Improved Tumour Targeting with Recombinant Antibody–Macrocycle 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 ORD*

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]

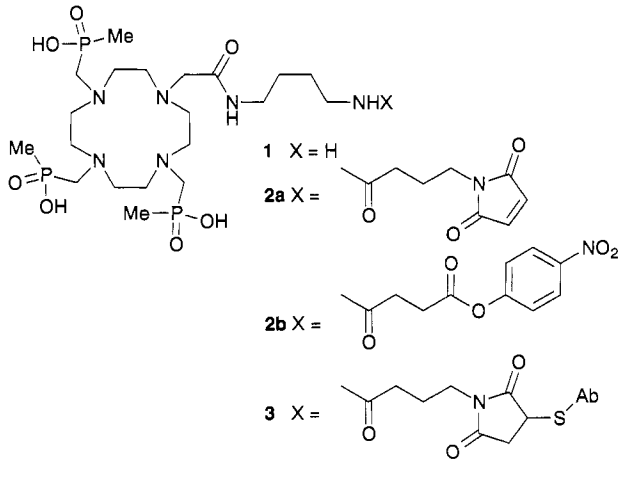
The three step synthesis of the amine functionalised macrocycle, **1**, in a 63% yield from 1,4,7,10-tetraazacyclododecane has been reported previously.[6] Reaction of the hydrobromide salt of **1** with the *N*-hydroxysuccinimidyl ester of 4-maleimidobutyrate (DMSO, *N*-methyl morpholine, 20 °C) gave 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 ([**2a**] = 5 μmol dm^{-3}, pH 6.5, 0.2 mol dm^{-3} NH_4 OAc) 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.

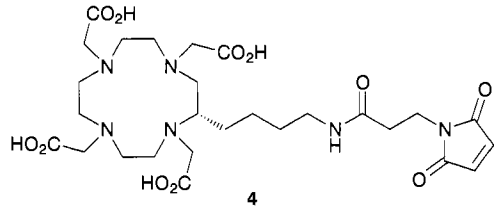
If the antigen-binding fragments (Fab′) of a monoclonal antibody are linked together, giving di, tri- or tetra-(Fab′)s, then faster blood clearance and greater avidity for the tumour-associated antigen ensues.[5] Given that a maleimide reacts quickly and selectively with a thiol residue, such as that 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]

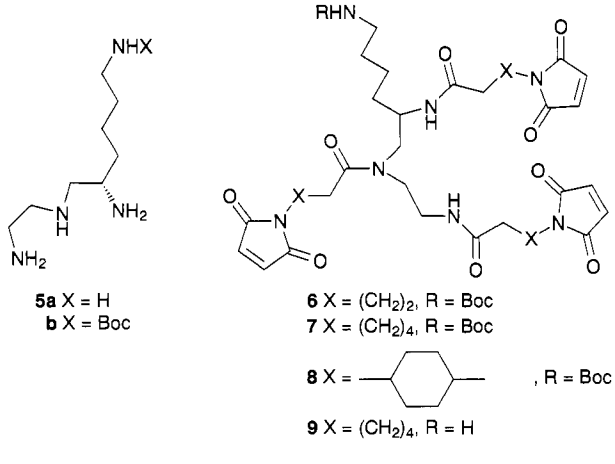
Complex[b]	$t_{\frac{1}{2}}$/h pH1	1.5	2.0
[Y.DOTA]$^-$	13	102	583
[Y.$N_4P_4Me_4$]$^-$	14	34	116
[Y.$N_4P_3CONBn_2$]	145	379	989
[Y.**2a**]$^-$	29	91	370
[Y.**4**]$^-$	71	254	1370

[a] Rates were determined as described in ref. 7 in glycine buffered solutions; ^{90}Y (β$^-$, $t_{\frac{1}{2}}$ 64 h). [b] $N_4P_4Me_4$ and $N_4P_3CONBn_2$ 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]





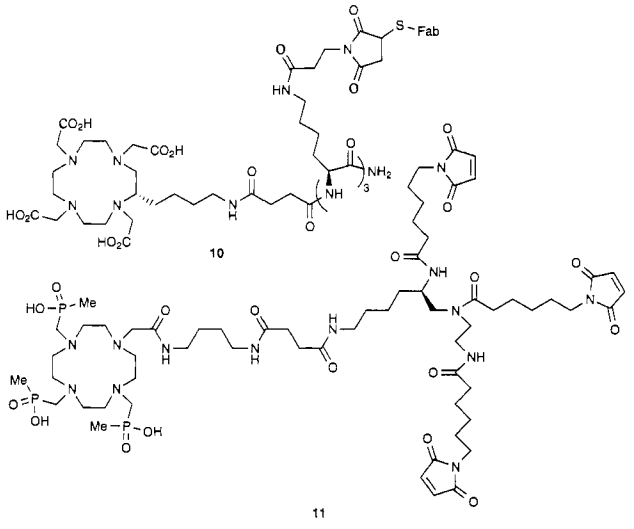
4



The tetraamine **5a**[12] was protected with copper(II) and reacted with BOCON (H_2O, dioxan, Et_3N) followed by treatment with H_2S to yield the mono-BOC amine **5b** (66%). Reaction of **5b** with the appropriate maleimido-acid chloride (CH_2Cl_2, Et_3N) gave the trimaleimides **6**, **7** and **8** in modest yield (16% typically, after HPLC purification). The differing length of the maleimide side-chains (C_3, C_5 *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(H_2O–DMF; 37 °C; 2 h) was monitored by analytical HPLC (Zorbax GF 250, 0.2 mol dm^{-3} phosphate buffer, pH 7.0, 1 cm^3 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 C_5-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 ^{90}Y-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 ^{90}Y 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.



