**Engineering a therapeutic IgG molecule to address cysteinylation, aggregation and enhance thermal stability and expression**

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**Introduction**

Recombinant monoclonal antibodies (mAbs) represent an established and growing class of therapeutics, with more than 20 mAbs approved for the treatment and prevention of disease. It is becoming increasingly apparent, however, that not all candidate mAbs emerging from the drug discovery process are suitable for commercial development, when considering their expression levels, stability and product homogeneity. In particular, the phenomenon of protein aggregation is a common issue that compromises the manufacture, storage, administration, biological activity and safety of biological drugs, including mAbs. In extreme cases, the consequences of biological drug aggregation can be severe. For example, aggregation of the anemia drug erythropoietin (EPO) was one of the factors implicated in EPO-derived immunogenicity that caused pure red cell aplasia, and subsequently fatalities, in patients. When antibodies are variable in their aggregation profile between production lots, costly monitoring and control procedures are necessary during the manufacturing process. There are many factors that can contribute to protein aggregation, including primary sequence, partial unfolding, post-translational modifications, hydrophobicity, charge, pH, temperature, protein concentration and formulation. Because mAbs are large multidomain proteins, the factors that lead to aggregation are complex and are generally not well understood. It is becoming standard practice in the industry to select lead antibodies based on both biological activity and aggregation profile. Aggregation propensity can be measured in a number of high throughput assays and predicted via in silico tools. If aggregation is identified, formulation development is routinely used to minimize aggregation following a ‘quality by design’ (QbD) approach. Nevertheless, there are limits to high concentration of antibody required to fill a 1 mL syringe with an effective dose.

Antibodies can undergo a variety of covalent and non-covalent degradation reactions that have adverse effects on efficacy, safety, manufacture and storage. We had identified an antibody to Angiopoietin 2 (Ang2 mAb) that neutralizes Ang2 binding to its receptor in vitro and inhibits tumor growth in vivo. Despite favorable pharmacological activity, the Ang2 mAb preparations were heterogeneous, aggregated rapidly and were poorly expressed. Here, we report the engineering of the antibody variable and constant domains to generate an antibody with reduced propensity to aggregate, enhanced homogeneity, elevated $T_m$, 26-fold improved level of expression and retained activity. The engineered molecule, MEDI-3617, is now compatible with the large scale material supply required for clinical trials and is currently being evaluated in Phase 1 in cancer patients. This is the first report to describe the stability engineering of a therapeutic antibody addressing non canonical cysteine residues and the design strategy reported here is generally applicable to other therapeutic antibodies and proteins.

**Keywords:** antibody, aggregation, expression, stability, cysteinylation

**Abbreviations:** Ang2, angiopoietin 2; mAb, monoclonal antibody; EPO, erythropoietin; FDA, Food and Drug Administration; QbD, quality by design; $V_H$, variable heavy; $V_L$, variable light

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Recombinant monoclonal antibodies (mAbs) represent an established and growing class of therapeutics, with more than 20 mAbs approved for the treatment and prevention of disease. It is becoming increasingly apparent, however, that not all candidate mAbs emerging from the drug discovery process are suitable for commercial development, when considering their expression levels, stability and product homogeneity. In particular, the phenomenon of protein aggregation is a common issue that compromises the manufacture, storage, administration, biological activity and safety of biological drugs, including mAbs. In extreme cases, the consequences of biological drug aggregation can be severe. For example, aggregation of the anemia drug erythropoietin (EPO) was one of the factors implicated in EPO-derived immunogenicity that caused pure red cell aplasia, and subsequently fatalities, in patients. Therapeutic antibody immunogenicity is rarely as severe as the case of EPO, but can still result in unfavorable outcomes, such as patients having to withdraw from therapy. The aggregation issue is somewhat exacerbated by the recent move, in the interests of patient convenience, toward subcutaneous self-administration of antibody drugs. In this case, the risk of aggregation is increased due to the high concentration of antibody required to fill a 1 mL syringe with an effective dose.

