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Hydrogen Peroxide Inducible JAK3 Covalent Inhibitor: Prodrug for the Treatment of RA with Enhanced Safety Profile

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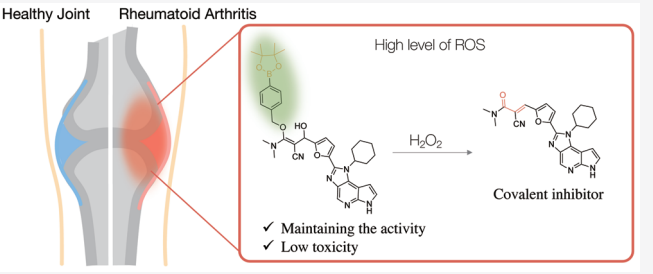
ABSTRACT: Selective inhibition of Janus kinases (JAKs) is an arising strategy in drug discovery. Covalent inhibitors targeting a unique cysteine in JAK3 exhibit ultraselectivity among JAK family members. However, safety and tissue specific concerns still remain. A prodrug of a known JAK3 covalent inhibitor sensitive to H_2O_2 was designed and synthesized and its therapeutic effect was evaluated in the CIA (collagen-induced arthritis) mice model of RA (rheumatoid arthritis). The prodrug strategy relied on the introduction of a hydrogen peroxide-sensitive borate trigger group to avoid random covalent binding to thiol functionalities in biomacromolecules. The results show that the prodrug can be activated and released under pathophysiological concentration of H_2O_2. In addition, the prodrug demonstrated stability to the physiological environment. In comparison to the parent compound, the prodrug showed a similar therapeutic effect in the CIA model but notably exhibited lower toxicity and a larger therapeutic window.



KEYWORDS: *Prodrug, H_2O_2, rheumatoid arthritis, JAK3 covalent inhibitor*

Rheumatoid arthritis (RA) is a systemic, chronic, progressive autoimmune disease,[1,2] which primarily affects the lining of the joints and causes erosion of the cartilage, bone damage, and deformity of joints at the later stages.[3,4] Common treatments shown to be effective against RA include TNF monoclonal antibodies and methotrexate.[5] However, not all patients respond adequately to these treatments.[6,7] Furthermore, while methotrexate is effective in treating rheumatism,[8] its mechanism of action is not fully understood at this time.[9,10] The JAK (Janus kinase) inhibitors, including tofacitinib (pan-JAK inhibitor)[11] and baricitinib (JAK1/2 inhibitor)[12,13] offer a novel and exciting opportunity for treating RA.[14,15] However, they carry the risk of undesirable side effects, including infection,[16,17] lipid effects,[18,19] anemia,[20,21] malignancies, and major adverse cardiovascular events.[22,23] This brings increasing attention to JAK selective inhibition.[4,24−26] Covalent inhibitors offer a solution to the JAK family selectivity issue due to a unique cysteine (Cys909) residue that only exists in JAK3 but not in JAK1/2.[27−29] Covalent JAK3 inhibitors may carry additional activities against kinases which also carry a cysteine ion in the equivalent position in their respective kinases (such as BTK and EGFR).[30−33]

In 2016, Michael Forster et al. developed a highly potent JAK3 covalent inhibitor (**1**) with an IC_{50} of 0.127 nM.[28] Our data suggested that **1** inhibits JAK3 in normal tissues and organs, which may cause hepatotoxicity and anemia. This compound contains a α-cyano-α,β-unsaturated ketone, a functional group which provides an opportunity to take advantage of a prodrug strategy, providing a high degree of tissue selectivity.[34] This can prevent the toxicity generated by inhibiting JAKs and other potential kinases in normal tissues and organs. The prodrug strategy is widely used to improve the safety and pharmacological properties of drugs. Among the various reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) plays important physiological roles in priming the immune system.[35−37] Due to oxidative stress, the expression levels of H_2O_2 are 100-fold greater in lesion tissues of immune diseases compared to normal tissue, with concentrations reaching up to 1 mM, and as a result several H_2O_2 responsive prodrugs have been developed.[38−45]

In 2018, Jorge Peiró Cadahía et al. reported a prodrug strategy that was applied to RA treatment.[37,46] The prodrugs they developed contain phenylboronic acids, a motif that can be activated by H_2O_2 (Figure 1). The parent drug will therefore be released in the presence of high concentrations of H_2O_2 in the pathological tissues. The strategy took advantage of the concentration differences of H_2O_2 in pathological tissues, making greater tissue accumulation of the drug possible.

