Preclinical to Clinical Translation of Tofacitinib, a Janus Kinase Inhibitor, in Rheumatoid Arthritis

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ABBREVIATIONS: AUC, area under the curve; AUC_d, AUC_drug; AUC_v, AUC_vehicle; AUC_50, AUC at which 50% of the maximum efficacy is achieved; BID, twice daily; C_ave, steady-state plasma concentration; C_ave_50, C_ave values achieving 50% of the maximal response; CCL, chemokine (C-C motif) ligand; CI, confidence interval; C_max, maximum plasma concentration; C_min, minimum plasma concentration; CRP, C-reactive protein; CXCL, chemokine (C-X-C motif) ligand; DAS, Disease Activity Score; DPBS, Dulbecco’s phosphate buffered saline; ED_50, dose achieving 50% of the maximal response; E_max,
maximal effect; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage colony-stimulating factor; IC₅₀, half-maximal inhibitory concentration; IFN, interferon; IFN-γ, interferon gamma; IL, interleukin; IP-10, interferon gamma-inducible protein 10; JAK, Janus kinase; LC/MS/MS, liquid chromatography tandem mass spectrometry; mCIA, murine collagen-induced arthritis; MCP-5, monocyte chemotactic protein 5; MIG, monokine induced by gamma interferon; MIP, macrophage inflammatory protein; mWB, mouse whole blood; PE, phycoerythrin; PK/PD, pharmacokinetic/pharmacodynamic; PO, per os; QD, once daily; RA, rheumatoid arthritis; SC, subcutaneous; STAT, signal transducers and activators of transcription; Th, T helper; Tyk2, tyrosine kinase 2.

Section: Drug Discovery and Translational Medicine
ABSTRACT

A critical piece in the translation of preclinical studies to clinical trials is the determination of dosing regimens which allow maximum therapeutic benefit with minimum toxicity. The preclinical pharmacokinetic/pharmacodynamic (PK/PD) profile of tofacitinib, an oral Janus kinase (JAK) inhibitor, in a mouse collagen-induced arthritis (mCIA) model was compared with clinical PK/PD data from patients with rheumatoid arthritis (RA). Preclinical evaluations included target modulation and PK/PD modeling based on continuous subcutaneous infusion or oral once- or twice daily (BID) dosing paradigms in mice. The human PK/PD profile was obtained from pooled data of four Phase 2 studies in patients with RA, and maximal effect models were used to evaluate efficacy after 12 weeks of tofacitinib treatment (1–15 mg BID). In mCIA, the main driver of efficacy was inhibition of cytokine receptor signaling mediated by JAK1 heterodimers, but not JAK2 homodimers, and continuous daily inhibition was not required to maintain efficacy. Projected efficacy could be predicted from total daily exposure irrespective of the oral dosing paradigm, with a total steady-state plasma concentration achieving 50% of the maximal response ($C_{ave50}$) of about 100 nM. Tofacitinib potency (ED$_{50}$) in clinical studies was approximately 3.5 mg BID (90% confidence interval: 2.3, 5.5) or total $C_{ave50}$ of about 40 nM, derived using disease activity scores from patients with RA. The collective clinical and preclinical data indicated the importance of $C_{ave}$ as a driver of efficacy, rather than maximum or minimum plasma concentration ($C_{max}$ or $C_{min}$), where $C_{ave50}$ values were within approximately 2-fold of each other.
Introduction

Cytokines are active mediators of innate and adaptive immunity and are involved in the development and homeostasis of hematopoietic cells. Type 1 and Type 2 cytokine receptors lack intrinsic enzymatic activity but instead utilize the Janus kinase (JAK) family of non-receptor tyrosine kinases to mediate downstream signaling following receptor-ligand binding (O'Shea, 1997; O'Shea, 2004).

JAKs constitutively associate with the intracellular domains of cytokine receptor chains and become activated upon receptor-ligand binding. Activated JAKs phosphorylate specific tyrosine residues on the cytokine receptor which then serve as docking sites for Src homology 2 domain-containing signal transducers and activators of transcription (STATs) – a family of cytoplasmic transcription factors – that are also tyrosine phosphorylated by JAKs (Shuai and Liu, 2003; Murray, 2007; Ghoreschi et al., 2009). STAT phosphorylation leads to their dimerization, nuclear translocation, and modulation of inflammatory gene transcription.

The JAK family comprises four members – JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2) – and cytokine receptors may utilize various JAK combinations to transmit cellular signals (Murray, 2007). Studies in knock-out mice have shown that deletion of these kinases can result in embryonic lethality, immunodeficiency, or impaired immune cell signaling and development (Shuai and Liu, 2003; Ghoreschi et al., 2009).

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation and joint destruction. Inflammatory cytokines are involved in the pathogenesis of RA, and JAK-STAT signaling plays an important role (Ivashkiv and Hu, 2003; Walker and Smith, 2005), as highlighted by constitutive STAT3 DNA-binding activity observed in RA synovial fluid (Wang et al., 1995). Patients with RA display one of two subsets of STAT-dependent
gene expression: one group consistent with inflammation and active immunity, and a second
group expressing genes important in tissue remodeling and a profile similar to that of
osteoarthritic tissues (Ivashkiv and Hu, 2003). Although the regulation of STAT activation is
complex, JAKs play an integral role in this process; therefore, selective inhibitors of JAK
have potential as treatments for RA.

