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Synthesis and evaluation of benzoxazole derivatives as 5-lipoxygenase inhibitors

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ABSTRACT

5-Lipoxygenase (5-LOX) is important enzyme in the biosynthesis of leukotrienes, and is a potential target in the treatment of asthma and allergy. We designed and synthesized a series of benzoxazoles and benzothiazoles as 5-LOX inhibitors. Fourteen compounds prepared showed the inhibition of LTC4 formation with IC_{50} value of 0.12–23.88 μM. Also two compounds **2d** and **2g** showed improved airway hypersensitiveness.

1. Introduction

Asthma is a predominantly inflammatory disease of the respiratory tract that has bronchial hyperreactivity and bronchospasm as a result. Increased number of inflammatory cells such as mast cells, eosinophils, neutrophils, and lymphocytes can be found in the bronchial mucosa from asthmatic patients.[1–3] 5-Lipoxygenase (5-LOX) is potential target in the treatment of asthma, atherosclerosis, prostate cancer, and allergy, because 5-LOX is required for the production of leukotrienes which are potent bronchocontraction and proinflammatory mediators.[4–9] Leukotrienes are short-lived, being rapidly converted to inactive metabolites, and prolonged expression of activity is dependent on continuous biosynthesis.[10] Thus their action can be blocked by two different approaches, using tissue cysteinyl luekotriene receptor antagonists (such as Pranlukast, Zafrilukast, and Montelukast) or enzyme 5-LOX inhibition (such as Zileuton).[11]

5-LOX has a molecular weight of 72–78 kDa and a single non-heme iron in the enzyme.[6] In resting cells, 5-LOX is localized in the cytosol or in the nucleus in some cell types. 5-LOX is activated by calcium, and once the cell is activated 5-LOX translocates to the nuclear envelope.[5,6] Then, it interacts with a small membrane protein designated 5-LOX activating protein (FLAP). This protein transfers arachidonic acid to 5-LOX and this interaction between the enzyme and FLAP being crucial for cellular leukotriene biosynthesis.[4,12] It was proposed that iron in the enzyme is bound by three permanent ligands, His-372, His-550, and Ile-673, and one exchangeable ligand, His-367. It was further proposed that a reaction intermediate replaces the exchangeable ligand during the catalytic cycle.[5,13] Therefore, compounds with affinity for the iron were proposed to displace the exchangeable ligand and thus inhibit catalysis. Inhibition of 5-LOX provided an advantage of independent of the evolving leukotriene receptor heterogenicity and ligand specificity.

A lot of hydroxamic acids and *N*-hydroxyurea derivatives are reported as iron-chelator inhibitors and Zileuton is the only 5-LOX inhibitor in market for the treatment of bronchial asthma. However it showed hepatic toxicities and drug interactions.[3,4,6] Recently 2-(substituted phenyl)benzimidazole derivatives are reported to have potent activity against IgE, cytokines, and CD23 for the treatment of allergy and asthma.[14,15] Some reported 5-LOX inhibitors containing indole or benzothiazole scaffold are shown in Figure 1.[16,17] In our study to develop novel agents for asthma, we designed and synthesized benzoxazole compounds as bioisosterer of benzothiazole, and proposed the affinity to 5-LOX iron as follows.

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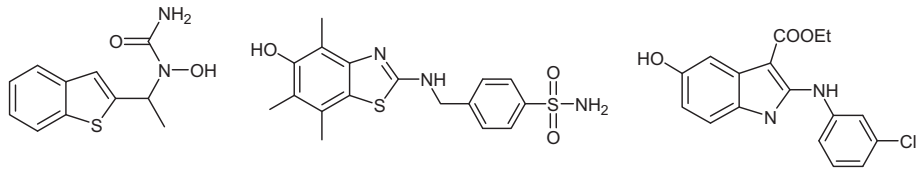
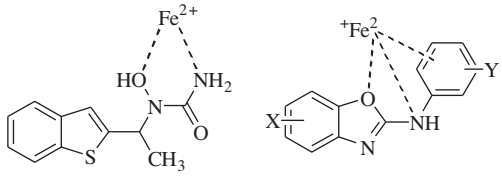


Figure 1. Structures of 5-LOX inhibitors.



Proposed affinity for the iron of 5-LOX and Zileuton, and designed compounds (**2a**–**n**).

2. Results and discussion

2.1. Chemistry

The reported method was employed for the synthesis of benzoxazoles.[18,19] 2-Aminophenol was reacted with various phenyl isothiocyanates to give the corresponding thioureas (**1a**–**n**). Benzoxazoles (**2a**–**n**) were prepared by acid catalyzed ring closure with oxidation from 2-hydroxyphenylthioureas (**1a**–**n**) (Scheme 1).

