Introduction

Bacterial infection is one of the most important threats to human health. Bacteria can enter the body through damaged skin and mucous membranes, triggering the specific defense responses of the immune system against the bacteria. Those defense systems are essential to ensure human survival, but severe systemic infections such as sepsis can induce an excessive or uncontrolled immune response that can lead to the Systemic Inflammatory Response Syndrome (SIRS), which is a life-threatening condition due to the occurrence of Multiple Organ Dysfunction Syndrome (MODS) [1,2].

Lipopolysaccharides (LPS) are the main pathogenic component of the outer membrane of Gram-negative bacteria and are the key to the host defense response to Gram-negative bacterial infection [3]. LPS action on membrane CD14 receptor on monocytes and macrophages requires the involvement of the LPS-Binding Protein (LBP), Toll-Like Receptor 4 (TLR4), and MD-2 to accomplish signal transduction [4-6]. Nevertheless, studies found that the immune cells do not respond or only weakly respond to LPS if only TLR4 is present, indicating that TLR4 must be assisted by MD-2 in order to recognize LPS [7]. In addition, blocking the combination of LPS with MD-2 is easier than blocking the combination of LPS with TLR4/MD-2 [8]. Therefore, how to develop a drug that can interfere with the combination of LPS with MD-2, or inhibit MD-2 binding to TLR4 may be an effective treatment strategy to control the inflammatory and immune over-responses caused by LPS.

Natural products and Traditional Chinese Medicine (TCM) have great structural and chemical diversity and are good sources of active compounds for drug discovery [9,10]. Cortex phellodendri is a plant used in TCM and has wide pharmacological activities including antioxidant [14], anti-dengue [15]. Studies only reported on the antioxidant [16], anti-inflammatory mechanism of its anti-inflammatory activity. Niloticin’s drug potential was analyzed using the Traditional Chinese Medicine Systems Pharmacology Database. Molecular docking and biolayer interferometry technology were used to explore the anti-inflammatory mechanism of niloticin by targeting myeloid differentiation protein 2 (MD-2), which mediates a series of Toll-Like Receptor (TLR) 4-dependent inflammatory responses. The cytokines involved in the LPS-TLR4/MD-2-NF-xB pathway were evaluated by ELISA, RT-PCR, and western blot. The results showed that niloticin has drug potential and could bind to MD-2. Niloticin had no impact on cell viability. Niloticin could significantly decrease the levels of NO, IL-6, TNF-α, and IL-1β (P<0.01) induced by LPS. IL-6, IL-1β, iNOS, TNF-α, and COX-2 mRNA expression levels were decreased by niloticin (all P<0.01). Compared with the control group, TLR4, p65, MyD88, p-p65, iNOS, TNF-α, and COX-2 mRNA expression levels induced by LPS were suppressed by niloticin (all P<0.01). In conclusion, niloticin is a potential MD-2 antagonist. It might interact with MD-2 to play an anti-inflammatory role by suppressing the activation of the LPS-TLR4/MD-2-NF-xB signaling pathway.

Keywords: Niloticin; Drug design; Anti-inflammation; Myeloid differentiation protein 2; Toll-like receptor 4; Lipopolysaccharides

Abstract

Niloticin is an active compound from Cortex phellodendri, but its anti-inflammatory activity has not yet been explored. The aim of the present study was to assess the drug potential of niloticin and to study the MD-2-targeting mechanism of its anti-inflammatory activity. Niloticin’s drug potential was analyzed using the Traditional Chinese Medicine Systems Pharmacology Database. Molecular docking and biolayer interferometry technology were used to explore the anti-inflammatory mechanism of niloticin by targeting myeloid differentiation protein 2 (MD-2), which mediates a series of Toll-Like Receptor (TLR) 4-dependent inflammatory responses. The cytokines involved in the LPS-TLR4/MD-2-NF-xB pathway were evaluated by ELISA, RT-PCR, and western blot. The results showed that niloticin has drug potential and could bind to MD-2. Niloticin had no impact on cell viability. Niloticin could significantly decrease the levels of NO, IL-6, TNF-α, and IL-1β (P<0.01) induced by LPS. IL-6, IL-1β, iNOS, TNF-α, and COX-2 mRNA expression levels were decreased by niloticin (all P<0.01). Compared with the control group, TLR4, p65, MyD88, p-p65, iNOS, TNF-α, and COX-2 mRNA expression levels induced by LPS were suppressed by niloticin (all P<0.01). In conclusion, niloticin is a potential MD-2 antagonist. It might interact with MD-2 to play an anti-inflammatory role by suppressing the activation of the LPS-TLR4/MD-2-NF-xB signaling pathway.