Tofacitinib (CP-690,550) is a potent and selective JAK inhibitor exhibiting functional
selectivity for signaling pathways mediated by JAK1 heterodimers (JAK1/3, JAK1/2,
JAK1/Tyk 2) over those of JAK2 homodimers (JAK2/2) in cells (Meyer et al., 2010). Studies
in animal models of arthritis have indicated that JAK inhibition reduces signs and histological
manifestations of arthritis (Milici et al., 2006; Milici et al., 2008; Lin et al., 2010; Fridman et
al., 2010). More specifically, tofacitinib treatment resulted in dose-dependent reduction in
joint inflammation in mouse and rat arthritis models (Milici et al., 2008; Meyer et al., 2010;
Ghoreschi et al., 2011; LaBranche et al., 2012). JAK inhibition by tofacitinib blocks signaling
through the common gamma chain-containing receptors for several cytokines, including
interleukin (IL)-2, -4, -7, -9, -15, and -21, and through receptors for cytokines such as IL-6,
interferon gamma (IFN-γ), and type 1 IFN (Meyer et al., 2010; Ghoreschi et al., 2011).

Tofacitinib was recently approved for the treatment of moderate to severe RA and is currently
in clinical development as a targeted immunomodulator for psoriasis, and inflammatory
bowel disease. In Phase 3 trials in patients with RA, tofacitinib demonstrated clinical efficacy
for signs and symptoms of RA, and improvements in physical function and disease activity
(van Vollenhoven et al., 2012; Fleischmann et al., 2012; Lee et al., 2012; Burmester et al.,
2013; van der Heijde et al., 2013; Kremer et al., 2013).

Understanding the preclinical to clinical pharmacokinetic (PK)/pharmacodynamic (PD)
translation of arthritis models provides insight into mechanisms of efficacy and optimal
dosing paradigms. This study aimed to provide an understanding of the PK/PD profile of tofacitinib in a murine arthritis model, and to compare this to the PK/PD profile in patients with RA.
Methods

The Pfizer Institutional Animal Care and Use Committee reviewed and approved the animal use in these studies. The Association for Assessment and Accreditation of Laboratory Animal Care International fully accredits the Pfizer animal care and use program.

Mouse JAK Target Modulation Study

Male DBA/1 mice (Harlan Sprague Dawley, Indianapolis, IN; or Dublin, VA) were left untreated, or administered either vehicle or a single oral (per os; PO) dose of tofacitinib 50 mg/kg and whole blood samples were collected in sodium heparin over various time points from 1 to 12 hours. To assess JAK1 heterodimer signaling, cells in whole blood were surface-stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 and phycoerythrin (PE)-conjugated anti-CD3 antibodies (BD Biosciences, Franklin Lakes, NJ); and stimulated with 100 ng/mL of either recombinant human IL-6 or mouse IL-15 (R&D Systems, Minneapolis, MN) or Dulbecco’s phosphate buffered saline (DPBS) for 20 minutes at 37ºC to assess JAK1 heterodimer signaling. Alternatively, cells in whole blood were surface-stained for monocyte markers using FITC-conjugated anti-CD11b (BD Biosciences) and PE-conjugated anti-F4/80 (eBiosciences, San Diego, CA) antibodies, and stimulated with 20 ng/mL recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems) or DPBS for 15 minutes at 37ºC to assess JAK2 homodimer signaling.

Following stimulation, red blood cells were lysed and the remaining cells fixed using Lyse/Fix Buffer (BD Biosciences). Cells were washed, permeabilized on ice in Perm Buffer III (BD Biosciences), and stained intracellularly for 30 minutes with AlexaFluor647-conjugated antibodies recognizing either phosphorylated STAT5 or STAT1 (pSTAT5 or pSTAT1; BD Biosciences).
Stained cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences), and list mode data were analyzed using FlowJo software (Tree Star; Ashland, OR). To identify CD8+ T cells, lymphocytes were selected via forward and side scatter, and the CD3+ CD8+ T cell population was gated. For monocytes, the CD11b+ F4/80+ cell population was gated. Vehicle-treated animals were used as an untreated control. pSTAT1 or pSTAT5 geometric mean fluorescence intensity (GMFI) was determined for each population from unstimulated and stimulated samples. Results were normalized to the response in untreated control mice as follows: % of control = [(stimulated sample GMFI – unstimulated sample GMFI) x 100]/[control stimulated GMFI – control unstimulated GMFI]. Reduced pSTAT signals were used as an indicator of JAK inhibition.

Plasma from the same animals was used to measure corresponding tofacitinib concentrations using liquid chromatography tandem mass spectrometry (LC/MS/MS).

