

## Histone Deacetylase Inhibitors Demonstrate Significant Preclinical Activity as Single Agents, and in Combination with Bortezomib in Waldenström's Macroglobulinemia

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### Abstract

We studied the role of histone deacetylase inhibitors in Waldenström's macroglobulinemia (WM). Gene expression profiling of bone marrow CD19+ cells from 30 patients and 10 healthy donors showed overexpression of HDAC4, HDAC9, and Sirt5, with validation of HDAC9 overexpression by q-PCR in primary and BCWM.1 cells. Suberoylanilide hydroxamic acid, trichostatin A, panobinostat, and sirtinol demonstrated dose-dependent killing of BCWM.1 cells. TSA showed the greatest potency with IC50 of 70 nM. Importantly, HDAC9 activity was decreased following TSA treatment suggesting an essential role for this HDAC in WM therapy. The combination of bortezomib plus HDAC inhibitors resulted in at least additive tumor cell killing in BCWM.1 cells. TSA and bortezomib-induced apoptosis depended on a similar set of caspase activation, whereas their effect on cell cycle regulators was distinctly different. These results provided a framework for examining HDAC inhibitors as monotherapy, as well as combination therapy with bortezomib in WM.

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### Introduction

Waldenström's macroglobulinemia (WM) is a low-grade lymphoproliferative disorder characterized by bone marrow (BM) infiltration of lymphoplasmacytic cells and production of monoclonal IgM protein.<sup>1,2</sup> Despite advances, WM remains incurable. Novel therapies are urgently needed.

Using gene expression profiling, we observed upregulation of HDACs in primary WM cells.<sup>3</sup> HDACs are involved in transcription regulation and signal transduction through modification of histones and non-histone proteins.<sup>4</sup> HDAC-specific inhibitors are investigated in other B-cell malignancies, alone and in combination therapy with bortezomib.<sup>5-9</sup> Bortezomib is active in both upfront and relapsed/refractory WM, producing 60%-80% response rate. We sought to delineate HDAC expression in WM, and to elucidate the effect of HDAC inhibitors by examining the activity of suberoylanilide hydroxamic acid (SAHA aka vorinostat), trichostatin A (TSA), panobinostat (LBH-589), and sirtinol as monotherapy and in combination with bortezomib in BCWM.1 and primary WM cells.

### Materials and Methods

#### Cells

BCWM.1 is derived from a patient with untreated WM.<sup>10</sup> Primary CD19+ WM cells were obtained from BM samples of previously treated patients. The patients were previously treated with fludarabine/rituximab (n = 3), cyclophosphamide-based rituximab therapy (n = 3), and bortezomib-based therapy (n = 1). Peripheral blood mononuclear cells (PBMC) were obtained from healthy subjects.

#### Reagents

SAHA, sirtinol, LBH-589, TSA, and bortezomib were commer-

cially obtained. A stock solution of each agent was stored at  $-80^{\circ}\text{C}$ ; they were diluted in culture medium immediately before use.

### Cellular Assays and Immunoblotting

WM cells were cultured with medium, HDAC inhibitors, or in combination with bortezomib. Cell viability, cell cycle analysis, apoptosis assays, cell lysate preparation, and immunoblotting were performed according to standard practice.<sup>11</sup>

Gene expression profiling was performed using Human Genome U133Plus2.0 Affymetrix arrays.

### Statistical Methods

Gene expression data from 30 WM patient and 10 HD BM samples were normalized using DNA-chip analyzer.<sup>12</sup> Normalized data was subjected to Bootstrap<sup>13</sup> and Monte Carlo<sup>14,15</sup> analyses using customized Perl scripts. The mean, standard deviation, Spearman correlation and sign test were calculated using Excel and R. A *P* value of  $\leq .05$  was deemed to be significant.

## Results

### HDAC Expression in Bone Marrow WM Cells and BCWM.1 Cells With q-PCR Validation (Table 2)

HDAC expression in WM CD19+ cells was significantly different from that of HD (*P* value of .0000154). WM CD19+ cells exhibited increased expression of HDAC4, HDAC9, and Sirt5. Quantitative PCR on BM CD19+ cells of 5 WM patients and 5 HDs showed upregulation in HDAC9 by 2.65-fold in WM cells, though HDAC4 and Sirt5 were the same. In BCWM.1 cells, HDAC expression level relative to ribosome-18s showed upregulation of HDAC9, HDAC11, and Sirt4.