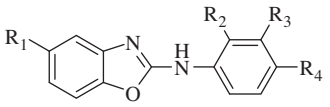
2.2. Evaluation of in vitro 5-LOX inhibition

The 5-LOX inhibition was measured the formation of LTC_4 in the bone marrow-derived mast cells (BMMC) as explained in Section 4. The ability of prepared compounds to inhibit 5-LOX was evaluated at 1 and 10 μM as a first screening. The IC_{50} of the most reported compounds was 0.1–10 μM (Table 1), and the most active compound **2g** showed 0.12 μM.

In general, the substitution on benzoxazole ring affected the 5-LOX inhibition. The introduction of nitro group on 5-position of benzoxazole clearly decreased the activity (**2e** < **2b**). However, 5-chloro benzoxazoles **2c** and **2d** showed almost same activity compared to their 5-H congeners **2a** and **2b**. The electron donating groups, such as 5-methyl and 5-methoxy, increased the activity (**2f** > **2a**, **2g** > **2b**, and **2k** > **2a**). This observation could be explained by the proposed affinity of 5-LOX iron to the ligands having the enhanced electron density as a result of the introduction of electron donating X and Y to the aromatic rings.

Also, the position of the substituent Y on aromatic ring might affect the 5-LOX inhibition, since there could be steric hindrance for *o*-position. The *p*-ethyl group on the phenyl ring increased the activity (**2b**, **2d**, and **2g**), but *o*-ethyl congener showed low activity (**2h**).

Table 1
Structure and in vitro 5-LOX inhibition of synthesized compounds



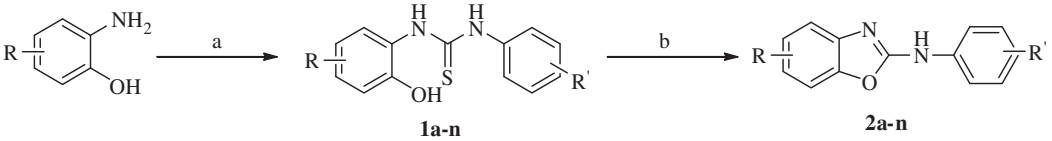
2a-n

Compound	R_1	R_2	R_3	R_4	IC_{50} (μM)
2a	H	H	H	H	8.91
2b	H	H	H	CH_2CH_3	1.21
2c	Cl	H	H	H	4.50
2d	Cl	H	H	CH_2CH_3	0.95
2e	NO_2	H	H	CH_2CH_3	23.88
2f	CH_3	H	H	H	4.60
2g	CH_3	H	H	CH_2CH_3	0.12
2h	CH_3	CH_2CH_3	H	H	5.66
2i	CH_3	H	H	SCH_3	7.31
2j	CH_3	H	OCH_3	H	6.83
2k	OCH_3	H	H	H	7.88
2l	OCH_3	H	H	$CH(CH_3)_2$	2.64
2m	OCH_3	H	H	SCH_3	2.77
2n	OCH_3	H	Br	H	2.03

2.3. Inhibition of allergen-induced airway responsiveness to methacholine

Airway hyperresponsiveness (AHR) is a hallmark of bronchial asthma. The in vivo efficacy of two compounds was tested in a murine model of airway hyperresponsiveness. Mice that had been sensitized to an allergen (ovalbumin, OVA) develop significantly greater airway resistance in response to methacholine than mice that have not exposed to OVA.

The ovalbumin-sensitized mice were challenged with aerosolized ovalbumin repeatedly for 3 days and lung resistance was measured before and after each dose of intravenous methacholine. In animals of the model group, airway hyperresponsiveness was determined by a significant increase in lung resistance PD_{50} value in addition to a significant increase in maximum lung resistance. The two compounds prepared, **2d** and **2g** were selected for this assay. The two compounds showed improved airway hypersensitiveness as shown in Figure 2. Treatment with **2d** and **2g** reduced the increases in Penh values after methacholine challenge compared with that of the OVA-treated group.



Scheme 1. Reagents and conditions: (a) phenyl isothiocyanates, CH_3OH, rt, 24 h; (b) KO_2, CH_3CN, rt, 12 h.

