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# Inhibition of NF-κB-Dependent Cytokine and Inducible Nitric Oxide Synthesis by the Macrocyclic Ellagitannin Oenothein B in TLR-Stimulated RAW 264.7 Macrophages

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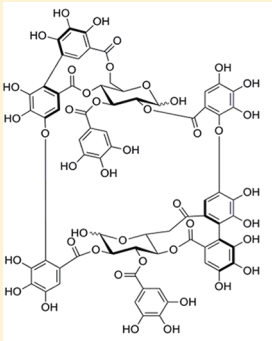
Supporting Information

**ABSTRACT:** Immunomodulatory effects of oenothein B (**1**), a macrocyclic ellagitannin from various Onagraceae species, have been described previously. However, the mechanisms underlying the anti-inflammatory activity of **1** have not been fully clarified. The effects of **1** were investigated on inducible nitric oxide synthase, TLR-dependent and TLR-independent signal transduction cascades, and cytokine expression using murine macrophages (RAW 264.7). Compound **1** (10−60 μg/mL) reduced NO production, iNOS mRNA, and iNOS protein levels in a dose-dependent manner, without inhibition of iNOS enzymatic activity. It reduced the binding of the NF-κB p50 subunit to the biotinylated-consensus sequence and decreased nuclear p65 translocation. Gallic acid as a subunit of the macrocyclic ellagitannin **1** showed a far lower inhibitory activity. Nitric oxide production was reduced by **1** after stimulation using TLR2 (Pam2CSK4) and TLR4 (Kdo2) agonists, but this compound did not inhibit inducible nitric oxide synthesis after stimulation using interferon-gamma. IL-1beta, IL-6, and TNF-alpha mRNA synthesis was clearly reduced by the addition of **1**. Oenothein B (**1**) inhibits iNOS after stimulation with LPS, TLR2, and TLR4 agonists via inhibition of TLR/NF-κB-dependent inducible nitric oxide and cytokine synthesis independent from IFN-gamma/JAK/STAT pathways. The full molecular structure of this macrocyclic ellagitannin seems to be required for its immunomodulatory actions.



**1**

Oenothein B (**1**) is a dimeric macrocyclic ellagitannin that has been isolated from several species of the Onagraceae, a widespread family of flowering plants, including evening primroses (*Oenothera* spp.) and common weeds such as the willow herbs (*Epilobium* spp.). *Oenothera* and *Epilobium* species have been used widely for various medicinal purposes. *Oenothera* spp. as well as *Epilobium* spp. contain polyphenolic compounds such as flavonoids and hydrolyzable tannins, including the dimeric macrocyclic ellagitannin **1**.[1−3] Compound **1** has been shown to be a constituent of extracts from *Oenothera biennis* L. defatted seeds,[4] and anti-inflammatory effects of **1** similar to the anti-inflammatory drug indomethacin have been described.[5] Numerous studies have been conducted to demonstrate the antiproliferative effect of **1**.[6−9] It shows inhibition of 5-alpha-reductase and aromatase, two enzymes involved in the etiology of benign prostatic hyperplasia.[10,11] Recently, immunomodulatory effects of **1** have been described,[12] although the mechanisms underlying the anti-inflammatory activity of oenothein B have not been fully clarified.

Nitric oxide (NO) is synthesized from L-arginine by the L-arginine−nitric oxide pathway.[13] A family of enzymes, termed the nitric oxide synthases (NOS), catalyze the formation of nitric oxide and citrulline from L-arginine, $O_2$, and NADPH.[14] The inducible isoform of NOS (NOS-2 or iNOS) generates large amounts of NO over a prolonged period of time through a $Ca^{2+}$-independent pathway.[15] iNOS is induced by lipopolysaccharide (LPS) or the combination of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-alpha), interleukin-1beta (IL-1beta), and interferon-gamma (IFN-gamma).[16] Inducible NOS expression has been observed in many cells, including murine macrophages.[17] Human iNOS is most readily observed in monocytes or macrophages from patients with infectious or inflammatory diseases, and the sustained production of NO endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminths, and tumor cells.[18]