**Murine Collagen-Induced Arthritis (mCIA)**

Collagen induced arthritis was generated following the protocol described by (Milici et al., 2008) with minor modifications as follows. Ten- to 12-week-old male DBA/1 mice were immunized intradermally (Day -21) at the base of the tail with 50 μg chicken type-II collagen (Western Institute for Biomedical Research, Salt Lake City, UT) emulsified in Complete Freund Adjuvant (Sigma Aldrich, St. Louis, MO). The mice were boosted intradermally again on Day 0 with 50 μg chicken type-II collagen in Incomplete Freund’s adjuvant (Sigma Aldrich).

Mice were evaluated for signs of arthritis on Day 21, 28, and 35, as described previously (Milici et al., 2008). To assess arthritis, all four paws on each mouse were examined and scored for severity. The mice were scored in a blinded manner (0-12) for signs of arthritis in each paw according to the following scale: 0 = no swelling or redness/normal paw; 1 =
swelling and/or redness in one digit; 2 = swelling and/or redness in two or more digits; and 3 = entire paw is swollen or red. The severity score was reported as the sum of all four paws for each mouse and severity was expressed as the average severity score for each group. The area under the curve (AUC) for each individual animal’s severity score time course in a group was calculated and average to obtain the group mean AUC. Plasma samples were collected at various time points for drug exposure and measured using LC/MS/MS.

Subcutaneous (SC) Dosing

In the mCIA studies, dosing was initiated prior to any visible signs of arthritis. Mini Alzet osmotic pumps loaded with vehicle (33% dimethyl sulfoxide in polyethylene glycol 300) or tofacitinib at doses of 1.5, 5, or 15 mg/kg/day were implanted subcutaneously on Day 1 and replaced on Day 19; mice received a total of 36 days’ infusion.

Oral Dosing

Compounds were prepared in the vehicle – 0.5% methylcellulose/0.025% Tween-20 – and sonicated to create a very fine aqueous suspension. Tofacitinib was administered by oral gavage (0.2 mL volume) either once daily (QD) or twice daily (BID) at doses from 1 to 100 mg/kg. A vehicle control was also administered for both BID and QD studies. A group of healthy mice (non-arthritic) were entered into each study and received tofacitinib 1 to 100 mg/kg QD or BID, or vehicle. Dosing of all mice was initiated on Day 1 before any visible signs of arthritis and continued until Day 35. Both BID and QD studies were repeated under the same conditions.

Preclinical PK/PD Modeling

Raw data from tofacitinib studies in the mCIA model were used in this assessment. PD area under the curve (AUC) values (severity score x day) were assessed by subtracting the mean
AUC for each treatment group from their respective mean vehicle control AUC. These values were normalized with the vehicle control AUC to yield a fractional PD AUC.

For SC pump studies, the mean fractional PD AUC values were plotted against their mean PK steady-state plasma concentration (C_{ave}) values and modeled with a best-fit maximal effect (E_{max}) non-linear regression model with a maximal PD AUC constraint of 1 (GraphPad Prism® v. 5.01). C_{ave} values achieving 50% of the maximal response (C_{ave50}), 95% confidence intervals (CIs), and r-squared values were determined.

For BID and QD PO studies, plasma profiles were modeled from sparse sampling using one-compartment PK, from which AUC values from time 0 to 24 hours were determined. The mean fractional PD AUC values were plotted against their mean PK AUC values, and modeled with a best-fit E_{max} nonlinear regression model with a maximal PD AUC constraint of 1 (GraphPad Prism® v. 5.01). AUC at which 50% of the maximum efficacy was achieved (AUC_{50}), 95% CIs, and r-squared values were determined. The AUC_{50} was converted back into its corresponding dose achieving 50% of the maximal response (ED_{50}), C_{ave}, maximum plasma concentration (C_{max}), and minimum plasma concentration (C_{min}) values.

**Plasma Biomarker PK/PD**

Arthritis was induced in male DBA/1 mice as described previously. On Day 21, mice were evaluated for signs of arthritis, and symptomatic mice were randomly sorted to ensure equal severity across all groups prior to treatment. Mice received a single dose of vehicle or tofacitinib at 10 or 50 mg/kg on Day 22. Subsets of six to seven mice were terminally bled 4, 12, 24, and 48 hours post-dose. Healthy mice (non-arthritic) were included in the study to provide baseline readouts and were administered vehicle only.
Plasma cytokine levels were detected using murine LincoPlex assays (Millipore Corp, St. Charles, MO) and Luminex 200 instrumentation (Luminex Corp. Austin, TX). Plasma drug levels were assessed using LC/MS/MS.

