Bispecific antibodies (BsAbs) represent an emerging class of biologics that achieve dual targeting with a single agent. Recombinant DNA technologies have facilitated a variety of creative bispecific designs with many promising therapeutic applications; however, practical methods for producing high quality BsAbs that have good product stability, long serum half-life, straightforward purification, and scalable production have largely been limiting. Here we describe a protein-engineering approach for producing stable, scalable tetravalent IgG-like BsAbs. The stability-engineered IgG-like BsAbs were envisioned to target and crosslink two TNF family member receptors, TRAIL-R2 (TNF-related apoptosis inducing ligand receptor-2) and LTβR (lymphotoxin-β receptor). Both N- or C-terminal BsAbs were active in inhibiting tumor cell growth in vitro, and with some cell lines demonstrated enhanced activity relative to the combination of parental Abs. Pharmacokinetic studies in mice revealed long serum half-lives for the BsAbs. In murine tumor xenograft models, therapeutic treatment with the BsAbs resulted in reduction in tumor volume either comparable to or greater than the combination of parental antibodies, indicating that simultaneously targeting and cross-linking receptor pairs is an effective strategy for treating tumor cells. These studies support that stability-engineering is an enabling step for producing scalable IgG-like BsAbs with properties desirable for biopharmaceutical development.

Introduction

Recombinant bispecific antibodies (BsAbs) are designed to perform as single agent, dual antigen-binding molecules. BsAbs can bind to different epitopes on the same antigen or to two entirely distinct antigens. For treating human disease, such as cancer, the biological consequence of engaging more than one epitope or target, for example cell membrane-associated receptors, with a BsAb may result in increased potency or efficacy due to: (1) enhanced avidity or specificity towards target or (2) simultaneous targeting of two distinct disease-related pathways resulting in an additive, synergistic or novel mechanism-of-action. Bispecific antibodies can also be employed for recruiting or redirecting a natural killer (NK) cell, T cell or monocyte/neutrophil cellular response to tumors by bringing these two cell types close together.1 BsAbs have also been used in pretargeting strategies where first the BsAb is administered to a patient such that it localizes to its target, and unbound BsAb is then allowed to clear from the bloodstream. A second agent, such as a derivatized cytotoxic or radionuclide, is subsequently administered whereupon it is captured by the immobilized BsAb concentrating the cell-killing agent to the target tissue.2
Over the last decade, a spectrum of innovative BsAb formats have been constructed, with single-chain Fv (scFv) and diabody fragments being extensively used as fundamental building blocks.\(^1,3,4\) A subgroup of these, designed as full-length IgG-like BsAbs, have the added advantage of preserving an intact Fc domain, thereby readily allowing purification, providing active cellular immune effector functions and imparting greatly extended serum pharmacokinetic properties.\(^5\) While many of these IgG-like BsAbs have shown promising activity in in vitro assays, low production yields, inadequate product quality and loss of activity or short serum half-life have largely remained significant impediments to conducting rigorous in vivo studies and advancing these compounds towards clinical development.

It is well documented that insufficient thermodynamic stability of Fv and scFv fragments can often limit scFv production, quality and yield.\(^6,7\) Many methods, including both rational design and molecular evolution techniques, have been described for improving the stability of scFvs.\(^6,8-10\) Given the interest in using scFv fragments as “building blocks” for constructing IgG-like BsAbs, we surmised that a scFv engineered to have sufficient stability should, upon incorporation into an IgG-like BsAb, result in a BsAb that also has improved and acceptable stability properties.

Our hypothesis that stabilization of scFv domains would enable production of therapeutically useful BsAb molecules was tested by evaluating a series of tetravalent IgG-like BsAbs constructed using scFvs selected with varying levels of resistance to thermal challenge. Here we report on the biochemical, biophysical and biological properties of BsAbs constructed from stabilized scFvs.

The stability-engineered BsAbs were built from two agonist antibodies targeting TNF family member receptors, TRAIL-R2 (TNF-Related Apoptosis Inducing Ligand Receptor-2) and LTβR (Lymphotixin-β Receptor) which are co-expressed on the surface of a variety of human tumor epithelial cell lines. The death domain containing TRAIL-R2 receptor represents an attractive new target in cancer, with early clinical trials of soluble TRAIL or agonist antibodies to TRAIL-R2 demonstrating acceptable safety and tolerability and promising signs of anti-tumor activity.\(^11\) LTβR, one of several non-death domain containing TNF family receptors, has shown preclinical efficacy in tumor xenograft models when targeted with an agonist antibody.\(^12\) We set out to construct a BsAb targeting TRAIL-R2 and LTβR to explore the possibility that a BsAb might trigger an enhanced, synergistic, or broader anti-tumor response than that achieved by treating with a mixture of the two antibodies.

The full-length anti-TRAIL-R2 antibody 14A2 was used as the IgG component of the BsAb, and the anti-LTβR antibody BHA10 served as a source for the stability-engineered scFv moiety. Once stabilized, the BHA10 scFv could be appended to either the amino- or carboxyl terminus of the 14A2 antibody heavy chain enabling scalable production of high quality tetravalent IgG-like BsAbs in a CHO cell expression system. The stability-engineered BsAbs demonstrated excellent serum half-lives in mice and showed anti-tumor activity in mouse tumor xenograft models. These results indicate that incorporating stability-engineered scFvs into IgG-like BsAb designs may greatly facilitate scalable evaluation of this novel class of biotherapeutics.

### Results

**Production of BsAb containing wild-type scFv.** The IgG-like BsAb design consisted of a scFv derived from the anti-LTβR humanized mAb, BHA10, genetically connected through a flexible linker to either the amino (N-BsAb) or carboxyl terminus (C-BsAb) of the full-length anti-TRAIL-R2 chimeric, IgG 14A2 (Fig. 1A). The BHA10 scFv was built in the VH\(\square\)VL orientation and joined in-frame via a (Gly\(_4\)Ser\(_4\)) linker to either the amino-terminal VH domain or the carboxyl end of the 14A2 IgG in the bicistronic mammalian expression vector pNS5KG1 as shown in Figure 1B. Plasmids were used to stably transfect CHO cells for protein production. Preliminary experiments with the C-BsAb containing wild-type BHA10 scFv revealed that a transfected pool of CHO cells secreted a moderate level of C-BsAb into the culture supernatant with an accumulated titer of approximately 40 mg per liter. However, nearly 40% of the Protein A purified BsAb was present as high MW aggregates (Fig. 1C), and even isolated monomeric BsAb containing wild-type scFv was still prone to forming aggregates (Bailly V, unpublished observation).

In order to determine whether the intrinsic stability of the scFv moiety might be a contributing factor to the poor quality of the wild-type C-BsAb, we compared the relative thermal stability of purified wild-type BHA10 scFv produced in E. coli to BHA10 FAb using differential scanning calorimetry. All four domains of the BHA10 FAb (V\(_H\), V\(_L\), C\(_{H1}\) and C\(_L\)) unfolded cooperatively with a T\(_m\) of 78°C (Fig. 2). Similar to other reported antibody fragments, the wild-type BHA10 scFv variable domains, lacking C\(_{H1}\) and C\(_L\), unfolded at much lower temperatures than the Fab.\(^13\) The V\(_L\) domain unfolded with a T\(_m\) = 68°C, while the V\(_H\) domain unfolded at a T\(_m\) = 58°C, twenty degrees lower than the observed unfolding transition of the BHA10 FAb. As expected, the measured calorimetric enthalpy of unfolding (\(\Delta H_m(T_m)\)) was also significantly reduced in the scFv format; \(\Delta H_m(T_m) = 260\) kcal/mol for the Fab; \(\Delta H_m(T_m) = 44\) kcal/mol for the V\(_L\) domain; and \(\Delta H_m(T_m) = 22\) kcal/mol for the V\(_H\) domain.