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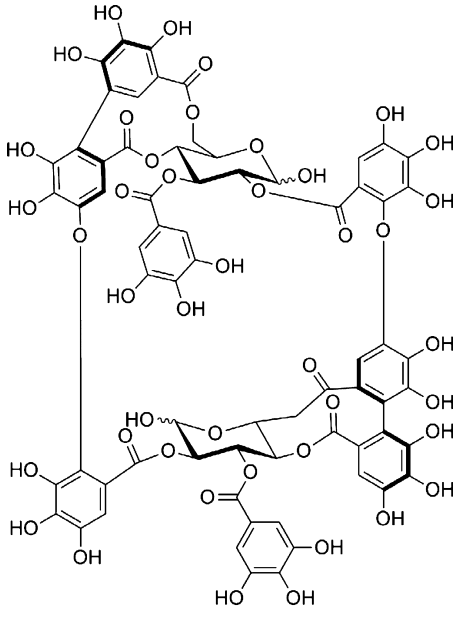
LPS initiates inducible nitric oxide synthesis through binding of specific molecular patterns (pathogen-associated molecular patterns, PAMPs) to Toll-like receptors (TLRs). LPS binding to its main receptor TLR4 is mediated by LBP (LPS-binding protein), CD14, and the small extracellular protein MD2 (lymphocyte antigen 96), a molecule that acts presumably by stabilizing TLR4 dimers and shows a highly homologous sequence to MD1 (lymphocyte antigen 86), both resembling leucine-rich repeat (LRR) molecules, which have been described in diverse organisms mediating innate defense mechanisms against pathogens.[19] The LPS-initiated signaling cascade leads to stimulation of myeloid differentiation primary response gene 88 (MyD88)-dependent pathways involving IL-1R-associated kinase (IRAK), Toll receptor IL-1R domain containing adapter protein (TIRAP), and TNF-alpha receptor-associated factor-6 (TRAF-6). Subsequently, this signal transduction cascade activates nuclear factor kappa-B (NF-κB), mitogen-activated protein kinase, and phosphatidylinositol 3-kinase/Akt pathways, with concomitant nuclear translocation of transcription factors such as the p65/p50 subunits of NF-κB, AP-1 (activating protein-1), and STAT1-alpha (signal transducers and activators of transcription).[20] On the other hand, iNOS transcription is increased by interferon-gamma via the IFN-gamma/JAK (Janus kinase)/STAT pathways.

The aim of this study was to investigate the effects of the macrocyclic ellagitannin oenothein B (**1**) on inducible nitric oxide production, iNOS expression, and iNOS activity, using murine macrophages (RAW 264.7). The actions of **1** on signal transduction cascades as a consequence of LPS, IFN-gamma, and TNF-alpha stimulation, TLR activation, and the influence on NF-κB-dependent cytokine expression (IL-1beta, IL-6, TNF-alpha) were characterized.



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## RESULTS AND DISCUSSION

In order to estimate the influence of **1** on inducible nitric oxide synthesis, murine macrophages were stimulated with LPS and nitrite was analyzed using the Griess reaction. Stimulated RAW 264.7 cells released large amounts of nitrite into the culture medium (116.9 ± 6.2 nmol of nitrite per mg of protein within 24 h versus 5.7 ± 0.6 nmol of nitrite per mg of protein for unstimulated control incubations). Incubation of activated RAW 264.7 cells with increasing amounts of **1** (20−60 μg/mL, 12.8−38.3 μM) was associated with a concentration-dependent reduction of inducible NO production (Figure 1). The extent of inhibition (percent inhibition) was 34.8 ± 0.9% for 20 μg/mL (12.8 μM), 64.6 ± 2.5% for 30 μg/mL (19.1 μM), and 87.9 ± 2.5% for 60 μg/mL (38.3 μM) of **1**. iNOS inhibition by **1** was significant statistically at all concentrations tested. The $IC_{50}$ value for **1** derived from dose−response curves was calculated to be 27.7 μg/mL (17.7 μM). The molecular structure of **1** isolated from *Epilobium* species was confirmed by $^{1}H$ and $^{13}C$ NMR spectroscopy in comparison with data reported in the literature.

