Scientia Pharmaceutica www.scipharm.at

Research article Open Access

Comparative Study on Inhibitory Activity of Zerumbone and Zerumbone 2,3-Epoxide on NF-κB Activation and NO Production

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Sci Pharm. 2009; 77: 589–595 doi:10.3797/scipharm.0907-16

Published: August 6th 2009 Received: July 23rd 2009
Accepted: August 6th 2009

This article is available from: http://dx.doi.org/10.3797/scipharm.0907-16

Abstract

In the present study the significant role of the α,β-unsaturated carbonyl structure in the anti-inflammatory activity of the natural humulane sesquiterpenoids zerumbone and zerumbone 2,3-epoxide was evidenced from a comparative study of the ability of zerumbone and zerumbone 2,3-epoxide to inhibit NF-κB activation and NO production in LPS (lipopolysaccharide)-stimulated RAW 264.7 cells. The IC_{50} of these compounds were 1.97 μM ± 0.18 and 30.11 μM ± 4.10 in the NF-κB activation assay and 3.58 μM ± 0.46 and 34.7 μM ± 3.72 in the nitric oxide production assay, respectively.

Keywords

Zerumbone • Zerumbone-2,3-epoxide • NF-κB • NO production

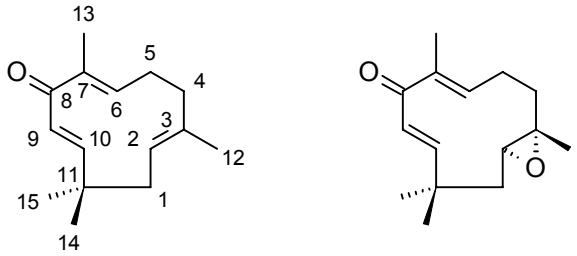
Introduction

Nuclear factor-κB (NF-κB) consists of homodimers and heterodimers of Rel/NF-κB proteins that bind to DNA target sites, where they directly regulate gene transcription. Activation of NF-κB has been showed to be involved in the pathogenesis of chronic inflammatory diseases, such as inflammatory bowel diseases and rheumatoid arthritis, and a variety of human cancers such as leukemias, lymphomas, and solid tumors [1]. Therefore, pharmacological intervention of NF-κB could be a valuable strategy to modulate

inflammatory processes as well as cancer. Several small molecular natural compounds have been reported to inhibit NF-κB at one or multiple activation steps of the signaling NF-κB pathway [2–4]. The natural humulane sesquiterpenoid zerumbone (**1**) (Fig. 1) was shown to modulate proinflammatory genes and cancer cell proliferation all of which are associated with the activation of NF-κB [5–7]. Zerumbone possesses a monocyclic eleven-membered ring containing a cross-conjugated dienone moiety, which is closely resembled to the α,β-unsaturated carbonyl group in the well-known NF-κB inhibitors such as *ent*-kaurane diterpenoids [8] or sesquiterpene lactones [9]. High biological activity seems to be dependent on the α,β-unsaturated carbonyl group of zerumbone [5] since this group can react with the cysteine sulfhydryl groups (Cys 38 and Cys 120) in the p65 subunit of NF-κB. Zerumbone and its natural analog zerumbone 2,3-epoxide (**2**) were isolated by us from the rhizomes of the Vietnamese *Curcuma zedoaria* species (Zingiberaceae), which is commercially available for the treatment of gastrointestinal disorders and ulcers [10, 11]. The significant role of the α,β-unsaturated carbonyl structure in the antiinflammatory activity of these humulane sesquiterpenoids can be further evidenced from a comparative study of the ability of zerumbone and zerumbone 2,3-epoxide to inhibit NF-κB activation and NO production in LPS (lipopolysaccharide)-stimulated RAW 264.7 cells.

Results and Discussion

Zerumbone (**1**) and zerumbone 2,3-epoxide (**2**) were examined for their dose-dependent effect on LPS (lipopolysaccharide)-induced NF-κB activation in RAW 264.7 cells, which were stably transfected with a plasmid containing κB elements linked to SEAP (secreted alkaline phosphatase) gene as NF-κB reporter gene. SEAP activity in the culture medium was measured after treatment without LPS or with LPS (1 μg/mL) in the presence of various concentrations of compounds **1** (0.3–30 μM) and **2** (1–100 μM).



