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Full Paper

Norkurarinol Inhibits Toll-Like Receptor 3 (TLR3)-Mediated Pro-inflammatory Signaling Pathway and Rotavirus Replication

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Abstract. This study examined the effect of norkurarinol on the toll-like receptor 3 (TLR3)-mediated signaling pathways and rotavirus replication. Norkurarinol, a lavandulylated flavanone, was isolated from the roots of *Sophora flavescens*, which has been shown to have anti-inflammatory activity. Norkurarinol suppressed the NF- κ B and AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) activity induced by poly(I:C), TLR3 ligand, in THP1-Blue-CD14 cells with IC₅₀ values of 20.9 μ M. Norkurarinol also significantly suppressed the mRNA expression of pro-inflammatory and adhesive molecules induced by poly(I:C) and rotavirus infection. Pretreatment of norkurarinol blocked the NF- κ B and AP-1 signaling pathway and the phosphorylation of MAPKs induced by poly(I:C). On the other hand, norkurarinol increased the level of IRF3 phosphorylation and IFN β expression in a dose-dependent manner. Moreover, norkurarinol inhibited the rotavirus-induced cytopathic effects. These results suggest that norkurarinol can modulate the TLR3-mediated inflammatory responses and rotavirus replication.

Keywords: norkurarinol, toll-like receptor 3 (TLR3), inflammatory response, rotavirus, dsRNA

Introduction

Toll-like receptors (TLRs) play a key role in regulating the activation of the innate immune response to microbial genomes such as viral dsRNA, ssRNA, viral DNA, and bacterial DNA (1, 2). Ten human TLRs have been characterized thus far (1). They are expressed mainly by cells involved in immune functions such as monocytes and macrophages (1, 2). Generally, TLRs can activate two branches of the downstream signaling pathways: myeloid differentiation factor (MyD)88-dependent and MyD88-independent pathways (3). MyD88 is a common downstream adaptor molecule for all mammalian TLRs with the exception of TLR3. TLR3 and TLR4 activate the MyD88-independent signaling pathway mediated

through the TIR domain containing adaptor inducing IFN β (TRIF)-dependent pathway (4 – 7). All TLRs and ligands engagements finally result in the production of downstream inflammatory cytokines and leukocyte adhesion molecules by the activation of nuclear factor κ B (NF- κ B) / activator protein-1 (AP-1)-dependent transcriptional pathways (8, 9). In particular, TLR3 is the receptor for viral dsRNA and the dsRNA mimic poly(I:C) (10). Most viruses synthesize dsRNA at some point during their replication cycle. Therefore, TLR3 is an important detector of viral infections and an initiator of the immune response.

The inflammatory response is useful for the defense against the invasion of viruses and bacteria. On the other hand, the excessive innate immune activation of pro-inflammatory cytokines can be harmful, leading to micro-circulatory dysfunction, tissue damage, shock, or even death of the host (11 – 14). Therefore, the excessive inflammatory response induced by the TLRs signaling pathways needs to be under tight control. If small mole-

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cule inhibitors can be found to modulate the TLRs-specific responses, it may assist in the development of therapeutic compounds to block excessive inflammation. Indeed, many studies demonstrated that certain phytochemicals including flavonoids and polyphenols possessing anti-inflammatory effects inhibit NF- κ B activation induced by a range of TLR agonists (15). Recently, the anti-inflammatory compounds auranofin and curcumin were reported to block TLR4/MyD88 interaction (7, 16).

As part of an ongoing search for inhibitors of TLRs activity, this study found that norkurarinol, a lavandulylated flavanone isolated from the roots of *Sophora flavescens*, has an inhibitory effect on TLR3 activation. Norkurarinol has many biological activities such as anti-oxidant, anti-bacterial, anti-influenza, and glycosidase inhibitory activities (17–21). Here, we report the first biochemical evidence that norkurarinol inhibits the TLR3-mediated pro-inflammatory signaling pathway and rotavirus-induced cytopathic effects.

Materials and Methods

Reagents and plasmid

Norkurarinol was isolated in our laboratory (Fig. 1) (20). Poly(I:C), anti- β -actin antibody, and secondary antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-NF- κ B p65 antibody was purchased from Abcam (Cambridge, MA, USA). Anti-phosphorylated p38, JNK, ERK1/2, IRF3, IKK α/β , and IKK γ were purchased from Cell Signaling Technology (Boston, MA, USA). The hnRNP_{C1/C2} antibody and c-Fos antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nucleofector kit V was purchased from LONZA (Cologne, Germany). The pcDNA3-TRIF-CFP plasmid was provided by Dr. Doug Golenbock (University of Massachusetts Medical School, Worcester, MA, USA) via Addgene (Addgene plasmid 13644; Cambridge, MA, USA). QUANTI-Blue, zeocin, and blasticidin were purchased from Invivogen (San Diego, CA, USA). All solvents were of analytical grade and obtained from Burdick & Jackson (Muskegon, MI, USA). All reagents were obtained from Sigma-Aldrich.

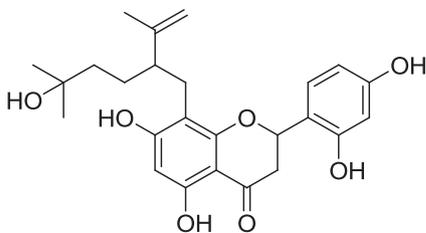


Fig. 1. The structure of norkurarinol.

