Humanization of the anti-CEA T84.66 antibody based on crystal structure data

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Chimeric T84.66 (cT84.66) is a monoclonal antibody (mAb) of high specificity and affinity for the tumor-associated carcinoembryonic antigen (CEA). Radiolabeled cT84.66 has demonstrated utility in the clinic as a reagent for the radioimmunoimaging and radioimmunoassay of CEA-positive colorectal and breast malignancies. To extend the therapeutic efficacy of T84.66, humanization by complementary determining region (CDR) grafting was employed. CDR grafting is a well-established technique, though often a series of framework back-mutations is required to restore high affinity. Recently, the crystal structure of the T84.66 diabody (scFv dimer) derived from the murine T84.66 mAb was determined, facilitating the humanization process by the availability of crystal structure data for both the graft donor and graft acceptor. A search of the Protein Data Bank revealed close structural similarity (r.m.s.d. of 1.07 Å) between the Fv of T84.66 and the Fv of 4D5v8, a humanized anti-p185HER2 antibody marketed as Herceptin® (Trastuzumab). This resulted in two humanized versions of the T84.66 M5A and M5B mAbs that differed only in the number of murine residues present in the C-terminal half of CDR-H2. Biochemical analysis and animal biodistribution studies were conducted to evaluate the humanized mAbs. The M5A, M5B and cT84.66 mAbs showed sub-nanomolar affinity for CEA and as radiolabeled mAbs exhibited specific tumor localization in tumor bearing mice. The T84.66 M5A mAb was selected for clinical development due to a slightly higher tumor uptake and a larger content of human residues, and was renamed hT84.66. A limited-scale production and was renamed hT84.66. A limited-scale production and characterization study demonstrated hT84.66’s ability to support clinical trials. Planned clinical trials will determine the effective utilization of this structure-based approach in the development of a promising new therapeutic.

Keywords: anti-CEA/CDR grafting/humanization/radioimmunotherapy/T84.66

Introduction

A key issue for in vivo therapeutic use of monoclonal antibodies (mAbs) has been the response of the human immune system to xenogeneic antibodies. Clinical studies with murine monoclonal antibodies have shown effective tumor targeting but resulted in rapid clearance of the murine antibody in subsequent doses due to the generation of a human anti-murine antibody (HAMA) immune response (Schroff, 1985; Shawler, 1985). Recombinant methodologies have generated human–murine chimeric mAbs that have a lower frequency of an immunological response, but have not eliminated the problem. The current generation of humanized mAbs approved for therapy are the result of grafting murine-derived CDRs onto a human antibody framework (Jones, 1986; Low, 1996). New technological advances which generate human antibodies from human immunoglobulin phage display libraries (Winter, 1994) or transgenic animals (Bruggemann, 1991; Mendez, 1997) are under current clinical evaluation.

T84.66 is a murine mAb with high specificity and affinity for carcinoembryonic antigen (CEA), a well characterized human tumor-associated antigen (Wagener, 1983). Radiolabeled T84.66 evaluated in the clinic was able to image 69% of primary colorectal carcinomas prior to surgery (Beatty, 1986) but a HAMA response was observed (Morton, 1988). Genes for T84.66 were cloned and a human murine chimeric version (cT84.66) was expressed in mammalian cells (Neumaier, 1990). In a pilot imaging study for colorectal disease using a single administration, only 1 out of 29 patients exhibited a human anti-chimeric antibody response (Wong, 1997). However, as multiple administration radioimmunotherapy trials (Wong, 1995, 1999) have proceeded, an increase in the frequency of a HACA response has been noted.

While focusing on reducing the immune response, a key requisite was to retain the high specificity and affinity of the parental T84.66 mAb. This discouraged the selection of an entirely new mAb from phage display libraries of human immunoglobulin genes or transgenic animals. As such, humanization via grafting of murine CDRs onto a human Fv framework was pursued. There are many examples in the published literature of antibody humanization via CDR grafting (O’Brien, 2003). However, a substantial loss of antigen binding affinity was frequently observed due to steric clashes between human framework and mouse CDR residues, which altered the conformation of the antigen binding loops. These unanticipated clashes are a result of molecular models being used to design the graft rather than actual crystal structures of the graft donor and graft acceptor molecules. A laborious and oftentimes random iterative process of introducing back-mutations is often required to restore key murine framework residues responsible for maintaining the correct loop conformation (Foote, 1992).

