Low dose histone deacetylase inhibitor, LBH589, potentiates anticancer effect of docetaxel in epithelial ovarian cancer via PI3K/Akt pathway in vitro

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\textbf{A B S T R A C T}

The purpose of this study was to investigate the effect of combination of LBH589 with docetaxel (DTX) on the growth and survival of epithelial ovarian cancer (EOC) cells in vitro and the possible mechanisms of chemo-sensitization of LBH589 in the combination treatment. The effect of LBH589 alone or in combination with DTX on four EOC cell lines (OVCAR-3, IGROV-1, A2780 and SKOV-3) was studied by MTT and clonogenic assays, acridine orange (AO)/ethidium bromide (EB) staining for apoptosis, Western blotting for apoptosis-related proteins, histone H3 and H4 proteins, DNA double strand break (DSB) repair marker and phosphorylation of Akt. LBH589 alone inhibited EOC cell proliferation in a time and dose-dependent manner. Low-dose of LBH589 (IC\textsubscript{20}) combined with DTX had an additive effect and greatly improved efficacy of DTX cell killing in EOC cells. Compared to DTX alone, the combination treatment with LBH589 and DTX induced more apoptosis and led to an increased and persistent DSB. Cell death following single or combined treatment was associated with the release of cytochrome c activity, increased caspase-3 (active) and PARP-1(cleaved), histone acetylation-related proteins and PI3K/Akt signaling pathway. Our results suggest that LBH589 enhances DTX-induced apoptosis in human EOC cells, and can be used in combination with DTX as an attractive strategy for treating human EOC.

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1. Introduction

Ovarian cancer is the leading cause of death among all gynaecological cancers in Western countries and in China. Around 90\% of human ovarian cancers are epithelial ovarian cancer (EOC), which originates from ovarian surface epithelial cells. Despite advances in surgery and chemotherapy over the past 20 years, overall survival has not changed significantly in patients with EOC and their overall 5-year survival is only 28\% [1]. Although current approaches yield responses in 60–80\% of patients with advanced stage disease, majority of EOC patients eventually relapse and become refractory to additional treatment. Approximately 70\% will experience a recurrence and ultimately die of the disease. Conventional cancer chemotherapy often results in severe side effects related to non-specific modes of action. Studies evaluating various cytotoxic agents in recurrent EOC have found response rates of 10–28\% with an accompanying progressive increase in the number of drug-resistant tumors [2]. Drug resistance and treatment toxicity are obvious limitations, and because continuous chemotherapy combined with or without targeting drug inevitably induces toxicity, patients often cannot continue with chemotherapy despite demonstrated evidence of systemic cancer control. As a result, there is an urgent need for innovative approaches for the management of this disease.

The search for agents effective in the treatment of either advanced or recurrent EOC has been disappointing. With the deep understanding of the tumor biology, there has been much interest in targeting specific pathways which are aberrant in the malignant cell by the use of new biological agents in addition to classical chemotherapy. One class of such agents is the histone deacetylase inhibitors (HDACIs) which have numerous off target effects in chemotherapy. One class of such agents is the histone deacetylase inhibitors (HDACIs) which have numerous off target effects in chemotherapy. One class of such agents is the histone deacetylase inhibitors (HDACIs) which have numerous off target effects in chemotherapy.

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LBH589 is a potent HDACi belonging to the structurally novel cinnamino hydroxamic acid class of compounds, which potently inhibits all Classes 1, 2 and 4 HDAC enzymes and has demonstrated anti-proliferative activity at low nanomolar concentrations against a broad range of tumor cell lines [8]. LBH589 alters gene expression to promote the up-regulation of proapoptotic genes and the down-regulation of anti-apoptotic genes [3], and mediates acetylation-dependent changes in nonhistone proteins involved in cell-cycle regulation, to regulate multiple oncogenic pathways [9]. It induces tumor cell-specific cytotoxicity and has demonstrated anti-tumor activity in several in vitro cancer cell lines and in vivo tumor models [10,11]. Moreover, in contrast to its effect on cancer cell lines, LBH589 is relatively sparing of normal cell lines [12] and reasonably well tolerated in patients [12]. These results support the hypothesis that this class of drug may be useful alone or in combination with other therapies to improve the treatment of the advanced EOC.

