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Cellular Anti-Inflammatory Activity of Novel I-κB Kinase Inhibitor

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ABSTRACT

We previously isolated oxo-cyclohexene-acetic acid,ketomycin, from a culture filtrate of *Actinomycetes* as an inhibitor of cancer cell migration and invasion. The mechanistic study revealed that it inhibited constitutively activated NF-kB and downstream MMP-9 and 11 expressions in human breast carcinoma cells. In the present research, we looked into the mechanism of NF-kB inhibition, and found that ketomycin inhibited I-kB kinase b activity in vitro. Moreover, physical binding between ketomycin and I-kB kinase b was confirmed by surface plasmon resonance. As an I-kB kinase inhibitor, ketomycin inhibited LPS-induced IkBa degradation, NF-kB activation, NO production, and IL-6 secretion at the nontoxic concentrations in mouse monocytic leukemia RAW264.7 cells. Thus, ketomycin is a new inhibitor of I-kB kinase b, and may be a candidate of non-steroidal anti-inflammatory drugs.

INTRODUCTION

Long-term use of steroids can suppress the immune system and may induce ulcers or gastrointestinal bleeding. Steroid therapy can also increase the risk of bone fractures. Other side effects of steroids include the thinning of the bones, influences to blood pressure causing arteriosclerosis, and glaucoma, in which pressure in the eye increases abnormally. Therefore, a search for nonsteroidal anti-inflammatory agents should be useful for the treatment of inflammatory diseases. Way of drug discovery includes screening from natural sources, chemical library, rational molecular design without computer and computer–assisted molecular design [1]. We employ screening from natural sources and rational molecular design. We have designed a novel NF-kB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), based on the natural compound epoxyquinomicin [2]. DHMEQ directly binds to and inactivates NF-kB components [3]. It inhibited cellular models of osteoporosis [4], psoriasis [5], nasal polyp [6], and peritoneal inflammation [7]. Moreover, DHMEQ ameliorated rheumatoid arthritis [8], sepsis [9], and graft rejection [10] in mice. Based on the structure of DHMEQ, we designed and synthesized its stable analog, (S)-β-Salicyloylamino-α-Exo-Methylene-γ–Butyrolactone (SEMBL) [11], and parasitenone, having the core structure of DHMEQ [12] as NF-kB inhibitors.More recently, we isolated cyclopenol and cyclopenin from a deep sea microorganism as inhibitors of cellular NF-kB. Among them, cyclopenin was found to ameliorate a fly Alzheimer

model, possibly acting as an anti-inflammatory agent [13]. We are also screening inhibitors of cancer cell migration. Recently, we isolated a known antibiotic, ketomycin (Figure 1A), from *Actinomycetes* as an inhibitor of human breast cancer MDA-MB-231 cell migration [14]. It inhibited cellular invasion and MMP-9, 11 expressions, by inhibiting cellular NF-kB in MDA-MB-231 cells. However, the upstream mechanism for the inhibition of cellular NF-kB has not been elucidated. In the present research we found that it directly inhibited I-kB kinase b (IKKb), the upstream signal of NF-kB. It also inhibited production of inflammatory mediators in mouse macrophage-like RAW264.7 cells.

