Suppression of Inducible Nitric Oxide Synthase Expression by Yakuchinones and Their Analogues

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Analogues of yakuchinones were synthesized as inhibitors of nitric oxide production in lipopolysaccharide-activated macrophage cell line, RAW 264.7 cells. We prepared stronger inhibitors than the original natural molecules, yakuchinones A and B reported from *Alpinia oxyphylla*. From the limited structural activity relation study of analogues, we concluded that the optimal length of linker between two aryl groups and the presence of enone moiety in the linker were identified as essential for the activity. The IC_{50} value of the most potent structure was 0.92 μM. The active analogues suppressed the expression of inducible nitric oxide synthase protein and mRNA.

Key words nitric oxide; yakuchinone analogues; inhibitor; iNOS; expression

Diarylheptanoid is a family of natural plant metabolites whose characteristic structural feature is the presence of two aromatic rings tethered by a linear seven-carbon chain. The diarylheptanoids exhibit a broad range of biological activities including anti-tumor,[1,2] anti-inflammatory,[3,4] anti-oxidant,[5,6] antihepatoxic,[7] antifungal,[8,9] and related effects. For instance, curcumin, a yellow pigment from tumeric (*Curcuma longa*, Zingiberaceae), has been shown to inhibit tumorigenesis during both initiation and promotion stages in several animal models, possibly through the inhibition of cyclooxygenase and lipoxygenase and blocking the formation of arachidonic acid metabolites.[10—12] It was reported that the substitution pattern on the aromatic moiety of curcumin has crucial effects on the gene expression of inducible forms of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS).[13] Yakuchinone A and yakuchinone B (Fig. 1), diarylheptanoids from *Alpinia oxyphylla* (Zingiberaceae) have been reported to show potent anti-inflammatory and anti-tumor promotional activities through the inhibition of COX-2 and iNOS expression.[14,15] Yakuchinone B and the structural analogues have been extensively studied as inhibitors of acyl-CoA: cholesterol *O*-acyltransferase that can be therapeutic agents for hypercholesterolemia and atherosclerosis.[16] Yakuchinone B has also been reported to show inhibitory activity of tyrosinase. The α,β-unsaturated carbonyl conjugated moiety and aromatic ring of yakuchinone B play important roles in the competitive inhibition of tyrosinase.[17] The overall biological activity of yakuchinone B is stronger than yakuchinone A that might come from the presence of enone group in the linker. We tried to optimize the structure of yakuchinone for the inhibitory activity of nitric oxide production in activated macrophages.

The critical role of nitric oxide (NO) in various pathological conditions has led to the discovery of new therapeutic agents. NO, a gaseous free radical, is produced through the oxidation of arginine by nitric oxide synthase (NOS).[18] The calcium-regulated constitutive isoforms, endothelial (eNOS) and neuronal (nNOS) have important roles in regulation of blood pressure and neurotransmission,[19] whereas the inducible isoform (iNOS) is calcium-independent and induced by LPS and various cytokines such as IFN-α, IL-1β, and TNF-α.[20] Low concentrations of NO produced by iNOS possess beneficial roles in antimicrobial activity of macrophages against pathogens,[21] while the overproduction of NO and its derivatives, such as peroxynitrite and nitrogen dioxide, have been suggested to be mutagenic *in vivo* and to provoke the pathogenesis of septic shock and various inflammatory processes.[22] Furthermore, NO and its oxidized forms have also been shown to be carcinogenic.[23] Thus, inhibitors of iNOS enzyme activity or its expression can be used as potential therapeutic tools for management of NO-related disorders.

In the present study, we tried to obtain potent inhibitors of iNOS expression by the structural modification of yakuchinone that can serve as a new lead for the development of anti-inflammatory drug. Our efforts toward the design of novel yakuchinone analogues have focused on the modification of the conjugation and the length of linker between two aromatic rings.

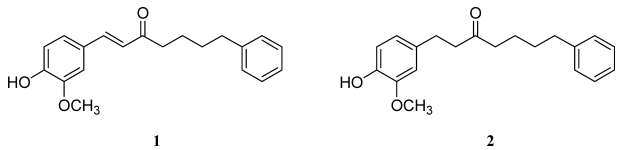

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Fig. 1. Structures of Yakuchinone B (**1**) and A (**2**)

Results and Discussion

For the evaluation of inhibitory activity of yakuchinone analogues against NO production, NO released from culture media was quantitated after incubation with samples during LPS-activation of RAW 264.7 cells. When the cells were treated with 1 μg/ml LPS for 20 h, the NO production was markedly increased to 30—40 μM, while basal level was 1.0—2.5 μM. The inhibitory potencies, expressed as the IC_{50} values, of the synthetic analogues are shown in Table 1. The most essential structural requirement for the activity is the enone moiety in the linker. The activity of compounds **1**, **4** and **7** are more potent than the respective non-enone type structures **2**, **5** and **8**, respectively. The length of linker is proportional to the activity up to six-carbon, and then the activity declined with the seven-carbon diarylheptanoid, compound **1**. Generally there are many reports of diarylheptanoids from plants with diverse biological activities. Diarylhexanoid can be another target for getting valuable bio-active structures. Compound **3** with enone moiety in the diaryl-