Inspired by this prodrug strategy, we designed and synthesized compound **2**, based on covalent JAK inhibitor **1**.[28] The H_2O_2-responsive prodrug **2** contains a borate ester, which

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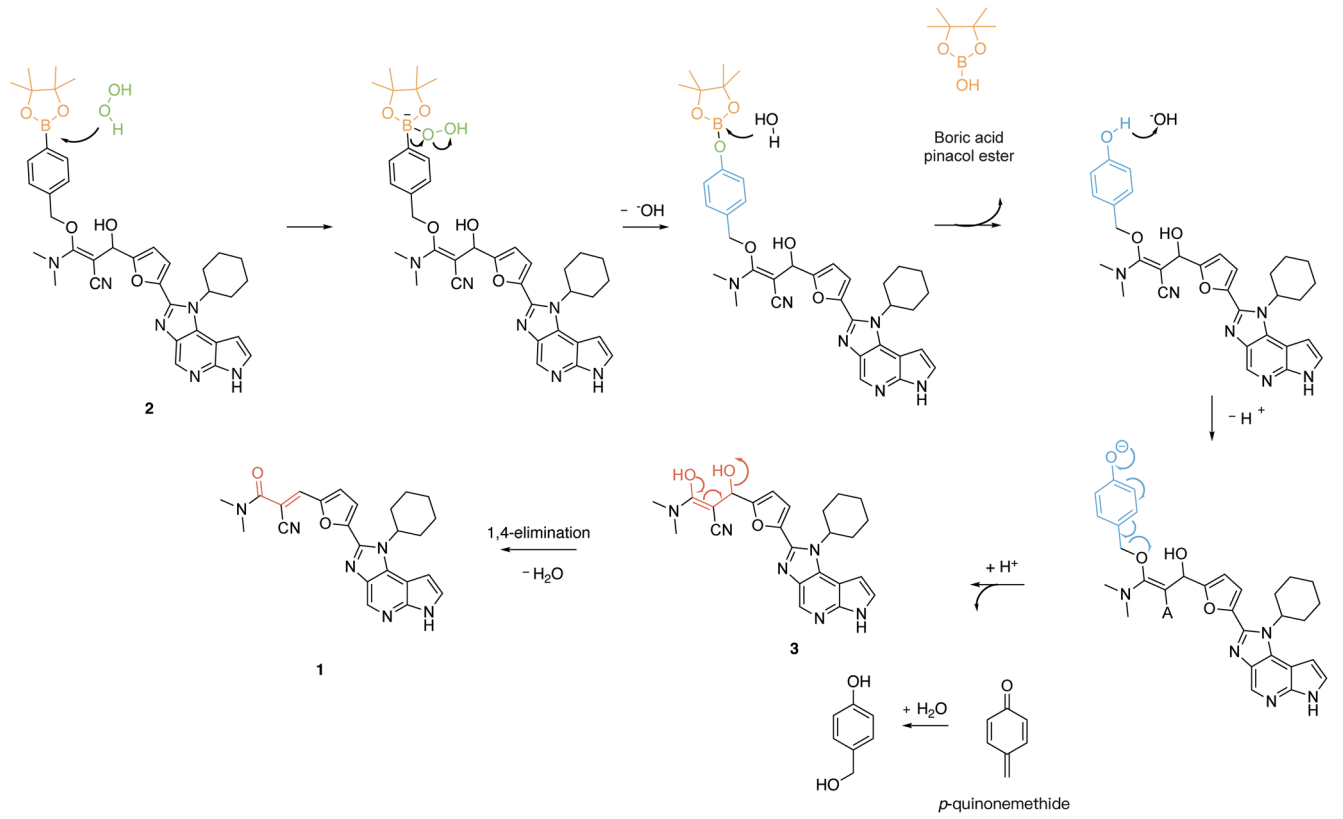
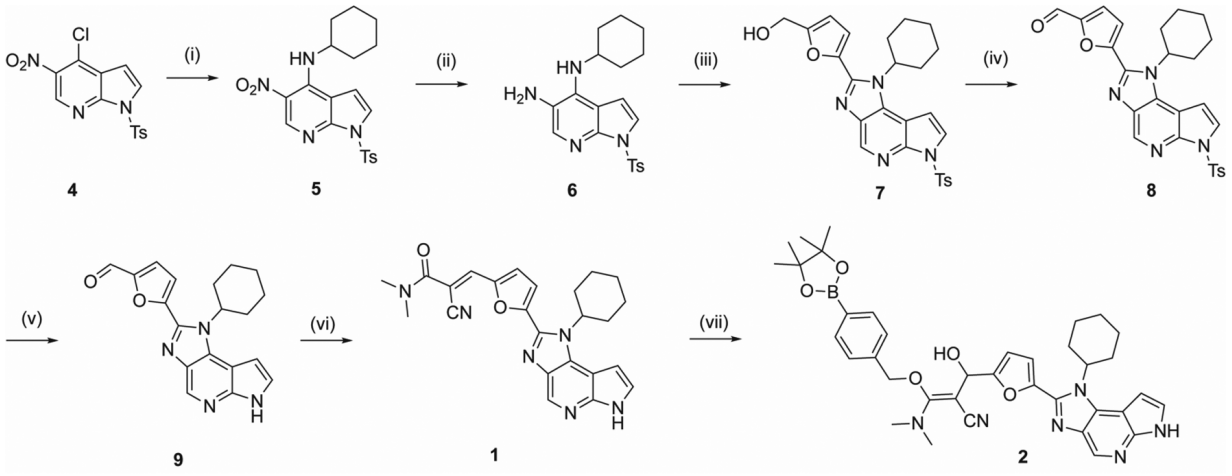


