MORINDONE FROM *MORINDA LONGISSIMA* INHIBITS CYTOKINE PRODUCTION IN TLR-COSTIMULATED MACROPHAGE MODEL POTENTIALLY THROUGH JNK/NF-κB/STAT1

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ABSTRACT

Morindone, an anthraquinone compound isolated from the roots of *Morinda longissima* Y.Z Ruan (Rubiaceae), is known for its diverse bioactive properties. This study aimed to investigate the unknown effects of morindone, isolated from *Morinda longissima*, on inflammatory cytokine production in macrophages costimulated with LPS and poly (I:C), a model mimicking bacterial and viral coinfection. The RAW264.7 cells were pretreated with 3, 10, 30, or 60 µM morindone for 30 min, followed by costimulation with LPS (1 µg/mL) and poly (I:C) (20 µg/mL). The cells were subsequently harvested for qRT–PCR at 3, 6, and 24 hours, and the supernatants were collected for ELISA at 24 hours. For the first time, morindone was demonstrated to inhibit the production of inflammatory cytokines IL-6, TNF-α, and IL-10 in a concentration-dependent manner in RAW 264.7 cells co-infected with bacteria and viruses using LPS and poly (I:C) with IC_{50} values of 3.2 ± 1.2 µM for IL-6, 2.2 ± 1.0 µM for TNF-α, and 9.1 ± 3.4 µM for IL-10. Morindone at 3, 10, 30, or 60 µM significantly inhibited the expression of *Jnk* by 17%, 26%, 53%, and 64%, *Nf-κb1* by 21%, 53%, 61%, and 66%, and *Stat1* by 22%, 26%, 57%, and 62%, respectively. These findings identify morindone as a promising candidate for targeting bacterial and viral co-infections.

MORINDONE TỪ *MORINDA LONGISSIMA* ỨC CHẾ SẢN SINH CYTOKINE TRÊN MÔ HÌNH ĐẠI THỰC BÀO ĐỒNG KÍCH THÍCH TLR THÔNG QUA JNK/NF-κB/STAT1

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TỪ KHÓA

Morindone
Morinda longissima
Cytokine viêm
Mô hình kích thích LPS-poly (I:C)
Đại thực bào

TÓM TẮT

Morindone là một hợp chất anthraquinone được phân lập từ thân rễ cây Nhó đông (*Morinda longissima* Y.Z Ruan, họ Rubiaceae) và đã được biết có nhiều hoạt tính sinh học. Mục đích của nghiên cứu này là tìm hiểu các hoạt tính mới của morindone tách từ cây *Morinda longissima* lên sự sản xuất các cytokine viêm ở đại thực bào đồng kích thích bởi LPS và poly (I:C), là mô hình đồng nhiễm vi khuẩn và virus. Tế bào RAW264.7 được xử lý trước 30 phút với 3, 10, 30, hoặc 60 µM sau đó được kích thích bởi LPS (1 µg/mL) và poly (I:C) (20 µg/mL). Các tế bào được thu sau 3, 6, 24 giờ cho qRT-PCR và dịch tế bào được thu sau 24h cho ELISA. Lần đầu tiên chứng minh được morindone có khả năng ức chế sản sinh các cytokine viêm IL-6, TNF-α, và IL-10 theo kiểu phụ thuộc nồng độ trên tế bào RAW 264.7 bị đồng kích thích nhiễm vi khuẩn và virus bằng LPS và poly (I:C), với giá trị IC_{50} tương ứng là 3,2 ± 1,2 µM với IL-6, 2,2 ± 1,0 µM với TNF-α và 9,1 ± 3,4 µM với IL-10. Hơn nữa, morindone ở nồng độ 3, 10, 30, hay 60 µM ức chế biểu hiện của các gen điều khiển sinh cytokine tương ứng với *Jnk* 17%, 26%, 53%, và 64%, *Nf-κb1* 21%, 53%, 61%, và 66%, và *Stat1* 22%, 26%, 57%, và 62%. Những kết quả này chứng minh morindone là một hợp chất triển vọng cho việc điều trị các bệnh nhiễm trùng đồng thời vi khuẩn và virus.

