Rational Design of Biobetters with Enhanced Stability

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ABSTRACT: Biotherapeutics are the fastest growing class of pharmaceutical with a rapidly evolving market facing the rise of biosimilar and biobetter products. In contrast to a biosimilar, which is derived from the same gene sequence as the innovator product, a biobetter has enhanced properties, such as enhanced efficacy or reduced immunogenicity. Little work has been carried out so far to increase the intrinsic stability of biotherapeutics via sequence changes, even though, aggregation, the primary degradation pathway of proteins, leads to issues ranging from manufacturing failure to immunological response and to loss of therapeutic activity. Using our spatial aggregation propensity tool as a first step to a rational design approach to identify aggregation-prone regions, biobetters of rituximab have been produced with enhanced stability by introducing site-specific mutations. Significant stabilization against aggregation was achieved for rituximab with no decrease in its binding affinity to the antigen. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:2433–2440, 2015

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INTRODUCTION

Although monoclonal antibodies (mAbs) have been on the market for only two decades, they are now the highest selling and fastest growing class of biologics, with 40 products representing more than a third of the biologics market.[1] With many of the earlier products soon reaching patent expiry, there is a large and rapidly growing interest in developing generic versions of biologics, which are referred to as biosimilars. Among the biologics pipeline, 36% of the products are expected to be biobetters.[2] Biobetters intend to be superior to the innovator product in such ways as an increase in clinical efficacy, safety, or ease of delivery. Biobetter mAbs are antibodies that target the same validated epitope as an innovator product, but have been engineered for improved properties. For example, engineered fragment crystallizable (Fc) domain can lead to improved half-life, slower degradation, or improved immune functions; optimization of glycosylation profiles can lead to enhanced cytotoxicity, which can also reduce immunogenicity; optimization of complementarity determining regions (CDRs) can increase efficacy and chemical modification or sequence alteration may increase mAbs stability.[3] Overall, biobetters will be a benefit to the patient, as they could present increased safety, enhance efficacy, reduced dose, and more convenient dosage formulation with opportunities for new delivery routes such as at home subcutaneous injection, with a reduced cost of treatment.

Despite the potential for engineering biobetters with enhanced stability, very little interest so far has been focused on this aspect. This is a golden opportunity, however, as enhanced stability can lead to new delivery routes, lower production costs, improved immunogenicity, and higher concentration formulations. Of the many forms of protein degradation, aggregation is arguably the most common and troubling, occurring in almost all phases of therapeutic protein development. Aggregation is also thought to be a major cause of immunogenicity and is affecting safety and efficacy of biotherapeutics.[4–6] Moreover, subcutaneous injection and new delivery routes (which require high-concentration liquid formulations) are becoming more desirable over intravenous infusions, making protein aggregation more and more important to reduce. From a regulatory perspective, the US FDA also recommends to monitor closely and minimize aggregation such that unwanted heterogeneity is avoided, immunogenicity reduced, and no product activity loss observed.[7]

In response to the need for enhanced stability, we developed in our laboratory in 2009 a computational tool termed spatial aggregation propensity (SAP).[8–10] This tool can predict aggregation-prone regions (APRs; i.e., exposed hydrophobic patches) on protein surfaces. In this study, we applied this technology to a therapeutic mAb, rituximab, with the aim of developing a biobetter with enhanced stability through selected mutations. Rituximab is a blockbuster therapeutic mAb targeting the protein CD20 displayed on the surface of B cells. Rituximab (Rituxan®/MabThera®) is indicated for the treatment of patients with B cell non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and some autoimmune conditions such as rheumatoid arthritis.[11–13] Rituximab is a chimeric mouse/human mAb consisting of a glycosylated IgG1 with human constant regions and murine light-chain and heavy-chain variable region sequences. Rituximab is currently formulated at a low 10 mg/mL concentration and has been previously shown to be aggregation prone,[14] making this mAb an excellent target for stability improvement.

Here, we report the application of our simulation method to characterize hydrophobic patches exposed on the rituximab fragment antigen binding (Fab) domain surface. A rational approach based on the computational predictions and various physical and qualitative factors was used to propose

Abbreviations used: APR, aggregation-prone region; CDR, complementarity determining region; Fab, fragment antigen binding; Fc, fragment crystallizable; mAbs, monoclonal antibodies; SEC-HPLC, size-exclusion high-performance liquid chromatography; SAP, spatial aggregation propensity; Φ_{eff}, effective hydrophobicity.

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enhanced versions of rituximab that could be more stable with respect to aggregation. We then demonstrated under accelerated conditions that a combination of our mutations enhance stability in real systems. With a minimal number of mutations, we observed a threefold reduction in the aggregation of rituximab. Our best biobetter presents partial humanization of the murine light chain and no loss in affinity to its target, CD20.

MATERIALS AND METHODS

Molecular Simulation

Fab domain structure of rituximab was obtained from PDB ID: 2OSL.[15] Hydrogen atoms are added to this structure at pH 7 using the PSFGEN plugin of the VMD.[16] The SAP values for the wild-type (WT) rituximab Fab domain were computed using this structure using the method described previously.[8] The SAP values for the mutated Fab domain (see Fig. 1c) were calculated after generating a structure of the mutated Fab domain using "mutate residue" plugin of VMD.

