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Identification and Characterization of TMI-1, a Novel Dual TACE/MMP Inhibitor for the Treatment of RA

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TNF-α, tumour necrosis factor-α; LPS, lipopolysaccharide; TACE, tumor necrosis factor-converting enzyme; MMP, metalloproteinase; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; IL, interleukin; RA, rheumatoid arthritis; CIA, Collagen-induced arthritis; TMI-1**,** 4-[[4-(2-butynyloxy)phenyl]sulfonyl]-N-hydroxy-2,2-dimethyl- (3*S*)-thiomorpholinecarboxamide

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Abstract

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 RA patients. Using a structure-based design approach, we have identified a novel dual TACE/MMP inhibitor 4-[[4-(2-butynyloxy)phenyl]sulfonyl]-N-hydroxy-2,2-dimethyl- (3*S*)-thiomorpholinecarboxamide (TMI-1). This molecule inhibits TACE and several MMPs with nanomolar IC_{50s} in vitro. In cell-based assays such as monocyte cell lines, human primary monocytes, and human whole blood, it inhibits LPS- induced TNF-α secretion at submicromolar concentrations, while there is no effect on the TNF-α mRNA level as judged by RNAase protection assay. The inhibition of LPS-induced TNF-α secretion is selective since TMI-1 has no effect on the secretion of other pro-inflammatory cytokines such as 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 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.

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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 pro-inflammatory 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; Feldmann et al., 2001). TNF-α also contributes to cartilage breakdown and bone erosion by stimulating the production of other pro-inflammatory cytokines, recruitment of inflammatory cells into the synovium of joints, and induction of degradative enzymes such as various MMPs (Choy and Panayi, 2001). The recent clinical success of anti-TNF-α agents such as the soluble TNF-α receptor (Enbrel^{TM}) (Moreland et al., 1997) and anti-TNF-α antibody (Remicade^{TM}) (Elliott et al., 1994a; Elliott et al., 1994b) 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 represent a highly desirable strategy for treating RA.

TNF-α is initially expressed on the cell surface as a 26 kD, 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 kD, 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$^{\Delta Zn/\Delta Zn}$ 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 (MMP) 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 angoigenesis in the formation of pannus in the joints of RA patients. Several MMP proteins or activities have been demonstrated to be elevated in the synovium of RA patients (Pap et al., 2000; Katrib et al., 2001; Tomita et al., 2002). Therefore, a dual TACE and MMP inhibitor 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-butynyloxy)phenyl]sulfonyl]-N-hydroxy-2,2-dimethyl- (3S)-thiomorpholine-carboxamide (TMI-1). Structure-based design led to this sulfonamide-hydroxamate inhibitor of TACE which has a P1' moiety that provides increased potency against TACE, both in enzyme and cellular assays. This compound inhibits TACE with an IC_{50} of 8.4 nM. It also inhibits several MMPs including MMP-1, -7, -9, -13, and -14 with nM activity. In various cell-based assays, including human whole blood, it inhibits LPS-induced TNF-α secretion at submicromolar concentrations while it has no effect on the TNF-α mRNA level as judged by RNAase 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 CIA models. Taken 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.

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Material and Methods

TACE and MMP Enzymatic Assays

A proprietary synthetic peptide of pro-TNF-α containing the minimal TACE cleavage sequence, Abz-LAQAVRSSSR-Dpa developed at Wyeth (Jin et al., 2002) and custom made 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 EGF-like domain, and the Crambin-like domain was used in the current study. The protein was expressed in CHO cells and purified by NiNTA and preparative SEC (Superdex 200 16/60) 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 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 should be very close to the Ki since the ratio of the substrate concentration to the Km 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 in Cambridge (MMP-1, -13) or purchased from Calbiochem (MMP-2, -7, -9) or Chemicon (MMP-14). A continuous assay was used in which the substrate is a synthetic peptide containing a fluorescent group (7-methoxycoumarin; Mca) which is quenched by energy transfer to a 2,4-dinitrophenyl group. When the peptide was cleaved by MMPs, an increase in fluorescence was observed. The substrate used was Mca-PQGL-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-AR-OH (denoted as Wammp-5, custom synthesized by AnaSpec, Inc.). The assays were carried out at room temperature in a buffer containing 50 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, and 0.005% Brij-35 as previously reported (Knight et al., 1992). Substrates were either colorimetric or fluorescent synthetic peptides purchased from either Bachem (MMP-1, -7, -9, -13, -14) or AnaSpec (MMP-2). The enzymatic reactions were initiated by adding the substrate to a final concentration of 20μM. The initial rate of the cleavage reaction was determined immediately after substrate addition. For most MMP assays reported here, the IC50 is approximately 2 fold of the Ki.

The ADAM-TS-4 (Aggrecanase-1) assay was performed using a fluorescent peptide Abz-TEGEARGSVI-Dap(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 $CaCl_2$, 0.1 % CHAPS, 5% glycerol. Total reaction volume is 100 μl. The recombinant Agg-1 proteins generated at Wyeth (final concentration of 5 μg/ml in the assay) were pretreated with the various concentrations of the compound for 10 –15 min at 37^{o}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 (GeminiXS from Molecular Devices, Inc).