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1. Introduction

The defense responses of the immune system to infection, commonly triggered by bacterial and viral pathogens, activate macrophages to release proinflammatory cytokines. The uncontrolled production of inflammatory cytokines such as IL-6, TNF-α, and IL-10, particularly under conditions known as "cytokine storms", can lead to severe tissue damage and organ failure [1].

Lipopolysaccharide (LPS), a component of Gram-negative bacterial outer membranes, activates inflammatory signaling through Toll-like receptor 4 (TLR4), leading to downstream activation of pathways including NF-κB, MAPKs (p38, JNK), and STATs [2], [3]. Poly(I:C), a synthetic analog of viral double-stranded RNA, triggers immune responses via TLR3, promoting the expression of antiviral and proinflammatory cytokines through NF-κB and other transcription factors [4].

Morindone is one of the major anthraquinones obtained from the roots of *Morinda longissima* Y.Z Ruan (Rubiaceae). It, along with other extracts from *Morinda longissima* roots rich in anthraquinone, has been reported to possess cytotoxic, -anti-viral properties against hepatitis B virus, and anti-inflammatory properties in *in vivo* and *in silico* models [5] – [8]. In previous studies, certain natural compounds have been shown to exhibit anti-inflammatory and antiviral properties. Costimulation of TLR-4 by LPS and TLR-3 by poly (I:C) has been documented in several cell types [9], [10]. In this study, we aimed to identify the unknown anti-inflammatory property of moridone involved in the production of inflammatory cytokines in macrophages under costimulation by LPS and poly (I:C), which mimics the coinfection of bacteria and viruses.

2. Research methods

2.1. Plant materials

The roots of *Morinda longissima* were collected in the Son La province, Vietnam. The plants were identified at Vietnam National Museum of Nature, VAST, Hanoi, Vietnam.

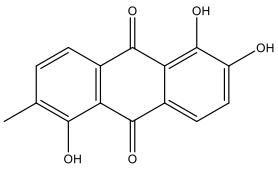

Figure 1. *Chemical structure of morindone isolated from Morinda longissima*

2.2. Apparatus

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Bruker Avance 500 MHz spectrometer. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained using a Varian FT-MS spectrometer and a MicroQ-TOF III mass spectrometer (Bruker Daltonics). Column chromatography was conducted on silica gel (Si 60 F254, 40–63 mesh; Merck), and all solvents were redistilled prior to use. Analytical thin-layer chromatography (TLC) was carried out on pre-coated silica gel plates (Si 60 F254; Merck). Spots were visualized under UV light at 254 and 365 nm or by spraying with 10% H_2SO_4 followed by heating with a heat gun.

2.3. Morindone extraction

Following the procedure described in an article published by Cuong et al. [11], morindone was isolated and its structure determined (Figure 1). Briefly, the dried and powdered roots of *M. longissima* (0.5 kg) were extracted with 96% ethanol at room temperature over a period of two days. The solvent was removed under reduced pressure to yield a black crude EtOH extract (20.0 g). This extract was suspended in a 1:1 (v/v) mixture of MeOH–H_2O and successively partitioned with EtOAc and H_2O. The resulting solvent-soluble fractions were concentrated under reduced pressure to yield the EtOAc-soluble fraction (5.0 g) and the H_2O-soluble fraction (10.0 g). The EtOAc fraction was subjected to normal-phase column chromatography (CC) on silica gel (Merck, 40–63 μm), eluted with a gradient of *n*-hexane–dichloromethane (4:1, 1:1, 3:4, v/v), followed by

dichloromethane, and then by a gradient of dichloromethane–methanol (20:1, 10:1, 2:1, v/v), yielding four subfractions. Subfraction 2 was further purified by CC on silica gel using *n*-hexane–acetone (4:1, v/v), yielding morindone (50 mg).

