Novel Oxicam Nonsteroidal Compound XK01 Attenuates Inflammation by Suppressing the NF- κ B and MAPK Pathway in RAW264.7 Macrophages

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Research Article

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Abstract

Traditional non-steroidal anti-inflammatory drugs (NSAIDs) show server adverse effects during clinical use, which limits their usage. Oxicams (e.g., piroxicam, meloxicam) are widely used as NSAIDs. However, selectivity to cyclooxygenase (COX) 2 may cause cardiovascular problems considering the long-term use of the drugs. Therefore, it is important to develop new non-steroidal compounds as anti-inflammatory drugs. In the present study, we evaluated the anti-inflammatory activity of a newly developed nonsteroidal drug XK01 *in vitro*. Our data showed that XK01 reduced the contents of nitric oxide (NO) and reactive oxygen species (ROS), and inhibited the transcription levels of tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and IL-1 β in a dose-dependent manner in lipopolysaccharide (LPS)-stimulated mouse RAW264.7 macrophages. XK01 showed no significant inhibitory effect on COX-1, but inhibited the expression of COX-2. At molecular level, XK01 prevented the translocation of p65 protein from the cytoplasm to the nucleus, and inhibited the phosphorylation of p65, I κ B, and MAPKs proteins. In addition, high concentration of XK01 also inhibited the phosphorylation of JNK, p38 and ERK, showing stronger effect than that of meloxicam. Our data indicate that XK01 inhibits the expression of inflammatory mediators and COX-2, and exhibits potential anti-inflammatory effects *in vitro* via suppressing the NF- κ B and MAPK pathway in RAW264.7 macrophages.

Introduction

Inflammation is a complex reaction of the immune system induced by stimuli, which provides self-protection for the host (Fornai et al., 2005). Macrophages play an important role in maintaining the homeostasis of the internal environment. Under the stimulation of pathogens, macrophages rapidly secrete active substances such as proteases and cytokines, phagocytose and digest cell debris or pathogens, and further activate immune cells or lymphocytes to respond to pathogens (Antman, 2017, Wiseman and Lombardino, 1981). Lipopolysaccharides (LPS) can activate the immune response and induce inflammation. LPS forms a complex with LPS binding protein (LBP) and pattern recognition receptors such as CD14 and Toll-like receptor 4 on the cell membrane surface of macrophages (Obremsky et al., 1994), which activates the adaptor protein myeloid differentiation protein (MyD88) and related kinases. Then turn on the NF- κ B signaling pathway to induce inflammatory response, regulating the synthesis and release of inflammatory factors including tumor necrosis factor (TNF), interleukins (ILs), nitric oxide (NO), cyclooxygenases (COX) and prostaglandin PGE2 (Duerden, 1975, Berte and Richelmi, 1988), causing symptoms such as fever, endothelial damage, capillary leakage, coagulation and microcirculation disturbances (Gagne et al., 2012).

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs with anti-inflammatory, antipyretic and analgesic effects (Ochi et al., 2014). When inflammation occurs, NSAIDs exert anti-inflammatory effect via blocking the synthesis of COX, thus the COX fails to catalyze the synthesis of cyclic endoperoxides from arachidonic acid (AA) to form PGE2. Traditional NSAIDs, such as diclofenac, piroxicam, and indomethacin are mostly non-selective COX inhibitors, which can inhibit COX-1 and COX-2, showing a stronger inhibitory effect on COX-1. Meloxicam blocks the production of COX-1-derived

prostaglandins (PGs) and disrupts the protective effect of COX-1 on the gastric mucosa, causing gastrointestinal side effects (Radia et al., 2012, Ahad et al., 2014). Studies have demonstrated that activation of NF- κ B promotes the expression of COX-2 (Ye et al., 2019). In contrast, MAPK family members induce COX-2 gene expression in various cell types such as macrophages, cardiomyocytes, human umbilical vein endothelial cells (HUVECs), and smooth muscle cells (Wang et al., 2014, Huang et al., 2013), and MAPKs regulate the cellularity of macrophages during stimulation (Rukoyatkina et al., 2013, Kuan et al., 2013).

Most NSAIDs are restricted by their adverse effects. Meloxicam, an important member of NSAIDs, exhibits good anti-inflammatory effects, gastrointestinal safety, and plays an increasingly important role in clinical practice because it preferentially inhibits COX-2 more than COX-1. However, long-term use of this drug is a challenge to cardiovascular health. In our previous work, we developed a novel oxicam-based non-steroidal compound XK01. In this study, we evaluated the anti-inflammatory effects of XK01 in mouse peritoneal mononuclear macrophage RAW264.7 cells, which were stimulated with LPS. XK01 showed strong anti-inflammatory effects by inhibiting the expression of inflammatory mediators and COX-1. Our data provided a theoretical basis for the further development of new non-steroidal anti-inflammatory drugs.

