Characterization of engineered anti-p185HER-2 (scFv-C_H3)_2 antibody fragments (minibodies) for tumor targeting

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An engineered antibody fragment (minibody; scFv-C_H3-y1 dimer, M_r 80 000) specific for carcinoembryonic antigen (CEA) has previously demonstrated excellent tumor targeting coupled with rapid clearance in vivo. In this study, variable (V) genes from the anti-p185HER-2 10H8 antibody were similarly assembled and expressed. Four constructs were made: first, the V genes were assembled in both orientations (V_L-linker-V_H and V_H-linker-V_L) as single chain Fvs (scFvs). Then each scFv was fused to the human IgG1 C_H3 domain, either by a two amino acid linker (ValGlu) that resulted in a non-covalent, hingeless minibody, or by IgG1 hinge and a GlySer linker peptide to produce a covalent, hinge-minibody. The constructs, expressed in NS0 mouse myeloma cells at levels of 20–60 mg/l, demonstrated binding properties equivalent to that for the parental 10H8 mAb (K_D ~2–4 nM) were equivalent to that for the parental 10H8 mAb (K_D ~1.6 M). Radioiodinated 10H8 hinge-minibody was evaluated in athymic mice, bearing C77/HER2 xenografts. Maximum tumor uptake was 5.6 (±1.61)% injected dose/g (ID/g) at 12 h, which was lower than that of the anti-CEA minibody, whereas the blood clearance (β-phase, 5.62 h) was similar. Thus, minibodies with different specificities display similar pharmacokinetics, while tumor uptake may vary depending on the antigen-antibody system.

Keywords: antibody fragments/biodistribution/breast cancer/HER2/minibody

Introduction

Monoclonal antibodies (mAbs) can be tailored to carry radio-isotopes, toxins or drugs for specific delivery to tumor cells. However, the application of intact antibody molecules (~150 kDa) as therapeutic or diagnostic molecules remains limited due to their size, as they have limited tumor penetration and clear relatively slowly from the circulation resulting in low tumor to blood uptake ratio. The development of recombinant DNA techniques has enabled production of antibody fragments of various sizes and avidities and in vivo evaluation in tumor xenograft models (Wu and Yazaki, 2000). Recombinant antibody fragments have been generated exhibiting high tumor retention and fast blood clearance. These are desirable properties for imaging that result in high tumor to blood ratios in mice bearing human tumor xenografts.

HER2/neu (c-erbB-2) is one of four closely related members in the EGF family of tyrosine kinase receptors. The transmembrane glycoprotein of 185 kDa (p185HER-2), encoded by the HER2/neu proto-oncogene, is overexpressed in 20–30% of breast cancers and its elevated expression is often associated with an unfavorable prognosis (Slamon et al., 1987). The relatively low expression of this antigen on normal tissues (Bercbuck et al., 1990; Press et al., 1990) makes it an attractive target. Herceptin (trastuzumab; Genentech, San Francisco, CA) is the humanized version of the 4D5 mAb (Carter et al., 1995; Willuda et al., 1996; Cobleigh, 1998) that has been approved by the FDA for the treatment of p185HER-2 positive tumors. Studies have suggested that this antibody can antagonize the constitutive growth-signaling properties of the p185HER-2 system, enlist immune cells to attack and kill the tumor target, and augment chemotherapy-induced cytotoxicity (Slawkowski et al., 1999). Moreover, successful treatment with Herceptin has been achieved in some women with overexpressing p185HER-2 breast cancers (Baselga et al., 1996; Cobleigh, 1998; Slamon et al., 2001; Vogel et al., 2002) demonstrating that p185HER-2 can be used selectively as a target in human cancers. However, the overall objective response rate to Herceptin in patients with p185HER-2 overexpressing tumors is only 15–20% (Cobleigh et al., 1999; Gilbert et al., 2003). Hence, there is a need for developing additional therapeutic as well as diagnostic agents.