2.4. Cell culture

The RAW264.7 cells (kindly provided by Prof. T. Kishimoto, Osaka University, Japan), were grown in complete RPMI 1640 medium (10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin). Cells were cultured until their density reached 80% confluence, then seeded into 24-well plates (5×10^5 cells/well). After adhesion, cells were treated with morindone (0, 3, 10, 30 or 60 μM). After 30 min, 1 μg/mL LPS (Sigma) and 20 μg/mL poly(I:C) (Sigma) were added. The plates were incubated at 37ºC in 5% CO_2. After 3 h, 6 h, and 24 h, the cells or the supernatants were collected for qRT-PCR or ELISA [12].

2.5. Cell viability assay

The macrophage cell line RAW264.7 (5×10^5 cells/well) were seeded in 96-well plates (Corning) and treated with 0–60 μM morindone for 24 h. Cell viability was assessed by MTT assay and expressed as a percentage of the control absorbance at 450 nm described previously [13]. Cell viability was calculated using a formular following:

Cell viability (%) = [(Absorbance of treated sample - Blank) / (Absorbance of control - Blank)] x 100.

2.6. ELISA

The levels of IL-6, TNF-α and IL-10 in the culture supernatants were measured via ELISA kits (Mabtech) following the protocol of manufacturer. The levels of cytokines were calculated from standard curves via an optical density (OD) reader with the absorbance at 450 nm. The values of IC_{50} were determined by using ImageJ 1.50i software (NIH).

2.7. Real-time qRT-PCR

RNA was isolated from cultured cells using total RNA extraction kit (iNtRON) according to the protocol of manufacturer. Total RNA (1 μg) was converted into cDNA via the RevertAid first strand cDNA synthesis kit (ThermoFisher) according to the protocol of manufacturer. PCR reactions were done by using green master mix (ThermoFisher). The sequences of primers used for qPCR are listed in Table 1.

Table 1. *Sequences of primers in qPCR* [14] – [17]

Genes	Forward primers	Reverse primers
Jnk	5'- CACCACCAAAGATCCCTGACA - 3'	5'- GCACCTAAAGGAGACGGCTG - 3'
Nf-κb1	5'- ATGGCAGACGATGATCCCTAC-3'	5'- TGTTGACAGTGGTATTTCTGGTG-3'
Stat1	5'- ATGTCTCAGTGGTACGAACTTC - 3'	5'- CTATACTGTGTTCATCATACTGTC - 3'
β-actin	5'- AGCCATGTACGTAGCCATCC - 3'	5'- CTCTCAGCTGTGGTGGTGAA - 3'

qPCR reactions and analyses were performed using the QuantStudio™6 PCR system. The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method [18].

2.8. Statistical analysis

All data are presented as means ± standard deviations (SDs) from three independent experiments. Statistical analysis was performed via Student's t test, and p values < 0.05 was considered significant.

3. Results and Discussion

3.1. Effect of Morindone on Viability of RAW264.7 Cells

As shown in Figure 2, morindone did not affect RAW 264.7 cell viability at concentrations of 3, 10, 30 or 60 µM (105 ± 4%, 106 ± 4%, 101 ± 7%, and 92 ± 2%, respectively) when compared with the control (100% ± 0%). These concentrations were therefore deemed non-toxic and used for subsequent experiments. Previous studies also demonstrated that morindone exhibited selective cytotoxicity— no cytotoxic effect in normal colon epithelial cells (CCD841 CoN), but significant effects in colorectal cancer cell lines (HT29, LS174T, HCT116) [5].

