Venetoclax and dinaciclib elicit synergistic preclinical efficacy against hypodiploid acute lymphoblastic leukemia

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Abstract

Hypodiploid acute lymphoblastic leukemia (ALL) is an aggressive blood cancer with a poor prognosis despite intensive chemotherapy or stem cell transplant. Children and adolescents with positive end-of-induction minimal residual disease have an overall survival lower than 30%. However, data regarding therapeutic alternatives for this disease is nearly non-existent, emphasizing the critical need for new or adjunctive therapies that can improve outcomes. We previously reported on the therapeutic efficacy of venetoclax (ABT-199) in hypodiploid B-lineage ALL but with limitations as monotherapy. In this study, we set out to identify drugs enhancing the anti-leukemic effect of venetoclax in hypodiploid ALL. Using a high-throughput drug screen, we identified dinaciclib, a cyclin-dependent kinase inhibitor that worked synergistically with venetoclax to induce cell death in hypodiploid cell lines. This combination eradicated leukemic blasts within hypodiploid ALL patient-derived xenografts mice with low off-target toxicity. Our findings suggest that dual inhibition of BCL-2 (venetoclax) and CDK9/MCL-1 (dinaciclib) is a promising therapeutic approach in hypodiploid ALL, warranting further investigation to inform clinical trials in this high-risk patient population.

Introduction

The outcome of therapy for childhood acute lymphoblastic leukemia (ALL) has shown significant improvement over time, with expected cure rates exceeding 85%.[1] However, despite intensive risk-adapted chemotherapy regimens, children with certain ALL subtypes continue to have very poor prognosis.[2] Hypodiploid ALL with fewer than 44 chromosomes presents a distinct genetic profile[3] and high relapse rate, particularly in patients who are slow to respond to induction.[2,4,5] Hypodiploid ALL is subclassified based on karyotype into high-hypodiploid (HH; 40-43 chromosomes), low-hypodiploid (LH; 32-39 chromosomes), and near-haploid (NH; 24-31 chromosomes), with an 8-year overall survival (OS) of 50%, 40%, and 34% respectively.[6] For patients with minimal residual disease (MRD) >0.01%, the 5-year event-free survival (EFS) is 27-29% and has not improved in children undergoing allogeneic hematopoietic cell transplantation (HCT).[2,4,5] These poor outcomes emphasize the need to identify curative therapies. Furthermore, the high rate of germline *TP53* mutations in LH B-lineage ALL (B-ALL) and its association with the cancer-predisposing Li-Fraumeni syndrome requires careful consideration, as such mutations may blunt the efficacy of therapies exerting p53-mediated mechanisms and increase potential acute and late toxicities of current cytotoxic therapies.[3]

In our previous study, we identified that the survival protein BCL-2 is significantly upregulated in hypodiploid leukemia.[7] Through *in vivo* studies, we demonstrated the

significance of BCL-2 as a viable therapeutic target.[7] We demonstrated rapid clearance of leukemic blasts from peripheral blood (PB), few off-target toxicities, and marked improvement in OS of patient-derived xenograft (PDX) mice treated with venetoclax (ABT-199) monotherapy, with survival of 85% *versus* only 15% in untreated mice. Subsequent clinical trials in adult patients with hematologic malignancies have shown the high therapeutic potential of venetoclax.[8-13] Through a multicenter collaboration, our studies contributed to the opening of the first phase I study of venetoclax combined with chemotherapy in pediatric patients with relapsed or refractory acute leukemias and other cancers (*clinicaltrials gov. Identifier: NCT03181126*).[8] However, our studies also demonstrated that venetoclax monotherapy was limited by persistence of leukemic blasts in liver and spleen, as well as positive minimal residual disease (MRD) in the bone marrow (BM) of most mice at end-of-therapy.

In order to inform a therapeutic approach that capitalizes on the rapid reduction of leukemic burden by venetoclax while overcoming its limitations as monotherapy, we set out to identify compounds that could synergize with venetoclax (either intrinsically or by having complementary tissue distribution), maximizing its anti-leukemic efficacy while preserving low off-target toxicity, with the ultimate goal to improve outcomes for hypodiploid B-ALL patients.

Methods

Preclinical trial design

Over 40 NSG mice of equal sex were injected with three distinct primary human hypodiploid leukemia samples. In order to enroll mice simultaneously in the trial, we selected those showing ≥1% hCD45 in PB or $5x10^7$ units by bioluminescent imaging 4 weeks after injection, 23 mice total (8 NH 1, 8 NH2 and 7 NH3). Mice were randomized to receive vehicle or drugs, either with dimethyl sulfoxide (DMSO) control, venetoclax monotherapy, dinaciclib monotherapy or combination therapy (venetoclax and dinaciclib). Venetoclax was delivered on a weekly schedule of 5 days on, 2 days off (50 mg/kg via oral gavage with a 3-day dose escalation starting at 12.5 mg/kg dose). Dinaciclib was delivered twice weekly (20 mg/kg intraperitonally) or as a combination of venetoclax and dinaciclib. Venetoclax was formulated as previously reported[10] and dinaciclib was reconstituted in 20% hydroxypropyl β-cyclodextrin.

High-throughput screening

High-throughput screening (HTS) was performed by the HTS Core Facility (Small Molecule Discovery Center) at the University of California, San Francisco (UCSF). NALM-16 cells were screened against a bioactive pathway inhibitor library (SelleckChem), including Food and Drug Adminstration (FDA)-approved anti-cancer agents and small molecule inhibitors. Cells were treated with each compound at 2-fold serial dilution doses starting from 20 mM and growth inhibition was measured at 48 and 96 hours (h). Growth inhibition was assessed using viability/apoptosis assays. The average Z' factor for the assay was 0.83+/-0.01. Scatterplot analysis of the average percentage growth inhibition was performed to identify compounds that significantly inhibited growth.

Lentiviral transduction

HEK293T cells were transfected with 2 μg of PMD2.G, 6 μg of psPAX2 and 8 μg of the transfer plasmid of interest using 48 μL TransIT LT1 (Mirus) as per their protocol. The supernatant was collected after 72 h, concentrated with Lenti-X Concentrator (Takara Bio, JP), and resuspended in 600 μl HEK medium (50x concentration).

Generation of stable isogenic cell lines with MCL-1 overexpression or knockout via short hairpin RNA

NALM-16 cells ($7x10^6$) were transduced with 250 μL of concentrated virus in the presence of polybrene (6 μg/mL). After 48 h, transduced cells were selected via EGFP fluorescence-activated cell sorting (FACS) sorting or puromycin selection (2 μg/mL). After 72 h, viable cells were isolated using a magnetic LeviCell sorting system (LevitasBio), washed, and resuspended in RPMI1640 plus 10% fetal bovine serum.

Patient-derived xenograft samples

PDX samples were obtained from St Jude Children's Research Hospital. All samples were de-identified and, thus this study was exempt from the UCSF Institutional Review Board. Tumor cells were previously transduced with lentiviral vCL20SF2-Luc2aYFP vector for stable expression of luciferase for live imaging.[7]

See the *Online Supplementary Appendix* for additional materials and methods.

