LP-118 is a novel B-cell lymphoma 2 / extra-large inhibitor that demonstrates efficacy in models of venetoclax-resistant chronic lymphocytic leukemia

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Abstract

Patients with chronic lymphocytic leukemia (CLL) respond well to initial treatment with the B-cell lymphoma 2 (BCL2) inhibitor venetoclax. Upon relapse, they often retain sensitivity to BCL2 targeting, but durability of response remains a concern. We hypothesize that targeting both BCL2 and B-cell lymphoma-extra large (BCLXL) will be a successful strategy to treat CLL, including for patients who relapse on venetoclax. To test this hypothesis, we conducted a pre-clinical investigation of LP-118, a highly potent inhibitor of BCL2 with moderate BCLXL inhibition to minimize platelet toxicity. This study demonstrated that LP-118 induces efficient BAK activation, cytochrome C release, and apoptosis in both venetoclax-naïve and -resistant CLL cells. Significantly, LP-118 is effective in cell lines expressing the BCL2 G101V mutation and in cells expressing BCLXL but lacking BCL2 dependence. Using an immunocompetent mouse model, Eμ-*TCL1*, LP-118 demonstrates low platelet toxicity, which hampered earlier BCLXL inhibitors. Finally, LP-118 in the RS4;11 and OSU-CLL xenograft models results in decreases in tumor burden and survival advantage, respectively. These results provide a mechanistic rationale for the evaluation of LP-118 for the treatment of venetoclax-responsive and -relapsed CLL.

Introduction

In chronic lymphocytic leukemia (CLL), upregulation of the anti-apoptotic protein B-cell lymphoma 2 (BCL2) allows cells to evade apoptosis by sequestering pro-apoptotic proteins containing BCL2 homology 3 (BH3) domains. Venetoclax is a first-in-class BCL2 inhibitor approved for the treatment of CLL in front-line and relapsed settings. In combination with anti-CD20 monoclonal antibodies, venetoclax produces response rates of over 80% and durable remissions.[1,2] However, patients progressing on venetoclax often do so due to one or more resistance mechanisms.[3-9]

One recurring mechanism of venetoclax resistance in CLL is the acquisition of a G101V point mutation in BCL2 resulting in a conformational change in the binding pocket which reduces its binding affinity for venetoclax.[5] Our group and others have identified other novel point mutations in *BCL2* in patients who relapsed with venetoclax therapy, including D103Y/E/V, F104L, L119V and duplication in R107_R110.[3,4,6] Aberrations in the tumor suppressor gene *TP53* are another marker of progression on venetoclax.[9] Lastly, resistance to venetoclax can arise from upregulation of other members

of the BCL2 family, including myeloid cell leukemia-1 (MCL1) through copy number gain or gene upregulation,[7,10] and B-cell lymphoma-extra-large (BCLXL) by protein overexpression.[11-13] Patients resistant to venetoclax can develop multiple resistance mechanisms simultaneously due to clonal evolution.[5,8] These resistance mechanisms suggest that targeting multiple pro-survival BCL2 family members would be of clinical benefit. Despite being attractive targets for venetoclax-resistant CLL, targeting MCL1 or BCLXL has had limited clinical utility due to on-target cardiac toxicity[14-16] or platelet toxicity,[17,18] respectively, with the latter causing dose-limiting thrombocytopenia. However, a recent trial combining venetoclax with low-dose navitoclax showed efficacy in patients with relapsed refractory acute lymphoblastic leukemia (ALL) without evidence of thrombocytopenia, suggesting that modulating BCLXL inhibition may allow successful therapeutic targeting.[19] Here, we characterize and demonstrate the preclinical efficacy of a new selective BCL2 inhibitor with moderate BCLXL targeting, LP-118, designed to minimize platelet toxicity and bypass various venetoclax resistance mechanisms. Most notably, we show that LP-118 is more potent than venetoclax in various BCL2-dependent models and has preclinical efficacy in venetoclax-resistant CLL. Therefore, LP-118 has the potential to treat patients with CLL, including those with venetoclax-relapsed disease, justifying the ongoing phase I trial.

Methods

Drug treatments and cell lines

LP-118 was provided for all studies by Newave Pharmaceutical Inc. Navitoclax (#S1001), venetoclax (#S8048), A-1331852 (#S7801), and Z-VAD-FMK (#S7023) were purchased from Selleckchem. Cell lines source and maintenance are detailed in the *Online Supplementary Methods*.

Biochemical

BCL2 tagged with 6XHIS (BPS Bioscience) and BCLXL (R&D systems) were pre-incubated with compounds for 15 minutes. BAK BH3 peptide labeled with anti-GST antibodies labeled with TAMRA and Tb-cryptate (CisBio) were incubated for one hour (hr) and read on an Analyst HT multimode plate reader (Molecular Devices).

Computational modeling

Docking of LP-118 (PubChem, ID 146663563) on murine BCL2 (UniProt, P10417) or human BCL2 (UniProt, P10415) was modeled using SiteMap module of Schrodinger Maestro Suite. BCL2 mutations (G101V, D103E) were modeled using Mutagenesis in Pymol. Docked molecules were ranked based on GlideScore.

Cytotoxicity assays

Studies and patient samples were used with approval and review by the Ohio State University Institutional Review Board with written consent from patients. BH3 profiling was performed as previously described by Letai *et al.*[20] and run on a CytoFLEX LX (Beckman Coulter). Primary CLL cells were treated with the indicated compounds and for the indicated timepoints, in suspension or co-cultures. For cytotoxicity assays, primary CLL cells were stained with Annexin V-FITC (Leinco), and TMRM (Thermo Fisher) or propidium iodide (PI) (Leinco) to examine apoptosis on the Gallios flow cytometer (Beckman Coulter). Primary CLL cells and RS4;11 cells were treated and stained with near-IR live / dead staining (Thermo Fisher), fixed and permeabilized with BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences), 0.125 μg of primary BAK (TC100) antibody (Enzo, Cat. #BML-SA298-0050) and Dylight 488 conjugated goat anti-mouse secondary antibody (Thermo Fischer, Cat. #35502), and analyzed on Gallios (Beckman Coulter) flow cytometer.

Mouse models

All experiments using animal models were carried out in accordance with the guidelines established by the Ohio State University and the Institutional Animal Care and Use Committee. Adoptive transfer of $CD5^+CD19^+$ cells carried out from spleens of an Eμ-*TCL1* mouse into C57Bl6 mice. At 10% of CLL cells in peripheral blood, mice were dosed daily via oral gavage. Female SCID mice (CB17/Icr-*Prkdc*^{scid}/IcrIcoCrl, Charles River) were engrafted with $1x10^7$ RS4;11 cells, injected subcutaneously on the right flank. At 100-150 mm^3 tumor volume, mice were drugged orally for 28 days. Tumors were measured twice a week then weekly after Day 63. NOG-F and NOG-M mice (Taconic, Albany, NY, USA) were engrafted with $1x10^7$ OSU-CLL cells intravenously. After four days, mice were treated once a day via oral gavage and tracked for overall survival. Early removal criteria are described in the *Online Supplementary Methods*.

Statistical analysis

Experiments with continuous variables were analyzed using mixed effect model, accounting for observational dependencies across treatment conditions, group comparisons, and trend tests IC_{50} obtained through non-linear mixed effect modeling. Skewed data such as cell counts were normalized by log transformation. Other data were analyzed following the scales demonstrated on the figures (subtracting DMSO or standardizing over DMSO). Survival data were analyzed by log rank test. Data were analyzed in SAS 9.4 (SAS Institute, Cary, NC, USA).
See the *Online Supplementary Methods* for more details.

