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Rational Combination Therapy for Melanoma with Dinaciclib by Targeting BAK-Dependent Cell Death

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ABSTRACT

Mutation of the oncogene BRAF is among the most common genetic alterations in melanoma. BRAF inhibitors alone or in combination with MEK inhibitors fail to eradicate the tumor in most patients due to combinations of intrinsic or acquired resistance. Therefore, novel strategies are needed to improve the therapeutic efficacy of BRAF inhibition. We demonstrated that dinaciclib has potent antimelanoma effects by inducing BAK-dependent apoptosis through MCL1 reduction. Contrary to dinaciclib, the inhibitors of BRAF/MEK/CDK4/6 induced apoptosis dominantly through a BAX-dependent mechanism. Although the combination of BRAF and MEK inhibitors did not exhibit additive antimelanoma effects, their combination with dinaciclib synergistically inhibited melanoma growth both *in vitro* and *in vivo*. Collectively, our present findings suggest dinaciclib to be an effective complementary drug of BAX-dependent antimelanoma drugs by targeting BAK-mediated apoptosis, and other such rational drug combinations can be determined by identifying complementary drugs activating either BAK or BAX.

Introduction

Cutaneous melanoma is one of the leading causes of cancer death with an increasing incidence worldwide (1). BRAF and NRAS are oncogenes frequently mutated in melanoma (2). Successful therapeutic advancements in the past decades have been achieved for monotherapies, including BRAF inhibitors (3); however, the benefits of BRAF inhibitor monotherapy are only temporary and almost all patients eventually relapse (3–5). Several acquired resistance mechanisms to BRAF inhibitors have been identified, some of which are related to reactivation of the downstream molecules MEK1/2. Although the combination therapy of a BRAF inhibitor with a MEK inhibitor was recently approved due to delayed acquired resistance (6), improvements are needed to combat intrinsic resistance to BRAF inhibition. Thus, additional therapeutic strategies are required for the optimal clinical effects of targeted therapy to be observed in patients with melanoma.

Apoptosis through the mitochondrial pathway is induced by multiple molecular-targeted drugs or anticancer drugs, which are regulated by BCL-2 family members (7). The activation and oligomerization of effectors of the BCL-2 family, BAX and BAK, play essential roles in this apoptotic pathway, and can be inhibited by the binding of antiapoptotic BCL2 family members (BCL2, BCL-w, BCL-xL, MCL1, and BCL2A1) with some selectivity; all five members bind to BAX, whereas BCL-xL, MCL1, and BCL2A1 bind to BAK (8). BAX/BAK inhibition by antiapoptotic BCL-2 family members is associated with intrinsic resistance to some molecular-targeted drugs or anticancer drugs in different malignancies. For example, inhibitors of the BCL2 family, such as obatoclax, increase the apoptotic rate in combination with some molecular-targeted drugs, including BRAF inhibitors (9). Thus, effective activation of both BAX and BAK may provide opportunities to overcome intrinsic resistance to drugs, resulting in improved outcomes for poor-prognosis patients with cancer.

The cyclin-dependent kinase (CDK) family is known to regulate cell-cycle progression or gene transcription (10). Although some CDK4/6 inhibitors, including palbociclib, have been approved by the FDA for some breast cancers, there are additional opportunities to target distinct CDKs such as CDK2 or CDK9 (11). CDK2 was reported to play an important role in melanoma growth and proliferation, but not in other cancers (12). On the other hand, CDK9 was demonstrated to regulate the transcription of MCL1, one of the important antiapoptotic BCL2 family members, in several cancers (13–15). In addition to cytostatic effects through cell-cycle arrest by some CDK inhibitors, cytotoxic effects have also been observed with these agents (16).

In this study, we found that dinaciclib functions as BAK-dependent anticancer drugs in human melanoma cells. In addition to cell-cycle arrest in melanoma cells, dinaciclib also activated the mitochondrial apoptotic pathway through MCL1 suppression. We further clarified that growth suppression with a BRAF inhibitor, vemurafenib, is dependent upon BAX in BRAF mutant human melanoma cells. MEK or CDK4/6 inhibitors also induced apoptosis in melanoma cells dominantly through a BAX-dependent mechanism. Furthermore, the combination of dinaciclib with these BAX-dependent inhibitors synergistically inhibited melanoma growth both *in vitro* and *in vivo*.

Materials and Methods

Reagents

Dinaciclib was purchased from MedChem Express, and CDK2/9 inhibitor (iCDK2/9; CAS No. 507487-89-0), Purvalanol A, and SNS032 were purchased from Merck Millipore. Atuveciclib and LDC000067 were purchased from Selleck. The BRAF V600E inhibitor vemurafenib and MEK inhibitor trametinib were purchased from LC

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Laboratories. For the *in vitro* experiments, all of the chemical inhibitors were formulated in DMSO and the final concentration of DMSO was <0.1%.

Plasmid preparation and lentivirus production

HA/MCL1 sequences from pcDNA3.1-HA/MCL1 (17) and E2-Crimson sequences from pCMV-E2 Crimson (Takara Bio) were subcloned into pENTR1A, and inserted into pLenti CMV Hygro DEST (w117-1), which was a gift from Dr. Campeau E. and Dr. Kaufman P. (Addgene plasmid no. 17454; ref. 18). Lentivirus particles were produced as described previously (9).

Cell cultures

Human melanoma cell lines A2058 and MeWo were from ATCC. A2058, M14, UACC257, UACC62, SK-MEL-2, and MeWo melanoma cell lines were cultured in RPMI1640. SK-MEL-28 melanoma cell line was cultured in DMEM. All of the media were supplemented with 2 mmol/L L-glutamine, 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. A2058/BAX$^{-/-}$ cells and A2058/BAK$^{-/-}$ cells were established using CRISPR-Cas9 as described previously (19). To establish A2058 cells stably expressing E2-Crimson or HA/MCL1, A2058 cells were infected with lentivirus particles and selected in 400 μg/mL of hygromycin for 2 weeks.

Cell growth assay

Human melanoma cells were seeded on 96-well plates (5×10^3 cells/well). After overnight culture, cells were treated with dinaciclib, CDK2/9 inhibitor, palbociclib, vemurafenib, or trametinib for 72 hours, and subjected to cell growth assays using the CellTiter-Glo 2.0 Luminescent Cell Viability Assay (Promega).

