Manufacturing history of etanercept (Enbrel®): Consistency of product quality through major process revisions

Brian Hassett, Ena Singh, Ehab Mahgoub, Julie O’Brien, Steven M. Vicik, and Brian Fitzpatrick

ABSTRACT

Etanercept (ETN) is a soluble protein that binds to, and specifically inhibits, tumor necrosis factor (TNF), a proinflammatory cytokine. ETN is synthesized in Chinese hamster ovary cells by recombinant DNA technology as a fusion protein, with a fully human TNFRII ectodomain linked to the Fc portion of human IgG1. Successful manufacture of biologics, such as ETN, requires sophisticated process and product understanding, as well as meticulous control of operations to maintain product consistency. The objective of this evaluation was to show that the product profile of ETN drug substance (DS) has been consistent over the course of production. Multiple orthogonal biochemical analyses, which included evaluation of attributes indicative of product purity, potency, and quality, were assessed on >2,000 batches of ETN from three sites of DS manufacture, during the period 1998–2015. Based on the key quality attributes of product purity (assessed by hydrophobic interaction chromatography HPLC), binding activity (to TNF by ELISA), potency (inhibition of TNF-induced apoptosis by cell-based bioassay) and quality (N-linked oligosaccharide map), we show that the integrity of ETN DS has remained consistent over time. This consistency was maintained through three major enhancements to the initial process of manufacturing that were supported by detailed comparability assessments, and approved by the European Medicines Agency. Examination of results for all major quality attributes for ETN DS indicates a highly consistent process for over 18 years and throughout changes to the manufacturing process, without affecting safety and efficacy, as demonstrated across a wide range of clinical trials of ETN in multiple inflammatory diseases.

Abbreviations: DMARD, disease-modifying anti-rheumatic drug; DP, drug product; DS, drug substance; ELISA, enzyme-linked immunosorbent assay; ETN, etanercept; Fc, crystallizable fragment; HIC, hydrophobic interaction chromatography; HCP, host cell protein; HPLC, high-performance liquid chromatography; IgG1, human type 1 immunoglobulin G; JIA, juvenile idiopathic arthritis; mAbs, monoclonal antibodies; MAH, market authorization holder; RA, rheumatoid arthritis; SAE, serious adverse event; TNF, tumor necrosis factor

Introduction

Targeting the pro-inflammatory cytokine tumor necrosis factor (TNF) has revolutionized the treatment of rheumatoid arthritis (RA) and other inflammatory diseases. With its approval by the European Medicines Agency (EMA) in 2000, etanercept (ETN) (Enbrel®) was one of the first TNF inhibitors to be approved in the European Union (EU) for the treatment of RA. ETN has since been approved for the treatment of other autoimmune diseases, including plaque psoriasis, psoriatic arthritis, ankylosing spondylitis, and non-radiographic axial spondyloarthritis, as well as for particular-course juvenile idiopathic arthritis (JIA), and the JIA categories extended oligoarthritis, enthesitis-related arthritis, and psoriasis arthritis.

ETN is a dimeric fusion protein consisting of the extracellular domain of human TNF receptor (TNFRII orp75), linked to the crystallizable fragment (Fc) of human type 1 immunoglobulin G (IgG1). ETN specifically inhibits TNF and binds to TNF (sTNF and tmTNF) reversibly in a 1:1 ratio. The Fc component of ETN contains the CH2 and CH3 domains and the hinge region, but not the CH1 domain of IgG.

ETN is a complex protein with a total of 934 amino acid residues (Mr ≈ 150 kDa). It is heavily glycosylated, containing both N- and O-linked oligosaccharides, which can potentially influence the structure, activity, signaling, clearance, and immunogenicity of such glycosylated proteins. TNF blockade with ETN modulates several biologic responses that are induced or regulated by TNF, including expression of adhesion molecules responsible for leukocyte migration, serum levels of cytokines (e.g., IL-6), and serum levels of matrix metalloproteinase-3.

