Impact of antibody framework residue V_H-71 on the stability of a humanised anti-MUC1 scFv and derived immunoenzyme

J Krauss1,7, MAE Arndt1,8, Z Zhu1, DL Newton1, BK Yu1, V Choudhry2, R Darbha3, X Ji3, NS Courtenay-Luck4,5, MP Deonarain5, J Richards5 and SM Rybak*,6

1SAIC, National Cancer Institute at Frederick, Frederick, MD 21702, USA; 2Laboratory of Experimental and Computational Biology, National Cancer Institute at Frederick, Frederick, MD 21702, USA; 3Macromolecular Crystallography Laboratory, National Cancer Institute at Frederick, Frederick, MD 21702, USA; 4Antisoma Research Ltd, West Africa House, Hanger Lane, Ealing W5 3QR, UK; 5Imperial College of Science, Technology & Medicine, London SW7 2AZ, UK; 6Developmental Therapeutics Program, National Cancer Institute at Frederick, Frederick, MD 21702, USA

Antigen binding, markedly stabilised the scFv while having only a minor effect on the binding affinity of the molecule. Because of its improved stability, the engineered fragment exhibited immunoreactivity with tumour cells even after 7 days of incubation in human serum at 37°C. It also showed, in contrast to the wild-type scFv, a concentration-dependent binding to the target antigen when displayed on phage. When fusing the scFv to the recombinant ribonuclease r prolactin RI, only the fusion protein generated with the stable mutant scFv was able to kill MUC1þ tumour cells with an IC50 of 80 nM. We expect this novel immunoenzyme to become a promising tool for the treatment of MUC1þ malignancies.

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The MUC1 membrane mucin glycoprotein is overexpressed in most adenocarcinomas (Taylor-Papadimitriou et al, 1999) and associated with poor prognosis in patients with many epithelial cancers, including colorectal and gastric carcinoma (Utsunomiya et al, 1998; Baldus et al, 2002). Recently, MUC1 was also found to be overexpressed in a variety of haematological malignancies including acute myelogenous leukaemia, chronic lymphocytic leukaemia, and multiple myeloma (Brossart et al, 2001). Since glycosylation of MUC1 in cancer cells is distinct from mucin expressed in healthy tissue (Hanisch and Muller, 2000), tumour-associated mucin represents a valuable target for diagnostic and therapeutic approaches with monoclonal antibodies (mAbs). Several mAbs have been raised against the highly conserved immunogenic core region of the extracellular domain of MUC1, which comprises tandem repeats of 20 amino acids (Gendler et al, 1998; Baldus et al, 2002). These include mAb HMFG1 (Taylor-Papadimitriou et al, 1991), which recognises the PDTR epitope of the protein core with high specificity (Price et al, 1990). HMFG1 becomes internalised after antigen binding (Aboud-Pirak et al, 1988) and thus provides a valuable tool for the selective delivery of cytotoxic agents into tumour cells. A 90Y-HMFG1 radioimmunoconjugate was employed in a phase I–II clinical trial in patients with ovarian cancer in an adjuvant setting. Intrapleural administration of a single dose of the reagent resulted in a >10 years long-term survival of 78% of these patients (Epnetos et al, 2000). A multicentre, multinational, phase III clinical trial utilising 90Y-HMFG1 for the adjuvant treatment of women with ovarian cancer completed enrolment in 2003, and first results are expected during 2004. To overcome the immunogenicity of mAb HMFG1 and make the antibody suitable for repeated systemic administration, a humanised version, designated huHMFG1, was generated by grafting the murine antigen-binding site into human antibody frameworks (Verhoeyen et al, 1993). huHMFG1 was shown to retain the antigen affinity and same specificity of the rodent ancestor. Results from a recently completed radioimmunoimaging study in breast cancer patients (Al-Yasi et al, 2002) showed the specific binding of huHMFG1 to tumour tissues. The humanised antibody is currently being evaluated as a therapeutic agent in a phase I clinical trial in patients with metastatic breast cancer. For many clinical applications, such as radioimmunoimaging, radioimmunotherapy, or administration of recombinant cytotoxic fusion proteins, the employment of small antigen-binding fragments such as single-chain Fv (scFv) antibodies or multivalent derivatives may have advantages over the use of antibodies in the IgG format. ScFv fragments penetrate solid tumour tissue more efficiently (Yokota et al, 1998) and are rapidly cleared from the circulation (Milenic et al, 1991). To exploit these advantages, huHMFG1 was reformatted into an scFv fragment. Although this construct exhibited appropriate antigen binding, its half-life was <2 h when incubated in human serum at 37°C. Low biophysical stability of
scFv fragments was shown to be associated with the failure to localise to xenografted tumour tissue in immunodeficient mice (Willuda et al., 1999). Thus, for clinical applications, sufficient stability of scFv fragments is of paramount importance.