Recently, the crystal structure of the murine T84.66 variable region was solved by X-ray diffraction analysis of the T84.66 diabody (scFv dimer) to a resolution of 2.6 Å (Carmichael, 2003). A search of the Protein Data Bank revealed close structural similarity to 4D5v8, a humanized anti-p185HER2 antibody marketed as Herceptin® (Trastuzumab). Computer graphics
visualization of the superimposed structures enabled the rational selection of graft splice junctions and framework back-mutations. Two versions were designed by CDR grafting: T84.66 M5A mAb and T84.66 M5B mAb. They differed only in the number of murine residues present in the C-terminal half of CDR-H2. Biochemical and animal biodistribution studies were used to compare the humanized mAbs to the chimeric version. The M5A, M5B and cT84.66 mAbs all had similar sub-nanomolar affinity for CEA and as radiolabeled mAbs exhibited specific tumor localization in athymic mice bearing CEA-positive-tumor xenografts. The T84.66 M5A was selected for pre-clinical production due to a slightly higher tumor uptake and a larger content of human residues and was renamed hT84.66 mAb. Scale-up production was conducted to demonstrate the capability of this clone to support clinical trials. Planned clinical trials will determine the effective utilization of this structure-based approach.

Materials and methods

**Humanization design**

Using molecular graphics technology, X-ray coordinates for a single T84.66 Fv unit were extracted from the coordinate set of the corresponding diabody structure (PDB file 1MOE). The Fv coordinate set was then submitted to VAST (Gibrat, 1996), a web-based least-squares structural alignment server, in order to identify a human or humanized Fv whose framework would serve as a suitable acceptor for the proposed CDR graft. Of the 20 antibodies that appeared in the list of homologous structures, five were selected for further consideration based on their high degree of alpha-carbon overlap with the query structure (r.m.s.d. between 1.00 and 1.25 Å). Next, residues defining the Kabat framework regions were identified and superimposed on the framework backbone atoms of the query structure (1312 atoms), resulting in r.m.s.d. values of 1.00 Å (PDB file 1CZ8), 1.00 Å (2FGW), 1.06 Å (1AD9), 1.06 Å (1FVC), and 1.06 Å (1BJ1). Given the closeness of these values, percent sequence identity (overall and framework only) was calculated as a second screening factor. The resulting values for 1CZ8 (63% overall, 54% framework), 2FGW, (61% and 54%), 1AD9 (65% and 60%), 1FVC (67% and 61%), and 1BJ1 (63% and 53%), suggested that 1FVC [humanized antibody 4D5, version 1.00 Å (2FGW), 1.06 Å (1AD9), 1.06 Å (1FVC), and 1.06 Å (1BJ1)].

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Visual inspection of the superimposed T84.66 and Herceptin Fv structures suggested that minimal disruption of the CDR loops could be achieved by deleting seven peptide segments (L24–L34, L50–L56, L66–L69, L89–L97, H30–H35, H50–H58, H93–H102) from the Herceptin structure (the graft acceptor Fv) and replacing the former with its murine equivalent (ala). The resulting humanized structure, M5A, was subjected to an energy minimization algorithm (conjugate gradients to a maximum derivative of 5.0 kcal/mol–Å) to optimize bond lengths and angles at the splice junctions. Including residues H59–H65 in the list of transplanted segments, so that the entire Kabat CDR-H2 loop from T84.66 was present in the grafted structure, resulted in a second humanized construct, M5B. To accommodate the additional segment, framework residue H67 (phe) was replaced with its murine equivalent (ala) to alleviate a steric clash with CDR-H2 residue H63 (phe).

**Molecular biology**

Splice overlap extension PCR (Horton, 1989) was used to create fully synthetic genes encoding the M5A and M5B Fv protein sequences. A schematic diagram of the PCR strategy is shown in Figure 1. Eight oligonucleotides (Integrated DNA Technologies, Inc., Coralville, IA) ranging in size from 79 to 89 bases were required for each domain construct. The degree of overlap between adjacent oligonucleotides corresponded to 30 base pairs. The PCR primer sequences for the variable light (VL) and variable heavy (VH) domains were:

