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A Novel Small-Molecule Inhibitor Targeting the IL-6 Receptor β Subunit, Glycoprotein 130

Soon-Sun Hong,*† Jung Ho Choi,‡§ Sung Yoon Lee,*† Yeon-Hwa Park,*† Kyung-Yeon Park,‡ Joo Young Lee,‡ Juyoung Kim,* Veeraswamy Gajulapati,‡ Ja-II Goo,‡ Sarbjit Singh,‡ Kyeeong Lee,‡ Young-Kook Kim,‡ So Hee Im,‡ Sung-Hoon Ahn,‡ Stefan Rose-John,** Tae-Hwe Heo,† and Yongseok Choi‡

IL-6 is a major causative factor of inflammatory disease. Although IL-6 and its signaling pathways are promising targets, orally available small-molecule drugs specific for IL-6 have not been developed. To discover IL-6 antagonists, we screened our in-house chemical library and identified LMT-28, a novel synthetic compound, as a candidate IL-6 blocker. The activity, mechanism of action, and direct molecular target of LMT-28 were investigated. A reporter gene assay showed that LMT-28 suppressed activation of STAT3 induced by IL-6, but not activation induced by leukemia inhibition by factor. In addition, LMT-28 downregulated IL-6-stimulated phosphorylation of STAT3, gp130, and JAK2 protein and substantially inhibited IL-6-dependent TNF-α cell proliferation. LMT-28 antagonized IL-6-induced TNF-α production in vivo. In pathologic models, oral administration of LMT-28 alleviated collagen-induced arthritis and acute pancreatitis in mice. Based on the observation of upstream IL-6 signal inhibition by LMT-28, we hypothesized IL-6, IL-6Ra, or gp130 to be putative molecular targets. We subsequently demonstrated direct interaction of LMT-28 with gp130 and specific reduction of IL-6/IL-6Ra complex binding to gp130 in the presence of LMT-28, which was measured by surface plasmon resonance analysis. Taken together, our data suggest that LMT-28 is a novel synthetic IL-6 inhibitor that functions through direct binding to gp130. The Journal of Immunology, 2015, 195: 237–245.
act in concert to cause destruction of cartilage and bone in the joints of patients with RA (17–19). IL-6 is also produced during pancreatitis (20, 21) and upregulated during cerulein-induced acute pancreatitis in mice (22). Anti-IL-6Ra Ab decreased the pancreatic edema in cerulein-induced acute pancreatitis in IL-6 transgenic mice (23). Recently, it was demonstrated that IL-6 trans signaling is an essential mediator of acute lung injury in severe acute pancreatitis, and therapeutic inhibition of the IL-6/STAT3 pathway may prevent severe acute pancreatitis–associated lethal acute lung injury (24).

Recently, IL-6 blockade by numerous Ab biologies has been suggested to be a potential and alternative therapy for inflammatory diseases that are refractory to conventional drugs. Clinical studies targeting IL-6 using anti–IL-6 Ab include olokizumab (CP6038), sirukumab (CNOT136), sintuximab (CNOT328), clazakizumab (BMS945429), PF-423691, elisilimomab (BE-8), and MEDIS117, and by anti–IL-6 Ab including ticiluzim (Actemra) and sarilumab (REGN88) are ongoing or complete (4). A recent clinical study showed that tocilizumab was superior to adalimumab, anti-TNF mAb, as monotherapy for patients with RA (25). Although anti–IL-6 therapies have achieved great commercial successes, mAb biologies have some limits to their clinical utility (26). The disadvantages of therapeutic Abs are the high cost, invasive route of administration, and high rate of immunogenicity. Therefore, the discovery of low m.w. inhibitors is warranted by their superiority in oral absorption, low toxicity, and low antigenicity. Nevertheless, information on clinical studies using small-molecule blockers directed against IL-6, IL-6Ra, or gp130 is extremely limited.

Madindoline A is a nontoxic small molecule from a natural source that shows IL-6/IL-6R blocking properties (27, 28). Unfortunately, madindoline A is produced in very low yield by fermentation and is difficult to synthesize chemically. It was shown that preincubation of cells with PMA inhibits IL-6–induced STAT3 activation (17, 29, 30). This negative effect was found to be dependent on the activation of MAPKs and de novo transcription and protein synthesis (19, 27). Recently, the discovery of a small-molecule inhibitor of gp130 (SC144) for the treatment of ovarian cancer was reported (31), but an evidence of direct interaction between SC144 and gp130 was still missing.

