



## The histone deacetylase inhibitor LBH589 enhances the anti-myeloma effects of chemotherapy *in vitro* and *in vivo*

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### ABSTRACT

Panobinostat (LBH589) is a potent histone deacetylase inhibitor (HDACi) that has shown anti-tumor activity in preclinical studies in both solid and hematological malignancies. We evaluated the anti-multiple myeloma (MM) effects of LBH589 alone and with melphalan or doxorubicin using MM cell lines and our human MM xenograft model LAG $\lambda$ -1. LBH589 treatment resulted in increased acetylation of histones, induction of caspase cleavage, inhibition of cell proliferation and synergistic anti-MM effects with melphalan or doxorubicin *in vitro*. LBH589 with melphalan or doxorubicin also showed significantly enhanced anti-myeloma activity *in vivo*. These findings provide the basis for clinical development of these combination therapies.

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### 1. Introduction

Multiple myeloma (MM) is a bone marrow-based malignancy of terminally differentiated plasma cells. It is a heterogeneous disease with specific subtypes based on unique gene-expression signatures [1,2]. Although survival has improved recently with the increasing number of effective therapies, patients eventually become resistant to all forms of therapy; and, thus, the disease remains incurable. As a result, there remains a need for new treatment options.

Histone acetylation, which is controlled by the balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities, is a major epigenetic modification that affects diverse cellular processes through regulation of gene transcription [3]. Although HDAC expression has not been directly linked with oncogenesis, it is well known that HDACs modulate the activities of many oncogenes and tumor suppressor genes [4]. In addition, many cancers are accompanied by genome-wide histone hypoacetylation [5]. HDAC inhibitors (HDACis) increase the amount of acetylated histones, transcription factors and other proteins that suppress tumor growth and survival; and, thus, are potential anti-cancer

agents [6]. Indeed, HDACis induce apoptosis and cell cycle arrest in many malignancies, including MM [7–9].

Melphalan is a member of the nitrogen mustard class of chemotherapeutic agents and exerts its anti-MM effects through alkylation of DNA [10]. It has been part of standard chemotherapy for the treatment of MM [11]. Another drug used to effectively treat MM is pegylated liposomal doxorubicin (PLD), which is doxorubicin hydrochloride encapsulated in liposomes for intravenous administration [12]. The anti-MM activity of both doxorubicin and melphalan was shown to be enhanced by the proteasome inhibitor bortezomib in preclinical studies [13], and this provided the rationale for the use of these combinations in clinical trials for MM patients [14–17]. These studies have led to the FDA approval of bortezomib with melphalan and prednisone for previously untreated MM patients and this proteasome inhibitor with PLD for MM patients with relapsed/refractory disease. Similarly, another agent active in the treatment of MM, thalidomide, has been shown to enhance the anti-MM effects of chemotherapy in preclinical studies and clinical trials [18,19].

LBH589 is a new member of the hydroxamate-derived family of HDACis which shows much more potency than other drugs in this family. It inhibits many different HDACs implicated in oncogenesis [20]. LBH589 induces protein acetylation in various cell types [21,22] and preclinical studies show its anti-tumor effects in many different tumors through inhibition of proliferation and induction of apoptosis [23–26]. Specifically, this HDACi demonstrates anti-

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tumor effects on the established human MM cell line (HMCL) MM1S [27–29].

In this study, we explored the anti-MM effects of the LBH589 alone and with melphalan or doxorubicin *in vitro* and this HDACi alone and with melphalan or PLD *in vivo* that was directly developed from a MM patient's bone marrow biopsy and has been maintained *in vivo* [30]. As an HDACi, LBH589 has been shown to increase acetylation of histone and tubulin in MM1S cells [27,28]. To verify whether LBH589 induces histone acetylation more generally in different types of MM cells, we determined the histone acetylation status in two other HMCLs, U266 and RPMI8226, following treatment with LBH589. We evaluated the anti-proliferative and pro-apoptotic effects of LBH589 alone or in combination with melphalan or doxorubicin *in vitro*. Furthermore, we determined whether LBH589 induced MM cell death in death receptor-dependent or -independent manners. We also evaluated the *in vivo* anti-MM effects of LBH589 alone or in combination with melphalan or PLD using our SCID-hu model of human MM, LAGλ-1.