The aim of this study was to generate a humanised anti-MUC1 scFv with sufficient antigen-binding and stability properties as required for clinical applications. Here we show that mutagenesis of human antibody framework 3 residue V_H-71Arg to the corresponding murine donor antibody site alanine (V_H-71Arg→Ala) dramatically increased the stability of the humanised scFv while only having a minor impact on the affinity of the molecule. As a consequence of its improved stability, only the robust mutant Ala dramatically increased the stability of the humanised scFv domains.

**MATERIALS AND METHODS**

**Identification of unusual framework residues**

To identify unusual residues within the human variable domain framework regions (FRs), which could possibly influence the structural integrity of the grafted antigen-binding site of the humanised scFv antibody, we aligned its amino-acid sequence to sequence reference templates. Mismatching 'key residues' (Chothia et al., 1989) between the frameworks of the murine donor antibody sequence and the human acceptor antibody sequence were identified and canonical-class assignments of the donor antibody complementarity determining regions (CDRs), L1–L3, H1, and H2, respectively, were determined by screening the sequence against sequence templates of antibody repertoires (Martin and Thornton, 1996) at http://www.bionf.org.uk/. Furthermore, uncommon residues at the V_H/V_L interface (Chothia et al., 1985) of the humanised scFv with a potential to compromise interdomain stability of the molecule were inspected.

**Generation of wild-type scFv huHMFG1 and scFv mutants**

To generate a humanised scFv, the variable light chain and the variable heavy chain of huHMFG1 were PCR amplified from plasmids pAS1 and pAS2 (Dr R Young, Antisoma Research Ltd), containing the complete light chain and heavy chain of the humanised antibody, respectively. Single-chain Fv variants with flanking NcoI and BamHI restriction sites were generated by PCR in either domain orientation and with different linker peptides (Table 1). Mutations were introduced by oligonucleotide directed mutagenesis and overlap extension PCR techniques as described previously (Arndt et al., 2003). Concentrations of purified scFvs were determined spectrophotometrically from the absorbance at A_280nm using the extinction coefficient μmg⁻¹cm⁻¹ = 1.67.

**Periplasmic expression and purification of scFv fragments**

Single-chain Fv fragments were expressed as soluble protein in the periplasm of the *Escherichia coli* strain TG1 (Strategene, La Jolla, CA, USA) and purified by immobilised metal chelate affinity chromatography (IMAC) and size-exclusion chromatography as described previously (Arndt et al., 2003). Concentrations of purified scFvs were determined spectrophotometrically from the absorbance at A_280nm using the extinction coefficient μmg⁻¹cm⁻¹ = 1.67.

**Figure 1** Schematic representation of fusion protein expression vector pBJR-2. Ap', ampicillin resistance gene; CoIE1, origin of DNA replication; c-myc, sequence encoding the c-myc epitope; His6, hexa-histidine encoding sequence; P/O, lac wild-type promoter/operator; rbs, ribosome-binding site; pelB, signal peptide sequence of bacterial pectate lyase; V_L, variable light chain; V_H, variable heavy chain; V_H'3, framework 3 residue 71; H/II, spacer connecting the ribonuclease with the scFv; (G6S)3, linker connecting the variable domains. Restriction sites for cloning are indicated.
containing 1.0 M NaCl. The protein solution was diluted 10-fold with 50 mM NaOAc, pH 5.0, immediately applied to a 2 ml SP-Sepharose column (Pharmacia Biotech Inc.) and eluted with a 200 ml linear NaCl gradient (0.1–0.7 M) in 50 mM NaOAc, pH 5.0.

**Cell-binding assays**

Specific binding of scFv constructs was determined by flow cytometry using the human MUC-1+ cell lines MCF7 (ATTG, Manassas, VA, USA) and SKOV-3 (ATTGC, and mouse myeloma B-cell line Sp2/0-Ag14 (ATTG) and human T-cell line Jurkat (ATTG) as a negative control. For the determination of antigen-binding affinity constants (Kd), MCF7 cells were used. Staining of antibody fragments and affinity measurements were performed as described previously (Benedict et al., 1997). Stained cells were analysed on a FACScan Flow Cytometer (BD Bioscience, San Jose, CA, USA), and median fluorescence intensity (MFI) was calculated using the CellQuest™ software (BD Bioscience). Background fluorescence was subtracted. Equilibrium constants were determined by using the Marquardt – Levenberg algorithm for nonlinear regression with the GraphPad Prism version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA).