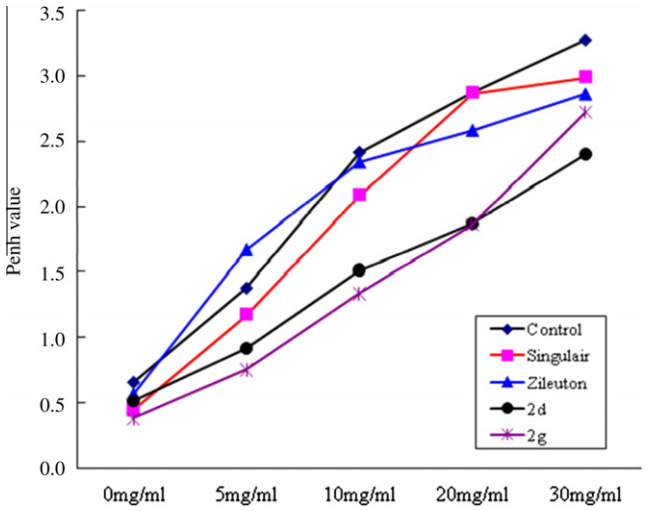


Figure 2. Effects on airway hyperresponsiveness (AHR). AHR was measured using plethysmography 24 h after the last ovalbumin (OVA)-challenged mice by increments of methacholine (0–30 mg/mL). Control was only vehicle in OVA-sensitized/challenged mice; others, compound (30 mg/kg) + OVA-sensitized/challenged mice.

2.4. Histological assay

Histopathology shows a marked peribronchial, perivascular, and parenchymal infiltration of inflammatory cells in the OVA group compared with control mice. Mice treated with 30 mg/kg of each compound showed decreased tissue infiltration by mononuclear cells and eosinophils compared with non-treated mice (Fig. 3). These results were confirmed by a reduction of the total histological score. In the compounds **2d** and **2g** treated OVA-induced model, the infiltration of eosinophil-rich leukocytes was attenuated compared to what was seen in OVA-treated mice. Among two compounds prepared, **2g** showed the most reduced total histological score. Zileuton showed a tendency to decrease tissue infiltration by inflammatory cells, but the decrease was not much different.

2.5. Measurement of BAL cytokines and OVA specific IgE levels

Th2 cells play a central role in the pathogenesis of allergic bronchial asthma, since each of their characteristic cytokines such as IL-4, IL-5, and IL-13 contributes to hallmarks of this disease, including airway eosinophilia, increased mucus production, production of allergen-specific IgE, and development of airway hyperresponsiveness.

Twenty-four hours after the last challenge, the mice were sacrificed via overdose of pentobarbital sodium. Blood was obtained and centrifuged. The serum levels of OVA-specific IgE were compared with the non-treated group (Fig. 4). The OVA-specific IgE levels in serum were dramatically increased in OVA-treated mice compared with PBS-treated mice. Treatment of **2d** reduced OVA-specific IgE levels compared with OVA-challenged group (25.44 ± 5.62 ng/mL vs 41.82 ± 10.57 ng/mL; P <0.05; control and test, respectively). Compound **2g** also showed a reduction of OVA-specific IgE levels (19.21 ± 5.21 ng/mL).

Also the levels of interleukin IL-4, IL-5, and IL-13 in serum were quantified in the supernatants of BAL fluids via enzyme

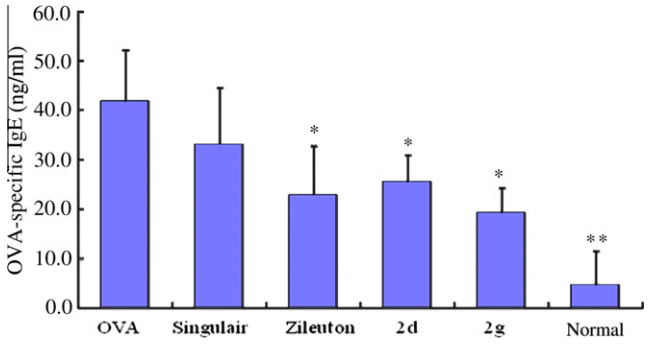


Figure 4. Effect on OVA-specific IgE levels in plasma. Plasma was collected 48 h after the last ovalbumin (OVA)-challenged mice. Each level was analyzed using ELISA (n = 5 or 6). Values are expressed as mean ± SEM (n = 6/group). *Significant difference from NC, P <0.005 and **significant difference from OVA, P <0.05.

