Identification and Characterization of 4-[[4-(2-Butynyloxy)phenyl]sulfonyl]-N-hydroxy-2,2-dimethyl-(3S)-thiomorpholinecarboxamide (TMI-1), a Novel Dual Tumor Necrosis Factor-α-Converting Enzyme/Matrix Metalloprotease Inhibitor for the Treatment of Rheumatoid Arthritis

Yuhua Zhang,¹ Jun Xu,¹ Jeremy Levin, Martin Hegen, Guangde Li, Heidi Robertshaw, Fionula Brennan, Terri Cummons, Dave Clarke, Nichole Vansell, Cheryl Nickerson-Nutter, Daephine Barone, Ken Mohler, Roy Black, Jerry Skotnicki, Jay Gibbons, Marc Feldmann, Philip Frost, Glenn Larsen, and Lih-Ling Lin


Received September 5, 2003; accepted December 18, 2003

ABSTRACT

Tumor necrosis factor (TNF)-α is a well validated therapeutic target for the treatment of rheumatoid arthritis. TNF-α is initially synthesized as a 26-kDa membrane-bound form (pro-TNF) that is cleaved by a Zn-metalloprotease named TNF-α-converting enzyme (TACE) to generate the 17-kDa, soluble, mature TNF-α. TACE inhibitors that prevent the secretion of soluble TNF-α may be effective in treating rheumatoid arthritis (RA) patients. Using a structure-based design approach, we have identified a novel dual TACE/matrix metalloproteinase (MMP) inhibitor 4-[[4-(2-butynyloxy)phenyl]sulfonyl]-N-hydroxy-2,2-dimethyl-(3S)-thiomorpholinecarboxamide (TMI-1). This molecule inhibits TACE and several MMPs with nanomolar IC₅₀ values in vitro. In cell-based assays such as monocyte cell lines, human primary monocytes, and human whole blood, it inhibits lipopolysaccharide (LPS)-induced TNF-α secretion at submicromolar concentrations, whereas there is no effect on the TNF-α mRNA level as judged by RNase protection assay. The inhibition of LPS-induced TNF-α secretion is selective because TMI-1 has no effect on the secretion of other proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and IL-8. Importantly, TMI-1 potently inhibits TNF-α secretion by human synovium tissue explants of RA patients. In vivo, TMI-1 is highly effective in reducing clinical severity scores in mouse prophylactic collagen-induced arthritis (CIA) at 5, 10, and 20 mg/kg p.o. b.i.d. and therapeutic CIA model at 100 mg/kg p.o. b.i.d. In summary, TMI-1, a dual TACE/MMP inhibitor, represents a unique class of orally bioavailable small molecule TNF inhibitors that may be effective and beneficial for treating RA.

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease that results in progressive joint destruction and substantial morbidity. Despite a tremendous effort made to understand its etiology over more than two decades, the precise cause of RA remains unresolved. In recent years, the proinflammatory cytokine TNF-α has been demonstrated to play a pivotal role in RA (Feldmann et al., 1998). Elevated concentrations of soluble TNF-α are found in the synovial fluid of RA patients (Feldmann et al., 1994, 2001). TNF-α also contributes to cartilage breakdown and bone erosion by...
stimulating the production of other proinflammatory cytokines, recruitment of inflammatory cells into the synovium of joints, and induction of degradative enzymes such as various matrix metalloproteases (MMPs) (Choy and Panayi, 2001). The recent clinical success of anti-TNF-α agents such as the soluble TNF-α receptor (Moreland et al., 1997) and anti-TNF-α antibody (Elliott et al., 1994a,b) has further validated TNF-α as an important therapeutic target for RA. However, despite the success of these biological agents in the treatment of RA, this class of agents has various limitations, including the requirement of parenteral injection, high cost, and the possibility of antibody formation against these agents. Hence, the development of orally bioavailable, small molecule inhibitors of TNF-α represents a highly desirable strategy for treating RA.

TNF-α is initially expressed on the cell surface as a 26-kDa, type II transmembrane pro-form. The membrane-bound pro-TNF-α can then be cleaved between Ala-76 and Val-77 by a Zn-metalloprotease, TNF-α converting enzyme (TACE), resulting in the formation of the 17-kDa, mature, soluble cytokine. TACE is a member of the ADAM (a disintegrin and metalloprotease-containing enzyme) family of proteases and is the predominant protease responsible for the generation of soluble TNF-α (Moss et al., 2001). Indeed, T cells derived from TACE−/− mice knockout mice have a 90% reduction in their ability to process pro-TNF-α (Black et al., 1997). Levels of TACE protein and its enzymatic activity in the synovial tissue of patients with RA are significantly higher than those of patients with osteoarthritis (Ohta et al., 2001). Therefore, synthetic TACE inhibitors, which inhibit the processing of pro-TNF-α on the plasma membrane, represent an appealing alternative to the neutralization of TNF-α by biological agents.

