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Development of a Janus Kinase (JAK) Inhibitor Prodrug for the Treatment of Rheumatoid Arthritis

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Abstract

While highly efficacious in treating rheumatoid arthritis (RA), the approved JAK inhibitor, Tofacitinib (Tofa, CP-690 550), has dose-dependent toxicities that limit its clinical application. In this study, we have examined whether a prodrug design that targets arthritic joints would enhance Tofa's therapeutic efficacy, which may provide opportunity for future development of safer Tofa dosing regimens. A prodrug of Tofa (P-Tofa) was synthesized by conjugating the drug to N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer via an acid cleavable carbamate linker. The therapeutic efficacy of a single dose of P-Tofa was compared to dose-equivalent daily oral administration of Tofa in an adjuvant-induced arthritis (AA) rat model. Saline treated AA rats and age-matched healthy rats were used as controls. Observational analyses support the superior and sustained efficacy of a single dose P-Tofa treatment compared to dose-equivalent daily Tofa administration in ameliorating joint inflammation. Micro-CT and histological analyses demonstrated that the P-Tofa treatment provided better structural preservation of joints than dose-equivalent Tofa. Optical imaging, immunohistochemistry (IHC) and fluorescence-activated cell sorting (FACS) analyses attribute P-Tofa's superior therapeutic efficacy to its passive targeting to arthritic joints and inflammatory cell-mediated sequestration. *In vitro* cell culture studies reveal that the P-Tofa treatment produced sustained inhibition of JAK/STAT6 signaling in IL-4 treated murine bone marrow macrophages (BMM), consistent with a gradual subcellular release of Tofa. Collectively, a HPMA-based nanoscale prodrug of Tofacitinib (P-Tofa) has the potential to enhance the therapeutic efficacy and widen the therapeutic window of Tofa therapy in RA.

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Graphical abstract

Keywords

Rheumatoid arthritis; Inflammation targeting; Prodrug; Tofacitinib; Janus kinase inhibitor; ELVIS mechanism

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory disorder that affects up to 1% of adults worldwide ^{1, 2}. The disease often leads to significant pain associated with progressive articular damage. At present, there is no cure for RA ^{3, 4}. The identification of the key role of intracellular kinase signaling pathways in the regulation of proinflammatory cytokines and immune cell activation has led to the recent development of orally available low molecular weight drugs that selectively target individual members of the Janus kinase pathway ⁵. As an emerging class of medication, Janus kinase (JAK) inhibitors offer an alternative for RA patients who have experienced severe side effects or are refractory to current treatments. Tofacitinib (Tofa, CP-690 550), is a JAK inhibitor that exhibits functional selectivity for JAK1/3 and JAK1/2 signaling pathways. It was approved by the US Food and Drug Administration in 2012 for the treatment of adults with moderate-to-severe RA who have had an inadequate response or who are intolerant to methotrexate (MTX). Recent results from randomized clinical trials indicate that Tofa, used either as monotherapy ^{6–10} or in combination with MTX ^{11–14} or other non-biologic disease-modifying anti-rheumatic drugs (DMARDs) ¹⁵, leads to clinical improvement compared to placebo or MTX alone in patients with moderate-to-severe RA.

As a potent suppressor of innate and adaptive immunity, Tofa has been associated with dose-dependent toxicity, including higher risk of infections, malignancy, liver toxicity and hematologic abnormalities, which at least in part can be attributed to its the ubiquitous biodistribution ¹⁶. We hypothesized that the development of a macromolecular prodrug of Tofa would modify its pharmacokinetics and biodistribution (PK/BD) pattern, favoring deposition in arthritic joints, according to the inflammation-targeting ELVIS mechanism (*Extravasation through Leaky Vasculature and Inflammatory cell-mediated Sequestration*). Specifically, after systemic administration, the macromolecular prodrug of Tofa would extravasate through the fenestrated vasculature featured at the inflammatory lesions and be swiftly internalized by the inflammatory infiltrates and local activated resident cells ¹⁷. Such inflammation-specific distribution pattern would widen Tofa's therapeutic window with sustained efficacy, providing the opportunity for future development of safer Tofa dosing

regimens. To test this hypothesis, a Tofa prodrug (P-Tofa) was synthesized by conjugating the drug to a water-soluble, biocompatible *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer via a hydrolysable carbamate linker (Scheme 1A, 1B). P-Tofa's therapeutic efficacy and the potential of toxicities were then evaluated in an adjuvant-induced arthritis (AA) rat model.

2. EXPERIMENTAL SECTION

2.1. Materials

N-(2-Hydroxypropyl) methacrylamide (HPMA)¹⁸, *S,S'*-bis(α , α' -dimethyl- α'' -acetic acid)-trithiocarbonate¹⁹, and *N,N*-dioctadecyl-*N'',N''*-bis(2-hydroxyethyl)-1,3-propanediamine (LA)^{20–22} were prepared as described previously. Tofacitinib and Tofacitinib citrate were purchased from JINLAN Pharm-Drugs Technology Co., Ltd (Hangzhou, China). IRDye® 800CW carboxylate was purchased from LI-COR, Inc. (Lincoln, NE, USA). Alexa Fluor® 647 NHS ester was purchased from Life Technologies, Inc. (Eugene, OR, USA). All other reagents and solvents, if not specified, were purchased from either Sigma-Aldrich (St. Louis, MO, USA) or Acros Organics (Morris Plains, NJ, USA). All compounds were reagent grade and used without further purification.

