Clinical applications of phage-derived sFvs and sFv fusion proteins

K.A. Chester*, J. Bhatia, G. Boxer, S.P. Cooke, A.A. Flynn, A. Huhalov, A. Mayer, R.B. Pedley, L. Robson, S.K. Sharma, D.I.R. Spencer and R.H.J. Begent
CRC Targeting and Imaging Group, Department of Oncology, RFUCMS, University College London, Royal Free Campus, London NW3 2PF, UK

Single chain Fv antibodies (sFvs) have been produced from filamentous bacteriophage libraries obtained from immunised mice. MFE-23, the most characterised of these sFvs, is reactive with carcinoembryonic antigen (CEA), a glycoprotein that is highly expressed in colorectal adenocarcinomas. MFE-23 has been expressed in bacteria and purified in our laboratory for two clinical trials; a gamma camera imaging trial using $^{123}$I-MFE-23 and a radioimmunoguided surgery trial using $^{125}$I-MFE-23, where tumour deposits are detected by a hand-held probe during surgery. Both these trials show MFE-23 is safe and effective in localising tumour deposits in patients with cancer. We are now developing fusion proteins which use MFE-23 to deliver a therapeutic moiety; MFE-23::CPG2 targets the enzyme carboxypeptidase G2 (CPG2) for use in the ADEPT (antibody directed enzyme prodrug therapy) system and MFE::TNFα aims to reduce sequestration and increase tumor concentrations of systemically administered TNFα.

Keywords: Antibody targeting, cancer, ADEPT, CEA, sFv, fusion protein

1. Introduction

Antibodies are highly specific recognition molecules which are increasingly being applied to target therapy in patients [1]. Single chain Fv antibody fragments (sFv), consisting of the variable heavy (VH) and variable light (VL) chain regions tethered by a flexible linker, are small fragments of antibody that retain full antigen binding capacity (Fig. 1). Since sFv are expressed as a single molecule they make ideal targeting units for fusion proteins with therapeutic as well as disease-localising properties. Moreover, recombinant sFvs can be displayed in functional form on the surface of filamentous phage, and this technology may be used to generate and select for antibodies with desired characteristics [2]. The phage approach is much more efficient than the screening approach used in hybridoma technology; whilst hybridoma screening allows investigation of $10^2$ to $10^3$ clones, phage technology selects antibodies with desired characteristics from $10^{10}$ or more clones. Pragmatically, the phage selection process is also advantageous as it favors stable antibodies which give high yields when expressed in bacteria and whose genes are already cloned.

2. Using phage technology to create a clinically useful sFv

Phage technology was used to produce MFE-23 [3] an sFv antibody with high affinity for carcinoembryonic antigen (CEA / CD66e). CEA is a tumour selective marker which is highly expressed on most gastrointestinal carcinomas and on a number of breast, lung and ovarian carcinomas. Monoclonal antibodies to CEA, and their chemically modified derivatives, have been used successfully in our laboratories to target colorectal tumours [4]. Our understanding of the parameters important for targeting this antigen forms the platform for current work with recombinant sFvs. MFE-23 anti-CEA is the most characterised of these sFvs and is the first sFv to be reported for use in clinical trials [5]. Here we use MFE-23 to illustrate the clinical applications of phage-derived sFvs and sFv fusion proteins.
MFE-23 was chosen from an anti-CEA sFv phagemid library constructed from antibody variable-region genes extracted from CEA-immunised mice [3]. The library contained $1.3 \times 10^7$ independent clones and was constructed by the two fragment assembly method [6] in pHEN phagemid [7] using murine V-region primers [8] to rescue the antibody genes (Fig. 2). MFE-23 was selected from this library by reacting the phage with low concentrations of biotinylated CEA followed by capture of the CEA binders on streptavidin-coated magnetic beads [3]. These conditions were predicted to favour selection of high affinity sFv [9] and, consistent with this, MFE-23 was shown to have high affinity for CEA after purification in soluble form (kD = 2.4 nM).