## 2. Materials and methods

### 2.1. Reagents and cell culture

LBH589 was supplied by Novartis Pharmaceuticals (Basel, Switzerland). Melphalan and doxorubicin were purchased from Sigma (St. Louis, MO) and PLD was donated by our clinic. The HMCLs RPMI8226 and U266 were obtained from ATCC and MM1S was kindly supplied by Steven Rosen, MD (Northwestern University, Chicago, IL). All cell lines were maintained in RPMI1640 (Omega Scientific, Tarzana, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin, in an atmosphere of 5% carbon dioxide at 37 °C.

### 2.2. Cell viability assay

MM cells were treated with anti-MM agents for 48 h. Cell viability was measured using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) added into culture for 1–4 h before reading the plate at 490 nm. Data was graphed using the mean ± SEM of six replicates.

### 2.3. Western blot analysis

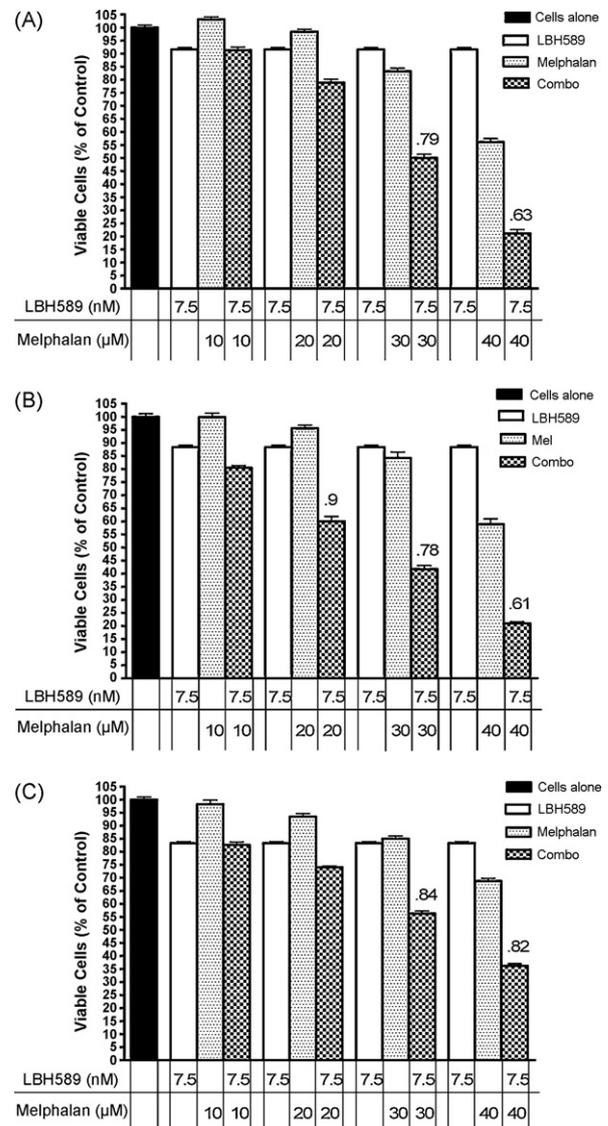
Western blotting was done according to standard protocols with the following antibodies: anti-caspase-3, -8, -9 and anti-PARP from Cell Signaling (Danvers, MA); anti-acetyl-histone H4, pan (Lys 5, 8, 12) from Upstate Biotechnology (Lake Placid, NY); anti-β-actin-HRP from Sigma (St. Louis, MO); anti-rabbit Ig-HRP and anti-mouse Ig-HRP from Jackson ImmunoResearch Laboratories (West Grove, PA). For re-blotting, membranes were incubated with Restore Western Blot Stripping Buffer from Pierce (Rockford, IL) at room temperature (RT) for 15 min, washed with PBS-Tween-20 and then incubated with another antibody.

### 2.4. Apoptotic assay using Annexin V staining

MM cells ( $5 \times 10^5$  mL<sup>-1</sup>) were treated with melphalan, doxorubicin or LBH589 or the combination of the HDACi with either chemotherapeutic agent for 24 h. After washing with PBS containing 1% FBS, cells were stained with 0.5 µl FITC-conjugated Annexin V (Biovision, Mountain View, CA) in 300 µl Annexin V binding buffer (Biovision, Mountain View, CA) for 5 min at RT. Cell death was determined using flow cytometric analysis with Cytomics FC500 and software CXP (Beckman Coulter, Miami, FL).