Docetaxel (DTX) has demonstrated significant activity in both pre-clinical and clinical studies for the treatment of numerous solid malignancies including EOC [13,14]. DTX combined with a platinum compound (such as carboplatin) has become the systemic chemotherapy of choice for primary EOC, with high efficacy. However, dose-related toxicity and the eventual development of resistance are major issues requiring attention in a gynaecologic oncology setting. Combination therapy specifically employing strategies such as a chemotherapeutic agent plus new small molecular inhibitor in addition to classical chemotherapy may reduce dose-limiting toxicity and improve treatment efficacy. A combination of LBH589 and DTX may provide a novel alternative treatment option.

In the current study, we examined the potential effect of LBH589 to augment the anti-tumor effect of DTX in primary and metastatic EOC cell lines and investigated the possible mechanisms of cell death and apoptosis. Our findings indicate that LBH589 combined with DTX exerted strong synergistic effects on EOC cells and induced more apoptosis via PI3k/Akt signaling pathway. LBH589 and DTX may provide a novel alternative treatment option.

2. Materials and methods

2.1. Drugs

DTX was purchased from Sigma–Aldrich Pty Ltd. (Castle Hill, NSW, Australia) and LBH589 was purchased from Selleck (Houston, TX, USA). The two drugs were first diluted in DMSO and then in growth medium. The final DMSO concentration was 0.01%.

2.2. Antibodies

Primary and secondary antibodies were purchased from different companies. The detailed source information and optimal Western blotting conditions for all the antibodies are listed in Table 1.

2.3. Cell lines

Primary (OVCAR-3, IGR-OV-1 and A2780) and metastatic (SKOV-3) EOC cell lines were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). All tissue culture reagents were supplied by the Invitrogen Australia Pty Ltd. (Melbourne, VIC, Australia), unless otherwise stated. OVCAR-3, IGR-OV-1, A2780 and SKOV-3 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 50 units/mL penicillin and 50 µg/mL streptomycin. All cell lines were maintained in a humidified incubator at 37 °C and 5% CO2. Sub-confluent cells cultured for 48 h without a change of medium were harvested by gently rinsing flasks twice with Dulbecco’s phosphate-buffer saline (DPBS) and then detached with 0.25% trypsin/0.05% EDTA in DPBS at 37 °C. Cells were collected and re-suspended in the appropriate buffer as described below.

2.4. In vitro cell cytotoxicity assay

Cell cytotoxicity was evaluated in EOC cell lines after LBH589 treatment using MTT assay following the published method [15]. Briefly, 2000 cells were seeded in 96-well plates incubated in culture media for 24 h. Cells were then treated with a range of concentrations of LBH589 (0–20 µM) or the same volume of DMSO control in fresh media for another 24, 48 and 72 h, respectively. The absorbance (OD) was read at 560 nm on a BIO-TEC micro-plate reader (Bio-RAD, Hercules, CA, USA). Each experiment was repeated at least three times. Results are represented as the OD ratio of the treated and vehicle control cells. The IC50 value (20% inhibitory concentration) of LBH589 at 24 h was calculated and chosen for the following experiments.

2.5. Colony forming assays

OVCAR-3, IGR-OV-1, A2780 and SKOV-3 cells were used for colony forming assays as described previously with minor modifications [15]. Briefly, 500 cells/well were seeded in six-well plates for 48 h at 37 °C, 5% CO2 and then treated with either a range of concentrations of DTX (10−7 to 10−12 M), combination (DTX plus LBH589 at the respective IC50 concentrations) or DMSO control. After 3 days treatment, the DTX or LBH589-containing media was replaced with fresh media and all cultures were incubated for an additional 7 days until colonies were large enough to be clearly discerned. After washing with DPBS, cells were stained with crystal violet (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia). The colonies, defined as groups of >50 cells, were scored manually with the aid of an Olympus INT-2 inverted microscope (Tokyo, Japan). Data from DTX, or DTX plus LBH589-treated cells were normalized against the DMSO vehicle treatment alone (scored as 100% colony forming ability). Cell survival curves were plotted, with mean and SD of at least three independent experiments.

2.6. Detection of apoptosis

OVCAR-3, IGR-OV-1, A2780 and SKOV-3 cells (5 × 104) were cultured in 6 well plates for 24 h and then treated with DMSO vehicle control, LBH589 (0.007–0.016 µM, IC50), DTX (0.00079–0.16 nM, IC50), or combined LBH589/DTX (0.0079–0.16 µM, IC50) and DTX (0.00079–0.16 µM, IC50) for 48 h. After treatments, apoptotic cells were observed by assessing nuclear morphology using acridine orange (AO)/ethidium bromide (EB) staining and ELISA test for cytochrome c activity.

