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LBH-589 (Panobinostat) potentiates fludarabine anti-leukemic activity through a JNK- and XIAP-dependent mechanism

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Abstract

Effects of the HDAC inhibitor LBH-589 (panobinostat) on fludarabine lethality toward acute myeloid leukemia (AML) cells were examined *in vitro* and *in vivo*. LBH-589 pretreatment sensitized U937, HL-60, and primary leukemia cells to fludarabine while blocking NF-κB activation accompanied by XIAP down-regulation and JNK activation. Pharmacologic or genetic JNK inhibition significantly attenuated LBH-589/fludarabine lethality, whereas XIAP over-expression diminished JNK activation and apoptosis. Combined *in vivo* treatment abrogated leukemia growth in a U937 xenograft murine model and substantially increased animal survival. These studies highlight the interplay between NF-κB activation, XIAP down-regulation, and JNK activation in anti-leukemic synergism between fludarabine and LBH-589.

Keywords

AML; histone deacetylase inhibitor; fludarabine

Introduction

Histone deacetylase inhibitors (HDACIs) consist of a group of agents that modify chromatin structure and, by extension, gene expression [1] and kill transformed cells through diverse mechanisms, including induction of oxidative DNA damage, up-regulation of death receptors, disruption of chaperone proteins, and interference with DNA repair, among numerous others [2-4]. Preferential lethality toward transformed cells has been attributed to

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failure to induce anti-oxidant or DNA repair responses [5; 6]. HDACIs have been approved for the treatment of CTCL [7] and have shown some, albeit limited single-agent activity in acute myelogenous leukemia AML [8].

Fludarabine is a purine nucleoside analog that kills transformed cells through multiple mechanisms, including incorporation into DNA, induction of DNA damage, inhibition of DNA replication, and inhibition of DNA repair, among others [9]. Its primary use to date has been in B-cell malignancies such as CLL and follicular lymphoma, where it is a mainstay of treatment [10]. However, it also displays activity against myeloid leukemia cells, and represents a component of the FLAG-Ida salvage regimen for patients with refractory AML [11]. Its activity in this setting may stem, at least in part, from potentiation of ara-C metabolism [12]. The factors that determine the sensitivity of AML cells to fludarabine have not been well defined.

Previous studies have shown that HDACIs potentiate the activity of certain DNA-damaging agents (e.g., topoisomerase inhibitors) in a sequence-dependent manner by promoting a more open chromatin structure, which allows the DNA greater access to agents such as VP-16 [13]. The mechanisms by which HDACIs enhance the anti-leukemic activity of nucleoside analogs are less clear. Previously, our group reported that MS-275 increased the anti-leukemic activity of fludarabine [14] and that prior exposure to the pan-HDACI LAQ-824 increased fludarabine lethality in diverse AML cell types through a mechanism involving potentiation of DNA damage [15]. However, the signaling events mediating this interaction have not been defined, nor has the *in vivo* relevance of this phenomenon been established. The primary goals of the present study were to define the role of certain stress-related pathways, particularly those related to NF- κ B and JNK (c-Jun N-terminal kinase) in fludarabine interactions with the more clinically relevant HDACI LBH-589[16], and to determine whether *in vitro* anti-leukemic synergism had *in vivo* implications.

Materials and methods

Cells and Cell Culture

Human AML (U937, M4-M5 with t[10;11][p13;q14]) and acute promyelocytic leukemia/APML (HL-60, M2 exhibiting loss and several rearrangements involving chromosome 5) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), and maintained in RPMI1640 medium containing 10% FBS as previously described [15]. U937 cells expressing TAM67 (mutated form of c-Jun in which the transactivation domain is deleted), XIAP, or shJNK1 were as previously described [17; 18].

Bone marrow (BM) or peripheral blood (PB) samples (>65% blasts) were obtained with informed consent, in accordance with the Declaration of Helsinki, from patients with histologically documented AML in relapse following induction therapy (FAB class M2; no known genetic abnormalities) undergoing routine diagnostic procedures with IRB approval (VCU IRB #HM 12517). Mononuclear cells were isolated by centrifugation at 400g for 30 min over Histopaque-1077 (Sigma Diagnostics, St. Louis, MO). Cell viability was regularly > 95%; all samples consisted of > 70% blasts. All experiments were performed at density of 1×10^6 cells/ml as described previously [19].

