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Design and synthesis of urea and thiourea derivatives and their inhibitory activities on lipopolysaccharide-induced NO production

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Abstract—Series of ureas and thioureas were designed and synthesized, and their inhibitory activities of NO production in lipopolysaccharide-activated macrophages were evaluated. We found several essential moieties in the structure of the prepared compounds for the activity. Thiourea derivatives revealed higher inhibitory activity than the corresponding urea derivatives. Among these compounds, **7e** having carboxymethyl group at N3 position of thiourea was the most potent in the inhibition of NO production. They inhibited NO production through the suppression of iNOS protein and mRNA expression.

The critical role of nitric oxide (NO) in various pathological conditions has led to the discovery of new inhibitors of NO production as potential therapeutic agents. NO, a gaseous free radical, is produced through the oxidation of L-arginine by three isoforms of nitric oxide synthase (NOS).[1] The constitutive NOS (cNOS) found in neuronal tissues (nNOS, type I) and vascular endothelium (eNOS, type III) is Ca^{2+}-dependent and releases small amounts of NO required for homeostatic function.[2] Meanwhile, inducible NOS (iNOS, type II), which can be induced by lipopolysaccharide (LPS) and various cytokines such as IFN-α, IL-1β, and TNF-α, is Ca^{2+}-independent and produces micromolar levels of NO.[3] Low concentrations of NO produced by iNOS possess beneficial roles in antimicrobial activity of macrophages against pathogens,[4] while the overproduction of NO and its derivatives, such as peroxynitrite and nitrogen dioxide, has been suggested to be mutagenic in vivo and to provoke the pathogenesis of septic shock and various inflammatory processes.[5] Furthermore, NO and its oxidized forms have also been known to be carcinogenic.[6]

Among the many strategies for providing rational control of NO levels, the efforts have been mainly directed toward development of selective inhibitor of iNOS that can be applied for the treatment of diseases accompanying high levels of NO. Regulation of iNOS can be achieved by the control of expression level and/or enzymatic activity of iNOS. Many classes of iNOS inhibitors can be classified according to their structural features, such as, amino acid analogues,[7] amino heterocycles,[8] amidines,[9] guanidines,[10] isoquinolinamines,[11] and isothiourea.[12,13] But most of the inhibitors are neither potent nor selective enough against NOS isoforms, that limited the application of them in vivo. Only one class of iNOS inhibitors, L-lysine analogue, was reported to enter the clinical trial in human.[14]

Urea was known to inhibit not only the activity of iNOS in macrophages during the uremia,[15] but also the expression of iNOS in LPS-activated macrophages.[16] Urea was suggested as an important modulator for renal function through the fine tuning of NO production.[17] Several groups have reported that urea and thiourea[18] or isothiourea[12,13,19] inhibit iNOS expression and/or NO production. Based on these reports, we tried to investigate urea and thiourea derivatives as novel inhibitors having mechanism for enzymatic inhibition and/or downregulation of iNOS expression.

Herein, we report the design and synthesis of urea and thiourea derivatives as depicted in Figure 1. Their effects on the NO production and expression of iNOS were evaluated in LPS-activated macrophage cell culture system.

Keywords: Urea; Thiourea; Carbazole; Nitric oxide synthase; Nitric oxide; Inhibitor.
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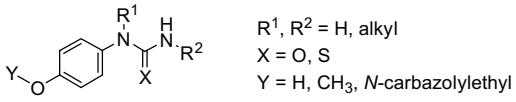


Figure 1. Representative structure of urea and thiourea.

