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INHIBITION OF NF-KAPPA B ACTIVATION BY 9-METHYLSTREPTIMIDONE ISOLATED FROM *STREPTOMYCES*

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Abstract – In the course of our screening of NF-κB inhibitors from microbial secondary metabolites, we isolated a piperidine compound, 9-methylstreptimidone, from the culture filtrate of *Streptomyces*. 9-Methylstreptimidone inhibited LPS-induced NO production and NF-κB-induced transcriptional activity. It showed selective toxicity on adult T-cell leukemia cells in which NF-κB is constitutively activated. Unlike cycloheximide it did not inhibit protein synthesis.

INTRODUCTION

NF-κB is the transcription factor that promotes expressions of cytokines such as interleukin 1 (IL-1)[1], IL-2, IL-6, IL-8, TNF-α[2]; cell adhesion molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1); viral proteins[3]; and anti-apoptotic proteins such as inhibitor of apoptosis proteins (IAPs)[4] and Bcl-XL[5]. In inflammatory sites NF-κB is often activated in inflammatory cells such as macrophages. NF-κB is usually located in the cytoplasm as an inactive form. Stimulants such as tumor necrosis factor (TNF)-α and lipopolysaccaride (LPS) induce nuclear translocation and activation of NF-κB. However, NF-κB is often constitutively activated in cancer and leukemia cells. The activated NF-κB can increase viability and metastatic activity of cancer cells increasing their malignant character. We previously designed and synthesized a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ).[6] It is being developed as an anticancer agent. However, more effective inhibitors of NF-κB may be further expected. Since, NF-κB inhibitors of low molecular weight should be useful as anti-inflammatory and anticancer agents, we looked for NF-κB inhibitors among microbial culture filtrates. As a result, we isolated 9-methylstreptimidone from the culture filtrate of *Streptomyces*.

RESULTS AND DISCUSSION

Screening system for NF-κB inhibitors. To obtain selective NF-κB inhibitors we employed multi-step screening system. Mouse macrophage-like cell line RAW264.7 cells produce nitrogen monoxide (NO) when they were stimulated with lipopolysaccharide (LPS), and this effect is mediated by NF-κB

activation.[7] Firstly, we tested the inhibitory activity of the sample on LPS-induced NO production. The toxicity was evaluated by MTT assay. Secondly the sample was evaluated by reporter-gene assay for NF-κB. The reporter DNA consisting of 3-tandem κB repeats and luciferase gene was transfected into human T cell leukemia Jurkat cells transiently. To exclude protein synthesis inhibitors the samples were also tested whether they inhibit leucine incorporation.

Purification of 9-methylstreptimidone. During the course of our screening for microbial secondary metabolites possessing NF-κB inhibitory activity, we isolated a 9-methylstreptimidone from the culture filtrate of *Streptomyces*. The culture filtrate (2L) of Lot 1848-26 provided by Microbial Chemistry Research Foundation was mixed with 2L of ethyl acetate and the ethyl acetate phase was obtained (× 2). The active ethyl acetate elutes were concentrated to dryness under reduced pressure to a dark yellow solid (50 mg), which was subjected to medium pressure liquid chromatography (MPLC) over silica gel using a 5% step gradient of ethyl acetate in n-hexane as the eluant. Active substance eluted out in 70% ethyl acetate in n-hexane afforded 10 mg of the crude material. The crude material was subsequently purified by preparative TLC with silica gel and CH_2Cl_2-MeOH-H_2O (10:0.5:0.05) as the developing solvent. Finally, the active substance was purified again by HPTLC on silica gel using $CHCl_3$-acetone (4:1) as developing solvent to give 2.2 mg of pure substances as colorless solid.

Structure determination. In order to define the structure of the active substance, ^{1}H-NMR, ^{13}C-NMR, mass spectrum (ESI mode), optical rotation and ultraviolet absorption spectrum were used. By the ESI-MS (m/z) spectrum its molecular mass was found to be 307, the UV λmax 235 nm and the ^{13}C-NMR showed that there were 17 carbon atoms. These data were used to computer data research on chemical data bank provided by Chapman & Hall/CRC. As shown in Table 1, the ^{1}H and ^{13}C spectral data of 9-methylstreptimidone were identical to the known antiviral antibiotic 9-methylstreptimidone (Figure 1). 9-Methylstreptimidone was first reported as antibiotic in 1974.[8]