### Effect of HDAC Inhibitors Alone and in Combination With Bortezomib on Cell Viability of BCWM.1 Cells

BCWM.1 cells were cultured with medium, and HDAC inhibitors at escalating doses for 24 hours. The inhibitory concentration for 50% reduction ( $\text{IC}_{50}$ ) in cell viability for SAHA, TSA, LBH-589, and sirtinol was 3.5  $\mu\text{M}$ , 70 nM, 0.8  $\mu\text{M}$ , and 30  $\mu\text{M}$ . BCWM.1 cells were next cultured with medium, HDAC inhibitors, or in combination with bortezomib for 24 hours (Figure 1A). The combinational effect of the drugs was determined using CalcuSyn.<sup>16</sup>

### Effect of TSA Alone and in Combination With Bortezomib on Primary WM Cell Cytotoxicity (Figure 1B)

BM CD19+ cells, isolated from 7 previously treated WM patients, were cultured with medium, TSA (20 nM), bortezomib (30  $\mu\text{g}/\text{mL}$ ) or both for 24 hours. A mean increase in apoptosis over untreated cells of 2.9%, 18.5%, and 23.2% was observed by Annexin V and PI (*P* < .04). In contrast, no increased apoptosis was observed in HD PBMC CD19+ cells.

### TSA Induced Apoptosis and $G_1/S$ Arrest in BCWM.1 Cells Are Uncoupled Processes

BCWM.1 cells treated with TSA showed  $49.16\% \pm 0.73\%$  arrest at the  $G_1/S$  phase, while  $39.7\% \pm 0.86\%$ ,  $40.08\% \pm 0.2\%$  and

Table 1

	Q-PCR Result	Bootstrap Result
HDAC 1	1.397557085	1.29
HDAC 2	0.511394563	0.736
HDAC 3	1.392329685	0.9565
HDAC 4	0.929942833	1.412
HDAC 5	0.761250529	0.949
HDAC 6	0.878930421	0.8463
HDAC 7	1.249	1.097
HDAC 8	0.63966073	0.973
HDAC 9	2.648834638	2.08
HDAC 10	1.287843228	0.9735
HDAC 11	0.722422547	1.0585
Sirt 1	1.325212994	0.587
Sirt 2	0.87323826	0.961
Sirt 3	0.750040668	1.326
Sirt 4	0.499997801	1.3695
Sirt 5	0.924749509	1.549
Sirt 6	1.188852011	0.868
Sirt 7	0.971039969	0.826

CD19+ bone marrow cells were isolated from 5 WM patients and 5 healthy donors (HD). The fold changes in expression level of the HDAC family members for WM patients versus healthy donors are shown in the column denoted as q-PCR result. The expression level of HDACs obtained from microarray analysis of bone marrow CD19+ cells isolated from 30 WM patients and 10 healthy donors was subjected to Bootstrap and Monte Carlo analyses. The fold changes in expression level of the HDACs from WM patients versus healthy donors are shown in the column denoted as Bootstrap result.

$41.13\% \pm 2.6\%$  of cells treated with medium, bortezomib, or both were arrested at the  $G_1/S$  phase. TSA exhibited 23.8% increase in  $G_1/S$  phase arrest compared to control, bortezomib or both.

