An increase in activity of the nuclear factor (NF)-κB family of eukaryotic transcription factors is a cardinal feature in the control of inflammation and oncogenesis. Cytokine-mediated activation of the NF-κB signaling axis involves phosphorylation and subsequent proteasome-mediated degradation of inhibitors of NF-κB (e.g. IκBα, -β, and -e), a critical step allowing for the rapid translocation of p65/Rel NF-κB heterodimers to the nucleus to activate gene expression (5, 6). Accordingly, fibroblasts isolated from IKKβ knock-out mice are defective in IKKβ-dependent NF-κB activation in response to either TNFα or interleukin (IL) 1 β (7). IKKγ mediates the recruitment of upstream activating kinases, including the NF-κB-inducing kinase (NIK) (8, 9), that modulate IKKβ activity (7, 10). This scaffolding protein also binds a number of molecules other than IKKKs that are involved in regulating IKK activity (11).

It has been found that the Ras superfamily of small GTP-binding proteins plays an important role in the regulation of a variety of cellular functions (12, 13). The activity of these small GTPases is regulated by signals originating from different classes of surface receptors, such as those for the proinflammatory cytokines TNFα and IL-1β (14). Engagement of small GTP-binding proteins (e.g. Rho, Ras, and Rac), whose proper membrane localization and function are dependent on isoprenylation, has emerged as an important signaling event in the promotion of an NF-κB-dependent program of gene expression that coordinately regulates inflammation and oncogenesis (15–19). Accumulating evidence indicates the clinical relevance of isoprenylation inhibitors to blockage of the geranylgeranylated Rho signaling does not affect TNFα activity of IKKβ (20, 21). Although selective protein farnesylation inhibitors have been shown to block nuclear targeting of NF-κB by oncogenic Ras (23). However, a recent study suggests that the Ras-related RhoB, an immediate-early inducible gene, inhibits NF-κB signaling in response to genotoxic stress or TNFα (24). Thus, the role of protein farnesylation in the regulation of NF-κB signaling has not been clearly defined.

The antibiotic manumycin A produced by Streptomyces parvulus is a potent and selective farnesyltransferase (FTase) inhibitor (25) that was shown previously to exert antitumor activity in vitro and in vivo in nude mouse xenograft models and to exhibit minor toxic side effects in vivo (26, 27). Incubation of human hepatoma HepG2 cells with manumycin A for 12 h induces caspase-mediated apoptosis (28). To understand further the regulatory role of protein farnesylation on NF-κB activity, we examined the effect of manumycin A on cytokine-induced NF-κB activation. In this study, we show that manumycin A exhibits rapid and potent inhibition of TNFα-induced phosphorylation of IκBα and NF-κB nuclear translocation and its binding to DNA by blocking the activity of IKKβ. The action of manumycin A was not derived from its role as FTase inhibitor but, instead, was dependent on its ability to promote stable homotypic dimerization of IKKβ and the dissociation of IKK from the adaptor protein IKKγ/NEMO. Our findings define a function for manumycin A in signal transduction other than as an FTase inhibitor and give new information on how it impairs NF-κB signaling by targeting IKKβ. Epoxyquinoid compounds may have therapeutic effects based in part on NF-κB inhibition.
Experimental Procedures

Plasmids—The eukaryotic expression vector for His-tagged NIK was provided by Zheng-gang Liu (National Institutes of Health, Bethesda); HA-tagged murine IKKβ and HA-tagged human IKKα were gifts from Hidekatsu Iha (National Institutes of Health), and FLAG-tagged IkBa was from Albert S. Baldwin (University of North Carolina). The expression plasmids of FLAG-tagged IKKβ and its mutant C179A in the pcDNA3.1 vector (Invitrogen) were obtained from Craig Creeds (Yale University, New Haven, CT). These constructs have been described previously (29). A double point mutant of FLAG-tagged IKKβ at Cys-662 and Cys-716 (termed C662A/C716A) has been provided by Tom Gilmore (Boston University) and checked by standard DNA sequencing.

Cell Lines and Cell Culture—CHO cells stably expressing the human insulin receptor (CHO-IR) were maintained in Ham’s F-12 medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The human hematoma cell line HepG2 was maintained in minimum Eagle’s medium containing 1 g/ml glucose, nonessential amino acids, and supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 10% FBS, and penicillin/streptomycin. Murine B16F10 melanoma cells were obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS and penicillin/streptomycin. For the preparation of rat hepatocytes, parenchymal liver cells were isolated from adult Fisher 344 male rats by in situ retrograde perfusion of the liver with collagenase (30). The cells were seeded onto collagen-coated dishes (Discovery Labware) and cultured for 4 h in William's E medium supplemented with 5% FBS, 2 mM l-glutamine, penicillin/streptomycin to allow attachment of adherent liver cells. The medium was then replaced with William’s E medium containing 5% FBS. After 18 h, the hepatocytes were subjected to 4 h of serum starvation before the addition of manumycin A (Calbiochem) and recombinant TNFα (R & D Systems) as indicated.

