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Attenuation of Murine Collagen-Induced Arthritis by a Novel, Potent, Selective Small Molecule Inhibitor of IκB Kinase 2, TPCA-1 (2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide), Occurs via Reduction of Proinflammatory Cytokines and Antigen-Induced T Cell Proliferation

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ABSTRACT

Demonstration that IκB kinase 2 (IKK-2) plays a pivotal role in the nuclear factor-κB-regulated production of proinflammatory molecules by stimuli such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 suggests that inhibition of IKK-2 may be beneficial in the treatment of rheumatoid arthritis. In the present study, we demonstrate that a novel, potent (IC_{50} = 17.9 nM), and selective inhibitor of human IKK-2, 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1), inhibits lipopolysaccharide-induced human monocyte production of TNF-α, IL-6, and IL-8 with an IC_{50} = 170 to 320 nM. Prophylactic administration of TPCA-1 at 3, 10, or 20 mg/kg, i.p., b.i.d., resulted in a dose-dependent reduction in the severity of murine collagen-induced arthritis (CIA). The significantly reduced disease severity and delay of disease onset resulting from administration of TPCA-1 at 10 mg/kg, i.p., b.i.d. were comparable to the effects of the antirheumatic drug, etanercept, when administered prophylactically at 4 mg/kg, i.p., every other day. Nuclear localization of p65, as well as levels of IL-1β, IL-6, TNF-α, and interferon-γ, were significantly reduced in the paw tissue of TPCA-1- and etanercept-treated mice. In addition, administration of TPCA-1 in vivo resulted in significantly decreased collagen-induced T cell proliferation ex vivo. Therapeutic administration of TPCA-1 at 20 mg/kg, but not at 3 or 10 mg/kg, i.p., b.i.d., significantly reduced the severity of CIA, as did etanercept administration at 12.5 mg/kg, i.p., every other day. These results suggest that reduction of proinflammatory mediators and inhibition of antigen-induced T cell proliferation are mechanisms underlying the attenuation of CIA by the IKK-2 inhibitor, TPCA-1.

Rheumatoid arthritis (RA) is a disease characterized by chronic inflammation of the joint, leading to progressive destruction of cartilage and bone. Migration of leukocytes to the synovium results in synovial hypertrophy and the production of proinflammatory mediators by both synoviocytes and leukocytes. These mediators are believed to be responsible for the subsequent cartilage destruction and bone erosion that characterizes the disease (Kingsley and Panayi, 1997; Hasunuma et al., 1998). Many of the proinflammatory molecules associated with RA, including TNF-α, IL-1, IL-6, IL-8, IFN-γ, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, cyclooxygenase (COX)-2, inducible nitric-oxide synthase, matrix metalloproteinase (MMP)-1, and MMP-9, are regulated by the Rel/NF-κB family of transcription factors (Pahl, 1999). Thus, members of this signaling pathway are potential targets for the development of novel RA therapeutics.

ABBREVIATIONS: RA, rheumatoid arthritis; IKK, IκB kinase; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; IL, interleukin; TPCA-1, 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide; LPS, lipopolysaccharide; CIA, collagen-induced arthritis; IFN, interferon; COX, cyclooxygenase; MMP, matrix metalloproteinase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DMSO, dimethyl sulfoxide; DMA, dimethylacetoacetamide; LNC, lymph node cells; ELISA, enzyme-linked immunosorbent assay; JNK3, c-Jun N-terminal kinase 3; AUC, area under the curve; NSAIDs, nonsteroidal anti-inflammatory drugs; DMARDs, disease-modifying antirheumatic drugs; GST, glutathione *S*-transferase; BSA, bovine serum albumin; DTT, dithiothreitol; HBSS, Hanks' balanced salt solution; APC, antigen-presenting cells; Th1, subset 1 of T helper cells.

In mammals, the Rel/NF-κB family consists of p50/p105 (NF-κB1), p52/p100 (NF-κB2), p65 (RelA), c-Rel (Rel), and RelB, which exist in the cell cytoplasm as homodimeric or heterodimeric complexes. The NF-κB dimer (classically p50/p65) is retained in the cytoplasm in an inactive form through its association with IκB (inhibitor of NF-κB) proteins. A variety of stimuli, including TNF-α and IL-1, are capable of inducing NF-κB activation. These agents initiate a signaling cascade leading to the phosphorylation of two N-terminal serine residues in IκB, which facilitates the ubiquitination and subsequent degradation of IκB by the 26S proteosome. Once released from IκB, NF-κB translocates to the nucleus, where it binds to a κB consensus sequence encoded within its target gene and initiates transcription (Makarov, 2001; Tak and Firestein, 2001).

Because the enzymes responsible for the ubiquitination of phosphorylated IκB are constitutively active, the phosphorylation of IκB is a critical regulatory step in IκB degradation and subsequent NF-κB activation. This phosphorylation event is catalyzed by the IκB kinase (IKK) complex, which consists of two enzymatically active kinases, IKK-1 (IKKα) and IKK-2 (IKKβ), and a regulatory subunit, NEMO (IKKγ) (Karin, 1999). Divergent physiological roles for the two kinases are suggested by targeted gene deletion studies in which IKK-2-deficient mice, but not IKK-1-deficient mice, exhibited significantly impaired TNF-α- and IL-1-induced NF-κB activation and IL-6 production (Li et al., 1999; Tanaka et al., 1999). These results suggest that IKK-2, rather than IKK-1, plays a critical role in the NF-κB-regulated production of proinflammatory molecules induced by stimuli such as TNF-α and IL-1 and thus is a relevant target for the development of an anti-inflammatory therapeutic.

Much evidence indicates a pivotal role for NF-κB in the etiology of RA. Nuclear localization of p50 and p65 has been shown to be significantly increased in synovial tissue from RA patients, compared with synovium from normal controls (Handel et al., 1995; Han et al., 1998). Similarly, it was demonstrated that fibroblast-like synoviocytes from RA synovium contain constitutively active NF-κB and spontaneously produce large quantities of IL-6, unlike fibroblast-like synoviocytes from osteoarthritis synovium (Miyazawa et al., 1998). In addition, a number of antirheumatic agents, including glucocorticoids, sulfasalazine, gold salts, leflunomide, and aspirin, are inhibitors of NF-κB activation (Makarov, 2001; Tak and Firestein, 2001), which may explain, at least in part, their anti-inflammatory effects.

Consistent with the data from human synovial tissue, increased NF-κB binding activity has been demonstrated in the synovium of mice and rats following the development of CIA, adjuvant arthritis, and streptococcal cell wall-induced arthritis (Tak and Firestein, 2001). Additional evidence implicating NF-κB in animal models of RA comes from the demonstration that in vivo administration of reagents exerting inhibitory effects at various points along the NF-κB signaling pathway resulted in a reduction of disease (Tak and Firestein, 2001). The results of studies specifically targeting IKK-2 suggest that this enzyme plays a pivotal role in the NF-κB-mediated inflammatory response underlying arthritis. Intra-articular injection of a wild-type IKK-2 gene into the joints of normal rats resulted in paw swelling and synovial inflammation, whereas transfer of a dominant-negative IKK-2 gene decreased the severity of rat adjuvant arthritis (Tak et al.,

2001). These studies suggest that inhibition of IKK-2 is a viable approach to the development of a novel therapeutic for RA.

