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Articles

Synthesis of Heterocycle-linked Thioureas and Their Inhibitory Activities of NO Production in LPS Activated Macrophages

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A series of thioureas were synthesized as inhibitors of NO production in lipopolysaccharide-activated macrophages. We investigated the effect of lipophilic moiety and *N*-substituents of the thioureas on the activity. Phenoxazine and carbazole-containing derivatives revealed higher activity than indole-containing thioureas. The appropriate spacer between lipophilic tail and thiourea head and methyl substituent at N3 position of thiourea brought beneficial effect on the inhibition of NO production. Among prepared compounds, phenoxazine-containing derivative **2a** was the most potent with 2.32 μM of IC_{50} value. RT-PCR analysis suggested that the prepared thioureas inhibited NO production through the suppression of iNOS mRNA expression.

Key Words: Thiourea, Nitric oxide, iNOS

Introduction

Nitric oxide (NO) is a ubiquitous biological messenger involved in a variety of physiological and pathophysiological processes. NO is synthesized by three types of nitric oxide synthases (NOSs) that catalyze the oxidation of L-arginine to L-citrulline as a co-product. Three quite distinct isoforms of NOS have been identified. The diverse biological role of NO depends on which subtype of enzyme is involved, and how much amounts of NO is synthesized.[1] Two isoforms found in neuronal tissues (nNOS, type I) and vascular endothelium (eNOS, type III) are constitutive NOS (cNOS). cNOS is intermittently activated by transient elevation of intracellular calcium level and releases small amounts of NO. The physiological concentrations of NO produced by cNOS have roles in the regulation of blood pressure and neurotransmission.[2] The third isoform is inducible NOS (iNOS, type II) that can be induced by lipopolysaccharide (LPS) and various cytokines such as IFN-α, IL-1β, and TNF-α. The iNOS produces a large amount of NO sustained over a long period of time after the enzyme induction by cytokines or endotoxins.[3] Low concentrations of NO produced by iNOS possess beneficial roles in host defence mechanism against pathogens,[4] while overproduction of NO by iNOS has been implicated in the pathogenesis of numerous inflammatory diseases such as rheumatoid arthritis, cancer, and atherosclerosis. Therefore, the inhibition of NO could be a good strategy for the treatment of diseases accompanying the overproduction of NO. There have been substantial efforts in the pharmaceutical industry to discover potent, selective iNOS inhibitors.[5-8]

The best-known inhibitors of iNOS were analogs of L-arginine such as N^G-methyl-L-arginine (L-NMA)[9] and N^G-nitro-L-arginine (L-NNA)[10] which exhibit poor selectivity for iNOS over eNOS. Recently, various small molecules of iNOS inhibitors that are structurally distinct from arginine have been reported such as amidines,[11] isoquinolinamines,[12] and isothioureas.[13,14] But most of the inhibitors are neither potent nor NOS isoforms selective enough to be applied *in vivo*.

Our initial search for iNOS inhibitors focused on urea derivatives since urea,[15] thiourea,[16] and isothiourea[13,14,17] have been reported to inhibit iNOS expression and/or NO production. Previously, we have reported urea, thiourea and isothiourea derivatives as inhibitors of NO production in LPS activated macrophages.[18,19] We further investigated structure-activity relationship of thiourea derivatives on the inhibition of NO production. Herein, we report the design and synthesis of modified thioureas. Structural feature of the thioureas are composed of lipophilic tail, spacer and thiourea head. Modification of the lipophilic group, spacer and substituents at nitrogen of thiourea were furnished as depicted in Figure 1. Their inhibitory activities for the NO production were evaluated in LPS-activated macrophage cell culture system.

