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Thienotriazolopyrimidine Derivatives Inhibit STAT3 Activation Induced by IL-6

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Interleukin-6 (IL-6) is pro-inflammatory cytokine that is secreted by immune and inflammatory cells such as macrophages and lymphocytes.[1] It responses to physiological and pathological processes such as viral infections, trauma, and other tissue damages, and it is an important mediator that could lead to inflammatory responses.[2,3] Many studies have reported that excess Il-6 were produced in inflammatory tissues related human disease such as rheumatoid arthritis, psoriasis, inflammatory bowel disease, osteoarthritis, and multiple myeloma.[4,5] It is also found in human atherosclerotic plaques, and is related to the impairment of endothelium-dependent dilatation in human veins.[6] Thus, IL-6 is a pivotal role in the pathogenesis of immune and inflammatory diseases. After IL-6 binds to its receptor, it induces the dimerization of gp130 (a signal transducing glycoprotein 130), and then leads the activation of STAT3 (signal transducer and signal activator of transcription 3). In general, the activation of STAT3 is one of the most important steps in the pro-inflammatory signals induced by IL-6.[7,8] Therefore, blocking IL-6 could be one of the more effective treatment methods against many human diseases related to IL-6.

Thienopyrimidines and their related derivatives have attracted great attention of many researchers due to a variety of biological activities, such as adenosine A1/A2a or A2a/A3 receptor antagonists, P2Y12 platelet aggregation inhibitor, and Aurora kinase inhibitor.[9-11] Also, they were reported to have various pharmacological activities including antibacterial, antiviral, antifungal, analgesic, anticancer, and anti-inflammatory properties.[12-15] Specifically, the sulfur-linked diheterocyclic compounds containing thienopyrimidines moiety were investigated for the development of antifungal agent.[16] On the other hand, triazolothiazole derivatives have also been reported to possess antibacterial, antiviral, and antimicrobial activities.[17-19] Therefore, we have designed and synthesized diheterocyclic compounds by the introduction of triazolothiazole moiety to thienotriazolpyrimidines ring through sulfur-linkage. It is expected to bring positive effects on diverse biological activities such as antimicrobial and anti-inflammatory activities. To begin with, the synthesized sulfur-liked diheterocyclic compounds were examined for the antibacterial activities against various bacteria such as *Bacillus megterium*, *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Enterococcus faecium*, and *Salmonella enterica*. Also, we verified whether they showed antimicrobial activities against other bacteria such as *Actinobacillus pleuropneumoniae*, *Haemophilus parasuis*, and *Pasteurella multocida*, which they are known as an important cause of porcine pleuropneumonia. Despite our expectations, the compounds **1-6** did not show the inhibitory activities against all bacteria. We further investigated whether they exhibited the anti-inflammatory activities. Fortunately, they have strong inhibitory activities on our assay when using IL-6-induced STAT3 activity. We have already reported the synthetic procedures and spectroscopic data (NMRs and MS) of the sulfur-linked diheterocyclic compounds **1-6**.[20] Therefore, we described the IL-6 induced luciferase activities and STAT3 phosphorylation of sulfur-linked dihetrocyclic compounds 1-6 as potential IL-6 inhibitors in this paper.

The synthesized sulfur-linked diheterocyclic compounds **1-6** were tested for their inhibitory activities on STAT3-dependent luciferase activity induced by IL-6, according to the reported method.[21] In brief, the Hep3B cells stably transformed with the pStat3-Luc plasmid were seeded on to 96-well culture plates at 2×10^4 cells/well. After 24 h, the cells were starved for 12 h and treated with IL-6 (10 ng/mL)

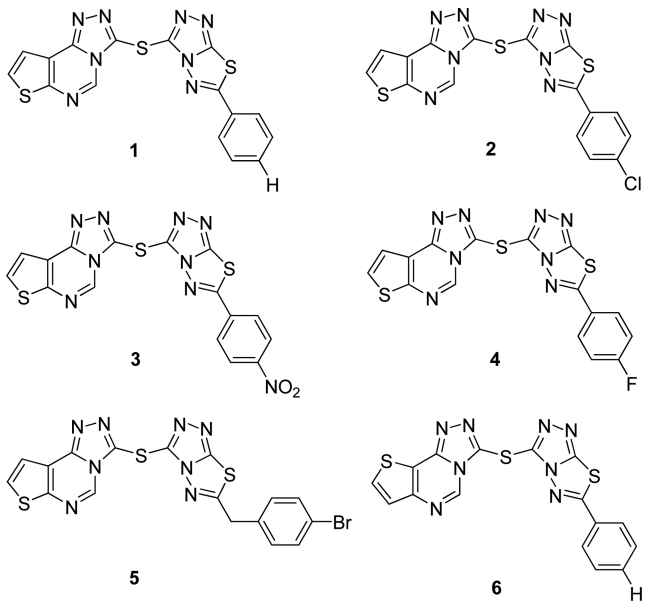

Figure 1. Chemical structures of compounds **1-6**.