EdU incorporation assay

The EdU incorporation assay was performed using a Click-iT Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, human melanoma cells were treated with 50 nmol/L dinaciclib for 12 hours. After treatment with 25 μmol/L EdU for 1 hour, cells were fixed and permeabilized, and stained by picolyl azide coupled to Alexa Fluor 647 dye to detect EdU. Then, cells were resuspended in 200 μL of PBS containing propidium iodide (50 μg/mL) and RNase A (100 μg/mL), and immediately analyzed by flow cytometry on the FACSCanto II (BD Biosciences). Data were analyzed by FlowJo software (TreeStar).

Western blotting

Whole cell lysates were prepared as described previously (9). Primary antibodies used were BAX (D2E11; #5023), BAK (D4E4; #12105), BCL2 (D55G8; #4223), BCL-xL (54H6; #2746), A1/Bfl-1 (#4647), BCL-w (31H4; #2746), MCL1 (D35A5; #5453), and PARP (#9542) from Cell Signaling Technology, HA from Roche, and α-tubulin (T9026) from Sigma-Aldrich. The band intensities were measured by ImageJ and normalized to that of each control lane.

Caspase-3/-7 activity assay

To measure the activity of caspase-3 and -7, the Caspase-Glo 3/7 assay system (Promega) was applied according to the manufacturer's instructions. Briefly, cells (5×10^3 cells/well in 96-well plates) were treated with each drug at the indicated doses for 24 hours and Caspase-Glo 3/7 reagent was then added. After a 30-minute incubation, caspase-3 and -7 activity was measured using the GloMax-Multi Detection system (Promega).

Annexin V/propidium iodide staining analysis

Apoptotic cells were determined using the FITC Annexin V apoptosis Detection Kit (BD Sciences) according to the manufacturer's instructions. Briefly, human melanoma cells were harvested after drug treatment for 24 hours, washed with PBS, and then resuspended in Annexin V-binding buffer containing FITC-conjugated Annexin-V and propidium iodide. Cells were incubated for 15 minutes at room temperature in the dark and immediately analyzed by flow cytometry on the FACSCanto II. Data were analyzed by FlowJo software.

RNA interference

siRNAs were purchased from Thermo Fisher Scientific. For knockdown of BAX or BAK, 12.5 nmol/L siRNAs for BAX (s1888, s1889), siRNAs for BAK (s1880, s1881), siRNAs for CDK2 (s204, s205), siRNAs for CDK9 (s2834, s2835), siRNAs for BRAF (s2080, s2081), or Silencer Select Negative Control #1 siRNA were reverse transfected to human melanoma cell lines by Lipofectamine/RNAiMax (Thermo Fisher Scientific). The cells were then treated with each drug after 48 hours transfection, and subjected to cell growth assays. For Western blotting, whole cell lysates were prepared after 96 hours transfection.

Real-time RT-PCR

Total RNA was prepared using the RNeasy Plus Mini Kit (Qiagen) and subjected to real-time PCR on an ABI Prism 7300 sequence detection system (Life Technologies). The expression levels of *MCL1, BCL2L1 (BCL-xL), BCL2L2 (BCL-w), BCL2*, and *BCL2A1* mRNAs were normalized to that of β-actin mRNA. The primers used were: 5′-TCG TAA GGA CAA AAC GGG AC-3′ (sense) and 5′-CAT TCC TGA TGC CAC CTT CT-3′ (antisense) for *MCL1* mRNA; 5′-CTG CTG CAT TGT TCC CAT AG-3′ (sense) and 5′-TTC AGT GAC CTG ACA TCC CA-3′ (antisense) for *BCL2L1* mRNA; 5′-TCA ACA AGG AGA TGG AAC CAC-3′ (sense) and 5′-ATA GAG CTG TGA ACT CCG CC-3′ (antisense) for *BCL2L2* mRNA; 5′-CTG AGT ACC TGA ACC GGC A-3′ (sense) and 5′-GAG AAA TCA AAC AGA GGC CG-3′ (antisense) for *BCL2* mRNA; 5′-CCC GGA TGT GGA TAC CTA TAA GGA GA-3′ (sense) and 5′-GTC ATC CAG CCA GAT TTA GGT TCA-3′ (antisense) for *BCL2A1* mRNA; and 5′-GCA CAG AGC CTC GCC TT-3′ (sense) and 5′-GTT GTC GAC GAC GAG CG-3′ (antisense) for β-actin mRNA.

In vivo xenograft model

Female 6-week-old C.B-17/lcrHsd-*Prkdc*^{scid} mice were purchased from Japan SLC. All experiments were approved and performed according to the guidelines of the Care and Use of Laboratory Animals of the University of Toyama. A2058 cells were inoculated subcutaneously (5×10^6 cells/ 100 μL PBS/mouse) into the mouse flank. Vemurafenib was dissolved in DMSO, followed by PBS, which was then injected daily intraperitoneally into mice at a dose of 50 mg/kg. Dinaciclib was dissolved in 20% hydroxypropyl-β-cyclodextrin, and then injected intraperitoneally into mice at a dose of 20 mg/kg every 3 days. The tumor volume was measured every 3 days starting from day 3. All the mice were sacrificed at day 23. The primary tumor was measured using a Vernier caliper square along the longer (a) and shorter (b) axis, and the tumor volume was calculated using the following formula: tumor volume (mm^3) = $ab^2/2$.

Statistical analysis

Statistical significance was calculated using GraphPad Prism software (GraphPad Software, Inc.). More than three means were composed using two- or one-way ANOVA with the Bonferroni correction,

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and two means were composed using the unpaired Student *t* test. $P < 0.01$ was considered statistically significant.

Results

Dinaciclib induced melanoma cell apoptosis

Considering the importance of CDK2 (12) and CDK9 (20–22) in many types of cancer, we first investigated the antitumor effects of two potent CDK2/9 inhibitors, dinaciclib (23) (**Fig. 1A**) and iCDK2/9 (compound #32 in ref. 24; Supplementary Fig. S1A), in a panel of human melanoma cell lines carrying activating mutations of BRAF V600E, NRAS Q61R, or BRAF/NRAS-wild type. Although dinaciclib is a known inhibitor of CDK1/2/5/9, iCDK2/9 has strong selectivity to CDK2/9. In all melanoma cell lines tested, both CDK inhibitors significantly suppressed melanoma cell growth in a dose-dependent manner. Consistent with previous reports (12, 24), dinaciclib induced cell-cycle arrest at the G_1 phase in all melanoma cell lines and also at the G_2 phase in some melanoma cells (**Fig. 1B**; Supplementary Fig. S1B). In addition to the induction of cell-cycle arrest, annexin V-positive apoptotic cells were increased by dinaciclib treatment in melanoma cells (**Fig. 1C**), suggesting that dinaciclib suppresses melanoma growth through cell-cycle arrest and the induction of apoptosis.