Experimental

The heterocycle-linked thioureas were prepared from appropriate lipophilic heterocyles such as indole, phenoxazine, and carbazole (Scheme 1). Linker was introduced by *N*-alkylation with iodoethanol and the hydroxyl group was mesylated to give compounds **6**. The resulted mesylates were treated with *p*-nitrophenol in the presence of NaH to afford corresponding nitro compounds **7**. Reduction of nitro group over 10% Pd/C under atmospheric pressure of hydrogen gas provided amines **8**, which

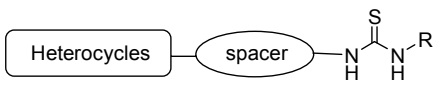
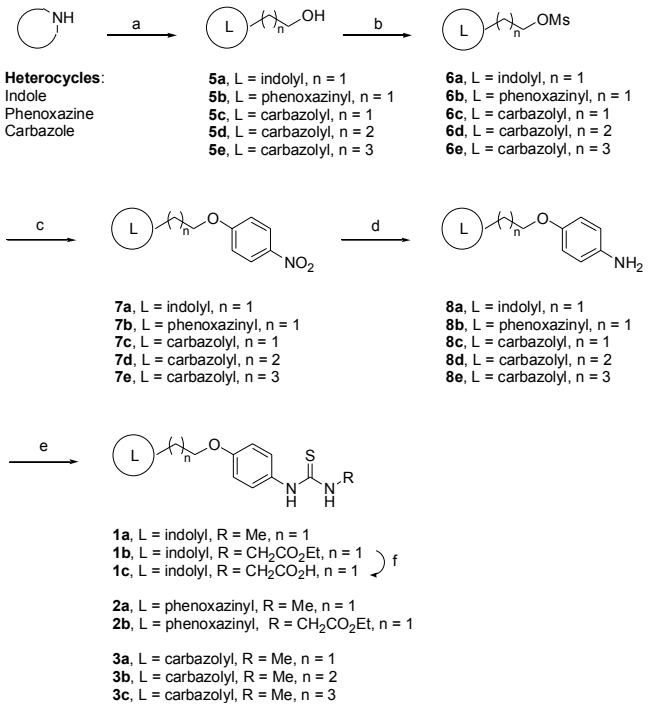
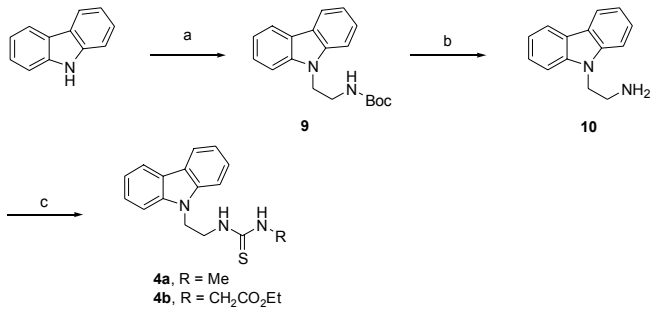


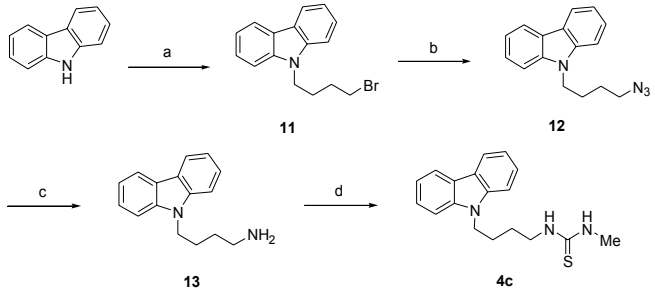
Figure 1. Representative structure of the prepared thioureas.



Scheme 1. Preparation of hetorocyle-linked phenylthiourea derivatives. Reaction conditions: (a) 2-Iodoethanol, NaH, DMF; (b) MsCl, TEA, DMF; (c) 4-Nitrophenol, NaH, DMF; (d) H_2, 10% Pd/C, THF; (e) SCNR, K_2CO_3, THF; (f) NaOH, THF/MeOH/H_2O.



Scheme 2. Preparation of carbazole-linked thiourea derivatives. Reaction conditions: (a) $Br(CH_2)_2NHBoc$, NaH, DMF; (b) TFA/MC=1:1; (c) **4a** : SCNMe, K_2CO_3, THF, **4b** : $SCNCH_2CO_2Et$, K_2CO_3, THF.