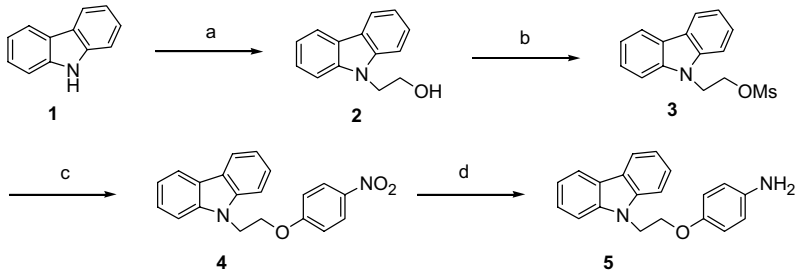
The preparation of the carbazole-linked urea and thiourea derivatives is outlined in Schemes 1 and 2. Hydroxy ethyl group was introduced at nitrogen of carbazole and the resulting alcohol **2** was mesylated to obtain compound **3**. Alkylation of 4-nitrophenol by treatment of compound **3** in the presence of NaH gave compound **4**. Following reduction of nitro compound **4** over 10% Pd/C under atmospheric pressure of hydrogen gas provided amine **5**. Condensation of amine **5** with the appropriate isocyanates or isothiocyanates offered the desired compounds **6** and **7**.

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, was stimulated with 1 μg/mL of LPS in the presence 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]

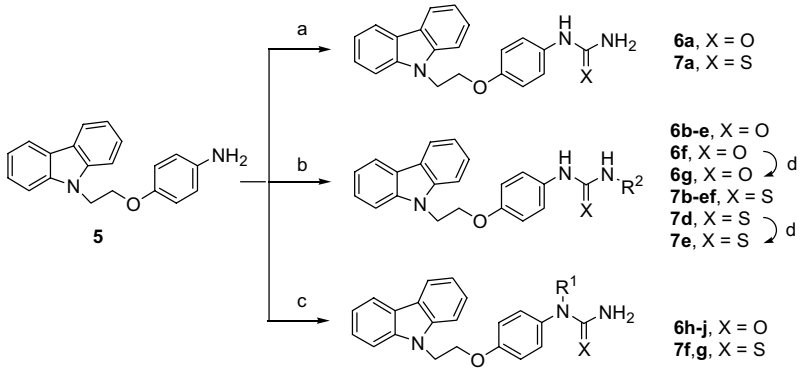
The inhibitory activities of the prepared compounds on the NO production are given in Table 1. Aminoguanidine, a well-known specific inhibitor of iNOS, was used as positive control that showed 85% inhibition of NO production at 0.1 μM. Most of the thiourea derivatives revealed higher activity than the corresponding urea derivatives. For example, thioureas **7a**, **7c**, **7e**, and **7f** showed significantly higher activities than ureas **6a**, **6b**, **6g**, and **6i**, respectively.

Effects of the alkyl substituents at the N1 and N3 position of urea **6a** and thiourea **7a** were investigated. Introduction of methyl group **6h** at the N1 position of urea **6a** lowered the activity, while introduction of bulkier substituent retrieved the activity. Compound **6i** with ethyl group showed the similar activity as **6a**. Meanwhile, activity of compound **6j** with cyclopropylmethyl at N1 became 2.5-fold higher than that of **6a**. On the other hand, thiourea derivatives **7f** substituted with ethyl and **7g** with cyclopropylmethyl at N1 of **7a** revealed 2-fold lower activity than **7a**.

The effects of substituents at N3 position were considerably different between urea and thiourea derivatives. While the substitution at N3 of urea derivatives **6b–6g** showed no significant effects on the activity, the alkyl substitution of thiourea greatly enhanced the activity. Compounds **7b**, **7c**, **7e** with methyl, ethyl, or carboxymethyl group at N3 revealed similar activity ranging from 80% to 90% inhibition of NO production at 5μM concentration. In both cases of ureas and thioureas, the carboxymethyl was the best substituent at N3 for the improvement of activity. IC_{50} values of **6j**, **7a–7c**, and

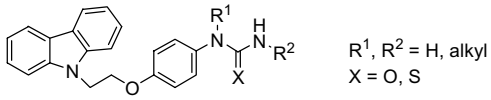


Scheme 1. Preparation of 4-(2-carbazol-9-ylethoxy)phenylamine. Reagents: (a) ICH_2CH_2OH, NaH, DMF; (b) MsCl, TEA, DMF; (c) 4-nitrophenol, NaH, DMF; (d) H_2, 10% Pd/C.



