N2733, 1-[3-(3-Pyridyl)-acryloyl]-2-pyrrolidinone Hydrochloride Inhibits LPS-induced TNF-α Production and Improves Survival in Endotoxemic Mice

Koichi Katsuyama,‡ Ryoutarou Kojima, Shinji Yokoyama, Makoto Yanai, Noriyoshi Sueda, Masanori Sugita, Kenichi Momose, and Hiroaki Yamada

Pharmaceutical Research Center, Nisshin Flour Milling Co., Ltd., 5-3-1, Tsurugaoka, Oh-machi, Irumagun, Saitama 356-8511, Japan

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N2733, 1-[3-(3-pyridyl)-acryloyl]-2-pyrrolidinone hydrochloride, was examined for its effect on TNF-α production by human myeloid THP-1 cells stimulated with lipopolysaccharide (LPS). N2733 inhibited LPS-induced release of TNF-α from THP-1 cells with an IC₅₀ of 11 μM. N2733 did not affect the cell viability at the concentration of 50 μM or 100 μM. This indicates that N2733 is a potent inhibitor for TNF-α production without severe cytotoxicity. N2733 was also studied in two murine endotoxin shock models induced with LPS. One model was DBA/2 mice injected with LPS (5.6 mg/kg, i.v.), which increased the serum level of TNF-α within 1 hr. Treatment of these mice with N2733 (100 mg/kg × 2, i.p.) decreased the serum level of TNF-α significantly. Another model was DBA/2 mice induced with LPS (30 mg/kg, i.v.), which reduced the survival rate to 30% during 7 days. Administrations of 30 mg/kg and 100 mg/kg N2733 (i.v.) restored the survival rates to 60% and 90% respectively. Our data demonstrate that N2733 inhibits LPS-induced TNF-α production, and this response is associated with an improvement in the survival rate of endotoxemic mice.

Key words: TNF-α; THP-1 cells; lipopolysaccharide; DBA/2 mice

TNF-α is one of the most studied proinflammatory cytokines.¹⁻³ TNF-α can be produced by stimulated monocytes and macrophages with various stimuli such as lipopolysaccharide (LPS), polyinosinic acid, and interleukins.⁴⁻¹¹ TNF-α is an important mediator in a variety of inflammatory diseases such as septic shock, rheumatoid arthritis, inflammatory bowel disease, and osteoporosis.¹⁻⁵ Moreover, administration of exogenous TNF-α also causes various responses including fever, hypotension, tissue injury, and death.¹⁻³ Inhibition of the action, synthesis, and release of TNF-α has been considered as an effective pharmacological target for medical treatments.⁶⁻¹⁰ In a search for an efficient anti-inflammatory drug without side effects such as cytotoxicity, we have found N2733 (Fig. 1), among about 250 compounds we tried in our laboratory, for its activity that inhibits TNF-α production from the LPS-stimulated human myeloid cell line THP-1. In this paper we report that N2733 inhibits TNF-α production in vitro by LPS-stimulated THP-1 cells. N2733 also suppresses the serum level of TNF-α in vivo in a murine endotoxin shock model induced with LPS. Furthermore, we demonstrate that N2733 improves survival rate of severe LPS-induced endotoxemic mice.

Materials and Methods

Materials. RPMI1640 medium was purchased from Bio Whittaker (MD, USA), and fetal calf serum (FCS) was obtained from Summit Biotechnology (CO, USA). Phorbol myristate acetate (PMA) and LPS (E. coli 0127: B8) were purchased from Sigma (MO, USA). Trichloroacetic acid (TCA) was from Nakarai (Kyoto, Japan). Human and murine TNF-α ELISA kits were from Genzyme (MA, USA). The Alamar Blue assay kit was from Bio Source International (IL, USA). A total RNA extraction kit and a cDNA synthesis kit were from Pharmacia (Tokyo, Japan). Specific TNF-α primers were from Genemed Biotechnology (CA, USA). [³H]leucine was from Amersham (Tokyo, Japan). Specific β-actin primers and N2733 were synthesized in our laboratory.

