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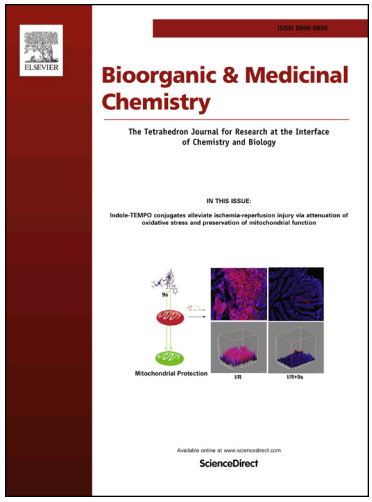

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Synthesis of benzoxazole derivatives as interleukin-6 antagonists

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Abstract

A growing number of studies have demonstrated that interleukin (IL)-6 plays pathological roles in the development of chronic inflammatory disease and autoimmune disease by activating innate immune cells and by stimulating adaptive inflammatory T cells. So, suppression of IL-6 function may be beneficial for prevention and treatment of chronic inflammatory disease. This study reports that a series of synthetic derivatives of benzoxazole have suppressive effects on IL-6-mediated signaling. Among 16 synthetic derivatives of benzoxazole, the compounds **4**, **6**, **11**, **15**, **17**, and **19** showed a strong suppressive activity against IL-6-induced phosphorylation of signal transducer and activator of transcription (STAT) 3 by 80-90%. While the cell viability was strongly decreased by compounds **11**, **17**, **19**, the compounds **4**, **6**, and **15** revealed less cytotoxicity. We then examined the effects of the compounds on inflammatory cytokine production by CD4+ T cells. CD4+ T cells were induced to differentiate into interferon (IFN)-γ-, IL-17-, or IL-4-producing effector T cells in the presence of either the compound **4** or the compound **7**. While the inactive compound **7** had no significant effect on the cytokine production by effector T cells, the active compound **4** strongly suppressed the production of inflammatory cytokines IFN-γ and IL-17, and also inhibited allergic inflammatory cytokines IL-4, IL-5, and IL-13 produced by effector Th2 cells. These results suggest that a benzoxazole derivative, compound **4** effectively suppresses IL-6-STAT3 signaling and inflammatory cytokine production by T cells and provides a beneficial effect for treating chronic inflammatory and autoimmune disease.

Key Words: Benzoxazole derivatives, IL-6-STAT3, IFN-γ, IL-17, IL-4

1. Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine that regulates pathological pain, neural signaling, inflammation and infection responses and also causes multidrug resistance in cancer. [1-4] IL-6 is produced by a variety of cell types including macrophages and T cells and functions through binding to its receptor (IL-6R). [5] Upon IL-6 binding, IL-6R undergoes dimerization with glycoprotein 130 (gp130) and activates Janus kinases and leads to the activation of phosphatase Src homology 2 domain-containing tyrosine phosphatase-2 (SHP-2) and mitogen-activated protein kinase (MAPK). IL-6 signaling recruits and phosphorylates the signal transducer and activator of transcription factor 3 (STAT3), which then activates transcription of inflammatory chemokine genes in the nucleus and causes inflammation by recruiting immune cells.[6] It turns out that IL-6 is aberrantly overproduced in chronic inflammatory disease like rheumatoid arthritis (RA). [7] IL-6 induces joint destruction by enhancing B cell maturation and chemokine-mediated recruitment of immune cells and also increases osteoclast differentiation causing osteoporosis. In addition, IL-6 stimulates Th17 cell differentiation by activation of STAT3 phosphorylation in T cells and accelerates inflammatory autoimmune response. Thus, it will be of great interest to find the way to block IL-6 signaling for treating RA.[8-10] Indeed, the neutralizing antibody against IL-6R, Tocilizumab has been approved for treating inflammatory diseases.[10-13] Tocilizumab binds both soluble and membrane-expressed IL-6R and inhibits IL-6-induced chronic inflammation.[14]

Not only tocilizumab, but sarilumab, ALX-0061, sirukumab, MEDI5117, clazakizumab, and olokizumab also block IL-6/IL-6R signaling and are used for treating arthritis. Furthermore, a variety of monoclonal antibodies such as infliximab, anakinra, and rituximab have explosively been developed as therapeutics for RA, which effectively block TNF-α, IL-1, and B cell maturation and are often used in combination with methotrexate. [9]

Although monoclonal antibodies have potential for treating arthritis, these are very expensive and only available as injection forms. Orally available small molecules are also under study, and recently Tofacitinib, an inhibitor of the enzyme janus kinase 3 (JAK 3), is developed and currently approved for the treatment of RA in the United States and other countries.[15] Other

small molecule inhibitors for RA, such as fostamatinib and VX-509 (JAK3 Inhibitor), [16-17] LX3305 (sphingosine-1-phosphate (S1P) lipase inhibitor)[18], and CCX354-C (CCR1 inhibitor)[19] are under development. Their structures are shown in Figure 1. Some side effects, such as liver test elevation and neutropenia are reported with tofacitinib, VX-509 and fostamatinib; lipid elevation with tofacitinib and VX-509; creatinine elevation and anemia with tofacitinib, and hypertension and diarrhea with fostamatinib.[15] A natural product compound, Madindoline A was reported as a highly selective inhibitor of IL-6, but cannot be developed into an effective drug, due to the scarcity of the natural resources and the complexity involved in the synthesis.[20]

