Venetoclax is a promising agent in the treatment of acute myeloid leukemia (AML), though its antileukemic activity is limited to combination therapies. Mcl-1 downregulation, Bim upregulation, and DNA damage have been identified as potential ways to enhance venetoclax activity. In this study, we combine venetoclax with the dual PI3K and histone deacetylase inhibitor CUDC-907, which can downregulate Mcl-1, upregulate Bim, and induce DNA damage, as well as downregulate c-Myc. We establish that CUDC-907 and venetoclax synergistically induce apoptosis in AML cell lines and primary AML patient samples ex vivo. CUDC-907 downregulates CHK1, Wee1, RRM1, and c-Myc, which were found to play a role in venetoclax-induced apoptosis. Interestingly, we find that venetoclax treatment enhances CUDC-907-induced DNA damage potentially through inhibition of DNA repair. In vivo results show that CUDC-907 enhances venetoclax efficacy in an AML cell line derived xenograft mouse model, supporting the development of CUDC-907 in combination with venetoclax for the treatment of AML.

Introduction

Venetoclax (ABT-199) is an oral, selective Bcl-2 inhibitor that was approved by the Food and Drug Administration (FDA) in November 2018 for use in acute myeloid leukemia (AML) patients aged 75 or over, or for whom has comorbidities which preclude the use of intensive induction chemotherapy, in combination with azacitidine or decitabine (hypomethylating agents), or low-dose cytarabine. We recently reported a reduction in Bcl-2/Bim binding in the presence of venetoclax and a resultant increase in the interaction between Bim and Mcl-1, especially in venetoclax-resistant AML models – a change which facilitates apoptotic evasion. Selective Mcl-1 inhibition appears sufficient to overcome this evasion. Triggering of DNA damage results in downregulation of Mcl-1, with functionally the same results (e.g., a relative excess of pro-apoptotic Bim in relation to the anti-apoptotic Mcl-1 and Bcl-2). We also found that Bcl-2 inhibition with venetoclax...
enhances DNA damage induced by DNA damaging agents in AML cells. Therefore, we hypothesized that simultaneously downregulating Mcl-1, upregulating Bim, and inducing DNA damage can maximally enhance venetoclax-induced cell death.

CUDC-907 (Fimepinostat) is an oral, dual inhibitor of PI3K and histone deacetylases (HDAC) presently under investigation in multiple phase I and II clinical trials in the context of multiple myeloma, solid tumors, and lymphoma (www.clinicaltrials.gov) – in the latter, it carries Fast Track designation from the FDA for use in adults with relapsed or refractory diffuse large B-cell lymphoma (DLBCL) (www.cancer.gov). CUDC-907 also shows promising antileukemic activity against preclinical models of AML, as demonstrated in our most recent studies. This activity appears to be at least partially mediated by the downregulation of key proteins involved in the cellular response to DNA damage such as CHK1 and Wee1, as well as ribonucleotide reductase catalytic subunit M1 (RRM1) and c-Myc. CUDC-907 also decreases Mcl-1 protein and increases Bim protein – findings that have been shown to contribute to this agent’s efficacy in lymphoma and AML. Importantly, these changes occur following downregulation of c-Myc, suggesting that early c-Myc downregulation may be the inciting event in the subsequent alterations in protein expression. Elevated or aberrant c-Myc expression and activation has been shown to be a key factor in AML leukemogenesis. Further, c-Myc has recently been identified as a highly statistically significant prognostic marker in AML, with notably shortened overall survival, event-free survival, and relapse-free survival in those with elevated levels of expression. Therefore, CUDC-907 would be an ideal compound to combine with venetoclax to enhance its antileukemic activity against AML.

To this end, we investigated the combination of CUDC-907 and venetoclax in preclinical in vitro and in vivo models of AML. The combination synergistically induces apoptosis in AML cell lines and primary AML patient samples ex vivo. CUDC-907 treatment enhances venetoclax activity in AML cells by altering Bim and Mcl-1 protein levels, downregulating c-Myc, and reducing DNA damage response proteins Wee1, CHK1, and RRM1. In vivo results show that CUDC-907 enhances venetoclax efficacy in an AML cell line derived xenograft model, suggesting that this combination has potential for the treatment of AML.