3. Clinical trials with MFE-23

Clinical grade MFE-23 was purified from bacterial supernatant by immobilised metal affinity chromatography (IMAC) and size exclusion. IMAC exploits the ability of histidines to bind metal ions so MFE-23 was first engineered to include a c-terminal hexahistidine tag to allow binding to chelated copper [10]. Production was according to Cancer Research Campaign Guidelines for the preparation of products derived from recombinant DNA technology for phase 1 trials [11]. The expression plasmid was pUC 119 and the bacterial host Escherichia coli TG1 cells. Details of the production procedure, including SOPs (standard operating procedures) have been published elsewhere [12] and can be used to illustrate practice of the Cancer Research Campaign Guidelines [11].

The first clinical trial aimed to test safety and efficacy of radiolabelled MFE-23 for imaging CEA-expressing tumours. Ten patients received $^{123}$I-MFE-23 prior to single photon emission computerised tomography (SPECT) imaging [5]. The median half lives of the $\alpha$ and $\beta$ phases of clearance from blood were 0.42 hours and 5.32 hours respectively. The antibody was stable in plasma and most of the antibody cleared from the circulation within 1 hour. MFE-23 localised in all known tumour deposits as illustrated in Fig. 3 which shows examples of gamma camera SPECT images obtained in patients with and without liver metastasis. The median peak tumour uptake of 2.4% of the injected radioactivity kg$^{-1}$ occurring 1 hour after injection was unexpectedly high in view of the rapid fractional blood clearance of MFE-23, but is consistent with good tumour penetration and efficient antigen binding of the sFv. The antibody injection was well tolerated by all
Fig. 2. Stages in sFv phage library construction are shown. Splenic lymphocytes are removed from an immunised mouse four days after the final antigen boost. mRNA is extracted and VH and VL cDNA independently produced using murine VH and VL primers in antibody framework region 4 [8]. The VH and VL fragments are subsequently amplified by PCR using murine V-region primers in frameworks 1 and 4 [8] and tethered by a (Gly₄Ser)₃ linker into the VH-VL sFv format using the two fragment assembly method [6]. To achieve cloning, the V-region primers were extended to create flanking SfiI/NotI restriction sites suitable for insertion into pHEN phagmid [7]. Cloned sFvs are displayed on the surface of phage as functional antigen-binding proteins; each phage carries the gene for an individual sFv, although it may express more than 1 copy of that sFv on its surface.

patients, and there was no evidence of anti-MFE-23 antibody formation in patients after 2 weeks. High kidney uptake was found to be a shortcoming in this trial.

A subsequent trial used ¹²⁵I-labeled MFE-23 for radioimmunoguided surgery (RIGS) of colorectal cancer [13]. RIGS is based on the preoperative injection of a radiolabelled antibody and intraoperative use of a hand-held gamma detecting probe. The technique has the advantage that the proximity of the probe exploits the inverse square law and therefore has the potential to detect small tumour deposits. Thirty four patients suffering from primary or metastatic colorectal or pancreatic cancer received ¹²⁵I-labeled MFE-23 at 24, 48, 72 and 96 hours prior to operation. Counts of three standard deviations above normal tissue were regarded as positive and results of probing were compared with histology for validation. Laboratory gamma counting acted as quality control and allowed calculation of the % injected dose/kg tissue, tumour to normal tissue ratio and tumour to blood ratio. MFE-23 showed good tumour localisation, comparison with histology showed 82.4% true positive, 3.9% true negative and 13.7% false negative findings. There were no false positive findings. A sensitivity of 100%, 62.5%, 95.7% and 84.6% at 24, 48, 72 and 96 hours, respectively was calculated [13]. Since tumour was removed during surgery, RIGS offered the opportunity to study uptake of ¹²⁵I-MFE-23 in excised tissues and localisation to tumour was confirmed in RIGS as illustrated in Fig. 4.

