Targeted Oncology
https://doi.org/10.1007/s11523-019-00638-4

ORIGINAL RESEARCH ARTICLE

Venetoclax Synergistically Enhances the Anti-leukemic Activity of Vosaroxin Against Acute Myeloid Leukemia Cells Ex Vivo

Fangbing Liu[1] · Tristan Knight[2,3] · Yongwei Su[1] · Holly Edwards[4,5] · Guan Wang[1] · Yue Wang[6] · Jeffrey W. Taub[2,3] · Hai Lin[7] · Liwei Sun[8] · Yubin Ge[3,4,5]

Abstract

Background The survival rate for acute myeloid leukemia remains unacceptably low, in large part owing to resistance to chemotherapy and high rates of relapse. There is an urgent need to develop new therapeutic modalities, in particular such that are tolerated by patients over the age of 60 years, who form the bulk of new acute myeloid leukemia diagnoses. Vosaroxin (SNS-595), a second-generation topoisomerase II inhibitor and DNA intercalating agent, shows promising preclinical and clinical activity against acute myeloid leukemia. Venetoclax (ABT-199), a selective Bcl-2 inhibitor, was recently approved for the treatment of acute myeloid leukemia.

Objective The objective of this study was to determine the anti-leukemic activity and the underlying molecular mechanisms for the combination of venetoclax and vosaroxin in acute myeloid leukemia cell lines and primary patient samples ex vivo.

Patients and Methods Using both acute myeloid leukemia cell lines and primary patient samples, annexin V/propidium iodide staining and flow cytometry analyses were used to quantify apoptosis induced by venetoclax or vosaroxin, alone or in combination, with subsequent western blotting analyses to assess levels of Bcl-2 family proteins. Alkaline comet assays were performed to quantify DNA damage induced by the two agents and to determine the effect of venetoclax on DNA repair. Finally, colony-forming assays were conducted on normal human CD34+ cord blood cells and primary acute myeloid leukemia patient samples to determine the effect of venetoclax and vosaroxin on normal hematopoietic and leukemic progenitor cells.

Results We found that venetoclax and vosaroxin synergistically induced apoptosis in multiple acute myeloid leukemia cell lines. Although vosaroxin could partially abrogate the increase of Mcl-1 protein induced by venetoclax, it could not abrogate the increased binding of Bim to Mcl-1 induced by venetoclax. Cooperative induction of DNA damage occurred within 8 h of treatment with venetoclax plus vosaroxin. Moreover, repair of DNA damage induced by vosaroxin was significantly attenuated by venetoclax. The combination also synergistically induced apoptosis in primary acute myeloid leukemia patient samples and significantly reduced the colony formation capacity of acute myeloid leukemia progenitor cells, while sparing normal hematopoietic progenitor cells.

Conclusions Vosaroxin and venetoclax synergistically induce apoptosis in acute myeloid leukemia cells and cooperatively target acute myeloid leukemia progenitor cells while sparing normal hematopoietic progenitor cells. Our results support the clinical testing of vosaroxin in combination with venetoclax for treating patients with acute myeloid leukemia, especially in the elderly population.

1 Background

Acute myeloid leukemia (AML) is an aggressive malignancy defined by explosive clonal proliferation of immature myeloid cells with a concomitant decrease in apoptosis [1]. Both pediatric and adult survival rates remain frustratingly low (5-year survival rates approximating 65% and 25%, respectively) [2]. For over 40 years, standard AML induction therapy has comprised cytarabine plus an anthracycline,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11523-019-00638-4) contains supplementary material, which is available to authorized users.

✉ Liwei Sun
Sunnylilwei@163.com

✉ Yubin Ge
gey@karmanos.org

Extended author information available on the last page of the article

Published online: 21 May 2019

Key Points

Venetoclax and vosaroxin synergize in inducing cell death in both acute myeloid leukemia cell lines and primary patient samples.

Venetoclax impairs the repair of DNA damage induced by vosaroxin in acute myeloid leukemia cells.

Limited analysis of primary acute myeloid leukemia patient samples and a normal CD34+ sample ex vivo suggest that a therapeutic window exists for the combination of vosaroxin and venetoclax.

e.g., the 7 + 3 regimen (7 days of cytarabine plus 3 days of an anthracycline, i.e., daunorubicin), followed by consolidation with a cytarabine-based regimen or allogeneic hematopoietic stem cell transplant [3]. However, this is an intensive regimen, and in patients over the age of 60 years (e.g., the largest population of patients with AML), and those with other comorbidities, 7 + 3 chemotherapy is not well tolerated because of its extensive toxicities and severe side effects [3]. The search for more efficacious and less toxic additions to the therapeutic armamentarium is therefore ongoing. Vosaroxin (SNS-595) and venetoclax (ABT-199) are two such promising agents.

Vosaroxin (SNS-595) is a topoisomerase II inhibitor and DNA intercalating agent. Clinically, vosaroxin has been shown to be well tolerated and moderately efficacious, particularly in older patients, but does not appear to be sufficient as monotherapy [4–7]. In July 2015, the US Food and Drug Administration advised against filing for approval and recommended that additional clinical trials were needed. Subsequently, in May 2017, its European Marketing Authorization Application was withdrawn following recommendations from the European Union Committee for Medicinal Products for Human Use because of the need for additional clinical data prior to further regulatory filings. Importantly however, despite the need for ongoing evaluation regarding its efficacy, vosaroxin is tolerable in elderly patients, especially in comparison to other topoisomerase II inhibitors. Organ-specific (including cardiac) toxicities do not appear to be increased [4–7]. Therefore, we suggest that while vosaroxin monotherapy shows limited therapeutic promise, its efficacy may be enhanced by combining it with a complementary agent, such as venetoclax, and that its true benefit may lie in the achievement of topoisomerase II inhibition without significant toxicity.

Venetoclax (ABT-199) is an oral selective Bcl-2 inhibitor and on 21 November, 2018 was granted accelerated approval by the US FDA for use as first-line up-front therapy in combination with low-dose cytarabine or the hypomethylating agents azacitidine or decitabine, for adults with newly diagnosed AML over the age of 75 years, or in those with comorbidities that otherwise preclude the use of intensive induction chemotherapy [8]. Venetoclax is well tolerated in this population, with relatively mild and largely hematological toxicities (e.g., neutropenia, thrombocytopenia); moreover, it is efficacious when combined with either of the above therapies, and phase III clinical trials are currently being conducted [8]. While there is insufficient clinical equipoise to conduct a trial of venetoclax monotherapy in patients with newly diagnosed AML, among patients with relapsed/refractory AML, it has demonstrated moderate efficacy in this challenging context [9].

Our laboratory has previously shown that venetoclax reduces the association of Bcl-2 with Bim and that once freed, a compensatory increase in Bim/Mcl-1 binding occurs, particularly in venetoclax-resistant AML cell lines, preventing the loss of mitochondrial membrane potential and reducing apoptotic potential [10]. Concurrent Mcl-1 inhibition (e.g., via selective Mcl-1 inhibitors such as A-1201447) is capable of diminishing this association and abrogates intrinsic venetoclax resistance [11]. Alternatively, induction of DNA damage, such as that which results following daunorubicin treatment, also reduces Mcl-1 levels, synergizing with venetoclax [12]. As noted, however, the toxicity and side-effect profile of anthracyclines may preclude their use (or continued use) in certain patients.

We therefore hypothesized that the combination of vosaroxin with venetoclax would demonstrate anti-leukemic efficacy via a combination of DNA damage and Bcl-2 inhibition, while also theoretically being more tolerable clinically; we propose to take advantage of the non-inferiority of vosaroxin to cytarabine [6] and the reduced side-effect profile to create a combination of agents with improved tolerability and preserved efficacy, particularly in frail populations, with sufficient clinical equipoise to justify translation to the clinical setting.

2 Methods

2.1 Drugs

Venetoclax and vosaroxin were purchased from Selleck Chemicals (Houston, TX, USA). Z-VAD-FMK was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Cell Culture

The AML cell lines, MV4-11, THP-1, and U937, were purchased from the American Type Culture Collection (2006, 2014, 2002, respectively; Manassas, VA, USA), OCI-AML3 was purchased from the German Collection of

Microorganisms and Cell Cultures (2011; DSMZ, Braunschweig, Germany), and MOLM-13 was purchased from AddexBio (2012; San Diego, CA, USA). All cell lines were cultured as previously described [13, 14] and tested monthly for the presence of mycoplasma utilizing the polymerase chain reaction method as described by Uphoff and Drexler [15]. All cell lines were authenticated in 2017 at the Genomics Core at Karmanos Cancer Institute using the PowerPlex® 16 System from Promega (Madison, WI, USA).

