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Therapeutics, Targets, and Chemical Biology

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The CDK9 Inhibitor Dinaciclib Exerts Potent Apoptotic and Antitumor Effects in Preclinical Models of MLL-Rearranged Acute Myeloid Leukemia

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Abstract

Translocations of the mixed lineage leukemia (*MLL*) gene occur in 60% to 80% of all infant acute leukemias and are markers of poor prognosis. MLL-AF9 and other MLL fusion proteins aberrantly recruit epigenetic regulatory proteins, including histone deacetylases (HDAC), histone methyltransferases, bromodomain-containing proteins, and transcription elongation factors to mediate chromatin remodeling and regulate tumorigenic gene expression programs. We conducted a small-molecule inhibitor screen to test the ability of candidate pharmacologic agents targeting epigenetic and transcriptional regulatory proteins to induce apoptosis in leukemic cells derived from genetically engineered mouse models of MLL-AF9–driven acute myeloid leukemia (AML). We found that the CDK inhibitor dinaciclib and HDAC inhibitor panobinostat were the most potent inducers of apoptosis in short-term *in vitro* assays. Treatment of MLL-rearranged leukemic cells with dinaciclib resulted in rapidly decreased expression of the prosurvival protein Mcl-1, and accordingly, overexpression of Mcl-1 protected AML cells from dinaciclib-induced apoptosis. Administration of dinaciclib to mice bearing MLL-AF9–driven human and mouse leukemias elicited potent antitumor responses and significantly prolonged survival. Collectively, these studies highlight a new therapeutic approach to potentially overcome the resistance of MLL-rearranged AML to conventional chemotherapies and prompt further clinical evaluation of CDK inhibitors in AML patients harboring MLL fusion proteins. *Cancer Res; 76(5); 1158–69. ©2015 AACR.*

Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia affecting adults accounting for approximately 25% of all leukemias in adults in the Western world (1). The combination of cytarabine (cytosine arabinoside) with an anthracycline is the standard-of-care for patients fit for induction therapy; nevertheless, treatment responses and outcome to this regimen vary from primary refractory disease to cure (2). Chromosomal abnormalities are found in 50% to 80% of AML cases with rising incidences in patients with secondary leukemia or older age. Frequently found abnormalities include chromosome loss or deletion (e.g., 5, 7, Y, and 9) and translocations such as t(16;16)/inv (16), t(8;21)(q22;q22); t(15;17)(q22;q21). These and other class II mutations occur concomitantly with class I mutations activating oncogenes such as *RAS*, *FLT3*, and *KIT* to cooperatively drive AML onset and progression (3).

Translocation of the mixed lineage leukemia (*MLL*) gene is an independent predictor of poor prognosis. *MLL* has been found in 73 different translocations and 54 partner genes have been cloned. MLL fusion proteins (MLL-FP) share a common structure with the respective partners invariably fused in-frame to MLL, resulting in maintenance of DNA-binding capacity but altered transcriptional regulatory capacity (4, 5). Many MLL-FPs recruit large macromolecular transcription regulatory complexes such as the super elongation complex (SEC) and the polymerase-associated factor complex (PAFc; refs. 6–10). Important epigenetic enzymes and regulatory proteins that interact with MLL-FPs as a part of the SEC, PAFc, or through direct interaction with MLL-FPs include the positive transcription elongation factor b (pTEFb) consisting of CDK9 and cyclin T1 that phosphorylates the carboxyl-terminal repeat domain (CTD) of RNA polymerase II, the acetylated lysine-binding bromodomain-containing "chromatin reader" protein BRD4, the methyltransferase PRMT1, the histone demethylase LSD1 (KDM1A), and the histone methyltransferase

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DOT1L (6–12). In addition, histone deacetylases (HDAC) can also associate with MLL-FPs (13). It is clear that not all MLL-FPs will recruit the same epigenetic or transcription-regulatory enzymes and therefore selective targeting of a specific enzyme(s) may be required to effectively treat AML driven by the different fusion proteins. For example, MLL-EEN and MLL-CBP do not recruit the SEC (12); however, MLL-EEN does recruit PRMT1 and requires this for oncogenesis (14).

Small molecules targeting DOT1L (15, 16), BRD4 (8, 17), LSD1 (18, 19), HDACs (20), and CDK9 (21) have been tested for activity against MLL-FP–driven AML in diverse *in vitro* and *in vivo* systems with varying effect. Herein, we have used sophisticated genetically engineered mouse models of MLL-AF9 AML and human leukemias expressing MLL-AF9 and other MLL-FPs to test the sensitivity of these tumors to a range of agents that target the epigenetic and transcriptional regulatory proteins recruited by these oncogenic fusion proteins. Using an *in vitro* screen, we found that the CDK inhibitor dinaciclib was consistently the most potent inducer of apoptosis of the MLL-AF9–driven leukemia cells. This apoptosis correlated with suppression of CDK9 enzymatic activity and downregulation of previously identified MLL-AF9 target genes *HoxA9* and *Meis1*, indicating CDK9 as an important mediator of the effect of dinaciclib. Consistent with prior reports in other malignancies (22–24), apoptosis induction by dinaciclib correlated with acute transcriptional downregulation of *Mcl-1* and retroviral overexpression of Mcl-1 rescued dinaciclib-induced death of MLL-AF9–driven AML cells. Importantly, dinaciclib was efficacious *in vivo* in mice bearing human and mouse MLL-AF9–driven AMLs and very clearly outperformed a chemotherapeutic regimen involving a combination of cytarabine and doxorubicin. Our data provide a strong rationale for the testing of dinaciclib in patients with AML driven by MLL-AF9 and other MLL-FPs that recruit pTEFb.

Materials and Methods

Experimental animals and materials

Retroviral constructs were previously described (25). C57BL/6 mice were purchased from The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) and NOD/SCID, IL2R γ chain-deficient (NSG) were bred in-house. All mice were used in accordance with the institutional guidelines of Peter MacCallum Cancer Centre (Melbourne, Australia). EPZ004777 was supplied by Epizyme, JQ1 was supplied by Dr. J. Bradner (Dana Farber Cancer Institute, Boston, MA), while panobinostat was provided by Novartis Pharmaceuticals Australia, and prepared as a 2 mg/mL solution in 5% dextrose/dH_2O (D5W). Dinaciclib was provided by Merck and prepared as a 2 mg/mL solution in 20% hydroxypropyl-beta-cyclodextrin solution. Cytarabine, doxorubicin, and cyclophosphamide were obtained from the Peter MacCallum Cancer Centre (Melbourne, Australia) and further diluted in PBS to a 12.5 mg/mL solution. Flavopiridol was purchased from Selleck Chemicals and prepared in sterile water as a 5 mmol/L stock, and palbociclib was generously provided by Shatha Abuhammad and Prof. Grant McArthur (Peter MacCallum Cancer Centre, Melbourne, Australia).

Transduction of fetal liver cells and reconstitution in irradiated mice

Primary leukemias were generated by the retroviral coexpression of MSCV-MLL-AF9-IRES-VENUS with MSCV-luciferase-IRES-$Nras^{G12D}$ or MSCV-luciferase-IRES-Cherry expression constructs as described previously (20). Transduced cells were used to reconstitute the hematopoietic compartment of each irradiated 6- to 8-week-old C57BL/6 mice. Mice were then monitored for disease onset and progression by the analysis of peripheral blood and bioluminescence imaging.

Isolation of primary murine leukemias

Spleens were harvested from primary leukemic mice at ethical disease burden. The spleens were then macerated and sifted through a 0.40-μm filter, centrifuged, and resuspended in fetal calf serum (FCS; Invitrogen Life Technologies)/dimethyl sulfoxide (DMSO; Life Technologies) before cryopreservation. For subsequent *in vitro* assays, thawed primary MLL-AF9 leukemia cells were cultured as described previously (20). Retroviral transduction of MLL-AF9 leukemia cells with the murine stem cell virus-internal ribosomal entry site-GFP (MSCV-I-GFP) *Mcl-1* construct was performed as described previously (26).