TACE shares a significant degree of structural homology with the matrix metalloproteases in enzyme structures, particularly around the zinc-containing active site. MMPs have also been implicated to play a critical role in both RA and OA (Shaw et al., 2000). They may contribute to joint destruction directly by degrading the cartilage and bone structure, or indirectly by promoting angiogenesis in the formation of pannus in the joints of RA patients. Several MMP proteins or activities have been demonstrated to be elevated in the synovial tissue of patients with RA are significantly higher than those of patients with osteoarthritis (Ohta et al., 2001). Therefore, synthetic TACE inhibitors, which inhibit the processing of pro-TNF-α, may be clinically beneficial in treating RA patients.

Here, we report the discovery of a potent, orally bioavailable, dual TACE/MMP inhibitor, 4-[(4-(2-butylnyloxy)phenyl)sulfonyl]-N-hydroxy-2,2-dimethyl-(3S)-thiomorpholinecarboxamide (TMI-1). Structure-based design led to this sulfonamide hydroxamate inhibitor of TACE, which has a PI moiety that provides increased potency against TACE, both in enzyme and cellular assays. This compound inhibits TACE with an IC50 value of 8.4 nM. It also inhibits several MMPs, including MMP-1, -7, -9, -13, and -14 with nanomolar activity. In various cell-based assays, including human whole blood, it inhibits lipopolysaccharide (LPS)-induced TNF-α secretion at submicromolar concentrations, whereas it has no effect on the TNF-α mRNA level as judged by RT-PCR protection assays. Furthermore, TMI-1 potently inhibits spontaneous TNF-α secretion by human synovium tissue explants of RA patients. In vivo, TMI-1 has been demonstrated to be highly effective in reducing LPS-induced TNF-α secretion in mouse serum and clinical severity scores in both prophylactic and therapeutic mouse collagen-induced arthritis (CIA) models. Together, TMI-1, a dual TACE/MMP inhibitor, represents a unique class of orally active small molecules that may be effective and beneficial for treating RA.

**Materials and Methods**

**TACE and MMP Enzymatic Assays.** A proprietary synthetic peptide of pro-TNF-α containing the minimal TACE cleavage sequence, Abz-LAQAVRSSSS-Dpa, developed at Wyeth (Jin et al., 2002) and purchased by AnaSpec, Inc. (San Jose, CA), was used as the substrate for measuring TACE activities. A segment of the extracellular portion of the human TACE that comprises the catalytic domain, the disintegrin domain, the epidermal growth factor-like domain, and the CUB-like domain was used in the current study. The protein was expressed in Chinese hamster ovary cells and purified by nickel-nitritotriacetic acid and preparative size exclusion chromatography (Superdex 200 16/60) or columns to near homogeneity. Compounds were tested for their ability to inhibit the cleavage of the substrate by the purified enzyme in a fluorescence-based fluorescence resonance energy transfer (FRET) assay. The human TACE protein (1 μg/ml) was pretreated with the inhibitors at various concentrations for 10 min at room temperature. The reaction was initiated by the addition of pro-TNF-α peptide (50 μM final concentration) to the TACE protein, and the increase in fluorescence was monitored at excitation of 320 nm and emission of 420 nm over a period of 10 min as described previously (Jin et al., 2002). Under this assay condition, the IC50 value should be very close to the K_i value because the ratio of the substrate concentration to the K_m is 1:10 in the assay.

For MMP assays described here, the source of enzymes was the recombinant human catalytic domain either prepared at Wyeth Research (Cambridge, MA) (MMP-1, -13) or purchased from Calbiochem (San Diego, CA) (MMP-2, -7, and -9) or Chemicon International (Temecula, CA) (MMP-14). A continuous assay was used in which the substrate is a synthetic peptide containing a fluorescent group (7-methoxyxocoumarin), which is quenched by energy transfer to a 2,4-dinitrophenyl group. When the peptide was cleaved, an increase in fluorescence was observed. The substrate used was 7-methoxyxocoumarin-PQGL-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-AR-OH (denoted as Wammp-5, custom synthesized by AnaSpec, Inc.). The assay buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM CaCl₂, and 0.005% Brij-35 was prepared (Knight et al., 1992). Substrates were either colorimetric or fluorescent synthetic peptides purchased from either Bachem (King of Prussia, PA) (MMP-1, -7, -9, -13, and -14) or AnaSpec (MMP-2). The enzymatic reactions were initiated by adding the substrate to a final concentration of 20 μM. The initial rate of the cleavage reaction was determined immediately after substrate addition. For most MMP assays reported here, the IC50 value is approximately 2-fold of the K_i value.

