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ORIGINAL RESEARCH

Nonclinical Evaluation of PF-06438179: A Potential Biosimilar to Remicade® (Infliximab)

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ABSTRACT

Introduction: PF-06438179, a potential biosimilar to Remicade® (infliximab, Janssen Biotech, Inc.), is a chimeric mouse–human monoclonal antibody targeting human tumor necrosis factor alpha (TNF).

Methods: Analytical (small subset reported here) and nonclinical studies compared the structural, functional, and in vivo nonclinical similarity of PF-06438179 with Remicade sourced from the United States (infliximab-US) and/or European Union (infliximab-EU).

Results: The peptide map profiles were superimposable, and peptide masses were the same, indicating identical amino acid sequences. Data on post-translational modifications, biochemical properties, and biological function provided strong support for analytical similarity. Administration of a single intravenous (IV) dose (10 or 50 mg/kg) of PF-06438179 or infliximab-EU to male rats was well tolerated. There were no test article-related clinical signs or effects on body weight or food consumption. Systemic exposures [maximum drug concentration (C_{max}) and area under the

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concentration–time curve (AUC)] in rats administered PF-06438179 or infliximab-EU were similar, with mean exposure ratio of PF-06438179 relative to infliximab-EU ranging from 0.88 to 1.16. No rats developed anti-drug antibodies. A 2-week IV toxicity study was conducted with once-weekly administration of 10 or 50 mg/kg of PF-06438179 to male and female rats. PF-06438179-related hyperplasia of sinusoidal cells occurred in the liver in rats administered 50 mg/kg, but was not adverse based on its minimal to mild severity. The no-observed adverse-effect level for PF-06438179 was 50 mg/kg. At this dose, C_{max} was 1360 μg/mL and AUC at 168 h was 115,000 μg h/mL on day 8.

Conclusions: The analytical and nonclinical studies have supported advancement of PF-06438179 into global comparative clinical trials.

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Keywords: Biosimilar; Infliximab; Liver; Monoclonal antibody; Remicade; Rheumatology; Toxicity; Toxicokinetics; Tumor necrosis factor

INTRODUCTION

Biosimilars are biopharmaceuticals that are approved by a regulatory agency and are highly similar to an approved marketed biologic product [1–3]. There is strong interest throughout the world in developing biosimilars to many currently marketed biopharmaceuticals, with the hope of reduced costs for medical care coupled with increased accessibility to these medicines.

Many countries, including those in the European Union (EU) and the United States (US), have created abbreviated approval pathways for biosimilars (compared with the requirements for innovative molecules) that require a comparison of the proposed biosimilar with the approved product, generally termed the reference product, to demonstrate biosimilarity. The EU defines the reference product as the product that has been granted marketing authorization in the EU [1]. In a similar manner, the US Biologics Price Competition and Innovation Act defines the reference product as the US Food and Drug Administration (FDA)-licensed product [2]. Under certain circumstances, both the European Medicines Agency (EMA) and FDA allow the use of data derived from human or animal studies comparing a proposed biosimilar to a reference product licensed in another region that has scientific and regulatory standards compatible with the International Conference on Harmonization [2, 4]. From a practical standpoint, this allows the possibility of conducting nonclinical in vivo studies with material from the EU or US to support global clinical trials. However, at the time of designing the nonclinical package in support of the development of PF-06438179, EMA guidance stipulated the use of the EU reference product, and hence, for the in vivo nonclinical studies, the EU reference product was employed as a comparator. Both regions suggest that biosimilar development should follow a stepwise approach, with a greater emphasis on in vitro assays, followed by generally limited or possibly no nonclinical in vivo studies prior to initiation of clinical trials in humans.

Remicade® (Infliximab; Janssen Biotech, Inc. Horsham, PA, USA) is a chimeric human–mouse monoclonal antibody (mAb) approved throughout much of the world for the treatment of a variety of inflammatory conditions, including Crohn's disease, pediatric Crohn's disease, ulcerative colitis, pediatric ulcerative colitis, rheumatoid

arthritis, ankylosing spondylitis, psoriatic arthritis, and plaque psoriasis [5]. The Fc region of infliximab is human, while the Fv region with the complementarity determining region (CDR) that binds human tumor necrosis factor alpha (TNF) is derived from the mouse. Infliximab binds soluble and transmembrane human TNF and blocks its interaction with p55 and p75 cell surface TNF receptors, thereby neutralizing the proinflammatory effects of TNF [6]. A number of nonclinical in vivo studies were conducted during the development of infliximab. These included single- and repeat-dose (up to five doses) studies in chimpanzees, single- and repeat-dose (up to seven doses) studies in rats, and a local tolerance study in rabbits [7]. Nonclinical toxicity studies were conducted primarily in chimpanzees because infliximab neutralizes human and chimpanzee TNF with similar potency. Infliximab does not have activity against mouse, rat, rabbit, pig, cotton top tamarin, marmoset (species not identified), pigtail macaque, rhesus monkey, cynomolgus monkey, or baboon TNF [7]. Although weak activity against dog TNF was observed (0.01% of potency for human or chimpanzee TNF), nonclinical testing of infliximab in this species was discontinued because immediate dermal hypersensitivity (urticaria and angioedema) was observed with the initial intravenous (IV) infusion [7]. In rats, minimal and reversible hepatic changes (Kupffer cell and hepatocellular hyperplasia) and slight reductions in erythrocyte counts, hemoglobin levels, and hematocrit values were observed after single or repeat daily doses at ≥10 mg/kg/day, but these findings were not considered relevant to humans, because they were considered to be related to the response of a normal rat reticuloendothelial system to large doses of chimeric (human–mouse) antibody, a foreign protein to this test animal [8]. Infliximab was well tolerated in chimpanzees, rabbits, and rats. Additional studies were conducted in mice using a surrogate anti-mouse TNF mAb that was reported to have similar pharmacodynamic and pharmacokinetic properties as infliximab [7]. No adverse infliximab surrogate-related findings were observed in 6-month repeat-dose or developmental and reproductive toxicity studies in mice using the surrogate anti-mouse TNF mAb up to 40 mg/kg [7].

PF-06438179 is an anti-TNF mAb currently under development as a potential biosimilar to Remicade. Consistent with current regulatory authority guidelines [1–3], a stepwise approach was used during development, whereby PF-06438179 was first evaluated for structural and functional similarity to the reference product in vitro. These analytical studies of PF-06438179 were performed side by side with Remicade sourced from the US (infliximab-US) [5] and the EU (infliximab-EU) [9]. Key attributes of primary structure, post-translational modifications, product purity, charge heterogeneity, and biological activity were shown to be similar [10]. Based on the comparative structural and functional results providing strong support for analytical similarity, of which only a small subset is outlined in this nonclinical paper, PF-06438179 was then evaluated in limited in vivo nonclinical studies. This study reports results comparing the structural, functional, and in vivo nonclinical attributes of PF-06438179 with those of the reference product infliximab. Overall, the data demonstrated analytical and nonclinical similarity of PF-06438179 to infliximab, and have supported global clinical trials with PF-06438179.