**Design, expression and characterization of stabilized BHA10 scFvs.** Based on the results from the DSC analysis, we hypothesized that enhancing the thermal stability of the BHA10 scFv might, in turn, lead to a reduced tendency of the IgG-like BsAb to form aggregates. A molecular model of the BHA10 scFv was used to visually inspect the integrity of the 15 amino acid (Gly\(_4\)Ser\(_4\)) linker used to connect the V\(_H\) and V\(_L\) domains on the flanking secondary structure elements as well as to determine the energetic contributions (Fig. 3A). The (Gly\(_4\)Ser\(_4\)) linker was found to be too short to adequately span the distance between the two variable domains possibly imposing structural constraints.\(^14\) Accordingly, we built and tested BHA10 scFvs containing a longer linker, (Gly\(_4\)Ser\(_4\)).

In addition, the model was used to evaluate pairs of cysteine residues substituted at framework positions V\(_H\)_44 and V\(_L\)_100, V\(_H\)_44 and V\(_L\)_105, and V\(_H\)_105 and V\(_L\)_43 that could participate in creating a stabilizing disulfide bond (Fig. 3A).\(^1\) The modeled conformation of the cysteines at positions V\(_H\)_44 and V\(_L\)_100 supported the geometry for forming a disulfide bond \([\delta(Sy - Sy) = 2.1\ \text{Å}, \delta(C\beta - C\beta) = 4.2\ \text{Å}, \delta(C\alpha - C\alpha) = 5.5\ \text{Å}]\). However,
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Figure 1. Design and production of IgG-like BsAbs. (A and B), Schematic diagrams of N- and C-BsAbs designs and mammalian expression vectors used for producing IgG-like BsAbs. Detailed components of the expression vectors are shown at the bottom of (B). (C), Analytical size-exclusion chromatography profile of C-BsAb constructed with wild-type BHA10 scFv following expression in CHO cells and purification on Protein A.

Figure 2. DSC analysis of BHA10 Fab and wild-type BHA10 scFv. Overlay of BHA10 Fab (dashed line) and wild-type scFv (solid line) thermograms. Unfolding transitions are measured at the endothermic peaks (transition midpoints) and reported as T_m values (°C).

Modeled cysteines at positions V_H44 and V_L105 appeared to be incompatible with the length of a disulfide bond δ(Cα - Cα) = 17.6 Å, and would likely require realignment of V_H FR4 region towards V_L.

Four plasmids were constructed from the parent BHA10 scFv (Fig. 3B). One scFv contained the longer (Gly4Ser)4 linker and the remaining three constructs each contained one of the three pairs of cysteine mutations described above. The variant BHA10 scFvs were expressed in the E. coli strain W3110 and culture supernatants containing secreted scFv proteins were analyzed by western blot. The scFv constructed with the (Gly4Ser)4 linker was produced by W3110 and the major protein product migrated according to its predicted molecular weight (~30 kDa, data not shown). ScFvs constructed with the different pairs of cysteine substitutions, however, varied greatly in levels and quality of proteins with only the BHA10 scFv containing the cysteine pair at positions VL100 and VH44 produced and fully intact (data not shown). We also tested the effect of combining the longer (Gly4Ser)4 linker with the cysteine substitutions at VL100 and VH44 in the BHA10 scFv. Supernatants containing the various engineered BHA10 scFvs were first compared to wild-type BHA10 scFv by determining the temperature (T_{50}) at which 50% of scFv molecules retained binding to LTβR antigen following thermal challenge. ScFvs were subjected to a range of temperatures spanning the thermal transition temperature of wild-type BHA10 scFv (previously determined to be T_{50} = 49°C). All of the engineered scFv molecules showed
Stability-engineered bispecific antibodies exposed hydrophobic surfaces such as those that occur in partially unfolded proteins. Wild-type BHA10 scFv containing the original (Gly4Ser)3 linker had higher baseline ANS binding compared to the engineered BHA10 scFvs at all temperatures below 37°C. Increasing the temperature led to a steady increase of ANS binding to wild-type BHA10 scFv up to the transition temperature (~60°C) indicative of additional exposure of hydrophobic sites during thermal denaturation. Increasing the length of the linker from (Gly4Ser)3 to (Gly4Ser)4 nearly halved the fluorescence signal at lower temperatures and had a sustained stabilizing effect at all temperatures approaching the Tₘ. Addition of the disulfide bond (BHA10-SS/GS4 scFv) had a stabilizing effect at temperatures below the 60°C transition temperature but also appeared to have some stabilizing effect at higher temperatures. BHA10-GS4 and BHA10-SS/GS4 demonstrated marked increases in ANS-binding improved resistance to thermal challenge relative to the wild-type scFv (Fig. 4A). The scFv with the longer linker (BHA10-GS4 scFv) showed a +4°C increase in thermal resistance relative to the wild-type BHA10 scFv in this assay. Introduction of the disulfide bond at positions V₄₁ and V₅₂/₅₃ (BHA10-SS scFv) improved scFv thermal resistance by +10°C. Combining the engineered disulfide bond with the longer linker (BHA10-SS/GS4 scFv) increased the Tₘ by as much as 13°C, resulting in a scFv that was equally resistant to thermal challenge as the BHA10 Fab.

To further assess the stability and conformation of wild-type BHA10 and the engineered BHA10-GS4 and BHA10-SS/GS4 scFvs, we examined temperature-dependent binding of the hydrophobic dye 1-anilino-8-naphthalene sulfonate (ANS) to the purified scFvs (Fig. 4B). The intrinsic fluorescence of ANS is quenched in aqueous solvent and increases when bound to exposed hydrophobic surfaces such as those that occur in partially unfolded proteins. Wild-type BHA10 scFv containing the original (Gly4Ser)3 linker had higher baseline ANS binding compared to the engineered BHA10 scFvs at all temperatures below 37°C. Increasing the temperature led to a steady increase of ANS binding to wild-type BHA10 scFv up to the transition temperature (~60°C) indicative of additional exposure of hydrophobic sites during thermal denaturation. Increasing the length of the linker from (Gly4Ser)₃ to (Gly4Ser)₄ nearly halved the fluorescence signal at lower temperatures and had a sustained stabilizing effect at all temperatures approaching the Tₘ. Addition of the disulfide bond (BHA10-SS/GS4 scFv) had a stabilizing effect at temperatures below the 60°C transition temperature but also appeared to have some stabilizing effect at higher temperatures. BHA10-GS4 and BHA10-SS/GS4 demonstrated marked increases in ANS-binding.

Figure 3. Molecular model and sequence of BHA10 scFv. (A) The scFv is in the V₄₁/V₅₂ orientation. V₄₁ is shown in red, V₅₂ is shown in blue, and the Gly/Ser linker is shown in red. Paired cysteine substitutions are shown at V₄₁ and V₅₂ positions 44 and 100, and 105 and 43, respectively. The V₄₁ 105C is shown unpaired as it utilizes V₅₂ 44C. (B) Amino acid sequence of wild-type BHA10 scFv. The Gene III signal peptide is shown underlined, (Gly4Ser)₃ linker is indicated in bold type, and the Enterokinase site, Myc and His tags are indicated in italics. Positions of cysteine substitutions are as follows—the V₄₁ substitution at position 44 is shown as a single underline and at position 105 as a double underline. The V₅₂ substitution at position 43 is shown as a single underline and at positions 100 and 105 as double underlines.
only as the scFv approached its $T_m$, suggesting that increases in ANS-binding occur only as the native folded states of the stabilized scFvs begin to unfold.