Cells and viruses

Three different cell lines, human monocytic cells (THP-1), THP1-Blue-CD14 cells, and fetal rhesus monkey kidney cells (TF-104), were used in this study. THP1-Blue-CD14 cells are stably transfected with the NF- κ B/AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter system (Invivogen). The THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. The THP1-Blue-CD14 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 200 μ g/mL zeocin, 100 μ g/mL blasticidin, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a 5% CO₂ incubator. The TF-104 cells were maintained in Eagle's minimum essential medium (EMEM) supplemented with 5% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 100 U/mL amphotericin B. The rotaviruses KJ56-1 (bovine rotavirus, G8P[7]) and 205-1 (porcine rotavirus, G5P[7]) were preactivated with 10 μ g/mL trypsin for 30 min at 37°C before inoculating them onto confluent TF-104 cells, and the infected cells were maintained in the presence of 1 μ g/mL trypsin (1:250). All cell culture reagents were obtained from Gibco BRL (Invitrogen, Carlsbad, CA, USA).

TLR3 activation assay

THP1-Blue-CD14 cells express TLR1-10, overexpress CD14, and are transfected with a reporter plasmid containing SEAP under the control of NF- κ B/AP-1-inducible promoter. TLR3 activation was determined by quantifying the SEAP secreted. Briefly, THP1-Blue-CD14 cells were seeded onto 96-well culture plates at 2×10^5 cells/well and the cells were treated with norkurarinol for 1 h before incubating them with poly(I:C) (50 μ g/mL). After 18-h incubation at 37°C in a 5% CO₂ atmosphere, 20 μ L of the cell suspension was added in new 96-well plates and mixed with 200 μ L of a QUANTI-Blue colorimetric assay reagent to detect SEAP. After incubation for 1 h at 37°C to allow color development, quantitative reading was performed at 655 nm using a microplate reader.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

For the anti-inflammatory effect of norkurarinol on poly(I:C)-stimulated cells, the THP-1 cells (5×10^5 cells/well) were treated with norkurarinol for 1 h before incubating them with poly(I:C) (50 μ g/mL). After 6-h incubation at 37°C in a 5% CO₂ atmosphere, the total RNA was isolated from the THP-1 cells. To determine the anti-inflammatory effect of norkurarinol on rotavirus-infected cells (22), TF-104 cells (5×10^5 cells/well) were treated with norkurarinol for 1 h before inoculating the virus.

The virus at 0.01 MOI (multiplicity of infection) were inoculated to TF-104 cells (5×10^5 cells/well) for 1 h with occasional rocking. The solution was removed and the cells were replaced with EMEM containing 1 $\mu\text{g}/\text{mL}$ trypsin and norkurarinol. The cultures were incubated for 24 or 48 h at 37°C under a 5% CO₂ atmosphere and the total RNA was isolated from the TF-104 cells. The total RNA was isolated using an RNeasy MinElute Cleanup kit (Qiagen, Germantown, MD, USA) including a DNase step. The total RNA concentration and quality were assessed on a 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized (TaqMan Reverse Transcription Reagents Kit; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed on a StepOne Plus real time thermal cycler using the SYBR Green PCR Master Mix Kit (Applied Biosystems). GAPDH was used as an endogenous reference to correct for the differences in the amount of RNA. The data was analyzed using the $2^{-\Delta\Delta\text{CT}}$ method (23). Table 1 lists the primer sequences.

Western blot analysis

The nuclear extracts of the THP-1 cells were prepared with NE-PER Nuclear Extraction Reagents (Thermo Scientific, Rockford, IL, USA) and the total protein of THP-1 and TF-104 cells was prepared using cell lysis buffer (Cell Signaling Technology). The nuclear extracts for an analysis of the NF- κ B subunit p65 and c-Fos and the total proteins for the analysis of mitogen-activated protein kinase (MAPKs), I κ B kinase (IKK) isoforms, and interferon regulatory factor 3 (IRF3) phosphorylation was subjected to western blot analysis with the primary antibodies (1:1000) and secondary antibodies (1:5000).

Cytopathic effect (CPE) inhibition assay

The CPE inhibition assay was performed, as described previously (22). Briefly, the TF-104 cell monolayer (1×10^5 cells/well) at near confluence was inoculated with the virus at 0.01 MOI for 1 h with occasional rocking. The solution was removed and the cells replaced with EMEM containing 1 $\mu\text{g}/\text{mL}$ trypsin and norkurarinol. The cells were incubated for 3–4 days at 37°C under a 5% CO₂ atmosphere until the cells in the infected, untreated control well showed complete viral CPE, as observed by optical microscopy. All compounds were assayed for virus inhibition in triplicate. After 3 days, 0.034% neutral red was added to each well of that plate and incubated for 2 h at 37°C in the dark. The neutral red solution was removed and the cells were washed with PBS (pH 7.4). A destaining solution (containing 1% glacial acetic acid, 49% H₂O and 50% ethanol) was added to each well. The plates were incubated in the dark for 15 min at room temperature. The absorbance was measured at 540 nm with a microplate reader.

Virus yield-reduction assay

The TF-104 cells were infected with the virus at a 0.001 MOI in 6-well plates. After 1 h of virus adsorption at 37°C, the cells were washed three times with PBS and cultured in a medium containing 30 μM norkurarinol. The untreated cell and virus controls were included. The supernatants were harvested after 72 h. The virus yields were determined using a 50% cell culture infective dose (CCID₅₀) assay in TF-104 cells.