**Clinical Exposure-Response Relationships**

Efficacy data from four double-blind, Phase 2, dose-ranging studies (A3921025 [NCT00413660], A3921035 [NCT00550446], A3921039 [NCT00603512], and A3921040 [NCT00687193]) were pooled. All studies were performed in accordance with the Declaration of Helsinki. These four studies evaluated tofacitinib 1–15 mg BID and placebo in patients with RA who had an inadequate response to other disease-modifying antirheumatic drugs. Efficacy data, measured by Disease Activity Score (28 joints, 3 components, C-reactive protein [DAS28-3(CRP)]) after 12 weeks of treatment were described using an E_{max} model (Suzuki et al., 2012).
Results

Mouse JAK Target Modulation Study

Mice were administered a single oral dose of tofacitinib and whole blood was sampled at varying time points for JAK target modulation and tofacitinib exposure. Inhibition of cytokine induced STAT signaling was used to assess JAK modulation by tofacitinib. Values were normalized to an untreated control group. The relationship between tofacitinib exposure and JAK target modulation in mice is shown in Fig. 1. Inhibition of cytokine receptor signaling directly correlated with tofacitinib plasma concentration, where inhibition of both JAK1/3/pSTAT5-mediated IL-15 signaling and JAK1/2/Tyk2/pSTAT1-mediated IL-6 signaling in CD8+ T cells was more pronounced and longer lasting than that of JAK2/2/pSTAT5-mediated GM-CSF signaling in monocytes. These results are consistent with the greater selectivity of the inhibitor for JAK1-containing heterodimers than for JAK2 homodimers. The profile in Fig. 1 also illustrates the rapid reversibility of JAK inhibition as tofacitinib plasma concentration declines with time.

Mouse CIA Efficacy Studies

Mouse CIA Summary

The collective summary of tofacitinib efficacy in mCIA following administration by SC pump, BID PO, and QD PO is summarized in Table 1.

SC Pump Studies

Disease severity score by study day was substantially reduced by SC administration of tofacitinib compared with vehicle (Fig. 2A). The exposure-response relationship of tofacitinib in mCIA following SC administration is shown in Fig. 2B, where the total and unbound $C_{ave50}$ was determined to be 44 nM (95% CI: 12, 76 nM) and 29 nM (95% CI: 8, 51 nM),
respectively. Plasma tofacitinib concentrations were in the region of that previously reported to achieve half-maximal inhibitory concentration (IC$_{50}$) of JAK1/3 signaling (whole blood IL-15 IC$_{50}$ 42 ± 12 nM; (Meyer et al., 2010)), indicating that tofacitinib efficacy was primarily achieved through inhibition of JAK1 heterodimers (Fig. 2C). Moreover, the previously reported tofacitinib concentration required to inhibit JAK2 homodimer signaling (whole blood GM-CSF IC$_{50}$ 4379 ± 655 nM; (Meyer et al., 2010)) was much higher than those observed in the present study, suggesting that JAK2 was not the primary mechanism of tofacitinib efficacy (Fig. 2C).

**Oral QD and BID Studies**

In contrast to SC pump studies, which highlighted the importance of inhibiting JAK1 heterodimer signaling, PO QD and BID dosing regimens were utilized to explore the relationship between JAK inhibition and efficacy, as well as the PK flexibility of target inhibition throughout the day. For BID dosing, severity scores by study day were reduced compared to vehicle by tofacitinib doses $\geq$ 3 mg/kg (Fig. 3A). The relationship between daily exposure (24-hour AUC) and efficacy ([AUC$_{vehicle}$-AUC$_{drug}$/AUC$_{vehicle}$]) is shown in Fig. 3B. The BID total and unbound $C_{ave}$ at the AUC$_{50}$ ($C_{ave50}$) was determined to be 90 nM (95% CI: 19, 160 nM) and 60 nM (95% CI: 13, 107 nM), respectively, consistent with previously reported in vitro whole blood JAK1 heterodimer IC$_{50}$ (Meyer et al., 2010). Efficacy was achieved via inhibition of heterodimeric JAK1, but not homodimeric JAK2 signaling (Fig. 3C), which corresponded with SC pump results (Fig. 2C). The profile of JAK1 inhibition varied widely over the course of the day, as indicated by the fluctuation of the plasma drug concentration. Tofacitinib plasma concentrations achieved mouse whole blood JAK1 heterodimer IC$_{50}$ coverage for about 12 hours at the ED$_{50}$, over a period of 24 hours; the total $C_{max}$ and $C_{min}$ values at the ED$_{50}$ (6 mg/kg) were 326 nM (unbound $C_{max}$ 218 nM) and 7 nM (unbound $C_{min}$ 5 nM), respectively (Fig. 3C).
The corresponding results for QD dosing are shown in Figs. 3D, 3E, and 3F. Severity scores during QD dosing (Fig. 3D) appeared more variable than those for BID dosing (Fig. 3A), and maximal efficacy was not achieved (Fig. 3E). The QD total and unbound $C_{\text{ave}50}$ was determined to be 128 nM (95% CI: 0, 295 nM) and 86 nM (95% CI: 0, 198 nM), respectively (Fig. 3F), which was consistent with the BID dosing regimen. However, $C_{\text{max}}$ and $C_{\text{min}}$ at the QD ED$_{50}$ (33.5 mg/kg) were 1140 nM (unbound $C_{\text{max}}$ 764 nM) and 0.03 nM (unbound $C_{\text{min}}$ 0.02 nM), respectively, which was greater than the $C_{\text{max}}$ and less than the $C_{\text{min}}$ of the BID ED$_{50}$. Over the 24-hour period, there was JAK1 heterodimer IC$_{50}$ coverage for about 8.5 hours at the ED$_{50}$ during the QD dosing schedule (Fig. 3F).