Aggregation in the manufacturing process can lead to unwanted heterogeneity in biological protein preparations. Pharmaceutical regulatory authorities, such as the United States Food and Drug Administration (FDA), recommend that heterogeneity be closely monitored and characterized to ensure consistent drug activity between manufacturing lots. When antibodies are variable in their aggregation profile between production lots, costly monitoring and control procedures are necessary during the manufacturing process. There are many factors that can contribute to protein aggregation, including primary sequence, partial unfolding, post-translational modifications, hydrophobicity, charge, pH, temperature, protein concentration and formulation. Because mAbs are large multidomain proteins, the factors that lead to aggregation are complex and are generally not well understood. It is becoming standard practice in the industry to select lead antibodies based on both biological activity and aggregation profile. Aggregation propensity can be measured in a number of high throughput assays and predicted via in silico tools. If aggregation is identified, formulation development is routinely used to minimize aggregation following a ‘quality by design’ (QbD) approach. Nevertheless, there are limits to...
the level of improvement that can be achieved by formulation changes alone. Up to 50% of manufactured product is wasted in some cases. Improved variable domain engineering strategies are important to address such issues early in the research phase of drug development to ensure the drug can meet the desired clinical performance.

In the current study, we focused on an antibody targeting angiopoietin 2 (Ang2), a soluble ligand for the Tie2 receptor and an import regulator of pathological angiogenesis and inflammation. The correlation between Ang2 expression in tumors with regions of high angiogenic activity and poor prognosis in many tumor types makes Ang2 an ideal drug target. We previously generated a human anti-Ang2 antibody that neutralizes Ang2 binding to the Tie2 receptor in vitro and inhibits angiogenesis and tumor growth in vivo and now is in clinical trials. Antibody development was hampered, however, by poor expression and aggregation caused in part by a non-canonical, unpaired Cys residue in the antibody variable domain. Surprisingly, this antibody emerged from a B cell hybridoma screening strategy that should in theory include an intrinsic selection for well-expressed, non-aggregating antibodies.

Antibody stability engineering strategies reported in the literature have focused on improving our general understanding of the residues linked to stability or using directed evolutionary strategies to identify aggregation resistant frameworks. Here, we started with an antibody with significant expression and aggregation liabilities and used a rational design approach to engineer the variable domain to reduce aggregation and improve expression. This is the first report of stability engineering addressing non-canonical Cys residues in an antibody and the strategy reported here is applicable to other proteins with unpaired Cys residues to enhance stability and therapeutic use.

### Results

**Characterization of Ang2 mAb.** The variable region genes from an anti-Ang2 hybridoma were cloned into a full-length human IgG2 vector and expressed in mammalian cells. Initial data from the expression and purification highlighted that the yield was significantly lower than expected (Table 1), but the mAb was greater than 99% monomer by SEC-HPLC (data not shown). Sequence analysis of the variable domains and comparison to human germline highlighted a non-canonical Cys at V\textsubscript{H} kappa position 49 and an additional non-germline residue at V\textsubscript{\lambda} position 37 (Fig. 1A). The presence of non-canonical Cys in antibody germline genes is relatively rare (Fig. 1B). The deconvoluted mass spectra of the N-deglycosylated IgG2 demonstrated heterogeneity of the antibody (Fig. 2A, upper trace). The theoretical mass of the mAb from the primary sequence is 145,460 Da. Unpaired peptide thiol groups can be modified by the addition of Cys (cysteinylation) or glutathione (glutathiolation) resulting in mass increases of 119 Da and 305 Da respectively. The mass changes from cysteinylation of both unpaired Cys and the combination of cysteinylation and glutathiolation can explain some of the additional peaks in spectra. In addition the degree of Cys modification was variable between antibody expression batches (Fig. S1).

The T\textsubscript{m} was calculated by DSC and an accelerated stability study performed to assess the effect of the free Cys on conformational stability and aggregation propensity (Table 1). In brief, the mAb was incubated at 10 mg/ml for two weeks and the proportion of monomer, aggregates and break down products were monitored and used to calculate the rate of aggregation. Ang2 mAb had a very significant rate of aggregation and a relatively low T\textsubscript{m}, 1 of 65°C (Fig. 2B).