In vitro dose response assays

Human THP-1, MV4-11, HL60, and Kasumi cell lines were obtained from the ATCC directly and minimally cultured in RPMI1640 (Invitrogen Life Technologies) + 10% FCS while murine MLL-AF9 leukemias were cultured in a DMEM supplemented with 10% FCS. Exponentially growing cells were seeded into 96 well-plates at 150,000 cells/150 μL. Inhibitors were serially diluted from appropriate starting concentrations. After 24-hour incubation, 50 μg/mL of propidium iodide (PI; Life Technologies) was added and the cells were analyzed via flow cytometry.

Patient-derived AMLs were described previously (27). The mouse bone marrow stromal cell line MS-5 was kindly provided by Prof. Mori (Niigata University, Niigata, Japan; ref. 28). MS-5 cells were seeded into 96-well plates at 10^4 cells per well in αMEM supplemented with 10% FBS, penicillin/streptomycin, and L-glutamine (2 mmol/L), and grown to confluence over 3 to 4 days. Human AML PDX cells (27) were retrieved from cryostorage and seeded onto the confluent MS-5 monolayer at 5×10^4 cells per well in Iscove's modified Dulbecco's medium containing 0.5% FBS, 1% penicillin/streptomycin, and L-glutamine. The next day, dinaciclib was added to duplicate wells at concentrations between 5 nmol/L and 10 μmol/L and plates were incubated for an additional 24 hours. Cells were then harvested with trypsin and stained with Annexin V-PE (BD Biosciences), an anti-human CD45-APC antibody, and 7AAD (BioLegend) according to the manufacturers' instructions. The stained cells were enumerated on a FACSCalibur flow cytometer and analyzed by CellQuest Pro v6.0 (BD Biosciences). Cells that were human $CD45^+$/Annexin V^-/$7AAD^-$ were gated as viable leukemia cells. IC_{50} was determined by cubic spline regression analysis using GraphPad Prism 5.0.

Apoptosis assays

For mitochondrial membrane potential assays, cells were incubated for 20 minutes with 50 nmol/L of tetramethylrhodamine (Abcam) before being resuspended in PBS for flow cytometry analysis. For the detection of subdiploid DNA content, cells were analyzed using the Nicoletti method (29). Phosphatidyl serine exposure was assessed by Annexin V antibody (Invitrogen Life Technologies) staining in buffer solution (10 mmol/L HEPES adjusted to pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$) as described previously (30). For caspase inhibition, cells were

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pretreated with 20 μmol/L of Q-VD-OPh as previously described (31). Flow cytometry was performed using a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.4 software (Tree Star).

Colony assays

Primary murine MLL-AF9 leukemia cells were plated on DNA grade agar (BD Biosciences) dissolved in DMEM onto non-tissue culture 6-well plates at 8,000 cells per well. For shRNA-mir knockdown experiments, cells were FACS-sorted for reporter expression prior to plating. After three weeks of incubation, viable colonies were identified using the MTT assay (32) prior to macrophotography and colony counting using Metamorph analysis software.

Western blot analysis

Western blot assays were performed as previously described (20) using antibodies against acetyl-histone H3 (Merck, 06-599), p-Rpb1 CTD (Ser2/5; Cell Signaling Technology, #4735), β-actin (Sigma-Aldrich #A2228), Mcl-1 (Rockland Immunochemicals Inc.), HSP90 (AC88, Enzo Life Sciences, Inc.), and the appropriate horseradish peroxidase–coupled secondary antibodies (Dako). All antibodies were used at 1:1,000 and detected with the ECL reagent by GE Healthcare.

In vivo studies

For murine MLL-AF9 treatment studies, 1×10^6 primary leukemia cells isolated from the spleens of terminally diseased mice were transplanted into 6- to 8-week-old C57BL/6 mice via tail vein injection. Dinaciclib and panobinostat treatment began 7 days posttransplantation via intraperitoneal (i.p.) injection. For panobinostat, mice were treated with 25 mg/kg Monday–Friday (M–F) for the first week and then 15 mg/kg M–F thereafter as described previously (20). For dinaciclib, mice were given 30 mg/kg every third day. To simulate cytosine arabinoside/anthracycline-based induction chemotherapy, mice were treated every 24 hours with 100 mg/kg of cytarabine from days 1 to 5 and 3 mg/kg of doxorubicin from days 1 to 3.

MV4-11 cells were transduced with MSCV-luciferase-IRES-Cherry vector and cherry-positive cells were sorted by flow cytometry and xenografted by tail vein injection into NSG mice two days after preconditioning with 5 daily doses of 150 mg/kg cyclophosphamide. Dinaciclib, 30 mg/kg, was administered every third day from day 7 posttransplant.

Treated mice were monitored by peripheral blood analysis and *in vivo* imaging using the IVIS100 bioluminescence imaging system (Perkin Elmer) as described previously (20).

Quantitative RT-PCR

RNA was prepared using TRIzol (Invitrogen, Life Technologies) or NucleoSpin RNA extraction kit, (Macherey-Nagel) according to manufacturer's instructions and cDNA was synthesized using TaqMan (Applied Biosystems, Life Technologies). Quantitative RT-PCR analysis was performed using SYBR green (Applied Biosystems) on an iCycler mounted with an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Signals were quantified using the $\Delta\Delta C_t$ method and normalized to L32 levels. Primer sequences (GeneWorks): *Mus musculus Hoxa9* [forward (F): GAG AGC GGC GGA GAC AAG CC; reverse (R): TCG TAC CTG CGG TCC CGT GT], *Mus musculus cMyc* (F: CAA ATC CTG TAC CTC GTC CGA TTC; R: CTT CTT GCT CTT CTT CAG AGT CGC), *Mus musculus Meis1* (F: ACA CAG TGG GGA TAA CAG CAG; R: TTT TTG TGA CGC TTT TTG TCC), *Homo sapiens CDK9* (F: AAA GTC TGC CAG CTT CAG GA; R: ACT TCT GCG AGC ATG ACC TT), *Mus musculus Mcl-1* (F: GGT GCC TTT GTG GCC AAA CAC TTA; R: ACC CAT CCC AGC CTC TTT GTT TGA), *Mus musculus Bcl-2* (F: ATG ACT GAG TAC CTG AAC CGG CAT; R: GGG CCA TAT AGT TCC ACA AAG GCA), *Mus musculus GAPDH* (F: CCT TCA TTG ACC TCA ACT AC; R: GGA AGG CCA TGC CAG TGA GC).

shRNA transfections

Platinum select MLP retroviral shRNA-mir vectors were obtained from Transomic Technologies. THP1 cells were incubated for 24 hours with 0.5 μg of plasmid DNA and 5 μL lipofectamine before FACS sorting for GFP positivity prior to further analyses. *CDK9* shRNA sequences were; *CDK9*#1 CAA CAC GAG AAT GTG GTC AAC ATA GTG AAG CCA CAG ATG TAT GTT GAC CAC ATT CTC GTG TTT; *CDK9*#2 CCA AGA TCC TTC AGC TTC TAA ATA GTG AAG CCA CAG ATG TAT TTA GAA GCT GAA GGA TCT TGA; *CDK9*#3 CCA CGA GAA TGT GGT CAA CTT ATA GTG AAG CCA CAG ATG TAT AAG TTG ACC ACA TTC TCG TGT.

Statistical analysis

Appropriate results were expressed in graphs with means ± SEM. Differences between groups of multiple samples from the same source were determined using one-way ANOVA and two-sample paired and nonpaired *t* tests were used to determine differences between groups. Statistical significance was set at $P < 0.05$. All statistics were performed on GraphPad Prism 6.01 with α at $P < 0.05$ for statistical significance.

