RESEARCH ARTICLE

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Design, synthesis and evaluation of (*R*)-3-(7-(methyl(7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)-5-azaspiro[2.4]heptan-5-yl)-3-oxopropanenitrile as a JAK1-selective inhibitor†

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Based on (*R*)-*N*-methyl-*N*-(5-azaspiro[2.4]heptan-7-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine as a core scaffold, we identified (*R*)-3-(7-(methyl(7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)-5-azaspiro[2.4]heptan-5-yl)-3-oxopropanenitrile [(*R*)-6c] as a JAK1 selective inhibitor. The structural design was based on the combination of tofacitinib's 7-deazapurine and 5-azaspiro[2.4]heptan-7-amine. Compound (*R*)-6c exhibited an IC₅₀ value of 8.5 nM against JAK1 with a selectivity index of 48 over JAK2. To optimize (*R*)-6c as a lead compound, we performed *in vitro* ADME, hERG, kinase profiling, and pharmacokinetic tests. Mouse and rat *in vivo* studies verified that (*R*)-6c exhibited desired efficacies in CIA and AIA models.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that affects approximately 1–2% of the world's population.^{1,2} Considerable work has been conducted in search of therapeutic targets^{3–5} and recently emerging molecular targets like cytokines,^{6,7} G-protein coupled receptors,⁸ and kinases^{9,10} have surfaced. Therapeutic agents against these targets are categorized as disease-modifying antirheumatic drugs (DMARDs).¹¹ The most commonly used drugs include conventional synthetic DMARDs, such as methotrexate, sulfasalazine, leflunomide, and so on.¹² However, they cannot be used for long-term treatment due to low therapeutic response and severe side effects. To overcome such limitations, researchers have developed biological DMARDs¹³ like etanercept, infliximab, and adalimumab. Although they exhibit higher efficacies than the synthetic ones, their applications also have several drawbacks such as high costs, efficacy limitations on single administration,¹⁴ limited intravenous (i.v.) administration accessibility,¹⁵ and so on.

To meet the unmet medical needs in RA, many researchers have concentrated their efforts on developing new synthetic DMARDs equipped with high efficacy, low cost, and a convenient administration route. As a result, the Janus kinase (JAK)/signal transducer and activator of the transcription (STAT) signal pathway has been identified as one of the new therapeutic targets. Janus kinases had first been isolated in 1989 (ref. 16) and their roles were discovered in 1994.¹⁷ Factors involved in the JAK-STAT signaling like the cytokines, receptors, STATs, and JAKs are related to many autoimmune diseases including rheumatoid arthritis, psoriasis, myelofibrosis, Crohn's disease, and ulcerative colitis. JAK3, out of the four isotypes, has received the most attention since it is mostly located in hematopoietic cells and affects the lymphoid cell function unlike others.^{18,19} In 2003, researchers at Pfizer reported tofacitinib as a JAK3 inhibitor;²⁰ however, later it was identified as a pan-JAK inhibitor.²¹ The fact that it suppresses all JAK-STAT signal pathways explains its excellent potencies in many preclinical²² and clinical trials.^{23–25} Finally, tofacitinib became the first US Food and Drug Administration (FDA) approved oral drug for the treatment of rheumatoid arthritis in 2012 with the trade name Xeljanz.²⁶

Using pan-JAK inhibitors like tofacitinib for treatment is accompanied by some side effects since they inhibit all JAK isoenzymes. In particular, preclinical studies^{27–29} and clinical trials^{30,31} have revealed adverse effects derived from JAK2 inhibition like anemia, neutropenia, increased low and high density lipoprotein cholesterol levels, and elevated triglyceride levels. In the case of tofacitinib, similar adverse events have also been reported.^{24,25,32} As a result, the European

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Medicines Agency (EMA) refused the marketing authorization of tofacitinib in Europe.³³ Therefore the search for JAK1-selective inhibition has received considerable attention³⁴ since it has been revealed that JAK1 inhibition plays a pivotal role in the efficacy of tofacitinib.³⁵

Among various JAK1-selective inhibitors,³⁶ Galapagos' filgotinib^{37,38} is known to be highly selective for JAK1 over JAK2 by 27.7 times. Its IC₅₀ values against IL-6/JAK1/pSTAT1 and GM-CSF/JAK2/pSTAT5 are 629 nM and 17 453 nM, respectively.³⁹ From collagen-induced arthritis (CIA) mouse and rat models, its efficacy was shown to be similar to that of etanercept, a TNF- α blocker.⁴⁰ Through the phase IIa proof-of-concept study, the hypothesis was proven that rheumatoid arthritis can be ameliorated by treatment with JAK1-selective inhibitors.⁴¹ In 2016, Galapagos and Gilead went into phase III clinical studies.^{42–44} Despite its advantages, the reported preclinical results indicated that it induced testicular toxicity in rats and dogs. Thus, the US FDA approved a lower male maximum clinical dosage than for the female one.⁴⁵ Therefore, new JAK1-selective drugs overcoming this toxicological weakness need to be developed. Another promising candidate compound in this class is upadacitinib in phase III by AbbVie.^{46,47} Although not much toxicological information on the preclinical and clinical trials of upadacitinib is available, its IC₅₀ values for JAK1 and JAK2 in cellular assay were reported to be 8 nM and 600 nM, respectively, indicating 74-fold selectivity.⁴⁸

We have initiated our investigation on new JAK1-selective inhibitors based on the 5-azaspiro[2.4]heptan-7-amine core structure for subjugating the filgotinib limitation. New lead compounds were obtained, which met the criteria set by us for treating rheumatoid arthritis. Herein we describe the design, synthesis and improved pharmaceutical efficacies of our inhibitors.