Drugs and Chemicals

LBH-589 was kindly provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ). Fludarabine was purchased from Sigma/Aldrich (St Louis, MO). The JNK inhibitor SP600125 was obtained from Alexis Corp. (San Diego, CA). Reagents were formulated in DMSO and stored at -20°C. Stock solutions were subsequently diluted with serum-free

RPMI medium prior to use to ensure the final concentration of DMSO did not exceed 0.02%.

Experimental Format

Logarithmically growing cells ($2.5\text{--}4.0 \times 10^5$ cells/ml) were exposed to various concentrations of LBH-589 for 24 hrs, after which fludarabine was either added or not added to the medium. After an additional interval (generally 24 hrs), cells were processed and assayed as previously described in detail [15].

Assessment of Apoptosis

Apoptosis was evaluated by annexin V/propidium iodide (PI) (BD PharMingen, Franklin Lakes, NJ) staining and flow cytometry as described [15].

Western Blot Analysis

Whole cell-pellets were washed in PBS, and lysed with loading buffer (Invitrogen, Carlsbad, CA) as previously described [15]. 30 μg of total protein for each condition were separated by 4-12% Bis-Tris NuPage precast gel system (Invitrogen) and electro-blotted to nitrocellulose. After incubation with the corresponding primary and secondary antibodies, blots were developed by enhanced chemiluminescence (New England Nuclear; Boston, MA). Primary antibodies were as follows: total JNK1, phospho-JNK, total and phospho-c-Jun (Ser73, Santa Cruz, Santa Cruz, CA), caspase-3 (BD Transduction Lab, San Diego, CA), Caspase-7 and -9 (BD Biosciences, San Jose, CA), Casapase-8 (ENZO Life Sciences, Plymouth Meeting, PA), PARP (Oncogene Inc., San Diego, CA), cleaved PARP (Cell Signaling, Beverly, MA), XIAP/hILP (BD Biosciences), $\gamma\text{H2A.X}$ (S139, Millipore, Billerica, MA), β -actin (Sigma) and α -tubulin (Oncogene Inc.). Secondary antibodies conjugated to horseradish peroxidase were from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

ELISA-based NF- κB activity analysis

RelA/p65-specific DNA binding activity in nuclear extracts was measured using Nuclear Extract and TransAM™ NF- κB p65 Chemi Kits (Active Motif, Carlsbad, CA), as we recently described [18].

Electrophoretic mobility shift assay (EMSA)

EMSA analysis was performed on nuclear extracts as we have previously described in detail [20].

Animal Studies

Human leukemia U937 cells (5×10^6 cells/mouse) were injected s.c. into the hind flank of athymic nude mice as we recently described [21]. Tumor growth was assessed 3-4 times/week by caliper, and tumor size was expressed in mm^3 using the standard formula: $\text{length} \times (\text{width})^2 \times 0.52$. Treatment was started after tumors developed and reached a size corresponding to approximately 250-350 mm^3 ; mice were divided in homogenous groups ($n = 4\text{--}5$ per group; control; LBH589, fludarabine; and LBH589 + fludarabine) according to tumor burden determined by size. Mice were treated daily with i.p. injections of LBH589 at 10 mg/kg/d, fludarabine at 50 mg/kg/d, or fludarabine with LBH-589. For the latter, LBH-589 was started 24 hr before fludarabine to mimic *in vitro* findings. Controls were treated with vehicle. Animals were injected daily for a maximum of 14 days or until tumors reached a size where animals required sacrifice.

Statistical Analysis

The significance of differences was determined by the Student's t-test. Kaplan Meier analysis was employed to monitor survival in various treatment groups.

Results

LBH-589 pre-treatment prevents fludarabine-mediated NF- κ B activation and promotes JNK activation and cell death in human leukemia cells

U937 cells were exposed to a minimally toxic concentration of LBH-589 (20 nM) for 24 hr followed by a marginally toxic concentration of fludarabine (0.8 μ M) for an additional 24 hr prior to determination of apoptosis by annexin V/PI staining, based on our observation that HDACI pretreatment substantially enhanced fludarabine lethality [15]. LBH-589-treated cells displayed a marked increase in cell death following fludarabine exposure compared to their untreated counterparts ($P < 0.001$; Fig 1A). Simultaneous administration as well as the reverse sequence (i.e., fludarabine followed by LBH-589) also increased cell death, although in the latter case, the extent of potentiation was less pronounced due to increased fludarabine lethality after a 48 h-exposure (data not shown). Administration of LBH-589 with or without fludarabine resulted in a marked increase in acetylation of histone H3 and tubulin (Fig 1B), indicating that LBH-589 targets both nuclear (class I) and cytoplasmic (class II) HDACs in this setting. In parallel studies, pretreatment of HL-60 promyelocytic leukemia cells with a marginally toxic concentration of LBH-589 (10 nM) significantly increased the lethality of 1.0 μ M fludarabine ($P < 0.001$ versus fludarabine alone; Supplemental Fig S1A). Similar effects on histone H3 and tubulin acetylation were also observed in HL-60 cells (Supplemental Fig S1B).