METHODS

Characterization Assays

Peptide Mapping Using Liquid Chromatography/Mass Spectrometry (LC/MS)

PF-06438179, infliximab-EU, and infliximab-US samples were denatured in 8-M guanidine HCl (Sigma-Aldrich), then reduced with 1,4-dithio-DL-threitol (DTT; Sigma-Aldrich) at 40 °C for 1 h and alkylated with iodoacetic acid, sodium salt (Sigma-Aldrich) at room temperature for 1 h. The reduced/alkylated samples were desalted using a fast desalting 10/100 GL column (GE Healthcare Life Sciences) into 50 mM tris buffer pH 8.2, and digested with trypsin (Roche) at 37 °C overnight. The resulting peptides were separated by reversed-phase high-performance liquid chromatography (HPLC) using a C_{18} column (3.5 μm, 2.1 × 250 mm; Waters BioSuite™) at 35 °C with an acetonitrile gradient containing trifluroacetic acid (Thermo Scientific). Absorbance was monitored at 214 nm using a Waters-2695 Alliance HPLC system equipped with an ultraviolet detector (Waters) and connected to an ultrahigh-resolution electrospray ionization quadrupole time-of-flight (UHR ESI-QTOF) mass spectrometer (Bruker Daltonics maXis) for identification.

Subunit Analysis Using LC/MS

PF-06438179, infliximab-EU, and infliximab-US samples were digested at pH 6.6 with the Ig-degrading enzyme of *Streptococcus pyogenes* (IdeS); FabRICATOR® IgG protease, Genovis AB. Following IdeS digestion, denaturation and disulfide bond reduction was carried out using guanidine and DTT. The resulting subunits were injected on a C4 reversed-phase column (Waters BEH300 C4, 1.7 μm, 2.1 × 100 mm) at a column temperature of 65 °C. Reversed-phase ultra-HPLC/electrospray ionization quadrupole time-of-flight (RP-UHPLC ESI-QTOF) mass spectrometry (MS) was performed on a Waters H-Class Acquity coupled to an UHR QTOF MS.

Imaged Capillary Isoelectric Focusing

For a quantitative assessment of charge isoforms by imaged capillary isoelectric (iCE) focusing, both native and CBP-treated (Sigma-Aldrich) PF-06438179, infliximab-EU, and infliximab-US samples were denatured using urea (Sigma-Aldrich) in methyl cellulose (ProteinSimple) and 4% Pharmalyte® pH 3–10 (GE Healthcare Life Sciences). Prepared samples were injected onto an FC-coated iCE cartridge (100 μm ID × 50 mm; ProteinSimple). Absorbance was monitored at 280 nm using a ProteinSimple iCE 280 system. The CBP enzyme was used to cleave the C-terminal lysine from the sample by incubating the mixture for 1 h at 25 °C.

Size Exclusion HPLC

Native PF-06438179, infliximab-EU, and infliximab-US samples were fractionated using a dihydroxypropane bonded silica column (8 mm × 300 mm; Waters YMC-Pack Diol-200) at 30 °C and a salt containing mobile phase at pH 5.0. The analysis was performed using isocratic flow conditions, and the absorbance was monitored at 280 nm using a Waters-2695 Alliance HPLC system equipped with an ultraviolet detector.

Biological Activity

Using an in-house validated assay, a serial dilution of each PF-06438179, infliximab-EU, and infliximab-US sample was prepared and incubated for 35 min at 37 °C and 5% carbon dioxide with recombinant human TNF (R&D Systems). Then, the content of each incubation

was added to a 96-well plate containing U937 cells and incubated for 2 h at 37 °C and 5% carbon dioxide. Caspase Glo® (Promega Corp.) reagent was added to the assay, lysing the cells and producing a luminescent signal proportional to the apoptotic population of cells. The luminescent intensity of each well in the plate was measured using a suitable plate reader. The dose–response plots were fit with a 4-parameter logistic (4PL) nonlinear regression model. Relative potency was calculated for test sample curves deemed parallel to reference material, using a half-maximal effective concentration (EC_{50}) ratio in a constrained 4PL fit.

In Vivo Animal Studies

The single- and repeat-dose studies were conducted in Sprague–Dawley (Crl:CD®[SD]) rats (Charles River Laboratories). All rats were acclimated to the laboratory environment for a minimum of 14 (single-dose study) or 13 (repeat-dose study) days prior to initiation of dosing. The IV route was chosen because it is consistent with the intended clinical route of administration and was used during the nonclinical program of infliximab. Toxicokinetic (TK) parameters were calculated from individual animal data using noncompartmental analysis (Watson LIMS, version 7.4.1; Thermo Inc). TK parameters included C_{max}, time to C_{max}, and AUC. Samples tested below the limit of quantification (<0.1 μg/mL in 100% serum) were assigned a value of 0 μg/mL for the TK calculations.

Single-Dose Study TK and Tolerability Study Comparing PF-06438179 with Infliximab-EU in Rats

The objective of this study was to determine the TK and tolerability of PF-06438179 compared with infliximab-EU when administered as a single dose to male rats (aged 9–10 weeks and weight at dose initiation, 337.9–392.6 g). PF-06438179 or infliximab-EU was administered by single IV bolus injection (volume of 5 mL/kg) to male rats ($n = 5$/group) at doses of 10 or 50 mg/kg. A separate group of five male rats received vehicle #1, and another group of five male rats received vehicle #2. Vehicles #1 and #2 consisted of excipients and buffers identical to those used with PF-06438179 and infliximab-EU, respectively, but with no active protein.

Following dose administration, the animals were retained for an 8-week observation and TK and anti-drug antibodies (ADA) sampling period. Clinical signs were assessed daily, and body weights and quantitative assessment of food consumption were recorded weekly through to day 57. For the TK comparison of PF-06438179 (vehicle #1 and PF-06438179 groups) versus infliximab-EU (vehicle #2 and infliximab-EU groups), approximately 0.25–0.55 mL of blood was collected from the jugular vein of each rat prior to dosing, and at 0.5, 4, 8, 24, 48, 96, 168, 336, 672, 1008, and 1344 h following the first and only dose (administered on day 1). The samples collected prior to dosing and at 1008 and 1344 h after dosing were also used for detection of serum ADA.

Serum PF-06438179 or infliximab-EU concentrations were determined using a validated enzyme-linked immunosorbent assay (ELISA). PF-06438179 or infliximab-EU was captured using immobilized recombinant human TNF adsorbed on a microtiter sample plate. Bound PF-06438179 or infliximab-EU was detected using a donkey anti-human IgG antibody conjugated with horseradish peroxidase and a 3,3′,5,5′-tetramethylbenzidine peroxidase substrate solution to generate a colorimetric readout. Sample concentrations

were determined by interpolation from a calibration curve generated using a 4PL curve-fitting program. Area under the concentration–time curve, AUC at 1344 h and extrapolated to infinity (AUC_{1344} and AUC_{inf}, respectively), was estimated using the linear trapezoidal rule.

Serum samples were analyzed for anti-PF-06438179 or anti-infliximab-EU antibodies using electro-chemiluminescent assays validated on the Meso Scale Discovery (MSD®) assay platform. Affinity-purified rabbit anti-infliximab-EU IgG was the positive control, and pooled normal SD rat serum was the negative control. Test samples were first incubated with biotinylated and ruthenylated PF-06438179 or infliximab-EU. Aliquots were added to the wells of a streptavidin-coated microtiter plate and, following a brief wash, tripropylamine-containing detection buffer was added; the resulting chemiluminescent signal that was proportional to the amount of ADA present was measured. Samples were tested using a tiered approach of screening and titer. The final assessment of induction of an ADA response for each animal was based on the comparison of the pre-dose and post-dose sample results.