4. Multivalent molecules

Effective targeted therapy relies on efficient antibody retention in tumour after clearance from normal tissue. Using image registration techniques which allow corre-
Excised tissue

Tumour

Phosphor Image

Fig. 4. Localisation of $^{125}$I-MFE-23 in liver metastasis surgically removed from a patient receiving RIGS. A photograph of the specimen is shown in (a) and the corresponding phosphor image, denoting radioactivity, is shown in (b).

relation of radiolabelled antibody distribution with tumour morphology [14] we have investigated the importance of valency with chemically modified anti-CEA fragments [15]. We found that increasing the number of binding arms (avidity/valency) in an antibody molecule increased the amount of antibody and its residence time in viable areas of tumour as shown in Fig. 5. This knowledge has formed our thinking about design of multivalent recombinant antibody-based molecules and led to the choice of divalent and trivalent molecules for therapy because an increase in antibody concentration in viable compared to necrotic areas signifies a distinct therapeutic advantage. We are thus investigating the possibility of increasing the avidity of MFE-23 by incorporation into high affinity dimers. This may be achieved by either using Chelating Recombinant Antibodies (CRAbs) which recognise and bind to adjacent, non-overlapping epitopes [16] or by increasing avidity using homodimers of MFE-23. To produce these molecules, we designed a vector, illustrated in Fig. 6, with the VH and VL domains of MFE-23 in ‘reverse’ orientation (VL-VH) downstream of restriction sites for insertion of a second sFv in the original VH-VL orientation [17]. To test the integrity of the vector the original VH-VL MFE-23 was cloned into the designed restriction sites to create a homodimer of MFE-23. Expression in Escherichia coli produced a protein that bound to CEA and gave an expected band at 57 kDa, as seen in Fig. 6. The vector can be used to search sFv libraries for a second anti-CEA sFv, which will give a CRAb effect when cloned alongside the reversed MFE-23. Alternatively a more structural approach may be taken to building sFv multimers. Using the crystal structure of MFE-23 [18] and the homology model of CEA [19], combined with epitope mapping techniques we have developed [20] it should be possible to map where MFE-23 binds to CEA. This information can be used to design CRAb candidates more selectively and to tailor linkers that will facilitate an avidity effect when binding CEA.

Another approach to increasing avidity is to create genetic fusion proteins of recombinant antibody fragments and therapeutic moieties which form multimers as described below.

5. Antibody directed enzyme prodrug therapy (ADEPT)

ADEPT is a two stage system for treatment of solid tumours. First, intravenously administered antibody delivers an enzyme selectively to a tumour deposit. When there is high tumour to plasma ratios of enzyme a relatively non-toxic prodrug is administered systemically. The prodrug is catalysed by the pre-localised enzyme to produce a potent cytotoxic agent in the tumour (Fig. 7). The active drug diffuses throughout the tumour mass, killing cells expressing tumour antigen and neighboring antigen-negative tumour cells (the bystander effect). Carboxypeptidase G2 (CPG2), a well characterised, homo-dimeric, bacterial enzyme with no mammalian equivalent [21–23] has been exploited to cleave glutamic acid from a variety of prodrugs to release potent nitrogen mustards for ADEPT [24]. The F(ab’)$_2$ fragment of A5B7 (an anti-CEA monoclonal antibody) conjugated to CPG2 has shown that CPG2 has great potential for ADEPT, in model systems [25, 26] and in the clinic [27,28]. However, a clearing antibody was needed to obtain high tumour to normal tissue ratios at early time points when the percentage injected dose in tumour is at the highest. The system also suffered from the complexity, expense and difficulty of obtaining a reproducible product with chemical conjugation of the components [29] and the F(ab’)$_2$-CPG2 conjugate was immunogenic. Since CPG2 naturally forms a non-covalent dimer, it was proposed that genetic fusion protein of MFE-23 antibody and CPG2 would give a dimeric molecule which would have po-
Fig. 5. The amount of antibody in viable parts of the tumour relative to necrotic regions. Regions were delineated using image registration which allows correlation of radiolabelled antibody distribution with tumour morphology [14]. The increase in avidity obtained with increasing valency leads to higher functional affinity and better retention in viable parts. The group with zero avidity contained pooled data from non CEA-specific whole IgG antibodies. The monovalent group contained pooled data from MFE-23 and A5B7 (monoclonal anti-CEA) Fab. The divalent group contained pooled data from A5B7-IgG, A5B7-F(\(ab'\))\(_2\) and chemically crosslinked divalent A5B7 Fab fragments [15]. The trivalent molecule was made from chemically crosslinked A5B7 Fab fragments [15].