We thank the BBSRC, EPSRC and the MRC for support, and Andrew Chapman for assistance with the immunoreactivity assays.

Received, 11th May 1995; Com. 5/02991K

Footnotes

† c.B.72.3[5] is a recombinant chimeric version of B72.3[13] 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 ^{125}I-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.

References

1 D. Parker, *Chem. Soc. Rev.*, 1990, **19**, 271; D. Parker, in *Comprehensive Supramolecular Chemistry*, ed. J. M. Lehn and D. N. Reinhoudt, 1996, Pergamon, vol. 17, ch. 10.

2 D. Parker and K. J. Jankowski, in *Advances in Metals in Medicine*, ed. B. A. Murrer and M. J. Abrams, Jai Press, New York, 1993, **1**, pp. 29–73.

3 D. Parker and A. T. Millican, *Int. Pat. Appl.*, WO 8901476, 1989; J. P. L. Cox, K. J. Jankowski, R. Kataky, D. Parker, N. R. A. Beeley, B. A. Boyce, M. A. W. Eaton, K. Millar, A. T. Millican, A. Harrison and C. A. Walker, *J. Chem. Soc., Chem. Commun.*, 1989, 797; C. J. Broan, E. Cole, K. J. Jankowski, D. Parker, K. Pulukkody, B. A. Boyce, N. R. A. Beeley, K. Millar and A. T. Millican, *Synthesis*, 1992, 63.

4 M. K. Moi, C. F. Meares and S. J. de Nardo, *J. Am. Chem. Soc.*, 1988, **110**, 6266.

5 D. J. King, A. Turner, A. P. H. Farnsworth, J. R. Adair, R. J. Owens, R. B. Pedley, D. Baldock, K. A. Proudfoot, A. D. G. Lawson, N. R. A. Beeley, K. Millar, A. T. Millican, B. A. Boyce, P. Antoniw, A. Mountain, R. H. J. Begent, D. Schochat and G. T. Yarranton, *Cancer Res.*, 1994, **54**, 6176.

6 K. P. Pulukkody, T. J. Norman, D. Parker, L. Royle and C. J. Broan, *J. Chem. Soc., Perkin Trans. 2*, 1993, 605.

7 C. J. Broan, J. P. L. Cox, A. S. Craig, R. Kataky, D. Parker, G. Ferguson, A. Harrison and A. M. Randall, *J. Chem. Soc., Perkin Trans. 2*, 1991, 87.

8 A. Harrison, C. A. Walker, K. A. Pereira, D. Parker, L. Royle, K. Pulukkody and T. J. Norman, *Magn. Reson. Imaging*, 1993, **11**, 761.

9 P. Wedeking, K. Kumar and M. F. Tweedle, *Magn. Reson. Imaging*, 1992, **10**, 641.

10 A. Harrison, C. A. Walker, D. Parker, K. J. Jankowski, J. P. L. Cox, A. S. Craig, J. M. Sansom, N. R. A. Beeley, B. A. Boyce, L. Chaplin, M. A. W. Eaton, A. P. H. Farnsworth, K. Millar, A. T. Millican, A. M. Randall, S. K. Rhind, D. Secher and A. Turner, *Nucl. Med. Biol.*, 1991, **18**, 469.

11 A. Turner, D. J. King, A. P. H. Farnsworth, S. K. Rhind, R. B. Pedley, J. Boden, R. Boden, A. T. Millican, K. Millar, B. A. Boyce, N. R. A. Beeley, M. A. W. Eaton and D. Parker, *Br. J. Cancer*, 1994, **70**, 35.

12 J. P. L. Cox, A. S. Craig, K. J. Jankowski, D. Parker, I. M. Helps, N. R. A. Beeley, B. A. Boyce, K. Millar, A. T. Millican and M. A. W. Eaton, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2567.

13 D. Colcher, D. Milenic, M. Roselli, A. Ranbitschek, G. Yarranton, D. J. King, J. Adair, N. Whittle, M. Bodmer and J. Schlom, *Cancer Res.*, 1989, **49**, 1738.

14 S. Welt, C. R. Divgi, F. X. Real, S. D. Yeh, P. Garin-Chesa, C. L. Finstad, J. Sakamoto, A. Cohen, E. R. Sigurdson, N. Kememy, E. A. Carswell, H. F. Oettgen and L. J. Old, *J. Clin. Oncol.*, 1990, **8**(11), 1894.