The Pharmacokinetics, Metabolism, and Clearance Mechanisms of Tofacitinib, a Janus Kinase Inhibitor, in Humans

Martin E. Dowty, Jinyan Lin, Tim F. Ryder, Weiwei Wang, Gregory S. Walker, Alfin Vaz, Gary L. Chan, Sriram Krishnaswami, and Chandra Prakash

Department of Pharmacokinetics, Dynamics, and Metabolism, Pfizer Inc, Andover, MA, USA
(M.E.D.); Pfizer Inc, Groton, CT, USA (J.L., T.R., W.W., G.S.W., A.V., C.P.); Department of Specialty Care Clinical Affairs, Pfizer Inc, Groton, CT, USA (G.L.C.); Department of Clinical Pharmacology, Pfizer Inc, Groton, CT, USA (S.K.)
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Corresponding author:

Martin E. Dowty, Pfizer Global Research & Development, Department of Pharmacokinetics, Dynamics, and Metabolism, 1 Burtt Rd, G2002G, Andover, MA 01810, USA
Tel: +1 978 247 2845, fax: +1 978 247 2842; email: Martin.Dowty@pfizer.com

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ABBREVIATIONS: ACN, acetonitrile; ARC, Accurate Radioisotope Counting; AUC, area under the plasma concentration–time curve; AUC₀-last, AUC from time 0 to the last time; AUC₀-∞, AUC from time 0 to infinity; AUCₜ-∞, AUC from time t to infinity; CLh, hepatic metabolism; Cmax, maximal observed plasma concentration; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; HLM, human liver microsome; HMBC, heteronuclear multiple bond correlation; HPLC, high performance liquid chromatography; HSQC, multiplicity edited heteronuclear single quantum coherence; JAK, Janus kinase; ke, terminal phase rate constants; LC, liquid chromatography; LSC, liquid scintillation counting; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ND, not detected; NMR, nuclear magnetic resonance; PK, pharmacokinetic; STAT, signal transducer and activator of transcription; t½, half-life; Tₘₐₓ, time to peak concentration; TOCSY, total correlation spectrometry; TRA, total radioactivity.
Abstract:

Tofacitinib is a novel, oral Janus kinase inhibitor. The objectives of this study were to summarize the pharmacokinetics and metabolism of tofacitinib in humans, including clearance mechanisms. Following administration of a single 50 mg $^{14}$C-labeled tofacitinib dose to healthy male subjects, the mean (standard deviation) total percentage of administered radioactive dose recovered was 93.9% (±3.6), with 80.1% (±3.6) in the urine (28.8% parent) and 13.8% (±1.9) in feces (0.9% parent). Tofacitinib was rapidly absorbed, with plasma concentrations and total radioactivity peaking at around 1 h after oral administration. The mean terminal phase half-life was approximately 3.2 h for both parent drug and total radioactivity. Most (69.4%) circulating radioactivity in plasma was parent drug, with all metabolites representing less than 10% each of total circulating radioactivity. Hepatic clearance made up around 70% of total clearance, while renal clearance made up the remaining 30%. The predominant metabolic pathways of tofacitinib included oxidation of the pyrrolopyrimidine and piperidine rings, oxidation of the piperidine ring side chain, N-demethylation and glucuronidation. Cytochrome P450 (CYP) profiling indicated that tofacitinib was mainly metabolized by CYP3A4, with a smaller contribution from CYP2C19. This pharmacokinetic characterization of tofacitinib has been consistent with its clinical experience in drug–drug interaction studies.
Introduction

The Janus kinase (JAK) pathway is commonly utilized by cytokines to mediate intracellular signaling. Following receptor–ligand binding, activated JAKs phosphorylate specific tyrosine residues on the receptor that serve as docking sites for signal transducers and activators of transcription (STATs). STATs are a family of cytoplasmic transcription factors that bind to phosphotyrosine residues via their Src homology 2 domains. STAT phosphorylation leads to dimerization and translocation to the nucleus, resulting in the modulation of gene transcription, in particular those involved in the inflammatory response (O'Shea, 1997; O'Shea et al., 2002; Shuai and Liu, 2003; Murray, 2007; Schindler et al., 2007; Ghoreschi et al., 2009).

Aberrant proinflammatory cytokine production, expression, and activation have been implicated in the pathogenesis of several autoimmune diseases (O'Shea, 2004), including rheumatoid arthritis (McInnes and Schett, 2007; McInnes and Schett, 2011) and psoriasis (Nograles et al., 2010). Therefore, inhibitors of the JAK-STAT pathway have therapeutic potential as immunomodulators (O'Shea, 2004).

Tofacitinib (3-[(3R,4R)-4-methyl-3-(methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl)amino)piperidin-1-yl)-3-oxopropanenitrile) is a novel, oral JAK inhibitor (Meyer et al., 2010b) that has been approved by the US Food and Drug Administration for the treatment of adults with moderately to severely active rheumatoid arthritis. In kinase assays, tofacitinib inhibits JAK1, JAK2, JAK3 and, to a lesser extent, tyrosine kinase 2. In cellular settings where JAKs signal in pairs, tofacitinib preferentially inhibits signaling by heterodimeric receptors associated with JAK3 and/or JAK1 with functional selectivity over receptors that signal via pairs of JAK2 (Meyer et al., 2010b). Inhibition of JAK1 and JAK3 by tofacitinib
blocks signaling through the common gamma-chain-containing receptors for several cytokines, including interleukin-2, -4, -7,-9, -15, and -21. These cytokines are integral to lymphocyte activation, proliferation, and function; thus, inhibition of their signaling may result in modulation of multiple aspects of the immune response (Meyer et al., 2010a).

An understanding of the pharmacokinetics (PK) and disposition of tofacitinib is important in order to identify the drug metabolic profile; to understand the PK of total radioactivity compared to parent compound and dose recovery; and to understand mechanisms of clearance, which can then inform the potential for drug–drug interactions. The PK of tofacitinib has been investigated in healthy volunteers (Krishnaswami et al., 2008b); patients with rheumatoid arthritis (Chow et al., 2008a), psoriasis (Gupta et al., 2011a), Crohn’s disease (Sandborn et al., 2011), ulcerative colitis (Sandborn et al., 2012), and in renal transplant recipients (Krishnaswami et al., 2008a). Tofacitinib PK were shown to be dose-proportional and characterized by rapid absorption and elimination, with a time to peak concentration (T_{max}) of approximately 0.5 h (range 0.3–1.0 h) and a terminal phase half-life (t_{1/2}) of approximately 3.3 h (S.D. 0.5 h) (Lawendy et al., 2009; Krishnaswami et al., 2009).