**Identification of improved variants enhancing expression and stability with no detrimental effect on immunogenicity and potency.** The non-canonical Cys residue was the most likely cause of the aggregation. Structural modeling indicated that it was likely surface-exposed and available for modification (Fig. 1C). The strategy used to engineer the mAb is outlined in Figure 1D. Nineteen variants of Ang2 mAb were screened for activity, mammalian expression and in silico immunogenicity. From these screens, two variants Cys49Asn and Cys49Thr were identified that had improved expression, retained bioactivity and reduced predicted immunogenicity (Fig. 3). To confirm that these variants had improved stability, a miniature accelerated stability study was performed at 1 mg/ml. The two variants retained greater than 90% monomer after 4 weeks, in contrast to the Ang2 mAb which lost 25% monomer (Fig. 4A). The V\textsubscript{H} was reverted to germline with the change Gly37Val which had no effect on potency (data not shown). At small scale the variants had improved expression relative to the Ang2 mAb. To further investigate the effect on mAb yield at larger scale, a 5 L transient expression and purification was performed. Both variants had improved expression relative to the Ang2 mAb but, surprisingly, expression of Cys49Thr was more than 10-fold greater than Ang2 mAb and Cys49Asn (Fig. 4B).

**Characterization of mAb2.** The variant Cys49Thr was characterized relative to the Ang2 mAb in more detail. As expected from previous results, it had significantly improved expression relative to the Ang2 mAb and retained affinity and potency (Table 1). The deconvoluted mass spectra of the N-deglycosylated IgG2 demonstrated that the heterogeneity of the antibody was significantly reduced (Fig. 2A). To assess the conformational stability

### Table 1. Enhanced profile of Cys49Thr mAb relative to Ang2 mAb

|                     | $K_D$ (pM) | IC\textsubscript{50} (nM) | % Agg/month at 40°C | T\textsubscript{m} 1 (°C) | Yield (mg/L) |
|---------------------|------------|---------------------------|---------------------|--------------------------|--------------|
| Ang2 mAb            | 24.5       | 0.2                       | 40                  | 65                       | 10           |
| Cys49Thr mAb        | 37         | 0.28                      | 0.2                 | 76                       | 260          |

Affinity ($K_D$) was measured by KinExA and activity (IC\textsubscript{50}) was measured in Ang2/Tie2 competition ELISA. The rate of aggregation (% Agg/month) was calculated from an accelerated stability study with IgG at 10 mg/ml in 10 mM histidine buffer pH 6 at 40°C. The mid-point of thermal unfolding (T\textsubscript{m}) was determined by differential scanning calorimetry. Yield was calculated following transient expression in mammalian cells.
and aggregation propensity of the engineered mAb, the $T_m$ was calculated by DSC and an accelerated stability study performed. In brief, the mAb was incubated at 10 mg/ml for two weeks and the proportion of monomer, aggregates and break down products were monitored and used to calculate the rate of aggregation. The $T_m$ was elevated by 11°C (Fig. 2B) and the rate of aggregation significantly reduced (Table 1). Finally, the variant Cys49Thr was converted from human IgG2 to a human IgG1 to remove the isomerisation liability. The IgG1 was characterized as the IgG2 and had an identical profile (data not shown).

