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Biochemical and Biophysical Research Communications 347 (2006) 797–802
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A small compound that inhibits lipopolysaccharide-induced tumor necrosis factor-α production

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Received 19 June 2006
Available online 5 July 2006

Abstract

Lipopolysaccharide (LPS) is critically involved in the inflammatory responses via generation of several pro-inflammatory cytokines. Since tumor necrosis factor-α (TNF-α) is one of the major pro-inflammatory cytokines which is induced by LPS treatment, the development of molecules capable of modulating LPS-induced TNF-α production is an issue of concern. We identified a novel synthetic compound that inhibits LPS-induced TNF-α production in human peripheral blood mononuclear cells (PBMCs). The active compound SM-7409 inhibited LPS-induced TNF-α production in a concentration-dependent manner, showing maximal activity at 5 μM. SM-7409 inhibited LPS-induced TNF-α mRNA transcript accumulation and protein expression. We also found that SM-7409 strongly inhibits LPS-induced extracellular signal-regulated protein kinase activity in PBMCs. Moreover, we found that SM-7409 strongly inhibits the LPS-induced other pro-inflammatory cytokines, such as interleukin (IL)-1β and IL-8 in PBMCs. SM-7409 also dramatically inhibits the LPS-induced TNF-α production in neutrophils. Taken together, our results demonstrate that SM-7409 is a synthetic compound that inhibits LPS-induced TNF-α production, and thus SM-7409 should be useful for the development of chemotherapies targeting LPS-mediated inflammatory responses.

Keywords: Lipopolysaccharide; TNF-α; Small compound; Peripheral blood mononuclear cells; ERK

Lipopolysaccharide (LPS) has been regarded as an important mediator involved in the inflammatory responses. LPS induces inflammatory responses via generation of several pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-8 [1–4]. These pro-inflammatory cytokines have a critical role in the shock, organ failure, and tissue destruction in inflammatory responses [5–7]. Since LPS is critically involved in inflammatory responses via the generation of pro-inflammatory cytokines, identification of molecules that inhibit LPS-induced cellular responses has been important for the treatment of inflammatory responses.

Chemical genomics using cell-based screening methods have the potential to modulate cellular responses [8–11]. Several successful outcomes have been reported with various purposes with screening of small synthetic compound libraries until now. They include the identification of an insulin mimetic small molecule with anti-diabetic activity in mice, a small non-peptidyl mimic of granulocyte-colony stimulating factor, a cyclin-dependent kinase inhibitor that induces cancer cell differentiation, and a direct phospholipase C activator [8–11]. These newly identified compounds can be utilized as modulators of specific cellular functions

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doi:10.1016/j.bbrc.2006.06.155

for research purposes, and eventually be developed as drug candidates.

In this study, we screened a chemical library consisting of more than 10,000 different species, in an effort to find a chemical that can inhibit LPS-induced TNF-α production in human peripheral blood mononuclear cells (PBMCs). As a result, we found a synthetic compound, SM-7409, that could stimulate PBMCs to inhibit the TNF-α production induced by LPS.

Materials and methods

Materials. LPS (*Escherichia Coli* O55:B5) was purchased from Sigma (St. Louis, MO). Compounds were purchased from Specs (Rijswijk, The Netherlands). RPMI 1640 from Invitrogen Corp. (Carlsbad, CA) and fetal bovine serum (FBS) from Hyclone Laboratories Inc. (Logen, UT). Rabbit anti-human antibodies to phospho-ERK and to ERK were from Cell Signaling Technology, Inc. (Beverly, MA), and horseradish peroxidase-conjugated antibody to rabbit IgG was purchased from Kirkegaard & Perry, Inc. (Gaithersburg, MD).

Isolation of leukocytes. Peripheral blood leukocyte concentrates were donated by healthy adults. PBMCs were separated on a Histopaque-1077 gradient. Human neutrophils were isolated according to the standard procedures of dextran sedimentation, hypotonic lysis of erythrocytes, and a lymphocyte separation medium gradient as described previously [12]. The isolated human leukocytes were then used promptly.