Strategy

Since tofacitinib is known to interact with the binding sites of ATP as a competitive inhibitor for JAK isozymes, we focused our attention on the structure of tofacitinib, which resembles adenosine triphosphate (ATP) minus the triphosphate group.⁴⁹ According to the X-ray crystallography of tofacitinib at JAK1 or JAK2 reported by N. K. Williams *et al.*,⁵⁰ the most important interaction of tofacitinib in its binding with JAKs appears to come from the pyrrolopyrimidine ring: it interacts with JAK1 with 4 hydrogen bonds and 11 van der Waals interactions. The next important interactions are 4 van der Waals interactions with four carbon atoms (C4–C7) at the piperidine ring. We envisioned that, to tune the inhibition selectivity for JAK1 over JAK2, new structural motifs could be explored for the substituted aminopiperidine moiety.

Synthesis

Various monocyclic and bicyclic nitrogen containing compounds 6a–6g were synthesized for the selection of the most

Fig. 1 Design strategy by changing the piperidine moiety A.

optimal scaffold at position A in Fig. 1, as shown in Scheme 1. Commercially available 4-amino-1-benzylpiperidine and (*R*)-3-amino-1-benzylpiperidine were converted to the *N*-ethyloxycarbonyl protected compounds, which were treated with LAH to result in the formation of methylamine derivatives 3a and 3b. The key pyrrolidine component of compound 6c, 5-((*R*)-1-phenylethyl)-5-azaspiro[2.4]heptan-7-amine, was prepared according to the method reported by Y. Kimura and colleagues.⁵¹ We obtained each diastereomer of 5-((*R*)-1-phenylethyl)-5-azaspiro[2.4]heptan-7-amine with carbon 7 as the epimeric center. Methylated compound 3c was obtained through the above method from 5-((*R*)-1-phenylethyl)-5-azaspiro[2.4]heptan-7-amine. Compound 3d was synthesized through debenzoylation of 2c. A bis(hydrochloric acid) salt form of bicyclic amine 3e, (*R,R*)-6-benzyl-octahydro-pyrrolo[3,4-*b*]pyridine dihydrochloride, was purchased from Sigma-Aldrich, USA. Commercially available compounds such as 4-hydroxypiperidine and *rac*-3-hydroxymethylpiperidine were converted to the corresponding benzylated amines, 3f and 3g.

The obtained amines and alcohols, 3a–3g, were used for the coupling with 6-chloro-7-deazapurine in the presence of K₂CO₃ in an aqueous solution, leading to compounds 4a–4g. We then performed debenzoylation of 4a–c and 4e–f using palladium on carbon and ammonium formate to remove benzyl and (*R*)-1-phenylethyl protection groups, obtaining 5a–c and 5e–f. In the case of 5d, the *N*-ethyloxycarbonyl group of 4d was deprotected with 1 N aqueous hydrochloric acid under reflux conditions. From compounds 5a–5g the corresponding amide coupling reactions were carried out with ethyl cyanoacetate in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at 80 °C, as shown in Scheme 1, leading to compounds 6a–6g. Since the inhibitor (*R*)-6c synthesized from 7(*R*)-5-((*R*)-1-phenylethyl)-7-amino-5-azaspiro[2.4]heptane (*R*)-1c showed the most promising inhibition selectivity between JAK1 and JAK2, we focused our efforts on compound (*R*)-6c. Amine (*R*)-4c was transformed into the final compounds 7–56 through various reactions at the pyrrolidine nitrogen.

Results and discussion

Enzyme assay

For the selection of a new scaffold, we first screened substituted piperidine and pyrrolidine scaffolds at position A

Methylation of primary amino groups

Compounds 1-3

Synthesis of inhibitors

Scheme 1 Synthesis of inhibitors containing various heterocyclic core units replacing the aminopiperidine unit of tofacitinib and substituted *N*-methyl-*N*-(5-azaspiro[2.4]heptan-7-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amines.

in Fig. 1 (Table 1). Each compound was evaluated for inhibition against JAK1 and JAK2 at a concentration of 1 μ M. In the case of 6a and 6f, the inhibition abilities against JAK1 and JAK2 do not appear to be influenced by the connecting atom (nitrogen vs. oxygen) at the C(4) position of piperidine. However, the amino-substitution position at the piperidine ring appeared to be an important factor for determining affinities for not only JAK1 but also JAK2. Between 6a and 6b, the substitution at the C(3) position of piperidine (6b) was more favoured for both JAK1 and JAK2 inhibitions than the substi-

tution at the C(4) position (6a) was. The substitution with the methoxy group (6g) was disfavoured for JAK1 affinity. In the case of 6e, introduction of the bicyclic (4a*R*,7a*R*)-octahydro-1*H*-pyrrolo[3,4-*b*]pyridine lowered the inhibition against JAK1. In introducing the 5-azaspiro[2.4]heptan-7-amine moiety, the substitution position was important. While compound 6c exhibited strong inhibition against JAK1, 6d displayed very low inhibition against JAK1. Though both 6b and 6c showed strong inhibition against JAK1, 6b showed high inhibition against JAK2 as well. Therefore, we selected 6c as our scaffold for further SAR studies for finding inhibitors with high JAK1/JAK2 selectivity.

Our further SAR study was based upon 5c as a scaffold for derivatives on the pyrrolidine nitrogen. When a racemic mixture (6g) was tested against the JAK1 isozyme (Table 2), it showed an IC_{50} value of 29 nM, proving that it could be used as a good lead for new JAK1 inhibitors. Then we investigated each enantiomer of 6c. Compound (*R*)-6c exhibited an IC_{50} value of 8.5 nM against JAK1, whereas an IC_{50} of 790 nM was observed with the enantiomeric (*S*)-6c. As a result, for further SAR studies, the (*R*)-configuration of 7-amino-5-azaspiro[2.4]-heptane was chosen.

