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Synthesis of Thienopyrimidine Derivatives as Inhibitors of STAT3 Activation Induced by IL-6

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A series of thienopyrimidine compounds (**6Aa–g** and **6Ba–d**) were synthesized and characterized by NMR spectroscopy and mass spectrometry. These compounds (**6Aa–g** and **6Ba–d**) potently inhibited STAT3 expression induced by IL-6 in a dose-dependent manner with IC_{50} values of 5.73–0.32 μM. Among the prepared thienopyrimidine derivatives, **6Aa**, **6Ab**, **6Ba** and **6Bc** significantly suppressed the phosphorylation of STAT3 and ERK1/2 stimulated by IL-6 in Hep3B cells. Furthermore, the synthesized compounds might be useful remedies for the treatment of inflammatory diseases by inhibiting the action of IL-6.

Keywords: Thienopyrimidine derivatives, interleukin-6, STAT3, anti-inflammation

Introduction

Pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8 play a central role in the induction of inflammation [1–3]. Among them, interleukin-6 (IL-6) is a pleiotropic cytokine that is secreted by immune and inflammatory cells, such as macrophages and lymphocytes; furthermore, it responds to both physiological and pathological processes, including viral infection, trauma and other tissue damage [4, 5]. In particular, many studies have reported that IL-6 is over-expressed in inflammatory tissues in several diseases, such as rheumatoid arthritis, psoriasis, inflammatory bowel disease, osteoarthritis and multiple myeloma [6–9]. Moreover, large quantities of IL-6 are found in human atherosclerotic plaques. After IL-6 binds to its receptor (IL-6R, a ligand-binding 80 kDa glycoprotein chain), it induces the homodimerization of a signal-transducing glycoprotein 130 (gp130), which in turn leads to the activation of the Janus kinase (Jak)/signal transducer and signal activator of transcription-3 (STAT-3) [10, 11]. Activation of IL-6/STAT3 is essential for mediating inflammatory signals via the expression of pro-inflammatory target genes, such as intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), C-reactive protein (CRP), monocyte chemotactic protein 1 (MCP-1), and IL-1β [12–14]. Therefore, the inhibition of IL-6-induced activation of STAT3 could be a good tool for screening effective therapeutic materials as well as target drugs for the treatment of diseases related to IL-6, including rheumatoid arthritis, atherosclerosis, diabetes, cancer and multiple myeloma [15].

Thienopyrimidines and their derivatives have been reported to have various biological activities, including as adenosine A_1/A_{2a} or A_{2a}/A_3 receptor antagonists, P2Y12

platelet aggregation inhibitors and Aurora kinase inhibitors [16–18]. Additionally, diheterocyclic compounds containing thienopyrimidine moieties have been investigated for the development of antifungal agents. As a result, they were shown to have various pharmacological activities, including antibacterial, antiviral, antifungal, analgesic, anticancer, and anti-inflammatory properties [19, 20]. The pyrimidine nitrogen of thienotriazolopyrimidine derivatives has been reported to be crucial for epidermal growth factor receptor (EGFR) ligand binding [21]. Phosphorylation of STAT3 at Y705 is mediated not only by the gp130/Jak pathway but also EGFR, which enhances immune response and cell proliferation without feedback from suppressors of cytokine signaling 3 (SOCS3), a negative regulator [22]. For that reason, we have designed and synthesized heterocyclic derivatives related to thienopyrimidines as potent STAT3 inhibitors [23–28].

In our search for potent IL-6/STAT3 inhibitors, we screened our in-house chemical library by using a STAT3-dependent luciferase assay involving the treatment of Hep3B cells with IL-6. Among the tested compounds, compound 6Aa exhibited potent inhibitory effects on IL-6-induced STAT3 activation. Therefore, we synthesized thienopyrimidine derivatives of **6Aa**, which we then evaluated for their inhibitory activities against STAT3 activation by IL-6. Herein, we describe the synthesis and biological activities of these thienopyrimidine derivatives as potential IL-6 inhibitors.