Cytokine assay. Cytokine measurement was performed as previously described [13]. PBMCs or neutrophils (3×10^6 cells/0.3 ml) were placed in RPMI 1640 medium (RPMI 1640 medium containing 5% FBS for neutrophils) in 24-well plates and kept in a 5% CO_2 incubator at 37 °C. After stimulation, cell-free supernatants were collected, centrifuged, and measured for TNF-α, IL-1β, or IL-8 by enzyme-linked immunosorbent assay (BD Biosciences Pharmingen, San Diego, CA) according to the instruction of the vender.

RT-PCR analysis. PBMCs (1×10^6 cells) were stimulated with 100 ng/ml of LPS for the indicated times. mRNA was isolated by using a QIAshredder and an RNeasy kit (Qiagen, Hilden, Germany). mRNA, M-MLV reverse transcriptase, and pd(N)6 primers (Gibco-BRL, Gaithersburg, MD) were used to obtain cDNA. The sequences of the primer used were as follows; TNF-α (444 bp product): forward, 5′-CAGAG GGAAGAGTCCCCCAG-3′; reverse, 5′-CCTTGGTCTGGTAGGAG ACG-3′. GAPDH (480 bp product): forward, 5′-CACAGTCCATGCC ATCACTG-3′, reverse, 5′-TACTCCTTGGAGGCCATGTG-3′. We ran 30 PCR cycles at 94 °C (denaturation, 1 min), 62 °C (annealing, 1 min), and 72 °C (extension, 1 min). PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Stimulation of cells with LPS for Western blot analysis. Isolated PBMCs (2×10^6) were stimulated with 100 ng/ml of LPS for 5 min in the absence or presence of SM-7409 or vehicle control. After stimulation, the cells were washed with serum-free RPMI 1640 medium and lysed in lysis buffer (20 mM Hepes, pH 7.2, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Detergent-insoluble materials were pelleted by centrifugation (12,000*g*, 15 min, at 4 °C), and the soluble supernatant fraction was removed and stored at either −80 °C or used immediately. Protein concentrations in the lysates were determined using Bradford protein assay reagent.

Electrophoresis and Western blot analysis. Proteins were separated in 10% SDS–polyacrylamide gel, and the proteins were blotted onto a nitrocellulose membrane, which was then blocked by incubating with TBST (Tris-buffered saline, 0.05% Tween-20) containing 5% non-fat dried milk. Subsequently, membranes were incubated with specific antibodies and washed with TBST. Antigen-antibody complexes were visualized after incubating the membrane with 1:5000 diluted goat anti-rabbit IgG antibody coupled to horseradish peroxidase and detected by enhanced chemiluminescence.

Statistics. The results are expressed as means ± SE of the number of determinations indicated. Statistical significance of differences was determined by ANOVA. Significance was accepted when $P < 0.05$.

Results

Identification of a synthetic that inhibits LPS-induced TNF-α production in PBMCs

Human PBMCs release basal levels of TNF-α when cultured in serum-free RPMI 1640 medium. When these cells were stimulated with LPS (100 ng/ml) for several lengths of time, TNF-α production was found to be dramatically enhanced in a time-dependent manner, showing maximal activity 12 h after stimulation (data not shown).

In this study, we screened around 10,000 chemicals in an effort to identify species that inhibit LPS-induced TNF-α production in PBMCs, and found several chemicals that do so at ca. 5 μM (data not shown), and among these, SM-7409 proved to be the most potent. Fig. 1A shows that SM-7409 greatly inhibited LPS-induced TNF-α production in a concentration-dependent manner. Vehicle (DMSO) for each concentration of SM-7409 did not affect LPS-induced TNF-α production (data not shown). We also found that