Discussion

Therapeutic proteins, including mAbs, are inherently heterogeneous and prone to degradation. The causes of heterogeneity include, but are not limited to, chemical modifications such as deamidation, oxidation and alternative disulfide connectivity. These chemical changes can lead to structural changes that impair the physical stability of the therapeutic protein. Unpaired Cys residues are very reactive and can potentially trigger aggregation through intramolecular scrambling or intermolecular disulfide formation.20-22 The Ang2 mAb non-canonical Cys is the cause for the mAb rapid aggregation as demonstrated by the accelerated stability study in Figure 4A. The molecular mechanism of aggregation was not investigated in detail; however, free sulfhydryl groups from unpaired canonical Cys residues are reported to result in decreased thermal stability.23 In the case of unpaired non-canonical Cys residues, there are three reports of such mAbs in the literature, MAB007 Cys in VH CDR3 position 104;24 OKT3 Cys in VH CDR3 position 100a25 and palivizumab Cys in VL CDR1 position 25.26 For these three antibodies, the unpaired Cys surprisingly had little effect on stability and aggregation. Palivizumab is a stable molecule,27 OKT3 reported < 1% aggregate after six months at 40°C28 and MAB007 had < 3% aggregate following storage for one month at 45°C.29 The only detrimental effect of the unpaired Cys was the cysteinylation-induced heterogeneity in MAB007.24 Another interesting parallel is the generation of site-specific antibody-drug conjugates based on the introduction of non-canonical Cys residues. Here, the additional Cys residues were modified with Cys or glutathione introducing heterogeneity, as observed for Ang2 mAb, which rendered the Cys inactive as a functional group for conjugation of the payload.30-32 The degree of Cys modification of Ang2 mAb was variable, dependent upon cell culture and process conditions. The affect of the Cys on stability is not reported; the affect of Cys on expression was variable and difficult to predict.32,33 A structural model of Ang2 mAb was generated and used as the basis for aggregation prediction using computational tools from Accelrys Software Inc. The Ang2 mAb was predicted to have a significant aggregation patch in VH CDR3 (Fig. S2). The mutation Cys49Thr reduced the predicted aggregation propensity score, while the VH germline change Gly37Val made no
human germline; however, there are examples of human germline genes encoding unpaired Cys residues, which will be present in platforms that use natural gene sources. The typical germline residue at position VL 49 is Tyr. The mutation Cys40Tyr did improve expression, but significantly reduced the potency of the mAb. The very large Tyr residue is likely to alter the conformation of the proximal VHCDR3 loop, highlighted in the model of Ang2 mAb. The other large, positively charged or aromatic amino acids also had a detrimental impact on potency, with the exception of Phe. Gly, Ser and Ala are amino acids typically used to replace Cys. Their impact on potency was within 2.5-fold, but only Ser and Ala improved expression. Ser and Ala are very small amino acids and, as expected, were potentially acceptable replacements; however, Ala increased the predicted immunogenicity while other variants, such as Asn, Thr, Asp and Glu, demonstrated lower predicted immunogenicity and improved potency relative to Ser.

Variants Asp and Glu had similar expression and potency to Ang2 mAb. Interestingly, the introduction of negatively charged residues, particularly Asp around VL CDR2, including position VL49, has been proposed as a general strategy to reduce the aggregation propensity of antibody scFv. To test whether this observation of scFv translated to full length IgG, we evaluated the variant Cys49Asp in the accelerated stability study. As predicted, Cys49Asp did have a reduced aggregation propensity relative to Ang2 mAb (Fig. S3). The molecular mechanism for this is unclear but this does confirm a preference for small negatively charged amino acids potentially influencing the localized environment, unlike supercharged proteins. This is the first report confirming that small charge changes around VL CDR2 translates from scFv to improved stability in full-length IgG molecules.

Figure 2. (A) Deconvoluted mass spectra of N-deglycosylated antibodies Cys49Thr mAb and Ang2 mAb. (B) Temperature induced unfolding of the Ang2 mAb and Cys49Thr mAb measured by DSC demonstrating the increased $T_m$.
Cys49Thr resulted in significantly higher expression than Cys49Asn is not obvious and attempts to predict in silico which mutations would be stabilizing were not successful. In silico tools to predict physicochemical behavior are generally improving but cannot yet replace empirical evidence when attempting to predict how mutations will affect large complex molecules such as IgG. As a final step to ensure Cys49Thr met the desired clinical performance, it was switched from an IgG2 to an IgG1. IgG2 have been shown to aggregate more than IgG1 in manufacture and are prone to disulfide rearrangement in vivo which can reduce activity.40,41

The systematic strategy reported here could be used to rationally engineer other antibody and protein therapeutics with unpaired Cys residues. Previous reports on addressing Cys based stability issues have assessed only a few variants, e.g., studies on IL-2 and G-CSF.42,43 The benefit of assessing all potential amino acid changes at a particular site with all the relevant attributes enables an optimal solution to be described by the molecule and the environment. In summary, this is the first report to describe the removal of a significant development liability from a therapeutic mAb candidate, enabling clinical development of the anti-Ang2 antibody. Given the description of other similar liabilities in both mAbs and therapeutic proteins, we believe the approach used here will be applicable to the development of future biopharmaceuticals.