Data analysis

All experiments were performed three times. The data is expressed as the mean \pm S.E.M. Statistical analysis was performed using Sigma Plot Statistical Analysis

Table 1. Primers used for real-time PCR analysis

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Human TNF- α	GCCCAGGCAGTCAGATCATCT	TTGAGGGTTTGCTACAACATGG
Human IL-6	GCAACACCAGGAGCAGCC	AACTCCTTCTCCACAAGCGC
Human MCP-1	GCAGAGGCTCGCGAGCT	ACAATGGTCTTGAAGATCACAGC
Human VCAM-1	GGGAGCTCTGTCACTGTAAG	ATCCGTATCTCCAAAAACT
Human ICAM-1	CCTTCCTCACCGTGTACTGG	AGCGTAGGGTAAGGTTCTTGC
Human IFN β	GATTACTCTAGCACTGGCTGG	CTTCAGGTAATGCAGAATCC
Human GAPDH	CTCAAGATCATCAGCAATGCCT	AAGTTGTCATGGATGACCTTGG
Monkey TNF- α	TTCTCGAACCCCAAGTGACAA	TTGGCCAGGAGGGCATT
Monkey IL-6	GGTACATCCTCGACGGCATC	CCAGTGCCTCTTTGCTGCTT
Monkey IFN β	TTCTCATCATGACCAACAAGTGCT	AGACAATTTGGCTCTGCATTATTTT
Monkey GAPDH	GCTGCTGAAGAGGCGCTT	GCTCCTCATCCATACCCTGATTTT

software. The differences between the group mean values were determined by one-way analysis of the variance followed by a two-tailed Student's *t*-test for unpaired samples, assuming equal variance.

Results

Norkurarinol inhibits the activation of TLR3 signaling induced by poly(I:C)

The individual TLRs interact with different combinations of adapter proteins and activate a range of transcription factors, such as NF- κ B, AP-1, and IRF3, driving the specific immune response (8, 24, 25). In particular, NF- κ B and AP-1 are common transcription factors activated by all TLRs eliciting conserved inflammatory pathway. Therefore, to determine if norkurarinol can modulate TLR3 activation, the SEAP reporter system employing poly(I:C), a TLR3 agonist, was used as a readout for the activation of TLR3. The THP1-Blue-CD14 cells were stimulated with poly(I:C) (50 μ g/mL) in the presence or absence of norkurarinol. Treatment with poly(I:C) alone for 18 h led to an up to 7-fold increase in SEAP activity. This effect was inhibited by the pretreatment of norkurarinol in a dose-dependent manner (Fig. 2) with IC₅₀ values of 20.9 μ M. Norkurarinol exhibited no cytotoxicity on the highest dose with the MTT assay (data not shown).

Effects of norkurarinol on TLR3-mediated pro-inflammatory and adhesive molecules expression

dsRNA stimulation of the TLR3-expressing cells up-regulates the expression of a wide range of NF- κ B and AP-1 target genes including multi pro-inflammatory and chemotactic cytokines such as TNF- α , IL-6, and MCP-1. To determine the effect of norkurarinol on mRNA ex-

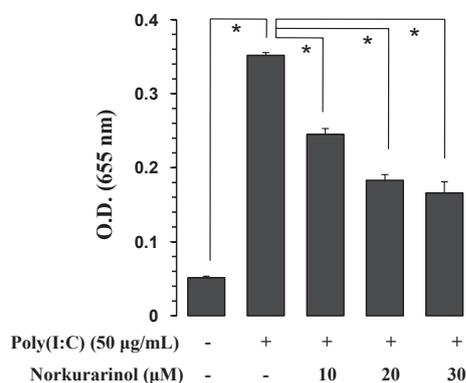


Fig. 2. Effects of norkurarinol on TLR3 activation. THP1-Blue-CD14 cells were treated with poly(I:C) (50 μ g/mL) in the presence or absence of norkurarinol for 18 h with the indicated concentrations, and TLR3 activation was assessed by measuring the levels of SEAP secreted in the supernatant by using QUANTI-Blue. **P* < 0.05.

pression of these molecules in poly(I:C)-stimulated THP-1 cells, the mRNA levels of TNF- α , IL-6, ICAM-1, VCAM-1, and MCP-1 were detected using real-time PCR. The THP-1 cells were stimulated with poly(I:C) in the presence or absence of norkurarinol. Treatment with poly(I:C) alone for 6 h led to an increase in TNF- α and IL-6 mRNA expression (31-fold and 33-fold, respectively), and these expressions were markedly inhibited by the pretreatment of norkurarinol (30 μ M) for 1 h before the treatment with poly(I:C) (Fig. 3a). Poly(I:C)-induced mRNA expression of VCAM-1, ICAM-1, and MCP-1 was also dramatically suppressed by the pretreatment with norkurarinol (30 μ M) (Fig. 3: b and c). On the other hand, a treatment with norkurarinol (30 μ M) alone did not lead to an increase in the expression of these inflammatory molecules. Because TLR3 uses a MyD88-independent signaling pathway in contrast to the other TLRs, we next examined whether the effect of norkurarinol is applicable to TLR2 signaling that is mediated by MyD88. Treatment of THP-1 with Pam3CSK4, a TLR2 agonist, induced mRNA expression of TNF- α and ICAM-1, while norkurarinol did not inhibit these mRNA inductions suggesting that this compound specifically inhibits TLR3 signaling, but not TLR2 (Fig. 3d).