Results

LP-118, a second generation novel BCL2 inhibitor with rationally designed BCLXL inhibition

LP-118 is a BCL2 potent inhibitor with moderate BCLXL inhibition, rationally designed to provide improved antitumor

efficacy relative to navitoclax while sparing platelets to reduce the risk of thrombocytopenia in the clinical setting. This was achieved through rational structure-based design using the platelet-sparing structure of venetoclax as the starting scaffold (Figure 1A, *Online Supplementary Figure S1A*). We tested whether LP-118 is a genuine BH3 mimetic for BCL2 and BCLXL. For such classification, compounds must occupy the BH3 binding pocket of anti-apoptotic proteins and inhibit their interaction with pro-apoptotic proteins, such as pore-forming protein BAK. A cell-free Fluorescence Resonance Energy Transfer (FRET) competitive assay was performed to evaluate the ability of LP-118 to displace BAK from BCL2, BCLXL, or MCL1. This *in vitro* biochemical study with LP-118 shows strong targeting of BCL2 (Figure 1B) with IC_{50} of 0.25 nM, compared to 0.34 nM for venetoclax and 0.75 nM for navitoclax. The BCLXL affinity of LP-118 (Figure 1C) is in-between navitoclax and venetoclax (IC_{50} = 3.76 nM, 0.9 nM and 34 nM, respectively; LP-118 *vs*. navitoclax $P<0.0001$; LP-118 *vs*. venetoclax $P<0.0001$). LP-118 does not inhibit MCL1 (IC_{50}>1 μM) (*Online Supplementary Figure S1B*), denoting selectivity for BCL2 and BCLXL. Furthermore, LP-118 has the highest percentage of inhibition of a recombinant BCL2 G101V peptide (IC_{50}= 1.6 nM, 6-fold shift relative to wild-type [WT]) (Figure 1D), compared to venetoclax (IC_{50}= 19.5nM, $P<0.0001$, 57-fold) and navitoclax (IC_{50}= 33.2 nM, $P<0.0001$, 44-fold). Computational modeling predicts docking of LP-118 at the BH3 binding groove of human BCL2 (*Online Supplementary Figure S1C*). GlideScores, an empirical measure of the free energy of binding, revealed a decreasing order of binding affinity for LP-118 with human BCL2 WT (-6.377 kcal/mol), D103E (-4.476 kcal/mol), and G101V (-3.995 kcal/mol), followed by murine WT BCL2 (-3.313 kcal/mol) (*Online Supplementary Figure S1C, D*).

Three CLL patient samples were collected at the point of clinical relapse during venetoclax treatment. Importantly, using an ion torrent panel of selected genes, we did not detect BCL2 mutations in these patients prior to venetoclax therapy or at the point of relapse on venetoclax (Table 1). This suggests the existence of alternative resistance mechanisms in these samples. To determine the functional dependence of these venetoclax-resistant cells on BCL2-family proteins for survival, we performed intracellular BH3 profiling (iBH3) (Figure 1E).[20-22] Initially, in six treatment-naïve CLL cells (Figure 1F, *Online Supplementary Table S1*), we observe dose-dependent sensitivity for BH3-only activator BIM ($P<0.0001$) and BH3-only sensitizer BAD ($P<0.0001$), indicating priming to undergo apoptosis upon liberation from their anti-apoptotic partner, BCL2, as has been previously established.[23] Unpaired venetoclax-resistant CLL cells (Figure 1G) show apoptotic priming, denoted by sensitivity to BIM peptide treatment ($P<0.0001$), but lack sensitivity to BAD ($P=0.0850$), consistent with resistance to BCL2 inhibition. There was no cytochrome C (Cyt C) release upon exposure to the MS-1 peptide in our cohort ($P=0.3643$). Statistical support for dose-dependent sensitivity to HRK was not established within this limited cohort of venetoclax-relapsed CLL ($P=0.3643$). However, unlike treatment-naïve CLL cells, which uniformly lacked HRK sensitivity, two out of three venetoclax-resistant CLL samples displayed significant Cyt C release upon HRK treatment (*Online Supplementary Figure S1E*) (HRK 1 μM: $P=0.0303$; 5 μM: $P=0.0152$; 10 μM: $P=0.0059$). Our findings suggest that venetoclax-relapsed CLL cells may employ multiple BCL2-family anti-apoptotic proteins to resist apoptosis. While BCL2- or MCL1-selective BH3 sensitizers (BAD and MS-1) may be ineffective, responses to a BCLXL sensitizer (HRK) are heterogenous. Given the retained sensitivity to BIM, the apoptotic threshold may be overcome by simultaneously inhibiting various survival proteins.

To evaluate the effect of *BCL2* mutations such as G101V on the survival dependence of cells on BCL2 family proteins, we conducted iBH3 profiling (Figure 1H) on RS4;11 cells overexpressing (OE) WT or $G101V^{OE}$ (*Online Supplementary Figure S1F*). WT cells are highly primed and BCL2-dependent, as indicated by Cyt C release after incubation with BIM and BAD peptide. Mutant $G101V^{OE}$ cells show decreased priming and reduced sensitivity to BAD ($G101V^{OE}$ *vs*. WT, at 1, 5, 10 μM: BIM $P<0.0001$, 0.1786, 0.7106; BAD $P<0.0001$, 0.01, 0.707). The moderate sensitivity of RS4;11 cells to the BCLXL selective peptide XXA1_Y4eK was not affected by the presence of G101V mutations ($G101V^{OE}$ *vs*. WT, at 1, 5, 10 μM: XXA1_Y4eK: $P=0.09$, 0.1786, 0.8799), and mutant cells remained insensitive to MCL1 inhibition by MS-1 peptide. This suggests that BCL2

Table 1. Clinical history and targeted sequencing panel of chronic lymphocytic leukemia (CLL) primary patient samples pre- and post-venetoclax relapse.

Patient	Sex	IGHV status	N of therapies before venetoclax	Amount of time on venetoclax in months	Relapse during or post-venetoclax treatment	Complex karyotype (≥3 abnormalities)	Mutations							
							Prior to venetoclax treatment				Post-venetoclax relapse			
							BCL2	*BTK*	*TP53*	*PLCG2*	*BCL2*	*BTK*	*TP53*	*PLCG2*
1	M	UM	8	32	During	Yes	No	Yes	No	No	No	Yes	No	No
2	M	UM	8	23	During	No	No	Yes	No	No	No	Yes	Yes	No
3	M	UM	5	46	During	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes

M: male; IGHV: immunoglobulin heavy chain variable region genes; UM: unmutated IGHV; *BCL2*: B-cell lymphoma 2; *BTK*: Bruton tyrosine kinase; *TP53*: tumor protein 53; *PLCG2*: phospholipase C gamma 2.

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Figure 1. LP-118, a novel inhibitor targeting BCL2 and BCLXL. (A) Chemical structure of LP-118. Key structural differences with its precursor, venetoclax, are highlighted in yellow. (B-D) Testing of LP-118, venetoclax, and navitoclax in time-resolved fluorescence energy transfer (TR-FRET) assay with human recombinant peptides. (B) BCL2 (N=3, independent experiments). (C) BCLXL, (N=3, independent experiments). (D) BCL2 G101V mutant (N=3, independent experiments). (E) Diagram of BH3-only peptides (pro-apoptotic proteins) and their binding interactions with anti-apoptotic protein targets.[20,22] (F) iBH3 profiling in treatment-naïve primary chronic lymphocytic leukemia (CLL) patient samples (N=6) showing cytochrome C (Cyt C) release. + Control is 25 μM alamethicin (black). - Control is 0.01 nM PUMA2A (purple). (G) iBH3 profiling in venetoclax-relapsed primary CLL patient samples (N=3). + Control is 25 μM alamethicin. – Control is 0.01 nM PUMA2A (purple). (H) iBH3 profiling in RS4;11 wild-type (WT) BCL2 cells and RS4;11 cells with a WT BCL2 and overexpressing (OE) BCL2 G101V mutation. Data included from 2 independent experiments with 3 replicates. - Control is 100 nM PUMA2A. + Control is 25 μM alamethicin. (A-G) Plots show mean ± Standard Deviation (SD). (H) Plots show mean from each independent experiment. Statistical analysis was performed and analyzed using the linear mixed effect model. ***P*≤0.01, *****P*≤0.0001.

and BCLXL remain excellent targets for G101V mutant cells. Together, these data indicate that LP-118 is a potent BCL2 inhibitor, and can target BCLXL and G101V-mutated BCL2 with higher potency than venetoclax. We predict that LP-118 could be useful in the CLL population of patients regardless of previous venetoclax treatment.