Results

Panobinostat and dinaciclib induce death of MLL-AF9 tumor cells *in vitro*

We previously developed a preclinical murine model of MLL-AF9–driven AML using retroviral vectors expressing MLL-AF9 linked to a GFP construct, and a luciferase reporter gene that is coexpressed with $NRAS^{G12D}$ or the fluorescent cherry protein (25). Previous studies have shown that these leukemias were relatively insensitive to conventional chemotherapeutics (25) and we therefore sought to identify therapeutic agents that would effectively kill MLL-AF9–driven AMLs. Three independently derived primary AMLs expressing MLL-AF9 with (MLL-AF9; $NRAS^{G12D}$) and without (MLL-AF9;cherry) coexpression of oncogenic RAS were subsequently used to test the cell death–inducing activity of small molecules targeting HDACs (panobinostat), CDK9 (dinaciclib), DOT1L (EPZ4777), and BET bromodomian proteins (JQ1), or the chemotherapeutic agents cytosine arabinoside and etoposide in a 24-hour assay. JQ1, EPZ4777, and etoposide had minimal effect on the short-term viability of all leukemias studied, while panobinostat and cytosine arabinoside were effective against certain leukemias and only at relatively high concentrations in the majority (Fig. 1). The most potent cell death responses were observed using dinaciclib, with this agent consistently inducing death of all six independently derived murine MLL-AF9 leukemias using the 24-hour assay (Fig. 1). Combination studies using dinaciclib and the two agents that demonstrated weaker antileukemic effects (panobinostat and cytosine arabinoside) did not reveal additive or synergistic activities between the agents. In fact, both panobinostat and cytosine

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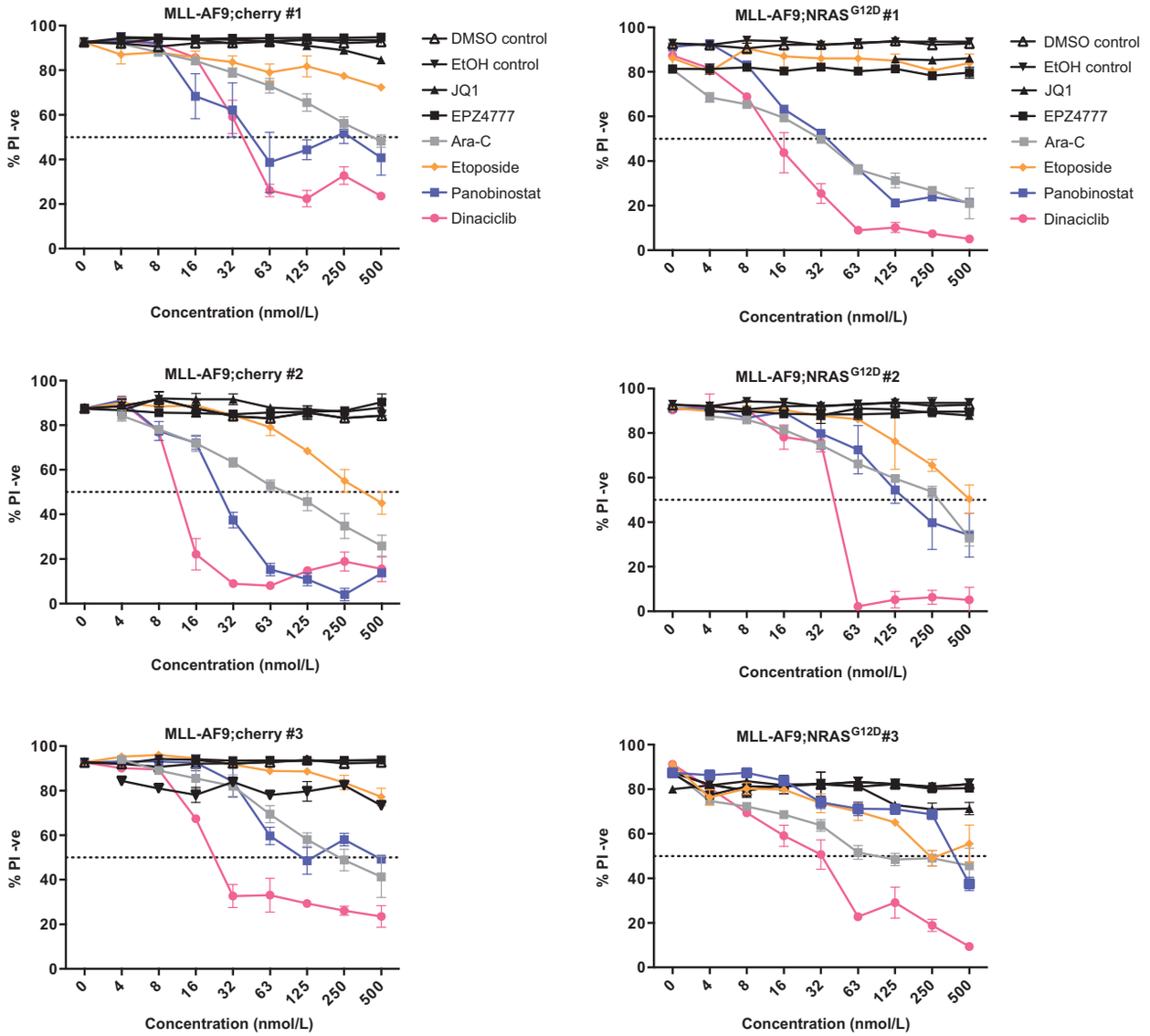

Figure 1.
MLL-AF9 murine leukemias show differential sensitivity to select targeting of different recruitment complex components. Three MLL-AF9 murine AMLs with (MLL-AF9;NRAS^{G12D}) and three without (MLL-AF9;cherry), oncogenic NRAS^{G12D}, were treated with various small-molecule inhibitors or chemotherapy at increasing concentrations for 24 hours, and then analyzed for viability via flow cytometry (PI exclusion). Data are presented as means ± SEMs from three different experiments. Ara-C, cytosine arabinoside.

arabinoside (Supplementary Fig. S1A and S1B) antagonized the apoptotic effects of dinaciclib.

We next tested our panel of agents for activity against primary murine leukemia expressing MLL-ENL and NRAS^{G12D} (MLL-ENL;NRAS^{G12D}) and against human AML cell lines expressing MLL-AF9 (THP-1) and MLL-AF4 (MV4-11) fusion proteins (Fig. 2A). In all instances, dinaciclib was the most potent death-inducing agent in a 24-hour cell death assay, indicating that CDK inhibition effectively kills tumors driven by different MLL fusion proteins. Interestingly, AML cell lines (Kasumi-1 and HL60) that do not express MLL-FPs were less sensitive to dinaciclib than THP-1 or MV4-11 cells (Fig. 2A). The sensitivity of MLL-rearranged leukemias to dinaciclib was further demonstrated using patient-derived AMLs (Fig. 2B). Dinaciclib reduced the viability of primary leukemias cultured *ex vivo*, with AMLs expressing MLL fusion proteins (AML-17 and AML-18) being particularly sensitive.