Several antibodies and fragments thereof have been developed to target p185HER-2 and evaluated for their tumor targeting properties in vivo. For example, recombinant single chain Fv (scFv; ~25 kDa) fragments composed of peptide-linked V_H and V_L domains, made from the anti-p185HER-2 4D5, 741F8 and C6.5 mAbs, demonstrate similar pharmacokinetics and tumor retentions (~1% injected dose per gram; ID/g) in mice bearing p185HER-2 positive tumor xenografts (Schier et al., 1995; Willuda et al., 2001). Shortening the peptide-linker between the V domains, forces the scFv to self-associate into a dimer (diabody; 55 kDa) (Holliger et al., 1993). These antibody fragments, including the anti-p185HER-2 C6.5 diabody (Adams et al., 1998), demonstrate a prolonged blood clearance that has resulted in tumor activity of >10% ID/g. The improved tumor retention has been attributed to the increase avidity rather than the increase in size (Adams et al., 1998), as monovalent Fab fragments that are of similar size do not provide a great increment in tumor retention compared with scFv (Milenic et al., 1991; Adams et al., 1993).

Multivalent scFv can also be accomplished by C-terminal addition of multimerization domains. For example, a bivalent scFv (~60 kDa) with a C-terminal synthetic helix–turn–helix...
domain, and a tetravalent scFv (~130 kDa) with C-terminal multimerization domain from p53 have been produced from 4D5 mAb (Willuda et al., 2001). However, the increased avidity and size of these fragments did not improve tumor retention, as the dimeric scFv reached ~1.5% ID/g and the tetravalent scFv ~4.3% ID/g in mice bearing p185HER-2 positive tumor xenografts (Willuda et al., 2001). The authors explain this as being due to possible instability and that the orientation and accessibility of the targeted epitope allowed the tetramer to bind to only two antigens on the target cells. We and other groups have used constant immunoglobulin domains from IgG1 CH3 (Hu groups have used constant immunoglobulin domains from IgG1 CH3 (Hu et al., 1996; Li et al., 1997; Olafsen et al., 1998) and IgE CH4 (Borsi et al., 2002) as dimerization domains to express intermediate-sized, bivalent scFv fragments. In a previous study, we targeted the carcinoembryonic antigen (CEA) expressed on colon cancer cells using an anti-CEA T84.66 minibody (scFv-CH3 fusion proteins, ~80 kDa) (Hu et al., 1996). Iodinated anti-CEA minibody demonstrated excellent tumor uptake (21.4–32.9% ID/g) (Hu et al., 1996; Wu and Yazaki, 2000; Yazaki et al., 2001) and a prolonged blood clearance as compared to its scFv and diabody counterparts in nude mice bearing CEA-positive tumor xenografts (Wu et al., 1996, 1999). Moreover, high-resolution microPET images of tumor xenografts were obtained with this fragment when labeled with the positron emitters, 11C (t1/2 = 12.7 h) (Wu et al., 2000) and 124I (t1/2 = 4.2 days) (Sundaresan et al., 2003).

In the present study, we have extended the minibody concept by making an analogous molecule with specificity for p185HER-2. The scFv was derived from the anti-p185HER-2 antibody, 10H8 (Park et al., 1999). A total of four 10H8 minibodies were made: first the V genes were assembled in both orientations as scFVs (V_L-linker-V_H and V_H-linker-V_L), and then each of these was fused to the C13 domain in two different ways. The various formats were made to provide insight into whether the orientation of the V domains and/or the presence/absence of a hinge influence the antigen binding properties. The minibody variants were characterized in vitro, and as a first step, one was further radioiodinated and evaluated for its in vivo application for targeting MCF7/HER2 xenografts in mice.

Materials and methods

Isolation of the V genes

Total RNA, extracted from the 10H8.C6.F12 hybridoma cell line (Park et al., 1999) using the Trizol Reagent (Life Technologies, Grand Island, NY), was utilized for cDNA synthesis. The V genes were isolated from cDNA by RT–PCR using sense primers annealing to the first framework (FR1) sequences in the V genes, and antisense primers annealing to the murine constant kappa or to the heavy-chain constant region (Dubel et al., 1994). The predicted primary amino acid sequences, obtained from DNA sequence analyses of cloned isolates, were compared to tryptic peptide sequences of the parental murine 10H8 mAb intact heavy and light chains (Figure 1) as described previously (Wu et al., 2001).