BID and QD studies were each repeated in separate experiments and results were consistent with the data discussed above (see Table 1). The exposure-response relationships for each of these studies overlapped with each other (Fig. 3G); yielding a combined $C_{\text{ave}50}$ value, which was similar to that of individual studies (Table 1).

**Mouse Plasma Biomarker PK/PD**

To understand the impact of drug concentration on various upstream and downstream inflammatory markers over time, CIA mice were treated with single oral doses of tofacitinib (10 and 50 mg/kg), and plasma biomarkers were examined for 48 hours (Fig. 4). While single doses of tofacitinib had no effect on disease severity, rapid reductions (within 12 hours) in plasma levels of disease-related inflammatory mediators were observed (Fig. 4). IL-6 (Fig. 4A) and KC/Chemokine (C-X-C motif) ligand 1 (CXCL1) (Fig. 4B) were significantly reduced within 4 hours of treatment with either tofacitinib dose and both proteins recovered to normal levels by 12 hours, in line with the short half-life of the drug (Fig. 4C).
Sustained reductions of T cell chemo-attractants, such as IFN-γ-inducible protein 10 (IP-10)/CXCL10 (Fig. 4D) and monokine induced by gamma interferon (MIG)/CXCL9 (Fig. 4E), were observed at 4 hours and 12 hours post-dose in mice treated with the 50 mg/kg dose, and the effect appeared to be diminished in mice treated with the lower dose. Monocyte chemotactic protein 5 (MCP-5)/chemokine (C-C motif) ligand 12 (CCL12), a lymphocyte and monocyte chemo-attractant produced by activated macrophages, was significantly reduced for at least 12 hours with both tofacitinib doses (Fig. 4F). Other monocyte chemotactic factors known to be elevated in mCIA, such as macrophage inflammatory protein (MIP)-1α/CCL3 and MIP-1β/CCL4, were not affected by single-dose tofacitinib treatment in this study (data not shown).

Clinical Exposure-Response Relationships

Collective non-compartmental PK modeling of available clinical data demonstrated that tofacitinib exhibited linear PK (Fig. 5). The clinical PK of tofacitinib displayed dose proportionality in various patient populations with a PO half-life of approximately 2–3 hours. JAK1 heterodimer IC₅₀ coverage, as determined previously (Meyer et al., 2010), was not continuous throughout the day, and there was little or no JAK2 homodimer IC₅₀ coverage. The JAK1 heterodimer and JAK2 homodimer IC₅₀ coverage for various clinical doses of tofacitinib are summarized in Table 2.

Dose-response profiles for mean DAS28-3(CRP) at Week 12 are shown in Fig. 6; all four studies showed dose-dependent responses in DAS28-3(CRP). Study-specific differences in placebo response and Eₘₐₓ were noted, while the potency (ED₅₀) of tofacitinib was similar across the four studies. The ED₅₀ estimate was 3.5 mg BID (90% CI: 2.3, 5.5) for mean DAS28-3(CRP) at Week 12, which corresponded to a total and unbound AUC of
approximately 1130 and 692 nM*hour, respectively. Incorporation of individual specific PK parameters (C_{max}, AUC, and C_{min}) did not provide further improvements to the model.
Discussion

The current studies have provided insight into the PK/PD profile of tofacitinib in preclinical models of arthritis and how that compares with recent clinical findings. Understanding the translation from preclinical to clinical studies has guided the determination of effective dosing regimens, and provided better understanding of efficacy mechanisms in RA.

The in vitro whole blood JAK potency profile previously described in mCIA models highlighted the importance of inhibition of JAK1 heterodimer signaling, but not JAK2 homodimer inhibition, for driving tofacitinib efficacy (Ghoreschi et al., 2011). In the current studies the relationship between tofacitinib plasma concentration and JAK1 heterodimer signaling was consistent with greater selectivity for JAK1-containing heterodimers than for JAK2 homodimers, and demonstrated the rapid reversibility of JAK inhibition as tofacitinib plasma concentration declines with time.

Tofacitinib efficacy following BID and QD oral administration was achieved with varying daily coverage of the JAK1 heterodimer IC50. The Cave values were comparable with both BID and QD dosing, whereas Cmax and Cmin at the QD ED50 were very different from the Cmax and Cmin of the BID ED50. These results suggest the importance of Cave as the driver of tofacitinib efficacy (independent of dosing paradigm) and a lack of dependence on Cmax or Cmin. Furthermore, over 24 hours, plasma concentrations of tofacitinib were at or above the mouse whole blood JAK1 heterodimer IC50 for about 8 and 12 hours at the QD and BID ED50, respectively, emphasizing that full inhibition of JAK1 heterodimer signaling was not necessary for efficacy.