Dinaciclib induced BAK-dependent melanoma cell apoptosis

As BAX and BAK are essential regulators of cancer cell apoptosis in response to some anticancer drugs, including MEK inhibitors or cisplatin (25, 26), we next examined the involvement of BAX- or BAK-dependent pathway(s) in the induction of apoptosis upon CDK2/9 inhibitor treatment of A2058 cells by knocking-out BAX (A2058/BAX$^{-/-}$) or BAK (A2058/BAK$^{-/-}$; ref. 19). As shown in **Fig. 2A**, four potent CDK2/9 inhibitors, dinaciclib, iCDK2/9 (27), SNS032 (CDK2/5/7/9 inhibitor; ref. 28), and LDC000067 (CDK2/9 inhibitor; ref. 29) had reduced inhibitory effects on the growth of A2058/BAK$^{-/-}$ cells compared with parental A2058 cells or A2058/BAX$^{-/-}$ cells. Furthermore, both the CDK2 inhibitor, purvalanol A (30), and CDK9 inhibitor, atuveciclib (31), showed BAK-dependent growth inhibition (Supplementary Fig. S1C). In contrast to dinaciclib and these potent CDK2/9 inhibitors, the CDK4/6 inhibitor, palbociclib, exhibited lower inhibitory effects on the growth of A2058/BAX$^{-/-}$ cells than on parental A2058 cells or A2058/BAK$^{-/-}$ cells (**Fig. 2A**). These results suggest that dinaciclib and these potent CDK2/9 inhibitors inhibit melanoma growth mainly through a BAK-dependent mechanism. Similar to the results in A2058/BAK$^{-/-}$ cells, both dinaciclib and iCDK2/9 had reduced inhibitory effect in the growth of three other melanoma cell lines (A2058, M14, SK-MEL-28, and MeWo) treated with siBAK, but not with siBAX (**Fig. 2B**; Supplementary Fig. S1D), although the growth inhibition by dinaciclib and iCDK2/9 was weakly rescued by BAX knockdown in SK-MEL-2. Consistent with the melanoma cell growth effects, we further demonstrated that both dinaciclib and iCDK2/9 caused less PARP cleavage and less caspase-3/7 activation measured using luminogenic caspase-3/-7 substrate or reduced annexin V-positive apoptotic cells in A2058 BAK$^{-/-}$ cells, respectively, than parental A2058 cells and A2058/BAX$^{-/-}$ cells (**Fig. 2C–E**; Supplementary Figs. S1E–S1G), suggesting that dinaciclib and iCDK2/9 induce melanoma cell apoptosis mainly through a BAK-dependent mechanism.

To directly examine the importance of CDK2 and/or CDK9 in melanoma cell growth via a BAK-dependent mechanism, we used A2058 cells treated with siRNAs against CDK2 or CDK9 instead of dinaciclib. Although knockdown of both CDK2 and CDK9 suppressed the growth of parental A2058 and A2058/BAX$^{-/-}$ cells, it did not affect the growth of A2058/BAK$^{-/-}$ cells (Supplementary Fig. S1H). Moreover, we observed the activation of BAK upon dinaciclib treatment in A2058 cells using a specific antibody detecting its conformational change on the mitochondrial membrane (**Fig. 2F**). Therefore, the inhibition of CDK2 and CDK9 by dinaciclib may induce BAK-dependent melanoma cell apoptosis.

Requirement of MCL1 for BAK-dependent melanoma cell apoptosis induced by dinaciclib

To assess the requirement of anti-apoptotic BCL-2 family members in BAK-dependent melanoma cell apoptosis induced by dinaciclib, we examined the expression of antiapoptotic BCL-2 family members (MCL1, BCL-xL, BCL-w, BCL2, and BCL2A1). As shown in **Fig. 3A**, both protein and mRNA expression of MCL1 were rapidly and strongly reduced by dinaciclib treatment, although that of some other BCL2 family members was also weakly reduced. The stronger reduction of MCL1 protein than of other BCL2 family members was also observed in A2058 cells treated with siCDK2, siCDK9, or their combination (Supplementary Fig. S1I). To clarify the functional importance of MCL1 in dinaciclib-induced apoptosis of melanoma cells, we established A2058 cells overexpressing MCL1 or E2-Crimson, which is a fluorescent protein, as a negative control, under the control of CMV promoter (A2058/MCL1 or A2058/E2-Crimson, respectively). A2058/MCL1 cells showed less response to dinaciclib as seen in the reduction of cell viability along with the sustained MCL1 expression, and impaired cleavage of PARP (**Fig. 3C**) compared with control A2058/E2-Crimson cells (**Fig. 3B**). These results indicate that dinaciclib induces BAK-dependent melanoma cell apoptosis through the suppression of MCL1 mRNA.

Inhibitors of BRAF and MEK induce BAX-dependent melanoma cell apoptosis

BRAF and MEK inhibitors are widely used to treat melanoma as monotherapy or in combination (6). Further, it is known that BRAF or MEK inhibition transiently induces MCL1 expression (32), leading to modest degrees of apoptosis of melanoma cells *in vitro* (33–36). Indeed, A2058/BAX$^{-/-}$ cells showed less sensitivity to vemurafenib at a lower dose of treatment (**Fig. 4A**) and there was a dominant role of BAX at a high-dose of vemurafenib treatment for induction of apoptosis in A2058/BAX$^{-/-}$ cells (**Fig. 4B**). Similar to vemurafenib treatment, we observed a dominant role of BAX in siBRAF-mediated growth suppression of A2058 melanoma cells (Supplementary Fig. S2). In addition to vemurafenib, a MEK inhibitor (trametinib) also suppressed the growth of A2058 (BRAF^{V600E}-mutant) and SK-MEL-2 (NRAS^{Q61R}-mutant) cells mainly in a BAX-dependent manner (**Fig. 4C**). These results demonstrate that inhibition of BRAF or MEK induces BAX-dependent melanoma cell apoptosis. Alternatively, BAK-dependent apoptosis may not be fully induced by BRAF or MEK inhibition.

