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(54) **Title:** IL-6R TARGETING COMPOSITIONS WITH REDUCED HETEROGENEITY IN FUNCTIONAL ATTRIBUTES

(57) **Abstract:** Present invention discloses an IL-6R targeting biosimilar composition with reduced heterogeneity in the functional attributes in vitro. The functional attributes includes binding activity and potency of the drug preparation. Reducing heterogeneity of functional attributes such that critical quality attributes are maintained within acceptable ranges ensures consistency in the product quality and thereby its therapeutic safety and efficacy.

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TITLE OF INVENTION

IL-6R TARGETING COMPOSITIONS WITH REDUCED HETEROGENEITY IN FUNCTIONAL ATTRIBUTES

FIELD OF THE INVENTION

The present invention relates to attributes of biologic pharmaceutical compositions comprising monoclonal antibodies. More particularly, the invention relates to functional attributes of an IL-6R targeting biosimilar preparation, with reduced heterogeneity in the said attributes.

BACKGROUND

Production of a stable protein biologic therapeutic preparation is a challenging exercise, the major reason being that the therapeutic protein is exposed to various modifying influences throughout its developmental stages (production, purification, formulation development etc.). During these different stages, the protein is prone to structural variations, which can lead to potential variations in the functional attributes. Hence it is important to ensure that these attributes are monitored and maintained within acceptable ranges with the aid of analytical methods. Depending upon the analytical evaluation (which often is carried out in parallel to development), methods associated with upstream, downstream and formulation development are modified such that heterogeneity of the drug preparation is minimized.

"Critical Quality Attributes" (CQA) of a biologic product is defined as a physical, chemical, biological, or microbiological property or characteristic that should be maintained within a certain range or distribution, to ensure homogeneity in the quality, safety, and efficacy of the product. Potential CQAs include charge variants, size variants, acidic/basic variants, glycosylation variants, structural variants and functional properties, among others. As variations in the CQA can impact the safety and efficacy of a biologic drug, research and development efforts towards its development focus towards reducing heterogeneity. Addressing this challenge is unique to every biologic product development – be it an innovator product or a biosimilar as the specific set of CQA varies with the type of protein under question and the associated manufacturing process conditions.

Tocilizumab (active ingredient of Actemra® and RoActemra®) is a recombinant humanized IgG1 monoclonal antibody that binds to human interleukin-6 receptor (IL-6R). Tocilizumab binds to both soluble IL-6R (sIL6-R) and membrane-bound IL-6R (mIL-6R), thereby inhibiting sIL-6R

and mIL-6R-mediated signaling events. Actemra® is approved by the US FDA for the treatment of Rheumatoid Arthritis (RA), Giant Cell Arteritis (GCA), Polyarticular Juvenile Idiopathic Arthritis (PJIA), Systemic Juveline Idiopathic Arthritis (SJIA) and Cytokine Release Syndrome (CRS).

To address the aforementioned challenge, the primary objective of present invention is to arrive at a stable tocilizumab drug preparation with reduced heterogeneity in the functional attributes of the preparation.

SUMMARY

Accordingly, present disclosure relates to a composition of tocilizumab drug preparation with reduced heterogeneity in the functional attributes *in vitro*. The functional attributes includes binding activity and potency of the drug preparation. This includes assays relating to inhibition of 'classical signaling' mechanism via membrane-bound IL-6R (mIL6-R), inhibition of 'trans-signaling' pathway via soluble IL-6R (s-IL6-R), Fc characterization through binding to FcγR1, FcRn, FcγRIIIA and C1q as well as estimation of CDC and ADCC activity upon binding.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Illustrates result of Complement Dependent Cytotoxicity (CDC) assay induced by rituximab and tocilizumab. The representative CDC dose response curves are shown in the figure.

Figure 2: Illustrates result of Antibody Dependent Cellular Cytotoxicity (ADCC) assay induced by rituximab and tocilizumab. The representative ADCC dose response curves are shown in the figure. The signal to noise ratio demonstrates the drug-induced cytotoxicity compared to baseline.