with or without the compounds **1-6** for 12 h. The STAT3-dependent promoter activity was measured according to the manufacturer's protocol. The inhibitory activities of compounds **1-6** as compared to genistein was used as a positive control which inhibited the STAT3-dependent luciferase activity with an IC_{50} value of 15 μM in this assay system.[22] Table 1 lists the biological data for compounds **1-6**. Compound **2**, which substituted hydrogen for chloride at 4-position of phenyl moiety, exhibited more inhibitory activities with 1.9-fold as compared with compound **1**. Also, the compound **5**, which had substituted chloride at benzene moiety, showed slightly improved inhibitory activities with IC_{50} values of 5.2 μM when compared to compound **1**. However, compound **4**, which had a fluoride at para position of phenyl moiety, led to a 2.1-fold decrease in the IL-6-induced STAT3 activity (IC_{50} value of 20.8 μM) when compared with compound **1**. Compound **3**, which substituted a nitro group for hydrogen at phenyl moiety, showed above 10-fold low inhibitory activity with IC_{50} value of > 100 μM than compound **1**. Also, the compound **6**, [3,2-*d*]thienopyrimidine analogue, showed 2.5-fold less inhibitory activity (IC_{50} value of 24.6 μM) than compound **1**, which suggested that [2,3-*d*]thieno-pyrimidine can improve the IL-6-induced STAT3 inhibitory activity (Figure 1 and Table 1). Specifically, compounds **2** and **5** have more potent inhibitory activities with 3-fold when compared to genistein used as a positive control.

In addition, we investigated whether compounds **1-6** show inhibitory effects on the phosphorylation of STAT3 induced by IL-6 in Hep3B cells. As shown in Figure 3, compounds **2** treatments of Hep3B cells for 20 minutes showed strong inhibitory activities on the phosphorylation of STAT3 by IL-6 at concentrations of 30 and 10 μM when compared with other compounds and genistein (100 μM). Also, the compounds **1** and **4** have more potent inhibitory activity at concentrations of 30 and 10 μM when compared to genistein. But, compounds **5** and **6** only showed the moderate inhibitory effects to phosphorylation of STAT3 at a concentration of 30 μM. None of the compounds tested had any cytotoxicity in Hep3B cells with MTT assay at these concentrations (data not shown).

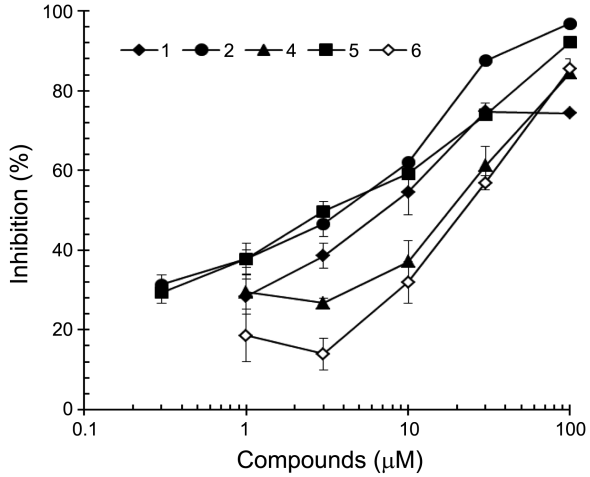

Figure 2. Inhibitory effects of compounds **1-6** on IL-6-induced STAT3 activation. Hep3B cells expressing stably pStat3-Luc were seeded on to 96-well culture plates at 2×10^4 cells/well. After 24 h, cells were performed with starvation for 12 h, and then treated with IL-6 (10 ng/mL) with or without sulfur-liked diheterocyclic compounds **1-6** for 12 h. Luciferase assay was performed with the kit from Promega according to the manufacturer's protocol. Data are presented as the mean ± SE (n = 3).

