Preclinical activity of LBH589 alone or in combination with chemotherapy in a xenogeneic mouse model of human acute lymphoblastic leukemia

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ABSTRACT
Histone deacetylases (HDACs) have been identified as therapeutic targets due to their regulatory function in chromatin structure and organization. Here we analyzed the therapeutic effect of LBH589, a class I-II HDAC inhibitor, in acute lymphoblastic leukemia (ALL). In vitro, LBH589 induced dose-dependent antiproliferative and apoptotic effects, which were associated with increased H3 and H4 histone acetylation. Intravenous (i.v.) administration of LBH589 in immunodeficient BALB/c-RAG2-/-γc-/- mice in which human-derived T and B-ALL cell lines were injected induced a significant reduction in tumor growth. Using primary ALL cells, a xenograft model of human leukemia in BALB/c-RAG2-/-γc-/- mice was established, allowing continuous passages of transplanted cells to several mouse generations. Treatment of mice engrafted with T or B-ALL cells with LBH589 induced an in vivo increase in the acetylation of H3 and H4, which was accompanied with prolonged survival of LBH589-treated mice in comparison with those receiving Vincristine and Dexametasone. Notably, the therapeutic efficacy of LBH589 was significantly enhanced in combination with Vincristine and Dexametasone. Our results demonstrate the therapeutic activity of LBH589 in combination with standard chemotherapy in pre-clinical models of ALL and suggest that this combination may be of clinical value in the treatment of patients with ALL.

Keywords: LBH589, Acute Lymphoblastic Leukemia, epigenetics, histone, mouse model
INTRODUCTION

Although it is tempting to consider acute lymphoblastic leukemia (ALL) as a curable disease due to the remarkable improvement in the cure rates observed in recent years, the prognosis of relapsed patients is still dismal (1). Almost 80% of children diagnosed with ALL become cured with modern risk-adapted therapies. However, more than 60% of adult patients will eventually relapse and most of them will succumb to their disease. This underlies the need for new therapeutic options for resistant patients. The role of recurrent chromosomal translocations in the pathogenesis of ALL has been clearly established (2) providing not only important prognostic information but also guiding the development of new treatments such as the use of tyrosine-kinase inhibitors (Imatinib or Dasatinib) in patients with t(9;22) (3). Other genetic alterations such as the overexpression of FLT3 receptor tyrosine kinase in mixed-lineage leukemia gene (MLL)-rearranged or hyperdiploid B-ALL (4) or the presence of NOTCH1-activating mutations in T-cell ALL are attractive candidates for targeted therapies with FLT3 or NOTCH inhibitors (γ-secretase inhibitors) (5). Indeed, the current view of the pathogenesis of ALL suggests that several genetic lesions need to act in concert to induce overt leukemia (1).

The classical view of cancer as a genetic disease has been challenged by the clear demonstration that epigenetic modifications can alter gene expression by mechanisms that do not affect the DNA sequence itself. Epigenetics plays an important role in the pathogenesis and prognosis of various tumors. The hypermethylation of DNA promoters and changes in histone modification patterns are the most frequently described abnormalities in tumor cells (6). We and others have extensively demonstrated that patients with ALL frequently show an abnormal hypermethylation of DNA promoter of tumor suppressor genes, miRNAs or genes involved in tumorigenic pathways such as WNT or Ephrin-Eph pathway, and that these changes are associated with prognosis of the disease (7-12).

Among the various epigenetic modifiers, histone deacetylases inhibitors (HDACi) have emerged as promising drugs for the treatment of a number of tumors (13). HDAC inhibitors are a class of anticancer agents that inhibit deacetylation of histones and non-histone cellular proteins, inducing hyperacetylation and an “open chromatin” configuration. In cancer, such hyperacetylation is associated with a greater transcriptional activity of silenced tumor suppressor genes. LBH589 (also called Panobinostat) is an HDAC inhibitor characterized as a pan-deacetylase inhibitor with activity against both class I and II HDACs. This drug has demonstrated a significant
activity against different tumors such as myeloma, acute myelogenous leukemia and T-cell lymphomas as well as breast, prostate, colon and pancreatic cancer (14, 15).

In this study, we investigate the role of the HDACi LBH589 using in vitro and in vivo models of ALL, and demonstrate that treatment of ALL cells with LBH589 induces a prolonged survival in a mouse model of human ALL. Also, we show that this effect is significantly improved when combined with currently active drugs used to treat ALL, providing the basis for using this combination in patients with ALL.