Transfections—Transient transfection of HepG2 cells was performed using Lipofectamine 2000 (Invitrogen). Cells were plated in duplicates, transfected with either pcDNA3.1 or expression plasmids encoding epitope-tagged NIK, IkBa, or various IKK constructs at a ratio of 2 μg of plasmid/35-mm dish, and analyzed after 48 h. In transfection assays, 0.25 μg of β-galactosidase plasmid was cotransfected with 1 μg of luciferase reporter plasmid controlled by four tandem copies of the κB-binding site consensus sequence inserted upstream of a minimal thymidine kinase promoter (31). CHO-IR cells were plated in duplicate and transfected using TransFast transfection reagent (Promega). Eighteen h later, cells were serum-starved for 3–4 h and then pretreated with 10 μM manumycin A for 1 h followed by the addition of 20 ng/ml TNFα. Cells were lysed after 24 h to determine luciferase and β-galactosidase levels by enzyme assay kits purchased from Promega. Luciferase activity was normalized to β-galactosidase activity as internal control.

Tumor Injection—C57BL/6 mice were purchased from The Jackson Laboratory. Mice were maintained on a 12-h light-dark cycle with ad libitum access to water and the standard NIH-07 diet. Animals were lightly anesthetized using isoflurane delivered via vaporizer. A dorsal area of skin, ~2 cm² in diameter, was shaved with Oster Golden A5 hair clippers. 10⁶ B16F10 melanoma cells suspended in 0.5 ml of sterile saline were inoculated by intradermal injection, as described previously (32), whereas saline-only injection was used in controls. Only one injection was required per animal. No restraint (other than being held in the investigator’s hand) was necessary during the injection. Tumors were measured using an acetate sheet on which circles of various diameters had been drawn. In addition, animals were monitored weekly for signs of morbidity, including abnormal appearance. After the tumors reached 25 mm in diameter, the animals were lightly anesthetized using isoflurane delivered via vaporizer followed by a single injection of manumycin A (7.5 μl of 10 mM) or Me₂SO (vehicle) into the tumor mass and incubated for 2 h. Freshly isolated tumors were collected, rinsed in phosphate-buffered saline, and snap-frozen in liquid nitrogen. The animal protocol was approved by the Animal Care and Use Committee of the National Institute on Aging, National Institutes of Health.

Farnesyltransferase Assay—The determination of FTase activity was performed according to the method of Goalstone et al. (33). In brief, lysates containing endogenous FTase were added to the reaction assay solution containing 50 mM Hepes, pH 7.5, 5 mM MgCl₂, 5 mM DTT, 100 nM Ras-CVLS (Calbiochem), and 100 mM ³H-farnesyli-phosphate (PerkinElmer Life Sciences). After incubation for 30 min at 37 °C, the reaction was terminated by the addition of acidified ethanol, and the reaction mixture was filtered through Whatman GF/C glass fiber filters and air-dried followed by quantitative measurement of tritiated product by liquid scintillation counting. Lysates were normalized for protein content using the Bio-Rad method.

Nuclear Extract Preparation and Electromobility Gel Shift Assay (EMSA)—Nuclear extracts from CHO-IR cells were prepared using the NE-PER™ extraction reagents according to the manufacturer’s protocol (Pierce). Protein concentrations were determined using the BCA method (Pierce), and extracts were stored at −70 °C until analysis. An aliquot of nuclear extracts containing 10 μg of protein was used for EMSA. Binding reactions proceeded for 20 min at room temperature in the presence of poly(dI-dC), using a biotinylated double-stranded oligonucleotide encompassing a putative κb site (sense, 5’-AATTGATAATGGCTGACATTCTCCAGGGCATGA; antisense, 5’-AGCCTGATGCCTGGGAAAGTCCCCTCAACTCGATG) or an oligonucleotide containing a mutation in the κb site (underlined nucleotides were replaced by T (sense) and A(antisense), respectively). For competition experiments, a 200-fold molar excess of unlabeled wild-type κb probe or the mutated κb probe was used. The oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). In some experiments, nuclear extracts were incubated for 30 min at room temperature with 2 μl of anti-p65 Rel (sc-8008X, Santa Cruz Biotechnology) prior to EMSA, as indicated in figure legends. DNA–protein complexes were separated by electrophoresis on native 6% polyacrylamide gels followed by electrotransfer onto Biodyne B™ nylon membrane (Pall Life Sciences, Ann Arbor, MI) and detection using streptavidin-based detection system (Pierce).

Western Blot Analysis—Unless otherwise indicated, cells were lysed directly in Laemmli sample buffer containing 5% 2-mercaptoethanol and 1 mM sodium orthovanadate. After heating at 70 °C for 10 min, proteins were separated by SDS-PAGE on 4–12% polyacrylamide gradient gel (Invitrogen) and electrotransferred onto 0.22-μm polyvinylidene difluoride (PVDF) membranes (Invitrogen). Immunoblot analysis was performed using specific primary antibodies, and bound antibodies were detected by the ECL method (Amersham Biosciences). Band intensities were quantitated by densitometry using the ImageQuant software (Amersham Biosciences). Antibodies used in Western blots were mAb anti-p65 Rel (BD Transduction Laboratories); mAb anti-lamin B2 (Novoceastra Laboratories); mAb anti-β-actin and rabbit anti-FLAG (Sigma); rabbit antibodies against p89 TFIH, IkBa, IKKβ, IKKα, and IKKγ (Santa Cruz Biotechnology); rabbit anti-p38 MAPK and rabbit antiphosphorylated IkBa (Ser-32) and ATF-2 (Thr-71) (Cell Signaling Technology); and rabbit anti-HA (Clontech).