Methods

See the Online Supplementary Appendix for a detailed description of the methods.

Clinical samples

Diagnostic blast samples were obtained from the First Hospital of Jilin University. Written informed consent was provided according to the Declaration of Helsinki. This study was approved by the Human Ethics Committee of The First Hospital of Jilin University. Clinical samples were screened for gene mutations by PCR amplification and automated DNA sequencing, and screened for fusion genes by real-time RT-PCR, as described previously. Patient characteristics are shown in the Online Supplementary Table S1. Samples were chosen based on availability of adequate sample at the time the assay was performed.

Annexin V/propidium iodide staining

Apoptosis was determined using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis Kit (Beckman Coulter, Brea, CA, USA), as described. Mean percentage of AnnexinV+/PI- (early apoptotic) and Annexin V+/PI- (late apoptotic and/or dead) ± standard error of the mean (SEM) from one representative experiment is shown.

Colony formation assay

Colony formation assays were carried out as previously described. Cells were treated with venetoclax and CUDC-907, alone or in combination, for 24 hours. Cells were washed three times with PBS, plated in MethoCult (catalog number 04434; Stem Cell Technologies, Vancouver, Canada) and incubated for 10–14 days, according to the manufacturer’s instructions. Colony forming units (CFU) were visualized utilizing an inverted microscope. Colonies containing over 50 cells were counted.

Leukemia xenograft model

Eight-week old immunocompromised triple transgenic NSG-SCID female mice (NSG, JAX #010062, non-obese diabetic scid gamma (NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg(CMV-IL3, CSF2, KITLG)1Eav/MloySzJ; Jackson Laboratory, Bar Harbor ME, USA) were injected intravenously with MV4-11 cells (1x10^6 cells/mouse; 0.2 mL/inj; day 0). On day 3, mice were randomized into No Rx control, 100 mg/kg/inj CUDC-907, 85 mg/kg/inj venetoclax, and 100 mg/kg/inj CUDC-907 + 85 mg/kg/inj venetoclax cohorts (5 mice/cohort). Drugs were prepared in 3% ethanol (200 proof), 1% Tween-80 (polyoxyethylene (20) sorbitan monooleate) and sterile water; all United States Pharmacopeia grade; v/v and administered orally. Mice were treated daily x 8 days given 4 days off and then treated x 6 days. Body weight and condition were assessed 1-2 times a day for the duration of study. Experimental endpoint and efficacy response was determined based on the median day for development of leukemic symptoms (hindleg weakness, >15% weight loss, metastatic spread to internal organs). All mice were provided food and water ad libitum, given supportive fluids and supplements as needed, and housed within an Association for Assessment and Accreditation of Laboratory Animal Care Internationa accredited animal facility with 24/7 veterinary care. In vivo experiments were approved by the Institutional Animal Care and Use Committee at Wayne State University.

Statistical analysis

Differences between treatment groups and/or untreated cells (comparison of the sum of Annexin V positive cells) were compared by pair-wise two-sample t-test or 1-way ANOVA with Bonferroni post hoc test (when comparing differences between three or more groups). Overall survival probability was estimated (Kaplan-Meier method) and statistical analysis was performed using the log-rank test. Statistical analyses were performed utilizing GraphPad Prism 5.0. Error bars represent ± SEM; significance level was set at P<0.05.

Results

CUDC-907 synergizes with venetoclax to induce apoptosis in acute myeloid leukemia cells and shows in vivo efficacy in an acute myeloid leukemia xenograft mouse model

