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Pyrogallin, an ATP-Competitive Inhibitor of JAK3

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The Janus kinase (JAK) family mediates important cytokine signaling proliferation, growth, hematopoiesis, and immune response in a variety of cells.[1,2] The JAK family consists of four different tyrosine kinases: TYK2, JAK1, JAK2, and JAK3. Activation of JAK1 and JAK3 induces phosphorylation of STAT (Signal Transducers and Activators of Transcription) proteins. The phosphorylated STAT proteins then dimerize and traffic into the nucleus in which they work as a transcription factor to regulate genes involved in various immune responses.[3] In contrast to other JAK kinases present in a wide range of cell lines, JAK3 is selectively expressed only in matured hematopoietic cells.[4] Thus, specific inhibition of JAK3 would have therapeutic potential in treatment of immune-related diseases including asthma and rheumatoid arthritis *via* immunosuppression without affecting cells and organ compartments except the immune system. In this context, pharmacological intervention of the JAK3 activity is being pursued to reduce organ transplant rejection and to treat T-cell-specific autoimmune diseases, including psoriasis, multiple sclerosis, inflammatory bowel disease, and rheumatoid arthritis. Several synthetic JAK3 inhibitors identified by pharmaceutical companies such as Pfizer, Rigel, and Pharmacopeia are currently in the process of clinical evaluation. In particular, Pfizer's CP-690550 is in Phase 3 for treatment of rheumatoid arthritis.[5] Although CP-690550 exhibits a high inhibitory potency against JAK3 (IC_{50} = 1 nM), its action is rather indiscriminate as it also affects activities of JAK1 and JAK2.[6,7] Accordingly, more efforts should be made toward developing new JAK3 inhibitors with improved specificity. Here, in an attempt to develop a lead compound for JAK3 inhibition by screening a chemical library, we have discovered pyrogallin as a JAK3 inhibitor. After examining its molecular mechanism, we further describe prospect to improve its potency as well as specificity for JAK3 inhibition.

First of all, the catalytic domain of JAK3 was required for setting up a kinase activity assay. In order to clone JAK3 gene for expression of the kinase, a cDNA encoding the catalytic domain (residues 812-1124) of human JAK3 was obtained from an adult human thymus cDNA library using two primers. The cDNA was subcloned into a transfer vector pAcG2T and expressed as a glutathione S-transferase (GST) fusion protein according to the previously reported procedure.[8] With the purified JAK3 protein, we screened a chemical library of 1040 compounds from the National Institute of Neurological Disorders and Stroke (NINDS) custom collection, since old drugs were often successfully rediscovered as new therapeutic tools in other diseases.[9] We employed a fluorescence polarization (FP)-based kinase assay for screening of the library. For the FP assay, a fluorescein-labeled JAK1 1015 - 1027 peptide (F-JAK3: fluorescein isothiocyanate-aminocaproic acid-CAGAGAIETDK EYYTVKD, with the phosphorylation site underlined, Peptron, Daejeon, Korea) was used as the substrate of JAK3 and a monoclonal antibody (PY20, Sigma-Aldrich, St. Louis, MO) for the phosphorylated tyrosine as the binding partner, respectively. In the designed assay, the presence of phosphotyrosine generated by JAK3 leads to binding of the peptides to the antibody, resulting in an increase of FP values. Such interaction assay based on FP measurements renders quantitative assessment of JAK3 activity, thereby enabling evaluation of inhibitors for the enzyme. The peptide substrate (F-JAK1) at the final concentration of 1 μM was incubated with 2.6 μg/mL recombinant GST-JAK3 in reaction buffer (50 mM Hepes, pH 7.5, 2 mM DTT, 1 mM EGTA, 10 mM $MgCl_2$, 0.01% Tween-20) containing 20 μM ATP in the presence of chemicals from the library for 20 min at 25 °C. The reaction mixtures containing DMSO alone with or without GST-JAK3 were included as positive or negative controls, respectively. The reactions were terminated by heating the reactants for 1 min at 95 °C, followed by dilution to a final peptide concentration of 100 nM in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.25% Nonidet P-40) in the presence of 100 nM PY-20. The mixtures were gently mixed and their FP values were measured. After subtracting the FP value of the negative control, the percentage of inhibition was calculated based on the FP value of the positive control. 14 compounds were selected in the primary screening with the threshold value set at 70% inhibition by 100 μM chemicals of the library (Fig. 1). These selected compounds were then subjected to secondary inhibition screening in the presence of 5 μM chemicals. In the secondary inhibition assays, we set the threshold at 20% inhibition to finally obtain 4 compounds (Fig. 2). To quantitatively compare the inhibitory potency, we determined IC_{50} values of those 4 compounds (Fig. 3) by analyzing FP changes with increasing chemicals. The most potent compound was identified as pyrogallin, showing about 6 μM of IC_{50}. Pyrogallin is a benzotropolone-containing compound, produced by oxidative dimerization of