1. Detection of nuclear morphology: Cells exposed to different treatments were stained with the DNA-binding agents AO/EB (Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia) as previously published [16] and examined with confocal microscopy (FV 300/FV500 Olympus, Tokyo, Japan). Apoptotic cells were characterized by morphology including nuclear condensation and fragmentation.

2. Detection of cytochrome c activity: Briefly, tryptophanized and non-adoriented cells were pooled, washed with cold DPBS at 1000g for 5 min at 4 °C as previously published [15]. The procedure was performed according to the manufacturer’s instructions (Cytochrome c Immunoassay Kit; R&D systems, Minneapolis, MN, USA). Sample absorbance was measured at 570 nm in a micro-plate reader (Bio-TEK, Winooski, Vermont, USA). A standard curve was constructed by plotting the absorbance values of diluted solutions of a cytochrome c standard. The results were compared with a standard curve and multiplied by the dilution factor. The amount was expressed as ng/mL.

2.7. Western blotting analysis

Western blotting analysis was used to determine the protein expression levels for apoptosis-related proteins, histone H3 and 4 proteins, DNA double strand break (DSB) repair marker and phosphorylation of Akt, mTOR and p70S6K as previously published [17]. Briefly, OVCAR-3, IGR-OV-1, A2780 and SKOV-3 cells (2 × 106) were plated in 10 cm dish for 24 h, then treated with DMSO vehicle control, LBH589, DTX, or combined LBH589 and DTX as described in the above section. Detection of apoptosis for 48 h. Whole cell lysate was separated by NuPAGE Novex 4–12% Bis–Tris gel electrophoresis and then transferred to polyvinylidene difluoride membrane. After blocking of non-specific sites with 5% skim milk, the membrane was incubated with specific antibodies at appropriate concentrations, followed by incubation with HRP-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit depend on the host species of primary antibody) (1:5000 dilution) (Table 1). ImageQuant LAS4000 system (GE Healthcare, USA) was used for image recording. Images were processed in Adobe Photoshop. Band densities of Western blotting were obtained using the Image J software (NIH, USA) and normalized by division with the respective β-tubulin band density.

2.8. Statistical analysis

All numerical data were expressed as averages (mean), and the standard deviation (SD) was calculated. The data from treated and control groups were compared using the two-tail student’s t test. All P values were 2-sided. One way ANOVA, fol-
after 24–72 h treatments and no cytotoxic effect was found for ranging from 0.002 to 6.3 (Fig. 1A). Each cell line displayed a variable response to LBH589 and dose-dependent cell proliferation inhibition by LBH589 were followed by the Dunnett’s post hoc test was performed to determine the significance of differences between different treatment groups. P < 0.05 was considered significant. All statistical analyses were performed using the GraphPad Prism 4.00 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Cytotoxicity of LBH589 alone on LBH589 alone in vitro

MTT assay was used to determine the effect of LBH589 treatment on single cell cytotoxicity. Our results indicated that time and dose-dependent cell proliferation inhibition by LBH589 were observed in OVCAR-3, IGROV-1, A2780 and SKOV-3 EOC cell lines (Fig. 1A). Each cell line displayed a variable response to LBH589 after 24–72 h treatments and no cytotoxic effect was found for vehicle control in all cell lines tested (data not shown), with IC50 ranging from 0.002 to 6.3 μM. IC50 values at 24, 48 and 72 h for four EOC cell lines are summarized in Table 2. The metastatic SKOV-3 cell line was shown to be less sensitive to LBH589 treatment compared to other three primary EOC cell lines except for IGROV-1 cell lines at 48 and 72 h. The IC50 values at 48 h were chosen for the following combination study.

