Novel Inhibitors of Cytokine-induced IκBα Phosphorylation and Endothelial Cell Adhesion Molecule Expression Show Anti-inflammatory Effects in Vivo*

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Jacqueline W. Pierce‡§, Robert Schoenleber¶, Gary Jesmok‡, Jennifer Best‡, Sarah A. Moore‡, Tucker Collins‡§, and Mary E. Gerritsen¶

From the §Vascular Research Division, Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115 and the ¶Bayer Corporation, West Haven, Connecticut 06516

We have identified two compounds that inhibit the expression of endothelial-leukocyte adhesion molecules intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selecin. These compounds act by inhibiting tumor necrosis factor-α-induced phosphorylation of IκB-α, resulting in decreased nuclear factor-κB and decreased expression of adhesion molecules. The effects on both IκB-α phosphorylation and surface expression of E-selectin were irreversible and occurred at an IC₅₀ of approximately 10 μM. These agents selectively and irreversibly inhibited the tumor necrosis factor-α-inducible phosphorylation of IκB-α without affecting the constitutive IκB-α phosphorylation. Although these compounds exhibited other activities, including stimulation of the stress-activated protein kinases, p38 and JNK-1, and activation of tyrosine phosphorylation of a 130–140-kDa protein, these effects are probably distinct from the effects on adhesion molecule expression since they were reversible. One compound was evaluated in vivo and shown to be a potent anti-inflammatory drug in two animal models of inflammation. The compound reduced edema formation in a dose-dependent manner in the rat carrageenan paw edema assay and reduced paw swelling in a rat adjuvant arthritis model. These studies suggest that inhibitors of cytokine-inducible IκBα phosphorylation exert anti-inflammatory activity in vivo.

The adhesion of circulating leukocytes to vascular endothelium is critical to inflammatory responses (reviewed in Refs. 1–3). Interaction of the selectin family of adhesion proteins and lectin counter-receptors is the predominant mechanism mediating initial adhesion between leukocytes and the vessel wall. The expression of endothelial-leukocyte adhesion molecule-1 (E-selectin, CD62E), vascular cell adhesion molecule-1 (VCAM-1, CD106), and intercellular adhesion molecule-1 (ICAM-1, CD54) on the surface of endothelial cells is elevated at sites of inflammation (2, 4). Induction of these molecules by tumor necrosis factor-α (TNFα) and other inflammatory cytokines is regulated at the level of gene transcription and requires binding of the transcription factor nuclear factor-κB (NF-κB) to the regulatory regions within the promoters of each of these genes (5–12).

The NF-κB/Rel transcription factor family plays an important role in cytokine-induced gene activation (13–15). The Rel family includes p50 (NFκB1), p52 (NFκB2), p65 (RelA), RelB, v-Rel, and c-Rel. In endothelial cells, the p50/p65 heterodimer is the predominant species that binds to κB consensus sequences in the VCAM-1, ICAM-1, and E-selectin genes and activates gene transcription. NF-κB is located in the cytoplasm of cells in an inactive form in association with the inhibitor IκB-α. In response to TNFα stimulation, IκB-α is phosphorylated on 2 serine residues (Ser-32 and Ser-36), ubiquitinated, and degraded by a proteosome-dependent pathway allowing active NF-κB to translocate to the nucleus where it can activate gene expression (16–23). Many NF-κB-dependent genes including the adhesion molecules and several cytokine genes are important mediators of inflammation (reviewed in Ref. 24). A diverse range of agents that block NF-κB signaling has been shown to decrease expression of adhesion molecules (25–31). Recently, several clinically important anti-inflammatory agents including glucocorticoids, salicylates, and nitric oxide have been reported to inhibit NF-κB-driven gene expression which may explain, at least in part, the anti-inflammatory actions of these drugs (24–26, 31–34). Thus, novel agents that block NF-κB-IκB-α signaling have the potential to inhibit a wide range of inflammatory processes.

In this study, we identified two novel pharmacologic agents that inhibit the TNFα-induced surface expression of ICAM-1, VCAM-1, and E-selectin in human endothelial cells. These compounds were examined for their effects on cytokine-induced NF-κB/IκB-α signaling. Both compounds decreased TNFα-induced nuclear translocation of NF-κB through inhibition of the TNFα-induced phosphorylation of IκB-α. Compound 1 selectively inhibited the TNFα-inducible phosphorylation of IκB-α without affecting the constitutive IκB-α phosphorylation. To determine whether these agents may inhibit other cellular phosphorylation events, we examined the effects of compound 1 on TNFα-induced activity of the stress-activated protein kinases, p38 and JNK-1. This agent increased the activity of p38 kinase and JNK-1 but had little or no effect on the activity of the MAP kinase, ERK-1. The agent was also examined for effects on protein tyrosine phosphorylation since agents that block tyrosine phosphorylation have been reported to inhibit NF-κB signaling and adhesion molecule expression (29). Treatment of endothelial cells with the test compound did not detectably inhibit protein tyrosine phosphorylation but rather resulted in an elevated level of a tyrosine-phosphorylated protein.
tein of molecular mass 130–140 kDa. We evaluated compound 2 in two animal models of inflammation. This agent reduced swelling in a dose-dependent manner in both the rat carrageenan paw edema assay and in a rat adjuvant arthritis model. Thus, we have identified a novel class of anti-inflammatory agents that act by selectively inhibiting the TNFα-induced phosphorylation of IκBα resulting in decreased expression of endothelial adhesion molecules.