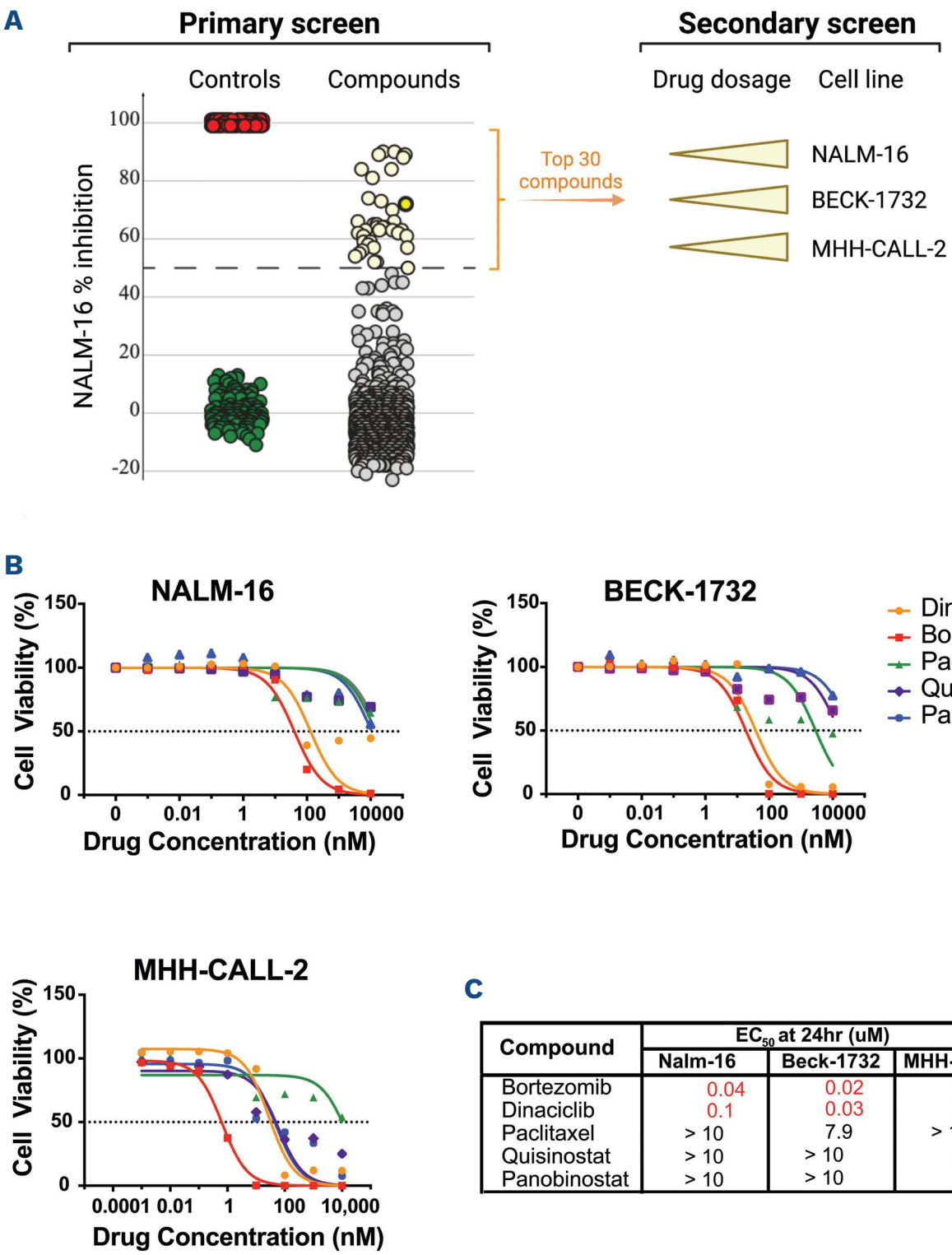
Results

High-throughput drug screening identifies compounds with high *in vitro* efficacy against hypodiploid acute lymphoblastic leukemia cell lines

In order to capitalize on the rapid reduction of leukemic burden with venetoclax, we set out to identify drugs with efficacy as single agents against hypodiploid B-ALL that could either synergize with venetoclax mechanistically or demonstrate efficacy both *in vitro* and through preclinical studies in internal organs where venetoclax showed suboptimal results. For this purpose, we performed an unbiased HTS, which utilized a library of 1,835

small molecules from the SelleckChem Bioactive Compound Library that included traditional chemotherapeutic agents, small molecules, and novel compounds. We first assessed growth inhibition to screen compounds at a fixed concentration (125 nM) against NALM-16 cells, a human near haploid pre-B-ALL cell line harboring a *TP53* mutation. At this dose, over 30 compounds demonstrated inhibitory efficacy greater >50% at 48 h (Figure 1A; *Online Supplementary Table S1*). Several drug classes were highly represented by those top 30 compounds including those targeting the proteasome, histone deacetylases (HDAC), cyclin-dependent kinases (CDK), and microtubules. We then assessed the efficacy of the top 30 drugs from this initial screen in all three available molecularly distinct NH pre-B-ALL cell lines (NALM-16, MHH-CALL-2, and BECK-1732) (Figure 1A; *Online Supplementary Figure S1A*). For this secondary screen, we ran dose response assays at 48 and 96 h (*Online Supplementary Figure S1B*). The half-maximal effective concentration (EC_{50}) of each compound was determined, as shown in the *Online Supplementary Table S2*. Twelve compounds representing six different drug classes showed EC_{50} values at or below 0.2 nM in all three cell lines.

The top five compounds (bortezomib, dinaciclib, paclitaxel, quisinostat, and panobinostat) representing four drug classes (proteasome, CDK, microtubule, and HDAC inhibitors) were selected based on their observed inhibitory efficacy in all three hypodiploid cell lines, known toxicity profile, and clinical availability to undergo further validation of their efficacy for hypodiploid ALL. Among them, dinaciclib (a CDK inhibitor) and bortezomib (a proteasome inhibitor) had the most profound impact starting at 24 h with EC_{50} values <100 nM as single drugs (Figure 1B, C; *Online Supplementary Figure S1C*).



Compound	EC_{50} at 24hr (uM)		
	Nalm-16	Beck-1732	MHH-CALL-2
Bortezomib	0.04	0.02	0.001
Dinaciclib	0.1	0.03	0.03
Paclitaxel	> 10	7.9	> 10
Quisinostat	> 10	> 10	0.06
Panobinostat	> 10	> 10	0.05

Figure 1. High-throughput screening identifies compounds with high *in vitro* efficacy against hypodiploid cell lines. (A) Primary drug screening was performed using NALM-16 cells dispensed in 384 plates and subjected to a library of small molecules/bioactive compounds, tested at 0.125 μM. After culturing, growth inhibition was measured using a luminescent cell viability assay and top hits were selected. Negative controls (green circles) represent untreated cells. Positive controls (red circles) represent wells without cells to approximate total inhibition. Hits (yellow circles) were selected from test compounds (grey circles) that exhibited greater than 50% inhibition. The 30 compounds with greatest proliferation inhibition were selected and used for a secondary screening using a range of concentrations in the three hypodiploid cell lines, NALM-16, BECK-1732 and MHH-CALL. (B) Graphs indicate % of viability in NALM-16, BECK-1732 and MHH-CALL-2 cells subjected to increasing concentrations of the top 5 compounds. (C) Half-maximal effective concentration (EC_{50}) values (μM) for the top 5 compounds in NALM-16, BECK-1732 and MHH-CALL-2 cells are shown at 24 hours.