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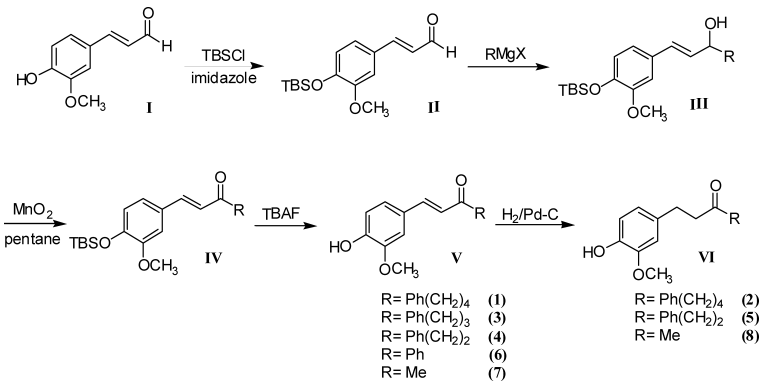
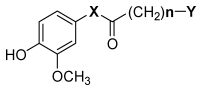


Chart 1. Preparation of Yakuchinone A, B, and Their Analogues

Table 1. Inhibitory Activities of Yakuchinone Analogues on NO Production in LPS-Activated Macrophages



Compounds	X	*n*	Y	IC_{50} (μM)[a]
1	CH=CH (*trans*)	4	Phenyl	1.38±0.35
2	CH_2–CH_2	4	Phenyl	16.81±1.47
3	CH=CH (*trans*)	3	Phenyl	0.92±0.07
4	CH=CH (*trans*)	2	Phenyl	3.73±0.14
5	CH_2–CH_2	2	Phenyl	>50
6	CH=CH (*trans*)	0	Phenyl	4.91±0.85
7	CH=CH (*trans*)	0	Methyl	9.62±1.26
8	CH_2–CH_2	0	Methyl	>50

a) IC_{50} values represents means±S.D. (n=3).

hexa-type linker between two aromatic rings showed the most potent inhibition of LPS-induced NO production (IC_{50}=0.92 μM). Compound with diaryl-type showed more potent activity compared with non-diaryl type (compound **6** *vs.* **7**).

These results indicate some structural requirements of yakuchinone analogues for the inhibition of LPS-induced NO production in macrophages as follows: (1) the enone moiety, especially the conjugated double bond located between aromatic ring and carbonyl group is essential; (2) the structure with diarylhexa type showed the most potent activity; (3) the diaryl structure tends to increase activity. To elucidate the activity mechanisms of active synthetic analogues, we examined the effects of diaryl compounds **1**—**6** on the expression of iNOS protein and mRNA in LPS-activated RAW 264.7 cells. The induced iNOS was detected in Western blot analysis after 20 h incubation with 1 μg/ml of LPS. Compounds **1**, **3**, **4** and **6** with enone moiety apparently reduced amounts of iNOS expression at 5 μM (Fig. 2(A)). At RT-PCR analysis, the level of iNOS mRNA expression was increased dramatically by LPS-activation for 6 h. The induction of mRNA was suppressed by the treatment of compounds **1**, **3**, **4** and **6** at 5 μM as shown in Fig. 2(B). The pretreatment of test compounds for 30 min prior to LPS-stimulation showed more distinct inhibition of mRNA expression than simultaneous treatment. The Western blot and RT-PCR analysis indicated that the inhibition of NO production by yakuchinone analogues was correlated with the suppression of the iNOS protein and mRNA expression. Yakuchinone A

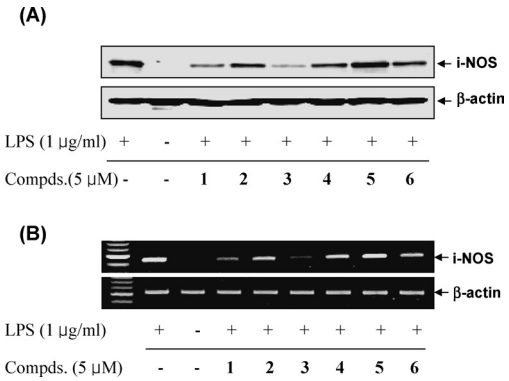


Fig. 2. (A) Effects of Yakuchinone Analogues on the Expression of iNOS Protein in LPS-Activated Macrophages

RAW 264.7 cells were treated with synthetic compounds **1**—**6** (5 μM) for 20 h during LPS (1 μg/ml) activation. Cell lysates were prepared and the iNOS and β-actin protein levels were determined by Western blotting. The blot is the representative result of three separate experiments.