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 lipopolysaccharide- (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 hrs 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 instruction (Biosource International, Inc.). The effect of TMI-1 on TNFRII shedding was determined in human whole blood after 4 hr of incubation with PMA (30 ng/ml). The supernatant was collected at the end of incubation period and the level of TNFRII was determined by an ELISA assay (Biosource International, Inc.).

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 DNAase. The generated macrophages, T cells, plasma cells, dendritic cells, and fibroblasts were treated with or without TMI-1 for 2 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 according to the manufacturer's instruction (Biosource International, Inc.).

RNAase Protection Assay

Raw cells were pretreated with compounds (TMI-1, PD98059, SB203580) for 1 hr at the various concentrations. Both PD98059 and SB203580 were purchased from Calbiochem. Cells were stimulated with 10 ng/ml of LPS overnight. The total RNA was extracted using TRIzol reagent from LifeTechnologies. 10 μg of total RNA from control and treated samples was hybridized with a ^{32}P-labeled mouse TNF-α probe (custom made by PharMingen) at 56°C overnight. The sample was digested with RNase, separated on a 6% polyacrylamide gel (Sequa Gel from National Diagnostics), and autoradiographed as described by the manufacturer's instruction (PharMingen).

LPS-induced Acute TNF-α Production in Mouse Sera

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Balb/CJ (H-2d) mice were dosed p.o. (five animals for each dose) with the compound at various concentrations one hour prior to the intra-peritoneal injection of LPS (40 ng/mouse). One hour after LPS injection, blood samples were drawn under Avertin anesthesia and the mice were sacrificed via CO_2 asphyxiation. TNF-α levels in serum samples were measured using a TNF-α ELISA assay.

Prophylactic Collagen-Induced Arthritis (CIA) in Mice

Prophylactic CIA studies were conducted with either LPS- or Collagen-boosts in DBA/1 mice. In LPS-boosted CIA model, the arthritis was induced in female mice, aged 6-8 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 of Mycobacterium tuberculosis H37 Ra. Mice were dosed with the TMI-1 twice a day, while control mice received vehicle only. The dosing began on day 18 prior to the onset of arthritis and continued for 17 days. On day 21, disease was induced by injecting 40 µg of LPS intraperitoneally. Inflammation in the paws of the type II collagen immune-mice was clearly observable 96 hours post LPS boost. Non-immune and CFA control mice did not exhibit paw inflammation. The disease severity of arthritis (Disease severity score) was assessed at different time points based on the appearance of each paw, and subjectively graded on a scale of 0 to 4 as follows:

0 = Normal appearance
1 = Erythema/edema in 1-2 digits
2 = Erythema/edema in more than 2 digits, or mild swelling in ankle/wrist joint
3 = Erythema/edema in entire paw
4 = Massive erythema/edema of entire paw extending into proximal joints, ankylosis, loss of function

The scores of each limb were summed, giving a maximum severity score of 16. Each data point represents the average of 15 mice.

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) in CFA. On day 21, the mice were boosted with 100 µg of bovine type II collagen in Incomplete Freund's Adjuvant (IFA). 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
4 Resolution of swelling, 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.

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Results

TMI-1 is a dual TACE and MMP inhibitor in enzymatic assays

TMI-1, a sulfonamide hydroxamate bearing a butynyloxy P1' group (Figure 1), is a dual TACE and MMP inhibitor in enzymatic assay in vitro. In a fluorescence-based 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 IC_{50} 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, -14 as well as ADAM-TS-4 (Aggrecanase-1) in vitro.

TMI-1 inhibits LPS-induced TNF-α secretion in cell-based assay

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 IC_{50} of 40 nM. In a human monocyte cell line, THP-1 cells, TMI-1 has an IC_{50} 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 (StemCell Technologies Inc) with more than 80% purity as determined by FACS analysis. The IC_{50} of TMI-1 in this assay is 190 nM. TMI-1 also inhibited the secretion of soluble TNF-α in a human whole blood assay with an IC_{50} 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 PMA-induced shedding of TNFR II in human whole blood with an IC50 of 0.72 μM.

The selectivity of TNF-α inhibition by TMI-1 over other pro-inflammatory 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).

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 LTA4 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

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tissue matrix. This synovium cell mixture (macrophages, T cells, plasma cells, dendritic cells, endothelial cells, and fibroblasts) spontaneously produces TNF-α and other pro-inflammatory 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 4 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 Figure 2. The data from all 4 RA patients are summarized in the table of Figure 2. An IC_{50} of less than 100 nM was derived from three out of four RA patient samples tested.

Treatment with TMI-1 in the synovium culture also reduced the level of other pro-inflammatory cytokines such as IL-1β, IL6, and IL8 to various degrees. An average of 30% reduction of IL-1β, IL-6, and IL-8 in the presence of 1 μM of TMI-1 was observed. This effect was likely due to the secondary effect of TNF-α-induced production of these cytokines since TMI-1 has no direct effect on the secretion of IL-1β, IL-6, or IL-8 induced by LPS in a 4 hr human whole blood assay in vitro as described above. Treatment with an-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 Figure 2.