To confirm apoptotic cell death following treatment with dinaciclib, we demonstrated phosphatidylserine exposure, DNA fragmentation, and mitochondrial outer membrane permeabilization in dinaciclib- and panobinostat-treated MLL-AF9;NRAS^{G12D} cells (Fig. 2C). Also consistent with apoptotic cell death, the pan-caspase inhibitor QVD-OPh rescued cell viability following dinaciclib exposure (Fig. 2D). Finally, both dinaciclib and panobinostat reduced the colony-forming potential of MLL-AF9;NRAS^{G12D} cells in a dose-dependent manner, with dinaciclib again being the more

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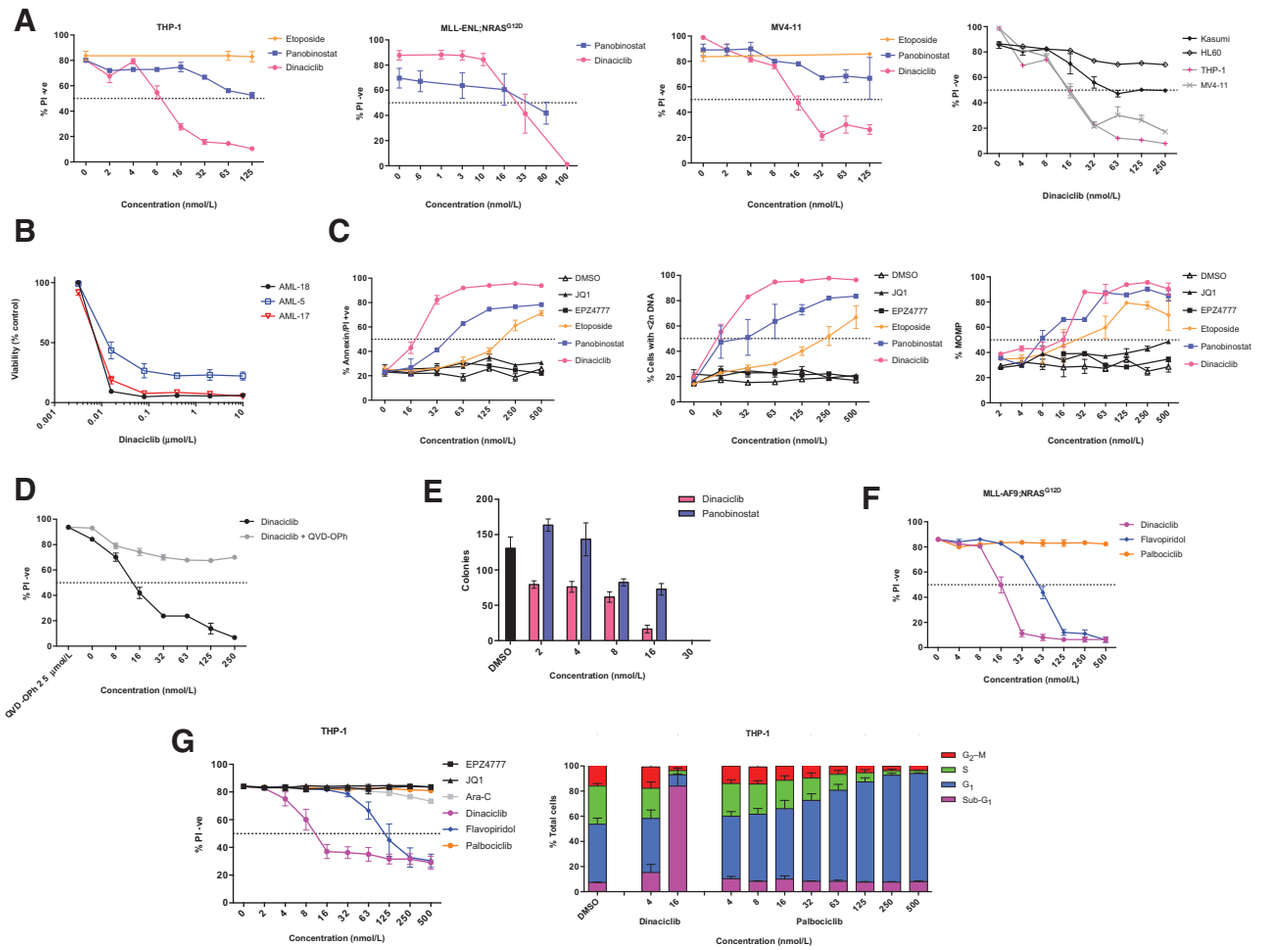

Figure 2.
Apoptosis observed with MLL-SEC targeting is selective according to presence or absence of the MLL rearrangement. A, human THP-1 and MV4-11 cells were treated with increasing concentrations of etoposide, panobinostat, and dinaciclib for 24 hours. Murine MLL-ENL;NRAS^{G12D} cells were treated with panobinostat and dinaciclib for 24 hours. Human Kasumi, HL60, THP-1, and MV4-11 cells were treated with increasing concentrations of dinaciclib for 24 hours. Viability was assessed by flow cytometry for PI exclusion. Data are presented as means ± SEMs ($n = 3$). B, patient-derived AML specimens cocultured with the mouse bone marrow stromal cell line MS-5 were exposed to dinaciclib for 24 hours prior to flow cytometric viability analysis using human CD45, Annexin V, and 7AAD. C, murine MLL-AF9;NRAS^{G12D} cells were treated with representative compounds for 24 hours. Flow cytometric evaluation for Annexin-V/PI positivity, DNA fragmentation, and mitochondrial outer membrane permealibilization was assessed as independent readouts for apoptosis. D, murine MLL-AF9;NRAS^{G12D} cells were cultured for 24 hours in the presence or absence of the pan-caspase inhibitor QVD-OPh prior to viability assessment (PI exclusion) by flow cytometry. E, murine MLL-AF9;NRAS^{G12D} cells were treated with the indicated concentrations of panobinostat or dinaciclib for 24 hours. The cells were washed and then plated in soft agar for 21 days and the colonies were counted. F, murine MLL-AF9;NRAS^{G12D} cells were treated with increasing concentrations of dinaciclib, flavopiridol, or palbociclib for 24 hours prior to viability analysis by flow cytometry for PI exclusion. G, human THP-1 cells were treated with various small-molecule inhibitors or chemotherapy at increasing concentrations for 24 hours and then analyzed for viability by flow cytometry for PI exclusion. Cells concurrently treated with palbociclib or dinaciclib were analyzed for cytostatis according to nuclear DNA content.

potent agent (Fig. 2E). To further probe the effect of CDK inhibition on the survival of MLL-AF9;NRAS^{G12D} cells, we tested the effect of flavopiridol, a small-molecule inhibitor of CDKs 1, 2, 4, 6, 7, 9, and 12 (33, 34) and the more selective CDK 4/6 inhibitor palbociclib (35). As shown in Fig. 2F, flavopiridol was less potent than dinaciclib at equimolar concentrations while palbociclib had no effect on the survival of MLL-AF9;NRAS^{G12D} leukemias. Similar responses were observed when MLL-AF9–expressing THP-1 cells were exposed to dinaciclib, flavopiridol, and palbociclib (Fig. 2G). These cells were more sensitive to dinaciclib than flavopiridol while palbociclib did not induce apoptosis but did mediate cell-cycle arrest at the G_1–S checkpoint (Fig. 2G).

Dinaciclib inhibits the activity of CDK9, reduces expression of MLL-FP target genes, and induces apoptosis through downregulation of Mcl-1

As dinaciclib was identified as the most potent apoptosis-inducing agent against MLL-driven leukemias, we sought to link this biologic response with the kinase-inhibitory activity of this agent. Dinaciclib potently inhibits CDK9 and phosphorylation of the RNA polymerase II CTD at Ser2 may be used as a biomarker of CDK9 activity (36). As shown in Fig. 3A, treatment of MLL-AF9;NRAS^{G12D} cells with dinaciclib resulted in a dose-dependent reduction in phospho-RNA Pol II. To demonstrate that dinaciclib downregulates MLL-AF9 target genes, qRT-PCR analysis was