Bliss scoring identifies dinaciclib as best combination partner with venetoclax

In order to evaluate whether any of the top five compounds identified in our screen acted synergistically with venetoclax, NALM-16 cells were treated with each compound alone and in combination with venetoclax. Dinaciclib showed the highest synergy with 97% growth inhibition in combination with venetoclax and a Bliss score of 0.32 (values >0 indicate synergy)[14] at 24 h (Figure 2A). We then validated the efficacy of venetoclax plus dinaciclib in all three hypodiploid cell lines using a serial 2-fold dilution ranging from 8-500 nM. Viability was measured at 24 and 48 h. Percentage of inhibition and BRAID synergy models[15] are represented with isobolograms in Figure 2B, C and *Online Supplementary Figure S2A*, with a positive κ value indicating synergy and a negative value indicating antagonism. This combination was highly synergistic (κ>1) in NALM-16 and MHH-CALL2 cell lines. Added synergy was not seen in BECK-1732, given that venetoclax monotherapy at low concentrations was highly efficacious in this cell line (*Online Supplementary Figure S2B*). Similarly, bortezomib showed great efficacy as a single agent in all three cell lines with marginal to no synergy when combined with venetoclax (*Online Supplementary Figure S2C*). Taken together, these results identified dinaciclib as the best agent to pair with venetoclax for further study in hypodiploid ALL cell lines and led to the pursuit of the mechanism underlying the synergistic combination of venetoclax with dinaciclib.

Dinaciclib exerts its inhibitory effect through CDK9 inhibition

Dinaciclib is a first-generation pan-CDK inhibitor with reported efficacy against CDK1, 2, 5, and 9.[16] While CDK1 regulates the G2/M cell cycle checkpoint, controlling cell division, and CDK2 regulates the RB1 cell cycle checkpoint to enter in S-phase, CDK5 and CDK9 have non-cell cycle functions.[17] We previously reported that hypodiploid B-ALL frequently presents with mutations in cell cycle regulators including *TP53* (mutated in 91% of LH patients) and *RB1* alterations (41% and 9% of LH and NH patients, respectively).[3] Furthermore, alterations in *CDKN2A*, encoding for the tumor suppressor p16, which modulates the G1 checkpoint, were seen in 24% of cases in hypodiploid ALL. In order to discern whether the sensitivity of hypodiploid ALL cells to dinaciclib was cell cycle-dependent, we initially subjected the three hypodiploid B-ALL cell lines to seven CDK inhibitors with a diverse spectrum of efficacy against CDK 1-9 at 24 and 48 h (Figure 3A). Of the seven compounds tested, only flavopiridol and SNS-032, both sharing the targets CDK2 and CDK9 with dinaciclib, showed high efficacy similar to dinaciclib against hypodiploid cell lines. Importantly, roscovitine, which shares CDK1, 2 and 5 targets with dinaciclib but not CDK9, did not affect cell viability. Moreover, inhibitors targeting CDK4/6 (i.e., abemaciclib, palbociclib and ribociclib), had minimal effect, supporting the lack of efficacy observed with cell cycle inhibitors (*Online Supplementary Figure S2D*). These results suggested that dinaciclib exerted its inhibitory effect in hypodiploid cells through inhibition of CDK9. In order to validate this observation, we subjected all three hypodiploid cells to NVP-2, a selective CDK9 inhibitor. NVP-2 showed similar efficacy as dinaciclib, as demonstrated by overlapping dose-dependent effects on proliferation and similar EC_{50} values (Figure 3B).

A

Compound name (125 nM)	% inhibition drugs alone	% inhibition + Venetoclax	Bliss score of combination	Targets
Dinaciclib	60	97	0.32	CDK1, 2, 5 and 9
Paclitaxel	35	68	0.19	Microtubules
Quisinostat	48	71	0.1	HDAC
Panobinostat	48	65	0.04	HDAC
Bortezomib	93	98	0.03	Proteasome

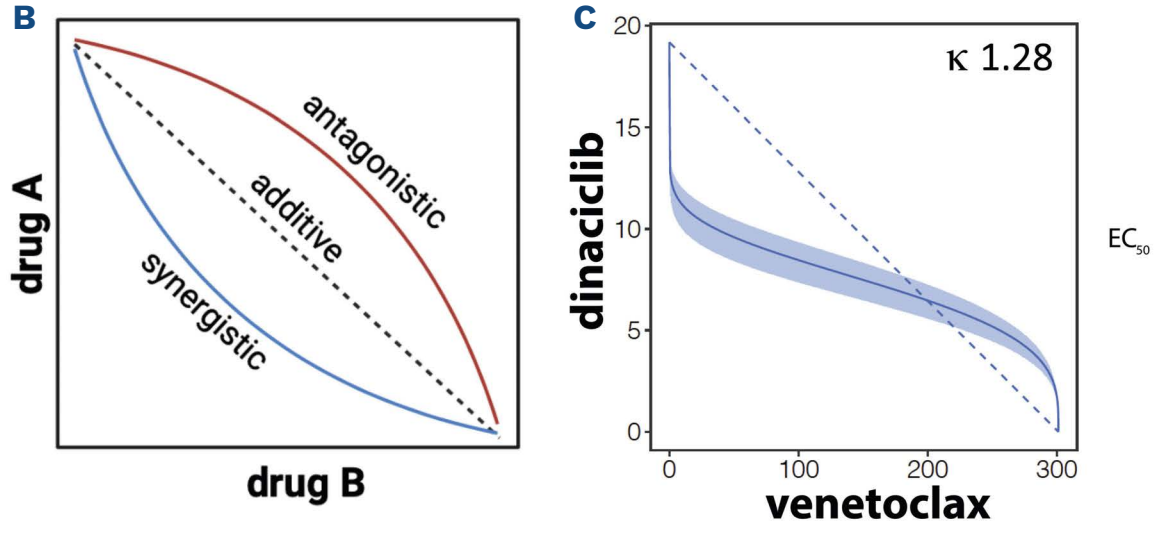


Figure 2. Dinaciclib demonstrates high synergy with venetoclax. (A) Summary of growth inhibition of indicated agents with and without venetoclax (nM) and Bliss score of the combinations in NALM-16 cells. (B) Isobologram illustration of synergy scores. (C) Synergy scores determined using an isobologram model, of NALM-16 cells treated for 24 hours with a combination of dinaciclib and venetoclax at 6 2-fold serial dilution doses ranging from 0.008-0.5 μM.