In the cases of *N*(5)-alkylated compounds 7, 8, and 9, inhibitor 9 having an *N*-benzyl group showed higher affinity to JAK1 than those with short alkyl groups but was disfavoured against JAK2 (120 nM in JAK1 vs. 98 000 nM in JAK2) (Table 3). The inhibitor with the benzylamine group displayed a selectivity index of 820 for JAK1 over JAK2.

As for inhibitors with amide groups at the pyrrolidine nitrogen, those with cyanoacetyl and azidoacetyl substitutions ((*R*)-6c and 10, respectively) were quite potent against JAK1 with IC_{50} values of 8.5 and 21 nM, respectively. A slightly larger isovaleric amide 11 exhibited comparable activity for JAK1 inhibition. It is interesting to note that two similarly sized amides, isobutyramide 12 and cyclopropanecarboxamide 13, exhibited similar JAK1 inhibitions. However, compound 12 showed a higher selectivity index against JAK2 than 13. This tells us that there may be a more sensitive structural interaction in JAK2 with the amide motif. Amides substituted with a polar group like compounds 14–16 did not show significant inhibition against JAK1. Aroyl amides 18–24 also showed IC_{50} values in the 100–1000 nM range except for the small 2-furanoyl amide 17, which gave an IC_{50} of 53 nM against JAK1. This indicates again that a large amide group at the pyrrolidine nitrogen is not tolerated well in the JAK1 binding site.

The transition from an amide to a thioamide lowered the inhibition activity against JAK1 but not so much against JAK2 that the inhibitor possessing 2-cyanoethanethioamide exhibited a lower selectivity index than that with 2-cyanoacetate ((*R*)-6c vs. 25). Introducing a urethane (26) into the pyrrolidine nitrogen resulted in considerable potency (IC_{50} = 34 nM) against JAK1 with a selectivity index of 35. Urea compounds (27–36), except for 31 and 36, exhibited two-digit nanomolar IC_{50} values against JAK1. For ureas made up with aliphatic amines, 1-butylurea 27 and

Table 1 Screening of the hydrophobic moieties (moiety A)

Compound		Inhibition% at 1 μ M	
		JAK1	JAK2
6a		49%	1%
6b		99%	83%
6c		95%	53%
6d		6%	10%
6e		54%	-7%
6f		35%	5%
6g		30%	10%

1-cyclohexylurea **28** showed similar affinities for JAK1 with IC_{50} values of 75 and 69 nM, respectively. However, compound **28** with a cyclic alkyl urea group was inferior in the JAK2 inhibition with an IC_{50} value of 4400 nM to the acyclic urea **27** with an IC_{50} of 2800 nM. Comparing 1-cyclohexyl urea **28** and phenyl urea **29** indicates that the inhibition abilities of the latter were higher against both JAK1 and JAK2 with IC_{50} values of 12 nM and 420 nM, respectively. Halide-substituted phenyl ureas **30–35** did not show any improvements in inhibitory activities compared to the parent phenyl urea, **29**. When the phenyl group of the phenyl urea was substituted with an *ortho*-phenyl group (compound **36**), the inhibition of JAK1 and JAK2 decreased precipitously with IC_{50} values of 1500 and 9100 nM, respectively, presumably due to increased steric hindrance.

In the case of sulfonamides, most compounds displayed strong inhibition against the two enzymes. Some inhibitors showed single digit nanomolar range IC_{50} values against JAK1. When amides and sulfonamides of similar sizes were

compared, in all cases the inhibitors possessing a sulfonamide showed increased affinities for JAK1: the IC_{50} values of **12** vs. **39**, **18** vs. **41**, **21** vs. **46**, and **22** vs. **47** were 170 vs. 20, 150 vs. 9.0, 100 vs. 5.8, and 190 vs. 9.8 nM, respectively. However, the sulfonamide inhibitors also increased their inhibition against JAK2, leading to lower selectivity indices than those of amide inhibitors. The JAK1 affinity appeared to be quite sensitive towards the substituent on benzene-sulfonamide (**42–50**): the *meta*-substitution gave the best inhibition whereas the *ortho*-substitution showed the lowest affinities for the JAK1 isozyme.

According to our enzyme assays, (*R*)-**6c** and **46** seemed to be more selective for JAK1 over JAK2 than filgotinib, of which the IC_{50} values are 10, 28, 810, and 116 nM for JAK1, JAK2, JAK3, and TYK2, respectively.⁴⁰ Therefore, we selected two representative compounds, (*R*)-**6c** and **46**, for additional evaluation against JAK3 and TYK2. The IC_{50} of (*R*)-**6c** for JAK3 and TYK2 were 1100 and 250 nM with selectivity indices of 130 and 29, respectively. For **46**, 110 and 25 nM IC_{50} values were observed for JAK3 and TYK2, respectively.

Docking simulation

To probe the validity of our approach with molecular modeling, we examined a flexible docking simulation of our representative compound (*R*)-**6c** with JAK1 (PDB ID: 3EYG) by AutoDock 4.2 (ref. 52) (Fig. 2). It appeared that its pyrrolopyrimidine moiety can bind to Ala906, Glu957 and Leu959 of JAK1 through hydrogen bonds and van der Waals interactions. Moreover, the (*R*)-5-azaspiro[2.4]heptan-7-amine moiety of (*R*)-**6c** was expected to interact with Val889 and Gly1020. As a result, the above strategy of modifying the aminopiperidine moiety seems to be suitable in finding a new JAK1-selective inhibitor.