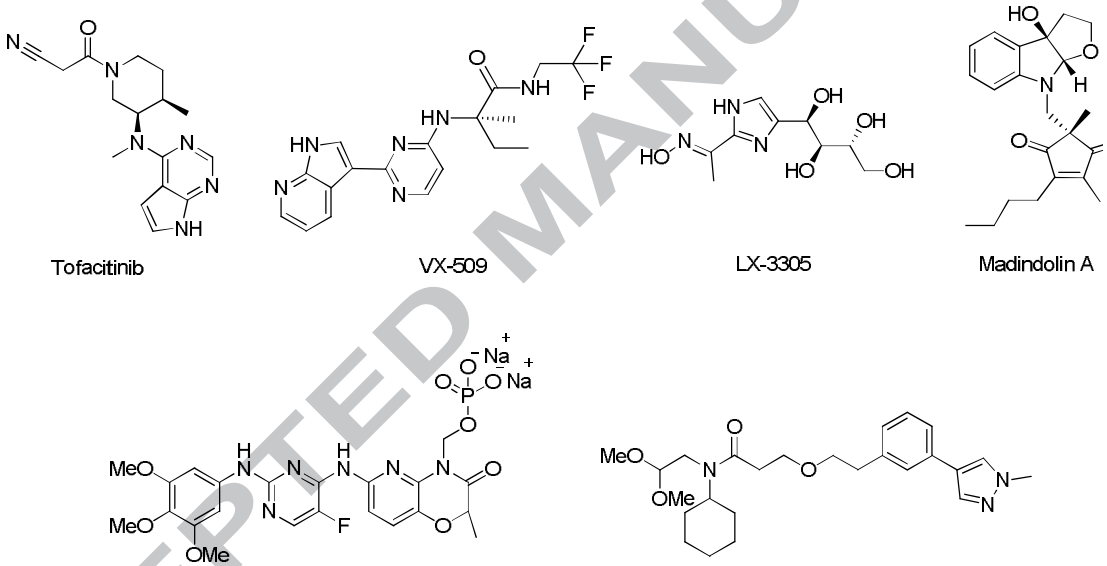


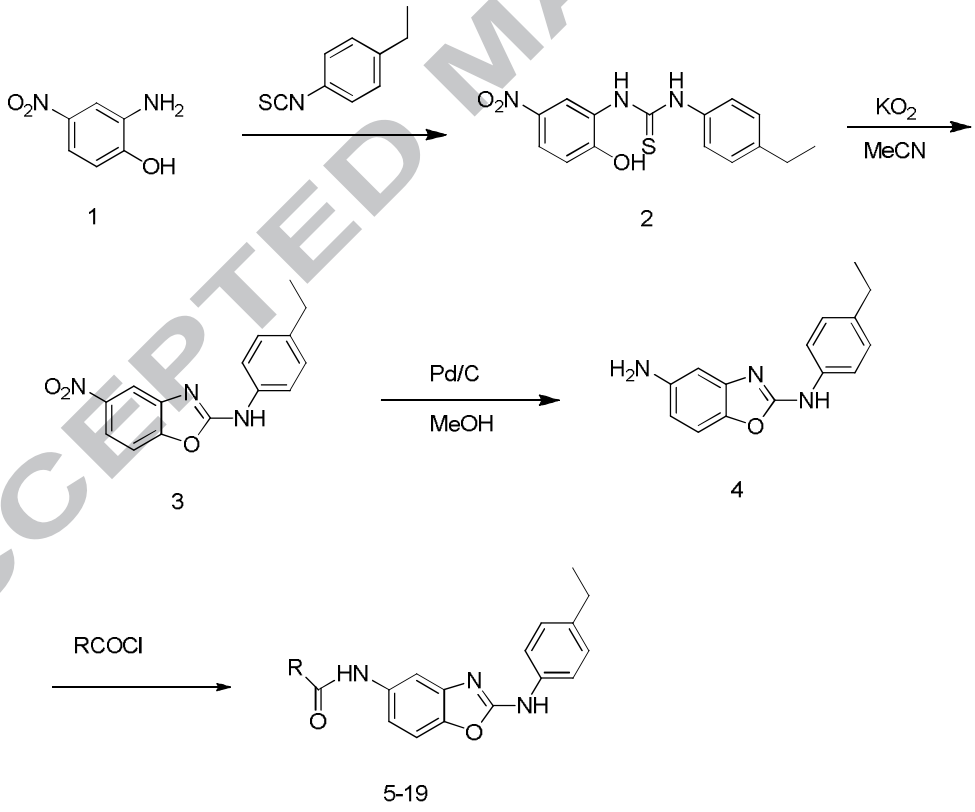
Figure1. Small molecules inhibitors for Rheumatoid Arthritis

Since only one oral drug for RA is approved in the market, it is highly desirable to design and identify novel, small molecule compounds as IL-6 inhibitors. Recently we have reported the benzoxazoles, benzisoxazoles, and benzthiazoles as 5-lypoxygenase inhibitors.[21-23] As our ongoing study on the use of benzoxazoles as anti-inflammatory agents, we have synthesized sixteen 4-amino benzoxazole derivatives as IL-6 inhibitiors in this study. The active compound **4** was evaluated further to elucidate the mechanism of action.

2. Results

2.1. **Synthesis of benzoxazole derivatives**

Various derivatives having the basic structure of benzoxazole were synthesized starting from the commercially available 2-amino-4-nitrophenol and 4-ethylphenyl isothiocyanate. The thiourea 2 was obtained in high yield (79%). The thiourea **2** was cyclized to benzoxazole **3** by oxidation with 5 eq KO_2 as previously reported.[21] This procedure was simple and mild to prepare benzoxazoles, with the per cent yields of the reactions ranges 80 to 90. The nitro group in **3** was reduced to amino group with catalytic hydrogenation using 5% Pd/C as catalyst to afford **4**. Then the amino group in **4** was reacted with various acid chlorides and N,N-diisopropylethylamine at room temperature to afford desired amides **5-19** in 35-77% yields.