Effects of norkurarinol on pro-inflammatory molecules expression in rotavirus-infected TF-104 cells

A rotavirus infection generates the production of viral dsRNA, which can induce pro-inflammatory molecules such as TNF- α and IL-6 (26). Therefore, here we investigated whether norkurarinol suppresses pro-inflammatory cytokines expression induced by a rotavirus infection. Rotavirus-infected cells increased the expression of TNF- α and IL-6, whereas the rotavirus-induced cytokine expression was reduced by the pretreatment with norkurarinol (Fig. 4: a and b), suggesting that norkurarinol might regulate pro-inflammatory cytokine production in response to rotavirus infection through the inhibition of the TLR3 signaling pathway.

Effects of norkurarinol on the TLR3-signaling through NF- κ B and MAPK/AP-1

TLR3 interacts with TRIF, which leads to the activation of NF- κ B and MAPK/AP-1 as well as the activation of IRF3. This ultimately produces pro-inflammatory cytokines (24, 25). We examined the effect of norkurarinol on the NF- κ B/AP-1 activation pathway induced by poly(I:C) in THP-1 cells. Treatment of THP-1 cells with poly(I:C) for 2 h induced NF- κ B p65 and c-fos, AP-1 subunit, translocation into the nucleus, whereas norkurarinol inhibited poly(I:C)-induced nuclear translocation of these factors in a dose-dependent manner (Fig. 5a). The effect of norkurarinol on the phosphorylation of κ B, an

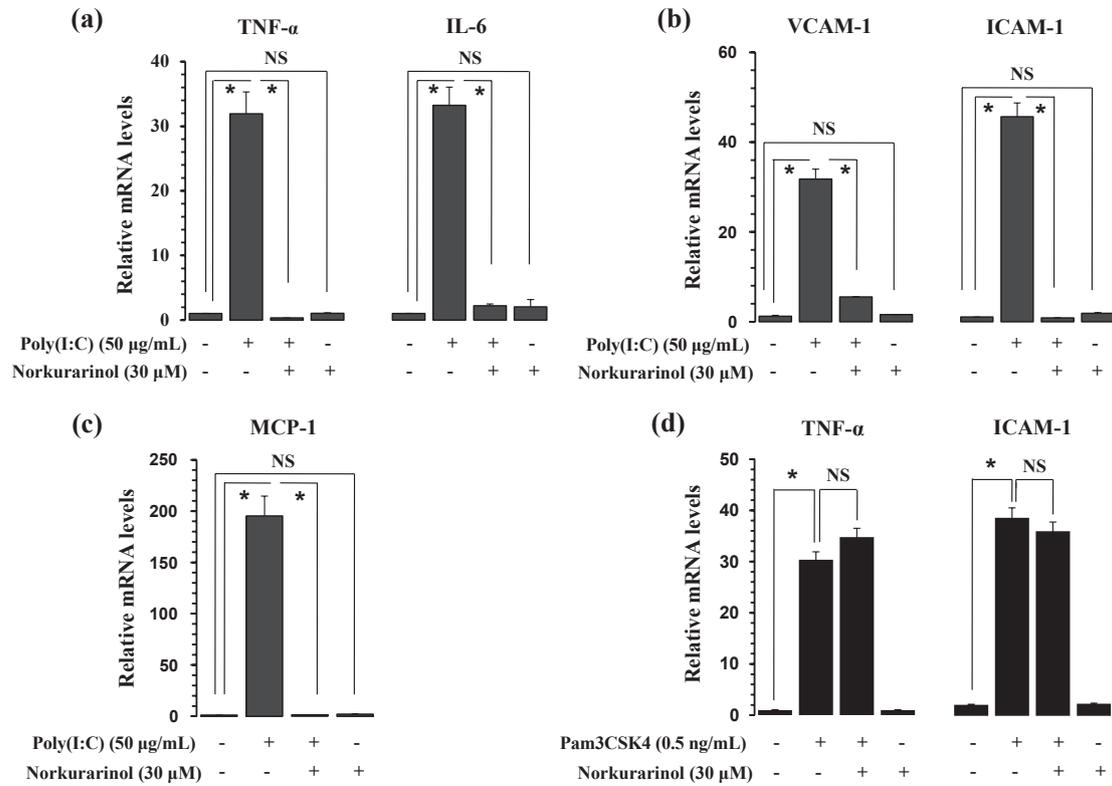


Fig. 3. Effects of norkurarinol on poly(I:C)-induced pro-inflammatory and adhesive molecule expression. THP-1 cells were incubated with poly(I:C) or Pam3CSK4 for 6 h in the presence or absence of norkurarinol. Total RNA was isolated and real-time PCR analysis of mRNA expression was performed. Data are represented as the relative expression of each mRNA normalized to GAPDH mRNA. All experiments were repeated more than 3 times. * $P < 0.05$, NS = Not significant.