Methods And Materials

Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and PBS were purchased from Hyclone (Logan, Utah). Fetal bovine serum (FBS) was from Gibco (Grand Island, NY, USA). LPS and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich Co. (St. Louis, Mo, USA). Enzyme-linked immunosorbent assay (ELISA) kits for measuring IL-6, TNF- α and PGE2 mouse ELISA kit was purchased from Elabscience (Wuhan, China). ROS kit and NO detection kit were purchased from Shanghai Beyotime (Shanghai, China). RNA extraction kit and RNA reverse transcription kit were purchased from Vazyme Biotech Co. (Nanjing, China). RT-PCR Mix and ultra-sensitive ECL chemiluminescence kit were acquired from BioRad Laboratories (Hercules, CA, USA). Antibodies for P38, p-P38, JNK, p-JNK were purchased from Signalway Antibody (California, US). Antibodies for p-NF- κ B, NF- κ B, ERK and p-ERK were purchased from Cell Signaling Technology (Beverly, MA, US).

Cell Culture

RAW264.7 cells were purchased from the Cell Resource Center, Shanghai Academy of Life Sciences, Chinese Academy of Sciences. The cells were maintained in a high-glucose DMEM supplemented with 10% FBS and 1% of an antibiotic mix, at 37°C in a humidified incubator with 5% CO₂.

Cell Viability Assay

MTT assay was conducted to evaluate the cytotoxicity of LPS, meloxicam (positive drug) and XK01. RAW264.7 cells were seeded in a 96-well plate and cultured for 24 h. Cells were treated with different concentrations of the drugs or vehicle (3 wells per dose) for 24 h. MTT solution was added to the cells. After incubating for 5 h, the medium was removed and 150 μ L of dimethyl sulfoxide was added to each well. To fully dissolve the formazan crystals, the plate was covered with silver paper and shaken on a shaker for 20 min. The absorbance (A) value at 490 nm was read using a microplate reader. The data from three independent experiments was processed by Prism (GraphPad Software, San Diego, CA) program.

NO assay

After treatment, sodium nitrite was used as a positive control. After treatment, Griess Reagent I (50 μ L per well) and Griess Reagent II (50 μ L per well) were added to the cells, respectively and incubated for 3 min in dark. Then the absorbance (A) at 540 nm wavelength was read using a multifunctional microplate reader to measure the content of NO.

ROS analysis

The cells were seeded in a 24-well plate and cultured for 24 h. Then, cells were culture in serum-free medium for 12 h followed by treatment with different concentrations of drugs, and LPS inducers were added. After 24 h, ROS fluorescent probe DCFH-DA was added and incubated for 30 min. Wash the cells with serum free medium to remove the remaining fluorescent probes. The fluorescence intensity was observed under a fluorescence microscope) and quantified using Image J software.

ELISA assay

After treatment, cells were collected and lysed by repeated freezing and thawing. The supernatant was collected after centrifuge. Levels of TNF- α , IL-1 β and IL-6 were measured using ELISA kits according to the manufacturer's instructions.

Real-time PCR

RNA was extracted by using the TRI reagent (Thermo Fisher Scientific, Waltham, MA). cDNA was generated by using the RNA reverse transcription kit. Quantitative RT-PCR was performed in a CFX96 real-time system (Bio-Rad, Hercules, CA). The specific sense and antisense primers were listed in Table 1. The PCR reaction system containing 12.5 μ L SYBR Green PCR master mix, 10.5 μ L of 1 μ M primer stock and 2 μ L of cDNA. The primers used in this study are listed in Table 1 (XXXXXX).

Table 1

Name	Forward	Reverse
GAPDH	ACCCCAGCAAGGACACTGAGCAAG	GGCCCCTCCTGTTATTATGGGGGT
TNF- α	CCCTCCTGGCCAACGGCATG	TCGGGGCAGCCTTGTCCCTT
IL-1 β	GCCTCGTGCTGTCTGGACCCATAT	TCCTTTGAGGCCCAAGGCCACA
IL-6	TGGGACTGATGCTGGTGACA	ACAGGTCTGTTGGGAGTGGT
COX-1	GCCCTTCAATGAATACCGAAAG	GGGTAGAACTCTAAAGCATCGA
COX-2	ATTCCAAACCAGCAGACTCATA	CTTGAGTTTGAAGTGGTAACCG
PGE ₂	TCTCATCGCACTGGCACTGTTG	AGGCAGGTTCCCAGCAGGTC