A

Compound	EC_{50} at 48h (μM)			Target
	NALM-16	1732	MHH-CALL2	
Dinaciclib	0.046	0.037	0.023	CDK1, 2, 5 & 9
Flavopiridol	0.303	0.260	0.143	CDK1, 2, 4, 6, 7, 9
SNS-032	0.482	0.295	0.158	CDK2, 7, 9
Abemaciclib	> 10	5.041	3.820	CDK4, 6
Palbociclib	> 10	> 10	3.308	CDK4, 6
Ribociclib	> 10	> 10	> 10	CDK4, 6
Roscovitine	> 10	> 10	> 10	CDK1, 2, 5

B

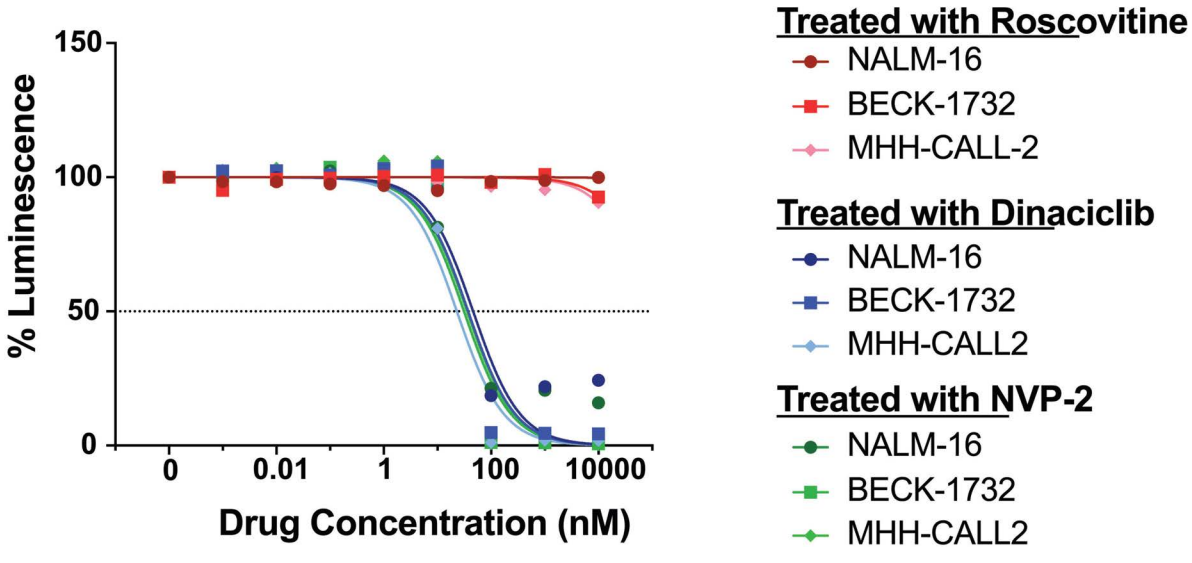


Figure 3. Dinaciclib exerts its inhibitory effect through CDK9 inhibition. (A) Summary of half-maximal effective concentration (EC_{50}) values in NALM-16, BECK-1732 and MHH-CALL-2 cells treated with inhibitors of CDK 1-9 at 48 hours (h). (B) Growth inhibition measured at 48 h using a luminescent cell viability assay in all 3 hypodiploid cells treated with the indicated agents, including NVP-2, a selective CDK9 inhibitor using 7 10-fold serial dilution doses ranging from 0.000001-10 μM.

Dinaciclib induces cell death rather than cell arrest in hypodiploid acute lymphoblastic leukemia

We then evaluated whether dinaciclib exerted a cytotoxic effect beyond a decrease in proliferation on hypodiploid ALL cells. Using an apoptosis assay, we observed an increase in the levels of active (cleaved) caspase 3 in NALM-16 cells exposed to increasing concentrations of dinaciclib for 24 h (*Online Supplementary Figure S3A*). Furthermore, we assessed the kinetics of apoptosis following dinaciclib treatment between 6 and 24 h, using induction of cleaved PARP and cleaved caspase 3 as the readout. Our data showed dose-dependent induction of cell death by 6 h, which was enhanced over 24 h (*Online Supplementary Figure S3B*). These findings further demonstrated the rapid cytotoxic activity of dinaciclib against hypodiploid cells *in vitro*.

Dinaciclib induces cell death in hypodiploid B-lineage acute lymphoblastic leukemia through inhibition of CDK9

In order to explore the underlying mechanism of the apoptotic effect of dinaciclib in hypodiploid ALL, and to better understand the biochemical basis behind the synergistic effect seen with venetoclax, we investigated the dose-dependent effects of dinaciclib on its reported targets (CDK1, 2 and 9) in NALM-16 cells. Based on our prior studies indicating high levels of the prosurvival proteins BCL-2, and MCL-1, and low levels of BCL-xL across all samples,[7] we also analyzed the levels of BCL-2 and MCL-1 following dinaciclib treatment (*Online Supplementary Figure S3C*). Increasing concentrations of dinaciclib resulted in a significant reduction in CDK9 levels but not CDK1/2 by 6 h at 10 nM drug. Consistent with the known mechanisms of CDK9 phosphorylation of RNA polymerase II (RNA pol II) at Serine 2[18] (indicating transcription elongation), a decrease in RNA pol II (Ser2) phosphorylation was observed, with concomitant MCL-1 downregulation, a gene reported to be under transcriptional regulation of CDK9.[16,17,19] BCL-2 levels were only reduced at high drug concentrations. Notably, the initial increase in MCL-1 at low nanomolar concentrations of dinaciclib did not correlate with changes in proliferation. The observation that MCL-1 levels are downregulated upon CDK9 inhibition were validated using NVP-2 with concomitant upregulation of the apoptotic marker cleaved PARP (*Online Supplementary Figure S4*).

We then sought to determine the time of onset and extent of apoptosis induced by dinaciclib and venetoclax alone and in combination (Figure 4). The rapid decrease of MCL-1 levels, particularly evident from 2 h, demonstrates dinaciclib as an effective MCL-1 inhibitor. Moreover, these data indicate that reduction in RNA pol II (Ser2) and decrease in MCL-1 precedes induction of apoptosis in the absence of BCL-2 inhibition. Importantly, however, in conditions where BCL-2 has been inhibited by venetoclax, the reduction of MCL-1 levels coincides with the induction of apoptosis and occurs at concentrations 10-fold lower.

These data suggest that dinaciclib inhibition primarily works through inhibition of MCL-1, and that in the absence of functional BCL-2 (e.g., in the presence of venetoclax), these cells become highly dependent on MCL-1 for survival. In order to validate such observations, we over-