In the current study, we characterize a novel, potent, selective small molecule inhibitor of IKK-2, TPCA-1 (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide). Prophylactic or therapeutic administration of TPCA-1 significantly reduced the severity of murine CIA. This modulation of disease was accompanied by decreased tissue levels of the proinflammatory cytokines, IL-1β, IL-6, TNF-α, and IFN-γ, as well as reduced T cell proliferation in response to antigen, suggesting that these mechanisms underlie the inhibition of CIA by TPCA-1.

Materials and Methods

Synthesis of TPCA-1. TPCA-1 was synthesized at GlaxoSmithKline by the Respiratory and Inflammation Center of Excellence for Drug Discovery (King of Prussia, PA). The 2-amino-5-(4-fluorophenyl)-3-thiophenecarboxamide precursor was prepared by the reaction of 4-fluorophenylacetaldehyde, 2-cyanoacetamide, sulfur, and triethylamine in dimethylformamide at 0°C, allowing the reaction to warm to room temperature overnight (Goudie, 1976). Treatment of 2-amino-5-(4-fluorophenyl)-3-thiophenecarboxamide with chlorosulfonylisocyanate in methylene chloride at 0°C followed by aqueous hydrolysis and subsequent recrystallization from ethanol provided TPCA-1.

IKK-2 Assay. Recombinant human IKK-2 (residues 1–756) was expressed in baculovirus as an N-terminal GST-tagged fusion protein, and its activity was assessed using a time-resolved fluorescence resonance energy transfer assay. In brief, IKK-2 (5 nM final) diluted in assay buffer (50 mM HEPES, 10 mM $MgCl_2$, 1 mM CHAPS, pH 7.4, with 1 mM DTT and 0.01% w/v BSA) was added to wells containing various concentrations of compound or dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) vehicle (3% final). The reaction was initiated by the addition of GST-IκBα substrate (25 nM final)/ATP (1 μM final), in a total volume of 30 μl. The reaction was incubated for 30 min at room temperature, then terminated by the addition of 15 μl of 50 mM EDTA. Detection reagent (15 μl) in buffer (100 mM HEPES, pH 7.4, 150 mM NaCl, and 0.1% w/v BSA) containing antiphosphoserine-IκBα-32/36 monoclonal antibody 12C2 (Cell Signaling Technology Inc., Beverly, MA), labeled with W-1024 europium chelate (PerkinElmer Wallac, Turku, Finland), and an allophycocyanin-labeled anti-GST antibody (ProZyme, San Leandro, CA) was added, and the reaction was further incubated for 60 min at room temperature. The degree of phosphorylation of GST-IκBα was measured as a ratio of specific 665-nm energy transfer signal to reference europium 620-nm signal, using a Packard Discovery plate reader (PerkinElmer Life Sciences, Buckinghamshire, UK).

Lipopolysaccharide-Induced Cytokine/Chemokine Production by Human Monocytes. Human monocytes were isolated from heparinized whole blood by positive selection using CD14+ microbeads (Miltenyi Biotec Inc., Auburn, CA). In brief, human whole blood was collected from healthy volunteers and diluted with an equal volume of HBSS (without Ca^{2+} or Mg^{2+}) containing 1 mM EGTA. Diluted blood was layered on a Ficoll-Hypaque gradient (Amersham Biosciences AB, Uppsala, Sweden) and centrifuged at 900g for 30 min. The resulting interface was removed and washed twice with HBSS containing 1 mM EGTA. Cell pellets were resuspended at 1.25×10^8 cells/ml in PBS containing 0.5% BSA and 2 mM EDTA. Cells were labeled with 20 μl CD14+ microbeads per 1×10^7 cells and incubated for 15 min at 6°C with frequent mixing. Labeled cells were washed once and resuspended at 1×10^8 cells/ml in chilled PBS containing 0.5% BSA and 2 mM EDTA. Cells were applied to magnetized columns using an autoMACS (Miltenyi Biotec Inc.), and separation was performed according to the manufacturer's instruc-

tions. The resulting monocyte population was >90% pure as assessed by differential staining.

Purified monocytes were washed twice with PBS containing 0.5% BSA and 2 mM EDTA and resuspended to 1×10^6 cells/ml in warm RPMI 1640 medium containing 10% FBS and L-glutamine. Monocytes were plated at 5×10^5 cells/well in 48-well tissue culture plates and incubated for 2 h at 37°C. Adhered monocytes were washed once with warm RPMI 1640 medium containing 10% fetal calf serum and L-glutamine, and TPCA-1 in 100% DMSO was added (0.1% DMSO final concentration). The monocytes were incubated with compound for 30 min at 37°C and then stimulated with 200 ng/ml LPS for 24 h. Plates were centrifuged at 500g for 10 min, and supernatants were removed and stored at −20°C until cytokine/chemokine evaluation was performed.

Mice. Male DBA/1 OlaHsd mice were obtained from Harlan UK Ltd. (Bicester, Oxon, UK) at 6 to 8 weeks of age. Mice were housed one per cage and fed standard rodent chow and water ad libitum.

Induction and Assessment of CIA. On day 0, 10- to 12-week-old male DBA/1 mice were immunized intradermally at the base of the tail with a total of 100 μl of complete Freund's adjuvant (Sigma-Aldrich) containing 200 μg of bovine type II collagen (Elastin Product Co., Owensville, MO) and 250 μg of *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). On day 21, mice were boosted intradermally with 100 μl of PBS containing 200 μg of bovine type II collagen. In all studies, TPCA-1 was administered in a vehicle consisting of 0.9% DMSO, 7% dimethylacetoacetamide (DMA) (Aldrich Chemical Co., Milwaukee, WI), and 10% Cremophor El (Sigma-Aldrich). Etanercept (Enbrel) (purchased commercially) was administered in PBS. Where the effects of TPCA-1 and etanercept were compared, both treatment groups as well as their relevant vehicle-treated control groups were included in the same study. For prophylactic studies, TPCA-1 in vehicle or vehicle alone was administered i.p., b.i.d., beginning on day 1. Etanercept in PBS or PBS alone was administered i.p. every other day beginning on day 1. The incidence of disease exhibited by both vehicle-treated control groups (PBS- and DMSO/DMA/Cremophor-treated mice) was 100%. For therapeutic studies, administration of TPCA-1, etanercept, or their respective vehicles, as described above, was initiated (day 1) once an animal exhibited a clinical score of "1" or greater for 2 consecutive days. Mice were scored daily for clinical symptoms of disease using a micrometer caliper to measure paw thickness. Each paw was assigned a score ranging from 0 to 4, based on the following criteria: 0, asymptomatic (paw thickness = 1.8 to 1.9 mm and no swollen digits); 1, paw thickness = 1.8 to 1.9 mm and one or more swollen digits; 2, paw thickness = 2.0 to 2.5 mm and one or more swollen digits; 3, paw thickness = 2.6 to 3.0 mm and one or more swollen digits; 4, paw thickness = 3.0+ mm and one or more swollen digits.