MATERIALS AND METHODS

Human samples and cell lines
Six ALL-derived cell lines TOM-1, REH, 697, SEM, TANOUE and MOLT-4, were purchased from the DSMZ (Deutsche Sammlung von Microorganismen und Zellkulturen GmbH). Cell lines were maintained in culture in RPMI 1640 medium supplemented with 10% fetal bovine serum and with 1% penicillin-streptomycin and 2% HEPES (Gibco-BRL) at 37°C in a humid atmosphere containing 5% CO2. To generate the mouse model, bone marrow or peripheral blood mononuclear cells were obtained at diagnosis from patients with ALL after signed informed consent had been obtained from the patient or the patient’s guardians, in accordance with the Declaration of Helsinki.

Reagent and cell drug treatment
The HDACi LBH589 was provided by Novartis Pharmaceuticals and diluted in saline solution for in vitro studies and in 5% dextrose (D5W) for in vivo experiments. Cell lines were treated with LBH589 at different concentration and maintained in culture for up to 4 days after which cells were washed in PBS and used for different assays. The half maximal inhibitory concentration (IC$_{50}$) values was determined using GraphPad Prism log (inhibitor) vs. response (variable slope) software (version 5, La Jolla, CA).

Protein extraction and western blot
Protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and incubated with a monoclonal antibody against described proteins. A detailed description is included in the supplementary materials and methods.
Cell proliferation

Cell proliferation was analyzed after 24, 48, 72 and 96 hours of *in vitro* treatment using the Celltiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) as previously described (8). All experiments were repeated three times.

Apoptosis assays

Apoptosis induction was analyzed using the ELISAPLUS 10x Cell Death Detection Kit (Roche), following the manufacturer’s instructions, the FITC-Conjugated Monoclonal Active Caspase-3 Antibody Apoptosis Kit (BD Pharmingen), and by detection of the 85 kDa fragment of PARP (8, 16).

SNP-chip-analysis

DNA from primary cells and tissues was extracted using QIAmp DNA Mini Kit following the manufacturer’s instructions (Qiagen). DNA was quantified using NanoDrop Spectrophotometer (NanoDrop Technologies). DNA from samples was analyzed on Affimetrix GeneChip Human mapping 250K SNP arrays (Santa Clara) capable of genotyping on average 250,000 SNPs according to the manufacturer’s protocol. Microarray data were analyzed for determination of both total and allelic-specific copy numbers using the Genotyping Console software (Affymetrix) as previously described (17).

DNA methylation array analysis

A HumanMethylation27 BeadChip (Illumina, Inc) was used to quantify DNA methylation. The panel is developed to quantify the methylation status of 27,578 CpG sites located within the proximal promoter of 14,475 well-annotated genes from the consensus coding sequence project as well as known cancer genes and miRNAs. The protocol was performed according to the manufacturer's instructions. The methylation status of a CpG was determined by the beta value calculation, which ranges from 0 for unmethylated CpGs to 1 for completely methylated CpGs.

Gene expression array analysis

Total RNA from primary cells and tissues was extracted with Ultraspec (Biotecx) following the manufacturer’s instructions after tissue lysates were prepared using a mechanical tissue homogenization by ultraturrax (IKA). RNA was quantified using NanoDrop Spectrophotometer (NanoDrop Technologies). Gene expression analysis was performed using GeneChip Human Gene 1.0 ST array (Affymetrix) following the
manufacturer’s instructions. Microarray data were normalized and analyzed as previously described (18)

**TaqMan Low Density Arrays**

A TaqMan Low Density Array A v2.1 (Applied Biosystems) was used for 377 human miRNA expression assays after treatment. A total concentration of 500ng of RNA was used for the array hybridization. Reverse transcription and real-time PCR reactions were done following the manufacturer’s instructions without product preamplification.

**Bioinformatic data analysis**

A detailed description of the bioinformatic data analysis is provided in the supplementary material and methods. Raw array data files were submitted to GEO and are available under the accession number GSE26807.