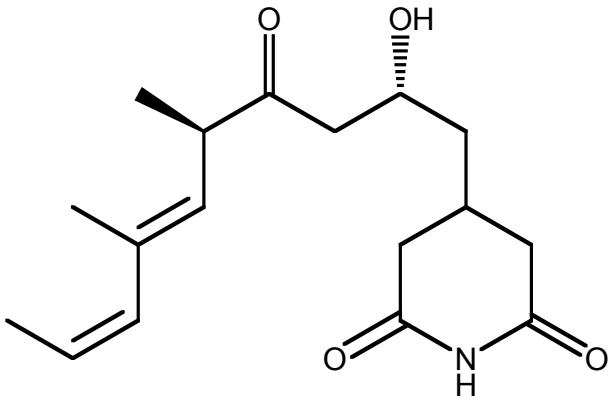

Figure 1 Structure of 9-methylstreptimidone

9-Methylstreptimidone inhibits LPS-induced NO synthesis and iNOS expression. We assessed the effect of 9-methylstreptimidone on the production of NO in LPS stimulated cells. LPS treatment increased NO production over the basal level at 24 hours. When RAW264.7 cells were pretreated with 9-methylstreptimidone for 2 hours prior to LPS stimulation the NO content in the conditioned media was decreased (Figure 2A). NO is produced by inducible NO synthase (iNOS) from L-arginine in RAW 264.7 cells. The promoter region of iNOS gene contains functional NF-κB binding sites. Treatment with 9-methylstreptimidone also led to a significant decrease of iNOS protein (Figure 2B) in a dose-dependent manner. At a concentration of 1.0 μg/mL, 9-methylstreptimidone decreased the expression of iNOS protein up to 95%.

Table 1 ^{1}H and ^{13}C NMR spectral data of 9-methylstreptimidone.

Position	^{1}H (300 MHz, $CDCl_3$) ppm Obs.	^{1}H (400 MHz, $CDCl_3$) ppm Reference[10]	^{13}C (300 MHz, $CDCl_3$) ppm Obs.	^{13}C (25 MHz, $CDCl_3$) ppm Reference[10]
1	1.58, 1.33 (2 x m)	1.61, 1.34 (2 x m)	40.7	40.9
2	4.09 (m)	4.12 (m)	64.8	64.8
3	2.59 (m)	2.57 (m)	47.1	47.3
4	-	-	212.8	212.5
5	3.45 (m)	3.44 (m)	46.9	46.9
6	5.17 (d 9.9 Hz)	5.17 (d, 9.6 Hz)	127.8	127.9
7	-	-	135.8	135.7
8	5.78 (11.7 Hz)	5.81 (d, 11.70 Hz)	132.7	132.7
9	5.49 (11.7, 7.2 Hz)	5.50 (dq, 11.7, 7.2 Hz)	125.4	125.3
1'	2.47 (m)	2.48 (m)	27.1	27.1
2'	2.76, 2.34 (2 x m)	2.76, 2.32 (2 x m)	38.4	38.4
3'	-	-	171.9	172.6
5'	-	-	171.9	172.6
6'	2.76, 2.34 (2 x m)	2.76, 2.32 (2 x m)	37.1	37.1
5-CH_3	1.18(d, 6.8 Hz)	1.18(d, 6.8 Hz)	14.7	14.7
7-CH_3	1.84 (s)	1.85 (s)	16.2	16.2
9-CH_3	1.78 (dd 7.2, 1.5 Hz)	1.78 (dd, 7.2, 1.5 Hz)	17.3	17.3
2-OH	3.23 (s)	3.41 (s)	-	-
4'-NH	7.89 (s)	-	-	-

9-Methylstreptimidone inhibits TNF-α-induced NF-κB activity in Jurkat cells. It is easier to transfect reporter DNA into Jurkat cells than into RAW264.7 cells. Jurkat cells are more sensitive to TNF-α than LPS to activate NF-κB. Therefore, we employed Jurkat cells with TNF-α to evaluate the effect on NF-κB transcriptional activity. When the cells were pretreated with 9-methylstreptimidone for 2 h prior to TNF-α stimulation, the luciferase activity was decreased in a dose dependent manner (Figure 3). Thus, 9-streptimidone was found to be an inhibitor of NF-κB.

9-Methylstreptimidone induced apoptosis in leukemia cells. TNF-α receptor signaling contains both anti-apoptotic NF-κB activation and apoptosis-inducing pathways. Actually, the TNF receptor has the FAS-like death domain. TNF-α alone did not induce apoptosis in Jurkat cells indeed. However, when the cells were pretreated with 9-methylstreptimidone for 2 hours prior to TNF-α, the cell viability was dominantly decreased in the dose dependent manner after 24 h (Figure 4A). Apoptosis was confirmed by nuclear condensation and degradation. MT-1 cells are one of the adult-T cell leukemia cell line, in which NF-κB is constitutively activated. We found that 9-methylstreptimidone alone selectively induced the cell death in MT-1 cells in 24 h (Figure 4B).