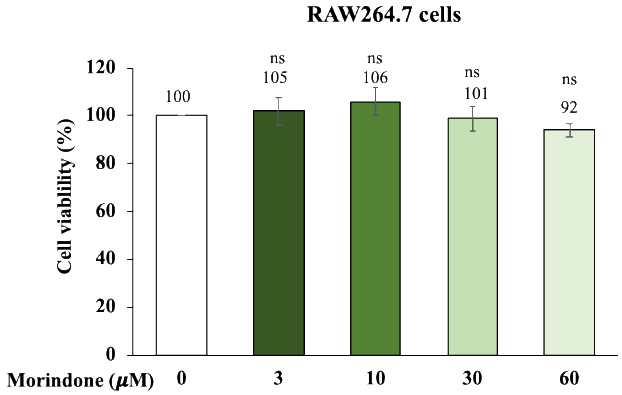

Figure 2. *Effect of morindone on the viability of RAW 264.7 cells. The cells were treated with different concentrations of morindone (3 - 60 µM). ns indicates not significant (p>0.05) versus the control group.*

3.2. Morindone Inhibits Cytokine Production in Macrophages Induced by LPS and poly (I:C)

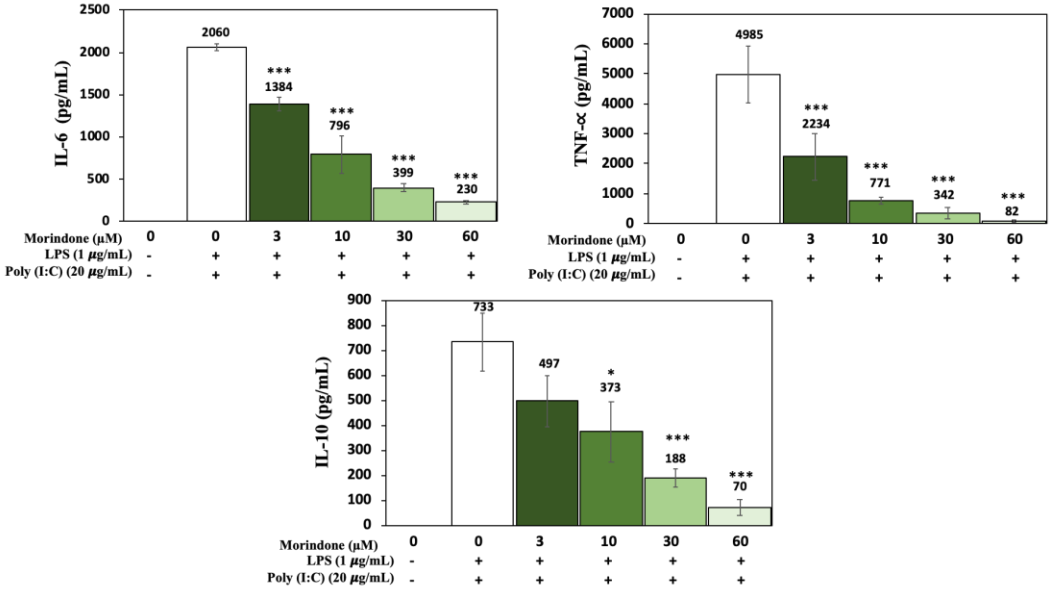

Figure 3. *Morindone decreased the levels of inflammatory cytokines including IL-6, TNF-α and IL-10 in RAW264.7 cells costimulated by LPS and poly (I:C). The cells were pretreated with various concentrations of morindone in the presence of 1 µg/mL LPS and 20 µg/mL poly (I:C) for 24 h. p < 0.05, *** p < 0.001 versus the LPS-poly (I:C) costimulated only group*

RAW264.7 cells were treated with morindone (3, 10, 30, 60 µM) for 30 min and then stimulated with LPS (1 µg/mL) and poly (I:C) (20 µg/mL) for 24 hours. As shown in Figure 3, morindone

suppressed the production of cytokines with the IC_{50} values of 3.2 ± 1.2 μM for IL-6, 2.2 ± 1.0 μM for TNF-α and 9.1 ± 3.4 μM for IL-10, respectively. Since cell viability remained above 90% in all treatment groups (Figure 2), the observed reduction in cytokines was attributed to morindone's anti-inflammatory effects, not cytotoxicity.