Rational combination therapy of melanoma with dinaciclib

To test rational combination approaches exploiting BAX- and BAK-induced apoptosis for developing an effective treatment against melanoma, we examined the antitumor effect of dinaciclib in combination with three BAX-dependent drugs (vemurafenib, trametinib, and palbociclib) *in vitro*. Although modest inhibition of cell growth was observed by single treatment with dinaciclib or vemurafenib, a synergistic antitumor effect of dinaciclib with vemurafenib in BRAF^{V600E}-mutated melanoma cell lines (A2058 and M14) was observed. Contrary to the BRAF^{V600E}-mutated melanoma cell lines, there was no obvious combination effect of dinaciclib with

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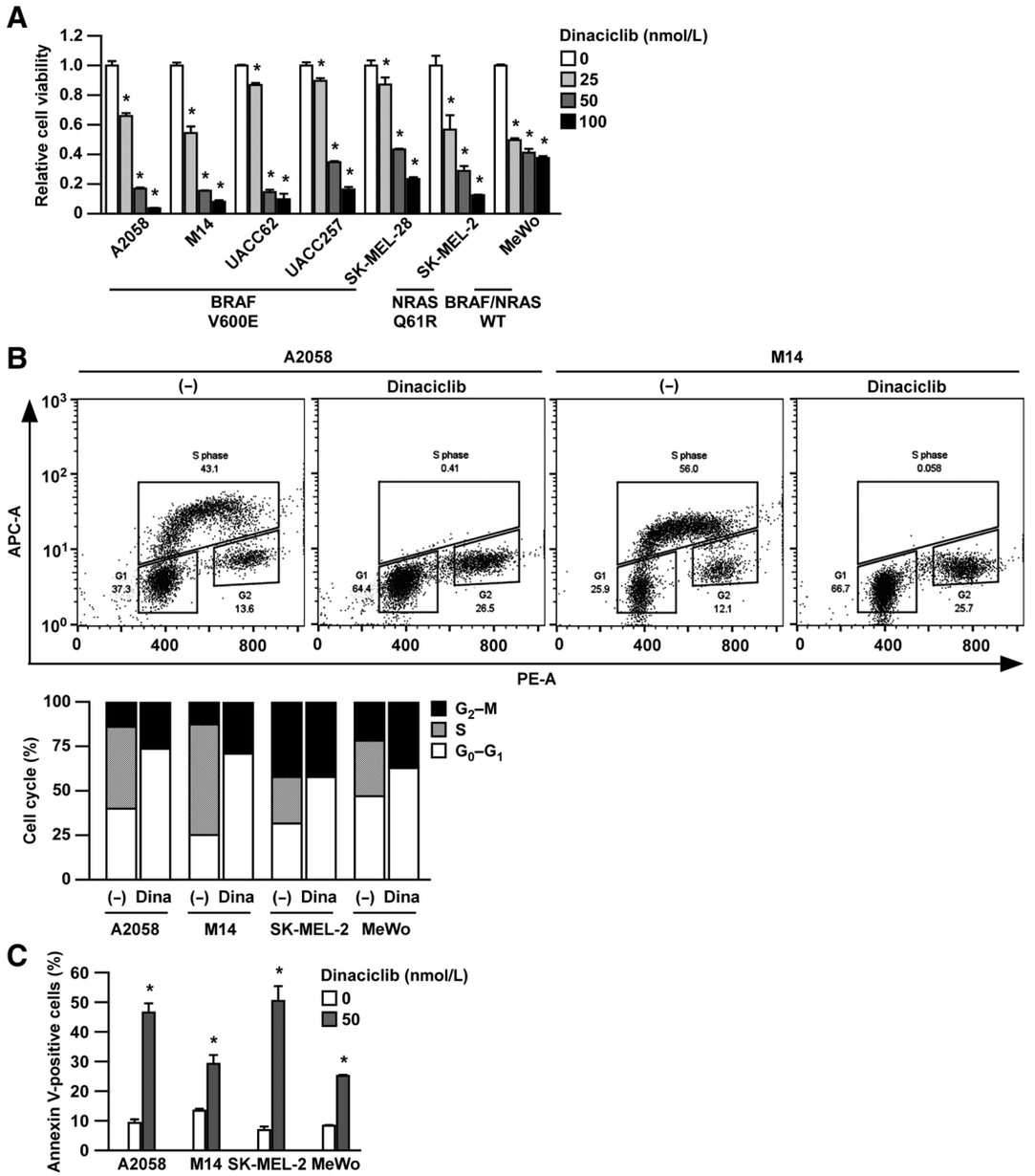

Figure 1.

Dinaciclib induced cell-cycle arrest and apoptosis in melanoma cells. **A,** Human melanoma cells were treated with dinaciclib at the indicated doses for 72 hours. After 72-hour incubation, cell viability was assessed by the CellTiter-Glo 2.0 assay. Relative growth to vehicle control is shown. Data are shown as the mean ± SD of at least three independent experiments. $^*P < 0.01$ by two-way ANOVA with Bonferroni correction compared with each cell line treated with vehicle. **B,** Human melanoma cell lines A2058 (BRAF mutant), M14 (BRAF mutant), SK-MEL-2 (NRAS mutant), and MeWo (BRAF/NRAS-wild type) were treated with 50 nmol/L dinaciclib for 12 hours. The treated cells were subjected to EdU incorporation assay. Top panels show the representative FACS patterns in A2058 and M14 cells. Bottom panels show the means of each phase in the cell cycle in four melanoma cell lines. **C,** All treated cells were subjected to annexin V/PI staining. Data are the means ± SD of at least three independent experiments. *, $P < 0.01$ by two-way ANOVA with Bonferroni correction compared with each cell line treated with vehicle.

vemurafenib in a $NRAS^{Q61R}$-mutated melanoma cell line (SK-MEL-2) or a BRAF/NRAS-wild-type melanoma cell line (MeWo; **Fig. 5A**). We further confirmed effects of the combination of dinaciclib and vemurafenib in the induction of melanoma cell apoptosis using annexin V-PI staining (Supplementary Fig. S3A). Importantly, the combination of trametinib with dinaciclib inhibited cell growth (**Fig. 5B**) or induced apoptosis (Supplementary Fig. S3B) more effectively than either single treatment, not only in $BRAF^{V600E}$-mutant melanoma cells, but also in $NRAS^{Q61R}$-mutant or BRAF/NRAS-wild type melanoma cells. We also observed more effective growth suppression of all these variants of melanoma cells by the combination of palbociclib, a BAX-dependent inhibitor (**Fig. 2A**), with dinaciclib as compared with either single treatment alone (**Fig. 5C**). Interestingly, the combination of vemurafenib with trametinib (both inhibit melanoma cell growth through