### 2.5. LAGλ-1 SCID-hu model

SCID mice implanted with LAGλ-1 tumor fragments developed measurable tumors and detectable levels of serum hIgG 7 days post-implantation [30]. At this time, animals were randomized into treatment groups ( $n=8$  mice/group), including control (vehicle alone), LBH589 (1 mg/kg daily, Monday to Friday), melphalan (1 mg/kg, once weekly), PLD (1 mg/kg, on three consecutive days weekly), LBH589 plus melphalan and LBH589 plus PLD. The doses and schedules of melphalan and PLD were based on previously published data from our laboratory [30]. All three drugs were administered intraperitoneally (i.p.). SCID mice bearing LAGλ-1 were bled retro-orbitally weekly. Serum hIgG levels were determined using human IgG ELISA kits according to the manufacturer's instruction manual (Bethyl Laboratories, Montgomery, TX). Absorbance at 450 nm with a reference wavelength of 550 nm was determined on a µQuant microplate spectrophotometer with KC Junior software



**Fig. 1.** Synergistic anti-myeloma activity of LBH589 in combination with melphalan. Myeloma cell lines U266 (A), RPMI8226 (B) and MM1S (C) cells were treated with a constant concentration of LBH589 and increasing concentrations of melphalan for 48 h. Cell viability was measured with the MTS assay. Bar graphs represent the percentage of cell growth with means of six replicates. CIs are shown above the bar for the synergistic results of the combination treatment.

(Bio-Tek Instruments, Winooski, VT). All animal study protocols were approved by the Institutional Animal Care and Use Committee.

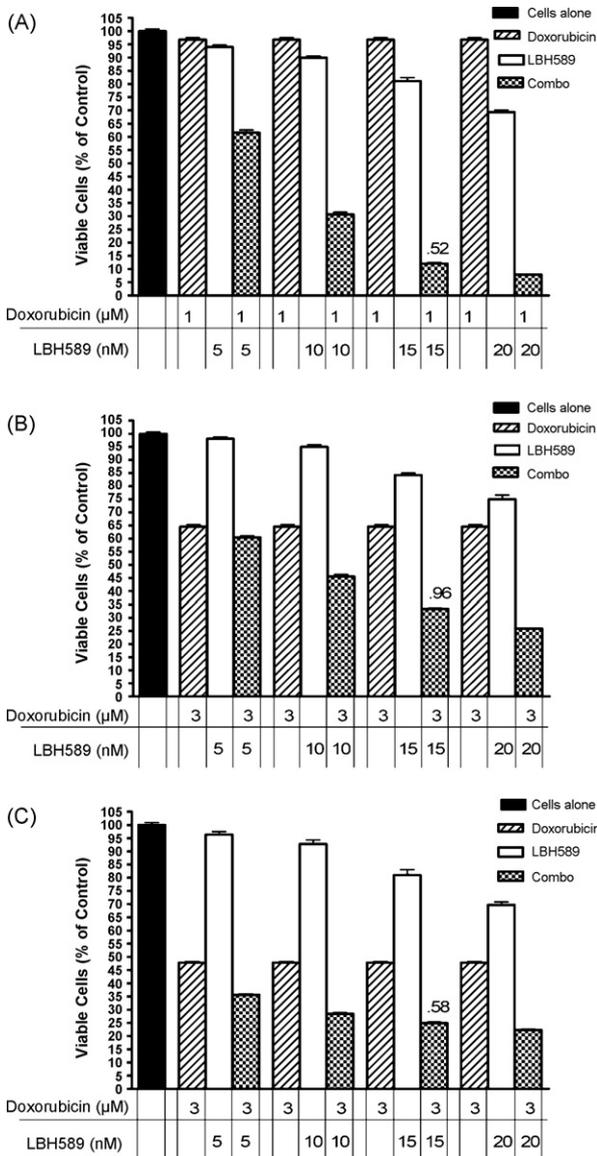
### 2.6. Statistical analysis

The Chou–Talalay method for quantitative analysis of dose–effect relationships was used to calculate a combination index (CI) for the *in vitro* studies [31]. CIs < 0.9 indicate synergistic effects and CIs 0.9–1.1 show additive effects. For *in vivo* studies, the Student's *t*-test was used and the minimal level of significance was considered to be  $P < 0.05$ .