LP-118 is cytotoxic to chronic lymphocytic leukemia cells *in vitro* and leads to mitochondrial cytochrome C release and apoptosis

To mechanistically understand how LP-118 affects apoptosis in CLL cells, we performed BAK activation, Cyt C release, and viability assays. Exposure of treatment-naïve CLL cells to LP-118 for 8 hr activates BAK proteins in the mitochondria at higher levels than venetoclax-treated cells (Figure 2A, 2B-left). Subsequently, at 12 hr, it releases more Cyt C, a marker of mitochondrial outer membrane permeabilization (Figure 2B-middle). To assess whether LP-118 is cytotoxic to primary CLL cells *in vitro*, we treated cells for 18 hr and performed TMRM and Annexin V staining. LP-118 significantly induces more apoptosis (IC_{50} = 0.056 nM) compared to venetoclax (IC_{50} = 3.8 nM) and navitoclax (IC_{50} = 10.09 nM) treatment in treatment-naïve CLL samples (Figure 2B-right) and was also cytotoxic to samples with *TP53* mutations (*Online Supplementary Figure S1G*). To determine whether LP-118 is more potent in cells resistant to venetoclax, we took samples from patients with CLL who clinically progressed with venetoclax. Venetoclax-resistant samples treated with LP-118 show significant increases in BAK transformation (Figure 2A, 2C-left) and more Cyt C release (Figure 2C-middle) than venetoclax, at matched concentrations. We demonstrate that LP-118 (IC_{50} = 0.34 nM) is superior to venetoclax (IC_{50} = 15.96 nM) and navitoclax (IC_{50} = 4.13 nM) at inducing apoptosis of venetoclax-relapsed CLL *in vitro* by TMRM and Annexin V staining (Figure 2C-right). Lastly, since caspase activation marks a late phase in mitochondrial apoptosis, we sought to determine if pan-caspase inhibition by Z-VAD-FMK could impede the cytotoxic effects of LP-118 on CLL cells. As expected, inhibition of caspases does not affect Cyt C release induced by LP-118, reflecting that Cyt C release occurs upstream of caspase activation. Notably, pan-caspase inhibition protects primary CLL cells from the cytotoxic effects of 4 nM LP-118 or venetoclax, as observed at 24 hr by CellTiter-Glo (Figure 2D, E). Taken together, these findings strongly suggest that LP-118 effectively kills CLL cells via mitochondrial apoptosis. The stromal microenvironment provides a survival advantage to CLL cells by means of anti-apoptotic and pro-survival signals including an increase in BCLXL.[12] Therefore, we wanted to investigate how LP-118, venetoclax and navitoclax would perform in the presence of stroma. To mimic the bone marrow niche, we co-cultured patient-derived CLL cells on monolayers of human bone marrow-derived cell lines, HS-5 or NK Tert, for 24 hr. Exposure to HS-5 stroma does not alter the sensitivity of CLL cells to venetoclax, navitoclax nor LP-118 (*Online Supplementary Figure S1H*). Notably, CLL cells co-cultured with NK Tert cells exhibit a downward shift in their sensitivity to venetoclax, from IC_{50} of 0.908 nM without stromal protection to 1.196 nM in co-cultures, and for navitoclax, from 5.90 nM to 15.97 nM (Figure 3A). LP-118 was potent against CLL cells in suspension and in stromal co-cultures, with IC_{50} of 0.081 nM and 0.075 nM, respectively. As observed by immunoblot, NK Tert stroma tended to increase the levels of BCL2 and BCLXL in CLL cells (Figure 3B). Together, these data indicate that LP-118 is cytotoxic to CLL cells that have not received prior treatment, as well as highly pretreated venetoclax-resistant samples, and it has the potential to overcome stromal protection driven by BCLXL upregulation.

LP-118 induces apoptosis in cell lines with the BCL2 G101V mutation or overexpression of BCLXL

We sought to determine whether LP-118 is effective in the presence of the venetoclax-resistant BCL2 G101V mutation. To model this in an isogenic system, BCL2 G101V was overexpressed (OE) in RS4;11 cells. A caveat of this model is that it expresses the G101V mutant construct in addition to endogenous WT BCL2, as confirmed by immunoblot (*Online Supplementary Figure S1F*). Compared to venetoclax, LP-118 produces significantly higher increases in active BAK at 4 hr in WT and $G101V^{OE}$ cells (Figure 4A-left). At 15 hr, LP-118 treatment also significantly releases more Cyt C from WT RS4;11 cells compared to venetoclax. Cells with a BCL2 $G101V^{OE}$ mutation are resistant to venetoclax; however, they release Cyt C following LP-118 treatment (Figure 4A-middle). Consistently, at 72 hr, TMRM / Annexin V staining shows that RS4;11 with WT BCL2 or $G101V^{OE}$ mutant BCL2 are more sensitive to LP-118