**In vivo experiments**

All animal studies had previous approval from the Animal Care and Ethics Committee of the University of Navarra, whereas experiments that used patient samples were approved by the Human Research Ethics Committees of University of Navarra. For the human subcutaneous ALL model, 6-week old female BALB/c-A-RAG2-/-γc-/- mice were subcutaneously inoculated in its back left flank with 1x10^6 human ALL viable cells in 100μl volume of saline solution. At least eight mice were included in each group. A group of healthy control BALB/c-A-Rag2-/-γc-/- mice was treated with increasing doses of LBH (1mg/kg, 5mg/kg, 10mg/kg and 20mg/kg). Doses were selected based on previously published studies (25) and administered intraperitoneally (i.p.) to determine the MTD of LBH589. Treatment was initiated 24 hours after injection of leukemic cells and included 3 cycles of 5 consecutive days of LBH589 with two days rest between cycles. Tumor size was analyzed every three days using the following method: V= Dxd^2/2, were D and d corresponding to the longest and shorter diameter, respectively. Mice were sacrificed 24 days after cell inoculation or when their tumor diameter reached 17mm.

Two human ALL xenograft mice models were generated by intravenously injection of 10x10^6 human primary cells diluted in 100μl of saline solution in the tail vein of a 6-week old female BALB/c-A-RAG2-/-γc-/- mice. Cells used for the T and B cell human ALL mice model (ALL-T1 and ALL-B1) were characterized by karyotype, immunophenotype and methylation profile (Table 1). After primary engraftment in mice, human blasts from mice spleen were isolated (>97% of human blasts) by Ficoll-Paque plus (GE
healthcare) and transplanted into the tail vein of new BALB/c-A-RAG2\(^{−/−}\)γc\(^{−/−}\) mice. Cells obtained from different generations of mice were compared with the initial patient sample by SNP arrays, methylation and mRNA expression arrays. Mice were divided in 4 treatment groups: 1) saline; 2) LBH589; 3) Vincristine and Dexametasone and 4) Vincristine-Dexamethasone and LBH589. Vincristine was administrated intravenously (i.v.) the first day of each cycle at 0.025 mg/kg and dexametasone was administrated intraperitoneally (i.p.) for 21 consecutive days at 1 mg/kg while LBH589 was administered i.p. at doses of 5mg/kg for 3 cycles of 5 consecutive days with two days rest between each cycle. For survival analyses, we used the date of: a) death from leukemia, b) sacrifice due to severe clinical symptoms or c) PB infiltration with more than 80% blasts.

**FACS analysis**
For immunephenotyping, 50-100μl of peripheral blood, bone marrow or 500.000 human blasts from mice spleen were isolated by ficoll-Paque plus (GE healthcare). Cells were labeled for 15 minutes with the following antibodies: rat anti-mouse CD45-PE (BD pharmingen), mouse anti-human CD5-APC (BD pharmingen) mouse anti-human CD45-PerCP (BD pharmingen), mouse anti-human CD22-PE (BD pharmingen) followed by 10 minutes incubation with 2ml of FACS lyses solution (1:10)(Becton Dickinson). Cells were washed with saline solution and centrifuged at 600g for 7 minutes. The supernatant was decanted and cells were fixed in 400μl of 0.4% paraformaldehyde. The analysis was done with FACSCalibur cytometer and Paint-A-Gate software (Bencton Dickinson).

**Histological and immunochemistry analysis**
Organs collected from mice were fixed in paraformaldehyde at 4% for 6-8 hours and washed twice with saline solution and stored in 70% ethanol. Samples were included in paraffin and 3μm serial sections were cut, deparafinated and stained with hematoxilin-eosin. For the immunohistochemical characterization, deparaffinated slides were heat treated using high or low PH solution (target retrieval solution low pH, En Vision FLEX, Dako) by means of the PTlink (Dako) for antigen unmasking. Immunohistochemistry was performed in the Autostainer PlusLink (Dako). Primary antibodies against active caspase 3 (diluted 1/150 for 30 minutes, R&D systems), Ki67 (Ready to use, for 20 minutes, Dako), CD45 (diluted 1/50 for 40 minutes, Dako) and CD19 (diluted 1/50 for 30 minutes, Dako) were used followed by secondary antibodies, goat anti Rabbit HRP, goat anti mouse HRP and biotinylated mouse IgG (MOM vector).
CD34 positive cell isolation and treatment
A detailed description of the CD34+ cells isolation and treatment protocols are provided in the supplementary material and methods.

