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Design, synthesis and activity of benzothiazole-based inhibitors of NO production in LPS-activated macrophages

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ABSTRACT

Series of benzothiazoles were synthesized and evaluated their inhibitory activities for NO production in lipopolysaccharide-activated macrophages. The most potent compound was the indole-containing benzothiazole **3c** with 4.18 μM of IC_{50}. The mechanistic study suggested that benzothiazoles inhibited NO production by the suppression of iNOS protein and mRNA expression. They also suppressed the expression of COX-2 through the NF-κB inactivation.

Nitric oxide (NO) is biosynthesized from L-arginine catalyzed by three isoforms of nitric oxide synthases (NOSs).[1] NO plays diverse biological roles depending on which subtypes of NOS are involved in its biosynthesis and which location is exposed to the released NO. Constitutive isoforms found in neuronal tissues (nNOS) and vascular endothelium (eNOS) are Ca^{2+}-dependent and release small amounts of NO required for homeostatic function.[2] Meanwhile, inducible NOS (iNOS) induced by lipopolysaccharide (LPS) and various cytokines such as IFN-α, IL-1β, and TNF-α is Ca^{2+}-independent and produces high levels of NO.[3] Overproduction of NO has been implicated in several pathophysiological states, for example, various inflammation,[4] septic shock, vascular dysfunction in diabetes, platelet aggregation, and cancer.[5] Cyclooxgenase-2 (COX-2) is another inducible enzyme that plays a significant role in the production of pro-inflammatory mediators,[6] while COX-1 is the constitutively expressed form in normal conditions. NO activates COX-2 resulting in the increased release of inflammatory prostaglandins (PGs). Therefore, the modulation of iNOS and COX-2 can be a good strategy for the management of diseases accompanying the overproduction of NO and PGs.

Early NOS inhibitors included the arginine derivatives such as N^G-methyl-L-arginine (L-NMA),[7,8] *N*-iminoethyl-L-ornitine (L-NIO),[9] N^G-nitro-L-arginine (L-NNA),[10] and L-thiocitrulline.[11] These substrate analogs have been subjected to both animal models and clinical trials to block overproduction of NO.[12,13] However, these compounds are not selective enough against NOS isoforms, which limited the application of them in vivo. Recent studies have focused on design and synthesis of non-amino acid analogs such as amino heterocycles,[14] amidines,[15] guanidines,[16] isoquinolinamines,[17] and isothioureas.[18,19] A number of these non-amino acid analogs have been reported as selective iNOS inhibitors.[20,21] Activation of iNOS enzyme requires homodimerization of NOS monomers and binding of cofactors such as FMN, FAD, NADPH, and tetrahydrobiopterin (BH_4) to enzyme. Thus, inhibition of dimerization would prevent overproduction of NO to attenuate NO-related damages. Several groups have reported potent inhibitors of iNOS dimerization.[22–25] It was reported that BBS-4, potent imidazole-based dimerization inhibitor disrupt the formation of NOS dimer by direct coordination of the imidazole to the heme iron in the active site of the enzyme.[26,27] However, BBS-4 have been found to be cross reactive to cytochrome P450.[28] Diverse efforts have been made to identify selective iNOS inhibitors with high safety profile for effective attenuation of NO overproduction.

In order to identify new class of iNOS inhibitors, our study was focused on the isosteric replacement of imidazole of the reported dimerization inhibitor with another heterocycle. After careful consideration of the structural features of several heterocycles, we decided to investigate benzothiazole-based compounds as novel inhibitors of dimerization and/or expression of iNOS. We initially expected that benzothiazole could function as a metal coordinator as imidazole did. It was supported by examination of the putative binding mode of the benzothiazole-based analogs through molecular docking analysis. We also anticipated the introduction of appropriate lipophilic groups to the benzothiazole scaffold might be beneficial for the activity and selectivity. Herein, we report the design and synthesis of 4-alkoxy substituted benzothiazoles

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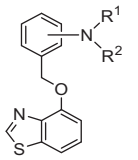


Figure 1. Representative structure of benzothiazole derivatives.