**EXPERIMENTAL PROCEDURES**

*Cell Culture, Cytokine Treatment, and Toxicity Assay—Human umbilical vein endothelial cells (HUVEC) were isolated and maintained in culture using previously described procedures (36). For experiments on cytokine induction, cells were exposed to recombinant human TNFα at a final concentration of 100 units/ml in complete media for the times indicated. The proteasomal inhibitor carboxenzyloxy-leucyl-leucyl-leucinal-H (MG115) was prepared as a 40 mM stock solution in MeSO and added to complete medium to a final concentration of 40 μM. Cell toxicity was assessed by morphology and by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (37).

**Cell Surface Fluorescent Immunoassay—**Cell surface binding assays were performed at 4 °C on viable human umbilical vein endothelial cell monolayers in microtiter plates, using saturating concentrations of monoclonal antibody supernatants and a secondary fluorescent-conjugated F(ab′)2, goat anti-murine IgG (Caltag Labs, San Francisco, CA) as previously detailed (38). Antibodies to E-selectin (H4/18), VCAM-1 (E/1), and ICAM-1 (Hu5/3) culture supernatants were kindly provided by Dr. Michael A. Gimbrone, Jr. Fluorescence intensities were determined using an automated microtiter plate reader (Pandex, Baxter Healthcare Corp.).

**Interleukin-6 and Interleukin-8 Assays—**The effects of compounds 1 and 2 on interleukin-6 (IL-6) and interleukin-8 (IL-8) production were evaluated on HUVEC that were grown to confluence on 96-well microtiter plates. The cells were preincubated with the drugs at concentrations of 0, 1, 5, 10, or 25 μM and then incubated with TNFα (10 units/ml) and drug for 16 h. The culture supernatants were removed and assayed for IL-6 and IL-8 content using enzyme-linked immunoassay kits from R & P Systems (Minneapolis, MN).

**Electrophoretic Mobility Shift Assay—**Nuclear extracts were prepared from test or control HUVEC in the presence of 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1.5 μg/ml pepstatin A, 40 μM ALLN (calpain inhibitor 1), 1 mM sodium orthovanadate, and 1 mM sodium fluoride as described previously (10). Oligonucleotides were gel-purified, annealed, and end-labeled with [α-32P]dCTP (50 μCi; specific activity of 3000 Ci/mmol, NEN Life Science Products) and the Klenow fragment of Escherichia coli DNA polymerase I. Binding reactions were performed in the presence of 10 mM Tris, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 1 μg of poly(dIdC) and electrophoresis was carried out as described previously (39). The following oligonucleotides were utilized: VCAM-1b (5′-CTGGGTTTTCCTCTGAA GATTCCCT-3′) and the complementary strand. Protein DNA complexes were resolved on 4% polyacrylamide gels.

**Western Blots—**Following experimental treatment of HUVEC, cytosolic and nuclear protein extracts were prepared, subjected to electrophoresis on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose in 25 mM Tris, 192 mM glycine, 5% methanol at 100 V for 1 h as described previously (10, 27). Anti-IκBα and anti-p38 antisera were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used at dilutions of 1:1000. Rabbit antisera directed against the phosphorlated p38 (Thr182) were obtained from New England Biolabs (Beverly, MA) and used at a 1:1000 dilution. Mouse anti-phosphotyrosine antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY) and used at 1:1000 dilution. Immunoreactive proteins were detected by enhanced chemiluminescence protocol (Amersham Corp.) using 1:10,000 horseradish peroxidase-linked donkey anti-rabbit or sheep anti-mouse secondary antisera. Blots were exposed to film for 1–15 min and then developed.