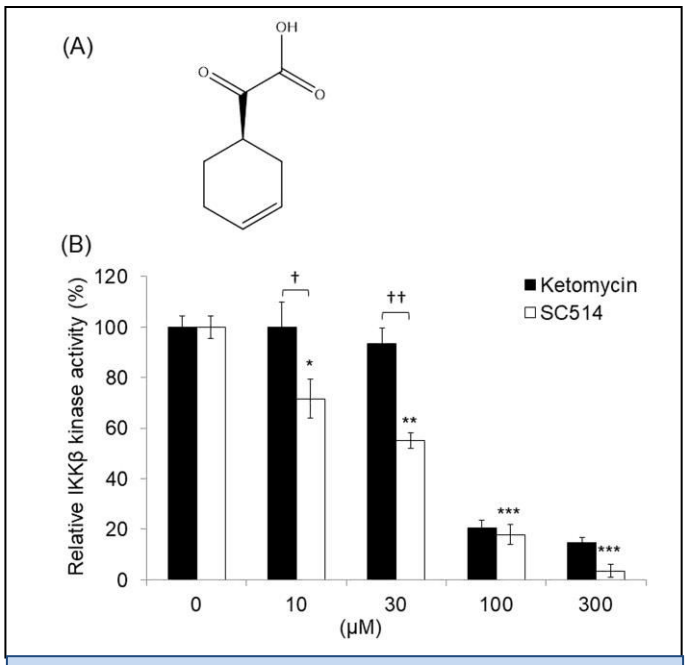

Figure 1: Inhibition of IKKβ in vitro by ketomycin. (A) Structure of ketomycin. (B) Inhibition of IKKβ in vitro by ketomycin. IKKβ(12.5 ng) and ATP (10 μM) were added. Values are the means ± SEM of 4 independent determinations. *, P≤0.05; **, P≤0.01; ***, P≤0.001 vs. control. †, P≤0.05;††, P≤0.01 vs. ketomycin.

MATERIALS AND METHODS

Chemical and cell culture

Ketomycin was purchased from Tecno Chem Co., Ltd., (Tokyo, Japan). Mouse monocytic leukemia RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco Thermo Fisher Scientific, Inc., Waltham, MA) at 37˚C in a humidified incubator with 5% CO_2 and 95% air. IKK inhibitorSC514waspurchased from Santa Cruz Biotechnology (Dallas, TX).

I-κB kinase activity

IKKβ Kinase Assay Kit (Promega, Madison, MI) was employed for the measurement of this activity. Briefly, ketomycin or SC514 solution (1 ml) was added for the final concentration of 10-300 μM with IKKβ solution (12.5 ng in 2 μl)and 2 μlof IKK peptide (1 μg)/ATP (10 μM) mix to 384well plate (Greiner Lumitrac™ 200,Tokyo, Japan) for the kinase reaction. After incubation at room temperature in 5 mlreaction mixture for 60 min, 5 μl of ADP Glo™ Reagent was added to delete the left ATP and incubated at room temperature. After 40 min, 10 μl of Kinase Detection Reagent was added for the luminescence detection and incubated at room temperature for 30 min. Luminescence (integration time 500 ms) was recorded by Spectra Max M5 (Molecular Devices, Tokyo, Japan).

Surface plasmon resonance (SPR) analysis

Interaction of the recombinant human IKKb (Signal Chem,WI) with ketomycin at different concentrations, IKKb inhibitor SC514 (Santa Cruz Biotechnology, Dallas, TX), and ATP (Promega, Madison, MI) were analyzed using a surface plasmon resonance biosensor (BIAcore T200; GE Healthcare, Uppsala, Sweden) [15,16]. Briefly, the recombinant human IKKb (10 mg/ml) was immobilized in flow cell 2 of the CM5 chip. Ethanolamine (1 M) solution was used as a control in flow cell 1 of the chip. IKKb solution (98ml) was injected into the activated CM5 chip at a flow rate of 10ml/min in 10mMsodium acetate buffer (pH 4.5) for 7 min. The immobilized chip was treated with 1M ethanolamine solution and washed with PBS.

Binding assays were carried out with a constant flow rate of 30 ml/min at 25˚Cduring both the association and dissociation phases using 10 mM HEPES buffer (pH 7.4) containing 0.15 M NaCl, 3 mM EDTA and 0.0005% Tween 20 (HBS-EP). ketomycin, SC514, ATP, or IKK peptide in the IKKβ Kinase Assay Kit in HBS-EP was injected into the IKKb ligand-immobilized flow cells for 60 s (association), followed by injection of HBS-EP buffer for 180s (dissociation). Regeneration of the sensor chip surface was accomplished by an injection of 20 ml of HBS-EP containing 2 M NaCl. The sensorgrams were recorded, and the kinetic parameters calculated from

association and dissociation curves were determined using BIA Evaluation 4.1 software (GE Healthcare).

The HPLC condition of IKKb for desalting was carried out as described below.IKKb (200 mlat 0.1 mg/mlin 10 mM sodium acetate buffer at pH 4.5) was applied to5 ml Hi Trap Desalting column (GE Healthcare Bio-Sciences KK, Tokyo, Japan). The flow rate was 1 ml/min with a pressure of 12 kg/cm^2 (0.5 MPa). The peak was detected under UV at 280 nm. The fraction was run at a rate of 0.l ml (0.1 min). Collected fractions at 1.9 min to 2.3 min (4 tubes, 0.4 ml) were used as the ligand in the following SPR analysis.