2.3 Clinical Samples

Acute myeloid leukemia blast samples derived from patients at either initial diagnosis or at relapse were obtained from the First Hospital of Jilin University, Changchun, China, following written informed consent as required by the Declaration of Helsinki. This study was approved by and carried out in accordance with the guidelines as set forth by the Human Ethics Committee of the First Hospital of Jilin University. All clinical samples were screened for the presence of both gene mutations and fusion genes, as previously described [13]. Patient characteristics are shown in Table 1. Acute myeloid leukemia blast samples were purified via standard Ficoll-Hypaque density centrifugation and cultured in RPMI 1640 with 20% fetal bovine serum supplemented with ITS (Sigma-Aldrich) and 20% supernatant of the 5637 bladder cancer cell line (as a source of granulocyte-macrophage colony-stimulating factor [16]), as described previously [13, 17]. Normal bone marrow mononuclear cells were purchased from Lonza (Walkersville, MD, USA) [13, 17].

2.4 Annexin V/Propidium Iodide Staining and Flow Cytometry Analysis

Following treatment with venetoclax, vosaroxin, both, or neither for up to 24 h, AML cells underwent flow cytometry analysis utilizing the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (Beckman Coulter; Brea, CA, USA) to identify the extent of drug-induced apoptosis, as previously described [18, 19]. All experiments utilizing the AML cell lines were performed three times in triplicate independently, and experiments using primary patient samples were performed once in triplicate because of a limited sample supply. Patient samples were selected based on the availability of an adequate sample for the assay. For both cell lines and primary patient samples, debris was gated out based on forward scatter vs. side scatter, with the remaining population analyzed for annexin V/PI staining. Apoptotic events are displayed as the mean percentage of Annexin V positive/PI negative (early apoptotic) and Annexin V positive/PI positive (late apoptotic and/or dead) cells ± the standard error from one representative experiment.

2.5 Western Blot Analysis

Whole cells were lysed by sonication in 10 mM Tris–Cl, pH 7.0, containing 1% SDS, protease inhibitors, and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis, electrophoretically transferred onto polyvinylidene difluoride membranes (Thermo Fisher

Table 1 Characteristics of patients from whom primary acute myeloid leukemia (AML) patient samples were isolated

Patient	Sex	Age (years)	Disease status	FAB subtype	Cytogenetics	Blast purity (%)	Gene mutation
AML#123	Male	24	Newly diagnosed	M5	46,XY	92.0	
AML#124	Male	19	Newly diagnosed	M4	46,XY	63.0	CEBPA double mutation, c-Kit, NRAS, GATA2, Flt3 S451F mutation
AML#127	Male	77	Newly diagnosed	M4	NA	NA	NA
AML#140	Female	33	Newly diagnosed	M3	NA	97.5	PML-RARα
AML#150	Male	48	Newly diagnosed	M2	46,XY	68.0	CEBPAdm, IDH2
AML#155	Male	68	Newly diagnosed	M5	46,XY	96.5	FLT3-ITD, NPM1, DNMT3A
AML#158	Male	53	Relapsed	M2	NA	98.0	NA
AML#159	Male	30	Newly diagnosed	NA	46,XY	95.5	FLT3-ITD, DNAH9, PHF6
AML#171	Female	59	Newly diagnosed	M2	47,XX,+8	60.5	FLT3-ITD, DNMT3A
AML#185	Female	37	Newly diagnosed	M2	46,XX	87.5	FLT3-ITD, NPM1, IDH2
AML#187	Male	50	Newly diagnosed	M4	47,XY,+8	96.5	FLT3-ITD
AML#188	Female	55	Newly diagnosed	M3	46,XX, t(15;17) (q22;q21)	93.0	FLT3-ITD, SH2B3

FAB French-American-British, *NA* not available

Inc., Rockford, IL, USA), and then immunoblotted using anti-Bcl-2 (12789-1-AP), anti-Bak (14673-1-AP), anti-Bax (50599-2-Ig), anti-Puma (55120-1-AP), anti-Noxa (17418-1-AP), anti-Mcl-1 (16225-1-AP; Proteintech Group, Chicago, IL, USA), anti-Bcl-xL (2764), anti-PARP (9542), anti-Bim (2819), anti-cleaved caspase-3 (9661, designated anti-cf caspase-3; Cell Signaling Technology, Danvers, MA, USA), or anti-β-actin (A2228; Sigma-Aldrich) antibody, as previously described [20]. Proteins were then visualized via the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE, USA), as described by the manufacturer. Western blots were performed at least three times, and a single representative blot is shown. For cell lines, three independent experiments were performed. Because of a limited number of cells obtained from primary patient samples, western blots of patient samples were from one independent experiment. Patient samples were selected on the basis of availability of an adequate sample for the analysis.

2.6 Alkaline Comet Assay

Following treatment with venetoclax, vosaroxin, both, or neither for up to 20 h, AML cells were subjected to alkaline comet assays, as previously described [21]. Using SYBR Gold (Life Technologies, Grand Island, NY, USA), slides were stained and then subsequently imaged using an Olympus BX-40 microscope equipped with a DP72 microscope camera and Olympus CellSens Dimension software (Olympus America Inc., Center Valley, PA, USA). Approximately 50 comets were scored per gel, using CometScore (TriTek Corp, Sumerduck, VA, USA). Median percentage of DNA in the tail was calculated and graphed as mean ± the standard error. Primary patient samples were chosen for this assay based on adequate sample availability.

2.7 Colony-Forming Assay

Primary AML patient samples and normal bone marrow mononuclear cells were treated with either venetoclax or vosaroxin, alone or in combination, for 24 h. Cells were then washed three times with phosphate-buffered saline and then plated in MethoCult (Catalog number 04434; Stem Cell Technologies, Vancouver, BC, Canada), which contains cytokines (recombinant human stem cell factor, recombinant human interleukin-3, human erythropoietin, and human granulocyte-macrophage colony-stimulating factor) to support the growth of human hematopoietic progenitor cells, incubated in a humidified atmosphere containing 5% CO_2/95% air at 37 °C for 14–16 days. Colony-forming units were visualized utilizing an inverted microscope, and colonies containing over 50 cells were counted. Technical triplicates were performed.

2.8 Statistical Analysis

Differences in cellular apoptosis (comparison of the sum of annexin V+ cells), colony-forming units, and the percentage of DNA present in the tail between treatment groups and/or untreated cells were compared by a pair-wise two-sample t-test or a one-way analysis of variance with Bonferroni post hoc test (when comparing differences between three or more groups). Statistical analyses were performed utilizing GraphPad Prism 5.0. Error bars represent ± standard error of the mean; significance level was set at $p < 0.05$.

3 Results

3.1 Venetoclax and Vosaroxin Synergistically Induce Apoptosis in Acute Myeloid Leukemia (AML) Cell Lines

To determine if the combination of vosaroxin and venetoclax synergistically induces apoptosis, we treated the AML cell lines THP-1, U937, OCI-AML3, MOLM-13, and MV4-11 with various concentrations of venetoclax or vosaroxin, alone or in combination, for 24 h. Drug-induced apoptosis was determined by utilizing Annexin V/PI staining and flow cytometry analyses. We then calculated the combination index (CI): CI = 1 representing additive effect, CI < 0.9 denoting synergy, and CI < 0.3 denoting strong synergy [22]. At 24 h, strong synergy was observed in MV4-11 (CI < 0.3), synergy in MOLM-13 (CI < 0.4), and moderate synergy in THP-1, U937, and OCI-AML3 (CI < 0.8, < 0.6, and < 0.6, respectively, Fig. 1a, b). Apoptosis was confirmed by western blotting of cleaved caspase 3 and PARP (Fig. 2a). We confirmed that apoptosis was at least partially caspase dependent using the pan-caspase inhibitor Z-VAD-FMK, which partially rescued AML cells from apoptosis induced by the two agents (Fig. 2b).