In this study, we discovered and synthesized a derivative of oxazolidinone, LMT-28, examined its anti-IL6 activities using in vitro and in vivo systems, and identified a molecular target. LMT-28 exhibited significant IL-6 inhibitory activity, noncytotoxicity, and oral availability in our system. Our data demonstrate that LMT-28 is the first synthetic IL-6 inhibitor that alleviates inflammation in mice and functions through direct interaction with gp130.

Materials and Methods

General

A UV spectrum was obtained using a Hewlett Packard model 8453 spectrophotometer. [1H]Nuclear magnetic resonance spectroscopy (NMR) (500 MHz), [13C]-NMR (75 MHz), distortionless enhancement by polarization transfer, heteronuclear multiple quantum correlation spectroscopy, and heteronuclear multiple bond correlation spectroscopy (HMB) spectra were obtained using a Varian 500 MHz NMR spectrometer. Electrospray ionization mass spectrometry was measured on a Finnigan navigator spectrometer. The semipreparative HPLC system consisted of a Shimadzu Model LC-6AD pump, SPD-10A detector, and C-8 RA recorder.

Synthesis of oxazolidinone derivatives

A stirred solution of 4-oxazolidinone (2.5 g, 13.6 mmol, 1.0 eq) in CH2Cl2 (60 ml) was added to Bu3BOTf (16.37 ml, 16.37 mmol, 1.2 eq) and diisopropylethylamine (3.31 ml, 19.0 mmol, 1.4 eq) at 0˚C. After stirring for 50 min, 2-methylene pentanal (3.53 g, 28.0 mmol, 2.0 eq) was added to the enolate reaction mixture at −78˚C. The reaction mixture was stirred at −78˚C for 20 min and allowed to warm to 0˚C. After stirring for 1 h at 0˚C, a pH 7 buffer solution (1 ml) and MeOH (2 ml) was added to the reaction mixture, followed by continuous addition of H2O2 (2 ml) and MeOH (2 ml) at the same temperature. After stirring the reaction mixture for 1 h at 0˚C, the solven was removed in vacuo, followed by addition of water to the remaining mixture. The mixture was then extracted with CH2Cl2 (5 × 50 ml). The combined organic layer was dried over anhydrous MgSO4, filtered, and concentrated in vacuo. The organic residue was purified by column chromatography on silica gel (using 14% ethyl acetate in hexane as eluting solvents) to afford (3.5 g, 84%) of 5d. [1H]-NMR (CDCl3, 300 MHz): 8.51 (1H, br s, C = CH2), 4.99 (1H, s, C = CH2), 4.50–4.70 (4H, m, -OCH2CH2OH), 4.12 (1H, dd, J = 17.4, 9.3 Hz), 2.58 (1H, dd, J = 9.3, 2.9 Hz), 1.59 (3H, s, C(CH3)2), 0.93 (3H, t, J = 7.3 Hz, -CH2CH2OH), 3.15 (1H, br s, C2H5), 2.39–2.40 (1H, m, -CH(CH3)2), 2.04–1.90 (2H, m, -C≡ CH2CH2CH2CH2CH2OH), 1.49–1.43 (2H, m, -C≡ CH2CH2CH2CH2CH2OH), 1.34–1.28 (4H, m, -C≡ CH2CH2CH2CH2CH2OH), 1.18 (3H, d, J = 7.3 Hz, -CH2CH2OH), 0.93 (3H, d, J = 7.1 Hz, -CH2CH2OH), 0.88 (3H, d, J = 7.1 Hz, -CH2CH2OH), 0.88 (3H, t, J = 5.4 Hz, -C≡ CH2CH2CH2CH2CH2OH). MS (ESI) mass to charge ratio 312.2(M+1), [13C]-NMR (CDCl3, 75 MHz): 0 177.6, 154.3, 147.9, 110.4, 72.8, 63.3, 58.3, 40.1, 33.7, 32.7, 31.5, 31.4, 28.3, 27.6, 24.4, 17.8, 14.0, 13.9, 10.5.

DNA constructs and luciferase assay

The luciferase reporter construct of pSTAT3-Luc containing the TA minimal promoter with four tandem STAT3-binding sites was purchased from Clontech Laboratories. This reporter construct was transfected into HepG2 cells (2 × 104 cells/200 μl/well) via lipofection using Lipofectamine (Invitrogen). For example, 0.1 μg pSTAT3-Luc was suspended with 6 μl serum-free MEM, and 1 μl Plus reagent was added. In a separate tube, 0.3 μl lipofectamine reagent was added to 6 μl MEM. After incubation for 15 min at room temperature, the two solutions were mixed with serum-free MEM in a total volume of 50 μl, incubated for an additional 15 min at room temperature, and then added to HepG2 cells, which were at 35 or 60% confluence, according to the manufacturer’s protocol. After incubation for 3–4 h, the medium was replaced with fresh medium. After incubation for an additional 24 h, the transfected cells were starved for 24 h with serum-free media containing 0.1% BSA and allowed to reach 70–80% confluence. They were then stimulated for 3 h with human rIL-6 (10 ng/ml; R&D Systems), LIF (10 ng/ml; R&D Systems), IL-11 (10 ng/ml; R&D Systems), OSM (20 ng/ml; Sigma-Aldrich) in the presence or absence of screening compounds. Luciferase assays were performed using a commercial available kit (Promega).