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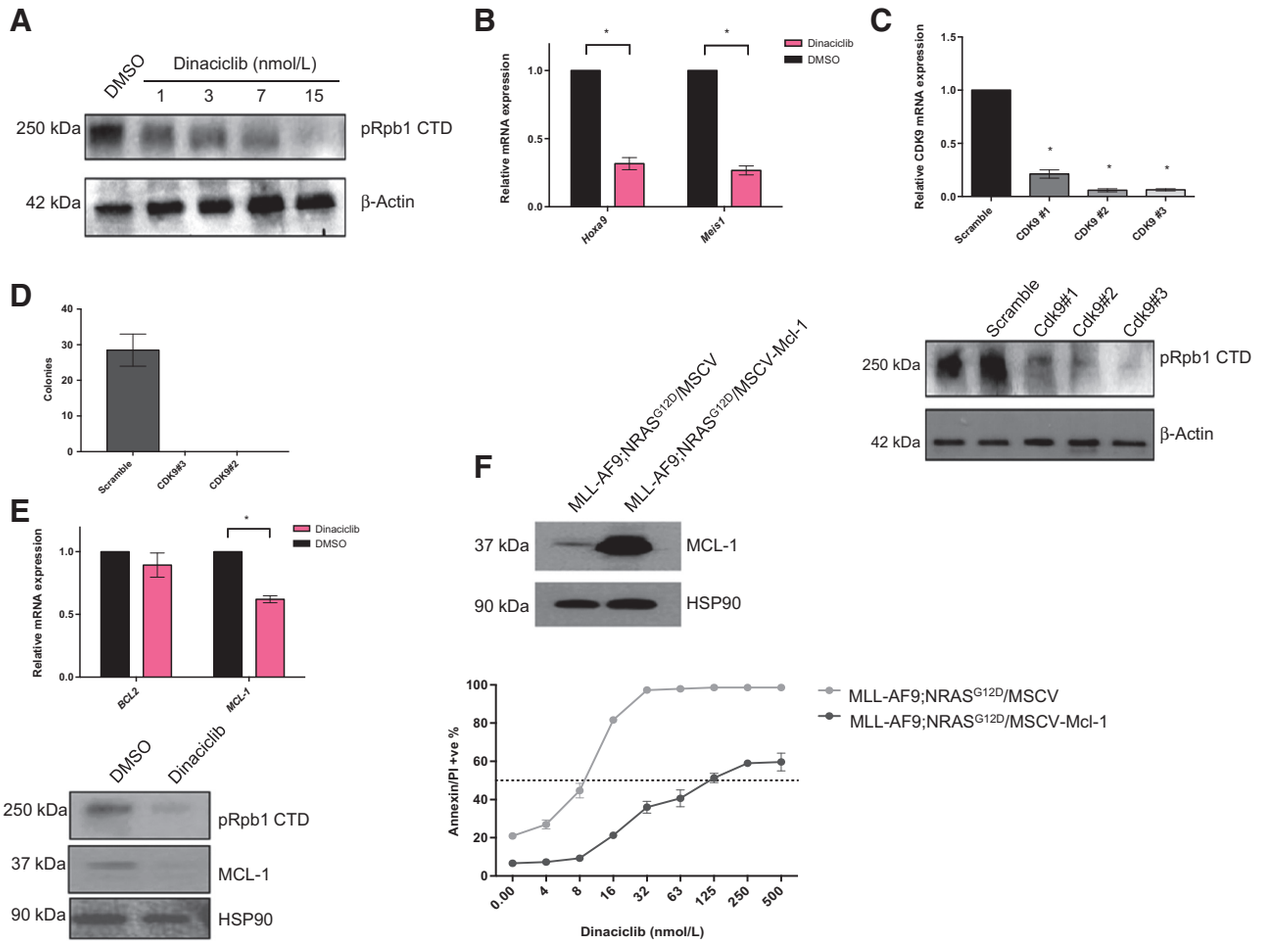

Figure 3.

CDK9 inhibition by dinaciclib phenocopies genetic depletion of CDK9 and is associated with reduced expression of MLL target genes and Mcl-1–dependent apoptosis induction. A, murine MLL-AF9;NRAS^{G12D} cells were cultured in the presence of dinaciclib for 6 hours prior to protein lysate preparation, then Western blotting for pRpb1 CTD (Ser2/5). β-Actin loading control shown. B, murine MLL-AF9;NRAS^{G12D} cells were cultured in the presence of dinaciclib for 6 hours prior to RNA extraction and subsequent assessment of *Hoxa9* and *Meis1* mRNA levels by qRT-PCR. (HOXA9, $P = 0.0001$; Meis1, $P \leq 0.0001$). C, THP-1 cells transfected with vectors expressing scramble (control) shRNA or three different shRNAs targeting CDK9 were isolated by FACS and *CDK9* mRNA (top) was determined by qRT-PCR. In these cells, reduced pRpb1 CTD (Ser2/5) was assessed by Western blot analysis (bottom). D, THP-1 cells transfected with vectors expressing scramble (control) shRNA or two different shRNAs targeting CDK9 were FACS sorted and then plated in soft agar. Viable colony numbers after 21 days of culture were assessed by MTT assay. E, MLL-AF9;NRAS^{G12D} leukemia cells were incubated with DMSO or 30 nmol/L dinaciclib for 3 hours and expression of *Mcl-1* and *Bcl-2* mRNA was assessed by qRT-PCR (top). Transcript levels represent fold-change compared with DMSO. Using this same treatment regimen, Mcl-1 protein expression and the phosphorylation status of RNA Pol II CTD was determined by Western blot analysis (bottom). F, MLL-AF9;NRAS^{G12D} leukemia cells were transduced with murine stem cell virus expressing empty vector control (MSCV) or *Mcl-1*. Western blot analysis displays Mcl-1 protein expression of cells transduced with representative vectors. MLL-AF9;NRAS^{G12D} leukemia cells transduced with MSCV or *Mcl-1* were incubated with dinaciclib for 24 hours prior to flow cytometric analysis of Annexin-V/PI positivity.

performed to assess the expression of *Meis1* and *Hoxa9*. Dinaciclib treatment of MLL-AF9;NRAS^{G12D} cells resulted in robust decrease in the expression of these MLL-AF9 targets following exposure for as little as 6 hours (Fig. 3B). To confirm that CDK9 was essential for the growth and survival of MLL-driven AML, we developed retroviral vectors expressing a GFP reporter gene and shRNAs targeting human CDK9. Transduction of THP-1 cells with retrovirus expressing a control (scrambled) shRNA had no effect on *CDK9* mRNA expression or phosphorylation of RNA Pol II (Fig. 3C). Importantly, knockdown of CDK9 using two different shRNAs reduced RNA Pol II CTD phosphorylation (Fig. 3C) and completely suppressed the colony-forming capacity of THP-1 cells (Fig. 3D). Taken together, these data indicate that pharmacologic inhibition or genetic depletion of CDK9 has a detrimental effect on the growth and survival of AML cells expressing MLL-FPs.

Expression of the antiapoptotic Bcl-2 family member Mcl-1 can be regulated by CDKs (37) and we have recently demonstrated that dinaciclib causes downregulation of Mcl-1 in primary mouse B-cell lymphomas (23). As shown in Fig. 3E, treatment of MLL-AF9;NRAS^{G12D} cells with dinaciclib for 3 hours resulted in a significant decrease in *Mcl-1* mRNA levels but no change in the related prosurvival gene *Bcl-2*. The dinaciclib-induced decrease in Mcl-1 levels resulted in decreased expression of the Mcl-1 protein concomitant with dinaciclib-mediated dephosphorylation of the RNA Pol II CTD (Fig. 3E). To demonstrate the functional

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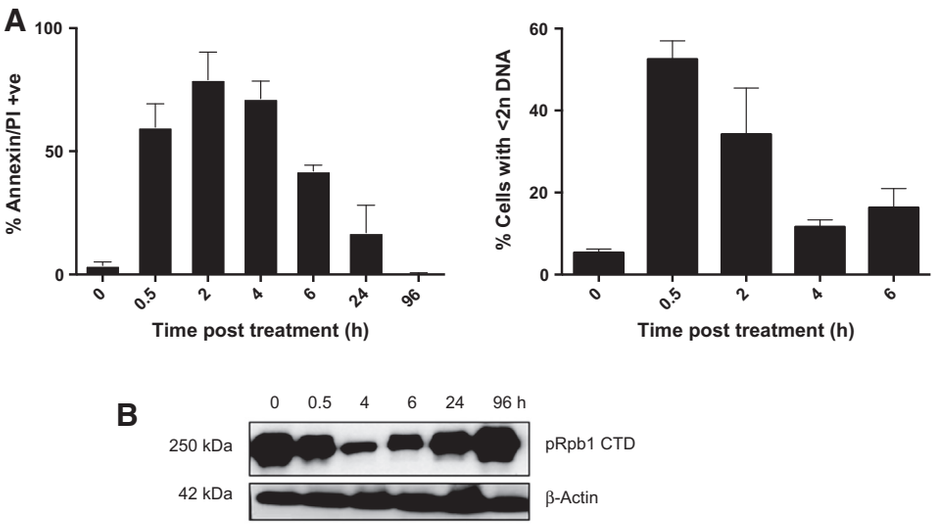