Scheme 2. Preparation of carbazole-linked urea and thiourea derivatives. Reagents: (a) NaCNX (X = O, S), AcOH, H_2O; (b) R^2NCX (X = O, S), K_2CO_3, THF; (c) i—$R^{1'}CHO$ ($R^{1'}$ = corresponding alkyl-CH_2), $NaCNBH_3$, CH_3CN; ii—NaCNX (X = O, S), AcOH, H_2O; (d) $Ba(OH)_2$, THF/MeOH/H_2O.

Table 1. Inhibitory activities of carbazole-linked phenylureas and phenylthioureas on the NO production in LPS-induced NO production



Compound	R^1	R^2	X	Inhibition[a] (%)	IC_{50}[b] (nM)
6a	H	H	O	24	
6b	H	Et	O	30	
6c	H	Pro	O	14	
6d	H	*i*-Pro	O	15	
6e	H	Ph	O	28	
6f	H	CH_2CO_2Et	O	38	
6g	H	CH_2CO_2H	O	43	
6h	Me	H	O	8	
6i	Et	H	O	25	
6j	Cyclopropylmethyl	H	O	61	3120 ± 250
7a	H	H	S	55	5310 ± 702
7b	H	Me	S	80	730 ± 180
7c	H	Et	S	90	901 ± 211
7d	H	CH_2CO_2Et	S	41	
7e	H	CH_2CO_2H	S	87	153 ± 83
7f	Et	H	S	33	
7g	Cyclopropylmethyl	H	S	34	

[a] Values mean the inhibition (%) of NO production at 5 μM concentration of compounds relative to the LPS control ($n = 3$).
[b] Values are means ± SD of three experiments.

7e, which showed more than 50% inhibitory responses at 5 μM, were determined as 3.12, 5.31, 0.73, 0.90, and 0.15 μM, respectively.

In order to find the influence of lipophilic tail on the activity, carbazolylmethyl group of thiourea derivatives **7b–7c** was eliminated. These compounds showed lower activities, less than 15% inhibition of NO production at 5 μM, than the corresponding carbazole-linked thioureas. It has been reported that a carbazole derivative inhibited iNOS expression in the LPS-activated macrophage.[22] Our results also demonstrated that both thiourea and carbazole moieties might play an important role for their activities although carbazole moiety devoid of thiourea group revealed no activity. We expect to potentiate the activity by the structural modification of thiourea and lipophilic segment.

For the further biological study of our derivatives, we examined the effects of **6j**, **7a–7c**, and **7e** on the expression of iNOS protein and mRNA in LPS-activated RAW 264.7 cells. The amounts of iNOS protein were analyzed in Western blot analysis after 20-h incubation with compounds during LPS (1 μg/mL) activation of macrophages.[23] Compounds **7b** and **7e** significantly reduced the amounts of iNOS at 10 μM (Fig. 2). At RT-PCR analysis,[24] the expression level of iNOS mRNA was increased markedly by LPS-activation for 6 h. Compounds **7b** and **7e** suppressed the induction of iNOS mRNA at 10 μM (Fig. 3). These results indicated that the inhibition of NO production by thiourea derivatives resulted from the suppression of iNOS protein and mRNA.

When we treated the compounds after the completion of iNOS induction by LPS (post-treatment), they showed