Western blot analysis

HepG2 cells were seeded in six-well plates at 1 × 104 cells/ml/well (MEM; Welgene, Gyeongsan, Korea) with 10% (v/v) FBS, streptomycin (100 U/ml), and penicillin (100 U/ml; Life Technologies). After seeding, cells were starved overnight, treated with LMT-28 for 1 h, and stimulated with IL-6 (10 ng/ml); LIF (10 ng/ml), or PMA (100 nM; Sigma-Aldrich) for 10 min.

Cells were treated with 5% cell lysis buffer (ProMega) for 30 min on ice with occasional vortexing. The cell lysates were resolved on 8% SDS-PAGE gels, followed by transfer to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked overnight in Tris-buffered solution (50 mM Tris- HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk at 4˚C and then incubated for 1–2 h with the appropriate primary Abs against p-STAT3 (Calbiochem), STAT3 (Calbiochem), p-ERK (Thermo Fischer Scientific), ERK (Thermo Fischer Scientific), p-JAK2 (Cell Signaling Technology), anti-p-gp130 (Cell Signaling Technology), or β-actin Ab (Santa Cruz Biotechnology) in Tris-buffered solution (TBST; 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk at room temperature. The membranes were washed (10 min, five times) and incubated for 1 h with HRP-conjugated secondary Abs diluted to 1:6000. After 10 washes, the membranes were incubated with ECL reagents (Amersham GM-CSF) and chemiluminescent signals were visualized using X-ray film. Western blot bands from three experiments were quantitated by densitometric analysis.

Bioassay with IL-6–dependent TF-1 cells

TF-1 cells were maintained in RPMI 1640 (HyClone Thermo Scientific) containing 10% FBS (HyClone Thermo Scientific), 1% penicillin-streptomycin (Corning), and 1 μg/ml GM-CSF (Peprotech). Inhibition of TF-1 cells without GM-CSF for 24 h, various concentrations of IL-6 (Peprotech) were added to 5 × 105 cells/well and incubated for 72 h. The EZ-Cytotox assay kit (Daeil Lab Service, Seoul, Korea) was used to measure...
cell proliferation. In brief, 10 μl kit reagent was added to each well and incubated for 4 h in a CO2 incubator. After incubation, OD was measured at 450 nm.

**IL-6 inhibitory bioassay**

After starvation of TE-1 cells for 24 h, various concentrations of LMT-28 (1, 10, 100, 1000, 10,000 nm) or tocilizumab (0.007, 0.07, 0.7, 7, 70 nm; Chugai Pharmaceutical, Tokyo, Japan) in the presence of IL-6 (1 ng/ml) were added to the cells and incubated for 3 d. An EZ-Cytoc assay kit was used to measure cell proliferation as described earlier.

**Quantification of mouse serum TNF-α**

C57BL/6 mice (6 wk old, male; San takito Bio Korea) were divided into four study groups (n = 3–5): vehicle, IL-6 alone, LMT-28 pretreatment (0.4 or 0.8 mg/kg) plus IL-6 injection. The mice were starved for 12 h and then orally treated with vehicle (carboxy methyl cellulose [CMC] 0.5%; Sigma-Aldrich), LMT-28 0.4 mg/kg, or LMT-28 0.8 mg/kg. After 1 h, each group was i.p. treated with vehicle (PBS; W elgene) or IL-6 (0.025 mg/kg). After a further 1 h, sera were obtained by retro-orbital bleeding and kept at −80 °C until use. Serum concentration of mouse TNF-α was measured using the ELISA Ready-SET-Go! kit (eBioscience) according to the manufacturer's procedures. Data were presented as mean ± SEM.

**Induction of collagen-induced arthritis in mice**

Six-week-old male DBA/1J mice were purchased from Shizukuwa (H amamatsu, Japan). Mice were housed at 22 °C with 12-h light/dark cycles and had free access to drinking water. Experiments were performed after acclimation for 1 wk. The animal study was performed in accordance with institutional guidelines, and the protocol was approved by the Institutional Animal Care and Use Committee. Collagen-induced arthritis (CIA) was induced as described previously (32). In brief, mice were intradermally injected in the tail with 100 μg type II collagen (CII) emulsified in an equal volume of CFA. Twenty-three days after administration of the first injection of collagen, the mice received an i.p. booster injection of 100 μg CII. LMT-28 (0.25 mg/kg), methotrexate (MTX; 2.5 mg/kg) as a reference drug, or vehicle (5% CMC) was orally administered once daily for 15 d after administration of the second injection of collagen.