Potential to overcome these obstacles. Recombinant fusion proteins can be reproducibly expressed and have the potential to be tailored to control pharmacokinetics and overcome hurdles such as immunogenicity.

6. MFE-23::CPG2 fusion protein produced in bacteria

MFE-23::CPG2 was constructed by fusing the gene for MFE-23 to the gene for CPG2 as outlined in Fig. 8. The fusion protein was expressed in Escherichia coli and purified using CEA affinity chromatography [30]. Efficacy of MFE-23::CPG2 delivery to tumours in vivo was assessed by measuring CPG2 catalytic activity in excised tissue after intravenous injection of purified MFE-23::CPG2 into nude mice bearing CEA-positive LS174T human colon adenocarcinoma xenografts [31]. Recombinant MFE-23::CPG2 cleared rapidly from circulation and catalytic activity in extracted tissues showed tumour to plasma ratios of 1.5 : 1 (6 h), 10 : 1 (24 h), 19 : 1 (48 h) and 12 : 1 (72 h). MFE-23::CPG2 catalytic activity was not retained in solid normal tissues resulting in excellent tumour to tissue enzyme ratios 48 h after injection. These were 371 : 1 (tumour to liver), 450 : 1 (tumour to lung), 562 : 1 (tumour to kidney), 1, 477 : 1 (tumour to colon) and 1, 618 : 1 (tumour to spleen). The favorable tumour : normal tissue ratios occurred at early time points when there was still 21% (24 h) and 9.5% (48 h) of the injected activity present/g tumour tissue. These results show that MFE-23::CPG2 delivers satisfactory quantities, with high tumour to normal tissue ratios, of CPG2 activity after a single injection to mice bearing human colorectal tumour xenografts. The supremacy of the recombinant fusion protein is illustrated when its localisation is compared with that of a chemical conjugate of CPG2 with the F(\(ab'\))\(_2\) fragment of A5B7 anti-CEA as shown in Fig. 9. Here we see over 10-fold improvement in tumour:normal tissue ratios with the fusion protein for all organs tested 24 hours after administration. In addition, there was substantially more enzyme delivered to the tumour by the fusion protein than the chemical conjugate (3.2% v. 21% injected activity/g tissue). The high tumour concentrations and selective tumour retention of active enzyme established that this recombinant fusion protein has potential to give improved clinical efficiency for ADEPT.

7. MFE-23::CPG2 fusion protein produced in yeast

To achieve yields of MFE-23::CPG2 which would be high enough to support a clinical trial, a yeast (Pichia pastoris) expression system was used. Yields of MFE-
23::CPG2 improved over 100-fold using this system and the resulting material was glycosylated. Biodistribution studies of enzyme activity in nude mice bearing the LS174T xenograft, given intravenous injection of MFE-23::CPG2 fusion protein (1000 units/kg), showed that glycosylated MFE-23::CPG2 cleared rapidly from plasma within 4–6 hours after injection. Enzyme activity persisted in tumours, however, (1.3 ± 0.1 units/g at 6 h) resulting in tumour to plasma ratio of 250 : 1. This allowed produg to be given as early as 4–6 hours after fusion protein injection, resulting in growth delay of the LS174T xenografts with minimal toxicity in mice [32].