Immunoprecipitation and IKK Assays—HepG2 cells were lysed in Nonidet P-40 lysis buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 1%
Nonidet P-40, 10% glycerol, 20 mM β-glycerophosphate, 1 mM DTT, 1 mM EDTA, 20 mM p-nitrophenyl phosphate, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1X protease mixture inhibitor set I (Calbiochem)), and the clarified lysates were incubated either with 4 μg of mAb anti-IKKβ or mAb anti-IKKγ (Oncogene) for 18 h at 4 °C with subsequent sedimentation of the immunocomplexes with agarose-bound protein G (Upstate Biotechnology). In other experiments, HepG2 cells transiently expressing epitope-tagged IKK constructs were lysed as shown above, and the clarified lysates were then incubated for 2 h at 4 °C with either anti-FLAG M2 affinity gel beads (Sigma) or mAb anti-HA (Covance) prebound to protein G–agarose. For experiments with murine B16F10 melanoma, samples of tumor tissues were homogenized in Nonidet P-40 lysis buffer and were centrifuged 10 min at 13,000 rpm. The supernatants (~500 μg of proteins) were incubated with 2 μg of rabbit anti-IKKβ/α (Santa Cruz Biotechnology) for 2 h followed by the addition of agarose-bound protein A/G beads (Upstate Biotechnology, Inc.) for an additional 90 min at 4 °C. In all instances, the beads were washed two times with Nonidet P-40 lysis buffer, two times with 20 mM Hepes, pH 7.6, 0.5 mM NaCl, and twice with kinase assay buffer (20 mM Hepes, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 2 mM DTT, 1 mM EDTA), according to the procedure of Chen et al. (34). The kinase reaction was performed using immunoprecipitates in a 20-μl total volume of kinase assay buffer containing 20 μM ATP, 6000 cpm/pmol [γ-32P]ATP (Amersham Biosciences), and 1 μg of GST-IκBα (Santa Cruz Biotechnology) for 20 min at 30 °C. The reaction was stopped by adding 12 μl of 3X reducing Laemmli sample buffer; the reaction mixtures were subjected to SDS-PAGE and transferred onto PVDF membrane, and then autoradiography with Hyperfilm (Amersham Biosciences) was performed.

In-gel Trypsin Digestion, LC-MS/MS Analysis and Identification of Proteins Present in FLAG Immunoprecipitates—After electrophoresis, the SDS-polyacrylamide gel was stained with Colloidal Brilliant Blue G (Bio-Rad), and the protein bands were excised and transferred to 0.5-ml Eppendorf tubes. The in-gel proteolytic digestion, LC-MS/MS analysis, and identification of the proteins was carried out by ProtTech, Inc. (Norristown, PA). In brief, each protein gel spot was destained and in-gel digested with modified sequencing grade trypsin (Promega), and the resulting peptide mixture was subjected to peptide sequencing by tandem mass spectrometry. An LCQ ion trap mass spectrometer (ThermoFinnigan) coupled on-line with a high pressure liquid chromatography system running a 75-mm inner diameter × 10-cm length 3-μm C18 capillary column was used. Data were acquired in a data-dependent mode. MS/MS spectra were used to search the nonredundant protein data base from GenBankTM with the ProQuest software suite (ProtTech, Inc.).

Conjugation of Manumycin A with Glutathione—Fifty nmo1 of manumycin A in MeOH was incubated with 3 eq of reduced glutathione in 20 mM Tris-HCl, pH 7.5, for 60 min at 37 °C. Twenty μl of the mixture was injected in the LC-MS system using methanol:water (1:1) as the mobile phase at a rate of 0.2 ml/min. ESI-positive mode. The LC-MS system was composed of a LC10AD pump (Shimadzu, Columbia, MD), ESA 540 auto-injector (ESA Inc., Chelmsford, MA), and Micromass Q-Tof mass spectrometer (Micromass, Beverly, MA). Data were recorded and processed using Mass-Lynx version 3.5 (Micromass).

RESULTS

Manumycin A Inhibits Farnesyltransferase Activity—The ability of manumycin A to inhibit FTase was examined in lysates from CHO-IR cells with [3H]farnesylpyrophosphate as a substrate. Control experiments showed an ~2.5-fold stimulation of FTase activity over non-stimulated cells after a 5-min challenge with insulin (data not shown). In contrast, pretreatment with manumycin A for 1 h elicited a 30% reduction in basal FTase activity and blocked the insulin-stimulated response. The concentration and duration of treatment with manumycin A used in these studies had no detectable effect on cell viability and did not cause cell rounding or alteration in the microtubule network.