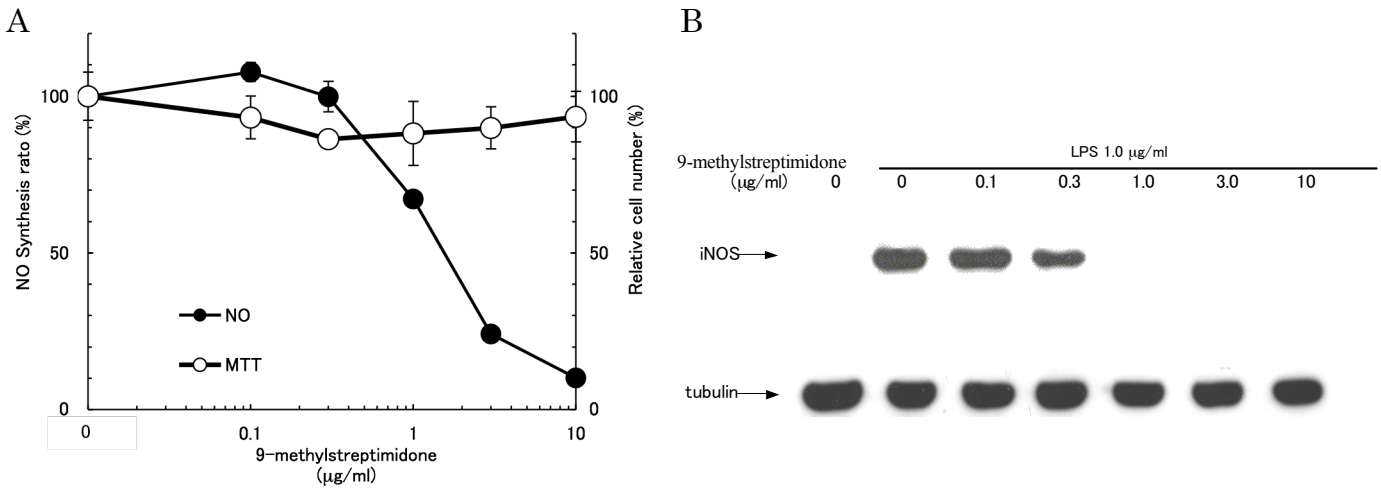

Figure 2 Inhibition of NO production and iNOS expression by 9-methylstreptimidone. (A) RAW264.7 cells in 96-well plate were pretreated with the chemical at the indicated concentrations for 2 h and then stimulated with LPS (1 μg/mL) for 24 h. The nitrite contents in the conditioned media were determined with Griess method. The cell viability was also assessed by the MTT method. All data are mean ±S.D. of three independent determinations. (B) The expression of iNOS protein in total cell extracts was analyzed by Western blotting.

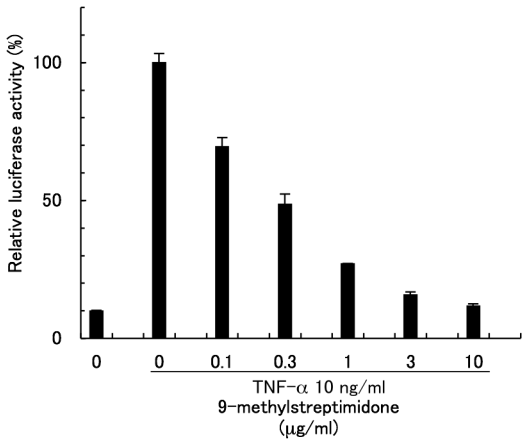

Figure 3 Inhibition of TNF-α-induced NF-κB activation by 9-methylstreptimidone in Jurkat cells. Jurkat cells were transfected with a reporter gene κB-luc vector. The transfected cells were pretreated with chemical as indicated for 2 h, stimulated with none or TNF-α (10 ng/mL) for 6 h and harvested for the luciferase assay. The results were expressed as percent control over the value obtained with the TNF-α (10 ng/mL). All data are means ± S.D. of three independent measurements.

9-Methylstreptimidone did not affect protein synthesis. Both 9-streptimidone and cycloheximide possess dicarbonylpyperidine structure. Then, we looked into the effect on protein synthesis in RAW 264.7 cells. The cells were pretreated with 9-methylstreptimidone for 2 h. Then the culture supernatant was replaced with serum-free medium, containing tritium-labeled leucine. The radioactivity was determined after 4 hours. Treatment of 9-methylstreptimidone did not lead to a significant decrease of protein synthesis distinct from cycloheximide (Figure 5).