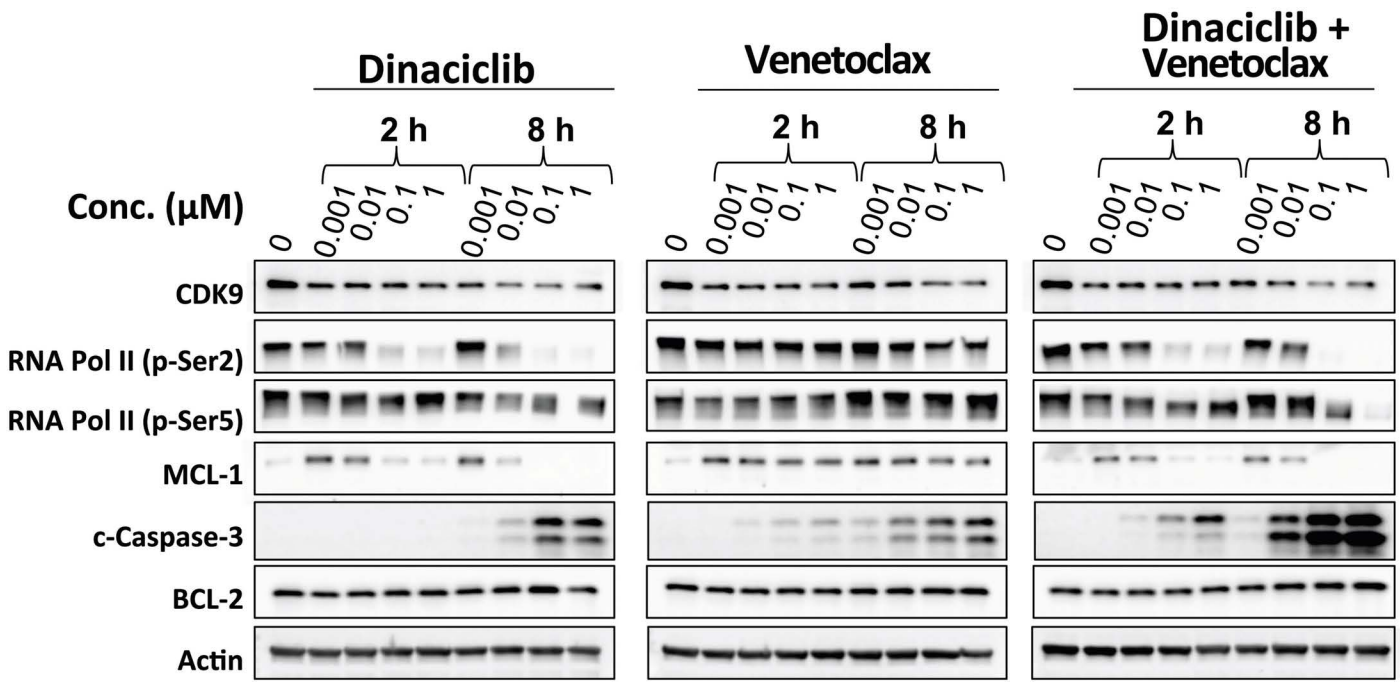


Figure 4. Apoptosis induction by dinaciclib, venetoclax and the combination in isogenic lines. NALM-16 cells were treated with dinaciclib, venetoclax or the combination for 2 hours and 8 hours. Levels of CDK9, RNA polymerase II phosphorylation on Ser 2 or Ser 5, prosurvival proteins MCL-1 and BCL-2, and the apoptosis marker cleaved caspase, are shown via western blotting.

expressed MCL-1, selected viable MCL-1-transduced cells (*Online Supplementary Figure S5*) and subjected them to dinaciclib, either alone or in combination with venetoclax. As shown in *Online Supplementary Figure S6A*, MCL-1 overexpression, while still under the control of CDK9, impairs the apoptotic effect of dinaciclib and venetoclax alone and more importantly the synergistic effect exerted by the two. Furthermore, downregulation of MCL-1 via short hairpin RNA recapitulated the dinaciclib enhancement of apoptosis by venetoclax as demonstrated by higher levels of cleaved caspase (and therefore apoptosis) at 10-fold lower concentrations (*Online Supplementary Figure S6B*).

Dinaciclib reduces viability in primary xenograft samples *ex vivo*

In order to further investigate the combination of dinaciclib and venetoclax in hypodiploid ALL cells, we queried the cancer dependency map (DepMap, http://depmap.org), an open-source repository of genetic and pharmacologic dependencies of cancer cell lines. At a gene expression level, both hypodiploid lines NALM-16 and MHH-CALL-2 showed higher co-expression of CDK9 and BCL-2 than most of the blood lineage cell lines (Figure 5). From a dependency perspective, we found the hypodiploid ALL cell line NALM-16 (*data not available* for MHH-CALL-2) to be highly genetically dependent on CDK9 compared to other ALL or AML, CLL and CML cell lines analyzed (*Online Supplementary Figure S7A*). Along these lines, MHH-CALL-2 cell line (*data not available* for NALM-16) is also more sensitive to dinaciclib and venetoclax compared to other blood cancer lines (*Online Supplementary Figure S7B*).

In order to validate the broader relevance of the CDK9 pathway in hypodiploid ALL, we looked at the RNA polymerase II/CDK9/MCL-1 complex in a gene expression database of hypodiploid B-ALL patients previously reported.[7] This database compared 89 hypodiploid (LH- and NH-) B-ALL samples to 24 non-hypodiploid B-ALL samples (*Online Supplementary Figure S8A*). This analysis demonstrated an overexpression of several genes within the mediator complex, working in coordination with CDK9 to mediate transcription, as well as MCL-1, in hypodiploid B-ALL compared to other diploid B-ALL (*Online Supplementary Figure S8A, B*). When analyzing transcript levels of MCL-1 across B-ALL of different genotypes we observed significantly elevated mRNA levels of *MCL-1* levels in LH, masked LH (mLH with chromosomal reduplication), NH and masked NH (mNH) leukemias (Figure 6A). These levels are as high, or higher than, BCL-2 levels (Figure 6B). Consistent with our previously reported protein levels, mRNA levels of the proapoptotic markers (*BIM*, *BAD* and *PUMA*) counteracting BCL-2 and MCL-1 are also elevated in hypodiploid B-ALL (*Online Supplementary Figure S9*).

In order to further validate dinaciclib as a potential therapeutic candidate, we tested its efficacy in five hypodiploid

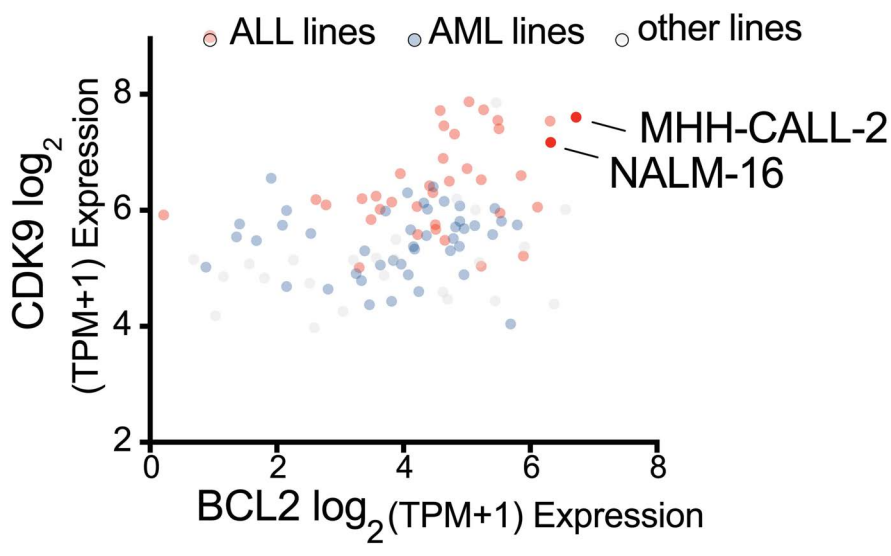


Figure 5. *CDK9* and *BCL-2* mRNA levels in hypodiploid acute lymphoblastic leukemia cell lines. Gene expression levels of *CDK9* and *BCL-2* in blood lineage cell lines (CCLE Expression 22Q2 Public dataset); values expressed as $\log_2$ transcript count per million (TPM); highlighted hypodiploid cell lines NALM-16 and MHH-CALL-2.