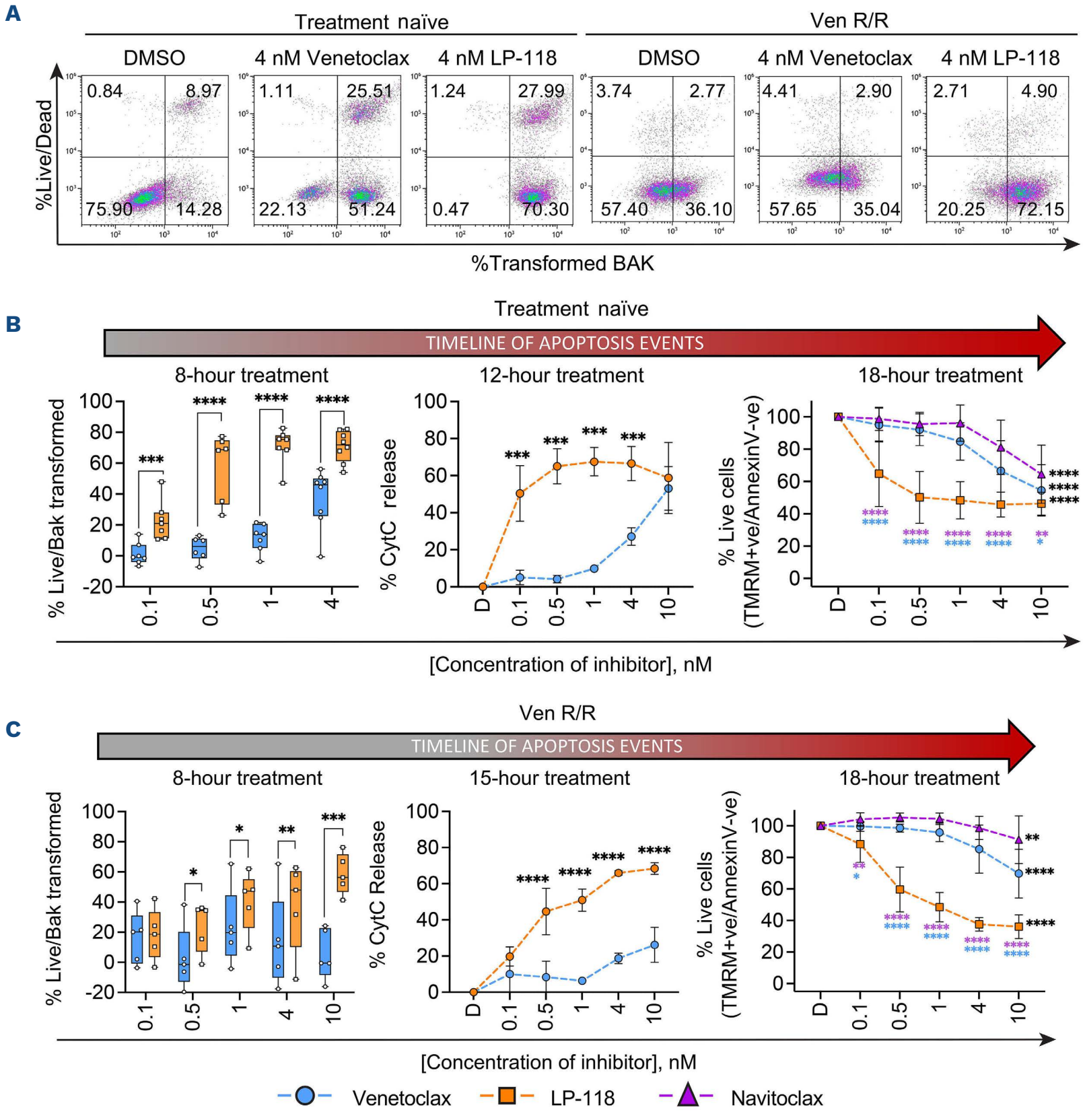

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Figure 2. LP-118 is cytotoxic to treatment-naïve and venetoclax-relapsed chronic lymphocytic leukemia cells. (A) Representative flow cytometry analysis of treatment-naïve chronic lymphocytic leukemia (CLL) cells or venetoclax-relapsed / -refractory (Ven R/R) CLL cells with *in vitro* treatment of DMSO, 4 nM venetoclax or 4 nM LP-118 for 8 hours (hr) and assayed for BAK activation. (B) (Left) Quantification of relative BAK activation in treatment-naïve CLL cells after treatment with 0.1-4 nM venetoclax or LP-118 for 8 hr. Plots display, N=8. ***P<0.001, ****P≤0.0001, mixed effect model. (Middle) Treatment-naïve primary CLL cells treated for 12 hr with concentrations of venetoclax or LP-118 ranging between 0.1 nM to 10 nM and assayed for cytochrome C (Cyt C) release. Plots display mean ± Standard Deviation (SD), N=4. ***P≤0.001, mixed effect model. (Right) Treatment-naïve primary CLL cells were treated with increasing concentrations of venetoclax or LP-118 and stained with Annexin V and TMRM followed by flow cytometry analysis. Plots display mean ± SD, N=7. *P≤0.05, **P≤0.01, ***P≤0.0001. (C) (Left) Quantification of relative BAK activation in relapse/refractory CLL cells after treatment with 0.1 nM to 10 nM venetoclax or LP-118 for 8 hr. Plots display mean ± SD, N=5. *P≤0.05, **P≤0.01, ***P≤0.001, mixed effect model. (Middle) Venetoclax-relapsed primary CLL cells treated for 15 hr with concentrations of venetoclax or LP-118 ranging between 0.1 nM to 10 nM and assayed for Cyt C release. Plots display mean ± Standard Error of Mean, N=3. ****P≤0.0001, mixed effect model. (Right) Venetoclax-relapsed primary CLL cells were treated with increasing concentrations of venetoclax or LP-118 and stained with Annexin V and TMRM followed by flow cytometry analysis. Plots display mean ± SD, N=4. *P≤0.05, **P≤0.01, ****P≤0.0001, mixed effect model. Overall trend analysis is indicated by black stars and individual comparisons are indicated by purple or blue stars. (D and E) Primary CLL cells were treated for 24 hr with DMSO, LP-118 1 nM, LP-118 4 nM, or venetoclax 4 nM, alone (-) or in presence of caspase inhibitor Z-VAD-FMK (+) and assayed for Cyt C release (D) and viability by CellTiter-Glo assay (E). Plots display mean ± SD, N=3 samples treated in triplicate. NS: not significant, *P≤0.05, **P≤0.01, ****P≤0.0001, mixed effect model.

(IC_{50s} = 1.93 nM and 15.67 nM, respectively) than venetoclax (IC_{50s} = 3.58 nM and 1324 nM, respectively) (Figure 4A-right). Together, these data demonstrate that LP-118 effectively induces apoptosis in cells harboring a G101V mutation.

Next, we investigated whether LP-118 can target cells resistant to venetoclax due to BCLXL dependence. Given that OSU-CLL cells express both BCL2 and BCLXL (*Online Supplementary Figure S1 I*), we generated OSU-CLL BCL2 knockout (KO) cells to model this feature. As assessed by CellTiter-Glo at 72 hr, venetoclax is unable to clear BCL2 KO cells, reaching a plateau at 50% killing above 200 nM. In contrast, LP-118 is effective at eliminating BCL2 KO cells with an IC_{50} of 29.5 nM *versus* 0.26 nM for WT cells (Figure 4B). To further characterize the relative potency of LP-118 in cells with increasing dependency on BCLXL, we used a panel of 5 lymphoid cell lines and determined their relative dependencies on BCL2 and BCLXL based on their sensitivity to venetoclax, navitoclax and A-1331852 (BCLXL inhibiting tool compound) at 24 hr of exposure, by CellTiter-Glo. The order of increasing BCLXL dependency is: RS4;11, LOUCY, CCRF-CEM, PF-382, and MOLT4 (Figure 4C). LP-118 (IC_{50} = 0.05 nM) has sub-nanomolar potency in RS4;11, surpassing the activity of venetoclax (IC_{50} = 4.7 nM) and navitoclax (IC_{50} = 42.1 nM), whereas A-1331852 is not effective at killing these BCL2-dependent cells. Notably, LOUCY is moderately sensitive to selective inhibitors of either anti-apoptotic protein (venetoclax IC_{50} = 125.1 nM and A-1331852 IC_{50}= 130.3 nM), and dual inhibitors are most potent at overcoming the apoptosis blockade. LP-118 (IC_{50} = 5.5 nM) surpasses the activity of navitoclax (IC_{50} = 47.4 nM) in this dual BCL2- / BCLXL-dependent cell line. Lastly, LP-118 retained efficacy to clear BCLXL-dependent cells resistant to venetoclax. Consistent with its low BCLXL binding affinity, LP-118 was less potent than navitoclax and A-1331852 in BCLXL-dependent cell lines (CCRF-CEM IC_{50s}= 979.9 nM, 491.9 nM and 13.9 nM, respectively; PF-382 IC_{50}= 812 nM, 378.7 nM and 46.61 nM, respectively; and MOLT4 IC_{50}= 626.8 nM, 290.7 nM and 5.5 nM, respectively). Our results indicate that LP-118 is successful in targeting 2 resistance mechanisms to venetoclax: BCL2 G101V mutations and increased dependence on BCLXL.

LP-118 has limited platelet toxicity *in vitro* and *in vivo*

We investigated whether LP-118 is toxic to human platelets, performing an MTS assay on normal donor platelets. The IC_{50} of LP-118 was determined to be 6360 nM, which is in-between that of venetoclax at 25000 nM and navitoclax at 170 nM (Figure 5A). Although venetoclax is clinically effective in treating patients with CLL, the well-established Eμ-*TCL1* adoptive transfer CLL mouse model is partially sensitive to single-agent venetoclax for short durations,[24] due to high dependence on MCL1.[25] Although this model cannot be used to investigate the efficacy of LP-118, due to lack of BCLXL dependence, we utilized this model which mimics the CLL microenvironment to evaluate T-cell, natural killer (NK)-cell, and platelet toxicity *in vivo*. WT C57BL/6NTac mice engrafted with Eμ-*TCL1* donor splenocytes were treated once daily via oral gavage with either vehicle (N=20), LP-118 (50 mg/kg, N=15), or venetoclax (50 mg/kg, N=15). As expected, all mice had similar peripheral $CD5^+CD19^+$ CLL cells throughout the course of treatment, and similar survival across groups (*Online Supplementary Figure S1J, K*). After six weeks of LP-118 treatment, there were significant decreases in $CD4^+$ (P=0.0434), $CD8^+$ (P<0.0001), and NK cells (P<0.001) compared to vehicle-treated mice, similar to venetoclax-treated mice (Figure 5B-D) suggesting that the effects of LP-118 on non-B-cell populations may be comparable to venetoclax and, therefore, clinically manageable. We found that LP-118 does not decrease platelet counts *in vivo* in these Eμ-*TCL1* engrafted mice (Figure 5E). Furthermore, platelet toxicity of LP-118 was preclinically evaluated in dogs, whose platelet counts normally range between 150 and 500x10^9/L. In a 14-day repeat-dose non-Good Laboratory Practice (GPL) toxicity study, platelet toxicity was induced with concentrations >3 mg/kg for navitoclax and >30 mg/kg for LP-118 (*Online Supplementary Figure S1L*), which corre-

sponds to 10-fold reduced platelet toxicity for LP-118 *in vivo*. No thrombocytopenia was observed for LP-118 doses within the 5 to 20 mg/kg range in a 28-day repeat-dose non-GLP toxicity study (*Online Supplementary Figure S1M*). Lastly, a 25 mg/kg single dose study revealed the maximum concentration of LP-118 in plasma (mean = 8 μg/mL) was reached 10 hr after treatment (*Online Supplementary Figure S1N*). A notable difference between venetoclax and LP-118 pharmacokinetics was seen at 30 hr post treatment where only venetoclax persisted in plasma, suggesting faster clearance for LP-118. In this study, the maximum reduction in platelets was 6%, 17%, and 97% for venetoclax, LP-118, and navitoclax, respectively. Therefore, LP-118 is an ideal candidate for clinical evaluation due to its minimal platelet targeting, and has a toxocity profile similar to that of venetoclax.