**Cytotoxicity assay**

Cytotoxicity of scFv-RNase fusion proteins towards target cells was analysed by a protein synthesis inhibition assay. In all, 2.5 × 10^4 MCF7 cells in a volume of 100 μl were plated in each well of a 96-well plate 24 h before treatment and the assay performed according to standard procedures as described previously (Newton et al., 1996). Experiments were performed at least twice. The concentration of fusion protein test sample that inhibits protein synthesis by 50% (IC50) was determined from semilogarithmic plots as percentage of [14C]leucine incorporation into mock-treated cells.

**Generation of computer homology models**

Three homologous models of scFv 4.9M were generated based on existing structures of scFv proteins (Berman et al., 2000) with either an Arg or an Ala at position VH-71 located in the heavy-chain FR 3. Two forms of huHMFG1-Arg71 with distinct conformations in the CDR-H1 (residues 31–35) and CDR-H2 (residues 50–65) regions were modelled based on observed conformations in scFv structures with an Arg at position VH-71. In one form, the side chain of VH-71Arg is exposed to the surface of the protein as a negative control. For the determination of antigen-binding affinity constants (Kd), MCF7 cells were used. Staining of antibody fragments and affinity measurements were performed as described previously (Benedict et al., 1997). The starting model was constructed with 1NQB fragments C121–C233 of the primary sequence similarity to scFv huHMFG1-Ala71 was modelled because all the scFv structures with a non-randomised linker peptide sequence were cloned into phagemid pCANTAB-5 (Amersham Pharmacia, Uppsala, Sweden) after the introduction of flanking restriction sites SfiI/NotI by PCR. Phagemid virions were prepared essentially according to standard methods as described (Sambrook et al., 1989; Marks et al., 1991).

**Expression of scFv fragments on phage**

Wild-type sequence scFv 4.10W and mutant scFv 4.9M encoding genes (Table 1) or wild-type scFv 17W with randomised linker peptide sequence were cloned into phagemid pCANTAB-5 (Amersham Pharmacia, Uppsala, Sweden) after the introduction of flanking restriction sites SfiI/NotI by PCR. Phagemid virions were prepared essentially according to standard methods as described (Sambrook et al., 1989; Marks et al., 1991).

**Phage ELISA**

The ability of phage-displayed scFvs for recognising the target antigen MUC1 was determined by whole-cell ELISA with MCF7 cells. In all, 1 × 10^6 cells were seeded in a 96-well plate (Nunc Nalgene, Roskilde, Denmark) and grown in DMEM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum until 60–70% confluent. Cells were fixed by adding paraformaldehyde diluted in PBS to a final concentration of 1% and incubated for 10 min at room temperature. After washing with PBS, a serial dilution of purified scFv phagemid virion or M13KO7 helper phage alone (Invitrogen) was added to the cells. Plates were gently shaken for 2 h at 37°C. After 6 × washing with PBS/ Tween 20 0.02%, rabbit anti-M13-HRP polyclonal antibody (1:5000; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) was added. The mixture was incubated at 37°C for 1 h. Cells were washed with PBS and bound phage scFvs were detected using ABTS (Sigma Chemicals, Saint Louis, MO, USA) as a substrate. After 30 min incubation, absorbance was read with an ELISA reader (Bio-Tek Instruments Inc., Winooski, VT, USA) at 405 nm.

**Biological stability**

Single-chain Fv fragments were incubated at 37°C in 90% human serum at a concentration of 20 μg ml⁻¹ for up to 7 days. Samples were taken at different time points and stored at −20°C. Binding activity of the samples to MUC1+ MCF7 cells was determined by flow cytometry. The MFI was determined as described above. Temperature-dependent degradation of monomeric scFvs was determined by incubation of samples at 37°C in 90% PBS at a concentration of 20 μg ml⁻¹ for 1 h, followed by analytical gel filtration on a calibrated Superdex 75 HR10/30 column (Amersham Pharmacia Biotech Inc.).