3.2 Vosaroxin Partially Abrogates Mcl-1 Induction by Venetoclax, but Fails to Prevent Mcl-1 Binding to Bim Released by Venetoclax in AML Cell Lines

Next, we examined the levels of the Bcl-2 family of proteins across AML cell lines post-drug treatment by western blot analysis. We identified consistent induction of Mcl-1 by venetoclax across the samples, and slight-to-moderate downregulation of Mcl-1 by vosaroxin. The combination of venetoclax and vosaroxin partially abolished Mcl-1 induction by venetoclax. In contrast, protein levels for Bcl-2, Bcl-xL, Bax, Bak, Bim, PUMA, and NOXA were largely unaffected by either agent, alone or in combination (Fig. 3a). Time-course experiments were performed to establish when

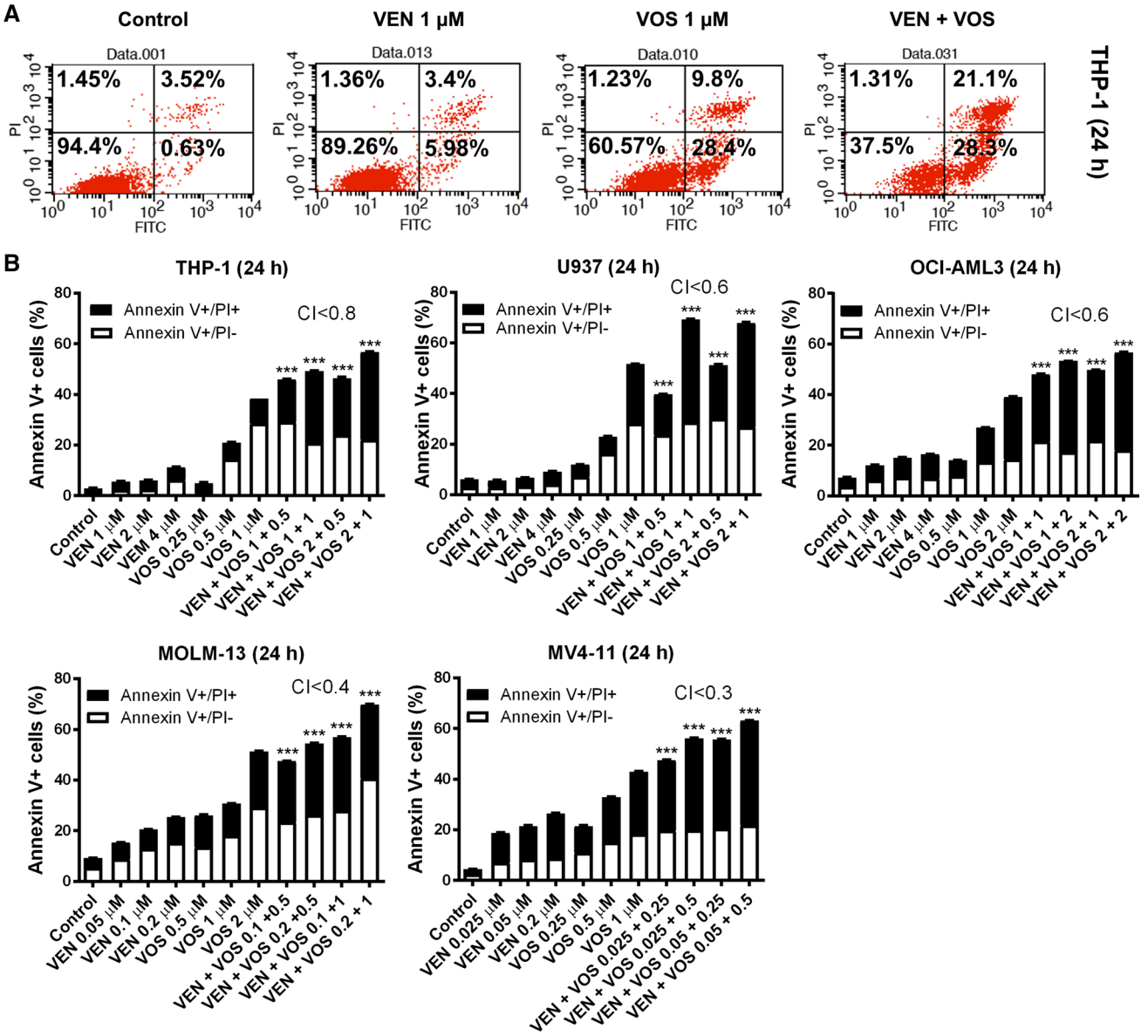

Fig. 1 Venetoclax (VEN) synergizes with vosaroxin (VOS) to induce apoptosis in multiple acute myeloid leukemia (AML) cell lines. **a**, **b** AML cell lines were treated with VEN and VOS, alone or in combination, for 24 h and then subjected to annexin V/propidium iodide (PI) staining and flow cytometry analyses. Combination index (CI) values were calculated using CompuSyn software. CI = 1, < 1, and > 1 indicate additive, synergistic, and antagonistic effect, respectively. *FITC* fluorescein isothiocyanate. ***$p < 0.001$ compared to single-drug treatment

the Mcl-1 alteration occurred, which was found to happen rapidly, with increased Mcl-1 protein induced by venetoclax being present within 4 h, which was at least partially abolished by vosaroxin (Fig. 3b–d). Co-immunoprecipitation was performed in a representative AML cell line THP-1. As expected, venetoclax treatment for 8 or 24 h markedly decreased Bim binding to Bcl-2 and dramatically increased Bim binding to Mcl-1. In contrast, vosaroxin did not have an obvious effect on Bim binding to either Bcl-2 or Mcl-1. Although vosaroxin partially to completely abolished Mcl-1 induction by venetoclax, it did not have any effect on the increased binding of Bim to Mcl-1 in the combination-treated cells (Fig. 3e, f). These results suggest that vosaroxin enhances the anti-leukemic activity of venetoclax through other mechanisms, rather than blocking venetoclax released Bim binding to Mcl-1.

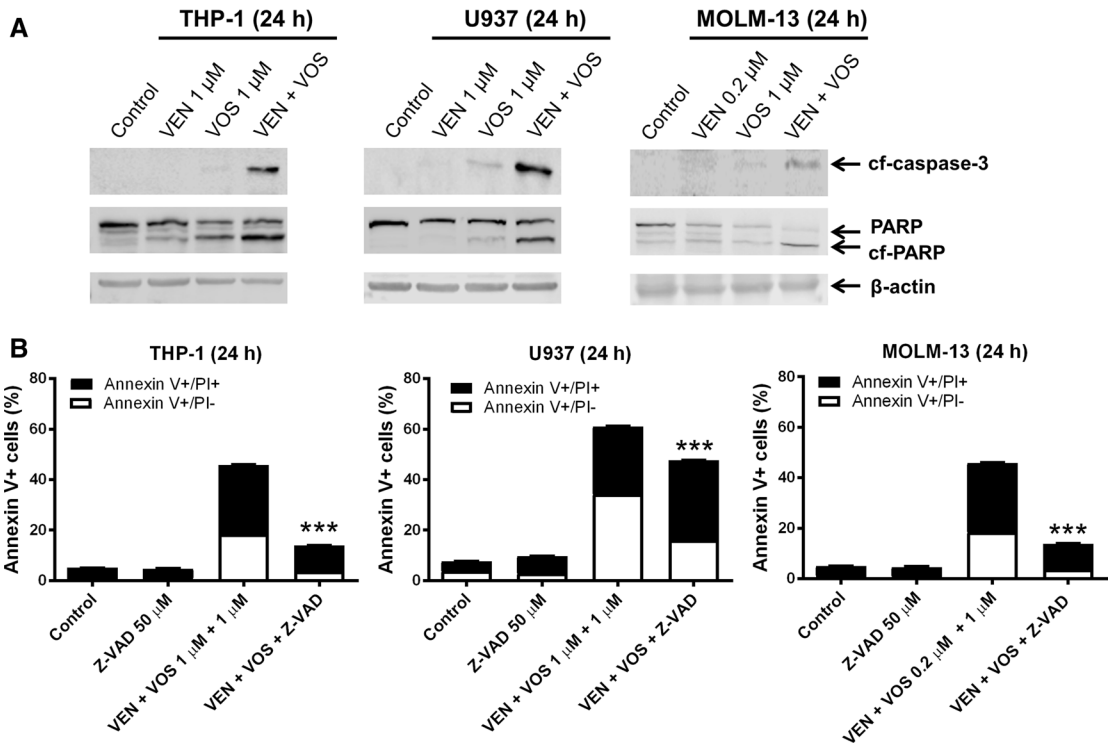

Fig. 2 Combined venetoclax (VEN) and vosaroxin (VOS) induce caspase-dependent apoptosis. **a** THP-1, U937, and MOLM-13 cell lines were treated with VEN and VOS, alone or in combination, for 24 h. Whole-cell lysates were subjected to western blotting and probed with the indicated antibodies. **b** THP-1, U937, and MOLM-13 cell lines were treated with Z-VAD-FMK, VEN and VOS, or in combination, for 24 h, and then subjected to annexin V/propidium iodide (PI) staining and flow cytometry analyses. *cf-caspase 3* cleaved caspase 3, *cf-PARP* cleaved PARP. ***$p < 0.001$ compared to VEN + VOS

3.3 Cooperative Induction of DNA Damage Coincides with Cooperative Induction of Apoptosis by Venetoclax and Vosaroxin in AML Cell Lines

In previous studies, we found that venetoclax can enhance the anti-leukemic activity of cytarabine and daunorubicin via enhancing DNA damage [10]. It is conceivable that venetoclax also enhances the anti-leukemic activity of vosaroxin through a similar mechanism. To investigate the occurrence and extent of DNA damage, we conducted alkaline comet assays following treatment with venetoclax or vosaroxin, alone or in combination, over 4–8 h. Increasing apoptotic induction was noted at 8 h of treatment initiation with the combination by annexin V/PI staining and flow cytometry analyses (Fig. 4a). At 4 h, the alkaline comet assay demonstrated significantly increased DNA strand breaks, as measured by the proportion of DNA in the comet tails with vosaroxin monotherapy and combined vosaroxin/venetoclax therapy, compared with venetoclax alone or the control (Fig. 4b, c). At 8 h, however, DNA damage was significantly increased via combination treatment, in relation to monotherapy with either agent alone (Fig. 4d, e). These changes correlated with the expected induction of apoptosis at 8 h, identified in Fig. 4a. Treatment with the pan-caspase inhibitor Z-VAD-FMK had no effect on DNA damage induced by the combination (Fig. S1 of the Electronic Supplementary Material [ESM]). We confirmed our findings in two additional cell lines. In MOLM-13 cells, induction of significant DNA damage was seen at 8 h, correlating with apoptosis (Fig. 4f–h). Similar results were obtained in the U937 cell line (Fig. 4i–k). Additionally, venetoclax enhanced DNA damage induced by daunorubicin and VP-16 in THP-1 cells (Fig. S2 of the ESM), further supporting the strategy of combining venetoclax with DNA damaging agents.