The current study was performed to better understand the PK, metabolism, and clearance mechanisms of tofacitinib in healthy human subjects. A single 50 mg dose of 14C-tofacitinib was orally administered to six human subjects. The urine, feces, and plasma were collected and assayed for radioactivity, and profiled for metabolites. The metabolites were separated on a reverse phase high performance liquid chromatography (HPLC) system and analyzed by HPLC-mass spectrometry (MS) and HPLC-tandem mass spectrometry (HPLC/MS/MS). Where possible, the proposed structures were supported by comparisons of their HPLC retention times and MS spectra with those of synthetic standards and the structure of M9 was confirmed by nuclear magnetic resonance (NMR). Attempts were also made to determine the
cytochrome P450 (CYP) enzyme responsible for the formation of major metabolites using human liver microsomes, recombinant CYP enzymes and isoform selective CYP inhibitors.
Materials and Methods

Compounds and Reagents

$^{14}$C-tofacitinib labeled at the C-6 of the pyrrolopyrimidine ring was synthesized by the radiochemistry group at Pfizer Global Research and Development (Groton, CT, USA) (Fig. 1). For administration in humans, $^{14}$C-tofacitinib (citrate salt) had a specific activity of 1.97 $\mu$Ci/mg salt form and radio and chemical purities of >99%. For in vitro metabolism experiments, $^{14}$C-tofacitinib had a specific activity of approximately 30 mCi/mmol and radio purity of >99%. Isoform selective CYP inhibitors furafylline (vs. 1A2), sulfaphenazole (vs. 2C9), (+)N-3-benzylnirvanol (vs. 2C19), quinidine (vs. 2D6) and ketoconazole (vs. 3A) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pooled human liver microsomes (HLMs) (HL-mix 13, 17.6 mg protein/ml, 0.32 nmol CYP/mg protein) were prepared and characterized by Pfizer Global Research and Development from 52 individual human livers (31 Male/21 Female). Respective $V_{\text{max}}$ (nmol/min/mg) and $K_{\text{m}}$ ($\mu$M) values for CYP specific substrates in HL-mix 13 were: phenacetin (1A2) 1.0/46; diclofenac (2C9) 2.6/5.9; tolbutamide (2C9) 0.1/251; mephenytoin (2C19) 0.1/55; bufuralol (2D6) 0.2/7.0; and testosterone (3A4) 4.1/77. Recombinant human CYP isoforms (1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) were obtained commercially from PanVera (Madison, WI, USA) and BD Gentest (San Jose, CA, USA). Commercially obtained chemicals and solvents were of HPLC or analytical grade.

Clinical Study Design and Sample Collection

The study was conducted at PPD Pharmaco (Austin, TX, USA) in six healthy male subjects between the ages of 18 and 55 years. Following a minimum 8-h fast, subjects were given a single 50 mg base equivalent dose of $^{14}$C-tofacitinib (citrate salt; ~164 $\mu$Ci radiolabeled
tracer) in 100 ml water in the morning. Water was provided ad libitum, beginning 1 h after dosing, and a standard meal was provided 4 h after dosing.

Urine and feces were collected prior to dosing and as passed from time of dosing until 240 h post-dose. The total weight of the urine and feces was recorded after each collection. Blood samples (~14 ml) were collected in tubes containing sodium heparin from each subject pre-dose (0 h) and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h post-dose for tofacitinib and total radioactivity (TRA) assessment. Additional blood samples (~40 ml) were collected for the identification of circulating metabolites at 0.5, 1, 2, 4, 8, and 12 h post-dose. Within 1 h of collection, blood samples were centrifuged (refrigerated) to obtain plasma.

**Radiochemical Analysis**

Analyses of radioactivity and sample preparation were conducted at Pfizer Global Research and Development. Aliquots of plasma (0.5 ml) or urine (0.1 ml) for each sampling time were mixed with 6 ml Ecolite (+) scintillation cocktail and analyzed by liquid scintillation counting (LSC) using a Wallac 1409 (Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA) liquid scintillation counter. Fecal samples were homogenized and air-dried overnight prior to combustion with a Packard 307 oxidizer equipped with Oximate 80 robotics. During combustion, liberated radioactive carbon dioxide was trapped in a column filled with 10 ml carbon dioxide absorbent (i.e. CarboSorb E) forming a carbamate. This carbamate was flushed from the oxidizer with the liquid scintillation cocktail Permafluor E+. The amount of radioactivity in fecal homogenates was determined using a Wallac 1409 liquid scintillation counter. Radioactivity less than twice the background value was considered to be below the
limit of determination. Samples collected prior to dosing were used as controls and counted to obtain a background count rate.

**Extraction of Metabolites from Biological Samples**

Urine samples collected from each subject from 0 to 24 h post-dose represented ≥96% of the excreted radioactivity. The pooled urine samples were flash frozen and lyophilized overnight. Sample residues were reconstituted in 0.2 ml acetonitrile (ACN): water (10:90). Fecal homogenates were pooled from each subject up to 144 h so that ≥90% of radioactivity was accounted for. Approximately 4 g of the pooled fecal samples were suspended in 20 ml ACN and 4 ml 1% formic acid. Suspensions were sonicated, vortexed, and centrifuged at 1800 g for 10 min. The pellets were further extracted with 20 ml ACN as described above and aliquots counted in a liquid scintillation counter. The mean recovery of radioactivity after extraction was 89%. The supernatants were evaporated to dryness under nitrogen in a Turbo Vap LV evaporator (Caliper Life Sciences Corporation, Hopkinton, MA, USA) and the residues reconstituted in 0.4 ml ACN: water (10:90). Aliquots (100 µl) were injected onto an HPLC/MS/MS system.

For profiling of circulating metabolites, plasma samples from each individual at 0, 0.5, 1, 2, 4, and 8 h post-dose were pooled according to the method of Hamilton et al. (Hamilton et al., 1981), i.e. 0.5, 1, 1.5, 3, 6, and 4 ml respectively. The pooled plasma was diluted with two volumes of ACN and the precipitated protein was removed by centrifugation. The pellets were extracted with an additional one volume of ACN. The mean recovery of radioactivity after extraction was 96%. The supernatants were concentrated to near dryness and the resulting residues were reconstituted in ACN: water (20:80). Aliquots (100 µl) were injected
on to an HPLC-Accurate Radioisotope Counting (ARC, AIM Research Company, NJ, USA) system for analysis.

**Tofacitinib Quantification**

Plasma concentrations of unchanged tofacitinib were determined at BAS Analytics (West Lafayette, IN, USA) by a validated HPLC/MS/MS assay. Briefly, human plasma or urine samples, fortified with internal standard (\(^{13}\text{C} \ ^{15}\text{N}\)) tofacitinib and 1% acetic acid, were extracted by solid phase extraction (Strata X-C 33 \(\mu\text{M}\) cation mixed-mode polymer 96 well plate; phenomenex.com), eluted with 13% ammonium hydroxide in methanol, evaporated to dryness and reconstituted with 50% methanol in water. The reconstituted sample was injected onto a Synergi\(^{\text{TM}}\) Polar-RP 4 \(\mu\text{m}\) 2.0 \(\times\) 50 mm LC/MS column (phenomenex.com), coupled to a Sciex 4000 MS/MS detector with TurboIonSpray ionization source using positive ion mode. The mobile phase was 40% 10 mM ammonium acetate and 60% methanol (with 0.05% formic acid) with a flow rate of 0.3 ml/min. MRM transitions were 313.4 \(\rightarrow\) 173.2 for tofacitinib and 316.3 \(\rightarrow\) 173.1 for the internal standard. The dynamic range of the assay was 1–100 ng/ml.