## 3. Results

### 3.1. LBH589 in combination with melphalan or doxorubicin demonstrates synergistic anti-MM effects *in vitro*

To determine whether LBH589 in combination with melphalan or doxorubicin would produce enhanced anti-MM effects, we assessed the concentration-dependent anti-MM activities of LBH589, melphalan or doxorubicin alone on MM1S, U266 and

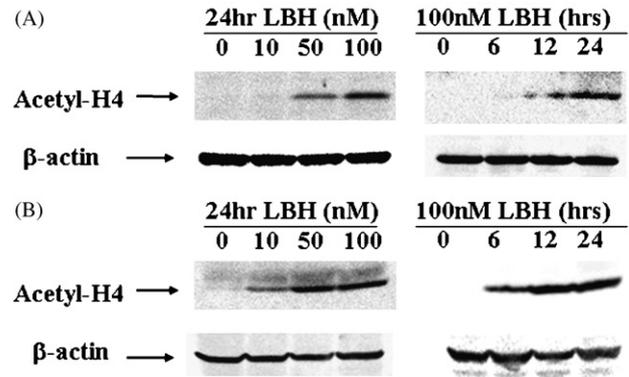


**Fig. 2.** Synergistic anti-myeloma activity of LBH589 in combination with doxorubicin. Myeloma cell lines U266 (A), RPMI8226 (B) and MM1S (C) were treated with increasing concentrations of LBH589 and constant concentrations of doxorubicin for 48 h. Cell viability was measured with the MTS assay. Bar graphs represent the percentage of cell growth with means of six replicates. CIs are shown above the bar for the synergistic results of the combination treatment.

RPMI8226 cells *in vitro* using an MTS assay (data not shown). Based on the IC values of these agents, we then evaluated the combined anti-MM effect of this HDACi with melphalan using these same cell lines. Synergism was demonstrated both when a constant concentration of melphalan and varying concentrations of LBH589 were used (data not shown) and when a constant concentration of LBH589 and varying concentrations of melphalan were used (Fig. 1A–C). All three MM cell lines showed markedly decreased viability and synergistic effects (CI < 0.9) when cells were treated with this combination compared to treatment with either drug alone, especially when cells were exposed to higher concentrations of melphalan (30 or 40 μM).

Synergistic anti-MM effects of LBH589 and doxorubicin were also observed when each MM cell line was exposed to a constant concentration of doxorubicin and varying concentrations of LBH589 (Fig. 2A–C). When three MM cell lines were exposed to a constant concentration of LBH589 and varying concentrations

**LBH589 Induced Histone H4 Acetylation in Myeloma Cells**



**Fig. 3.** LBH589-induced histone H4 acetylation in U266 and RPMI8226 myeloma cells. U266 (A) or RPMI8226 (B) cells growing in the exponential phase were treated with LBH589 for the indicated times and concentrations. Histone 4 acetylation was determined by Western blotting using an antibody against acetyl-histone 4. β-Actin was analyzed as a loading control.

of doxorubicin, enhanced effects were also observed (data not shown).