Two-Week Toxicity Study of PF-06438179 in Rats

The objective of this study was to determine the toxicity and TK of PF-06438179 following administration of 10 or 50 mg/kg (with a volume of 5 mL/kg) by IV injection once weekly (days 1, 8, and 15) for 2 weeks to male and female rats (aged 9 weeks and weight at dose initiation: male, 289.3–341.7 g; female, 203.7–252.4 g). In the toxicity arm of the study ($n = 10$ per sex/group), doses were administered on days 1, 8, and 15; in the TK arm (test article-dosed group, $n = 4$ per sex; control group, $n = 3$ per sex), doses were administered on days 1 and 8. Vehicle consisted of excipients and buffer, but no active protein.

Rats were monitored for clinical signs daily, and for changes in body weight, and food consumption, weekly, through day 15. Ophthalmic examinations were performed on the animals in the toxicity arm prior to the initiation of dosing (baseline) and on day 13. Hematology, coagulation, clinical chemistry parameters, and urinalysis were evaluated on day 16, just prior to necropsy on this day, which included full tissue collection, evaluations of organ weights, and macroscopic and microscopic observations. Clinical and anatomic pathology findings were peer reviewed. For the TK analysis in satellite animals, approximately 0.30–0.55 mL of blood was collected from each rat 5 days prior to the initiation of dosing (day −5) and at 1, 7, 24, 72, 120, and 168 h post-dose on days 1 and 8. In the vehicle control group, only the 1-, 7-, and 24-h post-dose samples were analyzed. Serum samples were analyzed for PF-06438179 using a validated ELISA assay and analysis methods as described above. AUC_{168} was estimated using the linear trapezoidal rule. For calculations of day 8 AUC_{168}, the serum concentration of PF-06438179 at time 0 was set to the day 1, 168-h post-dose concentration.

Compliance with Ethics Guidelines

In vivo animal studies were conducted in accordance with Good Laboratory Practice for Nonclinical Laboratory Studies regulations as set forth in the Code of Federal Regulations (21 CFR Part 58) and in accordance with current guidelines for animal welfare from the National Research Council for the Care and Use of Laboratory Animals. The procedures used in

these studies were reviewed and approved by the Institutional Animal Care and Use Committee.

RESULTS

Characterization Assessments

Peptide Mapping Using LC/MS

The chromatographic profiles for PF-06438179, infliximab-US, and infliximab-EU were essentially superimposable (Fig. 1). The identities of corresponding peptides by MS were consistent between PF-06438179, infliximab-US, and infliximab-EU, with 100% of the light chain and 98.7% of the heavy chain of the amino acid sequence of infliximab detected. The remaining undetected sequence corresponded to small peptides (two or fewer amino acids) that were not retained on the chromatographic column. For all three materials, the site of the *N*-linked glycosylation was determined to be on the H26 Fc peptide that contains the NST *N*-linked glycosylation consensus sequence. The predominant *N*-glycopeptides were G0F and G1F. For PF-06438179, the predominant C-terminal form of the heavy chain was des-lysine; however, for infliximab-US and infliximab-EU, the predominant C-terminal form was lysine. Minor modifications such as heterogeneity of C-terminal lysine are not expected to be clinically significant [11] and, therefore, were not considered to impact similarity [10]. The essentially superimposable chromatographic profiles of PF-06438179 against the reference products and consistent peak-by-peak peptide identifications by MS

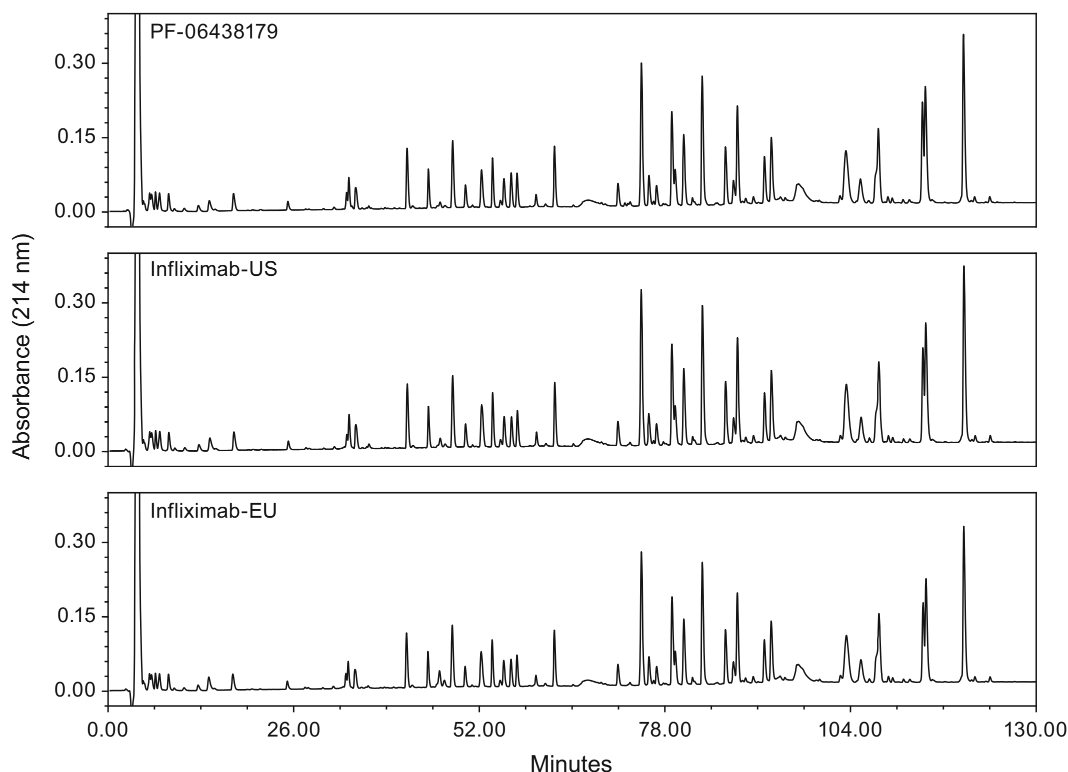

Fig. 1 Peptide map profiles (0–130 min) of PF-06438179, infliximab-US, and infliximab-EU. *infliximab-US* Remicade® sourced from the United States, *infliximab-EU* Remicade® sourced from the European Union

indicate that the primary sequences of PF-06438179, infliximab-US, and infliximab-EU are identical.

Subunit Analysis Using LC/MS

In the subunit analysis, the observed monoisotopic masses exhibited for the predominant isoforms of the scFc, Fd′, and light chain in each material were in excellent agreement with each other and the respective theoretical values (Fig. 2). The observed masses exhibited ≤1.2 ppm mass measurement errors, equivalent to a ±0.030 Da tolerance at 25 kDa, therefore allowing any single amino acid difference except Leu/Ile to be distinguished at the subunit level. For each subunit and domain, there was excellent agreement in the relative abundance of the individual isotopic species among each of the three materials, and with the respective theoretical isotopic distributions, indicating no subtle structural differences. The high accuracy of these mass and abundance measurements indicates that the amino acid composition of each subunit or domain of the three infliximab materials is identical and consistent with the established PF-06438179 sequence. The subunit analysis confirmed in each material that the scFc domain contained the expected IgG *N*-linked glycosylation and C-terminal heterogeneity with or without C-terminal lysine. As with the peptide map analysis, the major *N*-glycoforms for all three materials were G0F and G1F. No post-translational modifications were observed in Fd′ or in light chain in any of the three materials.

