Food and Agricultural Immunology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cfai20

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Version of record first published: 10 Feb 2010.

To cite this article: Thongchai Taechowisan, Asawin Wanbanjob, Pittaya Tuntiwachwuttikul & Jikai Liu (2010): Anti-inflammatory effects of lansai C and D cause inhibition of STAT-1 and NF-κB activations in LPS-induced RAW 264.7 cells, Food and Agricultural Immunology, 21:1, 57-64

To link to this article: http://dx.doi.org/10.1080/09540100903419592

Food and Agricultural Immunology
Vol. 21, No. 1, March 2010, 57–64

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(*Received 13 October 2009; final version received 16 October 2009*)

In inflammation, proinflammatory cytokines induce the formation of large amounts of nitric oxide (NO) by inducible nitric oxide synthase (iNOS), and compounds that inhibit NO production have anti-inflammatory effects. In the present study, we investigated the effects of lansai C and D on NO production in lipopolysaccharide-induced RAW 264.7 cells, and evaluated the mechanisms of action of the compounds. Lansai C and D inhibited iNOS protein and mRNA expression and also NO production in a dose-dependent manner. These compounds inhibited the activation of nuclear factor-κB, which is a significant transcription factor for iNOS and also inhibited the activation of the signal transducer and activator of transcription-1, another important transcription factor for iNOS. The present study characterises the effects and mechanisms of lansais on iNOS expression and NO production in activated macrophages. The results explain the pharmacological efficacy of lansais as anti-inflammatory compounds.

Keywords: anti-inflammatory activity; lansai C and D; NF-κB; RAW 264.7 cells; STAT-1

Introduction

Nitric oxide (NO) is produced from L-arginine by three nitric oxide synthase (NOS) enzymes; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Low physiological levels of NO are produced by constitutively expressed eNOS and nNOS, whereas iNOS is responsible for prolonged production of larger amounts of NO. iNOS is induced by bacterial products and inflammatory cytokines in macrophages and some other cells (Alderton, Cooper, & Knowles, 2001; Knowles & Moncada, 1994; Korhonen, Lahti, Kankaanranta, & Moilanen, 2005). NO production is increased in inflammation and has proinflammatory and regulatory effects (Bogdan, 2001; Yun et al., 2008). In addition, peroxynitrite formation in a reaction of NO and superoxide may lead to increased cytotoxicity. The experimental data support the idea that compounds inhibiting expression or activity of iNOS are potential anti-inflammatory agents (Connor et al., 1995; Evans & Whittle, 2001; Pelletier et al., 1998; Vallance & Leiper, 2002).

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ISSN 0954-0105 print/ISSN 1465-3443 online

DOI: 10.1080/09540100903419592
http://www.informaworld.com

Lansai C and D are phenolic compounds isolated from *Streptomyces* sp. SUC1 (Tuntiwachwuttikul, Taechowisan, Wanbanjob, & Thadaniti, 2008). These compounds have been reported to have anti-inflammatory properties by inhibition of proinflammatory cytokine production in lipopolysaccharide (LPS)-induced RAW 264.7 cells (Taechowisan, Wanbanjob, Tuntiwachwuttikul, & Liu, 2009). However, the molecular mechanisms explaining how lansai C and D suppress the inflammatory response are not known in detail. There are studies showing that certain lansai C and D down-regulate NO production in response to inflammatory stimuli (Taechowisan et al., 2009), but no more precise mechanisms of action are known.

In the present study, we investigated the effects of lansai C and D on iNOS expression and NO production in LPS-induced RAW 264.7 cells and evaluated the mechanisms of action of these affective compounds.

Materials and methods

Materials

Lansai C and D were isolated from *Streptomyces* sp. SUC1, carried out by the method of Tuntiwachwuttikul et al. (2008). Dulbecco's modified eagle medium (DMEM) and its supplements were obtained from Gibco BRL (Paislet, UK). All other reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

Cell culture

Murine macrophage RAW 264.7 cell line obtained from American Type Culture Collection (ATCC, MD, USA), was maintained in DMEM supplemented with 10% heat inactivated FBS, penicillin G (100 IU/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells (10^6/ml) were pre-incubated 2 h with lansai C and D (1, 2.5 and 5 μg/ml) and further cultured 24 h with LPS (1 μg/ml) in 24-well plates. Supernatants were removed at the allotted times and NO levels were quantified by immunoassay kits according to the manufacture's protocols (Assay Designs' Correlate-EIA™, Stressgen, USA).