Figure 1. Mechanism of H_2O_2-responsive prodrug.

Scheme 1. Synthetic Route of Prodrug[a]



[a]*Reagents and conditions:* (i), Cyclohexylamine, TEA, Me_2CHOH, 80 °C, yield 91%; (ii), H_2, Pd/C, EtOH, THF, 13 h, 50 °C, yield 94%; (iii), 5-hydroxymethyl-2-furaldehyde, DMF/H_2O, 10 min; $KHSO_5$, 1 h, yield 82%; (iv), Dess−Martin periodinane, dry DCM, 25 °C, 1 h, yield 82%; (v), NaOH, THF/MeOH, 50 °C, 5 h, yield 84%; (vi), *N,N*-Dimethyl-2-cyanoacetamide, acetic acid:Piperidine = 1:1, EtOH, 80 °C, 1 h, yield 24%; (vii) (a) K_2CO_3, H_2O, DMF, overnight; (b) 4-(Bromomethyl)benzene boronic acid pinacol cyclic ester, 30 min, yield 24%.

can be released into the RA pathological tissue and improve the accumulation of compound **1**. We proposed mechanisms of original drug release are as follows. Triggered by ROS, **2** converted from benzyloxyether of borate to enol structure **3**. Benzyloxyether of borate is converted to *p*-quinonemethide and metabolized in the body, while compound **3** is converted into the active molecule, compound **1**, by 1,4-elimination due to chemical instability.

Furthermore, it is shown that prodrug **2** has better membrane permeability than parent drug **1**. We evaluated the prodrug properties of compound **2**, including its antiproliferative activity, the inhibition against JAK-STAT (Signal Transducer and Activator of Transcription) signaling pathway, the *in vivo* antiarthritis efficacy, and preliminary safety. The results showed that compound **2** achieved the same therapeutic effects as the parent drug **1** at the same dose (10 mg/kg) and reduced toxicity.

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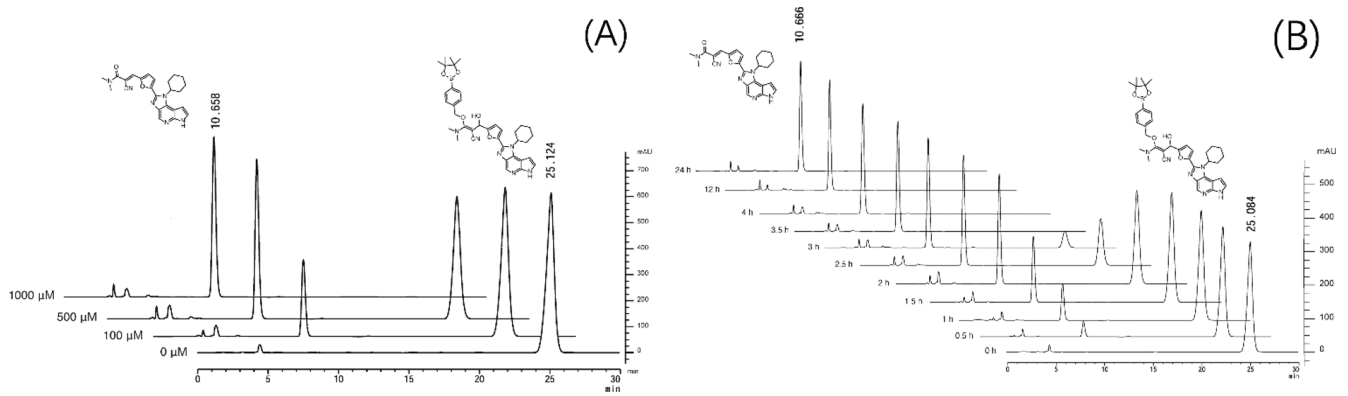

Figure 2. (A) Activation of prodrug after incubation with 0, 100, 500, and 1000 μM H_2O_2 respectively for 4 h at 37 °C. (B) Activation of prodrug after incubation with 1 mM H_2O_2 for 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 4, 12, 24 h respectively at 37 °C.