TNFα-induced NF-κB Nuclear Translocation and Transcriptional Activity Are Inhibited by Manumycin A—The translocation of NF-κB subunits to the nucleus is a critical determinant of NF-κB signaling. We examined the effect of manumycin A on the nucleocytoplasmic shuffling of the NF-κB p65 Rel subunit by immunoblot analysis. TNFα treatment elicited a rapid and time-dependent nuclear accumulation of NF-κB in CHO-IR cells, being first detected within 5 min and peaked at 30 min (Fig. 1, A and B). In contrast, 1 h of preincubation with 10 μM manumycin A increased the basal levels of nuclear p65 Rel ~1.3-fold but abolished the TNFα-stimulated NF-κB nuclear translocation (Fig. 1, A, upper panel, lanes 7 and 10 versus 6 and 9, and B, left panel). Under these experimental conditions, a significant accumulation of IκBα was observed in the nuclear extract of manumycin A–treated cells (Fig. 1, A, 2nd panel, and B, right panel). The levels of lamin B used as loading control for the nuclear fraction did not vary upon manumycin A addition (Fig. 1A, bottom panel). To determine whether manumycin A mediates its activity through induction of protein expression or inhibition of degradation of p65 Rel and/or IκBα, CHO-IR cells were treated with the protein synthesis inhibitor cycloheximide before the addition of manumycin A (Fig. 1C). Cycloheximide treatment had only a minimal effect on nuclear accumulation of p65 Rel and IκBα in response to manumycin A, and expression of these proteins was unchanged by the FTase inhibitor.

To test whether the defect in nuclear translocation of NF-κB was associated with impaired formation of NF-κB–DNA complexes, nuclear extracts from CHO-IR cells were subjected to EMSA to detect NF-κB complexes bound to a consensus κB probe. The binding of NF-κB appears as three bands in response to TNFα (Fig. 1D, lanes 1 and 4 versus lane 2), which are supershifted by anti-p65 Rel (lane 9, double asterisks) and that are competed with unlabeled probe (lane 6), indicating that complexes contained NF-κB. As expected, when the κB probe is mutated (mut), it failed to bind NF-κB (Fig. 1D, lane 8). All three bands were abrogated when nuclear extracts from manumycin A–treated cells stimulated with TNFα were tested (Fig. 1D, lane 5). Furthermore, CHO-IR cells incubated with 20 ng/ml TNFα for 24 h showed an ~4-fold increase of κB-luciferase reporter activity, which was blocked upon pretreatment with manumycin A (Fig. 1E). Taken together, these results demonstrated that manumycin A blocks activation of NF-κB by TNFα.

Impact of Manumycin A on Cytokine-dependent Phosphorylation of IκBα in Several Cell Types—Cytokine-mediated phosphorylation and subsequent proteolytic degradation of IκBα are critical steps for the translocation of NF-κB to the nucleus. As shown in Fig. 2A, treatment of CHO-IR cells with TNFα induced an increase in IκBα phosphorylation in a time-dependent manner, as assessed by immunoblotting cell lysates using an antibody specific for IκBα phosphorylated at Ser-32. Maximal activation was detected within 5 min of stimulation and declined thereafter, reaching its nadir by 30 min. TNFα elicited the degradation of IκBα from 15 min, with nearly complete proteolysis by 30 min (Fig. 2A, lower panel). Longer exposure to TNFα (1–2 h) induced de novo synthesis of IκBα protein. We then analyzed whether manumycin A interferes with the ability of TNFα to rapidly stimulate IκBα phosphorylation. Both the basal and TNFα-induced levels of phosphorylated IκBα were inhibited by manumycin A in a dose-dependent manner, with
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FIGURE 1. Manumycin A blocks TNFα-dependent nuclear redistribution of p65/RelA and gene induction through the IκB promoter. A, CHO-IR cells maintained in serum-free medium were left untreated (−, open bars) or treated (+, filled bars) with 10 μM manumycin A for 1 h prior to the addition of 20 ng/ml TNFα for the indicated periods of time. Cytoplasmic (lanes 1–5) and nuclear (lanes 6–10) fractions were immunoblotted using anti-p65 Rel (top panel) and anti-IκBα antibodies (middle panel). The amount of nuclear protein load was assessed by detection of the lamin B2 (bottom panel). The positions of p65 Rel, IκBα, and lamin B2 are indicated on the right margin, whereas the left margin indicates the M, × 10−5. B, bars represent quantitative analysis whereby the intensity of nuclear p65 Rel and IκBα proteins were normalized to that of lamin B2. The signal associated with control unstimulated cells was arbitrarily given a value of 1.0. The data are representative of two independent experiments. C, CHO-IR cells were pretreated with 10 μM cycloheximide for 1 h followed by the addition of 10 μM manumycin A for 20 min. Nuclear and cytoplasmic fractions were isolated and then immunoblotted with the indicated antibodies. The pattern of p65 TFIIH and β-actin immunostaining confirmed equal protein load. D, cells were treated with manumycin A for 30 min followed by the addition of TNFα for 15 or 30 min. Nuclear extracts were prepared, and NF-κB activation was determined by EMSA as described under “Experimental Procedures.” Nuclear extracts were incubated in the absence (lanes 1–8) or presence (lane 9) of anti-p65 Rel and subjected to EMSA using a biotinylated probe containing a wild-type κB consensus sequence (wt, lanes 1–7 and 9) or the same probe mutated at the κB site (mut, lane 8). Competition with excess of unlabeled wild-type and mutant probes was also carried out (lanes 6 and 7). ** Denotes binding of NF-κB to the κB probe supershifted with anti-p65 Rel. An identical result was obtained in an additional experiment. E, CHO-IR cells were transiently transfected with 4×κB-Luc reporter plasmid and pCMVSPORT β-galactosidase expression plasmid for 24 h. The cells were serum-starved for 4 h and then pretreated with manumycin A (Manu) for 30 min before the addition of TNFα for 24 h. Cells extracts were analyzed for luciferase activity and normalized for β-galactosidase. All values are expressed relative to control culture without treatment. Results are expressed as means ± S.E. of two experiments each performed in duplicate dishes.