2.2. Instruments

¹H and ¹³C NMR spectra were recorded on a 500 MHz NMR spectrometer (Varian, Palo Alto, CA, USA). The weight average molecular weight (M_w) and number average molecular weight (M_n) of copolymers were determined by size exclusion chromatography (SEC) using an ÄKTA FPLC system (GE Healthcare, Chicago, IL) equipped with UV and RI (KNAUER, Berlin, Germany) detectors. SEC measurements were performed on a Superdex 200 column (HR 10/30) with phosphate-buffered saline (PBS, pH=7.4) as the eluent. HPMA homopolymer (PHPMA) samples with narrow polydispersity were used as calibration standards. HPLC analyses were performed on an Agilent 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA) with a Hypersil™ ODS C18 Columns (Thermo Scientific, Waltham, MA). Histology slides were scanned with a VENTANA iScanner HT (Tucson, AZ, USA). Bone quality were analyzed using a Skyscan 1172 high resolution micro-CT system (Skyscan, Kontich, Belgium). A Faxitron® MX-20 Cabinet X-ray System (Tucson, Arizona, USA) was used to monitor the hard tissue decalcification progress. A Leica RM2255 rotary microtome (Leica Biosystems Inc., Buffalo Grove, IL, USA) was used for paraffin-embedded tissue sectioning. Tissue slides were analyzed using a ZEISS LSM 800 confocal microscope (Carl Zeiss Microscopy, LLC, Peabody, MA, USA). Live animals were imaged using Xenogen IVIS® Spectrum *in vivo* imaging system (PerkinElmer Inc., Waltham, MA, USA).

2.3. Synthesis of methacryloxyethyl chloroformate (HEMA-COCl)

Triphosgene (1710 mg, 5.7 mmol) in dichloromethane (4 mL, anhydrous) was added dropwise into a solution of hydroxyethyl methacrylate (HEMA, 500 mg, 3.8 mmol) and triethylamine (390 mg, 3.8 mmol) in dichloromethane (6 mL, anhydrous) maintained in an ice bath. The resulting mixture was stirred in the ice bath for 1 hr and then at room temperature for 1 hr. After removal of the solvent and excess phosgene using a rotary

evaporator, the resultant white residue was extracted with dry ether (5 mL). The solid was filtered and the filtrate was concentrated to give HEMA chloroformate as colorless oil (715 mg, 96% yield).

^1H NMR (500 MHz, CDCl_3): δ = 6.16 (m, 1H, =CH₂), 5.64 (m, 1H, =CH₂), 4.56 (t, J = 4.5 Hz, 2H, -CH₂-), 4.42 (t, J = 4.5 Hz, 2H, -CH₂-), 1.96 (s, 3H, -CH₃); ^{13}C NMR (125.7 MHz, CDCl_3): δ = 166.89 (O-C=O), 150.86 (Cl-C=O), 135.59 (=C), 126.62 (=CH₂), 69.02 (-CH₂-), 61.51 (-CH₂-), 18.21 (-CH₃).

2.4. Synthesis of Tofa-containing monomer (HEMA-Tofa)

HEMA-COCl (370 mg, 1.92 mmol) in dichloromethane (5 mL, anhydrous) was added dropwise into a solution of Tofa (500 mg, 1.60 mmol) and diisopropylethylamine (DIPEA, 310 mg, 2.40 mmol) in dichloromethane (15 mL, anhydrous) maintained in an ice bath. The reaction mixture was stirred in the ice bath for 1 hr before quenching with water (10 mL). The organic layer was separated and washed with brine, then dried over anhydrous magnesium sulfate. After removal of the solvent, the resulting light-yellow residue was subjected to flash column chromatography (CH_2Cl_2 : CH_3OH = 20:1) to afford the monomer as a white solid (715 mg, 95% yield).

^1H NMR (500 MHz, CDCl_3): δ = 8.44 and 8.42 (s, 1H), 7.41 and 7.40 (d, J = 4.2 Hz, 1H), 6.64 and 6.58 (d, J = 4.2 Hz, 1H), 6.16 (s, 1H), 5.60 (s, 1H), 5.08 (br, 1H), 4.71 (t, J = 3.6 Hz, 2H), 4.54 (t, J = 3.6 Hz, 2H), 4.05 (dd, J_1 = 13.2, J_2 = 4.2 Hz, 0.5H), 4.07-3.45 (m, 6.5H), 3.33 (s, 3H), 2.51 and 2.46 (p, J = 6.1, 1H), 1.94 and 1.96 (s, 3H), 1.89-1.85 (m, 1H), 1.76-1.66 (m, 1H), 1.09 and 1.06 (d, J = 7.1 Hz, 3H); ^{13}C NMR (125.7 MHz, CDCl_3): δ = 167.05 (O-C=O), 160.82, 160.38, 157.61, 157.52, 153.04, 153.01, 148.87 (N-C=O), 135.78, 126.36, 126.24, 121.60, 121.44, 114.33, 114.12, 106.21, 105.99, 105.17, 65.16, 65.06, 62.20, 61.98, 53.86, 53.58, 46.71, 43.74, 42.76, 39.48, 35.21, 34.75, 31.48, 31.22, 29.66, 25.20, 25.15, 18.25, 18.29 (CH₃), 14.25, 14.10. ESI-MS: $[\text{M}+\text{H}]^+ = 468.8$.

2.5. Synthesis of HPMA copolymer-Tofa conjugate (P-Tofa) via RAFT copolymerization

HPMA (3712 mg, 22.47 mmol) and HEMA-Tofa (750 mg, 1.60 mmol) were dissolved in anhydrous dimethyl sulfoxide (DMSO, 21 mL) with 2,2'-azobisisobutyronitrile (AIBN, 37.7 mg, 0.17 mmol) as initiator and *S,S'*-bis(α , α' -dimethyl- α'' -acetic acid)-trithiocarbonate (CTA, 33.7mg, 0.13 mmol) as the RAFT agent. The solution was purged with argon and polymerized at 60 °C for 40 hr. The resulting polymer was first purified by precipitation in acetone/diethyl ether (v/v = 1:1, 200 mL) twice to remove the unreacted low molecular weight compounds, and then dialyzed against ddH₂O. The molecular weight cutoff size of the dialysis tubing was 25 kDa of globular protein. The resulting solution was then lyophilized to afford the final P-Tofa (4.21 g).

To quantify Tofa loading, P-Tofa (1 mg/mL) was hydrolyzed in 0.01 N NaOH in CH₃OH overnight. The resulting solution was neutralized and analyzed with HPLC (mobile phase: acetonitrile/water = 3/1; detection, UV 284 nm; flow rate = 1 mL/min; injection volume = 20 μL). The analyses were performed in triplicate. The mean value and standard deviation were obtained with Microsoft Excel.