8. Reduced immunogenicity

Immunogenicity has been a major problem in ADEPT using CPG2 chemically conjugated to A5B7-F(ab')2. Treatment has so far been limited to 2 to 3 cycles within 2 weeks under cover of an immunosuppressive agent, cyclosporin [33]. As part of our program to identify and modify immunogenic sites on CPG2, a phage library of sFv antibodies to CPG2 was constructed from CPG2 immunised mice in the manner described for CEA in Fig. 2. Different sFvs reacting with the enzyme were identified by ELISA using CPG2 covered plates. One of these sFvs, CM79, inhibited a substantial part of the CPG2 binding by sera of patients who had developed an immune response to CPG2 during ADEPT. The discontinuous epitope on CPG2 that was recognised by CM79 was identified [20] using surface enhanced laser desorption ionisation affinity mass...
Antigen
Tumour cell
Enzyme
Antibody
ADEPT
Prodrug
Glutamate
Mustard drug
DNA cross links
Tumour cell

First Stage
Second Stage

Fig. 7. The two stage process of ADEPT is illustrated. In the first stage, antibody-enzyme conjugate is localised to antigen-bearing cancerous cells in the tumour and is allowed to clear from normal tissues. In the second stage, a non-toxic prodrug is administered. The prodrug is converted to active drug only in areas where the enzyme is localised. In the specific case of CPG2, which is a dimeric metalloenzyme, prodrug activation is achieved by cleavage of C-terminal glutamic acid to yield a potent cytotoxic mustard drug. The active drug kills antigen-positive cells and neighbouring antigen-negative cells, which overcomes problems of tumour heterogeneity and the difficulty of targeting to all cells in a tumour mass.

Fig. 8. The cartoon depicts stages in making an sFv-enzyme fusion protein for ADEPT. First, MFE-23, a CEA reactive sFv was selected from a phage library using biotinylated CEA and streptavidin-coated magnetic beads [3]. Second, MFE-23 was cloned as a direct fusion with the enzyme CPG2 and expressed in \textit{Escherichia coli} [30]. CPG2 forms a dimer in its natural state so the resultant fusion protein has potential avidity with respect to CEA binding. MFE-23:CPG2 is an effective delivery molecule for ADEPT [31,32].

spectrometry (SELDI-AMS) as follows: a metal chip coated with CM79 was used to capture CPG2 and the immune complex was subjected to selective proteolysis. During this process, CPG2 peptides reacting with the CM79 sFv are protected from proteolysis and these were subsequently desorbed from the chip and identified by their mass. Two peptides were found to form the epitope for CM79 and we were able to localise their position on the X-ray crystal derived model of CPG2 [22] where they were found to be adjacent on the
Fig. 9. Graph comparing performance (in separate experiments) of the recombinant fusion protein MFE-23::CPG2 with that of a chemical conjugate of CPG2 with the F(ab')₂ fragment of ASB7 anti-CEA. Tumour localisation was tested in vivo, 24 hours later, using the LS174T human colorectal xenograft model. 25U of enzyme activity were administered and enzyme levels in various excised organs measured by HPLC [31]. A 10 fold increase in tumour:normal tissue ratio is observed with the fusion protein for all organs tested.

surface of the enzyme. This epitope information was used to produce a series of CPG2 mutants which were tested for enzyme activity by a spectrophotometric assay and for CM79 antibody binding by ELISA. One of the CPG2 variants, where arginine 165 was replaced with an alanine, retained enzyme activity and had completely lost CM79 binding activity. The new variant of CPG2 is the first to prove that immunogenic sites of this clinically important enzyme can be removed without losing significant enzyme activity. Modifying the principle immunogenic sites on CPG2 may reduce overall immunogenicity but also gives potential to have alternative versions of the enzyme which can be given to patients who have an immune response to the original CPG2.

MFE-23 has not proved immunogenic in patients to date [5,13] but it is too early to predict immunogenicity of this sFv as part of MFE-23::CPG2 in ADEPT. Should this prove troublesome, the relevant epitopes on MFE-23 will be defined by SELDI as described above for CPG2, and a humanisation strategy will be applied based on the crystal structure of MFE-23 [18].