**Metabolite Quantification**

Metabolite quantification (urine and HLM) was performed by measuring radioactivity in the individual peaks that were separated by HPLC, using a \(\beta\)-RAM (IN/US, Tampa, FL, USA) and Laura program Version 3.1.1.39 (Lablogic System Ltd, UK). The \(\beta\)-RAM provided an integrated printout in counts per min and the percentage of the radio-labeled material, as well as peak representation. The \(\beta\)-RAM was operated in the homogeneous LSC mode with the addition of 3 ml/min of Tru-Count (IN/US, Tampa, FL, USA) scintillation cocktail to the effluent post-mass spectrometry (MS) detection. The metabolites in feces and plasma were
quantified by measuring the radioactivity in individually separated radioactive peaks using a liquid chromatography (LC)-AR system. The LC-ARC was operated in the homogeneous LSC mode with the addition of 2.5 ml/min of Tru-Count scintillation cocktail to the effluent post-UV detection.

**HPLC/MS/MS Analytics**

HPLC was conducted on a system that consisted of a HP-1100 solvent delivery system, a HP-1100 membrane degasser, a HP-1100 autoinjector (Hewlett Packard, Palo Alto, CA, USA) and a β-RAM. Chromatography was performed on a Kromasil (C-18) column (4.6 mm × 150 mm, 5 μm). The mobile phase was composed of 5 mM ammonium formate pH 3.0 (solvent A) and ACN (solvent B). A flow rate of 1.0 ml/min was maintained throughout the analysis. Analyses of the metabolites were performed on a Finnigan LCQ Deca LC/MS/MS (Finnigan, San Jose, CA, USA). The effluent from the HPLC column was split and approximately 50 μl/min was introduced into the atmospheric pressure ionization source via a pneumatically assisted electrospray interface. The remaining effluent was directed into the flow cell of the β-RAM. The β-RAM response was recorded in real time by the MS data system, which provided simultaneous detection of radioactivity and MS data. The electrospray interface was operated at 4500 V and the mass spectrometer was operated in the positive ion mode.

**Pharmacokinetic Data Analysis**

The following PK parameters were analyzed using the WinNonlin V3.2 program (Pharsight Corporation, Cary, NC, USA): maximal observed plasma concentration (C_max), T_max, t_1/2, area under the plasma concentration-time curve (AUC) from time 0 to the last time (AUC_0-last), and AUC from time t to infinity (AUC_t-∞). The C_max of tofacitinib or TRA (parent drug
equivalents) in plasma were estimated directly from the experimental data. Terminal phase rate constants ($k_e$) were estimated using least-squares regression analysis of the plasma concentration–time data obtained during the terminal log-linear phase and $t_{1/2}$ was calculated as $0.693/k_e$. AUC$_{0-last}$ was calculated using the linear trapezoidal rule. AUC$_{t-\infty}$ was estimated as $C_t/k_e$, where $C_t$ represents the concentration at time $t$ based on the aforementioned regression analysis. AUC$_{0-\infty}$ was estimated as the sum of AUC$_{0-t}$ and AUC$_{t-\infty}$ values.

**Nuclear Magnetic Resonance Characterization of Metabolite M9**

A dried sample containing metabolite M9 of tofacitinib, which had been purified via preparative HPLC from CYP2C19 incubations, was dissolved in 0.2 ml dimethyl sulfoxide (DMSO)-d$_6$ ‘100%’ (Cambridge Isotope Laboratories, Andover, MA, USA) under a dry argon atmosphere and placed in 3 mm NMR tubes. All NMR spectra were recorded on one of two systems. The first was a Bruker Avance 600 MHz (Bruker BioSpin Corporation, Billerica, MA, USA) controlled with Topspin V2.1 and equipped with a 5 mm triple resonance inverse cryoprobe. The second system was a Bruker Avance 600 MHz controlled by Topspin V1.3 and equipped with a 2.5 mm broadband inverse probe.

One-dimensional $^1$H spectra were recorded at 300 K, unless otherwise stated, using a sweep width of 7500 Hz and a total recycle time of 7 s. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1 Hz to enhance signal to noise. $^1$H data from the M9 isolate was also processed with resolution enhancement using a line broadening of -1 Hz and a Gaussian multiplication of 0.4. Spectra were referenced using residual DMSO-d$_6$ ($\delta = 2.49$ ppm relative to tetramethylsilane, $\delta = 0.00$ for $^1$H and $\delta = 39.5$ ppm relative to tetramethylsilane, $\delta = 0.00$ for $^{13}$C). The two-dimensional data (correlation spectroscopy, total correlation spectroscopy, multiplicity edited heteronuclear single quantum
coherence [HSQC], and heteronuclear multiple bond correlation [HMBC]) were recorded using the standard pulse sequences provided by Bruker. A 1000 × 128 data matrix was acquired using a minimum of four scans and 16 dummy scans. The data was zero-filled to a size of 1000 × 1000. A relaxation delay of 2 s was used between transients. A mixing time of 80 ms was used in the total correlation spectroscopy experiments.

**In Vitro Metabolism and CYP Phenotyping**

An initial assessment of the linearity of metabolite formation with respect to incubation time and microsomal protein content indicated that combined metabolite formation was linear up to a HLM protein concentration of 1.5 mg/ml for up to 30 min. For kinetics of metabolite formation studies in HLMs, incubations were performed in duplicate with reduced NADPH (1.3 mM) at 37°C in a shaking water bath. Samples were pre-incubated at 37°C for 5 min prior to the addition of NADPH. For kinetic studies, incubations contained HLMs (1.0 mg protein/ml), 0.1 M KH$_2$PO$_4$ buffer pH 7.4, MgCl$_2$ (3.3 mM), and $^{14}$C-tofacitinib (1.0, 5.0, 10, 20, 50, 100 or 150 µM). Samples were quenched with ACN (3.0 ml) after 30 min and centrifuged at 1800 g for 5 min. Supernatants were dried using an evaporative centrifuge set to 37°C, reconstituted in ACN:water (200 µl; 5:95), and analyzed directly by LC/MS/MS combined with a β-RAM, monitoring collective metabolite formation of M1, M5, M8, and M9. Apparent Michaelis-Menten constant ($K_m$) and maximum rate of reaction ($V_{max}$) values for HLMs were determined directly from a plot using a non-linear regression curve fitting in Sigma Plot 8.0.

For chemical inhibition studies in HLMs, the following inhibitors were utilized: furafylline (10 µM for 1A2), sulfaphenazole (10 µM for 2C9), quinidine (1 µM for 2D6), (+)N-3-benzylnirvanol (10 µM for 2C19) and ketoconazole (1 µM for 3A4). Incubations
(1 ml) were performed in duplicate as discussed above. Each incubation contained HLMs (1.0 mg protein/ml), KH2PO4 buffer pH 7.4 (0.1 M), MgCl2 (3.3 mM), 14C-tofacitinib (10 μM) and inhibitors. Furafylline was pre-incubated for 5 min with NADPH in buffer, prior to addition of tofacitinib. A control sample was also prepared without inhibitor. After 30 min, incubations were quenched and processed as above. For recombinant human CYP isoform studies, incubations (1 ml) were performed in duplicate as detailed above. Each incubation contained recombinant human CYP (25 pmol/ml), KH2PO4 buffer pH 7.4 (0.1 M), MgCl2 (3.3 mM) and 14C-tofacitinib (10 μM). Protein content was adjusted to 0.65 mg/ml using human CYP insect control (PanVera Lot#29870A). After 30 min, incubations were quenched and processed as above. In a separate study to further study metabolite identification, similar incubations using 50 pmol/ml of recombinant enzymes were carried out to 2 h.

