A Small Molecule Ubiquitination Inhibitor Blocks NF-κB-dependent Cytokine Expression in Cells and Rats*

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A small molecule inhibitor of NF-κB-dependent cytokine expression was discovered that blocked tumor necrosis factor (TNF) α-induced IkBα degradation in MM6 cells but not the degradation of β-catenin in Jurkat cells. Ro106-9920 blocked lipopolysaccharide (LPS)-dependent expression of TNFα, interleukin-1β, and interleukin-6 in fresh human peripheral blood mononuclear cells with IC50 values below 1 μM. Ro106-9920 also blocked TNFα production in a dose-dependent manner following oral administration in two acute models of inflammation (air pouch and LPS challenge). Ro106-9920 was observed to inhibit an ubiquitination activity that does not require βTRCP but associates with IkBα and will ubiquitinate IkBα S32E,S36E (IkBoee) specifically at lysine 21 or 22. Ro106-9920 was identified in a cell-free system as a time-dependent inhibitor of IkBoee ubiquitination with an IC50 value of 2.3 ± 0.09 μM. The ubiquitin E3 ligase activity is inhibited by cysteine-alkylating reagents, supported by E2UBCH7, and requires cIAP2 or a cIAP2-associated protein for activity. These activities are inconsistent with what has been reported for SCFTRCP, the putative E3 for IkBoee ubiquitination. Ro106-9920 was observed to be selective for IkBoee ubiquitination over the ubiquitin-activating enzyme (E1), E2UBCH7, nonspecific ubiquitination of cellular proteins, and 97 other molecular targets. We propose that Ro106-9920 selectively inhibits an uncharacterized but essential ubiquitination activity associated with LPS and TNFα-induced IkBoee degradation and NF-κB activation.

NF-κB is a heterodimeric transcription factor that regulates the transcription of many important mediators of inflammation including TNFα, IL-1β, IL-8, MCP-1, E selectin, ICAM-1, VCAM-1, MMP-1, and MMP-3 (1). Evidence is accumulating to suggest that blocking NF-κB will be an effective means to treat inflammatory diseases such as asthma, rheumatoid arthritis, and Crohn’s disease. Modulation of inflammatory diseases by blocking the action of NF-κB-dependent inflammatory mediators has been demonstrated with anti-TNFα therapies. Etanercept and Infliximab are efficacious for both rheumatoid arthritis and Crohn’s disease (2–4). Transrepression of NF-κB activation by glucocorticoids bound to the glucocorticoid receptor is proposed to contribute to the anti-inflammatory properties of glucocorticoids in asthma and arthritis (5, 6). Strategies that directly block NF-κB action have also shown efficacy in preclinical studies. Overexpression of IkBo inhibits both inflammatory and destructive mechanisms in rheumatoid synovium but spares anti-inflammatory mediators (7). NF-κB antisense has been effective in animal models of rheumatoid arthritis (8) and Crohn’s disease (9). NF-κB has also been shown to be important for osteoclast differentiation, leading to the suggestion that inhibitors of NF-κB may hold therapeutic potential in the treatment of osteoporosis and other bone diseases (10).

Transcriptional activation by NF-κB requires translocation of NF-κB from the cytoplasm to the nucleus (1, 11–13). The translocation into the nucleus is controlled by IkBo. IkBo binds to NF-κB in the cytoplasm and masks the NF-κB nuclear localization signal. NF-κB activation is initiated by many extracellular molecules, including LPS, TNFα, and IL-1β, through binding to their respective cell surface receptors. The binding initiates a signaling cascade that leads to the activation of the IkB kinase complex containing IKKα and IKKβ. Activation of the IKK complex requires phosphorylation of two serine residues located in the “activation loop” within the kinase domain of IKKα or IKKβ. Certain mitogen-activated protein 3-kinases (MEKK1, MEKK2, MEKK3, and NF-κB inducing kinase) are capable of phosphorylating these serines in vitro and activating NF-κB in transfection experiments. The activated IKK complex phosphorylates IkBo at serines 32 and 36. The phosphorylated IkBo is subsequently polyubiquitinated at lysine 21 or 22 and degraded by the proteasome. NF-κB is then released to translocate to the nucleus and promote gene expression.

The goal of our work was to block NF-κB-dependent gene expression via stabilization of the endogenous NF-κB-IkBα complex. Stabilization has been achieved in cells with mutations that prevent the phosphorylation at serines 32 and 36 (14–16), in cells with mutations that prevent the ubiquitination at lysines 21 or 22 (17, 18), or by inhibition of IkBo degradation by the proteasome (19, 20). Our approach was to target the ubiquitination of IkBo. Ubiquitination in general requires three enzymes: the ubiquitin-activating enzyme (E1), one of the multiple E2 ubiquitin-conjugating enzymes, and a ubiquitin E3 ligase (21–24). The ubiquitination of IkBo has been associated with a number of E2s including UBCH5b, UBCH5c, and CDC34/UBC3 (24) and an SCF E3 ligase complex in which IkBo binds to the F-box subunit known as βTRCP (25–27).