9. Selective delivery of tumour necrosis factor alpha (TNFα)

A potentially even more avidly binding fusion protein may be obtained when MFE-23 is linked to TNFα, a molecule which is trivalent in active form. Locoregional and intra-tumoural administration of TNFα has been shown to cause inhibition or regression of tumour growth in the clinic [34] but this potent anti-tumour activity of TNFα has not yet been exploited as a systemic agent in cancer therapy, mainly due to high levels of toxicity to normal tissues before a therapeutic dose has been achieved. We have constructed a genetic fusion of human recombinant TNFα with MFE-23. MFE-23::TNFα was expressed in bacterial inclusion bodies and successfully isolated, refolded and purified with a final yield of 28 mg/L. The MFE::TNFα monomer was shown to have an apparent molecular weight of 48 kDa by western blot under reducing conditions and the native molecule showed an FPLC elution profile consistent with trimer formation (144 kDa). The trimer possesses the antigen binding activity of the sFv and the cytotoxicity to WEHI 164 cells of rhTNFα. Radiolabelled MFE-23::TNFα localised effectively in nude (nu/nu) mice bearing human LS174T xenografts and tumour : tissue ratios of 21:1 and 60:1 were achieved at 24 and 48 hours (respectively) after intravenous injection [35] These studies indicate that MFE-23::TNFα will provide an effective means for systemically administered cancer therapy with TNFα.

10. Conclusions

The MFE-23 sFv has been successfully used to target CEA-expressing tumors in patients. MFE-23 will now be used as a targeting unit for multivalent fusion protein therapeutics with potential to selectively destroy cancerous tissue.
Acknowledgements

Work supported by the Cancer Research Campaign and the Ronald Raven Chair in Oncology Trust.

