Suberoylanilide Hydroxamic Acid Potentiates Apoptosis, Inhibits Invasion, and Abolishes Osteoclastogenesis by Suppressing Nuclear Factor-κB Activation*

Received for publication, July 5, 2005, and in revised form, November 18, 2005 Published, JBC Papers in Press, December 23, 2005, DOI 10.1074/jbc.M507213200

Yasunari Takada1, Ann Gillenwater2, Haruyo Ichikawa1, and Bharat B. Aggarwal1,2

From the Cytokine Research Laboratory, Departments of 1Experimental Therapeutics and 2Head and Neck Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Because of its ability to suppress tumor cell proliferation, angiogenesis, and inflammation, the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) is currently in clinical trials. How SAHA mediates its effects is poorly understood. We found that in several human cancer cell lines, SAHA potentiated the apoptosis induced by tumor necrosis factor (TNF) and chemotherapeutic agents and inhibited TNF-induced invasion and receptor activator of NF-κB ligand-induced osteoclastogenesis, all of which are known to require NF-κB activation. These observations corresponded with the down-regulation of the expression of anti-apoptotic (IAP1, IAP2, X chromosome-linked IAP, Bcl-2, Bcl-xL, TRAF1, FLIP, and survivin), proliferative (cyclin D1, cyclooxygenase 2, and c-Myc), and angiogenic (ICAM-1, matrix metalloproteinase-9, and vascular endothelial growth factor) gene products. Because several of these genes are regulated by NF-κB, we postulated that SAHA mediates its effects by modulating NF-κB and found that SAHA suppressed NF-κB activation induced by TNF, IL-1β, okadaic acid, dexamethasone, lipopolysaccharide, H2O2, phorbol myristate acetate, and cigarette smoke; the suppression was not cell type-specific because both inducible and constitutive NF-κB activation was inhibited. We also found that SAHA had no effect on direct binding of NF-κB to the DNA but inhibited sequentially the TNF-induced activation of IκBα kinase, IκBα phosphorylation, IκBα ubiquitination, IκBα degradation, p65 phosphorylation, and p65 nuclear translocation. Furthermore, SAHA inhibited the NF-κB-dependent reporter gene expression activated by TNF, TNFR1, TRADD, TRAF2, NF-κB-inducing kinase, IκB kinase, and the p65 subunit of NF-κB. Overall, our results indicated that NF-κB and NF-κB-regulated gene expression inhibited by SAHA can enhance apoptosis and inhibit invasion and osteoclastogenesis.

One of the modes of cancer therapy involves the manipulation of transcription regulated by histone acetylation by histone acetylase transfersases and deacetylation by histone deacetylases (1). Several inhibitors of histone deacetylases, including simple compounds such as butyrate, cyclic tetrapeptides, benzamides (e.g. MS-275), and hydroxamic acids (e.g. suberoylanilide hydroxamic acid; SAHA),3 are being considered as potential therapeutic agents with which to treat cancer. SAHA has been shown to induce differentiation, growth arrest, and apoptosis of transformed human cells in culture at micromolar concentrations. SAHA was originally identified based on its ability to induce differentiation of murine erythroleukemia cells (2). Subsequently, it was found to induce differentiation of human breast adenocarcinoma cells (3) and growth arrest in human prostate carcinoma (4), rhodomyosarcoma (5), and bladder transitional cell carcinoma cells (6). SAHA has also been shown to induce apoptosis in a wide variety of cells, including T cell leukemia (7, 8), acute promyelocytic leukemia (9), chronic myeloid leukemia (10, 11), promyelomonocytic leukemia (12), multiple myeloma (13), prostate carcinoma (4), and melanoma (14) cells. Furthermore, SAHA has been shown to suppress angiogenesis (15). How SAHA mediates its many effects is poorly understood. It has been shown to up-regulate the expression of the cyclin-dependent kinase inhibitor p21 (6, 16) and down-regulate the expression of caspase inhibitors (12, 14, 16), c-Myc (11, 17), cyclin D1 (10), daxx (9), and thioredoxin (18). SAHA also down-regulates the production of mediators of inflammation, including nitric oxide, tumor necrosis factor (TNF), interleukin (IL)-1b, and IL-12 (19).

Because genes involved in the regulation of apoptosis, proliferation, angiogenesis, and inflammation are regulated by the transcription factor NF-κB, we postulated that SAHA mediates its effects by modulating NF-κB activation and the associated gene expression. NF-κB activation is regulated by IκB kinase (IKK) and leads to IκBα phosphorylation, ubiquitination, and degradation and, sequentially, the release of NF-κB subunits p50 and p65 for translocation to the nucleus, binding to their consensus sequences, and induction of gene transcription.