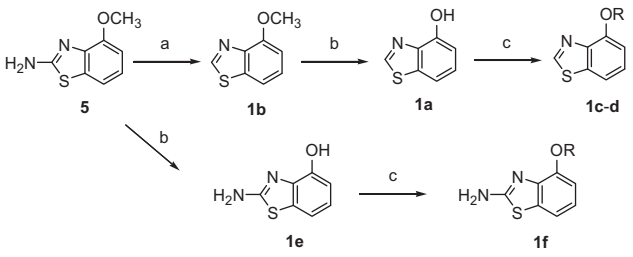
(Fig. 1), and their inhibitory activities of the NO production in LPS-activated macrophage. To understand the underlying mechanism of active compounds, we examined the expression of iNOS/COX-2 protein and mRNA, the degradation of I-κB-α, and the nuclear translocation of p65 subunit of NF-κB by Western blot analysis and RT-PCR.

First, simply substituted benzothiazoles were prepared to figure out the inhibitory activity of benzothiazole skeleton for NO production (Scheme 1). Commercially available 2-amino-4-methoxybenzothiazole (**5**) was converted to 4-methoxybenzothiazole **1b** by treating isoamyl nitrite. Deprotection of **1b** and **5** with BBr_3 gave corresponding 4-hydroxybenzothiazoles **1a** and **1e**, and the following alkylation of the resulting **1a** and **1e** with appropriate alkyl or benzyl halide provided **1b–d** and **1f**.

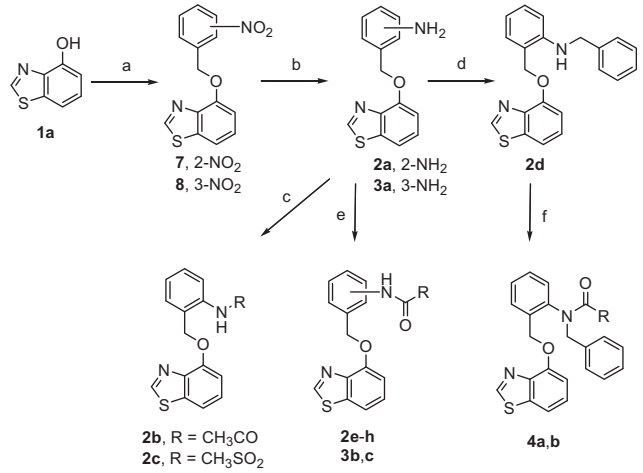
Next, amino-substituted 4-benzyloxybenzothiazole derivatives were synthesized as depicted in Scheme 2. *O*-benzylation of **1a** by treatment of 2- or 3-nitrobenzyl bromide in the presence of K_2CO_3 gave compounds **7** and **8**, respectively. The followed reduction of nitro compounds **7** and **8** over 10% Pd/C under atmospheric pressure of hydrogen gas provided amines **2a** and **3a**. Reductive amination of compound **2a** with benzaldehyde in the presence of $NaBH_3CN$ and AcOH gave compound **2d**. The amine **2a** was also acetylated and sulfonated to offer respective compounds **2b** and **2c**. Coupling reaction of the amine **2a** and **3a** with appropriate carboxylic acids using BOP as a coupling agent gave the amides **2e–h** and **3b**, **3c**. Amidation of secondary amine **2d** with corresponding acyl chlorides provided tertiary amides **4a**, **4b**.

The activities of the prepared compounds were evaluated for the inhibition of NO production in LPS-activated macrophages. Murine macrophage cell line, RAW 264.7 cells were stimulated with 1 μg/mL LPS in the presence of samples for 20 h. The amounts of NO released into culture media were determined by the Griess method[29] in the form of nitrite.[30]

The activities of the tested compounds were given in Tables 1 and 2. 4-Hydroxy or simple alkoxy substituted benzothiazoles revealed little activities. Also, the existence of 2-amino group did not show any difference on the activity. Activities of benzothiazole derivatives were improved by introducing substituted amines on the *ortho*- or *meta*-position of benzyloxy group as shown in Table 2. Effect of the *N*-substitution (R^1 and R^2) of aniline on the activity was investigated. Acetamide **2b** and sulfonamide derivatives **2c** showed no activities. *N*-benzylated compound **2d** showed the highest activity among the *N*-substituted compounds. Introduction

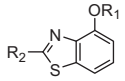


Scheme 1. Preparation of 4-alkoxybenzothiazole derivatives. Reagents: (a) isoamyl nitrite, dioxane; (b) BBr_3, DCM; (c) RBr, K_2CO_3, THF.