TMI-1 has no effect on TNF-α mRNA level

Since 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 RNAase 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, while incubation with two MAP kinase inhibitors, SB203580 and PD98059, that 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 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 hr prior to the i.v. injection of LPS. Blood samples were drawn one hour after LPS-stimulation and TNF-α levels in serum were determined by a TNF-α ELISA assay. As shown in Figure 4, TMI-1 treatment led to a dose-dependent inhibition of TNF-α production in this

model. At 25 mg/kg of TMI-1, complete inhibition of soluble TNF-α secretion was achieved. The ED_{50} of TMI-1 is 5 mg/kg in this model. The pharmacokinetic (PK) analysis at a single oral (IG) dose of 50 mg/kg in Balb/CJ mice indicates that TMI-1 has a Cmax of 3 μM, a half-life (T1/2) of 1.68 hr, an AUC of 1484 ng*/hr/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 collagen-induced arthritis (CIA) model

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. 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 po bid in this experiment. At a dose of 20 mg/kg po bid, 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 mg/kg 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 Figure 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 Figure 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 days treatment, although the extent of the reduction was less pronounced compared to that shown in the prophylactic model. This data suggests 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 IP injection of Enbrel (30 μg/mouse/day) produced efficacy comparable to TMI-1 at 10 mg/kg, bid, p.o. In the therapeutic model, once a day IP injection of Enbrel at 150 μg/mouse is more potent than TMI-1 (100

mg/kg, bid, p.o.) with a 74% of reduction of the disease severity scores on day 13 of the treatment in a separate study.

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Discussion

In this report we describe a novel, dual TACE/MMP inhibitor, TMI-1. While the majority of reported inhibitors of TACE are peptide-like molecules, TMI-1 is a sulfonamide hydroxamate bearing a butynyloxy P1' group. The P1' moiety of TMI-1 was designed by accounting for the unique size and shape of the S1' and S3' pockets of the enzyme(Maskos et al., 1998). The butynyloxy P1' moiety sits inside the channel connecting the S1' and S3' enzyme subsites and serves to enhance both enzyme and cellular inhibitory potency. Indeed, TMI-1 is a potent inhibitor of recombinant TACE (IC_{50}, 8.4 nM) and several MMPs, as well as a potent inhibitor of LPS-induced TNF-α release in a murine monocytic cell line (IC_{50}, 40 nM), human primary monocytes (IC_{50}, 190 nM), and human whole blood (IC_{50}, 300 nM). In vivo, TMI-1 significantly reduces systemic TNF-α release in mouse serum (ED_{50}, 5 mg/kg) 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 (IC_{50}<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 pro-inflammatory cytokines such as IL-1β, IL-6, IL-15, IL-17, IL-18 etc. 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 pro-inflammatory cytokines (TNF-α, IL1-β, IL15, 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 over-expressing non-cleavable TNF-α under the control of human β-globin promotor (Akassoglou et al., 1997). This study is, however, imperfect since the over-expression of

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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 non-cleavable 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 knock-in mice that express only the non-cleavable membrane-bound form of TNF-α were reported (Ruuls et al., 2001). Mice with this TNF-α knock-in did not develop arthritis spontaneously and had reduced disease severity in an experimental autoimmune encephalomyelitis (EAE) model. Furthermore, these mice were highly resistant in the CIA model (Sedgwick, personal communication). These results suggest that soluble TNF-α plays a dominant role in inflammation and autoimmune diseases while 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 TNFΔ1-9, K11E mutant (Decoster et al., 1998).

Whether the treatment with TACE/MMP inhibitors could lead to a massive build-up of pro-TNF-α on the cell surface raises another concern for these inhibitors. Although T cells derived from TACE knock-out 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 knock-in 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 (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).

As 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, RS-

130830, Prinomastat, CGS-27023) cause fibroplasias or musculoskeletal side effects in clinic, while 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 tox 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, while the broad-spectrum MMP inhibitor with TACE activity at the same exposure level did not (Drummond et al., 1999). Therefore, it is tempting to speculate 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.

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References:

Akassoglou K, Probert L, Kontogeorgos G and Kollias G (1997) Astrocyte-specific but not neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice. *J Immunol* **158**:438-445.

Beck G, Bottomley G, Bradshaw D, Brewster M, Broadhurst M, Devos R, Hill C, Johnson W, Kim H, Kirtland S, Kneer J, Lad N, Mackenzie R, Martin R, Nixon J, Price G, Rodwell A, Rose F, Tang J, Walter D, Wilson K and Worth E (2002) (E)-2(R)-[1(S)-(Hydroxycarbamoyl)-4-phenyl-3-butenyl]-2'-isobutyl-2'-(methanesulfonyl)-4-methylvalerohydrazide (Ro 32-7315), a selective and orally active inhibitor of tumor necrosis factor-alpha convertase. *J Pharmacol Exp Ther* **302**:390-396.

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ and Cerretti DP (1997) A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* **385**:729-733.

Butler D, RN M, M F and FM B (1995) Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. *Eur. Cytokine Netw.* **6**:225-230.

Chabaud M, Garnero P, Dayer JM, Guerne PA, Fossiez F and Miossec P (2000) Contribution of interleukin 17 to synovium matrix destruction in rheumatoid arthritis. *Cytokine* **12**:1092-1099.