The present study was designed to determine whether SAHA mediates its effects by suppressing the NF-κB pathway. Specifically, we investigated the effects of SAHA on the cellular responses that require NF-κB activation, the NF-κB activation pathway, and on NF-κB-regulated gene expression. Because TNF is a potent activator of the NF-κB activation pathway and because how TNF activates NF-κB is well characterized (20), we used this inducer to examine the mechanism of action of

3 The abbreviations used are: SAHA, suberoylanilide hydroxamic acid; NF-κB, nuclear factor-κB; IκB, inhibitory subunit of NF-κB; IKK, IκB kinase; SEAP, secretory alkaline phosphatase; PMA, phorbol myristate acetate; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; IAP, inhibitor-of-apoptosis protein; COX, cyclooxygenase; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; IL, interleukin; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay; TSA, trichostatin A; NaB, sodium butyrate; RANKL, receptor activator of NF-κB ligand; NIK, NF-κB-inducing kinase; PARP, poly(adenosine diphosphate-ribose)polymerase; MITT, 3-(4,5-dimethylthiazole)-2,5-diphenyl tetrazolium bromide.

1 This work was supported in part by a grant from the Clayton Foundation for Research, Department of Defense U. S. Army Breast Cancer Research Program Grant BC010610, National Institutes of Health PO1 Grant CA91844 on lung chemoprevention, and National Institutes of Health P50 Head and Neck SPORE Grant P50CA97007 (all to B. B. A.) and grants from the Odyssey Program and the Theodore N. Law Award for Scientific Achievement Fund from The University of Texas M. D. Anderson Cancer Center (to Y. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2 A Ransom Horne, Jr., Distinguished Professor of Cancer Research. To whom correspondence should be addressed: Cytokine Research Laboratory, Dept. of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Tel.: 713-792-3503/6459; Fax: 713-794-1613; E-mail: aggarwal@mdanderson.org.
SAHA. The results showed that SAHA potentiated apoptosis induced by TNF and chemotherapeutic agents and inhibited cell invasion and osteoclastogenesis. These effects were associated with the inhibition of NF-κB activation and NF-κB-regulated gene expression. The results of our studies provide a description of mechanisms through which SAHA could mediate its effects. The ability of SAHA to enhance apoptosis, suppress invasion, and inhibit osteoclastogenesis provides novel targets for cancer therapy.

MATERIALS AND METHODS

Reagents—SAHA was purchased from Midwest Research Institute, Kansas, MO (other suppliers of SAHA include Aton Pharma, Inc., Tarrytown, NY; BioVision, Mountain View, CA; Alexis Biochemicals, Grunberg, Germany). A 50-mM solution of SAHA was prepared in 100% Me2SO, stored as small aliquots at −20 °C, and then diluted as needed in cell culture medium. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of 5 × 10^8 units/mg, was kindly provided by Genentech (South San Francisco, CA). Cigarette smoke condensate, prepared as previously described (21), was kindly supplied by Dr. Chandra Gairola (University of Kentucky, Lexington, KY). Penicillin, streptomycin, Iscove’s modified Dulbecco medium, and FBS were obtained from Invitrogen. PMA, okadaic acid, H2O2, and anti-β-actin antibody were obtained from Sigma. Antibodies against p65, p50, IκBα, ICAM-1, c-Myc, cyclin D1, matrix metalloproteinase-9, poly(adenosine diphosphate-ribose)polymerase (PARP), IAP1, IAP2, Bcl-2, Bcl-xL, and TRAF1 and the annexin V staining kit were obtained from Cell Signaling (Beverly, MA). Anti-IKK-α, anti-IKK-β, and anti-FIAP antibodies were kindly provided by Imagenex (San Diego, CA).

Cell Lines—Human myeloid KBM-5 cells, mouse macrophage Raw 264.7 cells, human lung adenocarcinoma H1299 cells, human embryonic kidney A293 cells, and human squamous cell carcinoma MDA1986 and SCC-4 cells were obtained from American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove’s modified Dulbecco medium supplemented with 15% FBS. Raw 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium, H1299 cells were cultured in RPMI 1640 medium, and A293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. MDA1986 and SCC-4 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, nonessential amino acids, pyruvate, glutamine, and vitamins. All media were also supplemented with 100 units/ml of penicillin and 100 μg/ml of streptomycin.

Live and Dead Assay—To assess cytotoxicity, we used the Live and Dead assay (Molecular Probes, Eugene, OR), which determines intracellular esterase activity and plasma membrane integrity as previously described (22).

Cytotoxicity Assay—The effect of SAHA on the cytotoxic effects of TNF and chemotherapeutic reagents was determined by the 3-(4,5-dimethylthiazole)-2,5-diphenyl tetrazolium bromide (MTT) uptake method as previously described (22).

Annexin V Staining Assay—To determine the early effect of apoptosis, we used annexin V staining assay as previously described (22).

TUNEL Assay—We also assayed cytotoxicity by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) method as previously described (22).