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Study on Anti-Inflammatory Activity of Niloticin by Targeting MD-2 Based on Computer-Aided Drug Design and Biolayer Interferometry

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Abstract

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Keywords: Niloticin; Drug design; Anti-inflammation; Myeloid differentiation protein 2; Toll-like receptor 4; Lipopolysaccharides

Abbreviations

MD-2: Myeloid Differentiation Protein 2; SIRS: Systemic Inflammatory Response Syndrome; MODS: Multiple Organ Dysfunction Syndrome; LPS: Lipopolysaccharides; LBP: LPS-Binding Protein; TLR4: Toll-Like Receptor 4; TCM: Traditional Chinese Medicine; OB: Oral Bioavailability; DL: Drug-Likeness; ADME: Absorption, Distribution, Metabolism, and Excretion; TCMSP: Traditional Chinese Medicine Systems Pharmacology Database; MTT: Methylthiazolyterazolium; NO: Nitric Oxide; TNF: Tumor Necrosis Factor; IL: Interleukin; iNOS: inducible NO Synthase; COX: Cyclooxygenase; MW: Molecular Weight; AlogP: Partition Coefficient between Octanol and Water; Hdon: H-Bond Donors; Hacc: H-Bond Acceptors; RBN: Rotatable Bonds Number

Introduction

Bacterial infection is one of the most important threats to human health. Bacteria can enter the body through damaged skin and mucous membranes, triggering the specific defense responses of the immune system against the bacteria. Those defense systems are essential to ensure human survival, but severe systemic infections such as sepsis can induce an excessive or uncontrolled immune response that can lead to the Systemic Inflammatory Response Syndrome (SIRS), which is a life-threatening condition due to the occurrence of Multiple Organ Dysfunction Syndrome (MODS) [1,2].

Lipopolysaccharides (LPS) are the main pathogenic component of the outer membrane of Gram-negative bacteria and are the key to the host defense response to Gram-negative bacterial infection [3]. LPS action on membrane CD14 receptor on monocytes and macrophages requires the involvement of the LPS-Binding Protein (LBP), Toll-Like Receptor 4 (TLR4), and MD-2 to accomplish signal transduction [4-6]. Nevertheless, studies found that the immune cells do not respond or only weakly respond to LPS if only TLR4 is present, indicating that TLR4 must be assisted by MD-2 in order to recognize LPS [7]. In addition, blocking the combination of LPS with MD-2 is easier than blocking the combination of LPS with TLR4/MD-2 [8]. Therefore, how to develop a drug that can interfere with the combination of LPS with MD-2, or inhibit MD-2 binding to TLR4 may be an effective treatment strategy to control the inflammatory and immune over-responses caused by LPS.

Natural products and Traditional Chinese Medicine (TCM) have great structural and chemical diversity and are good sources of active compounds for drug discovery [9,10]. Cortex phellodendri is a plant used in TCM and has wide pharmacological activities including anti-inflammation [11], anti-apoptosis [12], anti-tumor [13], and anti-oxidation [14]. Niloticin (Figure 1A) is one of the active compounds found in Cortex phellodendri. Niloticin is a compound with good drug potential, high Oral Bioavailability (OB), and Drug-Likeness (DL) [15]. Studies only reported on the antioxidant [16], anti-respiratory syncytial virus [17], and anti-dengue [15] activities of...
niloticin, but its anti-inflammatory activity was not studied.

Thus, the aim of the present study was to assess the drug potential of niloticin and to study the MD-2-targeting mechanism of its anti-inflammatory activity. The results of the present study could lay the foundation for the development of niloticin as a new drug against SIRS.