Table 1. Inhibitory effects of compounds **1-6** on IL-6-induced STAT3 activation[a]

Compounds No.	IC_{50} (μM)[b]
1	9.9
2	5.1
3	> 100
4	20.8
5	5.2
6	24.6
Genistein[c]	15.0

[a]Data are mean ± standard error values of three replications. [b]IC_{50}: mean (50%) value of inhibition concentration. [c]Genistein was used as a positive control.

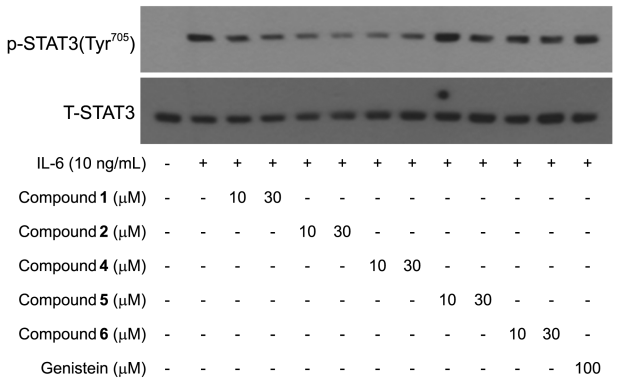

Figure 3. Effects of compounds **1**, **2**, **4**, **5**, and **6** on the STAT3 phosphorylation by IL-6. Hep3B cells were incubated with IL-6 for 20 minutes in the presence or absence of compounds **1**, **2**, **4**, **5**, and **6** at concentrations of 30 and 10 μM, and total cell lysates were isolated. Immunoblotting with anti-phospho STAT3 (Tyr705) and anti-total STAT3 IgG were performed on total protein (10 μg protein/lane).

In conclusion, among the tested sulfur-liked diheterocyclic compounds, compounds **1**, **2**, and **5** showed the most potent inhibitory activity on the STAT3-dependent luciferase assay when compared with genistein as a positive control. Compounds **4** and **6** had moderate inhibitory activities, and compound **3** had no inhibitory activity. Specifically, compound **2** had more inhibitory activity when compared with other compounds on IL-6-induced STAT3 phosphorylation in Hep3B cells. Based on these results, the synthesized compounds could be useful candidates for the therapeutic strategy of inflammatory diseases and could be the basis for the design of IL-6 inhibitors.

Experimental Section

Reagents and Chemicals. Recombinant human IL-6 was purchased from R&D systems (Minneapolis, MN, USA).

All reagents including genistein were obtained from Sigma-Aldrich Ltd (St Louis, MO, USA).

Cell Line and Cell Culture. Human hepatoma Hep3B cells were obtained from American Type Culture Collection (ATCC No. HB-8064, Rockville, MD) and were maintained in a DMEM medium, supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 50 mg/mL streptomycin, at 37 °C in a 5% CO_2 incubator. All cell culture reagents were obtained from GibcoBRL (Life Technologies, Cergy-Pontoise, France).

Establishment of Stable Cell Line Expressing pStat3-Luc. Hep3B cells were cotransfected with pStat3-Luc encoding the Stat3 binding site and pcDNA3.1 (+) carrying a hygromycin selection marker (Clontech laboratories, Palo Alto, CA) by using lipofectamin plus (Invitrogen, Carlsbad, CA, USA). Two days after the transfection, cells that stably expressed luciferase were selected by hygromycin treatment (100 μg/mL) and stable clones were expanded. Expression of luciferase in the clones stably expressing pStat3-Luc was confirmed by luciferase assays.

Luciferase Assay. Hep3B cells stably expressing pStat3-Luc were seeded on to 96-well culture plates at 2×10^4 cells/well. After 24 h, cells were performed with starvation for 12 h, and then treated with IL-6 (10 ng/mL) with or without compounds for 12 h. Luciferase assay was performed with the kit from Promega according to the manufacturer's protocol.

Western Blot Analysis. Total proteins were prepared from cells and subjected to Western blot with primary rabbit anti-phospho Stat3 (Tyr705) IgG, anti-total Stat3 IgG (1:1000), and secondary antibody (1:2000).