Invasion Assay—The membrane invasion culture system was used to assess cell invasion because invasion through the extracellular matrix is a crucial step in tumor metastasis. The BD BioCoat Tumor Invasion system is a chamber that has a light-tight polyethylene terephthalate membrane with 8-μm-diameter pores and is coated with a reconstituted basement membrane gel (BD Biosciences). A total of 2.5 × 10^4 H1299 cells were suspended in serum-free medium and seeded into the upper wells. After incubation overnight, cells were treated with 3 μM SAHA for 12 h and then stimulated with 1 nM TNF for a further 24 h in the presence of 1% FBS and the SAHA. The cells that invaded through the Matrigel were stained with 4 μg/ml of Calcein AM (Molecular Probes) in phosphate-buffered saline for 30 min at 37 °C and subjected to scan fluorescence with a Victor 3 luminometer (PerkinElmer Life Sciences).

Osteoclast Differentiation Assay—To determine whether SAHA could suppress RANKL-induced osteoclastogenesis, we cultured RAW 264.7 cells, which can differentiate into osteoclasts by RANKL in vitro (23).

Electrophoretic Mobility Shift Assay (EMSA)—To assess NF-κB activation, we performed EMSA as described previously (24).

Western Blot Analysis—To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts and fractionated them by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (25).

IKK Assay—To determine the effect of SAHA on TNF-induced IKK activation, IKK assay was performed by a method we described previously (26).

NF-κB-dependent Reporter Gene Expression Assay—The effect of SAHA on NF-κB-dependent reporter gene transcription induced by TNF and various genes was analyzed by secretory alkaline phosphatase (SEAP) assay as previously described (22).

Immunocytochemical Analysis of NF-κB p65 Localization—The effect of SAHA on the nuclear translocation of p65 was examined by immunocytochemistry as previously described (27).

Luciferase Assay—The effect of SAHA on COX-2 promoter activity induced by TNF was analyzed by luciferase assay as previously described (28).

RT-PCR Assay—The effect of SAHA on TNF-induced expression of COX-2 mRNA was analyzed using RT-PCR with β-actin as an internal control as previously described (28).

Chromatin Immunoprecipitation Assay—The effect of SAHA on NF-κB-binding to COX-2 promoter was analyzed by chromatin immunoprecipitation assay as described previously (29).

RESULTS

SAHA Potentiated Apoptosis Induced by TNF and Chemotherapeutic Agents—NF-κB activation inhibits apoptosis induced by TNF and chemotherapeutic agents (30). We investigated whether SAHA affects such apoptosis in KBM-5 cells by using the Live and Dead, PARP cleavage, annexin V staining, TUNEL staining, and MTT methods. The Live and Dead assay indicated that SAHA up-regulated TNF-induced cytotoxicity from 5 to 59% (Fig. 1A). Whether the enhanced cytotoxicity was due to apoptosis was investigated. TNF-induced caspase activation, as indicated by PARP cleavage, was potentiated by SAHA (Fig. 1B). The results of annexin V staining indicated that SAHA up-regulated TNF-induced early apoptosis (Fig. 1C), and the results of TUNEL staining also showed that TNF-induced apoptosis was enhanced by incubation with SAHA (Fig. 1D). Moreover, SAHA enhanced TNF-induced cytotoxicity as analyzed by the MTT method (Fig. 1E). This histone deacetylase inhibitor also enhanced the cytotoxic effects of cisplatin, 5-FU, doxorubicin, and taxol (Fig. 1, F–I, respectively). The results from all these assays together
suggest that SAHA enhances the apoptotic effects of TNF and chemotherapeutic agents.

**SAHA Suppressed TNF-induced Invasion Activity**—The mechanism of tumor metastasis has been studied, and it is known that matrix metalloproteinases, cyclooxygenases, and adhesion molecules play a major role in it (31). It is also known that TNF can induce tumor metastasis-related genes such as matrix metalloproteinase-9, COX-2, and ICAM-1 (30). To investigate the effect of SAHA on TNF-induced metastatic activity, we examined the invasive activity in vitro. For this study, we seeded the H1299 cells into the upper wells of a Matrigel invasion cham-
SAHA Inhibits NF-κB Activation and Potentiates Apoptosis

A

B

FIGURE 2. SAHA suppresses TNF-induced invasive activity and RANKL-induced osteoclastogenesis. A, H1299 cells (2.5 × 10⁴) were seeded into the upper wells of a Matrigel invasion chamber overnight in the absence of serum, pretreated with 3 μM SAHA for 12 h, treated with 1 nM TNF for 24 h in the presence of 1% serum, and then subjected to invasion assay. The value for no-SAHA and no-TNF was set to 1.0. B, RAW 264.7 cells (1 × 10⁴) were plated overnight, pretreated with 0.3 μM SAHA for 12 h, and then treated with 5 nM RANKL. 4 and 5 days later, cells were stained for tartrate resistance acid phosphatase-positive and evaluated for osteoclastogenesis. Photographs were taken after 5 days of incubation with RANKL.

older in the absence of serum. The cells were pretreated with SAHA and then treated with TNF in the presence of 1% serum and the SAHA. TNF induced 3.3-fold higher invasive activity, and SAHA suppressed it (Fig. 2A).