In order to determine if CUDC-907 has the capacity to enhance venetoclax-induced cell death, six AML cell lines were treated with venetoclax and CUDC-907, alone or combined, to determine the extent of the antileukemic
Figure 1. CUDC-907 and venetoclax synergistically induce apoptosis in acute myeloid leukemia cells in vitro and show in vivo efficacy in an acute myeloid leukemia cell line derived xenograft mouse model. (A) Acute myeloid leukemia (AML) cell lines were treated with vehicle control, venetoclax (VEN), CUDC-907 (CUDC), or in combination for 24 hours. Annexin V-FITC/PI staining was assessed by flow cytometry analysis. Mean percent Annexin V+ cells ± standard error of the mean are shown. Combination index (CI) values were calculated using CompuSyn software. ***P<0.001 compared to individual drug treatment. (B) AML cells were treated with CUDC-907 and venetoclax, alone or in combination, for 24 hours. Whole cell lysates were subjected to Western blotting and probed with anti-cleaved caspase 3 (cf-Caspase 3), -PARP (cf-PARP indicated cleaved PARP), or –β-actin antibody. (C) MV4-11 cells (1x10⁶ cells/mouse) were injected intravenously through the tail vein of immunocompromised NSGS mice. Three days post cell injection the mice were randomized (5 mice/group) and treated with vehicle control (3% 200 proof ethanol, 1% polyoxyethylene (20) sorbitan monooleate, and USP water), 85 mg/kg/inj venetoclax, 100 mg/kg/inj CUDC-907, or 85 mg/kg/inj venetoclax plus 100 mg/kg/inj CUDC-907 daily. The mice were treated for 8 consecutive days followed by 4 days off and then an additional 6 days. Body weight was measured on a daily basis and are graphed as mean ± standard error of the mean (panel C). Overall survival probability, estimated with the Kaplan-Meier method, is shown (panel D).
Figure 2. Legend on next page.
activity of the combination (cell line characteristics are shown in the Online Supplementary Table S2). Co-treatment of AML cells with CU DC-907 and venetoclax increased the proportion of Annexin V positive cells (indicative of apoptosis) significantly compared to single treatments. Western blots revealed increased cleavage of caspase 3 and PARP by combined drug treatments compared to single treatments (Figure 1B), demonstrating that the cells underwent apoptosis and further confirming the flow cytometry data. In order to test efficacy and tolerance of CU DC-907 and venetoclax co-treatment in vivo, immunocompromised NSGS mice were inoculated with MV4-11 cells and treated with vehicle control, 85 mg/kg/inj venetoclax, and/or 100 mg/kg/inj CU DC-907. The mice were treated for 8 days, given a 4-day break, and then treated for 6 additional days. The 4-
day break was given out of an abundance of caution since a higher dosed group from a concurrent trial (150 mg/kg CUDC-907) experienced moderate body weight loss (3%). Co-treatment of venetoclax with CUDC-907 prolonged median survival from 33 days for control subjects to 52.5 days with minimal weight loss (Figure 1D-E). Taken together, CUDC-907 enhances venetoclax activity in AML cell lines both in vitro and in vivo.

**Figure 3.** Legend on next page.

**CUDC-907 and venetoclax synergize in primary acute myeloid leukemia cells and co-operatively prevent colony formation of acute myeloid leukemia progenitor cells ex vivo**