In parallel with the increase in cell death and attenuation of NF- κ B activation, cells exposed to both LBH-589 and fludarabine displayed a pronounced increase in caspase-9 and PARP cleavage, associated with marked XIAP down-regulation (Fig 1C). The latter was accompanied by the appearance of an XIAP cleavage product. Consistent with earlier findings [20], LBH-589 induced activation of NF- κ B by ELISA assays in U937 cells (16 hr), although activity returned to basal levels over the ensuing 8 hr (Fig 1). Fludarabine treatment also induced pronounced NF- κ B activation which persisted for at least 24 hr. Interestingly, in cells pre-treated (24 hr) with LBH-589, in which activity had returned to baseline levels, fludarabine failed to trigger NF- κ B activation. EMSA assays confirmed that LBH-589 pretreatment sharply attenuated fludarabine-mediated increases in NF- κ B DNA binding activity (Fig 1D, lower panel).

Studies were then undertaken to assess the effects of LBH-589 treatment on fludarabine-mediated activation of the stress-related kinase JNK. U937 cells exposed to LBH-589 (20 nM) or fludarabine (0.8 μ M) individually minimally activated JNK, reflected by the expression of phospho-JNK (Fig 1E). However, exposure of LBH-589-pretreated cells to fludarabine resulted in the robust up-regulation of phospho-JNK. Parallel results were obtained when expression of phosphorylated c-Jun was monitored (Fig 1E; right panel). No change in total expression of JNK or c-Jun was noted. Similar results were observed in HL-60 cells (Supplemental Fig S1C), indicating that prior exposure of leukemia cells to LBH-589 diminishes fludarabine-mediated NF- κ B activation while increasing JNK activation.

LBH-589 increases fludarabine lethality in primary AML cells

Bone marrow (AML#1) and peripheral blood (AML#2) AML blasts were exposed sequentially to LBH-589 followed by fludarabine, as above. For both samples, exposure to 10 or 20 nM LBH-589 resulted in moderate toxicity (e.g., 35-40% cell death), whereas 0.5

or 1.0 μ M fludarabine induced minimal lethality (Fig 2A and 2B). However, sequential exposure of primary cells to LBH-589 followed by fludarabine resulted in cell death in essentially 100% of cells. Combined exposure also resulted in the pronounced activation/cleavage of caspase-8 and -3, accompanied by down-regulation of the NF- κ B target XIAP [20] (Fig 2B, right panel).

Activation of JNK plays a significant functional role in LBH-589/fludarabine anti-leukemic activity

Treatment of U937 cells with 20 nM LBH-589 (24 hr) followed by 0.8 μ M fludarabine (24 hr) in the presence of the JNK inhibitor SP600125 (2.5 μ M) [22] significantly reduced apoptosis compared to cells cultured in the absence of the JNK inhibitor ($P < 0.01$; Fig 3A). Similarly, TAM67 cells, which express a dominant-negative form of c-Jun [17], were significantly less sensitive to this regimen compared to empty-vector controls ($P < 0.05$; Fig 3B, left panel). Western blot analysis demonstrated diminished phosphorylation of c-Jun in TAM67 cells (Fig 3B, right panel). Following LBH-589/fludarabine treatment, TAM67 cells displayed diminished cleavage/activation of caspases-7, -3, and -9, and reduced PARP degradation, compared to controls (Fig 3C). Finally, U937 JNK1 knock down cells displayed a clear reduction in PARP cleavage compared to scrambled sequence controls (Fig 3D). In sharp contrast, LBH-589/fludarabine-induced expression of γ H2A.X, reflecting double-stranded DNA breaks, was not substantially reduced. These findings indicate a significant functional role for JNK activation in LBH-589/fludarabine lethality, and suggest that JNK activation occurs independently or downstream of DNA damage.