SAHA Suppressed RANKL-induced Osteoclastogenesis—It has been shown that RANKL can induce osteoclastogenesis through the activation of NF-κB (32, 33). We examined whether SAHA can suppress RANKL-induced osteoclastogenesis. For this study, we pretreated RAW 264.7 cells with SAHA and then treated them with RANKL for 4 or 5 days. We found that RANKL induced osteoclast differentiation, as indicated by the expression of tartrate resistance acid phosphatase-positive, and that SAHA suppressed it (Fig. 2B).

SAHA Repressed TNF-induced NF-κB-dependent Anti-apoptotic Gene Products—We found that SAHA potentiated the apoptotic effects of TNF. Because NF-κB regulates the expression of the anti-apoptotic proteins survivin, inhibitor-of-apoptosis protein 1/2 (IAP1/2), X chromosome-linked IAP, Bcl-2, Bcl-xL, TRAF1, and FLIP (30), we examined whether SAHA can modulate the expression of these anti-apoptotic gene products induced by TNF in KBM-5 cells. The results of Western blot analysis showed that TNF induced these anti-apoptotic proteins in a time-dependent manner and that SAHA suppressed it (Fig. 3A).

SAHA Repressed Expression of TNF-induced NF-κB-dependent Gene Products Involved in Cell Proliferation and Invasion—TNF has been shown to induce ICAM-1, matrix metalloproteinase-9, c-Myc, COX-2, cyclin D1, and vascular endothelial growth factor, which have NF-κB-binding sites in their promoters (30). We pretreated KBM-5 cells with SAHA and treated them with TNF for up to 24 h, and then we prepared whole-cell extracts and analyzed protein expression by Western blot analysis. TNF induced the expression of these proteins in a time-dependent manner, and SAHA suppressed it (Fig. 3B). These results further suggest a role for SAHA in blocking the TNF-induced NF-κB activation pathway.

SAHA Blocked NF-κB Activation Induced by IL-1β, Okadaic Acid, Doxorubicin, Lipopolysaccharide, H₂O₂, PMA, and Cigarette Smoke Condensate—IL-1β, okadaic acid, doxorubicin, lipopolysaccharide, H₂O₂, PMA, and cigarette smoke condensate are potent activators of NF-κB, but the mechanisms by which these agents activate NF-κB differ (30). We used EMSA to examine the effect of SAHA on the activation of NF-κB by these agents. Pretreatment of KBM-5 cells with SAHA suppressed the activation of NF-κB induced by all agents, including TNF (Fig. 4A). These results suggest that SAHA acts at a step in the NF-κB activation pathway that is common to all eight agents.

Inhibition of NF-κB Activation by SAHA Was Not Cell Type-specific—Distinct signal transduction pathways can mediate NF-κB induction in different cell types (34, 35). Because TNF is one of the most potent activators of NF-κB and because the mechanism of activation of NF-κB is relatively well established (20), we examined the effect of SAHA on TNF-induced NF-κB activation in human myeloid KBM-5 cells (Fig. 4B) and lung adenocarcinoma H1299 cells (Fig. 4C). These cells were pretreated with different concentrations of SAHA, treated with TNF, and subjected to EMSA. TNF activated NF-κB in both cell types, and SAHA strongly inhibited most of this activation. SAHA alone did not activate NF-κB.

Whether SAHA could suppress constitutive NF-κB activation was also examined by EMSA. Human squamous cell carcinoma MDA1986 cells and SCC-4 cells are known to express constitutively active NF-κB (36). Treatment of these cells with different concentrations of SAHA suppressed constitutive NF-κB activation (Fig. 4, D and E, respectively).

SAHA Inhibited TNF-dependent NF-κB Activation—Previous studies from our laboratory have shown that at a high concentration (1 nM), TNF can activate NF-κB within 5 min and that this induction is more intense than that obtained using a 10-fold lower concentration of TNF for a longer time (37). To determine the effect of SAHA on NF-κB activation at a high TNF concentration, KBM-5 cells were pretreated with SAHA, treated with up to 1,000 pM TNF, and then analyzed for NF-κB activation by EMSA. TNF at a concentration of 1 nM activated NF-κB activity strongly; however, cells pretreated with SAHA markedly inhibited this activation (Fig. 5A). These results show that SAHA is a potent inhibitor of TNF-induced NF-κB activation.