In order to enhance the clinical relevance of this study, primary AML patient samples were treated with CUDC-907 and venetoclax at the indicated concentrations for 24 hours. Annexin V/PI staining and flow cytometry analysis...
Figure 3. CUDC-907 upregulates Bim and downregulates Mcl-1 enhancing venetoclax activity against acute myeloid leukemia cells. (A) Acute myeloid leukemia (AML) cells were treated with vehicle control, venetoclax (VEN), CUDC-907 (CUDC), or in combination for up to 24 hours. Flow cytometry analysis of Annexin-V-FITC/PI staining was performed. Results are shown as mean percent Annexin V+ cells ± standard error of the mean (SEM). ***P<0.001 compared to single drug treatments. (B, C) AML cells were treated with vehicle control, venetoclax, CUDC-907, or in combination for 24 hours, and whole cell lysates were subjected to western blotting. Representative western blots are shown. The fold changes for the densitometry measurements, normalized to β-actin and then compared to no drug control, are indicated below the corresponding blot. Bim S, L, and EL indicate Bim short, long, and extra-long isoforms, respectively. (D, E) U937 and MOLM-13 cells were treated for 24 hours with vehicle control, venetoclax, CUDC-907, or in combination. Bcl-2 (left panel) or Bim (right panel) was immunoprecipitated from whole cell lysates. Western blots were probed with anti-Bim, -Bcl-2, or -Mcl-1 antibody. The fold changes for the densitometry measurements, normalized to β-actin and then compared to no drug control, are indicated below the corresponding blot. *Indicates the light chain of Bim or Bcl-2 antibody. (F) U937 cells were infected with NTC- (U937/NTC) or Bim-shRNA (U937/Bim) (panel F) or Precision LentiORF Mcl-1 (U937/Mcl-1) or RFP control (U937/RFP) (panel G) lentivirus particles overnight, then washed and incubated for 48 hours prior to adding puromycin or blasticidin, respectively, to the culture medium. The antibiotic-resistant cells were treated with vehicle control, venetoclax, CUDC-907, or in combination for 24 hours. Whole cell lysates were subjected to western blotting. Bim S, L, and EL indicate Bim short, long, and extra-long isoforms, respectively. The fold changes for the Mcl-1 or Bim densitometry measurements, normalized to β-actin and then compared to no drug treatment control, are indicated (top panel). Annexin V/PI staining and flow cytometry analysis results are shown (bottom panel). ***P<0.001 compared to NTC or RFP.
results demonstrate synergy between the two drugs (CI <0.6) in all of the patient samples collected (with the exception of AML#15, which did not have enough sample to assess synergy), including AML relapse samples (AML#10, AML#12, AML#13, and AML#21; Figures 2A; Online Supplementary Figure S1). In order to determine the effect of the combination on leukemic progenitor cells, colony forming assays were performed using primary AML patient samples. CUDC-907 alone significantly reduced colony forming capacity in all primary AML cells, with the exception of AML#19 (Figure 2B). Venetoclax also significantly reduced colony formation in the primary samples, except AML#20. Combination therapy significantly reduced colony forming capacity in all of the patient samples tested compared to individual drug treatment (Figure 2B). Normal bone marrow mononuclear cells (BMMNC) were also tested to determine whether healthy hematopoietic progenitors would be targeted by CUDC-907 and venetoclax therapy. These treatments did not significantly affect colony forming capacity of the normal BMMNC population.
Figure 4. Venetoclax enhances CUDC-907-induced DNA damage in acute myeloid leukemia cells. (A, B) Acute myeloid leukemia (AML) cells were treated with vehicle control, venetoclax, CUDC-907, or in combination for 16 or 24 hours, and whole cell lysates were subjected to western blotting. Representative western blots are shown. The fold changes for the densitometry measurements, normalized to β-actin and then compared to no drug control, are indicated below the corresponding blot. (C) AML cells were treated as in panels A, B. The levels of RPA32 and γH2AX bound to chromatin were analyzed by western blotting. Densitometry measurements normalized to histone H4 and then compared to vehicle control are presented below the corresponding blot. (D) AML cells and normal bone marrow cells were treated for 16 hours with vehicle control, venetoclax, CUDC-907, venetoclax + CUDC-907, or a positive control (20 μM daunorubicin for 4 hours). Representative alkaline comet assay images for MOLM-13 and AML#11 are shown. Representative images for U937 and normal bone marrow cells are shown in the Online Supplementary Figure S4. Data are graphed as median percent DNA in the tail from three replicate gels ± standard error of the mean (SEM). ns indicates not significant and ***P<0.001. (E) U937 cells were treated with venetoclax in the presence or absence of hydroxyurea (HU) or MK-1775 (MK) for 16 hours and then subjected to the alkaline comet assay (left panel). Data are graphed as median percent DNA in the tail from three replicate gels ± SEM. ns indicates not significant and ***P<0.001. Representative images are shown in the Online Supplementary Figure S5. The treated U937 cells were also subjected to Annexin V/PI staining and flow cytometry analysis (right panel). Mean percent Annexin V+ cells ± SEM are shown. ***P<0.01 compared to single drug treatments. (F) MOLM-13 cells were treated with or without CUDC-907 for 16 hours. The cells were washed with PBS three times and then split, half receiving fresh media and the other half receiving fresh media plus venetoclax. Cells were collected at 0, 4, 8, and 12 hours after addition of venetoclax. Alkaline comet assay results are shown as median percent DNA in tail from three replicate gels ± SEM. **P<0.01 and ***P<0.001 compared to CUDC-907 treatment. Representative images are shown in the Online Supplementary Figure S7.
(Figure 2C), suggesting that the combination spares normal hematopoietic progenitors. In order to further determine the effect of the combination therapy on leukemia progenitor/stem cells, primary AML cells were incubated with or without the human bone marrow stromal cell line HS-5. After allowing the cells to co-incubate for 24 hours, the cells were treated with CUDC-907 and venetoclax, alone or combined, for 24 hours and then measured the effects of these drug treatments on CD34+ AML progenitor/stem cells. CUDC-907 and venetoclax co-operated in inducing Annexin V positivity in these AML progenitor/stem cells in the absence of HS-5 cells (Online Supplementary Figure S2). Interestingly, the combination of CUDC-907 and venetoclax was still able to induce Annexin V positivity in these AML progenitor/stem cells even in the presence of HS-5 cells, though to a lesser extent.