**3.2. LBH589-induced histone H4 acetylation in myeloma cells**

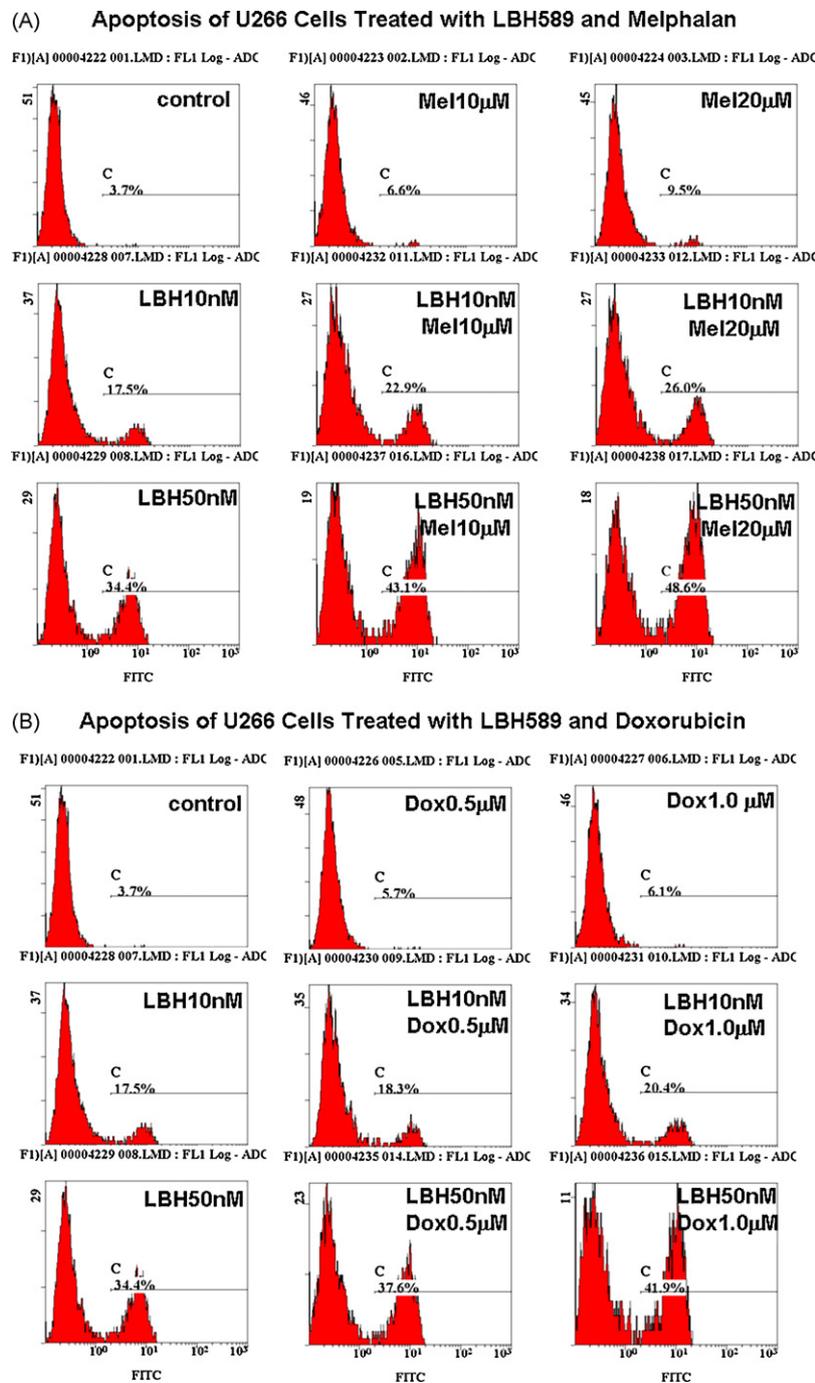
LBH589-induced histone acetylation in MM1S cells has been previously reported [27,28]. To determine histone H4 acetylation status following exposure to LBH589 in other MM cell lines, we determined the status of histone H4 acetylation in U266 and RPMI8226 cells following exposure to this HDACi. As shown in Fig. 3, LBH589-induced histone H4 acetylation in both cell lines in both a concentration- and time-dependent manner with RPMI8226 cells showing more acetylation following exposure to LBH589 than U266 cells.

**3.3. Detection of apoptosis with flow cytometric analysis**

Since the MTS assay measures the integrated consequence of cell cycle arrest and death, we assessed apoptosis of U266 cells following treatment with LBH589, melphalan, or doxorubicin alone, or the combinations of LBH589 with either melphalan or doxorubicin using flow cytometric analysis following Annexin V staining. LBH589 in combination with melphalan markedly increased apoptotic death in U266 cells compared to either drug alone, confirming that apoptosis contributes to the anti-MM effects when this HDACi is combined with melphalan (Fig. 4A). The combination of LBH589 and doxorubicin also showed enhanced apoptosis compared to either drug alone (Fig. 4B). However, this latter combination showed less of an increase in apoptosis than expected from the results from the MTS assay, suggesting that in addition to apoptosis, other mechanisms may contribute to the reduced numbers of U266 cells following their exposure to this HDACi with doxorubicin.

**3.4. Activation of caspases and PARP**

Given that apoptotic pathways contribute to LBH589-induced cell death, we then analyzed which of these pathways were involved in the cell death induced by LBH589. We first determined the activation of caspase 9, the activation marker for the mitochondrial pathway, in U266 myeloma cells upon drug treatment using Western blot analysis. Although melphalan alone at lower concentrations (10 or 20 μM) did not significantly activate caspase 9, it did enhance the activation of this caspase by LBH589 in a concentration-dependent manner (Fig. 5A). Doxorubicin induced caspase 9 activation also in a concentration-dependent manner