Figure 4.
Dinaciclib induces apoptosis *in vivo*. A, C57BL/6 mice transplanted with MLL-AF9;NRAS^{G12D} leukemia cells were treated with dinaciclib at 30 mg/kg and cells were harvested from the bone marrow and spleen at the indicated times. Cells were assessed for markers of apoptosis, including Annexin V staining and PI uptake (A; left) and DNA content (right); B, phosphorylation of RNA polymerase II CTD by Western blot analysis. The Western blot analysis is a representative of three independent experiments.

importance of dinaciclib-induced downregulation of Mcl-1, we used retroviral transduction to overexpress the protein in MLL-AF9;NRAS^{G12D} cells and observed the derived MLL-AF9;NRAS^{G12D};Mcl-1 cells to be protected from dinaciclib-induced apoptosis over a range of different drug concentrations (Fig. 3F). Taken together, these experiments indicate that the decrease in Mcl-1 expression following exposure to dinaciclib is important for the apoptotic effects of the compound.

Dinaciclib induces apoptosis of MLL-AF9–driven leukemia cells *in vivo*, resulting in a reduction in tumor burden and therapeutic efficacy

C57/BL6 mice were transplanted with *MLL-AF9;NRAS*^{G12D} tumors and following tumor establishment, cells were harvested at various times following dinaciclib treatment. Treatment of tumor-bearing mice for a little as 30 minutes resulted in an increase in apoptosis of *MLL-AF9;NRAS*^{G12D} tumors residing in the spleens of these animals as measured using Annexin V/PI staining and DNA fragmentation (Fig. 4A). Dinaciclib-mediated *in vivo* apoptosis occurred concomitantly with a reduction of phospho-RNA Pol II (Fig. 4B) providing a direct correlation between on-target molecular activity of the compound and antitumor biologic responses.

To determine the therapeutic potential of dinaciclib, C57BL/6 mice bearing established *MLL-AF9;NRAS*^{G12D} tumors were treated with vehicle or the compound at the maximum tolerated dose. Treatment with dinaciclib resulted in a significant decrease in tumor burden as measured by a decrease in white blood cell counts and a reduction in bioluminescence (Fig. 5A). Treatment of tumor-bearing mice with dinaciclib resulted in an overall survival benefit, with median survival times of 37 days in the Dinaciclib-treated cohort compared with 19 days for the vehicle-treated mice (Fig. 5A, $P \leq 0.0001$). The therapeutic effects of dinaciclib were greater than observed when mice bearing established *MLL-AF9;NRAS*^{G12D} tumors were treated with panobinostat or a chemotherapy regimen consisting of cytarabine and doxorubicin at the MTD (Fig. 5B and C). Treatment with panobinostat resulted in a median survival time of 19 days compared with the control cohort that had a median survival of 15 days. The chemotherapy regimen resulted in a median survival time of 20 days compared with 16 days for vehicle-treated mice. These results confirm the potent antitumor effects of dinaciclib and demonstrate a direct correlation between the potency of the compound *in vitro* with *in vivo* therapeutic effects.

To further demonstrate the therapeutic effects of dinaciclib against MLL-AF9–driven AML in the absence of an engineered *Ras* mutation, mice bearing established *MLL-AF9;cherry* leukemias were treated with dinaciclib or vehicle and the effects *in vivo* were monitored. Similar to the effects shown in Fig. 5A, treatment with dinaciclib significantly decreased leucocytosis and reduced tumor burden as demonstrated by decreased bioluminescence (Fig. 6A). Treatment with dinaciclib resulted in a significant prolongation of the survival of mice transplanted with MLL-AF9;cherry tumors (Fig. 6A, median survival of dinaciclib-treated mice = 86 days; median survival of vehicle-treated mice = 60 days, $P \leq 0.0002$). In contrast, the antitumor effects of panobinostat (Fig. 6B, median survival of panobinostat-treated mice = 62 days; median survival of vehicle-treated mice = 53 days) and chemotherapy (Fig. 6C, median survival of chemotherapy-treated mice = 71 days; median survival of vehicle-treated mice = 56 days) were markedly less robust than that observed with dinaciclib.

Therapeutic effects of dinaciclib against human MLL-fusion protein–driven AML tumors

The encouraging results using dinaciclib to treat mice bearing primary mouse MLL-AF9–driven tumors prompted us to further study the potential therapeutic efficacy of dinaciclib in a human MLL-translocated leukemia *in vivo*. Mice with established disseminated MV4-11;MSCV-Luciferase tumors were treated with dinaciclib or vehicle and tumor progression and overall survival of the treated mice were assessed. As shown in Fig. 7, treatment with dinaciclib resulted in a substantial decrease in white blood cell counts that corresponded with a significant survival advantage. Treatment with dinaciclib resulted in a median survival time of 52 days compared with the vehicle-treated control cohort that had a median survival of 33 days.

Discussion

MLL-FP–driven AML is associated with resistance to conventional chemotherapy and poor prognosis (38). A key oncogenic feature of MLL-FPs such as MLL-AF9 and MLL-ENL is their

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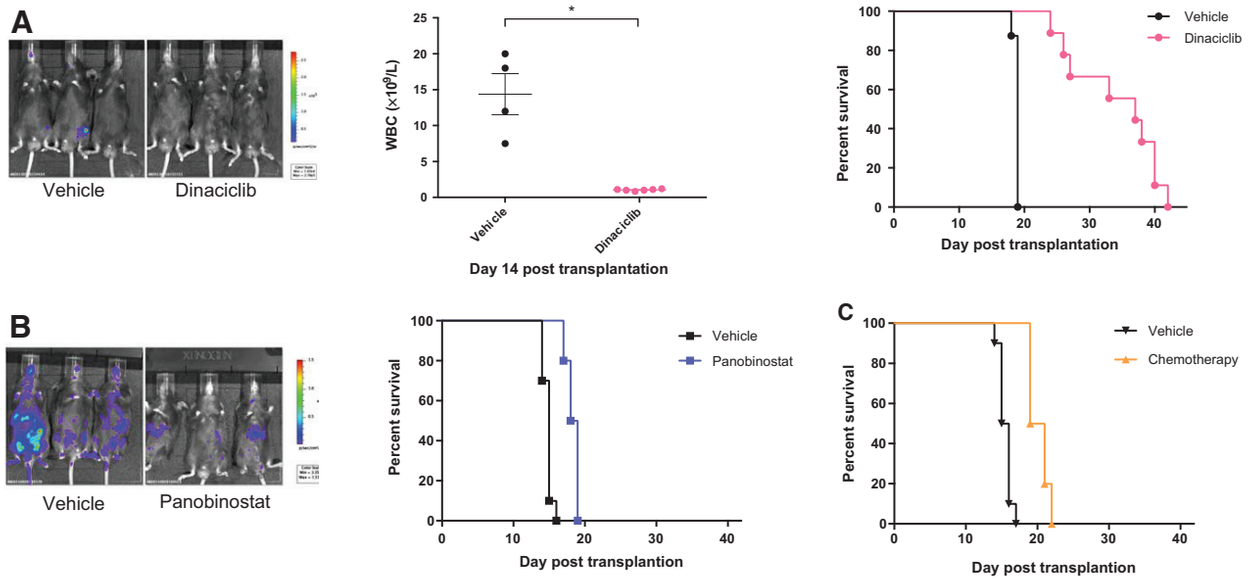