It should be noted that while time above the JAK heterodimer IC50 threshold is an appropriate method to assess efficacy within a defined dosing paradigm (BID or QD individually), these time periods are not equivalent between BID and QD dosing paradigms (Fig. 3C versus 3F).
For example, the time period when efficacy levels with the BID paradigm were above the JAK1 heterodimer IC₅₀ threshold were longer compared with the time period for similar levels of efficacy with QD dosing.

Using data from the murine models of inflammation reported here, QD dosing would be predicted to be equivalent in clinical efficacy to BID dosing at similar daily $C_{ave}$ values. The slower onset of efficacy relative to the daily fluctuations in PK plasma concentrations for tofacitinib suggests an indirect response relationship between drug PK and effects on arthritic tissues. Indeed, amelioration of disease does not appear to require continual JAK1 heterodimer inhibition and allows for short periods of drug absence. Single-dose tofacitinib data in mCIA demonstrated immediate effects of tofacitinib on plasma inflammatory mediators and in some cases sustained impact on chemotactic proteins, without an immediate bearing on the disease severity index. Rapid inhibition of IL-6, IP-10 and other inflammatory mediators has previously been demonstrated in mCIA and rat adjuvant-induced arthritis, and likely represents blockade of innate immune mechanisms (Ghoreschi et al., 2011; LaBranche et al., 2012). Sustained effects on innate immunity following tofacitinib administration would be consistent with a mechanism of prolonged modulation of transcriptional activity downstream of JAK signaling. Other mechanistic studies have indicated that tofacitinib inhibition of cytokine receptor signaling in T cells can ultimately lead to suppression of IL-12 or IFN-γ-dependent STAT1 activation during T helper 1 (Th1) cell differentiation, as well as reduced production of inflammatory Th17 cells generated in response to IL-6 and IL-23 (Ghoreschi et al., 2011). Additionally, tofacitinib has been shown to suppress the acute tumor necrosis factor response and enhance IL-10 levels following lipopolysaccharide administration in mice, consistent with JAK1-dependent inhibition of IFN-γ and STAT1 signaling (Ghoreschi et al., 2011). These effects were associated with improvement in severity scores in murine arthritis and reduced expression of STAT1-dependent genes.
Therefore, simultaneous control of signaling pathways involved in innate and adaptive immune responses by tofacitinib may explain the improvements observed in mCIA models and the clinical improvement reported in patients with RA treated with tofacitinib.

In our analysis, the human PK/PD profile was very similar to that of the mouse arthritis model in that efficacy was primarily driven via inhibition of JAK1 heterodimer signaling, with little to no inhibition of JAK2 homodimer signaling. Collectively, the preclinical and clinical data suggest that effective modulation of inflammation does not require continuous inhibition of JAK1 heterodimer signaling throughout the day. Results from preclinical experiments (collective unbound $C_{ave}$ 94 nM at ED$_{50}$) appear to translate within about 2–3 fold of results in patients with RA (unbound $C_{ave}$ approximately 40 nM at ED$_{50}$), as evidenced by the close prediction of the potency (ED$_{50}$) of tofacitinib (approximately 3.5 mg BID for DAS28-3[CRP] scores) observed in clinical trial patients with RA. Similar results were reported from the application of indirect response models to categorical efficacy data such as the American College of Rheumatology responses (Hutmacher et al., 2008). The efficacy of tofacitinib 5 and 10 mg BID doses, which provide approximately 60–80% of the maximum efficacy of tofacitinib, was confirmed in Phase 3 studies (van Vollenhoven et al., 2012; Fleischmann et al., 2012; Lee et al., 2012; Burmester et al., 2013; van der Heijde et al., 2013; Kremer et al., 2013). Additional studies are ongoing to further elucidate the cytokine profiles and the cell types involved in these arthritis models.

In summary, broad JAK1 heterodimer inhibition directly linked to tofacitinib PK is the main driver of efficacy in both preclinical murine arthritis models and clinical RA, where total drug exposure ($C_{ave}$), but not $C_{max}$ or $C_{min}$, is a predictor of projected efficacy. JAK1 heterodimer inhibition profiles vary over the day where continuous or high levels of inhibition are not required for effective RA treatment. These results demonstrate how
preclinical experiments can provide insight into efficacious dosing regimens, and may be a good indicator of clinical success for new chemical entities.

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Authorship contributions

Participated in research design: Dowty, Jesson, Ghosh, Lee, Meyer, Krishnaswami, Kishore

Conducted experiments: Dowty, Jesson, Ghosh, Lee, Meyer, Krishnaswami, Kishore

Contributed new reagents or analytical tools: Dowty, Jesson, Ghosh, Lee, Meyer, Krishnaswami, Kishore

Performed data analysis: Dowty, Jesson, Ghosh, Lee, Meyer, Krishnaswami, Kishore

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Footnotes

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**Figure legends**

**Fig. 1.** Relationship between tofacitinib pharmacokinetics and inhibition of JAK1/3 (IL-15), JAK1/2 or JAK1/Tyk2 (IL-6) and JAK2/2 (GM-CSF) signaling with time after a single dose of tofacitinib. DBA/1 mice were administered a single oral dose of vehicle or tofacitinib 50 mg/kg, and specific JAK signaling, as well as tofacitinib exposure were measured in blood for 12 hours post-dose. Values presented are the mean ± standard error of the specific cytokine stimulated response normalized as a percentage of the response in control mice, or mean ± standard error of tofacitinib plasma concentration. GM-CSF, granulocyte macrophage colony-stimulating factor; IL-15, interleukin 15; JAK, Janus kinase.