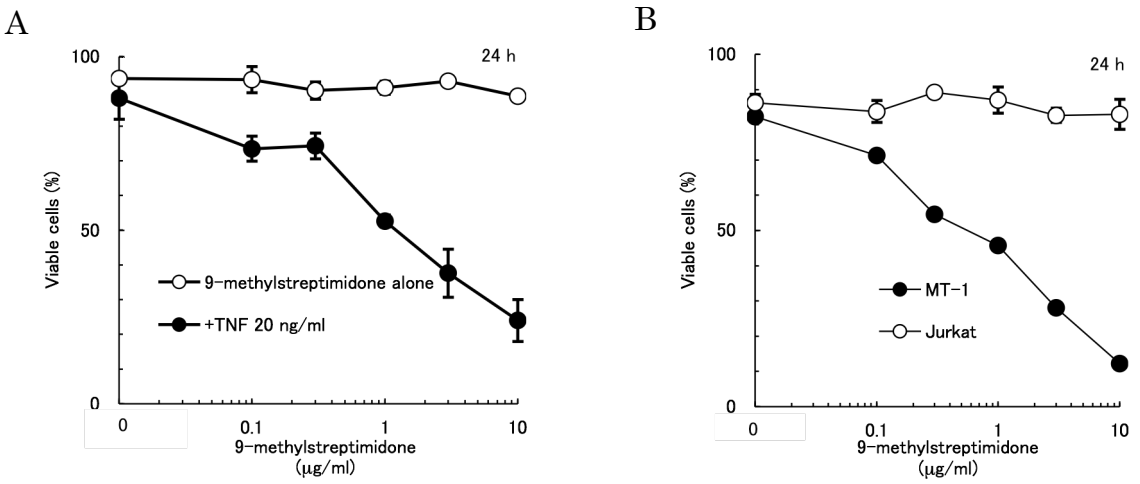

Figure 4 Induction of apoptosis by 9-methylstreptimidone in leukemia cells. (A) Jurkat cells were cultured with 9-methylstreptimidone with or without 20 ng/mL TNF-α for 24 h. then the viability was measured by trypan blue dye exclusion. (B) MT-1 or Jurkat cells were cultured with 9-methylstreptimidone for 24 h. The cell viability was measured by trypan blue dye exclusion.

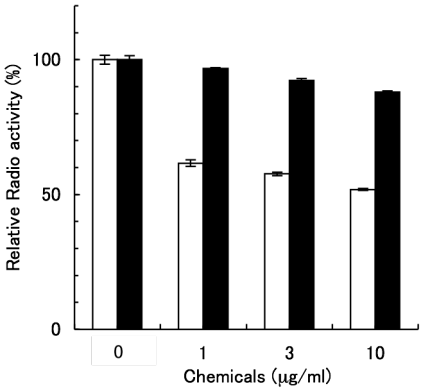

Figure 5. Effect of 9-methylstreptimidone on protein synthesis in RAW264.7 cells. The cells were treated with chemicals for 2 h. The culture supernatant was replaced with serum-free medium containing radioactively labeled leucine. After 4 h, the cells were immobilized and lysed. Liquid scintilation counter was employed to measure the intensity of radioactivity. All data are means ± S.D. of 3 independent measurements. White, cycloheximide; dark, 9-methylstreptimidone.

Discussion. We isolated 9-methylstreptimidone from the culture filtrate of *Streptomyces* as an inhibitor of LPS-induced NO production. It is likely to be a new NF-κB inhibitor. The mechanism of inhibition is being studied. 9-Methylstreptimidone was first isolated in 1974 from *Streptomyces* as an antibiotic.[8] Its absolute configuration was reported in 1976.[9] It is known to possess antiviral and interferon-inducing activity,[10] however, the NF-κB inhibitory activity has not been reported. We have first found that 9-mathylstreptimidone inhibits NF-κB and its mediated biological effects such as NO production and inhibition of apoptosis. 9-Methylstreptimidone induced apoptosis in Jurkat cells only in the presence of TNF-α, as DHMEQ.[11] It induced apoptosis selectively in adult T-cell leukemia cells as DHMEQ.[12] DHMEQ was effective to suppress various disease models in animals.[13] Therefore, 9-methylstreptimidone or its analogues may be useful as anti-inflammatory or anticancer agents, since another NF-κB inhibitor, DHMEQ, showed potent anti-inflammatory and anticancer activities in animal experiments.