Cell Viability. Hep3B cells were seeded at a plating density of 2×10^4 cells/well and cultured for 24 h to allow them to adhere to the plate. After 24 h, the culture medium changed to the serum free medium supplemented with the samples indicated dose. Following the culture with sample for 48 h, MTT (0.5 mg/mL) was added, and after 4 h of incubation at 37 °C, 200 μL of DMSO was added into each well. The absorbance of the samples at 540 nm was measured against a background control by using a 96-well plate reader. The percentage of viable cells under each treatment condition was determined according to the negative control.

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References

1. Shimaoka, M.; Xiao, T.; Liu, J. H.; Yang, Y.; Dong, Y.; Jun, C. D.; McCormack, A.; Zhang, R.; Joachimiak, A.; Takagi, J.; Wang, J. H.; Springer, T. A. *Cell* **2003**, *112*, 99.
2. Feldmann, M.; Brennan, F. M.; Maini, R. N. *Cell* **1996**, *85*, 307.
3. Carson, D. A.; Chen, P. P.; Kipps, T. J. *J. Clin. Invest.* **1991**, *87*, 379.
4. Jones, S. A.; Scheller, J.; Rose-John, S. *J. Clin. Invest*. **2011**, *121*, 3375.
5. Lebwohl, M.; Tyring, K. S.; Hamilton, K. T.; Toth, D.; Glazer, S.; Tawfik, H. N.; Walicke, P.; Wang, X.; Garovoy, R. M.; Pariser, D. N. *Engl. J. Med.* **2003**, *349*, 2004.
6. Bhagat, K.; Valance, P. *Circulation* **1997**, *96*, 3042.
7. Sebti, S.; Der, C. *Nat. Rev. Cancer* **2003**, *3*, 945.
8. Aaronson, D.; Horvath, C. *Science* **2002**, *296*, 1653.
9. Prasa, M. R.; Rao, A. R.; Rao, P. S.; Rajan, K. S.; Meena, S.; Madhavi, K. *Eur. J. Med. Chem*. **2008**, *43*, 614.
10. Kortum, S. W.; Lachance, R. M.; Schweitzer, B. A.; Yalamanchili, G.; Rahman, H.; Ennis, M. D.; Huff, R. M.; TenBrink, R. E. *Bioorg. Med. Chem. Lett*. **2009**, *19*, 5919.
11. McClellan, W. J.; Dai, Y.; Abad-Zapatero, C.; Albert, D. H.; Bouska, J. J.; Glaser, K. B.; Magoc, T. J.; Marcotte, P. A.; Osterling, D. J.; Stewart, K. D.; Davidsen, S. K.; Michaelides, M. R. *Bioorg. Med. Chem. Lett*. **2011**, *21*, 5620.
12. El-Sayed, W. A.; Ali, O. M.; Zyada, R. A.; Mohamed, A. A.; Abdel-Rahman, A. A. *Acta Pol. Pharm*. **2012**, *69*, 439.
13. Hafez, H. N.; Hussein, H. A.; El-Gazzar, A. R. *Eur. J. Med. Chem*. **2010**, *45*, 4026.
14. Zhu, W.; Liu, Y.; Zhai, X.; Wang, X.; Zhu, Y.; Wu, D.; Zhou, H.; Gong, P.; Zhao, Y. *Eur. J. Med. Chem*. **2012**, *57*, 162.
15. El-Tombary, A. A.; El-Hawash, S. A.; Habib, N. S.; Soliman, R.; El-Ashmawy, I. M.; Shaaban, O. G. *Med. Chem*. **2013**, *9*, 1099.
16. Chen, Q.; Zhu, X. L.; Jiang, L. L.; Liu, Z. M.; Yang, G. F. *Eur. J. Med. Chem*. **2008**, *43*, 595.
17. Khalil, N. S. *Eur. J. Med. Chem*. **2007**, *42*, 1193.
18. Omar, A.-M. M. E.; Aboulwafa, O. M. *J. Heterocycl. Chem*. **1986**, *23*, 1339.
19. Khalil, N. S. *Nucleosides Nucleotides Nucleic Acids* **2007**, *26*, 347.
20. Whang, J.; Song, Y.-H. *J. Heterocycl. Chem*. **2013**, *50*, 603.
21. Lee, S. W.; Yun, B. R.; Kim, M. H.; Park, C. S.; Lee, W. S.; Oh, H. M.; Rho, M. C. *Planta Med.* **2012**, *78*, 903.
22. Li, H. C.; Zhang, G. Y. *Acta Pharmacol. Sin.* **2003**, *24*, 1131.