Histone Deacetylase Inhibitors Activate NF-κB in Human Leukemia Cells through an ATM/NEMO-related Pathway

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This article has been withdrawn by the authors. The image used to represent U937 cells treated without TBAP for 4 h in Fig. 4A was reused to represent U937/3.1EV cells treated with LBH for 4 h in Fig. 7B. In Fig. 4C, lanes 1 and 2 of the left pIκK-β/α panel were duplicated, lanes 1 and 3 of the right pIκK-β/α panel were duplicated, lanes 4 and 5 of the left IκK-β/α panel were duplicated, and lanes 2–5 of the left actin panel were duplicated in lanes 2–5 of the right actin panel. The actin immunoblot in Fig. 6A was reused as the actin immunoblot in Fig. 7A. Part of the actin panels in Fig. 7A was reused as actin in Fig. 6B. In Fig. 6B, lanes 1 and 2 of the left actin panel were duplicated in lanes 2 and 3 of the right actin panel. In supplemental Fig. 1A, lane 3 of the actin immunoblot from Jurkat cells was reused as lane 1 of the actin immunoblot from HL-60 cells. In supplemental Fig. 3A, lanes 1 and 2 of the TRAF2 immunoblot were duplicated. The actin immunoblot from supplemental Fig. 4B was reused as the actin immunoblot in supplemental Fig. 5B.

Chromatin structure and gene expression are regulated by reversible acetylation of lysine residues in histone tails, a process comprising a component of the histone code (1). Histone acetylation is regulated reciprocally by histone deacetylases (HDACs) and histone acetyltransferases (2). Histone deacetylase inhibitors, a group of structurally diverse compounds, have shown encouraging activity in certain hematopoietic malignancies, including cutaneous T-cell lymphoma and acute leukemia (3, 4). Numerous mechanisms have been proposed to account for HDACI-mediated lethality, including oxidative damage, up-regulation of death receptors or proapoptotic genes, such as members of the tumor necrosis factor receptor superfamily, and activation of prosurvival genes, including antiapoptotic Bcl-2 family members (5). HDACIs also increase acetylation of various non-histone proteins including chaperone proteins (7), DNA repair proteins (3, 5, 6), and transcription factors (8, 10). These events can enhance HDACI antileukemic activity.

Mechanisms underlying histone deacetylase inhibitor (HDACI)-mediated NF-κB activation were investigated in human leukemia cells. Exposure of U937 and other leukemia cells to LBH-589 induced reactive oxygen species (ROS) followed by single strand (XRCC1) and double strand (γ-H2AX) DNA breaks. Notably, LBH-589 lethality was markedly attenuated through an ATM/NEMO/SCF(sumo)dependent process involving the induction of ROS and DNA damage and suggest that blocking NF-κB activation via the atypical ATM/NEMO nuclear pathway can enhance HDACI antileukemic activity.

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(casein kinase 2) (17). Notably, exposure of cells to HDACi results in p65 acetylation on lysine residues (e.g., Lys-221 and Lys-310), which diminishes binding of p65 to IkBα, enhances p65 nuclear translocation, and reduces p65 nuclear export while increasing p65 nuclear binding and transactivation (18, 19). HDACi-mediated acetylation of p65 and diminished affinity for IkBα may explain the more sustained activation of p65 that occurs with such agents compared with that triggered by TNFα (19).

A novel pathway of NF-κB activation, described recently, originates in the nucleus and is associated with DNA damage (20–22). Double-stranded DNA breaks initiate signals that trigger SUMOylation of nuclear-localized NEMO, preventing its nuclear export (23). Concomitantly, these breaks activate ATM (ataxia-telangiectasia mutant), which phosphorylates SUMO-modified NEMO, promoting the removal of SUMO and enhancing NEMO ubiquitination (24). Ubiquitinated NEMO then translocates to the cytoplasm, where it phosphorylates IKK in cooperation with ATM and the ELKS (glutamate-, NEMO then translocates to the cytoplasm, where it phosphor-

Although the contribution of HDACi-mediated acetylation to sustained p65 activation is well recognized (13, 18, 19), the mechanism by which HDACi initially trigger IKK and p65 has not yet been elucidated. However, the recent description of novel DNA damage/p65 activation pathway, as well as accumulating evidence that HDACi trigger oxidative stress (5, 26), raises the possibility that the two pathways may be related. To address this question, we used site-directed mutagenesis of the components of the DNA damage response system, particularly ATM and NEMO, in particular to test whether the present findings identify the ATM/NEMO pathway as a critical mediator of p65 activation in HDACi-treated cells exposed to HDACi. They also demonstrate that disruption of this pathway substantially lowers the threshold for HDACi-induced lethality.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—U937, HL-60, and Jurkat human leukemia cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), and maintained as described (27). Cells expressing various siRNAs were generated by transfection with an Amxax Nucleofector (Lonza, Conshohocken, PA) of pSilencer vector (Ambion, Austin, TX) harboring the following oligonucleotides: histone H1.2 (5‘-AAGAGCGTACGGGAGTTTGC-3’); ATM, NEMO, and scrambled control as described previously (28–30); pSilencer-siTRAF2 cells (kindly provided by Dr. M. Rahmani, Virginia Commonwealth University, Richmond, VA). All experiments utilized logarithmic phase cells (2.5 × 10⁵ cells/ml). Additional control cell lines were generated (31) including pSilencer siRNAs with two nucleotide changes from the target sequence described above (i.e. siH1.2-N, siNEMO-N, siTRAF2-N, and siATM-N; pSilencer vector) and a second specific siRNA using SureSilencing shRNA plasmids (i.e. shH1.2, shTRAF2, shNEMO, and shATM; SABiosciences, Frederick, MD). The corresponding sequences, cloned into a pSilencer vector harboring the following two-base mutated oligonucleotides (underlined), were: shH1.2-N, 5’-AAGAGCGTACGGGAGTTTGC-3’; siATM-N, 5’-AAGCGGCTGATCCGAGATCTC-3’; siTRAF2-N, 5’-CGCATGAAACATCGGAAG-3’; and siNEMO-N, 5’-AAGATTGTGATTGAGACCGTACGG-3’. Cells expressing a second specific siRNA were generated using SureSilencing (SABiosciences) shRNA plasmids: shH1.2, 5’-AAGGTTAGGAAGCCCAAGAAA-3’; shTRAF2, 5’-CAGGGGCAATATATTGAAGA-3’; shNEMO, 5’-AGAGGTCTCCTATGTGCAAAT-3’; shATM, 5’-GGCACTGCTATCCGAGAAG-3’; and scrambled control shC, 5’-GGAATCTCAATCTGCGATCATC-3’.