Choy EH and Panayi GS (2001) Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med* **344**:907-916.

Constantinescu CS, Grygar C, Kappos L and Leppert D (2001) Interleukin 15 stimulates production of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by human peripheral blood mononuclear cells. *Cytokine* **13**:244-247.

Conway JG, Andrews RC, Beaudet B, Bickett DM, Boncek V, Brodie TA, Clark Rl, Crumrine RC, Leenitzer MA, Mcdougald DL, Han B, Hedeen K, Lin P, Milla M, Moss M, Pink H, Rabinowitz MH, Tippin T, Scates PW, Selph J, Stimpson SA, Janet W and Becherer DJ (2001) Inhibition of Tumor Necrosis Factor-α Production and Arthritis in the Rat by GW3333, a Dual Inhibitor of TNF-α-Converting Enzyme and Matrix Metalloproteinases. *The Journal of Pharmacology and Experimental Therapeutics* **298**:900-908.

Crowe PD, Walter BN, Mohler KM, Otten-Evans C, Black RA and Ware CF (1995) A metalloprotease inhibitor blocks shedding of the 80-kD TNF receptor and TNF processing in T lymphocytes. *J Exp Med* **181**:1205-1210.

Decker T, Lohmann-Matthes ML and Gifford GE (1987) Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. *J Immunol* **138**:957-962.

Decoster E, Vanhaesebroeck B, Boone E, Plaisance S, De Vos K, Haegeman G, Grooten J and Fiers W (1998) Induction of unresponsiveness to tumor necrosis factor (TNF) after autocrine TNF expression requires TNF membrane retention. *J. Biol. Chem.* **273**:3271-3277.

Dekkers PE, Lauw FN, ten Hove T, te Velde AA, Lumley P, Becherer D, van Deventer SJ and van der Poll T (1999) The effect of a metalloproteinase inhibitor (GI5402) on tumor necrosis factor-alpha (TNF-alpha) and TNF-alpha receptors during human endotoxemia. *Blood* **94**:2252-2258.

Drummond AH, Beckett P, Brown PD, Bone EA, Davidson AH, Galloway WA, Gearing AJ, Huxley P, Laber D, McCourt M, Whittaker M, Wood LM and Wright A (1999) Preclinical and clinical studies of MMP inhibitors in cancer. *Ann N Y Acad Sci* **878**:228-235.

Elliott MJ, Maini RN, Feldmann M, Kalden JR, Antoni C, Smolen JS, Leeb B, Breedveld FC, Macfarlane JD and Bijl Hea (1994a) Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis. *Lancet* **344**:1105-1110.

Elliott MJ, Maini RN, Feldmann M, Long-Fox A, Charles P, Bijl H and Woody JN (1994b) Repeated therapy with monoclonal antibody to tumour necrosis factor alpha (cA2) in patients with rheumatoid arthritis. *Lancet* **344**:1125-1127.

Feldmann M, Brennan FM, Elliott M, Katsikis P and Maini RN (1994) TNF alpha as a therapeutic target in rheumatoid arthritis. *Circ Shock* **43**:179-184.

Feldmann M, Brennan FM, Foxwell BM and Maini RN (2001) The role of TNF alpha and IL-1 in rheumatoid arthritis. *Curr Dir Autoimmun* **3**.

Feldmann M, Charles P, Taylor P and Maini RN (1998) Biological insights from clinical trials with anti-TNF therapy. *Springer Semin Immunopathol* **20**:211-228.

Fernandes JC, Martel-Pelletier J and Pelletier JP (2002) The role of cytokines in osteoarthritis pathophysiology. *Biorheology* **39**:237-246.

Gracie JA, Forsey RJ, Chan WL, Gilmour A, Leung BP, Greer MR, Kennedy K, Carter R, Wei XQ, Xu D, Field M, Foulis A, Liew FY and McInnes IB (1999) A proinflammatory role for IL-18 in rheumatoid arthritis. *J Clin Invest* **104**:1393-1401.

Jin G, Huang X, Black R, Wolfson M, Rauch C, McGregor H, Ellestad G and Cowling R (2002) A continuous fluorimetric assay for tumor necrosis factor-alpha converting enzyme. *Anal Biochem* **302**:269-275.

Katrib A, Tak PP, Bertouch JV, Cuello C, McNeil HP, Smeets TJ, Kraan MC and Youssef PP (2001) Expression of chemokines and matrix metalloproteinases in early rheumatoid arthritis. *Rheumatology (Oxford)* **40**:988-994.

Knight C, Willenbrock F and Murphy G (1992) A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett* **296**:263-266.

Kobayashi H, Ohshima S, Nishioka K, Yamaguchi N, Umeshita-Sasai M, Ishii T, Mima T, Kishimoto T, Kawase I and Saeki Y (2002) Antigen induced arthritis (AIA) can be transferred by bone marrow transplantation: evidence that interleukin 6 is essential for induction of AIA. *J Rheumatol* **29**:1176-1182.

Kriegler M, Perez C, DeFay K, Albert I and Lu SD (1988) A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell* **53**:45-53.