Evidence for a role of XIAP down-regulation in JNK activation by the LBH-589/fludarabine regimen

The results of previous studies have shown that in the case of TNF α , JNK activation is negatively regulated by NF- κ B target genes, particularly XIAP [23]. Interestingly, LBH-589 and fludarabine alone moderately reduced XIAP levels in U937 (Fig 4A) and HL-60 cells (Supplemental Fig S1D). However, as shown in Fig 4A, sequential exposure to LBH-589/fludarabine virtually abrogated XIAP expression in U937 cells. Similar results were noted in HL-60 cells (Supplemental Fig S1D) and primary AML blasts (Fig 2B, right panels). A slight increase in an XIAP cleavage fragment was also observed with combined treatment (Fig 4A). To assess the functional significance of diminished XIAP expression, U937 cells ectopically expressing XIAP (U/XIAP) were employed (Fig 4B, inset). U/XIAP cells were significantly more resistant to LBH-589/fludarabine treatment compared to empty vector controls ($P < 0.01$; Fig 4B). Notably, the enhanced JNK activation observed following LBH-589/fludarabine exposure was substantially reduced in U/XIAP cells compared to controls (Fig 4C), suggesting that down-regulation of the NF- κ B-dependent target XIAP may contribute to JNK activation and cell death induced by the LBH-589/fludarabine regimen.

The effects of interruption of JNK on XIAP down-regulation were then examined. Following LBH-589/fludarabine treatment, both control and shJNK1 cells displayed marked XIAP down-regulation (Fig 4D). Concordant results were obtained in cells exposed to the JNK inhibitor SP600125 (Fig 4E). Collectively, these results, along with those shown in Fig 4C, argue that XIAP down-regulation occurs upstream of JNK activation in this system.

LBH-589 potentiates fludarabine anti-leukemic activity in vivo

To determine whether the LBH-589/fludarabine regimen exerted *in vivo* activity, a xenograft mouse model using U937 cells was employed analogous to one used in previous studies [24]. Following inoculation of 5×10^6 cells in the flanks of athymic nude mice and the appearance of tumors (7-10 days), mice were treated i.p. daily with fludarabine (50 mg/kg/

d), LBH-589 (10 mg/kg/d), or the combination for two weeks. LBH-589 treatment was initiated 24 hr before fludarabine to recapitulate the sequence employed in *in vitro* studies. Tumor size was monitored twice or three times per week for the first two weeks and weekly for the ensuing 7 weeks. LBH-589 alone had minimal effects on tumor growth (Fig 5A). Fludarabine alone suppressed the growth of tumors during the two week treatment schedule, but when discontinued, tumors regrew rapidly and mice had to be sacrificed within 10 days. However, combined treatment dramatically reduced the size of tumors to undetectable levels by day 7, and no regrowth was observed over the ensuing 7 weeks, even when treatment was discontinued at day 14. Survival was significantly greater with combined treatment versus single agent treatment ($P < 0.001$; Supplemental Fig S2A). Parallel studies were performed using U937/XIAP cells (Fig 5B). In mice treated with vehicle, tumors grew rapidly and all mice had to be sacrificed by day 14. However, virtually no tumor growth was observed in animals treated with LBH-589/fludarabine over the initial 14-day treatment period. Moreover, whereas variable regrowth of tumor occurred in 3 animals by day 35 (20 days post-treatment), none was seen in 3/6 animals at this interval. Treated animals did not display significant weight loss (e.g. $> 10\%$ body weight reduction) or other signs of toxicity (e.g., lethargy, etc.). Survival was significantly improved compared to controls for both groups ($P > 0.001$ in animals with no regrowth; $P < 0.01$ in animals with regrowth; Supplemental Fig S2B). These findings indicate that the LBH-589/fludarabine regimen displays significant anti-tumor activity in an *in vivo* xenograft model.

Discussion

The present results suggest an important functional role for perturbations in the NF- κ B-XIAP-JNK network in potentiation of fludarabine lethality by LBH-589 in human leukemia cells. Extensive cross-talk exists between the NF- κ B and JNK pathways, particularly in relation to ROS generation [25; 26] [27]. In this context, previous studies have shown that in leukemia cells exposed to HDACIs alone, NF- κ B plays an antioxidant role through the induction of MnSOD, which diminishes ROS generation, resulting in JNK activation [20]. Given evidence that oxidative injury plays a role in HDACI/fludarabine interactions [15], the present findings raise the possibility that analogous signaling events underlie HDACI-mediated potentiation of a cytotoxic nucleoside analog such as fludarabine.

