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Original Article

Synergistic anti-leukemic interactions between ABT-199 and panobinostat in acute myeloid leukemia *ex vivo*

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Abstract: Cure rates for acute myeloid leukemia (AML) remain suboptimal; thus new treatment strategies are needed for this deadly disease. Poor clinical outcomes have been associated with overexpression of the anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-xL, and Mcl-1, which have garnered great interest as therapeutic targets. While the Bcl-2-selective inhibitor ABT-199 has demonstrated promising preclinical anti-leukemic activities, intrinsic drug resistance remains a problem. In our most recent study, we identified Mcl-1 sequestration of Bim as a mechanism of intrinsic resistance to ABT-199 in AML cells, thus upregulating Bim could overcome such resistance. Histone deacetylase (HDAC) inhibitors (HDACI) are a class of agents that have been confirmed to upregulate Bim. This prompted our hypothesis that combining an HDACI with ABT-199 would overcome intrinsic resistance to ABT-199 and result in synergistic anti-leukemic activity against AML. In this study, we investigated the anti-leukemic activity of panobinostat, a pan-HDACI, in combination with ABT-199 in AML cell lines and primary patient samples. We found that the combined drug treatment resulted in synergistic induction of cell death in both AML cell lines and primary patient samples. Panobinostat treatment resulted in upregulation of Bim, which remained elevated in the presence of ABT-199. In addition, shRNA knockdown of Bim in AML cell lines significantly attenuated apoptosis induced by combined panobinostat and ABT-199. Our results provide compelling evidence that Bim plays a key role in the combined anti-leukemic activity of panobinostat and ABT-199 against AML, and support clinical evaluation of combined panobinostat and ABT-199 in the treatment of AML.

Keywords: ABT-199, Bcl-2, Bim, panobinostat, acute myeloid leukemia

Introduction

The five year survival rate for adults with acute myeloid leukemia (AML) is a dismal 25% [1]. Although the survival rate for the pediatric population is 65%, this significantly lags behind the overall survival rate for pediatric acute lymphoblastic leukemia (approximately 90%) [1]. Resistance to chemotherapy in leukemic cell line models and poor clinical outcomes of adult patients with AML have been associated with overexpression of the anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-xL, and Mcl-1, which have garnered great interest as potential therapeutic targets [2]. Inhibitors targeting multiple anti-apoptotic Bcl-2 family proteins have shown great potential in preclinical models but have limited clinical applicability due to increased incidence of thrombocytopenia; which was associated with inhibition of Bcl-xL [2]. Thus, attention has shifted to the Bcl-2-selective inhibitor ABT-199 (Venetoclax), which has shown excellent anti-leukemic activity against chronic lymphoblastic leukemia [3] and was approved by the US FDA in April 2016. Although ABT-199 has been shown to have promising preclinical effects on AML, intrinsic drug resistance remains a concern [4-6].

Mcl-1 has been identified as playing a key role in resistance to ABT-199 in AML and lymphoid malignancies [5, 7, 8]. In our most recent study,

Table 1. Patient characteristics for the primary acute myeloid leukemia patient samples

Patient	Gender	Age (year)	Disease status	FAB subtype	Cytogenetics	Blast purity (%)	Fusion gene/ Gene mutation
AML#18	Female	19	Relapsed	M2	46, XX, t(8;21)(q22;q22)	54	AML1-ETO
AML#19	Male	4	Newly diagnosed	M4	46, XY	40.5	
AML#21	Male	42	Newly diagnosed	M4	46, XY/44, XY, -17, -19, (11q-?)	51	MLL-ELL
AML#23	Male	60	Newly diagnosed	M5	48, XY, +2,+8, i(12)(q10)	81	MLL-AF10
AML#25	Female	12	Newly diagnosed	M3	46, XX, t(15;17)(q22;q21)	88	
AML#29	Male	9	Newly diagnosed	M4	46, XY, t(6;9)(p22;q34)	31	DEK-CAN
AML#30	NA	NA	NA	NA	NA	NA	NA
AML#31	Male	17	Newly diagnosed	M2	46, XY	68.5	CEBPA
AML#33	Female	76	Newly diagnosed	M5	46, XX	84.5	dupMLL, CEBPA
AML#34	Male	52	Newly diagnosed	M4	46, XY	96	DEK-CAN
AML#35	Male	65	Newly diagnosed	M5	47, XY, add(7q), -16, -17, +marx3	76	
AML#38	Male	27	Newly diagnosed	M3	46, XY, t(15;17)(q22;q21)	90.5	PML-RARα

Abbreviation: NA, not available.