**Immune Complex Kinase Assays—**Extracts were prepared from control and TNFα-treated HUVEC. Cells were solubilized with Triton lysis buffer (TLB, 20 mM Tris, pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). Extracts were centrifuged at 14,000 × g for 15 min at 4 °C. The JNK, p38, or ERK protein kinases were immunoprecipitated by incubation for 1 h at 4 °C with specific rabbit polyclonal antibodies bound to protein-A Sepharose (Pharmacia Biotech Inc.). The rabbit polyclonal JNK-1 and p38 antisera have been described (40). The immunoprecipitates were washed twice with TLB and twice with kinase buffer (20 mM Hepes, pH 7.4, 20 mM β-glycerophosphate, 20 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate). The kinase assays were initiated by the addition of 1 μg of substrate protein and 50 μM [γ-32P]ATP (10 Ci/mmol) in a final volume of 25 μl. The reactions were terminated by the addition of Laemmli sample buffer. Control experiments demonstrated that the phosphorylation reaction was linear with time for at least 30 min under these conditions. The phosphorylation of the substrate proteins was examined by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

**In Gel Kinase Assay—**In gel kinase assay for the proteins that phosphorylate IκBα was carried out according to the method of Hibi et al. (41) and as detailed below. Whole cell extracts were prepared from HUVEC treated with TNFα (100 units/ml) for 15 min in the presence or absence of compound 1 (20 μM, pretreatment for 1 h) as indicated. Proteins were separated on a 10% SDS gel containing 0.5 mg/ml HIS-Ilbα. Gels were washed two times in 20% propanol, 50 mM Hepes, pH 7.6, for 30 min and two times in buffer A (50 mM Hepes, pH 7.6, 5 mM 2-mercaptoethanol) for 30 min. A final buffer A containing 6 μl urea, 1 μl each in 3, 1.5, and 0.75 mM urea in buffer A and 0.05% Tween 20 and 1 h in buffer A with 0.05% Tween 20. The kinase assay was carried out for 1 h at 30 °C in the presence of 50 μg ATP, 5 μCi [γ-32P]ATP, 20 mM Hepes, pH 7.6, 20 mM MgCl₂, 20 mM β-glycerophosphate, 20 mM p-nitrophenyl phosphate, 1 mM sodium vanadate, 2 mM dithiothreitol. The gel was washed with 5% trichloroacetic acid and 1% sodium pyrophosphate, dried, and exposed to film. A separate gel with no HIS-Ilbα was assayed as a control.

**Carrageenan Paw Edema—**Male Harlan Sprague Dawley rats 150–175 g were used. A 1% suspension of carrageenan (Marine Colloids, Springfield, MA) in distilled water was administered to rats as 0.1 ml subplantar injection into the footpad of the right hind paw as described previously (42). One h prior to injection, rats were pretreated with a nonsteroidally with vehicle (polyethylylglycol 400 diluted 1:5 in 5% bovine serum albumin/H₂O) or a fine suspension of compound 2 (1, 5, or 50 mg/kg) in vehicle. A positive control group was also included in which rats were pretreated with 20 mg/kg ibuprofen. Four hours after carrageenan administration, the volume of the injected paw was measured by means of a water displacement plethysmograph. Edema volumes were determined immediately and at 1, 3, 4, and 24 h. Each group contained five animals. Data were analyzed by a one-way analysis of variance and, if indicated, differences between groups analyzed by Bonferroni’s modified t test. A p < 0.05 was considered significant.

**Adjuvant Arthritis—**Inbred, male Lewis rats 8–10 weeks of age weighing 250–275 g were obtained from Charles River (Wilmington, MA). Paw animals per group were used, and the animals were allowed to feed ad libitum on laboratory rat chow and water. Heat-inactivated Mycobacterium butyricum (Difco) was suspended at 10 mg/ml in mineral oil (Purepac Lubinol, Purepac Pharmaceuticals, Elizabeth, NJ) and administered as 0.1-ml injection (1 mg/animal) at the base of the tail. Paw volumes were determined by a water displacement plethysmograph as described above. Volumes were determined on the indicated dates and values compared with initial time 0 measurements. Vehicle (0.5% methyl cellulose) or drug (compound 2 or dexamethasone at the indicated concentrations) was administered once a day as an intraperitoneal injection (200 μl). Data from these studies are expressed as the mean difference in foot pad volume. At day 20 animals were sacrificed by CO₂ inhalation.

**RESULTS**

**Identification of Novel Inhibitors of ICAM-1, VCAM-1, and E-selectin Surface Expression—**Two structurally related compounds, compound 1 and compound 2 (Fig. 1), were identified as inhibitors after 15 min at 30 °C by means of the indicated procedures. Compound 1 and compound 2 inhibited the surface expression of all three adhesion molecules with
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IC₅₀ values in the range of 5–10 μM (Fig. 2). To determine whether the effects of the test compounds were reversible, we compared E-selectin levels in cells stimulated with TNFα in the presence of compound 1 (10 μM) with the levels obtained when the cells were pretreated with compound 1 followed by a 1 h “washout” period and then a 3-h stimulation with TNFα in the absence of test drug. Treatment of HUVEC with 10 μM compound inhibited E-selectin expression by 57% and a similar level of inhibition was seen when the drug was “washed out” prior to TNFα treatment (Fig. 3). Thus, compound 1 irreversibly inhibits surface expression of E-selectin. Other effects of compound 1 were reversible (see below) suggesting that the inability to reverse the inhibition of TNFα-induced E-selectin expression was not due to retention of the compound by the cell.