**Drugs and Chemicals**—LBH-589 (panobinostat) was provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ). Mn-TBAP was purchased from EMD-Calbiochem (Gibbstown, NJ). Vorinostat (SAHA) was provided by Merck (Whitehouse Station, NJ).

**Assessment of Apoptosis**—Apoptosis was evaluated by annexin V/propidium iodide (PI) (BD Biosciences) staining as described previously (12).

**Cell Cycle Analysis**—Cell cycle analysis by flow cytometry was performed using a BD Biosciences FACScan flow cytometer and Verity WinList software (Verity Software, Topsham, ME) as described previously (32).

**Western Blot Analysis**—Western blot was performed as described previously (32). The primary antibodies and dilutions used were: histone H1.2 (1:3000; Abcam, Cambridge, MA); γ-H2AX (1:2000; Upstate-Millipore, Billerica, MA); Bak (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); conformationally changed Bak-Ab1 (EMD-Calbiochem); actin (1:4000; Sigma-Aldrich); and ATM and pATM (1:1000; Cell Signaling Technology, Danvers, MA). Secondary antibodies conjugated to horseradish peroxidase were from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

**Immunoprecipitation Assay**—Analyses of protein complexes by immunoprecipitation including conformationally changed Bak were performed using CHAPS lysis buffer and immunomagnetic Dynabeads M-450 microspheres (Invitrogen) (26).
(Molecular Probes/Invitrogen). No positive cells were identified when specific antibodies were replaced by isotype-matched control antibody. Cells were covered with mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA). Images were captured using a Zeiss confocal laser microscope (Carl Zeiss, Yena, Germany) and LSM 510 software.

Detection of Single Strand DNA Breaks by Flow Cytometry—Single-stranded DNA breaks were determined with the ApoassDNA kit (Cell Technology, Mountain View, CA) and analyzed by flow cytometry as per the manufacturer's instructions.

Chromatin Immunoprecipitation Assay—After treatment, cells were processed with a two-step fixation method (33) using an NF-κB/p65 antibody (Upstate Biotechnology/Millipore). PCR amplification was performed using 1–2 μl of the bound fractions and 1/20th of the inputs. The MnSOD2 gene-specific PCR primer sequences were: NF-κB site-1, sense, 5'–CCTGT-AATCCCCAGCATTGG 3', and antisense, 5'–TGGATCCTCC-TGCTTAGCC-3'; NF-κB site-2, sense, 5'–TGGCTTACGCT-GTGAATCC-3'; and antisense, 5'–GGGTCCAAGCGATTGTCC-3'; and AP1 site-1, sense, 5'–GAGCCCCCCAGACTTTGT-CCTTC-3', and antisense, 5'–AGTCGATCTCGGGTTGGGATG-3'.

Extraction of RNA and Real-time Reverse Transcriptase-Polymerase Chain Reaction—Total RNA was extracted using the RNeasy Isolation Kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed in triplicate using the SensiMix One-Step PCR promoter was observed with primers corresponding to B site 1 (Fig. 1C, lower panel) and the recognition site for AP-1, was also amplification did not yield detectable PCR products. These findings may reflect the differential regulatory activity of both NF-κB sites in which the proximal site (NF-κB site 1) regulates basal expression, whereas the distal site (NF-κB site 2) is responsible for NF-κB-mediated inducible expression (39, 40); they are consistent with SOD2 mRNA induction by LBH-589 in U/EV control cells (Fig. 1C).

RESULTS
Induction of NF-κB Activity and Regulation of ROS by HDACIs—Previous studies have shown that HDACIs activate NF-κB in diverse cell types (11, 12, 34). To characterize this phenomenon in greater detail, a time course analysis of NF-κB activation was conducted (Fig. 1A) by ELISA (graph), electrophoretic mobility shift assay (inset, upper panel), and Western blot (p65/RelA translocation to the nuclear fraction; inset, lower panel). These studies demonstrated that exposure of human myeloid leukemia U937 cells to the pan-HDACI LBH-589 (20 nm) induced persistent NF-κB activation between 4 and 16 h. Similar results were observed with other HDACIs (e.g. vorinostat, LAQ-824, and sodium butyrate; data not shown) and other human lymphoblastic (Jurkat) and promyelocytic (HL-60) leukemia cells, as well as primary acute myeloid leukemia specimens (supplemental Fig. 1).

Generation of ROS has been implicated in HDACI-mediated lethality (12, 32, 35). Consequently, detailed time course studies were performed to characterize the effects of HDACIs on oxidative injury more fully. Exposure of U937 cells to LBH-589 induced an early, transient increase in ROS, which returned to baseline levels by 8 h (Fig. 1B), presumably reflecting induction of the ROS scavenger Mn-SOD2 (12) (Fig. 1B, inset). Because the SOD2 gene is an NF-κB target (36, 37), the association among LBH-589-induced ROS generation, NF-κB activation, and lethality was investigated in greater detail. To this end, ROS levels were monitored over time following LBH-589 (20 nm) treatment in empty vector-transfected U937 cells (U/EV) and in cells expressing an IκBa‘super-repressor’ (U/IκB), which lacks the serine 32 and 36 phosphorylation sites required for proteasomal degradation (38). Although ROS levels returned to basal levels after 6–8 h of LBH-589 exposure in control cells (U/EV; see Fig. 1B), they remained persistently elevated in U/IκB cells, consistent with a lack of Mn-SOD2 induction at both the protein (Fig. 1B, inset) and mRNA levels (Fig. 1C, upper panel). In contrast, U/EV cells exposed to LBH-589 displayed robust Mn-SOD2 induction (Fig. 1, B, inset (protein), and C, upper panel (mRNA)).

NF-κB involvement in the regulation of Mn-SOD2 induction was further investigated in U937 cells treated with LBH-589 for 2 and 6 h by chromatin immunoprecipitation assay. Cross-linked DNA-protein complexes were immunoprecipitated using an anti-p65/RelA antibody followed by PCR analysis with primers recognizing MnSOD2 gene promoter NF-κB proximal region (–164 to –146) responsible for basal regulation, and NF-κB enhancer distal region site 2 (–3326/34) (39, 40). A time-dependent association of p65/RelA with the promoter region corresponding to NF-κB site 1 (Fig. 1C, lower panel), the promoter region adjacent to site 2 (Fig. 1C, inset), and the recognition site for AP-1, was also amplified showed no changes. Template DNA obtained from a negative chromatin immunoprecipitation assay using nonimmune IgG did not yield detectable PCR products. These findings may reflect the differential regulatory activity of both NF-κB sites in which the proximal site (NF-κB site 1) regulates basal expression, whereas the distal site (NF-κB site 2) is responsible for NF-κB-mediated inducible expression (39, 40); they are consistent with SOD2 mRNA induction by LBH-589 in U/EV control cells (Fig. 1C).