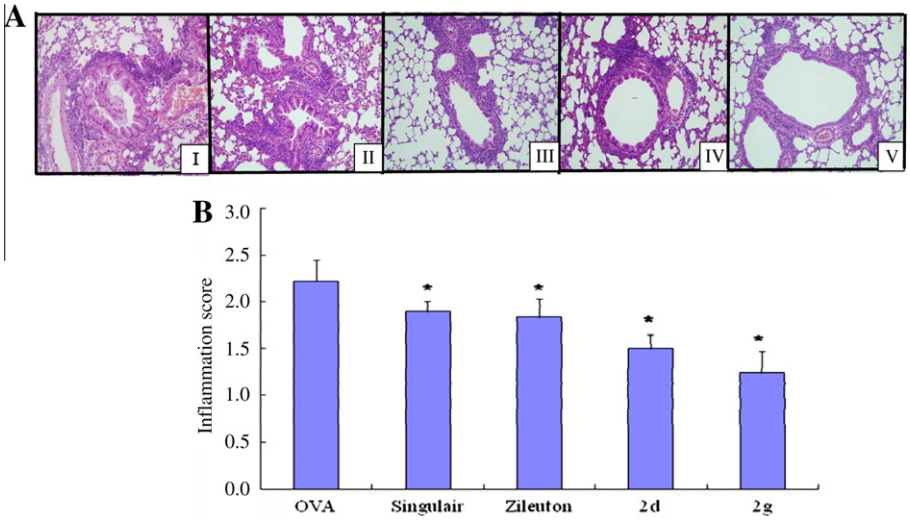


Figure 3. Effects on the recruitment of leukocytes in lung tissue. (A) Histological examination of lung tissue was performed 48 h after the last OVA challenge. Lung tissues were fixed, sectioned at 4 μm thickness, and stained with H&E solution (magnification ×200). (I) OVA-induced asthmatic control, (II–V) compound treated OVA-induced model, Montelukast (II), Zileuton (III), **2d** (IV), and **2g** (V), respectively. (B) Scoring the extent of inflammation by quantitative analysis of inflammatory-cell infiltration in lung sections were performed based on the methods of Myou et al.[18] OVA control, OVA-sensitized/challenged mice; others, each compound (30 mg/kg) + OVA-sensitized/challenged mice. *Significant difference from OVA control, P <0.01.

immunoassays, all of which were conducted in accordance with the manufacturer's instruction (Fig. 5). In **2d**-treated group, cytokine elevation was suppressed (IL-4, 163.49 ± 48.60 ng/mL; IL-5, 110.91 ± 23.12 ng/mL; IL-13, 71.91 ± 13.82 ng/mL). Compound **2g** also caused a reduction in IL-4 (198.34 ± 44.00 ng/mL), IL-5 (116.92 ± 33.55 ng/mL), and IL-13 (64.30 ± 17.83 ng/mL).

3. Conclusion

Fourteen compounds prepared showed the 5-LOX inhibition with IC_{50} value of 0.12–23.88 μM. The most active compound **2g** that have methyl group on 5-position of benzoxazole and *p*-ethyl group on the phenyl ring showed 0.12 μM. Selected two compounds **2d** and **2g** showed improved airway hypersensitiveness.