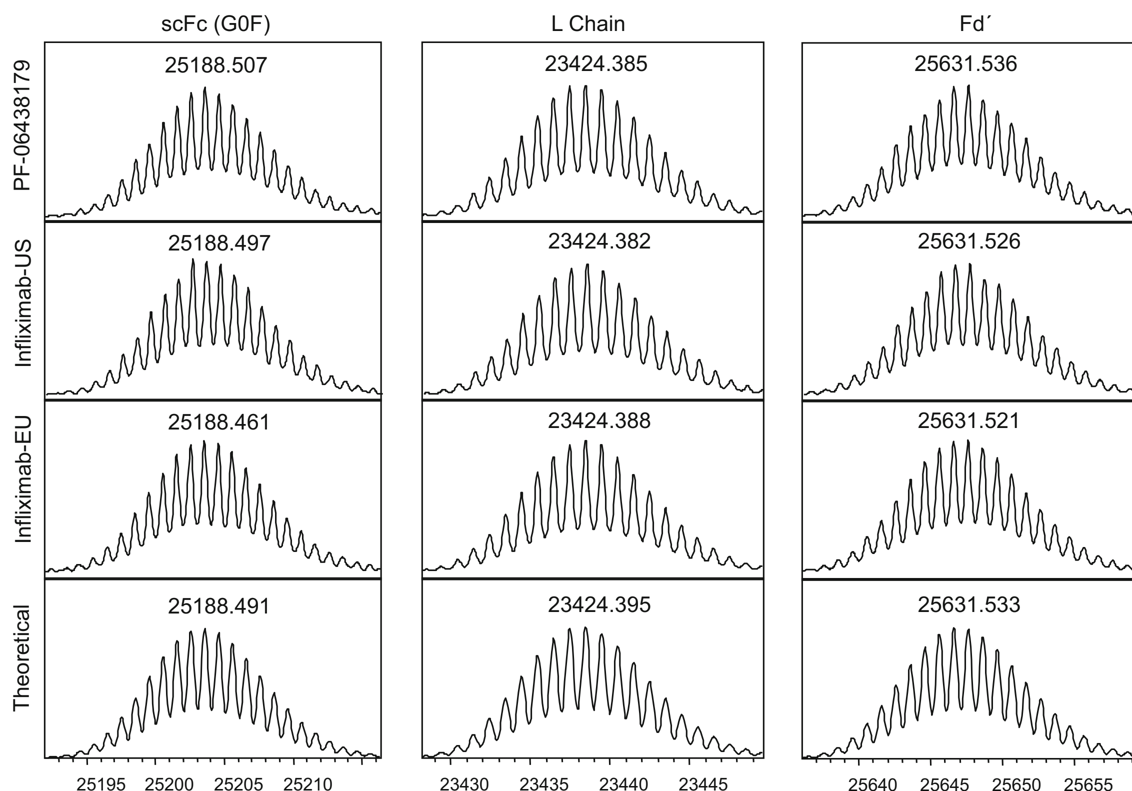

Fig. 2 Zero-charge mass spectra from liquid chromatography/mass spectrometry—subunit analysis of PF-06438179, infliximab-US, and infliximab-EU. *infliximab-US* Remicade® sourced from the United States, *infliximab-EU* Remicade® sourced from the European Union

iCE Focusing

The iCE profile consistently had three regions: acidic, main, and basic. The relative content of basic isoforms of PF-06438179 was less than that observed for the reference products (Fig. 3). The basic region primarily contained two peaks, which correlate to the presence of C-terminal lysine residues on the heavy chains. The two basic peaks were suspected to be mono-C-terminal lysine and di-C-terminal lysine species. This is consistent with the heavy chain C-terminal lysine observed by peptide map and subunit analyses. To confirm that the difference in the relative proportion of basic species between PF-06438179 and the reference products was related solely to C-terminal lysine, the materials were treated with carboxypeptidase B (CBP). The carboxypeptidase cleaves C-terminal lysine from the three materials, resulting in iCE profiles representative of the remaining charge heterogeneity. After treatment with CBP, the iCE profiles for PF-06438179 and two reference products were similar (Fig. 4), as were the relative abundance of acidic, main, and basic species (Table 1).

Size Exclusion HPLC

The size exclusion HPLC profile had consistently two peaks: high-molecular mass species (HMMS) and monomer. Low-molecular mass peaks were occasionally observed in the reference product, but levels were just above the detection limit of the method. The species under the HMMS peak was confirmed to be only dimer in all three materials (data not shown). Analysis of multiple lots of PF-06438179 and reference products

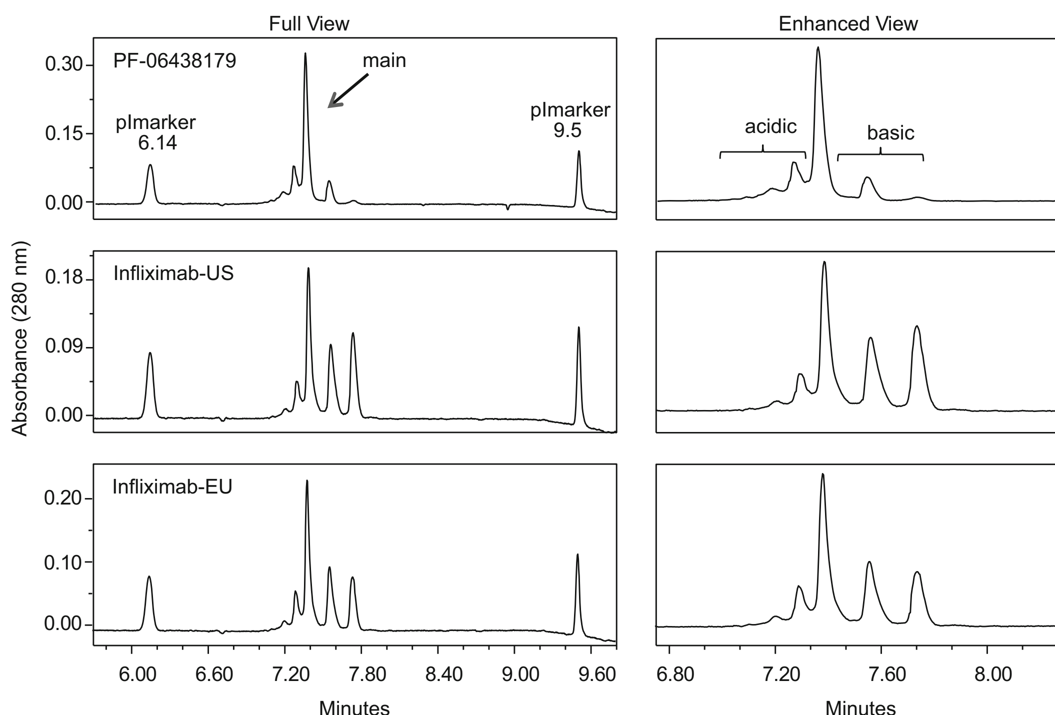

Fig. 3 Charge species profiles of PF-06438179, infliximab-US, and infliximab-EU obtained by imaged capillary isoelectric analysis. *infliximab-US* Remicade® sourced from the United States, *infliximab-EU* Remicade® sourced from the European Union