2.6. The synthesis of P-Tofa-APMA

To introduce fluorescent labels to the P-Tofa for biodistribution and immunofluorescence analysis, primary amine was introduced into P-Tofa using the following procedure: HPMa (700 mg, 4.89 mmol), HEMA-Tofa (142 mg, 0.3 mmol) and *N*-(3-aminopropyl)methacrylamide hydrochloride (APMA, 9.5 mg, 0.05 mmol) were dissolved in anhydrous dimethyl sulfoxide (DMSO, 6 mL) with 2,2'-azobis(isobutyronitrile) (AIBN, 7.11 mg, 0.04 mmol) as the initiator and *S,S'*-bis(α , α' -dimethyl- α'' -acetic acid)-trithiocarbonate (CTA, 6.79 mg, 0.02 mmol) as the RAFT agent, placed in an ampule, and purged with argon and polymerized at 60 °C for 40 hr. The resulting polymer was first purified by precipitation in acetone/diethyl ether (v/v = 1:1, 200 mL) twice to remove the unreacted low molecular weight compounds, and then dialyzed against ddH₂O. The amine content of the copolymer was determined as 3.56×10^{-5} mol/g using the ninhydrin assay.

2.7. The synthesis of P-Tofa-IRDye

To monitor its distribution after systemic administration, P-Tofa was labeled with IRDye[®] 800CW. The labeling procedure is briefly described as follows: IRDye[®] 800CW carboxylate (1.25 mg, 1.1 μ mol), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 1.56 mg, 8.1 mmol) and hydroxybenzotriazole (HOBt, 0.49 mg, 3.6 mmol) were dissolved in *N,N*-dimethylformamide (DMF, 1 mL) in dark at 21 °C and stirred for 30 min. DIPEA (7 mg, 0.054 mmol) and P-Tofa-APMA (50 mg, [NH₂] = 1.78 μ mol) in DMF (1 mL) were added into the reaction mixture and then stirred overnight. The reaction solution was dialyzed to remove DMF and low molecular weight reactants. P-Tofa-IRDye was then obtained via lyophilization with [IRDye[®] 800CW] = 6.88×10^{-6} mol/g of the conjugate.

2.8. The synthesis of P-Tofa-Alexa

To monitor the cellular sequestration of the P-Tofa in the rat after systemic administration, P-Tofa was labeled with Alexa Fluor[®] 647. The labeling procedure is briefly described as follows: Alexa Fluor[™] 647 NHS Ester (1 mg, 0.8 μ mol), DIPEA (3.5 mg, 0.027 mmol) and P-Tofa-APMA (50 mg, [NH₂] = 1.78 μ mol) were dissolved in *N,N*-dimethylformamide (DMF, 1 mL) in dark at 21 °C and stirred overnight. The reaction solution was dialyzed to remove DMF and low molecular weight reactants. P-Tofa-IRDye was then obtained via lyophilization with [Alexa] = 5.62×10^{-6} mmol/g of the conjugate.

2.9 *In vitro* Tofa release from P-Tofa

P-Tofa conjugates (~ 3 mg) were dissolved in 5 mL of buffer solutions with 0.2% sodium dodecyl sulfate (SDS, to create the “sink” condition) and different pH values (pH=7.4, pH=5.5, pH=10), and rats' serum (Invitrogen). The release experiments were conducted in a shaking water bath (37 °C, shaking rate 20 rpm). Hydrolysis samples (400 μ L) were withdrawn at designated time points. The released Tofa was extracted with ethyl acetate (1200 μ L), with a recovery rate at $94.3 \pm 0.48\%$. The solution of Tofa (300 μ L) was subsequently dried using a centrifugal evaporator. The sample was then stored at -80 °C for HPLC analysis as described above.

2.10. Treatment of adjuvant-induced arthritis (AA) rats

As described previously ²³, male Lewis rats (175–200 g) from Charles River Laboratories (Wilmington, MA, USA) were used to establish the adjuvant-induced arthritis (AA) rat model. The established AA rats were then divided into 3 groups: P-Tofa treatment (n = 10, single i.v. injection on day 14 post-induction, P-Tofa dose = 1 g/kg, dose equivalent of Tofa = 130.2 mg/kg), Tofa treatment (n=10, Tofa was suspended in 0.5% methylcellulose/0.025% Tween 20 (Sigma), once daily oral gavage for 21 days from day 14 post-induction, 6.2 mg/kg/day) ²⁴, and saline control (n=8). An additional group (n=5) of healthy rats were used as a negative control. A total of 33 rats were used in this experiment. Joint inflammation and body weight were monitored daily from day 11. Hematology profiles including absolute count of white blood cells (WBC), neutrophils (NE), lymphocytes (LY), monocytes (MO), eosinophils (EO) and basophils (BA) were analyzed using an HEMAVET 950 FS Hematology System (Drew Scientific Inc., Miami Lakes, FL, USA) every week post treatment initiation until the last date of free Tofa treatment. Blood was collected for liver enzyme analysis at necropsy. Liver function including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were analyzed using a DTX 880 Multimode Detector (Beckman Coulter, Jersey City, NJ, USA) at UNMC clinical test lab. All major organs were collected at the euthanasia and fixed with buffered formalin before paraffin embedding. Tissue sections (5 µm) were processed and H&E stained for histological evaluation by a pathologist (SML), who was blinded to the treatment group arrangement. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center or Hospital for Special Surgery.

2.11. Observational assessment of AA rats' joint inflammation

The articular index (AI) score was recorded during the treatment by the same observers (XW and GZ) as described previously ²³. An AI score was given to each hind limb from day 11 to day 56 post-arthritis induction. The AI scoring system is based on a 0–4 numeric system as the following: 0 = no signs of swelling or erythema; 1 = slight swelling and/or erythema; 2 = low-to-moderate edema and signs involving the tarsals; 3 = pronounced edema with limited use of the joint and signs extending to the metatarsals; 4 = excessive edema with joint rigidity and severe signs involving the entire hind paw. The sum of the two hind limb scores for each animal was recorded. Ankle diameter (medial to lateral) was measured using a digital caliper as confirmation of inflammation-associated edema/hyperplasia.