maximum inhibition at 10 μM (Fig. 2B, lanes 8 versus 5) and an IC50 of about 2 μM (Fig. 2C). Besides attenuating TNFα signaling, manumycin A also prevented IL-1β-dependent phosphorylation of IκBα in CHO-IR cells (Fig. 2D). Because the human hepatocyte-derived HepG2 cells have been shown previously to be responsive to TNFα in terms of NF-κB activation (35), this cell line was used to examine further the mechanism of NF-κB inactivation by manumycin A. The short term exposure of HepG2 cells to manumycin A led to a marked reduction in the levels of IκBα phosphorylation elicited by TNFα, with an effective concentration (10 μM) that was similar to that found in CHO-IR cells (Fig. 2E). Likewise, manumycin A effectively blocked TNFα signaling in a primary culture of rat hepatocytes (Fig. 2F). We confirmed that preincubation with the potent and selective geranylgeranyltransferase-I inhibitor GGTTI-2133 (up to 5 μM) had no effect on TNFα-mediated phosphorylation of IκBα (data not shown).

Members of other classes of FTase inhibitors were employed to compare their abilities to inhibit TNFα-induced phosphorylation of IκBα in HepG2 cells (Fig. 3A). In contrast to manumycin A, the compounds FTI, FTI-277, and (α-hydroxyfarnesyl) phosphonic acid had no inhibitory effects at concentrations that block FTase activity, indicating that manumycin A mediates its anti-inflammatory activity most likely in an FTase-independent manner. In the following experiments, the reversibility of manumycin action was tested upon removal of the drug from HepG2 cells for periods up to 3 h before the addition of TNFα (Fig. 3B). We observed that TNFα-induced IκBα phosphorylation was progressively restored with the removal of manumycin A from the incubation medium (Fig. 3, B and C). As shown in Fig. 3D, pretreatment of CHO-IR cells with manumycin A did not block TNFα-induced phosphorylation of the transcription factor ATF-2 at Thr-71 under conditions where IκBα phosphorylation was abolished. In addition, phosphorylation of
CREB and c-Jun in response to TNFα was further enhanced by pretreatment with manumycin A (Fig. 3D). These results indicate that the inhibitory effects of manumycin A on NF-κB activation pathways are selective rather than global.

A variety of upstream activating kinases, such as MEKK1, NIK, and NF-κB-activating kinase, participates in the signaling cascade leading to IKK complex activation (8, 36, 37). To test the possibility that manumycin A functions via inhibition of an upstream kinase, epitope-tagged
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NIK was coexpressed with FLAG-tagged IκBα in HepG2 cells. Overexpression of wild-type NIK is known to stimulate basal NF-κB activity, possibly because of NIK oligomerization (38). As shown in Fig. 4, manumycin A markedly inhibits constitutive and TNF-mediated IκBα phosphorylation elicited by ectopic NIK expression. Furthermore, manumycin A was also effective at blocking basal and TNFα-stimulated phosphorylation of IκBα in HepG2 cells coexpressing wild-type IKKβ and FLAG-tagged IκBα (Fig. 4). Because enforced oligomerization of IKKβ by upstream regulators can be sufficient for inducing activation of the IKK complex (39), our results provided evidence that manumycin A may alter some intrinsic properties within the IKK complex.

**Manumycin A Interferes with IKK Activity**—By using IKK immunocomplex kinase assays with GST-IκBα as a substrate, we found that TNFα evoked increases in IKK activity measured in either anti-IKKβ or anti-IKKγ immunoprecipitates of HepG2 cells (Fig. 5A). However, pretreatment with manumycin A blocked both the constitutive and TNFα-stimulated responses. Similar to the results observed in HepG2 cells, manumycin A abrogated the increase in TNFα-induced IKK activity levels in CHO-IR cells (data not shown). To study the mechanism whereby manumycin A may affect IKKβ function, we sought to transfect various IKKβ constructs into HepG2 cells before manumycin A treatment. Several structurally unrelated inhibitors, including arsenite, cyclic prostaglandins, and parthenolide, have been shown recently to target Cys-179 in the activation loop of IKKβ for their inhibitory activity (29, 40, 41). To determine whether the Cys-179 substitution to Ala (C179A) affected the sensitivity of IKKβ to manumycin A, epitope-tagged human wild-type and mutant IKKβ proteins from transfected cells were immunoprecipitated and examined for their constitutive autophosphorylation and exogenous kinase activity in vitro (Fig. 5B). We compared wild-type IKKβ with the C179A mutant and a mutant of IKKβ with both Cys-662 and Cys-716 replaced with Ala (C662A/C716A), and we found that IKKβ proteins exhibited varying sensitivities to manumycin A (C179A < WT ≤ C662A/C716A). Most unexpectedly, we noted on immunoblots the formation of a stable high molecular mass complex encompassing IKKβ upon manumycin A treatment of HepG2 cells expressing epitope-tagged IKKβ proteins, although the level was barely detectable with the C662A/C716A mutant (Fig. 5C). Ectopic expression of murine IKKβ but not human IKKα elicited formation of the complex in manumycin A-treated cells (data not shown and Fig. 5C). Thus, exposure of HepG2 cells to manumycin A results in the selective conjugation of IKKβ which requires Cys-662/716 residues.