Materials and Methods

Chemistry

Melting points were determined in capillary tubes on a Büchi apparatus and are uncorrected. Each compound in the reactions was monitored by thin-layer chromatography on Merck Kieselgel $60F_{254}$ and purified by column chromatography using Fuji silica gel (38−75 μm). The ^{1}H and ^{13}C NMR spectra were recorded using a Varian Inova 400NB FT-NMR spectrometer (400 MHz) with Me_4Si as the internal standard, and chemical shifts are given in ppm (δ).

General Procedures for the Synthesis of Compounds 6Aa-g and 6Ba-d

Preparation of **3a**/**3b**: A mixture of **1** (0.02 mol) or **2** (0.02 mol), the appropriate acetophenone (0.02 mol) and pyrrolidine (0.02 mol) in absolute ethanol (50 ml), was stirred and refluxed for 12 h. After work-up, the crude product was subjected to column chromatography on silica gel (dichloromethane/ethyl acetate) or recrystallization from ethyl acetate to give **3a** and **3b**.

Preparation of **4a**/**4b**: Dimethylformamide (0.06 mol) and phosphorus oxychloride (0.06 mol) were separately cooled to 0°C and then combined and stirred at the same temperature. A solution of **3a** (0.02 mol) or **3b** (0.02 mol) derivatives in DMF (10 ml) was added dropwise to the reaction mixture, which was then heated at 60°C for 5 h. After cooling to room temperature, the mixture was basified with a potassium carbonate solution and extracted with dichloromethane. The combined extracts were dried and concentrated to dryness. Purification by column chromatography on silica gel (dichloromethane/ethyl acetate) gave **4a** and **4b**.

Preparation of **5a**/**5b**: A mixture of **4a** or **4b** (0.02 mol), the appropriate 2-hydrazinylpyridine (0.02 mol) and piperidine (0.004 mol) in absolute ethanol (50 ml), was stirred and refluxed for 12 h. After cooling, the precipitate was isolated by filtration, washed with ethanol and petroleum ether, and recrystallized from DMF to give **5a** and **5b**.

Preparation of **6A**/**6B**: To a solution of **5a** (0.01 mol) or **5b** (0.01 mol) in dry THF (20 ml), iodosobenzene diacetate (0.01 mol) was slowly added. The reaction mixture was vigorously stirred for 3 h at room temperature. The solvent was evaporated, and the residue was purified by recrystallization from dichloromethane to give **6A** and **6B**.

*4-(4-([1,2,4]Triazolo[4,3-a]pyridin-3-yl)-3-phenyl-1H-pyrazol-1-yl)thieno[2,3-d]pyrimidine (**6Aa**)*. Yield: 80% as a brown solid; R_f = 0.24 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 224–225°C; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 9.31 (1H, s, H-7), 8.95 (1H, s, H-11), 8.61 (1H, d, *J* = 5.9 Hz, H-4), 7.87 (1H, d, H-29), 7.69 (1H, d, *J* = 5.9 Hz, H-3), 7.53 (3H, m, H-16, 20, 26), 7.39 (4H, m, H-17, 18, 19, 28), 6.67 (1H, t, H-27); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 172.1, 153.8, 152.2, 150.4, 150.1, 139.7, 132.0, 131.3, 129.4, 128.9, 127.3, 127.2, 127.1, 123.0, 122.7, 119.1, 116.5, 113.9, 108.2; EI-MS: *m/z* $[M]^+$: 395.1.

*4-(4-(5-Chloro-[1,2,4]triazolo[4,3-a]pyridin-3-yl)-3-phenyl-1H-pyrazol-1-yl)thieno[2,3-d]pyrimidine (**6Ab**)*. Yield: 76% as a yellow solid; R_f = 0.32 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 234–235°C; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 9.13 (1H, s, H-7), 9.04 (1H, s, H-11), 8.13 (1H, d, *J* = 5.9 Hz, H-4), 7.82 (1H, d, H-29), 7.65 (2H, m, H-16, 20), 7.60 (1H, d, *J* = 5.9 Hz, H-3), 7.32 (4H, m, H-17, 18, 19, 28), 6.80 (1H, d, H-27); ESI-MS: *m/z* $[M + H]^+$: 430.1.

