TO THE EDITOR:

Inhibiting casein kinase 2 sensitizes acute lymphoblastic leukemia cells to venetoclax via MCL1 degradation

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The treatment resistance and high morbidity associated with conventional chemotherapeutic treatments warrant new therapeutic approaches for B-cell precursor acute lymphoblastic leukemia (BCP-ALL), especially for relapses and adult patients. The selective BCL2 inhibitor venetoclax has demonstrated action against various hematological malignancies, is clinically approved for chronic lymphocytic leukemia, and has also shown remarkable efficacy in treatment of acute myeloid leukemia (AML). BCP-ALL response to venetoclax is heterogeneous, with the highest efficacy in preclinical models of rare molecular subtypes (TCF3-HLF-rearranged ALL3 and hypodiploid ALL4). Functional dependence on BCL2 has been identified as a major determinant of the venetoclax sensitivity of BCP-ALL. However, upstream regulation of BCL2 addiction is not well understood, and suitable targets to increase venetoclax efficacy via combination therapies are needed, to broaden clinical application for BCP-ALL.

To identify novel synergistic partners, we targeted candidate signaling pathways, with and without venetoclax, in BCP-ALL cell lines. This approach identified silmitasertib (CX-4945) as the most promising synergistic combination partner (Figure 1A; supplemental Figure 1A-B). Silmitasertib is a potent, selective, orally bioavailable, small-molecule inhibitor of the growth-stimulating, apoptosis-suppressing serine/threonine kinase casein kinase 2, which is overexpressed in acute and chronic leukemias. Silmitasertib performed well against preclinical leukemia models, and is the first casein kinase 2 inhibitor to enter phase 1/2 clinical trials for solid tumors and multiple myeloma.

BCP-ALL cell lines, representing molecular BCP-ALL subtypes, were treated with venetoclax, silmitasertib, or both before assessing viability (Figure 1A; supplemental Figure 1C). Combining a minimally effective silmitasertib concentration with venetoclax decreased viability across >5 venetoclax concentrations tested, allowing for an up to 99.9% dose reduction of the half-maximal effective concentration (EC50: 697 cell line; supplemental Table 1). This effect was most prominent in cell lines with a lower basal venetoclax sensitivity (NALM-6 and 697; EC50: ~5 μM) compared with cell lines with higher venetoclax sensitivity (HAL-01, NALM-16; EC50: ~0.5 μM). The combination effect analysis, using a Loewe model (Combeneffit)10 on 70 combined drug concentrations in each cell line, confirmed moderate to strong synergism in 4 of 6 cell lines, with up to 37 synergistic combined concentrations (Figure 1B; supplemental Figure 2A, supplemental Table 1). Flow cytometric analysis of annexin A5 and propidium iodide staining confirmed induction of apoptosis as the underlying cause of synergistic viability reduction (Figure 1C), independent of the time point tested (supplemental Figure 2B). To better reflect the biological and clinical heterogeneity of BCP-ALL, we treated 15 patient-derived BCP-ALL xenografts (Figure 1D; supplemental Table 2) and 14 primary patient samples (Figure 1E; supplemental Table 3) ex vivo. Comparison of the combined vs single venetoclax/silmitasertib treatments indicated a significantly reduced viability of BCP-ALL cells isolated from NSG mouse xenografts (Figure 1D; supplemental Figure 3A-B) and...
Figure 1. Synergistic effect of silmitasertib and venetoclax in BCP-ALL models. (A) Viability was assessed in BCP-ALL cell lines (WST-1 assay) 48 hours after treatment with serial dilutions of venetoclax alone or combined with minimally effective silmitasertib concentrations (left). Cell viability after treatment with a minimally effective

**Figure 1.** Synergistic effect of silmitasertib and venetoclax in BCP-ALL models. (A) Viability was assessed in BCP-ALL cell lines (WST-1 assay) 48 hours after treatment with serial dilutions of venetoclax alone or combined with minimally effective silmitasertib concentrations (left). Cell viability after treatment with a minimally effective
significantly increased apoptosis in short-term cultures of samples from patients with primary BCP-ALL (Figure 1E; supplemental Figure 4A-B). Combination effect analyses indicated an additive to synergistic interaction independent of the molecular driver subtype in these ex vivo culture systems (supplemental Figures 3B and 4C-D). BCP-ALL samples with a lower basal venetoclax sensitivity showed stronger synergistic responses (supplemental Figure 4E). Our data confirm the proapoptotic synergism achieved by combining venetoclax and silmitasertib in cell lines and clinically closer, patient-derived BCP-ALL samples and an overall higher susceptibility for synergistic interactions in BCP-ALL cells less responsive to venetoclax.