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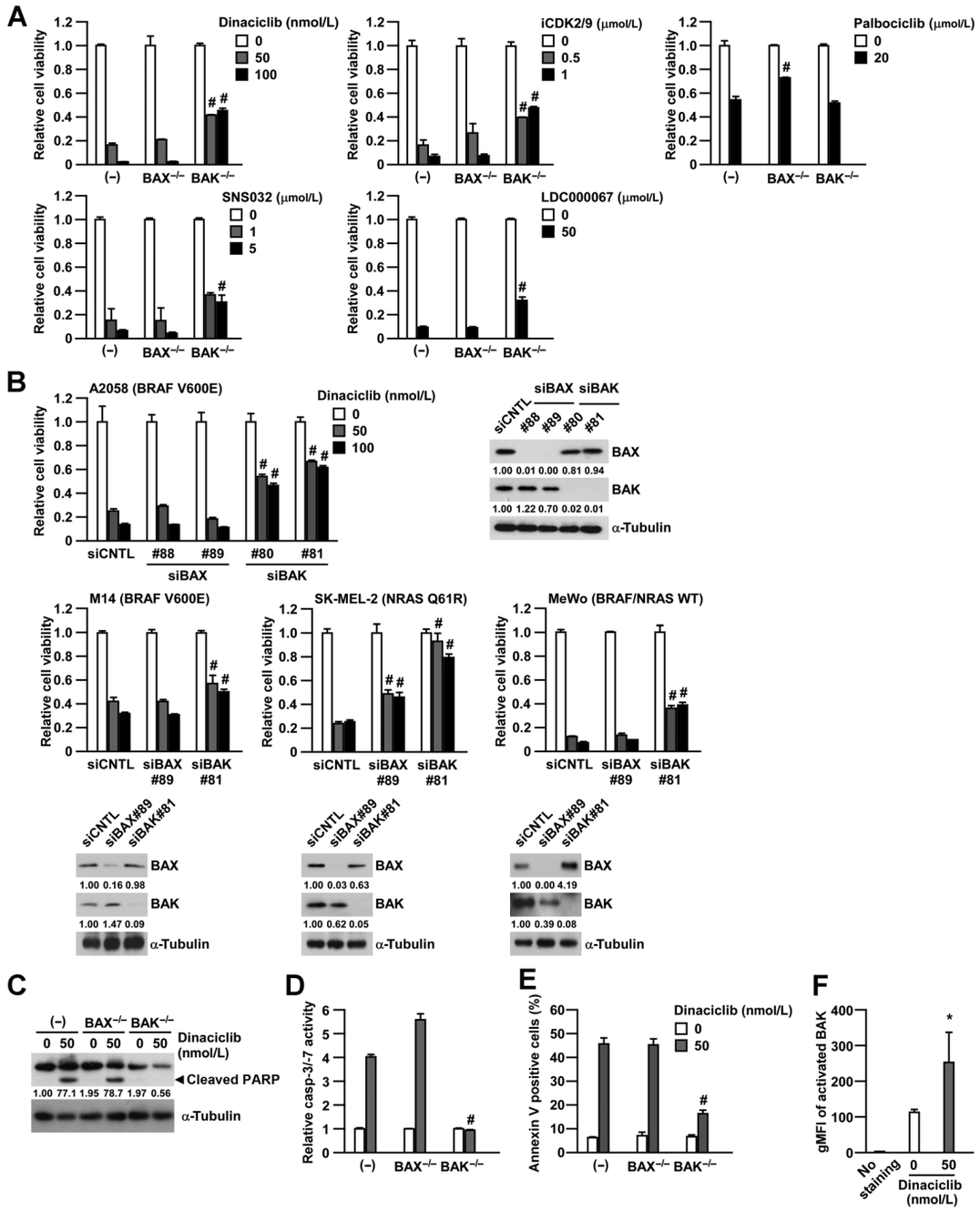

Figure 2.

BAK is required for dinaciclib-induced apoptosis in melanoma cells. **A,** Parental A2058 cells (-) or A2058 cells lacking BAX or BAK ($BAX^{-/-}$ or $BAK^{-/-}$) were treated with dinaciclib or palbociclib at the indicated doses for 72 hours. Cell growth was assessed by the CellTiter-Glo 2.0 assay. Data are shown as the means ± SD of at least three independent experiments. #, $P < 0.01$ by two-way ANOVA with Bonferroni correction compared with parental A2058 cells treated with each concentration of dinaciclib. **B,** A2058, M14, SK-MEL-2, and MeWo melanoma cells transfected with siCNTL, siBAX, or siBAK for 48 hours were treated with dinaciclib at the indicated dose for an additional 72 hours. Other conditions were similar to **A**. Whole cell lysates of transfected cells were subjected to Western blotting. The band intensities were measured by ImageJ, normalized to that of siCNTL-transfected cells, and shown below each panel. **C**–**E**, Parental A2058 cells, A2058 $BAX^{-/-}$, or A2058 $BAK^{-/-}$ cells were treated with 50 nmol/L dinaciclib for 24 hours. The treated cells were subjected to Western blotting (**C**), Caspase-Glo 3/7 assay (**D**), and annexin V/PI staining (**E**). The band intensities of cleaved PARP were measured by ImageJ, normalized to that of nonknockout cells with dinaciclib, and shown below each panel. #, $P < 0.01$ by two-way ANOVA with Bonferroni correction compared with parental A2058 cells treated with each concentration of dinaciclib. **F,** A2058 melanoma cells treated with 50 nmol/L dinaciclib for 24 hours were fixed, permeabilized, and stained using an antibody recognizing the conformational change of BAK. The BAK activation state was analyzed by flow cytometry. Data are shown as the means ± SD of at least three independent experiments. *, $P < 0.01$ by two-way ANOVA with Bonferroni correction compared with A2058 cells treated with vehicle.