The HPLC system consisted of a Surveyor auto sampler, quaternary solvent delivery pump, and a photodiode array detector (Thermo Fisher, San Jose, CA, USA). Chromatography was carried out on a Phenomenex Hydro RP HPLC column (4.6 mm × 150 mm, 4 μm) with a binary mixture of 5 mM ammonium formate (pH 3.0, solvent A) and ACN (solvent B). A flow rate of 1 ml/min was used for analysis. Identification of the metabolites was performed on a Thermo Fisher Quantum mass spectrometer operating with electrospray ionization. The effluent from the HPLC column was split and approximately 100 μl/min of flow was directed to the source. The remaining effluent was directed to the photodiode array and then to the β-RAM. The interface was operated at 3000 V and the spectrometer was operated in the positive ion mode with capillary temperature set at 270°C.
Results

Pharmacokinetic and Disposition Profiles

The absorption of tofacitinib was rapid, with plasma concentrations for both tofacitinib and TRA peaking at around 1 h after oral administration (Fig. 2). The PK parameters for tofacitinib and TRA are shown in Table 1. Mean (SD) C_{max} values for the parent drug and TRA were 397 (62) ng/ml and 611 (69) ng Eq/ml respectively. Mean (SD) AUC_{0-∞} values for tofacitinib and TRA were 1680 (380) ng·h/ml and 3440 (798) ng Eq·h/ml, respectively. The mean \( t_{1/2} \) was around 3.2 h for both tofacitinib and TRA.

After a single oral dose of $^{14}$C-tofacitinib in male subjects, TRA was excreted predominantly in the urine (Fig. 3). At 192 h after the dose, the mean (SD) cumulative excretion in the urine was 80.1% (3.6) and in the feces was 13.8% (1.9) (Table 2). In total, 93.9% (3.6) of the radioactive dose was recovered in urine and feces with a major portion of excreted radioactivity recovered during the first 24 h after dosing (i.e. 24-h recovery: urine 77.0% [3.5]; feces 1.6% [2.5]; total 78.6% [3.5]). Tofacitinib was the main component of drug-related material in urine at approximately 30%. Collectively, 12 metabolites were identified in urine and feces. The M9 metabolite was the most abundant in urine at approximately 20%. All other metabolites made up less than 10% of the dose. Representative HPLC radiochromatograms of tofacitinib urinary and fecal metabolites, following a single oral dose of $^{14}$C-tofacitinib in male subjects, are shown in Fig. 4A and Fig. 4B, respectively. A representative HPLC radiochromatogram of tofacitinib and circulating metabolites in the plasma of male subjects following a single oral dose of $^{14}$C-tofacitinib is shown in Fig. 4C.
Unchanged tofacitinib accounted for the majority (69.4% [8.5]) of the total circulating radioactivity (Table 3). All circulating metabolites made up less than 10% each of total circulating radioactivity.

**Metabolite Identification**

Structural determination was performed on 12 metabolites of tofacitinib (Table 4). The parent compound, tofacitinib, had a retention time of ~20.9 min using the HPLC conditions described. A protonated molecular ion of tofacitinib (m/z 313) was detected. The tofacitinib MS$^2$ spectrum displayed fragment ions at m/z 149, 165, 173, 229, 246 and 272 (Table 4). The diagnostic fragment ions at m/z 149 and 165 resulted from cleavage of the C-N bond between pyrrolo[2,3-d]pyrimidine methylamine and [piperidin-1-yl]-3-oxo-propionitrile with charge retention on both fragments. The other ions at m/z 272 and 246 were due to loss of CH$_3$CN and CO-CH$_2$CN, respectively, from the molecular ion at m/z 313.

**Metabolite M1**

M1 was detected in urine and plasma. M1 had a retention time of ~18.1 min using the HPLC conditions described and a full scan MS detected a protonated molecular ion of M1 at m/z 299. The m/z 299 protonated molecular ion was 14.0 Da lower than the parent drug, suggesting the loss of a methyl group. The MS$^2$ spectrum of m/z 299 showed fragment ions at m/z 135, 165, 215 and 232 (Table 4). The m/z 135 fragment ion was 14.0 Da lower than the parent drug, suggesting the loss of a methyl group from the nitrogen atom of the pyrrolo[2,3-d]pyrimidine methylamine moiety. The HPLC retention time and collision-induced dissociation product ion spectrum of M1 were similar to those of a synthetic standard. Based on these data, M1 was identified as the N-desmethyl-tofacitinib.
Metabolite M2

M2 was detected in urine, plasma and feces. M2 had a retention time of 17.1–18.1 min using the HPLC conditions described and the full scan MS detected a protonated molecular ion of M2 at \( m/z \) 304. The \( m/z \) 304 protonated molecular ion was 9.0 Da lower than the parent drug, and the MS\(^2\) spectrum of \( m/z \) 304 displayed fragment ions at \( m/z \) 149, 156, 173, 229, 246, 256 and 274 (Table 4). The fragment ions at \( m/z \) 229, 246 and 149 were similar to those observed for the parent drug, suggesting that modification of the side chain had occurred. The HPLC retention time and collision-induced dissociation product ion spectrum of M2 were similar to those of a synthetic standard. Based on these data, M2 was identified as 2-hydroxy-1-(4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl)-ethanone.

Metabolite M4

M4 was detected in urine and plasma. M4 had a retention time of 12.4–12.7 min using the HPLC conditions described and the full scan MS detected a protonated molecular ion of M4 at \( m/z \) 318. The \( m/z \) 318 protonated molecular ion was 5.0 Da higher than the parent drug, and the MS\(^2\)/MS\(^3\) spectra showed fragment ions at \( m/z \) 149, 173, 182, 229, 231, 246 and 274 (Table 4). The fragment ions at \( m/z \) 229, 246 and 149 were similar to those observed for parent drug, suggesting that modification of the side chain had occurred. The \( m/z \) 274 fragment ion, corresponding to a loss of carbon dioxide, suggested the presence of a carboxyl group. Treatment of the dried urine sample with 20% sulfuric acid in methanol resulted in the disappearance of the peak corresponding to M4 and formed a new peak that displayed a protonated molecular ion at \( m/z \) 332. The \( m/z \) 332 molecular ion was 14.0 Da higher than M4, suggesting the formation of a methyl ester on the carboxyl group. The MS\(^2\)/MS\(^3\) product ion spectra of methyl ester gave fragment ions at \( m/z \) 149, 173, 215, 229 and 272 (Table 4).
Based on these data, M4 was identified as 2-carboxy-1-(4-methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl)-ethanone.

**Metabolite M8**

M8 was detected only in urine. M8 had a retention time of ~24.9 min using the HPLC conditions described and the full scan MS detected a protonated molecular ion of M8 at m/z 329; the m/z 329 protonated molecular ion was 16.0 Da higher than the parent drug. The MS² spectra of m/z 329 displayed diagnostic fragment ions at m/z 165, 189, 245, 262 and 288 (Table 4). The ions at m/z 165 and 189 suggested the addition of an oxygen atom to the pyrrolopyrimidine moiety. Based on these data, M8 was tentatively identified as 3-(3-[(hydroxy-7H-pyrrolo[2,3-d]pyrimidin-4-yl)-methyl-amino]-4-methylpiperidin-1-yl)-3-oxo-propionitrile.