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‡ The abbreviations used are: TNF, tumor necrosis factor; IL, interleukin; LPS, lipopolysaccharide; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin ligase; DTT, dithiothreitol; PBMN, peripheral blood mononuclear cell; ELISA, enzyme-linked immunosorbent assay; MM6, Monomoae 6.
The work described in this report was initiated prior to the identification of IKK as the IkB kinase or SCF\(^{\text{IKK}}\) as the IkB ubiquitin ligase. Our approach utilized a phosphomimetic substrate of IkB (IκBα) to identify an active fraction in Jurkat cell lysates. The reconstituted fraction catalyzed IkB ubiquitination with the appropriate substrate selectivity and was used to identify inhibitors of IkB ubiquitination. The screening paradigm identified a number of inhibitors including Ro106-9920 that demonstrated the functional activity expected for an inhibitor of IkB ubiquitination. With the subsequent discovery of the SCF\(^{\text{IKK}}\) C installation of ly-Bspmid DNA was used as template in the PCR reaction to introduce the respective forward and reverse primers for UBCH5a (5'-GCG GGG GCC CTC C-3') and UBCH7 was also cloned into a pET30 vector. UBCH5a and UBCH7 were PCR-amplified from a human leukemia Jurkat cell 5' stretch cdNA library (CLONTECH, Palo Alto, CA) with the respective forward and reverse primers for UBCH5a (5'-CTA TGG ATC ACC CGC GTG AAG AGG ATT C-3') and UBCH7 (5’-CTA TGG ATC ACC CGC GTG AAG AGG AGG CTTG-3’). Both E2s were cloned into the NcoI site of pET30 vector. UBCH7 was also cloned into a NcoI site of the pET11. The resulting plasmids were transformed into BL21 (DE3) competent Escherichia coli cells (Novagen) for protein expression.

**Materials and Methods**

Mutagenesis, Cloning, and Expression—The IkBα coding sequence was cloned into the BamHI site of both the pET11 vector and the pET30 vector with an N-terminal His tag (NOVAGEN, Madison, WI). The pET11 IkBα was mutagenized following the USE mutagenesis method (Amersham Biosciences). The mutagenesis of serine 32 and 36 into glutamic acid (IkBα) was obtained with the mutagenic primer 5'-GCT GTC TTT CAT ctc GTC CAG GCC ttc GTC GTG GCG GTC GTC-3'. The subsequent mutagenesis of ly-Bspmid DNA was used as template in the PCR reaction to introduce the respective forward and reverse primers for UBCH5a (5’-GCG GGG GCC CTC C-3') and UBCH7 were PCR-amplified from a human leukemia Jurkat cell 5' stretch cdNA library (CLONTECH, Palo Alto, CA) with the respective forward and reverse primers for UBCH5a (5’-CTA TGG ATC ACC CGC GTG AAG AGG ATT C-3') and UBCH7 (5’-CTA TGG ATC ACC CGC GTG AAG AGG AGG CTTG-3’). Both E2s were cloned into the NcoI site of pET30 vector. UBCH7 was also cloned into a NcoI site of the pET11. The resulting plasmids were transformed into BL21 (DE3) competent Escherichia coli cells (Novagen) for protein expression.

**Protein Purification—**E. coli expressing His-tagged proteins were lysed with a Gaulin homogenizer (AVP Gaulin, Everett, MA) in 20 mM Tris, pH 8.0, 10% glycerol, 1% E2 UBCH7, 2 μg of E3, and 0.1 μg of E1, 0.5 μg of E2, and 1 μg of E3. The 125I-ubiquitin-IκBα was determined in γ-counter.

**E3 Immunodepletion—**The E3 fractions (20 μg) were incubated at 4°C with purified antibody (or rabbit serum) (2 μg) and 30 μl of prewashed protein G-Sepharose (Amersham Biosciences). After 24 h, the reactions were spun down at 1,500 rpm in a Sorvall H6000. The cleared E3 lysate was washed. The Sepharose beads were washed with 50 μl of phosphate-buffered saline and spun again. Fifty μl of wash solution was added to the sample and shaken for 1 h at room temperature. The supernatant was removed after centrifugation, and the beads were washed (four times) with 1 ml of 1% Nonidet P-40 in 60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9. The product was eluted with 20 μl of 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and the amount of 125I-ubiquitin-IκBα was determined in γ-counter.

**Equilibrium and Quantiﬁed Fluorescence and Assay—**The equilibrium and quantified fluorescence assay was conducted as reported by Gan et al. Black, high binding, 96-well plates (Greiner) were coated with 1 μg/ml of mouse anti-IκBα or rabbit serum (2 μg) and 30 μl of prewashed protein G-Sepharose (Amersham Biosciences). The europium signal was measured using a Victor 1420 Multilabel Counter (EG & G Wallac) following the addition of 100 μM of the enhancement solution (EG & G Wallac).

**E1/E2 Counterscreen—**The reactions were preincubated for 15 min at room temperature in 10 mM Hepes, pH 7.5, containing 0.8 μg of E1, 3 μg of E2, 30 μg of E3, 50 μl of binding buffer, and a mixture of ubiquitin and 125I-ubiquitin-IκBα in 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.05% Tween 20, washed twice with 20 mM Hepes, pH 7.5, 140 mM NaCl, 0.05% Tween 20, and washed once with 10 mM Tris, pH 7.5. Typically for IC\(_{50}\)_ determinations each well contained 0.2 μM of E1, 1 μM of E2, 1 μM of E3, and 0.05 μM of 125I-ubiquitin-IκBα in 25 mM Tris-HCl buffer, pH 7.5, containing 5 mM MgCl₂, 0.5 mM DTT, 1 mM ATP, 5 mM creatine phosphate, and 1 millimicron creatine phosphokinase. The 1-h incubation at 37°C was initiated with E3, stopped by aspiration, and washed with DELFIA wash buffer four times (EG & G Wall.ac).

**Nonspecific Ubiquitination Assay—**Non-specific ubiquitination was measured as the formation of ubiquitin conjugates from the S-100 fraction of Jurkat cell lysates. Jurkat S100 lysate (5 μg) was combined with 0.125 μl of E3, 3 μg of E2, 2 μg of E1, 1 μM of ubiquitin and 20 min with 5 μM urea (50 μl). The 125I-ubiquitin-(E2-His\(_6\))-thioesters were bound to nickel beads, washed, and counted as above.