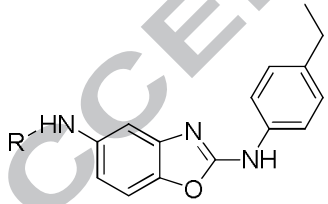
Scheme 1. Synthetic procedure for 4-aminobenzoxazole derivatives

2.2. **Inhibition of IL-6-STAT3 signaling by benzoxazole derivatives**

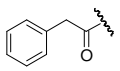
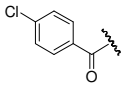
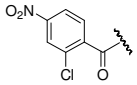
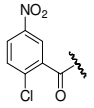
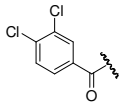
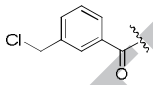
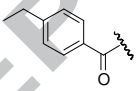
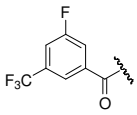
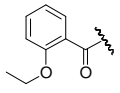
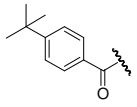
To identify small-molecule antagonists of IL-6, we screened our 16 synthetic compounds by measuring the effects of each compound on IL-6–induced luciferase expression in human hepatocarcinoma HepG2 cells transfected with p-STAT3-Luc. In brief, p-STAT3-Luc–transfected HepG2 cells were stimulated with IL-6 in the presence of screening compounds, and luciferase activities were measured.

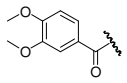
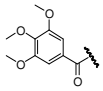
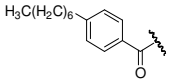
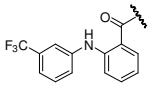
The IL-6 inhibition activities of the synthesized compounds are shown in Table 1. Synthesized benzoxazole derivatives were used for evaluatin of IL-6 inhibition activity at a concentration of 20μg/ml. Most of prepared compounds showed a more than 50% inhibition against IL-6, and six compounds (**4, 6, 11, 15, 17, 19**) showed a more than 80% inhibition. The active compounds, **4,** [*N*-(4-ethylphenyl)benzo[d] oxazole- 2,5-diamine], and **6**, [*N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)benzamide] showed excellent inhibitory activity (97.5% and 93.1%, respectively), of IL-6 at 20μg/ml *in vitro*. These compounds, **4** and **6** , exhibited the IC_{50} values of 18.9 μM and 5.8 μM, respectively, while the IC_{50} value of Madindolin A, a known selective inhibitor of IL-6, was 20.7 μM . The compounds, **4** and **6** showed low cytotoxicity in MTT assay (70.83% and 65.58%, respectively).

Table 1. The structures of prepared amides and their IL-6 inhibitory activity and cytotoxicity



Compound	R	IL-6 (STAT 3) Inhibition		MTT viability
		% (20μg/ml)	IC_{50} (μM)	(%)10μg/ml
4	H	97.5	18.9	70.82

5		61.7		-
6		93.1	5.8	65.58
7		12.1		-
8		20.0		-
9		65.6		-
10		69.3		-
11		91.7		41.09
12		63.3		-
13		50.2		-
14		58.0		-
15		86.5		59.79

16		26.2	-
17		83.7	16.41
18		57.2	-
19		84.6	38.25
20	Madindolin A		20.5

2.3. Suppression of inflammatory cytokine production by compound **4**

We have examined the effects of compounds **4** and **7** on inflammatory effector T cell functions. CD4+ T cells were induced to differentiate into Th1, Th17, or Th2 cells and additionally incubated with either compound **4** or compound **7**. Th1 cells that were stimulated with anti-TCR antibody and IL-12 produced signature cytokines of Th1 cells IL-2 and IFN-γ. Compound **4** substantially suppressed IL-2 and IFN-γ production by effector Th1 cells whereas compound **7** rather increased the level of IL-2 and IFN-γ (Fig. 2).

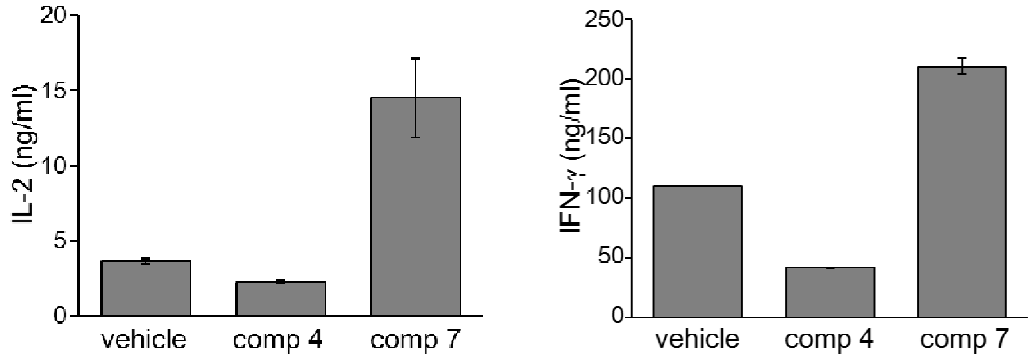


Figure 2. Effect of compound 4 and 7 on Th1 cell function

Inflammatory cytokine IL-17 that are produced by effector Th17 cells involved in arthritic pathophysiology was also suppressed by treatment with compound 4 but was not affected by compound **7** (Fig. 3). These results suggest that compound **4** may have a strong anti-inflammatory activity.

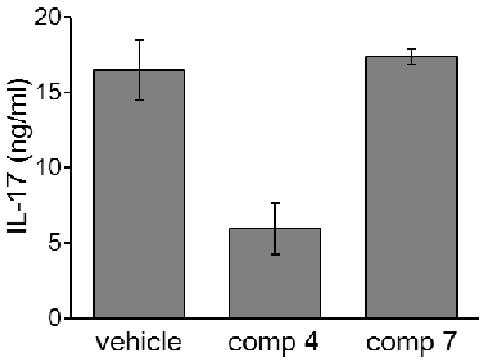


Figure 3. Effect of compounds **4** and **7** on IL-17 production

As Th2 cytokines such as IL-4, IL-5, and IL-13 are critically involved in the development of chronic inflammatory autoimmune diseases, we have also tested whether compounds **4** and **7** affect Th2 cytokine production. Th2 effector cells were generated from CD4+ T cells by treatment with IL-4. Cells were additionally incubated with either compound 4 or compound **7**. IL-4, IL-5, and IL-13 cytokines were all suppressed by compound **4** but were not influenced by treatment with compound **7** (Fig. 4). These results suggest that compound **4**, not compound **7** selectively suppressed inflammatory cytokines produced by Th1, Th2, and Th17.