Assembly of anti-HER2/neu minibodies, (scFv-CH3)2

The V genes were individually amplified and fused by overlap PCR to produce two scFv fragments with different orientation preceded by a light chain leader sequence. The scFvs, oriented as V_L–V_H and as V_H–V_L, with an 18 residue linker that was either GlySer rich (Figure 2A) or modified from Whitlow et al. (Figure 2B) (Whitlow et al., 1993; Wu et al., 2001) between the V domains, were fused directly to the human IgG1 CH3 domain via the two amino acids valine and glutamine (hingeless minibody) and indirectly via the human IgG1 upper and middle hinge and a lower GlySer linker peptide of 10 residues (hinge-minibody) by overlap PCR. These, when expressed, resulted in a non-covalent, hingeless dimer (Figure 2C), whereas the hinge-minibody is covalently bound through hinge disulfide bridges (Figure 2D). The four constructs were inserted as XbaI–EcoRI fragments into the mammalian expression vector, pEE12 (Lanza Biologics, Slough, UK), which contains the hCMV promoter for high expression (Cockett et al., 1991) and the glutamine synthetase gene for selection (Bebbington et al., 1992).

Expression, selection and purification

A total of 1 × 107 NSO mouse myeloma cells (Galfre and Milstein, 1981) were transfected with 40 μg of linearized vector DNA by electroporation and selected in glutamine-deficient media as described (Wu et al., 2001). Clones were screened for expression by ELISA, whereby the desired protein was captured by goat anti-human IgG (Fc specific) and detected by alkaline phosphatase-conjugated goat anti-human IgG (Fc specific) (both from Jackson ImmunoResearch Labs, West Grove, PA). Supernatants were also examined by western blot for size analysis, using the alkaline phosphatase-conjugated goat anti-human IgG (Fc specific). The highest producing clones were expanded. Minibodies were purified from cell culture supernatants, using a BioCAD 700E chromatography system (Applied Biosystems, Foster City, CA) as described (Yazaki et al., 2001a). Briefly, the supernatants were treated with 5% AG1-X8, 100–200 mesh (Bio-Rad Laboratories, Hercules, CA) overnight to remove phenol red and cell debris, then dialyzed versus 50 mM Tris–HCl, pH 8.5. Treated supernatant was loaded onto an anion exchange chromatography column (Source™ 15Q; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Proteins were eluted with a NaCl gradient from 0 to 0.2 M in the presence of 50 mM HEPES, pH 8.5. Eluted fractions, containing the desired protein, were subsequently diluted 5× in 50 mM MES (pH 6.5) and loaded onto a Macro-Prep Ceramic Hydroxyapatite column (Bio-Rad Laboratories) and eluted with a KPi gradient from 0 to 0.15 M in the presence of 50 mM MES, pH 6.5.

Biochemical characterization

Aliquots of eluted fractions from the columns were analyzed by SDS–PAGE on pre-cast 12% polyacrylamide Ready Gels (Bio-Rad Laboratories). The proteins were detected by staining with MicrowaveBlue™ (Protiga Inc., Frederick, MD). Samples were also subjected to size-exclusion HPLC on a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech AB) that was run isocratically in PBS, and elution time was compared to that of other minibodies. Fractions containing pure proteins were pooled, dialyzed against PBS and concentrated by Centriprep 30 (Amicon Inc., Beverly, MA). Concentrated protein was subjected to isoelectric focusing (IEF) for determination of pI using pre-cast gels (Novex, San Diego, CA) with IEF standards (Bio-Rad Laboratories). The final concentration of purified protein per milliliter was determined by running an aliquot of purified protein over a protein 1-agarose affinity column, measuring the protein peak by UV absorbance at
Characterization of engineered anti-p185HER-2 (scFv-C43)2 antibody fragments

280 nm as described (Yazaki et al., 2001a) and standardizing to a known concentration of an irrelevant anti-CEA minibody.