3.2. Effect of combination of LBH589 and DTX treatment on colony formation using EOC cell lines

 Colony formation assay was used to determine the long-term effect of single DTX or combination (LBH589 and DTX) on EOC cell growth. Our results indicated that single DTX or combination (low dose LBH589 with IC50 + DTX) reduced colony growth of four EOC cell lines in a dose dependent manner and that addition of LBH589 in the DTX treated cells led to more decreased colony forming ability (P < 0.05) (Fig. 1B). The effect of combined treatment with LHDS9 and DTX was 2.5–8 times that of DTX alone. After using the published method to determine synergy or additivity [18], we found that low dose LBH589 potentiates the cytotoxic effect of DTX in an additive manner. The IC50 values after single DTX or combination of LBH589 and DTX treatments in four EOC cell lines are summarized in Table 2. The most sensitive EOC cell line to the combination treatment is A2780 while the most resistant EOC cell line to the combination is SKOV-3. In this study, only colonies with >50 cells after treatment were counted as positive while small colonies <50 cells after treatment were not counted (Fig. 1C). The typical images for colony growth with different treatments and control are shown in Fig. 1D. There was a significant reduction in colony formation potential for the primary and metastatic cells with combined LBH589 and DTX treatment, compared to control, LBH589 or DTX alone (Fig. 1D).

3.3. Morphological changes and evaluation of apoptosis after single or combination treatment

To investigate whether apoptosis is the pathway of cell death after treatment, we observed cell morphological changes, used AO/EB assay to evaluate the proportion of apoptosis and used ELISA to examine cytochrome c activity in four EOC cell lines (OVCAR-3, IGROV-1, A2780 and SKOV-3). After treatment with LBH589 or DTX treatment, or combined treatment with LBH589 and DTX, obvious morphological changes were found in combination treated cells compared to those treated with single LBH589, DTX or vehicle control (Fig. 2A). Cells exposed to the combination treatment for 48 h displayed characteristic apoptotic morphology including rounding up and detachment from the flask surface, and condensed chromatin and cytoplasm (i.e., cells became rounded, shrunken, and detached), while untreated control did not exhibit apoptotic morphology (Fig. 2A). The characteristic morphological changes in treated cells were confirmed by AO/EB staining, which showed typical features of apoptosis especially in the combination treatment, including nuclear condensation and fragmentation (Fig. 2B). These changes did not occur in untreated control cells (Fig. 2B).

3.4. Effect of single and combination treatments on apoptosis-related proteins

To investigate the mechanisms of combination effect caused by LBH589 and DTX on EOC cells, we examined the apoptosis-related proteins after single or combination treatments. Our results indi-

**Table 1**

Antibodies used for Western blotting (WB).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Type</th>
<th>Dilution for WB</th>
<th>Incubation time (min)</th>
<th>Temperature (°C)</th>
<th>Application</th>
</tr>
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<td>MAb</td>
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<td>0/N</td>
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<td>WB</td>
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<td>MAb</td>
<td>1:1000</td>
<td>0/N</td>
<td>4</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit anti-human PARP (unchieved)</td>
<td>Epitomics, Inc.</td>
<td>MAb</td>
<td>1:1000</td>
<td>0/N</td>
<td>4</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit anti-human PARP (cleaved)</td>
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<td>MAb</td>
<td>1:1000</td>
<td>0/N</td>
<td>4</td>
<td>WB</td>
</tr>
<tr>
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<td>MAb</td>
<td>1:1000</td>
<td>0/N</td>
<td>4</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit anti-human bax</td>
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<td>MAb</td>
<td>1:1000</td>
<td>0/N</td>
<td>4</td>
<td>WB</td>
</tr>
<tr>
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<td>Pab</td>
<td>1:500</td>
<td>0/N</td>
<td>4</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit anti-human histone H4</td>
<td>Abcam</td>
<td>Pab</td>
<td>1:1000</td>
<td>0/N</td>
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<td>1:1000</td>
<td>0/N</td>
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<td>WB</td>
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<td>Mouse anti-human gamma H2A-X</td>
<td>Abcam</td>
<td>MAb</td>
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<td>0/N</td>
<td>4</td>
<td>WB</td>
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<tr>
<td>β Tubulin antibody</td>
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<td>O/N</td>
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<td>1:200</td>
<td>0/N</td>
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<td>1:1000</td>
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<td>MAb</td>
<td>1:1000</td>
<td>0/N</td>
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<td>WB</td>
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<td>Pab</td>
<td>1:200</td>
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<td>IgG</td>
<td>1:2000</td>
<td>1 H</td>
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<td>WB</td>
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<td>Goat anti-mouse IgG–HRP</td>
<td>Santa Cruz Biotechnology</td>
<td>IgG</td>
<td>1:2000</td>
<td>1 H</td>
<td>RT</td>
<td>WB</td>
</tr>
</tbody>
</table>