In addition to the mice that were scored throughout the experiment, at the designated time points during the course of disease, mice were removed from the experiment and used to measure cytokine/chemokine levels and p65 levels in the paw and the ex vivo antigen recall response by lymph node cells (LNC)/splenocytes. These mice were scored on a daily basis until their removal from the study, and the data were integrated into the analysis using the log rank test.

Quantitative Analysis of Blood TPCA-1 Levels. Quantitative analysis of TPCA-1 in blood samples was performed utilizing a high-performance liquid chromatography/dual mass spectrometry method. TPCA-1 was isolated from 25 μl of mouse blood (diluted with 25 μl of water) by protein precipitation and quantified with a Sciex API 4000 instrument with a turbo ionspray interface. Samples were injected onto a Luna C18 column (2×50 mm, 3 μm packing) under isocratic conditions [60:40, acetonitrile/10 mM ammonium formate (pH 3.0)] at a flow rate of 350 μl/min. Negative-ion multiple reaction monitoring was used for the dual mass spectrometry detection of TPCA-1, with a lower limit of quantitation of 10.0 ng/ml.

Preparation of Tissue for Evaluation of NF-κB Activation and Cytokine/Chemokine Measurement. Paw tissue was weighed and placed in a volume of PBS equal to 1 g/2 ml. The tissue was homogenized by Polytron (model PT 10/35; Brinkmann Instruments, Westbury, NY) and kept on ice during the processing. Samples were transferred to 1.5-ml conical tubes, and the tissue was centrifuged at 16,300g for 3 min at 4°C. The supernatant was then collected for cytokine/chemokine analysis. The pellet was used for preparation of nuclear extracts following published methods (Dignam et al., 1983; Osborn et al., 1989) with some modifications. In brief, the tissue was resuspended in 200 μl of buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) Nonidet P-40, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride]. The suspension was incubated on ice for 10 min and then centrifuged at 1020g for 10 min at 4°C. The supernatant was removed and labeled as the cytoplasmic extract. The remaining pellet was resuspended in 125 μl of buffer C [20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride] and gently mixed for 20 min on ice. The samples were then centrifuged at 16,300g for 10 min at 4°C, and the supernatant was removed and labeled as the nuclear extract. The samples were stored at −80°C until analysis.

Cytokine, Chemokine, and p65 Assays. Human TNF-α, IL-6, and IL-8 levels were measured using enzyme-linked immunosorbent assay (ELISA) kits purchased from BD Biosciences PharMingen (San Diego, CA). Paw tissue mediators were measured using mouse IL-1β, IL-6, TNF-α, and IFN-γ ELISA kits purchased from BioSource International (Camarillo, CA) and a mouse KC ELISA kit purchased from R&D Systems (Minneapolis, MN). Nuclear p65 levels were determined using the TransAM NF-κB p65 kit (Active Motif, Carlsbad, CA). Assays were performed according to the manufacturers' instructions.

Lymph Node and Spleen Cell Cultures. Preparation and culture of LNC and splenocytes were performed under sterile conditions. Inguinal lymph nodes and spleens were rinsed in HBSS containing penicillin (100 units/ml)-streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA) and gentamicin (50 μg/ml) (Sigma-Aldrich), (HBSS+), teased apart in 5 ml of HBSS+, and filtered through 50 μM nylon mesh. Samples were centrifuged at 500g for 10 min at 4°C, and the resulting LNC pellets were resuspended in 2 ml of HBSS+. Splenocyte pellets were resuspended in 9 ml of H_2O for 30 s, followed by the addition of 1 ml of 10× PBS. Splenocyte samples were centrifuged at 500g for 10 min at 4°C, and the resulting pellets were resuspended in 2 ml of HBSS+. LNC and splenocytes were counted, and cells from three mice were combined at a ratio of 80% LNC/20% splenocytes (each group of three mice considered an $n = 1$). In a volume of 200 μl of RPMI 1640 medium containing 10% FBS, penicillin (100 units/ml)-streptomycin (100 μg/ml), and gentamicin (50 μg/ml), 2×10^6 cells/ml were cultured in the presence or absence of bovine type II collagen (100 μg/ml). Following incubation for 72 h at 37°C in 5% CO_2, 1 μCi [^{3}H]thymidine was added to each well and the cells cultured for an additional 24 h. Cultures were harvested using a Packard Filtermate 196 (PerkinElmer Life Sciences, Boston, MA), and radioactivity was quantified using a Packard TopCount liquid scintillation counter.

Statistical Analysis. Statistical differences in p65 levels, cytokine/chemokine concentrations, and T cell proliferation were determined using the two-tailed Student's t test. Analysis of CIA clinical score data was performed by calculating the area under the curve (AUC) for each animal within a treatment group and then using the log rank test, a nonparametric test that allows for censored observations (i.e., for any animal removed before the completion of the study, the animal's complete AUC is considered to be at least as large as the partial AUC exhibited). Analysis of CIA disease onset data was performed using the log rank test. Values of $p < 0.05$ were considered significant.

Results

Characterization of a Potent, Selective, and ATP-Competitive Inhibitor of IKK-2. TPCA-1 (Fig. 1) was identified as a potent and selective inhibitor of IKK-2. In a time-resolved fluorescence resonance energy transfer assay, TPCA-1 inhibited human IKK-2 activity with an $IC_{50} = 19.5 \pm 1.7$ nM (representative experiment shown in Fig. 2). The results from 57 assays gave a mean $pIC_{50} = 7.74 \pm 0.18$ ($IC_{50} = 17.9$ nM). In addition, the compound was demonstrated to be ATP-competitive (data not shown).

Determination of the activity of TPCA-1 against ten selected kinases, as well as COX-1 and COX-2, showed the compound to be >550-fold selective for IKK-2 versus ten of these enzymes (Table 1). TPCA-1 exhibited IC_{50} values = 400 nM and 3600 nM against IKK-1 and JNK3, respectively, demonstrating it to be 22- and 200-fold selective, respectively, versus these kinases.

TPCA-1 Inhibits LPS-Induced TNF-α, IL-6, and IL-8 Production by Human Monocytes. To confirm the cell-based activity of TPCA-1, human peripheral blood monocytes were stimulated with LPS in the absence or presence of varying concentrations of the inhibitor, and cell supernants were assayed for cytokine/chemokine content. As shown in Fig. 3, TPCA-1 inhibited the production of TNF-α, IL-6, and IL-8 in a concentration-dependent manner, exhibiting IC_{50} values of 170, 290, and 320 nM, respectively. These results suggest that TPCA-1 effectively blocks the NF-κB signaling pathway in intact cells.