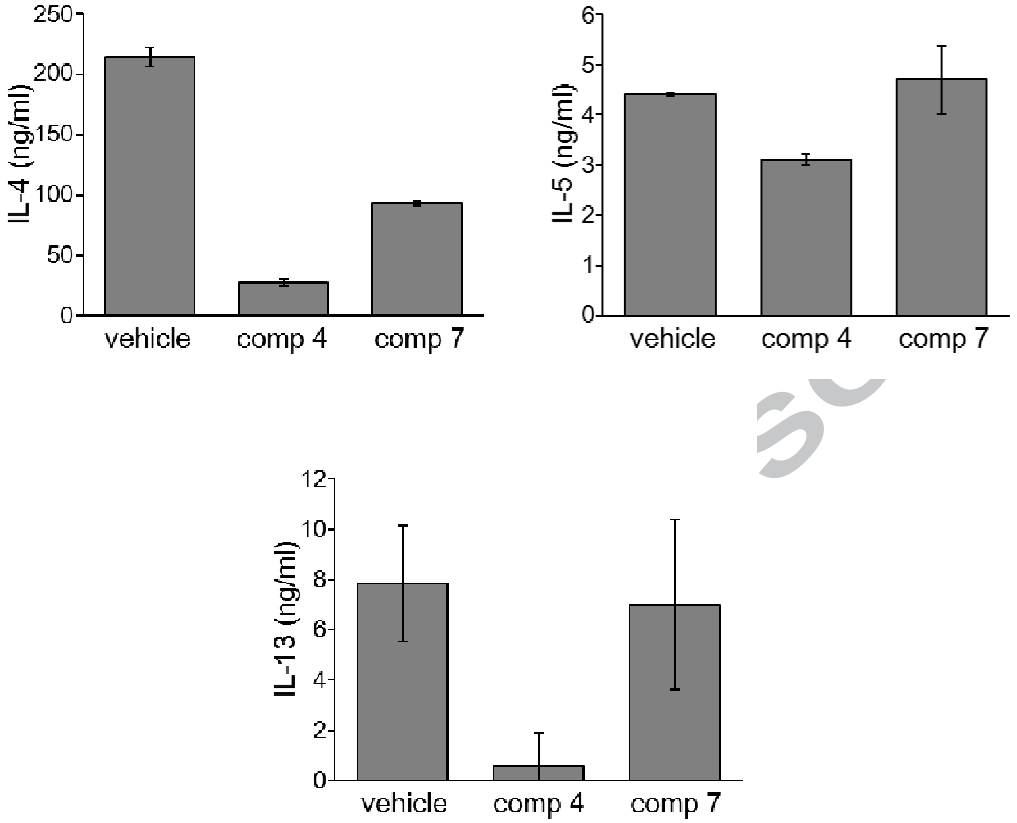


Figure 4. Effect of compounds **4** and **7** on Th2 cytokine production

3. Discussion

A pleiotropic proinflammatory cytokine IL-6 is a key player in inflammation, and IL-6 could be an attractive therapeutic targets, and previous studies indicated that IL-6 and the major down- stream effector STAT3 contributes to the pathogenesis of numerous human diseases such as RA.[24] IL-6 is involved in multiple immunologic processes such as T cell activation, B cell proliferation, initiation of acute-phase protein, and stimulation of hematopoietic precursor cell growth, differentiation, and trafficking.[25]

Sixteen 4-amino benzoxazole derivatives were synthesized and were tested for suppression of IL-6/STAT3 signaling. Benzoxazole derivatives are structurally related to biologically important bases and constitute a class of heterocyclic compounds that exhibit excellent druggability and substantial therapeutic activities. The prepared compounds showed 12 – 97% inhibition at 20μg/ml (Table 1). The highly active compounds **4** and **6** showed the IC_{50} values of 18.9 μM and 5.8 μM, respectively.

For structure activity relationship, 4-amino compound **4** showed good activity showing 97.5% inhibition. When amide group was introduced, both phenyl acetyl derivative (**5**) and phenyl derivative (**6**) showed good activity, 61.7% and 93.1%, respectively. The comparatively more potent phenyl derivatives were pursued on further studies. When the phenyl ring has para substitutions, such as chloro, ethyl, and heptyl, the activity was decreased (**6 > 7**, **12**, **18**). However the para-t-butyl congener **15** showed similar good activity (86.5%). The *m*-chloromethyl compound **11** showed good activity (91.7%), while *p*-chloro- or *m*, *p*-dichloro-compounds showed lower activity (**11 > 10 > 7).** A further significant increase in activity of **16** has been observed by introducing one more methoxy group to the *meta* position from the carbonyl moiety (**17> 16**). However, the introduction of electron withdrawing group such as nitro, fluoro- or trifluoromethyl group showed poor activity (**8, 9, 13**). The ortho (trifluoromethyl)phenylamino- substituted compound (**19**) showed good activity with 84.6% inhibition. Among the synthesized compounds, the lipophilic phenyl derivatives showed stronger activity, probably due to the inhibition of IL-6/GP130 protein-protein interaction, somewhat like Madindolin A.[26] Also the structural modification on *p*-substituted ethyl group at the aniline moiety is now undergoing and the result will be soon be reported.

IL-6 enhances B cell maturation and activates inflammatory T cell response by stimulation of

innate immune cells. In particularly, IL-17-producing Th17 cells are directly generated in response to IL-6 signaling. Effector T cells including Th1 and Th2 cells are known to be increased in chronic autoimmune pathology conditions such as RA, pancreatitis, multiple sclerosis, systemic lupus erythematosus, Crohn's disease, asthma, multiple myeloma, colorectal cancer, breast cancer, and lymphoma. Interestingly, a benzoxazole derivative, compound **4** substantially suppressed cytokines produced by effector Th 1, Th2, and Th17 cells while compound **7** reveals no suppressive activity. Compound **4** reveals a potent suppressive activity on IL-6-mediated STAT3 phosphorylation and the production of inflammatory cytokines produced by effector T cells, implicating its therapeutic potentials in treating chronic inflammatory and autoimmune disease. Therefore, application of compounds **4** or **6** to various human diseases is anticipated, especially inflammatory, autoimmune, and cancer conditions.