**RESULTS**

**Generation and characterisation of wild-type sequence scFvs**

In order to generate a small antigen-binding molecule to be used as a building block for the subsequent construction of recombinant fusion proteins, the variable domains of light and heavy chains of the humanised mAb huHMFG1 (Verhoeven et al., 1993) were recloned into a VH–VL oriented scFv format, separated by a synthetic 17-amino-acid linker peptide (clone 17W, Table 1). Although the scFv showed specific binding to MUC1+ tumour cells, its half-life in human serum at 37°C was less than 2 h (data not shown). To improve the stability of the wild-type scFv molecule by alteration of the linker peptide, this region was randomised by PCR and the resulting scFv fragments were recloned into a phagemid vector for display on phage. However, no clones could be enriched after panning the generated scFv library on MUC1+ MCF7 cells. These results indicate that the
linker-randomised scFvs were not displayed in a conformation for binding to native antigen. To further investigate whether the $V_L - V_H$ connecting linker peptide length or domain orientation accounted for the poor stability of the wild-type scFv, two further linker variants, $22W$ and $4.10W$, were generated (Table 1). Both constructs retained specific binding to MUC1$^+$ tumour cells but did not show an increase in their half-lives, indicating that changing the domain orientation or linker length was not sufficient to increase the stability of the molecules.

Analysis of huHMFG1 variable domain wild-type sequence

The amino-acid sequence of the humanised scFv antibody was analysed as described in Materials and Methods. We identified $V_H$-71, previously described as a ‘key residue’ for the CDR-H2 main-chain conformation (Chothia et al., 1989; Tramontano et al., 1990), to be potentially critical for maintaining the structural integrity of the grafted antigen-binding site of the wild-type scFv fragment. To study the effects of framework residue $V_H$-71 on antigen-binding and stability properties of the humanised scFvs, three mutant variants were generated by replacing arginine in the human framework 3 sequence with the murine donor antibody residue alanine (clones 17M, 22M, 4.9M; Table 1).

Effects of $V_H$-71Arg→Ala mutation on stability and specificity of scFv fragments

For initial characterisation, the mutant variant scFvs (clones 17M, 22M, 4.9M; Table 1) were IMAC purified. In flow cytometry, all three variants showed specific binding to MUC1$^+$ MCF7 cells. To assess the global stability, the engineered scFvs were incubated in human serum at 37°C for 12 h. The half-life of mutant 17M was not increased when compared with the corresponding wild-type scFv 17W (data not shown). In contrast, immunoreactivity with tumour cells at this time point was observed for mutants 22M and 4.9M, respectively. Monomers of scFvs 22M and 4.9M as well as their wild-type counterparts (clones 22W and 4.10W, respectively) were subsequently separated from higher molecular weight species by size-exclusion chromatography. Both purified wild-type and mutant scFvs exhibited specific binding towards MUC1$^+$ tumour cells as shown in Figure 2 for the example of constructs in the $V_H - V_L$ orientation. The stability of 22M and 4.9M monomers was determined by incubation of purified proteins in human serum at 37°C for various time periods. As demonstrated in Figure 3, both mutant scFvs exhibited dramatically improved stability when compared with the corresponding wild-type constructs. Nevertheless, both constructs showed a substantial decline in cell-binding activity within the first hour of serum incubation (4.9M, 40%; 22M, 51%) although they retained specific binding to tumour cells even after 7 days of serum incubation (Figure 3). To determine whether this phenomenon was induced by exposure of the scFvs to elevated temperature or to serum components, mutant scFv 4.9M was incubated in PBS for 1 h at 37°C and binding activity to MCF7 cells was analysed by flow cytometry. As shown in Figure 4A, the median fluorescence intensity dropped to a similar level whether the scFv was incubated in human serum or PBS at 37°C. Analytical size-exclusion chromatography revealed no degradation of the monomeric protein at this time point (Figure 4B), suggesting that the initial decrease in immunoreactivity was due to reduced binding affinity caused by exposure of the scFv to the elevated temperature.

Effects of $V_H$-71Arg→Ala mutation on affinity of scFv fragments

Binding affinity constants for the wild-type scFvs 22W and 4.10W, and mutant variant scFvs 4.9M and 22M with MCF7 cells was measured by flow cytometry after incubating the constructs at 37°C in human serum for various time periods. Binding activity is shown as per cent of the maximal MFI at time point zero.
CDR-H1 and CDR-H2 is dictated by the identity of residue $V_{H}-71$ but not the conformation of the two motifs: For the $V_{H}$-Ala71 model, it is 481 Å$^2$, which is significantly larger than for the two $V_{H}$-Arg71 models (384 and 398 Å$^2$ in Model-1 and Model-2, respectively).