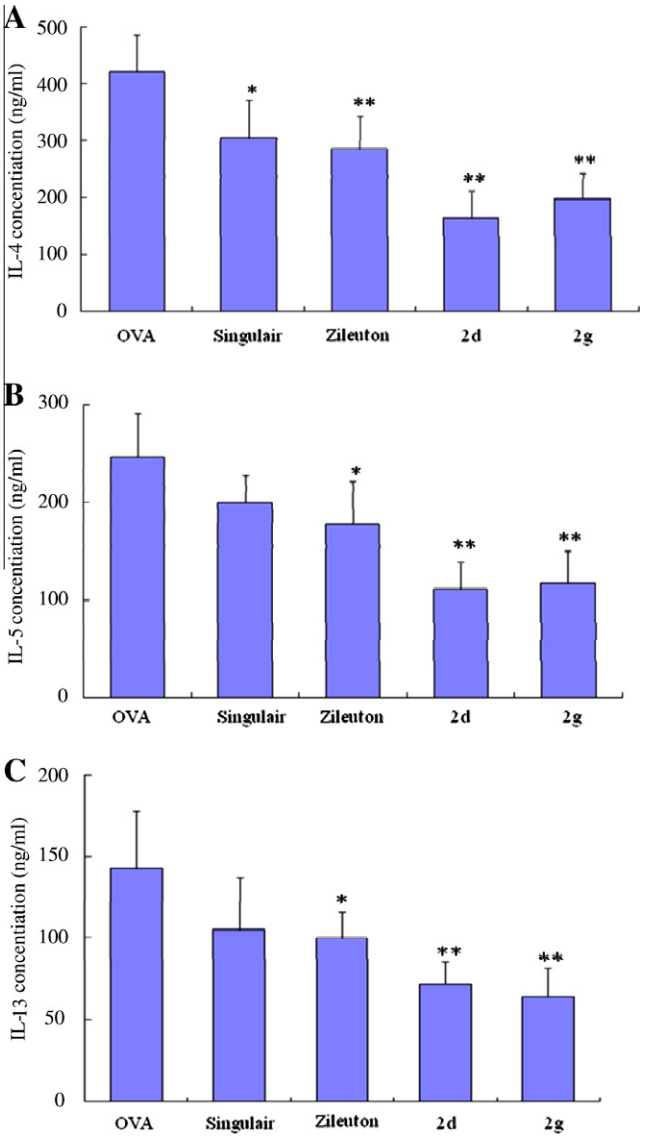


Figure 5. Cytokine levels in BALF of OVA-induced asthmatic model. Effect of four compounds with positive controls (Singulair and Zileuton) on cytokine levels in BALF. BALF was collected 48 h after the last ovalbumin (OVA)-challenged mice. Each level was analyzed using ELISA (n = 5 or 6). (A) Interleukin (IL)-4 level, (B) IL-5 level, (C) IL-13 level in BALF. OVA, OVA-sensitized/challenged mice; others, each compound (30 mg/kg) + OVA-sensitized/challenged mice. Values are expressed as mean ± SEM (n = 6/group). *Significant difference from NC, P <0.05 and **significant difference from OVA, P <0.005.

4. Experimental

All melting points were taken in Pyrex capillaries using electrothermal digital melting point apparatus (Büchi). NMR spectra were recorded on a 400 MHz Varian FT-NMR spectrometer using tetramethylsilane as an internal standard. Samples were dissolved in $CDCl_3$, Acetone-d_6 or DMSO-d_6. Mass spectra data were obtained on a Jeol JMS 700 high resolution mass spectrometer at the Korea Basic Science Institute (Daegu). Most of the reagents were purchased from Sigma–Aldrich Chemical Company. Purity of synthesized compounds was determined to be ⩾95% by analytical HPLC on a C-18 column eluted with a linear gradient of 10–90% acetonitrile in water containing 0.05% TFA (flow rate of 1 mL/min).

4.1. General procedure for the preparation of benzoxazoles

2-Aminophenol (0.92 mmol) and isothiocyanates (0.92 mmol) in methanol were stirred at room temperature for 1 day. The precipitate was collected by filtration and washed with ether to give the corresponding thioureas. A solution of *N*-(2-hydroxy-5-substituted phenyl)-*N*′-(substituted) thiourea (1 mmol) in CH_3CN (3 mL) was added to a heterogeneous solution of potassium superoxide (5 mmol) in CH_3CN (2 mL) at room temperature under dry nitrogen atmosphere. After being stirred well for 12 h at room temperature, the reaction mixture was poured into cold water, and extracted with dichloromethane. The solvent was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure to give product.

4.1.1. *N*-Phenylbenzo[*d*]oxazol-2-amine (2a)

White powder (31%), mp 180–182 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.47 (s, NH), 7.85 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 1H), 7.37 (t, J = 12.0 Hz, 3H), 7.21 (t, J = 12.0 Hz, 1H), 7.11 (t, J = 12.0 Hz, 1H), 7.04 (t, J = 12.0 Hz, 1H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 159.11, 148.59, 144.02, 139.98, 129.89, 124.92, 123.29, 122.68, 118.70, 117.91, 109.70; FABHRMS (m/z): 211.0871 (M^++1, $C_{13}H_{11}N_2O$ requires 211.0875).

4.1.2. *N*-(4-Ethylphenyl)benzo[*d*]oxazol-2-amine (2b)

White powder (51%), mp 123–125 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.51 (s, NH), 7.82 (d, J = 8.0 Hz, 1H), 7.42 (d, J = 8.0 Hz, 2H), 7.22 (d, J = 8.0 Hz, 2H), 7.04 (t, J = 12.0 Hz, 1H), 6.92 (d, J = 8.0 Hz, 1H), 6.84 (t, J = 12.0 Hz, 1H), 2.60 (q, J = 7.6 Hz, 2H), 1.19 (t, J = 7.6 Hz, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 159.29, 148.63, 144.14, 139.23, 137.66, 129.18, 124.86, 122.51, 118.88, 117.78, 109.63, 28.88, 16.34; FABHRMS (m/z): 239.1182 (M^++1, $C_{15}H_{15}N_2O$ requires 239.1184).

4.1.3. 5-Chloro-*N*-phenylbenzo[*d*]oxazol-2-amine (2c)

Yellow powder (41%), mp 183–186 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.60 (s, NH), 7.82 (d, J = 8.0 Hz, 1H), 7.42 (d, J = 8.0 Hz, 2H), 7.40 (s, 1H), 7.37 (d, J = 8.0 Hz, 1H), 7.30 (s, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 160.30, 147.40, 145.56, 139.55, 129.95, 129.81, 123.72, 122.41, 118.96, 117.68, 110.70; FABHRMS (m/z): 245.0482 (M^++1, $C_{13}H_{10}ClN_2O$ requires 245.0482).