References

[1] M.J. Glennie and P.W.M. Johnson, Clinical trials of antibody therapy, *Immunology Today* 21 (2000), 403–410.
[2] G. Winter, A.D. Griffiths, R.E. Hawkins and H.R. Hoogenboom, Making antibodies by phage display technology, *Annu. Rev. Immunol.* 12 (1994), 433–455.
[3] K.A. Chester, R.H. Begent, L. Robson, P. Keep, R.B. Pedley, J.A. Boden, G. Boxer, A. Green, G. Winter and O. Cochet, Phage libraries for generation of clinically useful antibodies, *Lancet* 343 (1994), 455–456.
[4] A. Mayer, K.A. Chester, A.A. Flynn and R.H. Begent, Taking engineered anti-CEA antibodies to the clinic, *J. Immunol. Methods* 231 (1999), 261–273.
[5] R.H. Begent, M.J. Verhaar, K.A. Chester, J.L. Casey, A.J. Green, M.P. Napper, L.D. Hope-Stone, N. Cushen, P.A. Keep, C.J. Johnson, R.E. Hawkins, A.J. Hilson and L. Robson, Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library, *Nat. Med.* 2 (1996), 979–984.
[6] R.E. Hawkins, D. Zhu, M. Oveck, G. Winter, T.J. Hamblin, A. Long and F.K. Stevenson, Idiotype vaccination against human B-cell lymphoma. Rescue of variable region gene sequences from biopsy material for assembly as single-chain Fv personal vaccines, *Blood* 83 (1994), 3279–3288.
[7] H.R. Hoogenboom, A.D. Griffiths, K.S. Johnson, D.J. Chiswell, P. Hudson and G. Winter, Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains, *Nucleic Acids Res.* 19 (1991), 4133–4137.
[8] H. Zhou, R.J. Fisher and T.S. Papas, Optimization of primer sequences for mouse scFv repertoire display library construction, *Nucleic Acids Res.* 22 (1994), 888–889.
[9] R.E. Hawkins, J.S. Russell and G. Winter, Selection of phage antibodies by binding affinity, Mimicking affinity maturation, *J. Mol. Biol.* 226 (1992), 889–896.
[10] J.L. Casey, P.A. Keep, K.A. Chester, L. Robson, R.E. Hawkins and R.H. Begent, Purification of bacterially expressed single chain Fv antibodies for clinical applications using metal chelate chromatography, *J. Immunol. Methods* 179 (1995), 105–116.
[11] R.H. Begent, K.A. Chester, T. Connors, D. Crowther, B. Fox, E. Griffiths, T.A. Hince, J.A. Ledermann, J.G. McVie and P. Minor, Cancer Research Campaign operation manual for control recommendations for products derived from recombinant DNA technology prepared for investigational administration to patients with cancer in phase I trials, *Eur. J. Cancer* 29A (1993), 1907–1910.
[12] A. Mayer, K.A. Chester, J. Bhatia, R.B. Pedley, D.A. Read, G.M. Boxer and R.H. Begent, Exemplifying guidelines for preparation of recombinant DNA products in phase I trials in cancer: preparation of a genetically engineered anti-CEA single chain Fv antibody, *Eur. J. Cancer* 34 (1998), 966–976.
[13] A. Mayer, E. Tsiompanou, D. O’Malley, G.M. Boxer, J. Bhatia, A.A. Flynn, K.A. Chester, B.R. Davidson, A.A. Lewis, M.C. Winslet, A.P. Dhillon, A.J. Hilson and R.H. Begent, Radioimmunoguided surgery in colorectal cancer using a genetically engineered anti-CEA single-chain Fv antibody, *Clin. Cancer Res.* 6 (2000), 1711–1719.
[14] A.A. Flynn, A.J. Green, G.M. Boxer, J.L. Casey, R.B. Pedley and R.H. Begent, A novel technique, using radioluminography, for the measurement of uniformity of radiolabelled antibody distribution in a colorectal cancer xenograft model, *Int. J. Radiat. Oncol. Biol. Phys.* 43 (1999), 183–109.
[15] J.L. Casey, D.J. King, L.C. Chaplin, A.M. Haines, R.B. Pedley, A. Mountain, G.T. Yarranton and R.H. Begent, Preparation, characterisation and tumour targeting of cross-linked divalent and trivalent anti-tumour Fab’ fragments, *Br. J. Cancer* 74 (1996), 1397–1405.
[16] D. Neri, M. Momo, T. Prospero and G. Winter, High-affinity antibody-binding by chelating-recombinant antibodies (CRAbs), *Journal of Molecular Biology* 246 (1995), 367–373.
[17] A. Hunalov, L. Robson, S.J. Perkins, R.H.J. Begent and K.A. Chester, A vector for expression of dimeric and chelating forms of anti-CEA scFvs, *British Journal of Cancer* 80 (1999), 92.
[18] M.K. Boehm, A.L. Corper, T. Wan, M.K. Sohi, B.J. Sutton, J.D. Thornton, P.A. Keep, K.A. Chester, R.H. Begent and S.J. Perkins, Crystal structure of the anti-(carcinoembryonic antigen) single-chain Fv antibody MFE-23 and a model for antigen binding based on intermolecular contacts, *Biochem. J.* 346(2) (2000), 519–528.
[19] M.K. Boehm, M.O. Mayans, J.D. Thornton, R.H. Begent, P.A. Keep and S.J. Perkins, Extended glycoprotein structure of the seven domains in human carcinoembryonic antigen by X-ray and neutron solution scattering and an automated curve fitting procedure: implications for cellular adhesion, *J. Mol. Biol.* 259 (1996), 718–736.
[20] D.I.R. Spencer, L. Robson, J. Bhatia, S.K. Sharma, N.P. Michael, N.R. Whitelegg, A.R. Rees, K.A. Chester and R.H.J. Begent, Identifying immunogenic sites on aeph enzymes CPG2 using a scFv phage library & SELDI (TM)-AMS, *British Journal of Cancer* 80 (1999), 94.
[21] M.P. Minton, T. Atkinson, C.J. Bruton and R.F. Sherwood, The complete nucleotide-sequence of the pseudomonas gene coding for carboxypeptidase G2, *Gene* 31 (1984), 31–38.
[22] S. Rowssell, R.A. Paapeit, A.D. Tucker, R.G. Melton, D.M. Blow and P. Brick, Crystal structure of carboxypeptidase G(2), a bacterial enzyme with applications in cancer therapy, *Structure* 5 (1997), 337–347.
[23] R.F. Sherwood, R.G. Melton, S.M. Alwan and P. Hughes, Purification and properties of carboxypeptidase G2 from Pseudomonas sp. strain RS-16. Use of a novel trazine dye affinity method, *Eur. J. Biochem.* 148 (1985), 447–453.
[24] C.J. Springer, P. Antonin, K.D. Bagshawe, F. Searle, G.M.F. Bisset and M. Jaran, Novel produgs which are activated to cytotoxic alkylating- agents by carboxypeptidase-G2, *Journal of Medicinal Chemistry* 33 (1990), 677–681.
[25] S.K. Sharma, K.D. Bagshawe, P.J. Burke, J.A. Boden, G.T. Rogers, C.J. Springer, R.G. Melton and R.F. Sherwood, Galactosylated antibodies and antibody-enzyme conjugates in antibody-directed enzyme prodrug therapy, *Cancer* 73 (1994), 1114–1120.
[26] S.K. Sharma, K.D. Bagshawe, C.J. Springer, P.J. Burke, G.T. Rogers, J.A. Boden, P. Antonin, R.G. Melton and R.F. Sherwood, Antibody-directed enzyme prodrug therapy (ADEPT): a three phase system, *Dis.Markers* 9 (1991), 225–231.
[27] K.D. Bagshawe, S.J. Sharma, C.J. Springer and P. Antonin, Antibody-directed enzyme prodrug therapy: a pilot clinical trial, *Tumor Targeting* 1 (1995), 17–29.
[28] K.D. Bagshawe and R.H.J. Begent, First Clinical experience with ADEPT, Drug Delivery Reviews 22 (1996), 365–367.