Cell Culture. Human THP-1 cells (ATCC TIB-202) were obtained through the American Type Culture Collection. THP-1 cells were maintained as subconfluent cultures in RPMI1640 medium with 10% FCS and 50 μg/mL 2-mercaptoethanol.

Measurement of TNF-α. For experiments using LPS as TNF-α stimulus, THP-1 cells were suspended in
RPMI1640 medium with 2% FCS and 50 μg/ml 2-mercaptoethanol. The cell suspension (1 x 10^5 cells/5 ml/well) was added to individual wells of 24-well flat-bottom plates (Corning, Tokyo, Japan) with PMA (1.5 μg/well). Cultures were incubated for 24 hr at 37°C. After incubation, the medium was removed from the well, and 0.5 ml of the fresh medium containing 10 μg of LPS with or without N2733 was added. Cultures were incubated for 24 hr at 37°C. After incubation, supernatants were collected and assayed for TNF-α by a commercial ELISA kit.

Analyses of the cell viability. For experiments on cell viability, the Alamar Blue assay kit was used. In brief, THP-1 cells were suspended in RPMI1640 medium with 2% FCS and 50 μg/ ml 2-mercaptoethanol. The cell suspension (1 x 10^6 cells/ml/well) was added to individual wells of 24-well flat-bottom plates with or without N2733. Cultures were incubated for 24 hr at 37°C. After incubation, 100 μl/well of the Alamar Blue solution was added to cultures. Cultures were treated for 3 hr at 37°C. After treatment, supernatants at 570/600 nm were measured.

Analyses of mRNA by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). THP-1 cells were suspended in RPMI1640 medium with 2% FCS and 50 μg/ml 2-mercaptoethanol. The cell suspension (1 x 10^6 cells/5 ml/bottle) was added to the 25-cm^2 bottles (Corning) with PMA (15 μg/bottle) and incubated for 24 hr at 37°C. After the incubation, the medium was removed from the bottle and 5 ml of fresh medium containing 100 μg of LPS with or without N2733 was added. Cultures were incubated for 24 hr at 37°C. After the incubation, total RNA was isolated using the guanidium/cesium method (Pharmacia QuickPrep total RNA Extraction Kit). First-strand cDNA was synthesized from 5 μg of total RNA with RTase and dT18 primer (Pharmacia First-Strand cDNA Synthesis Kit) in a final volume of 20 μl. Reverse transcription was done at 37°C for 1 hr, then heated at 90°C for 5 min and chilled on ice. PCR was then done by addition of 3 μl of first-strand cDNA to 47 μl of the PCR reaction mixture containing 1 x PCR buffer (Takara, Tokyo, Japan), 50 μM dNTP, 0.5 μM of specific primers for human TNF-α or β-actin, and 2.5 units of Taq polymerase (Takara EX Taq). Primers used were TNF-α forward 21-mer, 5’-ATG AGC ACT GAA AGC ATG ATC-3’; TNF-α reverse 23-mer, 5’-TCA CAG GGC AAT GAT CCC AAA GT-3’; β-actin forward 20-mer, 5’-GAC GGC CCC AGG CAC CA-3’; β-actin reverse 24-mer, 5’-CTT CTT AAT GTC ACG CAC GAT TTC-3’. Samples were treated by 30 cycles at 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. PCR products were analyzed by electrophoresis on 1% agarose gels and stained with ethidium bromide.