*4-(4-([1,2,4]Triazolo[4,3-a]pyridin-3-yl)-3-p-tolyl-1H-pyrazol-1-yl)thieno[2,3-d]pyrimidine (**6Ac**)*. Yield: 72% as a light brown solid; R_f = 0.31 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 250.1 – 250.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 9.28 (1H, s, H-7), 8.93 (1H, s, H-14), 8.59 (1H, d, *J* = 5.9 Hz, H-4), 7.86 (1H, d, H-26), 7.66 (1H, d, *J* = 5.9 Hz, H-3), 7.58 (1H, d, H-29), 7.49 (2H, d, H-16, 20), 7.25 (1H, t, H-27), 7.12 (2H, d, H-17, 19), 6.68 (1H, t, H-28), 2.34 (3H, s, CH_3); ESI-MS: *m/z* $[M + H]^+$: 410.1.

*4-(4-(5-Chloro-[1,2,4]triazolo[4,3-a]pyridin-3-yl)-3-p-tolyl-1H-pyrazol-1-yl)thieno[2,3-d]pyrimidine (**6Ad**)*. Yield: 84% as a light yellow solid; R_f = 0.25 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 280.2 – 280.9°C; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 9.16 (1H, s, H-7), 8.92 (1H, s ,H-11), 8.61 (1H, d, *J* = 5.9 Hz, H-4), 7.82 (1H, d, H-29), 7.67 (1H, d, *J* = 5.9 Hz, H-3), 7.37 (2H, d, H-16, 20), 7.26 (1H, t, H-28),

7.10 (2H, d, H-16, 18), 6.77 (1H, d, H-27) 2.32 (3H, s, CH_3); ESI-MS: *m/z* $[M + H]^+$: 444.1.

*4-(4-(5-Chloro-[1,2,4]triazolo[4,3-a]pyridin-3-yl)-3-(4-chlorophenyl)-1H-pyrazol-1-yl)thieno[2,3-d]pyrimidine (**6Ae**)*. Yield: 88% as a light yellow solid; R_f = 0.25 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 264–265°C; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 9.13 (1H, s, H-7), 8.98 (1H, s, H-11), 8.59 (1H, d, *J* = 5.9 Hz, H-4), 7.86 (1H, d, H-29), 7.49 (1H, d, *J* = 5.9 Hz, H-3), 7.44 (2H, d, H-16, 20), 7.31 (3H, m, H-17, 19, 28), 6.80 (1H, d, H-27); ESI-MS: *m/z* $[M + H]^+$: 464.0.

*4-(4-([1,2,4]Triazolo[4,3-a]pyridin-3-yl)-3-(3-chlorophenyl)-1H-pyrazol-1-yl)thieno[2,3-d]pyrimidine (**6Af**)*. Yield: 88% as a light yellow solid; R_f = 0.25 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 264–265°C; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 9.13 (1H, s, H-7), 8.98 (1H, s, H-11), 8.59 (1H, d, *J* = 5.9 Hz, H-4), 7.86 (1H, d, H-29), 7.49 (1H, d, *J* = 5.9 Hz, H-3), 7.44 (2H, d, H-16, 20), 7.31 (3H, m, H-17, 19, 28), 6.80 (1H, d, H-27); ESI-MS: *m/z* $[M + H]^+$ 430.8.

*4-(4-([1,2,4]Triazolo[4,3-a]pyridin-3-yl)-3-(4-chlorophenyl)-1H-pyrazol-1-yl)thieno[2,3-d]pyrimidine (**6Ag**)*. Yield: 76% as a light brown solid; R_f = 0.2 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 235–236°C; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 9.49 (1H, s, H-7), 9.02 (1H, s, H-11), 8.46 (1H, d, *J* = 5.9 Hz, H-4), 8.23 (1H, d, H-29), 8.12 (1H, d, *J* = 5.9 Hz, H-3), 7.83 (1H, d, H-26) 7.64 (2H, m, H-16, 20) 7.41 (3H, d, H-17, 18, 28), 6.92 (1H, t, H-27); ESI-MS: *m/z* $[M + H]^+$: 430.0.