In vitro ADME studies on (*R*)-**6c**, **38**, and **46**

Several *in vitro* ADME profiles such as plasma stability, protein binding, liver microsomal stability, Caco-2 permeability, and cytochrome P₄₅₀ inhibition for selected JAK1 inhibitors, (*R*)-**6c**, **38** and **46**, were investigated. First, over 98% of **38** and **46** remained in human and rat plasma for 120 minutes (Table 4). In the case of (*R*)-**6c**, 98.5% remained in human plasma for the same duration, showing similar stabilities to **38** and **46**. However, in rat plasma, 89.6% of (*R*)-**6c** remained after 120 minutes. Therefore, it can be concluded that most tested compounds remained as their parent drug structures in plasma. In human plasma protein binding tests (Table 5), the bound proportion of (*R*)-**6c** was 29.8%, which was similar to that of tofacitinib citrate and filgotinib, 39% and 31.8%, respectively.^{26,39} However, **38** and **46**, both of which have sulfonamide groups, showed higher protein binding – 44.4% and 87.4%, respectively. The results correlate well with their lipophilicities: the log*P* values of their neutral forms gradually increase in the order amide (*R*)-**6c**, aliphatic sulfonamide **38**, and aromatic sulfonamide **46**.

Table 2 Comparison of JAK1 IC_{50} values of 3-(7-(methyl(7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)-5-azaspiro[2.4]heptan-5-yl)-3-oxopropanenitrile racemate and enantiomers

Compound	Configuration at 7-position	JAK1 IC_{50} (nM)
6c	Racemate	29
(<i>R</i>)- 6c	(<i>R</i>)	8.5
(<i>S</i>)- 6c	(<i>S</i>)	790

Table 3 The IC₅₀ values against JAK1 and JAK2 and the selectivity indices of substituted (*R*)-*N*-methyl-*N*-(5-azaspiro[2.4]heptan-7-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amines

Cmpd	R	IC ₅₀ (nM)			Cmpd	R	IC ₅₀ (nM)		
		JAK1	JAK2	SI ^a			JAK1	JAK2	SI ^a
7		1400	30 000	21	26		34	1200	35
8		230	15 000	65	27		75	2800	37
9		120	98 000	820	28		69	4400	64
(<i>R</i>)-6c		8.5	410	48	29		12	420	35
10		21	250	12	30		23	1000	43
11		77	1100	14	31		120	4700	39
12		170	2800	16	32		40	1300	33
13		170	1200	7.1	33		54	2800	52
14		520	16 000	31	34		74	1300	18
15		670	13 000	19	35		85	1300	15
16		180	2500	14	36		1500	9100	6.1
17		53	930	18	37		470	11 000	23
18		150	6300	42	38		7.2	180	25
19		160	7800	49	39		20	130	6.5
20		140	6500	46	40		6.9	36	5.2
21		100	7400	74	41		9.0	51	5.7
22		190	4500	24	42		13	97	7.5
23		500	9100	18	43		2.8	8.4	3.0
24		420	11 000	26	44		9.4	50	5.3
25		20	160	8.0	45		50	480	9.6

Table 3 (continued)

Cmpd	R	IC ₅₀ (nM)			Cmpd	R	IC ₅₀ (nM)		
		JAK1	JAK2	SI ^a			JAK1	JAK2	SI ^a
46		5.8	59	10	52		9.3	180	19
47		9.8	1100	110	53		16	1400	88
48		26	470	18	54		17	150	8.8
49		1.4	5.5	3.9	55		15	150	10
50		6.3	30	4.8	56		31	260	8.4
51		12	66	5.5					

^a SI, Selectivity Index = JAK2 IC₅₀/JAK1 IC₅₀.

To probe the stability of the selected compounds in the liver first-pass, liver microsomal stabilities were examined (Table 6). The amount of remaining compound (*R*)-6c (94.6%) after 30 minute incubation in human liver microsomes was similar to that of filgotinib (87% after 60 minute incubation).³⁹ While compound 38 possessing an ethanesulfonamide group was less stable than filgotinib, with 79.7% remaining after 30 minutes, the *m*-cyanophenylsulfonamide-containing compound 46 was

heavily metabolized in human liver microsomes and only 6.0% remained after 30 min incubation (Table 7).

To probe drug-drug interaction possibilities, we screened compounds (*R*)-6c, 38, and 46 against representative CYP₄₅₀ isoforms at 10 μM concentrations of each compound (Table 8). CYP 2C19 and 2E1 were influenced at the same concentrations of (*R*)-6c. Compound 38 seemed to inhibit only the CYP 2E1 isoform at this concentration. However, 46 treatment was likely to affect many isoforms including CYP 1A2, 2C8, 2C9, 2C19, 2E1, and 3A4, which may lead to possible interactions with many drugs. The CYP 2E1 isoform, which (*R*)-6c, 38, and 46 commonly inhibited, has substrates for some anaesthetics like halothane, enflurane, methoxyflurane, sevoflurane, and so on.⁵³ Representative substrates of CYP 2C19 comprise proton

Fig. 2 Flexible docking simulation of (*R*)-6c with JAK1 (PDB ID: 3EYG) by AutoDock 4.2.⁵²

Table 4 Plasma stabilities of (*R*)-6c, 38, and 46

Compound	Human (% remaining)		Rat (% remaining)	
	30 min	120 min	30 min	120 min
(<i>R</i>)-6c	95.0	98.5	98.4	89.6
38	>100	>100	>100	>100
46	96.6	98.6	>100	>100