Analyses of total protein syntheses. THP-1 cells were suspended in RPMI1640 medium with 2% FCS and 50 μg/ml 2-mercaptoethanol. The cell suspension (1 x 10^6 cells/0.5 ml/well) was added to individual wells of 24-well flat-bottom plates with PMA (1.5 μg/well). Cultures were incubated for 24 hr at 37°C. After incubation, the medium was removed from the well, and 0.5 ml of the fresh medium containing 10 μg of LPS and [3H]leucine (1.5 μCi) in the absence or presence of N2733 was added. Cultures were incubated for 6 hr at 37°C. After the incubation, cells and medium were collected separately. 125 μl of 50% TCA was added to cells and medium respectively, and the precipitates were collected on Whatman GF/C filters. The filters were washed with 2 ml of 10% TCA and the radioactivity was counted.

In vivo slight endotoxin shock model. Female DBA/2 mice, 7-week-old (15-20 g, Sankyo Laboratories, Shizuoka, Japan) were used in groups of 10, 10, 10, 10, 10. One hundred mg/kg of N2733 dissolved in 0.1 ml of 0.9% saline was administered to the mice intraperitoneally. After 30 min, 5.6 mg/kg of LPS dissolved in 0.1 ml of 0.9% saline was injected intravenously. After 30 min, 100 mg/kg of N2733 was administered again. After 30 min, serum TNF-α levels were analyzed with a commercial TNF-α ELISA kit.

In vivo severe endotoxin shock model. Female DBA/2 mice, 7-week-old (15-20 g) were used in groups of 10, 10, 10, 10, 10. Thirty mg/kg or 100 mg/kg of N2733 dissolved in 0.1 ml of 0.9% saline was administered intravenously. Immediately, 30 mg/kg of LPS dissolved in 0.1 ml of 0.9% saline was injected intravenously. Mice were monitored continuously and survivors counted for 7 days.

Statistics and data analyses. Values were expressed as the means ± SE. Statistics and data analyses were done using Student’s t-test or the Logrank test. P values < 0.05 were considered significant.

Results and Discussion

Effects of N2733 on TNF-α production and cell viability in THP-1 cells

TNF-α production by THP-1 cells stimulated with LPS were 1.777 ± 0.004 ng/0.5 ml/24 hr (Fig. 2). TNF-α production by unstimulated THP-1 cells was not detected at 24 hr. The effects of N2733 on TNF-α release from stimulated THP-1 cells are shown in Fig. 2. N2733 inhibited TNF-α production in a concentration-dependent manner in stimulated cells. The IC50 of N2733 for TNF-α production was 11 μM.

To examine whether the inhibition of TNF-α production was due to the cytotoxicity by N2733, the effects of the compound on cell viability were measured (Table 1). As shown in Table 1, N2733 did not affect the viability of THP-1 cells at 50 μM or 100 μM. These results indicate N2733 is a potent inhibitor for TNF-α production without severe cytotoxicity.

Effects of N2733 on TNF-α mRNA expression and total protein syntheses in LPS-stimulated THP-1 cells

To discover the specific step of inhibition, the effects of N2733 on TNF-α mRNA signals in LPS-stimulated THP-1 cells were investigated under the same condi-
Inhibitory Effects of N2733 on TNF-α Production

Fig. 2. Effects of N2733 on TNF-α Production by LPS-Stimulated THP-1 Cells.

THP-1 cells (1 × 10⁵ cells/0.5 ml) were cultured for 24 hr at 37°C with PMA (1.5 μg/ml). After incubation, the medium was removed and PMA-treated cells were cultured at 1 × 10⁵ cells/0.5 ml with LPS (10 μg/0.5 ml) in the absence or presence of N2733 for 24 hr. After that, supernatants were assayed for TNF-α production by ELISA. TNF-α production by unstimulated THP-1 cells was not detected at 24 hr.

Table 1. Effects of N2733 on the Viability of THP-1 Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Absorbance (A₅₇₆-A₆₅₅)</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.543 ± 0.008</td>
<td>100.0 ± 1.5</td>
</tr>
<tr>
<td>N2733 50 μM</td>
<td>0.556 ± 0.040</td>
<td>102.4 ± 7.4</td>
</tr>
<tr>
<td>100 μM</td>
<td>0.551 ± 0.013</td>
<td>101.5 ± 2.4</td>
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</table>

The effects of N2733 on cell viability were measured by the Alamar Blue assay kit. N2733 was added to THP-1 cells in the medium and incubated for 24 hr at 37°C. After incubation, the Alamar Blue solution was added to cultures. 3 hr later, supernatants were read at 570/600 nm.