Figure 5. Legend on next page.
Figure 5. CUDC-907 synergizes with venetoclax by targeting Mcl-1 protein stability and transcriptionally regulating Bim, CHK1, Wee1, and RRM1. (A) Acute myeloid leukemia AML cells were treated for 24 h with vehicle control, venetoclax (VEN), CUDC-907 (CU DC), or in combination at the indicated concentrations. Total RNA was isolated and then Mcl-1, Bim, Wee1, CHK1, and RRM1 transcripts were determined by real-time RT-PCR. *P<0.05, **P<0.01, and ***P<0.001 compared to vehicle control. (B, C) MOLM-13 (panel B) and U937 (panel C) cells were treated with vehicle control, venetoclax, CUDC-907, or in combination for 12 hours, washed and then treated with 10 μg/mL cycloheximide for up to 2 hours. Western blots were probed with anti-Mcl-1 or -β-actin antibody. The fold changes for the densitometry measurements of Mcl-1 were normalized to β-actin and then compared to vehicle control. Representative western blots are shown on the left, while densitometry measurements are graphed and shown on the right. ns indicates not significant, *P<0.05, **P<0.01, and ***P<0.001. (D-F) MOLM-13 (panel D), U937 (panel E), and patient sample AML#23 (panel F) cells were treated with vehicle control, venetoclax, CUDC-907, or in combination with or without MG132, at the indicated concentrations, for 24 hours. Western blot analysis of whole cell lysates are shown. The fold changes for the densitometry measurements, normalized to β-actin and then compared to vehicle control, are shown.
CUDC-907 enhances venetoclax activity through upregulation of Bim and downregulation of Mcl-1

In order to begin to elucidate the mechanism of action of combined CUDC-907 and venetoclax, we treated AML cell lines for up to 24 hours to determine when enhancement of apoptosis occurs. Enhancement of venetoclax-induced apoptosis by CUDC-907 was detected as early as 12 hours in MOLM-13 and MV4-11 cells and 16 hours in U937 cells (Figures 3A; Online Supplementary Figure S3A). Based on these results, AML cell lines MOLM-13 and U937 were treated with the indicated concentrations for 16 and 24 hours to determine the effects on Mcl-1, Bim, and Bcl-2 protein levels. Consistent with our previous report, venetoclax treatment resulted in an increase of...
Mcl-1 protein in both cell lines, which was completely abolished by the addition of CUDC-907, even when CUDC-907 treatment alone only moderately downregulated Mcl-1 (Figures 3B; Online Supplementary Figure S3B). We did not detect an obvious increase of Bim after CUDC-907 treatment, though we have previously demonstrated that at higher concentrations increased Bim protein levels could be detected.11 However, in the combination treated samples, Bim levels were substantially increased compared to CUDC-907 treatment alone. Consistent with the cell line data, CUDC-907 treatment reduced Mcl-1 protein, which was further reduced by combined drug treatment in four primary AML samples. As in the MOLM-13 and U937 AML cell lines, venetoclax treatment of AML#1 also resulted in an increase of Mcl-1 protein, again it was completely abolished by CUDC-907 (Figure 3C). In contrast to our cell line data, CUDC-907 alone and its combination with venetoclax caused marked increase in Bim protein. For both AML cell lines and primary patient samples, Bcl-2 protein levels were unaffected.
by treatment with CUDC-907 and venetoclax, alone or in combination. Further, combination treatment resulted in increased cleavage of caspase 3 and PARP in both AML cell lines and primary patient samples, confirming the flow cytometry results shown in Figure 2A; Figure 3A; Online Supplementary Figure S1; Online Supplementary Figure S3A. In order to determine if combined CUDC-907 and venetoclax treatment disrupts the binding of Bim to Mcl-1 and Bcl-2, co-immunoprecipitation of Bcl-2 was performed using both U937 and MOLM-13 cells. CUDC-907 treatment caused increased binding of Bim to Bcl-2, while venetoclax treatment reduced Bim bound to Bcl-2 (Figure 3D-E). Co-immunoprecipitation of Bim revealed that venetoclax treatment increased binding of Bim to Mcl-1. CUDC-907 treatment reduced Bim bound to Mcl-1 in MOLM-15 cells, though not in U937 cells. However, combined treatment reduced both Bim bound to Bcl-2 and prevented increased Bim bound to Mcl-1 induced by venetoclax treatment. Knockdown of Bim (50%) significantly reduced apoptosis induced by CUDC-907 alone and in combination with venetoclax (Figure 3F). Mcl-1 overexpression also partially rescued cells from CUDC-907-induced apoptosis when treated alone and in combination with venetoclax (Figure 3G). Taken together, these results demonstrate that Bim and Mcl-1 play important roles in combined CUDC-907 and venetoclax treatment in AML cells. Further, CUDC-907 and venetoclax reciprocally overcome mechanisms of resistance to single drug treatment.