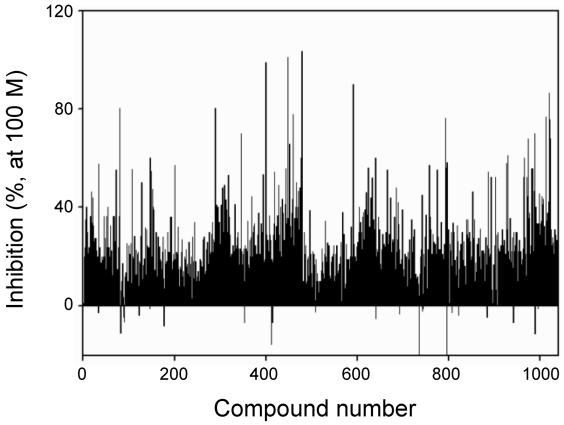


Figure 1. Screening of a library of 1040 chemicals for inhibition against JAK3.

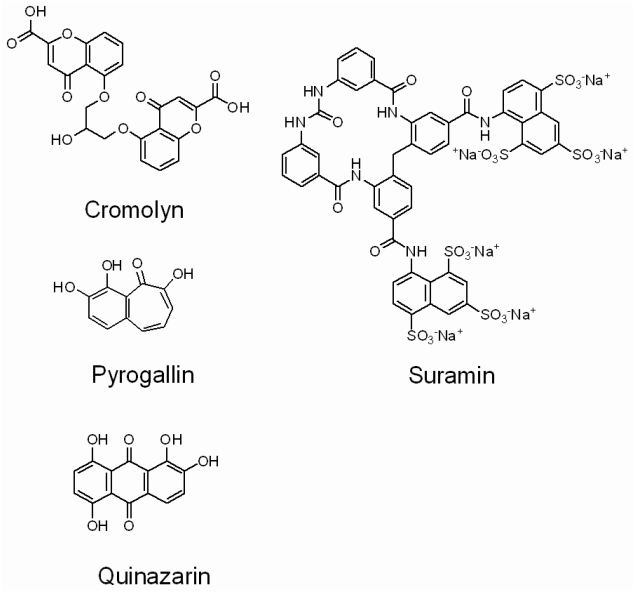
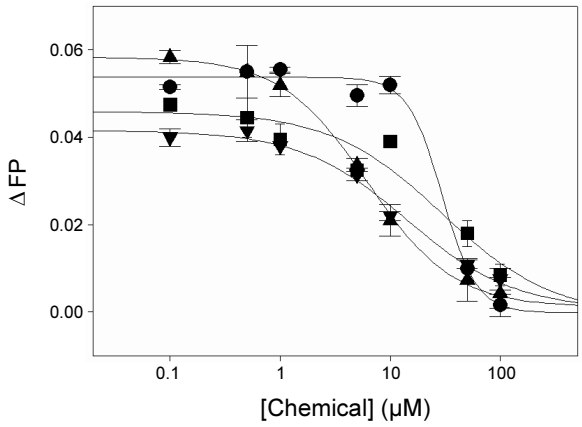


Figure 2. Chemical structures of JAK3 inhibitors identified by library screening.