**Evaluation of clinical arthritis score in CIA**

The severity of arthritis in the mice was evaluated in all four paws by three blinded observers. The results were assessed according to a previously described method (18). In brief, the severity was scored as follows: 0, normal; 1, mild, apparent swelling limited to individual digits; 2, moderate, redness and swelling of the ankle; 3, redness and swelling in the paw and in the digits; and 4, maximally inflamed leg with involvement of multiple joints. The arthritis score for each mouse was the sum of arthritis severity in all four paws, with the highest possible score being 16.

**Measurement of cartilage oligomeric matrix protein, serum amyloid P, and anti-CII IgG Ab**

Upon completion of the CIA experiment, serum samples were taken from the mice. The serum levels of cartilage oligomeric matrix protein (COMP), serum amyloid P (SAP), and anti-CII IgG Ab were then measured using commercially available ELISA kits according to the manufacturers’ instructions. Kits from Kamiya Biomedical were used for COMP and SAP assays, and the kit for anti-CII IgG Ab was obtained from Chondrex.

**Pancreatitis animal study**

Male BALB/c mice (6 wk old, weighing 22–26 g) were obtained from Orient Bio Animal (Korea). The animals were fed standard mice chow and tap water ad libitum, and were maintained with a 12-h dark/light cycle at 21°C. The animals were treated from 12 h before the experiment but had free access to drinking water. Experiments were performed after acclimation for 1 wk. The animal study was performed in accordance with institutional guidelines, and the protocol was approved by the Institutional Animal Care and Use Committee. Collagen-induced arthritis (CIA) was induced as described elsewhere (32). In brief, mice were intradermally injected in the tail with 100 μg type II collagen (CII) emulsified in an equal volume of CFA. Twenty-three days after administration of the first injection of collagen, the mice received an i.p. booster injection of 100 μg CII. LMT-28 (0.25 mg/kg), methotrexate (MTX; 2.5 mg/kg) as a reference drug, or vehicle (5% CMC) was orally administered once daily for 15 d after administration of the second injection of collagen.

**Determination of amylase activities**

Amylase activity was assessed using a commercial kit (Bioassay) based on the use of Cibacron blue-amylose as a chromogenic substrate. The soluble chromogen in 0.1 ml serum was measured spectrophotometrically at 580 nm. The absorbance was linearly correlated with enzyme activity.

**RNA extraction and RT-PCR**

Total RNA was extracted from pancreas samples with TRIzol reagent (Invitrogen) following the manufacturer’s protocol. An aliquot of total RNA was reverse transcribed using reverse transcriptase and amplified using Taq DNA polymerase (Promega). The expression level of all transcripts was normalized to that of GAPDH mRNA in the same tissue. PCR was performed with the following primers: IL-6 (5’-GAAAATG-GAAAGAGTTGGTCG-3’ for sense and 5’-ATTGGAATTGGGTAG-GAGG-3’ for antisense), TNF-α (5’-CTGATGACCCGCTTGAG-3’ for sense and 5’-TTGAGATCATCCGTTG-3’ for antisense), IL-1β (5’-TCATTGGCTGATGCTAGCCACCT-3’ for sense and 5’-CACCCTGCTTCACCA-TCTGTTG-3’ for antisense). PCR products were electrophoresed on a 1.5% agarose gel, and the results were recorded by an imaging system (Kodak Molecular Imaging Systems); bands were quantitated using densitometry.

**Western blot analysis of pancreas protein**

A total of 30 μg pancreas protein was separated by 12% SDS-PAGE, and the resolved proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 10 mm Tris-HCl containing 150 mm NaCl and 0.5% Tween 20 (TBST). After washing with TBST, the membrane was incubated with a 1:1000 dilution of primary Ab. After the thorough washing with TBST, a 1:2000 dilution of HRP-conjugated secondary Ab (New England Biolabs) in TBST was applied to the membrane and the blot was developed using an ECL detection kit (Amersham).

**Histopathological examination**

Pancreas samples were fixed in 10% buffered formaldehyde, embedded in paraffin, and sectioned. The 4-μm-thick sections were stained with H&E for routine histology. Sections were stained with hematoxylin for 3 min, washed, and then stained with 0.5% eosin for an additional 3 min. After washing with water, the slides were dehydrated in 70, 96, and 100% ethanol, and finally in xylene. Two expert pathologists blindly evaluated the degree of pancreatitis according to a strict standard. Histopathological changes were recorded and photographed using a camera system (Olympus BX51, Japan).