Notes: H: hour; MAb: monoclonal antibody; PAb: polyclonal antibody; O/N: overnight; PAb: polyclonal antibody; RT: room temperature.
cated that compared to vehicle control, single LBH589, DTX and combination of LBH589 and DTX induced increased cleavage of full length caspase-3, and PARP-1 but not pro-caspase-3 and caspase-7 in four EOC cell lines (Fig. 3A). Accordingly, the 85KD uncleaved form of PARP-1 was downregulated when the cleaved PARP-1 increased (Fig. 3A). The increased levels in caspase-3 (active) and PARP-1 (cleaved) were obvious (1–16-fold) in combination treatment compared to any single treatment in four EOC cell lines (Fig. 3A). We also analyzed the level of proteins that regulate cell survival and death and found reduced trend for Bcl-2 and increased trend for Bax from vehicle control, LBH589, DTX to combination treatment (LBH589 and DTX) in all four EOC cell lines (Fig. 3A).

Quantification of Western blot results in four EOC cell lines was shown in Supplementary data (Figs. S1–S4, Table S1).

3.5. Effect of single and combination treatments on histone acetylation-related proteins and DNA DSB marker

To investigate the mechanisms of chemo-sensitization effect caused by LBH589 on EOC cells, we examined the histone acetylation proteins after LBH589, DTX or combination treatments. Our results indicated that low-dose LBH589 treatment (IC_{50}) and combination of LBH589 and DTX induced acetylation of histone 3 (H3) and histone 4 (H4) in four EOC cells and the levels of H3 and H4

![Fig. 1. Effects of single LBH598 treatment by MTT assay and combination treatment with LBH589 and DTX by colony forming assay in EOC cell lines in vitro. (A) Cell growth for single LBH589 treatment was assessed by MTT assay. Representative survival curves at 24, 48 and 72 h are shown. (B) EOC cells were seeded in six-well plates and treated after 48 h with a range of DTX or combined LBH589 (IC_{20}) and a range of DTX for 3 days. Following treatments, cells were cultured in growth medium for 7 days. Colonies containing ≥50 cells were counted. Results are presented as a percentage of control colony formation (100%). [Mean% ± SD, n = 5, *P < 0.05 compared DTX alone to combination of LBH589 and DTX]. (C) Representative examples of colonies >50 (positive) or <50 (negative) cells are shown. (D) Typical images are shown for colony growth in DTX alone and combination treatment (LBH589 and DTX) in EOC cells. The images were taken by a Sony camera (Tokyo, Japan). The results are from three independent experiments (n = 3). DTX: docetaxel; VC: DMSO vehicle control.]
proteins were significantly increased (2–27-fold) in combination treatment or LBH589 treatment (2–23-fold) compared to vehicle and DTX; the levels of H3 and H4 expression were higher in combination treatment than those in any of single treatment (Fig. 3B), suggesting that LBH589 successfully inhibits HDAC activities in EOC cell lines and induces apoptosis pathway related to histone acetylation.

DNA DSB was examined using γH2AX as a marker by Western blotting. We found that single LBH589, DTX and combination induced increased γH2AX proteins (2–103 fold) compared to vehicle control and combination treatment induced more γH2AX proteins (Fig. 3C), suggesting that combination treatment induce more DNA DSBs. Quantification of Western blot results in four EOC cell lines were shown in Supplementary data (Figs. S1–S4, Table S1).

3.6. Effect of single and combination treatments on PI3K/Akt signaling pathway

PI3K/Akt signaling pathway is important for regulating cell growth and survival, particularly during tumor progression and
metastases. To investigate if PI3K/Akt signaling pathway is involved in single or combination treatment, we examined t-Akt/p-Akt, t-mTOR/p-mTOR and t-p70S6K/p-p70S6K expression using Western blotting analysis. Our results indicated that reduced p-Akt, p-mTOR and p-p70S6K levels were found in LBH589 or DTX treatments and that compared with the VC, obvious reduction of p-Akt (3–90%), p-mTOR (7–21%) and p-p70S6K (11–38%) was found in combination while no change was found in t-Akt, t-mTOR and t-p70S6K treatment.