Expression of scFv variants on phage

To examine the possibility of using the humanised scFv antibody as a template to select entire human fragments from phage display libraries by chain shuffling (Marks et al, 1992), both wild-type sequence scFv 4.10W and mutant 4.9M were recloned in a phagemid vector for display on phage. The titre of the concentrated phage stock was $10^{12}$–$10^{13}$ CFU ml$^{-1}$. In whole-cell ELISA, only the displayed mutant scFv 4.9M showed reactivity with MUC1$^+$ tumour cells in a concentration-dependent manner (Figure 6), indicating that the wild-type scFv was not appreciably expressed on phage.

Effect of $V_{H}$-71Arg → Ala mutation on cytotoxic properties of a recombinant immonozyme

To evaluate the stabilised humanised scFv antibody as a targeting moiety for the selective delivery of cytotoxic agents into tumour cells, a recombinant fusion protein comprising the recombinant ribonuclease $rap$L1 and scFv 4.9M was made. This construct could be purified to homogeneity and was active in killing MCF7 cells with an IC$_{50}$ of 80 nM (Figure 7). The cytotoxic activity of $rap$L1 in the fusion protein configuration was increased approximately 18-fold (IC$_{50}$ for native $rap$L1, 1500 nM). In


**VH-71 and stability of a humanised anti-MUC1 scFv**

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![Figure 7](image)

**Figure 7** Cytotoxicity of rapLRI-G4S-4.9M fusion protein. Various concentrations of the fusion protein or RNase alone were incubated with MCF7 cells for 3 days and protein synthesis measured by 

**DISCUSSION**

The aim of this study was to generate a functional scFv molecule from the clinically established humanised anti-MUC1 mAb huHMFG1. For clinical applications, scFv molecules must have sufficient affinity for the target antigen and remain stable in the human body for at least several hours. Failure to meet these criteria was shown to result in insufficient enrichment of scFv molecules in xenografted solid tumours in immunodeficient mice (Adams et al, 1998; Willuda et al, 1999), thus hampering future clinical applications.

We initially generated an scFv molecule from the variable domains of the humanised mAb huHMFG1 that specifically bound to MUC1+ tumour cells but did not exhibit sufficient stability. It has been reported that the length and amino-acid composition of scFv linker peptides can influence both the stability and solubility of scFv fragments (Robinson and Sauer, 1998). To assess whether the linker peptide or domain orientation may have contributed to the poor stability of the wild-type sequence scFvs, we systematically varied linker length, domain orientation, and eventually amino-acid composition of the linker by a phage display approach. None of these approaches, however, resulted in the generation of a stable molecule.

Sequence analysis of the humanised wild-type scFv revealed framework 3 residue VH-71Arg to be potentially critical for disrupting the structural integrity of the grafted mAb VH domain and for loss of antigen binding of a humanised anti-CD18 antibody (Hsiao et al, 1994). In this study, we also observed minor effects on antigen-binding properties of the VH-71Arg→Ala mutant scFv. Taken together, these data indicate that the residue type at VH-71 alone may not invariantly fix the spatial orientation of CDR-H1/CDR-H2 and thus may not always play an important role in influencing antigen-binding properties of an antibody. Surprisingly however, the mutant scFv fragments exhibited a 1.9-fold decline in antigen-binding affinity after short exposure to physiological body temperature. This phenomenon could be observed after preincubation at 37°C in either human serum or PBS. Because protein degradation could be excluded by analytical size-exclusion chromatography, it is conceivable that this phenomenon most likely occurred through a temperature-dependent conformational alteration of the antigen-binding site of the molecule.

The stabilised monovalent scFv antibody 4.9M exhibited 45-fold reduced binding affinity when compared with the parental humanised IgG. As shown for bivalent diabodies derived from scFvs with different affinities for binding to ErbB2, the increment in binding affinity upon dimerisation was greatest for the lowest affinity scFv (65-fold) and least for the highest affinity scFv (7.7-fold) (Nielsen et al, 2000). In keeping with this observation, a phage display library-selected Fab fragment with low affinity for binding to MUC1 exhibited an even 160-fold improved apparent $K_d$ after being re-engineered into a bivalent fully human IgG molecule (Henderikx et al, 2002). Similarly, reformattting the mutant scFv 4.9M characterised in this study into a bivalent diabody resulted in a molecule with high affinity binding to native MUC1 (Khu and J Krauss, unpublished results). These data indicate that the moderate binding affinity of the scFv and derived fusion proteins can be markedly enhanced by generating bivalent derivatives.