We also investigated the length of incubation required for SAHA to suppress TNF-induced NF-κB activation. KBM-5 cells were incubated with SAHA for up to 12 h, exposed to TNF, and subjected to EMSA. SAHA by itself did not activate NF-κB, and TNF-induced NF-κB activation was inhibited by SAHA at 12 h (Fig. 5B). Treatment of cells with 30 μM SAHA for 12 h had no effect on the cell viability as determined by the MTT method (see Table 1).

NF-κB is a complex of proteins in which various combinations of Rel/NF-κB protein constitute active NF-κB heterodimers that bind specific DNA sequences (35). To show that the band visualized by EMSA in TNF-treated cells was indeed NF-κB, we incubated nuclear extracts from TNF-stimulated KBM-5 cells with antibodies against the p50 (NF-κB1) or p65 (RelA) subunit of NF-κB. Both antibodies shifted the major band to a higher molecular mass (Fig. 5C), thus suggesting that the
TNF-activated complex consisted of p50 and p65 subunits. Preimmune serum had no effect on this band, excess (100-fold) unlabeled NF-κB caused complete disappearance of the band, and a mutant oligonucleotide of NF-κB did not affect NF-κB binding activity.

To determine whether the inhibitory activity of SAHA was due to inhibition of IκBα degradation, we pretreated KBM-5 cells with SAHA, exposed them to TNF for up to 60 min, and examined them for NF-κB activation by EMSA and for IκBα status in the cytoplasm by Western blot analysis. Activation of NF-κB was detected with increased TNF incubation times, and SAHA-pretreated cells showed dramatically decreased TNF-induced activation of NF-κB (Fig. 5D). The translocation of NF-κB to the nucleus is preceded by the proteolytic degradation of IκBα (35). In our study, TNF induced IκBα degradation in control cells within 5 min, but in SAHA-pretreated cells, TNF had no effect on IκBα degradation (Fig. 5E). These results indicate that SAHA inhibits both TNF-induced NF-κB activation and IκBα degradation.

SAHA Inhibited TNF-dependent IκBα Phosphorylation and Ubiquitination—To determine whether the inhibition of TNF-induced IκBα degradation was due to inhibition of IκBα phosphorylation and ubiquitination, we used the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal to block degradation of IκBα (38). KBM-5 cells were pretreated with SAHA, treated with 50 μg/ml N-acetyl-leucyl-leucyl-norleucinal for 30 min, exposed to TNF, and then examined for IκBα phosphorylation and ubiquitination status by Western blot analysis using an antibody that recognizes the serine-phosphorylated form of IκBα. TNF-induced IκBα phosphorylation was strongly suppressed by SAHA (Fig. 5F). The same membrane was reprobed with anti-IκBα antibody. The results show TNF-induced IκBα ubiquitination was also suppressed by SAHA (Fig. 5F).

SAHA Inhibited TNF-induced IKK Activation—IKK is required for TNF-induced phosphorylation of IκBα (35). Because SAHA inhibited the phosphorylation of IκBα, we determined its direct effect on TNF-
induced IKK activation in KBM-5 cells. Results from the immune complex kinase assay showed that TNF activated IKK as early as 5 min after TNF treatment but that SAHA strongly suppressed this activation (Fig. 5G). Neither TNF nor SAHA affected the expression of IKK-α or IKK-β proteins.

To evaluate whether SAHA suppressed IKK activity directly by binding the IKK protein or indirectly by suppressing the activation of IKK, we incubated whole-cell extracts from untreated and TNF-treated KBM-5 cells with up to 30 μM SAHA. Results from the immune complex kinase assay showed that SAHA did not directly affect the activity of IKK, suggesting that SAHA modulates TNF-induced IKK activation (data not shown).

SAHA Inhibited TNF-induced Nuclear Translocation of p65—To determine whether SAHA directly modified the binding of NF-κB complex to the DNA, we incubated nuclear extracts from TNF-treated KBM-5 cells with up to 30 μM SAHA and then analyzed DNA binding

FIGURE 4. A, SAHA blocks NF-κB activation induced by IL-1β, okadaic acid, doxorubicin, lipopolysaccharide, H₂O₂, PMA, and cigarette smoke condensate. KBM-5 cells were preincubated with 30 μM SAHA for 12 h and then treated with 0.1 nM TNF, 100 ng/ml of IL-1β or 10 μg/ml of lipopolysaccharide (LPS) for 30 min, 50 nM okadaic acid (OA) or 5 μM doxorubicin (Dox) for 4 h, 250 μM H₂O₂ for 2 h, or 15 μg/ml of phorbol 12-myristate 13-acetate (PMA) or 10 μg/ml of cigarette smoke condensate (CSC) for 1 h. The cells were then analyzed for NF-κB activation by EMSA. B, C, D, and E, SAHA suppresses TNF-induced NF-κB activation in MDA1986 cells (D) and SCC-4 cells (E) were incubated with different concentrations of SAHA for 12 h. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA.

