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Efficacy of panobinostat and marizomib in acute myeloid leukemia and bortezomib-resistant models

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ABSTRACT

Current relapse rates in acute myeloid leukemia (AML) highlight the need for new therapeutic strategies. Panobinostat, a novel pan-histone deacetylase inhibitor, and marizomib, a second-generation proteasome inhibitor, are emerging as valuable therapeutic options for hematological malignancies. Here we evaluated apoptotic effects of this combinatorial therapy in AML models and report earlier and higher reactive oxygen species induction and caspase-3 activation and greater caspase-8 dependence than with other combinations. In a bortezomib refractory setting, panobinostat induced high levels of DNA fragmentation, and its action was significantly augmented when combined with marizomib. These data support further study of this combination in hematological malignancies.

1. Introduction

Relapse in acute myeloid leukemia (AML) patients treated with standard regimens is a significant clinical problem [1]. In contrast to genetic aberrations that lead to irreversible structural DNA changes, epigenetic alterations result in loss or gain of gene function without modification of the DNA coding sequence in a manner that can be reversed pharmacologically to restore normal bone marrow function and achieve clinical disease response [2].

Histone acetylation is controlled by histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities [3]. Inhibition of HDAC activity modifies deregulated gene transcription in cancer cells, inducing growth arrest, differentiation, and apoptosis in a relatively selective manner in cancer versus normal cells [4,5]. Several structurally diverse classes of HDAC inhibitors (HDACi) have been developed. Among these, the pan-HDACi panobinostat, a novel derivative of cinnamic acid hydroxamate [6–8], is a highly potent inhibitor of all class I, II, and IV HDAC enzymes implicated in cancer development and progression [7,9]. In enzymatic assays, panobinostat inhibited HDACs 1, 2, 4, 8, 10, and 11 at IC_{50} values that were significantly lower than vorinostat, an FDA-approved pan-HDACi [6].

HDACi rarely exhibit efficacy as single agents, so combination strategies are being interrogated. A recent phase II trial reported encouraging results for the efficacy of panobinostat combined with the first-generation proteasome inhibitor bortezomib and dexamethasone in heavily pretreated bortezomib-refractory multiple myeloma (MM) patients [10]. The combination of panobinostat with bortezomib has yielded promising results in AML cell models [11]. Interestingly, prior work from our laboratory has shown that marizomib, a second-generation proteasome inhibitor, exhibits stronger synergy than bortezomib when combined with HDACi in leukemia cells [12,13].

Marizomib possesses several features that distinguish it from bortezomib. Unlike bortezomib, which was developed to primarily inhibit the chymotrypsin-like activity of the proteasome (the activity associated with the β5 catalytic subunit), marizomib can inhibit all three catalytic activities of the proteasome: the chymotrypsin-like, caspase-like, and trypsin-like activities. Marizomib has also been shown to rely more heavily on a caspase-8 dependent pathway to initiate cell death; caspase-8 has been shown to be critical

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for the synergy observed when marizomib is combined with HDACi in acute lymphoblastic leukemia (ALL) models [12]. Data recently published by Niewerth et al. [24] in acute leukemia cell lines showed that marizomib was effective in bortezomib-resistant human T-ALL cells, supporting the concept that these proteasome inhibitors trigger cell death in distinct ways [14].

In the present study, we evaluated panobinostat alone and in combination with marizomib to elucidate effects on cell proliferation, apoptosis, and drug-resistance in AML and bortezomib-resistant cell models. Furthermore, we investigated the molecular mechanisms underlying this combinatorial therapy.

2. Materials and methods

2.1. Cell lines and culture conditions

The human leukemia AML3 (human AML FAB-M4) and ML-1 (derived from an M5 AML relapse in a patient initially diagnosed with T-cell ALL) cell lines were purchased from ATCC (Manassas, VA). Cells were maintained in RPMI media supplemented with 0.01 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Cellgro, Mediatech, Herndon, VA). RPMI-8226 MM parental cells and bortezomib-resistant RPMI-8226vr10 cells were maintained in RPMI media with 2 mM L-glutamine containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Cellgro, Mediatech, Herndon, VA). Cells were maintained at 37 °C with 5% CO_2. Fresh peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque PLUS gradient (Amersham Biosciences, Uppsala, Sweden), as previously described [15].

2.2. Reagents

Panobinostat, vorinostat, and bortezomib were purchased from LC Laboratories (Woburn, MA). Marizomib was provided by Nereus Pharmaceuticals (San Diego, CA). The chymotrypsin-like activity fluorogenic substrate, suc-LLVY-amc, was obtained from AG Scientific (San Diego, CA). N-acetyl cysteine (NAC), was purchased from Sigma–Aldrich (St. Louis, MO). Hydroethidium (HEt) dye was obtained from Molecular Probes (Eugene, OR). The caspase-3 substrate, DEVD-amc, was obtained from Biomol International, LP (Plymouth Meeting, PA). Antibodies were purchased from the following sources: proteasome subunit β5 (Enzo Life Sciences, Inc., Farmingdale, New York), caspase-8 and caspase-3 (Cell Signaling Technology, Inc., Beverly, Massachusetts). The caspase inhibitors IETD-fmk and LEHD-fmk were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany).

2.3. Viability and DNA fragmentation analysis

Cells were plated at a density of 0.5×10^6 cells/mL in 12-well plates and treated with indicated doses of panobinostat, vorinostat, marizomib, and bortezomib for indicated times. Cell number and viability were analyzed by trypan blue exclusion measured on a Vi-CELL Cell Viability Analyzer (Beckman Coulter, Brea, CA). DNA fragmentation was assessed by staining cells with propidium iodide (PI) and analyzing samples on the FL-3 channel of a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ). The percentage of cells with fragmented DNA was determined by gating the subdiploid population using CellQuest Software (BD Biosciences, San Jose, CA).