Cytotoxicity assay

According to the method previously described, 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay was performed (Mosmann, 1983). MTT solution was added at a concentration of 50 μg/ml into each well, which also contain 1, 2.5 and 5 μg/ml of lansai C and D. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 μl DMSO. The optical density at 540 nm was determined with a microplate reader. The optical density of formazan formed in control (untreated) cells was taken as 100% of viability.

Preparation of cell lysates for inducible nitric oxide synthase (iNOS) Western blot

After the desired time of incubation cell lysates were prepared as described by Lahti, Sareila, Kankaanranta, and Moilanen (2006). The Coomassie blue method was used to measure the protein content of the samples (Bradford, 1976).

Preparation of nuclear extracts for p65 and signal transducer and activator of transcription (STAT)-1α Western blot

Cells were seeded on 10-cm dishes and were grown to confluence. Cells were incubated with the compounds for 30 minutes (p65) or for 6 hours (signal transducer and activator of transcription (STAT)-1α). After incubation, samples were prepared as described previously (Sareila et al., 2006). The Coomassie blue method was used to measure the protein content of the samples (Bradford, 1976).

Western blot analysis of inducible nitric oxide synthase (iNOS), p65 and signal transducer and activator of transcription (STAT)-1α proteins

Protein samples (20 μg) were separated by SDS-PAGE on 8% polyacrylamide gel and transferred to nitrocellulose membrane. Bound antibody – rabbit polyclonal antibodies for iNOS, STAT-1α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or for p65 subunit of nuclear factor-κB (NF-κB; Cell Signaling Danvers, MA, USA) – was detected by using goat anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and visualised by SuperSignal chemiluminescent substrate (Pierce, Cheshire, UK) and FluorChem 8800 imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). The quantitation of the chemiluminescent signal was carried out by using FluorChem software version 3.1.

RNA extraction and real-time RT-PCR of inducible nitric oxide synthase (iNOS) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNAs

RAW 264.7 cells stimulated with the compounds of interest were trypsinised after the desired time of incubation. Cell homogenisation, RNA extraction, reverse transcription and quantitative PCR were carried out following the methods of Lahti et al. (2006). The primer and probe sequences and concentrations were optimised according to the manufacturer's instructions in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C and were as follows: 5′-CCTGGTACGGGCATTGCT-3′, 5′-GCTCATGCGCCTCCTT-3′, (forward and reverse mouse iNOS primers, resp., both 300 nM); 5′-CAGCAGCGGCTCCATGACTCCC-3′ (mouse iNOS probe containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher, 150 nM); 5′-GCATGGCCTTCCGTGTTC-3′, 5′-GATGTCATCATACTTGGCAGGTTT-3′ (forward and reverse mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primers, resp., both 300 nM); 5′-TCGTGGATCTGACGTGCCGCC-3′ (mouse GAPDH probe containing 6-FAM as 5′-reporter dye and TAMRA as 3′-quencher, 150 nM). Results of iNOS mRNA levels were normalised against GAPDH mRNA in each sample.

Statistical analysis

Results are expressed as mean ± standard error of mean (SEM). The statistical significance of the detected differences was calculated by analysis of variance followed by Dunnett multiple comparison's test. Differences were considered significant when $p < 0.05$.

Results

The structures of lansai C and D are shown in Figure 1. Possible cytotoxic effects were tested by MTT assay. These compounds were not toxic to RAW 264.7 cells at the concentration 5 μg/ml.

Effects of lansai C and D on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 cells

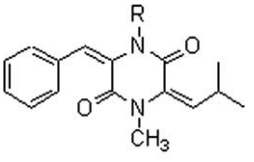
Untreated RAW 264.7 cells did not produce detectable amounts of NO during 24-hour incubation, but LPS (1 μg/ml) enhanced NO production significantly. In the first experiments, lansai C and D were used at 1, 2.5 and 5 μg/ml concentrations. These compounds inhibited LPS-induced NO production in a dose-dependent manner: lansai C ($IC_{50} \sim 2.63$ μg/ml) > lansai D ($IC_{50} \sim 3.42$ μg/ml) and also inhibited NO production by more than 50% at 5 μg/ml concentration (Figure 2).

Effects of lansai C and D on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) protein expression

The effects of lansai C and D inhibiting NO production were tested on iNOS protein expression by Western blot analysis. Unstimulated cells did not express detectable amounts of iNOS protein and LPS enhanced iNOS protein expression considerably. Lansai C and D inhibited LPS-induced iNOS protein expression (Figure 3).