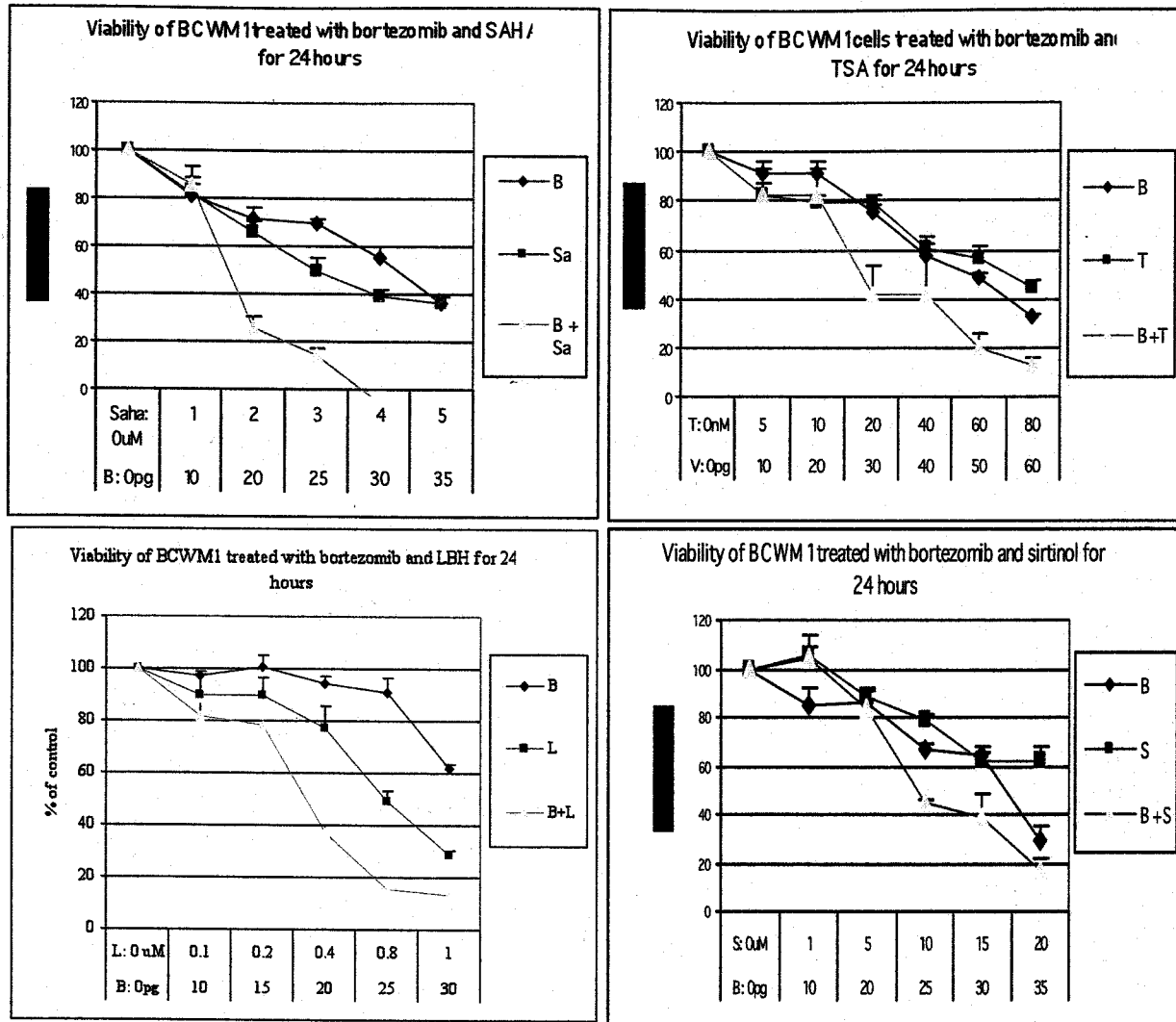
### Bortezomib and TSA-Induced Apoptosis in BCWM.1 Cells Depends on a Similar Set of Activated Caspases

To elucidate the apoptotic mechanism of HDAC inhibitors in WM, BCWM.1 cells were cultured using the above conditions prior to immunoblotting (Figure 2A). We observed increased cleavage of caspase 7, caspase 12, and PARP after treatment with TSA, bortezomib or both. The pattern of caspase activation was similar in cells treated with either TSA or bortezomib, indicating that TSA- and bortezomib-induced apoptosis depended on a similar set of caspases. Acetyl-histone 3 and acetyl-histone 4, substrates of HDAC9, were increased following treatment with TSA, bortezomib, or both, confirming a decreased activity of HDACs.

### $G_1/S$ Arrest Induced by TSA in BCWM.1 Cells Is Dependent on p15, p21, Cdk4/6, Cyclin D1 and Phospho-RB

Immunoblotting with cell cycle regulators was performed after BCWM.1 cells were cultured using the above conditions (Figure 2B). p15 was increased after treatment with TSA, bortezomib, or both compared to control. In bortezomib-treated cells, there was an increase in p16, while in TSA treated cells p16 increased at 6 hours but returned to baseline after 12 hours. After 12 hours, p21 and p27 were increased

Figure 1A



BCWM.1 cells were cultured with SAHA, TSA, LBH-589, or sirtinol alone, and in combination with bortezomib for 24 hours prior to cell viability MTT assay. One of the three repeated experiments is shown. Blue line: bortezomib; pink: HDAC inhibitors alone; green line: bortezomib plus HDAC inhibitors. 1B-b. The combination indexes of bortezomib and each of the HDAC inhibitors are tabulated.

in bortezomib-treated cells, consistent with other studies that p21 and p27 degradation is ubiquitin-proteasome pathway dependent.<sup>17,18</sup> In TSA treated cells, p21 was increased while p27 remained at baseline. Cdk4 and Cdk6 were unchanged after treatment with bortezomib, but decreased after treatment with TSA for 12 hours. Cyclin D1 and cyclin D3 were unaffected by bortezomib. TSA treatment resulted in reduction of cyclin D1, while cyclin D3 fluctuated over time. Phospho-RB was decreased and phospho-p53 remained unchanged following treatment with bortezomib, TSA, or TSA plus bortezomib.