Although residue VH-71 has been described as a major determinant for the CDR-H1 and CDR-H2 conformation and thus important for antigen binding, we are not aware of any reports indicating that this site also has an impact on the stability of antibody scFv fragments. As our data clearly show, the replacement of the bulky side-chain residue arginine by alanine at VH-71 dramatically increased the stability of the scFv fragments irrespective of the domain orientation. As a result, both mutant variants exhibited immunoreactivity with tumour cells even after 7 days of incubation in human serum at 37°C. The magnitude of stability improvement was surprising since the above-described location of VH-71 and its structural role did not necessarily suggest this residue to be of such critical importance for providing stability to the humanised scFv fragment. To study the consequences of the VH-71Arg→Ala mutation on the scFv structure, three computer homology models were generated. The models revealed the buried surface area between CDR-H1 and CDR-H2 to be significantly larger (approximately 20%) in the VH-71Ala variant, resulting in a markedly stabilised VH domain of the mutant scFvs.

Since previous attempts to display the wild-type scFv huHMFG1 on phage were unsuccessful, we analysed whether this failure was caused by the insufficient stability of the fragment. Indeed, our data show that only the stable mutant scFv recognised native
antigen, indicating that appropriate intrinsic stability of the molecule was absolutely required for being displayed on phage in correctly folded conformation. The possibility to express the mutant scFv on phage paves the way for generating entire human anti-MUC1 fragments by chain shuffling of a human phage display library.

Ribonucleases have become established potent anticancer reagents within the last few years. In a recently conducted multicentre phase II clinical trial, a ribonuclease very closely related to rapLRI used in this study, Ranpirnase (onconase), was administered to more than 100 patients with unspectacular malignant mesothelioma (Mikulski et al., 2002). Overall response rates of about 50% were achieved. When given intravenously on a weekly basis, in some cases for more than 3 years, onconase was remarkably well tolerated in the vast majority of patients, with no appreciable adverse side effects attributed to immunogenicity of the reagent. Factors accounting for the low immunogenic potential of onconase may include its considerable sequence homology to human ribonucleases and the small size, basicity, and lack of glycosylation of the molecule. RapLRI, as a very close homologue of onconase, might therefore not be expected to exhibit significant immunogenicity in humans despite its amphibian origin. We have previously shown that rapLRI exhibits cytotoxic properties comparable to those of onconase and that its potency could be dramatically increased both in vitro and in vivo after conjugation to mAbs (Hursey et al., 2002). In contrast to onconase, rapLRI does not require an N-terminal pyroglutamate in order to retain its full activity (Hursey et al., 2002), suggesting this ribonuclease to represent a most promising candidate for fusion with small-sized antibody fragments. By showing that only a recombinant rapLRI antibody fusion protein made with a stable scFv could be purified to homogeneity and exhibited potent cytotoxic activity, this study clearly illustrates the importance of addressing the stability properties of scFv fragments intended to be used for the generation of recombinant fusion proteins.

Since most recent results indicate strong in vivo activity of an immunoconjugate comprising the parental humanised mAb huHMFG1 and rapLRI in tumour-xenotransplanted immunodeficient mice (DL Newton, unpublished results), we believe that the novel, stable immunoenzyme derivative merits further investigation as a therapeutic agent for patients with MUC1+ malignancies.

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REFERENCES

Aboud-Pirak E, Sergent T, Otte-Slachmuylder C, Abarca J, Troout A, Schneider VJ (1988) Binding and endocytosis of a monoclonal antibody to a high molecular weight human milk fat globule membrane-associated antigen by cultured MCF-7 breast carcinoma cells. Cancer Res 48: 3188 – 3196

Adams GP, Schier R, Marshall K, Wolf EJ, McCall AM, Marks JD, Weiner LM (1998) Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. Cancer Res 58: 485 – 490

Al-Yasi AR, Krauss J, Schwarzenbacher R, Vu BK, Greene S, Rybak SM (2003) Generation of a highly stable, internalizing anti-CD22 single-chain Fv fragment for targeting non-Hodgkin’s lymphoma. Int J Cancer 107: 822 – 829