Scheme 3. Preparation of carbazole-linked thiourea derivatives. Reaction conditions: (a) 1,4-Dibromobutane, K_2CO_3, DMF; (b) NaN_3, DMF; (c) H_2, 10% Pd/C, THF; (d) SCNMe, K_2CO_3, THF.

were condensed with appropriate isothiocyanates to give the desired thioureas **1**-**3**.

Spacer-modified thioureas were also prepared to figure out the optimal size and type of spacer. Lipophilic moiety was directly connected to nitrogen of thiourea *via* methylene spacer without phenyl group. The preparation of directly *N*-substituted carbazolylethylthiourea is outlined in Scheme 2. *N*-alkylation of carbazole with Boc protected bromoethylamine and following deprotection of Boc group provided amine **10**. The obtained amine **10** was condensed with the appropriate isothiocyanates to give thioureas **4**. For the lengthening of spacer, carbazole was treated with 1,4-dibromobutane to afford compound **11**. Nucleophilic substitution of bromide **11** using NaN_3 gave azide **12**, which was reduced to amine **13**. Condensation of amine with methylisothiocyanate gave thiourea **4b**.

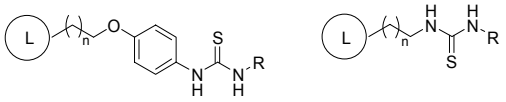
The inhibitory activity of NO production by prepared thioureas were determined by monitoring the amount of NO in cell culture media. In brief, murine macrophage cell line, RAW 264.7 cells were stimulated with 1 μg/mL of LPS in the presence or absence of samples for 20 h. The amounts of NO released into culture media were determined by the Griess method[20] in the form of nitrite.[21]

Results and Discussion

Our previous study with thiourea derivatives showed that the existence of suitable lipophilic moiety such as the carbazole group had beneficial effect on the inhibitory activity of NO production. We started with the SAR study of the lipophilic tail, and the results were summarized in Table 1. Activity of the thioureas was highly influenced by the modification of lipophilic moiety. When phenoxazine (**2a**) or carbazole (**3a**) moiety was replaced with indole (**1a**), the inhibitory activity toward NO production by **2a** or **3a** was decreased from 80% to 32% at 10 μM. Activity of other bicyclic benzoxazole-containing thiourea was as low as **1a** (data not shown). Regarding the N3 substituent of thioureas, the activities of methyl substituted derivatives were slightly higher than alkoxycarbonylmethyl substituted derivatives except **1b**. We next attempted to see the effect of spacer between lipophilic tail and thiourea head on the activity. Carbazole derivatives **3**, which have phenyl group in the spacer, were compared with compounds **4** in which the spacers were shortened by omitting the phenyl group. The activity of compounds **4** was lower than those of the corresponding compounds **3** having longer spacer. However, the activity of compounds **4a** was increased by homologation (**4b**) of the spacer with two methylene units. This result suggested that appropriate length of the spacer was necessary for the activity. Among the prepared thioureas, phenoxazine-containing derivative, **2a** was the most potent. IC_{50} values of compounds **2a** and **3a-c** were determined as 2.32, 3.29, 4.02 and 6.01 μM, respectively.

To elucidate the mechanism for the inhibition of NO production by thioureas, we examined the effects of **2a**, **2b**, **3b** and **3c** on the expression of iNOS mRNA in LPS-activated RAW 264.7 cells. At RT-PCR analysis,[22] the mRNA of iNOS was induced by the treatment of 1 μg/mL LPS for 6 hr. Curcumin was used as positive control in RT-PCR experiment. The treatment of **2a** and **3b** suppressed the expression of iNOS mRNA signi-

Table 1. Inhibitory activities of heterocycle-linked thioureas on the NO production in LPS-activated macrophages.