ALL cells depend on antiapoptotic signaling pathways for their survival, including signaling through the BH3 family members BCL2 or MCL1.13 We used BH3 profiling to assess MCL1 dependencies of xenografts derived from patients with BCP-ALL14 and observed a tendency toward higher MCL1 dependence in the less venetoclax-sensitive samples (Figure 2A), confirming previous reports of functional MCL1 dependence as an intrinsic resistance mechanism of BCL2 inhibition by venetoclax. Along this line, dynamic BH3 profiling5 analyzing the development of dependence on MCL1 in the presence of venetoclax, indicated significantly stronger dependence in a venetoclax-resistant cell line (697) than in a venetoclax-sensitive cell line (HAL-01; P < .05; Figure 2B; supplemental Figure 5). Remarkably, venetoclax treatment by itself upregulated MCL1 in different BCP-ALL cell lines with a dose-dependent increase in expression, which was maintained in the presence of the apoptosis inhibitor Q-VD (Figure 2C; supplemental Figure 6). Conversely, treatment with silmitasertib alone or in combination with venetoclax reduced MCL1 levels in BCP-ALL cell lines in a dose- and time-dependent manner, establishing a model of proapoptotic synergism, wherein venetoclax induces MCL1 upregulation as a mode of intrinsic apoptosis resistance that is blocked by cotreatment with silmitasertib (Figure 2C; supplemental Figures 6 and 7A-B). To confirm that effect, we overexpressed MCL1 in NALM-6 cells, which reduced sensitivity toward single treatments with venetoclax and silmitasertib (Figure 2D-E; supplemental Figure 7C-D) and nearly abolished the synergistic interactions (Figure 2D) of both cell viability/proliferation and induction of apoptosis (Figure 2E). Transcriptional sequencing confirmed the expected inhibition of AKT/mTOR signaling after silmitasertib and combined treatments but did not provide evidence of transcriptional regulation as a major contribution to the synergistic treatment effect (supplemental Figure 8).

On the posttranscriptional level, cotreatment with the proteasome inhibitor MG132 blocked MCL1 downregulation after silmitasertib treatment, suggesting that silmitasertib interfered with MCL1 protein stability (supplemental Figure 9A). Activation of glycogen synthase kinase 3β (GSK3B), via its dephosphorylation at residue serine 9 (S9), has been linked with priming MCL1 for proapoptotic degradation.18,19 We observed reduced GSK3B S9 phosphorylation after silmitasertib treatment alone or in combination with venetoclax (Figure 2C), suggesting that silmitasertib activates GSK3B to prime MCL1 for proapoptodal degradation, which results in synergistic induction of apoptosis when combined with venetoclax in MCL1-dependent cell lines. For functional validation of GSK3B in this context, we created GSK3B knockout clones from NALM-6 cells by CRISPR/Cas9 genome editing. In the absence of GSK3B protein expression, these cell lines retained sensitivity to single venetoclax and silmitasertib treatments which was comparable to NALM-6 wild-type (supplemental Figure 9B-C). However, the synergistic effect of combined treatment was markedly reduced in GSK3B knockouts (Figure 2D), confirming a functional dependency on GSK3B for the synergistic interaction. Our findings support a model in which silmitasertib induces the GSK3B activation that promotes MCL1 degradation by the proteasome to sensitize ALL cells to venetoclax-induced apoptosis. Our findings concur with similar observations made in a preclinical model of AML.20

To validate this synergistic interaction in vivo, we used an established xenograft model in zebrafish embryos,21 where BCP-ALL cells (cell line: SEM) are injected into the pericardium of immunosuppesss zebrafish embryos and bathed in the drug for 72 hours before flow cytometrically evaluating effects on BCP-ALL cells. Analyses showed an enhanced induction of apoptosis (P < .05) after combined silmitasertib/venetoclax treatment (Figure 2F) and confirmed in vivo the silmitasertib-induced downregulation of MCL1 as a functional underpinning of the synergistic treatment effect (supplemental Figure 10).

Various MCL1 inhibitors, in combination with venetoclax have proven effective in preclinical models of AML20 T-ALL,22 non-Hodgkin lymphomas,23 and high-risk BCP-ALL24,25 but none are currently approved for use in patients. In our study, combining the BCL2 inhibitor venetoclax with the casein kinase 2 inhibitor silmitasertib (currently in clinical trials) created synergism that reduced viability and enhanced apoptosis in BCP-ALL cell lines.
Figure 2. Silmitasertib enhances venetoclax-induced apoptosis via the GSK3B-MCL1 axis, and combined treatment is effective in a zebrafish xenograft model. (A) The association of MCL1 dependence assessed by basal BH3 profiling with venetoclax sensitivity. Patient-derived xenograft BCP-ALL samples were exposed ex vivo to the MS1 peptide specifically bound to MCL1, to assess MCL1 dependence (basal BH3 profiling). Spearman’s correlation was used to analyze correlation between venetoclax sensitivity and priming for MS1 (MCL1 dependence). Linear regression and 95% confidence intervals are shown. (B) BCP-ALL cell lines showing low or high...
patient-derived cells, and a zebrafish xenograft model of BCP-ALL. Antiproliferative and proapoptotic effects occurred across different concentration ranges and treatment times, caused mainly by destabilizing the antiapoptotic protein MCL1 via GSK3B (Figure 2G). The strongest synergy was observed in BCP-ALL cell lines and patient samples that were least sensitive to venetoclax, presenting a rationale for circumventing venetoclax resistance and improving efficacy against refractory and relapsed BCP-ALL.

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Figure 2 (continued) venetoclax sensitivity were exposed (4 hours) to venetoclax at their corresponding EC10 concentrations (697, 27 nM; venetoclax/HAL-01, 2 nM venetoclax) followed by exposure to increasing concentrations of the MCL1-specific inhibitor peptide MS1 or vehicle control (1 hour) before cytochrome C release was measured by flow cytometry to determine apoptosis. Data are shown as the mean of duplicates from 3 independent experiments for NALM-6 wild-type and MCL1- and/or GSK3B-depleted cell lines. ANXA5 fluorescence-activated cell sorting signal for NALM-6 wild-type cells treated with increasing concentrations of MS1 (Supplemental Figure 6A) is shown for the MS1 peptide. (G) Proposed mechanism by which silmitasertib sensitizes ALL cells to venetoclax.
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