**Indirect binding assays by flow cytometry**

Binding to antigen was initially assessed by indirect immunofluorescence on the human breast tumor cell line, MCF7/HER2 (gift of Dr Dennis J.Slamon, UCLA School of Medicine) (Pietras et al., 1995). Both supernatants from cells expressing minibodies and purified proteins were assayed. Briefly, 300 μl of 1 x 10^6 cells/ml was resuspended in supernatants, containing the protein of interest, or in PBS/2% FCS with 1–2 μg of purified protein and incubated on ice for 30 min. The cells were washed three times in PBS/2% FCS, and then incubated for an additional 30 min with FITC-conjugated goat anti-human IgG (Fc specific) (Jackson ImmunoResearch Labs). After a final wash, the cells were resuspended in PBS/2% FCS and analyzed on a MoFlo flow cytometer (Cytomation, Fort Collins, CO). Herceptin antibody was used as positive control and irrelevant anti-CD20 minibody was used as isotype control.

**Biotinylation of intact 10H8 mAb**

Protein (2 mg/ml) in 100 μl was incubated on ice for 2 h in the presence of a 20-fold molar excess of Sulfo-NHS-LC-Biotin reagent (Pierce, Rockford, IL). Excess biotin was removed using Quick Spin G-25 Sephadex columns (Boehringer Mannheim) prewashed with PBS. Biotin incorporation was determined by adding an aliquot of the biotinylated protein to the avidin-HABA [2-(4′-hydroxyazonbenzene)-benzoic acid] reagent and reading the absorbance at 500 nm, as described by the manufacturer (Pierce). The number of biotins per molecule was estimated to be 0.9, when assuming 80% recovery from the spin columns. Biotinylated 10H8 mAb was also analyzed by SDS–PAGE as described above, and by western blot in which the nitrocellulose membrane (Bio-Rad Laboratories), blocked with 5% non-fat dry milk (Carnation), was incubated with alkaline phosphatase-conjugated streptavidin (1:5000 dilution) (Jackson ImmunoResearch Labs) and analyzed on the tumor cells. The frozen sections were fixed in ethanol and incubated with biotinylated goat anti-human (H + L) or goat anti-rabbit antibodies (H + L) (1:400), respectively, using Vectastain Elite ABC kits (Vector Labs, Inc., Burlingame, CA). Biotinylated secondary anti-human antibody alone was used as the negative control.

**Biodistribution of 131I-10H8 hinge-minibody in tumor bearing mice**

Five days prior to injection of tumor cells, 7–8-week-old female athymic mice (NCI) were injected i.m. with 0.8–1.0 mg of Delestrogen (Bristol-Meyer Squibb Company, Princeton, NJ) in the shoulder to promote tumor growth. Two days later the mice were given a radiation dose of 200 rad in a Theratron-80 Co-60 unit (AECL/Theratronics International Ltd, Ontario, Canada). After an additional 2 days, 5 x 10^6 MCF7/HER2 cells were resuspended in 50% RPMI 1640 and 50% Matrigel (Becton Dickinson Labware, Bedford, MA) and were injected subcutaneously in the flank of the mice. At 14 days post-inoculation, mice bearing MCF7/HER2 xenografts were injected with 2.4 μCi (specific activity = 0.95 μCi/μg) of 131I-labeled V1–VH hinge-minibody via the tail vein. Groups of five mice were killed and dissected at 0, 2, 4, 12, 24 and 48 h post-injection. Major organs were weighed and counted in a gamma scintillation counter. Radioactive uptake in organs was corrected for radioactive decay and expressed as a percentage of injected dose per gram of tissue (%ID/g) and as a percentage of injected dose per organ (%ID/organ). Biodistribution data are summarized as mean and corresponding standard errors of the mean. Animal blood curves were calculated using the ADAPT II software (D’Argenio and Schumitzky, 1979) to estimate two rate constants (k) and associated amplitudes (Ai).

**Results**

**Isolation and analysis of 10H8 variable genes**

The 10H8 variable heavy and light chain genes, isolated from total RNA prepared from the 10H8 hybridoma cells, were
amplified by VHBi3d and HCRegi2 and VkBi7 and LCRegi2 primers, respectively (Dubel et al., 1994). The sequences were verified by comparison to tryptic peptides from the parental 10H8 antibody. The overlapping sequences are shown in bold letters in Figure 1, and they include the hypervariable regions CDR1 and CDR3 in the \( V_L \) chain and CDR1 and CDR2 in the \( V_H \) chain.