Baldus SE, Monig SP, Hanisch FG, Zilken G, Madejezick B, Thiele J, Schneider PM, Holscher AH, Dienes HP (2002) Comparative evaluation of the prognostic value of MUC1, MUC2, sialyl-Lewis(a) and sialyl-Lewis(x) antigens in colorectal adenocarcinoma. Histopathology 40: 440 – 449

Benedict CA, MacKrell AJ, Anderson WF (1997) Determination of the binding affinity of an anti-CD34 single-chain antibody using a novel, flow cytometry based assay. J Immunol Methods 201: 223 – 231

Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE (2000) The protein data bank. Nature Struct Biol 7: 521 – 523

Brossart P, Schneider A, Dill P, Schammann T, Grunebach F, Wirths S, Kanz L, Brügger AT, Rice LM (1997) Crystallographic refinement by simulated annealing: methods and applications. Methods Enzymol 277: 243 – 269

Carter P, Presta L, Gorman CM, Reddy JB, Hener D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci USA 89: 4285 – 4289

Chen S, Le SY, Newton DL, Maizel Jr JV, Rybak SM (2000) A gender-specific miRNA encoding a cytotoxic ribonuclease contains a 3’ UTR of unusual length and structure. Nucleic Acids Res 28: 2375 – 2382

Chothia C, Gelfand I, Kister A (1998) Structural determinants in the sequences of immunoglobulin variable domain. J Mol Biol 278: 457 – 479

Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, Air G, Sheriff S, Padlan EA, Davies D, Tulip WR, Colman PM, Spinelli S, Alzari PM, Poljak RJ (1989) Conformations of immunoglobulin hypervariable regions. Nature 342: 877 – 883

Chothia C, Novotny J, Brucoleri R, Karplus M (1985) Domain association of single-chain Fv antibodies. J Mol Biol 186: 651 – 663

Epenetos AA, Hird V, Lamberts H, Mason P, Coulter C (2000) Long term survival of patients with advanced ovarian cancer treated with intraperitoneal radioimmunotherapy. Int J Gynecol Cancer 10: 44 – 46

Eisner JM, 2003) Domain association in immunoglobulin molecules. The packing of variable domains. J Mol Biol 186: 651 – 663

Fleury D, Daniels RS, Skehel JH, Knossow M, Bizebard T (2000) Structural evidence for recognition of a single epitope by two distinct antibodies. Proteins 40: 572 – 578

Foote J, Winter G (1992) Antibody framework residues affecting the conformation of the hypervariable loops. J Mol Biol 224: 487 – 499

Gendler S, Taylor-Papadimitriou J, Duhig T, Rothbard J, Burchell J (1988) A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. J Biol Chem 263: 12820 – 12823

Hanisch FG, Muller S (2000) MUC1: the polymorphic appearance of a human mucin. Glycobiology 10: 439 – 449

Henderix P, Coolen-van Neer N, Jacobs A, van der Linden E, Arends JW, Mullberg J, Hoogenboom HR (2002) A human immunoglobulin G1 antibody originating from an in vitro-selected Fab phage antibody binds avidly to tumor-associated MUC1 and is efficiently internalized. Am J Pathol 160: 1597 – 1608

Holmes MA, Buss TN, Foote J (2001) Structural effects of framework mutations on a humanized anti-lysozyme antibody. J Immunol 167: 296 – 301
Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. 

Gene 77: 51 – 59

Hsiao KC, Bajorath J, Harris LJ (1994) Humanization of 60.3, an anti-CD18 antibody; importance of the L2 loop. Protein Eng 7: 815 – 822

Hussey M, Newton DL, Hansen HJ, Ruby D, Goldenberg DM, Rybak SM (2002) Specifically targeting the CD22 receptor of human B-cell lymphomas with RNA damaging agents: a new generation of therapeutics. Leuk Lymphoma 43: 953 – 959

Jones TA, Kjeldgaard M (1997) Electron-density map interpretation. Methods Enzymol 277: 173 – 208

Kipriyanov SM, Kupriyanova OA, Little M, Moldenhauer G (1996) Rapid detection of recombinant antibody fragments directed against cell-surface antigens by flow cytometry. J Immunol Methods 196: 51 – 62

Leger OJ, Rednok TA, Tanner L, Horner HC, Hines DK, Keen S, Saldanha J, Jones ST, Fritz LC, Bendig MM (1999) Humanization of a mouse antibody against human alpha-4 integrin: a potential therapeutic for the treatment of multiple sclerosis. Hum Antibodies 8: 3 – 16