Xenograft mouse models depict superiority of LP-118 compared to venetoclax *in vivo*

We wanted to study the efficacy of LP-118 *in vivo*, in cells harboring WT or G101V BCL2. Immunodeficient CB.17 SCID mice were engrafted with RS4;11 cells with WT or G101V^{OE} BCL2. After tumors reached an average size of 113 to 116 mm^3, WT RS4;11 mice were treated orally once daily with vehicle (N=7), 6.25 mg/kg LP-118 (N=7), or 6.25 mg/kg venetoclax (N=7) for 28 days. Mice were removed from study when tumors reached an endpoint volume of 2000 mm^3, or at 77 days, whichever occurred first. Upon stopping treatment, tumors from LP-118 treated mice did not show increased volume, while venetoclax-treated mice relapsed and exhibited increased tumor growth (Figure 6A). Mice treated with LP-118 had a significant survival advantage over vehicle-treated mice (median survival = 31.4 days; LP-118 *versus* vehicle *P*=0.0005) and venetoclax-treated mice (median survival = 59 days; LP-118 *versus* venetoclax *P*=0.0002) (Figure 6B). RS4;11 BCL2 G101V^{OE} cells were engrafted in mice and treated with vehicle, venetoclax (100 mg/kg, q.d., N=7) or LP-118 (50, 100 and 150 mg/kg, q.d., N=7) after reaching a tumor size of 131-134 mm^3. Contrary to venetoclax 100 mg/kg, mice treated with LP-118 at all doses exhibited a decrease in tumor growth (Figure 6C). All doses of LP-118 also resulted in a significant improved overall

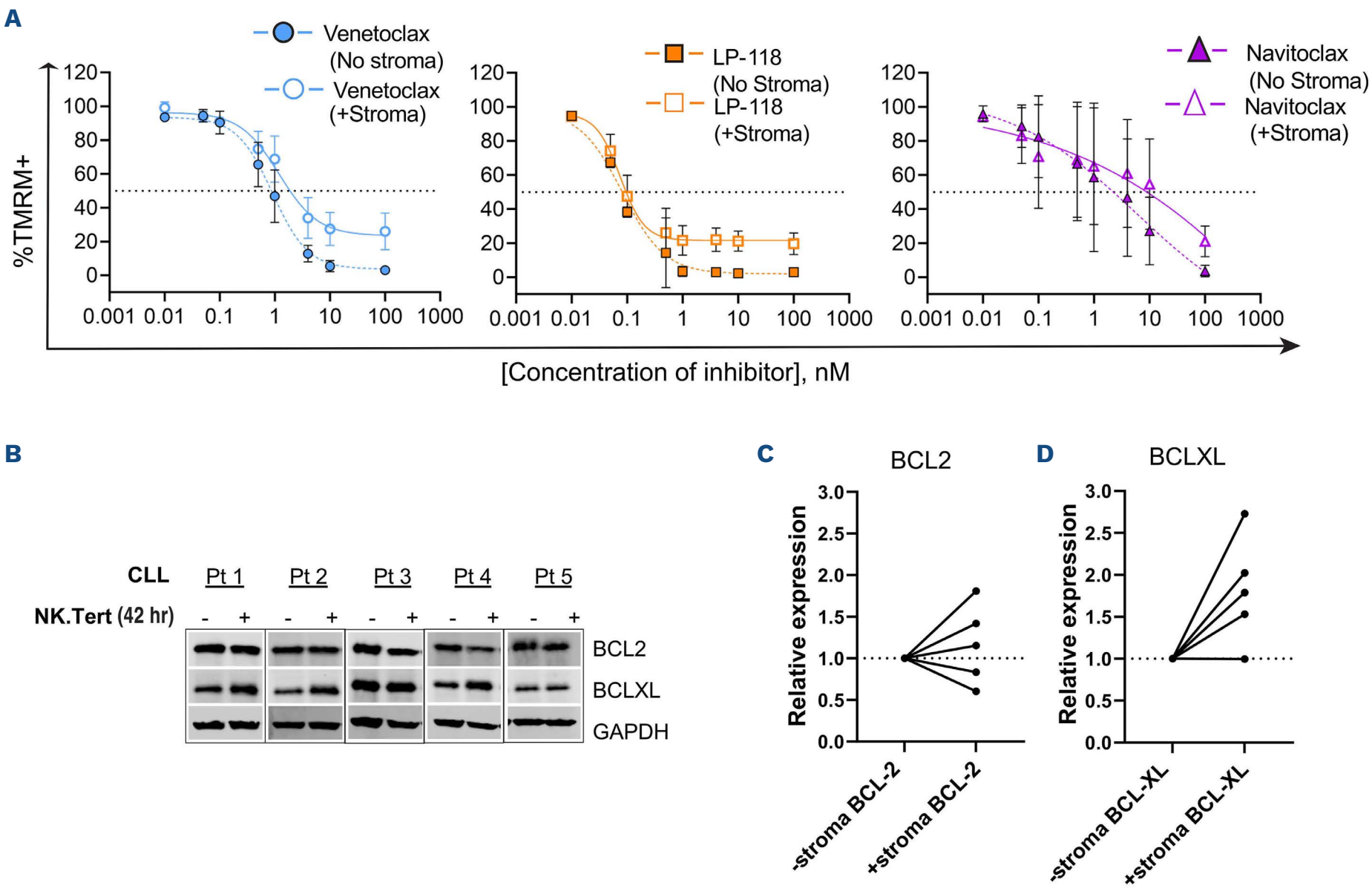
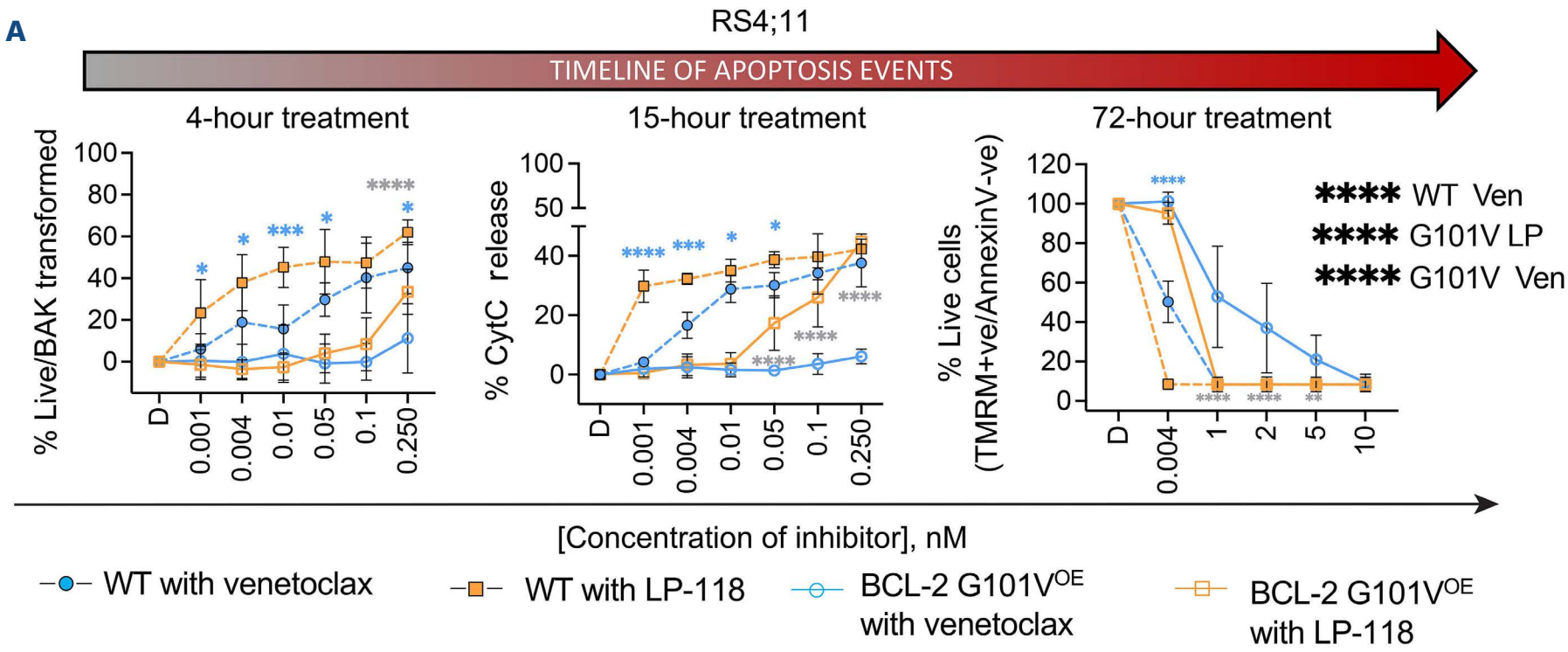


Figure 3. LP-118 induces apoptosis in chronic lymphocytic leukemia cells supported by NK Tert stroma. (A) Representative flow of N=6 primary chronic lymphocytic leukemia (CLL) samples cultured with or without NK Tert stroma with *in vitro* treatment of venetoclax, LP-118, or navitoclax. IC_{50} calculated by Nonlinear Regression. Plots display mean ± Standard Deviation. (B-D) Western blot, and quantification of BCL2 and BCLXL expression normalized to GAPDH, in primary CLL patient samples cultured with or without NK Tert stroma (N=5).