There was no detectable cytotoxicity as measured by MTT assay even after 16 h treatment of cells with this dose (10 μM) of the test compound (IC₅₀ in the MTT assay ranged from 25–38 μM). Thus, it is likely that the drug irreversibly modifies a cellular target.

We also determined whether the test compounds could inhibit E-selectin surface expression when added after initiation of TNFα treatment. Maximal inhibition occurred when the test compound was added from the start of the TNFα induction period, and no significant inhibition was observed when the test compound was added after 1 h (Fig. 3). These results are consistent with the drug acting rapidly and irreversibly within the 1st h of the TNFα induction.

Additionally, we determined whether the test compounds could inhibit cytokine production, as well as expression of leukocyte adhesion molecules. Compounds 1 and 2 also reduced TNFα-induced IL-6 and IL-8 production in a dose-dependent manner. This inhibition was greater than 50% at 10 μM and virtually complete at 25 μM (data not shown).

Inhibition of Nuclear NF-κB—The TNFα-induced expression of adhesion molecules E-selectin, VCAM-1, and ICAM-1 requires the transcription factor NF-κB (5–12). Therefore, we evaluated the test compounds for effects on nuclear translocation of NF-κB. We carried out electrophoretic mobility shift assay to determine the levels of NF-κB in nuclear extracts from HUVEC treated with TNFα in the presence of compound 1 or compound 2. As previously observed, TNFα-induced nuclear translocation of NF-κB occurs within 15 min in the absence of test compound (Fig. 4A, lane 2 and Fig. 4B, lane 2). At 20 μM, both test compounds completely inhibited nuclear NF-κB (Fig. 4A, lane 4, Fig. 4B, lane 3). A lower dose of compound 1 (10 μM) also reduced nuclear NF-κB (Fig. 4A, lane 3).

Inhibition of TNFα-inducible IxB-α Phosphorylation and Irreversible Stabilization of IxB-α—The TNFα-induced regulation of NF-κB involves the phosphorylation, ubiquitination, and degradation of the cytoplasmic inhibitor, IxB-α (16–23). In endothelial cells, the phosphorylation and degradation of IxB-α have been shown to occur within 15 min of TNFα treatment allowing NF-κB to translocate to the nucleus where it can activate gene expression (8). To determine whether the test compounds may affect TNFα-inducible phosphorylation and/or degradation of IxB-α, we examined the levels of IxB-α in the
cytoplasm of endothelial cells pretreated with increasing concentrations of test compounds and then stimulated with TNFα for 15 min. The results of Western blot analysis of endothelial cytoplasmic extracts using IκBα-specific antisera are shown in Fig. 5. A 37-kDa protein was detected in cytoplasmic extracts from unstimulated cells (Fig. 5, lane 1). Treatment of HUVEC with TNFα led to a rapid loss of IκB-α from the cytoplasm (Fig. 5, lane 2). Both test compounds stabilized IκB-α in a dose-dependent manner with an IC₅₀ value of approximately 10 μM (Fig. 5, lanes 3-5). There was a clear correlation between the concentration of drug that stabilized IκB-α, the concentration that inhibited nuclear levels of NF-κB, and the concentration that inhibited adhesion molecule expression. The levels of p38 were not significantly affected by the test compounds (Fig. 5) suggesting that these agents did not result in nonspecific effects on protein stability. In addition, these concentrations of test compounds did not affect protein synthesis as assessed by [⁹³⁷H]leucine incorporation into 5% trichloroacetic acid-precipitable protein.