The ADAM-TS-4 (Aggreganase-1) assay was performed using a fluorescent peptide Abz-TEGEOAR-SVa-Dnp-KK (denoted as WAAG-3R, custom synthesized by AnaSpec, Inc.). The assay buffer contains 50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM CaCl₂, 0.1% CHAPS, and 5% glycerol. Total reaction volume is 100 μl. The recombinant Agg-1 proteins generated at Wyeth Research (final concentration of 5 μg/ml in the assay) were pretreated with the various concentrations of the compound for 10 to 15 min at 37°C. The reaction was initiated by addition of the WAAG-3R substrate at a final concentration of 25 μM. The reaction is monitored at excitation of 340 nm and emission of 420 nm over a period of 30 min at 37°C in a fluorimeter (GeminiXPS; Molecular Devices Corp., Sunnyvale, CA).

**Cell-Based Assays.** The cell-based activity of TMI-1 was evaluated in human and murine monocyte cell lines, including THP-1 and RAW cells, human primary monocytes, and human whole blood for
the inhibition of LPS- or zymosan-induced TNF-α, IL-1β, IL-6, and IL-8 secretion. Cells were treated with LPS (100 ng/ml), zymosan (10 μg/ml) for 4 h in growth medium or human whole blood. At the end of the incubation period, the cells or the human whole blood were centrifuged at 1500 rpm for 15 min. The supernatants were collected and frozen at −80°C. The concentrations of soluble TNF-α and other cytokines were determined by an ELISA assay according to the manufacturer’s instructions (BioSource International, Camarillo, CA). The effect of TMI-1 on TNFRI shedding was determined in human whole blood after 4 h of incubation with phorbol 12-myristate 13-acetate (30 ng/ml). The supernatant was collected at the end of incubation period, and the level of TNFRI was determined by an ELISA assay (BioSource International).

Inhibition of TNF-α Secretion in Human Synovial Tissue Explants. The inflamed synovium tissues from the joints of RA patients were extracted as a by-product of joint replacement therapy with the consent of the patients. To liberate the cells from the connective tissue matrix, the synovium tissues were digested with collagenase IV and DNase. The generated macrophages, T cells, plasma cells, dendritic cells, and fibroblasts were treated with or without TMI-1 for 18 h before the onset of arthritis and cell culture. These synovium explant tissue cultures from RA patients secrete soluble TNF-α spontaneously with no external stimulation required. The supernatant was collected, and soluble TNF-α and IL-6 were detected by an ELISA assay according to the manufacturer’s instructions (BioSource International).

RNase Protection Assay. Raw cells were pretreated with compounds (TMI-1, PD98059, SB203580) for 1 h at the various concentrations. Both PD98059 and SB203580 were purchased from Calbiochem. Cells were stimulated with 100 ng/ml LPS overnight. The total RNA was extracted using TRIzol reagent from Invitrogen (Carlsbad, CA). Ten micrograms of total RNA from control and treated samples was hybridized with a 3²P-labeled mouse TNF-α probe (custom made by BD Pharmingen, San Diego, CA) at 56°C overnight. The sample was digested with RNase, separated on a 6% polyacrylamide gel (Sequa Gel; National Diagnostics, Manville, NJ), and autoradiographed as described by the manufacturer’s instructions (BD Pharmingen).

LPS-Induced Acute TNF-α Production in Mouse Sera. Balb/CJ (H-2d) mice were doped p.o. (five animals for each dose) with the compound at various concentrations 1 h before the i.v. injection of LPS (40 ng/mouse). One hour after LPS injection, blood samples were drawn under Avertin anesthesia, and the mice were sacrificed via CO₂ asphyxiation. TNF-α levels in serum samples were measured using a TNF-α ELISA assay.