IL-6, TNF-*α* and IL-10 are pro-inflammatory cytokines that mediate the development of various inflammatory diseases [19]. Our findings revealed that morindone has the potential to suppress inflammation via the inhibition of inflammatory cytokines such as IL-6, TNF-*α* and IL-10. In a previous study, anthraquinone compounds isolated from *Morinda longissima* were shown to have the anti-inflammatory efficacy based on their cyclooxygenase (COX)-1 and 2 inhibitory effects *in vitro* or their ability to bind to TNF-*α in silico* [7], [8]. In this study, the production of inflammatory cytokines in activated macrophages by TLR ligands may be modulated by signaling pathways including the JNK/NF-κB/STAT [20]. Therefore, we next investigated whether or not morindone regulates the expression of *Jnk*, *Nf-κb1* and *Stat1* in cytokine regulating pathways.

*3.3. Morindone inhibits the JNK/*NF-κB/*STAT signaling pathway in RAW264.7 cells costimulated by LPS and poly (I:C)*

To elucidate the detailed inhibitory mechanism by which morindone inhibits cytokine production, we examined the expression of the *Jnk*, *Nf-κb1* and *Stat1* genes in 1 μg/mL LPS-20 μg/mL poly (I:C)-costimulated RAW264.7 cells. The results in Figure 4 show that LPS together with poly (I:C) resulted in a 6-h peak in *Jnk* (1.7-fold), *Nf-κb1* (2.4-fold) and *Stat1* (2.2-fold) expression compared with the untreated control. Morindone at concentrations of 3, 10, 30, or 60 μM morindone significantly inhibited *Jnk* (17%, 26%, 53%, and 64%), *Nf-κb1* (21%, 53%, 61%, and 66%), and *Stat1* (22%, 26%, 57%, and 62%).

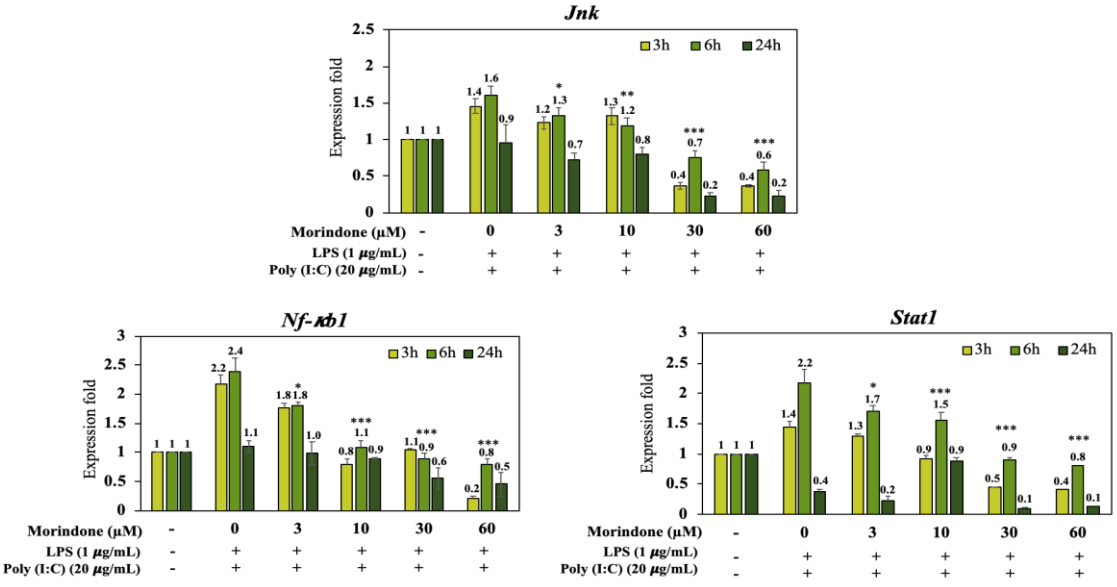

Figure 4. *Morindone reduced the expression of Jnk, Nf-κb1, and Stat1 in RAW 264.7 cells costimulated with LPS (1 μg/mL) and poly (I:C) (20 μg/mL). The cells were treated with various concentrations of morindone in the presence of LPS and poly (I:C) for 3, 6, or 24 h. * $p < 0.05$, *** $p < 0.001$ versus the LPS-poly (I:C)-costimulated only group.*