Statistical analysis
A detailed description is provided in the supplementary material and methods.

RESULTS

In vitro activity of LBH589 in ALL cells
We investigated the effect of treatment with LBH589 on six human derived ALL cell lines corresponding to the most representative translocations in B-cell ALL (TOM-1 t(9,22)(q32;q11), REH t(12;21)(p12;q22), 697 t(1;19)(q23;p13), SEM t(4;11)(q21;q23) and TANOUE t(8;14)(q24;q32)) and T-cell ALL (MOLT-4). In all analyzed ALL derived cell lines except to TANOUE cell line, the IC50 value of LBH589 were below 50nM (Figure S1). In order to establish the optimal dose of LBH589, apoptosis was measured in TOM-1 and MOLT-4 after treatment with doses of 1-100nM for 48 hours. At doses of 50nM a significant increase in cell apoptosis was observed in all leukemic cell lines by detection of active caspase-3 (Figure 1A), the increase in the 85-Kda fragment of PARP by western blot or the increased detection of oligonucleosomal fragments. Nevertheless some differences were found between cell lines (Figure S2). Treatment with LBH589 also markedly inhibited proliferation of ALL cells with an inhibition close to 100% after 4 days (Figure 1B and Figure S2). Inhibition of cell proliferation and increased apoptosis was associated with increased level of acetylation of histone H3 (AcH3) and histone H4 (AcH4) observed at doses of 50nM of LBH589 but not at lower concentrations (Figure 1C). As an early marker of DNA damaged and activation of DNA repair genes we examined the phosphorylation of H2AX (26) which was found to be up-regulated after treatment with LBH589 in a dose-dependent manner (Figure 1C). This suggests a link between disruption of DNA repair and apoptosis induced by LBH589 and establishes a dose of 50nM as the optimal dose in ALL cells.

While apoptosis of ALL cells was detected between 12 and 24 hours after treatment with LBH589, changes in acetylated H3 and H4 were detected as early as 2 hours (Figure 1D and 1E). Phosphorylation of H2AX was initially detected 12 to 24 hours after in vitro treatment with LBH589 depending on the cell line employed (Figure 1E). These results suggest that H3 and H4 acetylation precede DNA damaged and induction of apoptosis which might indicate that inhibition of HDAC are likely to be responsible at least in part for LBH589 induced apoptosis and inhibition of cell proliferation.
In vivo effect of LBH589 in a subcutaneous mouse model of ALL

The in vivo activity of LBH589 was initially examined in a subcutaneous ALL mouse model. The ALL cell lines TOM-1 and MOLT-4 were transplanted (1x10⁶ cell per animal) subcutaneously into the left flanks of 6-week-old female BALB/cA-Rag2⁻/⁻γc⁻/⁻. These cell lines develop into a rapidly growing tumor, as we have previously shown (8). A group of healthy control BALB/cA-Rag2⁻/⁻γc⁻/⁻ mice were treated with increasing doses of LBH589 (1, 5, 10 and 20mg/kg) administered i.p. in order to examine the maximum tolerated dose. Doses of 10mg/kg and 20mg/kg were associated with splenomegaly, weight loss and central nervous system abnormalities (Figure S3), while no adverse effects were observed at doses of 1 and 5mg/kg. Treatment with 5mg/kg of LBH589 was initiated 24 hours after injection of the leukemic cells and animals were monitored for 24 days. A significant inhibition of tumor growth was demonstrated in animals treated with LBH589 compared with control animals (P<0.01). Inhibition of leukemia cell growth was associated with an increase in the levels of acetylated H3 and H4 and an increase in phosphorylated H2AX as measured by western blot in the leukemic cells obtained after sacrifice of mice (Figure 2). These results suggest that LBH589 has a powerful antileukemic effect not only in vitro but also in vivo.

