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Charge variant analysis of proposed biosimilar to Trastuzumab

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ABSTRACT

Trastuzumab is a humanized monoclonal antibody (mAb) employed for the treatment of HER2 Positive Breast Cancer. A HER2 overexpressing tumor cell binds to Trastuzumab and attracts immune cells which lead to induction of Antibody Dependent Cellular Cytotoxicity (ADCC) by binding to Fc receptors (CD16a or FcγRIIIa) on an effector cell, such as natural killer (NK) cells.

The most commonly expressed receptor on NK cell is CD16a which binds to the Fc portion of Trastuzumab. The ligand-independent HER2–HER3 dimerization is the most potent stimulator of downstream pathways for regulation of cell growth and survival. An attempt has been made in this study to understand the impact of charge heterogeneity on the binding kinetics and potency of the monoclonal antibody. Trastuzumab has a pI range of 8.7–8.9 and is composed of mixture of acidic and basic variants beside the main peak. Ion exchange chromatography was used to isolate the acidic, basic, and main peak fractions from in-house proposed biosimilar to Trastuzumab and their activities were compared to the Innovator Trastuzumab Herclon®. Data from the mass analysis confirmed the potential modifications in both acidic and basic variant. Binding activity studies performed using Surface Plasmon Resonance (SPR) revealed that acidic variants had lesser binding to HER2 in comparison to the basic variants. Both acidic and basic variant showed no significant changes in their binding to soluble CD16a receptors. *In vitro* assay studies using a breast cancer cell line (BT-474) confirmed the binding potency of acidic variant to be lesser than basic variant, along with reduced anti-proliferative activity for the acidic variant of Trastuzumab. Overall, these data has provided meaningful insights to the impact of antibody charge variants on *in vitro* potency and CD16 binding affinity of trastuzumab.

1. Introduction

There were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide [1]. The high mortality rate of cancer serves as a reminder of the need for more effective therapies. Over the last few decades, monoclonal antibodies have made a major impact with respect to human therapeutics for cancer. There are currently several mAb-based products which have been approved by the regulatory agencies for the treatment of a wide range of human cancers [2,3]. Beside cancer, mAbs are also being used for treatment of several other diseases including autoimmunity, organ transplant rejection, inflammation and infection etc. [4].

Monoclonal antibodies are Immunoglobulin G (IgG) type of proteins (approximately 150 kDa), comprising a pair of heavy and light chains connected by disulfide bonds. The heavy chains constitute of a variable domain (V_H), a hinge and three constant (C_{H1}, C_{H2} and C_{H3}) regions. The light chains contain one variable (V_L) and one constant (C_L) region. Immunoglobulin structure can also be subdivided into Fragment antigen binding (Fab) and the Fragment crystallizable (Fc) domain. The specificity of Antibodies (Abs) is represented by the Fab region where the interaction between antigen and antibody takes place. Fc domain is responsible for effector function of the immunoglobulins [2]. The specificity and affinity of antibody-antigen interactions are essential for elucidating the biological activity of monoclonal antibodies. In general, Monoclonal

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antibodies function through three different mechanisms (a) by inducing Antibody-Dependent Cellular Cytotoxicity (ADCC) and Complement-Dependent Cytotoxicity (CDC), (ii) they directly target antigen present on tumor cells and (iii) can be conjugated to a lethal drug, toxin or radioisotope for enhanced specificity towards tumor cells [2].

In spite of their defined biological activities, heterogeneity has been observed very commonly in monoclonal antibodies. The heterogeneity of monoclonal antibodies can be introduced either by intracellular or extracellular process. Heterogeneity can also be introduced by incubation with buffers, during purification process and also during storage. Both enzymatic and non-enzymatic modifications including formation of disulfide bonds, glycosylation, *N*-terminal glutamine cyclization, C-terminal lysine processing, deamidation, oxidation, glycation, and peptide bond cleavage will contribute towards heterogeneity [5]. Chemical and enzymatic modifications such as deamidation and sialylation, respectively, result in an increase in the net negative charge on the mAbs and cause a decrease in their pI values, thereby leading to the formation of acidic variants. C-terminal lysine cleavage results in the loss of net positive charge and also leads to acidic variant formation [6]. Another mechanism for generating acidic variants is the formation of various types of covalent adducts, e.g., glycation, where glucose or lactose can react with the primary amine of a lysine residue during manufacturing process (in glucose-rich culture media) or during storage if a reducing sugar is present in the formulation. Formation of the basic variants can result from the presence of C-terminal lysine or glycine amidation, succinimide formation, amino acid oxidation or removal of sialic acid, which introduce additional positive charges or removal of negative charges; both types of modification cause an increase in pI values [5].