4.1.4. 5-Chloro-*N*-(4-ethylphenyl)benzo[*d*]oxazol-2-amine (2d)

Yellow powder (27%), mp 196–198 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.53 (s, NH), 7.72 (d, J = 8.0 Hz, 2H), 7.40 (s, 1H), 7.36 (d, J = 8.0 Hz, 1H), 7.22 (d, J = 8.0 Hz, 2H), 7.10 (d, J = 8.0 Hz, 1H), 2.60 (q, J = 8.0 Hz, 2H), 1.20 (t, J = 7.6 Hz, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 160.49, 147.44, 145.71, 139.70, 137.22, 129.76, 129.24, 122.23, 119.16, 117.55, 110.61, 28.89, 16.31; FABHRMS (m/z): 273.0794 (M^++1, $C_{15}H_{14}ClN_2O$ requires 273.0795).

4.1.5. *N*-(4-Ethylphenyl)-5-nitrobenzo[*d*]oxazol-2-amine (2e)

Yellow powder (81%), mp 161–163.5 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.55 (s, NH), 7.84 (d, J = 8.0 Hz, 1H), 7.43 (d, J = 8.0 Hz, 2H), 7.24 (d, J = 8.0 Hz, 2H), 6.72 (d, J = 8.0 Hz, 2H), 2.63 (q, J = 7.6 Hz, 2H), 1.19 (t, J = 7.6 Hz, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 161.42, 152.80, 145.09, 140.22, 136.78, 129.33, 119.43, 118.91, 112.75, 109.83, 28.90, 16.28; EI/MS (m/z): 283.0960 (M^+, $C_{15}H_{13}N_3O_3$ requires 283.0957).

4.1.6. 5-Methyl-*N*-phenylbenzo[*d*]oxazol-2-amine (2f)

White powder (86%), mp 205.0–209.8 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.34 (s, NH), 7.86 (m, 2H), 7.38 (m, 2H), 7.24 (d, J = 8.0 Hz, 1H), 7.05 (m, 1H), 7.03 (m, 1H), 6.94 (d, J = 8.0 Hz, 1H), 2.40 (s, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 159.24, 146.75, 144.18, 140.05, 134.44, 129.88, 123.33, 123.20, 118.64, 118.25, 109.09, 21.55; FABHRMS (m/z): 225.1023 (M^++1, $C_{14}H_{13}N_2O$ requires 225.1028).

4.1.7. *N*-(4-Ethylphenyl)-5-methylbenzo[*d*]oxazol-2-amine (2g)

White powder (79%), mp 184–186.2 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.21 (s, NH), 7.76–7.72 (m, 2H), 7.22–7.20 (m, 2H), 6.97–6.93 (m, 2H), 6.92–6.90 (m, 1H), 4.04 (q, J = 6.8 Hz, 2H), 2.38 (s, 3H), 1.36 (t, J = 6.8 Hz, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 159.43, 146.79, 144.29, 139.12, 137.73, 134.36, 129.15, 123.15, 118.82, 118.14, 109.02, 28.87, 21.56, 16.34; FABHRMS (m/z): 253.1345 (M^++1, $C_{16}H_{17}N_2O$ requires 253.1341).

4.1.8. *N*-(2-Ethylphenyl)-5-methylbenzo[*d*]oxazol-2-amine (2h)

Pale brown powder (92%), mp 127.3–128.4 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 8.50 (s, NH), 8.08 (d, J = 8.0 Hz, 1H), 7.30–7.24 (m, 2H), 7.01–6.98 (m, 2H), 6.91 (d, J = 8.0 Hz, 2H), 3.28 (s, 3H), 2.78 (q, J = 7.6 Hz, 2H), 1.24 (t, J = 7.6 Hz, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 160.66, 147.22, 144.28, 137.17, 136.12, 134.35, 129.75, 127.41, 125.36, 123.26, 122.98, 118.04, 109.03, 24.83, 21.55, 14.69; FABHRMS (m/z): 253.1346 (M^++1, $C_{16}H_{17}N_2O$ requires 253.1341).

4.1.9. 6-Methyl-*N*-(4-(methylthio)phenyl)benzo[*d*]oxazol-2-amine (2i)

Pale brown powder (69%), mp 187.0–190.8 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.45 (s, NH), 7.84–7.81 (m, 2H), 7.36–7.33 (m, 2H), 7.24 (d, J = 8.0 Hz, 2H), 6.94 (d, J = 8.0 Hz, 1H), 2.49 (s, 3H), 2.39 (s, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 159.14, 146.77, 144.12, 137.88, 134.48, 132.22, 129.31, 123.36, 120.27, 119.37, 118.24, 109.11, 21.55, 16.94; FABHRMS (m/z): 271.0905 (M^++1, $C_{15}H_{15}N_2OS$ requires 271.0905).