DESCRIPTION

Reducing heterogeneity of functional attributes of tocilizumab preparation such that CQAs are maintained within acceptable ranges ensures consistency in the product quality and thereby its therapeutic safety and efficacy. The present invention discloses a stable tocilizumab preparation with reduced heterogeneity in the functional attributes. Specifically, the functional attributes are binding properties and potency of the drug preparation.

To determine functional similarity, tocilizumab drug product composition (D_TC) was analyzed using various assays. As tocilizumab inhibits 'classical signaling' mechanism via membrane-bound IL-6R (mIL6-R), this attribute was demonstrated using the assay with human cell line DS-1. Next, as tocilizumab also binds to soluble IL-6R (s-IL6-R) and inhibits 'trans-signaling' pathway, ligand binding assay and cell-based signaling assay were performed. The Fc characterization of tocilizumab was demonstrated through binding to FcγR1. Although tocilizumab binds to FcγRIIIA and C1q, it does not induce antibody dependent cell cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) respectively. The binding to the FcγRIIIA and C1q proteins was demonstrated and lack of ADCC and CDC activities were shown through cell-based assays. Finally, as a surrogate to the pharmacokinetic properties, binding of tocilizumab to neonatal Fc receptor, FcRn, was demonstrated. In CDC & ADCC assays, even up to a concentration as high as 600 µg/mL of tocilizumab, the relative fluorescence units (RFUs) observed in the presence and absence of tocilizumab were similar. In contrast, Rituximab (positive control) showed a dose dependent cytotoxicity indicating that effector cells and other reagents used in the assay worked satisfactorily.

Detailed description of embodiments

In an embodiment, the invention discloses an IL-6R targeting biotherapeutic preparation for use in a method for treatment of a condition associated with IL-6R binding activity, wherein the preparation exhibits one or more of the following properties:

a) inhibits growth of an IL-6 dependent cell line with a relative potency of about 105% in a growth inhibition assay of the cell line;
b) binds to soluble IL-6 R with a relative binding capacity of about 100 % in an indirect binding ELISA assay;
c) inhibits IL-6/sIL-6R ά complex induced phosphorylation of signal transducer and activator of transcription-3 (STAT3) at Tyr705 at a relative potency of about 96% in a FRET-based assay;
d) binds FcγRI with a relative binding of about 99% in an indirect binding ELISA assay;
e) binds FcγRIIIa with an equilibrium dissociation constant (K_D) of about 128 nM as measured by surface plasmon resonance (SPR) method;
f) binds C1q in an ELISA at a relative binding capacity of about 97% in in an indirect binding ELISA assay;
g) does not exhibit complement dependent cytotoxicity (CDC) activity up to a concentration of about 600 µg/mL in an IL-6 dependent cell line;
h) binds to neonatal Fc receptor (FcRn) at a relative binding affinity of about 103% as measured by surface plasmon resonance (SPR) method; and
i) does not exhibit antibody dependent cell cytotoxicity (ADCC) activity up to a concentration of about 600 µg/mL in an IL-6 dependent cell line.

In another embodiment, the IL-6R targeting biotherapeutic preparation contains tocilizumab.

In another embodiment, the IL-6 dependent cell line is DS-1 cell line.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting the invention described herein.

Definitions

The term "biotherapeutic" or “biotherapeutic preparation” or “preparation” herein is used in the broadest sense and it covers proteins that are genetically engineered through recombinant DNA

technology, which are of therapeutic significance in the treatment of ailments. Biotherapeutics include monoclonal antibodies, fusion proteins, polyclonal antibodies, multispecific antibodies and antibody fragments so long as they exhibit the desired biological activity.

The term "about" refers to a range of values that are similar to the stated reference value to a range of values that fall within 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 percent above and below of the stated reference value.