*4-(4-([1,2,4]Triazolo[4,3-a]pyridin-3-yl)-3-phenyl-1H-pyrazol-1-yl)thieno[3,2-d]pyrimidine (**6Ba**)*. Yield: 72% as a light yellow solid; R_f = 0.12 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 244–245°C; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 9.25 (1H, s, H-7), 9.05 (1H, s, H-11), 8.17 (1H, d, *J* = 5.9 Hz, H-4), 7.84 (1H, d, H-29), 7.64 (3H, m, H-5, 16, 20), 7.58 (1H, d, H-26) 7.38 (3H, m, H-17, 18, 19), 7.27 (1H, m, H-28), 6.69 (1H, t, H-27); ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm): 164.1, 153.6, 153.1, 151.1, 150.5, 140.4, 139.6, 131.0, 130.8, 129.5, 128.9, 127.3, 127.1, 124.3, 122.7, 118.0, 116.5, 114.0, 108.6; EI-MS: *m/z* $[M]^+$: 395.0.

*4-(4-(5-Chloro-[1,2,4]Triazolo[4,3-a]pyridin-3-yl)-3-phenyl-1H-pyrazol-1-yl)thieno[3,2-d]pyrimidine (**6Bb**)*. Yield: 76% as an orange solid; R_f = 0.2 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 195–196°C; ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): 9.25 (1H, s, H-7), 9.05 (1H, s, H-11), 8.17 (1H, d, *J* = 5.9 Hz, H-4), 7.84 (1H, d, H-29), 7.64 (3H, m, H-5, 16, 20), 7.58 (1H, d, H-26) 7.38 (3H, m, H-17, 18, 19), 7.27 (1H, m, H-28), 6.69 (1H, t, H-27); EI-MS: *m/z* $[M + H]^+$ 429.1.

*4-(4-(5-Chloro-[1,2,4]Triazolo[4,3-a]pyridin-3-yl)-3-m-tolyl-1H-pyrazol-1-yl)thieno[3,2-d]pyrimidine (**6Bc**)*. Yield: 88% as a light brown solid, R_f = 0.2 (ethyl acetate/chloroform = 1/1, v/v), m.p. = 245–246°C; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 9.34 (1H, s, H-7), 9.05 (1H, s, H-11), 8.59 (1H, d, *J* = 5.9 Hz, H-4), 7.90 (1H, d, H-29), 7.68 (1H, d, *J* = 5.9 Hz, H-5), 7.39 (1H, t, H-28) 7.26 (1H, s, H-16) 7.06 (4H, m, H-17, 18, 20, 27) 2.44 (3H, s, CH_3); ESI-MS: *m/z* $[M + H]^+$ 444.0.

*4-(4-(5-Chloro-[1,2,4]triazolo[4,3-a]pyridin-3-yl)-3-(4-chlorophenyl)-1H-pyrazol-1-yl)thieno[3,2-d]pyrimidine (**6Bd**)*. Yield: 75% as a white solid; R_f = 0.25 (ethyl acetate/chloroform = 1/1, v/v); m.p. = 290–291°C; ^{1}H NMR (400 MHz, DMSO-d_6, δ ppm): 9.39 (1H, s, H-7), 9.10 (1H, s, H-11), 8.65 (1H, d, *J* = 5.2 Hz, H-4), 7.96 (1H, d, *J* = 9.2 Hz, H-27), 7.73 (1H, d, *J* = 5.2 Hz, H-5), 7.46 (5H, m, H-16,17,19,20,28), 7.18 (1H, d, *J* = 7.2 Hz, H-29); EI-MS: *m/z* $[M]^+$: 463.9.

Cell Line and Cell Culture

Human hepatoma Hep3B cells were obtained from American Type Culture Collection (ATCC No. HB-8064) and were maintained in DMEM 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). Recombinant human IL-6 was purchased from R&D Systems (USA). All reagents, including genistein and stattic, were obtained from Sigma-Aldrich Ltd. (USA).

Establishment of Stable Cell Line Expressing pStat3-Luc and Luciferase Assay

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) using lipofectamine plus (Invitrogen, USA). Two days after transfection, the cells that stably expressed luciferase were selected by hygromycin treatment (100 μg/ml), and stable clones were expanded. The expression of luciferase in these clones stably expressing pStat3-Luc was confirmed by luciferase assays.