Materials and Methods

Cells

The RAW 264.7 cells (#CL-0190) were obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and were cultured in RPMI 1640 medium (including 10% fetal bovine serum, 100 mg/L penicillin, and 100 U/ml streptomycin; GIBCO, Invitrogen Inc., Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO2 [18]. The sub-cultures were carried out when the cells reached 80-90% confluence.

Drug potential assessment

The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (http://lsp.nwu.edu.cn/tcmsp.php) can provide information on the Absorption, Distribution, Metabolism, and Excretion (ADME), Oral Bioavailability (OB), and Drug-Likeness (DL), and Lipinski’s rule of five for drug screening and evaluation [19] of a compound with potential biological effects [20,21]. In the present study, “niloticin” was entered and investigated.

Docking of niloticin to MD-2 protein

Niloticin was constructed in ChemBio3D Ultra 13.0 and minimized using the MMFF94x force field. The crystal structure of MD-2 (PDB code: 2Z65.pdb) was downloaded from the protein databank (www.rcsb.org). The protein structure was preprocessed with Autodock Tool (ADT) [22], including the addition of polar hydrogen atoms and the assignment of Kollman united partial charge. The docking calculation was done with Autodock Vina using the default parameters unless otherwise stated [23]. The center of the grid box (30×30×30 Å) was set on (23.36 Å, -6.98 Å, -3.97 Å) by referring to the position of the ligand co-crystallized MD-2. The conformation with the lowest binding energy was chosen as the final result. The binding mode was analyzed and visualized with ADT [22].

Bio-layer interferometry assay

The bio-layer interferometry assay was carried out by using an Octet K2 system (Pall ForteBio Corp., Menlo Park, CA). MD-2 (10µg/mL) was immobilized on NTA biosensors. The control was a set of sensors without protein. Niloticin was diluted into solutions in a buffer containing 10mM PBS pH 7.5, 0.5% Tween 20, and 0.5% DMSO, at concentrations of 3000, 1500, 750, 375, and 187.5 µM. A baseline step of 60 s, an association step of 45s, and a dissociation step of 60 s were acquired using the protein-loaded and the control sensors for each point of the concentration series. Data were processed to remove drift and well-to-well artifacts by using the ForteBio Data Analysis Software 10.0 (Molecular Devices, LLC, San Jose, CA, USA).

MTT assay for viability

The Methylthiazolyterazolium (MTT) assay was used to evaluate cell viability [24]. Cells (5×10^3 cells/well) were plated in a 96-well plate and acclimatized overnight at 37°C in an atmosphere with 5% CO2. Then, the adherent cells were treated with niloticin (8, 40, and 100 µg/mL; six wells per concentration per group) and co-incubated for 24 h. Then, MTT was added at 10:1 v/v (Sigma, St Louis, MO, USA) and incubated for 4h. The supernatant was discarded, and formazan crystals were dissolved by adding 150µL of DMSO. The OD value was measured at 570 nm using a microplate reader (Tecan Group Ltd., Mannedorf, Switzerland).

Cytokine measurement

RAW 264.7 cells (5×10^4 cells/well) were plated in a 96-well plate and cultured for 24 h at 37°C in a humidified atmosphere with 5% CO2. The experimental groups were the normal, model, and niloticin

Figure 1: Prediction of the interaction of niloticin with MD-2 and the effect of niloticin on cells. (A) The chemical structure of niloticin. (B) The binding mode of niloticin with MD-2 constructed in ChemBio3D Ultra 13.0 and minimized using the MMFF94x force field. (C) The effect of different concentrations of niloticin on the viability of RAW264.7 cells, determined by the MTT assay. (D) Analysis of niloticin interaction with MD-2 by bio-layer interferometry assay. The dissociation equilibrium constant in response to the concentration of niloticin varied from 0.0313, 0.0625, 0.125, 0.25, and 0.5 mM/mL.
Niloticin group was administered niloticin (8, 40, and 100 µg/mL; six wells per concentration per group) and then treated with 100 ng/mL LPS. The model group was treated with 100 ng/mL LPS. The normal group was treated with nothing. The cells were cultured for 24 h. Nitric Oxide (NO), Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-6, and IL-1β were analyzed in the supernatants of the cell cultures by ELISA according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA).