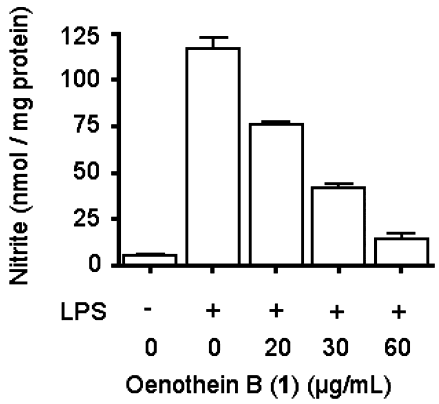


**Figure 1.** Inhibition of inducible nitric oxide production by oenothein B (**1**). LPS-stimulated cells were incubated with increasing amounts of **1** (20−60 μg/mL, 12.8−38.3 μM). After 24 h the culture medium was collected and assayed for nitrite per mg of cell protein. Data show the mean of triplicate measurements and are representative of three independent experiments. Error bars show the standard deviation.

To determine the effect of **1** on iNOS enzyme activity, cytosolic enzyme activity was measured. Enzymatic activity assays with **1** (60 μg/mL final concentration) showed no significant inhibition of iNOS enzyme activity compared to incubations without **1**. In contrast, 300 μM of the nitric oxide synthase inhibitor L-NAME (NG-nitro-L-arginine methyl ester) or the selective iNOS inhibitor W1400 (*N*-[[3-(aminomethyl)-phenyl]methyl]ethanimidamide, 50 μg/mL) reduced iNOS activity by more than 70% (data not shown).

To exclude the possibility that **1** interferes with the detection of nitrite by the Griess reaction or influences the stability of nitric oxide, compound **1** (0−60 μg/mL final concentration) was incubated with the nitric oxide donor linsidomine (2 mM) for 4 h at room temperature. Compound **1** had no statistically significant effect on the nitrite values measured. It was concluded that **1** does not scavenge NO or interfere with the detection of nitrite by the Griess reaction.

To exclude any cytotoxic response to **1** during the course of the experimental procedures, both trypan blue exclusion and MTT tests were applied, confirming the integrity of the cellular membranes and the intact mitochondrial respiration. The addition of **1** (20−60 μg/mL) did not show any reduction of membrane integrity and cellular metabolism/mitochondrial respiration (data not shown).

RAW 264.7 cells were stimulated using the TLR4 agonist Kdo2 (di[3-deoxy-D-manno-octulosonyl]-lipid A, 500 ng/mL)

and incubated with increasing amounts of **1** (30−60 μg/mL) for 24 h. The addition of **1** showed a dose-dependent inhibition of inducible nitric oxide synthesis (Figure 2A). Furthermore,