2.12. Micro-CT analysis of articular bone quality

Hind limbs were isolated after euthanasia and fixed with buffered formalin for no less than 48 hr. The left ankle joint bone quality was analyzed using a Skyscan 1172 micro-CT system. Micro-CT scanning parameters were set as follows: voltage, 70 kV; current, 142 µA; exposure time, 3650 ms; resolution, 13.1 µm; with aluminum filter (0.5 mm); rotation step = 0.4°; frame averaging = 6; random movement = 10; using 360° rotation scanning. Raw data were reconstructed using NRecon to obtain a visual representation of the results, and the volume rendering of the samples were performed via CTvox software (Skyscan). To quantitatively compare the four treatments, the entire calcaneus and the selected region of

interest (ROI) of the trabecular bone within the calcaneus (Figure 1) were used as the anatomical sites for micro-CT analyses. The ROI was defined by aligning the calcaneus bone along the sagittal plane using Dataviewer, with the ROI starts at the 75th slide away from the epiphyseal plate and continues for 76 slides (1.98 mm). The diameter of the cylindrical ROI was set at 1.00 mm. The morphometric parameters, such as percent bone volume (BV/TV), bone surface density (BS/TV), trabecular separation (Tb.Sp), trabecular number (Tb.N), bone mineral density (BMD), and trabecular thickness (Tb.Th) were calculated using CTAn (Skyscan).

2.13. Joint tissue histological evaluation

Right hind limbs were decalcified using 5% formic acid after fixation for histological analyses. Upon complete decalcification, the tissues were paraffin-embedded, sectioned (8 μ m) approximately 200 μ m apart, then H&E and Safranin O stained. Stained sections were histologically graded by a pathologist (S.M.L.), who was blinded to treatment groups²³, and then scanned using a high-throughput bright-field slide scanner. Each histopathologic feature was graded as follows: synovial cell lining hyperplasia (0 to 2); pannus formation (0 to 3); mononuclear cell infiltration (0 to 3); polymorphonuclear leukocytes infiltration in periarticular soft tissue (0 to 3); cellular infiltration and bone erosion at the distal tibia (0 to 3); and cellular infiltration of cartilage (0 to 2). Scores for all of the histopathologic features were summed for each animal.

2.14. P-Tofa biodistribution

P-Tofa was labeled with Irdye[®] 800CW (P-Tofa-Irdye) and administered i.v. to AA rats (n=5) on day 14 post-induction (P-Tofa-Irdye dose = 1 g/kg, Irdye dose = 3.4×10^{-7} mol Irdye/kg, Tofa dose = 130.2 mg/kg). Rats were then imaged with a Xenogen IVIS[®] Spectrum *in vivo* system under anesthesia at designated time points. The images were captured with the following conditions: Excitation: 778 nm (Filter: 745 nm); Emission: 794 nm (Filter: 800nm); exposure times: 2 s; Field of View: 24.5 cm; Binning Factor: 8; f Number: 2. The captured images were then analyzed using the Living Image 4.5 software (PerkinElmer Inc.). For *ex vivo* organ distribution analyses, P-Tofa-Irdye was administered i.v. to AA rats (n=3 per time point) on day 14 post-induction. Rats were perfused and euthanized at the designated time points. Major organs were then collected and imaged using a Pearl[®] Impulse small animal imaging system (LI-COR, Lincoln, NE, USA). The image acquiring conditions were set as dual channels (800 nm and white light) with 85 μ m resolution. The images for each rat were obtained using the same intensity scale with a common minimum and maximum value.

2.15. Immunohistochemically analysis of P-Tofa's cellular uptake within ankle joints

Alexa Fluor[®] 647-labeled P-Tofa (P-Tofa-Alexa) was administered i.v. to AA rats (n=5) on day 14 post-induction. Twenty-four hours later, rats were perfused and euthanized. Hind limbs were collected, fixed and decalcified using 14% EDTA solution (pH=7.4), paraffin embedded, sectioned (20 μ m). The slides were immunohistochemically stained with the following antibodies: mouse anti-rat CD68 (Bio-Rad, MCA341R, dilution 1:100) and rabbit anti-rat P4HB (Abcam, ab85564, dilution 1:50), respectively, overnight at 4 °C after antigen retrieval using sodium citrate buffer and blocked using 10% normal goat serum. Slides

incubated with mouse anti-rat CD68 were further incubated with Alexa Fluor 488-labeled goat anti-mouse secondary antibody (Thermo Fisher scientific, A11001, dilution 1:1000) and slides incubated with rabbit anti-rat P4HB were incubated with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (Thermo Fisher scientific, A11008, dilution 1:1000) for another 1 hr at 21 °C in the dark. The stained slides were imaged using a ZEISS LSM 800 confocal microscope after mounted in ProLong® Gold antifade mountant with DAPI (Thermo Fisher scientific, P36931, Waltham, MA).

2.16. *In vitro* macrophage cell culture

Primary bone marrow macrophages (BMMs) were isolated from 6–8 week old C57BL/6 mice ²⁵. BMMs were treated with Tofa (1 µM) or P-Tofa (Tofa equivalent = 16.1 µM) for 1 hr, after which 0.04 ng/mL, and 0.2 ng/mL of IL-4 was added for 24 hr. For “washout” experiments, a similar procedure was followed, except the P-Tofa/Tofa incubation time was increased to 24 hr, following which the cells were washed and incubated with fresh medium (without P-Tofa or Tofa) for 72 hr or 1 week, prior to the IL-4 challenge. RNA was isolated using RNAeasy kits (Qiagen, Redwood City, CA), in accordance with manufacturer’s recommendations and reverse transcribed using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, Waltham, MA, USA). Real-time qPCR was performed using the Maxima SYBR Green/Fluorescein qPCR Master Mix 2X (Thermo Scientific, Waltham, MA, USA) on a CFX96 real time thermocycler (Bio-Rad, Hercules, CA, USA) and the relative gene expression was measured using the standard Cq method and normalized to mouse GAPDH expression. Arg1, Ym1/2 and Fizz1 and their respective sequences are listed as follows:

Arg1 (GGAATCTGCATGGGCAACCTGTGT/
AGGGTCTACGTCTCGCAAGCCA),

Ym1/2 (GGGCATACCTTTATCCTGAG/CCACTGAAGTCATCCATGTC),

Fizz1 (TCCCAGTGAATACTGATGAGA/CCACTCTGGATCTCCCAAGA),

GAPDH (GGTGCTGAGTATGTCGTGGA/GTGGTTCACACCCATCACAA)

2.17. Serum cytokine measurements

CXCL10 protein levels in rat serum samples collected weekly from day 14 to day 56 were quantified by ELISA (Abnova, Rat CXCL10 ELISA Cat #KA2203, Taipei City, Taiwan). The assay was performed in duplicate using a two-fold dilution of serum according to the manufacturer’s instruction.