TLRs recognize molecules such as LPS, DNA, and RNA from bacteria and viruses as sensors for detecting possible infections. The recognition of TLR4 ligands, such as bacterial LPS, occurs at the cell surface, leading to the activation of the JNK and NF-κB pathways, while TLR3 is

expressed intracellularly within the endosomes to detect viruses, including the viral-mimic poly (I:C), initiating a signaling cascade that ultimately leads to the activation of both the JNK and NF-κB pathways [4], [21]. JNK/NF-κB signaling may be activated by various TLR stimuli, including bacterial and viral products, and initiates an immune cascade response for host defense, i.e., the production of inflammatory cytokines [1]. In addition, STAT1 is activated by TLR-mediated signaling via LPS or poly (I:C), leading to the activation of genes involved in inflammation. Both the NF-κB and STAT1 pathways contribute to the production of proinflammatory cytokines, such as IL-6, TNF-α, and IL-10, which are crucial for initiating the inflammatory response [22]. In our study, we found that morindone decreased *Jnk* expression, possibly followed by both *Nf-kb1* and *Stat1*. These results indicate that this compound may affect the network of JNK/NF-κB/STAT1 to regulate the production of cytokines (Figure 5).

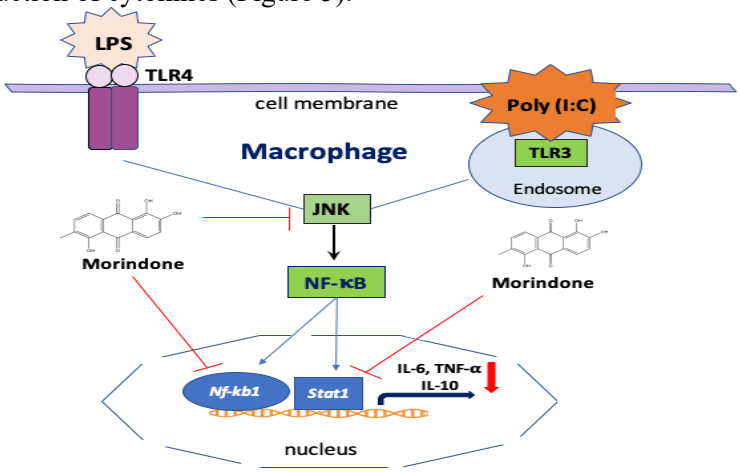

Figure 5. *Potential mechanism by which morindone suppresses cytokine production in LPS-poly (I:C)-costimulated macrophages*

4. Conclusion

Morindone exerted significant anti-inflammatory effects in RAW264.7 macrophages without affecting cell viability at concentrations up to 60 μM. Morindone suppressed the production of inflammatory cytokines, including IL-6, TNF-α, and IL-10, with low micromolar IC50 values, indicating potent activity. Mechanistically, morindone downregulated the expression of Jnk, Nf-κb1, and Stat1 in LPS and poly(I:C)-costimulated cells, suggesting that its inhibitory effects are mediated through suppression of the JNK/NF-κB/STAT signaling pathway. These findings highlight morindone as a promising natural compound with potential applications in managing inflammation in bacterial and viral coinfections, where macrophage activation and cytokine overproduction play central roles. The validation in animal models of infection and inflammation to establish in vivo efficacy, as well as mechanistic studies employing docking simulations to define direct molecular targets should be further investigation.

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