SAHA Inhibits NF-κB Activation and Potentiates Apoptosis
activity by EMSA. Our results showed that SAHA did not modify the DNA binding ability of the NF-κB complex (Fig. 6A), and we concluded that SAHA inhibits NF-κB activation indirectly rather than directly.

TNF induces the phosphorylation of p65, which is required for its transcriptional activity (39). After phosphorylation, the NF-κB p65 subunit is translocated to the nucleus. In our study, Western blot analysis
TABLE 1
Effect of SAHA on cell viability of human myeloid cells

Human myeloid KBM-5 cells (5,000 cells/well) were treated in triplicate with different concentrations of SAHA for the indicated times, and then cell viability was examined by the MTT method.

| Dose (μM) | 12 h | 24 h |
|-----------|------|------|
|           | Mean | S.D. | Percent | Mean | S.D. | Percent |
| 0         | 0.59 | 0.01 | 100     | 0.72 | 0.01 | 100     |
| 1         | 0.66 | 0.00 | 111     | 0.70 | 0.01 | 98      |
| 3         | 0.66 | 0.00 | 112     | 0.69 | 0.01 | 95      |
| 10        | 0.67 | 0.01 | 113     | 0.68 | 0.01 | 95      |
| 30        | 0.65 | 0.01 | 111     | 0.66 | 0.00 | 92      |
| 100       | 0.63 | 0.00 | 107     | 0.69 | 0.03 | 95      |

FIGURE 6. SAHA inhibits TNF-induced nuclear translocation of p65. A, direct effect of SAHA on the NF-κB complex. Nuclear extracts were prepared from untreated KBM-5 cells or cells treated with 0.1 nM TNF for 30 min, incubated for 30 min with the indicated concentrations of SAHA, and then assayed for NF-κB activation by EMSA. B, Western blot analysis of p65 using nuclear extracts. Cells were incubated with 30 μM SAHA for 12 h and treated with 0.1 nM TNF for the indicated times. Nuclear extracts were prepared and subjected to Western blot analysis using anti-p65 antibody. For loading control of nuclear protein, the membrane was blotted with anti-PARP antibody. C, Western blot analysis of p65 and phosphorylated p65 using cytoplasmic extracts. Cells were incubated with 30 μM SAHA for 12 h and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts were prepared and subjected to Western blot analysis using anti-p65 antibody and phospho-specific anti-p65 antibody. D, immunocytochemical analysis of p65 localization. Cells were incubated with 30 μM SAHA for 12 h and then treated with 1 nM TNF for 15 min. Cells were subjected to immunocytochemical analysis as described under “Materials and Methods.”
SAHA Inhibits NF-κB Activation and Potentiates Apoptosis

FIGURE 7. SAHA represses NF-κB-dependent reporter gene expression induced by TNF. A, SAHA inhibits the NF-κB-dependent reporter gene expression induced by TNF. A293 cells were transiently transfected with an NF-κB-containing plasmid for 24 h. After transfection, the cells were incubated with the indicated concentrations of SAHA for 12 h and then treated with 1 nM TNF for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity. B, SAHA inhibits the NF-κB-dependent reporter gene expression induced by TNF, TNFR1, TRADD, TRAF2, NIK, IKK, and p65. Cells were transfected with an NF-κB-containing plasmid alone or with the indicated plasmids. After transfection, cells were incubated with 3 μM SAHA for 12 h and then incubated with the relevant plasmid for an additional 24 h. For TNF-treated cells, cells were incubated with 3 μM SAHA for 12 h and then treated with 1 nM TNF for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity. C, SAHA inhibits COX-2 promoter activity induced by TNF. Cells were transiently transfected with a COX-2 promoter linked to the luciferase reporter gene plasmid for 24 h and then incubated with the indicated concentrations of SAHA for 12 h. Cells were then treated with 1 nM TNF for an additional 24 h, lysed, and subjected to a luciferase assay. Experiments in panels A–C are presented as means ± S.D. D, SAHA inhibits COX-2 mRNA expression induced by TNF. KBM-5 cells were pretreated with 3 μM SAHA for 12 h, treated with 1 nM TNF for 4 h, and then analyzed for COX-2 mRNA expression by RT-PCR. E, SAHA inhibits binding of NF-κB to the COX-2 promoter. Cells were pretreated with 3 μM SAHA for 12 h and then treated with 1 nM TNF for the indicated times. The proteins were cross-linked with DNA by formaldehyde and then subjected to chromatin immunoprecipitation assay using an anti-p65 antibody with the COX-2 primer. Reaction products were resolved by electrophoresis.

showed that TNF induced nuclear translocation of p65 in a time-dependent manner in KBM-5 cells, as early as 5 min after TNF stimulation (Fig. 6B). When the cells were pretreated with SAHA, TNF failed to induce nuclear translocation of p65. We also determined the effect of SAHA on TNF-induced phosphorylation of p65 in cytoplasm. Western blot analysis showed that TNF induced the phosphorylation of p65 within 5 min but that this phosphorylation gradually decreased and that SAHA strongly suppressed this phosphorylation (Fig. 6C).