Table 5 Plasma protein binding and log *P* values of (*R*)-6c, 38, and 46

Compound	Human (% bound)	Rat (% bound)	Log <i>P</i> (neutral X)
(<i>R</i>)-6c	29.8	18.7	1.69
38	44.4	49.5	2.07
46	87.4	85.8	3.69

Table 6 Liver microsomal stabilities of (*R*)-6c, 38, and 46

Compound	% Remaining after 30 min			
	Human (%)	Dog (%)	Rat (%)	Mouse (%)
(<i>R</i>)-6c	94.6	99.9	84.8	84.9
38	79.7	56.1	22.9	33.9
46	6.0	5.5	2.4	3.2

Table 7 Caco-2 permeabilities of (*R*)-6c, 38, and 46

Compound	P_{app} A to B (10^{-5} cm s $^{-1}$)	P_{app} B to A (10^{-5} cm s $^{-1}$)	Efflux ratio
(<i>R</i>)-6c	0.66	1.85	2.78
38	2.54	2.56	1.01
46	1.34	1.57	1.17

pump inhibitors including esomeprazole, lansoprazole, and so on. In the case of the proton pump inhibitors, their prescription frequencies are decreasing in recent times, so the combined prescription would not be a problem.

Due to high plasma protein binding, low liver microsomal stability, and high CYP₄₅₀ inhibition rates, we excluded compounds with sulfonamide groups from further studies. Therefore, (*R*)-6c was chosen for studies on hERG, kinase profiling, and *in vivo* efficacy tests.

Human ether-a-go-go related gene (hERG) potassium channel assays and kinase profiling

Next, we investigated the hERG binding of (*R*)-6c for cardiotoxicity prediction. The binding test was carried out with HEK293 cells according to the automated patch clamp method with an IC₅₀ value of 1.2×10^2 μ M. Under the same conditions, filgotinib gave an IC₅₀ value of 85 μ M. From these results, (*R*)-6c appears to be superior to filgotinib in cardiotoxicity at the same blood concentration predicted by the hERG assay.

Kinase inhibitors targeting an ATP-binding site show a probability of inhibiting other kinases in addition to the targeted one, which can result in some predictable side effects. Therefore, we screened (*R*)-6c against 323 kinases at a concentration of 10 μ M (Fig. 3). Under this condition, only four JAK family kinases including JAK1, JAK2, JAK3, and TYK2 showed over 90% inhibition. The kinases with 80–90% inhibition were only ROCK-IIIs derived from rats and humans.

Rodent pharmacokinetics of (*R*)-6c

To determine the dosage for *in vivo* efficacy tests, we investigated the pharmacokinetic profiles of (*R*)-6c in rats (Table 9). In the case of the oral administration at the 10 mg kg $^{-1}$ dose, the pharmacokinetic profiles of (*R*)-6c were obtained as follows: the maximum concentration (C_{max}) was 1000 ng mL $^{-1}$, the time to reach the maximum concentration (T_{max}) was 0.30 hours, the half-life ($t_{1/2}$) was 2.1 hours, and the area under the curve from time 0 to infinite time ($AUC_{0-\infty}$) was 2100 ng h mL $^{-1}$. However, the bioavailability of (*R*)-6c exceeded 100%. This is presumed to be caused by the use of

Fig. 3 The kinome tree of (*R*)-6c against 323 kinases at a concentration of 10 μ M drawn by means of the web accessible Kinome render program.⁵⁸

PEG400 as a vehicle to overcome poor water solubility, which apparently lowered the compound's i.v. exposure.⁵⁴ To improve (*R*)-6c exposure in the *in vivo* model, we made several different salts using hydrochloride, citric acid, and tartaric acid (Fig. 4). For the hydrochloride and citrate salts, their drug exposures increased by 26% compared to the free base form. However, the tartrate salt was relevantly less exposed than the free base in the oral administration. Moreover, the citrate form had the additive advantage that its half-life was prolonged to 3.6 hours. Hence, the citrate form appears to be the preferred formulation in oral administration.

In vivo efficacy studies on (*R*)-6c

Both collagen-induced arthritis (CIA) and adjuvant-induced arthritis (AIA) are well-established animal models for the testing and development of new RA therapeutics.^{55,56} We used these models to evaluate the efficacies for the treatment with (*R*)-6c in the free base form. In the mouse CIA study, the effect of (*R*)-6c treatment was evaluated by using the following indices: clinical arthritis score, paw volume, serum cytokine concentration, bone surface/volume ratio, and histopathological data of ankles. Filgotinib (100 mg per kg per day)⁴⁰ was used as a positive control. Treatment with (*R*)-6c (25, 50 or 100 mg per kg per day) resulted in significant attenuation of arthritis in DBA1/J mice compared to vehicle treatment

Table 8 The inhibition percentages against CYP₄₅₀ isoforms of (*R*)-6c, 38, and 46

Compound	CYP ₄₅₀ isoforms (% of control activity @ 10 μ M)							
	1A2	2A6	2B6	2C8	2C9	2C19	2D6	2E1
(<i>R</i>)-6c	92.5	92.0	91.5	81.0	84.4	66.0	91.6	50.3
38	99.9	96.7	>100	>100	90.7	93.8	>100	63.9
46	65.7	87.2	92.0	66.2	50.4	55.8	90.3	59.8

Table 9 Pharmacokinetic parameters of the free base and the salt forms of (*R*)-6c in Sprague-Dawley rats