Western blots

After treatment, cells were collected and washed with cold PBS. Cell pellets were resuspended in RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM sodium chloride, 1% NP-40, 0.2% SDS, 0.5% sodium deoxycholate, 0.1 mM EDTA and 1% protease and phosphatase inhibitors (Sigma-Aldrich). Lysates were centrifuged and supernatants were collected. Cell lysates (25 μ g) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P membranes; Millipore, Billerica, MA, USA). Membranes were blocked with blocking buffer (5% skim milk, 0.1% Tween-20 in PBS) for 1 h at room temperature. After incubation with primary antibodies overnight at 4°C, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, detected using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA) with the ODYSSEY Fc, Dual-Mode Imaging system (Li-COR, Lincoln, NE).

Statistical analysis

Data were expressed as means $\pm$ SD and were analyzed by two-tailed t-tests and two-way ANOVA. Data were given with 95% confidence intervals and were reported with corresponding P values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). GraphPad Prism 6 and SPSS 13.0 were used for all statistical analysis.

Results

XK01 showed low cytotoxic effects on RAW264.7 mouse macrophages cell lines

We firstly investigated the cytotoxic effect of XK01, meloxicam (Melo) and LPS on cell viability of RAW 264.7 mouse macrophages. The results showed that the cell viability was significantly decreased when the macrophages were treated with concentrations 50, 100 and 200 μ g/mL of XK01 (* $P < 0.05$ or *** $P <$

0.001), and the cytotoxicity is stronger at 200 µg/mL (Figure 1A). The positive control drug Melo showed similar cytotoxic effects as XK01 (Figure 1B). Moreover, LPS treatment with the indicated concentrations decreased the cell viability of the mouse macrophages (Figure 1C, $**P < 0.01$). Based on the results, a concentration of 100 µg/mL of XK01 or Melo was used as the maximum concentration in the subsequent experiments.

XK01 suppressed LPS induced ROS and NO production in RAW264.7 macrophages

The effects of XK01 on NO production in the LPS-induced RAW264.7 cells were examined. The results showed that after LPS treatment, the NO level was significantly increased in a dose-dependent manner (Fig. 2A, $**P < 0.01$ or $***P < 0.001$). Pretreatment with XK01 significantly reduced the NO production in the LPS-stimulated macrophages, and a high concentration (100 µg/mL) of XK01 reduced the NO production to 11.75 ± 0.07 µM, which was similar to that of the Melo (Fig. 2B). The effects of XK01 on LPS-induced ROS production were also detected. As shown in Fig. 2C, different concentrations of XK01 (6.25 µg/mL, 25 µg/mL, 100 µg/mL) as well 100 µg/mL of Melo significantly inhibited the production of ROS in LPS-induced RAW264.7 cells (Fig. 2C). The inhibitory effect of XK01 on ROS production was confirmed through observation of fluorescent signals using DCFH-DA as a probe. As shown in Figure 2D, no fluorescence was observed in the normal RAW264.7 cells. The fluorescence intensity was significantly increased when treated with LPS, which was gradually attenuated with the increasing concentration of XK01.

XK01 inhibited LPS-induced inflammatory cytokines secretion in RAW264.7 macrophages

We investigated the effects of XK01 on the mRNA expression of inflammatory cytokines in the LPS-induced macrophages as well levels of TNF-α and IL-1β in the cell culture supernatant. The transcriptional expression of TNF-α, IL-1β and IL-6 mRNA was significantly increased when the macrophages were treated with LPS alone when compared to the untreated cells. Pretreatment of XK01 or Melo significantly inhibited the mRNA expressions of the above mentioned inflammatory factors (Fig. 3A-C). The ELISA assay showed that LPS induced the secretion of TNF-α and IL-1β, which were also significantly decreased in the XK01-pretreated cells (Fig. 3D-E). The experimental results indicated that the new compound XK01 had a good inhibitory effect on the inflammatory cytokines release in macrophages. The inhibitory effects of high concentration XK01 was similar to that of 100 µg/mL of positive control Melo.

XK01 inhibited the expression of cyclooxygenase 2 induced by LPS in RAW264.7 macrophages.