Incorporation of revisions in the manufacturing processes of a biologic after initial regulatory approval is part of the life cycle management of a drug. These can range from relatively minor changes (e.g., a change in supplier of source materials) to more significant changes (e.g., introducing new purification steps).
and are governed by strict, regional-specific regulations. A review of authorized manufacturing changes for 29 therapeutic monoclonal antibodies (mAbs) with European Public Assessment Report (EPAR) documents from 1998 and 2014 showed the annual average number of approved changes categorized by risk status (low, moderate, or high) was 1.8 (range 0–3.71). These findings suggest such changes are not unusual, and the EMA is highly experienced in assessing and ensuring comparability of biologics pre- and post-manufacturing change. The International Conference on Harmonisation (ICH) guidelines (ICH Q5E) regulating this process reflect the complexity of the manufacture of biologics, in that demonstration of comparability does not necessarily mean the quality attributes of the pre- and post-change product are identical. Rather, they are required to be highly comparable, such that, based on the body of established knowledge and experience, it is possible to subjectively predict whether any differences in quality attributes will adversely affect the safety or efficacy of the drug product (DP).

Every batch of ETN drug substance (DS) is analyzed using multiple, orthogonal state of the art technologies, to evaluate the quality attributes that define the identity, strength, biological activity (potency and binding activity), purity (aggregated, misfolded, and clipped species), impurities (e.g., host cell proteins [HCP] and leached protein A), safety, physical characteristics, and overall quality profile. All batch-release test results must meet stringent specifications. Therefore, sophisticated controls are applied to the manufacturing process in order to ensure operation within tight operating ranges. The product quality parameters applied for batch-release testing have been selected following a development program, which includes extensive characterization of the process to facilitate understanding of the link between process performance and subsequent product quality profile. The product quality profile of the DS essentially defines the profile of the DP, as little change in quality attributes occurs through the DP manufacturing process.

The extent of the variance in quality attributes apparent for some marketed biologic products before and after a manufacturing change, illustrates the acceptable limits, within which, observed differences are considered by health authorities as not altering clinical profile. Pfizer is the market authorization holder (MAH) for Enbrel in the EU and the rest of the world (excluding US and Canada, where Amgen is the MAH for Enbrel). Amgen and Pfizer both presently manufacture ETN, and have business systems in place to ensure product consistency is maintained across the globe. US- and EU-sourced product have been shown to be indistinguishable from one another, based on their physicochemical and in vitro functional biological activity, consistent with a single product. Since 1998, the manufacture of ETN has been subject to various process changes, purposefully designed to enhance the efficiency of production, optimize process robustness, or adjust the raw material sources used to manufacture the DS. Information available from the EMA describes in the order of 20 process improvements that have been introduced in EU-manufactured ETN overall. Of these improvements, only three have been major process revisions, with each change being supported by individual comparability exercises to demonstrate that the pre- and post-change products are comparable.

Analytical comparability is demonstrated by evaluating all release test results against the pre-change historical normal values. Analytical comparability also includes a number of heightened characterization tests to confirm comparability with the reference standard. Heightened characterization includes analyses of primary, secondary, and tertiary structure, expected heterogeneity of the amino- and carboxy-termini, expected post-translational modifications, aggregation, fragmentation, molecular size, molecular charge distribution, and binding to multiple biological receptors, including the target molecule (TNF) and multiple Fcγ receptors.

The three major revisions to the original ETN DS manufacturing process were: 1) replacement of non-irradiated with γ-irradiated serum (2002); 2) addition of an extra purification step to decrease process impurities (protein A ligand) (2003); and 3) implementation of serum-free cell culture (2008). These changes were made proactively to improve the manufacturing process, and were not made as a consequence of any safety signals or concerns. Each of these changes in the manufacturing process was supported by an appropriate comparability package, including full product release and stability testing, together with a heightened characterization testing exercise and supportive clinical studies, where required, to meet the associated regulatory requirements.

To demonstrate consistency of product quality through each of these three major process revisions, data are presented for select analyses, which indicate the purity, levels of impurities, binding activity, potency, and overall product quality. The results span approximately 20 years of production across multiple sites and scales of manufacture, and demonstrate comparability before and after each process revision.

**Results**

Key quality attributes for ETN DS from >2,000 batches from three sites of commercial DS manufacture were assessed from 1998 to 2015. This assessment spanned implementation of the three major process revisions (Processes B, C, and D) to the original process (Process A), as well as manufacturing of ETN DP at three different sites (Table 1). The tests used for routine and heightened characterization of Enbrel quality attributes are listed in Table 2.

Results from a number of release tests to assess key quality attributes over the ETN DS life cycle (1998–2015) according to the manufacturing process (A−D) are shown in Fig. 1a-1d. Overlap in the periods during which the different processes were used in the manufacture of Enbrel reflects the commissioning and time for licensure for additional manufacturing sites, while implementing the transition from one manufacturing process to another. These changes in process and manufacturing sites were performed in line with the relevant health authority requirements. The results for purity (Fig. 1a, hydrophobic interaction chromatography [HIC] Peak 3), impurities (Fig. 1b, HCP), binding activity (Fig. 1c), cell-based potency (Fig. 1d), and quality (Fig. 2a-c, N-linked glycan profile) are highly comparable across each process revision.

