Evaluation of Antibody Properties and Clinically Relevant Immunogenicity, Anaphylaxis, and Hypersensitivity Reactions in Two Phase III Trials of Tralokinumab in Severe, Uncontrolled Asthma

Drug Safety

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Electronic Supplementary Material 1

1 Supplementary Methods

1.1 Determination of Sialic Acid Content

Sialic acid content determination was performed to measure the amount of two forms of sialic acid, N-acetylneuraminic acid (NANA) and N-glycolylneuraminic acid (NGNA). Sialic residues were released from tralokinumab by acid hydrolysis using 2 M acetic acid at 80°C for 2 h. The released NANA and NGNA molecules were fluorescently labeled with 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) at 60°C for 1.5 h. Separation was conducted using reverse phase high-performance liquid chromatography (RP-HPLC) with a C-18 3 µm, 4.6 mm x 7.5 cm CapCell Pak column (Shiseido Co, Ltd, Japan), followed by elution with a gradient of acetonitrile in methanol and water, and fluorescence detection. Concentrations of labeled NANA and NGNA were measured relative to an external standard curve, and were reported as a molar ratio to total protein.

1.2 Characterization of Fab Glycosylation

The Fab glycosylated species was prepared from tralokinumab using preparative size exclusion chromatography (SEC) with a TSK-gel G3000SWxL column, 7.8 mm x 30 cm (Tosoh Bioscience LLC, King of Prussia, PA, USA). Fab glycosylated mAb was eluted isocratically using 0.1 M sodium phosphate, 0.1 M sodium sulfate and 0.05% sodium azide, pH 6.8 at a flow rate of 1 ml/min with UV absorbance detection. The fraction enriched in Fab glycosylated mAb was eluted at ~8.4 min as the monomer pre-peak. The presence of Fab glycosylation in the enriched fraction was confirmed by intact mass analysis with a Synapt quadrupole time of flight (Q-Tof) mass spectrometer (Waters, Elstree, UK). Mass spectrometric data were collected in positive ion mode in m/z range 800–4500 and deconvoluted using MaxEnt 1 software (Waters, Elstree, UK). The starting material and the

monomer pre-peak were subsequently characterized by physicochemical and biological analyses.

The Fab glycosylated–enriched fraction was analyzed by non-reducing and reducing gel electrophoresis. Peptide mapping was used to identify N-glycosylation sites.

The bioactivity and Fab glycosylation level from tralokinumab drug substance and the enriched fraction were measured by a reporter gene assay and gel electrophoresis, respectively.

1.3 Characterization of Fc Glycosylation

The deglycosylated Fc species was prepared by treating tralokinumab with PNGase F, as in section 2.1 of the main manuscript, to remove all Fc oligosaccharides, and then purified using Protein A chromatography with a gradient of phosphate-buffered saline in 20 mM sodium phosphate, pH 3.0, followed by elution with 20 mM sodium phosphate, pH 3.0. Deglycosylation was confirmed by reduction by dithiothreitol (DTT) and gel electrophoresis (Protein LabChip electrophoresis system, PerkinElmer, Inc, Waltham, MA, USA) and intact mass analysis. Non-reduced deglycosylated Fc and deglycosylated Fc reduced by DTT at 37°C were separated by RP-HPLC with a PLRP-S column, 8 µm, 4000 Å, 2.0 x 150 mm (Agilent, Santa Clara, CA, USA) with a linear gradient of acetonitrile in 0.1% trifluoracetic acid in H_2O and analyzed with a Synapt Q-Tof mass spectrometer (Waters, Elstree, UK). Mass spectrometric data were collected in positive ion mode in m/z range 800–4500 and deconvoluted using MaxEnt 1 software (Waters, Elstree, UK). The deglycosylated material was tested by the reporter gene bioactivity assay and the FcRn binding assay.

1.4 Reporter Gene Bioactivity Assay

Biological activity of tralokinumab was assessed using a reporter gene assay. Serial dilutions of tralokinumab were prepared in cell culture medium; these were mixed 1:1 with 0.8 ng/ml interleukin (IL)-13 and aliquots were added to three flat-bottomed 96-well plates. Activity of tralokinumab was measured using HEK-Blue cells expressing the human *STAT6* gene and a

secreted alkaline phosphatase reporter gene induced by STAT-6 (InvivoGen, Toulouse, France). The concentration of STAT-6–induced secreted embryonic alkaline phosphatase in the supernatant was measured with QUANTI-Blue detection medium (InvivoGen, Toulouse, France). Absorbance was measured with a Molecular Devices Spectramax2 plate reader (Molecular Devices, San Jose, CA, USA).

1.5 Neonatal Fc Receptor Binding Assay

Recombinant human neonatal Fc receptor (FcRn) was amine coupled to a Biacore CM5 sensor chip (GE Healthcare Life Sciences, Chicago, IL, USA). A reference flow cell was created by amine coupling in the absence of FcRn. Samples of tralokinumab were prepared at 1000 nM in assay running buffer (50 mM sodium phosphate, 100 mM sodium chloride, 0.005% polysorbate 20, pH 6.0) and serial dilutions were prepared 1:2 from 1000–0.98 nM. Tralokinumab dilutions were injected across the FcRn and reference flow cells and binding was measured by surface plasmon resonance. After binding, the FcRn surface was regenerated with PBS, pH 7.4. Sensorgram data were processed using the Biacore evaluation software. Reference cell data and buffer data were subtracted from tralokinumab data and fitted to a heterogeneous ligand model that assumes that FcRn has a high and a low affinity binding site.