Thermal unfolding of the purified wild-type BHA10 and engineered BHA10-GS4 and BHA10-SS/GS4 scFvs was monitored by differential scanning calorimetry (DSC) (Table 1). DSC data was analyzed using the non-2 state curve-fitting model. All three scFv $V_H$ domains demonstrated nearly identical thermal transition temperatures. Incremental increases in $V_H$ stability were observed with BHA10-GS4 and BHA10-SS/GS4 scFvs suggesting that most of the stability gain in these scFvs was associated with stabilizing the folded state of the $V_H$ domain. Lastly, binding affinities of the engineered BHA10 scFvs to LTβR were measured by surface plasmon resonance. BHA10 GS4 and BHA10-SS/GS4 binding properties were found to be essentially unchanged (±2-fold) compared to wild-type BHA10 scFv and FAb (Table 1).

Expression, production and characterization of stability-engineered BsAbs. Wild-type and stability-engineered BHA10 scFvs were used to generate a panel of eight IgG-like BsAbs as listed in Table 2. Plasmids encoding the eight BsAbs were first used to transiently express protein in CHO cells. Supernatants containing BsAbs were collected and evaluated by western blot analysis. Both N- and C-heavy chain fusion proteins migrated according to their predicted molecular weights (calculated protein molecular weights ~77 kDa) (Fig. 5A). Light chain proteins also migrated according to their predicted molecular weights (~25 kDa). Western blot analysis showed that the stability-engineered BsAbs containing either the BHA10-SS or BHA10-SS/GS4 scFvs expressed well independent of whether the scFv was fused to the amino- or carboxyl-termini (Fig. 5A, lanes 4, 5, 8 and 9). Surprisingly, little to no secreted protein was detected for the non-stabilized N-BsAb or N-BsAb-GS4 constructs (Fig. 5A, lanes 6 and 7 compared to lanes 8 and 9). While intact heavy chain fusion protein was detected in supernatants from all four C-terminal BsAb transfections, the presence of a 55–60 kDa molecular weight immunoreactive byproduct was substantially reduced in supernatants from both of the disulfide-bonded (SS) stabilized scFv-containing BsAbs (arrow, Fig. 5A, lanes 2 and 3 versus lanes 4 and 5). While we did not further characterize the 55–60 kDa molecular weight byproduct, given that it was found to be immunoreactive with the anti-IgG detection reagent we speculate that the fragment contains heavy chain Fc sequences.

Based on these results, four stable CHO cell lines-N-BsAb-SS, N-BsAb-Ss/GS4, C-BsAb-SS and C-BsAb-SS/GS4 were established and chosen for scale-up BsAb production. Stability-engineered BsAb titers ranged from 16–87 mg/liter. Protein A eluates of the stability-engineered BsAbs versus wild-type C-BsAb were first examined by analytical size-exclusion chromatography (Fig. 5B). The chromatograms revealed that compared to the wild-type C-BsAb eluate, which previously had been shown to contain ~40% aggregates, the C-BsAb-SS eluate contained a reduced level of aggregates at ~20%, and the C-BsAb-SS/GS4 eluate contained the lowest level of aggregates at ~10%. In comparison, control 14A2 IgG contained 6–8% aggregates (data not shown). The Protein A eluates of N- and C-BsAb-SS/GS4 BsAbs were further...
Stability-engineered bispecific antibodies purified by anion exchange chromatography. Greater than 1 gram of highly purified material was readily obtained for each of the two BsAbs. Analytical size-exclusion chromatography and SDS-PAGE demonstrated ≥98% monomeric BsAb for both N- and C-BsAb-SS/GS4 BsAbs (Suppl. Fig. 1).

We were interested in determining whether the engineered modifications impacted the long-term stability of the N-BsAb-SS/GS4 and C-BsAb-SS/GS4 BsAb molecules, a critical attribute for a biological therapeutic. Real-time stability studies of the BsAbs stored at 4°C for three months were conducted under buffer conditions and protein concentrations described in the Methods. Protein quality was assessed for (1) aggregation/precipitation (2) polypeptide cleavage or proteolysis, and (3) post-translational modifications such as oxidation or changes in oligosaccharide distribution. SEC/light scattering analyses showed little evidence of aggregation or material loss that might occur through precipitation (Suppl. Table 1). Both the N-BsAb-SS/GS4 and C-BsAb-SS/GS4 BsAbs contained ≥96.7% monomer after three months storage at 4°C for both high and low protein concentrations as compared to 97.4% monomer for the BHA10 IgG control. Increases of 1–2% oligomeric material were detected at the end of the study for both BsAbs and were predominately composed of BsAb dimers. Neither of the BsAb samples accumulated detectable levels of high-order aggregates, even though this type of aggregate would be readily observed by light scattering detection. In comparison, the BHA10 IgG control antibody accumulated less than 1% dimers over the three month study. The integrity of the BsAbs was monitored by SDS-PAGE and LC/MS analyses. Non-reducing SDS-PAGE showed that the C-BsAb-SS/GS4 remained intact with no evidence of proteolysis observed during the three month study at 4°C (Suppl. Fig. 2). Analysis revealed extremely low and comparable levels of fragmentation by the end of the three month study for both the low and high concentration N-BsAb-SS/GS4 samples. LC/MS analyses of N-BsAb-SS/GS4 and C-BsAb-SS/GS4 were performed to detect any changes in protein molecular masses, an indication of potential degradation. Deconvoluted N-BsAb-SS/GS4 and C-BsAb-SS/GS4 light and heavy chain mass spectra showed no discernable modifications arising during the three month study indicating absence of chemical modifications that could adversely affect bispecific antibody function or stability (Fig. 6). Lastly, the two BsAbs were subjected to three cycles of freeze-thaw and agitation stress over 12 hours at room temperature. SEC/LS results showed a slight reduction of 0.2–0.6% in monomeric peak area for all samples with exception of the BHA10 antibody control (Suppl. Fig. 3 and Suppl. Table 2). Aggregates were mostly dimers as determined by SEC retention time as well as static light scattering detector (data not shown).

The results of the agitation studies yielded no visible precipitates. SEC/LS analyses showed a minor decrease of 1–1.5% in monomeric peak area for the two low concentration N-BsAb-SS/GS4 and C-BsAb-SS/GS4 samples (Suppl. Table 3). Reductions in monomeric peak areas were found to be inversely proportional with increases in BsAb dimers (data not shown).

In vitro activity of stability-engineered BsAbs. The N- and C-BsAb-SS/GS4 BsAbs were tested for dual binding activity

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**Table 2** Recombinant IgG-like BsAbs

| BsAb       | scFv moiety | (Gly₄Ser)n linker length | scFv V₁₀₀ and V₄₄ disulfide bond |
|------------|-------------|--------------------------|---------------------------------|
| N- BsAb⁹   | BHA10 wild-type | 15 aa                    | no                              |
| N- BsAb-GS4| BHA10 GS4    | 20 aa                    | no                              |
| N- BsAb-SS | BHA10-SS/GS4 | 15 aa                    | yes                             |
| N- BsAb-SS/GS4 | BHA10-SS/GS4 | 20 aa                    | yes                             |
| C- BsAbβ   | BHA10 wild-type | 15 aa                    | no                              |
| C- BsAb-GS4| BHA10 GS4    | 20 aa                    | no                              |
| C- BsAb-SS | BHA10-SS/GS4 | 15 aa                    | yes                             |
| C- BsAb-SS/GS4 | BHA10-SS/GS4 | 20 aa                    | yes                             |

⁹N- indicates scFv at the amino-terminus of the BsAb. βC- indicates scFv at the carboxyl-terminus of the BsAb.