LBH-589-induced NF-κB Activation Protects Cells from ROS-mediated DNA Damage and Cell Death—HDACI-mediated DNA damage has been described previously, and recent findings raise the possibility that ROS generation or perturbations in the DNA repair machinery may be involved in this process (5, 8, 26, 41). To characterize the relationship between these events in greater detail, evidence of oxidative DNA damage was monitored by confocal microscopic analysis of XRCC1, a component of the base excision repair system and early response protein recruited at the site of single strand breaks (SSBs), (42), as well as γ-H2AX, a hallmark of DNA double strand breaks (Molecular Probes/Invitrogen). No positive cells were identified when specific antibodies were replaced by isotype-matched control antibody. Cells were covered with mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA). Images were captured using a Zeiss confocal laser microscope (Carl Zeiss, Yena, Germany) and LSM 510 software.

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H1.2 expression and yielded results similar to those obtained with scrambled control siRNA oligonucleotide-transfected U937/siC cells (supplemental Fig. 2A).

To investigate the role of NF-κB activation by LBH-589 in these events, the presence of DNA SSBs was monitored by flow cytometry using specific anti-DNA SSBs antibodies in control (U/EV) or U/ικBα super-repressor cells exposed to LBH-589 in the presence or absence of Mn-TBAP. Shortly after the addition of LBH-589 (8 h), a modest increase in SSBs (e.g. to 116% of control values) was detected in U/EV control cells, whereas a pronounced increase (e.g. to 156% of controls) was observed in U/ικB cells (Fig. 2C). Significantly, SSBs were abrogated in both control and U/ικB cells by Mn-TBAP. U/ικB cells monitored for the transition from SSBs (XRCC1) to DNA DSBs (H2AX) by confocal microscopy showed that LBH-589 induced extensive DNA damage, reflected by early XRCC1 foci formation (4 h, green fluorescence) followed by a rapid transition to γ-H2AX foci (8 h, red fluorescence; Fig. 2D). Notably, these effects were also abrogated by Mn-TBAP (Fig. 2D). Analysis of DNA damage at subsequent intervals (e.g. 16–24 h) revealed that in the absence of NF-κB activation (i.e. in U/ικB cells), LBH-589-mediated DNA damage (γ-H2AX formation) was dramatically increased (Fig. 2E). In accord with the pronounced increase in cell death (Fig. 1D), LBH-589-mediated release of histone H1.2 into the cytosol was significantly increased in U/ικB cells, accompanied by the pronounced conformational change and activation of the H1.2 target, the proapoptotic protein Bak (Fig. 2E). Finally, consistent with the attenuation of LBH-589-mediated cell death observed in U/ικB cells exposed to Mn-TBAP (Fig. 1D), DNA damage (γ-H2AX), release of histone H1.2 into the cytosol, and activation of Bak were all significantly diminished by co-incubation of cells with Mn-TBAP (Fig. 2E). Collectively, these findings demonstrate that LBH-589-induced ROS generation plays an important functional role in triggering DNA damage, including induction of both DNA SSBs and DSBs as well as cell death. They also highlight the important cytoprotective role that NF-κB activation plays in regulating LBH-589-mediated ROS generation, the resulting induction of DNA damage, and apoptosis in leukemic cells.
ATM/NEMO-dependent NF-κB Activation by HDAC Inhibitors

**A** Control LBH-4h LBH-8h LBH-16h LBH-24h H$_2$O$_2$ - 20’
- TBAP + TBAP

**B**

![Graph showing % Cell Death](image)

- U/siC (expressing a sequence directed against histone H1.2)
- U/siH1.2 (24 h)
- U/siC
- U/siH589 (24 h)

**C**

![Western blot analysis](image)

**D**

| Control | LBH-4h | LBH-8h |
|---------|--------|--------|
| - TBAP  |        |        |
| + TBAP  |        |        |

**E**

| S100 | U937/EV | UI/I-B-SR |
|------|---------|-----------|
| C    | C       | C         |
| 16   | 24      | 16        |
| 24   |         | 24        |

**FIGURE 2. LBH-589-induced NF-κB activity in ROS-mediated DNA damage and cell death.** A, confocal microscopic analysis of U937 cells exposed to LBH-589 (20 μM) ± Mn-TBAP (400 μM). Antibodies: green fluorescence, XRCC1; red fluorescence, γ-H2AX; blue fluorescence, DAPI. Cells exposed to H$_2$O$_2$ (10 mM, 20 min) were used as a positive control. B, U/siC (stably expressing a scrambled sequence siRNA oligonucleotide) and U/siH1.2 (expressing a sequence directed against histone H1.2) were exposed to LBH-589 (20 μM) for 24 h and analyzed for cell death induction by flow cytometry (% annexin V/PI-positive cells). Values represent mean ± S.E., * p < 0.01. Inset, Western blot analysis (whole cell lysate). C, U/EV and U/IxB cells were incubated with 20 μM LBH-589 (8 h) ± Mn-TBAP (400 μM), labeled with an anti-DNA SSBs antibody, and analyzed by flow cytometry. Values represent percentage of cells displaying an increase in DNA SSBs. D, confocal microscopy of U937/IxB cells exposed to LBH-589 (20 μM) ± Mn-TBAP (400 μM) for the indicated intervals. E, U/EV and U/IxB cells were exposed LBH-589 (20 μM) and processed as needed (i.e. whole lysates, cytosolic S-100 fraction, and immunoprecipitation (IP)) to monitor protein levels (Western blot (WB)) of γ-H2AX, histone H1.2, and conformationally changed Bak, respectively. For the latter, IgG was used to confirm equivalent loading and transfer; for the former, β-actin was employed.