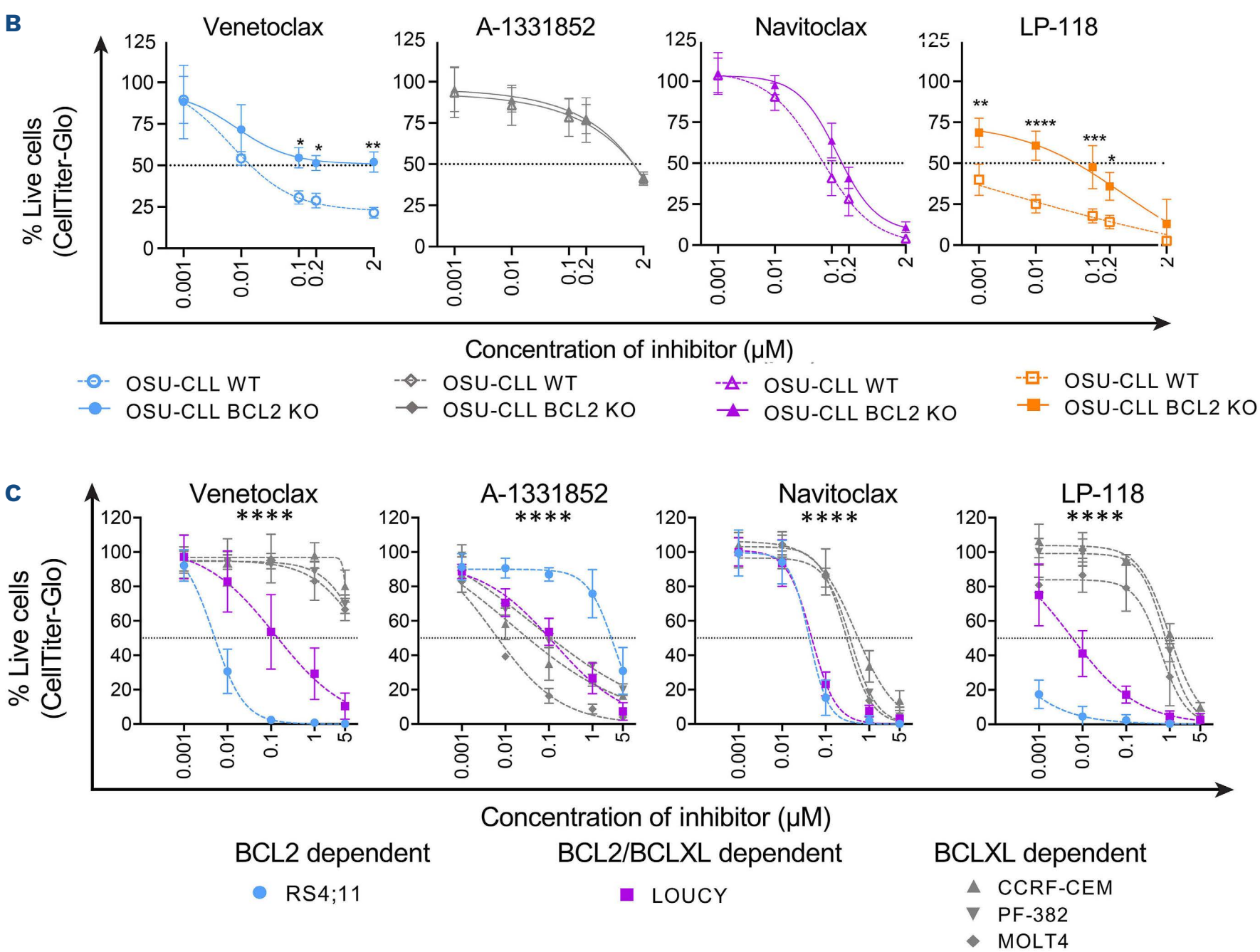


Figure 4. LP-118 targets RS4;11 cells with BCL2 G101V mutation and cells lines without strong BCL2 dependency. (A) RS4;11 cells with wild-type (WT) BCL2 or overexpressing (OE) G101V BCL2 (G101V^{OE}) were treated with increasing concentrations of venetoclax or LP-118. (Left) Cells were treated for 4 hours (hr) and stained with BAK antibody used to determine active BAK conformation and analyzed on a flow cytometer. Plots display mean ± Standard Deviations (SD), N=3 independent experiments. *P≤0.05,

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***P≤0.001, ****P≤0.0001, mixed effect model. Comparisons between the 2 treatment groups with WT BCL2 are in blue and between the 2 treatment groups with BCL2 G101V mutation are in gray. (Middle) RS4;11 cells were collected at 15 hr and stained with cytochrome C (Cyt C) and analyzed on a flow cytometer. Plots display mean ± SD, N=3 independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, mixed effect model. Comparisons between the 2 treatment groups with WT BCL2 are in blue and between the two treatment groups with BCL2 G101V mutation are in gray. (Right) Cells were collected at 72 hr and stained with Annexin V-FITC/TMRM for flow cytometry. Plots display mean ± SD, N=3 independent experiments. **P≤0.01, ****P≤0.0001, mixed effect model. Overall trend analysis is indicated by black stars and individual comparisons are indicated for comparisons between the 2 treatment groups with WT BCL2 in blue, and between the two treatment groups with BCL2 G101V mutation in gray. (B) OSU-CLL WT and BCL2 knockout (KO) cell lines were treated with venetoclax, A-1331852, navitoclax, or LP-118 for 72 hr, followed by CellTiter-Glo viability assay. Plots display mean ± SD, N=3 independent experiments in triplicate. *P≤0.05, **P≤0.01, mixed effect model. (C) RS4;11 (N=2), LOUCY (N=3), CCRF-CEM (N=3), PF-382 (N=3), and MOLT4 (N=3) cell lines were treated with venetoclax, A-1331852, navitoclax, or LP-118 for 24 hr, followed by CellTiter-Glo viability assay. Plots display mean ± SD from the indicated numbers of independent experiments, in triplicate. ****P≤0.0001, mixed effect model.

survival of mice *versus* venetoclax (50 mg/kg P=0.0104, 100 mg/kg P= 0.0012, 150 mg/kg P=0.0005) (Figure 6D). Taken together, both studies show that, at the same doses, LP-118 is more potent than venetoclax in reducing tumor burden and prolonging survival of mouse models of leukemia harboring either WT or G101V BCL2.

Next, we evaluated whether LP-118 is potent in a CLL cell line xenograft. Immunodeficient NOG mice were engrafted with OSU-CLL cells via tail vein injection. Four days post engraftment, we initiated daily treatment with vehicle (N=8), LP-118 (50 mg/kg, N=12), or venetoclax (50 mg/kg, N=8). Splenic involvement with leukemia was confirmed by flow