The expression of COX-1 and COX-2 mRNA and protein as well the PGE2 mRNA were evaluated in the LPS-induced RAW264.7 macrophages. After LPS stimulation, the expression levels of COX-2 mRNA as well the protein were significantly up-regulated, which were attenuated by pretreatment with XK01 or Melo (Fig. 4A, E, F). However, the expression of COX-1 showed no significant difference between the treatment

groups and the normal control group (Fig. 4B, C), which indicated that LPS, XK01 or Melo did not inhibit the COX-1 expression in the macrophages (Figure 4A-C). In addition, LPS stimulated the mRNA expression of PGE2, a target of COX; pretreatment with XK01 or Melo significantly decreased the PGE2 mRNA expression, and the inhibitory effects of 100 µg/mL XK01 and Melo were similar (Fig. 4D). Similar results were also observed by using immunofluorescence assay. As shown in Fig. 4G, the COX-2 signal was increased in LPS-induced cells, which was attenuated in the XK01 or Melo-pretreated cells. These results indicate that the new compound XK01 exerts the anti-inflammatory effect at least partially via inhibition of COX-2, and it might be a selective COX-2 inhibitor.

XK01 inhibited the activation of NF-κB induced by LPS in RAW264.7 macrophages

We evaluated the effects of XK01 pretreatment on NF-κB activation in LPS-induced macrophage cell lines. The results showed that XK01 intervention decreased the phosphorylation of p65 and IκBα induced by LPS in RAW264.7 cells (Fig. 5A-C). Moreover, LPS induced the nuclear p65 and suppressed the cytosolic levels in the macrophages, which means a large amount of p65 protein in the cytoplasm entered the nucleus after LPS stimulation. Pretreatment with a medium (25 µg/mL) or high concentration (100 µg/mL) of XK01 inhibited the nuclear translocation of the cytoplasmic p65 (Fig. 5D-F). IFA assay confirmed that translocation of NF-κB p65 from the cytoplasm to the nucleus was inhibited by pretreatment of XK01 or Melo in LPS-induced RAW264.7 macrophages (Figure 5G).

XK01 suppressed LPS-induced MAPK pathway

The mitogen-activated protein kinase (MAPK) family is widely involved in cell differentiation, metastasis, and inflammation. After LPS stimulation, the phosphorylation levels of ERK1/2, JNK and p38 in the MAPK pathway were significantly increased (Fig. 6). After cells were pretreated with XK01, a medium (25 µg/mL) or high concentration (100 µg/mL) of XK01 inhibited the phosphorylation of ERK1/2, JNK and p38 induced by LPS, displaying stronger inhibitory effects on p-ERK1/2 and p-JNK at the high concentration (Fig. 6).

Discussion

In this study, we investigated the effects of a newly developed nonsteroidal XK01 on inflammation induced by LPS *in vitro*. Similar to meloxicam, XK01 with a concentration of 50 µg/mL or more was low cytotoxic to the mouse macrophages. ROS production induced by LPS can elicit a pro-inflammatory response by upregulating levels of the pro-inflammatory cytokines that act as messengers in subsequent processes, and it interacts with NO to generate more lethal intermediates, leading to cytotoxic damage (Ko et al., 2017). Our data indicated that after pretreatment with different concentrations of XK01, IL-1β, IL-6, TNF-α, and NO levels were significantly decreased in a dose-dependent manner. ROS plays a major role in the immune system any pathogen attack. High level of ROS is always a result of immune dysfunction. The present findings indicated that XK01 exerted potent scavenging effect on cellular ROS production.

XK01 can inhibit the COX-2 at protein and mRNA levels in a dose-dependent manner. At the same time, the expression of the COX-2 product prostaglandin is also reduced, and XK01 at 100 µg/mL showed similar effects as positive control meloxicam. In the present study, XK01 showed no significantly effects on COX-1, which indicates that XK01 may have good gastrointestinal adaptability. The new compound XK01 may be a new NSAID of selective COX-2 inhibitor.

Nuclear factor NF-κB is an important transcription factor that controls the transcription of a variety of cellular genes such as COX-2, regulating the inflammatory response. (Kundu et al., 2006). The new compound XK01 significantly inhibited the phosphorylation of NF-κB upstream molecules, including IκBα and NF-κB p65. After translocation from the cytoplasm to the nucleus, they engage in transcriptional activation of target genes, various inflammatory factors are transcribed. A strong fluorescence intensity of NF-κB was detected in the nucleus in response to LPS exposure, which was blocked by XK01 pretreatment, indicating the XK01 prevented the entry of NF-κB into the nucleus. Therefore, the anti-inflammatory response of the new compound XK01 is regulated by NF-κB signaling pathway.

The MAPK signaling pathway, including ERK1/2, JNK and p38 MAPK, is widely involved in the regulation of cell life activities (Coskun et al., 2011). It well known that LPS activates the phosphorylated expression of p38, ERK and JNK in RAW264.7. Pretreatment with XK01 attenuated the activation of the indicated members of MAPK family. In this process, the effect of XK01 on p-ERK1/2 and p-JNK are more potential than on p-p38, and XK01 exerted a stronger effect when compared to the same dose of meloxicam.