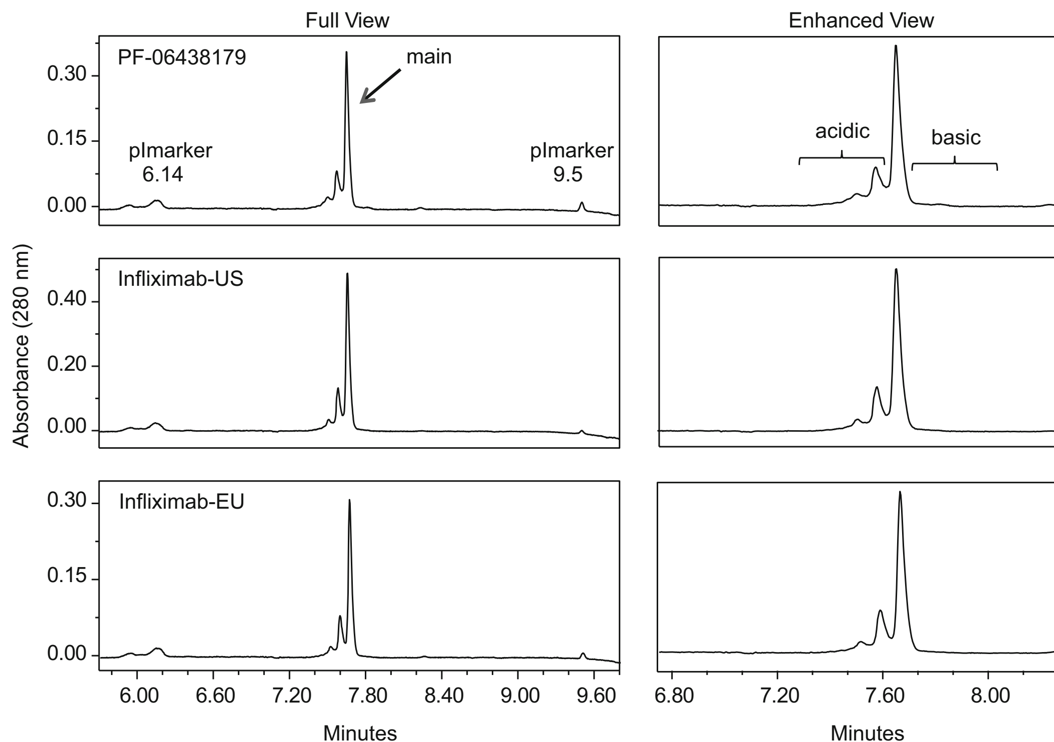

Fig. 4 Charge species profiles of carboxypeptidase B-treated PF-06438179, infliximab-US, and infliximab-EU obtained by imaged capillary isoelectric analysis. *infliximab-US* Remicade® sourced from the United States, *infliximab-EU* Remicade® sourced from the European Union

Table 1 Quantitation of charge species, obtained by imaged capillary isoelectric analysis of PF-06438179, infliximab-US, and infliximab-EU before and after treatment with CBP

	Before CBP treatment (%)			After CBP treatment (%)		
	Acid	Main	Basic	Acid	Main	Basic
PF-06438179	27	60	13	32	66	1.7
Infliximab-US	15	38	48	33	66	1.1
Infliximab-EU	18	41	41	33	66	1.0

CBP carboxypeptidase B, *infliximab-US* Remicade® sourced from the United States, *infliximab-EU* Remicade® sourced from the European Union

demonstrated that all three materials were primarily monomer with similar low levels of HMMS. Representative profiles are shown in Fig. 5.

Biological Activity

The functional similarity of PF-06438179, infliximab-US, and infliximab-EU was assessed by determining the ability of each to inhibit TNF-induced apoptosis in the U937 cell line, a human monocyte cell line that expresses TNF receptors. In this assay, PF-06438179 binds specifically to soluble TNF and blocks its interaction with cell surface TNF receptors on the U937 cell, thereby inhibiting TNF-induced apoptosis. The dose–response curves of

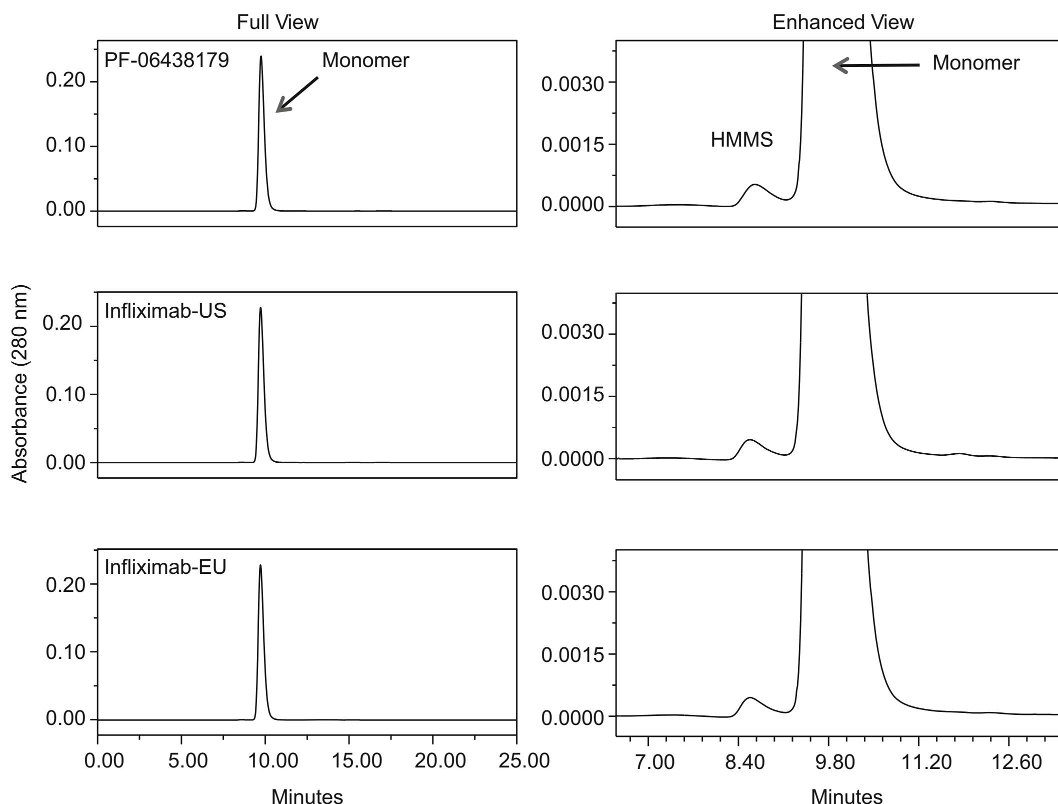

Fig. 5 Size exclusion high-performance liquid chromatography profiles of PF-06438179, infliximab-US, and infliximab-EU. *HMMS* High-molecular mass species, *Infliximab-US* Remicade® sourced from the United States, *infliximab-EU* Remicade® sourced from the European Union

