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Towards Selective Inhibitors of Janus Kinase 3: Identification of a Novel Structural Variation between Janus Kinases 2 and 3

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The mammalian Janus kinase (JAK) family of intracellular nonreceptor protein tyrosine kinases consists of four isoforms (JAK1, JAK2, JAK3 and TYK2) that play key roles in the cytokine-mediated JAK-STAT (signal transducer and activator of transcription) signaling pathway which controls survival, proliferation, and differentiation of a variety of cells.[1,2] Due to this pivotal role in cytokine signaling, JAKs have emerged as potential therapeutic targets for cytokine-related diseases. JAKs bind to different receptors. In particular, JAK3 interacts with cytokine receptors that contain the common gamma chain (γc).[3] The γc-receptors are the most important chemokine receptors because they bind interleukins (IL) -2, -4, -7, -9, -15 and -21, which are critical for development and function of lymphocyte. As a result, mutation of the γc-JAK3 signaling results in severe combined immunodeficiency (SCID) phenotype.[4,5] Likewise, humans or animals lacking JAK3 display severe combined immunodeficiency disease, indicating the crucial role of JAK3 in T-cell development and homeostasis of the immune system.[6] Also noteworthy is that, unlike other JAKs experiencing significant side effects due to their ubiquitous expression,[7] JAK3 is specifically expressed in activated immune cells, which renders JAK3 as an even more interesting target for immune-regulation. Thus, development of a selective JAK3 inhibitor has been anticipated to provide a novel immunosuppressive agent with an optimal therapeutic window. Several synthetic JAK3 inhibitors identified by pharmaceutical companies are currently in the process of clinical evaluation. In particular, CP-690,550, a highly potent inhibitor against JAK3 (IC_{50} = 1 nM), showed significant extension of life of the organ-transplanted animal[8,9] and demonstrated efficacy in phase III clinical trials for the treatment of rheumatoid arthritis[10,11] and rejection in kidney transplant patients.[12] Nevertheless, the difficulty of developing CP-690,550 as an immunosuppressant is related with the low selectivity between JAK3 and JAK2 (IC_{50} = 1 nM and 20 nM, respectively)[8] because concurrent inhibition of JAK2 would result in significant anemia particularly dangerous for patients under immunomodulating therapy.[13] Several pharmaceutical companies such as Merck,[14] Aventis,[15] and Procter and Gamble[16] have also reported potent JAK3 inhibitors but the selectivity between JAK3 and JAK2 has rarely been accomplished.

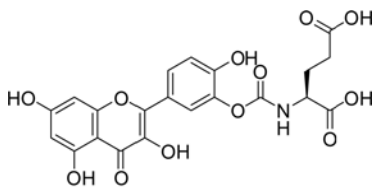

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Figure 1. Structure of quercetin-3-*O*-glutamate (**1**).

Obviously, the lack of selectivity of CP-690,550 and other ATP-competitive inhibitors must be attributed to the shared sequence homology (62%) between JAK3 and JAK2, along with a very high homology in the ATP-binding domain.[17-19] In this context, it is noteworthy that the recently published JAK3-selective inhibitor[20] include a unique phenyl-indolyl maleimide scaffold of which maleimide functionality is believed to play a key role in discriminating the ATP-binding site of JAK3 from that of JAK2. This result demonstrates that, in spite of high sequence homology between JAK3 and JAK2, it is still feasible to design a JAK3-selective inhibitor through incorporation of a novel ATP-mimic into the kinase inhibitor scaffold. Therefore, the purpose of this research was to identify a JAK3-selective scaffold which would provide selectivity over JAK2 to avoid potential side effects.

Setting out to explore the novel scaffold for JAK3-selective inhibitors, natural flavonoid drew our attention because it has been demonstrated to competitively bind at the ATP-binding site to inhibit various mammalian kinases.[21] The proven safety profiles of the natural flavonoids were also anticipated to provide the flavonoid-derived immunomodulators with additional benefits. In the previous proof-of-concept study, we tested our in-house flavonoid library for inhibition of JAK3. Among the 120 flavonoids tested, quercetin-3'-*O*-glutamate (**1**, Fig. 1)[22] showed 40% inhibition of JAK3 at 10 μM. Prompted by this result, we prepared a series of novel flavonoid derivatives and evaluated their JAK3-selective inhibitory activity. Herein we report *de novo* design, synthesis, and biological evaluation of novel JAK3-selective flavonol derivatives.