pyrogallol, a component of black tea.[10] Considering the structure of pyrogallin, we envisioned that pyrogallin might inhibit JAK3 by competing for the ATP binding site. We therefore tested whether the inhibitory effect of pyrogallin on the phosphorylation reaction involves its competition with ATP for the ATP binding site by performing kinase assays in the presence of constant amounts of pyrogallin with varying concentration of ATP. As shown in Figure 4, while pyrogallin at 5 or 10 μM inhibited the JAK activity in the presence of 10 μM ATP, the activity was recovered with increasing concentrations of ATP. This result suggests that pyrogallin works as an ATP-competitive inhibitor of JAK3. Although there was no previous study describing inhibitory effect of pyrogallin against kinases, purpurogallin, a structural analogue of pyrogallin is known to inhibit the tyrosine-specific protein kinase of the human *erb-b* oncogene product. When compared with purpurogallin in chemical structure, pyrogallin lack only one hydroxyl group. Having a similar structure to purpurogallin, pyrogallin might also have



Chemical	IC_{50} (μM)
Cromolyn	27.0 (2.49)[a]
Pyrogallin	6.37 (0.387)
Quinalizarin	16.8 (4.41)
Sumarin	13.5 (2.41)

[a]Error ranges from curve fitting.

Figure 3. Determination of IC_{50} values of chemicals selected against JAK3. Kinase reactions were performed with varying concentrations of the selected chemicals after the secondary screening. F-JAK1 peptide at 1 μM was reacted with 2.6 μg/mL GST-JAK3 in reaction buffer containing 20 μM ATP for 15 min at 25 °C. After reactions were quenched by heating for 1min at 95°C, the reactants were diluted in EBC buffer to yield a final peptide concentration of 100 nM in the presence of 100 nM PY-20, followed by FP measurements. Data were fit to obtain IC_{50} values of the JAK3 inhibitors: cromolyn (●), pyrogallin (■), quinalizarin (▲), or sumarin (▼). Each point represents the average of duplicate assays.

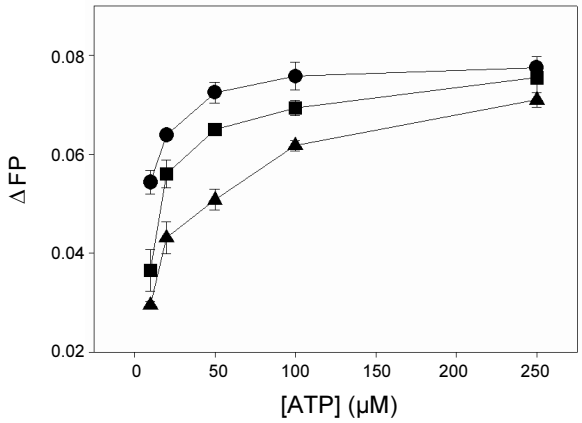


Figure 4. Pyrogallin as an ATP-competitive inhibitor of JAK3. 1 μM F-JAK1 peptide was reacted with 2.6 μg/mL GST-JAK3 in reaction buffer in the presence of 0 (●), 5 (■), or 10 μM (▲) pyrogallin with varying concentrations of ATP (10, 20, 50, 100, and 250 μM). After incubation for 20 min at 25 °C, 100 nM PY20 was added to reactants diluted to a final peptide concentration of 100 nM in EBC buffer, followed by FP measurements. Each point represents the average of duplicate assays.

an inhibitory potency against JAK3, a tyrosine kinase. Targeting ATP binding site has been a valuable starting point in the development of many successful drugs based on inhibition of kinases since all kinases share the homologous ATP binding site.

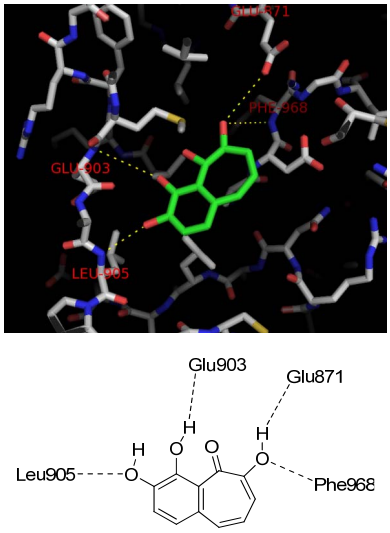


Figure 5. Docking study of pyrogallin into the ATP-binding site of JAK3. Carbon and oxygen atoms in pyrogallin were colored by green and red, respectively. Dashed lines indicated the potential interactions between pyrogallin and four key residues, which were highly conserved in JAK3 kinase. The figure was prepared by PyMol™.