To determine whether IKKβ is a direct target of manumycin A, we analyzed the effects of exogenous addition of the drug using wild-type FLAG-IKKβ immunoprecipitates. IKK autophosphorylation and kinase activity were efficiently inhibited with increasing concentrations of manumycin A (Fig. 5D, upper panel, lanes 1–4). When immunoprecipitates were incubated with manumycin A, the formation of IKKβ conjugates was also observed (Fig. 5D, middle panel, **). These effects of manumycin A were abrogated when the reducing agent dithiothreitol was present during the incubation period (Fig. 5D, lanes 5 and 6). Immunoblotting with anti-IKKβ following immunoprecipitation with anti-FLAG confirmed the formation of a stable high molecular mass form of IKKβ by manumycin A in the absence of DTT (Fig. 5D, bottom panel). Thus, manumycin A is able to impair IKKβ function in vitro in a thiol-dependent fashion.

Because TNFα-stimulated phosphorylation of IκBα was efficiently inhibited in murine B16F10 melanoma following addition of manumycin A (Fig. 5E), the in vivo evidence for the efficacy of this drug was then examined using mice xenografted with B16F10 tumors. An ∼35% reduction in IKK activity was observed upon injection of manumycin A into the tumors of five animals for 2 h (Fig. 5F). Taken together, these observations indicate that manumycin A can block IKK signaling both in vitro and in vivo.

To identify the nature of the IKKβ-containing complex, HepG2 cells were transfected with FLAG-tagged C179A mutant and then incubated with manumycin A. Anti-FLAG immunoprecipitates were resolved by SDS-PAGE, and protein bands on Colloidal Blue-stained gel (Fig. 6A) were subjected to in-gel digestion with trypsin, followed by peptide sequencing using tandem mass spectrometry (LC-MS/MS). Fig. 6B shows the sequence coverage determined by identified peptides from the LC-MS/MS experiment of FLAG-tagged C179A. The current experiment successfully identified 12 peptides from various regions of the IKK complex (39), our results provided evidence that manumycin A may alter some intrinsic properties within the IKK complex. To determine whether IKKβ, HepG2 cells were cotransfected with two IKKβ wild-type constructs with different epitope tags, and communoprecipitation assays were performed after cell treatment with manumycin A. We found that manumycin A caused covalent IKKβ dimerization in intact cells (Fig. 6C). Despite the lack of IKKα homodimer (Fig. 5C), formation of stable IKKβ/IKKα heterodimer was also observed in cotransfection experiments with the two IKK constructs (Fig. 6C), consistent with their selective capacities to react with manumycin A.