(B) Effects of Yakuchinone Analogues on the Expression of mRNA in LPS-Activated Macrophages

RAW 264.7 cells were pre-treated with synthetic compounds **1**—**6** (5 μM) for 30 min, and then treated with LPS (1 μg/ml) for 6 h. mRNA level for iNOS and β-actin were determined by RT-PCR from total RNA extracts. The gel shown is the representative result of three separate experiments.

and B have been reported to be strong inhibitors of COX-2 and iNOS expression through the suppression of NF-κB activation in 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treated mouse skin.[14] Here, we report the inhibitory effects of synthetic yakuchinone analogues on iNOS expression in activated macrophages. These results imply that the yakuchinone analogues may have anti-inflammatory and cancer chemopreventive potential through the similar mechanism of yakuchinones reported.

In conclusion, we prepared analogues of yakuchinone A and B, and evaluated the inhibitory activity of NO production in LPS-activated macrophages. Diarylhexanoid with enone moiety located in linker between two aromatic rings showed the most potent activity. These active compounds suppressed the LPS-induced upregulation of iNOS protein and mRNA. The optimized structures can be lead compounds for the development of anti-inflammatory and cancer chemopreventive drugs.

Experimental

Preparation of Yakuchinone Analogues The general synthetic route to yakuchinone A, B, and their analogues is outlined in Chart 1. The commercially available cinnamaldehyde **I** was protected with *tert*-butyldimethylsilyl

(TBS) chloride to give the TBS ether **II**. The alkylation of **II** with Grignard reagent, followed by oxidation of allylic alcohol functionality with MnO_2 gave the enone **IV**. Deprotection of compound **IV** with tetrabutylammonium fluoride (TBAF) in THF gave the enones **1**,[24] **3**,[25] **4**,[25] **6**[26] and **7**,[27] which were further reduced to the ketones **2**,[28] **5**[25] and **8**[29] by catalytic hydrogenation. Reaction progresses were checked by TLC and the structures of final products were confirmed by the analyses of NMR spectra.

Measurements of NO in LPS-Induced Murine Macrophages Murine macrophage cell line, RAW 264.7 cells, in 10% fetal bovine serum (FBS)-DMEM, were plated in 48-well plates (1.5×10^5 cells/ml), and then incubated for 24 h. The cells were replaced with fresh media containing 1% FBS, and then incubated in the presence or absence of test samples with 1 μg/ml of LPS for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite in culture supernatant. Samples (100 μl) of media were incubated with Griess reagent (150 μl)[30] at room temperature for 10 min 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 standard. Dose–response curves were prepared, and the results typically expressed as the IC_{50} values. IC_{50} represents the concentration required for 50% inhibition of NO production in LPS-activated RAW 264.7 cells. Percentage inhibition of NO production was calculated as followed equation; $100\times[OD_{LPS}-OD_{sample}]/[OD_{LPS}-OD_{media}]$.

Western Blot Analysis of iNOS Protein Expression RAW 264.7 cells (1.6×10^6 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. Sixty μg protein of cell lysates was applied on 8% SDS-polyacrylamide gels and transferred to PVDF membrane by standard method. The membrane was probed with antibody for anti-mouse iNOS (Transduction Laboratories, Lexington, KY, U.S.A.) and anti-β-actin (Sigma, St. Louis, MO, U.S.A.). The Western blot was visualized using an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience, Piscataway, NJ, U.S.A.) according to the manufacturer's instruction.

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 pre-treated with test compounds for 30 min and stimulated with LPS (1 μg/ml) for 6 h. After washing twice with PBS, total RNA was isolated from cell pellet, using an RNA isolation reagent (Trizol, Invitrogen, Carlsbad, CA, U.S.A.). 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, U.S.A.) 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, U.S.A.). The amplified PCR products were separated on 1% agarose gel.

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25) 1-(4-Hydroxy-3-methoxyphenyl)-6-phenylhex-1-en-3-one (>B>3): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.36 (1H, d, J=16.1), δ 7.24—7.10 (5H, m), δ 7.00 (1H, dd, J=8.1, 1.8), δ 6.96 (1H, d, J=1.8), δ 6.84 (1H, d, J=8.1), δ 5.82 (1H, s), δ 3.86 (3H, s), δ 2.64—2.56 (4H, m), δ 2.00—1.90 (2H, m).
(*E*)-1-(4-Hydroxy-3-methoxyphenyl)-5-phenylpent-1-en-3-one (**4**): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.48 (1H, d, J=16.1), δ 7.32—7.18 (5H, m), δ 7.07 (1H, dd, J=8.2, 1.9), δ 7.03 (1H, d, J=1.9), δ 6.92 (1H, d, J=8.2), δ 6.59 (1H, d, J=16.1), δ 5.90 (1H, s), δ 3.93 (3H, s), δ 3.00 (4H, m).
1-(4-Hydroxy-3-methoxyphenyl)-5-phenylpentan-3-one (**5**): ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.20—δ 7.06 (5H, m), δ 6.74 (1H, d, J=7.9), δ 6.59 (1H, d, J=1.9), δ 6.56 (1H, dd, J=7.9, 1.9), δ 5.41 (1H, s), δ 3.78 (3H, s), δ 2.81 (2H, t, J=7.4), δ 2.74 (2H, t, J=7.1), δ 2.66—2.58 (4H, m).
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