Scheme 2. Preparation of 4-aminobenzyloxybenzothiazole derivatives. Reagents: (a) 2- or 3-nitrobenzyl bromide, K_2CO_3, THF; (b) H_2, 10% Pd/C, THF/MeOH; (c) AcCl or CH_3SO_2Cl, TEA, DCM; (d) benzaldehyde, AcOH, $NaBH_3CN$, CH_3CN; (e) RCOOH, BOP, TEA, DCM; (f) RCOCl, DCM.

Table 1
Inhibitory activities of benzothiazole derivatives on the NO production in LPS-activated macrophages



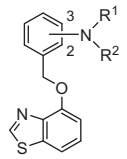
Compounds	R^1	R^2	Inh[a] (%)
1a	H	H	8.6
1b	CH_3	H	18.6
1c	Benzyl	H	12.3
1d	4-Methylbenzyl	H	10.4
1e	H	NH_2	11.4
1f	4-Methylbenzyl	NH_2	14.4

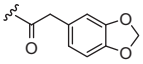
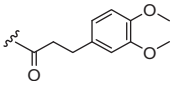
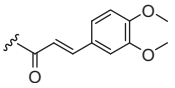
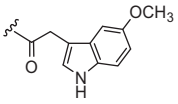
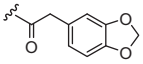
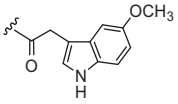
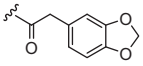
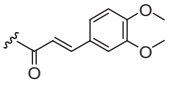
[a] Values mean the inhibition (%) of NO production at 20 μM concentration of compounds relative to the LPS control ($n = 3$).

of heterocycle-containing acyl group mostly enhanced activity except compound **2f**. The substituted positions of the compounds also influenced on the activity. Regarding the position of the phenyl substituents, *meta*-substituted amines **3a–c** revealed higher activity than the respective *ortho*-substituted amines **2a**, **2e**, and **2h**. Effect of the second introduction of benzyl substituent at nitrogen of amide was not clear. The introduction of lipophilic and bulkier tertiary amides increased the activity of **4a**, but decreased that of **4b**, comparing with the corresponding secondary amides, **2e**, **2g**. IC_{50} values of the representatively selected compounds **2d**, **2e**, **3b**, **3c**, **4a**, and **4b** were determined as 17.2, 72.3, 23.6, 4.2, 21.2, and 20.9 μM, respectively. Among the tested benzothiazoles, indole-containing compound **3c** was the most potent.

To elucidate the possible mechanism of the active benzothiazole compounds for the inhibition of NO production, further biological study was carried out. Western blot analysis[31] was performed to examine the effects of compounds **2d**, **3c**, and **4b** on the expression of iNOS and COX-2 protein in LPS-activated RAW 264.7 cells. A well known inhibitor of iNOS/COX-2 expression, curcumin was used as a positive control. The iNOS and COX-2 protein expression was markedly induced by LPS-activation. The treatment of 20 μM of compounds **2d**, **3c**, and **4b** decreased the expression of iNOS and COX-2 in correlation with their inhibitory potency of NO production. The most potent **3c** significantly inhibited the iNOS and COX-2 protein expression (Fig. 2). At RT-PCR analysis,[32] increased levels of iNOS and COX-2 mRNA

Table 2
Inhibitory activities of benzothiazole derivatives on the NO production in LPS-activated macrophages



Compounds	L[a]	R^1	R^2	Inh[b] (%)	IC_{50}[c] (μM)
2a	2	H	H	15.2	
2b	2	$COCH_3$	H	3.7	
2c	2	SO_2CH_3	H	0.4	
2d	2	Bn	H	55.8	17.2
2e	2		H	32.5	72.3
2f	2		H	11.6	
2g	2		H	44.7	
2h	2		H	36.4	
3a	3	H	H	32.9	
3b	3		H	58.9	23.6
3c	3		H	75.9	4.2
4a	2		Bn	54.9	21.2
4b	2		Bn	39.8	20.9

[a] L indicates the positions of amine substituents on the aromatic ring.
[b] Values mean the inhibition (%) of NO production at 20 μM concentration of compounds relative to the LPS control ($n = 3$).
[c] Values are means ± SD of three experiments. L indicates the position of substituent.