Figure 5.
Therapeutic effects of dinaciclib, panobinostat, and chemotherapy in mice bearing MLL-AF9;NRAS^{G12D} leukemias. C57BL/6 mice were transplanted with MLL-AF9;NRAS^{G12D} leukemia cells and treatment was initiated 7 days after transplantation. A, mice were treated with vehicle or dinaciclib (30 mg/kg every third day) and bioluminescence imaging was performed on day 14 following tumor cell transplantation (left). Mice were treated with vehicle or dinaciclib (30 mg/kg every third day) and assessed for peripheral white blood cell numbers on day 14 (middle) and overall survival (right). Dinaciclib-treated tumor-bearing mice had significantly fewer white blood cell numbers (day 14; $P = 0.0004$) and a Kaplan–Meier survival curve showed a significant difference ($P = < 0.0001$) in median survival for vehicle-treated mice (19 days) compared with dinaciclib-treated mice (37 days). B, mice were treated with vehicle or panobinostat (25 mg/kg M–F for the first week and then 15 mg/kg M–F thereafter) and bioluminescence imaging was performed on day 14 following tumor cell transplantation (left). Mice were treated with vehicle or panobinostat (25 mg/kg M–F for the first week and then 15 mg/kg M–F thereafter) and assessed for overall survival (right). A Kaplan–Meier survival curve showed median survival for vehicle-treated mice of 15 days compared with 18.5 days for panobinostat-treated mice. C, mice were treated with vehicle or chemotherapy (100 mg/kg of cytarabine from days 1–5 and 3 mg/kg of doxorubicin from days 1–3) and assessed for overall survival. A Kaplan–Meier survival curve showed median survival for vehicle-treated mice of 16 days compared with 20 days for chemotherapy-treated mice.

recruitment of large macromolecular complexes that mediate chromatin remodeling and regulate gene transcription initiation and elongation (6–10). Herein, we utilized primary mouse AMLs driven by MLL-AF9 or MLL-ENL and human AML cell lines harboring MLL-FPs to assess the apoptotic and therapeutic effects of small molecules that inhibit key epigenetic and transcriptional regulatory enzymes recruited by these oncogenic fusion proteins. Our initial screen using mouse AMLs driven by MLL-AF9 that were relatively chemorefractory indicated that dinaciclib, an inhibitor of CDKs 1, 2, 5, and 9, was the most potent inducer of apoptosis in a 24-hour assay. Interestingly, inhibition of DOT1L using EPZ004777 or BET bromodomain proteins with JQ1 did not induce apoptosis during the time course of our assays. Previous studies indicated that *in vitro* antitumor effects of EPZ004777 or JQ1 in similar models of MLL-AF9–driven AML are evident, albeit with slower kinetics than described herein (16, 17, 39).

Relatively selective killing of MLL-FP–driven AML by dinaciclib was demonstrated by comparing the effects against human AML cell lines harboring different oncogenic lesions. AMLs expressing MLL-AF9 (THP-1) and MLL-AF4 (MV4-11) fusion proteins were more sensitive than those expressing AML1-ETO (Kasumi) or with a complex karyotype including mutations in *TP53*, *NRAS*, and *CDKN2A* (HL60). This provides some evidence supporting the notion that the leukemias driven by MLL-FPs that recruit the CDK9-containing SECs are preferentially sensitive to CDK9 inhibition compared with other tumors where transcriptional elongation may not be as molecularly important. A recent functional genomics–based screen using gene knockdown technologies demonstrated that inhibition of CDK9 may be an effective therapeutic approach for Myc-driven hepatocellular carcinoma (HCC; ref. 40). Myc-regulated transcriptional elongation is mediated by the CDK9-containing pTEFb complex (41–44) and the anti-HCC effects observed following knockdown of CDK9 were phenocopied by knockdown of c-Myc (40). Consistent with the notion that targeting CDK9 is an effective strategy for the treatment of Myc-driven malignancies, we very recently demonstrated curative responses in dinaciclib-treated mice bearing Myc-driven B-cell lymphomas (23). Interestingly c-Myc expression is important for the maintenance of MLL-AF9–driven AML (8, 17, 45) and it is possible that dinaciclib-mediated inhibition of CDK9 had such a potent effect in our AML models because CDK9 is important for the oncogenic activity of both MLL-AF9 and c-Myc.

In addition to its crucial role in phosphorylating the CTD of RNA Pol II and regulating gene expression, CDK9 also functionally interacts with other cellular proteins including MyoD, p53, pRb, and c-Myc and can affect diverse biologic processes including cell differentiation, survival, and quiescence (46). Herein, we demonstrated that inhibition of CDK9 enzymatic activity resulted in rapid dephosphorylation of RNA Pol II and induction of apoptosis in AML cells and we identified *Mcl-1* as a gene crucial for this response. Proteins with relatively short half-lives such as Mcl-1 appear to be selectively reduced following CDK9 inhibition (23, 47–49). Dinaciclib treatment resulted in almost complete loss of Mcl-1 expression within 3 hours demonstrating that this

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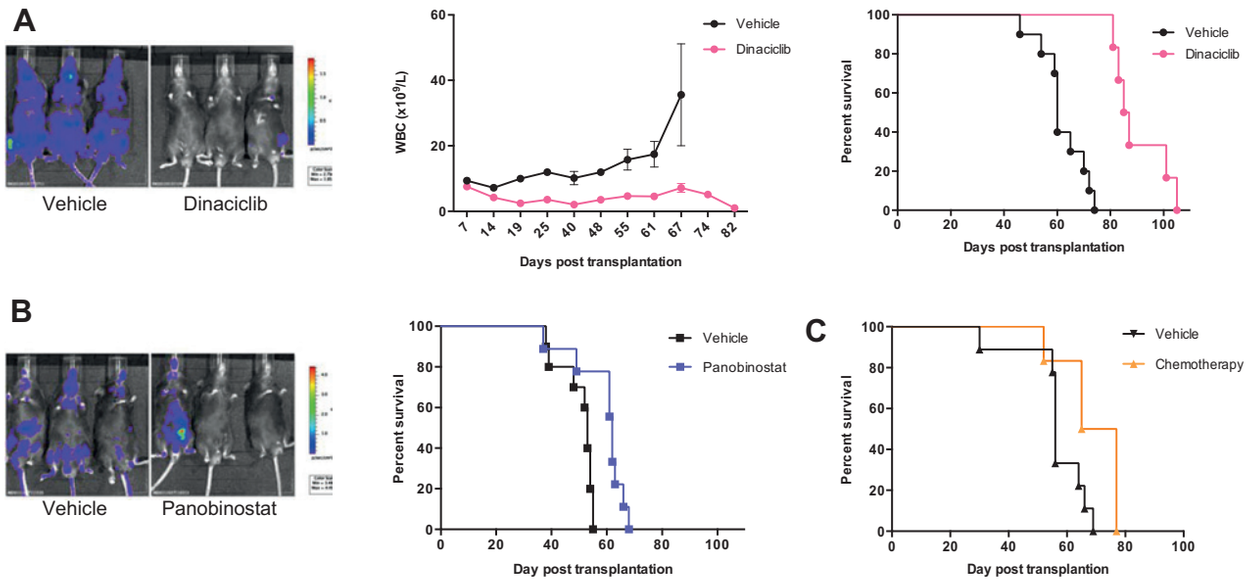