These variations in composition occur in many types of protein and can impact the activity and stability of biotherapeutics. As mentioned earlier, these variations are introduced either enzymatically during antibody expression or by spontaneous degradation during manufacturing and storage of the formulated material. Most degradation pathways occurring in proteins were also involved in charge-related heterogeneity of monoclonal antibodies. As more and more antibodies are characterized, the knowledge of modifications responsible for charge-related heterogeneity in antibodies will also increase. Therefore, the current challenge is to understand the effects that mAb micro-heterogeneity on the efficacy, potency, immunogenicity and clearance in patients [7–9]. Several articles have been published with respect to the charge variants of monoclonal antibodies which can be generated during the process [10–14]. In this paper, we decided to look at the acidic and basic variants of Trastuzumab and their effects on binding and potency.

Trastuzumab (brand name Herceptin®/Herclon®) is a recombinant humanized IgG1k monoclonal antibody glycoprotein that binds specifically to extracellular domain of human epidermal growth factor receptor 2 (HER-2) [15–17]. Overexpression of ErbB2 (HER-2), a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, occurs in 20–30% of invasive breast cancers, is associated with poor prognosis and rapid relapse. The major mechanisms of action of trastuzumab are believed to be by interfering with the dimerization of HER2 thus leading to abrogation of intracellular HER-2 signalling through pathways including PI3K/Akt and Ras/MAPK leading to cell cycle arrest, reduction in angiogenesis, inhibition of extracellular domain cleavage, and antibody-dependent cell-mediated cytotoxicity (ADCC) [18]. Clynes et al. demonstrated that the activity of Trastuzumab on breast cancer xenografts was attenuated in knock-out mice lacking activating FcγRIII (CD16) receptor [19]. Once the Fc receptor CD16 binds to the Fc region of Trastuzumab, the Natural Killer cell releases cytokines, thus killing the target cells, The charge variants for the study were isolated during the development of proposed biosimilar to Trastuzumab. The goal of this work is to separate the major charge variants (acidic, basic and main peak fractions) of the Trastuzumab, characterize some of their biophysical characteristics, their CD16 and HER2 binding activity and also their potency in an *in vitro* cell based assay.

2. Materials and methods

Trastuzumab innovator Herclon® (Roche) was procured and used for comparison in all the analytical assays.

2.1. Purification and isolation of charge variants

Chromatographic runs were performed with GE AKTA PURE 150 M system. All the columns were pre-equilibrated with respective equilibration buffers prior to loading. Clarified harvest sample was loaded onto XK26/40 MabSelect Sure Protein-A column at pH 7.2 and target protein (mAb) was eluted using low pH buffer (30 mM PBS, pH 3.0) followed by viral inactivation at pH 3.0 for 60 min. Virus inactivated protein-A elute was neutralized to pH 6.0 and then loaded onto XK26/20 AEX Q-Sepharose column in flow through (FT) mode. FT of AEX was loaded onto XK26/40 CEX SOURCE 30S column in binding and elute mode, column was re-equilibrated with loading buffer (50 mM Histidine, pH 6.0, 2 mS/cm) and washed with wash buffer (50 mM Histidine, pH 6.0, 5 mS/cm) for 3 column volumes (CVs) prior to elution. Elution was performed in linear gradient mode from 0 to 100% mobile phase B in 80 min where buffer A was 50 mM Histidine, 10 mM NaCl pH 6.0, 5 mS/cm and buffer B was 50 mM Histidine, 200 mM NaCl pH 6.0, 15 mS/cm respectively. Eluted fractions were collected and subjected to further ion-exchange analysis for determining percentage of acidic variants, main peak and basic variants in each fraction.

2.2. Protein concentration measurement of mAb fraction

The protein concentration of isolated charge variant fractions were determined by Protein-A affinity chromatography by using mAbPac Protein-A column (12 μm; 4 × 35 mm) on an Shimadzu Prominence-i HPLC system. Two mobile phases used were Dulbecco's Phosphate Buffered Saline (mobile phase −A) and 3% glacial acetic acid (mobile phase −B) at a flow rate of 2.5 ml/min. The gradient involved: 0% B for 0.5 min; a linear increase from 0% to 100% B in 0.25 min, a maintaining 100% B for 0.75 min, a linear decrease from 100% to 0% B in 0.5 min; and equilibration at 0% B for 1.0 min. The injection volume was set at 50 μL, column temperature was maintained at 25 °C and eluted fractions were detected at 280 nm. Chromatograms were integrated using Lab Solution Software and the concentration was estimated using Herclon Standard curve.

2.3. SDS PAGE analysis

Antibody samples were analyzed by SDS-PAGE under reducing and non-reducing conditions. 8% (v/v) resolving gel was used for non-reducing condition and 12% (v/v) resolving gel for the reducing condition. Tris Glycine SDS PAGE was performed on the Mini-PROTEAN system (Biorad) as per the supplier's protocol [19].

Polyacrylamide gels were prepared from acrylamide and bis-acrylamide stock solution (29:1), Stacking buffer (1 M Tris-HCl pH 6.8), Resolving buffer (2 M Tris-HCl pH 8.8), 10% SDS, APS and TEMED as the catalyst. Stacking gel was used at 4% concentration.

Antibody samples were mixed with 5X sample buffer (1 M Tris-HCl pH 6.8, 10% SDS, β-mercaptoethanol (in case of reducing