**Materials and Methods**

**Generation of IgG variants, expression and purification.** Site-directed mutagenesis was performed using the QuikChange system (Stratagene) according to the manufacturer’s instructions. Oligonucleotides were designed to mutate Ang2 mAb Vκ position Cys 49 to all other 19 amino acids and variable heavy (VH) chain Gly 37 to Val. The VH domain was cloned into a vector containing the human heavy-chain constant domains and regulatory elements to express whole IgG heavy chains in mammalian cells. Similarly, the variable light (VL) domain was cloned into a vector for the expression of the human light-chain (κ) constant domains and into regulatory elements to express whole IgG light chains in mammalian cells. To obtain IgG proteins, we transfected the heavy-chain and light-chain IgG-expressing vectors into HEK293/EBNA or CHO mammalian cells using standard methods. IgGs were expressed and secreted into the medium. Harvests were pooled and filtered prior to purification. Individual IgGs were purified using Protein A chromatography. Culture supernatants are loaded onto an appropriately sized column of Ceramic Protein A (BioSepra) and washed with 50 mM Tris-HCl (pH 8.0) and 250 mM NaCl. Bound IgG was eluted from the column using 0.1 M sodium citrate (pH 3) and neutralized by the addition of Tris-HCl (pH 9). The eluted material was buffer exchanged into phosphate-buffered saline (PBS) using Nap10 columns (Amersham). The concentration of IgG was determined at A280 using an extinction coefficient based on the amino acid sequence of IgG.

**Ang2-Tie2 competition binding ELISA.** Maxisorp plates (Nunc) were coated with 4 μg/ml human Tie2-Fc (R&D Systems), blocked with 0.5% BSA/0.1% Tween-20/PBS buffer and then washed with 0.05% Tween-20/PBS. Anti-Ang2 ranging from 0.4 to 6.6 pM in PBS was added to wells followed by 200 ng/ml biotinylated huAng2 (R&D Systems). Plates were washed, incubated with 1:5000 Streptavidin HRP (Pierce) and developed with TMB substrate (3, 3', 5, 5'-tetramethylbenzidine) solution (KPL). Reaction was stopped and read at OD 450 nm using a Molecular Devices VMAX plate reader (Sunnyvale).

**Affinity measurement.** The equilibrium binding constant (Kd) for the interaction of IgG with human Ang2 protein was measured using a KinExA platform (Sapidyne Instruments). Briefly, Ang2 protein was coated onto PMMA beads at a concentration of 5 μg/ml Ang2 using a protocol supplied by the instrument’s manufacturer. Afterwards, the beads were washed and blocked in PBS buffer, pH 7.4, containing 10 mg/ml BSA until used. Separately, batch volumes of IgG were prepared at 0.4 and 1 nM in HBS-P buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% P2O) + 0.01% BSA, then aliquoted into two separate sets of 13 tubes each. Human Ang2 protein was

Figure 3. Screening of the Ang2 mAb Vκ C49 variants as IgG for activity, predicted immunogenicity and yield. Activity was measured in Ang2/Tie 2 competition ELISA. Immunogenicity was assessed using AlgoNomics Epibase® and ranked for the number of predicted Tα epitopes. IgG yield was determined following transient expression.

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added to one tube from each series and then serially diluted across 11 of the remaining tubes, leaving one sample tube as an antibody-only control. The final Ang2 concentrations for the 40 μM and 1 nM IgG experiments ranged from 313 fM–16 nM and 980 fM–50 nM, respectively. These mixtures were then equilibrated at room temperature for 1–2 d. The Ang2-coated PMMA bead slurries were diluted to ~30 mLs with HBS-N instrument buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl) in a bead vial and affixed to the KinExA instrument. A user-defined timing program was then used to sequentially transfer the Ang2/PMMA beads to a capillary flow cell in the instrument, inject individual sample mixtures (plus or minus Ang2 protein) over the Ang2-coated beads. Following this, unbound sample solution was washed from the beads, and then a species-specific, Cy5-labeled antibody detection reagent, prepared at either 0.0625 μg/mL (1 nM IgG experiment) or 1 μg/mL (40 pM IgG experiment) in HBS-P buffer + 0.01% BSA beads were again washed to remove excess label, then the amount of fluorescence that remained associated with the beads was measured and the signal converted to percent free IgG. Between samples, the bead pack was flushed from the flow cell then replenished with more Ang2-coated beads in preparation for the next sample. An isotherm was then generated, which plotted the amount of free IgG detected at each concentration of Ang2. The resulting two binding curves were then evaluated in a dual curve analysis using the instrument’s evaluation software, from which the $K_D$ was derived.