**Surface plasmon resonance analysis**

To monitor the binding kinetics between LMT-28 and the extracellular domain of gp130 (Sino Biological, Beijing, China, or ANRT, Daejeon, Korea), we performed surface plasmon resonance (SPR) analysis using BIAcore T200 model (GE Healthcare) at 25°C with buffer HBS-EI+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% P-20) containing 5% DMSO (Sigma-Aldrich). The pH scouting for gp130 immobilization was performed in 10 mM acetate buffer at pH 4.0, 4.5, 5.0, 5.5. gp130 was immobilized on a CM5 sensor chip to the 3970 response unit (RU) with standard amine coupling at pH 4.5. LMT-28 was injected into the gp130-immobilized flow cell at concentrations of 100, 25, 12.5, 6.25, 3.125, and 1.563 μM with a flow rate of 30 μl/min for 240 s and allowed to dissociate for 900 s. T-200 BIAevaluation software was used to subtract references and determine the steady-state Kd. Between sample series, a solvent correction cycle was run to adjust for referencing errors caused by refractive index mismatches between the running buffer and sample.

The pH scouting for IL-6 or extracellular domain of recombiant human IL-6Rα (Peprotech) immobilization was performed in 10 mM acetate buffer at pH 4.0, 4.5, 5.0, 5.5. gp130 was immobilized on a CM5 sensor chip to the 3970 response unit (RU) with standard amine coupling at pH 4.5. LMT-28 was injected into the gp130-immobilized flow cell at concentrations of 100, 25, 12.5, 6.25, 3.13 μM with a flow rate of 30 μl/min for 240 s and allowed to dissociate for 900 s. T-200 BIAevaluation software was used to subtract references and determine the steady-state Kd. Between sample series, a solvent correction cycle was run to adjust for referencing errors caused by refractive index mismatches between the running buffer and sample.

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and allowed to dissociate for 600 s. Hyper–IL-6 (4, 2, 1, 0.5, 0.3, 0.1, and 0.06 μg/ml) was injected into the gp130-immobilized flow cell in the absence or presence of LMT-28 (200 μM) with a flow rate of 20 μl/min for 120 s and allowed to dissociate for 600 s. The pH scouting for Hyper–IL-6 immobilization was performed in 10 mM acetate buffer at pH 4.0, 4.5, 5.0, 5.5. Hyper–IL-6 was immobilized on a CM5 sensor chip with standard amine coupling at pH 4.5. gp130 (4, 2, 1, 0.5, 0.3, 0.1, and 0.06 μg/ml) was injected into the Hyper–IL-6-immobilized flow cell in the absence or presence of LMT-28 (200 μM) with a flow rate of 20 μl/min for 120 s and allowed to dissociate for 600 s.

Data analysis

Results are expressed as the mean ± SEM or SD, and data were compared for statistical significance with the Student t test. The p values <0.05, <0.01, and <0.001 were considered significant. LMT-28, the inhibitor that was identified in a primary screen by reporter gene assay, was validated by replicate testing in a five-point half-logarithmic or logarithmic dilution series, which allowed for determination of IC₅₀ values by nonlinear, least-squares fitting of dose–response curves using either Excel or Prism.

Results

Identification of LMT-28 as a specific blocker of IL-6 signaling by screening using a luciferase reporter gene assay

To identify small-molecule antagonists of IL-6, we screened our in-house chemical library containing >500 synthetic and 600 natural compounds by measuring the effects of each compound on IL-6–induced luciferase expression in human hepatocarcinoma HepG2 cells transfected with p-STAT3-Luc. In brief, p-STAT3-Luc–transfected HepG2 cells were stimulated with IL-6 in the presence of screening compounds, and luciferase activities were measured. Among the hit molecules, LMT-28, an oxazolidinone derivative
(Fig. 1A), showed the most potent activity and lowest cytotoxicity. LMT-28 reduced IL-6–induced luciferase activity by ∼90% at a concentration of 50 μM (Fig. 1B, left panel) and exhibited an IC₅₀ value of 5.9 μM. LIF binds to the heterodimer receptor complex of LIF receptor and gp130, and transduces a similar signal pathway with IL-6. However, because LMT-28 did not affect LIF-induced STAT3 activation (Fig. 1B, middle left panel), we postulated that LMT-28 targets the gp130 homodimer-inducing signaling. This hypothesis was confirmed by the observation that LMT-28 does not inhibit OSM stimulation (Fig. 1B, right panel) but does inhibit IL-11 stimulation on HepG2 cells (Fig. 1B, middle right panel).