First of all, the characteristic binding mode of **1** to the ATP-binding domain of the JAK3 was analyzed by molecular docking study. The enol-keto-enol functionality of the flavonol-amino acid conjugate **1** was shown to mimic other ATP-competitive kinase inhibitors to bind at the ATP binding site of the JAK3 through formation of hydrogen bonds to the hinge motif (Glu903 and Leu905; Fig. 2(a)). The other

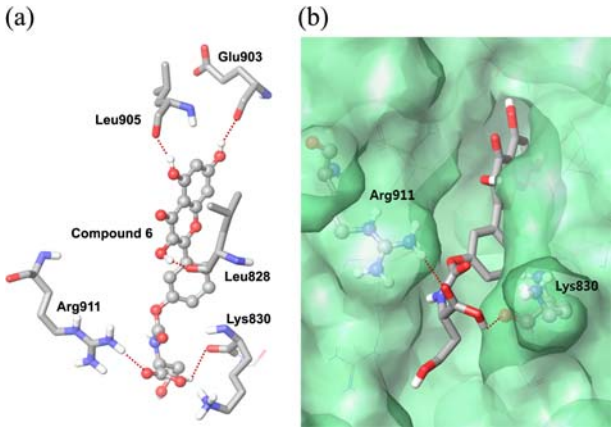


Figure 2. Binding mode of **1** to the ATP-binding site of JAK3.

part of **1** fits snuggly into the narrow ATP-binding site with the aspartic acid moiety connecting two enzyme residues, Arg911 and Lys830, *via* hydrogen bonds (Fig. 2(b)). While the hinge motif is shared by most kinases including JAKs, residues forming the remaining ATP-binding site are much more likely to vary between kinases (Fig. 2(b)).

Thus, the amino acid tether, attached to the key pharmacophoric hinge-binding motif, was anticipated to interact differently with JAK3 and JAK2 to provide JAK3-selectivity. These insights led us to design several quercetin analogues with different amino acids attached (Fig. 3). In particular, while keeping the polar groups (-OH and -NH_2), the length of the side chains were varied to find the optimum motif for selective binding to JAK3.

For preparation of flavonol-amino acid conjugates (**2-5**) thus designed, a well-known synthetic routine composed of fragmentation of quercetin (**6**) followed by acylation and ring closure was employed (Scheme 1). The perbenzylated quercetin was exposed to a strong base (18N KOH) at elevated temperature to give its fragmented product **7** which was coupled with 3-(methoxymethoxy)benzoic acid in the presence of EDC. Treatment of the resulting 3-methoxybenzoyl ester with K_2CO_3 and TBAB (tetra-*n*-butylammonium bromide) afforded the cyclized product, which was smoothly converted to the protected flavonoid **8** upon deprotection of the MOM protecting group with TFA (trifluoro acetic acid). Amino acids were then attached to the free phenol *via* a carbamate linkage. Conversion of amino acids *D*-O^{t}Bu-OAc-Ser and *D*-O^{t}Bu-OAc-Hse to the activated urethanes followed by condensation with free phenol **8** and

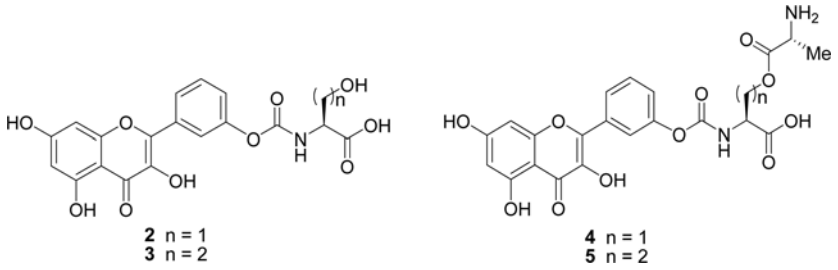
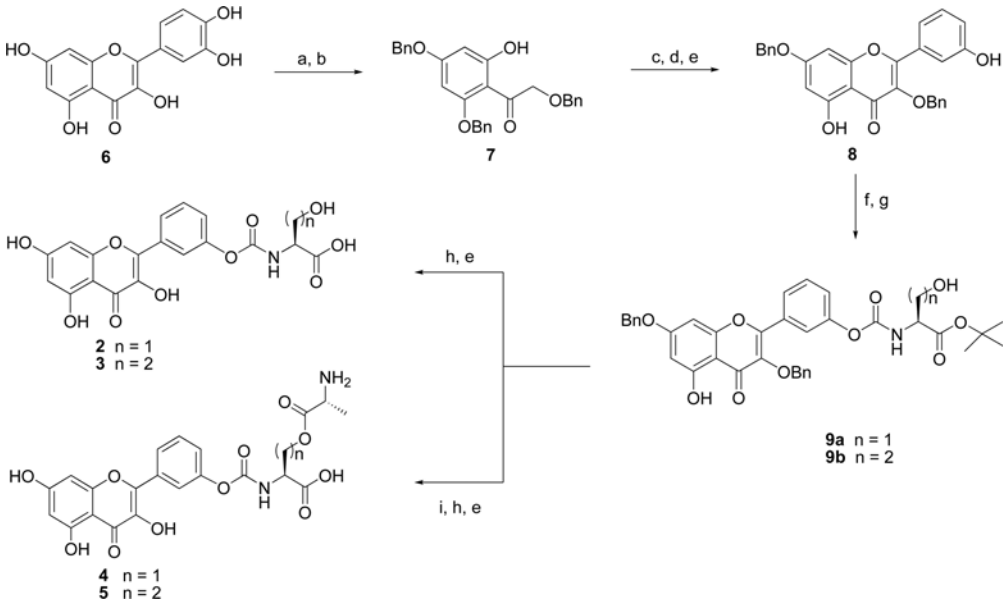