**Fig. 1.** Schematic diagram of PCR primer strategy. Splice overlap extension PCR was used to create fully synthetic genes encoding the M5A and M5B Fv protein sequences. Eight oligonucleotides ranging from 79 to 89 bases were used for each variable light and variable heavy domain.
DNA polymerase (New England Biolabs, Beverly, MA), 50 μ
and 7, whereas the final extension utilized primers 1 and 8.
primers (3 and 6). The third extension utilized primers 2
variable domain gene. The internal-most pair of primers (4 and
VH5A
VH4
VH3
VH2
VH8
VH7
VH6
VH5B
VH1

Bebbington, 1992), had been previously modified to contain
mine synthetase (GS) gene (Lonza Biologics, Slough, UK;
and ligated into one of two expression plasmids (pEE12 for
full-length synthetic genes were digested with
reaction were used as the template. Individually, the purified
product extracted from a 200 mg gel slice using a Qiaquik
the completed PCR reaction mix was electrophoresed on a 1%

Four SOE–PCR amplifications were required to build each
variable domain gene. The internal-most pair of primers (4 and
5) was amplified first. The resulting PCR product was gel
purified and further extended with the next set of external
primers (3 and 6). The third extension utilized primers 2
and 7, whereas the final extension utilized primers 1 and 8.
Each 50 μl reaction contained reaction buffer, 2 U of Vent
DNA polymerase (New England Biolabs, Beverly, MA),
amplification primers at 1 μM each and dNTPs at 200 μM.
Using a GeneAmp PCR 9600 thermocycler (Perkin Elmer,
Wellesley, MA), samples were heated for 2 min at 94°C, fol-
lowed by 30 cycles of heating for 30 s at 94°C, 30 s at 55°C
and 30 s at 72°C. After 30 cycles, the temperature was held constant
at 72°C for 10 min to ensure complete extension. In each case
the completed PCR reaction mix was electrophoresed on a 1%
agarose gel (Sigma Chemical, St Louis, MO), and the desired
product extracted from a 200 mg gel slice using a Qiaquik
column (Qiagen, Valencia, CA). For the second, third and
fourth reactions, 10 ng of purified product from the preceding
reaction were used as the template. Individually, the purified
full-length synthetic genes were digested with XbaI and XhoI,
and ligated into one of two expression plasmids (pEE12 for
VL; pEE6 for VH). These plasmids, which contain the glu-
tamine synthetase (GS) gene (Lonza Biologics, Slough, UK;
Bebbington, 1992), had been previously modified to contain
the cDNA corresponding to the constant regions of a human
IgG1 antibody. In the pEE12 light chain plasmid, residue L104,
whose codon is part of the XhoI restriction site, was mutated
from leucine to valine using a QuikChange kit (Stratagene,
San Diego, CA) in order to restore the Herceptin sequence
in this region. A dual chain plasmid was constructed by digest-
ing the pEE6 heavy chain plasmid with BglII and BamHI to
isolate the heavy chain gene, which was then ligated into the
BamHI site of the pEE12 light chain plasmid. The entire IgG1
gene was sequenced in both directions to confirm its identity.
Prior to electroploration, the dual chain plasmid was linearized
with SalI, filtered through a protein binding membrane to
remove the restriction enzyme (Millipore, Bedford, MA), etha-
nol precipitated, and resuspended in sterile water to a concen-
tration of 1 μg/μl.

Expression in mammalian cell culture
The dual chain pEE12/6 expression vector was electroplorated
to murine myeloma NS0 cells following previously described
procedures (Bebbington, 1992; Yazaki, 2001). Selection of
transfectants in glutamine-free culture media (JRH Biosci-
ences, Kenexa, KN) resulted in numerous clones, which were
screened using a recombinant CEA fragment (Young,
1998) based ELISA (Yazaki, 2001). The M5A mAb was pro-
duced in a Cell Pharm (CP) 2000 hollow fiber bioreactor
according to the operator’s manual (Biovest International,
Minneapolis, MN). The CP2000 was equipped with a single
20 sq. ft hollow fiber cartridge (MW exclusion 10 kDa) and
10 sq. ft oxygenator. The pH, glucose, lactate, ammonia and
antibody production levels were monitored every other day.
Adjustments were made to the incoming O2 and CO2 levels to
maintain the pH between 7.0 and 7.2. IMDM media
(Biowhittaker, Walkerville, MD) supplemented with 2%
fetal bovine serum (FBS) (HyClone, Logan, UT) was used in
the intracapillary space (ICS). Selective GS medium (JRH
Bioscience, Lenexa, KS) + 2% FBS was used in the extracap-
illary space (ECS). The ICS feed rate was 1.0–2.2 l/day and the
recirculation rate was 350–500 ml/min. An Autoharvester
(Biovest International) was connected to the ECS and
programmed at a rate of 30–80 ml per day.