**Metabolite M9**

M9 was detected in urine, plasma and feces. M9 had an HPLC retention time of 26.4–27.3 min and the full scan MS detected a protonated molecular ion of M9 (m/z 329). The m/z 329 protonated molecular ion was 16.0 Da higher than the parent drug, suggesting that a single site oxidation had occurred on the parent molecule. The MS² spectrum of m/z 329 displayed fragment ions at m/z 165, 189, 245, 262, 288 and 301 (Table 4). The m/z 165 and 189 fragment ions suggest the addition of an oxygen atom to the pyrrolopyrimidine moiety. The m/z 301 fragment ion, corresponding to a loss of carbon monoxide, suggested the addition of an oxygen atom at the pyrrole moiety. M9 was subsequently isolated and further characterized by NMR.

The ¹H spectrum of tofacitinib indicated the compound existed as a set of conformers at room temperature in DMSO. Evidence for this was indicated by the coalescence of the resonances
at 8.1, 7.2 and 4.9 ppm in a $^1$H variable temperature study ranging from 300 to 380 K (Fig. 5A). The $^1$H spectrum acquired for M9 at 300 K in DMSO also exhibited these spectral features and was therefore assumed to also be a mixture of conformers. The $^1$H spectrum of M9 indicated the absence of the two aromatic hydrogen atoms from the pyrrolopyrimidine of the parent compound (Fig. 5B). The resolution-enhanced $^1$H spectrum of M9 contained a set of nonequivalent methylene resonances between 3.7 and 4.0 ppm, each with coupling constants of 22 Hz. The HSQC data, when compared to tofacitinib, contained an additional cross peak with a chemical shift of 3.7–4.0 ppm in the hydrogen domain and $^{13}$C shift of 36.6 ppm (Fig. 6A). In the HMBC data these new hydrogen atoms correlated with three carbon chemical shifts; 94.7, 164.1 and 175.3 ppm (Fig. 6B). Considering the molecular weight of M9, there were two potential explanations for these data. Either C5 or the C6 of the pyrrolopyrimidine had been oxidized to a carbonyl. In either case the remaining other carbon, C5 or C6, becomes a methylene. If C5 is the site of oxidation, the expected carbonyl carbon chemical shift would be in the range of 190.0–200.0 ppm. However, if C6 is the site of oxidation the carbonyl carbon chemical shift would be significantly less, ranging from 165.0 to 175.0 ppm, and that of an amide. As stated above, the HMBC data contained a correlation from a new methylene resonance to a carbon with a chemical shift of 175.3 ppm. There was no evidence in the HMBC data of a carbon with a chemical shift greater than 180.0 ppm. All other acquired NMR data support the oxidation of the C6. Therefore, based on these data the structure of M9 was assigned as the 5,7-dihydro-6H-pyrrolo[2,3-d]pyrimidin-6-one (Fig. 6C).

Metabolite M11

M11 was detected in urine, plasma and feces. M11 had a retention time of 14.7–15.2 min using the HPLC conditions described and the full scan MS detected a protonated molecular ion of M11 at $m/z$ 345. The $m/z$ 345 protonated molecular ion was 32.0 Da higher than the
parent drug, suggesting that dihydroxylation had occurred on the parent compound. The MS² spectrum of m/z 345 showed fragment ions at m/z 165, 181, 278 and 317 (Table 4). The fragment ions at m/z 165 and 181 suggested the addition of an oxygen atom to both the piperidine and pyrrolopyrimidine moieties. The fragment ion at m/z 317, corresponding to loss of carbon monoxide, suggested the addition of an oxygen atom to the pyrrole moiety. Based on these data, M11 was tentatively identified as 3-(hydroxy-5-[(hydroxy-7Hpyrrolo[2,3-d]pyrimidin-4-yl)-methyl-amino]-4-methyl-piperidin-1-yl)-3-oxopropionitrile.

Metabolite M14

M14 was detected in urine and feces. M14 had a retention time of 9.2–11.2 min using the HPLC conditions described and the full scan MS detected a protonated molecular ion of M14 at m/z 345. The m/z 345 protonated molecular ion was 32.0 Da higher than the parent drug, suggesting that dihydroxylation had occurred on the parent drug. The MS² spectrum of m/z 345 displayed fragment ions at m/z 149, 197, 260, 278 and 327 (Table 4). The fragment ions at m/z 149 and 278 suggested that the dihydroxylation had occurred on the piperidine moiety. Based on these data, M14 was tentatively identified as 3-(dihydroxy-4-methyl- 5-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]piperidin-1-yl)-3-oxopropionitrile.

Metabolite M18

M18 was detected only in feces. M18 had a retention time of ~12.6 min, and co-eluted with M4 using the HPLC conditions described. A protonated molecular ion of M18 was detected at m/z 329, which was 16.0 Da higher than the parent drug. The MS² spectrum of m/z 329 showed fragment ions at m/z 149, 177, 244, 280, 294 and 311 (Table 4). The ions at m/z 149 and 244 suggested the addition of an oxygen atom to the piperidine moiety. Based on these
data, M18 was tentatively identified as 3-(hydroxy-4-methyl-5-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl)-3-oxo-propionitrile.

Metabolite M20

M20 was detected in urine and plasma. M20 had a retention time of ~14.9 min using the HPLC conditions described. A protonated molecular ion of M20 was detected at \( m/z \) 489. The \( m/z \) 489 protonated molecular ion was 176.0 Da higher than the parent compound, suggesting the addition of a glucuronide moiety. The MS\(^{2}\) spectrum of \( m/z \) 489 showed fragment ions at \( m/z \) 149, 165 and 313 (Table 4). The intense ion at \( m/z \) 313, corresponding to the loss of 176.0 Da, further suggested the presence of a glucuronide. The MS\(^{3}\) spectrum of \( m/z \) 313 displayed fragment ions at \( m/z \) 149, 165, 173, 229, 246 and 272, similar to those obtained for the parent drug. Based on these data, M20 was tentatively identified as the tofacitinib glucuronide. The exact position of glucuronide could not be determined by MS data.

Metabolite M22

M22 was detected only in feces. M22 had a retention time of ~31.4 min using the HPLC conditions described and the full scan MS detected a protonated molecular ion of M22 at \( m/z \) 343. The \( m/z \) 343 protonated molecular ion was 30.0 Da higher than the parent drug, and its MS\(^{2}\) spectrum displayed fragment ions at \( m/z \) 165, 179, 248 and 315 (Table 4). The fragment ions at \( m/z \) 165 and 179 suggested modification had occurred on both the piperidine and pyrrolopyrimidine moieties. Based on these data, M22 was tentatively identified as hydroxy-oxo-tofacitinib.
Metabolite M29

M29 was detected in urine and plasma. M29 had a retention time of ~15.2 min, and co-eluted with M11 using the HPLC conditions described. The full scan MS detected a protonated molecular ion of M29 at m/z 480, which was 176.0 Da higher than metabolite M2. The MS² spectrum of m/z 480 displayed an intense fragment ion at m/z 304 (Table 4). The intense ion at m/z 304, corresponding to a loss of 176.0 Da, suggested the presence of a glucuronide. The MS³ spectrum of m/z 304 showed fragment ions at m/z 149, 156, 173, 229, 246, 256 and 274, similar to those observed for M2 (Table 4). Based on these data, M29 was tentatively identified as the M2 glucuronide. The exact position of glucuronide could not be determined by MS data.