1, L = indolyl
2, L = phenoxazinyl
3, L = carbazolyl

4, L = carbazolyl

Compounds	R	n	Inh(%)[a] 10 μM	IC_{50} (μM)[b]
1a	Me	1	32	
1b	CH_2CO_2Et	1	43	
1c	CH_2CO_2H	1	6	
2a	Me	1	82	2.32 ± 0.29
2b	CH_2CO_2Et	1	41	15.17 ± 1.14
3a	Me	1	87	3.29 ± 0.55
3b	Me	2	77	4.02 ± 0.12
3c	Me	3	61	6.01 ± 0.17
4a	Me	1	23	31.62 ± 1.17
4b	CH_2CO_2Et	1	14	
4c	Me	3	50	13.04 ± 1.38

[a]Values mean the inhibition (%) of NO production of compounds relative to the LPS control (n = 3). [b]Values are means ± SD of three experiments.

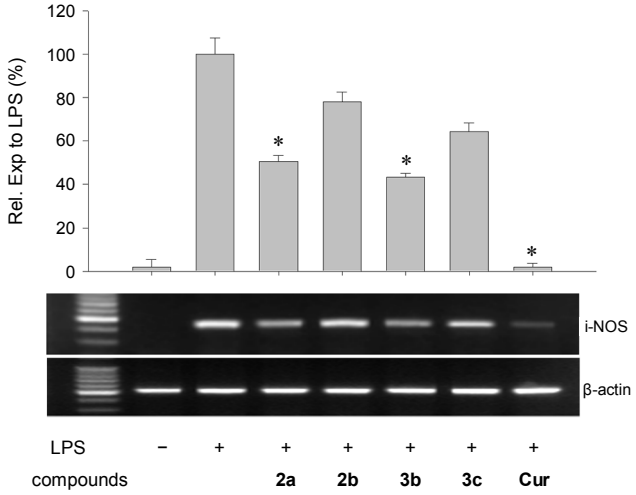


Figure 2. Effects of thioureas on the expression of iNOS mRNA in LPS-activated macrophages. RAW 264.7 cells were treated for 6 h with compounds **2a**, **2b**, **3b**, **3c** and curcumin (cur: as positive control)(10 μM) during LPS (1 μg/mL) activation. The mRNA levels of iNOS and β-actin were determined by RT-PCR from total RNA extracts. The relative iNOS 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.

ficantly at 10 μM (Figure 2). These results suggested that the inhibition of NO production by thiourea derivatives was due to the suppression of iNOS mRNA.

Conclusion

We prepared a series of heterocycle-linked thiourea derivatives and evaluated their inhibitory activities of NO production in LPS-activated macrophages. Several thioureas showed promising inhibitory activities of NO production. The SAR study demonstrated that the modification of lipophilic moiety, *N*-substituents of the thioureas, and the spacer between lipophilic tail and thiourea head highly influenced the inhibitory activity of NO production. Among prepared compounds, phenoxazine-containing derivative **2a** was the most potent with 2.32 μM of IC_{50} value. RT-PCR analysis suggested that the prepared thioureas inhibited NO production through the suppression of iNOS mRNA expression. The result provided our thiourea derivatives might serve as good leads for the development of therapeutic agents for the management of diseases accompanying overproduction of NO.

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Supporting Information. It is available on request from the correspondence author (Raok Jeon, Phone: 82-2-710-9571, Fax: 82-2-715-9571, E-mail: rjeon@sookmyung.ac.kr).

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21. 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. Culture media (100 μL) 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.
22. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of iNOS mRNA expression - RAW 264.7 cells (1.6×10^6 cells/60 mm dish) were stimulated for 6 h with LPS (1 μg/mL) in the absence or presence of test samples. 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 (Invitrogen, Carlsbad, CA) and random hexamer (Cosmo, Seoul, Korea). 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'-CCCTTCCGAAGTTTCTGGCAGCAG-3' and 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3', respectively. The sesnse and antisense primers for β-actin were 5'-TGTGATGGT GGGAATGGGTCAG-3' and 5'-TTTGATGTCACGCACGATT TCC-3', respectively. The PCR amplification was performed under following conditions; 25 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 55 ^{o}C for 30 s and extension at 72 ^{o}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 1% agarose gel.