Fig. 2. Morphological changes and apoptosis in EOC cell lines treated with single treatments (LBH589 or DTX) and combination treatment (LBH589 and DTX). (A) At 48 h post-treatment, treated cells displayed rounding up and detachment from the flask surface compared to vehicle-treated control cells. (B) Condensed and fragmented nuclear chromatin characteristic of apoptosis is clearly seen in treated cells using acridine orange/ethidium bromide staining and confocal microscopy (red) while VC-treated cells appeared normal (green). Magnification ×40 in A and ×60 in B. (C) Induction of cytochrome c release in EOC cells treated with LBH589, DTX or combined LBH589 and DTX compared to VC. Activity was measured using ELISA 48 h post-treatment. (Mean ± SD, n = 3. *P < 0.05 indicates single treatment alone compared to VC; and **P < 0.05 indicates combination treatment compared to LBH589 or DTX alone. The results were from three independent experiments (n = 3). DTX: docetaxel; VC: vehicle control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
and t-p70S6K (Fig. 3D). Quantification of Western blot results in four EOC cell lines was shown in Supplementary data (Figs. S1–S4, Table S1).

4. Discussion

In the present study, we investigated the effect of LBH589 alone and combination of LBH589 with DTX in killing EOC cells. Our results demonstrated that LBH589 inhibited the growth of OVCAR-3, IGROV-1, A2780 and SKOV-3 cells in a time and dose-dependent manner and at low doses LBH589 effectively killed EOC cells. In pre-clinical studies of EOC, a number of HDACIs have demonstrated impressive anti-proliferative (and generally nontoxic) effects against cultured cells and xenograft tumors. In one study, VPA (Depakote), impressively (\(-80\%\)) suppressed the growth of SKOV-3 EOC xenograft tumors, with significant p21 up-regulation in tumor tissues [5]. Despite encouraging pre-clinical studies, however, VPA has proved fairly disappointing in clinical trials for myeloid malignancies [19], diminishing optimism for its eventual use against human solid tumors. In another study, PXD-101 (belinostat), has demonstrated impressive anti-tumor effects against aggressive EOC xenografts [20,21] and is now in clinical trials. However, in contrast to hematologic malignancies, single-agent trials of HDACIs for solid tumors, including EOC, have only rarely demonstrated measurable patient responses [7].

HDACIs were able to induce the accumulation of acetylated histones in the chromatin of the p21WAF1 gene in human EOC cells. In xenograft models, some of HDACIs have demonstrated anti-tumor activity with only few side effects [5]. Some clinical trials demonstrated that HDACi drugs provide an important class of new mechanism-based therapeutics for EOC [22]. Qian et al. demonstrated that combination of PXD101 (HDACI) and carboplatin could enhance anti-tumor activity on human A2780 EOC xenografts [21]. Cooper et al. reported that combination of SAHA (HDACI) and paclitaxel increased survival of mice in a nude EOC animal model compared to single SAHA or paclitaxel alone [23]. Although HDACIs can be used for anti-cancer therapies, these agents may lack efficacy as monotherapies. It is generally agreed that HDACIs are most effective in combination with other agents [7].

LBH589 is a potent pan-HDACi, causing cell cycle arrest in G2/M and has been shown to kill tumor cell lines both in vitro and in vivo.
Using LBH589 to inhibit HDAC activity is a rational therapeutic strategy currently in clinical development. Recent studies have demonstrated synergistic anti-proliferative effects in cells co-treated with a chemotherapeutic drug and HDACi [25,26]. Ma et al. demonstrated that combination of cisplatin and low-dose LBH589 can overcome cisplatin-associated resistance in EOC cells [27]. However, the precise mechanism of LBH589 in these combination studies is still elusive.

The anti-tumorigenic properties of HDACIs are especially notable due to the fact that their cytotoxic effects are specific to cancer cells and not to normal cells or tissues. Compared with other anti-cancer agents, HDACIs are well tolerated with a good toxicity profile [28]. It was reported that HDACIs enhance the anti-tumor effects of DTX [29,30] in human prostate cancer. A phase I study of oral LBH589 alone and in combination with DTX has been performed in castration-resistant prostate cancer (CRPC) patients [29]. These remarkable properties of HDACIs have led many researchers to focus their study on the effects of HDACIs in combination with other chemotherapies.