**Manumycin A impairs IKK interaction with IKKγ**—Physical interaction between IKKα/IKKβ and the regulatory subunit IKKγ is critical for cytokine-induced NF-κB activation. The fact that manumycin A inhibited IKK activity may also indicate impairment in IKKβ-IKKγ association. To test this possibility, we immunoprecipitated FLAG-tagged IKKβ WT or C179A mutant from transfected HepG2 cells that were treated with manumycin A, and we performed immunoblotting using anti-IKKγ antibody (Fig. 7A). The results clearly showed that manumycin A was able to elicit a dose-dependent reduction in IKKβ interaction with IKKγ.
Manumycin A is an IKKβ inhibitor. A, serum-starved HepG2 cells were left untreated or treated with 10 μM manumycin for 30 min before the addition of 20 ng/ml TNFα for 5 min. Extracts were immunoprecipitated (IP) with a polyclonal antibody directed against IKKβ or IKKγ, and solid phase kinase assays were performed with [γ-32P]ATP and GST-IxkBα fusion protein as a substrate. Reactions were separated by SDS-PAGE, transferred to PVDF membrane, and exposed to film to detect phosphorylated GST-IxkBα (top panel). Immunoprecipitates were also probed with IKKβ and IKKγ antibodies to detect levels of both proteins (bottom panel). This experiment was repeated twice with similar results. B, HepG2 cells were transfected with plasmids encoding FLAG-tagged wild-type IKKβ (WT), C179A, or C662A/C716A IKKβ mutant for 48 h and then serum-starved for 3 h. Following the addition of 0, 10, or 20 μM manumycin A, whole cell lysates were prepared and incubated with agarose-linked FLAG M2 antibody. Immunoprecipitates were employed in kinase assays to measure substrate phosphorylation (top panel) and autophosphorylation of IKKβ constructs (middle panel). Moreover, membranes were probed with polyclonal anti-FLAG antibody to detect levels of IKKβ proteins (bottom panel). C, HepG2 cells were transfected with FLAG-tagged WT, C179A, C662A/C716A IKKβ constructs or HA-tagged IKKα, as indicated. Following manumycin A treatment, immunoprecipitates (IP) were prepared using FLAG M2 or HA antibody, and then immunoblotting (IB) was performed. Time of exposure for HA immunoblot was longer in an attempt to detect conjugation of IKKα. D, extracts of HepG2 cells overexpressing FLAG-IKKβ were incubated in the presence of anti-FLAG or control IgG (lane C). Immunoprecipitates were incubated for 30 min at room temperature with the indicated concentrations of manumycin A (0, 2, 10, or 20 μM) in the absence or the presence of 2 mM DTT. In vitro IKK activation was then tested as indicated earlier. The reaction mixtures were resolved by SDS-PAGE, transferred on PVDF membrane, followed by autoradiography (top panel). E, murine B16F10 melanomas were serum-starved for 4 h and then pretreated with vehicle (Me2SO) or manumycin A for 30 min followed by the addition of TNFα for 5 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-pIxBα and anti-IxBα. F, mice xenografted with murine B16F10 tumors received either vehicle (Me2SO) or manumycin A for 2 h, as discussed in the text. Anti-IKKβ and control (lane C) immunoprecipitates were prepared and assayed in the presence of [γ-32P]ATP and GST-IXBa as the substrate. The reaction mixtures were separated by SDS-PAGE, transferred on PVDF membrane, followed by autoradiography (top panel). The membrane was then probed using mouse anti-IKKβ (bottom panel). Bars represent the average ± S.E. of normalized IxBα phosphorylation relative to the vehicle control (equal to 1.0). #, p < 0.005; **, denotes stable high molecular mass form of IKKβ.
To determine whether impairment occurred in cells expressing endogenous levels of proteins, HepG2 cells were treated with vehicle or manumycin A for 30 min, and anti-IKKα immunoprecipitates were prepared after cell stimulation with TNFα. A constitutive association of IKKβ and IKKα with IKKγ was detected, and TNFα stimulation resulted in similar cosedimentation levels (Fig. 7, B and C). In the presence of manumycin A, IKKγ association was significantly reduced with respect to both IKKβ and IKKα. Membranes were reprobed with IKKγ antibody to demonstrate that comparable amounts of IKKγ had been immunoprecipitated in all conditions tested (Fig. 7B, lower panel). Similar to the results observed with HepG2 cells, manumycin A significantly reduced IKKγ association with IKKβ and IKKα in CHO-IR cells (data not shown).

Conjugation of Manumycin A with Glutathione—Our results demonstrated that DTT could neutralize manumycin A (Fig. 5D). Therefore, the modification of manumycin A by thiol was examined by mass spectrometry. Incubation of manumycin A with a 3-fold excess of reduced GSH resulted in the detection of three major components at m/z 615.1827 [M + H + 2Na + H₂O]⁺, 922.2802 [M + H + 2Na + H₂O + GSH]⁺, and 1229.1809 [M + H + 2Na + H₂O + 2GSH]⁺, indicating the addition of one or two molecules of GSH to manumycin A (Fig. 8A). The proposed mechanism of the reaction is illustrated in Fig. 8B.

DISCUSSION

The findings presented here provide the first evidence that manumycin A inhibits constitutive and TNFα-induced NF-κB activity by directly targeting IKKβ. This inhibition is rapid (within 30 min) and selective, as exposure to manumycin A results in normal and/or enhanced phosphorylation of other transcription factors (e.g. ATF2, c-Jun, and CREB) in response to TNFα. Furthermore, administration of manumycin A to mice xenografted with B16F10 tumors blocks IKK activation, which confirms our findings with cultured cell lines. Manumycin A, with its epoxyquinol core, offers the possibility of thiophilic attack (42) (Fig. 8), thus providing a mechanism for covalent homodimerization of IKKβ. Moreover, we show that manumycin A also triggers the dissociation of the noncatalytic subunit IKKγ (NEMO) from the two catalytic subunits of the IKK complex. These results suggest an important and novel property of manumycin A that is distinct from its role as a protein farnesyltransferase inhibitor.

A number of biologically active molecules, including aspirin, salicylate, sulindac, vitamin C, and thiol–reactive metal compounds have been reported to inhibit the catalytic activity of the IKK complex (43–46). More recently, parthenolide, a sesquiterpene lactone from the medicinal herb Feverfew, has been found to bind directly to and inhibit IKKα and IKKβ (29). More importantly, parthenolide and two other IKKβ inhibitors, including arsenite and cyclopentenone prostaglandins, were shown to interact with Cys-179 present in the activation loop of the kinase, and introduction of the C179A mutation renders IKKβ resistant to these inhibitors (29, 40, 41). Because Cys-179 has been reported to be the target of these inhibitors, we examined whether manumycin A alters autophosphorylation and homotypic dimerization of C179A. We found that the mutant sensitivity to manumycin A was
comparable with that of its wild-type counterpart with regard to dimer formation, thus indicating that this cysteine residue is not likely to play a role in manumycin A signaling.