2.18. Statistical methods

One-way analysis of variance (ANOVA), followed by Tukey’s post hoc test to account for multiple comparisons, was used for data analysis using GraphPad Prism Software. P-values 0.05 were considered statistically significant.

3. RESULTS

3.1. Characterization of P-Tofa

P-Tofa has a weight average molecular weight (M_w) of 30.4 kDa, a number average molecular weight (M_n) of 23 kDa and a polydispersity index (PDI) of 1.32, representing a narrow polydispersity. The Tofa drug loading in P-Tofa was found to be ~ 13 wt%. The *in vitro* Tofa release rate was found to be highly dependent upon buffer pH values (Figure 2). Both acidic and basic pH environments accelerated Tofa release, when compared to the release rate at pH 7.4. The presence of serum proteins in the releasing medium was also found to increase the Tofa release rate. Under each condition tested, intact Tofa was gradually released from P-Tofa. Interestingly, Tofa degradation was observed over time in the basic buffer (pH 10), which is in agreement with previous findings²⁶. During the course of the experiment (20 days), the release rates of Tofa from P-Tofa at pH = 5.5 and 7.4, in the rat serum averaged at ~2.5%, 1.5% and 2% of the loaded drug per day, respectively.

3.2. P-Tofa provides sustained amelioration of joint inflammation in AA rats

Ankle diameter and AI score of the Tofa-treated group exhibited a continuous decrease from day 15 to 34 post-arthritis induction, with an immediate flare upon cessation of oral Tofa on day 35 (Figure 3). A single injection of P-Tofa (dose equivalent to the entire Tofa treatment) resulted in greater reductions in ankle swelling and AI score from day 15 to 34, a difference that persisted to day 56. The ankle diameter of the P-Tofa group was significantly lower ($P < 0.05$) than both the Tofa group and saline group from day 14 to day 56; and significantly higher ($P < 0.05$) than the healthy control group from day 11 to day 56. No significant differences were found between the Tofa and saline groups except day 16 to day 22.

3.3. Histological Analysis of the Ankle Joints

Compared to healthy control animals, histological analyses revealed marked bone and cartilage destruction of the distal tibia, calcaneus and talus joints in the saline-treated group, with periosteal expansion and inflammatory cell infiltration. The Tofa-treated group exhibited histological findings similar to the saline group, consistent with a limited capacity in preventing joint bone erosion and cartilage damage. The single dose P-Tofa group, however, displayed markedly reduced joint damage and cellular infiltration, with bone and cartilage morphology maintained similar to that of the healthy rats (Figure 4A, 4B). The sum of the score from each animal was recorded and shown in Figure 4C. The statistically significant difference was found between Healthy vs. Saline, Saline vs. P-Tofa and P-Tofa vs. Tofa groups. No significant difference was found between P-Tofa vs. Healthy or Saline vs. Tofa groups.

3.4. Micro-CT evaluation of joint bone quality

The most severe bone damage was found in the saline group, with extensive erosion of the entire distal tibia (Figure 5A). Tofa-treated animals demonstrated reduced ankle bone erosion compared to the saline group. Six weeks following the single dose P-Tofa administration, there was only minor bone erosion. The quantitative analysis of the hind paw calcaneus trabecular bone (Figure 5B) micro-CT data shows that P-Tofa treatment preserved

the bone quality as evident in the morphometric parameters, such as percent bone volume (BV/TV), bone surface density (BS/TV), trabecular separation (Tb.Sp), trabecular number (Tb.N), bone mineral density (BMD), and trabecular thickness (Tb.Th) with their values similar to those observed for healthy controls; and significantly better than those observed for free Tofa-treated and the saline control. When the entire calcaneus bone was analyzed (Figure 5C), the Tofa and saline groups were found with significantly increased calcaneus tissue volume and calcaneus bone surface, and significantly decreased calcaneus bone volume percentage, when compared to the healthy and P-Tofa-treated groups.

3.5. Passive targeting and retention of P-Tofa in arthritic joints

Near infrared optical imaging analyses revealed that systemically administered P-Tofa-IRDye was mainly distributed to arthritic joints (Figure 6A). Signals observed at the ear and the base of the tail were attributed to the trauma from ear tag installation and inflammation associated with immunization. Signal intensity in the joints gradually decreased from $\sim 1 \times 10^9$ to $\sim 2 \times 10^8$ (p/sec/cm²/sr)/(uW/cm²) over 12-days. To validate live imaging results, major organs and both hind limbs were collected at necropsy and imaged *ex vivo*. The inflamed joints, especially the hind limb ankle joints, were the major sites of P-Tofa-IRDye distribution with moderate-to-high signal intensity also observed in the liver and kidneys (Figure 6B). The lack of fluorescent signal observation confirmed the absence of P-Tofa in the other organs. Semi-quantitative analyses of the optical imaging data corroborated this observation (Figure 6C).

3.6. Cellular distribution of P-Tofa

To identify the cell types that sequestered and retained P-Tofa within the joint, immunohistochemistry staining with a series of cell-specific markers was performed. Numerous P-Tofa-Alexa 647 (red fluorescence) positive cells were found in synovial tissues where they co-localized with P4HB⁺ (fibroblast) and CD68⁺ (monocytes/macrophages) cells, consistent with synovioocyte-mediated subcellular sequestration of P-Tofa-Alexa 647 (Figure 7).