Activation of NF- κ B i.e., by cytokines is a dynamic process which exhibits biphasic characteristics [28]. Initial activation of NF- κ B involves IKK activation, which phosphorylates I κ B α , targeting it for proteasomal degradation [29], leading to release of RelA, which is transported to the nucleus where it binds to DNA, resulting in the transcription of NF- κ B-dependent genes [30]. These events culminate in re-synthesis of I κ B α , which binds to and targets RelA for nuclear export, terminating the process [31]. HDACIs, in contrast to TNF α , induce prolonged NF- κ B activation due to RelA acetylation, which diminishes its affinity for I κ B α [32] [33]. It has recently been reported that HDACIs induce NF- κ B through oxidative injury and the atypical ATM/NEMO/SUMOylation DNA damage-related pathway [34] [35]. Regardless of the mechanism of initial induction, HDACI-mediated NF- κ B activation, although more sustained than that triggered by TNF α , is eventually reversed, i.e. declining to basal levels after 20 hr-exposure. Notably, after the NF- κ B response was abrogated (e.g., following 24 hr-pretreatment with LBH-589), in sharp contrast to untreated cells, fludarabine failed to trigger NF- κ B activation or subsequent JNK activation. This suggests that events responsible for termination of HDACI-mediated NF- κ B activation prevent NF- κ B induction by fludarabine, leading to enhanced lethality. Studies defining the mechanism for termination of the HDACI-induced NF- κ B response are underway.

The present findings demonstrate a significant functional role for JNK activation in potentiation of fludarabine lethality by LBH-589. Previous studies highlighted the importance JNK activation in cytokine-mediated transformed cell death, and its reversal by NF- κ B activation [26]. Analogously, NF- κ B inactivation i.e., by IKK or proteasome inhibitors, increase HDACI lethality toward human leukemia cells through a JNK-dependent mechanism [18; 20]. The findings that abrogation of NF- κ B activation by HDACI pre-treatment substantially enhanced fludarabine-mediated JNK activation, and that pharmacological or genetic interruption of JNK significantly attenuated LBH-598/fludarabine-mediated lethality, suggest that similar events occur in leukemia cells exposed to fludarabine. The mechanism by which NF- κ B prevents JNK activation may vary in different cell types. For example, in murine colon cells, this stems from reduction in ROS-mediated inactivation of MAP kinase phosphatases that dephosphorylate JNK [26]. Alternatively, in, MEF cells exposed to TNF α , this process involves the NF- κ B-dependent protein XIAP [23]. In the present studies, ectopic expression of XIAP substantially attenuated LBH-589/fludarabine-mediated JNK activation, and significantly reduced lethality, suggesting a similar mechanism. Given evidence that attenuation of HDACI-mediated NF- κ B activation i.e., by either IKK or proteasome inhibitors also diminishes XIAP expression [18; 20], it is possible that the failure of fludarabine to trigger NF- κ B activation in LBH-589-pretreated cells induces XIAP down-regulation, leading to enhanced JNK activation and lethality. In addition, XIAP has been implicated in NF- κ B activation [36], raising the possibility that XIAP down-regulation may also contribute to suppression of NF- κ B signaling. Finally, XIAP down-regulation, accompanied by cleavage, was most marked in cells exposed to both LBH-589 and fludarabine, raising the possibility that the latter phenomenon may amplify the regimen's lethality.

Results of *in vivo* studies demonstrated a striking increase in activity for the LBH-589/fludarabine regimen. Notably, administration of LBH-589 alone had minimal effects, whereas fludarabine alone significantly suppressed tumor growth during the administration interval. However, discontinuation of fludarabine resulted in a rapid re-growth of tumor within 7-10 days. In striking contrast, sequential administration of LBH-589 followed by fludarabine resulted in the virtual disappearance of tumors within 1 week. Significantly, after discontinuation of treatment, tumor re-growth did not occur by over the ensuing 50-day observation period, indicating profound *in vivo* effects on tumor cell survival. Interestingly, an identical regimen substantially inhibited the re-growth of XIAP-overexpressing tumors in 50% of inoculated mice, and eliminated detectable tumors in the remaining 50%. Consistent with the observation that ectopic expression of XIAP only partially (< 50%) protected cells from the LBH-598/fludarabine regimen *in vitro*. Collectively, these findings indicate that potentiation of fludarabine lethality by HDACIs [15] is not restricted to the *in vitro* setting, but also occurs *in vivo*.