**LBH-589-mediated NF-κB Activation Proceeds through a TNFα- and TRAF2-independent Process—**To identify signaling pathways involved in LBH-589-mediated NF-κB activation and to assess the involvement of the canonical, TNFα-related pathway, cells were exposed to LBH-589 in the presence or absence of TNF-soluble receptor (100 ng/ml), which antagonizes TNFα-related activity (44). Whereas the TNF-soluble receptor completely blocked TNFα-induced NF-κB activity (Fig. 3A, left panel, TNFα + SR), it had no effect on LBH-589-mediated NF-κB activation (Fig. 3A, right panel). To extend these findings to other receptor-mediated stimuli (45, 46), U937 cells expressing a siRNA directed against the adaptor and signaling protein TRAF2, a key intermediate in both the classical and alternative NF-κB signaling pathways (47, 48), were employed (Fig. 3B, inset, and supplemental Fig. 3). Consistent with the established cytoprotective role of TRAF2 in the canonical TNFα pathway (49), U937/siTRAF2 cells exposed to TNFα displayed significantly diminished NF-κB activation (Fig. 3B, left panel, and supplemental Fig. 3), which argues that HDACI-mediated NF-κB activation does not involve TNFα- or TRAF2 receptor-mediated signaling.

**HDACs Induce NF-κB Activation through an ROS-dependent Process—**The preceding findings (e.g. Fig. 1B) indicate that NF-κB activation played an important role in ROS regulation. On the other hand, previous studies have suggested a functional link between ROS generation and NF-κB activation (45), prompting us to investigate whether HDACI-mediated ROS production might be related to the induction of NF-κB. To this end, U937 cells were exposed to LHB-589 ± Mn-TBAP, which in contrast to other antioxidants such as N-acetylcysteine and pyrrolidine dithiocarbamate, known to...
ATM/NEMO-dependent NF-κB Activation by HDAC Inhibitors

Figure 3. Analysis of TNFα- and TRAF2-related signaling in LBH-589-mediated NF-κB activation. (A) NF-κB activity was determined by ELISA in nuclear extracts from U937 cells exposed to either TNFα (10 ng/ml, 2 h; left panel) or LBH-589 (20 nm) for the indicated intervals (right panel) ± TNFα-soluble receptor (SR; 100 ng/ml). B, U/siC or U/siTRAF2 cells (siRNA directed against TRAF2) were exposed to TNFα (10 ng/ml) for 2 or 24 h (cell death) and analyzed for NF-κB activity (ELISA, left panel) or cell death (annexin V/PI-positive cells) by flow cytometry (right panel). Inset, Western blot analysis of TRAF2 expression. Values represent mean ± S.E. *, p < 0.01.

modulate NF-κB activation directly, does not interfere with NF-κB activity (50, 51). Co-incubation of cells with 400 μM Mn-TBAP, a concentration that blocked LBH-589-induced ROS production (data not shown), prevented HDACI-mediated NF-κB activation, reflected by both p65/RelA ELISA (Fig. 4A, left panel) and p65 nuclear localization by confocal microscopy (Fig. 4A, right panels). In contrast, Mn-TBAP did not alter TNFα-induced NF-κB activation (Fig. 4B, left panel (p > 0.05)), nor did it affect LBH-589-mediated acetylation of histones H3 or H4 (Fig. 4B, right panel). Analysis of the NF-κB activation cascade in lysates obtained from LBH-589-treated cells cultured in the absence of Mn-TBAP revealed increased expression of the phosphorylated forms of IKKα/β within 4 to 8 h of exposure to drug (Fig. 4C). In marked contrast, Mn-TBAP-treated cells displayed no change in phospho-IKKα/β expression. The phospho-IKK target, IκBα, also exhibited pronounced phosphorylation following exposure to LBH-589 in the absence of Mn-TBAP (Fig. 4C, left panel) accompanied by modest reductions in the total levels of IκBα, presumably a consequence of ubiquitination and proteasomal degradation of the phosphorylated species (52). However, LBH-589-mediated phosphorylation of IκBα, as well as the reduction in total levels, was abrogated by Mn-TBAP (Fig. 4C). Activation of NF-κB following LBH-589 exposure was also manifested by increased mRNA expression of its target, the NFKBIA (IκBα) gene (Fig. 4D). Significantly, cells cultured in the presence of Mn-TBAP exhibited no changes in NFKBIA mRNA levels (Fig. 4D, right panel). Together, these findings implicate early ROS production in the activation of the IKK/IκBα cascade by HDACIs.

HDACIs Trigger NF-κB through a NEMO-dependent Process in Association with ATM Activation—Given evidence that oxidative stress can trigger the DNA damage-associated NF-κB response (16, 53), the relationship between HDACI-induced DNA damage and NF-κB activation was investigated. DNA damage-mediated NF-κB activation is dependent upon interactions between the ATM kinase and IKKγ/NEMO (20, 54). Phosphorylation of ATM, one of the initial kinases activated in response to DNA damage (55), was monitored in cells exposed to LBH-589. A rapid (i.e. 1 h) and sustained increase in the levels of phosphorylated ATM (pATM; Ser-1981) was observed by both confocal microscopy (Fig. 5A upper panel) and Western blot analysis (Fig. 5A, lower panel). Concomitantly, nuclear NEMO accumulation, determined by confocal immunofluorescence, occurred within 2 to 4 h of the addition of LBH-589 (Fig. 5B, upper panel). Time course immunoprecipitation analysis of NEMO/ATM interactions revealed that although ATM association with NEMO was undetectable in untreated cells, co-immunoprecipitating ATM sharply increased within 1 h of addition of LBH-589, and although subsequent declines were noted, persisted throughout the 8-h treatment interval (Fig. 5B, lower panel). Such findings suggest that as in the case of other genotoxic stimuli (16, 53), HDACIs activate the ATM/NEMO DNA damage-related pathway.
ATM/NEMO-dependent NF-κB Activation by HDAC Inhibitors

To investigate the functional role of ATM/NEMO interactions in NF-κB pathway activation, U937 cells stably expressing NEMO siRNA were generated. Because only a small fraction of the total pool of NEMO is involved in the activation of the ATM/NEMO/NF-κB pathway (20), two clones displaying only partial reductions in NEMO expression (Fig. 5C, inset [siN5 and siN12], and supplemental Fig. 4B) were selected to minimize effects on non-DNA damage-related NF-κB activity. Notably, siNEMO clones exhibited complete abrogation of LBH-589-mediated NF-κB activation compared with the responses of U937/siC control cells (Fig. 5C, left panel, and supplemental Fig. 4B) (supplemental Fig. 4A: cells expressing a corresponding two-base mutated siRNA directed against NEMO exhibited no decrease in expression and yielded results similar to those obtained with scrambled control siRNA oligonucleotide-transfected U937/siC cells). Consistent with its established NEMO dependence (56, 57), TNFα-induced NF-κB activation was attenuated in clones displaying partial reductions in NEMO levels; but in sharp contrast to the abrogation seen with HDACIs, these effects were very modest (Fig. 5C, right panel, and supplemental Fig. 4B). Such findings argue, albeit indirectly, against the possibility that knockdown of NEMO, the regulatory component of the IKKα-IKKβ-IKKγ complex, blocks HDACI-mediated NF-κB activation solely or primarily by disabling IKK.