Characterization of an in vivo xenogeneic model of human ALL in immune-deficient mice

In order to examine the efficacy of LBH589 in a more representative model, human ALL cells from patients with ALL were transplanted in BALB/cA-RAG2⁻/⁻γc⁻/⁻. A total of 10 million cells from a patient with T-ALL (ALL-T1) and a patient with B-ALL (ALL-B1) (Table 1) were administered intravenously into the tail vein of immunodeficient mice. Animals were monitored by immunophenotyping in peripheral blood (PB) and/or bone marrow (BM). Mice died of leukemia or were sacrificed when signs of overt leukemia were observed such as a percentage of human blasts higher than 80% in PB, weight lost higher than 20% and/or hunched posture. After being sacrificed, spleen blasts were re-transplanted in secondary and tertiary recipients. After 2-5 generations, leukemic cells were frozen, thawed and re-injected into new immune-deficient mice with development of leukemia (Figure S4). Kinetics of engraftment of leukemic cells was monitored in PB and BM by phenotyping while organ infiltration was analyzed by immunohistochemistry (Figure S5 and S6). Differences in the disease development were observed between ALL-T1 and ALL-B1, with faster development of the disease in the case of ALL-T1 (Figure S5). However, there were no differences in engraftment or
development of the disease according to whether secondary or later recipients were analyzed.

To characterize the *in vivo* model further, conventional karyotyping as well as genotyping, gene expression and methylation arrays were performed in fresh cells from patients and samples obtained from mice at different generations (generation 1 to 5). There were no significant differences in the genome, methylome or transcriptome between the original sample and the samples obtained after multiple generations, with the exception of minor differences between the original sample from ALL-T1 and the cells obtained after multiples transplants in mice, consistent with the presence of several clones at diagnosis with persistence of a single dominant clone after sequential transplants *in vivo* (Figure S7).

**LBH589 potentiates the *in vivo* effect of chemotherapy and prolongs survival in a mouse model of human ALL**

To determine the efficacy of LBH589 alone or in combination with drugs currently used for treatment of ALL, BALB/cA-RAG2<sup>−/−</sup>γ<sup>c−/−</sup> mice engrafted with ALL-T1 and ALL-B1 cells were treated with LBH589, Vincristine and Dexamethasone or a combination of LBH589 with Vincristine-Dexamethasone. A dose-finding study was previously performed in healthy BALB/cA-RAG2<sup>−/−</sup>γ<sup>c−/−</sup> mice establishing the following doses as the optimal non-toxic combination: 0.025 mg/kg (Vincristine), 1 mg/kg (Dexamethasone) and 5 mg/kg (LBH589) (Figure S8). The in vitro and in vivo hematopoietic toxicity associated with treatment with LBH589 or Vincristine plus Dexamethasone was not increased by the combination of the 3 drugs (Figure S9). Treatment was initiated when disease could be detected in PB by FACS (24 hours after injection of cells for ALL-T1 and between day 17 and 21 after injection for ALL-B1). LBH589 was administered i.p. on days 1-5, 8-12 and 15-19, Vincristine i.v. on days 1, 8 and 21 and Dexamethasone daily until day 21 i.p. (Figures 3A and 4A) and survival was compared in the four groups of animals: control (no treatment), LBH589, Vincristine-Dexamethasone and LBH589-Vincristine-Dexamethasone.

Treatment with either LBH589 or Vincristine-Dexamethasone significantly reduced the percentage of leukemic cells in the PB (Figure 3B) and BM (Figure 4B), but the effect was significantly greater in animals treated with the combination of the HDACi and chemotherapy (*P*<0.01). Similarly, a significant reduction in spleen size was found after treatment with the combination of drugs (Figures 3C and 4C) which was associated with an increase in acetylated H3 and H4 as well as in phosphorylation of H2AX in
leukemic cells from the spleen of animals treated with LBH589 (Figures 3D and 4D). Interestingly, treatment with LBH589 not only modified the acetylation of H3 and H4 but also induced significant changes in DNA methylation of leukemic cells obtained from the spleen of leukemic mice: ALL-T1 cells from mice treated with LBH589 clustered separately from untreated samples with hypomethylation of 59 genes and hypermethylation of 3 genes after treatment (Figure 3E and Table S1). Similarly, ALL-B1 cells from mice treated with LBH589 clustered separately from untreated samples showing 217 genes hypomethylated and 54 hypermethylated genes (Figure 4 and Table S2). As an example, genes that are known to be hypermethylated in ALL such as the Wnt inhibitor SFRP4 involved in WNT pathway (11) became hypomethylated after treatment with LBH589. Finally treatment with LBH589 and Vincristine-Dexamethasone prolonged survival of the leukemic mice in comparison with the control animals (P<0.05). Furthermore, there were statistically significant differences between animals treated with the combination of LBH589-Vincristine-Dexamethasone compared with any of the other groups (Figures 3F and 4F).