4. Experimental

4.1. Materials and methods.

Melting points were measured on an electro thermal digital melting point (Buchi, Germany) without calibration. ^{1}H NMR spectra were recorded on Varian NMR AS and Varian Unity Inova 400 MHz NMR spectrometers. Chemical shifts were reported in parts per million (δ) units relative to the solvent peak. The ^{1}H NMR data were reported as peak multiplicities:s for singlet; d for doublet; t for triplet; and m for multiplet. Coupling constants were recorded in hertz. Mass spectra data was obtained on an Agilent 6220 Accurate- Mass time- of- flight liquid chromatography/mass spectrometry (TOF LC/MS). Reagents were of commercial grade and were purchased from Sigma-Aldrich Co., Merck, and Ducksan Pure Chemical Co.

4.2. Procedure for synthesis of compounds

4.2.1. Synthesis of 1-(4-ethylphenyl)-3-(2-hydroxy-5-nitrophenyl)thiourea (2)

4-Ethylphenyl isothiocyanate (0.3g, 1.947 mmol, 1 eq,) was added to 2-amino-4-nitrophenol (0.318g, 1.947 mmol, 1eq) dissolved in 10mL of methanol, and stirred at room temperature for 24 h. The organic solvent was removed by evaporation under reduced pressure, and then the precipitate was washed with hexane to yield the title compound **2**.

Yellow-green powder (79%), mp 137-138℃; ^{1}H NMR(Acetone-d_6 400 MHz) δ9.48 (s, 1H), 9.35 (s, 1H), 8.76 (s, 1H), 7.97 (d, *J* = 9.8 Hz, 1H), 7.46 (d, *J* = 8.0 Hz, 2H), 7.28 (d, *J* = 8.8 Hz, 2H), 7.09(d, *J* = 9.2 Hz, 1H), 2.66 (q, *J* = 7.6 Hz, 2H), 1.23 (t, *J* = 7.6 Hz, 3H)

4.2.2. Synthesis of N-(4-ethylphenyl)-5-nitrobenzo[d]oxazol-2-amine (3)

In an ice bath under nitrogen gas, 15 mL of acetonitrile was slowly added to $K0_2$ (0.326 g, 4.58 mmol, 5 eq) with stirring. Then, the synthesized thiourea **2** (0.3g, 0.916 mmole, 1 eq,) in 20 mL of acetonitrile was slowly added to the solution. The mixture was reacted at room temperature for 16 h, and then the reaction mixture was diluted with 30 mL of ice water. This solution was extracted with dichloromethane, and washed two times with saturated aqueous NaCl solution. After drying with anhydrous MgSO4, the precipitate obtained by removing the solvent under reduced pressure was washed with EtOAc:hexane (1 : 10) to yield the title compound **3**.

Yellow powder (88%), mp 172-173℃;^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.81 (s, 1H), 8.23 (s, 1H), 8.11 (d, *J* = 8.8 Hz, 1H), 7.76 (d, *J* = 8.4 Hz, 2H), 7.62 (d, *J* = 9.2 Hz, 1H), 7.28 (d, *J* = 8.8 Hz, 2H), 2.65 (q, *J* = 7.6 Hz, 2H), 1.23 (t, *J* = 7.6 Hz, 3H).

4.2.3. Synthesis of *N*-(4-ethylphenyl)benzo[d] oxazole- 2,5-diamine (4)

Methanol 20 mL was added dropwise to the compound **3** prepared (1.31 mmol, 1 eq) and 5% Pd/C (0.4 g), and then the reaction mixture was charged with hydrogen and stirred at room temperature for 24 h. The reaction solution was filtered through celite, and then the solvent was removed from the filtrate under reduced pressure. Column chromatography with MC:MeOH (98:2) gave the title compound **4**.

Gray solid (88%), mp 143~145℃; ^{1}H NMR (Acetone-d_6, 400 MHz) δ9.17 (s, 1H), 7.74 (d, *J* = 8.4 Hz, 2H), 7.21 (d, *J* = 8.4 Hz, 2H), 7.04 (d, *J* = 8.8 Hz, 2H), 6.75 (s, 1H), 6.44 (d, *J* = 8.4 Hz, 1H), 4.44 (d, *J* = 10.4 Hz, 1H), 2.62 (q, *J* = 7.6 Hz, 2H), 1.21 (t, *J* = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{15}H_{16}N_3O$ (M^++H): 254.1293, Found: 254.1292.

4.2.4. General synthesis of *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-benzamides (5-19)

The compound **4** prepared (1 mmol, 1 eq) was dissolved with DMF (dimethylformamide, 3 mL), and substituted phenylacetyl chloride (1 mmol, 1 eq) and N,N-diisopropylethylamine (1 mmol, 1 eq) were added together thereto. The mixture was then stirred at room temperature for 16 h. To the reaction solution was added 10% aqueous HCl solution, and the mixture was extracted with 30 mL of EtOAc. The organic layer was washed with 10% aqueous HCl solution, and then two times with saturated $NaHCO_3$ solution and two times with saturated aqueous NaCl solution. After drying with anhydrous $MgSO_4$, the organic solvent was removed under reduced pressure, and column chromatography with MC:MeOH (98:2) gave the title compounds **5-19.**

4.2.4.1. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-2-phenylacetamide (5)

White solid (56%), mp >250℃;^{1}H NMR ($CDCl_3$, 400 MHz) δ7.51-7.46 (m, 2H), 7.44-7.40 (m, 2H), 7.37-7.33 (m, 2H), 7.24-7.16 (m, 4H), 7.05 (s, 1H), 6.92 (s, 1H), 3.77 (s, 2H), 2.64 (q, *J* = 7.3 Hz, 2H), 1.23 (t, *J* = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{23}H_{23}N_3O_2$ (M^++H): 372.1712, Found: 372.1714.