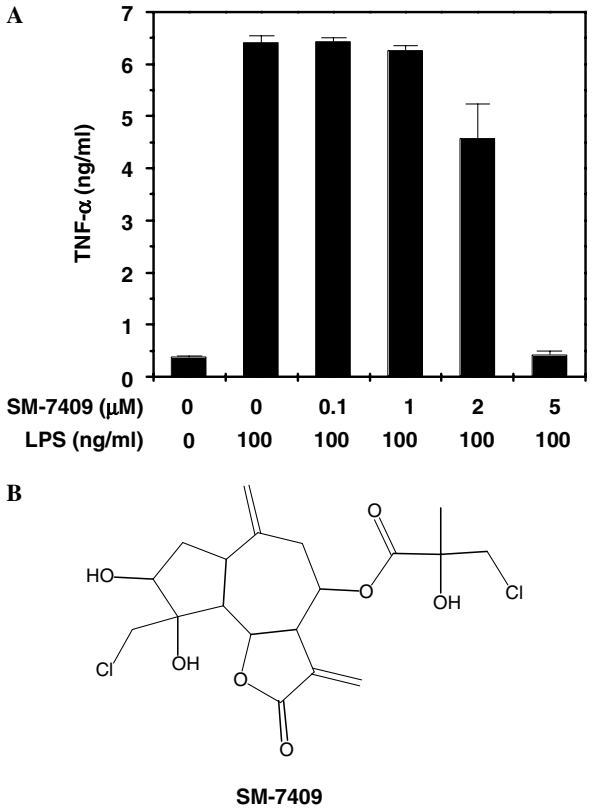

Fig. 1. Effect of SM-7409 on LPS-induced TNF-α production in PBMCs. Human PBMCs were treated with several concentrations of SM-7409 (0, 0.1, 1, 2, and 5 μM) or vehicle (DMSO) for 30 min prior to adding 100 ng/ml of LPS for 12 h (A). Secreted TNF-α was measured by ELISA. All data are presented as means ± SE of three independent experiments, each performed in triplicate. The chemical structure of SM-7409 (B).

many structurally different compounds did not affect LPS-induced TNF-α production up to 10 μM (data not shown). In fact, 5 μM of SM-7409 almost completely abrogated this cytokine production (Fig. 1A). Fig. 1B shows the chemical structure of SM-7409.

SM-7409 inhibits LPS-induced TNF-α production at the transcriptional level

The effect of LPS was investigated on the accumulation of the TNF-α mRNA transcript by RT-PCR. As shown in Fig. 2A, the stimulation of human PBMCs with 100 ng/ml LPS caused TNF-α mRNA transcript accumulation in a time-dependent manner; transcript levels were transiently increased after stimulating cells with LPS for 2–4 h (Fig. 2A). These results indicate that LPS-induced TNF-α production requires TNF-α transcriptional activation and de novo protein synthesis. To investigate the mechanism underlying LPS-induced TNF-α upregulation, we investigated whether SM-7409 acts at the transcriptional level. When human PBMCs were pretreated with SM-7409 prior to adding LPS, LPS-induced TNF-α mRNA accumulation was almost completely inhibited (Fig. 2B). These results indicate that SM-7409 inhibits LPS-induced TNF-α upregulation at a transcriptional level.

SM-7409 inhibits LPS-induced ERK phosphorylation in human PBMCs

The activation of LPS receptor induces extracellular signal-regulated protein kinase (ERK) activation in several cell types, including human PBMCs [14,15]. We observed that stimulation of PBMCs with 100 ng/ml of LPS induced ERK phosphorylation in a time-dependent manner, showing maximal activity at 5 min after stimulation (data not shown). Stimulation of PBMCs with 5 μM of SM-7409 did not stimulate ERK phosphorylation (data not shown). Since SM-7409 inhibited LPS-induced TNF-α induction and ERK is a well-known downstream signaling molecule of activated LPS receptor, we examined the effect of SM-7409 on the LPS-induced ERK phosphorylation. As shown in Fig. 3, preincubation of PBMCs with SM-7409 (5 μM) prior to addition of LPS inhibited LPS-induced ERK phosphorylation, suggesting SM-7409 may inhibit LPS signaling on the downstream of ERK in the cells.

SM-7409 inhibits LPS-induced IL-1β and IL-8 production in PBMCs

LPS stimulates IL-1β and IL-8 as well as TNF-α in PBMCs [16,17]. We investigated the effects of SM-7409 on LPS-induced IL-1β and IL-8 production in PBMCs. Stimulation of PBMCs with LPS (100 ng/ml) caused dramatic increases of IL-1β and IL-8 production from PBMCs (Fig. 4A and B). Preincubation of PBMCs with several concentrations of SM-7409 prior to adding LPS inhibits LPS-induced IL-1β and IL-8 production in PBMCs. Fig. 4A and B show that SM-7409 effectively inhibited LPS-induced IL-1β and IL-8 production in a concentration-dependent manner. Five micromolor of SM-7409 almost completely abrogated IL-1β and IL-8 production by PBMCs (Fig. 4A and B).