4.1.10. *N*-(3-Methoxyphenyl)-5-methylbenzo[*d*]oxazol-2-amine (2j)

Pale brown powder (72%), mp 164.8–166.4 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.41 (s, NH), 7.62 (m, 1H), 7.36–7.33 (m, 1H), 7.27–7.23 (m, 3H), 6.93–6.57 (m, 1H), 6.65–6.62 (m, 1H), 3.80 (s, 3H), 2.40 (s, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 161.54, 159.14, 146.71, 144.13, 141.16, 134.47, 130.64, 123.39, 118.32, 111.08, 109.11, 108.41, 104.91, 55.59, 21.54; FABHRMS (m/z): 255.1128 (M^++1, $C_{15}H_{15}N_2O_2$ requires 255.1134).

4.1.11. 5-Methoxy-*N*-phenylbenzo[*d*]oxazol-2-amine (2k)

White powder (75%), mp 213.7–214.5 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.43 (s, NH), 7.84–7.80 (m, 2H), 7.37–7.32 (m, 2H), 7.23 (d, J = 8.4 Hz, 1H), 6.99 (d, J = 2.4 Hz, 1H), 7.05–7.01 (m, 1H), 6.66 (dd, J = 2.4 and 8.4 Hz, 1H), 3.79 (s, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 159.83, 158.30, 144.95, 142.98, 139.97, 129.88, 123.26, 118.68, 109.61, 109.23, 102.90, 56.20; FABHRMS (m/z): 241.0980 (M^++1, $C_{14}H_{13}N_2O_2$ requires 241.0977).

4.1.12. *N*-(4-Isopropylphenyl)-5-methoxybenzo[*d*]oxazol-2-amine (2l)

Brown powder (63%), mp 129.6–132.5 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.35 (s, NH), 7.64–7.60 (m, 2H), 7.14–7.11 (m, 2H), 7.11 (d, J = 8.4 Hz, 1H), 6.87 (d, J = 2.8 Hz, 1H), 6.54 (dd, J = 8.4 and 2.8 Hz, 1H), 3.69 (s, 3H), 2.92 (m, 1H), 1.12 (s, 3H), 1.10 (s, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 160.07, 158.28, 145.10, 143.83, 143.02, 137.75, 127.69, 118.88, 109.52, 109.02, 102.83, 56.19, 34.33, 24.52; FABHRMS (m/z): 283.1447 (M^++1, $C_{17}H_{19}N_2O_2$ requires 283.1445).

4.1.13. 5-Methoxy-*N*-(4-(methylthio)phenyl)benzo[*d*]oxazol-2-amine (2m)

Pale brown powder (74%), mp 176.4–178.8 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.51 (s, NH), 7.84–7.81 (m, 2H), 7.36–7.33 (m, 2H), 7.26 (d, J = 8.8 Hz, 1H), 7.01 (d, J = 2.8 Hz, 1H), 6.69 (dd, J = 8.8 and 2.8 Hz, 1H), 3.83 (s, 3H), 2.49 (s, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 159.75, 158.31, 144.91, 142.99, 137.83, 132.29, 129.30, 119.41, 109.61, 109.26, 102.89, 56.20, 16.93; FABHRMS (m/z): 287.0854 (M^++1, $C_{15}H_{15}N_2O_2S$ requires 287.0848).

4.1.14. *N*-(3-Bromophenyl)-5-methoxybenzo[*d*]oxazol-2-amine (2n)

Pale brown powder (40%), mp 211.5–213.1 °C; ^{1}H NMR (Acetone-d_6, 400 MHz) δ 9.31 (s, NH), 7.72 (m, 2H), 7.33 (t, J = 8.0 Hz, 1H), 7.29 (d, J = 8.8 Hz, 1H), 7.23 (m, 1H), 7.07 (d, J = 2.4 Hz, 1H), 6.72 (dd, J = 8.8 and 2.4 Hz, 1H), 3.84 (s, 3H); ^{13}C NMR (Acetone-d_6, 100 MHz) δ 159.23, 158.38, 144.56, 142.87, 141.51, 131.58, 125.93, 123.24, 121.11, 117.44, 109.83, 109.80, 102.98, 56.22; FABHRMS (m/z): 319.0082 (M^++1, $C_{14}H_{12}BrN_2O_2$ requires 319.0086).

4.2. Determination of 5-lipoxygenase product LTC4 formation

Preparation of mouse bone marrow-derived mast cells (BMMC) were obtained from male Balb/c mice and cultured for up to 4 weeks in 50% enriched medium (RPMI containing 2 mM L-glutamine, 25 mM HEPES buffer, 2 mg/mL sodium bicarbonate, 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B) supplemented with 10% fetal bovine serum with IL-3 (Sigma I4144, 2 ng/mL).[20] Three weeks after the culture, more than 98% of BMMC was found in the cells as assessed by staining method with toluidine blue.