4.2.4.2. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)benzamide (6)

White solid (65%), mp 239-240℃;^{1}H NMR ($CDCl_3$, 400 MHz) δ7.91-7.89 (m, 2H), 7.82 (s, 1H), 7.70 (d, *J* = 2.0 Hz, 1H), 7.57-7.54 (m, 1H), 7.53-7.49 (m, 3H), 7.45 (dd, *J* =8.6 Hz, *J*= 2 Hz, 1H), 7.31 (d, *J*= 8.4 Hz, 1H), 7.23 (d, *J* = 8.4 Hz, 2H), 2.65 (q, *J* = 7.6 Hz, 2H), 1.25 (t, *J* = 7.6 Hz, 3H). HRFABMS Calcd for $C_{22}H_{21}N_3O_2$ (M^++H): 358.1556, Found: 358.1551.

4.2.4.3. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-4-chlorobenzamide (7)

White solid (40%), mp >250℃;^{1}H NMR ($CDCl_3$, 400 MHz) δ7.84 (d, *J* = 8.8 Hz, 2H), 7.76 (s, 1H), 7.69 (d, *J* = 1.6 Hz, 1H), 7.52-7.47 (m, 4H), 7.42 (d, *J* = 8.4 Hz, 1H), 7.31 (d, *J* = 8.4 Hz, 1H), 7.23 (d, *J* = 8.0 Hz, 2H), 6.90 (brs, 1H), 2.65 (q, *J* = 7.6 Hz, 2H), 1.24 (t, *J* = 7.6 Hz, 3H). HR-FABMSCalcd for $C_{22}H_{19}ClN_3O_2$ (M^++H): 392.1166, Found: 392.1161.

4.2.4.4. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-2-chloro-4-nitrobenzamide (8)

Yellow solid (35%), mp >250℃;^{1}H NMR (Acetone-d_6, 400 MHz) δ9.83 (s, 1H), 9.45 (s, 1H), 8.37 (s, 1H), 8.32 (d, *J* = 8.2 Hz, 1H), 7.96 (d, *J* = 8.4 Hz, 2H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.51 (d, *J* =8.8 Hz, 1H), 7.38 (d, *J* = 8.4 Hz, 1H), 7.24 (d, *J* = 8.4 Hz, 2H), 2.63 (q, *J* = 7.6 Hz, 2H), 1.22 (t, J = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{22}H_{18}ClN_4O_4$ (M^++H): 437.1017, Found: 437.1024.

4.2.4.5. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-2-chloro-5-nitrobenzamide (9)

White solid (35%), mp >250℃;^{1}H NMR (Acetone-d_6, 400 MHz) δ9.83 (s, 1H), 9.45 (s, 1H), 8.52 (s, 1H), 8.35 (dd, *J* = 9 Hz, *J* = 2.4 Hz 1H), 7.97 (s, 1H), 7.86 (d, *J* = 8.8 Hz, 1H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.50 (d, *J* = 8.8 Hz, 1H), 7.38 (d, *J* = 8.8 Hz, 1H), 7.24 (d, *J* = 8.4 Hz, 2H), 2.63 (q, *J* = 7.6 Hz, 2H), 1.22 (t, *J* = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{22}H_{18}ClN_4O_4$ (M^++H): 437.1017, Found: 437.1011.

4.2.4.6. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-3,4-dichlorobenzamide (10)

White solid (56%), mp >250℃;^{1}H NMR ($CDCl_3$, 400 MHz) δ8.00 (d, *J* = 2.0 Hz, 1H), 7.74-7.70 (m, 2H), 7.59 (d, *J* = 8.4 Hz, 1H), 7.51 (d, *J* = 8.4 Hz, 2H), 7.39 (d, *J* = 8.4 Hz, 1H), 7.31 (d, *J* = 8.6 Hz,1H), 7.23 (d, *J* = 8.4 Hz, 2H), 6.90 (brs, 1H), 2.65 (q, *J* = 7.6 Hz, 2H), 1.25 (t, *J* = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{22}H_{18}Cl_2N_3O_2$ (M^++H): 426.0776, Found: 426.0768.

4.2.4.7. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-3-(chloromethyl)benzamide (11)

White solid (47%), mp 194.5-195℃;^{1}H NMR ($CDCl_3$, 400 MHz) δ7.92 (s, 1H), 7.83 (d, *J* = 6.0 Hz, 1H), 7.71 (s, 1H), 7.59 (d, *J* = 8.0 Hz, 1H), 7.53-7.49 (m, 3H), 7.42 (dd, *J* = 8.8 Hz, *J* = 1.6 Hz, 1H), 7.31 (d, *J* = 8.4 Hz, 1H), 7.23 (d, *J* = 8.4 Hz, 2H), 4.66 (s, 2H), 2.65 (q, *J* = 7.6 Hz, 2H), 1.24 (t, *J* = 7.6Hz, 3H). HR-FABMS Calcd for $C_{23}H_{21}ClN_3O_2$ (M^++H): 406.1322, Found: 406.1320.

4.2.4.8. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-4-ethylbenzamide (12)

White solid (60%), mp >250℃; ^{1}H NMR (Acetone-d_6, 400 MHz) δ9.49 (s, 1H), 8.04 (d, *J* = 2.0 Hz, 1H), 7.94 (d, *J* = 7.2 Hz, 2H), 7.77 (d, *J* = 6.8 Hz, 2H), 7.55 (dd, *J* = 8.4 Hz, *J* = 2 Hz,

1H), 7.36 (d, *J* = 8.8Hz, 2H), 7.33 (d, *J* = 8.8 Hz, 1H), 7.24 (d, *J* = 8.8 Hz, 2H), 2.73 (q, *J* = 7.6 Hz, 2H), 2.63 (q, *J* = 7.6 Hz, 2H), 1.27-1.18 (m, 6H). HR-FABMS Calcd for $C_{24}H_{24}N_3O_2$ (M^++H): 386.1869, Found: 386.1867.