Luciferase Assay

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

Western Blot Analysis

Total proteins were prepared from the cells and were subjected to western blot analysis [29] with primary rabbit anti-phospho Stat3 (Tyr^{705}) IgG, anti-total Stat3 IgG (1:1000), anti-phospho ERK1/2, anti-total ERK (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 was changed to serum-free medium supplemented with the indicated doses of the test compounds. Following culture with the test compounds for 24 h, MTT (0.5 mg/ml) was added, and after 4 h of incubation at 37°C, 200 μl of DMSO was added to each well. The absorbance of each sample at 570 nm was measured against a background control using a 96-well plate reader. The percentage of viable cells from each set of treatment conditions was determined relative to the negative control.

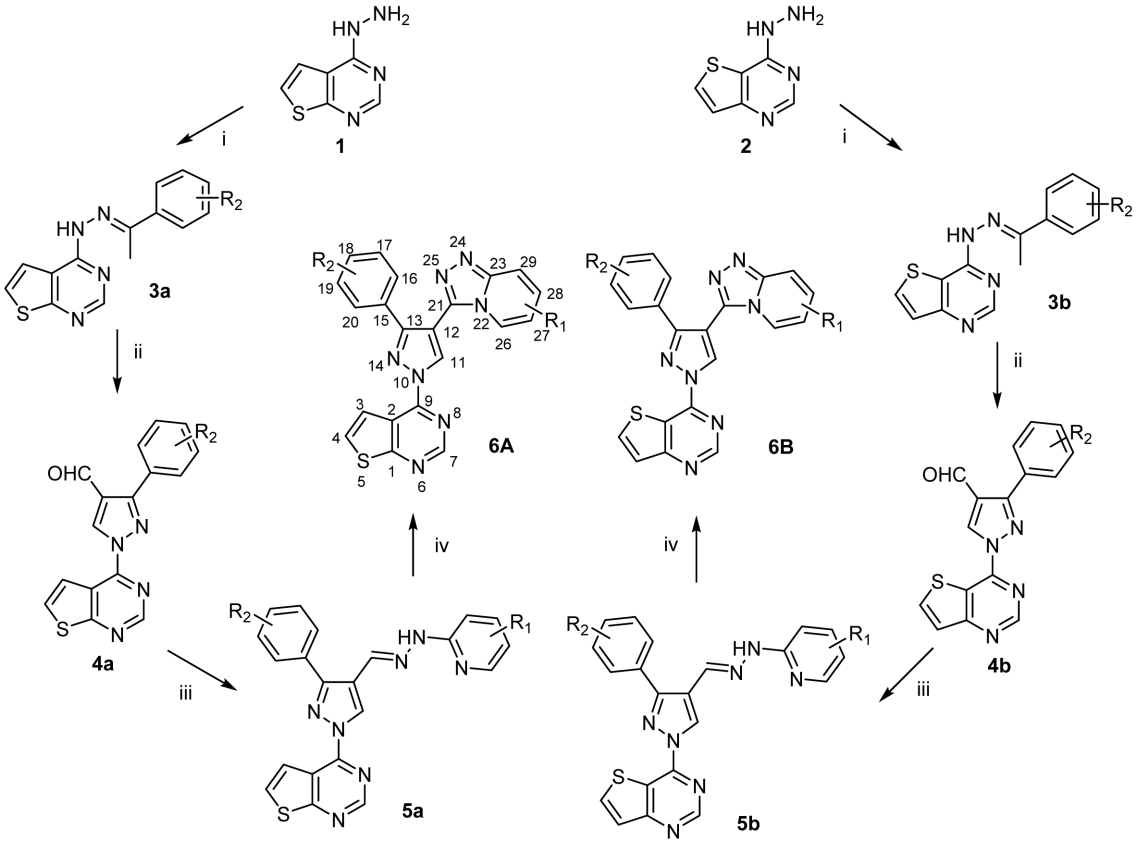
Results and Discussion

Chemistry

The preparation of **6A**/**6B** is outlined in Scheme 1. The synthesis of hydrazinylthienopyrimidines **1** and **2**, as starting materials, has been disclosed previously [23, 24]. The condensation of **1**/**2** with the appropriate acetophenone in hot ethanol containing pyrrolidine provided hydrazones **3a**/**3b**, which are then converted to 4a/4b in DMF/$POCl_3$ using a Vilsmeier-Haack reaction. Furthermore, pyrazole carbaldehydes **4a**/**4b** were treated with 2-hydrazinylpyridine to afford **5a**/**5b** via a standard methodology. Finally, the oxidative cyclizations of **5a**/**5b** to **6A**/**6B** were achieved using iodobenzene diacetate (Scheme 1).

IL-6-Induced STAT3-Dependent Promoter Activity

The inhibitory activities of synthesized [2,3-*d*]thienopyrimidine derivatives **6Aa–g** and [3,2-*d*]thienopyrimidine derivatives **6Ba–d** against STAT3 activation by IL-6 were evaluated according to the reported method [25]. In brief, Hep3B cells that had been stably transformed with the pStat3-Luc plasmid were stimulated with IL-6 (10 ng/ml) for 12 h in the presence or absence of compounds **6Aa–g** and **6Ba–d**. The Stat3-dependent promoter activity was