Venetoclax enhances CUDC-907-induced DNA damage in acute myeloid leukemia cells

Based on our previous study in which we show down-regulation of key DNA repair proteins by CUDC-907 treatment,10 we sought to determine if these changes also occurred in the presence of venetoclax. Consistent with our published work, CUDC-907 downregulated RRM1, CHK1, and Wee1, which was maintained by the combined treatment in both AML cell lines and primary patient samples (Figure 4A-B). CUDC-907 treatment increased chromatin-bound RPA32 and γH2AX, reflecting increased DNA replication stress and DNA damage (Figure 4C). Venetoclax treatment increased chromatin-bound RPA32 and γH2AX in MOLM-13 cells, though not in U937 cells. Combined CUDC-907 and venetoclax treatment resulted in more chromatin-bound RPA32 and γH2AX compared to single drug treatments. Alkaline comet assay results revealed that individual treatment with CUDC-907 induced DNA damage, while combined treatment significantly increased DNA damage compared to individual drug treatment for both AML cell lines and a primary AML patient sample (Figure 4D; Online Supplementary Figure S4). We previously reported that CHK1 plays a role in the mechanism of action of venetoclax in AML cells.7 In order to confirm the roles of Wee1 and RRMI in venetoclax activity, U937 cells were treated with the selective Wee1 inhibitor MK-1775 or the ribonucleotide reductase inhibitor hydroxyurea (HU) alone or combined with venetoclax, and then subjected to comet assays. As shown in Figure 4E and Online Supplementary Figure S5, Wee1 inhibition and HU treatment both induced DNA damage, which was significantly increased by the addition of venetoclax. HU and MK-1775 treatment increased the percent of Annexin V positive cells, which was significantly enhanced by venetoclax. In order to further confirm the role of Wee1, Wee1 was overexpressed in U937 cells (Online Supplementary Figure S6A), which partially prevented apoptosis induced by CUDC-907 alone and in combination with venetoclax (Online Supplementary Figure S6B). Further, overexpression of Wee1 decreased DNA damage induced by CUDC-907 treatment alone and in combination with venetoclax (Online Supplementary Figure S6C). These results show that Wee1 plays a role in apoptosis induced by CUDC-907 or combined CUDC-907 and venetoclax treatment in AML cells. In order to determine the effect of venetoclax on DNA damage, MOLM13 cells were treated with CUDC-907 for 16 hours, washed, given fresh media with or without venetoclax for up to 12 hours. Following CUDC-907 treatment, DNA damage decreased, suggesting that repair progressed after removal of CUDC-907. However, addition of venetoclax significantly slowed down the decrease of the percent DNA in the comet tails, indicating inhibition of DNA repair (Figure 4F; Online Supplementary Figure S7). Taken together, these results suggest that venetoclax impairs repair of DNA damage induced by CUDC-907 in AML cells.