4.3. Determination of LTC4

BMMC were suspended in the enriched medium at the cell density of 1×10^6 cells/mL, and were then incubated in a humidified 5% CO_2 incubator with or without sample in DMSO (final DMSO concentration was <0.5%) for 30 min at 37 °C. After the stimulation with stem cell factor (SCF, Sigma S9915, 100 ng/mL) for 20 min, the LTC_4 release in supernatants was measured by an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. All the experiments were carried out in triplicate and the inhibition of LTC_4 release was determined by calculating % reduction of LTC_4 release.[21]

4.4. Sensitization and airway challenge

Groups of mice (n = 5–6) were studied; they received the following treatment: (1) sham-sensitization plus challenge with phosphate-buffered saline (PBS; ipNeb); (2) sensitization plus challenge with OVA (ovalbumin: Sigma A5503; Sigma, St. Louis, MO) (ipNeb); (3) sensitization with OVA (ip) plus challenge with OVA (Neb) and samples (tiarellic acid or Zileuton, po). Briefly, mice were sensitized with intraperitoneal injection of 20 μg OVA, which was emulsified with 2 mg of aluminum hydroxide in 100 μl of PBS

buffer (pH 7.4) on days 0 and 11. The mice were challenged through the airways with OVA (1% in PBS) for 20 min using an ultrasonic nubuilizer (NE-U12; Omron Corp., Tokyo, Japan) on days 19, 20, 21, and 25 after the initial sensitization. The mice were sacrificed 48 h after the last challenge (day 32) to determine the suppressive effect of compounds on the airways of allergic asthma.

4.5. Determination of airway responsiveness

Twenty-four hours after the final aerosol challenge AHR was measured by using a whole-body plethysmography (OCP3000; Allmedicus, Korea).[22] Each mouse was placed in a brometric phethysmographic chamber and challenged with aerosolized PBS, followed by increasing concentrations of aerosolized methacholine (12.5, 25, and 50 mg/mL) for 3 min. Bronchoconstriction was recorded for additional 5 min at each concentration. The highest Penh value of each sample was obtained during each methacholine challenge, and expressed as a percentage of a basal Penh value in response to control (PBS) challenge.

The formula for enhanced pause value (Penh) is as follows:

$$[\text{expiratory time(Te)}/\text{relaxation time(RT)} - 1] \times [\text{peak expiratory flow(PEF)}/\text{peak inspiratory flow(PIF)}]$$

4.6. Histopathology studies

The lung tissue was fixed for 24 h on 10% neutral-buffered formalin. After being embedded in paraffin, the tissues were sliced made into 4-μm thickness sections, and stained with H&E solution (hematoxylin; Sigma MHS-16 and eosin; Sigma HT110-1-32). Subsequently, the stained tissue was mounted and cover-slipped with Dako-mounting medium (Dakocytomation; Denmark Carpinteria, CA). The degree of cell infiltration in the airway was scored in a double-blind test performed by two independent investigators.[23] The peri-bronchial and peri-vascular inflammation was evaluated by specific standard, that is, scoring of 0–3, 0, no cells; 1, a few cells; 2, a ring of cells one to five cell-layer deep; 3, a thick ring of cells more than five cell-layer deep. To evaluate the suppressive effect of the compounds on the leukocyte infiltration, the degree of inflammation was scored by quantitative analysis in lung tissues 48 h after the last challenge.

4.7. Effect on the OVA-specific IgE level

Forty-eight hours after the last challenge, the mice were sacrificed with an overdose of pentobarbital (Sigma P3761), and a tracheotomy was performed. After ice-cold PBS (0.5 mL) was instilled into the lungs, bronchoalveolar lavage fluid (BALF) was obtained by aspiration three times (total 1.5 mL) via tracheal cannulation. BALF was centrifuged and supernatants were collected and stored at −70 °C before use. The amount of IL-4, IL-5, and IL-13 in BALF was measured by a specific mouse ELISA kit (R&D Systems; Minneapolis, MN) according to the manufacturer's instructions. The detection limit of the assays was 250 pg/mL.

Plasma was obtained by cardiac puncture after the tracheotomy. Complementary capture and detection antibody pairs for mouse IgE antibodies were purchased from BD OptEIA (San Diego, CA), and the IgE enzyme-linked immunosorbent assay (ELISA) was performed according to the manufacturer's directions. Duplicate samples in plasma were diluted to 1:100. IgE levels in each sample were measured the optical density readings at 450 nm, and OVA-specific IgE concentrations were calculated from a standard curve, which was generated in case recombinant IgE (5–2000 ng/mL) was used.

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