### Discussion

This is the first study to report HDAC expression, and to elaborate on the role of HDAC inhibitors in WM. HDAC4, HDAC9 and Sirt5 were overexpressed in BM lymphoplasmacytic cells from WM patients by GEP. However, q-PCR only revealed an increase in HDAC9 in primary WM cells. Similarly, HDAC9 was overex-

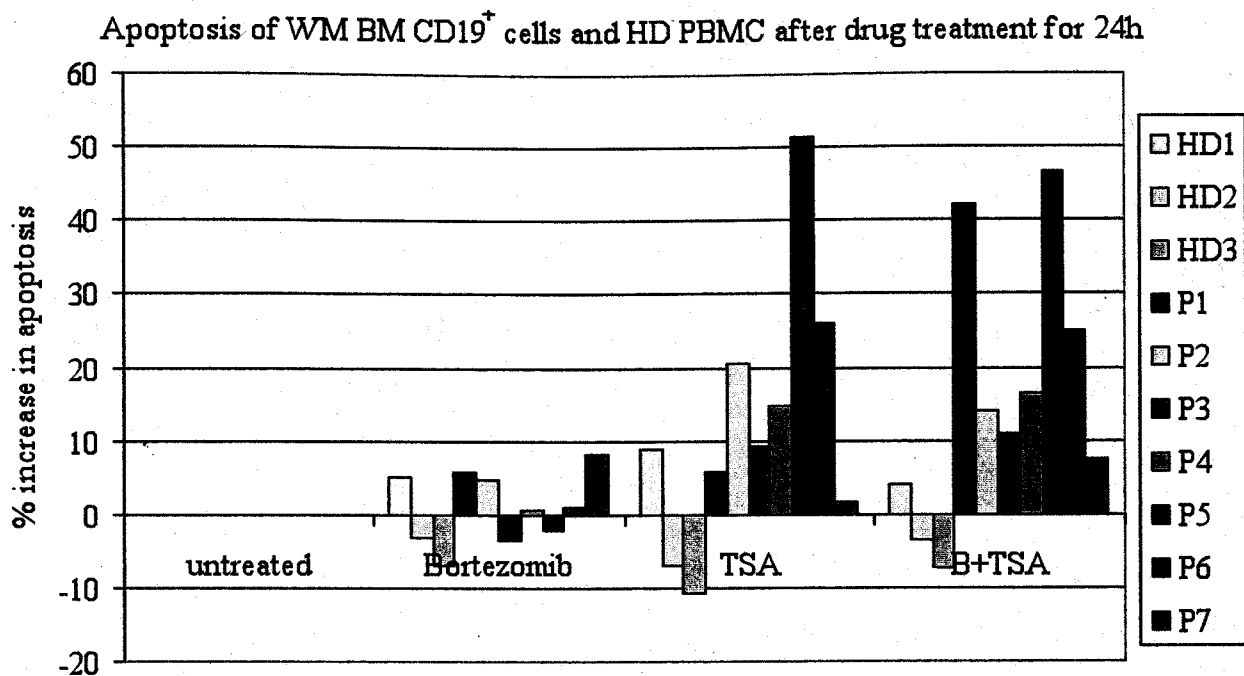
pressed in BCWM.1 cells. Among the HDAC inhibitors tested, TSA, a HDAC9 inclusive class II inhibitor exhibited the most potent apoptotic activity, occurring at nanomolar concentration, while the IC<sub>50</sub> of other HDAC inhibitors tested were in the micromolar range. TSA induced significant apoptosis in primary WM cells, but not in normal donor B cells.

SAHA, TSA, LBH-589, and sirtinol had at least additive anti-tumor effects when combined with bortezomib in BCWM.1 cells. TSA plus bortezomib accentuated primary

WM tumor cell killing compared to TSA alone (23.2% vs. 18.4%). While others have demonstrated increased anti-tumor activity with combinations of HDAC inhibitors and bortezomib by pre-clinical studies using multiple myeloma and mantle cell lymphoma, these studies have predominately relied on cell line data.<sup>7,19,20</sup>