CUDC-907 decreases Mcl-1 protein stability and transcriptionally regulates Bim, CHK1, Wee1, and RRMI, enhancing venetoclax activity in acute myeloid leukemia cells

In order to begin to determine if CUDC-907 modulates Mcl-1, Bim, CHK1, Wee1, and RRMI by altering transcription, transcripts were measured via real-time RT-PCR after drug treatment. In AML cell line U937 and primary patient sample AML#23, CUDC-907 treatment, both alone and in combination with venetoclax, significantly increased Mcl-1 transcripts, while in MOLM13 and AML#22 no significant change was detected (Figure 5A). CUDC-907 treatment alone and in combination with venetoclax significantly increased Bim and reduced CHK1, Wee1, and RRMI transcripts in all samples tested. These results suggest that the upregulation of Bim and downregulation of CHK1, Wee1, and RRMI by CUDC-907 treatment is likely through transcriptional mechanisms, while Mcl-1 is not. In order to determine if CUDC-907 and venetoclax have an impact on Mcl-1 protein stability, MOLM-13 and U937 cells were treated with CUDC-907 and venetoclax, alone or in combination, for 16 hours, washed, and then treated with cycloheximide (10 μg/mL) for up to 120 minutes. Western blots revealed that Mcl-1 levels decreased significantly faster in cells treated with CUDC-907 compared to control cells (MOLM13: 68 vs. 82 minutes, P=0.0189; U937: 82 vs. 94 minutes, P=0.0425). Venetoclax treatment significantly increased Mcl-1 half-life (MOLM13: 115 vs. 82 minutes, P=0.0005; U937: 115 vs. 94 minutes, P=0.0061), while combination treatment prevented venetoclax-induced Mcl-1 half-life increase (Figure 5B-C). Proteasome inhibition prevented downregulation of Mcl-1 by CUDC-907 both alone and in combination with venetoclax in AML cell lines and a primary AML patient sample (Figure 5D-F). Since phosphorylation of Mcl-1 at T163 has been shown to stabilize Mcl-1 by prolonging its half-life,12 we looked at phosphorylation of Mcl-1 post-drug treatment. CUDC-907 treatment alone and in combination with venetoclax reduced phosphorylation of Mcl-1 at T163 (Figure 5D-F). Taken together, these results suggest that CUDC-907 downregulates Mcl-1 by decreasing its protein stability in AML cells.
CUDC-907 downregulates c-Myc expression enhancing the antileukemic activity of venetoclax in acute myeloid leukemia cells

Our previous work shows that CUDC-907 inhibits the expression of c-Myc, which is an oncprotein that is frequently activated in AML cells and plays an important role in leukemogenesis. Western blot analyses revealed that CUDC-907 downregulation of c-Myc occurs in the presence or absence of venetoclax in AML cell lines and primary AML patient sample AML#24 (Figure 6A). c-Myc transcript levels were significantly reduced by CUDC-907 treatment alone and in combination with venetoclax (Figure 6B). In order to confirm the functional role of c-Myc, we transiently overexpressed c-Myc in U937 cells. Overexpression of c-Myc significantly reduced the effects of CUDC-907 alone and in combination with venetoclax (Figure 6C). Treatment with the c-Myc inhibitor 10058-F4 significantly enhanced venetoclax-induced apoptosis (Figure 6D). Further, c-Myc inhibition induced DNA damage that was further enhanced by combination with venetoclax (Figure 6E). These results indicate that c-Myc downregulation by CUDC-907 plays an important role in the enhancement of venetoclax-induced death of AML cells.

Discussion

We previously reported that Mcl-1 and Bim are important for the antileukemic activity of venetoclax and are also important for CUDC-907 activity in AML cells. In this study, we found that CUDC-907 treatment decreases Mcl-1 protein, overcoming a mechanism of resistance to venetoclax. Our previous study showing that inhibition of Mcl-1 enhances the antileukemic activity of venetoclax further supports that downregulation of Mcl-1 by CUDC-907 plays an important role in the combined antileukemic activity of CUDC-907 and venetoclax. Venetoclax prevents Bcl-2 from sequestering Bim, overcoming a mechanism of resistance to CUDC-907. In agreement with our previous study, Bim transcripts were increased after treatment with CUDC-907 alone and in combination with venetoclax (Figure 5A). Further, CUDC-907 treatment decreased Mcl-1 protein stability, which does not appear to be effected by venetoclax treatment. While Mcl-1 and Bim play important roles in CUDC-907 and venetoclax activity, knockdown of Bim and overexpression of Mcl-1 only partially prevented apoptosis induced by CUDC-907 alone and in combination with venetoclax, suggesting that other mechanisms exist.