Antibody quantitation
A Protein A affinity column (Poros 20A, Applied Biosystems,
Foster City, CA; 0.46 cm i.d.×10 cm h, 0.5 ml/min) was used for
antibody quantitation. The column was equilibrated in PBS,
sample loaded, washed with 0.02 M sodium citrate/0.02 M
sodium phosphate pH 7.4/0.5 M sodium chloride, and 0.1 M
sodium citrate. The antibody was eluted with a linear gradient
from 100% 0.1 M sodium citrate to 100% 0.1 M citric acid.
Absorbance was monitored at 280 nm and antibody concentra-
tion was determined by comparison to a standard.

Purification
Individually, the cell culture harvests were clarified by batch
treatment (5% w/v) with the anion exchanger, AG1x8 (Bio-
Rad Laboratories, Hercules, CA). Initial capture was on a
Prosep rA column (Millipore, 1 cm×10 cm, 2.5 ml/min) pre-
equilibrated with PBS. The clarified harvest was loaded, and
washed with 0.02 M sodium citrate/0.02 M sodium phosphate
pH 7.4/0.5 M sodium chloride. The antibody was eluted with a linear gradient
from 100% 0.01 M sodium phosphate, pH 7.4, to
100% 0.01 M sodium phosphate, pH 4.0. The eluted material
was collected in tubes containing 0.05 M sodium phosphate,
pH 8.0 (10% w/v). The Protein A eluted peak was diazed
versus 0.05 M sodium phosphate pH 5.5 overnight, prior

VH3
VH2
VH1
VH4
VH7
VH6
VH5B
VH1

VH2
VH1

VH3
VH2
VH1
to loading on a Source 15S cation exchange column (GE Biosciences, Piscataway, NJ; 0.4 cm × 10 cm, 2 ml/min). The mAbs were eluted with a linear gradient from 0 to 0.4 M sodium chloride/0.05 M sodium phosphate, pH 5.5. The eluted antibody was collected in tubes containing 1 M Tris, pH 8.0 (10% v/v). Assayed by SDS–PAGE and HPLC size exclusion, the antibody-containing fractions were pooled and dialyzed overnight versus PBS.

**Biochemical characterization**

Aliquots of the purified M5A, M5B and cT84.66 mAbs were electrophoresed by SDS–PAGE (Laemmli, 1970). Size-exclusion chromatography was carried out on a Superdex 200 HR10/30 column (GE Biosciences, 0.5 ml/min) run isocratic with PBS. The column was standardized with gel filtration standards (Bio-Rad Laboratories). The isoelectric point of each mAb was determined on pH 3–10 IEF gels (Novex Inc., San Diego, CA) and compared with IEF protein standards (Serva Electrophoresis, Heidelberg, Germany).

The mAb’s kinetic affinity of binding to purified CEA was determined by surface plasmon resonance (SPR) on a BIAcore 1000 (BIAcore AB, Uppsala, Sweden). CEA was biotinylated using the EZ-Link Sulfo-NHS-LC-Biotin kit (Pierce, Rockford, IL) and immobilized to a SA biosensor chip (BIAcore Inc.) in HBS buffer. Kinetic affinity analysis was performed at a series of mAb concentrations (3.12, 6.25, 12.5, 25, 50 and 100 nM) injected over a low density (175 RU) of immobilized CEA-biotin with regeneration by a single pulse of 6 M guanidine hydrochloride. The data were analyzed by BIAevaluation (v3.0) software using the bivalent analyte model to calculate \( K_A = k_{on}/k_{off} \). Protein concentrations were determined by amino acid analysis.

**Radiolabeling**

M5A, M5B and cT84.66 mAbs were radioiodinated by the Iodogen method as previously described (Wu, 1996). The purity of the radiolabeled mAb was determined by monitoring the radiochromatogram of tandem Superose 6 HR10/30 columns (GE Biosciences). For \(^{131}\)I labeling, the M5A mAb was conjugated with \( N \)-hydroxysuccinimidyldOTA (Macrocycles, Dallas, TX) using previously described conditions (Lewis, 2001).