The effects of test compounds on levels of IκB-α protein could be due to an inhibition of IκB-α phosphorylation or a block of degradation. To determine whether these agents affect IκB-α phosphorylation, we examined the IκB-α levels in cells treated with both compound 1 and the proteosome inhibitor carbenoxyl-leucinyl-leucinyl-leucinal-H (MG115) (Fig. 5, lanes 6 and 7). As previously reported (19, 22, 27), the proteosome inhibitor blocks the degradation of IκB-α, allowing visualization of the phosphorylated form of IκB-α, detected as a slower migrating band (Fig. 5, lane 7, IκB-α-P). The faster migrating form present in unstimulated cells is basally phosphorylated IκBα (18, 19). The IκB-α protein present in TNFα-stimulated cells treated with compound 1 appears as a single faster migrating band corresponding to basally phosphorylated IκB-α (Fig. 5, lane 5). Stimulation of cells with TNFα in the presence of both compound 1 and the proteosome inhibitor resulted in stabilization of the basally phosphorylated form of IκB-α (Fig. 5, lane 6). Little or no inducibly phosphorylated IκB-α protein was detected (Fig. 5, lanes 6 and 7, IκB-α-P), suggesting that compound 1 inhibits TNFα-inducible phosphorylation of IκB-α. Similar results were observed with compound 2 (data not shown). The IκB-α which has not undergone inducible phosphorylation is not targeted for degradation (19, 43). Thus, in contrast to the proteosome inhibitors that block degradation of the phosphorylated IκB-α, compounds 1 and 2 inhibit inducible phosphorylation of IκB-α. Compounds 1 and 2 may inhibit a TNFα-inducible kinase and/or activate a cellular phosphatase activity.

Because the effects of these agents on adhesion molecule expression were found to be irreversible (Fig. 3), we tested whether the stabilization of IκB-α was similarly irreversible.

The levels of cytoplasmic IκB-α were examined in HUVEC pretreated with compound 1 for 1 h and then incubated with media alone for 1 h prior to treatment with TNFα in the absence of drug. The wash out of this compound had little or no effect on the levels of IκB-α (Fig. 6, lanes 6 and 7). Thus, this drug appears to irreversibly stabilize IκB-α. Treatment of cells with the test compound 15 min after TNFα induction and immediately prior to preparation of extracts did not result in stabilization of IκB-α (Fig. 6, lane 8). Clearly, the drug blocks TNFα-induced phosphorylation and degradation of IκB-α in intact cells and not during extract preparation. The effects on both IκB-α phosphorylation and surface expression of E-selectin were rapid, irreversible, and occurred at an IC₅₀ of approximately 10 μM. This suggests that the inhibition of IκB-α phosphorylation and degradation causes the decrease in NF-κB, resulting in decreased transcription and surface expression of the adhesion molecules.

**Effect on Constitutive Phosphorylation of IκB-α—**The IκB-α protein is regulated by cytokine-inducible phosphorylation on Ser-32 and Ser-36 (16–23). In addition, the C-terminal sequences of IκB-α contain a consensus sequence for casein kinase II that may be important for basal phosphorylation of the IκB-α protein (19, 44–46). To determine whether compound 1 selectively inhibited TNFα-inducible phosphorylation of IκB-α or might also inhibit the activity of a constitutive IκB-α kinase that basally phosphorylates IκB-α, we carried out an in gel kinase assay for proteins that phosphorylate IκB-α (Fig. 7). Two proteins of molecular mass of approximately 36 to 41 kDa were observed in whole cell extracts. The activities of these kinases were unaffected by 15 min treatment with TNFα or by compound 1 (20 μM). The molecular weights of these kinases correspond to those expected for the catalytic subunits of casein kinase II; however, Bennett et al. (47) have recently described IκB-α kinases of similar molecular weight in endothelial cells that are distinct from casein kinase II. Our results suggest that compound 1 had no effect on the activity of these IκB-α kinases. Thus, compound 1 selectively inhibits the TNFα-inducible phosphorylation of IκB-α without affecting the constitutive IκB-α phosphorylation.

**Activation of Stress-activated Protein Kinases, p38 and JNK-1, with No Effect on ERK-1—**The treatment of endothelial cells with TNFα induces multiple signaling events that might be affected by the test compounds. It has been shown that TNFα treatment stimulates the stress-activated protein kinase cascade (40, 41, 48–53). The JNK-1 and p38 kinases are acti-
The level of ERK-1 activity detected in cells treated with TNF-α did not correlate with the inhibition of p38 phosphorylation. In contrast to the effects on adhesion molecule expression, the observed activation of p38, JNK-1, or ERK-1. The results are shown in Fig. 8.