Increased Nuclear Localization of NF-κB p65 in CIA. Based on the finding that IKK-2 plays a critical role in the NF-κB-mediated transduction of signals generated by the RA/CIA-associated cytokines, TNF-α and IL-1 (Li et al., 1999; Tanaka et al., 1999), it was of interest to characterize the kinetics of NF-κB activation during the development of CIA. DBA/1 mice were immunized (day 0) and boosted (day 21) with type II collagen, and tissue from all front and hind paws were collected on days 22, 30, and 40. As shown in Fig. 4A, clinical symptoms of CIA appeared at day 26 and increased thereafter, reaching peak severity between days 37 and 40. At day 22, nuclear extracts of paw tissue from collagen-immunized/boosted mice exhibited levels of p65 binding comparable to levels from naive mice (Fig. 4B). In contrast, p65 activity was significantly increased by day 30 of CIA, compared to naive controls, and elevated further by day 40 (Fig. 4B). This indicates that the kinetics of NF-κB activation correlate closely with the appearance and progression of the clinical symptoms of disease.
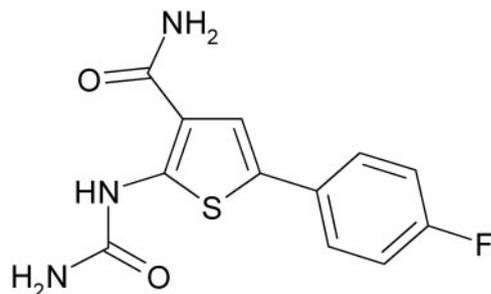
Fig. 1. The chemical structure of the IKK-2 inhibitor, 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide.
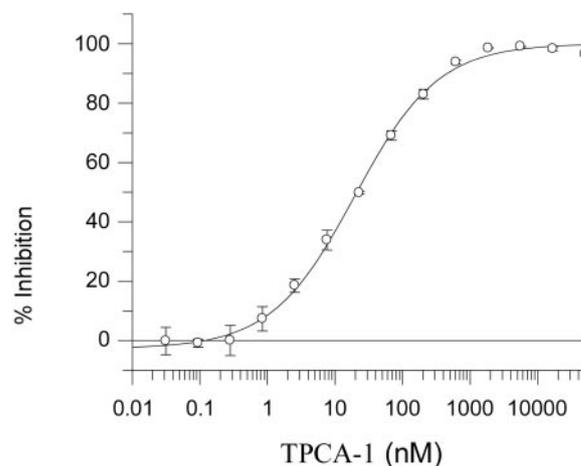
Fig. 2. TPCA-1 is a potent inhibitor of IKK-2 activity. C-terminal GST-tagged recombinant human IKK-2 (residues 1–756) was added to wells containing various concentrations of TPCA-1 or vehicle (DMSO), and the reaction was initiated by the addition of GST-IκBα substrate/ATP. Following termination of the reaction using EDTA, detection reagent containing europium-labeled antiphosphoserine-IκBα antibody and an allo-phycocyanin-labeled anti-GST antibody was added. The degree of phosphorylation of GST-IκBα was measured as a ratio of specific 665 nm energy transfer signal to reference europium 620 nm signal. Data are expressed as the mean percentage of inhibition of the vehicle-treated control group $\pm$ S.D. ($n = 4$ wells) and are representative of 57 independent experiments.

TABLE 1

Selectivity profile of 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide

Kinase assays were performed as described previously (Bain et al., 2003).

Enzyme	IC50
	μM
IKK-1	0.40
p38α	>16
p38β	>10
p38γ	>10
p38δ	>10
MAPKAPK2	>10
MKK1	>10
MAPK2	>10
COX-1	>50
COX-2	>50
JNK1	>10
JNK3	3.6

Prophylactic Administration of TPCA-1 Reduces the Severity and Delays the Onset of CIA. Given the association between NF-κB activation and the development of the clinical symptoms of CIA (Fig. 4), the effect of in vivo administration of TPCA-1 on murine CIA was explored. To determine the effect of prophylactic treatment of TPCA-1, the inhibitor was administered to collagen-immunized/boosted DBA/1 mice at 3, 10, or 20 mg/kg, i.p., b.i.d., from days 1 to 48. Blood concentrations of the inhibitor were measured in samples from three mice per dose, at 2 to 2.5 h following the first daily administration of TPCA-1, on days 4, 10, 15, 24, 31, 39, and 46. Administration of TPCA-1 at 3, 10, or 20 mg/kg resulted in blood levels ranging from 0.07 ± 0.01 to 0.17 ± 0.06, 0.25 ± 0.04 to 0.44 ± 0.09, and 0.79 ± 0.26 to 1.14 ± 0.13 μM, respectively.

As shown in Fig. 5, the severity of arthritis, represented by mean clinical score, was reduced in a dose-dependent manner, with administration of TPCA-1 at 20 or 10 mg/kg ($p < 0.01$ and $p < 0.05$, respectively), but not at 3 mg/kg, resulting
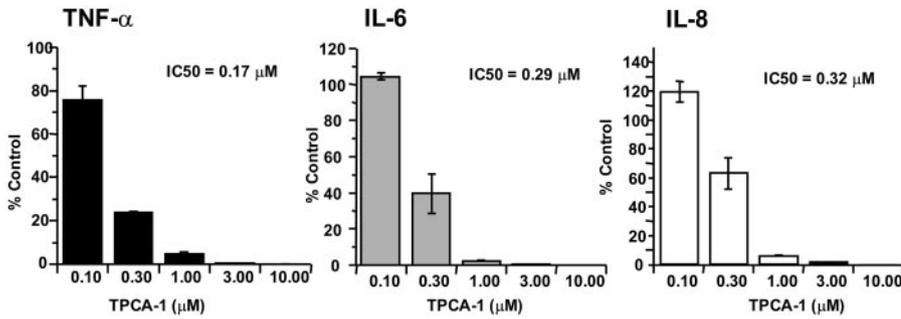
Fig. 3. TPCA-1 inhibits LPS-induced TNF-α, IL-6, and IL-8 production by human monocytes. Human peripheral blood monocytes were incubated for 30 min with various concentrations of TPCA-1 or vehicle (DMSO) and then stimulated for 24 h with LPS (200 ng/ml). Cell supernatants were assayed for TNF-α, IL-6, and IL-8 content. Each data point represents $n = 3$ wells and is expressed as the mean percentage of the vehicle-treated control group $\pm$ S.E.M. The results are representative of three independent experiments.

in a significantly decreased mean clinical score, compared to that of vehicle-treated mice.