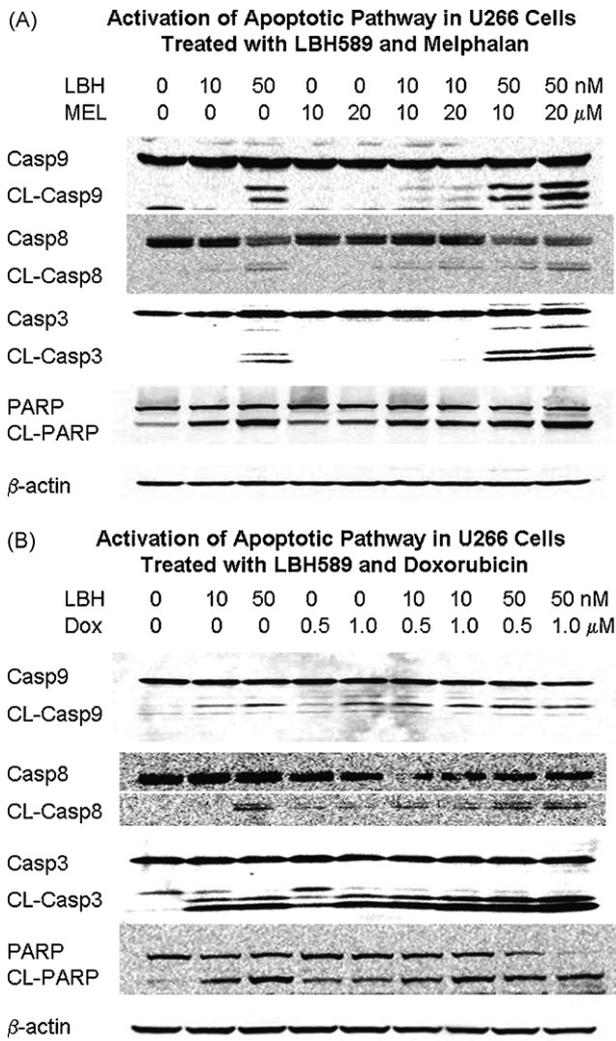
**Fig. 4.** Drug-induced apoptosis in U266 cells. U266 cells at exponential phase were treated for 24 h at the indicated concentrations of LBH589, melphalan (Mel) or the combination (A), or LBH589, doxorubicin (Dox) or the combination (B). Following labeling with Annexin V-FITC, apoptosis was determined using flow cytometric analysis. One representative set of data from four separate experiments is shown.

(Fig. 5B), and it also further enhanced the activation of this caspase that was induced by the HDACi (Fig. 5B).

Caspase 8 is the key caspase controlling the death receptor pathway. Upon drug treatment, this caspase was activated in U266 cells in a pattern similar to caspase 9 activation (Fig. 5A and B), suggesting that both the death receptor and mitochondrial pathways contribute to LBH589, melphalan and doxorubicin-induced U266 cell death. Consistent with the activation of both caspases 8 and 9, the downstream apoptotic executor common to both pathways, caspase 3, and its substrate PARP were cleaved accordingly (Fig. 5A and B).

### 3.5. Anti-MM effects of LBH589 alone and when combined with melphalan or doxorubicin *in vivo*

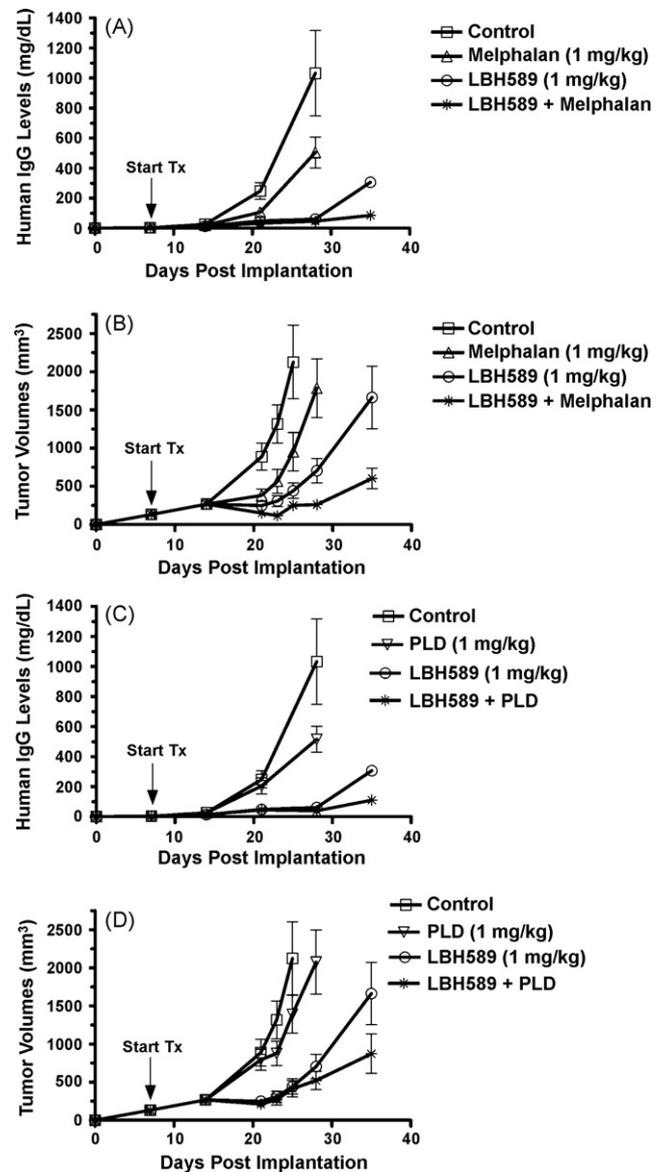
Our *in vitro* results showed that LBH589 increases the anti-MM effects of melphalan or doxorubicin. In order to determine whether similar effects occurred *in vivo*, we used our human MM xenograft model LAGλ-1 [30]. At the doses and schedules used, treatment with melphalan alone produced no anti-MM effects whereas treatment with LBH589 alone resulted in significant anti-MM activity compared to the control group. Fourteen days following initiation of treatment, mice treated with LBH589 alone showed a reduc-