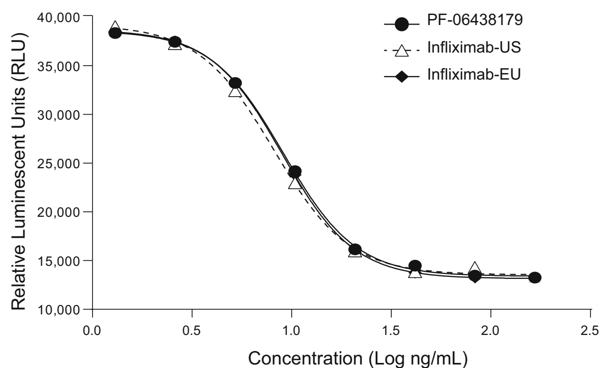

Fig. 6 Inhibition of cell apoptosis: dose–response curves for PF-06438179, infliximab-US, and infliximab-EU binding to tumor necrosis factor alpha. *infliximab-US* Remicade® sourced from the United States, *infliximab-EU* Remicade® sourced from the European Union

inhibition of cell apoptosis induced by PF-06438179, infliximab-US, and infliximab-EU were superimposable (Fig. 6), demonstrating functional similarity in their ability to bind TNF. Multiple lots of PF-06438179 and reference products had similar relative potencies (data not shown).

In Vivo Rat Studies

Based on the structural and functional similarity between PF–06438179, infliximab-US, and infliximab-EU, as demonstrated in the in vitro characterization assessments, limited in vivo studies were conducted in Sprague–Dawley rats. A comparative single-dose TK and tolerability study with PF-06438179 and one reference product (infliximab-EU) was conducted initially. A 2-week IV toxicity study was conducted later in the program based on a

specific request from a single Regulatory Authority.

Single-Dose Study TK and Tolerability of PF-06438179 Versus Infliximab-EU in Male Rats

Administration of PF-06438179 or infliximab-EU as a single IV bolus dose to male rats at 10 or 50 mg/kg was well tolerated, and the tolerability profiles of PF-06438179 and infliximab-EU were similar. All rats survived to the end of the study. There were no clinical signs attributed to administration of either PF-06438179 or infliximab-EU, and there were no PF-06438179- or infliximab-EU-related changes in body weight, body weight gain, or food consumption compared with the respective concurrent vehicle control groups. No clinical or anatomical pathology parameters were evaluated in this study.

There were no quantifiable concentrations of PF-06438179 or infliximab-EU in serum samples collected from male rats prior to dosing on day 1, or in samples analyzed from vehicle control groups. Exposure to study drug over the duration of the study was confirmed in samples following administration of PF-06438179 or infliximab-EU. Mean serum concentration–time profiles of PF-06438179 were similar to infliximab-EU at each dose following a single IV dose of 10 or 50 mg/kg (Fig. 7). Systemic exposure, as assessed by C_{max} and area under the concentration–time curve (AUC) in the PF-06438179 and infliximab-EU groups, were similar and increased with increasing dose (Table 1). Mean systemic exposure ratios C_{max} and AUC_{1344} and AUC_{inf}, respectively, ranged between 0.88 and 1.16 for PF-06438179 relative to infliximab-EU (Table 2). No induction of ADA was detected in rats receiving either PF-06438179 or infliximab-EU, nor was there a suggestion of ADA based on

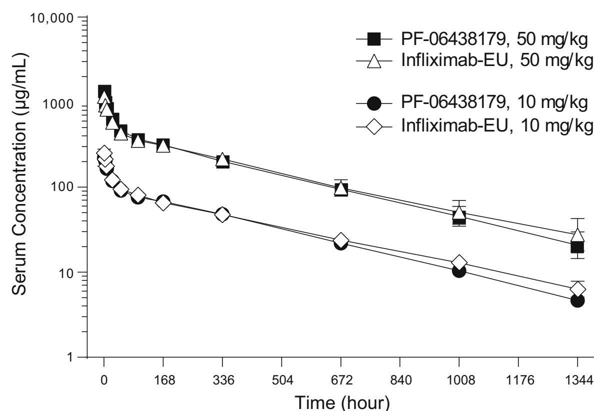

Fig. 7 Mean (standard deviation) serum concentration versus time profiles of PF-06438179 and infliximab-EU in rats following a single intravenous injection. *infliximab-EU* Remicade® sourced from the European Union

serum concentrations of the PF-06438179 or infliximab-EU.

Two-Week Toxicity Study of PF-06438179 in Rats

Repeated administration (dosing on days 1, 8, and 15) of PF-06438179 as an IV bolus injection to male and female rats at 10 or 50 mg/kg/dose was well tolerated, and all animals survived to their scheduled necropsy. There were no PF-06438179-related clinical signs or effects on body weight, body weight gain, food consumption, ophthalmology, coagulation, clinical chemistry, or urinalysis findings compared with those parameters for vehicle control group. PF-06438179-related findings included higher group mean neutrophil, monocyte, and large unstained cell counts in males administered 50 mg/kg, higher group mean fibrinogen in males and females administered 50 mg/kg, and lower group mean platelet counts in females administered 50 mg/kg (Table 3). These changes were not adverse, based on their small magnitude and/or the absence of any correlative clinical observations or macroscopic or microscopic effects. There were no adverse changes in hematology

Table 2 Toxicokinetic parameters of PF-06438179 compared with infliximab-EU: a single-dose study

Test article or reference	Dose (mg/kg)	C_{max} (μg/mL)	C_{max} ratio*	T_{max} (h)	AUC_{1344} (μg h/mL)	AUC_{1344} ratio*	AUC_{inf} (μg h/mL)	AUC_{inf} ratio[a]	$t_{½}$ (h)	CL (mL/min/kg)	V_{ss} (L/kg)
PF-06438179	10	225 ± 53.3	0.88	1.2 ± 1.6	45,000 ± 2000	0.96	47,100 ± 1730	0.93	301 ± 27.0	0.00354 ± 0.000131	0.0876 ± 0.0103
Infliximab-EU		256 ± 19.4		0.5 ± 0.0	47,100 ± 2700		50,400 ± 3870		349 ± 43.1	0.00332 ± 0.000246	0.0914 ± 0.00235
PF-06438179	50	1340 ± 113	1.16	0.5 ± 0.0	206,000 ± 23,700	0.99	215,000 ± 28,700	0.96	303 ± 45.8	0.00393 ± 0.000493	0.0912 ± 0.00788
Infliximab-EU		1160 ± 30.3		0.5 ± 0.0	209,000 ± 31,700		224,000 ± 42,000		355 ± 91.2	0.00383 ± 0.000761	0.0990 ± 0.00703

All values are mean ± standard deviation, $n = 5$/group

AUC_{1344} area under the concentration–time curve from 0 to 1344 h, *AUC_{inf}* area under the concentration–time curve from the first time point to infinity, *CL* clearance, *C_{max}* maximum drug concentration, *infliximab-EU* Remicade® sourced from the European Union, *$t_{½}$* half-life, *T_{max}* time to reach maximum concentration, *V_{ss}* volume of distribution

[a] Mean PF-06438179 divided by mean infliximab-EU

parameters. Compared with control, test article-related lower platelets (0.88 × control), and higher neutrophils (1.82 × control), monocytes (1.71 × control), large unstained cells (1.87 × control), and fibrinogen (1.23×) occurred at 50 mg/kg/dose, but these changes were not adverse based on the magnitude of the change and lack of any histological correlates. There were no test article-related differences in clinical chemistry or urinalysis parameters or test article-related macroscopic findings. PF-06438179-related microscopic changes were limited to minimal to mild sinusoidal cell hyperplasia in the liver in both males and females administered 50 mg/kg (Fig. 8; Table 4). Because of its minimal-to-mild severity and the lack of other effects on the liver, this change was not considered adverse.