RESULT AND DISCUSSION·

Synthesis of Prodrug. JAK inhibitor **1** was synthesized according to reported procedures.[28] The synthetic route began with the nucleophilic aromatic substitution of cyclohexylamine to give intermediate **5**, which was followed by reduction via Pd/H_2 to acquire intermediate **6**. Subsequently, **6** was transformed via an imidazole ring closure reaction to provide intermediate **7**, which was then oxidized by Dess−Martin periodinane, yielding aldehyde intermediate **8**. Intermediate **8** was deprotected, resulting in free pyridine **9**, which underwent a Horner−Wadsworth−Emmons reaction to provide intermediate ketone **1**. JAK inhibitor **1** was etherified with 4-(bromomethyl) benzene boronic acid pinacol ester to furnish enol ether **2** (Scheme 1).

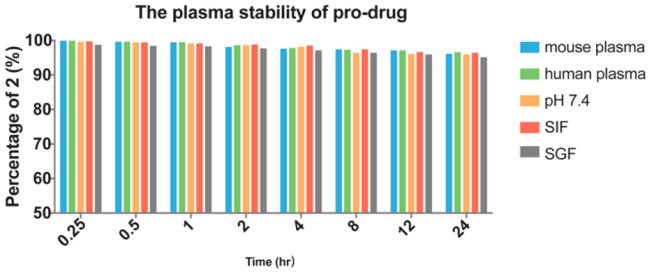

Figure 3. Percentage of prodrug **2** as a function of time after incubation with human, mouse plasma, simulated intestinal fluid (SIF), simulated gastric fluid (SGF), and phosphate buffer.

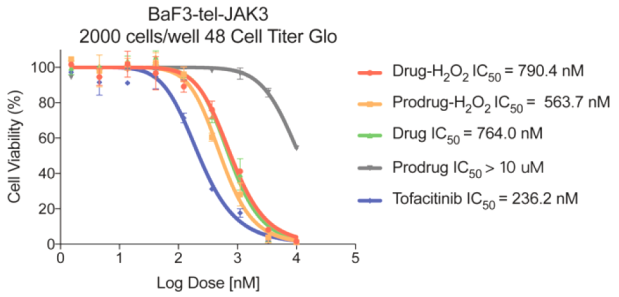

Figure 4. Cell viability profiles for BaF3-Tel-JAK3 cell line pretreated with/without H_2O_2 and treated with drug/prodrug of varied concentrations.

Table 1. Physicochemical Properties of Prodrug and Drug

Compound	M.W.	pK_a	Aqueous solubility at pH 7.4 (mg/kg)	Pe (10^{-6} cm/s) at pH 7.4
Drug **1**	428.5	10.11 ± 0.101	0.719	14.74 ± 0.34
Prodrug **2**	662.6	10.72 ± 0.334	0.011	56.38 ± 1.53

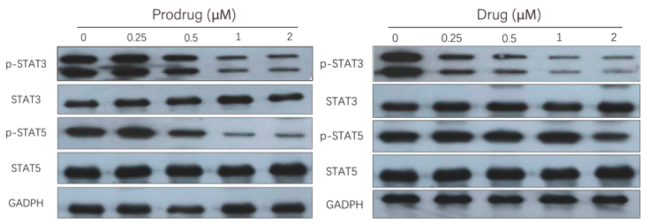

Figure 5. Phosphorylation level of STAT3 and STAT5 treated with prodrug and drug (concentrations increasing from 0, 0.25, 0.5, 1, to 2 μM).

Table 2. Pharmacokinetics Parameters of Drug/Prodrug in Healthy Rats

Parameter	Unites	Drug *i.p.* (20 mg/kg)	Prodrug *i.p.* (20 mg/kg)
$t_{1/2}$	h	2.36	2.92
C_{max}	μg/L	455.37	983.33
T_{max}	h	0.17	0.25
AUC	h·μg/L	331.91	963.50
$MRT_{(0-\infty)}$	h	3.84	2.38
Vz	L/kg	27.96	8.70
Clz	L/h/kg	6.02	2.10

Activation of Prodrug. We examined the effect of H_2O_2 concentration on prodrug activation. We evaluated the release of prodrug **2** using different concentrations of H_2O_2 (maximum concentration of 1 mM) solution (containing 30% PBS) for 4 h at 37 °C. As shown in Figure 2(A), prodrug can be released in a concentration-dependent manner. At a concentration of 0.1 μM H_2O_2, the prodrug was not released (no detection); at a concentration of 100 or 500 μM H_2O_2, prodrug was partially released (prodrug content, 27.1%). At the concentration of 1000 μM and higher, the prodrug was completely released (the parent drug could not be detected).