MTT assay

Cell viability was determined using an MTT assay. Following 24 h of incubation with or without test samples, the medium was replaced with 100 µl of fresh culture medium, and 10 µl of the 12 mM MTT stock solution was then added to each well. The cells were cultured at 37°C for 2 h, the supernatant was removed, and then 100 µl of DMSO was added to each well and mixed thoroughly. The cells were incubated at 37°C for another 10 min. The reaction products were quantified at 570 nm using a micro plate reader (Bio-Rad Laboratories Inc., Hercules, CA). The untreated cells were considered as having 100% viability. The results are expressed as the percentage of viable cells when compared with the control group.

Measurement of IkBa degradation

The amount of IkBa was measured by Western blotting. RAW264.7 cells were treated with ketomycin at 3 µg/ml (20mM)or SC514 at 5µM [17] for 2 h, then added with LPS at 10 ng/ml for a further15 min. Cells were lysed with sample buffer and boiled at 100°Cfor 5 min. Samples were electrophoresed on SDS-polyacrylamidegels with 10 % separating gel. Proteins were transferred to PVDF membranes and immunoblotted with IkBa antibody at 1000x dilution(Cell Signaling Technology, Danvers, MA) and β-actin antibody at 1000x dilution(Santa Cruz Biotechnology, Santa Cruz, CA). The bands were detected with Western Lightning Plus-ECL Enhanced Chemiluminescence Reagent (PerkinElmer, Waltham, MA), and the images were analyzed by Amersham Imager 600.

Measurement of NF-κB activity

The RAW264.7 cells in complete medium (2.5x10^6 cells) were grown in 60mm dishes. The following day, the cells were treated with ketomycinfor 2 h and stimulated with 10 ng/ml LPS for 15 min. The nuclear extracts were prepared with a Nuclear Extract kit (Active Motif Japan, Tokyo, Japan). The DNA binding activity of NF-κB in nuclear extracts was then measured with the Trans AM NF-κB p65 Transcription Factor Assay kit (Active Motif Japan, Tokyo, Japan).

Measurement of NO production

The cells were plated in a 96-well plate at a density of 1.0x10^5 cells/well and grown for 2 h to allow the cells to attach to the plate. Ketomycin was added to the culture for 30 min, then the cells were stimulated with *Escherichia coli*-derived LPS (Sigma, St. Louis, MO) at 10ng/ml. The cells were then incubated at 37°C for 24 h and subsequently chilled on ice. Subsequently, 100 µl of the culture supernatant was placed in duplicate in the wells of 96-well plates. To quantify nitrite, 50 µl of Griess reagent (1% sulfanilamide in 5% H_2PO_4 and 0.1% N-1-naphthyletylenediamide dihydrochloride) was added to each well. After 10 min, the reaction products were colorimetrically quantified at 570 nm using a microplate reader (Bio-Rad Laboratories). The concentrations of nitrite were calculated using a standard calibration curve.

Measurement of IL-6 secretion

The cells in complete medium (3x10^{5}cells/ml) were seeded in a 96-well plate, with each well receiving 200 µl of the cell suspension. The following day, the cells were treated with ketomycin for 2h and then stimulated with 10ng/ml LPS for 2 h. The cell-free medium was collected into a 1.5-ml tube and stored at -80°C prior to the assay. The concentration of IL-6 was quantified using the Mouse IL-6 ELISA Max Deluxe kit (Bio Legend, Inc., San Diego, CA). The wells were coated with the diluted capture antibody overnight, followed by being washed with the wash buffer, and then the thawed medium was added to the assay wells. The 200x diluted detection antibody was then added and incubated for 1h. The wells were then washed with PBS, the 1000x diluted avidin-HRP antibody was added at room temperature, and the plate was incubated for 30 min. The wells were washed again with PBS, and TMB substrate solution was added to the wells and incubated in the dark for 20 min. The color reaction was terminated by the addition of sulfuric acid, and the intensity was determined as absorbance (A_{450}-A_{570}) read on a 96-well microplate reader. The standard curve was prepared as indicated by the manufacturer. The

concentration of IL-6 was calculated from the standard curve each time.