We provide evidence that manumycin A uses a mechanism that is different from that of other compounds with anti-inflammatory properties in inhibiting IKKβ. Contrary to the action of parthenolide and other classes of metabolites, we show that manumycin A inhibits IKKβ, in part, through covalent dimerization of the enzyme. Given that manumycin A and other epoxyketone natural products and synthetic derivatives of these compounds present diverse arrays of electrophilic functionalities, one could expect that the alkylating properties of these antitumor antibiotics will vary widely. For example, a number of epoxyquinoids have been shown to inhibit NF-κB, whereas others are either inactive or exhibit low potency (47), thereby pointing to the role of the enantiomeric composition, number of reactive sites for nucleophilic attack, and/or the metabolite side chain. Microbial products such as jesterone, cycloepoxydon, epoxyquinol A, and a derivative from epoxyquinomicin C are known to inhibit activation of NF-κB and, consequently, to promote apoptotic cell death of different cancer cell lines (48–51). However, no direct targets have been identified. A previous report by Liang et al. (47) showed that the synthetic epoxyquinoid jesterone dimer elicits the formation of stable high molecular mass forms of IKKβ, whereas the parent compound jesterone had no activity. They suggested also that the synthetic jesterone dimer induces covalent IKKβ cross-linking, yet no direct biochemical evidence was provided to support this hypothesis. Our findings provide here the first indication that a natural monomeric epoxyquinoid, manumycin A, is capable of blocking NF-κB activation, in part, by promoting homotypic IKKβ dimerization likely through nucleophilic reaction on the epoxyquinoid core of manumycin A. This interaction is rather specific since manumycin A fails to elicit homodimerization of IKKα but promotes the formation of stable heterocomplexes between IKKβ and IKKα. These results are consistent with the presence of two distinct nucleophilic moieties on IKKβ, one of which is absent in IKKα.

It is likely that IKKβ is a direct target of manumycin A. The inhibition of IKK and promotion of homotypic dimerization of IKKβ are observed upon addition of manumycin A to IKKβ immunoprecipitates, but only if thiols are omitted from the assay. It is possible that in vitro reaction with DTT inactivates critical structural requirements necessary for manumycin A-induced IKKβ inhibition. Moreover, our mass spectrometry analysis revealed the formation of manumycin-glutathione adducts in vitro, consistent with the susceptibility of manumycin A to thiolic attack. It is unclear whether manumycin A targets IKKβ for conjugation to ubiquitin in intact cells. However, ubiquitination of IKKβ has been shown recently to depend upon signal-induced phosphorylation of the activation loop of the kinase (52).

The molecular mechanism of IKKβ inhibition by manumycin A remains unknown. Homotypic interactions allow IKKβ molecules to trans-autophosphorylate one another on their activation loops (53), inducing a conformational change that facilitates substrate access to the active site (54). One model for IKKβ inhibition may be that the binding of manumycin A could promote stable homotypic dimer formation that maintains the kinase activation loop in an inactive conformation. Indeed, very little, if any, of the ~220-kDa species was seen as a 32P-labeled band when in vitro kinase assays were performed using immunoprecipitates from IKKβ WT and C179A-transfected cells treated with manumycin A.4 However, the fact that manumycin A failed to promote significant formation of IKKα dimers despite inhibition in kinase activity does not support this hypothesis as the sole mechanism. Nevertheless, these results suggest that the binding of manumycin A hampers the events contributing to IKK autophosphorylation. On the other hand, IKKα protein may be lacking critical amino acid residue(s) that are the sites for covalent modification by manumycin A. Of note, IKKα lacks Cys-716 and the C662A/C716A mutant of IKKβ cannot proceed with efficient homotypic dimer formation in the presence of manumycin A. A simple interpretation of these results is that Cys-716 contributes to IKKβ reactivity and is positioned in close proximity to a second cysteine present in both IKKα and IKKβ.

Manumycin A binding could also induce dissociation of the IKK complex, leading to a decrease in IKK activity. Indeed, our data indicate that there is dissociation of IKKγ (NEMO) from the IKKα-IKKβ complex upon cell treatment with manumycin A. With the recent discovery of additional complex components, including protein phosphatases, heat shock proteins, and the adaptor protein CIKS (34, 55–58), the ability of manumycin A to interfere with their constitutive and inducible association with and regulation of the two catalytic subunits’ activity

4 M. Bernier and S. Kole, unpublished data.
FIGURE 8. Conjugation of GSH with manumycin A. A, manumycin A was incubated with 3 eq of GSH for 60 min at 37 °C, and the mixture was resolved by mass spectrometry. B, proposed mechanism of the reaction where two GSH molecules are forming an adduct with manumycin A.
remains a possibility and is the subject of current investigation in this laboratory.

Considerable work has been directed at identifying small molecules that inhibit IKKβ as possible targets for the development of anti-inflammatory and anti-neoplastic drugs. Of significance, an earlier report by Umezawa et al. (48) suggested that an epoxyquinomicin C derivative impairs the inflammatory signaling by inhibiting NF-κB in a murine rheumatoid arthritis model. In addition, manumycin A reduces the malignant potential of some transformed cells by inhibiting farnesyltransferase activity and gives new information on how it impairs NF-κB signaling in these cells.

Our data suggest that manumycin A may have therapeutic value in conditions associated with the pathologic activation of NF-κB.

Acknowledgments—We thank Drs. Baldwin, Ila, Li, Liu, Cross, and Gilmore for providing us with reagents. We gratefully acknowledge the contribution of Ruin Moaddel for help with the LC-MS analysis and Dr. Ranjan Sen for valuable insights.

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