Metabolite M31

M31 was detected only in urine. M31 had a retention time of ~23.3 min using the HPLC conditions described. The full scan MS detected a protonated molecular ion of M31 at m/z 320; the m/z 320 protonated molecular ion was 16.0 Da higher than metabolite M2. The MS² spectrum of m/z 320 showed fragment ions at m/z 156, 165, 190, 245, 262, 272, 290 and 292 (Table 4). The fragment ions at m/z 156 and 165 suggested that the addition of 16.0 Da had occurred at the pyrrolopyrimidine moiety. The fragment ion at m/z 292, corresponding to a loss of carbon monoxide, suggested the addition of an oxygen atom to the pyrrole moiety. Based on these data, M31 was tentatively identified as 2-hydroxy-1-(4-methyl-3-[methylhydroxy-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl)-ethanone.

In-Vitro Metabolism and CYP Profiling

HPLC radiochromatograms are shown in Fig. 6A to 6E. The HPLC radiochromatogram of 1⁴C-tofacitinib metabolites in HLM in the presence of NADPH showed the formation of a
number of primary metabolites as seen in vivo, including oxidation of the pyrrolopyrimidine ring, oxidation of the piperidine ring, oxidation of the piperidine ring side chain and N-demethylation (Fig. 7). In the absence of NADPH, no new metabolite peaks were generated indicating the dependency of NADPH in the metabolism of tofacitinib (Fig. 7). Turnover in recombinant human CYP isoforms was assessed in two separate experiments as shown in Table 5. In each case, turnover in CYP3A4 supersomes (Fig. 8A) was the greatest relative to the other CYP isoforms; this was followed by turnover in CYP2C19 supersomes (Fig. 8B) relative to the other CYP isoforms. Other CYP isoforms also demonstrated turnover, but at relatively lower levels in comparison to CYP3A4 and CYP2C19 (Fig. 9A and Fig. 9B). Considering relative activity factor (RAF) values (Walsky and Obach, 2004), indicates an approximate 70% contribution from CYP3A4 and approximate ≤5% from the remaining CYP enzymes.

Metabolite rate formation in HLM was consistent with Michaelis-Menten kinetics at substrate concentration range of 1–150 µM. The apparent $K_m$ and $V_{max}$ values (standard error) for the formation of these metabolites were determined to be 132.2 (12.2) µM and 517.3 (26.9) pmol/min/mg protein, respectively (Fig. 10). Estimating intrinsic clearance ($V_{max}/K_m$) at low substrate concentration yields a value of approximately 3.9 µl/min/mg protein, which scales to an in vivo hepatic clearance of approximately 2.1 ml/min/kg (~150 ml/min). Chemical inhibition studies indicated ketoconazole (1 µM) was able to inhibit approximately 72% of tofacitinib turnover in HLM, highlighting the importance of CYP3A. However, other chemical inhibitors against 1A2, 2C9, 2C19 and 2D6 had much lower effects on tofacitinib turnover – approximately 15%, 6%, 7% and 1% inhibition of tofacitinib turnover, respectively. As HLM turnover of tofacitinib was low (approximately 11%/30 min) in the
absence of inhibitors, the lower effects seen by the non-CYP3A chemical inhibitors made it
difficult to differentiate the relative importance of each of these isoforms.
Discussion

In this report, the PK and metabolism of tofacitinib, including clearance mechanisms, were evaluated in healthy human subjects and HLMs. Following administration of a single oral dose of $^{14}$C-tofacitinib 50 mg to healthy male subjects, the total percentage of administered radioactive dose recovered was 93.9%, indicating the mass balance of tofacitinib was well characterized. The absorption of tofacitinib was rapid, with plasma concentrations for tofacitinib and TRA peaking at around 1 h after oral administration. TRA eliminated in the urine was approximately 80%, indicating good oral absorption of tofacitinib. Clinical experience with tofacitinib has indicated dose-proportional oral PK over a range of 0.1 to 100 mg (Pfizer A3921002 unpublished data), which supports linearity of parent and metabolite profiles. The $t_\text{1/2}$ of both tofacitinib and TRA was similar, indicating no significant accumulation of metabolites in the body. The major component of drug-related material circulating in plasma was unchanged parent tofacitinib (69.4%). All metabolites in circulation were at levels <10% each of total drug-related activity, a threshold defining them as minor metabolites (Robison and Jacobs, 2009).

Clinically, tofacitinib has shown an absolute oral bioavailability of 74% and total plasma clearance of 413 ml/min (Gupta et al., 2011b). In the current study, the primary clearance mechanisms for tofacitinib in humans were determined to be renal clearance and CYP-mediated oxidation at approximately 30% and 70%, respectively. Renal (CLr) and hepatic (CLh) clearance can then be estimated to be 124 ml/min (1.8 ml/min/kg) and 289 ml/min (4.1 ml/min/kg) respectively, which can be used to further assess the PK and disposition properties of tofacitinib. Using a renal clearance of 124 ml/min indicates that tofacitinib has an apparent passive filtration component of clearance of approximately 18% of total clearance ($fu \times GFR = 76$ ml/min, where tofacitinib plasma fraction unbound ($fu$) = 0.6;
GFR [glomerular filtration rate] = 125 ml/min), with the remaining 12% attributed to apparent active processes (approximately 48 ml/min).

The current study indicated that the primary metabolic transformations of tofacitinib included oxidation of the pyrrolopyrimidine ring, oxidation of the piperidine ring, piperidine ring side chain oxidation and N-demethylation, which are consistent with CYP-mediated metabolism (Fig. 11). Scaled in vitro HLM turnover (CL = 150 ml/min) was within a 2-fold prediction of the calculated in vivo metabolic clearance (CL = 289 ml/min). Assuming first pass metabolism is driven primarily by hepatic clearance, a first-pass hepatic extraction value (E) can be estimated to be approximately 0.2 (where E=CLh/Q and liver blood flow (Q) = 1450 ml/min). In turn, considering oral bioavailability (F) of tofacitinib of approximately 74%, the fraction absorbed (Fa [absorption] × Fg [1-gut metabolism]) can be estimated to be approximately 0.93 from FaFg = F/(1-E), indicating a high level of intestinal availability.