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**Figure 5.** Design and expression of stability-engineered BsAbs. (A) Western blot analysis of BsAbs transiently expressed in CHO cells. Supernatant samples in the left panel are analyzed under non-reducing conditions and under reducing conditions in the right panel. Lane 1: MW marker, 2: C-BsAb, 3: C-BsAb-GS4, 4: C-BsAb-SS, 5: C-BsAb-SS/GS4, 6: N-BsAb, 7: N-BsAb-GS4, 8: N-BsAb-SS, 9: N-BsAb-SS/GS4. Arrow indicates presence of ~55–60 kDa unidentified immunoreactive species. (B) Normalized SEC profiles of stability-engineered C-BsAbs following Protein A chromatography. Sharp peaks eluting between 13–15 min represent monomeric BsAb. Broad peaks eluting between ~10.75–14 min represent aggregated protein. Blue dash-dot = C-BsAb, red dash = C-BsAb-SS, and green line = C-BsAb-SS/GS4.
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Figure 6. Intact mass analyses of N- and C-BsAb-SS/GS4. N- and C-BsAb-SS/GS4 stored for T = 0 and T = 3 months at 4°C were analyzed for proteolysis and posttranslational modification by LC/MS. Upper, N-BsAb-SS/GS light and heavy chains; lower, C-BsAb-SS/GS light and heavy chains. Both light chain and heavy chain masses are consistent with theoretical calculations based upon protein primary sequences. No significant changes in mass as results of modifications were detected. Typical IgG biantennary glycans with variable numbers of terminal galactose were observed such as G0, G1, and G2.

Figure 7. Dual-binding activity of stability-engineered BsAbs. Surface plasmon resonance analysis of stability-engineered BsAbs for dual-binding to TRAIL-R2 and LTβR. Biotinylated anti-His6 antibody was immobilized onto a streptavidin-labeled sensorchip followed by capture of TRAIL-R2-Fc-His6 (TRAIL-R2). (A) sensorgrams show subsequent additions of BsAbs and 0, 3, 10, 30, or 100 nM concentrations of sLTβR (LTβR). N-BsAb-SS/GS4 shown as dashed line and C-BsAb-SS/GS4 shown as solid line. (B) calculated kinetic rate constants. To assay for in vitro cellular activity, tumor cells that stain positive for expression of both TRAIL-R2 and LTβR by fluorescence cytometry (unpublished data) were treated with the stability-engineered BsAbs or with control mAbs (BHA10 and 14A2) alone or in combination, and cell viability was measured after 3–4 days in culture. In the human WiDr colon carcinoma cell line, the individual mAbs were effective in reducing cell number, while the combination of mAbs showed somewhat enhanced activity. Both the N- and C-terminal BsAbs showed activity comparable to the mAb combination (Fig. 8A), achieving similar total reduction in cell number as well as statistically similar IC50 values, that is, the concentration of antibodies resulting in 50% growth inhibition (IC50 = 18–36 pM). Preincubation of the

to the soluble receptor targets, TRAIL-R2 and LTβR, by surface plasmon resonance (SPR). N- and C-BsAb-SS/GS4 BsAb kinetic association/dissociation curves were generated to immobilized TRAIL-R2 followed by a secondary association/dissociation phase with soluble LTβR (Fig. 7). These results confirmed that both the N- and C-BsAb-SS/GS4 BsAbs simultaneously bound TRAIL-R2 and LTβR with nearly identical association and dissociation kinetics suggesting equivalent binding activity for both the N- and C-terminal BsAbs in this assay format. The control antibody BHA10 IgG showed no detectable binding to immobilized TRAIL-R2 while the control antibody 14A2 showed binding activity similar to both of the BsAbs towards TRAIL-R2 (data not shown).

Figure 8. Dual-binding activity of stability-engineered BsAbs. Surface plasmon resonance analysis of stability-engineered BsAbs for dual-binding to TRAIL-R2 and LTβR by fluorescence cytometry (unpublished data) were treated with the stability-engineered BsAbs or with control mAbs (BHA10 and 14A2) alone or in combination, and cell viability was measured after 3–4 days in culture. In the human WiDr colon carcinoma cell line, the individual mAbs were effective in reducing cell number, while the combination of mAbs showed somewhat enhanced activity. Both the N- and C-terminal BsAbs showed activity comparable to the mAb combination (Fig. 8A), achieving similar total reduction in cell number as well as statistically similar IC50 values, that is, the concentration of antibodies resulting in 50% growth inhibition (IC50 = 18–36 pM). Preincubation of the
Bispecific antibodies (BsAbs) with soluble receptor, either LTβR-Fc or TRAIL-R2-Fc, resulted in an expected reduction in activity in accordance with the contribution of the individual target (data not shown). In the MDA-MB231 breast tumor cell line, while treatment with the individual mAbs or the combination of mAbs revealed negligible activity, the C-BsAb-SS/GS4 BsAb had a pronounced effect (IC_{50} = 46 pM) in reducing tumor cell number (Fig. 8B). The N-BsAb-SS/GS4 BsAb, in contrast, exhibited no activity in the MDA-MB231 cell line. In the Me180 tumor cell line, both BsAbs showed stronger anti-tumor cell activity relative to the combination of mAbs, with the C-terminal version exhibiting greater activity (p = 0.03) relative to the N-terminal BsAb (Fig. 8C). Importantly, the BsAbs lacked detectable cytotoxic activity on a normal cell line (HUVEC) in this assay (Fig. 8D), despite the presence of both receptors on the cell surface as measured by fluorescence cytometry (data not shown), suggesting tumor specific activity of the BsAbs.

Stability-engineered BsAbs exhibit good pharmacokinetic properties and inhibit tumor cell growth in vivo. We next assessed the pharmacokinetic parameters of the stability-engineered N- and C- BsAbs. A single 10 mg/kg bolus of BsAb was administered by intraperitoneal injection into CB17-scid mice. The stability-engineered BsAbs exhibited in vivo half-lives comparable to that of standard monoclonal antibodies (N-BsAb-SS/GS4: T_{1/2} = 10.3 days; C-BsAb-SS/GS4: T_{1/2} = 15.1 days) (Table 3), as measured in an ELISA detecting IgG in the serum samples. Cmax values were 85.7 µg/ml and 105.7 µg/ml for N-BsAb-SS/GS4 and C-BsAb-SS/GS4 BsAbs, respectively. To confirm that the bispecific antibody remained intact in the serum, we employed an antigen-specific ELISA, using LTβR-Fc coated on the plate and detection with TRAIL-R2-Fc-His6. Serum concentration-time curves of the BsAbs detected by the antigen-specific ELISA were comparable to those in the standard IgG detection ELISA (Suppl. Fig. 4), indicating that the BsAbs remained functionally intact in the serum throughout the course of the study. Both BsAb designs bound mouse (Fig. 9A) and human (Fig. 9B) neonatal Fc receptor, FcRn, with affinities equivalent to that of native IgG antibodies. The observed affinities are consistent with the demonstrated IgG-like PK properties of the BsAbs. Next we proceeded to test for efficacy in a tumor xenograft model. Immunodeficient mice with established WiDr tumors were treated by intraperitoneal administration with stability-engineered BsAbs (1x/week at 2 mg/kg), or with control mAbs alone (2x/week at 2 mg/kg), or in combination (2x/week each at 1 mg/kg). Both N- and C- BsAbs were efficacious in achieving greater than 60% tumor growth inhibition in the mice (Fig. 10A). The in vivo results closely reflected those observed in WiDr cells in vitro, with the BsAbs showing comparable tumor inhibition to the combination of the individual antibodies, and enhanced activity (p < 0.05) relative to the individual antibodies.