were also decreased by treatment of compounds **2d**, **3c**, and **4b** (Fig. 3). These results suggested that the inhibition of NO production by the active benzothiazole derivatives was caused by the suppression of iNOS proteins and mRNA expression. When the compounds were treated after the completion of iNOS induction by LPS (post-treatment), they did not show significant inhibitory activity. This result confirmed that benzothiazole derivatives exhibited their activities mainly through the inhibition of iNOS expression not the function of enzyme. The gene expressions of iNOS and COX-2 are modulated by NF-κB, which can be activated by the degradation of inhibitor-κB (I-κB) and the followed translocation into nucleus. To verify further their inhibitory mechanism, we examined the effects of benzothiazole derivatives on I-κB-α degradation and nuclear translocation of p65 in LPS-activated macrophages. Compounds **2d**, **3c**, and **4b** decreased I-κB-α degradation and resulted in the decrease of nuclear translocation of NF-κB subunit, p65 (Fig. 4). Taking together the results from Western blot analysis and RT-PCR, benzothiazoles **2d**, **3c**, and **4b** decreased NO production by the suppression of iNOS protein and mRNA through the inhibition of NF-κB activation in LPS-activated macrophages.

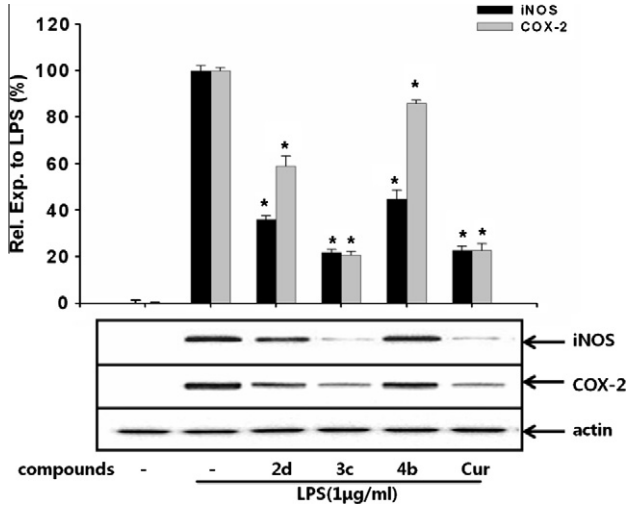


Figure 2. Effects of the prepared benzothiazoles on the expression of iNOS and COX-2 proteins in LPS-activated macrophages. RAW 264.7 cells were treated for 20 h with compounds (20 μM) **2d**, **3c**, **4b**, and curcumin during LPS (1 μg/mL) activation. Cell lysates were prepared and the iNOS, COX-2, and actin protein levels were determined by Western blotting. The relative iNOS, COX-2 protein levels were normalized with the respective amounts of actin. Values represent mean ± SD of three independent densitometric analyses of bands. * p <0.01 indicate significant difference between LPS alone control and sample treatment.

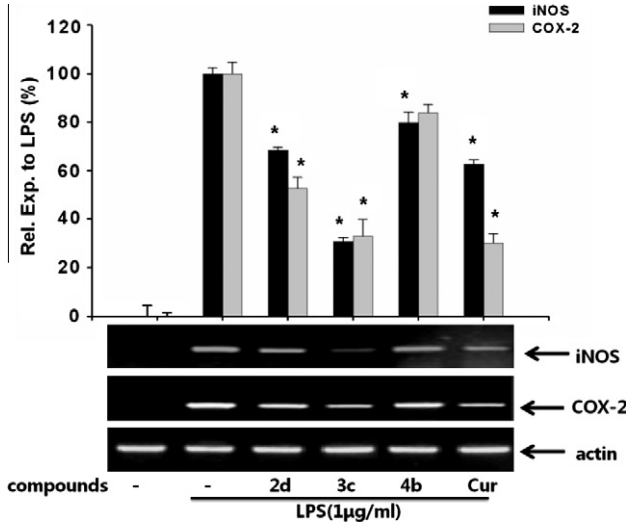


Figure 3. Effects of the prepared benzothiazoles on the expression of iNOS and COX-2 mRNAs in LPS-activated macrophages. RAW 264.7 cells were treated for 6 h with compounds (20 μM) **2d**, **3c**, **4b**, and curcumin during LPS (1 μg/mL) activation. The mRNA levels of iNOS, COX-2 and β-actin were determined by RT-PCR from total RNA extracts. The relative iNOS, COX-2 mRNA levels were normalized with the respective amounts of β-actin. Values represent mean ± SD of three independent densitometric analyses of bands. * p <0.01 indicate significant difference between LPS alone control and sample treatment.