	R_1	R_2
6Aa:	H,	H
6Ab:	2-Cl,	H
6Ac:	H,	4-CH_3
6Ad:	2-Cl,	4-CH_3
6Ae:	2-Cl,	4-Cl
6Af:	H,	3-Cl
6Ag:	H,	4-Cl

	R_1	R_2
6Ba:	H,	H
6Bb:	2-Cl,	H
6Bc:	2-Cl,	3-CH_3
6Bd:	2-Cl,	4-Cl

Scheme 1. Synthetic route for preparing the thienopyrimidine derivatives (**6Aa–6Ag** and **6Ba–6Bd**); (i) acetophenone derivative, pyrrolidine, EtOH, reflux; (ii) DMF, $POCl_3$, 60°C; (iii) 2-hydrazinylpyridine, piperidine, EtOH, reflux; (iv) iodobenzene diacetate, THF, rt.

Table 1. Inhibitory effects of compounds **6Aa–g** and **6Ba–d** on IL-6-induced STAT3 activation.[a]

Compound No.	IC_{50} (μM)[b]
6Aa	5.73 ± 0.04
6Ab	0.58 ± 0.01
6Ac	3.24 ± 0.04
6Ad	0.56 ± 0.02
6Ae	0.47 ± 0.01
6Af	0.45 ± 0.01
6Ag	2.94 ± 0.11
6Ba	3.61 ± 0.02
6Bb	0.68 ± 0.10
6Bc	0.88 ± 0.03
6Bd	0.32 ± 0.02
Genistein[c]	15.00 ± 0.53
Stattic[c]	0.26 ± 0.04

[a]Data are the mean ± standard error values of three replicates.

[b]IC_{50}: mean (50%) value of the inhibitory concentration.

[c]Genistein and stattic were used as positive controls.

then measured. The IC_{50} values of these compounds were calculated by determining the concentration needed to inhibit half of the maximum pSTAT3-Luc activation induced by IL-6. Genistein, a tyrosine kinase inhibitor [29, 30], and stattic, a selective STAT3 inhibitor [27], were used as positive controls, as they inhibited Stat3-dependent luciferase activity with IC_{50} values of 15.00 and 0.26 μM in this assay system, respectively. Table 1 lists the biological data for compounds **6Aa–g** and **6Ba–d**. Compound 6Aa, which was identified from the initial screening, exhibited stronger inhibitory activity than the other in-house chemicals on IL-6-induced STAT3 activation with an IC_{50} value of 5.73 μM.