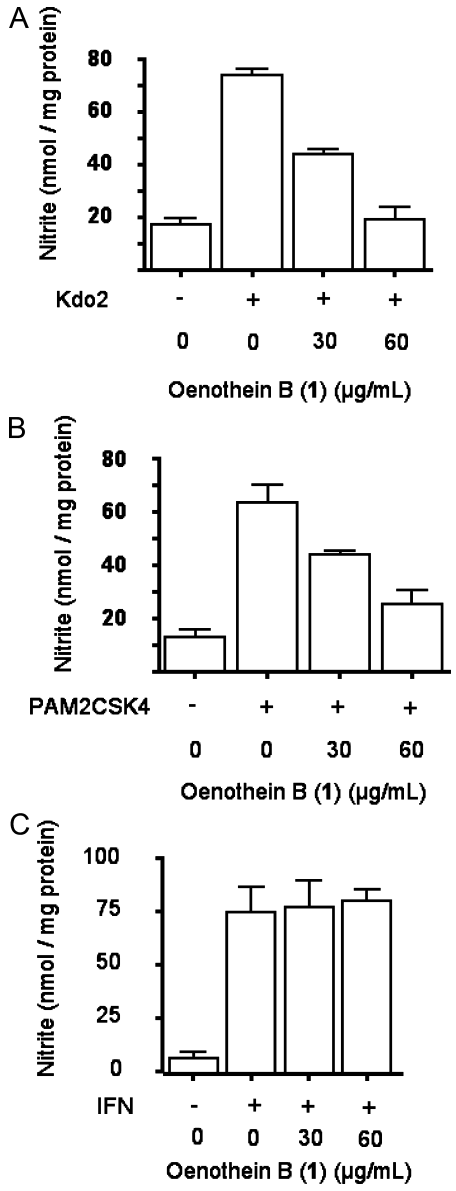


**Figure 2.** Inhibition of inducible nitric oxide synthesis by oenothein B (**1**) after stimulation with Toll-like receptor (TLR) agonists. RAW 264.7 cells were stimulated using Toll-like receptor-4 (TLR4) agonist Kdo2 (500 ng/mL, A), Toll-like receptor-2 (TLR2) agonist Pam2CSK4 (500 ng/mL, B), or IFN-gamma (100 U/mL, C) for 24 h. The addition of **1** showed a dose-dependent inhibition of inducible nitric oxide synthesis after stimulation of the TLR/NF-κB pathway.

RAW 264.7 cells were stimulated using the TLR2 agonist Pam2CSK4 (*S*-[2,3-bis(palmitoyloxy)-(2*R*,*S*)-propyl]-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysyl-(*S*)-lysine; 500 ng/mL) and incubated with increasing amounts of **1** (30−60 μg/mL) for 48 h. The addition of **1** was associated with a dose-dependent inhibition of inducible nitric oxide synthesis, as shown in Figure 2B. Compound **1** did not inhibit inducible nitric oxide synthesis after stimulation of the JAK-STAT pathway using interferon-gamma (100 U/mL, Figure 2C).

To test whether the entire molecule of **1** is required for iNOS inhibition, the effect of a molecular subunit (gallic acid) was tested. Incubation of LPS-stimulated RAW 264.7 cells with high concentrations of gallic acid (100−600 μM) was associated with a dose-dependent reduction of inducible NO production. The extent of inhibition (percent inhibition) was 10.7 ± 8.4% for 100 μM gallic acid, 24.7 ± 7.2% for 200 μM gallic acid, 32.4 ± 7.3% for 400 μM gallic acid, and 45.9 ± 3.4% for 600 μM gallic acid (data not shown). The $IC_{50}$ value for gallic acid derived from dose−response curves was calculated to be 631.6 μM and may be compared with that of **1** (17.7 μM). This provides evidence that biological actions of oenothein B (**1**) require its full molecular structure.

Figure 3 shows the Western blot analysis of inducible nitric oxide synthase expression in RAW 264.7 macrophages. Cells

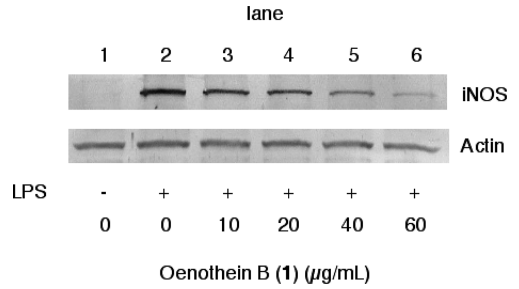


**Figure 3.** Western blotting analysis of iNOS protein expression. RAW 264.7 mouse macrophages were incubated with LPS and oenothein B (**1**) for 24 h, and Western blotting was performed as described in the Experimental Section. Lane 1: unstimulated control. Lane 2 (LPS-stimulated RAW 264.7 cells) shows a band with an estimated molecular mass of 130 kD (the known molecular mass of iNOS); lanes 3−6 (10−60 μg/mL compound **1**) show decreasing iNOS protein expression in a concentration-dependent manner. Beta-actin (43 kD) levels remained unchanged. Data shown are representative for three independent experiments.

were stimulated with lipopolysaccharide and incubated with increasing amounts of **1** (10−60 μg/mL) for 24 h. Immunoblotting showed a band with the estimated molecular mass of 130 kD (the known molecular mass of iNOS) in stimulated cells. An identical molecular mass was found by blotting against purified iNOS protein (not shown). Incubation with increasing amounts of **1** resulted in a dose-dependent decrease of iNOS protein expression. The beta-actin (43 kD) protein level remained unchanged. This shows that incubation with oenothein B is not associated with generalized reduction of protein expression. Figure 4 shows the semiquantitative competitive RT-PCR analysis of iNOS mRNA in RAW 264.7 cells. LPS-stimulated macrophages were incubated with increasing amounts of **1** (20−60 μg/mL) for 24 h. Analysis of iNOS mRNA levels showed a concentration-dependent reduction by **1** while actin mRNA levels remained unchanged. Thus, **1** did not cause toxic effects or a generalized decrease in mRNA transcription at the concentrations tested.