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ubiquitin. Following 1 h of incubation at 37 °C, the reactions were stopped with 50 μl of Laemmli SDS sample buffer, and the samples were electrophoresed on 10% Tris-Glycine gels (Novex) and visualized with autoradiography.

**Cytokine Production in Human Peripheral Blood Mononuclear Cells (PBMCs)—**Mononuclear cells were isolated from the fresh venous blood of human volunteers. The cells were washed three times with phosphate-buffered saline and resuspended at 1–5 × 10^6 cells/ml in RPMI 1640 medium containing 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. The cells were plated at 1 ml/well in 12-well plates and allowed to adhere for 90 min at 37 °C. The wells were then washed twice to remove nonadherent cells, before the addition of 1.5 ml of medium (RPMI 1640 containing 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate and 10% fetal calf serum) with or without various concentrations of the test compounds or solvent control (Me2SO). After 30 min, LPS was added to a final concentration of 1 ng/ml, and the cultures were incubated for an additional 3 h. The medium was then collected and frozen at −70 °C. Cytokine levels in the medium were subsequently measured using ELISA kits from PharMingen (San Diego, CA) (human TNFα and IL-6) and R & D Systems (Minneapolis, MN) (human IL-1β and IL-1ra). Compound cytotoxicity was assessed in parallel cultures, plated in 96-well dishes (100 μl/well), by the addition of 100 μl of a solution of 4 μM propidium iodide (Molecular Probes) in phosphate-buffered saline to the cells at the end of the assay. The cultures were then analyzed in a fluorescence plate reader; increases in the signal correlated with propidium-DNA interactions, an indication that the cytoplasmic membrane was no longer intact.

**IκBα Degradation Assay—**MM6 cells (1.5 × 10^5) were preincubated for 30 min with the inhibitor at 37 °C. The reaction was started by the addition of 10 μg/ml cycloheximide in Me2SO and 20 ng/ml TNFα (Promega). Following a 1-h incubation the cells were separated from the medium, resuspended in RIPA buffer (Roche Molecular Biochemicals), and broken by freezing and thawing three times at −80 °C. After centrifugation the supernatants were electrophoresed on 10% Tris-glycine gels, and the proteins were electrotransferred onto nitrocellulose paper. The Western blots were probed with a mouse antibody to IκBα (C-21, Santa Cruz Biotechnology) and detected with rabbit anti-mouse IgG horseradish peroxidase conjugate.

**β-Catenin Accumulation Assay—**Jurkat cells were incubated at 37 °C with inhibitors for 24 h and then were washed, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet, 0.5% sodium deoxycholate, 0.1% SDS, and complete protease inhibitor (Roche Molecular Biochemicals)), frozen and thawed three times, and the supernatant was analyzed for β-catenin by ELISA. β-Catenin was captured with a mouse anti-β-catenin antibody diluted 1:400 (Zymed Lab, sc-7199) diluted 1:1250, coupled to streptavidin-horseradish peroxidase conjugate (PharMingen).

**Animal Studies—**Hanover Wistar rats (Charles River Laboratories, Hollister, CA) were acclimated for 1 week prior to the initiation of experiments, at which time they weighed 165–205 g (males) or 90–120 g (females). Ro106-9920 was suspended in vehicle containing 0.9% NaCl, 0.5% sodium carboxymethylcellulose (type 7L; Aqualon, Wilmington, DE), 0.4% polysorbate 80, and 0.9% benzyl alcohol. Carrageenan air pouches were created by the subcutaneous injection of air (Hollister, CA) were acclimated for 1 week prior to the initiation of experiments, at which time they weighed 165–205 g (males) or 90–120 g (females). Ro106-9920 was suspended in vehicle containing 0.9% NaCl, 0.5% sodium carboxymethylcellulose (type 7L; Aqualon, Wilmington, DE), 0.4% polysorbate 80, and 0.9% benzyl alcohol. Carrageenan air pouches were created by the subcutaneous injection of air (Hollister, CA) was injected into the intracapsular area of the back on day 0. 1.5 h later. Vehicle or Ro106-9920 was orally administered 30 min prior to LPS challenge. After euthanasia, the blood was harvested via carotidcisaion, and the serum was isolated using serum separator tubes. The serum was stored frozen at −80 °C until ELISA analysis.

**Miscellaneous—**E1 was purified from rabbit liver according to the method of Haas and Bright (30). The phosphorilated peptide was synthesized by reported procedures (31, 32). Protein concentrations were determined by the Bradford method (Pierce). The antibodies used in this work were NEMO (sc-8330), SKP-1 (sc-7163), cIAP2 (sc-7944), IKKβ (sc-7607), and βTRCP (sc-8862) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); cullin-1 (rb-042) from Neomarkers ( Fremont, CA); cdc34 from Zymed Laboratories Inc. (San Francisco, CA); and nedd8 (210–194–R200) and sentrin (210–174–R200) from Alexis (San Diego, CA). Anti-rabbit βTRCP was custom prepared by Multiple Peptide Systems (San Diego, CA) to a peptide from the middle of the protein (CYTDDQIVGSLZDNKIKWD).