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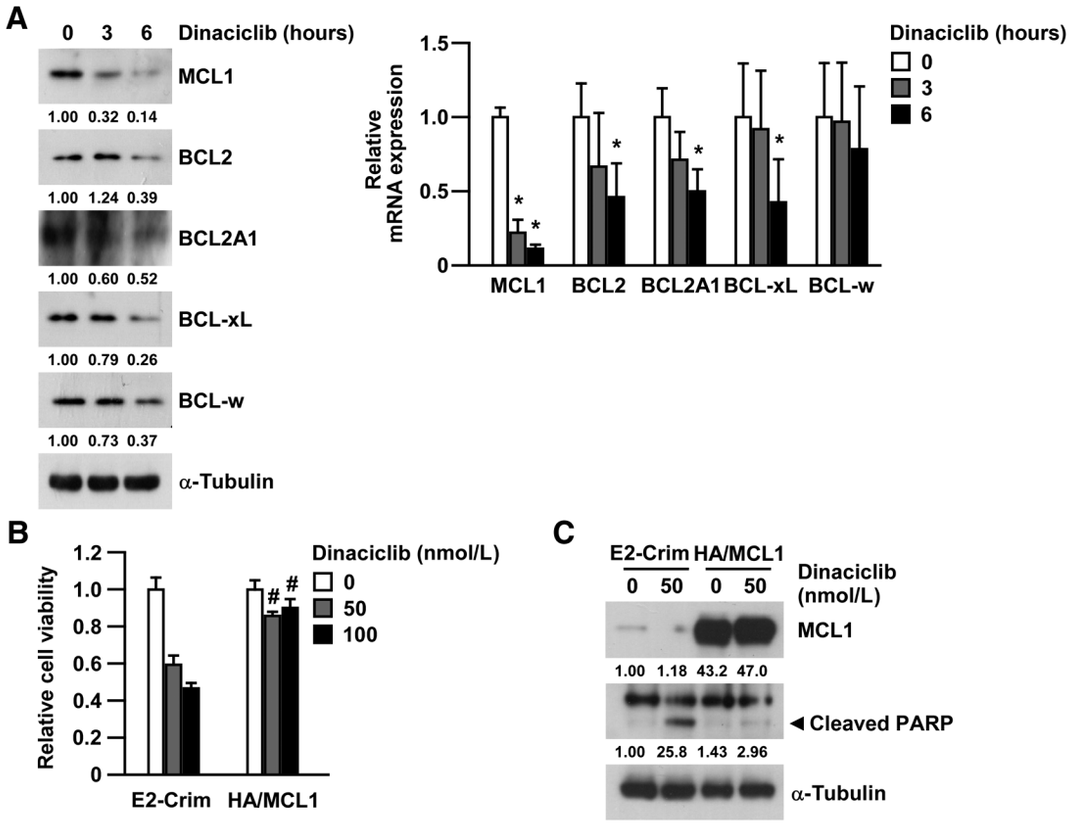

Figure 3.

The MCL1–BAK axis is required for growth suppression by dinaciclib in melanoma cells. **A,** A2058 melanoma cells were treated with 50 nmol/L dinaciclib for 3 or 6 hours. Whole-cell lysates were subjected to Western blotting (left). The mRNA levels were quantified by real-time RT-PCR (right). Relative mRNA expression was normalized to the value of each mRNA at 0 hours. Data are the means ± SD of at least three independent experiments. *, $P < 0.01$ by two-way ANOVA with Bonferroni correction compared with each mRNA expression level relative to vehicle control. **B** and **C**, A2058 melanoma cells stably overexpressing HA/MCL1 (HA/MCL1) or E2-Crimson (E2-Crim, as a control) were treated with dinaciclib at the indicated dose for 24 hours. Cell viability (**B**) and protein expression levels (**C**) were measured. Other conditions were similar to **Fig. 2A**.

BAX; **Fig. 4**) did not show any additional antimelanoma effects compared with either single treatment (**Fig. 5D**). In contrast with dinaciclib, the growth inhibition by either vemurafenib or trametinib could not be rescued by MCL1 overexpression (Supplementary Fig. 3C). Further, we also determined the significance of BAX and BAK for the combination of dinaciclib and vemurafenib in the melanoma growth suppression (**Fig. 5E**). Finally, we tested a rational combination of dinaciclib and vemurafenib in a mouse xenograft model using A2058 cells. Consistent with our *in vitro* findings, the growth of A2058 tumors was significantly suppressed by the combination of dinaciclib and vemurafenib as compared with either vemurafenib or dinaciclib treatment alone (**Fig. 5F**). These results indicate that the combination of dinaciclib and vemurafenib effectively suppresses the A2058 melanoma tumor growth *in vivo*. Collectively, these results strongly support use of a rational combination of BAK-dependent drug, dinaciclib, with BAX-dependent drugs, such as BRAF, MEK, or CDK4/6 inhibitor, as an attractive strategy for treating melanoma.

Discussion

CDKs are appealing molecular targets for numerous cancers due to their aberrant activation by their mutation or alterations of upstream inhibitory molecules such as p16, p21, or p27. Although CDK4/6 inhibitors, such as palbociclib, have been clinically used to treat some breast cancers, dinaciclib has not been approved for cancer therapy, although some clinical trials are ongoing. We demonstrated that dinaciclib suppresses melanoma cell growth by reducing MCL1 expression and inducing BAK-dependent cell death (**Fig. 2**). Consistent with our findings, MCL1 expression was reported as a predictive biomarker of dinaciclib for its responsiveness against solid tumor cells (37). Moreover, the molecular mechanism(s) that regulate the expression of MCL1 through CDK2 and CDK9 were previously reported (38, 39). CDK2 regulates the phosphorylation and stabilization of MCL1 (38), whereas CDK9 controls transcriptional regulation of *MCL1* mRNA expression (39), and these mechanisms may be related to the pharmacological effects of CDK2/9 inhibition in reducing MCL1 expression by dinaciclib and other potent CDK2/9 inhibitors (**Fig. 2A**; Supplementary Figs. S1D–S1G). Similarly, siRNAs against either CDK2 or CDK9 suppressed MCL1 expression in melanoma cells (Supplementary Fig. S1H). In addition to dinaciclib, both a CDK2 inhibitor and a CDK9 inhibitor also suppressed melanoma growth through BAK (Supplementary Fig. S1C). This implies that not only CDK9 but also CDK2 regulates the MCL1–BAK axis in melanoma. In addition to melanoma in this study, MCL1 addiction has been described in several cancers with *MCL1* gene amplification (40). Furthermore, genes related to MCL1 degradation, such as FBXW7,