To evaluate the activation of prodrug **2** under pathophysiological concentrations of H_2O_2, we examined the effect of time on prodrug activation.[37] As shown in Figure 2(B), the prodrug can be released in a time-dependent manner. At 0.5 h, the prodrug was released (content 7.8%), and the parent drug (content 78.1%) was detected. At 3.5 h, prodrug **2** had been completely released (no detection).

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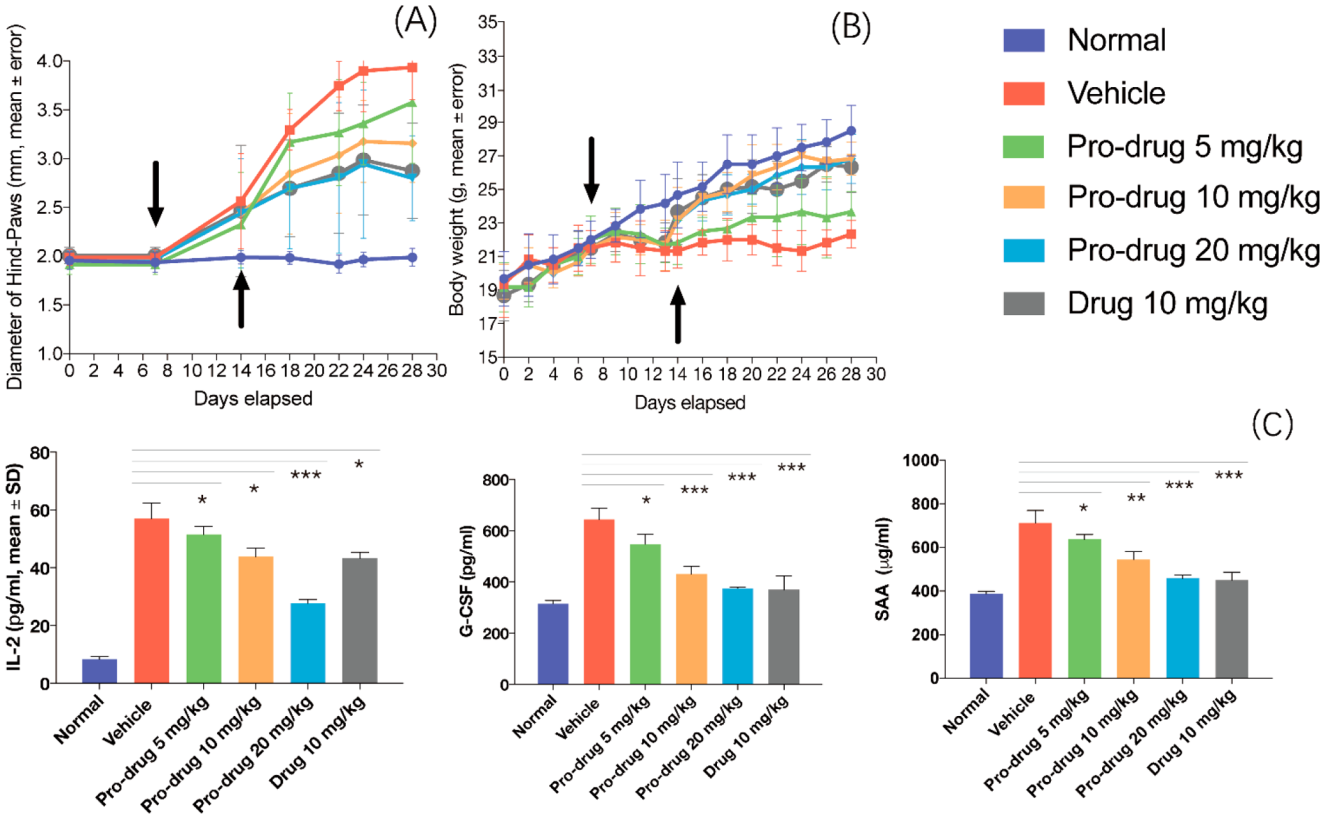

Figure 6. Antiarthritic Efficacy of prodrug *in vivo*. (A) Diameter of mice hind-paws during suppression of CIA development after treatment with prodrug (5, 10, and 20 mg/kg), drug (10 mg/kg), and vehicle (n = 6 per group). (B) Bodyweight during suppression of CIA development. (The up arrow is the time of the second immunization; the down arrow is the time of administration.) (C) The level of inflammatory factors (IL-2, G-CSF, and SAA) in blood in each group.