The term "IL-6 dependent cell line" refers to cell line that exhibits a dose dependent cell proliferation in response to IL-6 in the cell culture medium.

The term "relative potency" refers to activity of the preparation in the given context when compared to same or similar activity of the reference standard evaluated under similar test conditions.

The term "relative binding" refers to binding response of the preparation in the given context when compared to same or similar activity of the reference standard evaluated under similar test conditions.

EXAMPLES

During the development of the pharmaceutical preparation comprising tocilizumab, various parameters were analyzed and optimized. This is described as follows. Any methods and materials similar or equivalent to the scope of the disclosed invention described herein can be used in the practice of testing of the present invention.

Example 1: Estimation of relative potency of tocilizumab by IL6 induced growth-inhibition assay

An in-vitro cell based assay utilizing DS-1 cells was used to measure the potency of tocilizumab. DS-1 (ATCC® CRL11102™) is a human B-lymphocyte cell line that is known to express mIL-6R and is dependent on IL-6 for its growth. Briefly, varying concentrations of tocilizumab along with fixed concentration of recombinant human IL-6 were exogenously added to DS-1 cells in 96 well plates and incubated for approximately 72 hours in a humidified CO_2 incubator at 37°C. Post incubation, Cell Titer-Glo (a cell viability indicator) was used to detect live cells utilizing CellTitre-Glo® luminescent cell viability assay kit (Promega; G7571) by following manufacturers' instructions. The concentration of tocilizumab is inversely proportional to IL-6 induced proliferation of DS-1 cells and thus higher the concentration of tocilizumab, lesser is the IL-6 induced growth of cells.

Tocilizumab dose response curves were analyzed by a 4 parameter logistic fit using Softmax Pro software and the potency was determined relative to reference standard, tested in the same assay run.

Example 2: Estimation of rhuIL-6Rα receptor binding activity of tocilizumab using indirect binding ELISA

The binding of tocilizumab in samples to soluble IL-6R was assessed by an ELISA method. Briefly, recombinant human sIL-6R was coated onto the surfaces of wells of microtiter plates and samples containing tocilizumab was added at various concentrations followed by detection using a labelled secondary antibody. The observed absorbance at 450 nm is directly proportional to the binding of tocilizumab to the immobilized sIL-6R. The dose response curves were analyzed by a 4 parameter logistic fit using Softmax Pro software and the binding was determined relative to the reference standard, tested in the same assay run.

Example 3: Evaluation of Tocilizumab mediated inhibition of STAT3 phosphorylation induced by IL-6/sIL-6Rά complex

This method depicts the mechanism of action of tocilizumab by binding to soluble form of IL-6R and inhibiting IL-6 induced signal transducer and activator of transcription-3 (STAT3) phosphorylation primarily by trans-signaling mode. The endothelial cells are activated when soluble IL-6 receptor (sIL-6Rά) binds IL-6 resulting in STAT3 phosphorylation and therefore Human Umbilical Vein Endothelial Cells (HUVEC) were chosen for this assay. Briefly, varying concentrations of tocilizumab along with fixed concentration of sIL-6R were pre-incubated at 37°C with shaking at 300 rpm for approximately 45-60 minutes before addition of fixed concentration of IL-6. The incubation was continued post addition of IL-6 for another 30-45 minutes and then 100µL/well of reaction mixture was added to 96 well plate seeded with HUVEC cells. This method utilizes fluorescence resonance energy transfer (FRET) platform based Phospho-STAT3(Tyr705) cellular kit HTRF® (Cisbio; 62AT3PET) for detection of phosphorylated STAT3 at Tyr705. Manufacturer's instructions were followed.