To assess the effects of functional groups on the benzene and triazolopyridine moieties, we compared the inhibitory activity of compound **6Aa** with those of its analogues (**6Ab–6Ag**, Table 1). Of these compounds, 6Ab, in the hydrogen at the 2-position of the triazolopyridine moiety had been replaced with a chloride, exhibited an inhibitory activity 9.9-fold higher than that of compound **6Aa**. However, compound **6Ac**, which had a methyl group at the *para* position of the benzene moiety, only led to a 1.8-fold increase in IL-6-induced STAT3 activity (IC_{50} value of 3.24 μM) compared with **6Aa**. Also, compound **6Ag**, which possessed a chloride at the *para* position of the benzene moiety, demonstrated a higher inhibitory activity (IC_{50} value of 2.94 μM) than what was shown by compounds **6Aa** and **6Ac**. Additionally, we replaced the hydrogen at the 2-position of the triazolopyridine moieties of **6Aa**, **6Ac**, and **6Ag** with a chloride, and the inhibitory activities of the resulting compounds (**6Ab**, **6Ad**, and **6Ae**) were tested on IL-6-induced STAT3 activation. The results showed improved inhibitory activities with IC_{50} values (0.58, 0.56, and 0.47 μM, respectively) that were better than those of **6Aa**, **6Ac**, and **6Ag**. In addition, compound **6Af**, which possessed a chloro substituent at the *meta* position of the benzene moiety, showed an inhibitory activity 6.5-fold (IC_{50} value of 0.45 μM) more potent than that of **6Ag**, suggesting that *meta* substitution of the benzene moiety can impact the activity on IL-6-induced STAT3 activation (see Fig. S1). Moreover, we further evaluated the IL-6-induced STAT3 inhibitory activities of [3,2-*d*]thienopyrimidine analogues **6Ba-d** (Table 1**)**. Compound **6Ba** showed an inhibitory activity (IC_{50} value of 3.61 μM) 1.6-fold more potent than that of compound **6Aa**, suggesting that inclusion of a [3,2-*d*]thienopyrimidine moiety can improve the IL-6-induced STAT3 inhibitory activity (Table 1). Compound **6Bb**, which had a chloride at the 2-position of the triazolopyridine moiety, was 5.3-fold more potent than **6Ba** against IL-6-induced STAT3 activity (IC_{50} value of 0.68 μM); however, it showed a potency similar to that of compound **6Ab** (Table 1). Moreover, compound **6Bc**, which possessed a methyl substituent at the *meta* position of the benzene moiety, showed even stronger inhibitory activity, with an IC_{50} value of 0.88 μM, than compound **6Ba**. However, to our surprise, [3,2-*d*]thienopyrimidine analogues **6Bb** and **6Bc** did not show significantly more potent inhibitory activities compared to the [2,3-*d*]thienopyrimidine analogues (Table 1). Compound **6Bd**, which had a chloride substituent at the *para* position of the benzene moiety, was 10-fold more potent than **6Ba** against IL-6-induced STAT3 activity (IC_{50} value of 0.32 μM). Accordingly, the IL-6-induced STAT3 inhibitory activities of these thienopyrimidine derivatives may be affected by the chloride substituent at the 2-position of the triazolopyridine moiety and the chloride at the *meta* position of the benzene moiety (see Fig. S1).

IL-6-Induced STAT3 and ERK Phosphorylation Activity

We then investigated if compounds **6Aa–c** and **6Ba–c** could inhibit the phosphorylation of STAT3 induced by IL-6 in Hep3B cells. As presented in Figs. 1A and 1B, compounds **6Aa–c** and **6Ba–c** inhibited phosphorylation of STAT3 by IL-6 in Hep3B that had been treated with the test compounds for 20 min at concentrations of 30 and 10 μM. Moreover, compounds **6Ab** and **6Bc**, which possessed a