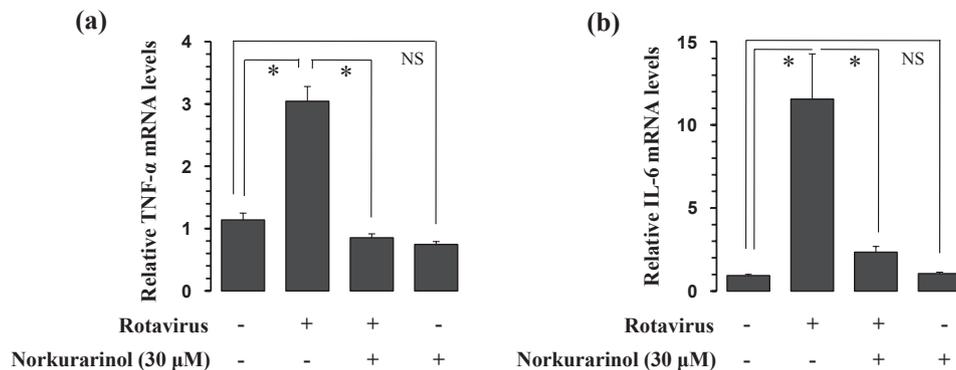


Fig. 4. Effects of norkurarinol on pro-inflammatory molecules expression in rotavirus-infected TF-104 cells. TF-104 cells were inoculated with rotavirus (KJ56-1) for 48 h in the presence or absence of norkurarinol. Total RNA was isolated and real-time PCR analysis of mRNA expression was performed. Data are represented as the relative expression of each mRNA normalized to GAPDH mRNA. All experiments were repeated more than 3 times. * $P < 0.05$, NS = Not significant.

upstream molecule of NF- κ B, was also examined. Treatment with poly(I:C) alone for 1 h induced the phosphorylation of I κ B, whereas norkurarinol inhibited the poly(I:C)-induced phosphorylation of I κ B in a dose-de-

pendent manner (Fig. 5b). IKK α and IKK β are upstream kinases of I κ B in the NF- κ B signaling pathway and phosphorylate I κ B, followed by NF- κ B activation (21). IKK γ is an important regulatory component of a high-

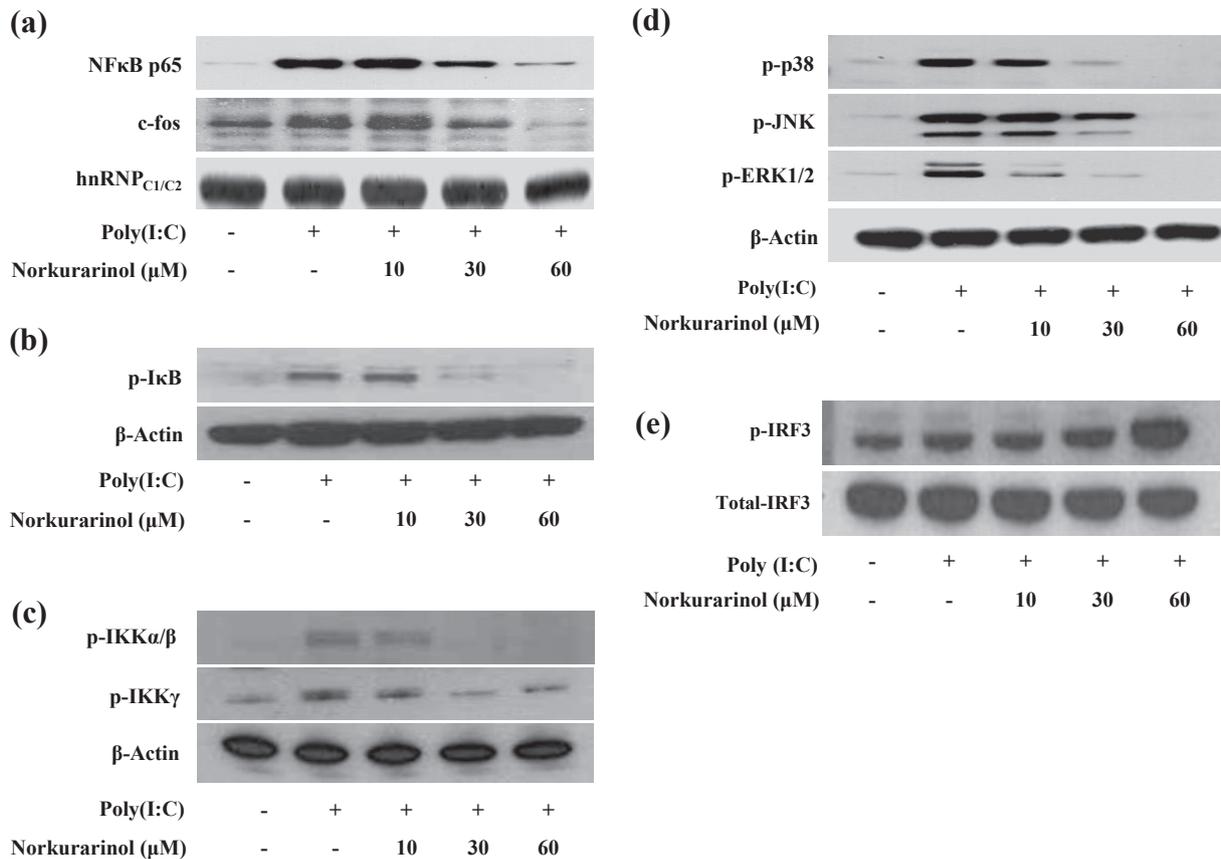


Fig. 5. Effects of norkurarinol on the TLR3 signaling pathway. THP-1 cells were incubated with poly(I:C) (50 μg/mL) in the presence or absence of norkurarinol for 2 h at the indicated concentrations. Nuclear protein and total protein were prepared from cells. Immunoblotting with the following antibodies was performed: a) anti-c-fos, anti-p65, and anti-hnRNP_{C1/C2}; b) anti-p-IκB and anti-β-actin; c) anti-p-IKKα/β, anti-p-IKKγ, and anti-β-actin; d) anti-p-p38, anti-p-JNK, anti-p-ERK1/2, and anti-β-actin; e) anti-p-IRF3 and anti-β-actin antibodies.