Consistent with the observation that knockdown of NEMO blocked HDACI-mediated NF-κB activation, siNEMO clones exposed to LBH-589 exhibited pronounced attenuation of Mn-SOD2 protein and mRNA expression (Fig. 6A, left panel, inset and bar graph), accompanied by persistent LBH-589-induced ROS accumulation, compared with U937/siC control cells (Fig. 6A, right panel). NEMO knockdown cells also exhibited increased DNA damage, manifested by the early (8 h) appearance of DNA SSBs, the subsequent appearance of DNA DSBs (γH2AX, 16–24 h), and the release of histone H1.2 into the cytosol (Fig. 6B, left panels). Finally, siNEMO cells displayed a pronounced increase in LBH-589 lethality compared with controls (Fig. 6B, right panel). Collectively, these findings indicate that NEMO plays an important functional role in diminishing HDACI lethality by permitting NF-κB activation and the resulting MnSOD2 induction, limiting ROS accumulation, and attenuating DNA damage.

NEMO SUMOylation Mutants Display Diminished NF-κB Nuclear Translocation/Activation and Reduced NEMO Nuclear Accumulation and ATM Interactions in HDACI-treated Cells—The rate-limiting step in NEMO-mediated DNA damage-related NF-κB activation is the addition of SUMO residues...
lysines 277 and 309, which prevents nuclear export of NEMO and permits ATM interactions (23, 25). To gain insights into the role of these events in HDACI actions, we transfected cells with mutant NEMO in which SUMOylation sites lysine 277 and 309 were replaced by alanines, either as single (K277A and K309A) or double (K277/309A) mutants. Expression of SUMOylation site-mutated NEMO was confirmed by Western blot using anti-V5 tagged antibodies (Fig. 7A, inset). Single SUMOylation site mutations (i.e. K277A or K309A) resulted in partial abrogation of LBH-589-induced NF-κB activation, whereas double mutant NEMO (U/K2–3) cells exhibited virtually complete NF-κB inactivation (Fig. 7A, left graph). In striking contrast, TNFα-induced NF-κB activation (ELISA) was unimpaired in SUMOlation mutant cells (Fig. 7A, right panel). Analysis of p65/RelA subcellular localization by confocal immunofluorescence microscopy revealed that although administration of LBH-589 resulted in the time-dependent nuclear translocation of p65/RelA in U/EV cells (i.e. 4–16 h), this process was abrogated in double SUMOylation mutant NEMO-expressing cells (U/K2–3, Fig. 7B). Consistent with a requirement for NEMO SUMOylation in ATM interactions (20), NEMO appeared early (e.g. within 2 to 4 h) in the nucleus of control cells following treatment with LBH-589 but was undetectable in U937/K277–309A cell nuclei (Fig. 7C). Consistent with this observation, a dramatic reduction in ATM co-immunoprecipitation with NEMO was observed in mutant U937/K277–309A (U/K2–3#1) cells exposed to LBH-589 compared with U/3.1EV control cells (Fig. 7D). Together, these findings indicate that SUMOylation plays an important functional role in NEMO nuclear translocation, ATM interactions, and NF-κB activation in HDACI-treated human leukemia cells.

Investigation of the functional implications of SUMOylation in HDACI responses revealed that expression of SUMOylation mutant NEMO, which substantially attenuated or abrogated HDACI-mediated NF-κB activation (Fig. 7A), markedly diminished expression of the NF-κB target, Mn-SOD2 (Fig. 8A, left panel), and resulted in sustained ROS accumulation (Fig. 8A, right panel) analogous to the effects of siRNA NEMO knockdown (Fig. 6A). Whereas exposure of U/EV control cells to LBH-589 induced a progressive increase in XRCC1 (green fluorescence), reflecting DNA SSBs, over the 4–8 h exposure interval, double mutant U/K277–309A cells displayed a very early (4 h) transition from DNA SSBs (XRCC1) to DNA DSBs (γH2AX, red fluorescence; Fig. 8B) and markedly enhanced LBH-589-induced apoptosis.
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A

LBH-589 (20nM)

U/siC  U/siN-5  U/siN-12

SOD2

actin

Mn-SOD2 mRNA (fold change)

C  16  24  C  16  24  C  16  24

B

U/siC - LBH-8h

U/siN12 - LBH-8h

γ-H2AX

H1.2

Histone

C  16  24  C  16  24

Anti-siDNA breaks (5h)

C  16  24  C  16  24

Cell Death

γ-H2AX


cells, clones

ATM (28) were generated (Fig. 9A, left panel, inset (U/siATM cells, clones #34 and #50), and supplemental Fig. 5B) (supplemental Fig. 5A: cells expressing the corresponding two-base mutated siRNA against ATM showed no decreased expression and displayed similar results to those obtained with scrambled control siRNA oligonucleotide-transfected U937/siC cells). As anticipated, ATM downregulation in U/siATM cells significantly increased cell sensitivity to the topoisomerase inhibitor and DNA-damaging agent, etoposide (supplemental Fig. 6A, VP16; p < 0.05 in each case). Notably, induction of NF-κB by LBH-589, reflected by p65/RelA activity (Fig. 9A, left panel (ELISA), and supplemental Fig. 5B) or nuclear localization (Fig. 9A, right panel (Western blot)), was essentially abolished in U937/siATM cells (U/ATM-50 and U/ATM-34) but was readily apparent in their empty vector counterparts. Significantly, NF-κB activation triggered by exposure of U937/siATM cells to TNFa was equivalent to that observed in scrambled sequence controls (Fig. 9A, lower right panel, and supplemental Fig. 5B). The failure of LBH-589 to induce NF-κB in U/siATM cells resulted in diminished induction of the NF-κB target Mn-SOD2 at both the mRNA (supplemental Fig. 6B) and protein levels (Fig. 9B, right panel) and persistent ROS accumulation (Fig. 9B, left panel). U/siATM cells exposed to LBH-589 also displayed early (8 h) evidence of enhanced DNA damage (e.g. DNA SSBs and DSBs; data not shown) compared with U/EV cells, as well as marked increases in cytosolic translocation of histone H1.2, Bak conformational change (Fig. 9C, left panel), and LBH-589-induced apoptosis (Fig. 9C, p < 0.002). In accord with these findings, U/EV cells displayed a robust increase in the NF-κB target gene NFκB1A (IkBa) mRNA in response to LBH-589, but this agent failed to increase IkBa mRNA levels in either the U/siATM clone or U937/IκB-SR cells used as controls (supplemental Fig. 6C, U/IκB-SR). Taken together, these results highlight a critical functional role for the oxidative DNA damage/ATM/NEMO pathway in initial NF-κB activation, as well as attenuation of ROS-mediated DNA damage and lethality by HDACIs in human leukemia cells.