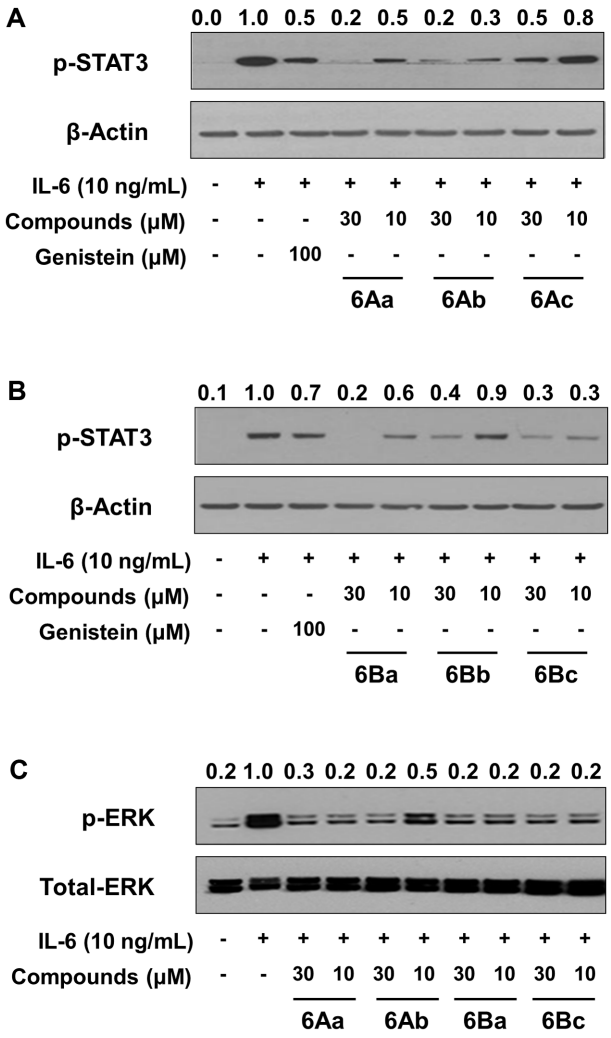


Fig. 1. Effects of derivatives synthesized from **6Aa** and **6Ba** on STAT3 and ERK phosphorylation by IL-6.

Hep3B cells were incubated with IL-6 for 20 min in the presence or absence of thienopyrimidine derivatives **6Aa–c** and **6Ba–c** at concentrations of 30 and 10 μM, respectively. Immunoblotting with anti-phospho STAT3 (Tyr705), anti-phospho ERK1/2, and anti-total ERK and anti-β-actin was performed on the total protein (10 μg protein/lane). The ratio of anti-phospho STAT3/β-actin and anti-phospho ERK1/2/total ERK were quantified using ImageJ software.

chloro substituent at the 2-position of the triazolopyridine moiety, showed higher inhibitory activities against IL-6-stimulated STAT3 phosphorylation compared to what was seen with compounds **6Aa** and **6Ba**. These data were in good agreement with the structure-activity relationship (SAR) indicated by the luciferase reporter assay.

ERK-MAPK is a downstream protein in EGFR signaling, and it is closely linked to the activation of STAT3 [32]. Thus, we tested whether compounds **6Aa**, **6Ab**, **6Ba** and **6Bc** could inhibit the phosphorylation of ERK1/2 induced by IL-6 in Hep3B cells. Compounds **6Ba** and **6Bc** potently inhibited the phosphorylation of ERK1/2 by IL-6 at concentrations of 30 and 10 μM (Fig. 1C); however, the inhibitory activity of compound **6Ab** was weaker than that of **6Aa** against IL-6-stimulated phosphorylation of ERK1/2 at a concentration of 10 μM (Fig. 1C). Accordingly, these results suggest that the STAT3 inhibitory activity of **6Ab** may exert its effect via other pathways, such as gp130/JAK, rather than the ERK-MAPK cascade.

Hence, none of the tested compounds showed any cytotoxicity toward Hep3B cells in the MTT assay (data not shown). Further synthetic studies on the optimization of thienopyrimidine derivatives based on SAR and detailed studies of the biological activities of these derivatives are now in progress.

In conclusion, the synthesized thienopyrimidine derivatives (**6Aa-h** and **6Ba-d**) exhibited potent inhibitory activity in the STAT3-dependent luciferase assay. Among these analogues, **6Aa**, **6Ab**, **6Ba** and **6Bc** inhibit the phosphorylation of STAT3 and ERK1/2 induced by IL-6 in Hep3B cells. Based on these results, these synthesized compounds could be useful candidates for the development of therapeutics for treating inflammatory diseases, and the SAR study could allow the design of IL-6 inhibitors.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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