SM-7409 inhibits LPS-induced pro-inflammatory cytokines production in neutrophils

Human neutrophils also play important roles in inflammatory responses caused by bacterial infection [3,18]. Neutrophil is one of the major cells which produce several

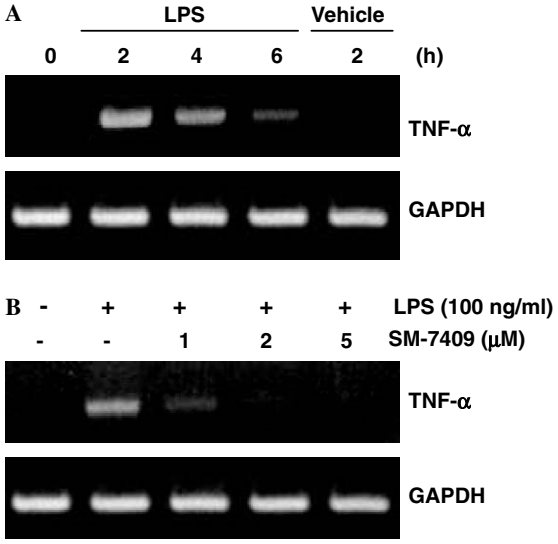

Fig. 2. Inhibition of LPS-induced TNF-α mRNA induction by SM-7409. PBMCs were stimulated with 100 ng/ml of LPS for 0, 2, 4, or 6 h and then harvested for RNA preparation (A). Cells were treated with LPS (100 ng/ml) in the absence or presence of several concentrations of SM-7409 (1, 2, and 5 μM) for 2 h, and then harvested for RNA preparation (B). RT-PCR was performed using specific primers for human TNF-α and GAPDH, and PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide. The data shown are of one representative experiment that was performed in quadruplicate (A,B).

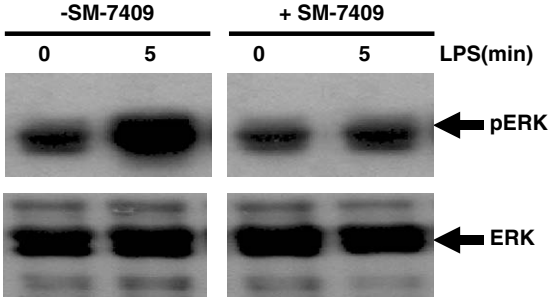

Fig. 3. Effect of SM-7409 on LPS-induced ERK phosphorylation. PBMCs were preincubated with vehicle (DMSO) or SM-7409 (5 μM) for 5 min, and stimulated with LPS (100 ng/ml) for 5 min. Each sample was harvested for Western blot analysis, and each sample (30 μg of protein) was subjected to 10% SDS–PAGE, and phosphorylated ERK levels were determined by immunoblot analysis using anti-phospho-ERK antibody. The results shown are representative of at least three independent experiments.