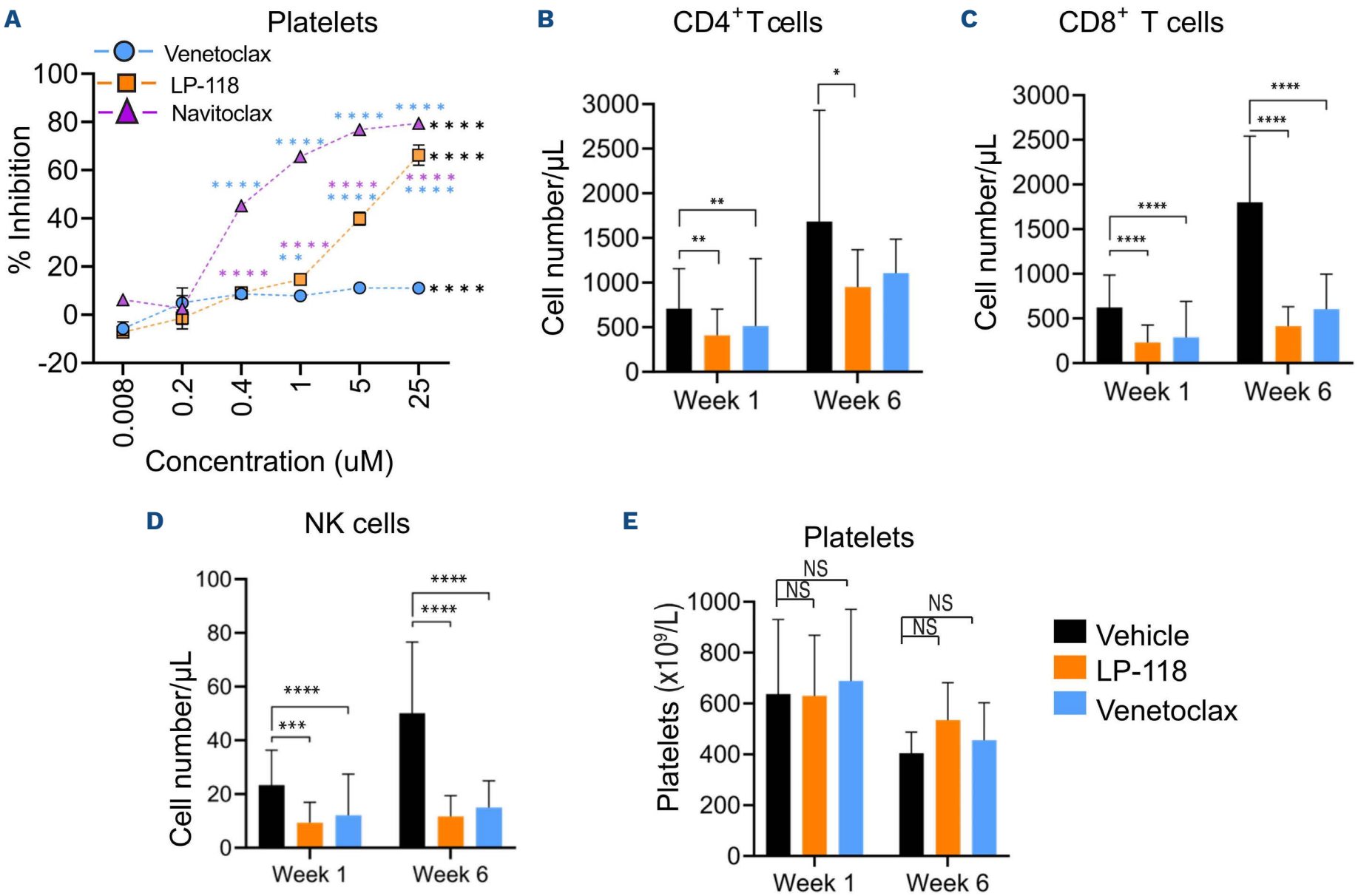

Figure 5. Effects of LP-118 on other immune cell types. (A) Human platelets were isolated and treated with venetoclax, LP-118 or navitoclax for 72 hours (hr). Inhibition of platelets was determined by MTS assay. Plots display mean±SD, N=3 healthy donors. ***P<0.001, ****P≤0.0001, mixed effect model. (B-D) Blood from Eμ-*TCL1* mice were stained for CD4+ T cells (B), CD8+ T cells (C), or NK cells (D) at week 1 and week 6 post treatment and analyzed via flow cytometry to identify the subsets of immune cells. Plots display mean±SD. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, mixed effect model. (E) Blood from Eμ-*TCL1* mice was also used and analyzed for platelet count using Complete Blood Count (CBC) machine. Plots display mean ± SD. *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, mixed effect model.

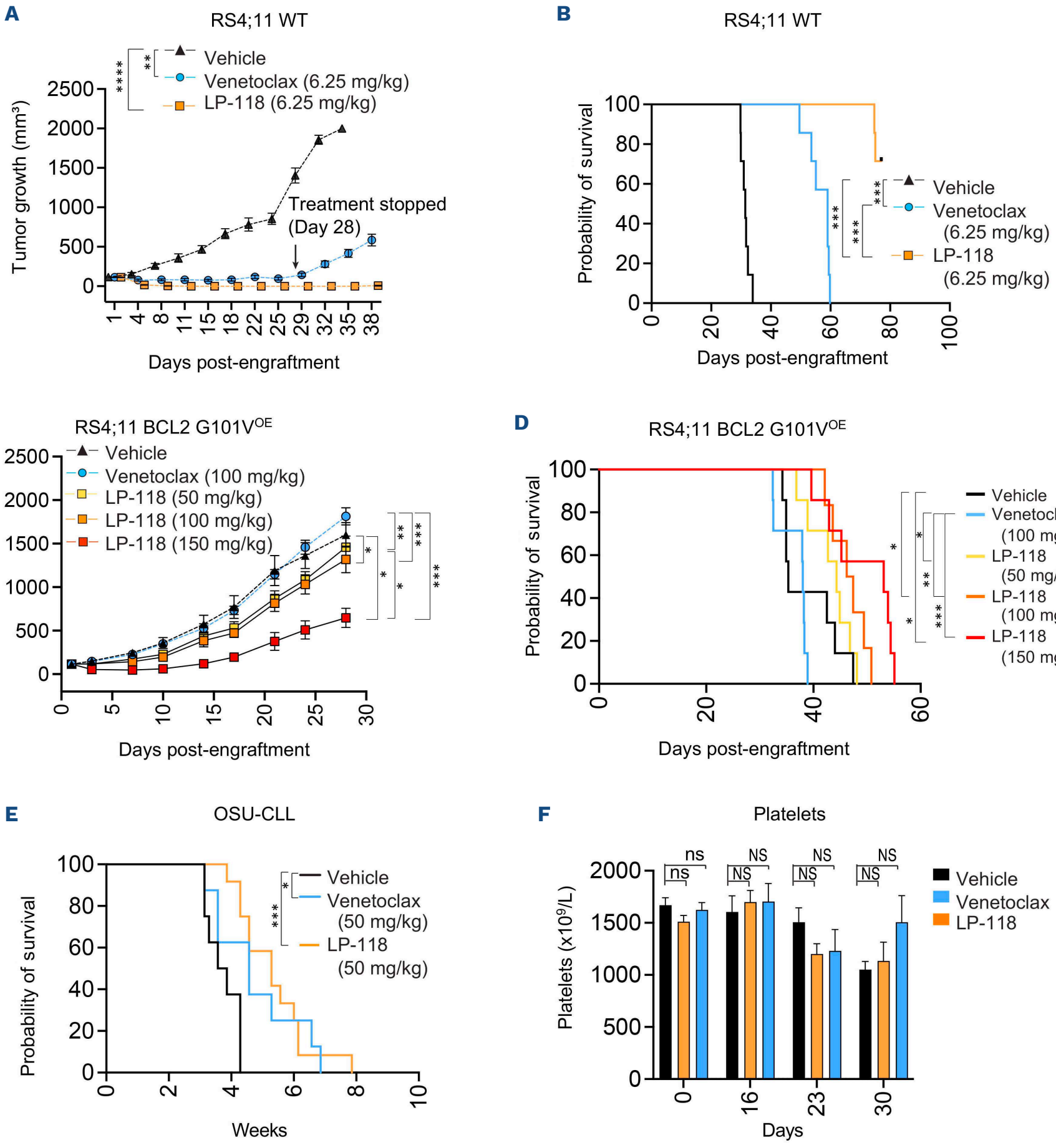

Figure 6. LP-118 decreases tumor size in RS4;11 xenograft and improves survival in OSU-chronic lymphocytic leukemia xenograft models. (A) NOG mice were engrafted with RS4;11 wild-type (WT) BCL2 cells and monitored for tumor volume. Data were cubic root transformed to minimize variance. Mixed effect model was used to compare tumor sizes among groups (**0.01≥*P*≥0.001, ****$P \leq 0.0001$). (B) Survival curve of NOG mice engrafted with RS4;11 WT BCL2 and treated with vehicle, venetoclax (6.25 mg/kg), or LP-118 (6.25 mg/kg). Log-rank Mantel-Cox test was performed (***$P \leq 0.0002$). (C) NOG mice were engrafted with RS4;11 cells overexpressing G101V mutant BCL2 (G101V^{OE}), treated with vehicle, venetoclax (100 mg/kg), or LP-118 (50, 100 or 150 mg/kg), and monitored for tumor volume. Data were cubic root transformed to minimize variance. Mixed effect model was used to compare tumor sizes among groups (*0.05≥*P*>0.01, **0.01≥*P*≥0.001, ***$P < 0.001$). (D) Survival curve of NOG mice engrafted with RS4;11 G101VOE BCL2 and treated with vehicle, venetoclax (100 mg/kg), or LP-118 (50, 100 or 150 mg/kg). Gehan-Breslow-Wilcoxon test was performed (*0.05≥*P*>0.01, **0.01≥*P*≥0.001, ***$P < 0.001$). (E) NOG mice were engrafted with OSU-CLL and monitored for survival until the endpoint of hind limb paralysis. Log-rank Mantel-Cox test was performed. (F) Complete blood cell (CBC) count of OSU-CLL engrafted NOG mice were used to determine platelet counts. Mixed effect model was used to compare among groups. NS: not significant, *0.05≥*P*>0.01, **0.01≥*P*≥0.001, ***$P < 0.001$. Ven: venetoclax; LP: LP-118.