In our previous study, we found that CHK1, Wee1, and RRM1 play important roles in the antileukemic activity of CUDC-907 against AML. We have also shown enhanced DNA damage in AML cells treated with combined venetoclax and DNA damaging agents and that CHK1 inhibition enhances the antileukemic activity of venetoclax. Pham and colleagues recently reported that venetoclax treatment activates the DNA damage response in lymphoma cells, though the mechanism is unknown. In agreement, we show that inhibition of Wee1 or RRM1 in combination with venetoclax co-operatively induce DNA damage in AML cells. Further, we found that venetoclax significantly prolonged persistence of CUDC-907-induced DNA damage, suggesting that Bcl-2 plays a role in the DNA repair.

There is preclinical evidence that CUDC-907 downregulates c-Myc in diffuse large B-cell lymphoma. Additionally, we previously reported that CUDC-907 downregulates c-Myc in vitro, in as little as 4 hours, and in vivo. This downregulation is maintained in the presence of venetoclax in vitro (Figure 6A). c-Myc overexpression partially rescues AML cells from both CUDC-907 monotherapy and combination therapy. Further, combination of venetoclax plus the c-Myc inhibitor 10058-F4 results in significant induction of apoptosis, supporting the assertion that c-Myc inhibition is a key mechanism underlying the combination’s synergy. Based on the roles PISK/mTOR and c-Myc play in proliferation, this combination may inhibit cell proliferation in addition to inducing apoptosis. Similar to our results, Cinar et al. reported that the c-Myc inhibitor 10058-F4 enhances venetoclax activity in double-hit and triple-hit lymphoma. Though the mechanism remains to be fully understood, c-Myc inhibition and venetoclax cooperatively induce DNA damage in AML cells (Figure 6E), suggesting that Bcl-2 is involved in the DNA damage response. Our data demonstrates that CUDC-907 can enhance venetoclax activity in vivo. The mice were given a 4-day break out of an abundance of caution since a higher dosed group in a concurrent trial (150 mg/kg CUDC-907, published in ) experienced moderate body weight loss (%). Additionally, treatment was stopped after 14 doses of CUDC-907, again due to the moderate body weight loss in the concurrent trial (150 mg/kg CUDC-907 group, which was completely reversible within 4 days). In hindsight, the body weight loss associated with combination treatment was minimal, suggesting that the 4-day drug holiday may not have been necessary or that we could have potentially given more than 14 doses. Nonetheless, our results show proof-of-concept; the combination of CUDC-907 and venetoclax increased the lifespan of the mice by %, while CUDC-907 and venetoclax alone increased the lifespan by % and %, respectively. Additionally, our unpublished data using the MV4-11-derived xenograft mouse model, shows that cytarabine in combination with venetoclax increased the lifespan of these mice by only % (unpublished data).

In summary, combined CUDC-907 and venetoclax shows great synergistic antileukemic activity against AML cells, at least partially mediated by Bim, Mcl-1, CHK1, Wee1, RRM1, and c-Myc (Figure 6F). Both CUDC-907 and venetoclax are orally available agents, which would simplify their administration in the clinical setting. More importantly, both agents are already in regular clinical use in experimental and non-experimental contexts. Venetoclax has been approved by the FDA for use in the context of AML, and CUDC-907 carries an FDA Fast Track designation for use in relapsed or refractory diffuse large B-cell lymphoma and a phase II trial is presently underway examining its efficacy in patients with MYC-altered relapsed/refractory diffuse large B-cell lymphoma. The results of this study support further development of this promising combination for the treatment of AML.

Contributions

XL, YS, KH, GM, RM, and GW performed the in vitro studies; LP, JK, SHD, and KW performed the in vivo mouse studies; LP, JY, GW, LZ, YW, HL, JWT and YG participated in the design and coordination of the study; XL, YS, KH, GM, HE, TK, LP, JK, SHD, KW, JY, RM, LZ, YW, HL, JWT and YG

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participated in the data analysis and interpretation; KH, GM, HE, TK and YG helped to draft the manuscript. All authors read and approved the final manuscript.

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