Effects of lansai C and D on lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) mRNA levels

iNOS mRNA was measured by quantitative real-time RT-PCR. Cells were incubated with LPS (1 μg/ml) or with LPS and the tested compounds (5 μg/ml) for 6 hours. This incubation time was chosen according to the time curve of iNOS mRNA, where the maximal iNOS mRNA levels were between 6 and 8 hours after addition of LPS. Untreated cell expressed very low levels of iNOS mRNA and LPS enhanced iNOS



lansai C : R = OH
lansai D : R = H

Figure 1. Structures of lansai C and D.

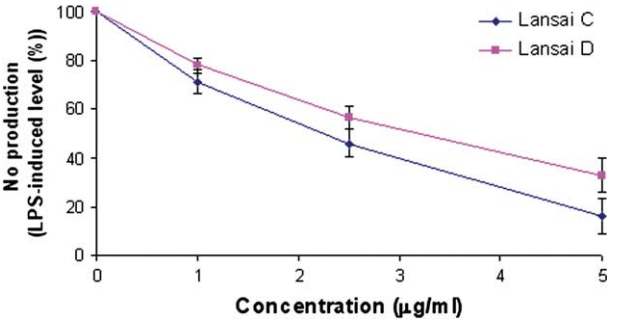


Figure 2. Effects of increasing concentrations of lansai C and D on LPS (1 µg/ml)-induced NO production in RAW 264.7 cells during a 24-hour incubation time.

mRNA expression considerably. Lansai C and D significantly lowered iNOS mRNA levels when measured after 6-hour incubation (Figure 4).

Effects of lansai C and D on lipopolysaccharide (LPS)-induced activation of transcription factors nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)-1

NF-κB and STAT-1 are important transcription factors for iNOS (Gao, Morrison, Parmely, Russell, & Murphy, 1997; Xie, Kashiwabara, & Nathan, 1994). Therefore, we measured the effects of lansai C and D on NF-κB and STAT-1 activations by measuring the nuclear translocation of the factors by Western blot. In unstimulated cells, low basal activity of NF-κB was detected and was significantly enhanced after LPS challenge. The maximal activation was found 30 minutes after LPS addition, and that incubation time was used in the subsequent studies. Lansai C and D inhibited the LPS-induced activation of NF-κB by more than 80 and 70%, respectively (Figure 5). Nuclear STAT-1 levels were significantly enhanced after LPS challenge. The maximal effect was found 6 hours after LPS addition, and that time point was chosen for subsequent studies. The LPS-induced activity of STAT-1 was 70 and 55% inhibition by lansai C and D, respectively (Figure 6).

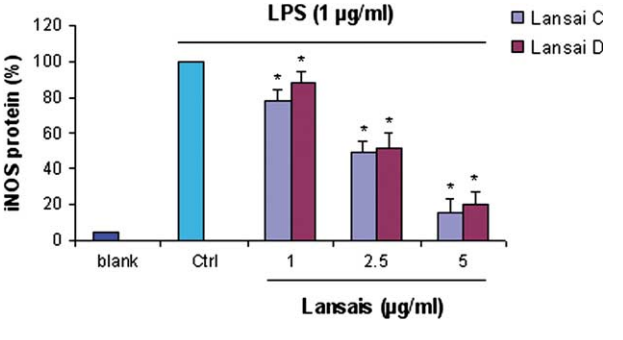


Figure 3. Effects of lansai C and D on LPS (1 µg/ml)-induced iNOS protein expression in RAW 264.7 cells during a 24-hour incubation time (*$p < 0.05$).

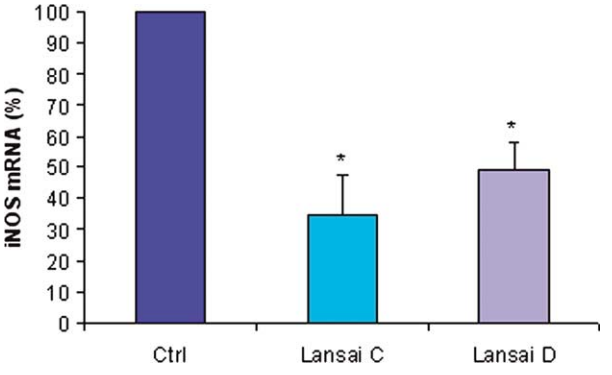


Figure 4. Effects of lansai C and D on iNOS mRNA expression. Cells were treated with LPS (1 μg/ml) or with LPS and the tested compound (5 μg/ml) and RNA was extracted after six hours. iNOS and GAPDH mRNA were measured by real-time RT-PCR and iNOS mRNA levels were normalised against GAPDH (*$p < 0.05$).