In a separate study, the effects of 10 mg/kg TPCA-1 administered i.p., b.i.d. from days 1 to 47, were compared to those of etanercept (recombinant human TNF receptor p75 Fc fusion protein; Enbrel), administered at 4 mg/kg, i.p., every other day from days 1 to 47. Previous studies established that when administered prophylactically, etanercept exhibits maximal efficacy in our CIA model under these conditions (data not shown). Similar to the results described above, administration of TPCA-1 at 10 mg/kg resulted in a significantly reduced mean clinical score compared to that of vehicle-treated mice ($p = 0.001$) (Fig. 6A). In addition, the time to onset of disease was significantly delayed as a result of treatment with TPCA-1 ($p < 0.001$) (Fig. 6B). Etanercept exhibited effects on disease comparable to those of TPCA-1 at 10 mg/kg, significantly reducing mean clinical score ($p < 0.001$) (Fig. 6C) and delaying time to onset of disease ($p < 0.001$) (Fig. 6D), compared to control animals.

Prophylactic Administration of TPCA-1 Reduces Nuclear Localization of p65 in CIA. To confirm the in vivo inhibition of NF-κB activation by TPCA-1, p65 levels were measured in the nuclear extracts of paw tissue from collagen-immunized/boosted DBA/1 mice following 38 days of prophylactic administration of TPCA-1 (10 mg/kg, i.p., b.i.d.) or of vehicle. As shown in Fig. 7, p65 nuclear localization was significantly inhibited in TPCA-1-treated mice compared to relevant vehicle-treated control mice. Mice receiving etanercept (4 mg/kg, i.p., every other day) also exhibited significantly decreased levels of p65 binding. These results suggest that inhibition of NF-κB activation is a likely mechanism through which TPCA-1, as well as etanercept, reduces the severity and delays the onset of CIA.
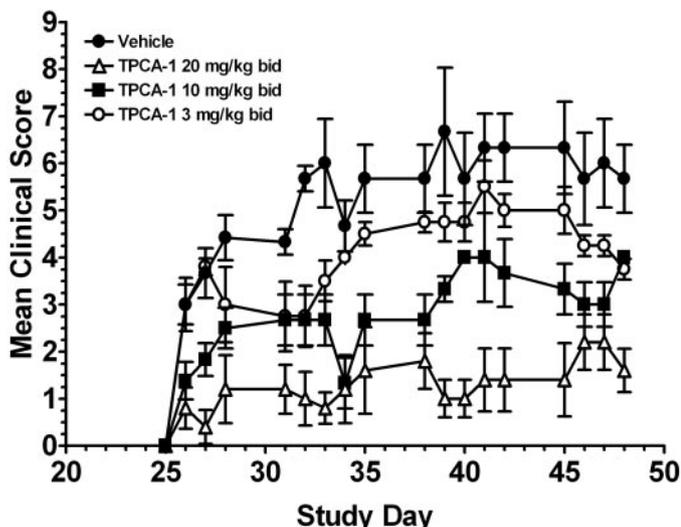
Prophylactic Administration of TPCA-1 Reduces Proinflammatory Cytokine/Chemokine Levels in CIA. Based on the fact that the genes encoding many of the proinflammatory cytokines/chemokines associated with RA and

CIA are regulated by NF-κB, we hypothesized that inhibition of expression of these mediators may be one mechanism by which TPCA-1 attenuates CIA. As illustrated in Fig. 7, following 38 days of prophylactic administration of TPCA-1 (10 mg/kg, i.p., b.i.d.) to collagen-immunized/boosted DBA/1 mice, paw tissue levels of IL-1β, IL-6, TNF-α, and IFN-γ were significantly inhibited compared to vehicle-treated control mice. A trend toward reduction in the levels of KC, a murine chemokine with sequence and functional homology to the human IL-8 family (Bozic et al., 1994), was observed in TPCA-1-treated mice, although this decrease did not reach statistical significance. Similar to the IKK-2 inhibitor, administration of etanercept (4 mg/kg, i.p., every other day) resulted in significantly decreased paw tissue levels of IL-1β, IL-6, TNF-α, and IFN-γ, as well as significantly reduced KC levels (Fig. 7).

Prophylactic Administration of TPCA-1 Attenuates Ex Vivo Antigen-Induced T Cell Proliferation in CIA. Previous studies have demonstrated that inhibition of the NF-κB signaling pathway in T cells via the T cell-specific expression of an IκBα transgene (Seetharaman et al., 1999) or the administration of a T cell-specific inhibitor of NF-κB (Gerlag et al., 2000) results in significant inhibition of murine CIA. To determine whether the TPCA-1-induced reduction in the severity of murine CIA and decrease in tissue proinflammatory mediators are accompanied by an inhibition in antigen-induced T cell proliferation, LNC and splenocytes were collected from collagen-immunized/boosted DBA/1 mice following 38 days of prophylactic administration of TPCA-1 (10 mg/kg, i.p., b.i.d.) or of vehicle. Cells cultured in the absence of the immunizing antigen, collagen, exhibited basal levels of proliferation that did not differ significantly between the vehicle-treated and TPCA-1-treated groups (Fig. 8). In contrast, cells from vehicle-treated mice cultured in the presence of collagen exhibited a robust antigen recall response, which was significantly reduced in cells derived from TPCA-1-treated mice (Fig. 8). These results indicate that in
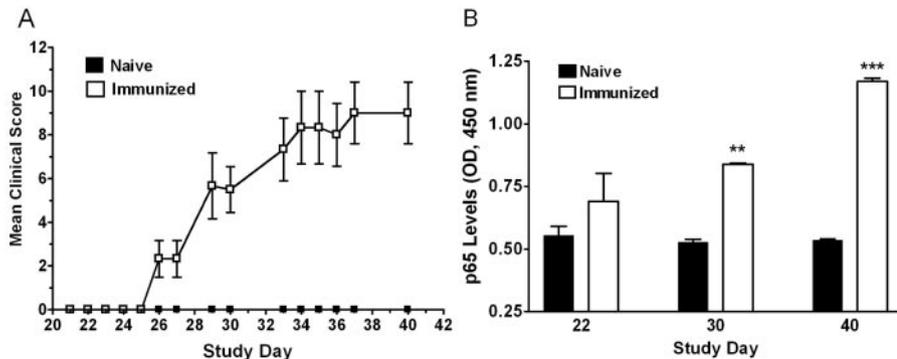
Fig. 4. NF-κB activation correlates with the development of the clinical symptoms of CIA. A, naive DBA/1 mice (■) (initial $n = 15$; final $n = 6$) and DBA/1 mice immunized on day 0 and boosted on day 21 with type II collagen (□) (inital $n = 15$; final $n = 6$) were scored for the clinical symptoms of disease. B, on days 22, 30, and 40, nuclear extracts of front and hind paw tissue from naive mice (■) (4 paws/animal pooled, $n = 3$ animals) and collagen-immunized/boosted mice (□) (4 paws/animal pooled, $n = 3$ animals) were assayed for p65 binding. Data are expressed as the mean $\pm$ S.E.M. ∗, $p < 0.05$; ∗∗, $p < 0.01$; and ∗∗∗, $p < 0.001$.