Figure 3. Structures of novel flavonoid-amino acid conjugates.



Reagents and conditions: a) BnBr, K_2CO_3, DMF, 80 °C; b) 18 N KOH, diethyleneglycol, pyridine, 120 °C; c) 3-methoxybenzoic acid, EDC, Oxyma, CH_2Cl_2, rt; d) TBAB, K_2CO_3, Toluene, 90 °C; e) TFA, 0 °C to rt; f) (4-NO_2-PhO)CO-NH-O^{t}Bu-OAc-Ser or (4-NO_2-PhO)CO-NH-O^{t}Bu-OAc-Hse, DIPEA, THF/DMF. 0 °C to rt; g) NH_3/MeOH, 0 °C; h) Pd/C, H_2, MeOH/THF, rt; i) *D*-*N*-Boc-Ala, EDC, Oxyma, CH_2Cl_2, rt.

Scheme 1. Synthesis of flavonol-amino acid conjugates (**2-5**).

Table 1. Inhibition of JAK3 and JAK2 by the flavonol conjugates

Flavonol Conjugates	% - Inhibition at 10 μM		Selectivity[b] (JAK3/JAK2)
	JAK3 (IC_{50}, μM)	JAK2	
2	42 ± 9 (–[a])	34 ± 3	1.2
3	47 ± 5 (8.9)	-12 ± 2	–[c]
4	59 ± 2 (–[a])	12 ± 6	4.9
5	70 ± 4 (2.5)	39 ± 4	1.8

[a]Not determined. [b]Selectivity = % inhibition of JAK3 at 10 μM/% inhibition of JAK2 at 10 μM. [c]JAK3 selective inhibition

deacetylation provided the flavonol-amino acid conjugates **9a** and **9b**, respectively. Sequential deprotection of benzyl and *tert*-butyl groups of **9a** and **9b** afforded the corresponding flavonoid-amino acid conjugates **2** and **3**. On the other hand, further condensation of **9a** and **9b** with *D*-O^{t}Bu-Ala gave serine or homoserine side chain-linked dipeptides which, upon removal of the protecting groups, were converted to the corresponding flavonoid-dipeptide conjugates (**4** and **5**).

JAK3-selectivity of the synthesized flavonol conjugates was then evaluated. *In vitro* kinase assay was performed by Upstate Ltd., in the presence of fixed concentrations of ATP at or near K_m for JAK3 of JAK2 using the KinaseProfiler™ Assay Protocols (Upstate Ltd.), and the results are summarized in Table 1. The flavonol conjugates **2** and **5** showed moderate inhibitory activity against both JAK3 (42% and 70% inhibition, respectively) and JAK2 (34% and 39% inhibition, respectively) with only slight JAK3-selectivity (1.2-1.8). However, to our surprise, the flavonol-L-Ser-L-Ala conjugate **4** as well as the flavonol-homoserine conjugate **3** did not show significant inhibition of the kinase activity of JAK2 (12% and −12% inhibition at 10 μM, respectively). In particular, compared with flavonol-serine conjugate **2**, the one-carbon homologue **3** showed similar inhibitory activity against JAK3 (42% *vs* 47%) but completely abolished inhibition of JAK2 (34% *vs* −12%). Thus, in spite of only moderate inhibitory activity (IC_{50} = 8.9 μM), the complete JAK3-selectivity of **3** deserves further notice in terms of developing a novel JAK3 selective scaffold.