Discussion

In a previous study, *Streptomyces* sp. SUC1 was isolated from the aerial roots of *Ficus benjamina*, the major active ingredients from the culture were identified as lansai A–D (Tuntiwachwuttikul et al., 2008). Lansai C and D have anti-inflammatory properties (Taechowisan et al., 2009). In the present study, we investigated the effects of lansai C and D on iNOS expression and NO production in activated macrophages. Both lansai C and D inhibited LPS-induced STAT-1 and NF-κB activations and iNOS expression. To our knowledge, their effects on STAT-1 and NF-κB activation have not been reported previously. Our results show that lansai C and D inhibit STAT-1 and NF-κB activations, which are implicated in their effects on iNOS expression. Inhibition of STAT-1 activation by lansai C and D is likely involved in the mechanisms by which it inhibits iNOS expression because JAK inhibitors AG-490 and WHI-P154 have been shown to inhibit iNOS expression

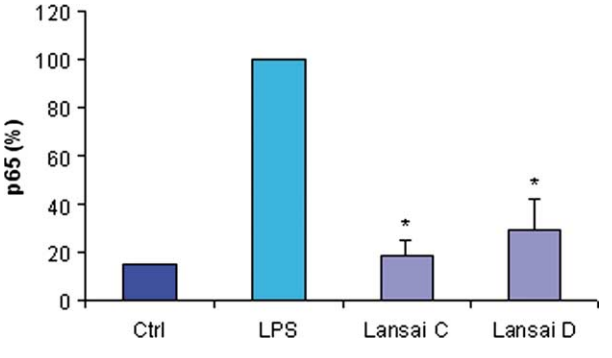


Figure 5. Effects of lansai C and D on LPS-induced NF-κB activation determined as nuclear translocation of NF-κB. RAW 264.7 cells were incubated for 30 minutes with LPS (1 μg/ml) or with LPS and the tested compound (5 μg/ml), and nuclear proteins were extracted. Western blot was used to detect the p65 subunits of NF-κB in the nuclear extract. p65 levels in LPS-treated cells were set at 100% and the other values were related to that (*$p < 0.05$ as compared to LPS-induced levels).

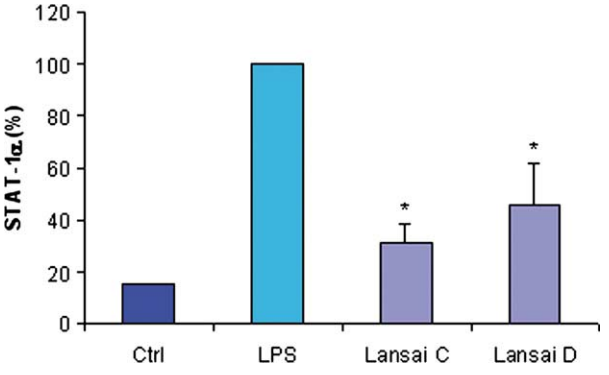


Figure 6. Effects of lansai C and D on LPS-induced STAT-1 activation determined as nuclear translocation of STAT-1α. RAW 264.7 cells were incubated for six hours with LPS (1 μg/ml) or with LPS and the tested compound (5 μg/ml), and nuclear proteins were extracted. Western blot was used to detect STAT-1α in the nuclear extract. STAT-1α levels in LPS-treated cells were set at 100% and the other values were related to that (*$p < 0.05$ as compared to LPS-induced levels).

along with their suppressive actions on STAT-1 activation (Salonen et al., 2006; Sareila et al., 2006; Wang et al., 2009). The mechanisms by which lansai C and D inhibit STAT-1 activation are not known, but may be associated with inhibition of phosphorylation of STAT-1 or its up-stream kinase JAK2 (Akiyama et al., 1987; Blanchette et al., 2009; Li, Zhang, Jin, Block, & Patel, 2007). Because NF-κB and STAT-1 are involved in the activation of several inflammatory genes, lansai C and D that inhibit activation of NF-κB and STAT-1 are likely to down-regulate production of an array of inflammatory mediators in addition to iNOS. Therefore, the present results offer an additional molecular mechanism for the anti-inflammatory action of lansai C and D.

In conclusion, we investigated the effects of lansai C and D on LPS-induced NO production and iNOS expression in activated macrophages. Both lansai C and D inhibited activation of both of the important transcription factors for iNOS, that is, STAT-1 and NF-κB. The results partly explain the anti-inflammatory effects of lansai C and D.

Acknowledgements

This work was supported by Thailand Research Fund MRG5180173 and The Faculty of Science, Silpakorn University. The authors are grateful to Dr Somkiat Thadaniti, Department of Chemistry, Faculty of Science, Silpakorn University, for measuring NMR and MS data.

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