**Mass spectrometry.** To profile the IgG, high resolution mass measurement is performed using a liquid chromatography mass spectrometry (LC-MS) method. The LC-MS system is consisted of a Waters Q-TOF (quadrupole and orthogonal acceleration time-of-flight) Premier™ mass spectrometer in conjunction with a Waters ACQUITY UPLC™ (ultra performance liquid Chromatography). To confirm the polypeptide structure, the samples are N-deglycosylated with PNGase F (Calbiochem) after adjusting the pH to 8.0 using 100 mM Tris buffer. Reversed-phased chromatography separation is performed on an Agilent Poroshell C3 75 mm × 2.1 mm column using mobile phase A of 0.01% TFA and 0.1% formic acid in water and mobile phase B of 0.01% TFA and 0.1% formic acid in acetonitrile. Sample is eluted using a fast linear gradient 5–80% mobile phase B over 3 min at a flow rate of 0.12 mL/min. Mass spectrometric data are collected at an m/z range of 2,000 to 4,500. The accurate mass of an antibody is obtained through the deconvolution of the mass spectrometric data using the MaxEnt I (Waters) software package.

**Accelerated stability assessment.** Purified antibody was tested for stability in an accelerated stability assay. Protein samples were stored at one mg/ml in PBS at 40°C for two weeks. At time zero and week two samples were analyzed by size exclusion chromatography (SEC) using an Agilent 1100 series HPLC system equipped with a TSKG3000SWXL column (Tosoh Bioscience). The column was equilibrated with 0.1 M sodium phosphate, 0.1 M sodium sulfate, 0.05% sodium azide, pH 6.8. Samples were injected onto the column and eluted at a flow rate of 1 mL/min with the equilibration buffer. Protein elution was monitored at A$_{280}$. For quantification purposes, all species eluted before and after the main peak were integrated, and represent aggregates and breakdown products respectively. To confirm the improved stability in a more relevant system antibody was stored at 10 mg/ml in 10 mM histidine pH 6.0 at 40°C for two weeks and the proportion of monomer, aggregates and breakdown products were measured as above. This was used to calculate the rate of aggregation as % aggregation/month after two weeks.

**Thermal stability.** To calculate the antibody Tm, differential scanning calorimetry (DSC) was performed using a VP capillary DSC system (MicroCal). The test samples were prepared in the buffer alone and in various excipient(s) of interest at a final protein concentration of one mg/mL. Change in the heat capacity (Cp) was measured as the samples were heated from 20°C to 90°C at a rate of 60°C /h. For each sample, a relevant buffer blank containing the same buffer and excipient concentration was used as a reference. Normalized Cp data were corrected for buffer baseline. Data analysis was performed using OriginLab® software (OriginLab Corporation). Raw data from the DSC run were fit using Origin scientific plotting software, which uses the Levenberg–Marquardt nonlinear least square method.

**Immunogenicity prediction.** The antibody V domains were scanned for the presence of putative T$_{	ext{h}}$-epitopes using Epibase®. In brief, the platform analyzes the HLA binding specificities of all possible 10 mer peptides derived from the target sequences.
Profiling was done at the allotype level of 20 BRB1, 7 DRB3/4/5, 14 DQ and 7 DP HLA receptors. Peptide corresponding to self peptides were treated separately as ‘germline-filtered’ peptides.

Disclosure of Potential Conflicts of Interest
All authors are current or former employees of MedImmune.

References
1. Schellekens H, Jukoot W. Erythropoetin-associated PRCA: still an unsolved mystery. J Immunotocological 2006; 3:123-30; PMID:16958692; http://dx.doi.org/10.1080/154769010600845567

2. Pointre Y, Commens SP, Calais G, Warier H, Platts-Mills TA. Fatal infusion reactions to cetuximab: role of immunoglobulin e-mediated anaphylaxis. J Clin Oncol 2012; 30:334; author reply 335; PMID:22165292; http://dx.doi.org/10.1200/JCO.2011.38.4701

3. Bennett NK, Heilig CE, Droll B, Wohlgemuth J, Armbruster FP, Heilig B. Immunogenicity, efficacy and adverse events of aludimumab in RA patients. Rheumatol Int 2007; 27:269-74; PMID:17067655; http://dx.doi.org/10.1007/s00296-006-0183-7

4. Anonymous. International conference on harmonisation: on guidance: specifications: test procedures and acceptance criteria for biotechnological/biological products. Notice Food and Drug Administration HHS Fed Reg 1999; 64: 44928-35