The JAK3-selectivity of **3** was then tackled by investigation of its binding modes to JAK3 as well as JAK2. Molecular docking study of **3** to the ATP binding site of the two JAK kinases was performed by automated docking program Glide. Interestingly, the docking pose of **3** to JAK3 (Fig. 4(a)) in comparison with that to JAK2 (Fig. 4(b)) revealed hetero unknown structural differences between the two kinases. Unlike other JAK3 inhibitors reported to date, the flavonol-homoserine conjugate **3** has a branched chain of which carboxylic acid functionality protrudes outside the ATP binding site (Fig. 4(a)). Upon binding to JAK3, the branched carboxylic acid group of **3** can be located in the crevice between Arg911 and Lys830 (Fig. 4(a)). In contrast, the corresponding residues in JAK2, Arg980 and Lys857, are hydrogen bonded to each other to close the carboxylate binding site (Fig. 4(b)). Due to the lack of carboxylate binding site, the conjugate **3** was shown to be docked to JAK2 with its amino acid side chain highly distorted (Fig. 4(b)),

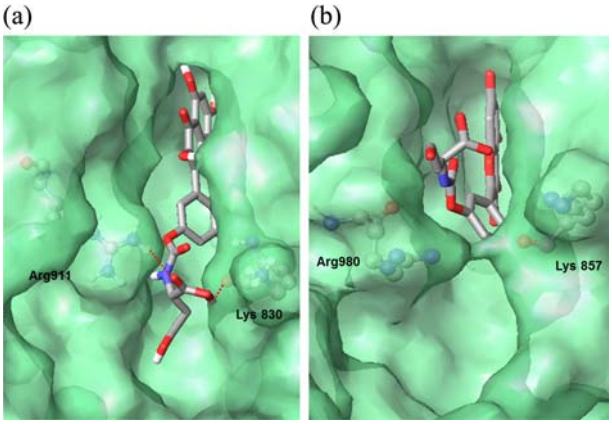


Figure 4. Binding site of compound **3** with JAK3 (a) and JAK2 (b).

which explains the abortive binding mode of **3** to JAK2 and thereby its lack of inhibitory activity against JAK2.

In summary, through modification of library hit compound, we discovered a novel flavonoid conjugate **3** with complete JAK3 selectivity. Docking study and binding mode analysis of **3** reveal hetero unknown structural difference around the ATP binding sites of JAK3 and JAK2, which can be targeted for development of novel JAK3-selective inhibitors.

Experimental Section

Amino Acid Derivatives of Flavonol (2 and 3). Palladium on charcoal (10%, 0.06 mmol) was added to a stirred solution of **9a** (0.6 mmol) or **9b** (0.6 mmol) in MeOH/THF (2:5 mL) under hydrogen atmosphere. The suspension was stirred for 3 h and then filtered through a plug of celite and eluted with acetone (50 mL). The filtrate was concentrated under reduced pressure, and the reaction mixture was dissolved in CH_2Cl_2 (5 mL). To this solution, TFA (1 mL) was added at 0 °C, and the solution was stirred for 1 h at room temperature. The resulting solution was concentrated under reduced pressure, and the residue was recrystallized from a mixture of acetone (0.5 mL) and CH_2Cl_2 (5 mL). The mixture was filtered, and the filter cake was washed with CH_2Cl_2 to afford **2** (230 mg, 0.55 mmol, 92% yield) or **3** (221 mg, 0.5 mmol, 83% yield) as yellow powder: For **2**, ^{1}H NMR (400 MHz, Acetone-d_6) δ 12.82 (s, OH), 7.64 (d, J = 8.05 Hz, 1H), 7.58 (dd, J = 2.2, 1.7 Hz, 1H), 7.48 (t, J = 8.1 Hz, 1H), 7.02 (dd, J = 8.1, 2.2 Hz, 1H), 6.74 (d, J = 2.1 Hz, 1H), 6.45 (d, J = 2.1 Hz, 1H), 4.03-4.12 (m, 1H), 3.50-3.68 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.4, 175.5, 164.2, 161.8, 160.5, 158.8, 150.0, 148.2, 136.6, 126.0, 125.5, 124.4, 120.8, 117.0, 105.2, 98.4, 94.1, 61.9, 60.4; LC/MS (ESI) *m/z* Found: 418.12 $[M+H]^+$; Calcd for $C_{19}H_{15}NO_{10}$: 417.07; For **3**, ^{1}H NMR (400 MHz, Acetone-d_6) δ 12.83 (s, OH), 7.64 (d, J = 8.1 Hz, 1H), 7.58 (dd, J = 2.2, 1.7 Hz, 1H), 7.48 (t, J = 8.1 Hz, 1H), 7.04 (dd, J = 8.1, 2.2 Hz, 1H), 6.75 (d, J = 2.1 Hz, 1H), 6.45 (d, J = 2.1 Hz, 1H), 4.03-4.12 (m, 1H), 3.50-3.71 (m, 2H), 2.02-2.15 (m, 1 H), 1.49-1.65 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.4, 175.7, 164.4, 161.8, 160.6, 158.8, 150.2, 148.4, 136.6, 126.1, 125.5, 124.4,