Fig. 3. Effects of N2733 on TNF-α (A, 702 bp) and β-actin (B, 540 bp) mRNA Expressions in LPS-Stimulated THP-1 Cells.

THP-1 cells (1 × 10⁵ cells/ml/bottle) were treated for 24 hr at 37°C with PMA (15 μg/bottle). After treatment, the medium was removed from the bottle and 5 ml of fresh medium containing 100 μg of LPS with or without N2733 was added. After 24 hr of LPS stimulation, total RNA was extracted and cDNA was synthesized with a commercial kit. cDNA pool was amplified with specific primers by PCR reaction. PCR products were analyzed by electrophoresis on 1% agarose gels. Lane 1 = Marker (Pharmacia 100 Base Pair Ladder); Lane 2 = Control (LPS alone); Lane 3 = LPS + 5 μM N2733; Lane 4 = LPS + 15 μM N2733; Lane 5 = LPS + 45 μM N2733.

Table 2. Effects of N2733 on Protein Synthesis in THP-1 Cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity in Medium (× 10⁴ dpm/well)</th>
<th>Radioactivity in Cells (× 10⁴ dpm/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>113.8 ± 13.8</td>
<td>147.5 ± 15.4</td>
</tr>
<tr>
<td>N2733 50 μM</td>
<td>113.8 ± 0.2</td>
<td>148.9 ± 3.8</td>
</tr>
<tr>
<td>100 μM</td>
<td>110.2 ± 14.3</td>
<td>153.8 ± 6.2</td>
</tr>
</tbody>
</table>

THP-1 cells were cultured for 24 hr at 37°C with PMA. After incubation, the medium was removed and PMA-treated cells were cultured with LPS and [3H] leucine in the absence or presence of N2733 for 6 hr. After incubation, cells and medium were collected separately. Cells and medium were added to 50% TCA separately, and the precipitates were collected on Whatman GF/C filters. The filters were washed with 10% TCA and the radioactivity was counted.

The effects of N2733 on TNF-α (702 bp) and β-actin (540 bp) mRNA signals are given in Fig. 3. N2733 did not affect β-actin mRNA expression even at 45 μM. TNF-α mRNA signals in LPS-stimulated THP-1 cells were reduced dose-dependently by N2733. The effects of N2733 on TNF-α mRNA expression seem to be corresponding to the reduction of protein levels (Fig. 2 and 3).

To investigate whether the inhibitory effect of N2733 on TNF-α production was specific or non-specific, analyses of total protein syntheses were examined. As shown in Table 2, N2733 did not affect intracellular or secretory protein synthesis in LPS-stimulated THP-1 cells. These results indicate that the suppressive effect of N2733 on TNF-α synthesis is a specific action. Our data demonstrate that N2733 inhibits TNF-α production at the transcriptional level.

TNF-α is synthesized as a precursor protein of M,26,000 (26 K), which is processed to a secreted 17 K mature form by proteolytic cleavage. The TNF-α precursor is cleaved to mature TNF-α by several matrix metalloprotease enzymes. Recently it was reported that metalloproteinase inhibitors blocked TNF-α processing activity.13-15) Especially, McGeehan et al. reported that GI 129471, a potent metalloproteinase inhibitor, suppressed TNF-α secretion from LPS-activated human peripheral blood monocytes.13) Still more, GI 129471 did not inhibit TNF-α mRNA accumulation in LPS-treated THP-1 cells. This indicates GI 129471 does not inhibit LPS signaling or TNF-α gene transcription. In contrast, N2733 suppressed TNF-α message production without affecting β-actin mRNA (Fig. 3). Therefore, N2733 inhibits TNF-α production by a distinct mechanism.