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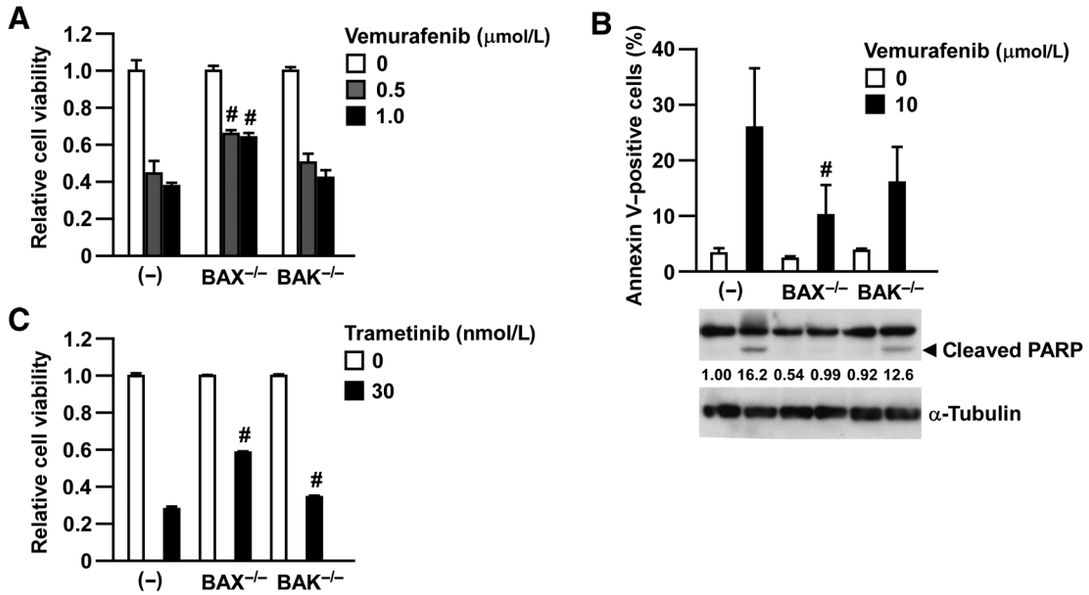

Figure 4.

Vemurafenib/Trametinib induces apoptosis through the activation of BAX in melanoma cells. **A,** Parental A2058, A2058 BAX$^{-/-}$, or A2058 BAK$^{-/-}$ cells were treated with vemurafenib at the indicated doses for 72 hours. Other conditions were similar to **Fig. 2A**. **B,** Parental A2058, A2058 BAX$^{-/-}$, or A2058 BAK$^{-/-}$ cells were treated with 10 μmol/L vemurafenib for 48 hours. Annexin V–positive cells (%) and protein expression levels were measured by flow cytometry (top) and Western blotting (bottom), respectively. Other conditions were similar to **Fig. 2C** and **2E**. **C,** Parental A2058, A2058 BAX$^{-/-}$, or A2058 BAK$^{-/-}$ cells were treated with trametinib at 30 nmol/L for 72 hours. Other conditions were similar to **Fig. 2A**.

have also been found to be mutated in several cancers (41, 42). In light of the observed reduction in the *MCL1* mRNA level (**Fig. 3A**), pharmacological suppression of MCL1 by CDK2/9 inhibition may be a valuable therapeutic strategy for melanoma and other cancers with aberrant MCL1 expression.

Targeting oncogenic BRAF mutations with small molecule inhibitors can lead to significant clinical responses in BRAF^{V600E}-melanoma (3–5). However, this strategy fails to eradicate tumors in most patients due to many resistance mechanisms, which include reactivation of the downstream kinases MEK1/2 (43). Although the combination of BRAF inhibition with MEK inhibition is known to improve the progression-free survival in melanoma patients compared with vemurafenib alone (6), their early survival rate was not significantly improved and eventual relapse remains common. In addition, the combination of BRAF inhibition with MEK inhibition did not have any additive effects on melanoma growth *in vitro* (**Fig. 5D**). These results suggest that intrinsic resistance is not targeted by this combination. To overcome their intrinsic resistance to BRAF inhibition, we focused on BAK-mediated cell death, which was not fully activated. We observed significant suppression of growth by vemurafenib combined with dinaciclib compared with the combination of vemurafenib and trametinib (**Fig. 5**), suggesting that inhibitors of CDK2/9, including dinaciclib, are effective and rational combination with BRAF inhibitors for melanoma treatment to overcome intrinsic resistance.

Our study supports the use of the combination of a BAK-activating drug with a BAX-activating drug as a rational therapeutic strategy for different cancers, but we focused on such a combination for melanoma. There are many strategies of drug combinations in cancer therapy. One is based upon combining drugs with different mechanisms of action such as cisplatin with paclitaxel for lung cancer. Although their mechanisms of action are different, these two drugs may activate the same downstream apoptotic effectors, BAX and BAK. Furthermore, in a combination containing one drug that activates both BAX and BAK, such as cisplatin (26), their combined effects may be weaker than expected, and adverse effects may be severe due to cytotoxicity in normal cells. Another combination strategy is based on an understanding of the acquired resistant mechanisms for the first drug, and supports the combination of vemurafenib with trametinib for melanoma. As acquired resistance can be caused by reactivation of upstream genes (e.g., NRAS; ref. 44), parallel genes (e.g., COT; ref. 45), or downstream genes (e.g., MEK1/2; ref. 46) relative to the target oncogene (e.g., BRAF V600E), the combination of two drugs may be based on targeting the common oncogenic pathway such as the MAPK pathway. Thus, for the activation of the same apoptotic effectors, BAX or BAK, as we observed with the combination of vemurafenib and trametinib (**Fig. 4**), there are no notable additive anti-tumor effects *in vitro* (**Fig. 5D**). Considering the clinical benefit of combined therapy with vemurafenib and trametinib over vemurafenib monotherapy (6), the advantage of the latter is difficult to predict without *in vivo* long-term study. However, our proposed rational combination provides a complementary approach to induce cancer cell death using both BAX-activating and BAK-activating drugs.