Real-time PCR analysis

The cells were prepared as for the cytokine assay, above. The Trizol reagent and ‘Turbo Dnase’ (Invitrogen Inc., Carlsbad, CA, USA) were used to extract total RNA and inactivate Dnase, respectively. Then, the Superscript First-Strand cDNA Synthesis Kit (Vazyme Biotech, Co., Ltd., Nanjing, China) was used to generate cDNA. The primer sequences of the primers for IL-1β, IL-6, TNF-α, inducible NO synthase (iNOS), Cyclooxygenase (COX)-2, and β-actin are presented in Table 1 [18,20]. Real-time PCR analysis (all reagents from Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) was performed as previously described [18]. Briefly, the thermal cycling conditions for the PCR reaction were 50ºC for 2 min followed by 40 cycles of 95ºC for 10 min, 95ºC for 30 s, and 60ºC for 30 s. The abundance of each gene was determined by the standard curve, and the values were normalized with β-actin. Each sample was repeated three times.

Western blot assay

The cells were prepared as for the cytokine assay, above. The RAW264.7 cells were lysed using the RIPA buffer, and the nuclear protein isolation-translocation assay kit was used for separating the nucleus and cytosol proteins according to the manufacturer’s instruction. The phosphatase inhibitor, PMSF, RIPA lysis buffer, and the BCA Protein Concentration Determination Kit were from Beyotime Institute of Biotechnology (Haimen, China). The 14-120 kD protein marker was from Beijing Quanjin Biotechnology Co., Ltd. (Beijing, China). The 10-250 kD protein marker was from Fermentas (Thermo Fisher Scientific, Waltham, MA, USA). Then, the protein concentration was determined, and the extracts were isolated and electro-blotted by 10% SDS-polyacrylamide gel electrophoresis and PVDF membranes. The membranes were blocked for 2 h at room temperature in blocking solution, followed by incubation with primary antibodies overnight at 4ºC: rabbit polyclonal anti-TLR4 (Wuhan Sanying Biotechnology Co., Ltd., Wuhan, China), rabbit polyclonal anti-MYD88 (Wuhan Sanying Biotechnology Co., Ltd., Wuhan, China), rabbit polyclonal anti-p-P65 (Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-p-P65 (Bioss Antibodies, Inc., Woburn, MA, USA), rabbit polyclonal anti-iNOS (Affinity BIO, Scoresby, Australia), and mouse monoclonal anti-β-actin (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). After incubation, TBST was used to wash the blots six times, and they were incubated for 2h with the secondary antibody at 37ºC: HRP-labeled goat anti-mouse and anti-rabbit secondary antibodies (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). Subsequently, the blots were washed and developed with a substrate for enhanced chemiluminescence (ECL; Pierce Chemical, Dallas, TX, USA). The Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify band intensities.

Statistical analysis

All data are presented as means ± standard deviations. One-way ANOVA with the LSD post hoc test was used to assess the differences among groups. All statistical analyses were carried out using SPSS 20.0 (IBM, Armonk, NY, USA). Two-sided P-values <0.05 were
considered statistically significant.

**Results**

**Drug potential assessment**

The important features related to ADME were provided by the TCMSP. Notably, the OB of niloticin was calculated to be 41.4%, and DL was 0.82, which suggested a good drug potential. In addition, the molecular weight (MW), partition coefficient between octanol and water (AlogP), H-bond donors (Hdon), H-bond acceptors (Hacc), and rotatable bonds number (RBN) of niloticin were 456.78 Da, 5.62, 1, 3, and 5.19, respectively, in line with the Lipinski’s principles [19,25].

**Molecular docking of niloticin**

To gain an insight into the interaction between niloticin and MD-2 at the molecular level, niloticin was docked to the LPS binding pocket in MD-2. The detailed binding mode is shown in Figure 1B. According to the docking results, niloticin had a wide contact with MD-2 by hydrophobic interaction with a binding energy of -10.0 kcal/mol. There were 12 residues involved in the interaction with niloticin, including ILE80, VAL135, TYR131, PHE151, ILE135, ILE32, ILE124, LEQ54, PHE121, LEU61, ILE63, and VAL135. No hydrogen-bonding interaction existed between niloticin and MD-2.