Maskos K, Fernandez-Catalan C, Huber R, Bourenkov GP, Bartunik H, Ellestad GA, Reddy P, Wolfson MF, Rauch CT, Castner BJ, Davis R, Clarke HR, Petersen M, Fitzner JN, Cerretti DP, March CJ, Paxton RJ, Black RA and Bode W (1998) Crystal structure of the catalytic domain of human tumor necrosis factor-alpha-converting enzyme. *Proc Natl Acad Sci U S A* **95**:3408-3412.

McInnes IB and Liew FY (1998) Interleukin 15: a proinflammatory role in rheumatoid arthritis synovitis. *Immunol Today* **19**:75-79.

Mohler KM, Sleath PR, Fitzner JN, Cerretti DP, Alderson M, Kerwar SS, Torrance DS, Otten-Evans C, Greenstreet T and Weerawarna K, et al. (1994) Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature* **370**:218-220.

Moreland LW, Baumgartner SW, Schiff MH, Tindall EA, Fleischmann RM, Weaver AL, Ettlinger RE, Cohen S, Koopman WJ, Mohler K, Widmer MB and Blosch CM (1997) Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N Engl J Med* **337**:141-147.

Moss ML, White JM, Lambert MH and Andrews RC (2001) TACE and other ADAM proteases as targets for drug discovery. *Drug Discov Today* **6**:417-426.

Mueller C, Corazza N, Trachsel-Loseth S, Eugster HP, Buhler-Jungo M, Brunner T and Imboden MA (1999) Noncleavable transmembrane mouse tumor necrosis factor-alpha (TNFalpha) mediates effects distinct from those of wild-type TNFalpha in vitro and in vivo. *J. Biol. Chem.* **274**:38112-38118.

Newton RC, Solomon KA, Covington MB, Decicco CP, Haley PJ, Friedman SM and Vaddi K (2001) Biology of TACE inhibition. *Ann Rheum Dis* **60**:iii25-32.

Ohta S, Harigai M, Tanaka M, Kawaguchi Y, Sugiura T, Takagi K, Fukasawa C, Hara M and Kamatani N (2001) Tumor necrosis factor-alpha (TNF-alpha) converting enzyme contributes to production of TNF-alpha in synovial tissues from patients with rheumatoid arthritis. *J Rheumatol* **28**:1756-1763.

Pap T, Shigeyama Y, Kuchen S, Fernihough JK, Simmen B, Gay RE, Billingham M and Gay S (2000) Differential expression pattern of membrane-type matrix metalloproteinases in rheumatoid arthritis. *Arthritis Rheum* **43**:1226-1232.

Rudek MA, Venitz J and Figg WD (2002) Matrix metalloproteinase inhibitors: do they have a place in anticancer therapy? *Pharmacotherapy* **22**:705-720.

Ruuls SR, Hoek RM, Ngo VN, McNeil T, Lucian LA, Janatpour MJ, Korner H, Scheerens H, Hessel EM, Cyster JG, McEvoy LM and Sedgwick JD (2001) Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. *Immunity* **15**.

Shaw T, Nixon JS and Bottomley KM (2000) Metalloproteinase inhibitors: new opportunities for the treatment of rheumatoid arthritis and osteoarthritis. *Expert Opin Investig Drugs* **9**:1469-1478.

Solomon KA, Covington MB, DeCicco CP and Newton RC (1997) The fate of pro-TNF-alpha following inhibition of metalloprotease-dependent processing to soluble TNF-alpha in human monocytes. *J Immunol* **159**:4524-4531.

Tomita T, Nakase T, Kaneko M, Shi K, Takahi K, Ochi T and Yoshikawa H (2002) Expression of extracellular matrix metalloproteinase inducer and enhancement of the production of matrix metalloproteinases in rheumatoid arthritis. *Arthritis Rheum* **46**:373-378.

Ziolkowska M, Koc A, Luszczykiewicz G, Ksiezopolska-Pietrzak K, Klimczak E, Chwalinska-Sadowska H and Maslinski W (2000) High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. *J Immunol* **164**:2832-2838.

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Figure Legends

Figure 1. Structure of 4-[[4-(2-butynyloxy)phenyl]sulfonyl]-N-hydroxy-2,2-dimethyl-(3S)-thiomorpholinecarboxamide (TMI-1).

Figure 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 4 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 DNAase. The resulting multi-cell mixture containing macrophages, T cells, plasma cells, dendritic cells, and fibroblasts were cultured in the presence of various concentrations of TMI-1 or DMSO vehicle control for 2 days. These synovium explants 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 is from the samples of patient SM1936. The percent of inhibitions of TNF secretion from all 4 patients tested is summarized in the table.

Figure 3. Effect of TMI-1 on the level of TNF-α mRNA in RNAase Protection Assay

RAW cells were pretreated with compounds (TMI-1, PD98059, SB203580) for 1 hr at the concentrations indicated. Cells were stimulated with 10 ng/ml of LPS for overnight. The total RNA was extracted and hybridized with a ^{32}P-labeled mouse TNF-α probe for overnight. The sample was digested with RNase and separated on a 6% polyacrylamide gel and autoradiographed. Data represent one of 3 similar experiments.