There were no consistent sex-related differences in systemic exposure [as assessed by C_{max} and AUC at 168 h (AUC_{168})]; therefore, group mean TK parameters are discussed and presented using combined data from both male and female rats within each dose group. Mean systemic exposure increased with increasing dose in an approximately dose proportional manner on days 1 and 8 (Table 5). Mean systemic exposure (AUC_{168}) was higher on day 8 relative to day 1, with day 8:day 1 exposure ratios of 1.7 and 1.6 at the 10 and 50 mg/kg doses, respectively. There was no suggestion of ADA based on consistent serum concentrations of PF-06438179 throughout the study.

Following once-weekly IV administration of PF-06438179, 50 mg/kg was identified as the no-observed adverse-effect level (NOAEL) based on lack of adverse findings in any in-life or postmortem evaluations in this 2-week toxicity study in rats. At this dose, the combined male and female C_{max} was 1360 μg/mL and the AUC_{168} was 115,000 μg h/mL on day 8 (Table 5).

Table 3 Control means and test article-related differences in hematology and coagulation parameters

Parameter	Male			Female		
	Dose (mg/kg)			Dose (mg/kg)		
	0	10	50	0	10	50
Platelet counts ($\times 10^3/\mu L$)	–	–	–	1086.1	–	0.88×
Neutrophils ($\times 10^3/\mu L$)	1.720	–	1.82×	–	–	–
Monocytes ($\times 10^3/\mu L$)	0.386	–	1.71×	–	–	–
Large unstained cell counts ($\times 10^3/\mu L$)	0.079	–	1.87×	–	–	–
Fibrinogen (mg/dL)	297.5	–	1.23×	254.3	–	1.23×

All values are mean control values and ratios relative to control mean
–, no test article-related finding present

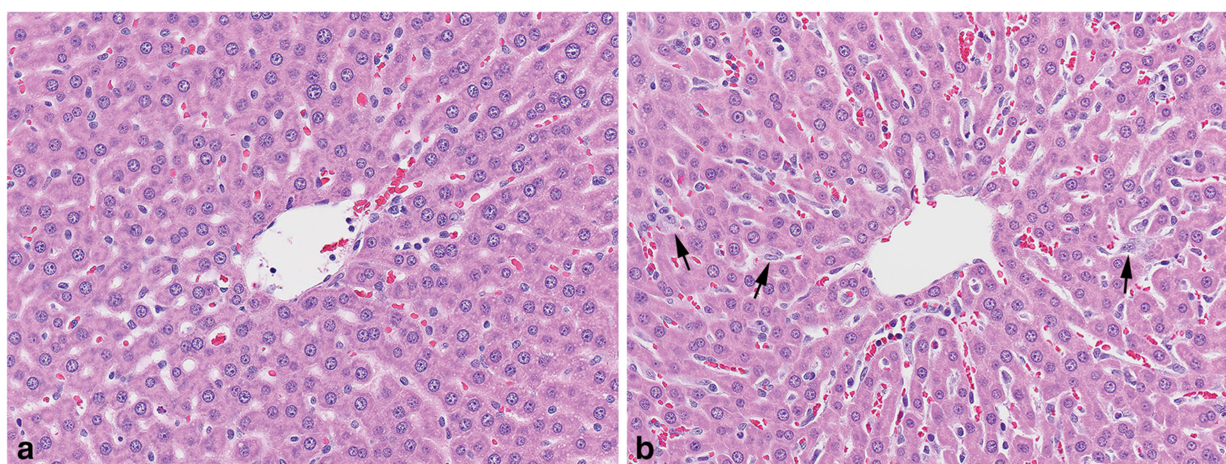

Fig. 8 Liver from female rats administered vehicle (**a**), or 50 mg/kg of PF-06438179 (**b**). Mild sinusoidal cell hyperplasia in the liver from the PF-06438179-dosed rat (several sinusoidal cells shown by arrows). A central vein is in the middle of each photomicrograph. ×20 objective

Table 4 Group incidences of test article-related microscopic findings

	Male			Female		
	Dose (mg/kg)			Dose (mg/kg)		
	0	10	50	0	10	50
Liver[a]	10	10	10	10	10	10
Hyperplasia: sinusoidal cell	NP	NP	9	NP	NP	9
Minimal (Grade 1)	NP	NP	8	NP	NP	6
Mild (Grade 2)	NP	NP	1	NP	NP	3

NP finding not present
[a] Number examined

Table 5 Toxicokinetic parameters of PF-06438179 following once-weekly intravenous administration of PF-06438179

Dose (mg/kg/dose)[a,b]	Study day	C_{max} (μg/mL)[c]	AUC_{168} (μg h/mL)	AUC_{168}/Dose (μg h/mL/mg/kg)
10	1	214 ± 13.8	15,600 ± 882	1560 ± 88.2
	8	304 ± 19.6	25,900 ± 1200	2590 ± 120
50	1	993 ± 107	72,300 ± 7420	1450 ± 147
	8	1360 ± 111	115,000 ± 12500	2310 ± 252

All values are mean ± standard deviation

AUC_{168} area under the concentration–time curve from time 0 to 168 h, C_{max} maximum drug concentration, T_{max} time to reach maximum serum concentration

[a] Animals were dosed on days 1 and 8

[b] Four animals per sex/dose group

[c] Mean T_{max} was observed at 1.0 h in all groups

DISCUSSION

In vitro analytical characterization, of which only a small subset is presented in this study, demonstrated that the proposed biosimilar, PF-06438179, is structurally and functionally similar to its reference products, infliximab-US and infliximab-EU, and that the reference products are similar to each other. Peptide mapping and subunit analyses confirmed that PF-06438179 has the identical primary structure as that of the reference product infliximab. Peptide mapping, subunit analysis, and iCE focusing confirmed that PF-06438179 has post-translational modifications similar to those of the reference products, with the exception of the levels of C-terminal lysine. This is a minor difference that is not expected to be clinically significant because the C-terminal lysine is rapidly processed in vivo with a half-life of 62 min [10, 11]. Size exclusion HPLC confirmed PF-06438179 has a product profile and a purity similar to those of the reference products. In the biological activity assay, PF-06438179 bound TNF, thus inhibiting TNF binding to its receptors on U937 cells and subsequently preventing TNF-related cell apoptosis. PF-06438179 had a similar dose response curve to that observed with infliximab-US and infliximab-EU. This side-by-side analytical characterization indicates that key structure/function attributes of reference product infliximab were successfully designed into PF-06438179 and provides strong support that PF-06438179 is analytically similar to infliximab-US and infliximab-EU. The analytical characterization also demonstrates that infliximab-EU is similar to infliximab-US.