**Bio-layer interferometry assay**

The global fitting analysis of a 1:1 binding interaction model in the Octet system was used to assay the affinity constants at several concentrations of niloticin to MD-2. The concentration of niloticin varied from 0.0313, 0.0625, 0.125, 0.25, and 0.5 mM/mL, and the KD value of niloticin was 7.16E-04 (Figure 1D), which indicated that niloticin could bind to MD-2 and block the binding of LPS with TLR4/MD-2.

**MTT assay for viability**

The MTT assay was used to determine cell viability. There was a numerical decrease in OD value with increasing concentrations of niloticin, but the differences were not statistically significant (Figure 1C). The results showed that niloticin had little effect on cell viability.

**The effect of niloticin on cytokines in RAW264.7 cells**

ELISA was used to detect the levels of NO, TNF-α, IL-6, and IL-1β in RAW264.7 cells treated with niloticin. The LPS group showed high levels of NO, IL-1β, IL-6, and TNF-α levels compared with the control group (all P<0.01). Cells treated with LPS and niloticin showed significantly decreased NO, IL-1β, IL-6, and TNF-α levels compared with the LPS group (all P<0.01), but the levels were still higher than in control cells (all P<0.05) (Figure 2A). There were numerical differences among the three niloticin doses, but the differences were not statistically significant.

**Real-time PCR**

IL-1β, TNF-α, iNOS, IL-6, and COX-2 mRNA expression levels were analyzed by real-time PCR. The LPS group showed high levels of iNOS, IL-1β, IL-6, TNF-α, and COX-2 mRNA expression compared with the control group (all P<0.01). Cells treated with LPS and niloticin showed significantly decreased iNOS, IL-1β, IL-6, TNF-α, and COX-2 mRNA expression compared with the LPS group (all P<0.01), but the levels were still higher than in control cells (all P<0.01) (Figure 2B).

**Western blot**

The protein expression levels of TLR4, MyD88, NF-κB p65, NF-κB p-p65, and iNOS were analyzed by western blot. The result indicated that compared with the control group, TLR4, MyD88, p65, p-p65, and iNOS expression levels were up-regulated in the LPS group (all P<0.01). Compared with the LPS group, TLR4, MyD88, p65, p-p65, and iNOS expression levels were up-regulated in the niloticin groups (all P<0.01). But the levels were still higher than in control cells (all P<0.01) (Figure 2C).

**Discussion**

Niloticin is an active compound from Cortex phellodendri [12-14], but its anti-inflammatory activity has not yet been explored. The aim of the present study was to assess the drug potential of niloticin and to study the MD-2-targeting mechanism of its anti-inflammatory activity using computer-assisted drug design and bio-layer interferometry technology. The results suggest that niloticin is a potential MD-2 antagonist. It might interact with MD-2 to play an anti-inflammatory role by suppressing the activation of the LPS-TLR4/MD-2-NF-κB signaling pathway. Figure 3 summarizes the possible mechanism of niloticin.

Poor pharmacodynamics, poor pharmacokinetics, and toxicity are the most important reasons for costs and delays in drug discovery.
and development. Therefore, it is believed that certain features of the drug discovery process should be given prioritized. For all pharmacokinetics properties, OB, DL, and Lipinski’s rules play vital roles in evaluating the efficacy of the drug distribution in systemic circulation [20]. In the present study, niloticin was in line with all these conditions, strongly suggesting that it has a good drug potential.