**Treatment with LBH589 is associated with a decrease in the NFκB pathway activity and downregulation of its target CDK6**

To gain insights into the mechanism of the anti-leukemic action of LBH589 in ALL, TOM-1 and MOLT-4 ALL cell lines were treated for 6 hours with LBH589 at 50nM, after which their transcriptomes were analyzed. Gene expression profiles and Venn analyses of both ALL cell lines identified a total of 930 genes significantly deregulated (536 up regulated and 394 downregulated genes; transcriptional changes in gene expression of B≥0). The analysis by gene ontology (GO) indicated that treatment of TOM-1 and MOLT-4 cells lines with LBH589 induced an enrichment of genes involved in chromatin modification (P=1.64X10⁻⁶), regulation of apoptosis/cell death (P=0.003) and regulation of transcription/gene expression (P=0.001) (Figure 5A). Using Ingenuity® Pathway Analysis (IPA) and the 930 differentially expressed genes 2 different networks in which the NFκB pathway was involved were identified (Genes related to cell death, cell-to-cell signaling, interaction and drug metabolism and metabolic disease). Both networks were further interconnected suggesting a potential role for the NFκB pathway in the anti-leukemic effect of LBH589 (Figure 5B). As shown in Figure 5C, a large number of known direct or indirect NFκB target genes (http://www.nf-kb.org), were significantly deregulated in T and B leukemia cells (27) including CDK6, a described target of NFκB and a gene previously described to be
regulated by miRNAs in ALL (8). No mutations of NFKB1, NFKB2 or CDK6 (data not shown) were found in ALL cell lines or in leukemic cells from ALL-B1 or ALL-T1.

In order to determine whether regulation of CDK6 in ALL, in addition to NFκB may be regulated by the expression of miRNAs, we compared the expression of 377 miRNAs after treatment of ALL cell lines with LBH589. Of the miRNAs differentially expressed after treatment with LBH589, a group of 6 miRNA (hsa-miR-124a, -139, -141, -145, -449b and -494) that have been shown to be putative regulators of CDK6 by the bioinformatic software miRGen (www.diana.pcbi.upenn.edu/miRGen.html) or found to regulate CDK6 by previous studies were up-regulated in TOM-1 and MOLT-4 (Figure 5D) (8, 28-30).

The analysis of CDK6 expression and its target Rb after treatment with LBH589 in vitro (cell lines) and in vivo (human leukemic blasts from the spleen) revealed a down regulation of CDK6 protein expression that was associated with a decrease in the levels of phosphorylated retinoblastoma (RB-P) in vitro and in vivo (Figures 5E and F). These results suggest that treatment with LBH589 leads to inactivation of the CDK6-Rb oncogeneic pathway, frequently over expressed in ALL (Figure 5G), through inactivation of the NFκB pathway and upregulation of miRNAs. Besides these pathways, LBH589 also induced an up-regulation of the pro-apoptotic Bcl2-member BIM (BCL2L11) which has also been described to be silenced by epigenetic mechanisms in other B-cell malignancies such as Burkitt’s lymphoma suggesting a common mechanism of apoptotic blockade in lymphoid malignancies that can be reverted by LBH589 (31).

**DISCUSSION**

Despite significant progress in the treatment of patients with ALL, more than 60% of adults will succumb to their disease underlining the requirement for new therapeutic strategies for these patients (32). The results of our study clearly establish the use of the HDAC inhibitor LBH589 as a clinically useful drug with a synergistic effect with standard chemotherapy in patients with ALL. Furthermore, the development of a human leukemia mouse model could be very useful for investigating the efficacy of new drugs in ALL.

The role of epigenetic regulation (hypermethylation of DNA and histone modifications) in the prognosis and pathogenesis of ALL has been clearly demonstrated by a number
of groups (8-11) (12, 33). It provides a basis for the use of demethylating agents such as 5-aza-2-deoxycytidine or decitabine in the treatment of ALL (34). While demethylating agents have been used and approved for different hematological malignancies such as AML and myelodysplastic syndromes (35) the experience in patients with ALL is very limited (36). On the other hand the inhibitors of histone deacetylases (HDACi) and specifically LBH589 (Panobinostat) have demonstrated preclinical activity in vitro and in vivo in a wide range of malignancies such as subcutaneous and Hodgkin lymphomas (37), multiple myeloma (25), melanomas (38), lung cancer (39), colon cancer cell lines (40), head and neck squamous cell carcinoma (41), glioma cells (42) and some hematological malignances (43). Similarly, the number of studies using LBH589 in ALL is very limited (13, 44).