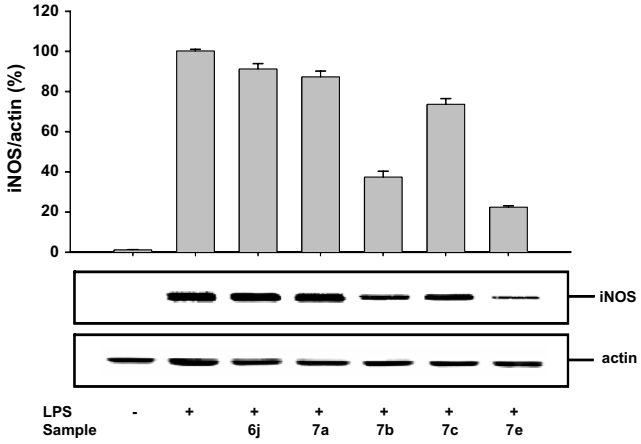


Figure 2. Effects of the prepared compounds on the expression of iNOS protein in LPS-activated macrophages. RAW 264.7 cells were treated for 20 h with compounds (10 μM) during LPS (1 μg/mL) activation. Cell lysates were prepared and the iNOS and actin protein levels were determined by Western blotting. The relative iNOS protein levels were normalized with the respective amounts of actin. Values represent means ± SD of three independent densitometric analyses of bands.

weak activity compared with the results of the co-treatment of compounds with LPS. Even the most potent compound **7e** showed 12% inhibition at 10 μM by post-treatment. These results suggested that thiourea derivatives exhibited their activities mainly through the inhibition of iNOS expression with marginal inhibition against enzymatic activity. It has been reported that urea itself inhibited the activity of iNOS by transcriptional[15] and post-transcriptional[16] mechanisms. Many of thioureas and isothioureas were reported[12,25] as inhibitors of iNOS enzyme devoid of controlling the expression step. In addition, a carbazole compound,

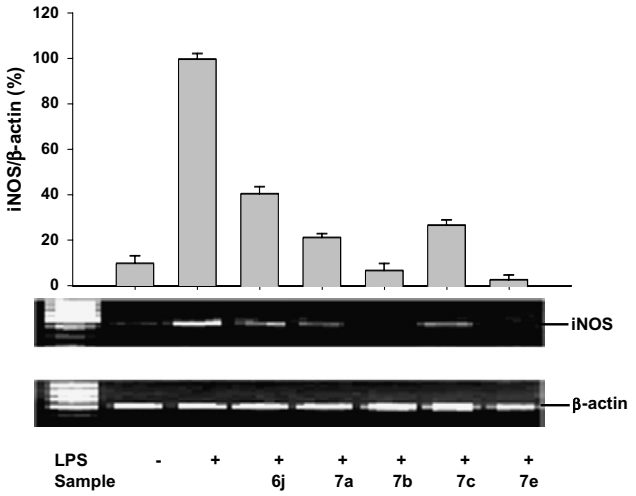


Figure 3. Effects of the prepared compounds on the expression of iNOS mRNA in LPS-activated macrophages. RAW 264.7 cells were treated for 6 h with compounds (10 μM) during LPS (1 μg/mL) activation. The mRNA levels of iNOS and β-actin were determined by RT-PCR from total RNA extracts. The relative iNOS mRNA levels were normalized with the respective amounts of β-actin. Values represent means ± SD of three independent densitometric analyses of bands.

9-(2-chlorobenzyl)-9*H*-carbazole-3-carbaldehyde, was reported as an inhibitor of iNOS mRNA expression through a signaling pathway that does not involve NF-κB pathway.[22] The exact difference between the mechanism of our compounds and that of the reported thioureas and carbazole derivative was not explained in this report. The study of the mechanism for the iNOS inhibition by our compounds might be worthy to pursue further.

In conclusion, we prepared a series of urea and thiourea derivatives and evaluated their inhibitory activities of NO production in LPS-activated macrophages. They suppressed the release of NO into culture media through the suppression of iNOS protein and mRNA expression. The SAR studies demonstrated that thiourea is superior to urea and N3 substitution of thiourea with alkyl group is highly beneficial for their activity. Further study of the other biological activities related with the overproduction of NO, and the detailed mechanism for the activities of these derivatives, is in progress. Our thiourea derivatives that can control the expression of iNOS can be good leads for the development of therapeutic agents for the management of NO-related diseases.

Acknowledgment

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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.2007.04.005.

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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, 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.
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23. 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 lysed with lysis buffer. Twenty μg 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.
24. 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 micrograms 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′-TGT GATGGTGGGAATGGGTCAG-3′ and 5′-TTTGATGT CACGCACGATTTCC-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.
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