Figure 5 shows the real-time RT-PCR analysis of IL-1beta (Figure 5A), IL-6 (Figure 5B), and TNF-alpha (Figure 5C) mRNA isolated from RAW 264.7 cells. MNE (mean normalized expression) values were calculated. IL-1beta, IL-6, and TNF-alpha were reduced by the addition of **1**. To test the influence of **1** on binding of the NF-κB p50 subunit to the biotinylated-consensus sequence, RAW 264.7 cells ($1 \times 10^6$) were seeded in six-well plates. LPS and increasing amounts of **1**

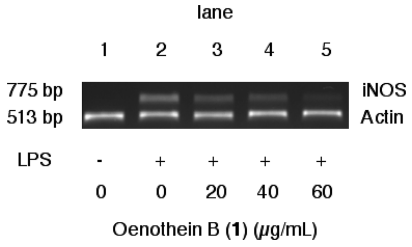


**Figure 4.** Effects of oenothein B (**1**) on iNOS mRNA. RAW 264.7 mouse macrophages were incubated with LPS and increasing amounts of **1** (20−60 μg/mL) for 24 h and showed dose-dependent inhibition of iNOS mRNA (775 bp) expression. Levels of beta-actin mRNA (513 bp) remained unchanged. One representative experiment out of three is shown.

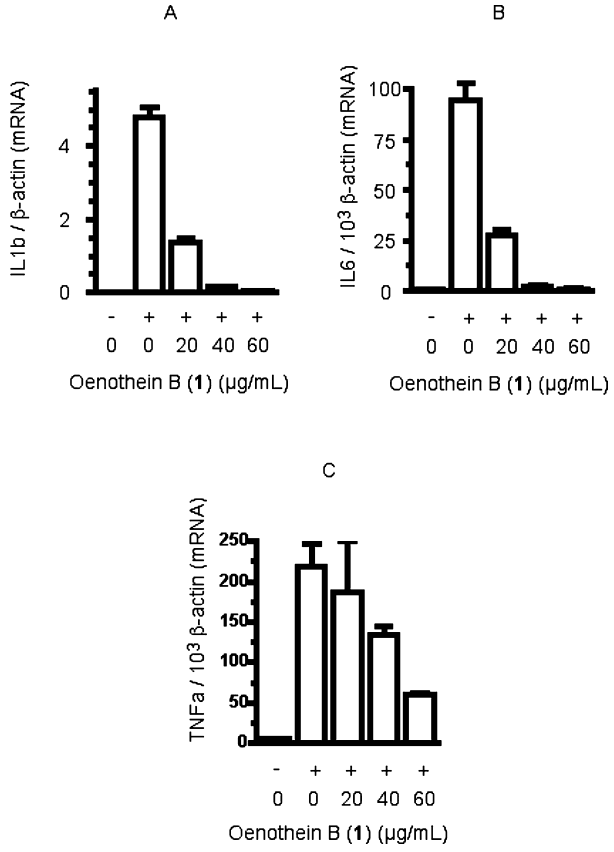


**Figure 5.** Real-time RT-PCR analysis. Effects of oenothein B (**1**) on mRNA expression of IL-1beta (A), IL-6 (B), and TNF-alpha (C) from RAW 264.7 cells. MNE (mean normalized expression) values were calculated. IL-6 and TNF-alpha mRNA transcription levels were normalized to 1000 copies of beta-actin. IL-1beta, IL-6, and TNF-alpha were clearly reduced by the addition of **1**.