Effects on Tyrosine Phosphorylation—Protein tyrosine kinase inhibitors have been reported to block NF-kB/IkBα signaling and inhibit adhesion molecule expression (29). To determine whether the selected agents may act as protein tyrosine kinase inhibitors, we assayed the tyrosine-phosphorylated proteins in whole cell extracts from endothelial cells treated with TNFα or TNFα and compound 1. Extracts were analyzed by Western blot with anti-phosphotyrosine antisera as described under “Experimental Procedures.” Multiple bands at a variety of molecular weights were reactive with the anti-phosphotyrosine antisera. There was not a general reduction in the pattern or intensity of the bands in compound 1-treated cells. Indeed, cells treated with TNFα and compound 1 showed a dramatic increase in a tyrosine-phosphorylated protein with a molecular mass of 130–140 kDa (Fig. 9). There was some increase in p38 phosphorylation without affecting other TNFα-induced phosphorylation events, we examined the effects of compound 1 on MAP kinase activity. We carried out Western blot analysis with antisera specific for the phosphorylated form of p38α to determine whether this agent affected the phosphorylation of the p38 kinase. Results are shown in the bottom panel of Fig. 5. There was a small increase in phosphorylation of p38 with TNFα alone and a marked increase in phosphorylation of p38 in HUVEC-treated with TNFα in the presence of 20 μM compound 1 (Fig. 5, lanes 2 and 5). In addition, there was a slight but detectable increase in p38 phosphorylation in HUVEC treated with compound 1 alone (data not shown). The level of p38 phosphorylation in cells treated with both compound 1 and TNFα was higher than that seen in with TNFα alone or compound 1 alone (Fig. 5, lanes 2 and 5). The total levels of p38 in these cells did not change. These results suggest that compound 1 stimulates TNFα-induced phosphorylation of p38 and that compound 1 does not globally inhibit all TNFα-induced phosphorylation. In contrast to the effects on IkBα and the effects on adhesion molecule expression, the observed activation of p38 phosphorylation was reversible with 1 h treatment in the absence of drug (data not shown). Thus, the increase in p38 phosphorylation did not correlate with the inhibition of adhesion molecule expression.

The effect of this compound on MAP kinase signaling was also measured by immunoprecipitation kinase assays for activity of p38, JNK-1, or ERK-1. The results are shown in Fig. 8. The level of ERK-1 activity detected in cells treated with TNFα in the presence of compound 1 (20 μM) was similar to the ERK-1 activity in cells treated with TNFα alone suggesting that compound 1 does not affect ERK-1 kinase activity (Fig. 8, lanes 2 and 3). The JNK-1 and p38 kinase activities were induced with 15-min TNFα treatment (Fig. 8, lane 2). The level of JNK-1 activity was somewhat enhanced in cells treated with TNFα in the presence of compound 1 (Fig. 8, lanes 2 and 3). Notably, there was a significant increase in the activity of the p38 kinase activity in cells treated with TNFα in the presence of 20 μM compound 1 (Fig. 8, lanes 2 and 3). This suggests that compound 1 stimulates the TNFα-induced p38 kinase and JNK-1 kinase activities with no detectable effect on ERK-1 kinase activity. This compound may activate signaling events upstream of p38 and JNK-1 that are distinct from the pathway activating ERK-1 (54, 55). Alternatively, the compound may inhibit a dual specificity phosphatase such as M3/6 which selectively regulates p38 and JNK-1 but not ERK-1 (56). Taken together, our Western blot and immunoprecipitation kinase assays suggest that the compound does not act as a global inhibitor of TNFα-induced phosphorylation events but selectively inhibits phosphorylation of IkBα.

Anti-inflammatory Actions of Compound 2—Compound 2 was evaluated in two in vivo models of inflammation. As shown in Fig. 10, compound 2 demonstrated a dose-dependent reduction in swelling in the rat carrageenan paw model. Compound 2 was also evaluated in established rat adjuvant arthritis (Fig. 11). In the vehicle-treated control group, the mean volume of both hind paws increased by 0.39 ± 0.15 ml. Compound 2,

![Image](78x600 to 278x729)

**Fig. 6. Irreversible stabilization of IκBα.** Top panel, lanes 1–6, Western blot analysis of IκBα levels in cytoplasmic extracts from HUVEC pretreated with the indicated concentrations of compound 1 for 1 h and then induced with 100 units/ml TNFα for 15 min are shown. Lane 7, HUVEC were pretreated with compound 1 (20 μM) for 1 h and then incubated with media alone for 1 h prior to induction with 100 units/ml TNFα for 1 h in the absence of compound 1. Lane 8, compound 1 (20 μM) was added after the 15 min treatment with TNFα, immediately prior to harvesting cells. Bottom panel, the same extracts from top panel were assayed for levels of p38 protein to normalize extracts.

![Image](351x622 to 521x729)

**Fig. 7. No effect on constitutive phosphorylation of IκBα.** Whole cell extracts were assayed by in gel kinase procedure as described under “Experimental Procedures.” Lane 1, untreated HUVEC; lane 2, HUVEC treated with TNFα (100 units/ml, 15 min). Lane 3, HUVEC pretreated with compound 1 (20 μM, 1 h) and TNFα (100 units/ml, 15 min) were fractionated on 10% SDS gels containing 0.5 mg/ml HIS-IκBα.