Structure of LMT-28

LMT-28 exhibited a molecular ion peak at mass to charge ratio 312.5 [M+H]⁺ in the electrospray ionization mass spectrometry, and the molecular formula was determined as C₁₇H₂₉NO₄ by high resolution electron ionization mass spectrometry. The UV spectrum exhibited λ_max at 210 and 213 nm. The structure was determined by NMR data including [¹H]-NMR, [¹³C]-NMR, distortionless enhancement by polarization transfer, HMBC, and heteronuclear multiple quantum correlation spectroscopy. In the [¹H]-NMR spectrum, the protons of the oxazolidinyl group (δ 4.47, 4.57), the proton of carbonyl region (δ 3.95), an oxygenated methane proton (δ 4.22), an angular methyl proton (δ 1.18), and three secondary methyl protons (δ 0.87, 0.90, and 0.93) were observed (Table I). Furthermore, the HMBC correlation between H 2.35 (H-5') and C 58.31 (C-4') confirmed that the isobutyl group was substituted at the C-4' position on the oxazolidine moiety. The characteristics of NMR data and other physicochemical data did not match any previously reported results. Thus, LMT-28 is a novel compound having a structure shown in Fig. 1A.

LMT-28 selectively inhibits IL-6–induced phosphorylation of STAT3, JAK2, and gp130

Because LMT-28 inhibited IL-6–induced luciferase activity, we investigated whether LMT-28 inhibits STAT3 phosphorylation. HepG2 cells were treated with 10 ng/ml IL-6 for 1, 5, 10, 30, or 60 min and p-STAT3 was detected by Western blotting (Fig. 1C, first panel). Cells were incubated with LMT-28 for 1 h, followed by treatment with IL-6 for 10 min. LMT-28 did not show any cytotoxic activity (data not shown). Western blot analysis indicated that preincubation with LMT-28 (1, 3, 10, 30, and 100 μM) resulted in dose-dependent inhibition of IL-6–induced p-STAT3 expression by LMT-28 was monitored.

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LMT-28 inhibits IL-6–induced proliferation of the human erythroleukemic cell line TF-1

The IL-6 dependency of TF-1 cell proliferation was confirmed and the optimal concentration for the inhibitory assay was determined (Fig. 1D). Serial dilutions of LMT-28 were applied to TF-1 cells in the presence of IL-6 (1 ng/ml), and a proliferation assay was performed after 72 h. The anti–IL-6Rα mAb tocilizumab is an immunosuppressive biologic and was used as a positive control (Fig. 1E, left panel). LMT-28 markedly inhibited IL-6–induced TF-1 proliferation with an IC₅₀ value of 7.5 μM (Fig. 1E, right panel).

LMT-28 inhibits IL-6–induced TNF-α production in mice

We designed an experiment to examine the IL-6 targeting activity of LMT-28 in vivo. TNF-α production was induced by IL-6, and the reduction of TNF-α expression by LMT-28 was monitored. The IL-6–induced TNF-α titer in mouse serum was reduced significantly by LMT-28 (0.8 mg/kg, per oral; Fig 2A).

LMT-28 alleviates CIA in mice

In preliminary study we investigated the individual effect of LMT-28 on CIA mice in the dose range of 0–0.5 mg/kg. A single treatment with LMT-28 exerted a maximal effect at a dose of 0.25 mg/kg and reached a plateau at a dose of 0.5 mg/kg. Mice were orally treated once daily for 15 d with a placebo control (0.5% CMC) or 0.25 mg/kg LMT-28 via oral gavage beginning on the day of the second collagen injection. MTX (2.5 mg/kg) was orally administered as a standard drug. Arthritis severity was evaluated daily over the same period. Arthritis scores substantially decreased in the group treated with LMT-28 (0.25 mg/kg) compared with the vehicle-treated control group (Fig. 2B, top panel). An ∼7-fold molar excess dose of MTX over LMT-28 is required to achieve equivalent arthritis scores. To assess the effect of LMT-28 on cartilage destruction, we analyzed the levels of serum COMP as a circulating marker of cartilage turnover, SAP, and anti-CII IgG Ab. Treatment of CIA mice with LMT-28 markedly reduced the serum levels of COMP by 50% (Fig. 2B, lower left panel), SAP by 55% (Fig. 2B, lower center panel), and anti-CII IgG by 62% (Fig. 2B, lower right panel) compared with each vehicle-treated group.