3.7. *In vitro* inhibition of JAK/STAT signaling by P-Tofa and Tofa

To assess the ability of P-Tofa and Tofa to inhibit JAK/STAT signaling, primary murine BMM were treated with or without P-Tofa or Tofa for 24 hr, then challenged with 2 different IL-4 concentrations (0.2 ng/mL and 0.04 ng/mL). qPCR analysis revealed that, as expected, IL-4 strongly induced expression of alternative macrophage activation markers Arg1, Ym1/2 and Fizz1, and both Tofa and P-Tofa pretreatment for 24 hr (Figure 8A) effectively repressed induction of all three genes. To evaluate if P-Tofa offers sustained anti-inflammatory activity, cells were pretreated with P-Tofa or Tofa, then washed and cultured for an additional 72 hr in the absence of the inhibitors, prior to IL-4 challenge. Notably, under these conditions, P-Tofa retained the ability to repress IL-4 signaling to a significantly greater extent than free Tofa (Figure 8B), suggesting that P-Tofa provides sustained efficacy via its cellular sequestration and subsequent subcellular Tofa release.

3.8. The impact of Tofa and P-Tofa treatments on serum levels of CXCL 10

To evaluate the effects of Tofa and P-Tofa treatments on systemic inflammatory cytokines, serum levels of CXCL10 were evaluated. As shown in Figure 9, CXCL10 levels were significantly elevated in arthritic rats at day 35 post arthritis induction, when compared to the healthy controls. A single dose of P-Tofa completely normalized the CXCL10 levels. Daily Tofa treatments also significantly decreased serum CXCL10 levels, though the levels were significantly higher compared to the P-Tofa group.

3.9. Preliminary toxicity assessment

Under the present dosing level, hematologic profiles of P-Tofa and Tofa treated animals (Table 1) were similar until week 3, when significantly lower total white blood cells (WBC), neutrophils (NE), eosinophils (EO) and basophils (BA) were observed in P-Tofa group. A small but significantly lower alkaline phosphatase (ALP) value was found in the P-Tofa group compared to the Tofa group at necropsy. There were no differences in AST or ALT levels. No histological abnormality (not shown) was found in major organs from P-Tofa group by the pathologist (SML) who was blinded to the group arrangement.

4. Discussion

In recent years, significant progress has been made in understanding the essential role of specific proinflammatory cytokines and other immunological processes responsible for RA initiation and progression^{27, 28}. Subsequent studies showed that the receptors for several proinflammatory cytokines exert their effects through the activation of intracellular signaling pathways mediated by a unique family of Janus kinases that phosphorylate downstream molecular targets that control key inflammatory and immunological processes^{5, 29}. This led to the development of Tofacitinib (Tofa, CP-690 550), the first selective JAK inhibitor tested in humans. Tofa inhibits both JAK3 and JAK1, to a lesser extent JAK2, and was found to be effective in disease suppression in a variety of clinical conditions and experimental models ranging from inflammatory arthritis, autoimmune disorders and transplantation. A major challenge in the development of JAK inhibitors for the treatment of inflammatory and autoimmune disorders has been their ubiquitous expression in multiple tissues and cell types, and the broad range of biological activities that they control. Thus, despite demonstrated efficacy in RA, their systemic administration is associated with many serious and potentially life threatening adverse side effects, including infections, malignancy, liver toxicity and hematologic abnormalities¹⁶. We hypothesized that selective targeting of Tofa to sites of joint inflammation might substantially enlarge the therapeutic window of Tofa, with the potential for an improved safety profile. We have previously shown in animal models that the macromolecularization of glucocorticoids significantly potentiates their therapeutic efficacy and reduces systemic toxicities^{23, 30–32}, via the ELVIS mechanism (*Extravasation through Leaky Vasculature and Inflammatory cell-mediated Sequestration*). Concurrently, for systemic inflammatory conditions, a fraction of the nanomedicine administered may also be sequestered by circulating white blood cells (WBC) and be actively transported to the inflammatory lesion.

Based upon these previous studies, we hypothesized that a macromolecular Tofacitinib prodrug would selectively target inflamed joints, followed by sequestration in resident cells and activation to release Tofa locally to suppress joint inflammation. Our initial attempt to conjugate Tofa to HPMA copolymer was challenging. The secondary amine in the pyrrole ring is the only available site for chemical conjugation. Given the regional pyrrole/pyrimidine-conjugation, this amine is not very reactive. Attempts to conjugate Tofa to HPMA copolymer via peptides or citraconic acid linkers failed. Eventually, Tofa was successfully conjugated to HEMA via a carbamate bond and the Tofa-containing monomer was copolymerized with HPMA (Scheme 1A, 1B). We purposely set the P-Tofa molecular weight (~ 30 kDa) lower than the glomerular filtration threshold of 45 kDa of HPMA copolymer to allow eventual renal clearance of the prodrug. There was an initial concern regarding the use of a carbamate linker as it has been known to be relatively stable *in vivo*³³. Enzyme catalyzed hydrolysis may be the reason of the accelerated Tofa release in the serum when compared to the pH 7.4 buffer (Figure 2)^{34, 35}. The *in vitro* release study, however, provided strong evidence that the prodrug can be gradually activated under acidic environments (e.g. inflammatory acidosis or lysosomal pH). The therapeutic activities of the prodrug were then established through a series of *in vitro/in vivo* experiments, which are discussed in detail in the following sections.