In summary, the present findings suggest a hierarchy of signaling events that may contribute to HDACI-mediated potentiation of fludarabine lethality. Specifically, they suggest that in HDACI-pretreated leukemia cells, attenuation of fludarabine-mediated NF- κ B activation through a yet to be defined mechanism diminishes expression of anti-apoptotic NF- κ B-dependent proteins, including XIAP [37]. Down-regulation of the latter releases inhibitory effects on JNK activation [23], which in turn plays a significant functional role in cell death. Notably, potentiation of fludarabine lethality by LBH-589 occurs in at least some primary AML samples, and combined treatment exhibited substantial *in vivo* efficacy. However, in view of the small sample size, a larger number of primary blast specimens will clearly be necessary to validate the present observations. Nevertheless, given evidence of the activity of fludarabine-containing regimens in the treatment of refractory AML [11], incorporating HDACIs into such approaches, if tolerable, warrants consideration. Based on the present

findings, post-treatment levels of XIAP and phospho-JNK in leukemic blasts represent plausible candidate pharmacodynamic determinants of regimen activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AML	acute myelogenous leukemia
CLL	chronic lymphocytic leukemia
CTCL	cutaneous T-cell lymphoma
LBH-589	panobinostat
FdA	fludarabine
HDACI	histone deacetylase inhibitor
XIAP	X chromosome-linked inhibitor of apoptosis
JNK	c-Jun N-terminal kinase
pJNK	phospho-c-Jun N-terminal kinase
PARP	poly ADP (adenosine diphosphate)-ribose polymerase
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum

Reference List

1. Lane AA, Chabner BA. Histone deacetylase inhibitors in cancer therapy. *J.Clin.Oncol.* 2009; 27:5459–5468. [PubMed: 19826124]
2. Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non histone proteins. *Gene.* 2005; 363:15–23. [PubMed: 16289629]
3. Chen CS, Wang YC, Yang HC, Huang PH, Kulp SK, Yang CC, Lu YS, Matsuyama S, Chen CY, Chen CS. Histone deacetylase inhibitors sensitize prostate cancer cells to agents that produce DNA double-strand breaks by targeting Ku70 acetylation. *Cancer Res.* 2007; 67:5318–5327. [PubMed: 17545612]
4. Almenara J, Rosato R, Grant S. Synergistic induction of mitochondrial damage and apoptosis in human leukemia cells by flavopiridol and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). *Leukemia.* 2002; 16:1331–1343. [PubMed: 12094258]
5. Ungerstedt JS, Sowa Y, Xu WS, Shao Y, Dokmanovic M, Perez G, Ngo L, Holmgren A, Jiang X, Marks PA. Role of thioredoxin in the response of normal and transformed cells to histone deacetylase inhibitors. *Proc.Natl.Acad.Sci.U.S.A.* 2005; 102:673–678. [PubMed: 15637150]
6. Lee JH, Choy ML, Ngo L, Foster SS, Marks PA. Histone deacetylase inhibitor induces DNA damage, which normal but not transformed cells can repair. *Proc.Natl.Acad.Sci.U.S.A.* 2010
7. Sasaki CY, Barberi TJ, Ghosh P, Longo DL. Phosphorylation of RelA/p65 on serine 536 defines an I{kappa}B{alpha}-independent NF-{kappa}B pathway. *J.Biol.Chem.* 2005; 280:34538–34547. [PubMed: 16105840]