120.8, 117.0, 105.2, 98.6, 94.2, 58.4, 51.9, 35.4; LC/MS (ESI) *m/z* Found: 432.12 $[M+H]^+$; Calcd for $C_{20}H_{17}NO_{10}$: 431.09.

Dipeptide Derivatives of Flavonol (4 and 5). To a stirred suspension of amino acid *tert*-butyl-esters of flavonol (**9a** and **9b**) (0.6 mmol) in CH_2Cl_2 (10 mL) was added EDC (350 mg, 1.8 mmol) followed by Oxyma (630 mg, 1.8 mmol). Upon complete dissolution, *D*-*N*-Boc-Ala (130 mg, 0.6 mmol) was added. The mixture was stirred at rt for 5 h, concentrated under reduced pressure, and purified by column chromatography on silica gel (3:1 = Hex:EtOAc) to give the desired product. The degassed suspension of flavonol conjugate obtained above was dissolved in a mixture of THF (3 mL) and MeOH (3 mL), treated with palladium on charcoal (10% w/w, 15 mg) under an atmosphere of hydrogen gas (balloon), and vigorously stirred for 2 h at room temperature. The reaction mixture was filtered through a short celite pad and concentrated under reduced pressure to give pale yellow syrup, which was used for the next step without further purification. The Boc-dipeptide flavonol conjugate obtained above was dissolved in CH_2Cl_2 (5 mL). To this solution, trifluoroacetic acid (1 mL) was added at 0 ºC, and the solution was stirred for 1 h at room temperature. The resulting solution was concentrated under reduced pressure, and the residue was recrystallized from a mixture of acetone (1 mL) and CH_2Cl_2 (10 mL). The mixture was filtered, and the filter cake was washed with CH_2Cl_2 to give flavonol dipeptide conjugate (**4** and **5**) as yellow solids: For compound **4**, ^{1}H NMR (400 MHz, Acetone-d_6) δ ^{1}H NMR (400 MHz, Acetone-d_6) δ 12.80 (s, OH), 7.64 (d, J = 8.2 Hz, 1H), 7.58 (dd, J = 2.1, 1.7 Hz, 1H), 7.46 (t, J = 8.1 Hz, 1H), 7.02 (dd, J = 8.1, 2.1 Hz, 1H), 6.74 (d, J = 2.1 Hz, 1H), 6.45 (d, J = 2.1 Hz, 1H), 4.00-4.16 (m, 2H), 3.40-3.60 (m, 2H), 1.28 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.4, 175.5, 171.5, 166.2, 161.8, 160.5, 158.8, 150.0, 148.2, 136.6, 129.0, 125.5, 124.7, 120.8, 117.0, 105.2, 98.4, 94.0, 61.9, 48.4, 33.4, 17.4; LC/MS (ESI) *m/z* Found: 489.15 $[M+H]^+$; Calcd for $C_{22}H_{20}N_2O_{11}$: 488.11; For compound **5**, ^{1}H NMR (400 MHz, Acetone-d_6) δ 12.82 (s, OH), 7.64 (d, J = 8.0 Hz, 1H), 7.58 (dd, J = 2.2, 1.7 Hz, 1H), 7.48 (t, J = 8.0 Hz, 1H), 7.04 (dd, J = 8.1, 2.2 Hz, 1H), 6.75 (d, J = 2.1 Hz, 1H), 6.45 (d, J = 2.1 Hz, 1H), 4.03-4.22 (m, 2H), 3.50-3.71 (m, 2H), 1.70-2.08 (m, 2H), 1.28 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.3, 175.8, 172.2, 162.1, 161.6, 160.4, 156.8, 150.2, 148.4, 136.6, 126.0, 125.5, 123.4, 120.8, 117.0, 104.2, 95.6, 94.2, 58.4, 61.2, 54.3, 35.4, 17.2; LC/MS (ESI) *m/z* Found: 503.24 $[M+H]^+$; Calcd for $C_{23}H_{22}N_2O_{11}$: 502.12.

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