molecular-weight complex (the IKK complex) that also contains two catalytic proteins known as IKKα and IKKβ. Although the phosphorylation of IKKγ at Ser376 has been shown to down-regulate the NF-κB signaling pathway, some groups reported that IKKγ is needed in the activation of NF-κB by a number of stimuli such as TNF-α, IL-1, HTLV1 Tax protein, LPS, and PMA (21). Treatment with poly(I:C) alone for 1 h induced the phosphorylation of IKKα/β and IKKγ, whereas norkurarinol inhibited the poly(I:C)-induced phosphorylation of IKKα/β and IKKγ (Fig. 5c). Furthermore, norkurarinol modulated the phosphorylation of MAPKs. Treatment with poly(I:C) alone for 1 h induced the phosphorylation of p38, JNK, and ERK1/2, whereas norkurarinol inhibited poly (I:C)-induced phosphorylation of p38, JNK, and ERK1/2 (Fig. 5d). In addition, effects of norkurarinol on the IRF3 pathway were investigated. Surprisingly, norkurarinol induced an increase in IRF3 phosphorylation rather than inhibiting IRF3 phosphorylation induced

by poly(I:C) (Fig. 5e).

Effects of norkurarinol on IRF3 phosphorylation and IFNβ expression

The phosphorylation of IRF3 leads to the formation of IRF3 dimers, followed by the nuclear translocation and transcription of genes such as IFNβ (27). To examine the capacity of norkurarinol to activate IRF3, the THP-1 cells and TF-104 cells were treated with norkurarinol for 2 h, and the total protein isolated was subjected to western blotting analysis with the anti-phospho-IRF3 antibody. Norkurarinol induced the phosphorylation of IRF3 in a dose-dependent manner in THP-1 (Fig. 6a) and TF-104 cells (data not shown). The effects of norkurarinol on the IFNβ mRNA expression in THP-1 cells were also investigated with real-time PCR. Consistent with IRF3 phosphorylation, norkurarinol induced IFNβ expression in a dose-dependent manner (Fig. 6b). We next examined whether IFNβ mRNA expression induced by poly(I:C) or

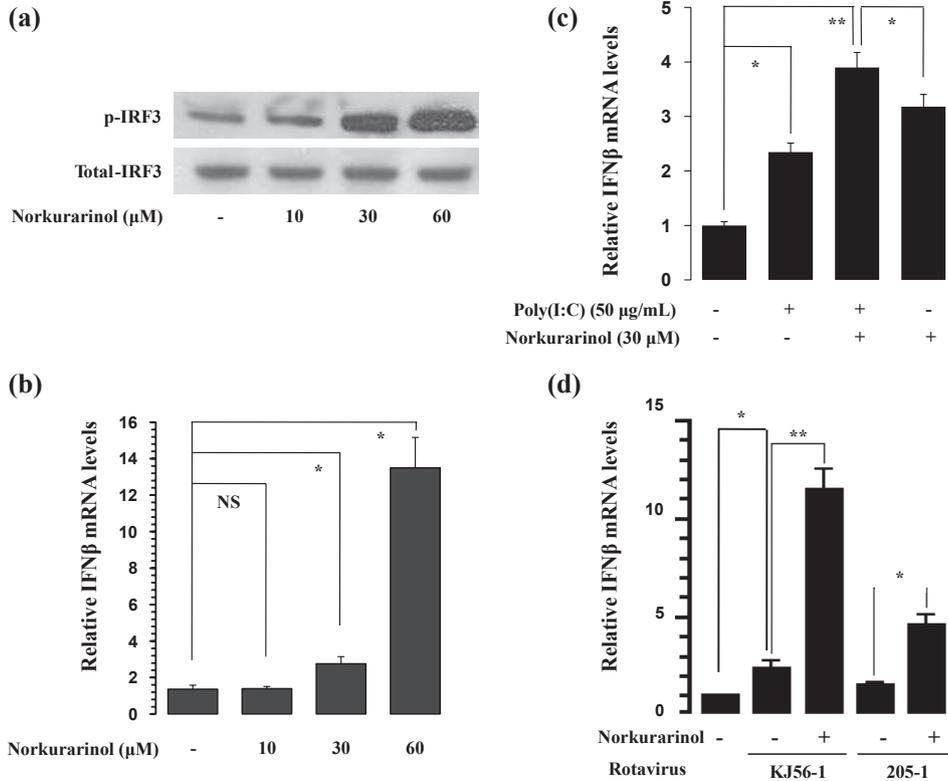


Fig. 6. Effects of norkurarinol on the IRF3 activation and IFN β production. a) THP-1 cells were incubated with norkurarinol for 2 h at the indicated concentrations, and total protein was isolated. Immunoblotting with anti-p-IRF3 and β -actin antibodies was performed on total protein (10 μ g protein/lane). b – d) Total RNA was isolated and cDNA synthesis and real-time PCR analysis were performed. Cells were incubated with norkurarinol for 6 h at the indicated concentrations (b). THP-1 cells were incubated with poly(I:C) (50 μ g/mL) in the presence or absence of norkurarinol for 2 h at the indicated concentrations (c). TF-104 cells were infected with 0.01 MOI rotaviruses for 1 h, and the cells were then incubated in the presence or absence of 60 μ M norkurarinol for 24 h (d). Data are represented as the relative expression of IFN β mRNA normalized to GAPDH. All experiments were repeated more than 3 times. **P* < 0.05, ***P* < 0.01, NS = Not significant.

virus infection is increased by the pretreatment with norkurarinol. Pretreatment with norkurarinol showed synergic effect on poly(I:C)-induced IFN β mRNA expression (Fig. 6c). This compound also markedly increased the IFN β mRNA expression in response to both rotavirus KJ56-1 and 205-1 infection (Fig. 6d).