ALL xenografted patient samples *ex vivo*, including three LH (harboring mutations in *TP53*) and two NH (harboring deletions in the Ras-GAP gene *NF1*) samples (*Online Supplementary Table S3*). Dinaciclib markedly reduced cell viability at low nanomolar concentrations within 24 h in the majority of the hypodiploid patient cells, with an observed

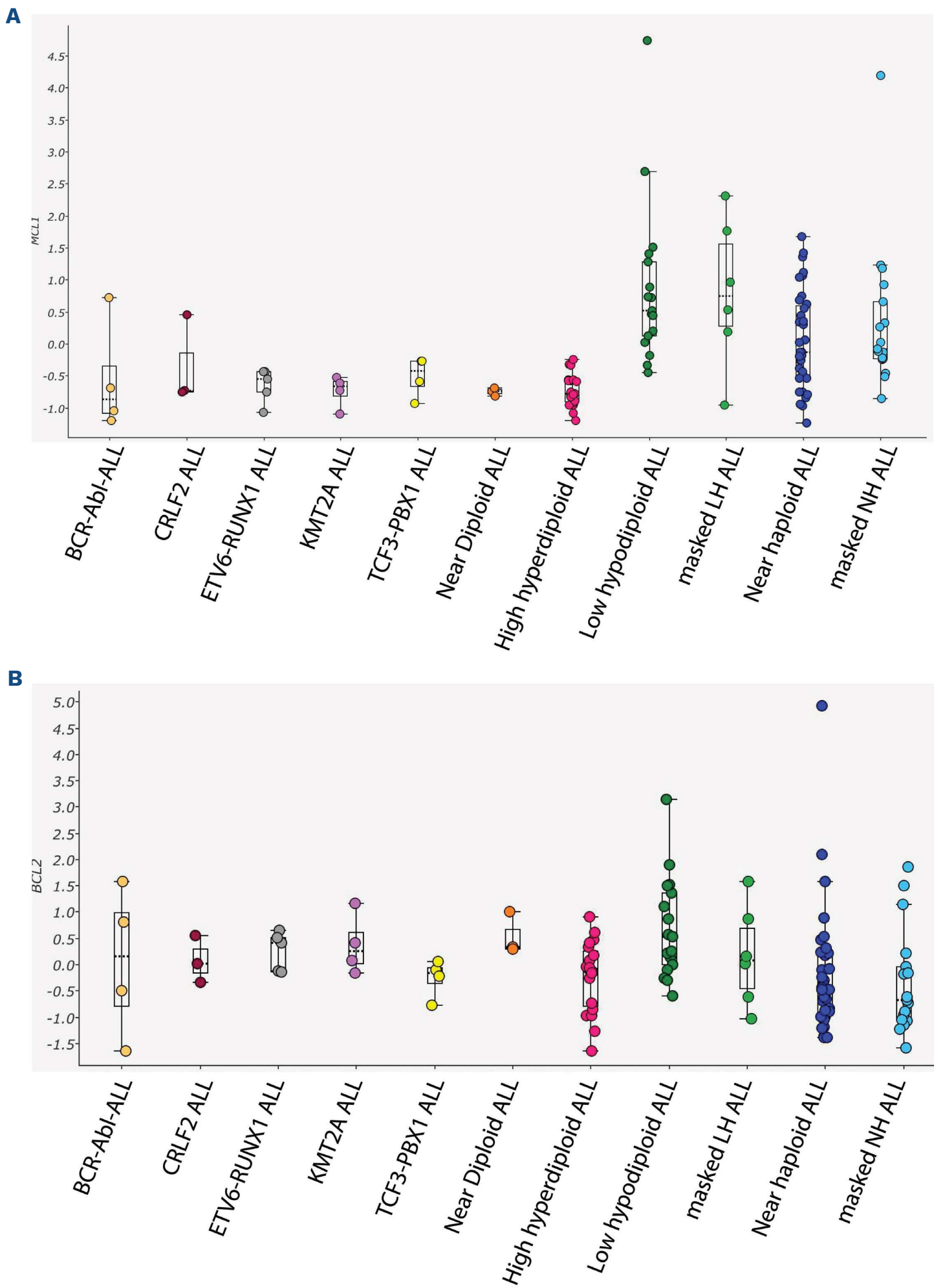


Figure 6. *MCL-1* and *BCL-2* levels in B cell acute lymphoblastic leukemia patients. Comparison of *MCL-1* (A) and *BCL-2* (B) gene expression in hypodiploid B-lineage acute lymphoblastic leukemia (B-ALL) subtypes (low-hypodiploid [LH], masked low-hypodiploid (mLH), near-haploid [NH] and masked near-haploid [mNH]) *vs.* diploid B-ALL of different genotypes obtained from microarray data (GSE23237).

increase in potency against NH *versus* LH cells (*Online Supplementary Figure S10*).

Preclinical efficacy of dinaciclib/venetoclax in hypodiploid acute lymphoblastic leukemia in patient-derived xenograft mice

Given our primary interest in identifying drugs with therapeutic potential against hypodiploid leukemia, we next assessed the overall efficacy of dinaciclib alone or in combination with venetoclax in reducing hypodiploid leukemic burden *in vivo*. For this purpose, we set up a preclinical trial using NH PDX models from NH samples tagged with a luciferase reporter as previously described. Upon engraftment, mice meeting enrollment criteria were randomly assigned to one of four treatment arms: untreated, venetoclax monotherapy, dinaciclib monotherapy or combination therapy with venetoclax and dinaciclib.

Consistent with previous results,[7] venetoclax alone showed rapid and persistent near-clearance of circulating blasts from PB in all mice (6/6) as measured by hCD45 surface marker expression via flow cytometry (Figure 7A [average] and *Online Supplementary Table S4* [by individual mice]) Such a response was accompanied by a significant reduction in overall leukemic burden throughout the trial as measured via bioluminescence imaging (BLI) (Figure 7B, C). Conversely, dinaciclib monotherapy had partial reduction in blasts from PB (3/6 mice) but showed limited effect on reducing leukemic burden as measured by BLI throughout the trial (Figure 7A-C). Consistent with our *in vitro* data however, the combination of venetoclax and dinaciclib showed a rapid and drastic overall reduction in both circulating blasts and overall leukemic burden in all mice (6/6) which persisted throughout the trial. Most importantly, end-of-trial analyses confirmed overall efficacy of the combination treatment, with a reduction of >97% of leukemic blasts from the bone marrow and liver of all mice as measured by %hCD45 (Figure 7C-E) as well as CD10, CD19 and CD20 (*data not shown*). Of note, two mice demonstrated greater than 2% hCD45/CD10/CD19-positive blasts within their spleens despite having 1% or less disease in all other organs. A single sample from each treatment arm was randomly selected for immunohistochemistry analysis at the end of the trial. Pathology analysis on these samples confirmed the complete clearance of leukemic blasts in spleen (*data not shown*) and liver in the mouse treated with combination therapy (Figure 7E).