**Generation of 10H8 minibodies, \((scFv-C_{H3})_2\)**

The 10H8 \( V \) genes were amplified by PCR and fused to the human IgG1 \( C_{H3} \) domain as described in Materials and methods, to generate four minibody variants that were expressed in murine NS0 myeloma cells. The expression levels were determined by ELISA to be between 20 and 60 mg/l among the best producing clones. Despite good expression, only 2–4 mg was recovered from 0.8 l, due to difficulty in separating the proteins from the contaminants on the anion exchange column. Analysis of the purified proteins by SDS–PAGE demonstrated that the two-step purification scheme yielded proteins that were >95% pure (Figure 3A). One of the hingeless minibody constructs, oriented \( V_H–V_L \), was consistently expressed at low levels and was therefore not included in most of the biochemical characterizations performed in this work.

**Fig. 1.** Predicted protein sequence for anti-p185\(^{HER-2}\) 10H8 light- and heavy-chain variable regions and partial confirmation by protein sequence analysis. The complementary determining regions (CDRs), according to Kabat’s definition (Johnson et al., 1996) are underlined. The sequences confirmed by protein sequencing of the parental intact 10H8 mAb, are indicated in bold lettering.

**Fig. 2.** Schematic diagram the anti-p185\(^{HER-2}\) 10H8 minibodies genes and the two versions with and without a hinge. (a) Minibody genes with the scFv orientated as \( V_L–V_H \) containing a GlySer-rich 18-residue linker. (b) Minibody genes with the scFv orientated as \( V_H–V_L \) containing a modified 218 (m218) Whitlow linker. Each gene includes a leader signal sequence (L) for secretion. (c) Hingeless minibody that assembles into 80 kDa dimers by hydrophobic interactions between the \( C_{H3} \) domains. (d) Hinge-minibody that assembles into covalently bound dimers through the cysteines in the hinge region.
exhibited a slightly larger apparent Stokes radius than the hingeless minibody, as it eluted at 18.1 min as opposed to 18.4 min (Figure 3C).

Functional characterization

Binding to target was initially shown indirectly by flow cytometry on MCF7/HER2 cells (Figure 4A). Strong binding of the minibodies was demonstrated and was equal to that seen with the Herceptin antibody. Specific binding was demonstrated by cell-based competition, in which radiolabeled V<sub>L</sub>–V<sub>H</sub> hinge-minibody was incubated simultaneously in the presence of a competitor at 100-fold concentration (Figure 4B). The results show that without competition (lane 1) the hinge-minibody exists in equilibrium between bound (gray) and unbound state (black). In the presence of unlabeled, parental, murine 10H8 mAb (lane 3), unlabeled hinge-minibodies (lanes 4 and 5) as well as the unlabeled hingeless minibody (lane 6), most of the labeled minibody is displaced, whereas in the presence of an irrelevant minibody (isotype control) (lane 2) and Herceptin (lane 7) there is no competition. Hence, the binding of 10H8 minibody is specific, essentially reproducing the binding of parental antibody, and it recognizes a different epitope on HER2/neu than the Herceptin antibody. The relative K<sub>D</sub> was estimated to be 1.6 nM for the parental 10H8 mAb and between 2 and 4 nM for the minibodies, based on the presence of competitors at different concentrations in an ELISA competition assay (Figure 4C). The affinity constant (K<sub>a</sub>) was measured by the Scatchard method to be 1.3 ± 0.7 × 10<sup>8</sup> M<sup>-1</sup> for the 131I-labeled 10H8 V<sub>L</sub>–V<sub>H</sub> hinge-minibody (Figure 4D).

The number of receptors on the MCF7/HER2 cells was calculated from the mean maximum binding capacity (B<sub>max</sub>) of two Scatchard plots to be 1.1 × 10<sup>7</sup> per cell which is in agreement with published receptor quantitation of these cells (Aguilar et al., 1999).