Sample	(<i>R</i>)-6c							
	Free base		Hydrochloride		Citrate		Tartrate	
Salt form								
Route	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.	P.O.	I.V.
<i>N</i> (S.D. rat)	4 M	4 M	4 M	4 M	4 M	4 M	4 M	4 M
Dose (mg kg ⁻¹)	10	5	10	5	10	5	10	5
<i>C</i> _{max} (ng mL ⁻¹)	1000		1300		1200		1000	
<i>T</i> _{max} (h)	0.30		0.40		0.50		0.40	
<i>t</i> _{1/2} (h)	2.1	1.2	1.1	0.60	3.6	4.3	1.0	1.0
AUC _{0→inf} (ng h mL ⁻¹)	2100	920	2400	1900	2400	2000	1900	1400
AUC _{0→t} (ng h mL ⁻¹)	1900	900	2400	1800	2400	2000	1900	1400
MRT (h)	3.1	1.1	1.6	0.7	2.3	1.2	1.5	0.9
<i>F</i> (%)	110		65		58		68	

(Fig. 5). Compound (*R*)-6c treatment ameliorated the clinical arthritis score, paw volume, serum IL-6 concentration, bone surface/volume ratio and histopathological parameters in a dose-dependent manner. Furthermore, at doses of 50 and

100 mg per kg per day, (*R*)-6c treatment exhibited more favourable results than filgotinib treatment did in clinical arthritis score, cytokine levels, bone surface/volume ratio and all histopathological features.

In the rat AIA study (Fig. 6), all treatments with test articles significantly suppressed the arthritis symptoms *versus* vehicle treatment for 14 days. The treatment with 20 mg per kg per day of (*R*)-6c demonstrated higher efficacy than that of filgotinib (20 mg per kg per day). The clinical arthritis scores at a dosage of 5, 10, and 20 mg per kg per day of (*R*)-6c reached 10.0, 10.6, and 9.0 values, respectively, and paw thicknesses were 10.33, 10.21, and 10.20 mm, respectively, at day 14. However, filgotinib showed a slightly inferior clinical arthritis score (10.2) and paw thickness (10.32 mm) (day 14) compared to (*R*)-6c at the same dosage.

Experimental section

Chemistry

The synthetic methods of representative compounds and the spectroscopic data of all compounds are described in the ESI.†

In vitro enzyme assays and kinase profiling

All enzyme inhibition assays including the kinase profiling results were obtained from commercially available kinase binding activity assays, KinaseProfiler™ services (Eurofins Scientific, UK).⁵⁷ All kinase binding activity assays were performed at *K*_m values for ATP. The 50% inhibitory concentration (IC₅₀) of each compound was determined with GraphPad Prism software. The kinome tree of the inhibition percentages of 323 kinases at a concentration of 10 μ M for (*R*)-6c was drawn by R. Najmanovich's Kinome Render web accessible tool.⁵⁸

Fig. 4 Plasma concentrations after a) intravenous injection and b) oral administration of the free base and the salt forms of (*R*)-6c in Sprague-Dawley rats.

Fig. 5 Effects of (R)-6c treatment on collagen-induced arthritis in DBA/1J mice: a) the clinical arthritis scores for 18 days, b) the volumes of right hind paws on days 1 and 15, c) and d) the concentrations of IL-6 and TNF- α , respectively, in the serums sampled after autopsy, e) the bone surface/volume ratios of right hind ankle joints measured by micro-CT, f) the histopathological semiquantitative scores of right hind ankle joints, g) the right hind ankle joint thicknesses, h) and i) the articular surface cartilage thicknesses (tibia and talus) in right hind ankle joints, and j) the numbers of inflammatory cells infiltrated in the right hind ankle joints. The significance symbols are as follows: ** = significantly different between G1 and G2 ($P < 0.01$), + = significantly different from G2 ($P < 0.05$), and ++ = significantly different from G2 ($P < 0.01$).

In vitro ADME assays

All *in vitro* ADME, including plasma stability, plasma protein binding, liver microsomal stability, Caco-2 permeability, and hERG assays, were performed by commercially available services at the New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation, South Korea and the Drug Discovery Platform Technology Group, Korea Research Institute of Chemical Technology, South Korea. The plasma stability, plasma protein binding, liver microsomal stability, Caco-2 permeability, and CYP₄₅₀ inhibition tests were analysed by LC-MS/MS, using a Nexera XR system (Shimadzu, Japan) equipped with a TSQ vantage triple quadrupole mass spectrometer (Thermo, USA). The column was a Kinetex XB-C18 column (2.1 $\times$ 100 mm, 2.6 μ m particle size; Phenomenex, USA) and the obtained data were analysed in the Xcalibur program (version 1.6.1).

Plasma stability assay

Human or rat plasma was treated with test articles at a concentration of 10 μ M. Procaine and diltiazem were used as

positive controls. The plasma tubes were incubated at 37 $^{\circ}$ C for 0, 30, and 120 minutes. Acetonitrile including an internal standard, chlorpropamide, was added to the tube, which was vortexed and centrifuged with a power of 14 000 rpm at 4 $^{\circ}$ C. After the centrifugation, the supernatant was analysed by LC-MS/MS.

Plasma protein binding test

The rapid equilibrium dialysis (RED) method was used for the plasma protein binding test. The positive controls were dexamethasone and warfarin. Human or rat plasma was treated with test articles at a concentration of 10 μ M. The same volumes of the treated plasma and phosphate-buffered saline (PBS, pH 7.4) were placed in the RED chamber. The chamber was incubated at 37 $^{\circ}$ C for 4 hours. The same volumes of the incubated plasma and buffer were sampled and the same volumes of buffer and blank plasma were added, respectively. Acetonitrile including an internal standard, chlorpropamide, was added to each sample tube, which was

Fig. 6 Effects of (R)-6c treatment on adjuvant-induced arthritis in Lewis rats: a) the clinical arthritis scores and b) the volumes of right hind paws. The data were measured twice per week for 14 days.

vortexed and centrifuged with a power of 14 000 rpm at 4 °C. After the centrifugation, the supernatant was analysed by LC-MS/MS.