ABT-199 treatment was found to result in dissociation of pro-apoptotic Bim from Bcl-2, demonstrating its canonical role in inducing apoptosis in AML cells [5]. However, ABT-199 treatment also resulted in increased sequestration of Bim by Mcl-1, preventing Bim from inducing apoptosis, while also resulting in increased Mcl-1 protein levels in ABT-199-resistant AML cells. These results suggest that Mcl-1 is a key player in the intrinsic resistance to ABT-199 in AML cells. CRISPR knockdown of Mcl-1 significantly enhanced ABT-199-induced apoptosis in AML cells, confirming its role in this aspect [5]. While there have been a number of reports focused on downregulating Mcl-1 [6, 8, 9], another approach to overcome this mechanism of intrinsic resistance to ABT-199 would be to increase Bim levels, leading to increased Bim/Mcl-1 ratio favoring apoptosis. Histone deacetylase (HDAC) inhibitors (HDACI) are a class of agents that have been demonstrated to upregulate Bim [10-12]. In particular, panobinostat, a highly potent pan-HDACI [13], has recently been approved by the US FDA for the treatment of multiple myeloma [14] and has been demonstrated to upregulate Bim in cancer cells [10, 12]. Therefore, panobinostat may overcome intrinsic ABT-199 resistance by increasing Bim protein levels. In this study, we investigated the combination of panobinostat and ABT-199 in AML cell lines and primary patient samples. We found synergistic induction of cell death by the combined drug treatment. In addition, we provided evidence that Bim played a key role in the combined anti-leukemic activity against AML. These findings support clinical evaluation of combined panobinostat and ABT-199 in the treatment of AML.

Materials and methods

Drugs

ABT-199 and panobinostat were purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture

THP-1 and U937 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in RPMI 1640 media with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 2 mM L-glutamine, plus 100 U/ml penicillin and 100 μg/ml streptomycin, in a 37°C humidified atmosphere containing 5% CO_2/95% air. The cell lines were tested for the presence of mycoplasma on a monthly basis.

Diagnostic blast samples were purified by standard Ficoll-Hypaque density centrifugation, then cultured in RPMI 1640 with 20% fetal bovine serum (Life Technologies) supplemented with ITS solution (Sigma-Aldrich, St. Louis, MO, USA) and 20% supernatant of the 5637 bladder cancer cell line (as a source of granulocyte-macrophage colony-stimulating factor) [5-7, 15].

Clinical samples

Diagnostic AML blast samples were obtained from the First Hospital of Jilin University, Changchun, China. Written informed consent