Prophylactic CIA in Mice. Prophylactic CIA studies were conducted with either LPS- or collagen-booster in DBA/1 mice. In LPS-boostered CIA model, the arthritis was induced in female mice, aged 6 to 8 weeks, by intradermal injection at the base of the tail with 100 μl (100 μg) of type II collagen emulsified in complete Freund’s adjuvant (CFA) supplemented with additional 2 mg/ml Mycobacterium tuberculosis H37 Ra. Mice were dosed with the TMI-1 twice a day, whereas control mice received vehicle only. The dosing began on day 18 post-H37 Ra and continued for 18 days in an ex vivo cell culture. These synovium explant tissue cultures from RA patients secrete soluble TNF-α spontaneously with no external stimulation required. The supernatant was collected, and soluble TNF-α and IL-6 were detected by an ELISA assay (BioSource International).

Therapeutic CIA Model. In the therapeutic CIA model, DBA/1LacJ male mice of age 8 weeks were immunized on day 0 with 100 μg of bovine type II collagen (Chondrex, Redmond, WA) in CFA. On day 21, the mice were boosted with 100 μg of bovine type II collagen in incomplete Freund’s adjuvant. The mice were monitored daily for signs of arthritis using an established scoring system. At the first sign of arthritis, the affected mice were assigned to a treatment group. The mice were treated for 14 days with vehicle control or the indicated compounds at 100 mg/kg p.o. b.i.d. The disease severity scoring system is as follows: 0, no arthritis; 1, one or two swollen digits; 2, three or more swollen digits or mild to moderate swelling of the entire paw; 3, extensive swelling of the entire paw; and 4, resolution of swelling, and ankylosis of the paw.

All paws were evaluated for each animal and the maximum score per animal was 16. Each data point represents the average of 15 mice.

Results

TMI-1 Is a Dual TACE and MMP Inhibitor in Enzymatic Assays. TMI-1, a sulfonamide hydroxamate bearing a butylnoxyloxy P1’ group (Fig. 1), is a dual TACE and MMP inhibitor in enzymatic assay in vitro. In a FRET assay using purified human catalytic domain of TACE and a pro-TNF-α peptide as a substrate, TMI-1 is shown to be a potent TACE inhibitor in the enzymatic assay with an IC₅₀ value of 8.4 nM (Table 1). As demonstrated in Table 1, TMI-1 also potently inhibits various MMPs, including MMP-1, -2, -7, -9, -13, and -14 as well as ADAM-TS-4 (Aggrecanase-1) in vitro.

TMI-1 Inhibits LPS-Induced TNF-α Secretion in Cell-Based Assays. The effect of TMI-1 on LPS-induced TNF-α secretion was determined in various cell-based systems. In a murine monocyte cell line, Raw cells, TMI-1 inhibited TNF-α secretion with an IC₅₀ value of 40 nM. In a human monocyte cell line, THP-1 cells, TMI-1 has an IC₅₀ value of 200 nM. The effect of TMI-1 was also tested in primary human monocytes. The human monocytes were purified from human Buffy coats using RosetteSep antibody cocktail (Stem Cell Technologies, Vancouver, BC, Canada) with more than 80% purity as determined by fluorescence-activated cell sorting analysis. The IC₅₀ value of TMI-1 in this assay is 190 nM. TMI-1 inhibited the secretion of soluble TNF-α in a human whole blood assay with an IC₅₀ value of 300 nM (Table 2). In addition, TMI-1 also inhibited the secretion of TNF-α induced by other stimuli such as zymosan (Table 2), suggesting the effect of TMI-1 is not specific to LPS stimulation. TMI-1 also inhibits the phorbol 12-myristate 13-acetate-induced shedding of TNFR II in human whole blood with an IC₅₀ value of 0.72 μM.

The selectivity of TNF-α inhibition by TMI-1 over other proinflammatory cytokines was examined in both purified human primary monocyte and human whole blood. TMI-1 had no effect on LPS-induced secretion of soluble IL-1β, IL-6, or IL-8 at concentrations up to 10 μM in both assays (data not shown).

Fig. 1. Structure of TMI-1.
TABLE 1
IC_{50} of TMI-1 in enzymatic assay in vitro
The TACE enzymatic activity was determined using a proprietary synthetic peptide of pro-TNF-α containing minimal TACE cleavage sequence Abz-LAQAVRSSSR-Dpa. A segment of the extracellular human TACE protein containing the catalytic and disintegrin domains expressed and purified in CHO cells was used in a fluorescence-based FRET assay. The recombinant human catalytic domains of all the MMPs were used in various MMP assays as described under Materials and Methods. These data are averages from three experiments.