**Fig. 2.** Efficacy of tofacitinib in mCIA following SC pump administration; (A) disease severity scores with time; (B) $E_{\text{max}}$ drug exposure-inflammation response relationship; (C) relationship of drug exposure and in vitro whole blood JAK inhibition. Dotted lines represent previously reported levels of tofacitinib to achieve IC$_{50}$ of JAK2 homodimer and IC$_{50}$ of JAK1/3 heterodimer (Meyer et al., 2010). AUC, area under the curve; AUC$_{d},$ AUC$_{\text{drug}}; $AUC_v, AUC_{\text{vehicle}}; C_{\text{ave}},$ steady-state plasma concentration; $E_{\text{max}},$ maximal effect; IC$_{50},$ half-maximal inhibitory concentration; JAK, Janus kinase; mCIA, murine collagen-induced arthritis; mWB, mouse whole blood; SC, subcutaneous. *$p \leq 0.05,$ **$p \leq 0.005,$ ***$p \leq 0.0005$ using ANOVA.

**Fig. 3.** Efficacy of tofacitinib in mCIA following oral BID (A–C) and QD (D–F) administration; (A and D) disease severity scores with time; (B and E) $E_{\text{max}}$ drug exposure-inflammation response relationship; (C and F) relationship of drug exposure over time and previously reported (Meyer et al., 2010) in vitro whole blood JAK inhibition, total $C_{\text{ave}50}$ is represented by the dotted line; (G) BID and QD composite $E_{\text{max}}$ drug exposure-inflammation response relationship. AUC, area under curve; AUC$_{d},$ AUC$_{\text{drug}};$
AUC<sub>v</sub>, AUC<sub>vehicle</sub>; AUC<sub>50</sub>, AUC at which 50% of maximum efficacy achieved; BID, twice daily; C<sub>ave50</sub>, steady-state plasma concentration at the AUC<sub>50</sub>; E<sub>max</sub>, maximal effect; IC<sub>50</sub>, half-maximal inhibitory concentration; JAK, Janus kinase; mCIA, murine collagen-induced arthritis; mWB, mouse whole blood; PO, per os; QD, once daily. *<em>p</em> ≤ 0.05, **<em>p</em> ≤ 0.005, ***<em>p</em> ≤ 0.0005, #<em>p</em> = 0.745 using ANOVA.

**Fig. 4.** Effect of single doses of tofacitinib 10 and 50 mg/kg on plasma tofacitinib concentration and various plasma inflammation biomarkers in mouse CIA versus time. Normal indicates levels in healthy (non-arthritic) mice. (A) IL-6; (B) KC/CXCL1; (C) tofacitinib exposure; (D) (IP-10/CXCL10; (E) MIG/CXCL9; (F) MCP-5/CCL12. *<em>p</em> < 0.05; **<em>p</em> < 0.005. CCL12, chemokine (C-C motif) ligand 12; CIA, collagen-induced arthritis; CXCL1, chemokine (C-X-C motif) ligand 1; IL-6, interleukin 6; IP-10, interferon gamma-induced protein 10; JAK, Janus kinase; MIG, monokine induced by gamma interferon; MCP-5, monocyte chemotactic protein 5.

**Fig. 5.** Human tofacitinib PK collective, non-compartmental PK modeling of clinical data for tofacitinib in patients with RA (plasma concentration versus time). Whole blood JAK IC<sub>50</sub> ranges derived from Meyer et al (Meyer et al., 2010). BID, twice daily; IC<sub>50</sub>, half-maximal inhibitory concentration; JAK, Janus kinase; PK, pharmacokinetics; RA, rheumatoid arthritis; WB, whole blood.

**Fig. 6.** Dose-response profiles for mean DAS28-3(CRP) at Week 12 in four Phase 2 studies of tofacitinib. BID, twice daily; CRP, C-reactive protein; DAS, disease activity score.
TABLE 1
Pharmacokinetic parameters of tofacitinib in the mouse CIA model following SC osmotic pump and BID and QD PO administration