cytometry at the end point, determined by hind limb paralysis.[26] Compared to vehicle, LP-118 and venetoclax significantly improved the survival of mice (P=0.0002) (Figure 6E). LP-118- and venetoclax-treated mice also maintained similar platelet counts throughout the course of treatment (Figure 6F). The data presented here indicate that LP-118 increases survival and decreases tumor growth in 2 leukemia xenograft models and can target cells harboring G101V mutant BCL2 *in vivo*.

Discussion

We characterized LP-118, a novel, highly potent, orally bioavailable, selective BCL2 inhibitor that also moderately targets BCLXL and can potentially overcome several venetoclax resistance mechanisms. LP-118 retains high binding affinity to WT BCL2 and is a more potent inhibitor of G101V mutant BCL2 than venetoclax. It shows improved platelet-sparing capability than navitoclax in human platelets *ex vivo* and Eμ-*TCL1* mice *in vivo*.

There are multiple mechanisms of venetoclax resistance that have been well-characterized. We showed that LP-118 is highly potent at inducing mitochondrial apoptosis in both treatment-naïve and venetoclax-relapsed CLL cells. BH3 profiling revealed that venetoclax-relapsed CLL may lose sensitivity to BAD peptide, which binds most tightly to BCL2. The observation that BCLXL targeting peptide HRK initiated apoptosis in some venetoclax-relapsed cells, but not in treatment-naïve CLL, suggests increased dependence on BCLXL at relapse. We also showed that LP-118 can induce apoptosis of venetoclax-resistant cell lines with high dependence on BCLXL, or with G101V BCL2 mutations. Additional studies are required to assess its potency against other BCL2 mutations, such as D103E which makes BCL2 more structurally similar to BCLXL.[3,4] *in vivo*, LP-118 improves the survival of RS4;11 WT, RS4;11 G101V^{OE} BCL2 and OSU-CLL xenografts. These results highlight the preclinical efficacy and success of LP-118 and warrant clinical evaluation to determine its potential to treat front-line or venetoclax-relapsed CLL.

A recent study described the few treatment options for patients who progress on venetoclax, and the limited and relatively ineffective therapeutic interventions for patients refractory to both BCL2 and BTK inhibitors.[27] Inhibitors of multiple BH3 proteins are attractive to overcome venetoclax resistance and must be carefully designed to limit on-target toxicities. For example, targeting MCL1 amplification and overexpression, venetoclax resistance factors in lymphoid malignancies,[7] will require strategies to circumvent cardiac side effects[14,15] such as CDK9 inhibition.[28] Clinical evaluation of first generation BCL2 / BCLXL inhibitors like navitoclax found on-target platelet toxicity leading to concentration-dependent thrombocytopenia that limit the clinical utility of these inhibitors.[17,18] LP-118 has low platelet toxicity, similar to venetoclax *in vitro* and *in vivo*, in Eμ-*TCL1* mice and dogs. Therefore, we predict LP-118 to have clinically manageable side effects.

Dependencies on various antiapoptotic proteins of the BCL2 family have been identified in a wide range of hematologic malignancies and solid tumors; therefore, LP-118 may also be of clinical interest in other cancers. Venetoclax has been approved by the US Food and Drug Administration for both CLL and acute myeloid leukemia (AML) and is currently under investigation for other non-Hodgkin lymphomas, T-cell lymphoma, acute lymphoblastic leukemia (ALL), and solid tumors. Moreover, in some solid tumors such as breast cancer[29] and colon cancer,[30] BCLXL is highly associated with resistance to therapy. Therefore, the high potency and broader activity of the BCL2 / BCLXL inhibitor LP-118 may have many areas of potential impact, surpassing more selective BH3 mimetics. These areas include ALL and venetoclax-resistant T-cell leukemia, as evidenced by the efficacy shown in RS4;11 and MOLT4 cells, respectively, among other cell lines.

While the preclinical data are encouraging, clinical trials will be required to determine whether the preclinical advantages seen with LP-118 compared to venetoclax are relevant in patients. Pharmacokinetics and platelet toxicity must be monitored carefully. These preclinical data support the ongoing phase I evaluation of LP-118 in CLL, as well as other hematologic malignancies.

Disclosures

JAW received research support from Verastem, Karyopharm, Morphosys, and Schrodinger, and has consulted for Pharmacyclics, Janssen, AstraZeneca, Arqule, Abbvie, Beigene, Loxo, Newave, and Genetech. DS is on the advisory board for Newave Pharma. KAR received research funding from Genentech, AbbVie, Janssen, and Novartis, consults for Genentech, AbbVie, AstraZeneca, Innate Pharma, Pharmacyclics, and Beigene, and received travel funding from Beigene. JAW and KAR are clinical scholars of the Leukemia and Lymphoma Society. ASK consulted for Abbvie, Beigene, Bristol-Myers Squibb, and Janssen. MSD reports receiving grant support paid to his institution and consulting fees from Ascentage Pharma, Astra-Zeneca, BMS, Genentech, MEI Pharma, Pharmacyclics, TG Therapeutics, and Verastem, and reports grant support paid to his institution from Surface Oncology, and consulting fees from AbbVie, Adaptive Biotechnologies, Aptitude Health, BeiGene, Celgene, Eli Lilly, Janssen, Merck, Research to Practice, and Takeda. JCB consulted for Acerta, AstraZeneca, Pharmacyclics, Astellas, Syndax, and Jazz Pharmaceuticals, and reports stock ownership in Vincerx Pharmaceutics. SAB consulted for Pharmacyclics, Janssen, Beigene, and Acerta / AstraZeneca. RL has membership on the Vincerx Pharma Inc. Board of Directors or advisory committees. YiC, SPA and YuC are employed by and hold a position on the Board of Directors of Newave Pharmaceutical Inc., the maker of LP-118, and report stock ownership in Newave Pharmaceutical Inc. YiC holds patents 10456397, 10377755, 10253029, 10239872, 10195200. YS and FT are em-

ployed by and hold a position on the Board of Directors of Lupeng Pharmaceutical Inc., and report stock ownership in Lupeng Pharmaceutical Inc.

Contributions

JR is responsible for conceptualization, methodology, investigation, formal analysis, visualization, and writing the original draft. DYDR is responsible for methodology, investigation, formal analysis, visualization, writing, reviewing and editing. EM is responsible for methodology, investigation, formal analysis, writing, reviewing and editing. THL, XM and SM are responsible for investigation, methodology, writing, reviewing and editing. CDW, JRS, AM, CL and AL are responsible for investigation. SS is responsible for computational modeling. EP and TK are responsible for investigation, methodology. GL is responsible for resources and investigation. KAR, ASK, NJ and WGW are responsible for resources, writing, reviewing and editing. SAB is responsible for resources. MCC is responsible for methodology. MSD is responsible for methodology, writing, reviewing and editing. RL is responsible for writing, reviewing and editing. JCB is responsible for supervision, writing, reviewing and editing. FT and YiC are responsible for conceptualization, methodology, resources, funding acquisition, supervision, writing, reviewing and editing. YuC and SPA are responsible for conceptualization, methodology, resources, supervision, writing, reviewing and editing. YS is responsible for conceptualization, methodology, supervision, writing, reviewing and editing. JAW and DS are responsible for conceptualization, funding acquisition, methodology, resources, supervision, visualization, and writing the original draft.

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Data-sharing statement

Any relevant and original data are available from the corresponding authors upon request.

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