In summary, a series of benzothiazole derivatives were identified as novel inhibitors of NO production in LPS-activated macrophages. Although they mostly revealed weak to moderate activity, several compounds showed promising activity. The SAR study suggested that amino-substituted 4-benzyloxy group on benzothiazole skeleton highly influenced on the activity. Among them, indole-containing compound **3c** was the most potent with 4.18 μM of IC_{50}. Biological study indicated that possible mechanism of the active benzothiazoles for inhibition of NO production was the suppression of iNOS protein and mRNA expression through inhibition of I-κB-α degradation and NF-κB activation. They also suppressed the expres-

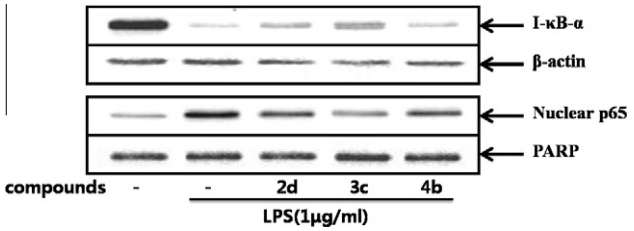


Figure 4. Effects of the prepared benzothiazoles on I-κB-α degradation and nuclear translocation of p65 in LPS-activated macrophages. Cells were pretreated with compounds (20 μM) **2d**, **3c**, and **4b** for 30 min and followed by further incubation with LPS (1 μg/mL) for 30 min. The protein levels of cytoplasmic I-κB-α and nuclear p65 were determined by Western blotting. The relative I-κB-α and p65 protein levels were normalized with the respective amounts of PARP.

sion of another inflammatory COX-2 through the same mechanism. Further study of the other biological activities related with the overproduction of NO including in vivo experiments are in progress. Our benzothiazole derivatives can be a promising lead for the development of therapeutic agents for the treatment of NO-related diseases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.112.

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30. Cell culture and nitrite assay in LPS-activated RAW 264.7 cells—Cells in 10% fetal bovine serum (FBS)-DMEM medium, were plated in 48-well plates (1×10^5 cells/mL), and then incubated for 24 h. The cells were replaced with fresh media with 1% FBS, and then incubated for 20 h in the presence or absence of test compounds with LPS (1 μg/mL). NO production in each well was assessed by measuring the accumulated nitrite in culture supernatant. Samples (100 μL) of media were incubated with Griess reagent (150 μL) for 10 min at room temperature in 96-well microplate. Absorbance at 570 nm was read using an ELISA plate reader. A standard calibration curve was prepared using sodium nitrite as a standard. A dose–response curve was prepared, and the results were typically expressed as IC_{50} values.
31. Western blot analysis of iNOS protein expression—RAW 264.7 cells (1.5×10^6 cells/60 mm dish) were stimulated with LPS (1 μg/mL) in the presence or absence of test compounds. After incubation for 20 h, the cells were washed and lyzed with lysis buffer. Twenty microgram protein of cell lysates was applied on 8% SDS–polyacrylamide gels and transferred to PVDF membrane by a standard method. The membrane was probed with antibody for anti-mouse iNOS (Transduction Laboratories, Lexington, KY) and anti-actin (Sigma, St. Louis, MO). The bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instruction.
32. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of iNOS mRNA expression—RAW 264.7 cells (1.8×10^6 cells/60 mm dish) were stimulated for 6 h with LPS (1 μg/mL) in the presence or absence of test compounds. After washing twice with phosphate buffered saline, total RNA was isolated from cell pellet, using an RNA isolation reagent (Trizol, Invitrogen, Carlsbad, CA). Two microgram of RNA was reverse transcribed into cDNA using reverse transcriptase and random hexamer. The PCR samples, contained in the reaction mixture, were comprised of mixture buffer, dNTP, Taq DNA polymerase (Promega, Madison, WI) and primers (sense and antisense). The sense and antisense primers for iNOS were 5′-ATGTCCGAAGCAAACATCAC-3′ and 5′-TAATGTCCAGGAAGTAGGTG-3′, respectively. The sense and antisense primers for β-actin were 5′-TGTGATGGTGGGAATGGGTCAG-3′ and 5′-TTTGATGTCACGCACGATTTCC-3′, respectively. The PCR amplification was performed under following conditions; 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, using thermal cycler (Gene Amp PCR system 2400, Applied Biosystems, Foster City, CA). The amplified PCR products were separated on a 2% agarose gel.