Antiviral effect of norkurarinol

A rotavirus is genetically comprised of 11 segments of dsRNA (28, 29). The purified genomic dsRNA from a rotavirus or reovirus are recognizable by TLR3 (10, 26). Therefore, this study evaluated the antiviral effect of norkurarinol against two types of rotaviruses, KJ56-1 (bovine rotavirus, G8P[7]) and 205-1 (porcine rotavirus, G5P[7]), using the viral CPE reduction assay in TF-104 cells. TF-104 cells were almost destroyed by the infection of rotaviruses, whereas the norkurarinol treatment (30 μ M) protected the TF-104 cells from the destruction induced by these rotaviruses (Fig. 7). In addition, the TF-104 cells infected with 0.001 MOI rotavirus (KJ56-1, G8P[7]) were incubated in a medium containing norkurarinol (30 μ M) for 72 h. The viral yield was estimated using a CCID₅₀ assay. The rotavirus titer (1.4×10^4 CCID₅₀) of the norkurarinol-treated cells was lower than that (4.1×10^9 CCID₅₀) of the untreated cells. This indicates that norkurarinol inhibits rotavirus shedding.

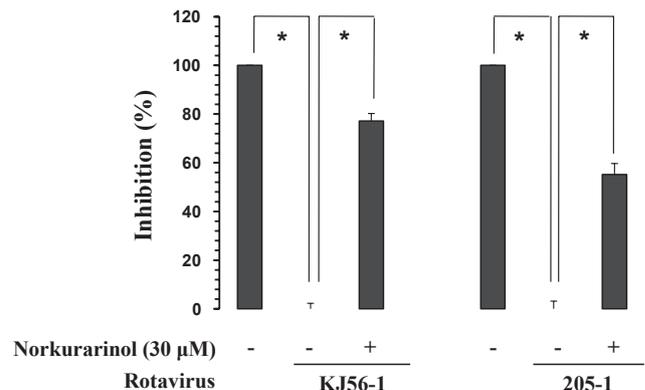


Fig. 7. Antiviral effect of norkurarinol. TF-104 cells were inoculated with two types of rotaviruses, KJ56-1 and 205-1, with or without norkurarinol (30 μ M). Three days after inoculation of rotaviruses, cytopathic effects were measured by adding neutral red solution. All experiments were repeated more than 3 times. **P* < 0.05.

Discussion

Our results provide the first evidence that norkurarinol can be a novel treatment for blocking the pro-inflammatory responses induced by a dsRNA virus infection and virus replication. Although current data does not identify

the direct target of norkurarinol on the TLR3 signaling pathway, norkurarinol blocked the inflammatory response induced by poly(I:C), a synthetic analogue of dsRNA, as well as the rotavirus-induced CPE. TLRs play a key role in regulating the activation of the innate immune response to microbial genomes such as viral dsRNA, ssRNA, viral DNA, and bacterial DNA (1, 2). Ten human TLRs have been characterized thus far. They are expressed predominantly by cells involved in immune functions such as monocytes and macrophages (1, 2). In particular, TLR3 is an intracellular TLR localized in endosomes and it binds to the dsRNA of viral origin (30–32). On the other hand, *Sophora flavescens* has been identified as having anti-inflammatory activities (17, 19, 21) and it has previously reported that polyphenols, pterocarpan, and flavanones from *Sophora flavescens* have glycosidase and neuraminidase inhibitory activities (18, 20). Recently, one article reported that matrine isolated from *Sophora flavescens* has protective effects against tissue damage caused by inflammation from NO-dependent vasomotion and has inhibitory effects on inflammatory cytokines induced by LPS (33). We showed here that norkurarinol isolated from *Sophora flavescens* might be a good material for specifically suppressing TLR3 signaling. Ligand interaction with TLR3 recruits the adaptor protein TRIF, which in turn activates NF- κ B, MAPK/AP-1, or IRF3 pathway. Norkurarinol effectively inhibited NF- κ B/AP-1-inducible SEAP activity induced by poly(I:C). This compound also completely inhibited the mRNA expression of pro-inflammatory cytokines and leukocyte adhesion molecules induced by poly(I:C) and rotavirus infection.

Although most TLRs share signaling components with the IL-1 receptor, including IL-1 receptor-associated kinase (IRAK), the cytoplasmic adaptor molecule MyD88, and TRAF6, TLR3 signals through a MyD88-independent pathway involving the adaptor molecule TRIF (30–34). In this study, we found that norkurarinol effectively inhibited the expression of TNF- α , IL-6, MCP-1, VCAM-1, and ICAM-1 induced by the TLR3 agonist, while it did not affect the expression of TNF- α and ICAM-1 induced by the TLR2 agonist (Fig. 3). TLRs use the different adaptor molecules which define the downstream signaling pathways, whereby they confer their specificity. Our results suggesting that norkurarinol might specifically inhibit TLR3 signaling through the suppression of the TRIF-dependent pathway. We found here that norkurarinol also substantially inhibited the expression of IL-6, MCP-1, and VCAM-1 induced by LPS, a TLR4 agonist (data not shown). Because TLR4 uses both MyD88-dependent and MyD88-independent (TRIF-dependent) pathways, norkurarinol might affect the TLR4 pathway through the suppression of TRIF. These results suggest-

ing that norkurarinol might be a specific inhibitor for the adaptor molecule TRIF. However, additional studies will be required to fully identify how norkurarinol inhibits TRIF.