Fig. 5. Prophylactic administration of TPCA-1 reduces the severity of CIA in a dose-dependent manner. DBA/1 mice immunized on day 0 and boosted on day 21 with type II collagen were administered vehicle (DMSO/DMA/Cremophor) (●), or TPCA-1 at 20 (△), 10 (■), or 3 (○) mg/kg, i.p., b.i.d., from days 1 to 48 and scored for the clinical symptoms of disease. Data are expressed as the mean ± S.E.M. (inital $n = 15$; final $n = 6$).

vivo administration of TPCA-1 attenuates ex vivo antigen-induced T cell proliferation in murine CIA.

Therapeutic Administration of TPCA-1 Reduces the Severity of CIA. To determine whether TPCA-1 is capable of modulating the severity of CIA when delivered therapeutically, administration of TPCA-1 (3, 10, or 20 mg/kg, i.p., b.i.d.) or of vehicle was initiated following the onset of clinical symptoms in collagen-immunized/boosted DBA/1 mice. Blood concentrations of TPCA-1 were measured in samples from three mice per dose at 2 to 2.5 h following the first daily administration of inhibitor (day 1) on days 4, 8, 15, 21, and 24. Administration of TPCA-1 at 3, 10, or 20 mg/kg resulted

in blood levels ranging from 0.13 ± 0.02 to 0.26 ± 0.09, 0.42 ± 0.15 to 1.05 ± 0.30, and 0.68 ± 0.25 to 2.50 ± 0.78 μM, respectively.

As shown in Fig. 9, therapeutic administration of TCPA-1 at 20 mg/kg ($p < 0.01$) (Fig. 9A), but not 10 (Fig. 9B) or 3 (Fig. 9C) mg/kg, significantly reduced the mean clinical score compared with that of vehicle-treated animals. Therapeutic administration of etanercept (12.5 mg/kg, i.p., every other day) also resulted in significant reduction of disease severity compared to that exhibited by vehicle-treated control mice ($p < 0.001$) (Fig. 9D).

Discussion

In this report, we identify a novel inhibitor of IKK-2, TPCA-1, and demonstrate its potency, selectivity, and cell-based activity. TPCA-1 originated from the optimization of an aminothiophene hit from high throughput screening of our compound collection against IKK-2 homodimer. Two other groups independently developed inhibitors in similar aminothiophene series (Kishore et al., 2003; Baxter et al., 2004). Subsequent structure-activity studies resulted in a series of 2-ureidothiophenes, an example of which is TPCA-1. The substitution of the 2-amino group in the initial hit series with a primary urea significantly increased the IKK-2 inhibitory activity. These studies also demonstrated that the 3-carboxamide significantly contributes to IKK-2 inhibition.

The identification of IKK-2 as the kinase primarily responsible for the NF-κB-regulated production of proinflammatory molecules induced by TNF-α and IL-1 suggested that TPCA-1 may be beneficial in the treatment of inflammatory diseases such as RA, leading us to test its activity in CIA. TPCA-1 reduced the severity and delayed the onset of murine CIA. In our studies, the significantly reduced severity of murine CIA resulting from prophylactic or therapeutic administration of the IKK-2 inhibitor is consistent with the results of a recent study demonstrating significant reduction of murine CIA
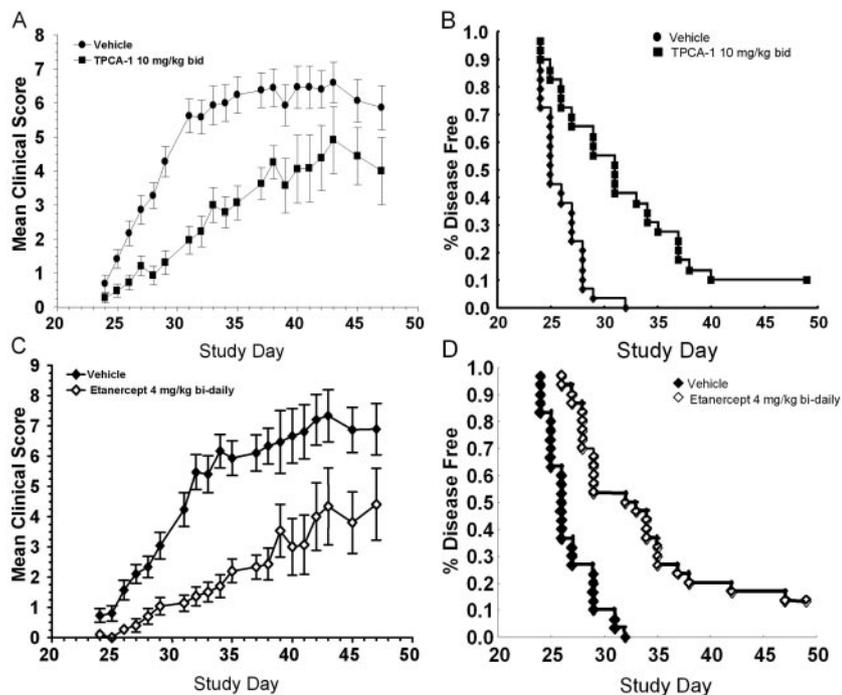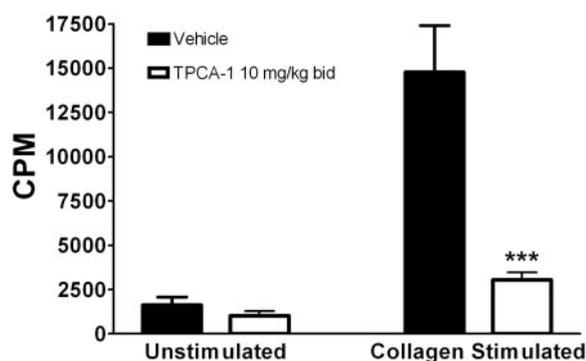