3.4 Venetoclax Impairs the Repair of DNA Strand Breaks Induced by Vosaroxin in AML Cells

To begin to understand how venetoclax enhances DNA damage induced by vosaroxin, we next sought to investigate the effect of venetoclax on the repair of DNA damage induced by vosaroxin. Following 4 h of exposure to vosaroxin alone, THP-1 cells were washed and re-suspended in fresh media and treated with venetoclax or vehicle control for up to 20 h; samples were taken at different times and subjected to alkaline comet assays. In the absence of venetoclax, DNA repair progressed rapidly. However, in the presence of venetoclax,

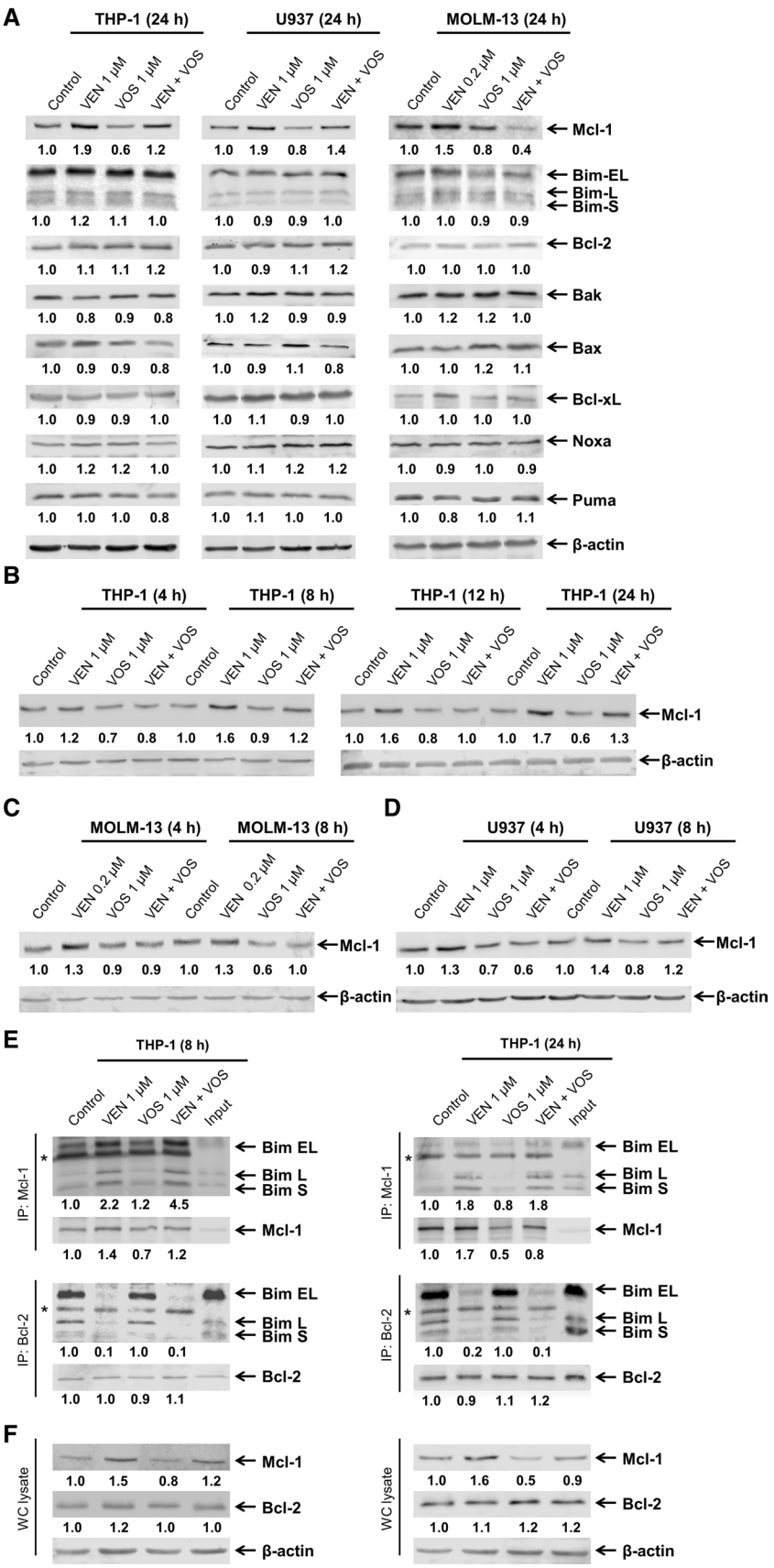

Fig. 3 Vosaroxin (VOS) decreases Mcl-1 protein levels but fails to prevent Mcl-1 binding to Bim released by venetoclax (VEN) in acute myeloid leukemia cell lines. **a**–**d** THP-1, U937, and MOLM-13 cells were treated with VEN and VOS, alone or combined, for up to 24 h. Whole-cell lysates were subjected to western blotting and probed with the indicated antibodies. **e** THP-1 cells were treated with VEN and VOS, alone or in combination, for 8 (left panels) or 24 h (right panels). Mcl-1 and Bcl-2 were immunoprecipitated from whole-cell lysates and then subjected to western blotting and probed with the indicated antibodies. Asterisk indicates the light chain of the anti-Mcl-1 or anti-Bim antibody. **f** THP-1 cells were treated with VEN and VOS, alone or combined, for 8 (left panels) or 24 h (right panels). Whole-cell lysates were subjected to western blotting and probed with the indicated antibodies. The fold changes for the densitometry measurements, normalized to β-actin and then compared to no drug control, are indicated below the corresponding blot