<table>
<thead>
<tr>
<th>Dosing paradigm</th>
<th>Mouse CIA efficacy parameters</th>
<th>24-hour AUC&lt;sub&gt;50&lt;/sub&gt; (nM x hour)</th>
<th>ED&lt;sub&gt;50&lt;/sub&gt; dose (mg/kg)</th>
<th>ED&lt;sub&gt;50&lt;/sub&gt; C&lt;sub&gt;max&lt;/sub&gt;/C&lt;sub&gt;min&lt;/sub&gt; (nM)</th>
<th>C&lt;sub&gt;ave&lt;/sub&gt;50 (nM)</th>
<th>95% CI</th>
<th>≥JAK1/3 IC&lt;sub&gt;50&lt;/sub&gt; Threshold&lt;sup&gt;a&lt;/sup&gt; (hours)</th>
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<tr>
<td>SC pump</td>
<td>Total</td>
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<td>NA</td>
<td>NA</td>
<td>44 (12, 76)</td>
<td>NA</td>
<td>NA</td>
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<td></td>
<td>Unbound&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>NA</td>
<td>NA</td>
<td>29 (8, 51)</td>
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<td>NA</td>
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<td>BID PO; Study 1</td>
<td>Total</td>
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<td>326/7</td>
<td>90 (19, 160)</td>
<td>12</td>
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<td>218/5</td>
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<td>BID PO; Study 2</td>
<td>Total</td>
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<td>12.8</td>
<td>394/10</td>
<td>115 (77, 151)</td>
<td>13</td>
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<td>40.5</td>
<td>2140/0.2</td>
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<td>1434/0.1</td>
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<td>BID/QD; All studies</td>
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<td>94 (52, 137)</td>
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\(^a\) JAK1/3 heterodimer human whole blood IC\(_{50}\) (IL-15-dependent pSTAT5) = 42 ± 12 nM.

\(^b\) Tofacitinib fraction unbound in mouse plasma = 67% (Pfizer data on file).

AUC\(_{50}\), area under the curve at which 50% of maximum efficacy is achieved; BID, twice daily; C\(_{ave50}\), steady-state plasma concentration achieving 50% of the maximal response; CI, confidence interval; CIA, collagen-induced arthritis; C\(_{max}\), maximum plasma concentration; C\(_{min}\), minimum plasma concentration; ED\(_{50}\), dose achieving 50% of the maximal response; IC\(_{50}\), half-maximal inhibitory concentration;
IL, interleukin; JAK, Janus kinase; NA, not applicable; PO, per os; QD, once daily; SC, subcutaneous; STAT, signal transducers and activators of transcription.
TABLE 2

Modeled human pharmacokinetic parameters of tofacitinib and estimation of JAK1 heterodimer or JAK2 homodimer IC$_{50}$ coverage. Observed clinical variation in pharmacokinetics was within about 30%.

<table>
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<tr>
<th>BID dose (mg)</th>
<th>Pharmacokinetic parameters</th>
<th>Approximate IC$_{50}$ coverage every 12 hours</th>
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<tr>
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<td>$C_{\max}$ (nM)</td>
<td>24-hour AUC (nM x hour)</td>
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<td>3.5</td>
<td>Total</td>
<td>130</td>
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<tr>
<td></td>
<td>Unbound</td>
<td>79</td>
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<tr>
<td>5</td>
<td>Total</td>
<td>186</td>
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<tr>
<td></td>
<td>Unbound</td>
<td>113</td>
</tr>
<tr>
<td>Time (min)</td>
<td>Total</td>
<td>Unbound</td>
</tr>
<tr>
<td>-----------</td>
<td>-------</td>
<td>---------</td>
</tr>
<tr>
<td>10</td>
<td>371</td>
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<td>135</td>
<td>83</td>
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<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0&lt;sup&gt;c&lt;/sup&gt;; 1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;; 2.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;; 6&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Tofacitinib fraction unbound in plasma = 61% (Pfizer data on file).

<sup>b</sup> JAK1/3 heterodimer human whole blood IC<sub>50</sub> (IL-15-dependent pSTAT5) = 56 ± 6 nM (Meyer et al., 2010).

<sup>c</sup> JAK2/2 homodimer human whole blood IC<sub>50</sub> (GM-SCF-dependent pSTAT5) = 1377 ± 185 nM (Meyer et al., 2010).

<sup>d</sup> GM-CSF stimulated myelomonocytic HUO3 cell IC<sub>50</sub> (JAK2 homodimer) = 324 nM (Changelian et al., 2003).

AUC, area under the curve; BID, twice daily; C<sub>ave</sub>, steady-state plasma concentration; C<sub>max</sub>, maximum plasma concentration; GM-CSF, granulocyte macrophage colony-stimulating factor; IC<sub>50</sub>, half-maximal inhibitory concentration; JAK, Janus kinase; IL, interleukin; STAT, signal transducers and activators of transcription.
Figure 1

- JAK1/3 (IL-15)
- JAK1/2 or JAK1/Tyk2 (IL-6)
- JAK2/2 (GM-CSF)
- Tofacitinib (μM)

% control stimulation vs. Plasma tofacitinib concentration (μM)

Hours post-dose
Figure 2

Panel A: Graph showing the severity score over study days for different doses of the drug (Vehicle, 1.5 mg/kg, 5 mg/kg, 15 mg/kg).

Panel B: Graph showing the efficacy (AUC - AUC_0) normalized by AUC_0 as a function of the average concentration (C_ave) in µM.

Panel C: Scatter plot showing plasma tofacitinib concentration (µM) on the y-axis and dose (mg/kg/day) on the x-axis. The IC_50 values for mWB, JAK2, homodimer, JAK1 and JAK3, and heterocomer are indicated.