**Animal studies**

Groups of 7- to 8-week-old female athymic mice (Charles River Laboratories, Wilmington, MA) were injected subcutaneously in the flank region with \( 10^6 \) LS174T human colon carcinoma cells obtained from American Tissue Culture Center (ATCC, Manassas, VA). After 10 days, when tumor masses were in the range of 100–300 mg, 1–3 \( \mu \)Ci of \(^{131}\)I-labeled M5A, M5B or cT84.66 per animal were injected into the tail vein. At time points 0, 6, 24, 48, 72 and 96 h, the animals were euthanized, necropsy performed and organs weighed and counted for radioactivity. All data are mean values and have been corrected for radiodecay back to the time of injection, allowing organ uptake to be reported as a percent of the injected dose per gram (% ID g\(^{-1}\)) with standard errors.

For animal imaging studies, tumor-bearing mice were sedated, 20 \( \mu \)Ci \(^{111}\)InDOTA-M5A were injected intravenously and the mouse was placed ventral side down on a high-resolution miniature scintigraphic camera, \( \gamma \) IMAGER (BIOSPACE Mesures s.a., Paris, France). The \( \gamma \) IMAGER system was redesigned for our mouse imaging requirements. Modifications consisted of a continuous 120 mm diameter, 4 mm thick cesium iodide crystal that allows a circular 100 mm diameter field of view, and a parallel hole collimator specifically designed for \(^{111}\)In imaging. The sedation and imaging were repeated on days 1, 2, 3, 6 and 7.

**Biostatistical analysis**

To compare differences in M5A, M5B, and cT84.66 mAb uptake over time, two-way analysis of variance (ANOVA) was performed. The interaction between time and antibodies was included in the statistical model along with main effects for time and antibody. Dependent variables compared using this model included the percent injected dose per gram for the organs: blood, liver, spleen, kidney, lung, tumor and carcass analyzed separately. Tumor to blood ratios as well as tumor masses were recorded. To compare differences between the antibodies at specific time points, the protected least significant difference procedure was used. All significance testing was done at the 0.01 level, using SAS software (SAS Inc., Cary, NC).
Results and discussion

Humanization design

The structure of murine T84.66 (as an scFv dimer) was previously determined by X-ray diffraction analysis to a resolution of 2.6 Å (Carmichael, 2003). The availability of structural data greatly facilitated a CDR-grafting approach toward humanization. Normally, suitable VL and VH acceptor sequences are selected based on homology searches of the Kabat immunoglobulin sequence database (Johnson, 2001). In this study, a suitable acceptor structure was selected based on a T84.66 homolog search of the Protein Data Bank (Bernstein, 1977) using a vector-based structural alignment program (Gibrat, 1996). This ensured that a cognate VL:VH pair would be selected with a domain-pairing angle that matched that of T84.66, preserving the relative orientation of the heavy and light chain CDR loops. Of the antibodies that had homologous structures, humanized antibody 4D5, version 8...
(anti-p185HER2, Herceptin, PDB file 1FVC) (Eigenbrot, 1993) was selected as the most appropriate framework provider. The superimposed structures revealed a high degree of overlap with a r.m.s.d. of 1.07 Å for 1326 backbone atoms. Furthermore, the angle of VL–VH domain pairing was essentially the same for both structures as seen from two orthogonal views in Figure 2.

In designing the humanized T84.66 antibody, many structural features of the graft donor and acceptor Fvs were examined in order to better understand how various framework residues influence the conformation of the CDR loops and vice versa. Examination of T84.66, the graft donor, revealed that CDR loops L2, L3, H1 and H2 had canonical structures, and could therefore be transplanted without inducing a major change in structure or a significant loss of antigen binding affinity, as demonstrated by a recent crystallographic comparison of two humanized Fvs with their murine counterparts (Banfield, 1997). However, CDR loops L1 and H3 were observed to be structurally unique and therefore required closer scrutiny to determine how their unusual conformations were maintained. The 11-residue CDR-L1 loop resembled a canonical 13-residue loop, but the deletion of two residues from its tip caused an unpredicted twist in this region and a