![Image](346x611 to 528x729)

**Fig. 8. Effects on TNFα induction of MAP kinases: JNK1, p38, and ERK1.** Immunoprecipitation, kinase assays on whole cell extracts. Lane 1, untreated; lane 2, HUVEC treated with TNFα (100 units/ml, 15 min); lane 3, HUVEC pretreated with compound 1 (20 μM, 1 h) and TNFα (100 units/ml, 15 min).
given intraperitoneally at 20 mg/kg, but not at 5 mg/kg, significantly reduced the mean paw edema of the rats, to levels similar to those observed with the positive control, dexamethasone, at 1 mg/kg intraperitoneally. Thus, this compound acted as an anti-inflammatory agent in both the rat carrageenan paw edema assay and in a rat adjuvant arthritis model. These studies suggest that novel pharmacologic agents that inhibit cytokine-inducible phosphorylation of IkB can act as anti-inflammatory agents.

The precise molecular target for these agents is not yet clear. While these drugs were shown to inhibit IkB-α phosphorylation, this may be the result of direct inhibition of a TNFα-inducible IkB-α kinase or due to inhibition of a signaling event upstream of the IkB-α kinase. Alternatively, the regulation of IkB-α phosphorylation involves cellular phosphatase activities that may be activated by these drugs (19). Once the TNFα-inducible IkB-α kinase(s) and regulatory phosphatase(s) are identified, it will be interesting to determine if these molecules are the direct target of compound 1 or 2. It has been observed that upstream activators of the MAP kinase pathway can induce NF-κB/IκB-α signaling, suggesting that the MAP kinase and NF-κB cascades share some common intermediates (57–61). Since TNFα signaling of MAP kinases was not inhibited by compound 1, the target for this drug is likely to be downstream of the events that are common to both NF-κB and MAP kinase signaling pathways. Recent reports have suggested that the TNFα signaling of NF-κB may occur by a ceramide-dependent mechanism, whereas the TNFα signaling of p38 and JNK-1 kinases in endothelial cells may be ceramide-independent (62). Therefore, ceramide-dependent protein kinases (63) and/or ceramide-dependent protein phosphatases (64) may be potential targets of the drug action.

In endothelial cells, cytokines increase superoxide anion production (30, 65), and reactive oxygen intermediates may act as an important regulator of NF-κB (reviewed in Ref. 66). A number of antioxidants have been reported to inhibit cytokine-induced IkB-α phosphorylation, nuclear translocation of NF-κB, and NF-κB-dependent transcription of VCAM-1 (24, 30, 67–70). However, it has been suggested that the expression of ICAM-1 and E-selectin may be less affected by antioxidants...
(30, 70, 71). The potential of the novel compounds described in this study to act as antioxidants by inhibiting the generation of reactive oxygen intermediates or by scavenging free radicals has not been evaluated.

Protein tyrosine kinase inhibitors have been shown to block phosphorylation of IκB-α and adhesion molecule expression. Recent reports suggest that reactive oxygen intermediates activate NF-κB by a tyrosine kinase-dependent mechanism (29, 72). It is possible that the compounds described in this study could act to inhibit a specific protein tyrosine kinase that is upstream of the IκB-α kinase; however, it does not appear that these compounds act as globally active tyrosine kinase inhibitors since we observed an increase in phosphotyrosine activity as measured by Western blot with anti-phosphotyrosine antibodies. In addition, these compounds activated tyrosine phosphorylation of p38 as measured by Western blot with phosphospecific antisera and stimulated p38 and JNK-1 activities.

Recent reports suggest that reactive oxygen intermediates act to inhibit a specific protein tyrosine kinase that is important mediators of inflammation in a variety of cell types. Understanding the mechanism by which these agents disrupt these molecules including ischemia, reperfusion injury, asthma, transplantation, inflammatory bowel disease, rheumatoid arthritis, and atherosclerosis.