STATISTICAL ANALYSIS

Statistical comparison was conducted using one way ANOVA and/or the Student's t-test where appropriate.

RESULTS

Inhibition of IKKβ by ketomycin *in vitro*

Since ketomycin (Figure 1A) inhibited constitutively activated cellular NF-kB and IKK phosphorylation in human breast carcinoma MDA-MB-231 cells [14], we studied the inhibitory effect of ketomycin on recombinant IKKβ. As a result, it inhibited IKKβ *in vitro* (Figure 1B), and the inhibitory activity was slightly weaker than that of positive control SC514.

Physical binding of ketomycin to I-kB kinase b

Next, we studied whether ketomycin directly binds to IKKβ employing surface plasmon resonance (Biacore). Recombinant I-kB kinase b was fixed to the sensor tip, and the change of the reflected light angle was measured by the addition of ketomycin. Physical binding is shown by the binding kinetics. Ketomycin (Figure 2A,B) gave stronger responses than IKK peptideor SC514 (Figure 2C). Addition of ATP did not change the response. Thus, ketomycin was shown to be a new inhibitor of IKKb that directly binds to the enzyme.

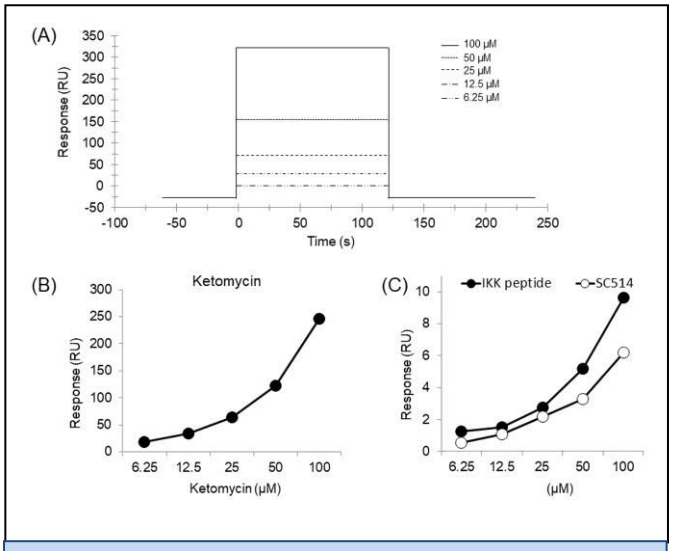

Figure 2: Binding kinetic analysis between ketomycin and IKKβ with SPR. (A,B) Dose effect of ketomycin on the response of binding kinetics on the IKKβ-immobilized chip in SPR. (C) Dose effects of IKK peptideand SC514on the response of binding kinetics on the IKKβ-immobilized chipin SPR.

Cellular anti-inflammatory activity of ketomycin in RAW264.7 cells

IKKb is an upstream signal of IkBa degradation and NF-kB activation. Since ketomycin was found to be its inhibitor, we next studied the effect on these activities in LPS-treated RAW264.7 cells. Ketomycin was not prominently toxic below 3 mg/ml at 2.5 or 24 hof incubation in RAW264.7 cells (Figure 3A). LPS induced degradation of IkBa prominently in 15 min. Ketomycin inhibited LPS-induced degradation of IkBa more strongly than SC514 (Figure 3B). Next, it inhibited LPS-induced NF-kB activation in 30 min (Figure 3C). Ketomycin also inhibited LPS-induced iNOS-mediated NO production (Figure 3D) and IL-6 secretionin 24 h (Figure 3E).