[29] R.G. Melton, J.M.B. Boyle, G.T. Rogers, P. Burke, K.D. Bagshawe and R.F. Sherwood, optimization of small-scale coupling of A5B7-MONOCLONAL antibody to carboxypeptidase-G2, Journal of Immunological Methods 158 (1993), 49–56.

[30] N.P. Michael, K.A. Chester, R.G. Melton, L. Robson, W. Nicholas, J.A. Boden, R.B. Pedley, R.H. Begent, R.F. Sherwood and N.P. Minton, In vitro and in vivo characterisation of a recombinant carboxypeptidase G2::anti-CEA scFv fusion protein, Immunotechnology 2 (1996), 47–57.

[31] J. Bhatia, S.K. Sharma, K.A. Chester, R.B. Pedley, R.W. Boden, D.A. Read, G.M. Boxer, N.P. Michael and R.H. Begent, Catalytic activity of an in vivo tumor targeted anti-CEA scFv:carboxypeptidase G2 fusion protein Int, J. Cancer 85 (2000), 571–577.

[32] S.K. Sharma, R.B. Pedley, J. Bhatia, N.P. Minton, K.A. Chester and R.H.J. Begent, in vivo characteristics of an engineered fusion protein for use in ADEPT, British Journal of Cancer 83 (2000), 71–76.

[33] K.D. Bagshawe and S.K. Sharma, Cyclosporine delays host immune response to antibody enzyme conjugate in ADEPT, Transplant. Proc. 28 (1996), 3156–3158.

[34] F.J. Lejeune, C. Ruegg and D. Lienard, Clinical applications of TNF-alpha in cancer, Curr. Opin. Immunol. 10 (1998), 573–580.

[35] S.P. Cooke, R.B. Pedley, R. Boden, P. Holliger, G. Winter, R.H.J. Begent and K.A. Chester, Tumour targeting using MFE-23:TNF alpha fusion protein, British Journal of Cancer 80(2) (1999), 85–85.