**Fig. 5.** Drug-induced activation of apoptotic pathways in U266 cells. U266 cells in the exponential phase were treated for 24 h at the indicated concentrations of LBH589, melphalan (MEL), or the combination (A), or LBH589, doxorubicin (Dox) or the combination (B). Caspases (Casp) and PARP cleavage (CL) were determined using Western blotting analysis.  $\beta$ -actin was analyzed as a loading control.

tion in human paraprotein levels ( $P=0.0038$ ; Fig. 6A) and tumor growth ( $P=0.0029$ ; Fig. 6B) compared to mice receiving vehicle alone. Not only did animals treated with the combination of melphalan and LBH589 show markedly lower levels of hlgG and smaller tumors than mice treated with melphalan or vehicle alone but they also showed decreased hlgG levels and tumor growth compared to LBH589 alone-treated mice (at day 35, hlgG:  $P<0.0001$ ; tumor volume:  $P=0.0277$ , Fig. 6B). For ethical reasons, all animals from the control and melphalan alone groups were euthanized at this time point because their tumors reached the 2 cm<sup>3</sup> tumor size.

Next, we evaluated the anti-MM effects of LBH589 and PLD. Although two weeks following the start of treatment, no effect on tumor volume or hlgG levels was observed among mice treated with PLD alone, animals treated with LBH589 alone or the combination of LBH589 and PLD showed markedly decreased hlgG levels and tumor volumes compared to the control groups (for LBH alone, hlgG:  $P=0.0063$ ; tumor volume:  $P=0.0036$  and for LBH589 and PLD, hlgG:  $P=0.0197$ ; tumor volume:  $P=0.0021$ ) (Fig. 6C and D). Notably, mice treated with the combination of LBH589 and PLD exhibited marked decreases in tumor size and hlgG levels ( $P=0.0008$ ) compared to mice treated with LBH589 on day 35 (Fig. 6C and D).



**Fig. 6.** Melphalan or PLD potentiates the anti-myeloma effects of LBH589 *in vivo*. Mice bearing LAG-1 tumors were treated with vehicle alone, LBH589 (1 mg/kg daily, Monday to Friday), melphalan (A and B, 1 mg/kg, once weekly), PLD (C and D, 1 mg/kg, on three consecutive days weekly) or the combination of LBH589 with either melphalan or PLD. All drugs were administered i.p. starting one week following tumor implantation. Serum hlgG levels (A and C) were determined with ELISA and tumor volumes (B and D) were measured using calipers weekly.

All mice treated with vehicle or PLD alone had to be euthanized because of high tumor burden by day 28.

In all treatment groups involved in these *in vivo* studies, the SCID mice did not demonstrate any obvious signs of toxicity or change in body weight. This preliminary data suggests that at doses and schedules showing these profound anti-MM effects *in vivo*, the regimens were well tolerated.

#### 4. Discussion

Previous studies have identified aberrant epigenetics as an important factor involved in oncogenesis. Chromosomal remodeling, genomic hypomethylation, promoter hypermethylation and histone hypoacetylation have been identified as characteristics of cancer cells [32]. Histone acetylation is a major epigenetic modification that affects gene expression and contributes to tumorigenesis.