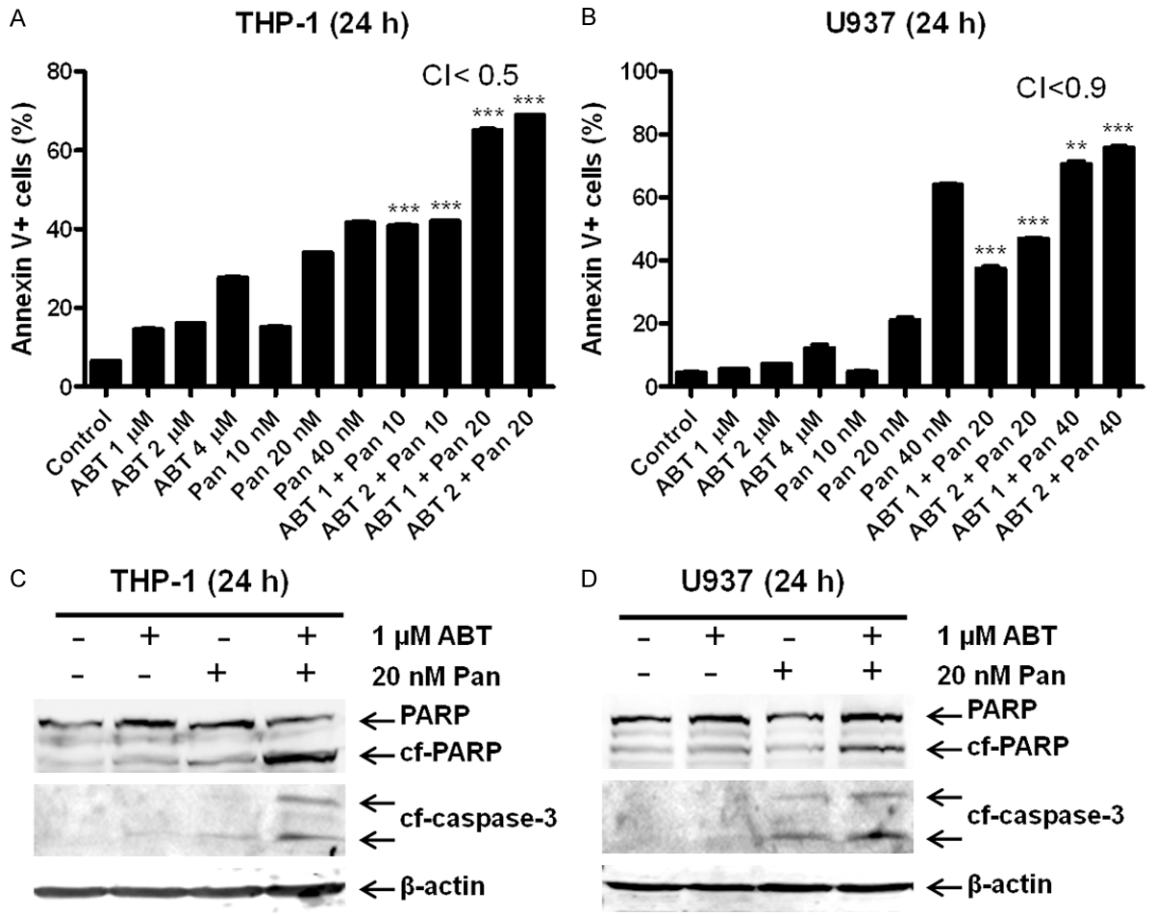

Figure 1. Synergistic induction of apoptosis by ABT-199 and panobinostat (Pan) in AML cell lines determined by annexin V/PI staining and flow cytometry and CompuSyn software analyses. (A and B) THP-1 (A) and U937 (B) cells were treated with ABT-199 and panobinostat, alone or in combination, for 24 h and then subjected to annexin V/PI staining and flow cytometry analysis. CI values were calculated using CompuSyn software. **indicates P<0.01 and ***indicates P<0.001. (C and D) Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies.

was provided according to the Declaration of Helsinki. Clinical samples were screened for gene mutations and fusion genes as previously described [5-7, 16, 17]. Patient characteristics are presented in **Table 1**.

In vitro cytotoxicity assays

In vitro cytotoxicities of the diagnostic AML blast samples were measured by using MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazoliumbromide, Sigma-Aldrich) assays, as previously described [18, 19]. Briefly, the cells were either treated with variable concentrations of ABT-199 or panobinostat alone, or in combination, for 72 hours. The cells were lysed using 10% SDS in 10 mM HCL. IC_{50} values were calculated as drug concentrations necessary to inhibit 50% proliferation compared to vehicle control treated cells. The IC_{50} values are means of duplicates from one experiment due to limited sample. Standard isobologram analysis was performed to determine the extent and direction of anti-leukemic interactions [20]. The IC_{50} values of each drug are plotted on the axes; the solid line represents the additive effect, whereas the points represent the concentrations of each drug resulting in 50% inhibition of proliferation. Points falling below the line indicate synergistic effect, whereas those above the line indicate antagonistic effect. Patient sample selection was merely based on sample availability.

Western blot analysis

Cells were lysed in Tris buffer (10 mM, pH 8.0) containing protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Whole cell lysates were subjected to SDS-polyacrylamide gel elec-