To test our hypothesis, a HPMA copolymer-based macromolecular prodrug of Tofacitinib (P-Tofa) was designed, synthesized and evaluated using an adjuvant-induced arthritis rat model. To establish the arthrotropism of P-Tofa according to our hypothesis, rats with established AA were administered P-Tofa-IRDye and its biodistribution and tissue-specific retention were evaluated using sequential *in vivo* NIR optical imaging. As shown in Figure 6A, the IRDye signal mainly localized in the arthritic limbs. Due to the limited tissue penetration depth of the NIR fluorescent signal³⁶, the distribution of P-Tofa-IRDye in the major organs and arthritic limbs were imaged *ex vivo* and analyzed semi-quantitatively using an LI-COR small animal imager. The results (Figure 6B, 6C) confirmed that the inflamed joints in the affected limbs were the major sites of P-Tofa-IRDye localization with additional distribution sites in the main clearance organs (i.e. liver and kidneys). Different from oral Tofa's clinical PK data³⁷, where no joint specific distribution of the drug has been observed, P-Tofa showed clear arthrotropism. On a cellular level, immunohistochemistry analysis of the decalcified arthritic joints revealed that the P-Tofa-Alexa was sequestered by fibroblast-like (P4HB+) and macrophage-like (CD68+) synoviocytes (Figure 7), providing direct evidence of P-Tofa's targeting to key cell types involved in the joint inflammatory pathology^{38–40}.

We hypothesized that the tissue and cellular specificity of systemically administered P-Tofa would lead to a potent and sustained anti-rheumatic effect. Our original dose equivalent treatment protocol was designed to terminate at day 35 post arthritis induction. The results (Figure 3A, 3B) established that a single dose of P-Tofa was effective in ameliorating joint inflammation and improving the articular index (AI) scores during this period of time. Having observed this initial beneficial effect, we further extended the observation period. To maintain dose equivalence, no additional Tofa was given to the Tofa group. Of importance, the single dose P-Tofa treated rats continued to show reduced joint inflammation and articular index score reduction until day 65 post arthritis induction, when signs of a minor

arthritis flare (e.g. a small increase of the arthritis score) was observed. Immediately after the cessation of Tofa treatment, a flare was detected in the Tofa treated group, which continued to worsen until the experimental endpoint (day 65 post arthritis induction). Tissue histopathology (Figure 4A, 4B) and micro-CT (Figure 5) analyses of the ankle joints isolated at the end point of the experiment demonstrated preservation of joint cartilage and subchondral bone integrity in the animals treated with the single dose P-Tofa. The Tofa-treated animals, in contrast, showed only moderate bone and cartilage protection when compared to the saline controls; but exhibited more extensive joint tissue damage compared to the P-Tofa-treated group.

In vitro cell culture studies were undertaken to compare the efficacy of P-Tofa and Tofa in inhibition of JAK/STAT signaling. Murine BMM were treated with P-Tofa or Tofa prior to challenge with IL-4. This cytokine signals via the JAK/STAT6 pathway and induces the expression of markers of alternative macrophage activation, including arginase-1 (Arg1), YM1/2, and Fizz1. qPCR analysis revealed that IL-4 strongly induced expression of Arg1, Ym1/2 and Fizz1. Tofa treatment for 24 hr effectively repressed induction of all three genes. P-Tofa was equally effective under these conditions (Figure 8A). To assess the relative efficacy of P-Tofa or Tofa to produce sustained inhibition of IL-4 induced JAK/STAT signaling, in a second set of experiments, cells were pretreated with P-Tofa or Tofa, then washed and cultured for an additional 72 hr in the absence of inhibitors, prior to IL-4 challenge. Of importance, under these conditions, the P-Tofa treatment produced sustained inhibition of IL-4 signaling (Figure 8B), whereas the Tofa treated cells became IL-4 responsive by 72 hr. These findings are consistent with sustained release of active drug from the P-Tofa and corroborate well with the *in vitro* data (Figure 2) showing the sustained release of free Tofa in acidic environments, present in the synovium of patients with active arthritis and in the subcellular lysosomal compartment in which the macromolecular prodrug is sequestered ³².

To further explore the impact of P-Tofa treatment, we measured the serum levels of CXCL10. In human studies, circulating CXCL10 as well as synovial expression of this chemokine has been shown to be sensitive to Tofa treatment ⁴¹ and it has been implicated as a major contributor to the recruitment and activation of immune cells involved in the local synovial inflammation. In this study, we found that CXCL10 levels were significantly elevated in arthritic rats, when compared to the healthy controls; and a single dose of P-Tofa completely suppressed the elevated serum CXCL10 levels at day 35 post induction of arthritis (Figure 9).

Our data attributes this superior and long-lasting therapeutic efficacy of P-Tofa to its passive targeting to sites of joint inflammation and synoviocyte-mediated local sequestration and sustained Tofa release, which is distinctively different from Tofa's pharmacokinetic profile ⁴². We detected lower WBC count and ALP levels in the P-Tofa group than the Tofa group at the later stage of the treatment. Though initially we considered these as signs of potential toxicities, further comparison with the healthy Lewis rats' hematology and biochemistry parameters (from Charles River) ⁴³ indicates that they are still within the normal range. Therefore, we consider the P-Tofa dosing at this level (130.2 mg/kg, Tofa equivalent, i.v. single dose), which is 20-times higher than the Tofa dose (6.2 mg/kg, daily oral gavage) used

in a preclinical therapeutic efficacy study in this particular animal model⁴⁴, may still be safe. The apparent reduction of the WBC count and ALP levels associated with P-Tofa treatment can be attributed to its higher efficacy in attenuation of the systemic inflammation^{45, 46}. Clearly, additional dose escalation and a more comprehensive toxicity studies are necessary to accurately define P-Tofa's safety profile. Given its superior and sustained therapeutic efficacy, we postulate that P-Tofa has significant potential for development as a treatment for RA.

5. CONCLUSIONS

In this study, we have developed a macromolecular prodrug of a Janus Kinase (JAK) inhibitor, Tofacitinib (P-Tofa) using a well-established, water-soluble and biocompatible *N*-(2-hydroxypropyl) methacrylamide (HPMA) copolymer. A single i.v. administration of P-Tofa provided superior and sustained therapeutic efficacy in an adjuvant-induced arthritis rat model, when compared to dose equivalent daily Tofa treatment. P-Tofa's significantly widened therapeutic window holds the promise for enhancing the clinical efficacy of Tofacitinib for the treatment of RA.

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Figure 1. Region of interest in micro-CT analyses

Gray color: rat calcaneus bone; Red color: cylindrical ROI within the calcaneus bone.