We then tested the C-BsAb-SS/GS4 BsAb in the MDA-MB231 xenograft model because in vitro data suggested improved potency of the BsAb relative to the combination of mAbs in this cell line. The C-BsAb-SS/GS4 BsAb indeed demonstrated significant anti-tumor activity (p < 0.001) achieving up to 50% tumor inhibition in the MDA-MB231 model (Fig. 10B), whereas the individual mAbs, and even the combination of mAbs, showed no efficacy. As was observed in vitro, the N-terminal BsAb did not inhibit tumor growth in the MDA-MB-231 model (data not shown).

Table 3  Pharmacokinetic parameters

| BsAb                | T1/2 (hr) | Cmax (µg/ml) | AUC (hr*µg/ml) | Vd* (L/kg) | Cl* (mL/min/kg) |
|---------------------|-----------|--------------|----------------|------------|-----------------|
| N-BsAb-SS/GS4       | 247.9     | 85.7         | 12724          | 0.171      | 0.008           |
| C-BsAb-SS/GS4       | 362.4     | 105.7        | 16486          | 0.175      | 0.0056          |

*Not corrected for bioavailability. T1/2, serum half-life; Cmax, maximum serum concentration; AUC, area under the curve; Vd, apparent volume of distribution; Cl, clearance.
Stability-engineered bispecific antibodies

Discussion

Here we show that enhancing the thermal stability of a scFv domain, a commonly used building block for constructing IgG-like BsAbs, greatly improves BsAb production and quality. Once sufficiently stabilized, scFv gene sequences can be appended to either the amino- or carboxyl-terminus of an IgG for producing biologically active, physically stable tetravalent IgG-like BsAbs. We found that stability-engineered BsAbs could be produced at reasonably high levels (16–87 mg/L) in a mammalian host cell line without gene amplification. The BsAbs could be purified to a high level of purity (>98%) using standard and scalable purification strategies and also demonstrated excellent biophysical stability. Only minor (≤2%) changes in protein quality, such as aggregation or precipitation, were detected over a three month storage period at 4°C in standard saline buffer. No chemical modifications, such as oxidized or deamidated amino acid residues, were detected. The oligosaccharide distribution pattern was indistinguishable from that of normal IgGs. The biophysical and biochemical behavior of the stability-engineered BsAbs under these conditions compares favorably to that of normal IgGs as no attempt was made to optimize formulation of the BsAbs. Pharmacokinetic studies showed that both the N- and C- BsAbs had long serum half-lives and remained functionally intact. The lengthy serum half-lives of the stability-engineered BsAbs is consistent with our finding that the BsAbs bind to the neonatal Fc receptor FcRn and therefore likely do not interfere with the IgG salvage pathway. Finally, both the in vitro and in vivo studies demonstrated that the BsAbs are biologically active with inhibition of tumor growth occurring at levels comparable to or greater than the combination of parental antibodies.

Numerous methods have previously been described for preparing recombinant IgG-like BsAbs with many showing excellent activity in vitro. However, preparation of IgG-like BsAbs that are suitable for preclinical evaluation in vivo has been particularly challenging, and few study results have been reported. Recently, a di-diabody IgG-like BsAb (assembled from two cross-over scFv chains) was described that was shown to simultaneously target and block activity of EGF and IGF-1 growth factor receptors expressed on the surface of cultured tumor cells. While the di-diabody BsAb was shown to be effective at inhibiting tumor growth in vivo in a mouse xenograft model, ≥210% activity to both receptors was reportedly lost within a 24 h period following administration, suggesting that the di-diabody construct may have lacked sufficient stability. More recently, a report by Wu and colleagues described a promising technology for producing dual-targeting N-terminal IgG-like BsAbs referred to as dual-variable domain (DVD) IgGs that were shown to exhibit favorable PK properties and in vivo activity comparable to that of the combined mAbs. The DVD technology links the V_L and V_H domains (Fv) specific to one target in series to the corresponding domains of an IgG specific to a second target using the naturally occurring conserved \textit{“elbow”} sequences located at the amino terminus of C_L and C_H\textsubscript{1}. It was proposed that the flexibility of the elbow sequences and absence of strong secondary structure allows for stable assembly of the added N-terminal Fvs.

In our study we provide proof-of-concept evidence that a sufficiently stable scFv can be a useful building block for producing high quality IgG-like BsAbs. Increasing the BHA10 scFv linker length from 15 to 20 amino acids resulted in a moderate increase in scFv thermal stability. Consistent with the computational model, the longer (Gly\textsubscript{3}Ser\textsubscript{3}) linker was likely relieving strain on the V_H domain, favoring a more stable scFv folded-state. Also consistent with scFv model, only the cysteine residues introduced at positions V_L\textsubscript{44} and V_H\textsubscript{100} between the BHA10 scFv V_H/V_L interface led to a stable, functional BHA10 scFv. However, given the likelihood that introducing disulfides may not be a general solution for stabilizing scFvs, we anticipate that linker length optimization combined with rational and molecular evolution strategies will make it possible to routinely improve scFv stability for producing quality IgG-like bispecific antibodies. While ongoing studies are addressing whether a threshold thermal transition value can be defined for selecting a suitably stabilized scFv for constructing a stability-engineered IgG-like BsAbs, we surmise that a T\textsubscript{m} value ≥65°C or a T\textsubscript{m} value ≥70°C may be desirable. Presumably, related approaches could be applied for

![Figure 9. Binding of BsAbs to neonatal Fc receptor. Binding of various concentrations of biotinylated FcRn-Fc to fixed concentrations of immobilized N-BsAb-SS/GS4 BsAb (●), N-BsAb-SS/GS4 BsAb (□), hlgG1 (♀) and mLgG1 (△) in an ELSA assay. Receptor complexes were washed with PBS, pH 6, detected by incubating with streptavidin-HRP conjugate and developed with peroxidase substrate. Plates were read at 450 nm. (A) mouse FcRn-Fc; and (B) human FcRn-Fc.](image-url)
stabilizing other immunoglobulin variable regions to be used for building IgG-like BsAbs such as domain antibodies, diabodies and tandem diabodies.

Antibody and antibody-derived therapeutics must have sufficient physical and chemical stability to progress towards clinical application. Preliminary stability studies of the N-BsAb-SS/GS4 and C-BsAb-SS/GS4 BsAbs showed good biophysical stability under conditions similar to those encountered during storage and handling of biotherapeutics. To fully validate the biophysical properties of our IgG-like BsAb molecules more comprehensive studies should be carried out. Such studies might include long-term stability studies at various temperatures in different formulation buffers. In addition, studies should be pursued to quantitatively investigate the effect of long-term storage on the binding activity of the BsAbs towards both target antigens.