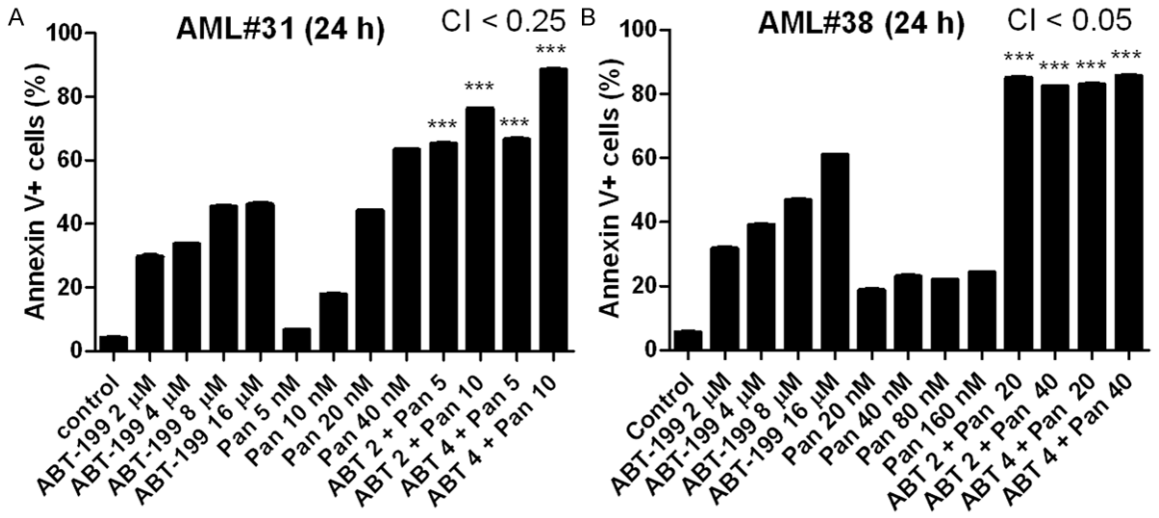

Figure 2. Synergistic induction of apoptosis by ABT-199 and panobinostat in primary AML patient samples determined by annexin V/PI staining and flow cytometry and CompuSyn software analyses. Primary AML patient samples, AML#31 (A) and AML#38 (B) were treated as indicated for 24 h and then subjected to annexin V/PI staining and flow cytometry analyses. CI values were calculated using CompuSyn software. ***indicates P<0.001.

trophoresis, electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Inc., Rockford, IL, USA) and immunoblotted with anti-Bim, -Bcl-2, -Mcl-1, -PARP, -Cleaved Caspase 3 (Cell Signaling Technology, Danvers, MA, USA), or -β-actin (A2228, Sigma-Aldrich) antibody, as previously described [18, 21, 22]. Immunoreactive proteins were visualized using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA), as described by the manufacturer. Western blots were repeated at least 4 times and one representative blot is shown. Densitometry measurements were made using Odyssey V3.0 (Li-Cor), normalized to β-actin first and then to the no drug treatment control (set as 1).

Apoptosis

AML cells were treated with variable concentrations of ABT-199 or panobinostat, alone or in combination, for 24 h, and then subjected to flow cytometry analysis to determine drug-induced apoptosis using the Annexin V-fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis Kit (Beckman Coulter; Brea, CA, USA), as previously described [18, 23]. Results were expressed as percent of Annexin V+ cells. AML cell line experiments were performed 3 independent times in triplicate and data presented are from one representative experiment, while the patient sample experiments were performed once in triplicate due to limited sample availability. The combination index (CI) values were determined using CompuSyn software. CI<0.9 indicates synergistic, 0.9<CI<1.1 indicates additive, and CI>1.1 indicates antagonistic anti-leukemic interactions [24].

Production of lentivirus particles and transduction of AML cells

The pMD-VSV-G and delta 8.2 plasmids were gifts from Dr. Dong at Tulane University. Bim and non-target control (NTC) shRNA lentiviral vectors were purchased from Sigma-Aldrich. Lentivirus production and transduction were carried out as previously described [25, 26]. Briefly, TLA-HEK293T cells were transfected with pMD-VSV-G, delta 8.2, and lentiviral shRNA constructs using Lipofectamine and Plus reagents (Life Technologies) according to the manufacturer's instructions. Virus containing culture medium was harvested 48 h post-transfection. Cells were transduced overnight using 1 mL of virus supernatant and 4 μg of polybrene and then cultured for an additional 48 h prior to selection with puromycin.