1 **2**

Fig. 1. Chemical structures of zerumbone (**1**) and zerumbone 2,3-epoxide (**2**)

As shown in Fig. **2**, zerumbone potently inhibited LPS-induced SEAP activity dose-dependently with an IC_{50} of 1.97 μM ± 0.18. Meanwhile, zerumbone 2,3-epoxide did weakly inhibit NF-κB activity with an IC_{50} value of 30.11 μM ± 4.10 (Fig. **2**), i.e. 15-fold greater than that of zerumbone.

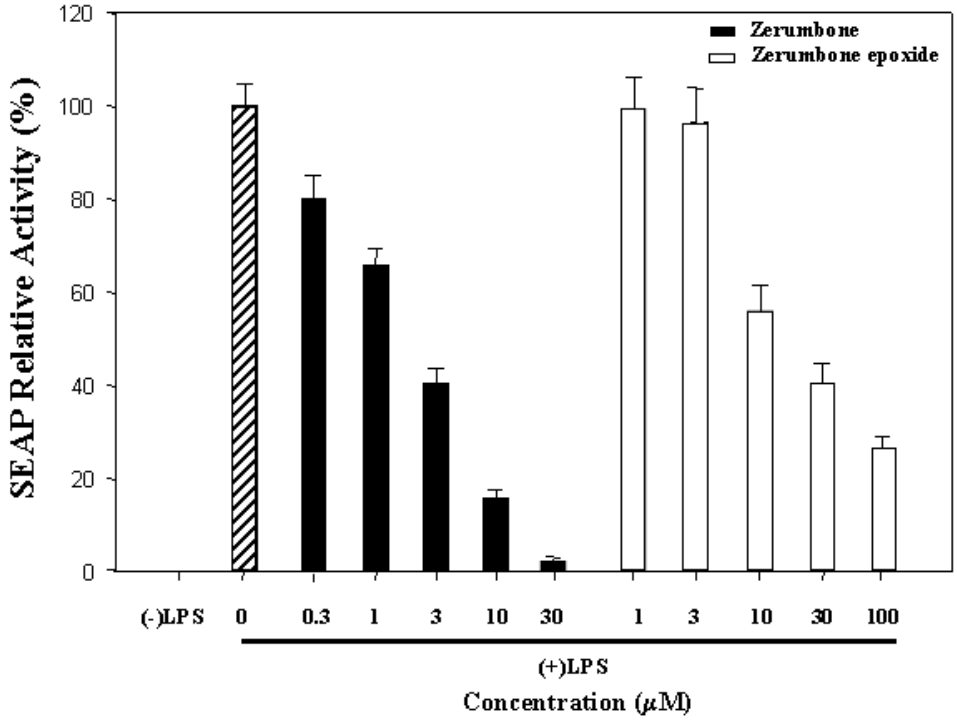


Fig. 2. Effect of zerumbone (**1**) and zerumbone 2,3-epoxide (**2**) on the activation of NF-κB. RAW 264.7 cells transfected with a plasmid containing eight copies of κB elements linked to SEAP gene were treated with the indicated concentrations of 1 and 2 and then stimulated with LPS. SEAP activity in the culture medium was measured as described in Experimental. Data represent mean ± SD of three independent experiments performed in triplicate.

