** with **9**, prepared by TFA deprotection of **8**, (DMSO, *N*-methyl morpholine, 25 °C) yielded the macrocycle-trimaleimide **11**, which was purified by reverse-phase HPLC, (25%). Incubation with h.A-33 Fab as described above afforded the desired macrocycle-tri-Fab'. The biodistribution of a ⁹⁰Y-labelled h.A-33 tri-Fab (based on **10**), carried out in nude mice bearing SW1222 tumour xenografts, revealed tumour to blood ratios of 6.33 : 1 and 28 : 1 at 24 and 48 h post injection, compared to values of 1.9 : 1 and 5.8 : 1 for the corresponding conjugate linked to the whole antibody, (h.A-33-IgG). Given the kinetic stability *in vivo* of the macrocyclic ⁹⁰Y complex, the enhanced immunoreactivity of the trivalent

recombinant antibody and the fact that these trivalent antibodies clear rapidly from the blood, generating high tumour : blood ratios,[‡] the characteristics of the recombinant antibody-macrocycle conjugates augur well for the clinical trials.

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Footnotes

[†] c.B.72.3⁵ is a recombinant chimeric version of B72.3¹³ which is an antibody which reacts with an antigen (TAG-72) found on human colorectal and breast neoplasms. The h.A-33 antibody recognises a glycoprotein found in >95% of colon cancers, affords higher tumour:tissue ratios and penetrates dense tumour tissue much more effectively.¹⁴

[‡] An ¹²⁵I-labelled h.A-33 tri-Fab, based on **7** or **10** showed ca. 7% of the injected dose per gram of blood in mice at 3 h, but only 0.15% of the injected dose gm⁻¹ tissue (blood) at 24 h. This compares to values of the order of 5–10% id.g⁻¹ blood at 24 h for a typical iodine radiolabelled whole antibody.

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