Fig. 6. Prophylactic administration of TPCA-1 reduces the severity and delays the onset of CIA in a manner comparable to a maximally effective dose of etanercept. A and B, DBA/1 mice immunized on day 0 and boosted on day 21 with type II collagen were administered vehicle (DMSO/DMA/Cremophor) (●) or TPCA-1 at 10 (■) mg/kg, i.p., b.i.d., from days 1 to 47 and scored for the clinical symptoms of disease. C and D, DBA/1 mice, immunized on day 0 and boosted on day 21 with type II collagen, were administered vehicle (PBS) (♦) or etanercept at 4 (◇) mg/kg, i.p., every other day from days 1 to 47 and scored for the clinical symptoms of disease. For A and C, data are expressed as the mean ± S.E.M. (initial $n = 30$, final $n = 15$). For B and D, data are expressed as the percentage of mice remaining free of disease (i.e., not exhibiting clinical symptoms for 2 consecutive days).
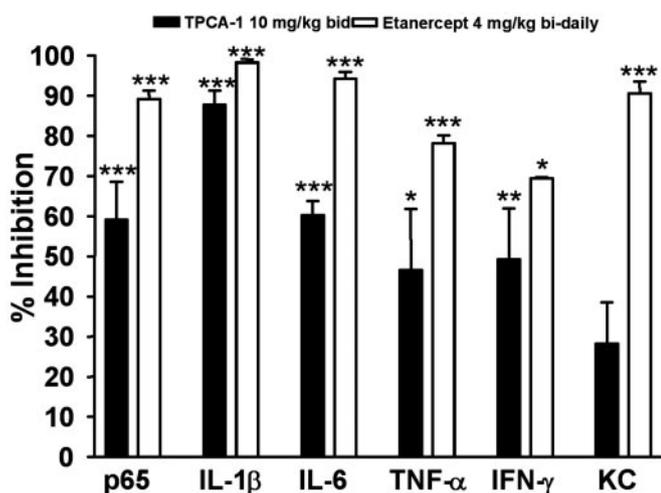
Fig. 7. Prophylactic administration of TPCA-1 or etanercept reduces p65 nuclear translocation and proinflammatory cytokine/chemokine accumulation in CIA. DBA/1 mice, immunized on day 0 and boosted on day 21 with type II collagen, were administered TPCA-1 in DMSO/DMA/Cremophor vehicle at 10 mg/kg, i.p., b.i.d., from days 1 to 37 (■); alternatively, collagen-immunized/boosted mice received etanercept in PBS vehicle at 4 mg/kg, i.p., every other day from days 1 to 37 (□). On day 38, nuclear extracts of front and hind paw tissue were assayed for p65 binding, and supernatants from tissue homogenates were assayed for IL-1β, IL-6, TNF-α, IFN-γ, and KC. For each treatment group, $n = 5$, where $n = 1$ is defined as paws from three animals pooled. Data are expressed as the mean percentage of inhibition of the relevant vehicle-treated control group ± S.E.M. ∗, $p < 0.05$; ∗∗, $p < 0.01$; and ∗∗∗, $p < 0.001$ compared to the relevant vehicle-treated control group.

following prophylactic or therapeutic administration of a selective quinoxaline IKK-2 inhibitor, BMS-345541 (McIntyre et al., 2003). We extend these observations, defining two mechanisms by which this inhibition of disease occurs, demonstrating that the TPCA-1-induced reduction of p65 nuclear translocation in vivo was accompanied by decreased protein levels of local NF-κB-regulated proinflammatory mediators, as well as by inhibition of collagen-induced T cell proliferation. In addition, the effects of TPCA-1 were shown to be

Fig. 8. Prophylactic administration of TPCA-1 reduces ex vivo antigen-induced T cell proliferation in CIA. On day 38, inguinal lymph nodes and spleens were collected from collagen-immunized/boosted DBA/1 mice treated from days 1 to 37 with vehicle (DMSO/DMA/Cremophor) (■) ($n = 9$) or TPCA-1 at 10 mg/kg (□) ($n = 9$), i.p., b.i.d. Following preparation of single cell suspensions, LNC and splenocytes from three mice were combined at a ratio of 80% LNC/20% splenocytes, giving an $n = 3$ pooled groups. LNC/splenocytes were incubated in the presence or absence of type II collagen (100 μg/ml) for 96 h with the final 24 h of incubation occurring in the presence of [³H]thymidine, and the incorporated radio-activity was quantified. Data are expressed as the mean ± S.E.M. ∗, $p < 0.05$; ∗∗, $p < 0.01$; and ∗∗∗, $p < 0.001$ compared with vehicle-treated control mice.

comparable to those of the antirheumatic drug, etanercept. Collectively, these results suggest that potent, selective, small molecule inhibitors of IKK-2 offer a promising approach to the development of novel therapeutics for RA.

The NF-κB family of transcription factors regulates the expression of a number of proinflammatory cytokines/chemokines associated with RA and CIA, including TNF-α, IL-1β, IL-6, IL-8/KC, and IFN-γ (Pahl, 1999). The ability of TPCA-1 to inhibit the LPS-induced production of TNF-α, IL-6, and IL-8 by human monocytes in vitro is consistent with the compound's potent inhibitory activity against recombinant human IKK-2 and demonstrates its cell-based activity. This modulation of proinflammatory mediator expression by TPCA-1 was observed in vivo as well, with TPCA-1-treated animals exhibiting significantly reduced paw tissue levels of IL-1β, IL-6, TNF-α, and IFN-γ, compared to vehicle-treated control animals. This observation suggests that reduction of proinflammatory mediators contributes to the TPCA-1-induced attenuation of CIA. In addition to exerting a direct inhibitory effect on the transcriptional regulation of these proinflammatory mediators, TPCA-1 may reduce cytokine expression indirectly through attenuation of proinflammatory cytokine cascades. In this regard, it has been demonstrated that IL-1β stimulates the production of IL-1β, TNF-α, IL-6, and IFN-γ (Dinarello, 1996), whereas TNF-α induces IL-1β, TNF-α, and IL-6 expression (Aggarwal et al., 2001). In addition, IFN-γ has been shown to induce the production of IL-1β and TNF-α (Collart et al., 1986).

It is of interest that administration of etanercept resulted in significantly reduced paw tissue levels of IL-1β, IL-6, TNF-α, IFN-γ, and KC. The down-regulation of proinflammatory cytokine cascades is believed to be an important mechanism underlying the clinical benefits of anti-TNF-α therapy in RA. It has been demonstrated that treatment of RA patients with etanercept results in significantly reduced plasma IL-6 levels (Feldmann et al., 1998); however, the studies described herein are, to our knowledge, the first comprehensive report of the effects of etanercept, as well as those of an IKK-2 inhibitor, on RA/CIA-associated proinflammatory mediators in vivo.

The fact that both TPCA-1 and etanercept significantly reduced IL-1β, IL-6, TNF-α, and IFN-γ levels in the paw tissue suggests that these therapeutic agents may modulate the same intracellular signaling pathway. This is supported by the observed decreases in p65 nuclear localization following in vivo administration of TPCA-1 and etanercept. While TPCA-1 exerts its effect on the NF-κB signaling pathway via attenuation of IKK-2-mediated phosphorylation of IκB, etanercept is presumably acting at a site more distal to the NF-κB/IκB complex, preventing TNF-α-induced signaling at the cell surface. Consistent with this hypothesis are reports of decreased DNA binding activity of NF-κB following in vitro or in vivo treatment with anti-TNF-α antibodies (Pimentel-Muiños et al., 1994; De Plaen et al., 2000).