The IgG-like BsAbs in this study were designed to target two TNF receptor family members, TRAIL-R2 and LTβR, to explore the possibility that a BsAb might trigger an enhanced or broader anti-tumor response than that achieved by treating with a mixture of the two antibodies. Significantly, the BsAbs exhibited enhanced anti-tumor activity relative to the combination of parental antibodies in a subset of cell lines in vitro. In vivo the enhanced activity of the C-BsAb was recapitulated in the MDA-MB231 model. Notably, normal HUVEC cells, despite being comparable to MDA-MB231 and Me180 in expression of both LTβR and TRAIL-R2 receptors on the cell surface and in their insensitivity to the mAbs, were uniquely insensitive to induction of cell death by the BsAbs, suggesting that tumor cells may be preferentially sensitive to the killing activity.

These studies suggest that the pronounced anti-tumor activity of the BsAbs may be due to crosslinking of LTβR and TRAIL-R2 receptors, an event which does not occur by simply combining the individual antibodies. We speculate that the anti-LTβR x anti-TRAIL-R2 BsAbs might be inducing the formation of non-physiological receptor complexes that promote novel or enhanced signaling events such as was described with Fas activation by varying the oligomer states of Fas ligand.26 Alternatively, the BsAb could alter signal intensity or quality by forcing receptor relocalization into a new environment such as a lipid raft or by altering the kinetics of receptor internalization.27 Further studies are warranted to gain a better understanding of the BsAb-mediated anti-tumor response. While the mechanism of cooperation is not fully understood, this is the first example of a BsAb with enhanced activity over the individual antibody combination, highlighting a potential feature of BsAb-based therapeutics using agonist antibodies.

While both the N- and C- BsAbs exhibited similar and equivalent dual binding activity to soluble TRAIL-R2 and LTβR receptors, the C-BsAb showed enhanced biological activity relative to the N-BsAb in some cell lines. It is possible that the differing activities of the N- and C-BsAbs may depend on the varying densities of LTβR and TRAIL-R2 receptors on the different tumor cell lines. The enhanced activity of the C-BsAb may also be explained by the possibility that the N-terminal configuration cannot fully engage and cross-link LTβR and TRAIL-R2 receptors due to close physical proximity and steric interference between the BHA10 scFv and the 14A2 Fab arm. We estimated that the 25 amino acid (G4S)5 linker used to append the BHA10 scFv to the amino terminus of 14A2 heavy chain generates about 50 angstroms distance between the complementarity determining regions of the 14A2 and BHA10 antibody binding domains and is expected to allow sufficient separation of the two sites. This suggests that the BsAb format (N- versus C-terminal BsAb)
and spatial orientation of the antibody binding domains may be critical for effective engagement of receptors on a cellular surface, although this may not be the case for targeting soluble antigens. We believe that the flexibility in design offered by this stability engineering approach is an important feature allowing assessment of various IgG-like BsAb formats to select a biologic with optimal therapeutic activity.

Materials and Methods

Cloning, design and stability engineering of BHA10 scFv. BHA10 scFv was cloned into the pBAD bacterial expression vector (Invitrogen, Carlsbad, CA) using the Polymerase Chain Reaction with primers 5’-CAG TAG CAT GCA GGT CCA ACT GGT GCA G-3’ and 5’-GTT CTA GAA AGC TTT TGT CGT CGT CTG CCT TGA TCT CCA CCT TGG TAG CCT G-3’. The PCR product was purified using the Millipore Ultrafree-DNA extraction kit (Millipore; Bedford, MA), digested with SpI I, made blunt-end by digesting with DNA Polymerase I in the presence of dNTPs, and then digested with Hind III. The blunt-ended/Hind III digested PCR product was ligated to Sca I/Hind III digested XL10-GOLD® (Stratagene). Paired cysteine substitutions were predicted using SCWRL3 algorithm.30 The models were subsequently refined in CHARMm and analyzed using Pymol implemented in Prime.29 The conformations of the side chains (Gly4Ser) loops were predicted using a hierarchical technique implemented in Prime.29 The conformations of the side chains were predicted using SWCRR3 algorithm.30 The models were essentially as described with the exception that no alkylating agents were used.33 LTβR-Fc was prepared as described.34

Thermal challenge assays. Plasmid DNAs encoding wild-type and engineered BHA10 scFvs were used to transform W3110. Colonies were grown overnight in SB expression media supplemented with 1% glycerol, 1% Triton X-100, 0.02% arabinose, and 50 μg/ml carbenicillin at either 37°C or 32°C.35 Bacterial supernatants were collected and heat-treated in either PCR strip tubes or 96-well plates (Applied Biosystems, Foster City, CA) for 60–90 min in a thermal cycler (iCycler, Bio-Rad, Gaithersburg, MD). Cooled samples were transferred to 96-well v-bottomed plates (Corning, Corning, NY) and centrifuged at 2,000 rpm, 4°C for 30 min (IEC Centra 8R, Thermo Electron, Waltham, MA). Supernatants were then assayed for binding to LTβR-Fc antibody by DELFIA. Maxisorp 96-well plates (Nalge Nunc, Rochester, NY) were coated with 1 μg/ml LTβR-Fc in 0.1 M sodium carbonate buffer, pH 9.5 overnight at 4°C, and then blocked with DELFIA assay buffer (DAB, 10 mM Tris HCl pH 7.4, 150 mM NaCl, 20 μM EDTA, 0.5% BSA, 0.02% Tween 20, 0.01% NaN3) for 1 h with shaking at room temperature and washed 3 times with DAB buffer without BSA (Wash buffer). Test samples diluted in DAB were added to plates in a final volume of 100 μl, incubated for 1 h with shaking at room temperature, and then rinsed with Wash buffer. Bound BHA10 scFv was detected by incubating with 40 ng/ml of Eu-labeled anti-His6 antibody followed by a wash step and addition of DELFIA enhancement solution (Perkin Elmer, Boston, MA). Plates were read using the Europium method on a Victor 2 (Perkin Elmer). For thermal gradients, the data was analyzed with Prism 4 software (GraphPad Software, San Diego, CA) using a sigmoidal dose response with variable slope as the model. The values obtained for the mid-point of the thermal denaturation curves are referred to as Tm values and are not construed as being equivalent to biophysically derived Td values.

Differential scanning calorimetry analyses. The thermal unfolding profiles of BHA10 Fab and scFv antibody fragments were measured using an automated capillary differential scanning calorimeter (capDSC; MicroCal LLC, Northampton, MA). Scans were performed at 1°C/min using the medium feedback mode for enhanced peak resolution. Protein concentrations ranged between 0.4–1.0 mg/ml. The scan range was 20–95°C. All 96-well plates containing protein were stored within the instrument at 6°C. Transition midpoints (Tm’s) were determined by fitting the curves using the non-two-state model with the Origin 7 software provided by the manufacturer.

ANS assay. The hydrophobic dye 1-anilino-8-naphthalene sulfonate was used to probe BHA10 scFv protein conformation. Twenty micrograms per milliliter scFv protein solutions in PBS were treated with 10 μM final concentration of dye and fluorescence measured on a J-810 circular dichroism spectropolarimeter (Jasco, Easton, MD) equipped with a thermoelectric peltier device and a fluorescence detector perpendicular to the light path.

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Excitation and emission monochromators were set to 370 nm (10 nm bandwidth) and 480 nm, respectively. Thermal denaturation was performed with a data pitch of 1.0°C and a temperature slope of 120°C/hour. Photomultiplier voltage was set to 600 V.