were added simultaneously to the culture medium for 1 h. Nuclear extracts were prepared, and NF-κB p50 binding activity was determined. Compound **1** (30, 60 μg/mL) reduced the binding of the NF-κB p50 subunit from nuclear extracts to the biotinylated-consensus sequence (Figure 6). Figure 7 shows the Western blot analysis against NF-κB (p65) subunit from nuclear extracts of RAW 264.7 cells. Immunoblotting against the NF-κB (p65) showed a band with an estimated molecular mass of 65 kD in nuclear extracts from stimulated RAW 264.7 mouse macrophages (lane 2). Nuclear extracts from unstimulated cells show a decreased translocation of NF-κB (p65, lane

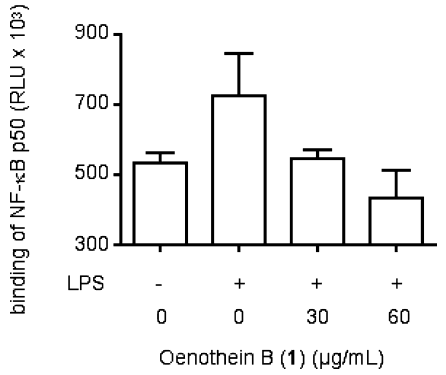


**Figure 6.** Effect of oenothein B (**1**) on NF-κB p50. RAW 264.7 cells ($1 \times 10^6$) were seeded in six-well plates. LPS and increasing amounts of **1** were added simultaneously to the culture medium for 1 h. Nuclear extracts were prepared, and NF-κB p50 binding activity was determined and expressed as relative light units (RLU).

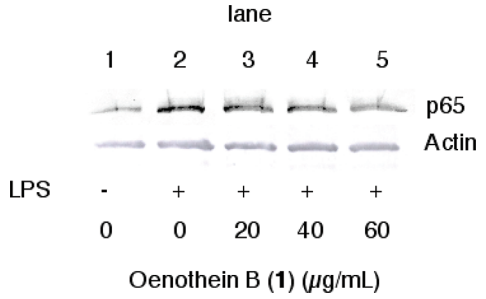


**Figure 7.** Western blot analysis against the NF-κB (p65) subunit. Macrophages were incubated with increasing amounts of **1** (20−60 μg/mL) for 30 min followed by LPS stimulation for 20 min. Nuclear extracts were prepared and Western blotting was performed. Incubation with increasing amounts of **1** resulted in a dose-dependent decrease of nuclear NF-κB (p65) protein (lanes 3−5). Data shown are representative for three independent experiments.

1). Incubation with increasing amounts of **1** resulted in a dose-dependent decrease of nuclear NF-κB (p65) protein (lanes 3−5). Similar inhibition of NF-κB (p65) nuclear translocation was found after NF-κB stimulation using TNF-alpha (50 ng/mL, data not shown).

Oenothein B (**1**) is a dimeric macrocyclic ellagitannin from *Oenothera* and *Epilobium* spp.[1−3] as well as *Eucalyptus* leaf extracts.[21] Ellagitannins are phenolic phytochemicals with well-known antioxidant and free-radical-scavenging properties. Their effects in preventing cardiovascular diseases have been studied previously. In vitro studies show that ellagitannins, at concentrations in the range 10−100 μM, show anti-inflammatory, antiatherogenic, antithrombotic, and antiangiogenic effects.[22] Compound **1** has been shown to be a constituent of extracts from *Oenothera biennis* defatted seeds.[4] Recently, immunomodulatory effects of **1** have been described,[12] although the underlying mechanisms have not yet been fully clarified. Previously reported effects of ellagitannins on NF-κB activation or inhibition remain to be confirmed and could be attributed to cell and species differences. In accordance with the present results, a statistically significant inhibition of inducible nitric oxide synthesis has been described previously by the addition of 20 μg/mL of compound **1** to RAW 264.7 macrophages[23] but without investigating the effects of higher amounts of this ellagitannin. The addition of 60 μg/mL **1** during the current experimental procedures almost


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completely abolished inducible NO synthesis. In addition, the inhibition of p65 nuclear translocation and decreased NF-κB binding activity by **1** was confirmed by Chen et al.[23] and is in agreement with our results. Further anti-inflammatory effects of **1** have been published. Thus, oenothein B (**1**) acts as a specific COX-1 inhibitor and inhibits MPO release from stimulated neutrophil leucocytes, analogous to the anti-inflammatory drug indomethacin.[5]

Clear data for the bioavailability of **1** are still missing. The inhibition of HIV and herpes virus replication by serum taken from mice after peroral treatment with **1** is suggestive that this ellagitannin may be bioavailable.[24] On the other hand, the size of the molecule suggests an intravenous route of application in case of any therapeutic application under clinical conditions. Available information about the metabolism of ellagitannins is sparse. The conversion of ellagitannins by the microflora of the gut to low-molecular-weight compounds that are well absorbed is currently under investigation.