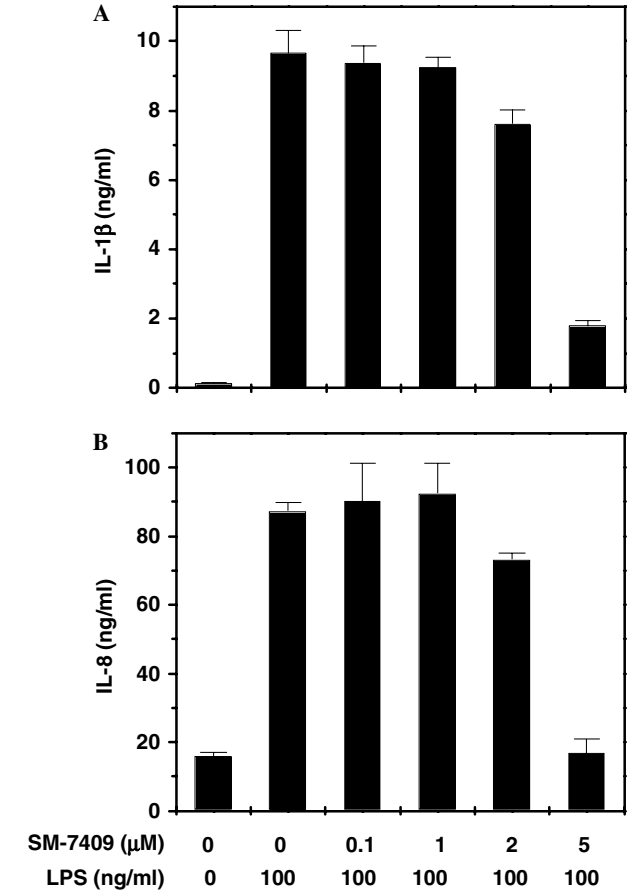

Fig. 4. Effect of SM-7409 on LPS-induced IL-8 and IL-1β production in PBMCs. Human PBMCs were treated with several concentrations of SM-7409 (0, 0.1, 1, 2, and 5 μM) or vehicle (DMSO) for 30 min prior to adding 100 ng/ml of LPS for 12 h. Secreted IL-1β (A) and IL-8 (B) were measured by ELISA. All data are presented as means ± SE of three independent experiments, each performed in triplicate.

pro-inflammatory cytokines against LPS stimulation [3]. We also observed that stimulation of human neutrophils with LPS induced dramatic increases of some pro-inflammatory cytokines, such as TNF-α, IL-8, and IL-1β (Fig. 5). We, then, examined whether SM-7409 blocks the LPS-induced pro-inflammatory cytokines production in human neutrophils. Pretreatment of several concentrations of SM-7409 before LPS stimulation elicited inhibitory effects on LPS-induced pro-inflammatory cytokines (TNF-α, IL-8, and IL-1β) production by neutrophils (Fig. 5). LPS-induced TNF-α and IL-8 productions were dramatically inhibited by 5 μM of SM-7409, however, LPS-induced IL-1β production was inhibited by 10–20 μM of SM-7409 (Fig. 5).