**RESULTS**

**Development and Characterization of Discovery Tools**

**E3 Fractionation—**The first step in efforts to discover inhibitors of IκBα ubiquitination was to develop a screening assay suitable to identify leads. IκBα ubiquitination requires phosphorylation of serines 32 and 36 and ubiquitination at lysine 38. To circumvent the need for phosphorylation, we engineered a protein in which serines 32 and 36 were mutated to glutamic acid. This protein was expressed with an N-terminal His6 tag, purified from E. coli and used in the presence of ATP, ubiquitin, rabbit E1, and human E2UBCH7 to screen protein fractions for an activity that would catalyze the formation of IκBα-ubiquitin conjugates. Jurkat cells were treated with TNFα for 5 min to degrade endogenous IκBα and lysed, and the S100 supernatant was fractionated over DE52 and DEAE. The activity eluted from the DEAE column in a sharp peak at ~0.22 M KCl (Fig. 1), immediately following E1. Western blots were run prior to pooling to ensure that E1 did not contaminate the fractions containing the E3 activity. The fractions from each peak were pooled and precipitated with 10% ammonium sulfate. Thioester assays showed that there were no detectable E1 or E2s in the final E3 fraction. Western blots showed the absence of endogenous IκBα (data not shown). Further attempts to purify the pooled activity resulted in a complete loss of activity.

**Substrate Specificity—**Validation of an IκBα ubiquitination enzyme assay requires a dependence upon phosphorylation of serines 32 and 36, ubiquitination at lysine 21 or 22, and the
formation of polyubiquitin chains (17, 18, 33). To this end we engineered, expressed, and purified His<sub>6</sub>-tagged wild type IκBα and IκBα S32E,S36E,K21R,K22R (IκBαerr). Neither wtIκBα nor IκBαerr were as efficient ubiquitination substrates for this system as IκBα (Table I). However, both wtIκBα and IκBαerr were inhibitors of IκBα ubiquitination with IC<sub>50</sub> values of 3.0 and 0.6 μM, respectively.

**E3 Characterization**—Subsequent to the completion of this work the identity of the IκBα E3 was discovered. The E3 was identified as a SCF type E3 with βTRCP as the F-box component (25–27). βTRCP has also been shown to be the F-box E3 component required for β-catenin ubiquitination (34, 35). βTRCP was detected in the batch DE52 flow-through and a 100 mM KC1 wash using a custom C-terminal peptide antibody and a N-terminal peptide antibody purchased from Santa Cruz Biotechnology. It was not detected in the 250 mM KC1 wash that contained the IκBα ubiquitination activity. The fractions that supported IκBα activity were analyzed by Western blot for the components of the E3 SCF<sup>βTRCP</sup> complex. Cul1 was observed in the fractions with IκBα ubiquitination activity (Fig. 1). However, βTRCP was not detected with either of the two antibodies. Cul1 showed two bands consistent with reports of neddylation (36). From these data we conclude that SCF<sup>βTRCP</sup> is not the E3 catalyzing the ubiquitination of IκBα.

Efforts to characterize the source of the E3 activity by Western blots showed cIAP2 to elute in the active fractions (Fig. 1). NEMO (IKKγ) was also detected in pooled E3 fraction. Cdc34, sentrin, and nedd8 were not detected. His-tagged IκBα pull down experiments also showed the presence of NEMO and IKKβ (data not shown). Immunodepletion of the E3 fraction with anti-cIAP2 decreased the activity to 31 ± 4.9% (n = 6) of fractions immunodepleted with rabbit serum. No decrease in activity was observed with immunodepletion using antibodies to TRAF6, TRAF2, IKKβ, IKKα, Skp1, NEMO, and MDM2. However, the addition of recombinant N-His<sub>6</sub>-tagged, purified cIAP2 to the reconstituted system in the presence or absence of the E3 fraction did not result in an increase in IκBα ubiquitination activity (data not shown). One caveat to the observation is that we did not verify that the expressed and purified cIAP2 was functionally active. In summary, we found that the IκBα ubiquitination activity co-purified with Cul1, Skp1, NEMO, and cIAP2 but not with βTRCP and required the presence of cIAP2 or a protein associated with cIAP2.

**Reaction Properties**—Reconstitution of IκBα ubiquitination was absolutely dependent on the partially purified E3, purified recombinant human E2 UBC17, purified rabbit E1, ubiquitin, ATP, and IκBα. The reaction was linear with time for over 60 min when initiated with E1 and E2 precharged with ubiquitin (Fig. 2). A lag in the initial velocity (~5 min) was observed when the complete reaction was initiated with ATP. The reaction was saturable with ubiquitin, E1, E2, and IκBα. The apparent binding constants for ubiquitin, E1, E2 UBC17, and IκBα, as determined by the saturation binding effect on IκBα ubiquitination, were 1.3 ± 0.28 μM, 2.6 ± 0.8 nM, 2.8 ± 0.44 μM, and 1.8 ± 0.28 μM, respectively. The binding constants were determined by fitting to the Michaelis-Menten equation. The apparent K<sub>m</sub> for IκBα ubiquitination of 1.8 μM is similar to the IC<sub>50</sub> values for wtIκBα and IκBαerr of 3.0 and 0.6 μM, respectively, and suggests that IκBα, wtIκBα, and IκBαerr all bind to the E3 with similar affinity. Therefore, the phosphomimetic glutamic acids at positions 32 and 36 in IκBα must increase the activity through an effect on k<sub>catalytic</sub>. From these results we conclude that the IκBα association with the E3 is independent of the phosphorylation state of serines 32 and 36.

The rates of IκBα ubiquitination were quantitated by the amount of labeled ubiquitin associated with IκBα bound to nickel beads (Table I). The nickel bead bound IκBα could also be separated by SDS gel electrophoresis and visualized by autoradiography. Fig. 3 shows the formation of high molecular mass IκBα-ubiquitin conjugates and the inhibition by a 14-mer phosphopeptide containing the IκBα ubiquitination recognition sequence. The high molecular mass bands >250 kDa and the smear between 30 and 250 kDa were excised from the gel and counted with scintillation spectroscopy. The percentage of inhibition by the peptide was similar to that obtained with the nickel bead assay. Fig. 3 is shown to emphasize 1) that the system catalyzes the polyubiquitination of IκBα, 2) that it is blocked in a dose-dependent manner by a peptide with the IκBα degradation sequence (IC<sub>50</sub> = 30 μM), and 3) that the quantitative data obtained with the nickel bead assay is consistent with the data observed on the SDS gels.