2.4. Cell lysates and Western blotting

Cells were lysed for 1 h in Triton X-100 lysis buffer (PBS containing 1% Triton X-100, 25 mM Tris, pH 7.5, and 150 mM sodium chloride) with a protease inhibitor cocktail tablet (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail 2 (Sigma–Aldrich, St. Louis, MO), followed by centrifugation. Lysates were collected and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene fluoride membranes and blocked for 1 h at room temperature with 5% milk in Tris-buffered saline with 0.05% Tween-20 (TBST). Membranes were incubated with primary antibodies (1:1000 dilutions) in 5% milk in TBST overnight at 4 °C. Membranes were incubated with corresponding secondary antibodies for 1 h at room temperature. Bands were visualized by chemiluminescent detection (GE Healthcare, Waukesha, WI). Densitometry was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

2.5. 20S proteasome activity assay

The fluorogenic peptide suc-LLVY-amc was used to measure chymotrypsin-like proteasome activity in leukemia cells, as previously described [16]. Cells were lysed by freezing and thawing in 20 mM Tris, pH 7.5, 0.1 mM ethylenediaminetetraacetic acid, pH 8.0, 20% glycerol, 0.05% Nonidet-P40, 1 mM 2-β mercaptoethanol, and 1 mM ATP. Lysates were centrifuged and supernatants were combined with fluorogenic peptides in 50 mM HEPES, pH 7.5, and 5 mM ethyleneglycoltetraacetic acid, pH 7.0. Samples were analyzed with a spectrofluorometer (SpectraMax Gemini EM; Molecular Devices, Sunnyvale, CA) at an excitation of 380 nm and an emission of 460 nm. The amount of fluorescence (amc) released correlates with the amount of proteasome activity of the specific proteolytic target. Fluorescence is expressed as relative fluorescent units (RFU).

2.6. Intracellular superoxide levels

HEt, a cell-permeable dye, was used to measure intracellular superoxide levels. Cells were harvested by centrifugation, the cell pellet was resuspended with 10 μM HEt in PBS and incubated for 30 min at 37 °C in the dark. Samples were harvested, resuspended with 500 μL PBS, and analyzed by measuring the fluorescence intensity on the FL-3 channel of a flow cytometer.

2.7. Caspase-3 activity assay

Caspase-3 activity was determined as previously described [12] using the fluorogenic substrate DEVD-amc. Cells were harvested and lysed in PBS by freezing and thawing on dry ice. Samples were aliquoted in triplicates in a 96-well plate with 150 μL DEVD buffer with 50 μM DEVD-amc. Release of fluorescence (amc) was measured with a spectrofluorimeter at an excitation of 355 nm and an emission of 460 nm. Fluorescence generated by the cleavage of fluorogenic peptide is proportional to caspase-3 activity.

2.8. Statistical analyses

Data presented are the mean (standard deviation [SD]) from three independent experiments performed in triplicate using GraphPad Prism version 6.0 for Windows (GraphPad Sofware, San Diego, CA). Student's *t*-tests were performed to determine statistically significant differences between samples. *p*-Values < 0.05 were considered statistically significant. Isobologram analyses using the Chou and Talalay [17] method with Calcusyn (Biosoft, Ferguson, MO) were used to determined synergy. A combination index (CI) value less than 1.0 indicates synergistic effects, a CI value equal to 1.0 indicates additive interactions, and a CI-value greater than 1.0 indicates antagonistic interactions.

3. Results

3.1. Panobinostat induces more DNA fragmentation and caspase-3 activation than vorinostat in AML and bortezomib-resistant cells

We examined the cytotoxic effects of panobinostat and vorinostat in AML cell lines and bortezomib-resistant MM cells. Both HDACi induced DNA fragmentation in a dose-dependent manner within 24 h. Panobinostat induced more DNA fragmentation than vorinostat, even at doses as low as 0.5 μM in AML cells and 0.05 μM in bortezomib-resistant cells (Fig. 1A). Since DNA fragmentation is a consequence of caspase-3 activation [18], we examined caspase-3 activity in ML-1 and RPMI-8226vr10 cells in response to equimolar doses (1 μM) of panobinostat and vorinostat. Panobinostat increased caspase-3 activity 4-fold compared to untreated controls in ML-1 cells and 2.5-fold in bortezomib-resistant cells; vorinostat only slightly increased caspase-3 activation in both cell lines (Fig. 1B). Results were further confirmed with detection of cleaved caspase-3 on Western blot (Fig. 1C).

3.2. Panobinostat overcomes β5 proteasome subunit overexpression associated with bortezomib resistance

Cell viability analysis revealed that ML-1 cells were more resistant to bortezomib than AML3 cells (Fig. 2A, top panels). Both proteasome inhibitors caused dose-dependent induction of DNA fragmentation at equimolar concentrations (500 nM for AML3 cells and 1 μM for ML-1 cells), with marizomib inducing significantly more DNA fragmentation than bortezomib in AML3 cells, and trending toward more death in ML-1 cells (72% vs. 51% in AML3 cells and 48% vs. 38% in ML-1 cells, respectively) (Fig. 2A, bottom panels).

Overexpression of the β5 proteasome subunit has been linked to bortezomib resistance [19]. Since ML-1 cells showed marked resistance to bortezomib, we examined whether these cells have