High-output production of nitric oxide (NO) produced by inducible form of nitric oxide synthase (iNOS) is considered as a promoter of tissue injury in inflammation. Since NF-κB is the major transcription factor involved in iNOS gene expression after exposure to various stimuli including LPS, the effect of zerumbone and zerumbone 2,3-epoxide on the NO production was measured in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with zerumbone (0.3–30 μM) and zerumbone 2,3-epoxide (1–100 μM) in the culture medium, followed by stimulation with LPS (1 μg/mL). As a parameter of NO synthesis, nitrite concentration was measured using the supernatant of RAW 264.7 cells by the Griess reaction. Consistent with the NF-κB inhibitory activity, both compounds inhibited nitrite production in a concentration-dependent manner with an IC_{50} of 3.58 μM ± 0.46 for zerumbone and 34.7 μM ± 3.72 for zerumbone 2,3-epoxide (Fig. 3). This is the first report on the NF-κB inhibitory activity of zerumbone 2,3-epoxide. It is noticeable that in both NF-κB activity and NO production assays almost no effect on cell viability was observed at concentrations up to 30 μM of zerumbone and 100 μM of zerumbone 2,3-epoxide in MTT-reduction assay (data not shown).

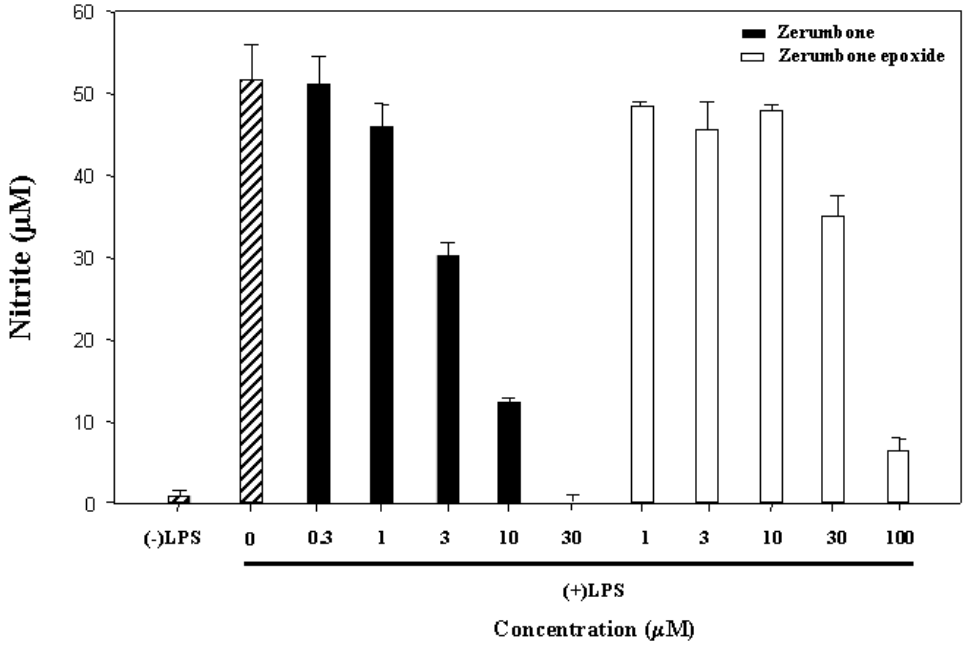


Fig. 3. Effect of zerumbone (**1**) and zerumbone 2,3-epoxide (**2**) on nitrite accumulation in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of 1 and 2 and then stimulated with LPS. NO in the culture medium was measured as described in Experimental. Data represent mean ± SD of three independent experiments performed in triplicate.

The presence of the cross-conjugated dienone is assumed to be the structural requirement for the inhibition of NF-κB activity of zerumbone [5] and its analog zerumbone 2,3-epoxide. However, the inhibitory effects of zerumbone 2,3-epoxide on both NF-κB activation and NO production were markedly weaker than those of zerumbone although the former possesses an additional epoxide moiety, which is considered as an electrophilic site for potential nucleophilic attack. This profound decrease in the inhibitory activity of zerumbone 2,3-epoxide on NF-κB activation and NO production may be associated with the change in the molecular geometry of zerumbone 2,3-epoxide due to the presence of the epoxide ring. A single-crystal X-ray analysis of the structure of zerumbone 2,3-epoxide [12] demonstrated a conformation in which the two methyl groups at C-3 and C-7 were located at one face of the ring while the epoxide oxygen lay on the opposite face, minimizing steric repulsion among these groups. No effective conjugation of the double bond with the carbonyl group can be formed since in comparison with the structure of zerumbone, the C-6/C-7 double bond of zerumbone 2,3-epoxide is significantly distorted from the plane of the dienone [12]. Therefore, C-6 of zerumbone 2,3-epoxide becomes less favorable Michael acceptor than that of zerumbone, resulting in the weaker inhibition of NF-κB activity and NO production.