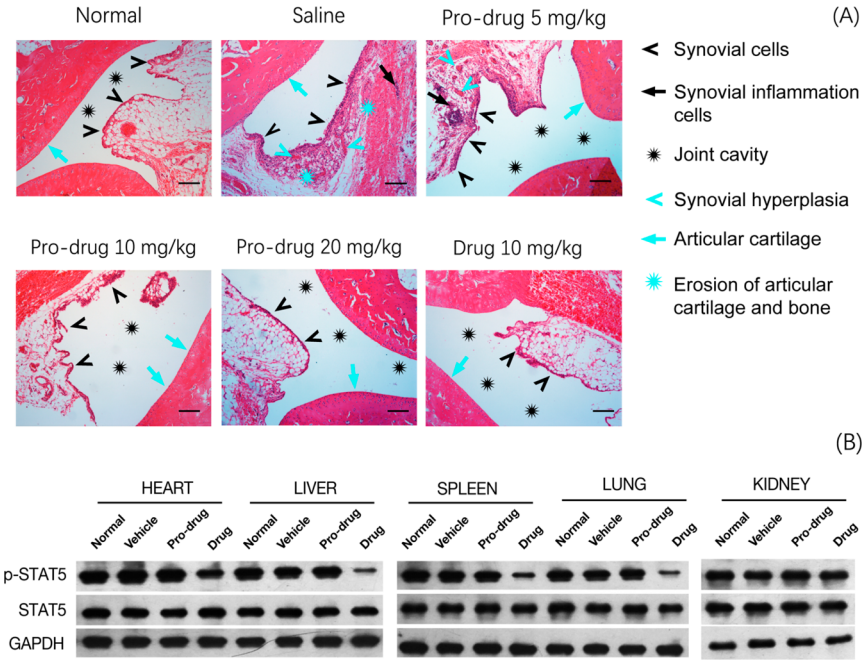

Figure 7. (A) HE analysis on ankle joint of each group. (B) Phosphorylation level of STAT3 and STAT5 in tissues (heart, liver, spleen, lung, and kidney) in normal group, vehicle group, pro-drug 20 mg/kg group, and drug 10 mg/kg group, respectively.

Chemical, Mouse Plasma, and Human Plasma Stability. To test the stability of prodrug **2** in saline, mouse plasma, human plasma, simulated intestinal fluid (SIF), and simulated gastric fluid (SGF), we evaluated prodrug content after exposure to plasma at 37 °C for different time points (0.25, 0.5, 1, 2, 4, 8, 12, and 24 h respectively) by HPLC (Figure 3). The experimental results show that prodrug **2** content is greater than 95% within 24 h, demonstrating good stability in all tests.

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Table 3. Drug/Prodrug Concentration in Ankle Joint in a CIA RA Model

Group	Homogenate concentration (μM)	
	Drug	Prodrug
Drug 20 mg/kg	1.07 ± 0.23	
Prodrug 20 mg/kg	1.13 ± 0.42	0.29 ± 0.09

Antiproliferative Activity, Physiochemical Properties, and Regulation of JAK-STAT Signal Pathway. We tested the antiproliferative activity of drug **1** and prodrug **2** in the presence and absence of H_2O_2 against BaF3-tel-JAK3 cells, a JAK3 kinase activity-dependent growth transgenic cell line (Figure 4).[47,48] The experimental results showed that the prodrug molecule can be released in the medium containing H_2O_2 and exert antiproliferative activity with an IC_{50} of 563.7 nM. Drug **1** and positive control to facitinib also exert antiproliferative activity with an IC_{50} of 790.4 nM, 236.2 nM, respectively. The prodrug showed no antiproliferative activity in the medium without H_2O_2 (IC_{50} > 10 μM, the original drug molecule **1** IC_{50} = 764.0 nM).

To show that prodrug **2** is superior to **1** in antiproliferative activity, we tested the physicochemical properties of these two compounds. We determined the acid dissociation constant (pK_a) and solubility using a Gemini Profiler instrument and tested the permeability (Pe) using a PAMPA instrument.[49] Shown in Table 1, prodrug **2** displays similar pK_a properties to drug **1**. While prodrug **2** demonstrates lower solubility than drug **1**, it exhibited more favorable membrane permeability.

To detect the effects of prodrug **2** and drug **1** on the signaling pathways of JAK3-STAT3 and JAK3-STAT5, we performed a western-blot assay based on BaF3-Tel-JAK3 cells. Shown in Figure 5, prodrug **2** and drug **1** have regulatory effects on both p-STAT3 and p-STAT5.