<table>
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<th>IC_{50} (nM)</th>
<th>TACE</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-7</th>
<th>MMP-9</th>
<th>MMP-13</th>
<th>MMP-14</th>
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TABLE 2
The effect of TMI-1 on the inhibition of TNF-α secretion in cell-based assays
The cell-based activity of TMI-1 was determined in murine and human monocyte cell lines, Raw cells, THP-1, human primary monocytes, and human whole blood for the inhibition of LPS or zymosan-induced TNF-α secretion. Cells were pretreated with TMI-1 or vehicle control at various concentrations for 1 h before addition of LPS or Zymosan. The cells were cultured for 4 h, and the supernatants were collected and frozen at −80°C. The concentration of soluble TNF-α was determined by an ELISA assay. In our systems, the range of soluble TNF-α induced by LPS (10 ng/ml) is 10 to 50 ng/ml in Raw cells, 5 to 15 ng/ml in human whole blood, and 2 to 10 ng/ml in human monocytes. In THP-1 cells, the range of soluble TNF-α induced by LPS (10 μg/ml) is 0.5 to 5 ng/ml. The range of soluble TNF-α induced by Zymosan (10 μg/ml) is 5 to 15 ng/ml in human whole blood. The data are averages from three experiments.

<table>
<thead>
<tr>
<th>Inducing Agent</th>
<th>Cell system</th>
<th>LPS</th>
<th>LPS</th>
<th>Human primary monocytes</th>
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<th>Human whole blood</th>
<th>LPS</th>
<th>Zymosan</th>
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<td>0.19</td>
<td>0.3</td>
<td></td>
<td>0.4</td>
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<tr>
<td>LPS</td>
<td>THP-1</td>
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To determine whether TMI-1 has an effect on other inflammatory pathways such as the arachidonic acid signaling pathway, we tested the effect of TMI-1 on LTE4 hydrolase, a Zn-metalloprotease involved in leukotriene biosynthesis in the arachidonic acid pathway. TMI-1 has no effect on the hydrolase activity of the enzyme at concentrations up to 10 μM in MC9 cells (data not shown).

**TMI-1 Inhibits the Generation of Soluble TNF-α in Synovium Culture of RA Patients.** The effect of TMI-1 on TNF-α production in synovium cultures of RA patients was examined in ex vivo experiments. To do this, the inflamed synovium from the joints of RA patients were removed as by-products of joint replacement therapy. The synovium tissue was then digested with collagenase IV and DNase to release the cells from the tissue matrix. This synovium cell mixture (macrophages, T cells, plasma cells, dendritic cells, endothelial cells, and fibroblasts) spontaneously produces TNF-α and other proinflammatory cytokines with no external stimuli required. The soluble TNF-α in the supernatant was determined by an ELISA assay after 2 days of culture with or without TMI-1 treatment. Treatment with TMI-1 markedly reduced the production of soluble TNF-α in samples of all four patients examined despite some variation in the level of TNF-α production in the synovium culture and the degree of inhibition between individual patients. A representative study (patient SM1936) is shown in Fig. 2. The data from all four RA patients are summarized in the table of Fig. 2. An IC_{50} value of less than 100 nM was derived from three of four RA patient samples tested.

Treatment with TMI-1 in the synovium culture also reduced the level of other proinflammatory cytokines such as IL-1β, IL-6, and IL-8 to various degrees. An average of 30% reduction of IL-1β, IL-6, and IL-8 in the presence of 1 μM TMI-1 was observed. This effect was likely due to the secondary effect of TNF-α-induced production of these cytokines because TMI-1 has no direct effect on the secretion of IL-1β, IL-6, or IL-8 induced by LPS in a 4-h human whole blood assay in vitro as described above. Treatment with a TNF antibody also led to similar reduction of the spontaneous secretion of IL-1β, IL-6, and IL-8 in this system as demonstrated previously (Butler et al., 1995). A representative experiment for IL-6 production is shown in Fig. 2.