LMT-28 ameliorates the progression of pancreatitis in mice

To investigate the protective effect of LMT-28 in a mouse model of cerulein-induced acute pancreatitis, we injected LMT-28 (0.25 or 1 mg/kg) orally 1 h before the first and fourth cerulein injections, respectively. Administration of LMT-28 decreased the activity of...
amylase, a pancreatic marker, in blood samples collected 3 h after the last cerulein injection (Fig. 2C, left panel). LMT-28 treatment also reduced the expression of the proinflammatory cytokines IL-1β, TNF-α, and IL-6 at the DNA and protein level, which was induced by cerulein treatment (Fig. 2C, center panel). Histopathological analysis of pancreas tissues showed that LMT-28 suppressed the formation of edema, inflammatory cell infiltration, and necrosis that were induced by cerulein treatment (Fig. 2C, right panel). Next, we studied the therapeutic effect of LMT-28 in a model in which we administrated LMT-28 (2 or 5 mg/kg) 1 h after the third and last cerulein treatments, respectively. LMT-28 showed therapeutic effect against pancreatitis as shown in the study for pancreatitis prevention (Supplemental Fig. 1).
identified. Based on the observation of upstream IL-6 signaling blockade by LMT-28, we suspected IL-6, IL-6Rα, and gp130 as potential candidate targets. To monitor the binding kinetics and affinity between LMT-28 and IL-6, IL-6Rα, or gp130, we performed an SPR assay. Recombinant gp130, rIL-6, or rIL-6Rα was covalently cross-linked to the dextran matrix of a CM5 sensor chip, and various concentrations of LMT-28 were passed on this surface and a reference surface. Representative reference-subtracted overlaid sensorgrams are displayed in Fig. 3A. Interestingly, LMT-28 bound to the extracellular domain of gp130 (Fig. 3A, left panel), but not to IL-6 (Fig. 3A, center panel) or to the extracellular domain of IL-6Rα (Fig. 3A, right panel). The binding affinity between LMT-28 and gp130 had a $K_D$ value of $7.4 \times 10^{-6}$ M derived from steady-state affinity determination. No binding of LMT-28 to IL-6 and IL-6Rα was observed, indicating that gp130 is the specific target of LMT-28.

After we confirmed that LMT-28 binds directly to gp130, we tested whether gp130-bound LMT-28 inhibits binding of the IL-6/IL-6Rα complex with gp130 using the BIAcore T200 system. Although LMT-28 did not affect IL-6 binding to chip-immobilized IL-6Rα (Fig 3B), LMT-28 substantially and dose-dependently inhibited the binding of the IL-6 plus IL-6Rα to chip-immobilized gp130 (Fig. 3C). In addition, we used Hyper–IL-6 instead of the soluble mixture of IL-6 and IL-6Rα to test the inhibitory activity of LMT-28. LMT-28 significantly inhibited Hyper–IL-6 binding to chip-immobilized gp130 (Fig. 3D), as well as gp130 binding to chip-immobilized Hyper–IL-6 (Fig. 3E).

**Discussion**

Deregulated formation of the IL-6/IL-6Rα/gp130 complex leads to a variety of clinical pathologic conditions, including inflammation and cancers. Various antagonists for IL-6, IL-6Rα, gp130, or downstream signaling molecules have been developed to treat IL-6–associated diseases. In fact, the anti–IL-6Rα mAb tocilizumab was approved in 2010 for the treatment of RA. However, targeting IL-6 or IL-6Rα with a specific mAb sometimes causes an increase in the systemic IL-6 level (33). Considering the multiple and shared networks with other cytokines, small-molecule inhibitors that target the intracellular IL-6 signaling pathway might evoke specificity and safety issues. Because gp130 is a common hub for transducing signals for IL-6 family cytokines, antagonism of gp130 has emerged as a potent therapeutic target for gp130-
mediated diseases. Although gp130 is clearly a promising target for inflammatory disease and cancers, to date no anti-gp130 Abs or gp130 inhibitors have been clinically developed.

In a screening for IL-6 blockers among synthetic chemicals, we identified LMT-28, an oxazolidinone derivative (Fig. 1A) that interferes with IL-6 stimulation by directly targeting gp130 (Fig. 3A) and blocking the association of the IL-6/IL-6Rα complex with gp130 (Fig. 3C–3E). Oxazolidinone derivatives are structurally related to biologically important bases and constitute a class of heterocyclic compounds that exhibit excellent druggability and substantial therapeutic activities, including antibiotic functions (34). Two small-molecule gp130 inhibitors, madindoline A (28, 35) and SC144 (31), have been reported. The natural compound madindoline A inhibits IL-6 activity in vitro and inhibits bone resorption in vivo (28), and appears to bind to the extracellular domain of gp130 (35). LMT-28 was superior to madindoline A in the context of activity (IC_{50} values 5.9 versus 22.8 μM, respectively, in our luciferase assay system), binding affinity (K_{D} values 7.4 versus 288 μM, respectively, in SPR assay system), and docking score energy (−3.895 versus −3.781 kcal/mol, respectively). Moreover, madindoline A is produced in very low yield in microbial fermentation and is difficult to synthesize chemically. SC144 inhibits STAT3 signaling and suppresses ovarian cancer cells (31). However, in contrast with LMT-28, SC144 induces gp130 phosphorylation, and physical interaction between SC144 and gp130 has not yet been demonstrated.