Figure 4. Effect of TMI-1 on LPS-induced TNF-α production in mouse sera

Balb/CJ (H-2d) mice were dosed p.o. with TMI-1 or vehicle control 1 hr prior to the i.v. injection of LPS (40 ng/mouse). One hour after LPS injection, blood samples were drawn and TNF-α level in serum was measured by TNF-α ELISA. Data represent one of 6 similar experiments.

Figure 5. Effect of TMI-1 in LPS-boosted prophylactic CIA model in mice

Arthritis was induced in female mice 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 of Mycobacterium tuberculosis H37 Ra. Mice were dosed with the TMI-1, b.i.d., while control mice received vehicle only. The dosing

began on day 18 prior to the onset of arthritis and continued for 17 days. On day 21, disease was induced by injecting 40 μg of LPS intraperitoneally. The clinical severity of arthritis was assessed based on the appearance of each paw, and subjectively graded on a scale of 0 to 4 as described in Material and Methods. The scores of each limb were summed, giving a maximum severity score of 16. Each data point represents the average of 15 mice. Data represent one of 5 similar experiments.

Figure 6. Effect of TMI-1 in therapeutic CIA Model in mice

DBA/1LacJ male mice of age 8 weeks were immunized on day 0 with 100 μg of bovine type II collagen (Chondrex) in CFA. On day 21, the mice were boosted with 100 μg of bovine type II collagen in Incomplete Freund's Adjuvant (IFA). 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 TMI-1 at 100 mg/kg p.o. b.i.d. Each data point represents the average of 15 mice.

IC_{50} (nM)

TACE	MMP-1	MMP-2	MMP-7	MMP-9	MMP-13	MMP-14	Agg-1
8.4	6.6	4.7	26	12	3	26	100

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 in Material and Methods. These data are averages from 3 experiments.

Inducing Agent	LPS	LPS	LPS	LPS	Zymosan
Cell System	Raw	THP-1	Human Primary Monocytes	Human Whole Blood	Human Whole Blood
IC_{50} (μM)	0.04	0.2	0.19	0.3	0.4

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 and THP-1, human primary monocytes, and human whole blood for the inhibition of lipopolysaccharide- (LPS) or zymosan-induced TNF-α secretion. Cells were pre-treated with TMI-1 or vehicle control at various concentrations for 1 hr before addition of LPS or Zymosan. The cells were cultured for 4 hrs 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 and 5 to 15 ng/ml in human whole blood, 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 3 experiments.

Figure 1

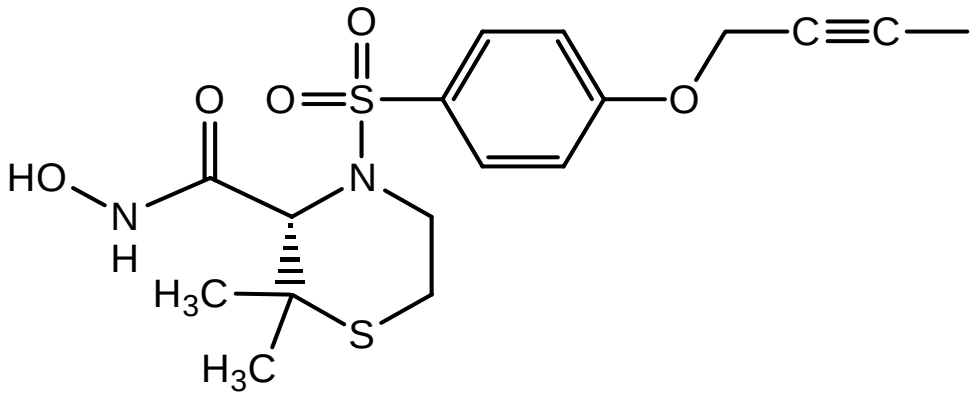
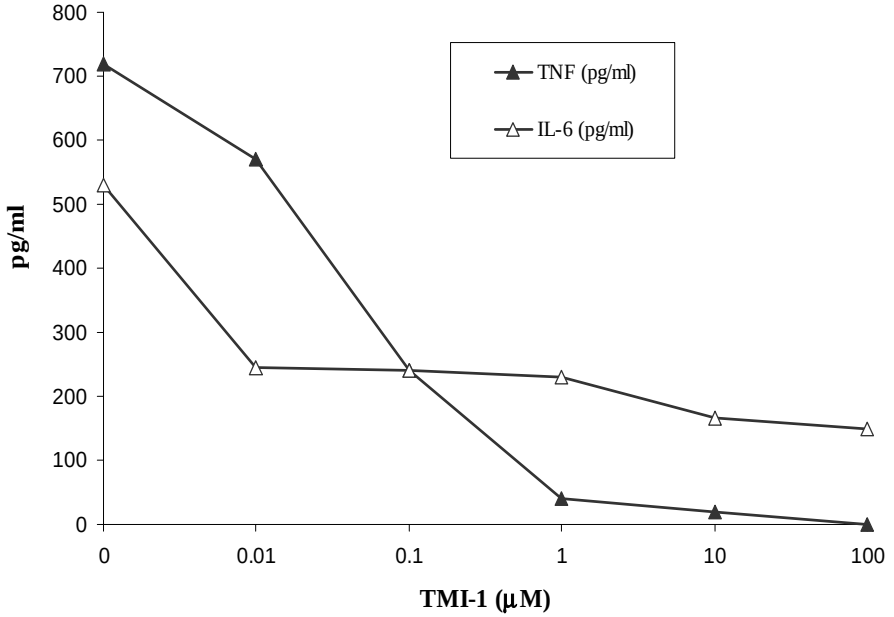