An immunocytochemical assay confirmed the effect of SAHA on the suppression of nuclear translocation of p65. In untreated KBM-5 cells, p65 localized in the cytoplasm, TNF induced its nuclear translocation, and SAHA suppressed its nuclear translocation to the nucleus (Fig. 6D).

SAHA Repressed TNF-induced NF-κB-dependent Reporter Gene Expression—Although we have shown by EMSA that SAHA blocked NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (40). TNF-induced NF-κB activation is mediated through sequential interaction with the TNF receptor. To determine the effect of SAHA on TNF-induced NF-κB-dependent reporter gene expression, we transiently transfected A293 cells with the NF-κB-regulated SEAP reporter construct, incubated them with up to 3 μM SAHA, and then stimulated them with TNF. We found that TNF induced NF-κB-regulated reporter gene expression and that SAHA suppressed it in a dose-dependent manner (Fig. 7A).

TNF-induced NF-κB activation is mediated through sequential interaction of the TNF receptor with TRADD, TRAF2, NIK, and IKK, resulting in phosphorylation of IκBα (41, 42). To determine the effect of SAHA on TNF-induced NF-κB-dependent reporter gene expression, A293 cells were transiently transfected with TNFR1-, TRADD-, TRAF2-, NIK-, IKK-, and p65-expressing plasmids and then monitored for NF-κB-dependent SEAP expression. Our results revealed that cells transfected with these plasmids showed NF-κB-regulated reporter gene expression and that all were suppressed by SAHA (Fig. 7B). These results suggest that the effect of SAHA occurs at a step downstream from p65.

SAHA Inhibited TNF-induced COX-2 Expression—TNF induces COX-2, which has NF-κB-binding sites in its promoter (30). Because down-regulation of NF-κB by SAHA suppressed the expression of NF-κB-regulated gene products, we examined the effect of SAHA on TNF-induced COX-2 promoter activity by using a COX-2 promoter-luciferase reporter plasmid. A293 cells were transiently transfected with this plasmid, pretreated with up to 3 μM SAHA, exposed to TNF, and then analyzed for luciferase activity by using whole-cell lysate. We found that TNF induced COX-2 promoter activity, which was sup-
pressed by SAHA in a dose-dependent manner (Fig. 7C). We also examined the effect of SAHA on induction of COX-2 mRNA transcript. KBM-5 cells were pretreated with SAHA and exposed to TNF for up to 4 h, and the total RNA was purified and subjected to RT-PCR. TNF induced COX-2 mRNA in a time-dependent manner, and treatment with SAHA inhibited it (Fig. 7D).

Whether the lack of TNF-induced COX-2 expression in SAHA-treated cells was due to suppression of NF-κB activation in vivo was examined by chromatin immunoprecipitation assay targeting NF-κB binding in the COX-2 promoter. KBM-5 cells were pretreated with SAHA, treated with TNF for up to 2 h, and cross-linked in situ with DNA-protein complexes, and the chromatin was isolated and sheared. Subsequently, the chromatin was immunoprecipitated with anti-p65 antibody, and the DNA was purified and subjected to PCR using COX-2 promoter-specific primer. We found that TNF induced NF-κB binding to COX-2 promoters in a time-dependent manner and that SAHA suppressed it (Fig. 7E). Overall, these results suggest that SAHA inhibits NF-κB-regulated gene expression by suppressing NF-κB binding to the portion of the COX-2 promoter.

**DISCUSSION**

This study was designed to determine whether SAHA mediates its effects by suppressing the NF-κB pathway. We found that SAHA potentiated the apoptosis induced by TNF and chemotherapeutic agents, inhibited TNF-induced invasion and RANKL-induced osteoclastogenesis, and down-regulated the expression of anti-apoptotic, proliferative, and angiogenic gene products. We also demonstrated that SAHA suppressed NF-κB activation induced by a wide variety of agents, inhibited both inducible and constitutive NF-κB activation, and inhibited IKK activation, IκBα phosphorylation, IκBα ubiquitination, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and NF-κB-dependent reporter gene expression.

Our observation that SAHA potentiated the apoptotic effects of TNF and chemotherapeutic agents agrees with results from other studies. Nakata et al. (43) reported that SAHA sensitized human malignant tumor cells to apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL)/APO2-L. Rueff et al. (7, 44) found that SAHA sensitized cells to various chemotherapeutic agents. Kim et al. (45) showed that pretreatment of cells with SAHA increased the killing efficiency of VP-16, ellipticine, doxorubicin, and cisplatin in cancer cell lines. Rueff et al. (7) implicated the role of P-glycoprotein and BID cleavage in cell chemosensitization to SAHA. Nimmanapalli et al. (46) found that SAHA enhanced imatinib-induced apoptosis of Bcr-Abl-positive human acute leukemia cells. Our own results implicate the role of NF-κB activation induced by cytokines and chemotherapeutic agents and are consistent with published reports that NF-κB activation can suppress apoptosis induced by cytokines (47–49) and chemotherapeutic agents (50–52). We also found that SAHA modulated the expression of anti-apoptotic genes regulated by NF-κB.