DTX is now considered the preferred chemotherapeutic agent for EOC. The most widely described mechanism by which DTX achieves this effect is through its activity as a mitotic spindle poison, disrupting microtubule dynamics and inducing G2/M cell cycle arrest, a downstream effect thought to be related to the phosphorylation of Bcl-2 [31]. In vitro data have suggested that DTX is more potent than paclitaxel in microtubule disruption [32]. It was reported that combinations of DTX with other agents could sensitize EOC cell responses and improve survival of patients with metastatic EOC [33]. In this study, we hypothesized that LBH589 could increase the sensitivity of EOC cells to DTX, as well as being directly cytotoxic to the cells. We examined the capacity of low dose LBH589 in potentiating the activity of DTX, which is the most effective chemotherapeutic agent for metastatic EOC until now.

Colony formation assay provides a more appropriate measure of the long-term effects of potential therapeutic agents, assessing the ability of cells to retain proliferative potential after treatment, a characteristic that clinically facilitates tumor recurrences in patients. In the current study, we showed that addition of low dose of LBH589 to different concentrations of DTX, the IC50 was greatly reduced in combination (13–40%) compared to DTX alone (Fig. 1B and Table 2), suggesting that LBH589 can increase the DTX sensitivity and the combination of LBH589 and DTX has an additive effect on EOC cells. Because the toxicity of LBH589 is much less than that of chemotherapeutic agents, this has implications clinically, where combined treatment using LBH589 and reduced dose of DTX may result in lower toxicity to EOC patients, with the same or even better clinical outcomes. These important results may potentially expand more clinical applications of LBH589 and benefit patients with advanced or recurrent EOC by increasing DTX sensitivity of tumors and minimizing its side effects. The enhanced response was observed for both primary and metastatic EOC cell lines, indicating that combined treatment could be a valid option for patients with localized and metastatic EOC disease.

As described above, single LBH589 treatment and combination of low dose LBH589 and DTX treatment could effectively inhibit EOC cell growth in vitro. While the mechanisms for this remain unclear, we conducted preliminary studies to determine whether apoptosis was involved, including AO/EB staining, ELISA for cytochrome c activity and Western blotting for caspasases, Bax/Bcl-2 and γH2AX expression. We found obviously increased AO/EB positive EOC cells in combination treatment compared to single DTX and LBH589 or vehicle control treatments (Fig. 2B), suggesting that combination treatment could induce more apoptosis. As activation of caspase-3 and cleavage of PARP-1 are markers for apoptosis, we also examined their expression and found more obviously increased expression of caspase-3 (active) and PARP-1 (cleaved) (13–32-fold and 6–145 fold, respectively), as well as decreased expression of full length PARP-1 (72–99% reduction) compared with VC control in combination treatment and no change in the expression of caspase-7 (active) in four EOC cell lines, suggesting that both caspase-3 and PARP-1 are involved in apoptosis induced by single and combination treatments.

In the present study, compared to vehicle treated control cells, we found a 1.5–4-fold increase in cytosolic cytochrome c activity in four EOC cell lines in response to single LBH589 and DTX alone treatment or combined treatment with LBH589 and DTX. The increase in both cytosolic cytochrome c activity (ELISA) and caspase-3 protein (Western blotting) is consistent with the cell cytotoxicity data and AO/EB results. Caspase-3 is a member of the apoptosis execution functional group of caspases, and is either partially or totally responsible for the proteolytic cleavage of many key proteins during apoptosis [34]. It is activated by proteolytic cleavage into two active subunits only when cells undergo apoptosis [35]. PARP-1 is a marker for apoptosis that can be immediately activated by DNA strand breaks; the degree of PARP-1 activation could be an important factor in the cell’s ability to repair the damage and survive or to die [36]. PARP-1 activation following limited DNA injury could constitute a signal to activate the repair and cell cycle control machineries.

The mitochondrial pathway is an important mechanism of apoptosis. Bax and Bcl-2 belong to a multigene family of proteins that play an important role in the regulation of apoptosis via mitochondrial pathway. The key element in mitochondria pathway is the efflux of cytochrome c from mitochondria to cytosol, where it subsequently forms a complex with Apaf-1 and caspase-9, leading to the activation of the caspase-3 [37]. The Bcl-2 family members regulate mitochondrial release of cytochrome c, mainly through alterations in levels of Bax and Bcl-2 proteins and increase of the ratio of Bax/Bcl-2 [38]. The exact mechanisms of action for the LBH589 on apoptosis are still unclear. One possibility is that it might work indirectly through Bcl-2 family-cytochrome c-caspase cascades or directly work on cytochrome c/caspase-3. Another possibility is that it might exert its action on other apoptosis related pathways. In our current results, we evaluated the Bcl-2 and Bax expression, and found the reduced Bcl-2 and increased Bax trend from vehicle control (VC), single LBH589 and DTX treatments to combination treatment in four EOC cell lines (Fig. 3A), however it is possible that other mechanisms are also involved in the LBH589-regulated apoptosis. The exact mechanisms of action of LBH589 on inducing apoptosis will be investigated in our future studies.