The in vitro CYP profiling results from the current study indicated the importance of CYP3A4 followed by CYP2C19 in tofacitinib metabolism, with ketoconazole inhibiting >70% of overall metabolism. These results are in agreement with those of Phase 1 clinical trials evaluating CYP3A4 involvement in tofacitinib metabolism via co-administration of the potent and moderate CYP3A4 inhibitors ketoconazole and fluconazole, respectively (Chow et al., 2008b; Gupta et al., 2012). Tofacitinib AUC and C_{max} values were increased by 79% and 27%, respectively, with fluconazole co-administration, and 103% and 16%, respectively, with ketoconazole co-administration (Chow et al., 2008b; Gupta et al., 2012). This AUC change is consistent with CYP3A4 involvement of approximately 50% in clearance and the C_{max} change is consistent with a calculated first-pass extraction ratio of approximately 0.2. The involvement of CYP3A4 has also been studied in Phase 1 studies of tofacitinib with concomitant administration of the CYP3A4 inducer rifampin. Following rifampin
administration, decreases in tofacitinib AUC\textsubscript{0-\infty} and C\textsubscript{max} of 84\% and 74\%, respectively, were observed (Lamba et al., 2012).

The predominance of CYP3A4 in tofacitinib clearance is also in agreement with a previously reported CYP2C19 polymorphism study with tofacitinib (Krishnaswami et al., 2009), which showed that subjects who were clinically poor CYP2C19 metabolizers (carriers of CYP2C19*2/*2, CYP2C19*2/*3, or CYP2C19*3/*3 alleles) had approximately 15\% and 17\% increases in tofacitinib C\textsubscript{max} and AUC\textsubscript{0-\infty}, respectively, compared with extensive metabolizers. Furthermore, pharmacogenomic studies have reported no differences in tofacitinib exposure and C\textsubscript{max} between the poor metabolizer phenotype and the ultra-extensive metabolizer phenotype for CYP2C19 following administration of rifampin (Lamba et al., 2012). The collective data show that CYP3A4 accounts for most of the 70\% of tofacitinib that is cleared by CYP metabolism, where CYP3A4 can be estimated to be approximately 50–55\% of overall total clearance.

The levels of all circulating metabolites of tofacitinib were <10\%, and were not clinically significant and did not require additional profiling. Some of the tofacitinib metabolites had definitive structural assignment, i.e. M1, M2, M4, and M9; however, others could only be defined as Markush structures. Further structural definition of these other metabolites was difficult due to their low detectable levels and therefore the significant difficulty in their isolation. Based on either metabolites synthesized (M1 and M2) or on close-in analogs made in drug discovery efforts (piperidine ring hydroxylations; pyrrole ring hydroxylation), the pharmacological activities of all metabolites including the low level oxidative metabolites are or are predicted to be less than one-tenth the potency of the parent molecule versus JAK1 and JAK3 (JAK1 and JAK3 enzyme IC\textsubscript{50} values: tofacitinib: 3.2 and 1.6 nM; M1: >10,000 and
8120 nM; and M2: 81 and 216 nM; piperidine or pyrrole ring hydroxylation analogs >1000 nM).

In summary, these studies provide a good understanding of tofacitinib PK, metabolism, and clearance mechanisms in humans. Tofacitinib was shown to be a well-absorbed drug with a predicted gut availability of 93%. Unchanged parent tofacitinib was the primary circulating species in plasma (approximately 70%), with all metabolites accounting for <10% of total drug-related activity. Clearance pathways of tofacitinib included approximately 30% renal and 70% hepatic metabolism, with most attributable to CYP3A4 turnover. Clinical experience with drug–drug interaction studies has supported this understanding of tofacitinib PK and disposition.
The authors would like to thank the A3921010 investigators and study team. In addition, the authors would like to thank Beth Obach for technical assistance.

**Authorship Contributions**

*Participated in research design:* Dowty, Ryder, Walker, Vaz, Chan, Krishnaswami, and Prakash

*Conducted experiments:* Lin, Ryder, Wang, Walker, Vaz, Chan, and Krishnaswami

*Contributed new reagents or analytic tools:* Lin, Ryder, Wang, Walker, Vaz, Chan, Krishnaswami, and Prakash

*Performed data analysis:* Dowty, Lin, Ryder, Wang, Walker, Vaz, Chan, Krishnaswami, and Prakash

*Wrote or contributed to the writing of the manuscript:* Dowty, Ryder, Walker, Vaz, Chan, Krishnaswami, and Prakash
References


Footnotes

These studies were sponsored by Pfizer Inc. Medical writing assistance was provided by Anne Marie Reid, PhD, from Complete Medical Communications and was funded by Pfizer Inc. M.E. Dowty, T. Ryder, G.S. Walker, A. Vaz, G.L. Chan, and S. Krishnaswami are full-time employees of Pfizer Inc. C. Prakash, J. Lin and W. Wang were full-time employees of Pfizer Inc at the time of the study.

Jinyan Lin is currently at Amgen, Thousand Oaks, CA, USA. Weiwei Wang is currently at Northeast Bioanalytical Laboratory, Hamden, CT, USA. Chandra Prakash is currently at Biogenidec, Cambridge, MA, USA.
Figure legends

Fig. 1. Chemical structure of tofacitinib. The asterisk designates the position of the $^{14}$C label.

Fig. 2. Mean (standard deviation) plasma concentration–time curves for tofacitinib (ng/ml) and total radioactivity (ng Eq/ml) in male subjects administered a single oral dose of $^{14}$C-tofacitinib 50 mg (n=6).

Fig. 3. Cumulative mean (standard deviation) recovery of administered radioactivity in urine and feces from male subjects administered a single oral dose of $^{14}$C-tofacitinib 50 mg (n=6).

Fig. 4. Representative HPLC radiochromatogram of (A) urinary (B) fecal and (C) circulating metabolites of tofacitinib from male subjects administered a single oral dose of $^{14}$C-tofacitinib 50 mg.

Fig. 5. (A) $^1$H Spectra of tofacitinib in a variable temperature study; and (B) $^1$H NMR spectra of tofacitinib M9 metabolite.

Fig. 6. (A) $^1$H-$^{13}$C HSQC spectra of tofacitinib and the tofacitinib M9 metabolite; (B) $^1$H-$^{13}$C HMBC NMR spectra of tofacitinib M9 metabolite; and (C) chemical structure of the M9 metabolite, as characterized by NMR.

Fig. 7. HPLC-radiochromatograms of metabolites of tofacitinib in human liver microsomes in the presence (top) and absence (bottom) of NADPH.

Fig. 8. HPLC-radiochromatograms of metabolites of tofacitinib in (A) recombinant human CYP3A4; and (B) recombinant human CYP2C19;

Fig. 9. HPLC-radiochromatograms of metabolites of tofacitinib in (A) recombinant human CYP2D6, 1A2 and 3A5; and (B) recombinant human CYP2E1, 2C8, 2C9 isoforms.
FIG. 10. Michaelis-Menten plot for the metabolism of tofacitinib in human liver microsomes.