Liver microsomal stability test

The liver microsomes of a human, dog, rat, or mouse (0.5 mg mL⁻¹), 0.1 M phosphate buffer, and a test article at a concentration of 1 μ M were placed in a tube. The positive control was verapamil. The tube was incubated at 37 °C for 5 minutes. NADPH regeneration system solution was added to the tube, which was incubated at 37 °C for 30 minutes. Acetonitrile, including an internal standard, chlorpropamide, was added to the tube, which was vortexed and centrifuged with a power of 14 000 rpm at 4 °C. After the centrifugation, the supernatant was analysed by LC-MS/MS.

Caco-2 permeability assay

In a 12-well transwell, 1×10^6 cells of Caco-2 cells (ATCC® HTB-37™) were seeded and they were grown for 3 weeks. The test article was diluted to a concentration of 25 μ M with transport buffer (10 mM glucose, 4 mM sodium bicarbonate, and 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in Hank's balanced salt solution, pH 7.4). The positive controls were caffeine, ofloxacin, and atenolol. The test article was added to one chamber of the apical and basolateral chambers and then the transport buffer was added to the other. During 60-minute incubation at 37 °C, samples from each chamber were taken every 15 minutes. The samples were diluted to a concentration of 5 μ M with acetonitrile in-

cluding an internal standard, chlorpropamide. The samples were analysed by LC-MS/MS.

CYP inhibition test

A mixture of human liver microsomes (0.25 mg mL⁻¹), 0.1 M phosphate buffer, each cocktail including substrates for CYP₄₅₀ isozymes, and the test article at a concentration of 0 or 10 μ M was incubated at 37 °C for 5 minutes. The cocktails are as follows: Cocktail A, phenacetin 50 μ M + coumarin 2.5 μ M + *S*-mephenytoin 100 μ M + dextromethorphan 5 μ M + midazolam 2.5 μ M; Cocktail B, bupropion 50 μ M + aminodauquine 2.5 μ M + tolbutamide 100 μ M + chlorzoxazone 50 μ M. Then NADPH generation system solution was added and it was incubated at 37 °C for 15 minutes again. After the incubation, the reaction was quenched with acetonitrile including chlorpropamide as an internal standard. It was centrifuged with a power of 14 000 rpm at 4 °C. After the centrifugation, the supernatant was analysed by LC-MS/MS.

Human ether-a-go-go related gene (hERG) potassium channel assay

hERG assay was performed with the automated planar patch clamp method in PatchXpress® 7000A (Molecular Devices, LLC., USA). HERG-HEK293 cells ($2-4 \times 10^6$ cells) were placed in a 384 well plate. Amphotericin B solution was added for the perforated patch clamp recording and then it was left to stand for 10 minutes. To measure the hERG normal current, the HEK293 cell membrane was held at -80 mV, and the current of the potassium channel was measured while the voltage was changed as follows: -40 mV for 100 milliseconds (ms), +40 mV for 500 ms, and -50 mV for 2 seconds. After measuring the normal current with the said method, the HEK293 cells were treated with the test article solution at the desired concentration. After 5 minutes, the current of the potassium channel treated with the test article was measured with the aforementioned method.

Pharmacokinetic study

Pharmacokinetic studies were performed on Sprague Dawley rats that were 7 to 8 weeks old. All groups consisted of four male rats fed freely for intravenous tests but had fasted for 16 hours beforehand per oral test. The dosages for the intravenous and oral tests were 5 and 10 mg kg⁻¹, respectively. The free base form of (R)-6c clearly dissolved under the vehicle condition of 10% ethanol and 90% PEG400 so a dose volume of 1 mL kg⁻¹ was used for the intravenous administration. For the oral administration, a suspension in corn oil was used, which had a 5 mL kg⁻¹ dose volume. The salt forms include hydrochloride, citrate, and tartrate, which clearly dissolved in 100% saline so that their dose volumes became 250 and 2500 μ L kg⁻¹ for IV and PO, respectively. After their administrations, the blood sampling was performed at 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours for IV and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 hours for PO. 20 μ L of the sampled plasma was diluted with 180 μ L of acetonitrile

containing an internal standard. It was then vortexed and centrifuged under 15 000 rpm at 4 °C. After the centrifugation, the supernatant was analyzed by LC-MS/MS, using a Nexera XR system (Shimadzu, Japan) equipped with a TSQ vantage triple quadrupole mass spectrometer (Thermo, USA). The column was a Kinetex XB-C18 column (2.1 × 100 mm, 2.6 μm particle size; Phenomenex, USA) and the pharmacokinetic parameters were obtained using the non-compartmental analysis model in Phoenix WinNonlin version 6.4 (Pharsight, USA). The animal care and procedures in this study were approved by the Animal Research Care Committee of the New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation. The committee assures that the welfare of the animals was maintained in accordance with the requirements of the Animal Welfare Act of the Republic of Korea.