**TMI-1 Has No Effect on TNF-α mRNA Level.** Because TMI-1 inhibits TACE and various MMPs in enzymatic assays in vitro, we investigated the possibility that the potent inhibitory effect of TMI-1 on TNF-α secretion in cellular assays may be due to its inhibitory effect on TNF-α transcription. TMI-1 was tested for its effect on TNF-α mRNA levels by an RNase protection assay in Raw cells. As shown in Fig. 3, stimulation with LPS (10 ng/ml, overnight) led to an approximate 5- to 10-fold increase of TNF-α mRNA. Treatment with TMI-1 at 1 μM did not have any effect on this LPS-stimulated increase of TNF-α mRNA levels, whereas incubation with two mitogen-activated protein kinase inhibitors, SB203580 and PD98059, which are known to inhibit TNF-α production at the post-transcriptional level, led to a significant inhibition of TNF-α mRNA levels. These results indicate that the mechanism for the inhibition of soluble TNF-α by TMI-1 is not due to the inhibition of mRNA transcription or to the destabilization of TNF-α mRNA and suggest that it is due to its activity on TACE.

**TMI-1 Inhibits TNF-α Production in an Acute LPS-Mouse Model.** To evaluate the ability of TMI-1 to inhibit soluble TNF-α secretion in vivo, we used an acute LPS model in Balb/CJ (H-2d) mice. Mice were dosed orally with TMI-1 1 h before the i.v. injection of LPS. Blood samples were drawn 1 h after LPS-stimulation and TNF-α levels in serum were determined by a TNF-α ELISA assay. As shown in Fig. 4, TMI-1 treatment led to a dose-dependent inhibition of TNF-α production in this model. At 25 mg/kg, complete inhibition of soluble TNF-α secretion was achieved. The ED_{50} value of TMI-1 is 5 mg/kg in this model. The pharmacokinetic analysis at a single oral dose of 50 mg/kg in Balb/CJ mice indicates that TMI-1 has a C_{max} value of 3 μM, a half-life (t_{1/2}) of 1.68 h, an area under the curve of 1484 ng · h/ml, and a bioavailability of 39%. Thus, TMI-1 is a potent, orally bioavailable inhibitor of TNF-α in vivo.

**TMI-1 Reduces Clinical Severity Scores in Prophylactic and Therapeutic CIA Models.** The efficacy of TMI-1 in the treatment of RA was evaluated in three different types of murine CIA models, a prophylactic model with either LPS- or type II collagen-boost, and a therapeutic model with type II collagen-boost.

Figure 5 shows the results of TMI-1 in a prophylactic CIA model.
model. Oral treatment with TMI-1 starting on day 18 led to a dose-dependent reduction of the symptomatic clinical severity scores. Efficacy was observed at both 10 and 20 mg/kg p.o. b.i.d. in this experiment. At a dose of 20 mg/kg p.o. b.i.d., treatment with TMI-1 caused a pronounced reduction of clinical severity scores with a relatively rapid onset. Microscopic evaluation of the joints also revealed protective effects of TMI-1 at 20 mg/kg against CIA as measured by the Mankin scores, histopathology scores, and the fat pad synovial histopathology and histomorphometry data (data not shown). In addition, the effect of TMI-1 in the prophylactic CIA model with a collagen boost was also evaluated. Oral treatment with TMI-1 (50 and 100 mg/kg) led to a clear reduction in clinical severity scores in the collagen-boosted CIA model (data not shown).

A more rigorous animal model for RA is the therapeutic CIA. In this model, the treatment with test compounds begins after all of the mice have developed symptoms of arthritis. In the study shown in Fig. 6, DBA/1LacJ male mice were immunized on day 0 with 100 μg of bovine type II collagen (Chondrex) in CFA, and the mice were boosted with 100 μg of bovine type II collagen on day 21. After the mice displayed arthritic symptoms, they were assigned to the treatment group and were dosed for 14 days either with vehicle control or TMI-1 at 100 mg/kg p.o. b.i.d. As seen in Fig. 6, TMI-1-treated mice began to show a clear difference from the vehicle control animals as early as 4 days after the treatment. The reduction of the disease severity score was maintained during the 14-day treatment, although the extent of the reduction was less pronounced compared with that shown in the prophylactic model. These data suggest that TMI-1 is efficacious in the therapeutic CIA model.

Enbrel, a soluble type II TNF receptor, was used as a positive control in both prophylactic and therapeutic CIA studies. In both models, Enbrel effectively blocked the disease severity scores and the efficacy was maintained through the course of the treatment. In the prophylactic model, an i.p. injection of Enbrel (30 μg/mouse/day) produced efficacy comparable with TMI-1 at 10 mg/kg p.o. b.i.d. In the therapeutic model, once a day i.p. injection of Enbrel at 150 μg/mouse is more potent than TMI-1 (100 mg/kg p.o. b.i.d.), with a 74% of reduction of the disease severity scores on day 13 of the treatment in a separate study.