4.2.4.9. *N*-(2- (4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-3-fluoro-5-(trifluoromethyl) benzamide (13)

White solid (77%), mp >250℃;^{1}H NMR (Acetone-d_6, 400 MHz) δ9.67 (s, 1H), 9.45 (s, 1H), 8.18 (d, *J* = 6.0 Hz, 1H), 7.99-7.95 (m, 2H), 7.77 (d, *J* = 6.4 Hz, 2H), 7.58-7.49 (m, 2H), 7.37 (d, *J* = 8.8Hz, 1H), 7.24 (d, *J* = 8.8 Hz, 2H), 2.63 (q, *J* = 7.6 Hz, 2H), 1.22 (t, *J* = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{23}H_{18}F_4N_3O_2$ (M^++H): 444.1335, Found: 444.1333.

4.2.4.10. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-2-ethoxybenzamide (14)

White solid (75%), mp 181.6-182℃; ^{1}H NMR (Acetone-d_6, 400 MHz) δ10.15 (s, 1H), 9.42 (s, 1H), 8.15 (dd, *J* = 7.6 Hz, *J* = 2 Hz, 1H), 8.02 (s, 1H), 7.54 (t, *J* = 7.9 Hz, 1H), 7.47 (d, *J* = 8.8 Hz, 1H), 7.26-7.21 (m, 3H), 7.13 (t, *J* = 7.6 Hz, 1H), 4.38 (q, *J* = 6.9 Hz, 2H), 2.64 (q, *J* = 7.6 Hz, 2H), 1.65 (t, *J* = 7.0 Hz, 3H), 1.23 (t, *J* = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{24}H_{24}N_3O_3$ (M^++H): 402.1818, Found: 402.1815.

4.2.4.11. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-4-tert-butylbenzamide (15)

White solid (60%);^{1}H NMR (Acetone-d_6, 400 MHz) δ9.47 (s, 1H), 9.40 (s, 1H), 8.04 (d, *J* = 2.0 Hz, 1H), 7.94 (d, *J* = 8.4 Hz, 2H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.58-7.54 (m, 3H), 7.33 (d, *J* = 8.4 Hz,1H), 7.24 (d, *J* = 9.0 Hz, 2H), 2.63 (q, *J* = 7.6 Hz, 2H), 1.36 (s, 9H), 1.22 (t, *J* = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{26}H_{28}N_3O2$ (M^++H): 414.2182, Found: 414.2186.

4.2.4.12. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-3,4-dimethoxybenzamide (16)

White solid (75%), mp >250℃; ^{1}H NMR (Acetone-d_6, 400 MHz) δ9.42 (s, 1H), 9.39 (s, 1H), 8.01 (d, *J* = 2.4 Hz, 1H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.64 (dd, *J* = 8.4 Hz, *J* = 2 Hz 1H), 7.61 (s, 1H), 7.50 (dd, *J* =8.6 Hz, *J* = 2 Hz, 1H), 7.32 (d, *J* = 8.8 Hz, 1H), 7.24 (d, *J* = 8.4 Hz, 2H), 7.06 (d, *J* = 8.4 Hz, 1H), 3.89 (s, 3H), 2.77 (s, 3H), 2.63 (q, *J* = 7.6 Hz, 2H), 1.22 (t, *J* = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{24}H_{24}N_3O_4$ (M^++H): 418.1767, Found: 418.1771.

4.2.4.13. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-3,4,5-trimethoxybenzamide (17)

White solid (66%), mp >250℃;^{1}H NMR (Acetone-d_6, 400 MHz) δ9.47 (s, 1H), 9.40 (s, 1H), 7.98 (d, *J* = 2.0 Hz, 1H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.45 (dd, *J* = 8.6 Hz, *J* = 2 Hz, 1H), 7.34-7.32 (m, 3H), 7.24 (d, *J* = 8.4 Hz, 2H), 3.91 (s, 3H), 3.80 (s, 3H), 2.78 (s, 3H), 2.63 (q, *J* = 7.6 Hz, 2H), 1.22 (t, *J* =7.6 Hz, 3H). HR-FABMS Calcd for $C_{25}H_{26}N_3O_5$ (M^++H): 448.1872, Found: 448.1868.

4.2.4.14. *N*-(2-(4-ethylphenylamino)benzo[*d*]oxazol-5-yl)-4-heptylbenzamide (18)

Pale brown solid (73%), mp 244.8-246℃; ^{1}H NMR (Acetone-d_6, 400 MHz) δ9.47 (s, 1H), 9.39 (s, 1H), 8.04 (d, *J* = 2.4 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 2H), 7.77 (d, *J* = 8.8 Hz, 2H), 7.55 (dd, *J* = 8.8 Hz, *J* = 2.4 Hz, 1H), 7.36-7.32 (m, 3H), 7.24 (d, *J* = 8.8 Hz, 2H), 2.70 (t, *J* = 7.6 Hz, 2H), 2.63 (q, *J* = 7.6Hz, 2H), 1.68-1.65 (m, 2H), 1.37-1.29 (m, 8H), 1.22 (t, *J* = 7.6 Hz, 3H), 0.89 (t, *J* = 7.6 Hz,3H). HR-FABMS Calcd for $C_{29}H_{34}N_3O_2$ (M^++H): 456.2651, Found: 456.2657.

4.2.4.15. 2-(3-(Trifluoromethyl)phenylamino)-*N*-(2-(4-ethylphenylamino) benzo[*d*] oxazol-5-yl)benzamide (19)

Pale brown solid (38%), mp 174-176℃;^{1}H NMR (Acetone-d_6, 400 MHz) δ9.71 (s, 1H), 9.51 (s, 1H), 9.42 (s, 1H), 7.97-7.96 (m, 1H), 7.86 (d, *J* = 7.6 Hz, 1H), 7.78-7.74 (m, 2H), 7.54-7.44 (m, 5H), 7.37-7.34 (m. 1H), 7.26-7.22 (m, 3H), 7.01 (t, *J* = 7.2 Hz, 1H), 2.63 (q, *J* = 7.6 Hz, 2H), 1.22 (t, *J* = 7.6 Hz, 3H). HR-FABMS Calcd for $C_{29}H_{24}F_3N_4O_2$ (M^++H): 517.1851, Found: 517.1854.