Pharmacokinetics. Prodrug **2** and drug **1** were selected for further *in vivo* pharmacokinetics studies in healthy SD rats. As shown in Table 2, drug and prodrug showed acceptable half-lives (2.36 and 2.92 h). In addition, prodrug showed a higher exposure level than drug. Both compounds showed low peak time (0.17 and 0.25 h). Prodrug showed different property of volumes of distribution compared to drug.

Antiarthritic Efficacy. To test whether this prodrug is effective *in vivo*, prodrug **2** was introduced into a mouse model of CIA rheumatoid arthritis that is commonly utilized in *in vivo* evaluation of rheumatoid drugs.[50] Additionally, the model is considered to have significant oxidative stress characteristics and has a high H_2O_2 concentration in joint tissues, claws, and limbs.[51,52] We randomly divided 30 male CIA Dba/1j mice of 8−10 weeks into five groups, each *i.p.* administered with saline, **2** (5 mg/kg, 10 mg/kg and 20 mg/kg), and **1** (10 mg/kg) once a day for a total of 14 days. During the experiment, we examined the mice's foot diameter and body weight, shown in Figure 6(A) and Figure 6(B), respectively. The experimental results show that the prodrug and the original drug have an immunosuppressive effect on the swelling of the feet in mice. The prodrug 20 mg/kg group and the original drug 10 mg/kg group resulted in the maximum inhibition of mice feet swelling. Additionally, the prodrug group demonstrated a dose-dependent inhibition of swelling of the mice feet.

After determining that prodrug **2** was effective in the treatment of RA *in vivo*, we tested the serum levels of inflammatory factors, namely Interleukin-2 (IL-2), Granulocyte-colony stimulating factor (G-CSF) and Serum amyloid A (SAA) in each group of mice. Shown in Figure 6(C), the prodrug administration group significantly showed a reduced number of inflammatory factors in the blood in a dose-dependent manner.

We performed HE (Hemotoxylin and Eosin) staining analysis on the ankle joint of each group of mice to determine the

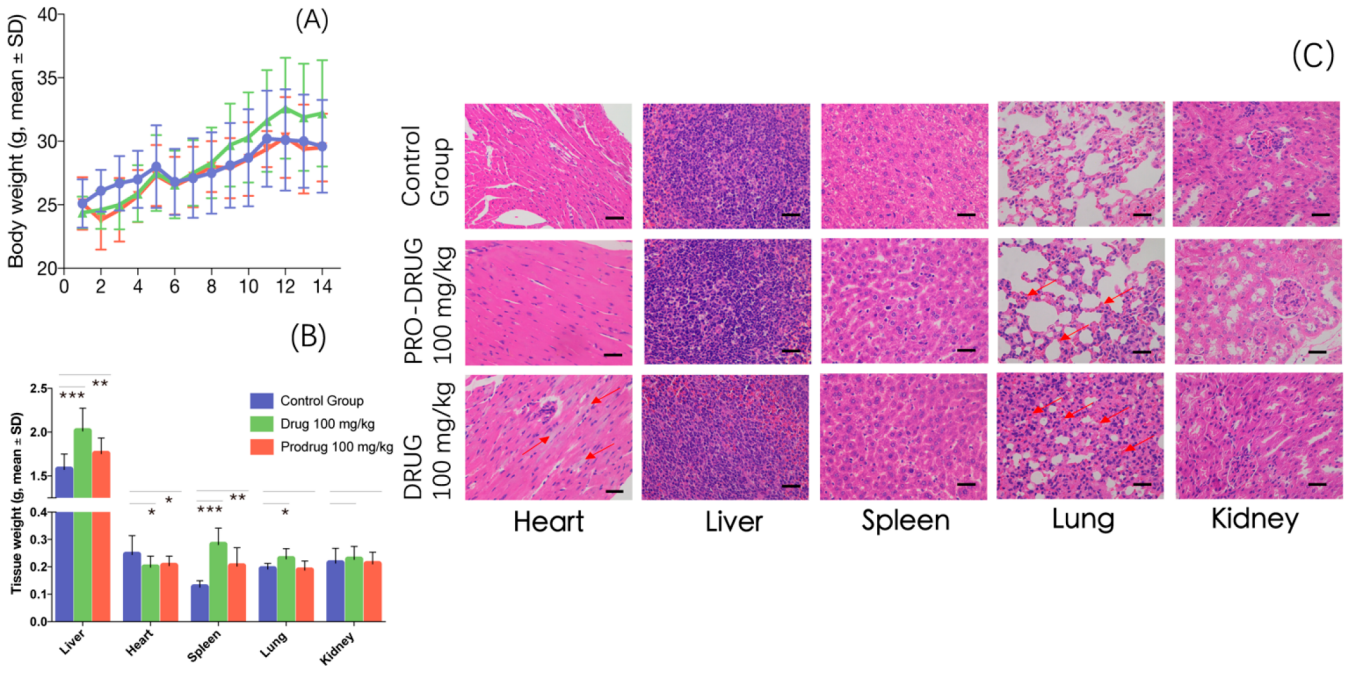

Figure 8. Subacute toxicity evaluation of Prodrug in healthy ICR mice (n = 10). The mice were treated with vehicle, prodrug (100 mg/kg), or drug (100 mg/kg), respectively. (A) Body weight change of each group during the treatment. (B) Tissue weight of each group after the treatment. (C) Representative images of heart, liver, spleen, lung, and kidney tissues stained by hematoxylin and eosin at 400×.