An important finding from our study is that the antileukemic effect of LBH589 is observed in every cytogenetic subtype of ALL including both T and B cell-ALL as well as patients with Philadelphia positive ALL. This is in agreement with recent studies, which demonstrate a synergistic effect of HDACi with tyrosine kinase inhibitors such as Imatinib or Dasatinib (45). Although mechanisms related to acetylation of other proteins may be involved in the effect of LBH589 (46), the time and dose dependent studies performed suggest that activation of acetylation of H3 and H4 are early events in ALL (2 hours after in vitro treatment). Interestingly, an increase in acetylation of H3 and H4 in leukemic cells was not only found in vitro but also in leukemic cells obtained from the spleen of treated mice.

The expression arrays performed after treatment with LBH589 provide an interesting insight into some of the putative pathways involved in the antileukemic effect induced by LBH589. The role of NFκB in the pathogenesis of certain subtypes of ALL such as T-ALL (27) or in the resistance to glucocorticoid has recently been described (47). Similarly, cyclins and cdk5 such as CDK6 have been implicated in the abnormal proliferation of ALL cells and establish these proteins as attractive targets (8). Although we have not demonstrated a direct relation between the two pathways, our results do suggest that they may be related and that activation of CDK6-Rb may be driven by the activation of the NFκB pathway. The differential expression of miRNAs after LBH treatment (hsa-miR-494 and hsa-miR-449a have been previously described as regulating CDK6 expression (28, 30)) indicates that LBH589 has a pleitropic effect on many genes and miRNA that may act in concert to induce the inhibition of CDK6 and decreased phosphorylation of Rb protein, leading to inhibition of proliferation.
The use of human ALL cells to engraft immune deficient mice has been previously exploited to assess the effect of new drugs in the treatment of ALL (48, 49) or even to predict the response of a specific patient to standard chemotherapy such as Vincristine and Dexamethasone (50). However, our approach differs from those of previous studies as we have developed a model that can be transplanted into multiple generations of animals without significant changes either in their genetic or epigenetic makeup. This makes our model particularly appropriate for long-term studies and for comparing different therapies. Whether this model is able to predict the efficacy of certain treatments in patients with ALL is yet to be demonstrated but the possibility of monitoring the disease in the PB and BM is advantageous for testing new therapeutic strategies.

Even though the combination of LBH589 with Vincristine and Dexamethasone was able to prolong survival, the mice eventually succumbed to their disease, consistent with the pattern we observed in patients with ALL, in which multiple courses of induction, consolidation and maintenance are required to treat the disease effectively. It is also plausible that this model may be helpful for testing treatments designed to mirror human ALL therapy, which would contribute significantly to the development of new therapies.

In conclusion, our results demonstrate that the addition of the HDACi LBH589 may form the basis of a novel treatment of patients with ALL as well as suggesting new candidate signal transduction pathways involved in the pathogenesis of ALL, such as CDK6, thereby providing a rationale for the use of CDK6 inhibitors in a combination of epigenetic drugs and standard therapy. The development of our human leukemic mouse model should facilitate the assessment of the efficacy of these new therapies for ALL, eventually enabling their clinical evaluation in ALL patients.
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CONFLICT OF INTEREST
The authors declare no conflict of interest.

Supplementary information is available at Leukemia's website.
REFERENCES


LEGEND TO THE FIGURES

Figure 1: \textit{In vitro} efficacy of LBH589 in ALL cell lines

(A) Apoptosis induced by LBH589 in TOM-1 and MOLT-4 cell lines measure by the activation of caspase-3 by FACS. (B) Survival of TOM-1 and MOLT-4 cells after treatment with LBH589 at doses of 50nM for 4 days. (C) Acetylation of H3 and H4 and phosphorylation of H2AX in TOM-1 and MOLT-4 cells after treatment with increasing concentrations of LBH589 measure by western blot analysis. (D) Time course analysis of activation of caspase-3 in TOM-1 and MOLT-4 ALL cells after treatment with 50nM of LBH589. (E) Acetylation of H3 and H4 and phosphorylation of H2AX in TOM-1 and MOLT-4 cells after treatment with LBH589 measure by western blot analysis. The mean±SD of at least 3 independent experiments are shown in A,B and D while a representative experiment is depicted in C and E. Total H3 was used as loading control in C and E.