Table 1. BIAcore analysis of the affinities of M5A, M5B and cT84.66 mAbs for CEA

<table>
<thead>
<tr>
<th>Antibody</th>
<th>$k_{\text{on}}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{\text{off}}$ (s$^{-1}$)</th>
<th>$K_A = k_{\text{on}}/k_{\text{off}}$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M5A</td>
<td>3.3x10$^5$</td>
<td>3.01x10$^{-5}$</td>
<td>1.1x10$^{10}$</td>
</tr>
<tr>
<td>M5B</td>
<td>4.88x10$^5$</td>
<td>2.56x10$^{-5}$</td>
<td>1.9x10$^{10}$</td>
</tr>
<tr>
<td>cT84.66</td>
<td>2.61x10$^5$</td>
<td>1.62x10$^{-5}$</td>
<td>1.6x10$^{10}$</td>
</tr>
</tbody>
</table>

SPR analysis by BIAcore was used to determine the kinetic affinities of the M5A, M5B and cT84.66 mAbs for CEA. Biotinylated CEA was coupled to a SA chip at a low density ($R_{\text{max}} = 175$ RU). Sensograms were analyzed using BIAeval 3.0 software.
disruption of the hydrogen bond network that stabilizes residues L30–L32. The structure of the 12-residue CDR-H3 loop was likewise not as predicted based on homologous structures, largely due to a rare proline residue located at framework position H94. Proline, lacking an amide hydrogen, is unable to participate in the hydrogen bonding network that normally stabilizes the base of most H3 loops and largely dictates their conformation (Morea, 1998). To compensate for its inability to hydrogen bond, the proline side chain joins a hydrophobic cluster formed by framework residues H2 (val), H4 (leu) and H27 (phe), plus CDR-H3 residues H99 (val) and H101 (tyr), thus causing the tip of the CDR-H3 loop (H98–H100A) to fold over rather than extend toward the combining site as anticipated for a loop of this length. We therefore made an effort to preserve all of these unique structural features in designing the humanized version of T84.66.

The structure of the graft acceptor was also closely examined to determine if any framework residues influenced the conformation of the CDR loops in an unexpected way. Structure data for Herceptin (as an Fv, determined to a resolution of 2.2 Å) (Carter, 1992; Eigenbrot, 1993), combined with site-directed mutagenesis data, confirmed the influence of five framework residues on CDR loop conformation and on relative binding affinity. Specifically, the authors of the study identified framework residues L66, H71, H73, H78 and H93 as being major determinants of CDR loop conformation. Arginine L66, which forms a salt bridge with the aspartate at L28 in CDR-L1 either interacts with antigen directly or stabilizes a specific conformation of CDR-L1 since replacing arginine with glycine results in a 4-fold decrease in binding affinity. Alanine H93, back-mutated to its mouse counterpart (serine), was...
shown to increase Herceptin’s affinity for antigen 2-fold. The structural consequences of humanizing framework residues H71, H73 and H78 in our hybrid construct could safely be ignored since these residues are the same in the donor and acceptor Fvs.

Upon superimposing the donor and acceptor Fv structures, it became clear that the peptide segments that needed to be transplanted did not necessarily correspond to the CDR loops defined by Kabat (1971) or Chothia (1987), nor the specificity determining region (SDR) loops defined by Padlan (1995). Rather, the segments simply corresponded to regions that differed in structure when the two Fv units were superimposed. In constructing the hybrid VL, the traditional Kabat CDR loop boundaries were not altered. However, a non-CDR peptide segment (L66–L69) from the donor was also transplanted onto the acceptor framework since residue L66 was shown to influence the conformation of Herceptin CDR-L1, as described above. In constructing the hybrid VH, all three CDR loop boundaries were altered. The Kabat definition of CDR-H1 (H31–H35) was expanded to include H30 since this residue packs against the tip of CDR-H2 in T84.66. For CDR-H2, there is a wide discrepancy between Kabat’s definition based on sequence hypervariability (H50–H65) and Chothia’s definition based on structure variability (H52–H56). To further complicate matters, only residues H50–H58 of Kabat CDR-H2 interact with antigen in cases where antibody–antigen co-crystals have been examined (Padlan, 1995; MacCallum, 1996). Note, however, that two independent humanization reports suggest that this is not always the case, since failure to retain murine residues at positions H59–H65 in the humanized construct reduced binding affinity over 1000-fold in each case (Eigenbrot, 1994; O’Connor, 1998). For this reason two humanized constructs were designed: T84.66 M5A, in which residues H59–H65 match the human acceptor sequence (Figure 3a), and T84.66 M5B, in which these residues match the mouse donor sequence (Figure 3b). Finally, based on our knowledge of the key structural role played by the rare proline at position H94 in T84.66, and the importance of H93 (Xiang, 1995), we expanded the Kabat definition of CDR-H3 to include framework residues H93 and H94. The CDR graft strategy is shown by sequence alignment in Figure 4.