We investigated the importance of cysteine to the functional activity by incubating the E3 fraction in the presence of N-ethylmaleimide and a cysteine reactive α-chloroketone diglycine peptide. The peptide was synthesized to mimic the C terminus of ubiquitin. We found that 100 μM of N-ethylmaleimide or the α-chloroketone blocked the activity when preincubated for 30 min with the E3 (Fig. 4). To ensure that the activity was not against the E1 or E2 components, DTT (5 mM) was added to quench the reaction prior to reconstitution. There was minimal loss of activity of E3-dependent activity when 5 mM DTT was preincubated with 300 μM of both compounds prior to addition of E3. These data show that the E3 activity is sensitive to cysteine alkylation reagents.

**E2 Specificity**—We evaluated three purified E2s (UBCH7, UBCH5a, and atUBC8) for their ability to support substrate-specific IκBα ubiquitination. All three E2s showed selectivity for ubiquitination of IκBα over wtIκBα (Table II). The selectivity was determined at saturating concentrations of the E2a. The overall activity was 4-fold greater for UBCH5a and atUBC8 as compared with UBCH7. However, the E3-independen-
ent ubiquitination (E1/E2 ubiquitination) was greater for UBCH5a and atUBC8 than UBCH7. UBCH7 was used in all subsequent studies because of its lower E3-independent, non-specific ubiquitination activity, which resulted in a lower background. We also observed that UBCH5a and UBCH7 with N-terminal His6 tags were unable to transfer ubiquitin to proteins in Jurkat cell lysates or to ubiquitinate IκBα, even though these E2s were able to form thioesters in the presence of E1, ATP and ubiquitin (data not shown). This is consistent with the data of Sullivan and Vierstra (37), which showed that the E2 N-terminal domain likely contains the E1-binding site. Therefore, nontagged E2 UBCH7 was used in all of the subsequent E3-dependent reactions.

The reaction products were isolated with nickel beads and either directly counted or run on a 14% Tris-glycine SDS-PAGE. The bands were excised, and the amount of radioactivity was quantitated.

The reaction was reacted with N-ethylmaleimide or the α-chloroketone-diglycine peptide for 15 min prior to quenching the reaction with 5 mM DTT. The E3 activity was then reconstituted as described under "Materials and Methods." As a control 5 mM DDT was added to 300 μM of inhibitor prior to addition to the E3 (open circle and open square).

Table II

| E2   | Relative rates | Selectivity |
|------|----------------|-------------|
|      | Specific       | Nonspecific |
|      | IκBα/ubq      | IκBα with E3/ubq |
| UBCH7| 1.0           | 15.2        |
| UBC5Ha| 4.8            | 2.3         |
| atUBC8| 3.8             | 1.9         |

**Discovery Paradigm**

The cell-free IκBα ubiquitin system was configured in a format suitable for time-resolved fluorescence spectroscopy to facilitate high throughput screening. The system catalyzed the addition of europium-labeled ubiquitin to IκBα bound to a 96-well plate. Compounds that inhibited that reaction were counter-screened for their ability to block the formation of His6-tagged UBCH7-thioesters. Compounds that inhibited E2 UBCH7 ubiquitin thioester formation were not studied further. Ro106-9920 (Fig. 5) was identified using the screening paradigm as an inhibitor of IκBα ubiquitination with an IC50 value of 2.30 ± 0.09 μM, n = 95 (Table III). It did not inhibit the formation of E1 or E2 thioesters or nonspecific ubiquitination of cellular proteins at concentrations up to 100 μM. The sulfone analog of Ro106-9920 (compound 1) had similar activity (IC50 = 6.9 μM), whereas the sulfide (compound 3) was inactive at 80 μM. The benzimidazole and tetrazole (compound 4) analogs were inactive, as was a ring open phenylazide analog (compound 5). The 4’-chlorophenyl analog (compound 2) had activity similar to that of Ro106-9920 (IC50 = 2.88 μM).

The key to developing an assay that was useful for discovering E3-selective compounds was to find the conditions where the E3 was rate-limiting. These conditions were achieved by increasing the concentrations of E1 and E2 UBCH7 to saturation and maximizing the ubiquitin concentration so that its consumption did not become rate-limiting. Under the conditions with which the reaction was run, E2 UBCH7 could be decreased by as much as 70% without dramatically influencing the overall rate. These conditions biased the reaction toward E3-dependent ubiquitination by increasing the window between inhibition of E1/E2- and E3-dependent ubiquitination. Another important factor was maintaining a good signal to noise ratio, which was defined as the ubiquitination in the absence versus presence of IκBα, or the comparison of IκBα ubiquitination with wtIκBα ubiquitination. The signal to noise ratio was solely dependent upon the E3 concentration. The amount of nonspecific ubiquitination increased at the expense of specific ubiquitination when the E3 concentration or the specific activity of the E3 preparation was too low. The relative increase in nonspecific ubiquitination decreased the signal to noise ratio and also resulted in an apparent upward shift of IC50 values of the E3-specific inhibitors. The shift in the IC50 proved to be the most robust measurement of the integrity of system. We believe that the decrease in the signal to noise ratio resulted in an apparent shift of the IC50 values, which in turn is due to the ability of the E2 to nonspecifically ubiquitinate other proteins present in the reaction. The increase in nonspe-
cific ubiquitination occurs when there is inadequate E3 available to direct the E2-conjugated ubiquitin to a specific substrate.