FIG. 11. Proposed metabolic pathways of tofacitinib in humans.
**TABLE 1.**

*Pharmacokinetic parameters for tofacitinib and total radioactivity in human subjects (n=6)*

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean (SD)</th>
<th>Mean (SD)</th>
<th>Mean (SD)</th>
<th>Mean (SD)</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{\text{max}}, \text{h}$</td>
<td>$C_{\text{max}}, \text{ng/ml}$</td>
<td>$\text{AUC}_{\text{0-last}}, \text{ng*Eq/h/ml}$</td>
<td>$\text{AUC}_{\text{0-\infty}}, \text{ng*Eq/h/ml}$</td>
<td>$t_{\frac{1}{2}}, \text{h}$</td>
</tr>
<tr>
<td>Tofacitinib</td>
<td>1.1 (0.5)</td>
<td>397 (62)</td>
<td>1670 (381)</td>
<td>1680 (380)</td>
<td>3.2 (0.6)</td>
</tr>
<tr>
<td>Total radioactivity</td>
<td>1.2 (0.4)</td>
<td>611 (69)</td>
<td>3120 (621)</td>
<td>3440 (798)</td>
<td>3.2 (0.5)</td>
</tr>
</tbody>
</table>

AUC, area under the plasma concentration–time curve; $\text{AUC}_{\text{0-last}}$, AUC from time zero to last visit; $\text{AUC}_{\text{0-\infty}}$, AUC from time zero to infinity; $C_{\text{max}}$, maximum plasma concentration; SD, standard deviation; $T_{\text{max}}$, time to $C_{\text{max}}$; $t_{\frac{1}{2}}$, plasma half-life.
### TABLE 2.

*Recovery of tofacitinib, metabolites, and total radioactivity in urine and feces following a single oral dose of $^{14}$C-tofacitinib 50 mg (n=6)*

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mean (SD) recovery in urine, % dose</th>
<th>Mean (SD) recovery in feces, % dose</th>
<th>Mean (SD) total recovery, % dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total radioactivity</td>
<td>80.1 (3.6)</td>
<td>13.8 (1.9)</td>
<td>93.9 (3.6)</td>
</tr>
<tr>
<td>Tofacitinib</td>
<td>28.8 (7.1)</td>
<td>0.9 (0.8)</td>
<td>29.7</td>
</tr>
<tr>
<td>M1/M2</td>
<td>3.6 (0.3)</td>
<td>ND</td>
<td>3.6</td>
</tr>
<tr>
<td>M2</td>
<td>ND</td>
<td>0.5 (0.1)</td>
<td>0.5</td>
</tr>
<tr>
<td>M4</td>
<td>8.2 (1.2)</td>
<td>ND</td>
<td>8.2</td>
</tr>
<tr>
<td>M4/M18</td>
<td>ND</td>
<td>3.4 (0.9)</td>
<td>3.4</td>
</tr>
<tr>
<td>M8</td>
<td>1.4 (0.5)</td>
<td>ND</td>
<td>1.4</td>
</tr>
<tr>
<td>M9</td>
<td>19.6 (2.2)</td>
<td>1.6 (0.4)</td>
<td>21.2</td>
</tr>
<tr>
<td>M11</td>
<td>ND</td>
<td>1.5 (0.3)</td>
<td>1.5</td>
</tr>
<tr>
<td>M11/M29</td>
<td>10.6 (1.9)</td>
<td>ND</td>
<td>10.6</td>
</tr>
<tr>
<td>M14</td>
<td>3.5 (0.9)</td>
<td>1.9 (0.3)</td>
<td>5.4</td>
</tr>
<tr>
<td>M20</td>
<td>2.2 (0.7)</td>
<td>ND</td>
<td>2.2</td>
</tr>
<tr>
<td>M22</td>
<td>ND</td>
<td>1.8 (0.4)</td>
<td>1.8</td>
</tr>
<tr>
<td>M31</td>
<td>1.4 (0.5)</td>
<td>ND</td>
<td>1.4</td>
</tr>
<tr>
<td>Unknown</td>
<td>ND</td>
<td>2.2 (0.6)</td>
<td>2.2</td>
</tr>
</tbody>
</table>

ND, not detected; SD, standard deviation
TABLE 3

Percentage of circulating tofacitinib and metabolites in plasma following a single oral dose of $^{14}$C-tofacitinib 50 mg (n=6)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Tofacitinib</th>
<th>M1/M2</th>
<th>M4</th>
<th>M9</th>
<th>M11/M20/M29</th>
<th>M14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) % of total</td>
<td>69.4</td>
<td>7.4</td>
<td>3.9</td>
<td>1.0</td>
<td>6.2</td>
<td>3.2</td>
</tr>
<tr>
<td>circulating activity</td>
<td>(8.5)</td>
<td>(3.6)</td>
<td>(1.6)</td>
<td>(0.5)</td>
<td>(1.4)</td>
<td>(2.1)</td>
</tr>
</tbody>
</table>

SD, standard deviation
### TABLE 4.

**Diagnostic MS product ions used for tofacitinib and proposed metabolite structures**

<table>
<thead>
<tr>
<th>ID</th>
<th>Structure and MS/MS product</th>
<th>Ion m/z</th>
<th>Diagnostic ions</th>
<th>MRM fragment ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tofacitinib</td>
<td><img src="image" alt="Tofacitinib Structure" /></td>
<td>313</td>
<td>MS²: 149, 165, 173, 229, 246, 272</td>
<td><img src="image" alt="Tofacitinib MRM Fragment" /></td>
</tr>
<tr>
<td>M1</td>
<td><img src="image" alt="M1 Structure" /></td>
<td>299</td>
<td>MS²: 135, 165, 215, 232</td>
<td><img src="image" alt="M1 MRM Fragment" /></td>
</tr>
<tr>
<td>M2</td>
<td><img src="image" alt="M2 Structure" /></td>
<td>304</td>
<td>MS²: 149, 156, 173, 229, 246, 256, 274</td>
<td><img src="image" alt="M2 MRM Fragment" /></td>
</tr>
</tbody>
</table>
M4

\[ \text{MS}^2/\text{MS}^3: 149, 173, 182, 229, 231, 246, 274 \]

M8

\[ \text{MS}^2: 165, 189, 245, 262, 288 \]

M9

\[ \text{MS}^2: 165, 189, 245, 262, 288, 301 \]

M11

\[ \text{MS}^2: 165, 181, 278, 317 \]
M29

480

MS²: 304

MS³: 149, 156,

173, 229, 246,

256, 274

M31

320

MS²: 156, 165,

190, 245, 262,

272, 290, 292

MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry
TABLE 5. Percentage of combined metabolite formation of tofacitinib in recombinant human CYP isoforms

<table>
<thead>
<tr>
<th>Observation time</th>
<th>Recombinant human CYP isoform relative % metabolite formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1A1</td>
</tr>
<tr>
<td>30 min</td>
<td>4.5</td>
</tr>
<tr>
<td>2 h</td>
<td>NA</td>
</tr>
</tbody>
</table>

CYP, cytochrome P450; NA, not available; NT, no significant turnover detected;
recombinant enzyme concentrations used were 25 pmol/1 ml for 30 min, and 50 pmol/1 ml for 2 h.
Figure 1
Figure 2

The 24- to 168-h time points for total radioactivity were below limits of detection (<25.6 ng Eq/ml). The 48- to 168-h time points for tofacitinib were below limits of detection (1 ng/ml).
Figure 3

[Graph showing recovered 14C-tofacitinib (% over hours post-dose (h)) for urine, feces, and total.]

- Urine
- Feces
- Total
Figure 5

NMR, nuclear magnetic resonance

A

B

ppm
Figure 8

A

B

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Figure 10

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
<th>±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$</td>
<td>517.3 pmol/min/mg protein</td>
<td>26.9</td>
</tr>
<tr>
<td>$K_m$</td>
<td>132.2 µM</td>
<td>12.2</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.997</td>
<td></td>
</tr>
</tbody>
</table>