Figure 2



TMI-1 (μM)	% Inhibition of TNFα Secretion			
	SM1936	SM2125	SM2126	SM2129
0.01	19	ND	ND	ND
0.1	67	74	64	0
1	96	80	63	14
10	100	81	65	38
100	100	90	100	100

Figure 3

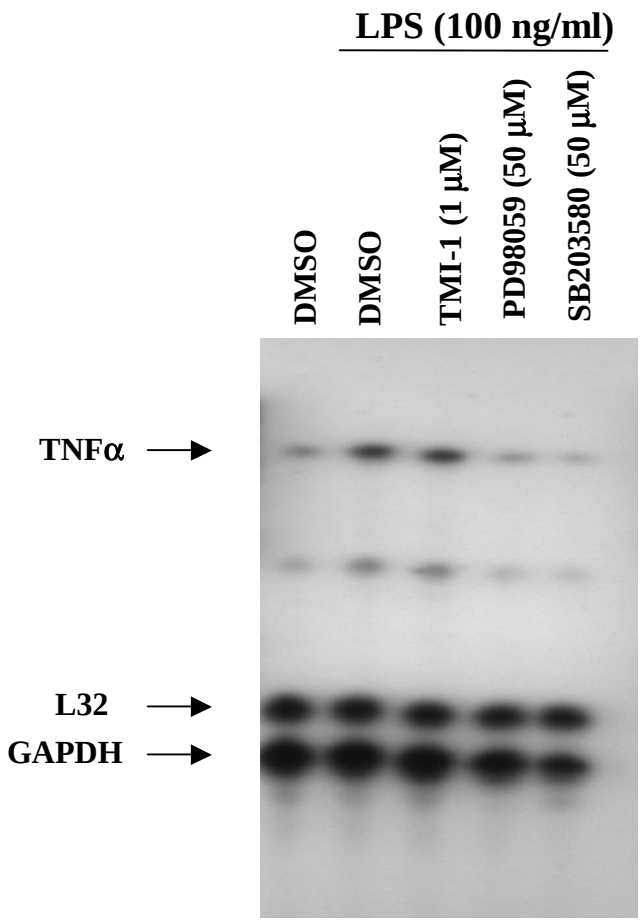


Figure 4

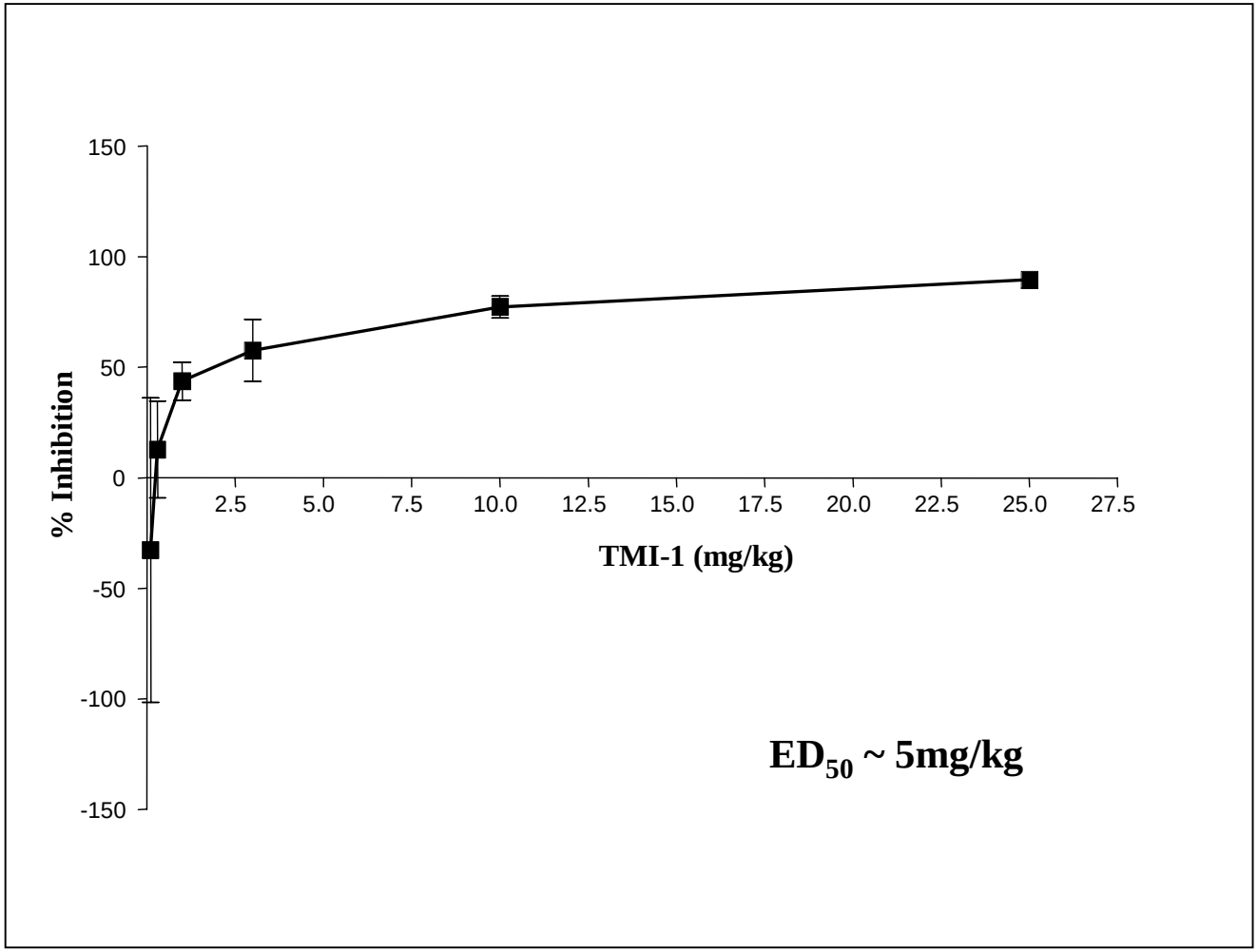


Figure 5

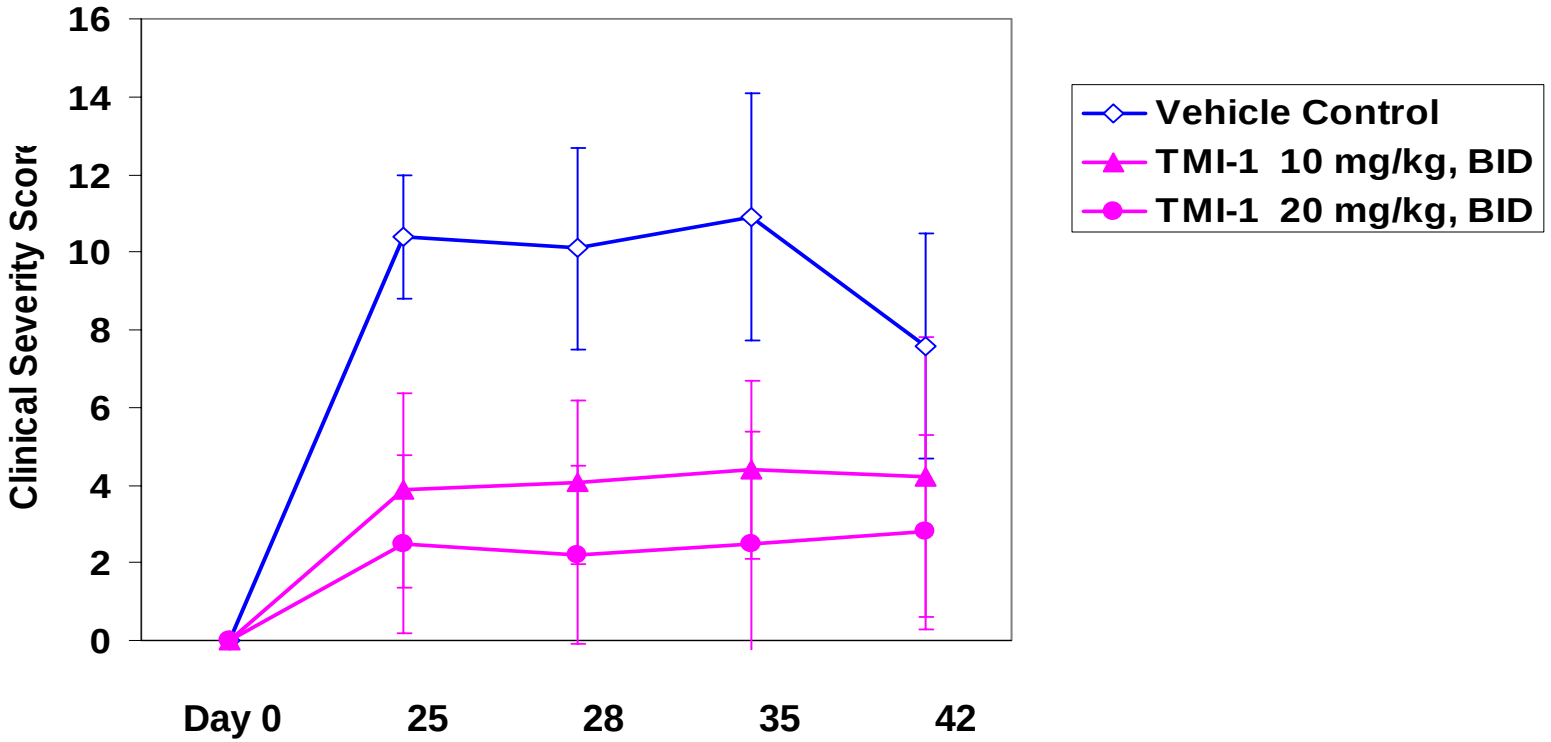


Figure 6