Time-dependent inhibition was observed when Ro106-9920 was added at the start of the reaction (Fig. 6, top panel). It took 10–20 min to achieve full inhibition with 10 µM of Ro106-9920. A more detailed analysis showed Ro106-9920 to have an apparent irreversible component to its mechanism of action (Fig. 6, bottom panel). The apparent irreversibility is evident from the parallel shift in the initial velocity curves at different E3 concentrations (38). A 100-fold difference in E2 concentration (0.1–10 µM) had no effect on the IC50 (data not shown).

The functional integrity of the system was tested by the ability of the inhibitors to block IxBoe degradation and NF-κB-dependent cytokine expression in stimulated lymphocytes. IxBoe degradation in MM6 cells treated with TNFα was blocked by Ro106-9920 at concentrations above 3 µM (Fig. 7). TNFα, IL-1β, and IL-6 expression was also inhibited in human PBMN by Ro106-9920 with IC50 values of 0.7, 0.6, and 0.7 µM, respectively (Table III). Ro106-9920 was less effective against IL-1ra (IC50 = 3.0 µM). The effect of Ro106-9920 on NF-κB-dependent cytokine expression was comparable with the proteasome inhibitor, lactacystin. The IC50 values for lactacystin were 1.8 ± 1.5, 2.0 ± 1.1, 2.2 ± 0.8, and >50 µM for TNFα,
IL-1β, IL-6, and IL-1ra, respectively. The cytotoxicity of Ro106-9920 was evaluated in Jurkat cells and PBMs by propidium iodide influx. No cytotoxicity was observed in PBMs following a 3.5-h incubation at 50 μM; however, cytotoxicity was observed in Jurkat cells after 2 h of incubation with >17.5 μM of Ro106-9920. The analogs of Ro106-9920 that were active in the cell-free system were equally effective inhibitors of cytokine expression in human PBMs, whereas the inactive analogs had no effect on the cytokine profiles (Fig. 7). These data show that the inhibitors of IκBαe ubiquitination do elicit the appropriate functional outcome with respect to blocking IκBα degradation and the suppression of NF-κB-dependent gene expression.

Ro106-9920 was evaluated for its ability to prevent β-catenin degradation in Jurkat cells. β-Catenin is phosphorylated by constitutively active GSK3β. This targets β-catenin for ubiquitination by SCFβTRCP and degradation by the proteasome. Incubation of Jurkat cells with the GSK3β inhibitor SB-216763 results in an accumulation of β-catenin (39); however, Ro106-9920 had no effect in this assay (Fig. 8). From these results we conclude that Ro106-9920 does not inhibit SCFβTRCP-mediated β-catenin ubiquitination in Jurkat cells.

Ro106-9920 was evaluated for its ability to inhibit cytokine production in two models of acute inflammation in the rat, the carrageenan air pouch, and systemic LPS. Following an oral dose of 10 or 100 mg/kg, a dose-dependent inhibition in serum TNFα was observed in both models (Table III). Ro106-9920 (100 mg/kg) also caused dose-dependent suppression of prostaglandin E2 levels in the air pouch, although this effect did not reach statistical significance (Table III). No acute toxicity was observed in these short-term animal models. From these results we conclude that Ro106-9920 blocks NF-κB-dependent gene expression in rats.

The selectivity of Ro106-9920 for other molecular targets was evaluated in screening assays provided by Cerep. Ro106-9920 was screened in 74 binding assays described as “Cerep high throughput profile” and 25 enzyme assays referred to as the “Cerep enzyme profile” (Table IV). Screening at a single 10 μM concentration in duplicate showed >40% inhibition of only the epidermal growth factor tyrosine kinase (63%), 5-lipooxygenase (89%), and inducible nitric-oxide synthase (111%).

**DISCUSSION**

The strategy employed to discover molecules with anti-inflammatory properties that would inhibit the production of NF-κB-dependent cytokines such as TNFα and IL-1β was to target the ubiquitination of IκBα. The first step was to develop tools and a screening paradigm to identify lead molecules. To this end we developed a cell-free assay for E3-dependent IκBαe ubiquitination, an E2-dependent assay as a selectivity filter, and a screening paradigm that effectively identified compounds with activity in cells and animals. Ro106-9920 was discovered to inhibit IκBα ubiquitination in a cell-free assay, to prevent IκBα degradation in MM6 cells, to have no effect on the degradation of β-catenin in Jurkat cells, to block the expression of the NF-κB-dependent cytokines TNFα, IL-1β, and IL-6 in human PBMs, and to lower the circulating levels of TNFα in LPS-treated rats as well as to reduce TNFα in exudate harvested from the air pouches of carrageenan-challenged rats.

The cell-free reaction utilized a mutant form of IκBα as the ubiquitination substrate, IκBαe, and a partially purified Jurkat cell lysate containing the E3 activity. The validity of the system to reconstitute IκBα ubiquitination was concluded by showing that 1) wtIκBα and IκBαeerr were poor substrates but good inhibitors for the reaction, 2) the reaction formed polyubiquitin chains in an E3-dependent manner, 3) the reaction was inhibited by the IκB phosphopeptide, and 4) an inhibitor of the cell-free reaction, Ro106-9920, was effective in blocking IκBα degradation in cells and NF-κB-dependent cytokine expression in cells and animals.

Ro106-9920 was observed to be selective for IκBαe ubiquitination over E1, E2UBCH7 nonspecific ubiquitination of cellular proteins, and 97 other molecular targets. Although Ro106-9920 does have activity against 5-lipooxygenase and inducible nitric-oxide synthase, zileuton, an inhibitor of 5-lipooxygenase, does not inhibit TNFα production in PBMs (40), and endogenous nitric oxide decreases TNFα in rat models of hepatic ischemia-reperfusion (41).