Statistical analysis

Differences in cell apoptosis between treated (individually or combined) and untreated cells were compared using the pair-wise two-sample

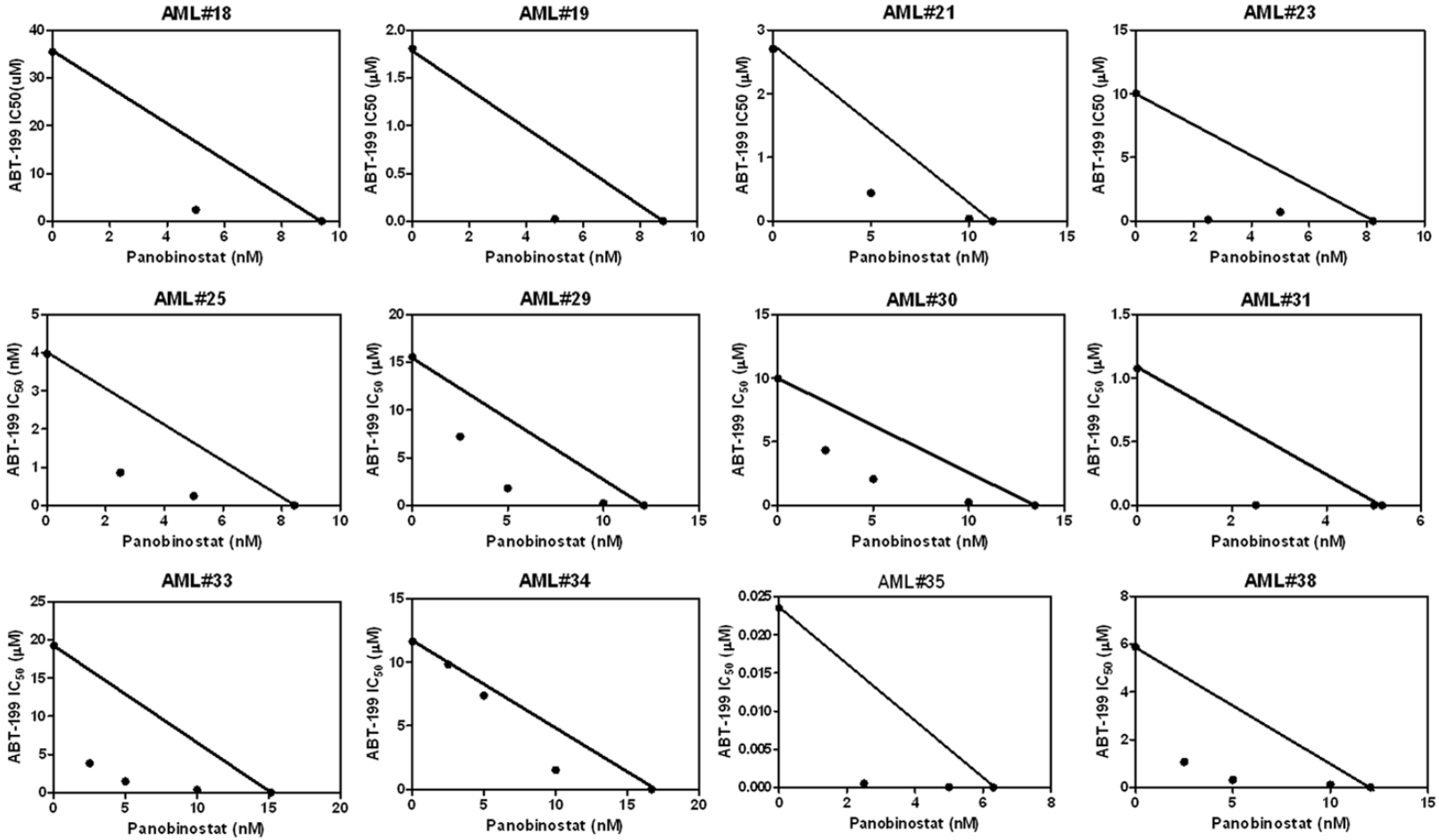

Figure 3. Panobinostat synergizes with ABT-199 in primary patient samples determined by MTT assays and standard isobologram analyses. Primary AML patient samples were treated with ABT-199 and panobinostat, alone or in combination, for 72 h and then viable cells were determined using MTT reagent. The IC_{50} values are means of duplicates from one experiment due to limited sample. Standard isobologram analysis was performed to determine the extent and direction of the anti-leukemic interaction between the two agents. The IC_{50} values of each drug are plotted on the axes; the solid line represents the additive effect, while the points represent the concentrations of each drug resulting in 50% inhibition of proliferation. Points falling below the line indicate synergistic effect, whereas those above the line indicate antagonistic effect.