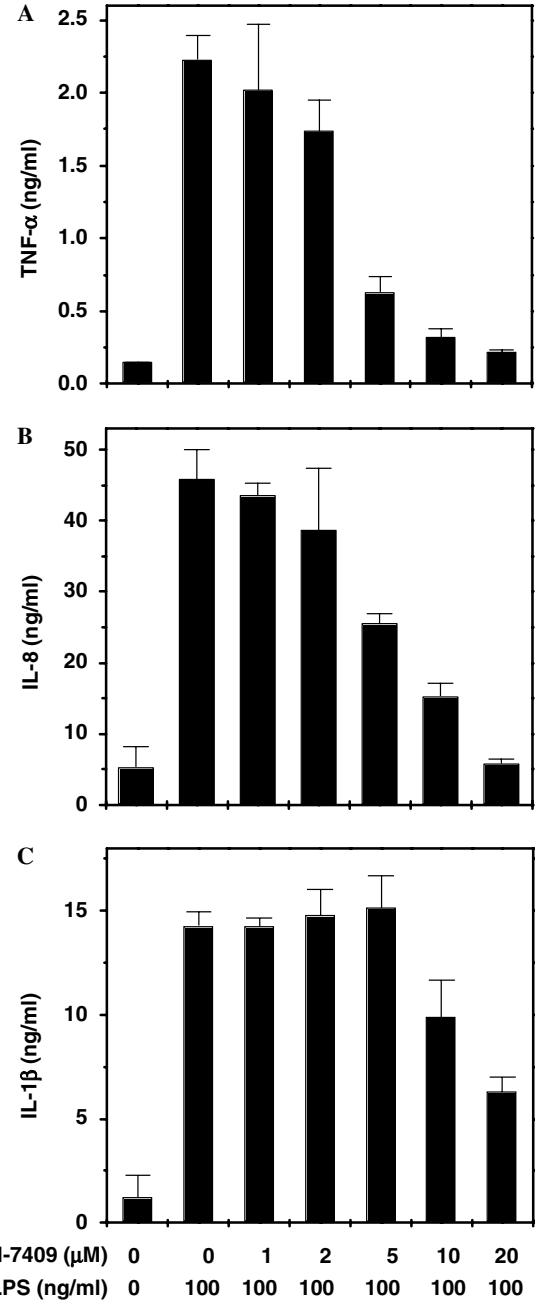

Fig. 5. Effect of SM-7409 on LPS-induced cytokines production in human neutrophils. Human neutrophils were treated with several concentrations (0, 1, 2, 5, 10, and 20 μM) of SM-7409 or vehicle (DMSO) for 30 min prior to adding 100 ng/ml of LPS for 12 h. Secreted TNF-α (A), IL-8 (B), and IL-1β (C) were measured by ELISA. All data are presented as means ± SE of three independent experiments, each performed in triplicate.

Discussion

In this study, we identified a synthetic compound SM-7409 that inhibits LPS-induced pro-inflammatory cytokines production in human PBMCs and neutrophils. By studying its mode of action, we found that SM-7409 inhibits LPS-induced ERK activity, which is crucially required for TNF-α expression in PBMCs. We also found that SM-7409 did not affect cell viability in PBMCs and neutrophils, ruling out the possibility that SM-7409 induces LPS-induced TNF-α production by inducing cell death.

According to the current view of the mechanism for host sense infectious pathogen, pathogen-associated molecular pattern is essential [19,20]. LPS, which is derived from gram-negative bacteria, is well-known ligand for TLR4 [21]. Since LPS is one of the most important pro-inflamma-

tory signals that contributes to the development of inflammatory responses [22], the development of molecule that blocks LPS-induced signaling has been regarded as an important issue. As mentioned above LPS acts on TLR4 in PBMCs and neutrophils [21,22]. So we examined whether SM-7409 inhibits LPS actions by affecting TLR4 expression in the cells. Flow cytometric analysis using anti-TLR4 antibody, we observed that SM-7409 did not affect the surface expression of TLR4 in PBMCs (data not shown). From the result we can rule out a possible effect of SM-7409 on the TLR4 endocytosis or degradation. Recently, it has become well known that commercially available LPS contain other bioactive components such as TLR2 ligands. We also found that SM-7409 did not affect the surface expression of TLR2 in PBMCs (data not shown). In terms of the chemical structure of SM-7409, SM-7409 has several hydrophilic groups. The molecular hydrophobicity was determined by calculating octanol–water partition coefficient $\log P$ at www.molinspiration.com. The $\log P$ value of SM-7409 is 0.805. This relatively low $\log P$ value suggests that SM-7409 may not have good permeability through biological membrane, and it may act on a putative target molecule in cell surface. Since we observed that SM-7409 did not affect TLR4 endocytosis in PBMCs, it will be interesting to reveal the role of other cell surface molecules, such as LPS binding proteins, MD-2, and CD14, on the inhibitory effect of SM-7409. Bearing in mind the crucial role of LPS in the regulation of pro-inflammatory cytokines, such as TNF-α, it is apparent that SM-7409 has the potential to be a useful lead compound for the development of anti-inflammatory drugs targeting LPS action.

On the signaling pathway for the activation of transcription factors involved in TNF-α induction by LPS, ERK has been reported to be essential for NF-κB activation. In the present study, we found that the stimulation of PBMCs with 100 ng/ml of LPS elicited ERK activation in a time-dependent manner, and that it shows maximal activity at 10 min (data not shown). This effect of SM-7409 on LPS-induced ERK activity was further investigated, and it was found that preincubating PBMCs with 5 μM of SM-7409 prior to adding LPS completely inhibited LPS-induced ERK activity (Fig. 3), which indicates that SM-7409 acts upstream of ERK in the LPS receptor signaling pathway. Identification of cellular target molecule for SM-7409 will be essentially necessary for the application of SM-7409 as a potential anti-inflammatory drug.

Acknowledgments

This work was supported by a Grant A060065 from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea, and by the Korea Science and Engineering Foundation through the Medical Science and Engineering Research Center for Cancer Molecular Therapy at Dong-A University.

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