The search and identification of target proteins is the first step in drug research and development. LPS is the most important pathogenic factor of endotoxemia and sepsis, leading to SIRS and MODS [26,27]. The key to the pathogenicity of LPS is that it can cause a cascade reaction of uncontrolled systemic inflammation by activating the TLR4 transmembrane signaling pathway, with TNF-α and IL-6 also playing important roles in the reaction [28]. Among signal proteins and receptors of the regulation pathway, MD-2 is the gatekeeper of TLR4 in LPS-induced inflammation signal, and it is considered as an indispensable receptor to activate the pathway [29]. The inflammatory response could be regulated by antagonizing the formation of the LPS-TLR4-MD-2 complex. Thus, MD-2 was used to dock niloticin by computer-aided drug design technology. In general, docking scores with binding energy ≤-5.0 kcal/mol indicate good compound-target interaction [30]. The binding energy between niloticin and MD-2 was only -10.0 kcal/mol, while the binding energy between LPS antagonist Eritoran to MD-2 was only -7.9 kcal/mol, indicating that niloticin can interact with MD-2 more strongly than LPS. Furthermore, MTI assay showed that niloticin has little effect on cell viability, suggesting that niloticin can be developed into a new drug based on MD-2 as a target. Therefore, the mechanism of niloticin was explored.

MD-2 and TLR4 on the surface of macrophages can form the TLR4-MD-2 complex, which recognizes and binds with LPS to activate the NF-κB signal transduction pathway to promote the expression of inflammatory factors such as TNF-α, IL-6, and IL-1β, further inducing the excessive inflammatory reaction [31]. In this study, niloticin could inhibit the release and expression of TNF-α, IL-6, and IL-1β. Moreover, NO would be released from macrophage cells stimulated by LPS to participate in the inflammation reaction [32]. In a pathological state, iNOS is the main source of NO [33]. Therefore, inhibition of NO release or iNOS expression might be important in the treatment of SIRS. In the present study, the release of NO and the expression of iNOS could be suppressed by niloticin. In addition, niloticin could also downregulate the expression of COX-2, which is related to the severity of inflammation [34]. A previous study showed that COX-2-deficient rats could delay the infiltration of leukocytes into important organs and resist infection mediated by LPS [35].

COX-2 is the specific target of many non-steroidal anti-inflammatory drugs, highlighting the importance of this protein in inflammatory responses. Therefore, any molecule that can decrease the expression of COX-2 can be a potent drug for inflammation modulation [36].

NF-κB activation can be caused by LPS, and this activation requires the involvement of Myd88 and TLR4 [37]. NF-κB can also be activated by proinflammatory factors, bacteria, and viruses [38]. Then, it interacts with the specific DNA sequence of the target gene, participates in inflammation, tumor cell proliferation and invasion, immunomodulation, angiogenesis, metastasis, and radiotherapy and chemotherapy resistance [39]. Western blot was used to evaluate the effect of niloticin on the LPS-TLR4/MD-2-NF-κB signaling pathway. The result indicated that niloticin could play an anti-inflammatory role by down-regulating the expression of P65, p-P65, TLR4, Myd88, and iNOS protein.

### Conclusion

In conclusion, niloticin is a potential MD-2 antagonist. It can interact with MD-2 to antagonize the binding of LPS to the TLR4/MD-2 complex, thus inhibiting the release of inflammatory factors (TNF-α, IL-6, and IL-1β) and NO through suppressing the activation of LPS-TLR4/MD-2-NF-κB signaling pathway. Of course, additional pre-clinical studies are necessary to determine the exact value of niloticin for the eventual management of SIRS.

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### Author Contributions

Y.X., G.C., and X.L. designed the study. Y.X., S.G., Y.L., M.Z., and G.C. performed the experiments. Y.X., S.G., Y.L. and G.C. collected and analyzed the data. Y.X., G.C., and X.L. wrote the paper.

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Table 1: Primer sequences for RT-PCR.

| Gene   | Sequences (5′→3′)                      |
|--------|----------------------------------------|
| IL-1β  | TCA GGC AGG CAG TAT CAC TC             |
| IL-6   | GTT GCC TTC TGG CTA GGA CTG AT          |
| TNF-α  | CGT CAG CGG ATT TGT CAT CT             |
| iNOS   | TGG CTC GCA GGA TGT ACC CT             |
| COX-2  | ATC ATA AGC GAG GAC CGT GG             |
| β-actin| CAC GAT GGA GGG GCC GGA GTC ATC        |
|        | TAA AGA CCT CTA TGC CAA CAC AGT        |

iNOS: inducible Nitric Oxide Synthase; COX-2: Cyclooxygenase 2.
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