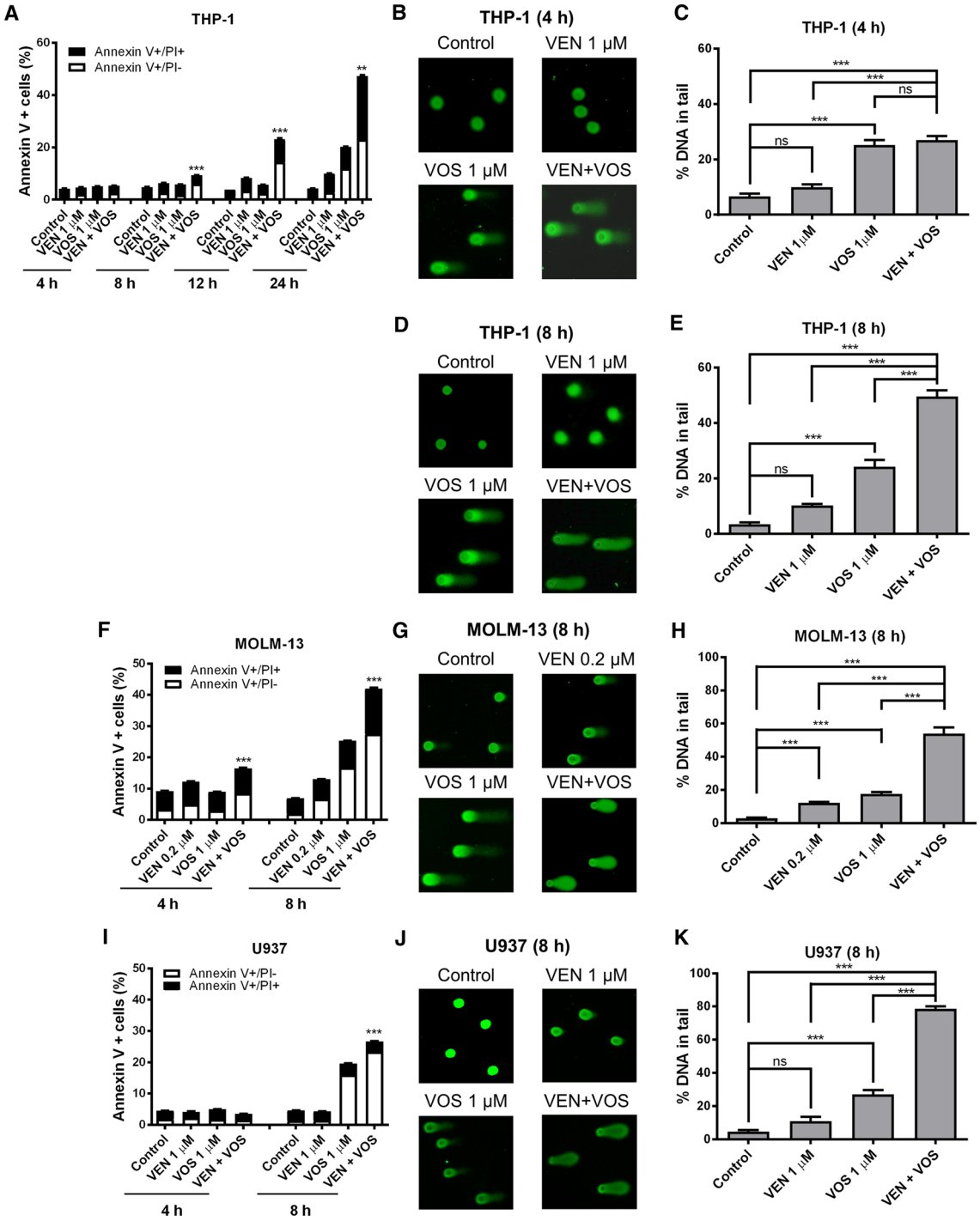

Fig. 4 Cooperative induction of DNA damage coincides with cooperative induction of apoptosis by venetoclax (VEN) and vosaroxin (VOS) in acute myeloid leukemia cell lines. **a** THP-1 cells were treated with VEN and VOS, alone or in combination, for up to 24 h and then subjected to annexin V-fluorescein isothiocyanate/propidium iodide (PI) staining and flow cytometry analyses. Mean percent annexin V+ cells ± standard error of the mean (SEM) are shown. $^{**}p < 0.01$; $^{***}p < 0.001$ compared to single-drug treatment. THP-1 cells were treated with VEN and VOS, alone or combined, for 4 h (**b**, **c**) or 8 h (**d**, **e**), and then subjected to alkaline comet analyses. Representative images are shown (**b**, **d**). Alkaline comet assay results are graphed as median percent DNA in the tail from four replicates gels ± SEM (**c**, **e**). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the indicated treatment. MOLM-13 (**f**–**h**) and U937 (**i**–**k**) cells were treated with VEN and VOS, alone or in combination, for up to 8 h and then subjected to annexin V-fluorescein isothiocyanate/PI staining and flow cytometry analyses. Mean percent annexin V+ cells ± SEM are shown; $^{***}p < 0.001$ compared to individual drug treatment (**f**, **i**). The treated cells were also subjected to alkaline comet analyses. Representative images are shown (**g**, **j**). Alkaline comet assay results are graphed as median percent DNA in the tail from four replicate gels ± SEM (**h**, **k**). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the indicated treatment

significantly more DNA was present in the comet tails, signifying that persistent inhibition of DNA repair was occurring (Fig. 5a, b). We then confirmed these findings in the additional AML cell line, MOLM-13, with near-identical results (Fig. 5c, d).

3.5 Venetoclax and Vosaroxin Synergistically Induce Apoptosis in Primary AML Patient Samples

We next performed annexin V/PI staining and flow cytometry analyses with multiple primary AML patient samples, using various concentrations of venetoclax or vosaroxin, alone or in combination, for 24 h and calculated CI values (Fig. 6a, b). Marked synergy was seen in all samples, with CIs ranging from <0.01 to <0.4. To confirm apoptosis induction, we performed western blotting analysis of cleaved caspase 3 and PARP in two representative samples with a sufficient number of cells, with findings being consistent with the AML cell lines (Fig. 6c). Next, alkaline comet assays were performed, showing significant increases in DNA strand breaks as determined by the percentage of DNA present in the comet tails, in the presence of combined venetoclax plus vosaroxin treatment, in comparison to either agent alone or a control (Fig. 7).

3.6 Venetoclax plus Vosaroxin Impairs the Colony-Forming Capacity of Leukemia Progenitor Cells, While Sparing Normal Hematopoietic Progenitor Cells

Finally, we performed colony-forming assays utilizing both primary AML patient samples and normal human CD34+ cord blood cells, subjecting them to treatment with venetoclax, vosaroxin, both, or neither, so as to jointly assess the efficacy of the combination against AML progenitor cells, and determine toxicity to 'bystander' bone marrow cells. Venetoclax treatment resulted in a significant reduction in AML colony-forming units in one of the three primary samples, while vosaroxin treatment resulted in a dramatic and significant decrease of AML colony-forming units in all the three samples. Consistent with the data shown in Fig. 5, the combination treatment resulted in significantly further decreased numbers of AML colony-forming units in comparison to either agent alone in two of the three primary patient samples (Fig. 8a). Neither agent, alone or in combination, displayed significant toxicity against healthy human CD34+ cord blood cells, with all assessed colony sub-types displaying similar numbers to the control untreated cells, irrespective of the treatment to which they had been subjected (Fig. 8b).

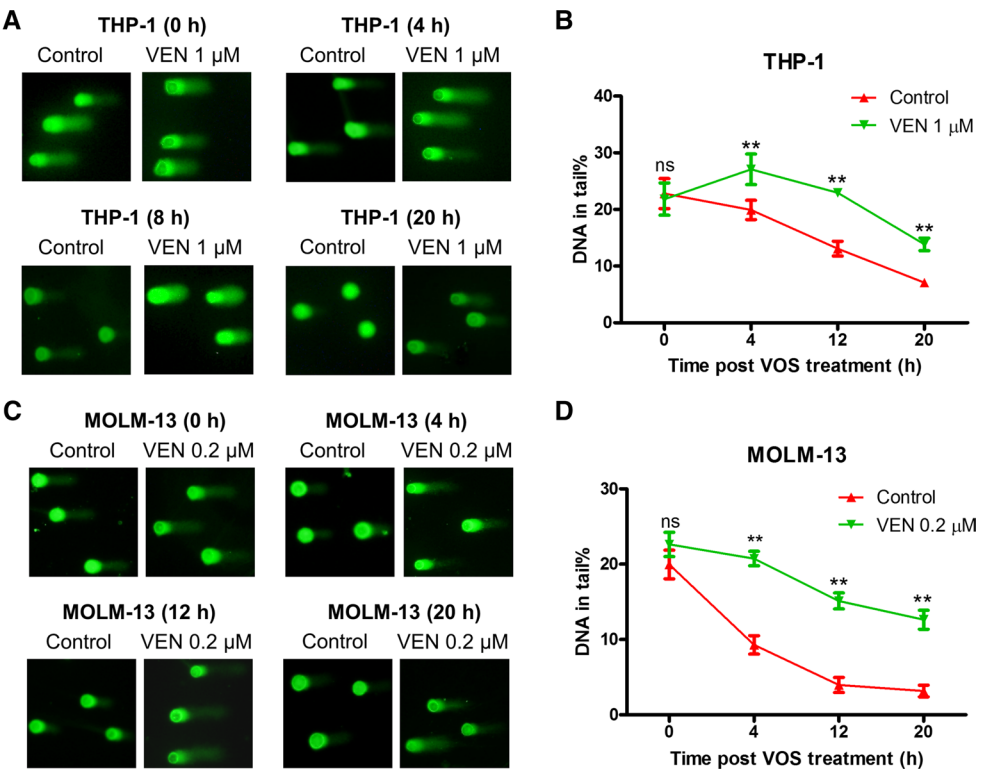

Fig. 5 Venetoclax (VEN) impairs the repair of DNA strand breaks induced by vosaroxin (VOS) in acute myeloid leukemia cells. **a**–**d** THP-1 (**a**) and MOLM-13 (**c**) cells were pretreated with VOS alone for 4 h, washed and re-suspended in fresh media, and then treated with VEN or vehicle control for up to 20 h. Cells were collected at different times and subjected to alkaline comet analyses. Representative images are shown. **b**, **d** Alkaline comet assay results are graphed as median percent DNA in the tail from four replicate gels ± standard error of the mean. $^{**}p<0.01$ and ns indicates not significant compared to control at the corresponding time point