Most viruses synthesize dsRNA at some point during their replicative cycle. The rotavirus is genetically comprised of 11 segments of dsRNA (28, 29). The purified genomic dsRNA from the rotavirus is recognizable by TLR3 (10, 26), suggesting that the genomic dsRNA and TLR3 signaling might be involved in viral pathogenesis. In addition, NF- κ B activation supported rotavirus replication by inhibiting premature apoptosis in the early stages of infection (35). Therefore, the inhibition of TLR3 signaling and the subsequent decrease in the pro-inflammatory responses can be a potential method of treating a dsRNA virus infection. The present study showed that norkurarinol blocked the NF- κ B and AP-1 signaling pathway as well as the phosphorylation of MAPKs induced by poly(I:C). On the other hand, norkurarinol failed to inhibit IRF3 phosphorylation induced by poly(I:C). Rather than blocking IRF3 phosphorylation, norkurarinol enhanced IRF3 phosphorylation and IFN β expression in a dose-dependent manner. It was reported that some cell types including hepatocytes contained two distinct antiviral signaling pathways leading to expression of type I IFNs, one dependent upon TLR3 and the other dependent on the retinoic acid-inducible gene I (RIG-I), with little cross-talk between these pathways (36). Activation of the RIG-I pathway by the intracellular presentation of dsRNA or another viral PAMP produced by replicating viruses may contribute to the initial induction of type I IFNs. Norkurarinol induced IFN β expression while suppressing the TLR3/TRIF-dependent pathway, suggesting that norkurarinol might induce IFN β expression through a TRIF-independent pathway such as the RIF-1 pathway. However, further studies are needed to explicitly determine the mechanisms by which norkurarinol induces IRF3/IFN β expression. IFN β has been reported to have the ability to diminish the production of TNF- α and IL-6 as well as inhibit virus replications (37, 38). The use of IFN β as immunotherapy has increased in recent years with an expanding list of potential applications.

In conclusion, this study provides the first biochemical evidence that norkurarinol can modulate TLR3 activation and block the NF- κ B, AP-1, and MAPK signaling pathways and subsequent inflammatory responses, which might prevent the prolonged inflammation responses induced by the synthetic analogue of dsRNA and rotavirus infection (summarized in Fig. 8). In addition, norkurarinol was found to have novel actions such as being a potent inducer of IRF3/IFN β expression and a modulator of rotavirus replication.

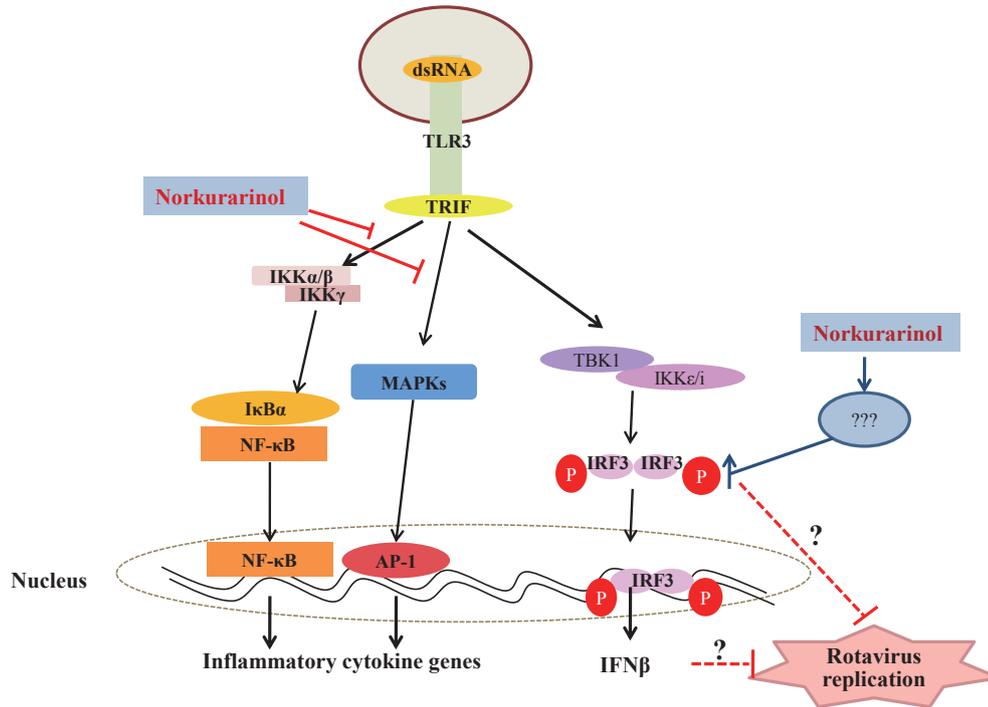


Fig. 8. Schematic depiction of norkurarinol actions on TLR3-mediated signaling pathway and rotavirus replication. Norkurarinol blocks dsRNA-induced TLR3 activation, leading to blockade of NF- κ B and MAPK/AP-1 signaling pathway and subsequent inflammatory responses. On the other hand, norkurarinol induces IRF3 phosphorylation and IFN β expression, and norkurarinol blocks rotavirus replication.

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