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Evaluation of Heavy-Chain C-Terminal Deletion on Product Quality and Pharmacokinetics of Monoclonal Antibodies

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ABSTRACT

Due to their potential influence on stability, pharmacokinetics, and product consistency, antibody charge variants have attracted considerable attention in the biotechnology industry. Subtle to significant differences in the level of charge variants and new charge variants under various cell culture conditions are often observed during routine manufacturing or process changes and pose a challenge when demonstrating product comparability. To explore potential solutions to control charge heterogeneity, monoclonal antibodies (mAbs) with native, wild-type C-termini, and mutants with C-terminal deletions of either lysine or lysine and glycine were constructed, expressed, purified, and characterized *in vitro* and *in vivo*. Analytical and physiological characterization demonstrated that the mAb mutants had greatly reduced levels of basic variants without decreasing antibody biologic activity, structural stability, pharmacokinetics, or subcutaneous bioavailability in rats. This study provides a possible solution to mitigate mAb heterogeneity in C-terminal processing, improve batch-to-batch consistency, and facilitate the comparability study during process changes.

Introduction

Therapeutic monoclonal antibodies (mAbs) have attracted significant attention due to their high specificity, reduced side-effects profile, and long-serum half-life requiring less frequent dosing compared with their small-molecule counterparts.[1] More than 40 mAbs and mAb fragments have been approved for use by the US Food and Drug Administration over the past 25 years.[2]

Due to the nature of their production, mAbs are more heterogeneous drug products.[3] Routine manufacturing of mAbs produces heterogeneous variants, which are commonly the result of modifications that occur during cell culture production due to enzymatic processes or spontaneous degradation. These variants can accumulate during production, purification, formulation, and storage,[4,5] which may be problematic for comparability testing during manufacturing process changes. Antibody charge variants, including

Abbreviations: ATA, anti-therapeutic antibody; CDC, complement-dependent cytotoxicity; CL, clearance; ELISA, enzyme-linked immunosorbent assay; Fab, fragment antigen-binding; Gly, glycine; GS, glutamine synthetase; HCCF, harvested cell culture fluid; HMWS, high−molecular weight species; HRP, horseradish peroxidase; iCIEF, imaged capillary isoelectric focusing; IEC, ion-exchange chromatography; IV, intravenous; Lys, lysine; MC, methyl cellulose; PAM, peptidylglycine α-amidating monooxygenase; PCR, polymerase chain reaction; PK, pharmacokinetic; Pro, proline; SC, subcutaneous; SEC, size exclusion chromatography; t_{max}, time of maximum serum concentration; TMB, tetramethylbenzidine peroxidase; UFDF, ultrafiltration and diafiltration; V_{ss}, volume of distribution at steady state; WT, wild type.

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acidic and basic variants, have been demonstrated not to affect the *in vitro* potency, FcRn binding affinity, or the pharmacokinetic (PK) properties of mAbs in rats.[6] However, variability in the level of charge variants from batch-to-batch, site-to-site, or process improvements still poses significant challenges for process comparability. A solution to minimize variations in the level of charge variants is an immediate need of the biopharmaceutical industry.

For human IgG1 antibodies, the amino acid sequence of the C-termini of the heavy chains is proline–glycine–lysine (Pro–Gly–Lys). Due to the endogenous carboxypeptidases during the production cell culture process, the terminal Lys residues are mostly removed, leading to the various isoforms of the C-terminal of the heavy chain.[7,8] The isoforms can carry 0, 1, or 2 heavy-chain C-terminal Lys (K) residues per antibody molecule (i.e., K0, K1, and K2 isoforms), which contributes to the formation of various levels of basic antibody charge variants.[9] The C-terminal Lys was shown to be rapidly cleaved by endogenous serum carboxypeptidase B *in vivo* after intravenous (IV) injection,[7,8] and therefore, this residue is generally absent from IgG found in serum.[10] It is long believed that C-terminal Lys does not affect antibody function given its spatial distance from the antigen-binding domain[11] and the CH2 regions involved with effector functions.[12-14] Recent findings by van den Bremer et al.,[15] however, demonstrated that C-terminal Lys residues may interfere with IgG hexamerization at the cell surface, which is critical for efficient C1q binding and subsequent maximal complement activation to elicit complement-dependent cytotoxicity (CDC). These findings demonstrated that C-terminal Lys may act as a molecular switch that requires clipping to activate the antibodies' full cytotoxic potential, which highlights the needs to manipulate the C-terminal "switch" to open up alternative ways to regulate CDC.[15]

C-terminal Pro-amidation was recently identified and characterized as another modification that exists as a basic charge variant of human IgG1.[16] Depending on the individual molecule, it constitutes either the minority[17] or majority[16] of the basic charge variants, in single- and double-amidation forms at the C-terminus of the heavy chains. The Pro-amidation reaction was catalyzed by peptidylglycine α-amidating monooxygenase (PAM), and copper was shown to be critical for the catalytic function of the PAM.[18] This amidation process is a 2-step reaction by PAM, which first involves removal of the Gly residue from the C-terminal, followed by the addition of an amide to the Pro residue.[19] Proline amidation of therapeutic antibodies is sensitive to the copper ion concentration in the production media during cell culture: the higher the Cu^{2+} ion concentration, the higher the detected level of Pro-amidation.[16] Other unknown factors including production scale and operational conditions, however, may also have some impact, making it difficult to control the level of Pro-amidation with copper, alone. Although this modification is not expected to affect biologic activity because of its location at the C-terminus of the heavy chain, its variability due to its sensitivity to copper concentration warrants more studies to better control this modification for a more consistent process in antibody production.