Results for the relative amount of misfolded and aggregated species, as measured by HIC Peak 3 (Fig. 1a), are consistent across each process variation, with minor variations between
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Maintenance of the structural consistency of ETN DS throughout the changes to the manufacturing process described here is reflected in a safety profile that has remained consistent with the approved product labeling. A wide range of clinical trials in a variety of inflammatory diseases. For instance, in an open-label extension, where patients with disease-modifying anti-rheumatic drug (DMARD)-refractory RA who completed initial ETN trials were offered the opportunity to continue treatment, rates of serious adverse events (SAEs), serious infections, cancer, and deaths were stable each year, through 8 years of ETN exposure. In addition, data from a long-term prospective study, with some patients receiving >10 years of treatment, indicated that ETN was well-tolerated and effective as a long-term, continuous therapy for the treatment of both patients with early RA and long-standing RA (Fig. 3); with the risk/benefit ratio of continuous long-term treatment remaining favorable. Consistency in activity is also evident from results from two large, prospective, 5-year, multicenter, observational registries (conducted from 2000 to 2008): RADIUS (Rheumatoid Arthritis DMARD Intervention and Utilization Study) 1, in which patients with RA required a change in treatment; and RADIUS 2, where patients commenced ETN treatment at study entry. In total, over 6,000 patients initiated ETN treatment in the RADIUS registries, and data showed that rates of SAEs, serious infectious events, and events of medical interest in the ETN-treated patients were comparable to those seen in clinical trials, and did not increase with ETN exposure. Maintenance of efficacy of ETN has also been demonstrated in the treatment of patients with plaque psoriasis in long-term open-label extensions to placebo-controlled trials. In patients receiving ETN (50 mg once-weekly), psoriasis activity and severity index (PASI) scores showed improvement from baseline of the extension study to 72 weeks. In a separate analysis of data from the open-label extension phases of two Phase 3 trials, ETN demonstrated sustained effectiveness (based on Physician Global Assessment [PGA] and Dermatology Life Quality Index scores) and a favorable safety profile for up to 4 years. This profile is also supported by 5-year data from an observational postmarketing safety surveillance registry, OBSERVE, in which the proportion of patients rated as clear/almost clear based on PGA, increased from 12% at baseline to 51% at month 6, and remained relatively stable thereafter. No new safety signals were observed in this study of long-term real-world ETN use.

Cumulatively, it is estimated that 28,123 subjects have participated in sponsor-initiated (Pfizer and Amgen) clinical trials, with 25,637 subjects exposed to ETN (unpublished data). There have been ~5.6 million patient-years of collective clinical experience with ETN across the various indications (unpublished data). In all of these patient populations, ETN (with or without methotrexate) effectively reduced signs and symptoms, disease activity and disability, and improved health-related quality of life, with these benefits being sustained during long-term treatment. During this time, changes to the manufacturing process (a familiar and carefully regulated aspect of the lifecycle of biologic products) have been implemented to ensure the
eficacy, safety, and quality of the ETN DP currently available to patients is no different compared to when it was first introduced.

Material and methods

The test methods used to assess attributes reflective of the product purity, level of impurities, binding activity, potency, and quality are described below. During validation of planned manufacturing process changes, Enbrel batches were tested against the existing reference standard derived from the licensed process, historical product trends, and in side-by-side batch analysis, where applicable. Following licensure of the process change, a new reference standard was typically prepared, in line with the recommended approach for biological products.22

Purity as measured by HIC high-performance liquid chromatography

HIC is performed to assess the purity of ETN DS and DP, and the extent of the related product species present. The method is used to resolve three variants of ETN, which differ in biological activity: Peak 1 is predominantly clipped species; Peak 2 is homogenous ETN; Peak 3 consists of misfolded species, aggregates, ETN fragments, and other process-related impurities. Results are expressed as relative percentage peak area.