5. Lowe D, Dudgen K, Rount R, Schofield P, Jerunics L, Christ D. Aggregation, stability, and formulation of human antibody therapeutics. Adv Protein Chem Struct Biol 2011; 84:41-61; PMID:21846562; http://dx.doi.org/10.1016/B978-0-12-386483-3.00004-5

6. Aucamp JP, Cosme AM, Lye GJ, Dalby PA. High-throughput measurement of protein stability in micrometer-sized. Biotechnol Bioeng 2005; 89:599-607; PMID:15672379; http://dx.doi.org/10.1002/bit.20397

7. Garadl P, Hegyi M, Bassarab S, Weissel M. A rapid, sensitive and economical assessment of monoclonal antibody conformational stability by intrinsic tryptophan fluorescence spectroscopy. Biotechnol J 2008; 3:1201-11; PMID:18702089; http://dx.doi.org/10.1002/biot.200800091

8. Goldberg DS, Bishop SM, Shah AU, Sathish HA. Formulation development of therapeutic monoclonal antibodies using high-throughput fluorescence and static light scattering techniques: Role of conformational and colloidal stability. J Pharm Sci 2010; 100: 1306-15; PMID:20906568; http://dx.doi.org/10.1002/jps.22371

9. Conley GP, Viswanathan M, Hou Y, Rank DL, Lindberg AP, Cramer SM, et al. Evaluation of protein engineering and process optimization approaches to enhance antibody drug manufacturability. Biotechnol Bioeng 2011; 108:2634-44; PMID:21618474; http://dx.doi.org/10.1002/bit.23230

10. Lauer TM, Agrawal NJ, Chennamsetty N, Egodage K, Lacy ER, Bishop SM, Shah AU, Sathish HA. Removal of cysteinylation from an unpaired sulfhydryl in the variable region of recombinant monoclonal IgG1 antibody improves homogeneity, stability, and biological activity. J Pharm Sci 2008; 97:775-90. PMID: 17786988

11. Mills TA. Fatal infusion reactions to cetuximab: role of anti-LINGO-1 monoclonal antibody Li33 by peptide mapping. Pharm Res 2012; 109:10879-84; PMID:22745168; http://dx.doi.org/10.1007/s10273-012-1087-9

12. Jespers L, Schon O, Famm K, Winter G. Aggregation-resistant domain antibodies selected on phage by heat denaturation. Nat Biotechnol 2004; 22:1161-5; PMID:15300234; http://dx.doi.org/10.1038/nbt1000

13. MedImmune LLC. Phase 1/1b, open-label, dose-escalation and expansion study to evaluate the safety and antitumor activity of MEDI617 as a single-agent or in combination therapy in adult subjects with advanced solid tumors. ClinicalTrials.gov Identifier: NCT01248994

14. Lajebin AE, Buijse AO, van den Bremer ET, Verwulgen AY, Bleeker WK, Thorpe SJ, et al. Therapeutic IgG4 antibodies engage in Fab-arm exchange with endogenous human IgG4 in vivo. Nat Biotechnol 2009; 27:961-7; PMID:19629083; http://dx.doi.org/10.1038/nbt.1553

15. Pepinsky RB, Silvian L, Berkowitz SA, Farrington G, Lugovskoy A, Wallace L, et al. Improving the solubility of anti-LINGO-1 monoclonal antibody Li33 by isotype switching and targeted mutagenesis. Protein Sci 2010; 19:954-66; PMID:20189683

16. Nakano K, Ishiguro T, Konishi H, Tanaka M, Sugimoto M, Sigo J, et al. Generation of a humanized anti-glycan 3 antibody by CDR grafting and stabilization optimization, Anticancer Drugs 2010; 21:107-16; PMID:20487643; http://dx.doi.org/10.1097/CAD.0b013e3283f5d60

17. Dudgeon K, Rount R, Kolmeijer I, Schofield P, Stolp J, Langley D, et al. General strategy for the generation of human antibody variable domains with increased aggregation resistance. Proc Natl Acad Sci U S A 2012; 109:10879-84; PMID:22745168; http://dx.doi.org/10.1073/pnas.1203867109

18. Voo HJ, Lottz MM, Jung JS, Mercurio AM. Carbohydrate-binding protein 35 (Mac-2), a lamellarin-binding lectin, forms functional dimers using cysteine 186. J Biol Chem 1991; 266:18419-22; PMID:19179666