Biochemical characterization

On SDS–PAGE (Figure 3A), under reducing conditions, the hinge-minibody migrated consistently with the expected M<sub>w</sub> of ~41 kDa (lane 5), while the hingeless minibody was slightly smaller and migrated consistent with the expected M<sub>w</sub> of ~39 kDa (lane 4). Under non-reducing conditions the covalently linked hinge-minibody migrated as a dimer with the expected M<sub>w</sub> of ~82 kDa (lanes 2 and 7). The constructs were subjected to IEF for estimation of the isoelectric point (pI) (Figure 3B). The pI for the hinge-minibody with a GS18 linker (V<sub>L</sub>–V<sub>H</sub>) was 7.4 (lane 4) and 7.8 with m218 linker (V<sub>H</sub>–V<sub>L</sub>; lane 3), whereas the pI for the hingeless minibody with GS18 linker (V<sub>H</sub>–V<sub>L</sub>) was 7.0 (lane 5). Size-exclusion chromatography further indicated that the hinge-minibody exhibited a slightly larger apparent Stokes radius than the hingeless minibody, as it eluted at 18.1 min as opposed to 18.4 min (Figure 3C).

In vivo biodistribution

The 131I-labeled 10H8 V<sub>L</sub>–V<sub>H</sub> hinge-minibody was assessed for its ability to target tumor in athymic mice bearing xenografts of p185<sup>HER-2</sup> overexpressing human breast cancer cells, MCF7/HER2. Tumor uptake reached a maximum of 5.59 (±1.69)% ID/g at 12 h which declined to 4.38 (±0.76)% ID/g at 24 h (Table I). The anti-p185<sup>HER-2</sup> hinge-minibody showed the same blood clearance pattern to that seen with the anti-CEA minibody (Figure 5), with the half-life in the β-phase being estimated to be 5.62 h compared to 6.99 h, respectively (Yazaki et al., 2001b). The slow clearance combined with low tumor uptake resulted in a modest tumor to blood ratio. At 12 h the tumor to blood ratio was 1:1, and reached a maximum of 3:1 at 48 h. Activities in other normal organs (liver, spleen and kidney) fell below 1% ID/g by 24 h to values essentially identical to those observed for the anti-CEA minibody (Yazaki et al., 2001b).

Immunohistochemistry of MCF7/HER2 tumors

MCF7/HER2 tumors from athymic mice not used in the biodistribution were removed and frozen. Sections were analyzed for expression of p185<sup>HER-2</sup> by IHC staining as shown in Figure 6. The staining pattern of the cell membrane by the 10H8 minibody (Figure 6B) matched that seen with the positive control (Figure 6C). Also, the staining intensity was
similar for the 10H8 minibody and the polyclonal anti-c-ErbB2 antibody. The negative control using biotinylated anti-human antibody only is shown in Figure 6A.

Discussion

Here, we describe the design, production and evaluation of intermediate-sized antibody fragments with specificity against p185HER-2. Four (svFv-C\textsubscript{H}3)\textsubscript{2} (minibody) fusions were made, including a version that was identical to a previously made anti-CEA minibody (Yazaki \textit{et al.}, 2001b). The intention was initially to evaluate whether the orientation of the V genes in the scFv fragment or the presence or absence of a hinge affected antigen binding. Three of the four versions expressed well allowing purification and characterization of binding to p185HER-2 by several methods, including flow cytometry on cells, competition ELISA and Scatchard. The different minibody versions demonstrated equal binding compared to the parental 10H8 mAb. In summary, no significant difference could be attenuated to the order of V regions, sequence of linkers and presence or absence of hinge. One minibody, V\textsubscript{L}–V\textsubscript{H} hinge, was further characterized in IHC assays, demonstrating an equal staining pattern compared to a commercial anti-p185HER-2 antibody.
Biodistribution and tumor targeting studies were evaluated in xenograft-bearing nude mice using radioiodinated 10H8 V₁–V₄ hinge-minibody that was similar in construction to the anti-CEA minibody. Although a positive tumor to blood ratio was achieved, the uptake in the tumor was unexpectedly low (max 5.6% ID/g at 12 h) in comparison to that observed with the anti-CEA minibody (max 21.4% ID/g at 12 h) (Wu and Yazaki, 2000). In contrast, biodistribution studies of intact anti-p185HER-2 antibody, 2C4, which binds to a different epitope of domain IV near the transmembrane domain. As a result, Herceptin probably provides steric hindrance, preventing proteolytic cleavage of the receptor. In our competition assay, we demonstrated that 10H8 antibody also recognizes a different epitope than that of Herceptin and would not necessarily inhibit shedding. Therefore, we addressed the possibility of shedding by assaying sera from separate untreated tumor-bearing mice for the presence of ECD using a commercial ELISA kit form Oncogene Research Products (San Diego, CA). Since no ECD was detected in this in vivo model (results not shown), the possibility of shed antigen being the cause for low tumor activity was dismissed.