EXPERIMENTAL

Materials. Cycloheximide was purchased from Wako. Recombinant human TNF-α was purchased from Sigama. Anti-iNOS antibody, anti-tublin antibody purchased from Amersham, Pharmacia. Anti-mouse-antibody derived from rabbit for iNOS and tublin were purchased from Amersham, Pharmacia.

Cell culture. MT-1 and Jurkat cells were grown in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 5% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 μg/mL kanamycin, 100 units/mL penicillin G, 30 μg/mL L-glutamine, and 2.25 g/L $NaHCO_3$. RAW264.7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 200 μg/mL kanamycin, 100 units/mL penicillin G, 600 μg/mL L-glutamine, and 2.25 g/L $NaHCO_3$.

Preparation of screening samples. Culture fluid of microorganisms was provided by Microbial Chemistry Research Foundation. The culture filtrate (500 μL) was mingled with n-BuOH in equal proportions to extract second metabolites from the fluid. After violently shaking, this mixture was centrifuged for 5 min at 13,000 rpm. Organic solvent phase was divided and evaporated in vacuo. Extract was dissolved in 200 μL MeOH.

NO assay. Cell suspension (100 μL) of RAW264.7 cells at concentration of 50% confluence was seeded into the 96-wells plate. Then chemicals or screening samples derived from microorganisms were added into the well plates. After 2 h, the cells were stimulated with LPS at 1 μg/mL and incubated for 24 h. Then 100 μL of the Griess reagent solution was added into each plate. The concentration of NO was obtained by measuring an absorbance of 570 nm.

MTT assay. Cell suspension (100 μL) of RAW264.7 cells at concentration of 50% confluence was seeded into the 96-wells plate. Then the samples were added into the well plates and incubated for 24 h. Then 10 μL of the MTT (3-[4,5-dimethylthiazol-2–yl]-2,5diphenyl tetrazolium bromide) solution was added into each plate and incubated for 4 h at 37 ℃, 5% CO_2. Subsequently, the culture supernatant was replaced with 100 μL DMSO to dissolve formazan crystal made from succinic dehydrogenase in the mitochondria and its substrate MTT. An absorbance of 570 nm was measured with a microplate reader.

Reporter gene assay with κB/luciferase. Jurkat cells were transfected with 2 μg of DNA consisting of three tandem κB repeats and the luciferase gene by the DEAE-dextran method. The transfected cells were seeded into 12-well plates at $1x10^6$ cells/well. Chemicals dissolved in methanol and TNF-α were added at 14 and 16 h, respectively. Six hours after the TNF-α addition, the cells were harvested and lysed, and the lysate was used for the luciferase assay with luciferin substrate buffer (20 mM Tricine-NaOH (pH 8.0), 1.07 mM magnesium carbonate hydroxide, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM CoA, 470 μM luciferin, 530 μM ATP). Luminescence was measured with a Lumat 9501 detector (Berthold).

Trypan blue dye exclusion assay. One ml of Jurkat or MT-1 cell suspension (2.0×10^5 cells/mL) was seeded into 24-well plate. Then, chemicals were added into each well and incubated for 24 h. The cell solution was collected into micro test tube and centrifuged at 3,500 rpm for 5 minutes. After the supernatant was removed, 80 μL medium solution and 20 μL trypan blue staining solution (4 mg/mL trypan blue, 9 mg/mL NaCl) was added into a new micro-test tube tube. Finally, whole and dead cell numbers were scored to calculate the cell viability.

Western Blotting. Jurkat cells were lysed with lysis buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 400 μM Na_3VO_4, 1% Nonidet P-40, 1 μg/mL leupeptin, 1 μg/mL aprotinin and 1 mM PMSF). Each extract (100 μg of protein) was fractionated on a polyacrylamide-SDS gel and then

transferred to a polyvinylidene difluoride membrane. The membrane was incubated for 1 h at room temperature for blocking in TBS buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl) containing 5% skim milk. After having been washed three times with 0.1% Tween 20 in TBS buffer the membrane was incubated for 1 h at room temperature with each antibody in TBS buffer. After three more washes with the TBS-Tween buffer, the membrane was incubated for 1 h at room temperature with anti-IgG rabbit antibody linked to horseradish peroxidase. Immunoreactive proteins were visualized by the ECL detection system.

Protein synthesis assay. RAW264.7 cells were seeded into 12-well plates at 80% confluence. Chemicals dissolved in MeOH were added to the cells for 2 h. Then, the culture medium was replaced with serum-free medium containing 1μCi of tritium-labeled leucine. After 2 h the cells were immobilized with 10% trichloroacetic acid and then they were lysed with 0.5 N NaOH. The radioactivity was measured by a liquid scintillation counter.

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