**Expression, characterization and production**

Fully synthetic genes encoding the M5A and M5B Fv protein sequences were constructed using a series of SOE-PCR reactions as outlined in Figure 1. Once constructed, the genes for the M5A and M5B variable domains were ligated to human IgG1 constant domains and the mAbs were expressed in murine myeloma NS0 cells (Bebbington, 1992; Yazaki, 2001). Purification by Protein A and cation exchange chromatography using standardized conditions resulted in highly purified antibodies. The M5A and M5B mAbs were subjected to biochemical characterization, with cT84.66 mAb serving as a control. Proper antibody assembly was confirmed by HPLC size exclusion chromatography after purification (Figure 5, left panel) and after radioiodination (Figure 5, right panel). The humanized M5A and M5B mAbs have an isoelectric point (pI) in the range of 8.4, showing a distinct migration from the parental T84.66 mAb (pI 7.4) toward the more alkaline Herceptin (pI > 9.0) (Figure 6). SPR analysis by BIAcore demonstrated subnanomolar affinity to CEA for the M5A, M5B and cT84.66 mAbs with $K_A = 1.1 \times 10^{10}$ M$^{-1}$, 1.9 $\times 10^{10}$ M$^{-1}$ and 1.6 $\times 10^{10}$ M$^{-1}$, respectively (Table I).

Animal biodistribution studies in tumor-bearing animals were conducted to determine the in vivo tumor targeting and
normal tissue distribution of the M5A and M5B mAbs. The results presented in Table II show that levels of M5A mAb uptake were not statistically different than that of cT84.66 for blood, tumor or any other organs. Similarly, M5B mAb overall was not statistically different than that of cT84.66 mAb with respect to uptake in the blood or any other normal organ. However, M5B had a slightly lower tumor uptake (p = 0.012) as shown in Figure 7. With comparable affinities, blood clearance and 100% immunoreactivity post-radiolabeling to soluble antigen in vitro (data not shown), it was unclear why M5B mAb had a lower tumor uptake than M5A and may be the result of experimental variation.

The M5A mAb was selected as the clinical development candidate based on a higher tumor uptake and a larger content of ‘human’ residues, and was renamed hT84.66. To determine the production capability of the hT84.66 clone, the best expressing clone was subcloned and expanded in a CP2000 hollow fiber bioreactor. A limited production run saw anti-CEA activity levels of 250 μg/ml, and resulted in a harvest of 640 mg. A portion of the harvest was purified, conjugated with the macrocycle 1,4,7,10-tetraazacyclododecane N,N',N'',N'''-tetraacetic acid (DOTA) (Lewis, 2001) and radiolabeled with 111In. The 111In-DOTA-hT84.66 was injected intravenously into athymic mice bearing LS174T xenografts. The mice were sedated and imaged using a high-resolution planar scintigraphic camera. The hT84.66 mAb displayed highly specific tumor localization and clearance from normal tissues over the course of 7 days (Figure 8). This combination of data is indicative of the therapeutic potential of the hT84.66 mAb.

Summary

The present humanization strategy was based on the requisite to retain our extensive clinical experience using the murine and chimeric T84.66 antibodies for radioimmunotherapy. Although newer humanization technologies have emerged in recent years, the CDR grafting approach preserved T84.66’s high specificity while reducing the potential for an immunological response. The previously obtained crystal structure data of the recombinant T84.66 diabody were instrumental in taking this approach. Interestingly, superimposing the structures revealed that the peptide segments that needed to be transplanted did not correspond to the common definitions of CDR loops. As a design process, the humanization of T84.66 is a success, as demonstrated by the fact that no tedious back-mutations were required to restore specificity or affinty. The question as to whether this newly engineered anti-CEA antibody will be immunogenic remains to be answered in the clinic. Radio-labeled hT84.66 mAb is a promising new therapeutic which may help in overcoming this challenge.

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