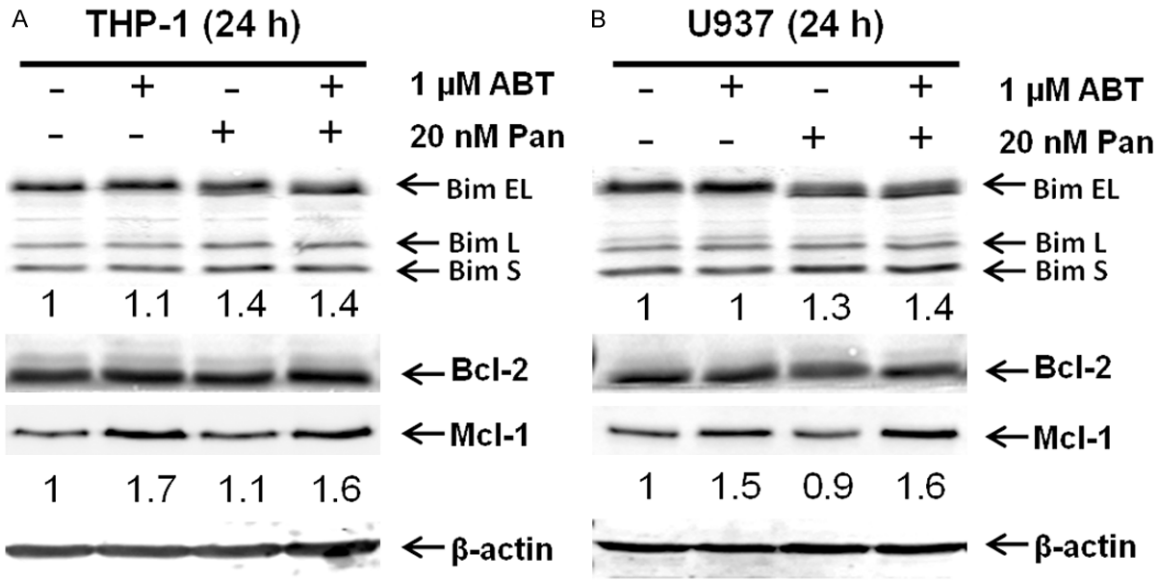

Figure 4. The effect of panobinostat on Bim expression in AML cell lines. THP-1 (A) and U937 (B) cells were treated with ABT-199 and panobinostat, alone or in combination, for 24 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies.

t-test. Statistical analyses were performed with GraphPad Prism 5.0. Error bars represent ± s.e.m.

Results

ABT-199 and panobinostat synergize in inducing apoptosis in AML cell lines and primary patient samples

To determine if panobinostat enhances ABT-199-induced apoptosis in AML cells, we treated two ABT-199-resistant AML cell lines, THP-1 and U937, (ABT-199 IC_{50}s of 2.4 mM for THP-1 and 13.5 mM for U937 cells, as determined previously by MTT assays [7]), with both drugs individually or simultaneously for 24 h. Annexin V/PI staining and flow cytometry analyses revealed significantly increased annexin V+ cells for the combined drug treatments compared to that for the individual drug treatments (**Figure 1A**, **1B**). This was accompanied by increased cleavage of caspase-3 and PARP in the combined treatments as compared to the individual drug treatments (**Figure 1C**, **1D**). These results demonstrate that the combined drug treatments caused significantly increased apoptosis in the two AML cell lines as compared to the individual drug treatments. Calculation of CI values by using the CompuSyn software showed that the anti-leukemic interactions between the two drugs were synergistic, as indicated by CI<0.9 (**Figure 1A**, **1B**).

To confirm that ABT-199 and panobinostat also synergize in primary AML patient samples, we tested the drugs in two freshly isolated AML blast samples (AML#31 and AML#38; samples were chosen based on availability of adequate number of cells for the assay) *ex vivo*. Interestingly, simultaneous combination of the two drugs caused strong synergistic induction of apoptosis in the two primary AML patient samples, as indicated by CI<0.25 (**Figure 2**).