The goal of the work presented here was to explore options for achieving superior batch-to-batch consistency of mAbs with regard to basic variants. The C-terminal Lys and Pro-amidation content of antibody products were controlled by recombinantly removing the Lys (−K) or Gly and Lys (−GK) residues before cell line development for mAb1 and mAb2. The materials were then purified and characterized for physiochemical properties and stability *in vitro*. The PK and immunogenicity of the −K and −GK antibody mutants after IV and subcutaneous (SC) administration were characterized and compared with their respective wild-type (WT) therapeutic IgG1 mAbs in the male Sprague–Dawley rat, which is a nonbinding species for mAb1 and a binding species for mAb2.

Experimental

Construction of Antibody Mutants and Purification of the Material

Antibody heavy-chain DNAs were constructed either without Lys (−K) or without Gly and Lys (−GK) by polymerase chain reaction. The −K and −GK polymerase chain reaction products were swapped back into the constant CH3 domains. Mutant DNA constructs were confirmed by Sanger DNA sequencing. WT antibody heavy chain, −K and −GK heavy chains, and the light chain were constructed into a glutamine synthetase drug selection vector. WT antibody and −K and −GK mutant antibodies for mAb1 and mAb2 were transfected into CHO-K1 cells. Three weeks after transfection, about 200 clones were picked from each antibody construct and evaluated for antibody production by enzyme-linked immunosorbent assay (ELISA). Based on the ELISA titer, top clones were pooled from each construct and scaled up for fed-batch shake flask production.

The harvested cell culture fluid for WT antibody and −K and −GK mutant antibodies for mAb1 and mAb2 was purified using Protein-A affinity chromatography (MabSelect SuRe; GE Healthcare Bio-Sciences AB, Uppsala, Sweden), cation exchange chromatography (Poros XS; Applied Biosystems, Carlsbad, CA, and GE Healthcare Bio-Sciences AB), and anion exchange chromatography (Q Sepharose FF; GE Healthcare Bio-Sciences AB). Protein A was operated in bind-elute mode with a Tris-sodium chloride buffer system at pH 7.7 and product elution at low pH using acetic acid. Cation exchange was operated in bind-elute mode with a sodium acetate buffer system at pH 5.5 and a conductivity gradient elution. Anion exchange was operated in flow-through mode with a Tris-sodium acetate buffer system at pH 8. The Q Sepharose FF pool was buffer exchanged and concentrated using an ultrafiltration and diafiltration step. The ultrafiltration and diafiltration pool was conditioned to achieve a final formulation of 25 mg/mL with a low level of endotoxin. Host cell protein was reduced to <2.5 ng/mg.

Size-Exclusion Chromatography

The mobile phase for size-exclusion chromatography (SEC) experiments was 200 mM potassium phosphate and 250 mM potassium chloride at pH 6.2. Before injection, the mAb samples were diluted to approximately 1 mg/mL with mobile phase. Injection volumes of 50 μL were used. SEC experiments were conducted on a 7.8 × 300 mm SW 3000XL column (Tosoh Bioscience, King of Prussia, PA) using a mobile phase flow rate of 0.5 mL/min. The column was placed in the thermal compartment where the temperature was controlled at 30°C. Column effluent was monitored at 280 nm for separation.

Imaged Capillary Isoelectric Focusing

Charge variant distribution was assessed using imaged capillary isoelectric focusing on an iCE280 analyzer (ProteinSimple, San Jose, CA) with a fluorocarbon-coated capillary cartridge (100 μm × 5 cm). The ampholyte solution consisted of a mixture of 0.35% methyl cellulose, 1.34% 3-10 carrier ampholytes, 1.34% 6.7-7.7 carrier ampholytes, pI markers (pI 6.61 and pI 9.22), and 10 mM L-arginine free base in purified water. The anolyte was 80 mM phosphoric acid, and the catholyte was 100 mM sodium hydroxide, both in 0.1% methyl cellulose. Samples were diluted, mixed with the ampholyte solution, and then focused by introducing a potential of 1500 V for 1 min, followed by a potential of 3000 V for 8 min. An image of the focused charge variants was obtained by passing 280 nm ultraviolet light through the capillary and into the lens of a charge-coupled device digital camera. To remove heavy-chain C-terminal Lys residue, carboxypeptidase B was added to each sample at the dilution