DISCUSSION

Inappropriate NF-κB activation represents a hallmark of numerous malignancies (59), including those of hematopoietic...
origin, particularly multiple myeloma and leukemia (60). Consequently, components of the IKK/NF-κB pathway have become the focus of interest as potential therapeutic targets (15, 46). Members of the NF-κB family are sequestered in inactive forms in the cytoplasm but are activated by diverse external stimuli (16, 61). Three "outside-in" pathways of NF-κB activation have been identified including the classical or canonical pathway, e.g. by TNFα; the alternative or non-canonical pathway, e.g. by CD-40 ligand, B-cell-activating factor, or lymphotxin-β; and the atypical pathway, e.g. by UV light (16). In contrast, the recently described unorthodox DNA damage pathway operates through an inside-out mechanism in which genotoxic or oxidative stress signals originating in the nucleus activate NF-κB (20, 21).

This involves nuclear export of two proteins, ATM and NEMO, which then activate cytoplasmic IKK complexes, leading to nuclear translocation of NF-κB and transcription of cytoprotective genes; this allows cells to survive otherwise lethal insults, e.g. DNA DSBs (20, 25). The present findings indicate that in addition to their cytoprotective actions in the face of genotoxic stress, components of the DNA damage pathway play important functional roles in the initial activation of NF-κB by HDACIs.

In addition to acetylating histones, HDACIs acetylate diverse proteins including transcription factors E2F, YY-1, and NF-κB (62). RelA acetylation plays an important role in regulating the degree and duration of NF-κB activation (13, 63) and is believed to play an important role in the sustained induction of this pathway by HDACIs (64, 65). However, these events do not address the issue of how HDACIs initially trigger activation of NF-κB. The present findings indicate that in certain human leukemia cells, initial RelA activation involves the ROS-dependent induction of DNA damage and proceeds through the atypical, NEMO/ATM-dependent NF-κB activation pathway. In support of this notion, the Mn-SOD2 mimetic, Mn-TBAP, blocked HDACI-mediated ROS generation, attenuated ROS-mediated DNA damage, and blocked activation of NF-κB, reflected by diminished RelA nuclear transport and DNA binding activity. Although it is known that HDACI-mediated oxidative injury contributes to the lethality (32, 35) and potentially the selectivity of these compounds (66), the present findings demonstrate that ROS generation also plays a central role in triggering the NF-κB cascade by HDACIs.

The present observations also provide a connection between ROS-mediated DNA damage and the ATM/NEMO-dependent induction of NF-κB by HDACIs. DNA DSBs activate ATM,

FIGURE 7. Mutation of NEMO SUMOylation sites Lys-277 and Lys-309 attenuates LBH-589-mediated NF-κB activation. A, U/EV (3.1cDNA-V5-His) cells or U937 stably expressing one of three mutated NEMO cDNAs (U/K277A, U/K309A, or the double mutant U/K277–309A) were treated with either 20 nM LBH-589 (left panel) or 10 ng/ml TNFα (2 h; right graph), and NF-κB activity was determined by ELISA. Inset, Western blot analysis of V5 tagged-mutated NEMO expression. Values represent mean ± S.E. for three separate experiments performed in triplicate, * p < 0.01, # p < 0.50, β, analysis of nuclear p65/RelA by confocal immunofluorescence (CIF). C, confocal microscopy analysis of nuclear-localized IKKγ/NEMO performed on U937 and U/K277–309A cells (clone #1) exposed to 20 nM LBH-589 (2–4 h). Confocal immunofluorescence, anti-IKKγ/NEMO; counterstain, DAPI.

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WITHDRAWN
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A

FIGURE 8. Cells expressing SUMO site-mutated NEMO display no evidence of knockdown of TRAF2, an important mediator of TNFα-related IKK activation (47, 48), markedly attenuated TNFα-related NF-κB activation and signaling but had virtually no effect on that initiated by LBH-589. Although the dependence of TNFα-induced NF-κB activation on NEMO is well established (56, 57), partial NEMO knockdown only modestly diminished NF-κB activation by this cytokine, but it essentially abrogated activation by HDACIs. Furthermore, knockdown of ATM or transfection of cells with SUMOylation-defective NEMO mutant protein ablated HDACI-mediated NF-κB activation and transcription of the NF-κB target genes Mn-SOD2 and IκBα but minimally affected TNFα responses. The finding that the loss of the NEMO SUMOylation signal accompanied by diminished association of NEMO with ATM specifically impaired HDACI-mediated NF-κB activation argues that in the case of HDACIs, disruption of the atypical DNA damage pathway, rather than dysregulation of the IKK complex, is primarily responsible for attenuated NF-κB responses.

ROS generation has been implicated in HDACI lethality in multiple earlier reports (32, 35, 66). Notably, in the present study, HDACI-induced ROS was clearly linked to the early appearance of XRCC1 complexes, indicating oxidative base damage, base excision repair, and DNA single strand breaks (42). Furthermore, HDACIs such as trichostatin A, SAHA, and MS-275 activate NF-κB (11, 12, 69), an event that attenuates lethality by promoting transcription of antiapoptotic target genes including XIAP, Bcl-xL, and Mn-SOD2 (12, 70, 71). In this context, the NF-κB-dependent induction of Mn-SOD2 attenuates TNFα (72) and HDACI lethality (11, 12). It is therefore significant that genetic disruption of the atypical DNA damage activation pathway (e.g. by ATM/NEMO knockdown or mutation) mimicked pharmacologic (e.g. Mn-TBAP) or genetic (e.g. IκBα super-repressor) interruption of NF-κB cascade in blocking Mn-SOD2 induction, thereby promoting sustained ROS generation and DNA damage. Such findings argue that the initial induction of ROS by HDACIs and the resulting DNA damage are critical for NF-κB activation, which, through induction of Mn-SOD2 and ROS elimination, limits further genotoxic stress and lethality. A corollary of this model is that interruption of HDACI-mediated NF-κB activation and potentiation of lethality may occur at two separate levels: (a) interference with IKK activation and/or RelA acetylation (11, 12); and (b) disruption of the ATM/NEMO DNA damage-related pathway (54, 60).