As more information becomes available regarding the characteristics of IκBα ubiquitination, it has become apparent that there were some inconsistencies between the biochemical properties of IκBαe ubiquitination and those reported for SCFβTRCP. Perhaps most disturbing was our inability to identify the F-box component βTRCP in the E3 fraction. βTRCP was observed to elute from the anion exchange resins at a lower ionic strength than the IκBαe ubiquitination activity. We were also unable to show an effect of Ro106-9920 on β-catenin degradation in Jurkat cells. Numerous reports have shown that βTRCP is the F-box component of the SCF complex that catalyzes the ubiquitination of β-catenin (34, 35). Another inconsistency was that E2UBCH7 supports specific IκBαe ubiquitination, whereas Ben-Neriah, Ciechanover, and co-workers (24, 42, 43) have previously shown that E2UBCH5b and E2UBCH5c supported specific IκBα ubiquitination and that E2UBCH7 supported nonspecific ubiquitination. They demonstrated IκBα ubiquitination in a cell-free assay using a fractionated E3 preparation isolated from HeLa cells and used in vitro translated, endogenously phosphorylated IκBα complexed to the NF-κB heterodimer as a substrate. Another inconsistency was the inhibition we observed with the thiol-modifying compounds. Strack et al. (27) reported that IκBα ubiquitination was not blocked by pretreatment of the glutathione S-transferase-βTRCP/Skp1 or His-Cul1/Rbx1 proteins with N-ethylmaleimide. Another inconsistency was the lack of accumulation of phospho-IκBα in Western blot analysis of IκBα levels in TNFα-stimulated MM6 cells treated with Ro106-9920. Kroll et al. (44) observed the accumulation of phospho-IκBα in TNFα-treated HeLa cells transfected with a F-box-deleted βTRCP mutant. Phosphorylated IκBα was also observed to accumulate in Jurkat cells transfected with a dominant negative βTRCP and stimulated with phorbol-ester and Ca++ ionophore (25). It could be that the phosphatase activity was greater in our systems or that Ro106-9920 blocks a step required for IKK activation. Altogether these data are inconsistent with what has been reported for SCFβTRCP. These data suggest that Ro106-9920 inhibits an ubiquitination activity in the NF-κB pathway that...
**TABLE IV**

*The effect of Ro106–9920 in receptor binding and enzyme assays*

The results are represented as the mean percentages of inhibition of control specific binding (receptors) or activity (enzymes) (n = 2) following incubation with 10 µM of Ro106–9920. The symbol − indicates less than 10% inhibition. The symbol + indicates a greater than 50% increase in binding or activity.

| Receptors | Inhibition | Receptors | Inhibition | Enzyme | Inhibition |
|-----------|------------|-----------|------------|--------|------------|
| A1        | −          | A2        | −          | PLA₂   | −          |
| A2a(h)    | −          | α1        | 16         | COX1   | +          |
| β2        | −          | β₁        | −          | COX2   | −          |
| AT1       | −          | AT2       | 24         | 5-Lipoxigenase | 89 |
| ANP       | −          | BZDcentral | 31       | PDE₁   | −          |
| B2        | −          | bombesin  | 14         | PDEII  | −          |
| CB¹central | −        | CB²peripheral | −      | PDEIV  | −          |
| CCKα      | 10         | CCKβ      | −          | ACE    | −          |
| D1        | −          | D2        | −          | ECE    | −          |
| D3        | 14         | D4.4      | −          | Elastin| −          |
| D5        | −          | DA uptake | 17         | Cathespin B | − |
| ET₁       | −          | ET₂       | −          | Cathespin G | − |
| GABA      | −          | GAL1      | −          | HIV-1 protease | − |
| PDGF      | −          | CXCR1     | −          | ACHase | −          |
| TNFα      | −          | TNFβ      | 19         | MAO-A  | −          |
| H1        | −          | H2        | −          | MAO-B  | −          |
| ML1       | −          | M₁        | −          | Tyrosine-OHase | − |
| M2        | −          | M₃        | −          | AT₃aNSN₈/K  | − |
| M₄        | −          | M₅        | −          | Myeloperoxidase | − |
| NK1       | −          | NK₁       | −          | Adenylated cyclase | − |
| NK3       | −          | Y₁        | 26         | Guanylate cyclase | − |
| Y₂        | −          | Neurotensin | −      | Protein kinase | − |
| δ         | 13         | ORL-1     | 27         | EGFR kinase | 63 |
| μ         | 23         | PCP       | −          | Phospholipase C | 33 |
| PACAP-null | −         | PGI₂      | −          | −      | −          |
| TXA₂      | −          | PGI₂      | −          | −      | −          |
| P.X.      | −          | P.Y       | +          | −      | −          |
| 5-HT₁ₐ    | −          | 5-HT₁₈    | 16         | −      | −          |
| 5-HT₂ₐ    | 10         | 5-HT₂₈    | −          | −      | −          |
| 5-HT₃₈    | −          | 5-HT₃₈    | −          | −      | −          |
| σ (nonselective) | − | Somatostatin | 10 | −      | −          |
| VIP       | −          | V₁        | −          | −      | −          |
| Ca²⁺ channel | −         | K⁺ channel voltage | − | −      | −          |
| C₁⁻  ionophore | − | Na⁺ channel site 2 | − | −      | −          |

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3. L. Scarafia, unpublished observations.
4. Z. J. Chen, personnel communication.