Figure 2: Anti-leukemic effect of LBH589 in a subcutaneous model of ALL in immunedeficient mice

Mice (n = 8) were injected subcutaneously with 1 x 10^6 TOM-1 or MOLT-4 cells and treatment with 5 mg/kg LBH589 was initiated 24 hours later. (A) Mouse model and treatment schedule summary. Tumor size was measured in treated and control animals at different times in TOM-1 (B) and MOLT-4 (C) transplanted animals. At sacrifice (day 24), the levels of acetylated H3 and H4 and phosphorylation of H2AX were assessed by western blot. The mean ± SD of tumor size are shown, while a representative picture of the treated mice and western blot is included.

Figure 3: Synergistic effect of LBH589 with Vincristine and Dexamethasone in a human ALL mouse model of T-ALL

(A) Treatment schedule in mice engrafted with human ALL-T1 cells. (B) FACS analysis of T-ALL human blasts in PB at different times after transplantation. (C) Spleen size at sacrifice in mice from control group, LBH589, Vincristine and Dexamethasone or with the three-drug combination. (D) Western blot analysis of acetylated H3 and H4 acetylation and H2A.X phosphorylation in leukemic cells from spleen after LBH589 treatment. (E) Heat map of hypermethylated and hypomethylated genes (red and green, respectively) in leukemic cells from spleen of mice treated with LBH589 or control mice. (F) Kaplan–Meier survival curves of leukemic mice after treatment with LBH, Vincristine and Dexamethasone, or with the combination of LBH with Vincristine and Dexamethasone.
Figure 4: Synergistic effect of LBH589 with Vincristine and Dexamethasone in a human ALL mouse model of B-ALL

(A) Treatment schedule in mice engrafted with human ALL-B1 cells. (B) FACS analysis of B-ALL human blasts in the BM of control mice or those treated with LBH589, Vincristine and Dexamethasone or LBH589 with Vincristine and Dexamethasone at sacrifice. (C) Spleen size at sacrifice in control group mice, LBH589, Vincristine and Dexamethasone or with the three-drug combination. (D) Western blot analysis of acetylated H3 and H4 acetylation and H2A.X phosphorylation in leukemic cells from spleen after LBH589 treatment. (E) Heat map of hypermethylated and hypomethylated genes (red and green, respectively) in leukemic cells from spleen of mice treated with LBH589 or control mice. (F) Kaplan–Meier survival curves of leukemic mice after treatment with LBH, Vincristine and Dexamethasone, or with the combination of LBH and Vincristine and Dexamethasone.

Figure 5: NFκB and its target CDK6 are downregulated after LBH589 treatment in ALL

(A) Venn analyses and gene ontology (GO) annotation of differentially expressed genes in three biological replicates of MOLT-4 and TOM-1 after treatment with 50nM of LBH589. (B) NFκB Ingenuity network obtained with the 930 differentially expressed genes in MOLT-4 and TOM-1 after treatment with 50nM of LBH589. Red: up regulated genes after treatment; Green: down regulated genes after treatment; White: genes used by IPA to build the network which have not been analyzed in our study. (C) Dendrogram of hierarchical cluster analysis based on NFκB pathway genes in three biological replicates of MOLT-4 and TOM-1 and these cell lines after treatment with 50nM of LBH589. Red: upregulated; Green: downregulated. (D) Dendrogram of hierarchical cluster analysis of differentially expressed miRNAs in three biological replicates of TOM-1 and MOLT-4 and these cell lines after treatment with 50nM of LBH589. Red: upregulated; Green: downregulated. (E) Western blot analysis of CDK6 and Rb-P levels in MOLT-4 and TOM-1 cell lines (upper panel) and (F) in human leukemic cells from spleen mice engrafted with ALL-T1 and ALL-B1 after treatment with LBH589 (C=control animals; T=mice treated with LBH589). β-Actin was used as a loading control. (G) Western blot analysis of CDK6 and Rb-P levels in healthy donor samples (HD) and primary samples of ALL. 1, 2 and 3: PB samples of healthy donors. T: ALL-T; B: ALL-B. GAPD was used as a loading control.