Each sample (5 μL; 2 mg/mL) was injected onto a TSKgel Butyl-NPR (Tosoh) (2.5 μm) analytical column (4.6 mm × 35 mm) at 35°C, connected to an HPLC system. Product-related impurities were separated by gradient elution over 50 minutes using mobile phase A (ammonium sulfate [475.9 g] and anhydrous disodium hydrogen phosphate [28.4 g] in 1,950 mL water for chromatography, adjusted to pH 7.0 with phosphoric acid and diluted to 2,000 mL with water for chromatography), and mobile phase B (anhydrous disodium hydrogen phosphate [28.4 g] in water for chromatography and diluted to 1,950 mL with water, adjusted to pH 7.0 with phosphoric acid and diluted to 2,000 mL with water). The flow rate was 1.0 mL/min, and chromatography was monitored by fluorescence detection (278 nm for excitation; 350 nm for emission). Elution of the protein molecules occurred in order of increasing hydrophobicity, as the salt concentration decreased throughout the run, separating the sample into HIC Peak 1, HIC Peak 2, and HIC Peak 3, which were integrated and reported as relative %Peak 1, %Peak 2, and %Peak 3.

Impurities as measured by enzyme-linked immunosorbent assay (ELISA)

A standard sandwich ELISA using polyclonal antibodies was used to quantify the amount of HCP in DS samples from a calibration curve prepared from CHO cell HCP, using a null vector (the reagents used are proprietary to Pfizer). The units are specific to this assay, and sample results are reported as ppm of protein (ng HCP/mg protein).

Binding activity as measured by ELISA

The receptor binding assay used was a quantitative solid-phase ELISA. ETN (TNFR:Fc) in samples and standards binds to
TNF adsorbed to the microplate wells and were detected using goat anti-human IgG antibody horseradish peroxidase conjugate (Sigma-Aldrich: #A0170). Binding activities are calculated based on the ratio of the ED_{50} values of the calibration curve relative to the control or sample curves.

**Potency as measured by cell-based bioassay**

The potency of ETN samples was quantified by measurement of their ability to neutralize TNF-mediated apoptosis in histiocytic lymphoma cell-line U937 (ATCC No. CRL-1593.2) via caspase activation. The U937 cells were incubated at 36.0–38.0°C for 30–60 min in a humidified incubator using 5% ± 2% CO_2 with varying dilutions of test and reference preparations of ETN, in the presence of TNF. They were then incubated with Caspase-Glo 3/7 assay test and reference preparations of ETN, in the presence of Flavobacterium meningosepticum ed from Flavobacterium meningosepticum; mobile phase B, acetonitrile) at a flow rate of 0.4 mL/minutes. Monitoring was by fluorescence detection (330 nm for excitation; 420 nm for emission). N-linked glycans are resolved into various neutral (Peaks 1–5) and sialylated (Peaks 6–9) species after enzymatic cleavage from the protein. Each peak is controlled by measurement of their ability to neutralize TNF-mediated apoptosis.

**Quality as measured by N-linked oligosaccharides map**

N-linked oligosaccharides are a post-translational modification and each ETN monomer has three sites for the addition of N-linked oligosaccharide structures. Each of the three N-linked oligosaccharides on the ETN protein can be any one of multiple different species. The characteristic profile for the nine pre-dominant species are routinely tested on every batch and must meet stringent acceptance ranges to ensure process consistency. N-linked glycans are chromatographically resolved into the various neutral (Peaks 1–5) and sialylated (Peaks 6–9) species after enzymatic cleavage from the protein. Each peak is controlled within a specified range.

0.25 M sodium phosphate, pH 7.5, (3 μL) and a 500,000 U/mL solution of peptide N-glycosidase F (2 μL) (New England Biolabs: #704L, Purified from Flavobacterium meningosepticum, free of proteases and Endo F activities) were added to the test solution (4 μL of 25 mg/mL solution). The mixture was incubated at 37°C for 20–24 h. The released N-glycans were labeled with 2-aminobenzamide. The labeled N-glycans were resuspended or diluted in water (100 μL) and separated by a reverse-phase HPLC (GlycoSepN HPLC column from Prozyme/ 5.0 μm) on an analytical column (4.6 mm × 250 mm) at 35°C. Components were separated by gradient elution of 80–20% of mobile phase B (mobile phase A, 0.5% formic acid in water [adjusted to pH with ammonia]; mobile phase B, acetonitrile) at a flow rate of 0.4 mL/minutes. Monitoring was by fluorescence detection (330 nm for excitation; 420 nm for emission). N-linked glycans are resolved into various neutral (Peaks 1–5) and sialylated (Peaks 6–9) species after enzymatic cleavage from the protein.

**Disclosure of potential conflicts of interest**

The authors are employees and shareholders of Pfizer.

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