Inhibition of NF-κB Activation

The results are represented as the mean percentages of inhibition of control specific binding (receptors) or activity (enzymes) (n = 2) following incubation with 10 µM of Ro106–9920. The symbol − indicates less than 10% inhibition. The symbol + indicates a greater than 50% increase in binding or activity.

| Receptors | Inhibition | Receptors | Inhibition | Enzyme | Inhibition |
|-----------|------------|-----------|------------|--------|------------|
| A1        | −          | A2        | −          | PLA₂   | −          |
| A2a(h)    | −          | α1        | 16         | COX1   | +          |
| β2        | −          | β₁        | −          | COX2   | −          |
| AT1       | −          | AT2       | 24         | 5-Lipoxigenase | 89 |
| ANP       | −          | BZDcentral | 31       | PDE₁   | −          |
| B2        | −          | bombesin  | 14         | PDEII  | −          |
| CB¹central | −        | CB²peripheral | −      | PDEIV  | −          |
| CCKα      | 10         | CCKβ      | −          | ACE    | −          |
| D1        | −          | D2        | −          | ECE    | −          |
| D3        | 14         | D4.4      | −          | Elastin| −          |
| D5        | −          | DA uptake | 17         | Cathespin B | − |
| ET₁       | −          | ET₂       | −          | Cathespin G | − |
| GABA      | −          | GAL1      | −          | HIV-1 protease | − |
| PDGF      | −          | CXCR1     | −          | ACHase | −          |
| TNFα      | −          | TNFβ      | 19         | MAO-A  | −          |
| H1        | −          | H2        | −          | MAO-B  | −          |
| ML1       | −          | M₁        | −          | Tyrosine-OHase | − |
| M2        | −          | M₃        | −          | AT₃aNSN₈/K  | − |
| M₄        | −          | M₅        | −          | Myeloperoxidase | − |
| NK1       | −          | NK₁       | −          | Adenylated cyclase | − |
| NK3       | −          | Y₁        | 26         | Guanylate cyclase | − |
| Y₂        | −          | Neurotensin | −      | Protein kinase | − |
| δ         | 13         | ORL-1     | 27         | EGFR kinase | 63 |
| μ         | 23         | PCP       | −          | Phospholipase C | 33 |
| PACAP-null | −         | PGI₂      | −          | −      | −          |
| TXA₂      | −          | PGI₂      | −          | −      | −          |
| P.X.      | −          | P.Y       | +          | −      | −          |
| 5-HT₁ₐ    | −          | 5-HT₁₈    | 16         | −      | −          |
| 5-HT₂ₐ    | 10         | 5-HT₂₈    | −          | −      | −          |
| 5-HT₃₈    | −          | 5-HT₃₈    | −          | −      | −          |
| σ (nonselective) | − | Somatostatin | 10 | −      | −          |
| VIP       | −          | V₁        | −          | −      | −          |
| Ca²⁺ channel | −         | K⁺ channel voltage | − | −      | −          |
| C₁⁻  ionophore | − | Na⁺ channel site 2 | − | −      | −          |

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does not require βTRCP but associates with IκBα and will ubiquitinate IκBα S32E,S36E (IκBα) specifically at lysine 21 or 22. The IκBα E3 ligase activity is inhibited by cysteine alkylating reagents, supported by E2UBCH7, requires cIAP2 or a cIAP2 associated protein for activity, and may precede the phosphorylation of IκBα by the IKK complex.

A number of proteins associated with the NF-κB pathway have the properties of ubiquitin E3 ligases including TRAFs (45, 46) and cIAP2 (47) or have been associated with ubiquitination including NEMO.² TRAF6 ubiquitination activity is sufficient to activate IKK through the TAK1 kinase (46). However, Ro106-9920 was not active against TRAF6-dependent ubiquitination.² cIAP2 has been reported to be critically involved in TNFα-induced NF-κB activation (48), and a cIAP2 mutant that lacked the RING domain blocked TNFα-induced NF-κB activity. We detected cIAP2 in the active E3 fraction. Immunodepletion with anti-cIAP2 reduced the IκBα ubiquitination activity by 70%, demonstrating cIAP2 to be essential and a promising candidate for the E3 ligase activity. However, reconstitution with recombinant cIAP2 purified from E. coli did not effect the IκBα ubiquitination activity. It may be that the purified recombinant cIAP2 was not functionally active or that the reaction requires additional components. Although cIAP2 is an enticing candidate for the IκBα E3 ligase, these data do not confirm the hypothesis.

Why was SCFβTRCP not identified? Why is the IκBα a substrate for the unidentified E3 ligase and what is the function of the unidentified ligase? The answer to why SCFβTRCP was not identified is most likely the use of E2UBCH7 as the E2 and IκBα as the substrate. Traenckner et al. (14) had previously observed in HeLa cell transfection studies that the double glutamic acid mutant did not stimulate IκBα degradation, and as previously noted, E2 UBCH7 was reported not to support specific IκBα ubiquitination (24, 42, 43). The specificity of SCFβTRCP-dependent IκBα ubiquitination must be rigorously dependent upon the nature of the E2 and the substrate. As to why IκBα is a substrate for the unidentified E3 ligase, it is possible that IκBα is a ubiquitination substrate in vitro because of features in its ubiquitination domain that are similar to those of the physiologically relevant substrate and/or that IκBα is a substrate for more than one E3. As to the function of the unidentified E3, the lack of accumulation of phosphorylated IκBα suggests that the E3 that catalyzes IκBα ubiquitination maybe involved in the activation of the IKK kinase. Chen et al. (28) (28) have reported an unidentified ubiquitin activity supported by E2UBCH5 that was required for activation of IKK. As far as we are aware the source of the ubiquitination activity described by Chen has not been identified.

In summary, the work described provides 1) a small molecule ubiquitin inhibitor, 2) evidence to show that a specific E3 ubiquitination activity can be blocked by small molecules with selectivity in respect to E1 and E2, and 3) evidence for the involvement of an unidentified E3 ligase in NF-κB activation.
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