**Discussion**

In this report, we describe a novel, dual TACE/MMP inhibitor, TMI-1. Although the majority of reported inhibitors of TACE are peptide-like molecules, TMI-1 is a sulfonamide hydroxamate, bearing a butynloxy P1 group. The P1 moi-

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<th>TMI-1 (μM)</th>
<th>% Inhibition of TNFα Secretion</th>
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<tr>
<td></td>
<td>SM1936</td>
</tr>
<tr>
<td>0.01</td>
<td>19</td>
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Fig. 2. Effect of TMI-1 on TNF-α and IL-6 secretion in human synovium culture of RA patients. The synovium tissues were extracted from the joints of four RA patients (SM1936, SM2125, SM2126, and SM2229) as a by-product of joint replacement therapy. Cells were released from the synovium connective tissue matrix by digestion with collagenase IV and DNase. The resulting multicell mixture containing macrophages, T cells, plasma cells, dendritic cells, and fibroblasts was cultured in the presence of various concentrations of TMI-1 or dimethyl sulfoxide vehicle control for 2 days. These synovium explant tissue cultures from RA patients secrete soluble TNF-α spontaneously with no external stimulation required. The supernatant was collected at the end of incubation period, and soluble TNF-α and IL-6 was detected by an ELISA assay. The data in the figure are from the samples of patient SM1936. The percentage of inhibitions of TNF secretion from all four patients tested is summarized in the table.
and is highly efficacious in the reduction of clinical symptoms in mouse prophylactic and therapeutic CIA models. More importantly, TMI-1 potently inhibits spontaneous TNF-α release from synovium explant cultures of RA patients (IC50 value <100 nM). This is the first demonstration of the activity in synovium cultures of RA patients for a dual TACE/MMP inhibitor.

Both TNF-α and various MMPs have been shown to play important roles in RA and OA (Feldmann et al., 1998; Shaw et al., 2000; Fernandes et al., 2002). In addition to the clinically validated role of TNF, other proinflammatory cytokines such as IL-1β, IL-6, IL-15, IL-17, and IL-18 are also believed to contribute to the progression of RA in a TNF-α-dependent or -independent manner (McInnes and Liew, 1998; Gracie et al., 1999; Ziolkowska et al., 2000; Kobayashi et al., 2002). A common activity shared by these proinflammatory cytokines (TNF-α, IL1-β, IL15, and IL-17) is to induce the production of various MMPs (Chabaud et al., 2000; Constantinescu et al., 2001), which can then directly or indirectly participate in progression of RA. A precise profile of individual MMPs desirable to be inhibited for RA treatment is currently not known, but expression of MMP-1, -2, -3, -8, -9, -13, and -14 has been shown to be elevated in RA synovial fluid (Katrib et al., 2001). Hence, a dual TACE and MMP inhibitor, such as TMI-1, may be effective and beneficial in treating RA and OA. In this context, several dual TACE/MMP inhibitors have recently been reported to be effective in various experimental models for RA (Conway et al., 2001; Beck et al., 2002).

One of the key questions that remains unresolved for the development of TACE inhibitors as therapeutic agents is whether membrane TNF-α plays a significant role in RA and other inflammatory diseases. Studies both in vitro and in vivo have shown that membrane TNF-α is biologically functional (Decker et al., 1987; Kriegler et al., 1988; Akassoglou et al., 1997). Controversial data on the involvement of membrane TNF-α in animal models of arthritis has been reported by several groups. The evidence of an in vivo role for membrane TNF-α in inflammation has come from transgenic mice overexpressing noncleavable TNF-α under the control of human β-globin promoter (Akassoglou et al., 1997). This study is, however, imperfect because the overexpression of the membrane TNF-α may enable immunological or pathological processes to be initiated that would not occur under physiological conditions. By contrast, an independent study that used a transgenic mouse model overexpressing a noncleavable membrane TNF-α in which TNF expression was controlled by the TNF-α promoter and the 3′AU-rich elements, did not support this finding. These mice express a moderate level of membrane TNF-α and did not spontaneously develop arthritis symptoms (Mueller et al., 1999). Recently, the knockin mice that express only the noncleavable membrane-bound form of TNF-α were reported (Ruuls et al., 2001). Mice with this TNF-α knockin did not develop arthritis spontaneously and had reduced disease severity in an experimental autoimmune encephalomyelitis model. Furthermore, these mice were highly resistant in the CIA model (J. Sedgwick, personal communication). These results suggest that soluble TNF-α plays a dominant role in inflammation and autoimmune diseases, whereas membrane-bound TNF-α may have a protective role. The molecular mechanism of this protective function of the membrane TNF-α is not yet understood, but it may be due to the induction of the TNF-α unresponsiveness...
by membrane TNF-α as previously reported by using an uncleavable TNFα1-9, K11E mutant (Decoster et al., 1998).