which in turn phosphorylates multiple proteins involved in DNA damage/repair and checkpoint responses (67). Recently, NEMO has been identified as a novel ATM substrate linking DNA damage to NF-κB stress responses through a complex and dynamic process (20, 23, 24). Genotoxic insults causing DNA DSBs induce SUMOylation of NEMO resident in the nucleus, blocking its export. Concomitantly, activated ATM allows removal of SUMO residues from NEMO, permitting NEMO ubiquitination (20, 68). The ATM-ubiquitinated NEMO complex then migrates to the cytoplasm, where it activates the IKK complex, leading to RelA nuclear transport and culminating in the induction of NF-κB-responsive genes. The identification of NEMO as an ATM substrate therefore provides a link between DNA damage responses and the cytoprotective NF-κB pathway through a nuclear-to-cytoplasmic signaling cascade (20). Consequently, ATM, in addition to its nuclear activity (67), may exert important cytoplasmic functions. A corollary of this concept is that under some circumstances, nuclear rather than extracellular signals may initiate the NF-κB activation cascade. The bulk of evidence indicates that in contrast to the cytokine TNFα, HDACIs act primarily through the latter pathway to induce NF-κB-dependent responses. This conclusion is based on evidence that knockdown of TRAF2, an important mediator of TNFα-related IKK activation (47, 48), markedly attenuated TNFα-related NF-κB signaling but had virtually no effect on that initiated by LBH-589. Although the dependence of TNFα-induced NF-κB activation on NEMO is well established (56, 57), partial NEMO knockdown only modestly diminished NF-κB activation by this cytokine, but it essentially abrogated activation by HDACIs. Furthermore, knockdown of ATM or transfection of cells with SUMOylation-defective NEMO mutant protein ablated HDACI-mediated NF-κB activation and transcription of the NF-κB target genes Mn-SOD2 and IκBα but minimally affected TNFα responses. The finding that the loss of the NEMO SUMOylation signal accompanied by diminished association of NEMO with ATM specifically impaired HDACI-mediated NF-κB activation argues that in the case of HDACIs, disruption of the atypical DNA damage pathway, rather than dysregulation of the IKK complex, is primarily responsible for attenuated NF-κB responses.

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The mechanism(s) by which HDACIs induce ROS and DNA damage remains to be fully elucidated. HDACIs regulate oxidative homeostasis by modifying antioxidant proteins such as MnSOD2 or Trx (66, 73), leading to ROS generation (26, 35). Because ROS are potent inducers of DNA damage (74), their lethality may be amplified by interference with DNA repair, either directly through acetylation of repair proteins (e.g. Ku70) (8, 26) or indirectly through down-regulation of repair genes (e.g. Rad51) (6). In this context, genetic ablation of HDAC3 has recently been shown to promote DNA damage and impair DNA double strand break repair (75). In addition, HDACI-mediated DNA damage (e.g. manifested by γH2AX formation) has been shown to be more pronounced in ATM-null fibroblasts than in their wild-type counterparts (5). The present results suggest an alternative and potentially complementary possibility, i.e. that interference with the ATM/NEMO DNA damage pathway, by blocking NF-κB activation, prevents the induction of NF-κB-dependent antioxidant proteins such as Mn-SOD2, resulting in sustained ROS accumulation and potentiation of DNA damage.

In summary, the present observations identify the ATM/NEMO DNA damage pathway as a critical mediator of NF-κB activation by HDACIs in human leukemia cells. They also provide further evidence of an important functional association between HDACI-induced ROS and DNA damage, as well as support for the notion that NF-κB activation plays a major role in protecting cells, via Mn-SOD2 induction, from genomic damage and apoptosis (76). The present findings now integrate previous observations implicating HDACI-related ROS generation and DNA damage with emerging evidence linking the pro-survival NF-κB pathway to the DNA damage response (20, 54). The recent identification of NEMO as an ATM substrate thus provides a connection between HDACI-mediated DNA damage responses and a nuclear-to-cytoplasmic signaling cascade that activates the IKK/NF-κB system (20, 53). According to this model, exposure of leukemic cells to HDACIs induces, perhaps by modulating the expression of antioxidant proteins (66), early ROS generation. ROS induce DNA damage, initially manifested as DNA SSBs and subsequently DSBs, which then trigger SUMOylation and nuclear trapping of NEMO as well as

FIGURE 9. Role of ATM in the HDACI-induced NF-κB response. A, U/siC and U/siATM (stably expressing siRNA directed against ATM; clones #34 and #50) cells were exposed to either 20 nM LBH-589 (left graph) or 10 ng/ml TNFα (2 h, right graph) and analyzed for NF-κB activity by ELISA and Western blot (for nuclear p65 expression; upper right panel). Inset, Western blot analysis of ATM expression in siC control and siATM cells (clones 34 and 50). Actin was used as the loading control. B, left panel, levels of ROS were determined in U/siC and U/siATM cells (clones 34 and 50) ± LBH-589 (20 nM, at indicated intervals) labeled with the oxidation-sensitive dye H2DCFDA (20 μM) and analyzed by flow cytometry. Values represent mean ± S.E. Right panel, analysis of Mn-SOD2 expression by Western blot in samples from U/siC and U/siATM cells (clones 34 and 50) ± LBH-589 (20 nM, 16 or 24 h). C, Western blot (WB) analysis of linker histone H1.2 released into the S-100 fraction (cytosolic fraction) and Bak conformational change (immunoprecipitation (IP)) in U/EV and U/siATM-34 cell lines treated with LBH-589 (16–24 h). IgG bands were used to confirm equivalent loading and transfer. Right graph, U/siC and U/siATM cells (clones 34 and 50) were exposed to LBH-589 (20 nM for 24 h) and monitored for cell death induction by flow cytometry (annexin V/PI). Results represent mean ± S.E. *, p < 0.01.
engagement of the DNA repair machinery. The latter involves ATM activation and ATM-mediated phosphorylation of NEMO (20, 67), which allows removal of SUMO residues promoting NEMO ubiquitination and ATM complex formation (20). NEMO-ATM complexes are then able to exit the nucleus and trigger IKK activation in the cytoplasm (20, 23), resulting in IκBα phosphorylation and proteasomal degradation (77). This leads in turn to the release and nuclear translocation of p65/RelA and transcriptional activation of multiple NF-κB-dependent genes, including the ROS scavenger Mn-SOD2 (78, 79), which eliminates ROS and limits further DNA damage and cell death. Such a model may have implications for attempts to enhance the antileukemic activity of HDACIs. For example, it has previously been shown that in such cells, interference with IKK activation (e.g. by IKK inhibitors), by blocking NF-κB activation, dramatically lowers the threshold for HDACI-mediated apoptosis (12). Interestingly, ATM and NEMO have recently been implicated in the constitutive NF-κB activation characteristic of certain malignant hematopoietic cells (e.g. acute myeloid leukemia and myelodysplastic syndrome cells) (58, 60). Thus, interference with ATM (e.g. by ATM inhibitors) (60) or other components of the atypical DNA damage-related NEMO pathway, by blocking NF-κB activation at the nuclear level, may enhance HDACI activity in these disorders. Efforts to test this hypothesis are currently under way.