Bacterial LPS is known to be a potent macrophage activator. LPS-activated macrophages induce immunoregulatory molecules, including IL-1, IL-6, TNF-α, tissue factor, and inducible nitric oxide synthase.22,23) The first step in LPS signal transduction is binding to the cell-surface Ag, i.e. CD 14. Binding of LPS to CD 14 induces the tyrosine phosphorylation of several cellular proteins, such as ones belonging to the src family, LPS-activated macrophages also phosphorylate and activate Raf-1/mitogen-activated protein kinases (MAP kinases), and then stimulate the transcription factors like myc, fos, NF-κB, and Elk-1.22,24) The inhibition of TNF-α production on addition of N2733 may be regulat-
ed the step(s) in LPS signal transduction, including tyrosine phosphorylation of a number of proteins, Raf/ MAP-kinases pathway, and several transcription factors. Our experiments here can not demonstrate how N2733 acts on TNF-α mRNA fully. Further studies are necessary to definitively demonstrate the mechanisms by which N2733 inhibits TNF-α production by LPS-stimulated THP-1 cells.

**Effects of N2733 on murine endotoxin shock models**

To evaluate the inhibitory activity in vivo, the effects of N2733 on TNF-α production in the LPS-induced endotoxin shock model were examined in mice (Table 3). Serum levels of TNF-α were not detected in unstimulated mice. As shown in Table 3, LPS (5.6 mg/kg, i.v.) increased serum levels of TNF-α greatly within 1 hr. N2733 (100 mg/kg × 2, i.p.) also inhibited LPS-induced circulating TNF-α levels in vivo significantly. We think that this result reflects the action of the compound in vitro (Fig. 2 and Table 3).

TNF-α is one of the potent multifunctional cytokines and produced predominantly by activated macrophages. To study whether our potent inhibitor of TNF-α production would improve the severe shock state in a murine endotoxin model, we examined the effects of N2733 on the survival rate in LPS-treated mice. As shown in Fig. 4, this model was made by the injection of DBA/2 mice with LPS (30 mg/kg, i.v.), which reduced the survival rate to 30% within 7 days. Administrations of 30 mg/kg and 100 mg/kg N2733 (i.v.) improved survival rates to 60% and 90% respectively. Increased level of TNF-α has been reported in experimental endotoxin shock animal models and in septicemic and septicemic patients. Still more, administration of neutralizing anti-TNF-α antisera to mice or rabbits was protective against the lethal responses induced with endotoxin. For these reasons, TNF-α suppression is thought to be an effective target for therapeutic treatments. In our study, N2733 could reduce LPS-induced TNF-α production and improve the survival rate in murine endotoxin shock models. Based on our data and evidence presented by other investigators, we suppose the existence of a causal relation between TNF-α production and endotoxin-induced mortality. Furthermore, we think that N2733 may become the novel lead compound for anti-inflammatory drugs. Further experiments on related compounds are now in progress.

### Table 3. Effects of N2733 on TNF-α Concentration in LPS-Treated Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>TNF-α Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>10</td>
<td>10.31 ± 0.99</td>
</tr>
<tr>
<td>LPS + N2733 (100 mg/kg × 2)</td>
<td>10</td>
<td>5.26 ± 0.37***</td>
</tr>
</tbody>
</table>

Female DBA/2 mice were administered test compounds at −0.5 hr. At time zero, LPS was injected. At 0.5 hr, drugs were administered again. At 1 hr, serum TNF-α levels were analyzed by the commercial ELISA kits. Serum TNF-α levels in unstimulated mice were not detected. Statistics and data analyses were done using Student’s t-test. ** Statistically significant from the LPS treatment at p < 0.001.

### References

duced hypothermia and serum TNF-α levels in CD-1 mice by various pharmaceutical agents. *Agents Actions*, 39, C52-C54 (1993).