Mouse collagen-induced arthritis

Male DBA1/J mice (6 weeks old) were purchased from Japan SLC, Inc. and all mice were housed under specific pathogen-free (SPF) conditions with free access to food and water. After 7 days of acclimation, the mice were immunized with 0.1 mL of an emulsion of a 1:1 mixture of type II collagen (2 mg mL⁻¹) and complete Freund's adjuvant by subcutaneous injection 1.5 cm distal from the tail base. After 21 days, the immunized mice were boosted by another injection with 0.1 mL of an emulsion of type II collagen and incomplete Freund's adjuvant. The emulsions were prepared according to manufacturer's instructions.⁵⁹ When all mice indicated signs of arthritis, treatment with test articles and assessment of arthritis were initiated (day 1). The immunized and boosted mice were randomized into 6 treatment groups (n = 10 each) and same-aged naive mice were assigned to a normal group (n = 5). All test articles or the vehicle were orally administered once daily and the clinical arthritis scores were assessed twice weekly for 18 days. Corn oil was used as the vehicle and all test articles were suspended in the vehicle. The test article doses were 25, 50, and 100 mg per kg per day for (R)-6c, and 100 mg per kg per day for filgotinib. Paw volumes were measured using a LE7500 plethysmometer (Panlab, Spain) on days 1 and 15. The severity of each paw was evaluated and scored according to the following criteria: 0 = normal; 0.5 = with redness of the toe, but not swollen; 1 = one toe inflamed and swollen; 2 = more than one toe, but not the entire paw, inflamed and swollen, or mild swelling of the entire paw; 3 = entire paw inflamed and swollen; 4 = very inflamed and swollen paw or ankylosed paw.⁶⁰ The clinical arthritis score was represented by the total score for each paw. On day 19, all individuals were sacrificed and autopsies were performed. Serum cytokines including IL-6 and TNF-α, were measured using ELISA kits (ProcartaPlex Mix and Match customized, Mouse 5 plex, BMS). For the histopathological studies, the right hind paws of each mouse were fixed with 10% formalin solution and hematoxylin-eosin staining was performed on the ankle and the third digit of the paw. The histopathological score was semiquan-

titatively measured according to the following criteria: 0 = normal; 1 = infiltration of inflammatory cells; 2 = synovial hyperplasia and pannus formation; 3 = bone erosion and destruction.⁶¹ The obtained images were analyzed using iSolution EL version 9.1 (IMT i-Solution Inc., Canada) and the micro-CT analyses of all individuals were performed using vivaCT 80 micro-CT (SCANCO Medical, Switzerland) to measure the bone surface/volume ratio. Student's *t*-test or one-way analysis of variance was performed to determine statistically significant differences. The data for clinical arthritis scores were statistically analyzed using the Kruskal–Wallis test or Mann–Whitney test where a significant difference was defined as *P* < 0.05. The experimental protocol of the mouse study was approved by the Animal Research Care Committee of Gyeonggi Biocenter (Approval No. 2015-11-0019). The committee assures that the welfare of the animals was maintained in accordance with the requirements of the Animal Welfare Act of the Republic of Korea.

Rat adjuvant-induced arthritis

AIA was induced in SPF Lewis LEW/SsNSlc rats (Japan SLC Inc., Japan). After 2 weeks of acclimation, 10 week old rats were immunized by the subcutaneous injection of 0.1 mL of complete Freund's adjuvant containing 10 mg mL⁻¹ of heat-killed mycobacterium (Chondrex, Inc., USA) 2.0 cm distal from the rat tail base. After 12 days of immunization (day 1), the rats were randomized into 6 treatment groups (n = 10 each) and they received test articles or the vehicle alone once daily for 14 days. Same-aged naive mice were assigned to a normal group (n = 5). Corn oil was used as the vehicle and the test article doses were 5, 10, and 20 mg per kg per day for (R)-6c, and 20 mg per kg per day for filgotinib. The clinical arthritis score and paw thicknesses were evaluated twice weekly for 14 days. The criteria for the clinical arthritis score are as follows: 0 = normal; 1 = mild edema or erythema; 2 = moderate edema; 3 = severe edema; 4 = ankylosis. The paw thicknesses were measured using a CD-15CPX electric caliper (Mitutoyo Corp., Japan). The Kruskal–Wallis test or one-way analysis of variance was performed to determine statistically significant differences, which were defined as *P* < 0.05. The experimental protocol of the rat study was approved by the Animal Research Care Committee of Qu-BEST BIO, Co., Ltd. (Approval No. QBSIACUC-A17001). The committee assures that the welfare of the animals was maintained in accordance with the requirements of the Animal Welfare Act of the Republic of Korea.

Conclusions

From the SAR studies hinged on the heterocyclic ring portion of the JAK1 inhibitors, we chose (R)-6c as our candidate for further evaluation and have shown the efficacy of (R)-6c through *in vitro* and *in vivo* tests. In the enzyme assays, the JAK1 IC₅₀ value was 8.5 nM and the selectivity index for JAK1 over JAK2 was 48, which was higher than that of filgotinib. In the kinase profiling, the inhibitory activities against other

kinases were lower than against the JAK series. From the above *in vitro* tests, we obtained highly JAK1-selective profiles for our inhibitor, which presumably would lead to lower toxicity.

In the *in vitro* ADME tests, its profiles were similar to those of filgotinib. The compound (**R**)-**6c** showed good human plasma stability along with two positive controls to exhibit similar profiles on the percentages bound to human plasma protein and the stability against human liver microsomes. Thus, there was moderate permeability from A to B in Caco-2 permeability tests like for filgotinib but a lower efflux ratio, so it seems to be more highly permeable to cells than filgotinib. In the CYP₄₅₀ isozyme screening, the compound showed inhibition of 2C19 and 2E1 isoforms at 10 μ M concentrations. In pharmacokinetic studies in rats through oral administration, the profiles of the free base were at acceptable levels.

In the *in vivo* studies, (**R**)-**6c** relieved the arthritis symptoms more than an equivalent dose of filgotinib, the JAK1-selective inhibitor belonging to the same category, did. Taken together, our present study indicates that (**R**)-**6c** has desirable physicochemical properties and efficacy *via* selective inhibition of the JAK1 pathway. These findings suggest that (**R**)-**6c** has therapeutic potential for the treatment of rheumatoid arthritis.

Conflicts of interest

The authors declare no competing interests.

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