It is widely recognized that CIA is a T cell-dependent, antigen-specific disease (Myers et al., 1997). It has been proposed that auto-antigen-specific T cells play a pivotal role in the etiology of RA as well (Panayi et al., 1992; Weyand and Goronzy, 1997). In the studies described herein, the inhibition of ex vivo collagen-induced T cell proliferation exhibited by TPCA-1-treated mice, compared to vehicle-treated mice, suggests that inhibition of antigen-induced T cell prolifera-
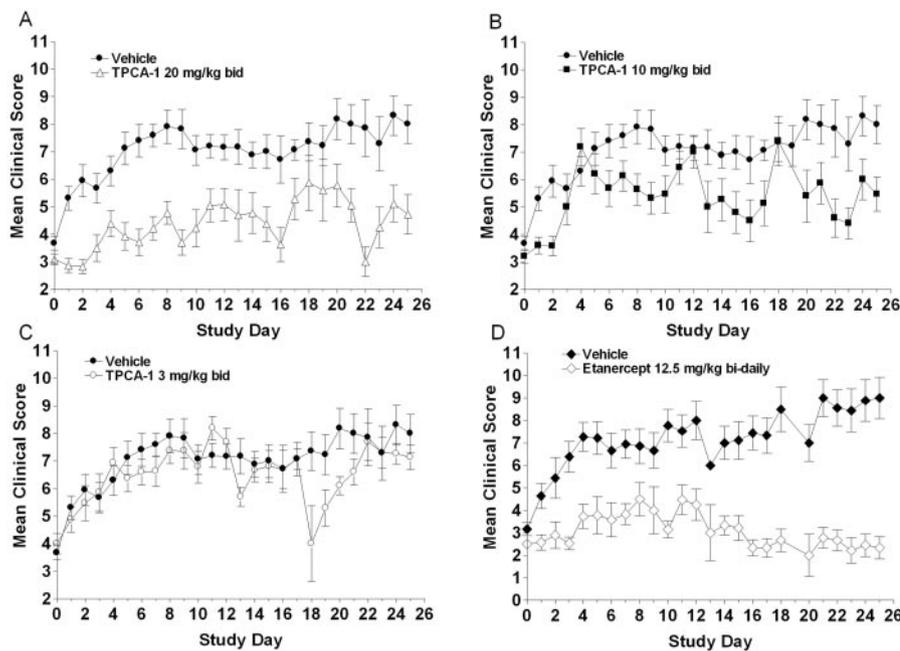
Fig. 9. Therapeutic administration of TPCA-1 or etanercept reduces the severity of CIA. Following exhibition of clinical symptoms for 2 consecutive days (days −1 and 0), collagen-immunized/boosted DBA/1 mice were administered vehicle or drug from days 1 to 25 and were scored for the clinical symptoms of disease. A–C, mice received vehicle (DMSO/DMA/Cremophor) (●) or TPCA-1 at 20 (△), 10 (■), or 3 (○) mg/kg, i.p., b.i.d. (n = 30) D, mice received vehicle (PBS) (◆) or etanercept at 12.5 (◇) mg/kg, i.p., every other day (n = 18). Data are expressed as the mean ± S.E.M.

tion is a mechanism underlying the beneficial effects of the IKK-2 inhibitor in CIA. It has been demonstrated that the decreased severity and incidence of murine CIA resulting from inactivation of NF-κB signaling, through the expression of an IκBα transgene, were accompanied by significantly reduced ex vivo collagen-induced T cell proliferation and IFN-γ production. In this study, expression of the IκBα transgene was restricted to T cells, suggesting that NF-κB signaling in T cells is critical to antigen-induced T cell proliferation (Seetharaman et al., 1999). The results of other studies support this finding, demonstrating that T cell-intrinsic NF-κB activation is required for antigen-induced T cell proliferation and the generation of a Th1 response (Artis et al., 2003; Corn et al., 2003). It is possible that inhibition of NF-κB signaling in antigen-presenting cells (APC) instead of, or in addition to, inhibition of NF-κB induction in T cells is responsible for the observed effects of TPCA-1 on collagen-induced T cell proliferation in murine CIA. Activation of the NF-κB signaling pathway has been shown to play a pivotal role in the antigen-presenting capacity of dendritic cells (Boffa et al., 2003; Ma et al., 2003; Yoshimura et al., 2003). More specifically, IKK-2 has recently been shown to be essential for dendritic cell antigen presentation to T cells (Andreakos et al., 2003). It is of interest that reduced susceptibility of c-Rel-deficient mice to the Th1-mediated disease, experimental autoimmune encephalomyelitis, was found to be a result of both defective T cell differentiation into Th1 cells and decreased IL-12 production by APC (Hilliard et al., 2002). Whether IKK-2 inhibitors act in a similar fashion, abrogating both T cell and APC function during antigen-induced T cell proliferation and differentiation, remains to be determined.

Currently, the two principal approaches to RA therapy consist of nonsteroidal anti-inflammatory drugs (NSAIDs), which interfere with prostaglandin production through the inhibition of COX enzymes, providing symptomatic relief, and disease-modifying antirheumatic drugs (DMARDs), which inhibit both the inflammatory and cartilage/bone destructive processes of RA. Over the last 10 to 15 years,

significant advances have been made in RA therapy. The development of NSAIDs that selectively inhibit COX-2 and spare COX-1 has reduced the gastrointestinal toxicity associated with nonselective NSAIDs. In addition, the recognition of the beneficial effects of aggressive DMARD therapy early in the course of the disease and the initiation of combination DMARD therapy, such as using methotrexate with cyclosporine or with sulfasalazine and hydroxychloroquine, has enabled DMARDs to be used to their maximum therapeutic potential (Saravanan and Hamilton, 2002; Smolen and Steiner, 2003). In the last 5 years, several new DMARDs have been approved for clinical use, including the nonpeptide immunomodulator leflunomide, the anti-TNF-α therapies etanercept, infliximab, and adalimumab, and the IL-1 receptor antagonist anakinra. Although TNF-α blockade, the aggressive use of DMARDs early in disease, and the initiation of combination DMARD therapy have been major advancements in the treatment of RA, it should be noted that all available therapies to date are associated with issues of efficacy and/or toxicity. Hence, a number of new targets, including proinflammatory cytokines/chemokines, adhesion molecules, and MMPs, are being pursued for the development of RA therapeutics (Smolen and Steiner, 2003). The fact that many of these molecules are regulated by the NF-κB family of transcription factors makes components of this signaling pathway, such as IKK-2, intriguing potential targets.

In summary, the studies presented in this paper demonstrate that a novel, potent, selective small molecule inhibitor of IKK-2, TPCA-1, significantly reduces the severity of murine CIA following prophylactic or therapeutic administration, exhibiting effects comparable to those of the antirheumatic drug etanercept. Inhibition of proinflammatory mediator accumulation and inhibition of collagen-induced T cell proliferation are likely mechanisms underlying modulation of CIA by the inhibitor. These results suggest that inhibition of IKK-2 may be a beneficial approach in the treatment of human disease.

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