Combination therapy with dinaciclib and venetoclax was well tolerated

During the first 7 days of combination therapy, the first two mice enrolled demonstrated fatigue and 5-7% weight loss, which improved with subcutaneous rehydration. For the remainder of the trial, all mice received a prophylactic subcutaneous fluid bolus on days 1 and 4 of the trial in conjunction with their first two doses of dinaciclib. This intervention significantly reduced these symptoms for all additional mice enrolled on the combination arm. Overall, there was limited hematologic toxicity with the combination of venetoclax and dinaciclib (*Online Supplementary Figure S11*).

Discussion

Hypodiploid B-ALL is an aggressive leukemia with a dismal prognosis for those with slow induction responses despite risk-stratified chemotherapy regimens or HCT. Given the median age of these patients, those surviving aggressive chemotherapy regimens or HCT face significant life-long morbidities. Additionally, due to the frequency of *TP53* mutations in LH ALL and its association Li-Fraumeni syndrome, these patients are at particularly high risk of therapy-related toxicities including treatment-related secondary malignancies.[3,20] For these reasons, we sought to identify novel or adjunctive therapies that could improve patient outcomes while limiting adverse events and avoiding agents known to increase the risk of secondary malignancies.

Our high-throughput screen identified several classes of drugs – including proteasome, HDAC, CDK and microtubule assembly inhibitors – with high efficacy against hypodiploid leukemia cell lines. The focus of this work was to further interrogate compounds showing synergistic efficacy with venetoclax to capitalize on the rapid reduction of leukemic burden while providing additional efficacy against hypodiploid ALL. Thus, we focused on the combination of dinaciclib and venetoclax as this combination showed the highest synergy *in vitro* that translated to the *in vivo* studies.

Dinaciclib, an inhibitor of CDK1, 2, 5 and 9, has shown efficacy in DLBCL, CLL, T-ALL, and small cell lung cancer.[19,21-23] It is well tolerated in phase I/II studies, with primary toxicities being transient laboratory abnormalities, tumor lysis syndrome, and limited marrow suppression.[24-26] Due to its efficacy in preclinical models and clinical trials it was granted orphan drug designation for CLL by the FDA, and it remains under active study in clinical trials for various malignancies. However, to our knowledge there are no reports of its potential efficacy in primary B-ALL *in vivo*. Our work indicates that dinaciclib exerts its antileukemic effect on hypodiploid ALL through inhibition of CDK9 but not CDK1 or 2. CDK9 inhibition leads to inhibition and downregulation of RNA polymerase II with concomitant downregulation of the prosurvival protein MCL-1 as reported elsewhere.[19,21,27-29] These data were confirmed with the use of another CDK9-specific inhibitor, NVP-2. We had previously reported that hypodiploid ALL cells display intermediate to high protein levels of MCL-17 and in this re-

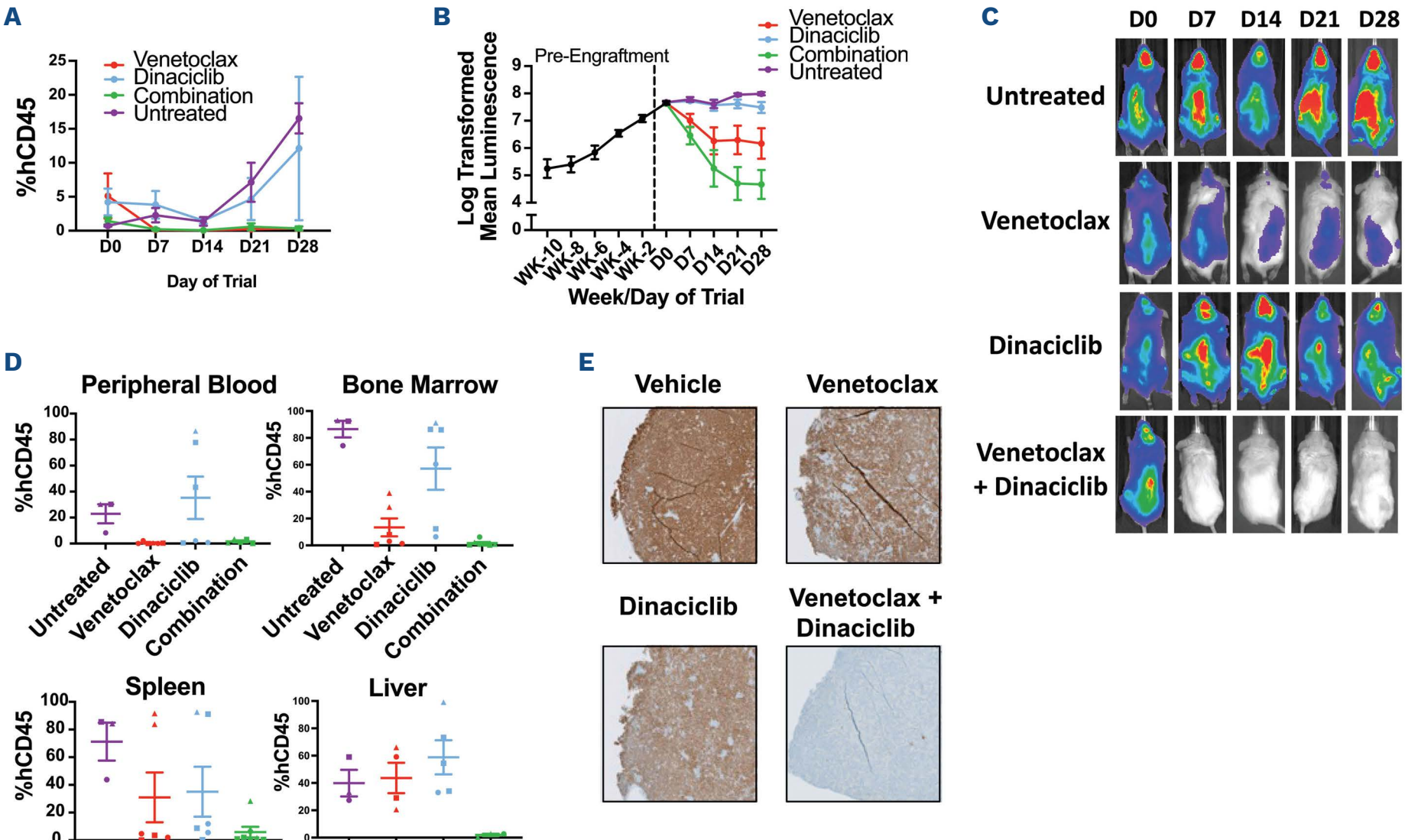


Figure 7. Preclinical study outcomes. (A) Average percentage of human CD45 (hCD45) in the peripheral blood of mice in each condition (5 vehicle and 6 in each drug condition) and (B) average of whole-body luciferase reporter expression, measured weekly in all 3 subtypes of hypodiploid acute lymphoblastic leukemia (ALL) patient-derived xenograft (PDX) mice treated on the preclinical trial, which included 4 treatment arms: untreated, venetoclax monotherapy, dinaciclib monotherapy or combination therapy with venetoclax and dinaciclib. Data displayed is an aggregate of all 3 near-haploid (NH) B-lineage All (B-ALL) sets. (C) All 3 hypodiploid leukemia primary samples (NH1, 2 and 3) were tagged with a luciferase reporter, prior to engraftment, and mice were imaged weekly using bioluminescent imaging before and during treatment. Data displays a single representative subject from each treatment arm, however, reflects the overall trend seen across all treatment groups. (D) Percentage of hCD45 in peripheral blood (from cardiac puncture), bone marrow, spleen and liver measured at the end of the trial in all 3 subtypes of hypodiploid ALL PDX mice (NH1 – circle, NH2 – square, NH3 -triangle) and compared across the 4 treatment arms. (E) Representative hCD45 immunohistochemistry stains of the liver tissue samples obtained from each arm of the trial at the end of therapy for NH3 mice.

port we indicate that this increase occurs at the transcriptional level.