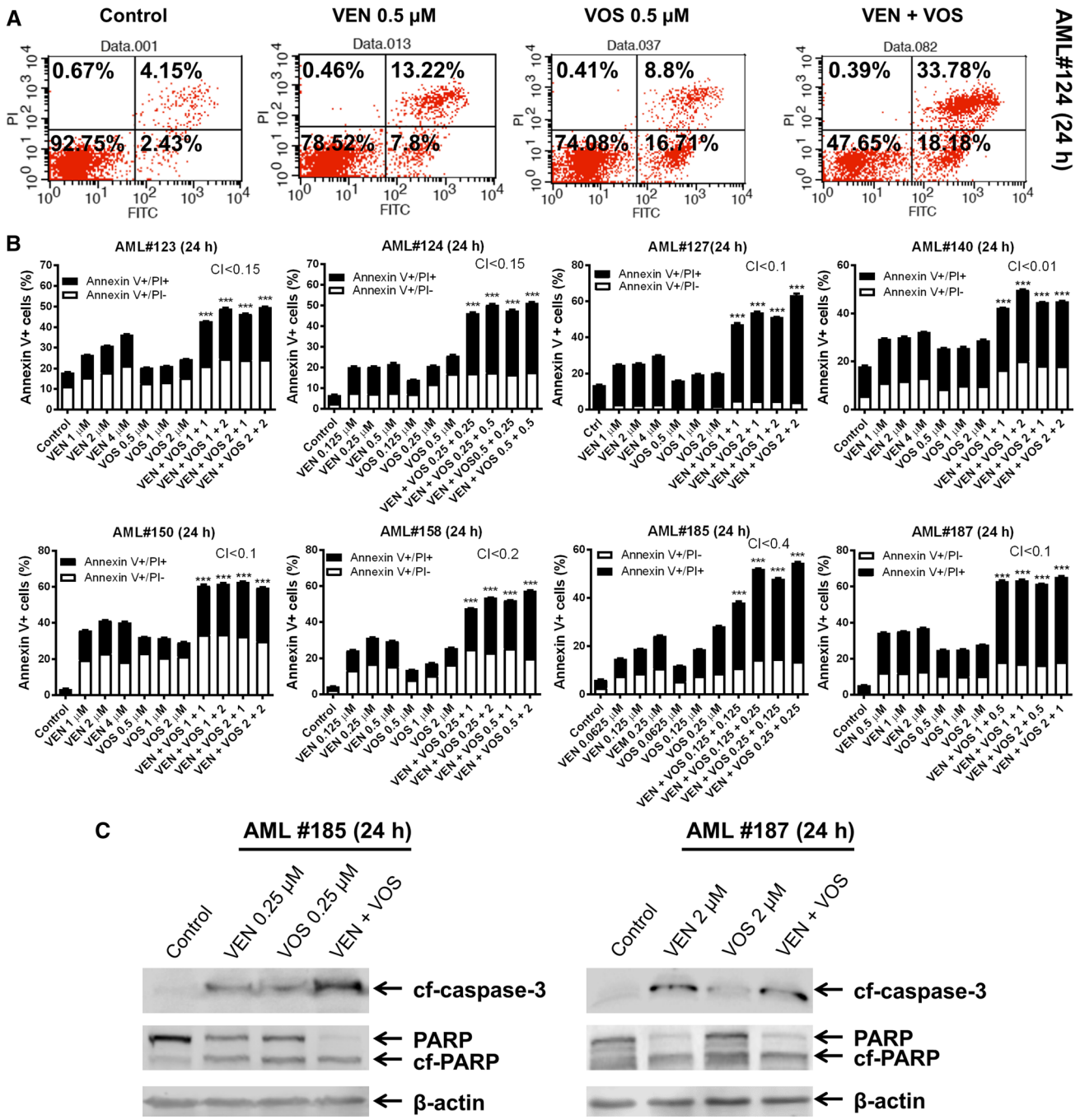

Fig. 6 Venetoclax (VEN) and vosaroxin (VOS) synergistically induce apoptosis in primary acute myeloid leukemia (AML) patient samples. **a**, **b** Primary AML patient samples were treated with VEN and VOS, alone or in combination, for 24 h, and then subjected to annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining and flow cytometry analyses. ***$p < 0.001$ compared to single-drug treatment. Combination index values were calculated using CompuSyn software. **c** Primary AML patient samples were treated with VEN and VOS, alone or in combination, for 24 h. Whole-cell lysates were subjected to western blotting and probed with the indicated antibodies

4 Discussion

Promising pre-clinical and clinical data exist to support the use of venetoclax in AML [9, 23, 24]. Clinical data regarding the use of vosaroxin are more mixed, but do suggest some efficacy in AML. At a minimum, vosaroxin does not appear to be inferior to current management options, particularly among patients who would not/do not tolerate more intensive chemotherapy [4–7]. Importantly, while vosaroxin does indeed carry some toxicities, predominantly gastrointestinal/

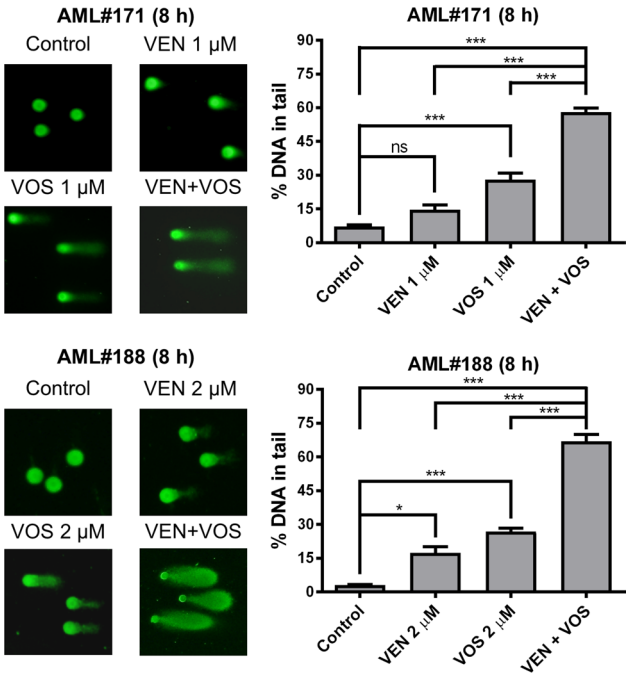

Fig. 7 Venetoclax (VEN) and vosaroxin (VOS) cooperatively induce DNA damage in primary acute myeloid leukemia (AML) patient samples. Primary AML patient samples were treated with VEN or VOS, alone or in combination, for 8 h, and then subjected to alkaline comet analyses. Representative images are shown (left panels). Alkaline comet assay results are graphed as median percent DNA in the tail from four replicate gels ± standard error of the mean (right panels). *$p < 0.05$; **$p < 0.01$;***$p < 0.001$ compared to the treatment group as indicated

hematological in nature, it lacks many of the more severe toxicities of other topoisomerase II inhibitors, particularly the anthracyclines, and is demonstrably well tolerated in patients over 60 years of age [4–7]. Despite AML having a median diagnostic age of 68 years, patients over the age of 60 years are known to fare significantly poorer than their younger counterparts and have a higher incidence of adverse cytogenetic abnormalities, such as FLT3-ITD [3, 25]. Therefore, older patients who may be most in need of intensive chemotherapy, may not be able to tolerate the treatments that are necessary to induce remission. As such, there is an urgent need for agents that not only display efficacy meeting or exceeding that of existing agents, but also demonstrate a favorable side-effect and toxicity profile.

The recent US Food and Drug Administration approval of venetoclax in AML was based on trials showing that combining venetoclax and a hypomethylating agent was safe and tolerable in patients 65 years of age or older, and achieved improvements in complete remission rates and overall survival [10, 11]. While displaying undeniable promise, there is considerable room for improvement [10, 11]. DNA-damaging agents synergize with venetoclax, as demonstrated in our previous studies [10, 12, 26]. Vosaroxin reduced the expression of Mcl-1, and abrogated most, if not all of the venetoclax-induced upregulation of Mcl-1. As such, this likely represents one point of synergy between the two agents. However, the addition of vosaroxin does not seem to impact the interaction between Mcl-1 and Bim, which is perhaps unsurprising given its non-Bcl-2-related mechanism.

The major synergistic mechanism we have identified is the cooperative induction of DNA damage by the two agents. Venetoclax alone does not cause overt induction/accumulation of DNA damage, while vosaroxin, congruent with its mechanism, is more effective at achieving this. However, we have identified the combination of both agents as being robustly and significantly more efficacious in inducing DNA damage, within 8 h of exposure. Similarly, venetoclax impairs the ability of AML cells to perform DNA repair. Along similar lines, prior work has established that γH2AX, an accepted biomarker for double-stranded DNA breaks, is markedly upregulated (signifying the presence of DNA damage) in the presence of venetoclax plus daunorubicin, compared with either agent alone [10]. While we are still working to identify the mechanism by which venetoclax impairs DNA repair, several potential explanations exist. Xiufeng et al. combined venetoclax with gemcitabine in human T-ALL cell lines and found synergistic cytotoxicity and apoptosis induction. Importantly, while neither agent alone inhibited RAD51/BRCA1-dependent DNA repair, the combination efficaciously disrupted this repair process, and also induced caspase 3/PARP activation [27]. It is also plausible that venetoclax inhibition of Bcl-2 interferes with its association with many proteins involved in DNA damage repair [28]. However, the precise mechanism by which this occurs remains to be elucidated and is the subject of ongoing work. p53 status and related p53-dependent mechanisms do not appear to play a major role in apoptotic induction by this combination. Of the cell lines we investigated, MOLM-13 is p53-wild type [29], while THP-1 and U937 are both p53-null [30].