Figure 2. *In vitro* Tofa release from P-Tofa at pH = 5.5, 7.4, 10.0 and in rat serum
The mean values and standard deviation were calculated with GraphPad Prism, n = 3.

Figure 3. P-Tofa can effectively amelioration of joint inflammation in an adjuvant-induced arthritis (AA) rat model

(A) The change of AA rats' left ankle joint size of different groups during the treatment study; (B) The change of articular index score of different groups during the treatment study. The arrow pointing up indicates the day when rats received the single P-Tofa injection and the daily oral Tofa treatment was initiated. The arrow pointing down indicates the day when rats received their last oral Tofa treatment. The prevention of arthritic ankle swelling by single injection of P-Tofa was sustained for 6 weeks from day 15 to day 56.

Figure 4. Histology evaluation of Tofa and P-Tofa therapeutic efficacy
(A) H&E-stained joint sections (10× and 40×). Cellular infiltration in periarticular soft tissue, bone and cartilage damage in Tofa and saline groups; (B) Safranin O-stained joint sections (10× and 40×). Ta, talus; Ti, tibia; CD, cartilage damage; CI, cell infiltration; BD, bone damage; (C) Semi-quantitative comparisons of histology scores of all treatment groups (*, $P < 0.05$, ANOVA).

Figure 5. Micro-CT analyses of the hind paw of the rats from different treatment groups
 (A) Representative 3-D reconstructed ankle joints from each treatment group. P-Tofa-treated rats were most similar structurally to the healthy group, while saline group exhibited extensive bone erosion. Significant bone damage was also found in the Tofa treated animals;
 (B) Bone morphometric parameters of the red cylinder ROI within calcaneus bone as shown in Figure 1; (C) Bone morphometric parameters of the entire calcaneus bone. No significant difference between the healthy and P-Tofa treated rats was found for all the parameters, indicative of the potent joint preservation capacity of the single dose P-Tofa treatment. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$)

Figure 6. Near infrared optical imaging-based analysis of P-Tofa biodistribution

(A) Representative IVIS images depicting P-Tofa-IRDye biodistribution in AA and healthy rats after systemic administration. Images obtained 1, 4, 7 and 12 days after one intravenous injection of P-Tofa-IRDye demonstrate its retention in arthritic joints; (B) Representative *ex vivo* optical imaging of major organs and limbs from AA rats at 1, 3 and 7-day post P-Tofa-IRDye administration; (C) Semi-quantitative analyses of P-Tofa-IRDye biodistribution. P-Tofa-IRDye signals were detected mainly in the arthritic joints, liver and kidneys.

Figure 7. Representative confocal microscopy of anti-CD68 and anti-P4HB antibody stained sections of decalcified ankle joints from AA rats following systemic administration of P-Tofa-Alexa

Each panel is composed of five subpanels: Antibody signal (green), P-Tofa-Alexa signal (red), DAPI signal (blue), a merged image at 200× magnification and a merged image at 630× magnification were shown. Co-localization of the red and green colors confirmed the internalization of the P-Tofa-Alexa by P4HB+ (fibroblast) and CD68+ (monocytes/macrophages) synoviocytes in the arthritic joints. White arrow points to the sites of colocalization.

Figure 8. qPCR analyses of expression of Arg1, Ym1/2 and Fizz1 from BMMs after treatment with IL-4

(A) Expression levels of IL4-induced genes after 24 hr Tofa or P-Tofa treatment; (B) Expression levels of IL4-induced genes after 24 hr Tofa or P-Tofa treatment, inhibitor washout and 72 hr additional culture without the inhibitors. (*, $P < 0.05$ versus no drug at same level of IL-4; ^, $P < 0.05$ versus Tofa at same level of IL-4)

Figure 9. Serum CXCL10 levels at days 35 from different groups of rats (*, $P = 0.05$; ****, $P = 0.0001$)

Scheme 1. A macromolecular prodrug of Tofacitinib (P-Tofa)

(A) The synthesis of HEMA-Tofa monomer; (B) The synthesis of P-Tofa.

Table 1

Hematologic profiles and liver function tests with P-Tofa and Tofa treatments.

	P-Tofa				Tofa		
	CBC						
	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks	
WBC (K/ μ L)	22.1 $\pm$ 6.1	14.9 $\pm$ 3.3	10.2 $\pm$ 2.7 *	24.6 $\pm$ 8.0	17.3 $\pm$ 5.9	19.1 $\pm$ 5.0	
NE (K/ μ L)	8.3 $\pm$ 3.0	5.0 $\pm$ 2.0	1.8 $\pm$ 1.7 *	11.0 $\pm$ 3.5	6.4 $\pm$ 4.1	7.7 $\pm$ 3.3	
LY (K/ μ L)	11.9 $\pm$ 3.0	7.7 $\pm$ 1.2	6.3 $\pm$ 2.0	12.0 $\pm$ 4.0	9.0 $\pm$ 3.3	9.9 $\pm$ 2.4	
MO (K/ μ L)	0.6 $\pm$ 0.3	1.2 $\pm$ 0.5	0.8 $\pm$ 0.5	0.7 $\pm$ 0.4	1.0 $\pm$ 0.4	0.6 $\pm$ 0.3	
EO (K/ μ L)	0.8 $\pm$ 0.6	0.6 $\pm$ 0.2	0.1 $\pm$ 0.1 *	0.7 $\pm$ 0.5	0.7 $\pm$ 0.4	0.6 $\pm$ 0.2	
BA (K/ μ L)	0.3 $\pm$ 0.3	0.3 $\pm$ 0.1	0.04 $\pm$ 0.04 *	0.3 $\pm$ 0.2	0.3 $\pm$ 0.2	0.3 $\pm$ 0.1	
Liver Function at Necropsy							
AST (U/L)		69.5 $\pm$ 2.0			67.6 $\pm$ 2.4		
ALT (U/L)		76.3 $\pm$ 4.7			76.6 $\pm$ 2.7		
ALP (U/L)		315.3 $\pm$ 29.4 *			347.3 $\pm$ 23.4		

* P 0.05, significantly lower than Tofa group