8. Garcia-Manero G, Yang H, Bueso-Ramos C, Ferrajoli A, Cortes J, Wierda WG, Faderl S, Koller C, Morris G, Rosner G, Loboda A, Fantin VR, Randolph SS, Hardwick JS, Reilly JF, Chen C, Ricker JL, Secrist JP, Richon VM, Frankel SR, Kantarjian HM. Phase 1 study of the histone deacetylase inhibitor vorinostat (suberoylanilide hydroxamic acid [SAHA]) in patients with advanced leukemias and myelodysplastic syndromes. *Blood*. 2008; 111:1060–1066. [PubMed: 17962510]
9. Gandhi V, Plunkett W. Cellular and clinical pharmacology of fludarabine. *Clin.Pharmacokinet*. 2002; 41:93–103. [PubMed: 11888330]
10. Ricci F, Tedeschi A, Morra E, Montillo M. Fludarabine in the treatment of chronic lymphocytic leukemia: a review. *Ther.Clin.Risk Manag*. 2009; 5:187–207. [PubMed: 19436622]
11. Jackson GH. Use of fludarabine in the treatment of acute myeloid leukemia. *Hematol.J*. 2004; 5(Suppl 1):S62–S67. [PubMed: 15079154]
12. Gandhi V, Plunkett W. Modulation of arabinosylnucleoside metabolism by arabinosylnucleotides in human leukemia cells. *Cancer Res*. 1988; 48:329–334. [PubMed: 3335008]
13. Marchion DC, Bicaku E, Daud AI, Richon V, Sullivan DM, Munster PN. Sequence-specific potentiation of topoisomerase II inhibitors by the histone deacetylase inhibitor suberoylanilide hydroxamic acid. *J.Cell Biochem*. 2004; 92:223–237. [PubMed: 15108350]
14. Maggio SC, Rosato RR, Kramer LB, Dai Y, Rahmani M, Paik DS, Czarnik AC, Payne SG, Spiegel S, Grant S. The histone deacetylase inhibitor MS-275 interacts synergistically with fludarabine to induce apoptosis in human leukemia cells. *Cancer Res*. 2004; 64:2590–2600. [PubMed: 15059916]
15. Rosato RR, Almenara JA, Maggio SC, Coe S, Atadja P, Dent P, Grant S. Role of histone deacetylase inhibitor-induced reactive oxygen species and DNA damage in LAQ-824/fludarabine antileukemic interactions. *Mol.Cancer Ther*. 2008; 7:3285–3297. [PubMed: 18852132]
16. Giles F, Fischer T, Cortes J, Garcia-Manero G, Beck J, Ravandi F, Masson E, Rae P, Laird G, Sharma S, Kantarjian H, Dugan M, Albitar M, Bhalla K. A phase I study of intravenous LBH589, a novel cinnamic hydroxamic acid analogue histone deacetylase inhibitor, in patients with refractory hematologic malignancies. *Clin.Cancer Res*. 2006; 12:4628–4635. [PubMed: 16899611]
17. Jarvis WD, Fornari FA Jr. Auer KL, Freermerman AJ, Szabo E, Birrer MJ, Johnson CR, Barbour SE, Dent P, Grant S. Coordinate regulation of stress- and mitogen-activated protein kinases in the apoptotic actions of ceramide and sphingosine. *Mol.Pharmacol*. 1997; 52:935–947. [PubMed: 9415703]
18. Dai Y, Chen S, Wang L, Pei XY, Kramer LB, Dent P, Grant S. Bortezomib interacts synergistically with belinostat in human acute myeloid leukaemia and acute lymphoblastic leukaemia cells in association with perturbation in NF-kappaB and Bim. *Br.J.Haematol*. 2011
19. Rahmani M, Anderson A, Habibi JR, Crabtree TR, Mayo M, Harada H, Ferreira-Gonzalez A, Dent P, Grant S. The BH3-only protein Bim plays a critical role in leukemia cell death triggered by concomitant inhibition of the PI3K/Akt and MEK/ERK1/2 pathways. *Blood*. 2009; 114:4507–4516. [PubMed: 19773546]
20. Dai Y, Rahmani M, Dent P, Grant S. Blockade of Histone Deacetylase Inhibitor-Induced RelA/p65 Acetylation and NF- κ B Activation Potentiates Apoptosis in Leukemia Cells through a Process Mediated by Oxidative Damage, XIAP Downregulation, and c-Jun N-Terminal Kinase 1 Activation. *Mol.Cell Biol*. 2005; 25:5429–5444. [PubMed: 15964800]
21. Dai Y, Chen S, Pei XY, Almenara JA, Kramer LB, Venditti CA, Dent P, Grant S. Interruption of the Ras/MEK/ERK signaling cascade enhances Chk1 inhibitor-induced-DNA damage in vitro and in vivo in human multiple myeloma cells. *Blood*. 2008; 112:2439–2449. [PubMed: 18614762]
22. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc.Natl.Acad.Sci.U.S.A*. 2001; 98:13681–13686. [PubMed: 11717429]
23. Tang G, Minemoto Y, Dibling B, Purcell NH, Li Z, Karin M, Lin A. Inhibition of JNK activation through NF-kappaB target genes. *Nature*. 2001; 414:313–317. [PubMed: 11713531]
24. Dai Y, Chen S, Shah R, Pei XY, Wang L, Almenara JA, Kramer LB, Dent P, Grant S. Disruption of Src function potentiates Chk1-inhibitor-induced apoptosis in human multiple myeloma cells in vitro and in vivo. *Blood*. 2011; 117:1947–1957. [PubMed: 21148814]