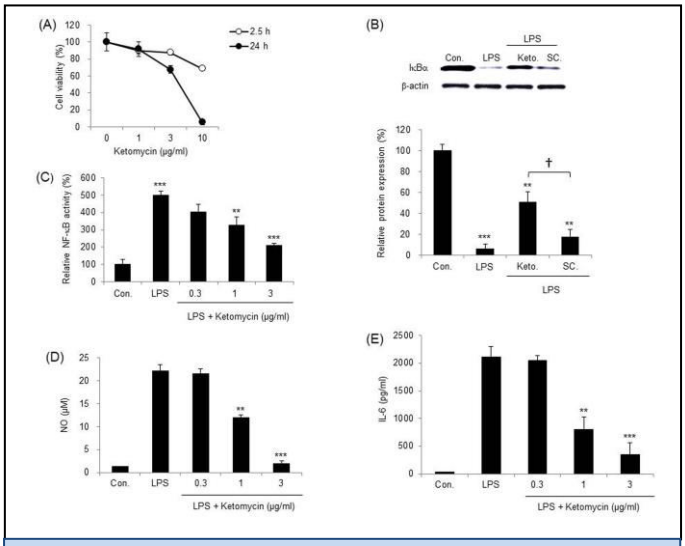

Figure 3: Cellular effects of ketomycin. (A) Effect of ketomycin on the viability of RAW 264.7 cells. The cells were incubated for 2.5 or 24 h. (B) Inhibition of LPS-induced IκBα degradation by ketomycin. (C) Inhibition of LPS-induced NF-κB activation by ketomycin. The cells were incubated for 30 min with LPS after 2 h incubation with ketomycin. (D) Inhibition of LPS-induced NO production by ketomycin. The cells were incubated for 24 h. (E) Inhibition of LPS-induced IL-6 secretion by ketomycin. The cells were incubated for 24h. Values are the means ± SEM of 5(A) or 4(B,C,D, and E) independent determinations.**, $P \leq 0.01$; ***, $P \leq 0.001$ vs. control.†, $P \leq 0.05$ vs. ketomycin.

DISCUSSION

Thus, ketomycin is considered to be a new, nonsteroidal anti-inflammatory agent that inhibits IKK β. There are several IKK inhibitors reported [18-21], although the structure of ketomycin is not related to any of them. Since the structure is different, the inhibitory machanism is likely to be different. IMD-0354 was

designed by analyzing a binding mode of aspirin to IKKb, and it inhibited neoplastic mast cell proliferation [18]. Active 2-benzamido-pyrimidines compounds that inhibited IKK inhibited the IkBa degradation and downstreamevents, like adhesion molecule expression [19]. The aminothiophene compound was an ATP-competitive inhibitor of IKKb [20], and inhibited LPS-induced production of TNF-α, IL-6, and IL-8 in human monocytes [21].

We employed commercially available SC514 as the positive control for IKKβ inhibitor [17]. Interestingly, the affinity of ketomycin to the enzyme is higher than that of SC514 (Figure 3B,C), and ketomycin more clearly inhibited LPS-induced IkBa degradation in cultured cells (Figure 3B).

Activation of inflammatory signals often accelerates neurodegenerative diseases [22]. We have isolated cyclopenin as a possible anti-inflammatory agent from deep sea microorganism [13]. Cyclopenin inhibited fly model of Alzheimer's disease. Therefore, ketomycin may also ameliorate in vivo models of neurodegenerative diseases.

We employed RAW264.7 cells for the evaluation of anti-inflammatory activity, because they possess macrophage phenotypes without any differentiation. They strongly respond to LPS, activating I-kB degradation. Inhibition of NF-kB signaling by ketomycin is unlikely to be cell dependent, since ketomycin lowered IKKb phosphorylation and NF-kB activity in human breast carcinoma cells [14]. However, we areplanning to employ mouse bone marrow-derived macrophages [23] for further research.

CONCLUSION

Ketomycin, a known antibiotic, was found to be a new inhibitor of IKKβ. It inhibited LPS-induced degradation of IkBa, NF-kB, NO production, and IL-6 secretion in macrophage-like mouse monocytic leukemia cells. Ketomycin has simpler structure than most nonsteroidal anti-inflammatory agents and steroids, and would be easily synthesized. It may be a candidate as a new, nonsteroidal anti-inflammatory agent.

AUTHOR CONTRIBUTIONS

Yinzhi Lin: Experimental design and practice, and manuscript preparation. Nobuo Sugiura: Experimental design and practice. Jun Ma: Experimental design. Kazuo Umezawa: Project design and manuscript preparation.

DECLARATION OF COMPETING INTEREST

K.U. belongs to the donated fund laboratory supported by Shenzhen Wanhe Pharmaceutical Co., Ltd, Shenzhen, China,Meiji Seika Pharma Co., Ltd,Tokyo, Japan, Fukuyu Medical Corporation, Nisshin, Japan, and BrunaiseCo., Ltd, Nagoya, Japan. Other authors declare no conflicts of interest.

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