Figure 6.
Therapeutic effects of dinaciclib, panobinostat, and chemotherapy in mice bearing MLL-AF9;cherry leukemias. C57BL/6 mice were transplanted with MLL-AF9;cherry leukemia cells and treatment was initiated 7 days after transplantation. A, mice were treated with vehicle or dinaciclib (30 mg/kg every third day) and bioluminescence imaging was performed on day 40 following tumor cell transplantation (left). Mice were treated with vehicle or dinaciclib (30 mg/kg every third day) and assessed for peripheral white blood cell numbers (middle) and overall survival (right). Dinaciclib-treated tumor-bearing mice had fewer white blood cell numbers and a Kaplan–Meier survival curve showed a significant difference ($P \leq 0.0002$) in median survival to death from leukemia for vehicle-treated mice (60 days) compared with dinaciclib-treated mice (86 days). B, mice were treated with vehicle or panobinostat (25 mg/kg M–F for the first week and then 15 mg/kg M–F thereafter) and bioluminescence imaging was performed on day 42 following tumor cell transplantation (left). Mice were treated with vehicle or panobinostat (25 mg/kg M–F for the first week and then 15 mg/kg M–F thereafter) and assessed for overall survival (right). A Kaplan–Meier survival curve showed median survival for vehicle-treated mice of 53 days compared with 62 days for panobinostat-treated mice. C, mice were treated with vehicle or chemotherapy (100 mg/kg of cytarabine from days 1–5 and 3 mg/kg of doxorubicin from days 1–3) and assessed for overall survival. A Kaplan–Meier survival curve showed median survival for vehicle-treated mice of 56 days compared with 71 days for chemotherapy-treated mice.

approach may be utilized to "indirectly" target Mcl-1. Recent elegant genetic studies shown the essential role of Mcl-1 in the development and maintenance of MLL-FP–driven AML (50). We demonstrated that downregulation of Mcl-1 by dinaciclib was functionally important as ectopic overexpression of Mcl-1 rescued tumor cells from dinaciclib-induced apoptosis. On the basis of these studies, we posit that downregulation of Mcl-1 could serve as a biomarker for response to dinaciclib and pharmacologic inhibition of CDK9 may be an attractive method to target Mcl-1 in AML and a range of other hematologic malignancies and solid tumors (51) that are dependent on sustained expression of this antiapoptotic protein.

In addition to CDK9, dinaciclib inhibits other CDKs (1, 2, 5) at approximately equipotent single-digit nanomolar concentrations

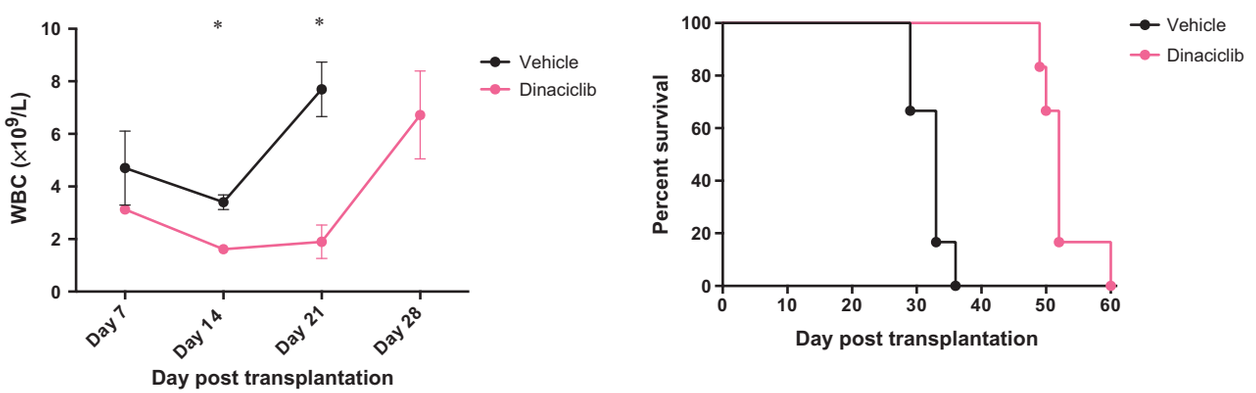

Figure 7.
Therapeutic effects of dinaciclib in NSG mice bearing MV4-11 xenografts. NSG mice transplanted with MV4-11;MSCV-luciferase cells and treatment was initiated 7 days after transplantation. Mice were treated with vehicle or dinaciclib (30 mg/kg every third day) and assessed for peripheral white blood cell numbers (left) and overall survival (right). Dinaciclib-treated tumor-bearing mice had significantly fewer white blood cell numbers (day 14; $P = 0.0004$) and a Kaplan–Meier survival curve showed a significant difference ($P = 0.0006$) in median survival for vehicle-treated mice (33 days) compared with dinaciclib-treated mice (52 days).

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(52) and was recently shown to bind to members of BET family of bromodomain-containing proteins, albeit in the higher micromolar range (53). Our data demonstrating that the antitumor effects of dinaciclib occurred concomitantly with inhibition of CDK9 activity as shown by reduced phosphorylation of RNA Pol II and that knockdown of CDK9 phenocopied the biologic (loss of AML clonogenic growth) and molecular (RNA Pol II dephosphorylation) effects of dinaciclib provide evidence that inhibition of CDK9 has important antileukemic consequences. Indeed, other agents capable of inhibiting CDK9 can also kill human AML cell lines and primary human leukemias *in vitro* (54–57). Comparison of the effects of dinaciclib with the broad CDK inhibitor flavopiridol and the more selective CDK 4/6 palbociclib revealed that flavopiridol also induced rapid apoptosis of MLL-AF9–driven AML cells, albeit at higher concentrations than dinaciclib, while palbociclib had no effect on cell survival. These data indicate that inhibition of CDK4 and/or CDK6 is not sufficient to phenocopy the effects of dinaciclib and provides further evidence supporting the role of CDK9 inhibition in mediating antileukemic apoptotic effects. Previous studies demonstrated that knockdown of CDK6 or inhibition of enzymatic activity resulted in delayed cell proliferation and induction of myeloid differentiation (58) as opposed to the apoptotic effects we observe using dinaciclib or flavopiridol. Collectively, these studies indicate that different CDKs likely play disparate roles in regulating proliferation, differentiation, and survival of MLL-rearranged AML.

Our *in vivo* data demonstrating the efficacy of dinaciclib in mice bearing disseminated syngeneic or xenogeneic MLL-FP–driven AML in the absence of overt toxicity provides preclinical evidence that targeting CDK9 in humans with AML should be considered for further evaluation. The antitumor effects using dinaciclib to treat mice bearing murine MLL-AF9 AML were significantly greater than the responses we observed using panobinostat or a chemotherapeutic regimen. These preclinical findings highlight the potential therapeutic value of dinaciclib for patients with AML harboring MLL translocations. Dinaciclib has already been shown to be deliverable in humans and initial clinical trials in patients with chronic lymphocytic leukemia (59), multiple myeloma (60), and solid tumors (61) have demonstrated the therapeutic effects of dinaciclib in those settings. A recent phase II study of dinaciclib including 14 patients with relapsed/refractory AML demonstrated dramatic reductions in blast counts in the majority of patients, associated with biochemical tumor lysis syndrome and acute reductions in Mcl-1 (62). However, formal remissions were not demonstrated due to pharmacokinetic limitations in heavily disease-burdened patients and the cohort was not enriched for *MLL*-translocated disease. Our studies provide evidence supporting the extended clinical utilization of dinaciclib in AML, especially in the context of MLL-FP–driven disease.

Disclosure of Potential Conflicts of Interest

R.W. Johnstone reports receiving a commercial research grant, has received speakers bureau honoraria, and is a consultant/advisory board member for Novartis. J. Shortt has received speakers bureau honoraria from Novartis. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Baker, G.P. Gregory, L., J.J. Hilton, E. Vidacs, E.M. Lee, R.B. Lock
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Baker, G.P. Gregory, L. Kats, J.J. Hilton, E. Vidacs, E.M. Lee, R.B. Lock, J. Shortt
Writing, review, and/or revision of the manuscript: A. Baker, G.P. Gregory, I. Verbrugge, E. Vidacs, R.B. Lock, J. Zuber, J. Shortt, R.W. Johnstone
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Baker, G.P. Gregory, E. Vidacs, E.M. Lee, J. Zuber
Study supervision: J. Shortt, R.W. Johnstone
Other (designed, conducted, and analyzed initial prepublication experiments (*in vitro* and *in vivo*) with A. Baker and R.W. Johnstone): I. Verbrugge

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