LMT-28 downregulates the phosphorylation of JAK2, gp130, and STAT3 that is induced by IL-6, but not that induced by LIF (Fig. 1B). Receptors of IL-6 type cytokines that share gp130 as a common subunit are divided into two types: homodimerization (e.g., IL-6 and IL-11) and heterodimerization (e.g., LIF and OSM) (36, 37). The mechanism of action of LMT-28 can be predicted from the differential effects on these two types of IL-6 type cytokines. Because IL-6 requires homodimerization of gp130 (13) and LIF requires heterodimerization between LIF receptor and gp130 (14), the mode of action of LMT-28 can be predicted as targeting the homodimerization of gp130. This theory was supported by our observation that LMT-28 also inhibited IL-11 activity (Fig. 1B). IL-11 also belongs to the same cytokine family at IL-6 and requires homodimerization of gp130 for its action. Consistent with this notion, this study demonstrates that LMT-28 interacts directly with gp130 (Fig. 3A), inhibits IL-6/IL-6Rα complex binding to gp130 (Fig. 3C–3E), and possibly inhibits gp130 homodimer-induced signaling (Fig. 1B).

Using an in vitro IL-6 bioassay system, we demonstrated inhibition of IL-6–induced cell proliferation by LMT-28 or toclizumab (Fig. 1E). In addition, LMT-28 specifically inhibits the in vivo activity of IL-6 (Fig. 2A). An important drug in the field of IL-6 blockade is the anti-IL-6Rα Ab tocilizumab, which is one of the new therapeutic approaches for RA (38). Instead of tocilizumab, which is not orally available, MTX was used as a positive control in our in vivo CIA model study (Fig. 2B). MTX is a disease-modifying antirheumatic drug and is used as conventional therapy for RA (39). LMT-28 is ~7-fold superior to MTX in terms of the required molar dose to achieve equivalent arthritis scores. This report describes a novel small-molecule inhibitor against RA with the underlying mechanism of directly targeting gp130. Like tocilizumab, LMT-28 has the potential to be a novel therapeutic for RA, but unlike tocilizumab, LMT-28 might be useful as a lead compound for the design of oral IL-6 blockers. Next, we evaluated the preventive and therapeutic effect of LMT-28 on acute pancreatitis as another inflammation model. In both studies, LMT-28 attenuated amylase activity, the expression of proinflammatory cytokines, and tissue inflammation (Fig. 2C, Supplemental Fig. 1). These results were consistent with previous reports in which anti-cytokine therapies against IL-6 showed a protective effect in experimental animal models of pancreatitis (23, 24). Taken together, LMT-28 seems to show preventive and therapeutic effects through targeting IL-6, as shown using anti–IL-6 Abs in other inflammatory diseases such as arthritis or colitis (40, 41).

IL-6 is a pleiotropic cytokine and contributes to the pathogenesis of numerous human diseases such as RA, pancreatitis, multiple sclerosis, systemic lupus erythematosus, Crohn’s disease, asthma, multiple myeloma, colorectal cancer, breast cancer, and lymphoma. Therefore, application of LMT-28 to various human diseases is anticipated, especially inflammatory, autoimmune, and cancer conditions. However, caution should be taken with any anti–IL-6 therapy, because complete or long-term inhibition of IL-6 may lead to negative side effects, including opportunistic infection, neuron degeneration, and heart failure.

Because other gp130 ligands can compensate for the inhibition of IL-6 signaling, gp130-targeted therapies have the potential to show more effective outcomes than anti–IL-6 or anti–IL-6Rα mAb therapies (12). However, considering the embryonic lethality of gp130 knockout mice (42) and the ubiquitous expression of gp130 in numerous human diseases such as RA, pancreatitis, multiple myeloma, colorectal cancer, breast cancer, and lymphoma, anti–IL-6 therapy, because complete or long-term inhibition of IL-6, has the potential to cause serious negative side effects. Therefore, the aim of gp130-targeted therapy should not be to completely eliminate the downstream signaling mediated by gp130, but rather to reduce it to normal levels.

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