**Affinity measurements.** Isothermal titration calorimetry (ITC) was used to measure the affinity of BHA10 FAb to LTβR-Fc. ITC was performed on a VP-ITC unit (MicroCal LLC) set to 30°C. Approximately 500 μl of a 7 μM LTβR-Fc solution was placed into the sample cell, and PBS dialysate was placed in the reference cell. A total of 234 μl of 70 μM BHA10 FAb was titrated into the sample cell in 7 x 10 μl, 12 x 7 μl, followed by 8 x 10 μl injections. The reaction stoichiometry was 1:1. ITC curves were analyzed using the Origin Software supplied by the manufacturer.

K<sub>D</sub> measurements of BHA10 scFv preparations were performed using surface plasmon resonance (SPR) on a Biacore 3000 instrument (Biacore Inc., Piscataway, NJ). All experiments were performed in HBS-EP buffer, pH 7.4. Twenty μg/ml biotinylated PENTA-His antibody (Qiagen) was immobilized onto a streptavidin-coated CM5 chip at a flow rate of 10 μl/min for approximately 1 min. 0.1 μM solutions of wild-type and engineered BHA10 scFvs were injected over the chip at a flow rate of 5 μl/min for 10 min. A series of LTβR-Fc solutions ranging from 1–200 nM were subsequently injected at a flow rate of 30 μl/min onto the scFv-coated surface. Background was subtracted using buffer only samples. Sensorgram curves were analyzed using the BiaEval 3.0 manufacturer’s software. K<sub>D</sub> values were calculated by fitting kinetic association and dissociation curves to a 1:1 Langmuir binding model.

**BsAb expression and purification.** Wild-type and engineered BHA10 scFv gene sequences were amplified in a two-step PCR reaction and used to construct a panel of BsAbs as N- and C-terminal fusions to the chimeric anti-TRAIL-R2 IgG (14A2) for expression in CHO cells using the mammalian expression vector pN5KG1. For the N-terminal bispecific constructs (N-BsAbs), a (Gly<sub>4</sub>Ser)<sub>3</sub> linker was used to connect the BHA10 scFvs to the mature amino terminus of 14A2 heavy chain. For the C-terminal bispecific constructs (C- BsAbs), a Ser<sub>5</sub> (Gly<sub>4</sub>Ser)<sub>3</sub> linker was used to connect the BHA10 scFvs to the carboxyl terminus of 14A2 heavy chain.

Plasmid DNAs were used to transform DHFR-deficient CHO DG44 cells for transient and stable production of antibody protein. For transient transfection 20 μg of plasmid DNA was transfected into 4 x 10<sup>6</sup> cells by electroporation and the cells cultured in CHO-SSFM II media containing 100 μM hypoxanthine and 16 μM thymidine (Invitrogen) for four days at 37°C. Transient protein expression was evaluated by western Blot. Briefly, supernatant samples were fractionated on 4–20% Tris-glycine SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-human IgG antibody-HRP conjugate. Membranes were processed using the ECL western blotting analysis system (Amersham ECL, GE Healthcare Life Sciences, Piscataway, NJ).

For stable protein production, transfected DHFR positive cell lines were adapted to serum-free conditions and scaled for antibody production. Supernatants from 11-day bioreactor runs were harvested and precleared by ultrafiltration. The BsAbs were affinity purified by Protein A Sepharose FF (GE Healthcare) chromatography. Bound antibodies were eluted with 0.1 M glycine, pH 3.0 and neutralized to approximately pH 8 by addition of Tris base. Eluants were diluted 10-fold into 50 mM Tris, pH 8.5 and proteins purified to homogeneity using a TMAE anion exchange column (Fractogel®, EMD Biosciences, San Diego, CA). BsAbs were dialyzed into PBS (Irvine Scientific, Santa Ana, CA). Endotoxin levels were assayed by Kinetic Quantitative Chromogenic LAL Analysis (Lonza, Allendale, NJ). Purity and percentage of monomer BsAb product was assessed by 4–20% Tris-glycine SDS-PAGE and analytical size-exclusion HPLC (SEC), respectively.

**BsAb dual binding assay.** Surface Plasmon Resonance (SPR) was used to measure dual binding activity of the BsAbs. Penta-His Biotin Conjugate antibody immobilized sensochips were prepared as described above. Twenty microliters of 50 nM TRAIL-R2-Fc-His<sub>6</sub> (R&D Systems, Minneapolis, MN) was injected at 2 μl/min flow rate. Flow rates were then increased to 10 μl/min. Seventy microliters of (1) HBS-EP buffer, (2) 14A2 antibody, (3) BHA10 antibody, (4) 25 nM N-BsAb-SS/GS4 or (5) 25 nM C-BsAb-SS/GS4 were injected followed by 10 minutes of dissociation in buffer prior to subsequent 70 μl injections of 0–100 nM dilutions of LTβR-Fc. Complexes were allowed to dissociate for approximately 8 additional minutes. Sensorgrams were processed using BiaEval software provided by the manufacturer and plotted using KaleidaGraph™ (Synergy Software, Reading, PA). K<sub>D</sub> values were calculated by fitting kinetic association and dissociation curves to a 1:1 Langmuir binding model.

**FcRn binding assay.** mAb or BsAb were coated on Nunc MaxiSorp 96-well plates at 3 μg/ml in PBS, pH 6 overnight at 4°C. The plates were washed once with PBS, pH 6 and blocked with 300 μl/well of 0.1 M sodium phosphate, 0.1 M sodium chloride, 0.05% Tween 20 and 0.1% gelatin, pH 6 for 2 h at room temperature. The plates were washed again with PBS, pH 6 and incubated with 100 μL of the appropriate concentration of recombinantly produced biotinylated mouse or human FcRn-Fc. The FcRn-Fc constructs contain mutations within the Fc that abrogate self-association of the FcRn with the Fc domains. After 1.5 h plates were washed with PBS, pH 6 and incubated with 100 μL of streptavidin HRP at room temperature for 1.5 h, washed with PBS, pH 6 and developed with TMB (3, 3′, 5′, 5′-tetramethylbenzidine) and read at 450 nm. A four-parameter logistic fit was used to model the binding data.

**Stability studies of BsAbs.** High and low concentration BsAb samples were formulated in PBS and used for 2–8°C stability studies. N-BsAb-SS/GS4 was used at 1.8 mg/ml and 10.3 mg/ml and C-BsAb-SS/GS4 at 5.0 mg/ml and 11.4 mg/ml. Initial (T = 0), intermediate (T = 1 week, 2 week, 1 month, 2 month) and final (T = 3 month) time point samples were analyzed immediately following sample collection. In addition, initial (T = 0) samples were frozen and stored at -70°C until thawed for secondary analyses at the end of the three month study. BHA10 IgG (8.7 mg/ml) was used as control and was similarly handled.

Protein aggregation or precipitation was monitored using SEC linked inline to a miniDawn light scattering detector and a rEX
refractive index detector (SEC/LS, Wyatt Technology Corp., Santa Barbara, CA). Light scattering data were analyzed using the Astra V software provided by the manufacturer. Twenty-five microgram samples were injected onto a TSK-gel G3000SWXL analytical SEC column (Tosoh Biosciences, Montgomeryville, PA) at 5 μl/min using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) pre-equilibrated with 10 mM phosphate, 150 mM NaCl, 0.02% azide at pH 6.8.