1.6 Determination of Disulphide Bonds and Site-Specific Free Thiol Concentration Using Lys-C Peptide Mapping

Tralokinumab samples were denatured by incubation in 100 mM phosphate buffer, 1% N-ethylmaleimide, 7 M guanidine HCl, pH 7.0 at 37°C for 30 min. The denatured mixture was diluted about four-fold with 100 mM phosphate buffer, 0.1 mM EDTA, and endoproteinase Lys-C was added at 1:10 enzyme:protein ratio. The reaction mixtures were incubated at 37°C overnight. Then the same amount of Lys-C was added and the samples were incubated again at 37°C for 4–6 h.

Following Lys-C digestion, half of each reaction mixture was reduced by adding 500 mM DTT. The reduced and non-reduced digests were separated by RP-HPLC using a C18 column, the mobile phase A was 0.02% TFA in H_2O and the mobile phase B was 0.02% TFA in acetonitrile. The peptides were eluted at a flow rate of 0.2 ml/min with a gradient of mobile phase B from 0% to 95% over 90 min, and were detected by an UV detector and an on-line mass spectrometer. Disulphide-bond linked peptides were those that could be seen in non-reducing runs and that disappeared under reducing conditions.

Site-specific free thiol concentration was determined from non-reduced Lys-C peptide mapping; free thiols were capped by N-ethylmaleimide (NEM). The percentage of NEM-capped cysteine represented the free thiol percentage of that cysteine residue under non-reducing conditions. The sum of free thiols for each cysteine residue multiplied by two equaled the percentage of total free thiols.

1.7 Generation and Characterization of Partially-Reduced Tralokinumab

Reduced and non-reduced control samples were prepared from tralokinumab. Reduced samples were prepared by diluting tralokinumab with Tris pH 8.0 buffer, adding 5 mM DTT and incubating at 37°C for 30 min. Control non-reduced samples were prepared by replacing DTT with water in the incubations. All samples were transferred by buffer exchange into formulation buffer (50 mM sodium acetate, 85 mM sodium chloride, 0.01% [w/v] Polysorbate 80, pH 5.5). Sample aliquots of 100 µl were placed in 1.5 ml Eppendorf tubes and stored either at –80°C (initial time point) or at room temperature for 1 month. At the end of the incubation period, all samples were analyzed by high-pressure SEC, non-reduced gel electrophoresis, and the reporter gene bioassay.

1.8 In-Vitro Fab-Arm Exchange

Tralokinumab and another IgG_4 therapeutic mAb, mavrilimumab (anti–granulocyte macrophage colony-stimulating factor receptor), were used to generate a Fab-arm

exchanged hybrid molecule under reducing conditions and non-reducing physiological conditions.

Glutathione-reduced tralokinumab and mavrilimumab were mixed together 1:1 and incubated at 37°C for 1 day. Non-reduced tralokinumab and mavrilimumab were mixed 1:1 or 1:5 at 25°C for 3 days or 8 days. Incubates were then separated by IEC with a Dionex ProPac WCX-10 4 mm x 250 mm column. Elution was carried out with 20 mM sodium acetate, pH 5.0 with a 0–90% gradient of 200 mM NaCl in elution buffer, followed by UV absorbance detection. Intact mass analysis, conducted as above, was used to confirm which eluted fractions contained tralokinumab, mavrilimumab and the hybrid antibody.

2 Supplementary Results

2.1 Disulphide-Bond Determination

Tralokinumab comprises two heavy chains and two light chains covalently linked with four inter-chain disulphide bonds, two in the hinge region and two in the Fab region, and 12 intra-chain disulphide bonds (see Electronic Supplementary Material 3). The identification of these disulphide bonds was confirmed by the disappearance of disulphide-containing peptides in the reducing Lys-C peptide map, compared with the non-reducing Lys-C peptide map (see Electronic Supplementary Material 4). The theoretical masses of disulphide bonded peptides were compared with observed masses (data not shown). Together these results confirmed that all the expected disulphide bonded peptides were present.

2.2 Free Thiol Concentration

Percentages of free thiols were greatest in the heavy chain constant domains of tralokinumab, being between 2.1% and 4.1% (see Electronic Supplementary Material 5). No free thiols were detected on the cysteines in the heavy chain variable domains and in the hinge region.

2.3 Titers for Persistent and Transient ADAs

In STRATOS 1, median (range) titers for participants with persistent positive post-baseline ADAs were 26.0 (<13–26) in the tralokinumab Q2W group, 19.5 (13–26) in the tralokinumab Q4W group, and 52.0 (<13–208) in the placebo group. Median (range) titers for those with transient post-baseline ADAs were 26.0 (26–26) in the tralokinumab Q2W group, 26.0 (26–26) in the tralokinumab Q4W group, and 13.0 (13–13) in the placebo group.

In STRATOS 2, median (range) titers for participants with persistent positive post-baseline ADAs were 26.0 (13–104) in the tralokinumab Q2W group and 13.0 (13–208) in the placebo group. Median (range) titers for those with transient post-baseline ADAs were 26.0 (<13–26) in the tralokinumab Q2W group and no participants had transient ADAs in the placebo group.