Example 4: Estimation of FcγRI receptor binding activity of tocilizumab using in-direct binding ELISA

The binding of tocilizumab to FcγRI was tested using ELISA. Briefly, ELISA measures the binding of tocilizumab to recombinant human FcγRI coated on the 96-well plate. Tocilizumab

bound to FcγRI is then detected with peroxidase-conjugated F(ab)'2 fragment specific to human IgG through catalysis of a tetramethylbenzidine (TMB) substrate. The intensity of the absorbance at 450 nm is directly proportional to the amount of tocilizumab bound to the FcγRI protein and was used to plot dose response curves. The dose response curves were analyzed by a 4 parameter logistic fit using Softmax Pro software and the binding was determined relative to the reference standard, tested in the same assay run.

Example 5: Estimation of FcγRIIIa(V) binding activity of tocilizumab using capture chemistry in SPR (Biacore 8K) based method

FcγRIIIa is a receptor belonging to the Fc-gamma class of receptors expressed on the surface of a number of immune cells and interacts with antibodies of immunoglobulin G isotype. It plays an important role in mediating ADCC of cells that are coated with IgG. Thus, it is important to study the FcγRIIIa binding. Using the Surface Plasmon Resonance (SPR) technique and software, the binding of tocilizumab in samples to FcγRIIIa (valine containing isoform) was estimated. Briefly, anti-Histidine antibody was immobilized on CM5 sensor chip to capture His tagged FcγRIIIa. Further, various concentrations of analyte were allowed to flow over the bound FcγRIIIa during the association phase, followed by buffer alone during the dissociation phase. Biacore™ 8K Evaluation Software was used to calculate the equilibrium dissociation constant (K_D) between antibody and FcγRIIIa.

Example 6: Estimation of C1q binding activity of Tocilizumab using indirect binding ELISA

C1q is a component of a larger protein complex, C1, which is part of the classical pathway of complement activation. Interaction of the Fc region of IgG or IgM antibodies with C1q initiates the complement activation pathway and results in an immune response. Using an ELISA, the binding of tocilizumab in sample to C1q was estimated. Briefly, various concentrations of tocilizumab in sample were coated onto the surface of 96-well plates and a fixed concentration of C1q was added to the antibody coated wells for binding. C1q protein bound to tocilizumab was detected by horseradish peroxidase-conjugated sheep anti-C1q antibody and subsequent catalysis of TMB substrate. Signal intensity was measured by absorbance at 450 nm and was used to plot 4-parametric logistic graphs of concentration versus mean absorbance. The signal intensity is directly proportional to the amount of tocilizumab bound to C1q protein.

Example 7: Evaluation of tocilizumab Complement Dependent Cytotoxicity (CDC) activity

Tocilizumab binds to the complement C1q protein, however, does not induce complement dependent cell cytotoxicity (CDC). Therefore, to confirm, different batches of tocilizumab sample were tested in CDC assay. DS-1 cells were incubated with various concentrations (600 to 0.06µg/mL) of the sample and baby rabbit complement. Dose-dependent cytotoxicity was evaluated by the uptake and metabolism of the redox dye, Alamar Blue, by viable cells. Rituximab was used as positive control in the assay using WIL2-S (human B-cells expressing CD20 protein) cells as target cells. In order to ensure the membrane IL-6 receptor expression and the associated downstream events are intact, the same DS-1 cells, as used in CDC assay, were tested in IL-6 growth inhibition bioassay.

Example 8: Evaluation of Tocilizumab mediated Antibody Dependent Cellular Cytotoxicity (ADCC)

Tocilizumab binds to FcγRIIIA, however, does not elicit antibody dependent cell cytotoxicity (ADCC). Here tocilizumab sample was tested in ADCC assay. Briefly, DS-1 cells (target cells) were incubated with various concentrations of the sample and peripheral blood mononuclear cells (PBMCs; effector cells) isolated from human peripheral blood. Dose-dependent cytotoxicity was evaluated by the activity of lactate dehydrogenase (LDH) released from lysed cells using a fluorogenic CytoTox reagent. The intensity of the relative fluorescence units (RFU) generated is directly proportional to the amount of LDH in the culture medium and also the number of lysed cells present in the sample. Rituximab was used as positive control in the assay using WIL2-S (human B-cells expressing CD20 protein) cells as target cells. In order to ensure the membrane IL-6 receptor expression and the associated downstream events are intact, the same DS-1 cells, as used in ADCC assay, were tested in IL-6 growth inhibition bioassay.