Our studies showed for the first time that SAHA inhibited TNF-induced invasion of tumor cells. The role of matrix metalloproteinase-9 and adhesion molecules in invasion has been demonstrated (53–55), and we found that the expression of these gene products was down-regulated by SAHA.

Our studies also demonstrated for the first time that SAHA abolished the RANKL-induced differentiation of monocytes into osteoclasts. This result is consistent with those of Rahman et al. (56), who observed that trichostatin A (TSA) and sodium butyrate (NaB), both inhibitors of histone deacetylase, suppressed the differentiation of monocytes into osteoclasts. This suppression was linked to the suppression of TNF-induced NF-κB activation by TSA and NaB.

We also showed for the first time that SAHA inhibited NF-κB activation induced by agents (e.g. TNF, IL-1β, PMA, lipopolysaccharide, and cigarette smoke condensate) that activate NF-κB through different mechanisms (21, 26, 57, 58). These results suggest that SAHA acts at a step common to all of these activators. In response to most of these stimuli, NF-κB activation proceeds through sequential activation of IKK, phosphorylation at serines 32 and 36 of IκBα, and ubiquitination at lysines 21 and 22 of IκBα, leading finally to degradation of IκBα and the release of NF-κB (35). We found that SAHA blocked NF-κB activation by inhibiting IKK. Whether other histone deacetylase inhibitors could suppress NF-κB activation is highly controversial. Some researchers have observed that NaB and TSA can suppress NF-κB activation (56, 59, 60), whereas Chen et al. (61) found that TSA enhanced TNF-induced NF-κB activation in human embryonic kidney 293 cells, and Mayo et al. (62) found no effect of TSA and NaB on NF-κB activation. Why there is such a discrepancy in results is not clear. Whether cell type, inhibitor dose, exposure length, type of NF-κB activator, or method used to determine NF-κB activation (e.g. reporter assay or DNA binding assay) affects the outcome requires investigation.

We clearly showed that SAHA alone did not affect DNA binding of NF-κB or NF-κB-dependent reporter gene expression and that it did not directly inhibit IKK activity. SAHA did, however, block TNF-induced activation of IKK, which led to suppression of IκBα phosphorylation, ubiquitination, and subsequently degradation. The phosphorylation and nuclear translocation of p65 were also abolished by SAHA. How SAHA inhibits the activation of IKK, which has been shown to phosphorylate p65 (63–66), is unclear. Numerous kinases, including Akt, have been implicated in the activation of IKK. NaB and TSA have been shown to inhibit Akt (62). Thus, SAHA might inhibit TNF-induced IKK activation by suppressing Akt, which has also been shown to phosphorylate p65 (41). Therefore, the suppression of p65 phosphorylation by SAHA could also be due to inhibition of Akt. Whether SAHA interferes with the TNF-induced NF-κB activation pathway by suppressing other, unknown kinases is uncertain. Using a photoaffinity labeling technique, Webb et al. (67) identified ribosomal protein S3 as a potential target of SAHA. Whether this protein has any role in NF-κB activation is unknown at present.

We showed that SAHA inhibited NF-κB-regulated gene transcription and NF-κB-regulated gene products involved in cell proliferation (e.g. cyclin D1 and COX-2), anti-apoptosis (e.g. survivin, IAP1, IAP2, X chromosome-linked IAP, Bcl-2, Bcl-xL, Bcl-1/A1, TRAF1, and FLIP), and invasion (matrix metalloproteinase-9 and vascular endothelial growth factor). To our knowledge, there is no other published report of the regulation of these gene products by SAHA. SAHA induction of cell cycle arrest (4, 68) may be due to down-regulation of cyclin D1, as we have described. The down-regulation of inflammatory cytokines TNF and IL-1 and of NO (19), c-Myc (17), and thioredoxin (18) by SAHA could also be due to suppression of NF-κB, as we have described. SAHA has also been shown to reduce acute graft-versus-host disease and preserve the graft-versus-leukemia effect (69); these effects may also be mediated through NF-κB suppression, as we have described.

Overall, the results of our studies provide a description of mechanisms through which SAHA could mediate its effects. The ability of SAHA to enhance apoptosis, suppress invasion, and inhibit osteoclastogenesis provides novel targets for cancer therapy.

**Acknowledgment**—We thank Elizabeth L. Hess for carefully proofreading the manuscript and providing valuable comments. We also thank Dr. Bryant Darnay for RANKL-protein.