Based on this similarity, only a limited in vivo nonclinical testing program was considered necessary. This strategy was consistent with global biosimilar guidance documents [2, 3, 12, 13] and shaped by interactions with the EMA (EU), FDA (US), and Pharmaceuticals and Medical Devices Agency (Japan). The nonclinical in vivo program initially conducted consisted of a single-dose IV TK/tolerability study in male rats to compare the similarity of PF-06438179 versus infliximab-EU. The plan to conduct only this single-dose study in male rats was based on several factors:

1. Infliximab is not pharmacologically active in typical nonclinical test species [7]. The only animal species demonstrating significant pharmacologic activity is chimpanzees. However, the use of chimpanzees is not considered ethically acceptable [14], and thus, a study in chimpanzees was not appropriate.
2. Conducting toxicity studies in pharmacologically nonrelevant species is generally discouraged as they can be misleading [15], and thus, repeat-dose toxicity studies in standard nonclinical species were not considered necessary.
3. No significant infliximab-related findings were reported in a number of studies with various species conducted by the originator, including in chimpanzees as well as in a variety of pharmacologically-nonrelevant species. Thus, conducting toxicity studies with PF-06438179 (± infliximab-US or infliximab-EU) would not be expected to demonstrate any findings, and such studies were not considered necessary.
4. Although the infliximab-CDR does not bind TNF from animal species other than chimpanzees, the neonatal Fc receptor (FcRn) from animal species including rats [16] does recognize the Fc fragment of human immunoglobulin (Ig) G, and thus, it is possible to characterize FcRn-mediated/nontarget-related clearance and TK and assess Fc-related functionality in animals, including rodents.
5. Infliximab clearance in humans decreases with increasing infliximab dose, likely related to saturation of target-mediated drug disposition (TMDD), and exposure appears to be linear at doses of 3–20 mg/kg [5], which is within the range of the approved/therapeutic dose of 3–10 mg/kg. Thus, nontarget-mediated mechanisms (i.e., FcRn recycling) play a more significant role in the overall pharmacokinetics (PK) profile of infliximab in humans relative to TMDD, and therefore, PK/TK studies in animal species that lack TMDD are relevant with respect to the human PK of infliximab at these or higher doses.
6. The use of rats (versus mice) enabled serial blood sampling for the evaluation of exposure and ADA response in individual animals over time.
7. Safety and exposure information was publically available from studies conducted previously with infliximab in rats.
8. Only one sex was considered necessary because no sex differences in TK were observed with the originator infliximab in rats [17]. In addition, there were no major weight-, age-, or sex-related differences in clearance or volume of distribution of infliximab in patients [5].
9. The use of only the EU-approved product (infliximab-EU) as the comparator in the TK/tolerability study was considered reasonable because the in vitro assays demonstrated that infliximab-licensed products sourced from the US or EU were similar to each other.
10. Overall, this scientifically based approach minimized animal use significantly, compared with a standard toxicology program.

Like the originator infliximab (Remicade) studies, there was no PF-06438179- or infliximab-EU-related toxicity observed during the single-dose study. The TK profile, systemic exposures, and ADA response of PF-06438179 were similar to those of infliximab-EU. Based on

these results, the tolerability, TK, and ADA responses of PF-06438179 were considered similar to those of infliximab-EU when administered as a single IV bolus dose to male rats at doses of 10 or 50 mg/kg. It should be noted that the absence or presence of ADA nonclinically is not predictive of responses in humans, and because immunogenicity is a key attribute for establishing biosimilarity between the proposed biosimilar and the reference product, it has been/is being monitored in completed and ongoing clinical studies with PF 06438179.

The strategy of conducting a single-dose TK and tolerability study in male rats was successful in allowing initiation of comparative clinical (phase I) trials in humans in the US, and is consistent with recent publications, suggesting that the requirement for nonclinical in vivo testing of biosimilars shifts from being a default requirement to being necessary only under selected situations where there is residual doubt regarding similarity that remains following in vitro testing [13, 18–20]. However, because pharmaceutical companies are seeking to develop biosimilar drug products globally, the overall program must meet the requirements of all countries where trials and registration are planned. During additional regulatory interactions, a 2-week toxicity study with PF-06438179 in both sexes was requested before clinical trials could proceed in one country/region. Based on this request, a 2-week IV toxicity study with PF-06438179 in male and female rats was conducted.

In the 2-week toxicity study in rats, once-weekly IV (bolus) administration of 10 or 50 mg/kg doses of PF-06438179 produced no adverse findings in any parameters evaluated. PF-06438179-related microscopic findings were limited to minimal to mild sinusoidal cell hyperplasia in the liver in both males and females administered 50 mg/kg. This finding was not adverse because of the minimal to mild severity and lack of other effects on the liver. The sinusoidal cell hyperplasia observed in this study was consistent with Kupffer cell hyperplasia observed in previous studies in rats following IV administration of Remicade at either single daily doses ≥10 mg/kg, or at seven daily doses ≥10 mg/kg/day [8], or the EU-approved biosimilar of Remicade (Remsima®, Celltrion Healthcare Co., Ltd.) at a dose of up to 40 mg/kg once weekly for 2 weeks [21]. This finding could have potentially resulted from the response of a normal rat reticuloendothelial system to large doses of chimeric (human–mouse) antibody, a foreign protein to this test animal [8], and therefore, it was not considered relevant to humans administered PF-06438179 or infliximab. Anti-drug antibodies were not evaluated in the 2-week study because there was no evidence of an ADA response in the single-dose study, and a lack of impact on serum concentrations of the study drug(s) in either study. Other test article-related differences in platelet counts, neutrophils, monocytes, large unstained cell counts, and fibrinogen were nonadverse, based on their small magnitudes and the lack of any correlative microscopic findings, such as inflammation in any organs. Minor hematologic findings (e.g., increases in reticulocytes and platelets at 50 mg/kg) have also been reported following administration of Remicade or Remsima to rats [17].

CONCLUSIONS

Overall, results with PF-06438179 demonstrate similarity to infliximab-US and infliximab-EU based on the in vitro analytical characterization evaluating protein structure, biological activity,

and in vivo studies evaluating tolerability, systemic exposure, and ADA responses. The test article-related clinical pathology findings observed with PF-06438179 were considered nonadverse due to their small magnitude and lack of any correlative microscopic effect. The microscopic findings observed in the study with PF-06438179 were also nonadverse and similar to microscopic findings reported in rats given Remicade and Remsima [8, 21]. The nonclinical in vivo data, when combined with the similarity demonstrated by the in vitro characterization, adequately supported the investigation of PF-06438179 as a potential biosimilar to Remicade in global comparative clinical trials.

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Compliance with Ethics Guidelines. In vivo animal studies were conducted in accordance with Good Laboratory Practice for Nonclinical Laboratory Studies regulations as set forth in the Code of Federal Regulations (21 CFR Part 58) and in accordance with current guidelines for animal welfare from the National Research Council for the Care and Use of Laboratory Animals. The procedures used in these studies were reviewed and approved by the Institutional Animal Care and Use Committee.

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