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Supplemental Figure 1. (A) Jurkat lymphoblastic leukemia and HL-60 promyelocytic leukemia cells were exposed to LBH-589 (20 nM) for the indicated intervals, after which nuclear extracts were prepared to monitor NF-κB activity using an ELISA-based procedure as described in Methods. Values represent the means ± S.E.M for three separate experiments performed in triplicate. (B) NF-κB activity was determined in nuclear extracts obtained from three primary AML patient samples treated with 20nM LBH-589 for 4, 8 and 16 h. Insets: Western blot analysis of gH2A.X expression was determined in lysates obtained from cell lines or patient samples after 16 h (HL-60, Jurkat) or 24 h (patient samples) exposure to LBH-589 (20 nM). Each lane was loaded with 30 µg of protein; blots were stripped and re-probed with an antibody directed against actin to ensure equivalent loading and transfer. Lower right panel: Confocal laser microscopic analysis of primary AML cells (Patient #1) exposed to LBH-589 (20 nM) for 24 h. After treatment, cytospin preparations were incubated with a specific anti-gH2AX (dsDNA breaks) antibody and viewed under 100x magnification. The results of a representative study are shown; two additional studies involving additional patient samples yielded similar results.
Supplemental Figure 2. A, U937/siC and U937 cells stably expressing a 2-nucleotide mutated siH1.2 negative control sequence directed against histone H1.2 (siHN, clones #3 and #8), and B, U937/shC cells (stably expressing a scrambled negative control sequence shRNA oligonucleotide) and U937/shH1.2 cells (expressing an alternative oligonucleotide directed against histone H1.2) were exposed to LBH-589 (20nM) for 24 h and analyzed for cell death induction by flow cytometry (% annexin V/PI-positive cells; values: Mean ± S.E.M.). *, P<0.01; Western blot analyses were performed involving the corresponding clones using whole cell lysates; each lane was loaded with 30 µg of protein; blots were stripped and re-probed with an antibody directed against actin to ensure equivalent loading and transfer. For all experiments, results represent the means ± S.D for three separate experiments performed in triplicate.
Supplemental Figure 3. A, U937/siC and U937 cells stably expressing pSilencer with a 2-nucleotide mutated negative control sequence siRNA directed against TRAF2 (siTN, clones #6 and #16), and B, shC (stably expressing a negative scrambled control shRNA oligonucleotide) and shTRAF2 (expressing an alternative oligonucleotide directed against TRAF2, clones shT-5 and shT-8) were exposed to TNFα (10 ng/ml) for 2h or 24h and analyzed for NF-κB activity (ELISA) or cell death (annexin V/PI-positive cells) by flow cytometry, respectively. Insets: Western blot analysis of TRAF2 expression were performed by using whole cell lysates; each lane was loaded with 30 µg of protein; blots were stripped and re-probed with an antibody directed against actin to ensure equivalent loading and transfer. C, shC and shTRAF2 cells were treated with LBH-589 (20nM, 8h) after which NF-κB activity was monitored by ELISA Values represent Mean ± S.E.M. *, P<0.01. For all experiments, results represent the means ± S.D for three separate experiments performed in triplicate.
Supplemental Figure 4. A, U937/siC and U937 cells stably expressing a 2-base mutated siNEMO negative control sequence directed against NEMO (siNN; clones #4 and #8), were exposed to either LBH-589 (20nM, 24h) or TNFα (10ng/ml, 2h) and analyzed for cell death (annexin V/PI-positive cells) by flow cytometry or NF-κB activity (ELISA), respectively. B, U937/shC (stably expressing a scrambled negative control sequence shRNA oligonucleotide) and U937/shN (expressing an alternative oligonucleotide directed against NEMO, clones #9 and #10) were exposed to either LBH-589 (20nM) or TNFα (10ng/ml) for 8h or 2 h, respectively and analyzed for NF-κB activity (ELISA); *, P<0.01. Western blot analyses were performed by using whole cell lysates; each lane was loaded with 30 µg of protein; blots were stripped and re-probed with an antibody directed against actin to ensure equivalent loading and transfer. For all experiments, results represent the means ± S.D. for three separate experiments performed in triplicate.
**Supplemental Figure 5.** A, U937/siC and U937 cells stably expressing a 2-base mutated siNEMO negative control sequence directed against ATM (siAN; clones #44 and #46), and B, U937/shC cells (stably expressing a negative scrambled control shRNA oligonucleotide) and U937/shATM cells (expressing an alternative oligonucleotide directed against ATM, clones # 13 and # 17) were exposed to either LBH-589 (20nM) or TNFa (10ng/ml) for 8h or 2 h, respectively and analyzed for NF-κB activity (ELISA); *, P<0.01. Western blot analyses were performed using whole cell lysates; each lane was loaded with 30 µg of protein; blots were stripped and re-probed with an antibody directed against actin to ensure equivalent loading and transfer. For all experiments, results represent the means ± S.D. for three separate experiments performed in triplicate.
Supplemental Figure 6. A, U/siC and U937 cells stably expressing a sequence directed against ATM (U/siATM; clones #34 and #50) were exposed to VP16 (10 mM) for 2 and 4 h, after which they were analyzed by flow cytometry to determine the percentage of annexin V/PI-positive cells. Results represent the means ± S.D. for three separate experiments performed in triplicate. *, significantly greater than values obtained for U/siC cells (P<0.05). B-C, Analysis of NFKB1A (IkBα) (B) and SOD2 (C) gene expression by Real-Time RT-PCR in samples from U/siC and U/siATM cells (clones #34 and #50) treated with 20nM LBH-589 for the indicated intervals. Real-Time RT-PCR values for each condition are expressed as amount of specific mRNAs/18S rRNA, normalized to levels corresponding to untreated control U/siC cells (C= 1). Results for U/IκB “super-repressor” cells are shown for comparison; *, significantly less than values for U/siC cells (P<0.01). For all experiments, values represent the means ± S.D for three separate experiments performed in triplicate.
Histone Deacetylase Inhibitors Activate NF-κB in Human Leukemia Cells through an ATM/NEMO-related Pathway
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