ABT-199 and panobinostat synergize in inhibiting cell proliferation in primary AML patient samples

To confirm the synergistic anti-leukemic interactions between ABT-199 and panobinostat in AML cells detected by annexin V/PI staining and flow cytometry analyses, MTT assays and standard isobologram analyses were performed in the AML#31 and AML#38 primary patient samples. As shown in **Figure 3**, simultaneous combination of the two drugs resulted in synergistic proliferation inhibition in the two primary patient samples.

To further enhance the clinical relevance of our study, we included 10 additional primary AML

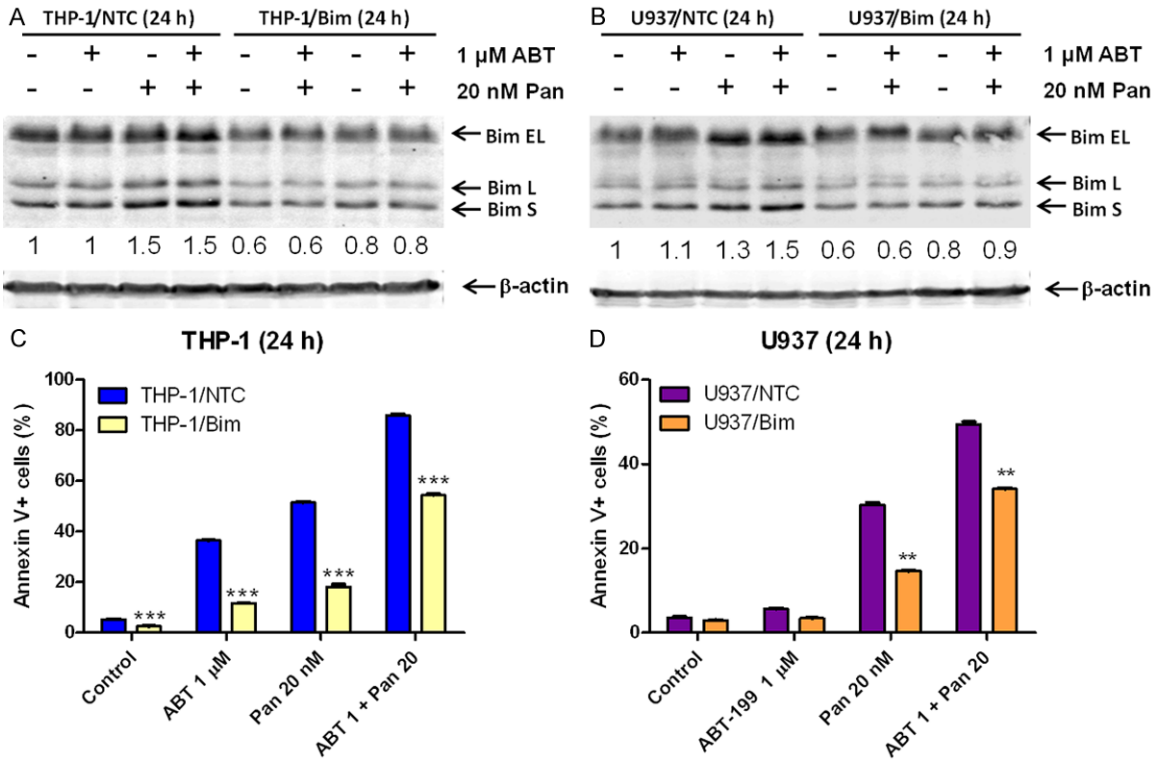

Figure 5. The role of Bim in apoptosis induced by ABT-199 and panobinostat, alone or in combination, in AML cell lines. (A and B) THP-1 (A) and U937 (B) cells were infected with non-target control (NTC) or Bim shRNA lentivirus particles overnight, then washed and incubated for 48 h prior to adding puromycin to the culture medium. The cells were then treated with ABT-199 and panobinostat, alone or in combination, for 24 h. Whole cell lysates were subjected to Western blotting. The fold changes for the Bim densitometry measurements, normalized to β-actin and then compared to no drug treatment control, are indicated. (C and D) THP-1/NTC, THP-1/Bim (C), U937/NTC and U937/Bim (D) cells were treated with ABT-199 and panobinostat, alone or in combination, for 24 h and then subjected to annexin V/PI staining and flow cytometry analyses. **indicates P<0.01 and ***indicates P<0.001.

patient samples in our drug interaction experiments. Due to limited sample availability, these additional 10 primary AML patient samples were tested by MTT assays and standard isobologram analyses, but not by annexin V/PI staining and flow cytometry analyses, which require large number of cells. Essentially the same results were obtained in the 10 additional samples as those in the AML#31 and AML#38 samples (**Figure 3**). These results further confirm the synergistic anti-leukemic interactions between ABT-199 and panobinostat in AML cells and the clinical relevance of our study.