Regarding the effectiveness of such combinations, the simultaneous use of two cytotoxic chemotherapeutic agents may cause severe side effects by affecting the growth of normal healthy cells. Considering the good antitumor efficacy at apparently well-tolerated doses in our *in vitro* experiments, at least one molecular-targeted drug that is specific to cancer cells may be necessary to reduce the cytotoxicity in the combination. Furthermore, the efficacy of each drug or their combination may be limited in cancers with genomic alteration of either BAX or BAK as observed in BAX-/BAK-knockout cells (**Fig. 2**, **3** and **5**). Indeed, BAX frameshift mutations are frequently found in DNA mismatch repair-deficient cancers, such as colon, gastric, and endometrial malignancies (47, 48), and frameshift/truncating

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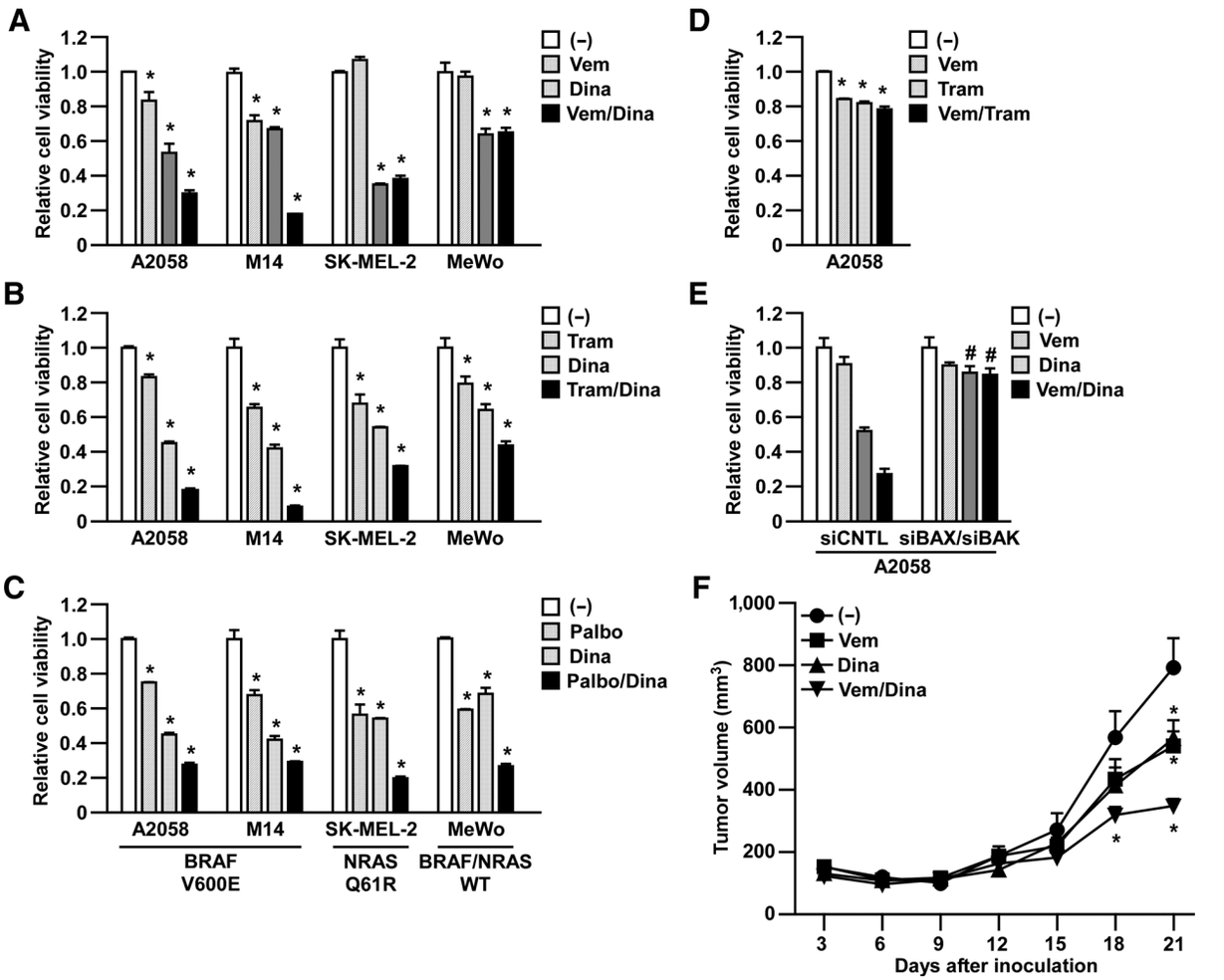

Figure 5.

Combination of dinaciclib with BAX-activating drugs in melanoma cells. **A–C**, Melanoma cell lines A2058, M14 (BRAF mutant), SK-MEL-2 (NRAS mutant), and MeWo (BRAF/NRAS-wild type) were treated with 1 μmol/L vemurafenib (Vem; **A**), 30 nmol/L trametinib (Tram; **B**), or 20 μmol/L palbociclib (Palbo; **C**) combined with 50 nmol/L dinaciclib (Dina) for 24 hours. Other conditions were similar to **Fig. 1A**. **D,** A2058 cells were treated with vemurafenib (Vem) combined with trametinib (Tram) for 24 hours. Other conditions were similar to **Fig. 1A**. **E,** A2058 melanoma cells transfected with siCNTL or siBAX/siBAK for 48 hours were treated with each drug for an additional 24 hours. Other conditions were similar to **A**. #, $P < 0.01$ by two-way ANOVA with Bonferroni correction compared with parental A2058 cells treated with each drug. **F,** A2058 melanoma cells were engrafted subcutaneously in SCID mice. Mice were randomly divided into 4 groups and treated with vehicle (-) as a control, vemurafenib (Vem), dinaciclib (Dina), or a combination of vemurafenib and dinaciclib (Vem/Dina). Tumor volumes were measured every 3 days by Vernier calipers, and the values were plotted as means ± SEM. *, $P < 0.01$ by two-way ANOVA with Bonferroni correction compared with the vehicle-treated group.

mutations of BAK have been observed in some cancers in TCGA data sets (49). Thus, combination therapies targeting BAX- and BAK-dependent cell death may have limitations in some cancer settings.

In summary, we identified the dinaciclib as an anti-melanoma drug candidate that regulates the MCL1-BAK axis, and it may be a potent complementary drug to the BRAF inhibitor vemurafenib for melanoma treatment to induce BAX-dependent cell death. We propose a novel approach to identify rational drug combinations for cancer treatment by examining cell intrinsic drug resistance or sensitivity and targeting proapoptotic BCL2 family members.

Disclosure of Potential Conflicts of Interest

D.E. Fisher has ownership interest (including patents) in Soltego Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: Y. Hayakawa, S. Yokoyama
Development of methodology: S. Yokoyama
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Xu, S. Eshima, S. Kato, Y. Hayakawa, S. Yokoyama
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Xu, Y. Hayakawa, S. Yokoyama
Writing, review, and/or revision of the manuscript: X. Xu, D.E. Fisher, Y. Hayakawa, S. Yokoyama
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Xu, S. Yokoyama
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Molecular Cancer Therapeutics

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