The increased expression of the prosurvival proteins MCL-1 and BCL-2 is a well-described feature of many malignancies, allowing for increased survival of cancer cells and resistance to therapy. Hypodiploid cells are characterized by high levels of both BCL-2 and MCL-1 at the protein level[7] and, as shown here, also at the transcript level. As we have previously reported, increased levels of BCL-2 with elevated levels of pro-apoptotic markers (BIM, BAD and PUMA) represent a biomarker signature for cellular sensitivity to venetoclax in hypodiploid cells[7] and other leukemias. Notably, elevated MCL-1 levels may result in acquired resistance to venetoclax by sequestration of pro-apoptotic mediators.[7,30,31] Importantly, for dinaciclib, while high BCL-2 levels in hypodiploid B-ALL may mediate resistance to dinaciclib as monotherapy, elevated MCL-1 levels may render these cells sensitive to this drug. Thus, this drug combination may sensitize cells with both survival proteins overexpressed and overcome the resistance to both drugs individually. Consistent with our findings, a recent study of venetoclax and S63845 (an alternate MCL-1 inhibitor) in diploid leukemias demonstrated synergistic anti-leukemia activity through the impaired binding of BCL-2 and MCL-1 to the apoptosis activator BIM.[32] Thus, for hypodiploid B-ALL, which has elevated levels of both BCL-2 and MCL-1, as well as the proapoptotic proteins (BIM, BAD, and/or PUMA) this drug combination may rep-

resent a promising targeted therapeutic approach.

Given the cytotoxic rather than cytostatic effect of both venetoclax and dinaciclib, this combination provides a strong rationale for a complementary and effective therapeutic approach. Indeed, our results indicate a potent and synergistic cytotoxic effect when combined and administered to hypodiploid cells. Importantly, this drug combination does not significantly inhibit BCL-xL, an activity linked to severe thrombocytopenia in clinical trials.[33] Consistent with this idea, we observed little thrombocytopenia in our preclinical study. Similarly, venetoclax has low off-target toxicity and is well-tolerated in clinical trials; its primary toxicity, tumor lysis syndrome, can be significantly reduced with gradual dose escalation and supportive care measures.

The significance of this work is the identification of a synergistic therapeutic approach against hypodiploid B-ALL *in vivo* by inhibiting both BCL-2 (venetoclax) and CDK9 (dinaciclib). This combination was remarkably effective in clearing leukemic blast cells from internal organs, overcoming the blast persistence observed when venetoclax was used as monotherapy. We note that the combination of venetoclax and dinaciclib is under study in an active clinical trial for acute myeloid leukemia in adults (*clinicaltrials go. Identifier: NCT03484520*). When available, information from this trial may allow repurposing this combination for hypodiploid ALL and other B-ALL subtypes that may show sensitivity to this combination *in vitro.*

While residual leukemic burden observed in solid organs (not in PB or BM) following venetoclax monotherapy may be unique to hypodiploid B-ALL,[7] it may represent a largely overlooked mechanism of resistance for some subsets of leukemia in clinical studies using venetoclax. Given the increasing number of clinical trials testing this drug, it would be important to evaluate such a possibility if patients subjected to venetoclax do not achieve complete remission.

Importantly, given that MCL-1 inhibition has been suggested to overcome MCL-1-mediated resistance to venetoclax, and the lack of FDA-approved MCL-1-targeted drugs, dinaciclib may represents an FDA-approved drug with therapeutic potential to overcome MCL-1-mediated resistance. While this study looked at venetoclax in combination with dinaciclib, the combination of venetoclax and anthracyclines warrants further investigation, given the reported inhibitory effect of doxorubicin and daunorubicin on MCL-1.[22] A synergistic anti-leukemic effect of venetoclax and daunorubicin would argue in favor of adding venetoclax to the traditional ALL induction backbone while dinaciclib is undergoing further study in the pediatric population.

Moreover, in addition to dinaciclib, this study identified the proteasome and histone deacetylases as potential targets for hypodiploid leukemia. While both families of proteins regulate a broad range of pathways, which could limit their therapeutic index, work is underway to explore the therapeutic relevance of bortezomib and HDAC inhibitors in treating hypodiploid leukemia.

In summary, our study identified a highly effective and synergistic drug combination, venetoclax and dinaciclib, for the treatment of hypodiploid B-ALL, an aggressive leukemia with few effective current therapies. Thus, while venetoclax monotherapy continues to demonstrate rapid clearance of leukemic blasts from the PB, the addition of dinaciclib led to complete resolution of the leukemic burden within the solid organs of most mice, which was not seen with venetoclax alone. This combination therapy was relatively well tolerated. This study also indicates that intermediate or high levels of BCL-2 and MCL-1 with concomitant levels of pro-apoptotic markers (BIM, BAD and/or PUMA), may represent biomarkers for response to this combination therapy or drugs with similar targets. Finally, the promising results presented in this study may prompt further studies to support the inclusion of hypodiploid and other B-ALL patients to clinical trials combining phase I/II drugs against BCL-2 (mainly venetoclax) and CDK9 (dinaciclib, alvocidib, flavopiridol, SNS-032) or MCL-1 (MIK-665). Ongoing research is warranted to confirm the efficacy of combination therapy in LH mouse models harboring *TP53* mutations as well as to address questions regarding response duration.

Disclosures

No conflicts of interest to disclose.

Contributions

The concept was developed by ED-F, HP, MRA and MLL. The methodology, validation and formal analysis was performed by HP, JF, SB, KKA, SM, CGW, BSB, BP, MW, KB, SX and ED-F. The investigation was done by HP, KKA, SM, CGW, MRA, MLL and ED-F. MRA and MLL provided resources. RPR, ABO, BP, CS and ED-F analyzed computational data. HP, ED-F, and MLL wrote the original draft. MRA, MLL and ED-F wrote, reviewed and edited the manuscript. ED-F visualized the research. ED-F and MLL supervised the research. ED-F and MLL acquired funding.

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Data-sharing statement

The authors are committed to the dissemination of data that may be requested. All information about patient subjects has been de-identified. Data will be shared via email or secure sharing providers (e.g., Box). Please contact the corresponding author.

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