Another explanation for the low tumor uptake may be antigen shedding, enabling formation of antigen–antibody complexes in blood and thus preventing efficient tumor targeting. Some patients with carcinomas that amplify and overexpress p185HER-2 have an elevated level of the HER2 extracellular domain (ECD) in their sera (Payne et al., 2000). A recent study demonstrated that Herceptin inhibits constitutive p185HER-2 cleavage/shedding, a property not shared by the anti-p185HER-2 antibody, 2C4, which binds to a different epitope (Molina et al., 2001). The structure of rat HER2 (neu) in complex with Herceptin Fab has recently been solved (Cho et al., 2003) showing that Herceptin binds to the C-terminal portion of domain IV near the transmembrane domain. As a result, Herceptin probably provides steric hindrance, preventing proteolytic cleavage of the receptor. In our competition assay, we demonstrated that 10H8 antibody also recognizes a different epitope than that of Herceptin and would not necessarily inhibit shedding. Therefore, we addressed the possibility of shedding by assaying sera from separate untreated tumor-bearing mice for the presence of ECD using a commercial ELISA kit form Oncogene Research Products (San Diego, CA). Since no ECD was detected in this in vivo model (results not shown), the possibility of shed antigen being the cause for low tumor activity was dismissed.

Finally, we cannot exclude the possibility that the minibody disassociates and/or becomes degraded before it reaches the tumor, although association is stabilized through disulfide bonds. However, this is unlikely as the 10H8 minibody blood clearance profile is identical to that of the anti-CEA minibody, suggesting that it remains intact in the circulation.
uptake and retention were modestly improved; however, kidney activity was elevated as well. The solution to this problem may be to utilize larger fragments (T.Olafsen et al., manuscript in preparation). It will also be important to generate and evaluate a non-internalizing minibody, and study additional p185HER-2 positive cell lines in order to define the parameters required for efficient targeting of p185HER-2 in vivo.

In summary, we have successfully made a set of minibodies with specificity for p185HER-2. The order of assembly of the variable regions and the presence or absence of the hinge do not seem to affect the activity and specificity in vitro. One of the minibodies was further investigated in vivo. It exhibited essentially the same clearance as previously demonstrated with an anti-CEA minibody, although the tumor uptake was lower than expected. Hence, antibody fragments of this format demonstrate similar pharmacokinetics, whereas their tumor uptake may vary depending on the tumor model. Future work will contribute to the further understanding of this tumor–antigen system.

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References


Fig. 6. IHC staining of frozen sections of MCF7/HER2 tumor (magnification ×20). (a) Negative control, i.e. secondary anti-human antibody only. (b) 10H8 V1–VH hinge-minibody. (c) Positive control, i.e. polyclonal rabbit anti-c-ErbB2.

These results verify the prolonged half-life of the minibody format, which should allow for a higher tumor uptake than that which we observed here. Since several studies have shown that higher tumor retention is observed with the radiometal labeled antibody when compared directly with the radiiodinated antibody (Stein et al., 1995; Sharkey et al., 1997; Garkavij et al., 1999) a logical next step would be to label the 10H8 hinge-minibody with radiometal. Indeed, in subsequent work, we have evaluated the biodistribution properties of radiometal-labeled 10H8 minibody and a second anti-Her2 minibody as well (T.Olafsen et al., manuscript in preparation). Tumor
Characterization of engineered anti-p185HER-2 (scFv-CH3)2 antibody fragments


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