HDACIs were discovered to increase histone acetylation through the inhibition of deacetylase enzymes [39]. In the current study, we also found low-dose LBH589 treatment (IC50) and combination of LBH589 and DTX induced acetylation of histone 3 (H3) and histone 4 (H4) in four EOC cells and the levels of H3 and H4 proteins were significantly higher in combination treatment or LBH589 treatment compared to vehicle and DTX treatment (Fig. 3B), suggesting that LBH589 successfully inhibits HDAC activities in EOC cell lines and induces apoptosis pathway related to histone acetylation.

In our study, γH2AX was used as a biomarker to measure DSB because γH2AX is a highly specific and sensitive molecular marker for monitoring both DSB initiation and resolution [40]. We found that phosphorylation of histone H2AX was enhanced by combination treatment with LBH589 and DTX in four EOC cells, suggesting that combination induces more DNA DSBs and further supporting that combination treatment is more effective than any single treatment (LBH589 or DTX). All above results from AO/EB staining, ELISA and Western blotting revealed that LBH589 alone and combination of LBH589 and DTX induced EOC cell apoptosis and the combination treatment can more effectively treat EOC cells in vitro.
The PI3K/Akt pathway is one of the most important pathways in ovarian cancer [41], and plays a critical role in the control of cell growth and proliferation [42]. The PI3K/Akt pathway is frequently overexpressed/activated in ovarian cancers [43]. It inhibits apoptosis as it inhibits cytochrome c release and expression of Bad and Bax [44]. Fraser et al. demonstrated that PI3K/Akt pathway has been shown to be constitutively active in ovarian cancer cell lines [45]. Huang et al. recently reported that using small interfering (siRNA) gene knockdown, down-regulating key components of the PI3K/Akt pathway can effectively induce EOC cell apoptosis and inhibit proliferation [46]. In the current study, to investigate whether PI3K/mTOR signaling pathway is involved in apoptosis induced by single LBH589589, DTX and combination of LBH589 and DTX treatments, we detected the change of p-Akt expression which is an important indicator and is activated in a PI3K-dependent manner by growth factors and cytokines, and is implicated in cell proliferation and survival. As downstream targets of Akt, mTOR and mTOR substrates are critical in cell growth and proliferation in the oncogenic process of PI3K/Akt induced signaling pathway. Our results indicate that obvious reduction of p-Akt, p-mTOR and p-p70S6K (a kinase which is acting on downstream of mTOR) was also seen in combination treatment and reduction of p-Akt, p-mTOR and p-p70S6K was seen in single LBH589 or DTX treatment; that simultaneously significant decrease of caspase-3 (active), PARP-1 (cleaved), Bcl-2 was found in the combination treatment, suggesting that PI3K/Akt signaling pathway is involved in single or combination treatments induced apoptosis and that this combination treatment may have effect on EOC cells via PI3K/Akt signaling pathway. The exact mechanisms of how PI3K/Akt signaling pathway plays a role in regulation of cell death in EOC cells will be investigated in future studies.

In summary, we have demonstrated for the first time that LBH589 inhibited EOC cell proliferation in a dose-dependent manner and low-dose LBH589 combined with DTX greatly enhanced killing of EOC cells. The putative mechanisms of the synergistic effect of this combination treatment in EOC cells include induction of apoptosis and more DNA damage via PI3K/Akt signaling pathway. Accordingly, we recommend that this combination be further evaluated in animal models of EOC and that it be considered for clinical trials. This combination treatment may provide a treatment strategy to improve anti-cancer efficacy while reducing the toxicity often seen in patients treated with DTX alone. In particular, our results may provide a basis for reducing the dose of DTX required, without adversely impacting on treatment efficacy. Additionally, since the mechanisms described are not EOC-specific, this combination may also prove beneficial for other malignancies commonly treated with DTX, including advanced prostate and breast cancers. Taken together, our data indicate that combined LBH589 and DTX (chemotherapy) offer opportunities for novel therapeutic strategies in EOC and other cancers.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.08.035.

References