The NCBI/IGBLAST webserver[17] was used for identifying the closest human germline sequence to the rituximab heavy-chain and light-chain Fv sequence. Germline sequence IGKV3 shares 62% sequence identity with LC Fv of rituximab. For the residues selected for mutation, we identify corresponding residues in the sequence 1GKV1 for the first round of mutations (V3Q, A9S, I10S, and V59S) and in the sequence 1GKV3 for the second round of mutations (I10T and V59D).

Cloning, Generation of Variants, Expression, and Purification of mAbs

The rituximab gene sequences were obtained from the innovator patent (US patent 5736137).[18] The genes were synthesized by GenScript® (Piscataway, New Jersey) and each subcloned in the GWIZ expression vector (Genlantis, San Diego, CA), resulting in the vectors gWiz-R-LC and gWiz-R-HC. Rituximab variants were generated by site-directed mutagenesis (primers synthesized by IDT), and confirmed by sequencing. Rituximab and variants were expressed by transient cotransfection of gWiz-R-LC and gWiz-R-HC vectors (0.5 g each) in the presence of polyethyleneimine (2 mg) into FreeStyle 293-F cells (1L) (Life Technologies, Carlsbad, CA) grown in GIBCO FreeStyle 293 Expression Medium (Life Technologies). After 6–8 days, the supernatant was filtered (0.22 μm) prior to purification. Expressed mAb (5–20 mg) was purified by affinity chromatography using protein A sepharose (GE Healthcare, Woburn, MA). Eluted samples (0.1 M sodium citrate pH 3.5) were further purified by cation exchange. The variants were then formulated in 20 mM histidine HCl pH 6.5 and concentrated to 20 mg/mL.

Accelerated Aggregation Study

An accelerated aggregation study of expressed WT rituximab and each variant were performed within 5 days after the purification by incubation of 20 mg/mL protein at 58°C (>10°C below melting temperature) using a Bio-Rad MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Aggregation was quenched at several time points by placing samples in ice. The amount of aggregation was determined by size-exclusion high-performance liquid chromatography (SEC-HPLC) performed on an Agilent 1200 LC (Agilent Technologies, Santa Clara, CA) using a Tosoh TSKgel super SW3000 (Tosoh Bioscience, Minato, TKY, Japan) column maintained at 22°C. The mobile phase was 150 mM potassium phosphate buffer pH 6.5, at a flow rate of 0.5 mL/min. Samples were diluted down to 10 mg/mL in 15 mM potassium phosphate buffer (pH 6.5) and were centrifuged for 3 min to pellet-insoluble aggregates prior to injection (5 μL). Area of peaks followed at 280 nm are integrated at each time point and the ratio of monomer peak to monomer peak area before incubation ($t = 0$) gives access to the relative amount of monomer left in solution. The mass balance with initial concentration allows the estimation of the amount of soluble and insoluble aggregates.

A standard error (±2.9%) for accelerated aggregation studies was measured for over 100 individual experiments. A propagation of error was applied to calculate standard deviations reported in the manuscript for the stabilization factors and the aggregation rate decrease. Aggregation rates were obtained by fitting the loss of monomers measured by SEC-HPLC to a second-order function to extract rate constants.

Antigen-Binding Assay

The cell-based assay was conducted to determine the dissociation constant (K_D) between the rituximab variants and the targeted antigen (CD20 protein) on the surface of B Cells. Raji cells, a Burkitt's lymphoma cell line, were obtained from Sigma–Aldrich and were grown as suspension cultures in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO) containing 2 mM L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin (Sigma–Aldrich), and 100 μg/mL streptomycin (Sigma–Aldrich) at 37°C, in humid air with 5% CO_2. An indirect immunofluorescence assay was conducted, where WT and variant rituximab at varying concentrations were incubated with Raji cells for 1 h at 4°C. Cells were washed with phosphate-buffered saline/1% bovine serum albumin and incubated with the fluorescently labeled secondary mAb, Alexafluor 488 goat anti-human IgG from Sigma–Aldrich, for 30 minutes at 4°C. Cells were then washed and analyzed by flow cytometry. For each antibody concentration, nonspecific binding was subtracted from the total binding counts to get specific bound counts. Data are represented as the fraction antigen bound (fluorescence of specific binding relative to the computed f_{max}; f_{max} is the maximum amount of fluorescence intensity associated with all antigen binding bound to the mAb).

RESULTS AND DISCUSSION

SAP Prediction and Residues Selection

A previously validated computational method, called SAP,[8] can determine potential APRs on the surface of mAbs by identifying the most hydrophobic dynamically exposed residues on the protein surface and is defined as (for details see previous work[8])

$$\mathrm{SAP}_{\mathrm{residue,\,j}} = \frac{1}{n_{\mathrm{atoms}}} \sum_{\text{all atoms in residue j}} \mathrm{SAP}_{\mathrm{atom,j}}$$

$$\phi_{\mathrm{eff,\ residue\ j}} = \frac{\text{SAA of side} - \text{chain atoms}}{\text{SAA of side} - \text{chain atoms of fully exposed residue}} \times \text{Residue hydrophobicity}$$

Reduction of exposed hydrophobic patches via mutation has been shown to increase colloidal stability, reduce aggregation,