Detailed mechanisms underlying the contribution of HDAC activities to tumor growth remain unclear although increased HDAC activities may increase susceptibility of chromosomes to tumor-promoting transcription factors and decreased stability of some tumor suppressor gene products such as p53 and E2F1 [33,34]. HDACs block deacetylases with increased acetylation of histones and other proteins resulting in activation of many tumor suppressor gene products; and therefore, are potential anti-cancer agents.

The HDACi LBH589 increases acetylation of histones and tubulin in HMCL MM1S *in vitro* [27,28]. In this study, we have similarly demonstrated histone 4 acetylation in the two additional HMCLs U266 and RPMI8226. We also demonstrated LBH589-induced apoptotic cell death using Annexin V staining and this effect was enhanced by melphalan or doxorubicin. LBH589 has been shown to enhance or synergize with some chemotherapeutic agents *in vitro*. We have now expanded these studies by showing synergism of LBH589 in combination with melphalan or doxorubicin on the HMCLs U266, RPMI8226 and MM1S cells. It is well known that p53 mutation is one major cause of tumorigenesis. We have observed that U266 cells are resistant to another HDACi, vorinostat, and several other HDACis (data not shown) but these cells are as sensitive as MM1S and RPMI8226 cells to the more potent HDACi LBH589. Since U266 cells are defective in p53 [35], our data also suggest that intact p53 is not required for LBH589 to be cytotoxic to MM cells.

Using MM1S cells, previous studies have shown that LBH589 induced both caspase-dependent as well as independent cell death with weak activation of caspases [28] whereas others have shown strong activation of caspases [27]. Using U266 cells, we analyzed the activation of caspases 8 and 9, the major caspases involved in the death receptor and mitochondrial pathways respectively. Our results demonstrate that both pathways were activated by LBH589, and the combination with melphalan or doxorubicin further enhanced the activation of caspases, suggesting that apoptotic cell death contributes to the anti-MM effect of LBH589. This is consistent with the effect of LBH589 on MM1S cells [27,28] and different from vorinostat, another HDAC inhibitor in the hydroxamate family, which shows caspase independent activity [36,37]. As the mitochondria is the convergence site of diverse apoptotic signaling pathways, current studies have not yet elucidated which specific factors trigger the initial cell death pathway.

Studies evaluating the combination of this HDACi with dexamethasone and bortezomib, or with dexamethasone and lenalidomide *in vivo* have been reported online [29]. However, these previous studies tested the anti-MM activity *in vivo* using cells derived from the myeloma cell line MM1S. In order to test the effects of this HDACi using MM derived directly from a MM patient, we tested the anti-MM effects of LBH589 using our mouse model of human myeloma LAG-1 that has only been maintained *in vivo* [30]. Single agent treatment with LBH589 in SCID mice bearing this human MM resulted in anti-MM effects. Notably, treatment of mice with this HDACi and either melphalan or PLD increased the anti-MM effects more than the effects of treatment with LBH589 alone or single agent chemotherapy. These findings suggest that LBH589 alone is active against MM and that it also shows enhanced anti-MM effects when combined with chemotherapy. Based on these preclinical results, we have initiated a Phase I trial evaluating the combination of LBH589 and oral melphalan for patients with relapsed or refractory MM with early promising results in heavily pretreated MM patients [38]. Similar strategies combining other newer therapeutic agents that have been shown to be effective as single agents for MM patients have demonstrated enhanced anti-MM effects when combined with chemotherapeutic agents including both melphalan and doxorubicin as demonstrated in our laboratory and others and in clinical trials [13–19].

The results from our *in vitro* and *in vivo* studies expand previous preclinical reports showing the anti-MM activity of HDACis alone

and their ability to enhance the efficacy of other drugs commonly used to treat MM patients. These results provide clear support for the further clinical development of this new agent both alone and in combination with melphalan or PLD for the treatment of MM patients.

### Conflict of interest

JRB has received consulting fees, honoraria and research support for clinical and laboratory research from Novartis Pharmaceuticals.

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**Contributions.** ES designed and carried out *in vivo* experiments, interpreted data and prepared the manuscript; JS and JS carried out *in vitro* experiments; ML and CW provided technical support; BB and HC helped in the data interpretation and reviewed the manuscript; ZWL designed and carried out the *in vitro* experiments, interpreted data and prepared the manuscript; JRB designed experiments, interpreted data and prepared the manuscript.

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