We were unable to identify any direct comparative studies between vosaroxin and other topoisomerase II inhibitors in either a clinical or preclinical setting. Although other combinations of venetoclax plus topoisomerase II inhibitors have been assessed in the literature [10, 31], the conduction of extrapolative comparisons is irreconcilably hindered by differences in dosages and concentrations used in the studies, which negate any possible value in performing such comparisons in the first place. As such, one major remaining question concerns the relative efficacy of combinations of topoisomerase II inhibitors with venetoclax. Concerning the already completed trials of vosaroxin, the worst-case scenario is that it results in non-inferior outcomes, while the best-case scenario is a small but significant improvement in outcomes, particularly among selected groups of patients. It therefore remains to be ascertained what the relative

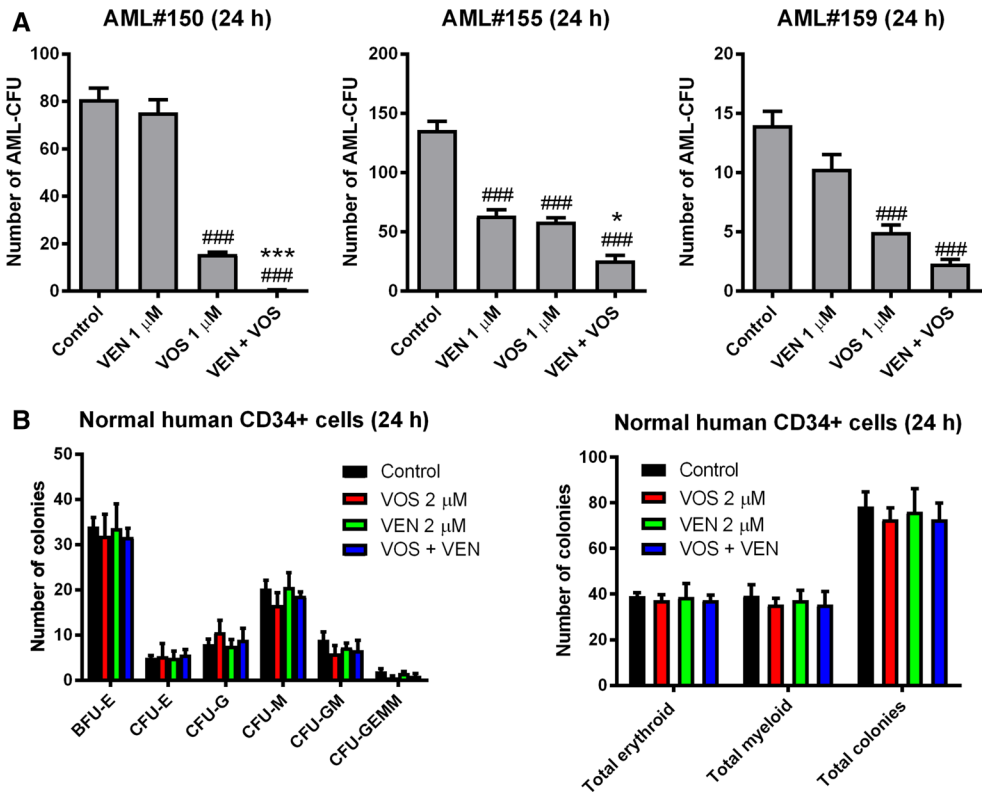

Fig. 8 Venetoclax (VEN) plus vosaroxin (VOS) impairs the colony-forming capacity of leukemia progenitor cells, while sparing normal hematopoietic progenitor cells. **a** Primary acute myeloid leukemia (AML) patient samples were treated with VEN and VOS, alone or in combination, for 24 h, and then plated in methylcellulose. After incubation for 2 weeks, the number of surviving AML cells capable of generating leukemia colonies [AML-colony-forming units (CFUs)] were enumerated. Data are presented as mean ± standard error of the mean $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, when compared to no drug treatment control; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, when compared to individual drug treatment. **b** Normal human bone marrow mononuclear cells from a single donor were cultured with VEN and VOS, alone or in combination, for 24 h, and then plated in methylcellulose. After incubation for 2 weeks, the number of surviving hematopoietic cells capable of generating colonies were enumerated. Total erythroid and myeloid colonies are presented as mean ± standard error of the mean. The number of BFU-E, CFU-E, CFU-G, CFU-M, CFU-GM, and CFU-GEMM colonies are presented as mean ± standard error of the mean

efficacies of the topoisomerase II inhibitors are, including vosaroxin, in relation to each other. Conversely, the combination of vosaroxin with other agents targeted against the Bcl-2 family warrants further exploration, we identified a small but significant downregulation of Mcl-1 induced by vosaroxin, within 8 h of exposure. It is possible that its combination with agents that directly inhibit Mcl-1 might result in anti-leukemic synergy. Although at a relatively immature phase in their development, the selective Mcl-1 inhibitors do demonstrate promising results, and several (AMG176, S64315/MIK665) are currently being evaluated clinically in patients with relapsed/refractory AML [32–34]. Moreover, it remains to be seen whether combinations with less selective inhibitors of the Bcl-2 family, such as navitoclax (also known as ABT-263; an inhibitor of Bcl-2, Bcl-xL, and Bcl-W) would be efficacious. While ABT-737 is known to cause a rapid marked thrombocytopenia, which is concentration dependent and dose limiting [35], its efficacy in multiple leukemia types has been established [36–38].

5 Conclusions

In combining venetoclax with vosaroxin, we identified potent anti-leukemic synergy in multiple AML cell lines and primary patient samples. One explanation for the synergistic anti-leukemic interaction is the cooperative induction of DNA damage. Additionally, combination therapy successfully blocks the colony-forming capacity of AML progenitor cells ex vivo, while sparing normal human hematopoietic progenitor cells. We therefore recommend that this combination be further evaluated in vivo in murine models to further assess efficacy, before a potential eventual clinical translation in patients otherwise unable to tolerate intensive AML therapy.

Author Contributions YG and LS designed the project and directed the study. FL and YS performed the experiments, data analysis, and interpretation. TK drafted the manuscript. FL, HE, and YG edited the manuscript. HL and YW provided the primary AML patient samples and participated in the coordination of the study. JWT, GW, HE, and YG participated in the data analysis and interpretation. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Funding This study was supported by Jilin University, Changchun, China, the Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, and by grants from the National Natural Science Foundation of China (NSFC 31671438 and NSFC 31471295), National Key Research and Development Program of China (2017YFC1702100), Hyundai Hope on Wheels, LaFontaine Family/U Can-Cer Vive Foundation, Kids Without Cancer, Children's Hospital of Michigan Foundation, Decerchio/Guisewite Family, Justin's Gift, Elana Fund, Ginopolis/Karmanos Endowment, and the Ring Screw Textron Endowed Chair for Pediatric Cancer Research. The funders had no role in the study design, data collection, analysis and interpretation of data, decision to publish, or preparation of the manuscript.

Conflict of interest Fangbing Liu, Tristan Knight, Yongwei Su, Holly Edwards, Guan Wang, Yue Wang, Jeffrey W. Taub, Hai Lin, Liwei Sun, and Yubin Ge have no conflicts of interest that are directly relevant to the contents of this article.