Whether the treatment with TACE/MMP inhibitors could lead to a massive buildup of pro-TNF-α on the cell surface raises another concern for these inhibitors. Although T cells derived from TACE knockout mice did show an increase in cell surface expression of pro-TNF-α (Black et al., 1997), and treatment with several broad-spectrum metalloprotease inhibitors also enhanced the expression of membrane TNF-α at the cell surface (Mohler et al., 1994; Crowe et al., 1995; Solomon et al., 1997), other dual TACE/MMP inhibitors and selective TACE inhibitors did not have a significant effect on cell surface TNF-α (Dekkers et al., 1999; Newton et al., 2001). The knockin mice with uncleavable membrane TNF-α under the control of a TNF-α promoter also did not show an accumulation of membrane TNF-α at the cell surface (Ruuls et al., 2001) in contrast to the transgenic mice overexpressing membrane TNF-α (Akassoglou et al., 1997). Our data in Raw cells and human synovium culture from RA patients (data not shown) also did not show a significant accumulation of pro-TNF-α in the cells treated with TMI-1. Thus, the accumulation of pro-TNF-α in the cells may be transient and was not detected in our experiments. Alternatively, the level of the membrane TNF-α may be tightly regulated. The membrane TNF-α may be rapidly degraded or internalized once it reaches a certain level on the cell surface. In support of this hypothesis, a previous report using a pulse-chase analysis in vitro in the presence of TACE inhibition has estimated that the majority (>85%) of pro-TNF-α that is not processed by the cells is rapidly degraded. In a human endotoxemia study in vivo, treatment with a broad-spectrum TACE/MMP inhibitor (GI5402) also did not lead to an accumulation of pro-TNF-α on the surface of circulating cells, including monocytes, lymphocytes, and granulocytes (Dekkers et al., 1999).

Because MMP is implicated in the remodeling of joint structure, an important concern for the development of a broad-spectrum TACE/MMP inhibitor is its speculative role in the induction of fibroplasias or other musculoskeletal side effects in vivo. It is known that some broad-spectrum MMP inhibitors with low or no TACE activity (Marimastat, Prinomastat, CGS-27023) cause fibroplasias or musculoskeletal side effects in clinic, whereas others (Trocade, BMS-275291, BAY 12-9566) did not (Rudek et al., 2002). Hence, whether the cause of fibroplasias is the inhibition of a particular MMP, or set of MMPs, or the inhibition of sheddases, is currently unknown. To examine the potential effect of TMI-1 on the induction of fibroplasias, we conducted a 28-day pilot study in CD VAF rats. We did not observe any signs of fibroplasias after treatment with TMI-1 at the doses up to 600 mg/kg/day in this 28-day study (data not shown). Similarly, a previous report that compared two broad-spectrum MMP inhibitors, one with and one without TACE activity, in a rat tendonitis model, also demonstrated that only the broad-spectrum MMP inhibitor without TACE activity induced the clinical signs of tendonitis, whereas the broad-spectrum MMP inhibitor with TACE activity at the same
exposure level did not (Drummond et al., 1999). Therefore, it is tempting to suggest that TACE activity may have a protective role against the development of fibroplasias. This may be due to the anti-inflammatory activity through the inhibition of soluble TNF and the additional protective, anti-inflammatory role of membrane TNF as discussed above.

In summary, we have reported here that TMI-1, a dual TACE/MMP inhibitor with concerted action of blocking the inflammatory cytokine TNF and the degradative enzyme MMP, has a potent activity in vitro and in the animal models of arthritis. Thus, it offers a great potential as a therapeutic agent in treating rheumatoid arthritis and other inflammatory diseases.

Acknowledgments


References


Dr. Yuhua Zhang, Wyeth Research, 200 Cambridge Park Dr., Cambridge, MA 02140. E-mail: yzhang@wyeth.com

Address correspondence to:

TACE Inhibitor for Treating RA 355


Dr. Yuhua Zhang, Wyeth Research, 200 Cambridge Park Dr., Cambridge, MA 02140. E-mail: yzhang@wyeth.com

Address correspondence to:

TACE Inhibitor for Treating RA 355