4.3 Activity assay

4.3.1. Inhibition assay of IL-6-induced phospho-STAT3 by compounds

HepG2 cells were plated onto 6-well plates with $5x10^5$/well density and cultured in MEM (Minimal Essential Medium, WelGENE Inc, Daegu, Korea) to 80% confluence, followed by the replacement with serum-free media for 6 h. Cells were treated with chemical compound for 1 h and incubated with IL-6 (20 ng/ml, BD Pharmingen, San Diego, CA) for an additional 10 min. Cell lysates were extracted with lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM PMSF, protease

inhibitor cocktail) and subjected to sodium dodesyl sulfate (SDS)-containing polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-phospho-STAT3 Ab (Cell signaling Technology, Danvers, MA). The band intensity of phospho-STAT3 was quantitatively calculated by scanning. IL-6-treated phospho-STAT3 band intensity was set to be 100% and relative intensity after treatment with compounds was calculated.

4.3.2 Cell viability assay

HepG2 cells were maintained in MEM medium supplemented with 10% fetal bovine serum and plated onto 96-well plates. Cells were incubated with different concentrations (1, 3, 10, 30, 100 μg/ml) of compounds for 48 h and additionally treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT; Sigma-Aldrich Korea, Seoul, South Korea) for 3 h. Cells were washed with PBS and then dissolved in DMSO (Sigma-Aldrich), followed by measurement of optical density at 570 nm using VERSA max microplate reader (Molecular Devices Inc., Sunnyvale, CA). Data are obtained from triplicates of three independent experiments and expressed as average ± SE. Statistical significance was calculated and considered at $P < 0.05$.

4.3.3 In vitro CD4+ T helper cell differentiation

CD4+ T cells were isolated from lymph node and spleen of C57BL6 mice and stimulated with anti-CD3 (2 μg/ml, BD Pharmingen, San Diego) and anti-CD28 (1 μg/ml) antibodies for 24 h. Cells were treated with compounds (10 μM) and then incubated with different sets of cytokine and anti-cytokine antibodies for inducing different T helper (Th) cell differentiation: Th1 cell differentiation was induced by the incubation with IL-12 (10 ng/ml) and anti-IL-4 (5 μg/ml) antibody; Th2 cells were induced by IL-4 (10 ng/ml) and IL-IFN-γ (1 μg/ml) antibody; and Th17 cells were differentiated by treatment with TGF-β (10 ng/ml), IL-6 (20 ng/ml), anti-IL-4 (5 μg/ml) antibody and anti-IFN-γ (5 μg/ml) antibody. Cells were replaced with compounds (10 μM) every other day and cultured for an additional 3-4 days under Th1-, Th2-, and Th17-differentiation conditions and re-stimulated with anti-CD3 (1 μg/ml) for 24 h. Cell supernatants were harvested and used for measuring cytokine expression.

4.3.4 Cytokine measurement by enzyme-linked immunosorbent assay (ELISA)

Enzyme immunoassay plates (BD Pharmingen) were coated with capturing antibody (1 μg/ml,

BD Pharmingen) against IL-2, IFN-γ, IL-4, or IL-17 and incubated with cell supernatants at 4°C overnight. The plates were washed and incubated biotinylated antibody (1 μg/ml, BD Pharmingen) for IL-2, IFN-γ, IL-4, or IL-17 for 2 h and subsequently peroxidase-conjugated streptavidin (0.5 μg/ml, BD Pharmingen). Peroxidase substrate was added to the plates after washing and optical density was measured using microplate reader (Molecular devices). The standard curve was obtained by measuring the optical density of serial dilutions of one known cytokine IL-2, IFN-γ, IL-4, or IL-17 (BD Pharmingen).

5. Conclusion

Benzoxazole derivatives were synthesized and their IL-6 inhibition activity was evaluated. Compound **4** and **6** appeared to be most potent (IC_{50} 18.9 μM, and 5.8 μM respectively) among the synthesized compounds. Those compounds have common characteristics of containing in 4-amino benzoxazoles in the structure. In addition, compound **4** significantly suppressed cytokines produced by Th1, Th2, and Th17 cells by inhibiting the development of effector T cells. These results indicate that compound **4** and **6** can be used as a lead compound for further development of new rheumatoid arthritis drugs which have aminobenzoxazole moieties.

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Graphical Abstract

Synthesis of benzoxazole derivatives as interleukin-6 antagonists

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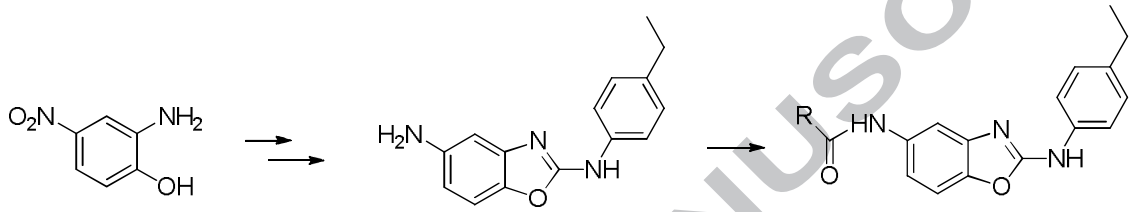

Highlights

1. Novel benzoxazole compounds effectively suppress IL-6-STAT3 signaling.
2. The compound **4** strongly suppressed the production of IFN-γ and IL-17.
3. Also **4** inhibited IL-4, IL-5, and IL-13 produced by effector Th2 cells.
4. Therefore **4** could be used for treating chronic inflammatory and autoimmune disease.