Example 9: Evaluation of FcRn binding activity of Tocilizumab using Biacore™ T200 based method

The neonatal Fc receptor (FcRn) is expressed on vascular endothelial cells and regulates the circulating half-life of IgG antibodies by receptor mediated re-circulation of antibodies to the extracellular space following fluid phase endocytosis.

FcRn binding was evaluated by a surface plasmon resonance (SPR) technique and Biacore™ T200 evaluation software was used to calculate the equilibrium dissociation constant (K_D)

between tocilizumab and the FcRn. Briefly, amine-coupling chemistry was used to immobilize purified recombinant FcRn receptor on the sensor chip. Various concentrations of tocilizumab were allowed to flow over the immobilized receptor during the association phase, followed by buffer during the dissociation phase. The sensorgrams of each sample were fitted by "two-state reaction" model to generate K_D between tocilizumab and the FcRn. The binding affinity of reference standard was considered as 100%. The relative binding affinity (RBA) data was calculated as: (K_D of Reference Standard / K_D of Test Sample) * 100.

Sl. No.	Attribute	Mean value (n=3)	SD	Min-Max
1	Relative potency* (%) in IL6 induced growth-inhibition assay	105	4	101-109
2	Relative s-IL-6R binding*(%)	100	3	97-103
3	Relative potency* (%) for inhibition of IL-6/sIL-6Rά complex-induced STAT3 phosphorylation	96	15	80-100
4	Relative FcγRI receptor binding* (%)	99	1	98-100
5	FcγRIIIa(V) binding (K_D) (nM)	128	2	126-130
6	Relative C1q binding* (%)	97	8	91-107
7	Relative FcRn binding* (%) affinity	103	2	102-106

Table 1: Provides claimed attributes of tocilizumab drug product composition (D_TC) with corresponding average values from three independent analyses of each attribute, with corresponding standard deviation (SD) and the minimum to maximum (Min-Max) values as indicated. The relative potency (Sl.No.1) was measured by IL6 induced growth-inhibition assay, rhuIL-6Rα receptor binding activity by indirect binding ELISA, inhibition of STAT3 phosphorylation using FRET-based assay, FcγRI receptor binding by indirect binding ELISA, FcγRIIIa(V) binding using capture chemistry in SPR (Biacore 8K) based method, C1q binding using indirect binding ELISA and FcRn binding using Biacore™ T200 based method as described herein. The asterisk(*), wherever applicable, indicates binding affinity(%) relative to the reference standard evaluated in the same assay.

CLAIMS

We Claim:

1. An IL-6R targeting preparation for use in a method for treatment of a condition associated with IL-6R binding activity, wherein the preparation exhibits one or more of the following properties:
   a) inhibits growth of an IL-6 dependent cell line with a relative potency of about 105% in a growth inhibition assay of the cell line;
   b) binds to soluble IL-6 R with a relative binding capacity of about 100% in an indirect binding ELISA assay;
   c) inhibits IL-6/sIL-6R ά complex induced phosphorylation of signal transducer and activator of transcription-3 (STAT3) at Tyr705 at a relative potency of about 96% in a FRET-based assay;
   d) binds FcγRI with a relative binding of about 99% in an indirect binding ELISA assay;
   e) binds FcγRIIIa with an equilibrium dissociation constant (K_D) of about 128 nM as measured by surface plasmon resonance (SPR) method;
   f) binds C1q in an ELISA at a relative binding capacity of about 97% in in an indirect binding ELISA assay;
   g) does not exhibit complement dependent cytotoxicity (CDC) activity up to a concentration of about 600 µg/mL in an IL-6 dependent cell line;
   h) binds to neonatal Fc receptor (FcRn) at a relative binding affinity of about 103% as measured by surface plasmon resonance (SPR) method; and
   i) does not exhibit antibody dependent cell cytotoxicity (ADCC) activity up to a concentration of about 600 µg/mL in an IL-6 dependent cell line.
2. The IL-6R targeting preparation as claimed in claim 1 wherein the preparation comprises tocilizumab.
3. The IL-6R targeting preparation as claimed in claim 1 wherein the IL-6 dependent cell line is DS-1 cell line.