In conclusion, the present data show an inhibition of TLR/NF-κB-dependent inducible nitric oxide and cytokine synthesis by oenothein B (**1**) independent from the IFN-gamma/JAK/STAT pathways, but requiring the full molecular structure of this macrocyclic ellagitannin for its biological effects, such as inhibition of NF-κB p65 and p50 activation.

## EXPERIMENTAL SECTION

**General Experimental Procedures.** Rabbit anti-iNOS polyclonal antibody was obtained from Alpha Diagnostic, San Antonio, TX, USA. Purified iNOS protein was supplied by Calbiochem, San Diego, CA, USA. Anti-rabbit IgG, BCIP, NBT, and MMLV-RT were obtained from Promega, Madison, WI, USA. Ellagic acid was purchased from ChromaDex (Santa Ana, CA, USA), and caffeic acid from Fluka (Steinheim, Germany). All solvents were HPLC grade. Cell culture materials were supplied by Greiner, Kremsmuenster, Austria. Recombinant mouse tumor necrosis factor-alpha (TNF-alpha) was purchased from eBioscience (San Diego, CA, USA), TLR4 agonist Kdo2-Lipid A was from Avanti Polar Lipids (Alabaster, AL, USA), TLR2 agonist Pam2CSK4 from InvivoGen (San Diego, CA, USA), and *Escherichia coli* lipopolysaccharide serotype 055:B5 (LPS) and all other chemicals were supplied by Sigma Chemical Co. (St. Louis, MO, USA). The isolation of oenothein B (**1**) was performed as described previously,[25] and a brief description is given in the Supporting Information. The structure of **1** was confirmed by $^{1}$H and $^{13}$C NMR spectroscopy. The purity of oenothein B (**1**) was over 95%, as shown by HPLC-DAD analysis.

**Analytical Procedures.** Preparation of nuclear extracts, binding of the NF-κB p50 subunit to biotinylated-consensus sequence, Western blotting, determination of cell viability, enzymatic activity assays, cell culture, and nitrite analysis were performed as described earlier.[26] The quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) used has been delineated previously.[27] For the qRT-PCR experiments, intron− or exon−exon boundary spanning custom-synthesized primer pairs (VBC-Biotech, Vienna, Austria) were either taken from the literature (when indicated) or self-designed. They were targeted against the sequences of murine IL-1beta (sense: 5′-TGTCTGAAGCAGCTATGGCAAC-3′, exon 2, antisense: 5′-CTGCCTGAAGCTCTTGTTGATG-3′, exon 4),[28] murine IL-6 (sense: 5′-TCTTGGGACTGATGCTGGTG-3′, exon 2, antisense: 5′-CAGAATTGCCATTGCACAACTC-3′, exon 2−3),[28] murine TNF-alpha (sense: 5′-CACGTCGTAGCAAACCACC-3′, exon 3−4, antisense: 5′-AGATAGCAAATCGGCTGACG-3′, exon 4), and the housekeeping gene beta-actin (ACTB) (sense: 5′-ATGGTGGGAATGGGTCAGAAG-3′, exon 3, antisense: 5′-TCTCCATGTCGTCCCAGTTG-3′, exon 3).[29]

**Data Analysis.** Statistical analyses were performed by use of ANOVA followed by posthoc analysis of unpaired data (Bonferroni correction for multiple comparisons). Statistical significance was defined as $p < 0.05$. When the SD is not displayed, it was smaller than the size of the symbol.

## ASSOCIATED CONTENT

### Supporting Information

Plant material, isolation procedures for oenothein B (**1**), and $^{1}$H NMR and $^{13}$C NMR data of oenothein B. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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