References

1. Marcucci G, Haferlach T, Dohner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. J Clin Oncol. 2011;29(5):475–86.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018;68(1):7–30.
3. Short NJ, Rytting ME, Cortes JE. Acute myeloid leukaemia. Lancet. 2018;392(10147):593–606.
4. Ravandi F, Ritchie EK, Sayar H, Lancet JE, Craig MD, Vey N, et al. Vosaroxin plus cytarabine versus placebo plus cytarabine in patients with first relapsed or refractory acute myeloid leukaemia (VALOR): a randomised, controlled, double-blind, multinational, phase 3 study. Lancet Oncol. 2015;16(9):1025–36.
5. Ravandi F, Ritchie EK, Sayar H, Lancet JE, Craig M, Vey N, et al. Durable overall survival benefit in patients $\geq$60 years with relapsed or refractory AML treated with vosaroxin/cytarabine vs placebo/cytarabine: updated results from the Valor Trial. Blood. 2016;128(22):903.
6. Dennis M, Russell N, Hills RK, Hemmaway C, Panoskaltsis N, McMullin MF, et al. Vosaroxin and vosaroxin plus low-dose Ara-C (LDAC) vs low-dose Ara-C alone in older patients with acute myeloid leukemia. Blood. 2015;125(19):2923–32.
7. Daver N, Kantarjian H, Garcia-Manero G, Jabbour E, Borthakur G, Brandt M, et al. Vosaroxin in combination with decitabine in newly diagnosed older patients with acute myeloid leukemia or high-risk myelodysplastic syndrome. Haematologica. 2017;102(10):1709–17.
8. Knight T, Luedtke D, Edwards H, Taub JW, Ge Y. A delicate balance: the BCL-2 family and its role in apoptosis, oncogenesis, and cancer therapeutics. Biochem Pharmacol. 2019;162:250–61.
9. Konopleva M, Pollyea DA, Potluri J, Chyla B, Hogdal L, Busman T, et al. Efficacy and biological correlates of response in a phase II study of venetoclax monotherapy in patients with acute myelogenous leukemia. Cancer Discov. 2016;6(10):1106–17.
10. Niu XJ, Zhao JY, Ma J, Xie CZ, Edwards H, Wang G, et al. Binding of released Bim to Mcl-1 is a mechanism of intrinsic resistance to ABT-199 which can be overcome by combination with daunorubicin or cytarabine in AML cells. Clin Cancer Res. 2016;22(17):4440–51.
11. Luedtke DA, Niu X, Pan Y, Zhao J, Liu S, Edwards H, et al. Inhibition of Mcl-1 enhances cell death induced by the Bcl-2-selective inhibitor ABT-199 in acute myeloid leukemia cells. Signal Transduct Target Ther. 2017;2:17012.
12. Xie C, Edwards H, Caldwell JT, Wang G, Taub JW, Ge Y. Obatoclax potentiates the cytotoxic effect of cytarabine on acute myeloid leukemia cells by enhancing DNA damage. Mol Oncol. 2015;9(2):409–21.
13. Niu X, Wang G, Wang Y, Caldwell JT, Edwards H, Xie C, et al. Acute myeloid leukemia cells harboring MLL fusion genes or with the acute promyelocytic leukemia phenotype are sensitive to the Bcl-2-selective inhibitor ABT-199. Leukemia. 2014;28(7):1557–60.
14. Su YW, Li XY, Ma J, Zhao JY, Liu S, Wang G, et al. Targeting PI3K, mTOR, ERK, and Bcl-2 signaling network shows superior antileukemic activity against AML ex vivo. Biochem Pharmacol. 2018;148:13–26.
15. Uphoff CC, Drexler HG. Detection of mycoplasma contaminations. Methods Mol Biol. 2005;290:13–23.
16. Quentmeier H, Zaborski M, Drexler HG. The human bladder carcinoma cell line 5637 constitutively secretes functional cytokines. Leuk Res. 1997;21(4):343–50.
17. Ma J, Li XY, Su YW, Zhao JY, Luedtke DA, Epshteyn V, et al. Mechanisms responsible for the synergistic antileukemic interactions between ATR inhibition and cytarabine in acute myeloid leukemia cells. Sci Rep. 2017;7:41950.
18. Xie CZ, Edwards H, Xu XL, Zhou H, Buck SA, Stout ML, et al. Mechanisms of synergistic antileukemic interactions between valproic acid and cytarabine in pediatric acute myeloid leukemia. Clin Cancer Res. 2010;16(22):5499–510.
19. Edwards H, Xie CZ, LaFiura KM, Dombkowski AA, Buck SA, Boerner JL, et al. RUNX1 regulates phosphoinositide 3-kinase/AKT pathway: role in chemotherapy sensitivity in acute megakaryocytic leukemia. Blood. 2009;114(13):2744–52.
20. Ge YB, Stout ML, Tatman DA, Jensen TL, Buck SA, Thomas RL, et al. GATA1, cytidine deaminase, and the high cure rate of Down syndrome children with acute megakaryocytic leukemia. J Natl Cancer. 2005;97(3):226–31.
21. Xie CZ, Drenberg C, Edwards H, Caldwell JY, Chen W, Inaba H, et al. Panobinostat enhances cytarabine and daunorubicin sensitivities in AML cells through suppressing the expression of BRCA1, CHK1, and Rad51. PLoS ONE. 2013;8(11):e79106.
22. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev. 2006;58(3):621–81.
23. DiNardo CD, Pratz K, Pullarkat V, Jonas BA, Arellano M, Becker PS, et al. Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia. Blood. 2019;133(1):7–17.
24. Wei A, Strickland SA, Roboz GJ, Hou JZ, Fiedler W, Lin TL, et al. Safety and efficacy of venetoclax plus low-dose cytarabine in treatment-naive patients aged $\geq$65 years with acute myeloid leukemia. Blood. 2016;128(22):102.
25. Creutzig U, Zimmermann M, Reinhardt D, Rasche M, von Neuhoff C, Alpermann T, et al. Changes in cytogenetics and molecular genetics in acute myeloid leukemia from childhood to adult age groups. Cancer. 2016;122(24):3821–30.
26. Zhao JY, Niu XJ, Li XY, Edwards H, Wang G, Wang Y, et al. Inhibition of CHK1 enhances cell death induced by the Bcl-2-selective

inhibitor ABT-199 in acute myeloid leukemia cells. Oncotarget. 2016;7(23):34785–99.
27. Xiufeng Z, Haijun Z, Silei B, Manman D, Yong Z, Lian Y, et al. Co-operation of ABT-199 and gemcitabine in impeding DNA damage repair and inducing cell apoptosis for synergistic therapy of T-cell acute lymphoblastic leukemia. Anticancer Drugs. 2019;30(2):138–48.
28. Laulier C, Lopez BS. The secret life of Bcl-2: apoptosis-independent inhibition of DNA repair by Bcl-2 family members. Mutat Res. 2012;751(2):247–57.
29. Drexler HG, Fombonne S, Matsuo Y, Hu ZB, Hamaguchi H, Uphoff CC. p53 alterations in human leukemia–lymphoma cell lines: in vitroartifact or prerequisite for cell immortalization? Leukemia. 2000;14(1):198–206.
30. Sugimoto K, Toyoshima H, Sakai R, Miyagawa K, Hagiwara K, Ishikawa F, et al. Frequent mutations in the p53 gene in human myeloid leukemia cell lines. Blood. 1992;79(9):2378–83.
31. Grundy M, Seedhouse C, Jones T, Elmi L, Hall M, Graham A, et al. Predicting effective pro-apoptotic anti-leukaemic drug combinations using co-operative dynamic BH3 profiling. PLoS ONE. 2018;13(1):e0190682.
32. Kotschy A, Szlavik Z, Murray J, Davidson J, Maragno AL, Le Toumelin-Braizat G, et al. The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. Nature. 2016;538(7626):477–82.
33. Bannister T, Koenig M, He Y, Mishra J, Spicer T, Minond D, et al. ML311: a small molecule that potently and selectively disrupts the protein-protein interaction of Mcl-1 and Bim: a probe for studying lymphoid tumorigenesis, probe reports from the NIH Molecular Libraries Program. Bethesda: National Center for Biotechnology Information; 2010.
34. Li Z, He S, Look AT. The MCL1-specific inhibitor S63845 acts synergistically with venetoclax/ABT-199 to induce apoptosis in T-cell acute lymphoblastic leukemia cells. Leukemia. 2019;33(1):262–6.
35. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. EABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med. 2013;19(2):202–8.
36. Kipps TJ, Eradat H, Grosicki S, Catalano J, Cosolo W, Dyagil IS, et al. A phase 2 study of the BH3 mimetic BCL2 inhibitor navitoclax (ABT-263) with or without rituximab, in previously untreated B-cell chronic lymphocytic leukemia. Leuk Lymphoma. 2015;56(10):2826–33.
37. Roberts AW, Seymour JF, Brown JR, Wierda WG, Kipps TJ, Khaw SL, et al. Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. J Clin Oncol. 2012;30(5):488–96.
38. Wilson WH, O'Connor OA, Czuczman MA, LaCasce AS, Gerecitano JF, Leonard JP, et al. Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. Lancet Oncol. 2010;11(12):1149–59.

Affiliations

Fangbing Liu[1] · Tristan Knight[2,3] · Yongwei Su[1] · Holly Edwards[4,5] · Guan Wang[1] · Yue Wang[6] · Jeffrey W. Taub[2,3] · Hai Lin[7] · Liwei Sun[8] · Yubin Ge[3,4,5]

1 National Engineering Laboratory for AIDS Vaccine, Key Laboratory for Molecular Enzymology and Engineering, The Ministry of Education, School of Life Sciences, Jilin University, Changchun, People's Republic of China

2 Division of Pediatric Hematology/Oncology, Department of Pediatrics, Children's Hospital of Michigan, Detroit, MI, USA

3 Department of Pediatrics, Wayne State University School of Medicine, Detroit, MI, USA

4 Department of Oncology, Wayne State University School of Medicine, 421 E. Canfield, Detroit, MI 48201, USA

5 Molecular Therapeutics Program, Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI, USA

6 Department of Pediatric Hematology and Oncology, The First Hospital of Jilin University, Changchun, People's Republic of China

7 Department of Hematology and Oncology, The First Hospital of Jilin University, Changchun, People's Republic of China

8 Research Center of Traditional Chinese Medicine, The Affiliated Hospital to Changchun University of Chinese Medicine, 1478 Gongnong Street, Changchun 130021, Jilin, People's Republic of China