Proteolysis and post-translational modifications were monitored using SDS-PAGE and liquid chromatography/intact mass spectral analyses (LC/MS, Agilent LC/MS/TOF TOF coupled to an Agilent 1100 LC system via an electrospray interface). For SDS-PAGE analysis, 5 μg of protein was loaded per lane. For LC/MS analysis, T = 0, T = 1 month, and T = 3 month samples were analyzed in both the non-reduced and reduced form. Reduced samples were prepared by dilution into a 50 mM DTT/4 M guanidine hydrochloride buffer for 1 hour at 37°C. HPLC Buffer A and Buffer B were 0.03% TFA in H2O and 0.025% TFA in acetonitrile, respectively. The flow rate was 100 μl/min. 7.5 μg of each sample (reduced and non-reduced) was injected onto a 2.1 x 50 mm Vydac C4 column and analyzed by Agilent ESI-TOF. A bind-and-elute method was used for non-reduced samples while a gradient method was used for reduced samples. Spectra were obtained using the Analyst and deconvoluted using the MaxEnt1 software packages included with the instrumentation.

For freeze-thaw analyses one set of high and low concentration BsAb samples and the BHA10 IgG control were stored at 4°C for the duration of the study. The remaining samples were frozen at -70°C for a minimum of one hour. The samples were removed from the freezer and allowed to stand at room temperature until completely thawed. Samples were then mixed, one set of samples moved to 4°C storage for the remainder of the study, and the remaining samples refrozen. This freeze-thaw sequence was repeated two additional times. Percent monomers were analyzed by SEC/LS.

Lastly, BsAb solutions and the BHA10 IgG control were subjected to agitation stress on an orbital shaker at 650 rpm for 16 h at room temperature. Percent monomers were analyzed by SEC/LS.

Tumor cell proliferation assay. The human tumor cell lines WiDr colon carcinoma (ATCC No. CCL-218), Me180 cervical carcinoma (ATCC No. HTB-33), and MDA-MB-231 breast carcinoma (generously provided by Dr. Dajun Yang, Ascenta Therapeutics, San Diego, CA) were cultured in MEM-Earles with 10% FCS, 2 mM L-Glutamine, 1X non-essential amino acids, 0.5 mM sodium pyruvate, and penicillin/streptomycin. Cell lines were rinsed once with PBS and cells released by digestion with trypsin. Cells were collected by centrifugation, resuspended in complete media, counted and seeded into 96-well tissue culture plates at 5,000 cells/well for WiDr and Me180; and 1,500 cell/well for MDA-MB-231. Human IFNγ (Biogen Idec, Corp.,) was added to the cell suspensions to result in a final cytokine concentration of 50–80 U/ml.39 Fifty microliters of the cell plus IFNγ suspension was mixed with 50 μl of 2X concentrated 3-fold serial dilutions of test antibodies prepared in complete media. The final concentrations of test antibodies typically ranged from 5,000 pM to 0.07 pM. Cells were grown for 4 d (WiDr and Me180) or 3 d (MDA-MB-231) at 37°C in a 5% CO2 humidified chamber and cell killing assessed by the addition of 20 μl/well Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay reagent (Promega Corporation, Madison, WI). Human umbilical vein endothelial cells (HUVEC) were used as control. Plates were read in a micoriter plate reader at 490 nm (Spectromax Plus, Molecular Devices, Sunnyvale CA). Data was graphed using Microsoft Excel (Microsoft Inc., Bellevue, WA).

In vivo PK analysis and tumor xenograft models. Mice were maintained in accordance with the Biogen Idec Institutional Animal Care and Use Committee, and city, state and federal guidelines for the humane treatment and care of laboratory animals. For pharmacokinetic (PK) studies a single bolus injection of 10 mg/kg (1 mg/ml) BsAb diluted in PBS was administered intraperitoneally (ip) into male CB17- scid mice (Harlan Sprague-Dawley, Inc.). Mice were sacrificed at 0, 0.5, 2, 6, 24, 48, 72, 96, 168, 240 and 336 h post-injection using three mice per timepoint for each BsAb. Serum samples were prepared for analysis by ELISA assay to quantify levels of the BsAbs. For the standard Ig detection ELISA, plates were coated with goat-anti-human IgG, blocked with PBS/1% BSA, and dilutions of serum containing the BsAbs were serially diluted in PBS/1% BSA, added to the plates and incubated. Captured antibodies were detected with a goat-anti-human kappa chain-HRP-linked antibody. Pharmacokinetic properties were calculated using noncompartmental module of WinNonLin (Pharsight Corp., Mountain View, CA). For the antigen-specific ELISA, a sandwich ELISA was employed. 96-well microtiter Immulon II plates were coated with 2 μg/ml LTβR-Fc overnight at 4°C. Nonspecific sites were blocked with 0.5% Nonfat Dry Milk in PBS plus 0.01% Thimerosal for 1 hour at 37°C. Serial dilutions of serum samples were added to duplicate wells and incubated for 1 hour at 37°C. Plates were washed and 100 μl of 100 μg/ml TRAIL-R2/Fc/His6 (R&D Systems, Minneapolis, MN) was added to the wells and the plate was incubated for 1 h at 37°C. LTβR-Fc: BsAb: TRAIL-R2/Fc/His6 complexes were detected with Penta-His Horseradish Peroxidase Conjugate (QIAGEN, Cat. 34460) and developed with TMB Peroxidase Substrate (Kirdgaard and Perry Labs, Cat. 50-76-00). Plates were read on a SpectraMax platereader and data plotted with the SoftMax Pro software provided by the manufacturer (Molecular Devices Corp., Sunnyvale, CA).

For tumor xenograft studies, WiDr human colon carcinoma (2 x 106 cells per mouse) cells were implanted subcutaneously into the flanks of athymic nude mice. Mice bearing tumors of approximately 100 mg in size were randomly divided into six groups of ten mice. IP treatments were administered, beginning on day 13 post-implantation, as follows: (1) pyrogen-free PBS; (2) BHA10 IgG, 2 mg/kg, 2x/wk; (3) 14A2 IgG, 2 mg/kg, 2x/wk; (4) N-BsAb/SS/GS4, 2 mg/kg, 1x/wk; (5) C-BsAb/SS/GS4, 2 mg/kg, 1x/wk; (6) BHA10 IgG + 14A2 IgG, 1 mg/kg each, 2x/wk. Tumor sizes and body weights were recorded bi-weekly. Study was terminated when average tumor size of vehicle group reached approximately
2,000 mg. Tumor volume was calculated using the formula: \( V = \frac{4}{3} \pi r^3 \) (L x W²/2).

MDA-MB-231 human breast carcinoma cells (2 x 10⁶ cells per mouse) were implanted subcutaneously into the flanks of athymic nude mice. The tumors were grown until day 13 at which point tumor-bearing mice with an average tumor size of approximately 168 mg were randomly assigned to treatment (N = 10) and vehicle control (N = 15) groups. Mice received antibodies and vehicle IP starting at day 13. Groups are shown as follows: (1) pyrogen-free PBS; 1x/wk; (2) BHA10 IgG, 2 mg/kg, 2x/wk; (3) 14A2 IgG, 2 mg/kg, 2x/wk; (4) C-BsAb-SS/GS4, 2 mg/kg, 1x/wk; (5) BHA10 IgG + 14A2 IgG, 1 mg/kg each, 2x/wk. Tumor sizes and body weights were recorded bi-weekly. Study was terminated when average tumor size of vehicle group reached approximately 2,800 mg.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/ MichaelsonMABS1-2-Sup.pdf

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