Panobinostat upregulates Bim expression in AML cell lines

To determine the effects of panobinostat on Bim expression in the absence or presence of ABT-199 in AML cells, the THP-1 and U937 AML cell lines were treated with panobinostat and ABT-199, alone or in combination, for 24 h and then whole cell lysates were subjected to western blotting. Bim protein levels were increased after treatment with panobinostat alone or in combination with ABT-199 (~30-40%, **Figure 4**), which is consistent with other studies reporting Bim upregulation following HDACI treatment [11, 27]. In contrast, Bcl-2 levels remained unchanged. As we found most recently [5], ABT-199 treatment resulted in increased Mcl-1 protein levels in relatively resistant AML cells, which remained elevated in the presence of panobinostat (**Figure 4**). These results indicate that even though panobinostat treatment does not block Mcl-1 upregulation induced by ABT-199, it upregulates Bim expression, ultimately resulting in increased ratio of Bim/Mcl-1 favoring apoptosis.

Bim plays a critical role in apoptosis induced by ABT-199 and panobinostat, alone or in combination

To confirm the functional role Bim plays in apoptosis induced by ABT-199 and panobinostat, alone or in combination, we used shRNA to knock down Bim in both THP-1 and U937 cells (**Figure 5A**, **5B**). Cells infected with non-target control shRNA (NTC) showed a similar induction of Bim by panobinostat, and the combination of ABT-199 and panobinostat resulted in similar synergistic induction of apoptosis. As expected, Bim knockdown (40% compared to the NTC) significantly attenuated apoptosis induced by combined panobinostat and ABT-199 (**Figure 5C**, **5D**). These results provide compelling evidence that panobinostat enhances ABT-199-induced apoptosis in AML cells through upregulation of Bim.

Discussion

In this study, we investigated the ability of panobinostat to overcome ABT-199 resistance in AML cells. We found that panobinostat synergistically enhanced ABT-199-induced apoptosis in two AML cell lines and two primary patient samples, which were relatively resistant to ABT-199. These results were further confirmed in 10 additional primary AML patient samples, as determined by MTT assays and standard isobologram analyses. Our results were similar to those of Chen et al. who used ABT-737, a Bcl-2 and Bcl-xL inhibitor, in combination with the HDACI suberoyl bis-hydroxamic acid (SBHA) and found that upregulation of Bim by SBHA contributed to the synergistic induction of cell death [11]. Another study, in mantle cell lymphoma, also found synergistic anti-cancer activity for ABT-263, which inhibits both Bcl-2 and Bcl-xL, in combination with suberoylanilide hydroxamic acid (SAHA) [27].

Consistent with other studies reporting Bim upregulation following HDACI treatment [11, 27] we confirmed that panobinostat treatment caused upregulation of Bim (30-40%) in AML cell lines. Our shRNA knockdown experiments convincingly revealed that Bim was a critical mediator of panobinostat enhancement on ABT-199-induced apoptosis. Although we did not investigate how panobinostat induces Bim expression, SAHA has been demonstrated to induce acetylation of the Bim, Bmf and NOXA promoters [27], and E2F1 has been shown to the increase of Bim expression following HDACI treatment [28], suggesting that increased transcription may be responsible for the upregulation of Bim by panobinostat.

However, it is important to note that although expression of the Bim shRNA in AML cell lines completely abolished Bim induction by panobinostat, it did not completely abolish drug-induced apoptosis suggesting that other factors contributed. Panobinostat has been demonstrated to affect numerous proteins which could also contribute to the enhanced cell death induced by ABT-199 [29].

In conclusion, we provide compelling evidence to support the development of panobinostat in combination with ABT-199 in the treatment of AML. Our cell line data demonstrates that ABT-199 synergizes with panobinostat in ABT-199 resistant cells. Furthermore, our MTT data using primary patient samples demonstrates that the combination also synergized in ABT-199 sensitive samples, suggesting that screening for ABT-199 resistance may not be necessary.

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Disclosure of conflict of interest

None.

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