19. Wootton SK, Yao D. Homo-eliganization of the protein reproductive and respiratory syndrome virus nucleocapsid protein and the role of disulide linkages. J Virol 2003; 77:4546-57; PMID:12663761; http://dx.doi.org/10.1128/JVI.77.7.4546-4557.2003

20. Trivedi MV, Laurence JS, Siahaan TJ. The role of thiol and disulfide on protein stability. Curr Protein Pept Sci 2009; 10:614-25; PMID:19538140; http://dx.doi.org/10.2174/138920309789630534

21. Lacy ER, Bristow BL, Clark S, Bhakta S, Leipold DD, Weir S, et al. Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. Nat Biotechnol 2008; 26:925-32; PMID:18641636; http://dx.doi.org/10.1038/nbt.1480

22. Younov V, Chenhamnetty N, Kayser V, Wallny HJ, Holb K, Trout BL. Design and application of antibody cysteine variants. Biotechnol J 2010; 21:385-92; PMID:20922944; http://dx.doi.org/10.1002/biot.200905090

23. Cordoba-Rodriguez RV. Aggregates in mAbs and recombinant therapeutic proteins: a regulatory perspective. BioPharm Int 2008; 21:44-53

24. Joshtbhoy MK, Luo Q, Nashed-Samuel Y, Wyppych J, Narhi LO. Classification and characterization of therapeutic antibody aggregates. J Biol Chem 2011; 286:25118-35; PMID:21454532; http://dx.doi.org/10.1074/jbc.M110.164957

25. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of protein pharmaceuticals. Nat Biotechnol 2009; 27:504-51; PMID:19364296; http://dx.doi.org/10.1038/nbt.1538

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Supplemental Materials
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37. Magdelaine-Beuzelin C, Kaas Q, Welbi V, Oltesser M, Jefferis R, Lefranc MP, et al. Structure-function relationships of the variable domains of monoclonal antibodies approved for cancer treatment. Crit Rev Oncol Hematol 2007; 64:210-25; PMID:17624800; http://dx.doi.org/10.1016/j.critrevonc.2007.04.011

38. Ehrenmann F, Kaas Q, Lefranc MP. IMGT/3Dstructure-DB and IMGT/DomainGapAlign: a database and a tool for immunoglobulins or antibodies, T cell receptors, MHC, IgSF and MHC class I/II. Nucleic Acids Res 2010; 38(Database issue):D301-7; PMID:19900967; http://dx.doi.org/10.1093/nar/gkp946

39. Pommie C, Levadoux S, Sahatier R, Lefranc G, Lefranc MP. IMGT standardized criteria for statistical analysis of immunoglobulin V-REGION amino acid properties. J Mol Recognit 2004; 17:17-32; PMID:14872534; http://dx.doi.org/10.1002/jmr.647

40. Franey H, Brych SR, Kolvenbach CG, Rajan RS. Increased aggregation propensity of IgG2 subclass over IgG1: role of conformational changes and covalent character in isolated aggregates. Protein Sci 2010; 19:1601-15; PMID:20556807; http://dx.doi.org/10.1110/ps.051489405

41. Liu YD, Chen X, Enk JZ, Plant M, Dillon TM, Hynn GC. Human IgG2 antibody disulfide rearrangement in vivo. J Biol Chem 2008; 283:29266-72; PMID:18713741; http://dx.doi.org/10.1074/jbc.M804787200

42. Ju G, Collins L, Kaffka KL, Tsien WH, Chizzonite R, Crowl R, et al. Structure-function analysis of human interleukin-2. Identification of amino acid residues required for biological activity. J Biol Chem 1987; 262:5723-31; PMID:3106342

43. Raso SW, Abel J, Barnes JM, Maloney KM, Pipes G, Treuheit MJ, et al. Aggregation of granulocyte-colony stimulating factor in vitro involves a conformationally altered monomeric state. Protein Sci 2005; 14:2246-57; PMID:16131655; http://dx.doi.org/10.1110/ps.051489405

44. Van Walle I, Gansemans Y, Parren PWHL, Stas P, Lasters I. Immunogenicity screening in protein drug development. Expert Opin Biol Ther 2007; 7:405-18; PMID:17309332; http://dx.doi.org/10.1517/14712598.7.3.405