Marks JD, Griffiths AD, Malmqvist M, Clackson TP, Bye JM, Winter G (1992) By-passing immunization: building high affinity human antibodies by chain shuffling. Biotechnology (NY) 10: 779 – 783

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol 222: 581 – 597

Martin AC, Thornton JM (1996) Structural families in loops of homologous proteins: automatic classification, modelling and application to antibodies. J Mol Biol 263: 800 – 815

Merritt EA, Bacon DJ (1997) Raster3D: photorealistic molecular graphics. Methods Enzymol 277: 505 – 524

Mikulski SM, Costanzi JJ, Vogelzang NJ, McCachren S, Taub RN, Chun H, Mittelman A, Panella T, Puccio C, Fine R, Shogen K (2002) Phase II trial of a single weekly intravenous dose of ranpirnase in patients with unresetable malignant mesothelioma. J Clin Oncol 20: 274 – 281

Milenic DE, Yokota T, Filpula DR, Finkelman MA, Dodd SW, Wood JF, Whitlow M, Snos P, Schlam J (1991) Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49. Cancer Res 51: 6363 – 6371

Newton DL, Xue Y, Olson KA, Fett JW, Rybak SM (1996) Angiogenin single-chain immunofusions: influence of peptide linkers and spacers between fusion protein domains. Biochemistry 35: 545 – 553

Nielsen UB, Adams GP, Weiner LM, Marks JD (2000) Targeting of bivalent anti-ErbB2 diabody antibody fragments to tumor cells is independent of the intrinsic antibody affinity. Cancer Res 60: 6434 – 6440

Pei XY, Holliger P, Murzin AG, Williams RL (1997) The 2.0-Å resolution crystal structure of a trimeric antibody fragment with noncognate VH-VL domain pairs shows a rearrangement of VH CDR3. Proc Natl Acad Sci USA 94: 9637 – 9642

Price MR, Hudecz F, O’Sullivan C, Baldwin RW, Edwards PM, Tendler SJ (1990) Immunological and structural features of the protein core of human polymorphic epithelial mucin. Mol Immunol 26: 795 – 802

Robinson CR, Sauer RT (1998) Optimizing the stability of single-chain proteins by linker length and composition mutagenesis. Proc Natl Acad Sci USA 95: 5929 – 5934

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

Suh SW, Bhat TN, Navia MA, Cohen GH, Rao DN, Rudikoff S, Davies DR (1986) The galactan-binding immunoglobulin Fab F339: an X-ray diffraction study at 2.6-Å resolution. Proteins 1: 74 – 80

Taylor-Papadimitriou J, Burchell J, Miles DW, Dalziel M (1999) MUC1 and cancer. Biochim Biophys Acta 1455: 301 – 313

Taylor-Papadimitriou J, Peterson JA, Arklie J, Burchell J, Ceriani RL, Bodmer WF (1981) Monoclonal antibodies to epithelium-specific components of the human milk fat globule membrane: production and reaction with cells in culture. Int J Cancer 28: 17 – 21

Tramontano A, Chothia C, Lesk AM (1990) Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins. J Mol Biol 215: 175 – 182

Utsunomiya T, Yonezawa S, Sakamoto H, Kitamura H, Aiko T, Tanaka S, Irimura T, Kim YS, Sato E (1998) Expression of MUC1 and MUC2 mucins in gastric carcinomas: its relationship with the prognosis of the patients. Clin Cancer Res 4: 2605 – 2614

Verhoeven ME, Saunders JA, Price MR, Marugg JD, Briggs S, Broderick EL, Eida SJ, Moeren AT, Badley RA (1993) Construction of a reshaped HMFG1 antibody and comparison of its fine specificity with that of the parent mouse antibody. Immunology 78: 364 – 370

Willsa J, Honegger A, Waiel R, Schubiger PA, Stahel R, Zangemeister-Wittke U, Pluckthun A (1999) High thermal stability is essential for tumor targeting of antibody fragments: engineering of a humanized anti-epithelial glycoprotein-2 (epithelial cell adhesion molecule) single-chain Fv fragment. Cancer Res 59: 5758 – 5767

Xiang J, Bia Y, Jia Z, Prasad L, Delbaere LT (1995) Framework residues 71 and 93 of the chimeric B72.3 antibody are major determinants of the conformaion of heavy-chain hypervariable loops. J Mol Biol 253: 385 – 390

Yokota T, Milenic DE, Whitlow M, Schlom J (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immuno-globulin forms. Cancer Res 52: 3402 – 3408