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REFERENCES

1. Springer, T. (1995) Annu. Rev. Physiol. 57, 827–872
2. Pober, J. S., and Cotran, R. S. (1990) Physiol. Rev. 70, 427–451
3. Carlos, T. M., and Harlan, J. M. (1994) Blood 84, 2068–2101
4. Wertheimer, S. J., Gualberto, A., Jewell, C. M., Cidlowski, J. A., and Baldwin, A. S. J. (1995) Mol. Cell. Biol. 15, 943–953
5. Richter, S. U. K. A., and Tolsis, A. K. J. (1989) US Patent 5,106,186
6. Gimbrone, M. (1976) in Progress in Hemostasis and Thrombosis (Spaet, T., ed) Vol. 3, pp. 1–28, Grune and Stratton, Inc., New York
7. Denizot, F., and Lang, R. (1986) J. Immunol. Methods 89, 271–277
8. Luccioni, F. W., Brock, A. F., Arnaout, M. A., and Gimbrone, M. A. (1989) J. Immunol. 142, 2257–2262
9. Sen, R. K., and Baltimore, D. (1995) Cell 66, 705–716
10. Raingeaud, J. S., Gupta, S., Rogers, J. S., Dickens, M., Ulevitch, R. J., and Davis, R. J. (1995) J. Biol. Chem. 270, 7420–7426
11. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Genes Dev. 7, 2135–2148
12. Gerrasen, M. E., Carley, W. W., Ranges, G. E., Shen, C.-P., Phan, S. A., Ligon, G. L., and Perry, C. A. (1995) Am. J. Pathol. 147, 278–292
13. Alkalay, I., Yaron, A., Hatakeyama, A., Arnaout, M. A., Gerlic, O., Pashut-Lavon, I., and Ben-Neriah, Y. (1995) Mol. Cell. Biol. 15, 1294–1301
14. McElhenny, J. A., Trushin, S. A., Bren, G. D., Chester, N., and Paya, C. V. (1996) Mol. Cell. Biol. 16, 899–906
15. Barrego, C. F., Stevenson, K. M., Schwart, E. M., and Verma, I. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7637–7641
16. Schwarz, R. M., Antwerp, D. V., and Verma, I. M. (1996) Mol. Cell. Biol. 16, 3554–3559
17. Bennett, B. L., Lacson, B. G., Chen, C. C., Cruz, R., Wheeler, J. S., Kletzien, R. F., Tomasselli, A. G., Heinrikson, R. L., and Manning, A. M. (1996) J. Biol. Chem. 271, 9685–9688
18. Derijard, B., Hibi, M., Wu, J.-H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037
19. Han, J., Lee, J.-D., Bibbs, L., and Ulevitch, R. J. (1994) Science 265, 808–811
20. Lee, C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., and Landwetter, S. W. (1994) Nature 372, 739–746
21. Kyratsios, J. M., Baeuerle, P., Nicolakis, E., Dai, T., Ruble, E., Ahmad, M. F., Verruch, J., and Woodgett, J. R. (1994) Nature 360, 595–599
22. Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994) Cell 78, 1027–1037
23. Sluss, H., Barrett, T., Derijard, B., and Davis, R. (1994) Mol. Cell. Biol. 14, 3876–3884
24. Davis, R. J. (1994) Trends Biochem. Sci. 19, 470–473
25. Raingeaud, J., Whitman, M. A., Barrett, T., Derijard, B., and Davis, R. J. (1994) Mol. Cell. Biol. 14, 1247–1255
26. Muda, M., Theodosiou, A., Rodrigues, N., Boschart, U., Camps, M., Gillieron, C., Davies, K., Ashworth, A., and Arkinstall, S. (1996) J. Biol. Chem. 271, 37205–37208
27. Read, M. A., Whitley, M., Collins, T., and Davis, R. J. (1994) J. Exp. Med. 179, 503–512
28. Schindler, U., and Bächler, V. R. (1994) Mol. Cell. Biol. 14, 5820–5831
29. Rodebush, M., Thanos, D., Read, M., Maniatis, T., and Collins, T. (1994) Mol. Cell. Biol. 14, 6440–6447
30. Hsu, J., Bächler, V., and Cao, Z. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11641–11645
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63. Liu, J., Mathias, S., Yang, Z., and Kolesnick, R. N. (1994) J. Biol. Chem. 269, 3047–3052
64. Hannun, Y. A. (1994) J. Biol. Chem. 269, 3125–3128
65. Matsubara, T., and Ziff, M. (1986) J. Immunol. 137, 3295–3298
66. Collins, T. (1993) Lab. Invest. 68, 499–508
67. Schreck, R., Rieber, P., and Baeuerle, P. (1991) EMBO J. 10, 2247–2258
68. Collins, T., Palmer, H., Whitley, M., and Neish, A. (1993) Trends Cardiovasc. Med. 3, 16–21
69. Finco, T., Beg, A., and Baldwin, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11884–11888
70. Marui, N., Offerman, M., Swerlick, R., Kunsch, C., Rosen, C., Ahmad, M., Alexander, R., and Medford, R. (1993) J. Clin. Invest. 92, 1866–1874
71. Orthner, C. L., Rodgers, G. M., and Fitzgerald, L. A. (1995) Blood 86, 436–443
72. Schieven, G., Kirihara, J., Myers, D., Ledbetter, J., and Uckun, F. (1993) Blood 82, 1212–1220
73. Singh, S., and Aggarwal, B. B. (1995) J. Biol. Chem. 270, 10631–10639
74. Menon, S. D., Guy, G. R., and Tan, Y. H. (1995) J. Biol. Chem. 270, 18881–18887
75. Iigo, Y., Takashi, T., and Tanatini, T. (1991) J. Immunol. 147, 4167–4171