To predict interactions of pyrogallin in the adenosine pocket of JAK3, the crystal structure of JAK3 complexed with AFN941 was utilized to gain insight into its possible binding mode in the active site of the kinase.[8] As shown in Figure 5, similar to the pattern observed in the case of AFN941, pyrogallin may inhibit JAK3 by adopting hydrogen bonds: two hydroxyl groups in the 6-membered ring in pyrogallin form hydrogen bonds with the carbonyl oxygen of Glu903 and the carbonyl oxygen with the amide nitrogen of Leu905 in JAK3. These amino acids typically have interactions with ATP in the kinase reaction.[12] Interestingly different from AFN941, pyrogallin appears to interact with a unique region in the active site. This hydrogen-bonding mode is similar to the previously suggested hydrogen bonds in the complex of JAK3 with another analog of staurosporine.[13] With conserving interactions with the ATP-binding pocket, unsubstituted sites of pyrogallin could be used for further chemical modification to achieve more specific inhibition against JAK3. Construction of a synthetic library of pyrogallin derivatives to this end is currently under way.

In summary, we have screened a chemical library to find hit compounds for development of JAK3 inhibitors. As a result, pyrogallin, a natural product was demonstrated to show an inhibitory effect on JAK3. Due to its structural similarity to ATP, pyrogallin likely inhibits JAK3 with an ATP-competitive mode. Based on the docking experiment, we further found that amino acid residues in the active site might interact with a certain point of pyrogallin, which would hold great promise for specific inhibition of JAK3 through elaborate chemical modifications.

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References

1. Aaronson, D. S.; Horvath, C. M. *Science* **2002**, *296*, 1653-1655.
2. O'Shea, J. J.; Pesu, M.; Borie, D. C.; Changelianl, P. S. *Nat. Rev. Drug. Disc.* **2004**, *3*, 555-564.
3. Chen, Z.; Lund, R.; Aittokallio, T.; Kosonen, M.; Nevalainen, O.; Lahesmaa, R. *J. Immunol.* **2003**, *171*, 3627-3635.
4. Leonard, W. J.; O'Shea, J. *J. Ann. Rev. Immunol.* **1998**, *16*, 293-322.
5. West, K. *Curr. Opin. Investig. Drugs* **2009**, *10*, 491-504.
6. Changelian, P. S.; Flanagan, M. E.; Ball, D. J.; Kent, C. R.; Magnuson, K. S.; Martin, W. H., *et al*. *Science* **2003**, *302*, 875-878.
7. Jiang, J.-K.; Ghoreschi, K.; Deflorian, F.; Chen, Z.; Perreira, M.; Pesu, M.; Smith, J.; Nguyen, D.; Liu, E. H.; Leister, W.; Costanzi, S.; O'Shea, J. J.; Thomas, C. J. *J. Med. Chem*. **2008**, *51*, 8012-8018.
8. Boggon, T. J.; Li, Y.; Manley, P. W.; Eck, M. J. *Blood* **2005**, *106*, 996-1002.
9. Chong, C. R.; Sullivan, D. J. *Nature* **2007**, *448*, 645-646.
10. Takino, Y.; Imagawa, H. *Agr. Biol. Chem.* **1964**, *28*, 125-130.
11. Abou-Karam, M.; Shier, W. T. *Phytother. Res.* **1999**, *13*, 337-340.
12. Zhang, X.; Gureasko, J.; Shen, K.; Cole, P. A.; Kuriyan, J. *Cell* **2006**, *125*, 1137-1149.
13. Yang, S.-M.; Malaviya, R.; Wilson, L. J.; Argentieri, R.; Chen, X.; Yang, C.; Wang, B.; Cavender, D.; Murray, W. V. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 326-331.