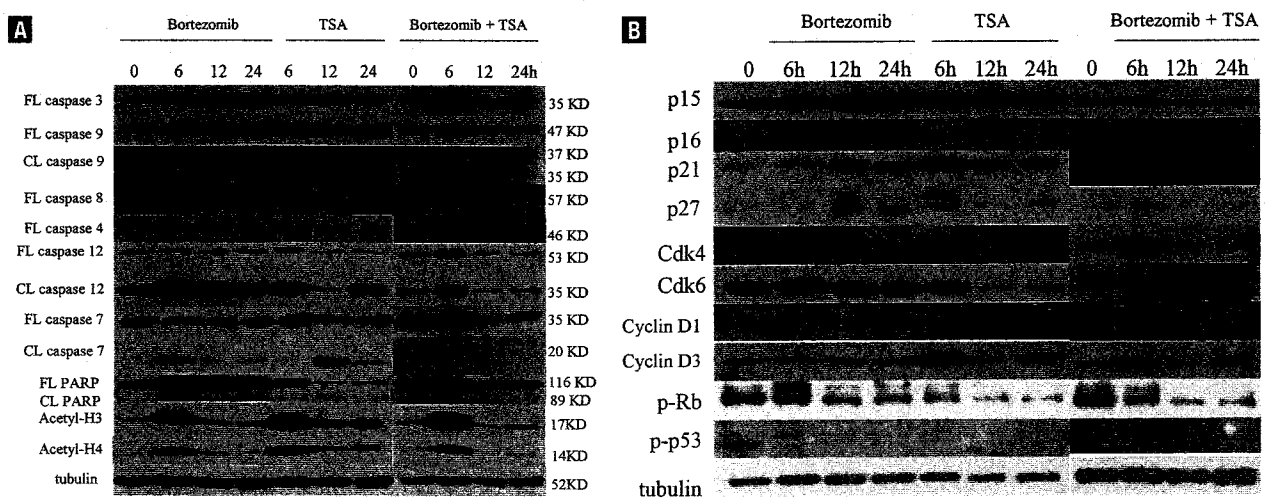
The execution of apoptosis in mammalian cells requires coordination between activation of initiator caspases and amplification

Figure 1B



CD19<sup>+</sup> cells from 3 healthy donor PBMCs and 7 WM bone marrow samples were isolated. CD19<sup>+</sup> cells were cultured with medium, bortezomib (20-100 pg), TSA (0.05-0.5  $\mu$ M), bortezomib plus TSA for 24 hours prior to Annexin V and PI staining. The percent change in apoptosis after the drug treatment compared to control medium is shown (HD, healthy donor; P, patient).

Figure 2



**A.** BCWM.1 WM cells were cultured with medium alone, bortezomib, TSA or both for 0, 6, 12, and 24 hours prior to immunoblotting with caspase and acetyl-H3 and acetyl-H4 antibodies.

**B.** BCWM.1 cells were cultured with medium, bortezomib, TSA or both for 0, 6, 12, and 24 hours prior to immunoblotting. Proteins involved in cell cycle control and check point regulation are shown.

of effector caspases.<sup>21</sup> TSA- and bortezomib-induced apoptosis in BCWM.1 cells are dependent on the activation of caspase 7, 9, and 12. The similar caspase activation patterns reflected a redundant effect of TSA- and bortezomib-induced caspase-dependent apoptosis.

While the pattern of caspase activation was similar in TSA and bortezomib-treated BCWM.1 cells, the effects on cell cycle regulation appeared quite different. These findings are consistent with previous reports that TSA-induced cell cycle arrest and apoptosis are partially

independent processes.<sup>22,23</sup> TSA treatment resulted in reduced cyclin D1, Cdk4 and Cdk6, and increased p15 and p21 protein. The increase of p15 and p21 further suppressed the kinase activity of Cdk4 and Cdk6, leading to a decrease in phospho-RB and subsequent sequestration of E2F, and eventual cell cycle arrest. The reduction in p16 and p27 is likely compensated by an increase of p15 and p21. The collective effect of G<sub>1</sub>/S arrest resulted from the alteration in cyclin D1, Cdk4, Cdk6, p15, p16, p21 and p27 are p53-independent.

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These results provided a framework for the examination of HDAC-inhibitors as monotherapy, as well as combination therapy with bortezomib in WM.

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## Authorship

JYS and SPT designed the experiments and prepared the manuscript. JYS and HYT performed the cell viability and apoptosis assays, cell cycle analyses, immuno-blotting and Q-PCR. EH performed the GEP study. KM, JYS, and ZRH conceptualized the methods for microarray analysis. ZRH and JYS performed the GEP analysis. LX, BZ, BC, MF, YZ, GY, SJR, XL, LI, PG, PS, RJM, CJP, NCM, and OO provided helpful discussions. We thank Philip Brodsky for assistance with graphics.

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