Conclusion

XK01 can reduce NO level, scavenge ROS, and down-regulated the levels of COX-2, TNF-α, IL-6, and IL-1β, which suggests that XK01 may be a potential anti-inflammatory agent. Regarding the molecular mechanisms, XK01 alleviates the inflammatory response via inhibition of the release of COX-2 and other inflammatory mediators, which was at least partially by down-regulating the activation of MAPK and NF-κB signaling pathways induced by LPS in RAW264.7 cells.

Abbreviations

COX
Cyclooxygenase
NSAID
Nonsteroidal anti-inflammatory drugs
ILS
Interleukin
ERK
Extracellular signal-regulated kinase
JNK
c-Jun N-terminal kinase

NF-κB
Nuclear factor-Kappa B
IκB
Inhibitory kappa B
TNF-α
Tumor necrosis factor-α

Declarations

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NOTES

The authors declare that they have no financial conflicts of interest relevant to this study.

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Figures

Figure 1

Cytotoxic effects of XK01 on RAW264.7 mouse macrophages cell lines. RAW264.7 cells were treated with XK01 (A), Meloxican (Melo, B) or LPS (C) at various concentrations as indicated. MTT colorimetric assay was used to determine the cell viability. Graphs show mean $\pm$ SEM of at least three independent experiments. * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$ obtained from Student's t-test.

Figure 2

XK01 inhibited NO and ROS production in LPS-activated RAW264.7 mouse macrophages cell lines. RAW264.7 cells were pre-treated with the indicated concentrations of XK01 or 100 μ g/ mL Melo followed by LPS stimulation. (A and B): Levels of NO were measured; C: Fluorescence intensity of ROS was quantified; D: Representative images of of fluorescent signal for ROS observed under microscope. Graphs show mean $\pm$ SEM of at least three independent experiments. * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$, ### $P < 0.001$ obtained from Student's t-test.

Figure 3

XK01 inhibits proinflammatory cytokine production in LPS-activated RAW264.7 mouse macrophages. RAW264.7 cells were pretreated with Melo and different concentrations of XK01 and then stimulated with LPS (10 μ g/mL). The transcription of inflammatory cytokines IL-1 β (A), TNF- α (B) and IL-6 (C) RNA were detected by real-time quantitative PCR, and the pro-inflammatory cytokines IL-1 β (D) and TNF- α (E) in conditioned medium were measured by ELISA. Graphs show mean $\pm$ SEM of at least three independent experiments. * P < 0.05. ** P < 0.01, *** P < 0.001, ### P < 0.001 from Student's t-test.

Figure 4

XK01 inhibited the expression of COX-2 and PGE2 in LPS-activated RAW264.7 mouse macrophages. RAW 264.7 Cells were pretreated with Melo and different concentrations of XK01 followed by LPS stimulation. A: Western blot was used to detect the expression of COX-1 and COX-2 proteins. B and F: Quantification of COX-1 and COX-2 proteins respectively. C-E: The mRNA expression of COX-1, PGE2 and COX-2 detected by real-time qPCR. G: COX-2 was detected by immunofluorescence assay. Graphs show mean $\pm$ SEM of at least three independent experiments. $^*P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{##}P < 0.01$ from Student's t-test.

Figure 5

XK01 pretreatment inhibited NF-κB signaling in LPS-activated RAW264.7 mouse macrophages.

RAW264.7 cells were pre-treated with different concentrations of XK01. A and D: Western blot was used to measure the expression of p-p65, p65, p-IκBα, nuclear and cytoplasmic p65; B, C, E and F: Quantification of p-p65/p65, p-IκBα/β-Action, p65/α-tubulin and p65/Histon H3; G: The effects of 100 μg/mL XK01 and Melo on NF-κB p65 nuclear translocation immunofluorescence assay. Graphs show mean ± SEM of at

least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, # $P < 0.05$, ## $P < 0.01$ from Student's t-test.

Figure 6

XK01 inhibited the expression of phosphorylated proteins of ERK1/2, JNK and P38 in MAPK. LPS-stimulated RAW264.7 cells were pre-treated with different concentrations of XK01. A: Western blot was used to detect the expression of ERK1/2, p-ERK1/2, JNK, p-JNK, P38 and p-P38; B-D: Quantification of p-ERK1/2/ERK1/2, p-p38/p38 and p-JNK/JNK. Graphs show mean $\pm$ SEM of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, ## $P < 0.01$ from Student's t-test.