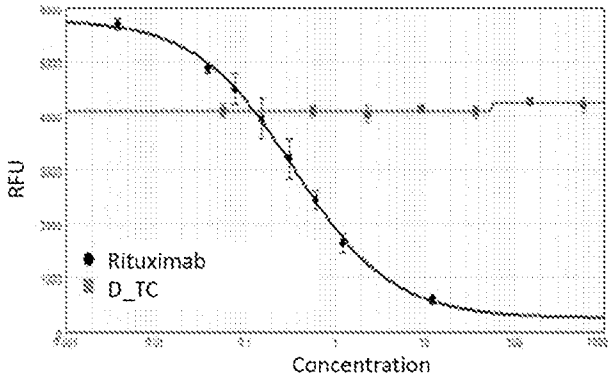

Figure 1

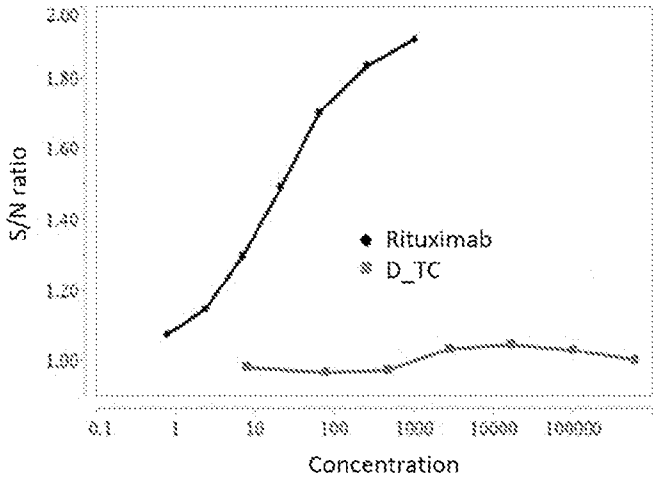
S/N ratio
2.00
1.80
1.60
1.40
1.20
1.00
Rituximab
D_TC
0.1
1
10
100
1000
10000
100000
Concentration

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2021/051179

A. CLASSIFICATION OF SUBJECT MATTER
C07K16/28,A61K39/00,A61K39/395 Version=2022.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PATSEER, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 20100316636 A1 (REGENERON PHARMACEUTICALS INC), 16 December 2010 (16-12-2010) Abstract, paragraphs [0005], [0018], [0036], [0046]-[0050], [0063] and [0074]	1-3
X	WO 2012118813 A2 (APEXIGEN INC.), 07 September 2012 (07-09-2012) Abstract, pages 6, 18, 30, 33 & 40, tables 2 & 3 and claims 28 & 29	1-3
X	WO 2018193471 A1 (DR. REDDY'S LABORATORIES LIMITED), 25 October 2018 (25-10-2018) Abstract, claims and examples	1-3
X	WO 2012064627 A2 (GENENTECH, INC. & F. HOFFMANN-LA ROCHE AG), 18 May 2012 (18-05-2012) Abstract, examples 4 & 6 and claims 18-25	1-3

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13-04-2022	13-04-2022
Name and mailing address of the ISA/ Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Authorized officer Raghavendra Madasetty Telephone No. +91-1125300200

INTERNATIONAL SEARCH REPORT
Information on patent family members

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PCT/IN2021/051179

Citation	Pub.Date	Family	Pub.Date
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