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pathological condition of CIA Dba/1j mice after administration, as shown in Figure 7(A). The synovial cells of the saline treated group were disordered and severely degenerated. The cartilage and bone were eroded, accompanied by synovial hyperplasia, and the vasodilatation in the synovium was congested. The synovial cells of **2** in the 20 mg/kg group were disordered and eroded, accompanied by synovial hyperplasia.

To examine the effects of prodrug **2** and drug **1** on JAK3-STAT5 signaling pathways in various tissues, such as heart, liver, spleen, lung, and kidney, we performed western-blot experiments on each tissue in the normal group, vehicle group, prodrug 20 mg/kg group, and drug 10 mg/kg group, respectively. As shown in Figure 7(B), the prodrug did not exhibit JAK3 inhibitory activity and down-regulate the phosphorylation level of STAT5 in other tissues, like the original drug.

In addition, we collected and ground the mice ankle joints of the prodrug 20 mg/kg group and drug 10 mg/kg group to determine the drug/prodrug at the site of action in CIA Dba/1j mice after administration. We detected the concentration of drug and prodrug, respectively. As shown in Table 3, the concentration of drug/prodrug in the ankle joint is greater than 1 μM, and most of the prodrug has been released to drug.

Preliminary Toxicity. To test the safety of prodrug **2** and drug **1** in mice, ICR mice were *i.p.* administered at a 5-fold effective dose (100 mg/kg) on a daily basis for 14 days. During the administration, we measured the body weight of ICR mice as indicated by Figure 8(A). Comparing the three groups of data, the prodrug group and the control group had similar body weight trends, but in the drug **1** group, the weight growth rate of the mice increased after the eighth day. In addition, we performed anatomy, weighing, and pathological analysis of the organs of the mice. The experimental results showed that the weight of the liver and spleen of the original drug group was increased compared with the control group and the prodrug group, and there was no significant difference in weight between other tissues. In the pathological section analysis, the original drug **1** has obvious inflammatory infiltration in the heart and lung, as indicated by the red arrows shown in Figure 8(C). Prodrug **2** was administered at a dose of 100 mg/kg in mice in a single administration for 14 days, demonstrating a favorable safety profile.

CONCLUSION

We have developed a novel JAK3 covalent inhibitor prodrug modification strategy based on H_2O_2 for the delivery of drug to tissues associated with RA. This blocked the high-reactivity α,β-unsaturated ketone group of the covalent inhibitor, while it maintained the activity and low toxicity of the original drug. Prodrug **2** can be activated by H_2O_2 and showed acceptable physicochemical properties, chemical stability, and pharmacokinetic properties. Prodrug **2** showed a therapeutic effect on the CIA model in the mice feet, while the JAK3-STAT signaling pathway was not affected in other tissues, especially the spleen and lung. Notably, compared to drug **1**, **2** exerts a good safety profile. This prodrug not only would benefit from further research on RA therapy but also provides a new avenue for the study of JAK3 covalent agents.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsmedchemlett.0c00323.

General considerations; details of chemical synthesis, activation assays, stability assay, physiochemical properties assay, *in vitro* studies including antiproliferative activity and western-blot assay, *in vivo* studies including pharmacokinetics, antiarthritic efficacy and preliminary toxicity assay; ^{1}H NMR and M/S (ESI) spectra of compounds **1** and **4**−**8**; and ^{1}H NMR, ^{13}C NMR, HRMS (ESI), and HPLC spectra of **2** (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

JAK, Janus kinase; CIA, collagen-induced arthritis; RA, rheumatoid arthritis; BTK, bruton's tyrosine kinase; EGFR, epidermal growth factor receptor; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; STAT, signal transducer and activator of transcription; SIF, simulated intestinal fluid; SGF, simulated gastric fluid; Pe, permeability; IL-2, interleukin-2; G-CSF, granulocyte-colony stimulating factor; SAA, serum amyloid A; TLC, thin-layer chromatography; TMS, tetramethylsilane; HRMS, high-resolution mass spectra; CFA, complete freund's adjuvant; HE, Hemotoxylin and Eosin

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