25. Greten FR, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell*. 2004; 118:285–296. [PubMed: 15294155]
26. Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell*. 2005; 120:649–661. [PubMed: 15766528]
27. Bubici C, Papa S, Dean K, Franzoso G. Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. *Oncogene*. 2006; 25:6731–6748. [PubMed: 17072325]
28. Ashall L, Horton CA, Nelson DE, Paszek P, Harper CV, Sillitoe K, Ryan S, Spiller DG, Unitt JF, Broomhead DS, Kell DB, Rand DA, See V, White MR. Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription. *Science*. 2009; 324:242–246. [PubMed: 19359585]
29. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat.Rev.Mol Cell Biol*. 2007; 8:49–62. [PubMed: 17183360]
30. Basseres DS, Baldwin AS. Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. *Oncogene*. 2006; 25:6817–6830. [PubMed: 17072330]
31. Karin M. Nuclear factor-kappaB in cancer development and progression. *Nature*. 2006; 441:431–436. [PubMed: 16724054]
32. Chen, Lf; Fischle, W.; Verdin, E.; Greene, WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science*. 2001; 293:1653–1657. [PubMed: 11533489]
33. Chen LF, Greene WC. Shaping the nuclear action of NF-kappaB. *Nat.Rev.Mol.Cell Biol*. 2004; 5:392–401. [PubMed: 15122352]
34. Wu ZH, Shi Y, Tibbetts RS, Miyamoto S. Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. *Science*. 2006; 311:1141–1146. [PubMed: 16497931]
35. Rosato RR, Kolla SS, Hock SK, Almenara JA, Patel A, Amin S, Atadja P, Fisher PB, Dent P, Grant S. Histone deacetylase inhibitors activate NF-kappaB in human leukemia cells through an ATM/NEMO-related pathway. *J.Biol.Chem*. 2010; 285:10064–10077. [PubMed: 20065354]
36. Gyrd-Hansen M, Darding M, Miasari M, Santoro MM, Zender L, Xue W, Tenev T, da Fonseca PC, Zvelebil M, Bujnicki JM, Lowe S, Silke J, Meier P. IAPs contain an evolutionarily conserved ubiquitin-binding domain that regulates NF-kappaB as well as cell survival and oncogenesis. *Nat.Cell Biol*. 2008; 10:1309–1317. [PubMed: 18931663]
37. Hua B, Tamamori-Adachi M, Luo Y, Tamura K, Morioka M, Fukuda M, Tanaka Y, Kitajima S. A splice variant of stress response gene ATF3 counteracts NF-kappaB-dependent anti-apoptosis through inhibiting recruitment of CREB-binding protein/p300 coactivator. *J.Biol.Chem*. 2006; 281:1620–1629. [PubMed: 16291753]

Figure 1.

LBH-589 blocks fludarabine-induced NF- κ B activation in association with XIAP downregulation, histone H3/tubulin acetylation, JNK activation, and apoptosis in U937 cells. C: control; LBH or L: LBH-589, Fd or F: fludarabine; C+C': control + unlabeled (cold) consensus oligonucleotide probes.

Figure 2. LBH-589 potentiates fludarabine lethality in primary AML blasts. BMMC: bone marrow mononuclear cells; PBMC: peripheral blood mononuclear cells; cf: cleavage fragment.

Figure 3. Interruption of the JNK signaling pathway significantly reduces lethality of the LBH-589/fludarabine regimen. U/EV and U/TAM67: U937 cells stably transfected with empty vector or mutated c-Jun; shNC and shJNK1: shRNA targeting negative control sequence or JNK1.

Figure 4. Overexpression of XIAP prevents JNK activation and cell death mediated by the LBH-589/fludarabine regimen, whereas interruption of the JNK signaling pathway fails to reverse XIAP down-regulation.

Figure 5.
In vivo anti-leukemic activity of the LBH-589/fludarabine regimen. D: day.