Experimental

Phytochemicals

Zerumbone (**1**) and zerumbone 2,3-epoxide (**2**) were isolated in pure form (GC analysis >98%) from the rhizomes of *Curcuma zedoaria* (Berg.) Roscoe (Zingiberaceae) growing in

Vietnam as previously reported [10]. The spectral data of **1** (colorless needles, mp 67–68° C) and **2** (colorless needles, mp 96–97° C) including IR, EIMS, ^{1}H NMR, ^{13}C NMR ($CDCl_3$) were identical with the reported values [13].

Cell lines and cell culture

RAW 264.7 cells obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dubelco's modified essential medium (Gibco/BRL, Gaithersburg, MD, USA) supplemented with penicillin (100 units/mL) – streptomycin (100 µg/mL) – L-glutamine (292 ng/mL) (Gibco/BRL, Gaithersburg, MD, USA) and 10% heat-inactivated fetal bovine serum (Gibco/BRL, MD, USA). Cells were incubated at 37° C in a humidified atmosphere containing 5% CO_2. RAW 264.7 cells transfected with NF-κB reporter construct were cultured in the same medium as RAW 264.7 cells, except for adding G418 (500 µg/mL) during maintenance.

NF-κB inhibitory activity assay

RAW 264.7 cells were transfected with a plasmid containing eight copies of κB elements linked to SEAP (secreted alkaline phosphatase) gene. Transfected cells were seeded in a 96-well plate at a density of 5×10^4 cells/well. After 3 h incubation at 37° C, the cells were treated with various concentrations of compounds tested and LPS (1 µg/mL) for 24 h. Then 100 µL of each culture supernatant was transferred to a new 96-well plate and heated at 65° C for 5 min. An additional 100 µL of 2 × SEAP assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, 20 mM L-homoarginine) was added to each well and incubated at 37° C for 10 min. The reaction was initiated by the addition of 20 µL of 120 mM p-nitrophenyl phosphate dissolved in 1 × SEAP assay buffer and incubated at 37° C. The absorbance of the reaction mixture was measured at 405 nm with a microplate reader (Molecular Devices Co., Menlo Park, CA, USA). Data represent mean ± SD of three independent experiments performed in triplicate.

Cell viability assay

Cytotoxicity of compounds was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO, USA)]-based colorimetric assay. Then 10 µL of MTT solution (5 mg/µL) was added to the remaining cells after SEAP assays. After incubation for 3 h at 37° C, the formed formazan crystals in viable cells were solubilized with 100 µL of DMSO and the absorbance of each well was read at 570 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA, USA). Cell numbers were obtained by standard curve.

Determination of NO production

RAW 264.7 cells were transferred in 96-well plates at a density of 1×10^5 cells/well. After 3 h incubation at 37° C, the cells were stimulated with LPS (1 µg/mL) for 24 h in the absence or presence of the compound tested. As a parameter of NO synthesis, nitrite concentration was measured in the supernatant of RAW 264.7 cells by the Griess reaction. Briefly, 100 µL of cell culture supernatant was reacted with 100 µL of Griess reagent (1:1 mixture of 0.1% N-(1-naphthyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid) in a 96-well plate, and absorbance was read with a microplate reader at 570 nm. The nitrite concentration in the supernatants was calculated by comparison with a

sodium nitrite standard curve. Data represent mean ± SD of three independent experiments performed in triplicate.

Acknowledgements

This study was supported by the Korea Science & Engineering Foundation (Seoul, Korea), International Foundation for Science (Stockholm, Sweden), and Vietnam National University, Hanoi (Hanoi, Vietnam).

Authors' Statement

Competing Interests

The authors declare no conflict of interest.

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